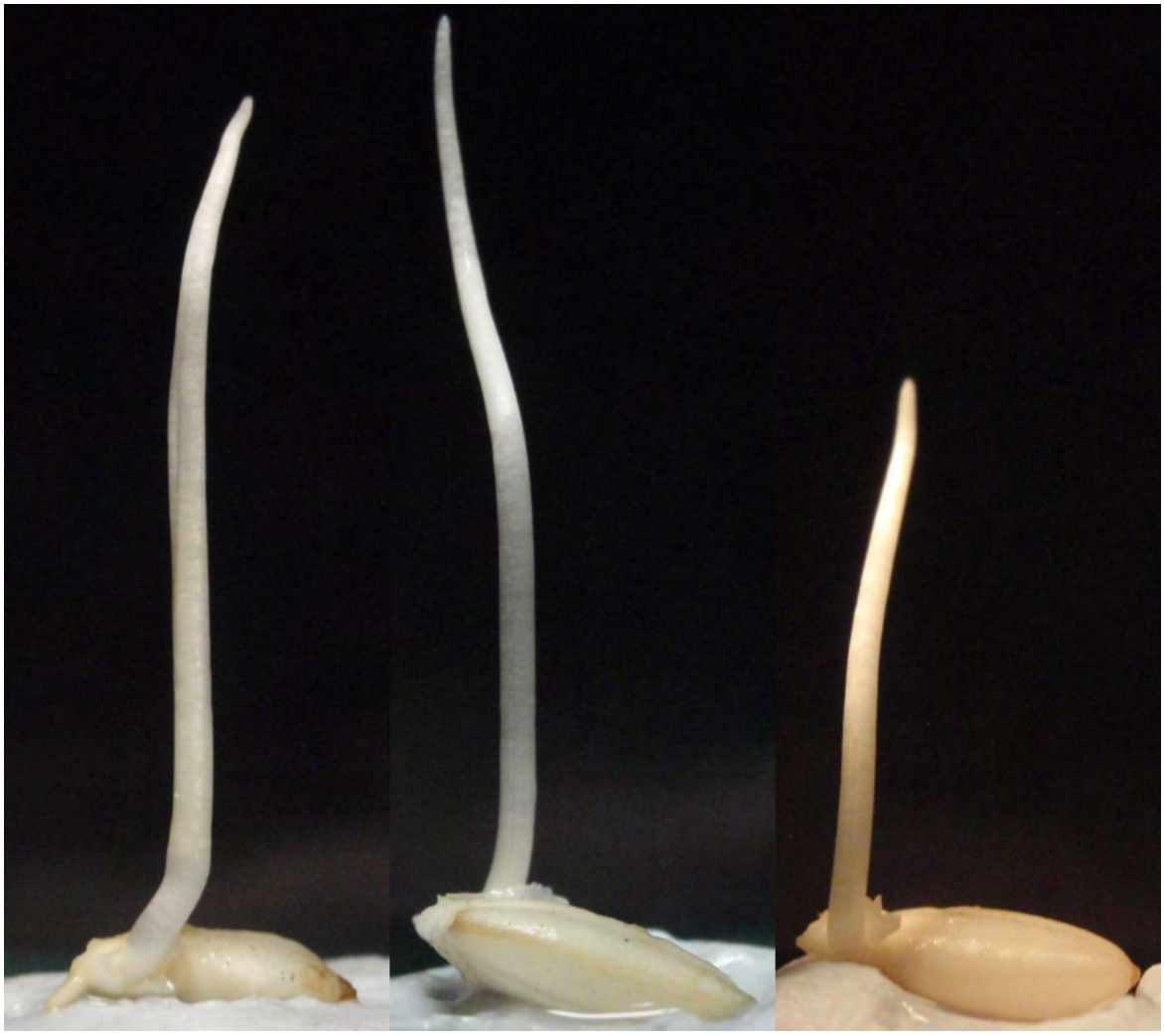
Differential Responses to Oxygen Deprivation in Rice Coleoptiles



Joshua Edwards BMath BSc(Hons)
Department of Biological Sciences
Macquarie University
Sydney, Australia

This thesis is presented for the degree of Doctor of Philosophy

July 2013

Department of Biological Sciences

Macquarie University

Sydney, New South Wales, Australia, 2109

Abstract

Rice (*Oryza sativa*), a major dietary staple for one-third of the world's people, is the only major cereal crop able to grow in the absence of oxygen (O_2). I examined the bio-energetic, transcriptomic and proteomic basis for this tolerance using rice coleoptiles grown in oxygenated (normoxia) and O_2-deprived (hypoxia and anoxia) conditions. I found that during hypoxia rice coleoptiles prioritise the utilisation of ATP by metabolic processes that allow for the rapid extension of the coleoptile, including cell wall synthesis, lipid synthesis and ion (K^+) uptake. By comparison, anoxic coleoptiles directed 52% of their available ATP towards protein synthesis while minimising the majority of other metabolic processes.

I also examined differences in the cellular growth profile and transcriptome (Chapter 3) and proteome (Chapter 4) between distal (tip) and basal (base) regions of coleoptiles grown in normoxic, hypoxic and anoxic conditions. The prioritisation of ATP towards protein, lipid and cell wall synthesis, ion uptake and the rapid elongation of hypoxic/anoxic coleoptiles (*cf.* normoxia) are reflected within the changes in the proteome and transcriptome, as are differences between the tip and base of the coleoptile, regardless of O_2 treatment.

The cellular growth profiles indicated that cells in the basal zone of coleoptiles divided and elongated faster than the distal tip cells, regardless of O_2 treatment. This was supported by differences in the transcriptome and proteome of coleoptile tips and bases: genes encoding for cellular division, cell wall synthesis and elongation and nucleic acid and protein synthesis were up-regulated in coleoptile bases compared to tips. Similar differences were observed in the proteome of tips and bases (Chapter 4).

The transcriptomic (Chapter 3) and proteomic (Chapter 4) differences between normoxic and hypoxic/anoxic coleoptile tips and bases were also examined. Hypoxic and anoxic bases (*cf.* normoxic bases) showed increased levels of genes related to

protein recycling, nucleic acid organisation and cell division and elongation. Hypoxic and anoxic tips (*cf.* normoxic tips) up-regulated genes related to RNA synthesis, editing and processing, protein synthesis/recycling and cell division and elongation, suggesting a higher rate of metabolic activity in these tissues than the normoxic tips. Proteins involved in ethanol fermentation were more highly expressed in hypoxia and anoxia (*cf.* normoxia). In hypoxic/anoxic tips, proteins involved in the stress response and the promotion of cell division were up-regulated whilst those involved in O_2-sensitive processes including lipid and jasmonate synthesis were down-regulated (*cf*. normoxic tips). Within hypoxic/anoxic bases, proteins involved in protein turnover and modification, promotion of cell division and elongation and response to abiotic stress were up-regulated (*cf*. normoxic bases).

I also examined the effects of the addition of low (2 mM) levels of exogenous nitrogen supply on coleoptiles grown in various O_2 treatments. Low levels of nitrate (NO_3) and ammonium (NH_4) did not significantly affect the growth of normoxic, hypoxic or anoxic coleoptiles at 3 days of treatment. This study was undertaken to examine the feasibility of using ^{15}N-labelled nitrogen sources as a means of examining differences in which proteins are being actively synthesised in the varying O_2 treatments. However, the inadvertent finding was that nitrogen had subtle effects on coleoptiles development, with ammonium tending to increase length at the expense of mass.

Finally, potential avenues for further research into tolerance of rice to O_2 deprivation and candidate genes for targeted genetic modification and breeding opportunities are discussed in Chapter 6.

Statement

I certify that all the published material in this thesis is of my own making unless stated otherwise. All cited material has been referenced in accordance with the policies of Macquarie University and no work has been previously submitted as a component of a degree at this or any other university or institution.

Joshua Edwards

Student number: 41686284

Professional Acknowledgements

Firstly, I would like to acknowledge the guidance and support provided by my supervisors Associate Professor Brian Atwell and Dr. Tom Roberts. Without your help, advice and support, this project would not have been possible.

For Chapter 2, I acknowledge the help of the following individuals and groups: Mr Michael Joseph (Sydney University) for the generous gift of isotopes; Dr Abdelbagi Ismail (International Rice Research Institute) for seed of Khaiyan and Khao Hlan On; Dr Narayana Upadhyaya (CSIRO) for seed of the PDC anti-sense mutant; and Professor Mikio Nakazono (Nagoya University) and Professor Hideo Matsumura (Shinshu University) for seed of the *rad* mutant and Kinmaze parent line. I also thank Dr Hank Greenway for comments on the experiments, Dr Jan Gebicki for reviewing an early draft of Chapter 2 and Denise Thomas from the Australian Proteome Analysis Facility (APAF) who conducted the amino acid analysis reported in Appendix A. Work conducted at APAF was facilitated using infrastructure provided by the Australian Government through the National Collaborative Research Infrastructure Strategy (NCRIS).

For Chapter 3 I acknowledge the following contributions to my research: Mr Muhammad Masood (Macquarie University) and Assoc. Prof. Brian Atwell performed the SEM described in Section 3.2.4; Professor Wendy Silk at the University of California (Davis) for comments on the growth kinematics; Dr Reena Narsai (Plant Energy Biology ARC COE, University of Western Australia) extracted RNA from frozen material sent to Perth in order to initiate the microarray study after unsuccessful attempts to send prepared RNA to Perth. Dr Narsai also performed initial analyses reported in Section 3.2.6 and aided equally in drafting the prospective publication that forms the basis of Chapter 3, by incorporating information from confidential databases held in the Centre of Excellence. I would also like to acknowledge Professor Jim Whelan (Plant Energy

Biology ARC COE, UWA) for the gift of 18 AffyMetrix Rice Genechips, valuable thoughts about the significance of the data and generous technical support through Dr Narsai's assistance.

For Chapter 4 I acknowledge the aid of Ms. Veronica Polaskova, Dr Charlie Ahn and Dr Alamgir Khan (APAF) for training and technical advice. I also thank Ed Breen for advice of the statistical analyses of spot differences.

For all chapters I would like to thank Associate Professor Brian Atwell (Macquarie University) and Dr. Thomas H Roberts (University of Sydney) for proof reading, editorial and content suggestions.

Finally, I would like to acknowledge the financial support provide by the Commonwealth Government through the Australian Post-graduate Award Scholarship scheme and the Department of Biological Sciences at Macquarie University for providing travel funds that allowed me to present my research at ComBio 2010 in Melbourne.

Personal Acknowledgements and Thanks

I would like to thank my wonderful fiancée Catherine for her love and support. You are the rock upon which I have built my home. Without you, I wouldn't have made it this far.

Additionally, I thank my parents for their care and encouragement, my supervisors Brian and Tom for their support and my many friends for remaining by my side despite my frequent lapses into immaturity.

Manuscripts and Presentations

Published

Edwards JM, Roberts TH, Atwell BJ (2012) Quantifying ATP turnover in anoxic coleoptiles of rice (*Oryza sativa*) demonstrates preferential allocation of energy to protein synthesis. Journal of Experimental Botany **63:** 4389-4402.

Manuscripts in preparation

Narsai R, Edwards JM, Roberts TH, Whelan J, Atwell BJ (2013) Tissue-specific transcriptomic responses to oxygen deficiency in rice coleoptiles. Manuscript in preparation.

Conference presentations

Edwards JM, Robert TH, Atwell BJ (2010) Surviving an "energy crisis": ATP utilisation in anoxic coleoptiles is dominated by protein synthesis. ComBio 2010. Melbourne, Australia.

1. Chapter 1 – Literature Review and General Introduction

1.1 Introduction

Terrestrial plants, unlike animals, spend their lifecycles immobilised by their root systems. Abiotic stresses such as extreme heat, cold, drought, nutrient deficiency or flooding/waterlogged soils cannot be avoided spatially, driving strong genetic adaptation to mitigate the impact of stress (Zhang *et al*., 2000). Plants in adverse environments have evolved developmental plasticity to acclimate to a wide variety of abiotic and biotic stresses (Sultan, 2000). However, acclimation carries a metabolic cost; changes in plant metabolism inevitably require changes to the expenditure of photosynthetic resources and generally, a growth or reproductive penalty results unless ATP can be synthesised more efficiently. One of the 'big questions' in plant biology, therefore, concerns energetics: How do plants produce and utilise the high-energy intermediates, particularly adenosine tri-phosphate (ATP), which form the energy currency of the metabolic and catabolic processes within the plant? And how do abiotic stresses influence this expenditure?

A common abiotic stress, particularly for plant roots, is O_2 deficit, brought about by events such as waterlogging of part of the soil profile after flash floods or prolonged periods of flooding (Drew, 1983). Bailey-Serres *et al*. (2012) places flooding as the second leading cause of yield loss in the USA (US$10-70 billion a year) while the 2011 floods in Queensland, Australia, destroyed an estimated $AUD500 million worth of crops (ABARES, 2011).

Flooding and submergence restrict the amount of O_2 that reaches plant tissues by slowing the diffusivity of O_2 (rate of O_2 movement under a standardised concentration gradient) by a factor of 10,000 (Drew, 1983). Oxygen is essential for production of ATP and the oxidation of NADH to NAD^+ because it is the final electron acceptor in oxidative

phosphorylation (Hatefi, 1985; Saraste, 1999). It also acts as a substrate for a range of other oxygenase reactions in living cells (Prescott and John, 1996). Carbohydrate breakdown by glycolysis and the tricarboxylic acid (TCA) cycle gives rise to the reducing power that is converted into the principal energy source for cellular growth and maintenance processes (Hatefi, 1985). Ultimately, ATP hydrolysis provides energy to many cellular processes, including the synthesis of new macromolecules (such as proteins, lipids and cellulose) (Penning de Vries, 1974; Mocquot *et al*., 1981), transport of molecules (Cheeseman *et al*., 1980; Drew *et al*., 1988) into and out of cells and maintenance of membrane potentials (Katou *et al*., 1982; Kojima *et al*., 1985). ATP deprivation, induced by an anoxic shock (for example), cause a rapid halt to energy-requiring cell processes such as biosynthetic reactions (Mocquot *et al*., 1981; Sachs and Ho, 1986; Vartapetian and Jackson, 1997; Geigenberger *et al*., 2000; Huang *et al*., 2005), leading to membrane depolarisation, irreversible cell and tissue damage and, eventually, tissue death. Such lowered rates of ATP synthesis result in what has been termed an energy crisis (Gibbs and Greenway, 2003). Tissues that exhibit high rates of growth and metabolic activity are typically those that are most affected by an energy crisis; for example, root tips of onion respire rapidly and have the greatest sensitivity to declining O_2 levels (Berry, 1949; Atwell *et al*., 1985). The sensitivity of respiring root tissues of non-hydrophytes to O_2 deficits is particularly evident in many commercially important species such as wheat, maize and barley, where prolonged soil waterlogging or flooding inevitably results in major crop losses (Drew, 1983; Bailey-Serres *et al*., 2012; Navrud *et al*., 2012). Thus, an insufficient supply of O_2, and thus inadequate ATP production, retards normal growth and alters development (Kordan, 1974), and may result in plant death.

Rice, a primary source of calories for food of ~50% of the world's population (FAO, 2007), is adapted to grow under low O_2, and hence low-energy, conditions. These

adaptations are both structural and metabolic. Structural adaptations include the formation of aerenchyma in root and internodes, which are specialised structures that allow internal O_2 diffusion, particularly of O_2 from shoots to roots (Jackson *et al*., 1985), and the rapid elongation of stem internodes during submergence seen in some cultivars (Métraux and Kende, 1983; Suge, 1985; Kende *et al*., 1998). Similarly, in some cultivars, the coleoptiles of submerged germinating rice seeds also elongate rapidly and have been characterised as a 'snorkel' because of their capacity to connect the organism with atmospheric O_2 (Kordan, 1974). These adaptations serve to increase transport of O_2 to the O_2-deprived tissue and thus improve respiratory activity. Metabolic adaptations include the maintenance of sufficient ATP turnover to enable tissues to survive (Atwell *et al*., 1982; Raymond *et al*., 1985; Reggiani *et al*., 1985), the maintenance of relatively stable cellular pH and membrane potentials (Menegus *et al*., 1991; Xia and Saglio, 1992; Setter and Ella, 1994; Setter *et al*., 1994*;* Felle, 2005; Kulichikhin *et al*., 2009) and the induction of genes and gene products involved in acclimation to an energy crisis (Sachs *et al*., 1980, 1996; Mocquot *et al*., 1981; Huang *et al*., 2005; Lasanthi-Kudahettige *et al.*, 2007).

The rapid turnover of high-energy intermediates has led to various ratios being used to express cell energy status. The term commonly used to express changes in energy status globally is the adenylate energy charge (AEC), the ratio of high-energy phosphate bonds (ATP + 0.5(ADP)) to total cellular levels of adenylates (ATP+ADP+AMP) (Atkinson, 1969; Raymond *et al*., 1985) and reflects the instantaneous metabolic state of the tissue (Krebs, 1973; Mocquot *et al*., 1981; Al-Ani *et al*., 1985; Reggiani *et al*., 1985). While this ratio is not a perfect absolute expression of cell energetics, it can be used through a time series to monitor relative changes in the sum of high-energy phosphate bonds in tissues undergoing an energy crisis (Atkinson, 1969; Raymond *et al*., 1985; Greenway and Gibbs, 2003). The limitation is that enzymes whose

activity is modulated by cell energy status are more likely to have evolved a response to ATP/ADP ratio or AMP levels than the composite expression of energy charge (Passonneau and Lowry, 1964).

This review will focus on three areas in plant energy metabolism:

1. Causes of an energy crisis in plants;
2. General responses of plants to an energy crisis, and
3. Use of rice as a model organism for studying plant adaptations to an energy crisis.

1.2 Energy crises

1.2.1 Causes of an energy crisis in plants

Numerous conditions can promote the development of an energy crisis in plants. These include exposure of the plant to anoxia, a deficit of substrates (carbohydrates or other carbon skeletons such as acetyl-CoA from lipids) and natural or synthetic inhibitors of the mitochondrial electron transfer chain, such as cyanide (Guy *et al*., 1989).

1.2.2 An O_2 deficit is the primary cause of an energy crisis

A deficit of O_2 is the most common environmentally induced cause of energy crisis in plants. Under normoxic conditions each mole of glucose metabolised allows production of between 24 and 36 moles of ATP (Gibbs and Greenway, 2003). Most of this ATP is produced by an H^+ gradient-driven ATP synthase in the inner membrane of the mitochondrion via the electron transport chain (Nicholls and Fergusson, 1992; Siedow and Umbach, 1995). The absence of O_2 blocks these processes (Figure 1), inhibiting oxidative phosphorylation. In the absence of O_2, the terminal electron acceptor of the mitochondrial electron transport chain cannot be re-oxidised (see Figure 1), leading to an excess of NADH in the cell and a deficit of oxidised pyridine nucleotides

to drive the further breakdown of substrates in the cytochrome chain (Krebs, 1973). Since hypoxia/anoxia prevents the oxygenic re-oxidation of NADH to NAD^+, tissues and cells subjected to this stress can survive only through re-oxidation of NADH to NAD^+ via fermentation. Energy metabolism can even be perturbed by de-coupling electron transport from ATP regeneration in the mitochondria, resulting in a decrease in the rate of ATP synthesis, energy charge and overall cellular ATP levels, causing an energy crisis in the *presence* of O_2 (Mocquot *et al*., 1981; Al-Ani *et al*., 1985; Reggiani *et al*., 1985; Gibbs and Greenway, 2003; Edwards *et al*., 2012).

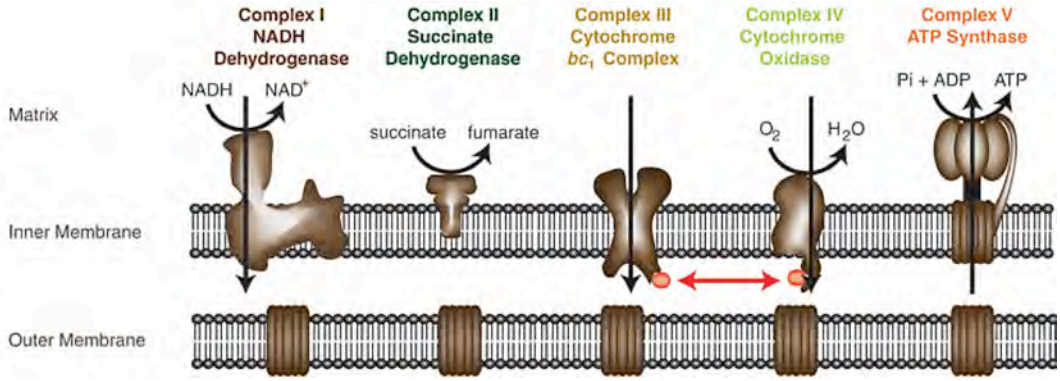


Figure 1. The molecular machinery of respiration in plant mitochondria. In normoxic conditions, O_2 acts as the terminal electron acceptor for the cytochrome c pathway (IV) allowing the efficient utilisation of pyruvate (pyr) in ATP production. In hypoxic and anoxic conditions this pathway is disrupted, severely compromising ATP regeneration (adapted from Jacoby *et al*., 2012).

Decreases in ATP synthesis rates have been observed in numerous plants and across a variety of plant tissues exposed to hypoxia or anoxia, including wheat seedlings (Waters *et al*., 1991), barley roots (Stoimenova *et al*., 2007), tobacco roots (Stoimenova *et al*., 2003), potato tubers (Rawyler *et al*., 1999), pea seeds (Raymond *et al*., 1985) and rice coleoptiles, roots and stems (Mocquot *et al*., 1981, Huang *et al*., 2005; Edwards *et al*, 2012).

Anoxia causes a decrease in the AEC of plant tissues. Raymond and Pradet (1980) observed a fall of greater than 50% in the AEC of lettuce seeds exposed to anoxia for 2 h compared to aerated controls. When Raymond *et al*. (1985) measured AEC in a wider range of species, they observed diverse responses of AEC to anoxia, with cereals

generally less affected than eudicots. Sieber and Braendle (1991) reported a decrease in the AEC of potato tubers exposed to anoxia compared to aerated controls. Anoxia also reduces absolute ATP levels within plant tissues; Kato-Noguchi and Morokuma (2007) reported a 48–74% reduction in the ATP levels of anoxia-treated rice coleoptiles.

Plant species can be classed as anoxia-intolerant or anoxia-tolerant, based on whether they are able to survive prolonged anoxia and a return to normoxic conditions without dying (Kennedy *et al.,* 1992; Gibbs and Greenway, 2003). Anoxia is a widely studied means of imposing an energy crisis because it acts directly on ATP production and is experimentally more straightforward to interpret than a shortage of substrates. Nonetheless, in evaluating the evidence available, care must be taken to ensure that the responses seen in anoxia-treated plants are due to a lack of ATP, not due to a shortage of O_2 for other reactions (such as ethylene biosynthesis by ACC oxidase) (He *et al*., 1996). Oxygen deficits can also be manifested as a shortage of energy for long-distance transport, in which case carbohydrate deficits rather than insufficient energy for biosynthesis might be observed, for example in maize root tips (Saglio, 1985).

1.2.3 Chemical means of inducing an energy crisis

As described above, a second means of inducing an energy crisis is through the use of poisons that uncouple oxidative phosphorylation from ATP synthesis in the mitochondria (see Figure 1), with such poisons including carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) (Cheeseman and Hanson, 1979; Andersson *et al*., 1987) and 2,4-dinitrophenol (DNP) (Abrams *et al*., 1990). Other compounds block the mitochondrial electron transport chain by preventing the progress of electrons through the pathway—potassium cyanide (KCN) (Bouny and Saglio, 1996; Blokhina *et al*., 2003; Yordanova *et al*., 2004) and sodium azide (NaN_3) (Rawyler *et* al., 1999; Tournaire-Roux *et al*., 2003), are two common examples. Yet more inhibitors are specific

to the non-phosphorylating alternative oxidase pathway, such as salicylhydroxamic acid (SHAM) (Vanlerberghe and McIntosh, 1997; Amor *et al*., 2000).

By uncoupling O_2 consumption from ATP production, FCCP and other compounds that act in a similar way cause an energy crisis similar to that caused by anoxia but with a paradoxically rapid consumption of O_2 (Blokhina *et al*., 2003). The *increase* in O_2 consumption is caused by removing the ADP limitation at the phosphorylation sites in mitochondria. Uncouplers have been used to study whether effects observed in anoxia are due to low ATP/NAD^+ levels or are regulated by a lack of O_2 (Cheeseman and Hanson, 1979; Andersson *et al*., 1987). A problem of the use of inhibitors is that it is difficult to remove them from tissues and thus to reverse their effects, thus precluding secondary damage to tissues. In contrast, it is possible to resupply O_2 to anoxic tissue and determine whether the tissue has survived the anoxia.

1.3 Plant cells during an O_2 deficit

This section discusses the general effects of an O_2 deficit on plants and several adaptations plants have made to survive energy crises. Much of the work cited below has been conducted with plant tissues exposed to hypoxia or anoxia. Metabolic changes, therefore, may not be strictly due to a lack of energy but may be attributable to a lack of O_2.

1.3.1 Plants regulate and maintain several key metabolic processes during an O_2 deficit

Atkinson (1969) speculated that a fall in ATP generation would be matched by a commensurate fall in energy utilisation. During an O_2 deficit, the low levels of ATP synthesis force plants to regulate strictly many of these processes. This regulation includes the continued production of ATP via substrate phosphorylation during glycolysis, a sharp reduction in protein production (Mocquot *et al*., 1981), regulation of

cellular pH (Sze *et al*., 1992) and the maintenance of cell membranes (Rawyler *et al*., 1999, 2002). These regulatory changes allow an optimisation of ATP turnover and increase the chance that tissues will survive the energy crisis.

1.3.2 ATP production continues via glycolysis and the fermentation pathways

During an anoxic energy crisis, ATP production continues by the glycolytic and fermentation pathways (Waters *et al*., 1991; Kennedy *et al*., 1992; Gibbs *et al*., 2000; Huang *et al*. 2008). During anoxia, three fermentative pathways consume pyruvate, resulting in the formation of ethanol, lactic acid and alanine (Waters *et al*., 1991; Kennedy *et al*., 1992; Ricard *et al*., 1994; Kato-Noguchi, 2001; Igamberdiev and Hill, 2004). The three pathways each consume different amounts of pyruvate: ethanol fermentation is dominant, consuming ~90% of the pyruvate produced by glycolysis, while together lactate and alanine fermentation consume ~10% (Smith and ap Rees, 1979; Ricard *et al.*, 1994; Gibbs *et al*., 2000). Both lactate and alanine present problems for plant tissues; lactate because it is a weak acid and alanine because it consumes nitrogen and yields no net energy.

In addition to consuming pyruvate, the ethanol and lactate fermentation pathways re-oxidise NADH to NAD^+, allowing glycolysis to continue (Drew, 1997). The accumulation of lactic acid, however, acidifies the cytoplasm of anoxic cells. Therefore ethanol fermentation, which has little or no negative effect on the cell, is favoured in the long term (Davies *et al*., 1980, Tadege *et al*., 1999).

Glycolysis and ethanol fermentation theoretically produce 2–3 moles of ATP per mole of glucose consumed, far less than the 24–36 moles of ATP per mole of glucose produced by oxidative phosphorylation (Gibbs and Greenway, 2003). However, a common adaptation to the lack of O_2 is an increase in the rate of carbohydrate

breakdown by glycolysis in anoxic tissue, commonly referred to as the 'Pasteur Effect' (Barker *et al*., 1964).

The Pasteur Effect has been reported in many plant tissues exposed to anoxia, including carrot cells (Faiz-ur-Rahman *et al*., 1974), beetroot storage cells (Zhang and Greenway, 1994), maize root tips (Neal and Girton, 1955) and rice shoots and coleoptiles (Menegus *et al*., 1991, Gibbs *et al*., 2000). For anaerobic glycolysis to sustain a rate of ATP synthesis equivalent to aerobic respiration would require consumption of eight times more glucose than in aerobic conditions (assuming three moles of ATP per mole of glucose in anoxia and 24 moles of ATP per mole of glucose in normoxia). The observed increases in glycolysis, however, can supply at best 35% that of the minimum rate of aerobic ATP synthesis (Greenway and Gibbs, 2003). Thus, even under ideal responses to anoxic conditions, increased rates of glycolysis are not able to compensate for the lack of ATP. This means that plants that have tolerance to low O_2 conditions are likely to have restrained ATP usage to allow ATP synthesis and utilisation to become optimal and improve survival in the anoxic environment.

1.3.3 Biosynthetic pathways are tightly regulated during an O_2 deficit

Biosynthetic processes that utilise ATP are a major energy sink and might therefore be expected to be tightly regulated during the hypoxic/anoxic energy crisis. Most of the normal metabolic and catabolic processes in plants consume ATP: protein synthesis requires 5–8 molecules of ATP per peptide bond formed (Amthor, 2000), DNA consumes ~38 molecules of ATP per DNA nucleoside added during DNA synthesis (Penning de Vries, 1974), lipids consume ~33 moles of ATP per mole of lipid (palmitate, 16:0) produced (Hespell and Bryant, 1979) and maintenance of cell turgor requires 1-3 moles of ATP per mole of osmoticum imported (Yeo, 1983).

Geigenberger (2003) reported a 7-fold decrease in total protein synthesis in potato tubers exposed to anoxia compared to normoxia. Chang *et al*. (2000) reported a

5-fold decrease in total protein synthesis in maize roots exposed to hypoxia compared to normoxic controls. Mocquot *et al* (1981) reported a 40% and 70% decrease in protein synthesis rates of anoxic rice seedlings and coleoptiles, respectively, compared to normoxic coleoptiles while Aspart *et al*. (1983) reported that mRNA synthesis in anoxic rice embryos stabilised at ~50% of its normoxic rate. Edwards *et* al. (2012) (Chapter 2) used a global energy analysis to demonstrate a 50–60% reduction in the protein synthesis rates in hypoxic and anoxic rice coleoptiles compared to normoxic controls. By down-regulating total protein synthesis in absolute terms, but not relative to other polymers, these seedlings can significantly reduce the demand for ATP under anoxia and enhancing survival.

Not all protein synthesis is halted under anoxia; approximately 20 'anaerobic proteins' are synthesised in increased amounts. The anaerobic proteins were first identified in maize where they account for more than 70% of translational activity in anoxia root tips (Sachs *et al*., 1980, 1996) and include proteins involved in the fermentation pathways (alcohol dehydrogenase (ADH), pyruvate decarboxylase (PDC), lactate dehydrogenase (LDH), alanine aminotransferase (ALT)) and glycolysis (sucrose synthase (SuSY), adolase (specifically fructose-bisphosphate aldolase, enolase))—see Gibbs and Greenway, 2003, Section 6.3 for a review. Chang *et al*. (2000) utilised ^{35}S-methionine labelling of maize root tips to detect newly synthesised proteins during anoxic and hypoxic conditions. These proteins included ADH1, PDC2, enolases, malate dehydrogenases (MDH) and aminotransferases. Similarly, Huang *et al*. (2005) utilised ^{35}S-methionine labelling of anoxic rice coleoptiles to study protein synthesis. They identified a number of newly synthesised proteins including ADH1, ADH2, aldolase, pyruvate orthophosphate dikinase (PPDK) and Mn-superoxide dismutase (Mn-SOD).

The observed changes in protein synthesis under low O_2 supply are due to a combination of transcriptional (Branco-Price *et al*., 2005; Liu *et al*., 2005; Lasanthi-

Kudahettige *et al*., 2007; Narsai *et al.*, 2009) and translational (Branco-Price *et al*., 2005; Howell *et al*., 2009; Narsai *et al*., 2009) regulation. Liu *et al*. (2005) examined transcriptional changes in whole hypoxic *Arabidopsis thaliana* seedlings and observed a greater than 2-fold increase in the transcript levels of the classic ANPs during hypoxia compared to normoxia. These included genes involved in glycolysis (*ADH, PDC*), sucrose synthases, bHLH transcription factors, adolase, enolase and aminotransferases. Lasanthi-Kudahettige *et al*. (2007) reported a similar increase in the transcript levels of a variety of genes in anoxic rice coleoptiles including glycolytic genes (*ADH1*, *ADH2*, *PDC1*, *PDC2*), genes encoding for expansins (*EXPA7* and *EXPB12*) and a variety of ethylene response factors. Both studies also reported an increase in the transcript level of genes encoding 'non-anaerobic proteins' whose metabolic activity has not been shown in hypoxic/anoxic tissues (Liu *et al*., 2005; Lasanthi-Kudahettige *et al*., 2007).

Branco-Price *et al*. (2005) reported that the majority of genes transcribed in *Arabidopsis* continue to be translated at lower levels in hypoxia compared to normoxia. In a follow-up study, Branco-Price *et al.* (2008) studied the changes in the whole and polysomal mRNA transcripts (i.e., associated with multiple ribosomes) of hypoxic *Arabidopsis* seedlings compared to normoxic controls. They identified that while 533 genes were up-regulated by exposure to hypoxia for 2 h, only 203 of these genes showed a similar increase in the ribosome-associated mRNA. They also found that only 435 of the 1444 genes up-regulated by nine hours of hypoxia had increased levels of polysomal mRNA (Branco-Price *et al*., 2008). Upon the return to normoxia, many genes whose translation was severely down-regulated under anoxia showed an increased rate of translation (Branco-Price *et al*., 2008), providing persuasive evidence that they were O_2-responsive.

These results taken together suggest that, under anoxia, plants reduce total protein synthesis in order to minimise ATP usage, while key proteins are still translated.

In addition, the transcript levels of genes required for the return to normoxia are increased, allowing their rapid translation upon re-oxygenation.

1.3.4 Cellular pH and membrane polarisation are perturbed during O_2 deficits

Maintenance of stable cellular pH and the 'strong ion' gradient across membranes is critical to maintain membrane charge and cell homeostasis (Felle, 2003). Within plant cells, cytoplasmic pH is maintained by the continuous action of tonoplast-bound H^+-ATPases continually transporting protons from the cytosol into the vacuole of the cell (Sze, 1985; Sze *et al*., 1992). The active pumping of H^+ from the cytosol to the vacuole aids in the maintenance of a near-neutral cytoplasmic pH. Additionally, the vacuole acts as a 'storage compartment' for a wide range of chemical ions and metabolites (particularly undissociated organic acids, ions, carbohydrates and amino acids) within the plant, with ATP-driven exchange of these playing a vital role in metabolic homeostasis (Wink, 1993).

An O_2 deficit, with its concurrent decrease in ATP synthesis, slows the functioning of these H^+-ATPases and decreases (acidifies) cytosolic pH. Cytosolic pH decreases rapidly (within minutes) by 1–2 pH units in potato (Rawyler *et al*., 1999), maize (Saint-Ges *et al*., 1991; Fox *et al*., 1995), rice (Menegus *et al*., 1991; Kulichikhin *et al*., 2009) and wheat (Menegus *et al*., 1991) tissues placed under hypoxic and/or anoxic conditions. In anoxia-intolerant plant tissues (e.g. wheat roots) cytoplasmic pH continues to drop until cell and tissue death occurs (Menegus *et al*., 1991; Greenway and Gibbs, 2003). In relatively anoxia-tolerant plant tissues (maize root tips, rice coleoptiles) the initial decrease in cytoplasmic pH is followed by an increase in cytoplasmic pH to a level slightly (0.5–1 pH units) below that of normoxic levels (Menegus *et al*., 1991; Xia and Saglio, 1992; Xia and Roberts; 1996; Greenway and Gibbs, 2003; Kulichikhin *et al*., 2009). This is what Felle (2005) termed the cytosolic pH 'set point'.

An O_2 deficit also perturbs vacuolar pH. Menegus *et al*. (1991) observed an increase of 0.2–0.4 pH units (alkalisation) in the vacuole of anoxic rice shoots. Kulichikhin *et al*. (2009) reported a rapid decrease of ~0.4 pH units in the vacuolar pH of anoxic rice after re-aeration. Such an alkalisation would aid in minimising or preventing the acidification of the cytoplasmic pH in anoxia compared to normoxia (Xia and Saglio, 1992). In comparison, there was no such alkalisation in the vacuole of anoxic wheat shoots, contributing to the continuing acidification of the cytoplasm (Menegus *et al*., 1991).

Irreversible acidification of the cytosol has been observed in numerous anoxia-intolerant species. Wheat shoots exposed to anoxia for more than 6 h show an irreversible acidification (pH 7.2 decreasing to pH 6.6) of the cytosol (Menegus *et al*., 1991). Xia and Roberts (1994) reported a similar acidification of the cytoplasm (pH 7.4 to pH 6.4) after 4 h of anoxia treatment. Xia and Roberts (1996) reported that lower external pH values (induced by weak acids) decreased the tolerance to anoxia of maize roots. Waters *et al*. (1991a, 1991b) found that anoxic wheat roots fared poorly at low external pH (4.0 *vs* 6.0) and showed a decreased rate of elongation.

The lowering of the cytoplasmic pH set-point from ~pH 7.5 to pH 7 to pH 6.5 in anoxia-tolerant species is thought to be an acclimative response to an O_2 deficit (Felle, 2005). Davies *et al*. (1974) found that the production of lactic acid by pea cells in anoxic conditions triggers a decrease in cytosolic pH and increases the enzymatic activity of PDC, thus transforming the tissue from lactate to ethanol fermentation. Similarly, a decrease in cytosolic pH decreases the acid load imposed on the cytoplasm by the vacuole and decreases the amount of ATP consumed by H^+-ATPases to maintain cytoplasmic pH.

Davies (1980) and Felle (2005) proposed that the decreased cytoplasmic pH set-point acts as a signal for low cellular energy levels, activating the processes necessary

for the adaptation to these low energy levels. Davies (1986) developed a general theory of pH-stat, in which H^+ exchange with carboxyl groups of various organic acids were purported to stabilise the pH of intracellular compartments. Accordingly, when Fox *et al*. (1995) halted the anoxic acidification of the cytoplasm of maize tips by the addition of methylamine, a lower rate of ethanol production than in the anoxic controls was observed.

1.3.5 Maintenance of cell membrane integrity and selectivity is essential for survival

Maintenance of cell membrane integrity and selectivity is essential for cellular survival during an energy crisis, and involves the maintenance of the membrane lipids and the transmembrane potential. Prolonged anoxia in many anoxia-intolerant species is associated with a loss of membrane integrity and selectivity. Loss of membrane integrity and selectivity were observed in wheat shoots (Menegus *et al.*, 1991) and roots (Greenway *et al*., 1992), barley roots (Hiatt and Lowe, 1967) and aged beetroots (Zhang *et al*., 1992) exposed to prolonged hypoxia or anoxia (Thomson *et al*., 1989).

Anoxia impacts the ability of plant tissues to maintain their lipid membranes. Rawyler *et al*. (1999) studied changes in membrane composition of potato cells exposed to anoxia and found an increase of the proportion of 'damaged' membranes (free fatty acids - FFA) correlated with a loss of membrane selectivity and the leakage of cell cytoplasm. A similar change was observed in wheat seedlings exposed to prolonged anoxia (Chirkova *et al*., 1989 cited by Blokhina *et al*., 2001). Rawyler *et al*. (2002) concluded that this increase in free fatty acids was tied to ATP synthesis rates: when ATP synthesis falls below a critical level, hydrolysis of lipids to free fatty acids disrupts membrane fluidity and leads to a loss of membrane integrity and selectivity.

Anoxia decreases the transmembrane potential of plant tissues (Gibbs and Greenway, 2003). Anoxic maize roots have a lower membrane potential than normoxic

controls (-100 mV *vs* -160 mV, respectively) (Cheeseman *et al*., 1980). Anoxia also decreased the membrane potential of potato cells compared to normoxic controls (-40 mV compared to -120 mV, respectively) (Katou *et al*., 1982; Kojima *et al*., 1985). In rice coleoptiles membrane potentials fell upon exposure to anoxia (from -130/-120 (control) mV to -98/-78 (anoxia) mV) but repolarised to normal levels within an hour (Zhang and Greenway, 1995). The decrease in the transmembrane potential disrupts the transport of ions, particularly K^+ and Na^+ (Cheeseman *et al*., 1980; Drew *et al*., 1988), into the cell, further disrupting the normal functioning of the tissue.

The precise means by which cells of anoxia-tolerant tissues maintain membrane integrity during an O_2 deficit are not well understood. As with other major metabolic pools, turnover of lipids requires metabolic energy and thus membrane function would be susceptible to energy crises. *De novo* synthesis of saturated lipids has been reported in anoxia-tolerant species such as *Echinochloa phyllopogon* (Kennedy *et al*., 1992) and *Iris pseudacorus* (Hetherington *et al*., 1982); however, synthesis of unsaturated lipids is prevented by the lack of O_2 (Vartapetian *et al*., 1978; Brown and Beevers, 1987). Further study in this area is required, in particular focussed on synthesis and recycling of lipids, changes in the membrane composition and electrolyte leakage in anoxia-tolerant species and tissues.

1.3.6 Causes of plant death during an O_2 deficit

Plant death during an O_2 deficit in anoxia-intolerant species is due to a loss of membrane integrity and selectivity, and coincides with a steady decline in cytoplasmic pH during prolonged anoxia.

A prolonged O_2 deficit results in an increase in the amount of FFA in cellular membranes (Chirkova *et al*., 1989 cited by Blokhina *et al*., 2001; Rawyler *et al*., 1999) and, consequently, a decrease in membrane fluidity and integrity (Rébeillé *et al*., 1980; Rawyler *et al*., 2002). The concurrent ATP shortage also results in a disruption of the

transmembrane potential in anoxia-intolerant species (Katou *et al*., 1982; Kojima *et al*., 1985) by disabling the membrane-bound H^+-ATPases in spite of pyrophosphate-driven transporters playing a potential mitigating role (Liu *et al*. 2010). This leads to a loss of the ability to maintain the ionic balance of the tissue (Cheeseman *et al*., 1980; Drew *et al*., 1988). This ionic disruption, combined with leaky membranes (Rawyler *et al*., 1999) and irreversible acidification of the cytoplasm (Menegus *et al*., 1991; Waters *et al*., 1991a, 1991b; Xia and Saglio, 1992; Xia and Roberts; 1996) lead to an inability to maintain intracellular potassium ion concentrations (Greenway and Gibbs, 2003) and eventual death of the plant.

In anoxia-tolerant plants, carbohydrate starvation is a likely cause of death during an O_2 deficit. Anoxia-tolerant tissues such as rice coleoptiles (Atwell *et al*., 1982; Setter *et al*., 1994a, 1994b; Gibbs *et al*., 2000) and roots (Webb and Armstrong, 1983) supplied with sufficient carbohydrates were able to survive a prolonged O_2 deficit, while a shortage of carbohydrate supply resulted in rapid tissue death (Setter *et al*., 1997). The ability of mature rice plants to survive a prolonged O_2 deficit (normally induced by flooding) is also dependent upon maintaining a sufficient carbohydrate supply to either elongate to break the surface of the water (Kende *et al*., 1998) or to enter a quiescent state until the flood passes (Settler *et al*., 1997; Das *et al*., 2005). In either case, carbohydrate status needs to be maintained (Webb and Armstrong, 1983).

1.4 Using rice coleoptiles as a model tissue for studying cell energetics during an O_2 deficit

1.4.1 The response of germinating rice to an O_2 deficit

Rice, uniquely amongst the major crop cereals of the world, has the ability to survive prolonged anoxia during germination, establishment and early growth (Taylor, 1942; Atwell *et al*., 1982). This is accomplished by a series of changes in rice seedling

morphology in response to O_2 availability (Figure 2). During germination in normoxic conditions, roots and coleoptiles emerge almost simultaneously. Shoots then emerge from the protective sheath of the coleoptile (Alpi and Beevers, 1983). During hypoxia only the coleoptile emerges from the seed and elongates rapidly to reach the surface of the water (Kordan, 1974), whilst in anoxia, coleoptile elongation proceeds slowly, acting as a 'snorkel' for O_2 transport from air to the developing seedling if the water surface is breached (Kordan, 1974; Jackson, 1985). Re-oxygenation allows normal seedling development to ensue (Alpi and Beevers, 1983; Perata and Alpi, 1993).

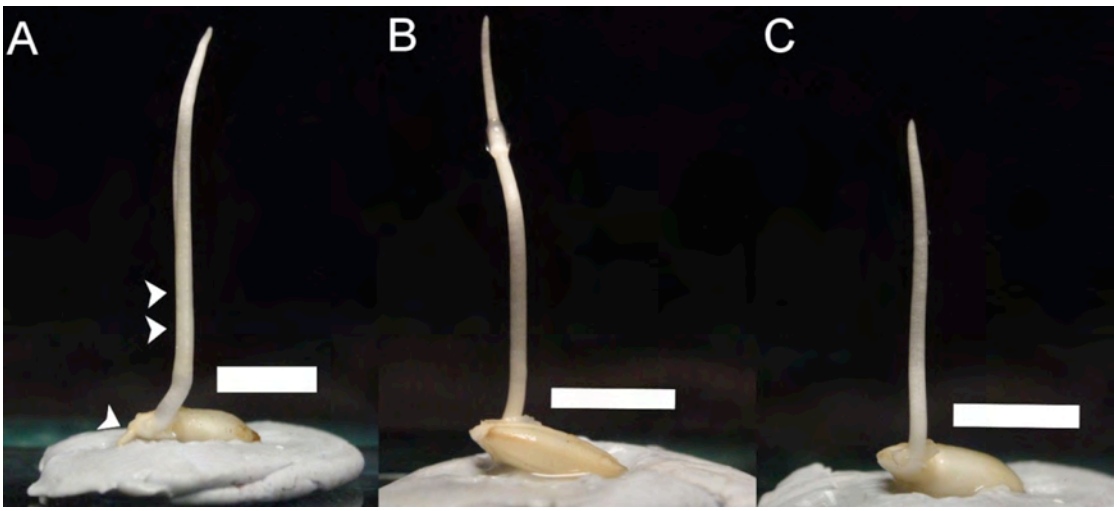


Figure 2. Three-day-old normoxic (A), hypoxic (B) and anoxic (C) coleoptiles. In the normoxic coleoptile, a radicle has emerged (single white arrow), defining the moment of germination, with elongation of the primary leaf to follow (double white arrow). In hypoxic and anoxic coleoptiles, the root and shoot fail to emerge while the coleoptile elongates, potentially reaching the water surface and acting as a 'snorkel' by providing O_2 to the developing seedling. White bars represent 5 mm.

The ability of the rice coleoptiles to grow during various levels of O_2 restriction, combined with their rapid elongation, and the ability to easily manipulate growth conditions makes them ideal for studying the effects of prolonged anoxia (causing an energy crisis).

1.4.2 Rice coleoptiles survive O_2 deficits by induction of adaptive genes

The growth of rice coleoptiles during O_2 deficits is based on a number of cellular responses that occur during prolonged hypoxia and anoxia. These include the synthesis

of many proteins additional to the anaerobically induced proteins that were identified more than 30 years ago (Sachs *et al*., 1980), stable cytosolic pH and retention of potassium ions based on membrane transport properties and stabilisation of membrane charge, ion selectivity during anoxia and a possible transition towards the use of PPi as an alternative energy currency in the place of ATP (Huang *et al*., 2008; Liu *et al*., 2010).

During an O_2 deficit, rice coleoptiles synthesise a wide range of proteins (Huang *et al*., 2005; Sadiq *et al*., 2011). In addition to the glycolytic anaerobic proteins identified by Sachs *et al*. (1980, 1996), anoxic rice coleoptiles synthesise additional proteins that play a putative role in energy conservation. The most extensive studies of the anoxic rice coleoptile proteome conducted to date were performed by Huang *et al*. (2005) and Sadiq *et al*. (2011). Huang *et al.* (2005) utilised ^{35}S-labelled methionine to examine the *de novo* synthesis of proteins in rice coleoptiles exposed to anoxia. They were able to identify 13 proteins differentially expressed between anoxic and normoxic rice coleoptile tips, including superoxide dismutase (SOD), a nucleoside diphosphate kinase (NDPK) and both ADH1 and ADH2. Sadiq *et al*. (2011) identified 33 proteins differentially expressed between normoxic and anoxic coleoptiles, including a number of heat-response, stress-response and ribosomal-component proteins.

Mocquot *et al*. (1981) and Huang *et al*. (2005) demonstrated that rice coleoptiles continued *de novo* protein synthesis during prolonged anoxia. In contrast, Kennedy *et al*. (1992) reported that many plant species rapidly cease protein synthesis on exposure to anoxia. This maintenance of relatively high levels of protein synthesis within rice compared to other species is indicative of its comparative tolerance to anoxia.

The wide range of changes in the proteome in anoxic rice coleoptiles is supported by similarly large changes in their transcriptome. In microarray experiments conducted by Lasanthi-Kudahettige *et al*. (2007), 3134 probe sets were identified that showed changed levels of expression (1364 increased, 1770 decreased) in anoxia compared to

normoxia. These included probe sets representative of genes key in the processes of glycolysis (PPi-PFK, adolase, hexokinase 7), fermentation (*PDC*, *ADH*), cell wall expansion (expansins A7 and B12), carbohydrate metabolism (sucrose synthase, amylase 3D) and a wide variety of ERF-like transcription factors including members of the bHLH and AP2 families.

1.4.3 Membrane physiology and integrity

Rice coleoptiles also have an enhanced ability to maintain a steady cytosolic pH and intact lipid membranes during prolonged anoxia. Kulichikhin *et al*. (2009) exposed excised rice coleoptiles to prolonged anoxia at pH 3.5 and found that, provided the coleoptiles were provided with 50 mM glucose, cells were able to maintain a cytosolic pH of 7.2. Furthermore, the coleoptile cells maintained a greatly lowered vacuolar pH (5.2) for periods of up to 90 h. This, combined with the findings of: (i) Huang *et al*. (2003) that anoxic rice coleoptiles are able to take up K^+ from their external media; (ii) Zhang and Greenway (1995) that anoxic rice coleoptiles are able to maintain their membrane potentials through a prolonged anoxia and (iii) Vartapetian *et al*. (1978) that anoxic rice coleoptiles produce saturated lipids during anoxia, point to the maintenance of cytosolic pH and cell membrane integrity, selectivity and potential as being key adaptations to the growth of rice coleoptiles during anoxia. This is despite the decrease in available ATP for these processes in anoxia.

