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Expression of the Enzymes of Nitrate Reduction during the Anaerobic Germination of Rice[1]

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During the anaerobic germination of rice (*Oryza sativa* L.), nitrate is translocated from the caryopsis and assimilated into the coleoptile (R. Reggiani, M. Mattana, N. Aurisano, A. Bertani [1993] Plant Cell Physiol 34: 379–383). Using antibodies against nitrate and nitrite reductases, proteins with the expected molecular mass were recognized by western blot analysis in extracts from 8-d-old rice coleoptiles. Both enzymes are de novo synthesized in 6- to 8-d-old seedlings, as shown by immunoprecipitation of radiolabeled proteins from young plants grown in the presence of [^{35}S]methionine. The anaerobic synthesis of both enzymes was enhanced by the addition of 5 mM KNO_3. The effect of exogenous nitrate on the expression of the corresponding genes in anaerobic rice coleoptiles was revealed by the analysis of their transcripts. The importance of the expression of these enzymes during the anaerobic development of rice seedlings is discussed.

Nitrate assimilation under anaerobic conditions is apparently severely inhibited and restricted to the reduction of nitrate to nitrite (Nance, 1948; Ferrari and Varner, 1971; Lee, 1978; Gray and Cresswell, 1983; Reggiani et al., 1985). Anaerobic nitrite production (Jaworski, 1971) or the loss of endogenous nitrate (Gray and Cresswell, 1984) has been used for the NR in situ assay in different plant organs (Ferrari et al., 1973). Recently, it has been shown that species capable of anaerobic germination (*Oryza sativa, Erythrina caffra*) reduce and assimilate nitrate (Kemp and Small, 1993; Reggiani et al., 1993a). Nitrate ions are stored in the embryonic axes and caryopses of *Erythrina* and rice, respectively. The caryopses of rice contain storage nitrate in amounts that vary among cultivars (Reggiani et al., 1993b).

The process of nitrate assimilation in rice coleoptile was estimated to account for about 10% of the nitrogen present in the amino acid pool during the first 4 d of anaerobic germination (Mattana et al., 1993). All the enzymes of nitrate reduction and assimilation (NR, NiR, GS, and Fd-GOGAT), with the exception of NADH-GOGAT, were present in the rice coleoptile (Mattana et al., 1993), and the two reductase activities were induced by supplying nitrate. As far as the reduction of nitrate to ammonium is concerned, recent evidence suggests anaerobic synthesis of the two reductases (NR and NiR) in rice coleoptile. First, both activities increased, on a tissue basis, during the anaerobic germination; second, a 24-h cycloheximide treatment strongly depressed NR and NiR activities (Reggiani et al., 1993a). The increase of NR activity during anaerobic germination was prevented by cycloheximide in *E. caffra* as well (Kemp and Small, 1993).

In the present study we investigated the synthesis of NR and NiR polypeptides during the anaerobic germination of rice with the use of antibodies raised against these proteins and cDNAs encoding NR and NiR polypeptides.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Seeds of rice (*Oryza sativa* L. cv Arborio) were sterilized and anaerobically germinated as reported previously (Reggiani et al., 1993a). The seeds were germinated for 8 d in the absence (control) or presence of 5 mM KNO_3. In some cases, nitrate was supplied later during germination as indicated below.

