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ORIGINAL RESEARCH

Stable Plastid Transformation in *Nicotiana benthamiana*

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Abstract Plastids from *Nicotiana benthamiana* were transformed with the vector for dicistronic expression of two genes—*aminoglycoside 3′-adenyltransferase* (*aadA*) and *green fluorescent protein* (*gfp*)—in the plastids of *Nicotiana tabacum*. Transplastomic shoots exhibited green fluorescence under UV light. Transformation efficiencies were similar between species. Although the border sequence (*trnI* and *trnA*) for homologous recombination to transform the plastid genome of *N. benthamiana* was identical to that sequence of *N. tabacum*, the exception was a 9-bp addition in the intron of *trnI*. This indicated that the *N. tabacum* sequence used as a border region for recombination was sufficient to insert the foreign gene into the target site between the *trnI* and *trnA* of *N. benthamiana* with similar efficiency. Southern blot analysis detected the presence of *aadA* and *gfp* between *trnI* and *trnA* in the plastid genome of *N. benthamiana*. Northern and western blot analyses revealed high expression of *gfp* in the plastids from petals and leaves. Our results suggest that the plastid transformation system established here is applicable to investigations of the interactions between plastid and nucleus in *N. benthamiana*.

Keywords *Nicotiana benthamiana* · Plastid transformation

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Abbreviations

aadA	aminoglycoside 3'-adenyltransferase
BA	6-benzyladenine
gfp	green fluorescent protein
MS	Murashige and Skoog
NAA	α-naphthaleneacetic acid

Plastid transformation provides many advantages over nuclear transformation. These include the prevention of gene flow to other plants due to maternal inheritance (Daniell 2002), a lack of gene silencing, and positional effects because integration of the foreign DNA is targeted instead to the precise region without disrupting unwanted genes (de Cosa et al. 2001; Daniell et al. 2002; Lee et al. 2003) and the insertion of multiple heterologous genes or operons under the control of a single regulatory sequence (de Cosa et al. 2001; Jeong et al. 2004). It is also useful for studies with reverse genetics of the functioning of unknown genes, ORFs, or sequences of a plastid genome that use targeted disruption. This system of stable and fertile transformation of higher plants, developed in the early 1990s, has been applied to major crops such as tomato, soybean, and cotton (Svab et al. 1990; Ruf et al. 2001; Dufourmantel et al. 2004; Kumar et al. 2004). However, because it has been performed with far fewer species than has nuclear transformation, this plastid transformation method must still be demonstrated in other plants of interest.

The short life cycle of *Nicotiana benthamiana* makes it a widely used model system for research at the nuclear, organellar, cellular, organ, or whole-plant levels. This species is amphiploid, with 38 chromosomes, and is thought to have resulted from the hybridization of *Nicotiana suaveolens* ($n=16$) and *Nicotiana debneyi* ($n=24$). It is a close relative of cultivated tobacco (*Nicotiana tabacum*) and a member of the Solanaceae that includes the important

crops of potatoes and tomatoes. This unique Australian native is the most popular experimental host for evaluating plant virology mainly because of its susceptibility to diverse viruses (Raquel et al. 2008). *N. benthamiana* is the best known model plant for virus-induced gene silencing (VIGS), a tool for functional analyses of genes and studies of plant disease (Baulcombe 1999). It is often used also for examining artificial miRNA-mediated resistance and viral protein-mediated cross-protection in plants (Lin et al. 2007).

Here, we developed a plastid transformation system with *N. benthamiana*. Our protocol may be useful for investigating interactions between plastid and nuclear genes and for monitoring gene expression in the plastid.

