
Nuclear transcriptional activity of the tobacco plastid *psbA* promoter

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ABSTRACT

The plastid *psbA* promoter of tobacco was used with the aim to construct plastid specific marker genes. Upon transfer to the tobacco nuclear genome the plastid promoter fragment appeared to specify a messenger RNA. Placed 5' to the *bar* or *nptII* coding sequences the level of expression is sufficient to obtain a selectable phenotype. The transcription start site in the nucleus is site specific and is located 4 nucleotides downstream relative to the start site used in plastids. Translational fusions of the *psbA* coding sequence with the *nptII* and *bar* coding sequences revealed that the *psbA* leader sequence and the *psbA* translation start codon, being the second ATG codon, are recognized by the plant cytoplasmic translation apparatus.

A promoter cassette utilisable in both *E. coli* and tobacco, was obtained by placing the CaMV 35S enhancer 5' to the *psbA* promoter.

INTRODUCTION

The principal function of plastids in higher plants is the fixation of CO₂ in chloroplasts of photosynthetically active tissue. However, plastids can also differentiate into other plastid types as chromoplasts or amyloplasts, to assume specialized physiological functions (1). Insight in the regulation of plastid differentiation and biosynthesis is limited. Plastids depend on nuclear genes for over 80% of their proteins, the remainder is synthesized within the organelle (2). Regulation of expression has been studied for some plastid genes in detail and appears to occur at both transcriptional and posttranscriptional level (3). Recently, the sequence analysis of two entire plastid genomes, *Nicotiana tabacum* and *Marchantia polymorpha*, has been completed (4, 5).

To identify plastid sequences important for gene regulation at DNA and/or RNA level, it is essential to study the expression of modified plastid genes *in vivo*. To this purpose the use of T-DNA transfer techniques to transform plastids was investigated (6). T-DNA cassettes were constructed that carried chimaeric reporter genes under the control of plastid promoters. The minimal plastid sequence required for promoter activity has been determined for a limited number of promoters *in vitro* (7) and these requirements may differ *in vivo*. Crucial to the success of plastid transformation is that the reporter genes confer resistance to the cell only when expressed in plastids.

In this report we demonstrate that unlike expected the tobacco plastid *psbA* promoter is not a plastid specific promoter but is also capable of supporting transcription initiation in the tobacco nuclear genome.

MATERIALS AND METHODS

DNA manipulations

All DNA manipulations were carried out essentially as described by (8). The *psbA* gene present on pTB8, was a gift of Dr. Sugiura, Nagoya University, Japan. The *psbA* promoter, leader and amino terminal codons were cloned as a SmaI-FnuDII fragment into the SmaI site of pUC19 (9), generating pUC19*psbA*. Translational fusions with the *bar* (10) and *nptII* (11) coding sequences were made by consequent cloning into the BamHI-HindIII site of pUC19*psbA*.

pGSC1700 (fig. 2a) is a binary plant vector derived from pGV1500 (12); the polylinker of pGV1500 was deleted by HpaI-HindIII digestion and replaced by the pUC19 PvuII-HindIII linker fragment generating pGSC1500. The β -lactamase gene of pUC19, which lacks the PstI site, was introduced to generate pGSC1600, and finally the pVS1 origin of replication and stability functions were introduced as a blunt BamHI-SacII fragment (12) into the unique PvuII site of pGSC1600. The recombinant plasmid carrying the pVS1 origin in the same orientation as in pGV943 (12) was partially digested by BamHI and filled in to remove the reconstituted BamHI, generating pGSC1700.

pGSC65, pGSC65-1, and pGSC65-2 are pGSC1700 derivatives carrying the *nptII* and *bar* reporter genes in a head to head orientation. 6 refers to a P_{35S}*nptII* fusion gene (10), 5, 5-1 and 5-2 refers to the P_{*psbA*}*bar* gene and promoter deletions StyI-SpeI and SmaI-BglII (filled in), respectively.

