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Comparison of Strength of Endogenous and Exogenous Gene Promoters in *Arabidopsis* Chloroplasts

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Abstract

We have focused on possible stronger promoters in the chloroplast: those of *psbA* encoding D1 protein of photosystem II reaction center, 16S rDNA in *rrn* operon, the bacterial fused promoter *tac*, and the bacteriophage T7 gene *ϕ10* in combination with transgenic T7 RNA polymerase (RNAP). *Arabidopsis* plants were made transgenic in the nuclear genome with the construct of a chimeric gene for T7 RNAP fused to a chloroplast transit peptide at its N-terminus placed under the control of CaMV 35S promoter. We have transiently expressed gene for β-glucuronidase (GUS) under control of the above promoters in the *Arabidopsis* chloroplast followed by particle bombardment. Expression in the chloroplast but not in the nucleus was confirmed histochemically and by treatment with α-amanitin. T7 promoter was the strongest among the examined promoters in the *Arabidopsis* chloroplast, being applicable to higher expression of foreign genes in the chloroplast with managed expression of T7 RNAP.

Introduction

The chloroplast of higher plants is an attractive target for genetic engineering (Bogorad, 2000; Heifetz, 2000). The higher expression in an ideally managed manner of endogenous and foreign genes in the chloroplast is desired for enhancement of photosynthetic productivity and efficient production of modified substances therein. However, strength of gene promoters for potentially higher expression of recombinant genes in the chloroplast has not intensively been compared under the common experimental condition.

It is recognized that promoters of *psbA* encoding D1 protein of photosystem II reaction center and of 16S rDNA in *rrn* operon are stronger than the others in the plastid genome, as revealed by run-on assay for determining the activity of transcriptional initiation in isolated chloroplasts of spinach (Deng *et al.*, 1987), tomato (Kobayashi *et al.*, 1990), *Arabidopsis thaliana* (Isono *et al.*, 1997), and tobacco (Sakai *et al.*, 1998). The promoter of *psbA* has typical -35 and -10 sequences (Gruissem and Zurawski, 1985) recognized by bacterial-type multimeric RNAP (plastid-encoded plastid RNAP, PEP) and transcribed by PEP (Satoh *et al.*, 1999). The promoter of 16S rDNA has both sequences recognized by PEP and nuclear-encoded plastid RNAP (NEP), respectively (Iratni *et al.*, 1997). The bacterial fused promoter *tac* which is stronger in *Escherichia coli*, may also facilitate transcription in the plastid to a higher extent due to its conserved sequences similar to *psbA* promoter (Amann *et al.*, 1983). The promoter of the bacteriophage T7 gene *ϕ10* (T7 promoter) that is applied for the highest expression of recombinant genes in *E. coli* in combination with T7 RNA polymerase (RNAP) (Studier *et al.*, 1990), is another choice.

We have here employed *Arabidopsis* that is suited for genetical dissection of plants and the entire nucleotide sequences of its nuclear genome have just been determined (The Arabidopsis Genome Initiative, 2000). *Arabidopsis* is also a member of the family Brassicaceae (Cruciferae), to which many vegetables such as cabbage, radish, rape, mustard, *etc.* belong. Therefore, the knowledge of manipulation of plastid genome of *Arabidopsis* is valuable both in basic and applied aspects.

We had initiated work to apply this T7 system to

enhancement of gene expression in the chloroplast of *Arabidopsis*, meanwhile this strategy was applied to tobacco and published by McBride *et al.* (1994). The promoter strength was determined by transient expression of the plastid by particle bombardment developed by Morikawa and his colleagues (Seki *et al.*, 1995, Inada *et al.*, 1997).

