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# Construction and Evaluation of Chloroplast Expression Vectors in Higher Plants

Po-Yen Chen, Yung-Ting Tsai and Kin-Ying To

## Abstract

Plastid transformation has a number of advantages in comparison with nuclear transformation. Currently, only tobacco (*Nicotiana tabacum*) is routinely used in plastid transformation. Here we constructed a series of chloroplast expression vectors specific for spinach (pCEV1), tomato (pCEV2 and pCEV3), and *N. benthamiana* (pCEV4). Selection marker aminoglycoside 3'-adenyltransferase (*aadA*) conferring spectinomycin resistance was used in pCEV1, pCEV2, and pCEV4, while selection marker neomycin phosphotransferase II (*nptII*) was used in pCEV3. The expression cassette in these vectors was integrated in the intergenic spacer between *trnI* and *trnA* of plastid genome via homologous recombination. Several transgenes, including a reporter gene encoding GFP:GUS fusion protein and genes from tomato (lycopene b-cyclase, z-carotene desaturase) and bamboo mosaic virus satellite RNA (encoding coat protein CP20), were independently cloned into some of these vectors. Transient GUS expression was detected in spinach leaves bombarded by pCEV1/GFP-GUS. Functional expression of selection markers *aadA* and *nptII* was demonstrated for spinach, tomato, and *N. benthamiana*. Seedling assay from T<sub>0</sub> self-pollinated plant of transplastomic *N. benthamiana* confirmed maternal inheritance of transgenes, and genomic PCR analysis confirmed integration of transgenic expression cassette into the plastid genome of *N. benthamiana*. Moreover, auxiliary vectors pECaad and pECnpt are also reported.

**Keywords:** bombardment, chloroplast transformation, homologous recombination, plastid transformation, transplastomic plant, vector construction

## 1. Introduction

Plastid transformation has a number of advantages in comparison with nuclear transformation. It has high levels of transgene expression, position effect or gene silencing is not found due to precise integration of the transgene into the host plastid genome via homologous recombination, and further, gene flow through pollen is not an issue due to maternal inheritance. Plastid transformation technology is important for analyzing the functions of chloroplast genes and has also been

widely used in mass production of foreign proteins for agronomic traits (such as insect resistance, herbicide resistance, disease resistance, drought resistance, salt resistance, phytoremediation, etc.), enzymes/biomaterials (such as cellulase, endo-glucanase, monellin, polyhydroxybutyrate, xylanase,  $\beta$ -glucosidase, pectin lyase, manganese peroxidase, superoxide dismutase, etc.), and pharmaceuticals/vaccine antigens (such as interferon- $\gamma$ , human serum albumin, human somatotropin, insulin-like growth factor, thioredoxin 1, basic fibroblast growth factor, aprotinin, cholera toxin, tetanus toxin, *Canine parvovirus*, anthrax protective antigen, dengue virus, etc.) over the past few decades [1–6]. In most cases, only tobacco (*Nicotiana tabacum*) is routinely chosen as a target plant in plastid transformation [1]. Besides tobacco, a few studies using tomato, lettuce, oilseed rape, potato, cabbage, cotton, carrot, *N. benthamiana*, etc., as target plants have also been reported to produce foreign proteins by plastid transformation technology [1–5, 7–10].

In nuclear transformation, one nuclear transformation vector can be applied to multiple plants. For example, a nuclear transformation vector pCHS [carrying the expression cassette for the selection marker neomycin phosphotransferase II (*nptII*) driven by nopaline synthase (*nos*) promoter and *Petunia* chalcone synthase (*chs*) driven by cauliflower mosaic virus 35S promoter] and an *Agrobacterium tumefaciens*-mediated method were employed to a range of plant species including the high-value medicinal plants *Echinacea purpurea* [11] and *E. pallida* [12], model plant tobacco [13], medicinal herb *Bidens pilosa* [14], and the floricultural plant *Cleome spinosa* [15]; the transfer DNA (T-DNA) containing the expression cassette in vector pCHS was integrated randomly in the nuclear chromosome of transgenic plants, and the foreign *Petunia chs* and selection marker *nptII* were detected and expressed. However, in plastid transformation, a plastid transformation vector has to be constructed for each target plant which is quite a laborious work. A typical plastid transformation vector consists of two components: (1) an expression cassette [a gene of interest (GOI) which is inserted between the plastid promoter and the plastid terminator, followed by a selection marker gene which is inserted between the plastid promoter and the plastid terminator] and (2) a targeting sequence for homologous recombination in the host plant plastid genome. The expression cassette is located between the left targeting region (LTR) and the right targeting region (RTR) in the vector, and this expression cassette is expected to integrate into the plastid genome of the host plant through double homologous recombination during plastid transformation [1, 3–5]. Thus, in comparison with random insertion in nuclear transformation, the expression cassette is precisely inserted at the predicted integration site of the plastid genome during plastid transformation. In theory, any plastid DNA fragment within the plant plastid genome can serve as a targeting region in plastid transformation, and the integration site for the foreign expression cassette into the plant plastid genome can be anywhere within the targeting region. In practice, only 10 or so plastid DNA sequences have been chosen as targeting regions in plastid transformation [3]. Among them, the plastid *trnI/trnA* sequence is the most frequently used as a targeting region, and the spacer between *trnI* and *trnA* is the most frequently used as the integration site [1, 3]. In addition, the selection marker aminoglycoside 3'-adenyltransferase (*aadA*) conferring resistance to spectinomycin or streptomycin is most frequently used in plastid transformation, although other selection markers such as antibiotic resistance (including *nptII*), herbicide resistance, photosynthesis, and metabolism have been reported [16]. More recently, a new selection marker, namely, aminoglycoside acetyltransferase/phosphotransferase, conferring tobramycin in tobacco plastid transformation has been developed [17].

Spinach (*Spinacia oleracea*), belonging to Chenopodiaceae, is a leaf vegetable which is an important source of vitamins and other essential nutrients in many countries. Callus induction and plant regeneration from different explants including leaves, cotyledons, and hypocotyls of several commercial spinach cultivars have been reported [18, 19]. For nuclear transformation, an *Agrobacterium*-mediated method was reported from leaf and hypocotyl explants of spinach cultivars Fall Green and High Pack, and transgenic spinach plants expressing cucumber mosaic virus coat protein were obtained [20]. In addition, *Agrobacterium*-mediated transformation was also established from cotyledon explants of spinach cultivars Longstanding Bloomsdale and Melody, and transgenic spinach plants expressing reporter gene encoding green fluorescent protein (GFP) were regenerated [21]. However with regard to plastid transformation, although the complete chloroplast genome of spinach has been published [22], plastid transformation in spinach has not yet been reported. To initiate plastid transformation in spinach, we constructed chloroplast expression vectors specific for spinach. During the construction, we found that we can easily replace the host plant targeting sequence, which is crucial for homologous recombination in plastid transformation, from spinach to other plant species. Tomato (*Solanum lycopersicum*) was chosen as another target plant in this study, since tomato is an important foodstuff worldwide. Although plastid transformation was reported in tomato cultivar Santa Clara [7], we still wanted to set up plastid transformation technology for tomato cultivar CL5915, a local Taiwan cultivar. *N. benthamiana* was the third target plant in this study. Similar to the widely used tobacco (*N. tabacum*), *N. benthamiana* has many advantages in cell culture such as being easy to regenerate, short lifetime, and self-pollination. Two more advantages in comparison with *N. tabacum* are the plant height and leaf size which are much reduced in *N. benthamiana*, allowing it to serve as model plant for fundamental research such as studies of host/virus interactions.

We want to set up chloroplast transformation technology in our laboratory. As a first step in achieving the goal, we need to construct various chloroplast expression vectors for different plant species. Here we describe the efficient construction of a series of chloroplast expression vectors specific for spinach (pCEV1), tomato (pCEV2 and pCEV3), and *N. benthamiana* (pCEV4). Selection marker *aadA* conferring spectinomycin resistance was used in pCEV1, pCEV2, and pCEV4, while selection marker *nptII* conferring kanamycin resistance was used in pCEV3. Subsequently, several transgenes, including reporter gene encoding GFP:GUS fusion protein and genes from tomato [lycopene  $\beta$ -cyclase (LCY),  $\zeta$ -carotene desaturase (ZDS)] and bamboo mosaic virus satellite RNA (encoding coat protein CP20), were independently cloned into some of these vectors. The reporter gene encoding GFP:GUS fusion protein was inserted into all plastid vectors, so that the expression of the transgene can be visualized by GFP fluorescence microscopy or GUS staining. LCY and ZDS are key enzymes in the carotenoid biosynthesis pathway. Previously, a few genes including *LCY* and *ZDS* have been cloned from tomato in our laboratory [23]. In this study, *LCY* and *ZDS* were selectively inserted into pCEV1 to investigate whether we can engineer spinach by using plastid transformation technology. CP20, encoded by bamboo mosaic virus satellite RNA, is the RNA-binding protein essential for virus infection of the host plant [24]. Thus, the coding region of CP20 with or without untranslated regions was inserted into pCEV4 for *N. benthamiana*. Accuracy of cloning GOI into these vectors has been demonstrated, and functional expressions of reporter and selection marker genes have also been confirmed. In addition, we further report two auxiliary vectors pECaad (carrying selection marker *aadA*) and pECnpt (carrying selection marker *nptII*) which may facilitate vector construction in other plant species.

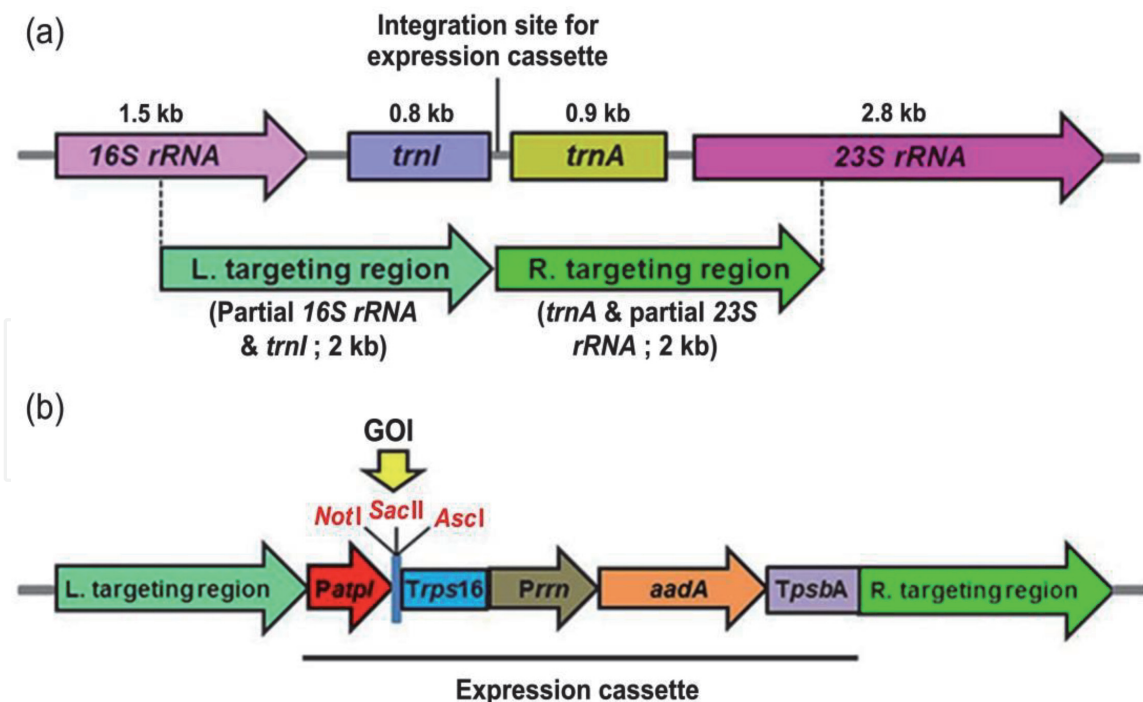
## 2. Materials and methods

### 2.1 Plant material and culture conditions

Seeds of spinach (*Spinacia oleracea*), tomato (*Solanum lycopersicum* var. CL5915), tobacco (*Nicotiana tabacum* var. W38), and *N. benthamiana* were individually sterilized by sequential treatment with 70% ethanol for 0.5 min and 1% sodium hypochlorite for 10 min, then washed thoroughly with sterile water, and germinated on MS basal medium (MS salts [25]; 3% sucrose; 0.8% Bacto agar; pH 5.7). The cultures were then incubated in a growth chamber at 22°C under a photoperiod of 16 h illumination ( $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) and 8 h darkness. In vitro explants were used to establish the plant regeneration and plastid transformation.

### 2.2 Construction of chloroplast expression vector pCEV1

Schematic representation of the cloning procedure used to obtain a chloroplast expression vector in this study is shown in **Figure 1**, and the list of the relevant primers used in this study is shown in **Table A1**. Genomic DNA from green leaves of the target plants was isolated by the cetyltrimethylammonium bromide (CTAB) method [26]. DNA fragments of *Prn*, *TpsbA*, *PatpI*, and *Trps16* were amplified in 50  $\mu\text{l}$  PCR mixture [ $1\times$  PCR buffer; 200  $\mu\text{M}$  dNPT; 0.5  $\mu\text{M}$  each of gene-specific forward and reverse primers; 500 ng tobacco (*N. tabacum*) genomic DNA; 2.5 U FastTaq DNA polymerase]. The PCR mixture was denatured at 94°C for 5 min prior to 35 amplification cycles (1 min at 94°C, 1 min at 55°C, 1 min at 72°C). Final extension reaction was performed at 72°C for 10 min. Following amplification, PCR



**Figure 1.**

Schematic representation of the targeting region, integration site, and expression cassette in the chloroplast expression vector. (a) Diagram showing chloroplast targeting sequence for homologous recombination. Left targeting region (ca. 2 kb) was composed of partial 16S rRNA and *trnI*, while the right targeting region (ca. 2 kb) was composed of *trnA* and partial 23S rRNA. Integration site for expression cassette was designed at the intergenic spacer between *trnI* and *trnA*. (b) Diagram showing the expression cassette between the left targeting region and the right targeting region. The expression cassette was composed of the synthetic unique cloning sites (*NotI*, *SacII*, *AscI*) which were inserted between *atpI* promoter and *rps16* terminator, followed by selection marker *aadA* which was inserted between *rrn* promoter and *psbA* terminator. Gene of interest (GOI) can be inserted into the expression cassette at the cloning sites.

products were analyzed on a 1% agarose gel, and PCR fragments were cut out of the gel, purified by the Zymoclean gel DNA recovery kit (Zymo Research, Orange, CA, USA), cloned into pGEM-T Easy vector (3015 bp; Promega), and then sequenced by an automatic DNA sequencer (ABI PRISM 377 DNA Sequencer, PerkinElmer). Stepwise protocols for cloning pCEV1 (Figure 2) were as follows.

