cDNA Clones for Corn Leaf NADH:Nitrate Reductase and Chloroplast NAD(P)^+ :Glyceraldehyde-3-Phosphate Dehydrogenase

Characterization of the Clones and Analysis of the Expression of the Genes in Leaves as Influenced by Nitrate in the Light and Dark

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ABSTRACT

cDNA clones were selected from a corn (Zea mays L.) leaf lambda gt11 expression library using polyclonal antibodies for corn leaf NADH:nitrate reductase. One clone, Zmnr, had a 2.1 kilobase insert, which hybridized to a 3.2 kilobase mRNA. The deduced amino acid sequence of Zmnr was nearly identical to peptide sequences of corn leaf NADH:nitrate reductase. Another clone, Zm6, had an insert of 1.4 kilobase, which hybridized to a 1.4 kilobase mRNA, and its sequence coded for chloroplastic NAD(P)^+:glyceraldehyde-3-phosphate dehydrogenase based on comparisons to sequences of this enzyme from tobacco and corn. When nitrate was supplied to N-starved, etiolated corn plants, nitrate reductase, and glyceraldehyde-3-phosphate dehydrogenase mRNA levels in leaves increased in parallel. When green leaves were treated with nitrate, only nitrate reductase mRNA levels were increased. Nitrate is a specific inducer of nitrate reductase in green leaves, but appears to have a more general effect in etiolated leaves. In the dark, nitrate induced nitrate reductase expression in both etiolated and green leaves, indicating light and functional chloroplast were not required for enzyme expression.

Higher plant NADH:NR (EC 1.6.6.1) is a molybdenum-flavoprotein with a polypeptide chain of 110,000 (4). Antibodies have been prepared for NR from many plants and have been used to select cDNA clones from λ-gt11 expression libraries (3, 6–8, 10). When NR cDNA clones were used for analysis, NR mRNA was low or absent without nitrate treatment but increased many-fold with nitrate (3, 6–8, 10). Another apparent regulator of NR expression is light (1, 4). In the dark, NR protein and activity are enhanced in etiolated corn leaves by nitrate, but levels remain low until light is given (16). Pulses of red and continuous far-red light, which induce the phytochrome system, enhanced NR, and it appeared that phytochrome mediated light enhancement of NR expression, but only in the presence of nitrate (15). We have isolated a cDNA clone for corn leaf NR and used it to study NR regulation by nitrate in the dark and light. In addition, a cDNA clone for GAPDH has been isolated and the expression of this light-regulated enzyme has been studied in parallel with NR.

MATERIALS AND METHODS

Growth of Plants and Enzyme Assays

Corn plants (Zea mays L. W64A × W182E) were imbibed in deionized H₂O for 24 h, planted in washed vermiculite, and grown at 30°C either in continuous darkness or light. Nitrate treatments consisted of Hoagland solution containing 35 mM NH₄NO₃ (16). Leaves were extracted and NR activity assayed as previously described (16). GAPDH activity was assayed via glyceral acid-dependent NADPH oxidation (11).

Immunoscreening of the Corn cDNA Library and Subcloning of Inserts

A corn leaf cDNA library prepared in the expression vector λ-gt11 was obtained from Dr. Steven Rothstein (CIBA-Geigy, Research Triangle Park, NC), which had been prepared with poly(A)+ RNA isolated from leaves of nitrate-induced corn plants of Funk G454. Escherichia coli Y1090 was infected with the library, lacZ fusion proteins were induced, and positive clones selected by screening with antibodies specific for corn leaf NADH:NR as described (7). Antibody-positive cDNA clones were grown up in minicultures, the DNA was isolated, and the insert was excised with EcoRI (13). Inserts were sized in 1% agarose gels, isolated by electroelution, and subcloned into pUC-12 (13).
DNA and RNA Blots

Insert DNA isolated from the pUC-12 subclones was radioactively labeled with $^{32}$P-dCTP by random primed DNA synthesis (Boehringer Mannheim Biochemicals) and was hybridized to nitrocellulose blots prepared from 1% agarose gels of the x-gt11 cDNA clones using a formamide/SDS protocol (13). RNA was isolated from liquid nitrogen frozen leaf tissue according to Logemann et al. (12) and poly(A)$^+$ RNA was isolated using HYBOND-mAP messenger-affinity paper (Amersham). RNA was denatured in formamide/formaldehyde, electrophoresed in 1% agarose/formaldehyde gels and transferred to nitrocellulose. Formamide/formaldehyde-denatured total RNA was applied to nitrocellulose with a BRL slot blot apparatus. RNA blots were baked in vacuo for 2 h at 80°C and prehybridized, hybridized, and washed as for DNA blots. Autoradiography was at −70°C for varying lengths of time (15).

