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Isolation of cDNA clones coding for spinach nitrite reductase: Complete sequence and nitrate induction

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Summary. The main nitrogen source for most higher plants is soil nitrate. Prior to its incorporation into amino acids, plants reduce nitrate to ammonia in two enzymatic steps. Nitrate is reduced by nitrate reductase to nitrite, which is further reduced to ammonia by nitrite reductase. In this paper, the complete primary sequence of the precursor protein for spinach nitrite reductase has been deduced from cloned cDNAs. The cDNA clones were isolated from a nitrate-induced cDNA library in two ways: through the use of oligonucleotide probes based on partial amino acid sequences of nitrite reductase and through the use of antibodies raised against purified nitrite reductase. The precursor protein for nitrite reductase is 594 amino acids long and has a 32 amino acid extension at the N-terminal end of the mature protein. These 32 amino acids most likely serve as a transit peptide involved in directing this nuclear-encoded protein into the chloroplast. The cDNA hybridizes to a 2.3 kb RNA whose steady-state level is markedly increased upon induction with nitrate.

Key words: Nitrite reductase – cDNA sequence – Nitrate assimilation – Nitrate induction – Transit peptide sequence

Introduction

Nitrate is the major source of nitrogen for higher plants, algae, and several bacteria and fungi. In higher plants the roots efficiently remove nitrate from the soil in a process facilitated by a carrier protein. In most plants, the bulk of the nitrate is then transported through the xylem to the leaves. Prior to its incorporation into amino acids, nitrate is reduced to nitrite by nitrate reductase (NR; EC 1.6.6.1) and nitrite is then reduced to ammonia by nitrite reductase (NiR; EC 1.7.7.1).

While in most plants studied the reduction of nitrate takes place primarily in green tissues, some reduction in non-green tissues has been shown to occur in many plants (for a general review see Haynes 1986). In those plants studied, the NR and NiR polypeptides isolated from non-green tissues seemed to be different from their counterparts in green tissues (Hucklesby et al. 1972; Dalling et al. 1973; Ishiyama and Tamura 1985). However, in spinach there is some immunological evidence that supports the notion that leaf and root NiR are actually the same protein (Hirasawa et al. 1984; Ferrario et al. 1983). In leaves, NR is found in the cytoplasm. However, NiR is a chloroplast-localized protein. Since it is a nuclear-encoded protein, it must be transported from the cytoplasm to the chloroplasts.

Nitrite reductase from green tissues has been purified to homogeneity from several species (reviewed in Guerrero et al. 1981; Serra et al. 1982; Small and Gray 1984; Ishiyama et al. 1985). The enzyme appears to be a monomer with a molecular weight between 60000 and 70000 daltons, depending on the species. It contains one siroheme and one tetranuclear iron-sulfur center as prosthetic groups (Lancaster et al. 1979; Vega et al. 1980). Reduced ferredoxin is the physiological electron donor, providing the six electrons necessary for the reduction of nitrite to ammonia (Vega et al. 1980).

The expression of NiR, as well as NR, has been shown to be induced by nitrate (Beevers and Hageman 1969). In those plant species studied, little NiR is expressed in the absence of nitrate. The addition of nitrate leads to an increase of approximately eightfold in the expression of the NiR protein (Gupta and Beevers 1984). However, the mechanism of this induction is still not known.

In this paper, we describe the cloning and analysis of a full-length cDNA coding for the entire NiR protein from spinach. The entire cDNA clone has been sequenced and codes for a precursor protein of 594 amino acids. This includes a transit peptide of 32 amino acids whose end-point has been delimited by sequencing the N-terminus of the mature protein. This transit peptide is almost certainly involved in the transport of the NiR protein from the cytoplasm into the chloroplast.

Materials and methods

Protein sequencing. Nitrite reductase (NiR) was isolated from spinach leaves following the procedure of Vega and Kamin (1977). Three nanomoles (165 μg) of purified NiR protein were reduced and carboxymethylated using standard procedures (Allen 1981). Peptides were generated by cleavage with cyanogen bromide and were isolated by reverse-phase HPLC using a Vydac C8 pH stable column on a Waters Model 590 HPLC. Peptides were sequenced on an Applied Biosystems Model 470A gas phase protein sequencer. The following oligopeptides were sequenced:

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MEEVDKSKHNKDDIDVRLKW;MDNVRNPVGNPL-AGIDPHE; MLEAFRDLGFRGNRQK.

