BIOLOGIA PLANTARUM 49 (2): 313-316, 2005

BRIEF COMMUNICATION

Use of a simple semiquantitative method for appraisal of green fluorescent protein gene expression in transgenic tobacco plants

M. HRAŠKA*, S. RAKOUSKÝ*[1] and T. KOCÁBEK

Institute of Plant Molecular Biology, Academy of Sciences of the Czech Republic, and University of South Bohemia, Faculty of Biological Sciences, Branišovská 31, České Budějovice, CZ-37005, Czech Republic*

Abstract

We have applied a simple method for evaluation of *gfp* gene expression in plants using a CCD camera and computerized processing of images. Transgenic tobacco plants were obtained by *Agrobacterium tumefaciens*-mediated transfer of plasmid T-DNA bearing a *m-gfp5-ER* sequence governed by the 35S promoter together with the *nptII* selectable marker gene. Presence of the *gfp* gene in plants was confirmed by a polymerase chain reaction method. Mean brightness values measured using image analysis software showed differences between transgenic and control plants and suggest the possibility of rapid selection of transgenic individuals among regenerants and their progenies.

Additional key words: CCD camera, computerized processing, *gfp* expression, image analysis, *Nicotiana tabacum.*

One of the main obstacles in plant transgenosis is the limited possibility of a simple and efficient selection of transgenic individuals among numerous plants of the first (T_0) generation of regenerants. Although relatively efficient systems (based on co-transfer of selectable or marker gene(s) with the gene of interest) allowing the selection on media containing antibiotics or other substances were developed (for review see Miki and McHugh 2004) those of different reasons are not generally applicable to a wide scale of materials. The aim of this work is to contribute to the development of selection schemes, which either alone or in a combination with selectable marker(s) would be applicable to any higher plant species and enable use of the automated or semi-automated computer driven basic laboratory instruments as well as preliminary evaluation of gene expression.

Many marker genes are used in plant molecular biology in the present time. One of these is the gene coding for green fluorescent protein (GFP) isolated from Pacific jellyfish *Aequorea victoria,* first described by Prasher *et al*. (1992). GFP became a powerful tool in biology for the studying gene expression, protein localization and transport, and many applications in plant research have been reported (Harper *et al*. 1999, Halfhill *et al*. 2001, Miki and McHugh 2004). Mostly, the application of GFP does not require a destructive preparation of studied samples as in the case of β-glucuronidase (GUS) assay and allows investigation of living organisms in real time (Hu and Cheng 1995). Many GFP variants have been created differing in their excitation and emission spectra or in their cell targets (Stewart 2001). In some cases it is suitable to study not only the presence and localization of GFP but also to quantify its activity. We have therefore, performed experiments with a view to detect the activity of *gfp* in transgenic tobacco plants by stereomicroscope and to use the computer-aided quantification of its signal.

Sterile true leaves detached from aseptically cultivated *Nicotiana tabacum*, cv. Petit Havana, SR1 WT plants (Maliga *et al*. 1973) were used for *Agrobacterium* mediated leaf disc transformation according to Horsch

Received 14 July 2004, *accepted* 24 November 2004.

Abbreviations: GFP - green fluorescent protein, MB - mean brightness, MS - Murashige and Skoog (1962) culture medium, PCR - polymerase chain reaction.

Acknowledgements: Authors are grateful to Dr. Vincent Lea, Molecular Research Group, NIAB Cambridge, UK for kind proofreading the manuscript. This work was supported by project # QE1123 of the Grant Agency of Czech Ministry of Agriculture.

