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Genetic mapping of the barley nitrate reductase-deficient *nar*1 and *nar*2 loci*

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Summary. The *nar*2 locus that codes for a protein involved in molybdenum cofactor function in nitrate reductase and other molybdoenzymes was mapped to barley chromosome 7. F_2 genotypic data from F_3 head rows indicated *nar*2 is located 8.4 ± 2.1 and 23.0 ± 4.6 cm from the narrow leaf dwarf (*nld*) and mottled seedling (*mt*2) loci, respectively. This locates the *nar*2 locus at 54.7 ± 3.1 cm from the short-haired rachilla (*s*) locus near the centromere of chromosome 7. Close linkage of *nar*2 with DDT resistance (*ddt*) and high lysine (*lys*3) loci was detected but could not be quantified due to deviations from the individual expected 1 : 2 : 1 segregations for the *ddt* and *lys*3 genes. Southern blots of wheat-barley addition lines probed with a nitrate reductase cDNA located the NADH : nitrate reductase structural gene, *nar*1, to chromosome 6.

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

Introduction

Nitrate reductase (NR) is the rate limiting enzyme involved in nitrogen assimilation that catalyzes the reduction of NO_3^- to NO_2^- (Beevers and Hageman 1969, 1980; Hewitt 1975). Several NR-deficient barley mutants have been isolated to aid in the characterization of the NR enzyme, its genetics and regulation (Tokarev and Shumny 1977; Warner et al. 1977; Kleinhofs et al. 1978, 1980; Bright et al. 1983). The mutants (symbolized as *nar*) belong to seven different complementation groups. The *nar*1 and *nar*7 loci are the NADH and NAD(P)H structural gene loci, respectively. Loci *nar*2–*nar*6 are involved with the molybdenum cofactor (MoCo) functions (A Kleinhofs, unpublished). All molybdenum cofactor mutants show pleiotropic deficiency for nitrate reductase and xanthine dehydrogenase (Kleinhofs et al. 1985). The function of the *nar*2 gene in relation to MoCo synthesis or activation is unknown at present; however, since mutant *nar*2a contains assembled flavohaemoprotein NR subunits (Narayanan et al. 1983, 1984) it may be concluded that the *nar*2 gene is not involved in molybdopterin synthesis but in some other aspect of MoCo formation (Wray 1986).

Preliminary linkage data indicated that the *nar*2 locus was weakly linked to the short-haired rachilla (*s*) locus on chromosome 7, and segregated independently of representative marker genes on the other six chromosomes (A Kleinhofs, unpublished). The *nar*2 locus was independent of the smooth awn (*r*) locus on chromosome 7. The *r* locus is distal to the *s* locus on the long arm of chromosome 7, suggesting *nar*2 is on the centromeric side of the *s* locus on 7L or on 7S.

Jensen (1981) and Tsuchiya (1985) have constructed detailed linkage maps of chromosome 7. Two genes, DDT resistance (*ddt*) (Shahla and Tsuchiya 1985) and erectoides (*ert* in cv Golden Promise) (Thomas et al. 1984) have been mapped to the short arm of chromosome 7. Pickering (1983) found the gene that controls partial incompatibility of *H. vulgare* cv Vada × *H. bulbosum* crosses (*inc*) closely linked with the *ddt* gene ($r = 0.11 \pm 0.03$). Whether the *inc* locus is on 7L or 7S is unknown. Other genes, including narrow leaf dwarf

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(*nld*), high lysine (*lys*3) Risø 1508 and mottled-2 (*mt*2) are located near the centromere on the long arm of chromosome 7 (Ullrich and Eslick 1977, 1978; Sozinov et al. 1979; Jensen 1981; Shahla and Tsuchiya 1984, 1985). The centromere of chromosome 7 is located between the *nld* and *ddt* loci (Shahla and Tsuchiya 1984, 1985) a map distance of only 15.2 cm (Jensen 1981).

Islam et al. (1976) and Fedak (1985) developed a set of wheat-barley addition lines that include individual barley chromosome pairs in a wheat background. This allows barley genes to be mapped to a chromosome if barley and wheat display restriction fragment length polymorphism for the gene. Saghai-Maroof et al. (1984) were able to map two ribosomal DNA genes, *rrn*1 and *rrn*2, to chromosomes 6 and 7 using these addition lines. The wheat-barley addition lines have also been used to confirm the location of the two structural genes for α-amylase (amy-2, amy-1) on chromosomes 1 and 6, respectively (Muthukrishnan et al. 1984).

The present investigation was carried out to map the *nar*2 locus on chromosome 7 and to determine the chromosomal location of *nar*1.