Another possible plant adaptation to anoxia involves the utilisation of PPi as an alternative energy currency to alleviate the anoxic ATP deficit. Huang *et al*. (2008) suggested that PPi, produced by a variety of cellular processes including protein synthesis, cell wall synthesis and nucleic acid synthesis (Maeshima, 2000), would decrease energy stress in anoxic rice coleoptiles by relieving demand on the ATP pool. A series of ATP-dependent enzymes can be functionally substituted by PPi-driven analogues during anoxia (Plaxton 1996; Huang *et al.,* 2005; Lasanthi-Kudahettige *et al*.,

2007; Liu *et al*., 2010). Igamberdiev and Kleczkowski (2011) reported that at pH 7.2—the lower end of the cytosolic pH range in anoxic rice coleoptiles (Kulichikhin *et al*., 2009)—the ΔG^0 of PPi is about 60% of that of ATP. Collectively, these findings suggest that there could be an adaptive response of anoxic rice coleoptiles towards an increased utilisation of PPi as a substitute for ATP for a variety of processes. This would allow the preferential utilisation of PPi in processes where there are parallel reactions using PPi instead of ATP, decreasing the pressure upon the availability of ATP (Huang *et al*., 2008). Reactions that can use PPi as an energy source are going to be particular interest in tissues such as rice coleoptiles, which grow at substantial rates in anoxia and thus have a source of PPi from biosynthetic reactions.

1.5 Research aims and objectives

Given the agricultural and economic importance of rice as a crop, my research was based on three key aims:

1. To examine and compare the response of the coleoptiles of a range of *O. sativa* genotypes to growth to hypoxic and anoxic environments;
2. To determine how the metabolism of rice coleoptiles grown in hypoxic/anoxic environments differs from those grown in a normoxic environment and;
3. To determine if and how differences in the transcriptome and proteome of hypoxic/anoxic coleoptiles (*cf*. normoxic coleoptiles) support the observed phenotypic differences.

Based on these aims, previous research and my own observations, I tested three main hypotheses during my research were:

1. That unique phenotypical response of rice coleoptiles to hypoxia/anoxia, the Snorkel Effect, would be supported by the prioritization of ATP to the metabolic processes need to support this rapid elongation (*cf.* normoxia);

2. That genes and proteins involved in the key processes identified during the examination of hypothesis 1 would be up-regulated in the the transcriptome and proteome of hypoxic/anoxic coleoptiles (*cf*., normoxic coleoptiles) and;
3. That there would be conserved differences in the transcriptome and proteome in the tips and bases of coleoptiles, regardless of O_2 supply.

1.6 Research methodologies and concluding remarks

Rice coleoptiles present a remarkable system for studying the adaptation of plant tissue to a prolonged O_2 deficit, especially with respect to bioenergetics. The relative ease of growing the tissue and exposing it to a variety of O_2 conditions (Atwell *et al.,* 1982) combined with the existence of a wide variety of cultivars with varying degrees of anoxia tolerance at germination (Setter *et al*., 1994a,b; Yamauchi and Biswas, 1997, Magneschi *et al*., 2009) make the rice coleoptile a unique system for studying the adaptation of bio-energetics of O_2-deficient plant tissues.

This thesis reports three independent approaches to studying the bioenergetics of anoxic rice coleoptiles. Primarily utilising the 'Amaroo' cultivar, studies were performed on the bio-energetic, genomic and proteomic basis of tolerance to O_2 deprivation in rice coleoptiles.

'Amaroo' is a cultivar of *O. sativa ssp. japonica* commonly grown commercially in Australia. It is a medium-grain rice derived from Californian germplasm and has a noted tolerance for submergence, exhibiting rapid coleoptile elongation when exposed to anoxia (Huang *et al.*, 2003, Millar *et al*., 2004; Liu *et al*., 2010).

Chapter 2 (Edwards *et al*., 2012) focuses on direct measurements of the rates of ATP generation (via respiration and/or fermentation) and utilisation (by metabolic processes) by normoxic, hypoxic and anoxic rice coleoptiles across a range of anoxia-tolerant and -intolerant cultivars. Budgets of ATP generation and utilisation (by the

major metabolic processes) for normoxic, hypoxic and anoxic coleoptiles of the cultivar Amaroo are presented and the implications for anoxia tolerance in plants are discussed.

Chapter 3 reports a study of changes in the transcriptome of hypoxic and anoxic rice cv. Amaroo coleoptiles compared to a normoxic control. Additionally, it examines differences between the basal (rapidly dividing and expanding) and apical (not rapidly dividing and expanding) tissues (Wada 1961; Furuya *et al*., 1969). Previous studies (Lasanthi-Kudahettige *et al*., 2007) have focussed solely on the intact coleoptile. Moreover, Lasanthi-Kudahettige *et al*. (2007) did not test the effect of severe hypoxia on rice coleoptiles, focusing solely on a normoxia/anoxia comparison. By examining differences between the basal and apical regions of coleoptiles across a variety of O_2 concentrations, as well as comparing differences in the transcriptome between basal and apical tissues across O_2 treatments, the present study was able to identify key adaptive genes. Specifically, genes associated with rapid elongation, particularly under hypoxia, could be identified. These genes may play an important role in regulating the survival of rice coleoptiles during an O_2 deficit.

Chapter 4 mirrors the work reported in Chapter 3 at the protein level. Huang *et al*. (2005) utilised ^{35}S-methonine to examine the *de novo* synthesis of protein in anoxic rice coleoptiles. While this technique allowed the quantification of proteins undergoing active synthesis during anoxia, like all previous studies, it was conducted on whole coleoptiles. Nor did it test the potential effects of hypoxia on the rice proteome. We chose to utilise 2D-DIGE (Differential In-Gel Electrophoresis) (Tonge *et al*., 2001) to examine the changes in the proteome of normoxic, hypoxic and anoxic rice coleoptile bases and tips.

Within growing rice coleoptiles, there is a distinct difference in the patterns of cell division and cell elongation between the basal (base) and distal (tips) regions (Wada, 1961; Furuya *et al*, 1969). In addition to examining the genomic and proteomic

basis for the bioenergetic differences between O_2 treatments reported in Chapter 2, Chapters 3 & 4 also report on the novel differences in gene and protein expression between the base and tips regions of normoxic, hypoxic and anoxic rice coleoptiles.

In conclusion, the rice coleoptile presents an excellent model for studying the bioenergetics of plant tissues during an O_2 deficit. Experiments with this model can contribute substantially to our understanding of how plants respond to an energy crisis.

2 Chapter 2 – ATP turnover in O_2-deprived coleoptiles

2.1 Introduction

The contents of this chapter were published in 2012 in the *Journal of Experimental Botany* (**63**(12): pp. 4389-4402) as a paper entitled: '*Quantifying ATP turnover in anoxic coleoptiles of rice (*Oryza sativa*) demonstrates preferential allocation of energy to protein synthesis*.'

This paper combined estimates of ATP production (derived by measuring rates of O_2 consumption and ethanolic fermentation) with estimates of ATP utilisation by key metabolic processes (rates determined using radio-isotope tracers and theoretical anabolic costs) to develop an ATP 'budget' for normoxic, hypoxic and anoxic rice coleoptiles. This is highly analogous to an economic budget wherein the inputs and outputs must match. It also examined rates of protein synthesis among a wide range of genetically contrasting cultivars, some highly adapted to anoxia and others extremely sensitive. The key findings of the paper are described below.

2.2 Key findings

Edwards *et al*. (2012) examined the production and utilisation of ATP by rice coleoptiles exposed to various O_2 availabilities. The key findings are:

- During steady-state hypoxia and anoxia (O_2 deprivation) the rates of major metabolic (protein, lipid, nucleic acid and cell wall synthesis) and transport processes (carbohydrate and nitrates) of rice coleoptiles are decreased compared to normoxic coleoptiles.
- The proportional of available ATP dedicated to these processes varies as O_2 availability decreases.
- In hypoxic coleoptiles compared to normoxic, absolute rates of cell wall and lipid synthesis do not decrease while protein synthesis is halved. In anoxic

coleoptiles, protein synthesis consumed the largest proportion of available ATP, indicating it was a critical process when an energy crisis took hold.

- In a comparison of seven rice cultivars with a range of tolerances to O_2 deprivation, the coleoptiles of tolerant cultivars showed high absolute rates of protein synthesis during hypoxia/anoxia, whilst those of intolerant cultivars showed significantly lower rates of protein synthesis in these conditions. However, the *proportion* of ATP expended in protein synthesis remained constant across all genotypes.

Note: We have identified a minor error in the Edwards et al. (2012) paper as reprinted below: Footnotes 'a' and 'c' in Table 7 should include the text 'ATP produced **minus** consumed' not 'ATP produced/consumed'.

2.3 Acknowledgements

All experimental work described in this paper was performed by myself. The paper was written by myself with Associate Professor Brian Atwell and Dr Thomas Roberts reviewed the drafts and providing suggestions and corrections for improvement.

2.4 Peer-reviewed publication

Journal of Experimental Botany, Vol. 63, No. 12, pp. 4389–4402, 2012
doi:10.1093/jxb/ers114 Advance Access publication 13 May, 2012
This paper is available online free of all access charges (see http://jxb.oxfordjournals.org/open_access.html for further details)

RESEARCH PAPER

Quantifying ATP turnover in anoxic coleoptiles of rice (*Oryza sativa*) demonstrates preferential allocation of energy to protein synthesis

Joshua M. Edwards[1], Thomas H. Roberts[2] and Brian J. Atwell[1,*]

[1] Department of Biological Sciences, Macquarie University, North Ryde, NSW 2109, Australia
[2] Department of Plant and Food Sciences, Faculty of Agriculture and Environment, University of Sydney, NSW 2006, Australia

* To whom correspondence should be addressed. E-mail: brian.atwell@mq.edu.au

Received 13 December 2011; Revised 22 March 2012; Accepted 23 March 2012

Downloaded from http://jxb.oxfordjournals.org/ by guest on July 22, 2013

Abstract

Oxygen deprivation limits the energy available for cellular processes and yet no comprehensive ATP budget has been reported for any plant species under O_2 deprivation, including *Oryza sativa*. Using 3-d-old coleoptiles of a cultivar of *O. sativa* tolerant to flooding at germination, (i) rates of ATP regeneration in coleoptiles grown under normoxia (aerated solution), hypoxia (3% O_2), and anoxia (N_2) and (ii) rates of synthesis of proteins, lipids, nucleic acids, and cell walls, as well as K^+ transport, were determined. Based on published bioenergetics data, the cost of synthesizing each class of polymer and the proportion of available ATP allocated to each process were then compared. Protein synthesis consumed the largest proportion of ATP synthesized under all three oxygen regimes, with the proportion of ATP allocated to protein synthesis in anoxia (52%) more than double that in normoxic coleoptiles (19%). Energy allocation to cell wall synthesis was undiminished in hypoxia, consistent with preferential elongation typical of submerged coleoptiles. Lipid synthesis was also conserved strongly in O_2 deficits, suggesting that membrane integrity was maintained under anoxia, thus allowing K^+ to be retained within coleoptile cells. Rates of protein synthesis in coleoptiles from rice cultivars with contrasting tolerance to oxygen deficits (including mutants deficient in fermentative enzymes) confirmed that synthesis and turnover of proteins always accounted for most of the ATP consumed under anoxia. It is concluded that successful establishment of rice seedlings under water is largely due to the capacity of coleoptiles to allocate energy to vital processes, particularly protein synthesis.

Key words: Anoxia, ATP utilization, hypoxia, *Oryza sativa*, rice.

Introduction

Rice (*Orzya sativa* L.) is a key food crop, providing a dietary staple for more than two billion people (FAO, 2004). Uniquely amongst major food crops, some rice cultivars have an extraordinary tolerance to anoxia at germination (Atwell *et al.*, 1982; Kawano *et al.*, 2002; Magneschi and Perata, 2009) and an ability to survive various periods of submergence up to maturity (Gibbs and Greenway, 2003; Xu *et al.*, 2006).

There are marked changes in rice seedling morphology in response to oxygen deprivation. During germination in normoxic conditions, roots and coleoptiles emerge almost simultaneously. Leaves then emerge from the protective sheath of the coleoptile (Alpi and Beevers, 1983). Under severely hypoxic conditions only coleoptiles emerge from seeds, elongating rapidly to reach the surface of the water (Kordan, 1974) while, in anoxia, preferential coleoptile elongation proceeds slowly, acting as a 'snorkel' for O_2 transport from air to a basal meristem if the water surface is breached (Kordan, 1974; Jackson, 1985). Re-oxygenation allows normal seedling development to ensue (Alpi and Beevers, 1983; Perata and Alpi, 1993).

The developmental differences described above are accompanied by biochemical and physiological phenomena that confer variation in anoxia tolerance between rice cultivars during germination (Atwell *et al.*, 1982; Setter *et al.*, 1994,

© 2012 The Author(s).
This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/3.0), which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

1997). These responses include the synthesis of the classic anaerobic response proteins (Sachs *et al.*, 1980; Mujer *et al.*, 1993; Matsumura *et al.*, 1998), higher rates of glycolysis in anoxia than in normoxia (the 'Pasteur Effect') (Crawford, 1977; Gibbs *et al.*, 2000; Gibbs and Greenway, 2003), and the high activity of fermentative enzymes (Gibbs *et al.*, 2000; Saika *et al.*, 2006). Despite these responses, ATP synthesis rates are at least 3-fold lower in anoxia compared with normoxia (reviewed by Gibbs and Greenway, 2003). Atkinson (1968) proposed a general hypothesis that lowering energy status in cells (e.g. in anoxia: Raymond *et al.*, 1985) should elicit two broad complementary responses in order to stabilize energy charge. First, ATP-regenerating pathways such as glycolysis become de-repressed to maximize energy production while ATP-utilizing pathways should decline, thereby conserving ATP. Such ATP-conserving events have been reported in protein synthesis (Mocquot *et al.*, 1981; Ishizawa *et al.*, 1999; Chang *et al.*, 2000; Geigenburger *et al.*, 2000), lipid synthesis (Rawyler *et al.*, 1999; Geigenburger *et al.*, 2000), and ion transport (Zhang and Greenway, 1995; Colmer *et al.*, 2001) but fine regulation of gene expression is only now being elucidated (Lasanthi-Kudahettige *et al.*, 2007; Narsai *et al.*, 2009). For example, diversity in the promoter region of six vacuolar pyrophosphatases in rice causes just one homologue to be preferentially transcribed (Liu *et al.*, 2010). Based on an analysis of biomass transfer from seed to seedling, Fox *et al.* (1994) made estimates of total ATP requirements for the growth of aerobic and anaerobic rice seedlings. However, a global picture of the co-ordination of energy allocation to individual ATP-consuming processes has not been reported for a single organ. Analysis of energy allocation to major processes leads us to the hypothesis that there is a *hierarchical* down-regulation of ATP consumption during periods of ATP shortage, as proposed by Atwell *et al.* (1982) and Greenway and Gibbs (2003). To test this hypothesis, an ATP budget was developed using *O. sativa cv.* 'Amaroo', a relatively submergence-tolerant cultivar grown commercially in Australia. This required detailed measurements of fermentation and respiration, fluxes of substrates into key biosynthetic pathways, and estimates of transport processes. Using bioenergetics data and theoretical estimates of ATP cost of various ATP-utilizing processes (Penning de Vries *et al.*, 1974; Amthor, 2000), the energy made in coleoptiles under steady-state oxygen deficits was apportioned to growth requirements. This question was further addressed by using radioactive tracers to estimate gross rates of protein biosynthesis in coleoptiles of another six rice genotypes (see the Materials and methods). Across all genotypes, our data suggest that protein synthesis becomes the predominant sink for ATP consumption in anoxic coleoptiles at the cost of almost all other biosynthetic processes.

Materials and methods

Plant material

Seven *O. sativa* genotypes were examined: Amaroo, an anoxia-tolerant line grown commercially in Australia; Khaiyan, an anoxia-tolerant line from Bangladesh; Khao Hlan On (KHO), an anoxia-tolerant line from the Myanmar; *rad*, a *r*educed *a*lcohol *d*ehydrogenase mutant (Matsumura *et al.*, 1998; Saika *et al.*, 2006); Kinmaze (the *rad* parent line); a pyruvate decarboxylase (PDC) T-DNA insertional knockout mutant (T-181-8-6-4) obtained from Dr Narayana Upadhyaya (CSIRO Division of Plant Industry, Canberra), and Nipponbare, the *PDC* mutant parent line.

Seeds were de-hulled and surface-sterilized [70% ethanol (v/v) for 1 min, washed in distilled H_2O for 1 min, 25% bleach (v/v) for 10 min, washed ×3 in sterile distilled H_2O for 1 min, 0.1% mercuric chloride (w/v) for 3.5 min, washed ×5 in sterile distilled H_2O for 1 min]. Seeds (excepting *rad*) then were grown in 2.0 l of solution containing 0.8 mM KH_2PO_4 and 0.5 mM $CaCl_2$ with air (normoxia), 3% O_2 in N_2 (v/v) (hypoxia) or N_2 (anoxia) bubbled through the solution at ~0.2 l h^{-1}. To grow coleoptiles of *rad* in hypoxia/anoxia, seeds were pre-germinated at 28 °C in Petri dishes containing moistened tissue paper. They were then transferred to the oxygen treatments as previously listed.

Growth measurements

Coleoptiles were collected from each treatment at 24 h periods, beginning 2 d after imbibition. Coleoptile lengths were measured (Fig. 1a) and then coleoptiles were excised and fresh weight determined (Fig. 1b). Coleoptiles were dried for 2 d at 60 °C and weighed to determine dry mass (Table 1). Dried coleoptiles were placed in 80% ethanol (v/v) and heated to 80 °C for 10 min to remove free amino acids. The ethanol was removed and samples were again dried at 60 °C for 24 h. Dried coleoptiles were weighed again to determine the ethanol-insoluble dry mass (Table 1).

Determination of protein content and amino acid analysis

Estimates of protein on a fresh weight basis were made in 3-d-old and 4-d-old coleoptiles of all cultivars by a phenol/chloroform extraction of total protein (Wang *et al*, 2003) and levels quantified by the Bradford assay (Pierce) as per the manufacturer's instructions. Nitrogen analysis was also made on 2–3 mg samples of 3-, 4-, and 5-d-old freeze-dried coleoptiles using a LECO CHN-900 to confirm the protein analyses above. Total protein by mass in each dried sample was estimated by a conversion factor from nitrogen to protein of 5.95 (Merrill and Watt, 1973). These data were used to estimate N demand by the growing coleoptile.

Analysis of total protein amino acid make-up and free amino acid levels in cv. Amaroo were determined from the same samples analysed above by reversed-phase UPLC performed by the Australian Proteome Analysis Facility (APAF, www.proteome.org.au). Results are shown in Supplementary Tables S1 and S2 at *JXB* online.

Determination of lipid content

Three- and 4-d-old coleoptiles from each treatment were excised, weighed, and freeze-dried. Total lipids in each sample were determined according to Lilitchan *et al.* (2008) with the exception that hexane:ethanol (19:1, v/v) was used to increase the extraction efficiency of phospholipids. Lipid content of each sample was determined by comparison with a standard curve of rice-bran oil dissolved in hexane:ethanol (19:1, v/v). Results are shown in Table 1.

Determination of nucleic acid content

Three- and 4-d-old coleoptiles were collected from each treatment and weighed. Total nucleic acids were extracted from coleoptiles (Kang and Yang, 2004). RNA and DNA were separated by the use of LiCl (Raha *et al.*, 1990). RNA/DNA quality and levels were determined spectrophotometrically. The results are shown in Table 1.

Downloaded from http://jxb.oxfordjournals.org/ by guest on July 22, 2013

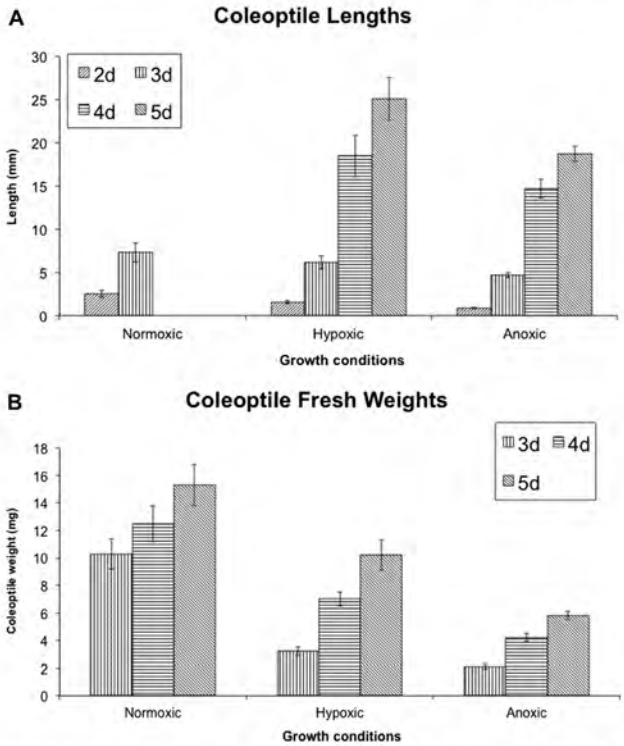


Fig. 1. Coleoptile lengths (A) and fresh weights (B) of rice seeds germinated in normoxic (aerated), hypoxic (3% O_2), and anoxic (N_2) solution. Coleoptiles (30–40) were collected from each treatment 2, 3, 4, and 5 d after imbibition. Lengths and fresh weights were determined. By 3 d after imbibition the coleoptiles of the normoxic seeds had begun to senesce. Length measurements were therefore halted at 3 d after imbibition in normoxic coleoptiles. Data are mean and SEM ($n \geq 3$).

Using the known genome size of *O. sativa* (403 Mbp) and the GC/AT ratio (Goff *et al.*, 2002) the mass of a single copy of the rice genome was calculated. To estimate cell numbers per coleoptile, total DNA content was divided by the theoretical estimate of DNA content per cell (see Supplementary Table S3 at *JXB* online). Total cell numbers were also estimated from previously published rice coleoptile cell dimensions (Wada, 1961) and measurements of coleoptile widths measured with micrometers (see Supplementary Table S3 at *JXB* online).

Estimations of cell number from coleoptile DNA content and the cell packing method were similar across all treatments (see Supplementary Table S3 at *JXB* online).

Oxygen utilization rates

Oxygen electrodes (Rank Brothers) were set up according to the manufacturer's instructions. To calibrate the electrodes, 5 ml of growth solution was placed into the electrode cuvette, the magnetic stirrer was engaged and the stable level achieved taken to be full saturation at 21% O_2. A small quantity of sodium dithionite was added to zero the electrode. This solution was removed and the electrode washed three times with fresh growth solution. Fresh solution that had been equilibrated for 4 h with either air or 3% O_2 (depending on the treatment) was used as the initial solution in the cuvette.

Three-, 4-, and 5-d-old coleoptiles (10–15 coleoptiles per replicate, three replicates per treatment) were excised, weighed, and placed into the electrode cuvette. Oxygen depletion curves were generated for each treatment and used to determine rates of O_2 utilization by coleoptiles in each treatment (Table 2 for Amaroo, Table 8 for other cultivars).

Determination of rates of ethanol fermentation

Three-day-old coleoptiles were excised from seedlings for each growth condition, weighed, and washed in distilled H_2O to remove residual ethanol. Groups of coleoptiles (~10 coleoptiles per group, three groups per treatment per biological replicate) were placed into 2 ml of distilled water in 5 ml Wheaton vials that previously had been previously been equilibrated for 4 h in an appropriate gaseous atmosphere (i.e. air, 3% O_2 or N_2) and were sealed for 1 h under each atmosphere. The O_2 concentration in the 3 ml headspace of the vials was sufficient to maintain a stable O_2 concentration in the bathing solution. 660 µl of 30% (w/v) $HClO_4$

Downloaded from http://jxb.oxfordjournals.org/ by guest on July 22, 2013

Table 1. Growth characteristics of coleoptiles grown in normoxic (aerated), hypoxic (3% O_2), and anoxic (N_2) solution
Coleoptiles were collected at 3, 4, and 5 d after imbibition and ethanol-insoluble dry weights (EiDW) were determined. Protein and free amino acid levels were determined by amino acid hydrolysis and water extraction, respectively, and were analysed via ultra-high performance liquid chromatography. Total DNA was extracted with phenol–chloroform and total amounts determined spectrophotometrically. Total lipids were determined by extracting powdered, freeze-dried material with hexane/ethanol (95:5%, v/v). (ND, not determined.) 'col' refers to 'coleoptile'. Data are mean and SEM ($n \geqslant 3$ for all samples). Statistically different data across treatments (Tukey-Kramer, $\alpha=0.05$) are indicated by different letters.

	Normoxic		Hypoxic		Anoxic	
Time (d)	**3**	**4**	**3**	**4**	**3**	**4**
Dry weight (mg col^{-1})	1.2±0.2 a	1.4±0.2 a	0.4±0.1 b	0.5±0.1 b	0.3±0.1 b	0.3±0.1 b
Ethanol-insoluble dry weight (mg col^{-1})	0.61±0.22 a	0.92 ±0.21 a	0.28±0.07 b	0.31±0.09 b	0.11±0.04 c	0.14±0.02 c
Protein (mg g^{-1} DW)	137±5 a	142±8 a	152±13 a	157±18 a	138±9 a	143±11 a
Free amino acids (mg g^{-1} DW)	16.1±0.8 b	ND	62.9±6.8 b	ND	41.2±2.8 b	ND
DNA (μg col^{-1})	1.2±0.6 a	ND	1.1±0.3 a	ND	0.8±0.1 a	ND
Lipid (mg g^{-1} DW)	109±16 a	93±12 a,b	122±22 a	88±9 b	106±19 a	81±13 a,b

Table 2. Rates of O_2 respiration, ethanol fermentation, and estimated ATP synthesis by rice coleoptiles grown in normoxic (aerated), hypoxic (3% O_2), and anoxic (N_2) solution
Coleoptiles were collected at 2, 3, and 4 d after imbibition. Coleoptiles from each treatment (10–15) were excised, weighed, and oxygen-use curves generated using an oxygen electrode. Rates of ethanol usage were determined for 3-d-old coleoptiles from each growth condition. Rates are given in the following units: O_2 consumption in nmol (O_2) g^{-1} (FW) min^{-1}; ethanol synthesis in μmol (ethanol) g^{-1} (FW) h^{-1}; ATP synthesis nmol (ATP) g^{-1} (FW) min^{-1}. Data are mean and SEM ($n \geqslant 3$) for all samples. Statistically different data across time (O_2 consumption) and treatments (ethanol synthesis) using the Tukey–Kramer test ($\alpha=0.05$) are indicated by different letters.

Treatment	O_2 consumption Age of coleoptile (d)			Ethanol synthesis	Estimation of ATP synthesis (3 d)
	2	**3**	**4**		
Normoxic	590±66 a	490±49 a	470±57 a	0.9±0.2 a	2471±249
Hypoxic	395±43 a	270±42 b	155±22 c	5.2±0.2 b	1520±213
Anoxic	ND	ND	ND	9.1±0.8 c	302±27

was added to the coleoptiles, which were left for 10 min on ice to allow ethanol to leach from the them. The solution was neutralized with 500 μl of 69% (w/v) K_2CO_3. Liquid was removed to Eppendorf tubes and spun at 5000 *g* for 2 min to precipitate $KClO_4$. An aliquot (0.5 ml) of the supernatant was added to 2 ml of 0.15 M $Na_4P_2O_7$ (sodium pyrophosphate)+0.15 M semicarbazide HCl+0.45 M glycine pH 9.0, 0.5 ml distilled H_2O, and 0.05 ml of 20 mg ml^{-1} NAD^+. The A_{340} of samples was measured, and then 2.5 IU of ADH was added to each sample. Samples were placed at 37 °C for 60 min and A_{340} measured again. Amounts of ethanol present in each sample were determined by comparison with a standard curve and were used to calculate rates of ethanol synthesis for each treatment on a fresh-mass basis (Table 2 for Amaroo, Table 9 for the other cultivars).

Estimation of rates of ATP synthesis

To estimate the rates of ATP synthesis, the rates determined above (oxygen consumption and ethanol synthesis) were converted into rates of ATP production. It was assumed that, in normoxic and hypoxic coleoptiles, the ATP:O_2 ratio was 5, based on rates of phosphorylation thought to be achieved in mitochondria (Gibbs and Greenway, 2003). In addition, each mole of ethanol produced was assumed to generate one mole of ATP in normoxia and hypoxia (Gibbs and Greenway, 2003) and two moles of ATP in anoxia through the engagement of PPi metabolism (Igamberdiev and Kleczkowski, 2011).

Using tracers to estimate 'instantaneous' rates of polymer synthesis

The choice of 4 h as a labelling period was made as a compromise between the need to label internal pools substantially while not allowing significant release of labelled product through degradation. Shorter labelling periods resulted in too few counts, while after 4 h, the most heavily labelled pool was the protein pool in normoxia where one-fifth of the endogenous pool was labelled. In the case of all other treatments and products, the 4 h exposure to tracers labelled a much smaller proportion of the endogenous pool. Therefore, it is concluded that instantaneous (gross) synthesis of these important polymers has been estimated rather than net synthesis in the steady-state.

$^{14}C/^{3}H$ amino acid tracer experiments

Three-day-old seedlings were placed in 10 ml of the appropriate growth solution that had been equilibrated for 4 h with

Downloaded from http://jxb.oxfordjournals.org/ by guest on July 22, 2013

a continuous gas supply specific to each treatment (air for normoxia, 3% O_2 for hypoxia, N_2 for anoxia) containing 5 mM of cold label and 10 nCi ml^{-1} of $^{14}C/^{3}H$ label as a tracer (~10 seedlings per vial, three vials per replicate, with three replicates per treatment per tracer). Seedlings were left to label in this solution for 4 h under the appropriate gas supply. Seedlings were removed from the labelling solution, washed three times with distilled H_2O and coleoptiles excised. Fresh weights for each replicate were determined and the excised coleoptiles were placed in solution containing 5 mM cold label overnight at 4 °C. The cold label was removed, coleoptiles were snap frozen in liquid N_2 and then freeze-dried. Freeze-dried coleoptiles were placed in 10 ml of 80% ethanol (v/v) and heated to 80 °C for 10 min to extract free amino acids. Ethanol was removed and coleoptiles dried in an oven at 60 °C for 60 min. 500 µl of 1 N NaOH was added to each sample to solubilize the protein and the mixture left overnight at 60 °C. 500 µl of 1 N HCl was added to each sample to neutralize the 1 N NaOH. Ten ml of BCS scintillation cocktail (Amersham) was added to each sample and total $^{14}C/^{3}H$ activity determined using a scintillation counter (Packard 2100TR-LSC). Total $^{14}C/^{3}H$ incorporation was calculated for each treatment. Using the known amino acid composition of proteins in each treatment (see Supplementary Table S2 at *JXB* online), total rates of amino acid incorporation into protein were estimated for each treatment. Samples were also taken for scintillation counting at the following stages in the experiment: from the stock solution; from the labelling solution after labelling was complete; from each of the washes, from the cold label when it was removed and from the ethanol after heating to determine leakage of $^{14}C/^{3}H$ activity during the extraction.

[^{3}H] thymidine tracer experiments

Three-day-old Amaroo seedlings were placed in 10 ml of the growth solution equilibrated for 4 h as above containing 1 mM of thymidine and 10 nCi ml^{-1} of [^{3}H] thymidine as a tracer (~10 seedlings per vial, three vials per replicate, with three replicates per treatment per tracer). Seedlings were left to label in this solution for 4 h in the appropriate gas supply. Seedlings were removed from the labelling solution, washed three times with distilled H_2O and coleoptiles excised. Fresh masses for each replicate were determined and the excised coleoptiles placed in solution containing 5 mM thymidine overnight at 4 °C. Total DNA was isolated as described previously. ^{3}H incorporation was determined for each treatment by scintillation counting (Packard 2100TR-LSC). Total rates of DNA synthesis for each treatment were estimated by allowing for the known GC/AT ratio of the *O. sativa* genome.

[^{14}C] sucrose tracer experiments

Three-day-old cv. Amaroo seedlings were placed into 10 ml of growth solution, equilibrated for 4 h as above, and supplemented with sucrose to a final concentration of 50 mM and 10 nCi ml^{-1} of [^{14}C] sucrose (~10 seedlings per vial, three vials per replicate, with three replicates for each treatment). Seedlings were left to label in this solution for 4 h in the appropriate gas supply. Seedlings were removed from the labelling solution, washed three times in distilled H_2O and coleoptiles excised. Fresh masses for each replicate were determined and the coleoptiles placed in solution containing 50 mM sucrose cold label overnight at 4 °C. The cold label was removed, coleoptiles were snap frozen in liquid N_2, and freeze-dried. Free amino acids, extractable proteins, cell wall mass, lipid and nucleic acid content was determined as described above. 200 µl of each extracted sample (except cell walls) were added to 800 µl of distilled H_2O. Ten ml of BCS scintillation cocktail (Amersham) was added to each sample and ^{14}C activity measured using a scintillation counter (Packard 2100TR-LSC). ^{14}C activity was converted to quantities of sucrose for each sample and rates of incorporation into each pool were determined.

Determination of K^+ uptake rates

Experiments were conducted to determine the rates of K^+/Rb^+ uptake by normoxic, hypoxic and anoxic cv. Amaroo coleoptiles.

Fresh boxes containing growth solution were prepared and gas (air, 3% O_2 or N_2) bubbled through them for 4 h. Three-day-old seedlings were transferred to this fresh solution containing 0.5 mM $CaCl_2$ and 0.25 mM KH_2PO_4. A lower external K^+ concentration than in the standard growth solution was used in order to preclude passive K^+ uptake. Two 50 ml samples were taken from the growth solution at the beginning of the experiment and each hour afterwards for 4 h. The coleoptiles for each treatment were then harvested and weighed. Total K^+ levels in each sample were determined using a flame photometer (Jenway PFP7) and rates of K^+ uptake by coleoptiles was determined by depletion of K^+ from the growth solution.

To determine Rb^+ uptake, a similar experiment was conducted but with the substitution of K^+ by adding 0.25 mM RbCl to the growth solution. Samples were taken every hour for 4 h and the coleoptiles harvested and weighed. Total Rb^+ levels in each sample were determined by flame photometry and rates of Rb^+ uptake by coleoptiles was determined by depletion of Rb^+ from the growth solution.

Statistical analysis

The raw data for Tables 1–6 and 8–10 were analysed with a single-factor ANOVA (across oxygen treatments for Tables 1–6 and across genotypes for Tables 8–10). The results of these ANOVAs were used to perform a Tukey–Kramer analysis (α=0.05) to determine significant differences.

Developing an ATP budget

To examine ATP utilization under different O_2 regimes, the various biosynthetic rates determined above were incorporated into an ATP budget.

Since it was not possible to measure rates of carbohydrate import from the seed to the coleoptile directly, a minimal estimate for carbohydrate import requirements was developed. It was assumed that the increase in EiDW between 3 d and 4 d (on a coleoptile basis) was wholly due to an increase in structural biomass such as cell walls. This increase would require an equivalent amount (by weight) of sucrose which was then converted to a rate of sucrose required for each treatment [in nmol (sucrose) g^{-1} (FW) min^{-1}]. For carbohydrate consumption by glycolysis, it was assumed that it was the sum of one-quarter of the rate of ethanol fermentation (one sucrose is consumed per four ethanol produced: Igamberdiev and Kleczkowski, 2011) plus one-twelfth of the rate of O_2 consumption (12 O_2 molecules are required to oxidize a single sucrose molecule completely: Fernie *et al.*, 2004). It was assumed that each unit of sucrose loaded into the coleoptile consumed 1 ATP and that unloading was symplastic (Scofield *et al.*, 2007).

Rates of amino acid synthesis were estimated by calculating the average rate of synthesis needed to supply the increase in soluble protein content seen in all treatments between 3 d and 4 d. Amounts of nitrate import needed to support this synthesis were estimated using the assumptions of a Jones Factor of 5.95 for rice (Merrill and Watt, 1973), that each nitrogen required was supplied by a single nitrate (NO_3^-) and that the ATP cost for nitrate transport was 1 ATP per nitrate (Lin *et al.*, 2000).

In developing this budget, a number of assumptions were made. (i) The cells of the coleoptiles were considered as a homogeneous mass, with no accounting made for the differences in ATP utilization between epidermal and cortical cells. (ii) Rates of biosynthesis estimated over 4 h labelling periods were assumed to represent steady-state rates in each treatment. (iii) In spite of a range of ATP costs associated with each biosynthetic process (e.g. longer lipids require greater amounts of ATP to synthesize)

Downloaded from http://jxb.oxfordjournals.org/ by guest on July 22, 2013

a single ATP cost was assumed for each process and was assumed to be invariant across treatments. (iv) Because of the differences in mass between coleoptiles in the three treatments, ATP utilization was normalized by converting all data to a rate of utilization per gram of fresh mass per minute.

ATP costs for each process in terms of moles of ATP consumed per mole of product synthesized were derived from the following sources: protein synthesis (per peptide bond formed and incorporating estimates of the ATP cost of protein breakdown and turnover)—5 moles ATP (Amthor, 2000); cell wall materials—3 moles ATP (Penning de Vries *et al.*, 1974); lipid synthesis (to produce palmitic acid)—21 moles ATP (Penning de Vries *et al.*, 1974; Goodwin and Mercer, 1985), and nucleic acid—4 moles ATP (Penning de Vries *et al.*, 1974).

Results

Coleoptile growth

Increases in length and fresh weight of Amaroo coleoptiles grown in normoxia, hypoxia or anoxia were measured from 2 d after imbibition (Fig. 1A, B). Hypoxic coleoptiles elongated approximately twice as fast as anoxic coleoptiles between 2 d and 5 d after imbibition (0.4 mm h^{-1} cf. 0.2 mm h^{-1}, respectively). Fresh weights were greatly reduced by oxygen deprivation, with a 2-fold difference in the increase in fresh weight for hypoxic compared to anoxic coleoptiles [0.15 mg (FW) h^{-1} cf. 0.08 mg (FW) h^{-1}, respectively].

Table 1 summarizes the effect of O_2 supply on the composition of Amaroo coleoptiles. The ethanol-insoluble dry weight (EiDW) of individual normoxic coleoptiles was approximately double that of hypoxic coleoptiles and 6–8-fold greater than that of anoxic coleoptiles. However, there was no significant difference between the three treatments in levels of protein, DNA or lipid on a dry weight basis. By contrast, anoxic and hypoxic coleoptiles had three to four times higher levels of free amino acids than normoxic coleoptiles, with alanine approximately 25-fold and 40-fold higher in anoxic and hypoxic tissues, respectively, than in normoxic coleoptiles (see Supplementary Table S1 at *JXB* online).

Normoxic coleoptiles, unlike hypoxic or anoxic coleoptiles, began to senesce 4 d after imbibition as shown by Kawai and Uchimiya (2000), determining that the detailed ATP budgets should be derived from coleoptiles 3 d after imbibition.

Estimating ATP synthesis by respiration and fermentation

Rates of O_2 uptake and ethanol synthesis of excised normoxic, hypoxic, and anoxic coleoptiles were measured in order to estimate rates of ATP synthesis in each treatment (Table 2). Rates of O_2 consumption decreased in both normoxic and hypoxic coleoptiles between the second and fourth day after imbibition, declining by approximately 20% in normoxic coleoptiles [590±66 to 470±57 nmol O_2 g^{-1} (FW) min^{-1}, respectively]) and by approximately 60% in hypoxic coleoptiles measured at 3% O_2 [395±75 to 155±39 nmol O_2 g^{-1} (FW) min^{-1}, respectively] over this period.

Rates of ethanol synthesis were higher in anoxic tissue [8.46±0.76 μmol (ethanol) g^{-1} (FW) h^{-1}] than either hypoxic [(5.15±0.20 μmol (ethanol) g^{-1} (FW) h^{-1}] or normoxic tissue [0.86±0.21 μmol (ethanol) g^{-1} (FW) h^{-1}]. A measurable but very low rate of ethanol synthesis during constant aeration suggested a failure of pyruvate to be fully oxidized in the TCA cycle and may be an indicator of localized hypoxia within normoxic coleoptiles or an imbalance between glycolysis and mitochondrial activity.

Estimated rates of ATP production [(nmol (ATP synthesized) g^{-1} (FW) min^{-1}] were 2464±249 (normoxic), 1520±213 (hypoxic), and 302±27 (anoxic). These values represent a 2-fold and an 8-fold decrease in ATP available under hypoxia and anoxia, respectively, compared with normoxia. The experiments described in the following paragraphs were used to derive estimates of total ATP consumption during normoxic, hypoxic, and anoxic conditions. By assuming that ATP production and consumption reach equilibrium in each steady-state O_2 treatment, energy costs could be ascribed to the major endergonic processes required for growth.

Estimating rates of protein synthesis

Estimates of the rates of protein turnover in normoxic, hypoxic and anoxic coleoptiles were made 3 d after imbibition (Table 3). Normoxic coleoptiles incorporated all radioactive amino acids two to three times faster than in hypoxic and anoxic coleoptiles [93±6 nmol amino acids g^{-1} (FW) min^{-1} in normoxic coleoptiles versus 41±2 and 32±2 nmol amino acids g^{-1} (FW) min^{-1} in hypoxic and anoxic coleoptiles, respectively]. The observed decrease in protein synthesis rates in anoxic coleoptiles compared with normoxic coleoptiles was proportionately less than the decrease in the estimated rates of ATP synthesis in anoxia,

Table 3. Rates of amino acid incorporation into protein by normoxic (aerated), hypoxic (3% O_2), and anoxic (N_2) grown coleoptiles as determined by [^{14}C/^{3}H]amino acid tracer experiments

Three-day-old seedlings (10–15) were labelled in solution containing 5 mM of cold label and 10 nCi ml^{-1} of [^{14}C/^{3}H] label as a tracer. Labelling period was 4 h. Total ^{14}C/^{3}H incorporation was determined for each treatment. Using the known amino acid composition of proteins in each treatment (see Supplementary Table S1 at *JXB* online), total rates of amino acid incorporation into protein were estimated for each treatment. Data are mean and SEM (n=3 for each label). Statistically different data across treatments (Tukey–Kramer, α=0.05) are indicated by different letters.

Label	Estimated incorporation of amino acid into protein (nmol aa g^{-1} FW min^{-1})		
	Normoxic	Hypoxic	Anoxic
[^{14}C]valine	83±12 a	40±6 b	30±4 b
[^{14}C]isoleucine	92±11 a	39±5 b	31±5 b
[^{3}H]leucine	105±15 a	45±7 b	35±6 b
Mean	93±6	41± 2	32±2

Downloaded from http://jxb.oxfordjournals.org/ by guest on July 22, 2013

suggesting that a greater proportion of available ATP was directed into protein synthesis during an O_2 deficit.

Estimating DNA synthesis rates by [^{3}H] thymidine incorporation

Experiments were conducted to determine rates of DNA synthesis via [^{3}H] thymidine incorporation (Table 4). Rates of DNA synthesis in hypoxic and anoxic coleoptiles were not significantly different and were approximately half those observed in normoxic coleoptiles [42±9, 51±17, and 90±6 ng (DNA) g^{-1} (FW) min^{-1}, respectively]. It should be emphasized that tissues of the first leaf, in which cell division was rapid, were removed from all coleoptile samples.

Table 4. Rates of DNA synthesis in normoxic (aerated), hypoxic (3% O_2), and anoxic (N_2) coleoptiles as estimated by [^{3}H]thymidine incorporation
Three-day-old seedlings (10–15) were placed in labelling solution containing 1 mM of thymidine and 1 nCi ml^{-1} of [^{3}H] thymidine as a tracer. Labelling period was 4 h. Total [^{3}H] thymidine incorporation was determined for each treatment. Using the known GC/AT ratio of the *O. sativa* genome, total rates of DNA synthesis were estimated for each treatment. Data are mean and SEM (n=3). Statistically different data across treatments (Tukey–Kramer, α=0.05) are indicated by different letters.

	Rate of [^{3}H]thymidine incorporation(ng DNA g^{-1} FW min^{-1})
Normoxic	90±6 a
Hypoxic	42±9 b
Anoxic	51±17 b

Table 5. Incorporation of sucrose into various biopolymer pools of normoxic (aerated), hypoxic (3% O_2), and anoxic (N_2) rice coleoptiles using a [^{14}C] sucrose tracer
Three-day-old seedlings (10–15) were placed into growth solution supplemented with 50 mM sucrose and 10 nCi ml^{-1} [^{14}C] sucrose. The labelling period was 4 h. Coleoptiles were frozen in liquid N_2 then freeze-dried. Free amino acids, extractable proteins, lipids, nucleic acids, and cell walls were extracted as described in the Materials and methods. ^{14}C activity was measured and converted to quantities of sucrose for each sample. Rates of incorporation into each pool were determined. Rates are in nmol (sucrose incorporated) g^{-1} (FW) min^{-1}. Data are mean and SEM (n=3). Statistically different data across treatments (Tukey–Kramer, α=0.05) are indicated by different letters.

Biopolymer pool	Normoxic	Hypoxic	Anoxic
Free amino acids	42.4±3.7 a	27.9±1.0 b	4.8±0.8 c
Extractable protein	35.4±4.0 a	19.9±2.2 b	4.4±0.9 c
Lipids	3.8±1.0 a	2.7±0.7 b	2.4±0.1 b
Nucleic acids	3.8±1.0 a	1.7±0.3 b	2.2±0.3 b
Cell walls	24.4±3.0 a	25.3±5.9 a	5.0±1.2

Incorporation of [^{14}C] sucrose to estimate biosynthesis

Experiments were conducted to estimate the rates of *de novo* synthesis of a range of metabolites from the dominant carbon source, using [^{14}C] sucrose as a tracer. Rates of incorporation of sucrose into free amino acids, proteins, lipids, nucleic acids, and cell wall materials were determined (Table 5).

Rates of [^{14}C] sucrose incorporation into both free amino acids and soluble protein were greatly reduced in hypoxic and anoxic coleoptiles compared with normoxic coleoptiles [42.4±3.7 and 35.4±4.0 (normoxic); 27.9±1.0 and 19.9±2.2 (hypoxic); 4.8±0.8 and 4.4±0.9 (anoxic) nmol (sucrose incorporated) g^{-1} (FW) min^{-1}]. [^{14}C] sucrose incorporation into lipids on a fresh weight basis was ~30% lower in both hypoxic and anoxic treatments compared with normoxic coleoptiles, although there was no significant difference between hypoxic and anoxic coleoptiles [2.7±0.7 and 2.4±0.1 versus 3.8±1.0 nmol (sucrose incorporated) g^{-1} (FW) min^{-1}, respectively].

[^{14}C] sucrose incorporation into total nucleic acids (RNA and DNA) was halved in hypoxic and anoxic treatments [1.7±0.3 and 2.2±0.3 nmol (sucrose incorporated) g^{-1} (FW) min^{-1}] compared with normoxic coleoptiles [3.8 nmol (sucrose incorporated) g^{-1} (FW) min^{-1}].

There was no significant difference in the amounts of [^{14}C] sucrose incorporated into cell walls between the normoxic and hypoxic coleoptiles [(24.4±3.0 and 25.3±5.9 nmol (sucrose incorporated) g^{-1} (FW) min^{-1}, respectively]. Rates of incorporation in anoxic coleoptiles [(5.0 ± 1.2 nmol (sucrose incorporated) g^{-1} (FW) min^{-1})] were ~20% of the rates in the presence of O_2.

Estimating costs of ion uptake using K^+/Rb^+ uptake

Experiments were conducted to estimate the costs of potassium acquisition by measuring the rates of K^+/Rb^+ uptake (Table 6). Both normoxic and hypoxic coleoptiles maintained a higher net uptake of K^+ [3.0±0.1 and 2.6±0.1 μmol K^+ g^{-1} (FW) h^{-1}] than anoxic coleoptiles [0.9±0.2 μmol K^+ g^{-1} (FW) h^{-1}]. Because K^+ uptake was measured

Table 6. Rates of Rb^+ and K^+ influx for coleoptiles grown in normoxic (aerated), hypoxic (3% O_2), and anoxic (N_2) solution
Three-day-old seedlings were transferred to fresh solution containing 0.25 mM of Rb^+ or K^+. Triplicate 50 ml samples were taken from the growth solution every hour for 4 h. Coleoptiles were excised and weighed. Total amounts of Rb^+/K^+ uptake were determined using a flame photometer. Data are mean and SEM (n=3). Statistically different data across treatments (Tukey–Kramer, α=0.05) are indicated by different letters.

Treatment	Rubidium influx (μmol Rb^+ g^{-1} FW h^{-1})	Net potassium influx (μmol K^+ g^{-1} FW h^{-1})
Normoxic	3.9±0.2 a	3.0±0.3 a
Hypoxic	3.6±0.3 a	2.6±0.1 a
Anoxic	0.8±0.2 b	0.9±0.2 b

Downloaded from http://jxb.oxfordjournals.org/ by guest on July 22, 2013

by depletion, the contribution of K^+ efflux to net uptake was accounted for by substituting Rb^+ for K^+ within the medium. This allowed us to estimate gross K^+ influx because Rb^+ mimics K^+ by entering through K^+ transporters (Etherton, 1967) but is not significantly effluxed in the 4 h time-course of the experiment. Rates of Rb^+ influx for normoxic and hypoxic coleoptiles were between four and five times greater than in anoxic coleoptiles [3.9±0.2, 3.6±0.3, and 0.8±0.2 μmol Rb^+ g^{-1} (FW) h^{-1}, respectively]. Low levels of efflux occurred when O_2 was present but not in anoxic coleoptiles.

Devising a budget of ATP consumption by rice coleoptiles

Significant differences were calculated in both the amounts and proportions of ATP allocated to each of the major ATP-consuming processes between the three O_2 treatments (Table 7). Key anabolic processes were analysed using substrates to estimate instantaneous rates of synthesis. Energy that was not accounted for by this reconciliation is identified in the last line of Table 7.

Protein synthesis consumed the largest proportion of available ATP in all three treatments [465, 205, and 160 nmol (ATP consumed) g^{-1} (FW) min^{-1} for normoxic, hypoxic, and anoxic coleoptiles, respectively]. Proportionally, however, the ATP utilized by protein synthesis in anoxic coleoptiles (~52%) was more then double that in normoxic coleoptiles (~19%) with a slightly smaller allocation observed in hypoxic coleoptiles (~14%).

