A simple and effective system for foreign gene expression in plants via root absorption of agrobacterial suspension

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Abstract

Due to the laborious and scale-up limitation we have developed a simple system named "root absorption" to express foreign proteins in plants successfully. It has been shown that GFP was expressed in tobacco plants by root absorbing the *Agrobacterium* suspension containing TMV-based P35S-30B-GFP vector. Various factors influencing the gene expression were studied including *Agrobacterium* cell density, seedling age, plant materials and inoculation conditions. This system has the special advantages as simple and convenient work process, ease to scale-up and higher level of expression than leaf infiltration. Interestingly, GFP was expressed at 24 h post-absorption. We assume that the root absorption system will facilitate the large-scale production of the recombinant pharmaceutical proteins in plants by means of transient expression.
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1. Introduction

Plants are the most economical producer of biomass in nature. They only require sunlight, water, carbon dioxide and minerals providing an optimal system for the expression of recombinant proteins free of contamination by bacterial toxins and animal pathogens. They also offer an eukaryotic protein modification machinery, allowing subcellular targeting, proper folding, and posttranslational modification (Cunningham and Porter, 1998).

The first pharmaceutical protein produced in plants was human growth hormone in transgenic tobacco in 1986 (Barta et al., 1986). In the past 20 years, extensive research has shown that a wide range of valuable proteins have been efficiently expressed in plants such as human serum proteins and growth regulators, antibodies, vaccines, industrial enzymes, biopolymers and molecular reagents in biology (Fischer and Emans, 2000; Giddings, 2001; Hood, 2002). Foreign proteins can be produced in plants by stable transformation or transient expression

using virus-based vectors (Walmsley and Arntzen, 2000). Stable integration is often much less efficient than transient expression (Janssen and Gardner, 1989). Virus vectors are advantageous when high level of gene expression is desired within a short time. Plant viral vectors are being successfully developed and exploited for the industrial-scale expression of heterologous proteins and as a research tool for gene function analysis.

Several plant virus vectors have been developed for the expression of foreign proteins, including tobacco mosaic virus (TMV), potato virus X (PVX) (Chapman et al., 1992; Baulcombe et al., 1995) and tobacco rattle virus (TRV). Among the several viral vector systems, tobacco rattle virus (TRV)-derived vectors are widely used because they produce mild symptoms on the host and TRV has a wide host range (Dinesh-Kumar et al., 2003; Liu et al., 2002; Ratcliff et al., 2001). The tobravirus expression vectors are able to express GFP in, for example, *Nicotiana* species, tomato, pea, Arabidopsis, and sugar beet. The TRV and TMV vectors were able to express GFP in roots efficiently (Anandalakshmi et al., 1998). When foreign genes were expressed as read-through fusions with viral proteins, the viral coat protein of tobacco mosaic virus (TMV) accumulated to as much as 10% of the dry weight of an infected leaf (Pogue et al., 1998; Copeman et al., 1969). As an expression

vector with characteristics of relatively high stability and high-level production of heterologous proteins, the TMV-based 30B vector has been available for the experimental use worldwide (Yusibov et al., 1997; Shivprasad et al., 1999; Nenchinov et al., 2000).

Recent years, several groups have shown that HC-Pro has functions as an effective suppressor of PTGS independently (MacFarlane and Popovich, 2000; Brigneti et al., 1998; Pruss et al., 1997). Kasschau and Carrington (1998) proposed that the absence of functional HC-Pro silencing was targeted against the viral RNA or a replication intermediate. The presence of a functional HC-Pro would suppress the silencing response and restore amplification. This suggests that the presence of HC-Pro could enhance the expression level of recombinant proteins.

Leaf infiltration is the most common method of agroinfection used for transient expression of foreign proteins. This method, however, has some limitations as it is laborious and difficult to scale-up. Here we describe a simple system of agroinoculation, named "root absorption". This system has been shown effectively for the expression of *gfp* gene by root absorbing *Agrobacterium* suspension containing P35S-30B-GFP. Furthermore, this system has some advantages like simple and convenient process, ease to scale-up and high level of expression than leaf infiltration.

2. Materials and methods

2.1. Plant materials

Nicotiana benthamiana (Nb) and *Nicotiana benthamiana* HC-Pro plants (transgenic *N. benthamiana* plants expressing VIGS suppressor HC-Pro, Nb-HC-Pro) were used as the host plants. The seeds of both plants were sown in soil. After 1 week, the seedlings were grown in pots at $25 \pm 3\,°C$ in a growth chamber under 16 h light/8 h dark cycle.

