

Cloning of DNA fragments complementary to tobacco nitrate reductase mRNA and encoding epitopes common to the nitrate reductases from higher plants

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Summary. Messenger RNAs encoding the nitrate reductase apoenzyme from tobacco can be translated in a cell-free system. Poly(A)⁺ mRNA fractions from the 23–32 S area of a sucrose gradient were used to build a cDNA library in the expression vector λ gt11 with an efficiency of cloning of approximately 10⁴ recombinants/ng mRNA. Recombinant clones were screened with a rabbit polyclonal antibody directed against the corn nitrate reductase, which cross reacts specifically with the nitrate reductases from dicotyledons. Among 240000 recombinant plaques, eight clones were isolated containing inserts of sizes ranging from 1.6 kb to 2.1 kb and sharing sequence homologies. Seven of these clones contained a common internal 1.6 kb *Eco*RI fragment. The identity of these clones was confirmed as follows. A fusion protein of 170 kDa inducible by IPTG and recognized by the rabbit nitrate reductase antibody was expressed by a lysogen derived from one of the recombinants. The antibodies binding the fused protein were eluted and shown to be inhibitory to the catalytic activity of tobacco nitrate reductase. Two monoclonal antibodies directed against nitrate reductase were also able to bind the hybrid protein. The 1.6 kb *Eco*RI fragment was sequenced by the method of Sanger. The open reading frame corresponding to a translational fusion with the β -galactosidase coding sequence of the vector shared strong homology at the amino acid level with the heme-binding domain of proteins of the cytochrome *b5* superfamily and with human erythrocyte cytochrome *b5* reductase. When the 1.6 kb *Eco*RI fragment was used as a probe for Northern blot experiments a signal corresponding to a 3.5 kb RNA was detected in tobacco and in *Nicotiana plumbaginifolia* mRNA preparations but no cross-hybridization with corn mRNAs was detected. The probe hybridized with low copy number sequences in genomic blots of tobacco DNA.

Key words: Nitrate reductase – cDNA expression cloning – Tobacco – Sequence – Cytochrome *b5*

Abbreviations: DMSO, dimethylsulfoxide; ELISA, enzyme-linked immunosorbent assay; IPTG, isopropyl β -D-thiogalactopyranoside; PMSF, phenylmethylsulfonyl fluoride; XGal, 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside

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Introduction

Nitrate reductase (NR) is a key enzyme involved in the first step of nitrate assimilation in plants (Kleinhofs et al. 1985), and is also found in bacteria and fungi. Although in these different organisms it catalyzes a similar reduction of nitrate involving NADH or NADPH as electron donor, the enzyme displays a variety of structures and dissimilarities. In *Escherichia coli* the enzyme is a heterotrimer bound to the bacterial membrane (Rasul Chaudhry and MacGregor 1953) and in *Chlorella* NR is an homotetramer (Howard and Solomonson 1982) found in the pyrenoid. In plants the enzyme is an homodimer possibly interacting with the chloroplast outer membrane. Apart from NADH, the three other cofactors involved in the reduction of nitrate by NR are FAD, cytochrome *b557* and the molybdenum cofactor. The NR from several plant species have been purified to homogeneity (Redinbaugh and Campbell 1985; Campbell and Wray 1983); and shown to catalyze, apart from nitrate reduction, other reactions, such as the reduction of ferric ion (Campbell and Redinbaugh 1984), which may be of physiological significance. The subunits of these plant NR have a molecular weight close to 110 kDa.

The regulation of NR activity in plants appears to be rather complex and many studies have been devoted to the description of this regulation (Hewitt 1975). For instance, the catalytic process of nitrate reduction takes place in the leaves of numerous plant species, but it can also occur exclusively in the roots of other species such as white lupin. Molecular tools have been lacking to study more deeply the features of these regulations and, for instance, NR monoclonal antibodies have been available only recently. Nucleic acid probes would be useful for studying the transcription of the NR structural gene under various physiological conditions. The isolation and characterization of a cDNA encoding more than 50% of the tobacco NR mRNA is presented here.

Materials and methods

Extraction and fractionation of poly(A)⁺ mRNA. Total RNA was extracted from leaf tissues according to a procedure derived from Chirgwin et al. (1979). Tobacco leaves were frozen in liquid N₂ and ground in a mortar. The powder was rapidly mixed with 100 ml of 50 mM Tris-HCl

buffer, pH 8.0, containing 10 mM EDTA, 5 M guanidinium thiocyanate, 2% sodium-N-lauroylsarcosine and 5% (v/v) 2-mercaptoethanol. After incubation at 65° C for 20 min and centrifugation at 20000 × *g* for 30 min, the supernatants were adjusted to 0.1 g/ml CsCl and layered on 12 ml cushions of 5.7 M CsCl, 50 mM EDTA, pH 8.0, in 30 ml polyallomer tubes. The tubes were centrifuged for 20 h at 25000 rpm in a SW27 rotor at 15° C. The RNA pellets were dissolved in 10 mM Tris, pH 8.0, 1 mM EDTA, 0.05% SDS and incubated at 55° C for 30 min in the presence of 200 µg/ml proteinase K. After PMSF treatment to inactivate proteinase K, samples were extracted by phenol, phenol/chloroform and chloroform, and ethanol precipitated. The yield was generally 200–300 µg total RNA per gram fresh tissue. Poly(A)⁺ mRNAs were purified by two cycles of selection on an oligo(dT) cellulose column (PL Biochemicals) according to the procedure of Bantle et al. (1978), including a step of denaturation of the RNA in 80% DMSO at 55° C for the second cycle of selection.

Poly(A)⁺ mRNAs represented generally 1.0%–1.2% of the total leaf RNA and contained less than 10% ribosomal RNA. Poly(A)⁺ RNAs (100 µg/tube) were fractionated on 5%–20% linear 12 ml sucrose gradients made in TE buffer (Tris-HCl 10 mM pH 8.0, EDTA 1 mM), by centrifugation for 16 h at 100000 × *g* in a SW40 rotor as previously described (Commère et al. 1986).

Cell-free translation of mRNAs and immunoselection of translation products. Total or fractionated poly(A)⁺ mRNAs were translated in a rabbit reticulocyte cell-free translation system (Amersham) using ³⁵S-labelled methionine (900 Ci/mmol, 1 mCi/ml, Amersham). Approximately 0.05–0.1 µg mRNA and 1.2 µCi ³⁵S-methionine were added per microlitre of lysate. After incubation for 1 h at 30° C, aliquots of the translation mixture were analysed by SDS-PAGE. Translation products corresponding to 50 µl of lysate were immunoselected for nitrate reductase polypeptide fragments by passing through a protein A-Sepharose 4B column on which 20 µl of NR-specific antiserum had been bound in TNE buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5% (w/v) Tween 20, 150 mM NaCl, 0.2% NaN₃). After adsorption of the lysate diluted in TNE buffer, the column was washed with TNE buffer containing 1 mM cold methionine and 1 M NaCl and then with 50 mM Tris-HCl buffer (pH 7.5). The protein A-bound material was then eluted and denatured in Laemmli buffer for SDS polyacrylamide gel electrophoresis (PAGE).

