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cis-Acting Elements for Light Regulation of Pea Ferredoxin I Gene Expression Are Located within Transcribed Sequences

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An intact pea gene encoding ferredoxin I (*Fed-1*) and several chimeric constructs containing portions of *Fed-1* were introduced into tobacco plants by *Agrobacterium*-mediated transformation. The intact gene was correctly transcribed and translated to produce a protein that was imported into the chloroplast and processed to its mature size. *Fed-1* mRNA accumulation in these plants was strongly light-dependent, as it is in pea leaves. In chimeric constructs, the *Fed-1* promoter was active but no light responses were seen, even when as much as 2 kilobases of 5′-flanking sequence were included. We also failed to observe clear light responses with a construct containing 3′-flanking sequences from *Fed-1* attached to a β-glucuronidase gene driven by the cauliflower mosaic virus 35S promoter. However, the transcribed portion of *Fed-1* conveyed normal light responsiveness when driven by the 35S promoter. The results are discussed in terms of the hypothesis that light determines *Fed-1* mRNA abundance by affecting RNA stability rather than by affecting transcription.

INTRODUCTION

Developmental changes in eukaryotic gene expression typically result from transcriptional changes mediated by promoters and enhancers (Ptashne, 1986; Maniatis, Goodburn, and Fischer, 1987; Guarente, 1988). However, alterations in mRNA stability can also play a large part in regulating gene expression, and, in some cases, stability seems to be the principle mechanism for altering steady-state mRNA levels. Examples include β-tubulin (Yen, Machlin, and Cleveland, 1988), transferrin receptor (Müller and Kühn, 1988), GM-colony stimulating factor (Schuler and Cole, 1988), and c-*myc* (Klinken et al., 1988). Most plant nuclear genes are also controlled primarily at the transcriptional level (Kuhlemeier, Green, and Chua, 1987). However, discrepancies between run-on transcription data and steady-state mRNA levels have led several laboratories to suggest that changes in mRNA stability may contribute to some responses (reviewed by Thompson, 1988), and others have suggested that post-transcriptional mechanisms play a major role in regulating chloroplast gene expression (e.g., Deng and Gruissem, 1987; Mullet and Klein, 1987; Woodbury et al., 1988).

In studies with etiolated pea seedlings, we have shown that the light responses of *Fed-1* (encoding ferredoxin I) differ from those of transcriptionally regulated genes such as *RbcS* and *Cab*. For example, *Fed-1* mRNA is induced much more rapidly (Kaufman, Briggs, and Thompson, 1985) and remains under phytochrome control longer (Kaufman et al., 1986). In addition, while *RbcS* and *Cab* transcript levels increase in both the nucleus and the cytoplasm, *Fed-1* transcript levels increase significantly only in the cytoplasm (Sagar et al., 1988). These differences are compatible with a post-transcriptional control mechanism for *Fed-1*.

In this paper, we show that a pea *Fed-1* gene is correctly transcribed and translated and shows normal light responses in transgenic tobacco plants. However, in contrast to all previous reports for light-regulated nuclear genes, 5′-flanking sequences do not mediate a major light response. Instead, we show that light responsiveness is conferred by sequences within the transcription unit and may involve alterations of *Fed-1* transcript stability.

RESULTS

Construction of Chimeric Genes

Subclones derived from λPS4601 (Elliott et al., 1989) were used to make the *Fed-1* constructs shown in Figure 1. The intact gene construct contains 3.6 kb of *Fed-1* sequence, running from approximately −2000 to +1600. The long

