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The use of nuclear-encoded sequences to direct the light-regulated synthesis and transport of a foreign protein into plant chloroplasts

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The light-inducible nuclear gene coding for the small subunit of ribulose-1,5-bisphosphate carboxylase (Rubisco), produces a precursor protein with an amino-terminal transit peptide which is transported into the plastids and cleaved by a specific proteinase. To test whether the promoter and transit peptide-coding sequences of the small subunit gene can be used to direct the light-inducible synthesis and transport of a foreign protein into chloroplasts, a chimaeric gene was constructed consisting of the promoter, first exon and intron as well as part of the second exon of the small subunit Rubisco gene fused to the amino-terminal end of the neomycin phosphotransferase II gene, (*npt*II) of Tn5. Tobacco tissue, as well as whole plants, into which this chimaeric gene was introduced, were resistant to kanamycin. The transcription of the chimaeric gene as well as the NPTII activity of the resulting fusion protein were shown to be light inducible. The fusion protein is processed and located within the chloroplasts of the transformed plants.

Key words: chloroplast protein transport/gene fusions/precursor proteins/plant cell transformation

Introduction

The experimentally controlled modification of chloroplast metabolism is of great importance for fundamental and applied reasons. It would, therefore, be of interest to be able to transform modified chloroplast genes or perhaps totally foreign genes into the plastid genome. However this strategy of direct DNA transfer has not been successful thus far. Another approach would be the generation of chloroplast mutants, as was done, for example, in *Chlamydomonas* (Spreitzer and Ogren, 1983; Dron *et al.*, 1983). However, the system in plants is highly buffered by the multiple copies of the genes involved. An alternative strategy was therefore developed based on the fact that many gene products are normally imported into chloroplasts. A rough estimate indicates that at least 70% of all the proteins in chloroplasts are coded for by nuclear genes (Ellis, 1981; Ellis and Robinson, 1984). Proteins made from these nuclear genes are transported into the plastid by the specific processing of a so-called transit peptide, which normally occupies the amino-terminal part of cytosolic precursors for chloroplast proteins (Schmidt *et al.*, 1979; Chua *et al.*, 1980). Two members of the nuclear-coded chloroplast proteins are very abundant and well characterized, namely the chlorophyll a/b binding proteins and the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase [Rubisco (EC 4.1.1.39)] (Schmidt *et al.*, 1981; Chua *et al.*, 1980). These polypeptides are synthesized as soluble precursors on cytoplasmic ribosomes (Apel and Kloppstech, 1978; Schmidt *et al.*, 1981) and are post-translationally translocated into chloroplasts. During or after translocation the NH_2-terminal transit peptides are proteolytically cleaved (Schmidt *et al.*, 1981) to yield the mature polypeptides. The mature A and B polypeptides associated with chlorophyll a and b are integrated into the thylakoid membrane. The mature polypeptide of the small subunit assembles with the large subunit to form functional Rubisco present as a soluble protein in the stroma of the chloroplast. The transit sequences of these two systems in *Pisum sativum* do not show any significant homology at the level of their amino acid sequence and they are of different size (Cashmore, 1984), suggesting specificity of a given transit peptide for the protein being transported. However, comparison of the amino acid sequences of small subunit precursor proteins from soybean (Berry-Lowe *et al.*, 1982), pea (Cashmore, 1983), duck weed (Stiekema *et al.*, 1983) and wheat (Broglie *et al.*, 1983) show high homology in a region surrounding the cleavage site of the precursor, whereas the homology within the rest of the transit sequence is less pronounced (Broglie *et al.*, 1983), notwithstanding the fact that the small subunit precursors of several higher plants can be transported into each other's chloroplasts (Chua and Schmidt, 1978). We hypothesized that a foreign protein similar in size and hydrophobicity could be transported if fused to the transit peptide and part of the mature protein, provided the entire region of high homology around the cleavage site were kept constant. Our colleagues at the laboratory of genetics in Gent on the other hand decided to test the possibility that only a transit peptide sequence is needed to transport a protein into chloroplasts. To test whether or not nuclear chimaeric genes coding for fusion proteins containing the transit peptide sequence would be effective in determining the synthesis, processing and transport of foreign proteins into chloroplasts, two different constructions were made: the first one, described in this paper, was designed to take into account the possibility that the entire region of high homology around the cleavage site would be a necessary requirement for transport and processing. The sequence coding for both the transit peptide and the first 22 amino acids of the small subunit gene (*rbcS*) from *P. sativum* (Cashmore, 1983) were fused to the coding region of the *npt*II gene. It was expected that the resulting fusion proteins would have neomycin phosphotransferase activity and would be easily detected in transformed plants. In the other construction (Van den Broeck *et al.*, 1985) the *npt*II coding sequence was fused directly to the transit peptide coding sequence such that the potential protein cleavage site did not contain any amino acids derived from the mature small subunit protein.

Results

Construction of plasmids pSNIP and pSNIF containing the chimaeric gene (tp-ss-nptII)

A genomic clone for one of the *rbcS* genes from pea was isolated, sequenced and made available by Dr. A.Cashmore, Rockefeller University, NY (pPSR6). From this clone the promoter signals (Cashmore, 1983; Herrera-Estrella *et al.*, 1984), the first exon coding for the *rbcS* transit peptide and the first two codons of the mature small subunit protein, followed by the first intron (83 bp) and part of the second exon (66 bp) coding for the amino terminus of the mature small subunit protein were fused *via* a *Sau*3A restriction endonuclease recognition site with the *Bam*HI site of the plasmid pKm109/9 (Reiss *et al.*, 1984b) which contains the coding region for the *npt*II gene from Tn5 (Beck *et al.*, 1982) (for further details see Figure 1a). The unmodified NPTII protein confers resistance for kanamycin to plants (Herrera-Estrella *et al.*, 1983; Fraley *et al.*, 1983; Bevan *et al.*, 1983) and it was expected that the fusion gene containing the transit sequence (57 codons) and 22 codons from the mature *rbcS* gene linked *via* six artificial codons with the second codon from the *npt*II gene (Figure 1b) would be similarily active. The size of the coding region of the *npt*II gene is 1130 bp. The fusion junction was verified (data not shown) by DNA sequencing (Maxam and Gilbert, 1977). The chimaeric protein should have a mol. wt. of 38 023 in the unprocessed and 32 298 in the processed form. Southern type (Southern, 1975) hybridisation data (Figure 2) established that transformed plant tissues contained the chimaeric gene constructs in the nuclear DNA and that no detectable DNA rearrangements had occurred during integration. A schematic representation of the results is given in Figure 3.

