The EMBO Journal vol.7 no.11 pp.3309 - 3314, 1988

A new locus (*NIA1*) in *Arabidopsis thaliana* encoding nitrate reductase

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Communicated by M.van Montagu

We have isolated two nitrate reductase genes and their corresponding cDNAs from *Arabidopsis thaliana*. Sequences of the two cDNAs, when compared to a sequence of a barley cDNA clone, confirm their identity as nitrate reductase clones and show that they are closely related. The two genes have been mapped using restriction fragment length polymorphisms; gNR2 is close to the previously identified *chl-3* locus and is probably identical to it, while gNR1 maps to a new locus (*NIA1*) on chromosome 1, near *gl-2*.

Key words: nitrate reductase/cDNA cloning/genomic clones/RFLP mapping

Introduction

Nitrate is the major source of nitrogen taken up from the soil by higher plants; however, prior to its incorporation into amino acids it must be reduced to ammonia. The nitrate reduction process requires a large amount ($8e^-$) of energy and the end product, ammonia, is toxic when accumulated. It is therefore important to optimize the rate of reduction in response to the availability of substrate and other factors which affect the physiological state of the plants, such as light, CO_2, temperature and age. The first enzyme in the nitrate reducing pathway, nitrate reductase (NR), is thought to play a key role in the rate of the reducing process (for review see Guerrero *et al.*, 1981).

Regulation of NR activity by its own substrate, nitrate, has been intensively studied (for review see Guerrero *et al.*, 1981). Inducible NR activity has been demonstrated in essentially all the plant species examined since it first was described in rice seedlings (Tang and Wu, 1957). Regulation of NR has also been demonstrated using a NR cDNA from barley; the steady-state NR mRNA level showed a marked increase in response to nitrate induction (Cheng *et al.*, 1986). Similar studies in squash and tobacco have also been reported (Crawford *et al.*, 1986; Calza *et al.*, 1987).

The genetics of NR have been extensively investigated in many plant species. In tobacco, barley and *Nicotiana plumbaginifolia*, a single locus has been characterized as the structural gene for NR (Warner *et al.*, 1977; Muller and Grafe, 1978; Gabard *et al.*, 1987). However, in *Arabidopsis* two loci, *chl-2* and *chl-3*, have been proposed as genes encoding NR (Braaksma and Feenstra, 1982). Here we report the molecular cloning of two *Arabidopsis* NR cDNAs and their corresponding genes, and the mapping of the genes using restriction fragment length polymorphisms (RFLP). One gene maps in the vicinity of *chl-3*, which is consistent with the genetic data. The other gene does not map to *chl-2*, but to a new locus near *gl-2*. This result not only indicates that a new locus encoding NR has been identified, but also raises interesting questions about the identity of *chl-2*.

Results

Isolation of initial cDNA clones

Arabidopsis poly(A)$^+$ RNA isolated from nitrate-induced shoots was size fractionated on a native sucrose gradient and the fractions containing the NR mRNA were identified by *in vitro* translation followed by immunoprecipitation using polyclonal antiserum raised against barley NR that cross-reacts with *Arabidopsis* NR on a Western blot (Kleinhofs *et al.*, 1985) and with native NR protein as shown in Figure 1a. A single gradient fraction enriched for NR mRNA was used for the construction of a cDNA library in λgt11. Approximately 10^5 recombinant plaques were screened with the NR antiserum and 12 independent clones identified. Clone λAtcNR/1 (cNR1) contains an insert of ~2.4 kb which hybridizes to a ~3.2-kb nitrate-inducible mRNA on