2.2. Infection vectors and antibody

Agrobacterium tumefaciens strain EHA105 was kept in our laboratory. The vector 30B-GFP was a gift from the Institute of Microbiology, Chinese Academy of Sciences. EHA105-30B-GFP and purified GFP proteins were previously prepared in our laboratory. Rabbit antisera against GFP and go an Rb IgG/HRP were purchased from TAKARA Company, Dalian, China.

2.3. Preparation of Agrobacterium suspension

EHA105-35S-30B cells were grown overnight at $28\,°C$ in 5 ml LB media containing Kanamycin ($50\,\mu g/ml$), Rifampicin ($50\,\mu g/ml$) and Tet ($5\,\mu g/ml$). Then 1 ml of the overnight culture was diluted with 50 ml LB containing the antibiotics, 10 mmol/l MES (pH 5.6) and $20\,\mu mol/l$ acetosyringone. The cultures were incubated overnight at $28\,°C$ with shaking at 280 r/min. *Agrobacterium* cells were collected by centrifugation at 3000 rpm at $4\,°C$, and resuspended in MMA medium (10 mmol/l $MgCl_2$, 10 mmol/l MES pH 5.6, $100\,\mu mol/l$ ace-

tosyringone), adjusted to OD_{600} value of 0.6, 0.8, 1.0, 1.2, 1.4, and 1.6, left at room temperature for 3 h without shaking before inoculation.

2.4. The root absorption procedure

For root absorption, 4–5 ml *Agrobacterium* suspension containing P35S-35S-30B vectors was placed into a 5 ml Eppendorf tube, and then the root part of each plant was placed into the MMA medium in the tube and cultured at $25 \pm 3\,°C$ in a growth chamber under 16 h light/8 h dark cycles.

2.5. GFP detection

Under illumination of a 100 W hand-held long-wave ultraviolet lamp (UV products, model B 100AP), GFP fluorescence in plants was visualized and photographed using a digital camera Nikon coolpix995.

2.6. Analysis of GFP in plant materials

For protein analysis, 100 mg of stems and leaves were ground in powder in liquid nitrogen, and then collected in an Eppendorf tube containing $100\,\mu l$ PBS. It was centrifuged at 1200 rpm for 5 min at $4\,°C$, the supernatant was collected and boiled for 5–6 min for SDS-PAGE, followed by Comassie Brilliant Blue R-250 staining and Western blotting analysis with Rabbit antiserum against GFP and go a Rb IgG/HRP.

2.7. RT-PCR analysis of gfp gene transcripts in plant roots, stems and leaves

The total RNA was isolated from roots, stems and leaves with Promega Reagent Kit. First-strand cDNA was synthesized using avian myeloblastosis virus (AMV) Reverse Transcriptase (Promega) according to the manufacturer's instruction. A fraction of the first-strand cDNA was used as a template in the following PCR procedures. Primer P1 (sense sequence: ggggtaccttatttgtatagttcatccatgc) and P2 (antisense sequence: cgggatccatgagtaaaggagaagaacttt) were used for detection of the *gfp* gene transcript. PCR amplification was performed with the following condition: $95\,°C$ for 5 min; 30 cycles at $95\,°C$ for 30 s, $52\,°C$ for 30 s, $72\,°C$ for 1 min, followed by a final incubation at $72\,°C$ for 5 min. The RT-PCR products were analysis by electrophoresis in a 1% agarose gel.

3. Results

3.1. Agrobacterium concentration

The root absorption system was performed in various *Agrobacterium* concentrations and it was found that when MMA $OD_{600} = 1.23$ GFP expression reached to 100% in Nb-HC-Pro plants investigated. The expression efficiency was determined by the percentage of plants showing green fluorescence after *Agrobacterium* treatments. At lower *Agrobacterium* concentration (MMA $OD_{600} = 0.58$), however, GFP expression frequency
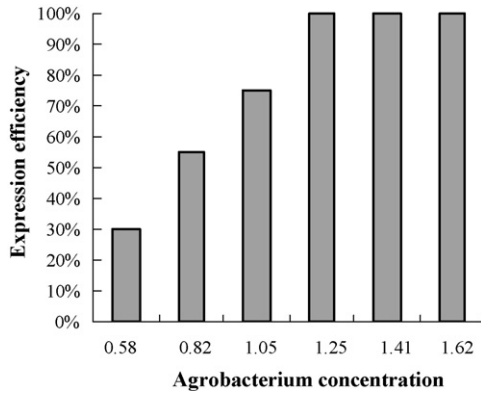
Fig. 1. The efficiency of GFP expression was influenced by *Agrobacterium* concentration in Nb-Hc-pro plants. The *Agrobacterium* concentration was determined by OD_{600} value.