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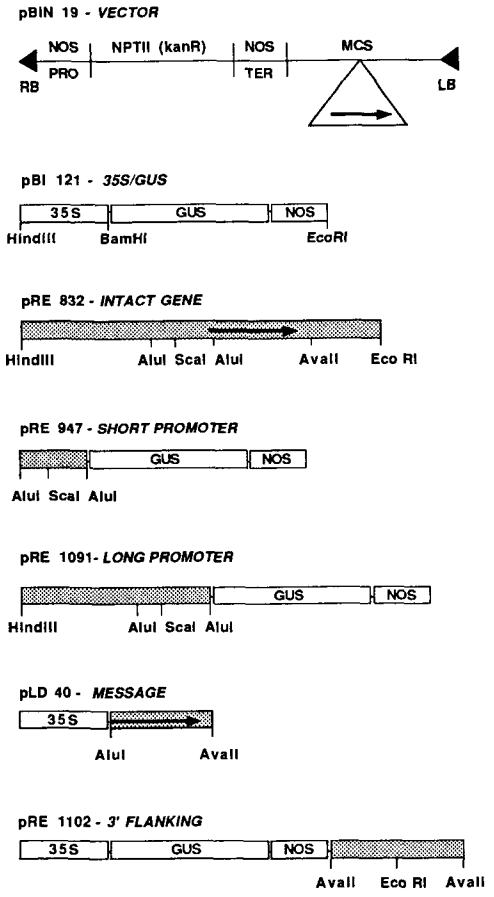

Figure 1. Constructs Used.

Various constructs were inserted into the binary vector pBIN19 (Bevan, 1984) as described in Methods. pBI121 (35S/GUS) and pBI101 (not shown) were obtained from Richard Jefferson. They are derivatives of pBIN19 containing the GUS gene of *E. coli*, with or without the CaMV 35S promoter/enhancer, respectively. pRE832 (intact gene) was constructed by inserting a 3.6-kb HindIII/EcoRI fragment (containing the *Fed I* gene and flanking sequences, approximately −2000 to +1600) into the HindIII/EcoRI sites of pBI101. pRE947 (short promoter) was constructed by cloning an AluI partial fragment (−494 to +5) from the *Fed-1* 5′-flanking region into the HindIII/BamHI sites of pBI101. pRE1091 (long promoter) was constructed by inserting a HindIII (approximately −2000) to AluI (+5) (partial) fragment from the *Fed-1* 5′-flanking region into the HindIII/BamHI sites of pBI101. pLD40 (message) was constructed by cloning an AluI (+5) (partial) to AvaII (+824) *Fed-1* fragment into pBI121 using BamHI/EcoRI. pRE1102 (3′-flanking) contains a 1.4-kb AvaII fragment (+824 to approx +2200), cloned into the EcoRI site of pBI121. Each of the six chimeric genes was incorporated into the binary vector pBIN19, which contains a bacterial neomycin phosphotransferase gene (*nptII*), providing a plant-selectable kanamycin resistance marker.

35S, CaMV 35S promoter/enhancer; NOS (or NOS TER), *nos* terminator sequences; NPTII, neomycin phosphotransferase gene; NOS PRO, *nos* promoter; MCS, multiple cloning site; LB, left border; RB, right border.

promoter construct contains *Fed-1* 5′-flanking sequence (from approximately −2000 to +5) upstream of the β-glucuronidase (GUS) gene of *Escherichia coli* with the nopaline synthase (*nos*) terminator. Similarly, the short promoter construct contains *Fed-1* 5′-flanking sequence (from −494 to +5) upstream of the GUS/*nos* fusion described above. The message construct includes an 800-bp fragment containing the cauliflower mosaic virus (CaMV) 35S enhancer and promoter (Jefferson, Kavanagh, and Bevan, 1987) placed immediately 5′ to the transcribed region of the *Fed-1* gene (+5 to +824). We also included 81 nucleotides of 3′-flanking sequences to direct polyadenylation of the *Fed-1* mRNA. The 3′-flanking construct contains *Fed-1* sequences between +824 and approximately +2200 inserted downstream from the *nos* terminator sequences of pBI121. The control gene, pBI121 (Jefferson, Kavanagh, and Bevan, 1987), consists of the CaMV 35S promoter upstream of the GUS/*nos* fusion described above.

Pea *Fed-1* mRNA and Protein Are Correctly Expressed in Transgenic Tobacco Plants

RNAs were isolated from the leaves of tobacco plants containing the intact pea *Fed-1* gene, and the transcript was mapped by RNase protection (data not shown). At both the 5′ end and 3′ end of the pea *Fed-1* transcript, we see the same sets of protected fragments that we observed in pea (Elliott et al., 1989), indicating that the pea gene is correctly transcribed in tobacco cells.

