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Molecular Cloning of Complementary DNA Encoding Maize Nitrite Reductase

MOLECULAR ANALYSIS AND NITRATE INDUCTION

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ABSTRACT

Complementary DNA has been isolated that codes for maize nitrite reductase (NiR) by using the corresponding spinach gene (E Back *et al.* 1988 Mol Gen Genet 212:20–26) as a heterologous probe. The sequences of the complementary DNAs from the two species are 66% homologous while the deduced amino acid sequences are 86% similar when analogous amino acids are included. A high percentage of the differences in the DNA sequences is due to the extremely strong bias in the corn gene to have a G/C base in the third codon position with 559/569 codons ending in a G or C. Using a hydroponic system, maize seedlings grown in the absence of an exogenous nitrogen source were induced with nitrate or nitrite. Nitrate stimulated a rapid induction of the NiR mRNA in both roots and leaves. There is also a considerable induction of this gene in roots upon the addition of nitrite, although under the conditions used the final mRNA level was not as high as when nitrate was the inducer. There is a small but detectable level of NiR mRNA in leaves prior to induction, but no constitutive NiR mRNA can be seen in the roots. Analysis of genomic DNA supports the notion that there are at least two NiR genes in maize.

Under most soil conditions, the reduction of nitrate to ammonia is the main source of reduced nitrogen for the biosynthesis of amino acids and other nitrogenous compounds in plants (15). The assimilatory pathway consists of three enzymic steps: NR[3] (EC 1.6.6.2), which reduces nitrate to nitrite; NiR (EC 1.6.6.4), which reduces nitrite to ammonia; GS (EC 6.3.1.2), which incorporates the ammonia into glutamate to make glutamine. NR has been localized in the cytoplasm, while NiR is found in the chloroplasts in green tissue and in proplastids or etioplasts in nongreen tissue (see 5 for a review).

NR and NiR activities have been shown to be induced by the addition of nitrate in a variety of plants and plant tissues (see 11 for a review). Furthermore, cDNA clones coding for NR isolated from several plant species have been used to demonstrate that the NR mRNA levels are increased with the addition of exogenous nitrate in barley (7), squash (8), and tobacco (6). Likewise, using a cDNA clone coding for spinach NiR, it has been demonstrated that in spinach the level of NiR mRNA also increases in the presence of nitrate (3). An *in vitro* translation assay has been used to demonstrate that the level of translatable NiR mRNA increases with the addition of nitrate in wheat (33) and pea (12).

Other environmental factors have been found to affect the levels of NiR in plant tissue. These include nitrite (2), light (28, 30), water stress (14, 16), and treatment with cytokinins (20, 31). It is generally assumed that the nitrate level is the primary inducer of NiR (and NR) expression, with the other environmental stimuli acting as modifiers of the expression of these genes.

NiR catalyzes one of the few known biological 6 e-transfer reactions during which no intermediates are released. Reduced ferredoxin is the physiological e-donor, contributing one e- at a time. NiR contains two prosthetic groups in close proximity, a 4Fe-4S center that appears to be the initial e- acceptor and a siroheme to which nitrite binds. Between one and three NiR isozymes have been reported in different plant species (13 and references therein). In maize two isozymes have been detected, although in one report the second isozyme was found in nongreen tissue only (9), while in the other report the second isozyme was found in green tissue only (19). This variability may reflect differences among maize cultivars.

In this report, we describe the isolation of maize NiR cDNA clones using the spinach cDNA clone as a heterologous probe. The DNA sequence has been determined and the ascribed amino acid sequence compared to the spinach gene. Initial studies on the induction of this gene at the mRNA level by nitrate and nitrite are described, and the copy number of this gene in the maize genome is determined.