Materials and Methods

Plastid Transformation of *N. benthamiana*

The plastid transformation vector pTIG, constructed for *N. tabacum* (Jeong et al. 2004), was used to transform the plastid genome of *N. benthamiana*. This vector has the *trnI* and *trnA* genes as a border sequence for homologous recombination, as well as the dicistronic construct of aadA–*gfp*-linked genes under the control of the *rrn* promoter and the *psb*A terminator cloned from the tobacco plastid genome. The transformation method used here was previously described by Jeong et al. (2004). Two-month-old plants of *N. benthamiana* were grown on an MS basal medium (Murashige and Skoog 1962). Leaf discs (approximately 1 to 2 cm long) were then placed abaxial side up on a shoot induction medium (SIM) that comprised an MS medium supplemented with 4.44 μM 6-benzylaminpurine and 0.54 μM α-naphthaleneacetic acid. At 4 to 8 weeks after bombardment, leaves from adventitious shoots with *gfp* fluorescence were cut into 2×2-mm explants and placed on a SIM to increase the homoplasmy level of the transgene in their plastids. Afterward, plastid-transformed shoots were selected by repeating the same procedure then transferred onto MS basal medium supplemented with 500 mg L^{-1} of spectinomycin to induce rooting. Plantlets were put into potting soil and held to maturity, flowering in a growth chamber (27°C and 16-h photoperiod under approximately 15 W m^{-2} from cool-white fluorescent lamps). Their T_1 seeds were germinated on an MS basal medium supplemented with 500 mg L^{-1} of spectinomycin. Protoplasts were isolated from the leaves of both plastid-transformed and wild-type (WT) plants, using an enzyme solution containing 2% cellulase Onozuka R-10 (Yakult) and 0.5% Macerozyme R-10 (Yakult) and incubating them at 25°C for 4 to 8 h. They were then observed under a fluorescence microscope (Axioskop, Zeiss) with a fluorescein isothiocyanate (FITC) set (excitation 450 to 490 nm, dichromic mirror 510 nm) and a bandpass filter (515 to 561 nm). This enabled us to distinguish transformed chloroplasts with the *gfp* from WT chloroplasts in the protoplasts.

PCR Amplification and Sequencing of *trnI* and *trnA* from *N. benthamiana*

Polymerase chain reaction (PCR) primers IL1 (5′-GCTCTA GATTCTCGACGGTGAAGTA-3′) and IR2 (5′-GGGGTA CCTTAAGGCTATGCCATCC-3′) were used to amplify the DNA fragment including the region of *trnI* and *trnA* in the plastid genome of *N. benthamiana*. Total DNA, primers, exTaq polymerase (TaKaRa), and the reaction buffer were incubated in a DNA thermal cycler (GeneAmp PCR system 9700; Applied Biosystems) under conditions of 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 3 min; then a final 10-min extension at 72°C. The amplified PCR product, with about 2 Kb of the WT, was purified and sequenced directly for verification with sequencing primers IL1, IR2, *trnI* F2 (5′-CTCAGCCACATGGA TAGTTC-3′), *trnA* R3 (5′-AGGGTTGAAGGGAGATAG-3′), and *trnA* RA (5′-AGAGTCTTT CAGTGGCACGTTTC-3′).

Southern Blot Analysis

Total genomic DNA was extracted from leaves of one WT and two independent transgenic plants with a DNeasy^{R} Plant Maxi Kit (Qiagen). Prepared DNA (5 μg) was digested with *Bgl*II and fractionated on a 1% agarose gel then transferred to a Zeta-Probe GT blotting membrane (Bio-Rad, USA). Afterward, 0.15 Kb of the *trnA*-specific DNA fragment, synthesized by PCR with pTIG as template, and primers *trnA* F1 (5′-TGCGATTACGGGTTGGATGT-3′) and *trnA* R1 (5′-GTTCTTGACAGCCCATCTTT-3′), was labeled with [α-^{32}P] dCTP. Prehybridization, hybridization at 65°C overnight, and washing of the membrane were performed according to the manufacturer's instructions.

Northern Blot Analysis

Following its extraction from leaves of the above plants with TRIzol™ Reagent (Invitrogen), total RNA (5 μg) was separated by agarose/formaldehyde gel electrophoresis and blotted onto a Zeta-Probe GT blotting membrane. A 0.5-Kb *gfp*-specific DNA fragment was amplified by PCR, using pTIG as template and *gfp* F (5′-GAA GGT GAT GCA ACA TAC GGA AAA-3′) and *gfp* R (5′-GTT TGT CTG CCG TGA TGT ATA CGT T-3′) primers. The 0.24-Kb *rbcL*-specific DNA fragment was PCR-amplified with genomic DNA from *N. benthamiana* as template and primers *rbcL* F (5′-CTTCTACTGGTACATGGACAACTGT-3′) and *rbcL*R (5′-CTCTACGTCTGGAAGATCTGCGAAT-3′). Amplified fragments were labeled with [α-^{32}P] dCTP. Prehybridization, hybridization at 65°C overnight, and membrane-washing were

performed according to the manufacturer's instructions. The membrane was probed initially with the *gfp* fragment then stripped and reprobed with the *rbcL* fragment.

