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Incorporation of nitrate nitrogen into amino acids during the anaerobic germination of rice

Short Communication

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Summary. Incorporation of $^{15}NO_3^-$ into amino acids was studied during the anaerobic germination of rice seeds. In treated coleoptiles, the label was incorporated into glutamine, glutamate, alanine, γ-aminobutyric acid (Gaba), arginine, aspartate and methionine. These findings are consistent with a primary incorporation of nitrate nitrogen into glutamine, glutamate and aspartate, and their further conversion to alanine, Gaba, arginine and methionine.

Keywords: Amino acids – Anoxia – Coleoptile – Nitrate nitrogen – Rice

Introduction

Rice seeds germinating in flooded soils may be subjected to anaerobiosis. Unlike most higher plants, rice is able to germinate in a strictly anoxic environment emitting only a white coleoptile (Opik, 1973). The anaerobic elongation of rice coleoptile is sustained by a continuous translocation of carbohydrates, amino acids and salts from the endosperm (Atweel et al., 1982). This flux of nutrients furnishes fermentable substrates and maintains turgor pressure at high levels for cell enlargement. Amino acids, among which alanine and γ-aminobutyric acid (Gaba) are the most representative (Reggiani et al., 1988), are important in maintaining the osmotic pressure in the anaerobically grown coleoptile (Menegus et al., 1984).

Recently, it has been established that nitrate ions are translocated from the caryopses (Reggiani et al., 1993b,c) and assimilated by the rice coleoptile during the anaerobic germination (Mattana et al., 1993). The enzymes of nitrate reduction (nitrate and nitrite reductases) and ammonia assimilation (cytosolic and plastidial glutamine synthetase) are anaerobically expressed in the coleoptile (Mattana et al., 1994a,b). In the rice coleoptile, most of the

nitrogen (both nitrate and ammonium) was channelled toward the synthesis of alanine and Gaba under anaerobic conditions, while a reduced amount of nitrate was incorporated into these two amino acids in air (Fan, 1994). The biosynthesis of many protein amino acids is compartimentalized into the plastids (Singh and Matthews, 1994) and, in the rice coleoptile, the amino acid demand for protein synthesis is as high in anoxia as in aerobic conditions (Aurisano et al., 1995). The objective of our work was to investigate the incorporation of nitrate nitrogen into the different amino acids during the anaerobic germination of rice.

Materials and methods

Seeds of rice (*Oryza sativa* L. var. Arborio) were sterilized and anaerobically germinated for 6 days following the method previously adopted (Reggiani et al., 1993b). Then, 1 mM $K^{15}NO_3$ was added for 2 additional days and the coleoptiles collected for analysis. Perchloric acid extracts and amino fractions were obtained as previously described (Reggiani et al., 1993b). The amino acid composition of the extract was determined by HPLC analysis of the o-phtaldialdehyde (OPA) derivatives according to Reggiani et al. (1993a). The separation of OPA derivatives was performed at a flow rate of 1.0 ml/min on a 250 × 4 mm Daltosil 100 ODS2 (4 μ) reverse-phase column (Serva). Two mobile phase were used: A) 50 mM Na-acetate buffer (pH 6.5) – tetrahydrofuran (97:3 v/v); B) methanol. Phase B was linearly increased from 0% to 70% in 35 min.

