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UTILIZING HETEROLOGOUS PROMOTERS TO EXPRESS GREEN FLUORESCENT PROTEIN FROM JELLYFISH IN TOBACCO CHLOROPLASTS

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Abstract

The green fluorescent protein (GFP) from the jellyfish (*Aequorea victoria*) has become a vital reporter not only to identify and screen transformed organisms including bacteria, animals and plants but also to study gene expression. A modified form of the green fluorescent protein was expressed in tobacco (*Nicotiana tabacum* var. Samsun) chloroplasts using both the bacterial as well as chloroplast specific promoters. A number of species-specific promoters have been used to express foreign DNA in chloroplasts, but there is no such report where DNA has been expressed in chloroplasts from bacterial promoters. This is the first report of stable expression of reporter gene (*gfp*) in chloroplasts using bacterial promoter. The GFP fluorescence was detected only in transformants where the *trc* promoter used to regulate *gfp*. In transformants where *gfp* was under the control of the chloroplast *rrn* promoter, fluorescence was comparable to controls without an introduced *gfp* gene. The transformed seedlings gave a green fluorescence after illumination with long-wave UV light. Fluorescence excitation and emission spectra of leaf extracts from the transformed plants confirmed the presence of GFP. Analysis of high expressing lines was carried out using Confocal Laser Scanning Microscopy. *Gfp* was found as a versatile and sensitive reporter and can be used to study promoters. The bacterial *trc* promoter appeared to be stronger than the chloroplast *rrn* promoter in *E. coli* as well as in chloroplasts of tobacco.

Introduction

The chloroplast gene expression and the inheritance of introduced genes in daughter plastids and cells can be studied by expressing reporter gene (s). A number of genes have been used to study gene expression in higher plants eg., the genes encoding β-glucuronidase (*uidA*, Jefferson *et al.*, 1986) and β-galactosidase (*lacZ*, Miller *et al.*, 1970), chloramphenicol acetyl transferase (*cat*) and neomycin phosphotransferase (*nptII*, Hererra-Estrella *et al.*, 1983; Fraley *et al.*, 1983), nopaline synthase (*nos*, Depicker *et al.*, 1983; Bevan *et al.*, 1983) and octopine synthase (*ocs*, Johnson *et al.*, 1974). Of these, *uidA* has been expressed transiently (Seki *et al.*, 1995) and stably in tobacco chloroplasts (Staub & Maliga, 1994). However, histochemical detection of GUS in chloroplasts requires prolonged incubation because the chloroplast envelope membranes act as a selective barrier to substrate penetration into the chloroplasts. The green fluorescent protein (*gfp*) of the jellyfish, *Aequorea victoria*, has recently been introduced as a reporter gene in plants (Baulcombe *et al.*, 1995; Chiu *et al.*, 1996; Haseloff *et al.*, 1997; Haseloff & Amos, 1995; Hu & Cheng, 1995; Niedz *et al.*, 1995; Rizzuto *et al.*, 1995, Khan & Maliga, 1999). *gfp* provides an easily scored genetic marker in plants and has major uses in monitoring gene expression and protein localization at high resolution. It allows direct imaging of the fluorescent gene product in living cells without the need for prolonged and lethal histochemical staining procedures (Chalfie *et al.*, 1994). The chromophore forms autocatalytically in the presence of oxygen and fluoresces green (508 nm) on absorption of blue or UV light of 395 nm. *gfp* is very stable to denaturants, such as 1% SDS, a range of pH and to proteases (Prasher *et al.*, 1992). This protein has successfully been

expressed in *E. coli* and chloroplasts of tobacco and rice (Khan & Maliga, 1999) using chloroplast specific expression signals. Several chloroplast promoters have been shown to direct the transcription initiation of reporter genes in prokaryotic cells (Boyer & Mullet, 1986; Erion *et al.*, 1983; Thompson & Mosig, 1988) on the basis of sequence similarities of putative chloroplast promoter regions with *E. coli* promoter regions and on the ability of prokaryotic RNA polymerase to recognize certain chloroplast promoters (Sugita & Sugiura, 1996; Erion *et al.*, 1983). In a previous study it was observed that bacterial promoter was able to express reporter gene *gfp* transiently (Hibberd *et al.*, 1998). It was therefore decided to examine the stable expression of *gfp* in chloroplasts under the control of such bacterial promoters.

