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A Nitrate-Inducible Ferredoxin in Maize Roots[1]

Genomic Organization and Differential Expression of Two Nonphotosynthetic Ferredoxin Isoproteins

Tomohiro Matsumura*, Hitoshi Sakakibara, Ryoji Nakano, Yoko Kimata[2], Tatsuo Sugiyama, and Toshiharu Hase

Division of Enzymology, Institute for Protein Research, Osaka University, 3-2 Yamadaoka, Suita, Osaka 565, Japan (T.M., T.H.); and Department of Applied Biological Sciences, School of Agricultural Sciences, Nagoya University, Nagoya 464-01, Japan (H.S., R.N., Y.K., T.S.)

We have identified and characterized a nitrate-inducible ferredoxin (Fd) in maize (*Zea mays* L.) roots by structural analysis of the purified protein and by cloning of its cDNA and gene. In maize Fd isoproteins are encoded by a small multigene family, and the nitrate-inducible Fd was identified as a novel isoprotein, designated Fd VI, which differed from any Fd I to Fd V identified to date. In the roots of seedlings cultured without nitrate, Fd VI was undetectable. However, during the induction of the capacity for nitrate assimilation, the amount of Fd VI increased markedly within 24 h. Concurrently, the level of transcript for Fd VI increased, but more quickly, reaching a maximal level within 2 h with kinetics similar to those of nitrite reductase and Fd-NADP$^+$ reductase. Fd III was constitutively expressed in roots, and no such changes at the protein and mRNA levels were observed during the nitrate induction. In the 5′ flanking region of the gene for Fd VI only, we identified NIT-2 motifs, which are widely found in genes for enzymes related to nitrogen metabolism. These data indicate that Fd VI is co-induced with the previously characterized enzymes involved in nitrate assimilation, and they suggest that the novel Fd isoprotein, distinct from the constitutively expressed Fd, might play an important role as an electron carrier from NADPH to nitrite reductase and other Fd-dependent enzymes in root plastids.

Higher plants have a set of enzymes that are involved in the assimilation of nitrate to amino acids, namely, NR, NiR, GS, and GOGAT, in both their photosynthetic and nonphotosynthetic organs (Oaks and Hirel, 1985; Kleinhofs and Warner, 1990; Lea et al., 1990). The enzymatic pathways require ATP, reducing power, and carbon skeletons for the reduction of nitrate to ammonia and the subsequent incorporation of ammonia into Glu. In roots these requirements are met by oxidation of carbohydrate, and this system is fundamentally different from that in leaves, where photosynthetic energy and metabolites are utilized directly. In root plastids the OPPP has been shown to be the source of reductants for NiR and GOGAT (Bowsher et al., 1989, 1992). NiR and GOGAT require six and two electrons, respectively, for their catalytic reactions, which are donated by Fd (Knaff and Hirasawa, 1991). Fd and FNR localized in root plastids seem to serve as a system for the donation of electrons from NADPH, derived from OPPP, to the Fd-dependent enzymes in a light-independent manner.

The expression of genes for nitrate-assimilatory enzymes is regulated by both external and internal factors, such as the availability of nitrate, light signals, and plant hormones (Hoff et al., 1994). Among the effects of these factors, the effects of nitrate on the induction of NR, NiR (Gowri et al., 1992; Crawford, 1995), and GS (Sakakibara et al., 1992; Redinbaugh and Campbell, 1993) have been studied extensively. More recently, evidence has been provided that Fd and FNR are also induced during nitrate assimilation (Bowsher et al., 1993; Aoki and Ida., 1994; Ritchie et al., 1994). For the assimilation of an adequate amount of nitrate in roots when nitrate is supplied, it may be physiologically important for plants to increase the otherwise low capacity of the reductant-supply system concomitant with the induction of nitrate-assimilatory enzymes.

A number of groups have investigated the Fd and FNR in roots (Wada et al., 1986, 1989; Kimata and Hase, 1989; Hirasawa et al., 1990; Morigasaki et al., 1990; Green et al., 1991), and it has been demonstrated that the molecular species of the root enzymes are distinct from their counterparts in leaves. Most plants examined to date have multiple Fd isoproteins, which can be divided into two main groups. One group, referred to as photosynthetic Fd, is distributed only in leaves and the expression is stimulated by light (Elliott et al., 1989; Kimata and Hase, 1989; Vorst et al., 1990; Hase et al., 1991; Bovy et al., 1995). The other group, referred to as nonphotosynthetic Fd, is distributed

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[2] Present address: Department of Biochemistry, School of Medicine, Keio University, 35 Shinanomachi, Shinjyuku-ku, Tokyo 160, Japan.