Materials and Methods

Construction of a plasmid for delivering T7 RNAP into the chloroplast

Binary vector pGA35T7 containing T7 RNAP (Studier *et al.*, 1990) fused to a chloroplast transit peptide (TP of RBCS-1A) (Krebbers *et al.*, 1988) at N-terminus was constructed as follows. The 210-bp sequence for the TP was amplified with total *Arabidopsis* DNA by PCR using 5'-primer containing an additional sequence (italicized) to generate the *Xba*I site (underlined): 5'-*TTTCTAGA*-CCTCAGTCACACAAAG-3', and 3'-primer containing the endogenous *Sph*I site (underlined): 5'-*T*TGCATGCAGTTAACTCTTCC-3'. To obtain the T7 RNAP coding region, colony-PCR was preformed using *E. coli* BL21 (Studier *et al.*, 1990) with 5'-primer containing the putative initiation codon (lowercase) and *Sph*I site (underlined): 5'-T*TGCATGC*AGAGGCACTAAatgAA-3', and 3'-primer containing the complementary sequence of the termination codon (lowercase) and *Sac*I site (underlined): 5'-*TTCCCGGGAGCTC*ttaAACGCG-AAGTCC-3'. The PCR product for the TP or T7 RNAP was digested with *Xba*I and *Sph*I, or *Sph*I and *Sac*I, respectively. These DNA fragments were ligated with pBI221 (Clontech) digested with *Xba*I and *Sac*I at the same time to make a plasmid harboring the P*CaMV35S-TP-T7 RNAP* construct (P, promoter; CaMV, cauliflower mosaic virus; referred as the p35T7 construct). The p35T7 construct was cut out with *Hin*dIII and *Eco*RI and ligated with the binary vector pGA28 after being cleaved with *Hin*dIII and *Eco*RI, that is a derivative of pGA469 possessing gene for neomycin phosphotransferase II for selection of *E. coli* clones and transgenic plant lines (An *et al.*, 1985) with an insert of synthetic multi-cloning region at its *Eco*RI site (**Fig. 1**).

Nuclear transformation of Arabidopsis

Agrobacterium tumefaciens GV3101 strain transformed with the pGA35T7 was employed for the *in planta*-vacuum infiltration (Bechtold *et al.*, 1993) to introduce P*CaMV35S-TP-T7 RNAP* construct into *Arabidopsis* (Bensheim, Nossen, and Columbia, which regenerate readily). The transformants were selected on the MS medium (Murashige *et al.*, 1962) containing 50 μg ml^{-1} kanamycin at the stage of seeds of the vacuum-infiltrated plant lines, and the presence of transgenes was confirmed by PCR. Wild-type *Arabidopsis* and its transformants with the P*CaMV35S-TP-T7 RNAP* construct were grown on the MS agar medium (Murashige *et al.*, 1962) without sucrose at 22 ℃ for 3 weeks under continuous white fluorescent light at 75 μmol m^{-2} sec^{-1} (3,000 lux).

Northern hybridization

Total cellular RNA was isolated from leaves of wild-type *Arabidopsis* and its transformants with Isogen (NipponGene, Toyama, Japan) according to the manufacture's instructions. Total RNA was electrophoresed in 1.5%-agarose gel containing 60 mM formaldehyde. The RNA was transferred to nylon membranes (Hybond-N+, Amersham) with 20 X SSC (3 M NaCl / 0.3 M Na citrate). The prehybridization and hybridization was performed at 65 ℃ . Gene-specific DNA probes were labeled with [α-^{32}P]dCTP by using a *Bca*BEST Labeling Kit (Takara). Membranes were washed with 2 X SSC containing 0.1% SDS for 10 min at 65 ℃ , and with 1 X SSC and 0.1 X SSC both plus 0.1% SDS for 15 min each at 65 ℃ . The radioactive signals were detected by BAS-2000 Bio-Imaging Analyzer (Fujix, Tokyo).

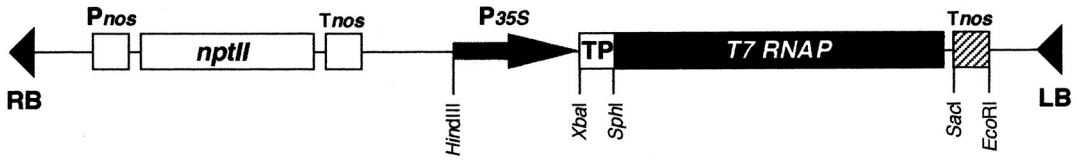

Fig. 1 Schematic representation of the structure of T-DNA region of the binary vector pGA35T7 harboring TP-T7 RNAP fusion gene.
The chimeric TP-T7 RNAP gene driven by CaMV 35S promoter was cloned into *Hin*dIII / *Eco*RI sites of binary vector pGA28 possessing neomycin phosphotransferase II gene. Details are described in the Materials and Methods. P*nos*, promoter of nopaline synthase gene; *nptII*, gene for neomycin phosphotransferase II; T*nos*, terminator of nopaline synthase gene; P*35S*, CaMV 35S promoter; TP, chloroplast transit peptide of RBCS-1A; *T7 RNAP*, gene for T7 RNAP.

