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Expression during Salt Stress and Nucleotide Sequence of cDNA for Ferredoxin-NADP$^+$ Reductase from *Mesembryanthemum crystallinum*[1]

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ABSTRACT

In the facultative halophyte *Mesembryanthemum crystallinum* (common ice plant) the enzyme ferredoxin-NADP$^+$-reductase (FNR) is coded for by a small family of 2 to 3 genes. We have determined the expression characteristics as the plants adapt to high salt and the nucleotide sequence of a full-length cDNA coding for the precursor of this chloroplast-located enzyme. On a developmental scale amounts of FNR transcripts and protein are highest in young emerging leaves. The FNR cDNA is a member of a class of genes whose expression is only slightly affected by salt stress. Even less pronounced than mRNA fluctuations, the amount of FNR protein is unaffected by salt stress. The longest FNR cDNA found was 1,419 nucleotides in size. It consisted of 74 nucleotides 5′-leader sequence, 1,095 nucleotides of protein coding sequence encoding 365 amino acids, and 247 nucleotides 3-region excluding a short poly(A$^+$) tail. As expected for a nucleus-coded chloroplast protein an amino terminal transit peptide (52 amino acids in length) was found. The mature FNR protein is predicted to contain 313 amino acids corresponding to a protein of M_r 35,713. The deduced amino acid sequence of the mature FNR protein is 93.2 and 85.9% identical to those of spinach and pea. The transit peptide of pea and spinach have 55.8 and 69.2% identity with that from ice plant.

We are studying changes in plant gene expression which are induced in response to environmental stresses such as drought and salt stress. The facultative halophyte *Mesembryanthemum crystallinum* (common ice plant) responds to salt stress by inducing the genes and enzymes of the CAM pathway (7, 13). Changes in the pathway of carbon assimilation from the common C_3-mode to CAM can be followed via the activity of a set of enzyme markers (24). The stress-induced expression of well studied biochemical markers (24), a defined procedure for stress induction (11, 13) and the demonstration of changes in gene expression make the ice plant a suitable model organism. Our studies of the changes in gene expression suggest that the expression of several of these enzymes is under transcriptional control. We have, in addition, shown that CAM induction is not developmentally programmed but environmentally controlled (21).

In our attempts to characterize genes whose mRNA levels are adjusted during the initial period of salt stress we have isolated cDNAs whose mRNA amounts change in stressed plants. While both up-regulated and down-regulated transcripts have been detected, the steady state levels of most transcripts do not change significantly in this halophyte. We are investigating members of each class. One of those mRNAs which appeared to be affected only marginally by stress was found to code for FNR.[3] FNR is a chloroplast localized protein which functions in the transfer of electrons from ferredoxin to NADP during photosynthesis. The biochemistry and kinetics of this reaction (27), some structural aspects of the enzyme, and the evolution of functional domains of different flavoproteins have been determined (15, 18). We present here the nucleotide sequence for FNR from *M. crystallinum*, including the sequence of its transit peptide which is essential for chloroplast import. We compare this sequence with the FNR sequences determined by peptide sequencing from spinach (9) and the cyanobacterium *Spirulina* sp. (26) and with the recently published cDNA sequences of pea (12) and spinach (8).

MATERIALS AND METHODS

Plant Growth

Mesembryanthemum crystallinum plants were grown in 1 L pots for 6 weeks in growth chambers, stressed by the addition of 500 mM NaCl, harvested and processed according to Ostrem et al. (13).

Library Construction

cDNA libraries in λgt11 were constructed as described (17).

