INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
1560–8530/2004/06–3–533–537
http://www.ijab.org

Expression of Green Fluorescent Protein Gene in Lychee (*Litchi chinensis* Sonn.) Tissues

D. PUCHOOA
Faculty of Agriculture, University of Mauritius Réduit, Mauritius
E-mail: sudeshp@uom.ac.mu

ABSTRACT

Green-fluorescent protein (GFP) gene expression was observed in tissues of lychee (*Litchi chinensis* Sonn.) after transformation using *Agrobacterium. In vitro* grown leaf tissues were used for transformation. After four weeks in culture, expression of GFP was apparent when the regenerated callus and the leaves were observed under fluorescence microscope fitted with a blue exciter filter, a blue dichroic mirror and a barrier filter. Although no transformed lychee plantlets were regenerated, screening for GFP gene expression may prove useful to improve transformation efficiency and to facilitate detection of transformed lychee plants.

Key Words: *Agrobacterium*; Green fluorescent protein; *Litchi chinensis* Sonn

INTRODUCTION

The economic importance of woody fruit species has led to selection and breeding over thousands of years. This practice has resulted in relatively few genotypes and therefore, in a restricted germplasm base. Such genetic uniformity has increased the vulnerability of these crops to insect pests and pathogens leading to excessive use of chemical pesticides (Norelli *et al*., 1994). Developments in biotechnology have provided an alternative approach to woody fruit crop improvement through the introduction of genes encoding desirable traits (Gmitter *et al*., 1992). Most research to date has focused on genes conferring resistance to viruses, bacteria, insects and fungi. Attention has also been given to genes that regulate columnar growth, rooting ability, freezing tolerance or toxin resistance. With these in hand, it has become possible to achieve crop improvements by genetic transformation, thus bypassing the long periods required for genetic crosses and selection. A limitation, however, has been the availability of methodology optimized for genetic transformation of woody fruit trees.

A range of dominant and selectable genetic markers and reporter genes for cells have been identified enabling one to select a marker that is most suitable for the plant species or tissues to be transformed. Commonly used reporters include gene encoding chloramphenicol acetyl transferase (CAT), β-glucuronidase (GUS), neomycin phosphotransferase (NPT-II), luciferase (LUC) and protein involved in the regulation of anthocyanin biosynthesis. This range of markers is important because plant species and particularly tissues of plant may differ widely in their sensitiveness to different selective agents. Furthermore, the choice of several markers increases the range of genetic manipulations and analyses that can be performed with transgenic plants. However, most of these systems either require exogenous added substrates and co-factors or are expensive and time-consuming (Stewart, 1996). Therefore, it is desirable to have a detection system which is easy, simple, real-time, non-toxic, independent on added substrates and one which could be used on any living tissues. Green fluorescent protein (GFP) may provide such a system.

Green fluorescent protein, GFP, is a spontaneously fluorescent protein isolated from coelenterates, such as the Pacific jellyfish, *Aequoria victoria* (Morin & Hastings, 1971). Its role is to transduce, by energy transfer, the blue chemiluminescence of another protein, aequorin, into green fluorescent light (Ward *et al*., 1982). The molecular cloning of GFP cDNA (Prasher *et al.,* 1992) and the demonstration by Chalfie that GFP can be expressed as a functional transgene (Chalfie *et al*., 1994) have opened exciting new avenues of investigation in cell, developmental and molecular biology. Green fluorescent protein has been expressed in bacteria (Chalfie *et al*., 1994), yeast (Kahana *et al*., 1995), slime mold (Moores *et al*., 1996), plants (Casper & Holt, 1996; Epel *et al*., 1996), drosophila (Wang & Hazelrigg, 1994), zebrafish (Amsterdam *et al.,* 1996), and in mammalian cells (Ludin *et al*., 1996; De Giorgi *et al.,* 1996). GFP can function as a protein tag, as it tolerates N- and C-terminal fusion to a broad variety of proteins many of which have been shown to retain native function (Cubitt *et al*., 1995; Olsen *et al.,* 1995; Moores *et al*., 1996). The enormous flexibility as a noninvasive marker in living cells allows for numerous other applications such as a cell lineage tracer, reporter of gene expression and as a potential measure of protein-protein interactions (Mitra *et al.,* 1996). Detection of intracellular GFP requires only irradiation by near UV or blue light and it is not limited by availability of substrates or co-factors. Thus, GFP should be able to provide an excellent means for monitoring gene expression

and protein localization in living cells.