Construction of cDNA libraries in λGT11. cDNAs were synthesized according to Gubler and Hoffman (1983) with the following modifications. Two hundred nanograms of total poly(A)⁺ mRNAs were heat-denatured at 70° C for 2 min in water, and reverse transcribed at pH 8.3 using 14 U of AMV reverse transcriptase (Genofit) in a final volume of 40 µl in the presence of 0.1 µg oligo(dT) (15 mers, Pharmacia) at 42° C for 40 min. The efficiency of synthesis of the first strand varied from 8%–25%. The second strand was synthesized from the first strand in the presence of 0.8 U of RNase H (BRL), and 4 U of *E. Coli* DNA ligase (Bio-labs) using 40 U of DNA Pol I holoenzyme (Amersham) in a 90 µl final volume. The incubation was performed at 12° C for 1 h and then at 16° C for an additional hour. The double stranded DNA was healed by incubation with 1 U of T4 DNA polymerase at 37° C for 15 min. The effi-

ciency of synthesis of the second strand was close to 100%. The cDNA obtained (approximately 100 ng) was subsequently methylated with 20 U of *Eco*RI methylase. To improve the efficiency of cloning (tenfold) a step of S1 nuclease treatment followed by a second healing step was included in the procedure of cDNA synthesis between the healing and methylation steps. cDNAs were treated with 2 U of S1 nuclease (Boehringer) in a volume of 100 µl for 15 min at 30° C in order to eliminate abnormal ends, as suggested by Lapeyre (1985). This resulted in a 5% decrease in the amount of high molecular weight material in the cDNA preparation. After addition of *Eco*RI linkers to the cDNA (phosphorylated 12 mers, PL Biochemicals) and two successive digestions with concentrated *Eco*RI, cDNAs were fractionated on a Sephacryl S300 column and the material corresponding to cDNAs larger than 0.5 kb was pooled. The cDNA was ligated with dephosphorylated λgt11 vector cut with *Eco*RI under conditions specified by Young and Davis (1983). The ligation mixture was packaged using commercially available packaging lysates (Gigapack, Clontech labs, CA). Libraries were checked for the proportion of recombinants on strain RY1088 in the presence of XGal and IPTG: they contained 70%–80% white plaques. A significant proportion of these white plaques detected in the first libraries turned to pale blue upon retesting on strain RY1090, and were lacking detectable inserts. This was found to be related to the poor elimination of small cDNA fragments and linkers by chromatography on Sepharose 4B columns and was improved by using Sephacryl S300. After optimization, yields were approximately 50000–100000 white plaques per nanogram cDNA, among which 50% contained detectable inserts.

A cDNA library enriched for high molecular weight mRNA sequences was made as follows. A sample of 100 µg of mRNAs was fractionated on a sucrose gradient from which the 23–32 *S* area was recovered (10 µg). This fraction was further fractionated in a 10 mM methylmercuryhydroxide 1.5% agarose gel. The area corresponding to the 2–5 kb molecular sizes was sliced out, mRNAs were eluted, phenol-extracted, and used (100 ng) to build a cDNA library according to the above procedure.

Amplification and immunological screening of libraries. Libraries were amplified on strain RY1088 at a density of 3 × 10⁴–1 × 10⁵ pfu per 90-mm dish. Plaques were allowed to grow at 42° C for 4 h, resulting in a 1000-fold amplification. Amplified libraries were plated on strain RY1090 at a density of 1.0–1.5 × 10⁴ pfu per 90-mm dish and allowed to grow at 42° C for 3–4 h. Nitrocellulose filters (Millipore HATF) were soaked in 10 mM IPTG and dried. Lawns overlaid with these filters were incubated overnight at 37° C and then cooled at 4° C. The corresponding filters were blocked with 3% BSA (bovine serum albumin) in TBS (50 mM Tris-HCl, pH 8.0, 150 mM NaCl) for 30 min, incubated with antiserum (1/200 diluted in TBS) for 1–2 h, and washed three times in TBS containing 0.05% Tween 20. Filters were incubated with 0.5 µCi ¹²⁵I-protein A in TBS containing 50 g/l skimmed milk and 0.05% Tween 20 for 1–2 h, washed three times in TBS, milk, Tween 20 and twice in TBS. All steps were performed at room temperature. Filters were autoradiographed on Kodak XAR5 film with Dupont Cronex amplifying screens for 18–24 h. An average of 1–3 spots corresponding to putative recombinants were detected per filter. Plugs corresponding to these spots were

taken with the wide end of Pasteur pipettes and incubated overnight at 4° C in 1 ml of lambda diluent (Tris-HCl 10 mM pH 8.0, MgSO₄ 10. Approximately 3000 plaques per plug were rescreened for confirmation. Two further rounds of screening at low density (100–200 plaques/dish) were required for the purification to homogeneity of recombinants.

Immunological reagents for the screening of libraries. The purification of nitrate reductase from corn and the obtainment of the rabbit polyclonal antibodies (α MNR) against this enzyme (Commère et al. 1986) have been described previously. To neutralize anti *E. coli* antibodies from the α MNR immunoglobulins, the serum was diluted fivefold in TBS-BSA and incubated with two volumes of an *E. coli* crude lysate (20 mg/ml protein). After incubation for 2 h at 4° C with swirling, the mixture was centrifuged in Eppendorf tubes and the procedure of lysate addition and clarification was repeated twice.

Analysis of hybrid proteins expressed by recombinant clones. Lysogens were obtained by infection of the *Hfl* strain RY1089 by recombinant phages. Bacterial extracts were obtained from lysogens induced or non-induced by IPTG as described previously (Riva 1985). Extracts were analysed by SDS-PAGE, Western blotting of the fractionated proteins on nitrocellulose by electrotransfer, and immunodetection with α MNR antiserum and a second antiserum from sheep directed against rabbit IgG coupled with peroxidase (Chérel et al. 1985).

Immunoselection of antibodies recognizing the hybrid proteins expressed by recombinants. Nitrocellulose transfers of lawns of strain RY1090 infected by recombinant phages were saturated for 1 h with 30 mg/ml BSA and after washes in TBS they were incubated in 5 ml of 1/100 diluted non-immune serum (α So) or α MNR antiserum. The antibodies adsorbed on the filters were eluted by a 30 s incubation in 5 ml of 0.2 M glycine-HCl buffer, pH 2.2. After removal of the filter the elution buffer was rapidly neutralized with Tris base and non-immune antiserum was added at a 1/200-fold dilution in a final 10 ml volume. Eluted proteins were concentrated and dialysed against PBS against PBS (Na-phosphate 10 mM pH 7.5, NaCl 150 mM) using an Amicon cell equipped with a PM30 membrane. Eluted antibodies were added to tobacco NR partially purified by affinity chromatography and kindly provided by T. Moureaux. After 5 min of incubation, NR activity was assayed as previously described (Chérel et al. 1986).

Recognition of the fusion protein in an ELISA test using monoclonal antibodies. The isolation and characterization of the monoclonal antibody 96(9)25, which inhibits the activity of various plant nitrate reductases, has been described previously (Chérel et al. 1985). Several monoclonal antibodies recognizing the tobacco enzyme have been obtained using a similar procedure (C. Meyer, unpublished results). The monoclonal antibodies 7(113)NP15 and 24(48)NP19 recognize native NR from tobacco but have no effect on the activity of the enzyme. Recognition of the fusion protein using monoclonal antibodies was performed using a two sites ELISA test according to a previously described procedure (Chérel et al. 1986). Reactants were added to the microtitration plates in the following order: monoclonal anti-