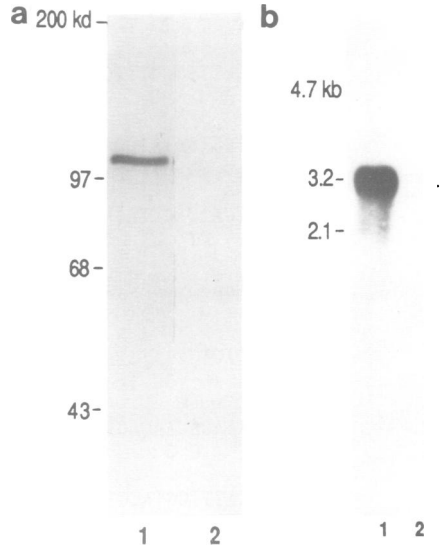

Fig. 1. Nitrate induction of NR mRNA and its translation product. (**a**) Immunoprecipitation of *Arabidopsis* NR by barley NR antiserum. poly(A)$^+$ RNA (200 ng) isolated from plants treated with media plus nitrate (**lane 1**) and minus nitrate (**lane 2**) was translated *in vitro* and the products immunoprecipitated with barley NR antiserum. Electrophoresis was in a 7.5% SDS–polyacrylamide gel. The positions of mol. wt standards (kd) are shown on the left from top to bottom: myosin (H-chain), 200 kd; phosphorylase B, 97 kd; bovine serum albumin, 68 kd; and ovalbumin, 43 kd. (**b**) Northern analysis of NR mRNA. Each lane contains 5 μg of total RNA from plants treated with media plus nitrate (**lane 1**) and minus nitrate (**lane 2**). RNA was denatured with formaldehyde and formamide, electrophoresed in a 1% agarose gel (Maniatis *et al.*, 1982), and transferred to a nylon filter. The filter was hybridized to the ^{32}P-labelled cDNA insert from cNR1.

a

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NR1 YRIGELITTGY...DSSPNVSVHGASNFGPLLAPIKELTPQKNIALVNPREKIPVRLIEKTSISHDVRKFRFALP
NR2           SSDS    N    S AVFS     G A  VR L      A V  Q V
BARLEY          . TGYN D SVHG S LSHLAPIREATKVAGAP   SS    V C  VD KEL     L

SEDQQLGLPVGKHVFVCANINDKLCLRAYTPTSAIDAVGHIDLVVKVYFKDVHPRFPNGGLMSQHLDSLPIG.SM
V  MV        I L  T           S TV V  YFE    I  GG            Y         T
 S  V        I    T DG   M       MV EI QFEL      R E  K       T Y E  QV S Y

IDIKGPLGHIEYKGKGNFLVSGKPKFAKKLAMLAGGTGITPIYQIIQSILSD.PEDETEMYVVYANRTEDDILVR
LE       V  L   S T H      D            V     A  K          I      E   L
  V      V  T R   VIN  QRR RR   IC  S    M  V  AV R Q       HL     S     L

EELEGWASKHKERLKIWYV...VEIAKEGWSYSTGFITEAVLREHIPEGLEGESLALACGPPPMIQFALQPNLEK
   D   EQYPD   V        S     A      S  IM     D  D SA  M           V
D  DR  AEYPD   V   IDQ KRPED  KF V  V  DI  A V   .GDDT           K  IS

MGYNVKEDLLIF
 Q  I   F
 K DMANSFIS
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b

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NR1  TCCTCAGAATCCAACAATACCTTGAAGAAATCTGTCTCATCTCCTTTCATGAACACTGCCTCGAAGATGTATTCA
NR2    GG T  CG GCCTCC  GTC A     G        GA G  G  T        AA TG          C  G

ATCTCTGAAGTTAGAAAACACAACACTGCTGACTCTGCATGGATCATCGTCCACGGTCACATCTATGACTGTACA
  G  C  G  C AG  G  T  TT G         TGC        T     T  A  T        T

CGTTTCTTGAAAGATCATCCAGGAGGCACAGATTCTATCCTGATAAACGCAGGTACAGATTGCACCGAAGAGTTT
  A   C T TG     C  G  T  TT G     A  T    C  T  T     G     T  G  G

GAAGCCATTCACTCTGACAAAGCCAAGAAGCTTTTGGAAGATTACCGTATCGGTGAACTCATCACCACTGG....
              G  T            A GC T  G                  G              TTAT

..CTACGACTCTTCC...CCTAACGTCTCAGTTCATGGTGCCTCAAACTTTGGTCCTTTGTTAGCTCCAATCAAA
TC  CT          TCG       AA   G     C   T A  CGC G GTTCT GC    G     C  TGG

