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

Kohki YOSHIMOTO, Mao SAKAIYA, Kyoichi ISONO and Hirokazu KOBAYASHI*

Laboratory of Plant Cell Technology, Graduate School of Nutritional and Environmental Sciences, University of Shizuoka, 52-1 Yada, Shizuoka 422-8526, Japan

1 Present address: Department of Molecular Embryology, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chu-o-ku, Chiba 260-8670, Japan

*Corresponding author E-mail address: hirokazu@ms.mail.shizuoka-ken.ac.jp

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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., 1985). 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 genetic 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 plastid 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 plastid**

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'-TTGAGCTAGTACTAAGAAAG-3', and 3'-primer containing the endogenous *Sph*I site (underlined): 5'-AGGTCATGCGATTACTCTTCTCC-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'-TTGAGCTAGTACTAAGAAAG-3', and 3'-primer containing the complementary sequence of the termination codon (lowercase) and *Sac*I site (underlined): 5'-AGGTCATGCGATTACTCTTCTCC-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 PCaMV35S-TP-T7 RNAP construct (P, promoter; CaMV, cauliflower mosaic virus; referred as the p35T7 construct). The p35T7 construct was cut out with *Hind*III and *Eco*RI and ligated with the binary vector pGA28 after being cleaved with *Hind*III 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 PCaMV35S-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 PCaMV35S-TP-T7 RNAP construct were grown on the MS agar medium (Murashige *et al.*, 1962) without sucrose at 22 °C 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 °C. Gene-specific DNA probes were labeled with [α-³²P]dCTP by using a BcaBEST Labeling Kit (Takara). Membranes were washed with 2 X SSC containing 0.1% SDS for 10 min at 65 °C, and with 1 X SSC and 0.1 X SSC both plus 0.1% SDS for 15 min each at 65 °C. The radioactive signals were detected by BAS-2000 Bio-Image 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.](image)

The chimeric TP-T7 RNAP gene driven by CaMV 35S promoter was cloned into HindIII / EcoRI sites of binary vector pOA28 possessing neomycin phosphotransferase II gene. Details are described in the Materials and Methods. Pnos, promoter of nopaline synthase gene; nptII, gene for neomycin phosphotransferase II; Tnos, terminator of nopaline synthase gene; P35S, 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 BamHI and SacI was ligated with the spinach rbcL 3' untranslated region of the hairpin structure, 5'-SacI-TAAACT-CCGCCCAACTTTTACTAAGAGTTGAATCGCAAATAAC-EcoRI-3', and inserted into BamHI and EcoRI 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 NcoI and PstI plus spinach rbcL 3' untranslated region of the hairpin structure derived from pUGUS-T by digestion with PstI and EcoRI, were inserted into NcoI and EcoRI 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 Smal and EcoRI was inserted into the blunt NheI site and the EcoRI site in pET-3d (Novagen) containing T7 promoter.

The vector, pGEM-5Zf(-) (Promega) was digested with SpeI, and ligated with 198-bp DNA fragment containing pshA promoter made of pTBA8 (Sugiura et al., 1986) by SpeI 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 BamHI and EcoRI, followed by blunting, was inserted into the blunt NcoI sites of pGAP, making pAGUS.

The 16S rDNA promoter was amplified with total Arabidopsis DNA by PCR using 5' primer, 5'-AACAGTGGCCCTTTGGCTTTTC-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 Sphi I site near their 3' end, generating the DNA fragment of one-A-protruding-5' end produced with the DNA polymerase during PCR and Sphi I digested 3' end, and ligated with Sphi I and SacI digested pGEM-5Zf(-) (Promega), producing pAP16S. The 16S rDNA promoter derived from pAP16S by digestion with NsiI and AccI to eliminate a transcribed region was blunt and ligated with the pT5GUS removed the tac promoter by digestion with SacI and Smal, followed by blunting to generate p16S5GUS.

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 SacI site (underlined), 5' CTTGAGCCATGAAACCCGAACGGTTATACG-3'; and 3' primer containing Smal site (underlined), 5' AGTCCGGGTGAAATTTGATACGCC-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 Smal site (underlined), 5' TACCATGCGCGGAGAGTCCCTTATACG-3'; and 3' primer containing SacI site (underlined), 5' ATGGTGCGACAGAGTGGCCTTGCGTTTCTC-3'. The PCR product for the tac promoter or uidA was digested with SacI and Smal, or Smal and SacI, respectively. The resultant DNA fragments were ligated with pUC19 digested with SacI and SacI to produce pT5GUS.

Transient expression

Arabidopsis was grown in Petri dishes at 22 °C 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 PCaMV35S-Luc-Tnos (PCaMV35S-Luc; Luc, for firefly luciferase; Kenneth et al., 1992) to internally standardize shooting efficiency. Biologic 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 °C for 24 hr under continuous light at 75 μmol m⁻² sec⁻¹, and were then subjected to histochemical and fluoromeric 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-base 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 (Nossem), 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 Isoplat (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'–TAACCCGGATGCAGATGAACACGATACCCGAAGACG–3' and 5'–TCCGGCTTGATTCTTGCAG–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.

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 addition to pT7-*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: a-amanitin, a specific inhibitor of nuclear RNAP II (Jendrisak, 1980); and cycloheximide, an 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 be transported into plastids on the basis of evidence: (i) the fact of DNA strand transfer 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 T7 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 PCaMV35S-Luc, incubated for 24 hr at 21 °C 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.

cell et al., 1993). The plasmid harboring PCaMV35S-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 PCaMV35S-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 PCaMV35S-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 be 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*.
plastids due to unecessity 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 \( \phi 10 \) (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 (Isoco et al., 1997). Since the transcription from \( tac \) promoter is interrupted by \( lac \) repressor encoded by \( lacI \) (Aman 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 (Sidedar 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'-adenyllyl 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 plastid 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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