pGSC54 is a pGSC1700 derivative carrying the P_{*psbA*}*bar* and P_{*psbA*}*nptII* reporter genes in a tail to head orientation, 3' to the *nptII* gene is located the 3' *rbcL* sequence as in (6). pGSCM100 and pGSCM102 are pGSFR280 (10) derivatives carrying the *psbA* leader, TATA- and CAAT-boxes at the position of the comparable 35S sequences. pGSCM100 was generated by exchange of the 126 bp BamHI-EcoRV 35S element with the 191 bp BamHI-SmaI *psbA* fragment of which the internal SpeI-XhoI fragment was deleted. pGSCM102 carries in addition in the EcoRV site the 300 bp 35S EcoRV-AccI enhancer element (13).

Plant DNA was prepared essentially as described by (14). Southern blot analyses and preparation of probes were according to Amersham protocols.

Tissue-culture

Cocultivation and leaf disc transformations of *Nicotiana tabacum* cv. Petite Havana SR1 (15) were carried out as described by (10). Transgenic SR1 are named SR1(T-X), where X refers to the T-DNA of the plasmid used.

Enzyme assays

Soluble protein was extracted according to (10). PAT assays were carried out as described by (10). NPTII assays were performed as described by (16).

Electroporation

Transient expression of plasmid vectors in SR1 protoplasts was done according to Denecke *et al.* (in preparation). 10⁶ protoplasts in 0.3 ml buffer (0.4 M sucrose, 80 mM KCl, 4 mM CaCl₂·2H₂O, 10 mM Hepes pH 7.2) were electroporated (field strength 400 V/cm) in the presence of 10 μ g plasmid DNA. After electroporation cells were suspended in 3 ml 0.4 M sucrose buffer (10) and kept for 16 hrs in the dark at 24°C. Cells were concentrated and used for either enzyme assays or RNA preparation.

RNA manipulations

RNA was extracted essentially as described by (17). Leaf or protoplast samples were frozen in liquid nitrogen, thawed in NTES buffer (0.1 M NaCl, 10 mM Tris pH 7.5, 1 mM EDTA, 1% SDS) and phenol/chloroform extracted. Nucleic acids were ethanol precipitated,

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CCGGGGCAAC CCACTAGCAT ATCGAAATTC TAATTTTCTG TAGAGAAGTC

      CAAT
CGTATTTTTC CAATCAACTT CATTAAAAAT TTGAATAGAT CTACATACAC

      -35      TATA      -10      RIS
CTTGGTTGAC ACGAGTATAT AAGTCATGTT ATACTGTTGA ATAAAAAGCC

TTCCATTTTC TATTTTGATT TGTAGAAAAC TAGTGTGCTT GGGAGTCCTT

GATGATTA~AA TAAACCAAGA TTTTACCATG ACTGCAATTT TAGAGAGACG

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fig. 1. Sequence of the *psbA* promoter region. Eucaryotic CAAT- and TATA-box consensus sequences and plastid -35 and -10 consensus sequences are indicated. The RNA Initiation Site (RIS) in plastids is underlined. The first codon of the *psbA* coding sequence is underlined, nucleotides in agreement with the translation initiation consensus of plants (22) are overlined, the start and stop codons of a small open reading frame upstream from the *psbA* coding sequence are indicated with a wavy line.

RNA was selectively precipitated in 2 M LiAc, o/n, 4°C. RNA was further purified by two ethanol precipitations and dissolved in H₂O at a concentration of 1 mg/ml. Northern blots were made according to Amersham protocols. The BamHI fragment of pGSFR280 carrying the *bar* coding sequence was cloned into vector pGEM2 (Promega Biotech.). Filters were hybridised with ³²P labeled riboprobes complementary to *bar* mRNA according to (17).

