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Isolation and Characterization of a Ferredoxin Gene from *Arabidopsis thaliana*[1]

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ABSTRACT

We report the cloning and characterization of an *Arabidopsis thaliana* (L.) Heynh. (Columbia ecotype) ferredoxin gene (*Fed A*). Sequence analysis of a genomic clone shows an intron-free, 444-base pair open reading frame which encodes a 96 amino acid mature ferredoxin polypeptide preceded by a 52 amino acid transit peptide. Comparison with other plant ferredoxin proteins suggests that *Fed A* encodes a leaf ferredoxin. Genomic Southern blot analysis indicates the presence of a second, weakly related gene, consistent with other reports of at least two ferredoxins in plants. The *Fed A* gene promoter contains two regions, ACGCCACGTGGTAGATAGGATT (G-I box) and CCACGCCATTTCCACAAGC (CCAC box), which are strongly conserved in both sequence and position between the *Arabidopsis* and pea ferredoxin genes. Similarities with other better characterized plant promoter elements are also discussed.

Ferredoxins are low mol wt iron-sulfur proteins which are involved in a variety of redox reactions in plants. In photosynthesis, ferredoxin has roles both in cyclic photophosphorylation and in the photoreduction of $NADP^+$. As an important component in the regulation of carbon metabolism, the ferredoxin/thioredoxin system reduces and activates key enzymes in the carbon cycle (1). Other roles for the protein in nitrogen fixation (29), nitrate reduction (19), glutamate synthesis (15), and sulfite reduction (24) indicate the importance of ferredoxin in the assimilation of nitrogen and sulfur compounds.

The expression of a nuclear-encoded ferredoxin gene has been shown to be under phytochrome control in pea (11). Unlike the products of other well-characterized light-regulated genes, the ferredoxin protein is found in both photosynthetic and non-photosynthetic tissue (32). We undertook the cloning and characterization of a ferredoxin gene from *Arabidopsis thaliana* as a first step in examining the expression of this distinct class of light-regulated genes within a facile genetic system.

MATERIALS AND METHODS

Library Screening and DNA Sequence Analysis

An amplified *Arabidopsis thaliana* (L.) Heynh. (Columbia ecotype) EMBL3 genomic library was screened with a random-primed (6) ^{32}P-labeled ferredoxin cDNA sequence from *Silene pratensis* (28) using standard techniques (18). Prehybridization and hybridization of the nitrocellulose filters were in 5× SSC (1× SSC: 0.15 M NaCl, 0.015 M Na citrate, pH 7.0), 3× Denhardt's solution (1× Denhardt's solution: 0.02% (w/v) Ficoll, 0.02% (w/v) PVP, 0.02% (w/v) BSA), 20 μg/mL denatured salmon sperm DNA, and 1% (w/v) SDS at 55 °C. Final wash conditions were 0.1× SSC at 55 °C for 1 h. Double-stranded DNA was sequenced in Bluescript (Stratagene) by the dideoxy chain termination method using [^{35}S]dATP and Sequenase (United States Biochemical Corp.) according to the manufacturer's protocol. Both strands were sequenced using synthetic oligonucleotides as primers and resolved on a 6% (w/v) denaturing (8 M urea) acrylamide gel. Sequence comparisons were done using the GAP and BESTFIT programs of the UWGCG Software Package (3).

DNA Preparation and Analysis

Arabidopsis DNA was isolated according to Landry *et al.* (14). DNA samples were digested with appropriate restriction enzymes, separated by gel electrophoresis, and transferred to GeneScreenPlus (New England Nuclear) by standard techniques (18). Blot hybridization using random-primed (6) *Fed A* was performed in 5× SSC, 3× Denhardt's solution, 1% (w/v) SDS, and 100 μg/ml denatured salmon sperm DNA at 65 °C for 16 h. Filters were washed for 60 min in 2 ×SSC, 0.1% (w/v) SDS at 65 °C for low stringency conditions and for 30 min in 0.1× SSC, 0.1% (w/v) SDS at 65 °C for high stringency conditions. Autoradiography was done at −70 °C with an intensifying screen.

