TITLE
Pursuing the identification of O₂ deprivation survival mechanisms in plants related to selective mRNA translation, hormone-independent cellular elongation and preparation for the arrival of oxygen.

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ADDENDUM TO:
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
Anoxia can occur in crop fields when flooding forms a physical barrier that reduces oxygen availability. Rice, but not wheat, can germinate and elongate its coleoptile under anoxia, providing an excellent model for understanding mechanisms of anoxia tolerance. We have shown differential molecular responses of rice and wheat coleoptiles to anoxia and discovered novel metabolic adaptations in amino acid metabolism for tissue tolerance. In this addendum, we elaborate on our discussion to speculate on the functions of differentially regulated proteins and their possible roles in selective transcription and translation, alternative elongation strategies and preparedness for exposure to air. In addition, it is thought that rapid growth is a stress avoidance strategy; if adequate coleoptile growth occurs then plants can outgrow floodwaters to resume or begin aerobic respiration. An innate response mechanism to the arrival of air, and the oxidative stress inherent to this, would therefore be necessary in survival beyond the alleviation of anoxia. Thus, we emphasize the importance of recognizing anoxia as a multi-stage stress where responses otherwise considered counter-intuitive may have evolved as preparative defenses for when exposure to air occurs.
INTRODUCTION
Anoxia is a debilitating consequence of crop flooding resulting in limited ATP production due to cessation of oxidative phosphorylation. As a result, glycolysis in combination with fermentation becomes relied upon as a vital, albeit less efficient, means of generating ATP. The wide spectrum of anoxia-tolerance within the plant kingdom affords an opportunity to study the mechanisms underlying success when O₂ is in short supply. We took this opportunity by studying two important crop species, rice and wheat, and found coleoptile proteome plasticity in an anoxia-tolerant rice variety (Amaroo), which was in opposition to what was observed in the coleoptiles of the Calingiri wheat variety. This plasticity was also reflected in the enhancement of glycolytic enzyme production and the accumulation of several amino acids in rice coleoptiles subjected to short-term anoxia. Again, this was less apparent in anoxia-intolerant wheat (Figure 1). We discovered accumulation of a set of enzymes involved in serine, glycine and alanine biosynthesis, which was consistent with the accumulation of these amino acids in anoxic rice coleoptiles (long or short-term anoxia). We also showed a benefit to cell integrity when externally supplying these amino acids to anoxic wheat coleoptiles. The discussion herein expands our discussion of new insights into mechanisms of anoxia tolerance in rice coleoptiles, focusing on four other aspects of rice coleoptile adaptation to anoxia, namely (1) selective transcription and translation (2) an alternative elongation strategy (3) accumulation of secretory proteins and (4) preparedness for exposure to air (Figure 1).

SELECTIVE TRANSCRIPTION AND TRANSLATION
The rice coleoptile is an ideal model to study anoxia tolerance due to its ability to survive and elongate when O₂ is unavailable. Using this model tissue, a microarray study measuring transcriptional changes during O₂ deprivation has been published. Core anaerobic and aerobic responses have been proposed based on transcriptional changes that occur during switch experiments, which suggests that selective transcription of genes under anoxia is likely to be linked with specific promoter responses. There is concern however over the degree of predictive power that these measurements offer since transcription does not necessarily result in translation and the accumulation of a functional protein product. When we compared published transcript fold differences with our protein abundance ratios, we found the correlation to be r=0.62 (supplemental Figure 3B). In other studies, poor correlations between protein and mRNA abundance changes have also been reported. For example, when Arabidopsis suspension cells responding to ABA treatment the correlation was r=0.66. But when considering only ABA-regulated gene products, the correlation improved (r=0.81). In an anaerobic yeast system, discrepancies between mRNA and protein ratios have also been described for proteins involved in purine nucleotide anabolism, amino acid metabolism, glycolysis/gluconeogenesis, aminoacyl-tRNA synthesis and electron transport/membrane-associated energy conservation. These results suggest the existence of selective translation and that this process might be more dominant under anoxia than in other circumstances in plants. In recent years, a study has shed light on the relationship between whole-cell and polysomal mRNA pool changes that occur in hypoxic Arabidopsis seedlings. This allows more confident inferences to be made that transcripts accumulating in these polysomal mRNA pools are actually translated into functional protein, whilst maintaining the in-depth coverage that microarray technology affords. The discrepancy between abundance changes in steady state and polysomal mRNA pools
during the hypoxic response strongly suggests that transcripts are differentially selected for translation, a process that would be beneficial in cases where ATP supply is diminished, as it is during anoxia. We could find no apparent relationship between this Arabidopsis translatome data (polysome loaded) and that of our rice proteome data (similar number of instances of co-directional and non-codirectional changes),\(^1\) with some notable exceptions including gene products involved in glycolysis, fermentation, amino acid biosynthesis and a few with unknown functions (Os08g04210, Os08g04250 and Os08g04240). We believe that a stronger correlation might well exist if anoxic rice coleoptile translatomes were studied.

