PIBB 生物化学与生物物理进展
Progress in Biochemistry and Biophysics
2011, 38(3): 269~279
www.pibb.ac.cn

Construction and Application of a Gateway Entry Vector With Rubisco Small Subunit Promoter and Its Transit Peptide Sequence*

MA Li[1], SONG Zhong-Bang[1], HU Qing-Quan[2], ZHAO Yue[1], NIAN Hong-Juan[1], YU Yong-Xiong[2], LI Kun-Zhi[1], CHEN Li-Mei[1]**

([1] *Biotechnology Research Center, Kunming University of Science and Technology, Kunming* 650224, China;
[2] *College of Zoological Science and Technology, Southwest University, Chongqing* 400716, China)

Abstract Gateway technology has been demonstrated to be easy and successful in construction of expression vectors for genes of interest. However, the present Gateway plant destination vectors do not contain any sequence for targeting expressed proteins of interest to chloroplasts. The *Xmn* Ⅰ restriction site was converted into a *Hin*dⅢ site in the Gateway entry vector pENTR-2B to generate pENTR*-2B and a Gateway entry vetcor designated as pENTR*-PrbcS-*T-GFP was constructed by subcloning the tomato Rubisco small subunit 3C promoter (PrbcS) and its transit peptide sequence (*T) as well as a GFP (green fluorescent protein) reporter gene into pENTR*-2B. These results demonstrated that the pENTR*-PrbcS-*T-GFP could be used to generate the plant expression vector *via* Gateway technology for targeting the expressed GFP into the chloroplasts of transgenic plant leaves. Similar results were also obtained for GUS (β-glucuronidase) reporter gene when it was used to replace the GFP gene in pENTR*-PrbcS-*T-GFP. These results indicated that pENTR*-PrbcS-*T-GFP can be generally applied to generate an entry vector for a target gene by replacement of the GFP with the target gene and the plant expression vector that serves to localize the expressed target protein in chloroplasts can be achieved rapidly *via* Gateway technology.

Key words Gateway technology, PrbcS promoter, entry vector, plant expression vector, chloroplast localization
DOI: 10.3724/SP.J.1206.2010.00362

In plant cells, chloroplasts are vital organelles in which many metabolic pathways take place [1–2]. To modify or alter the metabolic pathways in chloroplasts by genetic engineering, the target genes have to be expressed in leaf cells and the expressed proteins have to be targeted to chloroplasts. One way to target the protein of interest to chloroplasts is to attach a chloroplast transit peptide sequence upstream of the gene of interest. This allows synthesizing a precursor protein with the transit peptide, which will direct the protein to chloroplasts [3]. Alternatively, the gene of interest can be introduced into plastid genomes by a plastid transformation method and the protein of interest is synthesized by the plastid ribosomes [4-6]. However, current plastid transformation technology is only successful in a few species [7]. Thus, it is difficult to apply the technology extensively to localize target proteins into chloroplasts.

Ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) is a highly expressed protein in plant leaves, and comprises 40%～50% of leaf soluble proteins [8]. The promoters of Rubisco small subunit (rbcS) genes have been shown to be much more effective than CaMV 35S or other promoters to control target protein expression in plant leaves [9–11]. In addition, the transit peptide of rbcS has been proved to be able to target the

*This work was supported by grants from National Basic Research Program of China (2007CB108901), The National Natural Science Foundation of China (30670163) and The foundation of Yunnan Province and Kunming University of Science and Technology for Training Adult and Young Leaders of Science and Technology (2004PY01-5).

**Corresponding author.
Tel: 86-871-3801018-202, E-mail: chenlimeikm@yahoo.com.cn
Received: July 9, 2010 Accepted: November 16, 2010

rbcS into the stroma of chloroplasts[12]. Therefore, rbcS promoter and its transit peptide sequence are often used in genetic engineering to accomplish high expression of target genes in leaves and localize the expressed target proteins into chloroplasts[3, 13-17].

Gateway technology, developed based on a site-specific recombination mechanism between λ phage and *Escherichia coli* genomes [18], has been used generally and demonstrated to be a highly efficient gene-cloning tool[19-20]. Based on Gateway technology, a series of plant destination vectors for over-expression of a single gene or two to three genes simultaneously in transgenic plants have been developed [21-22]. Plant expression vectors for genes of interest can be constructed rapidly by using these destination vectors *via* Gateway LR reaction without the need for DNA digestion and ligation. However, chloroplast transit peptide sequences are lacking in these destination vectors. This limits the application of Gateway technology in plant chloroplast genetic engineering.

Recently, a unique set of plant destination vectors carrying multi-site Gateway cassette are developed, which provides users with easy to exchange promoter in a gene [23]. However, the use of a transit peptide sequence to localize the expressed target protein to chloroplasts requires the start codon (ATG) sequence of the cloned target gene to be in frame with the transit peptide sequence. Therefore, multi-site Gateway technology is neither suitable to construct such an expression vector which serves to localize the expressed target protein in chloroplasts.

In the present study, we developed a Gateway entry vector, pENTR*-PrbcS-*T-GFP, which contains tomato rbcS-3C promoter (PrbcS) and its transit peptide sequence (*T) as well as a reporter gene (GFP). GUS (β-glucuronidase) reporter gene was used to replaced the GFP gene in pENTR*-PrbcS-*T-GFP to generate a similar entry vector, pENTR*-PrbcS-*T-GUS. Using the two entry vectors, the plant expression vectors of GFP and GUS were constructed rapidly through Gateway LR reaction. Tobacco and geranium were transformed with the plant expression vectors of GFP and GUS to generate transgenic plants. The subcellular localization of GFP and expression pattern of GUS in transgenic plant leaves were investigated to evaluate the application potentiality of the pENTR*-PrbcS-*T-GFP entry vector.