[1] Corresponding author; fax: (+420) 38 777 2228, e-mail: srak@bf.jcu.cz

et al. (1985). *A. tumefaciens* strain LBA 4404 carrying binary vector pBINm-gfp5-ER (provided by J. Hasseloff, Department of Plant Sciences, University of Cambridge, UK) was used. The vector contains a modified *gfp* gene with endoplasmic reticulum (ER) retention signals mutated to allow visualization at UV (395 nm) or blue light (473 nm). Co-cultivated and control (non transformed) leaf disks were grown under *in vitro* conditions in a growth chamber (16-h photoperiod, 40 - 70 μmol(PAR) m^{-2} s^{-1}, 22 - 25 °C) on a basal Murashige and Skoog (1962; MS) medium supplemented with growth regulators and selective antibiotics (Horsch *et al*. 1985). Regenerated T_0 plants were maintained *in vitro* on a basal MS medium. Putative transformants (14 selected plants) and one control plant were transferred to soil and grown in a glasshouse under optimum temperature 21 - 23 °C. Dried seeds obtained from 12 selfed plants were surface sterilized with 70 % ethanol for 2 min and then for 45 - 65 min in commercial bleach containing 1.6 % m/v sodium hypochlorite and 0.1 % *Tween 20*. Seeds were then rinsed 4 times with sterile distilled water and sown dispersed in 0.125 % agar (*Difco Bacto Agar*, *Difco Laboratories*, Sparks, USA) on MS medium supplemented with 500 mg dm^{-3} kanamycin. Petri dishes (9 cm) with seeds were maintained for 5 weeks in a growth chamber under the same conditions as regenerated plants. Selected T_1 seedlings (11 + 1 control) were transferred to fresh MS without antibiotics. Plants thus obtained were used for further experiments. No visible differences in plant morphology and development between transgenic and control plants were observed either *in vitro* or *in vivo*

To confirm the presence of *gfp* gene in transgenic tobacco plants, DNA was isolated from 2-month-old leaves as described by Edwards *et al.* (1991). The *in vitro* plants used for analyses formed at that time only a ground rosette consisting of 3 - 5 true leaves. The primers *gfp0* (5´-ATG TTG CAT CAC CTT CAC CC) and *gfp1* (5´-AAG CTT ACA GTC TCA AAG ACC AAA G) were used to specifically amplify a region of 570 bp. PCR reactions were performed in 0.02 cm^3 reaction mixture containing buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3), 200 μM of each dNTP, 0.16 μM of each primer gfp01 and gfp1, 1 U of Taq polymerase (*Top-Bio*, Praha, Czech Republic) and approx. 50 ng DNA. The samples were amplified using 35 cycles (94 °C for 30 s, 52 °C for 30 s, 72 °C for 45 s) and analysed on 2 % agarose gels stained with ethidium bromide in TAE buffer. PCR product corresponding to *gfp* was found in all 11 putative transgenic samples, but not in the control sample (data not shown).

Other leaves detached from the same transgenic and of one control plant were used for further microscopic GFP study. Samples were studied in a normal position with the abaxial side uppermost on a Petri dish in a droplet of distilled water to prevent desiccation and following destruction of tissues. A *Leica MZ 12* stereo dissecting microscope equipped with a fluorescence module consisting of 100 W mercury lamp and GFP excitation and emission filters (*Leica*, Heerbrigg, Switzerland) integrated with CCD camera was used for the study. This set (excitation filter 480/40 nm, dichroic mirror 505 nm LP, barrier filter 510 nm LP), black-white CCD camera *Cohu* (San Diego, USA) permits the visualization and detection of GFP following tissue excitation by blue light. Emitted GFP signal was analysed by *Lucia® 4.71* software (*Laboratory Imaging*, Praha, Czech Republic). Leaves were analysed immediately after their separation from plants.

Objective magnification (1.6×) and 1.6 optical zoom were set up on the *Leica MZ 12* stereomicroscope. Two different approaches to obtain data were performed. First, each leaf was scanned from the tip to the base along the midrib. Second, the same leaf was than scanned around its perimeter, excluding the midrib conductive bundles. Mean brightness (MB) values were collected from a defined area (4.2 mm^2) and statistically processed using the Student's *t*-test by means of *Statistica®* software (*StatSoft*, Tulsa, USA). Two to three leaves of each plant were scanned. From each leaf at least 9 measurements were performed. Based on our finding from previous experiments that leaves of different non-transformed (control) plants show very low MB variability (data are not given here) only one plant was used as a control.

