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Inheritance and expression of NAD(P)H nitrate reductase in barley*

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Summary. NADH-specific and NAD(P)H bispecific nitrate reductases are present in barley (*Hordeum vulgare* L.). Wild-type leaves have only the NADH-specific enzyme while mutants with defects in the NADH nitrate reductase structural gene (*nar*1) have the NAD(P)H bispecific enzyme. A mutant deficient in the NAD(P)H nitrate reductase was isolated in a line (*nar*1a) deficient in the NADH nitrate reductase structural gene. The double mutant (*nar*1a;*nar*7w) lacks NAD(P)H nitrate reductase activity and has xanthine dehydrogenase and nitrite reductase activities similar to *nar*1a. NAD(P)H nitrate reductase activity in this mutant is controlled by a single codominant gene designated *nar*7. The *nar*7 locus appears to be the NAD(P)H nitrate reductase structural gene and is not closely linked to *nar*1. From segregating progeny of a cross between the wild type and *nar*1a;*nar*7w, a line was obtained which has the same NADH nitrate reductase activity as the wild type in both the roots and leaves but lacks NADPH nitrate reductase activity in the roots. This line is assumed to have the genotype *Nar*1*Nar*1*nar*7*nar*7. Roots of wild type seedlings have both nitrate reductases as shown by differential inactivation of the NADH and NAD(P)H nitrate reductases by a monospecific NADH-nitrate reductase antiserum. Thus, *nar*7 controls the NAD(P)H nitrate reductase in roots and in leaves of barley.

Key words: *Hordeum vulgare* – Mutants – Nitrate reductase

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Introduction

Nitrate reductase-deficient mutants are valuable tools for studying the genetic control of nitrate assimilation in eukaryotic organisms (for reviews see Cove 1979; Dunn-Coleman et al. 1984; Garrett and Amy 1978; Kleinhofs et al. 1983, 1985; Warner et al. 1985). In fungi, nitrate reductase (NR) is controlled by a single gene for the apoenzyme, up to five genes for the molybdenum cofactor (MoCo), and several genes regulating induction and repression (Cove 1979; Garrett and Amy 1978). Genetic regulation of NR in higher plants appears to be similar except that regulatory genes have not yet been identified (Kleinhofs et al. 1985). In barley, the MoCo is controlled by at least five genes (Kleinhofs et al. 1985; unpublished data).

The genetic regulation of nitrate assimilation in some higher plant species will probably be more complex than in fungi. For example, tobacco (*Nicotiana tabacum* L.), an allotetraploid, has duplicate genes for the NR apoenzyme (Müller 1983). Presumably other polyploids also have duplicate genes for NR and other enzymes. In addition, multiple forms of NR have been shown to be present in some plant species. NADH specific and NAD(P)H bispecific forms of NR have been reported in soybean (Jolly et al. 1976), maize (Redinbaugh and Campbell 1981), rice (Shen et al. 1976) and barley (Dailey et al. 1982b). In soybean, a mutant has been isolated which lacks the constitutive NAD(P)H NR but retains the inducible NADH NR (Nelson et al. 1983; Ryan et al. 1983).

Leaves of wild-type barley have only the NADH specific NR (Dailey et al. 1982a) while leaves of *nar*1 (NADH NR structural gene) mutants are deficient in the NADH NR but possess moderate levels of a NAD(P)H bispecific NR (Dailey et al. 1982b). The

NADH and NAD(P)H NRs have different kinetic, immunological and physical characteristics (Harker et al. 1986). Although the NAD(P)H NR activity in *nar*1 mutants is much lower than the NADH NR activity in the wild type, *nar*1 mutants are capable of good growth with nitrate as a nitrogen source (Oh et al. 1980; Warner and Kleinhofs 1981).

This report describes the isolation of a new barley mutant deficient in NAD(P)H NR and documents the presence of both NADH and NAD(P)H NRs in roots of wild-type barley.

