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Differential molecular responses of rice and wheat coleoptiles to anoxia reveal novel metabolic adaptations in amino acid metabolism for tissue tolerance

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ABSTRACT

Rice and wheat are the most important starch crops in world agriculture. While both germinate with an anatomically similar coleoptile, this tissue defines the early anoxia tolerance of rice and the anoxia intolerance of wheat seedlings. We combined protein and metabolite-profiling analysis to compare the differences in response to anoxia between the rice and wheat coleoptiles. Rice coleoptiles responded to anoxia dramatically, not only at the level of protein synthesis, but also at the level of altered metabolite pools, while the wheat response to anoxia was slight in comparison. We found significant increases in abundance of proteins in rice coleoptiles related to protein translation and antioxidant defence, and accumulation of a set of enzymes involved in serine, glycine and alanine biosynthesis from glyceraldehyde-3-phosphate or pyruvate, which correlates with an observed accumulation of these amino acids in anoxic rice. We show a positive effect on wheat root anoxia tolerance by exogenous addition of these amino acids, indicating their synthesis could be linked to rice anoxia tolerance. The potential role of amino acid biosynthesis contributing to anoxia tolerance in cells is discussed.

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INTRODUCTION

Rice and wheat are economically-important crops that are adversely affected by multiple environmental stresses. Both these monocotyledon grasses operate similar central metabolic processes, yet notably differ in aspects of their development and anatomy as well as in their optimal growth conditions; rice is typically cultivated in tropical regions on flooded/anaerobic soils whereas wheat is almost exclusively a dry-land winter crop (Nagai and Makino, 2009). Despite these differences, their critical role as the main source of nutrition for humanity makes the comparative study of these crops under yield-reducing stresses important. Furthermore the study of two species, as opposed to two cultivars of the same species, may be useful in highlighting mechanisms of anoxia adaptation in plants differing in the contexts of their domestication. Rice is an ideal model species for elucidating the mechanisms of anoxia tolerance in plants; its full genome sequence is available (Yu et al., 2002), it can survive under prolonged anoxia and it can even display elongation of its coleoptiles under anoxic conditions (Menegus et al., 1991; Perata et al., 1997). A critical aspect of rice anoxia tolerance is the induction of the starch-degrading enzyme α-amylase under anoxia, providing a continuing supply of substrates for metabolism (Perata et al., 1992). Rice growth under anoxia is largely restricted to the coleoptile, with root and leaf development halted in the absence of oxygen (Öpik, 1973). From an evolutionary perspective, successful coleoptile growth under anoxia provides rice seedlings with the opportunity to reach more air-saturated conditions above anaerobic mud or standing water (Kordan, 1974) and thus increases the chance of survival. In some regions of the world wheat also encounters waterlogging causing O_2 deficiency, but unlike rice, this normally leads to major or even total yield losses (Setter and Waters, 2003). Compared with rice seedlings, wheat seedlings are widely considered to be anoxia-intolerant, despite possessing an anatomically similar coleoptile tissue under normal growth conditions (Menegus et al., 1991). Although wheat seeds are starchy like rice, they cannot germinate and grow a coleoptile under anoxia due to an absence of the starch-degrading enzyme α-amylase in anaerobic seeds (Perata et al., 1992).

Without O_2, the glycolytic pathway that is linked with ethanolic fermentation is the predominant mechanism of energy production in plants (Gibbs et al., 2000; Bailey-Serres and Voesenek, 2008). However, there is still much less energy

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production during anoxia than in aeration per unit of carbohydrate metabolised. As a consequence, the synthesis rate of macromolecules such as proteins decrease well below that seen in aerated tissues (Alpi and Beevers, 1983). Even so, rice coleoptiles still exhibit a complex pattern of newly synthesized proteins under anoxia (Mocquot et al., 1981; Ricard and Pradet, 1989; Huang et al., 2005). Along with the classical anaerobic proteins first reported in maize (Sachs et al., 1996), anoxic rice coleoptiles also synthesize a range of proteins with unknown functions (Huang et al., 2005). To date, the identified anoxically-synthesised protein dataset in rice does not form complete biochemical pathways (Ricard et al., 1991; Huang et al., 2005). Evidently missing from this set are a range of enzymes in glycolysis and the enzymes that could explain the observed amino acid accumulation in rice coleoptiles under anoxia (Fan et al., 1997; Kato-Noguchi and Ohashi, 2006). It remains unclear whether these dramatically induced amino acid pools are derived from specific protein degradation under anoxia or *de novo* amino acid synthesis. With the improved techniques in protein identification using peptide mass spectrometry, it is feasible to analyze proteins on a large scale using combinations of gel-based or non-gel based methods to address these issues and provide an in-depth understanding of the mechanism(s) of anoxia tolerance. Direct comparisons of proteome responses that occur during anoxia in tolerant rice coleoptiles and intolerant wheat coleoptiles also provide an opportunity to differentiate proteome changes under anoxia associated with cellular stress and damage, from those associated with continued growth and adaptation.

At the metabolite level, the accumulation of fermentation end products such as ethanol, lactate and alanine, have been extensively studied in plants responding to O_2 deprivation (Raymond et al., 1985; Menegus et al., 1989; Menegus et al., 1991; Gibbs and Greenway, 2003). A recent metabolomic analysis of *Lotus japonicus* suggests that accumulation of succinate and alanine under low oxygen might function to generate ATP that is additional to what the glycolytic pathway offers (Rocha et al., 2010). In rice coleoptiles, the anaerobic assimilation of inorganic nitrogen into amino acids, particularly alanine and γ-aminobutyrate (GABA)/glutamic acid may serve to supplement ethanolic fermentation in sustaining glycolytic energy production (Fan et al., 1997). There are also several studies that report changes of carbohydrates (sucrose, glucose, fructose) and sugar-phosphates in coleoptiles and shoots (comprised of both leaves and coleoptiles) of rice seedlings in response to

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anoxia (Menegus et al., 1991; Guglielminetti et al., 1995; Huang et al., 2003). An analysis of the early germination stages of rice embryos at the metabolite level highlighted sets of 10 and 13 metabolites, respectively, as aerobic and anaerobic responders (Narsai et al., 2009). However, a broad picture of the changes of metabolites in the anoxic rice coleoptile itself remains unclear, and measuring only a few compounds, as has been reported in most of the earlier studies (Menegus et al., 1989; Menegus et al., 1991) makes it hard to understand the flow of both carbon and nitrogen between metabolic pools under anoxia. Furthermore, there are no reported studies on how wheat coleoptiles respond to anoxia across a broad set of metabolite pools.

In this study, we combined protein and metabolite-profiling analysis to compare the differences in response to anoxia between anoxia-tolerant rice coleoptiles and anoxia-intolerant wheat coleoptiles. Rice coleoptiles, but not wheat coleoptiles, responded to anoxia dramatically, not only at the level of new protein synthesis, but also at the level of altered metabolite pools. We also found significant increases in anoxic rice coleoptile proteins related to protein translation, such as 40S ribosomal proteins, initiation factor 4A and elongation factors. The possibility of selected mRNA translation and protein turnover in anoxic rice coleoptiles, but not in anoxic wheat coleoptiles which are remarkably unchanged, are discussed in light of the observed low correlation between protein abundance and reported gene expression data. A set of enzymes that increased in abundance in anoxic rice, a change that was not apparent in wheat, are involved in serine, glycine and alanine biosynthesis from glycolytic metabolites. This correlates with the observed accumulation of these amino acids in anoxic rice coleoptiles. The potential role of amino acid biosynthesis contributing to anoxia tolerance is discussed, and we show a positive effect on tolerance upon exogenous supplementation of these amino acids in wheat, but not in rice.

RESULTS

Physiological analyses highlight differences between responses of rice and wheat seedlings to anoxia.

Germination of the rice and wheat seeds used in this study under anoxic conditions replicated widely reported differences that rice can germinate and grow its

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coleoptile under anoxia, while wheat seeds fail to germinate under similar conditions ((Alpi and Beevers, 1983), **Supplemental Figure 1A**). We compared rice coleoptiles from seedlings germinated and grown under anoxia for 6 days to those under aeration for 4 days to characterise rice metabolism under prolonged anoxia. Rice coleoptiles from seedlings grown under aeration for 4 days followed by a 1-day-switch to anoxia were also studied. This allowed a comparison to be made between prolonged protein changes from germination and more rapid changes associated with the loss of oxygen from aerobic tissue. The treatment involving a switch to anoxia also generated a dataset comparable to publically-available microarray data characterising coleoptiles from 4-day-old anoxically-germinated rice seedlings (Lasanthi-Kudahettige et al., 2007). As they are unable to germinate under anoxia, we were constrained to studying wheat seedlings that were germinated and grown under aeration for 4 days and then switched to 1 day of anoxia. By using the Evans blue viability stain, a distinction in the anoxia tolerance of rice and wheat was confirmed in that the viability of rice roots was much greater than those of wheat after a switch from aeration to anoxia (**Supplemental Figure 1B**).

Detailed study of the 24 h-anoxic response in rice and wheat coleoptiles was performed tracking growth, sugar content and metabolic activities (**Table 1**). This showed that aerobic grown rice coleoptiles, but not wheat coleoptiles, displayed significant elongation after one day of anoxia (**Table 1**). Sugars are the primary carbon source for energy production via glycolysis and ethanolic fermentation. The sugar content of rice coleoptiles under anoxia for 6 days was considerably lower than that measured in 4 day-old aerobic coleoptiles (**Table 1**). The sugar content of coleoptiles, leaves and roots of both rice and wheat seedlings were also significantly lower even after a one-day switch from aeration to anoxia (**Table 1**). Induction of alcohol dehydrogenase (ADH) is a key step in the switch to anaerobic energy production, for it is ethanolic fermentation that re-generates NAD^+, an oxidant necessary for the continuation of glycolysis. In both anoxic rice and wheat coleoptiles, ADH activity was induced compared to the aerated control **(Table 1**). However the apparent inducibility of ADH activity during anoxia was greater in coleoptiles of rice (3.4-fold) than those of wheat (2.4-fold) (**Table 1**). The highest ADH activity recorded was observed in rice coleoptiles after 6 days under anoxia (**Table 1**). In the leaves and roots of both rice and wheat seedlings, ADH activities

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were also induced by anoxia, but the final specific activity of ADH was 5-10 fold higher in rice than in wheat **(Table 1**).

The 6-day-old anoxic rice coleoptiles were shorter than those from seedlings germinated and grown under aeration for 4 days (**Table 1**). This contrasts with previous reports that rice coleoptiles grown under anoxia were much longer than aerated ones (Atwell et al., 1982; Alpi and Beevers, 1983). The explanation for this difference could be the different cultivars used or the use of N_2 bubbling as a means to achieve anoxia, rather than stagnant conditions used in other studies (Magneschi et al., 2009). Bubbling removes other gases such as CO_2 and even ethylene produced by coleoptiles if trace amounts of O_2 are available, reducing the complexity of comparing anoxic and aerated conditions.

We also monitored the capacity for mitochondrial respiratory function by measuring tissue O_2 consumption rate. The rate of O_2 consumption in coleoptiles from seedlings continuously grown under anoxia, but returned to aeration for the measurements, was significantly less than that of continuously aerated coleoptiles (**Table 1**). This is consistent with the need for oxygen for the biosynthesis of haem groups for the cytochromes of the plant respiratory chain (Millar et al., 2004). The O_2 consumption rate capacity in roots and leaves from rice and wheat seedlings were significantly lowered by one day of anoxia (**Table 1**), indicating tissue adaptation to anoxia and/or damage or loss of mitochondrial function. Interestingly, this effect was more dramatic in rice than in wheat tissues. In comparison, the respiratory capacity of rice and wheat coleoptiles was not significantly affected by one day of anoxia (**Table 1**), suggesting no mitochondrial damage occurred during this period of anoxia and the immediate ability of mitochondrial function to return upon the aeration required for the measurements to be carried out. In conclusion, rice coleoptiles grown continuously under anoxia or aeration had significant differences in all the parameters investigated. When switched from aerated to anoxic conditions, coleoptiles of rice responded to anoxia to a greater degree than those of wheat. In comparison, leaves and roots of rice and wheat seedlings responded to anoxia similarly in all parameters investigated, distinct from the coleoptile response in both species, and despite the longer term differences noted from Evans blue viability staining of roots (**Supplemental Figure 1B**).

