Pergamon

0031-9422(94)00774-8

Phytochemistry, Vol. 38, No. 5, pp. 1147–1150, 1995

0031-9422/95 $9.50 + 0.00

ANAEROBIC ACCUMULATION OF 4-AMINOBUTYRATE IN RICE SEEDLINGS; CAUSES AND SIGNIFICANCE

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(*Received in revised form* 25 *July* 1994)

Key Word Index—*Oryza sativa*; Gramineae; rice; 4-aminobutyrate; anoxia; root; shoot; glutamate; protein; amino acids.

Abstract—Accumulation of 4-aminobutyrate is induced by anoxia in rice seedlings. The induction of 4-aminobutyrate accumulation in aerobic conditions by treatments with exogenous 4-aminobutyrate, Gabaculine and glutamate is well tolerated by the seedlings. The inhibition of protein synthesis in aerobic and anaerobic conditions by cycloheximide shows that this process competes with glutamate decarboxylase for glutamic acid. The sensitivity of the anaerobic 4-aminobutyrate accumulation to azaserine indicates that glutamate synthase is important in maintaining glutamate availability. The different tolerance to anoxia and protein metabolism of shoot and root of rice suggests that the causes leading to 4-aminobutyrate accumulation in these tissues are different. It is suggested that ammonia reassimilation in root plays an important role in the synthesis of 4-aminobutyrate.

INTRODUCTION

In the plant kingdom, the non-protein amino acid 4-aminobutyrate (Gaba) is ubiquitous and its concentration in the tissues is often similar to those of the normal protein amino acids [1, 2]. The physiological role of Gaba in plants has not been clearly established. This compound is present in transport fluids and can provide, through the Gaba shunt, carbon for energy production and nitrogen for amino acid biosynthesis [2]. In the central and peripheral nervous system of vertebrates, Gaba acts as an inhibitory neurotransmitter by increasing the membrane conductance to Cl^- ions and membrane polarization [3]. Evidence of a similar function in plants is lacking. Many reports indicate that the level of Gaba increases rapidly in plant tissues in response to various forms of stress [4–6] among which anaerobiosis is the condition inducing the largest accumulation [7, 8]. In wheat roots, the process of Gaba accumulation has been shown to be mediated by the phytohormone ABA [9].

Gaba is synthesized in plant tissues by the irreversible α-decarboxylation of L-glutamic acid in a reaction catalysed by glutamate decarboxylase (GDC). An alternative source of Gaba is the oxidation of polyamines but this is probably lower than for glutamate decarboxylation and null under anaerobiosis [7, 9]. The synthesis of Gaba has been suggested to be an adaptive response of plant tissues to stress-induced cellular acidosis [2]. The advantage of this process would be the concomitant H^+ consumption during decarboxylation which ameliorates the cytosolic acidification [10]. A decline in the cytosolic pH is a phenomenon extremely marked under oxygen deficit stress [11, 12].

This investigation used seedlings of rice which tolerate an anaerobic stress [12]. The coleoptile of rice is also capable of anaerobic elongation [13]. This study seeks to widen the knowledge about the role of Gaba in plants and the causes leading to its accumulation under anoxia.

RESULTS AND DISCUSSION

The effect of anaerobic conditions on the Gaba content in shoot and root of rice is shown in Fig. 1. As can be seen, 24 hr of anoxia induced an accumulation of *ca* 3.5 and 6.3 μmol g^{-1} fr. wt of Gaba in shoot and root, respectively. No change in Gaba concentration was observed after an additional day of growth in air. The large increase in Gaba content in anaerobiosis is in agreement with that previously described by us [7] and other authors [8, 14–16]. Moreover, the synthesis of Gaba under stress conditions, advantageous for the pH-stat of the cell [1, 2, 9], could also occur due to the low toxicity of this amino acid. To test this hypothesis, we carried out 24 hr treatments with exogenous Gaba (1 mM), Gabaculine (1 mM, an inhibitor of Gaba transaminase, Fig. 2, arrow H) or glutamate (1 mM) in order to increase the level of Gaba in non-stress conditions (aerobiosis). As can be seen in Fig. 1, the Gaba and glutamate treatments increased the concentration of this amino acid in both shoot and root. The exogenous supply of Gaba led to endogenous values similar to those observed in anaerobic shoot and root. The Gabaculine treatment induced Gaba accumulation