* Corresponding author; e-mail matumura/biochem@nms.ac.jp; fax 81–6–879–8613.

Abbreviations: FNR, Fd-NADP$^+$ oxidoreductase; GOGAT, glutamate synthase; GS, Gln synthetase; NiR, nitrite reductase; NR, nitrate reductase; OPPP, oxidative pentose phosphate pathway.

predominantly in nonphotosynthetic organs such as roots and mature fruits (Wada et al., 1986; Kimata and Hase, 1989; Green et al., 1991; Alonso et al., 1995).

In maize (*Zea mays* L.) four Fd isoproteins, designated Fd I, Fd II, Fd III, and Fd IV, have been identified at the protein level. Fd I and Fd II belong to the group of photosynthetic Fds, whereas Fd III and Fd IV are of the nonphotosynthetic type (Kimata and Hase, 1989). Three cDNAs, pFD1, pFD3, and pFD5, have been isolated (Hase et al., 1991). pFD1 and pFD3 encode Fd I and Fd III, respectively, and the Fd isoprotein corresponding to pFD5 has not yet been found. The transcript of pFD1 cDNA was detected only in leaves, and it accumulated considerably upon illumination. The mRNA for Fd III was detected both in roots and, to a lesser extent, in leaves, and light did not affect its accumulation.

In *Citrus sinensis* a cDNA for the nonphotosynthetic type of Fd (Fd1) was isolated (Alonso et al., 1995). The gene for Fd1 was shown to be expressed in petals and fruits, as well as in leaves and roots, and its expression was developmentally regulated during flower opening and fruit maturation. Furthermore, exogenous ethylene induced the expression of this gene.

With respect to the responses of Fd and FNR to the addition of nitrate to the plant-culture medium, an increase in the level of an Fd-like protein was reported in pea roots (Bowsher et al., 1993), but such nitrate-inducible proteins have not been characterized at the genetic level. The accumulation of FNR message upon treatment with nitrate was reported in maize and rice roots (Aoki and Ida., 1994; Ritchie et al., 1994), but we have found no reports of the response of the Fd message to nitrate.

In our previous study with maize seedlings, we showed that a distinct form of GS was induced in roots upon the application of nitrate or ammonia to the culture medium (Sakakibara et al., 1992). However, no significant change in the level of the nonphotosynthetic Fd III was observed at either the protein or the mRNA level. Assuming that an unidentified Fd isoprotein other than Fd III might be induced by nitrate, we attempted to identify and characterize such an Fd isoprotein. In this report, we demonstrate that a novel isoprotein, Fd VI, is induced on the top of the constitutive Fd III in maize roots. Analysis of a genomic clone for the gene for Fd VI revealed that putative nitrogen-responsive motifs are present in the 5′ flanking region of the gene.

MATERIALS AND METHODS

Maize (*Zea mays* L. cv Golden Cross Bantam T51) seedlings were grown in an aerated hydroponic system in 0.1× Hoagland solution (Arnon and Hoagland, 1940), without a nitrogen source, at 28°C for 4 d under continuous fluorescent light at an intensity of about 300 μE m^{-2} s^{-1}. The young seedlings were then transferred to the same medium supplemented with 10 mM $NaNO_3$ and allowed to grow under the same conditions for the indicated times.

Extraction of Proteins and Western Analysis

About 3 g of root tissue was ground with a mortar and pestle with 5% (w/w) Polyclar AT (Wako Pure Chemical Industries, Osaka, Japan) and a small amount of quartz sand in 2 volumes of buffer A (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5% [v/v] 2-mercaptoethanol, and 1 mM PMSF). The homogenate was centrifuged at 12,000*g* for 10 min at 4°C. The supernatant was fractionated by the addition of ammonium sulfate to 70% saturation and the precipitate was discarded after centrifugation at 12,000*g* for 10 min. The supernatant containing Fd was passed through a small column of DE-52 (Whatman), and the absorbed Fd was eluted with elution buffer (50 mM Tris-HCl, pH 7.5, and 700 mM NaCl) in a small volume. The partially purified Fd fractions were analyzed by PAGE on a nondenaturing gel as described previously (Kimata and Hase, 1989). Proteins on gels were stained with Coomassie brilliant blue or electrotransferred to a PVDF membrane (Immobilon, Millipore) for western analysis (Towbin et al., 1979). Immunological detection of Fd polypeptides was performed as described previously (Hase et al., 1991).