To determine whether transgenic plants containing the pea *Fed-1* gene correctly synthesize and process the pea ferredoxin I protein, stromal proteins from pea and tobacco chloroplasts were isolated, electrophoresed, blotted onto nitrocellulose, and probed with antibodies to pea ferredoxin. As shown in Figure 2, the mobilities of the pea and tobacco ferredoxins are different, allowing convenient analysis of proteins from both species on the same gel. In transformed tobacco plants, there are three bands, two that correspond to the endogenous ferredoxins, and one that co-migrates with the mature form of pea ferredoxin I. There are no bands visible that would correspond to unprocessed precursor or aberrant processing products. We conclude that the pea *Fed-1* message is properly translated, and the resulting protein taken up into the chloroplast and processed to its correct mature size.

Pea *Fed-1* mRNA Levels Are Light-Responsive in Transgenic Tobacco

Pairs of transgenic plantlets derived from cuttings were used to represent each original transgenic plant in light/dark experiments. After growth in continuous light to the 6-leaf stage, one set of cuttings was transferred to dark-

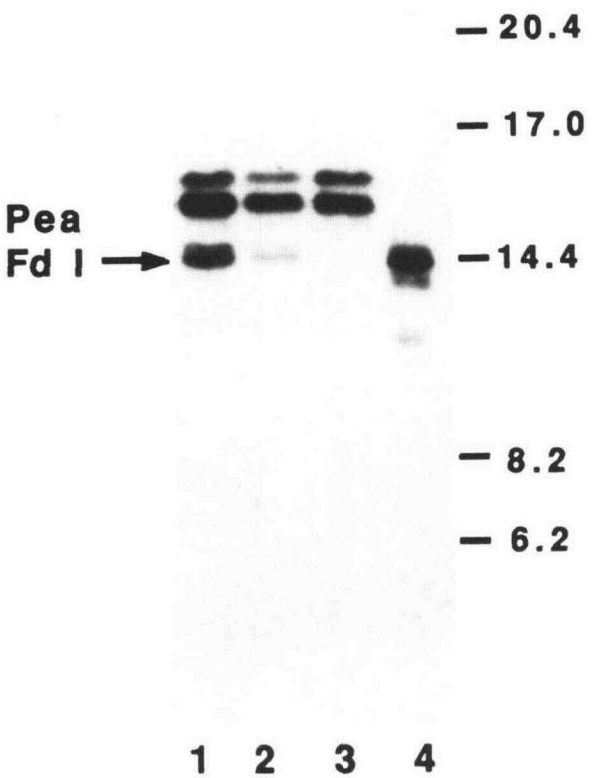

Figure 2. Pea Ferredoxin I (Fd I) Is Translated and Taken Up by Tobacco Chloroplasts.

Stromal proteins were isolated from the chloroplasts of two tobacco plants transformed with the intact pea *Fed-1* construct (lanes 1 and 2), from chloroplasts of a tobacco plant transformed with the 35S/GUS/*nos* construct (lane 3), and from pea chloroplasts (lane 4). Reduced, heat-denatured, and acetylated proteins were separated on a 20% polyacrylamide-SDS gel, blotted onto nitrocellulose, and reacted with an antibody to pea *Fed-1*, as described in Methods.

ness for 3 days while the other set remained in the light. After 3 days, one or two leaves 5 cm to 10 cm long were harvested, and total RNA was isolated. Pea *Fed-1* transcripts were assayed by RNA gel blot hybridization. Figure 3 shows there is strong light regulation of the intact pea gene in transgenic tobacco. Some cross-hybridization occurs between the pea probe and tobacco *Fed-1* mRNA, but the pea and tobacco mRNAs have different mobilities and can be distinguished at appropriate autoradiographic exposures. Some variability is seen in the absolute levels of expression from transformant to transformant, but, in all cases, the light/dark ratio is high.

