Anoxia Tolerance and Sugar Level in Roots and Coleoptiles of Aerobically Germinated Rice

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Abstract: The root and coleoptile growth of aerobically germinated rice (Oryza sativa L.) seedlings was suppressed by anoxic stress, but the suppression was more conspicuous in roots than in coleoptiles. In this study, the physiological and metabolic responses of roots to anoxia were compared with those of coleoptiles in aerobically germinated rice seedlings. ATP concentration in the coleoptiles was higher than that in the roots under anoxia. Alcohol dehydrogenase activity and ethanol concentration in the coleoptiles were also higher than in the roots under anoxia, which suggest that ethanolic fermentation may be more active in the coleoptiles than in the roots where glycolysis and ethanolic fermentation are the main source of ATP production. Activity of α-amylase, which mobilized reserved starch in the seeds, was not detected in the roots and coleoptiles of rice seedlings either under aerobic or anoxic conditions. However, α-amylase activity was found in endosperms of the rice seedlings even under anoxia. The α-amylase activity in anoxic endosperms was 64% of that in aerobic endosperms. Sugar concentration in the anoxic coleoptiles was higher than that in the anoxic roots. ATP and ethanol concentrations in the roots and coleoptiles of rice seedlings were correlated well with sugar concentrations in the roots and coleoptiles, which also showed a good correlation with anoxia tolerance of roots and coleoptiles with respect to elongation. These results suggest that the sugar level may be important for the anoxia tolerance of the roots and coleoptiles since the availability of sugar is essential for the operation of ethanolic fermentation leading to ATP production under anoxia, and it may serve to distinguish the anoxia tolerance of the roots and coleoptiles of the rice seedlings.

Key words: α-Amylase, Anaerobiosis, Anoxia tolerance, ATP, Ethanolic fermentation, Rice, Sugar.

Among cereals, only rice is able to germinate and elongate the coleoptiles under complete anoxia (Alpi and Beevers, 1983; Cobb and Kennedy, 1987; Lasanthi-Kudahettige et al., 2007). The tolerance of rice coleoptiles to anoxia was related to the ability to maintain active glycolysis, which replaces the Krebs cycle as the main source of ATP production under limited oxygen conditions (Drew, 1997; Vartapetian and Jackson, 1997; Tadege et al., 1999; Magneschi and Perata, 2009). Active glycolysis is linked predominately to ethanolic fermentation and rice coleoptiles display a strong ethanolic fermentation system under anoxia (Setter et al., 1997; Boamfa et al., 2003; Mustroph et al., 2006; Agarwal et al., 2007).

On the other hand, the roots of rice are as sensitive to anaerobiosis as those of other cereals such as maize, wheat and barley (Drew, 1997; Perata et al., 1997; Lasanthi-Kudahettige et al., 2007; Magneschi and Perata, 2009). Rice seeds germinated under anoxia or in water, develop longer coleoptiles than those germinated under aerobic conditions, while the roots fail to grow under the anoxia or in water (Alpi and Beevers, 1983). This phenomenon, called the “snorkel effect”, is thought to increase the possibility of the coleoptiles to contact with the water surface (Kordan, 1974), and is considered as a means to diffuse oxygen internally to the roots and endosperms (Turner et al., 1981). However, in these studies non-germinated rice seeds were exposed to anoxia or oxygen-limited conditions, and only the rice coleoptiles grew under oxygen-limited conditions (Perata et al., 1997; Gibbs et al., 2003; Lasanthi-Kudahettige et al., 2007; Magneschi and Perata, 2009). Therefore, the difference in anoxia tolerance between coleoptiles and roots of rice seedlings germinated under aerobic conditions is still not clear. In addition, even the coleoptiles of anoxia-intolerant cereals can grow under oxygen-limited conditions and ethanol production was observed in these coleoptiles when seeds...
were germinated under aerobic conditions and transferred to the oxygen-limited conditions (Raymond et al., 1985; Guglielminetti et al., 1995). These results indicate that these anoxia-intolerant cereals are also able to utilize sugars for glycolysis and ethanolic fermentation leading to ATP production even in the limited oxygen conditions. However, as noted by Goggin and Colmer (2007), the effects of anoxia on reserve carbohydrate catabolism in anoxia-intolerant cereals and organs germinated under aerobic conditions have not been evaluated. The objective of this study was to investigate the difference in the tolerance and reserve carbohydrate catabolism between anoxia-tolerant coleoptiles and anoxia-intolerant roots of rice seedlings germinated under an aerobic condition.