GAGCTAACTCCTCAAAAGAACATTGCTTTGGTCAACCCACGTGAGAAAATCCCGGTTAGGCTCATTGAGAAGACT
   GCG     GGTT G    C C        T  T  C  G CT   G        CAA   G C  A

TCGATCTCGCACGACGTTCGTAAGTTCCGATTCGCATTACCATCAGAAGATCAACAGCTTGGTTTACCGGTGGGG
  C  T  T  T  T        A     G  T  T     GGTT  G   ATGGTT  A  C        T  T

AAGCACGTTTTCGTTTGCGCCAACATAAACGACAAACTCTGTCTCAGAGCTTATACTCCCACCAGCGCCATCGAC
      A     C          C   C  T    G     C           C  A  A  G    A  G  T  T

GCGGTTGGTCATATCGACCTCGTCGTCAAAGTTTACTTCAAAGACGTTCATCCAAGGTTTCCCAATGGTGGACTC
 T      CT CT    G     G     GA       TGGC GT  C  C     A  C  T  C  C  G

ATGTCTCAACACTTAGACTCGTTACCAATCGGGTCAATGATAGATATCAAAGGTCCACTAGGGCACATCGAGTAC
         GT  C       T  G  T  A       CTT G  G  T     A   T  G  T   G  T     T

AAAGGCAAAGGCAACTTCCTGGTCAGCGGCAAACCTAAGTTTGCCAAGAAACTAGCAATGCTCGCCGGAGGAACA
CTC     G  T GT   AC   TCA   T     A         TG T   T G       T G  A  T     C

GGCATCACTCCTATCTACCAGATCATTCAATCTATATTGAGTGATCCAGAGGACGAAACCGAGATGTATGTGGTT
  A  A     GG T     A  T  C   G  C  TC C AG           T  G  T  A     C  CA

TACGCAAACAGAACCGAGGATGACATTCTTGTGAGGGAAGAGCTAGAAGGATGGGCTAGTAAGCATAAGGAGAGG
  T  T   C G         A  T     CC A     G  A  G  T  T     AGAGC AT CCC   CC

CTAAAGATTTGGTACGTCGTTGAAATCGCAAAGGAAGGTTGGAGTTACAGTACCGGGTTTATAACTGAAGCTGTG
T     G          A  G   TCA  T            GCA                 TT C  G  GA T

CTTAGGGAACATATCCCTGAAGGTTTGGAAGGCGAATCGCTAGCACTCGCATGCGGACCACCGCCTATGATTCAG
A GC A              T  A  A  T   TC G C  T  CA G  T           A  G

TTTGCGTTGCAGCCAAATCTAGAGAAGATGGGTTACAACGTGAAGGAGGATCTCTTAATCTTC
      G T     G   T G         CAA  T   A C         T    G  A
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Fig. 2. Sequence comparison. (**a**) Comparison of the partial amino acid sequences of cNR1, cNR2 and barley cDNA bNRp10 (Cheng *et al.*, 1986) deduced from their respective cDNA sequences. The last amino acid indicated is C-terminal. The cNR2 and barley sequences are shown only where they differ from cNR1. Appropriate gaps (indicated by dots) have been added to maximize the similarities between the sequences. (**b**) Comparison of the partial nucleotide sequences of cNR1 and cNR2. The last nucleotide shown corresponds to the end of the coding region. The cNR2 sequence is shown only where it differs from cNR1. Appropriate gaps (indicated by dots) have been added to maximize the similarities between the sequences.

Northern blot (Figure 1b). A comparison of the deduced amino acid sequences of cNR1 and a barley NR (Cheng *et al.*, 1986) cDNA clone indicate that they show 60% similarity (Figure 2a), confirming that cNR1 encodes a NR.