Sequences within the Transcription Unit Are Required for Normal Light Responses

To localize the region responsible for regulating the *Fed-1* gene, we constructed several chimeric genes and tested them in transgenic tobacco. Representative data for the constructs containing either 5′-flanking or 3′-flanking sequences are illustrated in Figure 4, which shows RNA gel blots of tobacco RNA hybridized with a probe from the GUS gene. Results from plants containing the short promoter construct are shown in Figure 4A. No consistent difference exists in the message levels between plants kept in the light or in the dark. The *Fed-1* sequence used in this construct (from −494 to +5) covers the regions shown to be sufficient to convey light responsiveness in pea *RbcS* (Fluhr and Chua, 1986; Fluhr et al., 1986) and *Cab* (Simpson et al., 1985; Simpson, Van Montagu, and Herrera-Estrella, 1986; Simpson et al., 1986). However, enhancer-like elements have also been found further upstream (as for example Stougaard et al., 1987; Castresana et al., 1988; Ueda et al., 1989), so we also tested plants containing an additional 1.5 kb more of the 5′-flanking sequence. This construct contained the entire 5′-flanking region present in the intact gene construct that we previously showed was normally light-responsive. However, results with this long promoter were essentially the same as those for the short promoter construct (Figure 4B). In all cases, the RNA levels obtained are similar for plants kept in the light or in the dark.

Similar results, with no clear light effect, were obtained for the 3′-flanking construct (Figure 4C), in which 3′-flanking sequences from *Fed-1* were placed downstream of the *nos* terminator in pBI121.

The only construct that showed a consistent, strong positive light response is the message construct, pLD40, in which transcribed sequences from *Fed-1* are driven by the 35S promoter/enhancer from pBI121. These results are shown in Figure 5, where they are presented together with data from plants containing the intact *Fed-1* gene. In both sets of plants, we can see very strong light/dark differences in *Fed-1* mRNA levels. In many cases, no mRNA is detectable in plants held in the dark, although low levels are sometimes seen in cases where mRNA levels in the light are unusually high. The strong positive light effects in these cases are particularly remarkable when compared with the negative light effects seen for GUS mRNA transcribed from the same 35S promoter shown in Figure 4D. We conclude that the *cis*-acting elements mediating the largest part of the *Fed-1* response

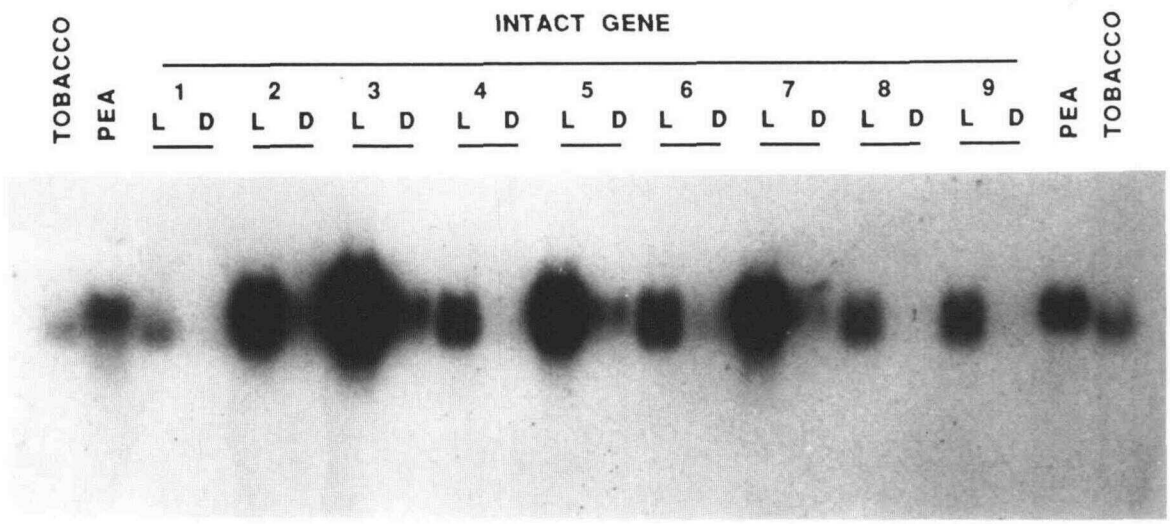

Figure 3. Light Responses of the Intact Pea Gene in Transgenic Tobacco.

