J. Plant Biotechnology (2005) Vol. 7 (2). pp. 97~103

Journal of Plant Biotechnology

Expression of the Green Fluorescent Protein (GFP) in Tobacco Containing Low Nicotine for the Development of Edible Vaccine

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Abstract

This study was carried out to obtain basic information for gene manipulation in potent edible tobacco (*Nicotiana tabacum* cv. TI 516). *N. tabacum* cv. TI 516 is a plant for a possible candidate to use as an edible vaccine, since it contains a low level of nicotine. The effective plant regeneration system through leaf disc culture was achieved using a MS basal medium supplemented with 0.1 mg 1^{-1} NAA and 0.5 mg 1^{-1} BA. In order to transform the *N. tabacum* cv. TI 516 with the green fluorescent protein (GFP) gene, *Agrobacterium tumefaciens* LBA 4404 containing the GFP gene was used. Genomic PCR confirmed the integration of the GFP gene into nuclear genome of transgenic plants. Expression of the GFP gene was identified in callus, apical meristem and root tissue of transgenic *N. tabacum* cv. TI 516 plants using fluorescence microscopy. Western blot analysis revealed the expression of GFP protein in the transgenic edible tobacco plants. The amount of GFP protein detected in the transgenic tobacco plants was approximately 0.16% of the total soluble plant protein (TSP), which was determined by ELISA.

Key words: *Agrobacterium*-mediated transformation; Edible tobacco; Edible Vaccine; Green fluorescent protein; Plant growth regulators; Plant regeneration.

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Received Dec. 17, 2004 ; Accepted Jun. 17, 2005

Introduction

Tobacco is particularly well suited to the task of producing vaccines and other specialized compounds. It is relatively easy and cheap to genetically alter tobacco. Tobacco plants also produce large amounts of biomass, about 180,000 pounds per acre per year. This raw tobacco leaf and other materials would be crushed and processed so the protein could be extracted. There has been a tobacco biomass research project at North Carolina State University for many years, demonstrating that it has vast food and medical potential from tobacco protein extracted from regularly mowed, densely planted acreage of high sugar, high protein, and no-nicotine tobacco. High in protein and fiber, these plants contain almost no nicotine and less sugar than the leaf tobacco that is used in cigarettes and cigars. Tobacco plants have been frequently used as a convenient model system for the development of edible vaccine because transgenic lines can be efficiently produced using an *Agrobacterium*-mediated transformation (Huang et al. 2001; Jani et al. 2004; Varsani et al. 2003; Zhang et al. 2002) or chloroplast transformation (Daniell et al. 2001; Kang et al. 2003; Tregoning et al. 2003) systems. However, the problem has always been the highly toxic nicotine in tobacco. This prevented it being used for an edible vaccine.

The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has become a very effective marker for use in plant genetic transformation research. It has proven to be a powerful tool in plant genetic transformation studies (Stewart 2001). Recently, there have been many reports on the expression of the GFP gene.

Ponappa et al. (1999) reported that the GFP gene was expressed in a stably transformed culture using an embryogenic suspension culture of soybean. Also, GFP fluorescence has been visualized in potato (Sidorov et al. 1999), brassica (Halfhill et al. 2001), and rose (Kim et al. 2004). Several other important modifications have been made to improve the GFP expression in plants. The commercially available (GFP) (clontech) has the S65T as well as the F64L and Y145F mutations and its human codon-optimized (Yang et al. 1996). The present studies were carried out to search for a tobacco that contained no-nicotine in order to screen for no-nicotine containing tobacco and develop transformation system carrying GFP as reporter gene, in turn can be applied for developing edible vaccine.

Materials and Methods

Search for tobacco containing low nicotine

The tobacco was searched by using the Germplasm Resources Information Network (GRIN), [Online Database] National Germplasm Resources Laboratory, Beltsville, Maryland, which was available at: http://www.ars-grin.gov/npgs/acc/acc_queries.html. GRIN web server provides germplasm information about plants, animals, microbes and invertebrates. This program is within the U.S. Department of Agriculture's Agricultural Research Service.

