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A new transient expression system for large-scale production of recombinant proteins in plants based on air-brushing an *Agrobacterium* suspension

Taicheng Jin [a], Jing Wang [a], Xiaojuan Zhu [b], Yanan Xu [a], Xiaofu Zhou [a,*], Liping Yang [a,**]

[a] The School of Life Sciences, Jilin Normal University, Siping 136000, China
[b] The Institute of Genetics and Cytology, Northeast Normal University, Changchun 130024, China

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ABSTRACT

Plant transient expression using virus-based vectors is advantageous when high level of gene expression is desired within a short time. In this study, a new system, named "air-brush," has been developed to facilitate a scale-up production of recombinant proteins in plants. GFP was expressed successfully in *Nicotiana benthamiana* (Nb) plants by air-brushing an *Agrobacterium* suspension that contained the TMV-based vector p35S-30B-GFP. Key factors influencing the gene expression were optimized, including the *Agrobacterium* cell density, seedling age, and the growth temperature of plant materials. In addition, the pharmaceutical protein human acidic fibroblast growth factor (ha FGF) was also expressed in Nb plants by the air-brush system. The results demonstrated that using this system is highly advantageous; it is convenient, quick, easily scaled-up, and has a higher expression efficiency than leaf infiltration.

1. Introduction

Nature's most economical producers of biomass are plants because their requirements are quite minimal: sunlight, water, carbon dioxide, and minerals [19]. Additionally, they also possess eukaryotic protein modification machinery, allowing for subcellular targeting, proper folding, and posttranslational modification [3]. Furthermore, in the past 20 years, extensive research has demonstrated that a wide range of valuable proteins have been efficiently expressed in plants, such as human growth regulators, antibodies, vaccines, industrial enzymes, biopolymers, and biological molecular reagents [6,8,11,9,13,5].

Using virus-based vectors to express foreign proteins in plants is advantageous for various reasons. For example, in stark contrast to the stable transformation system, foreign proteins that are expressed in plants by transient expression using virus-based vectors are not heritable by subsequent generations. Furthermore, virus vectors are particularly advantageous when high level of gene expression is desired within a short timeframe [16,19]. Moreover, many plant viruses have been designed as vectors for expressing foreign proteins, including the *tobacco mosaic virus* (TMV), *potato virus X* (PVX), [2,1,12], and *tobacco rattle virus* (TRV) [4]. Finally, plant viral vectors are being successfully developed and exploited for the industrial-scale expression of heterologous proteins.

However, this system remained restricted by laborious scale-up limitations; therefore, a simple system, named "root absorption," was developed for the expression of foreign proteins in plants [19]. Here, however, we described a more convenient system, named "air-brush," that facilitates the scale-up production of recombinant proteins in plants. The results of which demonstrated that GFP and the pharmaceutical protein ha FGF was expressed in Nb plants by air-brushing an *Agrobacterium* suspension that contained the TMV-based p35S-30B-GFP vector. This system is superior due to its special advantages; it is rapid, convenient, easily scaled-up, and attains a higher efficiency than leaf infiltration.

2. Materials and methods

2.1. Plant materials

Nicotiana benthamiana (Nb) plants were used as the host plants. Two weeks after the seeds were sown in soil, the resulting seedlings were grown in pots at 25 ± 3 °C in a growth chamber under a 16 h light/8 h dark cycle.

2.2. Infection vectors and antibody

The vector p35S-30B-GFP was given from the Institute of Microbiology at the Chinese Academy of Sciences (Beijing) [12]. The TMV-derived vector caused local symptoms to occur at 3 days

* Corresponding author. Tel.: +86 4343292019.
** Corresponding author.
E-mail addresses: zhouxiaofu@jlnu.edu.cn (X. Zhou), yangliping781124@163.com (L. Yang).