RNA gel blots of total RNA from plants containing the intact pea *Fed-1* gene were hybridized to an RNA probe prepared from the *Fed-1* cDNA clone pEA 46.2 (Dobres et al., 1987). Numbers 1 to 9 represent different primary transformants, while L and D denote light and dark treatments, respectively. Hybridization was at 65°C in 30% formamide, 5 × SSC. At this stringency, the RNA probe cross-hybridizes to tobacco *Fed-1* mRNA, but, on appropriate autoradiographic exposures, the pea and tobacco mRNAs can be distinguished by their different mobilities. The pea gene is expressed in plants 2 to 9, all of which show strong light responses.

are located within the region used to make the message construct. One or more such elements must reside within either the *Fed-1* transcription unit or the 81 nucleotides of 3′-flanking sequence containing polyadenylation signals.

DISCUSSION

We find no evidence of light-responsive enhancer sequences within 2 kb upstream of the *Fed-1* gene, an observation that contrasts sharply with results for other plant genes (reviewed by Kuhlemeier, Green, and Chua, 1987; Schell, 1987). As expected, we could show that this part of the *Fed-1* gene supports transcription (Figures 5A and 5B). However, none of the transformants containing only 5′ sequences shows clear light/dark expression differences. Since intact *Fed-1* genes in tobacco exhibit a very strong light response, our results with promoter constructs cannot be attributed to a failure of tobacco *trans*-acting factors to recognize *cis* elements in the pea gene. Instead, we believe that *cis* elements required for the major light effect on *Fed-1* must be located elsewhere than in the upstream region.

Internal enhancers are sometimes found in animal genes (e.g., Ford et al., 1988), but enhancers for RNA polymerase II have so far been reported only in introns and thus seem less likely in the intronless (Elliott et al., 1989) *Fed-1* gene. However, enhancers are also found 3′ to a number of animal genes (e.g., Choi and Engel, 1986; Bodine and Ley, 1987; Trainor, Stamler, and Engel, 1987), and it has recently been suggested that 3′ elements can affect transcriptional activity of a petunia *RbcS* gene (Dean et al., 1989). Thus, the possibility of a 3′ enhancer(s) in *Fed-1* deserves serious consideration. However, no clearly significant light effects were observed with the 3′-flanking construct (see Figure 4C).

We consistently see a lower level of GUS mRNA in light-grown plants containing the 35S/GUS/*nos* control construct (see Figure 4D). [Other investigators have observed similar responses with 35S-GUS constructs (N.-H. Chua, personal communication).] If this negative light effect results from increased turnover of GUS mRNA in the light, it might obscure a weak positive light effect on transcription in the *Fed-1* promoter or 3′-flanking constructs. Thus, our data do not exclude the possibility that a weak positive light effect is mediated by *Fed-1* flanking sequences. However, comparison of Figures 4 and 5 shows that any such effect must be quite small in relation to the large light responses seen with either the intact gene or the message construct.

By far the largest light effect was conveyed by sequences in the message construct, and, in fact, the responses of this construct are essentially identical to those of the intact gene with respect to both the absolute levels of mRNA accumulated and the magnitude of the light

effect. A transcriptional hypothesis to explain this result must postulate an element within the message construct that suppresses 35S transcriptional activity in the dark. In principle, such an element might exist within the 81 nucleotides of 3′-flanking sequence we included in the message construct to obtain proper polyadenylation, or *Fed-1* may contain an attenuator sequence similar to that described for c-*myc* in HeLa cells. (Bently and Groudine, 1988; Wright and Bishop, 1989). However, it is equally logical to suppose that transcription is not rate-limiting, and that light acts at a post-transcriptional step to increase the fraction of transcripts detectable on RNA gel blots. Such a mechanism would explain the remarkable quantitative and qualitative similarity in the behavior of the intact gene transcribed from its own promoter and the message construct driven by the stronger 35S promoter (see Figure 5).

Previously, we argued (Kaufman et al., 1986; Sagar et al., 1988; Thompson, 1988) that differences in the photophysiology of induction indicate that different genes respond to light by different biochemical mechanisms. The striking differences between *Fed-1* and genes such as *RbcS* and *Cab* provide strong support for this view. We are currently working to further define the *cis*-acting elements in *Fed-1* and to test directly our hypothesis that light affects stability of this mRNA. However, the absence of strong light-response elements 5′ to the *Fed-1* gene and their presence within the message construct provide strong evidence that *trans*-acting factors and induction mechanisms can differ for different light-regulated genes.

