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Incorporation of nitrate nitrogen in rice seedlings transferred to anaerobic conditions

Short Communication

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Summary. Incorporation of $^{15}NO_3^-$ into amino acids was studied in 3-day-old aerobic rice seedlings (with coleoptile and root) subjected for 24 h to anaerobic conditions. The incorporation of ^{15}N into glutamate, glutamine and alanine accounted for 89% and 84% of total incorporation in coleoptile and root, respectively. These findings indicate that, after the primary incorporation of ^{15}N into glutamate and glutamine, the main fate of nitrate nitrogen in rice seedlings subjected to anoxia is alanine.

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

Introduction

Nitrate assimilation is one of the main reductive processes occurring in plant tissues during aerobic metabolism. The importance of this pathway under anaerobiosis is controversial. Rice plants grow well and withstand anaerobiosis better with nitrate as the sole nitrogen source when cultured on liquid nutrient media (Malavolta, 1954). Under such condition, nitrate is better than ammonium with respect to plant growth, grain yield, some metabolic activities and cation absorption of the roots (Yamasaki and Seino, 1965).

Contrasting data are provided by the literature on the nitrate assimilation under anaerobic conditions. Investigations carried out on nitrate assimilation under anoxia have revealed that this pathway is partially or completely blocked at the nitrite reductase (NiR) step (Ferrari and Varner, 1971; Lee, 1978; Reggiani et al., 1985). Thus, the anaerobic nitrite production (Jaworski, 1971) or the loss of endogenous nitrate (Gray and Cresswell, 1984) has been used for the nitrate reductase (NR) *in situ* assay in different plant organs (Ferrari et al., 1973).

Assimilation of nitrate under anaerobic conditions was previously reported (Kemp and Small, 1993; Fan, 1994). In rice, it has been established that nitrate ions are assimilated by the rice coleoptile during the anaerobic germination (Reggiani et al., 1993, 1995). The enzymes for the nitrate reduction and further ammonia assimilation (NR, NiR, glutamine synthetase and ferredoxin-dependent glutamate synthase) are anaerobically expressed in the coleoptile (Mattana et al., 1994a,b; Mattana et al., 1996). However, the rice coleoptile of anaerobically germinating seeds is a highly tolerant tissue, being able to elongate in an oxygen-deprived environment (Opik, 1973). Most plant tissues (i.e. roots) are instead anoxia intolerant. The present study seeks to establish the capacity of anaerobic nitrate assimilation in rice seedlings germinated in air (seedlings with coleoptile and root) and then transferred to anoxia. This allows to compare the anaerobic nitrate assimilation in an intolerant tissue, such as rice root, with that described in the tolerant anaerobic rice coleoptile (Reggiani et al., 1995).

Materials and methods

The dehulled seeds of rice (*Oryza sativa* var. Arborio) were sterilized for 2 min in 70% (v/v) ethanol and for 30 min in 5% (w/v) calcium hypochlorite, each treatment followed by rinsing with distilled water. The seeds were germinated for 3 days on a sterile wet paper in Petri dishes at 29°C in the dark. Three-day-old seedlings were then placed in jars (500 seedlings/250 ml) and made anaerobic by flushing nitrogen gas (99.99% N_2) through the medium. After 3 h (in order to reach a complete anaerobic condition), 1 mM $K^{15}NO_3$ was added for 24 h and the coleoptiles and roots collected for analysis. Perchloric acid extracts and amino acid fractions were obtained as previously described (Reggiani et al., 1993). The amino acid analysis by HPLC of the o-phthalaldehyde (OPA) derivatives was performed according to the method of Jarrett et al. (1986) modified by Reggiani et al. (1995).

The amino acid fraction was freeze-dried before derivatization for gas chromatography-mass spectometry (GC-MS) analysis. Amino acids were derivatized with N-methyl-(*tert.*-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA) (Mawhinney et al., 1986; Reggiani et al., 1995). Derivatized samples were injected into a Hewlett-Packard 5985 B GC-MS apparatus operating in electron impact (EI) mode. GC analysis was performed as previously reported (Reggiani et al., 1995). 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 amino acid derivatives possess characteristic mass spectra with intense diagnostic ions, generally at $(M-57)^+$, which make them useful for selected ion monitoring (SIM)-GC-MS experiments (Patterson et al., 1993). The incorporation of ^{15}N into amino acids was calculated by integration of the areas obtained for selected ions for both labeled and unlabeled amino acids and expressing the data as atom % excess (Robinson et al., 1991). ^{15}N incorporation was considered relevant for amino acids exhibiting atom % excess >1. Incorporation of ^{15}N into amino acids in the rice coleoptile of anaerobic germinating seeds (thereafter named "anaerobic coleoptile") were obtained by elaborating data from Reggiani et al. (1995).

