

Effects of Anoxia on Amino Acid Levels in Rice Coleoptiles

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Abstract : Effects of anoxia on the levels of free-amino acids were investigated in the coleoptiles of rice (*Oryza sativa* L.) seedlings. Rice coleoptiles are able to grow in extremely low oxygen conditions. Anoxic stress increased the concentration of total free-amino acids in the coleoptiles. Alanine (Ala) and γ -aminobutyric acid (Gaba) were the main amino acids accumulated. After 48 h, Ala and Gaba concentrations in anoxic coleoptiles were 3.4- and 11.2-fold greater than those in non-stressed coleoptiles, respectively. Ala and Gaba represented 19 and 23 % of the amino acid pool in anoxic coleoptiles, respectively. Submergence stress also increased Ala and Gaba concentrations in rice coleoptiles. Since Ala and Gaba are bio-compatible solutes and their accumulation is known to stabilize osmotic potential and/or cytoplasmic pH in plant cells, these stress-induced amino acids may allow rice coleoptiles to make biochemical adjustment that enable them to cope with the stress conditions. Therefore, the ability to increase the concentrations of Ala and Gaba may be important for anoxic and submergence stress tolerance of rice seedlings.

Key words : alanine, amino acid, γ -aminobutyric acid, anaerobiosis, anoxia, fructose, glucose, *Oryza sativa* L.

Soil flooding or total submergence subjects plants to considerable periods of anaerobiosis, which suppresses plant growth and productivity (Crawford and Braendle, 1996; Vartapetian and Jackson, 1997). Anaerobiosis dramatically alters the pattern of gene expression and protein synthesis in many plants (Sachs et al., 1996), and many plants have evolved a series of adaptive physiological and biochemical changes which enhance their ability to survive adverse conditions (Ricard et al., 1994; Drew, 1997; Tadege et al., 1999). The metabolism of free-amino acids has been found to be markedly changed in response to anaerobiosis in several plant species (Wallace et al., 1984; Reggiani et al., 2000; Sato et al., 2002).

Rice has the ability to germinate and elongate its coleoptiles under extremely low oxygen conditions, even under vacuum, although most cereal seeds fail to germinate and quickly lose their viability in these conditions (Alpi and Beevers, 1983; Cobb and Kennedy, 1987). Rice coleoptiles survived more than 21 day under complete oxygen deprivation and their metabolic activity was high enough to allow RNA and protein synthesis (Cobb and Kennedy, 1987; Mujer et al., 1993; Umeda and Uchimiya, 1994). The tolerance of rice coleoptiles to oxygen deficiency is related to the ability to maintain glycolytic activity leading to ATP production (Guglielminetti et al., 1995; Perata et al., 1997; Gibbs et al., 2000; Rahman et al., 2001). In this regard, the coleoptiles of rice seedlings are the most studied, although no unique pathways of

metabolism have emerged to explain their tolerance to oxygen deficiency (Perata et al., 1997; Vartapetian and Jackson, 1997; Das and Uchimiya, 2002).

There is some information on the amino acid levels in rice roots (Reggiani et al., 1988, 2000) and shoots (Menegus et al., 1989) under anaerobiosis. However, little information is available with regard to the effects of anaerobiosis on amino acids in rice coleoptiles, although the coleoptiles are extremely tolerant to anaerobiosis. Here we investigated the effects of anoxic and submergence stresses on the free-amino acid levels in rice coleoptiles, and elucidated the role of anaerobic amino acid accumulation in anaerobic adaptation.

Materials and Methods

1. Plant materials and anoxic treatment

Seeds of rice (*Oryza sativa* L. cv. Nipponbare) were surface sterilized in an aqueous solution of 25 mM sodium hypochlorite for 15 min and rinsed four times with distilled water. Then, the seeds were germinated on two sheets of moist filter paper (No 1, Toyo Ltd., Tokyo, Japan) in darkness at 25°C in a growth chamber. After three days, twenty uniform seedlings were selected, transferred to 9 cm Petri dishes each containing two sheets of filter paper moistened with 10 mL distilled water. Then, the Petri dishes were placed into jars (5 L) at 25°C in the growth chamber described above. Distilled water (200 mL) was placed at the bottom of the jar to maintain humidity and the Petri dishes were elevated above the water as described by Kato-Noguchi (2002). A

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Abbreviations : Ala, alanine; Asp, aspartic acid; Gaba, γ -aminobutyric acid; Gln, glutamine; Glu, glutamic acid; Ile, isoleucine; Pro, proline; Ser, serine; Thr, threonine.