There was no statistically significant reduction in the absolute rate of ATP consumed by cell wall synthesis in hypoxic *vs.* normoxic coleoptiles [72 and 75 nmol (ATP consumed) g^{-1} (FW) min^{-1}, respectively]. However, the rate of ATP consumption by cell wall synthesis in anoxic coleoptiles was 15 nmol (ATP consumed) g^{-1} (FW) min^{-1}, one-fifth of that in the normoxic and hypoxic tissues.

Table 7. Breakdown of ATP costs associated with various biosynthetic processes for 3-d-old coleoptiles grown in normoxic (aerated), hypoxic (3% O_2), and anoxic (N_2) solution

Rates associated with each process are taken from Tables 2–6. The estimated costs for each process (given as moles of ATP consumed to produce one mole of product) were taken from the following: protein synthesis, Amthor (2000); cell wall synthesis, Penning de Vries (1974); lipid synthesis, Penning de Vries (1974) and Goodwin and Mercer (1985); nucleic acid synthesis, Penning de Vries (1974). ATP production via oxidative phosphorylation assumes an ATP:O_2 of 5 for normoxic and hypoxic coleoptiles, respectively (Gibbs and Greenway, 2003). Total rates of ATP generation/utilization were calculated by multiplying the rate of the process in each treatment by its associated ATP cost and are expressed on the basis of nmol (ATP consumed) g^{-1} (FW) min^{-1}. Carbohydrate import is expressed as sucrose equivalents. Nitrogen import is expressed as nitrate equivalent. Rates shown are the mean calculated rates for each process ($n \geq 3$ for all rates).

		Normoxic		Hypoxic		Anoxic	
ATP-generating processes	**ATP generated by process[a]**	**Rate of process in tissue[b]**	**Total ATP generated[c]**	**Rate of process in tissue[b]**	**Total ATP generated[c]**	**Rate of process in tissue[b]**	**Total ATP generated[c]**
Respiration (ATP:O_2)	5	490	2450±245	270	1350±210	N/A	N/A
Ethanol fermentation (ATP:ethanol)	1 (hypoxia) or 2 (anoxia)	14	14±3	85	85±3	151	302±26
Total ATP generated			**2464**		**1435**		**302**
ATP-consuming processes	**ATP cost of process[a]**	**Rate of process in tissue[b]**	**Total ATP cost[c]**	**Rate of process in tissue[b]**	**Total ATP cost[c]**	**Rate of process in tissue[b]**	**Total ATP cost[c]**
Protein	5	93	465±30	41	205±10	32	160±10
Cell wall	3	24	72±9	25	75±18	5	15±4
Lipid	18	4	72±18	3	54±13	2	36±2
Nucleic acid	4	6	24±4	2	8±1	1	4±1
K^+ influx	1	44	44±3	45	45±4	15	15±6
Carbohydrate (sucrose) import (from seed)	1	100	100±34	51	51±11	46	46±9
Nitrogen (nitrate) import (from seed)	1	22	22±8	24	24±7	9	9±2
Amino acid synthesis	4	17	68±15	18	72±12	7	28±6
Total ATP consumed			**867±122**		**534±76**		**313±40**
ATP generation/ consumption			**1597**		**901**		**−11**

[a] Units are mol (ATP produced/consumed) mol^{-1} (process product).
[b] Units are nmol (process product) g^{-1} (FW) min^{-1}.
[c] Units are nmol (ATP produced/consumed) g^{-1} (FW) min^{-1}.

Downloaded from http://jxb.oxfordjournals.org/ by guest on July 22, 2013

Table 8. Coleoptile length and fresh weight of 3-d-old Khaiyan, KHO, *rad*, *PDC*-insertional mutant, Kinmaze, and Nipponbare coleoptiles grown in normoxic (aerated), hypoxic (3% O_2), and anoxic (N_2) solution
Coleoptiles (30–40) were collected from each cultivar/treatment combination 3 d after imbibition. Lengths and fresh weights were determined. Data are mean and SEM ($n \geqslant 3$). Statistically different data across genotypes within a single O_2 treatment (Tukey–Kramer, α=0.05) are indicated by different letters.

	Normoxic		Hypoxic		Anoxic	
Genotype	**Length (mm)**	**Weight (mg)**	**Length (mm)**	**Weight (mg)**	**Length (mm)**	**Weight (mg)**
Khaiyan	16.3±0.5 a	8.8±1.4 a	36.8 ±2.1 a	4.6 ±0.6 a	41.2±2.0 a	5.4±1.1 a
KHO	16.5±0.9 a	9.1±0.9 a	37.0±1.6 a	5.6±1.2 a	38.4±4.6 a	4.7±1.3 a
Nipponbare	22.8±5.3 a	10.5±1.2 a	20.6±3.8 b	6.7±0.4 a	19.2±2.8 b	3.6±0.8 a
Kinmaze	22.0±2.8 a	7.8±0.9 a	19.2±2.6 b	6.2±0.8 a	15.6±2.5 b	3.8±0.9 a
PDC-insertional mutant	20.8±1.7 a	9.6±1.8 a	15.0±1.7 b	5.5±0.4 a	10.2±2.2 b	1.5±0.3 b
rad	21.8±5.3 a	6.4±1.3 a	4.8±0.4 c	0.9±0.3 b	3.6±0.3 c	1.0±0.4 b

ATP consumption by lipid synthesis was ~25% lower in hypoxic and anoxic compared to normoxic coleoptiles [54, 54, and 72 nmol (ATP consumed) g^{-1} (FW) min^{-1}, respectively]). Proportional to ATP production, lipid synthesis consumed approximately five times more ATP in anoxic coleoptiles compared to normoxic coleoptiles (14% and 3%, respectively).

ATP consumption by nucleic acid synthesis in hypoxic and anoxic coleoptiles was approximately one-third or one-sixth of that in normoxic coleoptiles [(8, 4 and 24 nmol (ATP consumed) g^{-1} (FW) min^{-1}, respectively]. However, there was no significant difference in the proportion of ATP allocated to nucleic acid synthesis amongst the three treatments and only a tiny proportion of the available ATP pool was expended making DNA.

Estimates of ATP consumption required to energize Rb^+ influx were similar between normoxic and hypoxic coleoptiles but significantly lower in anoxic coleoptiles [44, 45, and 15 nmol (ATP consumed) g^{-1} (FW) min^{-1}, respectively].

ATP was consumed in transporting carbohydrates from seeds to coleoptiles. ATP consumption estimates for transport were similar in all three treatments [157 to 187 nmoles (ATP consumed) g^{-1} (FW) min^{-1}]. ATP was also required to synthesize amino acids by assuming all N arrived in coleoptiles as nitrate ions. This component of ATP consumption fell dramatically in anoxic coleoptiles compared to hypoxic and normoxic coleoptiles [28, 72, and 68 nmol (ATP consumed) g^{-1} (FW) min^{-1}, respectively].

ATP production and consumption in anoxia-tolerant and -intolerant rice cultivars: using genetic variation in flood tolerance to assess cost of protein synthesis

Based on the dominant contribution of protein synthesis to ATP demand in anoxia (Table 7), further experiments were conducted to examine the cost of protein synthesis in coleoptiles of diverse anoxia-tolerant and -intolerant genotypes grown in normoxia, hypoxia, and anoxia. The six genotypes described in the Materials and methods were examined: Khaiyan, Khao Hlan On (KHO), *rad* (reduced *a*lcohol *d*ehydrogenase mutant), Kinmaze (the *rad* parent line), a pyruvate decarboxylase (*PDC*) T-DNA knockout mutant, and Nipponbare, the *PDC*-mutant parent line.

Table 8 shows there were no significant differences in the lengths or fresh weights of the coleoptiles of the six genotypes in normoxia. In hypoxia and anoxia, the two anoxia-tolerant lines (Khaiyan and KHO) produced coleoptiles that were significantly longer and weighed more than the less anoxia-tolerant lines (Kinmaze and Nipponbare). The genotypes with lesions in their fermentative pathways produced coleoptiles whose growth was significantly retarded compared with their parent lines.

Table 9 reports O_2 consumption, ethanol synthesis, and estimated rates of ATP production for the six genotypes. For the non-mutated lines (Khaiyan, KHO, Kinmaze, and Nipponbare), patterns of respiration and fermentation were broadly the same. The two genotypes with mutations in the fermentation pathway (*rad* and the *PDC*-knockout line) had significantly impaired ATP production because of low activities of ADH and PDC, respectively (Table 9, and Supplementary Table S4 at *JXB* online, respectively). The energy deficit in *rad* was approximately twice as severe as that in the *PDC*-knockout line.

Incorporation of [^{14}C] leucine was used to the assess rates of protein synthesis in the six genotypes (Table 10) based on preliminary experiments with several labelled amino acid species. Even in aerated solution, *rad* coleoptiles had low rates of protein synthesis, reflecting its intolerance to submergence. In anoxia, lesions in the fermentative pathway had a clear effect, reducing absolute rates of protein synthesis to less than 50% of those in non-mutated genotypes. These data give rise to an energy budget for protein synthesis (Table 11).

Discussion

During an O_2 deficit, alcoholic fermentation in anoxic tissues produces ATP at a rate that is, at best, one-third that of aerobic tissues (Gibbs and Greenway, 2003). This dramatic decrease in ATP production must be matched by an equivalent decrease in ATP consumption. Atkinson (1968) enunciated the concept of 'energy equilibrium',

Downloaded from http://jxb.oxfordjournals.org/ by guest on July 22, 2013

Table 9. Rates of O_2 consumption, ethanol fermentation, and estimated ATP synthesis by rice coleoptiles of anoxia tolerant (*) and intolerant (**) cultivars grown in normoxic (aerated), hypoxic (3% O_2), and anoxic (N_2) solution
Coleoptiles from each cultivar and treatment (10–15) were excised, weighed, and oxygen-use curves generated using an oxygen electrode. Rates of ethanol usage were determined for 3-d-old coleoptiles from each growth condition. Rates are given in the following units: O_2 consumption in nmol (O_2) g^{-1} (FW) min^{-1}; ethanol production in μmol (ethanol) g^{-1} (FW) h^{-1}; ATP synthesis nmol (ATP) g^{-1} (FW) min^{-1}. Data are mean and SEM (n >3) for all samples. Statistically different data across genotypes within a single set of measurements (Tukey–Kramer, α=0.05) are indicated by different letters.

	O_2 consumption		Ethanol production			ATP production		
Cultivar	**Normoxic**	**Hypoxic**	**Normoxic**	**Hypoxic**	**Anoxic**	**Normoxic**	**Hypoxic**	**Anoxic**
Khaiyan*	716±26 a	253±21 a	1.2±0.5 a	11.0±2.3 a	11.1±2.1 a	3600±146	1448±143	370±70
KHO*	590±29 a	244±32 a	1.1±0.4 a	10.1±0.4 a	10.5±2.5 a	2968±152	1388±167	350±83
Nipponbare	427±29 b	273±65 a	0.9±0.3 a	7.9±0.4 a	6.8±0.2 b	2138±151	1470±325	226±7
Kinmaze (*rad* parent line)	666±56 a	201±18 a	1.0±0.4 a	8.3±2.3 a	9.7±1.9 a	3346±287	1143±94	323±63
PDC-insertional mutant**	406±19 b	244±66 a	0.8±0.3 a	7.6±1.1 a	4.3±1.2 c	2043±101	1346±327	143±40
*rad***	403±38 b	180±29 a	0.2±0.2 b	2.3±0.6 b	2.3±0.4 d	2018±206	938±155	76±13

Table 10. Rates of amino acid incorporation into protein by normoxic (aerated), hypoxic (3% O_2), and anoxic solution-grown coleoptiles of anoxia-tolerant (*) and -intolerant (**) lines as estimated by [^{14}C]leucine tracer experiments
Three-day-old seedlings (10–15) were labelled in solution containing 5 mM of cold label and 10 nCi ml^{-1} of [^{14}C]leucine as a tracer. Labelling period was 4 h. Total ^{14}C incorporation was determined for each treatment. Data are mean and SEM (n=3). Statistically different data across genotypes within a single O_2 treatment (Tukey-Kramer, α=0.05) are indicated by different letters.

Cultivar	**Normoxic**	**Hypoxic**	**Anoxic**
Khaiyan*	102±9 a	44±8 a	41±6 a
KHO*	98±12 a	51±5 a	35±6 a
Nipponbare	72±11 a	38±4 a	31±6 a
Kinmaze (rad parent line)	63±3 a	35±7 a	26±8 a
PDC-insertional mutant**	69±9 a	31±7 a,b	12±6 b
*rad***	39±6 b	22±4 b	8±6 b

a general theory of ATP regeneration being up-regulated during an energy deficit and ATP utilization being simultaneously down-regulated such that the two reach a new equilibrium. If the sensitivity of each anabolic process to ATP supply were identical, ATP consumption might be expected to decrease uniformly as each process succumbs to anoxia. In reality, the single curves for 'ATP regeneration' and 'ATP utilization' (Atkinson, 1968) are composites of individual responses of processes to cell energy status. This postulate will be tested below.

In our study, ATP production decreased by one-half under hypoxia and by ~88% in anoxia when compared with normoxia. This detailed analysis of ATP consumption by Amaroo coleoptiles suggests that key processes accounted for about half of the available ATP in the presence of O_2 but all available ATP in anoxic coleoptiles. That is, all ATP produced in anoxia can be accounted for by the synthesis and turnover of just four polymer classes and potassium influx. Fox *et al.* (1994) calculated ATP requirements of 50.2 and 4.7 μmol ATP d^{-1} $seedling^{-1}$ to sustain seedling growth in aerobic and anaerobic seedlings, respectively. From Table 7, total ATP production by coleoptiles in these conditions was calculated as 35.3±3.5 and 2.7±0.2 μmol ATP d^{-1} $coleoptile^{-1}$. As Fox *et al.* (1994) was estimating ATP utilization based on biomass transfer from the seed to seedling, the concordance of these findings is remarkable.

Protein synthesis was strongly inhibited by hypoxia and anoxia, as reported earlier for rice seedlings in hypoxia (Mohanty and Ong, 2003) and coleoptiles in anoxia (Alpi and Beevers, 1983). However, the proportion of ATP estimated to support the synthesis and turnover of protein was at least three times greater in anoxic coleoptiles compared with those in the presence of O_2. Indeed, the gross rates of protein synthesis estimated to occur in anoxia (Table 3) could replace the entire protein complement approximately every 2.5 d. This accords with previously published work, suggesting that sustained gene expression and the synthesis of new proteins is critical for the acclimation of rice coleoptiles to prolonged O_2 deficits (Sachs *et al.*, 1980; Umeda *et al.*, 1994; Huang *et al.*, 2005; Lasanthi-Kudahettige *et al.*, 2007). The allocation of 52% of available ATP to a restricted complement of proteins under anoxia (Sachs *et al.*, 1980; Mujer *et al.*, 1993; Matsumura *et al.*, 1999) is clearly part of the acclimation response typical of rice coleoptiles.

Complex changes in transcriptional/translational patterns have been shown through studies on anaerobic rice coleoptiles. Lasanthi-Kudahettige *et al.* (2007) identified 3134 probe sets in microarray experiments that showed changed levels of expression (1364 increased, 1770 decreased) in anoxia compared with normoxia. Huang *et al.* (2005) similarly identified a number of unknown proteins by two-dimensional electrophoresis that were more abundant in anoxic coleoptiles than in normoxic controls. Of those that were identified, one vacuolar H^+-pyrophosphatase was highly expressed in coleoptiles of the flood-tolerant cultivar Amaroo when exposed to anoxia (Liu *et al.*, 2010). Similarly, the overexpression of *Sub1-A*, an ethylene response factor known to

Downloaded from http://jxb.oxfordjournals.org/ by guest on July 22, 2013

Table 11. ATP consumption ascribed to protein synthesis in rice coleoptiles of a variety of anoxia-tolerant (*) and -intolerant (**) cultivars grown in normoxic (aerated), hypoxic (3% O_2), or anoxic (N_2) solutions
ATP cost is given as nmol (ATP consumed) g^{-1} (FW) min^{-1}. Per cent available ATP was calculated as the estimated cost of protein synthesis divided by the total available ATP for each condition.

	Normoxic		Hypoxic		Anoxic	
Cultivar	**ATP cost**	**% Available ATP**	**ATP cost**	**% Available ATP**	**ATP cost**	**% Available ATP**
Khaiyan*	510	14.1	220	15.1	205	55.4
KHO*	490	16.5	255	18.4	175	50.0
Nipponbare	360	16.8	190	12.9	155	68.5
Kinmaze (rad parent line)	315	9.4	155	13.5	130	40.2
PDC-insertional mutant**	345	16.9	155	11.5	60	41.9
rad**	195	9.6	110	11.7	40	52.6

be induced by flooding in the highly flood-tolerant cultivar FR13A, conferred increased flood tolerance to transgenic lines compared with their flooding-intolerant parent lines (Xu *et al.*, 2006). The current study of ATP allocation, viewed alongside the strong induction of individual genes by anoxia, suggests that there may be a broader base of anoxically induced proteins than previously thought.

Further evidence for a shift in energy allocation can be seen from lipid synthesis, where the proportion of ATP directed to membrane synthesis doubles in anoxia compared with allocation in the presence of O_2. Membrane integrity is vital for survival in anoxia (Atwell *et al.*, 1982; Greenway and Gibbs, 2003; Felle, 2005). While free fatty acids increased in anoxic potato cell cultures (Rawyler *et al.*, 1999) indicating membrane damage (Crawford and Braendle, 1996), lipid metabolism in rice tissues is more robust under anoxia (Generosova and Vartapetian, 2005). However, unsaturated fatty acids were not synthesized in anoxic rice coleoptiles (Brown and Beevers, 1987), presumably because of the O_2 requirement of desaturase enzymes. Thus, even though the lipid pool was found to be preferentially synthesized in anoxic rice coleoptiles under an energy deficit, membrane composition could have been compromised.

ATP consumption based on estimates of carbohydrate transport from seeds to coleoptiles was similar in all three treatments, indicating that anoxia did not impede either carbohydrate transport or unloading. A Pasteur Effect is typically seen in rice coleoptiles, accounting for more rapid carbohydrate catabolism in anoxia (Setter *et al.*, 1987). In maize root tips, Saglio (1985) also showed that long-distance carbohydrate transport was not impaired by anoxia. If sugars were delivered to rapidly expanding coleoptile cells by a symplastic pathway (Scofield *et al.*, 2007), unloading could be expected to be relatively insensitive to anoxia.

Coleoptile growth was maintained even in anoxia, notwithstanding the ~50% fewer cells than in normoxic coleoptiles (Opik, 1973; see Supplementary Table S3 at *JXB* online). DNA synthesis was reduced in hypoxic as well as in anoxic coleoptiles. By contrast, cell elongation was rapid in hypoxia, as shown by a disproportionate length to biomass ratios (compare Fig. 1 with Table 1). Overall, this suggests that the energy cost of cell elongation is trivial compared with cell division.

Absolute rates of cell wall synthesis were identical in normoxic and hypoxic coleoptiles but were ~80% lower in anoxia (Table 5). Estimates of net K^+ import and K^+ influx using Rb^+ followed a similar response to O_2 as for cell wall synthesis, leading to the conclusion that turgor maintenance and coleoptile extension is robustly maintained in severe hypoxia, driving rapid elongation. The maintenance of tissue K^+ during growth in anoxia, and thus turgor pressure (Atwell *et al.*, 1982), relies, in part, upon the import of K^+ from seed reserves. This potentially lowers the costs of K^+ uptake from the bathing medium.

The energetics of coleoptile growth reflect the functional requirements of this organ. The lower ATP cost of synthesizing cell walls (cf. protein and lipid) and K^+ import, enable rapid coleoptile elongation, even when structural dry weight gain is compromised (Fig. 1a; Table 1). In turn, the rapid elongation of coleoptile cells (Wada, 1961) underpins the morphology classically known as the 'Snorkel Effect' (Kordan, 1974). In flooded rice beds, the decline in O_2 concentration is gradual and, therefore, maintenance of cell wall synthesis in severe hypoxia improves the chances of survival during subsequent anoxia (suggested by Atwell *et al.*, 1982). Even anoxic coleoptiles elongated at around 80% of the rates seen in hypoxic coleoptiles (Fig. 1), in spite of 80% less carbohydrate being directed to cell wall synthesis (Table 5). Reduced biosynthesis would conserve carbohydrates for accelerated glycolysis (the Pasteur Effect), sustaining the ATP production and turnover essential to survival in anoxia. By contrast, ATP requirements for maintenance in hypoxic and normoxic coleoptiles are easily accounted for within the 50% of ATP of unknown fate (Table 7).

To test the relationship between ATP production and consumption in *O. sativa* coleoptiles more rigorously, a range of genotypes with contrasting tolerance to anoxia was examined during germination and early seedling growth. By varying ethanol production the question of preferential ATP allocation could be tested more deeply.

Coleoptiles of all the genotypes without a lesion in energy production grew substantially in hypoxia/anoxia. The cultivars with the highest rates of ethanol synthesis (Khaiyan>KHO>Kinmaze>Amaroo) were also those that displayed the fastest growth in these conditions (Amaroo, Fig. 1; others, Table 8). Conversely the growth of coleoptiles

Downloaded from http://jxb.oxfordjournals.org/ by guest on July 22, 2013

of *rad* and the *PDC*-insertional mutant were compromised in anoxia (Table 8), with the *rad* seeds not even developing a coleoptile unless first pre-treated with 12 h of normoxia (see Materials and methods). These genetic contrasts afford an opportunity to test how a reduction in ATP consumption was achieved.

While absolute rates of protein synthesis halved in hypoxia compared with normoxia, the proportion of ATP dedicated to protein synthesis remained steady. By contrast, in anoxia the allocation of ATP to protein synthesis rose to 40–70% in all cultivars (Table 11). This establishes the preferential allocation of energy to protein synthesis during anoxia across many *O. sativa* genotypes regardless of submergence tolerance.

Notwithstanding these findings, those genotypes with compromised fermentative capacity (*rad* and *PDC*-insertional mutant) down-regulated protein synthesis as predicted by the Atkinson model (1968). While protein synthesis is obviously a high priority for survival in anoxia, cell maintenance processes which require ATP may be even more critical than protein synthesis.

The theoretical ATP deficit in anoxic coleoptiles suggests that there may be other unaccounted-for processes at work in the anoxic coleoptiles. These include the production of ATP by other processes within the tissue and the utilization of pyrophosphate (PPi) as an alternative energy currency during anoxic conditions. For example, Stoimenova *et al.* (2007) reported that mitochondria from rice roots were able to utilize nitrite as an alternative electron acceptor and produce ATP under anoxic conditions. This may explain the improvement in growth when anoxic seedlings were supplied with exogenous nitrate/nitrite (Trought and Drew, 1981; Prioul and Guyot, 1985). If nitrate were available from seed reserves (Reggiani *et al.*, 1995), it could act as a potential electron acceptor in rice coleoptiles.

Utilization of PPi as an alternate energy currency would also alleviate the anoxic ATP deficit reported in Table 7. Huang *et al.* (2008) suggested that PPi, produced by a variety of cellular processes including protein synthesis, cell wall synthesis, and nucleic acid synthesis (Maeshima, 2000), would decrease energy stress in anoxic rice coleoptiles by relieving demand on the ATP pool. A series of ATP-dependent enzymes can be functionally substituted by PPi-driven analogues during anoxia (Plaxton, 1996; Huang *et al.*, 2005; Lasanthi-Kudahettige *et al.*, 2007; Liu *et al.*, 2010). Igamberdiev and Kleczkowski (2011) reported that, at pH 7.2, the lower end of the cytosolic pH range in anoxic rice coleoptiles (Kulichikhin *et al.*, 2009), the ΔG^0 of PPi is about 60% of that of ATP. In the 'anoxic' energy budget reported in Table 7, even assuming the higher efficiency of four ATPs per glucose molecule consumed (Igamberdiev and Kleczkowski, 2011), all available energy from fermentation was accounted for. If the claim of Plaxton (1996) of a yield of five ATPs per glucose through PPi metabolism were accepted, energy yield would very slightly exceed utilization. Within the bounds of error, the budget shows that anoxic coleoptiles illustrate clearly the principle of balance between energy regeneration and utilization (Atkinson, 1968).

The bioenergetics of rice coleoptiles appears to be a paradigm for anoxia tolerance in higher plants. Bailey-Serres and Voesenek (2008) noted that plant responses to prolonged anoxia fall within two broad strategies: *escape* by rapid elongation to an O_2 source and *quiescence*, where energy allocation to maintenance dominates over growth. The cultivars Khaiyan and KHO employ the escape strategy, directing scarce energy resources in anoxia to the synthesis of key proteins, many of which remain unidentified. Coleoptiles of those genotypes with compromised fermentation scarcely grow in anoxia, thus employing the quiescence strategy. Their low rates of protein synthesis reflect this ecological characterization.

Supplementary data

Supplementary data can be found at *JXB* online.

Supplementary Table S1. Amino acid makeup of proteins of rice coleoptiles grown in normoxic (aerated), hypoxic (3% O_2), and anoxic (N_2) solution.

Supplementary Table S2. Free amino acid content of rice coleoptiles grown in normoxic (aerated), hypoxic (3% O_2), and anoxic (N_2) solution.

Supplementary Table S3. Total DNA content and estimates of cell number of 3-d-old coleoptiles grown in normoxic (aerated), hypoxic (3% O_2), and anoxic (N_2) solution.

Supplementary Table S4. Pyruvate decarboxylase activity in the *O. sativa PDC* mutant and its parent line (cv. Nipponbare).

Acknowledgements

The authors acknowledge the help of the following individuals and groups: Mr Michael Joseph (Sydney University) for the generous gift of isotopes; Dr Abdelbagi Ismail (International Rice Research Institute) for seed of Khaiyan and Khao Hlan On; Dr Narayana Upadhyaya (CSIRO) for seed of the *PDC* mutant; and Professor Mikio Nakazono (Nagoya University) and Professor Hideo Matsumura (Shinshu University) for seed of *rad* and Kinmaze. We also thank Dr Hank Greenway for comments on the experiments, Dr Jan Gebicki for reviewing an early draft of the paper, and Denise Thomas from the Australian Proteome Analysis Facility (APAF). Work conducted at APAF was facilitated using infrastructure provided by the Australian Government through the National Collaborative Research Infrastructure Strategy (NCRIS).

References

Alpi A, Beevers H. 1983. Effects of O_2 concentration on rice seedlings. *Plant Physiology* **71,** 30–34.

Amthor JS. 2000. The McCree–de Wit–Penning de Vries–Thornley respiration paradigms: 30 years later. *Annals of Botany* **86,** 1–20.

Downloaded from http://jxb.oxfordjournals.org/ by guest on July 22, 2013

Atkinson DE. 1968. Energy charge of the adenylate pool as a regulatory parameter. Interaction with feedback modifiers. *Biochemistry* **7,** 4030–4034.

Atwell BJ, Waters I, Greenway H. 1982. The effect of oxygen and turbulence on elongation of coleoptiles of submergence-tolerant and -intolerant rice cultivars. *Journal of Experimental Botany* **33,** 1030–1044.

Bailey-Serres J, Voesenek L. 2008. Flooding stress: acclimations and genetic diversity. *Annual Review of Plant Biology* **59,** 313–339.

Brown DJ, Beevers H. 1987. Fatty acids of rice coleoptiles in air and anoxia. *Plant Physiology* **84,** 555–559.

Chang WWP, Huang L, Shen M, Webster C, Burlingame AL, Roberts JKM. 2000. Patterns of protein synthesis and tolerance of anoxia in root tips of maize seedlings acclimated to a low-oxygen environment, and identification of proteins by mass spectrometry. *Plant Physiology* **122,** 295–318.

Colmer TD, Huang S, Greenway H. 2001. Evidence for down-regulation of ethanolic fermentation and K^+ effluxes in the coleoptile of rice seedlings during prolonged anoxia. *Journal of Experimental Botany* **52,** 1507–1517.

Crawford RMM. 1977. Tolerance of anoxia and ethanol metabolism in germinating seeds. *New Phytologist* **79,** 511–517.

Crawford RMM, Braendle R. 1996. Oxygen deprivation stress in a changing environment. *Journal of Experimental Botany* **47,** 145–159.

Etherton B. 1967. Steady-state sodium and rubidium effluxes in *Pisum sativum* roots. *Plant Physiology* **42,** 685–690.

FAO. 2004. The *state of food insecurity in the world*. Rome: FAO.

Felle HH. 2005. pH regulation in anoxic plants. *Annals of Botany* **96,** 519–532.

Fernie AR, Carrari F, Sweetlove LJ. 2004. Respiratory metabolism: glycolysis, the TCA cycle and mitochondrial electron transport. *Current Opinion in Plant Biology* **7,** 254–261.

Fox TC, Kennedy RA, Rumpho ME. 1994. Energetics of plant growth under anoxia: metabolic adaptations of *Oryza sativa* and Echinochloa phyllopogon. *Annals of Botany* **74,** 445–455.

Geigenburger P, Fernie AR, Gibon Y, Christ A, Stitt M. 2000. Metabolic activity decreases as an adaptive response to low internal oxygen in growing potato tubers. *Biological Chemistry* **381,** 723–740.

Generosova IP, Vartapetian BB. 2005. On the physiological role of anaerobically synthesized lipids in *Oryza sativa* seedlings. *Russian Journal of Plant Physiology* **52,** 481–488.

Gibbs J, Greenway H. 2003. Review: mechanisms of anoxia tolerance in plants. I. Growth, survival and anaerobic catabolism. *Functional Plant Biology* **30,** 353–353.

Gibbs J, Morrell S, Valdez A, Setter T, Greenway H. 2000. Regulation of alcoholic fermentation in coleoptiles of two rice cultivars differing in tolerance to anoxia. *Journal of Experimental Botany* **51,** 785–796.

Goodwin TW, Mercer EI. 1985. *Introduction to plant biochemistry*. New York, New York State, USA: Pergamon Press.

Goff SA, Ricke D, Lan TH, Presting G, Wang R, Dunn M, Glazebrook J, Sessions A, Oeller P, Varma H. 2002. A draft sequence of the rice genome (*Oryza sativa* L. ssp. *japonica*). *Science* **296,** 92–100.

Greenway H, Gibbs J. 2003. Review: mechanisms of anoxia tolerance in plants. II. Energy requirements for maintenance and energy distribution to essential processes. *Functional Plant Biology* **30,** 999–1036.

Huang S, Colmer TD, Millar AH. 2008. Does anoxia tolerance involve altering the energy currency towards PPi? *Trends in Plant Science* **13,** 221–227.

Huang S, Greenway H, Colmer TD, Millar AH. 2005. Protein synthesis by rice coleoptiles during prolonged anoxia: implications for glycolysis, growth and energy utilization. *Annals of Botany* **96,** 703–715.

Igamberdiev AU, Kleczkowski LA. 2011. Magnesium and cell energetics in plants under anoxia. *Biochemical Journal* **437,** 373–379.

Ishizawa K, Murakami S, Kawakami Y, Kuramochi H. 1999. Growth and energy status of arrowhead tubers, pondweed turions and rice seedlings under anoxic conditions. *Plant, Cell and Environment* **22,** 505–514.

Jackson MB. 1985. Ethylene and responses of plants to soil waterlogging and submergence. *Annual Review of Plant Physiology* **36,** 145–174.

Kang TJ, Yang MS. 2004. Rapid and reliable extraction of genomic DNA from various wild-type and transgenic plants. *BMC Biotechnology* **4,** 20.

Kawai M, Uchimiya H. 2000. Coleoptile senescence in rice (*Oryza sativa* L.). *Annals of Botany* **58,** 405–414.

Kawano N, Ella E, Ito O, Yamauchi Y, Tanaka K. 2002. Metabolic changes in rice seedlings with different submergence tolerance after desubmergence. *Environmental and Experimental Biology* **47,** 195–203.

Kordan H. 1974. The rice shoot in relation to oxygen supply and root growth in seedlings germinating under water. *New Phytologist* **73,** 695–697.

Kulichikhin KY, Greenway H, Byrne L, Colmer TD. 2009. Regulation of intracellular pH during anoxia in rice coleoptiles in acidic and near neutral conditions. *Journal of Experimental Biology* **60,** 2119–2128.

Lasanthi-Kudahettige R, Magneschi L, Loreti E, Gonzali S, Licausi F, Novi G, Beretta O, Vitulli F, Alpi A, Perata P. 2007. Transcript profiling of the anoxic rice coleoptile. *Plant Physiology* **144,** 218–231.

Lilitchan S, Tangprawat C, Aryusuk K, Krisnangkura S, Chokmoh S, Krisnangkura K. 2008. Partial extraction method for the rapid analysis of total lipids and [gamma]-oryzanol contents in rice bran. *Food Chemistry* **106,** 752–759.

Lin C-M, Koh S, Yu S-M, Lin T-Y, Tsay Y F. 2000. Cloning and functional characterization of a constitutively expressed nitrate transporter gene, *OsNRT1*, from rice. *Plant Physiology* **112,** 379–388.

Liu Q, Zhang Q, Burton RA, Shirley NJ, Atwell BJ. 2010. Expression of vacuolar H^+-pyrophosphatase (*OVP3*) is under control of an anoxia-inducible promoter in rice. *Plant Molecular Biology* **72,** 47–60.

Maeshima M. 2000. Vacuolar H^+-pyrophosphatase. *Biochimica et Biophysica Acta: Biomembranes* **1465,** 37–51.

Downloaded from http://jxb.oxfordjournals.org/ by guest on July 22, 2013

Magneschi L, Perata P. 2009. Rice germination and seedling growth in the absence of oxygen. *Annals of Botany* **103,** 181–196.

Matsumura H, Nirasawa S, Terauchi R. 1999. Transcript profiling in rice (*Oryza sativa* L.) seedlings using serial analysis of gene expression (SAGE). *The Plant Journal* **20,** 719–726.

Matsumura H, Takano T, Takeda G, Uchimiya H. 1998. *Adh1* is transcriptionally active but its translational product is reduced in a *rad* mutant of rice (*Oryza sativa* L.), which is vulnerable to submergence stress. *Theoretical and Applied Genetics* **97,** 1197–1203.

Merrill AL, Watt BK. 1973. Energy value of foods. Agriculture Handbook No. 74. United States Department of Agriculture.

Mocquot B, Prat C, Mouches C, Pradet A. 1981. Effect of anoxia on energy charge and protein synthesis in rice embryo. *Plant Physiology* **68,** 636–640.

Mohanty B, Ong BEEL. 2003. Contrasting effects of submergence in light and dark on pyruvate decarboxylase activity in roots of rice lines differing in submergence tolerance. *Annals of Botany* **91,** 291–300.

Mujer CV, Rumpho ME, Lin JJ, Kennedy RA. 1993. Constitutive and inducible aerobic and anaerobic stress proteins in the *Echinochloa* complex and rice. *Plant Physiology* **101,** 217–226.

Narsai R, Howell KA, Carroll A, Ivanova A, Millar AH, Whelan J. 2009. Defining core metabolic and transcriptomic responses to oxygen availability in rice embryos and young seedlings. *Plant Physiology* **151,** 306–322.

Öpik H. 1973. Effect of anaerobiosis on respiratory rate, cytochrome oxidase activity and mitochondrial structures in coleoptiles of rice (*Oryza sativa* L.). *Journal of Cell Science* **12,** 725–739.

Penning de Vries VFW, Brunsting A, Van Laar H. 1974. Products, requirements and efficiency of biosynthesis: a quantitative approach. *Journal of Theoretical Biology* **45,** 339–377.

Perata P, Alpi A. 1993. Plant responses to anaerobiosis. *Plant Science* **93,** 1–17.

Plaxton WC. 1996. The organisation and regulation of plant glycolysis. *Annual Review of Plant Physiology and Plant Molecular Biology* **47,** 185–214.

Prioul JL, Guyot C. 1985. Role of oxygen transport and nitrate metabolism in the adaptation of wheat plant to root anaerobiosis. *Physiologie Végétale* **23,** 175–185.

Raha S, Merante F, Proteau G, Reed JK. 1990. Simultaneous isolation of total cellular RNA and DNA from tissue culture cells using phenol and lithium chloride. *Gene Analysis Techniques* **7,** 173–177.

Rawyler A, Pavelic D, Gianinazzi C, Oberson J, Braendle R. 1999. Membrane lipid integrity relies on a threshold of ATP production rate in potato cell cultures submitted to anoxia. *Plant Physiology* **120,** 293–300.

Raymond P, Al-Ani A, Pradet A. 1985. ATP production by respiration and fermentation, and energy charge during aerobiosis and anaerobiosis in twelve fatty and starchy germinating seeds. *Plant Physiology* **79,** 879–884.

Reggiani R, Bertini F, Mattana M. 1995. Incorporation of nitrate nitrogen into amino acids during the anaerobic germination of rice. *Amino Acids* **9,** 385–390.

Sachs MM, Freeling M, Okimoto R. 1980. The anaerobic proteins of maize. *Cell* **20,** 761–767.

Saglio PH. 1985. Effect of path or sink anoxia on sugar translocation in roots of maize seedlings. *Plant Physiology* **77,** 285–290.

Saika H, Matsumura H, Takano T, Tsutsumi N, Nakazono M. 2006. A point mutation of *ADH1* gene is involved in the repression of coleoptile elongation under submergence in rice. *Breeding Science* **56,** 69–74.

Scofield GN, Hirose T, Aoki N, Furbank RT. 2007. Involvement of the sucrose transporter, OsSUT1, in the long-distance pathway for assimilate transport in rice. *Journal of Experimental Botany* **58,** 3155–3169.

Setter TL, Ella ES, Valdez AP. 1994. Relationship between coleoptile elongation and alcoholic fermentation in rice exposed to anoxia. II. Cultivar differences. *Annals of Botany* **74,** 273–279.

Setter TL, Ellis M, Laureles EV, Ella ES, Senadhira D, Mishra SB, Sarkarung SK, Datta S. 1997. Physiology and genetics of submergence tolerance in rice. *Annals of Botany* **79,** 67–77.

Setter TL, Waters I, Greenway H, Atwell BJ, Kupkanchanakul T. 1987. Carbohydrate status of terrestrial plants during flooding. In: Crawford RMM, ed. *Plant life in aquatic and amphibious habitats*. Special Publication No. 5. British Ecological Society. Oxford, UK: Blackwell Scientific Publications, 411–433.

Stoimenova M, Igamberdiev AU, Gupta KJ, Hill RD. 2007. Nitrite-driven anaerobic ATP synthesis in barley and rice root mitochondria. *Planta* **226,** 465–474.

Trought M, Drew M. 1981. Alleviation of injury to young wheat plants in anaerobic solution cultures in relation to the supply of nitrate and other inorganic nutrients. *Journal of Experimental Botany* **32,** 509–522.

Umeda M, Hara C, Matsubayashi Y, Li HH, Liu Q, Tadokoro F, Aotsuka S, Uchimiya H. 1994. Expressed sequence tags from cultured cells of rice (*Oryza sativa* L.) under stressed conditions: analysis of transcripts of genes engaged in ATP-generating pathways. *Plant Molecular Biology* **25,** 469–478.

Wada S. 1961. Growth patterns of rice coleoptiles grown on water and under water. *Scientific Reports of Tohoku University Series IV (Biology)* **27,** 199–207.

Wang W, Scali M, Vignani R, Spadafora A, Sensi E, Mazzuca S, Cresti M. 2003. Protein extraction for two-dimensional electrophoresis from olive leaf, a plant tissue containing high levels of interfering compounds. *Electrophoresis* **24,** 2369–2375.

Xu K, Xu X, Fukao T, Canlas P, Maghirang-Rodriguez R, Heurer S, Ismail AM, Bailey-Serres J, Mackill D. 2006. Sub1A is an ethylene-response-factor-like gene that confers submergence tolerance to rice. *Nature* **442,** 705–708.

Zhang Q, Greenway H. 1995. Membrane transport in anoxic rice coleoptiles and storage tissues of beetroot. *Functional Plant Biology* **22,** 965–975.

Downloaded from http://jxb.oxfordjournals.org/ by guest on July 22, 2013

3 Chapter 3 – An examination of the transcriptome of O_2-deprived rice coleoptiles

3.1 Introduction

Coleoptiles of rice (*Oryza sativa* L.) are a classical model for studies on growth and metabolism during O_2 deprivation. Specifically, this is because coleoptiles are able to grow at a modest rate when no O_2 is present and elongate most rapidly (up to 1 mm h^{-1}) in hypoxia, when compared with coleoptiles in aerated solution (Atwell *et al*., 1982; Alpi and Beevers, 1983). Rapid elongation under submergence is seen as an escape phenomenon and has therefore been referred to as the 'snorkel effect' (Kordan, 1974).

In recent years, a number of papers have been published examining cell and molecular responses to O_2 deprivation in rice seedlings (Huang *et al*., 2005; Lasanthi-Kudahettige *et al*., 2007; Narsai *et al*., 2009; Takahashi *et al*., 2011). A distinctive response to O_2 deficits in rice coleoptiles has been described at the gene level, including transcripts encoding for gene products involved in fermentation, cell wall expansion, carbohydrate and lipid metabolism and the induction of ethylene-response factors (Lasanthi-Kudahettige *et al*., 2007; Narsai *et al*., 2009). These studies have given valuable insights into metabolism of whole coleoptiles during an energy crisis. Curiously, none investigated the effects of hypoxia, even though this is the specific condition that elicits the 'snorkel effect' (Kordan, 1974). In addition, coleoptiles have developmental zones that can be exploited to understand growth but this has never been attempted.

The capacity of rice coleoptiles to grow in a range of O_2 concentrations from anoxia to normoxia provides an opportunity to investigate the effects of O_2 deprivation on gene expression and test how this interacts with cell maturation. Coleoptiles are almost completely undifferentiated, comprised overwhelmingly of functionally unspecialised

cortical and epidermal cells and have a distinct longitudinal gradient of growth rates (Wada, 1961; Furuya *et al*., 1969). Because cell elongation is not uniform along the coleoptile axis, this offers the opportunity to separate O_2 effects in rapidly elongating tissues from those in the tips where growth is slowing – that is, the interaction between O_2 and growth. Existing literature only offers hints of these growth gradients within coleoptiles. Setter and Ella (1994) showed that fermentation rates were greatest in the basal 3 mm of anoxic rice coleoptiles, contrasting with the 11–14 mm-long tips of anoxic coleoptiles which produced ethanol at only one-sixth the rate of the growing basal tissues. We hypothesise that such differences in metabolism along the length of the coleoptile should be accompanied by a change in the pattern of expressed genes.

Cell elongation remains incompletely understood at a mechanistic level. Rice coleoptiles were ideal experimental material because, compared to roots or rice internodes, their growth is highly dependent upon elongation of the large number of cells pre-formed in the embryo (Jones and Rost, 1989). Using time course experiments, Wada (1961), Öpik (1973) and Takahashi *et al*. (2011) observed that cell division within normoxic rice coleoptiles generally ceased 2.5 to 3 d after imbibition, resulting in an ultimate cell population of ~ 80,000 cells in normoxic coleoptiles and one-third less in anoxic coleoptiles (Edwards *et al*., 2012). Thus, by 3 d after germination coleoptile growth was almost completely a result of cell elongation.

Previous studies have focused on rice coleoptiles grown continuously or transiently in strict anoxia, while rapid elongation rates at very low O_2 levels (hypoxia) reported by Atwell *et al*. (1982) suggest that the effects of low O_2 levels on gene expression are likely to be qualitatively distinct. Edwards *et al.* (2012) identified significant differences in the utilisation of ATP by coleoptiles grown under hypoxia compared to normoxia or anoxia. We thus hypothesised that there would also be significant differences within the transcriptome in the hypoxic coleoptiles compared to

either their anoxic or normoxic counterparts. To examine these hypotheses, we used available scanning electron micrographs of epidermal cell lengths to calculate local growth rates and define the energy demands for growth in each zone of the coleoptile that we sampled. We then used microarrays to examine gene expression in basal segments (actively elongating) and distal segments (where cell expansion had almost ceased) of coleoptiles under normoxic, hypoxic (i.e. 3% O_2) and anoxic conditions.

By identifying a zone of elongation that was predominantly within basal tissues of the coleoptile, we complemented the work of Furuya *et al*. (1969) and Satler and Kende (1985). We identified several groups of genes commonly up-regulated in coleoptile tips compared to bases, regardless of O_2 treatment, while other changes were specific to the O_2 supply in the gas stream.

3.1.1 Experimental Aims

My research in this chapter was based around three key experimental aims:

1. To examine the changes in cell length in time and space along the axis of the elongating hypoxic coleoptile;
2. To investigate the differences in the transcriptome of the tips and bases of rice coleoptiles regardless of O_2 supply and;
3. To investigate the differences in the transcriptome between tissues (tips and bases) grown across differing levels of O_2-deprivation.

3.2 Materials and Methods

3.2.1 Plant growth

Seeds were de-hulled and surface sterilised by washing in: 70% ethanol (v/v) (1 min); dH_2O (1 min); 25% bleach (v/v) (10 min); 3 × dH_2O (1 min); 0.1% mercuric chloride (w/v) (3.5 min); 5 × dH_2O (1 min). Seeds were grown in 2 L of solution

containing 0.8 mM KH_2PO_4 and 0.5 mM $CaCl_2$ bubbled with air (normoxia), 3% O_2 in N_2 (v/v) (hypoxia) or N_2 (anoxia) at a rate of ~0.2 L h^{-1} for 3 d.

Coleoptiles were harvested 3 d after imbibition, and those from each O_2 treatment (air, 3% O_2 in N_2 and N_2) were dissected into tips (5–7 mm in length) and bases (5–7 mm in length) in batches of 10. Each batch was immediately placed into liquid nitrogen and snap frozen. Approximately 60–80 coleoptiles were harvested from each treatment/tissue sample and combined. Three replicates (of 60–80 coleoptiles) were harvested for each treatment/tissue combination.

3.2.2 Determination of protein content

Estimates of protein on a fresh weight basis were made in 3-d-old coleoptiles by a phenol/chloroform extraction of total protein (Wang *et al*., 2003) and levels quantified by Bradford assay (Pierce) as per the manufacturer's instructions.

3.2.3 Determination of nucleic acid content and calculation of energy use

Three- and 4-d-old coleoptiles were collected from each treatment and weighed. Total nucleic acids were extracted from coleoptiles as per Kang and Yang (2004). RNA and DNA were separated by the use of LiCl (Raha *et al*., 1990). RNA/DNA quality and levels were determined spectrophotometrically. Total ATP production for the coleoptile tips and bases in each treatment was estimated as described in Table 7 of Edwards *et al*. (2012).

3.2.4 Scanning electron microscopy

Hypoxic coleoptiles were grown to ½-, 1-, 2- and 3-d old. Coleoptiles were harvested and fixed in a mixture of 1.5% paraformaldehyde and 1.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Samples were critical-point dried and gold coated before examination under SEM. A sequence of overlapping images was taken from the base to the tip of the coleoptile. Because the mature cells were so long that few

individual cells fell entirely within a single image, a formula was devised to relate cell ends to cell length. Accordingly, average cell lengths were estimated as follows:

$\bar{x} = \frac{F \times L}{E}$ where *x* is the average cell length (μm)

F is the number of cell files per image

L is the length of the image (μm) and

E is the number of end cell walls per image.

3.2.5. RNA extraction and cDNA microarray analysis

Total RNA was isolated from coleoptile tips and bases using the Qiagen RNEasy Kit. For cDNA synthesis 400 ng of RNA was used as template, following the Affymetrix IVT express kit as per manufacturer's instructions. All samples were analysed in biological triplicate, as done previously (Narsai et al., 2011).

3.2.6. Data analysis

Microarray CEL files were first imported into the Affymetrix Expression Console software and MAS5 normalised to determine presence/absence calls for each probeset. All CEL files were then imported into Partek Genomics Suite (v. 6.6) (Missouri, USA) and GCRMA normalised. Only probesets that were present in two or more replicates were kept for further analysis, as done previously (Narsai et al., 2011). Raw data and differential expression analysis for all genes can be seen in Supplementary Table B1.

For differential expression analysis, the Cyber-T method incorporating false discovery rate correction (PPDE) was used, which relies on a Bayesian method to identify differentially expressed genes (Baldi and Long, 2001; Long et al., 2001). Only genes with $p < 0.05$ and PPDE >0.96 were considered to be differentially expressed, and these were filtered only to show probesets differentially expressed by >1.5 fold. These differentially expressed genes were used for the PageMan (v1.32) ORA_Fisher analysis, determining

over-represented functional categories (Usadel et al., 2006). In this way a z-score of >1.96 equates to significant over-representation at $p < 0.05$.

3.3 Results

3.3.1 Morphological differences in coleoptiles grown under different oxygen conditions

Oxygen supply had a dramatic effect on seedling morphology (Figure 1). Notably, normoxic seedlings were also developmentally distinct because a root emerged from the seed and the first leaf elongated within the coleoptile, whereas these events were suppressed under restricted O_2 supply (Figure 1).