Enzyme Extraction

Rice coleoptiles were ground with the following extraction buffer: 250 mM Tris-HCl (pH 8.6), 1 mM EDTA, 3 mM DTT, 1 μM Na_2MoO_4, 5 μM flavin adenine dinucleotide, 1 mM PMSF, and 10 μM antipain (Somers et al., 1983). The homogenate was centrifuged at 30,000*g* at 4°C for 20 min and the supernatant adjusted to 50% $(NH_4)_2SO_4$ saturation. After 30 min of stirring at 4°C, the solution was centrifuged at 12,000*g* for 15 min at 4°C. The supernatant and the pellet were separated and the supernatant was adjusted to 70% $(NH_4)_2SO_4$ saturation. The pellet was resuspended in one-seventh the original extraction volume in 250 mM K-phosphate (pH 7.5), 1 mM EDTA, 3 mM DTT, 5 μM Na_2MoO_4, 25 μM flavin adenine dinucleotide, and 10 μM antipain and the supernatant was desalted by a Sephadex G-25 column. The eluate from the column contained NR activity. After 70% $(NH_4)_2SO_4$ precipitation, the solution was centrifuged at 20,000*g* for 15 min at 4°C. The pellet was resuspended in 20 mM Tris-HCl (pH 7.6) and desalted by a Sephadex G-25 column. The eluate from this step contained NiR activity.

Western Blots

Twenty micrograms of protein from partially purified NR and NiR were loaded on ExcelGel SDS, 8 to 18% gradient

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Abbreviations: GOGAT, glutamate synthase; GS, glutamine synthetase; NiR, nitrite reductase; NR, nitrate reductase; SSC, saline sodium citrate.

acrylamide gels (Pharmacia, Uppsala, Sweden). After electrophoresis, the proteins were electroblotted to nitrocellulose membrane (Pharmacia, Uppsala, Sweden) using the Nova-Blot 2117 Multiphor semidry system (Pharmacia, Uppsala, Sweden). Transfer was performed at room temperature according to the manufacturer's instructions. After transfer the filters were rinsed and saturated overnight at room temperature in TBS-T (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% [v/v] Tween 20) containing 5% dry milk. Anti-barley NADH- and NAD(P)H-NR antibodies and anti-maize NiR antibody were kindly provided by Dr. A. Kleinhofs and Dr. L. Beevers, respectively. Antibodies were added at a 1:1000 dilution in TBS-T solution and adsorption was for 1.5 h; the resulting blots were washed exhaustively in TBS-T. The blots were incubated for 1 h with a 1:2500 dilution of the mouse anti-rabbit alkaline-phosphatase conjugate (Sigma). Excess secondary antibody was washed away with TBS-T. The blots were then pre-equilibrated in developing buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) and cross-reacting proteins were visualized by ProtoBlot System reagents (Promega, Madison, WI) dissolved in 30 mL of developing buffer.

Inhibition of Catalytic Activity by NR and NiR Antibodies

To test if the antibodies raised against the barley or maize enzymes would cross-react with the rice homologs, extracts were incubated for 5 min in the presence or absence of anti-NR, anti-NiR, or preimmune serum. NR and NiR activities were then assayed as previously described (Reggiani et al., 1993a).

Protein Labeling and Immunoprecipitation

The sterilized seeds were germinated in Petri dishes in anaerobic jars (Merck, Darmstadt, Germany) and anaerobic conditions were generated by BBL GasPak Plus (Becton Dickinson, Cockeysville, MD). After 6 d, the jars were put into a controlled chamber (AtmosBag, Aldrich Chimica, Milano, Italy) saturated with nitrogen gas. The seedlings were transferred into 500-mL bottles (450 seedlings per bottle) containing 200 mL of 1 mM Mes (pH 6.0), 0.5 mM $CaSO_4$, with or without 5 mM KNO_3. Then, 3.7 MBq L-[^{35}]Met (Amersham, Little Chalfont, UK) was added to each treatment and the seedlings were left for 2 additional d of anaerobic germination. At the end of the labeling period, the coleoptiles were homogenized in a cold mortar with extraction buffer. The homogenate was clarified by centrifugation at 12,000*g* at 4°C for 20 min. Immunoprecipitations with anti-NR and anti-NiR antibodies were performed using the method described by Gething et al. (1986). The final pellet was resuspended in sample buffer for SDS gels, boiled for 5 min, clarified, and then subjected to SDS-PAGE for analysis.

