Green Fluorescent Protein (GFP) in Vector Systems Played Sense Role of Epigenetic in Plants

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Abstract

The green fluorescent protein (GFP) of jellyfish (*Aequorea victoria*) has significant advantages over other reporter genes, because expression can be detected in living cells without any substrates. Recently, epigenetic phenomena are important to consider in plant biotechnology experiments for elucidate unknown mechanism. Therefore, soybean immature cotyledons were generated embryogenesis cells and engineered with two different gene constructs (pHV and pHVS) using gene gun method. Both constructs contain a gene conferring resistance to hygromycin (*hpt*) as a selective marker and a modified glycinin (11S globulin) gene (*V3-1*) as a target. However, *sGFP(S65T)* as a reporter gene was used only in pHVS as a reporter gene for study the relation between using *sGFP(S65T)* and gene silencing phenomena. Fluorescence microscopic was used for screening after the selection of hygromycin, identified clearly the expression of *sGFP(S65T)* in the transformed soybean embryos bombarded with the pHVS construct. Protein analysis was used to detect gene expression overall seeds using SDS-PAGE. Percentage of gene down regulation was highly in pHV construct compared with pHVS. Thus, *sGFP(S65T)* as a reporter gene in vector system may be play useful role for transgenic evaluation and avoid gene silencing in plants for the benefit of plant transformation system.

Key words: Epigenetics, Green fluorescence protein, Gene gun, Transgenic soybean.

Introduction

The advent of the technology to transform plants with DNA sequences such as the generation of transgenic plants, allowed an entirely new direction for the exploration of genetics.

GFP has grown in popularity as a reporter gene in molecular biology research. In plant transformation systems with the problem of low efficiency, the nondestructive analysis of putative transformed cells and tissues using a reporter gene such as the GFP (jellyfish green fluorescence protein) gene could significantly help to optimize transformation protocols and generate transgenic plants (Stewart 2001, El-Shemy et al. 2004, 2006, 2007)

With the successful development of procedures for transformation, transgenic plants and their progeny have been studied for continued stable expression of the foreign gene through several generations. These studies have revealed that in some cases the transgene expression was lost in a variable proportion of the progeny. This phenomenon, called ‘‘gene silencing’’, has been studied most extensively in dicot plants, such as tobacco, petunia, tomato, and buckweed (Matzke and Matzke 1995; Meyer 1995). Several mechanisms have been proposed to explain the phenomena of gene silencing (Matzke et al. 2001; Meyer 1995; Stam et al. 1997). Two types of silencing occur in plants and in other eukaryotes: transcriptional gene silencing (TGS) and posttranscriptional gene silencing (PTGS) (Carthew,2001; Waterhouse *et al.,* 2001a,b). Silencing at the transcriptional level is thought to occur primarily by methylation of promoter sequences, thereby interfering with assembly of the transcription factors and/or by attracting chromatin re-modeling proteins to these sites (Meyer, 2000; Wang and Waterhouse, 2002).

Gene silencing and its implications for transgene expression is an area of intense research at this time, and the reader is directed to recent reviews on this large subject (Meyer, 2000; Vance and Vaucheret, 2001; Wang and Waterhouse, 2002). For that, the

target of this study is identify the role of *sGFP(S65T)* to reduce or avoiding the gene silencing phenomena in transgenic plants.

Materials and methods

Construction of chimeric genes

Two plasmid vectors, pHVS and pHV, were constructed based on the pUC19 vector (Fig. 1 A and B). pHV contains the hygromycin phosphotransferase coding region, *hpt* (1.0kb), under regulatory control of the cauliflower mosaic virus (CaMV) 35S promoter, *35Spro*, and the modified proglycinin (A1aB1b) cDNA, *V3-1* (1.4kb), with a synthetic DNA encoding four continuous methionines. pHVS contains additionally a modified jellyfish green fluorescent protein coding region, *sGFP(S65T)* (0.8kb), under regulatory control of *35Spro* in the flanking region of the *V3-1* gene (El-Shemy et al, 2004,2006, 2007).

Initiation and proliferation of embryogenic cultures

Transformation and regeneration systems for soybean were optimized according to methods described elsewhere (Finer and Nagasawa 1988; Hadi et al. 1996; Sato et al. 1993, El-Shemy et al. 2004, 2006, 2007). Soybean plants, cv. Jack, were grown in soil in a glass-house controlled at 25 ºC under natural light. Developing green pods were obtained when the immature cotyledons were about 4-5 mm long. After sterilization of the pod surface with 70% ethanol followed by 3 rinses with sterile water, the immature cotyledons were extracted, the end removed together with the embryonic axis, and the sample placed flat side up on MSD40 medium consisting of MS salts (Murashige and Skoog 1962) and B5 vitamins (Gamborg et al. 1968) supplemented with 3% sucrose, 40mg/L 2,4-D, and 0.2% Gelrite (Wako Pure Chemical Industries, Osaka, Japan) (pH7.0).

Embryogenic tissues were initiated at 25 ºC under cool white fluorescent light (23/1 light regime, 5-10 μmol $m^{-2}s^{-1}$) for 3 to 4 weeks. Soybean embryogenic tissues were suspended and maintained in FN Lite liquid medium consisting of FN Lite macro salts, MS micro salts and B5 vitamins supplemented with 1 g/L asparagine, 5 mg/L 2,4-D, and 1% sucrose (pH 5.8) (Finer and Nagasawa 1988; El-Shemy et al.2004), and maintained by subculturing every week into 25 ml of fresh FN Lite liquid medium in a 100 ml flask (El-Shemy et al.2004, 2006).

Transformation by particle bombardment

Approximately 1 g of embryogenic suspension tissue was transferred to the center of MSD20 medium consisting of MS salts and B5 vitamins supplemented with 3% sucrose, 1 g/L asparagine 20mg/L 2,4-D, and 0.2% Gelrite (pH5.8) in a 9 cm petri dish. Bombardments were performed using a Biolistic PDS-1000/He Particle Delivery System (Bio-Rad, Richmond, CA, USA) according to the instruction manual. Each sample of embryogenic tissue was bombarded twice with a pressure of 1350 *psi*.

