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An NADH nitrate reductase gene copy appears to have been deleted in barley

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Abstract Cultivated barley, *Hordeum vulgare* L., has a single NADH nitrate reductase (NR) gene while diploid wheat, *Triticum monococcum*, and cultivated hexaploid wheat, *Triticum aestivum* L., have two NADH NR genes. To determine whether the NADH NR gene was duplicated since the divergence of *Triticum* from *Hordeum* or was deleted from barley, the *T. monococcum* NADH NR gene heme-hinge regions were sequenced and compared with the barley NADH NR gene sequence. Sequence identity and phylogenetic analyses showed that one of the *T. monococcum* NADH NR genes is more-closely related to the barley NADH NR gene than to the other *T. monococcum* NADH NR gene. The heme-hinge region of all three NR genes appeared to have evolved at a constant rate. These results suggest that the NADH NR gene duplicated before the divergence of *Triticum* and *Hordeum* and that a deletion resulted in the loss of one NADH NR gene from cultivated barley.

Key words *Hordeum* · *Triticum* · Grasses · Gene deletion · Gene duplication

Introduction

Eukaryotic nitrate reductases (NRs) catalyze the reduction of nitrate to nitrite and are classified into three groups based on reduced pyridine nucleotide dependency: NADH-specific (EC 1.6.6.1), NAD(P) H-bispecific (EC 1.6.6.2), and NADPH-specific (EC 1.6.6.3) (Guerrero et al. 1981). All eukaryotic NRs have three highly-conserved functional domains binding FAD, heme, and the molybdenum cofactor (Kleinhofs et al. 1989). The three functional domains are separated by two short, less-conserved, hinge regions (Warner and Kleinhofs 1992).

Cultivated hexaploid wheat (*Triticum aestivum* L.) has at least two NADH NR genes per haploid genome with high homology to the barley (*Hordeum vulgare* L.) NADH NR gene (Kleinhofs et al. 1988). Genetic mapping has placed the barley NADH NR gene on the short arm of chromosome 6 and the six hexaploid wheat cv Chinese Spring NADH NR genes on the short arms of chromosomes 6A, 6B, 6D, 7A and 7D and on the long arm of chromosome 4B (Kleinhofs et al. 1988).

Previous studies showed that *T. monococcum*, many wild *Hordeum* species, as well as many other grasses, have two NADH NR genes, while *Hordeum spontaneum* and *H. vulgare* have only one NADH NR gene (Zhou 1993). These data suggest a deletion of one NADH NR gene from cultivated barley after its divergence from *Triticum*. Alternatively, a relatively-recent duplication of an ancestral NADH NR gene could have occurred in *Triticum*. Although the existence of two NADH NR genes per haploid genome in many of the grass species examined (Zhou 1993) suggests that the first explanation is more likely, direct evidence is lacking. The deletion vs duplication hypotheses were examined by comparing the homology of the NADH NR gene sequences from *T. monococcum* and barley. If the *T. monococcum* NADH NR gene was duplicated before the divergence of the two species, then one of the two *T. monococcum* NADH NR genes would be expected to be more-closely related to the barley NADH NR gene than to the other *T. monococcum* NADH NR gene. If the *T. monococcum* NADH NR gene was duplicated after the divergence of the two species, then the two *T. monococcum* NADH NR genes should be more similar to one another than to the barley NADH NR gene. The sequence analyses suggest that a deletion resulted in the loss of one NADH NR gene from barley. These conclusions are, of course,

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dependent on equal rates of evolution of NR genes in these species.

Materials and methods

Genomic DNA was isolated from leaves as described previously (Zhou 1993). Genomic DNA of *T. monococcum* ssp. *monococcum*, digested with *DraI* and hybridized with a barley NADH NR gene probe, revealed three distinct bands of about 8, 5 and 3 kb (Zhou 1993). Hybridization with the barley NAD(P)H NR gene-specific probe revealed that the 5-kb band was due to the NAD(P)H NR gene. Thus the 8- and 3-kb bands were due to the NADH NR genes and these were designated Tmo2 and Tmo1 for identification purposes only. For large scale isolation, 15 µg of *T. monococcum* genomic DNA was digested twice for 24 h with excess *DraI* (60 U) and separated in an 0.8% low-melting-temperature (LMT) agarose gel. The sections of the gel corresponding to the NADH NR gene bands were excised and the DNA recovered using GELase (Epicentre Technologies, Madison, Wis.) according to the "fast protocol" of the manufacturer's instructions.

NADH NR gene-specific oligonucleotide primers were designed (Table 1) based on the barley NADH NR cDNA sequence (Schnorr et al. 1991) using the OLIGO program (Rychlick and Rhoads 1989). The primers were synthesized by the Laboratory of Biotechnology and Bioanalysis at Washington State University and purified as described previously (Zhou 1993).