We found several proteins involved in translation whose abundances were differentially regulated in anoxic rice coleoptiles. This includes ribosomal proteins (Os03g14530 up, Os11g29190 down) and elongation factors (Os03g08010 up, Os01g52470 up, Os02g32030 down), both of which could be involved in the process of selective translation.\(^1\) Such specificity has the potential benefit of preventing ATP exhaustion, which would otherwise occur if mRNAs were translated into proteins whose functions did not contribute to the plant's successful endurance of anoxia and beyond.

**ALTERNATIVE ELONGATION STRATEGY**

Auxin plays a significant role in coleoptile elongation under normoxic conditions. Free IAA induces a plasma membrane-bound H\(^+\)-ATPase pump, which causes cell wall acidification, a process that has been shown to activate cell wall-bound enzymes involved in cell wall loosening and cell extension.\(^12,\,13\) But under anoxic conditions, there is no synergistic effect of IAA on coleoptile elongation\(^14\) and the auxin-binding activities in anoxic coleoptiles decrease.\(^15\) We found that a putative IAA amino acid hydrolase 1 (ILR1; Os03g62060) is less abundant during anoxia when compared to aerated rice coleoptiles\(^1\) suggesting a higher prevalence of amino acid-conjugated IAA during O\(_2\) deprivation. Accumulation of IAA-Asp during anaerobic treatment has been previously observed\(^14\) and is thought to prevent IAA toxicity since IAA accumulates in the anoxic coleoptile to a greater degree than during aeration\(^16,\,17\) and can cause death in excised, aerated coleoptiles if concentrations are sufficiently high.\(^14\) It is unknown if IAA can cause death in anoxic coleoptiles but high concentrations (10\(^{-3}\) M) do appear to inhibit growth.\(^14\)

A low abundance of ILR1 in anoxic coleoptiles might prevent conjugate cleavage and thus prevent IAA growth inhibition or toxicity. In addition, anoxic coleoptiles experience an energy crisis under anoxia.\(^18\) Therefore IAA-induced cell elongation may not be an energetically efficient means of growth. ILR1 down-regulation could prevent conjugate cleavage and thus prevent the induction of an ATP consuming H\(^+\)-ATPase pump. In addition, removal of KCN or alleviation of anoxia has been shown to permit a growth burst in *Avena* coleoptiles,\(^12,\,13\) indicating that ATP availability is important. Alternatively, conjugate formation may also prevent oxidative damage of IAA.\(^19\) Oxidative damage to lipids appears to increase in anoxic coleoptiles returned to air\(^20\) and is a well-known consequence of rapid O\(_2\) exposure. Thus we speculate that protection of a growth-promoting hormone via down-regulation of ILR1, which would otherwise cleave these conjugates, could be beneficial to a seedling that is growing and recovering from anoxia.