Gene Isolation and Sequencing

Two full-length cDNA clones were sequenced. Inserts of cDNAs were recloned into Bluescript M13$^+$ and M13$^-$ (Stra-

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[3] Abbreviations: FNR, ferredoxin-NADP$^+$-reductase (EC 1.18.1.2); kbp, kilobase pair; PEPCase, phosphoenolpyruvate carboxylase; TP, transit peptide.

tagene, San Diego). Deletion subclones were generated according to Henikoff (6). For this purpose, plasmid DNA was cleaved with either *Hin*dIII and *Kpn*I, or with *Bam*HI and *Bst*XI. The DNA was digested with exonuclease III for 1.5 to 5 min at 37°C. The ends were rendered blunt with S1-nuclease and religated to circularize deletion subclones. The nucleotide sequences of full-length inserts and truncated fragments were determined using modified T7 polymerase (sequenase) (4, 20). Gels were dried and exposed to Kodak XOmat-AR film overnight.

Gene Expression Studies

RNA was isolated (13) from unstressed plants and plants stressed for the periods of time indicated in the legends of figures by the addition of 500 mM NaCl (11). Equal amounts of total RNA were separated electrophoretically in 1% agarose gels containing 10 mM methylmercury (11). Changes in the amount of mRNA were visualized by northern-type hybridization.

Gene Copy Number

Nuclear DNA was isolated according to Steinmüller and Apel (19). DNA fragments containing sequences from the coding region or 3′-flanking regions were radioactively labeled by nick-translation and hybridized to total DNA immobilized on nitrocellulose filters. The filters were washed at conditions of high stringency (2 × SSC, 0.1 × SSC, 58–60°C) and signals were visualized by autoradiography using Kodak XOmat-AR film.

Immunological Techniques

FNR isolated from spinach leaves (25) was used to generate antibodies in rabbits. Extracts from unstressed and stressed ice plants were separated by SDS/PAGE (sodium dodecylsulfate/polyacrylamide electrophoresis) and immunoreactive signals obtained as described (7, 13).

RESULTS

When ice plants are stressed with nutrient solution containing 500 mM NaCl, the change in complexity of the mRNA is reflected in a change of the banding pattern of the proteins synthesized from isolated poly(A^+) RNA in rabbit reticulocyte lysates (13). This change can also be visualized when radioactively labeled single-stranded cDNA derived from mRNA isolated from stressed or unstressed plants is hybridized against nitrocellulose filter replicas of cDNA sublibraries (not shown). We detected three classes of cDNAs. When compared to unstressed controls, hybridization signals either had intensified during the stress period, had remained unchanged, or had decreased. One clone, which was serendipitously obtained during screening for PEPCase-specific cDNAs, hybridized with a transcript of approximately 1,400 nucleotides in Northern-type hybridizations. Two cDNA clones for the 1.4 kbp transcript were sequenced and found to be full-length (Fig. 1). The sequences (Fig. 2) revealed mRNAs of 1,358 and 1,419 nucleotides, each containing a single long open reading frame (Figs. 2 and 3). The clones were distinguished by the length of their 5′-untranslated leader and the use of two different polyadenylation sites, as indicated in Figure 2; their protein coding regions were identical. Sequence identity of both the 5′- and the 3′-end indicated that the two cDNAs are derived from the same gene. The amino acid sequence encoded by these cDNAs is shown in Figure 3. Sequence comparisons (Los Alamos protein sequence database, release 5) showed that a long open reading frame had high amino acid homology with the protein sequences of FNR from spinach and *Spirulina* sp. This reading frame specifies 365 amino acids with a predicted molecular mass equivalent to 41,016 D. We have compared (Fig. 3) the ice plant coding region with the FNR protein sequences from spinach (369 amino acids [9]), *Spirulina* (294 amino acids [26]), and pea (360 amino acids [12]). Comparison of this FNR sequence and other FNR protein sequences showed 93.2% (spinach) and 43.4% (*Spirulina*) identity in the corresponding regions. Sequence homologies are very high in the known (15) $NADP^+$-binding and active site domains (underlined in Fig. 3). The spinach protein contains four amino acids not present in the sequence of ice plant and pea (amino acids +4 and +14 to +16). The ice plant FNR is longer than the sequenced FNR proteins by 52 amino acid. Most of the additional amino acids in the ice plant FNR can be attributed to a 52 amino acid chloroplast-specific TP sequence at the amino terminal end of the protein. This sequence is present in the pea FNR sequence. The pea amino acid sequence shows 81.6% overall identity with ice plant FNR. The hydrophilicity plot (10) of the ice plant FNR (not shown) reveals a protein of an overall hydrophilic nature. At the amino terminal end, coinciding with the transit peptide sequence, an extended hydrophobic region is calculated. Structure predictions (2) indicate a helical structure for this region.