### 2.2.1 Cloning of *Prrn-aadA-TpsbA* fragment into pUC19

- TpsbA* (222 bp) was inserted into pUC19: As mentioned above, PCR amplification was carried out using *TpsbA*-F (containing *Xba*I site) and *TpsbA*-R (containing *Pst*I site) as primers and 500 ng tobacco genomic DNA as template. After PCR amplification, PCR products were purified and cloned into pGEM-T Easy vector. Plasmid carrying *Xba*I-*TpsbA*-*Pst*I was isolated and double digested by *Xba*I and *Pst*I and then ligated with pUC19 (2686 bp; New England Biolabs) which was double digested by *Xba*I and *Pst*I, resulting in pUC19/*TpsbA*.
- Prrn* (219 bp) was inserted into pUC19/*TpsbA*: PCR amplification was carried out using *Prrn*-F (containing the *Kpn*I site) and *Prrn*-R (containing the *Bam*HI site) as primers and 500 ng tobacco genomic DNA as template. After PCR amplification, PCR products were purified and cloned into pGEM-T Easy vector. Plasmid carrying *Kpn*I-*Prrn*-*Bam*HI was isolated and double digested by *Kpn*I and *Bam*HI and then ligated with pUC19/*TpsbA* which was double digested by *Kpn*I and *Bam*HI, resulting in pUC19/*Prrn*-*TpsbA*.
- aadA* (792 bp) was inserted between *Prrn* and *TpsbA* in pUC19: PCR amplification was carried out using *aadA*-F (containing the *Bam*HI site) and *aadA*-R (containing the *Xba*I site) as primers and 500 ng plasmid DNA of

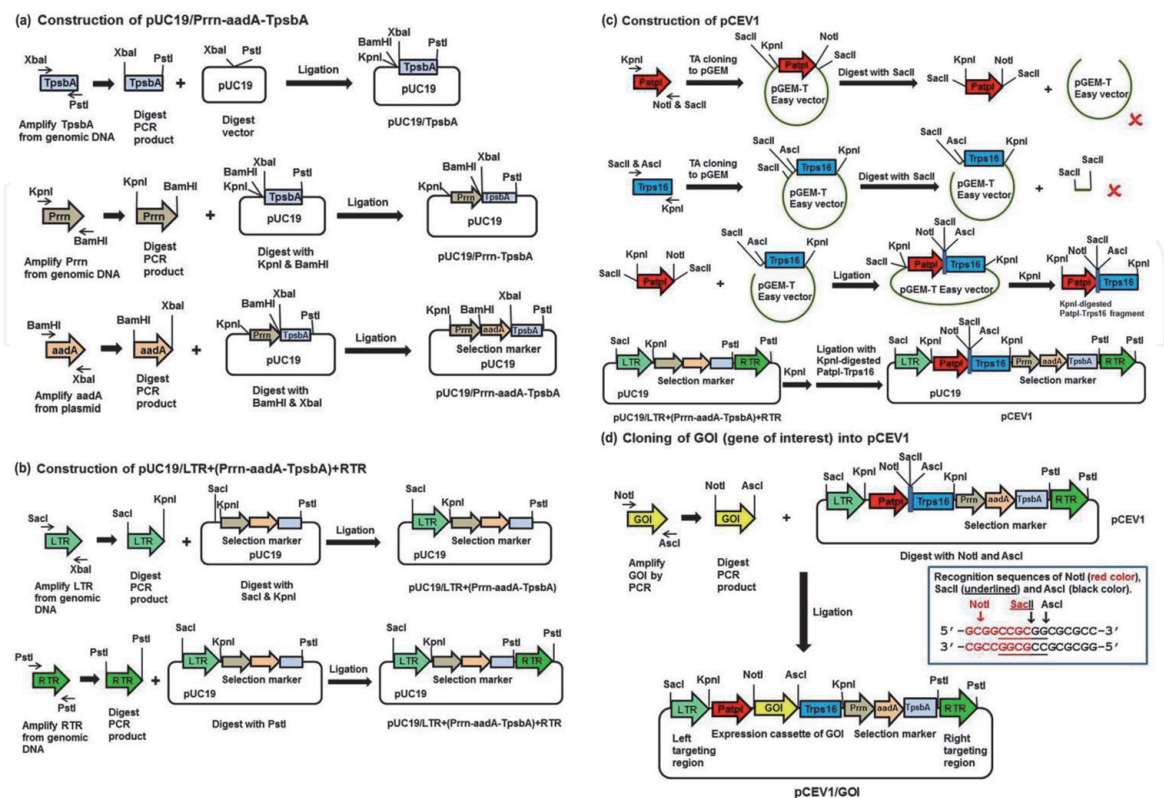


Figure 2.  
 Step-by-step cloning protocol.

pCR8 (Invitrogen) as DNA template. After PCR amplification, PCR products were purified and cloned into pGEM-T Easy vector. Plasmid carrying *Bam*HI-*aadA-Xba*I was isolated and double digested by *Bam*HI and *Xba*I and then ligated with pUC19/*Prrn-TpsbA* which was double digested by *Bam*HI and *Xba*I, resulting in pUC19/*Prrn-aadA-TpsbA*.

### 2.2.2 Cloning of spinach left targeting region (LTR; 2019 bp fragment containing partial 16S rRNA and full-length *trnI* in spinach chloroplast genome) into pUC19/*Prrn-aadA-TpsbA*

PCR amplification was carried out using 16S/*trnI*-F (containing the *Sac*I site) and 16S/*trnI*-R (containing the *kpn*I site) as primers and 500 ng of spinach (*S. oleracea*) genomic DNA as DNA template. After PCR amplification, PCR products were purified and cloned into pGEM-T Easy vector. Plasmid carrying *Sac*I-LTR-*Kpn*I was isolated and double digested by *Sac*I and *Kpn*I and then ligated with pUC19/*Prrn-aadA-TpsbA* which was double digested by *Sac*I and *Kpn*I, resulting in pUC19/LTR + (*Prrn-aadA-TpsbA*).

### 2.2.3 Cloning of spinach right targeting region (RTR; 2031 bp fragment containing full-length *trnA* and partial 23S rRNA in spinach)

PCR amplification was carried out using *trnA/23S*-F (containing the *Pst*I site) and *trnA/23S*-R (containing the *Pst*I site) as primers and 500 ng of spinach genomic DNA as DNA template. After PCR amplification, PCR products were purified and cloned into pGEM-T Easy vector. Plasmid carrying *Pst*I-RTR-*Pst*I was isolated and digested by *Pst*I and then ligated with pUC19/LTR + (*Prrn-aadA-TpsbA*) which was digested by *Pst*I, resulting in pUC19/LTR + (*Prrn-aadA-TpsbA*) + RTR.

### 2.2.4 Cloning of *PatpI*-(cloning sites)-*Trps16* into pUC19/LTR + (*Prrn-aadA-TpsbA*) + RTR

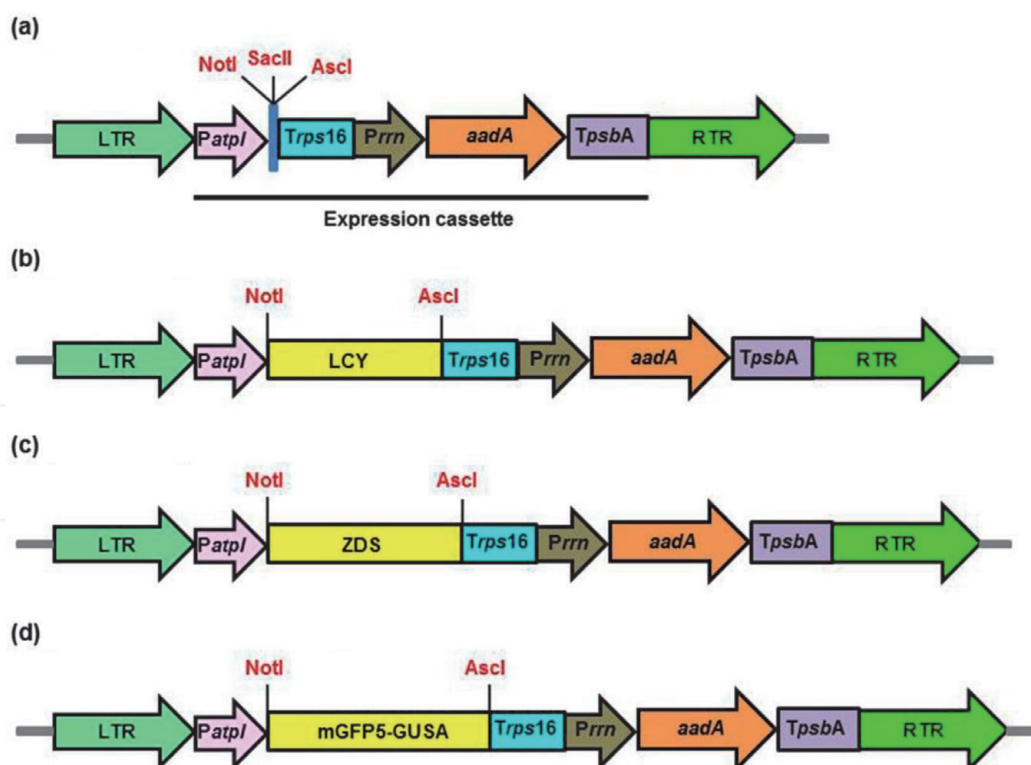
- a. Preparation of *PatpI* (248 bp) fragment: PCR amplification was carried out using *PatpI*-F (containing the *Kpn*I site) and *PatpI*-R (containing the *Not*I and *Sac*II sites) as primers and 500 ng tobacco genomic DNA as template. After PCR amplification, PCR products were purified and cloned into pGEM-T Easy vector.
- b. Preparation of *Trps16* (169 bp) fragment: PCR amplification was carried out using *Trps16*-F (containing the *Sac*II and *Asc*I sites) and *Trps16*-R (containing the *Kpn*I site) as primers and 500 ng tobacco genomic DNA as template. After PCR amplification, PCR products were purified and cloned into pGEM-T Easy vector.
- c. Preparation of *PatpI*-(cloning sites)-*Trps16* fragment in pGEM-T Easy vector: pGEM-T Easy vector carrying *PatpI* was digested by *Sac*II, a small 0.3 kb fragment containing ***Sac*II-*Kpn*I-*PatpI*-*Not*I-*Sac*II** (the 5' ***Sac*II-*Kpn*I** sequence was derived from multiple cloning sites in pGEM-T Easy vector and bold) and was isolated by gel electrophoresis and purified. Meanwhile, pGEM-T Easy vector carrying *Trps16* was digested by *Sac*II, the large fragment containing *Sac*II-*Asc*I-*Trps16-Kpn*I and almost complete pGEM-T Easy vector, was isolated by gel electrophoresis and purified, and then ligated with ***Sac*II-*Kpn*I-*PatpI*-*Not*I-*Sac*II** fragment. As a result, pGEM-T Easy vector

carrying *SacII-KpnI-PatpI-NotI-SacII-AscI-Trps16-KpnI* fragment was generated. The cloning site (*NotI-SacII-AscI*) is 16 bp in length.

- d. Cloning of *PatpI-Trps16* fragment into pUC19/LTR + (*Prrn-aadA-TpsbA*) + RTR: The plasmid pGEM-T Easy vector carrying *SacII-KpnI-PatpI-NotI-SacII-AscI-Trps16-KpnI* fragment was digested by *KpnI*; this *PatpI-Trps16* fragment was isolated and purified and then ligated with pUC19/LTR + (*Prrn-aadA-TpsbA*) + RTR which was digested by *KpnI*. As a result, pUC19 carrying LTR + (*PatpI-cloning site-Trps16*) + (*Prrn-aadA-TpsbA*) + RTR was generated, and this chloroplast expression vector for spinach was designated as pCEV1 (8375 bp; **Figure 3a**).

### 2.3 Cloning of GOI into pCEV1

PCR amplification was carried out using LCY-F (containing *NotI* site) and LCY-R (containing *AscI* site) as primers and 500 ng plasmid DNA of our tomato cDNA clone encoding lycopene  $\beta$ -cylase (LCY, 1503 bp; Accession no. EF650013) as DNA template. After PCR amplification, PCR products were purified and cloned into pGEM-T Easy vector. Plasmid carrying *NotI-LCY-AscI* was isolated and double digested by *NotI* and *AscI* and then ligated with pCEV1 which was double digested by *NotI* and *AscI*, resulting pCEV1/LCY (9878 bp; **Figure 3b**). Similarly, PCR amplification was carried out using ZDS-F (containing *NotI* site) and ZDS-R (containing *AscI* site) as primers and 500 ng plasmid DNA of our tomato cDNA



**Figure 3.** Chloroplast expression vector pCEV1 and its derivatives specific for spinach. (a) pCEV1. Cloning sites (*NotI*, *SacII*, *AscI*) were inserted between *atpI* promoter and *rps16* terminator. (b) pCEV1/LCY. The cDNA fragment (1503 bp) encoding lycopene  $\beta$ -cylase (LCY) from tomato was cloned into pCEV1 which was double digested with *NotI* and *AscI*, resulting in pCEV1/LCY. (c) pCEV1/ZDS. The cDNA fragment (1767 bp) encoding  $\zeta$ -carotene desaturase (ZDS) from tomato was cloned into pCEV1 which was double digested with *NotI* and *AscI*, resulting in pCEV1/LCY. (d) pCEV1/GFP-GUS. The DNA fragment (2568 bp) encoding mGFP5-GUSA fusion protein from plasmid pCAMBIA1304 was cloned into pCEV1 which was double digested with *NotI* and *AscI*, resulting in pCEV1/GFP-GUS.



clone encoding  $\zeta$ -carotene desaturase (ZDS, 1767 bp; Accession no. EF650012) as DNA template. After PCR amplification, PCR products were purified and cloned into pGEM-T Easy vector. Plasmid carrying *NotI*-ZDS-*AscI* was isolated and double digested by *NotI* and *AscI* and then ligated with pCEV1 which was double digested by *NotI* and *AscI*, resulting in pCEV1/ZDS (10,142 bp; **Figure 3c**). For cloning the gene (2568 bp) encoding mGFP5-GUSA fusion protein, PCR amplification was carried out using mGFP5-F (containing *NotI* site) and GUSA-R (containing *AscI* site) as primers and 500 ng plasmid DNA of pCAMBIA1304 (Center for the Application of Molecular Biology to International Agriculture, Australia) as DNA template. After PCR amplification, PCR products were purified and cloned into pGEM-T Easy vector. Plasmid carrying *NotI*-GFP-GUS-*AscI* was isolated and double digested by *NotI* and *AscI* and then ligated with pCEV1 which was double digested by *NotI* and *AscI*, resulting in pCEV1/GFP-GUS (10,943 bp; **Figure 3d**).

## 2.4 Construction of chloroplast expression vectors pCEV2 and pCEV3 and their derivatives

### 2.4.1 Cloning of expression cassette [(*PatpI*-*Trps16*) + (*Prrn-aadA*-*TpsbA*)] into pUC19

PCR amplification was carried out using *SacI*+*PatpI*-F (containing the *SacI* site) and *TpsbA*+*PstII*-R (containing the *PstII* site) as primers and 500 ng pCEV1 plasmid DNA as template. After PCR amplification, PCR products (1.7 kb) were purified and double digested by *SacI* and *PstII* and then ligated with pUC19 (ampicillin resistance marker; 2686 bp) which was double digested by *SacI* and *PstII*, resulting in pUC19/(*PatpI*-*Trps16*) + (*Prrn-aadA*-*TpsbA*). As described in pCEV1, cloning sites (*NotI*, *SacII*, *AscI*) for GOI are located between *PatpI* and *Trps16*. Thus, the resulting plasmid carrying expression cassette for GOI and *aadA* selection marker on pUC19 was designated as pECaad (4343 bp).

### 2.4.2 Cloning of tomato LTR (containing partial 16S rRNA and full-length *trnI*; 1994 bp) into pECaad

PCR amplification was carried out using *SacI*+16S/*trnI*-F (containing the *SacI* site) and Le 16S/*trnI*+*SacI*-R (containing the *SacI* site) as primers and 500 ng tomato (*S. lycopersicum* var. CL5915) genomic DNA as template. After PCR amplification, PCR products (2 kb) were isolated and cloned into pGEM-T Easy vector. Plasmids carrying *SacI*-LTR-*SacI* were isolated and digested by *SacI* and then ligated with pECaad which was digested by *SacI*, resulting in pUC19/LTR + (*PatpI*-*Trps16*) + (*Prrn-aadA*-*TpsbA*).