**ACCUMULATION OF SECRETORY PROTEINS**
We found three unknown proteins encoded very closely together on chromosome 8 of rice (Os08g04210; Os08g04240 and Os08g04250) that were highly accumulated in anoxic rice coleoptiles1 and in fact their transcripts are hugely up-regulated by anoxia at 1007, 525 and 248-fold, respectively.4 These three proteins share high similarity at the amino acid sequence level (80-85%) suggesting they originate from tandem duplication events in the evolutionary history of rice. These rice proteins contain the DUF26 domain of unknown function and are annotated as putative cysteine-rich repeat secretory protein 55 precursors based on rice genome annotation project (http://rice.plantbiology.msu.edu; Osa1 release 6.1). An Arabidopsis thaliana gene, At5g48540 is an orthologue to these three genes. The At5g48540 transcript is upregulated in Arabidopsis root cultures and in polysomal mRNA pools of seedlings exposed to low oxygen stress.11,21 This gene encodes two DUF26 domains, which are also common to the plasmodesmata-located protein (PDLP1) family.22 Knockout studies implicate several PDLP1 members in reducing “plasmodesmal trafficking potential” pointing to their role in regulating cell-to-cell cross talk. At5g48540 bears an N terminal signal peptide for secretion and accordingly is annotated as a 33 kDa secretory protein.21 However, At5g48540 lacks a transmembrane domain and forms “large unresolved bodies in the apoplast” as opposed to being localised in the plasmodesma. The latter two features exclude At5g48540 from the PDLP1 family. Nevertheless, the extracellular location of At5g48540, as well as the shared presence of the DUF26 domain between Arabidopsis and rice make these O2-responsive orthologues intriguing targets for further study.

PREPAREDNESS FOR EXPOSURE TO AIR

We propose that accumulation of proteins that seem unnecessary to anoxia itself could be beneficial for when plants are returned to air, and this future role is a driver for their conservation of expression during anoxia. It is evident that removal of flood-induced anoxia can actually result in a “stress shift”. Rapid exposure to O2 can cause accumulation of reactive oxygen species23 and in the case of de-submergence, drought stress.24 Thus the survival of a plant during flood-induced anoxia will rely on tolerance mechanisms to O2 deprivation itself as well as the stresses imposed when air returns. This kind of ‘preparation’ would be essential if, for example, plants were exhausting energy reserves to aid rapid growth that would enable contact with air (referred to as the snorkel effect25). We speculate that proteins like peroxiredoxin (Os07g44430), whose known function is to detoxify peroxides, accumulate during anoxia in rice coleoptiles to increase survival chances for when the plant can gain access to O2. It is also hypothesised that late embryogenesis abundant proteins (LEA3; Os05g46480) whose transcripts are elevated during de-submergence may benefit survival rate when plants are suddenly removed from a wet environment.24 Our proteome analysis indicates that the LEA3 protein accumulates in coleoptiles of anoxically-germinated rice seedlings.1 This could lay down the foundation plants need to prepare for when floods subside as well as allowing a prompt up-scaling of LEA3 translation if drought stress eventuates. We are of the opinion that the pursuit of identifying survival mechanisms requires approaching O2 deprivation in a holistic manner, that is by considering both anoxia itself and the inevitable return of air.
REFERENCES


Figures:

**Figure 1.** Differential response of rice and wheat coleoptiles to anoxia. Rice coleoptiles but not wheat coleoptiles dramatically respond to anoxia at the level of physiology, the metabolome and the proteome. Under anoxia, rice coleoptiles enhance glycolysis and fermentation for ATP production. Glycolytic intermediates can also be used for synthesis of serine, glycine and alanine. We speculate that through selective transcription and translation, anoxic rice coleoptiles may utilise an alternative elongation strategy and prepare themselves for the arrival or return of air, which further enhances their tolerance.
Anoxia

Rice coleoptiles  Wheat coleoptiles

Physiology
Metabolome
Proteome

Large response  Small response

Enhanced glycolysis and fermentation.
Enhanced amino acid metabolism.
Enhanced protein synthesis.
Selective transcription and translation?
Alternative elongation strategy?
Inhibition of cell-to-cell trafficking?
Preparedness for the arrival of air?

Enhanced fermentation.
Moderate changes in amino acid metabolism.
Limited protein synthesis.

Anoxia tolerant  Anoxia intolerant