Construction of plasmids for monitoring promoter strength by transient expression

The DNA fragment for *uidA* derived from pBI221 (Clontech) by digestion with *Bam*HI and *Sac*I was ligated with the spinach *rbcL* 3'-untranslated region of the hairpin structure, 5'-*Sac*I-TAAACT-CGGCCCAATCTTTTACTAAAAGGATTGAGC-CGAATACA-*Eco*RI-3', and inserted into *Bam*HI and *Eco*RI sites in pUC18 to produce pUGUS-T.

The plasmid pT7GFP to express GFP(S65T) (Heim *et al.*, 1995) under the control of the T7 promoter was constructed as follows. The DNA fragment for GFP(S65T) from the plasmid digested with *Nco*I and *Pst*I plus spinach *rbcL* 3'-untranslated region of the hairpin structure derived from pUGUS-T by digestion with *Pst*I and *Eco*RI, were inserted into *Nco*I and *Eco*RI sites of pET-3d (Novagen) containing T7 promoter .

Plasmid of pT7GUS containing T7 promoter and GUS gene (*uidA*) was constructed as follows. The DNA fragment for *uidA* and spinach *rbcL* 3'-untranslated region of the hairpin structure derived from pUGUS-T digested with *Sma*I and *Eco*RI was inserted into the blunted *Nhe*I site and the *Eco*RI site in pET-3d (Novagen) containing T7 promoter.

The vector, pGEM-5Zf(+) (Promega) was digested with *Spe*I, and ligated with 198-bp DNA fragment containing *psbA* promoter made of pTBa8 (Sugiura *et al.*, 1986) by *Spe*I digestion, resulting in generation of pGAP. The DNA fragment for *uidA* and spinach *rbcL* 3'-untranslated region of the hairpin structure derived from pUGUS-T digested with *Bam*HI and *Eco*RI, followed by blunting, was inserted into the blunted *Nco*I sites of pGAP, making pAGUS.

The 16S rDNA promoter was amplified with total *Arabidopsis* DNA by PCR using 5'-primer, 5'-AAGAGTGGCCTTGCGTTTCTC-3'; and 3'-primer, 5'-GGCGATTACTAGCGATTCCT-3' synthesized according to the corresponding maize sequences (Schwarz and Kössel, 1980). The PCR products were cleaved at endogenous *Sph*I site near their 3' end, generating the DNA fragment of one-A-protruding-5'-end produced with the DNA polymerase during PCR and *Sph*I-digested 3' end, and ligated with *Sph*I and *Sac*I-digested pGEM-5Zf(-) (Promega), producing pAP16S. The 16S rDNA promoter derived from pAP16S by digestion with *Nsi*I and *Acc*I to eliminate a transcribed region was blunted and ligated with the pTGUS removed the *tac* promoter by digestion with *Sac*I and *Sma*I, followed by blunting to generate p16SGUS.

The *tac* promoter was amplified with pMAL-p2 vector (New England Biolabs) by PCR using 5'-primer containing an additional sequence (italicized) to make the *Sac*I site (underlined), 5'-CTT<u>GAGCTCA</u>TGAAACCAGTAACGTTATACG-3'; and 3'-primer containing *Sma*I site (underlined), 5'-*AG*T<u>CCCGGG</u>TGAAATTGTTATCCGCTC-3'. To obtain the *uidA* coding region, colony-PCR was preformed using *E. coli* JM109 with the 5'-primer containing the initiation codon (lowercase) and *Sma*I site (underlined), 5'-*TACCA*TATG<u>CC-CGGG</u>AGGAGTCCCTTatgTTACG-3'; and 3'-primer containing *Sal*I site (underlined), 5'-*ATTGT*-<u>CGAC</u>AGGAGAGTTGTTGATTCATTG-3'. The PCR product for the *tac* promoter or *uidA* was digested with *Sac*I and *Sma*I, or *Sma*I and *Sal*I, respectively. The resultant DNA fragments were ligated with pUC19 digested with *Sac*I and *Sal*I to produce pTGUS.