RNA Preparation

Total *Arabidopsis* RNA was prepared according to Sharrock and Quail (26) with the following modification. Frozen leaf samples (12 g) were extracted in 60 mL of extraction buffer (100 mM Tris-HCl (pH 7.6), 100 mM NaCl, 50 mM EGTA, 1% (w/v) SDS, 1.4 g/L diethyldithiocarbamate) with an equal volume of phenol:isoamyl alcohol:chloroform (25:1:25, v/v). Poly(A)$^+$ RNA used for S1 nuclease and primer extension analyses was prepared according to Lissemore *et al.* (16).

S1 Protection Assay

A synthetic oligonucleotide complementary to the first 20 bases of the ferredoxin protein coding sequence was 5′ end-

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labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase (18). *Fed A* plasmid (3 μg) was denatured and 3 pmol of labeled primer were annealed to the template in 10 mM Tris-HCl (pH 8.0), 60 mM NaCl, and 66 mM $MgCl_2$. The four deoxynucleotides (30 mM each, final concentration) and 5 units Klenow (Bethesda Research Laboratories) were added and reacted at room temperature for 1 h. The reaction was terminated by phenol/chloroform extraction and ethanol precipitation and digested with *Acc*I to linearize the plasmid. The product was run on a 6% (w/v) denaturing acrylamide gel and a single 500 base band was visualized by autoradiography, cut from the gel and eluted in 500 mM NH_4 acetate, 1 mM EDTA, and 0.1% SDS. The single-stranded primer extension product (32,000 cpm) was precipitated with 1 μg of poly(A)$^+$ RNA from *Arabidopsis* leaves and 20 μg of glycogen (Boehringer Mannheim Biochemicals), and resuspended in hybridization buffer (400 mM NaCl, 40 mM Pipes (pH 6.4), 1 mM EDTA, 50% deionized formamide) and incubated 16 h at 37 °C. Ten volumes (100 μL) of cold S1 nuclease buffer (250 mM NaCl, 30 mM Na acetate (pH 5.5), 1 mM $ZnSO_4$, 10 μg/mL denatured salmon sperm DNA) containing S1 nuclease (Bethesda Research Laboratories) at a final concentration of 250 units/mL were added and reacted for 1 h at 37 °C. To precipitate the protected fragments, 30 μL of S1 termination buffer (2.5 M NH_4 acetate, 50 mM EDTA, 400 μg/mL tRNA) and 300 μL of ethanol were added. Half the precipitate was loaded onto a 6% (w/v) denaturing (8 M urea) acrylamide gel and visualized by autoradiography.

Primer Extension Analysis

Primer extension analysis was according to Twell *et al.* (31). One pmol of the same ^{32}P 5′ end-labeled oligonucleotide used in the S1 protection assay was annealed to 1 μg of poly (A)$^+$ RNA (from white light-grown shoots) at 37 °C for 3 h. The primer was extended with AMV reverse transcriptase at 37 °C for 45 min. Following RNA base hydrolysis, the product was run on a 6% (w/v) denaturing (8 M urea) acrylamide gel and visualized by autoradiography.

RESULTS AND DISCUSSION

Genomic Cloning and Southern Analysis

Three 16 kb clones from an EMBL3 genomic library of *Arabidopsis thaliana* were isolated using a cDNA probe coding for the amino terminal region of the mature *Silene pratensis* ferredoxin (28). Restriction digests showed all three clones to be identical. One representative clone (λ402) was restriction mapped (Fig. 1A) and a 1.5 kb *Hin*cII-*Eco*RI segment was identified as the sole hybridizing fragment and subcloned into the Bluescript vector pBSKS$^-$. The 1.5 kb fragment, shown to contain the entire ferredoxin protein coding region and termed the *Fed A* gene (see below), was used to probe an *Arabidopsis* genomic Southern blot and washed with increasing stringency. Under low stringency conditions two bands were seen in each of the five restriction digest lanes (Fig. 1B). The stronger bands correspond in size with those obtained from comparable digestions of the λ402 clone (data not shown). The weaker signals were lost with a higher stringency wash (Fig. 1C) suggesting the presence of a second sequence with limited similarity to *Fed A*. This result is in accord with recent reports of two or more ferredoxins in most plant species (23, 32).