Materials and methods

Crosses and classification of genotypes

The parents and the chromosome 7 marker genes used in this study are listed in Table 1. F_2 plants were grown in the field in 1985 and visually scored for *nar*2a (yellow-green seedlings), *nld* (narrow leaf dwarf) and *mt*2 (mottled leaves) phenotypes. Individual plants were also assayed for NR activity. Heads were harvested from each F_2 plant and seed was sown as F_3 head rows in the field in 1986. Rows were scored for *nar*2a, *nld* and *mt*2 phenotypes as seedlings. F_3 plants from the Risø 1508 × *nar*2a cross were sprayed with a suspension of 12.5% active ingredient DDT and 0.5% Tween 20 at approximately the 4–5 leaf stage. After 14 days, plants were scored for chlorosis of sprayed leaves (Ramage 1984). F_3 seed harvested from F_2 plants were scored for the *lys*3 phenotype. Seeds homozygous for *lys*3 were characterized by a shrunken endosperm with a noticeable depression on the dorsal or germ side. In addition, classification for segregation of the *lys*3 trait in the F_3 seeds was done by a modified turbidity test (Jensen 1979). Seeds were cut in half and the end distal to the embryo crushed and soaked overnight in 2 ml of 50% v/v isopropanol in H_2O plus 0.5% v/v 2-mercaptoethanol. Three milliliters of 0.1 *M* NaCl were added, mixed and allowed to stand overnight. Homozygous *lys*3 seeds gave a faint turbidity while all other genotypes gave a clear solution with agglutinated material on the bottom of the tube.

In a few instances F_3 head rows could not be clearly classified in the field for *ddt* or *mt*2 genotypes. In these cases reserve seed was screened in a growth chamber or greenhouse.

Estimation of linkage

Compiled F_3 data for each cross were analyzed using Linkage-1 (Suiter et al. 1987). The recombination percentage, r, was converted to map distance, D, using the Kosambi function to correct for undetected double crossovers. Estimating the chromosomal location of *nar*2 was accomplished using the maximum likelihood method (see Jensen and Jorgensen 1975).

Table 1. Parents used in crosses for mapping *nar2*

Line	Genotype	Phenotype
Nagaoka Dwarf	*nld nld; Nar2 Nar2*	narrow leaf dwarf
Risø 1508	*lys3 lys3; ddt ddt; Nar2 Nar2*	high lysine, DDT resistant
Montcalm	*mt2 mt2; Nar2 Nar2*	mottled seedling
Az34	*Nld Nld; Lys3 Lys3; Ddt Ddt; Mt2 Mt2; nar2a nar2a*	nitrate reductase-deficient

Southern hybridizations

Wheat-barley addition lines were obtained from Islam (Waite Agricultural Research Institute, University of Adelaide, Adelaide, South Australia). DNA for Southern blot analysis was isolated using the procedure of Saghai-Maroof et al. (1984). Restriction enzyme digested DNA was separated on 0.7% agarose gels and transferred to GeneScreen membrane (NEN Research Products, Boston, Mass) using the alkaline transfer method (Chomczynski and Qasba 1984). After transfer, the membrane was neutralized in 0.1 *M* Na_2HPO_4 buffer pH 7.2 for 5–10 min. The DNA was covalently bound to the membrane by UV irradiation for 5 min. The filter was then rinsed in H_2O and prehybridized for 5 min at 65 °C in 20 ml hybridization buffer (1% BSA, 1 m*M* EDTA, 0.5 *M* Na_2HPO_4 pH 7.2, 7% SDS). Hybridization was 18 h at 65 °C in 10 ml hybridization buffer plus 100 ng nick translated 1.1 kb Pst I fragment of bNRp10 ($1–2 \times 10^8$ cpm/μg) (Cheng et al. 1986). Washes were as follows: two times with 250 ml wash no. 1 (0.5% BSA, 1 m*M* EDTA, 40 m*M* Na_2HPO_4 pH 7.2, 5% SDS), and 4 times with 250 ml wash no. 2 (1 m*M* EDTA, 40 m*M* Na_2HPO_4 pH 7.2, 1% SDS). Wash solutions were brought to 68 °C then added to the filter and agitated at room temperature for 15 min. Filters were autoradiographed on Kodak XAR-5 X-ray film (Eastman Kodak Company, Rochester, NY) with Cronex hi-plus intensifying screens (E. I. DuPont De Nemours and Co., Inc., Wilmington, Del) at −70 °C for 1–5 days.