Results and discussion

In Table 1 are shown the amino acid levels in aerobic and anaerobic rice tissues. Anoxia determined a significant increase in the level of total amino

Table 1. Effect of 1 mM $K^{15}NO_3$ on the amino acid content in coleoptile and root of 3-day-old aerobic rice seedlings subjected for 24 h to anaerobic conditions

Amino acid	Coleoptile			Root		
	Aerobiosis[1]	Anaerobiosis		Aerobiosis	Anaerobiosis	
		$-K^{15}NO_3$	$+K^{15}NO_3$		$-K^{15}NO_3$	$+K^{15}NO_3$
		(μmol/g FW)				
Ala	1.77 ± 0.12	6.53 ± 0.69	7.33 ± 0.37	1.94 ± 0.17	4.59 ± 0.18	5.40 ± 0.10
Gaba	0.37 ± 0.03	1.03 ± 0.03	1.17 ± 0.05	0.54 ± 0.04	1.81 ± 0.04	2.03 ± 0.08
Glu	1.74 ± 0.13	0.50 ± 0.02	0.78 ± 0.05	2.15 ± 0.19	0.70 ± 0.02	0.87 ± 0.03
Gln	0.97 ± 0.08	0.48 ± 0.01	0.56 ± 0.04	0.76 ± 0.06	0.37 ± 0.02	0.55 ± 0.03
Asp	0.71 ± 0.05	0.12 ± 0.01	0.20 ± 0.02	0.88 ± 0.07	0.20 ± 0.02	0.30 ± 0.03
Asn	1.36 ± 0.10	0.49 ± 0.02	0.81 ± 0.04	1.15 ± 0.11	0.57 ± 0.01	0.83 ± 0.03
Met	0.17 ± 0.02	0.21 ± 0.01	0.29 ± 0.03	0.06 ± 0.01	0.07 ± 0.01	0.06 ± 0.01
Arg	0.10 ± 0.01	0.09 ± 0.01	0.14 ± 0.01	0.40 ± 0.03	0.45 ± 0.02	0.39 ± 0.03
Ser	0.75 ± 0.08	0.81 ± 0.05	1.00 ± 0.06	0.67 ± 0.06	0.76 ± 0.06	0.81 ± 0.05
His	0.07 ± 0.01	0.06 ± 0.01	0.08 ± 0.01	0.15 ± 0.02	0.17 ± 0.02	0.17 ± 0.01
Gly	0.17 ± 0.02	0.23 ± 0.03	0.27 ± 0.03	0.30 ± 0.02	0.34 ± 0.02	0.34 ± 0.02
Thr	0.31 ± 0.02	0.38 ± 0.03	0.40 ± 0.03	0.38 ± 0.04	0.43 ± 0.05	0.50 ± 0.06
Tyr	0.10 ± 0.01	0.08 ± 0.01	0.35 ± 0.02	0.19 ± 0.02	0.22 ± 0.01	0.30 ± 0.03
Val	0.32 ± 0.03	0.39 ± 0.02	0.65 ± 0.04	0.20 ± 0.01	0.23 ± 0.01	0.39 ± 0.02
Lys	0.31 ± 0.02	0.29 ± 0.02	0.46 ± 0.03	0.61 ± 0.05	0.69 ± 0.04	0.86 ± 0.04
Phe	0.09 ± 0.01	0.10 ± 0.01	0.17 ± 0.01	0.15 ± 0.01	0.17 ± 0.03	0.14 ± 0.02
Ile	0.10 ± 0.01	0.12 ± 0.02	0.14 ± 0.01	0.26 ± 0.03	0.29 ± 0.02	0.31 ± 0.03
Leu	0.19 ± 0.02	0.23 ± 0.03	0.26 ± 0.02	0.50 ± 0.04	0.57 ± 0.06	0.55 ± 0.05
Total	9.60	12.14	15.06	11.29	12.63	14.80

[1] Amino acid levels before the imposition of the stress. These levels did not show significant changes after 24 h in well-aerated conditions. Values are the mean of three replicates ±SE. Proline is not detectable as OPA derivative.

acids and, in particular, of alanine and Gaba, while a decrease in the level of glutamate, aspartate and their amides was observed. In coleoptile and root transferred for 24 h in anaerobiosis, nitrate induced an increase in total amino acids by 24% and 17% in coleoptile and root, respectively (Table 1). Independently from the nitrate treatment, about 50% and 36% of total amino acids in coleoptile and root, respectively, was alanine. In anaerobic rice roots, an increase in total amino acids and alanine in the presence of nitrate was previously described (Reggiani et al., 1985). On the contrary, in the coleoptile of anaerobically germinated rice seeds, the supply of nitrate led to a decrease in total amino acids and alanine while increased the level of Gaba, arginine and glutamine (Reggiani et al., 1995). In coleoptile and root of seedlings transferred to anoxia, the level of almost all the other amino acids was increased by the nitrate treatment (Table 1).