Fig. 2. Comparison of GFP expression efficiency in seedlings at different ages.

was reduced to 50%. At higher *Agrobacterium* concentration ($1.23 < MMA\ OD_{600} < 1.62$), no destructive symptoms in the plants were observed in root absorption system (Fig. 1).

3.2. The expression level influenced by seedling age

In order to determine the influence of seedling age in expression efficiency, seedlings at quadrifiliate phase (phase I), quinquefoliate phase (phase II) and hexaphyllous phase (phase III) were used as the host materials with MMA $OD_{600} = 1.2$ in this system. The expression efficiency of phase I, phase II and phase III was 90%, 95% and 20%, respectively. The results showed that phase I and phase II were superior to phase III in GFP expression determined by the number of plants showing green fluorescence after root absorption of *Agrobacterium* suspension (Fig. 2).
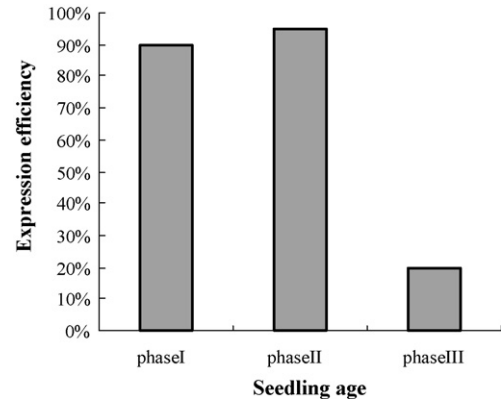
3.3. The influence of seedling roots in GFP expression

In seedlings with root-removal, green fluorescence was clearly visualized in upper leaves of Nb-HC-Pro plants treated by *Agrobacterium* suspension containing P35S-30B-GFP vectors at 5 days post-absorption (dpa) under UV illumination (Fig. 3a), diffused green fluorescence of GFP was observed in old leaves at 7 dpa (Fig. 3b), and green fluorescence was then detected in new rootlets (Fig. 3c, left). *Agrobacterium* suspension containing P35S-30B vectors was used as the control (Fig. 3c, right).

In seedlings with roots retained it was interesting to find that green fluorescence was mainly visualized in stems and young leaves of Nb-HC-Pro plants as early as 24 h post-absorption (Fig. 3d and e), green fluorescence was diffused in old leaves on 2-day post-absorption (Fig. 3f) and intact plants were totally green on 5 dpa (Fig. 3g). GFP expression in seedlings with roots was faster than in seedlings with root-removal.
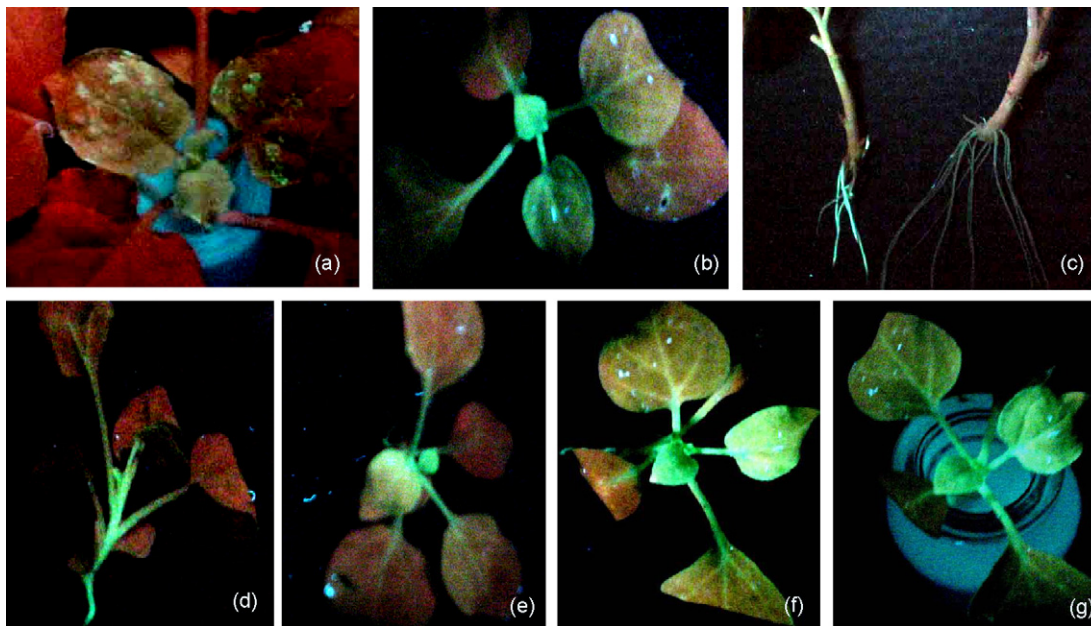