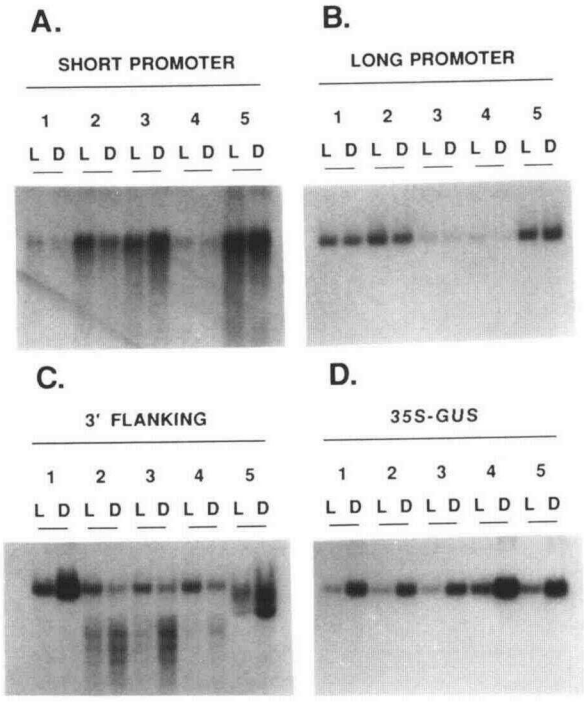

Figure 4. Expression of Constructs not Showing Strong Positive Light Responses.

RNA gel blots of total RNA from plants containing the short and long promoter constructs, the 3′-flanking sequence construct, or the 35S/GUS/*nos* construct were hybridized with an RNA probe synthesized from a 570-bp PstI/EcoRV fragment at the 5′ end of the GUS gene. Growth of plants, light treatments, and hybridizations were as described for Figure 3, except that the hybridization solution contained 50% formamide instead of 30%.

METHODS

Construction of Chimeric Genes

The intact gene construct (pRE832) was made by cloning a 4.8-kb EcoRI fragment of λPS4601 (Elliott et al., 1989) into pBSM13− (Stratagene, San Diego, CA) to produce the subclone pRE80. A 4.0-kb HindIII/EcoRI fragment of pRE80 containing the *Fed-1* gene and flanking sequences was then cloned into the HindIII/EcoRI sites of pBI101.

The short promoter construct (pRE947) was made using an AluI partial digestion. pRE80 was digested with Sau3A, and an 800-bp fragment containing a portion of the *Fed-1* gene and 513 bp of 5′-flanking sequence was subcloned into the BamHI site of pBSM13− to produce the plasmid pRE282. The insert from pRE282 was then released from the multiple cloning site by digestion with HindIII and EcoRI, and gel-purified. An AluI partial digest of this insert was then shotgun-cloned into the SmaI site of pBSM13−, and a plasmid containing the sequences from +5 to −494 was identified. This plasmid (pRE737) was digested with EcoRI, the ends were filled in with the Klenow fragment of DNA polymerase I and digested with BamHI, and then the insert gel was purified. The purified insert was ligated with T4 DNA ligase into pBI101, which had been prepared by digesting with HindIII, filling in the ends with Klenow, and digesting with BamHI, which allows the ligation of the EcoRI site flanking the insert to the HindIII site of pBI101.

The long promoter construct (pRE1091) was made as follows: pRE80 was double-digested with EcoRV and PstI and deleted using the exonuclease III/mung bean nuclease protocol of Henikoff (1984). One of the resulting plasmids (pRE672) contained *Fed-1* sequences from +29 to approximately −2800. This plasmid was then digested with ScaI and EcoRI (releasing sequences from −2800 to −420), and the fragment was gel-purified. The *Fed-1* 5′ region (−2800 to +5) was reconstructed by cloning the fragment from pRE672 into pRE737 digested with EcoRI and partially digested with ScaI. A BamHI/HindIII insert from the reconstructed promoter (pRE1062) was cloned into the BamHI/HindIII sites of pBI101 to produce pRE1091.