Materials and Methods

Generation of reporter gene constructs: Two chimeric *aadA* gene constructs were used for selection of chloroplast transformants. The first construct consists of the bacterial *aadA* gene (Hollingshead & Vapnek, 1985) encoding aminoglycoside 3'-adenylyltransferase and giving resistance to spectinomycin and streptomycin (Goldschmidt-Clermont, 1991) obtained as an 800 bp *Nco*I-*Pst*I fragment from pUC-atpX-AAD (Goldschmidt-Clermont, 1991). pUC-atpX-AAD contains the *aadA* gene inserted at *Nco*I and *Pst*I sites between the *Chlamydomonas reinhardtii atpA* promoter and the *rbcL* terminator in pUC18. The 800 bp *Nco*I-*Pst*I fragment was inserted between the *trc* promoter and *rrnB* terminator in pKK233-2 (Amann & Brosius, 1985) digested with *Nco*I and *Pst*I. The resulting plasmid was called pMSK1. The second *aadA* construct was obtained from pZS197 which contains a 793 bp *SphI*-*Xba*I fragment of the *aadA* gene inserted between the tobacco chloroplast ribosomal RNA promoter and the tobacco *psbA* terminator (Svab & Maliga, 1993).

A 740 bp *Eco*RI-*Sac*I fragment containing *gfp* was excised from pBIN 35S-mGFP5 (Haseloff & Amos, 1995) and inserted at the same sites in pNtcC1, a modified pSP73 plasmid containing restriction sites for *Not*I, *Nhe*I and *Nco*I between the *Bgl*II and *Cla*I sites in the polylinker (P. J. Linley, unpublished). The resulting plasmid was named pMSK14. The *rrn-aadA-psbA3'* cassette was excised from pZS197 as a 1.3 kb *Bam*HI fragment. The fragment was gel purified and inserted into pMSK14 at the *Bam*HI site; the generated plasmid was called [illegible]. To insert the *trc* promoter upstream of the *gfp* gene, it was necessary to carry out several more cloning steps to provide suitable restriction sites. It was not possible to use the 52 bp *Hin*cII-*Nco*I fragment from pMSK1 because of an internal *Nco*I site in the *gfp* gene. A 906 bp *Bam*HI-*Eco*RV fragment containing *petD*, excised from pTB22, which contains a 4.8 kb insert from a partial *Bam*HI digest ([illegible] and 196 bp *Bam*HI fragments) in pBR322 (Sugiura *et al.*, 1986), was inserted at the same sites in pBCSK⁺ to yield pMSK7. The 1.6 kb *Hin*cII fragment containing the *aadA* gene with the *trc* promoter and the *rrnB* terminator was excised from pMSK1 and inserted into pMSK7 at the *Eco*RV site (compatible with *Hin*cII) to make pMSK8. The plasmid pMSK12 was generated to allow excision of the *trc* promoter as a *Not*I-*Nco*I fragment to control *gfp* expression. The plasmid pMSK12 was generated by excising a 860 bp *Hin*dIII fragment containing the *trc* promoter and *aadA* coding region from pMSK8 and inserting it into the *Hin*dIII site of pBCSK⁺ so that the *Not*I site was upstream of *trc* promoter. To control expression of the *gfp* coding

region, the bacterial *trc* promoter was excised from pMSK12 as a 180 bp *Not*I-*Nco*I fragment and inserted into pNtcC1 at the same sites; the resulting plasmid was called pMSK16. The *trc* promoter was excised from pMSK16 with *Not*I and *Eco*RI and inserted into the same sites of pMSK15 to give pMSK17. A 2.3 kb *Not*I-*Sal*I fragment from pMSK17 was inserted at the same sites of pNtcT3 (P. J. Linley, unpublished) to generate a final transformation vector named pMSK18 which contains the *trc-gfp-rrn-aadA-psbA*3' genes (Fig. 1). The plasmid pNtcT3 contains a double-stranded oligonucleotide with sites for *Not*I, *Hin*dIII, *Sal*I, *Stu*I and *Sma*I inserted at the *Mfe*I site in the 2.9 kb *Eco*RI-*Bgl*II fragment of the tobacco chloroplast genome (position 138447-141382, Shinozaki *et al.*, 1986) in pSP73.