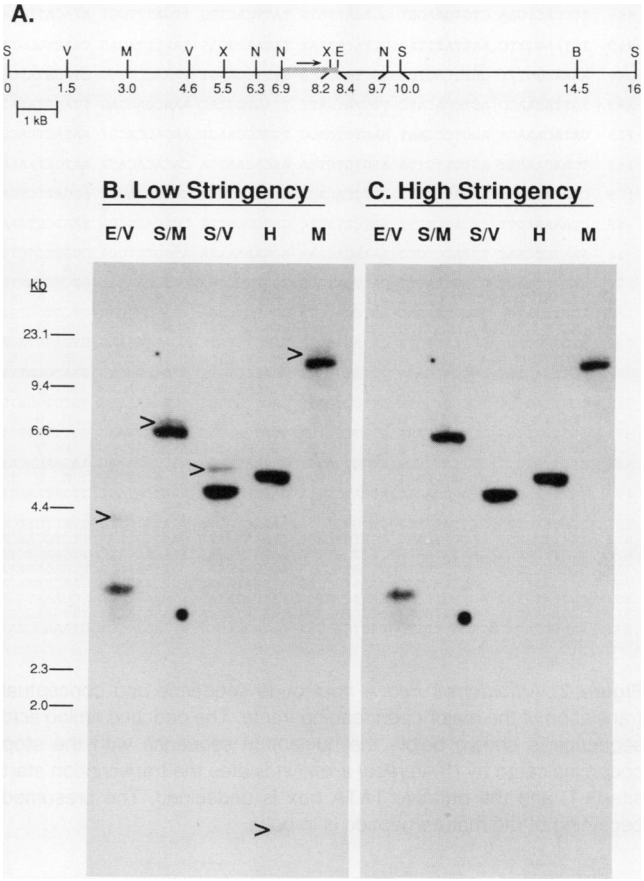

Figure 1. *Arabidopsis* ferredoxin genomic clone and Southern blot analyses. (A) Restriction map of the 16 kb insert from genomic clone λ402. The position of the *Fed A* gene (*shaded box*) in the clone is positioned above a scale showing the cumulative size of the restriction fragment lengths, beginning at *Sal*I(0) at the left. *Arrow* indicates the direction of transcription. *Hin*cII sites were mapped only within the *Bam*HI(3.0)/*Sal*I(10.0) segment and *Eco* RI, *Eco*RV and *Xba*I were mapped only between *Sal*I(0) and *Sal*I(10.0). (B and C) *Arabidopsis* genomic Southern blot probed with *Fed A*. Genomic DNA (3 μg) was restricted with the enzymes indicated and loaded in each lane. Molecular size scale is shown at the left. (B) Hybridization remaining after low stringency wash (60 min at 65 °C, 2 × SSC, 0.1% (w/v) SDS). Arrowheads indicate positions of weakly hybridizing bands. (C) Hybridization remaining after high stringency wash (30 min at 65 °C, 0.1 × SSC, 0.1% (w/v) SDS). E, *Eco*RI; *H, Hin*d III; M, *Bam*HI; N, *Hin*cII; S, *Sal*I; V, *Eco*RV; X, *Xba*I.