Effect of plant growth regulators on plant regeneration

To investigate the effect of plant growth regulators (PGRs) on plant regeneration, leaf tissues from an *in vitro* germinated plant were cut into 5~6 segments (6 x 6 mm) and they were cultured on a MS medium with various PGRs (2,4-D, NAA, kinetin and BA). After 30 days of culturing, the frequency of shoot formation was examined and the number of induced shoots per explant was counted.

Response of kanamycin concentration for selection

To investigate the adequate concentration of antibiotics for the transformation of edible tobacco, leaf segments were cultured on the medium supplemented with 0.1 mg l^{-1} NAA, 0.5 mg l^{-1} BA plus various concentrations of kanamycin (0, 50, 100, 150, or 200 mg l^{-1}, respectively). After 30 days of culturing, the number of induced shoots was counted.

Vector construction

The GFP gene was PCR-amplified with template DNA from the plasmid pCMS-EGFP (Clontech, USA) with a PCR forward primer 5'- GGA TCC ATG GTG AGC AAG GGC GAG GAG-3', and the reverse primer 5'- GGT ACC TTA TCT AGA TCC GGT GGA TC-3'. PCR reaction was carried out with 100 ng of genomic DNA / 40 pmol primers / 200 μM dNTPs / 1 x Taq polymerase buffer (10 mM Tris-HCl pH 8.8, 50 mM KCl, 0.1% TritonX-100) / 1.5 mM $MgCl_2$ / 2U Taq Polymerase in a total reaction volume of 50 μl. Amplification used a program of denaturing at 94℃ for 2 min followed by 30 cycles of 94℃ for 1 min, 55℃ for 1 min, and 72℃ for 1 min and a final extension step of 72℃ for 7 min. The PCR product was cloned into the pGem T-Easy vector (Promega, USA), yielding the pMYO37 plasmid. To ensure accurate gene amplification, both strands of the GFP gene were sequenced using primers that were specific for the T7 and SP6 promoters. The GFP gene was cloned into the plant expression vector pMY27 (Lee et al. 2001). The plasmid pMYO37 was digested with *Bam*HI and *Kpn*I restriction enzymes and the GFP fragment was ligated into the equivalent sites in the plant expression vector at a position downstream of the CaMV 35S promoter and TMV omega-prime leader (the transcriptional and translational enhancer), and upstream of the nopaline synthase (NOS) terminator, thereby yielding pMYO38 (Figure 1).

Plant transformation

The seeds of *Nicotiana tabacum* cv. TI 516 were first

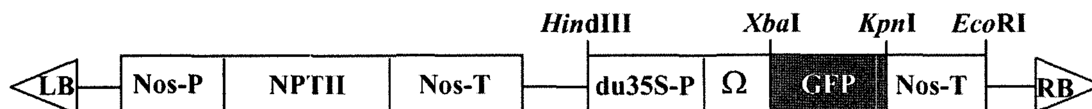

Figure 1. Plant expression vector containing the GFP gene used in transformation of *N. tabacum* cv. TI 516. Nos-P *nos* gene promoter; NPTII, neomycin phosphotransferase gene; Nos-T, *nos* gene terminator; du35S-P, duplicated cauliflower mosaic virus 35S promoter; Ω, TMV Omega-prime leader.

