

REVIEW

## Rice Germination and Seedling Growth in the Absence of Oxygen

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- **Background** Higher plants are aerobic organisms which suffer from the oxygen deficiency imposed by partial or total submergence. However, some plant species have developed strategies to avoid or withstand severe oxygen shortage and, in some cases, the complete absence of oxygen (tissue anoxia) for considerable periods of time.
- **Scope** Rice (*Oryza sativa*) is one of the few plant species that can tolerate prolonged soil flooding or complete submergence thanks to an array of adaptive mechanisms. These include an ability to elongate submerged shoot organs at faster than normal rates and to develop aerenchyma, allowing the efficient internal transport of oxygen from the re-emerged elongated shoot to submerged parts. However, rice seeds are able to germinate anaerobically by means of coleoptile elongation. This cannot be explained in terms of oxygen transport through an emerged shoot. This review provides an overview of anoxic rice germination that is mediated through coleoptile rather than root emergence.
- **Conclusions** Although there is still much to learn about the biochemical and molecular basis of anaerobic rice germination, the ability of rice to maintain an active fermentative metabolism (i.e. by fuelling the glycolytic pathway with readily fermentable carbohydrates) is certainly crucial. The results obtained through microarray-based transcript profiling confirm most of the previous evidence based on single-gene studies and biochemical analysis, and highlight new aspects of the molecular response of the rice coleoptile to anoxia.

**Key words:** Anoxia, coleoptile, fermentative metabolism, germination, hypoxia, *Oryza sativa*, rice.

### INTRODUCTION

Rice (*Oryza sativa*) is an essential food crop for billions of people. It can cope with a wide range of environmental conditions and, in particular, with low (hypoxia) or absent oxygen (anoxia), experienced during direct sowing in paddy field as a consequence of soil flooding (Yamauchi *et al.*, 2000). Soil flooding is one of the most important abiotic constraints on rice yields, with complete submergence of plants being particularly serious for rice farmers in the rainfed lowlands of humid and semi-humid tropics of Asia (Jackson and Ram, 2003). Although seeds of the vast majority of higher plant species fail to germinate under anaerobic conditions, rice germinates successfully even when deprived of oxygen completely (anaerobiosis) to create the metabolic state of anoxia (reviewed by Perata and Alpi, 1993). According to Raymond *et al.* (1985), seeds can be grouped into classes on the basis of their responses to oxygen availability. Starchy seeds were shown to be especially tolerant of anaerobiosis because they are able to maintain a high energy metabolism under oxygen deficiency when compared with fatty seeds. However, amongst starchy seeds there is considerable variation in the ability to germinate when anoxic. While declining oxygen concentrations negatively affect oat and barley germination (which starts with root emergence), rice behaves differently – root growth is suppressed while shoot growth increases as the oxygen concentration declines (Tsuji, 1973; Alpi and Beevers, 1983). Indeed, when germinated without oxygen, the final length of rice coleoptiles can exceed the length of aerobic coleoptiles whilst root

and primary leaf fail to grow (Fig. 1; Alpi and Beevers, 1983). This phenomenon is thought to increase the probability of the hollow coleoptile making contact with better-aerated water surface (the Snorkel effect; Kordan, 1974) thereby allowing oxygen to diffuse internally to the root and endosperm (Turner *et al.*, 1981) and supporting more complete and vigorous seedling establishment. This review article examines the biochemistry and molecular biology that underpins this important adaptive feature that is extant in all rice cultivars to a varying extent.

### COLEOPTILE ELONGATION UNDER ANOXIA

Several hypotheses have been proposed to explain the anoxic growth of the rice coleoptile (reviewed by Masuda *et al.*, 1998), and considerable variation exists among rice genotypes in coleoptile extension during anoxia (Setter *et al.*, 1994). Differences in the ability to elongate the coleoptile under anoxia might influence crop establishment in submerged fields, since coleoptile extension plays a key role in enabling the seedlings to make contact with the atmosphere and thus to gain access to O<sub>2</sub> (Huang *et al.*, 2003). However, there is, in general, a negative correlation between elongation underwater and survival, because the metabolic costs of rapid elongation shorten survival times by competing with cell maintenance processes for limited energy (reviewed by Jackson and Ram, 2003). Tsuji (1973) suggested that the lack of a normal aerobic auxin breakdown results in a longer anoxic coleoptile, but there is still no clear link between auxins and anaerobic coleoptile growth because the hormone cannot act in oxygen-free coleoptiles (Pegoraro *et al.*, 1988). Anoxia induces a decrease in the activity of cell wall-bound peroxidase, and

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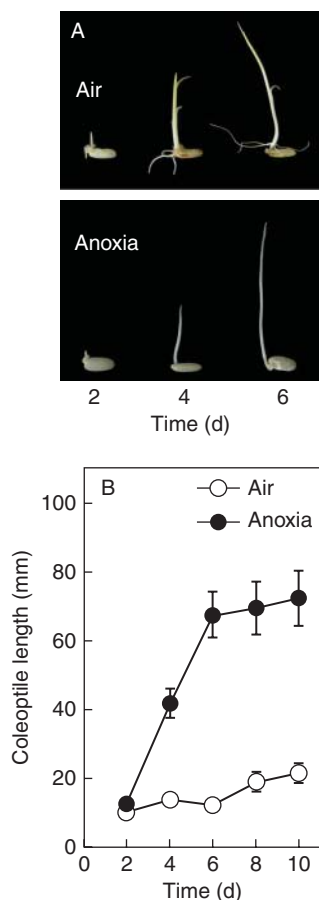


FIG. 1. Aerobic versus anoxic germination in rice ('Arborio Precoce'). (A) Rice seedlings 2, 4 and 6 d after sowing. Under aerobic conditions both roots, coleoptile and primary leaf are produced while anoxia-germinated seedlings lack primary leaf and roots and only the coleoptile grows. (B) Rice coleoptiles length under aerobic (Air) and anaerobic (Anoxia) conditions. Under anoxia, coleoptile length is, on average, three times longer than under aerobic conditions 10 d after sowing. Data are means of 60 measurements  $\pm$  s.d.

a correlation between this activity and coleoptile elongation has been observed (Lee and Lin, 1995a). Peroxidases are involved in the formation of diferuloyl cross-links to matrix polysaccharides which affects cell wall extensibility, and anoxia may indirectly lower peroxidase activity through a decrease in the internal abscisic acid (ABA) concentration (Fry, 1979) arising from the oxygen requiring step in ABA biosynthesis (Creelman *et al.*, 1987).

Recently Mattana *et al.* (2007) suggested the transcription factor Myb1eu as promoter of coleoptile growth and accelerator of the fermentative metabolism in anoxic coleoptiles. Tolerance of the rice coleoptile may be due to a shift from cell division (high energy-requiring) to cell expansion (Atwell *et al.*, 1982), which involves processes that are less energy-requiring than protein synthesis, such as cell wall synthesis and solute uptake (De Vries, 1975). Cosgrove's model (Cosgrove, 1998) of cell wall extension involves expansins, which are cell wall proteins that disrupt hydrogen bonds between cellulose and hemicellulose. Such expansins are encoded by a large

superfamily consisting of two major divisions ( $\alpha$ - and  $\beta$ -expansins). Expansin activity is enhanced by apoplastic acidification, which in anoxic tissue could be due to putrescine-stimulated  $H^+$ -ATPase activity on the plasma membrane (Reggiani *et al.*, 1992). A strong increase in *EXPA2* and *EXPA4* mRNA levels in rice coleoptiles under hypoxic conditions has been reported (Huang *et al.*, 2000), in addition to the well-recognized role in internode elongation for deepwater rice (Cho and Kende, 1997), suggesting a role for these expansins in the elongation of submerged rice coleoptiles. *EXPA7* and *EXPB12* may be involved in the elongation of the rice anoxic coleoptile (Lasanthi-Kudahettige *et al.*, 2007), based on their expression profile, correlating with the anoxic elongation of the coleoptile. However, there is still no comprehensive analysis of the role of expansins in relation to the diverse behaviour of rice cultivars in the anoxic elongation of the coleoptile.