The incorporation of $^{15}NO_3^-$ into amino acids in 3-day-old seedlings transferred for 24 h in anaerobic conditions was analysed using SIM-GC-MS (Table 2). In aerobic seedlings transferred to anoxia, the incorporation of ^{15}N into glutamate and glutamine (first products of NH_4^+ assimilation through the glutamine synthetase/glutamate synthase cycle) was significantly higher than

Table 2. Incorporation of ^{15}N into amino acids after treatment with 1 mM $K^{15}NO_3$ of 3-day-old aerobic seedlings transferred for 24 h in anaerobic conditions

Amino acid[1]	Anaerobic coleoptile[2]		Transferred coleoptile		Transferred root	
	Atom % excess	nmol/g FW	Atom % excess	nmol/g FW	Atom % excess	nmol/g FW
Ala	1.2	47.1	3.3	240.4	1.9	102.6
Gaba	5.1	44.0	1.5	17.1	1.5	31.1
Glu	2.8	3.6	14.9	116.1	13.2	114.8
Gln	1.7	5.4	14.5	81.4	5.7	31.2
Asp	2.3	1.7	3.9	7.7	5.3	15.8
Asn	<1		2.1	17.0	<1	
Arg	5.0	36.8	3.7	5.2	<1	
Met	2.5	10.5	1.7	5.0	1.8	1.1
Total		102.0		489.9		296.6

[1] Amino acid showing atom % excess <1 were omitted, [2] data for coleoptile of anaerobically germinating rice seeds were elaborated from Reggiani et al. (1995).

in the coleoptile of anaerobically germinated seeds. The final product of the glutamine synthetase/glutamate synthase cycle is glutamic acid. We have estimated, elaborating data on the amino acid content in the anaerobic coleoptile (Reggiani et al., 1995), that the level of glutamate in this last tissue was similar to that found in coleoptile and root of seedlings transferred to anoxia (Table 1). As a consequence of this, the higher label incorporation into this amino acid would suggest that, in transferred seedlings, the assimilation of nitrate nitrogen is faster.

The incorporation of label into alanine resulted higher in coleoptile and root of transferred seedlings than in the anaerobic coleoptile (5.1 and 2.2 times higher in coleoptile and root, respectively). Under anoxia, alanine is continuously synthesized from glutamate through a reaction catalyzed by alanine aminotransferase (Reggiani et al., 1988). The reasons of this are the greater pyruvate availability for transamination and the utilization of α-ketoglutarate in NH_4^+ (re)assimilation (Reggiani et al., 1988). Considering that, in tissues subjected to anaerobiosis, pyruvate is made available by the stimulation of glycolysis, it could be suggested that a faster fermentation in seedlings transferred to anoxia than in anaerobically germinated seeds occurred. Aspartate, which derives by transamination from glutamate, resulted more labeled in transferred tissues than in the anaerobic coleoptile (Table 2). This may be a consequence of the higher ^{15}N incorporation into glutamate. However, in transferred seedlings, the incorporation of label into aspartate was lower in coleoptile than in root and, in the former, incorporation into asparagine was detected. Thus, in this tissue a part of aspartate might be utilized for the synthesis of asparagine. The incorporation into Gaba, arginine and methionine was lower in coleoptile and root of transferred seedlings than in the anaerobic coleoptile (Table 2). The first two amino

acids belong to the glutamate family while the latter belongs to the aspartate family (Singh and Matthews, 1994). The lower label incorporation into these three amino acids led, as a consequence, that almost all the ^{15}N in seedlings transferred to anoxia was recovered into alanine, glutamate and glutamine (89% and 84% in coleoptile and root, respectively). This is different from that found in the coleoptile of anaerobically germinated rice seeds in which the ^{15}N into alanine, glutamate and glutamine accounted for only 55% of total amino acid labelling (Reggiani et al., 1995). The data here presented would indicate that, in aerobic seedlings subjected to an anaerobic stress, the metabolism of nitrate nitrogen is almost all described in terms of alanine synthesis. The faster fermentation above hypothized for these seedlings in comparison with those anaerobically germinated would produce both ATP and NADH for nitrate assimilation and pyruvate for transamination.

The complete assimilation of nitrate ions by an anoxia-intolerant tissue such as the rice root indicates that the pathway of assimilation is not blocked at the NiR step as suggested by some authors (Ferrari and Varner, 1971; Lee 1978). This leads to the conclusion that the *in situ* assay for NR, based on the nitrite production, is an underestimation of the NR activity of the tissue.

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