sterilized in 70% ethyl alcohol for 30 sec, then surface-sterilized in 2% sodium hypochlorite solution for 13 minutes. Finally, the seeds were rinsed three or four times in sterile distilled water. They were germinated on a MS medium (Murashige and Skoog 1962) without plant growth regulators under 16 h of light (45 μ mol m^{-2} s^{-1}, cool white fluorescent lamps) at 25±1℃. All media were supplemented with 8 g l^{-1} agar and 30 g l^{-1} sucrose, adjusted to pH 5.8 prior to adding agar, and autoclaved at 121℃ for 15 minutes. The plasmid pMYO38 was introduced into *Agrobacterium tumefaciens* LBA4404 (Hoekema et al. 1983) along with the helper plasmid pRK2013 using the tri-parental mating method. Five ml culture of *A. tumefaciens* containing the GFP gene was grown overnight in a LB medium supplemented with 50 mg l^{-1} kanamycin in the dark at 28℃. *Agrobacterium* suspensions (OD 1.0 at 600 nm) were harvested and diluted with 20 ml MS liquid medium. For transformation of edible tobacco, leaves were pre-cultured on a MS medium with 0.1 mg l^{-1} NAA and 0.5 mg l^{-1} BA. After 2 days of pre-culture, leaf pieces were then fully submerged into 20 ml of the *Agrobacterium* suspension in 100 x 25 mm Petri dishes for 15 minutes and blotted with sterile filter paper and co-cultured on a MS medium with 0.1 mg l^{-1} NAA and 0.5 mg l^{-1} BA for 2 days. For shoot regeneration, the explants were transferred to a MS selection medium (SM) containing 0.1 mg l^{-1} NAA, 0.5 mg l^{-1} BA, 100 mg l^{-1} kanamycin, and 300 mg l^{-1} cefotaxime. Every 4 weeks the explants were transferred to a fresh SM. For shoot elongation and rooting, the regenerated shoots induced from the explants were transferred to MS medium containing 100 mg l^{-1} kanamycin and 300 mg l^{-1} cefotaxime, without PGRs. Putative transgenic plantlets were acclimated on pots containing vermiculite and perlite

Genomic DNA isolation and PCR analysis

Genomic DNA was isolated from the young leaves of putative transgenic plants using the DNeasy plant mini kit (Qiagen) after grinding them with a mortar and pestle. PCR analysis was accomplished using the primers and reaction conditions as described in the section of 'Vector construction'. PCR products were electrophoresed on a 1.0% agarose gel, stained with ethidium bromide, and examined on a UV transilluminator.

Visual detection of the GFP

To identify the expression of the GFP gene in the transformants, we observed calluses, roots and apical meristem tissues of the transformed plants using fluorescent microscopy (Axiolab, ZEISS).

Western blot analysis

The transgenic leaf tissues were homogenized in the extraction buffer (200 mM Tris-Cl, pH 8.0, 100 mM NaCl, 400 mM sucrose, 10 mM EDTA, 14 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 0.05% Tween-20). The tissue homogenate was centrifuged twice at 17000 x g to remove insoluble cell debris. An aliquots of supernatant were resolved by 12% SDS-PAGE and transferred onto Hybond C membranes (Promega) in transfer buffer (50 mM Tris, pH 8.3, 40 mM glycine, 0.04% SDS, 20% methanol) using a mini-transblot apparatus. To prevent non-specific antibody reactions, the membranes were blocked with a 5% non-fat milk powder in TBST with gentle agitation overnight. The membrane was incubated for 2 h at room temperature in 1:1000 dilution of GFP antibody in a TBST antibody dilution buffer containing 2.0% nonfat dry milk and then washed three times with a TBST buffer. The membrane was incubated for 2 h in 1:7000 dilution of anti-mouse IgG conjugated with alkaline phosphatase (Promega S372B) in a TBST buffer and washed three times with a TBST buffer, and once with a TMN buffer. After washing, the color was developed with BCIP/NBT (USB) in a TMN buffer.

ELISA

To determine the GFP protein levels expressed in transgenic tobacco plants, ELISA assay was performed. Total soluble proteins were extracted by grinding the leaf tissues of transgenic and wild-type plants. The powder was then homogenized with a bicarbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6). Total soluble protein (TSP) samples from transgenic and wild-type plants were coated at 100 μl $well^{-1}$ into a 96-well microtiter plate along with purified GFP, and the plates were incubated overnight. The plate was washed three times with PBST, and the background was blocked with a 1% (w/v) BSA solution in PBS for 2 h at 37℃ followed by washing the plates three times with PBST. The plate was incubated with a 1:500 dilution of mouse anti-GFP antibody in 0.01 M PBS containing 0.5% BSA for 2 h at 37℃ and washed three times with PBST. The plates were finally incubated with a 1:8000 dilution of anti-mouse IgG conjugated with horseradish peroxidase (Sigma A9044) in 0.01 M PBS containing 0.5% BSA and washed three times with PBST buffer. The plates were developed by the addition of 100 μl $well^{-1}$ of TMB substrates (PharMingen 2606 and 2607KC) for 30 min at room temperature in the dark. The plate was measured at 405 nm and quantified by comparison with known amounts of GFP-antibody complex.

