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The expression of foreign gene under the control of cauliflower mosaic virus 35s RNA promoter

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ABSTRACT

The promoter region of cauliflower mosaic virus (CaMV) 35s RNA was employed to construct an intermediate expression vector which can be used in Ti plasmid system of Agrobacterium tumefaciens. The original plasmid, which contains a polylinker between CaMV 35s RNA and its 3' termination signal in pUC18 was modified to have another antibiotic resistance marker (kanamycin resistance gene Km^r) to facilitate the selection of recombinant with Ti plasmid. Octopine synthase (ocs) structural gene was inserted into this vector downstream of CaMV 35s RNA promoter. This chimaeric gene was introduced into integrative Ti plasmid vector pGV3850, and then transformed into Nicotiana tobaccum cells. A binary plasmid vector was also used to introduce the chimaeric gene into tobacco cells. In both cases, the expression of ocs gene was demonstrated. The amount of octopine was much more than the nopaline synthesized by nopaline synthase (nos) gene transferred at the same time with Ti plasmid vector. This demonstrated that CaMV 35s RNA promoter is stronger in transcriptional function than the promoter of nos in tobacco cells.

Key words: *Agrobacterium tumefaciens, gene expression, cauliflower mosaic Cirus 35s RNA promoter.*

INTRODUCTION

During the past decade, extensive research on the Ti plasmid of Agrobacterium tumefaciens has brought development of useful and efficient vectors to introduce foreign genes into dicotyledonous plants. The system has taken advantage of the natural process by which A. Tumefacienstransforms plant cells. A. tumefaciens has

been shown to transfer a small region of Ti plasmid into plant and the transferred DNA(T-DNA) integrates into plant chromosomes at different sites. Any gene inserted in T-DNA can thus be introduced into plant cells. Many vectors which contain chimaeric gene fusions with T-DNA have been constructed. The nos promoter, ocs promoter and CaMV promoters were used in these vectors to initiate transcription of bacterial genes and eukaryotic genes in the transformed plants[15].

Cauliflower mosaic virus is one of only a few doublestranded DNA viruses in plants. Its promoter directs the synthesis of 35s RNA in infected plant cells. This promoter has been used for expression of various genes in transformed plants. Compared with the widely used plant transcriptional initiation signals used in vitro, such as nos promoter and ocs promoter, CaMV 35s RNA promoter was considered as the strongest one in model plants. Thomas Hohn et al. have constructed a vector which contains a polylinker between 35s RNA promoter and the 3' termination signal in pUC18[2]. They inserted foreign genes downstream of the promoter, and demonstrated transient expression of foreign genes in plant protoplasts. We modified this vector to construct an intermediate vector which can be used in Ti plasmid system. The new vector contains another antibiotic resistance marker, in addition to ampicillin resistant gene to facilitate the selection of recombinant with Ti plasmid. Using this vector, we introduced ocs gene into plant cells and detected its expression in transformed cells.

MATERIALS AND METHODS

1. Bacterial strains and plasmids used. (see Table 1)

Table 1. Bacterial strains and plasmids used

Names	Some characters and usages	Origin
E. coli JM101	△(lac pro) supE, thi , F' proAB traD36, lacl^{q}Z△ M15 Recipient for transformation	Gronenborn and Messing [6].
E. coli MC1061	△(lacIPOZYA), X74, galU, galK strA$^-$ hsdR△(ara leu) Recipient for transformation	Casadaban and Cohen [7].
A. tumefaciens C58C1	Rif^r, cured strain	This lab (unpublished)
A. tumefaciens A281	Helper strain for plant transformation	An *et al.* [8]

(to be continued)

(continued)

pKC7	Ap^r, Km^r, Cloning vector	Rao and Roger[9].
pDH51	Ap^r, Plasmid containing CaMV 35s RNA promoter fragment.	Thomas *et al.* [2].
pNO1	Ap^r, Plasmid containing ons gene	Herrela-Estrela[10].
pEND4K	Km^r, Cm^r, Binary plasmid vector	Klee[11].
pGV3850	Ap^r, Modified plant transformation vector	Zambryski et al. [12].
pGJ28	Km^r/Nm^r, co1D replication Mob+, helper plasmid	Van Haute [l3].
R64drd11	Tc^r/Sm^r, Iq, Tra+ Helper plasmid	Van Haute [13].
pRK2073	Str^r, helper plasmid	Ditta [14]

2. Media

LB medium[15] was used for *E.coli*. LB and AB minimum medium[16] were used for Agrobacterium *tumefaciens*. Plant tissue was grown on MS medium[17] with appropriate amount of relevant hormones.