The complexity of the FNR gene family in *M. crystallinum* was estimated by genomic Southern hybridizations (Fig. 4). Nuclear DNA was digested with several restriction endonucleases, among them endonucleases whose recognition sites had been found in the cDNA sequences. Two probes were used, one specific for the 5′-untranslated leader and the coding region, while the second probe was specific for the 3′-untranslated region alone (Fig. 1). Radioactively labeled DNA fragments were hybridized against filter-immobilized nuclear DNA fragments. The probe of the coding region revealed one to three DNA fragment bands in the hybridization (Fig. 4, panel A). The 3′-end probe invariably hybridized to one band (Fig. 4, panel B). From the sizes of DNA fragments which are bordered by recognition sites known from sequencing it could be estimated that at least two introns are present in the genes. The sizes of the introns combined must be less than 0.8 kbp. The low complexity of hybridizing bands, irrespective of the combinations of restriction endonucleases used to digest the genomic DNA, suggested that FNR constitutes a small family of genes. As indicated by the few bands produced, the FNR genes are similar including regions within about 2 kbp on either side of the coding region. Copy number was estimated by dot-blot hybridization (not shown). A reconstitution of signals for 10, 5, 2, 1, and 0.5 gene equivalents was compared with the signal strength obtained with nuclear DNA. For this

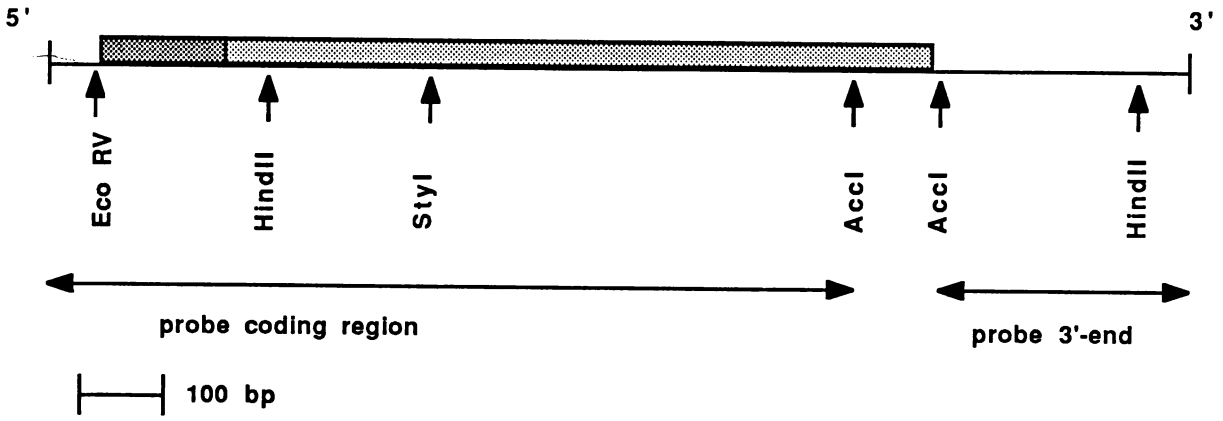

Figure 1. Structure of FNR cDNA clones. The cDNAs were cloned into the *Eco*RI-site of Bluescript M13±. The protein coding region is represented by the hatched areas. Several restriction endonuclease recognition sites are indicated. The amino terminal darkly hatched area represents the transit peptide. Deletions for sequencing were generated from both ends of the cDNAs. Probes used for hybridizations are indicated.

estimation a (haploid) genome size obtained by sorting of fluorescence-stained ice plant nuclei in the presence of mouse nuclei of known complexity (EJ DeRocher, D Galbraith, personal communication) of 350,000 kbp was assumed. Signals of equal intensity were obtained for the cloned cDNA and nuclear DNA at 1.5 to 3 genes per haploid genome in different experiments. The intensity with which the 3′-end probe hybridized to nuclear DNA and the cDNA clones showed that the equivalent of one to two genes hybridized.

