5' proximal sequences of a soybean ribulose-1,5-bisphosphate carboxylase small subunit gene direct light and phytochrome controlled transcription

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ABSTRACT

Two closely related ribulose-1,5-bisphosphate carboxylase small subunit (SSU) genes, SRS1 and SRS4, are transcribed at high levels in soybean plants in response to light. Transgenic petunia plants containing 5' sequences from SRS1 or SRS4 fused to the polypeptide encoding region of a neomycin phosphotransferase (NPTII) gene exhibit selectable kanamycin resistance. Deletion of three ATG codons from the region preceding the normal NPTII translation start site has little effect on the levels of kanamycin resistance in transformed plants. Run-on transcription assays in isolated nuclei demonstrate that transcription of the SRS1/NPTII chimera and the native petunia SSU11A gene subfamily is light regulated and under phytochrome control in leaves of transgenic plants. In young expanding leaves of fully light grown plants, transcription of these genes is markedly reduced within minutes of far-red treatment, while ribosomal DNA and actin gene transcription remains unchanged. This is analogous to the transcriptional response we observed for SRS1 and SRS4 in soybean seedlings. These data suggest (1) that transcription of SSU genes in both soybean and petunia require the continued presence or synthesis of phytochrome in the P_{fr} form and (2) that 5' sequences are sufficient to direct the phytochrome controlled transcriptional response of the SRS1 gene. In fully expanded mature leaves we found the transcription rates of the native SSU11A gene subfamily, the chimeric SRS1/NPTII gene, the rDNA genes, and several other control genes to be reduced markedly after far-red treatment or after extended periods of darkness. The contrast between results in young and mature leaves is discussed.

INTRODUCTION

The small subunit of the chloroplast enzyme ribulose-1,5-bisphosphate carboxylase (SSU) is transcribed in the nucleus and translated on cytoplasmic ribosomes (1). It is encoded by a small multigene family in all plants examined (2, 3). We have isolated two genes, SRS1 and SRS4, encoding SSU in soybean (2, 4, 5). Both genes have been sequenced and partially characterized and have been found to share extensive homology at the nucleotide level, even in introns and flanking regions (4,5). SRS1 and SRS4 account for 3-4% of total transcription in nuclei from light grown soybean seedlings and approximately 80% of the SSU clones in a soybean leaf cDNA library (6).

Evidence for light controlled transcription of SRS1 and SRS4 (4,6) and small subunit genes from Lemna (7) and pea (8) has been provided by in vitro transcription experiments in isolated nuclei. Experiments with SSU genes from pea (9,10,11) and tobacco (12) and with the soybean gene, SRS1 (13,14), in transgenic plants and plants cells suggest that 5' flanking sequences control steady state mRNA and protein levels by acting as light inducible promoters and/or enhancers.

In this manuscript we report the use of run-on transcription assays in nuclei from transgenic petunia plants to demonstrate that a 754 bp fragment from SRS1, containing sequences from -694 to +60 (relative to the start of transcription), controls the transcription of a neomycin phosphotransferase (NPTII) gene in response to white and far-red light. The transcription of this SRS1/NPTII chimera, the native petunia SSU11A gene subfamily and several other control genes has been examined in young and old leaves.

MATERIALS AND METHODS

Transcription Run-on Assays in Petunia

Isolation and labeling of petunia nuclei was by the method of Berry-Lowe and Meagher (4) with the following exceptions. A large excess of dry ice was used as the leaves were powdered by 60 sec of grinding in a Braun coffee grinder. This material was thawed slowly, suspended in Honda buffer and filtered through cheesecloth. The initial homogenization step and pelleting of nuclei used for soybean (4) was omitted due to excessive shearing of petunia nuclei. Homogenization in liquid can result in greater than 99% loss of petunia nuclei. The filtrate (10 ml) was layered directly onto 18 ml Percoll gradients. The bottom layer was changed to 2.7M sucrose (3M sucrose containing 1/10 volume of 10X Gradient Buffer) to prevent pelleting of the petunia nuclei. Nuclei were washed once in 10-20 ml Honda buffer and once in 10-20 ml 20% nuclei resuspension buffer. Approximately 0.4×10^6 nuclei are present in each petunia leaf and typically 35% of these are recovered from the Percoll gradients. DNA dot blots were prepared by suction blotting of denatured DNA onto nitrocellulose as described in Berry-Lowe and Meagher (4). All hybridizations using labeled RNA from these nuclei with filters were carried out at 56°C in 50% formamide and 5 X Denhardt's solution (15). Filters were washed 4X, 15 min each in 0.2 X SSC, 0.1% SDS at 56°C. Young expanding leaves are defined as leaves less than 2 cm in length near the apical meristem of vigorously growing plants. Old leaves are defined as those approximately 4 cm in length and below the apical meristem.