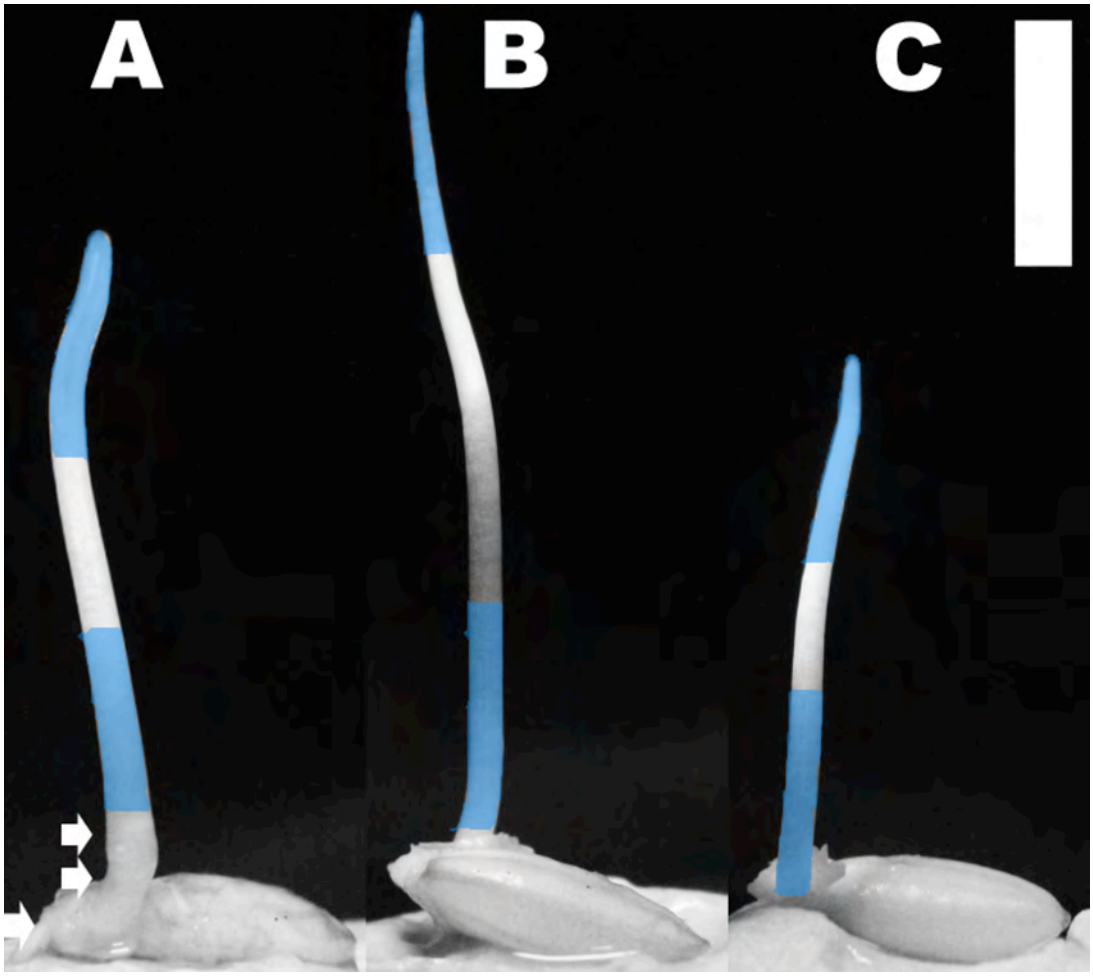


Figure 1. Representative normoxic (A), hypoxic (B) and anoxic (C) coleoptiles. The normoxic coleoptile shows the normal emergence of the root (single white arrow) and the leaf of the growing seedling (double white arrow). Pale blue areas show the tissue harvested for mRNA extraction. The white bar is 5 mm long.

In order to examine differences at a metabolic level, ATP production, protein, RNA and DNA content of the tips and bases under normoxic, hypoxic and anoxic

conditions were examined (Table 1). Under all conditions, bases had 60–200% more soluble protein per unit of tissue volume than tips (p <0.01 in all three O_2 treatments), presumably due to vacuolisation as basal cells elongated rapidly and were displaced from their initial position adjacent to the seed (Table 1; Figure 2). Rates of expansion varied across O_2 treatments, with hypoxic coleoptiles elongating the most over the 3 d post-germination (Figure 1) and thus having the greatest dilution of soluble protein in the tips (Table 2). Both DNA and RNA content showed a similar pattern, being higher in the bases than the tips across all treatments (Table 1). Within each O_2 treatment total RNA content was 2–3× greater in the bases than in the tips (Table 1). Estimated ATP production per unit fresh weight was consistently 20% lower in tips than in bases in each O_2 treatment, although these differences were not statistically significant. These estimates are based on rates of fermentation and do not take into account the role of pyrophosphate or the haemoglobin-NO cycle in energy production. Nonetheless, O_2 deficits reduced ATP yields dramatically, as would be expected and would have therefore commensurately reduced rates of ATP turnover. Overall, the observation that there was more protein, DNA and RNA content in basal tissues compared to apical tissues reflects the smaller cells, higher proportion of cytosol and greater metabolic activity in the rapidly elongating coleoptile bases (Figure 2).

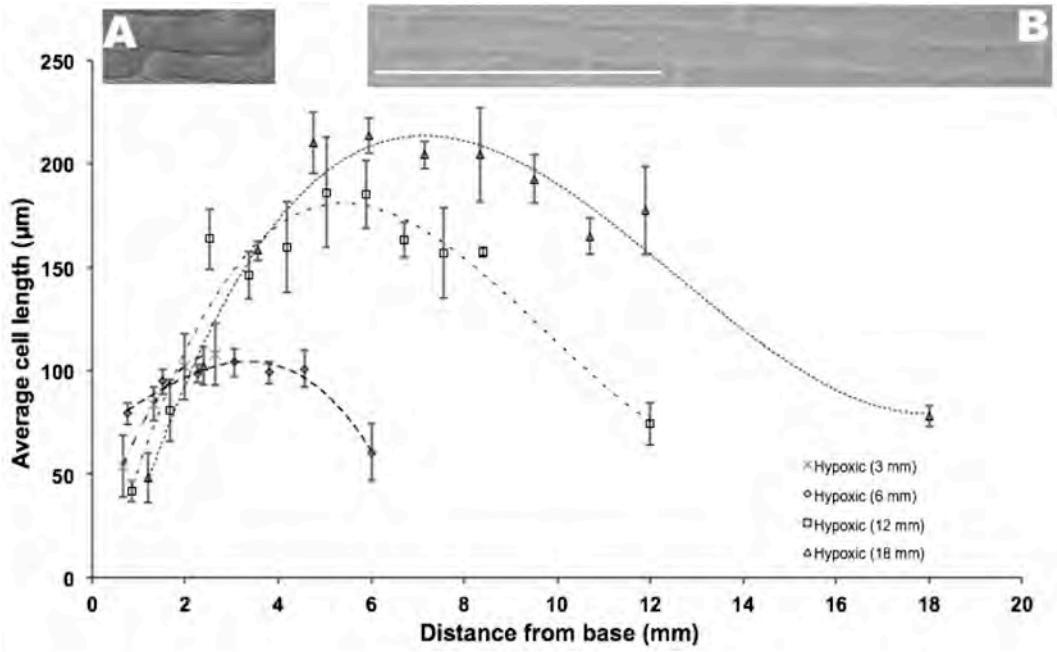


Figure 2. Cell sizes and relative growth rates (insert) of 6 mm, 12 mm and 18 mm old hypoxic rice coleoptiles. Rice seedlings were grown for 24 (3 mm) 36 (6 mm), 48 (12 mm) or 72 (18 mm) hours in stagnant growth solution. Rice coleoptiles were harvested and prepared for scanning electron microscopy. Cell lengths for each time point were measured at set distances along the rice coleoptile. Representative cells from the base (A) and tips (B) of 12 mm long hypoxic coleoptiles are shown. The white scale bar represents 50 µm. Data are mean and SEM.

Table 1. Protein, RNA and DNA content of the tips and bases of normoxic, hypoxic and anoxic coleoptiles. Coleoptiles were grown for three days in normoxic (aerated), hypoxic (3% O_2 in N_2) or anoxic (N_2) growth solution. The tips (5–6 mm) and bases (5–6 mm of coleoptiles nearest the seed) were harvested and snap frozen. Soluble protein was extracted with a phenol double extraction; RNA was extracted using a Qiagen RNeasy mini-kit, and DNA was extracted with a phenol chloroform extraction (after Kang and Yang, 2003). Data shown are mean and SEM.

	Normoxic		Hypoxic		Anoxic	
Tissue Contents	**Base**	**Tips**	**Base**	**Tips**	**Base**	**Tips**
Protein (mg soluble protein g^{-1} FW)	7.0 ± 1.1	4.4 ± 0.6	7.2 ± 1.6	2.4 ± 0.8	4.4 ± 0.9	1.8 ± 0.6
RNA (µg RNA g^{-1} FW)	460 ± 60	154 ± 11	537 ± 28	247 ± 28	575 ± 51	177 ± 17
DNA (µg DNA g^{-1} FW)	150 ± 45	120 ± 39	148 ± 38	103 ± 32	135 ± 29	89 ± 41
ATP production (nmol ATP g^{-1} FW min^{-1})	2600 ± 535	2100 ± 450	1836 ± 300	1421 ± 221	380 ± 67	290 ± 45

Micrographs showed that cells in the mid-region of coleoptiles were longest, regardless of age (Figure 2), with the epidermal cells in the most distal tip region intrinsically short. Maximal cell length was ~100 µm in the 24- and 36-h-old coleoptiles and 150–200 µm in the 48- and 72-h-old coleoptiles (Figure 2). In order to estimate elongation rates during coleoptile development, localised growth rates were carefully

analysed from the slopes of curves in Figure 2. Coleoptile elongation from 6 to 18 mm coincided with rapid elongation of cells within the basal third of the coleoptile (see steep gradient in Figure 2). Cell lengths in our study increased rapidly from nearest to the seed, peaking approximately one-third of way along the coleoptile as measured from the base, with final cell lengths and elongation rates declining in the tip region (Figure 2).

3.3.2 Tissue-specific responses to oxygen deprivation

In order to examine the tissue-specific transcriptomic differences under O_2 limitation, tips and bases were harvested from normoxic, hypoxic and anoxic conditions in biological triplicate (Figure 3A). This design allowed multiple comparisons of tips and bases, both within and across the different O_2 conditions (Figure 3A). Following GeneChip RMA (GC-RMA) normalisation, Principal Component Analysis (PCA) was carried out to visualise global differences between samples (Figure 3B). This analysis revealed some distance (within the analysis space) between bases and tips, supporting the idea that there are tissue-specific differences in the base and tip transcriptomes under each O_2 treatment. When the numbers of differentially expressed genes (DEGs) were compared between bases and tips (base *vs* tips), 1728 genes were differentially expressed under all three O_2 conditions, representing O_2-independent, tissue-specific differences in expression (1011 + 717; Figure 4). In addition to tissue-specific differences, a clear distance was also seen between normoxia (red) and both hypoxia (light purple) and anoxia transcriptomes (dark purple) (PC1, y-axis; Figure 3B). This supports previous studies showing significant transcriptomic differences between the normoxic and anoxic transcriptomes in rice (Lasanthi-Kudahettige *et al*., 2007; Narsai *et al*., 2009). Additionally, with the increased tissue-specific resolution in this study, tissue-specific transcriptomic differences were unveiled between the tips and bases under low-O_2 conditions (PC2, x-axis; Figure 3B). Notably, similarity between the hypoxic and anoxic responses in the tips was also evidenced by PCA; hence these grouped closer

together (Figure 3B). Similarly, closer grouping was also observed between the hypoxic and anoxic bases, suggesting overlapping transcriptomic responses to these treatments as well (Figure 3B).

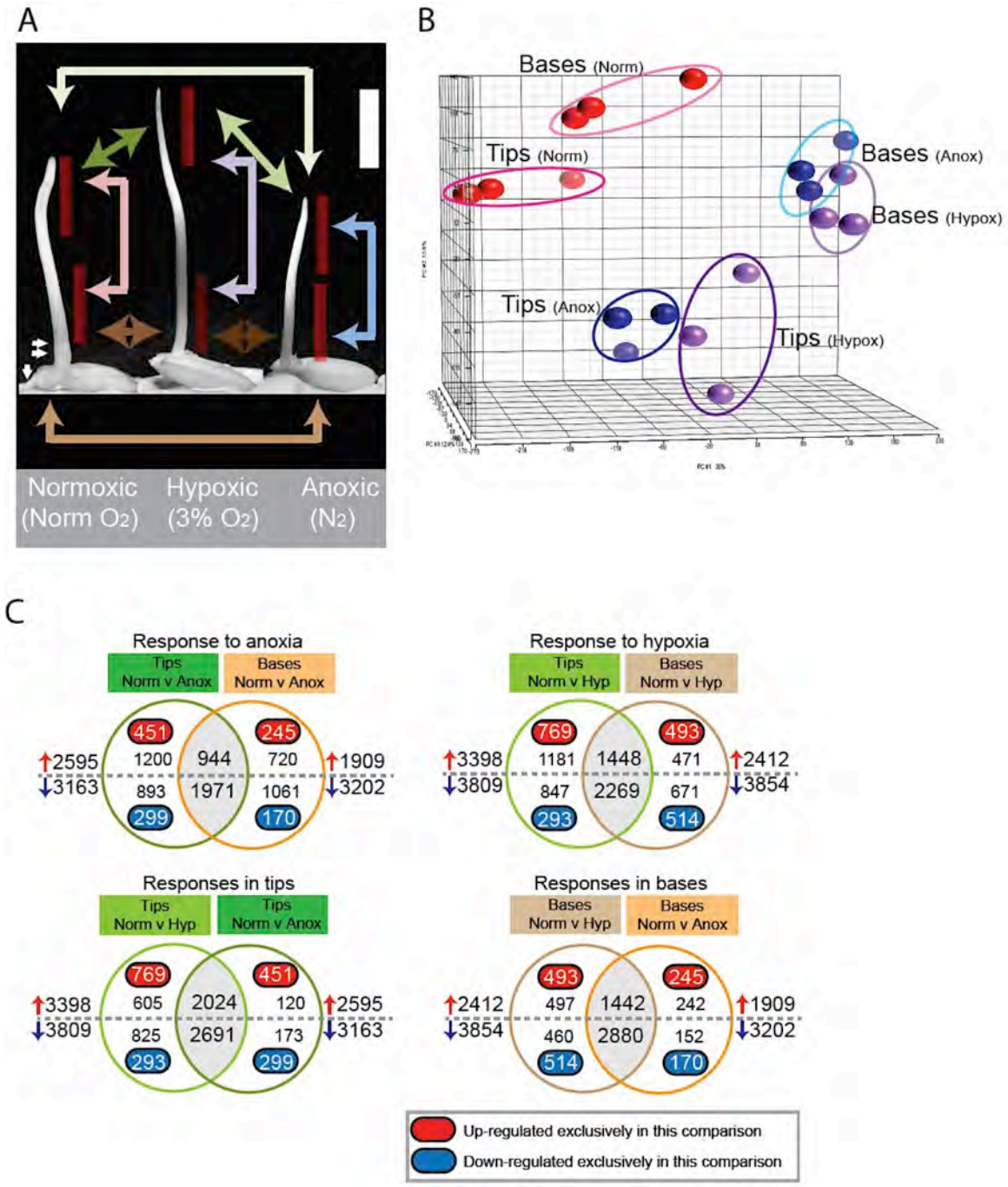


Figure 3. Overview of microarray analysis. A) The comparisons carried out for differential expression analysis. B) Principal Component Analysis of GC-RMA normalised microarray data. C) Venn diagrams show overlaps in transcriptomic responses under the O_2-deprived conditions in both tissue types.

In order to determine the basis of the differences observed by PCA analysis, differential expression analysis was carried out and the responses were compared

(Figure 3C). Firstly, the response to anoxia was compared between tips and bases, revealing 944 up-regulated and 1971 down-regulated genes that were responsive in both bases and tips (Figure 3Ci). Similarly, 1448 genes were up-regulated and 2269 genes were down-regulated in both tips and bases under hypoxic conditions (Figure 3Cii). Interestingly, regardless of tissue type, it was observed that the number of differentially expressed genes (DEGs) (using normoxia as the baseline) was higher in response to hypoxia than in response to anoxia.

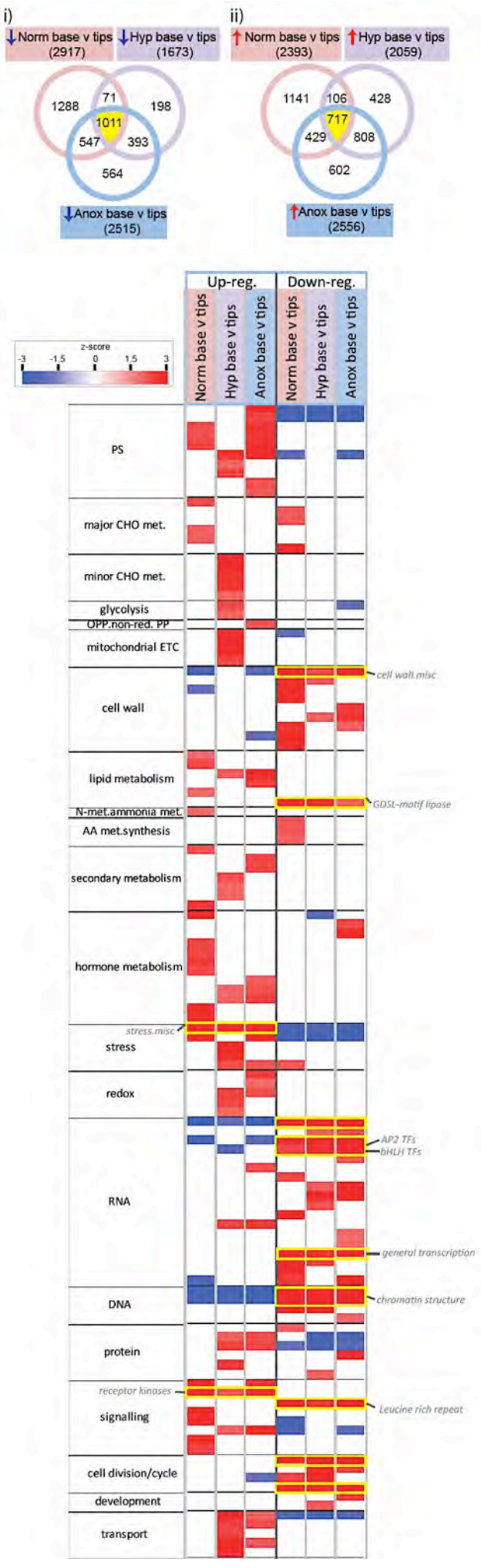
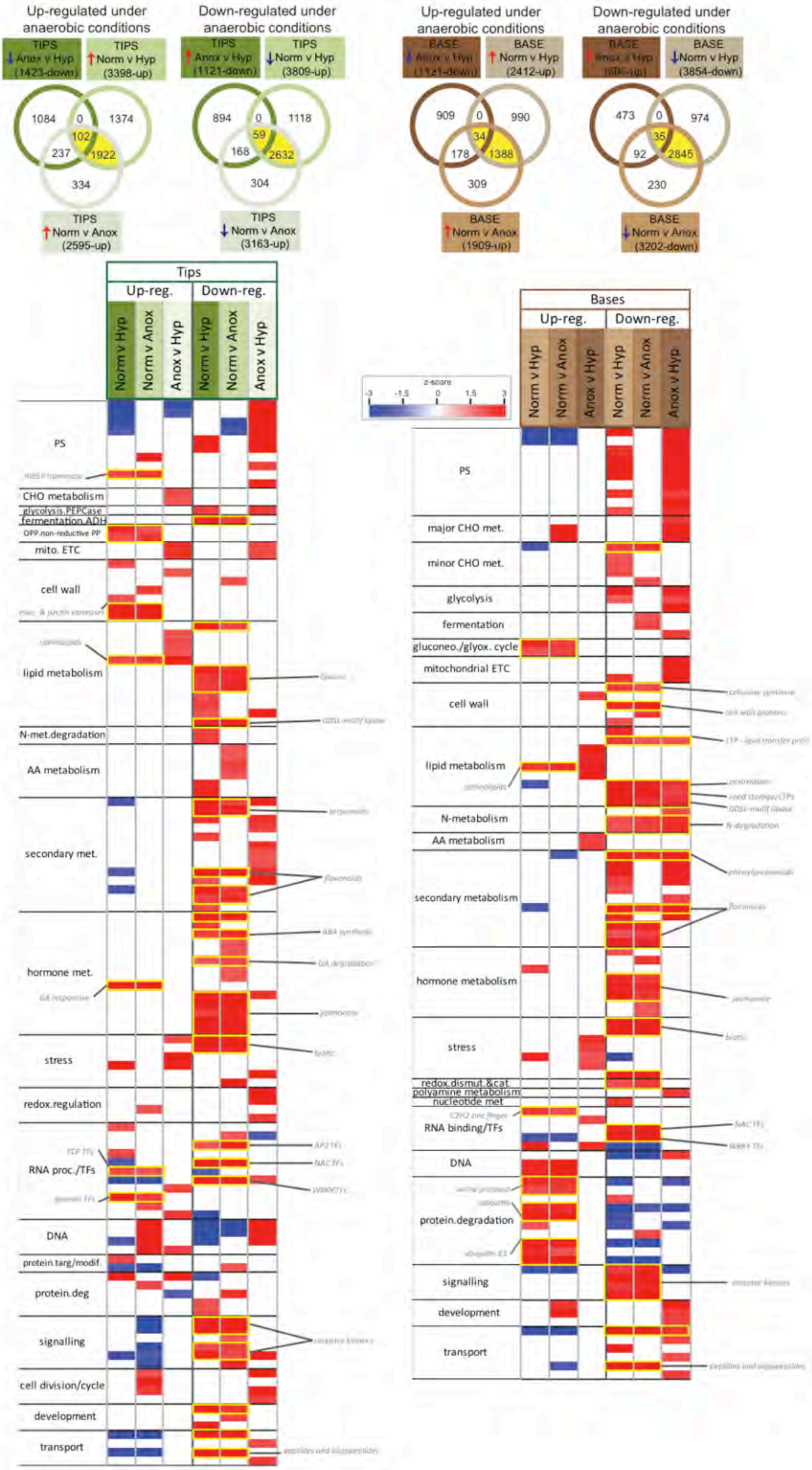


Figure 4. Differential gene expression between bases and tips, independent of O_2 conditions. (A) Venn diagram showing the overlap of probesets identified as (i) up-regulated or (ii) down-regulated in coleoptile tips compared to bases. The overlapping genes in yellow represent tissue-specific differences between bases and tips, irrespective of O_2 conditions. (B) Under/over-representation of functional categories of the differentially expressed probesets, as determined by PageMan analysis.

When the DEGs in the tips were compared in response to hypoxia and anoxia, 4715 genes (2024 + 2691) were overlapping, representing 82% of all DEGs in response to anoxia (Figure 3Ciii). Similarly, 84% of all DEGs in response to anoxia in the bases also overlapped with the hypoxia-responsive genes (Figure 3Civ), indicating that while a smaller number of genes were responsive to anoxia compared to hypoxia, a significant (z-score, $p < 0.05$) percentage (>80%) of these showed overlapping responses. In contrast, the overlapping genes only represented 65% and 69% of all hypoxia-responsive genes in the tips and bases, respectively (Figure 3Ciii, iv). In addition, 1063 DEGs (769 + 293) in the tips were responsive to hypoxia only (Figure 3Ciii) and a similar number was observed in the bases (Figure 3Civ). This indicated that the hypoxic coleoptiles displayed a larger transcriptomic response compared to those in anoxia and that ~15% of the DEGs are responsive to hypoxia only.

Given these observations of a larger response to hypoxia in bases and tips (Figure 3Ciii, iv), we carried out Pageman over-representation analysis to determine the functions of the genes that showed overlapping, or hypoxia-specific responses (Figure 5). In this way, it was shown that genes encoding pectin esterases, sphingolipid-metabolism functions, GA-responsive proteins and TCP transcription factors were up-regulated in response to both hypoxia and anoxia in the tips, while the genes encoding lipases, terpenoids, flavonoids and ABA/GA metabolism were down-regulated (Figure 5). Thus, overall, the up-regulation of genes of several categories in tips can be grouped under the common theme of growth maintenance or growth promotion. Specifically, it is interesting to see that several transcription factors belonging the TCP family are up-regulated—these are dealt with more fully in the Discussion.



Up-regulated under anaerobic conditions
Down-regulated under anaerobic conditions
TIPS
BASE
Norm v Hyp
Anox v Hyp
Norm v Anox
Tips
Bases
Up-reg.
Down-reg.
z-score
PS
CHO metabolism
glycolysis.PEPCase
fermentation.ADH
mito. ETC
cell wall
lipid metabolism
N-met.degradation
AA metabolism
secondary met.
hormone met.
stress
redox.regulation
RNA proc./TFs
DNA
protein.targ/modif.
protein.deg
signalling
cell division/cycle
development
transport
major CHO met.
minor CHO met.
glycolysis
fermentation
gluconeo./glyox. cycle
mitochondrial ETC
N-metabolism
secondary metabolism
hormone metabolism
redox.dismut.&cat.
polyamine metabolism
nucleotide met.
RNA binding/TFs
protein.degradation

(Previous page) Figure 5. Genes up-regulated in tips and bases under anaerobic conditions. (A) Venn diagrams showing the overlap of probe IDs identified as up-regulated in coleoptile tips under anaerobic conditions and (B) the over- and under-representation of functional categories of said probe IDs as determined by PageMan analysis. (C) Venn diagrams showing the overlap of probe IDs identified as up-regulated in coleoptile bases and (D) representation of functional categories of these probe IDs, as determined by PageMan analysis.

Notably, while the responses to hypoxia and anoxia in the coleoptile bases were similar to those in the tips, additional base-specific responses were also seen. Namely, the significant induction of protein degradation functions and suppression of lipid transfer proteins, peroxidases, seed storage proteins and stress-responsive genes including NAC and WRKY transcription factors (Figure 5). Analysis of the specific changes that take place in bases yields only ~ 100 genes that were strictly considered to be down-regulated in a base-specific manner and, interestingly, these tended to encode lipid metabolism functions. Overall, the more base-specific up-regulation of protein degradation coupled with the down-regulation of amino acid degradation suggests that amino acids may be recycled for protein synthesis in the bases under low-O_2 conditions.

3.3.3 Hypoxia-specific responses

Given the increased growth and cell lengths observed under hypoxia compared to anoxia (Figure 1 and 2), and the well-documented phenomena behind these, it is clear that significant molecular re-programming occurs during changes in O_2 conditions. This is also supported by the larger transcriptomic responses seen under hypoxia compared to anoxia (Figure 3C). Thus, these hypoxia-specific responses were examined in greater detail, revealing changes in cell wall, secondary metabolism, stress and photosynthesis functions (Figure 5; Figure 6). One of the strongest and most notable hypoxia-specific responses is the down-regulation of genes encoding photosystem components (Figure 6). Specifically, nine DEGs encoding photosystem II components were down-regulated under hypoxia, while only one of these was also down-regulated under anoxia (Figure 6). This gene, LOC_Os01g41710.1, which encodes the chlorophyll a-b binding protein 2, was down-regulated 15-fold in the tips and 24-fold in the bases under hypoxia, but was

down-regulated only 2-fold in the bases under anoxia (Figure 6). Similarly, three genes encoding photosystem I polypeptide subunits were all only down-regulated under hypoxia, with one of these (LOC_Os04g33830.1) showing an 18-fold and 13-fold reduction in the hypoxic tips and bases, respectively (Figure 6). To a lesser extent, a hypoxia-specific down-regulation (*cf*. anoxia) was also seen for genes encoding 6-phosphofructokinase (PPFK) in the glycolysis pathway and the internal NADH-ubiquinone oxidase in the alternate mitochondrial respiratory pathway (Figure 6). As well as these direct energy-related functions, it was interesting to find that 15 of the 19 differentially expressed peroxidases under hypoxia were differentially expressed in both tips and bases (*cf.* normoxia), while only two of these were differentially expressed in the same manner in the tips under anoxia (Figure 6). Similarly, four genes encoding acyl-CoA dehydrogenase showed a greater base-specific down-regulation under hypoxia compared to anoxia (Figure 6). In addition to these, several genes encoding secondary metabolism functions, such as phenylpropanoid and flavonoid metabolism, also showed hypoxia-specific down-regulation (Figure 6). Lastly, a collection of genes involved in development were down-regulated specifically under hypoxia, including genes encoding embryogenesis-associated and senescence-related proteins (Figure 6). Collectively, these findings suggest a controlled suppression of the aforementioned energy-related functions under hypoxia that is not observed under anoxia.

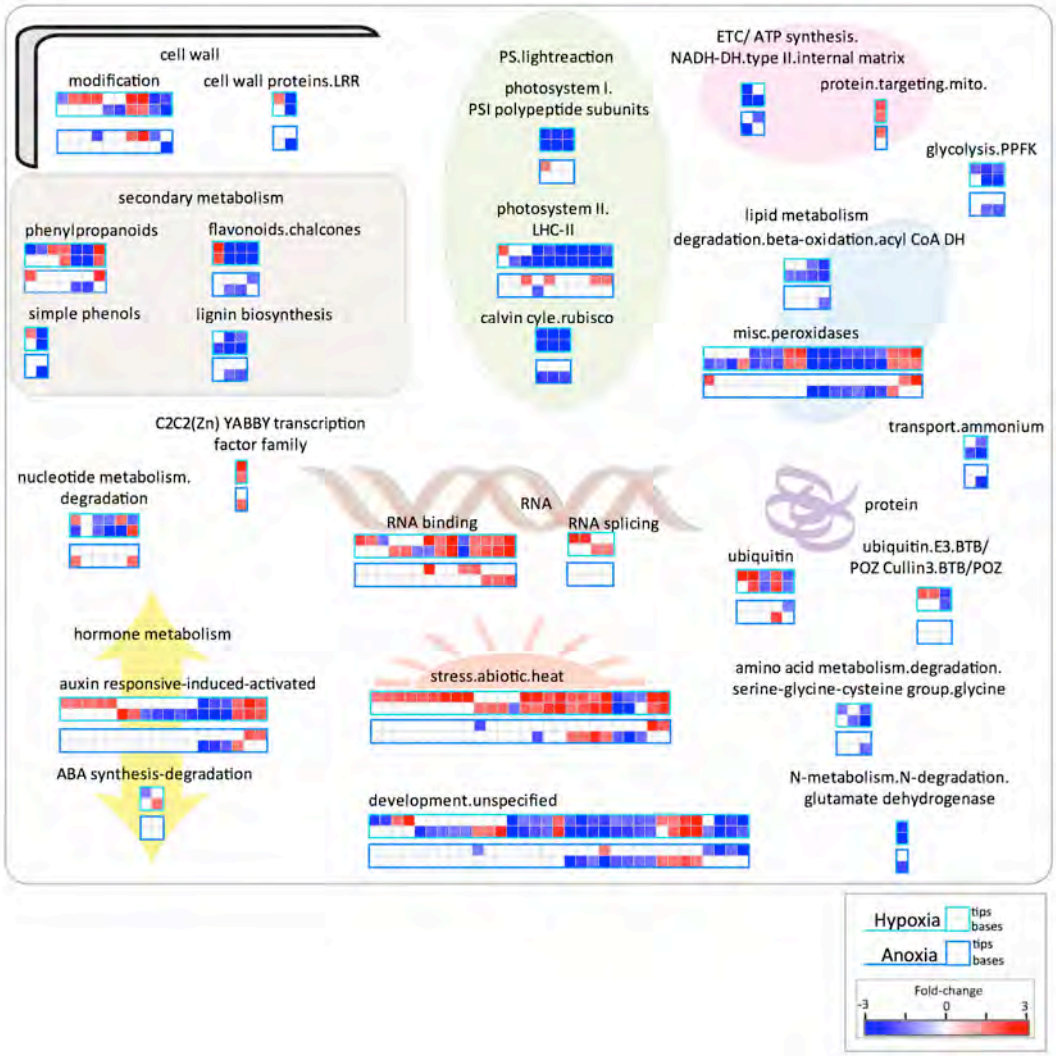


Figure 6. Different transcriptomic changes in response to hypoxia compared to anoxia. Significant fold-changes are shown for genes that were significantly differentially expressed in response to hypoxia (Norm v Hyp) and anoxia (Norm v Anox). Only genes encoding functional categories specifically affected by hypoxia (tips and/or bases) are shown.

While more genes showed hypoxia-specific down-regulation compared to anoxia, there were specific functions, particularly RNA-related functions, that were up-regulated under hypoxia (Figure 6). These included a number of genes encoding RNA binding and splicing functions that were up-regulated under hypoxia only (Figure 6). For example, a gene (LOC_Os05g24160.1) containing a double-stranded RNA binding motif was up-regulated 8- and 10-fold in the hypoxia treated tips and bases, whilst only a two-fold induction was seen in the anoxia-treated bases only (Figure 6). Similarly, 26 heat shock proteins (HSPs) were differentially expressed under hypoxia and only seven of these were responsive in the same manner under anoxia (Figure 6). For example, while the

two genes (LOC_Os04g36750.1 and LOC_Os11g13980.1) encoding 22-kDa class IV HSPs were induced 6- and 8-fold under hypoxia in the tips and bases, respectively, no differential expression was seen under anoxia (Figure 6), suggesting these to be more hypoxia-specific responses.

3.3.4 Common responses to oxygen limitation

When the responses to hypoxia and anoxia in both tips and bases were overlapped, common changes were revealed including the up-regulation of 33 genes encoding pentatricopeptide repeat (PPR) proteins (Figure 7).

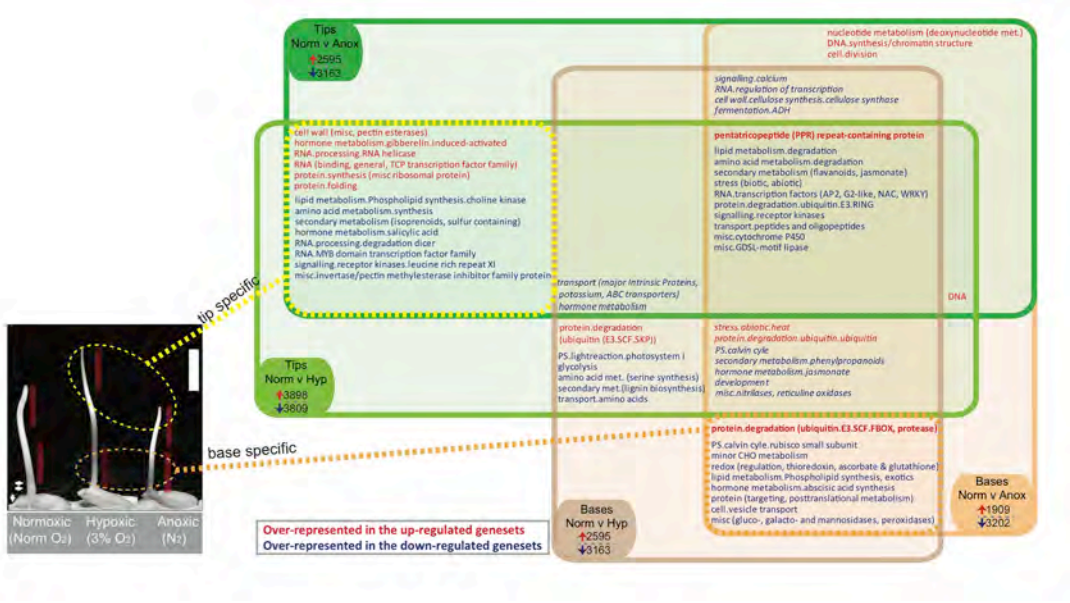


Figure 7. Over-represented functional categories in differentially expressed gene-sets for tips and bases. Pageman analysis was carried out and the common significantly over-represented functional categories ($p < 0.05$) are shown for the up-regulated genesets (in red) and down-regulated genesets (blue). The functional categories are shown within a Venn diagram to reveal common and distinct over-represented functional categories within the different regions (i.e. tips and/or bases).

For transcripts that are down-regulated in abundance in both tips and bases under O_2 limitation, a common theme seems to be a suppression of the stress response. While this suppression seems to occur across multiple stresses, it is notable that biotic stress as a category is over-represented. Thus NAC, AP2 and WRKY transcription factors, protein degradation factors (E3 ring), flavonoids and jasmonates, and signalling receptor kinases associated with stress, are all down-regulated. Thus it appears that the

stress response is being suppressed and, as this is a common response to both hypoxia and anoxia, it may represent a cellular response in order to maintain rapid growth under hypoxia (Figure 1B). This might aid the escape strategy as stress responses are typically accompanied by a cessation of growth.

3.4 Discussion

The data reported provide three areas for further exploration and discussion: (1) global differences in genes expression between coleoptile tips and bases (regardless of O_2 treatment); (2) similarities between the response of coleoptile tips and bases to O_2 deprivation and (3) the unique response of coleoptiles to hypoxia compared to anoxia.

3.4.1 Cell elongation and division in rice coleoptiles

Elongation was fastest in hypoxia (Figure 1), with Edwards *et al*. (2012) (Chapter 2) reporting that elongation rates in steady-state hypoxia were 200% and 50% faster than for coleoptiles growing in normoxia or anoxia, respectively. Hypoxic (3% O_2) coleoptiles are also heavier than their anoxic counterparts but not the normoxic coleoptiles, even after enclosed leaves were removed.

Epidermal cell lengths were measured in hypoxic coleoptiles from unstirred solutions in order to infer elemental rates of elongation continuously along coleoptiles of various lengths (Figure 2). Hypoxic coleoptiles were used for this purpose because they elongated very rapidly for many days and could be imaged *in vivo* in related experiments that are not reported in this thesis. Micrographs showed that cells in the mid-region of coleoptiles were longest, regardless of age (Figure 2), with the epidermal cells in the most distal tip region intrinsically short in spite of being the oldest cells, as reported previously (Wada, 1961). Coleoptile elongation from 6 to 18 mm over one day (Edwards *et al*., 2012) coincided with rapid elongation of cells within the basal third of the coleoptile (see steep gradient in Figure 2). As in the analysis of Furuya *et al*. (1969),

cell lengths in our study increased rapidly from nearest to the seed, peaking approximately one-third of way along the coleoptile as measured from the base, with final cell lengths and elongation rates declining in the tip region (Figure 2). New embryonic cells were continuously recruited into the coleoptile, with separate kinematic analysis (Atwell, pers. comm.) showing that these basal segments elongated at rates up to 20–30% per hour (refer to micrographs A & B in Figure 2).

To provide some mechanistic basis for rapid growth in coleoptile basal cells, we analysed tissue-specific (base *vs* tips) transcriptomes of 12–18 mm-long (~3-day old) coleoptiles for microarray analysis in all three treatments (Figure 1). This approach was apposite as it enabled tissues to be compared under three O_2 regimes with a precise knowledge of local elongation rates (*cf*. Lasanthi-Kudahettige *et al*., 2007; Takahashi *et al*., 2011). Furthermore, coleoptiles sampled at age 3 d had not begun to senesce, a process which begins in the days to follow in distal tissues of normoxic coleoptiles (Kawai and Uchimiya, 2000).

Previous studies of gene expression in rice coleoptiles have focused on intact coleoptiles excised from seedlings that were grown in either normoxic or anoxic conditions (Lasanthi-Kudahettige *et al*., 2007; Narsai *et al*., 2009), without addressing the differential growth along the coleoptile axis (Wada, 1961; Furuya *et al*., 1969). Here, we examined changes in the transcriptome of the distal zone ('tips') and basal zone ('bases') of rice coleoptiles grown under three O_2 regimes, including severe hypoxia (one-seventh of the O_2 available in full aeration). This design enabled us to examine two biological phenomena and their interactions. We examined gene expression patterns that characterise the most rapidly elongating cells in the basal zones with those approaching maturity in the apical zones. Secondly, we compared the transcriptomes of tissues growing under severe hypoxia with those in normoxia or anoxia in order to identify the genes required for accelerated growth in submerged coleoptiles. This also

gave the opportunity to study the effects of O_2 deprivation by comparing gene expression patterns in hypoxia and anoxia with those in normoxic tissues. Finally, the expression of genes peculiar to the interaction between growth and energetics could be investigated through this study.

The observations in Figure 2 help quantify the complex spatial distribution of cell division and elongation in the coleoptile segments we used for microarray analysis. With this knowledge, we determined which functional categories were over-represented in the list of genes differentially up-regulated in coleoptile bases compared with tips, regardless of O_2 treatment (Figure 3). Five broad functional categories were over-represented in the genes commonly up-regulated in the bases regardless of O_2 treatment: genes involved in cell wall synthesis and modification; structural organisation and replication of DNA; regulation of cell division and the cell cycle and members of the AP2 and bHLH transcription factor families.

3.4.2 Cell wall synthesis

Plant cell walls consist of cellulose microfibrils connected by a web of polysaccharide and peptide crosslinks (Cosgrove, 2005). Cell elongation involves three processes: the weakening of crosslinks between cellulose microfibrils, turgor-driven expansion and deposition of cross-linking polymers and new cellulose microfibrils (Cosgrove and Jarvis, 2012). Genes involved in the biosynthesis of these key molecules (cellulose) are over-represented amongst those up-regulated in the bases compared to the tips (Supplementary Table B1).

Amongst the over-represented genes were a number of β-1,4-glucanases and pectin esterases (Supplementary Table B1). β-1,4-glucanases act as cellulases, capable of hydrolysing the bonds between adjacent xyloglucans within cellulose microfibrils (Cosgrove, 2005). A rice mutant line lacking a functional *OsGLU3*, which encodes a β-1,4-

glucanase, showed reduced root elongation compared to the wild-type. This was due to decreased cell elongation (Zhang *et al*., 2012). Similarly, loss-of-function mutations in *OsGLU1* result in decreased cell elongation in rice stems compared to the wild-type (Zhou *et al*., 2006). Pectin esterases (PE) act to break the pectin crosslinks between cellulose microfibrils within the cell wall, weakening and loosening the cell wall to allow cell elongation to occur (Cosgrove, 2005). Gao *et al*. (2009) showed that mutations affecting *D88*, a rice pectin esterase, led to a dwarf phenotype caused by decreased cell elongation within the stem. Derbyshire *et al*. (2007) showed that two *Arabidopsis* mutant lines with non-functional PEs showed reduced cell elongation in the hypocotyl. Finally, Gou *et al*. (2007) noted the over-expression of a wide variety of PEs in the elongation fibres of cotton seeds.

A large number of these genes encoding for α- and β-expansins, which promote cell elongation (Cosgrove, 2005), were also amongst the over-represented genes. The correlation between the presence of expansins and rapid elongation in rice internodes has been demonstrated in deepwater rice (Cho and Kende, 1997a; Cho and Kende, 1997b; Lee and Kende, 2001); submerged internodes were enriched in expansins compared to non-elongating internodes. Huang *et al*. (2000) also reported that the rice expansin *Os-EXP4* was strongly expressed in submerged coleoptiles. Choi *et al*. (2003) constructed over-expression and anti-sense lines for the rice expansin gene *OsEXP4*, demonstrating that expansin overexpression lines had coleoptiles one-third longer than the wild-type, with a corresponding increase in cell size, while anti-sense lines gave proportionately lower dimensions for coleoptile length and cell size.

A third group of cell wall metabolism genes that were strongly over-represented in bases were the cellulose synthase-like genes. This is an extensive family in rice (Hazen *et al.,* 2002), with members playing key roles in the formation of normal morphology of the root hair (Kim *et al*., 2007) and cell walls (Burton *et al*., 2006, Li *et al.*,

2009). Cellulose content is central to cell wall integrity (Cosgrove, 2005) and the polarity of growth through microfibril orientation (Sugimoto *et al*., 2001), even though anoxic coleoptiles invested a small proportion of total biomass in cell walls (Edwards *et al*., 2012) and therefore had low cellulose content (Alpi and Beevers, 1983).

In addition to the continuing extension of existing cells, coleoptiles increase their length through growth of newly divided cells. Edwards *et al.* (2012) reported the continual synthesis of DNA in rice coleoptiles regardless of O_2 supply. Additionally, as the cells elongating at the base of the coleoptile reach their maximum length (200–250 μm (Wada, 1961; Furuyu *et al*., 1969)), new cells would be required to allow coleoptile elongation to proceed.

Figure 4 shows genes with functions related to DNA synthesis and chromatin structure. Those with a function in DNA synthesis include DNA polymerase, DNA helicase, DNA primase, DNA exonuclease and components of the origin recognition complex (Bryant *et al*., 2001). A large number of genes encoding histones, proteins involved in the structural remodeling of genetic material during cell division, were also up-regulated in the bases of the coleoptiles. Two genes that encode for cell division control-proteins, CDC6 and CDC45, (Os01g63710 and Os11g03430 respectively), increased their relative abundance 1.6- to 3-fold and 1.7-to 7-fold respectively. These two genes interact with other members of the origin recognition complex to regulate DNA replication, with the replacement of *Cdc6* by *Cdc45* resulting in the recruitment of DNA polymerase to begin DNA replication (Mimura and Takisawa, 1998; Mimura *et al*., 2000; Mori *et al*., 2005). Genes encoding for proteins regulating the *onset* of cell division were also strongly over-represented in the up-regulated genes basal genes, including a large number of members of the cyclin family.

In addition to the above genes, a number of genes related to controlling the formation of the mitotic spindle (Os04g40940) and maintaining chromosomal cohesion

during mitosis were more highly represented in the basal tissues. One of these genes (Os05g41750) is homologous to *AtSMC3,* a gene that is required for successful segregation of chromosomes (Lam *et al*., 2005). Taken together, these differences in gene expression (bases *vs* tips) suggest greater rates of cell division within the bases of the coleoptiles compared to the tips.

3.4.3 Transcription factors

bHLH and AP2 transcription factor families were also significantly over-represented in the bases of coleoptiles from each O_2 treatment. The bHLH family of transcription factors contains at least 167 members in rice (Li *et al*., 2006). They serve a wide variety of roles in plants, including driving the response to phosphate starvation (Yi *et al*., 2005), control of shoot formation (Komatsu *et al*., 2003) and, in an antagonistic relationship with HLH transcription factors, control of tissue elongation, with bHLH factors acting to prevent elongation (Zhang *et al*., 2009). Rice plants transformed with an anti-sense construct against a bHLH inhibitor gene were significantly shorter than wild-type coleoptiles, while those over-expressing the inhibitor where significantly longer (Zhang *et al*., 2009). Those genes up-regulated in the bases appear to be members of the HLH sub-family, suggestive of an inhibition of bHLH activity and consistent with the elongation of cells reported in Figures 2 and 3.

The second major family of transcription factors over-represented in the bases is the AP2 family. This is a large family of ethylene response factors in rice, which have been implicated in control of responses to cold (Ito *et al*., 2006), drought (Shinozaki *et al*., 2007) and bacterial and nematode infection (Gutterson and Reuber, 2004). Two of the AP2 genes up-regulated in all the bases show extremely high sequence similarity to two *Arabidopsis* genes *BABY BOOM* (Os02g40070) and *AINTEGUMENTA* (Os03g12950), which have been found to control (and promote) the proliferation of new cells (Mizukami and Fischer, 2000; Passarinho *et al*., 2008).

Taken together, these patterns of gene expression suggest a greater degree of cell wall synthesis, modification and extensibility (cell elongation) and cell division in the base of the coleoptile compared to the tip, regardless of O_2 treatment. When considered alongside Figures 2 and Furuya *et al*. (1969), we conclude that, regardless of O_2 availability, the basal regions of submerged rice coleoptiles are the primary site of cell elongation and division in the growing coleoptile.

3.4.4 Effects of oxygen deprivation

In addition to examining universal changes between the tips and bases of coleoptiles, we examined the impact of hypoxia on the transcriptomes of coleoptile tissues to determine how they differ from anoxic and normoxic coleoptiles, which grew much more slowly. Chapter 2 shows that ATP utilisation of hypoxic coleoptiles was intermediate between that of normoxic and anoxic coleoptiles but not uniformly for each metabolic process. For example, rates of cell wall synthesis, K^+ influx, nitrate import and amino acid synthesis were not significantly different from those of normoxic coleoptiles, whereas protein synthesis, nucleic acid synthesis and carbohydrate import were closer to those of anoxic coleoptiles. We hypothesised that this metabolic 'compromise', which enabled rapid turgor-driven cell elongation, would be reflected in gene expression patterns.

Microarrays clustered into three distinct groups: normoxic tips/bases, hypoxic/anoxic tips and hypoxic/anoxic bases (Figure 3B). Based on this analysis, we further examined which groups were over-represented in a four-way comparison (Figure 7): genes up-regulated by both hypoxia and anoxia in comparison to normoxia in a tip-specific, base-specific and whole-tissue sense, and those up-regulated in either hypoxia *or* anoxia in comparison to normoxia.

3.4.5 Tip-specific changes

Within the tips, genes involved in cell wall loosening and synthesis (see previous section) as defined by Cosgrove (2005) were over-represented.

Genes associated with protein synthesis, particularly those encoding ribosomal proteins, and folding (post-translational modification), were also over-represented in anoxic and hypoxic rice coleoptile tips compared to normoxic tips. During anoxia, translation of key proteins is a result of the formation of polysomal complexes translating key anaerobic genes (Taliercio and Chourey, 1989; Fennoy and Bailey-Serres, 1995; Bailey-Serres *et al*., 2009). This minimises the cost of protein synthesis by reducing the amount of mRNA required for translation (Bailey-Serres *et al*., 2009). Additionally, genes encoding for post-transcriptional RNA editing, particularly the RNA helicases, which play a key role in nuclear mRNA export (Xiong *et al.*, 2001*)* and regulation of translation (Jankowsky and Bowers, 2006), were also over-represented.

The TCP transcription factor family is also over-represented (Figure 5) in O_2-deprived coleoptile tips. These transcription factors contain domains similar to the bHLH transcription factors discussed above; they are frequently associated with cell division (Kosugi and Ohashi, 1997). Two members of the family, PROLIFERATION CELL FACTOR 1 and 2 (PCF1 and PCF2), are vital for the function of meristematic cells within rice (Kosugi and Ohashi, 1997), while a third OsTB1 regulates lateral branching via repression of axillary meristems (Takeda *et al*., 2003). TCP transcription factors are also involved in maintenance of the circadian clock in *Arabidopsis* (Pruneda-Paz *et al*., 2009; Giraud *et al*., 2010). Additionally, it has also been shown that TCP binding sites are enriched in genes encoding ribosomal proteins (Trémousaygue *et al*., 2003; Tatematsu *et al*., 2008), which is evident here, as 45 genes annotated as encoding ribosomal proteins are also up-regulated, as well as transcripts encoding proteins involved in protein folding. The resulting effect of the up-regulation of these genes is that several

transcripts annotated as being involved in cell wall synthesis/remodeling are also up-regulated. Thus, overall growth processes appear to be up-regulated in tips.

The over-representation of these functional categories in hypoxic and anoxic coleoptile tips compared to normoxic coleoptile tips reflects the fact that, at the time of harvesting, normoxic coleoptiles would be expected to have been in an early stage of senescence (Kawai and Uchimiya, 2000), while the hypoxic and anoxic coleoptiles were capable of elongating for several days longer (Atwell et al., 1982). Consistent with this, normoxic coleoptile tips had less RNA: levels expressed in μg RNA per gram fresh weight were 154 (normoxic); 247 (hypoxic) and 177 (anoxic) (Table 1). Accordingly, hypoxic and anoxic tips had higher expression levels of genes encoding for ribosomal proteins than normoxic coleoptiles, suggesting sustained metabolic activity in these O_2-deprived tissues.