3. Enzymes

Restriction enzymes and other enzymes were purchased from commercial sources and used according to the producer's recommendation.

4. Isolation of DNA.

Bacterial plasmid was isolated according to Birnboim and Doly[18]. Large scale isolation was done according to Maniatis et al.[19]. DNA sample was purified by ultracentrifugation in CsCl (1 g/ml) and ethidium bromide(2.5 μg/ml) solution at 400,00 rpm (rotor Ti50, Beckman L8-M ultracentrifuge) for 40 hr. Agrobacterium total DNA was isolated according to Dhaese[20].

5. Transformation of E. coli.

Transformation of *E.coli* competent cells was done according to Maniatis *et al*. [21].

6. Plasmid mobilization from E. coli to Agrobacterium.

The plasmid was mobilized from *E. coli* to Agrobacteria according to Van Haute *et al*. [13].

7. DNA Hybridization

Southern transfer was done according to Southern *et al*.. DNA probe was labeled by nick translation with (α -^{32}p) ATP (From Amersham).

8. Plant tissue transformation.

Plant transformation was done according to Tang Ti *et al*. (to be published in Acta Phytophy-

siologia Sinica). After leaf was sterilized, lower epidemis of Nicotiana tobaccum was torn off and leaf pieces were put on MS medium containing cefotaxime (0.5mg/ml) and 6BA (1μg/ml). Then a few drops of overnight bacterial suspension culture were applied to leaf surface. The plant tissue was incubated at 28℃.

9. Opine assay.

Opine was detected by a modified method from Otten et a1.[22]. Plant tissue was cut into small pieces and incubated in an assay buffer [Arginine (0.05M), Na_2HPO_4/NaH_2PO_4 (0.1M, pH 7.0)] at 28℃ overnight. The tissue was ground and then centrifuged at 8,000 rpm. The supernatant was used for opine assay.

RESULTS

1. Construction of intermediate vector pBW1.

Part of the sequences between the left border and right border of T-DNA was replaced by pBR322 sequence in modified Ti plasmid vector pGV3850. Those intermediate vectors that contain pBR322 sequence can integrate into pGV3850 by homologous recombination. Because there is an ampicillin resistance marker in pGV3850 itself, it would be better that the intermediate vector had another antibiotic resistance marker to select for the recombinant. We used Km^r in pKC7 as such a selective marker. We first digested pKC7D with SalI and self-ligated with T-DNA ligase to get plasmid pKC7D which contains no BamHI site and is more convenient for future cloning. Then we inserted the EcoRI fragment containing 35sRNA promoter and terminator from pDH51 into EcoRI site of pKC7D to get the intermediate vector pBW1 (Figs. 1,2,3).

