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, Zmnrl, had a 2.1 kilobase insert, which hybridized to a 3.2 kilobase mRNA. The deduced amino acid sequence of Zmnrl was nearly identical to peptide sequences of corn leaf NADH:nitrate reductase. Another clone, Zm5, 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^2 (EC 1.6.6.1) is a molybdohemoflavoprotein 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 imibed in deionized H_2O 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 NH_4NO_3 (16). Leaves were extracted and NR activity assayed as previously described (16). GAPDH activity was assayed via glyceric 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).

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2 Abbreviations: NR, nitrate reductase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.13); kb, kilobase pair.
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 \(\lambda\)-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 SstI 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 \(\lambda\)-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 pZmrn1. The 1.4 kb insert was subcloned into pUC-12 and designated pZm6.

Characterization and Nucleotide Sequencing of pZmrn1

Southern blots showed that the two 2.1 kb inserts cross-hybridized, whereas neither of the smaller inserts hybridized to pZmrn1 (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 pZmrn1 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 Zmrn1 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, pZmrn1 is an authentic corn leaf NADH:NR cDNA clone. However, pZmrn1 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 chloroplast GAPDH with a reported precursor polypeptide of 45 kD (5). Furthermore, by comparing
Transit Peptide Sequence (66 Residues for corn)

Zm6

\[
ggtcgtgccgct ATG GCC TCG TCC ATG GCC TCG TCC ACC ACC GTG CCA CTC CAG CAG GGG
AAG ACC TCT TCT AG GTC AGC AAC
\]

Tobacco GapA (Partial Sequence)

\[
AAn Ser Ser Leu Gin Val Ser Asn
\]

Gly Gly Leu Ser Glu Phe Ser Gly Leu Arg Ser Ser Ala Ser Leu Pro Met Arg Arg Asn
GGC GCC CTG TCC GAG TCT GCC CAG GCC TCG TCT GGG TCG CCA ATG GCC CAG GGG
AAA A C T A T A G C A T A C T G C A
A Lys Phe Thr Ser Ala Ile Phe Gly Lys

Ala Thr Ser Asp Asp Phe Ser Met Ser Ala Val Ser Phe Arg Thr His Ala Val Gly Thr Ser
GCC ACC TCC GCC ACC TCC GCC TCG TCC ACC GCC TCC ACC ACC GCC TGG GCC TGG
A T T --- T C G C T T T T G C C T C A A T C T A T A --- G A
Thr Asn --- Leu Leu Val Ala Gin Ser Val Ile Gly

Gly Gly Pro Arg Ala Pro Thr Glu Ala

GGG CCC CGG CCG GCC CCG ACC GAC GAA
G AAC AGC AA A GA GTA GT
Asn Ser Lys Gly Val Val

Amino Terminal Sequence (Residues 1 to 59)

Lys Leu Lys Val Val Ala Ile Asn Gly Phe Gly Arg Ile Gly Arg Asn Phe Leu Arg Cys Trp
AAG CTG AAC ATG GCC ACC ACC GCC ACC ACC ACC GCC ACC GCC ACC ACC ACC CTG GCC TGG
T A A T A T A A G T A T T A T

His Gly Arg Gly Asp Ala Ser Pro Leu Asp Val Val Ala Ile Asn Thr Gly Val
CAC GCC GCC GCC GCC GCC GCC TCG CAC GTC ACC GTCACC ACC ACC GCC GCC GCC GCC ACC ACC GCC
T T A G A A A --- T T T T T T T Lys

Lys Gin Ala Ser His Leu Leu Lys Tyr Asp Ser Thr Leu Gly Ile Phe Asp Ala Asp
AAG CAG GCC TCA CTG CTC ACC TAG GAC GCC ACC GCC ACC GCC ACC GCC GCC GCC GCC
A C T T T T T T T C T T Lys

Carboxyl Terminal Sequence (Residues 234 to 337) and 3' Untranslated Region

Arg Val Pro Thr Pro Asn Val Pro Ser Val Val Asp Leu Val Val Gin Val Ser Lys Thr
CGG GTG CCC ACC ACC ACC GCC ACC GCC ACC ACC ACC GCC ACC GCC ACC ACC ACC GTG GCC TGG
T A T G T T T C A A A

Leu Ala Glu Glu Val Asn Gin Ala Phe Arg Asp Ala Ala Ala Asn Gin Leu Leu Thr Gly Ile
CTC GCC GAG GAG GTG ACC CAG GCC CTC GCC GCC GCC GCC GCC GCC ACC GCC GCC GCC GCC
T T T A T G C T A T A G G T T T G A
Phe Lys

Leu Glu Val Cys Asp Val Pro Leu Val Asp Phe Arg Cys Ser Asp Ser Val Ser
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T T A A A A A A A C A G T G T G A A
Glu

Thr Ile Asp Ala Ser Leu Thr Met Val Met Gly Asp Asp Met Val Lys Val Ile Ser Trp
ACC GCC GCC TCC ACC ACC ATG GCC ACC GCC ACC ACC GCC GCC ACC ACC GCC GCC GCC GCC
A T T T A T T T T A T T A T T T T G T T A A T Ala

Tyr Asp Asn Glu Trp Gly Tyr Ser Gin Arg Val Asp Leu Ala Asp Ile Cys Ala Asn
TAC GAC AAC GAG TGG GCC GCC GCC GCC GCC GCC GCC GCC GCC GCC GCC GCC GCC GCC GCC
A T T A A A A A G T T T T T G T T A A A A Ala

Gln Trp Lys

CAG TGG AGG tgaagctgtcatggttaaataaaagactttgaatcctggtcttcagactttgtaatcctggtcttcagaggtttgggat--cac
A atc gaa aac g c t g t a t t t c t g t a c t t c t a c t c
ctcagcgtcggcgttcctcggtggctgctgtcggaatccggtggaatctggttccttcggtggtctgcttacctgtctgtcgtggtctggtttgatg
A t t t t t t t t t t t t t t t t t t t t t t t g g g g g t t c

Figure 2. Nucleotide sequence of Zmnf1 and partial corn NR amino acid sequence compared to the sequences of the corn NADH:NiF peptides, chicken Cyt b5, and human NADH:Cyt b5 reductase. Sequences of corn NADH:NiF 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 b5 and human NADH:Cyt b5 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 Zmnf1, 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.
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 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 authenticated 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 b_{5} (14). The carboxy-terminal sequence of corn leaf NADH:NR (residues 350-617) has 45.5% identity with mammalian NADH:Cyt b_{5} 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 chloroplastic 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.
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

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* D and L are used to designate the plants treated with nitrate in the dark and light, respectively.  
* Percentages of mRNA are expressed relative to the highest level in the light as determined by densitometry of the autoradiograms.  
* Units for activities are: NR, nmol nitrite produced/min and GAPDH, μmol NADPH oxidized/min.

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

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* D and L are used to designated the plants treated with nitrate in the dark and light, respectively.  
* Percentages of mRNA are expressed relative to the highest level in the light as determined by densitometry of the autoradiograms.  
* Units for activities are: NR, nmol nitrite produced/min and GAPDH, μmol NADPH oxidized/min.

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 expression (Tables I and II). We found that NR mRNA increased to high levels in the dark when either etiolated or green leaves were given nitrate. Thus, neither light nor functional chloroplasts are absolutely required for obtaining a high 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.

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LITERATURE CITED

light control of mRNAs coding for angiosperm glyceraldehyde-3-phosphate dehydrogenases. Proc Natl Acad Sci USA 79: 7624–7628