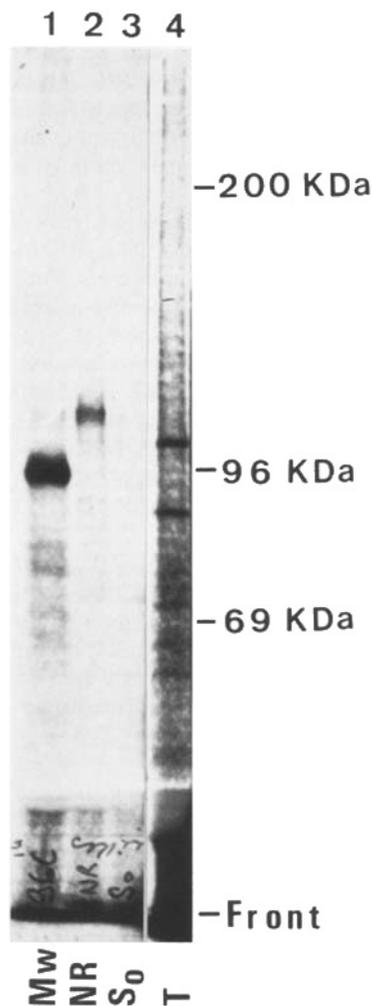


Fig. 1. Cell-free translation in a reticulocyte system of the messenger RNA encoding for the nitrate reductase (NR) apoenzyme. Total poly(A)⁺ mRNAs were isolated from young tobacco leaves and translated in vitro. Translation products were fractionated on a 5% polyacrylamide denaturing gel. Lane 1, [¹⁴C]-phosphorylase B; lane 2, translation products immunoselected with polyclonal antibody α MNR; lane 3, translation products immunoselected with a non-immune rabbit polyclonal antibody (α So); lane 4, total translation products

body from ascitic fluid, lysogen *E. coli* extract, α MNR antiserum and anti-rabbit IgG alkaline phosphatase conjugate.

Phage DNA preparation, subcloning of inserts and DNA sequencing. Bacterial and phage procedures were essentially as in Miller (1972). The strains used are described by Huynh et al. (1985). Phage production, purification and DNA extraction were performed according to the procedure of Huynh et al. (1985) described for λ gt10.

All recombinant DNA manipulations were performed using standard methods (Maniatis et al. 1982). *Eco*RI fragments excised from the vector were subcloned in the dephosphorylated *Eco*RI site of pUC9 according to standard procedures. The bacterial host strains for pEMBL vectors (Dente et al. 1985) were TG2 or JM109 (Yanish-Perron et al. 1985). The cDNA insert of clone 13–29 contains a unique *Kpn*I site. It was subcloned as a 2.5 kb *Kpn*I/*Kpn*I (in λ gt11) fragment in both orientations into the *Kpn*I site of pEMBL18 and as a 1.2 kb *Kpn*I/*Sst*I (in *lacZ* of λ gt11)

fragment in the *KpnI/SstI* sites of pEMBL18. The 120 bp of cDNA sequences contained in the *KpnI/SstI* fragment were turned round by cloning into the *EcoRI/XbaI* sites of pEMBL19.

Ordered deletions of the *KpnI* fragment were generated by the ExoIII, S1 nuclease method (Henikoff 1984). The single-stranded plasmid templates were prepared for sequencing mainly as described previously (Dente et al. 1985; Messing 1983), except that a single bacterial colony containing a recombinant pEMBL plasmid was first grown overnight in M9 minimal medium (Messing 1983) containing 100 µg/ml ampicillin. DNA sequencing by chain termination (Sanger et al. 1977) and labelling with ³⁵S-nucleotides was performed using standard methods (Messing 1983). A synthetic oligonucleotide and the λ reverse primer (Biolabs) were used to complete the sequence. Ninety-eight percent of the sequence was determined from both strands.

Computer programs. Sequence data treatments were performed with the BISANCE package, using the CITI2 facilities on a DPS8 computer, and the programs of R. Staden (MRC, Cambridge, UK) and M. Kanehisa (NIH, Bethesda, USA).

Northern and Southern blot experiments. Messenger RNAs were fractionated by electrophoresis in 10 mM methylmercuryhydroxide 1.5% agarose gels for the accurate estimation of molecular weights, or in 3.0% formaldehyde 1.5% agarose gels for routine experiments. After fractionation, mRNAs were transferred to Hybond C (Amersham) membranes as described previously (Thomas 1980).

Total DNA was extracted from young tobacco leaves by a previously published procedure (Dellaporta et al. 1983), purified by CsCl gradient equilibrium centrifugation and used for Southern blot analysis as described previously (Deshayes et al. 1985).

Northern and Southern blots were probed with the nick-translated 1.6 kb *EcoRI* fragment from phage 13–29 purified by sucrose gradient centrifugation, or with nick-translated pTA71 carrying a 9 kb wheat rDNA insert (Gerlach and Bedbrook 1979). Hybridization was performed in 50% formamide at 45° C and washed under stringent conditions (3 washes at room temperature in 2 × SSC, 0.1% SDS, followed by two 30-min washes at 65° C in 0.2 × SSC, 0.1% SDS; Maniatis et al. 1982). SSC buffer is 0.1 M NaCl and 0.1 M sodium citrate, pH 7.0.

Results

Immunological detection of cell-free translation products of the NR mRNA

Immunological screening of a cDNA expression library requires that the new translation product can be recognized by specific antibodies raised against polypeptide chains matured in eucaryotic cells. A polyclonal antibody against corn NR (αMNR) could be used to immunoselect a tobacco NR polypeptide chain among the cell-free translation products of total poly(A)⁺ RNAs extracted from leaves (Fig. 1). The signals obtained were very weak, NR representing 2–5/10000 of total tobacco soluble leaf proteins. Cell-free translation products migrated close to the 110 kDa enzyme subunits. Similar results were obtained previously with corn NR mRNA (Commère et al. 1986).

Building of a leaf tobacco cDNA library in λgt11

The cloning vector λgt11 (Young and Davies 1983) was chosen for the construction of cDNA libraries since it was expected that the frequencies of NR cDNAs would be very low, and large libraries required to be screened. cDNA libraries were first built from unfractionated mRNA preparations. The initial efficiencies of cloning and the proportions of true recombinants were low (<10000 clones/ng cDNA) when the Gubler and Hoffmann (1983) procedure was followed for the synthesis of cDNAs. As described previously, the efficiency of cloning was increased by including a mild S1 nuclease treatment between the healing and methylation steps of cDNA synthesis, and allowed the routine obtaining of cloning efficiencies higher than 10⁵ recombinants/ng cDNA.

Since the mRNA encoding for NR was expected to be of a size larger than 3 kb, mRNA preparations were fractionated on a sucrose gradient, and molecules with a sedimentation constant in the 23–32 S range were collected. These mRNAs were further fractionated on a methylmercuryhydroxide gel and the mRNAs migrating in the 3–4 kb area were used to build a cDNA library (4 × 10⁵ clones, 70% recombinants). These two steps of purification represented a five- to tenfold enrichment of NR mRNA.

Immunological screening of the library

Although it was specific for tobacco NR in tobacco leaf extracts, the antibody αMNR recognized several *E. coli* proteins in Western blot experiments (see Fig. 3). This resulted in high background levels when libraries were immunologically screened. The antibody αMNR was therefore incubated with large amounts of *E. coli* crude protein extracts, and confirmed to be still able to detect tobacco NR at the nanogram level. Among a total of 40 clones initially selected by screening 240000 recombinant plaques, 11 clones expressing reproducibly a signal upon rescreening were further purified to homogeneity. This eventually required, for the highly expressing clone 13–29, the plating, picking and amplification of isolated plaques in the absence of IPTG induction to prevent the appearance of non-expressing clones during plaque growth (results not shown).