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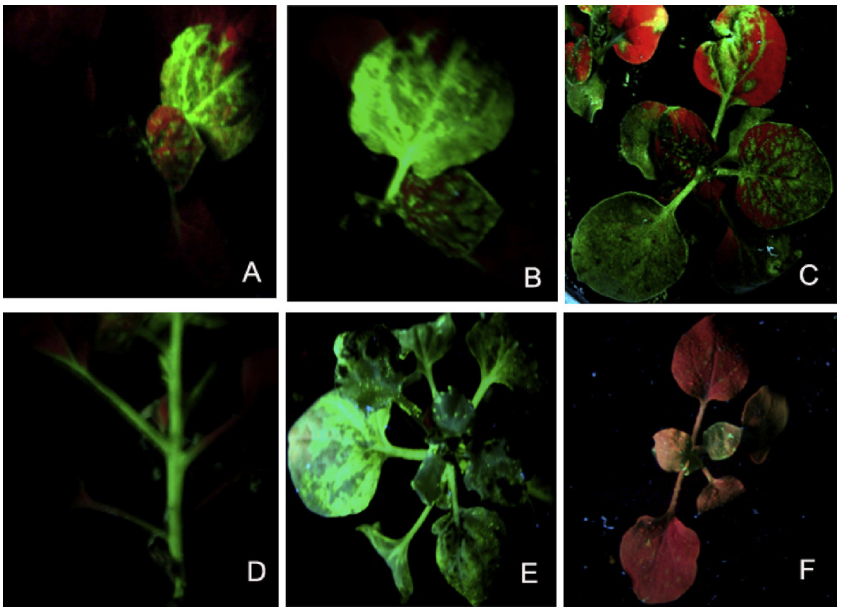

Fig. 1. GFP expression in *Nicotiana benthamiana* (Nb) plants.
A and B: Green fluorescence was visualized in the Nb plants' new leaves on 3 dpi and 4 dpi; C and D: diffused green fluorescence from GFP was detected in old leaves and stems on 5 dpi; E: the highest GFP accumulation appeared in both leaves and stems on 7 dpi; F: Nb inoculated by an *Agrobacterium* suspension that contained p35S-30B vectors was used as the control.

post-inoculation (dpi) and then led to systemic symptoms at 7 dpi. Our lab possessed the *Agrobacterium tumefaciens* strain EHA105. Furthermore, both the EHA105-p35S-30B-GFP cells and the purified GFP proteins were prepared in our laboratory. Rabbit antisera against GFP and Goat Anti-Rabbit IgG/HRP were both purchased from the TAKARA Bio Inc. (Changchun, China).

2.3. The air-brush procedure

The EHA105-p35S-30B and EHA105-p35S-30B-GFP cells were grown overnight at 28 °C in 5 ml of LB medium containing both Kanamycin (50 g/ml) and Rifampicin (50 g/ml). Then, 1 ml of the overnight culture was diluted with 50 ml of LB that contained the following components: the antibiotics, 10 mmol/l MES (pH 5.6), and 20 μmol/l acetosyringone. The cultures were incubated overnight at 28 °C with shaking at 280 rpm. The *Agrobacterium* cells were collected by centrifugation at 3000 rpm at 4 °C and then resuspended in MMA medium (10 mmol/l $MgCl_2$, 10 mmol/l MES (pH 5.6), and 100 μmol/l acetosyringone). Next, the culture was adjusted to optical density values at 600 nm (OD_{600}) of 0.6, 0.8, 1.0, 1.2, 1.4, and 1.6 and subsequently left at room temperature for 2–3 h.

The Nb leaves of 4-week-old plants were agroinoculated with the *Agrobacterium* suspension. Briefly, the suspension of the *A. tumefaciens* strain EHA105 contained the p35S-30B-GFP vectors with an OD_{600} of 0.6, 0.8, 1.0, 1.2, 1.4, and 1.6, mixed with 1% carborundum. The suspension was placed into a 250 ml air-brush tube and then sprayed onto the leaves using the air-brush technique Whitham et al., 1999 at a 75–80 psi export pressure during spraying. The inoculated plants were covered with black plastic bags for 24 h for recovery [10]. Next, they were cultured at 25 ± 3 °C in a growth chamber under 16 h light/8 h dark cycles.

2.4. GFP detection

Using a 100 W hand-held long-wave ultraviolet lamp (UV products, model B 100AP) for illumination, the GFP fluorescence in plants was visualized and photographed using a digital camera (Nikon Coolpix 995).