Results of the *t*-test showed pronounced differences of MB values between transgenic and non-transgenic (control) plants (Table 1). All transgenic leaves provided visual fluorescence when compared to the control leaves (Fig. 1). Fluorescence outcomes from the area close to the midrib demonstrate considerable variability in MB values between individual measurements. This observation corresponds with the high level of standard deviation (SD) of MB among tested samples. Data collected from the perimeter of each leaf, excluding the midrib part, demonstrate lower levels of SD, except plants 4 and 10. However, differences of SDs in these two cases were not significant. High SD in the medial region of leaves are most probably caused by the influence of the midrib, which exhibits stronger fluorescence than the surrounding tissue. Although leaves of the same physiological age were used, some of them had a stronger midrib than others, which resulted in a higher fluorescence and thus also in higher MB values. This discrepancy was avoided by collecting data from the leaf perimeter, which had lower SD. We suggest that areas away from the midrib should be selected for further use and development of this method.

Data also showed differences in MB values within transgenic leaves from different plants. For example, the fluorescence from leaf 10 was practically invisible and MB was also very low, whereas fluorescence and MB from plant 3 was extremely high. This is probably due to

Table 1. Mean brightness (MB) values of fluorescence signals obtained as summarized outcomes from *Lucia® 4.71* software following the scanning of transgenic and control leaves. Two to three leaves of each plant were scanned. From each leaf two groups of data were collected (with and without midrib) and at least 9 measurements were performed (C denotes control, * - MB values significantly different at $P \leq 0.01$ from corresponding ones measured on a control plant).

Plant number	1	2	3	4	5	6	7	8	9	10	11	C
MB from the medial part of leaves (including the midrib)												
MB ± SD	134.03 ±26.00*	11.92 ±5.11*	226.55 ±24.29*	13.58 ±4.74*	87.72 ±26.19*	7.18 ±5.05*	143.85 ±57.09*	95.86 ±43.52*	78.14 ±9.36*	1.12 ±0.17	134.98 ±23.79*	0.91 ±0.14
MB from the periphery of leaves (excluding the midrib)												
MB ± SD	137.16 ±15.82*	11.12 ±2.02*	219.38 ±14.60*	13.92 ±3.09*	46.16 ±6.76*	18.64 ±7.75*	86.04 ±14.45*	55.46 ±10.36*	47.73 ±7.69*	1.33 ±0.20*	69.49 ±10.74*	0.47 ±0.11

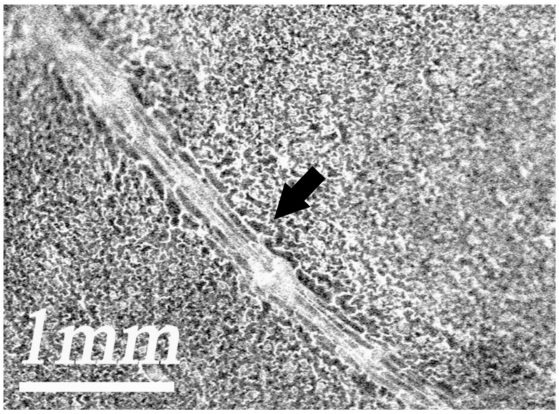

Fig. 1. Example of fluorescence image obtained after the illumination of tobacco leaves with 480/40 nm light using *Leica MZ 12* stereomicroscope equipped with a fluorescence module (objective magnification - 1.6×, optical zoom - 1.6×) and black-white CCD camera *Cohu*. Sample of transgenic plant 1 emits high fluorescence signal (white spots) opposite to the control plant tissue, which is undistinguishable of the low background noise. Region free of the midrib is presented here. *Arrow* indicates the position of conductive bundles on abaxial side of the leaf surface of transgenic plant.

different copy numbers of transgenes inserted in the plant genome and/or their total expression level, which may be influenced by gene silencing (Voinnet and Baulcombe 1997). This idea could be confirmed by Southern blot analyses and quantification of recombinant protein. However, these analyses were not performed, because the aim was to show whether this approach for studying *gfp* expression is possible.