Peptide Sequence Comparisons and Transcript Mapping

The nucleotide sequence of the 1.5 kb *Fed A* fragment contains a single large open reading frame of 444 bp (Fig. 2). The 148 amino acid polypeptide encoded by this region has extensive similarity to other known ferredoxin sequences (Fig. 3). The 52 amino terminal residues are similar in length and

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-469  GTCGACTGAA GTGTGAAGGT GGAGATTATG TATTCACTTG TTGATTTGGT ATACATTCTA
-409  TGTAAGGTTC AATTATTTAC GTTATATAAT TATAATGGAG TAATTTACAG TAATTGGGTT
-349  AAAATGGTTT GATTCGGTCA GGTTGATACG GTTTGGAAGT TAAACCCGGC CTAGATATGA
-289  TGTTACAACC AGTCCACATC TTTTATGATT TTAGTGGAAC AAACGAAGAG TTATTTAGAC
-229  GATACAAACA AGGTCCGAAT AAGTGTGAGC TGTCCCAAGT AAGACCACGT AATACTCACC
-169  TCAACAAGAT AGTGTTCTTA AAGTGTGTCA AACACAATCA CACACACACA AATCATAAAA
-109  CACAAAGACG ATAATCCATC GATCCACAGA ATAGACGCCA CGTGGTAGAT AGGATTCTCA
 -49  CTAAAAAGTT CTCACCTTTT AATCTTTCTC CACGCCATTT CCACAAGCCA TAATCCTCAA
  12  AAATCTCAAC TTTATCTCCC AAAACACAAA ACAAAAAAAA ATGGCTTCCA CTGCTCTCTC
                                                  M  A  S  T  A  L  S
  72  AAGCGCCATC GTCGGAACTT CATTCATCCG TCGTTCCCCA GCTCCAATCA GTCTCCGTTC
       S  A  I  V  G  T  S  F  I  R  R  S  P  A  P  I  S  L  R  S
 132  CCTTCCATCA GCCAACACAC AATCCCTCTT CGGTCTCAAA TCAGGCACCG CTCGTGGTGG
       L  P  S  A  N  T  Q  S  L  F  G  L  K  S  G  T  A  R  G  G
 192  ACGTGTCACA GCCATGGCTA CATACAAGGT CAAGTTCATC ACACCAGAAG GTGAGCTAGA
       R  V  T  A  M  A  T  Y  K  V  K  F  I  T  P  E  G  E  L  E
 252  GGTTGAGTGT GACGACGACG TCTACGTTCT TGATGCTGCT GAGGAAGCTG GAATCGATTT
       V  E  C  D  D  D  V  Y  V  L  D  A  A  E  E  A  G  I  D  L
 312  GCCTTACTCT TGCCGTGCTG GTTCTTGTTC GAGCTGTGCT GGTAAAGTTG TGTCTGGATC
       P  Y  S  C  R  A  G  S  C  S  S  C  A  G  K  V  V  S  G  S
 372  TGTTGATCAG TCTGACCAGA GTTTCCTTGA TGATGAACAG ATTGGTGAAG GGTTTGTTCT
       V  D  Q  S  D  Q  S  F  L  D  D  E  Q  I  G  E  G  F  V  L
 432  CACTTGTGCT GCTTACCCTA CCTCTGATGT TACCATTGAA ACCCACAAAG AAGAAGACAT
       T  C  A  A  Y  P  T  S  D  V  T  I  E  T  H  K  E  E  D  I
 492  TGTTTAAGCC TCACCTACTC ACCAGCTTTT GATGGTTTAA AAATCATGTC TTTATAAATT
       V  *
 552  TCACATTTTG GGTTGAGTTT GTTGTTACTA AAAACTATTG TTATCTGTTG TTATTGTTCC
 612  TGGTTTGGCT CACCATCAAT CGATGACATT TTAAACTATG CAACTGCAAA TTCTGCAACA
 672  CTTTCGATGA GAATCTAACA TTATCGTTTA AACATTGGAA ATACATTTTC TTGAAGTCTA
 732  GCTAGCTTTG GTTTGTAGTT CTTATTCTGA ACTCAACAAT CATCAAAGTA TCAAGAAAAA
 792  TCCGATTTGC GAGCAATTGT GAAATCTTAG ATTGATAAAT TCTCTAGAAA GAACTATACA
 852  TGTTATTTGT AGCTATAGAA GAAACTATGG AATCTTATAC TCTAATTAAG CCCAAATGGC
 912  AAGAGAAGAC ATGAGTCCAG CTCCTAGGAA CAACATTACA TAAGATACAA TTTGAAGCCT
 972  AAAGTTACAC CTCATTTTTG TACTCAAGAA ATCAGCAGCT ATGAGATCCA CTAAAGCCAT
1032  GTACACAAGA ATTC
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Figure 2. *Arabidopsis Fed A* nucleotide sequence and conceptual translation of the major open reading frame. The deduced amino acid sequence is shown below the nucleotide sequence with the stop codon indicated by (*). *Vertical arrow* indicates the transcription start site (+1) and the putative TATA box is underlined. The presumed beginning of the mature peptide is in bold.