### 2.4.3 Cloning of tomato RTR (containing full-length *trnA* and partial 23S rRNA; 2004 bp) into pUC19/LTR + (*PatpI*-*Trps16*) + (*Prrn-aadA*-*TpsbA*)

PCR amplification was carried out using Le *PstI*+*trnA*/23S-F (containing the *PstI* site) and *trnA*/23S+*PstI*-R (containing the *PstI* site) as primers and tomato (*S. lycopersicum* var. CL5915) genomic DNA as template. After PCR amplification, PCR products (2 kb) were isolated and cloned into pGEM-T Easy vector. Plasmid carrying *PstI*-LTR-*PstI* was isolated and digested by *PstI* and then ligated with pUC19/LTR + (*PatpI*-*Trps16*) + (*Prrn-aadA*-*TpsbA*) which was digested by *PstI*. As a result, pUC19 carrying LTR + (*PatpI*-*Trps16*) + (*Prrn-aadA*-*TpsbA*) + RTR was generated, and this chloroplast expression vector for tomato was designated as pCEV2 (8353 bp).

#### 2.4.4 Cloning of GOI into pCEV2

As described in pCEV1, for cloning the gene (2568 bp) encoding GFP:GUS fusion protein, PCR amplification was carried out using mGPF5-F (containing *NotI* site) and GUSA-R (containing *AscI* site) as primers and 500 ng plasmid DNA of pCAMBIA1304 as DNA template. After PCR amplification, PCR products were purified and cloned into pGEM-T Easy vector. Plasmid carrying *NotI*-GFP-GUS-*AscI* was isolated and double digested by *NotI* and *AscI* and then ligated with pCEV2 which was double digested by *NotI* and *AscI*, resulting in pCEV2/GFP-GUS (10,921 bp).

#### 2.4.5 Construction of pCEV3 and pCEV3/GFP-GUS

To clone the *nptII* gene (795 bp), PCR amplification was carried out using BamHI+NPTII-F (containing *BamHI* site) and XbaI+NPTII-R (containing *XbaI* site) as primers and 500 ng pBI121 (GenBank Accession no. AF485783; [27]) plasmid DNA as template. After PCR amplification, PCR products (0.8 kb) were purified and double digested by *BamHI* and *XbaI* and then ligated with pECaad [i.e., pUC19/(PatpI-Trps16) + (Prrn-aadA-TpsbA)] which was double digested by *BamHI* and *XbaI*. Thus, the *BamHI*-aadA-*XbaI* fragment was replaced by the *BamHI*-*nptII*-*XbaI* fragment. As a result, pUC19/(PatpI-Trps16) + (Prrn-*nptII*-TpsbA) was generated and designated as pECnpt (4346 bp). Again, as described in pCEV1, cloning sites (*NotI*, *SacII*, *AscI*) for GOI are located between PatpI and Trps16. The rest of the protocol for cloning tomato LTR and RTR sequences was the same as described for pCEV2. Finally, the new chloroplast expression vector, carrying *nptII* gene as selection marker for kanamycin resistance, for tomato was designated as pCEV3 (8356 bp). Using the same strategy, the gene encoding mGFP5-GUSA fusion protein was inserted into pCEV3, resulting in pCEV3/GFP-GUS (10,924 bp).

### 2.5 Construction of chloroplast expression vector pCEV4 and its derivatives

Similar to pCEV2, *N. benthamiana* (Accession no. FJ217346) LTR (containing partial 16S *rRNA* and full-length *trnI*; 1995 bp) was first cloned into pECaad [i.e., pUC19/(PatpI-Trps16) + (Prrn-aadA-TpsbA)] as follows: PCR amplification was carried out using Nb-*SacI*+16S/*trnI*-F (containing the *SacI* site) and Nb-16S/*trnI*+*SacI*-R (containing the *SacI* site) as primers and 500 ng *N. benthamiana* genomic DNA as template. After PCR amplification, PCR products (2 kb) were isolated and cloned into pGEM-T Easy vector. Plasmid carrying *SacI*-LTR-*SacI* was isolated and digested by *SacI* and then ligated with pECaad which was digested by *SacI*, resulting in pUC19/LTR + (PatpI-Trps16) + (Prrn-aadA-TpsbA). Then, cloning of *N. benthamiana* RTR (containing full-length *trnA* and partial 23S *rRNA*; 1908 bp) into the above plasmid pUC19/LTR + (PatpI-Trps16) + (Prrn-aadA-TpsbA) was described as follows: PCR amplification was carried out using Nb-*PstI*+*trnA*/23S-F (containing the *PstI* site) and Nb-*trnA*/23S + *PstI*-R (containing the *PstI* site) as primers and 500 ng *N. benthamiana* genomic DNA as template. After PCR amplification, PCR products (1.9 kb) were isolated and cloned into pGEM-T Easy vector. Plasmid carrying *PstI*-RTR-*PstI* was isolated and digested by *PstI* and then ligated with pUC19/LTR + (PatpI-Trps16) + (Prrn-aadA-TpsbA) which was digested by *PstI*. As a result, pUC19 carrying LTR + (PatpI-Trps16) + (Prrn-aadA-TpsbA) + RTR was generated, and this chloroplast expression vector for *N. benthamiana* was designated as pCEV4 (8246 bp).

For cloning of GOI into pCEV4, PCR amplification was carried out using *NotI*-BaMV\_S-F (containing the *NotI* site) and BaMV\_S\_CP-*AscI*-R (containing the *AscI* site) as primers and 500 ng plasmid pBS2-8 DNA carrying bamboo mosaic virus

strain S complete genome (6366 bp; GenBank Accession number AF018156) as DNA template. After PCR amplification, PCR products (753 bp containing enzyme cutting sites) for coding region of CP20 were purified and cloned into pGEM-T Easy vector. Plasmid carrying *NotI*-BaMV\_S\_CP-*AscI* was isolated and double digested by *NotI* and *AscI* and then ligated with pCEV4 which was double digested by *NotI* and *AscI*, resulting in pCEV4/BaMV (8975 bp). In addition, PCR amplification was carried out using *NotI*-BSL6-F (containing the *NotI* site) and BSL6-*AscI*-R (containing the *AscI* site) as primers and 500 ng plasmid pBSL6 DNA [carrying 5'-untranslated region (5'-UTR), coding region of CP20, and 3'-UTR] as DNA template. After PCR amplification, PCR products (861 bp containing enzyme cutting sites) of BSL6 (i.e., CP20 containing 5'-UTR and 3'-UTR) were purified and cloned into pGEM-T Easy vector. Plasmid carrying *NotI*-BSL6-*AscI* was isolated and double digested by *NotI* and *AscI* and then ligated with pCEV4 which was double digested by *NotI* and *AscI*, resulting in pCEV4/BSL6 (9083 bp). For cloning the gene encoding mGFP5-GUSA fusion protein, the same strategy was used as for pCEV1. In brief, PCR products of *NotI*-GFP-GUS-*AscI* were isolated and double digested by *NotI* and *AscI* and then ligated with pCEV4 which was double digested by *NotI* and *AscI*, resulting in pCEV4/GFP-GUS (10,814 bp).

## 2.6 Bombardment and transient GUS expression in spinach leaves

Spinach (*S. oleracea* cv. Green Giant; Known-You Seed Company, Taiwan) young leaves were excised from in vitro-grown plantlets and inoculated on culture medium [MS salts and vitamins (Duchefa Biochemie B.V., The Netherlands); 3% sucrose; 0.4 mg l<sup>-1</sup> NAA; 1.0 mg l<sup>-1</sup> BA; 1.0 mg l<sup>-1</sup> GA<sub>3</sub>; 0.8% Bacto agar, pH 5.8] in the dark for 3 days. Then, leaves were bombarded by the biolistic bombardment device (PDS-1000/He Particle Delivery System, Bio-Rad) with 1.25 mg of 0.6 μm gold particles (Bio-Rad) coated with 5 μg of plasmid DNA (pCEV1/GFP-GUS or pCAMBIA1305.1) using 1100 psi rupture disks (Bio-Rad). The distance from the uncovered Petri dish containing the leaf sample to the top of the PDS chamber was set to 9 cm, and the vacuum was set to 27 mm Hg. After bombardment, the samples were kept in the dark for 1 day.

GUS activity in bombarded leaves was determined according to the histochemical procedure [28]. Briefly, spinach leaves were stained in staining buffer [100 mM phosphate buffer; pH 7.0; 0.5 mM ferrocyanide; 0.5 mM ferricyanide; 10 mM EDTA, pH 8.0; 0.1% Triton X-100; 1 mM Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, USB)] and incubated overnight at 37°C. Chlorophylls from leaf tissue were removed by soaking in 70% ethanol several times.

## 2.7 Plastid transformation in spinach and tomato

Leaf segments were cut from in vitro-grown plantlets of spinach and inoculated on SO culture medium (MS salts and vitamins; 3% sucrose; 0.5 mg l<sup>-1</sup> 2,4-D; 2 mg l<sup>-1</sup> kinetin; 0.8% Bacto agar; pH 5.8) for 1 day in a growth chamber with a cycle of 8 h light at 22°C and 16 h dark at 20°C. Conditions for bombardment were as described above, except that plasmid pCEV1/GFP-GUS, pCEV1/LCY, or pCEV1/ZDS was used in each individual experiment. After bombardment, the sample was cultured in the dark for 4 days. Then, the bombarded leaf segments were trimmed into small pieces of 2–4 mm<sup>2</sup>, transferred onto SO selection medium (SO culture medium supplemented with 300 mg l<sup>-1</sup> spectinomycin), and cultured for 1 month in a growth chamber with a cycle of 8 h light at 22°C and 16 h dark at 20°C. Survival green calli were picked up and transferred onto fresh SO selection medium and cultured for another 1 month in the same growth chamber.

For plastid transformation in tomato, leaf segments were cut from plantlets of tomato (*S. lycopersicum* cv. CL5915) grown in vitro and inoculated on SL culture medium (MS salts and vitamins; 3% sucrose; 0.2 mg l<sup>-1</sup> 2,4-D; 0.1 mg l<sup>-1</sup> kinetin; 0.8% Bacto agar; pH 5.8) for 1 day in a growth chamber with a cycle of 8 h light at 22°C and 16 h dark at 20°C. Conditions for bombardment were as described above, except that plasmid pCEV2/GFP-GUS or pCEV3/GFP-GUS was used in each individual experiment. After bombardment, the sample was cultured in the dark for 3 days. Then, the bombarded leaf segments were trimmed into small pieces of 2–4 mm<sup>2</sup>, transferred onto SL selection medium (MS salts and vitamins; 3% sucrose; 1 mg l<sup>-1</sup> NAA; 1 mg l<sup>-1</sup> zeatin; 0.8% Bacto agar; pH 5.8), and supplemented with 300 mg l<sup>-1</sup> spectinomycin (for pCEV2/GFP-GUS) or 50 mg l<sup>-1</sup> kanamycin (for pCEV3/GFP-GUS) for 1 month in the same growth chamber. Survival green calli were picked up and transferred on fresh selection medium for another 1 month.

## 2.8 Plastid transformation and selection of transplastomic lines in *N. benthamiana*

Leaf segments were cut from in vitro-grown plantlets of *N. benthamiana* and inoculated on NB culture medium (MS salts and vitamins; 3% sucrose; 0.1 mg l<sup>-1</sup> NAA; 1.0 mg l<sup>-1</sup> BA; 0.8% Bacto agar; pH 5.8) for 1 day in a growth chamber with a cycle of 8 h light at 22°C and 16 h dark at 20°C. Conditions for bombardment were previously described, except that plasmid pCEV4/BaMV or pCEV4/BSL6 was used in each individual experiment. After bombardment, the sample was cultured in the dark for 3 days. Then, the bombarded leaf segments were trimmed into small pieces of 2–4 mm<sup>2</sup>, transferred onto NB selection medium (NB culture medium supplemented with 300 mg l<sup>-1</sup> spectinomycin), and cultured for 1 month in a growth chamber with a cycle of 8 h light at 22°C and 16 h dark at 20°C. Survival green shoots were excised and transferred onto MS basal medium for rooting. The rooted plantlets were transferred into pots and grown in a greenhouse.

## 2.9 Transplastomic plant verification by PCR analysis

Transplastomic plant verification was carried out with putative transformants and wild-type *N. benthamiana* plants by PCR analysis. Total genomic DNA was extracted from green leaves of putative transformants and wild-type plants using the CTAB method [26]. PCR was carried out with the following sets of primers (Table A1): BSL-F and BSL-R for amplification of 837-bp-long DNA fragment corresponding to the 5' UTR coding region and 3' UTR of coat protein (CP20) in BaMV strain S (GenBank Accession no. AF018156), BaMV-F and BaMV-R for amplification of 729-bp-long DNA fragment corresponding to the coding region of CP20 in BaMV strain S, TpsbA-F and trnI-trnA-r for amplification of 351-bp-long DNA fragment corresponding to the truncated sequence composed of the transgenic TpsbA and partial RTR of *N. benthamiana* chloroplast genome, and trnA-f and 23S-rRNA-r for amplification of 342-bp-long DNA fragment located within the RTR of *N. benthamiana* chloroplast genome. PCR reactions were performed under the following conditions: (a) denaturation for 5 min at 94°C; (b) 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C; and (c) extension for 10 min at 72°C. Following amplification, PCR products were analyzed by electrophoresis.

## 2.10 Seedling assay for spectinomycin resistance

T<sub>1</sub> seeds from a self-pollinated transplastomic plant were sterilized in 70% ethanol for 0.5 min followed by 1% sodium hypochlorite for 10 min and washed

thoroughly with sterile distilled water. The sterile seeds were germinated on a medium containing MS basal medium supplemented with  $300 \text{ mg l}^{-1}$  spectinomycin in a  $25^\circ\text{C}$  growth chamber under 16 h illumination for 4 weeks. Seedlings with green cotyledons were considered to be resistant, while those with white cotyledons were sensitive.

### 2.11 GenBank accession numbers

Nucleotide sequences in this study were submitted into GenBank with the following accession numbers: pCEV1, KY930497; pCEV2, KY930498; pCEV3, KY930499; pCEV4, KY930500; pECaad, KY930501; pECnpt, KY930502; *Nicotiana benthamiana* chloroplast DNA fragment containing partial 16S *rRNA*, *trnI*, *trnA*, and partial 23S *rRNA*, KY930503.