In order for a plant containing the transgene to fluoresce green under UV or blue light though, the GFP protein must be highly expressed to mask the pink auto-fluorescence of chlorophyll. The wild type GFP gene has been modified by Jim Haseloff (mGFP4) by altering codons at the site of prior mis-splicing and now provides stable and high expression in transgenic plants (Haseloff *et al*., 1996). mGFP4 provides a several fold increase in protein expression over native GFP because it has altered codons at prior mis-spliced sites (Haseloff *et al*., 1997). Therefore, plants containing high amounts of fluorophore-active GFP can be distinguished from non-transgenics by a visual screening using a portable hand-held UV light (Stewart, 1996).

MATERIALS AND METHODS

Plant materials. Leaf pieces of *in vitro* cultures of *Litchi chinensis* Sonn., variety 'Tai So' were used as explant materials. They were cultured on Murashige and Skoog (1962) basal medium supplemented with 225 mg L^{-1} of each ascorbic and citric acid, 30 g L^{-1} sucrose, 2,4-D (4.52 μM), BAP (2.22 μM) and solidified with 0.25% Phytagel for callus initiation for four weeks followed by culturing onto the regeneration medium which contained all of the callus induction medium but replacing 2,4-D (4.52 μM) and BAP (2.22 μM) with 13.31 μM BAP and 3.42 μM IAA. Cultures were maintained at a temperature of 25 ± 2°C with a 16 hour photoperiod (photon flux of 27 μE m^{-2} s^{-1}).

Bacterial strains. The following bacterial strains were used:

i) *Agrobacterium* strain LBA 4404 carrying the disarmed Ti plasmid pAL 4404

ii) *E. coli* strain HB 101 carrying the mobilization plasmid pRK 2013 and

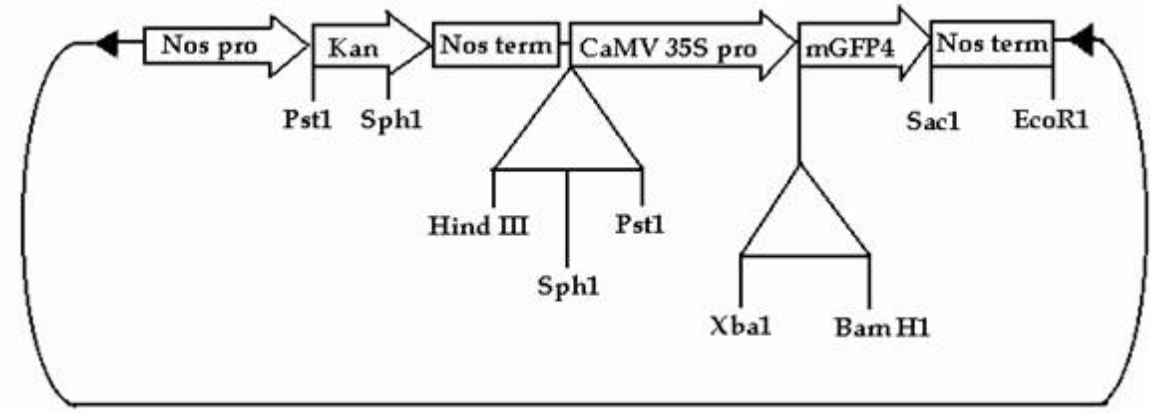
iii) *E. coli* strain HB 101 carrying the pBin 35 S mGFP 4 plasmid of interest

The mGFP 4 sequence is cloned into a Bam H1 Sac1 fragment of pBin 121 (replacing the GUS gene) and positioned adjacent to the 35 promoter. Sequences for efficient translation in both *E. coli* (ribosome binding sequence) and plants (AACA at –4 to –1) are positioned upstream of the AUG (Fig. 1).