composition to transit peptide sequences identified from other ferredoxins (Fig. 3A). These presequences play a role in directing the nuclear-encoded proteins to their appropriate positions in the chloroplast (12). They are cleaved from the precursor polypeptide once inside the plastid, resulting in the mature protein. The processing site for the *Arabidopsis Fed A* ferredoxin was inferred by comparison with numerous ferredoxin protein sequences (21) which begin at alanine residues homologous in position to that indicated in Figures 2 and 3B. Unlike the transit peptides of SSU[2] and CAB, which share extensive regions of identity (12), the four known ferredoxin presequences are highly similar only near the putative proteolytic processing site (Fig. 3A). Recent studies using deletion mutations of the *Silene* ferredoxin transit peptide lend further support to the importance of the residues near the cleavage site for proper binding and import (27). The absence of a strong amino acid sequence consensus among the ferredoxin presequences allows that identity in function may instead derive from secondary structure. However, computer program analyses of the four transit sequences were unable to uncover consistent similarities in secondary structure (data not shown). A similar result has been reported for presequences of other chloroplast proteins (12).

Comparison of the remaining 96 amino acids with a sampling of known ferredoxin polypeptides clearly demonstrates that *Fed A* encodes a plant ferredoxin (Fig. 3B). The deduced mature protein is highly similar to previously sequenced ferredoxin polypeptides and is nearly identical (96%) to a ferredoxin from the leaves of the related mustard, *Raphanus sativus* (radish) (Fig. 3B). At least two isoforms of ferredoxin have been found in most plant and algal species but their localization and significance have not been clear (23). Recently, three ferredoxin isoforms were purified from radish roots and two from leaves (32). Amino acid comparisons showed that these root and shoot ferredoxins were most similar to other isoforms derived from within their respective tissue groups, although differences among the five in electron-donating functions could not be seen *in vitro*. *Arabidopsis Fed A* shares 92 of 96 amino acids with the radish leaf isoform and only 62 of 96 residues with the closest root isoform (Fig. 3B), strongly suggesting that *Fed A* encodes a leaf-type ferredoxin. Studies are underway to directly determine tissue specific expression of this gene.

The transcription start site of *Fed A* was mapped by S1 nuclease protection and primer extension analyses using poly $(A)^+$ RNA from shoots of mature green plants (Fig. 4). Both methods gave identical results, indicating the absence of introns within the 5′ untranslated region. The multiple bands in the S1 lane are most likely due to limited digestion of the end of the RNA/DNA duplex by the nuclease. Based on these results, it is concluded that a single ferredoxin-specific message extends to a position 51 bases upstream of the ATG codon and begins 27 bases downstream of a putative TATA box (TAAT) (Fig. 2).

