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Nitrate reductases in hexaploid and tetraploid wheats and *Aegilops*

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Summary. Nitrate reductase activity (NR activity), protein content (NR protein) and polypeptides were compared in shoots of *Triticum aestivum* ssp. *vulgare* (L.) cv Fidel (bread wheat, AABBDD genome), *Triticum dicoccum* cv Vernal (AABB genome), *Aegilops squarrosa* var. *strangulata* (DD genome) and the amphiploid 365 (AABBDD genome), produced by crossing *T. dicoccum* cv Vernal and *Ae. squarrosa* var. *strangulata*. Constitutive NR protein and activity were found in shoots of all seedlings grown without nitrate, with the highest activity in the bread wheat. The inducible NR protein and activity developed upon the addition of nitrate. A 116-K polypeptide was identified as the main component of the NR from the bread wheat, while a faint, sometimes discernable 94-K band appeared on Western blots. Only one NR polypeptide could be identified in *Ae. squarrosa* – the 94 K. An intermediary situation was observed with the tetraploid *T. dicoccum* and the amphiploid: The 94-K polypeptide was the only one separated from NR of seedlings grown in the absence of nitrate. The 116-K polypeptide appeared after the addition of nitrate. The intensity of its band on the gel increased with the duration of the nitrate treatment. When comparing *Ae. squarrosa* and *T. dicoccum*, the constitutive isozyme (94-K polypeptide) was found in the D as well as in the AB genomes, while the inducible NR (116-K polypeptide) was absent from the D genome. Addition of the D genome into the AB genome slightly reinforced the expression of the inducible form (AB genome expression) in the amphiploid wheat. We postulate that the inducible form of NR in the bread wheat resulted from an evolutionary selection pressure favoured by cultivation.

Key words: Constitutive enzyme – Genome – Inducible enzyme – Nitrate – Pressure selection

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Introduction

Bread wheats resulted from the evolution and domestication of the allopolyploid *Triticum aestivum* (L.) Thell. ssp. *vulgare* (Vill.) M.K. (AABBDD, $2n=6x=42$). *Triticum aestivum* ssp. *vulgare* have arisen initially by crosses of wild diploid species ($BB \times AA$) yielding tetraploid wheats (AABB genomes, $2n=4x=28$). Subsequently, tetraploid wheats have hybridized with a diploid grass, *Aegilops*, with a genome DD ($2n=2x=14$) (Vedel et al. 1978), to yield the genome AABBDD of the contemporary cultivated species *Triticum aestivum*. Wheat contains up to three related (homeologous) forms of each gene, one on each of the component genomes, A, B or D, leading to multiple forms of several enzymes.

Two NADH-NR isozymes (EC 1.6.6.1.) have been reported for wheat. Because of their different sensitivity to the nitrate present in the nutrient solution, they were designated a constitutive and an inducible NR (Heath-Pagliuso et al. 1984; Kavanagh and Jones 1987). The structural genes for the constitutive NR subunit were shown to be carried on group 3 chromosomes, and those for the inducible NR on group 5 chromosomes (Moynihan and Jones 1987).

The aim of the present work was to determine whether both the constitutive and the inducible forms of NR protein in the bread wheat were present and active in the tetraploid and diploid ancestors, or whether they were the result of selection pressure. The strategy used for the study of the genetic transmission of the NR characters was to compare the effect of nitrate nutrition on NR activity, NR protein content and NR polypeptide of a cultivated wheat (*Triticum aestivum* L. cv Fidel, AABBDD) with those of the tetraploid wheat, *T. dicoccum* cv Vernal (AABB), the diploid, *Aegilops squarrosa* var. *strangulata* (DD) and the amphiploid (AABBDD)

produced by the hybridization *T. dicoccum* × *Ae. squarrosa*.

Materials and methods

Plant material

Triticum dicoccum cv Vernal 1263, cultivated wheat at the tetraploid level (AABB) and the wild *Aegilops squarrosa* var. *strangulata* 1270 (DD) were the gift of Dr. Kihara (National Institute of Genetics, Japan). The bread wheat *Triticum aestivum* (L.) cv Fidel was purchased at Ets Ringot (La Chapelle d'Armentières, France). The amphiploid 365 is the C_2 generation produced by the crossing *T. dicoccum* cv Vernal × *Ae. squarrosa* var. *strangulata* 1270, followed by colchicine treatment of the F_1 generation for doubling the chromosomes. The hexaploid nature of the seedlings was under cytogenetic control.