Isolation of genomic clones

The DNA insert of clone cNR1 was used as a probe to screen an *Arabidopsis* genomic cosmid library constructed in pOCA18 (N.Olszewski *et al.*, personal communication). Two overlapping clones were identified that hybridize to the cDNA probe. One of these clones, pAtgNR/2 (gNR2), was further characterized and shown to contain a ~4-kb *Hin*dIII insert which hybridized to the cNR1 probe (data not shown). However, the restriction map of the cDNA (cNR1) differs from that of the genomic clone (gNR2) (Figure 3a), suggesting that the genomic clone does not encode cNR1. In an effort to identify the corresponding genomic clone, cNR1 was used to screen a second genomic library (a *Mbo*I partial digest of *Arabidopsis* DNA cloned in the λ vector EMBL3). Two overlapping clones distinct from gNR2 were identified. Clone λAtgNR/1 (gNR1) was further characterized by restriction mapping and Southern blot anlaysis. cNR1 hybridizes to a single *Eco*RI fragment of ~3.5 kb (data not shown) and to two *Hin*dIII fragments in gNR1: a ~5-kb internal fragment (site H to H^2, Figure 3a) and a ~15-kb fragment which also contains vector DNA (Figure 3a). This is consistent with the hybridization pattern observed on a genomic Southern blot (Figure 3c). Interestingly, when the restriction map of cNR1 was compared with the map of genomic clone gNR1, a *Hin*dIII site (H^1) was found to be missing in the genomic clone (Figure 3a). Both fragments a (5′ end/H^1) and b (H^1/H^2) of the cDNA, cNR1, hybridized to fragment B (R^1/H^2) of the genomic clone, gNR1. On the other hand, fragment c (H^2/3′ end) of cNR1 hybridized only to fragment C (H^2/R^2) of gNR1 (see Figure 3a; data not shown). These results are consistent with the possibility that the additional *Hin*dIII site (H^1) present in the cDNA is formed by splicing, a conclusion confirmed by sequence data (Figure 3b).

Isolation of cDNA encoded by gNR2

To isolate the cDNA corresponding to gNR2, a *Bam*HI/*Hin*dIII fragment (fragment D, Figure 3a) from the genomic clone, gNR2, was used as a probe to screen a cDNA library made from total poly(A)$^+$ RNA from *Arabidopsis* roots. Two classes of clones were identified: one class gave strong hybridization signals and the other gave weak signals. The class which hybridizes weakly has the same restriction pattern as cNR1. Among the strongly hybridizing clones, λAtcNR/2 (cNR2) contained a ~2.9-kb insert and was further characterized. As expected, cNR2 hybridizes to a single ~4-kb *Hin*dIII fragment from gNR2 (Figure 3a) as well as *Arabidopsis* genomic DNA (Figure 3c).

The nucleotide sequences of the 3′ ends (~1 kb) of the

Fig. 3. Restriction maps and Southern analysis. (**a**) Restriction maps of the cDNAs λAtcNR/1 (cNR1) and λAtcNR/2 (cNR2) and their corresponding genes λAtgNR/1 (gNR1) and pAtgNR/2 (gNR2) respectively are illustrated. The sites are designated as follows: H, *Hin*dIII; R, *Eco*RI; and B, *Bam*HI. Sites and fragments discussed in the text are indicated by numbers and letters respectively. (**b**) *Hin*dIII (H^1) site formed by splicing. Sequence shown in upper-case is the coding region and the intron is shown in lower-case. Arrows indicate the presumed splice sites. The *Hin*dIII site (AAG CTT) formed after intron excision is underlined. (**c**) Genomic Southern analysis using cNR1 and cNR2 as probes. *Arabidopsis* DNA (1 μg) was digested with *Hin*dIII, subjected to gel electrophoresis and then hybridized to ^{32}P-labelled cDNAs by Southern blot analysis. **Lane 1** was hybridized to cNR1 and **lane 2** to cNR2. The positions in the gel of a λ *Hin*dIII marker digest are shown on the left.

coding regions of the two cDNAs (cNR1 and cNR2) were compared (Figure 2b). Although they are clearly different, the levels of similarity (72% nucleotide and 78% amino acid) between the two show that they are closely related (Figure 2a,b). Sequence data also confirm that gNR1 encodes cNR1 and gNR2 encodes cNR2 (data not shown).