## 3. Results and discussion

### 3.1 Construction of spinach chloroplast expression vectors

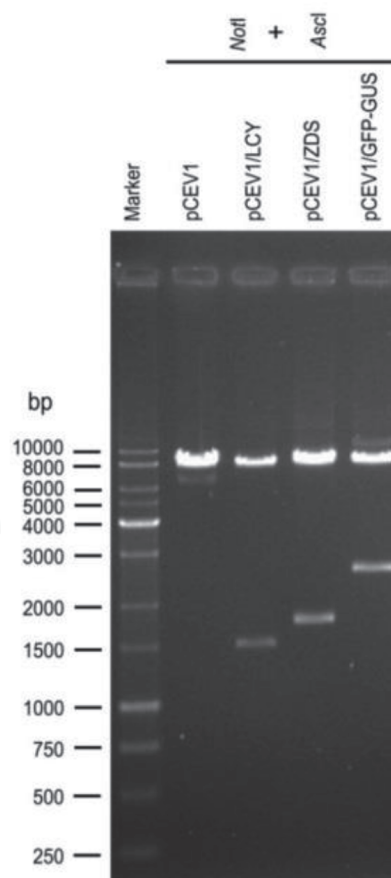
Although the complete chloroplast genome of spinach has been published [22], plastid transformation in spinach has not yet been reported. As a first step in achieving plastid transformation in spinach, spinach-specific chloroplast expression vectors must be constructed. As shown in **Figure 1**, the integration site for the expression cassette is designed at the spacer region between *trnI* and *trnA*. This integration site at *trnI/trnA* has been commonly used in constructing chloroplast expression vectors in different plants [1]. In addition, the *trnI* gene contains the chloroplast replication origin which might promote replication of foreign vectors within chloroplasts and enhance the transgene integration [1, 29]. Another feature of our chloroplast expression vectors is the use of longer sequences for targeting via homologous recombination: the left targeting region (ca. 2 kb) contains partial 16S *rRNA* and full-length *trnI*, whereas the right targeting region (ca. 2 kb) contains full-length *trnA* and partial 23S *rRNA* (**Figure 1**). Targeting sequences are usually 1 kb each in size and are located on both sides of the expression cassette [1]. However, a 4 kb DNA fragment containing 16S *rRNA-trnI/trnA-23S rRNA* (i.e., 2 kb each on either side of the expression cassette), which is twice the size of the flanking region commonly used in chloroplast transformation, was used in cotton chloroplast transformation [9]. It has been reported that the frequency of homologous recombination is linearly reliant on the length of the homologous sequences [30]. Thus, in this study, each of the left/right targeting sequences was increased to 2 kb (total 4 kb in length), similar in size to that described in cotton chloroplast transformation [9].

Next, each DNA fragment including the left and right targeting regions, synthetic unique cloning sites (*NotI*, *SacII*, *AscI*) flanked by *atpI* promoter and *rps16* terminator, as well as *aadA* selection marker gene flanked by *rrn* promoter and *psbA* terminator (**Figure 1**) were amplified by PCR and cloned and inserted into pUC19 according to a step-by-step protocol (**Figure 2**), and finally a chloroplast expression vector for spinach (designated as pCEV1) was constructed (**Figure 3a**). Any GOI may be cloned into pCEV1 by using the restriction enzyme *NotI*, *SacII*, or *AscI*. However in this study, pCEV1 was double digested by *NotI* and *AscI* and ligated with a PCR fragment of the GOI carrying *NotI* or *AscI* restriction site at each end. As a result, pCEV1/LCY [carrying full-length cDNA (1503 bp) encoding LCY from tomato], pCEV1/ZDS [carrying full-length cDNA (1767 bp) encoding ZDS from tomato], and pCEV1/GFP-GUS [carrying mGFP5-GUSA fusion protein gene

(2568 bp) from pCAMBIA1304] were constructed (**Figure 3**). To check the accuracy of the cloning sites, plasmid DNA was isolated from *E. coli* carrying pCEV1, pCEV1/ZDS, pCEV1/LCY, or pCEV1/GFP-GUS and double digested with *Not*I and *Asc*I. As shown in **Figure 4**, only a large fragment (it should be 8367 bp) was detected in pCEV1 after double digestion was carried out; the small fragment (it should be 8 bp) was not observed. As predicted, the same size large fragment (it should be 8367 bp) was detected in pCEV1/ZDS, pCEV1/LCY, or pCEV1/GFP-GUS; however, different sizes of small fragment including coding regions of LCY (1503 bp), ZDS (1767 bp), and GFP-GUS (2568 bp) were easy to detect and match according to the prediction (**Figure 4**). Thus, we concluded that spinach chloroplast expression vector pCEV1 and its derivatives were successfully constructed.

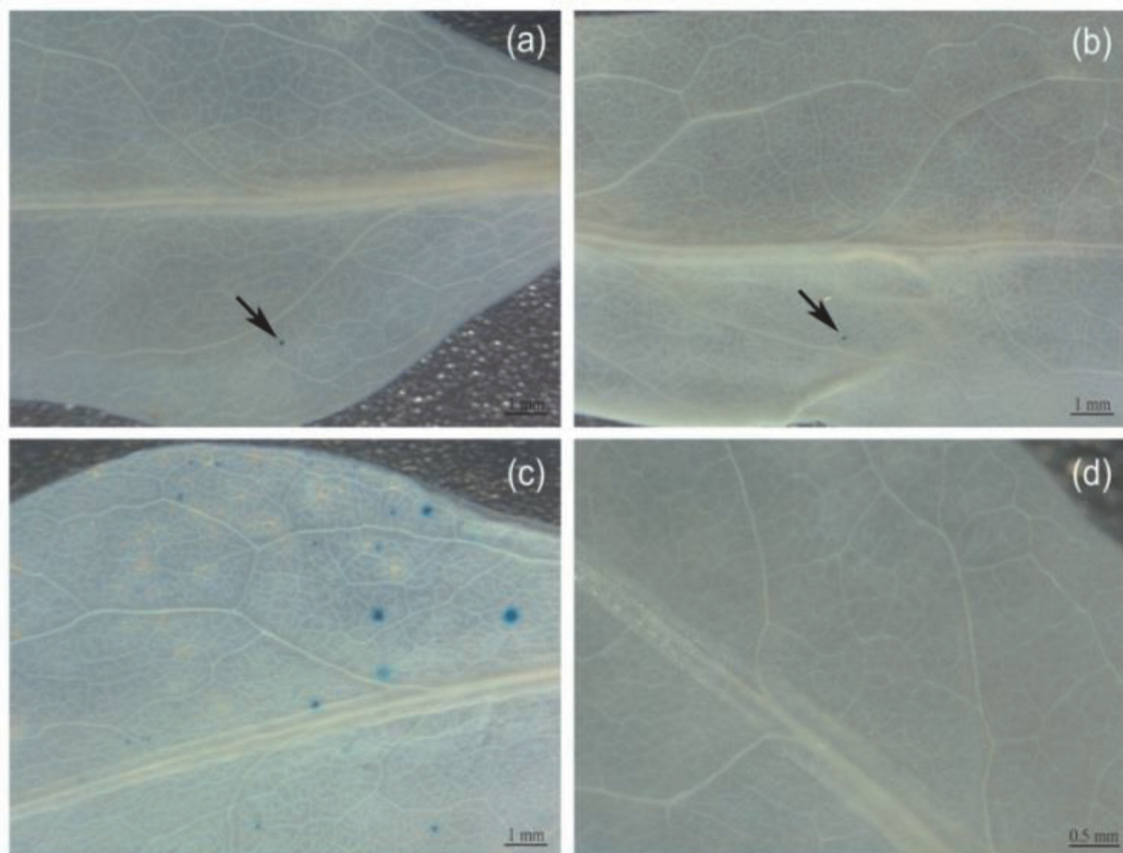
### 3.2 Transient GUS expression and development of plastid transformation in spinach

To evaluate the functionality of the constructed vector, spinach chloroplast expression vector pCEV1/GFP-GUS [carrying a mGFP5-GUSA fusion protein gene driven by plastid *atpI* promoter (PatpI) from tobacco] (**Figure 3d**) or positive nuclear control pCAMBIA1305.1 [carrying GUSPlus driven by cauliflower mosaic virus (CaMV) 35S promoter] was used to independently bombard the spinach leaves followed by GUS staining. As shown in **Figure 5d**, no blue spots were detected in leaf samples without bombardment (negative control). Only one to two



**Figure 4.** Restriction endonuclease analysis for the cloning sites in pCEV1 and its derivatives. Three milligrams of vector DNA was double digested with *Not*I (10 U) and *Asc*I (10 U) for 2 h at 37°C, and then the restriction fragments were separated by 0.8% agarose gel electrophoresis in TAE buffer. After digestion, the expected fragments (in bp) for each vector were as follows: pCEV1, 8367 and 8; pCEV1/LCY, 8367 and 1511; pCEV1/ZDS, 8367 and 1775; and pCEV1/GFP-GUS, 8367 and 2576. The observed fragments in each digested vector were the same sizes as predicted.

very small blue spots per leaf were detected in leaves bombarded with pCEV1/GFP-GUS (**Figure 5a** and **b**). This is because to display blue spots, the golden particles carrying pCEV1/GFP-GUS need to pass through the cell wall and then the cell membrane and then the chloroplast membrane into the chloroplast of a cell, for which the probability is small, so the tiny but condensed blue spots indicated that GUS expression was indeed detected within chloroplasts. On the other hand, more than 10 blue spots per leaf were detected when bombardment was carried out with pCAMBIA1305.1, and the sizes of the blue spots were much larger than those blue spots bombarded with pCEV1/GFP-GUS (**Figure 5c**). It is worth pointing out that pCAMBIA1305.1 is a cloning vector for stable plant transformation. Just like other pCAMBIA vectors, the T-DNA region (containing selection marker *nptII* gene, expression cassette for GOI and reporter gene *GUSPlus*) in pCAMBIA1305.1 will integrate into the plant nuclear genome via a stable transformation process. However in the bombardment experiment followed by transient GUS staining assay, cytosolic GUS activity can also be detected when plant tissue is bombarded with pCAMBIA series (such as pCAMBIA1305.1), since the *gus* gene in the transformation vector is driven by non-tissue specific and constitutive CaMV 35S promoter. Particularly, CAMBIA's *GUSPlus* is a patented reporter gene isolated from *Staphylococcus* species with some properties that are superior to the conventional *E. coli gusA* gene (<http://www.cambia.org>). Previously, Ye et al. [31] reported that a chloroplast expression vector pHD203-GUS [carrying *gus* and chloramphenicol acetyltransferase (*cat*) genes driven by a double chloroplast *psbA* promoter fragment from pea (in opposite orientation)] and a positive nuclear control pBI505 (carrying a *gus* gene driven by a double CaMV 35S promoter plus a leader sequence



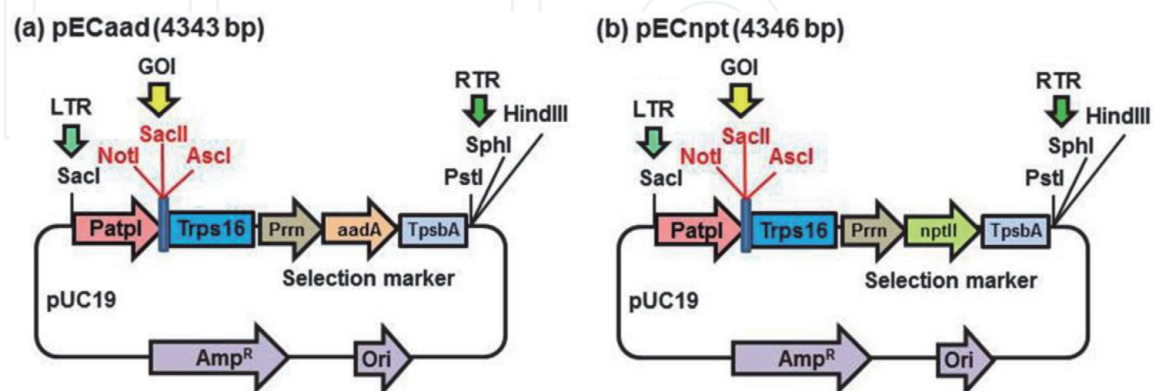
**Figure 5.** Transient GUS expression. Spinach leaves were bombarded with golden particles coated with equal amounts (5 mg) of plastid vector pCEV1/GFP-GUS (a, b) or nuclear vector pCAMBIA1305.1 (c). After bombardment, samples were kept in the dark for 1 day, and then GUS staining was carried out. Spinach leaf without bombardment was used as a negative control (d).

from alfalfa mosaic virus) were separately bombarded into tobacco suspension cells, followed by GUS staining, and examined by microscopy. Cells bombarded with pBI505 showed high levels of GUS expression, as indicated by blue spots, and were distributed evenly throughout the cytosol of the transformed cells, whereas pHD203-GUS was expressed in chloroplasts. Chloroplast transient expression rates appeared to be 40–50-fold or even 300–400-fold lower than nuclear transformation rates [31]. Later, the same chloroplast expression vector pHD203-GUS together with positive (pBI221) and negative (pUC18) controls was used to develop an electroporation-mediated method for the study of foreign gene expression within spinach chloroplasts, and this study found that both GUS and CAT activities were detected in chloroplasts electroporated with pHD203-GUS but not with both controls; the expression of GUS protein in pHD203-GUS-treated chloroplasts was further confirmed by Western blot analysis [32]. Taken together, our observation in transient GUS expression is consistent with what was previously described under microscopy [31] and confirms that pCEV1/GFP-GUS is a functional vector.

Next, leaves from in vitro-grown plantlets of spinach (*S. oleracea* cv. Green Giant) were excised and bombarded with pCEV1/LCY, pCEV1/ZDS, and pCEV1/GFP-GUS individually. Almost all leaf segments became larger but turned brown in color after incubation for 1 month in selective medium, suggesting that the screening medium is effective (Figure A1b). Eventually, a few green calli were identified (small panel in Figure A1b) from hundreds of explants after bombardment. Currently, plant regeneration from spectinomycin-resistant calli is in progress.

### 3.3 Construction of chloroplast expression vectors for tomato and *N. benthamiana*

In order to rapidly construct other chloroplast expression vectors for other plant species (i.e., tomato and *N. benthamiana*), a DNA fragment (1667 bp) containing expression cassettes of *atpI* promoter and cloning sites for GOI and *rps16* terminator, followed by selection marker *aadA* gene, was amplified by a PCR strategy using a pCEV1 as a DNA template. After double digestion with *SacI* and *PstI*, the PCR product was ligated with pUC19 which was double digested with *SacI* and *PstI*, resulting in a new auxiliary vector pECaad (Figure 6a). Accuracy of the inserted DNA fragment has been confirmed by DNA sequencing. Thus, tomato LTR



**Figure 6.** Schematic representation of the auxiliary vectors pECaad (a) and pECnpt (b). Selection marker aminoglycoside 3'-adenyltransferase (*aadA*; 792 bp) was used in pECaad (a), and selection marker neomycin phosphotransferase (*nptII*; 795 bp) conferring kanamycin resistance was used in pECnpt (b). Besides the selection marker genes, the other sequences in both vectors were the same. The left targeting region (LTR) from the plastid genome of plant species can be inserted into these vectors at the *SacI* site, while the right targeting sites from pUC19. Gene of interest (GOI) can be cloned into these vectors by using our cloning sites (*NotI*, *SacII*, *AscI*).



(containing partial 16S *rRNA* and *trnI*) can be inserted into the *SacI* site of the 5' terminus by using forward primer **SacI+16S/trnI-F** and reverse primer **Le-16S/trnI+SacI-R** and tomato DNA as a DNA template for PCR amplification; and tomato RTR (containing *trnA* and partial 23S *rRNA*) can be inserted into the *PstI* site of the 3' terminus by using forward primer **Le-PstI+trnA/23S-F** and reverse primer **trnA/23S+PstI-R** and tomato DNA as a DNA template for PCR amplification. As a result, chloroplast expression vector pCEV2 for tomato was constructed (**Figure A2**). To clone the GOI into pCEV2, the PCR fragment, carrying GFP:GUS fusion protein gene with *NotI* at one end and *AscI* at the other end, was inserted into pCEV2 which was double digested with *NotI* and *AscI*, resulting in pCEV2/GFP-GUS (**Figure A2**).

