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Tissue-specific expression directed by an *Arabidopsis thaliana* pre-ferredoxin promoter in transgenic tobacco plants

Oscar Vorst, Frans van Dam, Renske Oosterhoff-Teertstra, Sjef Smeekens and Peter Weisbeek
Department of Molecular Cell Biology and Institute of Molecular Biology, University of Utrecht, Padualaan 8, 3584 CH Utrecht, Netherlands

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Abstract

We have isolated and analyzed a pre-ferredoxin gene from *Arabidopsis thaliana*. This gene encodes a 148 amino acid precursor protein including a chloroplast transit peptide of 52 residues. Southern analysis shows the presence of a single copy of this ferredoxin (Fd) gene in the *A. thaliana* genome. Its expression is tissue-specific and positively affected by light. Response times, both to dark and light conditions, are remarkably rapid.

A chimeric gene consisting of a 1.2 kb Fd promoter fragment fused to the β-glucuronidase reporter gene was transferred to tobacco. This fusion gene is expressed in a tissue-specific way; it shows high levels of expression in green leaves, as compared to root tissue.

Introduction

Ferredoxin is a low molecular weight (ca. 10 kDa) iron-sulphur protein, present in all photosynthetic organisms. Its active center is a [2Fe-2S] cluster, chelated by four conserved cysteine residues [8]. Ferredoxin functions as an electron carrier in the photosynthetic electron transport chain of the chloroplast, where it is active at the reducing site of PSI. It plays a central role in the distribution of reducing power over several different processes in the cell. Usually two types of ferredoxins (I and II) are present in the same species. These two types differ considerable (ca. 25%) at the amino acid level, but share many structural and functional properties [4, 25].

Although the plant ferredoxin is functional in the stroma of the chloroplasts, it is encoded in the nucleus as a precursor protein [22]. This pre-apoferredoxin possesses an N-terminal extension, the transit peptide, involved in chloroplast import [23, 24]. Following post-translational translocation across the chloroplast envelope, the transit peptide is removed by a stromal protease [19] and the iron-sulphur cluster enzymatically inserted [26].

The expression of ferredoxin was shown to be light-regulated in pea [3, 12, 13]. Exposure of dark grown plants to 24 h of white light results in a 10-fold increase of the ferredoxin mRNA level. A pulse of red light causes a rapid response, resulting in a 2-fold increase in transcript levels within one to two hours. Because this response can be cancelled by far-red light, phytochrome is believed to be involved in this regulation.

To study the genomic organization and expres-

sion regulation of ferredoxin in higher plants we isolated an *Arabidopsis thaliana* Fd gene. The isolated gene is uninterrupted and encodes a precursor similar in structure to the *Silene pratensis* preferredoxin. Southern analysis shows the presence of a single copy of the Fd gene per haploid genome. Fd expression in *A. thaliana* appeared to be tissue-specific and light-regulated. A 1.2 kb promoter fragment was capable of directing high-level, tissue-specific expression in transgenic tobacco plants.

Materials and methods

Plant material

Arabidopsis thaliana strain Columbia was grown in soil at 25 °C under continuous fluorescent light (seedlings) or in the green house (mature plants).

Nucleic acid manipulation

All DNA manipulations were performed essentially as described [15].

Library construction and screening

A. thaliana genomic DNA isolated essentially according to Murray and Thompson [18], was partially digested with *Sau* 3A and ligated to λEMBL3 arms [27]. The library was packaged and amplified by transfecting *Escherichia coli* VCS257. Ca. 15000 recombinant phages were screened by plaque hybridization using a random-primer-labelled probe [5]. This probe consisted of a *Bal* I-*Nco* I insert derived from pFD1, encoding the *Silene pratensis* Fd mature protein [22]. The filters were hybridized in 50% formamide, 5 × SSC, 50 mM sodium phosphate pH 6.5, 1 mM EDTA, 0.1% SDS, 0.1 mg/ml denatured salmon sperm DNA and 5 × Denhardt's solution at 42 °C overnight. Next, the filters were washed three times in 5 × SSC at 60 °C and exposed. Positive plaques were rescreened at lower density.