Analysis of recombinant clones

Purified clones were tested for the presence of DNA inserts by *EcoRI* digestion. Seven clones (13–18, 13–20, 13–27, 13–29, 13–33, 13–34, 13–36) contained *EcoRI* inserts of similar size (1.6 kb). They gave consistently signals of differing intensities upon immunological detection. A strong signal was detected for clone 13–29 and to a lesser extent for clone 13–28, whereas other clones gave low signal intensities. The 1.6 kb *EcoRI* fragment from clone 13–36 was subcloned in pUC9 (Vieira and Messing 1982) and used as a probe for the study of insert homologies among the ten other recombinants. Seven clones cross-hybridized with this probe. Six of them (13–18, 13–20, 13–27, 13–29, 13–33, 13–34) had been classified as containing an insert of similar size, suggesting that these inserts were possibly identical. Clone 13–28 contained a 2 kb *EcoRI* insert. The structure and orientation of cDNAs in the cloning site of the vector were studied using the restriction enzymes *KpnI* and *SstI*. It was found that the size of inserts in some of the recombinants was larger than 1.6 kb and that the 1.6 kb *EcoRI*

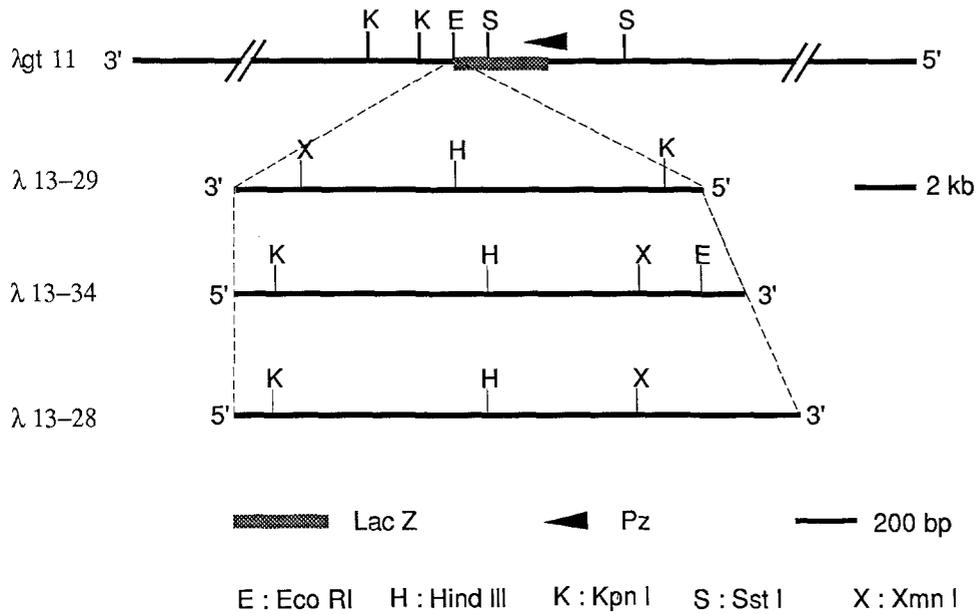


Fig. 2. Map of the inserts of three clones cross-hybridizing with the 1.6 kb *Eco*RI fragment from phage λ 13-34. The area corresponding to the cloning site of the vector has been *magnified*. Orientations of the inserts have been preserved. Pz, *lacZ* promoter

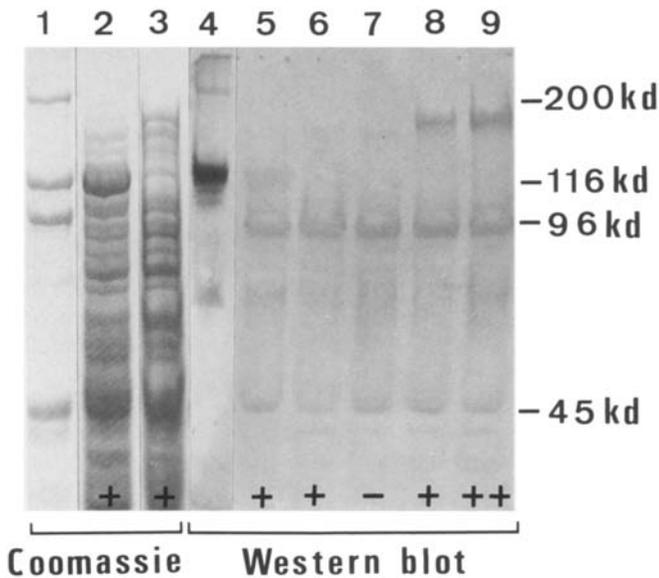


Fig. 3. Western blotting analysis of fusion proteins synthesized by various lysogens. Extracts were prepared from cultures induced for the lytic phase in the absence (-, 1 h) or presence (+, 1 h; ++, 2 h) of IPTG. After SDS-PAGE, gels were either stained with Coomassie blue (Lanes 1-3) or transferred on a nitrocellulose sheet (lanes 4-9). Blots were revealed with the antiserum α MNR. Two bands corresponding to the immunodetection of *E. coli* proteins were detected in all extracts. Lanes 2 and 5, λ gt11; lane 6, clone 13-36; lanes 3, 7, 8 and 9, clone 13-29. Lane 4 corresponds to purified corn nitrate reductase and lane 1, molecular weight markers

fragment was therefore most probably internal to the cDNAs and flanked by short cDNA fragments which were not seen on ethidium bromide stained *Eco*RI digests of recombinant phage DNAs. Inserts were found in both orientations; there was no obvious correlation between the orientation of inserts and the antigenicity of recombinant clones. Physical maps of three of these inserts are presented in Fig. 2. From their comparison it can be concluded that clones 13-28 and 13-34 contain 3' sequences lacking in

clone 13-29, and that they differ as regards the presence of an *Eco*RI site, suggesting an heterogeneity of the NR mRNA population. The remaining three recombinant clones contained small heterologous inserts, which are still under study.

Expression of a fusion protein in the recombinant clone 13-29

Lysogens of phages 13-29 and 13-36 carrying inserts in opposite orientations were constructed in strain RY1089. These lysogens were tested for the expression of a hybrid protein by Western blot analysis (Fig. 3). Using the polyclonal antibody α MNR, a protein of an approximate molecular weight of 175 kDa was found in the extracts of a lysogen for phage 13-29 induced with IPTG, but not in the non-induced controls. This hybrid protein was also detected after Coomassie blue staining of the corresponding SDS-PAGE fractionated extracts (Fig. 3). No hybrid protein was detected in a lysogen for phage 13-36. These results are in agreement with the insert of clone 13-29 being integrated in frame with the coding sequence of β -galactosidase and encoding a polypeptide chain of an approximate size of 60 kDa, which is in reasonable agreement with the size of the corresponding cDNA insert. The isolation by immunological screening of clone 13-36 carrying an insert oriented opposite to the β -galactosidase gene, and the study of the corresponding lysogen, confirm the weak expression of inserted sequences under the control of another phage promoter in λ gt11, as previously discussed (Lapeyre 1985). The absence of detection of hybrid proteins in Western blots of extracts from a lysogen of the recombinant clone 13-36 may be attributed to the low level of expression of this hybrid protein. On the basis of these results, phage lambda 13-29 was chosen for further studies and the corresponding insert was subcloned.

