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Controlled expression of plastid transgenes in plants based on a nuclear DNA-encoded and plastid-targeted T7 RNA polymerase

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ABSTRACT **Phage T7 RNA polymerase has been used extensively in *Escherichia coli* for high-level expression of selected genes placed under the control of the phage T7 gene *10* promoter. We have constructed an analogous system for use in plastids of higher plants. A T7 RNA polymerase chimeric gene containing a cauliflower mosaic virus 35S promoter and a tobacco ribulose-bisphosphate carboxylase/oxygenase small-subunit chloroplast transit-peptide sequence was introduced into tobacco by nuclear transformation. Stable plastid transformation of tobacco expressing the T7 RNA polymerase activity with a T7 promoter/β-glucuronidase (GUS) reporter gene construct resulted in expression of GUS mRNA and enzyme activity in all tissues examined. Expression of GUS activity was extremely high in mature leaves, moderate in young leaves and petals, and low in stems, roots, and developing seeds. Plastid transformation of wild-type tobacco with the same chimeric GUS gene resulted in undetectable levels of GUS mRNA and enzyme activity. Genetic crosses demonstrated that a silent T7/GUS reporter gene could be activated in the F_1 generation by transmission of an active nuclear T7 RNA polymerase gene from the male parent.**

The plastids of higher plants are an extremely attractive target for genetic engineering. Plant plastids (chloroplasts, amyloplasts, chromoplasts, etc.) are the major biosynthetic and photosynthetic centers of the cell, responsible for production of important compounds such as amino acids, complex carbohydrates, fatty acids, and pigments. Various plastid types are derived from proplastids located in meristematic cells and thus have the same genetic content. Stable transformation of plastid genomes has been achieved in the green alga *Chlamydomonas* (1) and in higher plants (2) through homologous recombination of a selectable marker delivered to the plastid by particle-gun bombardment. Plant cells have been found to contain up to 50,000 copies of the chloroplast genome (3). The plastid genome is circular and varies in size between plant species from 120 to 217 kbp due mainly to the presence of a large inverted repeat which is typically 20–30 kbp but can range from 0 to 76 kbp (4). This makes it possible by plastid transformation to engineer plant cells to maintain any introduced gene at an extremely high copy number, potentially resulting in a very high level of foreign gene expression. The complete genome sequences of plastids from liverwort (5), rice (6), and tobacco (7) have been determined. These analyses reveal that plastid genomes contain close to 120 genes, with approximately half involved in transcription/translation and one-quarter involved in photosynthesis. DNA sequence and biochemical data reveal a striking similarity of the plastid organelle's transcriptional and translational machinery and cognate initiation signals to those found in prokaryotic systems. In fact, many plastid-derived promoter sequences can direct expression of reporter genes in prokaryotic cells (8). In addition, plastid genes are often organized into polycistronic operons as are genes in prokaryotes.

Despite the apparent similarities between plastids and prokaryotes, they exhibit fundamental differences in the methods used to control gene expression. As opposed to the transcriptional control mechanisms typically observed in prokaryotes, plastid gene expression is controlled predominantly at the level of translation (9, 10) and mRNA stability (8) by trans-acting, nuclear DNA-encoded proteins.

A major drawback in the engineering of plastid gene expression is the lack of tissue-specific developmentally regulated control mechanisms. It is possible that unregulated modification of plastid metabolism and/or the introduction of new biochemical pathways could result in the inability to obtain viable plants. One way to address this problem would be to specifically trans-activate a silent plastid-borne transgene by tissue-specific expression of a nuclear-encoded and plastid-directed RNA polymerase (RNAP). The polymerase chosen for this purpose should have a high degree of specificity for the promoter element associated with the plastid transgene. To establish such a system, a β-glucuronidase (GUS) reporter gene under control of the phage T7 gene *10* promoter was introduced into the plastid genome of plants. GUS expression was dependent on nuclear-encoded plastid-targeted T7 RNAP activity.