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Quantitative proteomic analysis of coleoptiles show a significant rice response but a minimal wheat response to anoxia

A number of molecular responses that underlie the differences noted in Table 1 have been investigated in published reports (Menegus et al., 1991; Perata et al., 1992). There are also a number of studies on global gene expression in response to anoxia or O_2 deficiency in plants including rice (Lasanthi-Kudahettige et al., 2007; Narsai et al., 2009), and Arabidopsis (Klok et al., 2001; Branco-Price et al., 2005; Liu et al., 2005; Loreti et al., 2005; Mustroph et al., 2010). But so far, information on the changes of protein abundances in response to anoxia is limited (Mocquot et al., 1981 ; Ricard and Pradet, 1989; Huang et al., 2005). Because there were such dramatic differences at the physiological level between 4-d aerated and 6-d anoxic coleoptiles (**Table 1)**, we started analysing differences in protein profiles using Differential in Gel Electrophoresis (DIGE) which is based on staining protein samples with different fluorescent dyes (**Figure 1A**). Out of 1259 protein spots detected on IEF/SDS-PAGE gels (pI range 3-10), 164 (13.3%) protein spots met the criteria of significant 2-fold differences in protein abundance in three replicate gels ($P<0.05$). 107 of these protein spots were more abundant under anoxia and 57 more abundant under aeration (**Figure 1A**). This suggested a significant difference in the protein profiles of samples from 6-day anoxic and 4-day aerated rice coleoptiles. We also compared rice coleoptiles from 4-day aerated seedlings with those grown in the same control conditions but switched to an additional 1-day anoxia **(Figure 1B**). There were 1245 protein spots detected and 67 (5%) of these met the above-mentioned significance criteria (**Figure 1B**). 85% of these changing protein spots were more abundant in coleoptiles subjected to the anoxic switch (**Figure 1B**), suggesting a rapid 24 h response to anoxia at the protein level. This is in agreement with the results of van Dongen and colleagues (2009) where they reported a tendency for gene expression to increase in response to 0.5-48 h of hypoxia as opposed to cessation of transcription in roots of Arabidopsis seedlings (van Dongen et al., 2009). We then compared coleoptile proteomes of wheat using the same anoxic-switch experimental setup used in rice (**Figure 1C**). According to the same significance criteria, only 5 (0.4%) of the 1245 protein spots detected differed in abundance between the two treatments (**Figure 1C**), suggesting a very limited response to anoxia at the protein level in wheat coleoptiles.

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We then conducted two additional independent analyses to further quantify the difference between 4-d-old aerated and 6-d-old anoxic coleoptiles in order to overcome limitations of the 3-10NL DIGE gel analysis. We used a broader pH range to show more basic protein spots in a DIGE analysis (using pI 3-11 gels) and a non-gel based iTRAQ (isobaric Tag for Relative and Absolute Quantitation) experiment to remove the bias against protein size and solubility that are inherent to isoelectric focusing based analysis. Out of the 1007 spots detected in the pI 3-11 DIGE, 140 (13.9%) spots met the criteria of significance. 46 of these prospective proteins were more abundant under anoxia and 94 more abundant under aeration (**Supplemental Figure 2 and 4**). Using iTRAQ analysis, we identified 142 proteins, 126 of which could be quantified in a 3-biological replicate experiment comparing coleoptile proteomes extracted from 6-d anoxic and 4-d aerated rice seedlings (**Supplemental Table 1**). Among them, 34 were significantly more abundant under anoxia and 29 were significantly more abundant under aeration (**Supplemental Figure 2**). The fold differences in protein abundance as revealed by iTRAQ were proportional to those revealed by the DIGE analysis, with the r^2 being 0.61(**Supplemental Figure 3A**). However the DIGE analysis resolved much larger fold differences between the two treatments than those calculated in the iTRAQ analysis (**Supplemental Figure 3A**). For example, according to the DIGE analysis, peroxiredoxin (Os07g44430.1) was reported to be 3.38-22.69 fold more abundant in 6-d anoxic rice coleoptiles than those of the 4-d aerated control whereas this difference was only 2.48 fold according to the iTRAQ analysis (**Table 2**) Similar discrepancies between the linearity of responses by the two methods have been previously reported (Wu et al., 2006).

Identified proteins in rice coleoptiles with changes in abundance under anoxia

The identified proteins with significant changes in abundance between aerated and anoxic treatments are listed in **Table 2.** The protein identification evidence from all protein analysis is shown in **Supplemental Table 1** and further details for iTRAQ data analysis are provided in **Supplemental Table 2**. We have incorporated microarray data (4-d-old anoxic and 4-d-old aerobic rice coleoptiles from Lasanthi-Kudahettige et al., 2007) into **Table 2** and **Supplemental Table 1** for further comparison. The enzymes detected which are involved in glycolysis, fermentation and amino acid biosynthesis were also incorporated into a metabolite pathway map

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in **Figure 2**. We could not identify the 5 protein spots that were significantly different between treatments in wheat coleoptiles due to their very low abundance in gels.

Enzymes involved in glycolysis and ethanolic fermentation

We identified the accumulation of enzymes involved in ethanolic fermentation such as ADH1 (Os11g10480) and pyruvate decarboxylase 1 (PDC1; Os05g39310) in anoxic rice coleoptiles (**Table 2, Figure 2),** consistent with published reports (Mocquot et al., 1981; Ricard and Pradet, 1989; Bailey-Serres and Chang, 2005; Huang et al., 2005; van Dongen et al., 2009). Significantly increased abundance of enzymes involved in multiple steps of glycolysis were also identified and listed in **Table 2 and Figure 2**. Those enzymes were PPi-fructose-6-phosphate 1-phosphotransferase beta subunit (Os06g13810) fructose-bisphosphate aldolase (Os05g33380, Os01g67860, Os10g08022), triosephosphate isomerase (Os01g05490), glyceraldehyde-3-phosphate dehydrogenase (Os04g40950, Os02g38920, Os08g03290), phosphoglycerate kinase (Os02g07260), phosphoglucomutase (Os03g50480), 2,3-bisphosphoglycerate-independent phosphoglycerate mutase (Os01g60190) and enolase (Os10g08550) (**Table 2, Figure 2)**. Exceptions to these anoxia increases were Fructokinase-2 (Os08g02120), which was observed to decrease in abundance under anoxia as well as discrepancies in the direction of change for phosphoglucomutase (Os03g50480) between the DIGE and iTRAQ analyses.

We also identified two sucrose synthase enzymes (Os03g28330, Os06g09450) that were significantly more abundant in rice coleoptiles of anoxically-germinated seedlings (**Table 2**). Our data agree with reports that under anoxia, there is a switch from invertase to sucrose synthase as a means of degrading sucrose into sugars that can then enter the glycolytic pathway (Guglielminetti et al., 1995).

These observations reinforced the evidence for general enhancement of glycolysis and ethanolic fermentation for rice coleoptiles during adaptation to anoxia, and highlight gene-specific identification of changing proteins that would promote a Pasteur effect. Notably, only about two-thirds of the transcripts for these glycolytic proteins are transcriptionally more abundant under anoxia in rice (Lasanthi-

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Kudahettige et al., 2007). But notably the decreased abundance of fructokinase-2 was also seen at the transcript level under anoxia (**Table 2**).

Enzymes involved in amino acid metabolism

We identified several enzymes involved in the synthesis of Ser and Gly that increased in abundance under anoxic stress, notably, D-3-phosphoglycerate dehydrogenase (PGDH; Os04g55720), phosphoserine aminotransferase (PSAT; Os03g06200) and serine hydroxymethyltransferase (SHMT; Os12g22030) (**Table 2, Figure 2**). Alanine aminotransferase 2 (AlaAT; Os01g25130) was also more abundant in anoxic rice coleoptiles. Glutamine synthetase root isozyme 3 (Os02g50240) was less abundant under anoxia when compared to aeration and different isoforms of 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase (methionine synthase or MS; Os12g42876 up in DIGE, Os12g42876 / Os12g42884 down in iTRAQ) showed discrepancies in their direction of change. Methionine generation is particularly interesting as this amino acid is involved in the synthesis of ethylene, a plant hormone involved in submergence-induced gene expression (Fukao et al., 2006; Xu et al., 2006). Methionine could only be detected in anoxically-switched coleoptiles and did not appear to accumulate (**Table 3).** The enzyme involved in the first step of ethylene production, *S*-adenosylmethionine synthase (Os05g04510), was also detected in our iTRAQ analysis (**Table 2**). However it was significantly less abundant in coleoptiles of anoxically-germinated rice, an observation in line with the O_2-dependence of the ethylene biosynthetic pathway.

Enzymes or proteins involved in ROS detoxification

Reactive oxygen species (ROS) play an important role in signalling under O_2 deficiency (Baxter-Burrell et al., 2002; Bailey-Serres and Chang, 2005). We identified several proteins involved in ROS degradation that changed in abundance. As examples, peroxiredoxin (Os07g44430) was significantly more abundant in rice coleoptiles derived from 6-d-old anoxic seedlings than in 4-d-old aerated seedlings (**Table 2**). In concordance, the transcript for this gene was 32-fold higher in abundance in anoxic rice coleoptiles (Lasanthi-Kudahettige et al., 2007). Peroxiredoxin is an antioxidant enzyme that can reduce both H_2O_2 and alkyl hydroperoxides. In contrast, we identified another H_2O_2 decomposing enzyme,

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ascorbate peroxidase (Apx; Os03g17690; Os07g49400) that was less abundant in anoxic coleoptiles (**Table 2**). The Bowman-Birk type Trypsin Inhibitor (BBTI; Os01g03340) found to decrease in abundance under anoxia may have other functions beyond its role in proteolysis. BBTIs have been reported to act as monodehydroascorbate reductases and dehydroascorbate reductases in etiolated mung bean seedlings (Hou et al., 2000) and roots of sweet potato (Hou and Lin, 1997), and thus can be involved in regeneration of ascorbate. These results suggest that rice coleoptiles may use different detoxification systems under anoxia/hypoxia and re-aeration to those used during continual aeration. It has been reported that anoxia can cause an increase in H_2O_2 in the rice root apoplast and plasma membrane (Blokhina et al., 2001), suggesting a protective function of these antioxidant defense enzymes in anoxic rice coleoptiles. However, we have previously measured markers for lipid oxidation and demonstrated that damage was lower in anoxic coleoptiles relative to aerobic or re-oxygenated coleoptiles (Millar et al., 2004) suggesting that either (1) oxidative stress under anoxia is minimal and that Prx have a protective role upon the return of O_2 or (2) Prx has a function under anoxia that we have not anticipated. The former seems more likely given that O_2 is necessary for the formation of ROS. Interestingly, MnSOD was detected in two DIGE analyses and although statistically significant in its accumulation under 6d anoxia ($p<0.05$), MnSOD did not meet all of the criteria set for significance, specifically the abundance change did not exceed two (**Table 2**), indicating the quantitatively differential role of peroxiredoxin in responding to the availability of O_2. What is clear from the literature is that O_2 deprivation perturbs the redox status of cells; whether it be ROS levels (Blokhina et al., 2001), oxidative damage (Blokhina et al., 1999; Millar et al., 2004) or regulation of genes and small-molecule antioxidants involved in redox regulation (Yan et al., 1996; Biemelt et al., 1998; Blokhina et al., 2000; Blokhina et al., 2003; van Dongen et al., 2009).

Proteins involved in the process of translation

Selective translation of cytoplasmic mRNAs in plants under O_2 deficiency have been discussed (Bailey-Serres, 1999). We identified several proteins in the iTRAQ analysis involved in translation processes, which were more abundant in 6-d-old anoxic coleoptiles relative to the control. Those proteins were elongation factor 1-alpha (Os03g08010); elongation factor 2 (Os01g52470) and S10/S20 domain

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containing ribosomal protein (Os03g14530) **(Table 2)**. Others involved in translation that were less abundant under anoxia were 40S ribosomal protein S5 (Os11g29190) and elongation factor (Os02g32030). Whether or not these abundance changes in translational machinery are responsible for the perturbations in the levels of glycolytic, amino acid biosynthetic and ROS-defense proteins is currently unknown.

Other proteins of interest

The lower abundance of IAA-amino acid hydrolase precursor (ILR1; Os03g62060) we report under anoxia can be related to a long history of research on auxin-regulated coleoptile elongation dating back to the famous Went experiment (Went, 1942). The IAA-amino acid hydrolase is involved in cleavage of conjugates between IAA and amino acids (Bartel and Fink, 1995). The dramatic decrease of ILR1 (**Table 3**) could indicate that IAA is maintained in its conjugate form under anoxia. Microarray data also suggest that the transcript of this protein was dramatically down-regulated (271-fold) in anoxic rice coleoptiles (Lasanthi-Kudahettige et al., 2007). This supports the observation that auxin-binding activities were decreased in anoxic coleoptiles (Mapelli and Locatelli, 1995) and that there was no synergistic effect of IAA and anaerobiosis on rice coleoptile elongation (Pegoraro et al., 1988). The repressive effect of anoxia on auxin-related genes has also been observed in Arabidopsis through a global gene expression analysis (Loreti et al., 2005).