Calculations

The method for calculating the amount of transcription based on ribosomal DNA transcription has previously been described in detail (4). The rDNA clone, pSR1.2B3, contains 1.05 kb of sequence internal to the soybean 18S rDNA gene in pBR322. The NPTII clone, pMON1001 contains 1 kb of coding sequence and 3.7 kb of vector sequences. The 5.0 kb pMON9503 plasmid contains approximately 0.5 kb of petunia SSU11A mRNA sequence.

RESULTS

The soybean genes, SRS1 and SRS4, are closely related members of a multigene family encoding the small subunit of ribulose-1,5-bisphosphate carboxylase (2,5). Transcription of these genes is low in darkness and high in the light and the transcriptional induction exhibits phytochrome control (4,6). Furthermore, in fully light grown seedlings transcription is reduced to the levels found in etiolated plants by far-red light treatment. The 5' upstream regions of these genes contain both classical transcriptional regulatory sequences and other potential regulatory sequences which may be involved in light and phytochrome mediated regulation (2, 5). Therefore, we have used the 5' regions of SRS1 and SRS4 to direct the transcription of a bacterial gene in a heterologous plant system and thereby assay directly which elements of transcriptional control are present.

Plant Transformation of a Soybean SSU Fusion to NPTII.

A 754 bp MboI fragment from SRS1 (2,4) containing 5' upstream sequences, the promoter region, the mRNA leader, and 14 bp of peptide coding sequence (-694 to +60) was fused to the neomycin phosphotransferase (NPTII) coding sequences and nopaline synthase (NOS) gene 3' untranslated sequences from pMON129 (16). The construction of pMON141 containing this SRS1/NPTII chimera, an intact NOS gene and functions required for integration into the T-DNA region of the *Agrobacterium* Ti plasmid are shown in Fig. 1. *Petunia* (Mitchell) protoplasts were transformed via the cocultivation method (17) and calli were selected for kanamycin resistance. Those calli undergoing the "short" transfer (17) and lacking phytohormone genes were regenerated into plants. Southern analysis of DNA from plants transformed with pMON141 confirmed that the plants contained only one or a few copies of the DNA inserted at a single site. Nopaline was detected (18) in leaves from transformed plants, indicating expression of the NOS gene on the construct (data not shown). Cuttings from a single transformed plant, T141-2, were propagated

A.

B.

and used in all experiments, along with nontransformed control *Petunia* (Mitchell) plants.

Transcription Assays in Young Transgenic *Petunia* Leaves.

Transcription run-on assays using nuclei from T141-2 plants were used to demonstrate that the light and phytochrome control of transcription resides in the 754 bp 5' fragment of the SRS1 gene. Alpha ^{32}P UTP labeled RNA from transgenic *petunia* plant nuclei was used to probe dot blots containing DNA from the SRS1/NPTII chimera and various control genes. Transcription of various structural genes in young expanding leaves was normalized to control rDNA expression as reported in Berry-Lowe and Meagher (4) for young soybean seedlings. It can be seen in Fig. 2 that significant levels of SRS1/NPTII transcription occur in young light-grown leaves relative to transcription of the rDNA control. Transcription of the SRS1/NPTII chimera can be normalized to rDNA transcription by comparing dots of similar intensity and making the appropriate corrections for the dilution, the amount (μg) of each plasmid loaded, the portion of the gene on the plasmid and by assuming that rDNA accounts for approximately 50% of total transcription (4). We estimate that the SRS1/NPTII chimera accounts for approximately 0.2% of total transcription in the light. It should be noted that SRS1 accounts for at least 1% of total transcription in soybean (4). Transcription of the NOS gene contained on the construct can also be detected, but at lower levels than for the SRS1/NPTII

