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Transgenic expression of two marker genes under the control of an *Arabidopsis rbcS* promoter: Sequences encoding the Rubisco transit peptide increase expression levels

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Summary. Chimeric gene constructs were made in which two reporter genes, the *neo* and *bar* genes, encoding neomycin phosphotransferase II and phosphinothricin acetyl transferase, respectively, were placed under the control of the promoter of *ats1A*, one of four genes encoding the ribulose-1,5-bisphosphate carboxylase (Rubisco) small subunit (SSU) in *Arabidopsis thaliana*. In one set of constructs the fusions were made at the initiation codons, while in the second set the sequences encoding the *ats1A* transit peptide were included. Significantly higher steady-state levels of RNA and protein were observed in leaves of transgenic plants carrying the latter constructions. Individual transgenic plants varied in their degree of tissue specific expression of the chimeric genes as well as in absolute levels of expression. Preliminary results suggest that the *ats1A* promoter may be only weakly responsive to phytochrome.

Key words: Rubisco – Small subunit – Transgenic plants – Chimeric genes – Transit peptide

Introduction

A large number of chimeric genes composed of various promoters, genes and termination signals have been introduced into both dicots and, more recently, some monocots, using either vectors based on *Agrobacterium* Ti plasmids or direct DNA transfer methods (Fraley et al. 1986). In many cases, regulation of an intact gene transferred to another plant species is similar to that in the homologous system (see Shah et al. 1987 for review). By using easily assayable marker genes it has been shown that a promoter confers similar expression patterns in a transgenic plant, and regulatory regions of the promoter have been defined by deleting portions and observing the effect on gene expression (Kuhlemeier et al. 1987; Willmitzer 1988). Such studies have greatly expanded the understanding of plant promoter structure and function.

The choice of promoter to direct expression of foreign genes in transgenic plants will depend on the objective, and thus it is important to develop an array of promoters with well-defined characteristics. Promoters commonly used for this purpose include the promoter from the nopaline synthase gene of the T-DNA of *Agrobacterium tumefaciens* Ti plasmid (Herrera-Estrella et al. 1983), the bidirectional TR promoter from the *A. tumefaciens* T-DNA (Velten et al. 1984), the 35S (Odell et al. 1985) and 19S (Koziel et al. 1984) promoters from cauliflower mosaic virus (CaMV). Other promoters originate from abundantly expressed light-regulated plant genes such as chlorophyll *a/b* binding protein promoters or ribulose-1,5-bisphosphate carboxylase (Rubisco) small subunit (SSU) gene promoters (Herrera-Estrella et al. 1984; Jones et al. 1985; Morelli et al. 1985; Simpson et al. 1986).

Rubisco is the bifunctional enzyme which catalyzes the initial carbon dioxide fixation step in the Calvin cycle and functions as an oxygenase in photorespiration. In higher plants it consists of eight each of two subunits, a large subunit encoded by the chloroplast genome and the SSU polypeptides encoded in the nuclear genome (Ellis 1981). The SSU polypeptides are formed as precursors containing an amino-terminal extension termed a transit peptide, which is involved in the transport of the SSU polypeptide into the chloroplast after which it is cleaved off (Ellis 1981). In most plant species analyzed thus far, the SSU polypeptide is encoded by multigene families (Manzara and Gruissem 1988). The role of the different *rbcS* genes is not completely understood, but it is shown that different members of the same family show varying levels of expression and different patterns of tissue specific and light regulated expression (Dean et al. 1985; Fluhr and Chua 1986; Fluhr et al. 1986; Sugita and Gruissem 1987).

Rubisco is the most abundant protein found in plant leaves, representing up to 50% of the soluble protein in these organs (Kung 1976). While stable mRNAs could also be in part responsible for the high protein levels observed, the promoters of its genes have been attractive candidates for expression of genes at high levels. The large gene families encoding SSU polypeptides found in most plants, with their varying patterns of expression, have made the choice of an *rbcS* promoter for use in chimeric constructs difficult. *Arabidopsis thaliana* provides an interesting model due to its small genome, which has less repetitive DNA and smaller gene families than other species (Pruitt and Meyerowitz 1986). Recently, the four member *rbcS* gene family of *Arabidopsis* has been characterized (Krebbers et al. 1988). In this work the expression of chimeric genes under control of the promoter of one of these genes, *ats1A*, in transgenic tobacco plants is analyzed. The promoter was fused to two marker genes. Examination of expression in leaves at both RNA and protein levels reveals that the *ats1A* promoter is capable of directing higher levels of expression than the *nos*, 35S

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and TR1′ promoters. Moreover, the presence of the transit peptide sequence in the chimeric construct had a significant influence on the steady state levels of RNA and protein. Analysis of expression of the chimeric genes in different tissues in some cases reveals an unexpected pattern. Light regulation of chimeric *ats1A* genes has also been studied.