Also of interest was the finding that several proteins with unknown functions accumulated under anoxia. These proteins are annotated as protein kinases that contain the domain of unknown function 26 (DUF26). Their transcript fold increases under anoxia range from 248-1007X (Os08g04250; Os08g04210; Os08g04240 (Lasanthi-Kudahettige et al., 2007)). In addition, the Arabidopsis orthologue (At5g48540; **Supplemental Table 3)** is upregulated in response to 2 and 9 h hypoxia both within the total and polysomal mRNA pool of Arabidopsis seedlings (Branco-Price et al., 2008). Notably, we found anoxic accumulation of two group 3 late embryogenesis abundant proteins (LEA; Os05g46480; Os02g15250) as well as embryonic protein DC-8 (Os03g07180). LEAs are hydrophilic unstructured proteins rich in Gly, Ala and Ser (Baker et al., 1988; Campos et al., 2006). It has been reported that an LEA protein (Os04g52110) accumulates in anoxic rice embryos (Howell et al., 2007) and other groups have demonstrated inducibility of GUS

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reporters when fused to a carrot group 3 LEA promoter under hypoxia, salinity and dehydration (Siddiqui et al., 1998). Recently it was shown that *SUB1A* increased the accumulation of transcripts encoding for proteins involved in dehydration tolerance. Most interestingly, the *LEA3* transcript level increased to a greater degree during de-submergence in M202(*Sub1*) rice relative to wild type M202 (Fukao et al., 2011). This is especially interesting given that dehydration is a stress inherent to desubmergence. This *LEA3* transcript actually showed a decrease during submergence, a change in opposition to what we have found. This may be attributable to the use of different cultivars, the measurement of transcript levels and not protein abundance levels, and that submergence and anoxia are not perfectly comparable. Despite these differences, in our experimental system it is tempting to speculate that LEA up-regulation is a response that provides a protective and anticipatory function for when plants return to air. Clearly, LEAs are stress responsive, however the role of these proteins in anoxic environments awaits further insights into their molecular function.

Metabolomic analysis reveals a greater response to anoxia in rice than in wheat coleoptiles

To investigate the impact of the changes in primary metabolism on metabolite pools, we considered the overall changes in the GC-MS profiles of primary metabolites in wheat and rice coleoptiles exposed to anoxia. Consistent with the physiological and proteomic data, there were dramatic differences in metabolite profiles between rice coleoptiles derived from 4-day-old aerated and 6-day-old anoxic seedlings, with very high accumulation of amino acids under anoxia (**Table 3, Figure 2**). Many of these responses were also observed in rice coleoptiles that were switched to anoxia for 1 d, although these tended to be considerably more subtle (**Table 3, Figure 3**).

A number of major differences observed in 6-d-old anoxic seedling coleoptiles relative to 4-d-old aerobic seedling coleoptiles were not observed at all as responses in switched seedling coleoptiles (eg. 18 to 45-fold increases in 3-phosphoglycerate, β-alanine, 2-aminoadipate, and a remarkable 560-fold increase in urate; **Supplemental Table 7**). Moreover, some metabolites responded in opposite directions to the two treatments (eg. arginine, glutamate, homoserine, lysine and

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tyrosine; **Supplemental Table 7**). These discrepancies are consistent with oxygen-dependent biogenesis of cellular components involved in regulation of these metabolites in rice.

The wheat coleoptile metabolite profile also responded to anoxia (**Table 3, Supplemental Table 4, Figure 3**). Certain features were reproducibly found to be common to responses of wheat and rice coleoptiles to 1d anoxic transfer. These included accumulations of GABA, glycine, isoleucine, proline, threonine, succinate and putrescine and decreases in aspartate, glutamate, fructose, ribose, trehalose, citrate, isocitrate, citramalate, glucarate, malate, glycerate, threonate and cytosine (**Table 3, Supplemental Table 5**). However, in wheat, these responses tended to be much less pronounced than those observed in anoxically-switched rice coleoptiles (**Table 3, Figure 3**).

While the majority of metabolite responses to the 1-day anoxic shift were common to both species (**Figure 3** and **Table 3**), we did identify a number of species-specific responses which may be linked to the differential anoxia-tolerance of these species (**Supplemental Table 5, Supplemental Table 6**). Rice-specific responses included moderate-to-strong increases in serine, alanine, leucine and tryptophan and decreases in arginine, methionine, tyrosine, ornithine, 6-phosphogluconate and aconitate (with the aconitate response being the most consistently strong between experiments). Wheat-specific responses included moderate increases in β-alanine, 4-hydroxycinnamate and shikimate; strong increases in urate and moderate decreases in 4-hydroxyproline and sucrose. Interestingly, a small number of metabolites responded moderately strongly in opposite directions between the two species. For example, α-aminobutyrate and phosphate increased in rice while decreasing in wheat while, conversely, lysine, phenylalanine, xylose, 2-oxoglutarate and ascorbate decreased in rice while increasing in wheat. The distinctive and significant accumulation of alanine, glycine and serine in rice was consistent with our evidence of increased abundance of enzymes in these pathways in rice (**Figure 3**).

Surprisingly, L-alanine did not significantly differ in abundance between control and anoxically-switched wheat coleoptiles (**Table 3**), contradictory to a previous report where alanine levels accumulated in wheat shoots to the same degree as that of rice

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shoots after 8h of anoxia (Menegus et al., 1989) as well as a range of reports from other species (van Dongen et al., 2009; Narsai et al., 2011). Such a difference might be explained by differences in the experimental system, the specific dissection of the coleoptile tissue used in this report, or the timing of the amino acid accumulation. For example, accumulation of alanine in roots of Arabidopsis was found by treatment with 48 h of 4 % and 8 % oxygen but not when the concentration of oxygen was reduced to 1 % (van Dongen et al., 2009). To consider the last of these we repeated metabolite profiling at 4h after the switch to anoxia in both rice and wheat, but again we saw an increase in L-alanine in rice, but not in wheat (**Table 3**).

In addition to changes in amino acids, variations in intermediates in the TCA cycle were also observed. In the TCA cycle, the step converting succinate into fumarate by succinate dehydrogenase (SDH) requires the operation of electron transport chain and reduction of O_2 to water. Without O_2, the TCA cycle will stop at SDH and succinate will accumulate, as we observed in both rice and wheat coleoptiles (**Table 3, Figure 3)** and as other studies have reported (Menegus et al., 1991; Fan et al., 1997; Rocha et al., 2010). This claim was also supported by the decrease in other TCA cycle intermediates such as malate and citrate in both anoxically-switched cereals, yet contradicted when observing 6-d-old anoxic rice coleoptiles (**Table 3, Figure 2 and 3**) and suggests that the other intermediates in the TCA cycle were utilised under anoxia. The advantage of accumulation of succinate under anoxia has been widely discussed in the context of the extra ATP production that can result (Gibbs and Greenway, 2003; Bailey-Serres and Voesenek, 2008; Rocha et al., 2010).

Under prolonged anoxia, higher abundances of sucrose, glucose-6-phosphate (G-6-P) and fructose-6-phosphate (F-6-P) in rice coleoptiles were observed (**Table 3 and Figure 3**). Rice coleoptiles treated with shorter periods of anoxia (24h or 4h) revealed no significant differences in these sugars between stress and control treatments. In wheat however, a 4h anoxic treatment resulted in significant decreases in all three sugars and the 24h treatment, in a decrease of sucrose (**Table 3 and Figure 3**). Decreases in the levels of the transportable sugar sucrose as well as decreases in glycolytic intermediates might be indicative of a delayed transition to anaerobic metabolism in wheat when compared to rice.

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Database-driven metabolic phenotype analysis reveals conserved and divergent responses to low oxygen in rice and wheat.

Having established that wheat and rice coleoptiles display differential responses to oxygen deprivation, we thought it would be informative to compare these responses to those previously observed in other species. To this end, we used the *PhenoMeter* tool of *MetabolomeExpress* (https://www.metabolome-express.org; see Methods section for details) to search the *MetabolomeExpress* database of metabolic phenotypes, *MetaPhenDB* (see Methods), for previously reported metabolic phenotypes of statistically-significant qualitative overlap (co-directional responses) or inverse overlaps (opposite-direction responses) with the rice and wheat responses to anoxia that we report.

As expected, the metabolic responses of rice and wheat coleoptiles to anoxic transfer retrieved significant hits ($p < 0.05$; Fisher's Exact Test) to a number of previously-reported responses to oxygen-deprivation (Gibon et al., 2002; Narsai et al., 2009; Narsai et al., 2010; Rocha et al., 2010) summarised in **Supplemental Table 9**) while retrieving very few matches to any of the many other functionally less closely related metabolic responses in the *MetaPhenDB* database (see **Supplemental Table 9** for detailed results, including Fisher's Exact Test p-values). In addition, a number of species-specific positive and negative hits were also observed to diverse treatments (**Supplemental Table 8**). For example, rice gave highly-significant positive hits to the responses of Arabidopsis cell suspensions to inhibition of the mitochondrial electron transport chain complex I (Garmier et al., 2008; Garmier et al., 2008) while wheat did not give any significant hits to this phenotype. Conversely, only wheat gave significant positive hits to low-oxygen responses of potato tubers (Geigenberger et al., 2000), castor bean (*Ricinus communis*) phloem (van Dongen et al., 2003) or the sulphur-depletion mediated hypoxia response of the *Chlamydomonas reinhardtii stm6* mutant (Timmins et al., 2009; Timmins et al., 2009) (**Supplemental Table 8**). In two cases - waterlogging of *Populus x canescens* roots (Kreuzwieser et al., 2009) and low-oxygen treatment of Arabidopsis roots (van Dongen et al., 2009) - rice gave positive hits while wheat gave inverse hits (**Supplemental Table 8**) indicating significant divergence between rice and wheat in metabolites that define these responses. Given the large difference

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that these two species display in their responses to anoxia at the metabolite level we wanted to consider whether the ability to generate a particular metabolite pool contributes to anoxia tolerance and we set out to test this hypothesis.

Amino acid-induced improvement of cell integrity in wheat under anoxia.

A range of reports in mammalian cells have highlighted that exogenous addition of glycine, serine and/or alanine can enhance the survival of cells to O_2 deprivation (Brecht and Groot, 1994; Tijsen et al., 1997; Wang et al., 2010). To test whether the differential accumulation of these amino acids could be part of plant anoxia tolerance and to define the functional importance of the divergence of rice and wheat metabolic responses to anoxia, we supplemented the media used for rice and wheat growth under anoxia. We supplemented with a combination of amino acids and assessed plant performance with the Evans blue root cell viability assay after 3 days in anoxia (**Figure 4A**). This showed that amino acid supplementation significantly increased cell viability in wheat but not in rice roots, consistent with the differential accumulation of these amino acids in rice. To confirm this finding from whole-wheat seedlings, we used measurements of electrical conductivity as a direct indicator of electrolyte leakage and thus cell integrity from anoxically-treated seedlings in the presence or absence of these three amino acids in several different combinations (**Figure 4B**). This showed that the combination of Ser/Ala/Gly significantly lowered electrolyte leakage, as did Ser/Ala, but the presence of only one of the amino acids did not protect wheat seedlings from electrolyte leakage. We also confirmed the absence of this positive effect in anoxia-tolerant rice seedlings. This suggests that the disparity between rice and wheat metabolite pool responses (**Figure 3**) may contribute to the degree of anoxia tolerance and that partial generation of these pools (via exogenous supplementation) in sensitive plants can improve cellular integrity.

DISCUSSION

We have analysed the differential responses of coleoptiles from rice and wheat seedlings to anoxia at the physiological, metabolomic and proteomic levels. Our data suggest that despite wheat having an anatomically similar coleoptile, it responds to

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anoxia to a lesser degree at a molecular level than the coleoptiles of rice. Our findings are consistent with previous reports that rice seedlings were much more tolerant to anoxia than wheat seedlings and that this involves an adaptive response (Menegus et al., 1989; Menegus et al., 1991). Our results also suggest that the changes in the capacity of metabolic pathways, via alterations in protein synthesis or degradation rates, are important at least in the rice coleoptile, for anoxia tolerance.