Transient expression

Arabidopsis was grown in Petri dishes at 22 ℃ for 3 weeks under continuous white fluorescent light at 75 μmol m^{-2} sec^{-1}, and used for particle bombardment. About 10 leaves were placed onto moist filter paper prior to bombardment. Each sample was then bombarded with gold particles (1 μm in diameter) coated with each the *uidA* construct and pJD301 harboring P*CaMV35S-Luc*-T*nos* (P*CaMV35S-Luc*; *Luc*, for firefly luciferase; Kenneth *et al.*, 1992) to internally standardize shooting efficiency. Biolistic PDS-1000/He Particle Delivery System (Bio-Rad) was employed at 1,100 psi at 9-cm distance. After bombardment, samples were placed onto the medium and incubated at 21 ℃ for 24 hr under continuous light at 75 μmol m^{-2} sec^{-1}, and were then subjected to histochemical and fluorometric GUS assays. Histochemical and fluorometric GUS assays were performed basically by the procedure described by Inada *et al.* (1997).

Results

Transgenic Arabidopsis lines expressing chloroplast-targeted T7 RNAP

To establish bacteriophage T7 RNAP system in the plastid of *Arabidopsis*, the construct for T7 RNAP fused to a chloroplast transit peptide at its N-terminus (**Fig. 1**) was introduced into *Arabidopsis* by *A. tumefaciens*-mediated transformation. Seven kanamycin-resistant lines were obtained. The integration of gene for chimeric T7 RNAP and its expression in the transgenic lines was confirmed by genomic PCR and Northern hybridization, respectively. Approx. 300-bp PCR product for T7 RNAP gene was detected in all the transgenic lines (**Fig. 2**). Transcripts for T7 RNAP were detected in 3 transgenic lines, B3 (Bensheim), N3 (Nossen), and

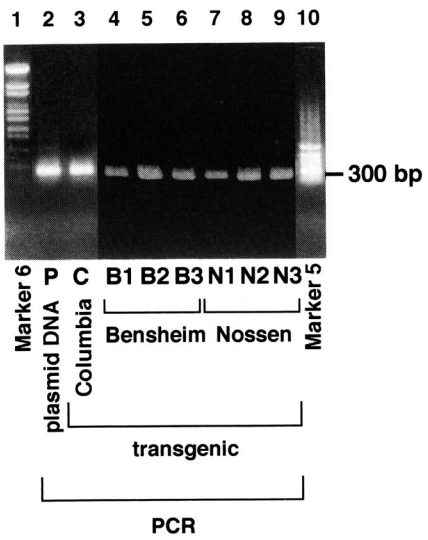

Fig. 2 Integration of exogenous gene for TP-T7 RNAP in the genomic DNA of transgenic lines of *Arabidopsis*.

Total cellular DNA was isolated from *Arabidopsis* leaves grown for 3 weeks with Isoplant (NipponGene) and subjected to PCR with *TakaraTaq* (Takara) following the suppliers' instructions. Primers for PCR of T7 RNAP were designed to amplify 300-bp DNA fragment on the basis of nucleotide sequence of coding region, 5'-TAACCCGGGATGCAGATGAAC-ACGATTAACATCGCTAAGAACG-3' and 5'-TCCGGCTTGATTTCTTGCAG-3'. PCR products were electrophoresed in 1%-agarose gel (Agarose HS, NipponGene), stained with ethidium bromide. The band in lane 2 was amplified with prepared DNA of the binary vector pGA35T7 as a control. DNA size markers (Marker 5 and Marker 6, NipponGene) were electrophoresed in lanes 1 and 10, respectively.

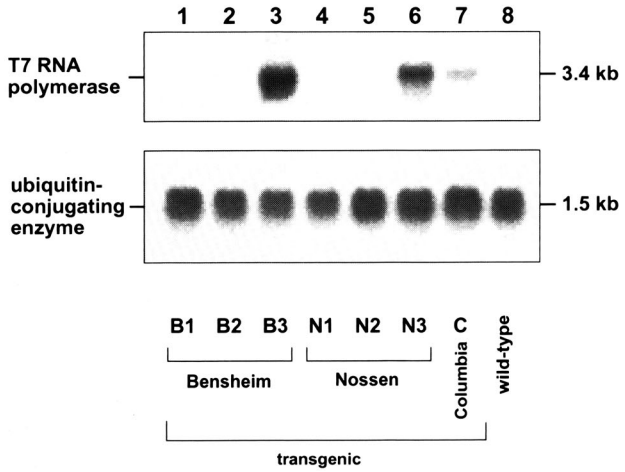

Fig. 3 Expression of T7 RNAP gene in the tansgenic lines.