Promoter Comparisons

Nucleotide sequences 5′ to protein coding regions have been shown to control gene transcription. Strongly conserved short sequence elements (boxes) have been found to be shared

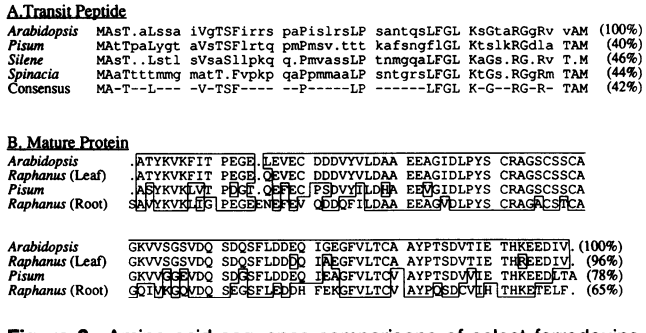

Figure 3. Amino acid sequence comparisons of select ferredoxins. (A) Optimal alignment of the putative *Fed A* transit peptide with comparable regions from *Pisum* (5), *Silene* (28) and *Spinacia* (33) based on cDNA and genomic sequences. Agreement at three of four residues was required for consensus. Final alignments were done by eye. (B) Comparison of the deduced amino acid sequence of *Arabidopsis Fed A* ferredoxin with that from *Pisum* (5) and with root and leaf ferredoxin protein sequences from *Raphanus* (radish) (32). A second root-specific radish ferredoxin sequence has been reported (32) and shares a similar identity to *Fed A* as the one shown here. The radish leaf ferredoxin has four microheterogeneities (32) which do not significantly alter the relative similarities shown above. Percent identity to the *Arabidopsis* sequence is shown at right in (A) and (B).

[2] Abbreviations: SSU, ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit protein; CAB, Chl *a/b* binding protein.