Primer extension

5 pmoles of the 225 bp Sall-BglI fragment of pGSFR280 was dephosphorylated with bovine alkaline phosphatase (BAP) and end labeled using γ -³²P-ATP (\pm 3000 Ci/mmol) and T4 polynucleotide kinase according to (8). The DNA was denatured and single stranded labeled primer was gel purified. 0.5 pmoles primer were incubated with 5 μ g RNA in 0.25 M KCl, 2 mM Tris pH 7.9, and 0.2 mM EDTA for 3 hrs, at either 50, 55 and 60 °C. Extension was done in 10 mM MgCl₂, 5 mM DDT, 20 mM Tris pH 8.3, 0.33 mM dNTP, and 100 μ g/ml actinomycin-D with 10 units AMV reverse transcriptase at 37°C for 45'. Nucleic acids were ethanol precipitated and dissolved in 3 .l 0.1M NaOH, 1mM EDTA and left at r.t. for 30' before formamide was added. The length of the cDNA was determined with the help of a known sequence ladder.

RESULTS

The tobacco psbA promoter bears features of an RNA polymerase II promoter

A chimaeric gene comprising the tobacco *psbA* plastid transcription and translation initiation signals fused to the *npfII* (neomycin phosphotransferase) coding region of Tn5 was inserted on small T-DNA vectors with the aim of obtaining plastid transformants by *Agrobacterium tumefaciens* mediated T-DNA transfer (6). These *Agrobacterium* strains were used in transformation experiments of SR1 protoplasts which yielded significant numbers of phenotypically resistant plants.

The seven transgenic SR1 plants studied, carried two to four copies of the T-DNA in the nuclear genome. NPTII-assays showed that all transformants produced a low level of active fusion product of the expected size. NPTII could not be detected by Western blot analysis, implying that its contribution to the total soluble protein pool was less than 0.01%. The gene product was present in total leaf extracts but not in purified plastids. This result

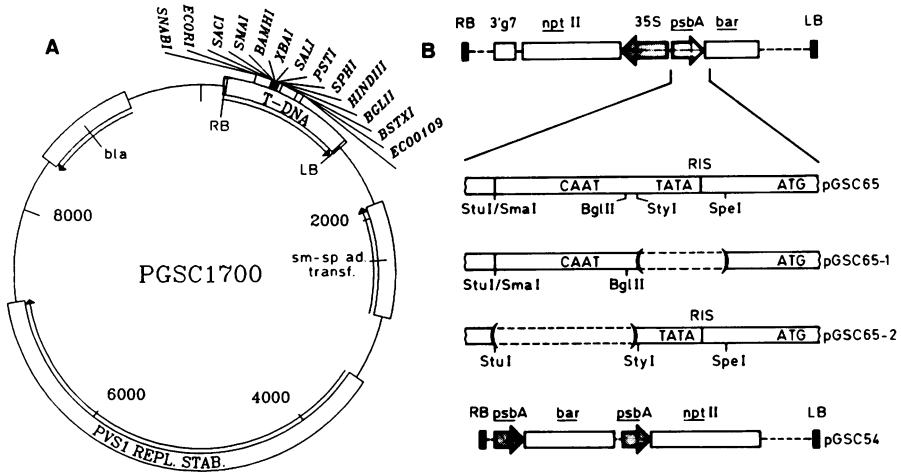


fig. 2. Schematic outline of pGSC1700 and the T-DNA constructions pGSC65, pGSC65-1, pGSC65-2 and pGSC54. CAAT- and TATA-box and restriction sites chosen for deletion generation are indicated.

indicated that either the *psbA* promoter is not plastid specific but is recognized by the RNA polymerase II transcription complex as well, or that the regions upstream from the *psbA* promoter located either on the T-DNA or on the plant nuclear genome, contain an RNA polymerase II transcription start site.