Subcloning and sequence analysis

DNA restriction fragments were isolated and subcloned in pEMBL vectors [2] and ssDNA was prepared. This was used for sequence analysis by the dideoxy chain termination method [20], using either synthetic oligonucleotides or the M13 primer. Both strands were sequenced at least once.

RNA isolation and transcript analysis

Total RNA was isolated using aurintricarboxylic acid (Sigma A-1895) as RNase inhibitor, as described by Kuhlemeier *et al.* [14]. To prepare a single-stranded probe the coding region primer 5′GGAGCTGGGGAACGACG (positions 49–65; Fig. 2) was annealed to pFd2.11 (consisting of the 1.26 kb *Hin*c II-*Xba* I fragment cloned into the multiple cloning site of pEMBL19) ssDNA. A labelled second strand was synthesized in 10 mM Tris-HCl pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 0.07 mM each of dATP, dGTP and dTTP, 0.007 mM dCTP and 10 μCi ^{32}P-dCTP (3000 Ci/mmol) with Klenow fragment at 30 °C and subsequently digested with *Hin*c II. The resulting 582 nt coding strand fragment was separated by electrophoresis on a denaturing 5% polyacrylamide gel and hybridized with total leaf RNA (25 μg) at 48 °C for 16 h in a 20 μl volume [15]. Next, the hybridization mixture was diluted with 200 μl S1 nuclease buffer containing 200 units S1 nuclease, and incubated at 35 °C for 1 h. The reaction was extracted with phenol and ethanol-precipitated with 5 μg carrier tRNA. The length of the protected fragment was determined on a 6% sequencing gel with the sequencing ladder of pFd2.11 primed with the same oligonucleotide, as a reference.

Gene transfer

A novel *Xba* I-site, 5 bp upstream of the ferredoxin start codon, was created by oligonu-

cleotide-directed mutagenesis. This required the change of ACAAAA to TCTAGA (positions −10 to −5; Fig. 2). The 1.2 kb fragment resulting from digestion with *Xba* I and *Hinc* II (partial) was cloned in the pEMBL19 *Xba* I and *Hinc* II sites. In this way the promoter could be transferred to the pBIN19-derived vector pBI101.1 [11] as a *Hind* III-*Xba* I fragment, placing the GUS gene under its control (pFd2.64).

Binary vectors were transferred from *E. coli* JM101 into *Agrobacterium tumefaciens* LBA4404 [9] by triparental mating, with HB101/pRK2013 providing the mobilization functions. Leaf disks of *Nicotiana tabacum* cv. Petit Havana SR1 were transformed using the leaf disk procedure [10]. Selection for transformed calli was done on an MS agar medium [17] containing 300 μg/ml kanamycin, 500 μg/ml carbenicillin, 0.01 μg/ml naphtaleneacetic acid and 2 μg/ml benzylaminopurine. Shoots were rooted on MS medium without hormones and antibiotics. From each construct at least 10 individual transformants were regenerated, transferred to soil and grown in a growth chamber under a 14 h/10 h light/dark cycle at 24–26 °C and 55–65% relative humidity. Southern blotting showed the presence of one to three integrations in most transformants.

β-Glucuronidase assay

A 10–12 cm leaf of each transformed tobacco plant was harvested 25 days after transfer to soil and frozen on dry ice. Frozen tissue (stored at −70 °C) was ground in 50 mM sodium phosphate (pH 7.0), 10 mM EDTA, 0.1% Triton X-100, 0.1% sarkosyl and 10 mM β-mercaptoethanol. Enzymatic reactions were incubated at 37 °C in the same buffer, containing 1 mM 4-methylumbelliferyl-β-D-glucuronide as a substrate, and were stopped by adding 100 μl of the reaction mixture to 2 ml 0.2 M Na_2CO_3. Formation of 4-methylumbelliferone was quantified by measuring fluorescence at 450 nm (under excitation at 365 nm). The protein content of the extracts was determined according to Bradford [1].