#### FERMENTATIVE METABOLISM

When oxygen is limited, mitochondrial respiration is negatively affected and the NADH produced by the glycolytic pathway is re-oxidized through the fermentative pathway, whose main derivatives are lactate and ethanol (Fig. 2; Perata and Alpi, 1993). Lactate is produced from pyruvate by the action of lactate dehydrogenase (LDH), while ethanol production is the consequence of the decarboxylation of pyruvate to acetaldehyde [catalysed by pyruvate decarboxylase (PDC)] followed by the reduction of acetaldehyde to ethanol [catalysed by alcohol dehydrogenase (ADH)]. This switch to anaerobic metabolism allows the production of the ATP necessary for the survival of the cell (reviewed by Perata and Alpi, 1993). The anaerobic pathway produces, however, only two ATP per molecule of glucose, when compared with about 38 ATP through aerobic respiration. Under anoxia and hypoxia, protein synthesis is thus redirected through transcriptional control to the production of, mostly, only those enzymes which are essential for the metabolism of carbohydrates (reviewed by Perata and Alpi, 1993) and the fermentative pathway, such as sucrose synthases, PDC, ADH and LDH (Sachs, 1994). Two regions (anaerobic response element), which are upstream of the transcription site of several genes coding for anaerobic polypeptides (ANPs), may control the accumulation of these peptides under anoxia (reviewed by Sachs, 1991). Sequences showing homology with the anaerobic response element have been found in several anoxia-inducible genes such as LDH, ADH, sucrose synthase and aldolase genes (reviewed by Sachs, 1991).

Plants tolerant of anaerobiosis are usually able to maintain an active fermentative metabolism, essential for the production of ATP, for a relatively long time. In some circumstances, a 'Pasteur effect' may be evident. This is defined as an acceleration of carbohydrate consumption that can be expected to increase the energy production (Waters *et al.*, 1991), with a 2- to 3-fold increase in glycolytic flux under anoxia with respect to the aerobic control (Gibbs *et al.*, 2000). Despite this, coleoptiles of anoxia 'tolerant' rice genotypes produce 4.4–9.0 times less ATP in

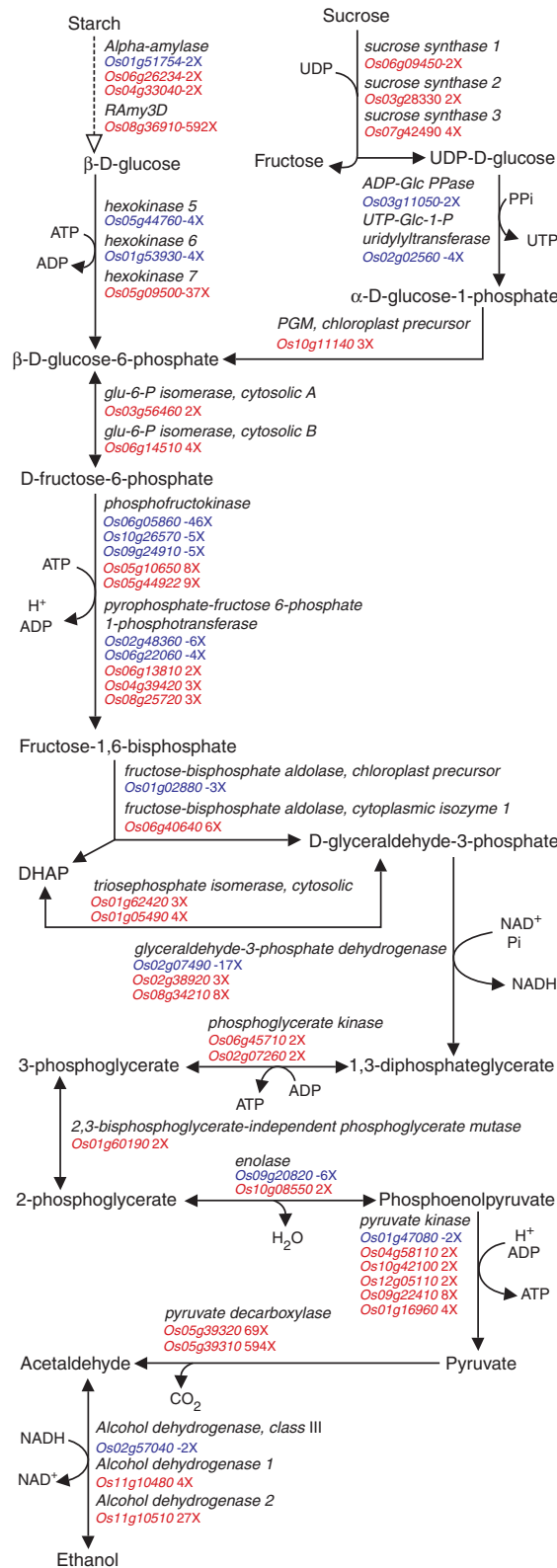


FIG. 2. Conversion of carbohydrate reserves into ethanol and NADH reoxidation under anoxia. Differentially expressed genes derived from probesets showing an FDR (False Discovery Rate) lower than 5% and involved in starch degradation, glycolysis and ethanolic fermentation are shown together with their fold (x) of induction. Down-regulated genes are reported in blue, while up-regulated genes are listed in red. Dotted arrows represent reactions that involve more than one step, i.e. starch degradation. Most of the reactions are strongly up-regulated under anoxia and, although some genes are repressed, their function is very likely to be counterbalanced by the high fold of induction of other isoforms. Data elaborated from Lasanthi-Kudahettige *et al.* (2007, supplemental table S2); metabolic pathways modified from Gramene ([www.gramene.org](http://www.gramene.org)).

anoxia than in air (Gibbs and Greenway, 2003). However, unlike in other plant tissues, the energy charge  $[(ATP + \frac{1}{2}ADP)/(ATP + ADP + AMP)]$  in rice coleoptiles recovers to a fairly high value (0.80) that approaches those growing in air after a marked decline in the initial transfer to anaerobic conditions. This suggests that the maintenance of a high energy charge may account for the success of this rice tissue under anoxia (Mocquot *et al.*, 1981).

Ethanol fermentation is important for hypoxia/anoxia tolerance, as highlighted by studies on *adh* null mutants in several different plant species (Jacobs *et al.*, 1988), though the correlation between the level of ADH induction and the tolerance to anoxia has been controversial (McManmon and Crawford, 1971; Harberd and Edwards, 1982; Agarwal *et al.*, 2007). Alpi and Beevers (1983) observed a decrease in several enzymatic activities during anoxic rice germination, but not for ADH, whose increase was first reported by Hageman and Flesher (1960) and is now considered as a common response of plants to anaerobic conditions, although there are some exceptions (reviewed by DeLisle and Ferl, 1990). In rice, anoxic seed germination as well as the transfer of 20-d-old plants to an anaerobic environment induces both *ADH1* and *ADH2* genes (Lasanthi-Kudahettige *et al.*, 2007). Saika *et al.* (2006) characterized a point mutation of the *ADH1* gene in rice. In this *reduced adh activity (rad)* mutant, coleoptile elongation is repressed under submergence, in agreement with the results obtained by Rahman *et al.* (2001) using an *ADH1*-antisense transformant. These results indicate that ADH activity is essential for coleoptile elongation in submerged rice, and this correlation appears to be linked to the lower ATP production in the mutant (Saika *et al.*, 2006). This is also likely to apply to anaerobic coleoptiles. Energy supply is indeed a key factor for rice tolerance to oxygen deficiency, and differences among rice cultivars (Atwell *et al.*, 1982; Setter *et al.*, 1994) as well as experiments with mutants characterized by low metabolic ability strongly support this evidence.

Even though ethanol fermentation is essential for ATP production and  $NAD^+$  regeneration under low oxygen conditions, high ethanol fermentation rates are not always beneficial for the plant. Indeed, although a high ethanol production under anaerobiosis is often associated with a sufficient ATP production, this fast carbohydrate consumption inevitably leads to carbohydrate depletion (Mustroph *et al.*, 2006). A positive correlation between ethanol production and anoxia tolerance has been reported for rice (Setter *et al.*, 1994; Gibbs *et al.*, 2000), whereas in anoxia-intolerant species such as tobacco and cotton, over-expression of PDC or ADH decreases anoxia tolerance (Ellis *et al.*, 2000). DeLisle and Ferl (1990) observed that the concentrations of ethanol required for the inhibition of growth (30–170 mM) are rarely observed. This may be due to ethanol diffusion from cells into the surrounding media. Moreover, the anoxic production of ethanol in rice coleoptiles is higher than that observed in anoxia-intolerant cereals, suggesting that ethanol toxicity is not the primary cause of anoxia-sensitivity (Alpi and Beevers, 1983). Only a high and non-physiological concentration of ethanol, when exogenously supplied, can mimic the