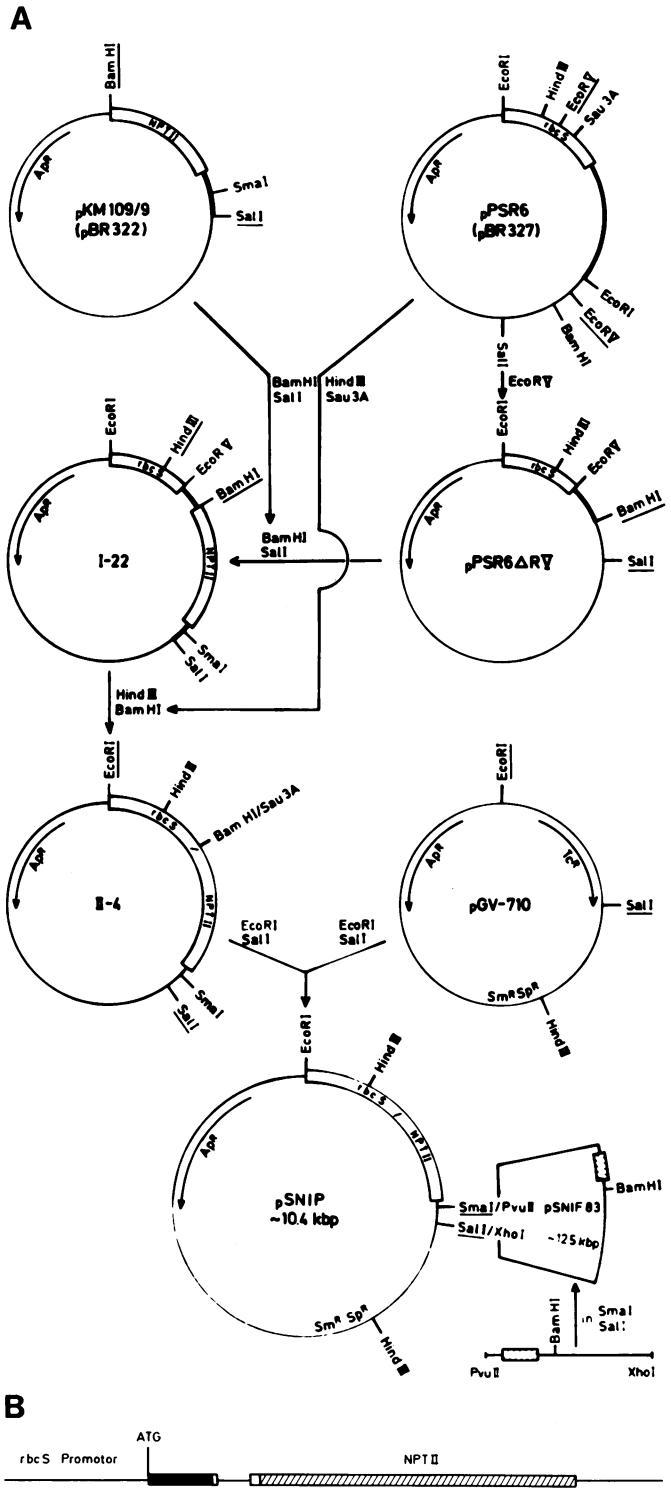

Fig. 1. (**A**) Construction of the chimaeric *rbcS-npt*II genes pSNIP and pSNIF. A *Bam*HI-*Sal*I fragment from pKM109/9 (Reiss *et al.*, 1984b) containing the entire coding region from a modified *npt*II gene from Tn5 (Beck *et al.*, 1982) was inserted in plasmid pPSR6 delta RV next to a 950-bp DNA fragment (*Eco*RI-*Eco*RV) containing the promoter region and the 5′ end of the *rbc*S gene resulting in plasmid I-22. In this plasmid the *Hind*III-*Bam*HI fragment was replaced by a *Hind*III-*Sau*3A fragment (53 bp) from the original *rbcS* clone (pPSR6) to form the plasmid II-4 containing the fusion gene. The pBR derived region in II-4 was exchanged against an *Eco*RI-*Sal*I fragment from pGV710 in order to introduce streptomycin and spectinomycin resistance to be used as a marker to select for co-integration of this final plasmid [pSNIP (10.4 kbp)] with the Ti-plasmid in *Agrobacterium*. Plasmid pSNIF (12.3 kbp) was constructed by replacement of the *Sma*I-*Sal*I fragment of pSNIP with an *Pvu*II-*Xho*I fragment from the octopine synthase gene from plasmid pAGV40 (Herrera-Estrella *et al.*, 1983; De Greve *et al.*, 1983) harboring the polyadenylation site of that gene next to a *Bam*HI restriction site. (**B**) Structure of the *rbcS-npt*II chimaeric gene. The black bar represents the transit-peptide sequence with the first ATG, the white area (two codons in exon 1 and 22 codons in exon 2) is interrupted by the first intron and represents the mature *rbcS* sequence. The hatched part represents the *npt*II sequence.