Confirmation of the identity of the cloned cDNAs by immunoselection of antibodies inhibiting the activity of tobacco NR

Although the antiserum α MNR used for the screening procedure was reasonably specific towards tobacco NR, this

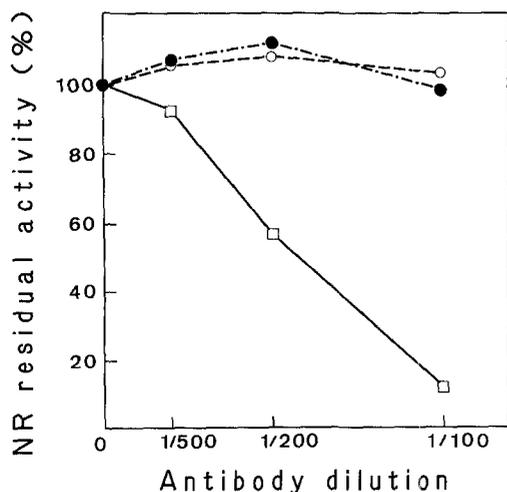


Fig. 4. Inhibition of the catalytic activity of tobacco nitrate reductase (NR) by immunoglobulins immunoselected by adsorption on plaques formed by phages λ gt11 and 13-29 grown in the presence of IPTG. Immunoglobulins were added in the enzyme incubation mixture at the indicated dilution, based on the initial amount of antiserum used for adsorption on phage plaques. Immune antiserum α MNR adsorbed on λ gt11 plaques (\bullet) or on recombinant clone 13-29 plaques (\square). Non-immune antiserum α So adsorbed on plaques from clone 13-29 (\circ). A small but significant stimulation of NR activity was induced by adding non-immune antiserum (α So) in the preparation of nitrate reductase. This is attributed to a stabilizing effect of serum proteins on the enzyme. The specific activity of the extract was $0.4 \mu\text{M NO}_2/\mu\text{g protein per minute}$

Table 1. Recognition of the hybrid-protein, expressed by clone 13-29, in an ELISA test using monoclonal antibodies directed against nitrate reductase (NR)

Monoclonal antibody	Specificity	Bacterial extract tested after IPTG induction ^a	
		RY1089 (λ gt11) ^b	RY1089 (λ 13-29) ^b
96(9)25	native corn and tobacco NR	0.006	0.457
7(113)NP15	native tobacco NR	0.019	0.004
24(48)NP19	native tobacco NR	0.031	0.272
GET	swine coronavirus	0.003	0.057

A double site ELISA was performed as described in Materials and methods.

^a Extracts were prepared as for Western blotting experiments, and clarified by a 15 min centrifugation in a Beckman airfuge before use. Extracts corresponding to 10^8 cells were diluted to 1 ml and used for ELISA measurements.

^b Results are expressed in OD measurements of alkaline phosphatase activity after 1 h of incubation. Background levels in the absence of bacterial extract, corresponding to OD values ranging from 0.140 to 0.170, have been subtracted

antiserum may have contained some antibodies recognizing other tobacco proteins. This could lead to the isolation of so-called "false positives". To test this possibility, *E. coli* strain RY 1090 lawns were infected by phages λ gt11 and 13-29. After induction with IPTG the synthesized proteins were transferred on nitrocellulose filters. Control (α So) and α MNR polyclonal antibodies were incubated with the filters

and the corresponding immunoselected immunoglobulins were eluted from the filters at low pH. These selected antibodies were then assayed for their ability to inhibit the nitrate reducing activity of tobacco NR. As shown in Fig. 4, a 40% inhibition of tobacco NR activity was obtained with a 1/200 dilution of the selected immunoglobulins, in reasonable agreement with the inhibitory activity of the α MNR serum (70% inhibition with a 1/200 dilution of this serum). Proteins expressed by clone 13-29 could bind specifically antibodies inhibitory to the tobacco NR in the α MNR serum. This suggests also that the amount of hybrid protein available for immunoselection was in excess of the amount of immunoglobulins to be adsorbed in the serum. Indeed we found that α MNR antiserum used for the confirmation of immunologically positive clones could not be used for further screening of new recombinants, probably due to a depletion of immunoglobulins directed against NR.

Recognition of the hybrid protein expressed by clone 13-29 by monoclonal antibodies directed against NR

Three NR monoclonal antibodies raised against *N. plumbaginifolia* and corn NR and a control monoclonal antibody directed against a swine coronavirus (Table 1) were used to characterize further the hybrid proteins. Among the four monoclonal antibodies tested, clones 96(9)25 and 24(48)NP19 specifically recognized antigenic determinants from an extract of a lysogen for clone 13-29, made in the bacterial strain RY1089. No significant recognition was detected for clone 7(113)NP15 and GET. The monoclonal antibody 96(9)25 has a higher affinity for tobacco NR than the monoclonal antibody 24(48)NP19. The recognition of the hybrid protein by these two monoclonal antibodies suggests a similar situation. At least two different epitopes can be detected on the hybrid protein that are also recognized on tobacco NR. The absence of the epitope recognized by the monoclonal antibody 7(113)NP15 shows also, as expected, that tobacco NR and the hybrid protein are antigenically different, due to a lack of part of the NR coding sequence in the cloned cDNA.

Nucleotide sequence of clone 13-29; analysis of the deduced amino acid sequence

The nucleotide sequence of clone 13-29 is shown in Fig. 5. This partial cDNA is 1662 bp long, including the *EcoRI* sites, and does not contain polyadenylation sequences. Although the cDNA was cloned in the vector after methylation and linker addition, no linker sequences are found at the ends of the cDNA. This strongly suggests that the methylation step had not worked properly and that an internal *EcoRI* fragment of the cDNA had been cloned. Among the six possible reading frames of the sequence only one is uninterrupted by stop codons and encodes a polypeptide chain of 554 amino acids with a molecular mass of 62175 kDa. This open reading frame corresponds to a translational fusion with the flanking β -galactosidase sequence of the vector, and represents about 55% of the expected size of the tobacco nitrate reductase apoenzyme. Codon usage is biased (data not shown); the CUG codon for Leu and UCG for Ser are never used.

A computer search was undertaken for homologies to sequences in the PIR data base (release 11) or deduced from open reading frame sequences in Genbank (release