When total RNA from the leaves of plants of different ages were compared, transcript levels were highest in young emerging leaves and declined approximately fivefold, as revealed by liquid scintillation counting of excised bands, in older leaves (Fig. 5A). The expression of the cDNAs was studied by Northern hybridization of total RNA from unstressed plants and from plants subjected to 500 mM NaCl for up to 4 weeks. A gene-specific probe consisting of the 3′ noncoding region (*Acc*I-*Eco*RI, 271 bp; Fig. 1) resulted in signals as they are shown in Figure 5B. Similar hybridization intensities were obtained with a probe for the coding region (*Eco*RI-*Acc*I, 1,029 bp; Fig. 1) (data not shown). During stress the gene-specific probe (Fig. 5B) showed that the amount of FNR mRNA remained approximately constant during the stress, or, at most, varied by a factor of two. After a longer period of stress (Fig. 5B, lane 7) FNR mRNA amounts consistently higher than prestress values were observed. These slight fluctuations in transcript levels and the late increase varied between experiments, although the general behavior was identical. The variations appeared to be correlated with differences in light intensity in the greenhouse and the growth chambers used rather than with stress.

FNR expression was also studied at the protein level. Polyclonal rabbit anti-FNR antibodies raised against the spinach enzyme (25) were used. As with the gene specific probes for the mRNA, high amounts of FNR protein could be demonstrated in young unstressed plants (Fig. 5C). As the plants aged, the level of FNR protein declined. Salt stress over a period of 10 d did not significantly alter the amount of the enzyme. The antibody reveals several polypeptides close together in spinach protein extracts (not shown) and in extracts from ice plant, corresponding to molecular masses of approximately 35,000, 32,000, and 31,000, respectively. Such complexity has been observed previously for FNR from several sources (5, 12), indicating that FNR exists in more than one form. This result may be produced by the presence of isoenzymes, by degradation or posttranslational processing. While the overall amount of FNR did not change during stress under our conditions, the relative amounts of these three polypeptides changed during stress and development (Fig. 5C, compare lanes 1 and 9/10).

DISCUSSION

We are interested in studying the changes of expression of genes during the salt stress-induced build-up of CAM in *M. crystallinum*. This has led to the characterization of cDNAs which code for major enzymes of this pathway such as phosphoenolpyruvate carboxylase (PEPCase; [11, 13]). Transcripts for one PEPCase isoenzyme increase approximately 50-fold (11) over 5 d of stress. Other cDNAs were found that belong to transcript classes whose amounts either do not change during stress or which appear down-regulated. One cDNA, specific for a transcript whose abundance did not change significantly after salt application, encoded FNR, a component in the light reactions of photosynthesis. In addition, no change in FNR protein was observed accompanying the adaptation of the plant to high salt. While this pattern is not interesting as such, the demonstration that control of the pathway specifying primary reactions of photosynthesis is not affected by salt is an important documentation. The lack of adaptation in gene expression during salt stress is supported by the observation that amounts of several chloroplast transcripts do not change either (our unpublished data). Interpreting this finding, it appears that in this facultative halophyte, in contrast to the enzymes regulating carbon flow (11, 13, 14, 17), gene expression for enzymes of the photosynthetic light reactions need only fine-tuning under conditions which constitute a severe stress for glycophytes. The specific cDNA