Bombarded tissues were resuspended in the FN Lite medium. One week after bombardment the embryogenic tissues were transferred to fresh FN Lite medium containing 15mg/L hygromycin B (Roche Diagnostics, Mannheim, Germany). The bombarded tissues were transferred to fresh antibiotic-containing FN Lite medium weekly for an additional 3 weeks. Then, white lumps of tissue that contained bright green lobes of embryogenic tissue were selected and transferred to fresh FN Lite medium containing 30mg/L hygromycin B. The hygromycin tolerant tissues were selected and resuspended in fresh antibiotic-containing FN Lite medium weekly for 3 additional weeks (El-Shemy et al 2004, 2006).

Hygromycin tolerant embryos were subcultured in FNL0S3S3 liquid medium, which contained FN Lite macro salts, MS micro salts and B5 vitamins supplemented with 1 g/L asparagine, 3% sucrose, and 3% sorbitol (pH 5.8). Three weeks after the suspension, excess liquid of the developing embryos was withdrawn with sterile filter paper, and the embryos were placed in dry petri dishes for 3 to 5 days. After the desiccation treatment, the embryos were placed on MS0 medium containing MS salts, B5 vitamins, 3 % sucrose, and 0.2 % Gelrite (pH5.8). The germinating plantlets were transferred to 1/2 B5 medium. After root and shoot elongation, plantlets were transferred to pots containing soil, and maintained under high humidity. Plantlets were gradually adapted to ambient humidity and placed in the glass-house.

GFP detection

The presence of sGFP(S65T) was detected by blue light excitation (Chiu et al. 1996). Embryos, cotyledons, leaves of regenerated plants and seeds were observed using a microscope (Leica Microsystems, Wetzlar, Germany) with a filter set providing 455-490 nm excitation and emission above 515 nm.

PCR and Southern blot analysis

Total DNA was isolated from soybean leaves by the method of (Draper and Scott 1988), and used to investigate the presence of the transgenes. PCR analysis was conducted to screen transformed plants in a 20 µl reaction mixture containing 10 ng of genomic DNA, 200 µM of each dNTP, 0.2 µm of each primer, and 2.5 units of Ampli-taq Gold polymerase (Applied Biosystems, Foster City, CA USA) in the corresponding buffer. Reaction were hot-started (9 min at 94°C) and subjected to 30 cycles as follows: 30 sec at 94°C; 1 min at 55°C; and 1 min at 72°C. The last extension phase was prolonged to 7

min at 72°C. The primer set for *hpt* was designed for amplification of a 560 bp fragment; sequences are 5'-ATCCTTCGCAAGACCCTTCCT-3' (35S promoter) and 5'-GGTGTCGTCCATCACAGTTTG-3' (*hpt*). The primer set for *V3-1* was designed for amplification of a 1403 bp fragment; sequences are 5'-TTCAGTTCCAGAGAGCAGCAGCCT -3' and 5'-CTGATGCATCATCATCTGAGG -3'. That for *sGFP(S65T)* was designed for amplification of a 708 bp fragment; sequences are 5'- AAGGTACCGGATCCCCCCTCAGAA -3' and 5'- AAGAGCTCCGATCTAGTAACATAGATGACACC -3'.

Southern blot analysis was conducted to confirm the stable integration of transgenes into soybean. Total DNA (10 μg) was digested with the restriction enzyme, *Sac*I, and digested DNA was separated by electrophoresis in a 1% agarose gel and transferred onto a hybond N+ membrane (Amersham Biosciences, Buckinghamshire, England). Labeling and detection were conducted following the protocol of ECL direct nucleic acid labeling and detection (Amersham Biosciences). DNA fragments of the *V3-1* gene, *sGFP(S65T)* gene, and *hpt* gene were amplified from the plasmids with the same primer sets used for the PCR analysis, and served as hybridization probes on Southern blot membranes.

Analysis of seed proteins in transgenic soybean

The subunit composition of seeds from individual transgenic soybean was analyzed by SDS-PAGE (Laemmli 1970). A total globulin fraction was extracted from soybean seed meal by grinding with 50 mM Tris-HCl, pH 8.0. The supernatant was obtained after centrifugation at 15,000*g* for 10 min. The protein concentration was determined with a BCA protein assay (Pierce, Rockford, IL, USA) kit using bovine serum albumin as a

standard. The protein solution was mixed with the same amount of a twice-concentrated SDS sample buffer containing 2-mercaptoethnol. The proteins (25 μg) from each sample were separated on a gels (Tricine-SDS-PAGE and SDS-PAGE) containing 12 % (w/v) acrylamide and 0.2 % (w/v) bis-acrylamide, and were stained with Bio-Safe CBB G-250 stain (Bio-Rad).

Results and Discussion

The potential of GFP appears, is greater than its current applications as researchers seeking to characterize GFP under novel conditions reveal new uses for the protein (Stewart 2001, Richards et al 2003, El-Shemy et al 2004, 2006). This increased potential is especially pertinent to plants, since it is often desirable to quantify gene expression on the plant and in the field.

Embryogenic cells induced from a soybean cultivar, Jack, were transformed by microparticle bombardment with the pHVS, which contains a modified globulin gene, a selectable marker gene *hpt* and a reporter gene *sGFP(S65T*). To optimize the conditions for particle delivery into the embryogenic tissues, transient expression of the *sGFP(S65T)* gene was detected one day after bombardment with a fluorescent microscope. According to the intensity and number of foci expressing GFP, we decided on a pressure of 1350 psi and distance of 6 cm for the delivery. Expression of *sGFP(S65T)* in soybean was also monitored during the selection with hygromycin and development of plants (Fig. 2).