Poly(A) RNA isolation. Spincah leaves were frozen in liquid nitrogen and stored at −80° C. Between 10 and 15 g of tissue were added to a mixture of 50 ml buffer (50 mM Tris-HCl, pH 8.0; 4% sodium para-aminosalicylate; 1% sodium 1,5-naphthalenedisulphonate) and 50 ml of water-saturated phenol. The mixture was homogenized with a Polytron (Kinematica, Luzern) for 2 min at maximum speed and shaken for 10 min at 300 rpm. After the addition of 30 ml $CHCl_3$, the mixture was shaken for another 10 min and centrifuged at 7000 rpm in a GSA rotor (Sorvall) for 20 min. The aqueous phase was re-extracted with phenol/$CHCl_3$ and then extracted with $CHCl_3$. To isolate poly(A)$^+$ RNA, the aqueous phase was adjusted to 20 mM Tris-HCl, pH 7.5; 500 mM LiCl; 1 mM EDTA; 0.1% SDS. About 1.5 ml oligo(dT) cellulose was added and the suspension shaken gently for 10 min at room temperature. After pelleting the oligo(dT) cellulose at 100 *g*, the slurry was poured into a disposable column and poly(A)$^+$ RNA was eluted as described (Maniatis et al. 1982).

Northern and Southern blot hybridization. For Northern blots, poly(A)$^+$ RNA was denatured with formaldehyde, electrophoretically separated on agarose gels and blotted onto nitrocellulose filters (Maniatis et al. 1982). The filters were prehybridized in 5 × SSCP; 10 × Denhardt's; 0.05% pyrophosphate; 100 µg/ml denatured salmon sperm DNA at 63° C for at least 4 h and then were hybridized overnight at 42° C in 50% formamide; 5 × SSC; 10 × Denhardt's; 100 µg/ml denatured salmon sperm DNA, and radioactively labeled denatured DNA probe. DNA was radioactively labeled with ^{32}P using a nick translation kit (Amersham International) to a specific activity of $1–4 \times 10^8$ cpm/µg. Southern blots were prepared by standard procedures. Filters containing DNA were prehybridized and hybridized in 50% formamide; 1 M NaCl; 50 mM Pipes, pH 7.0; 0.5% Sarcosyl; 5 × Denhardt's; 10 mM EDTA; 100 µg/ml denatured salmon sperm DNA. Dextran sulfate (10%) was included in the hybridization buffer only. Filters were washed, dried and subjected to autoradiography on Kodak XOM film as described (Maniatis et al. 1982).

Construction and screening of the cDNA library. Poly(A)$^+$ RNA was isolated from spinach leaves 2 h after nitrate induction. Double-stranded cDNA was synthesized using a variation of the Okayama and Berg (1982) procedure. The following modifications in the procedure were used. First strand cDNA was primed with oligo(dT). Double-stranded cDNAs were treated with *Eco*RI methylase and S-adenosylmethionine, and the ends were blunted with T4 DNA polymerase I. After *Eco*RI linkers were ligated to the ends, cDNAs longer than 500 bp were isolated from a low melting temperature agarose gel and inserted into the *Eco*RI site of λ GT11 (Young and Davis 1983).

The cDNA library was screened with NiR antiserum as described by Young and Davis (1983). NiR antiserum was diluted 1:3,000 and preincubated with *Escherichia coli* lysate (Bio-Rad) diluted 1:600. Alkaline phosphatase-conjugated anti-rabbit antibodies were used as the second antibody.

