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Complementary DNA Cloning and Characterization of Ferredoxin Localized in Bundle-Sheath Cells of Maize Leaves[1]

Tomohiro Matsumura[2], Yoko Kimata-Ariga*, Hitoshi Sakakibara, Tatsuo Sugiyama, Hiroshi Murata, Toshifumi Takao, Yasutsugu Shimonishi, and Toshiharu Hase

Division of Enzymology (T.M., Y.K.-A., T.H.), and Division of Organic Chemistry (H.M., T.T., Y.S.), Institute for Protein Research, Osaka University, 3-2 Yamadaoka, Suita, Osaka, 565–0871 Japan; and Department of Biological Mechanisms and Functions, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya, 464–8601 Japan (H.S., T.S.)

In maize (*Zea mays* L.) two leaf-specific ferredoxin (Fd) isoproteins, Fd I and Fd II, are distributed differentially in mesophyll and bundle-sheath cells. A novel cDNA encoding the precursor of Fd II (pFD2) was isolated by heterologous hybridization using a cDNA for Fd I (pFD1) as a probe. The assignment of the cDNAs to the Fds was verified by capillary liquid-chromatography/electrospray ionization-mass spectrometry. RNA-blot analysis demonstrated that transcripts for Fd I and Fd II accumulated specifically in mesophyll and bundle-sheath cells, respectively. The mature regions of pFD1 and pFD2 were expressed in *Escherichia coli* as functional Fds. Fd I and Fd II had similar redox potentials of −423 and −406 mV, respectively, but the K_m value of Fd-NADP$^+$ reductase for Fd II was about 3-fold larger than that for Fd I. Asparagine at position 65 of Fd II is a unique residue compared with Fd I and other Fds from various plants, which have aspartic acid or glutamic acid at the corresponding position as an electrostatic interaction site with Fd-NADP$^+$ reductase. Substitution of asparagine-65 with aspartic acid increased the affinity of Fd II with Fd-NADP$^+$ reductase to a level comparable to that of Fd I. These structural and functional differences of Fd I and Fd II may be related to their cell-specific expression in the leaves of a C_4 plant.

Maize (*Zea mays* L.), a typical C_4 plant, is characterized by the compartmentation of carbon assimilation into two differentiated photosynthetic cells, MC and BSC. Atmospheric CO_2 is first incorporated into oxaloacetate in the MC cytosol and is successively reduced to malate with the consumption of NADPH in the MC chloroplasts (Hatch, 1987, 1992). The malate is then transported into the BSC chloroplasts, where it is decarboxylated, with the concomitant formation of NADPH. The released CO_2 is incorporated into glycerate-3-P by the C_3 cycle (Hatch, 1987, 1992). In comparison with the MC chloroplasts, the BSC chloroplasts have a limited capacity for the photosynthetic formation of NADPH because of the deficiency of PSII (Edwards and Walker, 1983), and the NADPH generated by malate decarboxylation is not enough to reduce all of the glycerate-3-P to triose phosphate in the BSC chloroplasts. Therefore, a large proportion of the Pi has to be exported to the MC chloroplasts, which are rich in NADPH, to be reduced (Hatch, 1987, 1992). The triose phosphate thus formed in MC returns to BSC.

Some other NAD(P)H-requiring processes are also restricted to MC in maize leaves. The reduction of nitrate occurs exclusively in MC (Moore and Black, 1979). Recently, a study of the compartmentation of antioxidants showed that glutathione reductase and dehydroascorbate reductase, which function together to generate reduced glutathione at the expense of NADPH, were almost exclusively localized in MC (Doulis et al., 1997). The metabolic compartmentations of carbon and nitrogen assimilations and of the antioxidant process probably developed to adapt to the lower availability of NADPH in BSC.

On the other hand, BSC chloroplasts produce ATP required to drive the C_3 cycle by cyclic electron flow via PSI, irrespective of the absence of PSII (Edwards and Walker, 1983; Asada et al., 1993).

Fd, an electron-transfer protein, occupies a key position both for transferring the photoreducing power to FNR, hence the formation of NADPH, and for mediating the cyclic electron flow around PSI (Arnon, 1989). Therefore, the information above suggests that the function of Fd in MC and BSC could be partly differentiated. In addition to the photosynthetic electron-transfer process, there are several other redox enzymes requiring Fd as an electron donor, such as nitrite reductase, sulfite reductase, glutamate synthase, fatty acid desaturase, and Fd/thioredoxin reductase (Knaff, 1996). Nitrite reductase is restricted to MC (Moore and Black, 1979; Schmuts and Brunold, 1985), but sulfite reductase (Schmuts and Brunold, 1985) and glutamate synthase (Sakakibara et al., 1992) are distributed in both types of cells. Information concerning the localization of other enzymes is not yet available.

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[2] Present address: Department of Biochemistry and Molecular Biology, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo, 113–8602 Japan.

* Corresponding author; e-mail a-yoko@protein.osaka-u.ac.jp; fax 81–6–879–8613.

Abbreviations: BSC, bundle-sheath cell(s); ESI, electrospray ionization; FNR, Fd-NADP$^+$ oxidoreductase; LC, liquid chromatography; MC, mesophyll cell(s); PEPC, PEP carboxylase; UTR, untranslated region.