Figure 1. A. Physical map of pMON141. Construction of a soybean SSU promoter chimera with the *E. coli* kanamycin resistance gene NPTII and its manipulation into an *A. tumefaciens* Ti plasmid. A 754 bp MboI fragment from the soybean SSU SRS1 gene clone, pSRS2.1 (2, 4), was subcloned into the BglII site of the pKC7 vector plasmid (26) to form pMON121. This SRS1 fragment contains 694 bp of 5' DNA including the TATA box and other potential regulatory sequences, the start of transcription, the 46 bp mRNA leader, the ATG initiation codon (black box) and the next 11 bp of coding sequence. This sequence was excised from pMON121 as part of an EcoRI/BclI fragment and fused to a BglII/EcoRI fragment from pMON129 (16) containing the neomycin phosphotransferase (NPTII) gene coding region and a nopaline synthase gene (NOS) 3' polyadenylation site. This SRS1/NPTII/NOS chimeric EcoRI fragment was cloned into the EcoRI site of pMON120 in the orientation shown to yield pMON141. The chimeric gene and NOS have the same orientation on the plasmid. Plasmid pMON141 was recombined via triparental mating into the homologous region (LIH) in the Ti plasmid, pTiB6S3 (17), to yield the construct pTiB6S3::pMON141 shown at the bottom of the figure. This recombination event results in there being two right borders in the Ti plasmid (right pointing triangles). Transformed cells resulting from the long transfer of DNA from the left border (left pointing triangle) to the second right border cannot be regenerated into plants. Transformed cells resulting from the short transfer of DNA from the left border to the first right border do not carry the hormone biosynthetic genes and are capable of regeneration into plants. B. Sequence of the SRS1/NPTII junction in pMON141.

Figure 2. Transcription of a SRS1 gene chimera in young expanding petunia leaves in light and in response to darkness and far-red light. The autoradiographs are of nitrocellulose filters containing plasmid DNAs applied in a two fold dilution series and probed with the transcription run-on products described below. Plasmid DNA: vector pBR322 (322), internal fragment of the soybean 18S rDNA gene in pSR1.2B3 (rRNA), neomycin phosphotransferase protein coding portion of the gene chimera in pMON1001 (NPTII), nopaline synthase gene in pMON41 (NOS) and the 3' end of the petunia SSU gene, SSU11A, in pMON9503 (ssPET3'). The rDNA plasmid is present in 1 μ g amounts in the first dot. All other plasmids are present in 5 μ g amounts in the first dot. Filters in each experiment were hybridized with equal counts of radioactive RNA from [α - 32 P] UTP labeled nuclei isolated from plants grown in the light (Lt), grown in the light and placed in darkness for four days (Lt→Dk 4 days), or grown in the light, treated for 15 min with far-red light and placed in darkness for 2 hours (Lt→Fr→Dk2hrs) or 12 hours (Lt→Fr→Dk12hrs). The dilutions shown can be normalized to equivalent amounts (equal dot intensities) of rRNA synthesis.

chimera. High levels of transcription are detected for the endogenous petunia SSU gene, SSU11A (19) and its closely related subfamily members (20). We estimate that this subfamily accounts for approximately 5% of the total transcription. The UTP labeled RNA does not hybridize with plasmid pBR322 DNA. In non-transformed control plants no NPTII or NOS transcription is detected (not shown).