The genomic DNA recovered from each excised gel section was amplified by the polymerase chain reaction (PCR) with the primers NR 1395 + 25 and NR 2070 − 20 (Table 1). The PCR amplification was carried out as described previously (Zhou 1993) except that the annealing temperature was 60 °C. All stocks for PCR amplification were made, and all procedures were performed, with the precautions against DNA contamination suggested by Kwok and Higuchi (1989).

PCR products were purified by electrophoresis on a 1.5% LMT-agarose gel. The target band was excised, recovered as above and used for DNA sequencing. DNA sequences of the amplified fragments were determined from both directions with four internal primers (Table 1) using the double-stranded DNA cycle sequencing system (Bethesda Research Laboratories) in a programmable temperature cycler (Model 50, Coy Inc.) for 30 cycles at 95 °C for 1 min, 60 °C for 1 min, and 70 °C for 1 min. Electrophoresis was performed at 60 W constant power on 6% polyacrylamide (19 parts acrylamide: 1 part bisacrylamide) and 42% urea gels.

Sequence data were analyzed in a DEC VAX 11/785 and 386-25 MHz Personal Computer. The number of nucleotide substitutions was computed using Nei and Gojobori's (1986) method after the sequences were aligned by the program PILEUP in the Genetics Computer Group software package (Devereaux et al. 1984). In order to examine whether NR genes evolved at a constant rate, the relative-rate test (Sarich and Wilson 1967) was performed as described by Li and Tanimura (1987). The phylogenetic trees were constructed by the maximum parsimony method, maximum likelihood method, and UPGMA (unweighted pair-group method using arithmetic means), using the programs in the PHYLIP phylogeny inference package (Felsenstein 1989).

Table 1 Oligonucleotide primer sequences and location

Designation[a]	Primer sequence
NR 1395 + 25	5'GGGCATGATGAACAACTGCTGGTTC3'
NR 2070 − 20	5'GATGTGCTTGCCGACGGGGA3'
NR 1560 + 20	5'GCTCAAGCGGAGCACGTCCA3'
NR 1900 − 19	5'CCTTCGTCGCCTCGCGGAT3'
NR 1754 + 20	5'GCACCGAGGAGTTCGACGCC3'
NR 1716 + 20	5'GCCGGGGTGGTCCTTGAGGA3'

[a] The primer designation numbers correspond to the barley NADH NR cDNA sequence (accession number: X57844)

Results and discussion

The barley NADH NR gene and one of the *T. monococcum* NADH NR genes (Tmo2) shared higher amino-acid and nucleotide sequence identities than were observed between the barley NADH NR gene and the other *T. monococcum* NADH NR gene or between the two *T. monococcum* NADH NR genes (Table 2). The phylogenetic trees constructed with the UPGMA, maximum likelihood, and maximum parsimony methods also clustered one of the *T. monococcum* NADH NR genes (Tmo2) more closely with the barley NADH NR gene

Table 2 The amino-acid sequence identity (above diagonal) and nucleotide sequence identity (below diagonal) of the heme-hinge region of NR genes from *T. monococcum* and barley (*Nar1* and *Nar7* are the NADH and NAD(P)H NR genes of *H. vulgare* respectively. Tmo1 and Tmo2 are abbreviations used to designate the sequences of the two NADH NR genes of *T. monococcum*, respectively)

Genes	*Nar1*	*Nar7*	Tmo1	Tmo2
Nar1		71.2	90.7	97.7
Nar7	78.9		68.0	71.2
Tmo1	86.6	74.0		88.4
Tmo2	97.5	78.9	86.6	