Transcriptional vs translational control of rice anoxia response

There is an increasing body of literature on the transcriptional response of rice to anoxia that shows great complexity in the response (Howell et al., 2007; Lasanthi-Kudahettige et al., 2007; Narsai et al., 2009) and the role of differential translation of only an active pool of RNAs during germination and anoxia further complicates its interpretation (Branco-Price et al., 2008). We have extracted published gene expression data from 4-d-old anoxic and aerobic rice seedlings (Lasanthi-Kudahettige et al., 2007), to make comparisons with our current protein profiling data. Overall, the correlation between differences in protein abundance and differences in mRNA expression in anoxically-germinated rice vs. aerated rice was poor (r^2=0.39 when comparing log_{10} ratios; **Supplemental Figure 3B**). However, there were some positive correlations observed where the direction of change in response to anoxia or aeration was the same for both the protein and its transcript (**Table 2).** For example, peroxiredoxin (Os07g44430) and protein kinases (Os08g04250; Os08g04210; Os08g04240) were highly accumulated under anoxia alongside clear up-regulation of the corresponding transcripts (**Table 2**). The Bowman-Birk type bran trypsin inhibitor (BBTI; Os01g03340) and IAA-amino acid hydrolase ILR1 (Os03g62060) were less abundant under anoxic conditions, which was in concordance with the extracted transcript data indicating their strong down-regulation (**Table 2**). Such results suggest that these particular proteins might be regulated at the transcriptional level. However, another BBTI was reported as more abundant in 6d- anoxic coleoptiles according to iTRAQ quantitation (Os01g03360) despite its transcript showing the opposite direction of change under anoxia (Table 2).

Selective mRNA translation under O_2 deficiency has been previously observed in plants (Bailey-Serres, 1999). In Arabidopsis, it was recently reported

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that selective mRNA translation coordinates “energetic and metabolic adjustments” to O_2 deficiency and recovery (Branco-Price et al., 2008). This claim was also supported by our proteomic data with accumulation of proteins from the glycolytic pathway in anoxic rice coleoptiles (**Table 2, Figure 3**). For example, fructose-bisphosphate aldolase (Os05g33380) and glyceraldehyde-3-phosphate dehydrogenase cytosolic 3 (GAPDH; Os04g40950; Os08g03290) were significantly more abundant at the protein level, while the extracted microarray data indicated that both genes were not responsive to anoxia (**Table 2**). Although, other isoforms of GAPDH (Os02g38920) showed abundance differences that were in agreement at the protein and mRNA level. This suggests that the selected translation of different mRNAs might require modification of the cytosolic ribosome. However, the actual mechanism of selective translation in plants remains unknown. Matching of the rice genes studied here to their Arabidopsis orthologues, showed no apparent correlation between the rice proteins whose abundance was not reflected in rice transcript data with evidence for ribosomal loading of orthologous mRNA under anoxia in Arabidopsis (**Supplemental Table 3**).

Alternatively, difference in protein abundance between treatments could be accounted for by alterations of the rate of synthesis and/or degradation of each protein. The abundance of cytosolic APX (Os03g17690) was significantly decreased without any apparent change in gene expression (**Table 2**), suggesting that the translation of this gene was inhibited by some downstream consequence of anoxia, or that this protein underwent selective degradation. The mechanism of selective protein degradation under anoxia also deserves further investigation. Because the wheat coleoptile proteome was largely unchanged even after 24 h of anoxia (**Figure 1C**), selective mRNA translation or protein degradation might not be occurring as frequently in this species as is apparent in rice coleoptiles. The consequence of a smaller upstream response in regulation of translation and protein turnover in wheat coleoptiles under anoxia was also reflected in metabolic and physiological responses.

Amino acid metabolism is perturbed during anoxia

The accumulation of amino acids in anoxic rice and wheat coleoptiles is consistent with well-documented observation of this phenomenon when plants are exposed to differing degrees of O_2 deprivation (Fan et al., 1997; Kato-Noguchi and

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Ohashi, 2006; Narsai et al., 2009; van Dongen et al., 2009; Rocha et al., 2010). We also detected the accumulation of enzymes involved in alanine, serine and glycine biosynthesis concomitant with the accumulation of those amino acids (**Figure 2**). The proposed benefit of accumulation of alanine under O_2 deprivation in different plant species has been discussed in detail (Gibbs and Greenway, 2003; Bailey-Serres and Voesenek, 2008). Also, Ala synthesis through AlaAT does not contribute to the oxidation of NADH as does lactate or ethanol production but rather serves as a retainable carbon source upon return to air (Good and Crosby, 1989; Miyashita et al., 2007). However, the role of glycine and serine accumulation is less clear. The transcripts for a number of these biosynthetic proteins are more abundant under anoxia ((Lasanthi-Kudahettige et al., 2007), **Table 2**) indicating that amino acid synthesis rather than protein degradation is likely to be responsible. But to our knowledge direct evidence for the benefits of feeding exogenous amino acids to seedlings growing under anoxia in an anoxia-intolerant but not an anoxia-tolerant species (**Figure 4**) has not previously been reported.

We initiated these exogenous feeding experiments on the basis of an intriguing report on the positive effects of glycine, serine and alanine on mammalian cells under hypoxic stress. Of the 23 standard amino acids tested, only glycine, L-alanine and L-serine provided significant protection from hypoxic injury of cultured hepatocytes (Brecht and Groot, 1994). Over some years hypoxic or energy deficiency injury to hepatocytes and kidney tubules have been treated with glycine as a method of cell preservation (Weinberg et al., 1991; Carini et al., 1997; Tijsen et al., 1997). Although the literature agrees that protection by glycine is not simply an enhancement of the energetic state of the hypoxic cells, the mechanism of protection is still unclear. Research favours two different mechanisms associated with the modification of the rise in intracellular Na^+ during hypoxia due to energy induced loss of Na^+-K^+-ATPase activity, indirectly via the activation of glycine receptor neurotransmitters (Carini et al., 1997), or directly by blocking non-selective sodium transport (Frank et al., 2000)

Subsequent literature examination also shows that while addition of a range of amino acids into external medium can result in cytoplasmic acidification of plant cells (Felle, 1981), the addition of alanine and serine does not acidify the cytoplasm, but instead results in a pH increase of some 0.2~0.3 units (Felle, 1996). This suggests a selective benefit of these amino acids in avoiding cytoplasmic acidification under

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anoxia. Additionally, serine is the entry point for sphingolipid biosynthesis in plants. The transcript of the gene controlling the first step of sphingolipid biosynthesis, the condensation of palmitate and serine to form 3-keto-dihydrosphingosine (serine palmitoyltransferase SPT, Os01g70370), was up-regulated 20-fold in anoxic coleoptiles (Lasanthi-Kudahettige et al., 2007) and was classified as a core anaerobic responder in germinating rice embryos (Narsai et al., 2009). Recent research in *C. elegans* (Crowder, 2009; Menuz et al., 2009) suggests that ceramides play a critical role in anoxia tolerance. The possible role of serine in ceramide biosynthesis through SPT in plant adaptation to anoxia deserves further investigation to identify novel mechanisms conferring anoxia tolerance.

Hence there are a range of possible explanations for the beneficial effects of combinations of alanine/serine/glycine on plant cell anoxia tolerance through retention of carbon skeletons, modification of biosynthetic processes and cellular ion balance.

Conclusion:

In summary, our study reinforced the importance of glycolysis and ethanolic fermentation in the adaptation to anaerobiosis and suggests that glycolysis might also be important in providing substrates for amino acid synthesis. Rice, but not wheat coleoptiles responded to anoxia dramatically at the physiological, proteomic and metabolomic levels in concordance with the respective tolerance and intolerance of these species to anoxia. Further investigation into the role of machinery differences in selected mRNA translation and/or protein turnover between rice and wheat coleoptiles is needed based on the targets identified here. We provide novel protein and metabolite evidence of the enhancement of serine/glycine biosynthesis as well as support observations that alanine accumulates in anoxic rice. We also show a benefit for wheat by exogenous application of these amino acids and highlight a range of mechanisms that could be responsible for conferring anoxia tolerance.

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Methods

Plant Material

Dehulled rice (*Oryza sativa L. cv* Amaroo) and wheat seeds (*Triticum aestivum L. cv* Calingiri) were surface sterilised for 10 min using 50 % [v/v] NaOCl and then thoroughly rinsed with ddH_2O. Fifty to 75 seeds were placed in conical flasks containing 250 mL culture medium (0.5 mM MES, 0.4 mM $CaSO_4$, pH 6.5) and bubbled with air or N_2 (6-7 L min^{-1}). Plastic tubing delivered the gas to the seeds and the system was sealed using parafilm and aluminium foil. Seedlings were grown in the dark at 30˚ C for (1) 4 days under aeration, (2) 4 days under aeration with an additional 1 day switch to anoxia or (3) 6 days anoxia. To ensure that stress conditions were anoxic, O_2 concentrations were monitored and after 10 min of N_2 bubbling the O_2 concentration was below the level of detection. This was measured using the LabQuest® Vernier O_2 meter with a sensitivity of 0.01 % O_2. Rice seed was kindly provided by the New South Wales Department of Primary Industries (Yanco, NSW), and wheat seed by the Western Australian Department of Agriculture and Food (Perth, WA)

Evans blue viability stain

This protocol was adapted from the method described by Baker and Mock (Baker and Mock, 1994). Fresh tissues were excised from seedlings, weighed out (0.05-0.1 g) and placed in a 10 mL Falcon tube containing 100 µL ddH_2O. 2 mL of 0.25 % [w/v] Evans blue was added to each sample and horizontally-laying tubes were shaken at room temperature for 20 min at 300 rpm. The stain was rinsed from tissues in a sieve until the water ran clear, snap frozen and then ground in a 2 mL eppendorf microfuge tube containing a carborundum ball for 3 min at 17 shakes/s. 0.5 mL of 1 % [w/v] SDS was added and samples ground for 3 min again. 1 mL ddH_2O was added to samples, which were then centrifuged at 8800 x g for 3 min. The absorbance of diluted supernatants (1:3) were measured at 600 nm. The average masses of tissue samples (0.075 g) were used to normalise absorbance measurements so that different samples could be compared (n=3).

Measurement of electrical conductivity

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We measured electrical conductivity (EC) as an estimation of membrane integrity (Yan et al., 1996). This was done in whole rice and wheat seedlings germinated and grown for 4 days in aeration and subsequently transferred to anoxia or air for 3 days in fresh culture media (0.5 mM MES, 0.4 mM $CaSO_4$, pH 6.5). Some samples were supplemented with the amino acids L-alanine, L-serine and/or glycine at 10mM concentrations. Seedlings were then washed with dH_2O, patted dry with tissue paper and placed in 15 mL falcon tubes containing 10 mL ddH_2O for one hour at 19.5˚ C. The EC of these solutions were measured (C_1, TPS Aqua-C conductivity-TDS-temperature meter). Samples were then microwaved for 2 minutes. Care was taken to ensure boiling of each sample. After one hour the EC was measured again (C_2) at 19.5˚ C and used as the denominator in the calculation of percent electrolyte leakage.

ADH activity

Measurement of ADH activity was performed as described by Waters et al. (Waters et al., 1991), briefly protein was extracted by grinding ~70 mg snap-frozen plant tissue with acid-washed sand and 1 mL extraction buffer at (125 mM MES, 110 mM NaCl, 1 mM EDTA, 0.5 mM TPP, 2.5 mM $MgSO_2$ and freshly added DTT at 2 mM, pH 6.8). After centrifugation at 10,000 g for 4 min at 4˚ C the supernatant was removed ready for analysis. The ADH activity was measured at 340 nm in a 1 mL cuvette in reaction media (10 mM acetaldehyde, 50 mM TES, 0.17 mM NADH, pH 7.5).

Carbohydrate measurement

Carbohydrate levels were measured using a modified method (Trevelyan and Harrison, 1952). Tissue extracts were prepared by heating 20 mg samples submerged in 2 mL of 80 % ethanol at 70˚ C for 20 min in a tightly sealed tube. Extracts were then removed from tissue and 100 µL of extract was added into 1 mL of freshly prepared anthrone reagent (mixture of 0.2 g anthrone to 100 mL of 70 % [v/v] sulphuric acid). After vortexing, samples were boiled at 100˚ C for 10 min exactly and promptly placed in an icy water bath for 5 min and then removed and stored at RT for 5 min before measuring absorbance at 627 nm.

O_2 uptake measurements

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O_2 uptake measurements followed a procedure described previously (Lee et al., 2008) using a computer-controlled Clark-type O_2 electrode unit. Slight modifications include the use of ~90 mg fresh tissue and 2 mL of O_2-saturated buffer composed of 5 mM KH_2PO_4, 10 mM TES, 10 mM NaCl, 2 mM $MgSO_4$, pH 7.2.

Protein purification

Snap-frozen coleoptiles were ground with acid-washed sand and a solubilising solution (7 % [w/v] SDS, 125 mM Tris-HCl and 10 % [w/v] β-mercaptoethanol at a 5:8 [w/v] ratio, pH 7). Protein purification was carried out using the chloroform-methanol method (Wessel and Flügge, 1984). Protein pellets were then incubated with 80% acetone for 1 h at -20˚ C. The solution was centrifuged at 14,000 rpm for 10 min at 4˚ C and the pellets were air-dried. Protein was re-solubilised with rehydration buffer (RB; 6 M urea, 2 M thiourea, 2 % [w/v] CHAPS, 2 % IPG buffer [v/v] and 18 mM DTT) for preparative gels or lysis buffer (LB; 6 M urea, 2 M thiourea, 2 % [w/v] CHAPS and 40 mM Tris) for DIGE gels by shaking in an orbital rocker at 1400 rpm at 25˚ C for 45 min. Centrifugation at 20, 000 x g for 15 min was then carried out. Protein was quantified using 2D Quant Kit (GE Healthcare).