Complete plastid genome sequences have been reported for tobacco (*N. tabacum*; NC\_001879), spinach (*S. oleracea*; NC\_002202), and tomato (*S. lycopersicum*; NC\_007898) but not for *N. benthamiana*. However, partial chloroplast sequence of *N. benthamiana* 16S-23S ribosomal RNA intergenic spacer including *trnI* and *trnA* genes has been determined (1874 bp; FJ217346). In order to prepare a longer targeting sequence (ca. 4 kb in total) in *N. benthamiana*, forward and reverse primers were designed within 16S *rRNA* and 23S *rRNA*, respectively, according to the plastid genome of *N. tabacum*, and total genomic DNA from *N. benthamiana* was used as a DNA template for PCR amplification. The PCR product was cloned and then sequenced. As expected, a longer targeting sequence (3893 bp) containing partial 16S *rRNA*, *trnI*, *trnA*, and partial 23S *rRNA* in *N. benthamiana* was obtained. As shown in **Figure A3**, 9 bp (GTTCGGCCT) insertion was conducted in *N. benthamiana* as compared to *N. tabacum* plastid genome. The same 9 bp insertion, located in the intron of *trnI* in *N. benthamiana*, has been reported [33]. In addition, two more nucleotides, indicated by the first and second arrow in **Figure A3**, were changed between *N. tabacum* and our *N. benthamiana* sequence. Moreover, five unknown nucleotides (labeled as "N") in the published *N. benthamiana* sequence (FJ217346) were determined in our *N. benthamiana* sequence. These five nucleotides in our *N. benthamiana* sequence were identical to *N. tabacum* (**Figure A3**).

Using the strategy in pCEV2, LTR and RTR from *N. benthamiana* were rapidly amplified by PCR and then inserted into pECaad, resulting in chloroplast expression vector pCEV4 for *N. benthamiana* (**Figure A4**). Similarly, GOI regarding *gfp:gus*, coat protein CP20 gene with or without 5'- and 3'-untranslated region (UTR) from BaMV satellite RNA was cloned separately into pCEV4 which was double digested with *NotI* and *AscI*, resulting in pCEV4/GFP-GUS, pCEV4/BSL6 (CP20 containing 5'- and 3'-UTR) and pCEV4/BaMV (CP20 only), respectively (**Figure A4**). Therefore, the GOI can be inserted into our chloroplast expression vectors pCEV1, pCEV2, and pCEV4 specific for spinach, tomato, and *N. benthamiana*, respectively. Moreover, although the *trnI/trnA* fragment (here we used the longer sequence of partial 16S *rRNA-trnI/trnA*-partial 23S *rRNA*) is one of the most frequently used targeting region in plastid transformation [3, 5], if a researcher wants to use another targeting region, such as *rbcl/accD*, *rpl32/trnL*, *petA/psbJ*, *trnfM/trnG*, *trnN/trnR*, *atpB/rbcL*, *rps7/ndhB*, or *ycf3/trnS* [5], such chloroplast targeting sequence can be amplified and cloned into our auxiliary vector pECaad (**Figure 6a**) which carries the expression cassette for the GOI and selection marker *aadA* on pUC19. We believe that our auxiliary vector pECaad will be of benefit for constructing different targeting regions for plastid transformation.

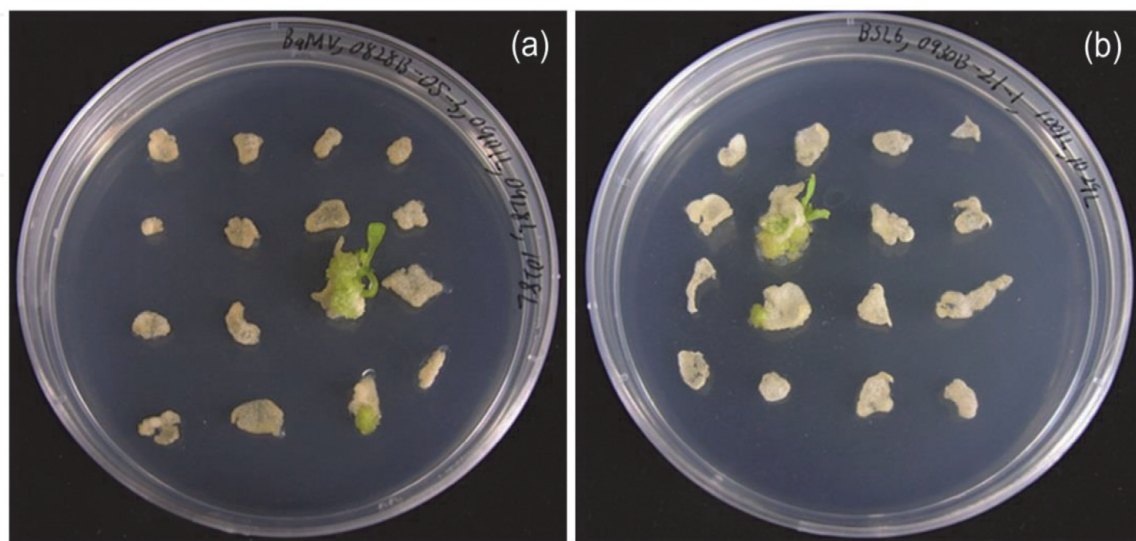
For selection in plastid transformation, *aadA* gene conferring spectinomycin resistance is widely used and is the most efficient selective marker [2]; however, *nptII* gene conferring kanamycin resistance has also been developed for tobacco plastid transformation [34]. Thus, besides the selection marker *aadA* gene which has been used in pCEV1, pCEV2, pCEV4, and their derivatives, we also constructed *nptII* gene as a selection marker for tomato plastid transformation. In brief, PCR-

amplified BamHI-nptII-XbaI fragment was subcloned and replaced the BamHI-aadA-XbaI fragment in pECaad, generating another auxiliary vector pECnpt (**Figure 6b**) which carries an expression cassette for GOI and *nptII* selection marker on pUC19. Then, tomato LTR and RTR were sequentially cloned into pECnpt, generating a new chloroplast expression vector pCEV3 for tomato plastid transformation by using selection marker *nptII* gene (**Figure A2**). Similarly in pCEV2, the gene encoding GFP:GUS fusion protein was inserted into pCEV3 with double digestion by *NotI* and *AscI*, resulting in pCEV3/GFP-GUS (**Figure A2**).

### 3.4 Bombardment, selection, and verification of transplastomic *N. benthamiana* lines

To further evaluate the functional expression of our constructs, plasmids pCEV4/BaMV and pCEV4/BSL6 were bombarded separately into *N. benthamiana*. After bombardment, almost all leaf segments turned brown and finally died in NB selection medium containing  $300 \text{ mg l}^{-1}$  spectinomycin for 1-month culture. From a total of 344 leaf segments we used, only 1 shoot was regenerated using pCEV4/BaMV as a transforming vector (**Figure 7a**), and also only 1 shoot was regenerated from a total of 340 leaf segments examined using pCEV4/BSL6 as a transforming vector (**Figure 7b**). These shoots were cut and transferred into MS basal medium without spectinomycin for rooting. Finally, two putative transplastomic plantlets, one from pCEV4/BaMV and another from pCEV4/BSL6, were obtained and transferred into a greenhouse. Thus, the transformation efficiency for both vectors pCEV4/BaMV and pCEV4/BSL6 was 0.3%.

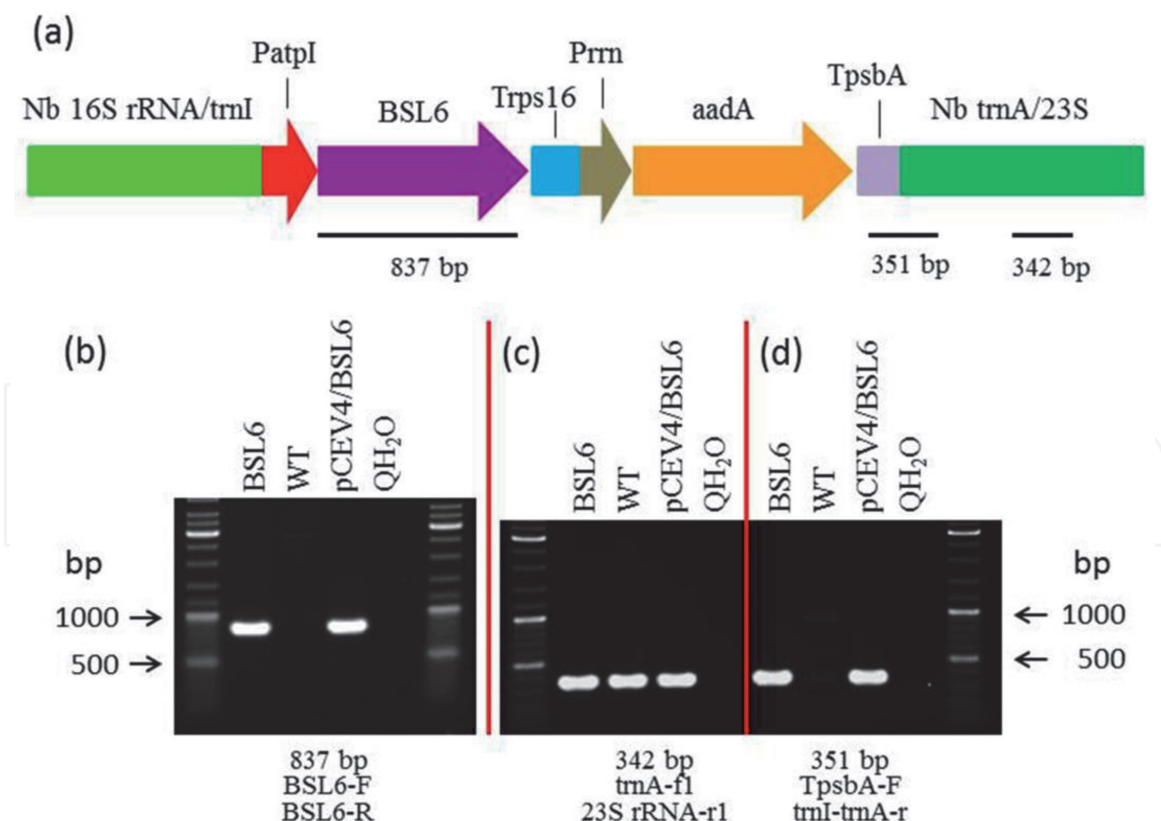
For the putative transgenic line transformed by pCEV4/BSL6, genomic DNA was isolated from leaves of the putative line and wild-type *N. benthamiana*, and PCR amplification was carried out. A unique band of 837 bp, representing CP20 coding region flanked by 5'-UTR and 3'-UTR in the transforming vector pCEV4/BSL6, was detected in samples from the transplastomic line BSL6 as well as the positive control vector pCEV4/BSL6 (**Figure 8b**). No PCR product was detected in the WT plant (**Figure 8b**). To demonstrate the quality of DNA we isolated, a primer set amplifying a 342 bp fragment between *trnA* and 23S *rRNA* of plastid genome in *N. benthamiana* was



**Figure 7.** Selection of transplastomic *Nicotiana benthamiana* lines. Vector DNA of pCEV4/BaMV (a) or pCEV4/BSL6 (b) was used to bombard leaf segments of *N. benthamiana*. After bombardment, leaf segments were trimmed into small pieces and transferred onto NB selection medium containing  $300 \text{ mg l}^{-1}$  spectinomycin for 1 month. Green shoots from selection medium were cut and transferred onto MS basal medium (without spectinomycin) for rooting.

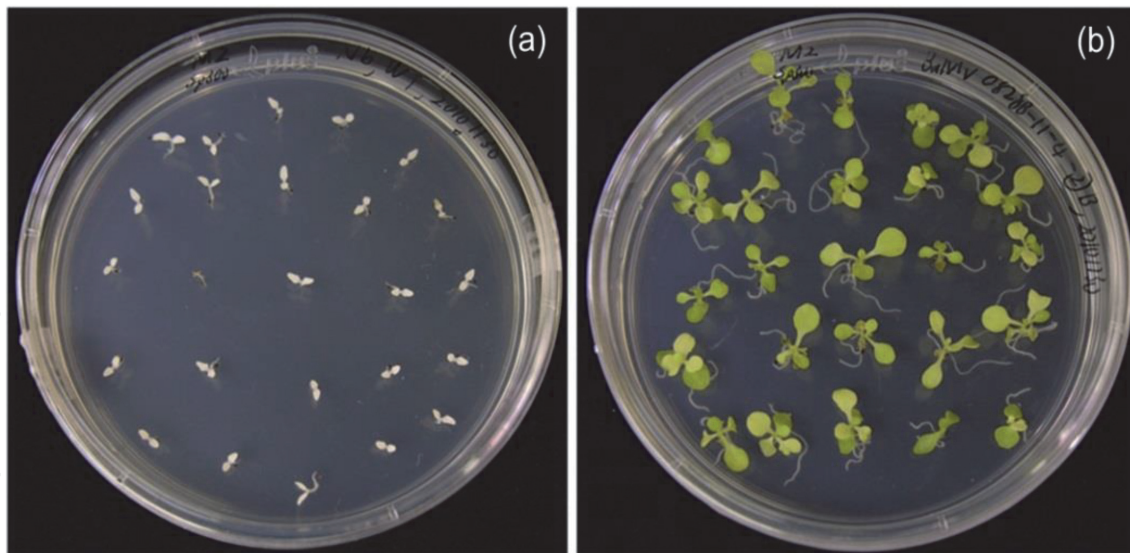
designed (**Figure 8a**). As expected, a unique band (ca. 0.3 kb) was detected in our transplastomic plant BSL6, the WT and the positive control vector pCEV4/BSL6 (**Figure 8c**). To further confirm the integration site in the transplastomic line, forward primer TpsbA-F was designed within the terminator region of *psbA* in the vector pCEV4/BSL6, and reverse primer trnA-r was located at the *trnA* of the right targeting region in the vector pCEV4/BSL6. As predicted, a unique band of 351 bp was detected in transplastomic plant BSL6 but not in the WT (**Figure 8d**). In conclusion, the expression cassette carrying CP20 encoding gene (with 5'- and 3'-UTR) and *aadA* selection marker gene flanked by LTR (i.e., Nb 16S rRNA/*trnI*) and RTR (i.e., Nb *trnA*/23S) was indeed integrated into the *trnI/trnA* integration site by homologous recombination of plastid genome in transplastomic line BSL6. Unfortunately, serious infection by microorganisms occurred in this plant and it finally died.

For the putative transplastomic line which was transformed by pCEV4/BaMv, self-pollinated seeds from the T<sub>0</sub> transplastomic line BaMV as well as WT seeds were sterilized and then germinated on MS basal medium supplemented with 300 mg l<sup>-1</sup> for 4 weeks. As shown in **Figure 9b**, all T<sub>1</sub> seedlings from the BaMV plant examined were resistant to spectinomycin, confirming an important characteristic of maternal inheritance in plastid transformation. By contrast, all seedlings from the WT were sensitive to spectinomycin (**Figure 9a**). To further confirm the transgene, three spectinomycin-resistant seedlings were randomly picked up from the selection plate, transferred onto MS basal medium without spectinomycin, and then grown in a greenhouse. Genomic DNA was isolated from these transplastomic T<sub>1</sub> as well as WT plants, and PCR analysis was then carried out (**Figure 10**).