3.4.6 Base-specific changes

Within the hypoxic and anoxic bases there is a consistent over-representation of genes involved with the degradation and re-cycling of protein, including ubiquitin and a broad range of proteases consistent with a high rate of protein degradation and turnover. Hypoxic and anoxic coleoptiles maintain a rate of gross protein synthesis 44% and 34% (respectively) of that of normoxic coleoptiles (Edwards *et al*., 2012) even though the net increase in protein content of the anoxic coleoptile does not exceed one-quarter of that in normoxic coleoptiles (Mocquot *et al*., 1981; Alpi and Beevers, 1983; Edwards *et al*., 2012). Given the high cost of amino acid synthesis (Penning de Vries, 1974) compared to the degradation and re-synthesis of protein (Amthor, 2000), and the relatively high rates of protein synthesis in these tissues, turnover of existing proteins would act to significantly decrease ATP utilisation.

Finally, regardless of tissue, there is a consistent over-representation of the members of the pentatricopeptide repeat-containing proteins (PPR) family in the genes

up-regulated by hypoxia and anoxia. Members of this large family of genes in plants are associated with editing or processing of organelle mRNA molecules (Andres *et al*., 2007; Delannoy *et al*., 2007) including stabilisation (Mancebo *et al.*, 2001; Yamazaki *et al*., 2004), splicing and post-transcriptional processing (Meierhoff *et al*., 2003; Ellis *et al*., 2004) and degradation/stabilisation (Akagi *et al*., 2004; Komori *et al*., 2004; Wang *et al*., 2006). They have been found to have a broad range of roles in organelle development in plants (Lurin *et al*., 2004; Delannoy *et al*., 2007) and many are responsive to a variety of other stress treatments (Yan *et al*., 2006; Ahsan *et al*., 2007a; Jain *et al*., 2007). Within rice, members of the PPR family are essential for proper mitochondrial (Kazama and Toriyama, 2003; Kocabek *et al*., 2006) and chloroplast development (Gothandam *et al*., 2005). An analysis of the 33 PPR genes that are up-regulated in transcript abundance reveal that they are generally widely expressed and have previously been documented to be induced under O_2 deprivation in rice (Lasanthi-Kudahettige *et al*., 2007), However a comparison to *Arabidopsis* orthologs reveals that while many are also widely expressed, they do not appear to be induced by O_2 limitation but are induced by a variety of other stresses (Phee *et al*., 2004; Baxter *et al*., 2007). Notably, for both rice and *Arabidopsis*, this set of proteins is reported to be expressed during germination (Ashan *et al*., 2007b); however during germination under anaerobic conditions and O_2 deprivation, they are expressed in rice but not in *Arabidopsis*.

3.4.7 Genes over represented in anoxic tissues

Genes related to DNA synthesis/structure and the cell cycle are significantly over-represented in the genes up-regulated in anoxic tissues compared to normoxic tissues. This is suggestive of continued DNA turnover or cell division in the anoxic tissues compared to the normoxic tissues at the time they were sampled. At approximately 3 d post-imbibition in this experiment when mRNA was extracted, cell division had ceased in normoxic coleoptiles (Wada, 1961; Furuya *et al*., 1969; Takahashi *et al*., 2011) and

some of the tip cells were on the brink of senescence (Edwards *et al*., 2012). Given the continued slow growth of anoxic coleoptiles past 3 d and their continued synthesis of new DNA (Edwards *et al*., 2012), it is consistent that genes for cell division continued to be expressed.

3.4.8 Genes regulated by hypoxia but not anoxia

As mentioned previously (Figure 3), ~15% of the genes regulated by hypoxia (*cf.* normoxia) were not correspondingly regulated by anoxia. By mapping these genes to functional categories (Figure 6), we were able to examine the differences between the hypoxic and anoxic response in coleoptiles.

Physiologically, hypoxic coleoptiles are longer than anoxic coleoptiles (Figure 1 and Edwards *et al*., 2012), elongate at a faster rate (0.5 mm h^{-1} *vs* 0.1 mm h^{-1} in anoxia) and have a higher biomass-to-length ratio than anoxic coleoptiles, hence being structurally stronger (Edwards *et al*., 2012). They are also metabolically different from anoxic coleoptiles with higher rates of cell wall (5×), amino acid (3×) and protein (1.5×) synthesis as well as higher rates of K^+ import from the surrounding media (3×) (Edwards *et al*., 2012). Hypoxia therefore, appears to elicit a unique response in rice coleoptiles distinct to that of anoxia.

The distinct differences between the changes in gene expression in hypoxic and anoxic coleoptiles (*cf*. normoxia) support the physiological changes referred to above. Numerous families of genes were up- and down-regulated by hypoxia (*cf*. normoxia) but not anoxia (Figure 7). Those up-regulated by hypoxia (but not anoxia) include heat stress genes, RNA binding and splicing genes and genes involved in protein degradation (ubiquitin pathway). Those primarily down-regulated by hypoxia (*cf*. normoxia) (but not anoxia) include the peroxidases, photosystem II genes and a broad grouping of developmental genes. Genes involved in cell wall modification and auxin response were

both up- and down-regulated in hypoxia (*cf.* normoxia) but were similarly affected in anoxia.

A wide range of heat stress protein (HSP) genes were up-regulated by hypoxia, including the 16.9- and 17.4-kDa class 1 genes, the 17.5-kDa class 2 and 70-kDa HSPs. HSPs are a broad family of genes that act primarily as molecular chaperones and maintain cellular membrane integrity (Feder and Hofmann, 1999; Wang *et al*., 2004). This gene family has previously been reported as being induced by anoxia in rice (Lasanthi-Kudahettiege *et al*., 2007) as well as being induced by hypoxia in *Arabidopsis* (Liu *et al.*, 2005). Banthi *et al*. (2008) reported that pretreatment of *Arabidopsis* seedlings with either heat stress or hypoxia improved survival in anoxia to the same degree. Banthi *et al*. (2010) showed that *Arabidopsis* heat shock factor A2 (*ATHsfA2*) was required for these pretreatments to work: *ATHsfA2* knockout mutants exhibited no advantage in anoxic survival after either hypoxic or heat stress pretreatments. Similar inductions of HSP genes by hypoxia/anoxia are conserved across the plant kingdom (Mustroph *et al*., 2010). Based on these previous findings, it appears that these genes may be playing two roles in hypoxia: acting as metabolic chaperones and in an acclimative role to increase anoxia tolerance. The first role is relatively straight forward: the metabolic differences between normoxic and hypoxic coleoptiles would require the synthesis and cellular localisation of a new suite of proteins. The second depends upon the ecology of rice: in field conditions such as submergence or in fully-inundated soils, anoxia is preceded by a period of hypoxia as O_2 supplies are consumed. By synthesising these genes and proteins while ATP production is still relatively high (hypoxia *cf.* anoxia) (Edwards *et al*., 2012) rice coleoptiles would improve their ability to survive (and elongate out of) the prolonged anoxia to follow.

The genes involved in RNA binding and splicing include genes encoding for a ribonucleoprotein (LOC_Os07g46820) and pre-mRNA slicing factor 18

(LOC_Os03g49430) and 31 (LOC_Os07g04850) and protein splicing factor, arginine/serine-rich 7 (LOC_Os02g39720). Together, they play an important role in the alternate splicing and translation of mRNA in rice (Lorkovic *et al*., 2000; Isshiki *et al*., 2006). Together with the large number of ribosomal proteins that are up-regulated in the tips of hypoxic coleoptiles (but not anoxic coleoptiles) they can help maintain the higher rates of protein synthesis reported in hypoxic coleoptiles (*cf*. anoxic coleoptiles) (Edwards *et al*., 2012). The proteins involved in RNA binding and splicing may also play a role in controlling the formation of polysomes, a key means by which protein expression is focussed during hypoxia (Branco-Price *et al*., 2008).

Genes involved in the ubiquitin pathway were also up-regulated in hypoxia (*cf*. normoxia) but not in anoxia. The bases of hypoxic coleoptiles contained more protein than their anoxic counterparts while the tips did not (Table 1). On a whole-coleoptile scale, there was also no significant difference in protein content (Edwards *et al*., 2012) between the two treatments. Thus, in order to maintain the higher rates of protein synthesis a source of amino acids is required. Increased activity of the ubiquitin pathway would provide such a source of amino acids (Moon *et al.,* 2004) to support this protein synthesis.

Genes encoding peroxidases were broadly down-regulated by hypoxia (*cf.* normoxia) but not anoxia. Ismail *et al*. (2009) reported that peroxidase activity decreased cell wall extensibility in rice while Lee and Lin (1996) reported that peroxidase enzymatic activity decreased in elongating tissues (normoxic shoots and roots, anoxic coleoptiles) but increased in non-elongating tissue (anoxic shoots and roots). MacAdam *et al*. (1992a,b), working in *Festuca arundinacea* (tall fescue), reported that cessation of leaf cell expansion coincided with the secretion of soluble cationic peroxidases in the leaf apoplasm. By maintaining low levels of peroxidase gene expression (and likely enzyme activity) hypoxic coleoptiles would increase their cell

wall extensibility (*cf*. normoxic and anoxic coleoptiles), further supporting the physiology reported above.

Genes associated with the photosystem II (PSII) light harvesting complex (LHCII) were strongly down-regulated in hypoxia (*cf*. normoxia) in both tips and bases by (2–15-fold). LHCII is a membrane-bound component of a super-complex of proteins that form PSII (reviewed by Kouřil *et al*., 2012). Inada *et al*. (1998) reported the existence of chloroplasts with well-developed thylakoid systems in 3-d old normoxic coleoptiles. Kordan (1976) reported that normoxic coleoptiles grown in light contain chlorophyll (suggesting active photosynthesis) while those grown in anoxia do not contain chlorophyll. Additionally, while dark-grown normoxic coleoptiles did not contain chlorophyll, they had a light yellow-green colour, consistent with the presence of portions of the photosystem being expressed within the coleoptile. From these previously published results, hypoxic coleoptiles down-regulating the production of LHCII components (*cf*. normoxic coleoptiles) would reduce demand on both limited amino acid and ATP supplies, allowing them to be directed into other metabolic processes. This further illustrates the fine control exhibited on gene expression by the hypoxic coleoptile.

Genes involved in the modification of the cell wall were both up- and down-regulated in response to hypoxia (*cf*. normoxia) but not anoxia. They primarily encode expansins, which are proteins that aid the extension of cell walls (Cosgrove, 2000). There are also differences in the expression of these genes between tips and bases (Figure 6). β-expansin A1 (LOC_Os03g01270) was down regulated (~1.6×) in both tips and bases while α-expansin 1 (LOC_Os05g19570) was down-regulated 2.2× in bases only. In comparison, α-expansins 10 (LOC_Os03g21820), 13 (LOC_Os04g49420) and 15 (LOC_Os02g51040) were all up-regulated in hypoxic tips (1.7×, 1.6× and 2.0× respectively), while β-expansin 4 (LOC_Os03g44290) was up-regulated 1.6× in hypoxic

bases. Lee and Kende (2001) reported that β-expansin 4 expression was increased by submergence in elongating rice stems. Lee and Kende (2002) reported that α-expansins 13 and 15 were expressed in roots of deep-water rice and that α-expansin 10 was expressed constitutively with its highest levels in rapidly elongating stem sections. The differential regulatory (up- or down-regulated) and spatial patterns of the α- and β-expansin expression showcase the complex role these genes play in cell elongation, the tight regulatory control on their expression and highlight the need for further research into their precise roles in hypoxic coleoptile elongation.

Changes in the expression of a range of auxin responsive/induced/activated genes are also induced by hypoxia (*cf*. normoxia) but not anoxia. Those up-regulated include auxin-induced growth promoters (LOC_Os06g47290 and LOC_Os02g49460) in the tips and members of the Small Auxin-Up RNAs (OsSAUR) (LOC_Os09g26610, LOC_Os09g37330, LOC_Os09g37446 and LOC_Os09g37480) in the bases. Down-regulated in both tips and bases were a large number of the Aux/IAA family genes including *IAA2*, *IAA11*, *IAA22* and *IAA23*. Both IAA11 and IAA23 have been shown to play a role in cell division: an *IAA11* gain of function mutant inhibited the formation of lateral root primordia (Zhu *et al*., 2012) while a loss-of-function mutation in *IAA23* led to the loss of identity of the quiescent centre of roots (Jun *et al*., 2011). The OsSAUR as synthesised in response to auxin appear be localised to the nucleus and have a poorly understood function within the cell (Jain *et al*., 2006). Within *Arabidopsis* over-expression of the *SAUR19* subfamily promotes cell expansion throughout the plant (Spartz *et al*., 2012) while the *SAUR63* subfamily promotes elongation of the hypocotyl (Chae *et al*., 2012). Cells in the base of hypoxic coleoptiles elongate more rapidly than those in the tip (Figure 2). Based on the evidence reported in *Arabidopsis*, it is likely that the *OsSAUR* genes reported enhance cell elongation in the hypoxic coleoptile bases.

3.5 Conclusions

This chapter reports on the most extensive study on the changes of gene expression in rice coleoptiles under decreasing O_2 supply to date. Additionally, it also reports on the intra-coleoptile differences in gene expression between the distal (tip) and basal (base) regions of the coleoptile. Based on these findings, we concluded that the base of the coleoptile, regardless of O_2 supply, shows clear signs of being the locus of cell division and elongation within the coleoptile, that there are clear differences in response of coleoptile tips and bases to hypoxia and anoxia and finally, that hypoxia induces unique changes in gene expression that maximise the rate of cell (and thus coleoptile) elongation compared to either normoxia or anoxia.

4. Chapter 4 - An examination of the proteome of O_2-deprived rice coleoptiles

4.1. Introduction

Oxygen (O_2) deprivation causes a large and immediate decrease in the rate of ATP production by slowing or halting oxidative phosphorylation (Gibbs and Greenway, 2003). In partial compensation, fermentative pathways become active, increasing the rate of glycolysis (the Pasteur Effect) to produce up to one-third of the energy available in aerobic tissues. To match this decreased ATP production, Atkinson (1969) proposed that there would be a matching decrease in ATP utilisation.

Edwards *et al*. (2012) (Chapter 2) examined the production and utilisation of ATP using a notional budget in normoxic, hypoxic and anoxic rice coleoptiles. It was found that the absolute rates of the major metabolic (protein, nucleic acid, lipid, amino acid and cell wall synthesis) and transport (carbohydrate and nitrate) processes were down-regulated in hypoxic and anoxic coleoptiles compared to normoxic coleoptiles. Importantly, however, the *relative* proportion of ATP directed towards each of these processes was not the same between the three O_2 treatments. When hypoxic, anoxic and normoxic coleoptiles were analysed, not only was protein synthesis identified as the metabolic process consuming the largest proportion of available ATP, but the percentage of ATP utilised ranged from 14% and 19% in hypoxia and normoxia, respectively, to 53% of available ATP in anoxia (Edwards *et al.*, 2012).

Given the proportionally large amount of ATP dedicated to protein synthesis during O_2 deprivation, we examined the differences in the proteome of normoxic, hypoxic and anoxic coleoptiles. Furthermore, to allow comparison to our transcriptome analysis reported in the previous chapter (Chapter 3), we divided coleoptiles into distal ('tip')

and basal ('base') sections. This afforded a developmental comparison at the gene and protein levels.

4.1.1. The proteome of anoxic plants

During O_2 deprivation, early proteomic studies have shown that plant tissues relatively well adapted to hypoxia preferentially synthesise proteins involved in the maintenance of cellular pH and ATP production. In early studies on maize, Sachs *et al*. (1980) determined that only 21 distinct proteins (including ADH1 and ADH2), termed the ANaerobic Proteins (ANPs), accounted for greater than 70% of all protein synthesis in anoxic maize root tips. Chang *et al*. (2000) was able to identify a large number of these ANPs, including glycolytic/fermentative proteins such as pyruvate decarboxylase 2 (PDC2), enolase 1 and 2 and UDP-glucose pyrophosphorylase, as well as proteins involved in the maintenance of cellular pH (glutamate dehydrogenase and malate dehydrogenase).

4.1.2. Previous proteomics studies on rice coleoptiles

Rice is the cereal most tolerant to growth, development and survival during prolonged O_2 deprivation. In addition to the ANPs described above, rice has been found to synthesise a wide variety of proteins beyond the classic ANPs. Huang *et al*. (2005) utilised ^{35}S-methionine labelling to examine the *de novo* synthesis of proteins in anoxic rice coleoptiles. In addition to classic ANPs such as ADH1, ADH2 and fructose 1,6-bisphosphate aldolase, identified proteins included a glycine-rich RNA-binding protein, manganese superoxide dismutase (Mn-SOD), pyruvate orthophosphate dikinase (PPDK) and a nucleoside disphosphate kinase as being induced by anoxia.

Sadiq *et al*. (2011) also studied changes in the proteome of anoxic rice coleoptiles. Apart from the ANPs discussed (PDC2), members of the heat-shock protein family were identified as being induced by anoxia. Sadiq *et al.* (2011) also compared

changes in protein levels with corresponding changes in gene transcripts encoding these proteins. They found that increase in mRNA levels were matched by increases in protein levels while decreases in mRNA levels were matched by decreases in protein levels. These changes, however, were normally non-identical in magnitude (e.g. for the universal stress protein, a 6× increase in protein levels (anoxia *vs* normoxia) was accompanied by a 600× increase in mRNA levels), suggesting control of protein synthesis was being exerted at the post-translational level, possibly via the action of micro RNAs (Dugas and Bartel, 2004) or by regulation of polysome formation. Branco-Price *et al*. (2008) reported such control of translation by polysome formation in hypoxic *A. thaliana* seedlings where less than half of the genes identified as being present (via cDNA microarray) in the whole mRNA population were found in the population of polysomal mRNA (mRNA that is actively being translated by the polysomes).

4.1.3. Two-dimensional differential in-gel electrophoresis (DIGE)

In order to examine differences in the proteome of the tips and bases of O_2-deprived coleoptiles, we used differential in-gel electrophoresis (DIGE). In a DIGE experiment, protein samples (of a known amount) from the various tissues and treatments to be analysed are labelled with one of three fluorescent dyes (Cy2, Cy3 and Cy5). A 'standard' is created by pooling equal amounts of each sample. In this study, triplets consisting of equal amounts of labelled standard and two samples were then combined and run on a single 2D-acrylamide gel. This process was repeated with each set of samples (Lilley and Dupree, 2006).

4.1.4. Experimental Aims

My research in this chapter was based around three key experimental aims:

1. To investigate the differences in the proteome of the tips and bases of rice coleoptiles regardless of O_2 supply;
2. To investigate the differences in the proteome between tissues (tips and bases) grown across differing levels of O_2-deprivation and;
3. To examine the correlation between changes in mRNA levels and corresponding changes in protein levels.

4.2. Materials and Methods

4.2.1. Plant material

Seeds were de-hulled and surface sterilised (70% ethanol (v/v) for 1 min, washed in distilled H_2O for 1 min, 25% bleach (v/v) for 10 min, washed 3 × in sterile distilled H_2O for 1 min, 0.1% mercuric chloride (w/v) for 3.5 min, washed 5 × in sterile distilled H_2O for 1 min). Seeds were placed in 2 L of solution containing 0.8 mM KH_2PO_4 and 0.5 mM $CaCl_2$ with air (normoxia), 3% O_2 in N_2 (v/v) (hypoxia) or N_2 (anoxia) bubbled through the solution at ~0.2 L h^{-1} for 3 d. Coleoptiles were harvested, divided into tips (apical 5–6 mm) and bases (basal 5–6 mm), snap frozen in liquid N_2 and stored at -80°C.

4.2.2. Protein extraction

Coleoptile tips and bases from each treatment were ground to a powder under liquid N_2. Total soluble protein was extracted from the bases and tips of coleoptiles by an SDS-phenol extraction (Wang *et al.*, 2003).

4.2.3. Two dimensional gel electrophoresis

Protein pellets were resolublised in 500 µL of 2D buffer (5 M urea, 2 M thiourea, 2% (w/v) CHAPS, 2% (w/v) sulfobetaine 3–10, 1% [w/v] carrier ampholytes [Amersham Biosciences, GE], 40 mM Tris, Milli-Q water) and protein concentrations determined by Bradford assay (Bio-Rad) as per the manufacturer's instructions.

Equal amounts of protein from each sample (50 µg) were mixed. The volume of this mixture was increased to 300 µL by the addition of fresh 2D buffer.

Standard samples were reduced with 5 mM tributylphosphine (TBP), alkylated in 10 mM acrylamide and incubated at room temperature for 1 h.

Samples (300 µL) were loaded onto pH 4–7 and pH 5–8 18-cm immobilised pH gradients (IPG) strips (Bio-Rad) via passive hydration for 4 h or until no further liquid was visible in the loading tray. Iso-electric focusing (IEF) of the IPG strips was performed overnight using a stepwise protocol (300 V for 1 h, 300V – 8000V for 8 h (gradient), 8000 V for 12 h) to 135 kVh.

After IEF, the strips were stored at -80°C for 1 h, thawed at room temperature and equilibrated in 2D equilibration buffer (6 M urea, 2% SDS, 0.375 M Tris/HCl pH 8.8, 20% glycerol, 5 mM TBP, 2.5% acrylamide) for 15 min, with two changes of buffer (15 min each). IPG strips were embedded on top of 8–18% gradient polyacrylamide gels (17 × 17 cm) using hot agarose (0.5% agarose, 0.001% bromophenol blue, 192 mM glycine, 0.1% SDS, 24.8 mM Tris base pH 8.3). The gels were electrophoresed in Protean II multi-cell tanks (Bio-Rad) using a power supply (Bio-Rad Power Pac 3000) set at 5 mA per gel for 30 min, and then at 20 mA per gel for approximately 16 h, or until the dye front had run off the gel (overnight).

Gels were removed from their casts and placed into fixing solution (50% methanol, 10% acetic acid) for at least 1 h. Gels were transferred to Coomassie staining solution (20% methanol, 7% acetic acid, 0.2% Coomassie Blue G-250) overnight and then destained (10% methanol, 2% acetic acid) until the protein spots were visible. Based on results obtain from these gels, the pH 4.7 strips were used subsequently for the 2D-DIGE analysis.

4.2.4. Two-dimensional differential in-gel electrophoresis

Protein samples were extracted as per section 3.2.2 and re-suspended in a minimal 2D buffer (5 M urea, 2 M thiourea, 2% (w/v) CHAPS).

Proteins were labelled according to the manufacturer's instructions with the exception that the CyDye:protein ratio was 200 pmol:100 µg of protein. Aliquots of labelled protein (50 µg) from each of the six samples were combined to form 300 µg of standard for the DIGE gels. Proteins were labelled as per Table 1.

Table 1. Protein sample and corresponding dye mixtures loaded onto 2D gels for tip and base DIGE experiments.

Samples loaded onto each gel						
Cy dye	Gel 1	Gel 2	Gel 3	Gel 4	Gel 5	Gel 6
Cy2	Standard	Standard	Standard	Standard	Standard	Standard
Cy3	Anoxic base	Hypoxic base	Normoxic base	Anoxic tip	Hypoxic tip	Normoxic tip
Cy5	Hypoxic base	Normoxic base	Anoxic base	Hypoxic tip	Normoxic tip	Anoxic tip

Aliquots (100 µg) of protein from each sample were combined as per Table 1 to create the protein loaded onto each gel. The labelled, pooled protein samples were made up to a volume of 300 µL using fresh minimal 2D buffer. Samples were reduced with 5 mM TBP and alkylated with 10 mM acrylamide.

Pooled samples were loaded on pH 4–7 IPG strips (Bio-Rad). The re-hydrated strips were focused and the first and second dimensional electrophoresis was run as in Section 3.2.3.

Gels were removed from their casts, washed for 2 × 15 min in 1% acetic acid and placed in MilliQ water. The DIGE gels were imaged at three wavelengths corresponding to the emission peaks of the three dyes (C2, 510nm; Cy3, 570nm; Cy5, 670 nm) using a Typhoon Variable Imager at a resolution of 50 µm and the quantitative intensity data loaded into analysis software (Progenesis SameSpot). The average spot volume for a standard channel (Cy2, 510 nm) was calculated and used as a baseline value for all other protein levels. Similar spot volumes were calculated for each of the treatment samples

and normalised to the standard value. Those spots with a significant difference in volume between samples were labelled, marked and extracted from the gel for identification via mass spectrometry.

Images were loaded into Progenesis SameSpots software (v4.5). All gels images passed quality control. Fifteen to twenty large spots present on the Cy2 images of each gel were aligned to the reference image (Cy2 channel of Gel 1). The same spots were used to manually align the Cy3 and Cy5 channels for each gel to their respective Cy2 channel. Gels were automatically aligned and protein spots detected. Protein spots were quality checked manually.

The channels of each gel were assigned to the appropriate treatment (as Table 1) and the volumes of each spot were calculated (relative to the standard). Eighty-eight spots were identified as exhibiting at least a 1.5-fold-change between two treatments ($p<0.05$) and were selected for further analysis.

4.2.5. Protein extraction and identification

Gels 2 and 5 were selected for spot cutting, stained with Sypro Ruby Stain (Invitrogen) as per the manufacturer's instructions and visualised using a Typhoon Variable Imager at a resolution of 50 μm. The Sypro Ruby stained gels were scanned on an automated spot cutter (Bio-Rad, EXQuest) and only spots that could be clearly seen (48 in total) were cut from the gels. Gel plugs were destained with 200 μL of wash solution (50% [v/v] acetonitrile (ACN) and 25 mM ammonium bicarbonate) and placed onto an orbital shaker (37°C, 300 rpm) for 3 × 10 min. Excess wash was removed and the plugs were left to dry for 1 h at 37°C.

An aliquot (8 μL) of 15 ng μL^{-1} sequencing-grade trypsin (Promega) in 25 mM ammonium bicarbonate, pH 7.8, was added to each well. Plugs were incubated for 1 h at 4°C to allow the trypsin to be absorbed. Excess trypsin was removed, and the gel plugs

were sealed and incubated overnight at 37°C. Peptides were extracted in 10 µL of extraction solution (0.1% trifluoroacetic acid [TFA]) with the aid of a water bath sonicator (Transsonic 700/H; Elma) for 20 min.

ZipTips were activated by aspirating and dispensing 10 µL of 70% ACN and 0.1% TFA three times. The tips were washed with 0.1% TFA in the same manner. Peptide extraction solution (8 µL) was taken up into the ZipTip at least ten times to concentrate the peptides onto the column. The tips were further washed three times with 10 µL of 0.1% TFA.

Four microliters of extraction solution (4 mg mL^{-1} matrix, α-cyano-4-hydroxycinnamic acid, 70% ACN, and 0.1% TFA) were drawn up into the ZipTip. The extraction solution in the tip was drawn up and down at least five times until a drop formed at the end of the tip. Two microlitres of this solution was spotted onto a designated circle marked on the ABI plate. A standard (prep mix with matrix) was also spotted following each sample on the ABI plate to externally calibrate using near-point calibration with four peptide standards (bradykinin, angiotensin I, neurotensin and adrenocorticotropic hormone fragment).

Samples were dried and analyzed using an Applied Biosystems 4700 MALDI MS/MS apparatus with time of flight (TOF)/TOF optics in reflector mode for positive ion detection. A Nd.YAG laser with wavelength of 355 nm and repetition rate of 200 Hz was used. All MS spectra resulted from accumulation of 4,000 laser shots (20 subspectra were accumulated with 200 shots per subspectrum). Laser intensity varied between 3,000 and 4,000 instrument units. Data were collected over a mass range of 750 to 3,500 Da. Mass spectral data were collected using the following criteria for peak detection: for MS, mass range 500 to 4,000 Da, maximum 30 peaks per 200 Da, minimum signal-to-noise ratio 20, minimum area 200, maximum peak/spot 200; for MS/MS, mass range 60 Da to precursor mass−15 Da, maximum 20 peaks per 200 Da, minimum signal-to-noise

ratio of 18, minimum area 300, maximum peak/spot 60. These peak mass lists were converted into Mascot- and The GPM-compatible text files.

MASCOT (Matrix Science) was used to identify each spot, using the SwissProt database. Thirty spots with good matches ($p<0.05$) were identified and listed alongside their respective spot volumes (Table 2).

4.3. Results

4.3.1. Identity of differentially expressed protein

Twenty-three spots were identified as being differentially expressed in our experiment (Table 2). The table also shows the confidence value of the identity and the relative fold changes of the spot across the treatments. Of the 23 proteins identified, 22% were related to glycolysis or ethanol fermentation, 26% were stress response proteins, 4% were lipid synthesis proteins, another 4% were cell cycle proteins, 13% were related to protein synthesis or modification, 9% were related to ATP synthesis and 22% performed a variety of miscellaneous functions.

4.3.2. Relative fold changes

The ratio of the protein and mRNA fold changes for each 23 spots were identified as being differentially expressed in our experiment was calculated (Table 3). As Table 3 shows, the fold change in protein levels generally followed the change in mRNA levels although it was often 2-3x greater. Four proteins, 17.9 kDa stress protein, the late embryogenesis abundant protein, the OsTCTP homologue and cyanate hydratase, showed large (>5x) differences between the protein and mRNA fold changes.

Table 2. Proteins identified as being differentially expressed in rice coleoptiles grown under different O_2 treatments. Protein identities were determined by searching the SwissProt database limited to *Oryza sativa*/*Viridiplantae*. The column 'Gene identification' lists the LOC_Os number or the ORF name for proteins identified as *O. sativa* proteins. For proteins identified as non-*O. sativa* homologues, it lists the NCBI GenBank ID or recognised name for the gene. Accession number is the internal reference for the SwissProt database. Coverage is the percentage of the protein covered by MS peptide fragments. Spot # is the Progenesis SameSpot assigned spot number used during gel analysis (see text). Relative fold change is the relative change in protein expression (spot volume) in the following comparisons: aerated tips (AT) *vs* aerated bases (AB); hypoxic tips (HT) *vs* hypoxic bases (HB); N_2-treated tips (NT) *vs* N_2-treated bases (NB); AT *vs* HT; HT *vs* NT; AT *vs* NT; AB *vs* HB; HB *vs* NB and AB *vs* NB. A positive number indicates that the protein was more abundant in the first of the listed samples (numerator); a negative number indicates that the protein was more abundant in the second sample comparison (denominator). The magnitude of the number indicates relative fold change between the two samples (n=2 for each sample). Fold changes in bold indicate those determined as significant by a two-way ANOVA as described in the text.

Gene identification	**Protein identification**	**Accession number**	**Coverage (%)**	**Spot #**	**Relative fold change**								
					O_2 treatments			***Coleoptile tips***			***Coleoptile bases***		
	Glycolytic and fermentative proteins				*AT/AB*	*HT/HB*	*NT/NB*	*AT/HT*	*HT/NT*	*AT/NT*	*AB/HB*	*HB/NB*	*AB/NB*
ZmADH1	Alcohol dehydrogenase 1 OS=*Zea mays*	P00333	3	1729	**-1.8**	-1.0	-1.1	**-2.7**	-1.4	**-3.7**	**-1.6**	-1.5	**-2.3**
LOC_Os10g08550	Enolase OS=*Oryza sativa* ssp. japonica	Q42971	7	1000	1.8	-1.1	-1.3	1.2	-1.2	1.0	**-1.6**	-1.4	**-2.3**
LOC_Os01g66940	Fructokinase-1 OS=*Oryza sativa* ssp. indica	A2WXV8	16	1577	-1.2	1.1	-1.2	1.2	**2.1**	**2.5**	**1.6**	**1.6**	**2.5**
OsI_025158	Nucleoside diphosphate kinase 1 OS=*Oryza sativa* ssp. indica	A6N0M9	17	2069	1.5	-1.5	1.4	-1.0	-1.0	-1.1	**-2.4**	**2.0**	-1.2
OsI_025158	Nucleoside diphosphate kinase 1 OS=*Oryza sativa* ssp. indica	A6N0M9	25	2176	**-3.3**	**-4.1**	**-2.2**	-1.4	**-1.6**	**-2.2**	**-1.7**	1.2	-1.5
	Stress response genes												
LOC_Os01g04370	16.9 kDa class I heat shock protein 1 OS=*Oryza sativa* ssp. japonica	P27777	29	1996	1.0	**3.3**	1.1	**-1.9**	**3.1**	**1.6**	**1.7**	1.1	**1.8**
LOC_Os03g15960	17.9 kDa class I heat shock protein OS=*Oryza sativa* ssp. japonica	Q84Q77	24	1903	**-9.4**	**-4.6**	**-24.4**	**-3.8**	**6.3**	**1.7**	**-1.9**	1.2	**-1.6**
X66874	Heat shock 70 kDa protein, mitochondrial OS=*Phaseolus vulgaris*	Q01899	2	2299	1.1	-1.4	-1.3	1.2	-1.2	-1.1	-1.3	-1.2	**-1.5**
LOC_Os01g24710	Salt stress-induced protein OS=*Oryza sativa* ssp. indica	A2WPN7	22	1751	**3.3**	1.5	**3.2**	**2.2**	-1.1	**1.9**	-1.1	**2.00**	**1.9**
LOC_Os01g24710	Salt stress-induced protein OS=*Oryza sativa* ssp. indica	A2WPN7	33	1482	**2.2**	-1.2	**-2.0**	1.2	1.3	**1.5**	**-2.3**	-1.2	**-2.8**
OsI_020113	Late embryogenesis abundant protein, group 3 OS=*Oryza sativa* ssp. indica	A2Y720	7	1868	1.7	1.3	-2.0	**-4.2**	**2.8**	-1.5	**-5.8**	1.1	**-5.1**
	Lipid synthesis and modification												
LOC_Os08g23810	Enoyl-[acyl-carrier-protein] reductase [NADH] 1,chloroplastic OS=*Oryza sativa* ssp. japonica	Q6Z0I4	20	1549	**2.2**	**-2.2**	**-1.9**	**1.7**	**1.6**	**2.6**	**-2.8**	1.8	-1.6

	Cell cycle proteins												
LOC_Os11g43900	Translationally-controlled tumor protein homolog OS=*Oryza sativa* ssp. japonica GN=TCTP	P35681	5	1948	**-14.9**	**-3.7**	**-2.1**	**-2.1**	-1.4	**-3.0**	2.0	1.2	**2.4**
	Protein synthesis and modification												
LOC_Os03g26970	Proteasome subunit alpha type-2 OS=*Oryza sativa* ssp. indica	A2YVR7	8	1736	**-2.9**	-1.1	**-3.2**	**-3.0**	**4.9**	1.6	-1.1	1.6	1.5
LOC_Os11g09280	Protein disulfide isomerase-like 1-1 OS=*Oryza sativa* ssp. japonica	Q53LQ0	7	2297	-1.1	**-2.1**	**-1.9**	-1.4	1.2	-1.2	**-2.6**	1.3	**-2.0**
LOC_Os11g09280	Protein disulfide isomerase-like 1-1 OS=*Oryza sativa* ssp. japonica	Q52PJ0	7	1766	1.3	**-3.6**	**-1.6**	1.3	-1.8	-1.4	**-3.6**	1.3	**-2.8**
	ATP synthesis												
X58498	ATP synthase subunit beta, mitochondrial OS=*Hevea brasiliensis*	P29685	11	1129	1.9	-1.3	-1.8	1.0	1.1	1.1	**-2.4**	-1.3	**-3.1**
X58498	ATP synthase subunit beta, mitochondrial OS=*Hevea brasiliensis*	P29685	13	2271	**2.1**	-1.2	-1.7	1.2	-1.2	1.0	**-2.2**	-1.6	**-3.5**
	Miscellaneous												
LOC_Os05g25850	Superoxide dismutase [Mn], mitochondrial OS=*Oryza sativa* ssp. japonica	Q43008	37	1808	-1.1	**-3.3**	-1.3	**-3.3**	**3.2**	-1.0	**-3.3**	1.6	**-2.1**
LOC_Os04g09920	9-beta-pimara-7,15-diene oxidase OS=*Oryza sativa* ssp. japonica	Q0JF01	2	1976	1.1	-1.0	-1.3	**2.4**	**2.0**	**4.8**	**2.0**	1.7	**3.5**
LOC_Os04g38870	14-3-3-like protein GF14-B OS=*Oryza sativa* ssp. japonica	Q7XTE8	15	1725	**-2.5**	1.8	-1.3	**-2.8**	**2.7**	-1.0	1.7	1.1	1.9
LOC_Os03g17690	L-ascorbate peroxidase 1, cytosolic OS=*Oryza sativa* ssp. indica	A2XFC7	12	1749	**-5.1**	**-2.4**	-1.7	1.1	1.1	1.1	**2.3**	1.5	**3.4**
LOC_Os10g33270	Cyanate hydratase OS=*Oryza sativa* ssp. indica	A2Z8F9	5	2004	**-7.9**	**-2.9**	**-3.7**	**-5.6**	1.6	**-3.6**	**-2.1**	1.2	-1.7

Table 3. Ratio of the relative folds changes at the protein and gene level of proteins identified as differentially expressed by DIGE analysis. Protein identities were determined by searching the SwissProt database limited to *Oryza sativa*/*Viridiplantae*. The column 'Gene identification' lists the LOC_Os number or the ORF name for proteins identified as *O. sativa* proteins. For proteins identified as non-*O. sativa* homologues, it lists the NCBI GenBank ID or recognised name for the gene. Accession number is the internal reference for the SwissProt database. Coverage is the percentage of the protein covered by MS peptide fragments. Spot # is the Progenesis SameSpot assigned spot number used during gel analysis (see text). The ratio of the fold changes is calculated by the fold change at the protein level divided by the fold change at mRNA level in the following comparisons: aerated tips (AT) *vs* aerated bases (AB); hypoxic tips (HT) *vs* hypoxic bases (HB); N_2-treated tips (NT) *vs* N_2-treated bases (NB); AT *vs* HT; HT *vs* NT; AT *vs* NT; AB *vs* HB; HB *vs* NB and AB *vs* NB.. The magnitude of the ratio signifies the fold change was greater in protein (magnitude >1) or at the mRNA level (magnitude <1). A negative number indicates that the observed fold change was in different directions. For example a fold change of -2 in the AT/AB comparison means that the protein fold change was twice that of the mRNA but that the changes were observed in the opposing tissues (tips versus base). ND indicates that a particular comparison was not calculated.

Gene identification	**Protein identification**	**Accession number**	**Coverage (%)**	**Spot #**	**Ratio of Fold Change (Protein fold change : mRNA fold change)**								
					O_2 treatments			***Coleoptile tips***			***Coleoptile bases***		
	Glycolytic and fermentative proteins				*AT/AB*	*HT/HB*	*NT/NB*	*AT/HT*	*HT/NT*	*AT/NT*	*AB/HB*	*HB/NB*	*AB/NB*
ZmADH1	Alcohol dehydrogenase 1 OS=*Zea mays*	P00333	3	1729	1.7	1.0	1.1	2.3	-1.3	3.4	1.8	-1.4	2.2
LOC_Os10g08550	Enolase OS=*Oryza sativa* ssp. japonica	Q42971	7	1000	-1.7	1.1	1.3	-1.1	-1.2	-1.0	1.7	-1.4	2.2
LOC_Os01g66940	Fructokinase-1 OS=*Oryza sativa* ssp. indica	A2WXV8	16	1577	1.0	-1.0	1.1	-0.7	1.6	-2.0	-2.4	1.2	-2.2
OsI_025158	Nucleoside diphosphate kinase 1 OS=*Oryza sativa* ssp. indica	A6N0M9	17	2069	-1.1	1.4	-1.3	0.7	-1.0	0.8	2.6	-2.0	1.1
OsI_025158	Nucleoside diphosphate kinase 1 OS=*Oryza sativa* ssp. indica	A6N0M9	25	2176	2.5	3.8	2.0	1.0	-1.6	1.7	1.9	-1.2	1.4
	Stress response genes												
LOC_Os01g04370	16.9 kDa class I heat shock protein 1 OS=*Oryza sativa* ssp. japonica	P27777	29	1996	0.6	2.4	0.8	-0.9	2.3	0.5	1.0	0.8	0.8
LOC_Os03g15960	17.9 kDa class I heat shock protein OS=*Oryza sativa* ssp. japonica	Q84Q77	24	1903	7.8	3.9	23.5	2.0	4.9	-1.1	2.6	1.1	1.2
X66874	Heat shock 70 kDa protein, mitochondrial OS=*Phaseolus vulgaris*	Q01899	2	2299	ND	ND	ND	ND	ND	ND	ND	ND	ND
LOC_Os01g24710	Salt stress-induced protein OS=*Oryza sativa* ssp. indica	A2WPN7	22	1751	3.3	1.4	3.0	2.2	-1.1	1.9	-1.1	2.0	1.8
LOC_Os01g24710	Salt stress-induced protein OS=*Oryza sativa* ssp. indica	A2WPN7	33	1482	2.2	1.2	-1.9	1.2	1.3	1.5	-2.2	-1.2	-2.6
OsI_020113	Late embryogenesis abundant protein, group 3 OS=*Oryza sativa* ssp. indica	A2Y720	7	1868	0.9	0.9	-1.2	3.6	2.5	1.4	9.4	0.8	4.2
	Lipid synthesis and modification												
LOC_Os08g23810	Enoyl-[acyl-carrier-protein] reductase [NADH] 1,chloroplastic OS=*Oryza sativa* ssp. japonica	Q6Z0I4	20	1549	-1.4	2.0	1.4	-1.0	1.2	-2.0	3.2	1.7	1.5

	Cell cycle proteins												
LOC_Os11g43900	Translationally-controlled tumor protein homolog OS=*Oryza sativa* ssp. japonica GN=TCTP	P35681	5	1948	14.8	3.7	2.1	2.0	1.4	2.9	-2.1	-1.2	-2.3
	Protein synthesis and modification												
LOC_Os03g26970	Proteasome subunit alpha type-2 OS=*Oryza sativa* ssp. indica	A2YVR7	8	1736	2.7	-1.1	-3.2	3.0	4.8	-1.6	-1.0	-1.6	1.4
LOC_Os11g09280	Protein disulfide isomerase-like 1-1 OS=*Oryza sativa* ssp. japonica	Q53LQ0	7	2297	1.0	-2.1	1.8	1.2	1.1	1.1	2.8	1.3	1.9
LOC_Os11g09280	Protein disulfide isomerase-like 1-1 OS=*Oryza sativa* ssp. japonica	Q52PJ0	7	1766	-1.2	-3.5	1.5	-1.1	-1.6	1.3	3.8	1.3	2.7
	ATP synthesis												
X58498	ATP synthase subunit beta, mitochondrial OS=*Hevea brasiliensis*	P29685	11	1129	ND	ND	ND	ND	ND	ND	ND	ND	ND
X58498	ATP synthase subunit beta, mitochondrial OS=*Hevea brasiliensis*	P29685	13	2271	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Miscellaneous												
LOC_Os05g25850	Superoxide dismutase [Mn], mitochondrial OS=*Oryza sativa* ssp. japonica	Q43008	37	1808	1.1	3.0	1.2	-3.3	3.2	-1.0	3.5	1.6	2.0
LOC_Os04g09920	9-beta-pimara-7,15-diene oxidase OS=*Oryza sativa* ssp. japonica	Q0JF01	2	1976	0.4	0.9	-0.6	0.2	-0.8	1.0	0.5	-1.6	0.9
LOC_Os04g38870	14-3-3-like protein GF14-B OS=*Oryza sativa* ssp. japonica	Q7XTE8	15	1725	-2.4	1.8	-1.3	2.7	2.6	1.0	-1.8	1.1	-1.8
LOC_Os03g17690	L-ascorbate peroxidase 1, cytosolic OS=*Oryza sativa* ssp. indica	A2XFC7	12	1749	4.7	2.1	1.5	1.0	-1.0	1.1	2.2	-1.4	-3.4
LOC_Os10g33270	Cyanate hydratase OS=*Oryza sativa* ssp. indica	A2Z8F9	5	2004	0.1	0.4	0.3	0.2	1.6	0.3	2.1	1.1	0.6

4.4. Discussion

4.4.1. Glycolytic and fermentative proteins

The abundance of specific proteins involved in glycolysis and ethanol fermentation was affected by both the position on the coleoptile that was sampled and O_2 concentration. For example, alcohol dehydrogenase 1 (ADH1) was induced by low O_2 status and was more abundant in the coleoptile bases. Even in normoxic coleoptiles, ADH1 levels were significantly higher in bases than in tips, indicating a constitutive presence of this enzyme in this anaerobically adapted organ (Gibbs *et al*., 2000). Notwithstanding, this protein was more abundant in hypoxic and anoxic coleoptiles compared to normoxic coleoptiles in both tips (2.7 and 3.7-fold, respectively) and bases (1.6 and 2.3-fold, respectively). Such an observation concurs with both microarray probe sets (Chapter 3, Supplementary Table B1) and published data that report that anoxia strongly induces ADH1 in rice coleoptiles (App and Meiss, 1958; Mohanty *et al*., 1993; Gibbs *et al*., 2000; Huang *et al*., 2005). This relative increase in ADH1 in low O_2 conditions corresponds to an increase in the rate of ethanol production, thereby increasing ATP availability and relieving the low-O_2 'energy crisis'.

During normoxia, rice *cv.* 'Amaroo' exhibits strong constitutive expression of ADH1 (Edwards *et al*., 2012). Lower ADH1 levels in tips than bases could be indicative of lower energy demands within the tips, where cells are close to their ultimate length; this contrast in energy demand was implied by the 10-fold higher rate of ethanol synthesis in basal zones of rice coleoptiles (Setter and Ella, 1994). This differential is also consistent with the observations of Kawai and Uchimiya (2000) and Edwards *et al*. (2012) that tips

of 3-d-old coleoptiles begin to senesce, accompanied by a decreased rate of ATP regeneration.

Expression levels of enolase (phosphopyruvate hydratase) were altered by both tissue position and exposure to low O_2 levels. Enolase is the penultimate enzyme in glycolysis, converting 2-phosphoglycerate (2-PG) to phosphoenolpyruvate (PEP). Increases in enolase protein abundance (Sachs *et al*., 1980; Fox *et al*., 1995) and enzymatic activity (Fox *et al*., 1995; Ishizawa *et al*., 1999; Mustroph and Albrecht, 2003) have been reported as a response to O_2 deprivation. In our experiment, levels of enolase protein were statistically higher in hypoxic and anoxic bases compared to normoxic bases (1.6 and 2.3-fold, respectively). There were no significant differences in enolase levels between tips across O_2 treatments or between tips and bases within O_2 treatments, in contrast to ADH1 levels.

The increase of enolase activity in O_2-deprived bases (compared to normoxic bases) could result in a net increase in the rate of glycolysis (commonly referred to as the 'Pasteur effect') if its control co-efficient (Kacser *et al*., 1995) made it a rate-determining enzyme. This would enhance the net rate of ATP generation and ensure sufficient ATP for the sustained cell division and elongation in hypoxic and anoxic coleoptiles compared to normoxic coleoptiles.

The relative increases in ADH1 and enolase protein levels in hypoxic and anoxic bases (compared to normoxic bases) were almost identical. Anoxia lowers the cytoplasmic pH set point (long-term average of cytoplasmic pH) of rice cells from pH 7.5 to pH 7.0 (Menegus *et al*., 1991). Cytoplasmic acidification increases the catalytic activity of PDC (Davies *et al*., 1974), the enzyme immediately prior to ADH in the ethanol fermentation pathway. The effect of cytoplasmic acidification of enolase has not been studied in plants but in yeast (*Saccharomyces cerevisiae*) enolase exhibits maximum activity at ~pH 7, with activity decreasing rapidly with pH (Pampulha *et al*., 1990). The

higher activities of both PDC and enolase at pH 7 (relative to higher and lower pH values) would increase the rate of glycolysis in hypoxic and anoxic coleoptiles, supporting the increased rates of ethanol fermentation observed in hypoxic and anoxic coleoptiles (compared to normoxic coleoptiles).

Fructokinase 1 (FK1) enzyme levels decreased in anoxia (compared to hypoxia and normoxia) in both coleoptiles tips and bases. Such a decrease was not seen in *OsFK1* transcript, which remained at a constant level throughout both bases and tips in all three O_2 treatments (Chapter 3). Fructokinase 1 is one of two isoforms in rice, FK2 being the other (Jiang *et al*., 2003; Guglielminetti *et al*., 2006). Both act to phosphorylate fructose to fructose-1-phosphate in sink tissues, consistent with its expression in the faster growing normoxic coleoptiles. The two isoforms have different kinetic profiles, with OsFK1 having lower affinity for fructose than OsFK2 (K_m = 3.3 mM and 0.34 mM fructose, respectively). Additionally OsFK1 activity is inhibited by high fructose levels (>50 mM) while OsFK2 activity is not (Jiang *et al*., 2003). Guglielminetti *et al.* (2006) found that anoxia lead to lower *OsFK1* transcript levels in anoxic rice coleoptiles compared to normoxic ones. The V_{max} of OsFK1 and OsFK2 are comparable at 0.8 and 0.7 nkat.mg^{-1} protein, respectively (Jiang *et al*., 2003).

During normoxia, cytosolic sucrose within coleoptiles is degraded through a combination of invertase and sucrose synthase (SuSy) activity (Guglielminetti *et al*., 1995). During hypoxia and anoxia, this balance is perturbed towards SuSy activity (Guglielminetti *et al*., 1995; Wang *et al*., 1999). This requires one less ATP than invertase for sucrose to enter the glycolytic pathway. This shift would result in an increase in intracellular fructose levels and a repression of OsFK1 activity. The low K_m of OsFK2 (0.34 mM fructose), combined with a V_{max} comparable to that of OsFK1, would result in the rapid conversion of fructose to fructose-1-phosphate, which in turn would aid in

reducing the amount of ATP required to allow sucrose degradation for glycolysis (Plaxton, 2010).

Two protein spots (#2069, #2176) were identified as rice nucleoside diphosphate kinase 1 (OsNDPK1). They exhibit different expression patterns: spot #2176 was higher in volume in bases than in tips (3.3-, 4.1- and 2.2-fold in normoxia, hypoxia and anoxia, respectively), regardless of O_2 treatment. The volume of spot #2176 was also higher in anoxic tips than normoxic (2.2-fold) and hypoxic (1.7-fold) tips. The other kinase (#2069) showed a different expression pattern: there were no significant differences between tips and bases (within a single O_2 treatment) or within the tips (between various O_2 treatments). Bases of the hypoxic coleoptiles contained approximately twice the amount of the protein (spot #2069) as found in either normoxic (2.4-fold) or anoxic (2.0-fold) coleoptile bases.