Materials and methods

Plasmid constructions. The plasmid pATS3 (Timko et al. 1988; Krebbers et al. 1988) contains a 1.8 kb *Eco*RI fragment encoding part of the *A. thaliana rbcS ats1A* gene including the promoter region, the transit peptide encoding sequence, and a portion of the sequence encoding the mature polypeptide. Four chimeric constructs were made with the *ats1A* promoter: pGS1400 and pGS1401 contain promoter fusions with *neo*, and pGSFR1400 and pGSFR1401 with *bar*. DNA manipulations and cloning were carried out as described in Maniatis et al. (1982). All promoter fusions were confirmed by DNA sequencing (Maxam and Gilbert 1980).

A. pGS1401. Plasmid pGSST*neo*2 contains the *neo* gene followed by the 3′ end of the octopine synthase gene (Van den Broeck et al. 1985). It was derived from pKM109-9 (Reiss et al. 1984a), in which seven extra codons were inserted between the first and second codons. The extra amino acids were shown not to affect the enzyme's activity. At its 5′ end the *neo* coding sequence is flanked by a *Bam*HI site. To fuse the *ats1A* promoter to the *neo* gene, the *Eco*RI – *Bam*HI (the latter site made blunt using Klenow DNA polymerase) fragment of pGSST*neo* was replaced by the *Eco*RI-*Sph*I fragment of the *ats1A* gene, consisting of the promoter region and the transit peptide encoding sequence. A synthetic oligonucleotide (5′-GATCCATG-3′), complementary to both *Sph*I and *Bam*HI protruding ends, was used to fuse the transit peptide and *neo* gene in-frame. Finally, the *Eco*RI – *Sal*I fragment containing this gene fusion was isolated, the ends filled-in with Klenow DNA polymerase, *Bgl*II linkers attached, and the entirety cloned into pGV825 digested with *Bam*HI and *Bgl*II (Deblaere et al. 1985).

B. pGS1400. In order to make fusions with the promoter at the initiation codon of the *rbcS* gene, a *Kpn*I site was created immediately downstream of the initiation codon by site-directed mutagenesis (Stanssens et al., in preparation), leaving the nucleotide sequence in front of the ATG intact. The 3′ overhang of this site was removed using Klenow DNA polymerase and the *neo* gene was fused in-frame by exchanging the *Eco*RI – *Bam*HI fragment containing the SSU promoter and transit peptide of pGS1401 for the *Eco*RI – *Kpn*I Klenow-treated fragment carrying the *ats1A* promoter fragment.

C. pGSFR1401. A *bar* gene cassette was obtained from pGSFR1 (De Block et al. 1987). An *Nco*I site was created at the initiation codon of the gene by site-directed mutagenesis, yielding pGSFR2. pATS3 was digested with *Sph*I, treated with Klenow DNA polymerase and ligated to the filled-in *Nco*I ends of pGSFR2. This yielded an exact fusion between the transit peptide and the *bar* gene.

D. pGSFR1400. An exact fusion at the initiation codon with the *bar* gene was obtained by ligating a *Kpn*I fragment containing the *ats1A* promoter treated with Klenow DNA polymerase to the *bar* gene in pGSFR2, which was digested with *Nco*I and made blunt-ended with S1 nuclease. The construction is such that no extra amino acids are added to the *bar* gene product.

Both pGSFR1400 and pGSFR1401 were inserted between the T-DNA borders of pGSC1702. The latter is a binary vector derived from pGSC1700 (Cornelissen and Vandewiele 1989), which contains the 3′ ends of the T-DNA gene 7 (Velten and Schell 1985).

Plant transformation and regeneration of transformants. The chimeric genes flanked by the octopine T-DNA borders of a co-integration type vector (pGS1400 and pGS1401) or of a binary vector (pGSFR1400 and pGSFR1401) were mobilized into *Agrobacterium* recipient strain C58C1Rif^R (pGV2260) (Deblaere et al. 1985) by triparental mating. The structure of the co-integrates was confirmed by Southern analysis of total DNA prepared from *Agrobacterium* according to Deblaere et al. (1987). Mobilization of binary plasmids were checked by restriction digests of plasmid minipreparations from *Agrobacterium*. Leaf discs of *Nicotiana tabacum* cv Petit Havana (SR1) were infected with the different *Agrobacterium* strains as described (Deblaere et al. 1987). Transformed shoots were selected on shoot inducing medium containing either 100 μg/ml kanamycin (pGS1400 and pGS1401) or 50–100 μg/ml phosphinothricin (pGSFR1400 and pGSFR1401) (De Block et al. 1987). Transformed shoots were rooted on a hormone free medium.