Western Blot Analysis

Total soluble protein was extracted from leaf, petal, and root tissues with a buffer of 50 mM Tris [tris(hydroxymethyl)-aminomethane]-HCl (pH7.5), 2 mM EDTA, 0.5 mM EGTA, and 1% Triton X-100 (*v*/*v*). Proteins (10 μg) were separated by standard SDS-PAGE on 12% (*w*/*v*) polyacrylamide gels and transferred to PVDF membranes (Amersham-Pharmacia Biotech, USA). Immunodetection by chemiluminescence was performed with GFP antibody (Sigma, USA) according to the manufacturer's instructions (ECL-Plus; Amersham-Pharmacia Biotech). To compare GFP accumulations among plastid sources, relative intensities of the protein bands were analyzed by Quantity One® software (Bio-Rad).

Results and Discussion

trnI and *trnA* Sequences from The Plastid Genome in *N. benthamiana*

Sequences for the border regions of plastid transformation vectors for specific species should be determined prior to vector construction in order to establish a stable and high-efficiency system because of a mechanism for homologous recombination. Efficiency decreases drastically when a petunia or tobacco border region is used to transform the plastids of tobacco or potato, respectively (Sidorov et al. 1999; DeGray et al. 2001). Our vector was originally constructed for use with *N. tabacum*. However, the plastid genome sequence of *N. benthamiana* has not been reported previously. In most of the higher plants, the gene order and sequence in the ribosomal RNA operon region, including 16S rRNA, *trnI*, *trnA*, and 23S rRNA, are highly conserved. To determine the sequences of *trnI* and *trnA*, we designed putative universal PCR primers IL1 and IR2 to amplify and sequence the DNA fragment that included *trnI* and *trnA* from the plastid genome of *N. benthamiana*. An approximately 2-Kb fragment was examined. Except for a 9-bp (GTTCGGCCT) addition to the intron of *trnI* (Fig. 1), *N. benthamiana* has sequences for those *trnI* and *trnA* regions that are identical to those of *N. tabacum* (GenBank Accession No. FJ217346).

Production of Transplastomic Plants from *N. benthamiana*

Transplastomic plants of *N. benthamiana* were produced with a transformation efficiency similar to that of *N. tabacum*. Green spots or calli formed on leaf explants in 30% of the Petri dishes after 4 to 8 weeks of culture