Fd is present as isoforms in most of the higher plants examined to date. In maize four Fd isoproteins (Fd I to Fd IV) were found in young seedlings (Kimata and Hase, 1989), and a new nitrate-inducible isoprotein (Fd VI) has recently been identified in roots (Matsumura et al., 1997). Two of them (Fd I and Fd II) are restricted to leaves, and their accumulation is induced by light. Thus, they are referred to as photosynthetic Fd (Kimata and Hase, 1989; Hase et al., 1991a). The others are distributed in other organs such as roots and mesocotyls. Curiously, Fd I and Fd II were found to be distributed differentially between MC and BSC (Kimata and Hase, 1989), and it was presumed that the differential localization of Fd I and Fd II might be related to the differences in the electron transfer and metabolic processes between MC and BSC.

We previously obtained a cDNA for Fd I from a cDNA library of maize leaves (Hase et al., 1991a; accession no. M73829). In the present study we isolated a cDNA encoding Fd II and demonstrated that the transcripts for Fd I and Fd II are cell-specifically accumulated in MC and BSC, respectively. By using recombinant proteins of Fd I, Fd II, and their mutants, we found a functional difference between Fd I and Fd II in the electron-transfer ability toward FNR and propose that specific amino acid residues of the two Fds are responsible for their differential affinities with FNR. Our results indicate that the two Fds may be functionally differentiated to gear the demand for NADPH supply, which is different between the chloroplasts of the two types of photosynthetic cells in a C_4 plant.

MATERIALS AND METHODS

Plant Materials

Maize (*Zea mays* L. cv Golden Cross Bantam T51) plants were grown in the field for a few weeks or in a greenhouse for 10 to 12 d under natural illumination. Total green leaves were used for preparation of Fd isoproteins, and the second and third leaves of the seedlings were used for the extraction of total RNAs.

Purification of Fd Isoproteins from Maize Leaves

About 250 g of leaf tissue was homogenized with a Waring blender in 1 L of an ice-cold extraction buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.5 mM EDTA, 0.5% [v/v] 2-mercaptoethanol, and 1 mM PMSF) containing 10% (w/v) Polyclar AT. The homogenate was filtered through two layers of Miracloth (Calbiochem). The leaf debris were ground further in a mortar with a pestle in a small volume of extraction buffer containing quartz sand. The combined filtrate was centrifuged at 10,000*g* for 10 min at 4°C. The supernatant was fractionated by the addition of ammonium sulfate to 70% saturation, and the resulting precipitated material was removed by centrifugation at 12,000*g* for 10 min. The supernatant containing Fd isoproteins was passed through a small DE-52 column (Whatman), and absorbed proteins were eluted with 50 mM Tris-HCl, pH 7.5, and 700 mM NaCl. Fd isoproteins were isolated by successive chromatography on a Sephadex G-75 column (Pharmacia), a DE-52 column, and a Phenyl-Superose column (Pharmacia) as described previously (Hase et al., 1991a; Matsumura et al., 1997).

Screening of a cDNA Library, Subcloning, and Sequencing

A cDNA library, constructed in pUEX1 vector (Amersham) with poly(A^+) RNA prepared from maize seedlings (Sakakibara et al., 1991), was screened by colony hybridization (Sambrook et al., 1989) with full-length pFD1 cDNA (Hase et al., 1991a) as a probe. Positive clones were further screened with the 3′ UTR of pFD1 to distinguish clones for Fd I from clones for other Fd isoproteins. The probes were labeled by random priming (Feinberg and Vogelstein, 1984) in the presence of [α-^{32}P]dCTP. The probed filters were washed under low-stringency conditions in 2× SSC (300 mM NaCl and 30 mM sodium citrate, pH 7.0) and 0.1% (w/v) SDS at 42°C for the first screening and under high-stringency conditions in 0.1× SSC (15 mM NaCl and 1.5 mM sodium citrate, pH 7.0) and 0.1% (w/v) SDS at 50°C for the second screening. The filters were then subjected to autoradiography.

Insert DNAs were excised from the recombinant plasmids by *Bam*HI digestion and subcloned into M13mp19 or pUC19 for sequencing. DNAs were sequenced with a dideoxynucleotide-sequencing kit (Prism with TaqFS, Applied Biosystems) and an automated DNA sequencer (model 370A, Applied Biosystems).

Extraction of RNA and Blot Analysis

Total RNAs from whole leaves, MC protoplasts, and BSC strands were prepared as described previously (Sakakibara et al., 1995). Total RNA (10 μg) was separated by electrophoresis on a 1% (w/v) agarose gel containing 6.3% (v/v) formaldehyde (Sambrook et al., 1989) and blotted onto nylon membranes (Hybond-N^+, Amersham). The blots were probed with various cDNA fragments that had been labeled with [α-^{32}P]dCTP. Hybridization and washing of the filters were performed as described previously (Hase et al., 1991a).

The probes for Fd I and Fd II mRNA were prepared from the 3′ UTR of pFD1 (*Sac*I/*Eco*RI) and pFD2 (*Nhe*I/*Bam*HI), respectively (Fig. 1A). The probes for PEPC and Rubisco small subunit mRNA were prepared from pM52 (*Eco*RI fragment; Izui et al., 1986) and pZmSSu1025 (*Eco*RI/*Hin*dIII fragment; Matsuoka et al., 1987), respectively.