effects of anoxia (reviewed by Perata and Alpi, 1993). Furthermore, ethanol-injuries on carrot cell culture may be due to acetaldehyde rather than ethanol *per se*, since acetaldehyde is able to create adducts with cellular proteins (Perata *et al.*, 1986; Perata and Alpi, 1991). In the rice genome, as well as in other grasses, at least two ADH genes are present (*ADH1* and *ADH2*) that display distinct patterns of expression (Gaut *et al.*, 1999; Lasanthi-Kudahettige *et al.*, 2007). The activity of ADH present in aerobic plant tissue appears to be high enough already to support the rate of ethanol production under fermentative conditions. This means that it is not clear what role is played by the increased ADH activity under oxygen deficiency (Roberts *et al.*, 1989). Mustroph *et al.* (2006) analysed the fermentative activities of shoots and roots in rice (tolerant to anoxia) and wheat (anoxia-intolerant) seedlings germinated in air and then transferred to anoxia. Their results showed that in rice roots, PDC and ADH activities increased up to 5-fold, though this was not observed in wheat roots. Furthermore, in rice shoots ADH and PDC activities increased 2- and 6-fold, respectively, whereas in wheat shoots only a 3-fold decrease in the fermentative activities was observed. Kato-Noguchi (2006) demonstrated that the transfer of rice seedlings to anoxia increased PDC and ADH activity, with PDC activity being well below that of ADH, suggesting that PDC is the limiting factor for ethanol fermentation in rice coleoptiles. In the same paper, no significant increases in LDH activity were reported, in accordance with the lack of lactate accumulation. Additionally, anoxia rapidly increased the accumulation of alanine and induced the activity of the enzyme responsible for its production (alanine aminotransferase) thus catalysing the transamination of pyruvate to alanine. Kato-Noguchi (2006) reported that 92% of pyruvate is converted into ethanol, 1% into lactate and 7% into alanine. This means that ethanol fermentation accounts for most of the pyruvate consumption, thus leading to  $NAD^+$  recycling, and minimizing cytoplasm acidosis (see 'pH-regulation and cytoplasm acidosis'). As reported by Lasanthi-Kudahettige *et al.* (2007), two differentially expressed PDC genes are up-regulated, with *Os05g39310* being induced at the highest level (594-fold). Differentially expressed ADH genes appear to be less up-regulated than PDC genes, with the highest up-regulated ADH gene (*Os11g10510*, *ADH2*) induced by 27-fold. No differentially expressed LDH genes were detected by Lasanthi-Kudahettige *et al.* (2007). On the other hand, alanine aminotransferase genes are down-regulated in anoxic coleoptiles (*Os07g01760*). This data is based on expression analysis and does not actually contradict the enzymatic activities measured by Kato-Noguchi (2006), since the level of expression is not directly related to the activity of the enzyme because of differences in the translation efficiency.

Under aerobic conditions rice shoots contain seven times more soluble carbohydrates than roots, whereas in wheat the content in shoots is three times higher (Mustroph *et al.*, 2006). During a 4-h anaerobic treatment, high fermentation rates resulted in a 40% decline in carbohydrate content for both roots and shoots, with a two times higher carbohydrate

depletion observed for rice shoots, which is a possible consequence of the higher initial sugar content (Mustroph *et al.*, 2006). In shoots, 20 times and four times more ethanol was found in anoxic rice and wheat, respectively, indicating a more efficient fermentative metabolism in rice. After 4 h of oxygen deficiency, the ATP content decreased by 30 % in rice and by 50 % in wheat, while after a 24-h anoxic treatment the ATP content decreased almost to zero in rice roots and in wheat roots and shoots, while rice shoots still retained 30 % of the aerobic ATP level (Mustroph *et al.*, 2006). Sucrose and starch are metabolized differently in rice compared with wheat and barley (Guglielminetti *et al.*, 1995a, 1997; Perata *et al.*, 1996, 1998). In agreement with this observation, ethanolic fermentation is comparable among the three cereals only during the first days under anoxia, when the presence of soluble sugars reserves allows the fermentative metabolism to proceed. But, only in rice does fermentation actively continue for several days (Guglielminetti *et al.*, 2001). The high fermentation rate of rice seedlings, together with a higher content of soluble carbohydrate and an optimized ATP use during anoxia may explain the success of this species when compared with anoxia-intolerant cereals (Mustroph *et al.*, 2006). Of course, prolonged periods of anaerobiosis can result in carbohydrate starvation, with negative consequences in rice too. However, the lower tolerance of wheat to anoxia does not appear to be directly linked to the amount of endogenous carbohydrate, since wheat seedlings do not consume all the stored carbohydrates, probably because of the low glycolytic enzyme activities (Albrecht *et al.*, 2004).

#### pH-REGULATION AND CYTOPLASM ACIDOSIS

Reduced lactate production and improved pH control is often recognized as part of the strategy that confers anoxia tolerance in plants. Davies (1980) proposed a pH-dependent regulation of the fermentative pathway. According to this hypothesis, PDC is not active under normoxic conditions due to a cytoplasmic pH which is higher than optimal for PDC activity. At the onset of anaerobiosis, LDH can, on the other hand, operate to recycle pyridine nucleotides through the lactic fermentation pathway. Lactate production would then acidify the cytoplasm thereby activating PDC, thus furnishing ADH with its substrate pyruvate, leading to ethanol production. This shift to ethanol production is not always associated with a decrease in cytoplasmic pH, suggesting that multiple mechanisms are likely involved in the induction of alcoholic and lactic fermentation (Saintges *et al.*, 1991; Albrecht and Vartapetian, 1992). The lower  $K_m$  of plant pyruvate dehydrogenases for pyruvate with respect to PDC, and the internal pyruvate concentration in plant tissues (between 0.1 and 0.4 mM) are consistent with the preferential entrance of pyruvate into the TCA cycle at the aerobic pH. However, when respiration is blocked, pyruvate concentration increases (Good and Muench, 1993), thus becoming available for the PDC reaction (Tadege *et al.*, 1999). In rice, the presence of pyruvate at 3 mM concentration can overcome the lag phase in PDC activity, as

expected by the lower  $K_m$  of PDC for pyruvate, suggesting that pyruvate concentration is more important than pH (Tadege *et al.*, 1999). The lag phase for ethanol production observed at the onset of anoxia thus does not seem to be strictly related to a drop in cytoplasm pH, but may be the consequence of the time required for the build-up of pyruvate (Tadege *et al.*, 1999). Several authors (Menegus *et al.*, 1991; Saintges *et al.*, 1991; Fox *et al.*, 1995; Gout *et al.*, 2001) suggested that the initial acidification is not the consequence of lactate production, but of the protons release accompanying the Pi-liberating breakdown of NTP, which was observed immediately after plants were transferred to anaerobiosis. In this case, ethanolic fermentation might be activated even without the initial production of lactate, as observed in rice coleoptiles (Kato-Noguchi, 2006).

Another cause of cytoplasmic acidosis may be the leakage of  $H^+$  from the vacuole. In fact, under hypoxic conditions vacuolar pH increases are inversely related to cytoplasmic pH decreases in maize root tips (Roberts *et al.*, 1984b). Active  $H^+$  transport from cytoplasm to vacuoles is an important mechanism by which the cells regulate their intracellular pH. By lowering the free energy for hydrolysis of ATP, hypoxia negatively affects the maintenance of the proton gradient across the tonoplast; consequently, tonoplast permeability to protons results in cytoplasm acidification (Roberts *et al.*, 1984a).

Regulation of acidosis may be critical for anoxia tolerance in plant species (Roberts *et al.*, 1985). Experiments with an *ADH1* null mutant (*adh1*) of maize (Roberts *et al.*, 1984b) revealed the absence of pH regulation and a very low ATP production through fermentation. The authors attributed the lower tolerance of the *adh1* to hypoxia to the inability of the mutant to regulate its cytoplasmic pH. This is presumably due to a continued, though lower, rate of lactate production, which is only able to fulfil the energetic needs of the hypoxic root tips for short periods of time. However, anoxic rice seedlings show a very low activity of LDH (Rivoal *et al.*, 1991) and a lower production of lactate (Menegus *et al.*, 1991). Even though there is no ubiquitous quantitative correlation between lactate production/accumulation and cytoplasmic acidification (Felle, 2005), a low level of LDH activity in rice may stay alongside its tolerance to anoxia (Rivoal *et al.*, 1991), as well as lactate efflux outside the tissues as observed in maize by Xia and Saglio (1992), though the use of metabolic energy for such transport appears unlikely (Felle, 2005). The lack of correlation between lactate production and cytoplasm acidification is not surprising, in fact lactate is only one of the factors involved in the process of acidification (reviewed by Felle, 2005).

The differing abilities of plants to cope with or avoid cytoplasm acidification seems to correlate with the differences observed in hypoxia/anoxia tolerance (Perata and Alpi, 1993). It is noteworthy that in stems of *Potamogeton pectinatus* – that elongate even more dramatically than the rice coleoptile does – cytoplasmic pH is very tightly regulated with almost no cytoplasmic acidification and is associated with an invigorated rate of sugar consumption (Summers and Jackson, 1994; Summers *et al.*, 2000).