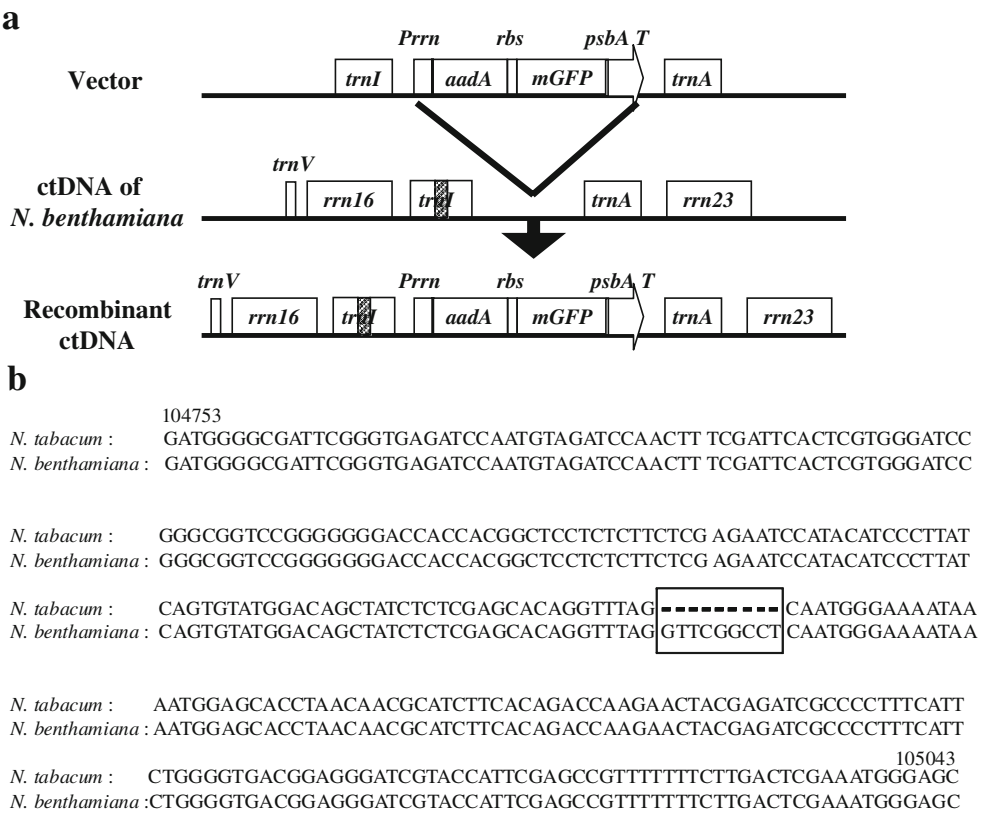

Fig. 1 Plastid transformation of *N. benthamiana* via homologous recombination. **a** Schematic diagram showing transformation and targeted region in plastid genome. Hachured box refers to 9-bp insertion into intron of *trnI* in genome. **b** Comparison of partial sequence for *trnI* between *N. tabacum* and *N. benthamiana*. Inserted nine nucleotides in *N. benthamiana* are marked by box; numbers above sequence correspond to position within *N. tabacum* plastid genome sequence (GenBank Accession No. Z00044)

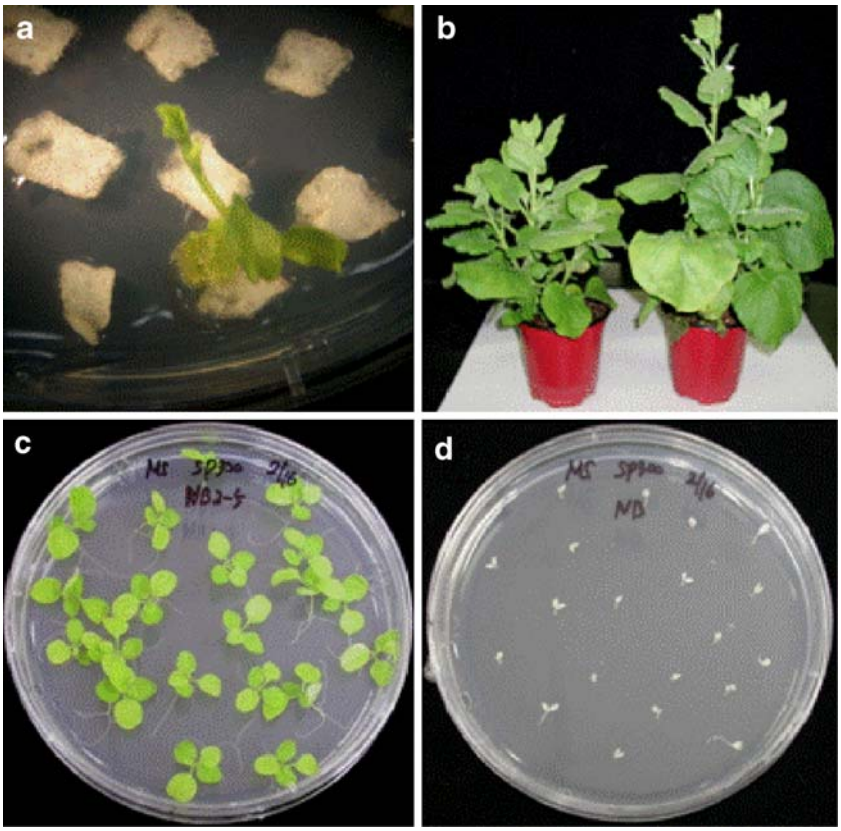

Fig. 2 Production of transplastomic *N. benthamiana* plants. **a** shoot regeneration on selection medium. **b** T_0 transplastomic plants in soil. **c**, **d**, T_1 seed germination on MS medium containing 500 mg L^{-1} of spectinomycin