Sequencing

Inserts of pUC-12 subclones were mapped with restriction endonucleases and appropriate Sst I and Aval fragments were cloned in pUC-12. Nucleotide sequences were determined using Sequenase (U.S. Biochemicals) with double-stranded DNA of pUC-12 clones, which were denatured with NaOH, and a dideoxynucleotide protocol with $^{35}$S-dATP. Corn leaf NADH:NR was purified to homogeneity by immunoaffinity chromatography (4). Amino acid sequence determinations were done by the Biotechnology Unit of CIBA-Geigy, Research Triangle Park, NC. Direct sequencing of the protein yielded no results, which indicated a blocked N-terminal residue. After the protein was cleaved with endoproteinase Lys-C, peptides were separated by reverse-phase high-pressure liquid chromatography and some were sequenced.

RESULTS

Selection of cDNA Clones from a Corn Leaf λ-gt11 Expression Library

Four clones were selected by screening lacZ fusion proteins with monospecific, polyclonal rabbit anti-NR. Two cDNA clones had inserts of 2.1 kb, while the other two had smaller inserts of 1.4 and 0.8 kb. One of the large inserts was subcloned into pUC-12 and designated pZmnrl. The 1.4 kb insert was subcloned into pUC-12 and designated pZm6.

Characterization and Nucleotide Sequencing of pZmnrl

Southern blots showed that the two 2.1 kb inserts cross-hybridized, whereas neither of the smaller inserts hybridized to pZmnrl (data not shown). A Northern blot of poly(A)$^+$ RNA isolated from nitrate-induced corn leaves had a band at about 3.2 kb which hybridized to radioactively labeled pZmnrl insert (Fig. 1). This RNA could not be detected in poly(A)$^+$ RNA isolated from leaves of plants not given nitrate. This is the size of mRNA expected for corn leaf NR which has a 110 kD polypeptide and about the same size as found for mRNAs hybridizing to NR clones from other plants (3, 4, 6–8). The DNA sequence of Zmnrl is presented in Figure 2 along with the amino acid sequence deduced from its open reading frame, which contains 617 residues. The amino acid sequence of corn leaf NADH:NR was partially determined and these sequences are underlined in Figure 2. The only differences found between the deduced sequence and the peptides were in two residues where the cDNA coded for an amide residue and the peptides contained an acid residue, which may be explained by hydrolysis of these amides to acids during the purification of the corn leaf NR since it involved elution from the immunoaffinity column at pH 11 (4). Thus, pZmnrl is an authentic corn leaf NADH:NR cDNA clone. However, pZmnrl represents only about two-thirds of the complete NR sequence based on the full-length clone isolated for Arabidopsis NR with a deduced amino acid sequence of 917 residues (8).

Partial Nucleotide Sequencing of Zm6

The 5' and 3' termini of Zm6 were sequenced (Fig. 3). Deduced amino acid sequences were compared to the Protein Sequence Database of the Protein Identification Resource, which revealed Zm6 coded for a GAPDH with a signal peptide. A Northern blot confirmed that pZm6 insert hybridized to a 1.4 kb mRNA (Fig. 1), which is the size mRNA expected for corn chloroplastic GAPDH with a reported precursor polypeptide of 45 kD (5). Furthermore, by comparing
Transit Peptide Sequence (66 Residues for corn)