MATERIALS AND METHODS

DNA and RNA Manipulations. Restriction digests, blunt-end treatments with Klenow DNA polymerase, ligations, and Southern and Northern blot analysis were according to Maniatis *et al.* (11). PCRs were done in 100 μl under the standard conditions recommended by Perkin–Elmer/Cetus in a thermal cycler (GeneAmp system 9600) set for 25 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec. Total plant DNA and RNA were prepared (12, 13) and their quantities were determined spectrophotometrically. RNA dot blot assays were carried out by fixing 0.5 μg and 5 μg of each total RNA sample to nylon membranes, hybridizing the RNA with a GUS-specific probe, and washing the filters in 0.5× standard saline citrate (SSC) at 60°C. Quantitative Northern blot analysis was performed on 5 μg of total RNA from stem, root, petal, and seed tissue and 0.5 μg of total RNA from mature leaves. The radioactive signals were detected with an Ambis 4000 radioanalytical imaging system.

Plasmid Constructions. Binary vector pCGN4026 containing a modified T7 RNAP gene was constructed as follows. Plasmid pAR3283 (14) was digested with *Bgl* II/*Eco*RI, and a synthetic adapter (top strand, 5′-GATCTGGATCCAACACGATTAACATCGCTAAGAACG-3′; bottom strand, 5′-AATTCGTTCTTAGCGATGTTAATCGTGTTGGATCCA-3′) was introduced. In the new plasmid, pCGN4023, the

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Abbreviations: CaMV, cauliflower mosaic virus; GUS, β-glucuronidase; RNAP, RNA polymerase; SSU, ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit.

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adapter restores the wild-type amino-terminal coding region of the T7 RNAP gene except that the methionine start codon has been replaced by a *Bam*HI restriction site. The *Bam*HI fragment from pCGN4023 containing the coding region for T7 RNAP was then cloned into pCGN3672 to give a T7 RNAP expression cassette consisting of a double cauliflower mosaic virus (CaMV) 35S promoter (−526 to −90 and −363 to +2 relative to the transcription start site; J. K. O'Neal, personal communication); the tobacco ribulose-1,5-bisphosphate carboxylase/oxygenase small-subunit (SSU) 5′ untranslated region, the sequence encoding the chloroplast transit peptide, intron I, and the first 12 amino acids of mature SSU; and a 260-bp *nos* 3′ untranslated region (15). The resulting plasmid, pCGN4025, was then digested with *Hin*dIII/*Bgl* II and the T7 RNAP expression cassette was cloned into binary vector pCGN1559 (16) digested with *Hin*dIII/*Bam*HI to create pCGN4026. pCGN1559 contains a 35S/*nptII* chimeric gene for kanamycin selection in tissue culture.