Materials and methods

Mutant *nar*1a (formerly Az12) was subjected to mutagenesis with sodium azide (Kleinhofs et al. 1978). Mutagenized seed (M_1 generation) were space-planted in the field to produce M_2 seed. M_2 seedlings were screened for in vivo NR activity by a modification of the procedure previously described (Warner et al. 1977). Two leaf discs were removed from the primary leaves of 7-day-old seedlings with a paper punch. The leaf discs were placed in a microtitration plate well containing 0.3 ml NR assay medium (0.1 M potassium nitrate; 0.1 M potassium phosphate, pH 7.0; 0.01% Tergitol NPX), vacuum infiltrated, incubated in the dark for 30 min, and assayed for nitrite (Warner et al. 1977). Seedlings deficient in NR activity were transplanted and grown to maturity with urea as a nitrogen source.

Progeny from selected plants and segregating generations from crosses were grown in growth chambers (Warner et al. 1974) and assayed for in vitro NADH and NADPH NR activities (Dailey et al. 1982a). NR activities are expressed in units (μmoles nitrite produced/g fresh weight · h). All NADPH NR assays were conducted in the presence of 1.8 μg/ml lactic dehydrogenase and 0.5 mM pyruvate to competitively eliminate NADH produced by phosphatase conversion of NADPH and NADH. A monospecific, polyclonal antiserum against NADH NR was raised as previously described (Somers et al. 1983).

Table 1. NADH and NADPH nitrate reductase activities in roots and leaves of Steptoe, *nar*1a and *nar*1a; *nar*7w seedlings

Part	Cofactor	Nitrate reductase activity (Units)		
		Steptoe	*nar*1a	*nar*1a;*nar*7w
Leaves	NADH	61.5	3.08	0.12
	NADPH	0.37	5.48	0.15
Roots	NADH	2.09	0.94	0.06
	NADPH	1.29	1.82	0.08

Table 2. Segregation for NADH nitrate reductase activity in leaves of F_2 and F_3 seedling from Steptoe × *nar*1; *nar*7w. F_2 families were classified as HH if all F_3 seedlings had high activity (greater than 40% of Steptoe), as LL if all seedlings had low NR activity (less than 20% of Steptoe) or as HL if seedlings segregated for high or low activity

F_2 plants		F_2 families		
NR Act.	No.	HH	HL	LL
Units		No.		
35–40	2	2	0	0
30–34	7	5	2	0
25–29	8[a]	1	6	0
20–24	10	0	10	0
15–19	4	0	4	0
9–14	1	0	1	0
5–9	1	0	0	1
1–4	4	0	0	4
0–0.9	2	0	1[b]	1

[a] One F_2 plant did not produce seed

[b] Abnormal segregation; probably due to seed mixture or outcrossing

Results and discussion

A mutant was selected from an M_2 population obtained by mutagenesis of *nar*1a seeds. This mutant was designated *nar*1a;*nar*7w after biochemical and genetic analyses revealed the presence of a gene specific to the NAD(P)H NR. Mutant *nar*1a;*nar*7w lacked most of the NAD(P)H NR present in *nar*1a and the NADH NR present in the wild type (Table 1). Xanthine dehydrogenase and nitrite reductase activities in *nar*1a;*nar*7w, *nar*1a and Steptoe were all similar (data not shown). These results indicate that mutations in *nar*1a;*nar*7w are not in loci controlling the MoCo. In fungi, the pleiotropic loss of NR and nitrite reductase activities is diagnostic of a regulatory gene mutation while the pleiotropic loss of NR and xanthine dehydrogenase activities is evidence for a defective MoCo gene (Cove 1979; Garrett and Amy 1978; Kleinhofs et al. 1985). NR activity can be restored in crude extracts of most MoCo mutants by in vitro complementation with a MoCo donor such as milk xanthine oxidase (Kleinhofs et al. 1985; Narayanan et al. 1984). In barley only loci controlling the MoCo and the NADH NR apoenzyme have been previously uncovered (Kleinhofs et al. 1985). Therefore, the gene controlling the NAD(P)H NR is representative of a new NR locus and is designated *nar*7.