Figure 3. Transcription of a SRS1 gene chimera in mature petunia leaves in the light and in response to darkness and far-red light. The autoradiographs are of nitrocellulose filter imprints of plasmid DNAs applied in a two fold dilution series and probed with labeled RNA. Plasmid DNA: as in Fig. 3 plus petunia actin gene in pPAC2 (PAC2), petunia extensin-like gene in p2BamR1 (HGRP1), and petunia glycine rich protein gene in p7D3-1200 (GRP1). The rDNA plasmid is present in 0.1 μ g amounts in the first dot, tenfold less than in Fig. 3 above. This allows the lower levels of transcription measured in mature leaves to be normalized to rDNA transcription. The petunia SSU plasmid is present in 1 μ g amounts in the first dot. All other plasmids are present in 5 μ g amounts in the first dot. Filters in each experiment were hybridized with equal counts of radioactive RNA from [α - 32 P] UTP labeled nuclei isolated from plants grown in the light (Lt), grown in the light and placed in darkness for four days (Lt→Dk 4 days), or grown in the light, treated for 15 min with far-red light and placed in darkness for 15 min (Lt→Fr→Dk15') or 2 hours (Lt→Fr→Dk2hrs).

If these transgenic plants are treated with 15 minutes of far-red light and then placed in darkness for either 2 or 12 hours, transcription of the petunia SSU11A gene and subfamily the SRS1/NPTII chimeric gene are reduced to undetectable levels in young leaves, whereas rDNA transcription is essentially unaffected (Fig. 2). Exposure of petunia plants to two hours of darkness with no far-red treatment has little effect on transcription levels in young leaves, parallel to what was observed in soybean seedlings. Light grown plants were placed in darkness for 4 days, reducing transcription of the native SSU11A gene subfamily and the SRS1/NPTII chimera in young leaves to the levels observed after 15 minutes of far-red light followed by 2 hours of darkness (Fig. 2). Treatment with far-red light or 4 days of darkness result in at least a 32 and 128 fold reduction in transcription rate for the SRS1/NPTII chimera and the native SSU11A subfamily respectively. These data suggest that some or all of the sequences responsible for light regulation and phytochrome control of transcription of SRS1 (4) are present in the 754 bp 5' region.

Transcription Assays in Mature Transgenic Petunia Leaves.

The response of transcription to darkness and far-red light is different in mature leaves than in young expanding leaves as is shown in Fig. 3. High levels of transcription were observed for all genes examined in young or mature light grown leaves. When fully light grown plants were placed in the darkness for 4 days the levels of petunia SSU or SRS1/NPTII transcription in young or mature leaves were reduced to the lowest levels measured. However, in contrast to the high levels of transcription observed under these conditions for rDNA genes in young expanding leaves, there is a low or undetectable level of rDNA transcription in mature leaves from the same plants. Transcription of a petunia actin gene (Baird and Meagher, unpublished), a petunia extensin-like gene (Condit, unpublished) and a petunia glycine rich protein gene, GRP1 (21), now included as controls, was also undetectable in mature leaves from plants treated with 4 days of darkness, whereas substantial levels are detected in mature leaves in the light.

What is also surprising is the extreme sensitivity of all specific transcription in mature leaves to far-red light. Mature leaves from plants treated with 15 minutes of far-red light and placed in darkness for 15 minutes shut down transcription of the petunia SSU11A gene subfamily and the SRS1/NPTII chimera, as expected. In addition, a slow reduction in transcription is observed in mature leaves for genes which appear to be constitutively expressed in young expanding leaves. Ribosomal DNA and actin

transcription, for example, are slightly reduced after far-red treatment and 15 minutes of darkness. Transcription of all genes examined is at least 100 fold lower if the far-red treatment is followed by 2 hours of darkness. These extremely low levels of specific transcription following far-red light treatment for all of the genes examined is surprising. Incubation of these nuclei with [α - 32 P] UTP resulted in relatively normal levels of incorporation into total RNA.

Kanamycin Resistance of Petunia Containing pMON141 and Other SSU Constructions.

The construction of the gene chimera in pMON141 resulted in the presence of three ATG codons following the transcription initiation site and preceeding the correct NPTII ATG initiation codon (Fig. 1). The first of these is the ATG initiation codon for the SRS1 polypeptide. This and the second ATG, which is contained in the SRS1 coding sequence, are both in frame with the NPTII polypeptide, and are followed by an in-frame stop codon. The third ATG codon was created in the construction and is out of frame. Despite this potential for incorrect initiation, significant kanamycin resistance is obtained in plants containing the SRS1/NPTII chimeric gene. Protoplasts and leaf discs derived from pMON141 transformed plant cells will form shoots on media (17) containing 50 and 300 μ g/ml kanamycin, respectively, whereas protoplasts and leaf discs from control petunias fail to form shoots readily on media containing 30 and 150 μ g/ml kanamycin, respectively.