Total cellular RNA (8 μg per lane) from leaves of wild-type *Arabidopsis* (lane 8) and transgenic lines (lanes 1 to 7) were subjected to Northern hybridization with a gene probe for T7 RNAP. The cDNA for ubiquitin-conjugating enzyme, which is constitutively expressed (M. Shimizu and H. Kobayashi, unpublished), was used as an internal control for equivalent amounts of RNA loaded into lanes.

C (Columbia) among 7 transformant lines (**Fig. 3**). Segregation of the selectable marker gene *nptII* indicated the integration of the transgene at multiple loci in the genome in some lines other than these (data not shown), suggesting that cosuppression of gene expression may occur in the lines. Among the lines homozygous for the transgene, *T7 RNAP*, it was most highly expressed in line B3, which was used for further experiments.

To examine whether T7 RNAP system can work, GFP(S65T) (Heim *et al.*, 1995) and GUS gene (*uidA*) were employed for monitoring the expression. Either GFP(S65T) or *uidA* construct under the control of T7 promoter (pT7GUS or pT7GFP, respectively) was transiently expressed in leaves of line B3 and the wild-type line after particle bombardment. Green fluorescence of GFP(S65T) was detected at the same intracellular compartment as that generating red chlorophyll fluorescence (**Fig. 4**, panels **A** to **C**), indicating that GFP(S65T) is located in the chloroplast. The leaves after bombardment with pT7GUS were stained with X-gluc, and only chloroplasts in cells of line B3 hit by particles were turned blue (**Fig. 4**, panel **D**), whereas those in wild-type leaves were not (data not shown). The GUS activity was further determined fluorometrically (**Fig. 5**). The GUS activity in leaves depended on the host plant line harboring T7 RNAP.

Evaluation of gene promoter strength

In order to evaluate strength of different promoters in chloroplasts of *Arabidopsis*, we made 3 new constructs in addtion to P*T7-uidA*, in which *uidA* was placed under the control of tobacco *psbA* promoter, *Arabidopsis* chloroplast 16S rDNA promoter, and bacterial *tac* promoter. To exclude the expression of plastid genes in the nucleus by particle bombardment as pointed out in case of *psbA* (Cornelissen and Vandewiele, 1989), two inhibitors were employed: α-amanitin, a specific inhibitor of nuclear RNAP II (Jendrisak, 1980); and cycloheximide, a inhibitor of protein synthesis on 60S subunit of cytoplasmic ribosomes to which the inhibitor binds (Lord *et al.*, 1991). When DNA is delivered into cells, DNA must to be transported into plastids on the basis of evidence: (i) the fact of DNA strand transferred into intact chloroplasts (Cerutti and Jagendorf, 1995), and (ii) success in plastid transformation with polyethylene glycol treatment (O'N-