Segregation of progeny for NR activity from a cross between Steptoe and *nar*1;*nar*7w indicated that the *nar*1 and *nar*7 genes act independently. Among F_2 progeny, 31 seedlings had high and 7 seedlings had low NR activity (Table 2). This is a good fit to a 12:4 ratio ($X^2 = 0.88$; $P = 0.75$–0.90). F_2 individuals classified as low NR ranged from the phenotypes of *nar*1a to *nar*1a; *nar*7w. Further subclassification of the low NR individuals was not possible due to continuous variation and the small sample size. Individuals heterozygous for *nar*7 appeared to have NR activity intermediate to *nar*1a and *nar*1;*nar*7w. NR activities of individual F_3 seedlings confirmed most of the F_2 classifications. F_2 individuals with highest NR activities tended to be homozygous for NADH NR while the F_2s with intermediate ac-

Table 3. Observed and predicted NADH nitrate reductase activities in leaves of *nar*1 and *nar*7 genotypic combinations

Genotype	Line	Relative nitrate reductase	
		Observed	Predicted
*Nar*1*Nar*1*Nar*7*Nar*7	Steptoe	100	
*Nar*1*Nar*1*Nar*7*nar*7			100
*Nar*1*Nar*1*nar*7*nar*7	F_3 selection	100	
*Nar*1*nar*1*Nar*7*Nar*7	Step. × *nar*1a	60[a]	
*Nar*1*nar*1*Nar*7*nar*7	Step. × *nar*1a;*nar*7w	57	
*Nar*1*nar*1*nar*7*nar*7			60
*nar*1*nar*1*Nar*7*Nar*7	*nar*1a	5	
*nar*1*nar*1*Nar*7*nar*7			3
*nar*1*nar*1*nar*7*nar*7	*nar*1a;*nar*7w	< 1	

[a] See Kleinhofs et al. 1980

tivities segregated in the F_3 (Table 2). Kleinhofs et al. (1980) showed that *nar*1 is codominant and that the heterozygote had about half the NR activity of the wild type. The low NR individuals exhibited a range of NR activities with the extremes typical of *nar*1 and *nar*1; *nar*7w. Heterozygotes at *nar*7 appeared to have intermediate NR activity suggesting that *nar*7 is also codominant.

The observed segregation patterns are consistent for two independent loci where one locus has a much greater influence on the observed phenotype than the other locus. In seedling leaves, the NR activity under control of the NADH NR structural gene (*nar*1) is much greater than the NAD(P)H NR activity controlled by *nar*7 (Table 1). Furthermore, the NAD(P)H NR is not expressed in leaves in the presence of the NADH NR (Dailey et al. 1982b). Therefore, 75% of the F_2 progeny from *nar*1a;*nar*7w × Steptoe would be expected to have high NADH NR activity. Individuals with low NR activity would be expected to segregate for the *nar*1 and *nar*7 genotypes. Observed and predicted NADH NR activities of the *nar*1 and *nar*7 genotypic combinations are presented in Table 3. The observed segregations are in agreement with this model.

Leaves of Steptoe barley have only the NADH NR (Dailey et al. 1982a), while roots have NR activity with both NADH and NADPH (Table 1). The NADPH NR to NADH NR ratio of 0.62 was an indication that roots of the wild type contained a mixture of the two enzymes because the NAD(P)H enzyme of *nar*1a has about 1.8 times more NR activity with NADPH than with NADH (Harker et al. 1986), while the NADH enzyme has very low NR activity with NADPH (Table 1; Dailey et al. 1982a).

Further evidence that roots of Steptoe barley have both NRs was provided by the differential inactivation

Table 4. Inactivation of NADH and NADPH nitrate reductase activities from Steptoe and *nar*1a by an antiserum raised against NADH nitrate reductase. Crude extracts were incubated for 60 min at 0°–2 °C with 12 µl/ml preimmune serum (PI); or with 6 or 12 µl/ml NADH-nitrate reductase antiserum (ANR)