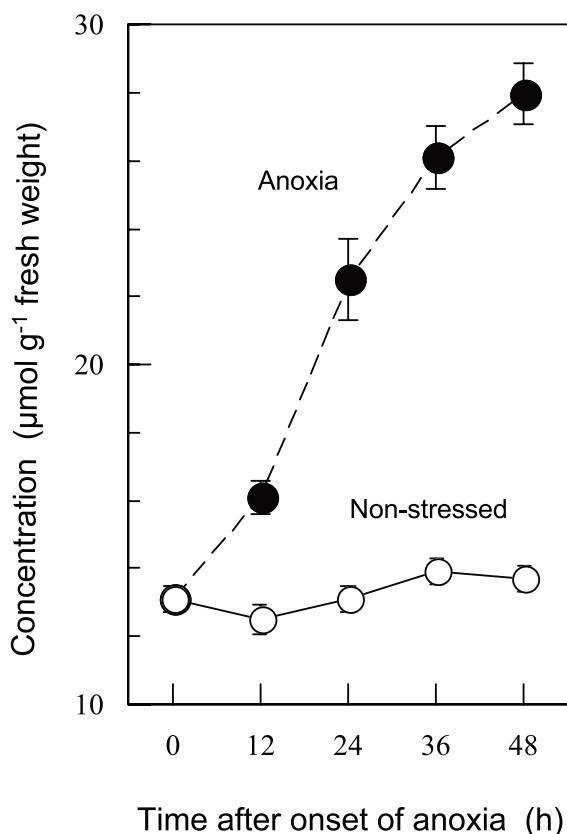


Fig. 1. Effects of anoxic stress on the concentrations of total free-amino acids in rice coleoptiles. Three-day-old rice seedlings were exposed to anoxic stress for 48 h. Non-stressed seedlings were grown in air for 48 h. Means \pm SE from five independent experiments are shown.

stream of N₂ was passed continuously through the jar at a rate of 200 mL min⁻¹. Non-stressed seedlings were supplied with air flowing at 200 mL min⁻¹. For determination of amino acids and carbohydrates, the coleoptiles of rice seedlings were harvested at 0, 12, 24, 36 and 48 h after the onset of anoxia, frozen immediately with liquid N₂ and stored at -80°C until extraction.

2. Submergence treatment

Three-day-old rice seedlings were grown as described above, and then uniform seedlings were transferred to plastic containers (120 × 98 × 86 (height) cm), 50 seedlings in each container, and completely submerged in water. The water surface was covered with a sheet of plastic film as described by Muench et al. (1993), and the containers were kept in darkness at 25°C in the growth chamber.

3. Determination of amino acids and carbohydrates

Frozen coleoptiles (10 coleoptiles for one determination) were powdered in liquid nitrogen using a mortar and a pestle. The powder was homogenized with 15 mL of 70 % aqueous ethanol. After incubation at 80°C for 20 min, the homogenate was centrifuged at 15,000

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Amino acids	Anoxia (µmol g ⁻¹ fresh weight)	Non-stressed (µmol g ⁻¹ fresh weight)
Asp	1.45 \pm 0.14	1.13 \pm 0.12
Glu	2.37 \pm 0.17	2.01 \pm 0.19
Gln	1.34 \pm 0.13	0.84 \pm 0.11
Ser	2.67 \pm 1.97	1.91 \pm 0.15
Thr	0.71 \pm 0.18	0.61 \pm 0.09
Ala	5.12 \pm 0.27	1.51 \pm 0.17
Pro	1.75 \pm 0.19	0.98 \pm 0.12
Gaba	6.15 \pm 0.29	0.54 \pm 0.09
Ile	0.35 \pm 0.01	0.29 \pm 0.05
Others	5.41 \pm 0.41	3.91 \pm 0.37

xg for 15 min and the supernatant was evaporated *in vacuo* to give an aqueous residue. The residue was shaken against an equal volume of ethyl ether, and the aqueous phase was evaporated to dryness *in vacuo*. Then, the residue was resolved in 0.02 mM hydrochloric acid and applied into auto amino acid analyzer (Model L-8500, Hitachi). The amount of every amino acid was determined by comparison with a standard solution containing all the amino acids as described by Reggiani et al. (1988). Coleoptile powder was prepared as described above and glucose and fructose were extracted and determined as described by Guglielminetti et al. (1995).