IEF/SDS-PAGE gel separations

For preparative gels, 800 μg of protein re-solubilised in RB containing equal amounts of both samples was loaded onto IEF strips (3-10 NL, 3-11 NL, 24 cm, GE Healthcare) and separated for 24 h (Up to 50 μA/strip, 5 W, 21˚ C. Six-step program parameters were: 30 V for 12h step and hold (stp), 500V for 1h stp, 1000V for 1h gradient (grd), 3000 V for 2h grd, 8000 V for 2h grd and 8000 V for 6h stp). IEF strips were then dipped in 1 X gel buffer and placed on top of a 12 % acrylamide gel and run at 45 mA / gel for 6-7 h. For DIGE gels (Eubel et al., 2007), 50 μg of treated, control and a 1:1 internal standard mixture of the above mentioned coleoptile proteins were labelled separately with 400 μM of fluorescent CyDye. The labelling procedure was carried out according to the recommendations of GE Healthcare. Samples were pooled and separated in the manner that preparative gels were. DIGE gels were scanned using a Typhoon™ laser scanner (GE Healthcare) and quantitative analysis carried out using DeCyder™ software package (v 6.5, GE Healthcare). Three independent dye-swapping replicates were carried out. Statistically significant spots were selected for MS identification according to their

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appearance in 9 / 9 fluorescent images, a ratio of abundance difference ≥ 2 and a p-value ≤ 0.05. Both DIGE and preparative gels were Coomassie stained and de-stained.

Protein identification using mass spectrometry

Selected protein spots were excised from gels and digested in-gel as described previously (Taylor et al., 2005). Vacuum dried samples were re-dissolved in 5 % [v/v] acetonitrile and 0.1 % [v/v] formic acid for analysis on an Agilent XCT Ultra Ion Trap mass spectrometer (Agilent Technologies) and MS/MS spectra exported for data analysis. MS spectra were examined against an in-house rice database of the TIGR Rice Pseudomolecules and Genome Annotation and mitochondrial and plastid protein sets (Rice v6) using version 2.2.03 (Matrix Science) of the Mascot search engine. The following settings were selected for searching; MS error tolerance of ±1.2 Da, MS/MS error tolerance of ±0.6 Da, maximum missed cleavages tolerated as 1, two variable modifications including Carbamidomethyl (C) and Oxidation (M), peptide charge as 2^+ and 3^+ and finally, the instrument selected as ESI-TRAP. After results were retrieved from the Mascot search engine, 'Require bold red' and 'Standard scoring' checkboxes were selected with the ion score cut-off set at 37, the 'Significance threshold' set at p<0.05 and the 'Max. number of hits' set at AUTO. MS spectra files are available for analysis via the Proteome Commons Tranche Project under hash Wiq7A0erU/p/zv9lJSKf+5pyjbFZXnERbYRQYwrglOB3N/kmT/Sp9qJ4ksGmr9J76Am ipUl3xMLTO1I07LFWa19V0K8AAAAAAAACEg==

Metabolite extraction, GC-MS sample and data analysis

Metabolite extraction from coleoptiles followed a modified procedure described previously (Howell et al., 2009). Tubes containing ground tissue samples (100 mg of stressed or non-stressed coleoptiles) and grinding balls (cooled) were placed in a liquid N_2-cooled solid rack. 0.5 mL of cold Metabolite Extraction Medium (85 % [w/v] HPLC-grade methanol, 15 % [w/v] untreated MilliQ water and 100 ng μL^{-1} ribitol) was added to each tube, immediately vortexed and then shaken at 1400 rpm for 20 min at 65˚ C. To pellet cell debris, samples were centrifuged at 20 000 x g for 10 min. 60 µL aliquots of extract were dried down in a vacuum centrifuge for approximately 2 h. 20 µL of 20 mg mL^{-1} methoxylamine.HCl (98 % purity, Sigma) was added to each

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of the dried samples. Samples were then shaken at 1400 rpm for 90 min at 30˚ C. To each sample, 30 µL of *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (derivatisation grade, Sigma) was added followed by shaking again at 1400 rpm for 30 min at 37˚ C. After this, 10 µL *n*-alkane standard mix (0.029 % [v/v] *n*-dodecane, 0.029 % [v/v] *n*-pentadecane, 0.029 % [w/v] *n*-nonadecane, 0.029 % [w/v] *n*-docosane, 0.029 % [w/v] *n*-octacosane, 0.029 % [w/v] *n*-dotriacontane, and 0.029 % [w/v] *n*-hexatriacontane dissolved in anhydrous pyridine) was added and vortexed. Samples were transferred to GC-MS amber vials with screw-top seals and low-volume inserts (Agilent Technologies). These were then incubated for 4 h at room temperature for equilibration. Analysis of samples on the GC-MS followed the procedure described previously (Howell et al., 2009). GC/MS data were collected using Chemstation GC/MSD Data Analysis Software (Agilent Technologies). Raw GC-MS data pre-processing and statistical analysis were performed using METABOLOME-EXPRESS software (version 1.0, http://www.metabolome-express.org). Detailed methods have been reported (Carroll et al., 2010).

Metabolic phenocopy analysis using the MetabolomeExpress PhenoMeter

To systematically characterise relationships between the metabolic phenotypes observed in this study and metabolic phenotypes reported in previous studies, we used the *PhenoMeter* tool of *MetabolomeExpress* (https://www.metabolome-express.org) to search the *MetabolomeExpress* metabolic phenotype database, *MetaPhenDB*, for metabolic phenotypes having statistically significant qualitative overlap with the responses observed in this study (submitted as a batch of 'bait' responses). The *PhenoMeter* uses the following procedure for each bait response. The bait response is compared with each and every reference response in *MetaPhenDB*, one at a time. Each comparison is done by first counting: 1) the number of metabolites increased (ie. having a fold-change > 1) in both bait and reference; 2) the number of metabolites decreased in both bait and reference; 3) the number of metabolites increased in bait but decreased in reference; and 4) the number of metabolites decreased in bait but increased in reference. These counts were then used as input in a two-tailed Fisher's Exact test to calculate the p-value indicating the probability of obtaining the observed positive (co-directional response) or negative (inverse) response overlap by chance alone. A p-value of >0.05 was used to indicate statistically significant overlaps or inverse overlaps. To minimise

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biases caused by the presence of different sets of 'unknown' metabolites in metabolic phenotypes acquired from different studies, only metabolites of known structure (and hence having the same name in each study) were considered in comparisons. Only metabolites present in both bait and reference were considered. So that the anoxia responses observed in this study could be compared with previously published plant responses to anoxia and hypoxia, we added the complete set of 36 metabolic phenotypes associated with 7 peer-reviewed publications (Geigenberger et al., 2000; Geigenberger et al., 2000; Gibon et al., 2002; Gibon et al., 2002; van Dongen et al., 2003; van Dongen et al., 2003; Branco-Price et al., 2008; Branco-Price et al., 2008; Timmins et al., 2009; Timmins et al., 2009; van Dongen et al., 2009; van Dongen et al., 2009; Rocha et al., 2010; Rocha et al., 2010) from other groups presented in a recent review (Narsai et al., 2010) of the topic to *MetaPhenDB* prior to *PhenoMeter* analysis. At the time of analysis, *MetaPhenDB* contained 12379 publicly-available metabolite response statistics representing 116 metabolic phenotypes including oxygen-deprivation related metabolic phenotypes for a total of six plant species (*Arabidopsis thaliana*, *Solanum tuberosum*, *Lotus japonicus*, *Populus x canescens*, *Ricinus communis* and *Oryza sativa*) in addition to metabolic phenotypes associated with a wide variety of other environmental, developmental and genetic perturbations.

ITRAQ analysis

Total protein extracts were prepared, grinding the tissue in a Retch homogeniser and solubilisation with 2D lysis buffer (8M urea, 2% (w/v) CHAPS, 22mM DTT, 40mM Tris). The homogenate was vortexed for 5 min and then centrifuged at 15,000 rpm (x *g*) for 15 min at 4°C. The supernatant was collected and the concentration of total protein was determined using a reducing-agent compatible BCA assay (Pierce). For each sample, a total of 100 µg of protein was precipitated by the addition of four volumes of cold acetone and stored in -20°C overnight. The precipitated protein was then resuspended in dissolution buffer, denatured, and cysteines blocked according to the manufacturer's instructions (AB Sciex). Each sample was then digested with 20 µL of 0.25µg/µL trypsin (Invitrogen) at 37°C overnight and labelled with the iTRAQ tags in triplicate. iTRAQ reagents were resuspended in 50 µl of 2-propanol and added to each sample, pH adjusted and allowed to incubate at RT for 2 hours. The labelled samples were pooled prior to further analysis. To remove excess

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labelling reactants and to reduce interference of salts during LC-MS/MS analysis, the pooled samples were diluted 4-fold with SCX buffer A (10mM KH_2PO_4 in 25% acetonitrile at pH 3.0) and subjected to strong cation exchange (SCX) chromatography using an OPTI-LYNX cartridge (Optimize Technologies). The eluent was dried in a vacuum concentrator and stored at -20°C for LC-MS/MS analysis.

Samples were analysed on an Agilent 6510 Q-TOF mass spectrometer with an HPLC Chip Cube source. The Chip consisted of a 160 nl enrichment column (Zorbax 300SB-C18 5μm) and a 150 mm separation column (Zorbax 300SB-C18 5μm) driven by a Agilent Technologies 1100 series nano/capillary liquid chromatography system. Peptides were loaded onto the trapping column at 4 μl/min in 5% (v/v) acetonitrile and 0.1% (v/v) formic acid with the chip switched to enrichment and using the capillary pump. The chip was then switched to separation and peptides were eluted during a 1 hour gradient (5% (v/v) acetonitrile – 60% (v/v) acetonitrile) using the nano pump at 300 nl/min, directly into the mass spectrometer. The Q-TOF was run in positive ion mode and MS scans run over a range of *m/z* 275-1500 and at 4 spectra/s. Precursor ions were selected for autoMS/MS at an absolute threshold of 500 and a relative threshold of 0.01, with max 3 precursors per cycle, and active exclusion set at 2 spectra and released after 1 min. Precursor charge-state selection and preference was set to $[M+H]^{2+}$ and then $[M+H]^{3+}$ and precursors selected by charge then abundance. Resulting MS/MS spectra were searched against the TIGR Rice Pseudomolecules and Genome Annotation and mitochondrial and plastid protein sets (Rice_osa6) using version 2.2.03 (Matrix Science) of the Mascot search engine. The following settings were selected for database searching; MS error tolerance of ±100 ppm, MS/MS error tolerance of ±0.5 Da, maximum missed cleavages tolerated as 1; fixed modifications methylthio (C), iTRAQ8plex (N-term), iTRAQ8plex (K) and variable modifications carbamidomethyl (C) and oxidation (M) iTRAQ8plex (Q), peptide charge as ≥ 2+ and finally, the instrument selected as ESI-Q-TOF. The resulting searches were then exported and all peptides identified ($p<0.05$) were extracted to create an exclusion list for the subsequent run. All 5 runs were performed and combined using mzdata Combinator v.1.0.4 (The West Australian Centre of Excellence in Computational Systems Biology) for database searching as outlined below. MS spectra files are available for analysis via the Proteome Commons Tranche Project under hash

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5AJQpzyi1I5adgPNIGdJ+oQf8nlIoXnjLVhePv9x39srDtRpuZe9gQu9ij62NLKetNEdx6t1M
qirlvSglVAAUHcQZAYAAAAAAAABcA==

Quantitation was carried out using default settings in Mascot v.2.2.03 (Matrix Science) for protein identifications as outlined above and quantitation on isobaric mass tags (iTRAQ) at the peptide level. In more detail, ratios for individual peptide matches are obtained from peptides meeting the minimum criteria outlined above and are then combined to determine ratios for proteins hits using a weighted average. Outlier removal was carried out by Dixon’s method for up to 25 data points per protein or by Rosner’s method where >25 data points were present and normalization carried out by median ratio. Values are reported as geometric mean (geometric standard deviation) and those significantly different from 1 at a 95% confidence interval are marked (*) (**Supplemental table 1**). For proteins reported to have a non-normal distribution, the geometric standard deviation was determined manually. Here a geometric mean for the individual peptide ratios and a 95% CI window was calculated as a *t*-test in Analyse-it v.2.21.