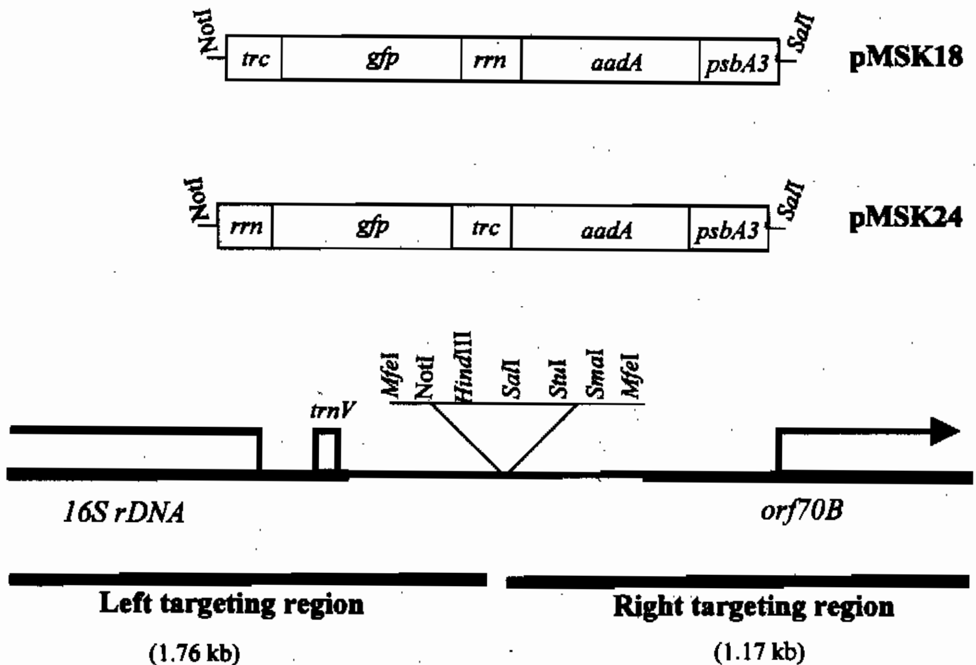

Fig. 1. **Plastid transformation vectors containing *gfp* as a reporter gene under heterologous promoters.** The transformation vectors, pMSK18 and pMSK24, target the reporter and selection genes into the inverted repeat region. Shown are the component parts of the vectors, *trc* and *rrn* (promoters), *psbA3"*- (terminator), *gfp* (reporter), *aadA* (selectable marker gene), sequences of inverted repeat region of plastome used as flanks, *16SrRNA*, *ORF70B*, for homologous recombination and restriction enzyme sites, *Eco*RI, *Not*I, *Nco*I, *Sac*I, *Bam*HI and *Bgl*II. In plasmid pMSK18 *gfp* is expressed from the *trc* where as in pMSK24 from *rrn* promoters.

A second construct with *gfp* inserted under the control of the chloroplast ribosomal RNA promoter was also generated. The *rrn* promoter was amplified from pZS197 (Svab & Maliga, 1993) by PCR using a forward primer (5'-GCTGGGCGGCCGC CGTTCAATG-3', *Not*I site underlined) and a reverse primer (5'-CGCTTCTGCCATGG TTCCCTCCC-3', *Nco*I site underlined) which varied from the chimeric *rrn* promoter used in pZS197 at nucleotides 134 and 135 from TT to CC. A PCR product of 160 bp was obtained and digested with *Not*I and *Nco*I. The digested product was inserted into the