1 Materials and methods

1.1 Bacterial strains and plasmids

E. coli DH5α (Invitrogen) was used for DNA construction and plasmid amplification. The pUC118-PrbcS-T-rbcS-3C vector containing the tomato PrbcS promoter and the transit peptide sequence in a 1.7 kb *Hin*dⅢ restriction fragment[12] was digested with *Sph* Ⅰ to remove rbcS-3C and self-ligated to generate pUC118-PrbcS-T. pGFP and Gateway entry vectors, pENTR-2B and pENTR-GUS, were purchased from Invitrogen. The Gateway plant destination vector pK2GW7 was purchased from VIB/Gent (Belgium). pPZP211 [24] was used to construct light inducible plant expression vectors for GFP and GUS. *Agrobacterium* strain C58C1 (pMP90) was used for plant transformation.

1.2 Site-directed mutagenesis

Mutagenesis was carried out in pENTR-2B and pUC118-PrbcS-T with a quickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. The mutagenic sense primer *Nco* Ⅰ5 (ATTCTATAATACCGCC*ATGGC-TTCTTCAGTA) and the antisense mutagenic primer *Nco* Ⅰ3 (TACTGAAGAAGCCATG*GCGGTATTA-TAGAAT) were used to introduce the *Nco* Ⅰ site into pUC118-PrbcS-T. Similarly, the mutagenic sense primer *Hin*d Ⅲ5 (AAAGCAGGCTGGCGCCGGA*A*GC*TTTTCAGTCGACTGGATCCG) and the mutagenic antisense primer *Hin*d Ⅲ3 (CGGATCC-AGTCGACTGAAAAG*CT*T*CCGGCGCCAGCC-TGCTTT) were used to convert the *Xmn* Ⅰ site to a *Hin*dⅢ site in pENTR-2B. Asterisks on the upper right corner of the residues mark the mutation sites. Following mutation, plasmids were digested with restriction enzymes and subsequently confirmed by sequence analysis.

1.3 TA cloning and TOPO cloning

The GFP gene was amplified from pGFP *via* PCR using a sense primer GFP5 (caccgcATGcGTAAAGG-AGAAGAACTTTTC) containing a *Sph* Ⅰ site and an antisense primer GFP3 (ggatccCTATTTGTATAGT-TCATCCATGCC) with a *Bam*H Ⅰ site. Similarly, the GUS gene was amplified from pENTR-GUS using a sense primer GUS5 (gcATGcTCCGTCCTGTAGAA-ACCC) containing a *Sph* Ⅰ site and an antisense primer GUS3 (ggatccTTATTGTTTGCCTCCCTG)

with a *Bam*H Ⅰ site. The PCR products were subcloned into a TA cloning vector, pUCm-T (Biocolor production, China), to generate pUCm-T-GFP and pUCm-T-GUS, respectively. The blunt end of GFP PCR product was obtained using high fidelity DNA polymerase, KOD (TOYOBO), and subcloned into the pENTR/SD/D-TOPO cloning vector (Invitrogen) to generate a Gateway entry plasmid pENTR-GFP.

1.4 Gateway LR reaction

pENTR-GFP and pENTR-GUS entry clones, and destination vector pK2GW7 were purified using a plasmid purification kit. The Gateway LR Clonase Ⅱ Enzyme Mix kit (Invitrogen) was used to perform the LR reaction according to the manufacturer's instructions. The reaction mixture was incubated overnight at 25℃ and transformed into *E. coli* DH5α competent cells. The recombination clones were selected on LB-medium plates with 50 mg/L spectinomycin (Spe).

1.5 Plant transformations

The plant expression vectors were transferred into *Agrobacterium tumefaciens* C58C1 (pMP90) by electroporation using Gene Pulser (BioRad) with parameters of 200 Ω and 2.5 kV/0.2 cm. *A. tumefaciens* transformants were selected on LB-agar plates containing 100 mg/L Spe. Tobacco (*Nicotiana tabacum* cv. Xanthi) was transformed following the leaf disk co-cultivation protocol of Horsch *et al* [25]. Transformation of geranium (*Pelargonium* sp. frensham) was performed following the leaf petiole co-cultivation protocol of Krishnaraj *et al*[26].

1.6 Growth and maintenance of transgenic plants

For aseptic growth of tobacco and geranium, the resistant shoots were first selected on MS medium with Kanamycin (Kan, 50 mg/L) and then transferred to MS medium (pH 5.7) containing sucrose (3%) and grown in a growth chamber at 25℃ under constant light. For the growth on soil, the plantlets with roots were first selected on Kan-containing medium and then transplanted to pots with 1/2 perlite plus 1/2 organic soil.

1.7 Genomic PCR and RT-PCR analysis

Genomic DNA was prepared from plant leaves as described by Murray and Thompson[27]. To detect GFP and GUS gene integrations in transgenic plants, 100 ng genomic DNA was used as the templates for genomic PCR (30 cycles) analysis with GFP5/GFP3 primers and GUS5/GUS3 primers, respectively.

Total RNA was isolated from plant leaves with TRIzol Reagent (Invitrogen) and used to synthesize cDNA using the RevertAid™ M-MuLV Reverse Transcriptase Kit (Fermentas). RT-PCR (30 cycles) analysis was performed with Ex-taq DNA polymerase (TaKaRa) using cDNA as the template.