Purification of Fd Isoproteins from Roots

Approximately 1.5 kg of frozen root tissues was ground to a fine powder in liquid nitrogen in a Waring blender. The tissues were macerated in 4 L of buffer A with 5% (w/w) Polyclar AT using a polytron homogenizer (Kinematica, Littau, Switzerland), and then they were homogenized thoroughly with a mortar and pestle. The homogenate was filtered through two layers of Miracloth (Calbiochem) and centrifuged at 10,000*g* for 10 min at 4°C. The resulting supernatant was saturated to 70% with ammonium sulfate and centrifuged. The supernatant was passed through a small column packed with an excess amount of DE-52. The absorbed proteins were eluted with the elution buffer. The eluted proteins were chromatographed on a column of Superdex pg200 (fast protein liquid chromatography system; Pharmacia), which was equilibrated and developed with buffer B (50 mM Tris-HCl, pH 7.5, and 150 mM NaCl). The Fd-containing fraction was loaded onto a Resource-Q column (fast protein liquid chromatography system) and eluted with a linear gradient of NaCl from 150 to 500 mM in buffer B. Two peaks of Fd were obtained and each Fd fraction was further purified on a Phenyl Superose column (fast protein liquid chromatography system) with a linear gradient of ammonium sulfate (3–0 M) in 50 mM Tris-HCl (pH 7.5).

For small-scale purification, Fd isoprotein after the gel-filtration step was chromatographed on a Mono-Q column in the SMART system (Pharmacia).

Determination of Amino-Terminal Sequences of Fd Isoproteins

About 10 μg of Fd isoprotein was precipitated by adding TCA to a final concentration of 10% (w/v). The precipitate was collected by microcentrifugation and washed briefly with water. The pellet was dissolved in 1 mM NaOH and the denatured Fd polypeptide was analyzed on a gas-phase amino acid sequencer (model 476A, Applied Biosystems).

Screening of a cDNA Library

A cDNA library, constructed in λgt11 with poly(A)$^+$ RNA prepared from nitrate-induced maize roots (Sakakibara et al., 1995), was screened by plaque hybridization (Sambrook et al., 1989) with full-length pFD3 cDNA (Hase et al., 1991) as a probe. Positive clones were further screened with the 3′ untranslated region of pFD3 (nucleotides 509–705; Hase et al. [1991]) as a specific probe for Fd III, to distinguish clones for Fd III from clones for other Fd isoproteins. The probes were labeled by the random-primed method in the presence of [α-^{32}P]dCTP (Feinberg and Vogelstein, 1984). The probed filters were washed under low-stringency conditions in 2× SSC and 0.1% (w/v) SDS at 42°C and subjected to autoradiography.

Construction and Screening of a Maize Genomic Library

Maize genomic DNA was prepared from etiolated shoots as described by Richards (1987). After partial digestion with *Sau*3AI, the DNA was size-fractionated to yield fragments of 10 to 20 kb in length, which were ligated into the λEMBL3 vector (Sambrook et al., 1989). Screening of genes for Fd was performed by nucleic acid hybridization, as described above.

Subcloning and Sequencing

Insert DNAs were excised from recombinant phage by appropriate restriction endonucleases and subcloned into pUC19 for sequencing analysis. DNAs were sequenced by the chain-termination method (Sanger et al., 1977) with an automated DNA sequencer (A.L.F., Pharmacia).

Extraction of RNA and Northern Analysis

Total RNA from maize roots was prepared as described by Wadsworth et al. (1988). The RNAs were separated by electrophoresis on 1% (w/v) agarose gel that contained 6.3% formaldehyde (Sambrook et al., 1989) and blotted onto nylon membranes (Hybond-N+, Amersham). The blots were probed with various cDNA inserts that had been labeled with [α-^{32}P]dCTP. cDNAs for FNR, NiR, and ubiquitin, which had originally been cloned by Ritchie et al. (1994), Lahners et al. (1988), and Liu et al. (1995), respectively, were isolated from the maize root cDNA library with appropriate oligonucleotide DNAs as the probes. The probes were synthesized on the basis of the published sequences. Hybridization and washing of the filters were performed as described previously (Hase et al., 1991).