The construction of the plastid transformation vector pCGN4276 was as follows. The phage T7 polymerase gene *10* promoter and entire 5′ untranslated region of gene *10* were PCR amplified from plasmid pET3a (17) by using top-strand primer 5′-GGGAAGCTTGCGAAATTAATACGACTCAC-3′ and bottom-strand primer 5′-CCCCCATGGGTATATCTCCTTCTTAAAG-3′ and cloned into pUC120 (*Hin*dIII/*Nco* I) to give pCGN4028. A 3′ region was created by fusing the *psbA* 3′ untranslated region [base pairs 533–435 of the tobacco plastid genome sequence (7)] and the T7 gene *10* terminator from pET3a (17) in a two-step PCR. The *psbA* 3′ untranslated region was amplified with the top-strand primer 5′-GGGGAATTCGATCCTGGCCTAGTCTATAAG-3′ and bottom-strand primer 5′-GGTTATGCTAGTTATTGCTCAAAAGAAAAAAAGAAAGGAGC-3′; using *Nicotiana tabacum* cv. Xanthi total DNA as template. The T7 gene *10* terminator was amplified with top-strand primer 5′-GCTCCTTTCTTTTTTTCTTTTGAGCAATAACTAGCATAACC-3′ and bottom-strand primer 5′-CCCCTGCAGCCGGATATAGTTCCTCC-3′ using pET3a as template. Five microliters of each of these reaction products was mixed into a final reaction mixture in which a fusion product was amplified by using the top strand primer for the *psbA* 3′ region and the bottom-strand primer for the T7 gene *10* terminator. The fusion product was then digested with *Eco*RI/*Pst* I and cloned into pBluescript II KS(−) (Stratagene) cut with *Eco*RI/*Pst* I to create pCGN4027. Through further manipulation, the T7 promoter (*Hin*dIII/*Nco* I) and *psbA* 3′/T7 terminator (*Eco*RI/*Pst* I) were combined with an *Nco* I–*Eco*RI GUS gene fragment (*uidA* locus of *E. coli*) from pKiwi101 (18) to give pCGN4055. Plasmid pCGN4055 was then cut with *Hin*dIII/*Pst* I and the T7 5′::GUS::*psbA* 3′/T7 3′ expression cassette was cloned into *Hin*dIII/*Pst* I-digested pOVZ44B (gift of Pal Maliga, Waksman Institute, Rutgers University, New Brunswick, NJ) to create pCGN4276 (see Fig. 2). Plasmid pOVZ44B, a vector designed for integration of chimeric genes into the tobacco plastid genome by homologous recombination, contains a streptomycin/spectinomycin selectable marker, *aadA*, expressed from the tobacco 16S rRNA promoter, *rrn*, in a cassette with an *rps16* 3′ region. This cassette is inserted upstream of the *trnV* locus in the intergenic region between *trnV* and *rps12* such that >2 kb of homology exists between the vector and target plastid DNA.

Plant Transformation. Nuclear transformation was performed by cocultivation of tobacco (*N. tabacum* cv. Xanthi N/C) with *Agrobacterium tumefaciens* LBA4404/pCGN4026 and selection of kanamycin-resistant plants, as described (19).

Plastid transformation was carried out as follows. Tungsten microprojectiles (1.0 μm) were coated with pCGN4276 according to Maliga (20) and used to bombard mature leaves, placed abaxial-side-up on RMOP medium [Murashige and Skoog salts (21) with benzylaminopurine (1 μg/ml), 1-naphthaleneacetic acid (0.1 μg/ml), sucrose (30 mg/ml), and 0.7% phytagar] (2), with the Bio-Rad PDS 1000 He system (22). Development of transformed plants on RMOP medium supplemented with spectinomycin dihydrochloride (500 μg/ml) and subsequent subcloning on the same selective medium were done according to Svab *et al.* (2). Selected plants were rooted in Murashige–Skoog medium (21) containing indolebutyric acid (1 μg/ml), spectinomycin dihydrochloride (500 μg/ml), and 0.6% phytagar.

Enzyme Assays. GUS was assayed as described (15). T7 RNAP was assayed by incorporation of [α-^{32}P]UTP into pBluescript II KS(−) transcripts from 5 μl of crude leaf extract in a 25-μl reaction mixture with 40 mM Tris·HCl (pH 7.9), 8 mM $MgCl_2$, 5 mM dithiothreitol, 4 mM spermidine hydrochloride, 0.4 mM NTPs, and 2.5 μCi of [α-^{32}P]UTP (1 μCi = 37 kBq) (23). Crude extracts were prepared by grinding 2-cm-diameter leaf disks in 200 μl of 50 mM sodium phosphate, pH 7.0/10 mM EDTA/10 mM 2-mercaptoethanol.

Crosses and Segregation Assays. Spectinomycin-resistant plants were transplanted into soil and grown with a 16 hr/8 hr light/dark photoperiod at 24°C. Flower buds were emasculated 2 days before opening and fertilized with donor pollen. Mature seeds were collected 4 weeks after pollination. Seeds were surface-sterilized as above and plated aseptically on medium with 0.2× Murashige–Skoog salts and allowed to germinate. After 12 days, the seedlings were histochemically stained for GUS activity (15).