Preparation of plant extracts and gene product quantitation. Tissues of transformed plants were ground in 2 volumes (w/v) of 50 mM TRIS-HCl pH 6.8, containing 0.13 mg/ml leupeptin, and 1% β-mercaptoethanol. The extracts were clarified by centrifugation (12000 × *g*). The protein concentration of the supernatant was determined by modified Bradford assay kit (Bio-Rad) as specified by the manufacturers. Aliquots of plant tissue extracts corresponding to 25–75 μg of total extracted protein were fractionated either in a 10% non-denaturing gel, for neomycin phosphotransferase II (NPT II) activity detection, or in 12.5% SDS-polyacrylamide gels for Western blotting. NPT II activity was measured essentially as described by Reiss et al. (1984b). The results were quantified by localizing the portions of the filters containing the phosphorylated kanamycin sulfate by autoradiography, cutting these out and measuring Cerenkov counts. Western blots were done as described by Towbin et al. (1979) using 50–75 μg of protein. The nitrocellulose filter was first treated with a rabbit polyclonal antiserum against phosphinothricin acetyl transferase (PAT) or NPT II, and then with alkaline phosphatase labelled anti-rabbit antibody after which the reaction color development solution (Bio-Rad) was used as a substrate. PAT activity assays were done as described by De Block et al. (1987). In all Western blots and enzyme activity assays, equal amounts of protein per sample were used.

Plant RNA and DNA preparation: Hybridization techniques. Tobacco leaf RNA was prepared as described (Jones et al. 1985). Total RNA (20 μg) was electrophoresed in a 1.2% agarose-formaldehyde gel and blotted to Hybond N membranes (Amersham). Hybridizations were done in 2 × SSPE (Maniatis et al. 1982), 0.1% SDS, 0.02% Ficoll, 0.02% polyvinylpyrolidone and 50 μg/ml sonicated herring sperm DNA at 60° C. Filters were washed at 65° C for 10 min each in 5 × SSPE; 1 × SSPE, 0.1% SDS; and 0.1 × SSPE,

0.1% SDS. ^{32}P-labeled RNA probes complementary to the *bar* or *neo* mRNAs were made using the pGEM system (Promega).

Leaf DNA of individual transformed plants was isolated according to Dellaporta et al. (1983). After digestion and electrophoresis in a 1% agarose gel, Southern blots were performed as described (Meinkoth and Wahl 1984) and the filters probed with nick-translated DNA (Maniatis et al. 1982) fragments containing the *bar* or *neo* genes.

S1 nuclease mapping of the 5′ ends of transcripts from chimeric genes in transformed tobacco plants. Total RNA was prepared from light-grown leaf tissues of transformed plants (Cashmore 1982). An approximately 1900 bp *Eco*RI–*Bam*HI fragment from pGS1401 was used as a probe, and S1 nuclease protection experiments carried out as described in Krebbers et al. (1988).

Light regulation studies. Seeds were surface sterilized for 20 min in bleach, rinsed 3 times with water, and sown on Murashige and Skoog salts (Flow Laboratories) medium supplemented with 1.5% sucrose and 0.8% agar. They were grown in a dark incubator at 25° C for 18 days, after which light treatments were carried out as described by Rethy et al. (1983). The fluence rate of the broad band red light was 19.56×10^{-6} mol/m^2 per s; that of the broad band far-red light was 7.5×10^{-6} mol/m^2 per s. Plants were exposed to red or far-red light for 10 min, or red light for 10 min followed by far-red light for 10 min, then kept in the dark for a further 24 h after which RNA was prepared. Plants treated with white light were left in the light for 24 h.

Results

Experimental strategy

To study expression of chimeric genes under control of the *ats1A* promoter in transgenic plants, two promoter cassettes were created as shown in Fig. 1A. A *Kpn*I site was introduced at the initiation codon by site-directed mutagenesis. Gene fragments can be exactly fused at the ATG upon cleavage with *Kpn*I and removal of the protruding 3′ end. Secondly, a *Bam*HI site was generated at the transit peptide cleavage site, which allows the construction of gene fusions at the transit peptide sequence. The two different promoter cassettes were combined with two heterologous genes (Fig. 1B): the *neo* gene encoding neomycin phosphotransferase II (NPT II), which confers resistance to aminoglycoside antibiotics such as kanamycin and G418 (Beck et al. 1982), and the *bar* gene, encoding phosphinothricin acetyl transferase (PAT; Thompson et al. 1987), which confers resistance to the herbicidal compounds phosphinothricin (PPT) and bialaphos. pGS1400 and pGSFR1400 have the reporter genes fused at the *ats1A* initiation codon (no extra amino acids were added during the construction), while pGS1401 and pGSFR1401 are fusions at the transit peptide cleavage site. These plasmids carrying the chimeric genes between the T-DNA border repeats were mobilized into an appropriate *Agrobacterium* strain and used to transform tobacco. Transformed shoots were selected on medium containing either kanamycin or phosphinothricin. Tests of leaf discs from the resulting plants on higher, varying levels of the appropriate selective agent showed differences in maximum resistance levels between different regenerants,