MATERIALS AND METHODS

Plant Growth Conditions. Maize seeds (*Zea mays* Funk inbred 6N603) were germinated in distilled water-soaked germination paper in the dark for 3 to 4 d. Seedlings having roots approximately 4 cm long were inserted into slits cut into packing foam (Fidelity Products Co.). The seedlings were grown hydroponically by floating the foam pads on the surface of the media. The seedlings were grown with no added nitrogen source and were then induced with nitrate (20 mM $CaNO_3^-$, 10 mM MES, pH 5.8 [22]) when 10 to 12 cm tall. Circulation was provided by a submersible pump (Little Giant Series One). The plants were grown under a 16-h light/8-h dark light regime.

Isolation of cDNA Clones and DNA Sequencing. Double-stranded cDNA was made from poly A^+ RNA isolated from nitrate-induced maize leaf material (*Zea mays* Funk line G450 grown in a vermiculite/sand mixture) using the method of Okayama and Berg (26). EcoRI linkers (New England Biolabs) were added, and the cDNA was cloned into λGT11 (36). Nitrocellulose filter duplicates of the phage cDNA clones were hybridized

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[3] Abbreviations: NR, nitrate reductase; NiR, nitrite reductase; GS, glutamine synthetase.

with the nick-translated spinach NiR cDNA clone pCIB400 (3) in 50% formamide, 2 × standard saline citrate (SSC), 0.2% SDS, and 5 mM EDTA (21) at 42°C. The filters were washed in 2 × SSC, 0.2% SDS, and 5 mM EDTA at 50°C and subjected to autoradiography. Positive plaques were purified and the inserts subcloned into pUCI9 (New England Biolabs). DNA sequencing was carried out using the dideoxy chain termination method (29) using either single-stranded M13 DNA or double-stranded plasmid DNA as template. The entire sequence was confirmed by sequencing both strands.

RNA Isolations and RNA Blot Analysis. Plants were harvested, fast frozen in liquid N_2, and stored at −90°C. Five grams of either leaves or roots were added to 10 mL of extraction buffer (50 mM Tris-HCl, pH 8.0, 4% sodium *p*-aminosalicylate, 1% sodium 1,5-naphthalenedisulfonate), and 10 mL of buffer-saturated phenol. The mixture was homogenized with a Brinkman polytron and then shaken for 20 min at 300 rpm. After the addition of 10 mL of chloroform, the mixture was shaken for an additional 10 min prior to centrifugation at 7000 rpm in an SS34 rotor (Sorvall). The aqueous phase was reextracted with 10 mL of chloroform and made 2 M with LiCl. After an overnight precipitation at 4°C, the RNA was sedimented in an SW41 rotor (Beckman) at 25 K for 2 h. The RNA was resuspended in 1% SDS, 5 mM EDTA, 20 mM NaOAc, 40 mM Tris-HCl, pH 7.5, and was precipitated with ethanol. The resulting pellet was resuspended in dH_2O and reprecipitated with ethanol. The pellet was again resuspended in dH_2O and the concentration of the RNA determined spectrophotometrically. The quality of the RNA was assessed by electrophoresis on a 1.0% agarose gel and examination of the intactness of the ribosomal RNA by EtBr staining. Twenty μg of total leaf RNA and 10 μg of total root RNA were subjected to electrophoresis through a 1.2% agarose, 2.2 M formaldehyde gel, and the RNA was blotted onto nitrocellulose. The probe used for hybridization was an isolated fragment from pCIB801 containing 1579 base pairs of the maize NiR cDNA sequence. Hybridization conditions were the same as for the isolation of the cDNA clones. The filters were washed in 0.1 × SSC, 0.1% SDS at 55°C.

DNA Isolation and DNA Blot Analysis. Maize DNA from the inbred lines was obtained from S. Evola. Five micrograms of DNA were digested with restriction enzymes and subjected to electrophoresis in a 0.6% agarose gel. The hybridization conditions used were those of Klessig and Berg (18), with the filters being washed under the same conditions used for the RNA blot hybridizations.