Materials and Methods

1. Plant material and anoxic treatment

Caryopses of rice (Oryza sativa L. cv. Nipponbare) were surface sterilized in an aqueous solution of 25 mM sodium hypochlorite for 15 min, rinsed four times in distilled water, and germinated on two sheets of filter paper (No 1; Toyo Ltd., Tokyo) for three days in darkness at 25°C. Uniform seedlings were then transferred to 9-cm Petri dishes (10 seedlings per dish) each containing two sheets of filter paper moistened with 10 mL of distilled water, and subjected to anoxic treatment. The Petri dishes were placed into 5-L jars at 25°C. Aerobic seedlings were supplied with air flowing at 200 mL min⁻¹ for 24 and 48 hr. Aerobic seedlings were killed by adding 2 mL of 40 mM dinitrosalicylic acid solution containing 400 mM NaOH and 1 M KNa tartrate, and then placing it immediately into a boiling water bath for 5 min. After dilution with distilled water, absorbance at 530 nm of the reaction mixture was measured and reducing power evaluated using a standard curve obtained with glucose.

2. Measurements of root and coleoptile length

The length of seminal roots and coleoptiles of rice seedlings at the start and the end of the 24- and 48-hr anoxic treatment was measured with a ruler, and elongation of the roots and coleoptiles during the treatment was determined.

3. Extraction and determination of ATP and ethanol

After a 48-hr anoxic treatment, rice seedlings were killed with liquid N₂ at −80°C and stored at −80°C until extraction. Ten roots or coleoptiles of the seedlings for one sample. Appropriate dilutions of the enzyme preparations were made, and 0.2 mL of the diluted preparations of the enzyme were added to 0.5 mL of 100 mM Na-acetate (pH 6.0) containing 10 mM CaCl₂. The reaction was initiated with 0.5 mL 2.5% (w/v) soluble starch. After incubation at 37°C for 15 min, the reaction was terminated by adding 0.5 mL of 40 mM dinitrosoalicylic acid solution containing 400 mM NaOH and 1 M KNa tartrate, and then placing it immediately into a boiling water bath for 5 min. After dilution with distilled water, absorbance at 530 nm of the reaction mixture was measured and reducing power evaluated using a standard curve obtained with glucose.
6. Extraction and determination of sugars

After a 48hr anoxic treatment, coleoptiles, roots and endosperms of rice seedlings were ground to a powder as described above and then extracted in 80% (v/v) ethanol. The sugar concentration in the extract was determined as hexose units using anthrone (Yemm and Wills, 1954; Huang et al., 2003). The starch in the rice coleoptiles and roots was less than 5% of the total sugars (data not shown) as described by Huang et al. (2003).

7. Statistical analysis

The experiments were repeated three times with 50 plants in each experiment for the measurement of coleoptile length. The experiments were repeated three times with four assays in each experiment for the determination of ATP, ethanol, ADH, α-amylase and sugar. The significance of differences was examined using Tukey’s multiple test.

Results and Discussion

1. Anoxia tolerance

Rice seedlings were subjected to anoxic stress for 24 or 48 hr and the effects of the stress on the root and coleoptile elongation were determined (Fig. 1). Anoxic stress inhibited the elongation of the roots and coleoptiles. The elongation of roots during 24-hr and 48hr anoxic treatment was 42 and 34% that of roots under aerobic conditions, respectively, whereas the elongation of coleoptiles during 24- and 48-hr anoxic treatment was 86 and 78% that of coleoptiles under aerobic conditions, respectively. These results suggest that, although the contribution of cell elongation and division to total elongation of coleoptiles and roots at this growing stage of rice may be different, the anoxia tolerance with respect to the elongation was much greater in the coleoptiles than in the roots. In addition, the inhibitory effect of 48-hr anoxic stress on the root and coleoptile elongation in percentage was greater than that of 24-hr anoxic stress.

2. ATP concentration

Anoxic stress reduced ATP concentrations in roots and coleoptiles of rice seedlings (Fig. 2). The ATP concentration in roots and coleoptiles under anoxia was 13 and 37% that under aerobic conditions, respectively. Thus, the coleoptiles maintained a higher level of ATP than the roots under anoxic conditions. ATP in the roots and coleoptiles under anoxia was probably produced by anaerobic glycolysis, since ATP production by oxidative phosphoryzation is negligible under anaerobiosis (Kennedy et al., 1992; Greenway and Gibbs, 2003; Magneschi and Perata, 2009). Thus, glycolysis under anoxia may be greater in the coleoptiles than in the roots.