Results and Discussion

The concentration of total free-amino acids in coleoptiles of rice seedlings was 13.1 µmol g⁻¹ fresh weight at time 0 (Fig. 1), and did not change significantly in air (non-stressed coleoptiles). Anoxic stress increased the free-amino acid concentration. At 48 h after the onset of anoxia, the concentration in anoxic coleoptiles became 27.3 µmol g⁻¹ fresh weight, which was two-fold greater compared to non-stressed coleoptiles.

Table 1 shows free-amino acid concentrations in rice coleoptiles in anoxia and non-stressed conditions. The concentrations of all amino acids were greater in anoxic coleoptiles than in non-stressed coleoptiles, and marked difference was found between the two treatments in the concentrations of alanine (Ala) and γ -aminobutyric acid (Gaba). Ala and Gaba made up 19 and 23%, respectively, of the amino acid pool in anoxic coleoptiles.

The concentrations of Ala and Gaba increased rapidly in anoxic coleoptiles, while they remained unchanged in non-stressed coleoptiles (Fig. 2). After 48 h, Ala and Gaba concentrations in anoxic coleoptiles were 3.4- and 11.2-fold, respectively,

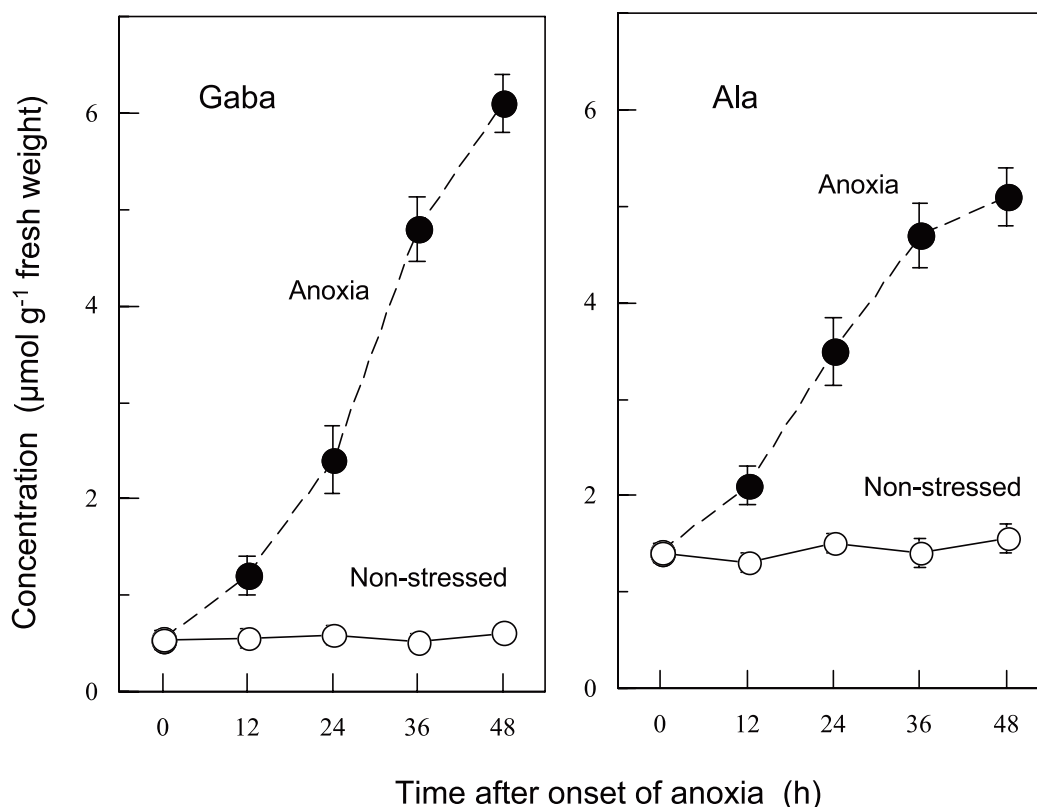


Fig. 2. Changes in the concentrations of Ala and Gaba in rice coleoptiles after the onset of anoxic stress. Three-day-old rice seedlings were subjected to anoxic stress. Non-stressed seedlings were kept in air. Means \pm SE from five independent experiments are shown.