Reduction and Carboxymethylation of Fd I and Fd II and Analysis of Proteolytic Fragments by Capillary LC/ESI-MS

About 100 μg of Fd I and Fd II purified from leaves was denatured with 5% (w/v) TCA. The apoproteins were reduced and carboxymethylated according to the method of Crestifield et al. (1963) and dried after removal of excess reagents by dialysis against water. The carboxymethylated proteins (3 nmol each) were dissolved in 50 μL of 100 mM Tris-HCl, pH 9.5, and digested with lysylendopeptidase (30 pmol) for 12 h at 37°C. The resulting digests were loaded onto a capillary column of C_{18} and eluted with a linear

gradient of 10% to 60% acetonitrile while monitoring at 214 nm. LC/ESI mass spectra were obtained with a double-focusing mass spectrometer (model JMS-HX/HX110A, JEOL) equipped with an ESI source (Analytica of Branford, Branford, CT) by directing the effluent from the UV detector to the ESI source (Sakakibara et al., 1996).

Construction of Expression Plasmids of Fd I and Fd II and Site-Directed Mutagenesis

The pTrc99A expression vector (Pharmacia) was digested with *Pst*I and *Hin*dIII, end-filled with Klenow fragment, and self-ligated to delete the *Pst*I site in the polylinker region. The resulting vector, pTrc99A-1, was used for the construction of the expression plasmids for Fd I and Fd II. A pair of primers, ATTACCATGGCCAC-CTACAACGTGAAGCTGA and ATTATGCATGCTTA-CATGAACAGTGCT, was used to amplify a DNA fragment containing the mature region of pFD1. The PCR fragment was digested with *Sph*I, end-filled with Klenow fragment, digested with *Nco*I, and inserted into the *Nco*I/*Sma*I site of pTrc99A-1 to construct the Fd I expression plasmid pTMmFD1. A unique *Pst*I site was present at the same position of pFD1 and pFD2, and the amino acid sequences of Fd I and Fd II were identical in the region from the N terminus to the residue corresponding to the *Pst*I site. Thus, a DNA fragment from the *Pst*I site to an *Nhe*I site in the 3′ UTR of pFD2 was replaced with the *Pst*I/*Xba*I fragment of pTMmFD1 to obtain the Fd II expression plasmid pTMmFD2. We also altered the codon usage of amino acids near the translation start site of pTMmFD1 and pTMmFD2 to obtain higher expression efficiency.

Site-specific mutants of Fd I and Fd II at position 65 were prepared with the sequential PCR method (Ausbel et al., 1991). The synthetic oligonucleotides used for PCR were ACAGACCATGGCTACT, CATGAATGATTATGCGCCGG, ACCTCAACGACGGCCAGAT, and CTGGCCGTCGTT-GAGGTAGCT for the preparation of Fd I(D65N) and GAC-CTGCCCTTCTCCTG, TCTTCTCTCATCCGCCA, TCCTC-GACGACAACCAG, and GTCGT/CGAGGAAGCTCT for the preparation of Fd II(N65D). Underlined bases denote mismatches with the wild-type sequence.

Expression and Purification of Recombinant Fd Isoproteins

Escherichia coli JM105 cells were transformed with the expression plasmids for Fd I or Fd II. The transformants were grown overnight at 37°C in Luria-Bertani medium containing 50 μg/mL ampicillin. The seed cultures were inoculated into 8 L of the same medium at 1% volume. The cells were grown at 37°C with vigorous aeration for 2 h. Then, isopropyl-β-D(−)-thiogalactopyranoside was added to a final concentration of 0.5 mM. After further cultivation for 8 to 12 h, the cells were collected by centrifugation at 5,000*g* for 10 min and stored at −30°C. The frozen cells were suspended in 50 mM Tris-HCl, pH 7.5, containing 25% (w/v) Suc, 0.5% (v/v) 2-mercaptoethanol, and 0.5 mM PMSF and treated with lysozyme at a concentration of 0.5 mg/mL on ice for about 10 min. The cell suspension was diluted 2-fold with 50 mM Tris-HCl, pH 7.5, 60 mM NaCl, and 3 mM EDTA, and then −30°C acetone was added at a final concentration of 40% (v/v). The resulting homogenate was centrifuged at 10,000*g* for 10 min. The supernatant was passed through a DEAE-cellulose column, and the absorbed Fd proteins were eluted with 50 mM Tris-HCl, pH 7.5, 700 mM NaCl. Further purification of Fd isoproteins was carried out as described above.

Measurement of Oxidation-Reduction Potentials of Fds

Oxidation-reduction potentials of Fd I and Fd II were measured by cyclic voltammetry using an In_2O_3 electrode modified with poly-L-Lys as described previously (Taniguchi et al., 1997).

Assay for Electron-Transfer Activity

Electron-transfer activity of Fd was assayed by measuring the rate of Cyt *c* reduction, as described previously (Hase et al., 1991b). The reaction mixture contained (in a total volume of 800 μL) 0.5 mM NADPH, 40 nM FNR, 0.2 mM Cyt *c*, 50 mM Tris-HCl (pH 7.5), and 100 mM NaCl. The reaction was initiated by the addition of Fd at final concentrations from 5 to 100 μM. Cyt *c* reduction was measured by monitoring the increase in A_{550}.