RESULTS

Construction and Analysis of Transgenic Tobacco Lines Expressing Chloroplast-Targeted T7 RNAP. To determine whether any gene could be expressed in plastids under control of the *E. coli* phage T7 gene *10* promoter, plant lines expressing chloroplast-targeted T7 RNAP activity were established. To this end a binary vector, pCGN4026, harboring a T7 RNAP chimeric gene with signal sequences for plastid targeting was constructed. The chimeric T7 RNAP gene, lacking its ATG start codon, is expressed from an enhanced CaMV 35S promoter (24) as a translational fusion to the tobacco SSU transit peptide and the first 12 amino acids of mature SSU. Use of the SSU chloroplast transit peptide plus mature protein sequences is responsible for efficient import of foreign protein into the plastid (25). Due to the approximately constitutive nature of the 35S promoter, T7 RNAP activity was expected to be present to some degree in plastids of most plant tissues. Twenty-one kanamycin-resistant pCGN4026 tobacco lines were generated by *Agrobacterium*-mediated transformation. T7 RNAP activity was detected in the leaf tissue from 10 of the primary transformants (T_1 generation), varying from 0.01 (40% above background level) to 2.25 units/μg of total protein (data not shown).

Kanamycin germination assays were conducted on the seeds from self-pollinated T_1 plants positive for T7 RNAP activity to determine those segregating 3:1 for the linked resistance gene. Three plants, nos. 3, 9, and 11, met this criterion and were used to generate homozygous lines for further experiments. T7 RNAP assays of leaf extracts prepared from these homozygous lines indicated that line 4026-3 expressed the lowest level of polymerase activity (Table 1).

Establishment of Tobacco Lines Homoplasmic for a T7 RNAP-Dependent GUS Expression Marker. Introduction of a marker gene into the plastid genome was carried out to test for trans-activation by the plastid-localized T7 RNAP activity. The GUS gene (*uidA* locus of *E. coli*) was chosen as it codes for an enzyme that is a simple biochemical marker for plants and that has been shown to be expressed in the plastid under the control of a *psbA* promoter and 3′ region (10). The GUS gene coding sequence is located downstream from the *E. coli* phage T7 gene *10* promoter and 5′ untranslated regions

Table 1. T7 RNAP activity in homozygous 4026 tobacco lines

Tobacco clone	Specific activity, unit/μg of protein
4026-3	0.3
4026-9	1.0
4026-11	0.8

One unit of T7 RNAP is the enzyme activity that incorporates 1 nmol of UMP into acid-precipitable RNA products within 60 min at 37°C. Unit values for T7 RNA polymerase were derived by comparison with a purified enzyme preparation.

such that the start codon for gene *10* is the start codon of GUS. The T7 gene *10* 5′ untranslated region was retained because this region has been shown in *E. coli* to be important for efficient translation of chimeric mRNAs (26). The 3′ region of the marker gene construct contains the *psbA* 3′ regulatory element shown to be important in stabilizing mRNA in green tissues (27) as well as the Rho-independent phage T7 gene *10* terminator to prevent transcription beyond this cistron. The chimeric GUS marker was introduced into the tobacco plastid homology vector pOVZ44B and the resulting plasmid, pCGN4276 (Fig. 1), was used to transform plastids of tobacco by particle-gun delivery of DNA-coated tungsten microprojectiles. Since integration into the plastid genome occurs by homologous recombination and the target site is near the rRNA operon in the inverted repeat, two copies of the transgene per plastid genome are expected (2). Following a second round of shoot formation and spectinomycin selection, two independently isolated, fertile, transplastomic lines in the T7 RNAP-producing background (4026-3) were generated in addition to three fertile, transplastomic lines in the Xanthi control background (containing no T7 RNAP activity). These tobacco lines were found to be >95% homoplasmic by Southern blot analysis (Fig. 1; probe A), where the introduction of a new *Bam*HI site by the transgene changed the size of the probed fragment from 3.3 kb to 0.8 kb in the transplastomic lines. The presence of a 5.8-kb GUS-specific band indicated that the chimeric GUS gene was integrated intact into the plastid genome (Fig. 1; probe B). Interestingly, fertile transformants were not obtained in the high-level T7 RNAP-producing line 4026-9 under conditions similar to those used for the 4026-3 transformants.

