J. Appl. Hort., 6(1): 11-15, January-June, 2004

Expression of green fluorescent protein gene in litchi (*Litchi chinensis* Sonn.) tissues

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Abstract

Green-fluorescent protein (GFP) gene expression was observed in tissues of litchi (*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 litchi plantlets were regenerated, screening for GFP gene expression may prove useful to improve transformation efficiency and to facilitate detection of transformed litchi plants.

Key words: *Agrobacterium*-mediated transformation, green-fluorescent protein, *in vitro* leaf explants, *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 as an alternative approach to woody fruit crop improvement through the introduction of genes encoding desirable traits (Hammerschlag and Litz, 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.

Methods available for plant transformation are arranged into three main groups: those using biological vectors (virus-or *Agrobacterium*-mediated transformation), direct DNA transfer techniques (chemical, electrical or microlaser induced permeability of protoplasts or cells) and non-biological vector systems (microprojectiles, microinjection). *Agrobacterium* shows an advantage over other methods since it targets transgenes to the nucleus and integrates them into the host DNA. *Agrobacterium* vectors have been most frequently used in woody fruit crops, achieving a higher level of stable transformants as compared to that obtained with biolistics (Mehlenbacher, 1995). Nevertheless, particle bombardment has proved to be efficient for the transformation of peach, papaya and grapevine.

A critical step in the development and evaluation of transformation strategies for plants is the construction of vectors which include one or more genetic markers that allow transformed plant cells to be either selected or screened. 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), α-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 and Hastings, 1971). Its role is to transduce, by energy transfer, the blue chemiluminescence of another protein, aequorin, into green fluorescent light (Ward, 1979). 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 and Holt, 1996; Epel *et al.*, 1996), drosophila (Wang and 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 (Moores *et al.*, 1996; Cubitt *et al.*, 1995; Olsen *et al.,* 1995). 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).

The bioluminescent jellyfish *Aequorea victoria* produces light when calcium binds to the photoprotein aequorin (Cody *et al.,* 1993). Although activation of aequorin *in vitro* or in heterologous cells produces blue light, the jellyfish produces green light. This is the result of a second protein in *A. victoria* that derives its excitation energy from aequorin (Morise *et al.*, 1974). Purified GFP, a protein of 238 amino acids (Prasher *et al.*, 1992) absorbs blue light (maximally at 395 nm with a minor peak at 470 nm) and emits green light (peak emission at 509 nm with a shoulder at 540 nm). This fluorescence is very stable. The GFP chromophore is derived from the primary amino acid sequence through cyclization and oxidation of the protein's own Ser-Tyr-Gly sequences at positions 65-67 (Cody *et al.*, 1993). GFP expressed in prokaryotic (*E.coli*) and eukaryotic (*Caenohabditis elegans*) cells is capable of producing a strong green fluorescence when excited by blue light. Because this fluorescence requires no additional gene products from *A. victoria*, chromophore formation is not species-specific and occurs either through the use of ubiquitous cellular components or by autocatalysis (Chalfie *et al.,* 1994). According to Cody *et al.* (1993), formation of the final fluorophore requires molecular oxygen and proceeds with a time constant (~4h at 22°C). 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.

Physical and chemical studies of purified GFP have identified several important characteristics. It is highly resistant to denaturation requiring treatment with 6 M guanidine hydrochloride at 90°C or pH of <4.0 or >12.0. Partial to near total renaturation occurs within minutes following reversal of denaturing conditions by dialysis or neutralization (Ward and Bokman, 1982). Circular dichroism predicts significant amounts of sheet structure that is subsequently lost on denaturation (Ward and Bokman, 1982). Over a non-denaturing range of pH, increasing pH leads to a reduction in fluorescence by 395 nm excitation and an increased sensitivity to 475 nm excitation (Ward *et al.*, 1982). Reduction of purified GFP by sodium dithionite results in a rapid loss of fluorescence that slowly recovers in the presence of room air. While insensitive to sulphydryl reagents such as 2-mercaptoethanol, treatment with the sulphydral reagent dithiobisnitrobenzoic acid (DTNB) irreversibly eliminates fluorescence (Inouye and Tsuji, 1994).