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Table 1 Growth, sugar concentration, O_2 consumption rate and Alcohol Dehydrogenase (ADH) activity of coleoptiles from rice and wheat seedlings exposed to aeration or anoxia.

Parameters	**Treatments**	**Rice**			**Wheat**		
		Coleoptiles	Leaves	Roots	Coleoptiles	Leaves	Roots
Growth (mm)	4 d Air	11.9 ± 0.9	--	--	28.1 ± 2.7	--	--
	4 d Air + 1 d N_2	14.2 ± 1.0*	--	--	26.2 ± 1.5	--	--
	6 d N_2	7.1 ± 0.7*	--	--	--	--	--
	6 d N_2 + 1 d Air	9.7 ± 0.7^	--	--	--	--	--
Sugar concentration (mg hexose g^{-1} FW)	4 d Air	44.8 ± 1.4	55.8 ± 2.1	28.0 ± 2.8	37.0 ± 0.7	64.9 ± 2.5	12.4 ± 1.2
	4 d Air + 1 d N_2	21.6 ± 0.9*	31.3 ± 0.7*	13.5 ± 0.7*	26.4 ± 1.2*	40.4 ± 1.0*	3.4 ± 0.3*
	6 d N_2	3.5 ± 0.2*	--	--	--	--	--
	6 d N_2 + 1 d Air	7.7 ± 1.1^	--	--	--	--	--
O_2 consumption rate (nmol min g^{-1} FW)	4 d Air	126.3 ± 5.1	302.2 ± 10.8	398.8 ± 20.2	140.6 ± 6.0	478.8 ± 36.2	436.8 ± 14.4
	4 d Air + 1 d N_2	104.7 ± 2.8*	132.7 ± 20.0*	82.1 ± 1.2*	146.1 ± 8.9	336.7 ± 10.1*	177.3 ± 1.0*
	6 d N_2	67.4 ± 6.0*	--	--	--	--	--
	6 d N_2 + 1 d Air	134.0 ± 11.8^	--	--	--	--	--
ADH activity (unit mg^{-1} protein)	4 d Air	0.75 ± 0.06	0.94 ± 0.07	0.94 ± 0.05	0.25 ± 0.01	0.09 ± 0.01	0.32 ± 0.02
	4 d Air + 1 d N_2	2.68 ± 0.13*	3.42 ± 0.04*	5.22 ± 0.70*	0.59 ± 0.01*	0.28 ± 0.01*	1.08 ± 0.01*
	6 d N_2	6.61 ± 0.48*	--	--	--	--	--
	6 d N_2 + 1 d Air	4.38 ± 0.24^	--	--	--	--	--

* indicates that the p-value of continuously anoxic/anoxically-switched samples is <0.05 when compared with continuously aerated samples

^ indicates that the p-value of aerobically-switched samples is <0.05 when compared with continuously anoxic samples

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Table 1: Growth, sugar concentration, O_2 consumption rate and Alcohol Dehydrogenase (ADH) activity of coleoptiles from rice and wheat seedlings exposed to aeration or anoxia. Rice seeds were germinated and grown under aeration for 4 days (control), anoxia for 6 days (anoxically-germinated; 6 d N_2) or 4 days in aeration followed by 1 day of anoxia (anoxically-switched; 4 d Air + 1 d N_2). Wheat seeds were treated with control conditions or were anoxically-switched.

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			DiGE: 3-10 NL			DiGE: 3-11 NL			DiGE: 3-10 NL			iTRAQ		Array
			6d N_2 / 4d Air			6d N_2 / 4d Air			4d Air 1d N_2 / 4d Air			6d N_2 / 4d Air		4d N_2 / 4d Air
Functional Category	*Os* Gene No.	Protein ID	Spot	FAD	p-val	Spot	FAD	p-val	Spot	FAD	p-val	TGM	Sig.	FAD
Sugar Metabolism, Glycolysis, Fermentation & TCA Cycle	Os03g28330.1	sucrose synthase	-	X	X	-	X	X	-	X	X	**1.6**	S	**2**
	Os06g09450.1	sucrose synthase	-	X	X	-	X	X	-	X	X	**1.7**	S	**2**
	Os01g60190.1	iPGAM	31	**3.3**	**4.E-04**	20	**5.7**	**5.E-04**	27	1.3	1.E-01	-1.0	N/S	**2**
	Os01g60190.1	iPGAM	32	**4.3**	**4.E-06**	21	**6.1**	**3.E-05**	26	**1.5**	**9.E-03**	-1.0	N/S	**2**
	Os10g08550.1	enolase	33	**7.5**	**5.E-09**	18	**5.7**	**9.E-04**	28	**2.4**	**7.E-04**	**1.2**	S	**2**
	Os10g08550.1	enolase	34	**2.2**	**3.E-05**	19	**2.8**	**2.E-03**	29	**1.5**	**8.E-04**	**1.2**	S	**2**
	Os08g02120.1	fructokinase-2	-	X	X	-	X	X	-	X	X	***-2.2***	S	**-2**
	Os05g33380.1	aldolase	16	**4.7**	**5.E-06**	-	X	X	5	**2.1**	**3.E-04**	**2.0**	S	N/S
	Os01g67860.1	aldolase	-	X	X	-	X	X	-	X	X	**2.2**	S	N/S
	Os10g08022.1	aldolase	-	X	X	-	X	X	-	X	X	**2.3**	S	X
	Os04g40950.1	GAPDH	6	**2.2**	**7.E-04**	6	**2.4**	**2.E-03**	4	1.1	8.E-02	**1.4**	S	N/S
	Os04g40950.1	GAPDH	10	**3.6**	**7.E-05**	-	X	X	11	1.0	7.E-01	**1.4**	S	N/S
	Os04g40950.1	GAPDH	-	X	X	8	**2.6**	**1.E-03**	-	X	X	**1.4**	S	N/S
	Os02g38920.1	GAPDH	18	**13.2**	**5.E-06**	-	X	X	9	**1.7**	**2.E-03**	**1.8**	S	**3**
	Os02g38920.1	GAPDH	17	**6.7**	**4.E-08**	-	X	X	8	**1.7**	**1.E-02**	**1.8**	S	**3**
	Os08g03290.1	GAPDH	15	**2.5**	**6.E-04**	-	X	X	6	**1.2**	**4.E-02**	X	X	N/S
	Os08g03290.2	GAPDH	-	X	X	-	X	X	-	X	X	**1.3**	S	N/S
	Os03g50480.1	phosphoglucomutase	14	**6.3**	**2.E-05**	-	X	X	25	**2.7**	**2.E-03**	***-1.1***	S	N/S
	Os02g07260.1	phosphoglycerate kinase	28	**5.3**	**1.E-04**	-	X	X	31	**2.4**	**1.E-02**	**1.2**	S	**2**
	Os01g05490.1	triosephosphate isomerase	40	**2.9**	**5.E-05**	-	X	X	36	**1.2**	**9.E-03**	**1.4**	S	**4** & N/S
	Os06g13810.1	PPi-phosphofructokinase	23	**2.8**	**2.E-02**	-	X	X	19	**2.1**	**9.E-04**	**1.4**	S	**2**
	Os05g39310.1	pyruvate decarboxylase 1	-	X	X	-	X	X	-	X	X	**2.0**	S	**594**
	Os11g10480.1	alcohol dehydrogenase 1	12	**6.0**	**2.E-05**	-	X	X	12	**3.6**	**4.E-05**	**2.2**	S	**4**
	Os01g46070.1	malate dehydrogenase	8	***-1.5***	***2.E-02***	-	X	X	2	***-1.3***	***2.E-03***	***-1.1***	S	N/S
Amino Acid Synthesis	Os02g50240.1	glutamine synthetase	38	***-2.3***	***7.E-05***	-	X	X	34	***-1.3***	***3.E-04***	***-2.3***	N/S	**-2**
	Os12g42876.1	MetSyn	-	X	X	-	X	X	-	X	X	***-1.7***	S	X
	Os12g42884.1	MetSyn	-	X	X	-	X	X	-	X	X	***-1.7***	S	**-2** & N/S
	Os12g42876.1	MetSyn	25	**2.3**	**2.E-03**	-	X	X	17	**3.3**	**2.E-03**	X	X	X
	Os12g42876.1	MetSyn	26	1.1	6.E-01	-	X	X	18	**2.7**	**3.E-03**	X	X	X
	Os03g06200.1	PSAT	5	**4.7**	**9.E-07**	5	**6.5**	**1.E-03**	-	X	X	1.7	N/S	**5**
	Os03g06200.1	PSAT	-	X	X	9	**5.6**	**1.E-03**	-	X	X	1.7	N/S	**5**
	Os12g22030.1	SHMT	19	1.6	9.E-02	-	X	X	14	**2.0**	**4.E-04**	X	X	N/S
	Os04g55720.1	3-PGDH	24	**5.8**	**2.E-05**	-	X	X	20	**2.0**	**1.E-03**	**1.9**	S	**4**
	Os10g25130.1	alanine aminotransferase	-	X	X	15	**2.5**	**4.E-03**	-	X	X	X	X	N/S
Stress Responsive	Os07g44430.1	peroxiredoxin	4	**5.5**	**6.E-05**	4	**11.0**	**5.E-05**	-	X	X	**2.5**	S	**32**
	Os07g44430.1	peroxiredoxin	3	**3.4**	**2.E-03**	3	**22.7**	**1.E-06**	-	X	X	**2.5**	S	**32**
	Os05g25850.1	Mn superoxide dismutase	1	**1.6**	**1.E-02**	1	**1.9**	**1.E-02**	-	X	X	1.4	N/S	N/S

	Os03g17690.1	ascorbate peroxidase	39	***-4.6***	***3.E-06***	-	X	X	37	***-2.3***	***1.E-04***	***-1.8***	S	N/S
	Os07g49400.1	ascorbate peroxidase	-	X	X	-	X	X	-	X	X	***-1.9***	S	N/S
	Os03g07180.1	embryotic protein DC-8	-	X	X	10	**3.5**	**4.E-05**	-	X	X	X	N/S	X
	Os03g07180.1	embryotic protein DC-8	-	X	X	11	**3.9**	**3.E-05**	-	X	X	X	N/S	X
	Os03g07180.1	embryotic protein DC-8	-	X	X	12	**3.6**	**4.E-05**	-	X	X	X	N/S	X
	Os03g07180.1	embryotic protein DC-8	-	X	X	13	**4.7**	**1.E-04**	-	X	X	X	N/S	X
	Os05g46480.1	LEA gp 3	2	**4.1**	**7.E-07**	2	**7.1**	**2.E-04**	-	X	X	X	X	***-2***
	Os02g15250.1	LEA domain-containing	-	X	X	14	**5.9**	**8.E-05**	-	X	X	-	X	X
Translation	Os03g14530.1	S10/S20 ribosomal protein	-	X	X	-	X	X	-	X	X	**1.3**	S	**2**
	Os11g29190.1	40S ribosomal protein S5	-	X	X	-	X	X	-	X	X	***-1.3***	S	**2**
	Os03g08010.1	elongation factor 1-alpha	-	X	X	-	X	X	-	X	X	**1.3**	S	N/S
	Os01g52470.1	elongation factor	-	X	X	-	X	X	-	X	X	**1.4**	S	X
	Os02g32030.1	elongation factor	-	X	X	-	X	X	-	X	X	***-1.1***	S	N/S
Miscellaneous	Os08g04210.1	protein kinase	-	X	X	-	X	X	-	X	X	**3.2**	S	**1007**
	Os08g04250.1	protein kinase	-	X	X	-	X	X	-	X	X	**2.3**	S	**248**
	Os08g04240.1	protein kinase	-	X	X	-	X	X	-	X	X	**2.8**	S	**525**
	Os04g56430.1	CRK5	-	X	X	-	X	X	-	X	X	***-1.9***	S	**3**
	Os01g03340.1	BBTI4	11	***-6.0***	***2.E-06***	-	X	X	-	X	X	***-1.9***	S	***-3 & -2***
	Os01g03360.1	BBTI5	-	X	X	-	X	X	-	X	X	**1.1**	S	***-2***
	Os03g62060.1	IAA-amino acid hydrolase	36	***-5.3***	***3.E-04***	17	***-4.6***	***6.E-03***	32	-1.0	4.E-01	***-3.0***	S	***-271***
	Os05g04510.1	S-adenosyl-Met synthetase	-	X	X	-	X	X	-	X	X	***-1.6***	S	N/S

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Table 2: Proteomic analysis of rice coleoptiles in response to anoxia or switch from air to anoxia. Proteins selected from DIGE corresponding to Fig. 1A and 1B and iTRAQ (**Supplemental Table 2**) were identified by MS/MS (Protein ID) with corresponding *Oryza sativa* gene numbers (*Os* gene no.). Details of the matched protein size, number of peptides identified and percentage coverage are shown in **Supplemental Table 1**. Transcript abundance differences between anoxic (4d) and aerated (4d) rice coleoptiles derived from independent microarray data (Lasanthi-Kudahettige et al., 2007) have been incorporated (significant increase (positive, bold), significant decrease (negative, bold italics)). Protein spots chosen for MS/MS analysis met the following criteria in at least one analysis: a protein abundance difference ≥ 1.5 where proteins were higher in anoxic samples (positive, bold) and those higher in aeration (negative, bold italics), $p < 0.05$ and an abundance high enough in preparative gels for subsequent MS identification. *Abbreviations*- Os, *Oryza sativa*; FAD, fold abundance difference; TGM, transformed geometric mean; Sig., significantly different from 1 or not; S, significant; N/S, not significant; X, no data; iPGAM, 2,3-bisphosphoglycerate-independent phosphoglycerate mutase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MetSyn, cobalamin-independent methionine synthase; PSAT, phosphoserine aminotransferase; SHMT, serine hydroxymethyltransferase; 3-PGDH, D-3-phosphoglycerate dehydrogenase; LEA, late embryogenesis abundant; BBTI, Bowman-Birk type bran trypsin inhibitor precursor.