2.5. Analysis of GFP in plant materials

For protein analysis purposes, 100 mg of the leaves were ground into a powder in liquid nitrogen and then collected in an Eppendorf tube. The powder was stirred into an extraction buffer of 20 mmol/L sodium phosphate (pH 7.4) that contained 0.6 mol/l NaCl; after stirring, the mixture was centrifuged for 20 min at 4 °C [13]. The resulting supernatant was collected and then boiled for 5–6 min for use in SDS-PAGE and then transferred onto nitrocellulose membranes for immunoblotting; the electrophoresis was followed by Comassie Brilliant Blue R-250 staining and Western blotting analysis [5] with rabbit antiserum against GFP and Goat Anti-Rabbit IgG/HRP. The murine polyclonal antibodies against GFP were generated in our laboratory. GFP protein-bound antibodies were visualized by chemiluminescent detection (Super-Signal WestPico Trial kit from Pierce). The resulting Western blot image

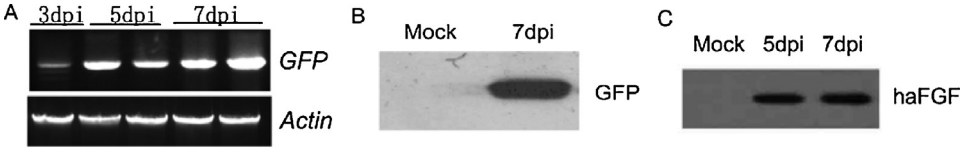

Fig. 2. The semi-quantitative RT-PCR and Western blotting results for the Nb plants.
A: Sq-RT-PCR analysis of GFP expression in Nb plants. *Actin* served as a control; B: Western blotting analysis of GFP with rabbit anti-GFP sera. 7 dpi, Western blotting products produced from Nb plants expressing GFP on 7 dpi; mock served as the control, Western blotting products of Nb plants inoculated on 7 dpi by the *Agrobacterium* suspension that contained p35S-30B vectors; C: Western blotting analysis of ha FGF, Western blotting products of Nb plants inoculated on 5 dpi and 7 dpi; mock served as the control.

was used to analyze the protein expression quantity using Bandscan software.

2.6. RT-PCR analysis of GFP gene transcripts in plants

The leaves' total RNA was isolated using the Trizol Reagent. First-strand cDNA was synthesized using the avian myeloblastosis virus (AMV) Reverse Transcriptase (TAKARA Bio Inc., Changchun, China) according to the manufacturer's instruction. Then, a fraction of this first-strand cDNA was used as a template in the following PCR procedures: Primers P1 (5′-GGGGTACCTTATTTGTATAGTT-CATCCATGC-3′) and P2 (5′-CGGGATCCATGAGTAAAGAGGAA-GAACTTTT-3′) were used to detect the *GFP* gene transcript through using the DNA polymerase Ex Taq (TAKARA Bio Inc., Changchun, China). The RT-PCR products were analyzed by electrophoresis in a 1% agarose gel [19]. The gene *Actin* served as the control.

3. Results

3.1. GFP expression in Nb plants

Green fluorescence was clearly visualized in the upper leaves of the Nb plants that had been treated with the *Agrobacterium* suspension that contained the p35S-30B-GFP vectors at both 3 days post-inoculation (dpi) and 4 dpi under UV illumination (Fig. 1A and B). Moreover, diffused green fluorescence from the GFP was then observed in old leaves and stems at 5 dpi (Fig. 1C and D), and furthermore, green fluorescence was totally detected in the intact plants at 7 dpi (Fig. 1E). An *Agrobacterium* suspension that contained the p35S-30B vectors was used as the control (Fig. 1F).

3.2. GFP and ha FGF expression were detected in Nb plants

The semi-quantitative RT-PCR (sq-RT-PCR) results for the *GFP* gene transcripts were a further source of verification for the systemic spread of the foreign gene in plants. The Nb plants that were inoculated with the *Agrobacterium* suspension that contained the p35S-30B-GFP vectors on 3 dpi, 5 dpi, and 7 dpi were used as the plant materials from which the total RNA was extracted. *Actin* served as a control (Fig. 2A).

Through analysis, we determined that the highest GFP accumulation generally appeared in both the leaves and stems on 7 dpi. The GFP production in the Nb plants that had been inoculated on 7 dpi with the *Agrobacterium* suspension that contained the p35S-30B-GFP vectors was detected via a Western blot analysis of the total plant proteins. The control was the total soluble proteins of the Nb plants that had been inoculated on 7 dpi with the *Agrobacterium* suspension that contained the p35S-30B vector (Fig. 2B). These results demonstrate that the GFP protein's maximal expression was achieved on 7 dpi.