For a plant containing the transgene to fluoresce green under UV or blue light, 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 & Skoog's (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 mM), BAP (2.22 mM) 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 mM) and BAP (2.22 mM) with 13.31 µM BAP and 3.42 µM IAA. Cultures were maintained under white fluorescent light at a photon flux of 27 µE m^{-2} s^{-1}at 16 hour light / 8 hour dark period at a temperature of 25 ± 2°C.

Bacterial strains: The following bacterial strains were used: *Agrobacterium* strain LBA 4404 carrying the disarmed Ti plasmid pAL 4404, *E.coli* strain HB 101 carrying the mobilization plasmid pRK 2013 and *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 S 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 28°C to avoid curing of the Ti plasmid. *E. coli* cultures were

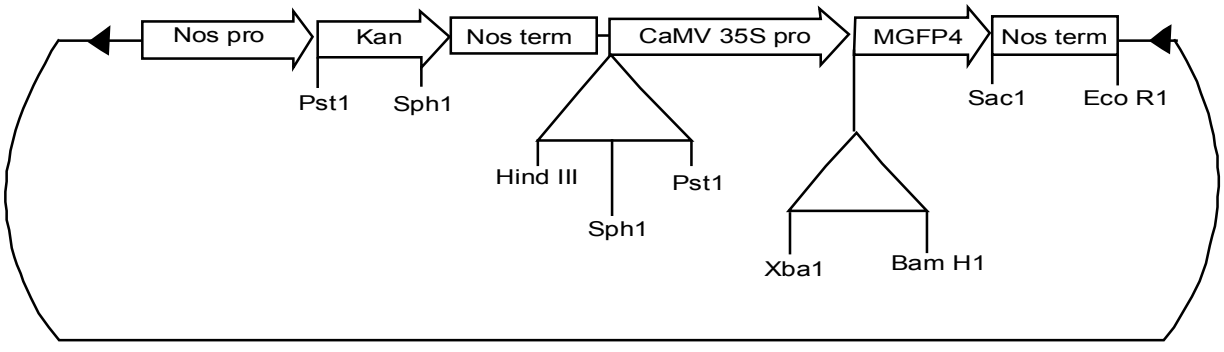


Fig. 1. Schematic diagram of pBin 35 S mGFP 4 plasmid

started from a glycerol stock. They were grown separately in 10 ml of LB broth plus 50 mg l^{-1} kanamycin at 37°C. 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°C overnight. The following daythe 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°C 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°C, the plates were stored at 4°C. Frozen stocks were also made from an overnight culture and kept at –70°C.

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 mM), BAP (2.22 mM) 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 g l^{-1} sucrose, 2,4-D (4.52 mM), BAP (2.22 mM) 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 mgL^{-1} of each ascorbic and citric acid, 30 g l^{-1} sucrose, IAA (3.42 mM), BAP (13.31 mM) 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 light / 8 hour dark period at a 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 and discussion

The sensitivity of the *in vitro* grown leaf cultures of *Litchi chinensis* Sonn. to kanamycin is shown in Table 1.

Table 1. Kanamycin sensitivity of in vitro grown leaves of litchi

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.

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. The concentration of vancomycin to be used was also determined by a similar experiment (Table 2).

Table 2. Effect of vancomycin concentration on *Agrobacterium tumefaciens*

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

On the basis of the above findings, the concentration of kanamycin and vancomycin used in the growth medium following transformation, were 50 and 300 mg l^{-1}, respectively. At this combination, callus regeneration frequency was 100% after four weeks in culture. 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 3).

Table 3. Effect of increasing kanamycin concentration on regeneration capacity

Kanamycin concentration (mg l^{-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.