The amino acid fraction was freeze-dried before derivatization for gas chromatography-mass spectrometry (GC-MS) analysis. Appropriate amounts of the freeze-dried amino acid fraction (ca. 1.5 mg) were placed into 1 ml screw-cap derivatization vials. Thirty μl of trifluoroacetic acid (TFA) and 150 μl of N-(*tert.*-butyldimethylsilyl)-N-methylfluoroacetamide (MTBSTFA) were sequentially added to the vials at 0°C. The reaction mixture was then maintained at room temperature for 1 h. Aliquots (1–2 μl) of the solution containing the derivatives were injected directly into the gas-chromatograph. In order to improve the conditions for the derivatization of the more basic amino acids, histidine, lysine and arginine, N,N-dimethylformamide (DMF) was used as solvent in place of TFA. In these experiments the vials were heated at 150°C for 2 h. Derivatized samples were injected into a Hewlett-Packard 5985 B GC-MS apparatus operating in electron impact (EI) mode. The gas-chromatograph was equipped with a SUPELCO SPB-50 fused silica capillary column (30 m × 0.32 mm i.d., 0.25 μm film thickness). The oven was programmed from 130 to 270°C at 7°C/min holding the initial temperature for 5 min. Mass spectral data were obtained under the following conditions: ionizing electron energy, 70 eV; emission current, 0.3 mA; ion source temperature, 200°C; scanning rate 1.5 scan/s over the mass range m/z 50–600. The *tert.*-butyldimethylsilyl-amino acids (tBDMS-AA) derivatives possess characteristic mass spectra with intense diagnostic ions, generally at $(M\text{-}57)^+$. This makes them very useful for selected ion monitoring (SIM) GC-MS experiments. ^{15}N incorporation was calculated after integrating the areas obtained for selected ion for both labeled and unlabeled amino acids and expressing the data as atom % excess (Robinson et al., 1991). ^{15}N incorporation was considered not significant for amino acids exhibiting atom % excess <1.

Results and discussion

The amino acid composition of the coleoptile extract was characterized by an elevated level of alanine (about 37% of the amino acid pool) while all the other amino acids were below 10% of the total amino acid fraction (Table 1).

Table 1. Effect of 1 mM $K^{15}NO_3$ on the amino acid content of 8-day-old anaerobic rice coleoptiles

Amino acid	Control (nmol/coleoptile)	$K^{15}NO_3$ (nmol/coleoptile)
Ala	49.75 ± 1.88 (36.8)	41.01 ± 1.38 (35.3)
Gaba	7.87 ± 0.31 (5.8)	9.19 ± 0.32 (7.9)
Glu	1.41 ± 0.04 (1.0)	1.35 ± 0.04 (1.2)
Gln	2.96 ± 0.07 (2.2)	3.39 ± 0.12 (2.9)
Asp	0.83 ± 0.02 (0.6)	0.80 ± 0.02 (0.7)
Asn	3.68 ± 0.11 (2.7)	3.32 ± 0.08 (2.9)
Gly	9.47 ± 0.34 (7.0)	5.48 ± 0.22 (4.7)
Met	6.17 ± 0.30 (4.6)	4.57 ± 0.14 (3.9)
Arg	1.66 ± 0.08 (1.2)	7.87 ± 0.50 (6.8)
Ser	5.24 ± 0.24 (3.9)	5.65 ± 0.27 (4.9)
Thr	2.64 ± 0.21 (2.0)	3.02 ± 0.20 (2.6)
His	3.03 ± 0.11 (2.2)	2.21 ± 0.13 (1.9)
Lys	10.44 ± 0.73 (7.7)	4.57 ± 0.14 (3.9)
Tyr	3.68 ± 0.22 (2.7)	3.11 ± 0.14 (2.7)
Phe	5.75 ± 0.27 (4.3)	5.68 ± 0.15 (4.9)
Trp	1.08 ± 0.08 (0.8)	0.99 ± 0.05 (0.9)
Val	1.53 ± 0.13 (1.1)	1.30 ± 0.12 (1.1)
Leu	11.15 ± 0.80 (8.3)	7.38 ± 0.65 (6.3)
Ile	6.81 ± 0.23 (5.0)	5.41 ± 0.40 (4.7)

In parentheses are the relative percentages of each amino acid. Values are the mean of four replicates ± SE. Proline is not detectable as OPA derivative.

A net synthesis of alanine occurs under anoxia by transamination, since pyruvate is more available and α-ketoglutarate is channelled towards ammonia reassimilation (Reggiani et al., 1988). The nitrate treatment induced a reduction in the absolute amount of most amino acids while increased markedly the level of arginine (6.21 nmol/coleoptile) and, to a lesser extent, Gaba and glutamine (1.32 and 0.43 nmol/coleoptile, respectively).