Triparental mating procedure. The vector was introduced into *Agrobacterium tumefaciens* strain using the triparental mating procedure. The detailed mating procedures are as follows: overnight cultures of *E. coli* and *Agrobacterium* were started. The *A. tumefaciens* cultures were started from colonies taken from Luria-Bertani (LB) agar plates containing Tryptone (10 g L^{-1}), NaCl (10 g L^{-1}), agar (15 g L^{-1}) and rifampicin (150 mg L^{-1}) and grown in 10 mL of LB broth containing 150 mg L^{-1} rifamicin at 28ºC to avoid curing of the Ti plasmid. *E. coli* cultures were started from a glycerol stock. They were grown separately in 10 mL of LB broth plus 50 mg L^{-1} kanamycin at 37^0C. The following day, the cells were diluted back and grown to log phase. One mL from each of the cultures were mixed together in a sterile polystyrene tube, centrifuged and resuspended in 2 mL of 10 mM $MgSO_4$. The mixture was transferred into a 5 mL syringe connected to a filter apparatus. The cells were collected on the filter disc and the latter was aseptically transferred onto fresh, non-dried LB agar plates which were incubated at 28^0C overnight. The following day the filters were removed and placed into sterile tubes containing 2 mL of 10 mM $MgSO_4$. The tube was vortexed to remove the cells from the filter disc. A 0.1 mL aliquot of the cells was spread on a freshly prepared LB agar selection plate containing 50 mg L^{-1} kanamycin and 150 mg L^{-1} rifampicin. Thus selection was only for *Agrobacterium* cells containing the plasmid of interest. The plates were incubated at 28^0C for four days. After this period several colonies appeared. The colonies were streaked onto freshly prepared LB plates containing 50 mg L^{-1} kanamycin and 150 mg L^{-1} rifampicin. After an incubation period of 2 days at 28^0C, the plates were stored at 4^0C. Frozen stocks were also made from an overnight culture and kept at -70^0C.

Transformation, culture and selection of transformed tissues. Prior to the transformation experiments, the kanamycin sensitivity of the *in vitro* grown leaves were tested by growing them in the presence of different concentrations of kanamycin (0, 25, 50, 75 and 100 mg L^{-1}) and determining the regenerative capacity after four weeks in culture. For *Agrobacterium* infection, the *in vitro* grown leaf explants were transferred to a petri dish containing *Agrobacterium* infection medium (AIM) which consisted of MS salts and vitamins supplemented with 225 mg L^{-1} of each ascorbic and citric acid, 30 g L^{-1} sucrose, 2,4-D (4.52 μM), BAP (2.22 μM) plus 1.0 mL of bacterial suspension. The latter was obtained by growing single colonies of the *Agrobacterium* in LB broth containing the appropriate antibiotics to ensure maintenance of the Ti plasmid followed by centrifugation for 3 min at 16,000 rpm and re-suspending the cells in 1.0 mL of LB. The petri dish was gently shaken for 20 min at room temperature. The leaves were incubated on medium containing MS salts and vitamins, 225 mg L^{-1} of each ascorbic and citric acid, 30 gL^{-1} sucrose, 2,4-D (4.52 μM), BAP (2.22 μM) and solidified with 0.25% Phytagel. After co-cultivation for 2 days, the leaves were washed by gentle shaking for 30 min in a petri dish containing 5 mL of the liquid culture medium. This treatment was repeated five more times to remove the bacteria as completely as possible. After blotting on sterile filter paper (Whatman No. 1) to remove extra moisture, the leaves were transferred to the regeneration medium (MS salts and vitamins, 225 mg L^{-1} of each ascorbic and citric acid, 30 g L^{-1} sucrose, IAA (3.42 μM), BAP (13.31 μM) solidified with 0.25% Phytagel) containing 50 mg L^{-1} kanamycin and 300 mg L^{-1} vancomycin. The cultures were transferred to new plates every four weeks and cultured under white fluorescent light at a photon flux of 27 μE m^{-2} s^{-1}at 16 hour photoperiod at a