RESULTS

NiR cDNA Sequence and the Deduced Amino Acid Sequence. An amplified cDNA bank made from maize leaf poly A^+ RNA cloned into λGT11 (36) was screened for plaques hybridizing to the spinach NiR cDNA clone (3). Fourteen hybridizing plaques were isolated out of the 270,00 screened. Positive plaques were purified and the longest cDNA inserts cloned into pUC19. The entire nucleotide sequence of one of the two largest inserts (pCIB808) was determined and is shown in Figure 1. The portions of the other cDNA clone sequenced were identical to the corresponding regions of pCIB808. The cDNA insert in pCIB808 is 1850 base pairs long, which is approximately 150 bases shorter than the mRNA. The sequence is 66% homologous to the spinach NiR cDNA sequence, with this similarity extending virtually throughout the length of the protein coding sequence. The G/C content of the maize cDNA clone is high (69.5%) compared to the spinach gene (46%). In the maize gene, 559 codons end in G or C while only 10 end in A or T. In contrast, the spinach gene has 264 codons ending with G or C and 330 ending with A or T.

NiR has been found to be localized in chloroplasts in green tissue and the plastid fraction in roots (see 5 for a review). As expected, the spinach protein has a leader peptide at its N-terminal end, which presumably is involved in the transport of this protein into chloroplasts (3). By analogy to the spinach gene, the maize NiR cDNA clone pCIB808 codes for the entire mature protein coding sequence (Figs. 1 and 2). (It was not possible to obtain N-terminal sequence information for the maize NiR protein.) The spinach protein is cleaved after an Arg residue which is also present in the maize sequence. The coding sequence for the rest of the chloroplast signal peptide is not present in this clone. The amino acid sequence of the mature maize NiR protein shows an 86% similarity with the spinach protein when analogous residues are included. When only identical residues are included the protein sequences have a 75% similarity. For the first 21 amino acid residues at the N-terminal end of the spinach and maize mature proteins, there is considerably less similarity.

There are two prosthetic groups at the active site of NiR, a 4Fe-4S center (1) and a siroheme (25). It has been shown that four cysteine sulfurs 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 (32). It could be shown that the four cysteines in positions 473, 479, 514, and 518 of the spinach NiR sequence are almost certainly involved in the binding of the cofactors (23, 27). As can be seen in Figure 2, these cysteines are conserved in the maize sequence in exactly the same location in the protein.

Gene Copy Number. In order to determine the copy number of the NiR gene in maize, DNA from five different highly induced inbred lines was digested with either *Bgl*II or *Hind*III, neither of which cleaves within the NiR cDNA clone. DNA blot hybridization analysis of this DNA using the maize NiR cDNA clone as the probe is shown in Figure 3. As can be seen, three of the five inbred lines have two hybridizing fragments in both digests, while the 232 line has a single *Bgl*II band and two *Hind*III fragments. Only the 32 line has a single hybridizing band in each digest. However, when the DNA was digested with EcoRI each of the lines had at least two hybridizing fragments (data not shown). The simples explanation for these results is the presence of two NiR genes per haploid genome. This would correlate with the presence of two NiR isozymes in maize. It is still not possible to eliminate the possibility that there is a single gene coding for NiR, since it is possible that all of the restriction enzyme cleavage sites tested could be in parts of the gene not present in the cDNA clones. Furthermore, it is possible that some maize lines might possess a single gene. However, preliminary results in analyzing NiR genomic clones support the presence of two different genes (our unpublished results).

Induction of the NiR Gene by Nitrate and Nitrite. Maize seedlings were grown hydroponically 7 to 10 d postgermination in the absence of nitrate. Under these conditions in the absence of an exogenously supplied nitrogen source, the plants grow with no outward signs of stress for approximately 14 to 17 d postgermination. Control plants were harvested just prior to the addition of nitrate to a final concentration of 20 mM. The nitrate-induced plants were harvested 90 min after the nitrate was added. RNA was isolated from the induced and uninduced leaves and roots. Using the cDNA clone as a probe, the amount of NiR mRNA was analyzed as shown in Figure 4. In the roots there is generally no detectable NiR message under the experimental conditions utilized prior to the addition of nitrate and there is a considerable induction. In the leaves, a small but reproducible amount of NiR mRNA can be detected in uninduced plants. Even so, there is a large and rapid induction of this gene at the mRNA level in the presence of nitrate. The nitrocellulose filters were reprobed with a soybean actin cDNA probe to ensure that an equal amount of mRNA was present in each lane (data not shown).