Northern Blot Analysis for NR

For the RNA isolation, seedlings were anaerobically germinated for 8 d and then incubated for 5 h in the presence or absence of 5 mM KNO_3. Total RNA was isolated using the RNAfast isolation system (Molecular Systems, San Diego, CA). The poly(A)$^+$ RNA was prepared using the Oligotex-dT mRNA Kit (QIAGEN, Chatsworth, CA). Five micrograms of poly(A)$^+$ RNA were denatured using formamide and formaldehyde, subjected to electrophoresis, and then transferred to a Hybond-N nylon membrane (Amersham) according to Sambrook et al. (1989). The filters were prehybridized in a solution containing 5× Denhardt's solution (Denhardt, 1966), 6× SSC (1× SSC = 150 mM NaCl, 15 mM Na citrate, pH 7.0), 0.5% (w/v) SDS, 100 μg mL^{-1} denatured salmon-sperm DNA, and 40% (v/v) formamide at 42°C for 2 h, and then hybridized at 53°C overnight in the same buffer but also containing 15% (v/v) formamide. The hybridization was performed with a tobacco NR cDNA probe (clone 13–19 pBMC102010) kindly provided by Dr. M. Caboche (Calza et al., 1987). Filters were first washed at room temperature in 5× SSC, 0.1% SDS, and 2× SSC, 0.1% SDS. Then the filters were washed twice for 30 min in 2× SSC, 0.1% SDS at 53°C. Before rehybridization, the probe was stripped from the membrane with 0.1% SDS at 70°C.

Northern Blot Analysis for NiR

Twenty micrograms of total RNA were denatured, subjected to electrophoresis, and hybridized as described above. The hybridization was performed with a maize cDNA probe (clone pCIB808), which was a kind gift of Dr. Lisa Jackman. Filters were washed at a higher stringency corresponding to 0.2× SSC and 0.1% SDS at 53°C.

RESULTS

Specificity of NR and NiR Antibodies

The NR and NiR antibodies raised against barley and maize, respectively, were assayed for their ability to inhibit the activity of the two homologous enzymes in rice coleoptiles (Fig. 1). Both NR and NiR activities were inhibited by the heterologous antibodies. An almost complete inhibition was obtained with a 1:100 dilution of both sera. No inhibition was observed by a preimmune serum.

Analysis of NR and NiR Proteins in Rice Coleoptile

Western blot analyses using NR and NiR antisera were performed on extracts from aerobic and anaerobic rice coleoptiles (data not shown). The NADH-NR antiserum recognized in aerobic coleoptile extracts three cross-reacting polypeptides of 113, 96, and 92 kD, whereas in anaerobic coleoptile extracts only one band with a molecular mass of 92 kD was evident (data not shown). A similar result was obtained using a NAD(P)H-NR antiserum. NiR antiserum revealed the presence, in both aerobic and anaerobic rice coleoptile extracts, of a single band of 62 kD (data not shown).

Anaerobic Synthesis of NR and NiR in Rice Coleoptile

The de novo synthesis of NR and NiR in rice coleoptiles was investigated in extracts from 6-d-old seedlings anaerobically grown for 2 additional d in the presence of [^{35}S]Met. After immunoprecipitation, the labeled proteins were separated by SDS-PAGE. Two labeled proteins with apparent