Results

The results of the phenotypic classification of F_3 lines are shown in Table 2. The classification of F_2 genotypes based on F_3 phenotypes corresponded exactly with F_2 phenotypic classifications (data not shown). For all five linkage relationships tested the χ^2 was significant with $P < 0.001$, indicating that the two loci were not segregating independently. Linkage was detected in all crosses studied. The *nar*2 locus was linked to the *nld* and *mt*2 loci with map distances of 8.38 ± 2.06 and 22.96 ± 4.61, respectively (Table 2). Map distances for the *lys*3/*nar*2a, *ddt*/*nar*2a and *lys*3/*ddt* were questionable due to a deficiency in *lys*3 *lys*3 genotypes among

Table 2. Observed frequency of F_2 genotypes from selected crosses as determined by F_3 phenotypes

Genotype	Cross				
	nld/ nar2a	*mt2/ nar2a*	*ddt/ nar2a*	*lys3/ nar2a*	*lys3/ ddt*
−−/++	15	17	1	6	2
−−/+−	5	9	6	0	3
−−/−−	0	1	0	0	3
+−/++	3	10	1	1	5
+−/+−	48	24	57	57	53
+−/−−	2	2	1	2	3
++/++	0	2	1	0	1
++/+−	6	4	3	2	3
++/−−	22	14	24	27	22
Total	101	83	94	95	95
D±SD	8.38 ±2.06	22.96 ±4.61	7.25 ±2.00	2.69 ±1.19	13.04 ±2.79

Table 3. Partitioning of χ^2 analysis of each cross

Cross	Partition	df	χ^2	*P*
nld×*nar2a*	Seg *Nld:nld*	2	3.57	0.25–0.10
	Seg *Nar2:nar2a*	2	1.51	0.50–0.25
	Linkage	4	114.90	<0.001
	(Total)	(8)	119.98	<0.001
mt2×*nar2a*	Seg *Mt2:mt2*	2	4.45	0.25–0.10
	Seg *Nar2:nar2a*	2	2.64	0.50–0.25
	Linkage	4	42.47	<0.001
	(Total)	(8)	49.56	<0.001
ddt×*nar2a*	Seg *DDT:ddt*	2	25.66	<0.001
	Seg *Nar2:nar2a*	2	15.51	<0.001
	Linkage	4	75.75	<0.001
	(Total)	(8)	116.92	<0.001
lys3×*nar2a*	Seg *Lys3:lys3*	2	17.72	<0.001
	Seg *Nar2:nar2a*	2	15.75	<0.001
	Linkage	4	156.63	<0.001
	(Total)	(8)	190.10	<0.001
lys3×*ddt*	Seg *Lys3:lys3*	2	10.95	0.005–0.001
	Seg *Ddt:ddt*	2	17.87	<0.001
	Linkage	4	59.67	<0.001
	(Total	(8)	88.49	<0.001

Risø 1508×*nar*2a progeny. Partitioning of the χ^2 for these linkage relationships indicated that the individual loci did not segregate in the expected 1 : 2 : 1 ratios (Table 3). While the Risø 1508×*nar*2a data were not used to map the *nar*2 locus, it is important to note that linkage was detected between *nar*2a, *ddt* and *lys*3.

The location of the *nar*2 locus with respect to markers on chromosome 7 is shown in Fig. 1. By using Jensen's (1981) chromosome 7 map and map distances we were able to locate the *nar*2 locus at 54.7±3.1 cm from *s* near the centromere of chromosome 7 between *nld* and *ddt*.

No linkage was detected between the *nar*1 locus and male sterile genes *msg* 1, 2, 5, 6, 10, 19, 24, which represent all 7 barley chromosome pairs. Linkage data with several translocations and *nar*1 were also inconclusive (A Kleinhofs, unpublished). Therefore, we determined the chromosomal location of the *nar*1 locus using wheat-barley addition lines.

DNA of the wheat and barley parents digested with Bam HI exhibited a noticeable restriction fragment polymorphism when probed with a *nar*1 specific cDNA clone (Fig. 2). The addition line, which contains barley chromosome 6, gave the restriction fragment pattern expected for a plant containing both wheat and barley *nar*1 genes. All other addition lines contained only the wheat *nar*1 restriction fragment pattern. No barley chromosome 5 addition line was tested since incompatibility genes on this chromosome have prevented their construction in a wheat background.

The chromosome 6 addition line was confirmed to contain barley chromosome 6 by probing Eco RI digested addition line DNAs with the Eco RI-Hind III α-amylase gene fragment of p141.17. This fragment contains a partial cDNA clone of the barley low pI type I α-amylase gene which has previously been shown to be located on chromosome 6 (Muthukrishnan et al. 1984).

Discussion

The location of *nar*2 on chromosome 7 was determined using standard genetic mapping techniques. The *nar*2 locus was located in the area of the centromere. The marker loci *nld* and *mt*2 were found to be 8.4±2.1 and 23.0±4.6 cm from *nar*2, respectively. It is not known, at present, whether this locates *nar*2 on the long or short arm of chromosome 7. Shahla and Tsuchiya (1984, 1985), using telotrisomic 7S, were able to show that *nld* is on the long arm of chromosome 7 while *ddt* is on the short arm. Tsuchiya (1985) indicated that the centromere is between *nld* and *ddt*. Pickering (1983) found *ddt* and *inc* to be closely linked ($r=0.11\pm0.03$); thus, *nar*2 and *inc* may also be linked.