In a separate experiment, the plants were induced with 20 mM

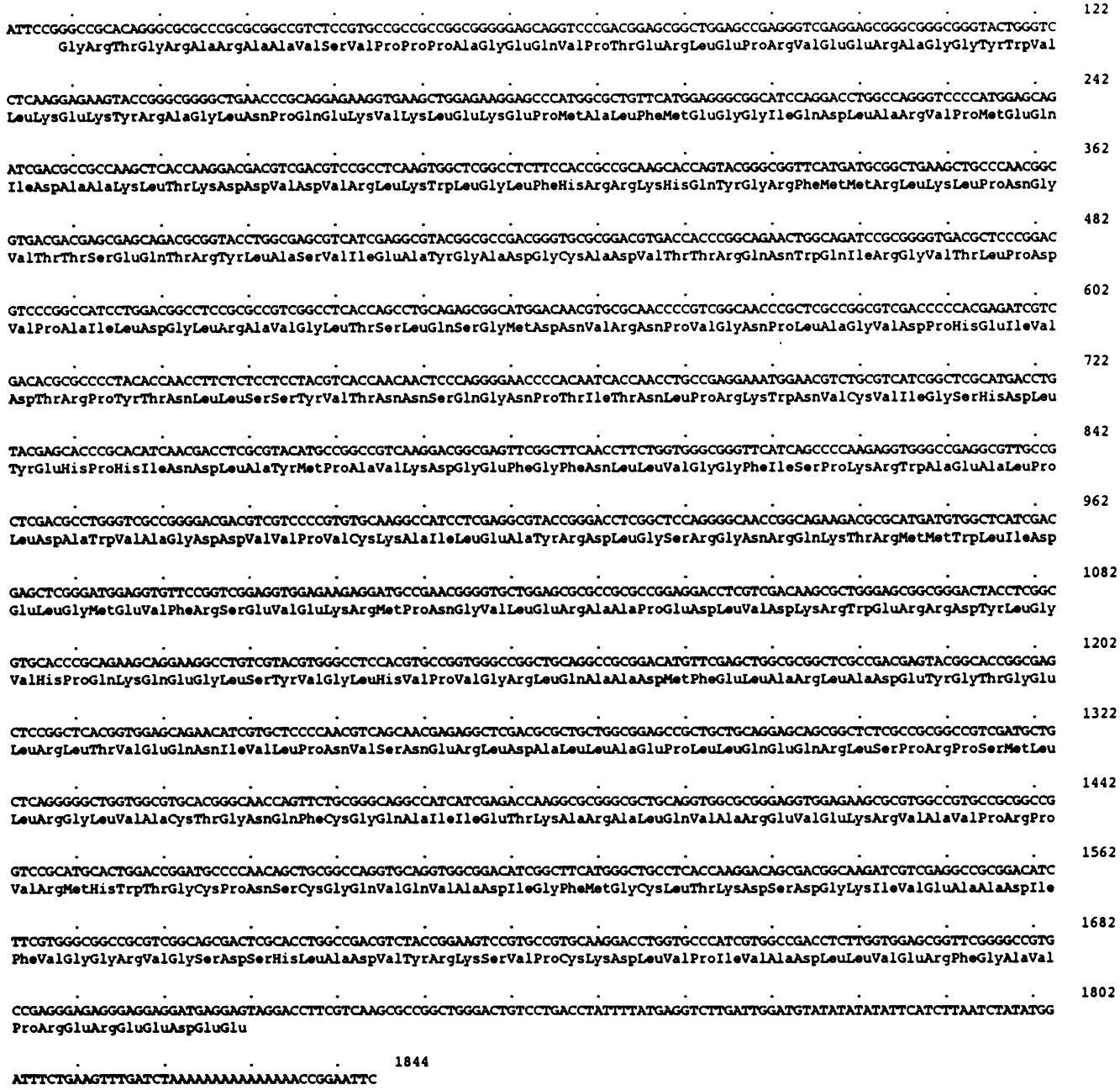

FIG. 1. DNA sequence and deduced amino acid sequence of the maize NiR cDNA clone pCIB808. The cDNA clone pCIB808 was sequenced using the technique of Sanger *et al.* (29). The nucleotide sequence is presented above the deduced amino acid sequence.