Results

Isolation of a genomic clone coding for pre-ferredoxin

A genomic library was constructed by cloning a partial *Sau* 3A digest of total *Arabidopsis* DNA in the lambda vector EMBL3. A previously isolated cDNA clone encoding the *Silene pratensis* ferredoxin [22] was used as a heterologous hybridization probe for screening the library. A single positive plaque was selected. Restriction mapping and Southern blot analysis showed this clone (Fd2) to contain a 7.0 kb *Pst* I fragment strongly reacting with the *Silene* probe. This 7.0 kb fragment was subcloned in pEMBL18 (resulting in pFd2.1) and a 1.7 kb region covering the whole coding region and 0.9 kb of the 5′ flanking sequence was sequenced.

Sequence analysis was performed by the dideoxy chain termination method with the use of overlapping subclones and synthetic oligonucleotides. In this way both strands were sequenced at least once (Fig. 1). The resulting nucleotide sequence along with the deduced amino acid sequence is presented in Fig. 2. The uninterrupted open reading frame of 148 codons shows extensive similarity at the amino acid level to both the *Silene* (67%) and the spinach (64%) ferredoxin precursor and hence encodes a pre-ferredoxin. Comparison with the spinach pre-ferredoxin [28] and published mature ferredoxin sequences suggests that the processing site is located between the methionine and alanine at position 52 and 53. In this way the precursor is divided in a 52 amino acids long N-terminal transit peptide (5.3 kDa), involved in chloroplast import, and a mature protein of 96 amino acid residues (10.3 kDa). As in other nuclear-encoded chloroplast proteins, the transit peptides are rather poorly conserved when compared to the mature polypeptides: 48% and 76%, respectively, between the *Silene* and *Arabidopsis* ferredoxins.

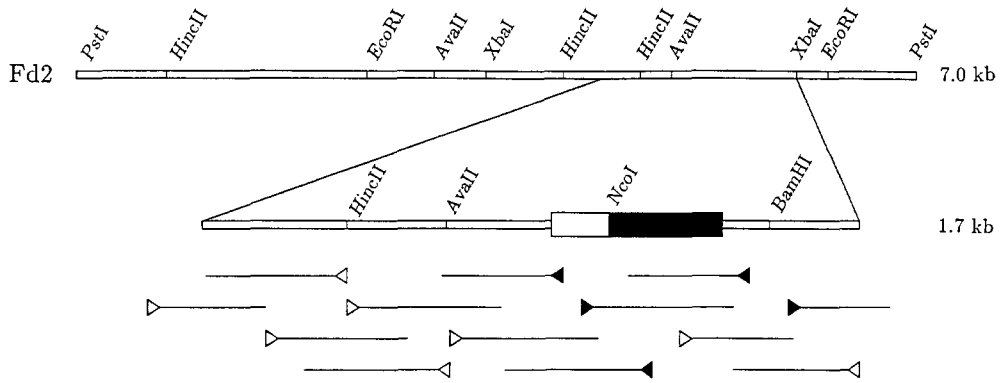

Fig. 1. Restriction map of the Fd2 genomic clone. The enlarged fragment indicates the region that has been sequenced. The open and closed triangles represent the use of synthetic oligonucleotides and M13 primer, respectively. The mature ferredoxin is indicated by a filled box, the transit peptide by an open one.

Genomic organization

To study the organization of the ferredoxin gene, the 410 bp *Nco* I-*Bst* NI fragment of pFd2.1, encoding the mature ferredoxin, was used as a specific probe. Southern blot analysis was performed on total *Arabidopsis* DNA digested with *Xba* I, *Eco* RI or *Hin*d III. All three digests show only one hybridizing band, under moderately stringent conditions (final wash at 60 °C in 0.5 × SSC, 0.1% SDS; Fig. 3). The molecular weight of these bands is as predicted from the Fd2 clone: 2.5 kb, 3.8 kb and more than 6.2 kb, respectively. These data suggest the presence of only one copy of the isolated Fd gene in the *Arabidopsis* genome as has been observed in *Silene* [22].

Transcript analysis

Northern blot analysis of total RNA isolated from different *Arabidopsis* tissues shows only one Fd-specific band. The 5′ end of this transcript was defined by nuclease S1 mapping. Total RNA was hybridized to a homogeneously labelled antisense ssDNA fragment, extending from nucleotide position − 517 to + 65. As shown in Fig. 4 a single set of bands is protected from S1 digestion, of which the major one has a length of 115 nucleotides. Since the probe includes 65 bases of the coding sequence the 5′ untranslated leader measures 50 nucleotides.