Plant transformation

To introduce the chimaeric genes in the nuclear genome of plants, the plasmid was inserted into the T-DNA of pGV3851 and of pGV3850, both derivatives of the Ti plasmid pTIC58, in which parts of the T-DNA were substituted by pBR322 (Zambryski *et al.*, 1983, 1984). The T-DNA of pGV3851 still contains the genes coding for transcripts 4, 6a and 6b (Willmitzer *et al.*, 1983) which results in a teratoma-like growth of the transformed tissue (Joos *et al.*, 1983), whereas all tumor-controlling genes have been eliminated in pGV3850 with the result that plant cells transformed with this vector can differentiate and grow as normal plants (Zambryski *et al.*, 1983; De Block *et al.*, 1984). The gene constructions were introduced into pGV3850 and pGV3851 Ti plasmids by homologous recombination after mobilization from *Escherichia coli* to *Agrobacterium* with the help of plasmids R64*drd*11 and GJ28 (Van Haute *et al.*, 1983). Co-integrates were selected on spectinomycin- and streptinomycin-containing plates and their structure verified by Southern blot hybridization (Southern, 1975) using various parts of the constructions as probes (data not shown).

The chimaeric genes were introduced into *Nicotiana tabacum* cv. Wisconsin 38 or SR1 by inoculation of a wounded plantlet or by co-cultivation of protoplasts with *Agrobacter-*

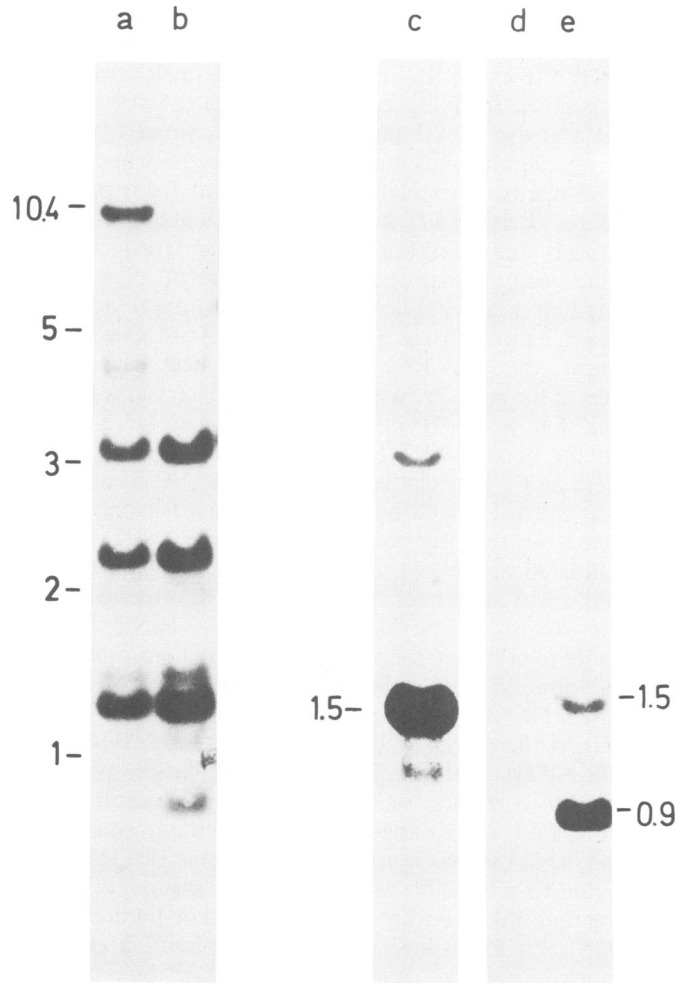

Fig. 2. Hybridization of different probes to nuclear DNA from transformed (pGV3851::pSNIP) (**a, c** and **e**) and untransformed (**b** and **d**) tobacco. In Southern hybridization experiments (Southern, 1975), **lane a** and **b** resolve several bands of different size resembling the small subunit gene family when a 661-bp *Eco*RV-*Ava*III DNA fragment from the genomic small subunit clone was used as probe (Cashmore, 1983). An additional band of 1.04 kbp reveals the chimaeric gene fragment in **lane a**. In **lanes c, d** and **e** DNA was digested with *Pst*I and *Eco*RI and either the promoter region of the small subunit gene (972-bp *Eco*RI/*Hind*III fragment) (**lane c** and **d**) or the coding region of the *npt*II gene (1000 bp *Bam*HI/*Sma*I fragment from plasmid pKM109/9) were used as probes. In **lane c** a strong signal is observed at 1.5 kbp (transformed) and no signal is detected from untransformed material (**lane d**). Weak signals in **lane c** are most likely due to cross-hybridization of endogenous *rbcS* sequences or incomplete digestion of the DNA. In **lane e** a band of 0.9 kbp lights up the internal *Pst*I fragment of the *npt*II gene and the weaker band shows again the 1.5-kbp fragment seen in **lane c**, due to small overlap between the probe and the promoter region of the chimaera.

ium. Transformed material obtained by wounding was screened for the presence of nopaline synthase activity (Otten, 1982), a co-transferred marker. Transformants (pGV3851::pSNIP) grew on 250 μg/ml kanamycin as green teratoma tissue, suggesting that a functional chimaeric gene was present and transcribed. In co-cultivation experiments, *N. tabacum* SR1 protoplasts were incubated with *Agrobacterium* containing (pGV3850::SNIF) and selected after 2 weeks with 100 μg/ml kanamycin. From nine individual colonies which were positive when tested for NPTII activity, one was chosen and regenerated to a fully normal looking plant. These results suggested that transcripts from the chimaeric genes were properly processed, transported out of the nucleus and translated into a functionally active protein.