nitrite rather than nitrate. When RNA from induced and uninduced roots was analyzed, the nitrite also was found to be capable of inducing the NiR mRNA (see Fig. 5). The leaves showed strong signs of nitrite toxicity under these conditions and showed only a small amount of induction (data not shown). The induction in the roots was not as great as when nitrate was used as the inducer. An exposure time three times longer was required to get approximately the same signal when compared to plants induced with nitrate. This could be due to the general lack of health in the plants or could be a sign that nitrite is not a primary inducer of this gene (as discussed below).

DISCUSSION

A cDNA coding for maize NiR has been cloned using the spinach gene as a heterologous probe. The DNA sequence of the cDNA clones from the two species has a 66% homology that is fairly constant throughout the gene. Most of the variability between the two genes is in the nucleotides used in the third codon position. The maize gene has a striking third codon usage pattern with 559/569 being either a G or a C. A preference for G or C in the wobble position has also been found for the genes coding for maize phospho(enol)pyruvate carboxylase (17), alcohol dehydrogenase (10), and glutathione-S-transferase (24). However, this preference is not seen in the gene coding for the ATP/ADP translocator (4). The functional reason, if any, behind this G/C bias is unclear. It could in theory have an effect on the secondary structure of the mRNA and consequently the rapidity with which it is degraded. In fact, the NiR mRNA does appear to have a very short half-life after nitrate induction (our unpublished results).

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                                    ↓
  3 ..........................GRTGRARAAVSVPPPAGEQV 22
                                |  ||  |   ||  |  |
  1 MASLPVNKIIPSSTTLLSSSNNNRRRNNSSIRCQKAVSPAAETAAVSPSV 50

 23 PTERLEPRVEERAGGYWVLKEKYRAGLNPQEKVKLEKEPMALFMEGGIQD 72
    |||||||||| |||||||| || |||| |||||||||| |||||||  |
 51 DAARLEPRVEER.DGFWVLKEEFRSGINPAEKVKIEKDPMKLFIEDGISD 99

 73 LARVPMEQIDAAKLTKDDVDVRLKWLGLFHRRKHQYGRFMMRLKLPNGVT 122
    ||  |  |||||  |  ||||||||||||||||||||||||||||||||||
100 LATLSMEEVDKSKHNKDDIDVRLKWLGLFHRRKHHYGRFMMRLKLPNGVT 149

123 TSEQTRYLASVIEAYGADGCADVTTRQNWQIRGVTLPDVPAILDGLRAVG 172
    ||||||||||||  || ||||||||||||||||| |||||  ||  ||  ||
150 TSEQTRYLASVIKKYGKDGCADVTTRQNWQIRGVVLPDVPEIIKGLESVG 199

173 LTSLQSGMDNVRNPVGNPLAGVDPHEIVDTRPYTNLLSSYVTNNSQGNPT 222
    |||||||||||||||||||||||||||||||||||| ||| ||  ||
200 LTSLQSGMDNVRNPVGNPLAGIDPHEIVDTRPFTNLISQFVTANSRGNLS 249

223 ITNLPRKWNVCVIGSHDLYEHPHINDLAYMPAVKDGEFGFNLLVGGFISP 272
    |||||||| |||||||||||||||||||| ||| ||||||||||||
250 ITNLPRKWNPCVIGSHDLYEHPHINDLAYMPATKNGKFGFNLLVGGFFSI 299

273 KRWAEALPLDAWVAGDDVVPVCKAILEAYRDLGSRGNRQKTRMMWLIDEL 322
    ||  |||||||||| |||||||||||||||||||| ||||||  |||||||||
300 KRCEEAIPLDAWVSAEDVVPVCKAMLEAFRDLGFRGNRQKCRMMWLIDEL 349