The message construct (pLD40) was made by digesting pRE80 with XhoI, SstI, and EcoRI and then cloning the fragment containing the *Fed-1* gene into pBSM13− digested with SstI and EcoRI. The resulting plasmid (pRE387) was digested with AvaII (nearest site 3′ to the transcript), filled in with Klenow (to allow cloning into pBS multiple cloning site), and then digested with BamHI (releasing *Fed-1* sequences from vector). The insert was gel-purified,

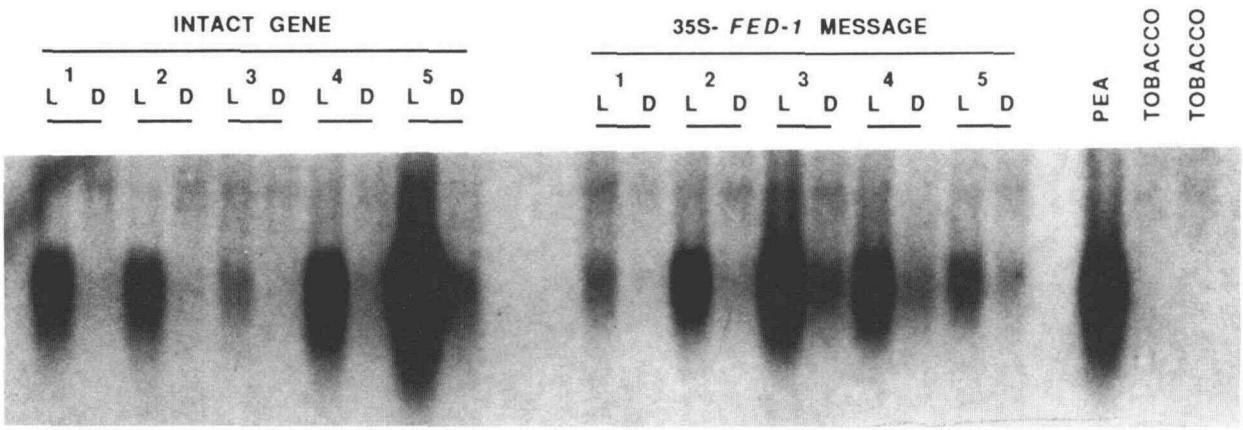

Figure 5. Light Responses in Transgenic Tobacco Plants.

RNA samples from plants containing either the intact *Fed-1* gene or the message construct were hybridized with an RNA probe prepared from the *Fed-1* cDNA. Growth of plants, light treatments, and hybridizations were as described for Figure 3, except that a higher hybridization stringency (50% formamide) was used to suppress cross-hybridization to tobacco *Fed-1* mRNAs. The pea and tobacco lanes contain RNAs isolated from the leaves of light-grown untransformed plants.

partially digested with AluI, and shotgun-cloned into the SmaI site of pBSM13−. An insert from one of the resulting plasmids (pLD40), which contained sequences from +5 to +824, was recloned into the BamHI/EcoRI sites of pBI121.

For the 3′-flanking construct (pRE1102) a 4.8-kb XhoI fragment was subcloned from λPS4601 into pBSM13−. The resulting plasmid was digested with AvaII, filled in with Klenow, and cloned into the SmaI site of pBSM13−. The resulting plasmid (pRE928) was digested with PvuII and PstI and the insert subcloned into the SmaI/PstI sites of pKSM13+ (Stratagene), creating another EcoRI site. The insert of the resulting plasmid (pRE1046) was then released by partial digestion with EcoRI, and the insert was gel-purified. This EcoRI fragment was then cloned into the EcoRI site of pBI121.