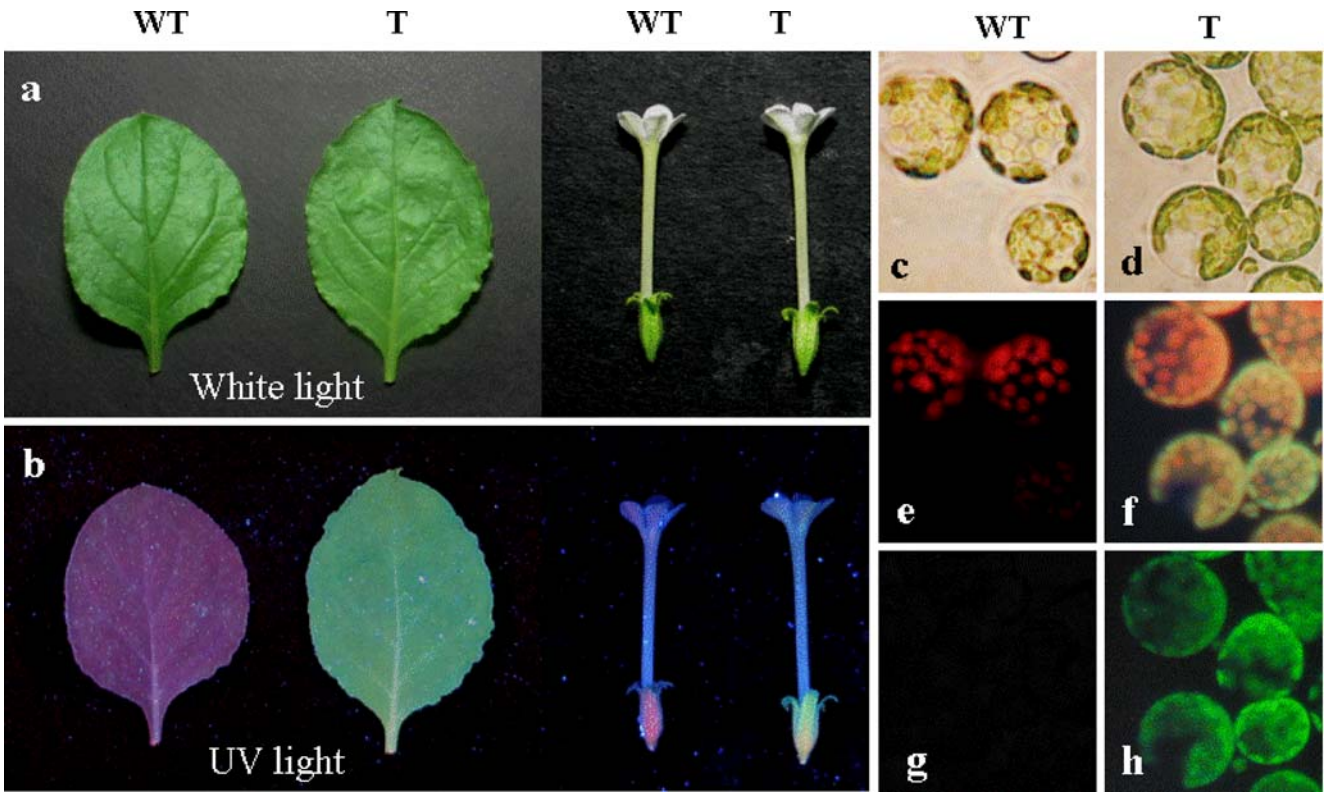

Fig. 3 GFP fluorescence. **a, b** Comparison of fluorescence under UV light in transplastomic leaf and flower and under white light as control. **c**–**h** WT and transgenic (T) chloroplasts under white, UV, or UV light using FITC filter