Culture and nitrate treatment

Seedlings were germinated and grown in vermiculite, either in the dark or under a 16-h photoperiod. They were watered with N-free nutrient solution (Talouizte et al. 1984). When they were 7 days old, 10 m*M* nitrate was added to the nutrient solution. Experiments were run with seedlings treated for 0, 1, 2 or 3 days with nitrate. Sampled shoots were frozen in liquid nitrogen and stored at −80 °C until needed.

Preparation of crude extracts

All steps were performed at 5 °C. Frozen shoots were ground in liquid nitrogen. The powder was extracted in (6 ml/g fresh weight) 25 m*M* Tris-HCl buffer, pH 8.5, containing 1% BSA, 1 m*M* leupeptin, 250 μ*M* paramethyl sulfonyl fluorure, 1 m*M* EDTA, 10 m*M* cysteine, 1 m*M* dithiothreitol, 20 μ*M* FAD, 1 μ*M* sodium molybdate, as described by Nakagawa et al. (1984) and modified by Wray and Kirk (1981). After centrifugation at 20,000 g for 30 min, the supernatant was designated the crude extract and used for measurement of NR activity, assay of NR protein and analysis of the NR polypeptide.

Assay methods

The crude extract was assayed for NADH : NR activity within 30 min after completion of its preparation. Activity was measured at 30 °C according to the method of Wray and Filner (1970). One unit of activity is the amount catalyzing the production of 1 μmol of nitrite/min.

NR protein was estimated with a two-site ELISA test, as described by Chérel et al. (1986) and adapted to wheat NR by Ouhmidou et al. (1989). The mAb ZM 96(9)25 was the first coating reagent. A rabbit immunoserum produced against wheat leaf nitrate reductase (Soualmi and Champigny 1986) was the second antibody. The goat anti-rabbit immunoglobulin G (IgG) conjugated with alkaline phosphatase (Biosys) was the final antibody. The phosphatase reaction developed in the presence of para-nitrophenyl phosphate (Sigma) and the color density was estimated at 405 nm.

Gel immunodiffusion

NR in the crude extracts from the *Aegilops* and the wheats here studied was assayed by immunodiffusion on agar plates (1% agarose, in 50 m*M* borate buffer, pH 8.4) according to Ouchterlony et al. (1949) in the presence of rabbit immunoserum against NR from leaves of *T. aestivum* cv Fidel. Staining was with Coomassie brilliant blue R 250.

Electrophoresis and electrophoretic transfer blotting

Before SDS/PAGE electrophoresis, the crude extract was denatured in Tris-HCl buffer, pH 6.8, containing 2% sodium dodecyl sulfate, 5% mercaptoethanol, 0.02% bromophenol blue, 10% glycerol, at 95 °C for 5 min. SDS/PAGE on slab gels (7.5% polyacrylamide) was performed according to Laemmli (1970). The detection of protein bands was by staining with Coomassie brilliant blue R 250. Apparent molecular masses were obtained by comparison with protein standards (Pharmacia Fine Chemicals).

For immunoblotting, the method of Towbin et al. (1979) was used. The proteins separated by SDS/PAGE were transferred to nitrocellulose sheets (0.45 μm, Schleicher and Schüll) by electrotransfer at 0.15 V/cm^2. Immunodetection was with a rabbit immunoserum produced against wheat shoot NR, followed by an anti-rabbit IgG-peroxidase conjugate. For staining, the nitrocellulose sheet was incubated in 4-chloro-1-naphtol (Merck, 3 mg/ml) and H_2O_2.

Results and discussion

NR in leaves of N-starved seedlings

The NR protein and NR activity were evident in N-starved green seedlings grown under a natural day/night regime (Fig. 1). Thus, *Ae. squarrosa, T. dicoccum,* the amphiploid and the bread wheat contained a constitutive NR, the synthesis and activity of which were possible in the absence of nitrate. This observation has already been shown in barley, wheat and oat seedlings (Heath-Pagliuso et al. 1984).

Effect of nitrate on NR activity and protein

When the N-starved green seedlings were treated with K-nitrate, both NR activity and NR protein level increased (Fig. 1). Except for cv Fidel, which had a high NR activity already before the treatment, the enhancement of the activity was greater than the increase of the protein level, which indicates that the effect of nitrate was not only through the induction of the enzyme apoprotein synthesis, but also via the activation of a NR cross-reacting inactive protein. It has been shown previously with maize (Oaks et al. 1988) that there are active and inactive forms of NR, and that the inactive form could be induced by contaminating NO_3^- found in sand or vermiculite. Conversion of the inactive form into an active form of NR could be by oxidation of the protein, a phenomenon well documented in Chlorella (Pistorius et al. 1976) and also observed in wheat (Aryan et al. 1983), or by induction and insertion of the Mo-cofactor synthesis (Mendel et al. 1982).