RESULTS

Cloning and Characterization of cDNAs for Fd Isoproteins in Maize Leaves

Fd I and Fd II have the same N-terminal sequences up to residue 19 (Hase et al., 1991a), and antibodies raised against Fd I cross-reacted with Fd II (Kimata and Hase, 1989), indicating a close sequence similarity between the two Fds. A cDNA, pFD1, encoding a precursor of Fd I was previously isolated (Fig. 1A; Hase et al., 1991a). To obtain a cDNA clone for Fd II, a cDNA library prepared from maize leaves was screened by heterologous hybridization with a combination of the entire region and the 3′ UTR fragment of pFD1 as the probes. Twenty positive clones were obtained from 5×10^4 colonies by hybridization with the full-length cDNA. Six of these clones, which did not hybridize with the 3′ UTR fragment, were selected as candidates for clones encoding Fd II. The 5′ and 3′ terminal sequences of the six clones were determined. Four encoded the same Fd polypeptide, whose C-terminal Leu residue agreed with that of Fd II. In the remaining two clones, one was the same clone as pFD5 previously reported (Hase et al., 1991a; accession no. M73828) and the other was an unidentified new clone for Fd.

The longest cDNA clone among the first four, designated pFD2 (Fig. 1A), was completely sequenced (Fig. 1B). The insert DNA of pFD2 was composed of 804 bp, including a 39-bp poly(A^+) tail, and contained an open reading frame encoding 140 amino acids. A stretch of amino acids from residues 45 to 63 in the deduced sequence coincided with the N-terminal sequence of Fd II (Hase et al., 1991b). The

additional 44 residues upstream of the mature protein appeared to be a putative transit peptide, and no start codon was present in the same frame further upstream. The length of the putative transit peptide was similar to those (approximately 40–50 residues) for the various Fds deduced from cDNA sequences.

MS Analysis of Fd I and Fd II

The assignment of pFD1 and pFD2 as the genes for Fd I and Fd II, respectively, was made based only on the terminal sequences of the two Fds. To obtain further structural information about Fd I and Fd II, we carried out MS. Fd I and Fd II purified from maize leaves (Fig. 2A) were reduced and carboxymethylated. They were then digested with lysylendopeptidase and subjected to capillary LC/ESI-MS. The digests of both Fds were separated into three major peaks (Fig. 2B) and their M_rs were measured. Lysylendopeptidase cleaves the polypeptide bond at the C-terminal side of Lys. Thus, the determined M_rs of each of the three peptides derived from Fd I and Fd II could be equated to those of the segments of the deduced amino acid sequences of pFD1 and pFD2, as shown in Table I. We concluded that pFD1 and pFD2 encoded Fd I and Fd II, respectively.

Amino acid sequences of the precursors of Fd I and Fd II are compared in Figure 1C. The mature and transit peptide regions showed 88% and 52% identity, respectively. The two Fds were similar, especially at the N-terminal region of the mature proteins, which were identical up to residue 26. Fd I was longer by two residues at the C terminus than Fd II.

Cell-Specific Expression of Fd I and Fd II in Maize Leaves

To investigate the expression of Fd I and Fd II genes in MC and BSC in leaves, total RNAs were isolated from whole leaves, MC, and BSC. RNA-blot analysis was performed using the restriction fragments at the 3′ UTR of pFD1 and pFD2 as gene-specific probes (Fig. 1A). cDNAs for PEPC and the Rubisco small subunit were used as the marker probes for MC and BSC, respectively. As shown in Figure 3, the transcripts of the two Fd genes accumulated differently in the two types of photosynthetic cells. The probes for pFD1 and pFD2 hybridized almost exclusively with the RNA from MC and BSC, respectively.

Expression of Fd I and Fd II cDNAs in *E. coli*

We first developed a large-scale expression system for the Fds in *E. coli* (Fig. 4). The mature region of pFD1 was amplified by PCR using a pair of primers with concomitant creation of the initiation Met codon at the N terminus. This fragment was inserted under the control of the *trc* promoter of pTrc99A-1 to yield pTMmFD1. Next, the third nucleotides (mostly C or G) within the first 11 codons were substituted synonymously with A or T to give pTMmFD1-1. These changes in codon usage might reduce the secondary structure within the mRNA around the translation start site, thereby increasing translation efficiency in *E. coli* (De Boer and Hui, 1990). Figure 5 shows an overexpression of the Fd I polypeptide. Yields of Fd I with pTMmFD1 and pTMmFD1-1 were about 0.2 and 10 mg, respectively, from 1 L of bacterial culture.

The expression plasmids for Fd II, pTMmFD2 and pTMmFD2-1, were constructed by a simple replacement of the *Pst*I/*Xba*I fragment of pTMmFD1 and pTMmFD1-1 with the *Pst*I/*Nhe*I fragment of pFD2. pTMmFD2-1 also showed considerably higher expression of Fd II than pTMmFD2, as expected (Fig. 5C).

