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Full Length Research Paper

Transient expression of acidic fibroblast growth factor in pea (*Pisum sativum* L.) plants

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Nowadays, there are many therapeutic proteins produced in different host plants in transient easy to perform, short production cycle, efficient and inexpensive. In this study, the modified pea early browning virus (PEBV) vector containing GFP and acidic fibroblast growth factor (aFGF) was introduced into pea plants by leave injection. GFP expression was first observed in the treated leaves 8 to 10 days post-inoculation (dpi) and reached to the highest accumulation in both leaves and stems in 12 to 14 dpi. We suggested that 12 to 14 dpi were the best harvesting time for foreign proteins. The aFGF expression in pea plants has also been confirmed by Western blot.

Key words: Pea plants, acidic fibroblast growth factor, leave injection, plant bioreactor.

INTRODUCTION

Transient expression system due to high levels of protein expression in short time has become a popular way to express foreign proteins (Fischer et al., 2004). So far, there are several inoculation methods that have been used for transient expression. Early in 1986, Dawson confirmed that the plants infected with the cDNA transcripts of tobacco mosaic virus appeared identical to the parental virus (Dawson et al., 1986). After that mechanically inoculation method was developed to express foreign proteins such as antiviral and antitumor proteins in squash plants (Arazi et al., 2002) and HIV-1 in spinach plants (Karasev et al., 2005), foot-and-mouth disease virus epitopes in tobacco plants (Wu et al., 2003). Turpen et al. (1993) first inoculated tobacco plants with *Agrobacterium tumefaciens* containing cDNA of tobacco mosaic virus and this was the first time to introduce *Agrobacterium* for transient expression. The vacuum infiltration was one of the *Agrobacterium* mediated transient expression methods developed by Kapila in 1997 (Kapila et al., 1997) and this method mainly works on the intact leaves. So far, many host plants were chosen to express foreign proteins in this way (Hulla et al., 2005; Li et al., 2007). *Toxoplasma gondii* dense granule Gra4 antigen was expressed in tobacco leaves (Ferraro et al., 2008) and a human IgG1 k anti-tissue factor antibody (hOAT) was produced in lettuce (Negrouk et al., 2005). Leave injection was another effective way to introduce *Agrobacterium* with foreign gene into plant leaves (Jia et al., 2003; Constantin et al., 2004). By this way, the human acidic fibroblast growth factor has been expressed in tobacco plants in our lab (Liu et al., 2007) and Li et al. (2004) expressed a human lactoferrin N-lobe in *Nicotiana benthamiana* with potato virus X.

Virus based protein expression produced the systemic spread of virus as well as the rapid expression so that recombinant protein was produced in every cell. MacFarlane (2000) inoculated the pea plants with PEBV-GFP (pea early browning vector) transcripts, in this vector, GFP is placed under control of the TRV coat protein promoter, substituting the nematode transmission genes and the GFP produced fluorescence also in the noninoculated leaves 5 to 7 dpi (days post inoculation). PEBV is a rod-shaped virus with an RNA genome consisting of RNA1 and RNA2 and belongs to the same genus as TRV (MacFarlane, 2010; MacFarlane and Popovich, 2000; Ratcliff et al., 1999; Wang et al., 1997).

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Abbreviations: PEBV, Pea early browning virus; **aFGF,** acidic fibroblast growth factor; **dpi,** days post inoculation.