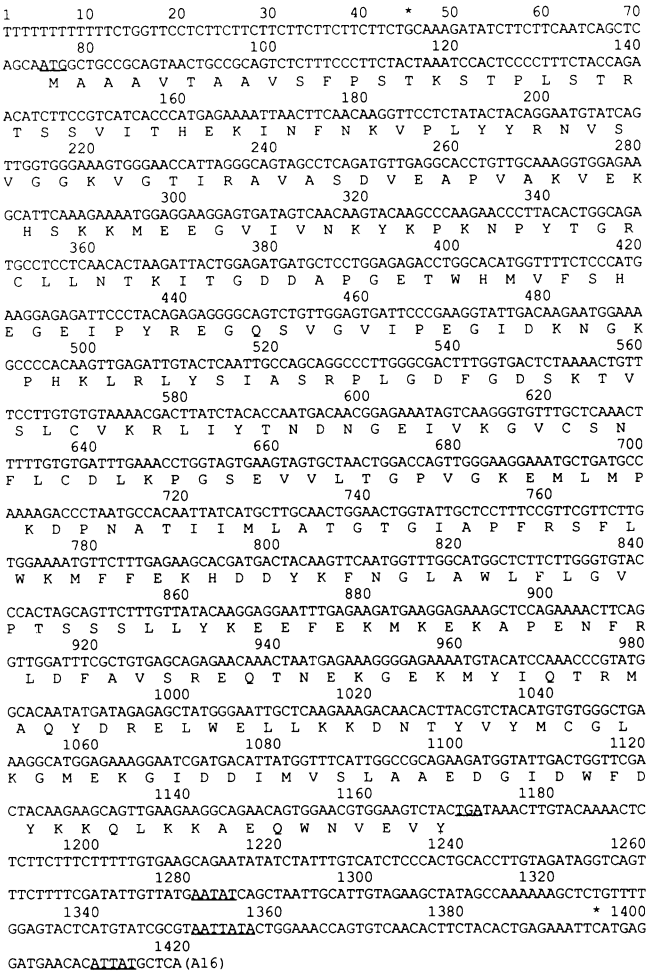

Figure 2. Nucleotide sequence and deduced amino acid sequence of FNR cDNA. No differences between this clone and another FNR cDNA in the coding regions were found. The initiation codon, ATG, the termination codon, TGA, and putative polyadenylation sites are underlined. Stars (*) denote the first and last nucleotide of the second, shorter cDNA sequence.