Transgenic Plants

Transgenic tobacco plants were produced using the leaf disc method of transformation (Horsch et al., 1985). The binary vector constructs were conjugated into *Agrobacterium* using the methods of Bevan (1984). Leaf discs from surface-sterilized leaves (almost fully expanded) from 3-inch- to 4-inch-tall tobacco plants (*Nicotiana tabacum* SR1 cv Petite Havana) were infected with the *Agrobacterium tumefaciens* LBA 4404 (Hoekema et al., 1983) containing the binary vector pBI19 (Bevan, 1984) or a derivative containing one of the genes being tested. After 48 hr on plates containing MS salts (Murashige and Skoog, 1962) and B5 vitamins (Gamborg and Eveleigh, 1968) plus 1.25 mg/L benzyladenine, the discs were transferred to the same medium supplemented with 300 μg/mL kanamycin and 500 μg/mL carbenicillin or cefotaxime. After several weeks, one shoot was removed from each disc. After several additional weeks, shoots were transferred to rooting medium (the same medium but without benzyladenine or kanamycin). After rooting, each plant was cut in half at the third or fourth node, and the cuttings were allowed to regenerate the missing shoot or roots prior to transfer to soil. The plantlets were grown at 22°C in continuous white light (photosynthetic photon flux density, 400 nm to 700 nm: 350 mmol m^{-2} sec^{-1} to 400 mmol m^{-2} sec^{-1}; photomorphogenic irradiance, 700 nm to 850 nm: 7 W m^{-2}) until they were 15 cm to 25 cm tall with approximately six leaves. At this point, one member of each pair of cuttings was transferred to a loosely covered plastic box inside a completely dark room for 3 days. The other was kept in continuous white light. After 3 days, leaves 5 cm to 10 cm long were harvested and used for RNA isolation. Between six and 20 different pairs of transformants were examined. Seed collected from these plants showed inheritance of kanamycin resistance consistent with the presence of T-DNA at one or more loci (M. Gallo and W.F. Thompson, unpublished data).

RNase Protection Mapping and RNA Gel Blots

RNAs were isolated as described by Kaufman, Briggs, and Thompson (1985). RNase protection experiments were performed as described by Elliott et al. (1989). ^{32}P-labeled RNA probes hybridizing to the region from +127 to −119 and between +582 and +827 were synthesized from appropriate subclones in the plasmid vector pBSM13−, using either T7 or T3 RNA polymerase (Melton et al., 1984). These probes were hybridized to 20 μg of total RNA and digested with RNase A and T1, and the products analyzed on a 6% polyacrylamide gel.

For RNA gel blots, 5-μg aliquots of total RNA were glyoxylated and electrophoresed according to Maniatis, Fritsch, and Sambrook (1982) and transferred to GeneScreen membrane with 25 mM sodium phosphate, pH 7.0. Blots were baked 2 hr at 80°C under vacuum and prehybridized overnight in 50% (or 30%) formamide, 5 × SSC, 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 2.2 mM sodium pyrophosphate, 1% polyvinylpyrrolidone (PVP

40,000), 1% Ficoll, 1% bovine serum albumin, 600 μg/mL calf liver RNA. Hybridization was in the same buffer for 6 hr at 65°C, using RNA probes prepared as described above. The filters were then washed twice for 15 min at room temperature in 2 × SSC, 0.1% SDS, and twice for 15 min at 65°C in 0.5 × SSC, 0.1% SDS.

Immunoblots

Chloroplasts from the leaves of 10-day-old pea and 2-month-old tobacco plants were isolated by the method of Grossman et al. (1982), and stromal extracts were prepared by lysis of the chloroplasts in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA (with several seconds of vortexing) followed by removal of the membranes by centrifugation at 10,000 *g* for 4 min at 4°C. The supernatant was mixed with an equal volume of 2 × sample buffer (4% SDS, 100 mM dithiothreitol, 10% glycerol, 130 mM Tris-HCl, pH 8.4). After heating at 65°C for 30 min, 1/20 volume of 0.25 M iodoacetamide was added and the sample was heated at 42°C for 15 min and then frozen immediately. The samples were electrophoresed for 24 hr on a 20% pH 8.8 polyacrylamide (acrylamide:bisacrylamide, 29:1) running gel overlaid with a 5% pH 6.1 stacking gel (White and Green, 1987), electroblotted onto nitrocellulose membranes at 220 mA overnight in 50 mM sodium acetate, pH 7.0, and probed with an anti-pea ferredoxin as described in White and Green (1987). Briefly, blots were incubated for 1 hr in blocker in PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4), incubated with a rabbit antibody to pea ferredoxin (diluted 1/2000) in blocking solution for 2 hr, washed three times, incubated in goat anti-rabbit IgG conjugated to alkaline phosphatase diluted 1/1000 in blocking solution for 1 hr, and washed three times. Wash solutions consisted of 1 × PBS, 0.05% Tween 20. Blots were then washed 10 min in 50 mM Tris-HCl, pH 8.0, followed by the addition of substrate (0.1% w/v disodium naphthol AS MX phosphate, 0.2% w/v Fast Red TR salt; both from Sigma) dissolved in the same buffer.

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