In this experiment, the conditions for delivery of particles coated with plasmids according to the transient expression was successfully optimized of the *sGFP(S65T)* gene (Fig. 2). GFP can partially replace antibiotic selection and be of great use when the organogenesis or conversion of transformation procedures is inefficient under antibiotic or herbicide

selection (Stewart 2001). It could be helpful in isolating events during the early stages of transformation experiments as described here. An example of this was the transformation of sugarcane with *sGFP(S65T)* (Elliot et al. 1998)

Transformation was achieved by coating each plasmid, either pHV or pHVS, onto the particles and bombarding embryogenic tissues. Hygromycin-resistant cells were selected then matured in FNL0S3S3 liquid medium and germinated on MS0 media. All regenerated plants obtained from the two constructs were confirmed the presence of *hpt* gene by PCR analysis. Out of a total of 122 regenerated plants obtained from the introduction of pHVS, 82 plants produced an expected band with a 0.5 kbp of PCR product within the *hpt* gene (Table 1). On the other hand, 29 of 98 regenerated plants obtained from the introduction of pHV yielded the 0.5 kbp *hpt* fragment in PCR analysis (Table 1). PCR analysis for *sGFP(S65T)* genes was conducted in the soybean plants that yielded a *hpt* band to confirm the presence of all transgene cassettes (Fig. 3). The expression of sGFP(S65T) was detected in about 52 % of the *hpt*-positive soybeans engineered using pHVS (Fig. 3).

Silencing was first observed concerning transgene expression in plants about 10 years ago,with a report that transformation of petunia with extra copies of the chalcone synthase gene could result in a block in expression of both the transgene and the corresponding endogenous gene (Napoli et al., 1990, Lessard et al, 2002). This phenomenon was termed cosuppression (Napoli et al., 1990). Suppression of endogenous glycinin in the transformed soybean was frequently observed on the introduction of the modified glycinin *V3-1* gene (Table 1).

Southern blot analysis was performed to confirm the integration, and to estimate

the copy numbers of transgenes. Total genomic DNA, which was isolated from transgenic plants to as certain the presence of all transgene cassettes by PCR (Fig. 3), was digested with *SacI*, and hybridized with one of the three probes for *hpt* and *sGFP(S65T)* (Fig. 4). All the transformants analyzed here yielded one to seven bands hybridized with the *hpt* probe in addition to a common band at around 6.6 kbp (Fig. 4 A). The *V3-1* gene was altered genetically from a proglycinin (A1aB1b) cDNA, which ordinarily exists in soybean. Therefore, untransformed plants also gave multiple bands, indicating that these bands would correspond to the endogenous glycinin genes. The transgenic plants gave additional bands resulting from the integration of the *V3-1* gene (Fig. 4 B). On the other hand, DNA isolated from untransformed plants hybridized with the *sGFP(S65T)* probe, even though GFP is not derived from plants. This maybe caused by unspecific hybridization of the probe with soybean genomic DNA. The individual plants exhibited different banding patterns, confirming that they resulted from different events.

The effects of transgenic copy number on the level of gene expression are known to be complex. Though it was anticipated that the increase of transgene copy number would increase the expression level (Dai et al. 2001; El-Shemy et al.2004, 2006,2007), it is now known that gene co-suppression phenomena frequently occur in transgenic plants with repeated transgenes or an unusual structure such as inverted repeats (Vaucheret et al. 1998). Such events are likely to be powerful inducers of co-suppression and methylation (Luff et al. 1999). Transgenic plants lacking all subunits of glycinin had multiple copies of the *V3-1* gene and small fragments hybridized with a *V3-1* probe (Fig. 4).

The accumulation of glycinin was confirmed by SDS-PAGE analysis of the globulin fraction extracted from transgenic seeds (Fig. 5). The modified glycinin *V3-1*

could not be distinguished from endogenous glycinin subunits by the SDS-PAGE, because the modified glycinin contains only six additional amino acids in the basic subunit. However, the glycinin subunit polypeptides in some transformants were intensely stained with CBB compared to non-transformants (Fig. 5). This may be due to the accumulation of the modified glycinin *V3-1* in transgenic seeds. On the other hand, some transgenic soybeans lack all subunits of glycinin, suggesting the transgene may cause the suppression of endogenous glycinin genes by the effect of gene silencing (Table 1, Fig. 5 and Fig. 6). The ratio of gene silencing was lower in transformants engineered with the pHVS construct and selected based on GFP expression than transgenic soybean engineered with the pHV construct.

It is suggested that the transgene causes the effects of gene silencing. There is some predictability to silencing. Counter intuitively, an increasing copy number of a transgene can correlate with an increased risk of silencing (Lessard, et al 2002). However, determining whether silencing will occur in particular transgenic plants is still largely an empirical problem, requiring testing of transgene expression in the individual plants. Two general approaches can be used to avoid problems with silencing (Lessard, et al 2002). First, the use of gene delivery methods, such as *Agrobacterium*-mediated transformation, that result in integration of relatively few copies of a transgene into the genome,can minimize problems with silencing (Dai *et al.,* 2001). Second,the use of constructs in which matrix attachment regions flank the transgene may also minimize silencing (Spiker and Thompson,1996). The results expected that the transgenic soybeans accumulating the modified glycinin *V3-1* to have a higher level of methionine than nontransformants (Fig. 5). Up to date, gene silencing was seen as a problem for plant

genetic transformation, as it prevented reliable expression of a desired phenotype within transgenic plants (Taylor and Fauquet, 2002). However, with increasing knowledge of the mechanisms underlying this phenomena, and realization that it can be utilized to down-regulate native genes within the plants, and it will become a powerful tool in future transgenic applications (Vance and Vaucheret, 2001; Lessard et al, 2002).

Silencing effect appeared stochastic in nature, with patterns and variegation appearing in the tissues of the transformed plants, much like previous observations of somaclonal variation and paramutation (Grant-Downton and Dickinson 2005). This ‘co-suppression’ of an endogenous gene by extra copies of the same gene was also accompanied by observations that insertion of entirely foreign coding gene sequences did not necessarily lead to their expression (Grant-Downton and Dickinson 2005). Other experimentation with transgenic technology led to the discovery that this gene silencing effect on endogenous genes by inserted DNA was more consistent when the inserted gene copy was in reverse or ‘antisense’ orientation (van der Kroll et al., 1988). Perhaps more remarkable was the realization that a whole copy of the gene was not even required to elicit this effect (Grant-Downton and Dickinson 2005). Clearly, adding new fragments of DNA to the genome not only resulted in the silencing of their own expression, but also the specific silencing of endogenous genomic sequences to which they were homologous (Grant-Downton and Dickinson 2005). An intact copy with no nucleotide mutation was being ‘silenced’ by the presence of a foreign sequence at another site in the same genome. Exactly how this extra DNA was delivered via *Agrobacterium* or biolistic integration, (Grant-Downton and Dickinson 2005).