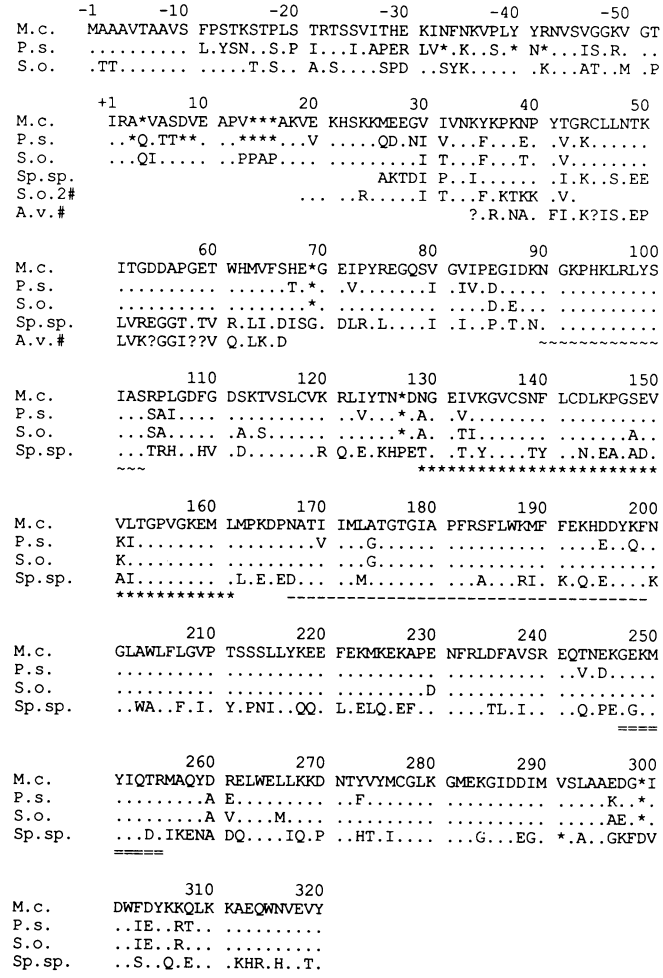

Figure 3. Comparison of amino acid sequences of FNR from *M. crystallinum*, *Spinacia oleracea*, *Spirulina* sp., and *Pisum sativum*. The ice plant (M.c.) sequence denoted (−1 to −52) represents the putative amino acid sequence of the transit peptide for chloroplast import. The actual site of processing has not been determined. (#), Amino terminal sequences available from spinach [5] and *A. variabilis* (16); (*), amino acid omissions for maximum alignment; (?), unidentified amino acids from peptide sequencing; (·), identical amino acids in other sequences. Sp.sp., *Spirulina* sp (23, 26); S.o., *S. oleracea* (8, 9); P.s., *P. sativum* (12). The underlined sequence stretches represent the locations of the ferredoxin binding site (~ ~) (27), NADP-PPi binding region (***) (15), NADP$^+$ binding region (---) (2, 15, 18), and the active site domain (===) (1), respectively.

sequenced is most likely represented by a single gene (Fig. 4). The expression of FNR is restricted to green tissue, as we have not observed any mRNA for FNR in RNA isolated from roots of either stressed and unstressed plants.

The assignment of the sequenced cDNA as coding FNR protein is based on overall sequence identities (12), and also the homologies of the documented active site domains and nucleotide binding domains of other FNR and other oxidoreductases (1, 3, 9, 15, 23). The localization of the protein in chloroplasts was corroborated immunologically (not shown). The ice plant FNR contains codons at the amino terminus which have no counterpart in the sequenced proteins from spinach and *Spirulina*. We assign the 52 amino terminal codons (−1 to −52, Fig. 3) of ice plant FNR to a TP based on sequence comparisons with other transit peptide sequences for nucleus-coded chloroplast-localized proteins (22). Without having actually determined the cleavage site, we localize it at the sequence threonine-isoleucine (amino acids −52/+1), based on the presence of the sequence, −SVGGKV−, immediately amino terminal to the putative cleavage site. This sequence is reminiscent of the sequence, −SGGVRQG−, preceding the cleavage site of the pea *rbcS* (small subunit of ribulose-1,5-biphosphate carboxylase/oxygenase, SSU) and other small subunit genes (22). It appears that the presence of amino acids that are not bulky, followed closely by charged amino acids is necessary in this region. At least part of this sequence is required for correct cleavage of pea pre-SSU proteins (22). By comparison with a variety of transit peptide sequences, this FNR-TP resembles most closely transit peptides for soluble stromal chloroplast proteins. This agrees with the fact that most of FNR is loosely bound to ferredoxin on