Accumulation of GUS mRNA Requires T7 RNAP. To determine T7 RNAP-dependent transcription of the GUS gene, total cellular RNA samples from leaves 4–8 cm in length of one clone each of 4276 Xanthi and 4276/4026-3 were subjected to Northern analysis with a 519-bp GUS-specific probe (Fig. 2). A single abundant mRNA band of the expected size (2.1 kb) was present only in the 4276/4026-3 RNA sample. This indicates that transcription of the GUS transgene is indeed dependent on the presence of T7 RNAP. To show that T7 RNAP had not affected the levels of other transcripts in the two genetic backgrounds, a duplicate filter was hybridized with an *aadA* gene-specific probe (789 bp) and the GUS filter was rehybridized with a 1.2-kbp *psbA*-specific probe. The results show that the genetic background has no effect on the relative transcript levels of either the linked *aadA* transgene or the unlinked *psbA* gene. Ethidium bromide staining of the formaldehyde gel prior to transfer of the RNA showed that slightly more RNA had been loaded in the 4276/Xanthi lane, explaining why the *aadA* and *psbA* bands were slightly more intense in that lane. Curiously, an *aadA*-specific transcript of 1.3 kb was detected in both lanes in addition to the expected 0.9-kb band. This may be the result of either the *aadA* transcript not being processed within the boundaries of the *rps16* 3′ region or the initiation of transcription occurring upstream within the GUS coding region.

GUS Activity Is Expressed from the Plastid Transgene and Is Dependent on T7 RNAP. The 5′ untranslated region of T7