The cDNA library was screened with oligonucleotide probes according to the procedure of Wood et al. (1985). The following mixed oligonucleotide probes were synthesized on an Applied Biosystems Model 380A DNA synthesizer and used as hybridization probes:

1. oligoNiR1 (20-mer; 64-fold redundancy)

```
   Lys His Asn Lys Asp Asp Ile
5'-AAA CAC AAC AAA GAC GAC AT-3'
     G   T   T   G   T   T
```

2. oligoNiR2 (18-mer; 64-fold redundancy)

```
   Met Glu Glu Val Asp Lys
3'-TAC CTT CTT CAA CTA TTT-5'
         C   C   C   G   C
                 G
                 T
```

3. oligoNiR3 (14-mer; 32-fold redundancy)

```
   Met Asp Asn Val Arg
5'-ATG GAC AAC GTA AG-3'
        T   T   C C
                G
                T
```

The mixed olignucleotide probes were end-labeled to a specific activity of 1×10^6 cpm/pmole with $\gamma[^{32}P]$ATP (> 5000 Ci/mmol; New England Nuclear) and T4 polynucleotide kinase (Maniatis et al. 1982). Nitrocellulose filter replicas of the cDNA clones were prehybridized overnight at 60° C in 6 × SSC; 0.5% SDS; 0.1% sodium pyrophosphate; 100 µg/ml denatured salmon sperm DNA; 5 × Denhardt's solution. The filters were then hybridized overnight at 42° C in 6 × SSC; 5 × Denhardt's solution; 0.1% sodium pyrophosphate; 20 µg/ml tRNA, and 5×10^6 cpm/ml radioactive DNA probe. The filters were washed for 1 h at 48° C in 6 × SSC; 0.1% sodium pyrophosphate, dried and autoradiographed.

DNA sequencing. DNA was sequenced using the dideoxy chain-termination technique (Sanger et al. 1977). DNA was sequenced either as single-strand DNA in the M13 system or as double-strand DNA plasmids. Some regions of the DNA sequence were confirmed or completed using the following synthetic oligonucleotide primers as indicated in Fig. 1: 5′-CATCAACGACATTAC-3′; 5′-GATTTATCAACTTCC-3′; 5′-CTTACATTGTCCATC-3′; 5′-CAAGGTCATTGATGT-3′; 5′-CTTGCTTACATGCCT-3′; 5′-TCCTAGATGCGAGTC-3′; 5′-AGTTAACAAGAACAG-3′.

Computer sequence analysis. All DNA and protein sequence analysis was carried out on a Digital VAX 11/750 computer using the University of Wisconsin Computer Genetics Group program.

mRNA induction experiments with spinach. Spinach (*Spinacia oleracea* var. Dark Green Bloomsdale) was grown in a phytotron in 52 × 26 × 6 cm plastic trays in a mixture of vermiculite no. 2/peat moss/sand (1:1:1) containing all major and minor nutrients necessary for plant growth except for nitrate. Growth conditions were: 8.5 h day at 22° C and 50000 lx, 15.5 h night at 18° C, 54% humidity. The seedlings were fertilized every 3rd day with Peter's Plant Starter (NH_4-PO_2-K: 9−45−15; Peter's Plant Products, Cambridge, Mass) at 1 g/l; 450 ppm N). For induction, 25 mM $Ca(NO_3)_2$ was given at time 0, followed by another application of nitrate after 24 h.

Results

Isolation of a cDNA clone containing the complete protein coding region of nitrite reductase

Nitrite reductase (NiR) was isolated from spinach leaves following the procedure of Vega and Kamin (1977). This protein preparation when analyzed by SDS polyacrylamide gel electrophoresis gave a single polypeptide band of 61000 molecular weight and was at least 90% pure. Antibodies were raised in rabbits against the non-denatured NiR protein. This antibody preparation when incubated with total spinach protein immobilized on a nitrocellulose filter (Western blot) reacted with two polypeptides. One of these was the correct molecular weight for NiR, while the other had an apparent molecular weight of 70000. Several peptide fragments of the purified NiR protein were partially sequenced, and based on their amino acid sequences, three mixed oligonucleotide probes were synthesized (see Material and methods).