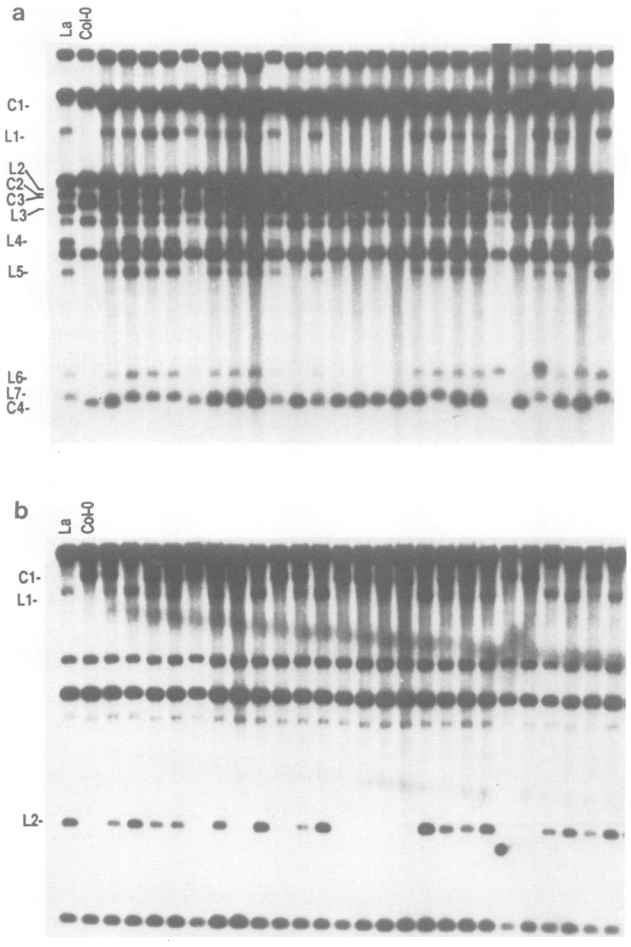

Fig. 4. Southern blots of the RFLP analysis. (**a**) *Arabidopsis* genomic DNA (1 μg) was digested wtih *Hin*dIII, subjected to gel electrophoresis and probed with genomic clone gNR2 by Southern blot analysis. Col-0 (Columbia) and the *erecta* mutant of Landsberg (La) were the two parental lines. The remaining lanes contain DNA made from pooled F_3 plants which in turn were derived from single F_2 plants. The polymorphic bands are indicated. (**b**) The same DNA preparations as in (a) were used except digestion was with *Bcl*I. The blot was probed with genomic clone gNR1. Only DNAs from the progeny of crosses between the male parent Col-0 and the multi-marker strain of La carrying the visible markers *ch-1*, *ap-1* and *gl-2* (all on chromosome 1) are shown.

RFLP mapping

Chlorate-resistant mutants deficient in NR activity have been isolated in *Arabidopsis* (Oostindier-Braaksma and Feenstra, 1973; Braaksma and Feenstra, 1982) and two mapped loci, *chl-2* on chromosome 2 and *chl-3* on chromosome 1, have been suggested to encode NR structural genes. To test whether gNR1 and gNR2 correspond to *chl-2* and *chl-3*, the chromosomal locations of the two genomic clones were determined by RFLP analysis.

Genomic DNA from two *Arabidopsis* lines, Columbia (Col-0) and Landsberg *erecta* (La) was digested with *Hin*dIII and *Bcl*I. *Hin*dIII-digested DNA showed 11 polymorphic bands between the two *Arabidopsis* lines when gNR2 was used as a probe for Southern blot analysis (Figure 4a, lanes marked La and Col-0); *Bcl*I-digested DNA showed three polymorphic bands when gNR1 was used as a probe (Figure 4b, lanes marked La and Col-0). The remaining lanes in Figure 4a and b represent the RFLP patterns of 25 F2 plants (DNA is from pooled F3 plants; see Materials and methods for details) obtained from the progeny of a cross between the male parent Col-0 and the multi-marker strain of La carrying the visible markers *ch-1*, *ap-1* and *gl-2* (all on chromosome 1). RFLPs of both genes segregated in a Mendelian fashion and are therefore monogenic alleles (Figure 4a and b; Table I).