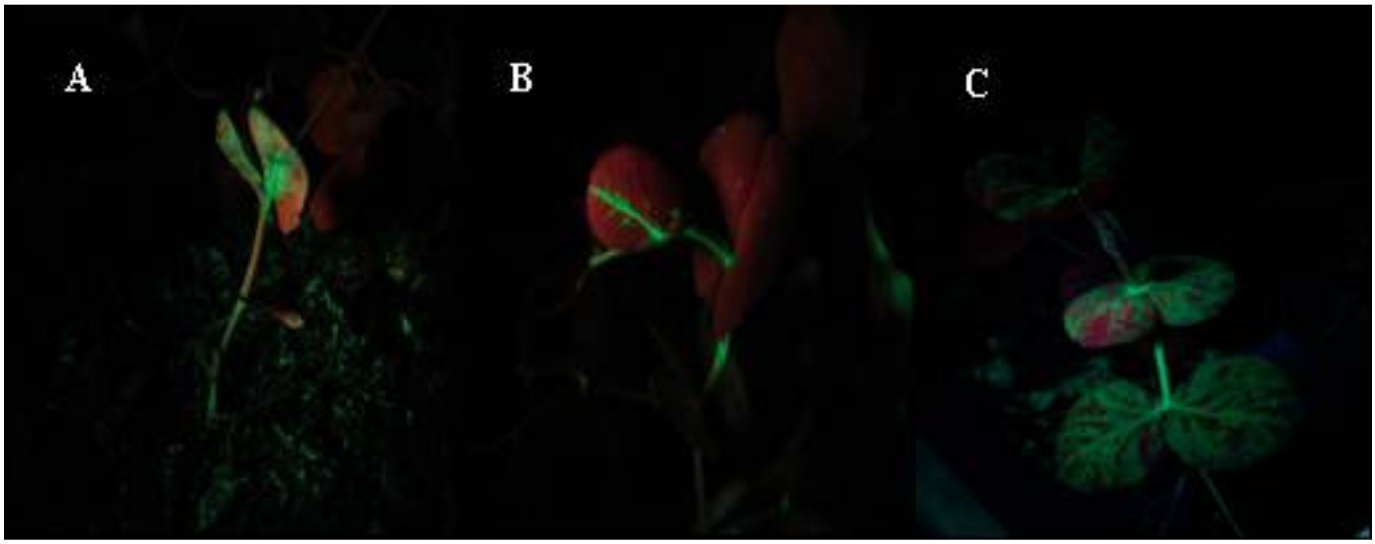

Figure 1. GFP expression in pea plants after agroinfection with GV3101-pCAPE2-GFP and GV3101-pCAPE1, (A) 8 dpi, GFP first expressed in the inoculated leaves; (B) 10 dpi, GFP appeared in the noninoculated leaves and stems; (C) 13 dpi, GFP diffused intensively in the noninoculated leaves.

It has also been modified as VIGS vector derived from pCAMBIA 1300. The expression cassette of RNA1 and RNA2-GFP has been named as pCAPE1 and pCAPE2-GFP, respectively, cloned under control of the 35S promoter and the NOS terminator (Constantin et al., 2004).

Acidic fibroblast growth factor (aFGF) is a member of a large FGF family consisting of group of structurally related polypeptides. It plays important roles in various stages of development and morphogenesis and also in angiogenesis and wound healing processes (Basilico and Moscatelli, 1992; Burgess and Maciag, 1989; Pellegrini et al., 2000; Yamashita et al., 2000). In our experiments, the PEBV expression vector containing aFGF was inoculated into pea (*Pisum sativum* L.) plants by leaf injection and aFGF expression was confirmed by Western blot analysis successfully. This implies that there is an important commercial values for pea plants as bioreactor due to the fact that the most leguminous plants have high quality protein content.

MATERIALS AND METHODS

Plasmid construction

The pCAPE2 vector (pCAPE1 and pCAPE2-GFP provided by Dr. Gabriela Constantin) was modified by a BglII site since the NcoI site was not suitable for the aFGF (A01474) sequence (pET28-aFGF, provided by Jilin Agricultural University, Changchun, China). Primers to amplify the aFGF fragment with unique BglII and EcoRI sites were designed. The forward and reverse primers were 5'-GGAAGATCTACATGGCTAATTACAAGAAGCC-3' and 5'-CGGAA TTCTTAATCAGAAGAGACTGGCAGG-3'. The PCR fragment between BglII-EcoRI digestion site was subcloned into pCAPE2 by the replacement of GFP sequence and this new vector was named pCAPE2-aFGF.