The incorporation of $K^{15}NO_3$ into amino acids of 6-day-old anaerobically grown rice coleoptiles was analysed using SIM-GC-MS (Table 2). Glutamine and glutamate, the first products of ammonia assimilation through the glutamine synthetase/glutamate synthase pathway, were significantly labeled. However, the low ^{15}N incorporation into these two amino acids (58 and 38 pmol/coleoptile for glutamine and glutamate, respectively) suggests that glutamate is rapidly metabolized. The label from nitrate was accumulated mainly in Gaba and alanine (471 and 504 pmol/coleoptile, respectively). These data agree well with a previous report showing that, in anoxic rice coleoptiles, nitrate reduction was enhanced and alanine and Gaba were the main products of nitrate assimilation (Fan, 1994). The relatively low atom % excess for alanine in comparison with Gaba is explained by the fact that alanine accounts for about 35–37% of the total amino acid pool, whereas Gaba is only 8–9% (Table 1). Considering that alanine and Gaba are accumulated as anaerobic products at different levels (Table 1), the incorporation of nitrate nitrogen

Table 2. Incorporation of ^{15}N into amino acids after 2 days treatment with 1 mM $K^{15}NO_3$ of 6-day-old anaerobic rice coleoptiles

Amino acid	Diagnostic ion (m/z)	Incorporation of ^{15}N	
		atom % excess[1]	pmol/ coleoptile
Ala	260	1.23	504
Gaba	274	5.12	471
Glu	432	2.83	38
Gln	431	1.70	58
Arg	442	5.00	394
Asp	418	2.31	18
Met	320	2.46	112
Asn	417	ns[2]	–
Gly	246	ns	–
Ser	390	ns	–
Thr	404	ns	–
His	440	ns	–
Lys	431	ns	–
Tyr	466	ns	–
Phe	336	ns	–
Trp	375	ns	–
Val	288	ns	–
Leu	302	ns	–
Ile	302	ns	–
Pro	286	ns	–

[1] atom % excess >1 was considered significant; [2] not significant

might be higher into Gaba than into alanine. This fact could be associated with the increase in the level of Gaba observed in rice seedlings supplemented with 1 mM $K^{15}NO_3$ (Table 1). Both alanine and Gaba are synthesized in the cytoplasm suggesting that most glutamate left the plastid after ammonia assimilation (therefore α-ketoglutarate has to be imported into the plastid). High ^{15}N incorporation was also observed into arginine (394 pmol/coleoptile), suggesting that the marked effect of nitrate ions on arginine level (Table 1) may be attributed to a stimulation of its biosynthesis from glutamate. Arginine is an important amino acid in the anaerobic rice coleoptile, being precursor for the synthesis of putrescine, a compound involved in the elongation of the coleoptile (Reggiani et al., 1989). The pathway of arginine biosynthesis from glutamate is restricted to plastids, with the conversion of citrulline to arginine occurring in the cytoplasm (Bryan, 1990). Label was also detected into aspartate and methionine (the latter belonging to the aspartate family). The ^{15}N incorporation into methionine (112 pmol/coleoptile), although 4–5 times lower than the incorporation into alanine and Gaba, would indicate a significant anaerobic synthesis of this amino acid. This last datum is strengthened by the label into aspartate, whose precursor. Since methionine is synthesized in the cytoplasm from homocysteine produced in the plastid

(Anderson, 1990), aspartate is likely synthesized from glutamate by the plastidic aspartate aminotransaminase. The role of methionine in the anaerobic metabolism of the rice coleoptile is still uncertain. These data taken as a whole would indicate that glutamine, glutamate and aspartate are the primary products during the assimilation of the nitrate nitrogen. The final fate for the nitrate nitrogen in the anaerobic rice coleoptile would be the incorporation into alanine, Gaba, arginine and methionine.

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