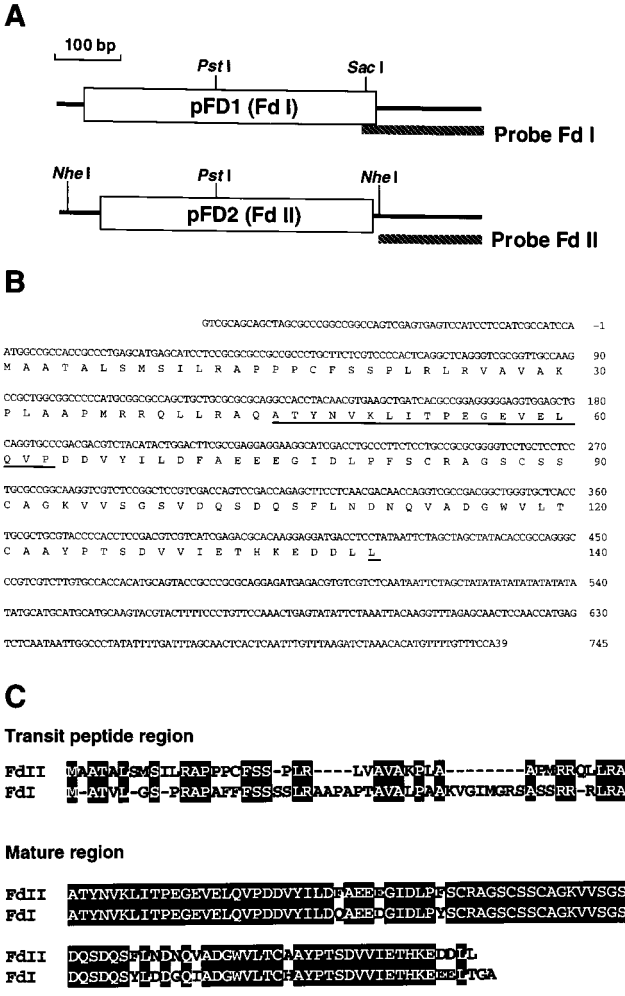

Figure 1. Restriction map of maize Fd cDNAs (A), nucleotide sequence of the cDNA designated pFD2 (B), and comparison of the deduced amino acid sequences of maize Fd I and Fd II (C). A, The coding region is represented by an open box. Positions of the gene-specific probes that were used for RNA analysis are indicated by hatched bars. B, The amino acid sequence encoded by the open reading frame is shown below the nucleotide sequence. The determined N-terminal amino acid sequence and C-terminal residue of the mature form of Fd II (Hase et al., 1991a) are underlined. C, The amino acid sequence of maize Fd II deduced from pFD2 is compared with that of maize Fd I (Hase et al., 1991a). Gaps, denoted by dashes, have been inserted to achieve maximum homology. Identical amino acid residues between Fd I and Fd II are indicated by white letters on a black background.

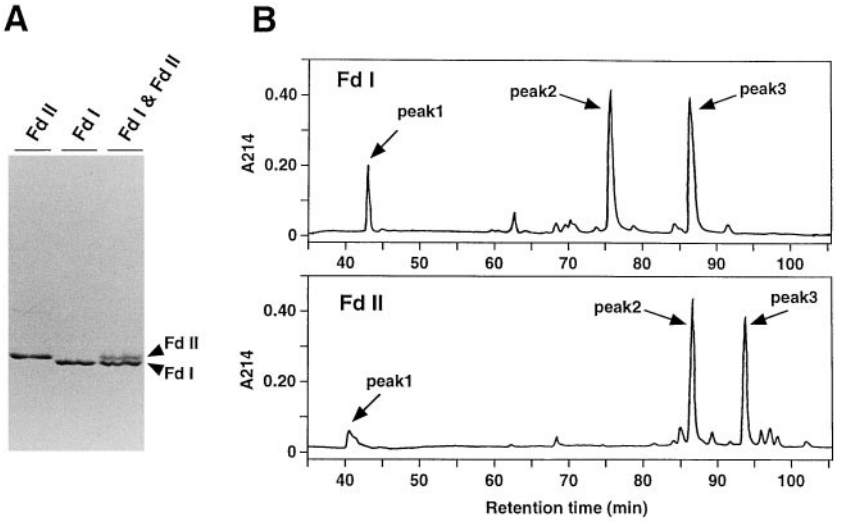

Figure 2. Peptide mapping of Fd I and Fd II prepared from maize leaves. A, Purified Fd I and Fd II (2 μg each) and their mixture were subjected to nondenaturing PAGE with a linear gradient of 15% to 25% acrylamide (Kimata and Hase, 1989) and stained with Coomassie brilliant blue. B, The purified Fds were carboxymethylated and digested with lysylendopeptidase. The resulting peptides were subjected to capillary LC/ESI-MS. Three major peaks were obtained from the samples of both Fd I and Fd II, and their M_rs were measured (Table I).

Electron-Transfer Ability of Fd I and Fd II

Fd I and Fd II were purified from the recombinant *E. coli* as functional molecules with a [2Fe-2S] cluster. The recombinant Fds were indistinguishable from the authentic Fds in maize leaves in terms of their absorption spectra and mobilities on nondenaturing PAGE (data not shown). Redox potentials of Fd I and Fd II were determined to be −423 and −406 mV, respectively, by cyclic voltammetry. These values were within the range of those reported for various photosynthetic Fds from plants and algae (Cammack et al., 1977). The electron-transfer abilities of Fd I and Fd II were assayed in a reconstitution system of Cyt *c* reduction by FNR (Fig. 6). Fd I showed higher electron-transfer activity than Fd II at all Fd concentrations examined. This was mainly due to the difference in the affinity of FNR to the Fds. The K_m was about 3 times higher for Fd II than for Fd I, whereas V_{max} did not show a large difference between the two Fds (Table II).