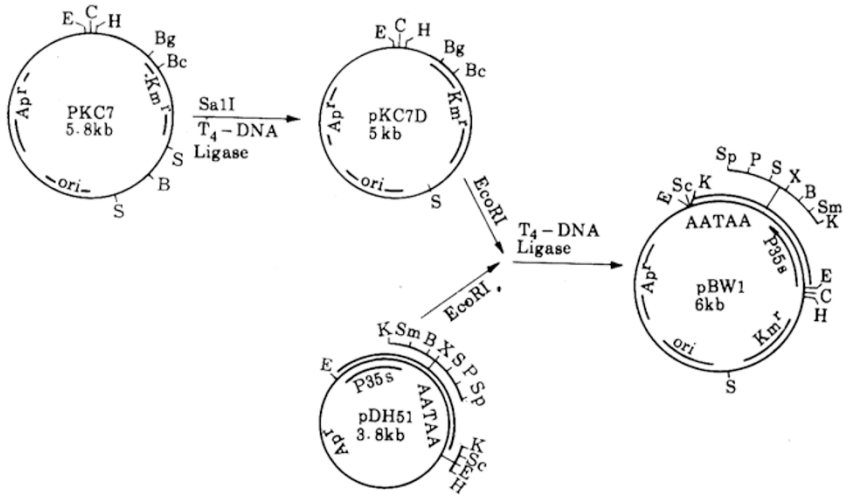

Fig. 1 Construction of pBW1.
pKC7 was digested with SalI and self-ligated with T_4-DNA ligase. The recombinant plasmid, pKC7D, contains no BamHI site. The EcoRI fragment from pDH51 containing CaMV 35s RNA promoter was then inserted into the EcoRIsite of pKC7Dtoget pBW1. B. BamH I; Bc, Bcl I; Bg, Bgl II; C, ClaI; E, EcoR I: H, HindIII; K, Kpn I; P, Pst I; S.Sal I; Sc, SacI; Sm, SmaI; Sp, SphI; X, XbaI.

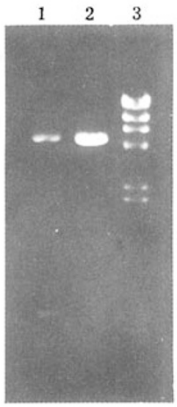

Fig. 2 Demonstration of the construction of pKC7D.
1. SalI digested pKC7.
2. SalI digested pKC7D.
3. HindIII digested λ DNA.

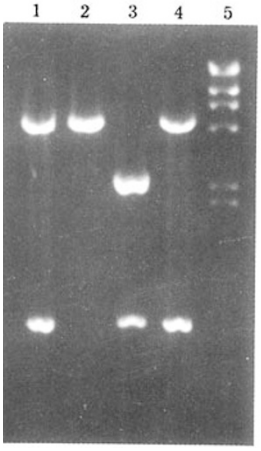

Fig. 3 Demonstration of the construction of pBW1.
1. EcoRI digested pBW1.
2. EcoRI digested pKC7D.
3. EcoRI digested pDH51.
4. HindIII and SacI digested pBW1.
5. Hind III digested λ DNA.

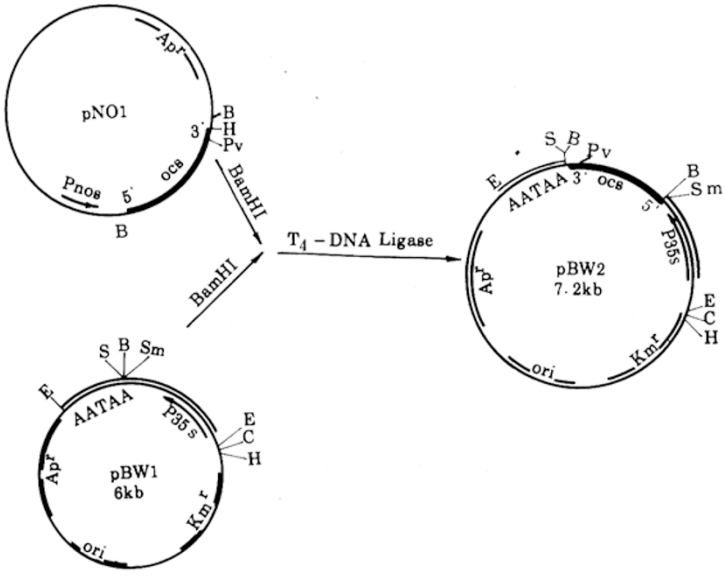

Fig. 4
Construction of pBW2.
pNO1 and pBW1 were digested with BamHI and then ligated with T_4-DNA ligase.
The recombinant plasmid was digested with PvuII and SmaI to determine the orientation of the ocs gene. B, BamHI: C, ClaI; E, EcoRI; H, Hind III; Pv, PvuII; S, SalI; Sm, SmaI.