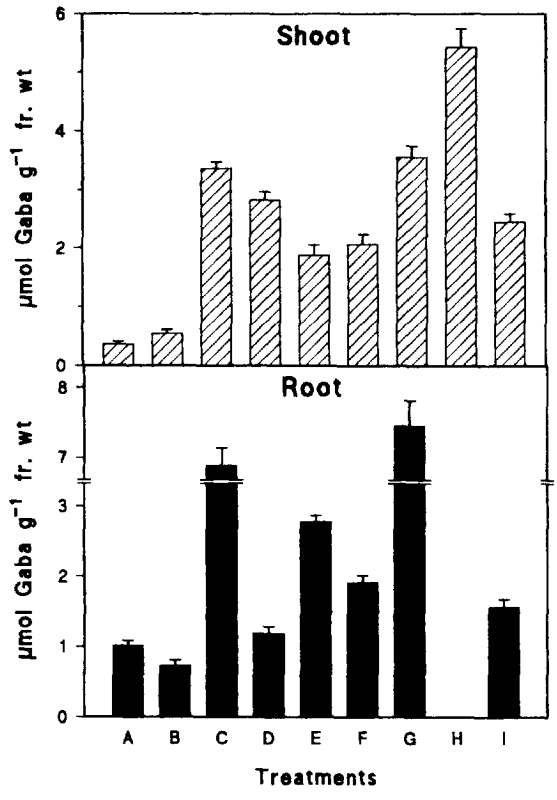

Fig. 1. The level of 4-aminobutyrate in shoot and root of rice seedlings subjected to different aerobic and anaerobic treatments. Each bar is the mean of three independent experiments and the SE are indicated. Treatments are as follows: A = 3-day-old seedlings; B = + 24 hr air; C = 24 hr air + 1 mM Gaba; D = 24 hr air + mM Gabaculine; E = 24 hr air + 1 mM glutamate; F = 24 hr air + 0.5 $\mu g\,ml^{-1}$ CHX; G = 24 hr anoxia; H = 24 hr anoxia + 0.5 $\mu g\,ml^{-1}$ CHX; I = 24 hr anoxia + 1 mM AZA.

only in rice shoot, suggesting that the metabolism of Gaba in roots may be slow. The increase of the Gaba concentration in the two tissues affected neither their growth in fr. wt (Table 1) nor the appearance of the seedlings (not shown). This indicates that Gaba is probably not toxic for plant tissues at the concentrations found under stress conditions. The higher level of Gaba in the glutamate treatment suggests that the availability of glutamate for decarboxylation is one of the factors which could control the formation of Gaba under stress conditions. However, the exogenous supply of glutamate induced a lower Gaba accumulation than anoxia, suggesting that in this last condition glutamate is still available for decarboxylation. In Fig. 3 can be seen the effect of anoxia on the level of total amino acids in shoot and root. In both tissues, there was an increase of amino acids of *ca* 2.5-fold in comparison with the aerobic control. This accumulation of amino acids has been shown to contribute to the osmotic potential of the tissue, compensating for the decrease in soluble hexose sugars which occurs during anoxia [17]. A bigger amino acid pool may increase the availability of glutamate both by transamination and ammonia reassimilation (Fig. 2, arrows C–F).

A factor affecting the demand of glutamate in plant tissues is protein metabolism [1]. Protein synthesis would compete with GDC for glutamic acid (Fig. 2). In general, a stress condition decreases the rate of protein synthesis consequently lowering the demand for glutamate. When the synthesis of protein was inhibited in aerobic rice seedlings by 0.5 $\mu g\,ml^{-1}$ cycloheximide (CHX), the level of protein was decreased in both shoot and root (Fig. 4). Moreover, the increase in fr. wt was prevented (Table 1), the concentration of amino acids increased (Fig. 3) and accumulation of Gaba was induced in both tissues (Fig. 1). This treatment would confirm that the inhibition

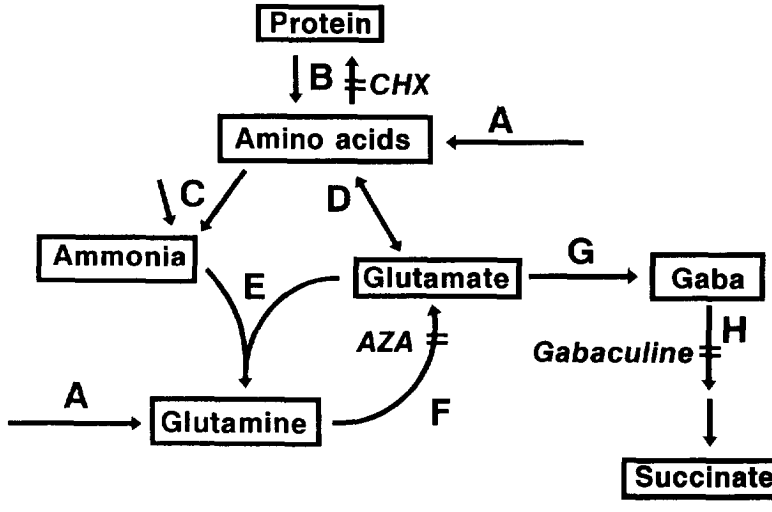

Fig. 2. Metabolic pathways connected with the metabolism of Gaba. The sites of action for the inhibitors CHX, AZA and Gabaculine are indicated. The arrows represent: A = translocation from the endosperm; B = protein metabolism; C = catabolism of nitrogenous compounds; D = transaminations; E = glutamine synthetase; F = GOGAT; G = GDC; H = Gaba transaminase.