Fourteen visible markers, distributed on the five *Arabidopsis* chromosomes, were used to map gNR1 and gNR2. Two of the markers, *cp-2* and *ch-1*, are closely linked to *chl-2* and *chl-3* respectively (Koornneef *et al.*, 1983; Koornneef, 1987). Linkage analysis of the RFLPs relative to the visible markers (Table II) was used to ascertain the approximate map positions of the two genes. NR clone gNR2 maps close to *ch-1* (2 ± 2% recombination frequency) at a map position on chromosome 1 (*NIA2*) that suggests that gNR2 is the *chl-3* gene. gNR1 maps to chromosome 1 in the vicinity of *gl-2* (2 ± 2% recombination frequency) and shows no linkage to either *chl-2* or *chl-3*. We have designated this locus as *NIA1*.

Discussion

The following lines of evidence support the conclusion that both cDNAs encode NR. First, the cDNA clone cNR1 was initially identified by immunoscreening with barley NR-

Table I. Mendelian segregation of RFLPs in the NR genes

Gene	RFLP bands	Segregation ratio		χ^2	Probability
		Observed	(Theory)		
NR1	C1	84:33	(3:1 = 87.75:29.25)	0.64	0.42
	L2	84:33	(3:1 = 87.75:29.25)	0.64	0.42
	C1 + L2	33:51:33	(1:2:1 = 29.25:58.5:29.25)	1.92	0.38
NR2	C4	87:30	(3:1 = 87.75:29.25)	0.03	0.87
	L5	84:29	(3:1 = 84.75:28.25)	0.03	0.87
	C4 + L5	29:53:30	(1:2:1 = 28.0:56.0:28.0)	0.34	0.84

Number of progeny in each case is the number of plants scored for the band(s) in question. Single bands behave as dominant markers: the expected ratio is 3:1 (band present:band absent). Columbia bands (C) and Landsberg bands (L) scored together behave as co-dominant markers: the expected ratio is 1:2:1 (C:C+L:L). Probability values >0.05 indicate that the observed ratios are consistent with the expected ratios.

Table II. Linkage analysis between NR genes and visible markers

Gene	Visible markers	Recombination frequency (%)	χ^2 associated	Probability
NR1	ch-1	27.6 ± 7.7	16.3	<0.05
	ap-1	10.3 ± 4.6	41.4	<0.05
	gl-2	2.0 ± 2.0	62.2	<0.05
	cp-2	unlinked	1.4	0.84
NR2	ch-1	2.0 ± 2.0	48.3	<0.05
	ap-1	19.3 ± 6.3	19.0	<0.05
	gl-2	28.1 ± 7.8	12.3	<0.05
	cp-2	unlinked	1.5	0.82

χ^2 associated is the total χ^2 adjusted for deviations of each individual marker from Mendelian segregation. Probability values <0.05 indicate deviation from non-linkage, i.e. linkage. Pooled F_3 plants from 25 individual F_2 plants were used to calculate the recombination frequency for each linkage group.

specific antiserum. The antiserum reacted with a single band of ~110 kd on a Western blot with total *Arabidopsis* protein (Kleinhofs *et al.*, 1985) and with a similar size product of *in vitro* translation (Figure 1a). Second, cDNA cNR1 hybridized to a single band of ~3.2 kb which is nitrate inducible on a Northern blot (Figure 1b). This is the approximate size of NR mRNA in barley, squash and tobacco (Cheng *et al.*, 1986; Crawford *et al.*, 1986; Calza *et al.*, 1987). Third, the sequence comparison (Figure 2a) between the two *Arabidopsis* cDNAs and the barley cDNA clearly shows that all three are closely related (60% amino acid similarity). Additional circumstantial evidence comes from the results of the RFLP mapping. One of the genomic clones, gNR2, maps in the region where the *chl-3* mutant (designated as one of the structural genes of NR) has been previously located. This supports the conclusion that the genomic clone gNR2 is a gene coding for NR.