atcggc	GAA TTC CCT TGT AGA GAA TTG CCC GTT ACG CTT GTT TGT GCT GGC AAT CGA AGG AAA GAA CAG AAC ATG GTT AAA CAA ACC ATT	90
	Glu Phe Pro Cys Arg Glu Leu Pro Val Thr Leu Val Cys Ala Gly Asn Arg Arg Lys Glu Gln Asn Met Val Lys Gln Thr Ile	28
GGT TTC AAC TGG GCC GCC GCT GCC GTT TCA ACA ACG ATA TGG CGC GGG GTA GCC CTC CGC GCT TTG CTA AAA GGG TGC GGT GTT TTT AGC	180	
Gly Phe Asn Trp Gly Ala Ala Ala Val Ser Thr Thr Ile Trp Arg Gly Val Pro Leu Arg Ala Leu Leu Lys Arg Cys Gly Val Phe Ser	58	
AAG AAT AAA GGG GCG CTT AAT GTT TGC TTC GAA GGA GCT GAT GTC TTG CCC GGA GGT GGT GGT TCA AAG TAT GGA ACC AGC ATT AAG AAG	270	
Lys Asn Lys Lys Gly Ala Leu Asn Val Cys Phe Glu Gly Ala Asp Val Leu Pro Gly Gly Gly Gly Ser Lys Tyr Gly Thr Ser Ile Lys Lys	88	
GAA TTT GCA ATG GAT CCA GCA CGA GAT ATC ATC GTA GCC TAC ATG CAG AAC GGA GAA AAA TTG GCA CCC GAC CAC GGG TTT CCA GTA CGA	360	
Glu Phe Ala Met Asp Pro Ala Arg Asp Ile Ile Val Ala Tyr Met Gln Asn Gly Glu Lys Leu Ala Pro Asp His Gly Phe Pro Val Arg	118	
ATG ATA ATT CCA GGA TTC ATT GGA GGA AGA ATG GTG AAA TGG ATA AAG AGG ATT ATA GTC ACC ACC CAA GAA TCA GAC AGC TAT TAT CAT	450	
Met Ile Ile Pro Gly Phe Ile Gly Gly Arg Met Val Lys Trp Ile Lys Arg Ile Ile Val Thr Thr Gln Glu Ser Asp Ser Tyr Tyr His	148	
TTC AAG GAC AAT AGA GTT CTT CCT CCC CAT GTT GAT GCT GAA CTT GCA AAT ACC GAA GCA TGG TGG TAC AAG CCA GAG TAT ATC ATC AAT	540	
Phe Lys Asp Asn Arg Val Leu Pro Pro His Val Asp Ala Glu Leu Ala Asn Thr Glu Ala Trp Trp Tyr Lys Pro Glu Tyr Ile Ile Asn	178	
GAG CTT AAT ATT AAC TCT GTC ATT ACG ACG CCG TGT CAT GAA GAA ATT TTG CCA ATT AAC GCC TGG ACG ACT CAG CGA CCT TAC ACG TTG	630	
Glu Leu Asn Ile Asn Ser Val Ile Thr Thr Pro Cys His Glu Glu Ile Leu Pro Ile Asn Ala Trp Thr Thr Gln Arg Pro Tyr Thr Leu	208	
AGC GGC TAT TCT TAT TCT GGC GGA GGG AAA AAA GTA ACG CGA GTA GAA GTG ACG TTG GAT GGA GGA GAA ACA TGG CAA GTT AGC ACA CTA	720	
Arg Gly Tyr Ser Tyr Ser Gly Gly Gly Lys Lys Val Thr Arg Val Glu Val Thr Leu Asp Gly Gly Glu Thr Trp Gln Val Ser Thr Leu	238	
GAT CAC CCA GAG AAG CCC ACC AAA TAT GGC AAG TAC TGG TGT TGG TGC TTT TGG TCA CTC GAG GTT GAG GTG TTA GAC TTG CTC AGT GCT	810	
Asp His Pro Glu Lys Pro Thr Lys Tyr Gly Lys Tyr Trp Cys Trp Cys Phe Trp Ser Leu Glu Val Glu Val Leu Asp Leu Leu Ser Ala	268	
AAA GAA ATT GCT GTT CGA GCT TGG GAT GAG ACC CTC AAT ACT CAA CCC GAG AAG CTT ATT TGG AAC GTC ATG GGA ATG ATG AAT AAT TGC	900	
Lys Glu Ile Ala Val Arg Ala Trp Asp Glu Thr Leu Asn Thr Gln Pro Glu Lys Leu Ile Trp Asn Val Met Gly Met Met Asn Asn Cys	298	
TGG TTC CGA GTA AAG ATG AAT GTG TGC AAG CCT CAC AAG GGA GAG ATT GGA ATA GTG TTT GAG CAT CCG ACT CAA CCT GGA AAC CAA TCA	990	
Trp Phe Arg Val Lys Met Asn Val Cys Lys Pro His Lys Gly Glu Ile Gly Ile Val Phe Glu His Pro Thr Gln Pro Gly Asn Gln Ser	328	
GGT GGA TGG ATG GCG AAG GAG AGA CAT TTG GAG ATA TCA GCA GAG GCA CCT CAA ACA CTA AAG AAG ACT ATC TCA ACT CCA TTC ATG AAC	1080	
Gly Gly Trp Met Ala Lys Glu Arg His Leu Glu Ile Ser Ala Glu Ala Pro Gln Thr Leu Lys Lys Ser Ile Ser Thr Pro Phe Met Asn	358	
ACA GCT TCC AAG ATG TAC TCC ATG TCC GAG GTC AGG AAA CAC AGC TCT GCT GAC TCT GCT TGG ATC ATA GTC CAT GGT CAT ATC TAT GAC	1170	
<u>Thr Ala Ser Lys Met Tyr Ser Met Ser Glu Val Arg Lys His Ser Ser Ala Asp Ser Ala Trp Ile Ile Val His Gly His Ile Tyr Asp</u>	388	
GCC ACG CGT TTC TTG AAA GAT CAC CCT GGT GGG ACT GAC AGC ATT CTC ATC AAT GCT GGC ACT GAT TGC ACT GAG GAA TTT GAT GCA ATT	1260	
<u>Ala Thr Arg Phe Leu Lys Asp His Pro Gly Gly Thr Asp Ser Ile Leu Ile Asn Ala Gly Thr Asp Cys Thr Glu Glu Phe Asp Ala Ile</u>	418	
CAT TCT GAT AAG GCT AAG AAG CTC TTG GAG GAT TTC AGG ATT GGT GAA CTC ATA ACT ACT GGT TAC ACC TCT GAC TCT CCT GGC AAC TCC	1350	
<u>His Ser Asp Lys Ala Lys Lys Leu Leu Glu Asp Phe Arg Ile Gly Glu Leu Ile Thr Thr Gly Tyr Thr Ser Asp Ser Pro Gly Asn Ser</u>	448	
GTG CAC GGA TCT TCT TCC TTC AGC AGC TTT CTA GCA CCT ATT AAG GAA CTT GTT CCA GCG CAG AGG ACT GTG GCC CTA ATT CCA AGA GAG	1440	
<u>Val His Gly Ser Ser Ser Phe Ser Ser Phe Leu Ala Pro Ile Lys Glu Leu Val Pro Ala Gln Arg Ser Val Ala Leu Ile Pro Arg Glu</u>	478	
AAA ATC CCA TGC AAA CTC ATC GAC AAG CAA TCC ATC TCC CAT GAT GTT AGG AAA TTT CGA TTT GCA TTG CCC TCT GAG GAT CAA GTC TTG	1530	
<u>Lys Ile Pro Cys Lys Leu Ile Asp Lys Gln Ser Ile Ser His Asp Val Arg Lys Phe Arg Phe Ala Leu Pro Ser Glu Asp Gln Val Leu</u>	508	
GGC TTG CCT GTT GGA AAA CAT ATC TTC CTC TGT GCC GTT ATT GAC GAT AAG CTC TGC ATG CGC GCT TAC ACG CCT ACT AGC ACC ATC GAT	1620	
<u>Gly Leu Pro Val Gly Lys His Ile Phe Leu Cys Ala Val Ile Asp Asp Lys Leu Cys Met Arg Ala Tyr Thr Pro Thr Ser Thr Ile Asp</u>	538	
GAG GTG GGG TAC TTC GAG TTG GTT GTC AAG ATA TAC TTC AAA GGA ATT cca	1671	
<u>Glu Val Gly Tyr Phe Glu Leu Val Val Lys Ile Tyr Phe Lys Gly Ile</u>	554	

Fig. 5. Nucleotide sequence of the cDNA insert from recombinant phage λ 13-29 and the predicted amino acid sequence of the cDNA-encoded part of the fusion protein expressed by this phage. The nucleotides (upper line) and the amino acids (lower line) are numbered on the right. The nucleotides types with lower case letters correspond to flanking β -galactosidase sequences. The underlined sequences refer to the two domains analysed in Fig. 6

46). As shown in Figs. 5 and 6, two stretches of high homology were found. From position 360 to 435 the tobacco nitrate reductase showed sequence homology ranging from 35%–50% with proteins from the cytochrome *b5* superfamily (Guiard and Lederer 1979a). Lê and Lederer (1983) have isolated, by chymotrypsin proteolysis, the heme-binding domain from *Neurospora crassa* nitrate reductase and shown its homology with the cytochrome *b5* family. Homology between the tobacco nitrate reductase amino acid sequence and the fungal nitrate reductase heme-binding domain is also found, as expected.

From residues 476 to 552 the tobacco nitrate reductase amino acid sequence showed 57% identity with the N-terminal sequence of the human cytochrome *b5* reductase (Yubisui et al. 1984). This FAD flavoenzyme is known to form

a binary complex with cytochrome *b5*, performing electron transport from NADH for various redox physiological processes. No definite homologies were found with other polypeptides in the sequence data banks or with selected sequences, such as the partial coding sequence for the alpha subunit of *E. coli* respiratory nitrate reductase.