Anoxia-tolerant rice shoots held their anoxic pH close to the aerobic pH over many hours, whereas anoxia-intolerant wheat shoots rapidly acidified after a few hours of anoxia (Felle, 2005). It has been suggested that the H<sup>+</sup>-consuming ATP synthesis accompanying ethanolic fermentation plays a role in ameliorating cytoplasmic pH (Gout *et al.*, 2001). Indeed, a partial pH recovery in rice is attributed to H<sup>+</sup>-consuming ATP synthesis accompanying fermentative metabolism, although an increase in alanine and 4-aminobutyrate (GABA) concentration may also counteract the acidification effect of lactate (Menegus *et al.*, 1991; Reggiani *et al.*, 2000).

#### NITRATE REDUCTION MAY PREVENT CYTOPLASM ACIDOSIS

Several plant species have been studied in order to elucidate the role of nitrate under anoxia (Reggiani *et al.*, 1985; Fan *et al.*, 1997; Polyakova and Vartapetian, 2003), and a possible function of nitrate reduction as an alternative for NADH and NADPH re-oxidation under hypoxia has been proposed (Reggiani *et al.*, 1985; Roberts *et al.*, 1985; Fan *et al.*, 1997). Reggiani *et al.* (1993d) showed that nitrate is utilized during the anaerobic germination of rice, and a role for the pH-regulation of nitrate was subsequently suggested for rice coleoptiles (Kaiser and Brendlebehnisch, 1995; Fan *et al.*, 1997). An increased nitrate supply seems to prolong the survival of anoxic maize root tips (Roberts *et al.*, 1985), and experiments on tobacco transformants lacking nitrate reductase showed a higher production of ethanol and lactate in the transformants, resulting in a more pronounced acidifying metabolism compared with the wild type (Stoimenova *et al.*, 2003).

Nitrate reductase and nitrite reductase activities are essential for the assimilation of nitrate nitrogen into organic compounds, and the fact that these enzymes are present in the anoxic rice coleoptiles suggests that these organs assimilate nitrate (Reggiani *et al.*, 1993c; Mattana *et al.*, 1994). Lasanthi-Kudahettige *et al.* (2007) reported the up-regulation of two differentially expressed genes coding for nitrate reductase (*Os08g36480* and *Os08g36500*), while no differentially expressed genes were observed for nitrite reductase. The increase in nitrate reductase activity during anaerobic germination is prevented by cycloheximide, suggesting *de novo* synthesis of this enzyme, which is also supported by the presence of the respective mRNA (Mattana *et al.*, 1994). After its reduction to ammonium, nitrate can be assimilated through the glutamate synthase and glutamine synthetase pathway or by glutamate dehydrogenase (Reggiani *et al.*, 1993c). Lasanthi-Kudahettige *et al.* (2007), however, did not identify differentially expressed genes for both glutamate synthase and glutamate dehydrogenase under anoxia. Additionally, putative root glutamine synthetase (*Os03g12290*) is repressed by anoxia in the rice coleoptile (Lasanthi-Kudahettige *et al.*, 2007).

Nitrate reductase is reversibly activated at low pH (Kaiser and Brendlebehnisch, 1995), and since nitrate reduction is proton consuming, it could play a role in the regulation of cytoplasmic pH (Botrel *et al.*, 1996; Botrel and Kaiser, 1997; Ratcliffe, 1999; Greenway and Gibbs, 2003).

However, as outlined by Libourel *et al.* (2006), nitrate reduction generates protons when the only source of reducing power is glycolysis (Gerendás and Ratcliffe, 2002). The reasons for the beneficial effect of nitrate on pH regulation under anoxia are still largely unknown. Ivanov and Andreev (1992) reported negative effects of exogenous nitrate on growth and energy metabolism of rice, pea and wheat seedlings under strict anoxia, whereas Vartapetian (2005), using the same experimental conditions, observed a delay in mitochondrial ultrastructure changes for detached coleoptile in the presence of exogenous nitrate, in agreement with results of Reggiani *et al.* (1993d). While Ivanov and Andreev (1992) believed that exogenous nitrate had an adverse effect explained by a competition between nitrate reductase and ADH for reducing power (NADH), Polyakova and Vartapetian (2003) did not notice any adverse effect and, on the contrary, suggest that the protective action of nitrate could be explained by its role as a terminal acceptor of electrons in the absence of molecular oxygen. Since hardly any of the plants are able to assimilate the nitrate-derived nitrogen under anoxia (Mattana *et al.*, 1994), the potential ability of rice coleoptiles to perform this reduction may be part of the mechanisms that allow this species to germinate under anoxia (Reggiani *et al.*, 1993c).

Libourel *et al.* (2006) suggested an alternative hypothesis for the role of nitrate under anoxia in maize root, based on the observation that micromolar levels of nitrite can confer the same effect as nitrate itself. The reduction of nitrite to NO would consume H<sup>+</sup> but the low nitrite concentration observed seems to rule out a possible metabolic effect. On the other hand, an NO-dependent signal may be responsible for the reduction in metabolic rates and a consequent decrease in acidification.

Overall, the most probable positive effect of nitrate reduction in anoxic coleoptile is related to the prevention of the negative effects of cytoplasm acidification, since in this tissue nitrate undergoes reduction through an assimilatory pathway to NH<sub>4</sub><sup>+</sup> and amino acids (Vartapetian, 2005).

#### HYPOXIA-DEPENDENT ACCLIMATION TO ANOXIA

A hypoxic treatment can confer higher tolerance to a subsequent hypoxic/anoxic stress in *Arabidopsis thaliana* (Ellis *et al.*, 1999). In *A. thaliana*, *de novo* protein synthesis is required for acquired tolerance, since the addition of cycloheximide during the hypoxic pre-treatment completely blocked the ability of plants to acclimate to hypoxic stress (Ellis *et al.*, 1999). In rice too, seedlings exposed to hypoxia have been observed to be more tolerant of the subsequent anoxic stress (Ellis and Setter, 1999). Shoot tissues are often more tolerant than roots (Saglio *et al.*, 1988), and Menegus *et al.* (1991) confirmed this phenomenon in rice as well, leading to questions on how these differences can be explained biochemically (Mustroph *et al.*, 2006). According to Greenway and Gibbs (2003) low-oxygen intolerant species lack the ability to direct three processes that have energy-consuming priority under anoxia: (1) maintenance of membrane selectivity and integrity; (2) *de*

*novo* synthesis of specific proteins; (3) maintaining a safe cytoplasmic pH. Thus, hypoxia-induced acclimation to subsequent anaerobiosis would seem to be due to an increase in glycolysis and a more effective directing of carbohydrates to this pathway, resulting in a different energy flow (reviewed by Felle, 2005). Xia and Roberts (1994) reported that hypoxic pre-treatment prior to anoxic stress increased lactate secretion into the medium, reduced intracellular lactate concentrations, regulated pH better and improved survival under anoxia. But the biochemical and metabolic mechanisms underlying this phenomenon are not known.

#### PYROPHOSPHATE AS AN ALTERNATIVE ENERGY SOURCE

As explained above, ATP-consuming processes are often repressed under low oxygen (Pradet and Raymond, 1983) and one way this can happen is when pathways dependent on pyrophosphate [inorganic diphosphate (PPi)] are activated to replace ATP-consuming ones. An example of this switch is the preferential use of the sucrose synthase pathway instead of invertase route to generate fructose 6-phosphate the starting point of glycolysis. This saves ATP usage 1.5- to 2-fold (Mertens, 1991), depending on pyrophosphate availability. Processing of fructose 6-phosphate to fructose 1,6-bisphosphate by ATP-dependent phosphorylation by phosphofructokinase (Plaxton, 1996) can also be substituted by a PPi-dependent reaction. This is catalysed by pyrophosphate:fructose 6-phosphate 1-phosphotransferase (PFP), induction of which has been reported in anoxic rice coleoptiles (Gibbs *et al.*, 2000). The advantage of PFP rather than the ATP-dependent phosphofructokinase is that, under anoxia, PFP action can result in a saving of up to 50% in ATP yield during glycolysis through the use of PPi as a phosphate donor (Mertens *et al.*, 1990). As reported by Weiner *et al.* (1987), pyrophosphate is produced by a wide range of reactions and is usually present in large amounts in the cytosol. For example, pyruvate orthophosphate dikinase (PPDK) catalyses the reversible reaction  $\text{pyruvate} + \text{ATP} + \text{PPi} \rightarrow \text{PEP} + \text{AMP} + \text{PPi} + 2\text{H}^+$ , thus producing PPi (Moons *et al.*, 1998). Alternatively, PPi might be produced by PFP (reviewed by Greenway and Gibbs, 2003). The substrate cycle pyruvate kinase–pyruvate orthophosphate dikinase in anoxic rice coleoptiles also results in the production of PPi. Although energy release by PPi hydrolysis is almost half the energy produced by ATP-hydrolysis, PPi concentration in the cytoplasm is stable, at least in hypoxic tissue (Geigenberger *et al.*, 2000). This may mean that PPi could be an alternative source of energy for enzymes such as UDPG-pyrophosphorylase (coupled to sucrose synthase), phosphofructokinase and tonoplasmic  $\text{H}^+$  pyrophosphatase (Mustroph *et al.*, 2005),