In a number of plant species the enzyme EC 1.6.6.1 is nitrate inducible, uses NADH as electron donor and appears to be a homodimer composed of 100- to 115-kd subunits (Solomonson, 1975; Campbell and Wray, 1983; Sommers *et al.*, 1983; Nakagawa *et al.*, 1985; Streit *et al.*, 1987). However, there is no published detailed study of *Arabidopsis* NR enzymes. It is therefore appropriate to ask whether the two *Arabidopsis* genes encode two different subunits of the same enzyme or two separate enzymes. Another NR (EC 1.6.6.2), which utilizes both NADPH and NADH as electron donors but prefers NADPH, has been purified from soybean (Streit *et al.*, 1987). In other plant species such as corn, rice and barley, NADPH−NR activities have also been found (Shen *et al.*, 1976; Campbell, 1978; Dailey *et al.*, 1982). This enzyme (EC 1.6.6.2) is constitutively expressed in soybean and is the major form (Jolly *et al.*, 1976). In the other plant species examined, the inducible NR (EC 1.6.6.1) is the major form (Streit *et al.*, 1985). Additionally, in soybean there is a third NR which is constitutively expressed and uses NADH as electron donor. All three soybean enzymes have similar subunit sizes (107−109 kd) and each NR is a homodimer (Streit *et al.*, 1987). If the situation is similar in *Arabidopsis*, the two *Arabidopsis* genes probably represent two different NR proteins rather than two different subunits of the same enzyme.

NR mutants in *Arabidopsis* have been isolated by selection for a chlorate-resistant phenotype. Both *chl-2* and *chl-3* have been postulated to encode NR structural genes since neither is a nitrate uptake mutant nor defective in the molybdenum cofactor and both have reduced levels of NR activity as compared to wild-type. For example, *chl-3* mutants have low but substantial amounts of NR activity (20% of wild-type) and are able to use nitrate as the only nitrogen source (Braaksma and Feenstra, 1982). Our RFLP mapping indicates that one of the genomic clones (gNR2;*NIA2*) maps close to, or is identical with *chl-3*. However, the other genomic clone (gNR1) does not map near the *chl-2* locus on chromosome 2, but rather at a new position on chromosome 1 (*NIA1*). It is not clear why no NR mutations have been mapped to this locus. One can speculate that besides reducing nitrate the gene product of *NIA1* plays another essential role in the survival of plants, and mutations of this locus would therefore be lethal. It could also be that the gene is expressed tissue specifically such that the chlorate tretment is not detrimental in the presence of *NIA1* protein.

Since both *chl-3* and *NIA1* encode NR apoproteins, the *chl-2* mutants require explanation. It is possible that there is a third NR gene in *Arabidopsis* which has not yet been isolated. By Southern blot analysis cNR1 and cNR2 hybridize only to bands in *Arabidopsis* DNA that are also present in gNR1 and/or gNR2 even under low-stringency conditions (data not shown). Therefore, if there were a third gene, it would have to be quite divergent in sequence from either gNR1 or gNR2. A more interesting interpretation would be that the *chl-2* locus encodes a gene which regulates one or more of the NR structural genes and is not a NR structural gene itself, a possibility currently being tested by analyzing the expression of the two genes in the mutant plants.

Materials and methods

Nucleic acid extractions

Arabidopsis DNA was extracted as described (Watson and Thompson, 1986). RNA was extracted by the guanidinium/phenol method (Feramisco *et al.*, 1982) except that the extraction procedure was carried out at 4°C. Poly(A)$^+$ RNA was selected by oligo(dT)−cellulose chromatography (Aviv and Leder, 1972).

Two genomic libraries were used: a *Mbo*I partial digest of *Arabidopsis* DNA cloned in the λ vector EMBL3 (a gift from Dr B.Hauge) and a *Taq*I partial digest of *Arabidopsis* DNA cloned in the cosmid vector pOCA18 (a gift from Dr N.Olszewski).