A cDNA library was constructed from poly(A)$^+$ RNA from leaves of nitrate-induced spinach and was screened for the presence of NiR-cDNAs with the NiR-antiserum and the mixed oligonucleotide probes. Recombinant phage (200000) were initially screened with the radioactively-labeled mixed oligonucleotide probe oligoNiR1, a 20-mer with 64-fold redundancy (see Materials and methods). To minimize the number of false positives, the filters were washed at 57° C in the presence of 3 M tetramethylammonium chloride (Wood et al. 1985). In theory, under these conditions 19 out of 20 bp would need to be homologous in order to form a stable hybrid. Thirty six plaques showed a hybridization signal on duplicate filters and were rescreened with the mixed oligonucleotide probes oligoNiR2 and oligoNiR3. One plaque that hybridized to all 3 probes was isolated and called pCIB401. In an alternative approach, recombinant phage (120000) were screened with NiR antiserum with 35 plaques showing a positive signal. Nine were randomly chosen and rescreened with the oligonucleotide probes oligoNiR1 and oligoNiR2. Two of the 9 clones hybridized to both oligonucleotide probes confirming the presence of NiR-cDNA. Both clones were isolated and called pCIB400 and pCIB402 respectively. The remaining 7 clones failed to hybridize with the oligonucleotide probes, but all cross-hybridized to each other and hence seem to represent a gene or gene family. This gene probably codes for the 70 kDa protein of unknown function which reacts with the NiR antiserum.

Restriction maps were prepared from the three isolated NiR-cDNA clones (pCIB400, pCIB401 and pCIB402). All three cDNA inserts were found to have an identical restriction enzyme pattern and varied only slightly in length. The map of one of the clones, pCIB400, is shown in Fig. 1. Its entire DNA sequence was determined and is shown in Fig. 2. This cDNA is 2015 bp long and has an open reading frame of 594 amino acids starting with a potential translation initiation codon. The protein sequence derived from the DNA sequence confirmed the presence of all three amino acid sequences determined by direct protein sequencing (underlined sequences in Fig. 2), with the exception of one amino acid. At position 221 the sequenced protein shows a valine rather than an isoleucine. This difference is probably due to a point mutation in the DNA, reflecting allelic variation in the protein between different spinach cultivars.

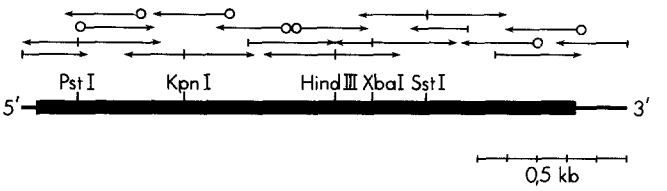

Fig. 1. Restriction map of the nitrite reductase cDNA clone pCIB400. The *wide bar* represents the translated region, the *narrow bar* untranslated regions. The dideoxy chain-termination method of Sanger et al. (1977) was used to determine the DNA sequence. The sequencing strategy is outlined by *arrows*. A *circle* at the base of an *arrow* indicates where a synthetic oligonucleotide primer homologous to the cDNA has been used to prime the sequencing reaction

Neither a poly(A) tail nor a polyadenylation signal resembling the animal consensus sequence AATAAA is in the NiR−cDNA of pCIB400. In the other two NiR−cDNA clones (pCIB401 and pCIB402) where only the ends of the cDNA were sequenced, both the poly(A) tail and the AATAAA sequences were also absent. Moreover, at the 3′-end the three NiR-cDNA inserts end at different locations (see Fig. 2) and it is not possible to postulate where the NiR-mRNA ends in vivo.

Amino acid sequence of the NiR protein

The cloned NiR-cDNA codes for a precursor protein that is 594 amino acids long, with a deduced molecular weight of 66394 daltons. In order to determine the length of the mature protein, the N-terminus of the isolated NiR protein was sequenced. According to the sequencing data, about 25% of the protein started with the cysteine at position 33, 50% with the alanine at position 36 and 25% with the valine at position 37. The most likely interpretation of these data is that the mature NiR protein starts with a cysteine at position 33 (arrow in Fig. 2). The two other start sites are probably artifacts resulting from protease activity that was present during protein isolation with several amino acids being digested away at the N-terminal end.

Starting with the cysteine at position 33 the mature NiR protein is 562 amino acids long and has a deduced molecular weight of 62883 daltons. This value corresponds well with the apparent molecular weight of 61000 daltons observed by us (data not shown) and others (Vega and Kamin 1977; Ho and Tamura 1973). The amino acid composition of the mature NiR protein predicted from the DNA sequence is very similar to that determined for the protein by Vega and Kamin (1977).