For detection of 0.7 kb GFP transcript, GFP5 and GFP3 primers were employed. For detection of GFP constitutive transcript, the forward primer attB1 (ACAAGTTTGTACAAAAAAGCA) or rbcS5 (GACAAAAGAGTACATCAACCCC) and the reverse primer GFP3 were used. GUS5 and GUS3 primers were used for GUS transcript detection. ACT5 primer (ATGGCGGATGGGGAGGACATTC) and ACT3 primer (ATATGCCAGTTTCTCTTTAAC) were used to amplify actin mRNA (AB158612) as an internal reference in tobacco. TUB5 primer (TCCTCCCGGGATCCCAGCATAC) and TUB3 primer (GCCCTTAATTCCATCTCGTC) were used to amplify tubulin mRNA (EF202092) as an internal reference in geranium.

1.8 GFP visualization

Tobacco leaf discs and geranium leaf petiole segments with calli were placed on a slide and observed with a stereo-microscope under white light. Following white light observations, GFP fluorescence was visualized under long-wave ultraviolet (UV) light produced from a hand held UV lamp. Tobacco leaf discs and geranium leaf petiole segments were photographed using a digital camera under white light and UV light.

The green fluorescence of GFP and the red fluorescence of chlorophyll were monitored using a confocal laser-scanning microscope (FV1000S, Olympus, Japan) with Kr/Ar laser excitation. GFP and chlorophyll fluorescence were induced at an excitation wavelength of 488 nm, and detected at emission wavelengths of 500～515 nm and 630～680 nm, respectively.

1.9 GUS histochemical staining assays

Histochemical staining for GUS was performed as described by Zhao *et al*[28]. Mature leaves from tobacco plants grown aseptically on MS medium were dipped in GUS staining solution (80 mmol/L sodium phosphate buffer (pH 7.0), 0.4 mmol/L potassium ferricyanide, 0.4 mmol/L potassium ferrocyanide, 8 mmol/L EDTA, 0.05% Triton X-100, 0.8 g/L 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc)) with a brief vacuum infiltration for 30 min, and then incubated at 37℃ overnight. GUS staining solution was replaced with

70%～100% ethanol and incubated at 37℃ for 3 h to remove leaf chlorophyll.

2 Results

2.1 Construction of entry vectors

A *Hin*d Ⅲ-digested 1.7 kb fragment (PrbcS-T) containing tomato rbcS-3C promoter (PrbcS) and its transit peptide sequence (T) was used to construct pENTR*-PrbcS-*T-GFP entry vector (Figure 1a). To construct a plant expression vector for a target gene whose expression was driven by the PrbcS promoter and the expressed proteins were retained in the cytoplasm, an *Nco* Ⅰ site was introduced into the start codon region of the transit peptide by site-directed mutagenesis in pUC118-PrbcS-T. The resulting plasmid was referred as pUC118-PrbcS-*T. The Gateway entry vector pENTR-2B was used as the backbone for construction of pENTR*-PrbcS-*T-GFP. To ligate the promoter-transit peptide fragment, PrbcS-*T, from pUC118-PrbcS-*T into pENTR-2B, we converted the *Xmn* Ⅰ site to a *Hin*d Ⅲ site in the pENTR-2B by site-directed mutagenesis. The resulting plasmid was assigned as pENTR*-2B. The PrbcS-*T fragment was subsequently excised from pUC118-PrbcS-*T and ligated into pENTR*-2B between *Hin*d Ⅲ and *Eco*R Ⅰ sites to produce an intermediate vector designated as pENTR*-PrbcS-*T.

To construct a plant expression vector for a target gene with pENTR*-PrbcS-*T *via* Gateway LR reaction and investigate the function of PrbcS-*T in this entry vector, a GFP reporter gene was inserted downstream of PrbcS-*T in pENTR*-PrbcS-*T. The GFP fragment was excised from pUCm-T-GFP and ligated between the *Sph* Ⅰ and *Bam*H Ⅰ sites to yield the pENTR*-PrbcS-*T-GFP. In this entry vector, the *Sph* Ⅰ site was still remained in the ATG start codon region of the GFP gene while its 3′ end mulitcloning site contained eight restriction sites (Figure 1a). As such, for the entry vector construction of a target gene, it is only necessary to add the *Sph* Ⅰ restriction site to the 5′-end of the target gene and one of the eight restriction enzymes to its 3′-end *via* PCR amplification and then replace GFP with the target gene by restriction/ligation. Because the size of this entry vector is small, subcloning a target gene into pENTR*-PrbcS-*T-GFP to replace GFP is easy by restriction/ligation strategy.

To determine if the pENTR*-PrbcS-*T-GFP could be generally applied to construct entry vectors for other target genes, we replaced GFP in the entry vector with GUS to generate a similar entry clone. The GUS DNA fragment was excised from pUCm-T-GUS and ligated into pENTR*-PrbcS-*T-GFP between *Sph* Ⅰ and *Bam*H Ⅰ sites to yield pENTR*-PrbcS-*T-GUS entry clone (Figure 1b).