The higher concentration of kanamycin (75 and 100 mg l^{-1}) seemed to have a negative effect on regeneration as most of the explants formed green callus only. 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

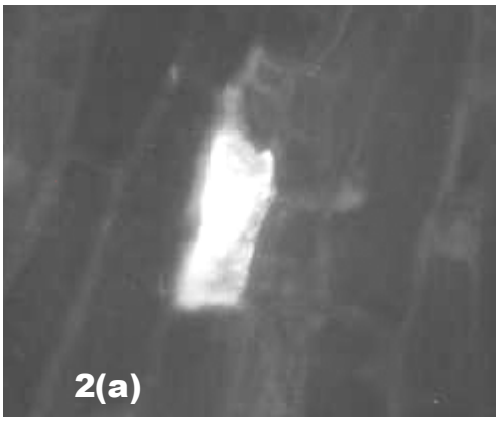
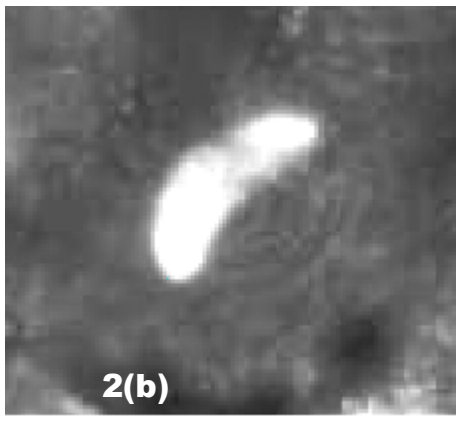


Fig. 2. Single transformed cells (2a) and 2(b)



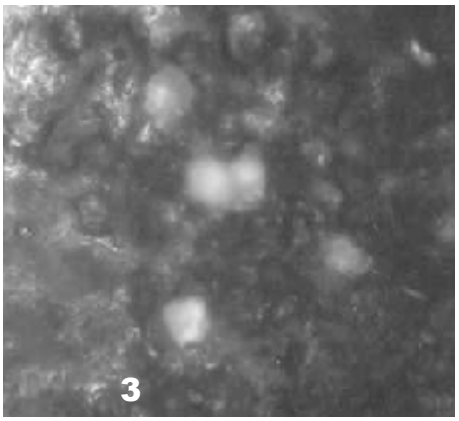


Fig. 3.Transformed cells embedded in tissue

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).

Results showed that the GFP system provides a convenient indicator of transformation. This study also demonstrates that the GFP gene from *A. victoria* can be ex-pressed transiently and stably in tissues of litchi 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. Expression level seems to be low in plant cells (Haseloff and Amos, 1995) compared to bacterial cells where expression level of 75% of total protein has been obtained (Crameri *et al.*, 1995 and Heim *et al.*, 1995). Several methods have been reported to increase *Agrobacterium* virulence and improve transformation efficiency. Addition of phenolic compounds such as acetosyringone either to bacterial growth media or during tissue co-culture, can improve transformation efficiency by stimulating induction of the bacterium virulence genes. Acetosyringone, as well as the osmoprotectants betaine phosphate and proline, have been reported to improve transformation efficiency in apple when added to the virulence induction medium (James *et al.*, 1993). According to Dandekar (1992), there are two important issues that must be considered in the process of regenerating transgenic plants from *Agrobacterium*-mediated transformation, the regenerating cells must be accessible to *Agrobacterium* and the plants must originate from single cells. Improved regeneration systems for litchi is required as the callus may be a source of somaclonal variation, requiring extensive field tests to ensure that regenerated plants are true to type. In general, regeneration of transgenic fruit trees is a problem. Antibiotics used to kill *Agrobacterium* have also been found to affect regeneration (Sain *et al.*, 1994). This may explain the low regenerability observed in this study using higher concentrations of vancomycin.

The ability to monitor constitutively expressed GFP in plants has many potential uses such as developing and optimizing transformation methods by continuously monitoring transformation events at different stages, and non-destructive identifying transformed cells or cell lines. This also enables the removal of all un-transformed material (usually the large majority of tissue) at an early stage and facilitate the prediction of the number of likely transformants. This methodology has not been possible using GUS histochemical assays which are toxic, or luciferase assays which frequently result in the reversion of embryogenic sugarcane calli to non-regenerative forms (Bower *et al.*, 1996). 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. Combinations of any selection marker genes with GFP in detecting transgenic cells should lead to more widespread use in developing plant transformation in litchi protocols, which is proven to be difficult than other crops.

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