Light induction of the chimaeric gene

Poly(A)$^+$ and poly(A)$^-$ RNA from wild-type and from transformed tissues (pGV3851::pSNIP) was isolated and analysed by Northern gel hybridizations. When the coding region of the *npt*II gene (*Bam*HI-*Sma*I fragment from pKM109/9) was used as a probe, a complex hybridization pattern was observed with RNAs ranging between 5500 nucleotides and 8000 nucleotides in size. These RNAs were detected in light-grown teratomas only. Four days of darkness after a day/night rhythm of 12 h resulted in a marked decrease of the signals (Figure 4). The very large size of these transcripts probably results from the fact that no proper polyadenylation and transcription termination site was introduced near the translation termination signal. No signals of comparable size or strength were observed in wild-type Wisconsin 38 tobacco or in material obtained from a plant transformed with the pGV3850 vector only (Figure 4). To compare the light-dependent transcription of the chimaeric gene with that of both the endogenous *rbcS* gene and the chloroplast gene coding for the large subunit of Rubisco (*rbcL*), poly(A)$^+$ and poly(A)$^-$ RNA from light- and dark-grown teratoma were hybridized to specific probes for each of these genes. The results are illustrated in Figure 5A. Signals of the endogenous *rbcS* transcripts (850 nucleotides) were observed at the expected position. Similarly, a transcript of 1750 nucleotides was observed when a *rbcL*-specific probe was used (Zurawski *et al.*, 1981). The results suggest that the promoter of the *rbcL* gene, which resides in the chloroplasts, is less sensitive to light stimuli than both the endogenous *rbcS* and the newly introduced chimaeric gene. Dot-blot experiments were included to quantify these results (Figure 5B). The same probes were used as mentioned before. Individual dots were cut out and the radioactivity counted. A difference of ~25-fold was measured between poly(A)$^+$ RNA from light- and dark-grown teratoma shoots probed with either

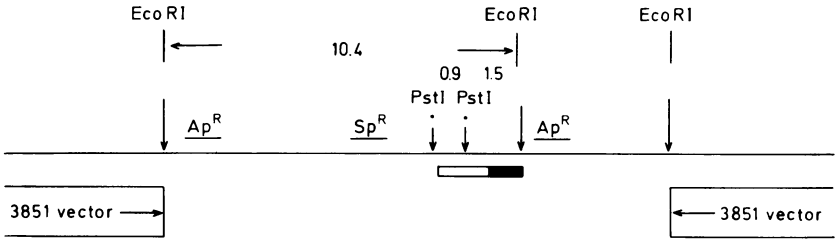

Fig. 3. Schematic representation of the organization of the gene fusion and the flanking vector sequences. Sizes are indicated in kbp. The chimaeric *rbcS-npt*II coding region is indicated by an open bar, the 5'-flanking sequence by a closed bar. *Eco*RI and *Pst*I indicate restriction endonuclease sites. SpR and ApR represent antibiotic resistance markers against spectinomycin and ampicillin. Numbers indicate the size of fragments obtained in the Southern experiments (Figure 2). The DNA fragments between the gene fusion and the right part of the T-DNA represent the pBR322 sequences present in the vector pGV3851.

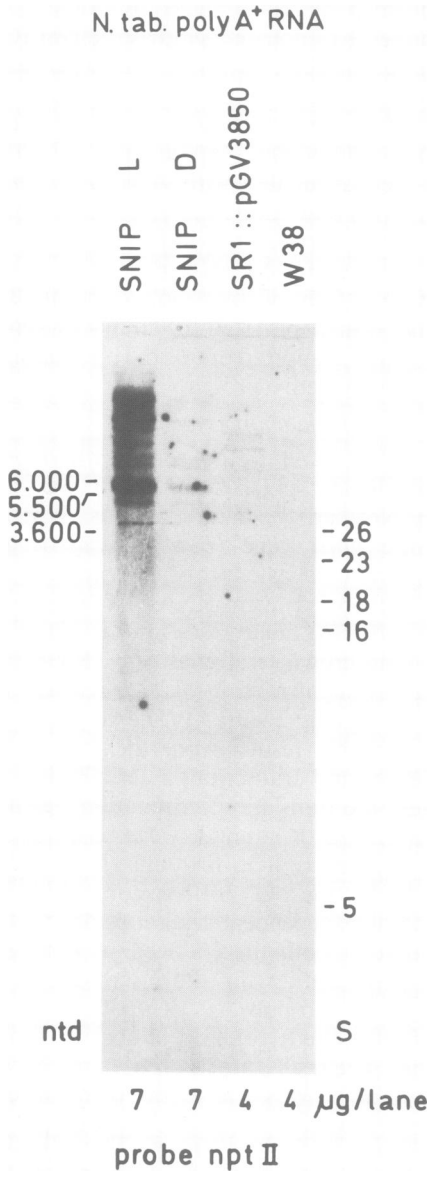

Fig. 4. Transcriptional activity of *rbcS* promoter. RNA was separated in denaturing 1% agarose gel and transferred to nitrocellulose filters which were probed with different parts of the construction. The coding region of the *npt*II gene (*Bam*HI-*Sma*I fragment from pKM109/9) was used as a probe. **Lane 1**: RNAs from light grown teratoma shoots. **Lane 2**: RNAs from plant material kept in darkness for 4 days after a day/night rhythm of 12 h. **Lane 3**: RNAs from plant leaves transformed with pGV3850. **Lane 4**: RNAs from wild-type Wisconsin 38. Weak signals in the latter are probably due to contaminating material in the probe which hybridizes to mRNA which is transcribed through the pBR322 sequences from a promoter active in the T-DNA or near the position of insertion in the plant chromosome. Numbers on the left indicate size in nucleotides, numbers on the right refer to the Svedberg values of RNA markers.

rbcS or *npt*II sequences. In contrast, the difference is only 5-fold for poly(A)$^-$ RNA specific for *rbcL* sequences. These results support the Northern experiments indicating that the transcripts of the chloroplast gene coding for the large subunit is less sensitive to influence of light in comparison with the nuclear gene for the small subunit. In addition, it seems that the pea *rbcS* promoter of the introduced chimaeric gene has a sensitivity to different light regimes which is comparable with that of the endogenous promoter or promoters measured in the tobacco teratoma tissue.