323 GMEVFRSEVEKRMPNGVLERAAPEDLVDKRWERRDYLGVHPQKQEGLSYV 372
    |||  ||||||||||| |||||  ||||||  |||||||||||||||||||
350 GMEAFRGEVEKRMPEQVLERASSEELVQKDWERREYLGVHPQKQQGLSFV 399

373 GLHVPVGRLQAADMFELARLADEYGTGELRLTVEQNIVLPNVSNERLDAL 422
    ||||||||||| || ||||||| || |||||||||||||||  | |||  |
400 GLHIPVGRLQADEMEELARIADVYGSGELRLTVEQNIIIPNVVENSKIDSL 449

423 LAEPLLQEQRLSPRPSMLLRGLVACTGNQFCGQAIIETKARALQVAREVE 472
    |  |||| |  | || |  |||||||||||  |||||||||||||||  |  |||
450 LNEPLLKE.RYSPEPPILMKGLVACTGSQFCGQAIIETKARALKVTEEVQ 498

473 KRVAVPRPVRMHWTGCPNSCGQVQVADIGFMGCLTKDSDGKIVEAADIFV 522
    | | | |||||||||||||||||||||||||||||||||||  |||  |||||||
499 RLVSVTRPVRMHWTGCPNSCGQVQVADIGFMGCMTRDENGKPCEGADVFV 548

523 GGRVGSDSHLADVYRKSVPCKDLVPIVADLLVERFGAVPREREEDEE 569
    |||||||||||||||| |||||||||||||||| |||||||||| |
549 GGRIGSDSHLGDIYKKAVPCKDLVPVVAEILINQFGAVPREREEAE* 595
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FIG. 2. Amino acid sequence comparison between the spinach and maize NiR proteins. The best alignment of the maize NiR amino acid sequence (top line) with the spinach NiR sequence (bottom line [3]) is shown. No gaps were required to best align the sequences. When analogous amino acid residues are counted (as shown here), there is an 86% similarity between the two sequences. When only identical amino acids are included, there is a 75% similarity. The arrow denotes the cleavage site in the spinach NiR polypeptide between the chloroplast signal peptide and the mature protein.

The deduced amino acid sequences of the proteins from the two species show a high degree of conservation, with an 86% similarity when analogous amino acids are included. If only identical amino acids are counted, then the proteins are 75% similar. This conservation almost certainly implies that most alterations in the amino acid sequence would lead to a decrease in enzymatic function. The only region of the protein where considerable variation has occurred is at the N-terminal end of the mature protein. It has been shown, at least for the small subunit of ribulose-bisphosphate carboxylase, that the N-terminal region of the mature protein is required for efficient partitioning of the protein into chloroplasts (35). Therefore this region of the NiR protein may not be involved in catalytic activity. The four cysteine residues that have been implicated in prosthetic group binding are present in the same position in the maize as in the spinach protein (27). Furthermore, the sequence around these residues is highly conserved. However, since virtually the entire protein is highly conserved, it is difficult to conclude that these residues have any particular importance in enzyme activity from a simple sequence comparison.