Zm6  Met Ala Ser Ser Met Leu Ser Ala Thr Val Pro Leu Gin Gin Gly
  ggtcgtgacg ATG GCC TCG ATG CTC ACC GCT ACC GTG CCA CTC CAG CAG GGG
  AAG TCT TCT TAG GTC AGC AAC
Tobacco GapA (Partial Sequence)
  Asn Ser Ser Leu Gin Val Ser Asn
  Gly Gly Leu Ser Gly Leu Arg Ser Ser Ala Ser Leu Pro Met Arg Arg Asn
  GCC GCC CTG TTC GAG TTC GGG CTT CAG ACC GCC TCG ATG CCA GCG CGG
  AAA CTA CTA T A A G GCC C T C AT A GTC A T A C T G A A A
  Lys Phe Thr Ser Ala Ile Phe Gly Lys
  Ala Thr Ser Asp Asp Phe Met Ser Ala Val Ser Phe Arg Thr His Ala Val Gly Thr Ser
  GCC ACC GCC AGC TTC ATG GCC GTC TCC ATC ACC GAC GCC GTC ATC AGC
  A T T --- T G C C T T T T GCC TCAA TCT TAT A --- GA
  Thr Asn
  Leu Leu Val Ala Gln Ser Val Ile Gly
  Gly Gly Pro Arg Ala Pro Thr Glu Ala
  GCC GCC CCC CGG GCC GCC CGG AGC GAG GCA
  G AAC AGC AA A GA GTA GT C
  Asn Ser Lys Gly Val Val

Amino Terminal Sequence (Residues 1 to 59)
  Lys Leu Lys Val Ala Ile Asn Gly Phe Gly Arg Ile Gly Arg Asn Phe Leu Arg Cys Trp
  AAG CTG AAG GTG GCC ATC AAC GGG TTC GCC ACC ATC GCC AAG TCT CTG GGC TCG
  T A A T A T A A G T G T T T T T A T
  His Gly Arg Gly Asp Ala Ser Pro Leu Asp Val Ile Ala Ile Asn Thr Gly Val
  CAC GCC GCC GCC GCC TCG CCC GTC GAC GTC GCC ATC ACC GAC ACC GGA GCC GTC
  T T A G AAA --- T T T T T T T T
  Lys Gln Ala Ser His Leu Leu Lys Tyr Asp Ser Thr Leu Gly Ile Phe Asp Ala Asp
  AAG CAG GCC CCC CTG CTC ATG AGC TAC GCC TCC GAG GTC TTC GCC ACC ATC GCC
  A C T T T T G C T T A G T T T T T T
  Carboxyl Terminal Sequence (Residues 234 to 337) and 3' Untranslated Region
  Arg Val Pro Thr Pro Asn Val Ser Val Val Val Ser Leu Leu Val Val Gin Val Ser Lys Thr
  CGG GTG CCC ACC CGG AAC GTC TCC GTC GTC GAC CTC GTC CTG GAC CAG GCT
  A A G ACC T A T G T T T T C A A
  Leu Ala Glu Glu Val Asn Glu Ala Ala Asn Leu Thr Gly Ile CTC GCC GAG GAG GTG AAC CAG GCC TCC GCC GCC GAC ACC GAC ATC
  T T T A T G C T A T A G G T T A G A A T
  Phe Lys
  Leu Glu Val Cys Asp Val Pro Leu Val Asp Phe Arg Cys Ser Asp Ser Val Ser
  CTC GAG GTC TGC GCC GTG CCG CTC GTG TCT GAA GCC TGC GTC TTC GCC
  T T T AA A A A C AGT T G A A
  Glu
  Thr Ile Asp Ala Ser Leu Thr Met Val Met Gly Asp Met Val Ile Ser Trp
  ACC ATC ACC GCC TGC CTC ACC ATG GCC ATG GCC ATG GCC GAC ATC ATC TCG
  T G T T T A A T A T A T T T T G T
  Ala
  Tyr Asp Asn Glu Trp Gly Tyr Ser Gin Arg Val Ala Asp Leu Ala Ala Asn
  TAC GCC AAC TGG GCC TTC ATG CAG CTC GAT GTC GAC GTC CTC ATC CAC GCC
  T T A T A A G T T T T G T T T A
  Glu Trp Lys
  CAG TGG TAC GGG GAT CTT ATG GAT GTT GAT TAT TAT CTT
  Glu
  Figure 2. Nucleotide sequence of Zmnrl and partial corn NR amino acid sequence compared to the sequences of the corn NADH:NR peptides, chicken Cyt Dβ and human NADH:Cyt b reductase. Sequences of corn NADH:NR peptides are underlined and were identical to the deduced amino acid sequence except at residues 443 and 556, which were Asp and Glu, respectively, in the peptides. Gaps placed in the sequences to maximize homology between chicken Cyt Dβ and human NADH:Cyt b reductase are indicated by . . . , except at residues 133 to 139 of the reductase sequence where it has 6 more residues than NR, which are not shown (14, 20). In the 3' untranslated nucleotide sequence of Zmnrl, a putative polyadenylation signal is underlined.