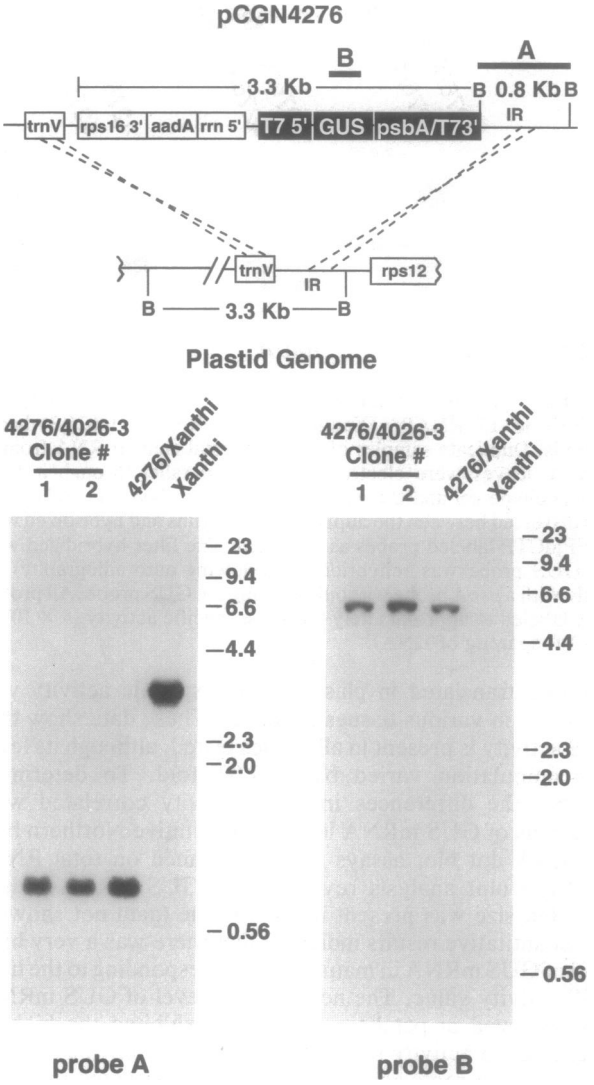

FIG. 1. Creation of homoplasmic tobacco lines containing a T7 RNAP-dependent GUS expression marker. (*Upper*) Incorporation of the spectinomycin-resistance marker *aadA* and T7 promoter/GUS reporter gene into the tobacco plastid genome by homologous recombination (double crossover). Upper line represents the incoming DNA donated by pCGN4276 and the lower line represents the plastid genome target region. rrn 5′, rRNA operon promoter; rps16 3′, 3′ region of the 30S-ribosomal-subunit protein 16 gene; trnV, tRNA valine gene; rps12, 30S-ribosomal-subunit protein 12 gene; IR, intergenic region. The T7/GUS cassette is described in *Materials and Methods* and *Results*. Expected sizes of *Bam*HI (B) fragments are shown for the incoming DNA as well as the wild-type DNA. The combined size of the two chimeric genes is indicated. Also shown are the location of the two probes, A and B (represented as solid bars) used for Southern analysis. (*Lower*) Southern blot hybridization. Probe A determines the degree of transformation (homoplasmy) and probe B reveals the presence of the GUS gene. The 4276/4026-3 clones 1 and 2 represent two independent spectinomycin-resistant transformants in *N. tabacum* cv. Xanthi line 4026-3. 4276/Xanthi represents a spectinomycin-resistant transformant in wild-type *N. tabacum* cv. Xanthi. The control DNA was from untransformed Xanthi. DNA molecular size markers are in kilobase pairs. Approximately 2 μg DNA for each sample was digested with *Bam*HI, electrophoresed through 1% agarose, transferred to Nytran+ (Schleicher & Schuell), and hybridized with [α-^{32}P]dCTP-labeled probes A and B.

gene *10* has a stem–loop structure at the very 5′ end followed by a sequence containing a translational enhancer (26), a strong prokaryotic ribosome-binding site, and the start codon. To demonstrate that T7/GUS transcripts containing this specialized prokaryotic untranslated leader sequence

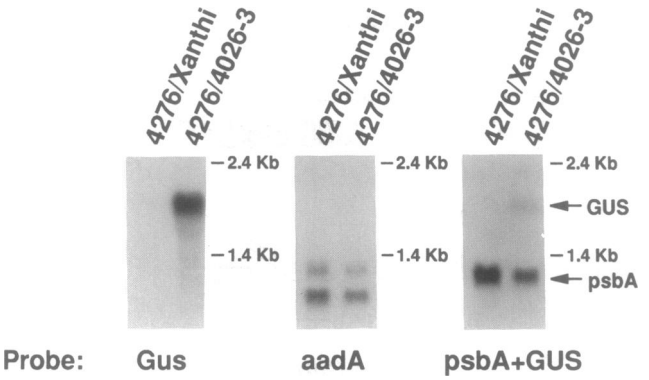

FIG. 2. The presence and relative levels of GUS, *aadA*, and *psbA* mRNAs in T7 RNAP$^-$ (Xanthi) and T7 RNAP$^+$ (4026-3) backgrounds. Duplicate samples of ≈5 μg of total cellular RNA from 4- to 8-cm leaves were electrophoresed in parallel through a 1.5% formaldehyde gel and the RNA was blotted to Nytran+. The filter was dissected between the duplicate sample runs and hybridized with [α-^{32}P]dCTP-labeled probes as indicated. The filter hybridized with the GUS probe was rehybridized (following autoradiography) directly with a *psbA* probe without stripping the GUS probe. All probes were labeled to approximately the same specific activity (5 × 10^8 to 1 × 10^9 cpm/μg of DNA).

could be translated in plastids, GUS specific activity was measured in various tissues (Table 2). These data show that GUS activity is present in all tissues tested, although its level of accumulation varied by >10,000-fold. To determine whether the differences in GUS activity correlated with variations of GUS mRNA levels, quantitative Northern blot and RNA dot blot assays were performed on total RNA. Northern blot analysis revealed that GUS mRNA of the expected size was present in each tissue (data not shown). The quantitative results indicated that there was a very high level of GUS mRNA in mature leaf, corresponding to the high GUS activity value. The next highest level of GUS mRNA was observed in petal tissue, which exhibited the highest level of GUS activity for a non-leaf tissue. Enzyme activity was lowest in roots and seeds, which accumulated the least amount of GUS mRNA. The extremely high level of GUS activity observed in mature leaves compared to young leaves and other tissues can be explained not only by a high level of GUS mRNA but also by the fact that mature leaf chloroplasts exhibit an enhanced translational capacity and ability to accumulate proteins relative to other plastid types (28).