Inspection of the *psbA* promoter sequence shows the presence of a perfect eucaryotic TATA-box (18) at position -27 relative to the start site of transcription which the plastid RNA polymerase uses *in vivo* (19), whereas at -80 a perfect CAAT-box (20) is found (fig. 1). When correctly spaced, these two components are sufficient in animal cells for accurate, low frequency transcription initiation by RNA polymerase II (21). The expected start site of transcription by RNA polymerase II, which in plants is located at a mean distance of 32 ± 7 nucleotides downstream from the TATA-box (22), will approximately coincide with the start site of the plastid RNA polymerase. This indicates that the leader sequence of the *psbA* messenger produced in the nucleus corresponds to the plastid counterpart. This leader contains a small open reading frame which may negatively interfere with cytoplasmic translation initiation at the *psbA* coding sequence (23). The sequences flanking the translation start site of the *psbA* coding region have a significant homology with the consensus postulated for plants (22, 24), suggesting it may function as a suboptimal site for translation initiation.

Vectors to test nuclear transcriptional activity of the psbA promoter

The production of *nptII* messenger can be explained either by transcriptional read through or transcription initiation at the *psbA* sequence. To distinguish between these possibilities, total RNA of 4 transgenic plants was analysed. Due to too low steady state levels of the *nptII* mRNA, it was in this way not possible to obtain any information (data not shown). Therefore, T-DNAs were constructed with which the transcriptional activity of the *psbA* promoter sequence could be monitored (fig. 2).

On pGSC65 a divergent promoter element is constituted by a head to head fusion of the cauliflower mosaic virus (CaMV) 35S promoter (25) and the *psbA* promoter. The 35S promoter is fused to the *nptII* coding sequence and can be used to select transgenic SR1.

Table 1. Expression of hybrid 355 *nptII*, *psbAnptII*, *psbAbar* and *psbAbar* deletion mutant genes in transgenic SR1

T-DNA construction	Expressions in tobacco	
	NPTII	PAT
pGSC65	+	+
pGSC65-1	+	-
pGSC65-2	+	-
pGSC54	+	+

Transcription initiation promoted by the *psbA* region can be monitored by PAT (phosphinotricin acetyl transferase) production, which is specified by the *bar* (bialophos resistance, 26) coding sequence. Due to the inverted orientation of the *nptII* gene relative to the *bar* gene, no selective pressure is applied to enrich for promoter tagging events (27, 28) leading to a PAT phenotype. Therefore, a transcriptional read through initiated by an upstream plant promoter faces minimally a separation of 2 kb between promoter and *bar* coding sequence.

In addition, two T-DNA constructions identical to pGSC65 were made which lack either the TATA-box region (pGSC65-1) or the CAAT-box region (pGSC65-2). The rationale being that deletion of either box would significantly reduce PAT expression, thus supporting their functional importance.

On pGSC54 two chimaeric genes producing respectively PAT and NPTII, and both under the control of the *psbA* promoter, are placed in a head to tail configuration. In order to avoid the introduction of any cryptic promoter the distance between the two coding regions was kept to a minimum, and no further 3' gene signals were included. The addition of these signals is not essential because cryptic signals 3' to the coding region will be found (29). A transcript produced by a T-DNA sequence or plant promoter upstream from the reporter coding sequences would be polycistronic. No evidence exists that eucaryotic polycistronic mRNAs produce the second protein efficiently (30). Therefore, in SR1(T-GSC54) plants expressing both marker proteins at comparable levels, expression of the

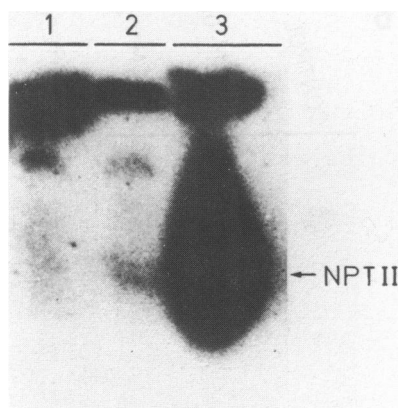


fig. 3. Detection of NPTII activity in leaf extracts of SR1 (1), SR1 containing T-DNA of pGSC54 (2), or pGSC65 (3). The concentration of soluble protein of the samples was normalized with Biorad assays. The NPTII activity of sample 2 is several magnitudes less than of sample 3.

distal coding sequence is likely to be due to a promoter element located between the two reporter coding sequences.