The 5′ and 3′ flanking sequences

In addition to the coding region 870 bp of the 5′ and 350 bp of the 3′ flanking region were sequenced. In Fig. 2 the consensus promoter elements are overlined. A TATA box-like sequence (TTTTAAT) is present at 34 bp upstream of the transcription startsite. However, an obvious CAAT box is absent. At position − 122 a G box (5′CACGTGGCA; [7]), present in many other plant genes [21], can be discerned. A stretch of twelve alternating purine and pyrimidine residues, a potential Z-DNA-forming sequence, is located at position − 182. Upstream of the TATA box two 5′GATA sequences can be identified, which modulate expression levels in *Petunia Cab* genes [6].

Regulated expression of Fd in Arabidopsis

The pattern of Fd expression in *Arabidopsis* was studied at the RNA level. Steady-state messenger levels were analysed in different tissues and under various light regimes by northern blotting. To this purpose *Arabidopsis* plants were grown to maturi-

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                                                                  GAAAAGA

-869 AGAGAGAAGTGAGAATCGTCTCTGTTTTCAGAAACTCTGAAAAACGTTTATGACCACGTGTTTTTCCAGAAATGATTGA

-790 TTTTATTCTTTTTATTAAAATTTAATACTTTATCTAAATTCAATTAAAATAAGCAATATTTTATTCATGAGAAATTCTT

-711 TTTTGAGAATCAACCGATGTAGATGGTCTCATACTCTACTCTGTTGATTGTGTTTAAGTTTCTGAGGATTTTTCTACTT

-632 TCCGACGTTATGCCAAGAGGCTGGTCTTCACTAGAAAACTACTTCCACCCAATTCAAGCAAGTATGACCTCTTCTCCCC

-553 ACAATTTATTCATGTACTGAAAGGCCATTAGAAGTTGACTGAAGTGTGAAGGTGGAGATTATGTATTCACTTGTTGATT

-474 TGGTATACATTCTATGTAAGGTTCAATTATTTACGTTATATAATTATAATGGAGTAATTTACAGTAATTGGGTTAAAAT

-395 GGTTTGATTCGGTCAGGTTGATACGGTTTGGAAGTTAAACCCGGCCTAGATATGATGTTACAACCAGTCCACATCTTTT

-316 ATGATTTTAGTGGAACAAACGAAGAGTTATTTAGACGATACAAACAAGGTCCGAATAAGTGTGAGCTGTCCCAAGTAAG

-237 ACCACGTAATACTCACCTCAACAAGATAGTGTTCTTAAAGTGTGTCAAACACAATCACACACACACAAATCATAAAACA

-158 CAAAGACGATAATCCATCGATCCACAGAATAGACGCCACGTGGTAGATAGGATTCTCACTAAAAAGTTCTCACCTTTTA

 -79 ATCTTTCTCCACGCCATTTCCACAAGCCATAATCCTCAAAAATCTCAACTTTATCTCCCAAAACACAAAACAAAAAAAA

   1 ATG GCT TCC ACT GCT CTC TCA AGC GCC ATC GTC GGA ACT TCA TTC ATC CGT CGT TCC CCA
       M   A   S   T   A   L   S   S   A   I   V   G   T   S   F   I   R   R   S   P

  61 GCT CCA ATC AGT CTC CGT TCC CTT CCA TCA GCC AAC ACA CAA TCC CTC TTC GGT CTC AAA
       A   P   I   S   L   R   S   L   P   S   A   N   T   Q   S   L   F   G   L   K

 121 TCA GGC ACC GCT CGT GGT GGA CGT GTC ACA GCC ATG GCT ACA TAC AAG GTC AAG TTC ATC
       S   G   T   A   R   G   G   R   V   T   A   M   A   T   Y   K   V   K   F   I

 181 ACA CCA GAA GGT GAG CTA GAG GTT GAG TGT GAC GAC GAC GTC TAC GTT CTT GAT GCT GCT
       T   P   E   G   E   L   E   V   E   C   D   D   D   V   Y   V   L   D   A   A

 241 GAG GAA GCT GGA ATC GAT TTG CCT TAC TCT TGC CGT GCT GGT TCT TGT TCG AGC TGT GCT
       E   E   A   G   I   D   L   P   Y   S   C   R   A   G   S   C   S   S   C   A