Plant materials and growth condition

The seeds of pea plant (*P. sativum.*) were obtained from the local market. The seeds were sowed and plants were grown in a plant growth chamber at 25°C under a 16 h cool fluorescent light/8 h dark cycle. *Agrobacterium* inoculation was performed when the plants have 2 to 3 expanded leaves.

Agroinfection of pea plants

The binary vectors pCAPE2-aFGF, pCAPE2-GFP and pCAPE1 were introduced into *Agrobacterium tumefaciens* strain GV3101 by freeze-thaw method (Holsters et al., 1978). Individual clones were grown in 200 ml LB with 50 ug ml^{-1} rifampicin, 50 ug ml^{-1} kanamycin and 25 ug ml^{-1} tetracycline at 28°C for 12 to 16 h with shaking. At OD_{600}= 1.0-1.5 the bacteria were harvested by centrifugation (3500 *g*) at room temperature. Cells were resuspended in infiltration medium (10 mM NaCl, 1.75 mM $CaCl_2$, 100 uM acetosyringone and 250 ul L^{-1} Tween 20, pH 5.6) and incubated at room temperature for 90 min without shaking. *Agrobacterium* cultures harboring pCAPE1, pCAPE2-GFP, pCAPE1 and pCAPE2-aFGF were mixed 1:1 v/v with infiltration media prior to inoculation. Infiltration was performed using a 2 ml syringe without needles as the plants with 2 to 3 whole expand leaves. GFP fluorescence was observed in seedlings using a 100 W, long-wave UV lamp (Black Ray model B-100 AP; Ultra-Violet Products, Upland, CA, U.S.A.). Plants were photographed with a digital camera (Sony DSC-F828) mounted with yellow filters (Figure 1).

Analysis of GFP expression frequency

The percentage of plants expressing GFP (GFP expression frequency) was used to estimate the treatment efficiency during 15 days post expression period. More than 20 plants were used in each treatment with three repeats (Figure 2).

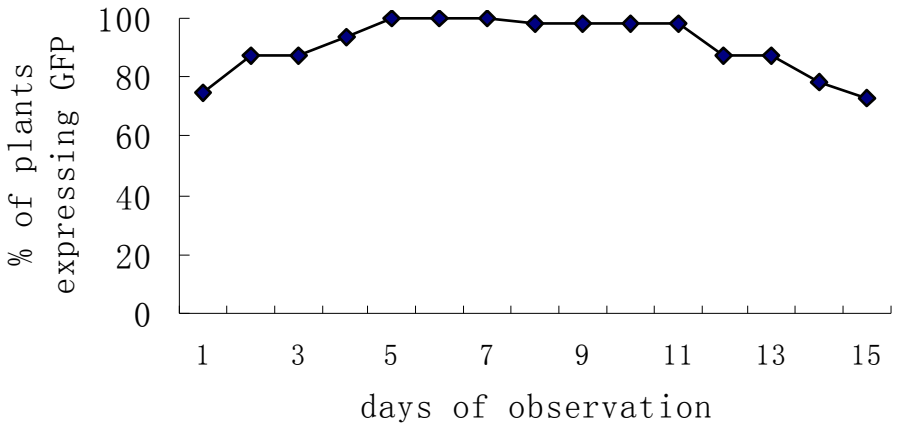

Figure 2. Analysis of GFP expression frequency. 1 to 4 days of observation more than 75% plants expressed GFP, 5 to 7 days of observation (12 to 14 dpi) GFP expressed in all the plants and GFP tended to decrease from the 8th days of observation.