The chimaeric psbA gene is expressed in the nucleus

The T-DNAs of pGSC65 and pGSC54 were stably introduced in SR1 protoplasts via the cocultivation transformation method (10). The T-DNAs of pGSC65-1 and pGSC65-2 were introduced into SR1 via the leaf disc transformation method (10). Transformants

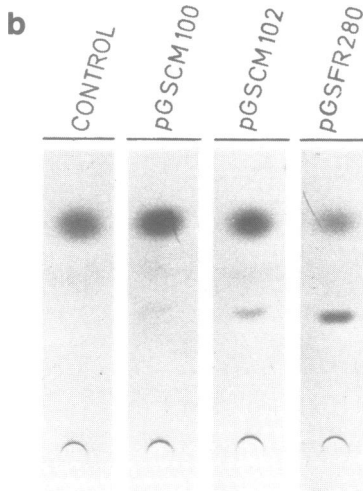
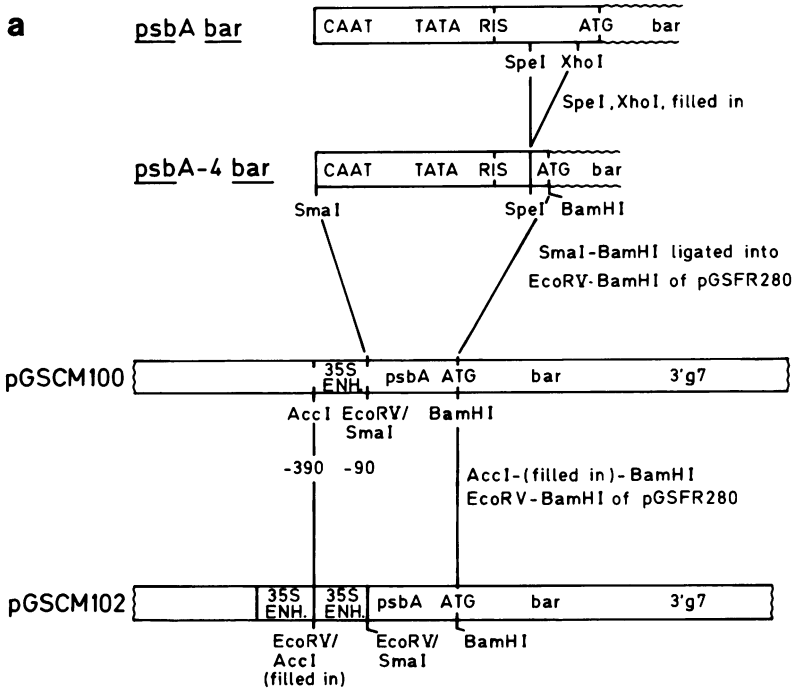




fig. 4. 4a: schematic presentation of the construction of pGSCM100 and pGSCM102, 4b: PAT activity directed by the three constructions in a comparative transient expression assay, 4c: detection of *bar* mRNA from RNA extracts of SR1 16 hours after electroporation in the presence of pGSCM102 (2) or pGSFR280 (3), (1) refers to the SR1 control.

were identified by their ability to grow on medium containing 100 mg/l kanamycin. The two cocultivation experiments yielded an equivalent number of resistant calli. The expression capability of the 35S *nptII* gene is well documented (10). Therefore, the comparable transformation efficiencies suggest that in the case of the SR1 plants transformed by pGSC54 the *nptII* gene is driven by a promoter located on the T-DNA. Southern analysis of two plants of both lines showed the presence of intact T-DNA copies in the nuclear genome (not shown).