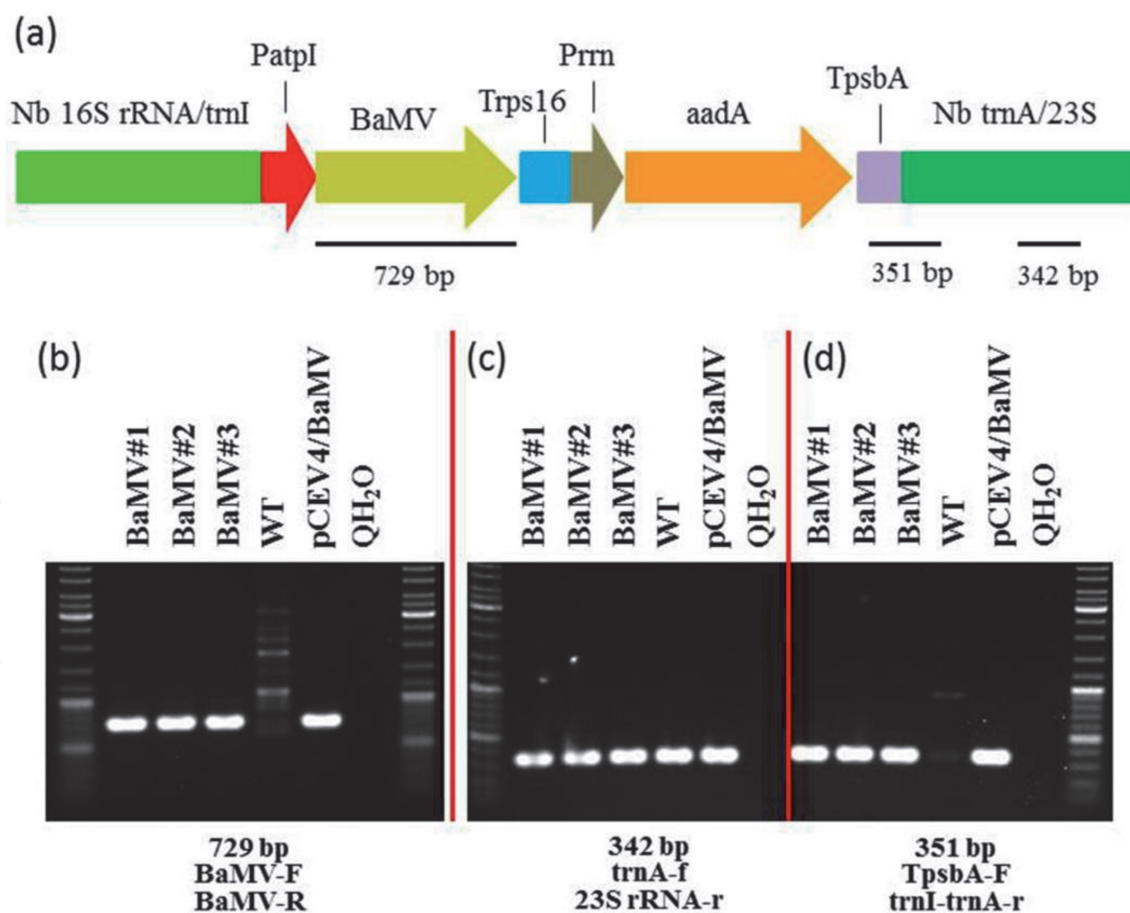


**Figure 8.**

PCR analysis of T<sub>0</sub> transplastomic *Nicotiana benthamiana* plant bombarded by pCEV4/BSL6. (a) Schematic representation of pCEV4/BSL6. Sizes and locations of gene-specific PCR products were indicated. (b) PCR analysis for transgene BSL6 (837 bp). The PCR product was only detected in the transplastomic line but not in the WT plant. (c) PCR analysis of the internal *trnA*/23S rRNA region (342 bp). PCR products were detected in both WT and transplastomic lines. (d) PCR analysis for the flanking region (351 bp). The PCR product was only detected in the transplastomic line but not in the WT plant. “QH<sub>2</sub>O” in each panel represented the negative control to which no DNA was added in the reaction mixture for PCR amplification.



**Figure 9.** Seedling assay of *T*<sub>0</sub> transplastomic *Nicotiana benthamiana* plant bombarded by pCEV4/BaMV. Seeds from the WT (a) and the transplastomic plant (b) were harvested, sterilized, and then sown on MS basal medium containing 300 mg l<sup>-1</sup> spectinomycin for 4 weeks. (a) All seedlings from the WT plant showed spectinomycin-sensitive phenotype. (b) All seedlings from the transplastomic plant showed spectinomycin-resistant phenotype.



**Figure 10.** PCR analysis of three random-selected *T*<sub>1</sub> transplastomic *Nicotiana benthamiana* plants bombarded by pCEV4/BaMV. (a) Schematic representation of pCEV4/BaMV. Sizes and locations of gene-specific PCR products were indicated. (b) PCR analysis for transgene BaMV (729 bp). PCR products were only detected in three transplastomic lines but not in the WT plant. (c) PCR analysis of the internal trnA/23S rRNA region (342 bp). PCR products were detected in WT and three transplastomic lines. (d) PCR analysis of the flanking region (351 bp). PCR products were detected in three transplastomic lines but not in the WT plant. “QH<sub>2</sub>O” in each panel represented the negative control to which no DNA was added in the reaction mixture for PCR amplification.

A unique band of 729 bp, which is the size of CP20 encoding gene (without 5'-UTR and 3'-UTR) in the transforming vector pCEV4/BaMV, was detected in all T<sub>1</sub> transplastomic lines BaMV#1, BaMV#2, and BaMV#3 but not in the WT plant (**Figure 10b**). Again, the same PCR primer set was employed for the amplification between *trnA* and 23S *rRNA* in the plastid genome of *N. benthamiana*. As expected, a unique band of 0.3 kb was detected in all three transplastomic lines and the WT plant (**Figure 10c**). A unique band of 351 bp, representing the flanking sequence around the integration site at the RTR (i.e., Nb *trnA*/23S), was only detected in the three transplastomic lines but not in the WT sample (**Figure 10d**). Here we clearly demonstrated the expression cassette carrying CP20 encoding gene and *aadA* gene was integrated into the *trnI/trnA* site of the plastid genome in *N. benthamiana* via homologous recombination and the transgene was transmitted into progeny by maternal inheritance.

### 3.5 Bombardment in tomato

After bombardment, almost all tomato leaf explants turned brown and finally died in SL selection medium containing 300 mg l<sup>-1</sup> spectinomycin (for pCEV2/GFP-GUS) or 50 mg l<sup>-1</sup> kanamycin (for pCEV3/GFP-GUS). Eventually, at least two green calli were obtained for each construct (**Figure A5**) from hundreds of explants examined. Currently, plant regeneration from spectinomycin-resistant calli is also in progress.

## 4. Conclusion

Here we constructed a series of chloroplast expression vectors specific for spinach (pCEV1), tomato (pCEV2 and pCEV3), and *N. benthamiana* (pCEV4). Common features of these vectors are described as follows: (1) sequence of cloning sites (*NotI*, *SacII*, *AscI*) for the GOI is located between plastid *atpI* promoter and plastid *rps16* terminator; (2) selection marker *aadA* or *nptII* is located between plastid *rrn* promoter and plastid *psbA* terminator; (3) longer LTR (ca. 2 kb) contains partial 16S *rRNA* and *trnI* in the plastid genome; (4) longer RTR (ca. 2 kb) contains *trnA* and partial 23S *rRNA* in the plastid genome; (5) the integration site for the expression cassette is located in the intergenic spacer between *trnI* and *trnA*; and (6) the backbone plasmid is pUC19 (carrying ampicillin resistant marker gene).

Another feature of this study is the construction of the so-called “auxiliary vector” pECaad (**Figure 6a**) and pECnpt (**Figure 6b**). Briefly, DNA fragment of expression cassette, which consists of (1) GOI cloning sites (*NotI*, *SacII*, *AscI*) flanked by *atpI* promoter and *rps16* terminator and (2) selection marker gene *aadA* or *nptII* flanked by *rrn* promoter and *psbA* terminator, was ligated with pUC19 (carrying ampicillin resistant marker gene), generating pECaad or pECnpt. LTR can be inserted into the 5' terminus of the expression cassette, RTR can be inserted into the 3' terminus of the expression cassette, and GOI can be cloned into the cloning sites (*NotI*, *SacII*, *AscI*) within the expression cassette. Thus, a new vector suitable for other new plant species can be rapidly and efficiently constructed. We believe that our auxiliary vectors pECaad and pECnpt will aid the faster construction of new chloroplast expression vectors for other plant species.

## Acknowledgements

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## Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
<i>aadA</i>	aminoglycoside 3'-adenyltransferase
BA	N <sup>6</sup> -benzyladenine
GA <sub>3</sub>	gibberellic acid
GFP	green fluorescent protein
GOI	gene of interest
GUS	β-glucuronidase
LCY	lycopene β-cyclase
LTR	left targeting region
MS	Murashige and Skoog (1962)
NAA	α-naphthaleneacetic acid
<i>nptII</i>	neomycin phosphotransferase II
RTR	right targeting region
ZDS	ζ-carotene desaturase

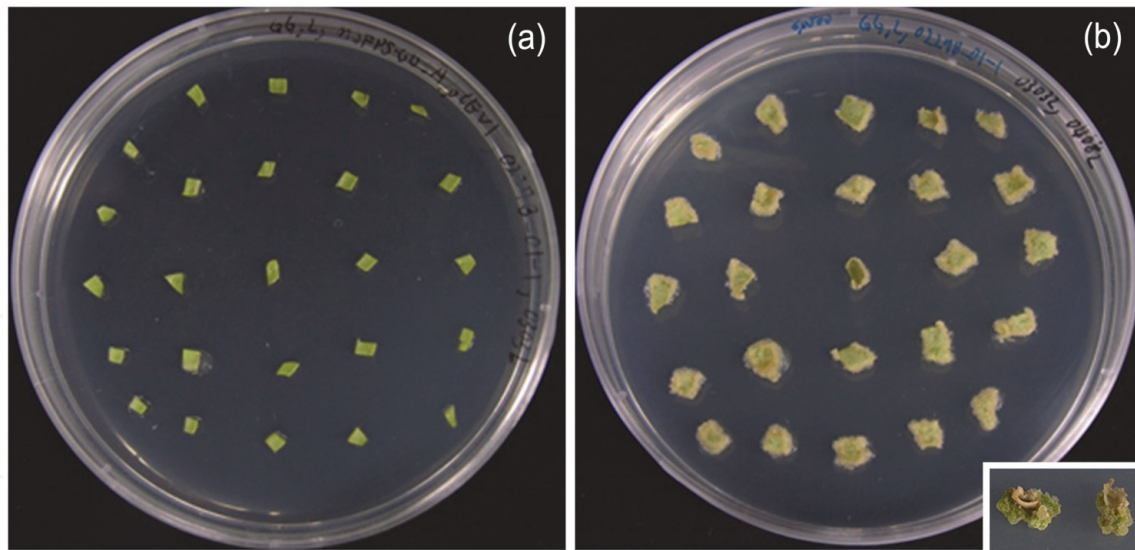
## Appendix

Name of primer	Sequence (5'→3')	PCR size (bp)	Purpose
<b>SacI</b> +16S/trnI-F	AAGAGCTCTACCAAGCTGGAGTACGG	2020	Cloning of pCEV1 and derivatives
16S/trnI+ <b>KpnI</b> -R	AAGGTACCTGGGCCATCCTGGACTTGAACCAG		
<b>PstI</b> +trnA/23S-F (PstI)	AACTGCAGCTGCGCCAGGGAAAAGAATAAGAA	2032	
trnA/23S+ <b>PstI</b> -R	AACTGCAGACCGCTCTCGCGGCCCGCACCGAA		
<b>aadA</b> -F1 (BamHI)	CGGGATCCATGAGGGAAGCGGTGATCCC	808	
<b>aadA</b> -R1 (XbaI)	GCTCTAGATTATTTGCCGACTACCTTGGTGAT		
<b>TpsbA</b> -F (XbaI)	GCTCTAGAGATCCTGGCCTAGTCTATAGG	238	
<b>TpsbA</b> -R (PstI)	AACTGCAGAAACAAATACAAAATCAAAAT		
<b>Prrn</b> -F (KpnI)	GGGGTACCATCAGTTCGAGCCTGATTATCCCTAAG	235	
<b>Prrn</b> -R (BamHI)	CGGGATCCGTAGACAAAGCGGATTCGGAA		
<b>PatpI</b> -F (KpnI)	AAGGTACCTCTAGCTATATAAGAAAATCCTTG	268	
<b>PatpI</b> -R ( <b>NotI</b> , <i>SacII</i> )	AACCGCGCCGCTGCCTTGCCCTCTGAAAAAATTG		
<b>Trps16</b> -F ( <i>SacII</i> , <b>AscI</b> )	AACCGCGCGCGCCTCAACCGAAATTCAATTAAGGA	191	
<b>Trps16</b> -R ( <b>KpnI</b> )	AAGGTACCGTTATAGAACACGGAATTCAATGG		
<b>NotI</b> + <b>LCY</b> -F	AAGCGGCCGCATGGATACTTTGTTGAAAACCCC	1523	
<b>LCY</b> - <b>AscI</b> -R	AAGGCGCGCCTCATTCTTTATCCTGTAACAAATTG		