Results and Discussion

Effect of plant growth regulators on plant regeneration

To investigate the effect of PGRs on plant regeneration from the *in vitro* culture of edible tobacco, the *in vitro* cultured leaf segments were cultured on a MS medium with 2,4-D, NAA, BA and kinetin at various concentrations (0.1 to 1.0 mg 1^{-1}). After 14 days of culturing, calli were induced vigorously and the protuberances appeared at the cut end of the explants. They developed into shoots on the media with PGRs of various concentrations and showed normal growth. Especially, more shoots with normal growth were induced on the MS medium with 0.1 mg 1^{-1} NAA and 0.5 mg 1^{-1} BA. Shoot formation rate and the number of shoots per explant were examined after 45 days of culturing. When explants were cultured on the MS medium with 0.1 mg 1^{-1} NAA and 0.5 mg 1^{-1} BA, the shoot formation rate was higher than those on MS medium containing 2,4-D in combination with kinetin. Also, on the medium with 0.1 mg 1^{-1} NAA and 0.5 mg 1^{-1} BA, the number of shoots per explant was greater than those on MS medium with 2,4-D and kinetin. This result shows that the combination of 0.1 mg 1^{-1} NAA and 0.5 mg 1^{-1} BA was more effective for shoot regeneration in the edible tobacco (Table 1). The PGRs used in the regeneration of *N. tabacum* cv. TI 516 is not completely consistent with those in the *N. tabacum* cv. Havana. In Havana (Kwon et al. 2003), plants were regenerated on MS medium with 0.1 mg 1^{-1} NAA and 1.0 mg 1^{-1} BA. For shoot elongation and rooting, induced shoots were then transferred to the hormone free MS medium.

Response of kanamycin concentration for transformation

Since the kanamycin sensitivity affects the recovery of transformed plants and varies widely among different tissue and species, kanamycin sensitivity should be determined at the initial stage of developing a plant transformation system (Colby and Meredith 1990). To investigate the adequate concentration of antibiotics for the transformation of *N. tabacum* cv. TI 516, leaf segments were cultured on medium supplemented with 0.1 mg 1^{-1} NAA, 0.5 mg 1^{-1}BA and various concentrations of kanamycin (0, 50, 100, 150 or 200 mg 1^{-1}, respectively). After 15 days of culturing, the cut end of the explants became brown on the medium containing a low concentration (50 mg l^{-1}) of kanamycin, and there was no response at higher than 100 mg 1^{-1} of kanamycin concentration. After 30 days of culturing, the number of living segments that induced protuberance and induction rate was examined. Callus induction rate was 5%, and no shoots were observed on the medium with more than 100 mg 1^{-1} of kanamycin (Table 2). As a result, we considered that an adequate concentration of kanamycin for selection was 100 mg 1^{-1}. This data will provide a valuable transformation system for incorporation of a useful gene in edible tobacco.

Plant transformation and visual detection of the GFP

Kanamycin concentration used for the transformation of edible tobacco plants was determined as 100 mg 1^{-1} for the selection of transformants, because direct shoot regeneration and callus formation on excised leaf segments was inhibited at this level. Leaf segments were co-cultivated with

Table 1. Effect of plant growth regulators on shoot regeneration from leaf segments of *N. tabacum* cv. TI 516 after 45 days of culture.

Plant growth regulators (mg 1^{-1})				Callus induction (%)	Shoot formation(%)	No. of shoots /explant
2,4-D	kinetin	NAA	BA			
control				13	0	0
0.1	0.5			43	21	0.4
0.1	1.0			60	55	0.9
0.5	1.0			17	0	0
1.0	2.0			27	8	0.1
		0.1	0.5	97	94	4.2
		0.1	1.0	90	83	2.0
		0.5	1.0	93	88	3.4
		1.0	2.0	90	86	2.4

MS basal medium was used

Table 2. Response of leaf segments of *N. tabacum* cv. TI 516 sensitive to kanamycin concentration.