Nucleoside diphosphate kinases (NDPKs) act to transfer a phosphate group from UTP to ADP, resulting in the formation of ATP and UDP (Paxton, 2010). Three classes of NDPKs have been identified in plants and are known to have differing intracellular localisation, with class I thought to be cytosolic (Glavis *et al*., 1999) while class II and class III NDPKs have been localised to the chloroplast (Choi *et al*., 1999) and intermembrane space of the mitochondria (Sweetlove *et al*., 2001), respectively. One NDPK (in class I), has been identified in rice (Yano *et al*., 1993) and found to be essential for coleoptile elongation during submergence (Pan *et al*., 2000).

The changes in protein expression reported above suggest accelerated degradation of sucrose to fructose and glucose in the bases of hypoxic coleoptiles compared to either normoxic or anoxic coleoptiles. During low O_2 conditions (hypoxia and anoxia) in rice coleoptiles, it is thought that sucrose degradation shifts from the invertase pathway to the SuSy pathway. Guglielminetti *et al*. (1995) reported such a change in anoxic rice seedlings. Hirose *et al*. (2008) reported that in hypoxic rice seedlings, levels of *SUS2*

were 3-fold greater than in normoxic controls. This is supported by our observed changes in proteome of the hypoxic and anoxic bases: levels of the low affinity OsFK1 fall, while it appears that levels of OsFK2 remain stable to allow effective scavenging and phosphorylation of fructose to fructose-1-phophate. Simultaneously, the OsNDPK1 (spot #2069) is strongly up-regulated in the bases of hypoxic coleoptiles. The system described by these changes in shown in Figure 1 (adapted from Paxton, 2010). The decrease in OsFK1 levels (relative to normoxia) (number 2 in Figure 1) would allow OsFK2 to fulfil this role more efficiently. The increased levels of OsNDPK1 (spot #2069) (number 3 in Figure 1) would allow the maintenance of sufficient regeneration of ADP to ATP via conversion of high-energy phosphate from UTP. Supply of PPi could be provided by a variety of metabolic processes including protein synthesis, mRNA synthesis or cellulose synthesis.

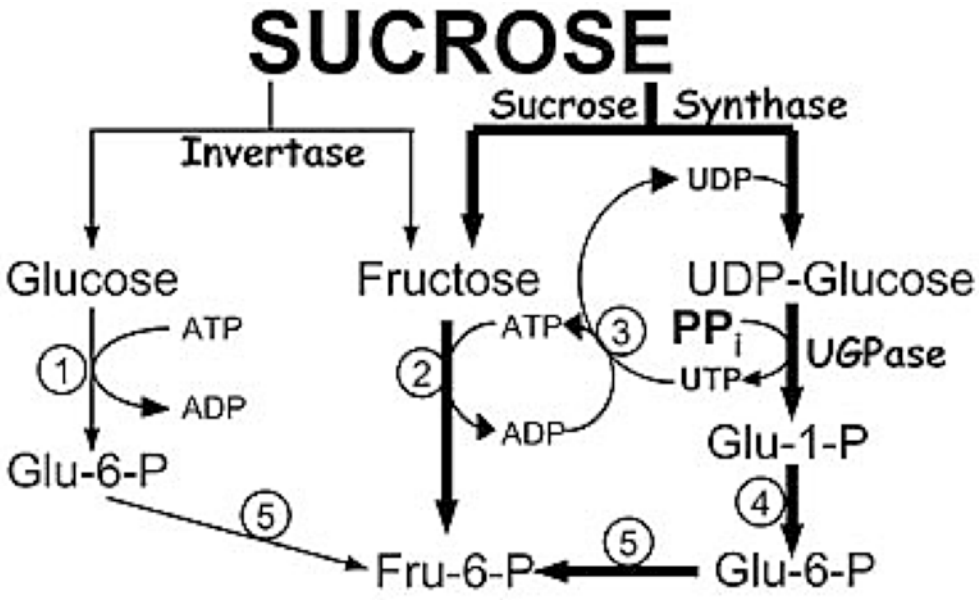


Figure 1. A model for changes in sucrose degradation in O_2-deprived rice coleoptile bases (1, hexokinase; 2, fructokinase; 3, nucleoside diphosphate kinase; UDP-glucose pyrophosphorylase; 5, phosphoglucose isomerase). Adapted from Paxton, 2010.

4.4.2. Stress-response genes

Our experiments identified a variety of stress-related proteins as being differentially expressed based on tissue position and O_2 treatment.

Three protein spots (#1996, #1903 and #2299) were identified as heat shock proteins (HSPs), namely 16.9- and 17.9-kDa class I HSPs (spots #1996 and #1903, respectively) and a 70-kDa mitochondrial heat shock protein (spot #2299). The 16.9- and 17.9-kDa HSPs showed different expression patterns. The 16.9-kDa protein was strongly expressed in hypoxic tips (compared to both normoxic and anoxic tips) but was more abundant in normoxic bases than their hypoxic and anoxic counterparts. The 17.9-kDa HSP was strongly preferentially expressed within coleoptile bases compared to tips, regardless of O_2 treatment. When comparing the same tissues across O_2 treatments, the 17.9-kDa HSP was more strongly expressed during O_2 deprivation (hypoxia and anoxia) compared to normoxia in both tips and bases.

The HSPs are a large group of proteins within rice, consisting of several large families, each with many members (Hu *et al*., 2009). They act as molecular chaperones, with the various gene families acting to ensure correct protein folding, assembly, localisation, secretion and degradation (Vierling, 1991). In addition to responding to heat stress (Lin *et al*., 2005), HSPs have been found to be induced by a wide range of abiotic stresses including drought, cold, salinity and oxidative stress (Vierling 1991; Ueda *et al*., 2006; Hu *et al*., 2009). One isoform of the rice 17.9-kDa HSP has been shown to be highly expressed in rice tissue undergoing rapid cell division and expansion (Sarkar *et al*., 2009). The expression pattern of the 17.9-kDa class I HSP in our experiments; i.e. strongly expressed in bases (dividing and elongating) compared to tips (very few if any cell division and slowing elongation) of coleoptiles, is suggestive of a similar function. However, more importantly, the expression of this small HSP was highly up-regulated in tips and, to a lesser degree, bases of hypoxic coleoptiles

(compared to those in normoxia), therefore appearing to be most represented in the most rapidly elongating tissues. It is highly likely that such a small protein interacts with larger HSPs, similar to what has been observed for the Group I chaperones, HSP60 and its 10-kDa partner (Wang *et al*., 2004).

Expression levels of two salt stress-induced proteins were also significantly affected by changes in O_2 levels. The first protein (Spot #1751) was more highly expressed in the tips compared to the bases in all O_2 treatments. Anoxia down-regulated its expression (compared to normoxia and hypoxia) in tips and bases. Conversely, the second protein (spot # 1482) showed a change in relative expression between tips and bases: in normoxia it was twice as abundant in tips (relative to bases) while in anoxia this ratio was reversed. It was also more abundantly expressed in low O_2 (hypoxia and anoxia) in the base of coleoptiles. In that rice coleoptiles are not typically exposed to salinity, nor particularly tolerant to it, these proteins probably play some role unrelated to their name, such as in tissue maturation or abiotic stress response.

The changes in protein expression of the first protein list in Table 2 (spot #1751) are consistent with a protein that is down-regulated by exposure to low O_2 levels, possibly a transcription factor or regulatory element, while the changes in the expression of the second protein (spot #1482) is consistent with induction by O_2 deprivation. Both of these proteins merit further investigation. Such changes are hard to interpret using our current knowledge of the molecular regulation of rice tissue growth and O_2-dependent metabolism. However, they do highlight that the contrasts in gene and protein expression within rice coleoptiles can be highly treatment- and tissue-specific.

A group 3 Late-Embryogenesis Abundant (LEA) protein was regulated by O_2 availability. Within coleoptile tips, it was 4.1 and 2.8-fold more abundant in hypoxia

than in normoxia and anoxia, respectively. In coleoptile bases, in was 5.8 and 5.0-fold more abundant in hypoxia and anoxia than in normoxia.

Within rice, the transgenic expression of the LEA family confers tolerance to a large range of abiotic stresses including drought (Cheng *et al*., 2002; Babu *et al*., 2004) and salt stress (Xu *et al*., 1996). This protection is primarily due to LEAs stabilising and preventing the degradation of the cell membranes (reviewed by Shih *et al*., 2008) by acting either as protective proteins during desiccation or by preventing membrane degradation.

The high level of the LEA we identified in O_2-deprived bases and hypoxic tips would corroborate the membrane protection role described above. During anoxia (and to a lesser extent hypoxia) lipid synthesis slows, compared to normoxia. Additionally, in the absence of O_2, the synthesis of unsaturated lipids is halted. Rawyler *et al*. (1999) noted that, during anoxia, there was an increase in the free fatty acid content of the cell membranes of anoxic potato cells. If LEA were playing a protective role in hypoxic and anoxic coleoptiles, the decrease in lipid degradation would aid the growth of the coleoptile.

4.4.3. Cell cycle related proteins

A protein (spot #1948) identified as a translationally-controlled tumour protein (TCTP) was altered by both tissue position and O_2 treatment. It was consistently higher in bases (compared to tips) regardless of O_2 supply (14.9, 3.7 and 2.1-fold in normoxia, hypoxia and anoxia, respectively). Comparing tips and bases across O_2 treatments, TCTP expression was 2–3-fold higher in hypoxic and anoxic tips (compared to normoxia) but was ~2-fold higher in normoxic bases (compared to hypoxia and anoxia).

Members of the TCTP family in plants play a role in the regulation of cell division at the mitotic stage. In *Arabidopsis*, TCTP is strongly expressed in rapidly dividing

tissues such the apical meristems, auxiliary meristems and the developing embryo (Berkowitz *et al*., 2008). Disruption of AtTCTP (via the use of RNAi) resulted in a slowing of growth and development compared to wild-type plants (fewer and smaller leaves, delayed onset of flowering, shorter roots and defects in pollen tube formation) (Berkowitz *et al*., 2008). Brioudes *et al.* (2010) showed that these defects were caused by disruptions in cell division: two *AtTCTP* mutant lines exhibited slowing of mitosis, marked by decreases in the synthesis and recruitment of key cyclins. Interestingly, in these lines, ultimate cell size was unaffected, suggesting that the effect of TCTP lies solely within the regulation of cell division, not expansion.

The basal tissue of rice coleoptiles is the primary site of cell division and expansion of dormant cells in the seed embryo (Wada, 1961; Furuya *et al*., 1969; Takahashi *et al*., 2011) (see Chapter 3 for more details). Additionally, the cells of normoxic rice coleoptiles have largely ceased dividing within 3 d of germination (Kordan, 1976; Mocquot *et al*., 1977; Sakai *et al*., 2006; Takahashi *et al*., 2011), whereas some cells in hypoxic and anoxic coleoptiles are still actively dividing until at least 4 d (Sakai *et al*., 2006, Edwards *et al*., 2012). Assuming that OsTCTP plays a similar role to AtTCTP, its expression patterns correlate with the known physiology of the rice coleoptiles. Its high levels in the bases of coleoptiles (compared to tips), regardless of O_2 treatment, correlate with these tissues being the primary sites of cell division within the coleoptile. Edwards *et al*. (2012) reported that the rate of DNA synthesis (a first approximation for the rate of cell division) was 2 and 4-fold greater in normoxic coleoptile compared to hypoxic and anoxic coleoptiles, respectively. This matches the relative expression levels of TCTP reported above (Table 2) and is suggestive of a higher rate of cell division in this tissue. Given the essential role of TCTP in cell division (Brioudes *et al*., 2010), the higher TCTP levels in O_2-deprived tips (compared to

normoxic tips) may be suggestive of a longer period of cell division in O_2-deprived tips (compared to normoxia).

It has been suggested that TCTP may play a role in inhibiting programmed cell death in plants, similar to its role in animals (Liao *et al*., 2009; Gupta *et al*., 2013). If this is the case, the decrease in TCTP level observed in normoxic tips (compared to hypoxic/anoxic tips) could account for the senescence of this tissue observed to begin at ~3 d in normoxic coleoptiles (Edwards *et al*., 2012).

4.4.4. Protein synthesis and modification proteins

Three proteins involved in the synthesis or modification of other proteins were identified as differentially regulated: proteasome subunit α type-2 and two distinct spots identified as protein-disulfide isomerase-like 1-1 (spot #1736 and #2297/1766 respectively).

The two polypeptides identified as protein disulfide isomerase (PDI)-like 1-1 (spot #2297 and 1766) exhibited similar expression profiles: they are up-regulated 2–3-fold in the anoxic and hypoxic bases (compared to their respective tips) and are up-regulated 2–3-fold by O_2 deficiency in the coleoptile base (compared to the normoxic bases). PDIs act to isomerise disulfide bonds within proteins, facilitating protein folding and targeting (Chivers and Raines, 1997). Defects in a PDI gene in rice prevent the development of the seed storage protein glutelin during seed filling (Takemoto *et al*., 2002). Homologous genes are involved in the post-translation folding of defense/stress related proteins in wheat (Ray *et al*., 2003) and *Oldenlandia affinis* (Gruber *et al*., 2007). They are also known to play a regulatory role in cell death: a PDI (ATPDI5) in *Arabidopsis* acts to regulate programmed cell death by chaperoning and controlling cysteine protease activity (Ondzighi *et al*., 2008).

The proteasome subunit α type-2 was 3-fold more abundant in the bases of normoxic and anoxic coleoptiles (compared with their tips) but was uniformly expressed in the hypoxic coleoptiles. It was ~3 and 5-fold more abundant in the hypoxic tips than the normoxic and anoxic tips, respectively. Differences in its expression across O_2 treatments in the bases were not statistically different. The proteasome is a multi-protein, ATP-dependent complex (Coux *et al*., 1996; Hilt and Wolf, 1996) that acts to degrade proteins targeted to it by ubiquitination.

The strong expression of a component protein of the proteasome complex, along with proteins (PDIs) frequently found to regulate its activity, in the basal tissue of the coleoptiles (in normoxia and anoxia) and throughout the hypoxic coleoptile is consistent with the physiology of these expanding tissues. In normoxic and anoxic coleoptiles, the basal tissues are the location of cell division and expansion (Wada, 1961; Furuya *et al*., 1969) (see Chapter 3 for details). These processes require a constant supply and turnover of amino acids that proteasome activity can provide (Nocker *et al*., 1996). Similarly, the high levels of proteasome subunit α type-2 expression throughout hypoxic coleoptile are consistent with the unique physiology of this organ, which elongates at extraordinary rates with little cell division and at low O_2 status (and thus ATP) (Opik, 1973; Takahashi *et al.*, 2011). The proteasome activity would allow rapid recycling of non-essential protein, minimising the amount of ATP required for amino acid synthesis (Penning de Vries, 1974) and allowing it to be directed towards other essential processes.

4.4.5. ATP synthesis proteins

Two proteins were identified as similar to the mitochondrial ATP synthase subunit β from *Hevea brasiliensis* (Para rubber tree) (spot #1129 and #2271). They

exhibited similar expression profiles with both being up-regulated 2–3.5-fold in hypoxic and anoxic bases (compared to normoxic bases).

It is strange that the two proteins above, which are strongly associated with aerobic metabolism (Nishizawa and Hirai, 1989), should be induced by strict anoxia. Whether this is an adaptation for the return of coleoptiles to aerobic conditions cannot be determined from our data but this could be tested in subsequent experiments. They may play a role in active ATP synthesis. Stoimenova *et al*. (2007) reported that during strict anoxia mitochondria isolated from rice roots were able to produce ATP using nitrite as a terminal electron acceptor. Gupta and Igamberdiev (2011) proposed a model in which, during hypoxia/anoxia, externally facing NADH and NADPH dehydrogenases transfer electrons to ubiquinone and onto the electron transfer chain. In the absence of O_2, NO_2^- acts as the terminal electron acceptor for the system. Such a system would produce a similar amount of ATP as that supplied by glycolysis (Stoimenova *et al*., 2007; Gupta and Igamberdiev, 2011). The expression of a specialised ATP synthase (or components thereof) under anoxia would be consistent with this still unproven mechanism of energy production under O_2 limitation.

4.4.6. Miscellaneous proteins

Five miscellaneous proteins were identified as being differentially regulated by coleoptile tissue and O_2 supply.

A rice mitochondrial manganese superoxide dismutase (SOD) (spot #1808) was up-regulated 2–3-fold in hypoxic tips and bases (compared to both normoxic and anoxic bases). It was three-fold higher in the hypoxic bases than hypoxic tips. SODs act to detoxify superoxide oxygen within plant tissue, acting as part of the plant anti-oxidant defence system (Bowler *et al*., 1994). Previous work has identified increased levels of

SOD in rice tissue (coleoptiles and roots) exposed to hypoxia and anoxia (Drew, 1997; Ushimaru *et al*., 1999; Huang *et al*., 2005).

Submerged, hypoxic rice coleoptiles provide the classic case of the 'snorkel effect' (Kordan, 1974), in which coleoptiles rapidly elongate to break the surface of the water and allow sufficient O_2 supply to reach the developing seedling. By expressing relatively large amounts of SOD, when hypoxic coleoptiles reach the air (containing O_2) and conduct it throughout the 'snorkel', they might be able to prevent extensive oxidative damage by a sudden excess of electrons. Hunter *et al*. (1983) noted that of two species of *Iris, I. germanica* and *I. pseudacorus*, the more anoxia-tolerant *I. pseudocorus* showed higher levels of SOD activity during anoxia and lower levels of damaged lipid membranes upon re-aeration than *I. germanica*. Similarly, the higher relative levels of SOD in hypoxic bases (compared to tips) would endow greater protection on the actively dividing and expanding cells found in this area.

A second antioxidant protein, L-ascorbate peroxidase 1 (APX), was identified as being differentially regulated by both position and O_2 treatment. It was more abundant in coleoptile bases than tips regardless of O_2 supply (5, 2.4 and 1.7-fold in normoxia, hypoxia and anoxia, respectively). It was also more highly expressed in normoxic tips than either hypoxic (2.3-fold) or anoxic (3.4-fold) tips. Its levels were 2.3 and 3.4-fold higher in normoxic bases (than hypoxic or anoxic bases), respectively. APX acts to detoxify H_2O_2 by conversion to water via the oxidation of ascorbate (Asada, 1992). The fall of APX levels in hypoxic and anoxic bases (compared to normoxia) is likely linked to the reduction of potential oxidative damage while coleoptiles remain in these conditions. The higher expression levels of APX in coleoptile bases, particularly in normoxic coleoptiles, is likely due to the growth patterns described previously. This is because at the site of the majority of cell division, maintenance of a strong set of antioxidant systems is essential to prevent oxidative damage, particularly to the DNA of

new cells. It remains unclear why proteins that fulfil different anti-oxidant roles might be differentially expressed in the hypoxic and anoxic coleoptiles.

Spot #1749 was identified as 9-β-pimara-7,15-diene oxidase. Its expression levels were unaffected by tissue position but were strongly down-regulated (2–5-fold) in anoxia (both tips and bases) compared to both normoxia and hypoxia. 9-β-pimara-7,15-diene oxidase is an enzyme involved in the synthesis of diterpenes (Chu and Coates, 1992). Molecules of this family have been identified as playing a role in the defence response of rice plants infected with sheath blight (Sekido *et* al., 1986; Kumar *et al*., 2003). As it catalyses an oxidative step in the synthesis of a variety of diterpenes, anoxia would completely inhibit its action. The down-regulation of this protein is an example of the fine control over translation exhibited by the rice coleoptile during anoxia: in order to preserve the low amounts of available ATP, synthesis of proteins that are not playing a crucial role in survival are blocked.

Protein spot #1725 was identified as 14-3-3-like protein GF14-b. It was strongly (2.5-fold) up-regulated in normoxic bases (compared to normoxic tips). It was also strongly up-regulated in hypoxic tips compared to normoxia (2.8-fold) and hypoxia (2.7-fold). It should be recalled that tips of hypoxic coleoptiles do participate in rapid elongation under the conditions of these experiments but will have been growing for longer than their counterpart base tissues. The 14-3-3-like proteins are a large family of adaptor/chaperone proteins that aid the activity of regulatory kinases and the transduction of signal cascades in a large variety of cellular metabolic processes including aiding in the regulation (inhibition and promotion of) of plasma membrane H^+-ATPase, nitrate reductase and sucrose phosphate synthase pathways (Palmgren *et al*., 1998; Chung *et al*., 1999). OsGF14-b has been identified as being down-regulated in the shoots of rice plants exposed to high levels of selenium (Wang *et al*., 2012). Rohila *et al*. (2006) identified it as directly interacting with two receptor kinases (encoded at loci

Os03g61060 and Os01g10450). The kinase encoded for by Os03g61060 was also shown to interact directly with glutamate decarboxylase (GAD) (Os08g36320), a plasma membrane proton effluxing ATPase (Os04g56160) and a cysteine proteinase inhibitor (CPI) (Os01g58890). Likewise, the kinase encoded for by Os01g10450 also interacted with GAD (as above) as well as the histone H2A (Os10g28230) and thioredoxin (Os08g29110). The genes encoding these proteins are all either known to be regulated in hypoxic/anoxia rice plants by being induced (GDC in rice) (Reggiani *et al*., 1988), by being inhibited (the ATPase and CPI) or by acting as in a key role in the regulatory network responding to O_2 shortage (thioredoxin) (Blokhina and Fagerstedt, 2010). The effect of disrupting the function of GF14-b is unknown but merits further investigation.

The final miscellaneous protein spot (#2004) was cyanate hydratase. It was strongly affected by both tissue position and O_2 supply. It was consistently higher in coleoptile bases than tips regardless of O_2 supply (7.9, 2.9 and 3.7-fold in normoxia, hypoxia and anoxia, respectively). In tips, it was 5.6-fold in hypoxia and 3.6-fold higher in anoxia than normoxia. In bases, it was 2-fold higher in hypoxia than normoxia. Cyanate hydratase acts to converts cyanate and bicarbonate into ammonia and carbon dioxide (Sung and Fuchs, 1988). Rotini and Marino (1957) noted that potassium cyanate inhibited mitosis in *Allium cepa*. Smith and Thompson (1971) showed that potassium cyanate decreased rates of nitrate reductase activity in barley root tips by 59% (compared to controls). The presence of cyanate hydratase in the rapidly dividing tissues (bases) would serve to prevent any toxic cyanate, effects from occurring during mitosis. The increased levels of cyanate hydratase in O_2-deprived tissues (compared to normoxia) would protect the functioning of nitrate reductase, a protein whose expression increases in associated with O_2 deprivation in these tissues (Mattana *et al*., 1994).

In conclusion, the observed changes in specific protein abundance in our experiments demonstrate the dynamic nature of the rice coleoptile proteome: both different levels of external O_2 supply and tissue position within the coleoptile caused large-scale proteomic changes. Additionally the changes reported above (Table 2) suggest that hypoxia is not merely a transitional stage between normoxia and anoxia but instead elicits a unique response in rice coleoptiles.

4.4.7. Changes in the proteome and transcriptome are often poorly matched

To complement our microarray experiment (Chapter 3), we examined differences in the proteome of the tips and bases of normoxic, hypoxic and anoxic rice coleoptiles (Table 2). Such an approach, with gene expression and protein data on tightly defined growing zones across treatments gave an opportunity to assess the regulation of phenotype in a very precise manner. This was considered important especially because levels of gene expression as measured by microarrays often correlate poorly with protein measurements using proteomic techniques (Gygi *et al*., 1999) (see Table 3).

As can be seen from Table 3, the observed fold changes in protein expression often do not correlate well, with 59% (107/180) of the comparisons featuring protein:mRNA ratios of less than 2 whilst a further 37% (67/180) of the comparisons had a ratio of less than 5. Only 4% (6/180) were greater than a 5-fold difference protein and mRNA levels. Branco-Price *et al*. (2008) showed that in hypoxic *Arabidopsis* seedlings only 23% of mRNAs (of the total expressed population) were strongly associated with polysomes and thus rapidly translated to proteins. One of the proteins that showed a higher protein:mRNA ratio was TCTP (Table 3). AtTCTP expression is known to be strongly correlated to the formation of polysome in proliferating cells in *Arabidopsis* (Berkowitz *et al*., 2008). Similarly, the 17.9 kDa heat shock protein shows a

23-fold difference in protein and mRNA changes, suggestive of the formation of polysomes on its' mRNA. Finally, cyanate hydratase shows a much larger (3-10 times) increase in its' mRNA levels (*cf*. protein levels), particularly when normoxic tips are compared to either normoxic bases or hypoxic/anoxic tips. This could be indicative of an exclusion from the formation of polysomes in normoxic tissues and recruitment into polysomes during O_2 deprivation.

5 Chapter 5 – The effect of differing exogenous N supplies on O_2-deprived rice coleoptiles

5.1 Introduction

As established, during an O_2 deficit, the ability of rice coleoptiles to produce ATP is severely retarded (Mocquot *et al*., 1981; Guglielminetti *et al*., 1995; Gibbs *et al*., 2000; Edwards *et al*., 2012). Indeed, while in strict anoxia rice coleoptiles could support an ATP regeneration rate that is up to 35% that of their normoxic counterparts by increasing their consumption of carbohydrate by glycolysis 8-fold (Gibbs and Greenway, 2003), actual rates of ATP synthesis are probably reduced by about 90% in anoxia. Any means that would allow a reduction of carbohydrate consumption while aiding in either ATP production or maintenance of cellular homeostasis would aid in surviving the O_2 deficit. Possible energy sources that could supplement fermentation are pyrophosphate-driven metabolism (Huang *et* al., 2008; Liu et al., 2010) and the haemoglobin (Hb)-nitrous oxide (NO) cycle (Igamberdiev and Hill, 2004; Igamberdiev *et al*., 2005).

Utilisation of endogenous and exogenous nitrogen (N) sources, particularly in the form of nitrate, may provide such a means. The mobilisation of endogenous inorganic N and organic molecules such as amino acids from seed reserves is vital for germination and coleoptile growth in anoxic conditions (Reggiani *et al*., 1993a). During anoxia, rice coleoptiles exhibit an increase in nitrate reductase activity (Reggiani *et al*., 1993a, 1993b) and readily take up exogenous nitrate and incorporate it into protein (Fan *et al*., 1997). Vartapetian and Polyakova (1999) showed that supplying anoxic coleoptiles with exogenous nitrate had a protective effect on their mitochondria, preventing degradation of the mitochondrial membrane compared to controls. Polyakova and Vartapetian (2003) concluded that nitrate was acting as a terminal electron receptor for the mitochondrial electron transfer chain in anoxic coleoptiles. Stoimenova *et al*. (2007)

demonstrated that, in the absence of O_2, mitochondria isolated from rice roots were able to use nitrite generated from nitrate reductase (Reggiani *et al*., 1993b) as a terminal electron acceptor to allow production of ATP. Similarly, Igamberdiev *et al*. (2005) also suggested that a cycle could exist in hypoxia whereby NO would be oxidised by Hb to form nitrate (NO_3^-) and allow the reduction of NADH to NAD^+.

The protective qualities of nitrate on mitochondrial membranes noted above and the nitrite-driven production of ATP would supplement fermentative ATP in anoxic rice coleoptiles and might allow coleoptiles to elongate enough to survive.

In order to determine if the supply of exogenous nitrate affected the growth of anoxic rice coleoptiles, we supplied low levels of exogenous nitrate to seedlings germinating in anoxic conditions. Since anoxic coleoptiles are known to incorporate exogenous ammonia into their proteins (Fan *et al*., 1997), we used three external N supplies: 2 mM ammonium phosphate (to test the effect of ammonium on the coleoptiles), 2 mM sodium nitrate (to test the effect of nitrate) and 1 mM ammonium nitrate (to test the combined effect). This resulted in 2 mM exogenous N being available to the coleoptiles regardless of the treatment. This experiment was, in addition, a preamble to attempting quantitative proteomics in which we labelled with exogenous ^{15}N and separated proteins. These quantitative data remain unresolved.

Additionally, since the ability of rice coleoptiles to elongate under anoxia varies with cultivar (Edwards *et al*., 2012), we decided to incorporate four cultivars into our experimental design: Khao Hlan On (KHO) an extremely flood-tolerant Burmese rice (Ismail *et al*., 2009), Amaroo a commercial Australian cultivar with moderate flood tolerance (Edwards *et al*., 2012), *rad*, a *r*educed *a*lcohol *d*ehydrogenase mutant known to be extremely intolerant to flooding and Kinmaze, the *rad* parent line (Matsumura *et al*., 1998).

5.1.1. Experimental Aims

The work reported on in this chapter had two key experimental aims:

1. To examine the effect of varying exogenous N supplies on the growth of *O. sativa*. Cv 'Amaroo' coleoptiles grown under various levels of O_2-deprivation and
2. To examine the effect of variation in *O. sativa* genotype in the response to exogenous N supply.

5.2 Methods and Materials

5.2.1 Plant Growth

Seeds of four genotypes of *Oryza sativa* (Amaroo, KHO, Kinmaze and *rad*) were surface sterilised as described in Edwards *et al*. (2012). Seeds were grown using the setup described there, with the exception that the growth solution (containing KH_2PO_4; $CaCl_2$) was either un-supplemented with N (control) or supplemented with exogenous N (2 mM ammonium phosphate, 2 mM sodium nitrate or 1 mM ammonium nitrate). Plants were grown in strict anoxia (using a pure N_2 supply bubbled through the growth solution) or normoxia (air bubbled through the growth solution).

5.2.2 Measurements of coleoptile length and weight

Coleoptile lengths (in mm) (Table 1 and 2) and weights (Supplementary Tables D1 & D2) of each cultivar in each treatment were measured at daily intervals for 3 d beginning 3 d after imbibition. Means and standard errors of the data were calculated. From this data, the weight-to-length ratio was calculated for each treatment (Tables 3 & 4).

5.2.3 Statistical analysis of coleoptile length and weight

In order to determine any interaction between harvest age, cultivar and treatment on coleoptile length, a three-way ANOVA was conducted using these three items as the

factors. Based on this analysis it was determined that cultivar ($\omega^2 = 0.42$) and harvest age ($\omega^2 = 0.35$) were the major contributors to variation within the data. The interaction of these two factors also produced the third strongest influence on variance ($\omega^2 = 0.12$)

On this basis, further two-way ANOVAs were conducted for each cultivar (using harvest age and treatment as factors) and harvest age (using cultivar and treatment as factors). From these, it was determined that the ANOVAs across harvest age (using cultivar and treatment as factors) were able to explain the variance within the data to the greatest extent and were used for further analysis.

From the harvest age ANOVAs, a Fisher LSD test was conducted within each factor (cultivar and treatment) to determine statistically different means. This is shown below (Table 1 and 2).

5.3 Results

There was significant variation between the lengths of coleoptiles at equivalent ages, both across treatments within a cultivar and between cultivars within a single treatment in both anoxia and normoxia (Table 1 and 2).

In anoxia (Table 1), KHO consistently produced the longest coleoptiles regardless of age or N supply. Amaroo and Kinmaze were generally statistically indistinguishable in all treatments except when grown in 2 mM ammonium phosphate when Amaroo coleoptiles were longer than those of Kinmaze 4 and 5 d after germination (15.0 ± 2.6 *vs* 10.7 ± 1.5 mm and 25.5 ± 2.1 *vs* 21.8 ± 2.9 mm for Amaroo *vs* Kinmaze at 4 and 5 d, respectively). Within all four cultivars grown in anoxia, the 1 mM ammonium nitrate-treated coleoptiles were consistently significantly longer than those either in 2 mM ammonium phosphate or 2 mM sodium nitrate. When N was supplied uniquely as nitrate *or* ammonium, coleoptile lengths were generally statistically not different to one

another but they were consistently longer than the control coleoptiles. The *rad* coleoptiles were the shortest of all, regardless of treatment or age.

Coleoptiles grown in normoxia showed a different growth pattern (Table 2) to those grown in anoxia. Coleoptiles of Amaroo and KHO were longer than those of both Kinmaze 3 and 4 d, regardless of treatment. In turn, Kinmaze coleoptiles were longer than those of *rad*. Comparing individual cultivars across N treatments at a single time-point, there was no variation in either Amaroo or KHO at any time. The 2 mM ammonium phosphate and 1 mM ammonium nitrate treatments showed an effect in Kinmaze and *rad*: coleoptiles grown in these conditions were longer than control coleoptiles in all time-points, suggesting a specific response to ammonium. By 5 d, large numbers of Amaroo and KHO coleoptiles were visibly beginning to senesce, splitting from the tip and curling back down the emerging shoot, regardless of N treatment; thus elongation had probably naturally terminated. While the control and sodium nitrate-treated Kinmaze were also senescing, none of the ammonium phosphate or ammonium nitrate-treated coleoptiles had begun to senesce and were twice the length of the control and sodium nitrate-treated coleoptiles. None of the *rad* coleoptiles appeared to be senescing, regardless of treatment.

Table 1. Lengths (mm) of coleoptiles of various rice cultivars grown in strict anoxia with a variety of exogenous N sources. Data shown are means ± SEM. Superscripts denote statistically different results as determined by a Fisher LSD test. *Numbers* designate differences within rows, *letters* differences within columns within a single time point (3, 4 or 5 d).

	Treatment			
	Control (no exogenous N)	2 mM ammonium phosphate	2 mM sodium nitrate	1 mM ammonium nitrate
Cultivar	*3-d old coleoptiles*			
Amaroo	4.3 ± 1.5^{1,a}	5.4 ± 1.6^{1,a}	4.8 ± 2.4^{1,a}	5.7 ± 1.6^{1,a}
KHO	5.7 ± 1.6^{1,a}	9.5 ± 2.3^{2,b}	10.9 ± 2.8^{2,b}	15.0 ± 3.7^{3,b}
Kinmaze	4.1 ± 1.3^{1,a}	5.4 ± 1.4^{1,2,a}	5.8 ± 1.3^{2,a}	6.4 ± 1.1^{2,a}
rad	0.6 ± 0.7^{1,b}	0.6 ± 0.7^{1,c}	0.4 ± 0.7^{1,c}	0.9 ± 0.9^{1,c}
	4-d old coleoptiles			
Amaroo	13.1 ± 1.8^{1,a}	15.0 ± 2.6^{1,2,a}	14.9 ± 1.6^{1,2,a}	17.2 ± 1.9^{2,a}
KHO	17.0 ± 2.9^{1,b}	19.6 ± 3.9^{2,b}	21.7 ± 3.2^{2,b}	26.6 ± 4.3^{3,b}
Kinmaze	10.6 ± 1.7^{1,c}	10.7 ± 1.5^{2,c}	13.8 ± 2.3^{3,a}	15.9 ± 1.9^{4,a}
rad	1.2 ± 0.6^{1,d}	1.3 ± 0.8^{1,d}	1.6 ± 0.9^{1,c}	1.7 ± 1.0^{1,c}
	5-d old coleoptiles			
Amaroo	17.1 ± 3.2^{1,a}	25.5 ± 2.1^{2,a}	25.2 ± 2.9^{2,a}	31.2 ± 5.5^{3,a}
KHO	34.7 ± 3.7^{1,b}	40.0 ± 4.5^{2,b}	35.5 ± 7.2^{1,b}	51.7 ± 5.4^{3,b}
Kinmaze	17.2 ± 2.5^{1,a}	21.8 ± 2.9^{2,c}	20.5 ± 3.0^{2,a}	30.0 ± 3.1^{3,a}
rad	3.2 ± 1.6^{1,c}	4.1 ± 1.5^{1,d}	4.1 ± 1.4^{1,c}	5.2 ± 1.3^{1,c}

Table 2. Lengths (mm) of coleoptiles of various rice cultivars grown in normoxia with a variety of exogenous N sources. Data shown are means ± SEM. Superscripts denote statistically different results as determined by a Fisher LSD test. *Numbers* designate differences within rows, *letters* differences within columns within a single time point (3, 4 or 5 d).

	Treatment			
	Control (no exogenous N)	2 mM ammonium phosphate	2 mM sodium nitrate	1 mM ammonium nitrate
Cultivar	*3-d old coleoptiles*			
Amaroo	16.3 ± 3.1^{1,a}	21.5 ± 2.1^{1,a}	17.2 ± 4.6^{1,a}	23.2 ± 2.1^{1,a}
KHO	15.6 ± 2.7^{1,a}	13.5 ± 1.4^{1,b}	20.1 ± 5.1^{2,a}	18.8 ± 1.4^{2,a}
Kinmaze	9.8 ± 2.0^{1,b}	14.2 ± 2.5^{1,2,b}	18.8 ± 3.0^{2,a}	19.1 ± 1.4^{2,a}
rad	1.5 ± 0.4^{1,c}	3.8 ± 1.1^{2,c}	2.2 ± 1.2^{1,2,b}	6.8 ± 1.6^{3,c}
	4-d old coleoptiles			
Amaroo	32.0 ± 2.8^{1,a}	33.3 ± 2.1^{1,a}	29.9 ± 1.9^{1,a}	37.9 ± 5.9^{1,a}
KHO	31.7 ± 1.7^{1,a}	34.4 ± 1.8^{1,a}	28.2 ± 2.2^{1,a}	33.2 ± 2.6^{1,a,b}
Kinmaze	21.4 ± 1.7^{1,b}	27.3 ± 2.1^{1,2,a}	20.6 ± 1.2^{1,b}	31.3 ± 1.4^{2,b}
rad	8.7 ± 1.1^{1,c}	11.8 ± 1.9^{1,2,b}	8.3 ± 2.5^{1,c}	14.8 ± 1.9^{2,c}
	5-d old coleoptiles			
Amaroo	38.8 ± 2.5^{1,a}	35.9 ± 4.4^{1,a,b}	38.3 ± 4.1^{1,a}	43.4 ± 8.9^{1,a}
KHO	31.8 ± 3.6^{1,a}	41.8 ± 4.2^{1,b}	32.0 ± 5.8^{1,a}	42.2 ± 6.8^{1,a}
Kinmaze	19.0 ± 2.5^{1,b}	36.2 ± 4.7^{2,b}	19.0 ± 2.5^{1,b}	42.6 ±4.7^{2,a}
rad	12.2 ± 1.3^{1,c}	30.1 ± 3.1^{2,a}	11.9 ± 2.0^{1,c}	26.3 ± 1.8^{2,b}

We also examined the weights of anoxic and normoxic coleoptiles grown with a variety of N supplies. From these weights we calculated the weight-to-length ratios of the various treatments (Table 3 and 4). Proportional error in the weight-to-length ratios was calculated as the sum of the proportional errors in the length and weight.

Table 3. Fresh weight-to-length ratio (mg (FW) mm^{-1}) of coleoptiles of various rice cultivars grown in strict anoxia with a variety of exogenous N sources. Data shown are calculated ratios and errors.

	Treatment			
	Control (no exogenous N)	2 mM ammonium phosphate	2 mM sodium nitrate	1 mM ammonium nitrate
Cultivar	*3-d old coleoptiles*			
Amaroo	0.27 ± 0.07	0.25 ± 0.05	0.28 ± 0.09	0.35 ± 0.07
KHO	0.23 ± 0.05	0.16 ± 0.03	0.13 ± 0.03	0.11 ± 0.03
Kinmaze	0.24 ± 0.5	0.24 ± 0.4	0.23 ± 0.04	0.23 ± 0.04
rad	0.50 ± 0.57	0.51 ± 0.51	0.70 ± 0.70	0.38 ± 0.32
	4-d old coleoptiles			
Amaroo	0.20 ± 0.02	0.21 ± 0.03	0.22 ± 0.02	0.20 ± 0.03
KHO	0.15 ± 0.02	0.13 ± 0.03	0.12 ± 0.02	0.10 ± 0.02
Kinmaze	0.20 ± 0.03	0.18 ± 0.02	0.19 ± 0.02	0.19 ± 0.02
rad	0.38 ± 0.13	0.32 ± 0.14	0.29 ± 0.12	0.29 ± 0.12
	5-d old coleoptiles			
Amaroo	0.17 ± 0.03	0.16 ± 0.01	0.15 ± 0.01	0.13 ± 0.02
KHO	0.09 ± 0.01	0.08 ± 0.01	0.09 ± 0.02	0.06 ± 0.01
Kinmaze	0.12 ± 0.01	0.13 ± 0.01	0.14 ± 0.02	0.12 ± 0.01
rad	0.21 ± 0.08	0.20 ± 0.05	0.19 ± 0.04	0.19 ± 0.03

Table 4. Fresh weight-to-length ratio (mg (FW) mm^{-1}) of coleoptiles of various rice cultivars grown in normoxia with a variety of exogenous N sources. Data shown are calculated ratios and proportional errors.

	Treatment			
	Control (no exogenous N)	2 mM ammonium phosphate	2 mM sodium nitrate	1 mM ammonium nitrate
Cultivar	*3-d old coleoptiles*			
Amaroo	0.28 ± 0.11	0.20 ± 0.04	0.26 ± 0.10	0.17 ± 0.03
KHO	0.21 ± 0.07	0.22 ± 0.05	0.15 ± 0.08	0.17 ± 0.02
Kinmaze	0.40 ± 0.16	0.26 ± 0.09	0.20 ± 0.07	0.19 ± 0.03
rad	0.92 ± 0.46	0.36 ± 0.20	0.50 ± 0.41	0.21 ± 0.09
	4-d old coleoptiles			
Amaroo	0.19 ± 0.03	0.19 ± 0.02	0.19 ± 0.02	0.14 ± 0.02
KHO	0.13 ± 0.01	0.17 ± 0.02	0.13 ± 0.01	0.12 ± 0.01
Kinmaze	0.29 ± 0.04	0.25 ± 0.04	0.32 ± 0.04	0.20 ± 0.02
rad	0.32 ± 0.08	0.22 ± 0.07	0.30 ± 0.18	0.18 ± 0.04
	5-d old coleoptiles			
Amaroo	0.32 ± 0.04	0.27 ± 0.07	0.27 ± 0.06	0.32 ± 0.13
KHO	0.21 ± 0.05	0.13 ± 0.03	0.17 ± 0.06	0.15 ± 0.05
Kinmaze	0.53 ± 0.14	0.25 ± 0.06	0.57 ± 0.15	0.18 ± 0.10
rad	0.34 ± 0.08	0.14 ± 0.03	0.37 ± 0.13	0.18 ± 0.02

In anoxic coleoptiles, within a single genotype, the weight-to-length ratio generally decreased from age 3 to 5 d (Table 3). Across genotypes, the KHO coleoptiles consistently had the lowest weight-to-length ratios, regardless of age or treatment. The Amaroo and Kinmaze coleoptiles had similar weight-to-length ratios, again regardless of age or treatment. Finally, coleoptiles of the *rad* mutant showed the highest weight-to-length ratios at all timepoints and in all treatments.

Normoxic coleoptiles showed a range of response to the supply of exogenous N (Table 4). As in anoxic coleoptiles, KHO coleoptiles had the lowest weight-to-length ratio, regardless of age or treatment (with one exception: 3-d-old Amaroo coleoptiles treated with ammonium phosphate had a lower weight-to-length ratio than the similarly aged and treated KHO coleoptiles). Coleoptiles of the *rad* mutant had the highest weight-to-length ratios at 3-d but by 5-d had achieved weight-to-length ratios similar to the other three cultivars. Across the treatments, the changes exogenous N supply had no significant effect on the weight-to-length ratio of either Amaroo or KHO coleoptiles. The weight-to-length ratios of Kinmaze and *rad* coleoptiles were significantly decreased (2–3-fold) by the addition of 1 mM ammonium phosphate to the growth medium.

5.4 Discussion

The ability of rice coleoptiles to emerge and elongate under restricted O_2 supply varies dramatically based on cultivars and the availability of exogenous nutrients and endogenous solutes such as K^+ and sugars (Atwell *et al*., 1982; Huang *et al*., 2003; Huang *et al*., 2005). Depending on the cultivar, rice coleoptiles exposing to an O_2 deficit (such as during submergence) respond with one of two broad strategies: a Low Oxygen Escape Strategy (LOES) or a Low Oxygen Quiescence Strategy (LOQS) (Bailey-Serres and Voesenek, 2008). LOES-adapted rice coleoptiles elongate at rates of up to 1 mm h^{-1} (Atwell *et al*., 1982; Alpi and Beevers, 1983; Edwards *et al*., 2012) in a tropic response whereby they reach the surface of the water and obtain an unrestricted O_2 supply – the 'snorkel effect' (Kordan, 1974). LOQS-adapted rices minimise carbohydrate consumption and survive, resuming growing after a prolonged submergence. Edwards *et al*. (2012) suggest that these two strategies are not mutually exclusive: hypoxic (one-seventh normal O_2 levels) rice coleoptiles pursue a primarily LOES strategy, whereas coleoptiles in anoxia primarily pursue a LOQS strategy; they still elongate slowly at a

fraction the rate of hypoxic coleoptiles, allowing them potentially to reach the water surface.

The addition of an exogenous N source (either nitrate, ammonia or both) greatly increased the rate of coleoptile elongation in anoxia in all cultivars except *rad*. This is indicative of a transition from a predominately LOQS to a LOES. The rates of elongation and coleoptile lengths were similar to those observed in hypoxic coleoptiles (without exogenous N) for Amaroo, KHO and Kinmaze (Edwards *et al*., 2012). The relative inability of *rad* to elongate even in the presence of exogenous N is likely due to the lesion in ethanolic fermentation in this mutant (Saika *et al*., 2006; Edwards *et al*., 2012).

The shift from a LOQS to a LOES for anoxic rice coleoptiles supplied with exogenous N is most clearly seen in KHO. Anoxic KHO coleoptiles supplied with exogenous ammonium nitrate had weight-to-length ratios of 0.11 mg mm^{-1} compared to 0.23 mg mm^{-1} at 3 d. This was maintained until 5 d when the respective ratios were 0.06 and 0.9 mg mm^{-1}.

The effect of exogenous N on anoxic elongation appears not to be affected by the N source (nitrate *vs* ammonia) (Table 1), suggesting that both oxidised and reduced N can promote the elongation of anoxic rice coleoptiles. Interesting, the supply of both ammonia and nitrate (in the form of ammonium nitrate) had an additive effect on elongation and final coleoptile length compared to ammonia or nitrate alone, suggesting that these compounds may act through separate pathways to enhance elongation. The effect was not due to difference in N supply *per se* as total available exogenous N was the same in each treatment.

Within normoxic coleoptiles (Table 2), elongation rates (and onset of senescence) in Amaroo and KHO coleoptiles appeared to be unaffected by an exogenous N supply. Normoxic Kinmaze and *rad* coleoptiles were affected by the addition of an exogenous ammonium supply; by 5 d in both cultivars an exogenous supply of ammonium (as

either ammonium phosphate or ammonium nitrate) doubled coleoptile length compared to either control or sodium nitrate-fed coleoptiles. This doubling in length was matched by an approximate halving of the weight-to-length ratio in both genotypes (Table 4). Additionally, exogenous ammonium specifically prevented the onset of senescence in Kinmaze coleoptiles, suggesting a developmental effect that is independent of growth. This deserves further attention, with regard for the low energy requirement for ammonium uptake.

The increased rates of coleoptile elongation in ammonium-treated normoxic Kinmaze and *rad* coleoptiles are hard to explain. The weight-to-length ratios of 5-d old normoxic Kinmaze and *rad* coleoptiles supplied with ammonium were similar to those of 4- and 5-d-old anoxic Kinmaze and *rad* coleoptiles (regardless of N supply). This suggests that an O_2-independent response is causing the elongation of these coleoptiles. This response also appears to be ATP-independent: *rad* is genetically identical to Kinmaze excepting a non-functioning ADH1 (Saika *et al*., 2006) but both show a similar response to exogenous ammonium in normoxia.

Since only ammonium-treated normoxic *rad* and Kinmaze show this response, ammonium or one of its by-products may play a role regulatory role in coleoptile elongation. Sasakawa *et al*. (1978) observed that rice roots in air-bubbled solution had increased ammonium uptake compared to roots in N_2-bubbled solution. Fan *et al*. (1997) reported that during normoxia, 3× more ammonium is incorporated into alanine than into glutamate, whereas nitrate is incorporated into alanine and glutamate in approximately equal amounts. Alanine, which is known to accumulate in anoxic coleoptiles (Reggiani *et al*., 1995; Kato-Noguchi 2006; Edwards *et al*., 2012), may play a role in influencing the elongation of the Kinmaze and *rad* coleoptiles. Overall, the reason for which coleoptiles (ammonium-treated Kinmaze and *rad*) supplied with abundant O_2

(in solution) would elongate at such a rapid rate (*cf*. their control and sodium nitrate treated counterparts) is unknown and merits further investigation.

6 Chapter 6 - General Discussion

6.1 Introduction

Rice (*Oryza sativa*) provides a staple food for more than three billion people worldwide (FAO, 2007). Compared to other major cereal crops, which are predominantly grown under dryland conditions (wheat, maize and barley), the cultivation of rice is in wet soils largely in the Third World is time-consuming, labour intensive and less amenable to mechanisation (Khush, 1997; Higham and Lu, 1998). However, rising labour costs even in the poorest countries have motivated researchers to find ways to replace traditional transplantation of seedlings into paddies with a means of direct sowing into water. This has rapidly heightened the focus of recent research on the germination and establishment of seedlings under low-O_2 conditions (reviewed by Miro and Ismail, 2013) while driving the identification of QTLs and prospective genes conferring submergence tolerance at germination.

In low O_2 conditions produced in the field by sowing into paddies or slow moving/stagnant floodwater, coleoptiles of some rice cultivars rapidly elongate in order to reach atmospheric O_2, while shoot and root growth is suppressed. This rapid elongation has been replicated in laboratory conditions and varies based on genotype (Atwell *et al*., 1982; Alpi and Beevers, 1983; Magneschi *et al*., 2009). This phenomenon was called the 'snorkel effect' by Kordan (1974), referring to the ability of coleoptiles to access atmospheric O_2 from stagnant deep solutions.