The three additional upstream ATG codons were deleted by in vitro mutagenesis to produce pMON259 (Fig. 4). pMON259 contains the same sequence information as pMON141 except that it lacks the last 15 bp of the SRS1 mRNA leader and the extra ATG codons, and retains only 10 bp of the NPTII leader. Tissue from petunia containing this construct is only slightly more resistant to kanamycin than is pMON141-transformed tissue.

A third construct, pMS7L (pMS7+) (Fig. 4), has been made in which the SRS1 upstream promoter region in pMON259, up to a HindIII site 9 bp prior to the start of transcription, has been replaced with a homologous 5' upstream HindIII fragment (-672 to -9) from SRS4 (5). This construct retains the SRS1 mRNA leader sequences present in pMON259. The SRS4 5' promoted NPTII chimera also allows for regeneration and growth on media containing 300 μ g/ml kanamycin. Regeneration and growth on this level of kanamycin is indistinguishable from that of pMON259 transformed tissue.

Figure 4. Physical maps of junction between SSU and NPTII contained in pMON141, pMON259 and pMS7L (pMS7+). The *Bgl*III (*) site was created in the construction by *in vitro* mutagenesis.

DISCUSSION

Isolation of Transcriptionally Active Petunia Nuclei

In attempting to extend our transcription studies from soybean to petunia we encountered numerous initial difficulties due to the instability of petunia nuclei. Loss of up to 99% of the nuclei during liquid homogenization, as monitored by fluorescent microscopy (4), was not prevented by changing the ionic strength, detergent or sucrose concentration of the Honda buffer. This difficulty has not been encountered with soybean or maize (22) nuclei. However, efficient grinding of frozen leaves appears to be sufficient to release petunia nuclei from the membranes and cell walls of tissue, even though we found this treatment alone did not work with soybean. Pelleting and resuspension of petunia nuclei can also cause significant disruption of the nuclei and must be done with some care and as few times as possible. The abbreviated procedure described in the Materials and Methods usually results in the recovery of 3×10^6 nuclei from twenty leaves, an approximately 35% recovery. If this number of nuclei are labeled with 250 μ Ci of [α - 32 P] UTP (3000 Ci/mole) then 2 to 8×10^6 cpm are incorporated and recovered in RNA by this procedure.

Differences in the Transcriptional Response of Young and Mature Leaves.

We have observed differences in the transcriptional response of specific

genes between fully expanded mature petunia leaves and young expanding petunia leaves or soybean seedlings. Transcription of rDNA is not affected by far-red light treatment or exposure to darkness in soybean seedlings or in young expanding petunia leaves. However, transcription of the rDNA genes and all other genes examined was reduced significantly in mature leaves from petunia plants shifted to darkness for 4 days or treated with far-red light. Based on UTP incorporation, the nuclei were equally active in transcription in all plant samples following these various light treatments. The size of the radioactive RNA products from all petunia samples was equivalent although transcripts from petunia were smaller than those from soybean (4). The filters were probed with approximately equal numbers of counts. Our inability to detect any specific transcription for the variety of genes examined in far-red or dark treated mature leaves is an enigma. Perhaps the majority of transcription in far-red or dark treated mature leaves produces nonspecific RNA. In *E. coli* starved for nutrients rDNA transcription and protein synthesis are quickly shut down (23). It is possible that nutrient pools are more substantial in young seedlings and young leaves than in mature leaves which export most of their carbon and energy to other parts of the plant. Transcription and processing in mature leaves might therefore be more sensitive to the changes in the state of energy and carbon that must occur after extended periods of darkness or exposure to far-red light.

Light Regulated Transcription of SSU in Petunia

We have used a gene fragment from a highly expressed petunia SSU gene, SSU11A (19), to examine the light regulated transcription of native SSU genes in transgenic petunia plants. The high degree of homology between the six members of the subfamily to which SSU11A belongs (20) suggests that these experiments assay transcription of several closely related subfamily members. These experiments show that petunia SSU expression is controlled at the level of transcription over at least a 128 fold range in response to light, a greater response than has been demonstrated in soybean, *Lemna* and pea. The observation that far-red light rapidly shuts down SSU transcription as well as transcription of the SRS1/NPTII chimera in fully light grown petunia plants parallels our earlier work in soybean seedlings (4) and indicates that this may be part of a general phytochrome response in a variety of plants.