RNA size fractionation and construction of cDNA libraries

RNA size fractionation, construction of a cDNA library and the immunoprecipitation conditions were essentially the same as described in Cheng *et al.* (1986). Total poly(A)$^+$ RNA was fractionated on a native sucrose gradient (Cashmore, 1979). *In vitro* translation conditions were as described by the suppliers (New England Nuclear or Bethesda Research Laboratories). cDNAs were synthesized according to Gubler and Hoffman (1983) and, after *Eco*RI linker addition, cloned into bacteriophage λgt11 (Huynh *et al.*, 1985). Antiserum was diluted 1:1000 to screen for the recombinant bacteriophages expressing nitrate reductase protein. The unfractionated cDNA library made from total poly(A)$^+$ RNA of *Arabidopsis* roots was a gift of Dr Kaye Peterman.

DNA probes

The hybridization probes were made with gel-purified DNA inserts and labelled with ^{32}P by the random priming method (Feinberg and Vogelstein, 1983) except for the genomic probes (gNR1 and gNR2) used for RFLP analysis which were labelled using the entire clone after first digesting the λ DNA (λAtgNR/1) and the cosmid DNA (pAtgNR/2) with *Hin*dIII prior to labelling. The specific activities of the labelled probes were usually ~5 × 10^8 c.p.m./μg DNA.

Hybridization conditions

Nylon filters ('Biotrans' from ICN Biomedicals, Inc.) with bacterial colonies or phage plaques were heat treated to denature and immobilize the DNA (Maas, 1983). Southern blot and Northern blot analysis was performed using the UV cross-linking procedure described by Church and Gilbert (1984). Hybridization was done at 65°C in 0.5 M Na_2HPO_4, pH 7.2, 1 mM EDTA, 7% SDS and 1% BSA for 16–24 h. The filters were washed twice at room temperature for 15 min and twice for 15 min each at 57°C for Southern blots and 70°C for Northern blots in 40 mM Na_2HPO_4, 1 mM EDTA and 1% SDS.

Nucleotide sequence analysis

cDNAs were subcloned in the *Eco*RI site of the 'Bluescript' plasmid (Stratagene). Unidirectional deletion clones were generated by *Exo*III digestion. Plasmid DNAs were prepared by the alkaline method (Birnboim and Doly, 1979) and were sequenced using the dideoxynucleotide chain termination method (Sanger *et al.*, 1977).

Growth of plants

Plants used in RNA analysis were *A.thaliana* (Columbia line; Col-0) grown in vermiculite/perlite mixture under continuous illumination. Induced plants were irrigated with 1/4 Hoagland's solution and uninduced plants with 1/4 Hoagland's solution in which $(NH_4)_2SO_4$ replaced the KNO_3.

Plant crosses

The Columbia line (Col-0) was used as the male parent and five different marker strains of the Landsberg *erecta* line (La) were used as the female parents. Each of the five marker strains contained multiple phenotypic markers on each chromosome (e.g. chromosome 1: *ch-1*, *ap-1* and *gl-2* plus the *er* mutation on chromosome 2; chromosome 2: *cp-2*, *er*, *as* and *cer-8*; etc). F_1 plants were allowed to self-fertilize to give rise to F_2 populations. F_3 plants were grown from seeds of individual F_2 plants to reveal the genotypes of the F_2 plants. Genomic DNA was prepared from pools of F_3 plants obtained from individual F_2 plants and used in the RFLP segregation analysis.

Computer analysis

DNA sequence data were assembled and analyzed with the programs of Staden and the University of Wisconsin Genetics Computer Group. χ^2 values for segregation and independent assortment of RFLP alleles were calculated on a graphic spread-sheet, Graphic Outlook©. Recombination values were calculated by the maximum likelihood method using a computer program developed by P.Stam (Koornneef *et al.*, 1983).

Acknowledgements

We wish to thank Fred Ausubel and David Moore for critical reading of the manuscript. We also thank Martin Koornneef for the mutant seeds; Andy Kleinhofs for antiserum; Brian Hauge, Neil Olszewski and Kaye Peterman for the libraries; Bill Loos for computer analysis. This work was supported by a grant from Hoechst AG.

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Received on June 28, 1988; revised on August 16, 1988