Biomolecular techniques allow for the insertion of the gene for GFP right before the stop codon (Chalfie et al. 1994). The cell would keep making hemoglobin, but before reaching the stop codon it would make the GFP (Chalfie et al. 1994, Fig. 7).

There were three reasons Prasher thought that GFP could potentially become a significant tracer molecule. Firstly, if enough protein with attached GFP were made, it should be easy to detect and to trace it as it moved through the cell, because irradiating the cell with ultra violet light would cause the GFP attached to the protein to fluoresce. Secondly, Shimomura had shown in 1974 that GFP was a fairly small protein. This GFP was important because a small protein attached to the protein of interest was less likely to hinder its proper function. Its small size would also allow it to follow the fused protein, especially in organelles like neurons, whereas the diffusion of large proteins would be difficult. Thirdly, it had been shown that once GFP was made in the jellyfish, it was fluorescent. Most other bioluminescent molecules require the addition of other substances before they glow. For example, aequorin will glow only if calcium ions and coelenterazine have been added, and firefly luciferase requires ATP, magnesium, and luciferin before it luminesces. This would make GFP a much more versatile tracer than either aequorin or firefly luciferase, which were being used as tracers. Besides attaching GFP to a protein and making it a fluorescent tag, Prasher also thought that GFP could potentially be a very useful reporter molecule. For activate protein production DNA promoters are used, these are sequences of DNA next to genes that contain the information about where and when the gene should be read and make the protein. If GFP is linked to a specific promoter then it will be expressed in place of the protein, showing

where and when the gene of interest is switched on (Douglas Prasher, from Zimmer, Marc website, Fig. 7).

Therefore, *sGFP(S65T)* can be effectively used to select the transformants expressing all the gene cassettes (El-Shemy et al.2004, 2006, 2007). The transgenic plants expressing *sGFP(S65T)* grew and reproduced normally, and the GFP expression was inherited without any abnormalities. In conclusion, using *sGFP(S65T)* as a reporter gene was reduced or avoiding the gene silencing in transgenic soybean .

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Figure Legends

Fig. 1 Structures of plasmid constructs for soybean transformation. **A**, pHVS contains a modified glycinin gene, *V3-1*, between *hpt* as a selectable gene and *sGFP(S65T)* as a reporter gene. **B**, pHV contains *V3-1* flanked by *hpt* as a selectable gene. Restriction sites are indicated.

Fig. 2 Visualization of *sGFP(S65T)* expression in transformed soybean plantlets. Embryos (**A**), leaves (**B**) and Seeds (**C**).

Fig. 3 Detection of foreign genes in transgenic soybean plants by PCR: *hpt* (A), and *sgfp* (B) genes. lane 1 (untransformed control), 2-15 (transformed soybeans with pHVS and pHV), and 16 (plasmid with pHVS)

Arrowheads expected 560 bp and 708 bp fragments of *hpt* and *sgfp* genes.

Fig. 4 Southern blot analysis of soybeans transformed with pHVS and pHV. Total DNA was digested with *Sac*I and loaded in lane N (untransformed control), 1-4 (transformed soybeans with pHVS), and 5-7 (transformed soybeans with pHV). Separated DNA was transferred to a nylon membrane and hybridized with each probe containing the coding region of *hpt* (**A**), and *sGFP(S65T)* (**B**).

Fig. 5 Tricine-SDS-PAGE (A) and SDS-PAGE (B) analysis of components of seed storage proteins in transformed soybean. Globulin fractions were isolated from QF2 (mutant line lacking 7S and 11S globulin), Jack (untransformed control) and transformants, 25 μg of each fraction was fractionated by SDS-PAGE and then stained with CBB.

Fig. 6 Detection of storage proteins (11S globulin) in soybean transgenic seed proteins(Immune-blotting). QF2 (mutant line lacking 7S and 11S globulin), Jack

(untransformed control) and transformants. lane 1 (transformed soybeans with pHVS), lane 2(transformed soybeans with pHV) and lane 3 (transformed soybeans with pHVS).

Arrowheads expected fragments of 11S globulin (Jack, untransformed control, lane 1,3 transformed soybeans with pHVS).

QF2 and Lane 2 transformed soybeans with pHV showed silencing of internal 11S globulin.

Fig. 7 Insertion of GFP gene into DNA. Douglas Prasher GFP School, (*Science* 263, 802–805, 1994). http://www.conncoll.edu/ccacad/zimmer/GFP-ww/GFP-1.htm

Table 1 Gene silencing efficiency in soybean by using two vector systems

Construct	pHVS	pHV
Regenerated plants	122	98
hpt-positive by PCR	82	29
Transgenic plants[1)]	49(29[2)]/20[3)])	21
Absence of glycinin	24(10[2)]/ 14[3)])	15
Gene Silencing %	58.5(34.4[2)] / 70[3)])	71.4

1) Transformats giving *hpt* and *sGFP* bands by PCR analysis
2) Transgenic soybean expressing s*GFP*
3) Transgenic soybean not expressing s*GFP*