		6d N_2 / 4d air		4d Air + 1d N_2 / 4d Air				4d Air + 4h N_2 / 4d Air			
		Rice		Rice		Wheat		Rice		Wheat	
	Metabolite	RV	p-val	RV	p-val	RV	p-val	RV	p-val	RV	p-val
Amino acids & polyamines	L-proline	**98.6**	**5.8E-06**	**8.7**	**2.5E-02**	**2.6**	**1.7E-03**	0.5	4.9E-01	0.5	1.4E-01
	L-leucine	**43.8**	**1.1E-05**	**11.6**	**1.7E-03**	0.6	6.6E-02	0.6	4.6E-01	0.1	6.0E-01
	β-alanine	**43.4**	**1.4E-08**	0.7	2.6E-01	**1.0**	**3.3E-03**	0.9	4.4E-01	**0.9**	**4.9E-04**
	L-alanine	**42.6**	**2.3E-06**	**43.2**	**8.1E-04**	0.1	6.3E-01	**6.9**	**1.5E-03**	0.5	3.5E-01
	L-glutamic acid	**36.5**	**1.7E-06**	-11.7	6.9E-02	-0.9	5.8E-02	-39.2	7.7E-02	-0.4	2.8E-01
	L-tyrosine	**22.5**	**4.4E-06**	***-1.2***	***4.3E-03***	0.4	2.7E-01	-2.3	9.6E-02	0.2	3.0E-01
	2-aminoadipic acid	**17.3**	**6.6E-07**	-1.0	8.1E-02	-0.1	7.4E-01	0.1	7.4E-01	-0.4	1.6E-01
	L-arginine	**15.6**	**1.2E-05**	***-0.7***	***2.7E-03***	0.7	1.0E-01	0.0	9.6E-01	0.1	5.7E-01
	L-arginine	**15.6**	**1.2E-05**	***-0.7***	***2.7E-03***	0.7	1.0E-01	0.0	9.6E-01	0.1	5.7E-01
	L-homoserine	**15.5**	**1.9E-02**	***-0.9***	***1.0E-02***	0.6	3.2E-01	-0.9	9.6E-02	-0.2	3.2E-01
	L-isoleucine	**12.0**	**3.2E-05**	**5.2**	**6.6E-04**	0.7	1.1E-01	0.3	4.7E-01	0.3	3.3E-01
	L-lysine	**10.9**	**3.6E-05**	***-2.0***	***3.1E-03***	**1.5**	**3.5E-02**	-1.8	1.1E-01	0.5	1.0E-01
	L-valine	**10.2**	**5.8E-07**	**5.5**	**3.4E-07**	-0.1	6.6E-01	-0.1	8.8E-01	0.3	4.2E-01
	L-serine	**9.0**	**5.9E-12**	**2.0**	**2.3E-03**	***-0.8***	***4.4E-03***	0.1	8.3E-01	-0.4	4.2E-01
	γ-aminobutyric acid	**8.9**	**1.5E-08**	**8.1**	**1.2E-03**	**0.4**	**3.5E-03**	2.3	1.4E-01	**0.6**	**8.6E-04**
	L-methionine	**6.0**	**2.8E-02**	***-1.9***	***3.9E-02***	0.6	1.4E-01	-123.6	5.4E-02	-0.2	4.4E-01
	L-tryptophan	**5.2**	**1.8E-05**	**4.6**	**1.7E-02**	0.6	1.9E-01	-0.4	3.5E-01	0.5	8.8E-02
	L-threonine	**2.4**	**1.1E-06**	**2.1**	**6.4E-06**	0.1	4.3E-01	0.6	1.4E-01	0.5	1.2E-01
	glycine	**1.9**	**2.5E-03**	**3.7**	**1.2E-03**	**1.4**	**4.4E-03**	0.6	1.9E-01	0.4	2.0E-01
	L-glutamine	**1.8**	**4.6E-04**	***-0.5***	***4.4E-03***	-1.4	9.0E-02	-0.2	2.3E-01	***-1.5***	***2.0E-03***
	4-hydroxyproline	**1.4**	**1.1E-06**	-0.1	4.9E-01	-0.1	4.9E-01	0.1	5.0E-01	***-0.9***	***2.2E-02***
	ornithine	**0.6**	**3.5E-03**	***-1.3***	***2.8E-04***	0.4	1.8E-01	***-10.0***	***4.3E-02***	-0.9	1.2E-01
	L-asparagine	-0.6	4.5E-01	0.0	9.7E-01	-0.2	5.9E-01	***-40.0***	***4.7E-02***	0.1	6.1E-01
	L-aspartic acid	-1.5	1.5E-01	-16.6	2.2E-01	***-1.7***	***3.7E-02***	***-448.4***	***4.7E-02***	***-3.2***	***1.2E-03***
	L-phenylalanine	N/D	N/D	-2.1	1.5E-01	**0.9**	**5.3E-03**	-0.2	2.1E-01	0.4	6.6E-02
	L-α-aminobutyric acid	N/D	N/D	**2.5**	**1.5E-04**	-2.7	2.7E-01	0.2	4.6E-01	***-1.2***	***2.4E-03***
	putrescine	**52.4**	**6.9E-08**	**7.9**	**1.1E-04**	**1.7**	**4.2E-03**	0.2	5.9E-01	-0.2	1.5E-01
Sugars & glycolytic substrates	6-phosphogluconate	**9.7**	**1.0E-03**	-0.8	3.6E-01	-1.6	1.1E-01	***-1.4***	***3.9E-02***	***-1.0***	***1.0E-02***
	D-ribose	***-0.8***	***9.2E-05***	***-1.2***	***9.2E-04***	***-1.3***	***3.1E-03***	***-0.5***	***2.9E-04***	***-0.6***	***1.3E-02***
	trehalose	***-1.0***	***2.3E-02***	***-2.1***	***3.1E-02***	***-1.6***	***3.3E-04***	-0.3	1.8E-01	***-0.9***	***1.9E-03***
	D-xylose	***-2.7***	***5.8E-06***	-0.5	1.4E-01	0.1	6.4E-01	0.1	2.2E-01	0.1	4.0E-01
	3-phosphoglyceric acid	**18.0**	**1.3E-04**	-0.5	5.5E-01	-0.2	7.6E-01	-0.3	5.6E-01	-0.2	1.2E-01
	sucrose	**4.8**	**3.4E-04**	0.7	9.3E-02	***-5.6***	***3.6E-05***	-0.3	1.3E-01	***-11.5***	***1.5E-03***
	fructose-6-phosphate	**2.9**	**2.9E-04**	0.1	8.7E-01	-0.3	2.8E-01	-0.2	3.4E-01	***-1.2***	***8.8E-03***
	glucose-6-phosphate	**2.8**	**4.4E-05**	-0.2	6.9E-01	-0.5	1.0E-01	-0.1	1.7E-01	***-1.4***	***2.2E-03***

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	D-fructose	***-2.4***	***9.9E-07***	***-0.3***	***1.7E-05***	-0.4	3.9E-01	0.0	8.1E-01	***-0.4***	***3.8E-02***
	D-glucose	***-4.2***	***2.1E-05***	***-0.4***	***8.8E-05***	***-0.3***	***6.3E-03***	-0.1	2.0E-01	0.1	4.9E-01
TCA cycle substrates & other acids	succinate	**116.5**	**1.8E-08**	**3.5**	**4.2E-03**	**1.0**	**1.1E-03**	**6.5**	**1.5E-03**	***-1.1***	***2.7E-03***
	fumarate	**7.0**	**2.9E-07**	-0.7	2.7E-01	-0.2	1.6E-01	1.1	5.1E-02	-0.4	1.7E-01
	aconitate	**4.3**	**5.3E-04**	***-2.8***	***3.2E-02***	-0.5	1.5E-01	***-1.6***	***1.1E-02***	-0.2	2.4E-01
	citrate	**2.5**	**4.0E-05**	11.7	5.5E-02	***-1.5***	***8.3E-03***	***-3.2***	***1.3E-02***	***-0.7***	***1.4E-04***
	malate	0.0	1.5E-01	***-2.2***	***8.8E-05***	***-1.0***	***3.1E-05***	-0.1	9.2E-02	0.0	7.5E-01
	isocitrate	-0.1	1.9E-01	-2.1	8.7E-02	***-0.5***	***3.7E-02***	***-5.3***	***6.0E-03***	***-0.5***	***1.1E-03***
	2-oxoglutarate	***-2.2***	***1.3E-03***	***-3.8***	***2.6E-02***	-0.3	1.8E-01	***-24.5***	***6.4E-04***	***-0.8***	***2.8E-03***
	glycerate	***-1.8***	***1.3E-04***	***-1.5***	***2.6E-06***	***-0.9***	***7.3E-03***	***-0.5***	***1.6E-02***	***-0.2***	***8.9E-03***
	threonate	***-118.6***	***1.0E-05***	***-1.1***	***7.0E-04***	***-0.2***	***4.3E-03***	**0.3**	**8.7E-03**	-0.2	2.0E-01
	ascorbate	N/D	N/D	***-1.8***	***3.8E-04***	1.2	2.2E-01	-0.1	2.0E-01	-0.1	2.7E-01
	glucarate	**0.9**	**1.1E-04**	***-0.3***	***3.0E-02***	***-0.4***	***3.9E-03***	-0.2	1.9E-01	-0.2	2.1E-01
	citramalate	-0.5	5.7E-02	***-0.8***	***1.9E-02***	-0.2	6.8E-02	0.2	6.1E-02	***-1.4***	***4.9E-03***
	4-hydroxycinnamate	N/D	N/D	-0.1	3.0E-01	**0.5**	**1.2E-03**	**0.2**	**2.2E-02**	**0.8**	**8.0E-04**
	urate	N/D	N/D	0.2	5.8E-01	**15.2**	**2.9E-03**	-1.4	3.3E-01	**15.4**	**3.4E-04**
Other	shikimic acid	-0.7	1.0E-03	-0.1	4.1E-01	4.5	6.2E-02	0.0	9.3E-01	3.4	1.5E-01
	phosphate	**1.6**	**5.5E-08**	**0.3**	**2.9E-02**	-0.1	6.0E-01	-0.1	6.2E-01	-0.2	2.4E-01
	cytosine	N/D	N/D	***-1.6***	***2.0E-04***	***-0.6***	***1.5E-05***	-0.2	4.0E-01	-0.3	3.0E-01

Table 3: Metabolomic analysis of rice and wheat coleoptiles in response to anoxia. Rice plants were grown under (1) 4 days of aeration (2) 6 days of anoxia (3) 4 days of aeration and then 4 hours of anoxia or (4) 4 days of aeration and then 1 day of anoxia. Wheat plants were grown under all treatments except number 2. Coleoptiles tissues were separated from leaves for analysis. Complex polar metabolite extracts were taken from all tissues and analysed by GC/MS. Raw GC/MS data processing and statistical analyses were then carried out using MetabolomeExpress software (Ver. 1.0; http:// www.metabolome-express.org). Metabolite signal intensity ratios were calculated by dividing the mean tissue mass- and internal standard normalised signal intensity for each metabolite in treated samples by its corresponding value in control samples (see columns labelled 'RV'). The statistical significance of each ratio was tested by Welch's t-test ($P < 0.05$; $n = 5$; p-values <0.05). Values highlighted in bold are significantly more abundant in anoxic coleoptiles whereas negative bold italics indicates a metabolite that is more abundant under aeration with P-values that meet a threshold for significance <0.05. *Abbreviations-* RV, response value; N/D, no data.