*Not*I and *Nco*I sites of pMSK14, which contains the *gfp* coding region. The resulting plasmid was called pMSK22. The *rrn-gfp* cassette was removed from pMSK22 as a 925 bp *Not*I-*Sal*I fragment and inserted into the same sites of pNtcT3; the resulting plasmid was named as pMSK23. The *aadA* cassette containing the *trc-aadA-rrnB* fusion was isolated as a 1.6 kb *Hin*cII fragment from pMSK1 and inserted downstream of the *gfp* cassette at a *Stu*I site in pMSK23. The orientation of the inserted fragment was confirmed by diagnostic restriction enzyme digestions with *Nco*I, *Not*I and *Pst*I. The final transformation vector was called pMSK24 (Fig. 1).

Chloroplast transformation and selection of transformants: The transformation vectors pMSK18 and pMSK24 were introduced into leaves of seedling-derived tobacco plants by the biolistic process. Bombarded leaves were placed on RMOP agar plates without antibiotic. After two days, leaves were sectioned and 5 mm leaf sections were placed on the same medium containing 500 mg/l spectinomycin. Most of the leaf sections bleached within 4-5 weeks, but green calli and shoots were observed on some leaf sections within 4-9 weeks. Usually green shoots arose by direct organogenesis on the leaves, but shoots were also produced from green callus. Regenerated shoots were shifted to MS medium containing 500 mg/l spectinomycin for leaf proliferation and rooting. The 5 mm leaf sections from the primary transformants were placed on the same RMOP selective medium for further regeneration and the same MS medium with spectinomycin for root initiation. All regenerated shoots produced roots. Transformants were analysed for the presence of the *gfp* gene by using long-wave UV light as well as Confocal Laser Scanning Microscopy.

Tracking GFP expressing chloroplasts by fluorescence: Calli and leaf sections from transformed plant leaves were examined for *gfp* expression in chloroplasts using confocal laser scanning microscopy (MRC-1024 Confocal Image System, Bio-Rad Laboratories, Hercules, California, USA). Using this system, a green colour image can be obtained at 488-514 nm, a red image in the rhodamine channel at 560-580 nm represents chlorophyll fluorescence. A merged image in the third channel, showed both *gfp* and chlorophyll fluorescence at the same time. Moreover, seedlings were illuminated with a hand-held long-wave UV lamp (Model B 100AP, UV Products, Upland, California, USA) and photographed with a Canon EOS 1000 using 100 macro lens using Fuji film.

Confirmation by fluorescence spectroscopy: Fluorescence excitation and emission spectra of transformed plant leaf extracts for the presence of *gfp* were obtained using a Perkin-Elmer LS50 luminescence spectrometer with a cuvette. Leaf tissue (0.1 g) was ground in 3 ml grinding buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 10 mM dithiothretiol, 18% (v/v) glycerol) in a mortar with a pestle and centrifuged at 4000 *g* for 5 min to remove debris. The supernatant was diluted 10-fold with same buffer and was scanned using excitation at 395 nm for the emission spectrum and detection at 509 nm for the excitation spectrum. *E. coli* cells transformed with pMSK18, pMSK24 and pSP73 were obtained from colonies visualized by a long-wave UV lamp and were streaked on agar plates and grown overnight at 37°C. A single colony was picked and grown overnight in liquid LB medium (5 ml) with shaking at 37°C. Cells obtained from 500 ml of culture were suspended in 1 ml lysis buffer (50 mM Tris-HCl pH 8.0, 1 mM EDTA, 100 mM NaCl) followed by the addition of 10 µl of lysozyme (10 mg/ml) for 1 h and then centrifuged at 11,600 *g* for 10 min. The supernatant was diluted 10-fold with the same lysis buffer and used for fluorescence measurements as above.