Fig. 1

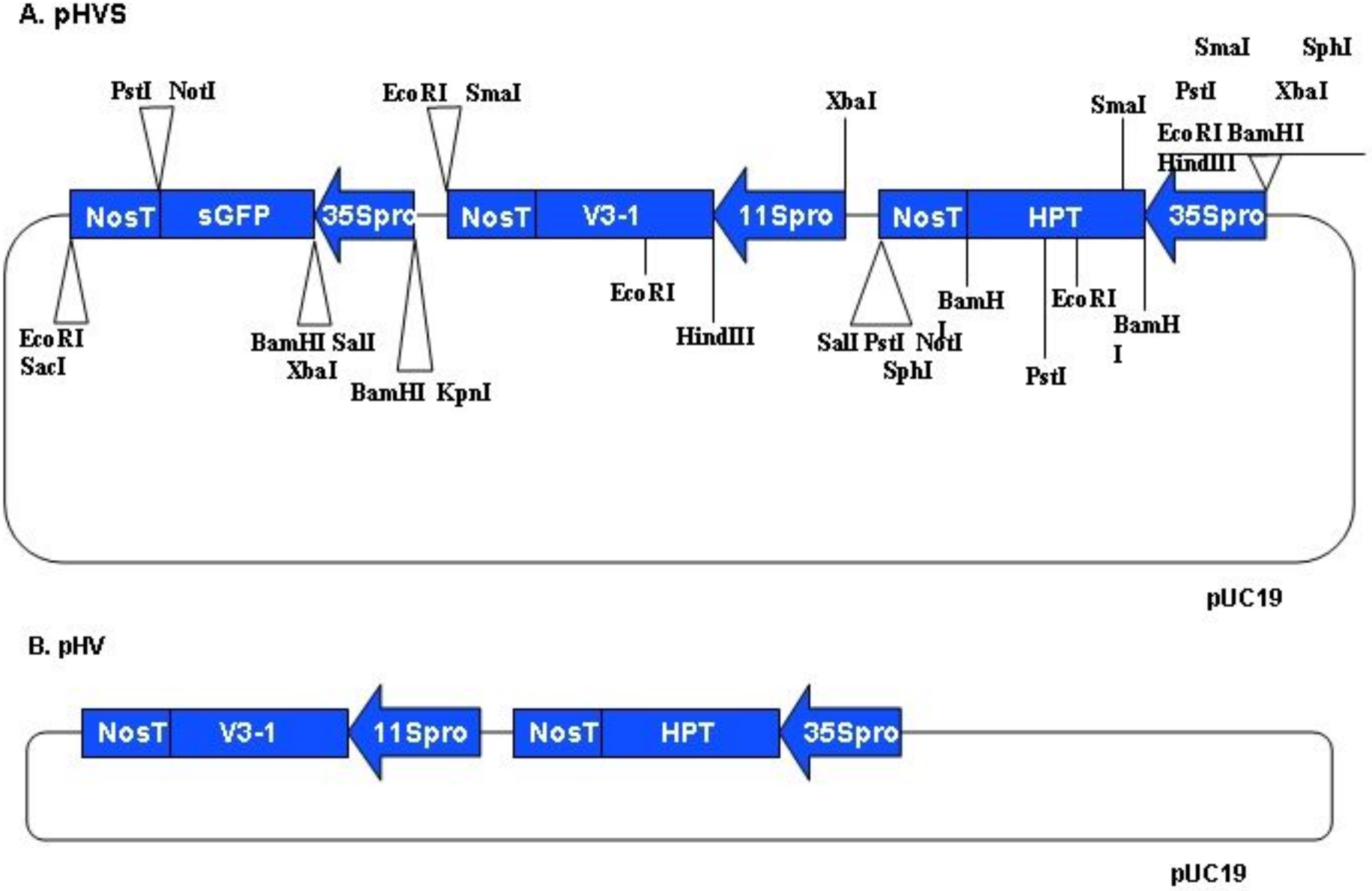

A. pHVS
PstI NotI
EcoRI SmaI
XbaI
SmaI
SmaI SphI
PstI XbaI
EcoRI BamHI
HindIII
NosT
sGFP
35Spro
NosT
V3-1
11Spro
NosT
HPT
35Spro
EcoRI
SacI
BamHI SalI
XbaI
BamHI KpnI
EcoRI
HindIII
SalI PstI NotI
SphI
BamH
I
PstI
EcoRI
BamH
I
pUC19
B. pHV
NosT
V3-1
11Spro
NosT
HPT
35Spro
pUC19