Figure 3. Partial nucleotide sequence of Zm6 and partial corn chloroplastic GAPDH amino acid sequence compared to the sequences of tobacco GapA. Where the corn and tobacco sequences are identical, spaces were placed in the tobacco sequence such that only differences are shown (18). Bases and amino acid residues missing from one of the sequences as compared to the other are indicated by --. Putative polyadenylation signals are underlined.

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the sequences of Zm6 to those for tobacco gapA, gapB, and gapC sequenced by Shih et al. (18), it was possible unequivocally to identify pZm6 as a cDNA clone for a chloroplast NAD(P)H:GAPDH (Fig. 3). After we had isolated and partially sequenced Zm6, the sequence of corn leaf chloroplastic NAD(P)+:GAPDH was published for a cDNA clone designated Zm57 (2). The partial sequence of Zm6 is identical to that reported for Zm57 except Zm6 codes for the full signal peptide and Zm57 has 86 bases more in the 3′ untranslated region (2). However, Zm6 has a poly(A) tail and an apparent polyadenylation signal (which is underlined in Fig. 3), and thus appears to code for a near full-length clone of corn leaf chloroplastic GAPDH. From the combined results on Zm6 and Zm57 (2), corn chloroplast GAPDH mRNA appears to have two polyadenylated forms. Several other plant genes have been found to be polyadenylated at multiple sites (9). Since there is no indication that the anti-NR antibodies used here are contaminated with antibodies for GAPDH (16), it appears that NR and GAPDH may have an epitope in common. However, since their amino acid sequences are not similar to any significant degree, this conclusion is only tentative.

Expression of NR and GAPDH

In etiolated leaves, NR activity was not detected initially, while NR mRNA and GAPDH mRNA and activity levels were low (Table I). When nitrate was given and the plants kept in the dark, NR and GAPDH mRNA levels increased in parallel, while the activity levels increased to a smaller degree. When nitrate was given in the light, NR mRNA increased by about eight-fold during the first 2 h and reached its highest level at 12 h (Table I). NR activity had a slower increase and did not reach its maximum until 24 h. GAPDH mRNA level also increased about eight-fold in 2 h and peaked at 12 h, while GAPDH activity increased more slowly reaching its highest level at 24 h (Table I). Thus, for etiolated plants given nitrate in the dark or light, the patterns of NR and GAPDH expression were very similar.

In green leaves, NR mRNA and activity were low initially (Table II). When nitrate was given in the dark, NR mRNA increased 6-fold by 4 h, but decreased thereafter. NR activity lagged behind and reached its highest level at 8 h with a 7-fold increase. In contrast, GAPDH mRNA and activity levels were initially high and changed only slightly during the nitrate treatment in the dark (Table II). In the light, NR mRNA increased 6-fold during the first 2 h of nitrate treatment and reached its highest level at 4 h (Table II). NR activity again lagged behind and reached its highest level at 8 h with a 15-fold increase. GAPDH mRNA increased only slowly in the light, reaching a 4-fold higher level at 12 h and GAPDH activity increased only slightly. Thus, for green plants given nitrate in the dark or light, the patterns of NR and GAPDH expression were very different.

DISCUSSION

cDNA clones for higher plant NR have been isolated and characterized (3, 6–8, 10). However, the cDNA clone for corn leaf NADH:NR described here is the first one to be authentified by comparison to amino acid sequences of the enzyme (Fig. 2). The 617 residues of corn NR now known are 74.6% identical with tobacco NR and 72.4% identical with Arabidopsis NR, while the dicot NR forms are 80.5% identical in this region of their sequences (3, 8, 10). Major differences between corn and dicot NR are found in the nucleotide sequences, which are reflected in codon usage. ZmnrI uses almost exclusively codons ending in G and C (90%), while the coding sequences for the dicot NR do not show this codon bias. This codon bias is also found for corn GAPDH clones and is illustrated clearly in the comparison of the partial nucleotide sequence we have determined for Zm6 and the corresponding sequence of tobacco gapA (Fig. 3). Codon bias is found in some genes of monocots, but not all, and may be characteristic of specific classes of genes (2).