 301 GGT AAA GTT GTG TCT GGA TCT GTT GAT CAG TCT GAC CAG AGT TTC CTT GAT GAT GAA CAG
       G   K   V   V   S   G   S   V   D   Q   S   D   Q   S   F   L   D   D   E   Q

 361 ATT GGT GAA GGG TTT GTT CTC ACT TGT GCT GCT TAC CCT ACC TCT GAT GTT ACC ATT GAA
       I   G   E   G   F   V   L   T   C   A   A   Y   P   T   S   D   V   T   I   E

 421 ACC CAC AAA GAA GAA GAC ATT GTT TAA GCCTCACCTACTCACCAGCTTTTGATGGTTTAAAAATCATGTC
       T   H   K   E   E   D   I   V   U

 486 TTTATAAATTTCACATTTTGGGTTGAGTTTGTTGTTACTAAAAACTATTGTTATCTGTTGTTATTGTTCCTGGTTTGGC

 565 TCACCATCAATCGATGACATTTTAAACTATGCAACTGCAAATTCTGCAACACTTTCGATGAGAATCTAACATTATCGTT

 644 TAAACATTGGAAATACATTTTCTTGAAGTCTAGCTAGCTTTGGTTTGTAGTTCTTATTCTGAACTCAACAATCATCAAA

 723 GTATCAAGAAAAATCCGATTTGCGAGCAATTGTGAAATCTTAGATTGATAAATTCTCTAGA
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Fig. 2. Nucleotide and derived amino acid sequence of the *Arabidopsis* pre-ferredoxin gene Fd2. Numbering of the nucleotides is relative to the translation initiation codon. The consensus promoter elements are overlined. The start of transcription is marked by dots and the potential Z-DNA forming region is boxed. The probable processing site of the pre-ferredoxin is indicated by an arrowhead.

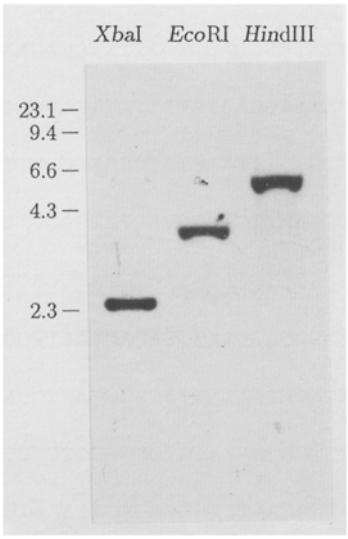

Fig. 3. Southern blot of *Arabidopsis* DNA. Total DNA (10 μg) was digested with *Xba* I, *Eco* RI or *Hind* III, separated on a 1.0% agarose gel and transferred to nitrocellulose. The filter was hybridized to the 410 bp *Nco* I-*Bst* NI Fd2 probe at 42 °C in 50% formamide, 5 × SSC, 50 mM sodium phosphate pH 6.5, 1 mM EDTA, 0.1% SDS, 0.1 mg/ml denatured salmon sperm DNA and 5 × Denhardt's solution. The final washing was at 60 °C in 0.5 SSC, 0.1% SDS.

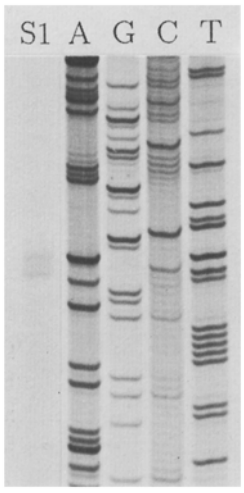

Fig. 4. S1 nuclease analysis of the Fd2 transcription start site. A homogeneously labelled 582 nt long ssDNA probe (position − 517 to + 65) was hybridized to 25 μg *Arabidopsis* total RNA. The DNA-RNA hybrids were digested with S1 nuclease. The protected fragments were resolved on a sequencing gel next to a sequencing ladder resulting from pFd2.11 annealed to the same primer (position + 48 to + 65) as used for probe generation.

ty in the greenhouse and total RNA was isolated from the different tissues. As shown in Fig. 5A, steady-state Fd mRNA levels are higher in green tissue (rosette, stem leaves and stem) and whole flowers than in non-green tissue such as root and seeds.