In order to further confirm the obtained results, the experiment was repeated using a second recombinant protein ha FGF. It was demonstrated that ha FGF was successfully expressed in Nb plants using the air-brushing system. The ha FGF produced in the Nb plants was detected, and again, a Western blot was employed to analyze the total plant proteins; mock served as a control that the total soluble proteins from the Nb plants inoculated on 7 dpi with the *Agrobacterium* suspension that contained the p35S-30B vectors (Fig. 2C).

3.3. The influence of Agrobacterium concentration on expression efficiency

After performing the air-brush system with various *Agrobacterium* concentrations (MMA OD_{600}), it was found that

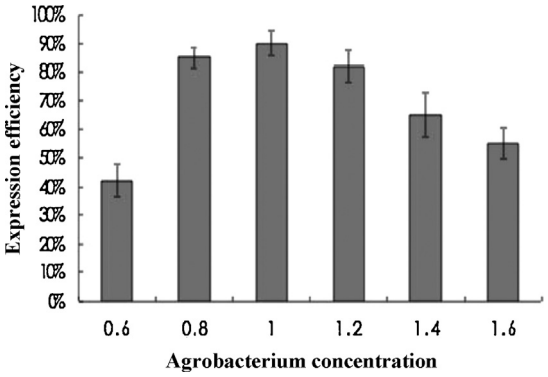

Fig. 3. The GFP expression efficiency was influenced by the *Agrobacterium* concentration (concentration determined by the OD_{600} value) in Nb plants.

the GFP expression reached a maximum efficiency [90%] in the investigated Nb plants when MMA OD_{600} = 1.00 as well as when the plant materials were cultured at 28 °C in a growth chamber. The expression efficiency was determined according to the percentage of plants that exhibited green fluorescence after the *Agrobacterium* treatments. At lower *Agrobacterium* concentrations (MMA OD_{600} < 0.80), the GFP expression frequency reduced to 42% (Fig. 3). Furthermore, at higher *Agrobacterium* concentrations (MMA OD_{600} > 1.20), the plants' GFP expression frequency was also reduced in the air-brush system.

3.4. The influence of growth temperature on expression efficiency

In order to determine whether growth temperature influences expression efficiency, the GFP expression efficiency was examined in relation to growth temperature in the system. When MMA OD600 = 1.0 and the growth temperatures were set to 22 °C, 25 °C, 28 °C, 31 °C, and 34 °C, the GFP expression efficiency was 65%, 80%, 92%, 75%, and 68%, respectively (Fig. 4). This data reveals that 28 °C was superior to both the lower temperature (22 °C) as well as the higher temperature (34 °C) in terms of GFP expression efficiency, which was determined by the number of plants that exhibited green fluorescence with the *Agrobacterium* suspension on 7 dpi.

3.5. The influence of seedling age on expression efficiency

In order to determine whether seedling age influences expression efficiency, seedlings at the quadrifiliate phase (phase I), the quinquefoliate phase (phase II), and the hexaphyllous phase (phase III) were used as the plant materials with the *Agrobacterium* suspension (MMA OD_{600} = 1.0) and a growth temperature of 28 °C in the system. The expression efficiency of phase I, phase II, and phase III was 84%, 92%, and 65%, respectively. These results reveal that phase I and phase II are superior to phase III in terms of GFP

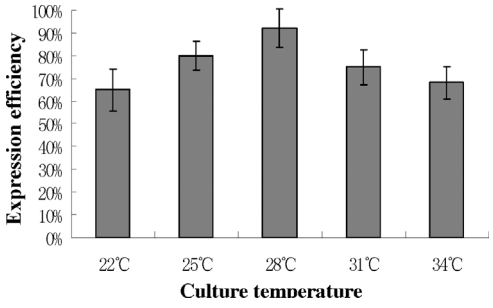

Fig. 4. Comparison of GFP expression efficiency in Nb plants at different temperatures with the same *Agrobacterium* concentration (OD_{600} = 1.0).