Fig. 3. GFP expression in root-removal experiments and in plants with roots retained. Green fluorescence was visualized in root-removal experiments (a) in new leaves of Nb-HC-Pro plants on 5 dpa, (b) in old leaves on 7 dpa, (c) in new rootlets (left), and control (right); the green fluorescence of GFP was observed in plants with roots retained (d and e) at 24 h post-absorption, (f) in old leaves on 2 dpa and (g) intact plants on 5 dpa.
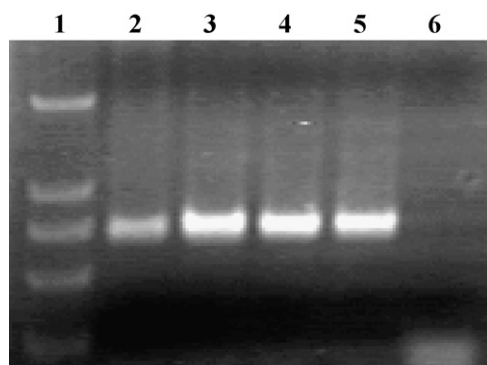
Fig. 4. The RT-PCR results in Nb-HC-Pro plants. Lane 1, molecular weight marker (D2000), lane 2, positive control, lanes 3–5, RT-PCR products of roots, stems and leaves, respectively, and lane 6, negative control.

The RT-PCR results for *gfp* gene transcripts provided further evidence for systemic spread of the foreign gene. The plasmid P35S-30B-GFP was used as positive control. Nb-HC-Pro plants infected by *Agrobacterium* suspension containing P35S-30B vectors was used as negative control (Fig. 4).

3.4. The expression level of GFP was different in Nb and Nb-HC-Pro plants

Nicotiana bonthanmiana (Nb) and *Nicotiana benthamiana* helper component-proteinase (Nb-HC-Pro) plants were used as the host plant to express GFP. The GFP expression was more effective in Nb-Hc-pro plants than in Nb plants infected by *Agrobacterium* suspension containing p35S-30B-GFP vectors. We compared the time course of Nb-HC-Pro plants and Nb plants for GFP expression, and found that the GFP expression in Nb-HC-Pro plants was faster for about 3–5 days than in Nb plants indicated by the green fluorescence.

GFP produced in Nb and Nb-HC-Pro plants was detected when the total plant proteins was analyzed by SDS-PAGE (Fig. 5a) and Western blotting (Fig. 5c). Total soluble proteins
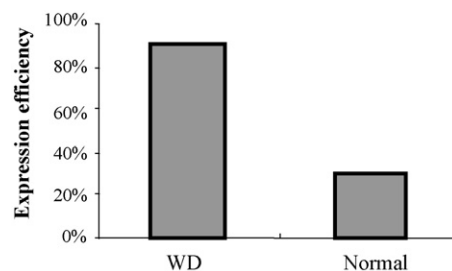

Fig. 6. Comparison of GFP expression efficiency between water deficiency (WD) and normal watering condition in Nb-HC-Pro plants.

of different *Agrobacterium* concentrations (MMA OD$_{600}$) from *Agrobacterium* suspension containing P35S-30B-GFP vectors was the control of total soluble proteins from leaves of Nb-HC-Pro plants infected by *Agrobacterium* suspension containing P35S-30B-GFP vectors (Fig. 5b). GFP expression level in Nb-HC-Pro plants was higher than in Nb plants also confirmed by extracted proteins in addition to the green fluorescent intensity. The amount of GFP in Nb-HC-Pro plants was estimated to be 8% of the total soluble plant proteins, however, be 5% in Nb plants.