Genotype	Part	Serum		Nitrate reductase activity		
				NADH	NADPH	NADPH/NADH
		µl/ml		Units		Ratio
Steptoe	Leaf	PI	12	57.8	0.26	< 0.01
		ANR	6	41.6	0.02	< 0.01
		ANR	12	18.4	0.02	< 0.01
	Root	PI	12	7.1	1.79	0.25
		ANR	6	1.7	1.44	0.85
		ANR	12	1.23	1.43	1.16
*nar*1a	Leaf	PI	12	2.72	6.04	2.22
		ANR	6	1.7	3.32	1.95
			12	1.45	2.72	1.88
	Root	PI	12	1.87	3.49	1.87
		ANR	6	1.53	3.06	2.00
		ANR	12	0.97	1.86	1.92

of NADH and NADPH NR activities by the NADH NR antiserum (Table 4). Both the NADH and NAD(P)H enzymes are inactivated by the NADH NR antiserum but the NAD(P)H NR requires several times more antiserum for inactivation than does the NADH NR (Harker et al. 1986). Therefore, in mixtures of the two enzymes, the NADH NR is preferentially inactivated and the NADPH/NADH NR ratio is increased. Since the ratio changed only in the roots of the wild type and not in the leaves of the wild type nor in the leaves or the roots of *nar*1a (Table 4), both the NADH and NAD(P)H NRs must be present in roots of the wild type.

The knowledge that roots of the wild type have both NRs was used to demonstrate that *nar*1 and *nar*7 genes act independently and to isolate a line having normal NADH NR activity but lacking the NAD(P)H NR. Progeny of the nine F_2 individuals homozygous for leaf NADH NR were assayed for root NADPH NR activity. Of these, eight segregated for the presence or absence of NAD(P)H NR and one was homozygous for the presence of NAD(P)H NR activity (Table 5). The combined segregation of the eight lines was 44 seedlings with NADPH NR and 19 without NADPH NR activity. This is a good fit to a 3:1 ratio ($X^2 = 0.89$; $P = 0.75$–0.90). Individual F_3 seedlings homozygous for NADH NR in leaves and lacking NADPH NR activity in roots were transplanted and allowed to produce seed. These lines have wild-type levels of NADH NR in both the roots and leaves but lack the NADPH NR activity present in the roots of the wild type (Table 6).

Table 5. NADPH nitrate reductase activity in roots of F_3 seedlings from F_2 families (Steptoe × *nar*1a; *nar7*w) homozygous for NADH nitrate reductase

Line	Nitrate reductase activity (units)					
	5–6	4–4.9	3–3.9	2–2.9	1–1.9	0–0.9[a]
	No. of seedlings					
84-106-1	6	3	1			
84-105-12	4		1			5
84-104-1	1	1	2	1		4
84-106-6			3		3	2
84-106-10		4	1		1	2
84-106-11	4	1	1	1	1	2
84-106-12	1	1		2	2	4
84-106-3			1		8	1
Steptoe	2	8	12		1	

[a] Observed activities all less than 0.5 units

Table 6. NADH and NADPH nitrate reductase activities of a line selected for the absence of root NADPH nitrate reductase activity from *nar*1a; *nar7*w × Steptoe

Part	Cofactor	Nitrate reductase activity (Units)	
		Steptoe	*Nar*1*Nar*1*nar7nar7*
Leaves	NADH	38.6	39.4
	NADPH	0.1	0.1
Roots	NADH	6.24	4.22
	NADPH	2.20	0.08

These results indicate the *nar7* locus controls the NAD(P)H NR but not the NADH NR. The presence of xanthine dehydrogenase activity in *nar*1a;*nar7*w and NADH NR activity in the reselected lines (Table 6) exclude a MoCo function for the *nar7* locus. The *nar7* locus is probably the NAD(P)H NR structural gene but regulatory functions specific to the NAD(P)H NR cannot be ruled out even though nitrite reductase is present in all genotypes.