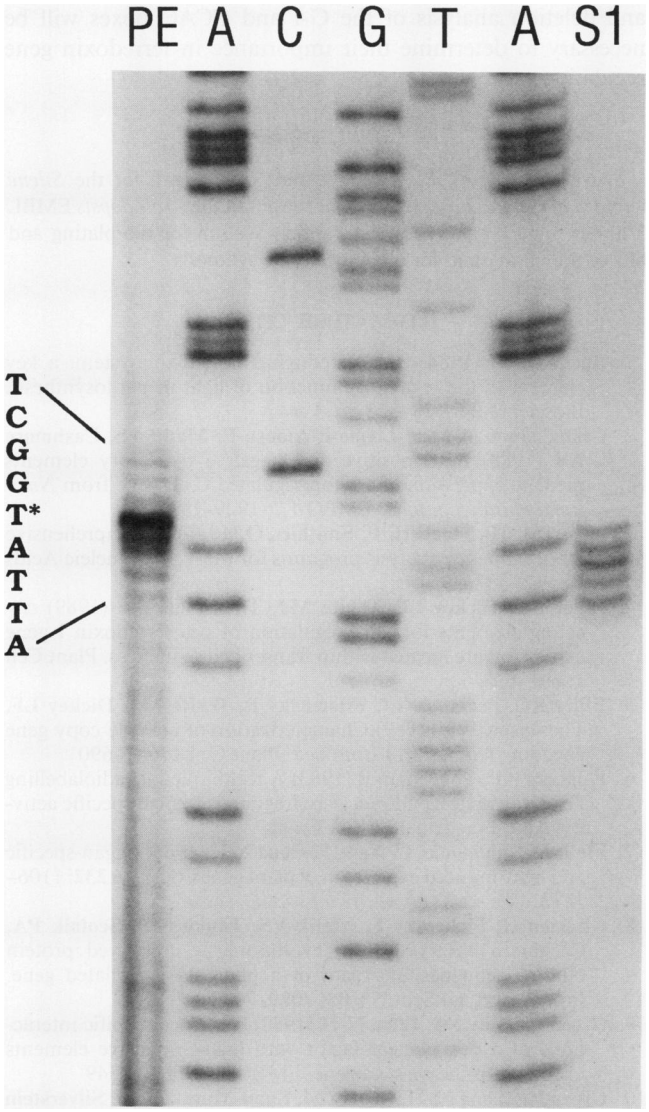

Figure 4. S1 nuclease protection (S1) and primer extension (PE) analyses of the 5′ end of the *Arabidopsis Fed A* transcript. Reaction products were run on either side of a dideoxy sequencing ladder (A C G T A) on a 6% (w/v) denaturing gel. The 20 base oligonucleotide primer used for the sequencing and primer extension reactions, and to produce the S1 nuclease protection probe was complementary to the *Fed A* coding strand between +52 and +71. A portion of the gene sequence, complementary to the transcribed strand, is shown to the left. Both methods indicated the same transcription start site (T*) 51 bases upstream of the initiation codon.

among different plant species, often between promoters of genes with similar modes of regulation (*e.g.* SSU and CAB) (8, 20, 22). These elements are presumed to play key roles in gene regulation and some have been shown to bind nuclear proteins *in vitro* and stimulate or enhance transcription in transgenic plants (2, 10, 30). To determine whether similar sequence elements are present in the ferredoxin gene, computer-based comparisons were made between the putative *Fed A* promoter (−1 to −469) and a number of plant promoter consensus sequences (2, 7, 8, 17, 25). The pea *Fed-1* promoter (5), the only other ferredoxin gene sequence reported from higher plants, was also included in the comparison. Figure 5 shows only the most significant similarities to emerge.

The *Fed A* sequence between −53 and −71 shows a high degree of similarity to the core regions of the previously reported G and I boxes (Fig. 5A) (8). The G box contains a 6 bp palindrome, CACGTG, which has been identified in a wide range of plant promoters and which can bind specific nuclear factors from a wide range of species (including *Arabidopsis*) as assayed by gel shifts (8, 25, 30). The GATAAG region of the I box is highly conserved among many SSU genes (8) and recently has been shown to be involved in the control of the *Arabidopsis rbcs1-a* gene (R. Donald, personal communication).

Most striking is the coincidence of both boxes within a region of the *Fed A* promoter (−54 to −75) that also bears a high degree of identity to a stretch of the *Fed-1* promoter of pea (−44 to −65) (Fig. 5C). This 22 bp motif is largely a hybrid of G and I box sequences which we term the G-I box. The pea and *Arabidopsis* G-I boxes differ from the GATAAG consensus of the I box by a conservative A to G base change (GATAGG), and *Fed-1* replaces the CACGTG consensus with CACCTG. Methylation at the CpG of the CACGTG