Although the cDNA clone obtained for corn NADH:NR is not full-length, functionally significant homology in amino acid sequence between NR and two mammalian proteins, which has been noted in other studies of NR cDNA clones (3, 8, 10), can be recognized for corn NR (Fig. 2). An approximately 80 amino acid residue region near the middle of the corn NR sequence (residues 250–330) has 41 to 47% identity with the amino acid sequences of mammalian Cyt b5 (14). The carboxy-terminal sequence of corn leaf NADH:NR (residues 350–617) has 45.5% identity with mammalian NADH:Cyt b5 reductase (EC 1.6.2.2), a flavoprotein (20).

Regulation of NR by its substrate nitrate and the influence of light on this process have been studied by this laboratory and others (1, 14, 16). The studies focused on NR activity changes until recently when immunochemical methods were used to evaluate NR protein (4, 16). The cloning of NR led to analysis of NR mRNA levels in nitrate-induced and uninduced plants (3, 6–8, 10). The data presented here analyze NR induction by nitrate with respect to time and these kinetic patterns provide more detail on the process (Tables I and II). In addition, the availability of a GAPDH clone allowed us to study an important carbon metabolism enzyme’s expression in parallel with a key enzyme of nitrogen metabolism. We had expected that GAPDH expression might be slower than NR when etiolated leaves were greening in the light, since the induction of GAPDH by light in dark-adapted tobacco leaves was slower for chloroplast forms versus the cytosolic form (17). But we found that NR and chloroplastic GAPDH increased in parallel (Table I). Especially interesting was the induction by nitrate of both NR and GAPDH in the dark in etiolated leaves, where we had expected to find specific enhancement of NR. This effect of nitrate on both nitrogen and carbon metabolism enzymes may be due to the change in availability of nitrogen to previously N-limited tissue as was observed for several other carbon metabolism enzymes in corn leaves (19). Nitrate was a specific inducer of NR in green leaves where GAPDH had already been expressed during greening, but GAPDH expression was enhanced at longer times (Table II). These results reinforce the previous observation that nitrogen may be a regulatory factor in expression of carbon metabolism enzymes (19).

A central question in the regulation of NR expression is the role of light. We have recently shown that the phytochrome system appears to mediate light regulation of NR
chloroplasts  

expression in squash cotyledons, but only when nitrate was present (15). The results presented here reinforce the concept of nitrate as the dominant factor in controlling the level of NR mRNA. However, the levels of NR activity are lower in the dark than light, which indicates that light and/or functional chloroplasts may play a role in production of enzyme activity. Since the levels of Mo-pterin in leaves are altered only by nitrate and not light (4), the availability of the other cofactors (flavin and heme-Fe) and/or the stability of the enzyme may be influenced by light.

ACKNOWLEDGMENTS

We thank Drs. Peg Redinbaugh, Ron Cannon, and other members of Professor John Scandalios’s laboratory, Genetics Department, North Carolina State University, for assistance with this study. Dr. Steven Rothstein, Department of Molecular Biology, University of Guelph, Ontario, Canada, is gratefully thanked for his help with the cloning of nitrate reductase.

LITERATURE CITED


Table I. For Etiolated Corn Leaves, Time Courses of Changes in NR and GAPDH mRNA Levels and Activities after Treatment with Nitrate in Dark and Light

Six d after imbibition, etiolated plants were given Hoagland solution containing 35 mN NH4NO3 and maintained in darkness or transferred to light. Leaves were harvested at various times and extracts assayed for NR and GAPDH activities. Total RNA was isolated from frozen leaf tissue and hybridized to radioactively labeled inserts of pPmaw and pPm6 using separate slot blots. For NR, 2 and 8 μg of total RNA were used for each time point; 0.5 and 2.5 μg of total RNA were used for GAPDH. The experiment was done twice and representative results are shown.

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<th>Activity D*</th>
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* D and L are used to designate the plants treated with nitrate in the dark and light, respectively.  
  a Percentages of mRNA are expressed relative to the highest level in the light as determined by densitometry of the autoradiograms.  
  c Units for activities are: NR, nmol nitrite produced/min and GAPDH, μmol NADPH oxidized/min.
light control of mRNAs coding for angiosperm glyceraldehyde-3-phosphate dehydrogenases. Proc Natl Acad Sci USA 79: 7624-7628