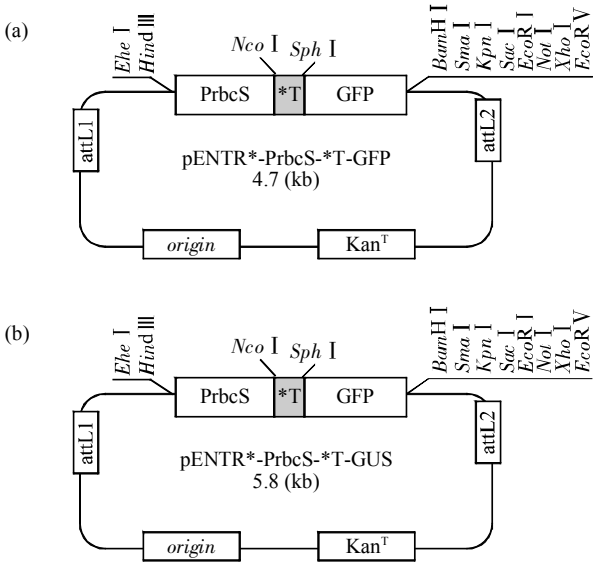

Fig. 1 Schematic diagrams of the GFP entry vector (a) and the GUS entry vector (b)

In pENTR*-PrbcS-*T-GFP and pENTR*-PrbcS-*T-GUS, GFP and GUS were fused directly to the modified transit peptide sequence at the ATG initiation codon; attL1 and attL2, two specific attachment sites for Gateway LR reaction; Kan, kanamycin resistance gene; PrbcS, tomato rbcS-3C promoter; *T, tomato rbcS-3C transit peptide sequence with the *Nco* Ⅰ site in the ATG initiation codon; GFP, protein-coding region of a gene for green fluorescence; GUS, protein-coding region of a gene for β-glucuronidase.

2.2 Construction of plant expression vectors

Via Gateway LR reaction, PrbcS-*T-GFP segment from pENTR*-PrbcS-*T-GFP and PrbcS-*T-GUS segment from pENTR*-PrbcS-*T-GUS were subcloned into the destination vector, pK2GW7, to yield the plant expression vectors, pK2-35S-PrbcS-*T-GFP (Figure 2b) and pK2-35S-PrbcS-*T-GUS (Figure 2e), respectively. In the pK2-35S-PrbcS-*T-GFP and pK2-35S-PrbcS-*T-GUS expression vectors, the GFP and GUS genes were directly downstream of PrbcS-*T and the 35S promoter was located upstream of the PrbcS promoter.

To compare the expression efficiency of the Gateway expression vectors, pK2-35S-PrbcS-*T-GFP and pK2-35S-PrbcS-*T-GUS, with that of the light inducible plant expression vectors constructed *via*

restriction/ligation strategy, the PrbcS-*T-GFP and PrbcS-*T-GUS DNA fragments were excised from pENTR*-PrbcS-*T-GFP and pENTR*-PrbcS-*T-GUS and inserted into pPZP211 between the *Hin*d Ⅲ and *Bam*H Ⅰ sites to yield pPZP211-PrbcS-*T-GFP (Figure 2c) and pPZP211-PrbcS-*T-GUS (Figure 2f), respectively.

The 35S promoter is ubiquitously employed in widely available plant expression vectors for transgenic plant generation [21–22, 29]. Thus, the constitutive plant expression vectors of GFP and GUS were also constructed as the controls. The GFP from pENTR-GFP and the GUS from pENTR-GUS was integrated into pK2GW7 to replace the ccdB gene *via* Gateway LR reaction to yield pK2-35S-GFP (Figure 2a) and pK2-35S-GUS (Figure 2d), respectively. In pK2-35S-GFP and pK2-35S-GUS, the expression of both GFP and GUS was directly under the control of the 35S promoter.

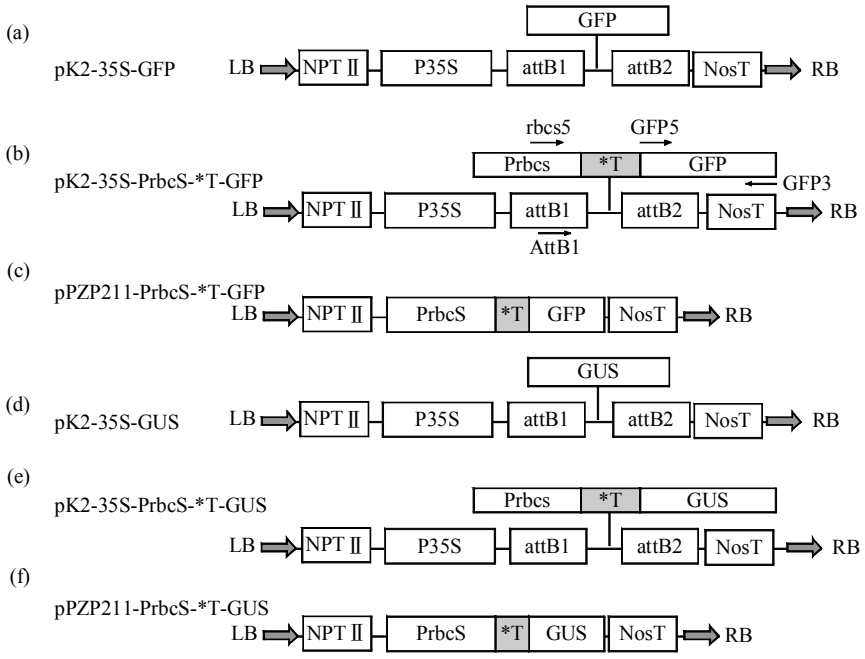

Fig. 2 Diagrams of DNA constructs in plant expression vectors

(a, b, d, e) Constructs were inserted into the Gateway destination vector pK2GW7. (c, f) Constructs were inserted into a small binary vector pPZP211. PrbcS, *T, GFP and GUS are described in Figure 1; LB and RB, left border sequence and right border sequence, respectively; NPT Ⅱ, kanamycin-resistance gene; P35S, CaMV 35S promoter sequence; attB1 and attB2, two specific attachment sites for Gateway BP reaction; NosT, nopaline synthase terminator sequence; Arrows indicate the positions of the primers used in PCR and RT-PCR analysis.