Fig. 1. Schematic diagram of pBin 35 S mGFP 4 plasmid (Haseloff *et al.*, 1996)



temperature of 25 ± 2°C. The kanamycin concentration was gradually increased by 25 mg L^{-1} during each subculture to 100 mg L^{-1}. As control *in vitro* grown leaves, which were not infected with *Agrobacterium* were cultured on the same growth medium as that used for transformation but without any antibiotics.

The regenerated callus and the leaves were observed under an Olympus BH-2 fluorescence microscope fitted with a blue exciter filter (IF-490), a blue dichroic mirror (OM 500 (0-515) and a barrier filter 0.530 (Olympus Optical Co., Tokyo, Japan) to detect for any fluorescence.

RESULTS

The sensitivity of the *in vitro* grown leaf cultures of *Litchi chinensis* Sonn. to kanamycin is shown in Table I. The concentration of vancomycin to be used was also determined by a similar experiment (Table II).

As there is a high risk of escapes using a low concentration of kanamycin in the selection of transformants, the concentration of kanamycin was gradually increased during each subculture after every four weeks, to 75 mg L^{-1} and eventually to 100 mg L^{-1}. At this concentration of kanamycin, transformants survived (Table III).

The regenerants were checked for the expression of the green fluorescent protein (GFP) gene by irradiation with UV light. No glowing plantlets or tissues were observed. However, all kanamycin resistant regenerants which were observed under the fluorescent microscope, were found to contain GFP gene activity (Fig. 2). In most cases the transformed cells were embedded in the tissue and therefore looked blurred (Fig. 3).

DISCUSSION

In this work, the application of Green fluorescent protein (GFP) gene as a screenable marker in the transformation of lychee was tested. The first stage involved determining the sensitivity of the *in vitro* grown leaf cultures of *Litchi chinensis* Sonn. to kanamycin. Culturing was completely inhibited by 50, 75 and 100 mg L^{-1} kanamycin. However, when only 25 mg L^{-1} or less of kanamycin was used, callus culture could be initiated. For the selection of transformed cell lines, 50 mg L^{-1} of kanamycin was therefore used. Similarly the concentration of vancomycin to be used was also determined. On the basis of the findings, the concentration of kanamycin and vancomycin used in the growth medium following

Table I. Kanamycin sensitivity of *in vitro* grown leaves of lychee

Kanamycin concentration (mg L^{-1})	Surviving callus (%)
0	100
25	24
50	0
75	0
100	0

The figures shown are an average of ten replicates per treatment and 5 leaf explants per replicate

Table II. Effect of vancomycin concentration on *Agrobacterium tumefaciens*

Vancomycin concentration (mg L^{-1})	*Agrobacterium* growth
0	Overgrowth
100	55%
200	25%
300	No growth

Table III. Effect of increasing kanamycin concentration on regeneration capacity

Kanamycin concentration (mgL^{-1})	Regeneration after four weeks in culture (%)
50	60
75	45
100	10

The figures shown are an average of five replicates per treatment and 5 explants per replicate