RESULTS

Accumulation of a Novel Fd Isoprotein in Roots during Treatment with Nitrate

The Fds from leaves of young maize seedlings can be separated into at least four molecular species, Fd I, Fd II, Fd III, and Fd IV, as a consequence of their different mobilities during PAGE on a nondenaturing gel. In roots of such seedlings grown without nutrients, Fd III is detected as the sole major species (Kimata and Hase, 1989; Hase et al., 1991). It has been reported that an Fd-like protein is inducible by nitrate in pea roots (Bowsher et al., 1993), but, in our preliminary study, the level of Fd III did not change in maize roots upon treatment with nitrate. To examine whether the accumulation of other Fd isoproteins occurred upon treatment with nitrate, we analyzed the steady-state levels of Fd isoproteins in roots at various times after the addition of nitrate to the culture medium. As shown in Figure 1A, the level of a novel polypeptide with a lower electrophoretic mobility increased from an undetectable level to one similar to that of Fd III within 24 h. The polypeptide was found to be cross-reacted with antibodies raised against Fd III (Hase et al., 1991) in the western analysis (Fig. 1B). The mobility of this nitrate-induced polypeptide on the gel did not correspond to those of any isoform of Fd I through Fd IV identified to date (Kimata and Hase, 1989). These results imply that a novel Fd isoprotein accumulated in roots during nitrate induction.

Purification and Structural Analysis of Fd Isoproteins from Roots

To identify the nitrate-inducible Fd isoprotein, we subjected the total Fd fraction from nitrate-treated roots to further purification as described in "Materials and Methods." The Fd fraction was separated into two peaks during chromatography on an anion-exchange column when the absorbance of the eluate was monitored at 280, 330, and 420 nm (Fig. 2A). Fds in peaks I and II were collected separately and each was further purified to homogeneity. The absorption spectra of both Fds were typical of plant-type Fds, with maxima at 460, 422, and 330 nm in the visible region (data not shown). Each of the final preparations yielded a single band after PAGE on a nondenaturing gel, and the mobilities of Fds from peaks I and II corresponded to those of the nitrate-inducible Fd and Fd III, respectively (Fig. 2B). The two Fds were nearly in equal amounts in the nitrate-treated roots (Figs. 1A and 2A). The lower signal for the nitrate-inducible Fd in the western blot (Fig. 2B) was prob-

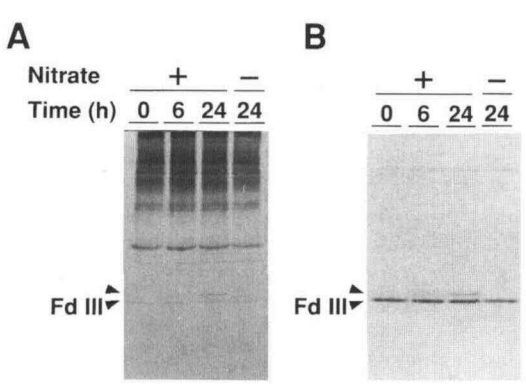

Figure 1. Effects of nitrate in the growth medium on the accumulation of Fd isoproteins in maize roots. Total proteins were extracted from roots at the indicated times after the start of treatment with nitrate (10 mM $NaNO_3$). Partially purified Fd fractions, corresponding to 0.5 g fresh weight of tissue, were subjected to nondenaturing PAGE. Proteins were visualized by staining with Coomassie brilliant blue (A), or by immunolabeling with anti-maize Fd III antibodies (B).