2.3 GFP expression in transgenic calli

To verify the functions of the GFP expression vectors, we transformed tobacco and geranium with the three GFP expression vectors, pK2-35S-PrbcS-*T-GFP, pK2-35S-GFP and pPZP211-PrbcS-*T-GFP, respectively. Each vector was transformed with 40 to 50 tobacco leaf discs and 50 to 60 leaf petiole fragments of geranium, respectively.

A UV-lamp was used to visualize GFP transient expression in tobacco and geranium calli. The results showed that 70%～80% of the transformed tobacco leaf discs and 60%～70% of the transformed geranium leaf petiole fragments displayed GFP fluorescence spots. Typical tobacco leaf discs and geranium leaf petiole fragments that exhibited GFP fluorescence spots are showed in Figure 3. The results indicated that GFP intensity in pK2-35S-PrbcS-*T-GFP tobacco and geranium calli (Figure 3b and 3e) was the same as that in pK2-35S-GFP calli (Figure 3c and 3f) and pPZP211-PrbcS-*T-GFP calli (Figure 3a and 3d). GFP spots were absent in untransformed tobacco and geranium (wild type (WT), negative control) calli (Figure 3g and 3h) under the same conditions. This data indicated that the GFP gene from pK2-35S-PrbcS-*T-GFP was expressed transiently in most calli generated from the transformed plant tissues.

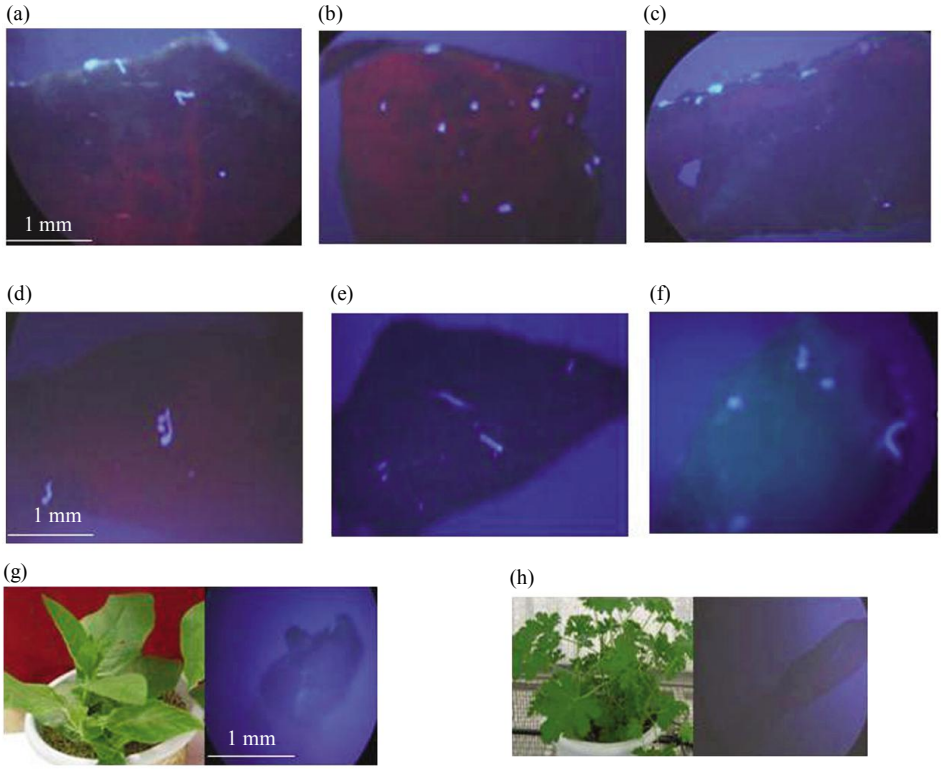

Fig. 3 GFP fluorescence spots in transgenic tobacco and geranium calli

(a) pPZP211-PrbcS-*T-GFP tobacco calli. (b) pK2-35S-PrbcS-*T-GFP tobacco calli. (c) pK2-35S-GFP tobacco calli. (d) pPZP211-PrbcS-*T-GFP geranium calli. (e) pK2-35S-PrbcS-*T-GFP geranium calli. (f) pK2-35S- GFP geranium calli. (g) WT tobacco. (h) WT geranium.

2.4 Integration and transcription of GFP in transgenic plants

Transgenic GFP shoots were generated from tobacco leaf discs and geranium leaf petioles that exhibited GFP fluorescence at the callus stage. 15 to 20 lines of transgenic tobacco and 10 to 15 lines of transgenic geranium were obtained for each vector, respectively. Using GFP5 and GFP3 primers, PCR and RT-PCR analysis was carried out to confirm the integration and transcription of GFP gene in transgenic plants. Most of the transgenic tobacco and geranium lines showed positive results. Figure 4a, 4b and 4c collects a part of the representative results for the investigated plants. The data indicated that the molecular size (0.7 kb) of the PCR products for all transgenic plants was the same as that of the positive control. However, the corresponding PCR products could not be detected for the negative control. Comparable to pPZP211-PrbcS-*T-GFP and pK2-35S-GFP, GFP from the pK2-35S-PrbcS-*T-GFP vector was normally incorporated into the genome of transgenic plants and transcribed normally. Furthermore, all transgenic plants exhibited a normal phenotype when grown on soil or MS agar medium.

The 35S promoter is upstream of the PrbcS promoter in the pK2-35S-PrbcS-*T-GFP expression vector. Therefore, we performed a second RT-PCR analysis for the transgenic tobacco using the forward primer attB1 downstream of the 35S promoter or rbcS5 in the PrbcS promoter region and the reverse primer GFP3 (Figure 2b). This approach enabled us to determine if there was any constitutive GFP transcript produced under the control of the 35S promoter in the pK2-35S-PrbcS-*T-GFP transformants. The results showed that the constitutive GFP transcript was only appeared in pK2-35S-GFP transgenic tobacco (Figure 4d) but not in pK2-35S-PrbcS-*T-GFP transgenic tobacco (Figure 4e). The data suggested that in pK2-35S-PrbcS-*T-GFP transformants, GFP expression was driven by PrbcS promoter but not 35S promoter.