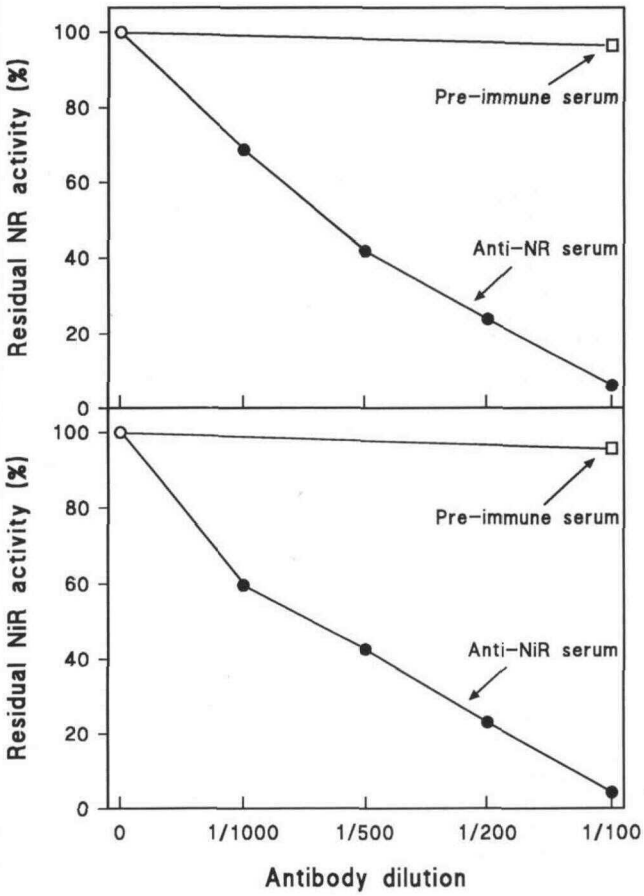

Figure 1. Inhibition of the catalytic activity of rice NR (upper panel) and NiR (lower panel) by anti-barley NR and anti-maize NiR, respectively.

molecular masses of 96 and 92 kD were immunoprecipitated by anti-NR (Fig. 2A). The immunoprecipitation with anti-NiR showed a single labeled protein of 62 kD (Fig. 2B).

To detect the effect of exogenous nitrate on the de novo synthesis of NR and NiR, the 6-d-old anaerobic seedlings were fed with [^{35}S]Met and with 5 mM KNO_3. As shown in Figure 2, the amount of immunoprecipitated NR and NiR was higher in extracts from nitrate-treated seedlings than in control seedlings.

Anaerobic Expression of mRNA for NR and NiR

To investigate the presence of mRNA for NR and NiR in anaerobic rice seedlings and the effect of exogenous nitrate, 8-d-old seedlings were incubated for 5 h with or without 5 mM KNO_3. The northern blot analysis of poly(A)$^+$ RNA showed the accumulation of a 3-kb RNA in nitrate-treated seedlings, whereas the transcript was not evident in uninduced seedlings. The size of this transcript was as expected for an mRNA encoding the approximately 100-kD NR subunit (Fig. 3A). The effect of nitrate on the accumulation of

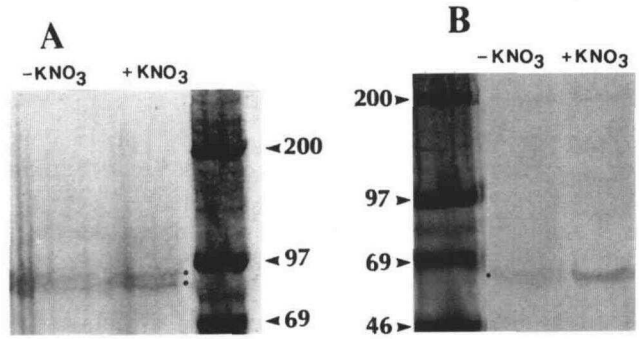

Figure 2. Immunoprecipitation of in vivo [^{35}S]Met-labeled proteins with anti-barley NR (A) and anti-maize NiR (B). The lanes represent uninduced and induced (without and with 5 mM KNO_3) anaerobic rice coleoptiles, respectively. Molecular masses of labeled markers are indicated in KD.

NiR mRNA was similar to that observed for NR mRNA (Fig. 3B). The NiR transcript was evident in nitrate-treated seedlings and was approximately 2 kb. In uninduced seedlings, only a pale band was detectable.