Table 1. Fresh weight of shoot and root of rice seedlings in aerobic and anaerobic treatments

Treatment	Shoot*	Root*
Control (3 days)	16.7 ± 1.3	3.03 ± 0.10
24 hr air	31.2 ± 1.6	4.70 ± 0.12
24 hr air + Gaba	30.8 ± 0.9	5.40 ± 0.29
24 hr air + Gabaculine	34.3 ± 0.6	5.10 ± 0.50
24 hr air + glutamate	33.4 ± 1.0	4.10 ± 0.11
24 hr air + CHX	17.2 ± 1.4	4.30 ± 0.40
24 hr anoxia	26.5 ± 2.0	3.30 ± 0.15
24 hr anoxia + CHX	16.6 ± 1.1	
24 hr anoxia + AZA	25.1 ± 1.3	4.20 ± 0.30

*The values are expressed as mg fr. wt tissue^{-1}.

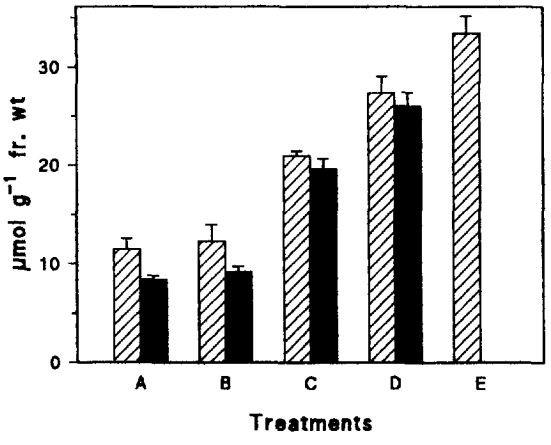

Fig. 3. The level of amino acids in shoot and root of rice seedlings subjected to different aerobic and anaerobic treatments. Each bar is the mean of three independent experiments and the SE are indicated. Treatments are as follows: A = 3-day-old seedlings; B = + 24 hr air; C = 24 hr air + 0.5 µg ml^{-1} CHX; D = 24 hr anoxia; E = 24 hr anoxia + 0.5 µg ml^{-1} CHX. Hatched and solid bars are shoot and root, respectively.

of protein synthesis greatly affects the availability of glutamate. Anoxia influenced the protein metabolism of the two tissues in a different way (Fig. 4). The level of protein was similar in aerobic and anaerobic shoots, while it was reduced by *ca* 70% after 24 hr of anoxia in root. This agrees with previous observations in which, under oxygen deficit stress, protein synthesis in rice was drastically reduced in root, while it was only slightly decreased in shoot [18]. The different protein metabolism in shoot and root can be associated with their different behaviour in anaerobic conditions (Table 1). The rice root requires oxygen in order to grow while the coleoptile elongates [19]. In rice root, there was a decrease in protein content of 1.1 mg g^{-1} fr. wt and an increase in the concentration of amino acids of 16.9 µmol g^{-1} fr. wt (Figs 3 and 4). In a previous work, we have estimated that a decrease of 1 mg of protein would lead, in rice root, to an increase of 16.2 µmol of amino acids [7]. These data suggest that the increase of the amino acid pool in root is

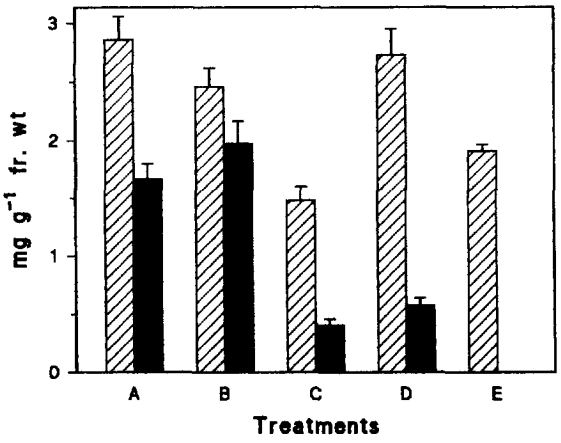

Fig. 4. The level of protein in shoot and root of rice seedlings subjected to different aerobic and anaerobic treatments. Each bar is the mean of three independent experiments and the SE are indicated. Treatments and symbols as in Fig. 3.