The NiR precursor protein is 32 amino acids longer than the mature protein. These additional amino acids precede the mature protein and most likely serve to act as a transit peptide sequence in directing and NiR protein into the chloroplast (see Discussion).

Induction of NiR-mRNA by nitrate

The induction of NiR-mRNA by nitrate was investigated in spinach seedlings that were grown on ammonia as the sole source of nitrogen (see Materials and methods). After approximately 5 weeks of growth (2–4 leaf stage), the induction of NiR-mRNA was initiated by watering the spinach seedlings with 25 mM $Ca(NO_3)_2$ while the control seed-

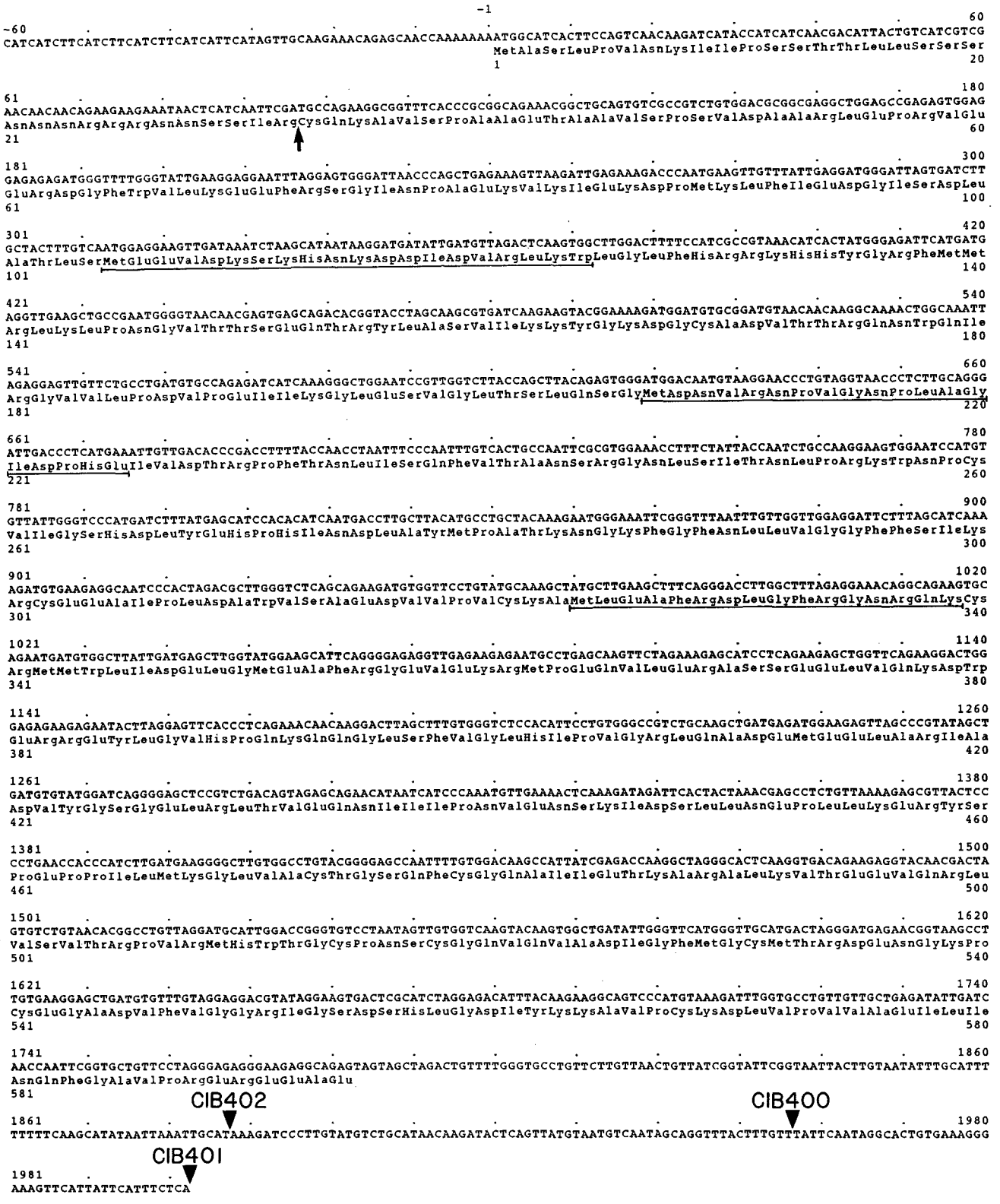

Fig. 2. DNA sequence and deduced amino acid sequence of the nitrite reductase (NiR)-cDNA clone pCIB400. Amino acids confirmed by protein sequencing are *underlined*. At position 221 the sequenced protein shows a valine rather than an isoleucine. The mature NiR protein starts with the cysteine at position 33 (*arrow*). The nucleotide residues from position 1957 to 2002 are from the cDNA clone pCIB401. The 3′ ends of the three cDNA inserts sequenced are indicated by *arrowheads*

lings were only treated with water. The plants were kept under continuous light for 100 h and poly(A)$^+$ RNA was then isolated from its leaves. The RNA was used for a Northern blot analysis with ^{32}P-labeled NiR-cDNA as a probe. The autoradiograph of the Northern blot hybridization is shown in Fig. 3. A single NiR-mRNA of about 2.3 kb in length is present in the poly(A)$^+$ RNA of spinach leaves. The NiR-mRNA band was increased considerably