NR polypeptide identification and effect of nitrate

In immunodiffusion assays, the complete fusion of single precipitin bands indicated similar antigenic properties on *Ae. squarrosa,* the tetraploid and the hexaploid wheats

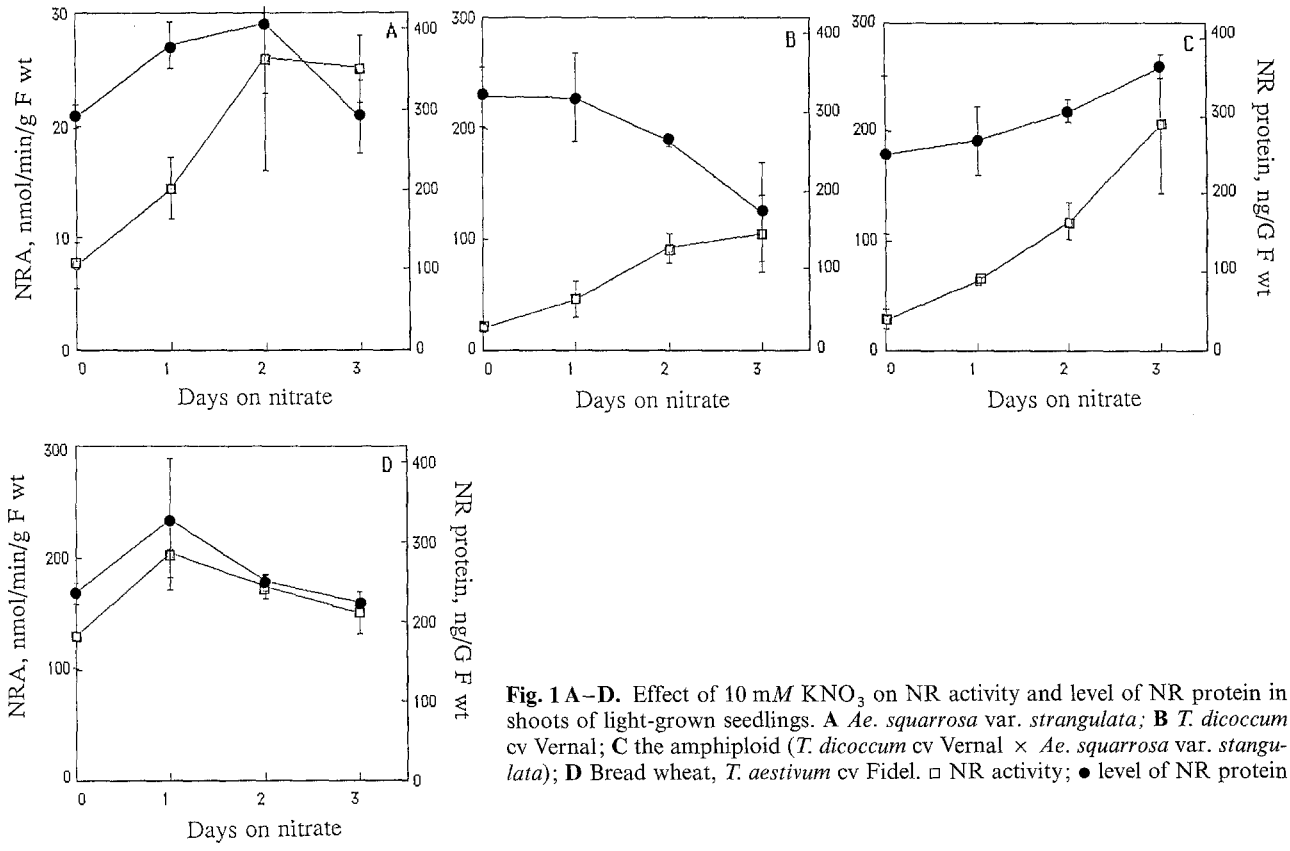

Fig. 1 A–D. Effect of 10 m*M* KNO_3 on NR activity and level of NR protein in shoots of light-grown seedlings. **A** *Ae. squarrosa* var. *strangulata;* **B** *T. dicoccum* cv Vernal; **C** the amphiploid (*T. dicoccum* cv Vernal × *Ae. squarrosa* var. *stangulata*); **D** Bread wheat, *T. aestivum* cv Fidel. □ NR activity; ● level of NR protein