#### THE SUCROSE SYNTHASE PATHWAY: CHANNELLING SUCROSE IN GLYCOLYSIS THROUGH AN ENERGY-CONSERVING PATHWAY

Sucrose synthase catalyses the reversible conversion of sucrose and UDP to UDP-glucose and fructose (Ricard

*et al.*, 1998). Sucrose synthase might have an important role in the tolerance of rice and maize roots to anoxia because this enzyme is responsible for channelling sucrose into anaerobic metabolism, rather than invertase, whose activity is depressed by anoxia in rice seedlings (Guglielminetti *et al.*, 1995a; Perata *et al.*, 1997). Furthermore, sucrose breakdown through the invertase and hexokinases pathway requires two molecules of ATP for the conversion of sucrose to Glc6P + Fru6P, while, as already explained, the sucrose synthase pathway coupled with UDPglucose-pyrophosphorylase consumes only one molecule of PPi for each sucrose molecule (Mustroph *et al.*, 2005). The importance of sucrose synthase in sucrose hydrolysis in maize (Ricard *et al.*, 1998) and *Potamogeton distinctus* (pondweed; Harada *et al.*, 2005) has been demonstrated. Ricard *et al.* (1998) found that a maize sucrose synthase double mutant showed higher levels of sucrose under oxygen deficiency compared with the wild type, suggesting that sucrose synthase has a role in sucrose utilization under fermentative metabolism conditions. Furthermore, the presence of sucrose synthase activity is associated with improved anoxic tolerance in maize seedlings (Ricard *et al.*, 1998). In rice, several enzymatic activities required for the sucrose synthase pathway (essentially sucrose synthase and fructokinase) were found to be induced by anaerobic treatment. On the other hand, no such induction was observed in wheat and barley grains under anoxia (Guglielminetti *et al.*, 1995a; Perata *et al.*, 1996). Experiments with radiolabelled sucrose suggested that only rice is able to convert sucrose to  $\text{CO}_2$  under anoxia, with efficiency comparable to (or even higher than) that of the embryos incubated in air (Guglielminetti *et al.*, 2001). In wheat and barley this conversion is five times less efficient under anaerobiosis, probably as a result of lower sucrose degradation, since all three of these cereals are able to convert exogenous glucose and fructose to ethanol efficiently under anoxia (Guglielminetti *et al.*, 2001). Rice's greater capacity to induce sucrose synthase at both the transcriptional and translational levels, when compared with maize (Ricard *et al.*, 1991), suggests that this enzyme plays an important role in rice tolerance to anaerobiosis (Perata *et al.*, 1997).

#### AMINO ACID ACCUMULATION AND CELL HOMEOSTASIS

Anoxia results in the accumulation of free amino acids in rice coleoptiles (Alpi and Beevers, 1983; Menegus *et al.*, 1984; Reggiani *et al.*, 1993c; Lee and Lin, 1995b). These amino acids may have a functional role in maintaining the high osmotic pressure (low solute potential) typical of anoxia-grown rice coleoptiles (Menegus *et al.*, 1984). During the first 4 d of anoxic germination, the accumulation of amino acids is known to derive from the assimilation of the nitrate stored in the seeds (Mattana *et al.*, 1993), whereas later, most of the amino acids present in the rice coleoptiles probably come from the breakdown of storage protein followed by the translocation of amino acids (Reggiani *et al.*, 1993c).

Besides quantitative changes, a shift in the composition of the amino acid pool is observed under anoxia in rice seedlings, with a decrease in the levels of glutamate, aspartate and their amides, and an accumulation of alanine, GABA and the diamine putrescine (Reggiani *et al.*, 1989b), synthesized directly from glutamate. Alanine and GABA are the two bio-compatible solutes that represent up to 50 % of the amino acid pool in rice seedlings after a few hours of anaerobiosis (Reggiani *et al.*, 1989b).

Alanine is synthesized through transamination (with glutamate as the amino group donor) in a reaction catalysed by alanine aminotransferase and may contribute efficiently to anoxia tolerance (Ricoult *et al.*, 2005). The activity of alanine aminotransferase is enhanced by anaerobiosis, resulting in an accumulation of alanine in several plant tissues (Muench and Good, 1994; Ricoult *et al.*, 2005). Although the reaction is fully reversible, alanine synthesis is favoured, because  $\gamma$ -ketoglutarate is continuously removed from re-synthesizing glutamate by the glutamine synthetase/glutamate synthase cycle (Reggiani *et al.*, 1988). Alanine production has been demonstrated in rice seedlings (Reggiani *et al.*, 1989b).

Another compound that accumulates under anoxic stress conditions is the non-protein amino acid GABA (Aurisano *et al.*, 1995). The synthesis of GABA in rice coleoptiles would be advantageous for the concomitant  $H^+$  consumption during the decarboxylation of L-glutamate and the additional low toxicity of these amino acids (Aurisano *et al.*, 1995). The synthesis of putrescine and polyamines in higher plants from the amino acids arginine and ornithine is catalysed by arginine decarboxylase and ornithine decarboxylase, respectively (Reggiani *et al.*, 1989b). The increased arginine decarboxylase activity due to the synthesis of the enzyme under anaerobic conditions is related to the tolerance of plant tissues to an oxygen deficit, as reported for seedlings of rice, barnyard grass (*Echinochloa crus-galli*), maize, rye, barley and wheat (Reggiani, 1994). The role of arginine decarboxylase could be related to different biochemical reactions. First, decarboxylation reactions result in the consumption of hydrogen ions, which is beneficial under anoxic conditions for the attenuation of the cytoplasm acidosis, as suggested for arginine and glutamate decarboxylation (Menegus *et al.*, 1991; Reggiani, 1994; Reggiani *et al.*, 1993a). Secondly, the accumulation of putrescine may help the cell to maintain the ionic balance, counteracting the accumulation of organic acids such as lactate and succinate (Reggiani *et al.*, 1993b). This hypothesis is supported by evidence that the level of putrescine is inversely associated with  $K^+$  content (Reggiani *et al.*, 1993b). Furthermore, putrescine may be involved in the elongation of the rice coleoptile under anoxia (Reggiani *et al.*, 1989a).

To summarize, anoxic conditions in rice seedlings lead to an increase in the level of total amino acids, thus compensating for the osmotic potential that cannot rely simply on soluble hexose, which is consumed at a high rate in the process of energy-formation (Bertani *et al.*, 1981; Menegus *et al.*, 1984; Kennedy *et al.*, 1992; Drew, 1997).

## GIBBERELLIN-INDEPENDENT $\alpha$ -AMYLASE INDUCTION IN ANOXIC RICE SEEDLINGS

An advantage of rice over the other cereals seems to be its ability to form a complete set of starch-degrading enzymes, such as  $\alpha$ - and  $\beta$ -amylases, debranching enzymes, maltase, and the enzymes needed for the complete degradation of starch and its subsequent utilization through glycolytic flux in the absence of oxygen (Perata *et al.*, 1992a; Perata and Alpi, 1993; Guglielminetti *et al.*, 1995a, b; Loreti *et al.*, 2003b). Amongst these enzymes,  $\alpha$ -amylase plays a major role in starch degradation (Sun and Henson, 1991). They are produced in rice seeds under anoxia (Perata *et al.*, 1992a) but not in anoxia-intolerant cereals such as wheat, barley, oat and rye (Guglielminetti *et al.*, 1995b). In the absence of  $\alpha$ -amylase, starch is not degraded, and anoxia-intolerant cereals such as wheat and barley soon suffer from sugar starvation, and eventually die (Perata *et al.*, 1996).