Fig 2

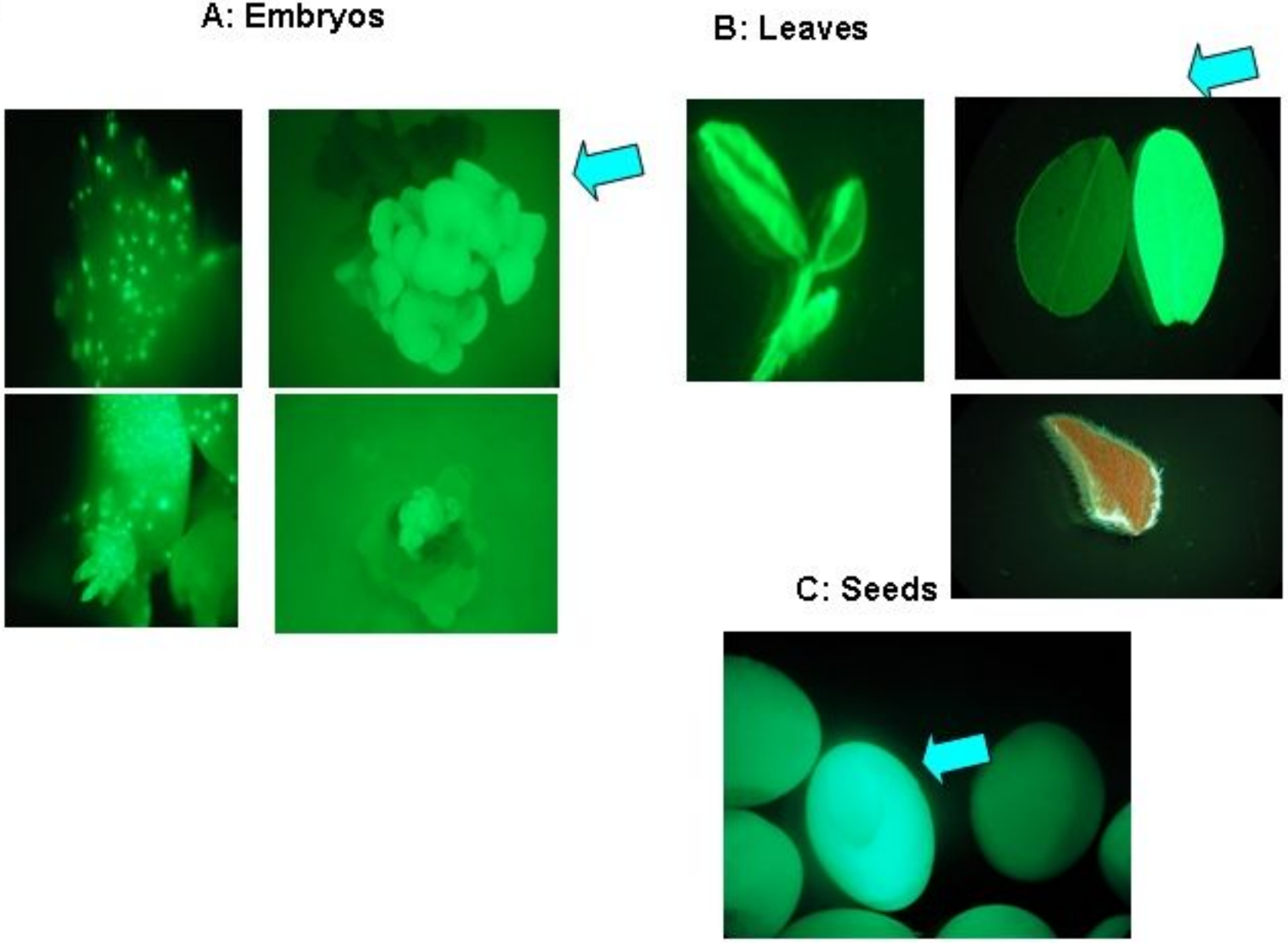


Fig 3

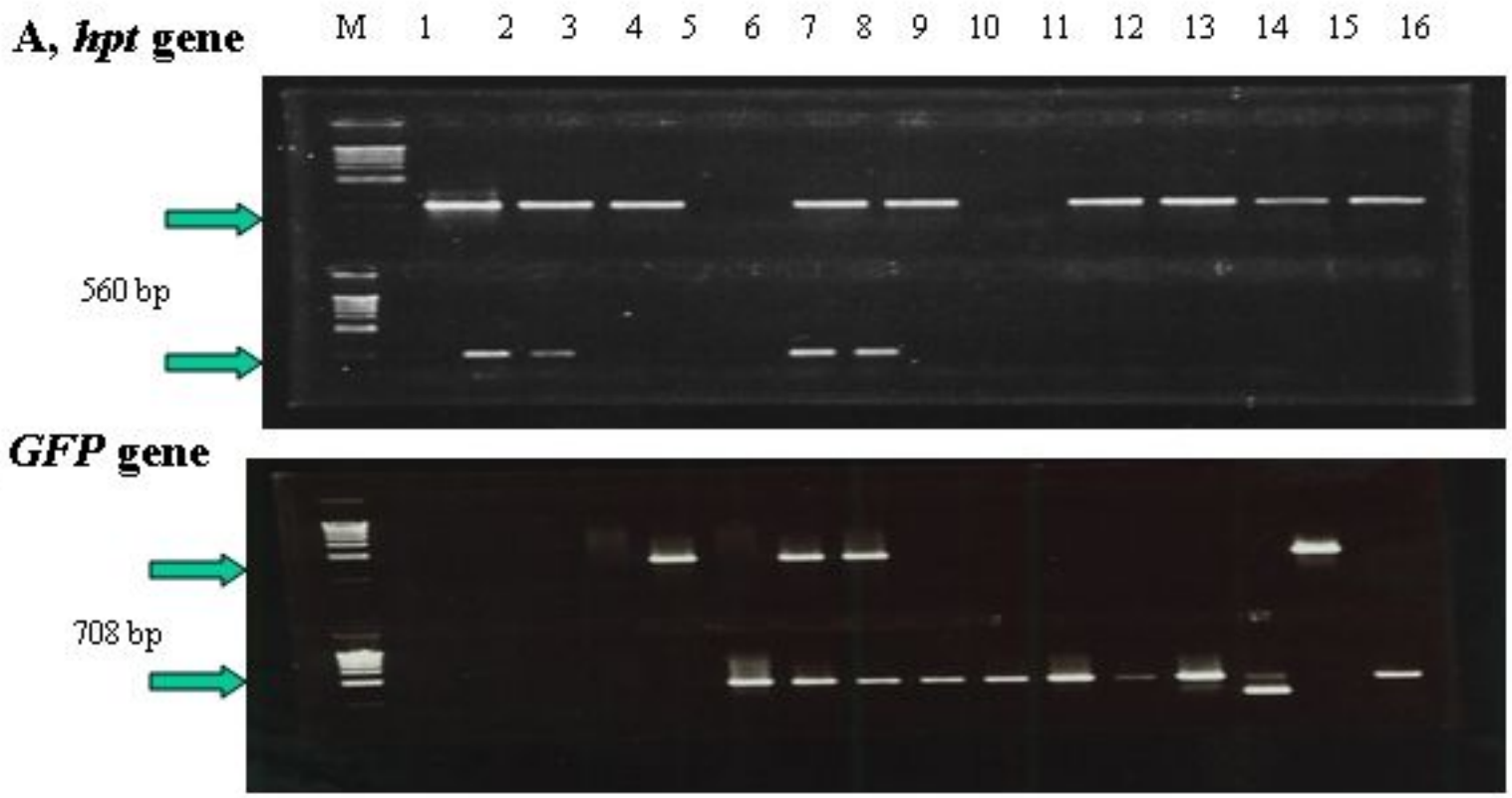

A, hpt gene
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
560 bp
B, GFP gene
708 bp