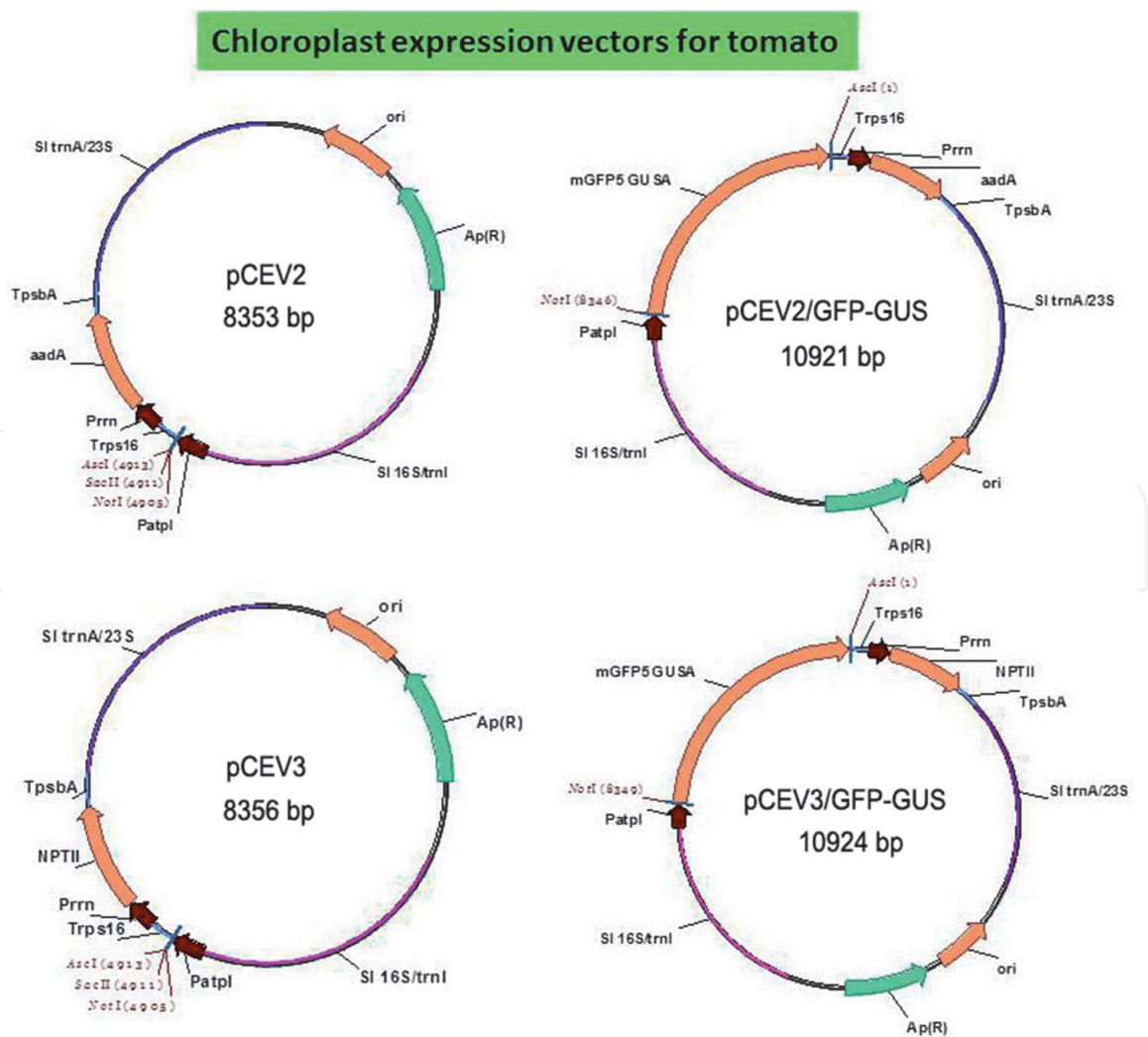
Name of primer	Sequence (5'→3')	PCR size (bp)	Purpose
<b>NotI</b> +ZDS-F	AAGCGGCCGCATGGCTACTTCTTCAGCTTATCTT	1787	
ZDS- <b>AscI</b> -R	AAGCGCGCCTCAGACAAGACTCAACTCATCAG		
<b>NotI</b> -mGFP5-F	GGTTGCGGCCGCATGGTAGATCTGACTAGTAAAGG	2592	
GUSA- <b>AscI</b> -R	GATAGGCGCGCCTCACACGTGGTGGTGGTGG		
<b>SacI</b> +PatpI-F	AAGAGCTCTCTAGCTATATAAGAAATCCT	1700	Cloning of pCEV2, pCEV3, and derivatives
TpsbA- <b>PstI</b> -R	AACTGCAGAAACAAATACAAAATCAAAT		
<b>SacI</b> +16S/trnI-F	AAGAGCTCTACCAAGCTGGAGTACGGTAGG	2010	
Le-16S/trnI + <b>SacI</b> -R	AAGAGCTCTGGGCCATCCTGGACTTGAAC		
Le- <b>PstI</b> +trnA/23S-F	AACTGCAGCTGCGCCAGGGAAAAGAATAG	2020	
trnA/23S+ <b>PstI</b> -R	AACTGCAGACCGCTCTCGCGCCCCGCACCGAA		
<b>BamHI</b> +NPTII-F	AAGGATCCATGATTGAACAAGATGGATTG	811	
<b>XbaI</b> +NPTII-R	AATCTAGATCAGAAGAACTCGTCAAGAAG		
Nb- <b>SacI</b> +16S/trnI-F	AAGAGCTCTACCAAGCTGGAGTACGGTAGG	2005	Cloning of pCEV4 and derivatives
Nb-16S/trnI + <b>SacI</b> -R	AAGAGCTCTGGGCCATCCTGGACTTGAACC		
Nb- <b>PstI</b> +trnA/23S-F	AACTGCAGCTGCGCCAGGGAAAAGAATAGA	1918	
Nb-trnA/23S + <b>PstI</b> -R	AACTGCAGACCGCTCTCGCGCCCCGCACCG		
<b>NotI</b> -BaMV_S-F	GGTTGCGGCCGCATGTCTGGAGCTGGAACGG	753	
BaMV_S_CP- <b>AscI</b> -R	GATAGGCGCGCCTTAGTCTGATGTTGGTTCGG		
<b>NotI</b> -BSL6-F	GGTTGCGGCCGCAGAAACTCACC GCAACGA	861	
BSL6- <b>AscI</b> -R	GATAGGCGCGCCCATCTTTTAACGTCTTTATTTCGG		
BSL6-F	GAAAACCTCACC GCAACGA	837	Transgenic plant verification
BSL6-R	CATCTTTTAACGTCTTTATTTCGG		
BaMV-F	ATGTCTGGAGCTGGAACGG	729	
BaMV-R	TTAGTCTGATGTTGGTTCGGG		
TpsbA-F	GATCCTGGCCTAGTCTATAG	351	
trnI-trnA-r	ACCCGTAATCGCAACGAC		
trnA-f	GGATGTCAGCGGTTTCGAGTC	342	
23S rRNA-r	CCTGCCCATGGATTTCAGCAG		

**Table A1.**

List of PCR primers for vector construction and transgenic plant verification.



**Figure A1.**  
 Development of stable plastid transformation in spinach. Plastid vectors *pCEV1/LCY*, *pCEV1/ZDS*, and *pCEV1/GFP-GUS* were delivered individually into spinach leaves by bombardment. (a) After bombardment, leaf segments were cultured onto callus induction medium (MS salts and vitamins; 3% sucrose; 1 mg l<sup>-1</sup> BA; 0.4 mg l<sup>-1</sup> NAA; 0.8% Bacto agar; pH 5.8) supplemented with 300 mg l<sup>-1</sup> spectinomycin. (b) After culturing for 1 month, almost all the leaf pieces turned brown in color and finally died. Occasionally, green calli from a few leaf pieces were observed after several bombardments and then picked up for plant regeneration, as indicated in the small panel in (b).



**Figure A2.**  
 Construction of chloroplast expression vectors for tomato.



*N. tabacum* CTACCAAGCTGGAGTACGGTAGGGGACAGGGAAATTCGGTGGAGCGGTGAAATCGTAGAGATCGGAAAGAACCAACCGCGAAAGCACTCTGTGG  
*N. benthamiana* CTACCAAGCTGGAGTACGGTAGGGGACAGGGAAATTCGGTGGAGCGGTGAAATCGTAGAGATCGGAAAGAACCAACCGCGAAAGCACTCTGTGG  
*N. benthamiana\** -----

*N. tabacum* GCCGCACTGACACTGAGAGACGAAAGCTAGGGGAGCGAATGGGATTAGATACCCAGTAGTCTAGCCGTAACAGATGGATACTAGGCGCTGTGGTAT  
*N. benthamiana* GCCGCACTGACACTGAGAGACGAAAGCTAGGGGAGCGAATGGGATTAGATACCCAGTAGTCTAGCCGTAACAGATGGATACTAGGCGCTGTGGTAT  
*N. benthamiana\** -----

*N. tabacum* CGACCCGTGCAGTGTAGTAAACGCTTAAGTATCCCGCTGGGGAGTACGTTCCGAAGAATGAAACTCAAAGGAATTGACGGGGGCGCCACAAGCG  
*N. benthamiana* CGACCCGTGCAGTGTAGTAAACGCTTAAGTATCCCGCTGGGGAGTACGTTCCGAAGAATGAAACTCAAAGGAATTGACGGGGGCGCCACAAGCG  
*N. benthamiana\** -----

*N. tabacum* GTGGAGCATGTGGTTAATTCGATGCAAAGCGAAGAACTTACCAGGGCTTGACATGCCCGAATCCTCTTGAAGAGAGGGGTGCCTTCGGGAACGGG  
*N. benthamiana* GTGGAGCATGTGGTTAATTCGATGCAAAGCGAAGAACTTACCAGGGCTTGACATGCCCGAATCCTCTTGAAGAGAGGGGTGCCTTCGGGAACGGG  
*N. benthamiana\** -----

*N. tabacum* ACACAGGTGGTGCATGGCTGTCTGACGCTGCGCTAAGGTGTGGGTTAAGTCCCGCAACGAGCGCAACCCCTGTTTGTAGTCCCATCGTTGAGTTT  
*N. benthamiana* ACACAGGTGGTGCATGGCTGTCTGACGCTGCGCTAAGGTGTGGGTTAAGTCCCGCAACGAGCGCAACCCCTGTTTGTAGTCCCATCGTTGAGTTT  
*N. benthamiana\** -----

*N. tabacum* GGAACCTGAAACAGACTGCCGTGATAAGCCGGAGGAGGTGAGGATGACGTAAGTATCATGCCCTTATGCCCTGGGCGACACGCTGTACAATGG  
*N. benthamiana* GGAACCTGAAACAGACTGCCGTGATAAGCCGGAGGAGGTGAGGATGACGTAAGTATCATGCCCTTATGCCCTGGGCGACACGCTGTACAATGG  
*N. benthamiana\** -----

*N. tabacum* CCGGGACAAAGGGTTCGCGATCCCGCGAGGGTGTAGTAAACCCAAAACCCGCTCAGTTCGGATTGCAAGCTGCAACTCGCTGCATGAAGCCGGAATC  
*N. benthamiana* CCGGGACAAAGGGTTCGCGATCCCGCGAGGGTGTAGTAAACCCAAAACCCGCTCAGTTCGGATTGCAAGCTGCAACTCGCTGCATGAAGCCGGAATC  
*N. benthamiana\** -----

*N. tabacum* GCTAGTAATCGCCGTCAGCCATACGGCGGTGAATTCGTTCCCGGCCCTGTACACACCGCCCTCACACTATGGGAGCTGGCCATGCCGAAGTCGTTA  
*N. benthamiana* GCTAGTAATCGCCGTCAGCCATACGGCGGTGAATTCGTTCCCGGCCCTGTACACACCGCCCTCACACTATGGGAGCTGGCCATGCCGAAGTCGTTA  
*N. benthamiana\** -----

*N. tabacum* CCTTAACCGCAAGGAGGGGATGCCAAGGCGAGGCTAGTACTGGAGTGAAGTCTAACAGGTAGCCGCTACTGGAAGTGGGCTGGATCACCTCCTT  
*N. benthamiana* CCTTAACCGCAAGGAGGGGATGCCAAGGCGAGGCTAGTACTGGAGTGAAGTCTAACAGGTAGCCGCTACTGGAAGTGGGCTGGATCACCTCCTT  
*N. benthamiana\** -----

*N. tabacum* TTCAGGGAGGCTAATGCTTGTGGGATTTTGGTTTGACACTGCTTCACACCCCAAAAAA---GAAGGGAGCTACGCTGAGTTAACTTGGAGATGG  
*N. benthamiana* TTCAGGGAGGCTAATGCTTGTGGGATTTTGGTTTGACACTGCTTCACACCCCAAAAAA---GAAGGGAGCTACGCTGAGTTAACTTGGAGATGG  
*N. benthamiana\** -----

*N. tabacum* AAGTCTTCTTCTTCTCGACGGTGAAGTAAGCAAGCTCATGAGCTTATATCCTAGGTCGGAACAAGTTGATAGGACCCCTTTTTTACGTCGCCA  
*N. benthamiana* AAGTCTTCTTCTTCTCGACGGTGAAGTAAGCAAGCTCATGAGCTTATATCCTAGGTCGGAACAAGTTGATAGGACCCCTTTTTTACGTCGCCA  
*N. benthamiana\** -----

*N. tabacum* TGTTCGCCCGTGTGGCGACATGGGGCGAAAAAGAAAGAGAGGGATGGGTTTCTCTCGCTTTTGGCATAGCGGGCCCGAGTGGGAGGCTCGCACG  
*N. benthamiana* TGTTCGCCCGTGTGGCGACATGGGGCGAAAAAGAAAGAGAGGGATGGGTTTCTCTCGCTTTTGGCATAGCGGGCCCGAGTGGGAGGCTCGCACG  
*N. benthamiana\** -----

*N. tabacum* ACGGGCTATAGCTCAGTGTAGAGCGCCCTGATAATTCGCTGTTGCTTGGCTGTGAGGGCTCTCAGCCACATGGATAGTTCAATGTGCTCAT  
*N. benthamiana* ACGGGCTATAGCTCAGTGTAGAGCGCCCTGATAATTCGCTGTTGCTTGGCTGTGAGGGCTCTCAGCCACATGGATAGTTCAATGTGCTCAT  
*N. benthamiana\** -----

*N. tabacum* CGGCGCTGACCTGAGATGTGGATATCCAAGGCACATAGCATGGCTACTCTCTGTTGCAACCGGGTTTGAACCAAACTCCTCTCAGGAGGA  
*N. benthamiana* CGGCGCTGACCTGAGATGTGGATATCCAAGGCACATAGCATGGCTACTCTCTGTTGCAACCGGGTTTGAACCAAACTCCTCTCAGGAGGA  
*N. benthamiana\** -----

*N. tabacum* TAGATGGGCGATTTCGGGTGAGATCCAATGTAGATCCAACCTTCGATTACTCTGTTGGATTCGGGCGGTCGGGGGGACACCAGGCTCCTCTCTCT  
*N. benthamiana* TAGATGGGCGATTTCGGGTGAGATCCAATGTAGATCCAACCTTCGATTACTCTGTTGGATTCGGGCGGTCGGGGGGACACCAGGCTCCTCTCTCT  
*N. benthamiana\** -----

*N. tabacum* CGAGAATCCATACATCCCTTATCAGTGTATGGACAGTATCTCTCGACAGGTTAG-9-bp---CAATGGGAAAATAAATGGAGCAACTAACAA  
*N. benthamiana* CGAGAATCCATACATCCCTTATCAGTGTATGGACAGTATCTCTCGACAGGTTAG-9-bp---CAATGGGAAAATAAATGGAGCAACTAACAA  
*N. benthamiana\** -----

*N. tabacum* GCATCTTCCAGACCAAGAACTACGAGTACGCGCCCTTTCATCTGGGTCAGCGAGGATCGTACCATTTCGAGCCGTTTTTTCTTGACTCGAAATGGGA  
*N. benthamiana* GCATCTTCCAGACCAAGAACTACGAGTACGCGCCCTTTCATCTGGGTCAGCGAGGATCGTACCATTTCGAGCCGTTTTTTCTTGACTCGAAATGGGA  
*N. benthamiana\** -----

*N. tabacum* CGAGGTTTGAAGAAAGGATCTTAGAGTGTCTAGGGTGGGGCAGGAGGCTCTTAAACCGCTCTTTTTTCTTCTCATCGGAGTATTTTCCAAAGACTTG  
*N. benthamiana* CGAGGTTTGAAGAAAGGATCTTAGAGTGTCTAGGGTGGGGCAGGAGGCTCTTAAACCGCTCTTTTTTCTTCTCATCGGAGTATTTTCCAAAGACTTG  
*N. benthamiana\** -----

*N. tabacum* CCAGGTAAGGAAGAGGGGGGAACAAGCACCTTGGAGAGCGCAGTACAACGGAGAGTTGATGCTGCTTCGGGAAGGATGAATCGCTCCGAAAAGG  
*N. benthamiana* CCAGGTAAGGAAGAGGGGGGAACAAGCACCTTGGAGAGCGCAGTACAACGGAGAGTTGATGCTGCTTCGGGAAGGATGAATCGCTCCGAAAAGG  
*N. benthamiana\** -----

*N. tabacum* AATCTATTGATCTCTCCCAATTTGGTTGACCGTAGTGGCATGATTTACTTCAACGGGCGAGGCTCTGGTTCAAGTCCAGGATGGCCAGCTGCGCCAG  
*N. benthamiana* AATCTATTGATCTCTCCCAATTTGGTTGACCGTAGTGGCATGATTTACTTCAACGGGCGAGGCTCTGGTTCAAGTCCAGGATGGCCAGCTGCGCCAG  
*N. benthamiana\** -----

*N. tabacum* GGAAAAGATAGAAGAAGCATCTGACTACTTATGATGCTCCACTTGGCTCGGGGGATATAGCTCAGTTGGTAGAGCTCCGCTTTCGAATTTGGGTG  
*N. benthamiana* GGAAAAGATAGAAGAAGCATCTGACTACTTATGATGCTCCACTTGGCTCGGGGGATATAGCTCAGTTGGTAGAGCTCCGCTTTCGAATTTGGGTG  
*N. benthamiana\** -----