Among the 11 residues that differ between mature Fd I and Fd II (Fig. 1C), Asn-65 in Fd II is unique. Residue 65 is evolutionarily conserved as either Asp or Glu in all other Fds. Some of the acidic residues at the surface of Fd may interact with FNR (Knaff, 1996), and the acidic residue at position 65 seemed to be one such residue. Therefore, the weaker interaction of Fd II with FNR may be due to the presence of Asn instead of Asp or Glu at position 65. To verify this possibility, we prepared site-directed mutants of Fd I (D65N) and Fd II (N65D), and the electron-transfer activities of the mutants were compared with those of the wild-type Fds (Fig. 6). The activity of Fd II (N65D) increased to a level comparable with that of Fd I. Conversely, the activity of Fd I (D65N) was decreased. Again, these changes were reflected in the values of K_m (Table II). This result confirmed that the amino acid at position 65 conferred a significant difference between Fd I and Fd II with respect to their interaction with FNR.

DISCUSSION

We have isolated and characterized two maize Fd cDNAs, pFD1 and pFD2, the transcripts of which specifically accumulated in MC and BSC, respectively. From the analysis of the purified Fds by MS, we concluded that pFD1 and pFD2 encoded the precursors of Fd I and Fd II, respectively. Previous analysis with nondenaturing PAGE indicated that Fd I was distributed in the chloroplasts of both MC and BSC, whereas Fd II was localized only in the chloroplasts of BSC (Kimata and Hase, 1989). The present study demonstrated that the BSC-specific expression of Fd II was determined at the level of mRNA accumulation. However, the MC-specific accumulation of the transcript for Fd I was in contradiction to our previous interpretation

Table I. *Theoretical and measured M_rs of proteolytic fragments of carboxymethylated Fd I and Fd II*

The observed M_rs of each of three polypeptides obtained by the digestion of Fd I and Fd II with lysylendopeptidase (shown in Fig. 2) were determined by LC/MS. The theoretical values of the polypeptides were calculated from the deduced amino acid sequences of pFD1 and pFD2.

Peak No.[a]	Fd I			Fd II		
	Peptide[b]	Observed value	Theoretical value	Peptide[b]	Observed value	Theoretical value
1	Ala-1–Lys-6	694.3	694.8	Ala-1–Lys-6	694.6	694.8
2	Val-51–Ala-98	5224.3	5224.6	Val-51–Leu-96	5041.6	5040.4
3	Leu-7–Lys-50	4860.8	4861.1	Leu-7–Lys-50	4879.0	4878.3

[a] Numbering of peaks is taken from Figure 2. [b] Numbering of amino acid residues is based on the sequence of the mature region of Fds shown in Figure 1C.

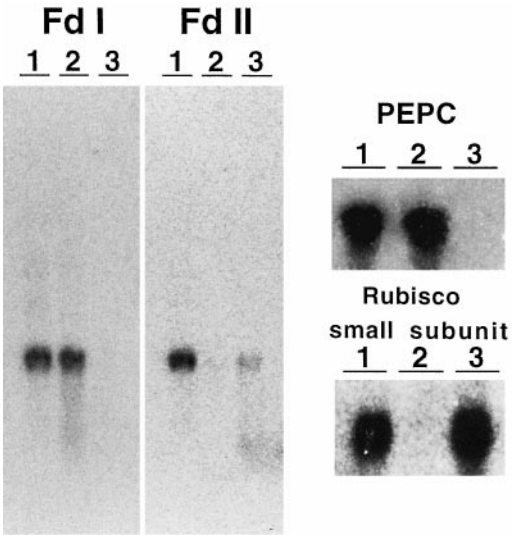

Figure 3. RNA analysis of Fd I and Fd II transcripts in MC and BSC. Total RNAs (10 μg each) prepared from maize whole leaves (lane 1), MC protoplasts (lane 2), and BSC strands (lane 3) were subjected to electrophoresis on an agarose gel containing formamide and transferred to nylon membranes. The blots were probed with ^{32}P-labeled probes for genes for Fd I, Fd II, PEPC, and the Rubisco small subunit (see "Materials and Methods").

of Fd I distribution. The most probable explanation for this discrepancy is that another Fd isoprotein(s) with an electrophoretic mobility corresponding to Fd I may be present in BSC. Our preliminary results indicated that two minor Fds (other than Fd I to IV and VI) were present in leaves and that their amounts were variable with growth stages and/or environmental conditions. Such candidates could be the product of pFD5, whose transcript was detected in the leaves of greening seedlings (Hase et al., 1991a), or the unidentified Fd cDNA clone obtained in this study.