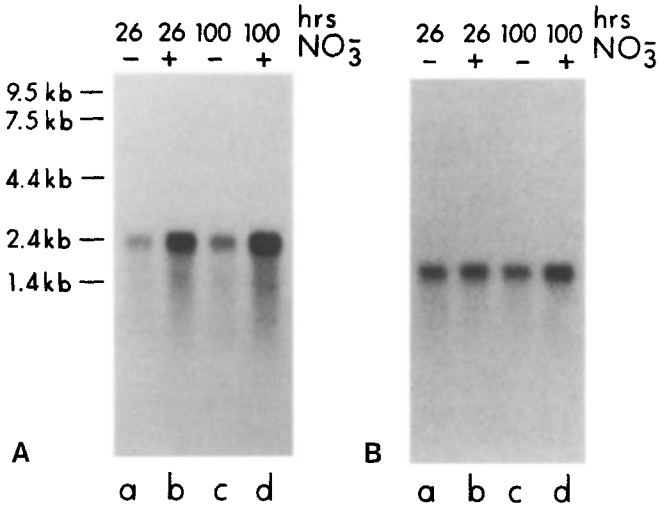

Fig. 3A, B. Induction of nitrite reductase (NiR) mRNA by nitrate. Poly(A)$^+$ RNA (10 μg) from plants treated with nitrate (+) or with H_2O (−) were loaded on a 0.8% agarose gel, electrophoresed and transferred to nitrocellulose. **A** The nitrocellulose filter was hybridized to ^{32}P-labeled insert DNA from the NiR-cDNA pCIB400 (3×10^6 cpm/ml; 2×10^8 cpm/μg) and washed under high stringency conditions in 0.1 × SSC; 0.1% SDS at 60° C. **B** The same filter was rehybridized to ^{32}P-labeled insert DNA of the actin gene pSAc3 (Shah et al. 1982) from soybean (3×10^5 cpm/ml; 10^8 cpm/μg). The filter was washed under low stringency conditions in 2 × SSC; 0.1% SDS at 50° C. Molecular size markers were a RNA ladder from BRL

in the samples that were treated with nitrate for 26 h or 100 h (lanes b, d) as compared to the controls (lanes a, c). A significant amount of NiR-mRNA is, however, present in our uninduced RNA samples. This level possibly reflects a constitutive level of NiR-mRNA in leaf cells. Alternatively, we cannot rule out the possibility that, under the experimental conditions used, trace amounts of nitrate were available to the plants, inducing the NiR-mRNA to a low level. When the same filter was rehybridized with a heterologous actin cDNA probe from soybean all lanes showed roughly the same hybridization signal (Fig. 3B). These results show that nitrate induction leads to a marked increase in the steady-state level of NiR-mRNA after 100 h.