This thesis examined some of the underlying mechanisms that have evolved into these exquisite adaptations to O_2 deficits. Several approaches were used, based on coleoptiles of rice (*cv.* Amaroo) subjected to O_2 deprivation: (a) metabolic (Chapter 2), (b) transcriptomic (gene expression) (Chapter 3) and (c) proteomic (Chapter 4) responses. Finally, in a smaller final chapter dealing with alternative electron acceptors,

the effect of various exogenous sources of nitrogen on O_2-deprived coleoptiles was reported. In the General Discussion, we discuss five major points: broad-ranging questions about energy turnover in O_2-deprived coleoptiles in the face of reduced ATP availability; the basis of the 'snorkel effect' in hypoxic coleoptiles; the disparate metabolic, gene expression and proteomic profiles of coleoptiles tips and bases; the characteristics that make rice coleoptiles an ideal model organism for studying the response to an O_2 shortage in plants and the phenomena which can be elucidated in future research using this system.

6.2 ATP production and utilisation: a delicate balance

Atkinson (1968) proposed a model linking ATP production and utilisation, proposing that any decrease in ATP production would be matched by an equivalant decrease in ATP-utilising processes. Chapter 2 uses the most comprehensive energy budget yet attempted for an O_2-limited organ to demonstrate that rice coleoptiles support this model, at least in semi-quantitative terms. As O_2 supply (and ATP production) decreased (normoxia → hypoxia → anoxia) there was decrease in ATP utilisation across a range of the principal energy-consuming processes (Edwards *et al*., 2012). The amount of ATP that could be assigned to the major metabolic processes that were measured here provided an insight into how coleoptiles acclimated to O_2 deficits. In normoxia and hypoxia, two-thirds of (estimated) available ATP could not be accounted for by the major metabolic processes measured, presumably because there were many metabolic pathways engaged but not measured (e.g. secondary metabolism, transport of ions that were not measured). By contrast, in anoxia, *all* of the available ATP was accounted for by eight metabolic processes (protein, cell wall, lipid, nucleic acid and amino acid synthesis; carbohydrate and nitrate import from the seed and K^+ influx). This demonstrates that in periods of ATP shortage, rice coleoptiles tightly matched ATP

production to utilisation, as suggested by Atkinson (1968). This finding would not be possible in a tissue unable to grow in anoxia. More significantly, these energy budgets establish clearly, for the first time, that protein synthesis is preferentially maintained when O_2 supply fails (consuming 52% of available ATP), to the detriment of other processes. This is phenomenon is at the core of expression of the key 'adaptive genes' discussed below.

6.3 Hypoxia induces a unique response in rice coleoptiles

Hypoxia (3% O_2) produces a unique response in rice coleoptiles compared to either normoxia (air) or anoxia (N_2), namely the 'snorkel effect' described above. This response has its origins in the ecology of rice as a marsh grass, where rapidly elongating coleoptiles can reach atmospheric O_2 and normal seed development can occur (Sweeney and McCouch, 2007).

6.3.1 Comparisons with normoxic and anoxia

Hypoxic rice coleoptiles had intrinsically distinct metabolic, gene expression and proteomic profiles when compared to coleoptiles in normoxia and anoxia. For example, hypoxic coleoptiles had metabolic properties of both normoxic and anoxic coleoptiles. Rates of cell wall synthesis, amino acid synthesis, nitrate import and K^+ influx were identical to normoxic coleoptiles but much higher than in anoxic coleoptiles. Conversely, rates of protein synthesis, nucleic acid synthesis and carbohydrate import in hypoxic coleoptiles were closer to those of anoxic coleoptiles (*cf*. normoxic) while rates of lipid synthesis hypoxia were midway between those of normoxic and anoxic coleoptiles.

At the level of gene expression, hypoxic coleoptiles were distinct from both normoxic and anoxic coleoptiles. Comparing the sets of genes up-regulated by hypoxia and anoxia (*cf*. normoxia) ~20% of the genes up-regulated by hypoxia were unaffected by anoxia (Figure 3, Chapter 4). Genes showing an up-regulation during hypoxia

exclusively include those related to cell wall modification (pectin esterases and expansins), protein degradation (members of the proteasome, ubiquitin and PPR) and general stress response (Figures 4 & 5, Chapter 4). Similarly, of genes down-regulated by hypoxia or anoxia (*cf.* normoxia) ~12% were down-regulated by hypoxia alone (Figure 3, Chapter 4). Chief amongst these were the peroxidases, noted to decrease cell wall extensibility in rice (Ismail *et al*., 2009). Peroxidase enzyme activity decreased in anoxic rice coleoptiles (*cf*. normoxia), which elongate in response to anoxia, but increased in anoxic roots where elongation is retarded (Lee and Lin, 1995).

When gene products were measured by comparative proteomics, hypoxic coleoptiles showed an increase in a number of proteins involved in energy production and others previously associated with elongation in rice tissues including the 16.9-kDa and 17.9-kDa HSPs, LEA3 and the 14-3-3-like protein GF14-B as discussed in Chapter 4.

6.3.2 Hypoxic coleoptiles have evolved for rapid elongation

Hypoxic coleoptiles have evolved to elongate as rapidly as their metabolism could sustain, achieving rates of up to 0.5 mm h^{-1} (*cf*. 0.3 mm h^{-1} for normoxic coleoptiles) (Edwards *et al*., 2012). Slower rates were observed in normoxic coleoptiles because their growth terminated early as leaves emerged, and in anoxic coleoptiles, which were severely energy deprived; hypoxic coleoptiles elongated at 4× and 1.5× the rates of anoxic and normoxic coleoptiles, respectively. This is reinforced by the experimental observations in Chapters 2 to 4. Metabolically, hypoxic coleoptiles (*cf.* anoxic coleoptiles) allocated available ATP to the processes required for rapid elongation: cell wall synthesis to strengthen the long fragile organs, lipid synthesis to maintain cell membrane integrity and K^+ (and other solute) uptake for turgor maintenance. In the microarray study of hypoxic coleoptiles, high levels of gene expression were associated with cell wall modification/loosening (pectin esterases and expansins) and synthesis (cellulose synthases), uptake of ions and lipid synthesis. This strategic expression of

genes is clearly under regulatory controls that allow low, but not negligible, levels of O_2 to be detected. Presumably, multiple anaerobically response elements in the rice genome have evolved to permit this subtle response (see Liu *et al*., 2010 for examples of such motifs).

6.4 Tips and bases: a tale of two tissues

In addition to highlighting the unique nature of the hypoxic response in rice coleoptiles, our research allowed the examination of differences between the tips and bases of rice coleoptiles, highlighting the difference between these two tissues. This difference in most clearly seen in normoxic coleoptiles, where the tips begin to senesce while the bases may still be elongating (Inada *et al*., 1998; Kawai and Uchimiya, 2000; Edwards *et al*., 2012).

Extension of the rice coleoptile requires two processes: cell division and cell elongation. Cell division has been reported previously in rice coleoptiles regardless of O_2 supply (Öpik, 1973; Kordan 1976; Edwards *et al*., 2012), as has cell elongation (Wada 1961; Furuya *et al*., 1969; Takahashi *et al*., 2011). The localisation (if any) of these processes, however, has been a matter of some debate (Wada 1961, Furuya *et al*., 1969; Atwell *et al.*, 1982). Our research suggests that these processes (cell division and elongation) are largely confined to the base of the tissue based on three observations. Firstly, we observed that the greatest difference in cell size within hypoxic coleoptiles was located in the basal 5 mm of the coleoptiles, regardless of size (Figure 2; Chapter 3). Secondly, genes encoding for the machinery of cell division (chromatin organisation, DNA polymerase and cell cycle promoters) are highly up-regulated in bases (*cf*. tips) (Supplementary Figure B1, Chapter 3). Finally, the rice homologue of the *Arabidopsis* protein TCTP, which is known to promote cell division (Berkowitz *et al*., 2008; Brioudes

et al., 2010), was most strongly expressed in the base of coleoptiles (*cf.* the tips) regardless of O_2 treatment.

6.5 Rice coleoptiles provide an unique model system for studying O_2 deprivation in plants

Rice coleoptiles provide a unique experimental system for investigating the response of plant tissues to low O_2 conditions. In addition to experimental priorities; i.e., the ability to produce large amounts of tissue in 3–4 d, the ease with which the material can be harvested and with which the external O_2 conditions can manipulated, there are around 120,000 genotypes of *O*. *sativa* in the IRRI Genebank as well as 24 wild relatives in the genus *Oryza*. Tolerance of rice coleoptiles to exposure to O_2-limited conditions varies dramatically among naturally occurring genotypes (Setter *et al*., 1994; Gibbs *et al*., 2000; Magneschi *et al*., 2009; Edwards *et al*., 2012; Miro and Ismail, 2013) with a wide mutational population also available. By exploiting this variation in an experimental system, a wide range of responses to O_2 deprivation can be examined readily (see Chapters 2 and 5 for examples).

6.6 Future research directions

Our study of the response of rice coleoptiles to O_2 deprivation opens numerous future avenues of research including investigating the regulation of mRNA translation by polysome formation and investigating the regulation of protein and mRNA turnover in rice coleoptiles. These events are unlikely to be synchronous, with considerable modification of mRNA and modification of protein turnover rates likely.

We studied the changes in the mRNA of normoxic, hypoxic and anoxic rice coleoptiles at the whole mRNA population level (Chapter 4). While this provides information on the broad-scale changes in the transcriptome of the rice coleoptile, it ignores any translational regulation of the mRNA population by polysomal formation.

Branco-Price *et al*. (2008) isolated polysomal mRNA in *Arabidopsis* using His-tagged ribosomes. They showed that in hypoxic seedlings only 23% of mRNAs (of the total expressed population) were associated with polysomes and thus translated to proteins. While no rice lines with His-tagged ribosomes have been reported, it is possible to isolate polysomal mRNA in plants by the use of differential centrifugation (Pawlowski *et al*., 1994; Mustroph *et al*., 2009). The approach of tissue and O_2 treatments combinations reported in Chapter 4 would allow us to determine the overall role of polysomal formation in regulating gene expression in O_2-deprived rice coleoptile tissues.

A further area for research involves investigating the turnover of the protein and mRNA pools in O_2-deprived coleoptiles. Based on the numbers reported in Edwards *et al*. (2012) a single normoxic or hypoxic coleoptile could turn over their protein complement in ~27 h compared to 33 h for anoxic coleoptiles. Plant proteins have been found to have a wide range of half-lives from just 20 min (Kende, 1993) to over 100 h Vierstra (1994) with a median half-life for total protein of 5–8 d (Vierstra, 1996). Given the great variance in plant protein half-lives, further research into protein turnover of rice coleoptile proteins is required. At present, we are undertaking quantitative proteomic studies to determine the rates of ^{15}N-labelled ammonium nitrate incorporation into the protein spots identified as being differentially regulated in Chapter 3.

7 Bibliography

ABARES (2011) ABARES assesses impact of floods on agricultural, mining commodities. *In*. Australian Bureau of Agricultural and Resource Economics and Sciences.

Ahsan N, Lee D-G, Lee S-H, Kang KY, Lee JJ, Kim PJ, Yoon H-S, Kim J-S, Lee B-H (2007) Excess copper induced physiological and proteomic changes in germinating rice seeds. Chemosphere **67:** 1182-1193.

Ahsan N, Lee S-H, Lee D-G, Lee H, Lee SW, Bahk JD, Lee B-H (2007) Physiological and protein profiles alternation of germinating rice seedlings exposed to acute cadmium toxicity. Comptes rendus biologies **330:** 735-746.

Akagi H, Nakamura A, Yokozeki-Misono Y, Inagaki A, Takahashi H, Mori K, Fujimura T (2004) Positional cloning of the rice *Rf-1* gene, a restorer of BT-type cytoplasmic male sterility that encodes a mitochondria-targeting PPR protein. Theoretical and Applied Genetics **108:** 1449-1457.

Al-Ani A, Bruzau F, Raymond P, Saint-Ges V, Leblanc JM, Pradet A (1985) Germination, respiration, and adenylate energy charge of seeds at various oxygen partial pressures. Plant Physiology **79:** 885-890.

Alpi A, Beevers H (1983) Effects of O_2 concentration on rice seedlings. Plant Physiology **71:** 30-34.

Amor Y, Chevion M, Levine A (2000) Anoxia pretreatment protects soybean cells against H_2O_2-induced cell death: possible involvement of peroxidases and of alternative oxidase. FEBS Letters **477:** 175-180.

Amthor JS (2000) The McCree–de Wit–Penning de Vries–Thornley respiration paradigms: 30 years later. Annals of Botany **86:** 1-20.

Andersson B, Aw T, Jones DP (1987) Mitochondrial transmembrane potential and pH gradient during anoxia. American Journal of Physiology-Cell Physiology **252:** C349-C355.

Andres C, Lurin C, Small ID (2007) The multifarious roles of PPR proteins in plant mitochondrial gene expression. Physiologia Plantarum **129:** 14-22.

Angers S, Li T, Yi X, MacCoss MJ, Moon RT, Zheng N (2006) Molecular architecture and assembly of the DDB1–CUL4A ubiquitin ligase machinery. Nature **443:** 590-593.

App AA, Meiss AN (1958) Effect of aeration on rice alcohol dehydrogenase. Archives of Biochemistry and Biophysics **77:** 181-190.

Asada K (1992) Ascorbate peroxidase–a hydrogen peroxide‐scavenging enzyme in plants. Physiologia Plantarum **85:** 235-241.

Aspart L, Got A, Delseny M, Mocquot B, Pradet A (1983) Adaptation of ribonucleic acid metabolism to anoxia in rice embryos. Plant Physiology **72:** 115-121.

Atkinson DE (1968) Energy charge of the adenylate pool as a regulatory parameter. Interaction with feedback modifiers. Biochemistry **7:** 4030-4034.

Atwell B, Thomson C, Greenway H, Ward G, Waters I (1985) A study of the impaired growth of roots of *Zea mays* seedlings at low oxygen concentrations. Plant, Cell & Environment **8:** 179-188.

Atwell B, Waters I, Greenway H (1982) The effect of oxygen and turbulence on elongation of coleoptiles of submergence-tolerant and-intolerant rice cultivars. Journal of Experimental Botany **33:** 1030-1044.

Bailey-Serres J, Lee SC, Brinton E (2012) Waterproofing crops: Effective flooding survival strategies. Plant Physiology **160:** 1698-1709.

Bailey-Serres J, Sorenson R, Juntawong P (2009) Getting the message across: cytoplasmic ribonucleoprotein complexes. Trends in Plant Science **14:** 443-453.

Bailey-Serres J, Voesenek L (2008) Flooding stress: acclimations and genetic diversity. Annual Review of Plant Biology **59:** 313-339.

Baldi P, Long AD (2001) A Bayesian framework for the analysis of microarray

expression data: regularized t-test and statistical inferences of gene changes. Bioinformatics **17:** 509-519.

Banti V, Mafessoni F, Loreti E, Alpi A, Perata P (2010) The heat-inducible transcription factor *HsfA2* enhances anoxia tolerance in *Arabidopsis*. Plant Physiology **152:** 1471-1483.

Barker J, Khan M, Solomos T (1966) Mechanism of the Pasteur effect. Nature **211:** 547-548.

Baxter CJ, Redestig H, Schauer N, Repsilber D, Patil KR, Nielsen J, Selbig J, Liu J, Fernie AR, Sweetlove LJ (2007) The metabolic response of heterotrophic *Arabidopsis* cells to oxidative stress. Plant Physiology **143:** 312-325.

Berkowitz O, Jost R, Pollmann S, Masle J (2008) Characterization of TCTP, the translationally controlled tumor protein, from *Arabidopsis thaliana*. Plant Cell **20:** 3430-3447.

Berry LJ (1949) The influence of oxygen tension on the respiratory rate in different segments of onion roots. Journal of Cellular and Comparative Physiology **33:** 41-66.

Blokhina O, Fagerstedt KV (2010) Reactive oxygen species and nitric oxide in plant mitochondria: origin and redundant regulatory systems. Physiologia Plantarum **138:** 447-462.

Blokhina O, Virolainen E, Fagerstedt KV (2003) Antioxidants, Oxidative Damage and Oxygen Deprivation Stress: a Review. Annals of Botany **91:** 179-194.

Blokhina OB, Chirkova TV, Fagerstedt KV (2001) Anoxic stress leads to hydrogen peroxide formation in plant cells. Journal of Experimental Botany **52:** 1179-1190.

Bouny JM, Saglio PH (1996) Glycolytic flux and hexokinase activities in anoxic maize root tips acclimated by hypoxic pretreatment. Plant Physiology **111:** 187-194.

Bowler C, Van Camp W, Van Montagu M, Inzé D, Asada K (1994) Superoxide

dismutase in plants. Critical Reviews in Plant Sciences **13:** 199-218.

Branco-Price C, Kawaguchi R, Ferreira RB, Bailey-Serres J (2005) Genome-wide analysis of transcript abundance and translation in *Arabidopsis* seedlings subjected to oxygen deprivation. Annals of Botany **96:** 647-660.

Branco‐Price C, Kaiser KA, Jang CJ, Larive CK, Bailey‐Serres J (2008) Selective mRNA translation coordinates energetic and metabolic adjustments to cellular oxygen deprivation and reoxygenation in *Arabidopsis thaliana*. Plant Journal **56:** 743-755.

Brioudes F, Thierry A-M, Chambrier P, Mollereau B, Bendahmane M (2010) Translationally controlled tumor protein is a conserved mitotic growth integrator in animals and plants. Proceedings of the National Academy of Sciences of the United States of America **107:** 16384-16389.

Brown DJ, Beevers H (1987) Fatty acids of rice coleoptiles in air and anoxia. Plant Physiology **84:** 555-559.

Bryant JA, Moore K, Aves SJ (2001) Origins and complexes: the initiation of DNA replication. Journal of Experimental Botany **52:** 193-202.

Burton RA, Wilson SM, Hrmova M, Harvey AJ, Shirley NJ, Medhurst A, Stone BA, Newbigin EJ, Bacic A, Fincher GB (2006) Cellulose synthase-like *CslF* genes mediate the synthesis of cell wall (1, 3; 1, 4)-ß-D-glucans. Science **311:** 1940-1942.

Chae K, Isaacs CG, Reeves PH, Maloney GS, Muday GK, Nagpal P, Reed JW (2012) *Arabidopsis* SMALL AUXIN UP RNA63 promotes hypocotyl and stamen filament elongation. Plant Journal **71:** 684-697.

Chandra Babu R, Zhang J, Blum A, David Ho T-H, Wu R, Nguyen H (2004) *HVA1*, a LEA gene from barley confers dehydration tolerance in transgenic rice (*Oryza sativa* L.) via cell membrane protection. Plant Science **166:** 855-862.

Chang WW, Huang L, Shen M, Webster C, Burlingame AL, Roberts JK (2000) Patterns of protein synthesis and tolerance of anoxia in root tips of maize seedlings acclimated to a low-oxygen environment, and identification of proteins by mass spectrometry. Plant Physiology **122:** 295-318.

Cheeseman JM, Hanson JB (1979) Energy-linked potassium influx as related to cell potential in corn roots. Plant Physiology **64:** 842-845.

Cheeseman JM, Lafayette PR, Gronewald JW, Hanson JB (1980) Effect of ATPase inhibitors on cell potential and K^+ influx in corn roots. Plant Physiology **65:** 1139-1145.

Cheng Z, Targolli J, Huang X, Wu R (2002) Wheat LEA genes, PMA80 and PMA1959, enhance dehydration tolerance of transgenic rice (*Oryza sativa* L.). Molecular Breeding **10:** 71-82.

Chirkova TV, Sinyutina NF, Blyudzin YA, Barsky IE, SV S (1989) Phospholipid fatty acids of root mitochondria and microsomes from rice and wheat seedlings exposed to aeration or anaerobiosis. Russian Journal of Plant Physiology (in Russian) **36:** 126-134.

Chivers PT, Raines RT (1997) General acid/base catalysis in the active site of *Escherichia coli* thioredoxin. Biochemistry **36:** 15810-15816.

Cho H-T, Kende H (1997) Expression of expansin genes is correlated with growth in deepwater rice. Plant Cell **9:** 1661-1671.

Cho H-T, Kende H (1997) Expansins and internodal growth of deepwater rice. Plant Physiology **113:** 1145-1151.

Choi D, Lee Y, Cho H-T, Kende H (2003) Regulation of expansin gene expression affects growth and development in transgenic rice plants. Plant Cell **15:** 1386-1398.

Choi G, Yi H, Lee J, Kwon Y-K, Soh MS, Shin B, Luka Z, Hahn T-R, Song P-S (1999) Phytochrome signalling is mediated through nucleoside diphosphate kinase 2.

Nature **401:** 610-613.

Chu M, Coates RM (1992) Partial synthesis of 9, 10-syn-diterpenes via tosylhydrazone reduction:(-)-(9. beta.)-pimara-7, 15-diene and (-)-(9. beta.)-isopimaradiene. Journal of Organic Chemistry **57:** 4590-4597.

Chung H-J, Sehnke PC, Ferl RJ (1999) The 14-3-3 proteins: cellular regulators of plant metabolism. Trends in Plant Science **4:** 367-371.

Colmer TD, Huang S, Greenway H (2001) Evidence for down‐regulation of ethanolic fermentation and K^+ effluxes in the coleoptile of rice seedlings during prolonged anoxia. Journal of Experimental Botany **52:** 1507-1517.

Cosgrove DJ (2000) Loosening of plant cell walls by expansins. Nature **407:** 321-326.

Cosgrove DJ (2005) Growth of the plant cell wall. Nature Reviews Molecular Cell Biology **6:** 850-861.

Cosgrove DJ, Jarvis MC (2012) Comparative structure and biomechanics of plant primary and secondary cell walls. Frontiers in Plant Science **3:** 204.

Coux O, Tanaka K, Goldberg AL (1996) Structure and functions of the 20S and 26S proteasomes. Annual Review of Biochemistry **65:** 801-847.

Crawford RMM (1977) Tolerance of anoxia and ethanol metabolism in germinating seeds. New Phytologist **79:** 511-517.

Crawford RMM, Braendle R (1996) Oxygen deprivation stress in a changing environment. Journal of Experimental Botany **47:** 145-159.

Das KK, Sarkar RK, Ismail AM (2005) Elongation ability and non-structural carbohydrate levels in relation to submergence tolerance in rice. Plant Science **168:** 131-136.

David G. Nicholls SSJF (1992) Bioenergetics 2. Academic Press, London; San Diego.

Davies DD (1980) Anaerobic metabolism and the production of organic acids. *In* DD Davies, ed, The Biochemistry of Plants, Vol 2. Academic Press, London, pp 581-

611.

Davies DD (1986) The fine control of cytosolic pH. Physiologia Plantarum **67:** 702-706.

Davies DD, Grego S, Kenworthy P (1974) The control of the production of lactate and ethanol by higher plants. Planta **118:** 297-310.

De Vries F, Brunsting A, Van Laar H (1974) Products, requirements and efficiency of biosynthesis a quantitative approach. Journal of Theoretical Biology **45:** 339-377.

Delannoy E, Stanley W, Bond C, Small I (2007) Pentatricopeptide repeat (PPR) proteins as sequence-specificity factors in post-transcriptional processes in organelles. Biochemical Society Transactions **35:** 1643-1647.

Derbyshire P, McCann M, Roberts K (2007) Restricted cell elongation in *Arabidopsis* hypocotyls is associated with a reduced average pectin esterification level. BMC Plant Biology **7:** 31-36.

Drew M (1983) Plant injury and adaptation to oxygen deficiency in the root environment: a review. Plant and Soil **75:** 179-199.

Drew M, Guenther J, Läuchli A (1988) The combined effects of salinity and root anoxia on growth and net Na^+ and K^+-accumulation in *Zea mays* grown in solution culture. Annals of Botany **61:** 41-53.

Drew MC (1997) Oxygen deficiency and root metabolism: injury and acclimation under hypoxia and anoxia. Annual Review of Plant Biology **48:** 223-250.

Dugas DV, Bartel B (2004) MicroRNA regulation of gene expression in plants. Current Opinion in Plant Biology **7:** 512-520.

Edwards JM, Roberts TH, Atwell BJ (2012) Quantifying ATP turnover in anoxic coleoptiles of rice (*Oryza sativa*) demonstrates preferential allocation of energy to protein synthesis. Journal of Experimental Botany **63:** 4389-4402.

Ellis TP, Helfenbein KG, Tzagoloff A, Dieckmann CL (2004) *Aep3p* stabilizes the mitochondrial bicistronic mRNA encoding subunits 6 and 8 of the H^+-

translocating ATP synthase of *Saccharomyces cerevisiae*. Journal of Biological Chemistry **279:** 15728-15733.

Escobar Galvis ML, Håkansson G, Alexciev K, Knorpp C (1999) Cloning and characterisation of a pea mitochondrial NDPK. Biochimie **81:** 1089-1096.

Etherton B (1967) Steady state sodium and rubidium effluxes in *Pisum sativum* roots. Plant Physiology **42:** 685-690.

Faiz-ur-Rahman A, Trewavas A, Davies D (1974) The Pasteur effect in carrot root tissue. Planta **118:** 195-210.

FAO (2007) The State of Food Insecurity in the World. *In*, Rome.

Feder ME, Hofmann GE (1999) Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. Annual Review of Physiology **61:** 243-282.

Felle HH (2005) pH regulation in anoxic plants. Annals of Botany **96:** 519-532.

Fennoy SL, Bailey‐Serres J (1995) Post‐transcriptional regulation of gene expression in oxygen‐deprived roots of maize. Plant Journal **7:** 287-295.

Fernie AR, Carrari F, Sweetlove LJ (2004) Respiratory metabolism: glycolysis, the TCA cycle and mitochondrial electron transport. Current Opinion in Plant Biology **7:** 254-261.

Fox G, McCallan N, Ratcliffe R (1995) Manipulating cytoplasmic pH under anoxia: a critical test of the role of pH in the switch from aerobic to anaerobic metabolism. Planta **195:** 324-330.

Fox TC, Kennedy RA, Rumpho ME (1994) Energetics of plant growth under anoxia: metabolic adaptations of *Oryza sativa* and *Echinochloa phyllopogon*. Annals of Botany **74:** 445-455.

Furuya M, Pjon CJ, Fujii T, Ito M (1969) Phytochrome action in *Oryza sativa* L. III. The separation of photoperceptive site and growing zone in coleoptiles, and auxin

transport as effector system. Development, Growth & Differentiation **11:** 62-76.

Gao Z, Qian Q, Liu X, Yan M, Feng Q, Dong G, Liu J, Han B (2009) *Dwarf 88*, a novel putative esterase gene affecting architecture of rice plant. Plant Molecular Biology **71:** 265-276.

Geigenberger P (2003) Response of plant metabolism to too little oxygen. Current Opinion in Plant Biology **6:** 247-256.

Geigenberger P, Fernie AR, Gibon Y, Christ M, Stitt M (2000) Metabolic activity decreases as an adaptive response to low internal oxygen in growing potato tubers. Biological Chemistry **381:** 723-740.

Generosova I, Vartapetian B (2005) On the physiological role of anaerobically synthesized lipids in *Oryza sativa* seedlings. Russian Journal of Plant Physiology **52:** 481-488.

Gibbs J, Greenway H (2003) Review: Mechanisms of anoxia tolerance in plants. I. Growth, survival and anaerobic catabolism. Functional Plant Biology **30:** 353-353

Gibbs J, Morrell S, Valdez A, Setter T, Greenway H (2000) Regulation of alcoholic fermentation in coleoptiles of two rice cultivars differing in tolerance to anoxia. Journal of Experimental Botany **51:** 785-796

Giraud E, Ng S, Carrie C, Duncan O, Low J, Lee CP, Van Aken O, Millar AH, Murcha M, Whelan J (2010) TCP transcription factors link the regulation of genes encoding mitochondrial proteins with the circadian clock in *Arabidopsis thaliana*. Plant Cell **22:** 3921-3934.

Goff SA, Ricke D, Lan T-H, Presting G, Wang R, Dunn M, Glazebrook J, Sessions A, Oeller P, Varma H (2002) A draft sequence of the rice genome (*Oryza sativa* L. ssp. japonica). Science **296:** 92-100.

Goodwin TW, Mercer EI (1985) Introduction to plant biochemistry. Pergamon Press, New York, New York State, USA.

Gothandam KM, Kim E-S, Cho H, Chung Y-Y (2005) OsPPR1, a pentatricopeptide repeat protein of rice is essential for the chloroplast biogenesis. Plant Molecular Biology **58:** 421-433.

Gou J-Y, Wang L-J, Chen S-P, Hu W-L, Chen X-Y (2007) Gene expression and metabolite profiles of cotton fiber during cell elongation and secondary cell wall synthesis. Cell Research **17:** 422-434.

Greenway H, Gibbs J (2003) Review: mechanisms of anoxia tolerance in plants. II. Energy requirements for maintenance and energy distribution to essential processes. Functional Plant Biology **30:** 999-1036.

Greenway H, Waters I, Newsome J (1992) Effects of anoxia on uptake and loss of solutes in roots of wheat. Functional Plant Biology **19:** 233-247.

Gruber CW, Čemažar M, Clark RJ, Horibe T, Renda RF, Anderson MA, Craik DJ (2007) A novel plant protein-disulfide isomerase involved in the oxidative folding of cystine knot defense proteins. Journal of Biological Chemistry **282:** 20435-20446.

Guglielminetti L, Morita A, Yamaguchi J, Loreti E, Perata P, Alpi A (2006) Differential expression of two fructokinases in *Oryza sativa* seedlings grown under aerobic and anaerobic conditions. Journal of Plant Research **119:** 351-356.

Guglielminetti L, Yamaguchi J, Perata P, Alpi A (1995) Amylolytic activities in cereal seeds under aerobic and anaerobic conditions. Plant Physiology **109:** 1069-1076.

Gupta KJ, Igamberdiev AU (2011) The anoxic plant mitochondrion as a nitrite: NO reductase. Mitochondrion **11:** 537-543.

Gupta M, Yoshioka H, Ohnishi K, Mizumoto H, Hikichi Y, Kiba A (2013) A Translationally Controlled Tumor Protein Negatively Regulates the Hypersensitive Response in *Nicotiana benthamiana*. Plant and Cell Physiology *doi: 10.1093/pcp/pct090.*

Gutterson N, Reuber TL (2004) Regulation of disease resistance pathways by AP2/ERF transcription factors. Current Opinion in Plant Biology **7:** 465-471.

Gygi SP, Rochon Y, Franza BR, Aebersold R (1999) Correlation between protein and mRNA abundance in yeast. Molecular and Cellular Biology **19:** 1720-1730.

Hatefi Y (1985) The mitochondrial electron transport and oxidative phosphorylation system. Annual Review of Biochemistry **54:** 1015-1069.

Hazen SP, Scott-Craig JS, Walton JD (2002) Cellulose synthase-like (CSL) genes of rice. Plant Physiology **128:** 336-340.

He C-J, Morgan PW, Drew MC (1996) Transduction of an ethylene signal is required for cell death and lysis in the root cortex of maize during aerenchyma formation induced by hypoxia. Plant Physiology **112:** 463-472.

Hespell RB, Bryant MP (1979) Efficiency of rumen microbial growth: influence of some theoretical and experimental factors of YATP. Journal of Animal Science **49:** 1640-1659.

Hetherington AM, S Hunter MI, Crawford RM (1982) Contrasting effects of anoxia on rhizome lipids in *Iris* species. Phytochemistry **21:** 1275-1278.

Hiatt A, Lowe RH (1967) Loss of organic acids, amino acids, K^+, and Cl^- from barley roots treated anaerobically and with metabolic inhibitors. Plant Physiology **42:** 1731-1736.

Higham C, Lu T-D (1998) The origins and dispersal of rice cultivation: Rice domestication. Antiquity **72:** 867-877.

Hilt W, Wolf DH (1996) Proteasomes: destruction as a programme. Trends in Biochemical Sciences **21:** 96-102.

Hirose T, Scofield GN, Terao T (2008) An expression analysis profile for the entire sucrose synthase gene family in rice. Plant Science **174:** 534-543.

Howell KA, Narsai R, Carroll A, Ivanova A, Lohse M, Usadel B, Millar AH, Whelan J

(2009) Mapping metabolic and transcript temporal switches during germination in rice highlights specific transcription factors and the role of RNA instability in the germination process. Plant Physiology **149:** 961-980.

Hu W, Hu G, Han B (2009) Genome-wide survey and expression profiling of heat shock proteins and heat shock factors revealed overlapped and stress specific response under abiotic stresses in rice. Plant Science **176:** 583-590.

Huang J, Takano T, Akita S (2000) Expression of α-expansin genes in young seedlings of rice (*Oryza sativa* L.). Planta **211:** 467-473.

Huang S, Colmer TD, Millar AH (2008) Does anoxia tolerance involve altering the energy currency towards PPi? Trends in Plant Science **13:** 221-227.

Huang S, Greenway H, Colmer T (2003) Responses by coleoptiles of intact rice seedlings to anoxia: K^+ net uptake from the external solution and translocation from the caryopses. Annals of Botany **91:** 271-278.

Huang S, Greenway H, Colmer TD, Millar AH (2005) Protein synthesis by rice coleoptiles during prolonged anoxia: implications for glycolysis, growth and energy utilization. Annals of Botany **96:** 703-715.

Hunter MIS, Hetherington AM, Crawford RM (1983) Lipid peroxidation—a factor in anoxia intolerance in *Iris* species? Phytochemistry **22:** 1145-1147.

Igamberdiev AU, Baron K, Manac'H-Little N, Stoimenova M, Hill RD (2005) The haemoglobin/nitric oxide cycle: involvement in flooding stress and effects on hormone signalling. Annals of Botany **96:** 557-564.

Igamberdiev AU, Hill RD (2004) Nitrate, NO and haemoglobin in plant adaptation to hypoxia: an alternative to classic fermentation pathways. Journal of Experimental Botany **55:** 2473-2482.

Igamberdiev AU, Kleczkowski LA (2011) Magnesium and cell energetics in plants under anoxia. Biochemical Journal **437:** 373-379.

Inada N, Sakai A, Kuroiwa H, Kuroiwa T (1998) Three-dimensional analysis of the senescence program in rice (*Oryza sativa* L.) coleoptiles. Planta **206:** 585-597.

Ishizawa K, Murakami S, Kawakami Y, Kuramochi H (1999) Growth and energy status of arrowhead tubers, pondweed turions and rice seedlings under anoxic conditions. Plant, Cell & Environment **22:** 505-514.

Ismail AM, Ella ES, Vergara GV, Mackill DJ (2009) Mechanisms associated with tolerance to flooding during germination and early seedling growth in rice (*Oryza sativa*). Annals of Botany **103:** 197-209.

Isshiki M, Tsumoto A, Shimamoto K (2006) The serine/arginine-rich protein family in rice plays important roles in constitutive and alternative splicing of pre-mRNA. Plant Cell **18:** 146-158.

Ito Y, Katsura K, Maruyama K, Taji T, Kobayashi M, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2006) Functional analysis of rice DREB1/CBF-type transcription factors involved in cold-responsive gene expression in transgenic rice. Plant and Cell Physiology **47:** 141-153.

Jackson MB, Fenning TM, Jenkins W (1985) Aerenchyma (gas-space) formation in adventitious roots of rice (*Oryza sativa* L.) is not controlled by ethylene or small partial pressures of oxygen. Journal of Experimental Botany **36:** 1566-1572.

Jacoby RP, Li L, Huang S, Pong Lee C, Millar AH, Taylor NL (2012) Mitochondrial Composition, Function and Stress Response in PlantsF. Journal of Integrative Plant Biology **54:** 887-906.

Jain M, Nijhawan A, Arora R, Agarwal P, Ray S, Sharma P, Kapoor S, Tyagi AK, Khurana JP (2007) F-box proteins in rice. Genome-wide analysis, classification, temporal and spatial gene expression during panicle and seed development, and regulation by light and abiotic stress. Plant Physiology **143:** 1467-1483.

Jain M, Tyagi AK, Khurana JP (2006) Genome-wide analysis, evolutionary expansion,

and expression of early auxin-responsive *SAUR* gene family in rice (*Oryza sativa*). Genomics **88:** 360-371.

Jankowsky E, Bowers H (2006) Remodeling of ribonucleoprotein complexes with DExH/D RNA helicases. Nucleic Acids Research **34:** 4181-4188.

Jiang H, Dian W, Liu F, Wu P (2003) Isolation and characterization of two fructokinase cDNA clones from rice. Phytochemistry **62:** 47-52.

Jones TJ, Rost TL (1989) The developmental anatomy and ultrastructure of somatic embryos from rice (*Oryza sativa* L.) scutellum epithelial cells. Botanical Gazette**:** 41-49.

Jun N, Gaohang W, Zhenxing Z, Huanhuan Z, Yunrong W, Ping W (2011) OsIAA23‐mediated auxin signaling defines postembryonic maintenance of QC in rice. Plant Journal **68:** 433-442.

Kacser H, Burns J, Fell D (1995) The control of flux. Biochemical Society Transactions **23:** 341-366.

Kang T-J, Yang M-S (2004) Rapid and reliable extraction of genomic DNA from various wild-type and transgenic plants. BMC Biotechnology **4:** 20.

Kato-Noguchi H (2001) The importance of ethanolic fermentation for primary root growth of germinating rice under anoxia. Plant Growth Regulation **35:** 181-185.

Kato-Noguchi H (2006) Pyruvate metabolism in rice coleoptiles under anaerobiosis. Plant Growth Regulation **50:** 41-46.

Kato-Noguchi H, Morokuma M (2007) Ethanolic fermentation and anoxia tolerance in four rice cultivars. Journal of Plant Physiology **164:** 168-173.

Katou K, Tomiyama K, Okamoto H (1982) Effects of hyphal wall components of *Phytophthora infestans* on membrane potential of potato tuber cells. Physiological Plant Pathology **21:** 311-317.

Kawai M, Uchimiya H (2000) Coleoptile senescence in rice (*Oryza sativa* L.). Annals of

Botany **86:** 405-414.

Kawano N, Ella E, Ito O, Yamauchi Y, Tanaka K (2002) Metabolic changes in rice seedlings with different submergence tolerance after desubmergence. Environmental and Experimental Botany **47:** 195-203.

Kazama T, Toriyama K (2003) A pentatricopeptide repeat-containing gene that promotes the processing of aberrant *atp6* RNA of cytoplasmic male-sterile rice. FEBS Letters **544:** 99-102.

Kende H (1993) Ethylene biosynthesis. Annual Review of Plant Biology **44:** 283-307.

Kende H, van der Knaap E, Cho H-T (1998) Deepwater rice: a model plant to study stem elongation. Plant Physiology **118:** 1105-1110.

Kennedy RA, Rumpho ME, Fox TC (1992) Anaerobic metabolism in plants. Plant Physiology **100:** 1-6.

Khush GS (1997) Origin, dispersal, cultivation and variation of rice. *In* Oryza: From Molecule to Plant. Springer, pp 25-34.44.

Kim CM, Park SH, Je BI, Park SH, Park SJ, Piao HL, Eun MY, Dolan L, Han C-d (2007) *OsCSLD1*, a cellulose synthase-like D1 gene, is required for root hair morphogenesis in rice. Plant Physiology **143:** 1220-1230.

Kocábek T, Řepková J, Dudová M, Hoyerová K, Vrba L (2006) Isolation and characterization of a novel semi-lethal *Arabidopsis thaliana* mutant of gene for pentatricopeptide (PPR) repeat-containing protein. Genetica **128:** 395-407.

Kojima H, Katou K, Okamoto H (1985) Homeostatic regulation of membrane potential by an electrogenic ion pump against change in the K^+ concentration of the extra- and intra-organ perfusion solutions. Plant and Cell Physiology **26:** 351-359.

Komatsu K, Maekawa M, Ujiie S, Satake Y, Furutani I, Okamoto H, Shimamoto K, Kyozuka J (2003) *LAX* and *SPA*: major regulators of shoot branching in rice. Proceedings of the National Academy of Sciences of the United States of America

100: 11765-11770.

Komori T, Ohta S, Murai N, Takakura Y, Kuraya Y, Suzuki S, Hiei Y, Imaseki H, Nitta N (2004) Map‐based cloning of a fertility restorer gene, *Rf‐1*, in rice (Oryza sativa L.). Plant Journal **37:** 315-325.

Kordan H (1974) The rice shoot in relation to oxygen supply and root growth in seedlings germinating under water. New Phytologist **73:** 695-697.

Kordan HA (1976) Oxygen as an Enviromnental Factor in Influencing Normal Morphogenetic Development in Germinating Rice Seedlings. Journal of Experimental Botany **27:** 947-952.

Kosugi S, Ohashi Y (1997) PCF1 and PCF2 specifically bind to cis elements in the rice proliferating cell nuclear antigen gene. Plant Cell **9:** 1607-1619.

Kouřil R, Wientjes E, Bultema JB, Croce R, Boekema EJ (2012) High-light vs. Low-light: Effect of light acclimation on Photosystem II composition and organization in *Arabidopsis thaliana*. Biochimica et Biophysica Acta (BBA)-Bioenergetics **3:** 411-419.

Krebs H (1973) Pyridine nucleotides and rate control. *In* DD Davies, ed, Rate Control of Biological Processes, 27th Symposium of the Society for Experimental Biology. Cambridge University Press, Cambridge, England., pp 299-318.

Kulichikhin KY, Greenway H, Byrne L, Colmer TD (2009) Regulation of intracellular pH during anoxia in rice coleoptiles in acidic and near neutral conditions. Journal of Experimental Botany **60:** 2119-2128.

Kumar KK, Poovannan K, Nandakumar R, Thamilarasi K, Geetha C, Jayashree N, Kokiladevi E, Raja JA, Samiyappan R, Sudhakar D (2003) A high throughput functional expression assay system for a defence gene conferring transgenic resistance on rice against the sheath blight pathogen, *Rhizoctonia solani*. Plant Science **165:** 969-976.

Lam WS, Yang X, Makaroff CA (2005) Characterization of *Arabidopsis thaliana* SMC1 and SMC3: evidence that AtSMC3 may function beyond chromosome cohesion. Journal of Cell Science **118:** 3037-3048.

Lasanthi-Kudahettige R, Magneschi L, Loreti E, Gonzali S, Licausi F, Novi G, Beretta O, Vitulli F, Alpi A, Perata P (2007) Transcript profiling of the anoxic rice coleoptile. Plant Physiology **144:** 218-231.

Lee T-M, Lin Y-H (1996) Changes in soluble and cell wall-bound peroxidase activities with growth in anoxia-treated rice (*Oryza sativa* L.) coleoptiles and roots. Plant Science **106:** 1-7.

Lee Y, Choi D, Kende H (2001) Expansins: ever-expanding numbers and functions. Current Opinion in Plant Biology **4:** 527-532.

Lee Y, Kende H (2002) Expression of α-expansin and expansin-like genes in deepwater rice. Plant Physiology **130:** 1396-1405.

Li M, Xiong G, Li R, Cui J, Tang D, Zhang B, Pauly M, Cheng Z, Zhou Y (2009) Rice cellulose synthase‐like D4 is essential for normal cell‐wall biosynthesis and plant growth. Plant Journal **60:** 1055-1069.

Li X, Duan X, Jiang H, Sun Y, Tang Y, Yuan Z, Guo J, Liang W, Chen L, Yin J (2006) Genome-wide analysis of basic/helix-loop-helix transcription factor family in rice and Arabidopsis. Plant Physiology **141:** 1167-1184.

Liao M, Li Y, Wang Z (2009) Identification of elicitor‐responsive proteins in rice leaves by a proteomic approach. Proteomics **9:** 2809-2819.

Lilitchan S, Tangprawat C, Aryusuk K, Krisnangkura S, Chokmoh S, Krisnangkura K (2008) Partial extraction method for the rapid analysis of total lipids and γ-oryzanol contents in rice bran. Food Chemistry **106:** 752-759.

Lilley KS, Dupree P (2006) Methods of quantitative proteomics and their application to

plant organelle characterization. Journal of Experimental Botany **57:** 1493-1499.

Lin C-M, Koh S, Stacey G, Yu S-M, Lin T-Y, Tsay Y-F (2000) Cloning and functional characterization of a constitutively expressed nitrate transporter gene, *OsNRT1*, from rice. Plant Physiology **122:** 379-388.

Liu F, VanToai T, Moy LP, Bock G, Linford LD, Quackenbush J (2005) Global transcription profiling reveals comprehensive insights into hypoxic response in Arabidopsis. Plant Physiology **137:** 1115-1129.

Liu Q, Zhang Q, Burton RA, Shirley NJ, Atwell BJ (2010) Expression of vacuolar H^+-pyrophosphatase (OVP3) is under control of an anoxia-inducible promoter in rice. Plant Molecular Biology **72:** 47-60.

Long AD, Mangalam HJ, Chan BY, Tolleri L, Hatfield GW, Baldi P (2001) Improved Statistical Inference from DNA Microarray Data Using Analysis of Variance and A Bayesian Statistical Framework ANALYSIS OF GLOBAL GENE EXPRESSION IN ESCHERICHIA COLIK12. Journal of Biological Chemistry **276:** 19937-19944.

Lorković ZJ, Wieczorek Kirk DA, Lambermon MH, Filipowicz W (2000) Pre-mRNA splicing in higher plants. Trends in Plant Science **5:** 160-167.

Lurin C, Andrés C, Aubourg S, Bellaoui M, Bitton F, Bruyère C, Caboche M, Debast C, Gualberto J, Hoffmann B (2004) Genome-wide analysis of Arabidopsis pentatricopeptide repeat proteins reveals their essential role in organelle biogenesis. Plant Cell **16:** 2089-2103.

MacAdam JW, Nelson CJ, Sharp RE (1992) Peroxidase activity in the leaf elongation zone of tall fescue I. Spatial distribution of ionically bound peroxidase activity in genotypes differing in length of the elongation zone. Plant Physiology **99:** 872-878.

MacAdam JW, Sharp RE, Nelson CJ (1992) Peroxidase activity in the leaf elongation zone of tall fescue II. Spatial distribution of apoplastic peroxidase activity in

genotypes differing in length of the elongation zone. Plant Physiology **99:** 879-885.

Maeshima M (2000) Vacuolar H^+-pyrophosphatase. Biochimica et Biophysica Acta (BBA)-Biomembranes **1465:** 37-51.

Magneschi L, Kudahettige RL, Alpi A, Perata P (2009) Expansin gene expression and anoxic coleoptile elongation in rice cultivars. Journal of Plant Physiology **166:** 1576-1580.

Magneschi L, Perata P (2009) Rice germination and seedling growth in the absence of oxygen. Annals of Botany **103:** 181-196.

Mancebo R, Zhou X, Shillinglaw W, Henzel W, Macdonald PM (2001) BSF Binds Specifically to the bicoidmRNA 3′ Untranslated Region and Contributes to Stabilization ofbicoid mRNA. Molecular and Cellular Biology **21:** 3462-3471.

Matsumura H, Nirasawa S, Terauchi R (1999) Transcript profiling in rice (*Oryza sativa* L.) seedlings using serial analysis of gene expression (SAGE). Plant Journal **20:** 719-726.

Matsumura H, Takano T, Takeda G, Uchimiya H (1998) *Adh1* is transcriptionally active but its translational product is reduced in a *rad* mutant of rice (*Oryza sativa* L.), which is vulnerable to submergence stress. Theoretical and Applied Genetics **97:** 1197-1203.

Mattana M, Coraggio I, Bertani A, Reggiani R (1994) Expression of the enzymes of nitrate reduction during the anaerobic germination of rice. Plant Physiology **106:** 1605-1608

Meierhoff K, Felder S, Nakamura T, Bechtold N, Schuster G (2003) HCF152, an Arabidopsis RNA binding pentatricopeptide repeat protein involved in the processing of chloroplast psbB-psbT-psbH-petB-petD RNAs. Plant Cell **15:** 1480-1495.

Menegus F, Cattaruzza L, Mattana M, Beffagna N, Ragg E (1991) Response to anoxia in rice and wheat seedlings changes in the pH of intracellular compartments, glucose-6-phosphate level, and metabolic rate. Plant Physiology **95:** 760-767.

Merrill AL, Watt BK (1973) Energy value of foods, Agricultural Handbooks No. 74. United States Department of Agriculture.

Métraux J-P, Kende H (1983) The role of ethylene in the growth response of submerged deep water rice. Plant Physiology **72:** 441-446.

Millar AH, Trend AE, Heazlewood JL (2004) Changes in the mitochondrial proteome during the anoxia to air transition in rice focus around cytochrome-containing respiratory complexes. Journal of Biological Chemistry **279:** 39471-39478.

Mimura S, Masuda T, Matsui T, Takisawa H (2000) Central role for *cdc45* in establishing an initiation complex of DNA replication in Xenopus egg extracts. Genes to Cells **5:** 439-452.

Mimura S, Takisawa H (1998) Xenopus *Cdc45*-dependent loading of DNA polymerase α onto chromatin under the control of S-phase Cdk. EMBO Journal **17:** 5699-5707.

Miro B, Ismail AM Tolerance of anaerobic conditions caused by flooding during germination and early growth in rice (*Oryza sativa* L.). Frontiers in Plant Science **4:** 269.

Mizukami Y, Fischer RL (2000) Plant organ size control: *AINTEGUMENTA* regulates growth and cell numbers during organogenesis. Proceedings of the National Academy of Sciences of the United States of America **97:** 942-947.

Mocquot B, Pradet A, Litvak S (1977) DNA synthesis and anoxia in rice coleoptiles. Plant Science Letters **9:** 365-371.

Mocquot B, Prat C, Mouches C, Pradet A (1981) Effect of anoxia on energy charge and protein synthesis in rice embryo. Plant Physiology **68:** 636-640.

Mohanty B, Ong BEEL (2003) Contrasting effects of submergence in light and dark on pyruvate decarboxylase activity in roots of rice lines differing in submergence tolerance. Annals of Botany **91:** 291-300.

Mohanty B, Wilson PM, Rees Ta (1993) Effects of anoxia on growth and carbohydrate metabolism in suspension cultures of soybean and rice. Phytochemistry **34:** 75-82.

Moon J, Parry G, Estelle M (2004) The ubiquitin-proteasome pathway and plant development. Plant Cell **16:** 3181-3195.

Mori Y, Yamamoto T, Sakaguchi N, Ishibashi T, Furukawa T, Kadota Y, Kuchitsu K, Hashimoto J, Kimura S, Sakaguchi K (2005) Characterization of the origin recognition complex (ORC) from a higher plant, rice (*Oryza sativa* L.). Gene **353:** 23-30.