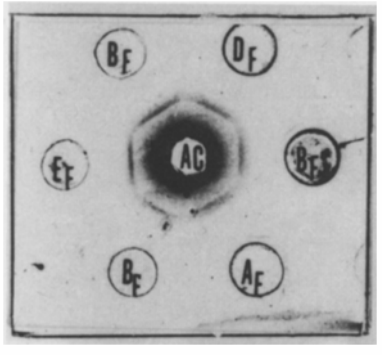

Fig. 2. Ouchterlony diffusion of NR with immunoserum prepared from the bread wheat, *T. aestivum* cv Fidel leaf NR (central well AC). Surrounding wells were filled with crude extracts from: (A_F) the amphiploid wheat; (B_F) *T. aestivum* cv Fidel; (D_F) *T. dicoccum* cv Vernal; (E_F) *Ae. squarrosa* var. *strangulata*

(Fig. 2). This validated the immunochemical study of the NR from all plants using the same immunoserum against the cultivated wheat NR.

SDS-PAGE of proteins of green leaves of *Ae. squarrosa, T. dicoccum* and both hexaploid wheats, sampled before and after addition of 10 m*M* nitrate, showed two polypeptides, of nuclear weight (MWt) 116 K and 94 K. They were designated NRa and NRb, respectively (Fig. 3). The two polypeptides were not identified in the leaves of each species under all conditions. The NRb band was the only one visible in *Ae. squarrosa* preparations. Its intensity increased over the two days of nitrate treatment. In the tetraploid *T. dicoccum* and the amphiploid, NRb was the only band in the N-starved samples. After treatment with nitrate, the NRa band appeared. Its intensity increased with duration of the treatment, while NRb remained fainter. In the leaves of *T. aestivum,* NRa was the main, and often the only discernable NR polypeptide, whether the seedlings were grown on N-free or nitrate-solution. That NRb was a proper poylpeptide from an individual NR and not an artefact resulting from NRa breakdown by degradating enzymes which would be more active in *Ae. squarrosa* and in the N-starved wheats than in the cv Fidel, was shown by comparison of the SDS-PAGE patterns of *Ae. squarrosa, T. aestivum* and a mixture of crude extracts from both seedlings. The intensity of the NRa band in the lanes containing the *T. aestivum* proteins was similar, whether or not these proteins had been mixed with the proteins from *Ae. squarrosa* (Fig. 4).

Based on the analysis of the data concerning NR activity, NR protein level and the identity of the polypeptides in N-starved or NO_3^--fed leaves from the diploid *Ae. squarossa,* the tetraploid *T. dicoccum* and their am-

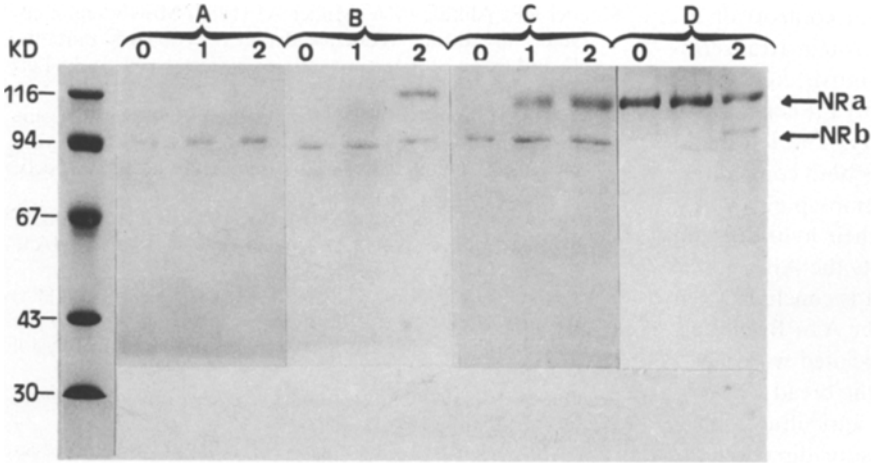

Fig. 3 A–D. Effect of NO_3^- on the form of NR polypeptide in green shoots of **A** *Ae. squarrosa* var. *strangulata;* **B** *T. dicoccum* cv Vernal; **C** the amphiploid (*T. dicoccum* × *Ae. squarrosa*); **D** Bread wheat *T. aestivum* cv Fidel. *All lanes 0,* no nitrate (before the treatment); *all lanes 1,* 1 day on 10 m*M* KNO_3; *all lanes 2,* 2 days on 10 m*M* KNO_3. SDS-PAGE western blots were made from denatured crude extracts. Blots were developed with rabbit antiserum against wheat leaf NR and goat IgG conjugated to peroxidase