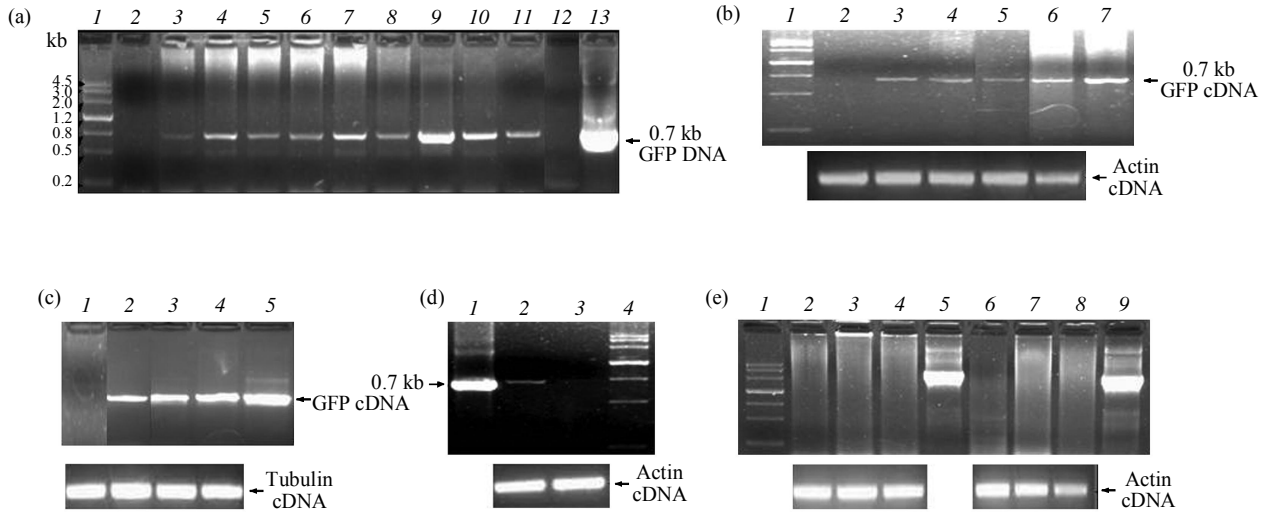

Fig. 4　Integration and transcription of GFP in transgenic plants

(a) PCR analysis for detection of GFP integration in transgenic tobacco and geranium. PCR was performed as described in **Materials and methods**. *1*: DNA marker; *2*: WT tobacco line (negative control); *3* and *4*: pK2-35S-GFP tobacco lines; *5* and *6*: pK2-35S-PrbcS-*T-GFP tobacco lines; *7* and *8*: pPZP211-PrbcS-*T-GFP tobacco lines; *9*: pK2-35S-GFP geranium line; *10*: pK2-35S-PrbcS-*T-GFP geranium line; *11*: pPZP211-PrbcS-*T-GFP geranium line; *12*: WT geranium line (negative control); *13*: Positive control (with pENTR*-PrbcS-*T-GFP as the template). (b) RT-PCR analysis for detection of GFP transcription in transgenic tobacco. RT-PCR was performed as described in **Materials and methods**. Transcript levels of the actin gene were used as an internal amplification control and are shown in the lower panel. *1*: DNA marker (the size of each band is described in (a)); *2*: WT tobacco line; *3*: pK2-35S-GFP tobacco line; *4* and *5*: pK2-35S-PrbcS-*T-GFP tobacco lines; *6*: pPZP211-PrbcS-*T-GFP tobacco line; *7*: Positive control. (c) RT-PCR analysis for detection of GFP transcription in transgenic geranium. RT-PCR was performed as described in (b). *1*: WT geranium line; *2*: pK2-35S-GFP geranium line; *3*: pK2-35S-PrbcS-*T-GFP geranium line; *4*: pPZP211-PrbcS-*T-GFP geranium line; *5*: Positive control. (d) RT-PCR analysis for detection of GFP constitutive expression in transgenic pK2-35S-GFP tobacco. RT-PCR was performed as described in (b) with attB1 and GFP3 primers. *1*: Positive control (with pK2-35S-GFP as the template); *2*: pK2-35S-GFP tobacco line; *3*: WT tobacco line (negative control); *4*: DNA marker (the size of each band is described as in (a)). (e) RT-PCR analysis for detection of GFP constitutive expression in transgenic pK2-35S-PrbcS-*T-GFP tobacco lines. RT-PCR was performed as described in (b). *1*: DNA marker (the size of each band is described in (a)); *3* and *4*: PCR analysis with attB1 and GFP3 primers; *7* and *8*: PCR analysis with rbcS5 and GFP3 primers; *2* and *6*: WT; *5* and *9*: Positive control (with pK2-35S-PrbcS-*T-GFP as the template).