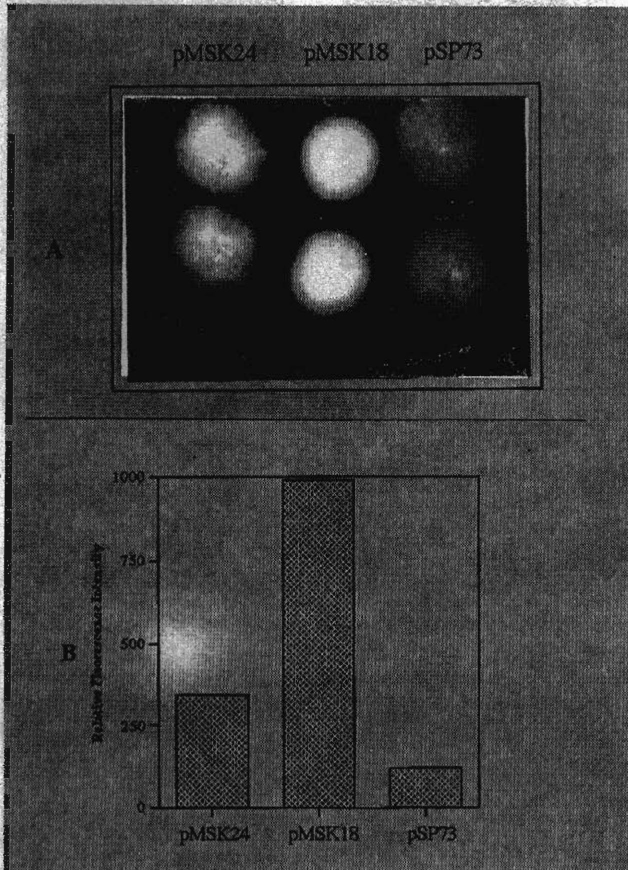

Fig. 2. **Expression of *gfp* in *E. coli* cells.** A) *E. coli* DH5α cells were electroporated with pMSK24, pMSK18 and pSP73 (plasmid without *gfp*) and grown on agar plates at 37°C overnight. Plates were illuminated with UV light using a long-wave UV lamp. B) The relative fluorescence intensity of cells expressing *gfp* under *trc* promoter (column 1), under *rrn* promoter (column 2) and without *gfp* (column 3) was measured using Perkin-Elmer LS50 luminescence spectrometer.

Results

Expression of *gfp* in *E. coli*: To confirm that the *gfp* constructs were able to produce functional green fluorescent protein, expression was examined in *E. coli*. The colonies containing the constructs pMSK18 and pMSK24 fluoresced green on excitation with long-wave UV light but those containing pSP73 did not fluoresce (Fig. 2A). Liquid cultures (5 ml) of all three strains were grown in the presence of ampicillin (50 mg/ml) at 37°C for 2-3 h to an OD_{600} of 0.5 measured using a Perkin-Elmer Lambda 9 spectrophotometer. The relative fluorescence intensities at 508 nm after excitation at 395 nm were measured using a Perkin-Elmer LS spectrofluorimeter. GFP fluorescence was 6-fold higher in cells transformed with pMSK18 where expression of *gfp* was controlled by the bacterial *trc* promoter compared with cells transformed with pMSK24, where *gfp* expression was controlled by the chloroplast ribosomal RNA promoter (Fig. 2B). This confirms that the chimeric *gfp* constructs were functional and suggests that the *trc* promoter is stronger than chloroplast *rrn* promoter in *E. coli*.