Analysis of PAT activity of transgenic SR1 carrying T-DNAs of pGSC65, pGSC65-1, pGSC65-2 and pGSC54 revealed that only transgenic plants carrying the intact *psbA* region express PAT to a detectable level (table 1). This indicates the functional importance of both the CAAT and the TATA region for PAT expression. The most likely interpretation is that the *psbA* sequence functions as a nuclear promoter and that the deletions affect transcription initiation.

The frequency of recovery of kanamycin resistant transgenic SR1 carrying the T-DNA of pGSC54 and the fact that the ten plants tested from this experiment produce both PAT and an NPTII protein of the expected size (fig. 3), implicate the presence of two functional eucaryotic promoters 5' to the coding sequences on the T-DNA. These results, therefore, strongly suggest that both coding regions are transcribed by polymerases that initiated at the *psbA* promoters.

To ascertain that the interpretations are correct and that the deletions made indeed are promoter mutations, the transcription start site at the *psbA* element had to be determined.

The site of transcription initiation at the psbA promoter in the nucleus

The *psbA* region carries at a proper spacing two typical consensus sequences, which are essential for accurate, site specific transcription initiation by RNA polymerase II. Since the steady state transcript level established by such promoter is very low, transcriptional enhancers can be used to obtain higher steady levels of further identical RNAs. The CaMV 35S enhancer was placed 5' to the *psbA* promoter in an attempt to improve its efficiency

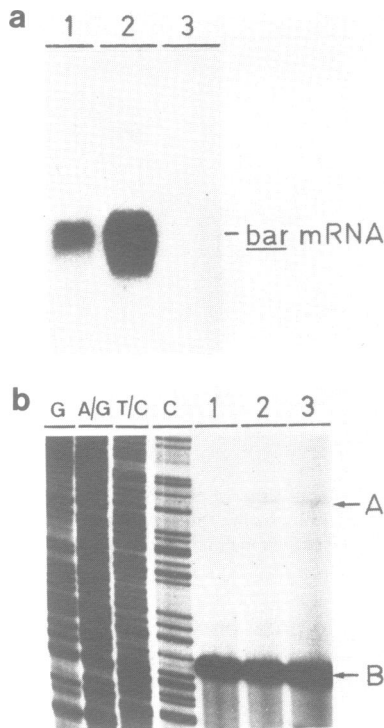


fig. 5. 5a: Detection of *bar* mRNA in RNA prepared from transgenic SR1 carrying T-DNA of pGSCM100 (1) and pGSCM102 (2) and from untransformed SR1 (3). RNA of 5 independent regenerants of each type was pooled to obtain a more quantitative view. 5b: Primer extension assay to localize the transcription start site at the hybrid 35S-*psbA* promoter. The sequencing tracks are indicated. 1, 2, 3 refer to annealing temperatures of 60, 55, and 50 °C, respectively. A and B refer to extended primer and primer, respectively.

(fig. 4a; 13), generation a chimaeric 35S *psbA bar* gene. This construction, pGSCM100 and the parental 35S promoter construction (pGSFR280, 10) were transiently expressed in SR1 protoplasts. The expression level of PAT directed by pGSCM100 is only tenfold below that of pGSFR280 (fig. 4b). This is an increase of several orders of magnitude with respect to the original *psbA bar* gene and it is interpreted as an elevated frequency of transcription initiation at the *psbA* transcription start site. To further optimise the hybrid promoter, the 35S enhancer element was duplicated (fig. 4a, construction pGSCM102). Figure 4b shows that duplication of the enhancer element results in a several fold increase of PAT activity. RNA analysis of protoplasts electroporated in the presence of pGSCM102 and pGSFR280 revealed that the *bar* mRNAs specified by both genes are of a comparable length (fig. 4c). Since both genes are identical apart from the region upstream of the coding region, the corresponding length of the mRNAs indicates that indeed transcription initiates at the *psbA* sequence.