Table 2. GUS activity and mRNA levels in transplastomic tobacco

Tissue*	Specific activity,† nmol/min per mg		Relative mRNA level in
	4276/4026-3	4276/Xanthi	4276/4026-3‡
Mature leaf	2.0 × 10^5	0	240
Young leaf	1167	0	7
Stem	13	0	7
Root	3	0	1
Petal	497	0	24
Seed	8	0	(1)

*Mature leaf tissue refers to fully expanded leaves (≈15 cm long); young leaf tissue refers to leaves <2 cm long. Root tissue consisted of the entire root system. Petal tissue consisted of the pink portion of the corolla after the flower had completely opened. Seeds were analyzed 12–15 days after pollination.

†4-Methylumbelliferone (nmol) produced per minute per milligram of total soluble protein. Values are the average of three replicate assays. The background activity for each tissue type (typically ≤0.05 nmol) was measured from untransformed Xanthi control plants and subtracted from the values obtained for the corresponding tissue samples of the above homoplasmic lines.

‡From RNA dot blots and Northern blots. Values are the average of radioactive counts from two dot blot assays and one Northern blot normalized to the value (designated as 1) obtained for seed RNA.

Activation of a Silent Plastid-Borne T7/GUS Reporter Gene via Sexual Transmission of the T7 RNAP Chimeric Gene. To demonstrate that the reporter gene behaves as a maternally inherited character, several crosses were conducted. Seeds derived from these crosses were germinated and the seedlings were scored for GUS activity by histochemical staining (15) (Fig. 3). A self-cross for 4026-3/4276 resulted in 129 positives and no negatives, as expected since the nuclear-encoded polymerase is homozygous. The wild-type nucleus/4276 plastid (female) × 4026-3 nucleus (homozygous)/wild-type plastid (male) cross resulted in 192 positives and no negatives, while for the reciprocal cross there were no positives in 119 seedlings. An outcross between homozygous 4026-3/4276 (female) and wild-type Xanthi male resulted in 135 out of 135 seedlings tested having a slightly less blue phenotype than the seedlings from the female parent, implying a T7 RNAP dosage effect in the heterozygotes. The reverse cross yielded no positives for the 143 seedlings examined. These data confirm that the reporter gene is maternally inherited in all crosses and that it can be successfully activated by sexual transmission of an active gene encoding plastid-targeted T7 RNAP.

DISCUSSION

We have demonstrated the manipulation of plastid transgene expression via the action of nuclear-encoded and plastid-targeted T7 RNAP. The plastid-borne GUS reporter gene, under control of the phage T7 gene *10* promoter and 5′ untranslated region, was expressed in all plastid types examined when incorporated into a tobacco line containing an active T7 RNAP gene. GUS activity was extremely high in fully expanded leaves—40-fold higher than that reported by others expressing the GUS gene in tobacco plastids from the strong *psbA* promoter (10). GUS is the major protein in this tissue, 20–30% of the total soluble protein as judged by Coomassie blue-stained SDS/polyacrylamide gels (data not shown). The levels of GUS activity in nonphotosynthetic tissues are much lower but comparable to levels obtained by nuclear transformation with a GUS transgene driven by the strong CaMV 35S promoter (15, 29).