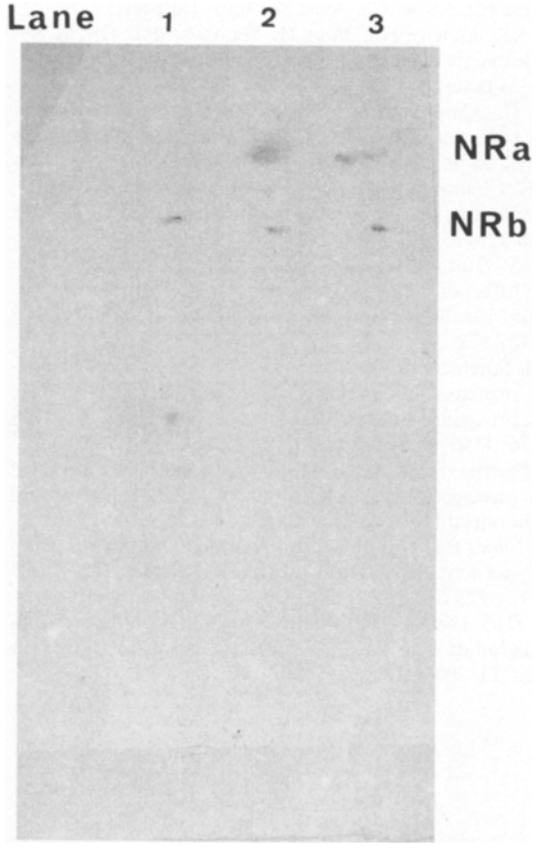

Fig. 4. SDS-PAGE western blots of denatured crude extracts from: (*lane 1*) *Ae. squarrosa* var. *stangulata;* (*lane 2*) *T. aestivum* cv Fidel; (*lane 3*) *Ae. squarrosa* mixed with *T. aestivum* cv Fidel. The blots were developed with the mAB ZM 96 (9) 25 against corn leaf NR and goat anti-mouse IgG conjugated to peroxidase

phiploid (Figs. 1 and 3), we propose the following interpretation: NRb, the only peptide present in N-starved leaves would be the polypeptide of a constitutive NR of low specific activity, or (and) a precursor of NR, synthesized in the absence of nitrate, slowly in the dark, faster in the light. NRa, polypeptide of the inducible form of NR, could be synthesized de novo and (or) also produced by the activation of a precursor protein. As in corn, high levels of nitrate would be necessary for converting an inactive NR cross-reacting protein to a form of the enzyme capable of reducing nitrate (Oaks et al. 1988). The difficulty in detecting the *T. aestivum* NRb polypeptide could be accounted for by the high sensitivity of the bread wheat NR to trace amounts of nitrate, as mentioned above.

The presence of two NR isozymes has been reported for wheat and other cereal species (Heath-Pagliuso et al. 1984; Kavanagh and Jones 1987; Remmler and Campbell 1986). Further characterization of the barley isozymes leads to the conclusion that one of them was the commonly reported inducible NR and the other one a constitutive NR, or a form induced at extremely low levels of NO_3^- (Heath-Pagliuso et al. 1984). The study described here extends this interpretation to the wheat NR. It shows, in addition, that the constitutive NR is active in vivo and can in some species be the only NR present in the leaves of seedlings grown in the light with no nitrate or with extremely low levels of nitrate nutrition.

Conclusion

Our results extend to wheat what was previously shown for barley, that the two NR isozymes which differ by their MWt are under different regulatory control. The possibility of two genes for NR, as established with barley by mutation methodology (Kleinhofs et al. 1985), sustains this conclusion.

The 94-K isozyme synthesized in the dark, and in the light in the absence of nitrate, is a constitutive form of NR. Since there are antigenic similarities, it may also be the precursor of the inducible form of NR. The 116-K isozyme depends on NO_3^-, which either activates the con-

stitutive precursor (post-transcriptional control) or induces the de novo synthesis of the protein (transcriptional/translational control). The primitive diploid *Ae. squarrosa* (DD genome) synthesized only the constitutive form, which has a low specific activity. The tetraploid *T. dicoccum* (AABB genomes) contains both constitutive and inducible forms of NR. The amphiploid wheat (AABBDD genomes) resulting from their hybridization (*T. dicoccum* × *Ae. squarrosa*) exhibits the AB genome characters relative to the NR forms. It is concluded that the inducible form is associated with the A or B genome, while only the constitutive NR is associated with the D genome. The sensitivity of the NR of the bread wheat to trace amounts of nitrate suggests that agricultural selection could represent a selective pressure directing the change or evolution of a species.

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