Northern blot analysis of mRNAs hybridizing with the 1.6 kb EcoRI fragment

Messenger RNAs were extracted from tobacco, *Nicotiana glauca* and corn leaves of plants grown on a nitrate-containing medium. Northern blots of mRNAs fractionated on formaldehyde-agarose gels were probed with

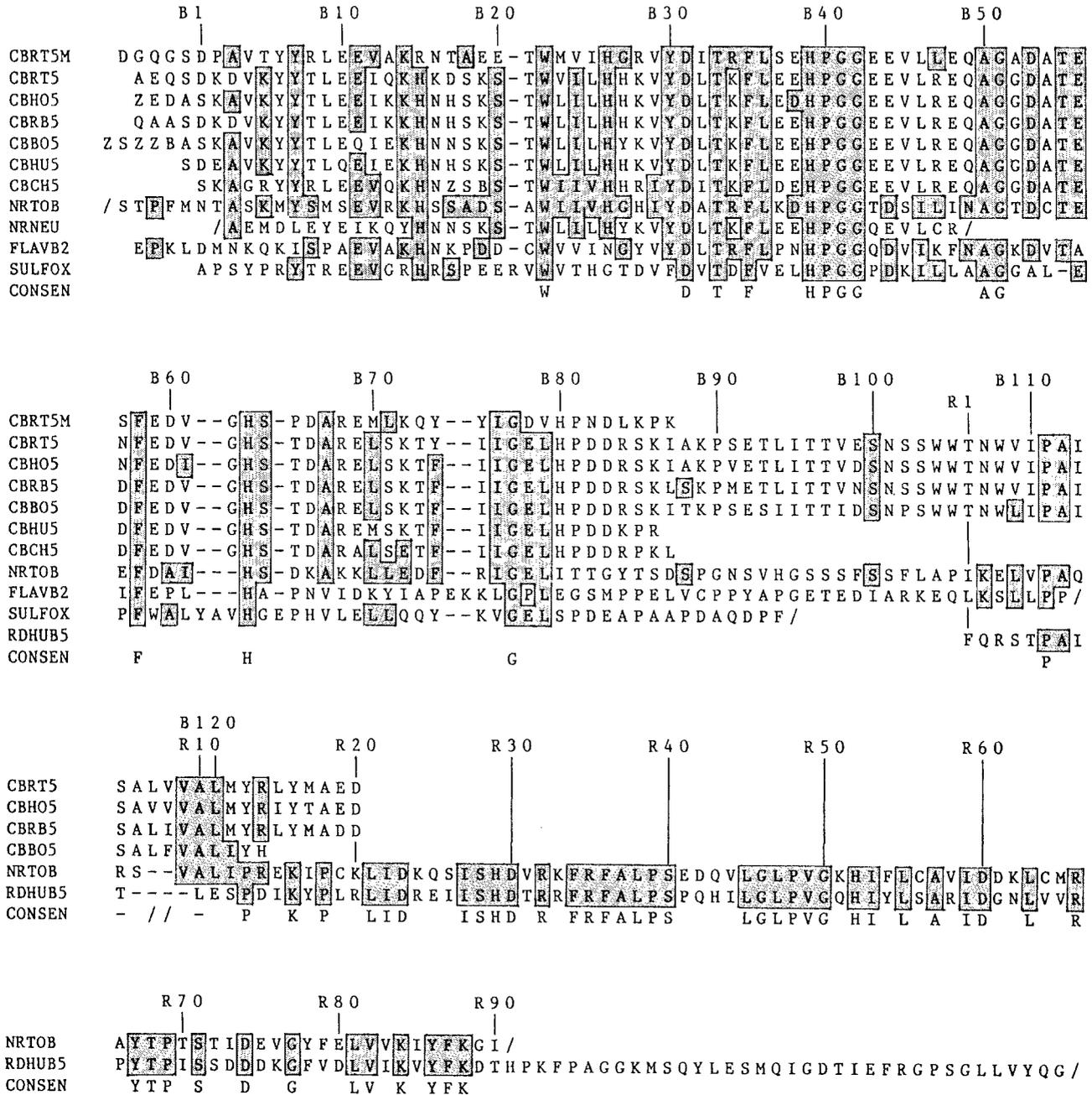


Fig. 6. Alignment of the predicted amino acid sequence of tobacco nitrate reductase (NR) in the heme-binding domain (B1 to B120) and FAD/NADH domain (R1 to R90) with proteins of the cytochrome *b5* superfamily and with human erythrocyte cytochrome *b5* reductase (RDHUB5). Identity between any sequence and tobacco NR is shown by boxed residues. Residues of the heme-binding domain have been numbered as conventional for bovine cytochrome *b5* (CBB05, Ozols and Strittmatter 1969) with the two heme-liganding histidines numbered His-39 and His-63 stars. The FAD/NADH domain has been numbered from the N-terminal residue of cytochrome *b5* reductase. CBRT5M, rat mitochondrial cytochrome *b5* (Lederer et al. 1983); CBRT5, rat microsomal cytochrome *b5* (Ozols and Heinemann 1982); CBHO5, horse microsomal cytochrome *b5* (Ozols et al. 1976); CBRB5, rabbit microsomal cytochrome *b5* (Tsugita et al. 1970); CBHU5, human microsomal cytochrome *b5* (Nobrega and Ozols 1971); CBCH5, chicken microsomal cytochrome *b5* (Nobrega and Ozols 1971); NRTOB, tobacco nitrate reductase (this paper); NRNEU, *Neurospora crassa* nitrate reductase (Lê and Lederer 1983, and unpublished data); FLAVB2, yeast flavocytochrome *b2* (Lederer et al. 1985); SULFOX, chicken sulfite oxidase (Guiard and Lederer 1979b); CONSEN, consensus sequence

the 1.6 kb cDNA insert (Fig. 7). An hybridization signal was detected after 3 days of exposure in tobacco and *N. plumbaginifolia* mRNA preparations. No signal was detected even after prolonged exposure in the case of corn mRNAs. Hybridization of the same blots with a rDNA wheat repeat unit showed that mRNAs homologous to the

1.6 kb insert were migrating slightly faster than the 28 S ribosomal RNA. A size of 3.5–3.6 kb was assigned to the mRNA when migrations were performed in methylmercuryhydroxide gels under fully denatured conditions in the presence of single-stranded DNA molecular weight markers.

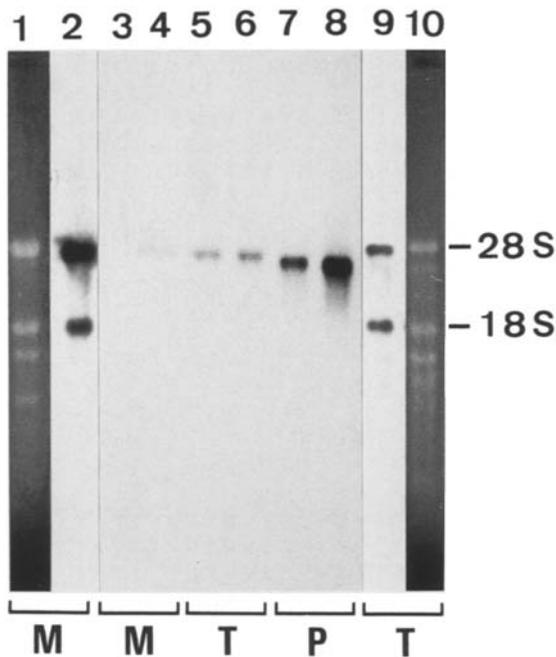


Fig. 7. Northern blot analysis of mRNA sequences hybridizing with the 1.6 kb *Eco*RI insert from phage λ 13–29. Total RNA was extracted from the leaves of tobacco (*T*), *N. plumbaginifolia* (*P*) and corn (M) plantlets grown on a nitrate-containing medium. Poly(A)⁺ mRNAs were purified from these samples. Lanes 1 and 10, total corn RNA (5 μ g) and total tobacco RNA (2 μ g) stained with ethidium bromide; lanes 2 and 9, the same samples probed on Northern blots with the wheat rDNA fragment from plasmid pAT71; lanes 3–8, Northern blots of poly(A)⁺ mRNAs probed with the 1.6 kb *Eco*RI fragment from phage 13–36 subcloned in pUC9 (sp. act. 10^9 cpm/ μ g, 1 week autoradiography). Lanes 3 and 4, 1 and 2 μ g corn mRNA; lanes 5 and 6, 1 and 2 μ g tobacco mRNA; lanes 7 and 8, 2 and 5 μ g *N. plumbaginifolia* mRNA