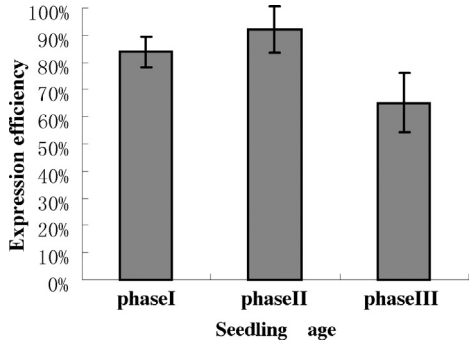

Fig. 5. Comparison of GFP expression efficiency in seedlings at different ages.

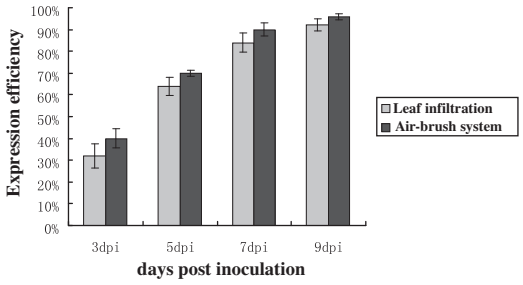

Fig. 6. The efficiency of GFP expression by the air-brush system compared with leaf infiltration.
Compared with leaf infiltration, the efficiency of GFP expression obtained by the air-brush system was higher than leaf infiltration method on 3 dpi, 5 dpi, 7 dpi and 9 dpi with the same *Agrobacterium* concentration (OD_{600} = 1.0) and at the same incubate temperature (28 °C).

expression, as determined by the number of plants exhibiting green fluorescence after air-brushing with the *Agrobacterium* suspension (Fig. 5).

3.6. Comparison between the air-brush system and leaf infiltration on GFP expression efficiency

A comparison was conducted in order to determine the difference in GRP expression efficiencies of the air-brush system and leaf infiltration. To do so, the efficiency of GFP expression was detected in Nb plants using each of the two different methods. Compared to the leaf infiltration, the GFP expression efficiency produced by the air-brush system was higher than that produced by the leaf infiltration method on 3 dpi, 5 dpi, 7 dpi, and 9 dpi using the same *Agrobacterium* concentration (OD_{600} = 1.0) and the same incubation temperature (28 °C) (Fig. 6).

4. Discussion

Transient expression has been demonstrated to result in the efficient expression of a wide range of valuable proteins in plants [15,9,14]. Of the agroinoculation methods used for the transient expression of foreign proteins, leaf infiltration is the most common [17,7,18]. Having said that, a new, more simplistic system, named "root absorption," was developed to express foreign proteins in plants [19]. In this paper, however, we present a more convenient alternative. In order to facilitate a scaled-up production of recombinant proteins, we have developed the air-brush system to express foreign proteins in Nb plants. In this study, GFP and the other protein ha FGF were successfully expressed by air-brushing the *Agrobacterium* suspension that contained the TMV-derived vectors.

Moreover, various factors capable of influencing gene expression were optimized, including the *Agrobacterium* cell density, seedling age, and the plants' incubation conditions. In addition, a variation of two other parameters can directly influence the system's gene expression, such as the percentage of carborundum and the pressure used during spraying. Further, we found that no destructive symptoms in the plants were observed after inoculation when the *Agrobacterium* suspension was mixed with 1–5% carborundum; however, the tobacco leaves exhibited serious damage when this carborundum percentage was increased to 10%. In addition, it has been shown that no destructive symptoms in the post-inoculation plants were observed when a 75–80 psi export pressure was used. However, when a higher pressure (95–100 psi) was used during spraying, obvious necrosis symptoms were observable in the plants.

The proposed system is advantageous because it presents a rapid, simple work process. For instance, when the *Agrobacterium* suspension was prepared beforehand, it only took twenty minutes to spray hundreds of plants with an *Agrobacterium* suspension that contained the vector p35S-30B-GFP. Therefore, it is clear that when a large number of plants need to be agroinoculated, the new system is more advantageous and applicable for transiently expressing foreign proteins. Furthermore, the system is also suitable for other virally-derived vectors, such as PVX and TRV. We assume that the new system will facilitate a scale-up production of recombinant proteins in plants by means of a transient expression system.