2. Construction of CaMV 35s-ocs chimaeric gene.

In order to study the expression of foreign gene under the control of 35s RNA promoter, octopine synthase structural gene was inserted downstream of the promoter in pBW1. The pNO1 plasmid containing ocs gene was digested with BamHI and the resulted 1.2kb fragment was inserted into BamHI site of pBW1. The orientation of *ocs* was examined by digestion with PvuII and SmaI, because there is a PvuII site near the 3' end of the *ocs* gene (Figs. 4,5).

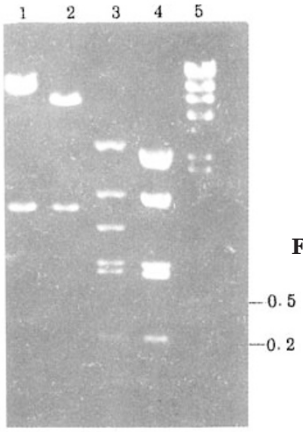

Fig. 5 Demonstration of construction of pBW2.
1. BamHI digested pNO1.
2. BamHI digested pBW2.
3. PvuII and SmaI digested pBW2.
4. PvuII and Sina I digested pBW1.
5. Hind III digested λ DNA.

3. Mobilization of plasmid pBW2 into A. tumefaciens and transformation of plant tissue.

Plasmid pBW2 was mobilized into A *tumefaciens* C58C1 (pGV3850) with the help of plasmid pGJ28 and R64drd11, and integrated into Ti plasmid pGV3850. The transconjugant was selected on LB medium containing ampicillin (100 mg/l), kanamycin (100 mg/l) and rifampicin(50mg/l). Using *ocs* genefragment as probe, we have detected the *ocs* gene in the transconjugant total DNA (Fig. 6).

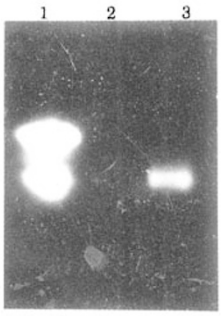

Fig. 6 Southern analysis of DNA from *Agrobacterium* C58 (pBW2::pGV3850) The probe was BamHI fragment from pNO1.
1. pNO1 digested with BamHI.
2. DNA from C58 (pGV3850 digested with BamHI.
3. DNA from C58 (pGV3850::pBW2) digested with BamH I.

The Agrobacterial suspension culture was used to transform tobacco leaves Shoots grow at the sites of inoculation after three weeks of cultivation on MS medium containing cefotaxime (0.5 mg/ml) and 6BA (1 μg/ml). Octopine and nopaline

were detected in some shoots. The ammount of octopine detected is much more than that of nopaline (Fig. 7).

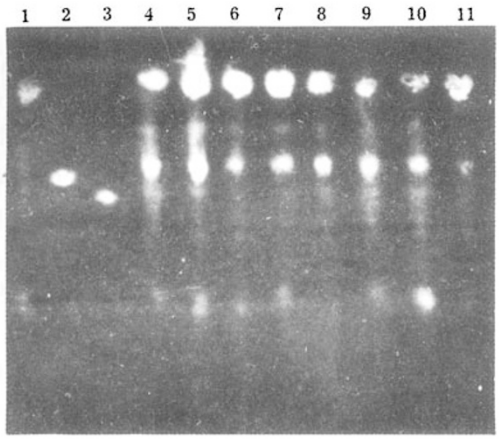

Fig. 7 Detection of octopine and nopaline.
1. Untransformed plant tissue.
2. Standard octopine.
3. Standard nopaline.
4-11. Transformed plant shoots.