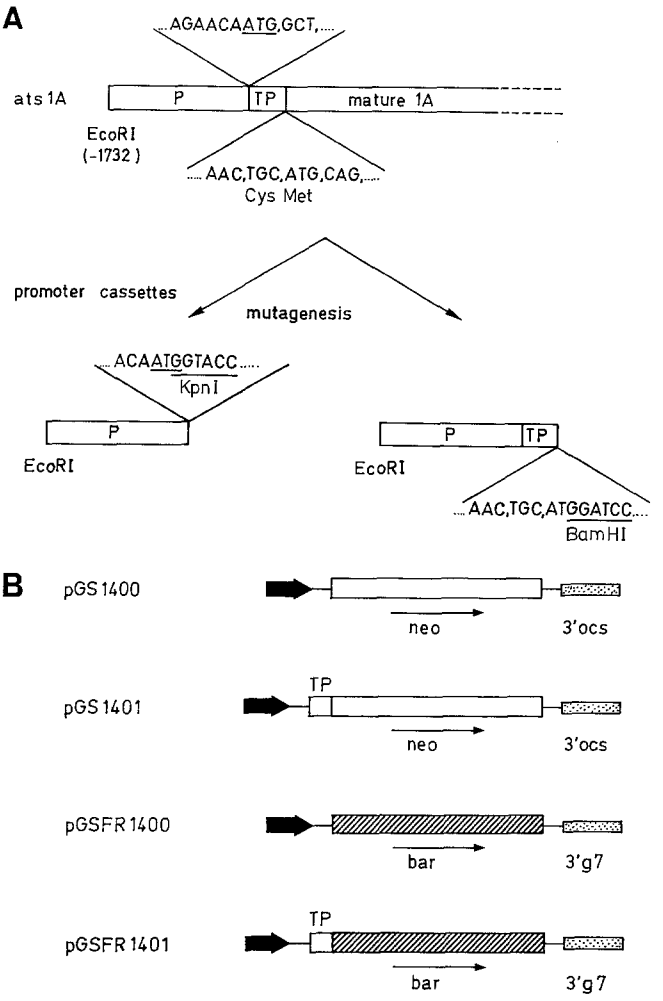

Fig. 1A and B. Schematic diagrams of *ats1A* promoter cassettes and chimeric constructs. **A** Structure of the *ats1A* gene. P indicates the promoter and upstream regions and TP the transit peptide. The sequences at the initiation codon and the transit peptide cleavage site are shown. A unique *Eco*RI site is located at position −1732. The promoter cassette contains a *Kpn*I site at the initiation codon and the promoter-transit peptide cassette contains a *Bam*HI site immediately after the transit peptide cleavage site. **B** Chimeric constructs with the *ats1A* promoter. The *neo* and *bar* genes were fused to both cassettes described above. pGS1400 and pGSFR1400 carry the respective genes fused at the initiation codon, whereas pGS1401 and pGSFR1401 have the genes fused at the transit peptide cleavage site. The *heavy arrow* indicates the position and direction of transcription of the promoter. The *neo* gene is represented by the *open box*, the *bar* gene by the *hatched box*. The 3′ untranslated ends of octopine synthase (3′ ocs) and the T-DNA gene 7 (3′ g7) are represented by *dotted boxes*

suggesting that the original selection did not unduly bias the experiment in favor of those transformants generating the highest levels of expression. For convenience, the plasmid designations are used to refer to the corresponding chimeric genes.

Expression of ats1A-neo *and* ats1A-bar *chimeric genes at the protein level*

Enzymatic activity tests and Western blotting were used to analyze the expression of NPT II driven by the *ats1A* promoter. NPT II activities measured in extracts of tobacco

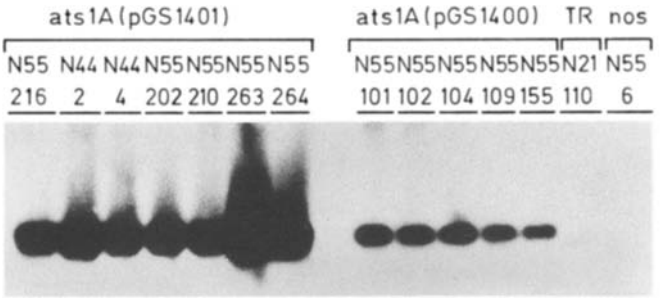

Fig. 2. Neomycin phosphotransferase II (NPT II) expression levels in total leaf extracts of transgenic plants carrying pGS1401 and pGS1400. An NPT II enzymatic assay is shown. The numbers preceded by N identify individual plants. The slots labeled TR and nos contain extracts from plants carrying chimeric *neo* genes driven by those promoters

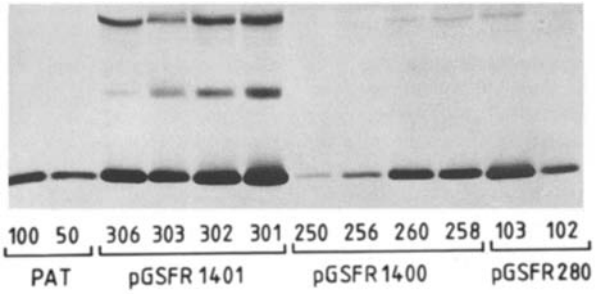

Fig. 3. Phosphinothricin acetyl transferase (PAT) expression levels in total leaf extracts of transgenic plants carrying pGSFR1401, pGSFR1400 and pGSFR280. A Western blot using a polyclonal PAT antiserum is shown. The slots labeled PAT were loaded with 100 and 50 ng of PAT, respectively

leaves from the subset of randomly chosen plants transformed with pGS1400 and pGS1401 are shown in Fig. 2. The tests showed higher enzymatic activity in transgenic plants in which the chimeric gene included the transit peptide encoding sequence (pGS1401) than in those without (pGS1400). Semi-quantitative analysis of the enzymatic activities revealed a 4- to 10-fold higher level of NPT II activity in plants expressing pGS1401 relative to those transformed with pGS1400. Similar results were obtained when Western blots of total leaf extracts were done (not shown).