Fig 4

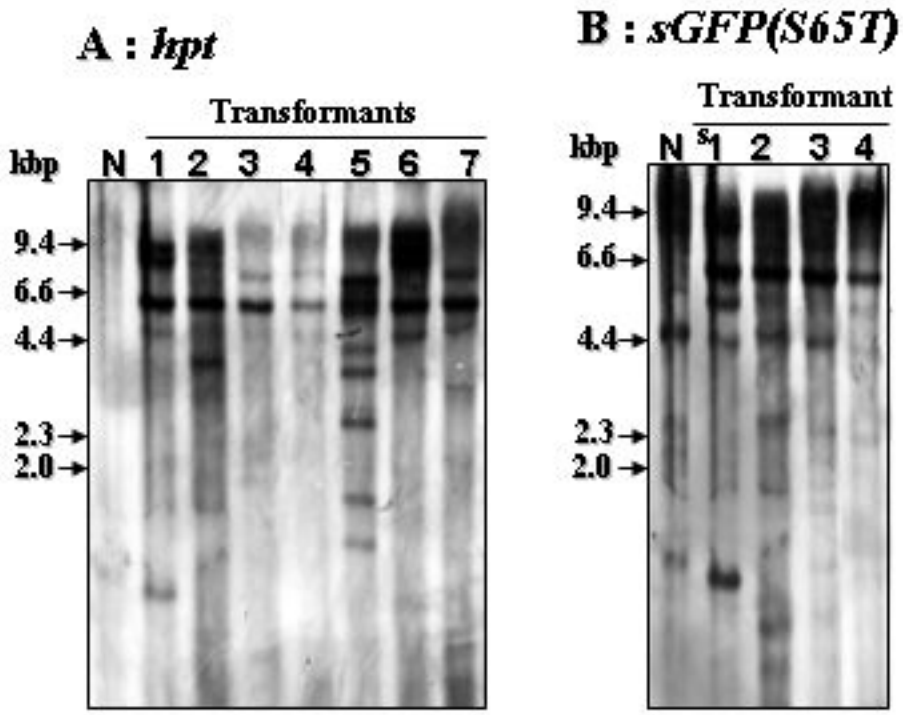

A : hpt
Transformants
kbp
N
1
2
3
4
5
6
7
9.4
6.6
4.4
2.3
2.0
B : sGFP(S65T)
Transformant
kbp
N
S1
2
3
4
9.4
6.6
4.4
2.3
2.0

Fig 5

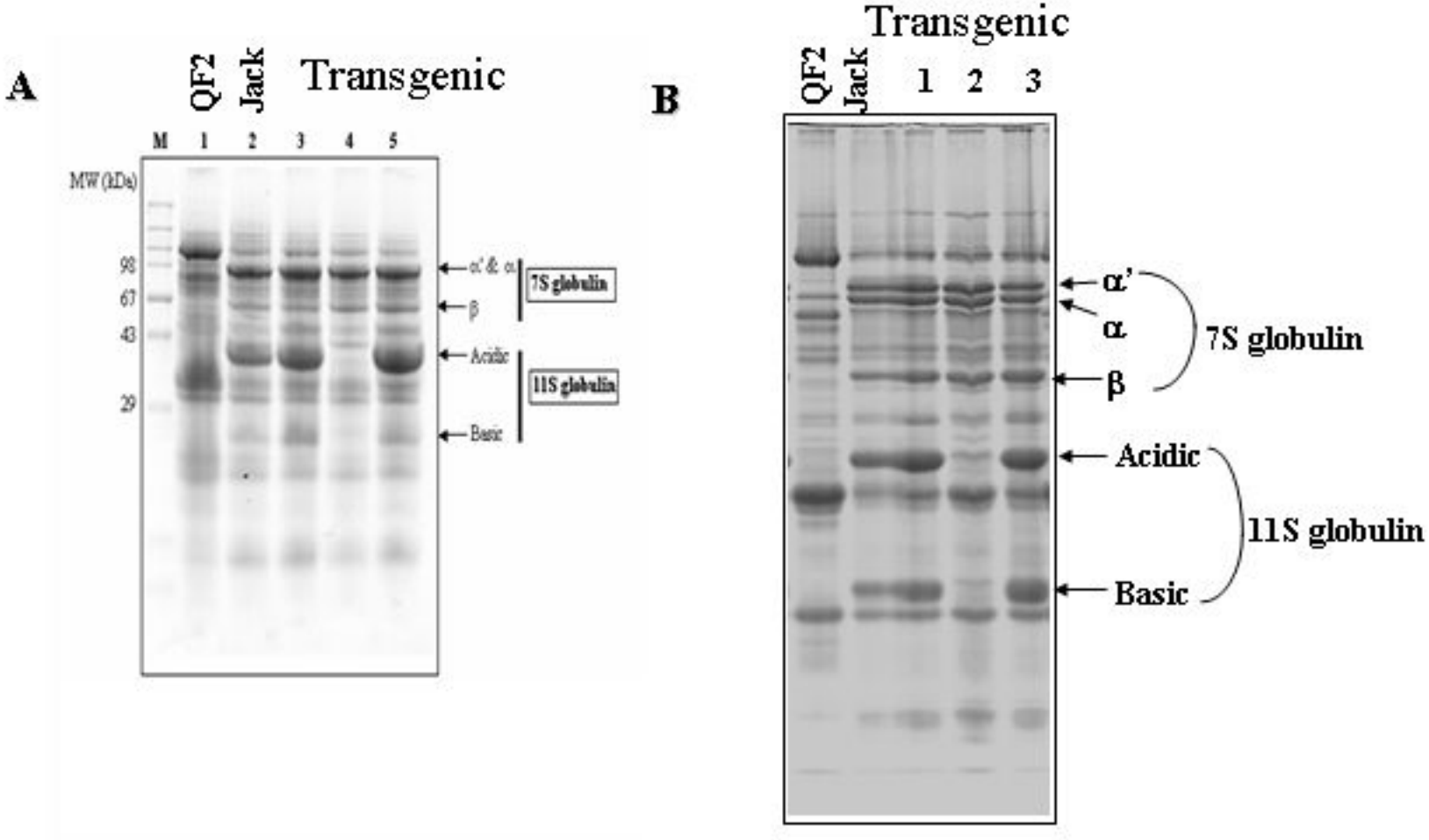

A
QF2
Jack
Transgenic
M
1
2
3
4
5
MW (kDa)
98
67
43
29
α' & α
β
Acidic
Basic
7S globulin
11S globulin
B
Transgenic
QF2
Jack
1
2
3
α'
α
β
Acidic
Basic
7S globulin
11S globulin