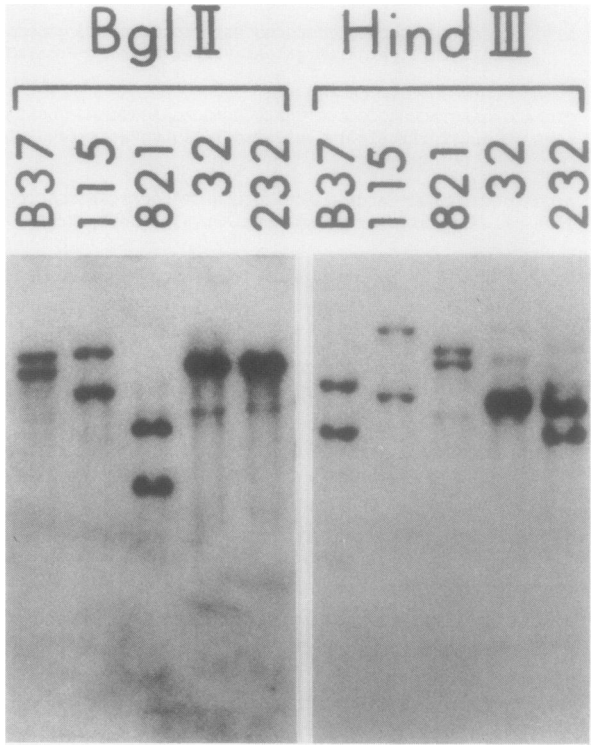

FIG. 3. Genomic DNA analysis of NiR genes in inbred maize lines. Five highly inbred lines of maize were analyzed for the presence of NiR genes. Five μg of DNA were digested either with the restriction endonuclease HindIII or BglII. The DNA was subjected to electrophoresis on a 0.6% agarose gel, and the DNA was blotted onto nitrocellulose. The NiR cDNA sequences from pCIB801 were nick-translated and used as a probe. The resulting autoradiograph is shown. The corn inbred lines are abbreviated as follows: B37- B37, 115- T 115, 821- New York 821, 32-Pa 32, 232- Tenn. 232.

From DNA blot analysis it appears as if there are two NiR genes per haploid genome in maize. Preliminary analysis of genomic clones supports this conclusion (our unpublished results), although further work is required to answer this point conclusively. This is in contrast to what has been found for spinach where there is a single gene per haploid genome (E Back, unpublished results). There are two reports that there are two NiR isozymes in maize (9, 19). Given the similarity between the spinach and the maize NiR genes, one would assume that the genes coding for the two NiR isozymes would be similar enough to each other to be detected by hybridization. If so, each of the two genes seen would code for one of the isozymes. It has been reported that there are differences in the regulation of the different isozymes (19). It will be interesting to see whether these differences can be detected at the transcriptional level.

The levels of the NR and NiR proteins have been shown to increase in the presence of nitrate in a variety of plants. The level of spinach NiR mRNA was shown to increase about fourfold with the addition of nitrate (3). However, given the spinach growth conditions, it was difficult to ensure that absolutely no nitrate was present in the medium. Use of a hydroponic system in the present study to grow the maize seedlings ensured very little nitrate contamination. In the leaf material, there is a low and reproducible level of constitutive NiR mRNA present. The presence of constitutive NiR activity has been reported for a