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References

[1] D.C. Baulcombe, S. Chapman, S. Santa Cruz, Jellyfish green fluorescent protein as a reporter for virus infections, Plant J. 7 (1995) 1045–1053.
[2] Chapman, T. Kavanaph, D. Baulcombe, Potato virus X as a vector for gene expression in plants, Plant J. 2 (1992) 549–557.
[3] C. Cunningham, J.R. Porter, Recombinant Proteins from Plants: Production and Isolation of Clinically Useful Compounds, Totawa, NJ, Humana Press, 1998, pp. 203–227.
[4] S.P. Dinesh-Kumar, R. Anandalakshmi, R. Marathe, M. Schiff, Y. Liu, Virus-induced gene silencing, Methods Mol. Biol. 236 (2003) 287–294.
[5] Y.J. Fan, W. Li, J.J. Wang, J.Y. Liu, M.Y. Yang, D. Xu, X.J. Zhu, X.Z. Wang, Efficient production of human acidic fibroblast growth factor in pea (*Pisum sativum* L.) plants by agroinfection of germinated seeds, BMC Biotechnol. 11 (2011) 45.
[6] R. Fischer, N. Emans, Molecular farming of pharmaceutical protein, Transgenic Res. 9 (2000) 277–299.
[7] R. Fischer, E. Stoger, S. Schillberg, P. Christou, R.M. Twman, Plant-based production of biopharmaceuticals, Curr. Opin. Plant Biol. 7 (2) (2004) 152–158.
[8] G. Giddings, Transgenic plants as protein factories, Curr. Opin. Biotechnol. 12 (2001) 450–454.
[9] Y. Gleba, V. Klimyuk, S. Marillonnet, Magnifection: a new platform for expressing recombinant vaccines in plants, Vaccine 23 (17–18) (2005) 2042–2048.
[10] H. Chen, Z. Zhang, K. Teng, J. Lai, Y. Zhang, Y. Huang, Y. Li, L. Liang, Y. Wang, C. Chu, H. Guo, Q. Xie, Up-regulation of LSB1/GDU3 affects geminivirus infection by activating the salicylic acid pathway, Plant J. 62 (2010) 12–23.
[11] E.E. Hood, From green plants to industrial enzymes, Enzym. Microb. Technol. 30 (2002) 279–283.
[12] H.G. Jia, Y.Y. Pang, R.X. Fang, Agroinoculation as a simple way to deliver a tobacco mosaic virus-based expression vector, Acta Bot. Sin. 45 (7) (2003) 770–773.
[13] J.Y. Liu, P.D. Ma, Y. Sun, M.Y. Yang, L.P. Yang, Y.X. Li, Y.F. Wu, X.J. Zhu, X.Z. Wang, Expression of human acidic fibro-blast growth factor in *Nicotiana benthamiana* with a potato-virus-X-based binary vector, Biotechnol. Appl. Biochem. 48 (3) (2007) 143–147.
[14] J. Li, M. Chen, X.W. Liu, H.C. Zhang, F.F. Shen, G.P. Wang, Transient expression of an active human interferon-beta in lettuce, Sci. Hort. 112 (2007) 258–265.

[15] Y. Li, Y. Geng, H. Song, G. Zheng, et al., Expression of a human lactoferrin N-lobe in *Nicotiana benthmiana* with potato virus X-based agroinfection, Biotechnol. Lett. 26 (12) (2004) 953–957.
[16] V. Negrouk, G. Eisner, H.I. Lee, K. Han, D. Taylor, H. Wong, Highly efficient transient expression of functional recombinant antibodies in lettuce, Plant Sci. 169 (2005) 433–438.
[17] R.M. Twyman, E. Stoger, S. Schillberg, P. Christou, R. Fischer, Molecular farming in plants: host systems and expression technology, Trends Biotechnol. 21 (12) (2003) 570–578.
[18] A. Varsani, A. Williamson, D. Stewart, E. Rybicki, Transient expression of Human papillomavirus type 16 L1 protein in *Nicotiana benthamiana* using an infectious tobamovirus vector, Virus Res. 120 (2006) 91–96.
[19] L.P. Yang, H.W. Wang, J.Y. Liu, L. Li, Y.J. Fan, X. Wang, Y.H. Song, S.G. Sun, L. Wang, X.J. Zhu, X.Z.H. Wang, A simple and effective system for foreign gene expression in plants via root absorption of agrobacterial suspension, J. Biotechnol. 134 (3–4) (2008) 320–324.