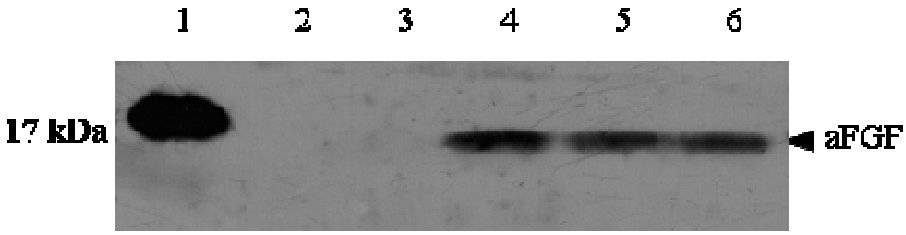

Figure 3. Western blot of aFGF. Lane 1, commercial aFGF; lane 2, protein from GV3101 (contain pCAPE1 + pCAPE2-aFGF); lane 3, proteins from noninoculated pea plants; lanes 4, 5, 6 proteins from plants agroinfected with GV3101-aFGF and GV3101-pCAPE1.

Western blot analysis of aFGF

The plant materials were frozen in liquid nitrogen and pulverized. The powder was stirred in extraction buffer of 20 mmol/l sodium phosphate (pH 7.4) containing 0.6 mol/l NaCl, then centrifuged at 20 817 *g* for 20 min at 4℃. The supernatants were analyzed by 15% SDS PAGE and then transferred to nitrocellulose membranes for immunoblotting. The murine polyclonal antibodies against aFGF were made in our laboratory. The proteins were extracted from GV3101 and non-inoculated pea plants were used as the negative control (Figure 3).

RESULTS

GFP expression in agroinfected pea plants

The GFP expression was visible in the plants agro-infected with GV3101-pCAPE1 and GV3101-pCAPE2-GFP. The green fluorescence was first visualized in the treated leaves at 8 to 10 dpi and on the next day, GFP was detected in stems and upper non-agroinfected leaves of 75% plants. In the following two days, the diffused GFP was more intensive in leaves, GFP fluorescence was visualized in all the plants at 12 to 14 dpi and the highest accumulation of GFP appeared in the stems and leaves at this period. GFP tended to decrease at 15 to 16 dpi; and in 18 to 20 dpi, the GFP fluorescence almost disappeared. We suggested that 12 to 14 dpi was the best time for protein harvesting.

Western blot analysis of aFGF

For Western blotting, the total soluble protein expressed in plants agroinfected with GV3101-pCAPE1 and pCAPE2-aFGF showed a single band in Western hybridization and the total soluble proteins harvested from GV3101 and non-inoculated plants did not react with anti-aFGF antibody. The Western blot image was used to analyze protein expression quantity by Bandscan software and 1% of the plant total soluble protein was yielded in this system.

DISCUSSION

Legumes are important crops in agriculture due to their ability to form nitrogen-fixing symbiosis with rhizobia and

their nutritional value. Some legume plants cannot only be used as forage crop but also as an expression system to produce pharmaceuticals, for example, monoclonal antibodies and blood substitutes produced in alfalfa (Vlahova et al., 2005) and some of the legumes plants including *P. sativum* are difficult to transform (Udvardi, 2001). In our experiments, pea plants were used as bioreactor to transient express a human acidic fibroblast growth factor. This is a new alternative crop for pharmaceutical production since the tobacco plants contain nicotine and other toxic alkaloids, which should be removed during downstream processing steps.

There are two strategies for expression of pharmaceutical protein in plants, stable plant transformation and transient plant expression. Compared with the stable transformation, the later has the advantages of simple manipulation, rapid analysis and high expression efficiency. Turpen (1993) and Marillonnet (2005) have demonstrated that agroinfection by DNA viruses is much more effective than the wild-type virus. After infiltration of the plants with the *Agrobacterium* containing the modified viral vector, the virus were multiplied and spread in most nutritorium of the plants, the target gene is transcribed by the viral RNA replication and translated into protein in the cytoplasm (Porta et al., 1996), there is no chromosomal integration and more ecological safety in transient expression plants (Gleba, 2007; Negrouk, 2005). In this study, the pea plants were inoculated with the modified PEBV vectors and GFP was used as reporter gene, the recombinant protein expression was observed under UV illumination. Not only the highest accumulation of GFP but also the highest protein expression frequency appeared in 12 to 14 dpi, so we harvested the aFGF protein at 12 to 14 dpi. These results showed that the pea plants could be a new alternative crop as plant bioreactor.

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