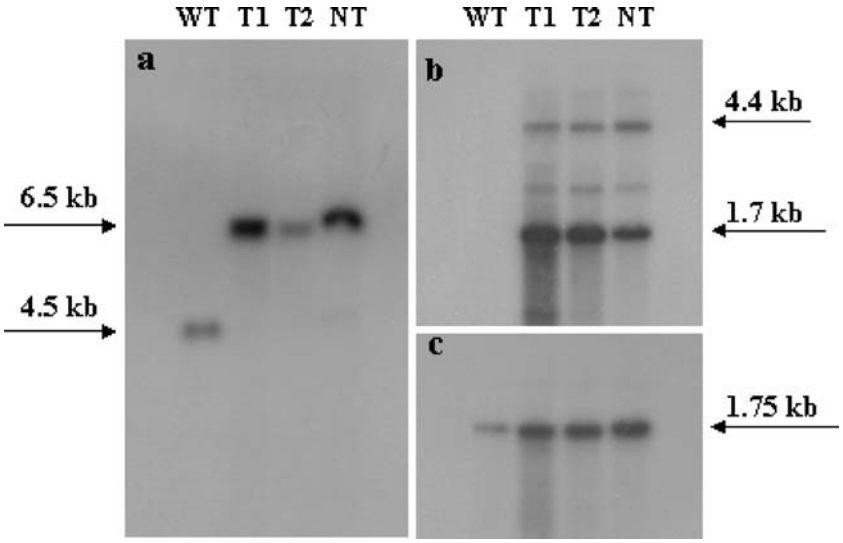

Fig. 4 Insertion and expression of transgene in transplastomic *N. benthamiana* plants. **a** Southern blot of WT and T1 and T2 tobacco indicating homoplasmy. **b** Northern blot showing GFP expression in transplastomic plants. **c** *rbcL* expression as loading control for northern blot hybridization. WT, wild type; T1 and T2, independent transplastomic lines of *N. benthamiana*; NT, transplastomic line of *N. tabacum*

following bombardment (Fig. 2a). Although this efficiency was lower than the >40% found with *N. tabacum*, we obtained 22 independent shoots from 15 bombardments in *N. benthamiana* versus 34 shoots from 20 bombardments in *N. tabacum*. When subcultured on the fresh SIM, green calli developed into shoots that emitted green fluorescence under UV light, indicating that they were transplastomic. Adventitious shoots were repeatedly cut and placed on fresh SIM to increase homoplasmy in second- and third-round selections. Those shoots were then rooted and transferred to soil for growth to maturity (Fig. 2b). T_1 seeds were germinated on an MS medium containing 500 mg L^{-1} of spectinomycin. After 2 weeks, spectinomycin-resistant seedlings were green and showed vigorous growth (Fig. 2c) compared with the bleached WT plants (Fig. 2d).

Transgene Expression in Plastid-Transformed Plants

Transplastomic leaves and flowers exhibited green fluorescence whereas WT leaves showed red autofluorescence under UV light (Fig 3a, b). Although the green fluorescence from transplastomic petals was not as high as that from their sepals, it was distinguishable from the WT. Chloroplasts in isolated protoplasts from selected plant leaves were yellow under UV light and green under fluorescent light with a FITC filter, whereas chloroplasts from the WT were red under UV and dark with the FITC filter (Fig. 3c–h). This indicated that *gfp* was properly and highly expressed in the transformed plastids of *N. benthamiana* and that its expression facilitated the tracking of cells.

PCR detected the presence of *aadA* and *gfp* in DNA extracts from plants with spectinomycin resistance and GFP fluorescence. The PCR with IL1 and IR2 primers revealed a 2-Kb amplified DNA band in the WT but a 4-Kb band and no 2-Kb WT band in transplastomic plants, thereby demonstrating homoplasmy for the transgenes (data not shown). Southern blot analysis with a *trn*A probe detected an approximately 4.5-Kb band from the WT and an approximately 6.5-Kb band from two independent transplastomic plants, indicating that *aadA* and *gfp* were correctly inserted between *trnI* and *trnA* in the plastid genome (Fig. 4a). Northern blot analysis with a *gfp* probe produced more than three bands, including strong 1.7-Kb