The influence of different light regimes was studied in young seedlings. Six-day-old seedlings, grown at 25 °C under continuous fluorescent light, show a rapid decrease of Fd mRNA within two hours after transfer to the dark (Fig. 5B). A minimum at 4% of the light-level is reached in 24 hours. When these plants are placed in the light again, after 3 days of dark treatment, a very rapid increase is observed: 10-fold within 3 hours. The normal light-dependent level is reached in 24 hours.

Tissue-specific expression in transgenic tobacco plants

To study the effects of the *Arabidopsis* Fd promoter on expression regulation in transgenic tobacco plants, a chimeric gene consisting of a 1.2 kb Fd promoter fragment, including the transcription start site, and the coding region of the reporter gene β-glucuronidase (GUS) was constructed. For this purpose a novel *Xba* I site at positions − 10 to − 5 (Fig. 2) was created by oligonucleotide-directed mutagenesis. The resulting 1.2 kb *Hinc* II-*Xba* I fragment was cloned in the pBIN19-derived vector pBI101.1 [11], placing the GUS gene under its control (pFd2.64). Transformed tobacco plants containing this fusion gene (Fd-GUS) were obtained by infection of leaf disks with *A. tumefaciens* harbouring pFd2.64, followed by selection for kanamycin-resistant calli and regeneration of plants. In addition plants were transformed with a 35S CaMV promoter-GUS fusion (35S-GUS; pBI121) and a promoterless GUS gene (0-GUS; pBI101.1).

To examine the activity of the Fd promoter in transgenic plants, GUS activity was measured in 10–12 cm leaves of 29 individual transformants in the case of the Fd-GUS and 10 plants for each of the 35S-GUS and 0-GUS constructs. Both Fd-

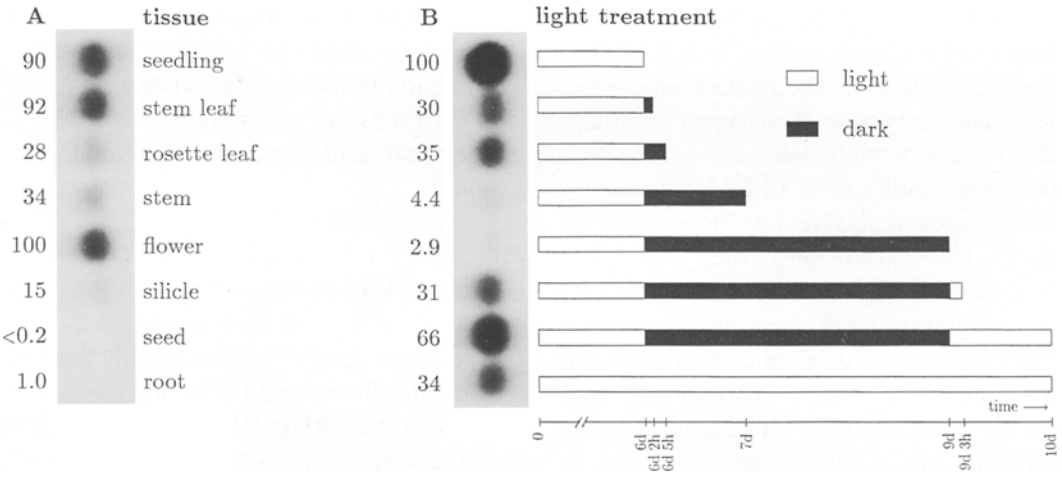

Fig. 5. Expression of the Fd2 gene in *Arabidopsis* in different tissues (A) and under different light regimes (B) analysed by northern blotting. Total *Arabidopsis* RNA (25 μg) isolated from the different tissues was separated on a 1.0% formaldehyde agarose gel and blotted onto nitrocellulose. The filters were probed with a 410 bp *Nco* I-*Bst* NI restriction fragment, encoding the *Arabidopsis* Fd mature protein. The final wash was at 60 °C in 0.5 × SSC, 0.1% SDS. The relative intensity of the bands was determined on a LKB Ultroscan XL.