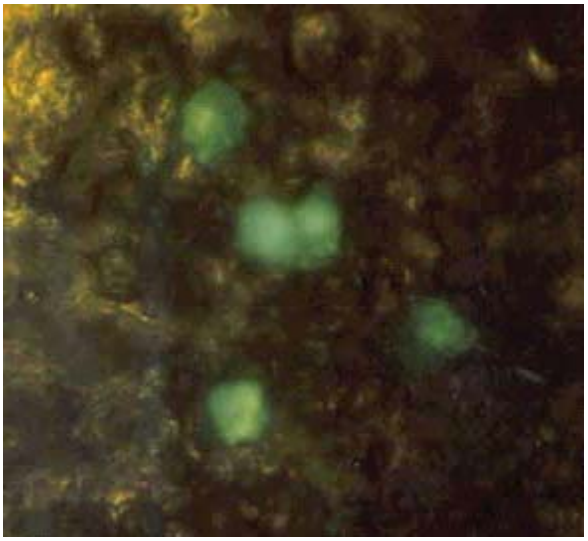
Fig. 2. Single transformed cells

transformation, were 50 and 300 mgL^{-1} respectively. At this combination, callus regeneration frequency was 100% after four weeks in culture.

However, to avoid the risk of escapes using a low concentration of kanamycin, its concentration was gradually increased to 100 mgL^{-1}. The higher concentration of kanamycin (75 & 100 mgL^{-1}) seemed to have a negative effect on regeneration as most of the explants formed green callus only. The green fluorescence was clearly visible under UV light in transformed lychee tissues. To reduce the chlorophyll auto-fluorescence problem, filters designed for GFP detection were used. These filters are known to block red light without affecting the green fluorescence derived from GFP expression. Blue exciter filter (IF-490), a blue dichroic mirror (OM 500 (0-515) and a barrier filter 0.530 (Olympus Optical Co., Tokyo, Japan) was used to detect for fluorescence.

The results show that the GFP system provides a convenient indicator of transformation. This study also demonstrates that the GFP gene from *A. victoria* can be expressed transiently and stably in tissues of lychee using *Agrobacterium.* However, further investigations need to be carried out to optimise the explants and *Agrobacterium* contact time to obtain a high expression as possible - this incubation period is likely to differ for each plant species. The ability to monitor constitutively expressed GFP in plants has many potential uses such as developing transformation methods by continuously monitoring transformation events at different stages, and non-destructive identifying transformed cells or cell lines (Lawton *et al*., 2000). Expression level seems to be low in plant cells (Haseloff & Amos, 1995) compared to bacterial cells where expression level of 75% of total protein has been obtained (Crameri *et al*., 1995; Heim *et al*., 1995). The GFP gene may also be a useful reporter/marker in monitoring in vivo gene expression spatially and temporally at the sub-cellular and whole plant levels (Lawton *et al*., 2000). Combinations of any selection marker genes with GFP in detecting transgenic cells should lead to more widespread use in developing plant transformation protocols in lychee.

Fig. 3. Transformed cells embedded in tissue



Acknowledgements. The author wishes to thank Prof. K Lindsey for supervising this work, Dr. J. Ward for the bacterial strains and the Faculty of Agriculture, University of Mauritius for supporting this work.

REFERENCES

Amsterdam, A., S. Lin, L. Moss and N. Hopkins, 1996. Requirements for green fluorescent protein detection in transgenic zebrafish embryos. *Gene,* 173: 99–103

Casper, S. and C. Holt, 1996. Expression of the green fluorescent protein–encoding gene from a tobacco mosaic virus–based vector. *Gene,* 173: 69–73

Chalfie, M., Y. Tu, G. Euskirchen, W. Ward and D. Prasher, 1994. Green fluorescent protein as a marker for gene expression. *Sci.,* 263: 802–5

Crameri, A., E.A. Whitehorn, E. Tate and W.P.C. Stemmer, 1995. Improved Green Fluorescent Protein by Molecular Evolution using DNA shuffling. *Nat. Biotech.,*14: 315–9

Cubitt, A., R. Heim, S. Adams, A. Boyd, L. Gross and R. Tsien, 1995. Understanding, improving and using green fluorescent proteins. *Trends in Biotech.,* 20: 448–55