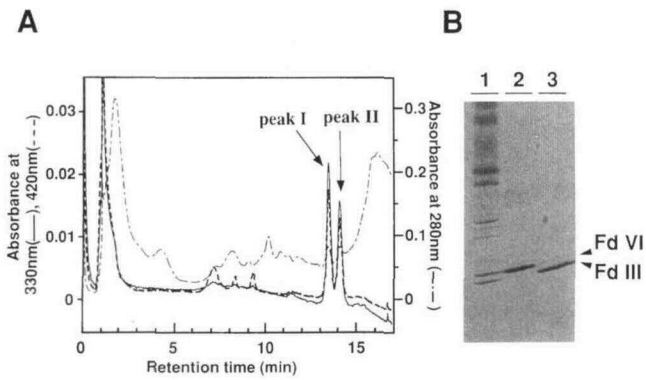

Figure 2. Separation of Fd isoproteins from nitrate-treated maize roots. A mixture of partially purified Fd isoproteins was chromatographed on a Mono-Q column while monitoring the A_{280}, A_{330}, and A_{420} with the SMART system (A). The purified Fd isoproteins were electrophoresed on nondenaturing PAGE and stained with Coomassie brilliant blue (B). Lane 1, Partially purified Fd fraction (30 μg of protein); lane 2, the purified Fd from peak I (2 μg of protein); and lane 3, the purified Fd from peak II (2 μg of protein).

ably due to a weak cross-reactivity of our antibodies raised against Fd III.

The amino-terminal sequences of the Fds in peaks I and II were determined to be AAAVLHKVKLVGPDGTEHEF and AVYKVKLVGPEGEEHE, respectively. In a comparison with reported amino acid sequences of maize Fd isoproteins, we found that the amino-terminal structure of the Fd in peak I was unique, and that of the Fd in peak II was identical to that of Fd III. These results indicated that a novel Fd isoprotein was induced by nitrate in roots and was distinct from the constitutively expressed Fd III. We designated the new isoprotein Fd VI.

Cloning of a cDNA and a Genomic DNA for Nitrate-Inducible Fd VI

Since the similarity in sequence between Fd VI and Fd III suggested that Fd VI was a member of the group of nonphotosynthetic Fds, as is Fd III, we screened a λgt11 cDNA library that had been prepared from nitrate-induced maize roots with the coding region of Fd III cDNA as a probe under low-stringency conditions. About 50 clones were obtained from 5×10^5 recombinant plaques. Then, we excluded clones for Fd III from the positive clones by the hybridization method using the 3′ untranslated region of Fd III cDNA as a specific probe for Fd III (Hase et al., 1991). The remaining 19 clones were further analyzed and each gave almost identical restriction patterns with various restriction enzymes (data not shown). The complete sequence of the insert of the respective longest cDNA clone, designated pFD6, was determined (Fig. 3). The pFD6 insert contained 845 nucleotides, including an 8-nucleotide poly(A) tail. An open reading frame encoding 155 amino acids was found in the cDNA, and the deduced amino acid sequence contained a stretch of 20 residues, which coincided with the amino-terminal sequence of Fd VI, starting from an Ala residue at position 56. Thus, we concluded that pFD6 encoded a precursor of Fd VI.

The genomic clone GFD6, isolated from a maize genomic library constructed with the λEMBL3 vector, contained the Fd VI gene. The nucleotide sequences of the coding and flanking regions were determined as shown in Figure 3. The nucleotide sequence of the cDNA was identical to that of the corresponding genomic clone, with the exception that the intron sequence was included in the 5′ untranslated region of the cDNA. The length of the intron in the Fd VI gene was 364 bp (Fig. 3); the intron began with nucleotides GT and ended with AG.

The amino acid sequence of Fd VI is compared with those of other Fds in Figure 4. A polypeptide encoded by