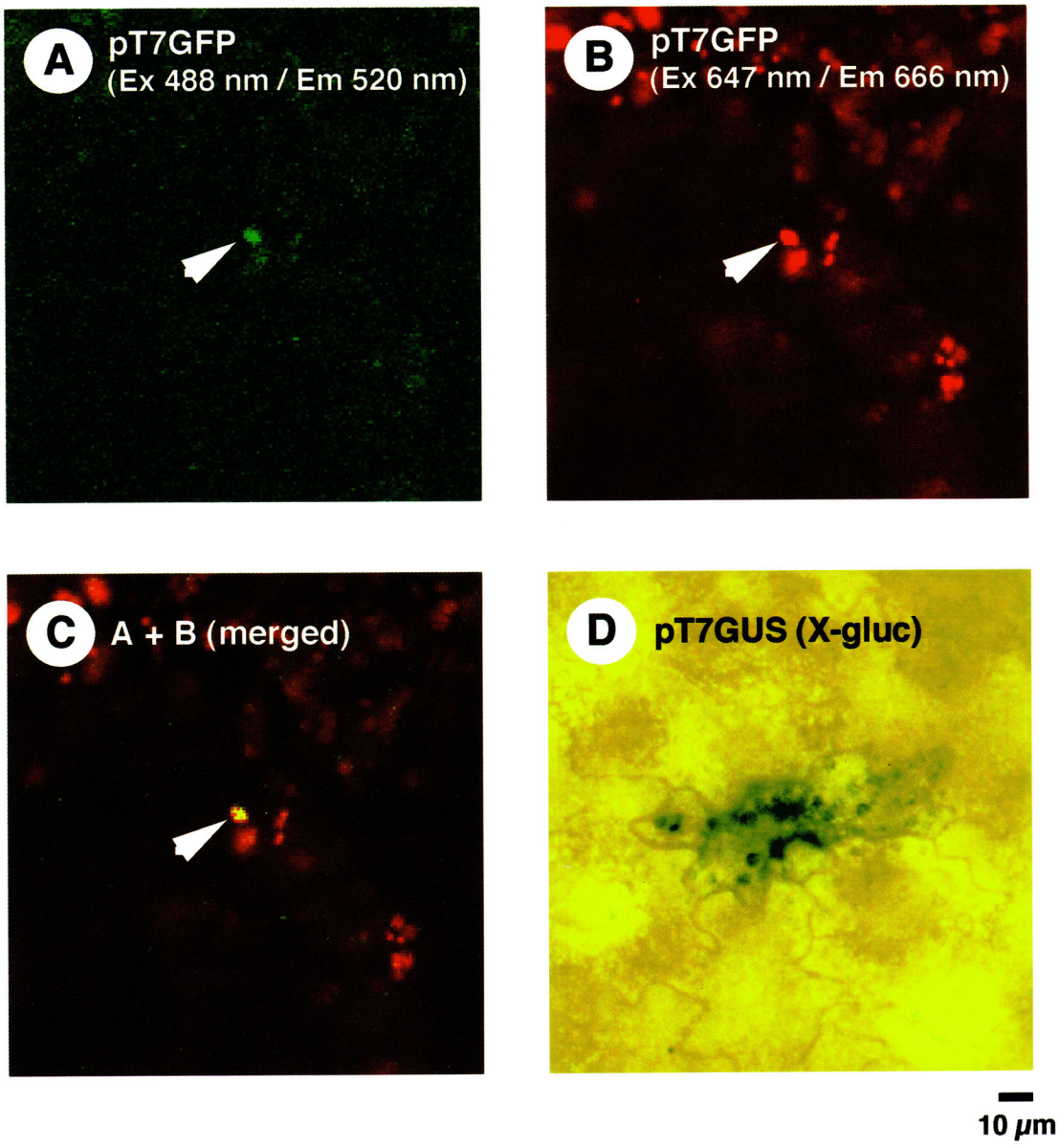

Fig. 4 Localization of GUS and GFP expressed under the control of the T 7 promoter.
Leaves of the transgenic line B3 harboring T7 RNAP were bombarded with either pT7GUS or pT7GFP, incubated for 24 hr at 21°C under continuous light, and then assayed. Leaves were observed using the MRC-1024 Confocal Imaging System (Bio-Rad) with excitation at 488 nm and emission at 520 nm for detection of GFP(S65T) (A), as well as excitation at 647 nm and emission at 666 nm for chlorophyll fluorescence(B). A merged image is represented in panel C. Localization of GUS activity was detected with X-gluc essentially as described by Inada *et al.*(1997) (D).

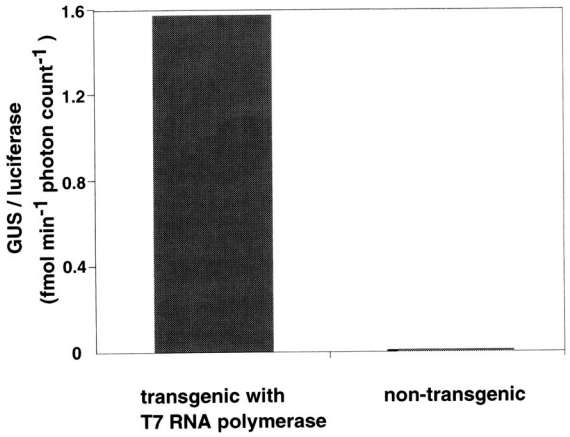

Fig. 5 The GUS activity transiently expressed under the control of T7 promoter in the transgenic line expressing T7 RNAP.

Leaves of transgenic and wild-type *Arabidopsis* were bombarded with pT7GUS mixed with the plasmid harboring P*CaMV35S-Luc*, incubated for 24 hr at 21 ℃ under continuous light, and subjected to GUS and luciferase assays. The ordinate axis represents "pmol min^{-1} μg $protein^{-1}$" for GUS per "photon count μg $protein^{-1}$" for luciferase.