DeGiorgi, F., M. Brini, C. Bastianutto, R. Marsault, M. Montero, P. Pizzo, R. Rossi and R. Rizzuto, 1996. Targeting aequorin and green fluorescent protein to intracellular organelles. *Gene,* 173: 113–7

Epel, B., H. Padgett, M. Heinlein and R. Beachy, 1996. Plant virus movement protein dynamics probed with a GFP–protein fusion. *Gene,* 173: 75–9

Gmitter, F.G, J.W. Grosser and G.A. Moore, 1992. Citrus. *In*: Hammerschlag, F.A. and R.E. Litz (eds.). *Biotechnology of Perennial Fruit Crops*. pp: 23–45. CAB International Press.

Haseloff, J. and B. Amos, 1995. Green Fluorescent Proteins in plants. *Trends in Genetics,* 11: 328–9

Haseloff, J., K. Siemering, S. Hodge, R. Golbik, D. Prasher, 1996. The green fluorescent protein gene must be modified for use as a vital marker in *Arabidopsis thaliana*. *Plant Physiol.,* 111: 17

Haseloff, J., K.R. Siemering and D.C. Prasher, 1997. Removal of cryptic intron and sub–cellular localization of green fluorescent protein are required to mark transgenic *Arabidopsis* plants brightly. *Proc. Natl. Acad. Sci.* 94: 2122–7

Heim R., A.B. Cubitt and R.Y. Tsein, 1995. Improved Green Fluorescence. *Nature,* 373: 663–4

Kahana, J., B. Schapp and P. Silver, 1995. Kinetics of spindle pole body separation in budding yeast. *Proc. Natl. Acad. Sci*., 92: 9707–11

Lawton, R., S. Winfield, D. Hanery, A.S. Bhagsari and S.K. Dhir, 2000. Expression of Green–Fluorescent Protein Gene in Sweet Potato Tissues. *Plant Mol. Biol. Rep*., 18: 139a–139i.

Ludin, B., T. Doll, R. Meill, S. Kaech and A. Matus, 1996. Application of novel vectors for GFP–tagging of proteins to study microtubule–associated proteins. *Gene,* 173: 107–11

Mitra, R., C. Silva and D. Youvan, 1996. Fluorescence resonance energy transfer between blue–emitting and red–shifted excitation derivatives of the green fluorescen t protein. *Gene,* 173: 13–7

Moores, S., J. Sabry and J. Spudich, 1996. Myosin dynamics in live *Dictyostelium* cells. *Proc. Natl. Acad. Sci.* 93: 443–6

Morin, J. and J. Hastings, 1971. Energy transfer in a bioluminescent system. *J. Cell Physiol.,* 77: 313–8

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

Norelli, J.L., H.S. Aldwinckle, L. Destéfano–Beltran and J.M. Jaynes, 1994. *Euphytica,* 77: 123–8

Olsen, K., J. McIntosh and J. Olmstead, 1995. Analysis of MAP4 function in living cells using green fluorescent protein (GFP) chimeras. *J. Cell Biol.,* 130: 639–50

Prasher, D.C., V.K. Eckenrode, W. Ward, F. Prendergast and M. Cormier, 1992. Primary structure of the *Aequorea victoria* green–fluorescent protein. *Gene,* 111: 229–33

Stewart (Jr.), C.N, 1996. Monitoring transgenic plants using in vivo markers. *Nat. Biotech.,* 14: 682

Wang, S. and T. Hazelrigg, 1994. Implications for bcd mRNA localization from spatial distribution of exu protein in *Drosophila oogenesis*. *Nature,* 369: 400–3

Ward, W., H. Prentice, A. Roth, C. Cody and S. Reeves, 1982. Spectral perturbations of the *Aequoria* green fluorescent protein. *Photochem. Photobiol*., 35: 803–8

(Received 23 April 2004; Accepted 12 May 2004)