3. ADH activity and ethanol concentration

Anoxic stress increased ADH activity, which catalyzes the conversion of acetaldehyde to ethanol, in roots and coleoptiles in rice (Fig. 3). The ADH activity in the roots and coleoptiles under anoxia was 23- and 38-fold, respectively, that under aerobic conditions. However, the
Elevated ADH activity in the coleoptiles was much higher than that in the roots.

Ethanol concentrations were low in roots and coleoptiles of rice seedlings under aerobic conditions and were increased under anoxic conditions (Fig. 4). This result indicates that the anoxic stress probably activates ethanolic fermentation in the roots and coleoptiles. However, the increase in the ethanol concentration in the coleoptiles was 3-fold that in the roots. The concentration of ethanol and the activity of ADH in anoxic coleoptiles and roots (Figs. 3 and 4) suggest that the ethanolic fermentation is probably more active in the coleoptiles than in the roots.

In anaerobic plant tissue, glycolysis is linked predominantly to ethanolic fermentation and this is also to the case in rice (Setter et al., 1997; Boamfa et al., 2003; Agarwal et al., 2007). The ethanolic fermentation pathway allows the continuation of glycolysis owing to pyruvate consumption and recycling NAD$^+$ when oxygen supply is limited and glycolysis becomes the main source of ATP (Drew, 1997; Saglio et al., 1999; Tadege et al., 1999). In addition, the importance of ethanolic fermentation under anaerobiosis has been demonstrated by studies on ADH null mutant of several plant species (Matsumura et al., 1995; Ellis et al., 1999; Saika et al., 2006). Therefore, activation of ethanolic fermentation is considered to be one of the strategies for plants to survive under anaerobiosis (Ricard et al., 1994; Drew, 1997; Tadege et al., 1999; Gibbs and Greenway, 2003; Magneschi and Perata, 2009).

### Table 1. Effect of 48-hr anoxia on α-amylase activity in roots, coleoptiles and endosperms of rice seedlings.

|                  | Aerobic | Anoxia |
|------------------|---------|--------|
| Root             | n.d     | n.d    |
| Coleoptile       | n.d     | n.d    |
| Endosperm        | 3.7 ± 0.3 a | 2.4 ± 0.2 b |

Means ± SE from three independent experiments with four assays for each determination (n = 12) are shown.

### 4. α-Amylase activity

α-Amylase activity was not detected in either the roots or coleoptiles of rice seedlings under either aerobic or anoxic conditions. The activity was found in endosperms of rice seedlings under both conditions although the activity in the anoxic endosperm was 64% of that in the aerobic endosperms (Table 1). In contrast to rice endosperms, α-amylase activity was not found in anoxic endosperms of wheat and barley seedlings (Guglielmimetti et al., 1995; Perate et al., 1997).

A set of enzymes, α-amylase, β-amylase, α-glucosidase and debranching enzyme, is needed for starch degradation, but only α-amylase is considered to play a major role in the degradation of reserved carbohydrate to utilize sugars under anoxia (Sun and Henson, 1991; Guglielmimetti et al., 1995).
Conclusion
Anoxic stress inhibited the elongation of roots and coleoptiles of rice seedlings, but the elongation during the stress was greater in coleoptiles than in roots (Fig. 1). Anoxic treatment decreased ATP concentration in the roots and coleoptiles. However, the coleoptiles maintained a higher level of ATP than the roots under anoxia (Fig. 2). The ADH activity and ethanol concentration in the roots and coleoptiles were increased by anoxic treatment, but the increase was greater in the coleoptiles than in the roots (Fig. 3 and 4), which suggests that ethanolic fermentation is probably more active in the coleoptiles than in the roots.

Sugar concentration was also higher in the coleoptiles than in the roots under anoxia (Fig. 5), suggesting that sugar from the endosperms may be transported to the coleoptiles preferentially than to the roots under anoxia. The availability of sugar is essential for glycolysis and fermentation leading to ATP production under anoxia (Perata et al., 1997; Saglio et al., 1999; Gibbs and Greenway, 2003; Mustroph et al., 2006). ATP and ethanol concentrations in the roots and coleoptiles of rice seedlings are closely correlated with sugar concentrations in the roots and coleoptiles (Figs. 2, 3 and 4), which show a high correlation with anoxia tolerance with respect to the elongation. Therefore, the sugar level may be important for the anoxia tolerance in rice roots and coleoptiles and may serve to distinguish their anoxia tolerance.

In the intensive irrigated rice cultivation systems in Japan, rice is often sown by “anaerobic seeding” below the soil surface (Yamauchi et al., 1993). Anoxia tolerance is thus essential where this sowing method.
is used. An assessment of the genetic diversity for the sugar level in rice seedlings is important for germplasm improvement of rice cultivars.

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