The production of different  $\alpha$ -amylase isoforms (Yamaguchi *et al.*, 1995) has been reported in anoxic rice seedlings (Perata *et al.*, 1997). Rice  $\alpha$ -amylases are encoded by at least ten genes, grouped into three subfamilies: *Amy1* (A-B-C), *Amy2* (A) and *Amy3* (A-B-C-D-E-F) (Rodriguez *et al.*, 1992). Isoforms A and B (encoded by the *RAmy1A* gene) have been observed in extracts from both aerobic and anaerobic seedlings, whereas isoforms G and H (encoded by the *RAmy3D* gene) have only been identified in anoxic rice seedlings (Perata *et al.*, 1997). These observations are in agreement with those of Huang *et al.* (1999), who observed *RAmy1A* repression by anoxia, whereas *RAmy3D* is anoxia-induced. *Ramy1A* and *RAmy3D* differ in their respective regulation mechanisms: *Ramy1A* is hormonally modulated and *RAmy3D* sugar modulated (Morita *et al.*, 1998; Loreti *et al.*, 2003b). *RAmy1A* is readily induced by gibberellin (GA) hormone under aerobic conditions, but only limited transcript accumulation is observed under anoxia (Perata *et al.*, 1993). *RAmy3D* does not possess a GA-response *cis*-acting element in its promoter and thus cannot be induced by GA in any circumstances (Morita *et al.*, 1998; Loreti *et al.*, 2003b). Under aerobic conditions *RAmy3D* is expressed at low levels, whereas *RAmy3D* mRNA is easily detectable in the anoxic embryo-less half grain (Loreti *et al.*, 2003b), confirming that embryo-derived GAs are not essential for the induction of this gene. The GA-deficient mutant *Tan-ginbozu* allowed the evaluation of the importance of GA-induced  $\alpha$ -amylase on rice grain germination (Loreti *et al.*, 2003a). Although the induction of GA-dependent  $\alpha$ -amylase genes in the mutant was dramatically reduced in the absence of exogenous GAs, the *Tan-ginbozu* mutant germinated promptly under anoxia, suggesting that other  $\alpha$ -amylase genes, that are not GA-modulated, are sufficient for triggering the production of enough  $\alpha$ -amylase to efficiently degrade starch and allow germination (Loreti *et al.*, 2003b). Thus, the germination of rice grains under anoxia is GA-independent, and the vigorous expression of *RAmy3D* compensates for the lower expression of the GA-modulated *RAmy1A* (Loreti *et al.*, 2003b).

Exogenous glucose can inhibit the induction of *RAmy3D*, thus explaining the aerobic repression of *RAmy3D*. Under

aerobic conditions, the strong and rapid GA-dependent expression of *RAmy1A* in the aleurone layer results in a high sugar concentration which represses *RAmy3D* expression (Loreti *et al.*, 2003b). On the other hand, under anoxia, the level of soluble sugars drops (Guglielminetti *et al.*, 1995a; Perata *et al.*, 1996), and sugar starvation induces the expression of *RAmy3D*, suggesting that a direct anaerobic induction of *RAmy3D* is unlikely (Loreti *et al.*, 2003b). *RAmy3D* expression is also strongly up-regulated in anoxic rice coleoptiles (Lasanthi-Kudahettige *et al.*, 2007). Overall, *RAmy3D* expression appears to be of importance for rice germination under anoxia, at least during the first days of anaerobiosis, since *RAmy1A* expression takes place at a level comparable with aerobic expression only after 6 d of anoxia (Perata *et al.*, 1993). These results suggest that *RAmy3D* and *RAmy1A* co-operate in the process of anoxic starch degradation, with *RAmy3D* allowing the rice grains to degrade starch during the initial stages of germination when the induction of *RAmy1A* is slowed down by GA-unavailability and/or insensitivity (Loreti *et al.*, 2003a).

#### SUCROSE SYNTHESIS UNDER OXYGEN DEPRIVATION

During germination, the breakdown product of starch (glucose) is absorbed through the epithelium of the embryo to the scutellum where sucrose synthesis takes place (Nomura *et al.*, 1969). This disaccharide is very likely transported into the growing tissues of the developing seedlings, thus furnishing carbohydrate as substrate for glycolysis and fermentation.

The synthesis of sucrose in germinating rice seeds under anoxia is due to the simultaneous induction of sucrose-phosphate synthase and glucose-6-phosphate isomerase (Bertani *et al.*, 1981; Ricard *et al.*, 1991; Guglielminetti *et al.*, 1995a, 1999). Although sucrose synthesis under anoxia represents a metabolic cost, it is tempting to speculate that sucrose availability is of importance to allow transport of carbon units resulting from starch degradation to the growing coleoptile. Anoxia has a negative effect on sucrose-phosphate synthase activity in anoxic barley grains (Guglielminetti *et al.*, 1999). Guglielminetti *et al.* (1999) observed sucrose synthesis in isolated anoxic rice embryos fed with glucose, indicating that *in vivo* conversion of glucose into sucrose takes place. On the other hand, barley grains were not able to synthesize sucrose under anoxia. The role of sucrose synthase in sucrose synthesis appears to be marginal under anoxia, possibly because of the pH optimum of this enzyme (7.5–8; Avigard, 1982), although sucrose phosphate synthase, which has a broader pH optimum (6–8; Avigard, 1982), could be active even at the sub-acidic pH typical of the anoxic condition (Roberts *et al.*, 1984b). Sucrose synthase and sucrose phosphate synthase may thus be involved in the synthesis of sucrose under aerobic conditions, while sucrose synthesis under anoxia appears to be strictly linked to the activity of sucrose-phosphate synthase (Guglielminetti *et al.*, 1999). However, under anoxia, sucrose content in embryos from germinating rice grains does not increase, possibly because there is less glucose available and

because of its concomitant conversion into ethanol (Guglielminetti *et al.*, 1999). Sucrose synthesis is even more affected in anoxia-intolerant cereals such as barley, as sucrose disappeared in barley embryos when the tissue is incubated under anoxic conditions, even in the presence of exogenous glucose (Guglielminetti *et al.*, 1999).

#### HEXOKINASES UNDER ANOXIA

Hexokinase catalyses the production of hexose-6-phosphate from hexoses such as glucose or fructose. Guglielminetti *et al.* (2000) identified six isoforms of hexokinase in rice embryos: three glucokinases (*GK1*, *GK2* and *GK3*), two hexokinases (*HK1* and *HK2*) and one fructokinase (*FK1*). The same authors then identified a second fructokinase, and the respective names were changed to *OsFK1* and *OsFK2*. *OsFK1* is preferentially expressed, both at a transcriptional and translational level, under aerobic conditions, whereas *OsFK2* is induced by anoxia (Guglielminetti *et al.*, 2006). Since sucrose hydrolysis by means of sucrose synthase releases fructose and UDP-glucose, fructokinase plays a key role in driving this hexose into glycolysis. Lasanthi-Kudahettige *et al.* (2007) showed that only *OsHXX7* (*Os05g09500*) is up-regulated in anoxic rice coleoptiles 4 d after germination, probably as a consequence of sugar starvation, since this hexokinase gene was shown to be sugar-repressed (Cho *et al.*, 2006).

#### RICE IS ABLE TO SUSTAIN MITOCHONDRIAL ULTRASTRUCTURE UNDER ANOXIA

Mitochondria are usually negatively affected by anoxia probably because metabolism carried out by these organelles is strictly connected to the availability of oxygen. However, anoxia-germinated seedlings of rice develop coleoptiles that preserve the intact structure and potential functional activity of most of the sub-cellular organelles, including mitochondria, which are capable of oxidative phosphorylation during post-anaerobic re-oxygenation (reviewed by Vartapetian, 2005). A lower level of cytochromes has been observed in anoxically grown rice, but mitochondrial cytochrome content increases rapidly (i.e. within 24 h) on air re-adaptation from anoxia (Millar *et al.*, 2004).

Rice coleoptile ultrastructure is also resilient under secondary anoxia, i.e. when seedlings that were germinated aerobically were transferred to an anaerobic environment for several days (Vartapetian, 2005). However, high anoxia-resistance of mitochondrial ultrastructure is lost if coleoptiles are detached from the rest of the seedling (reviewed by Vartapetian, 2005). Damage is evident even after relatively short-term anoxia, but tolerance can be restored by the addition of exogenous glucose. This suggests that the availability of readily fermentable carbohydrates, which fuel the anaerobic metabolism, is crucial for maintaining a sub-cellular ultrastructure in rice (Perata *et al.*, 1992a).

Fox and Kennedy (1991) observed that tricarboxylic acid cycle enzymes succinyl-CoA synthase and citrate synthase behave similarly in aerobically and anaerobically grown

rice seedlings, whereas the activities of 2-oxoglutarate dehydrogenase complex, aconitase, isocitrate dehydrogenase and fumarase were reduced in anaerobic seedlings. The microarray analysis of Lasanthi-Kudahettige *et al.* (2007) reveals differentially expressed genes involved in TCA. Among these genes *Os04g32020* (2-oxoglutarate dehydrogenase E1 component) and *Os01g14580* (putative isocitrate dehydrogenase) were anoxia-down regulated, in agreement with data based on enzymatic activities.