Features of fusion proteins

To detect the expected fusion protein formed between the transit peptide, the NH_2-terminal region of the mature small subunit and the NPTII protein in plants, we developed an assay detecting the phosphotransferase II activity in crude extracts of plants. The method was adapted from published procedures (Reiss *et al.*, 1984a) and eliminates most of the endogenous self-phosphorylating proteins which interfere with the assay by proteinase K treatment. The results presented in Figure 6 demonstrate that NPTII activity is detected in a crude extract (lane 4) of leaves of tobacco plants containing the pGV3850::pSNIF construct. The activity migrates in the gel assay with a mobility which is intermediate between that of the TP-NPTII fusion protein (35.5 kd) and that of the normal NPTII protein (29 kd) from extracts of *E. coli* (lane 1). The relative mobility of the NPTII activity in lane 4 is consistent with our conclusion that it represents the processed form of the precursor protein (SS-NPTII) which has a theoretical mol. wt. of 32 298. Since the polarity index (Capaldi and Vanderkooi, 1972) of the three proteins is 41 for NPTII, 40 for SS-NPTII and 41 for TP-NPTII, it is legitimate to compare the three proteins by their mobility on native polyacrylamide gels (see Figure 6B). Indeed the unprocessed TP-SS-NPTII protein has a mol. wt. of ~38 000 and would therefore presumably migrate more slowly than the TP-NPTII marker. The SS-NPTII fusion protein is degraded *in vitro* after isolation yielding active subfragments with a mobility which approaches that of the normal NPTII enzyme. That the lower mol. wt. spots seen in Figures 6A and 7 are due to unspecific degradation was shown by demonstrating that this and other NPTII fusion proteins are actually degraded *in vitro* in both bacterial and plant extracts (data not shown, Reiss *et al.*, 1984b). Incubation in the presence of protease inhibitors could not completely prevent this degradation. No activity was detected in control extracts from tobacco lacking the TP-SS-NPTII chimaeric gene (lane 3). The SS-NPTII activity observed in crude extracts can also be detected in isolated chloroplasts (lane 2). The relative amount of activity detected in the chloroplasts is significantly less than the activity observed in crude extracts. This could be due to leakage of the activity out of the chloroplasts during chloroplast isolation. Indeed the procedure used to isolate chloroplasts leads, in our hands and with this particular plant material, to a substantial damage of the chloroplasts. More than 90% of the chloroplast material is either visibly damaged or runs at a reduced density in the Percoll gradients. Further manipulations during recovery and concentration prior to the NPTII assay could contribute to further minor damage leading to significant loss of the protein by leakage. Our observations do not exclude the possibility that although all of the precursor TP-SS-NPTII protein is processed to the SS-NPTII form, it is not actually all transported *in vivo* into the stroma of the chloroplasts. However, our data clearly demonstrate that at least some of the processed SS-NPTII protein is within the stromal fraction of the chloroplasts. Indeed the activity associated with the chloroplasts was shown to be located within the stroma by demonstrating that broken chloroplasts did not contain any detectable NPTII activity and that the NPTII activity in intact chloroplasts could not be eliminated by trypsin treatment (data not shown). Further evidence that the detected SS-NPTII activity was derived from the introduced light-inducible chimaeric gene was obtained by demonstrating that the activity was significantly