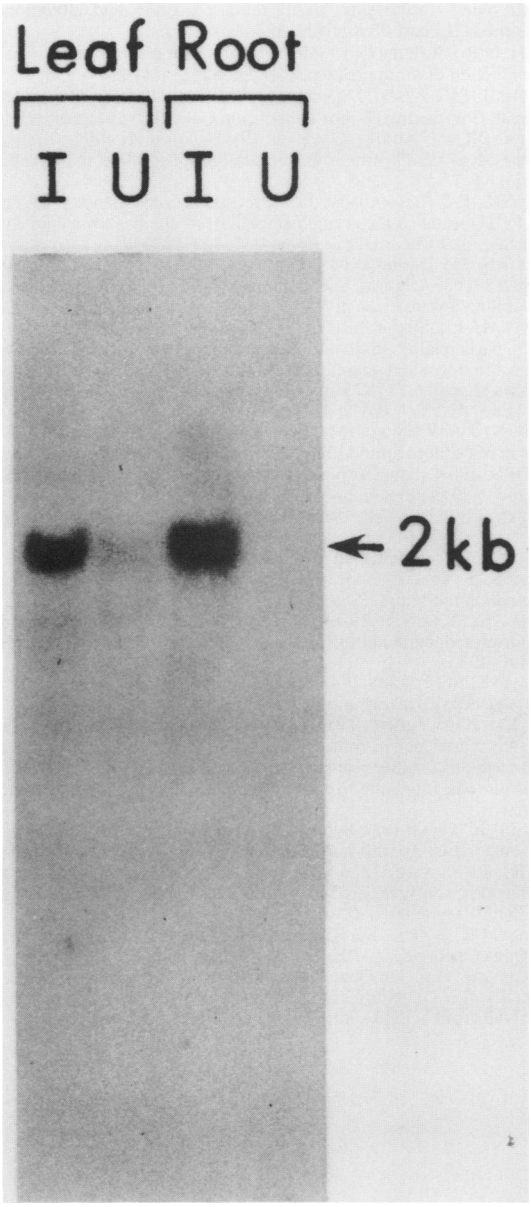

FIG. 4. Nitrate induction of the NiR gene in green tissue and roots. Total RNA was isolated from maize plants induced with nitrate (harvested 90 min after nitrate treatment) or from uninduced plants. The total RNA from either roots (10 μg) or leaves (20 μg) was blotted onto nitrocellulose. The filter was probed with the nick-translated NiR cDNA probe, washed at high stringency, and autoradiographed for 16 h. The NiR mRNA was found to be approximately 2 kb in length as determined by comparison with stained molecular weight standards (Bethesda Research Laboratories). Autoradiography was for 24 h. I, RNA isolated from nitrate-induced plants; U, RNA from uninduced plants.

variety of plant species (see, for example, 27); given the toxicity of nitrite to the plant, it seems reasonable that this should be the case. One possibility that cannot be eliminated at this time is that one of the NiR genes is expressed at a low, but constitutive level, while the other gene would be completely regulated by nitrate. Finally, it is impossible to eliminate the possibility that there is a small amount of nitrate present in the system either from the seed or in the medium.

It has been reported that upon feeding of nitrite to barley leaves, a significant amount of the nitrite is oxidized to nitrate in the plant. Furthermore, it was found that the amount of NiR

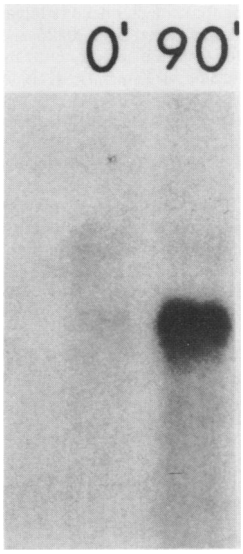

FIG. 5. Nitrite induction of the NiR gene in root tissue. Plants induced with nitrite harvested 90 min after induction or uninduced plants were harvested. Ten μg of total root RNA were subjected to electrophoresis and blotted onto nitrocellulose. The NiR cDNA sequence from pCIB801 was used as a probe. Autoradiography was for 3 d. 0′, RNA from nitrite-induced plants; 90′, RNA from uninduced plants.

enzyme activity did not increase until nitrate was detected (2). Therefore, even though exogenously added nitrite clearly induces expression of NiR mRNA synthesis, it is difficult to know whether nitrite actually acts as a second inducer of this gene. The amplitude of the induction with nitrite was not as great as with nitrate (about one-third to one-half as much). However, this could be due to nitrite toxicity. Only a small increase in the level of NiR mRNA was seen in the leaves of nitrite-treated plants. However, nitrite toxicity was especially acute in this tissue.

The first two enzymes in the nitrate assimilatory pathway, NR and NiR, are both clearly regulated by nitrate at the transcriptional level (see 5 for a review). They are also regulated by a variety of other environmental factors, such as water stress (14, 16) and light (28, 30), and possibly internal stimuli such as cytokinins (20, 31). However, it is still not clear whether the regulation of these genes has any effect on the ability of plants to use field nitrate efficiently. Calculations have been made that for maize demonstrate the presence of an excess of both enzymes compared to substrate under most physiological conditions (34 and R. Volk, personal communication). However, it is difficult to extrapolate these data to what occurs in a developmentally complex organism. With the isolation of these genes and the ability to make transgenic plants with defined changes, it should be possible to ask whether the flux of substrate is limiting at either of these steps in the pathway. Furthermore, it will be possible to analyze whether altering the regulation of these genes in an otherwise isogenic background has any effect on nitrate utilization efficiency.

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