We found that Fd I possessed higher electron-transfer ability than Fd II in the reconstitution assay in which FNR catalyzes the Fd-dependent transfer of electrons from NADPH to Cyt *c*. However, the redox potentials of the two Fds were comparable. As shown in Table II, there was a 3 times greater difference in K_m values between Fd I and Fd II, indicating a lower affinity of Fd II toward FNR. Because electron transfer in the above assay system was in the reverse direction of the noncyclic electron flow of photosynthesis, we checked the activities of the two Fds in the photoreduction of $NADP^+$ using illuminated thylakoid membranes and confirmed that the activity of Fd I was also higher (to a similar extent) than that of Fd II (data not shown).

A subsequent study with site-directed mutagenesis of Fd I and Fd II showed that the lower affinity of Fd II toward FNR was attributed to the presence of Asn-65. Fd is known to form an electrostatically stable 1:1 complex with FNR (Knaff, 1996), and chemical modification of spinach Fd indicated that several acidic residues on the surface, Asp-26, Glu-29, Glu-30, Asp-34, Asp-65, and Asp-66, were involved in the binding to FNR (De Pascalis et al., 1993). These six acidic residues are also conserved in the two maize Fds, except for Asn-65 in Fd II (Fig. 1C). The acidic residue corresponding to Asp-65 is well conserved among amino acid sequences of more than 60 Fds from plants and algae (Matsubara and Saeki, 1992). Therefore, this structural uniqueness of Fd II tempted us to hypothesize that Asn-65 may confer distinct biological properties for the function of Fd II in BSC.

The differential distribution of Fds in the two types of photosynthetic cells seems to be a general phenomenon in C_4 plants of the NADPH-malic enzyme subtype. We have

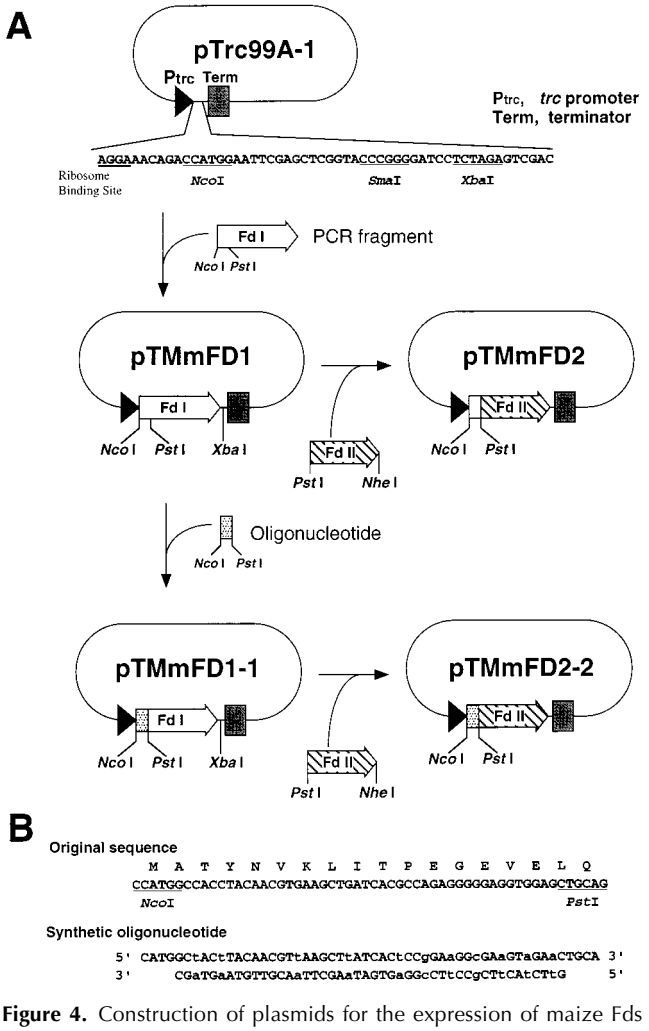

Figure 4. Construction of plasmids for the expression of maize Fds (A) and the sequence of synthetic oligonucleotides for overexpression (B). pTrc99A-1, with a deletion of the *Pst*I site in the polylinker region of the expression vector pTrc99A, was used for the construction. The region of pFD1 corresponding to the mature Fd I was amplified by PCR and inserted under the control of the *trc* promoter of pTrc99A-1 to yield pTMmFD1, as described in "Materials and Methods." Codon usage of amino acids near the translation site of pTMmFD1 was altered by replacing the *Nco*I/*Pst*I fragment with synthetic oligonucleotides shown in B to give pTMmFD1-1. Nucleotides altered from the original ones are denoted by lowercase letters. The expression plasmids for Fd II, pTMmFD2 and pTMmFD2-1, were constructed by replacing a *Pst*I/*Xba*I fragment of pTMmFD1 and pTMmFD1-1 with a *Pst*I/*Nhe*I fragment of pFD2, respectively.