The differences observed in GUS activity between tissues could not always be explained by changes in GUS mRNA levels. In mature leaf the level of GUS activity was disproportionately higher than could be accounted for by the increase in mRNA accumulation. This is likely to be caused by a large increase in translational activity and protein accumulation capacity associated with maturing chloroplasts (28). This would be in contrast to the presumed low translational activity of nonphotosynthetic plastids such as amyloplasts (30) and chromoplasts (31, 32). The large, 10- to 240-fold increase in GUS mRNA accumulation in mature leaf as compared to non-leaf tissues could be partially accounted for by the T7/GUS transgene, which has a *psbA* 3′ regulatory region known to result in a 6-fold increase in mRNA accumulation in photosynthetically active plastids. Also, the plastid DNA template level is 8- to 30-fold higher in mature leaves than in nonphotosynthetic tissues such as roots (30, 33). Petal tissue contains a high number of chromoplasts per cell (31) and thus a higher amount of GUS mRNA and protein is accumulated relative to the other nonphotosynthetic tissues. It is likely that expression of T7 plastid transgenes in plastids other than chloroplasts can be enhanced by modifying the 3′ untranslated segment of the RNA to appropriately change mRNA stability and/or by increasing the level of T7 RNAP in a tissue-specific manner. However, the level of

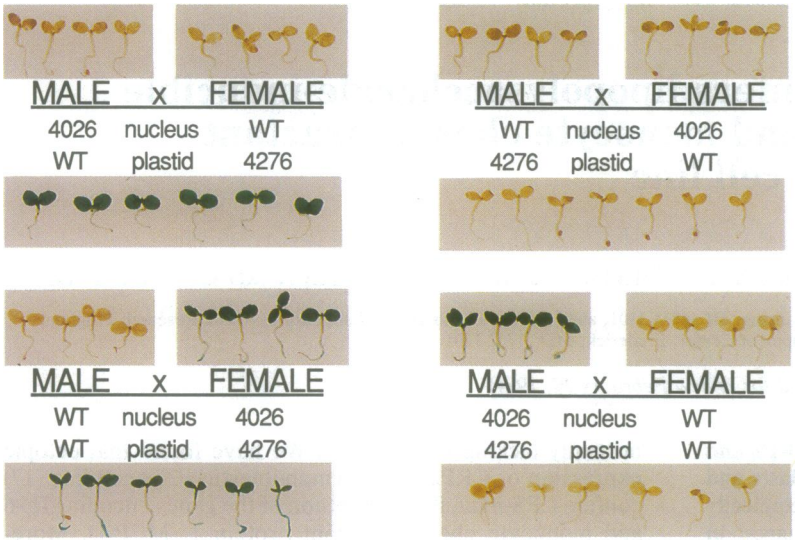

FIG. 3. Inheritance pattern of GUS marker and T7 RNAP genes as determined by sexual crosses. Seeds were germinated and seedlings were histochemically stained for GUS activity. (*Upper*) Reciprocal crosses between a plant homozygous for a T7 RNAP locus (4026) and a plant homoplasmic for a T7 promoter-driven GUS gene (4276). WT, wild type. (*Lower*) Reciprocal crosses between a wild-type plant and a plant homozygous for the T7 RNAP locus and homoplasmic for the T7 promoter-driven GUS gene. Representative seedlings of the parents of individual crosses are displayed above each label, while progeny seedlings of each cross are shown below each label.

expression will also be determined by the DNA template level and translational capacity of such plastids.

We have established that a plastid-encoded monocistronic mRNA can be synthesized by T7 RNAP and that this message is fully capable of being translated in plastids of different tissues within the developing plant. Plants can now be designed to express T7 RNAP in a tissue- or induction-specific manner limiting expression of the plastid transgene appropriately. This may allow modification of plastid metabolism in selected plastid/tissue types without altering normal plant growth and development. This may not be possible with light-regulated plastid-transgene expression in photosynthetic tissues (10). The next goal will be to introduce into plastids polycistronic operons encoding multiple traits and/or novel complex biochemical pathways. Operons with the potential for improving plant properties already exist in bacteria and could possibly be inserted into the plastid genome under the control of the T7 promoter without further modification.

The availability of other single-subunit RNAPs having a high degree of specificity for their corresponding promoters, such as phage T3 and SP6 RNAPs (34, 35), will eventually allow the expression of different sets of genes at different times and/or tissues in the plant.

Finally, genetic modifications introduced by this technology cannot be pollen transmitted, since plastids in most plants species are maternally inherited. This allows efficient integration of silent plastid-borne transgenes into hybrid breeding programs and subsequent protection of novel traits.

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