Discussion

Several lines of evidence indicate that we have cloned a cDNA whose nucleotide sequence codes for the entire NiR precursor protein from spinach:

1. The deduced protein sequence coded for by the cDNA contains the three sequences of amino acids obtained by partially sequencing oligopeptides of the NiR protein.
2. The antibodies raised against purified NiR protein cross-reacted with the protein coded for by the isolated cDNAs in λ GTII.
3. The size and the amino acid composition of the mature NiR protein deduced from our cDNA are very close to the values published earlier for spinach NiR (62900 vs 61000; Vega and Kamin 1977; Ho and Tamura 1973).
4. NiR has been reported to be induced by nitrate (Beevers and Hageman 1969). On a Northern blot our cDNA hybridizes to a mRNA that is markedly increased after the seedlings have been treated with nitrate.

We have cloned an almost full length copy of the NiR-mRNA. As determined by Northern blot analysis (Fig. 3), the length of the NiR-mRNA is 2.3 kb while our cDNA is 2,061 nucleotides long. At present we cannot say whether the remaining 240 or so nucleotides are missing from the 5′ end, the 3′-end or from both ends of the NiR-mRNA, since either end is known at this time. Three independent NiR-cDNAs (pCIB400, pCIB401, pCIB402) have been sequenced at their 3′-ends. All three NiR-cDNAs ended at different sites (see Fig. 2). Moreover, none of them had a poly(A) tail nor did they show the consensus animal polyadenylation signal sequence (AATAAA). However, several other plant genes have been reported to deviate from the AATAAA consensus sequence (reviewed in Heidecker and Messing 1986).

NiR is a nuclear-encoded chloroplast protein. Therefore, one would expect that the mature NiR protein would be preceded by a transit peptide directing the protein into the chloroplast. In order to define the transit peptide, we have determined the N-terminal amino acid sequence for the mature NiR protein. The evidence suggests that the cysteine at amino acid position 33 (see Fig. 2) is the N-terminal amino acid. There is only one potential initiation codon upstream of the cysteine in the open reading frame of the NiR-cDNA and this ATG is surrounded by a plant translation initiation consensus sequence (Lütcke et al. 1987). Starting at the upstream methionine, the NiR precursor carries an N-terminal extension of 32 amino acids which almost certainly functions as a transit peptide.

Transit peptides have been found for a number of proteins studied which are transported into the chloroplast (compared in Karlin-Neumann and Tobin 1986; see also Smeekens et al. 1986). Transit peptide sequences have been shown to be necessary and sufficient for the transport of proteins into chloroplasts (Van den Broeck et al. 1985; Schreier et al. 1985; Broglie et al. 1984; Robinson and Ellis 1984). Furthermore, a transit peptide coded for by a gene from one plant can be recognized by chloroplasts from distantly related species (Chua and Schmidt 1978; Coruzzi et al. 1983; Mishkind et al. 1985; Van den Broeck et al. 1985; Schreier et al. 1985; Broglie et al. 1984). Nevertheless, little amino acid homology is seen among transit peptides from different species. A common amino acid framework among nuclear encoded chloroplast proteins has been hypothesized (Karlin-Neumann and Tobin 1986). However, a comparison of this amino acid framework with the transit peptides from NiR and from plastocyanin (Smeekens et al. 1985; 1986) shows very few conserved residues (Fig. 4). The sequences presented for the light-harvesting chlorophyll a/b-protein (LHCP) II and for the small subunit (SSU) of ribulose 1,5-bisphosphate carboxylase are a consensus from 12 and 13 different transit peptides, respectively. Despite some amino acid homology among individual transit peptide sequences, only the two initial amino acids methionine and alanine are strictly conserved in every known chloroplast transit peptide. While all transit peptides known so far are positively charged, this alone cannot determine whether a protein will be transported into chloroplasts, since mitochondrial transit peptides are also positively charged.