DISCUSSION

Nitrate reduction and assimilation under anaerobic conditions has been suggested for *E. caffra* and rice (Kemp and Small, 1993; Reggiani et al., 1993a). In previous work we showed that the rice coleoptile was able to incorporate $^{15}NO_3$ nitrogen into amino acids under anaerobic conditions (Reggiani et al., 1993b). Moreover, the activity of NR, NiR, GS, and Fd-GOGAT increased during germination. In particular, nitrate induced NR and NiR activities in rice coleoptile even under anaerobic conditions. The presence of enzyme activity does not reveal whether the enzyme polypeptides were synthesized during anaerobiosis or if pre-existing polypeptides

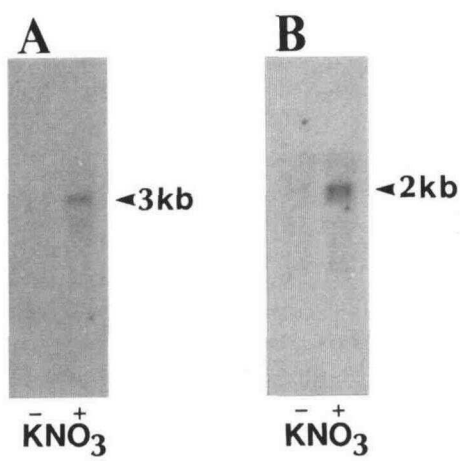

Figure 3. Nitrate induction (5 mM KNO_3) of NR (A) and NiR (B) transcripts in anaerobic rice coleoptiles. The lanes represent uninduced and induced coleoptiles, respectively.

were activated to yield enzyme activity. Preliminary evidence of de novo protein synthesis in sustaining NR and NiR activities arose from cycloheximide treatments of anaerobic rice seedlings (Mattana et al., 1993). By immunoprecipitation with specific antibodies, we detected newly synthesized NR and NiR proteins (Fig. 2). For NR, two labeled proteins were identified, probably corresponding to the two isoforms, NADH-specific and NAD(P)H-bispecific protein, described in rice (Hamat et al., 1989). Three NR isoforms were isolated from soybean (Caboche and Rouzé, 1990), and, in rice, at least three different NR-encoding genes were described (Hamat et al., 1989). The significance of the presence of several NR isoforms in plants is not understood, but different isoforms may be required to optimize nitrate reduction under varying physiological conditions (Hoff et al., 1992).

Synthesis of NiR under anaerobiosis is shown by the appearance of a polypeptide with the molecular mass expected for this enzyme (62 kD) that was recognized by specific antibodies (Fig. 2B). The presence of mRNA coding for both the reductases (Fig. 3) further supports the hypothesis for de novo synthesis of NR and NiR during anaerobic germination of rice. Exogenous nitrate modulates the protein synthesis and the mRNA transcription for NR and NiR proteins (Figs. 2 and 3). This response to nitrate is well documented for many plant species (Melzer et al., 1989; Redinbaugh and Campbell, 1991; Warner and Kleinhofs, 1992), but is here described for anaerobic conditions.

In anoxia, only a few metabolic pathways are active through the expression of a restricted number of proteins (Sachs et al., 1980). Both NR and NiR polypeptides are synthesized during the anaerobic development of rice seedlings. Our data support the hypothesis of a common regulation for NR and NiR expression (Faurè et al., 1991). In plant tissues, constitutive levels of NR and NiR are often present. However, supply of nitrate results in a substantial increase in activity that is due to de novo synthesis of the two proteins (Small and Gray, 1984). As can be observed in Figure 2, the uninduced seedlings showed constitutive amounts of both enzymes. This agrees with previous results on the basal activity of NR and NiR (Reggiani et al., 1993a, 1993b).

Since the stored nitrate is translocated from the caryopsis to the coleoptile during the anaerobic germination of rice (Mattana et al., 1993), the basal level of NR and NiR could be due to the ion availability. If this is the case, expression of NR and NiR during the anaerobic germination of rice may be enhanced by increasing the storage nitrate in the caryopsis.

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