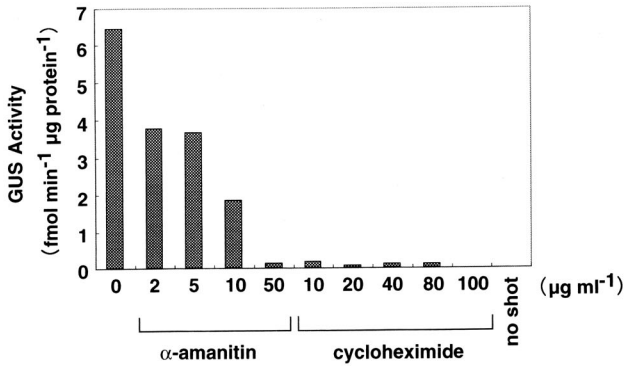

Fig. 6 Effects of α-amanitin and cycloheximide on GUS activity expressed under the control of CaMV 35S promoter in the nucleus.

Experimental details of the transient expression are described in the Materials and Methods. After particle bombardment with pBI221(Clontech) containing *uidA* under the control of CaMV 35S promoter, leaves were incubated in the presence of different concentrations of α-amanitin or cycloheximide for 24 hr at 21 ℃ under continuous light, followed by fluorometric determination of GUS activity.

eill *et al.*, 1993). The plasmid harboring P*CaMV35S-Luc* (Kenneth *et al.*, 1992) that was expressed in the nucleus was employed as an internal standard for gene delivery into cells. The GUS activity in the *Arabidopsis* leaves bombarded with P*CaMV35S-uidA* was strongly inhibited even with 10 μg ml^{-1} of cycloheximide (**Fig. 6**). However, we considered that cycloheximide was not suitable for inhibition of the expression in the nucleus, since leaves on the medium containing cycloheximide were bleached. By contrast, 50 μg ml^{-1} of α-amanitin inhibited the expression of *uidA* under the control of CaMV 35S promoter, but the leaves kept green. Therefore, 50 μg ml^{-1} of α-amanitin was used for further experiments to monitor *uidA* expression in chloroplasts of *Arabidopsis*.

Leaves bombarded with pT7GUS (T7 promoter), pAGUS (*psbA* promoter), p16SGUS (16S rDNA promoter), or pTGUS (*tac* promoter) mixed with P*CaMV35S-Luc* were divided into halves and placed on medium with or without α-amanitin. Both activities of GUS and luciferase were then determined. Leaves incubated on the medium containing α-amanitin accounted for expression in chloroplasts with detection of GUS activity, and for inhibition of expression in nuclei with luciferase activity. On the other hand, leaves incubated on the medium without α-amanitin were used as an internal standard for gene delivery into cells with luciferase activity. Each GUS activity was standardized with efficiency of gene delivery into cells as represented by luciferase activity (**Fig. 7**). Since 50 μg ml^{-1} of α-amanitin efficiently inhibited nuclear gene expression (**Fig. 6**), the results shown in **Fig. 7** indicate that *uidA* expression after bombardment with pT7GUS, pAGUS, p16SGUS, or pTGUS may ascribed to expression in plastids and reflect the promoter strength therein. The T7 promoter had the obviously most intense activity among promoters for *psbA*, 16S rDNA, and *tac* in chloroplasts of *Arabidopsis*.

Discussion

It has been shown that, *tac*, a fused promoter of prokaryotic origin (Amann *et al.*, 1983) can function more efficiently than the plastid promoter of *psbA* (**Fig. 7**). This result proves that the nature of transcriptional machinery is basically common to that in prokaryotes, and PEP can efficiently recognize promoters consisting of typical -35 and -10 sequences. Among the examined promoters, T7 promoter was stronger in *Arabidopsis* chloroplasts engineered to possess T7 RNAP than the promoters of endogenous plastid genes (**Fig. 7**). In *E. coli*, the chloroplast *psbA* promoter has been reported to be more efficient than the T7 promoter with T7 RNAP (Brixey *et al.*,1997). This result could be explained by interference with cell growth in supplementation with isopropylthio-β-D-galactoside (IPTG) to induce the gene expression, leading to the death of cells harboring the plasmid. By contrast, the system with T7 RNAP can work well in *Arabidopsis*