4. Transformation of plant with binary plasmid vector.

We cloned the chimaeric *35s-ocs* gene between T-DNA borders in binary plasmid pEND4K which contains *chimaeric nos*-NTPII gene highly expressed in plants. pBW2 was digested with Sal I and inserted into the unique SalI site in pEND4K to get plasmid pBW3 (Fig.8). pBW3 was mobilized into *Agrobacterium* A281 (pTiBo

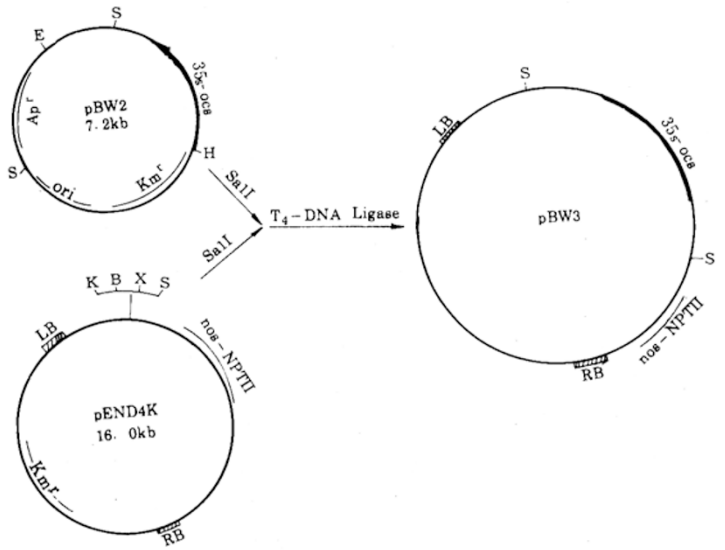

Fig. 8 Construction of pBW3.
pBW2 was digested with Sal I and ligated with Sal I digested pEND4K to get pBW3. B, BamHI; E, EcoRI: K, KpnI; S, Sal I; X, XbalI: LB, left border; RB, right border.

542) by the help of plasmid pRK2073 via triparential procedure [13]. The bacterium was selcted on AB minimum medium containing ampicillin (100 mg/l) and kanamycin (100 mg/l). The presence of pBW3 in the *Agrobacterium cells* was confirmed by plasmid minipreparation (data not shown).

Overnight culture of A281 (pTiBo542, pBW3) was used to transform plant leaves. Calli formed on the surface of the leaves after 10 days of cultivation on MS medium containing cefotaxime and 6BA. The calli were then transferred to MS medium containing no hormone but kanamycin. After 10 days, shoots formed and half of them were albinous and died soon, but others were green and normal. After the green shoots were transferred to fresh medium of the same composition for more than a week, we used them for octopine and nopaline assay and found that all of the kanamycin resistance shoots produced octopine (Data not shown).

DISCUSSION

We report the results of experiment designed to introduce foreign gene into plants, to get them expressed and to regenerate plants directly from the transformed tissue. With the development of Ti plasmid as plant transformation vector, it's no longer difficult to introduce genes into dicotyledonous plants. Since not all foreign genes inserted into plant genome will carry apromoter functional in plants, plant recongnizable promoter is required for the expression of foreign genes.

The nos promoter was used to direct the expression of bacterial genes including some economically useful genes. CaMV 35s RNA promoter was also used extensively. Ti plasmid vector pGV3850 contains a *nos* gene in its modified T-DNA region. This *nos* gene was transferred to plant cells at the same time with foreign chimaeric gene. Fig. 7 shows the detection of octopine and nopaline in transformed plant cells. The octopine synthesized by chimaeric gene was much more than the nopaline synthesized by *nos* gene. This demonstrates that the CaMV 35s RNA promoter is stronger than that of *nos*. This character of CaMV 35s RNA promoter will be benefitial to us when we try to make foreign genes highly expressed.

We used both integrative and binary Ti plasmid vectors to introduce chimaeric gene into plant cells. In the first case, we used *nos* gene as a marker to select the transformed cells. This selection method is time consuming. When we used the binary plasmid vector pEND4K, which contains a chimaeric *nos*-NTPII gene, transformed plant cells were kanamycin resistance. All the shoots resistance to kanamy*cin* produced octopine. While in the first case, we used no kanamycin as a selective marker, less than 10% of the shoots examined produced octopine.

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