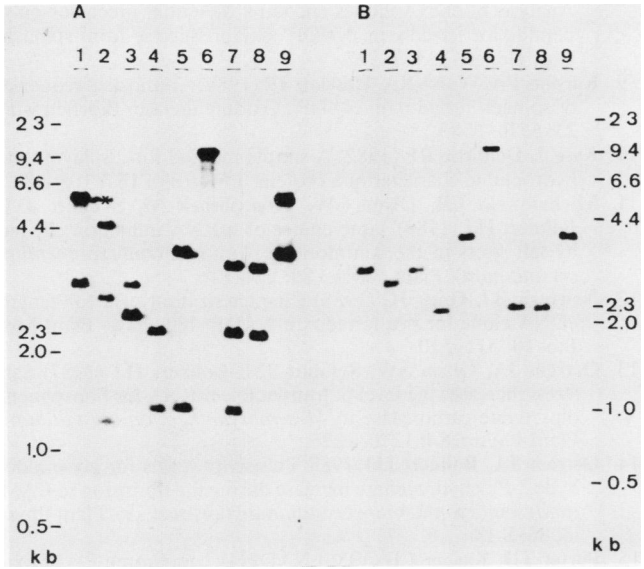

Figure 4. Determination of the copy number of FNR in *M. crystallinum* DNA. Ice plant nuclear DNA (4 μg/lane) was digested with restriction endonucleases and DNA fragments were separated by agarose gel electrophoresis (1% agarose). DNA fragments, after transfer to nitrocellulose filters, were probed with FNR coding region (1028 bp fragment) in panel A and with 3′ noncoding region (285 bp fragment) in panel B. The following restriction endonucleases were used. *Pst*I (lanes 1); *Pst*I × *Sty*I (lanes 2); *Eco*RV × *Pst*I (lanes 3); *Eco*RV × *Eco*RI (lanes 4); *Eco*RV × *Sty*I (lanes 5); *Eco*RV (lanes 6); *Eco*RI (lanes 7); *Eco*RI × *Sty*I (lanes 8); *Sty*I (lanes 9). The star (*) in panel A denotes hybridization to a fragment due to incomplete digestion of the DNA. Two different gels were used for this figure.