5' Proximal Soybean Sequences Direct Light and Phytochrome Controlled Transcription

The upstream regions from four SSU genes and two chlorophyll

a/b-binding protein (Cab) genes from other laboratories have been shown to direct the light regulated expression of bacterial reporter genes in transgenic callus or plants. Upstream sequences from -973 to -4 of the pea Rubisco ss gene, *rbcS ss3.6*, regulate chloramphenicol acetyl transferase (CAT) activity and steady state mRNA levels in tobacco callus in a manner consistent with the presence of a light regulated enhancer in this region (10). Two other pea genes, *rbcS-3A* and *rbcS-E9*, contain sequences from -410 to -48 and -317 to -82, respectively, that function as light induced enhancers and confer phytochrome regulation on CAT levels in transgenic petunia plants (11). Sequences from -1,038 to +32 of a tobacco SSU gene, *rbcs-8B*, control CAT transcript levels in response to light in transgenic tobacco and petunia plants (12). In addition, upstream sequences from a wheat Cab gene, *Cab-1*, have been shown to direct the phytochrome controlled expression of CAT levels in transgenic tobacco seedlings (24), while the region from -347 to -100 of a pea Cab gene, *AB80*, acts as a light-inducible enhancer and tissue-specific silencer in a NOS/NPTII fusion in transgenic tobacco plants (25).

Upstream sequences from *SRS1* have previously been shown to direct steady state mRNA expression of reporter genes in response to light in transgenic plants and plant cells. A 1.1 kb 5' fragment, including 36 bp of the 45 bp mRNA leader, was fused to the NPTII gene, introduced into soybean cotyledons, and transformed callus was shown to contain light regulated steady state levels of chimeric mRNA (13). In addition, a chimera containing 900 bp of *SRS1* upstream sequences, including 38 bp of the mRNA leader, fused to a NOS gene has been shown to express NOS activity in *Kalanchoe* cells in a light dependent manner (14).

In this study we have shown that *SRS1* sequences direct the light regulated and phytochrome mediated transcription of an NPTII gene in transgenic petunia plants. A 5' gene fragment from *SRS1* (from -694 to +60), containing upstream flanking sequences, the promoter, the mRNA leader and a portion of the first exon was fused to the coding region of NPTII in order to assay this region of soybean DNA for light regulatory functions. In transformed petunia plants transcription of the *SRS1*/NPTII chimera is regulated in the same manner as the *SRS1* gene in soybean and parallels transcription of a native petunia small subunit gene subfamily. Transcription is much higher in the light than in darkness, as expected. Most significantly, transcription of the chimera is rapidly shut down in plants treated with far-red light, demonstrating that at least a portion of the light and phytochrome response of the *SRS1* gene is controlled on this 754 bp piece of DNA. This result and the observation that

transcription of these genes gradually decreases in the absence of white light imply that phytochrome control is closely linked to transcription and that transcription of these genes may require the continued presence or synthesis of phytochrome in the P_{fr} form (4). The similar levels of kanamycin resistance obtained for the SRS1/NPTII and SRS4/NPTII chimeras and the high degree of sequence homology between SRS1 and SRS4 suggest that the light and phytochrome controlled transcriptional response of SRS4 is also controlled by sequences contained within the 700 bp upstream region.

Conclusion

Light, darkness and far-red light treatments all affect the transcription of SRS1/NPTII chimeric gene and a native petunia SSU gene subfamily. Transcription run-on assays have confirmed the immediate negative effects of far-red light treatments on transcription of the native SSU11A gene subfamily in fully light grown plants parallel to our earlier work in soybean (4). We demonstrate that this response is controlled by 5' proximal sequences in the soybean gene, SRS1. A striking difference is observed between young expanding and fully expanded mature leaves in the transcriptional response of all genes examined.

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