greater than those in non-stressed coleoptiles. Effects of submergence stress on the concentrations of Ala and Gaba in rice coleoptiles were also examined since submergence tolerance is the essential feature of rice coleoptiles (Setter et al., 1997; Kato-Noguchi, 2001). The submergence stress increased Ala and Gaba concentrations in rice coleoptiles (Table 2). At 48 h, Ala and Gaba concentrations became 2.1- and 6.9-fold of the initial levels, respectively. Thus, submergence stress as well as anoxic stress increased Ala and Gaba concentrations in rice coleoptiles (Fig. 2 and Table 2).

Concentrations of glucose and fructose were determined in rice coleoptiles. After the onset of anoxia, glucose and fructose decreased rapidly (Fig. 3). This indicates that these carbohydrates may be consumed rapidly by anaerobic respiration because under limited oxygen supply, glycolysis and ethanolic fermentation accelerate and replace the Krebs cycle as the main source of ATP in many plant cells (Kennedy et al., 1992; Drew, 1997). The production of ATP by oxidative phosphorylation in anoxia is negligible relative to that by glycolysis and fermentation (Drew, 1997; Vartapetian and Jackson, 1997; Tadege et al., 1999). On the other hand, the reduction of these carbohydrate concentrations might lower the osmotic potential in the cells of rice coleoptiles.

Gaba is a four-carbon non-protein amino acid

Table 2. Concentrations of Ala and Gaba in rice coleoptiles after the onset of submergence stress. Three-day-old rice seedlings were subjected to submergence stress. Means \pm SE from five independent experiments are shown.

Amino acids	Concentration ($\mu\text{mol g}^{-1}$ fresh weight)		
	0 h	24 h	48 h
Ala	1.51 ± 0.17	2.12 ± 0.19	3.13 ± 0.34
Gaba	0.54 ± 0.09	1.41 ± 0.15	3.72 ± 0.29

found in all prokaryotic and eukaryotic organisms. In higher plants Gaba is synthesized primarily through the H^+ -consuming α -decarboxylation of Glu in the reaction ($\text{Glu} + \text{H}^+ \rightarrow \text{Gaba} + \text{CO}_2$) catalyzed by Glu decarboxylase (EC. 4.1.1.15). Gaba synthesis increases rapidly in response to a variety of environmental signals, including hypoxic, cold, drought and mechanical stresses, a result attributed to the stimulation of Glu decarboxylase activity by Ca^{2+} /calmodulin or H^+ (Bown and Shelp, 1997; Shelp et al., 1999). Accumulation of this amino acid takes place without changes in the quantity of Glu decarboxylase protein (Serraj et al., 1998).

Glu decarboxylase activity is not only stimulated by H^+ but it also consumes H^+ as described above, which may play an important role of pH stabilization in plant