#### THE ANAEROBIC SYNTHESIS OF LIPIDS IS INVOLVED IN CELL MEMBRANE TURNOVER

Some authors have hypothesized that the hydrogenation of unsaturated fatty acids and the biosynthesis of lipids are adaptive mechanisms under anaerobic stress because they function as terminal acceptors of respiratory electrons and protons (Kennedy *et al.*, 1991; Fox *et al.*, 1994). As reviewed by Vartapetian (2005), no changes in fatty acid composition of lipids and in the ratio unsaturated:saturated fatty acids are observed during the anaerobic germination of rice seedlings, thus anaerobic synthesis of lipids and hydrogenation of unsaturated fatty acids is unlikely to act as an alternative mechanism of electron acceptance. On the other hand, anaerobic production and accumulation of lipids in the primary leaves of *Echinochloa phyllopogon* are regarded as an adaptive mechanism of this plant, which is tolerant of anoxia. It seems probable that lipid biosynthesis and accumulation in *Echinochloa phyllopogon* is a mechanism through which this plant re-oxidizes pyridine nucleotides, thus by-passing the major problem of anaerobiosis (Kennedy *et al.*, 1991). However, the rice coleoptile requires anaerobic synthesis of lipids. This synthesis is an adaptive mechanism to achieve the turnover of saturated fatty acids, phospho-, glyco- and neutral lipids under extreme conditions of oxygen deficiency, thereby stabilizing cell membranes under strict anoxia (Vartapetian, 2005). The maintenance of electrogenic membrane potentials in anoxic rice coleoptiles is evidence of the conservation of the integrity of cellular membranes, and ATP shortage may be responsible for the partial repolarization of the  $E_m$  following anoxia (Zhang and Greenway, 1995).

#### ANAEROBIC GENE EXPRESSION AND PROTEIN SYNTHESIS

Plants respond to low oxygen stress by regulating gene expression at both transcriptional and translational levels (reviewed by Bailey-Serres and Chang, 2005). The synthesis of most of the aerobic proteins is repressed, probably as a mechanism of energy conservation, since mRNA levels are sometimes comparable. On the other hand, the induction of ANPs, which are often involved in carbohydrate metabolism, takes place (Sachs *et al.*, 1980; Das and Uchimiya, 2002; Bailey-Serres and Chang, 2005). Lasanthi-Kudahettige *et al.* (2007) observed that anoxic coleoptiles show increased expression of 1364 probe sets and decreased expression of 1770 probe sets, supporting the evidence that anoxia does not only result in a down-regulation of unnecessary genes. Several of the up-regulated probe sets were found to encode

for glycolytic enzymes, and many genes involved in pyruvate metabolism were reported to be strongly up-regulated in anoxic coleoptiles.

In the rice cultivar FR13A, Huq and Hodges (1999) identified two cDNA fragments (anaerobically inducible early, *AIE*), which are induced in adult plants subjected to anoxia earlier than *ADHI* and *PDC*. Treatment with cycloheximide revealed that *AIE* transcript accumulation under anoxia requires new protein synthesis, although a role of these genes in submergence tolerance when some oxygen is present has been ruled out by the fact that both the flooding-tolerant FR13A and the flooding-sensitive IR54 cultivars display a comparable induction (Huq and Hodges, 1999). *AIE* induction seems to be anoxia-specific, since other stress conditions did not increase *AIE* transcript accumulation (Huq and Hodges, 1999). *AIE* genes are amongst the up-regulated genes of rice coleoptiles ('Nipponbare') germinated and grown for 4 d under anoxia, as observed by the microarray experiments of Lasanthi-Kudahettige *et al.* (2007). Although several ANPs have still to be identified (Chang *et al.*, 2000), most of the known ANPs seem to be enzymes of glycolysis, ethanol formation and related processes of carbohydrate metabolism and aerenchyma formation (Loreti *et al.*, 2003a).

#### RE-AERATION INDUCES REACTIVE OXYGEN SPECIES (ROS) PRODUCTION

ROS are likely to be produced immediately anaerobic plant tissues are exposed to normal oxygen tensions, thus causing severe peroxidation (Biemelt *et al.*, 1998). Furthermore, Santosa *et al.* (2007), suggested that lipids also peroxidize under hypoxic (but not anoxic) conditions, and not only after re-exposure of rice seedlings to air.

Several antioxidative enzymes are involved in the detoxification of these ROS in plants (Asada and Takahashi, 1987). As shown by Ushimaru *et al.* (1992), underwater-germinated rice seedlings lack these antioxidative systems which are, however, rapidly synthesized after the seedlings have been exposed to air, with activities exceeding the control level. Lasanthi-Kudahettige *et al.* (2007) reported a strong down-regulation of a catalase (*Os02g02400*) in anoxic rice coleoptiles. However, when submerged seedlings were exposed to air, the activities of catalase exceeded those detected in aerobically grown controls (Ushimaru *et al.*, 1994), suggesting that the  $H_2O_2$ -degradation system is rapidly reconstituted under aerobic conditions. It is thus likely that, in the absence of oxygen, the production of  $H_2O_2$  is negligible, making catalase superfluous in the anoxic coleoptile. The down-regulation of catalase may be an energy-saving strategy to produce only housekeeping enzymes and those needed for anaerobic metabolism (Perata and Alpi, 1993; Fukao and Bailey-Serres, 2004). Assuming that hydrogen peroxide can act as a second messenger in low-oxygen signalling regulating gene expression (Fukao and Bailey-Serres, 2004; Bailey-Serres and Chang, 2005), another explanation may be that the decline in catalase activity contributes to the build-up of  $H_2O_2$  to the level required for it to act as a signal. Although the adaptive

value of catalase action has not been established experimentally for anoxic coleoptiles it is notable that a line of rice (FR13A) with superior tolerance of submergence as a small seedling is able to suppress lipid peroxidation possibly by means of re-directing superoxide to other less damaging reactions. These may involve  $H_2O_2$  catalase and synthesis of sub-toxic amounts of acetaldehyde from ethanol (Boamfa *et al.* 2005; Santosa *et al.*, 2007).

Several other differentially expressed genes coding for antioxidant enzymes include superoxide dismutase, ascorbate peroxidase, dehydroascorbate reductase and glutathione reductase. Each of these has been identified in anoxic rice coleoptiles (Lasanthi-Kudahettige *et al.*, 2007). Two genes coding for superoxide dismutase (*Os03g22810* and *Os07g46990*) are down-regulated and two (*Os06g05110* and *Os03g11960*) are up-regulated under anoxia. Genes coding for ascorbate peroxidase and monodehydroascorbate reductase are unaffected by anoxia whereas a gene coding for dehydroascorbate reductase (*Os05g02530*) and a gene coding for glutathione reductase (*Os02g56850*) are down-regulated by anoxia. The antioxidative system therefore seems to be regulated in response to changes in oxygen tension in rice seedlings.

#### ALDEHYDE DEHYDROGENASE (ALDH) ACTIVITY INCREASES UPON RE-AERATION

Besides the production of ROS, re-aeration also induces the production of acetaldehyde, arising from the oxidation of ethanol (Zuckermann *et al.*, 1997; Boamfa *et al.*, 2003). Acetaldehyde can be toxic for the cell since it tends to form adducts with proteins and DNA (Perata *et al.*, 1992b). Another explanation for its toxicity may be that acetaldehyde can donate electrons for the formation of ROS via xanthine oxidase (Mustroph *et al.*, 2006). Mustroph *et al.* (2006) compared the production of ethanol and acetaldehyde in rice and wheat seedlings subjected to 4 h of anoxic treatment and then re-exposed to air for another 4 h under darkness. A transient burst of acetaldehyde was observed in shoots of both species, but afterwards acetaldehyde levels dropped to the initial value. Acetaldehyde production under anoxia correlates with the activity of PDC rather than that of ADH, thus a transient acetaldehyde production takes place as a possible outcome of an imbalance between the activities of PDC and ADH (Mustroph *et al.*, 2006). Alternatively Zuckermann *et al.* (1997) proposed that the  $H_2O_2$ -dependent, catalase-controlled peroxidation of ethanol upon re-aeration may explain post-anoxic acetaldehyde production.