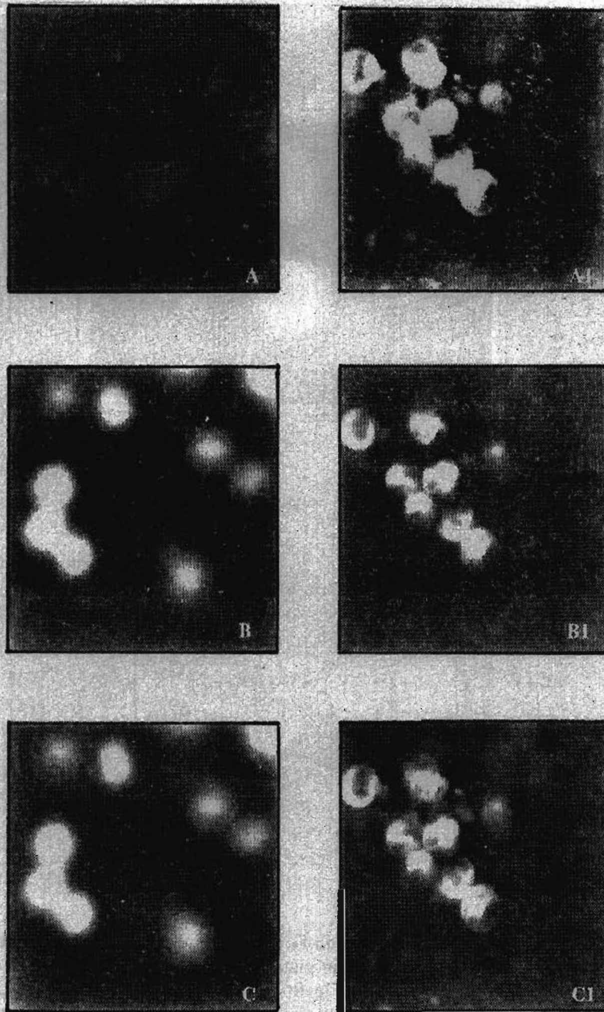

Fig. 3. Confocal micrographs of chloroplasts expressing *gfp*. Spectinomycin-resistant calli obtained from tobacco leaves bombarded with pMSK18 and pMSK5 (a control plasmid containing *aadA* gene for spectinomycin resistance without *gfp*) were examined for *gfp* expression usinf confocal laser scanning microscopy. A) *gfp* fluorescence visualized in the fluorescein channel, B) chlorophyll fluorescence in the rhodamine channel, and C) a merged image of the fluorescin and rhodamine channels. Callus obtained with pMSK5 (A-C) and pMSK18 (A1-C1) was viewed at 400X magnification and zoomed 15X using computer software facilities.

Chloroplast transformation and regeneration of transformants: Chloroplast transformation to examine expression of *gfp* was carried out using pMSK18 and pMSK24. Leaves were placed adaxial side up on Whatman 3MM paper on RMOP medium for bombardment. Ten leaves were bombarded with each construct and selection of spectinomycin-resistant calli and shoots was carried out on RMOP medium. Two resistant calli and one green shoot were recovered in 4-9 weeks after bombardment with construct pMSK18, and one resistant callus and one green shoot obtained from

bombardment with pMSK24. Calli were analysed for *gfp* expression during the first round of selection, because they were growing very slowly and there was no organogenesis to generate plants. The cells from calli were examined by laser-scanning confocal microscopy rather than by conventional epifluorescence microscopy. Confocal microscopy produced three types of images that were viewed on a computer screen attached to the microscope. There was a green colour image obtained in the fluorescein channel from callus from pMSK18 (Fig. 3A1). This represents *gfp* expression. A red image in the rhodamine channel represents chlorophyll fluorescence (Fig. 3B1). A merged image was obtained in a third channel, showing both GFP and chlorophyll fluorescence. GFP fluorescence was quite clear at the boundaries of the chloroplasts looking green whereas the combination of GFP and chlorophyll fluorescence produced an orange colour over the rest of the chloroplasts (Fig. 3C1). In some cells most of the chloroplasts showed GFP fluorescence, while in other cells none of the chloroplasts showed GFP fluorescence.