Kanamycin conc. (mg 1^{-1})	No. of explants	No. of explants inducing callus (%)	No. of shoots
0	20	20 (100)	48
25	20	15 (75)	6
50	20	1 (5)	0
100	20	0(0)	0
150	20	0(0)	0
200	20	0(0)	0

MS medium supplemented with 0.1 mg 1^{-1} NAA and 0.5 mg 1^{-1} BA was used.

an *A. tumefaciens* strain LBA4404 containing the GFP gene. After 2 days of co-cultivation, explants were cultured on SM with 100 mg 1^{-1} of kanamycin to select the kanamycin-resistant shoots and 300 mg 1^{-1} cefotaxime to eliminate *A. tumefaciens*. After 3 weeks of culturing, calli were induced at the cut end of the explants, and they were green-fluoresced when observed under fluorescent microscopy (Figure 2A). Also, putative transformed shoots were induced from the transformed callus or directly differentiated at the cut end of the explant, and they showed normal growth in the SM. Apical meristems expressing GFP were detected on the leaf segments that were transformed by *A. tumefaciens* containing GFP after 45 days of culturing (Figure 2C). No green fluorescence was observed in the untransformed leaf segments.

The confidence of GFP detection is elevated by the ability to discern cellular detail in the optically clear fluorescent cells (Elliott et al. 1999). GFP expression was visually reduced in the chlorophyll-containing tissue such as well-developed leaves and observed in some stomatal guard cells of older leaves (Kim et al. 2004). We also observed the expression of GFP in the apical meristem tissue rather than leaf tissue. For rooting, shoots were transferred to a MS medium containing 100 mg 1^{-1} kanamycin and 300 mg 1^{-1} cefotaxime without PGRs (RM, rooting medium). Putative transformed shoots were rooted in RM for one week of culture, and the expression of GFP was detected on the roots of the transformed plant. The roots of transgenic plants also fluoresced (Figure 2B). The tissues of the putative transgenic plant were bright green, while the leaves of the untransformed plantlets were reddish purple due to chlorophyll autofluorescence when illuminated by a fluorescent microscope lamp. Untransformed explants produced substantially dark red chlorophyll when observed under the dissecting fluorescence microscope and were easily distinguished from the light to bright green of the transgenic plants. Therefore, the use of GFP as a marker to identify transgenic shoots in combination with kanamycin selection after transformation appears to be an effective procedure for identifying transgenic tissue if you use a fluorescence dissecting microscope. GFP positive tissue was easier to identify in calluses or young plantlets than in the older and more developed parts of the regenerating shoots. Halfhill et al. (2001) reported that GFP fluorescence has been shown to be visible in young leaves, stem, veins, and flowers of *Brassica*. This could be due to the chlophyll autofluorescence of the higher cytoplasmic density in young tissues compared to the more vacuolated older tissues. Most of the transgenic plants appeared to be morphologically normal. Finally, 10 normal plantlets among the regenerated plantlets were transplanted on pots with vermiculite and perlite. They were all acclimated in green house.

PCR analysis

In addition to the expression of the GFP gene and resistance to kanamycin, stable transformation of the edible tobacco plants grown in the greenhouse was confirmed by PCR analysis. PCR products showed bands of 720 bp in size in lanes of the transgenic plants, no bands were found in lane of control plant (Figure 3). PCR analysis demonstrated that the GFP gene was stably integrated into the plant's nuclear genome. Therefore, it suggests that the selection based on GFP expression could be utilized to identify the transgenic tobacco plants.

Figure 2. Expression of GFP gene in (A) callus, (B) root and (C) apical meristem of *N. tabacum* cv. TI 516.

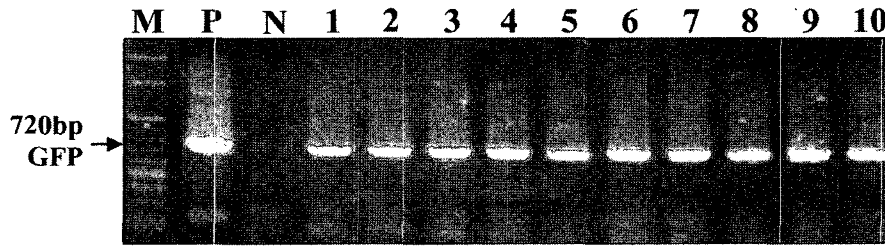

Figure 3. Genomic PCR analysis of transgenic plants. P, positive control (plasmid); N, non-transgenic plant; line No. 1~10, transgenic plants.