Southern blot analysis of genomic sequences homologous to the 1.6 kb EcoRI fragment

The genomic complexity of the sequences homologous to the 1.6 kb *Eco*RI fragment was studied using DNA extracted from tobacco. The results of a Southern blot performed under high stringency conditions are presented in Fig. 8. Signals corresponding to low copy numbers of the corresponding sequences were detected in the digests, as indicated by a comparison with hybridization signals obtained by probing with a rDNA wheat repeat unit. When genomic DNA was cut by *Eco*RI a signal was detected in the positions of a 3.4 kb and a 5.0 kb fragment and a weak hybridization was also observed corresponding to a 2.9 kb fragment. Two functional structural genes are assumed to be present in the tobacco genome (Müller 1983). Taking into account that the 1.6 kb *Eco*RI fragment is internal to the cDNA, this suggests that intervening sequences must be present in the corresponding area of at least one of the two structural genes.

Discussion

The present report provides evidence for the cloning of cDNAs encoding a part of a protein antigenically related to tobacco nitrate reductase. A family of clones was isolated by immunological screening of a cDNA library built in

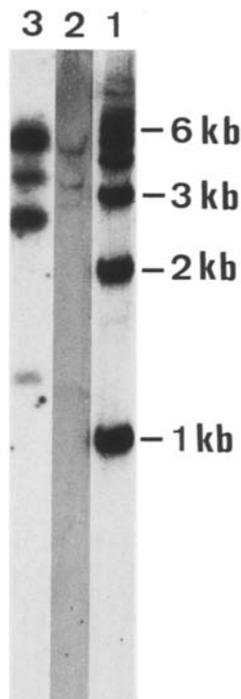


Fig. 8. Southern blot analysis of tobacco genomic sequences homologous to the 1.6 kb *Eco*RI insert from phage λ 13–29. Lane 1, molecular weight markers; lane 2, 10 μ g of tobacco genomic DNA digested with *Eco*RI, probed with the 1.6 kb *Eco*RI fragment (sp. act. 10^9 cpm/ μ g, 3 days autoradiography); lane 3, 1 μ g of the same DNA cut by *Eco*RI probed with the wheat rDNA sequences from plasmid pTA71 (sp. act. 10^8 cpm/ μ g, 1 day autoradiography)

the expression vector λ gt11. These recombinant clones encode polypeptide fragments sharing homologies with the active site of the nitrate reductase from tobacco. The cloned fragments hybridize with a 3.5 kb mRNA species and to low copy number genomic sequences.

These cloned sequences have been confirmed to correspond to fragments of the nitrate reductase mRNA by sequence data analysis. Plant and fungal nitrate reductases contain a cytochrome *b557* domain (Redinbaugh and Campbell 1985). As shown by Lê and Lederer (1983) by amino acid sequencing, the heme-binding domain of *Neurospora crassa* nitrate reductase is a member of the cytochrome *b5* superfamily. Our results show that the tobacco enzyme also shows a significant homology with different members of the superfamily. The cytochrome *b5* homology entirely covered the heme-binding domain of the various proteins: we thus propose that this sequence defines the heme-binding domain of tobacco nitrate reductase. All the 13 conserved residues of the superfamily (Lederer et al. 1983) are present in our tobacco NR sequence, which appears to be closer to chicken cytochrome *b5* than to any other member of the superfamily. The relative dissimilarity between the plant and fungal nitrate reductases indicates a divergent evolution for that enzyme. Since X-ray determinations of the spatial structure of beef microsomal cytochrome *b5* (Mathews et al. 1971) and flavocytochrome *b2* (Xia et al. 1987) have been performed, it is hoped from the observed homology that the three dimensional conformations will be predictable.

A significant homology of the coding sequence of the cloned cDNA with human flavoprotein cytochrome *b5* re-

ductase was also detected. This protein is known to catalyse the reduction of cytochrome *b5*, using NADH as an electron donor and FAD as a redox intermediate. This may be functionally compared to the catalytic reaction of nitrate reduction involving NADH, FAD, and cytochrome *b5* as cofactors. The suggestion is that this second homologous sequence is the N-terminal moiety of the NADH/FAD domain of tobacco nitrate reductase. Nitrate reductase would therefore appear to be a protein in which the heme-binding site and the reducing flavoprotein domain lie side by side in the polypeptide chain. To the best of our knowledge no sequences are available for the molybdopterin-binding domain from redox enzymes, which would be helpful to further analyse the functional structure of assimilatory nitrate reductases.

Nitrate reductase represents a small proportion (2–5/10000) of the soluble proteins. Cell-free translation data and Northern experiments suggest that the corresponding mRNA accounts for approximately 1/10000 of total poly(A)⁺ mRNA. The frequency of immunologically positive clones isolated from the cDNA library is 1/20000. This value seems low since this library was made from purified mRNAs and should contain one NR cDNA among 1000–2000 recombinants. When the library was screened by hybridization with the 1.6 kb *Eco*RI fragment it was found that indeed the proportion of inserts homologous to the probe was larger (1/5000). We conclude that most of these cDNAs were too small to express an immunogenic protein. The library was screened with a polyclonal antibody raised against the corn NR. The preferential isolation of relatively similar cDNAs may reflect the limited number of epitopes that can be recognized on tobacco NR by an antiserum raised against a heterologous NR. Previous work has shown that among six different epitopes of the corn NR identified by a family of monoclonal antibodies, only one epitope, a conformational epitope involved in the catalytic activity of the enzyme, was found on the tobacco enzyme (Chérel et al. 1985). This epitope is apparently also present on the hybrid protein expressed by recombinant clone 13–29. It will be of interest to see whether the hybrid protein is able to catalyze some of the oxidoreductive steps involved in nitrate reduction by nitrate reductase.

The NR structural gene is one of the few examples of plant genes for which it is possible to select for or against its expression. Furthermore the impairment in nitrate assimilatory functions still allows the regeneration of fertile plants. Numerous NR mutants have been isolated in our laboratory and are currently being characterized. A probe corresponding to part of the NR apoenzyme coding sequence will be useful to analyse these mutants which belong to seven different complementation groups (Müller AJ and Gabard J, unpublished results; Gabard et al. 1987), and to study the NR structural gene. Genomic clones homologous to this probe have been isolated and are under study.

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Note added in proof

The sequence of xanthine dehydrogenase, a molybdopterin binding enzyme was published recently (Keith TP, Riley MA, Kreitman M, Lewontin RC, Curtis D, Chambers G 1987) Sequence of the Structural Gene for Xanthine Dehydrogenase (*rosy* Locus) in *Drosophila melanogaster* (*Genetics* 116:67–73). No striking homologies were found between the partial protein sequence of nitrate reductase and this sequence.