ALDH converts acetaldehyde to acetic acid and this enzyme may help to protect the plant from post-hypoxically produced acetaldehyde (Tsuji *et al.*, 2003a). Whilst ALDH activity in submerged plants is comparable with that of the aerobic control, the activity of the enzyme increases gradually upon re-aeration (Tsuji *et al.*, 2003a). Rice has two mitochondrial ALDH genes, named *ALDH2a* and *ALDH2b* (Tsuji *et al.*, 2003b). Submergence represses the transcription of *ALDH2b* and induces the transcription of *ALDH2a*, whereas re-aeration results in an increased level of *ALDH2b* and in a decline in *ALDH2a* mRNA (Tsuji *et al.*, 2003a). These data suggest that *ALDH2a* contributes

to the increase in the total ALDH activity after the re-aeration of rice plants (Tsuji *et al.*, 2003a). Indeed, the increase in *ALDH2a* transcript under submergence does not increase *ALDH2a* protein, suggesting that selective translation takes place. However, the *ALDH2a* mRNA might be pooled for the preparation of the upsurge to acetaldehyde after re-aeration (Zuckermann *et al.*, 1997), and the post-anoxic increase in *ALDH2a* protein supports this evidence (Tsuji *et al.*, 2003a). Microarray data are in agreement with this observation, highlighting that a gene coding for a mitochondrial ALDH (*ALDH2a*, *Os02g49720*) is induced in anoxic rice coleoptiles (Lasanthi-Kudahettige *et al.*, 2007). Cytosolic ALDH does not seem to be involved in detoxification since the mRNA level of both *ALDH1a* and *ALDH1b* decreases following submergence (Tsuji *et al.*, 2003a). A lower acetaldehyde accumulation in rice shoots may thus contribute to the higher tolerance of this species to anaerobic and post-anoxic conditions.

#### THE ROLE OF HORMONES

Although extension of all vegetative organs of the shoot of the vast majority of rice lines can be stimulated by ethylene (reviewed by Jackson, 2008), little is known about the role of plant hormones in anoxic rice germination and coleoptile elongation.

Under anoxia the role of ethylene is still not clear. Since ethylene biosynthesis requires molecular oxygen for the activity of the enzyme ACC oxidase (Bleecker and Kende, 2000), the reaction is unlikely to take place under strict anaerobic conditions. Most of the literature available regarding ethylene physiology under low oxygen refers to plants, including rice, under hypoxia or submergence. The production of ethylene by coleoptiles is slowed by partial oxygen shortage (Raskin and Kende, 1984). Under hypoxic conditions, the rapid elongation of coleoptiles of rice seedlings is possibly due to an interaction between ethylene and indolacetic acid (IAA), the former prolonging the IAA-dependent elongation of rice coleoptiles (Ishizawa and Esashi, 1984). The increase in IAA level found in coleoptiles of intact seedlings during anaerobic treatment could be due to a translocation from the endosperm, in which the hormone is contained in a great quantity (Pegoraro *et al.*, 1988). IAA and anaerobiosis have, however, no synergistic effect on rice coleoptile elongation (Pegoraro *et al.*, 1988).

Horton (1991) showed that GA, ethylene and IAA enhanced coleoptile growth under anaerobic conditions but not under anoxia, whereas ABA and kinetin were inhibitory. Indeed, anoxia-germinated as well as aerobic rice seedlings subjected to anoxia release free ABA in the culture media with time (Mapelli *et al.*, 1995). This suggests that the higher coleoptile length achieved under anoxia is possibly linked to a faster ABA release in the medium which would thus avoid the negative effects of this hormone (Mapelli *et al.*, 1995). In fact, when seedlings of wheat (anoxia intolerant) are subjected to anoxia, this release of free ABA is not observed (Mapelli *et al.*, 1995). Furthermore, the enhancement of coleoptile growth might be linked to a lower ABA synthesis under anoxia

(Hoffmannbenning and Kende, 1992). It is difficult, however, to envisage a clear role of plant hormones in order to explain the anoxic rice coleoptile elongation. Since the anoxic seed and coleoptile make no ethylene and are not responsive to the gas, the phenomenon of anoxic germination and coleoptile growth should not be confused with ethylene-promoted coleoptile and leaf extension which takes place in submerged rice when enough oxygen is present to allow ethylene to be made and to act (reviewed by Jackson, 2008). The recent physiology, QTL analyses, sequencing and functional analysis of the *Sub1* locus in rice are thus not relevant to anoxic rice growth, although they help explain submergence-stimulated elongation where some oxygen is present. Rice's capacity to germinate under complete anoxia cannot therefore be explained in terms of *Sub1* genes. The 'M202' and 'Nipponbare' cultivars, neither of which has the *Sub1A* gene (Fukao *et al.*, 2006; Xu *et al.*, 2006), display a vigorous germination under anoxia (Fig. 3).

#### SIGNALLING PATHWAYS IN THE ANOXIC RICE SEEDLINGS

Although oxygen sensors have been described in bacteria, yeast, insects and mammalian cells, the identity of oxygen sensor(s) in plants is still not clear. However, haem, or protein that encloses a haem cofactor, are possible candidates (reviewed by Dat *et al.*, 2004). Non-symbiotic haemoglobins are induced under hypoxia (Taylor *et al.*, 1994) and plant haemoglobins may play a role in modulating low oxygen responses, as low oxygen concentration

induces the class-1 haemoglobin gene *GLB1* in arabidopsis (Trevaskis *et al.*, 1997; Klok *et al.*, 2002). Furthermore, transgenic plants overexpressing *GLB1* showed increased survival under severe hypoxia (Hunt *et al.*, 2002). However, because non-symbiotic haemoglobins have a very high affinity for O<sub>2</sub> that exceeds that of mitochondrial cytochrome oxidase, and also displays a slow O<sub>2</sub> dissociation rate, they are unlikely to function as O<sub>2</sub> transporters or as low-oxygen sensors. However, non-symbiotic haemoglobins may act as NO-detoxifying agents under hypoxia, where conspicuous amounts of NO are generated (reviewed by Perazzolli *et al.*, 2005).

Microarray data have revealed several transcription factors whose expression increases in response to various regimes of oxygen deprivation in arabidopsis and rice, such as heat shock factors, ethylene response-binding proteins, MADS-box proteins, AP2 domain, leucine zipper, zinc finger and WRKY factors (Loreti *et al.*, 2005; Lasanthi-Kudahettige *et al.*, 2007). Furthermore, genes that encode putative components of the signal transduction pathway have been identified, such as calcium binding proteins, protein-modifying enzymes (i.e. receptor-like kinases and MAP kinases) and also already known components such as the gp91\_phox subunit of the NAD(P)H oxidase (reviewed by Bailey-Serres and Chang, 2005). Lasanthi-Kudahettige *et al.* (2007) demonstrated the induction of several ethylene responsive factors (ERF) and heat shock factors in anoxic rice coleoptiles. ERF-like transcription factors are induced both in *A. thaliana* and rice, and these genes may play a role in the growth of coleoptiles if the plants regain access to oxygen and the capacity to synthesize ethylene.

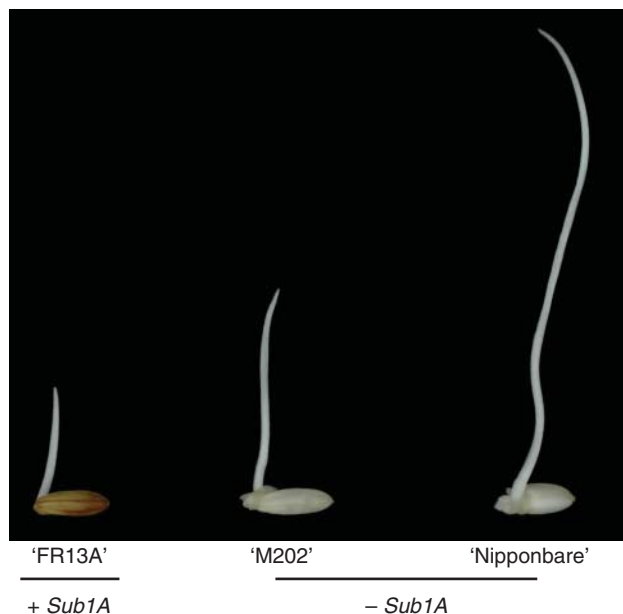


FIG. 3. Anoxic germination of rice cultivars lacking *Sub1A* (*- Sub1A*; 'M202' and 'Nipponbare') in comparison to 'FR13A' (*+ Sub1A*). Both 'M202' and 'Nipponbare' display a vigorous germination when compared with 'FR13A', suggesting that the *Sub1A* gene cannot explain the ability to germinate under anoxia.

#### CONCLUSIONS

Since the biochemical characterization of anoxic rice germination by Alpi and Beevers in 1983, research into rice adaptation to low oxygen has provided many new insights. Microarrays have led to new tools that can investigate the anaerobic response of rice. Many questions are, however, still unanswered.

T-DNA tagged rice mutant and Full-length cDNA Over-eXpresser (FOX) rice lines are promising genomic tools for the identification of genes that reside behind a particular phenotype (Hsing *et al.*, 2007; Nakamura *et al.*, 2007). The study of those lines displaying an abnormal phenotype in terms of anoxic germination behaviour might bring new clues on the molecular mechanisms that allow rice to germinate and elongate the coleoptile under complete anoxia. Furthermore, understanding how oxygen-sensing mechanisms function remains a fascinating challenge.

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