Western blot analysis

Western blot analysis was performed to detect the presence of the GFP protein, using the leaf tissues of two transgenic tobacco plant lines. Plant lines No. 1 and 2 showing vigorous growth were selected for GFP protein characterization. Western blot analysis revealed the GFP protein synthesized in plants with a molecular weight of 32 kDa, which is the same size as the *E. coli*-expressed GFP. The level of GFP protein accumulation did not significantly differ in the two transgenic tobacco plants and no GFP protein was detected in the non-transgenic plant (Figure 4).

ELISA

To obtain quantitative estimation of GFP protein level in the tobacco leaf tissues of transgenic plants, an ELISA test was performed. The amount of plant GFP protein was estimated by comparing the relative light units (RLU) from a known amount of bacterial GFP protein-antibody complex with that emitted from a known amount of transformed plant total soluble protein. The amount of GFP detected was expressed as a percentage of total soluble plant protein (% TSP) in the sample. Optimal concentrations of soluble protein loaded in the wells of the microtiter plate yielded GFP protein levels reaching 0.16% of total soluble protein in the plant line No. 2 of tobacco leaf samples. Transgenic plant line No. 2 exhibited a higher GFP protein accumulation than line No. 1 (Figure 5).

The goal of this study was to introduce GFP protein gene and express it in the transgenic edible tobacco using an *Agrobacterium*-mediated transformation system, and we investigated the possibility of an edible vaccine using the edible tobacco plants. As a result, we obtained the transformed tobacco plants synthesizing 0.16 % GFP of TSP (total soluble protein). The normal use of plants as human foods and as animal feed, with the production of vaccine subunit components in plant tissues, should allow vaccines to be produced at a fraction of the cost of other approaches. Therefore, these transgenic edible tobacco plants using an *Agrobacterium*-mediated transformation system will provide a valuable tool for the development of edible vaccines.

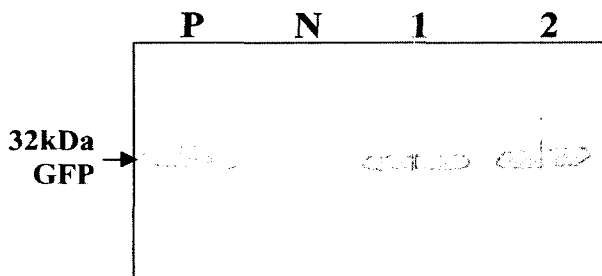

Figure 4. Western blot analysis of transgenic plants indicating the expression of the GFP protein. P, purified bacterial GFP protein; N, TSP (total soluble protein) of non-transgenic plant; 1 and 2, TSP of two transgenic plant lines.

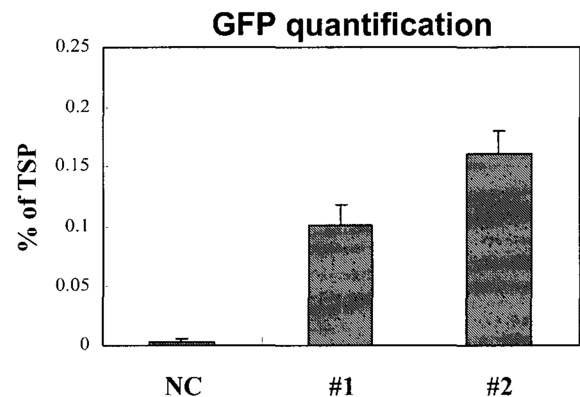

Figure 5. ELISA for the determination of the GFP protein level in the leaves of transgenic tobacco plants. NC, non-transgenic plant; #1 and #2, two transgenic plants (lines No. 1 and 2, respectively).

Acknowledgment

This work was supported by Korean Research Foundation Grant (KRF-2002-070-C00069).

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