GUS and 35S-GUS plants showed considerable levels of GUS expression in leaves. GUS activity appeared to be 4 times higher in leaves expressing Fd-GUS than in the 35S-GUS controls (4.4 vs. 1.0 nmol MU/min per mg protein). The expression in roots of Fd-GUS plants is only 6% of that in leaves. The 35S-GUS constructs however are much higher expressed in roots, giving rise to 6 times the activity measured in leaf tissue (Fig. 6). The 0-GUS plants show no activity in leaf tissue, as compared with untransformed tobacco leaves (2×10^{-3} nmol MU/min per mg protein).

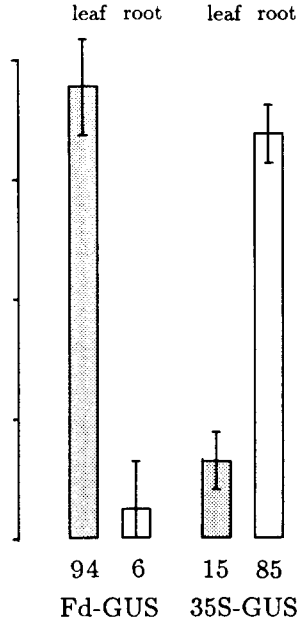

Fig. 6. Relative β-glucuronidase activity in root and leaf tissue of Fd-GUS and 35S-GUS tobacco plants. Root and leaf activity together were taken as 100% for each individual. Standard deviations are indicated with bars.

Discussion

We have isolated a pre-ferredoxin gene from *Arabidopsis thaliana* and analyzed its organization and expression. Only one copy of the isolated pre-ferredoxin gene (Fd2) seems to be present in *Arabidopsis*. In many higher plant species however, two types of ferredoxin are known [4, 25]. Genes encoding a second type of ferredoxin may be present, but not detected by Southern blotting, because of too low a similarity with the isolated gene.

The expression of the Fd2 gene in *Arabidopsis* is regulated in a tissue-specific and light-dependent way. As expected, steady-state mRNA levels are high in photosynthetically active parts as seedlings, stems and both stem and rosette leaves.

In whole flowers, however, the highest relative expression level is observed (Fig. 5A). Light has a clear positive effect on the relative amount of Fd2 messenger in young *Arabidopsis* seedlings (Fig. 5B): 3 hours of white light after a three days dark treatment results in a 10-fold increase. A comparable rapid response has been reported from pea [3, 12, 13]. Not only when transferred from the dark to the light, but also in the reverse situation, adaptation to the new environment is rapid. Only 30% of the original transcripts is left after two hours in the dark. This means that, assuming the complete shut-off of transcription, the Fd mRNA has a half-life time of about 70 minutes under these conditions. This relative instability of the Fd messenger is confirmed by the observation of a considerable (5-fold) circadian fluctuation in *Silene* (J. Hageman, unpublished results). One can wonder about the function of such a rapid response at the RNA level in relation to the relative stability of the mature protein.

In the promoter region of the Fd2 gene some consensus elements involved in transcription can be discerned. A G-box is found at position − 122. Such a sequence is not only present in other light- or UV-regulated genes but also in promoters from other inducible genes: *adh*, *patatin* and *rolbc* [21]. At position − 182 a potential Z-DNA-forming sequence is located that may be involved in gene expression. The functional significance of such a Z-element in the nopaline synthase promoter has recently been shown [16]. GATA boxes, as present upstream from the TATA box, are important for the quantitative expression of the *Petunia Cab22R* gene. A 2 bp substitution in one of these sequences resulted in a fivefold reduction in expression [6].

A 1.2 kb promoter fragment fused to the β-glucuronidase gene gives rise to tissue-specific expression in transgenic tobacco plants. In young leaves (10–12 cm) GUS activity is 4.4 nmol MU/min per mg protein (29 individual transformants tested). In roots the measured activity is only 6% of that in leaves (Fig. 6). This difference cannot completely be attributed to a lower stability of β-glucuronidase in roots, because transgenic plants carrying a GUS gene under control of the 35S CaMV promoter show a much higher activity in roots than in leaves. Apparently signals directing tissue-specific expression of the *Arabidopsis* Fd2 gene are present in a 1.2 kb promoter fragment and they are functional in tobacco plants.

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