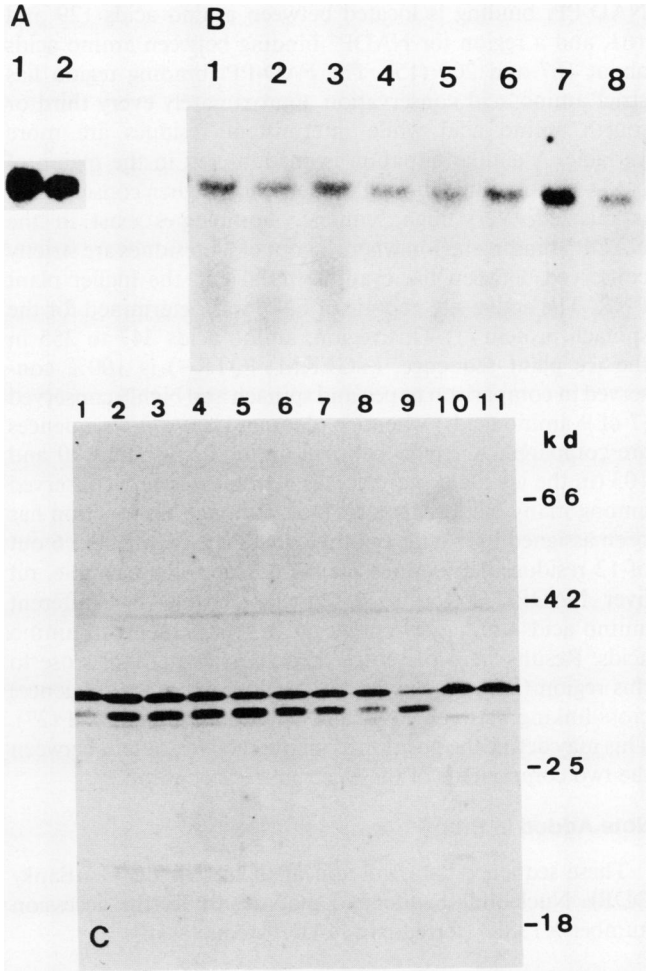

Figure 5. Expression of FNR mRNA during salt stress and at different plant age. A, Radioactively labeled cDNA was hybridized to poly(A^+) RNA (5 μg/lane) separated on a 1% agarose gel from unstressed young (4 weeks) plants (lane 1) and plants 10 weeks old (lane 2). B, A DNA fragment 285 bp in size containing a gene-specific region of FNR, was hybridized to total RNA (15 μg/lane). Lanes 1 to 6, 2 to 7 d of stress; lane 7, 10 d of stress; lane 8, 4 weeks of stress. C, FNR protein amount in *M. crystallinum*. Forty μg of total protein, solubilized in the presence of 20 mM EDTA in the homogenization buffer, was loaded in each lane. Proteins were separated in SDS/polyacrylamide (12.5%) gels and electroblotted onto nitrocellulose filters. Transfer efficiency was monitored by staining of the gels after transfer. FNR was identified using antibodies raised against purified spinach FNR (25). Lanes 1 to 6, extract from plants stressed by the addition of 500 mM NaCl for 2 to 7 d; lane 7, plants stressed for 10 d; lane 8, plants stressed for 28 d; lane 9, extracts from stressed (10 d) flowers; lane 10, extract from seedlings (4 weeks old, no stress); lane 11, extract from mature plants (7 weeks old, grown hydroponically without stress). The antibody binds to one protein band equivalent to molecular mass 35 kD and two bands close together, equivalent to approximately 32 and 31 kD, respectively. Similar size heterogeneities have been observed in other plants (5).

the surface of the thylakoid membrane and may be removed by mild EDTA treatment (25).

Discounting the TP sequence, ice plant FNR is still longer than the spinach protein at the amino terminus of the mature protein by 2 amino acids and longer than *Spirulina* FNR by 24 amino acids. This is likely due to additional processing of the enzyme or to the loss of amino terminal amino acids during purification. FNR amino termini have been reported from *Anabaena variabilis* (16) and for another spinach enzyme (5). In both sequences homologies place the peptides at a position that apparently is not at the very amino terminal end of the proteins which again points to posttranslational processing and the occurrence of isoforms (5). The partial spinach sequence (5) differs from the completely sequenced spinach FNR (9) and appears to be derived from an isogene of spinach FNR. When ice plant FNR and the partial sequence from *Anabaena* are compared, homologies are slightly less pronounced than between *Spirulina* and ice plant. The mature FNR is negatively charged (−1 at pH 7.0), while the transit peptide is positively charged (+5 at pH 7.0). The hydropathy plot of TP-FNR is compatible with the soluble nature of the enzyme. Structure predictions (10) indicate several of the observed β-sheets at the carboxy-terminus (18). Predicted structural features of the transit peptide region include an α-helix within the amino-terminal 20 amino acids, a feature that appears in structure predictions of many chloroplast signal sequences.

Sequence homologies are very obvious in some regions of the proteins. Among the functional domains identified for several oxidoreductases a region for FAD-PPi binding is located between amino acids 51 and 85, a region identified as

NAD-PPi binding is located between amino acids 129 and 161, and a region for $NADP^+$ binding between amino acids about 167 and 200 (15). The FAD-PPi binding region has strict amino acid conservation approximately every third or fourth amino acid while intermittent residues are more variable. A similar situation is encountered in the region of NAD-PPi binding, although a generally higher conservation is realized. Very high sequence homologies exist in the $NADP^+$-binding region where 26 out of 34 residues are strictly conserved between the cyanobacterial and the higher plant FNR. The active site peptide of FNR was determined for the spinach protein (1) This region, amino acids 247 to 255 in the ice plant sequence, (–GEKMYIQTR–) is 100% conserved in comparison to pea and spinach and highly conserved (7 of 9 amino acids) when ice plant and *Spirulina* sequences are compared. A region between amino acids about 90 and 103 (in the ice plant sequence) is extremely highly conserved among many oxidoreductases (15), although no function has been assigned to it. This putative domain is identical in 6 out of 12 residues between ice plant FNR and, for example, rat liver NADPH P-450 oxidoreductase, while the different amino acids are largely conservative replacements of amino acids. Results were presented recently showing that close to this region (amino acids 76 to 95 of the ice plant sequence) cross-linking between FNR and ferredoxin is observed (27). This may define the position as an interactive domain between the two components of the electron transfer complex.

Note Added in Proof

These sequence data will appear in the EMBL/GenBank/DDBL Nucleotide Sequence Databases under the accession number X13884 (ferredoxin $NADP^+$ reductase).

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