Many other systems for studying fluorescence events are now available. They include the use of various high-sensitivity UV lamps, spectrofluorometers, scanning laser systems and GFP meters (Millwood *et al*. 2003). However, all these instruments, designated for detection and quantification of various fluorescent compounds, are expensive and can be used only for the purposes they were developed for. The detecting set we used here is designated for universal image analyses and represents a new potential utilisation for laboratories equipped with this arrangement and adds value to this set. It was demonstrated here in accordance to other authors (Soukupová and Albrechtová 2003) that results of signal quantification with image analysis may greatly depend on the character and procedure used. If it is done in full respect of conditions and knowledge of plant anatomy it can gain reliable and interesting results. The preliminary results presented in this paper showed, that the detection and quantification of GFP signal from transgenic plant tissue by CCD camera and *Lucia® 4.71* imaging software is possible and promising.

References

Edwards, K., Johnstone, C., Thompson, C.: A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. - Nucl. Acids Res. **19**: 1349, 1991.

Halfhill, M.D., Richards, H.A., Mabon, S.A., Stewart, C.N., Jr.: Expression of *GFP* and *Bt* transgenes in *Brassica napus* and hybridization with *Brassica rapa*. - Theor. appl. Genet. **130**: 659-667, 2001.

Harper, B.K., Mabon, S.A., Leffel, S.M., Halfhill, M.D., Richards, H.A., Moyer, K.A., Stewart, C.N., Jr.: Green fluorescent protein as a marker for expression of a second gene in transgenic plants. - Nature Biotechnol. **17**: 1125-1129, 1999.

Horsch, R.B., Fry, J.E., Hoffmann, N.L., Eicholtz, D., Rogers, S.G., Fraley, R.T.: A simple and general method for transferring genes into plants. - Science **227**: 1229-1231, 1985.

Hu, W., Cheng, C.-L.: Expression of *Aequorea* green fluorescent protein in plant cells. - FEBS Lett. **369**: 331-334, 1995.

Maliga, P., Sz.-Breznovits, A., Márton, L.: Streptomycin-resistant plants from callus culture of haploid tobacco. - Nature **244**: 29-30, 1973.

Miki, B., McHugh, S.: Selectable marker genes in transgenic plants: applications, alternatives and biosafety. - J. Biotechnol. **107**: 193-232, 2004.

Millwood, R.J., Halfhill, M.D., Harkins, D., Russotti, R., Stewart, C.N., Jr.: Instrumentation and methodology for quantitative GFP fluorescence in intact transgenic plants. - Biotechniques **24**: 638-643, 2003.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue cultures. - Physiol. Plant. **15**: 473-497, 1962.

Prasher, D.C., Eckenrode, V.K., Ward, W.W., Prendergast, F.G., Cormier, M.J.: Primary structure of the *Aequorea victoria* green-fluorescent protein. - Gene **111**: 229-233, 1992.

Soukupová, J., Albrechtová, J.: Image analysis – tool for quantification of histochemical detections of phenolic compounds, lignin and peroxidases in neeedles of Norway spruce. - Biol. Plant. **46**: 595-601, 2003.

Stewart, C.N., Jr.**:** The utility of green fluorescent protein in transgenic plants. - Plant Cell Rep. **20**: 376-382, 2001.

Voinnet, O., Baulcombe, D.C.: Systemic signalling in gene silencing. - Nature **389**: 553, 1997.