To determine if this difference is specific for the *neo* constructs, randomly chosen plants transformed with pGSFR1400 and pGSFR1401 were similarly analyzed for *bar* gene expression. Analysis of crude leaf extracts by Western blotting revealed the synthesis of a polypeptide which co-migrates with purified PAT. Extracts from four plants from each population are shown in Fig. 3. Data obtained from a larger number of plants by spectrophotometric quantitation of PAT activity in leaf extracts are presented in Table 1. Again, the highest expression was observed in plants transformed with the constructs containing the transit peptide-encoding sequence (pGSFR1401), the mean expression level of the latter being 14 times that of plants expressing pGSFR1400. The expression level in some cases was sufficient to give a just visible band on Coomassie blue stained SDS-polyacrylamide gels, which in the gel system used corresponds to approximately 1% of total cellular protein (data not shown).

Table 1. Phosphinothricin acetyl transferase (PAT) activity in individual transformants

pGSFR1401		pGSFR1400	
Plant	Activity	Plant	Activity
300	390	250	4
301	370	251	20
302	460	252	12
303	135	253	20
304	596	254	45
305	174	255	12
306	228	256	10
307	490	258	88
308	506	260	43
309	372	261	49
310	973		
311	222		
312	766		
Mean	435		30

Numbers under plant refer to individual plant numbers. PAT activity is given in 10^{-3} unit/mg extracted protein

In Western blot analysis it is observed that NPT II from plants carrying pGS1400 and pGS1401 (not shown), and PAT from plants carrying pGSFR1400 and pGSFR1401 (Fig. 3), comigrate, and immunogold experiments reveal that both reporter proteins are located in the chloroplasts (E. De Almeida and G. Engler, unpublished results). These experiments suggest that transport of the reporter proteins into chloroplasts and cleavage of the transit peptide occurs.

Expression of ats1A-neo *and* ats1A-bar *chimeric genes at the RNA level*

To determine if the difference in the expression pattern at the protein level was reflected at the RNA level, Northern analyses were carried out. The analyses of total RNA of leaves from plants transformed with pGS1400 and pGS1401 are shown in Fig. 4A and from plants transformed with pGSFR1400 and pGSFR1401 in Fig. 4B. The differences seen at the protein level are also reflected at the RNA level; the chimeric genes including transit peptide-encoding sequences show higher steady state RNA levels in leaves than those without.

S1 nuclease protection experiments (not shown) demonstrate that the transcripts derived from the chimeric genes initiate at the same position (approximately 28 nucleotides upstream from the initiation codon) as in the *ats1A* gene (Krebbers et al. 1988).

Comparison of NPT II expression directed by different promoters

The expression level of NPT II directed by the *ats1A* promoter was compared with that directed by the 35S, TR1′ and *nos* promoters. Plants were transformed with plasmids which contain chimeric *neo* genes cloned in the T-DNA expression vectors pGSH150 and pGSJ280 (Deblaere et al. 1987). Total leaf extracts of independently isolated transformants were analyzed at the RNA and protein levels. In general, at the protein level the *ats1A* promoter showed much higher levels of expression compared to TR1′ and

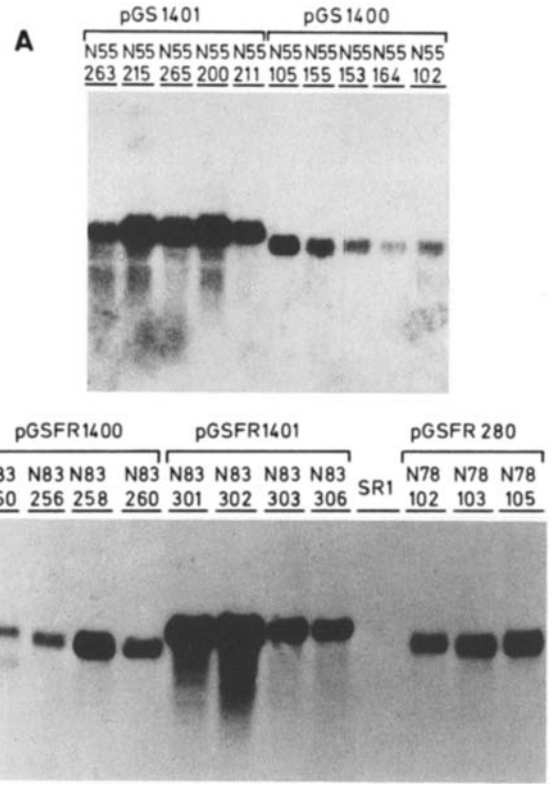

Fig. 4A and B. Northern blot analysis of *ats1A* chimeric gene expression. Total RNA extracted from leaves of transgenic tobacco plants expressing the constructs indicated was probed with labeled *neo* (**A**) and *bar* (**B**) antisense RNA. The numbers preceded by N identify individual plants, and SR1 indicates a lane containing RNA extracted from an untransformed plant