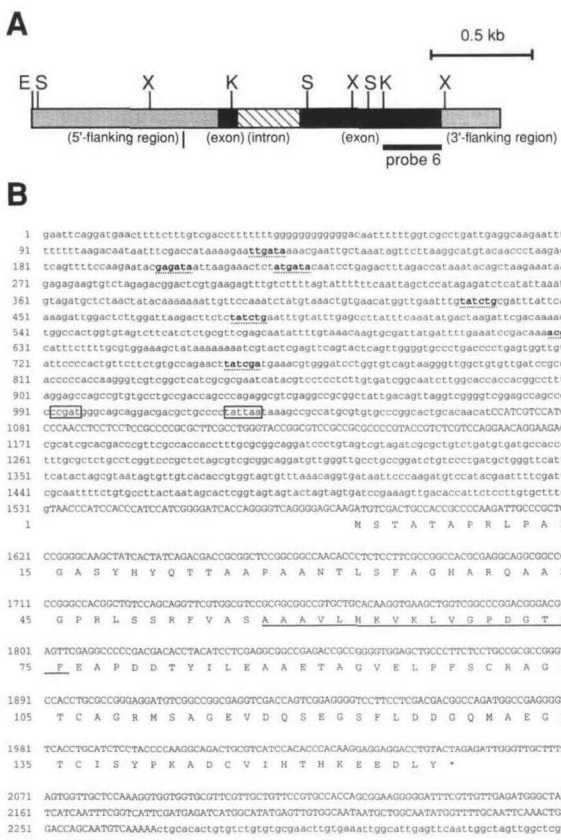

Figure 3. Restriction map (A) and nucleotide sequence of the cDNA designated pFD6 and of the corresponding the genomic clone, GFD6 (B). A, The position of the probe that was used for northern analysis is indicated by a heavy black bar (probe 6). E, *Eco*RI; S, *Sal*I, K, *Kpn*I; and X, *Xho*I. B, The amino acid sequence encoded by the open reading frame is shown below the nucleotide sequence. Sequences of the intron and untranscribed regions are denoted by lowercase letters and that of the exon by uppercase letters. The amino acid sequence found in the amino-terminal region of the mature form of Fd VI is underlined. The putative TATA box and CCAAT box are boxed. The putative binding sites for the NIT-2 protein factors were searched using the TFSEARCH program of GenomeNet (Genome Research Network, Japan) and they are underlined with dotted lines. The transcriptional start site of the Fd VI gene has not been determined. The position of the probe that was used for northern analysis is nucleotides 1972 to 2268.

the Fd VI cDNA has 55 more amino acid residues at the amino-terminal region than the isolated protein. These sequences might be a transit peptide, which is most probably necessary for the import into plastids. The sequence of the transit peptide shows no significant homology to sequences of other Fds, but it shares common features of import-related sequences: it is rich in hydroxylated amino acids and small hydrophobic amino acids such as Ala, Ser, and Thr, and it has a net positive charge (Keegstra et al., 1989). A putative processing site is located between Ser-55 and Ala-56. The mature polypeptide of Fd VI consists of 100 amino acids, including two additional Ala residues at the amino terminus. The mature region of Fd VI shows 57 and 56% identity with other two photosynthetic-type Fds, maize Fd I and spinach Fd I, respectively, and 78 and 68% identity with two other nonphotosynthetic-type Fds, maize Fd III and Fd1 from *C. sinensis*, respectively. Four Cys residues, Cys-98, Cys-103, Cys-106, and Cys-136, which are essential for the formation of a 2Fe-2S cluster, are conserved.

Differential Expression of Two Fd Genes in Roots in Response to Induction by Nitrate

The steady-state level of the transcript of the Fd VI gene in roots during induction by nitrate was analyzed by northern blotting and compared with the transcripts of genes for Fd III, NiR, and FNR (Fig. 5). The NiR gene was established to be one of the nitrate-responsive genes (Privalle et al., 1989), and root FNR was also recently revealed to be nitrate-inducible in maize (Ritchie et al., 1994). To distinguish between the transcripts for Fd III and Fd VI, we used the *Kpn*I fragment of pFD6 (probe 6 in Fig. 3A) and the *Xho*I fragment of pFD3 as described in "Materials and Methods" as the specific probe for each gene. The transcript for Fd VI, about 1 kb in length, accumulated markedly within 2 h of the start of exposure to nitrate (Fig. 5A). The accumulation pattern was very similar to those of the transcripts for NiR and FNR, suggesting that the gene for Fd VI is a nitrate-responsive gene. The level of the transcript for Fd III did not change during the induction period (Fig. 5B).

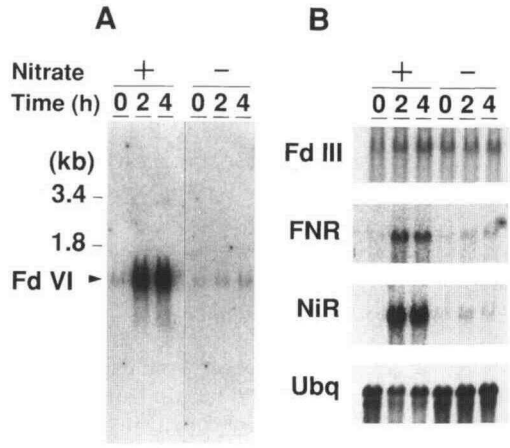

Figure 5. Effects of nitrate on the accumulation of transcripts for Fd VI and related proteins in maize roots. Total RNA (10 μg), prepared from maize roots at the indicated times after the start of treatment with nitrate (16 mM $NaNO_3$), was subjected to electrophoresis on an agarose gel and transferred to nylon membranes. The blots were probed with ^{32}P-labeled probes for the genes for Fd VI (A), Fd III, FNR, and NiR (B) (see "Materials and Methods"). The extent of migration of ribosomal RNAs is indicated on the left. The probe for ubiquitin (Ubq) mRNA was used as an internal standard.