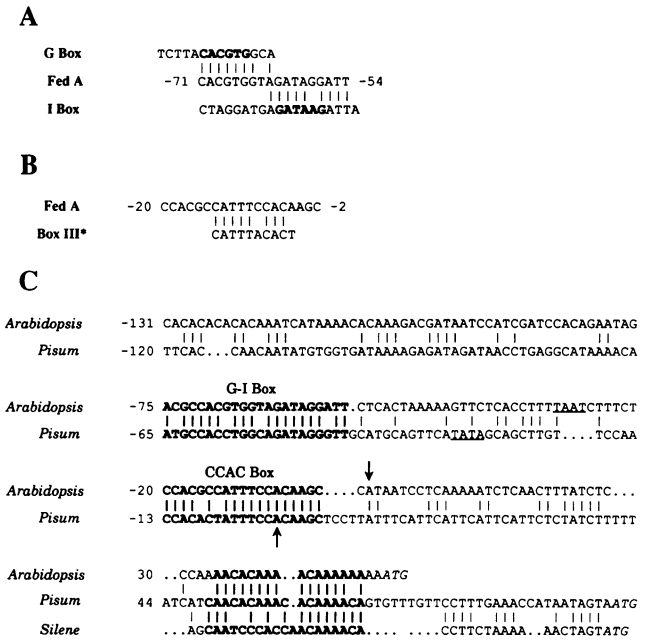

Figure 5. Comparison of *Fed A* 5′ flanking sequences with similar regions of select genes. (A) Optimal alignment of *Fed A* G-I box with conserved sequences of the G and I boxes (8) and (B) pairing of the *Fed A* CCAC box with Box III* from the *rbcs-3a* SSU promoter of pea (13). (C) Alignment of *Arabidopsis* and pea (5) ferredoxin 5′ flanking sequences. The highly conserved G-I and CCAC boxes are in bold. The putative TATA boxes are underlined and *vertical arrows* indicate transcription start. The *Silene* ferredoxin sequence (28) is included in the comparison of the CA-rich leader (in bold) present near the translation start in all three genes. The pea-*Silene* alignment is from Elliot *et al.* (5). The initiator ATG is italicized.

motif has been shown to inhibit tobacco nuclear factor binding *in vitro* (30), but more complete, base-for-base mutation analyses have not been reported for these two motifs. In pea, a region 5′ to the *Fed-1* coding sequence (−23 to −494) which contains the G-I box was shown by gel shift assays to specifically bind one or more protein factors from pea nuclei (4). However, subsequent deletion of the G-I box did not affect the binding assay results.

The G-I box lies 30 to 33 bases upstream of a stretch of 19 bases that is nearly identical between the pea and *Arabidopsis* ferredoxin promoters, which we have termed the CCAC box (Fig. 5B). It lies at or near the transcription start site of both genes (*Fed A*: −2 to −20; *Fed-1*: +4 to −15) and contains a central TTT core flanked on both sides by an 8 bp CA-rich stretch. Within this region the sequence CCAC, which is half the G box dyad (see 25), occurs twice. A similar region is conserved in pea SSU genes (Box III*) (Fig. 5B), but is located much further upstream (*rbcS-3a*; − 257 to −248) and lacks the CCAC repeat (13). Competition studies and DNase I footprinting experiments have shown that boxes III*, III, II* and II bind the same factor (GT-1) from pea nuclei in a light-independent manner (9).

The positions of the G-I and CCAC motifs relative to each other are highly conserved in both genes (*Fed A*: 33 bp; *Fed-1*: 30 bp). This distance is *ca.* three turns of a DNA helix and could permit an interaction between factors which might bind the two boxes that is dependent on a specific spatial relationship. Deletions and insertions which alter the relative spacing between the two elements should prove fruitful.

A third region of similarity is found within the 5′ untranslated leader of pea, *Arabidopsis* and *Silene* ferredoxins (Fig. 5C). A highly CA-rich sequence lies near the ATG initiator codon of all three transcripts and is similar in composition and position to sequences found in *Arabidopsis cab2* and *cab3* genes (22). A 34 nucleotide deletion of the *cab3* gene which included this region reduced the expression of a reporter construct by six-fold in transgenic plants (22). Experiments are in progress to determine the functional significance of this sequence for ferredoxin.

The G-I and CCAC boxes described above illustrate the high degree of sequence conservation between specific promoter regions of the pea and *Arabidopsis* ferredoxin genes. Both boxes also share sequence similarity with regions of other plant promoters shown to bind nuclear proteins and therefore are candidates for elements which may be essential to the control of ferredoxin gene transcription. Initial reports indicated a phytochrome control of message abundance for pea ferredoxin (11) and one possibility is that these upstream boxes are involved in light-regulated gene transcription. However, Elliot *et al.* (4) have reported that light-regulated accumulation of ferredoxin mRNA is not controlled by 5′ genomic sequence elements in pea. Instead, they concluded that light regulatory elements reside within transcribed sequences and possibly involve changes in message stability. In addition, GBF and GT-1, which have been shown to bind to G Box and Box III* sequences (*i.e.* the core elements of the G-I and CCAC boxes), respectively, are present in nuclear extracts from both light and dark grown tissue and their role in light-regulated gene expression is unclear (8, 9). Further mutation and deletion analysis of the G-I and CCAC boxes will be necessary to determine their importance in ferredoxin gene regulation.

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