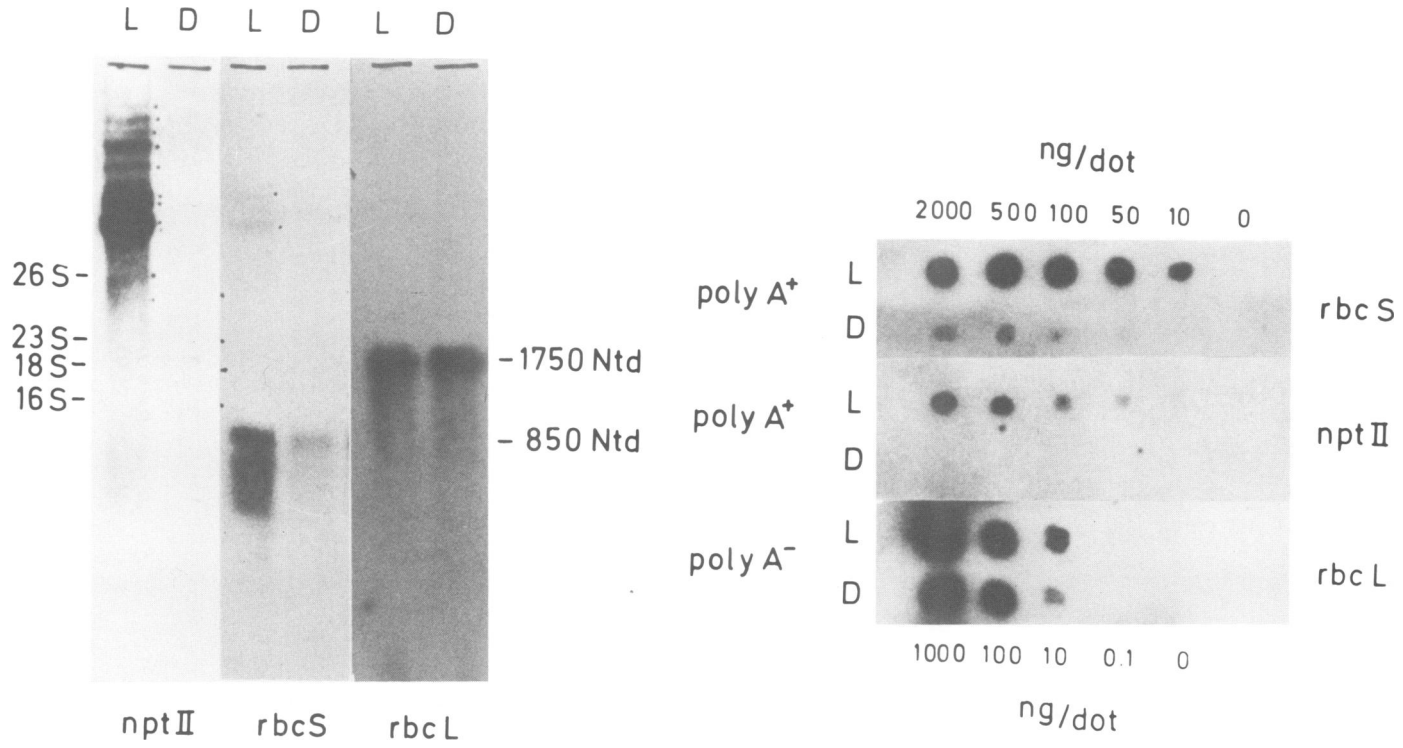

Fig. 5. **(A)** Comparison of light dependence of *rbcS* and *rbcL* promoters. Poly(A)$^+$ RNA from teratoma shoots grown in a daily rhythm of 12 h light/dark (L) and material kept subsequently for 4 days in the dark (D) were hybridized to an *npt*II-specific probe (see Figure 4) and to a *rbc*S-specific probe (see Figure 2). The endogenous *rbcS* transcripts are observed at the position of 850 nucleotides. Poly(A)$^-$ RNA was analysed with the same technique probed with a 1750-bp fragment from a *rbcL* gene (Zurawski *et al.*, 1981). Numbers on the left refer to Svedberg values of RNA markers or to the size of the mRNA (right). **(B)** Dot-blot hybridization to RNA from transformed (pGV3851::pSNIP) plant material. L indicates light grown material in 12 h light/dark cycle. D indicate subsequent growth in the dark for 4 days. Single dots were cut out and radioactivity measured.

reduced when tobacco plants containing the pGV3850::pSNIF construct and grown in the green house in a 12 h light/dark regime (Figure 7, lane 3) were transferred for 96 h to complete darkness (Figure 7, lane 2).

Discussion

The use of *Agrobacterium* vectors to transfer and express genes in plant cells has been amply documented (Caplan *et al.*, 1983; Zambryski *et al.*, 1983, 1984; Herrera-Estrella *et al.*, 1983, 1984). Our results demonstrate that this approach can be extended to target a foreign protein for a specific cell compartment, namely the chloroplast. An existing transport system which imports a nuclear-encoded gene product into the stroma of the chloroplast was used for this purpose. In this study we have designed a chimaeric gene consisting of the light-inducible promoter sequence, the transit peptide coding sequence plus the first intron and the coding sequence of the first 22 amino acids of the small subunit of ribulose-1,5-bisphosphate carboxylase from pea fused to the coding sequence of the neomycin phosphotransferase II from the *E. coli* transposon Tn5. The results demonstrate (i) that the gene fusion is integrated in the nuclear DNA of tobacco without rearrangement of the DNA, and (ii) that the transcription of this chimaeric gene is regulated by light. It is important to note that the induced transcription of this introduced gene is as efficient as that of the endogenous small subunit gene(s) and rather more efficient than previously observed in tobacco with another chimaeric gene using the same pea small subunit promoter (Herrera-Estrella *et al.*, 1984). Possibly the higher level of induced steady-state mRNA in these tissues is due to improved mRNA stability. The presence of one intron in the transcript derived from this transit peptide small subunit neomycin phosphotransferase chimaeric gene (*tp-ss-npt*II) and the absence of any intron in the construction described by Herrera-Estrella *et al.* (1984), might explain an increased stability of this RNA (Hamer and Leder, 1978). Our observations also demonstrate that the pea small subunit promoter can be active in leaves of normal tobacco plants. This is in contrast to previous observations in several laboratories which indicated that the pea small subunit promoter, while active in tobacco tissue cultures and teratomas, was inactive in leaves of normal plants. Possibly a position effect is involved in this phenomenon.

The chimaeric *tp-ss-npt*II gene in (pGV3851::pSNIP) did not contain a polyadenylation or a transcription termination signal, which probably explains the observed very large transcripts. We assume that transcription termination occurs at a number of possible sites in the flanking plant DNA sequence (Dhaese *et al.*, 1983).

(iii) The chimaeric *tp-ss-npt*II gene contains an intron and we assumed that the tobacco system would correctly recognize and use the splicing signals of the mRNA coded for by the heterologous gene. Our results suggest that splicing occurs since we obtained functional fusion proteins. The intron sequence (Cashmore, 1983) contains two or more translation stop codons in each reading frame and a shift in register; it therefore seems unlikely that a functional fusion protein could be made without correct splicing of the mRNA.

(iv) The translation product of the chimaeric *tp-ss-npt*II is transported to the chloroplasts and processed. The processing of the precursor of the small subunit of the ribulose-1,5-bis-