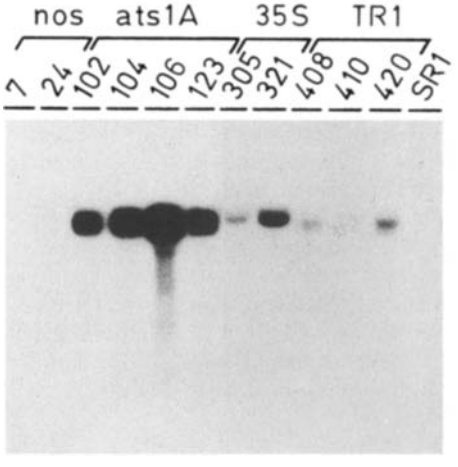

Fig. 5. Comparison of NPT II RNA levels produced by the *ats1A*, 35S, TR1′ and *nos* promoters in transgenic tobacco. Northern blot analysis of total leaf RNA was done using a labeled *neo* antisense probe. The plant used for the *ats1A*-driven RNA carried pGS1401. Numbers identify individual plants

the *nos* promoters (e.g. Fig. 2). Northern analysis of total RNA from leaves showed that the *ats1A* promoter produced significantly higher levels of steady state RNA than the other promoters (Fig. 5). Similar results were obtained with the *bar* chimeric genes. Comparison of plants expressing pGSFR1400 and pGSFR1401 with tobacco plants transformed with a CaMV 35S-*bar* construct (pGSFR280, De Block et al. 1987) indicates that plants carrying pGSFR1401 have PAT activity levels averaging at least an order of magnitude higher than the 49×10^{-3} units/mg protein found for those carrying the CaMV 35S constructs (Fig. 3; Table 1; De Block et al. 1987; due to clerical error the activity is labeled as being 10 times too high in that report), while those carrying pGSFR1400 have an average activity of the same order of magnitude as that found in the 35S plants. Southern analysis showed that the differences in expression of both NPT II and PAT were not due to multicopy inserts (data not shown).

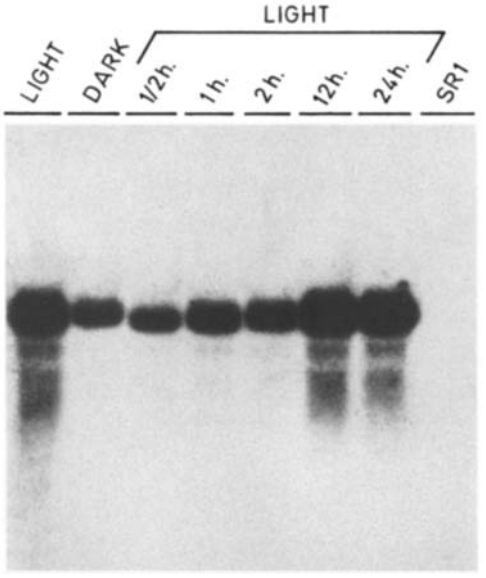

Fig. 6. Kinetic analysis of the effect of light on steady state levels of RNA encoded by the *ats1A-neo* chimeric gene. Plants transformed with pGS1401 were grown in light, placed into dark for 5 days, and moved into the light again. Total leaf RNA was extracted before the plants were moved into the dark (light), at the end of the period in the dark (dark) and subsequently after 0.5 (1/2), 1, 2, 12 and 24 h. The filter was probed with labeled NPT II antisense RNA. SR1 indicates a lane containing RNA extracted from an untransformed plant

ats1A-neo *chimeric genes are light inducible*

In order to examine the light inducibility of chimeric genes under the control of the *ats1A* promoter, adult plants grown in the greenhouse carrying pGS1400 and pGS1401 were moved to the dark for 5 days and then moved into the light. RNA was extracted and a Northern analysis performed. During the dark period expression dropped, but significant levels are still observed (Fig. 6). It rises again when plants are moved into the light, reaching the initial level after 2–12 h.

Phytochrome as well as other photoreceptors has been implicated in the regulation of *rbcS* genes; in dark grown seedlings, red light induction of genes can be reversed with a brief pulse of far-red light (Tobin 1981; Sazaki et al. 1983; Thompson et al. 1983; Bennett et al. 1984; Kaufman et al. 1984; Fluhr and Chua 1986). To determine if a similar response occurs, 18-day-old dark grown seedlings transformed with pGS1401 were irradiated with red, red fol-