The chloroplasts from a control callus gave only a very weak signal in the fluorescein channel (Fig. 3A) that might be spillover of chlorophyll fluorescence. This callus is from a control plant obtained by bombardment of tobacco leaves with a control construct containing no *gfp* and was obtained by growing a leaf section of 5x5 mm on RMOP medium containing spectinomycin (500 mg/l). Fluorescence in the merged fluorescein and rhodamine channels (Fig. 3B) was identical to that in the rhodamine channel (Fig. 3C), indicating that the fluorescence was principally from chlorophyll. The cells from the callus obtained with pMSK24 produced images in three channels which were comparable to control plant (Data not shown). In transformants grown in tissue-culture on spectinomycin, GFP fluorescence was masked by chlorophyll. It was therefore decided to produce seeds from these spectinomycin-resistant green shoots to examine GFP fluorescence. Three-week-old progeny seedlings, grown on MS-agar medium containing spectinomycin (500 mg/l), of the primary transformants obtained with pMSK18 and pMSK24 were examined with a hand-held long-wave UV lamp. Some seedlings, obtained from the pMSK18 transformant, produced a green fluorescence, although in the majority of the seedlings chlorophyll fluorescence masked the GFP fluorescence (Fig. 4). None of the seedlings from pMSK24 showed a visible GFP fluorescence. To confirm that the fluorescence was due to GFP, leaf extracts from positive seedlings from the pMSK18 transformant were prepared by grinding leaves (0.1 g) in 3 ml grinding buffer. After centrifugation the supernatant was diluted 10-fold with buffer and the excitation and emission spectra measured using a Perkin Elmer LS 50 spectrofluorimeter. *E. coli* cell extracts were also prepared by growing a single colony of cells containing *gfp* (pMSK18) in liquid LB medium (5 ml) overnight at 37°C. Cells from 500 μl were lysed, centrifuged and the supernatant diluted 10-fold with lysis buffer. The emission spectrum was determined on illumination with 395 nm light, and the excitation spectrum was determined by monitoring emission at 509 nm. These are the peak emission and excitation wavelengths for GFP (Morise *et al.*, 1974; Ward *et al.*, 1980). From the scans, it is clear that the excitation and emission spectra are similar in both *E. coli* and leaf extracts. An additional peak at 680 nm was observed in the excitation spectrum of leaf extracts; this is probably due to chlorophyll fluorescence (Data not shown). The excitation and emission spectra of leaf extracts from the transformant obtained with pMSK18 confirmed the presence and expression of *gfp* in these plants.

Fig. 4. **Seedlings expressing *gfp* illuminated using long-wave UV light.** Seedlings were grown on MS-agar plates containing spectinomycin and illuminated using hand-held long-wave UV lamp. Seeds were obtained from transformants pMSK18 grown in green house.

Discussion

Chloroplast transformation in tobacco (*Nicotiana tabacum* L. var. Samsun) was established and was used to examine expression of the *gfp* reporter gene in chloroplasts. In these studies a modified *gfp* gene, version *gfp*5, which lacks a cryptic intron sequence (Haseloff & Amos, 1995) was used for expression in two different systems under two different promoters, viz., *trc*, a bacterial promoter, and *rrn*, the chloroplast ribosomal RNA promoter. *gfp* was expressed in both bacterial and chloroplast systems. The reason for examining the *E. coli* transformants was to determine whether the *gfp* constructs were functional. In *E. coli* colonies where *gfp* expression was controlled by the *trc* promoter, an intense fluorescence was detectable by eye using a long-wave UV lamp. However, in cells where *gfp* was under the control of the chloroplast ribosomal RNA promoter, the expression was much lower. A weak autofluorescence from cells without GFP was also observed. *gfp* expression in *E. coli* cells was six-fold higher under the control of the *trc* promoter than under the control of the chloroplast *rrn* promoter. It would suggest that the *gfp* constructs were functional and the *trc* promoter is apparently stronger than the *rrn* promoter, provided the plasmid copy number was the same in both transformed lines.

In chloroplasts of tobacco plants transformed with the *trc-gfp* construct, reasonable levels of GFP fluorescence were observed using laser-scanning confocal microscopy. GFP fluorescence was detected only in transformants where *gfp* was regulated by the *trc* promoter. In transformants where *gfp* was under the control of the chloroplast *rrn* promoter, fluorescence was comparable to controls without an introduced *gfp* gene. From these experiments it is concluded that *gfp* is a versatile and sensitive reporter and can be used to study promoters. The bacterial *trc* promoter appeared to be stronger than the chloroplast *rrn* promoter in tobacco chloroplasts.

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