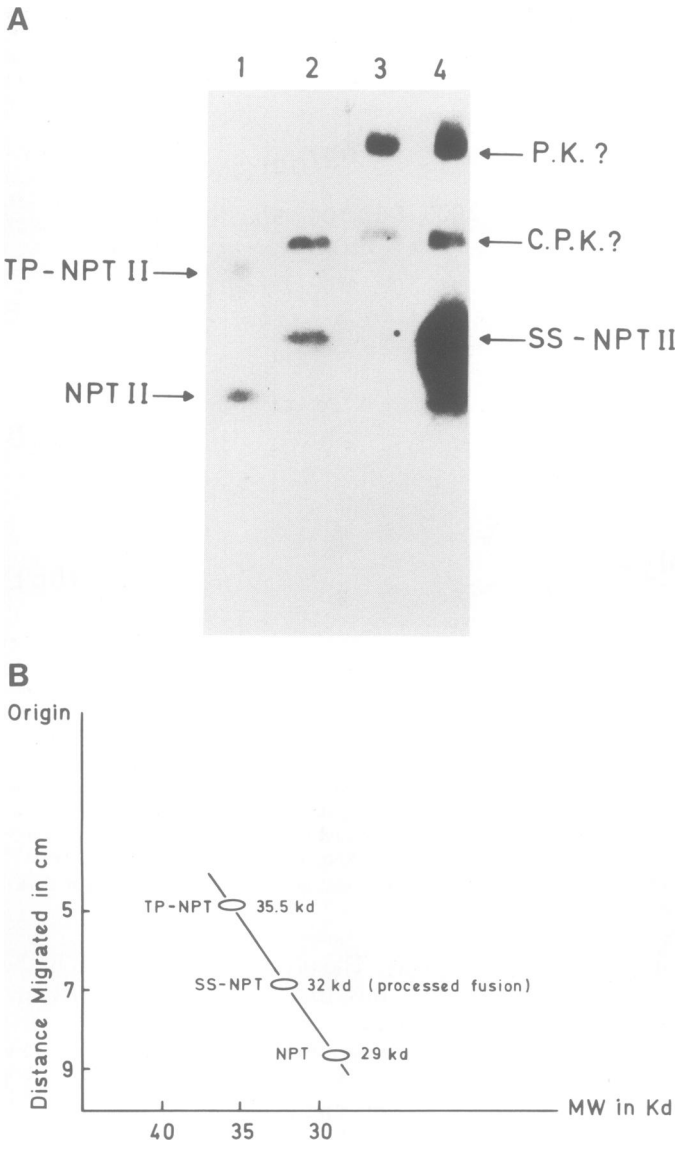

Fig. 6. Demonstration of transport of TP-SS-NPTII precursor in chloroplasts of tobacco plants containing the pGV3850::pSNIF construct. **(A) Lane 1**: extracts from *E. coli* pGLT neo1 expressing a TP-NPTII protein (Van den Broeck *et al.*, 1985) and *E. coli* pKM2 containing the Tn5 encoded NPTII enzyme. **Lane 2**: neomycin phosphotransferase activity in chloroplasts purified from leaves of tobacco plants containing the chimaeric *tp-ss-npt*II gene. **Lane 3**: crude extract from leaves of a control SR1 tobacco plant. **Lane 4**: crude extract from leaves of tobacco plants containing the chimaeric *tp-ss-npt*II gene. The P.K? band is presumed to be due to a cytoplasmic self-phosphorylating protein and C.P.K? is presumed to be due to a chloroplast self-phosphorylating protein. **(B)** Graphic display of the relative mobility of the different NPTII activities detected in **A**. As described in the Results it is legitimate to make the assumption that these proteins are separated according to mol. wt. on these native gels because of their very similar polarity index (Capaldi and Vanderkooi; 1972).

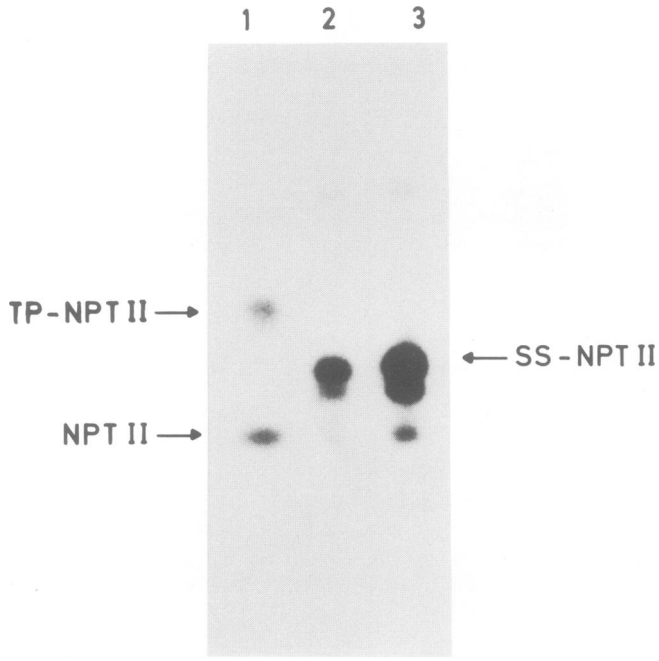

Fig. 7. Light-dependent expression of the SS-NPTII fusion protein. **Lane 1**: as for Figure 6A. **Lane 2**: as for Figure 6A lane 4 except for the fact that the plants were kept in complete darkness for 96 h before extraction. **Lane 3**: as for Figure 6A, lane 4.

phosphate carboxylase is catalysed by a processing enzyme and requires at least two steps (Robinson and Ellis, 1984). In view of the high level of conservation in the amino acid sequence of the transit peptide and of the mature small subunit protein near the cleavage site, it was assumed that these amino acid residues played an important role in the translocation process (Broglie *et al.*, 1983; Timko and Cashmore, 1983). Our data demonstrate that the chimaeric *tp-ss-npt*II gene, which upon expression yields a fusion protein with a transit peptide and the conserved amino acid sequence flanking the processing site, is indeed translocated to the chloroplasts and is processed to yield a fusion protein located in the stroma, consisting of the NH_2-terminal end of the small subunit protein and an active NPTII protein. This SS-NPTII fusion protein migrates in the gel NPTII-assay with an electrophoretic mobility which is intermediate between the TP-NPTII (35.5 kd) and that of the original NPTII activity (29 kd). This mobility is in very good agreement with the mol. wt. (32 298) of the SS-NPTII fusion protein. Our results indicate that this fusion protein, which confers kanamycin resistance to the transformed tobacco plants, is located within the chloroplasts and leaks out when the chloroplasts are broken. However, the results obtained by Van den Broeck *et al.* (1985) demonstrate that the NPTII component of a precursor protein which contains only the transit peptide sequence immediately fused to the NPTII protein and thus missing part of the conserved amino acid sequence flanking the processing site, is equally translocated across the chloroplast envelope and apparently properly processed. The latter results indicate that the transit peptide sequence alone is sufficient to both transport and process precursor proteins into chloroplasts. The function, if any, of the conserved amino acid sequence around the processing site, is therefore unclear. The results presented here, together with those of Van den Broeck *et al.* (1985), open the way for important fundamental studies and agricultural applications involving chloroplast functions. Indeed it would seem to be possible to introduce foreign proteins into the plant cell organelles either as such or as fusions with proteins which are coded for by nuclear genes and normally transported into the plant cell chloroplasts. This may lead to a better understanding of the role played by various domains of transported proteins interacting with plastid-coded proteins. The approach described here probably does not apply for chloroplasts only but might be generalised to several cellular

organelles and compartments. Indeed Hurt *et al.* (1985) have independently demonstrated that chimaeric genes can be used to direct the transport of foreign proteins into yeast mitochondria and that the cleavable prepiece of an imported mitochondrial protein is sufficient to direct this transport. Whether transit peptides can also be used to direct proteins into membranes such as the thylakoid membrane is unclear. A better candidate to resolve this question is probably the transit peptide of the chlorophyll a/b-binding protein.