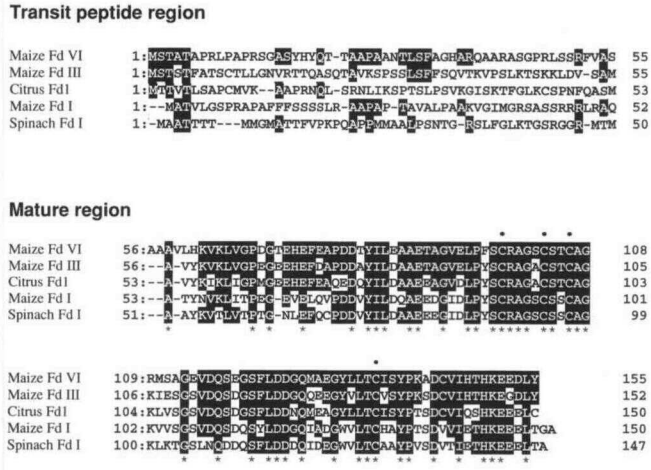

Figure 4. Comparison of amino acid sequences of Fd isoproteins from various plant species. The deduced amino acid sequence of maize Fd VI is compared with the sequences of maize Fd I and Fd III (Hase et al., 1991), spinach Fd I (Wedel et al., 1988), and *C. sinensis* Fd1 (Alonso et al., 1995). Gaps, denoted by dashes, have been inserted to achieve maximum homology. Amino acid residues identical to those in Fd VI are indicated by white letters on a black background. Amino acids that are identical in all sequences are marked by asterisks (*), and the four conserved Cys residues that are required for the formation of a 2Fe-2S cluster are marked by dots (·).

DISCUSSION

A novel Fd isoprotein, Fd VI, was purified from maize roots, and both a cDNA and a genomic clone encoding Fd VI were isolated. During the induction of nitrate-assimilatory capacity in roots, the amount of Fd VI increased from an undetectable level to a level similar to that of the Fd III in roots (Fig. 1). We also confirmed that the accumulation of Fd III was not affected by nitrate. In pea roots, a 14.5-kD protein with antigenic sites common to Fd from pea leaves increased severalfold upon induction by nitrate (Bowsher et al., 1993). However, chemical identification of this protein has not yet been reported.

The data presented here demonstrate that, in maize roots, a nitrate-inducible Fd isoprotein is distinct from the constitutively expressed Fd isoprotein. Such nitrate dependency suggests a basic difference between the regulation of synthesis of the two nonphotosynthetic Fds in roots. This regulation seems to be achieved at the level of transcription. Using gene-specific probes, with which we were able to detect mRNAs for Fd III and Fd VI separately, we found that the accumulation pattern of the two

transcripts was completely different. Only the level of the mRNA for Fd VI increased markedly after induction with nitrate, reflecting and preceding the change in the level of the protein (Figs. 1 and 5). The response of the Fd VI gene to a supply of nitrate was rapid, and the accumulation of the transcript for Fd VI was coordinated with that of the transcripts for root FNR and NiR. This phenomenon of the nitrate-induced expression of the Fd VI gene was also observed in leaves (H. Sakakibara, T. Hase, unpublished results).

With respect to nitrogen regulation of gene expression, it is noteworthy that upstream of the transcribed region of the Fd VI gene there are multiple sequences identical or homologous to the motif for the binding site of NIT-2 (Fig. 3), which is a global, regulatory factor of nitrogen metabolism in fungi (Fu and Marzluf, 1990). We have obtained a genomic clone encoding Fd III (accession no. AB001387), and found that there is no such sequence in the Fd III gene. The NIT-2 motif has been widely found in genes for the enzymes involved in nitrogen metabolism (Marzluf, 1993). In higher plants these motifs have been found in the 5′ flanking regions of the genes for NR (Lin et al., 1994) and NiR (Tanaka et al., 1994) in *Arabidopsis thaliana*, and for FNR in *Oryza sativa* (Aoki et al., 1995), as listed in Table I. However, the importance of the NIT-2 motif is still controversial because none of the NIT-2 motifs found in the Arabidopsis NR genes is located within the promoter regions, which are necessary and sufficient for induction by nitrate. A comparative analysis of the Fd VI and Fd III genes might help us to identify *cis* elements that are involved in the nitrogen-dependent expression of certain genes.