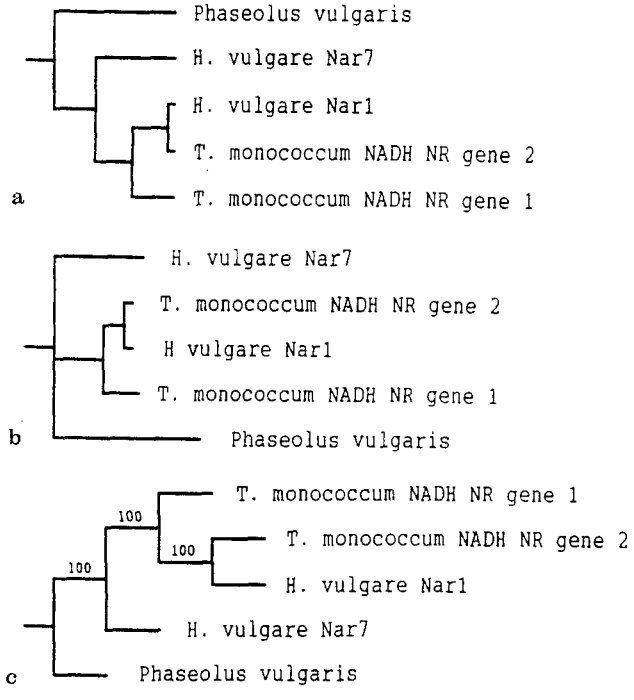

Fig. 1a–c Phylogenetic relationships of *T. monococcum* and barley NR genes. The trees were constructed with UPGMA (**a**); the maximum likelihood method (**b**); and the bootstrap parsimony method (**c**). The barley NAD(P)H NR gene sequence and bean and barley NADH NR sequences are from Miyazaki et al. (1991), Hoff et al. (1991), and Schnorr et al. (1991), respectively. In the bootstrap tree, the numbers above the horizontal lines are bootstrap confidence estimates on the branches in 100 replicates

Table 3 Relative-rate test of the NR gene heme-hinge fragments (K_{12} or K_{13} is the number of nucleotide substitutions per site between "species" 1 or "species" 2 and the reference "species" 3. SE is the standard error of ($K_{12} - K_{23}$). The null hypothesis is $K_{13} - K_{23} = 0$. Pvu is the NADH NR gene of *Phaseolus vulgaris*. Other abbreviations are in Table 2 footnote)

"Species" 1	Species" 2	"Species" 3 reference	Nonsynonymous substitution	Overall substitution
			$K_{13} - K_{23} \pm SE$	$K_{13} - K_{23} \pm SE$
Tmo1	Tmo2	Pvu	-0.021 ± 0.021	0.014 ± 0.031
Tmo1	*Nar1*	Pvu	-0.007 ± 0.019	0.014 ± 0.031
Tmo1	*Nar7*	Pvu	-0.050 ± 0.030	-0.009 ± 0.041
Tmo2	*Nar1*	Pvu	0.014 ± 0.018	0.000 ± 0.014
Tmo2	*Nar7*	Pvu	-0.029 ± 0.039	-0.023 ± 0.039
Nar1	*Nar7*	Pvu	-0.043 ± 0.038	-0.023 ± 0.030

than with the other *T. monococcum* NADH NR gene (Tmo1) (Fig. 1a–c). Although only a small portion of the NR genes was sequenced, the barley *Nar1* and *Nar7* comparison based on this short sequence is representative of that based on the entire gene sequence (Miyazaki et al. 1991). Thus, there is reason to believe that the data for the *T. monococcum* genes are also representative.

The sequence identity and phylogenetic analyses are concordant in suggesting that one of the *T. monococcum* NADH NR genes (Tmo2) is more-closely related to the barley NADH NR gene than to the other *T. monococcum* NADH NR gene (Tmo1). These data imply that the NADH NR gene duplicated before the divergence of *Triticum* and *Hordeum* with subsequent deletion of one NADH NR gene in cultivated barley. The time of the deletion of the second NADH NR gene in barley appears to be relatively recent. This is suggested by our observation of the presence of two NADH NR genes in *H. bulbosum* (unpublished), a species closely related to *H. vulgare* (Dewey 1984; Jorgensen 1986).

The conclusion that a barley NADH NR gene was deleted may be invalid if the NR genes evolved at different rates. This question is particularly relevant in the absence of knowledge on whether both or either of the two *T. monococcum* NR genes are expressed. The constancy of evolutionary rate among the NR gene heme-hinge regions was examined with the relative-rate test (Table 3). No significant deviation from the expectation that species 1 and 2 show equal divergence from the reference species was observed, suggesting that the nonsynonymous and overall nucleotide substitution rates of the NR gene heme-hinge regions were constant. These data lend support to the above conclusion that the barley NADH NR gene is more-closely related to one of the *T. monococcum* genes than the two *T. monococcum* genes are to one another.

The barley NADH NR gene deletion hypothesis is also supported by our previous results, showing the existence of two or more NADH NR genes per haploid genome in many grass species, including wheat, rice, sorghum, rye, millet, bamboo, many wild *Hordeum* species and some turf and forage species (Zhou 1993). An NADH NR gene duplication before grass speciation seems more likely than multiple duplications in all of the above species.

Phylogenetic analyses showed that the *T. monococcum* and barley NADH NR genes are clustered separately from the barley NAD(P)H NR gene (Fig. 1a, b, c), suggesting that the *T. monococcum* and barley NADH NR genes are more-closely related to each other than to the barley NAD(P)H NR gene. This implies that the duplication of the progenitor NR gene leading to NADH and NAD(P)H NR genes occurred before the duplication of the NADH NR genes.

In conclusion, sequence analyses indicate that the NADH NR gene duplicated before the divergence of *Triticum* from *Hordeum* and probably before the radiation of most grass species. The occurrence of a single NADH NR gene in barley is the exception and probably resulted from a deletion event in that species.

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