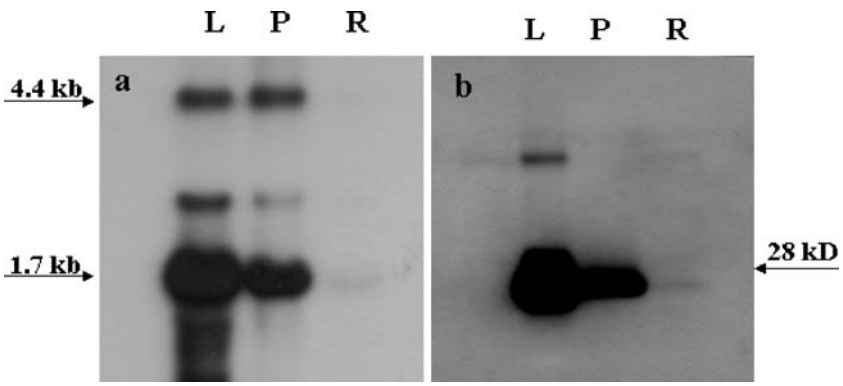

Fig. 5 *GFP* expression in plastids from different organs. **a** Northern blot for leaf, petal, and root from transplastomic *N. benthamiana.* **b** GFP production and accumulation. L, leaf; P, petal; R, root

and weak higher bands from the independent transformants (Fig. 4b). We compared *rbcL* transcription to show the loading amount of plastid RNA (Fig. 4c). Detection of several transcripts probed with *gfp* was attributed to the transgene in this vector system that is also expressed via read-through transcription by an intrinsic 16 S rRNA promoter (Jeong et al. 2004), as well as polycistronic transcriptional units with overlapping mRNAs (Sugita and Sugiura 1996).

GFP Expression in Various Plastids from Transplastomic Plants

Transcription and translation are much more active in the chloroplasts than in other plastids, including the chromoplasts and leucoplasts. The expression of foreign proteins in tomato fruits is up to 50% of that measured in leaves (Ruf et al. 2001). To compare transgene expression in the plastids of organs from transplastomic *N. benthamiana*, we studied *GFP* expression, including transcription and translation, in fully opened petals, leaves, and roots from 2-month-old plants. Very low levels of mRNA and GFP protein were detected in the roots (less than 2% of that in the leaves). This finding is consistent with a report that 100-fold less GFP protein is accumulated in the amyloplasts of potato tubers compared with the leaves (Sidorov et al. 1999). In carrot, the use of a full 16S rRNA promoter with a T7 10 5'UTR increases foreign expression in the chromoplasts of the taproot (53.1%; 74.8% BADH activity) compared with the leaf chloroplasts (100%). However, expression of GFP in our petals was as high as about 40% of that measured in the leaves, based on total RNA-loaded (Fig. 5a). Moreover, GFP accumulation in the petals was as much as about 37% of that in the leaves (Fig. 5b), suggesting that petal chromoplasts are also good candidates when using this system to study foreign gene transcription and translation because GFP mRNA and the protein expressed in those chromoplasts are still much higher than the cytosolic expression in a nuclear transgenic plant.

Plastid Transformation System in *N. benthamiana*

Here, we established a plastid transformation system for *N. benthamiana* that utilizes the same vector as for *N. tabacum*. We believe this is the first report of stable transformation for plastids in *N. benthamiana*. A comparable selection period, 4 to 8 weeks, was used for transgenic shoots of both species. Transformation efficiency was also similar between the two, except for the 9-bp addition in the intron sequence of the border region for *trnI*. This indicated that the *N. tabacum* sequence used here was sufficient for inserting a foreign gene into the target site between *trnI* and *trnA* of *N. benthamiana*. Expression of foreign genes has been reported for root and tuber amyloplasts, leaf chloroplasts, and fruit and petal chromoplasts (Ruf et al. 2001; Daniell et al. 2002). Furthermore, expression of an AADA-GFP fused protein has been found in petal chromoplasts from tobacco without a comparable level of expression in different plastids (Khan and Maliga 1999). Here, we observed that transgene expression, including transcription and translation, was active in both the petal chromoplasts and the leaf chloroplasts, which suggests that plastid transformation engineers the flower petal for metabolic changes or protein overexpression in the chromoplasts.

A number of studies about functional genomics using VIGS and plant disease are performed with *N. benthamiana* as the model species because of its easy nuclear transformation, small plant size, short life cycle, and the availability of adequate mass from just a single plant. The plastid transformation system for *N. benthamiana* described here may also be useful to investigations of the interactions between plastid and nuclear genes as well as for examining gene regulation and expression in the plastid.

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