Another interesting feature of the structure of the gene for Fd VI is the presence of the intron in the 5′ untranslated region. No introns were found in the genes for photosynthetic Fds from pea (Elliott et al., 1989), Arabidopsis (Vorst et al., 1990), or maize (T. Hase, Y. Kimata, unpublished results). It remains to be determined whether the intron in this region is present in the genes for nonphotosynthetic Fds from other plant species.

The deduced amino acid sequence of Fd VI indicates that this protein is synthesized as a precursor with a transit peptide of 55 amino acids, which could potentially function as a signal for transport to plastids. The precursor protein of Fd III is imported into plastids (Suzuki et al., 1991). The sequences of Fd III and Fd VI are 78% homologous in the mature regions. Both Fd III and Fd VI must function in root plastids. It was proposed previously that the OPPP might be involved in nitrate assimilation, with respect to the supply of NADPH as a reducing power (Suzuki et al., 1985; Bowsher et al., 1989, 1992), and, moreover, that a major function of root Fd and FNR might be to transfer electrons from the NADPH to Fd-dependent enzymes such as NiR and GOGAT in root plastids. The data obtained in this study indicate that there is a specific combination of isoproteins of Fd and FNR in roots. Their patterns of induction by nitrate coincide with those of nitrate-assimilatory enzymes such as NiR and NR.

Under nitrogen-limiting conditions the constitutive Fd III must function as an electron carrier for Fd-dependent enzymes involved in metabolism other than nitrate assimilation, such as sulfite reductase and fatty acid desaturase. If there is only one form of FNR in roots in which expression is strictly regulated by nitrate, and if there is no physiological way to reduce Fd other than through the FNR/NADPH system, then how would the constitutive Fd be reduced in nitrogen-limiting conditions? Although we have no conclusive answer to this question, it is likely that there is a nitrate-noninducible combination of Fd and FNR isoproteins, because FNR activities are detected in the crude extracts prepared from nitrogen-limiting root tissues of pea (Bowsher et al., 1993) and maize (T. Hase, T. Matsumura, unpublished results). Moreover, we have recently obtained a novel cDNA for FNR from a cDNA library from maize roots that is distinct from the nitrate-inducible FNR (Ritchie et al., 1994). It remains to be seen whether the FNR encoded by the cDNA is an isozyme corresponding to the FNR activity in the nitrogen-limiting root tissues.

Nitrate reduction appears to take place in all cell types in maize roots when nitrate is at a high level (Rufty et al., 1986). The expression of nitrate transporters is cell-type-specific in tomato roots (Lauter et al., 1996). Although it is unknown whether the two Fds are distributed in the same or in different root tissues, the molecular diversity and differential expression of the Fd III and Fd VI during induction of the capacity for assimilation of nitrate is probably associated with specialized roles in the partitioning of electrons to Fd-linked enzymes, or with the fine control of the total amount of Fd required to meet the elevated demand for reducing power. We are now trying to produce recombinant molecules of the two Fds to study their biochemical characteristics.

Table I. *The presence of binding motifs of NIT-2 in the 5′ upstream regions of various genes*

Putative binding sites for NIT-2[(AGT)(CTA)GATA or TATC(TAG)(ACT)] were located with the TFSEARCH program (version 1.3) of GenomeNet (Genome Research Network, Japan) with a threshold score of 85.0.

Gene (Species)	Upstream Region Searched	No. of Putative Binding Sites	Inducibility by Nitrate	Reference	Data Base Accession No.
Fd VI (Z. mays)	1–1063	7	+	This study	AB001386
Fd III (Z. mays)	1–965	None	−	This study	AB001387
FNR (O. sativa)	1–1122	4	+	Aoki et al. (1995)	D38445
NRI (A. thaliana)	1–1449	12	+	Lin et al. (1994)	S76264
NR2 (A. thaliana)	1–1600	15	+	Lin et al. (1994)	S76563
NiR (A. thaliana)	1–1247	11	+	Tanaka et al. (1994)	D14824

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