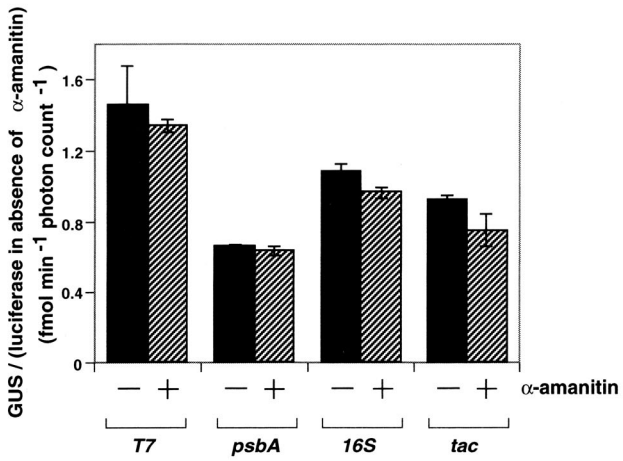

Fig. 7 Strength of gene promoters in transient expression of *Arabidopsis* chloroplasts.

Leaves transgenic with pGA35T7 were bombarded with four *uidA* expression constructs with different promoters mixed with the plasmid harboring P*CaMV35S-Luc*. The leaves were divided into halves and placed on the medium with or without 50 μg ml^{-1} of α-amanitin, incubated for 24 hr at 21 ℃ under continuous light, and then assayed for GUS and luciferase activities. The ordinate axis represents "pmol min^{-1} μg $protein^{-1}$" for GUS per "photon count μg $protein^{-1}$" for luciferase.

plastids due to unnecessity of supplementation with IPTG in the present study.

It has recently been reported that structures around initiation codon of mRNA, the ribosome-binding site in the leader of bacteriophage T7 gene ϕ*10* (Staub *et al.*, 2000) and the 5'-translational control region of *rbcL* (Kuroda and Maliga, 2001), are crucial for the higher activity of translation of foreign proteins in tobacco chloroplasts. Instead of engineering of translational regulation, choice of promoters for expressing recombinant genes is rather critical for turning on or off gene expression. Expression of *psbA* is controlled in light- and organ-dependent manners, although that of 16S rDNA was less specifically to kinds of organs (Isono *et al.*, 1997). Since the transcription from *tac* promoter is interrupted by *lac* repressor encoded by *lacI* (Amann *et al.*, 1983), it may become possible to switch on and off the gene expression when introducing *lacI* gene into the plants in combination with supply of IPTG. When we regulate expression of T7 RNAP to be transported into the plastid in employment of promoters either specific to organs or growth stages, or activated by environmental signals such as light, temperature, drought, salt, and so on, the expression driven by T7 promoter in chloroplasts may be critically controlled.

We judge that the T7 system is useful for stable transformation of the plastid genome of *Arabidopsis*. Plastid transformation in higher plants has been so far reported for tobacco (Svab and Maliga, 1993), potato (Sidorov *et al.*, 1999), and rice (Khan *et al.*, 1999). Although plastid transformation of the model plant *Arabidopsis* has been reported (Sikdar *et al.*, 1998), the frequency of plastid transformation of *Arabidopsis* was much lower than that of tobacco and regenerated *Arabidopsis* were infertile. Why is it difficult to stably introduce genes into *Arabidopsis* plastids? Some reasons are considered: (i) low efficiency of plant regeneration, (ii) low contents of chloroplasts, (iii) small volumes of chloroplasts, and (iv) unrecoverable damage with spectinomycin treatment. Plastid transformation of higher plants is accomplished through selection of transformants at a heteroplasmic stage to the homoplasmic population of cells using a selectable maker, gene for aminoglycoside 3"-adenylyl transferase (*aadA*) that conferred resistance to spectinomycin and streptomycin (Prentki *et al.*, 1991). *Arabidopsis* and *Brassica napus* suffer unrecoverable damage by spectinomycin resulting in maintenance of chlorophyll-deficient phenotype after eliminating the antibiotic, whereas the damage of tobacco is recoverable (Zubko and Day, 1998). Therefore, enhancement of expression of *aadA* is one of strategies for stable transformation of plastids of *Arabidopsis* and others plant species.

The present investigation may promise that the employment of *aadA* as a selectable marker under the control of the T7 promoter must increase the efficiency of transformation of *Arabidopsis* plastids. Stable transformation of the plastids genome of *Arabidopsis* may contribute to elucidation of interaction of genes encoded in plastid and nuclear genomes for photosynthesis, because *Arabidopsis* has advantages in investigations in the aspect of molecular genetics. This strategy is also applicable to other vegetable species in the family Brassicaceae.

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