*N. tabacum* TTGCGATTACGGGTGGATGCTAATTTGCCAGGCGTAATGATAGTATCTTGACTGAAACCGGTGGCTCAGTTTCTTAAGTAATGGGAAGAGGACC  
*N. benthamiana* TTGCGATTACGGGTGGATGCTAATTTGCCAGGCGTAATGATAGTATCTTGACTGAAACCGGTGGCTCAGTTTCTTAAGTAATGGGAAGAGGACC  
*N. benthamiana\** -----

*N. tabacum* GAAACGTGCCACTGAAAGACTCTACTGAGACAAAGATGGGCTGTCAAGAAGCTAGAGGAGTAGGATGGCAGTTGGTTCAGATCTAGTATGGATCGTACA  
*N. benthamiana* GAAACGTGCCACTGAAAGACTCTACTGAGACAAAGATGGGCTGTCAAGAAGCTAGAGGAGTAGGATGGCAGTTGGTTCAGATCTAGTATGGATCGTACA  
*N. benthamiana\** -----

*N. tabacum* TGGACGGTAGTGGAGTCGGCGGCTCTCCAGGGTTCCTCATTTGAGATCTCTGGGGAAGAGGATCAAGTTGGCCCTTGGCAACAGCTTGTATGCACTAT  
*N. benthamiana* TGGACGGTAGTGGAGTCGGCGGCTCTCCAGGGTTCCTCATTTGAGATCTCTGGGGAAGAGGATCAAGTTGGCCCTTGGCAACAGCTTGTATGCACTAT  
*N. benthamiana\** -----

*N. tabacum* CTCCTTCAACCTTTGAGCGAAATCGCGAAAAGAAAGAAAGAAATCCATGGACCGACCCATCATCTCCACCCGTAAGAACTACGAGATCACCC  
*N. benthamiana* CTCCTTCAACCTTTGAGCGAAATCGCGAAAAGAAAGAAAGAAATCCATGGACCGACCCATCATCTCCACCCGTAAGAACTACGAGATCACCC  
*N. benthamiana\** -----

*N. tabacum* CAAGGACGCTTCGGCATCCAGGGTTCAGGACCGACCATAGAACCTGTTCAATAAGTGAACGCAATAGCTGTCCGCTCTCAGGTTGGGCGAGTCAGG  
*N. benthamiana* CAAGGACGCTTCGGCATCCAGGGTTCAGGACCGACCATAGAACCTGTTCAATAAGTGAACGCAATAGCTGTCCGCTCTCAGGTTGGGCGAGTCAGG  
*N. benthamiana\** -----

*N. tabacum* TCGGAGAAGGGCAATGACTCATTAGTTAGATAGGATTTCAACTCAGACCTTTTGGAGTGGATTTGAGAAGAGTTGCTCTTTGGAGAGACAGTA  
*N. benthamiana* TCGGAGAAGGGCAATGACTCATTAGTTAGATAGGATTTCAACTCAGACCTTTTGGAGTGGATTTGAGAAGAGTTGCTCTTTGGAGAGACAGTA  
*N. benthamiana\** -----

*N. tabacum* CGATGAAAGTTGTAAGCTGTGTTCCGGGGGGATTTTGTCTATCTTGGCCTCTATGGTGAATCAGTCGGGGACCTGAGAGGCGGTGGTTTACCCTG  
*N. benthamiana* CGATGAAAGTTGTAAGCTGTGTTCCGGGGGGATTTTGTCTATCTTGGCCTCTATGGTGAATCAGTCGGGGACCTGAGAGGCGGTGGTTTACCCTG  
*N. benthamiana\** -----

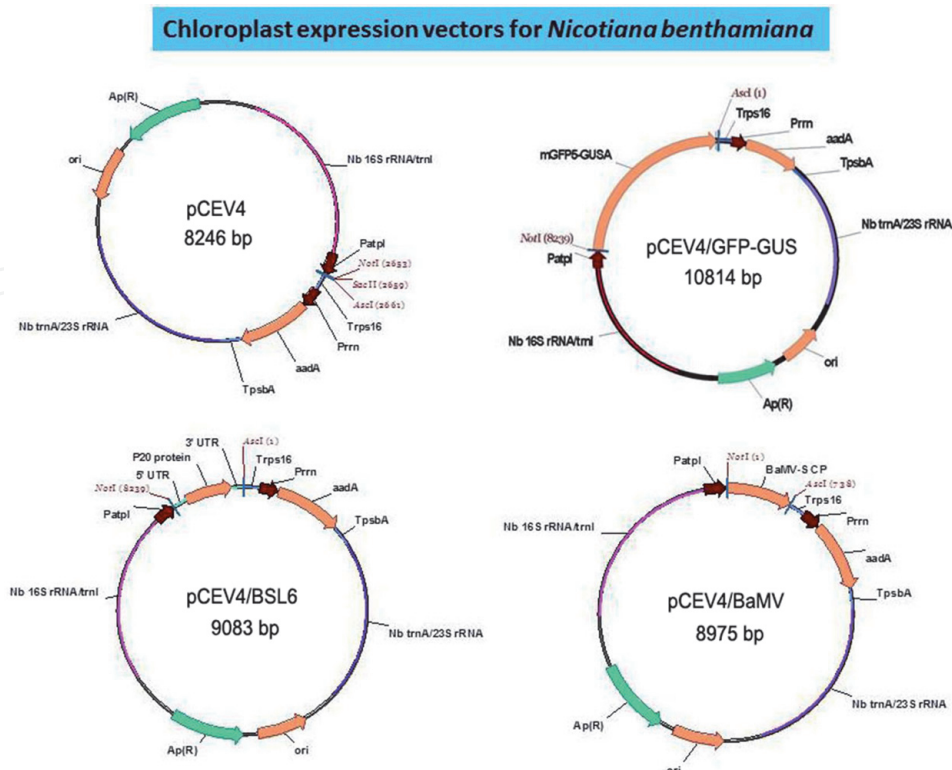
*N. tabacum* CGGCGGATGTCAGCGGTTGAGTCCGCTTATCTCCAACCTGTAAGCTTAGCCGATACAAAGCTTTATGATAGCACCAATTTTCGATTTCGGCGGTTTCG  
*N. benthamiana* CGGCGGATGTCAGCGGTTGAGTCCGCTTATCTCCAACCTGTAAGCTTAGCCGATACAAAGCTTTATGATAGCACCAATTTTCGATTTCGGCGGTTTCG  
*N. benthamiana\** -----

*N. tabacum* ATCTATGATTATCATTTCATGGAGTTGATAAGATCCATCCATTTAGCAGCACCTTAGGATGGCATAGCCCTAAAAGTGAAGGGCGAGGTTCAAACGAGG  
*N. benthamiana* ATCTATGATTATCATTTCATGGAGTTGATAAGATCCATCCATTTAGCAGCACCTTAGGATGGCATAGCCCTAAAAGTGAAGGGCGAGGTTCAAACGAGG  
*N. benthamiana\** -----

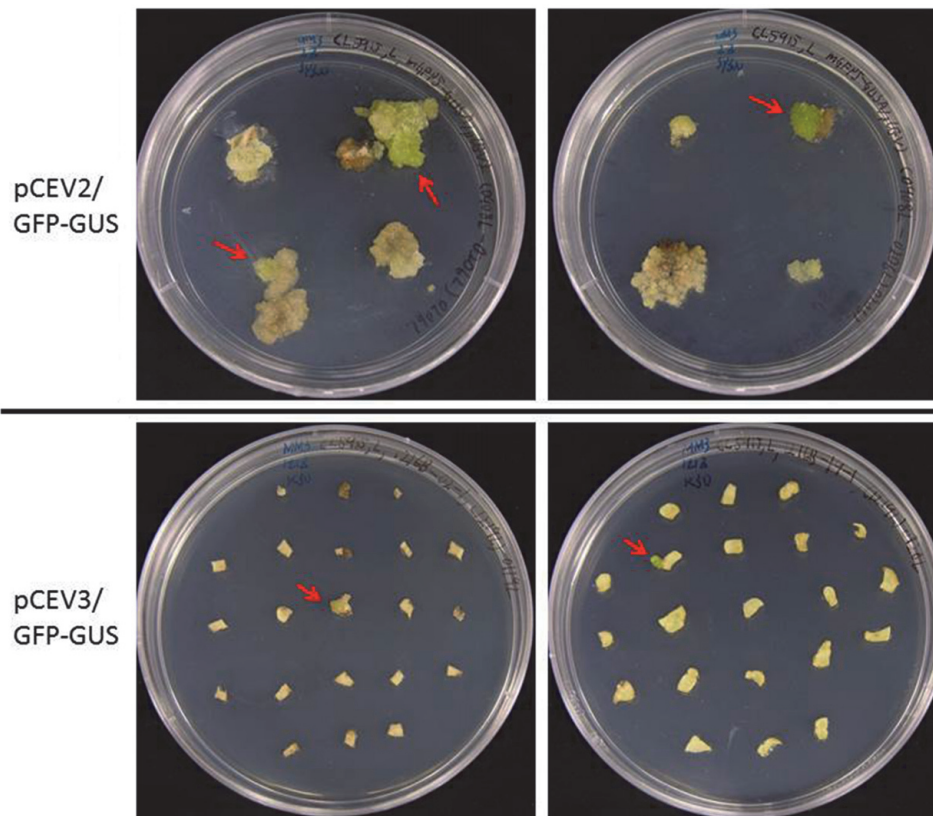
```

N. tabacum AAAGCCTTACGGTGGATACCTAGGCACCCAGAGACGAGGAAGGGCGTAGTAATCGACGAATGCTTCGGGGAGTTGAAAATAAGCATAGATCCGGAGATT
N. benthamiana AAAGCCTTACGGTGGATACCTAGGCACCCAGAGACGAGGAAGGGCGTAGTAATCGACGAATGCTTCGGGGAGTTGAAAATAAGCATAGATCCGGAGATT
N. benthamiana* -----
N. tabacum CCCGAATAGGGCAACCTTTGCAACTGCTGTAANTCCATGGGCAGGCAAGAGACAACCTGGCGAATGAAACATCTTAGTAGCCAGAGGAAAAGAAAGCA
N. benthamiana CCCGAATAGGGCAACCTTTGCAACTGCTGTAANTCCATGGGCAGGCAAGAGACAACCTGGCGAATGAAACATCTTAGTAGCCAGAGGAAAAGAAAGCA
N. benthamiana* -----
N. tabacum AAAGCGATTCCCGTAGTAGCGGGAGCGGAAATGGGAGCAGCCTAAACCCTGAAAACGGGGTTGTGGGAGAGCAATACAAGCGTCGTCTGCTAGGCGAAG
N. benthamiana AAAGCGATTCCCGTAGTAGCGGGAGCGGAAATGGGAGCAGCCTAAACCCTGAAAACGGGGTTGTGGGAGAGCAATACAAGCGTCGTCTGCTAGGCGAAG
N. benthamiana* -----
N. tabacum CAGCCCGAATGCTGCACCCTAGATGGCGAAAGTCCAGTAGCCGAAAGCATCACTAGCTTATGCTCTGACCCGAGTAGCATGGGGCACGTGGAATCCCGTG
N. benthamiana CAGCCCGAATGCTGCACCCTAGATGGCGAAAGTCCAGTAGCCGAAAGCATCACTAGCTTATGCTCTGACCCGAGTAGCATGGGGCACGTGGAATCCCGTG
N. benthamiana* -----
N. tabacum TGAATCAGCAAGGACCACCTTGCAAGGCTAAATACTCCTGGGTGACCGATAGCGAAGTAGTACCGTGAGGGAAAGGGTGAAAAGAACCCCATCGGGGAGT
N. benthamiana TGAATCAGCAAGGACCACCTTGCAAGGCTAAATACTCCTGGGTGACCGATAGCGAAGTAGTACCGTGAGGGAAAGGGTGAAAAGAACCCCATCGGGGAGT
N. benthamiana* -----
N. tabacum GAAATAGAACATGAAACCGTAAGCTCCCAAGCAGTGGGAGGAGCCAGGGCTCTGACCCGCTGCTGTTGAAGAATGAGCCGGGACTCATAGGCAGTGGC
N. benthamiana GAAATAGAACATGAAACCGTAAGCTCCCAAGCAGTGGGAGGAGCCAGGGCTCTGACCCGCTGCTGTTGAAGAATGAGCCGGGACTCATAGGCAGTGGC
N. benthamiana* -----
N. tabacum TTGGTTAAGGGAACCCACCGAGCCGTAGCGAAAGCGAGTCTTCATAGGGCAATTGTCACCTGTTATGGACCCGAACCTGGGTGATCTATCCATGACCAG
N. benthamiana TTGGTTAAGGGAACCCACCGAGCCGTAGCGAAAGCGAGTCTTCATAGGGCAATTGTCACCTGTTATGGACCCGAACCTGGGTGATCTATCCATGACCAG
N. benthamiana* -----
N. tabacum GATGAAGCTTGGGTGAAACTAAGTGGAGGTCGGAACCGACTGATGTTGAAGAATCAGCGGATGAGTTGGTTAGGGGTGAAATGCCACTCGAACCCAGA
N. benthamiana GATGAAGCTTGGGTGAAACTAAGTGGAGGTCGGAACCGACTGATGTTGAAGAATCAGCGGATGAGTTGGTTAGGGGTGAAATGCCACTCGAACCCAGA
N. benthamiana* -----
N. tabacum GCTAGCTGGTTCTCCCGAAATGCGTTGAGGCGCAGCAGTGTACTGGACATCTAGGGGTAAGCACTGTTTCGGTGCGGGCGCGAGAGCGGT
N. benthamiana GCTAGCTGGTTCTCCCGAAATGCGTTGAGGCGCAGCAGTGTACTGGACATCTAGGGGTAAGCACTGTTTCGGTGCGGGCGCGAGAGCGGT
N. benthamiana* -----
    
```

**Figure A3.** Sequence comparison for the targeting region (partial 16S rRNA, trnI, trnA, and partial 23S rRNA) between *N. tabacum* and *N. benthamiana*. *N. tabacum* sequence was extracted from NCBI Accession no. NC\_001879. *N. benthamiana* sequence (3893 bp) was our PCR product. *N. benthamiana*\* sequence (1874 bp) was the partial sequence of 16S-23S ribosomal RNA intergenic spacer from NCBI with the Accession no. FJ217346. A different nucleotide was indicated by red arrow and labeled with a red letter. Common region in these three sequences was highlighted in yellow color.



**Figure A4.** Construction of chloroplast expression vectors for *Nicotiana benthamiana*.



**Figure A5.**

Selection of survival calli in tomato plastid transformation. Plastid vector pCEV2/GFP-GUS (carrying selection marker aadA) or pCEV3/GFP-GUS (carrying selection marker nptII) was bombarded into tomato leaves. Leaf segments were cultured for 1 month on callus induction medium (MS salts and vitamins; 3% sucrose;  $1 \text{ mg l}^{-1}$  NAA;  $1 \text{ mg l}^{-1}$  Zeatin; 0.8% Bacto agar; pH 5.8) supplemented with  $300 \text{ mg l}^{-1}$  spectinomycin (for pCEV2/GFP-GUS) or  $50 \text{ mg l}^{-1}$  kanamycin (for pCEV3/GFP-GUS). Most of the leaf pieces turned brown and finally died. Several survival green calli (as indicated by red arrows) were observed and then picked for shoot regeneration.

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