Materials and methods

Strains and plasmids

E. coli DH1 was used for *in vitro* transformation. *Agrobacterium* C58CIRif was the receptor strain in all bacterial conjugations. The conjugation followed the protocol described by Van Haute *et al.* (1983) and Zambryski *et al.* (1984).

DNA techniques

Restriction endonucleases and other DNA modifying enzymes were used as recommended by the manufacturers. Other techniques were used as described by Maniatis *et al.* (1982).

Nopaline assay

The presence or synthesis of nopaline due to expression of the *nos* gene in transformed calli and regenerating shoots from these calli was monitored according to Otten (1982).

Plant transformation

Small axenically growing plants were kept in 1/2 M + S medium (Murashige and Skoog, 1962) in jars and were inoculated after decapitation with *Agrobacterium* strains as described (Zambryski *et al.*, 1984). Wound calli were removed and put on medium containing 0.2 mg/l benzaminopurine and 0.6 mg/l indoleacetic acid and 0.5 mg/ml cefotaxime (Hoechst). After ~4 weeks the callus material was transferred to hormone-free medium and emerging shoots were tested for nopaline production. Nopaline synthase-positive shoots were propagated and tested on 100 – 500 μg/ml kanamycin. Teratoma shoots which grew on concentrations of 100 μg/ml or higher were used for analysis. Protoplasts were kept in co-culture with *Agrobacteria* according to Marton *et al.* (1979) with modifications described by Hain *et al.* (1985).

Analysis of DNA and RNA

DNA was isolated according to Bedbrook (1981) from preparations of nuclei. The DNA was digested with restriction endonucleases (10 – 30 μg/lane, overnight digestion with a 3-fold excess of enzymes), separated on agarose gels according to size and transferred to nitrocellulose filters (Thomas, 1983). Hybridization with radioactive probes was performed in 50% formamide, four times SSC, 10 times Denhardt's solution, 0.2% SDS and 0.1 mg/ml calf thymus DNA at 50°C for 48 h (Bohnert *et al.*, 1982). The filters were washed twice for 15 min each in 50% formamide, four times SSC at the hybridization temperature, followed by washing in 50% formamide, three times SSC at room temperature (1 – 4 h) and twice SSC at room temperature (1 h). Dot-blot hybridizations were performed according to Thomas (1983) with DNA amounts covering a range equivalent from 1000 to 0.1 gene copies per sample. Hybridization was as described above. RNA was isolated according to Chirgwin *et al.* (1979), and separated into poly(A)$^+$ and poly(A)$^-$ RNA by passage over oligo d(T)-cellulose (Collaborative Research, type III) following the procedure of Aviv and Leder (1972). RNAs were separated according to size in 1% agarose gels containing 5 mM methylmercury hydroxide (Bailey and Davidson, 1976) and transferred to nitrocellulose paper (Thomas, 1983). Hybridizations with ^{32}P-labelled, nick-translated probes were carried out as described (Bohnert *et al.*, 1982); between 2 and 3 x 10^6 c.p.m./lane were used.

Neomycin phosphotransferase activity assay

The assay was adapted for plant extracts from a procedure worked out for bacterial and animal cell lysates (Reiss *et al.*, 1984a). Between 20 and 100 mg of tissue from transformed plants was crushed in 0.1 ml buffer (10% glycerol, 5% β-mercaptoethanol, 62.5 mM Tris/HCl, pH 6.8, 50 μg/ml bromophenol blue and 0.1% SDS). Several protease inhibitors were used in an attempt to inhibit specific and unspecific proteases. Aprotinin (trade name Trasylol) was used at a final concentration of 100 μg/ml in water. *p*-Hydroxy-mercuribenzoate (PHMB) was used at a concentration of 1 mM, ε-amino-n-caproic-acid and 1,10-phenanthroline were added to a final concentration of 5 mM. Protease inhibitors were used according to Gray (1982). Crystalline phenylmethylsulfonyl fluoride (PMSF) was added immediately before use at a concentration of 100 μg/ml. The cleared homogenate (5 min, 13 000 r.p.m., Eppendorf centrifuge) was loaded onto 10% non-denaturing polyacrylamide gels (Laemmli, 1970; without SDS). After electrophoresis the buffer in the gel was exchanged against 67 mM Tris/maleate, 42 mM $MgCl_2$, 400 mM NH_4Cl, pH 7.1, and the acrylamide gel was covered by an agarose gel (1%) containing kanamycin-sulfate (1 mg/ml) and [γ-^{32}P]ATP (5 μCi/ml of a specific activity of 2000 – 3000 Ci/mmol) in the same buffer as the polyacrylamide gel. The gel-sandwich was covered by Whatman P81 paper, Whatman 3MM paper, and paper towels. After 3 h the P81 paper was incubated for 30 min in a solution containing 1% SDS and 1 mg/ml proteinase K in water at 60°C and subsequently washed several times in 10 mM phosphate buffer (pH 7.5) at 80°C, dried and exposed to Kodak XR5 film for up to 48 h. The principle of this method is the binding of kanamycin to the phosphorylated DEAE paper by which the positions in the gel are revealed where a kanamycin phosphorylating activity migrated. The additional proteinase treatment suppresses signals of plant activities which after phosphorylation bind to P81 paper but do not phosphorylate kanamycin.

Isolation of chloroplasts

Chloroplasts were isolated from 1 – 2 g of leaves of transformed plants. Structurally intact chloroplasts were collected from Percoll (Pharmacia) gradients (Ortiz *et al.*, 1980). The washed chloroplasts were concentrated by centrifugation, lysed and then used for the *in situ* demonstration of NPTII activity as described above. Trypsinisation of chloroplasts was performed according to Bartlett *et al.* (1982).

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