2.5　GFP subcellular localization in transgenic geranium mesophyll cells

To confirm the subcellular localization of the expressed GFP protein, we examined the distribution patterns of GFP fluorescence in the mesophyll cells of transgenic geranium lines in which GFP transcription was confirmed by RT-PCR analysis under a confocal laser-scanning microscope. Positive results were obtained following observations with an excitation wavelength at 488 nm. In WT plants, chlorophyll autofluorescence (red) was observed at emission wavelengths greater than 560 nm, but no signal was detected for GFP at emission wavelengths of 505～530 nm (Figure 5a～c). In pPZP211-PrbcS-*T-GFP mesophyll cells, GFP fluorescence (green) and chlorophyll autofluorescence were co-localized in chloroplasts (Figure 5d～f). In pK2-35S-PrbcS-*T-GFP mesophyll cells, GFP fluorescence distribution patterns (Figure 5g～i) were similar as those in pPZP211-PrbcS-*T-GFP mesophyll cells. The data indicated that GFP was also co-localized with chlorophyll autofluorescence in the chloroplasts of pK2-35S-PrbcS-*T-GFP mesophyll cells. These observations suggested that the expressed GFP protein encoded by GFP gene from the pK2-35S-PrbcS-*T-GFP vector could be targeted directly into the chloroplast stroma of transgenic geranium leaves.

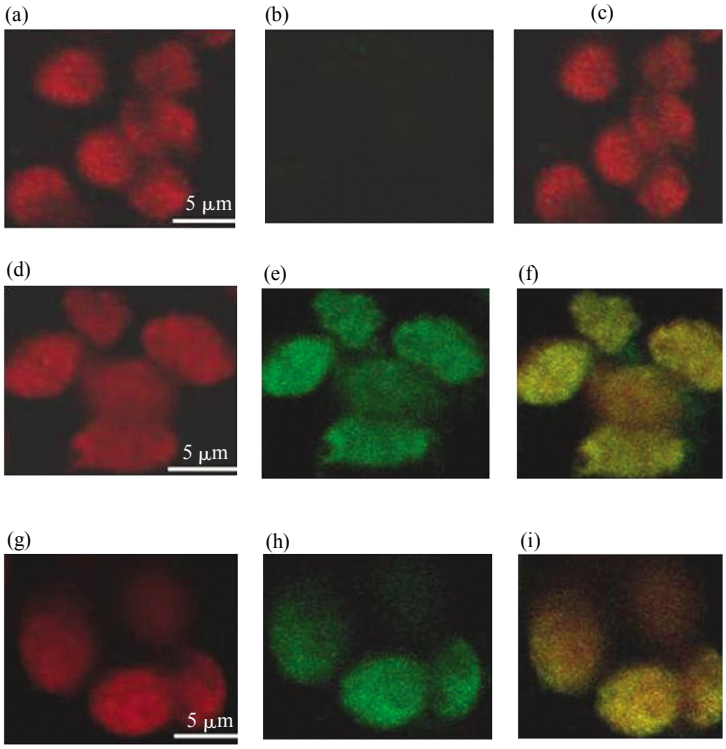

Fig. 5　Chloroplast localization of GFP in transgenic geranium leaves

Fresh leaves were transversely sectioned and immediately mounted between a glass slide and cover slip. The green fluorescence of GFP and the red fluorescence of chlorophyll were monitored separately using a confocal laser-scanning microscope. (a, b, c) WT leaf. (d, e, f) pPZP211-PrbcS-*T-GFP leaf. (g, h, i) pK2-35S-PrbcS-*T-GFP leaf.　(a, d, g) Red chlorophyll fluorescence. (b, e, h) Green GFP fluorescence. (c, f, i) Two fluorescence images were overlaid.

2.6　GUS staining in transgenic tobacco leaves

The three GUS expression vectors, pK2-35S-PrbcS-*T-GUS, pK2-35S-GUS and pPZP211-PrbcS-*T-GUS, were used to transform tobacco, respectively. Genomic PCR and RT-PCR analysis with GUS5 and GUS3 primers verified that GUS gene was integrated into the genomes and transcribed in the transgenic tobacco lines (data not shown).

The transgenic GUS tobacco lines confirmed by RT-PCR analysis were used to conduct histochemical GUS staining analysis to determine if high GUS expression could be achieved in the pK2-35S-PrbcS-*T-GUS transgenic tobacco leaves. Determination of GUS activity distribution patterns in tobacco leaves required whole leaf samples. However, an entire tobacco leaf from soil grown plants was too large to be uniformly stained. Therefore, we selected the third leaf from the top of the plants (5 to 7 cm high) grown on MS agar medium for GUS staining analysis. We observed that no variation in the staining intensity of the same transgenic GUS lines, but variability was noticeable in the three different transgenic tobacco lines (data not shown). Figure 6 collects a part of the

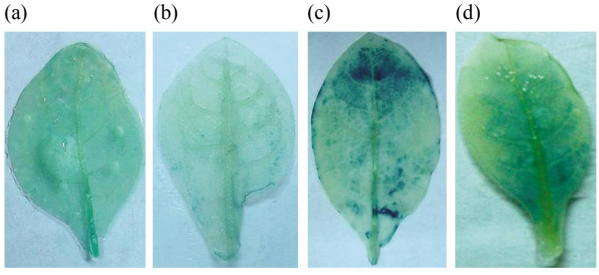

Fig. 6　GUS staining in transgenic tobacco leaves

GUS staining was performed as described in the **Materials and methods**. (a) WT leaf. (b) pK2-35S-GUS leaf. (c) pPZP211-PrbcS-*T-GUS leaf. (d) pK2-35S-PrbcS-*T-GUS leaf.

representative results for the stained leaves. Leaves representing pK2-35S-PrbcS-*T-GUS (Figure 6c) and pPZP211-PrbcS-*T-GUS (Figure 6d) were extensively and deeply stained. The data indicated that GUS protein expression levels in pK2-35S-PrbcS-*T-GUS leaves were similar to those in pPZP211-PrbcS-*T-GUS leaves. Moreover, difference in the blue spot distribution mode between the pPZP211-PrbcS-*T-GUS leaf and the pK2-35S-PrbcS-*T-GUS leaf was observed. In the pK2-35S-PrbcS-*T-GUS leaf, blue staining was intensified in the regions close to the main vein and faint in the areas close to the leaf margin (Figure 6d). In the pPZP211-PrbcS-*T-GUS leaves, blue spots were weak in the regions proximal to the petiole and dark blue in the regions distal to the petiole (Figure 6c). The pK2-35S-GUS leaf was poorly stained (Figure 6b) and the WT leaf was not stained (Figure 6a).