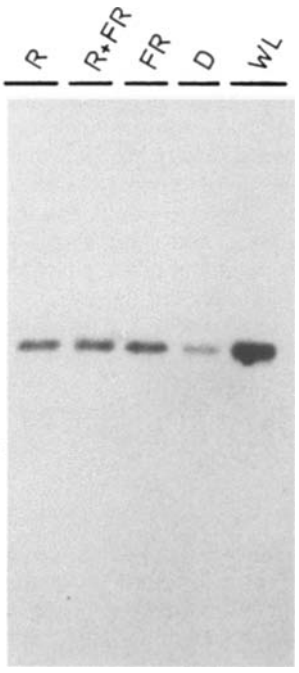

Fig. 7. Effects of red, far-red and white light on NPT II RNA levels driven by the *ats1A* promoter in etiolated seedlings of plants transformed with pGS1401. At 18 days old, seedlings were irradiated with 10 min red (R), 10 min red followed by 10 min far-red (R + FR) or 10 min far-red (FR) light. After irradiation, the seedlings were kept in darkness for 24 h. Seedlings were also exposed to 24 h of white light (WL) or continuously kept in darkness (D). After these treatments, RNA was isolated and subjected to Northern analysis using labeled *neo* antisense RNA as probe

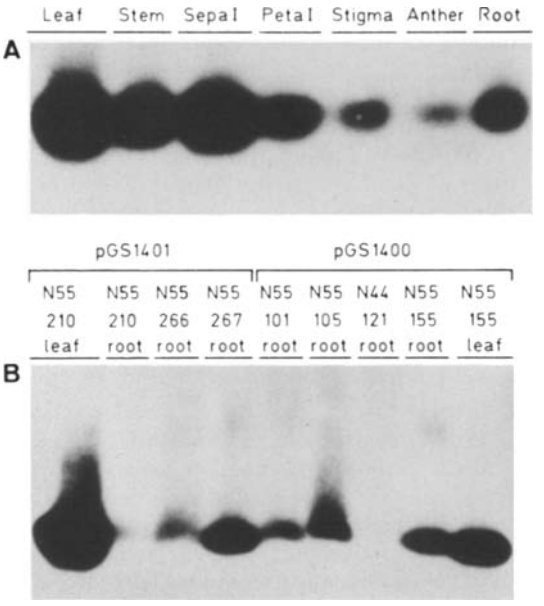

Fig. 8A and B. Organ-specific expression in transgenic tobacco. **A** NPT II enzymatic assay of protein extracts from the indicated plant tissues from plants transformed with pGS1401. Extracts from several plants were pooled. **B** As in **A**, but from roots or leaves of individual plants, identified by the numbers preceded by N, transformed with pGS1401 or pGS1400

lowed by far-red, far-red and white light. After irradiation, the seedlings were kept in darkness for 24 h, after which RNA was prepared. Northern analysis showed a small increase of NPT II RNA after red light irradiation. On the other hand, a small increase of NPT II RNA was also observed in seedlings exposed to only far-red light compared to the dark control and a great increase was observed when the seedlings where exposed to white light (Fig. 7). Thus classical phytochrome regulation could not be demonstrated, but it is possible that a very low fluence response is occurring (see Discussion).

Analysis of the tissue specificity of chimeric genes driven by the ats1A *promoter*

The tissue specificity of expression of the chimeric genes was examined by analyzing pooled floral parts, stems, seed pods and roots of several plants transformed with pGS1400 and pGS1401 for NPT II activity. It was clearly most abundant in the green tissues (leaves, stems and sepals) tested. Lesser but still significant amounts were observed in petals, anthers and stigmas (Fig. 8A). A surprisingly high NPT II activity was observed in the roots. To determine if this was a general phenomenon, roots from individual plants, all of which had high expression levels in leaf tissue, were prepared. Unexpectedly, a wide range of expression was observed (Fig. 8B). These results were confirmed by a Western blot done on roots obtained from transgenic plants expressing the *bar* gene (data not shown). Thus, the organ specificity of heterologous gene expression can vary between individual transformants (see Discussion).

Discussion

Studies on the expression of chimeric genes based on the *Arabidopsis rbcS ats1A* promoter in tobacco have been described. Two markers, *neo* and *bar*, were expressed as unmodified proteins (plasmids pGS1400 and pGSFR1400) or as fusion proteins with the transit peptide of the SSU gene attached at their amino-terminal ends (pGS1401 and pGSFR1401).

Inclusion of transit peptide encoding sequences affects expression levels regardless of the reporter gene used

When expression levels of the reporter genes fused at the initiation codon or at the transit peptide are compared, the latter are found to have significantly higher expression levels at both the protein level (Figs. 2, 3, Table 1) and the RNA level (Fig. 4). This effect was observed independently of the marker gene expressed. There are several possible explanations for this phenomenon, including higher stability of an mRNA molecule which includes the transit peptide encoding sequence, or a higher rate of translation of those transcripts, which may in turn stabilize the RNAs. It cannot be excluded that the transit peptide encoding sequences themselves have intrinsic transcriptional enhancing or mRNA stabilizing capabilities. This effect may not be specific to the transit peptide sequences, but due to the presence of eukaryotic sequences between the initiation codon and the prokaryotic *neo* and *bar* genes. Further experiments are required to distinguish between these possibilities. No obvious clues can be found in the sequences flanking

the initiation codon, which have been shown to be important in determining the final levels of expression, illustrated in plants by Jones et al. (1988). The sequences in the two 1401 constructs (AACAATGGCT) and the two 1400 constructs (AACAATGGAC for the *bar* fusion , AACAATGGAT for the *neo* fusion) all match the consensus sequences found in a series of plant genes (AACAATGGC; Lutcke et al. 1987). In plants expressing the transit peptide fusions the product appears to be transported to the chloroplast, but it is difficult to envision how the transit peptide itself or the transport process could affect transcriptional activity.