Mujer CV, Rumpho ME, Lin J-J, Kennedy RA (1993) Constitutive and inducible aerobic and anaerobic stress proteins in the *Echinochloa* complex and rice. Plant Physiology **101:** 217-226.

Mustroph A, Albrecht G (2003) Tolerance of crop plants to oxygen deficiency stress: fermentative activity and photosynthetic capacity of entire seedlings under hypoxia and anoxia. Physiologia Plantarum **117:** 508-520.

Mustroph A, Juntawong P, Bailey-Serres J (2009) Isolation of plant polysomal mRNA by differential centrifugation and ribosome immunopurification methods. *In* Plant Systems Biology. Springer, pp 109-126.

Mustroph A, Lee SC, Oosumi T, Zanetti ME, Yang H, Ma K, Yaghoubi-Masihi A, Fukao T, Bailey-Serres J (2010) Cross-kingdom comparison of transcriptomic adjustments to low-oxygen stress highlights conserved and plant-specific responses. Plant Physiology **152:** 1484-1500.

Narsai R, Howell KA, Carroll A, Ivanova A, Millar AH, Whelan J (2009) Defining core

metabolic and transcriptomic responses to oxygen availability in rice embryos and young seedlings. Plant Physiology **151:** 306-322.

Narsai R, Law SR, Carrie C, Xu L, Whelan J (2011) In-depth temporal transcriptome profiling reveals a crucial developmental switch with roles for RNA processing and organelle metabolism that are essential for germination in Arabidopsis. Plant Physiology **157:** 1342-1362.

Navrud S, Tuan TH, Tinh BD (2012) Estimating the welfare loss to households from natural disasters in developing countries: a contingent valuation study of flooding in Vietnam. Global Health Action **5.**

Neal MJ, Girton RE (1955) The Pasteur effect in maize. American Journal of Botany **42:** 733-737.

Nicholls DG, Ferguson SSJ (1992) Bioenergetics 2. Academic Press, London; San Diego.

Nishizawa Y, Hirai A (1989) The nucleotide sequences and expression of genes for the beta and epsilon subunits of ATP synthase from rice (*Oryza sativa* L.). Idengaku Zasshi **64:** 223-229.

Ondzighi CA, Christopher DA, Cho EJ, Chang S-C, Staehelin LA (2008) Arabidopsis protein disulfide isomerase-5 inhibits cysteine proteases during trafficking to vacuoles before programmed cell death of the endothelium in developing seeds. Plant Cell **20:** 2205-2220.

Öpik H (1973) Effect of anaerobiosis on respiratory rate, cytochrome oxidase activity and mitochondrial structures in coleoptiles of rice (*Oryza sativa* L.). Journal of Cell Science **12:** 725-739.

Palmgren MG, Fuglsang AT, Jahn T (1998) Deciphering the role of 14-3-3 proteins. Experimental Biology Online **3:** 1-17.

Pampulha M, Loureiro-Dias M (1990) Activity of glycolytic enzymes of *Saccharomyces cerevisiae* in the presence of acetic acid. Applied Microbiology and Biotechnology

34: 375-380.

Pan L, Kawai M, Yano A, Uchimiya H (2000) Nucleoside diphosphate kinase required for coleoptile elongation in rice. Plant Physiology **122:** 447-452.

Passarinho P, Ketelaar T, Xing M, van Arkel J, Maliepaard C, Hendriks MW, Joosen R, Lammers M, Herdies L, den Boer B (2008) *BABY BOOM* target genes provide diverse entry points into cell proliferation and cell growth pathways. Plant Molecular Biology **68:** 225-237.

Passonneau JV, Lowry OH (1964) The role of phosphofructokinase in metabolic regulation. Advances in Enzyme Regulation **2:** 265-274.

Pawlowski K, Kunze R, De Vries S, Bisseling T (1994) Isolation of total, poly (A) and polysomal RNA from plant tissues. *In* Plant molecular biology manual. Springer, pp 231-243.

Perata P, Alpi A (1993) Plant responses to anaerobiosis. Plant Science **93:** 1-17.

Phee BK, Cho JH, Park S, Jung JH, Lee YH, Jeon JS, Bhoo SH, Hahn TR (2004) Proteomic analysis of the response of Arabidopsis chloroplast proteins to high light stress. Proteomics **4:** 3560-3568.

Plaxton WC (1996) The organization and regulation of plant glycolysis. Annual Review of Plant Biology **47:** 185-214.

Plaxton WC (2010) Metabolic Flexibility Helps Plants to Survive Stress. *In* Chapter 11, Plant Physiology Online.

Polyakova L, Vartapetian B (2003) Exogenous nitrate as a terminal acceptor of electrons in rice (*Oryza sativa*) coleoptiles and wheat (*Triticum aestivum*) roots under strict anoxia. Russian Journal of Plant Physiology **50:** 808-812.

Prescott AG, John P (1996) Dioxygenases: molecular structure and role in plant metabolism. Annual Review of Plant Biology **47:** 245-271.

Prioul J-L, Guyot C (1985) Role of oxygen transport and nitrate metabolism in the

adaptation of wheat plant to root anaerobiosis. Physiologie végétale **23:** 175-185.

Pruneda-Paz JL, Breton G, Para A, Kay SA (2009) A functional genomics approach reveals CHE as a component of the Arabidopsis circadian clock. Science **323:** 1481-1485.

Raha S, Merante F, Proteau G, Reed JK (1990) Simultaneous isolation of total cellular RNA and DNA from tissue culture cells using phenol and lithium chloride. Gene Analysis Techniques **7:** 173-177.

Rawyler A, Arpagaus S, Braendle R (2002) Impact of oxygen stress and energy availability on membrane stability of plant cells. Annals of Botany **90:** 499-507.

Rawyler A, Pavelic D, Gianinazzi C, Oberson J, Braendle R (1999) Membrane lipid integrity relies on a threshold of ATP production rate in potato cell cultures submitted to anoxia. Plant Physiology **120:** 293-300.

Ray S, Anderson JM, Urmeev FI, Goodwin SB (2003) Rapid induction of a protein disulfide isomerase and defense-related genes in wheat in response to the hemibiotrophic fungal pathogen *Mycosphaerella graminicola*. Plant Molecular Biology **53:** 741-754.

Raymond P, Al-Ani A, Pradet A (1985) ATP production by respiration and fermentation, and energy charge during aerobiosis and anaerobiosis in twelve fatty and starchy germinating seeds. Plant Physiology **79:** 879-884.

Raymond P, Pradet A (1980) Stabilization of adenine nucleotide ratios at various values by an oxygen limitation of respiration in germinating lettuce (*Lactuca sativa*) seeds. Biochemical Journal **190:** 39-44.

Reggiani R, Bertini F, Mattana M (1995) Incorporation of nitrate nitrogen into amino acids during the anaerobic germination of rice. Amino Acids **9:** 385-390.

Reggiani R, Cantu CA, Brambilla I, Bertani A (1988) Accumulation and interconversion of amino acids in rice roots under anoxia. Plant and Cell

Physiology **29:** 981-987.

Reggiani R, Mattana M, Aurisano N, Bertani A (1993) The rice coleoptile: an example of anaerobic nitrate assimilation. Physiologia Plantarum **89:** 640-643.

Reggiani R, Mattana M, Aurisano N, Bertani A (1993) Utilization of stored nitrate during the anaerobic germination of rice seeds. Plant and Cell Physiology **34:** 379-383.

Ricard B, Couee I, Raymond P, Saglio P, Saint-Ges V, Pradet A (1994) Plant metabolism under hypoxia and anoxia. Plant Physiology and Biochemistry **32:** 1-10.

Rohila JS, Chen M, Chen S, Chen J, Cerny R, Dardick C, Canlas P, Xu X, Gribskov M, Kanrar S (2006) Protein–protein interactions of tandem affinity purification‐tagged protein kinases in rice. Plant Journal **46:** 1-13.

Rotini O, Marino G (1957) The antimitotic activity of cyanates on the bulbs of *Allium cepa* L.. Archives of Biochemistry and Biophysics **69:** 346-352.

Sachs MM, Freeling M, Okimoto R (1980) The anaerobic proteins of maize. Cell **20:** 761-767.

Sachs MM, Ho T-HD (1986) Alteration of gene expression during environmental stress in plants. Annual Review of Plant Physiology **37:** 363-376.

Sachs MM, Subbaiah CC, Saab IN (1996) Anaerobic gene expression and flooding tolerance in maize. Journal of Experimental Botany **47:** 1-15.

Sadiq I, Fanucchi F, Paparelli E, Alpi E, Bachi A, Alpi A, Perata P (2011) Proteomic identification of differentially expressed proteins in the anoxic rice coleoptile. Journal of Plant Physiology **168:** 2234-2243.

Saglio PH (1985) Effect of path or sink anoxia on sugar translocation in roots of maize seedlings. Plant Physiology **77:** 285-290.

Saika H, Matsumura H, Takano T, Tsutsumi N, Nakazono M (2006) A point mutation

of *Adh1* gene is involved in the repression of coleoptile elongation under submergence in rice. Breeding Science **56:** 69-74.

Saint‐Ges V, Roby C, Bligny R, Pradet A, Douce R (1991) Kinetic studies of the variations of cytoplasmic pH, nucleotide triphosphates (31P‐NMR) and lactate during normoxic and anoxic transitions in maize root tips. European Journal of Biochemistry **200:** 477-482.

Saraste M (1999) Oxidative phosphorylation at the fin de siecle. Science **283:** 1488-1493.

Sarkar N, Kim Y-K, Grover A (2009) Rice sHsp genes: genomic organization and expression profiling under stress and development. BMC Genomics **10:** 393.

Sasakawa H, Yamamoto Y (1978) Comparison of the uptake of nitrate and ammonium by rice seedlings influences of light, temperature, oxygen concentration, exogenous sucrose, and metabolic inhibitors. Plant Physiology **62:** 665-669.

Satler SO, Kende H (1985) Ethylene and the growth of rice seedlings. Plant Physiology **79:** 194-198.

Scofield GN, Hirose T, Aoki N, Furbank RT (2007) Involvement of the sucrose transporter, OsSUT1, in the long-distance pathway for assimilate transport in rice. Journal of Experimental Botany **58:** 3155-3169.

Sekido H, Endo T, Suga R, Kodama O, Akatsuka T, Kono Y, Takeuchi S (1986) Oryzalexin D (3, 7-dihydroxy-(+)-sandaracopimaradiene), a new phytoalexin isolated from blast-infected rice leaves. Journal of Pesticide Science **11.**

Setter T, Ella E (1994) Relationship between coleoptile elongation and alcoholic fermentation in rice exposed to anoxia. I. Importance of treatment conditions and different tissues. Annals of Botany **74:** 265-271.

Setter T, Ella E, Valdez A (1994) Relationship between coleoptile elongation and alcoholic fermentation in rice exposed to anoxia. II. Cultivar differences. Annals of

Botany **74:** 273-279.

Setter T, Ellis M, Laureles E, Ella E, Senadhira D, Mishra S, Sarkarung S, Datta S (1997) Physiology and genetics of submergence tolerance in rice. Annals of Botany **79:** 67-77.

Setter TL, Waters I, Greenway H, Atwell BJ, T K (1987) Carbohydrate status of terrestrial plants during flooding. *In* RMM Crawford, ed, Plant life in aquatic and amphibious habitats. Blackwell Scientific Publications, Oxford, UK, pp 411-433.

Shih M-D, Hoekstra FA, Hsing Y-IC (2008) Late embryogenesis abundant proteins. Advances in Botanical Research **48:** 211-255.

Shinozaki K, Yamaguchi-Shinozaki K (2007) Gene networks involved in drought stress response and tolerance. Journal of Experimental Botany **58:** 221-227.

Sieber M, Braendle R (1991) Energy metabolism in rhizomes of *Acorus calamus* (L.) and in tubers of *Solanum tuberosum* (L.) with regard to their anoxia tolerance. Acta Botanica **104:** 279-282.

Siedow JN, Umbach AL (1995) Plant mitochondrial electron transfer and molecular biology. Plant Cell **7:** 821-831.

Smith AM, ap Rees T (1979) Pathways of carbohydrate fermentation in the roots of marsh plants. Planta **146:** 327-334.

Smith F, Thompson JF (1971) Regulation of nitrate reductase in excised barley roots. Plant Physiology **48:** 219-223.

Spartz AK, Lee SH, Wenger JP, Gonzalez N, Itoh H, Inzé D, Peer WA, Murphy AS, Overvoorde PJ, Gray WM (2012) The *SAUR19* subfamily of SMALL AUXIN UP RNA genes promote cell expansion. Plant Journal **70:** 978-990.

Stoimenova M, Igamberdiev AU, Gupta KJ, Hill RD (2007) Nitrite-driven anaerobic ATP synthesis in barley and rice root mitochondria. Planta **226:** 465-474.

Stoimenova M, Libourel I, Ratcliffe R, Kaiser W (2003) The role of nitrate reduction

in the anoxic metabolism of roots II. Anoxic metabolism of tobacco roots with or without nitrate reductase activity. Plant and Soil **253:** 155-167.

Suge H (1985) Ethylene and gibberellin: regulation of internodal elongation and nodal root development in floating rice. Plant and Cell Physiology **26:** 607-614.

Sugimoto K, Himmelspach R, Williamson RE, Wasteneys GO (2003) Mutation or drug-dependent microtubule disruption causes radial swelling without altering parallel cellulose microfibril deposition in Arabidopsis root cells. Plant Cell **15:** 1414-1429.

Sultan SE (2000) Phenotypic plasticity for plant development, function and life history. Trends in Plant Science **5:** 537-542.

Sung Y-C, Fuchs JA (1988) Characterization of the cyn operon in *Escherichia coli* K12. Journal of Biological Chemistry **263:** 14769-14775.

Sze H (1985) H^+-translocating ATPases: advances using membrane vesicles. Annual Review of Plant Physiology **36:** 175-208.

Sze H, Ward JM, Lai S (1992) Vacuolar H^+-translocating ATPases from plants: structure, function, and isoforms. Journal of Bioenergetics and Biomembranes **24:** 371-381.

Tadege M, Dupuis I, Kuhlemeier C (1999) Ethanolic fermentation: new functions for an old pathway. Trends in Plant Science **4:** 320-325.

Takahashi H, Saika H, Matsumura H, Nagamura Y, Tsutsumi N, Nishizawa NK, Nakazono M (2011) Cell division and cell elongation in the coleoptile of rice *alcohol dehydrogenase 1-deficient* mutant are reduced under complete submergence. Annals of Botany **108:** 253-261.

Takeda T, Suwa Y, Suzuki M, Kitano H, Ueguchi‐Tanaka M, Ashikari M, Matsuoka M, Ueguchi C (2003) The *OsTB1* gene negatively regulates lateral branching in rice. Plant Journal **33:** 513-520.

Takemoto Y, Coughlan SJ, Okita TW, Satoh H, Ogawa M, Kumamaru T (2002) The

rice mutant *esp2* greatly accumulates the glutelin precursor and deletes the protein disulfide isomerase. Plant Physiology **128:** 1212-1222.

Taliercio EW, Chourey PS (1989) Post-transcriptional control of sucrose synthase expression in anaerobic seedlings of maize. Plant Physiology **90:** 1359-1364.

Tatematsu K, Nakabayashi K, Kamiya Y, Nambara E (2008) Transcription factor *AtTCP14* regulates embryonic growth potential during seed germination in Arabidopsis thaliana. Plant Journal **53:** 42-52.

Taylor DL (1942) Influence of oxygen tension on respiration, fermentation, and growth in wheat and rice. American Journal of Botany**:** 721-738.

Thompson SM, Gahwiler BH (1989) Activity-dependent disinhibition. II. Effects of extracellular potassium, furosemide, and membrane potential on ECl-in hippocampal CA3 neurons. Journal of Neurophysiology **61:** 512-523.

Tonge R, Shaw J, Middleton B, Rowlinson R, Rayner S, Young J, Pognan F, Hawkins E, Currie I, Davison M (2001) Validation and development of fluorescence two‐dimensional differential gel electrophoresis proteomics technology. Proteomics **1:** 377-396.

Tournaire-Roux C, Sutka M, Javot H, Gout E, Gerbeau P, Luu D-T, Bligny R, Maurel C (2003) Cytosolic pH regulates root water transport during anoxic stress through gating of aquaporins. Nature **425:** 393-397.

Trémousaygue D, Garnier L, Bardet C, Dabos P, Hervé C, Lescure B (2003) Internal telomeric repeats and ‘TCP domain’ protein‐binding sites co‐operate to regulate gene expression in *Arabidopsis thaliana* cycling cells. Plant Journal **33:** 957-966.

Trought M, Drew M (1981) Alleviation of injury to young wheat plants in anaerobic solution cultures in relation to the supply of nitrate and other inorganic nutrients. Journal of Experimental Botany **32:** 509-522.

Ueda A, Kathiresan A, Bennett J, Takabe T (2006) Comparative transcriptome analyses of barley and rice under salt stress. Theoretical and Applied Genetics **112:** 1286-1294.

Umeda M, Hara C, Matsubayashi Y, Li H-H, Liu Q, Tadokoro F, Aotsuka S, Uchimiya H (1994) Expressed sequence tags from cultured cells of rice (*Oryza sativa* L.) under stressed conditions: analysis of transcripts of genes engaged in ATP-generating pathways. Plant Molecular Biology **25:** 469-478.

Usadel B, Nagel A, Steinhauser D, Gibon Y, Bläsing OE, Redestig H, Sreenivasulu N, Krall L, Hannah MA, Poree F (2006) PageMan: an interactive ontology tool to generate, display, and annotate overview graphs for profiling experiments. BMC Bioinformatics **7:** 535.

Usadel B, Nagel A, Thimm O, Redestig H, Blaesing OE, Palacios-Rojas N, Selbig J, Hannemann J, Piques MC, Steinhauser D (2005) Extension of the visualization tool MapMan to allow statistical analysis of arrays, display of coresponding genes, and comparison with known responses. Plant Physiology **138:** 1195-1204.

Ushimaru T, Kanematsu S, Shibasaka M, Tsuji H (1999) Effect of hypoxia on the antioxidative enzymes in aerobically grown rice (*Oryza sativa*) seedlings. Physiologia Plantarum **107:** 181-187.

Vanlerberghe GC, McIntosh L (1997) Alternative oxidase: from gene to function. Annual Review of Plant Biology **48:** 703-734.

Vartapetian B, Mazliak P, Lance C (1978) Lipid biosynthesis in rice coleoptiles grown in the presence or in the absence of oxygen. Plant Science Letters **13:** 321-328.

Vartapetian BB, Andreeva IN, Generozova IP, Polyakova LI, Maslova IP, Dolgikh YI, Stepanova AY (2003) Functional electron microscopy in studies of plant response and adaptation to anaerobic stress. Annals of Botany **91:** 155-172.

Vartapetian BB, Jackson MB (1997) Plant adaptations to anaerobic stress. Annals of

Botany **79:** 3-20.

Vartapetian BB, Polyakova LI (1999) Protective effect of exogenous nitrate on the mitochondrial ultrastructure of *Oryza sativa* coleoptiles under strict anoxia. Protoplasma **206:** 163-167.

Vierstra RD (1994) Phytochrome degradation. *In* Photomorphogenesis in Plants. Springer, pp 141-162.

Vierstra RD (1996) Proteolysis in plants: mechanisms and functions. Plant Molecular Biology **32:** 275-302.

Wada S (1961) Growth patterns of rice coleoptiles grown on water and under water. Scientific Reports of Tohoku University Series IV (Biology) **27:** 199-207

Wang A-Y, Kao M-H, Yang W-H, Sayion Y, Liu L-F, Lee P-D, Su J-C (1999) Differentially and developmentally regulated expression of three rice sucrose synthase genes. Plant and Cell physiology **40:** 800-807.

Wang W, Scali M, Vignani R, Spadafora A, Sensi E, Mazzuca S, Cresti M (2003) Protein extraction for two‐dimensional electrophoresis from olive leaf, a plant tissue containing high levels of interfering compounds. Electrophoresis **24:** 2369-2375.

Wang W, Vinocur B, Shoseyov O, Altman A (2004) Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. Trends in Plant Science **9:** 244-252.

Wang Y-D, Wang X, Wong Y-s (2012) Proteomics analysis reveals multiple regulatory mechanisms in response to selenium in rice. Journal of Proteomics **75:** 1849-1866.

Wang Z, Zou Y, Li X, Zhang Q, Chen L, Wu H, Su D, Chen Y, Guo J, Luo D (2006) Cytoplasmic male sterility of rice with boro II cytoplasm is caused by a cytotoxic peptide and is restored by two related PPR motif genes via distinct modes of

mRNA silencing. Plant Cell **18:** 676-687.

Waters I, Kuiper P, Watkin E, Greenway H (1991) Effects of anoxia on wheat seedlings I. Interaction between anoxia and other environmental factors. Journal of Experimental Botany **42:** 1427-1435.

Waters I, Morrell S, Greenway H, Colmer T (1991) Effects of anoxia on wheat seedlings II. Influence of O2 supply prior to anoxia on tolerance to anoxia, alcoholic fermentation, and sugar levels. Journal of Experimental Botany **42:** 1437-1447.

Webb T, Armstrong W (1983) The effects of anoxia and carbohydrates on the growth and viability of rice, pea and pumpkin roots. Journal of Experimental Botany **34:** 579-603.

Wink M (1993) The plant vacuole: a multifunctional compartment. Journal of Experimental Botany **44:** 231-231.

Xia J-H, Roberts JK (1996) Regulation of H^+ extrusion and cytoplasmic pH in maize root tips acclimated to a low-oxygen environment. Plant Physiology **111:** 227-233.

Xia J-H, Saglio PH (1992) Lactic acid efflux as a mechanism of hypoxic acclimation of maize root tips to anoxia. Plant Physiology **100:** 40-46.

Xiong L, Gong Z, Rock CD, Subramanian S, Guo Y, Xu W, Galbraith D, Zhu J-K (2001) Modulation of Abscisic Acid Signal Transduction and Biosynthesis by an Sm-like Protein in *Arabidopsis*. Developmental Cell **1:** 771-781.

Xu D, Duan X, Wang B, Hong B, Ho T-HD, Wu R (1996) Expression of a late embryogenesis abundant protein gene, *HVA1*, from barley confers tolerance to water deficit and salt stress in transgenic rice. Plant Physiology **110:** 249-257.

Xu K, Xu X, Fukao T, Canlas P, Maghirang-Rodriguez R, Heuer S, Ismail AM, Bailey-Serres J, Ronald PC, Mackill DJ (2006) *Sub1A* is an ethylene-response-factor-like gene that confers submergence tolerance to rice. Nature **442:** 705-708.

Yamauchi M, Biswas J (1997) Rice cultivar difference in seedling establishment in flooded soil. Plant and Soil **189:** 145-153.

Yamazaki H, Tasaka M, Shikanai T (2004) PPR motifs of the nucleus‐encoded factor, *PGR3*, function in the selective and distinct steps of chloroplast gene expression in Arabidopsis. Plant Journal **38:** 152-163.

Yan S-P, Zhang Q-Y, Tang Z-C, Su W-A, Sun W-N (2006) Comparative proteomic analysis provides new insights into chilling stress responses in rice. Molecular & Cellular Proteomics **5:** 484-496.

Yano A, Shimazaki T, Kato A, Umeda M, Uchimiya H (1993) Molecular cloning and nucleotide sequence cDNA encoding nucleoside diphosphate kinase of rice (*Oryza sativa* L.). Plant Molecular Biology **23:** 1087-1090.

Yeo AR (1983) Salinity resistance: Physiologies and prices. Physiologia Plantarum **58:** 214-222

Yi K, Wu Z, Zhou J, Du L, Guo L, Wu Y, Wu P (2005) *OsPTF1*, a novel transcription factor involved in tolerance to phosphate starvation in rice. Plant Physiology **138:** 2087-2096.

Yordanova RY, Christov KN, Popova LP (2004) Antioxidative enzymes in barley plants subjected to soil flooding. Environmental and Experimental Botany **51:** 93-101.

Zhang J, Klueva NY, Wang Z, Wu R, Ho T-HD, Nguyen HT (2000) Genetic engineering for abiotic stress resistance in crop plants. In Vitro Cellular & Developmental Biology-Plant **36:** 108-114.

Zhang J-W, Xu L, Wu Y-R, Chen X-A, Liu Y, Zhu S-H, Ding W-N, Wu P, Yi K-K (2012) OsGLU3, a putative membrane-bound endo-1, 4-beta-glucanase, is required for root cell elongation and division in rice (*Oryza sativa* L.). Molecular Plant **5:** 176-186.

Zhang L-Y, Bai M-Y, Wu J, Zhu J-Y, Wang H, Zhang Z, Wang W, Sun Y, Zhao J, Sun X

(2009) Antagonistic HLH/bHLH transcription factors mediate brassinosteroid regulation of cell elongation and plant development in rice and Arabidopsis. Plant Cell **21:** 3767-3780.

Zhang Q, Greenway H (1994) Anoxia tolerance and anaerobic catabolism of aged beetroot storage tissues. Journal of Experimental Botany **45:** 567-575.

Zhang Q, Greenway H (1995) Membrane transport in anoxic rice coleoptiles and storage tissues of beetroot. Functional Plant Biology **22:** 965-975.

Zhang Q, Lauchli A, Greenway H (1992) Effects of anoxia on solute loss from beetroot storage tissue. Journal of Experimental Botany **43:** 897-905.

Zhou H-L, He S-J, Cao Y-R, Chen T, Du B-X, Chu C-C, Zhang J-S, Chen S-Y (2006) OsGLU1, a putative membrane-bound endo-1, 4-ß-D-glucanase from rice, affects plant internode elongation. Plant Molecular Biology **60:** 137-151.

Zhu Z-X, Liu Y, Liu S-J, Mao C-Z, Wu Y-R, Wu P (2012) A gain-of-function mutation in *OsIAA11* affects lateral root development in rice. Molecular Plant **5:** 154-161.

Appendix A – Supplementary material for Chapter 2

The contents of this Appendix are formed by the supplementary material originally published with Edwards *et al.* (2012).

Table S1. The amino acid makeup of proteins of rice coleoptiles grown in normoxic (aerated), hypoxic (3% O_2) and anoxic (N_2) solution. *O. sativa* (cv. Amaroo) seeds were de-hulled and surface sterilised. Seeds were grown in 2 L of solution with 3% O_2, N_2 or atmosphere (aerated) gas bubbled though the solution at 0.2 L h^{-1}. Coleoptiles were collected at 3 d after imbibition, freeze dried and powdered. Amino acid make-up of total protein was determined by the Australian Proteome Analysis Facility.

	anoxic		***hypoxic***		***normoxic***	
Amino acid	*Amino acid content (mg g^{-1})*	*% by weight*	*Amino acid content (mg g^{-1})*	*% by weight*	*Amino acid content (mg g^{-1})*	*% by weight*
Histidine	3.7	1.9	5.8	1.9	3.7	2.0
Serine	7.6	6.1	11.7	6.0	7.8	6.7
Arginine	10.3	4.6	16.5	4.8	10.0	4.8
Glycine	8.7	10.6	13.1	10.4	7.3	9.6
Aspartic acid	14.2	8.6	21.7	8.5	18.4	12.0
Glutamic acid	18.6	10.1	28.9	10.1	20.6	12.0
Threonine	6.6	4.6	10.1	4.5	7.0	5.2
Alanine	18.0	17.8	30.1	19.1	9.4	10.0
Proline	7.6	5.5	11.1	5.2	7.1	5.5
Lysine	10.1	5.5	15.0	5.3	10.3	6.1
Tyrosine	5.1	2.2	7.5	2.1	4.4	2.0
Methionine	2.8	1.5	4.5	1.5	2.9	1.7
Valine	9.1	6.4	13.9	6.3	8.9	6.8
Isoleucine	6.2	3.8	9.5	3.8	6.4	4.3
Leucine	11.9	7.3	18.0	7.2	11.9	7.9
Phenylalanine	7.3	3.5	11.2	3.4	7.0	3.6
TOTAL	147.9	100.0	228.5	100.0	143.2	100.0

Table S2. Free amino acid content of rice coleoptiles grown in normoxic (aerated), hypoxic (3% O_2) and anoxic (N_2) solution. *O. sativa* (cv. Amaroo) seeds were de-hulled and surface sterilised. Seeds were grown in 2 L of solution with 3% O_2, N_2 or atmosphere (aerated) gas bubbled though the solution at 0.2 L h^{-1}. Coleoptiles were collected at 3 d after imbibition, freeze dried and powdered. Free amino acid make-up of each sample was determined by the Australian Proteome Analysis Facility.

	anoxic		***hypoxic***		***normoxic***	
Amino acid	*Amino acid content (mg g^{-1})*	*% by weight*	*Amino acid content (mg g^{-1})*	*% by weight*	*Amino acid content (mg g^{-1})*	*% by weight*
Histidine	0.24	0.4	0.76	0.8	0.23	1.2
Asparagine	1.18	2.3	2.59	3.3	4.32	26.4
Serine	2.03	4.9	2.20	3.5	0.81	6.2
Glutamine	1.34	2.3	1.15	1.3	2.44	13.5
Arginine	0.37	0.5	1.37	1.3	0.16	0.7
Glycine	1.64	5.5	1.65	3.7	0.16	1.8
Aspartic acid	0.90	1.7	1.30	1.6	1.85	11.3
Glutamic acid	5.03	8.7	5.15	5.8	1.76	9.7
Threonine	0.31	0.7	0.82	1.1	0.45	3.0
Alanine	20.50	58.5	33.80	63.0	0.82	7.4
Proline	1.33	2.9	1.78	2.6	0.35	2.5
Cysteine	0.03	0.1	0.09	0.1	0.00	0.0
Lysine	0.29	0.5	0.59	0.7	0.26	1.4
Tyrosine	1.40	2.0	1.78	1.6	0.29	1.3
Methionine	0.24	0.4	0.43	0.5	0.09	0.5
Valine	1.65	3.6	2.21	3.1	0.83	5.7
Isoleucine	0.40	0.8	0.85	1.1	0.48	3.0
Leucine	1.29	2.5	2.49	3.2	0.48	3.0
Phenylalanine	1.00	1.5	1.70	1.7	0.26	1.3
Tryptophan	0.11	0.1	0.17	0.1	0.07	0.3
Total	41.26	100.0	62.89	100.0	16.10	100.0

Table S3. Total DNA content and estimates of cell number of 3 d old coleoptiles grown in normoxic (aerated), hypoxic (3% O_2) and anoxic (N_2) solution. *O. sativa* (cv. Amaroo) seeds were de-hulled and surface sterilised. Seeds were grown in 2 L of solution with 3% O_2, N_2 or atmosphere (aerated) gas bubbled though the solution at 0.2 L h^{-1}. Coleoptiles were collected at 3 d after imbibition, total DNA extracted by phenol-chloroform extraction and quantified spectrophotometrically. This total was used to estimate total cell number per coleoptile. Total cell number was also estimated based on the known dimensions of the coleoptile and cells within it (Wada, 1961).

Treatment	*DNA Content (ng DNA per coleoptile)*	*Cell number (cells per coleoptile) by DNA content*	*Cell number (cells per coleoptile) By cell packing methods*
Normoxia	1.2 ± 0.6	74000 ± 17000	78000 ± 12000
Hypoxia	1.1 ± 0.3	68000 ± 18000	59000 ± 13000
Anoxia	0.8 ± 0.1	50000 ± 6000	49000 ± 8000

Table S4. PDC activity in the *O. sativa* PDC mutant and its parent line (*cv.* Nipponbare). *O. sativa* seeds were de-hulled and surface sterilised. Seeds were grown in 2 L of solution with N_2 or gas bubbled though the solution at 0.2 L h^{-1}. Coleoptiles were collected at 3 d after imbibition, total protein extracted and PDC activity determined as per Gibbs *et al*. (2000).

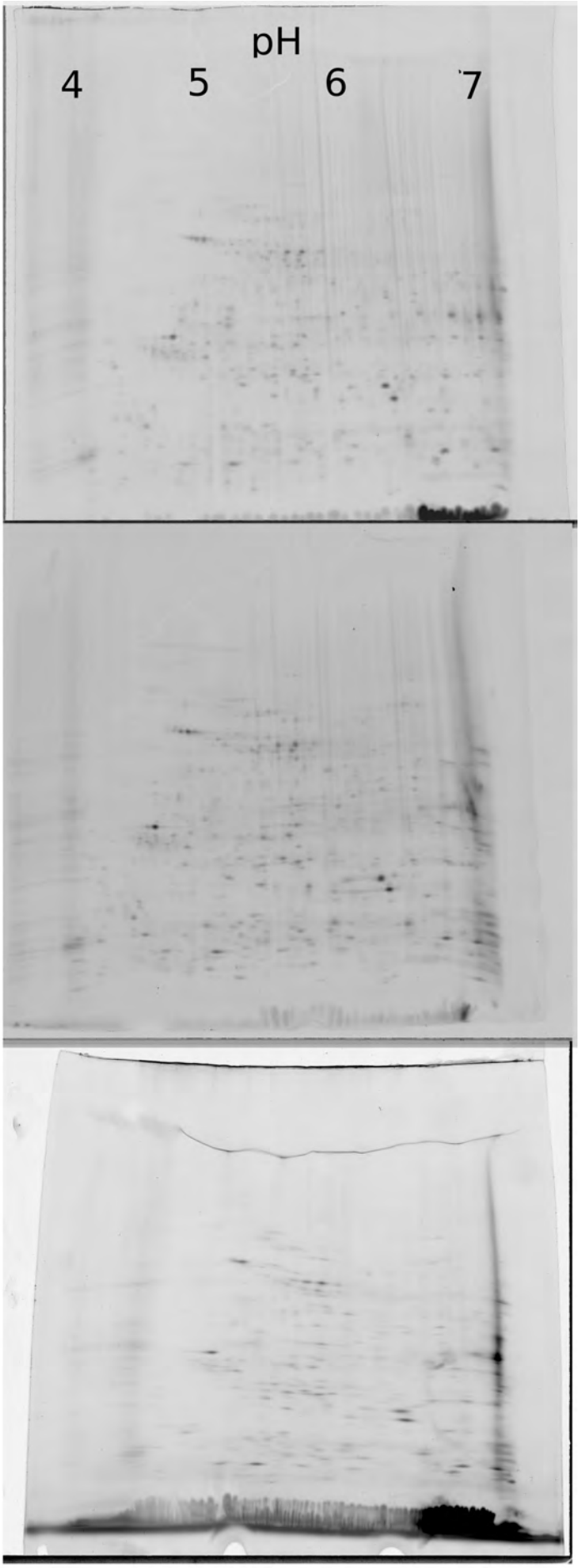
Line	PDC Activity (nmol NADH g^{-1} (FW) min^{-1})
PDC mutant	50 ± 14
Nipponbare	245 ± 39

Appendix B

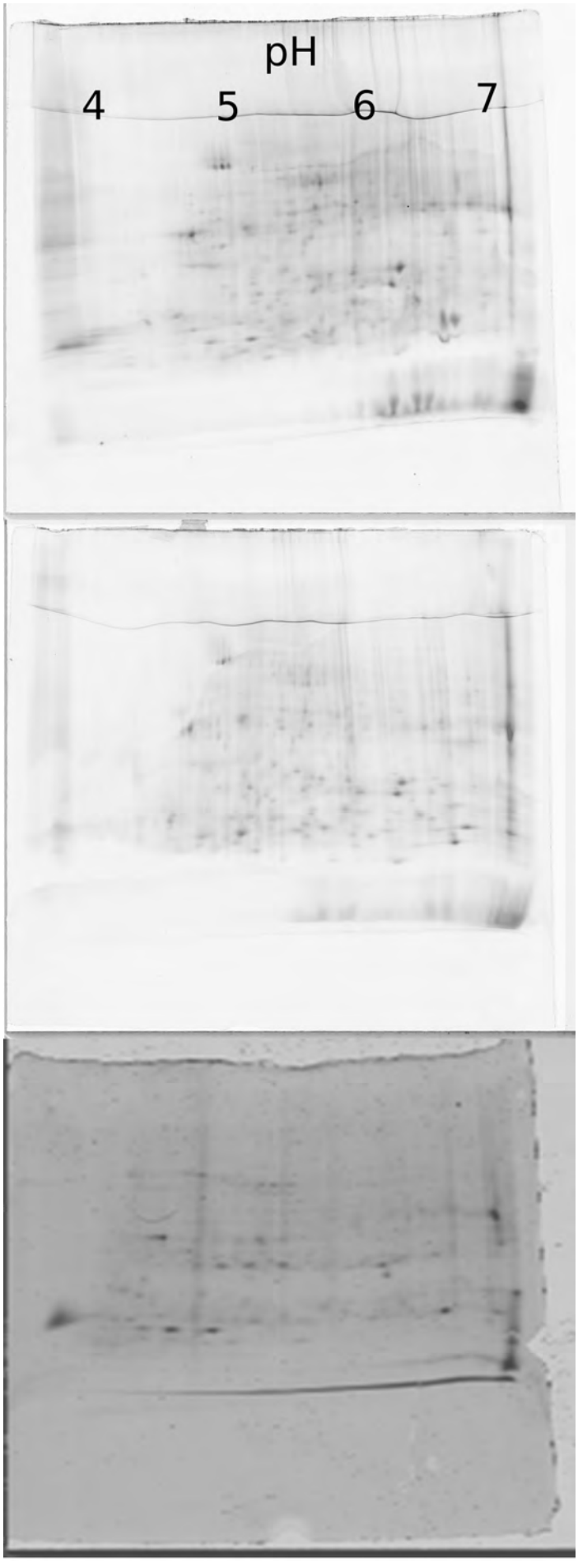
Supplementary Table B1. (Found on DVD in directory label Appendix B) This table contains the raw expression data and relative fold changes for cDNA microarray experiment described in Chapter 3.

Appendix C

Supplementary Table C1. (Found on DVD in directory labeled Appendix C) The table lists the spot numbers and their corresponding well ID/ MS/MS spectra id.
Sub-appendix CI. (Found on DVD in directory labeled Appendix C\I) This directory contains the MS/MS spectra (labeled by well ID) for each of the 48 spots cut from gels 2 and 5.
Sub-appendix CII. (Found on DVD in directory labeled Appendix C\II) This directory contains the Mascot protein reports for each of the spots (labeled by well ID) for each of the spots identified in Chapter 4.
Sub-appendix CIII. (Found on DVD in directory labeled Appendix C\III) This directory contains the Progenesis Samespot report for the DIGE experiment reported on in Chapter 4.



Supplementary Figure C1. The CyDye2 (standard) channels of gels 1, 2 and 3 (top to bottom) (basal protein samples) as described in Chapter 4. A higher resolution version of this image is included on the data DVD.



Supplementary Figure C2. The CyDye2 (standard) channels of gels 4, 5 and 6 (top to bottom) (distal tissue samples) as described in Chapter 4. A higher resolution version of this image is included on the data DVD.

Figure C3. Spot locations on gel 1. See Table C1 for corresponding spot ids.

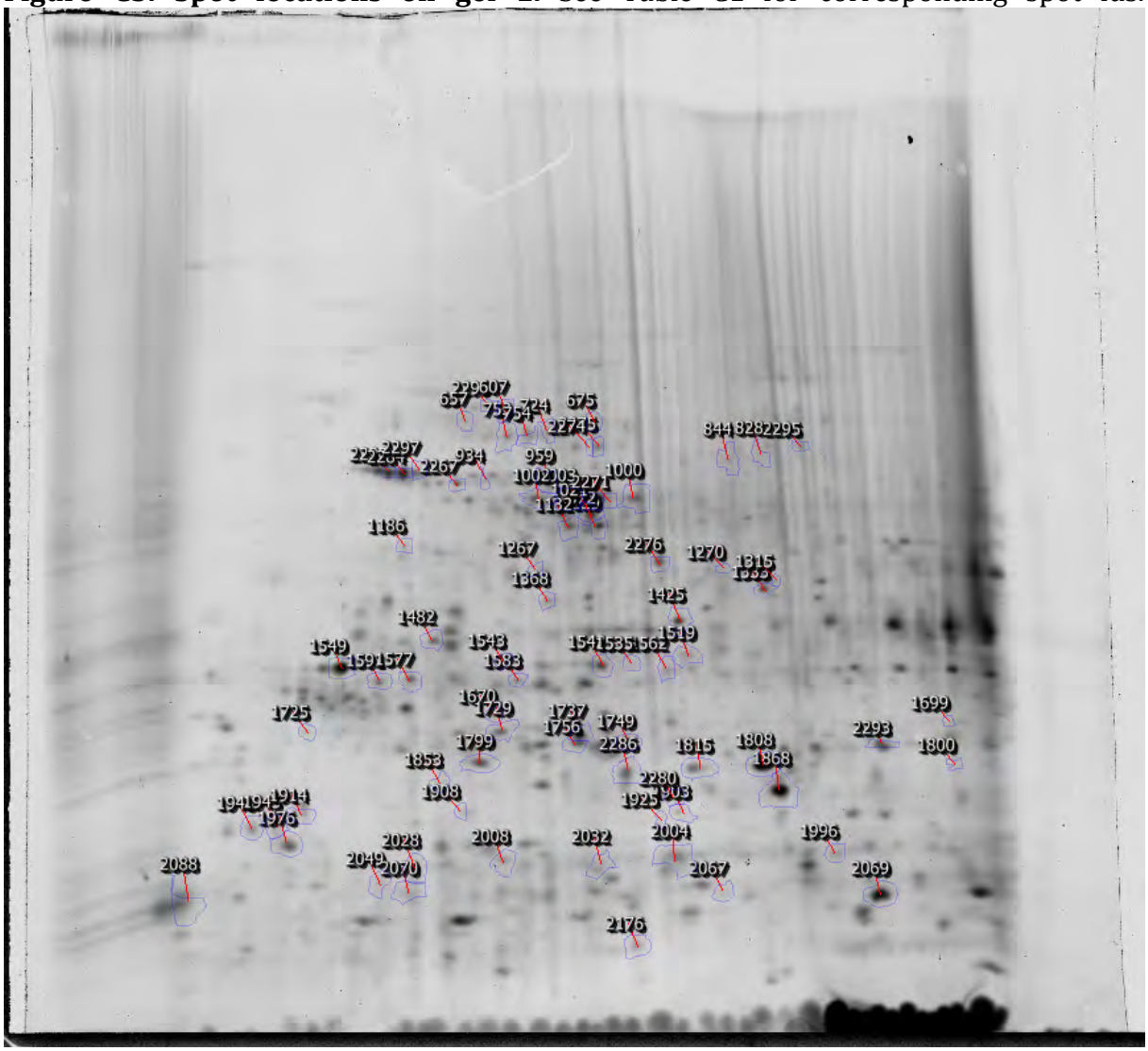
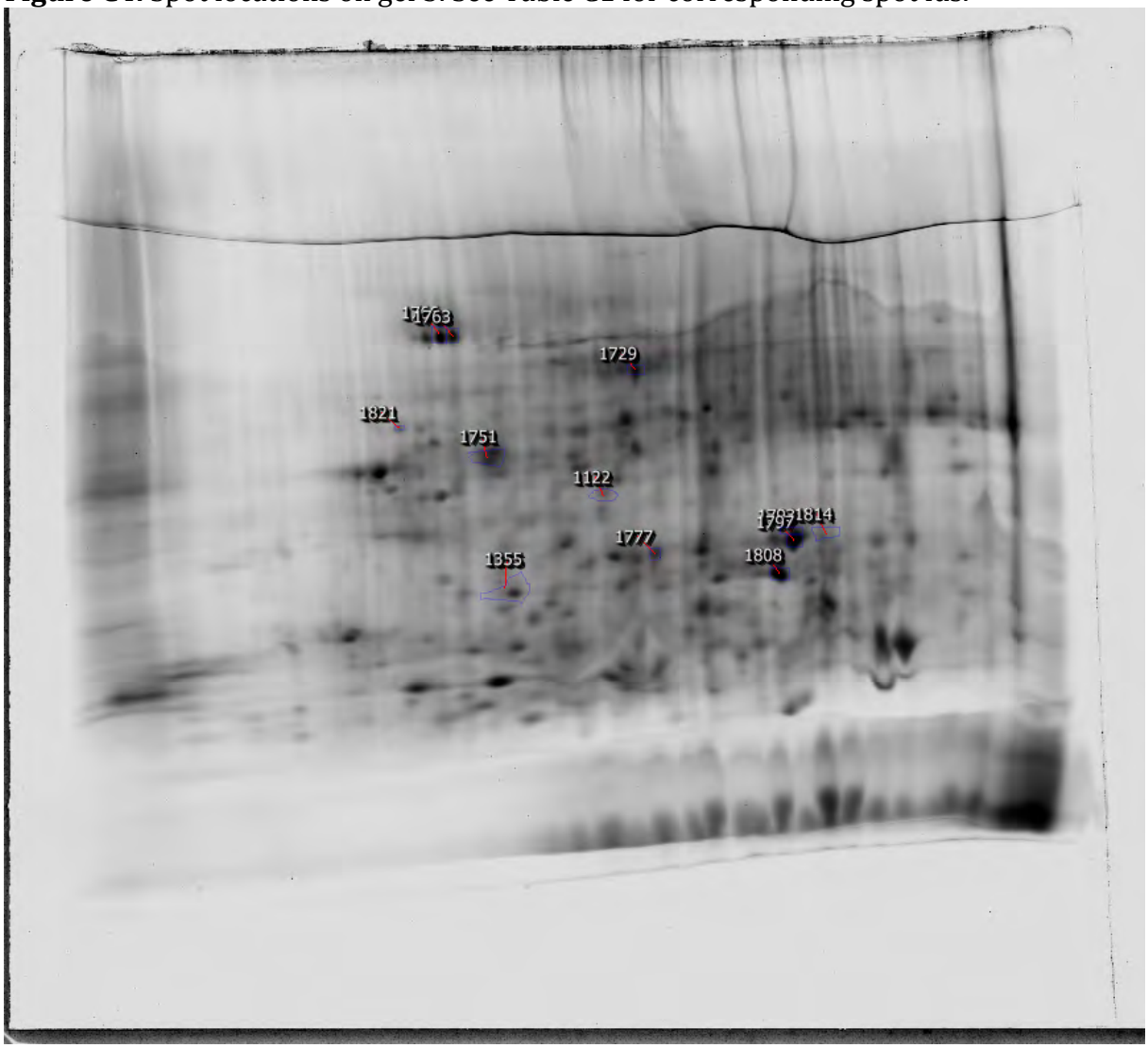


Figure C4. Spot locations on gel 5. See Table C1 for corresponding spot ids.



Appendix D

Table D1. Fresh weight (mg per coleoptile) of coleoptiles of various rice cultivars grown in strict anoxia with a variety of exogenous N sources. Data shown is mean and standard deviation.

	Treatment			
	Control (no exogenous N)	2 mM ammonium phosphate	2 mM sodium nitrate	1 mM ammonium nitrate
Cultivar	*3-d old coleoptiles*			
Amaroo	1.1 ± 0.4	1.4 ± 0.4	1.4 ± 0.7	2.0 ± 0.5
KHO	1.3 ± 0.4	1.5 ± 0.4	1.4 ± 0.4	1.6 ±0.4
Kinmaze	1.0 ± 0.3	1.3 ± 0.3	1.4 ± 0.4	1.5 ± 0.2
rad	0.3 ± 0.4	0.3 ± 0.4	0.3 ± 0.5	0.3 ± 0.4
	4-d old coleoptiles			
Amaroo	2.6 ± 0.4	3.2 ± 0.6	3.3 ± 0.3	3.5 ± 0.4
KHO	2.5 ± 0.4	2.5 ± 0.5	2.6 ± 0.4	2.5 ± 0.4
Kinmaze	2.1 ± 0.3	1.9 ± 0.3	2.7 ± 0.4	3.1 ± 0.4
rad	0.5 ± 0.2	0.4 ± 0.3	0.5 ± 0.3	0.5 ± 0.3
	5-d old coleoptiles			
Amaroo	2.9 ± 0.6	4.1 ± 0.3	3.9 ± 0.4	4.2 ± 0.7
KHO	3.3 ± 0.3	3.3 ± 0.4	3.3 ± 0.7	3.3 ± 0.7
Kinmaze	2.1 ± 0.3	2.9 ± 0.4	2.9 ± 0.4	3.5 ± 0.4
rad	0.7 ± 0.3	0.8 ± 0.3	0.8 ± 0.3	1.0± 0.2

Table D2. Fresh weight (mg per coleoptile) of coleoptiles of various rice cultivars grown in normoxia with a variety of exogenous N sources. Data shown is mean and standard deviation.

	Treatment			
	Control (no exogenous N)	2 mM ammonium phosphate	2 mM sodium nitrate	1 mM ammonium nitrate
Cultivar	*3-d old coleoptiles*			
Amaroo	4.6 ± 0.9	4.2 ± 0.4	4.5 ± 1.2	4.0 ± 0.4
KHO	3.3 ± 0.4	3.0 ± 0.3	3.1 ± 0.8	3.2 ± 0.9
Kinmaze	3.9 ± 0.8	3.7 ± 0.6	3.8 ± 0.6	3.5 ± 0.3
rad	1.4 ± 0.3	1.4 ± 0.4	1.1 ± 0.3	1.4 ± 0.3
	4-d old coleoptiles			
Amaroo	6.1 ± 0.5	6.3 ± 0.4	5.7 ± 0.4	5.4 ± 0.4
KHO	4.2 ± 0.2	4.2 ± 0.9	4.8 ± 1.1	4.7 ± 0.8
Kinmaze	6.2 ± 1.8	6.7 ± 1.2	6.5 ± 1.9	6.3 ± 2.1
rad	2.8 ± 0.6	2.6 ± 0.7	2.5 ± 0.4	2.6 ± 0.4
	5-d old coleoptiles			
Amaroo	12.4 ± 0.8	9.7 ± 1.2	10.4 ± 1.1	14.0 ± 2.9
KHO	6.6 ± 0.5	5.6 ± 1.6	5.5 ± 1.5	5.4 ± 1.0
Kinmaze	10.1 ± 1.4	8.9 ± 1.3	10.9 ± 2.0	7.8 ± 1.9
rad	4.2 ± 0.7	4.1 ± 0.8	4.4 ± 1.1	4.7 ± 0.6