almost completely explained by proteolysis (Fig. 2, arrow B). In shoot, the level of amino acids increased by 15.9 µmol g^{-1} fr. wt without any fall in protein content (Figs 3 and 4). In this case, it is evident that a translocation of amino acids from the endosperm occurs. In rice shoot, the flux of amino acids from the endosperm is necessary to sustain the protein synthesis and growth of the coleoptile [13]. Consequently, protein synthesis in the shoot competes with GDC for the glutamate pool and this could explain the smaller accumulation of Gaba in shoot than in root (Fig. 1). To confirm this, the protein synthesis of the shoot was inhibited by CHX during a 24 hr anaerobic treatment. The supply of CHX led to the death of the rice root (checked by regrowth) and, consequently, this treatment for the root was omitted. In shoot, the level of protein was decreased by 30% in comparison with the anaerobic control (Fig. 4), while Gaba and amino acids were increased (Figs 1 and 3). This further supports the hypothesis that protein metabolism strongly affects the amino acid pool and the glutamate availability.

An alternative source of glutamate is the glutamate synthase (GOGAT) reaction (Fig. 2, arrow F). The GOGAT enzyme converts glutamine (which can derive from ammonia reassimilation or translocation, Fig. 2, arrows A and E) in glutamic acid. To establish the role of this reaction in the anaerobic Gaba accumulation, the GOGAT inhibitor azaserine (AZA) was supplied at a concentration of 1 mM to rice seedlings subjected to anaerobic conditions. In Fig. 1 is shown the level of Gaba in AZA-treated anaerobic seedlings. AZA inhibited the accumulation of Gaba in both shoot and root, but the effect was stronger in root.

CONCLUSIONS

The anoxia-induced Gaba accumulation is a process important for the pH-stat of the plant cell [1, 2, 7, 10].

Besides this, the data here shown indicate that the tissues accumulated Gaba, since they are able to tolerate high concentrations without visible damage. This could exclude any physiological role of Gaba *per se* in plant tissues and further support the hypothesis of an advantage under stress in its biosynthesis [2, 10]. Glutamate, the direct precursor of Gaba, appeared to have a different origin in shoot and root of rice seedlings subjected to anaerobic conditions. Rice shoots carried out an efficient protein synthesis which competes with GDC for glutamate. However, the translocation of amino acids from the endosperm (Fig. 2, arrow A), required for the coleoptile elongation [13], makes glutamate available also for decarboxylation. The sensitivity to AZA of the anaerobic Gaba accumulation in shoot, suggests that glutamine is among the amino acids translocated. In roots, the increase of the amino acid pool was mainly due to proteolysis. As a consequence, the glutamate in root could be derived: (i) directly by proteolysis; (ii) by transamination from other amino acids; (iii) by reassimilation of ammonia released from the catabolism of nitrogenous compounds (Fig. 2, arrows B–F). The high sensitivity of the anaerobic Gaba accumulation to AZA suggests that the reassimilation of ammonia in root is an important source of glutamate for GDC.

EXPERIMENTAL

Dehulled seeds of rice (*Oryza sativa* L. cv. Arborio) were sterilized and germinated as previously described [20]. Three-day-old seedlings were subjected to aerobic or anaerobic conditions. Fifteen seedlings were transferred into a jar containing 100 ml of a soln with the following composition: 1 mM Mes-Tris buffer (pH 5.5), 0.5 mM $CaSO_4$. Aerobic treatments were carried out at 25° under continuous stirring for 1 day in the dark. For anaerobic treatments, the jars were put into an anaerobic jar (Merck) and anoxia generated by BBL GasPAK Plus (Becton Dickinson).

In some experiments, 1 mM Gaba, 1 mM Gabaculine (Sigma) [21], 1 mM glutamate or 0.5 $\mu g\,ml^{-1}$ CHX were added to aerobic treatments, and 1 mM AZA or 0.5 $\mu g\,ml^{-1}$ CHX were added to anaerobic samples. After the treatments, the tissues were excised and ground in a mortar with 0.6 M $HClO_4$ (100 mg fr. wt ml^{-1}) and the homogenate centrifuged at 13 000 *g* for 15 min. Supernatant (100 μl) was dansylated and Gaba separated on a HPTLC plate of silica gel 60 by the method of ref. [19]. Gaba was identified by comparison of R_f values for unknowns and standard.

The level of amino acids in $HClO_4$ extracts was determined by the ninhydrin method using L-leucine as a standard [22]. Protein content was determined on 20 mM Mes-Tris (pH 6) extracts by Coomassie protein assay reagent (Pierce) using BSA as standard.

Acknowledgements—Research supported by National Research Council of Italy, Special project RAISA Subproject N. 2 Paper No. 1944.

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