The T-DNAs of pGSC100 and pGSC102 were stably introduced in SR1 by T-DNA transfer. Analyses of five transgenic plants of each type confirmed the observation seen with the electroporation experiment that duplication of the 35S enhancer further increases expression of the chimaeric *bar* gene (fig. 5a). RNA extracted from these plants was used for

primer extension analysis. As shown in figure 5b it revealed that in the nucleus transcription is initiated at a single site. The calculated length of the extended primer locates the nuclear transcriptional start site 4 nucleotides downstream from the transcription start site used in plastids and 31 nucleotides downstream from the TATA-box (fig. 1). The first nucleotide of the transcript is an A, as is the case for most of the nuclear transcripts (22).

DISCUSSION

The promoter sequence of the *Nicotiana tabacum psbA* gene was used to construct chimaeric reporter genes with the aim to obtain selectable genes for plastid transformation (6). The reporter genes were introduced in tobacco by T-DNA mediated transfer. The experiments suggested that the *psbA* promoter is transcriptionally active when it is introduced into the tobacco nuclear genome. Inspection of the sequence revealed the presence of two eucaryotic, cis-acting elements: the CAAT- and TATA-box. Fused to the *nptII* or *bar* coding sequences the transcriptional activity of the promoter is sufficient to provide the cell a selectable phenotype, but the transcriptional activity is too low to produce detectable mRNA levels. Therefore, three approaches were used to ultimately demonstrate that the cassette provides a site for transcription initiation.

First, T-DNA cassettes were designed and tested on which the *psbA* promoter was placed in a position that efficiency of transformation, gene expression, and gene product size could only be explained if indeed the *psbA* sequence would act as a nuclear promoter.

Secondly, deletions were made in the *psbA* promoter sequence to remove the regions in which the CAAT- and TATA- box are located. These mutants displayed at a protein level significantly lower expression which indicates the functional importance of the two regions for expression.

Thirdly, the transcriptional activity of the chimaeric *psbA bar* gene was dramatically increased by placing 5' to the *psbA* promoter one and two copies of the 35S enhancer. RNA extracted from transgenic SR1 carrying these 35S *psbA bar* genes was used to determine the 5' end of the *bar* transcript in order to deduce the start site of transcription. In eucaryotic protein coding genes the TATA-box plays a major role in the determination of the exact site of transcription initiation, which means that the addition of the 35S enhancer should affect the frequency of initiation only. Indeed, primer extensions revealed that in the nucleus transcription is initiated within the *psbA* promoter region at a single site 4 nucleotides downstream from the site used in plastids. This site is in agreement with the expected spacing between transcription start site and TATA-box (22).

The 5' sequence of the messengers specified by the *psbA* promoter in the two cellular compartments is nearly identical. Since the translation machinery of the organelles operate in completely different fashions, it is interesting that although the *psbA* coding region starts at the second ATG, this ATG is utilised as a translation start codon in the cytoplasm. The original scope of the experiments was to assess whether plastids can be transformed using T-DNA transfer techniques in combination with plastid specific marker genes. T-DNA transfer is highly efficient for nuclear transformation: in cocultivation experiments with tobacco protoplasts up to 20–30% of the cells become stably transformed. Therefore, a thight control of expression of any plastid specific marker gene is required to be able to identify and select for plastid transformation events. Clearly, the tobacco *psbA* promoter is unsuitable for this purpose.

The bifunctionality of the 5' part of the *psbA* gene may be coincidental. However, it could be argued that the data favour the hypothesis that during evolution the plastid genome has lost coding capacity due to cytoplasmic complementation. Cross-hybridisation and

sequence analyses demonstrated DNA exchange between nuclear, mitochondrial and plastid genomes at low frequencies (31–33).

The hybrid 35S *psbA* promoter is efficient in transcription initiation in both *E. coli* and plant cells. This aspect adds to vectors equipped with this hybrid promoter the unique advantage that any coding sequence placed downstream from the promoter can be expressed in the two systems. It offers the possibility to perform with a single vector cDNA cloning, expression and immuno-detection in *E. coli*, and stable or transient plant cell transformation.

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