3 Discussion

Gateway technology allows the user to clone the target genes easily into specifically designed plasmids without the need for DNA restriction [30]. Thus, we inserted the PrbcS promoter-transit peptide fragment into the multicloning sites of a modified Gateway entry vector, pENTR*-2B, to generate pENTR*-PrbcS-*T-GFP. The evidence from the present study demonstrated that pENTR*-PrbcS-*T-GFP can be generally used to generate an entry vector for a target gene by replacement of GFP with the target gene. From the entry vector, the plant expression vector that serves to localize the expressed target protein to chloroplasts can be achieved rapidly *via* Gateway technology. Furthermore, the target gene can be integrated into the genome of transgenic plants and efficiently expressed in transgenic calli (transient expression) and transgenic plant leaves (stable expression).

The tomato rbcS-3C transit peptide has been demonstrated to be able to localize the expressed fusion proteins into the chloroplast stroma in transgenic plants [13, 16-17]. Our observation under the cofocal laser-scanning microscope verified that the transit peptide sequence from the pENTR*-PrbcS-*T-GFP entry vector specifically targeted the expressed GFP protein to chloroplasts.

GUS staining patterns in the leaf of the transgenic tobacco generated with pK2-35S-PrbcS-*T-GUS were similar to that of pPZP211-PrbcS-*T-GUS leaf. Blue spots appeared in chloroplast-containing mesophyll tissues and not visible in the leaf veins, indicating expression of GUS protein in the transgenic tobacco leaves. However, blue staining density was not uniformly distributed in the pPZP211-PrbcS-*T-GUS leaf and the pK2-35S-PrbcS-*T-GUS leaf (Figure 6). The previous studies showed that the rbcS promoter functioned in the tissues containing chloroplasts and was light inducible[31-35]. The possibility for our results can be envisaged: the number of chloroplasts differed in various leaf parts since the plants were grown in boxes and the leaves might not have received uniform light under our experimental conditions. Therefore, GUS gene expression levels and GUS protein accumulation were variable throughout the leaves.

Utilization of the entry vector will expand the application of Gateway technology and the plant destination vectors created by VIB/Gent, which provides convenience for chloroplast genetic engineering. In fact, we have successfully constructed plant expression vectors for several target genes using the pENTR*-PrbcS-*T-GFP entry vector. These plant expression vectors have been used to conduct genetic engineering on the CO_2 fixation pathway in transgenic plants. Currently, VIB/Gent (Belgium) developed several new Gateway destination vectors for promoter analysis [22]. The application of our entry vector and these destination vectors facilitates the construction of the plant expression vectors without the 35S promoter.

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含有 Rubisco 小亚基启动子和转运肽序列的通路克隆入门载体的构建和应用 *

马　莉[1)]　宋中邦[1)]　胡清泉[2)]　赵　玥[1)]　年洪娟[1)]　玉永雄[2)]　李昆志[1)]　陈丽梅[1)**]

([1)] 昆明理工大学生物技术研究中心，昆明 650224；[2)] 西南大学动物科学与技术学院，重庆 400716)

摘要　Gateway(通路克隆)技术是最近开发出来的一种分子克隆技术，其特点是操作简单、省时高效，已经成功应用于很多基因表达载体的构建．然而，现有的通路克隆植物表达载体不包含任何将表达蛋白定位到叶绿体中的序列．将通路克隆入门质粒载体 pENTR-2B 的 *Xmn* Ⅰ位点改造成 *Hin*dⅢ位点，产生入门载体 pENTR*-2B，然后将番茄 1, 5 二磷酸核酮糖羧化酶(Rubisco)小亚基 3C 的启动子(PrbcS)及其转运肽序列(*T)和绿色荧光蛋白(GFP)报告基因亚克隆到 pENTR*-2B 中，构建通路克隆入门载体 pENTR*-PrbcS-*T-GFP．实验结果证实，用 pENTR*-PrbcS-*T-GFP 和通路克隆的植物表达载体进行 LR 反应，构建 GFP 的光诱导型植物表达载体，可以成功地将表达的 GFP 定位到转基因植物的叶绿体中．利用 β- 葡糖苷酸酶(GUS)报告基因替代该入门载体中的 GFP 基因做试验也得到相似的结果．这说明用目的基因替换该入门载体中的 GFP 可以构建目的基因的入门载体，然后用通路克隆技术可以快速构建其光诱导型植物表达载体，将表达的目的蛋白定位到转基因植物或组织细胞的叶绿体中．

关键词　通路克隆技术，1, 5 二磷酸核酮糖羧化酶小亚基 3C 启动子，入门载体，植物表达载体，叶绿体定位

学科分类号　Q78，Q94　　**DOI**: 10.3724/SP.J.1206.2010.00362

* 国家重点基础研究发展计划(2007CB108901), 国家自然科学基金(30670163)和云南省中青年学术与技术带头人培养费(2004PY01-5)资助项目.

** 通讯联系人.

Tel: 0871-3801018-202, E-mail: chenlimeikm@yahoo.com.cn

收稿日期：2010-07-09，接受日期：2010-11-16