Deficiencies in the homozygous *lys*3 genotype have been observed previously (Ullrich and Eslick 1977, 1978; Sozinov et al. 1979). Sozinov et al. (1979) found that *lys*3 *lys*3 types were not as viable and did not always germinate. They corrected the deficiency by multiplying the number of observed *lys*3 *lys*3 individuals in each genotypic class by an average coefficient Km. Km was the percentage reduction in viability as determined by germination studies. This type of correction of our data was not possible due to the extremely low number of observed individuals of the parental *lys*3 *lys*3 genotype. We therefore used only the

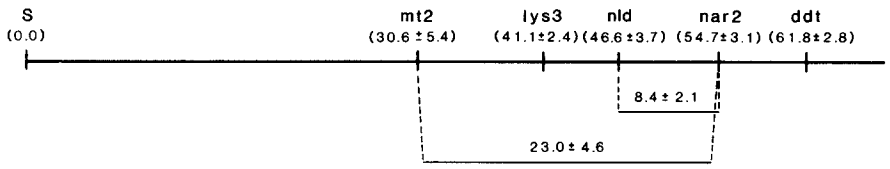

Fig. 1. Linear order of marker loci around centromere of chromosome 7. *Bold line* and *values* based on maximum likelihood estimated of linkage intensity and from Jensen (1981). *Thin lines* and *values* are observed map distances (cm) from our data

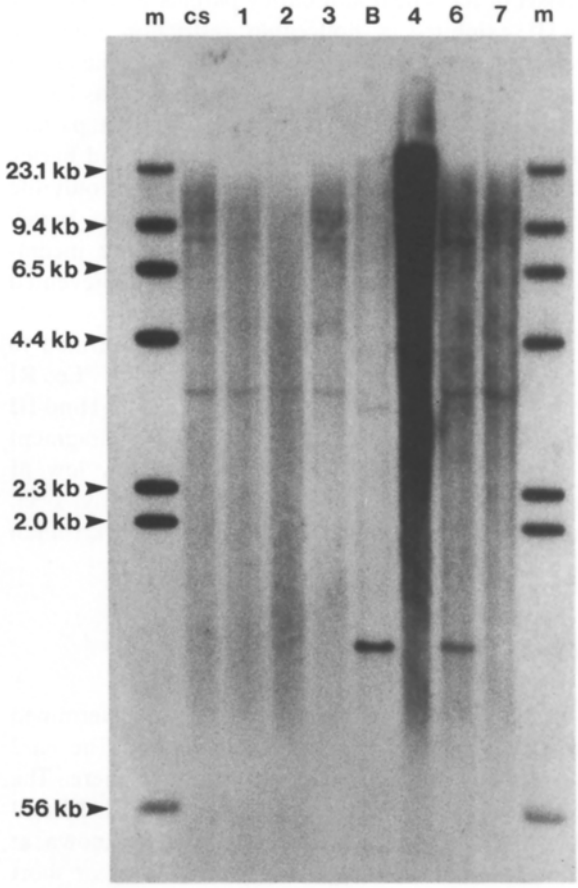

Fig. 2. Autoradiograph of gel of wheat-barley addition line DNAs probed with Bst I fragment of bNRp10. Twelve micrograms Bam HI digested DNA per lane, except marker lanes. *Lanes m* Hind III digested end-labelled lambda DNA; cs Chinese Spring wheat; *1* chromosome 1 addition line; *2* chromosome 2 addition line; *3* chromosome 3 addition line; *B* Betzes barley; *4* chromosome 4 addition line; *6* chromosome 6 addition line; *7* chromosome 7 addition line

nld/*nar*2a and *mt*2/*nar*2a linkage data to locate the *nar*2 locus on chromosome 7.

While we were unable to detect linkage of *nar*1a with marker genes and translocations representing each of the barley chromosomes, we were able to determine the location of the *nar*1 locus using wheat-barley addition lines. Wheat-barley DNA polymorphism for the *nar*1 gene allowed the mapping of *nar*1 to barley chromosome 6.

It is unlikely that more than one NADH : NR structural gene exists in the barley genome since 22 mutants of this type are all allelic to *nar*1a. The Pst I fragment of the NR cDNA clone bNRp10, used as probe in these experiments, codes for part of the NADH : NR structural gene, not the NAD(P)H : NR structural gene (Cheng et al. 1986). We have no evidence of hybridization of the Pst I fragment of bNRp10 with the NAD(P)H : NR gene. Thus we feel confident that the hybridization seen in these Southern blots represents the NADH : NR structural gene, *nar*1.

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