3.5. GFP expression level was increased in water deficiency treatment

It was assumed that the GFP expression was due to the natural ability of active absorbing *Agrobacterium* suspension containing the virus vectors in root-absorption system. The water deficiency treatment was therefore performed and the enhancement of gene expression efficiency was resulted in this experiment (Fig. 6). Nb-Hc-pro plants were not watered for 7–10 days before inoculation (the Nb-HC-Pro plants with normal watering were used as control). GFP expression frequency was 90% and 30% in Nb-HC-Pro plants for water deficiency treatment and the control (MMA medium OD$_{600}$ = 0.5), respectively. The expression level was 60% different. This result suggested that gene expres-
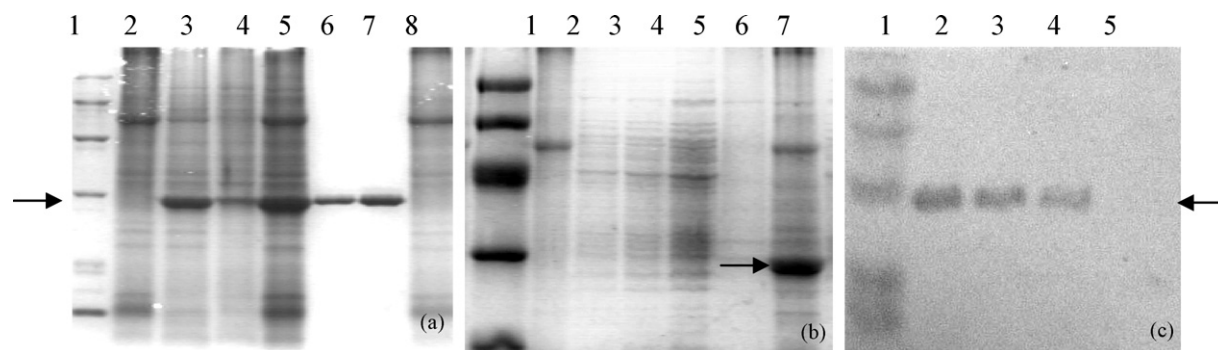

Fig. 5. (a) GFP protein analysis by 12% SDS-PAGE. Lane 1, molecular weight marker; lanes 2 and 8, total soluble proteins from leaves of Nb infected by *Agrobacterium* suspension containing P35S-30B vectors (control); lanes 3 and 5, total soluble proteins from stem and leaves of Nb-HC-Pro plants expressing GFP; lane 4, total soluble proteins from stems and leaves of Nb expressing GFP; lanes 6 and 7, purified GFP protein (1.4 ng, 2.8 ng). (b) Protein analysis by 12% SDS-PAGE. Lane 1, molecular weight marker; lanes 2, 6, total soluble proteins from leaves of Nb infected by *Agrobacterium* suspension containing P35S-30B vectors (control); lanes 3, 4, 5, total soluble proteins from *Agrobacterium* suspension containing P35S-30B-GFP vectors; lane 7, total soluble protein from leaves of Nb-HC-Pro plants expressing GFP. (c) Western blotting analysis of GFP with rabbit anti-GFP sera. Lane 1, molecular weight marker; lane 2, purified GFP protein; lane 3, total proteins from Nb-HC-Pro plants expressing GFP; lane 4, total soluble proteins from Nb plants expressing GFP; lane 5, total soluble proteins from leaves of Nb plants infected by *Agrobacterium* suspension containing p35S-30B vectors (control).

sion by root absorbing system may need to accumulate a certain amount of Agrobacteria.

4. Discussion

The pharmaceutical proteins can be efficiently expressed in plants via virus vector infection. We have developed the root absorption system, a simple protocol of agroinoculation to expression foreign proteins by tobacco mosaic virus (TMV)-derived vectors. This method has been proved effectively for the expression of GFP by root absorbing *Agrobacterium* suspension containing P35S-30B-GFP vectors. Compared with leaf infiltration technique, this novel method was a simple and convenient process, and ease to scale-up. Moreover, the expression level obtained by root absorption was higher than leaf infiltration method. It is assumed that the root absorption system may facilitate a rapid, large-scale and high expression level manner for the production of recombinant proteins in plants in the future.

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