Given the primary amino acid sequence for NiR, one can hypothesize which amino acids are involved in the active center of the enzyme. NiR has as cofactors a 4Fe−4S center (Aparicio et al. 1975; Lancaster et al. 1979) and a

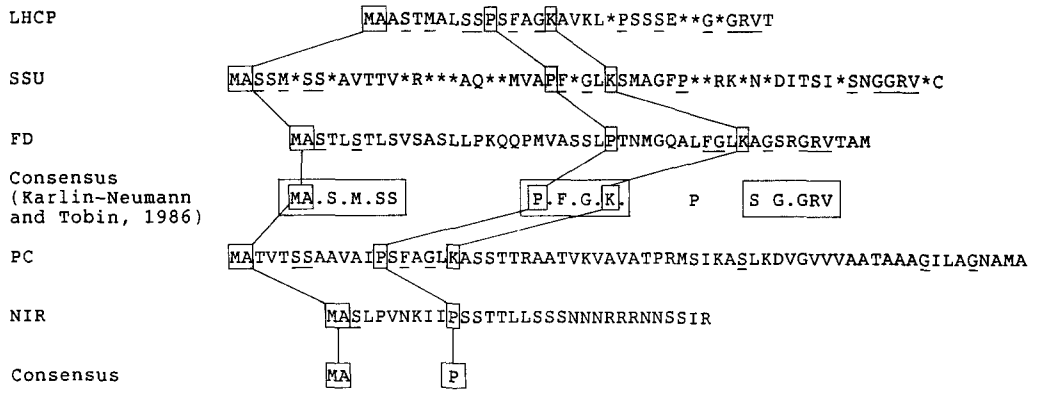

Fig. 4. Comparison of chloroplast transit peptide sequences. The sequence for the light-harvesting chlorophyll a/b-protein (LHCP) II is a consensus of 12 different transit peptides from 5 plant species. The sequences for the small subunit (SSU) of ribulose 1,5-bisphosphate carboxylase/oxygenase is a consensus of 13 different transit peptides from 5 plant species and from *Chlamydomonas*. An *asterisk* indicates a non-conserved residue at the position. (For details see Karlin-Neumann and Tobin 1986.) *Solid lines* enclose conserved residues. *Underlined* residues correspond to those found in the consensus of Karlin-Neumann and Tobin (1986). The sequences of ferredoxin (FD) and plastocyanin (PC) are from Smeekens et al. (1986) and Smeekens et al. (1985), respectively. Nitrite reductase (NIR)

siroheme (Murphy et al. 1974) which are both part of the active center (Cammack et al. 1978). Four cysteine sulfurs are expected to bind the tetranuclear iron cluster, with four bridging sulfur ligands, to the protein. One of these cysteine sulfurs is also probably bound to the siroheme iron (Siegel et al. 1987). The four cysteines in positions 473, 479, 514 and 518 have been shown to be those most likely involved in the binding of the cofactors (McRee et al. 1986; Ostrowski et al. 1987).

Nitrate induction of the expression of the NR and the NiR proteins is well documented for a number of plant species as has been reviewed by Guerrero et al. (1981). However, the mechanism of induction is still unknown. Recently, cDNA clones that encode part of the NR-mRNA have been isolated from barley (Cheng et al. 1986) and squash (Crawford et al. 1986). With the cDNA as probes, both groups were able to show an increase in steady-state NR-mRNA level in response to nitrate induction. In this manuscript, we show that the steady-state level of NiR-mRNA is also increased upon nitrate induction. It still remains to be determined for both genes whether this increase in mRNA is due to changes in transcription rate, altered RNA processing, or decreased mRNA degradation.

The isolated NiR-cDNA clone can be used in the analysis of several important molecular processes. The regulatory elements for NiR can be studied in an attempt to elucidate the mechanism of nitrate-inducible expression of the gene. It should also be possible to see whether in spinach, as well as in other plant species, the same NiR gene is expressed in the leaves as in the roots. Furthermore, the functional dissection of the NiR transit peptide sequence might help in better understanding the mechanism of transport of proteins into chloroplasts. Finally, nitrite reduction has been the object of considerable biochemical interest and it should now be possible through the use of in vitro mutagenesis and expression of this gene in bacteria to delineate the important amino acids for enzymatic function.

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