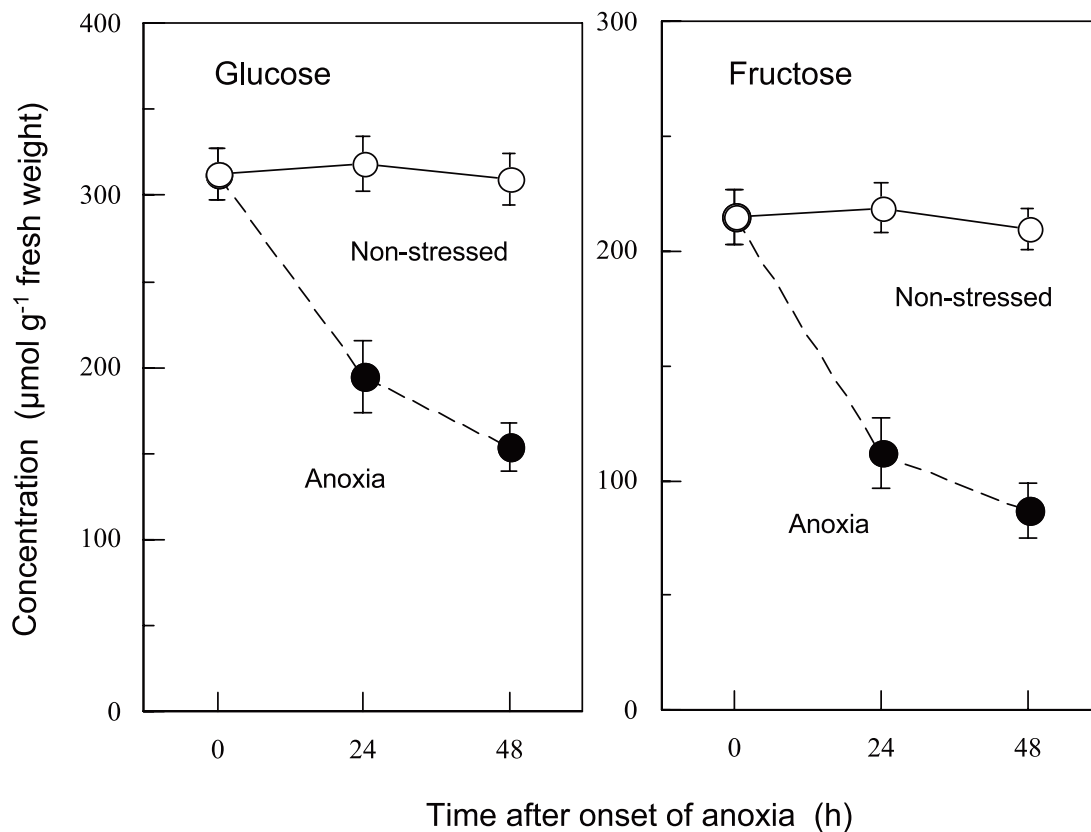


Fig. 3. Changes in the concentrations of glucose and fructose in rice coleoptiles after the onset of anoxic stress. Three-day-old rice seedlings were subjected to anoxic stress. Non-stressed seedlings were kept in air. Means \pm SE from five independent experiments are shown.

cells. Changes in cytoplasmic pH and Gaba level in asparagus cells exposed to weak acids were monitored, and it was found that decrease in cytoplasmic pH by the acid treatment increased Gaba accumulation (Crawford et al., 1994). Cytoplasmic pH and Gaba level in carrot cells were also measured by *in vivo* ³¹P and ¹⁵N-NMR spectroscopy (Carroll et al., 1994). In their research, exogenously applied ammonium chloride caused a decline of cytoplasmic pH by 0.2 units, followed by an accumulation of Gaba. The activity of Glu decarboxylase increased with reduced pH, and declined as the pH recovered. Thus, Gaba accumulation may ameliorate acidification.

Anoxic stress was reported to reduce cytoplasmic pH by 0.4 - 0.8 pH units in most plant cells including rice coleoptiles (Saint-Ges et al., 1991; Fox et al., 1995). This reduction of pH could elevate Gaba concentration by stimulating Glu decarboxylase activity concomitant with consumption of H⁺, which may contribute to regulation of pH in cytoplasm of plant cells under anoxic condition. Avoiding the cytoplasmic acidosis is essential for plant survival in anoxic conditions (Drew, 1997).

Ala is converted from pyruvate by Ala aminotransferase (EC. 2.6.1.2). The activity of Ala aminotransferase was enhanced by anaerobiosis and the concentration of

Ala was increased in several plant tissues (Good and Crosby, 1989; Muench and Good, 1994). Ala and Gaba are bio-compatible solutes and their accumulation may be a response to the decrease in osmotic potential. In fact, the anaerobic sugar level strongly decreased as a consequence of faster carbohydrate consumption (Fig. 3), and it is likely that amino acid synthesis serves to counteract a decrease in osmotic potential (Reggiani et al., 1988; Aurisano et al., 1995).

In response to anoxic and submergence stresses, amino acids, the concentrations of Ala and Gaba, in rice coleoptiles rapidly increased (Fig. 2 and Table 2). These stress-induced amino acids may allow rice coleoptiles to make biochemical adjustment that enable them to cope with the stress conditions. It was reported that submergence tolerance of cv. Yukihihikari coleoptiles is greater than that of cv. Sasanishiki coleoptiles (Kato-Noguchi, 2001). Therefore, the concentration of Ala and Gaba might increase greatly in cv. Yukihihikari coleoptiles than in cv. Sasanishiki coleoptiles under the stress conditions. An assessment of the genetic diversity of this trait may be important for germplasm improvement of rice cultivars, where rice seeds are sown by "anaerobic seedling" in soil (Yamauchi et al., 1993).

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