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Figure Legends

Figure 1. Differential in gel electrophoresis (DIGE) on two-dimensional IEF/SDS gels. Comparisons were made between coleoptile proteomes of rice seedlings treated with 4 days of aeration versus (A) 6 days of anoxia as well as (B) 4 days of aeration with an additional 1 day under anoxia. Wheat responses to anoxia were also analysed (C) by comparing coleoptiles from seedlings treated in the same way as in (B). The top panels are gel images of each fluorescence signal, and the bottom panel a combined image electronically overlaid using ImageQuant TL software (GE Healthcare). Yellowish spots represent proteins of equal abundance between the two samples. The numbered arrows indicate proteins identified by MS (listed in **Table 2**) with abundances that were significantly different between treatments (identified in all 9 gel images, $p<0.05$, abundance difference > 1.5). Below the DIGE image is a Venn diagram representing the percentage of protein spots significantly changing in abundance between the two treatments. The percentage of protein spots significantly more abundant under anoxia or aeration are on the left- or right-hand side of each Venn diagram, respectively. The percentage of proteins that did not significantly differ in abundance lie in the middle.

Figure 2. Effect of prolonged anoxia on carbohydrate metabolism, glycolysis, fermentation, amino acid metabolism and the TCA cycle in rice coleoptiles. Rice seeds were germinated and grown under anoxia for 6 days or aeration for 4 days. The green and red boxes represent metabolites significantly more abundant under aeration and anoxia, respectively ($p<0.05$). The yellow boxes represent metabolites whose abundances are unchanged. Enzyme names on arrows are also coloured in this fashion. The numbers on the top left side of each box represent the response value (RV) of the corresponding metabolite (anoxic/aerated) in rice coleoptiles. All data were extracted from **Table 2** and **Table 3**. (**Metabolite abbreviations**: Glucose-6-P, glucose-6-phosphate; fructose-6-P, fructose-6-phosphate; fructose-1,6-bis-P, fructose-1,6,- bisphosphate; G-3-P, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetonephosphate; 1,3-PGA, 1,3-bisphosphoglycerate; 3-PGA, 3-phosphoglycerate; 2-PGA, 2- phosphoglycerate; PEP, phosphoenolpyruvate; GABA, 4-aminobutyrate; SSA, succinic semialdehyde. **Protein abbreviations**: SS, sucrose synthase; FK, fructokinase; PGM, phosphoglucomutase; PFK-PPi, PPi-fructose-6-phosphate 1-phosphotransferase; Aldolase, fructose-bisphosphate aldolase; TPI, triosephosphateisomerase; 3-PGDH, D-3- phosphoglycerate dehydrogenase; PSAT, phosphoserine aminotransferase; SHMT, serine hydroxymethyltransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase, PGK, phosphoglycerate kinase; iPGAM, 2,3-bisphosphoglycerate-independent phosphoglycerate mutase; AlaAT, alanine aminotransferase; PDC, pyruvate decarboxylase; ADH, alcohol dehydrogenase; MDH, malate dehydrogenase; MetSyn, 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase (cobalamin-independent methionine synthase); GlnSyn, glutamine synthetase).

Figure 3. Effect of a one-day anoxic switch on carbohydrate metabolism, glycolysis, fermentation, amino acid metabolism and the TCA cycle in rice and wheat coleoptiles. Rice and wheat seeds were germinated and grown under aeration for 4 days or for 4 days with a switch to one day of anoxia. Green and red boxes represent metabolites significantly more abundant during aeration or the anoxic switch, respectively ($p<0.05$). The yellow boxes represent metabolites whose abundances are unchanged. Enzyme names that accompany arrows are also coloured in this fashion for the rice response only (anoxia-responsive proteins were not identified in wheat). The numbers on the top left and right side of each square represent the response value (RV) of the corresponding metabolite (anoxia/aeration) in rice and wheat coleoptiles, respectively. All data were extracted from **Table 2** and **Table 3**. (For abbreviations see **Figure 2**).

Figure 4. The effect of exogenous amino acid feeding on cell integrity after prolonged anoxia in wheat and rice seedlings. Rice and wheat seeds were germinated and grown under 4 days of aeration. Fresh culture media in the presence or absence of 10 mM of Ala, Ser and/or Gly

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was then added to seedlings. Seedlings were returned to 3 days of aeration (green) or transferred to 3 days of anoxia (non-supplemented in red; supplemented in dark red). Roots were then analysed using the Evans Blue viability stain (A; n=3). An increase in cell death is proportional to increased absorbance at 600 nm. Cell membrane permeability in whole rice and wheat seedlings was also analysed (B; n=10-23). This was done by measuring electrical conductivity (EC) after seedlings were incubated in ddH_2O for one hour (C_1). A second measurement was taken after sample boiling (C_2) to obtain the proportion of cell leakage in different samples. Larger C_1/C_2 values indicate higher electrolyte leakage and thus lower cell integrity. *** indicates $p<0.001$, ** indicates $p<0.01$ and * indicates $p<0.05$ when compared to anoxic seedlings that were not supplemented (red bars).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table 1: Entire list of rice coleoptile proteins significantly changing between treatments that were identified by DIGE and iTRAQ proteomic analyses.

Supplemental Table 2: Quantitative analysis of protein abundance from 4 days aerated and 6 days anoxic rice coleoptiles using isobartic Tag for Relative and Absolute Quantitation (iTRAQ).

Supplemental Table 3: Comparison of steady-state and polysomal Arabidopsis mRNAs under aeration and hypoxia whose genes are orthologous to rice genes encoding proteins significantly changing in abundance in at least one of our rice proteome analyses.

Supplemental Table 4: Whole set of relative metabolite levels in wheat and rice coleoptiles under aerated and anoxic conditions.

Supplemental Table 5: Comparison of metabolite responses between wheat and rice coleoptiles from seedlings switched to 1d of anoxia in two independent experiments.

Supplemental Table 6: Grouping of metabolite responses in coleoptiles of 4-day-old rice and wheat seedlings transferred to anoxia for 1 day (anoxic switch conditions)

Supplemental Table 7: Comparison of metabolite responses in coleoptiles of anoxically-switched and anoxically-germinated rice seedlings.

Supplemental Table 8: Metabolic phenocopy analysis - comparison of the low-oxygen responses of rice and wheat coleoptiles with low-oxygen and respiratory-perturbation responses in other species and tissues.

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Supplemental Table 9: Expanded results of MetabolomeExpress PhenoMeter analysis.

Supplemental Figure 1. Germination, growth and cell integrity of rice and wheat under anoxia.

Supplemental Figure 2. Number of protein spots detected in each proteomic analysis that significantly differed in abundance between treatments.

Supplemental Figure 3. Correlations between protein abundance detected from iTRAQ and DIGE, and between transcript and protein abundance differences between continuously anoxic and aerated rice coleoptiles.

Supplemental Figure 4. Differential in gel electrophoresis (DIGE) on two-dimensional 3-11 NL IEF/SDS gels.

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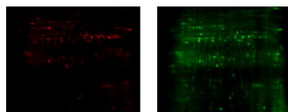

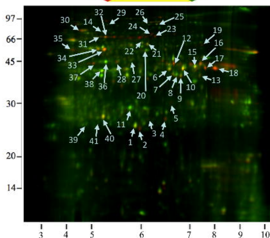

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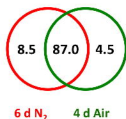

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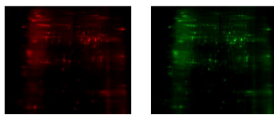
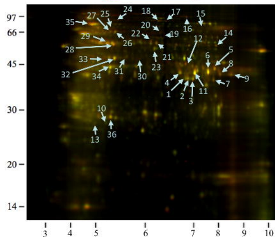
n=1259





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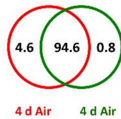

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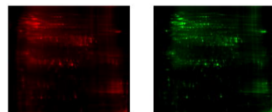
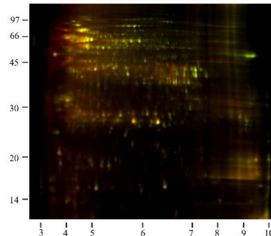
n=1245





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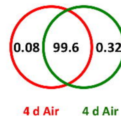

4 d Air 1 d N_2 4 d Air

n=1245

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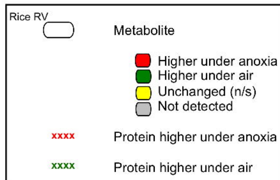
Rice: 6 d Anoxia / 4 d Air



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-4.2 glucose
2.8 glucose-6-P
-2.4 fructose
FK
2.9 fructose-6-P
SS
PGM*
PFK-PPi
UDP-glucose
fructose-1,6-bis-P
aldolase
3-phosphoserine
PSAT
3-phosphohydro -pyruvate
3-PGDH
G-3-P
TPI
DHAP
GAPDH
glycolysis
9.0 Ser
Cys
1.9 Gly
1,3-PGA
PGK
18.0 3-PGA
PGM*
2-PGA
enolase
42.6 Ala
AlaAT
PEP
-0.7 shikimate
chorismate
5.2 Trp
Phe
22.5 Tyr
43.8 Leu
pyruvate
PDC
acetaldehyde
ADH
ethanol
10.2 Val
TCA cycle
acetyl CoA
2.5 citrate
-0.6 Asn
-1.5 Asp
oxaloacetate
4.3 aconitate
MDH
-0.1 isocritrate
15.5 homoserine
10.9 Lys
0.0 malate
1.8 Gln
His
MetSyn*
GlnSyn
2.4 Thr
6.0 Met
-2.2 2-oxoglutarate
36.5 Glu
98.6 Pro
7.0 fumarate
12.0 Ile
17.3 2-aminoadipic acid
8.9 GABA
0.6 ornithine
succinyl-CoA
116.5 succinate
SSA
15.6 Arg
52.4 putrescine

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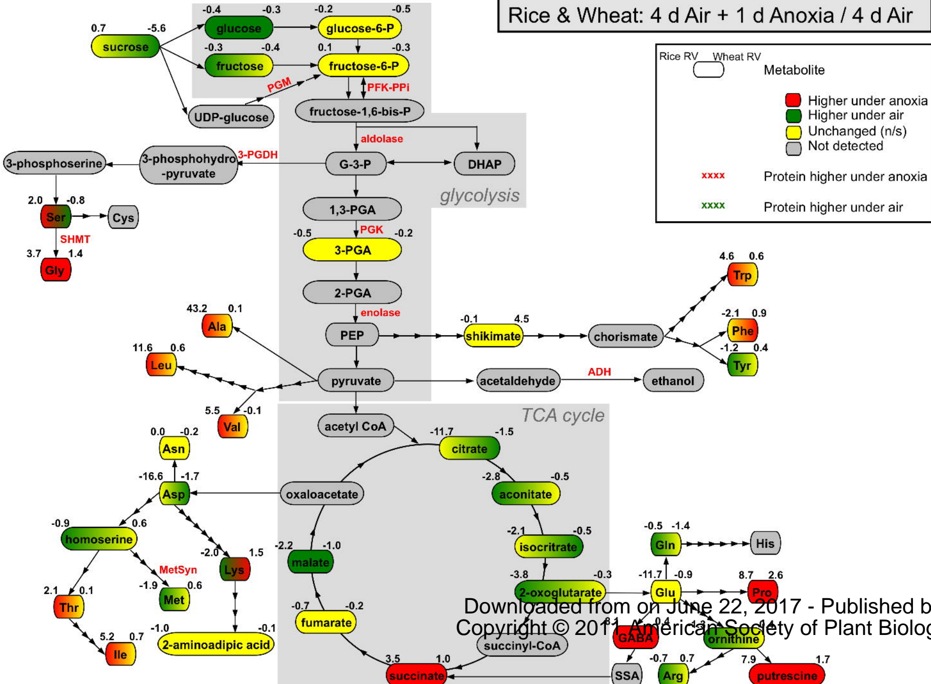

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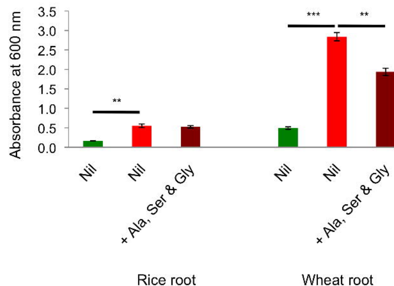

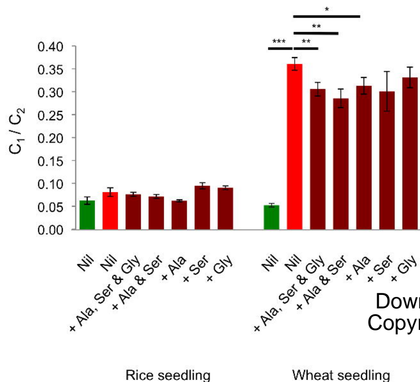

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