Fig 6

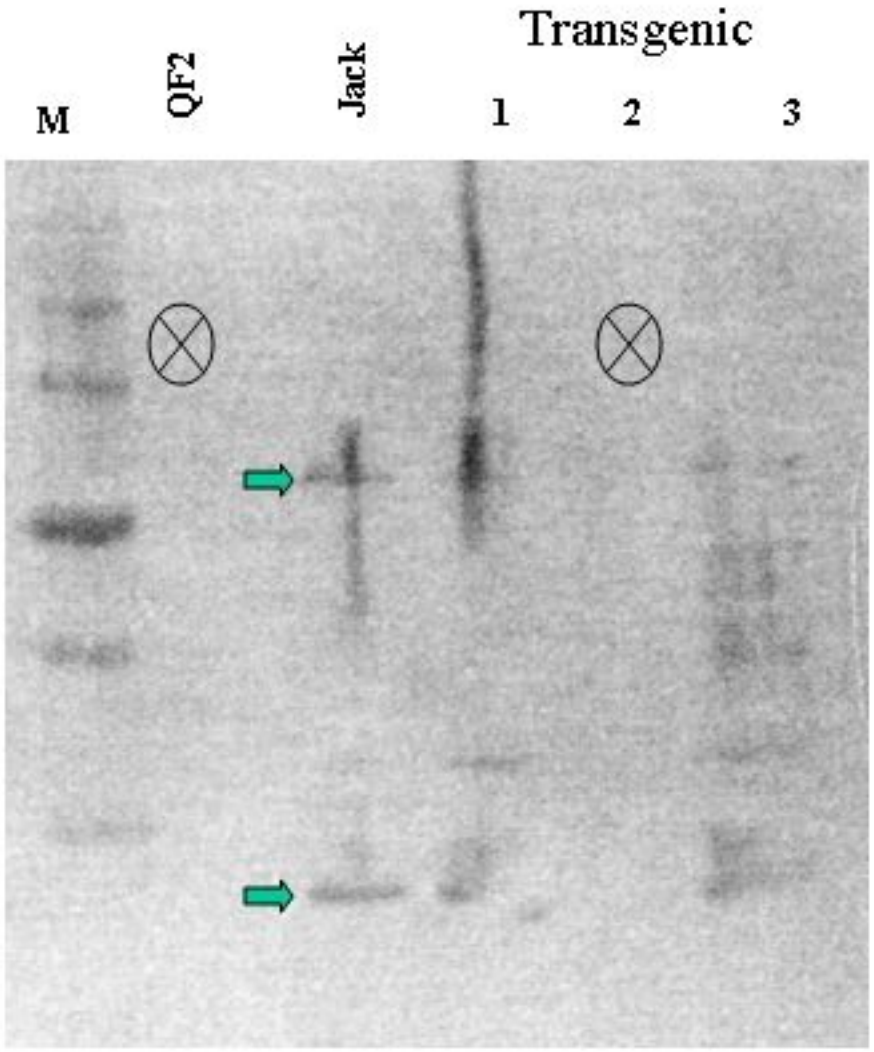
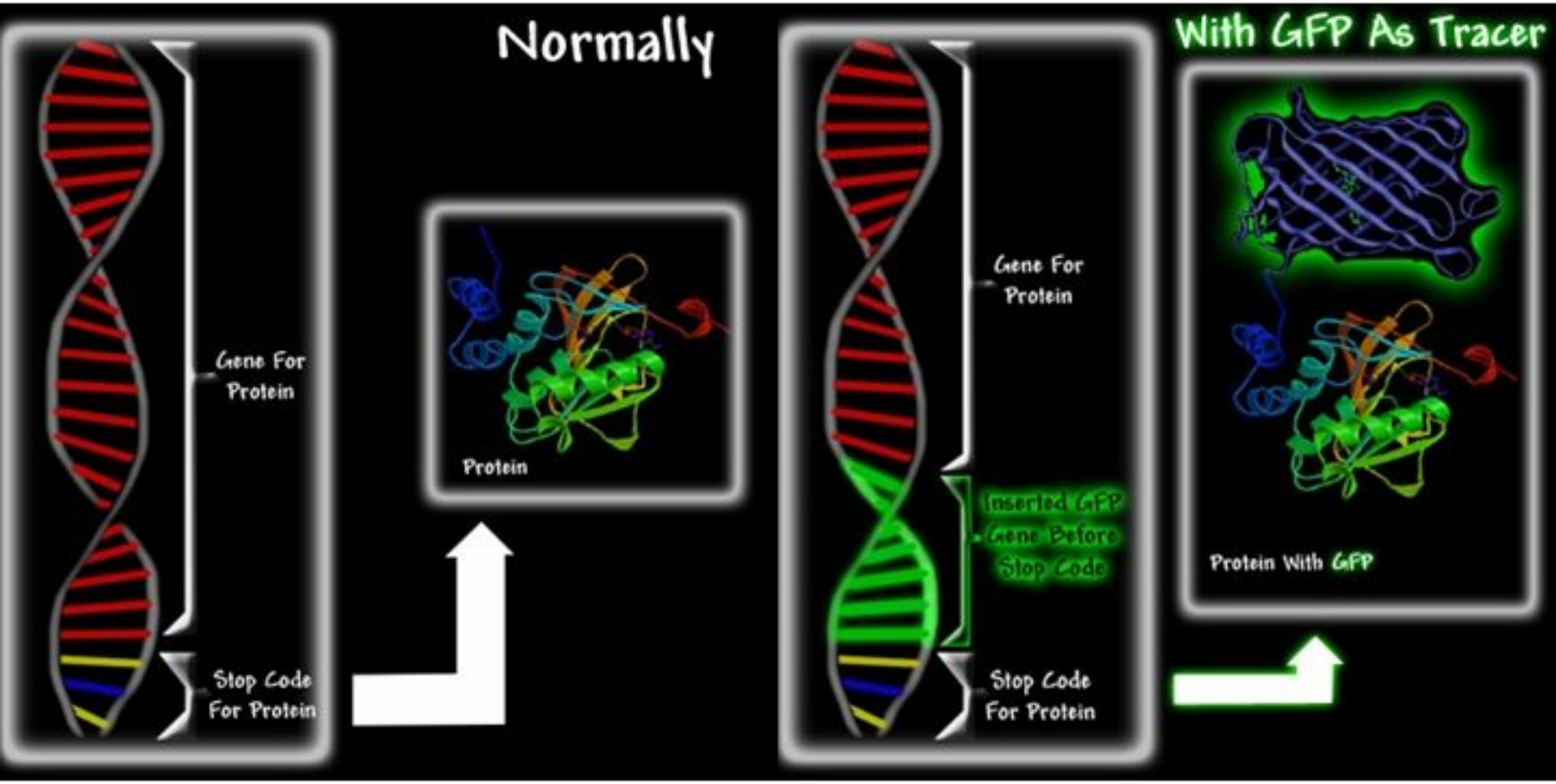


Fig 7


Normally
Gene For Protein
Stop Code For Protein
Protein
Gene For Protein
Inserted GFP Gene Before Stop Code
Stop Code For Protein
With GFP As Tracer
Protein With GFP