Comparison of different promoters expressing the *neo* gene at both the RNA and protein level shows that, in leaves, *ats1A* directed higher levels of expression relative to the 35S, TR1′ and *nos* promoters in that orders (Figs. 2, 3) when the transit peptide encoding sequences were included, and levels roughly equal to that of 35S when they were not included. A similar overall view was observed in a broader comparative analysis of transgenic tobacco plants transformed with nearly identical chimeric *bar* gene constructs under the control of the same four promoters (Denecke et al., in preparation), and the ranking of the latter three is in agreement with the results of Sanders et al. (1987) and Harpster et al. (1988). Tumer et al. (1987) showed the level of expression of the AMV coat protein when expressed under the control of the CaMV 35S promoter to be 0.1–0.4% of total soluble protein. In plants carrying pGSFR1401 it is estimated that expression levels reach up to 1% of total soluble protein.

Chimeric genes driven by ats1A *are stimulated by light*

When using a promoter to express chimeric genes, it is important to be able to predict its developmental patterns of expression, and to determine if the chimeric gene will be regulated as expected. Chimeric genes driven by *rbcS* promoters would be expected to be light inducible and largely specific to green tissues. Experiments carried out to see whether the *ats1A-neo* chimeric gene is light inducible in transgenic tobacco plants were done in two ways because different types of light regulation have been reported using different experimental systems (Tobin 1981; Sazaki et al. 1983; Thompson et al. 1983; Bennett et al. 1984; Fluhr and Chua 1986). In the first case, leaves of light-grown plants placed in darkness for 5 days showed a significant decline in the RNA steady state level but a basal and relatively high level of RNA was still present during the dark period in these plants. This result indicates that the chimeric gene *ats1A-neo* is not strictly dependent on light (Fig. 6).

In a second set of experiments, the induction of the *ats1A-neo* chimeric gene by light in etiolated seedlings was examined. Previous work has shown that *rbcS* is often under strict phytochrome control in etiolated tissues (Tobin 1981; Sazaki et al. 1983; Thompson et al. 1983; Bennett et al. 1984; Fluhr and Chua 1986). A low level of transcripts was observed in etiolated seedlings not exposed to the light (Fig. 7). Light is thus not absolutely required to initiate RNA synthesis in the etiolated seedlings. While exposure to white light results in a rapid increase in mRNA levels (Fig. 7), classical phytochrome regulation could not be demonstrated. Similar results are obtained when the *ats1A* gene is studied in *Arabidopsis* itself (E. De Almeida, unpublished results). These data suggest that chimeric genes driven by the *ats1A* promoter are regulated by light in a fashion similar to the endogenous gene in *Arabidopsis*. It is possible that the *ats1A* gene is regulated by multiple photoreceptors, as reported for the pea *ss3.6* gene (Simpson et al. 1986). Studies of individual members of *rbcS* multigene families have shown that not all *rbcS* genes are strictly phytochrome regulated (Simpson et al. 1986; Sugita and Gruissem 1987). While the classical phytochrome response could not be demonstrated for the *ats1A-neo* gene, the small increase in expression at the RNA level seen in the etiolated seedlings exposed to red, red plus far-red or far-red light (Fig. 7) is consistent with a very low fluence phytochrome response as reported for the *cab* genes (Kaufman et al. 1984). To confirm this requires further work with both *Arabidopsis* itself and the transgenic plants carrying these constructs.

Tissue specificity may vary between individual plants

The tissue specificity of the chimeric genes in transgenic tobacco plants was studied. NPT II was found in all the organs of pooled transformed plants tested, with the highest expression in green tissues (Fig. 8A). Other workers have shown that some *rbcS* genes are expressed in tissues other than leaves (Coruzzi et al. 1984; Dean et al. 1985; Fluhr et al. 1986). However, the unusually high expression in roots made it desirable to examine this phenomenon in individual plants. Surprisingly, a wide variation was observed between individual plants, all of which had comparable levels of expression in the leaves (Fig. 8B). Presumably, similar plant to plant variation is responsible for the expression levels observed with pooled petals, anthers, and stigmas. These results indicate that not only is there variation in levels of expression between individual plants, as reported by other workers (Nagy et al. 1985; An 1986; Kay et al. 1987; Gidoni et al. 1988), but that organ specificity of heterologous gene expression can vary between independent transgenic plants. As has been suggested for varying levels of expression in individual plants (Jones et al. 1985), such variation in tissue specificity may depend on the chromosomal environment in which a gene integrates. These observations add a new level of complexity to the analysis of transgenic plants, and imply that care must be taken in choosing individual transgenic plants to be used for further work.

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