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References

Cove DJ (1979) Genetic studies of nitrate assimilation in *Aspergillus nidulans.* Biol Rev 54:291–327

Dailey FA, Kuo T, Warner RL (1982a) Pyridine nucleotide specificity of barley nitrate reductase. Plant Physiol 69:1196–1199

Dailey FA, Warner RL, Somers DA, Kleinhofs A (1982b) Characteristics of a nitrate reductase in a barley mutant deficient in NADH nitrate reductase. Plant Physiol 69:1200–1204

Dunn-Coleman NS, Smarrelli J Jr, Garrett RH (1984) Nitrate assimilation in eukaryotic cells. Int Rev Cytol 92:1–50

Garrett RH, Amy NK (1978) Nitrate assimilation in fungi. Adv Microbiol Physiol 18:1–65

Harker AR, Narayanan KR, Warner RL, Kleinhofs A (1986) NAD(P)H bispecific nitrate reductase in barley leaves: partial purification and characterization. Phytochemistry 25:1275–1279

Jolly SO, Campbell W, Tolbert NE (1976) NADPH- and NADH-nitrate reductases from soybean leaves. Arch Biochem Biophys 174:431–439

Kleinhofs A, Warner RL, Muehlbauer FJ, Nilan RA (1978) Induction and selection of specific gene mutations in *Hordeum* and *Pisum.* Mutat Res 51:29–35

Kleinhofs A, Kuo T, Warner RL (1980) Characterization of nitrate reductase-deficient barley mutants. Mol Gen Genet 177:421–425

Kleinhofs A, Taylor J, Kuo TM, Somers DA, Warner RL (1983) Nitrate reductase genes as selectable markers for plant cell transformation. In: Lurquin PF, Kleinhofs A (eds) Genetic engineering in eukaryotes. Plenum Press, New York, pp 215–231

Kleinhofs A, Warner RL, Narayanan KR (1985) Current progress towards an understanding of the genetics and molecular biology of nitrate reductase in higher plants. In: Miflin BJ (ed) Oxford surveys of plant molecular and cell biology, vol 2. Oxford University Press, Oxford, pp 91–121

Müller AJ (1983) Genetic analysis of nitrate reductase-deficient tobacco plants regenerated from mutant cells. Evidence for duplicate structural genes. Mol Gen Genet 192:275–281

Narayanan KR, Müller AJ, Kleinhofs A, Warner RL (1984) In vitro reconstitution of NADH: nitrate reductase in nitrate reductase-deficient mutants of barley. Mol Gen Genet 197:358–362

Nelson RS, Ryan SA, Harper JE (1983) Soybean mutants lacking constitutive nitrate reductase activity. I. Selection and initial plant characterization. Plant Physiol 72:503–509

Oh JY, Warner RL, Kleinhofs A (1980) Effect of nitrate reductase deficiency upon growth, yield, and protein in barley. Crop Sci 20:487–490

Redinbaugh MG, Campbell WH (1981) Purification and characterization of NAD(P)H: nitrate reductase and NADH: nitrate reductase from corn roots. Plant Physiol 68:115–120

Ryan SA, Nelson RS, Harper JE (1983) Soybean mutants lacking constitutive nitrate reductase activity. II. Nitrogen assimilation, chlorate resistance, and inheritance. Plant Physiol 72:510–514

Shen T-C, Funkhouser EA, Guerrero MG (1976) NADH- and NAD(P)H-nitrate reductases in rice seedlings. Plant Physiol 58:292–294

Somers DA, Kuo T-M, Kleinhofs A, Warner RL (1983) Nitrate reductase-deficient mutants in barley. Immunoelectrophoretic characterization. Plant Physiol 71:145–149

Warner RL, Kleinhofs A (1974) Relationships between nitrate reductase, nitrite reductase, and ribulose diphosphate carboxylase activities in chlorophyll-deficient mutants of barley. Crop Sci 14:654–658

Warner RL, Kleinhofs A (1981) Nitrate utilization by nitrate reductase-deficient barley mutants. Plant Physiol 67:740–743

Warner RL, Lin CJ, Kleinhofs A (1977) Nitrate reductase-deficient mutants in barley. Nature 269:406–407

Warner RL, Kleinhofs A, Narayanan KR (1985) Genetics, biochemistry, and physiology of nitrate reductase-deficient mutants in barley. In: Harper JE, Schrader LE, Howell RW (eds) Exploitation of physiological and genetic variability to enhance crop productivity. Am Soc Plant Physiol, Rockville, Maryland, pp 23–30