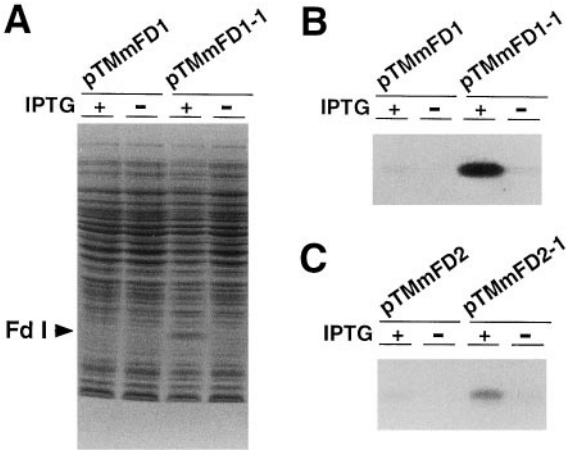

Figure 5. Expression of maize Fds in *E. coli* JM105. Cells were transformed with the Fd-expression plasmids pTMmFD1, pTMmFD1-1, pTMmFD2, and pTMmFD2-1 and grown in the presence (+) or absence (−) of isopropyl-β-D(−)-thiogalactopyranoside (IPTG), as described in "Materials and Methods." Whole cells from 10 μL of each bacterial culture were subjected to SDS-PAGE, and proteins were visualized by staining with Coomassie brilliant blue (A) or by immunolabeling with antimaize Fd I antibodies (Kimata and Hase, 1989; B and C).

found that C_4 plants of this subtype other than maize, such as *Sorghum vulgare* and *Pennisetum typhoides*, also have MC- and BSC-specific Fds (T. Hase, unpublished results). It would be interesting to examine whether the structural and functional differences found in the maize Fds are also found in these C_4 plant Fds.

The data presented here raised the following questions. First, what is the physiological significance for the inferior function of the BSC-specific Fd toward FNR? In maize almost all of the capacity for noncyclic electron flow is localized in MC chloroplasts, and the BSC chloroplasts are

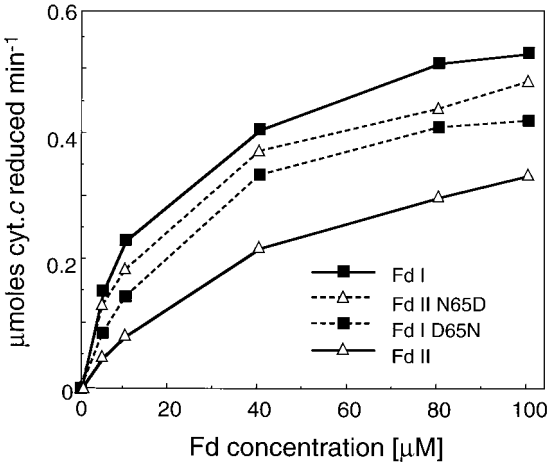

Figure 6. Electron-transfer activity of wild-type and mutant Fds. Electron-transfer activity of Fd was assayed by measuring the rate of Cyt *c* (cyt.*c*) reduction as described in "Materials and Methods." The values represent the amount (in micromoles) of Cyt *c* reduced in 800 μL of a reaction per minute. A typical example of three independent experiments is shown.

Table II. *Kinetic parameters of FNR with wild-type and mutant maize Fds*

These data were extracted from the Fd saturation curves as exemplified in Figure 6. V_{max} and K_m for Fds were determined from a double-reciprocal (Lineweaver-Burk) plot. The values are means ± SD of three independent determinations.

Fd Species	K_m	V_{max}	k_{cat}
	μM	μmol Cyt *c* min^{-1}	s^{-1}
Wild-type Fd I	13.2 ± 0.8	0.521 ± 0.04	271 ± 21
Fd I D65N	24.1 ± 2.0	0.456 ± 0.06	238 ± 31
Wild-type Fd II	43.5 ± 2.2	0.430 ± 0.01	224 ± 5
Fd II N65D	15.0 ± 1.1	0.488 ± 0.02	254 ± 10

limited in their capacity for the formation of NADPH because of the deficiency of PSII. In fact, major metabolic processes requiring NADPH, such as carbon and nitrogen assimilation and the antioxidative pathway, are restricted to MC. Therefore, the BSC-specific Fd II may not be needed to function as an efficient electron carrier for the formation of NADPH mediated by FNR. We have observed a decreased amount and activity of FNR in BSC compared with MC (T. Hase, unpublished results).

What, then, is the role of the BSC-specific Fd? Fd is known to provide electrons to several assimilatory enzymes and the thioredoxin/Fd system in response to the level of reducing power generated by photosynthesis. Because the content of Fd II in BSC on a chlorophyll basis is comparable to or even higher than that of Fd I in MC (Kimata and Hase, 1989), Fd II seems to have a major function other than electron donation to FNR. A possible candidate is the cyclic electron flow of photosynthesis to generate membrane potential, which forms the ATP required to drive the C_3 cycle in the BSC chloroplasts. Although this electron flow was demonstrated to be dependent on Fd in maize thylakoid membranes (Miyake et al., 1995), we have no in vitro assay system to examine whether Fd II is a superior molecule to mediate cyclic electron flow. So far, Fd-dependent enzymes specific to or predominant in BSC are not known. Nitrite reductase is known to occur exclusively in MC (Schmutz and Brunold, 1985), whereas sulfite reductase (Schmutz and Brunold, 1985) and Fd-dependent glutamate synthase (Sakakibara et al., 1992) are distributed in both MC and BSC. Our preliminary results indicate that substitution of Asp-65 of Fd I with Asn-65 does not affect the activity for Fd-dependent glutamate synthase. Thus, the region of Fd involved in the recognition of FNR and Fd-dependent glutamate synthase is different. It is therefore important to obtain more information about the interaction of Fd and Fd-dependent enzymes to understand the functional characteristics of the Fd isoproteins.

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