Journal of Biochemistry and Molecular Biology, Vol. 39, No. 1, January 2006, pp. 61-67

JOURNAL OF Biochemistry and Molecular Biology

Molecular Control of Gene Co-suppression in Transgenic Soybean via Particle Bombardment

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Received 27 September 2005, Accepted 21 October 2005

Molecular co-suppression phenomena are important to consider in transgene experiments. Embryogenic cells were obtained from immature cotyledons and engineered with two different gene constructs (pHV and pHVS) through particle bombardment. 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. *sGFP(S65T)* as a reporter gene was, however, inserted into the flanking region of the *V3-1* gene (pHVS). Fluorescence microscopic screening after the selection of hygromycin, identified clearly the expression of *sGFP(S65T)* in the transformed soybean embryos bombarded with the pHVS construct. Stable integration of the transgenes was confirmed by polymerase chain reaction (PCR) and Southern blot analysis. Seeds of transgenic plants obtained from the pHV construct frequently lacked an accumulation of endogenous glycinin, which is encoded by homologous genes to the target gene *V3-1*. Most of the transgenic plants expressing *sGFP(S65T)* showed highly accumulation of glycinin. The expression of *sGFP(S65T)* and *V3-1* inherits into the next generations. *sGFP(S65T)* as a reporter gene may be useful to increase the transformation efficiency of transgenic soybean with avoiding gene co-suppression.

Keywords: Gene co-suppression, Green fluorescence protein, Particle bombardment, Transgenic soybean

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Introduction

The difficulties of transformation and plant regeneration in soybean retard the improvement of agronomic traits in addition to studies on gene function. *Agrobacterium tumefaciens* and particle bombardment mediated transformation systems have been commonly used to make transgenic soybean (Hinchee *et al*., 1988; McCabe *et al*., 1988; Finer and Mcmullen 1991). Particle bombardment-meditated transformation can be an efficient method for soybean transformation, and is often genotype-independent (Aragao *et al*., 2000). On the other hand, particle bombardment tends to result in the integration of multiple copies of and rearrangement of transgenes, sometimes leading to their co-suppression (Vaucheret *et al*., 1998; Dai *et al*., 2001). 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, referred to as "gene co-suppression", has been studied most extensively in dicot plants, such as tobacco, petunia, tomato, and buckweed (Matzke and Matzke 1995; Meyer 1995). Transgene suppression and the associated co-suppression of homologous endogenous genes have been observed in plants with multiple copies and or high-level expression of the transgenes. Several mechanisms have been proposed to explain the phenomena of co-suppression (Meyer 1995; Stam *et al*., 1997; Matzke *et al*., 2001). Silencing phenomena are important to consider in transgene experiments. Two types of silencing occur in plants as well as 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).
Co-supression operates at the RNA level, and involves the production of double stranded RNA which acts as a trigger to initiate degradation of a target RNA, thereby resulting in gene silencing (Vance and Vaucheret, 2001). 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). 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). Therefore, construct containing the target gene between a modified GFP, *(sGFP(S65T))*, gene as a reporter gene and the hygromycin phosphotransferase gene (*hpt*) as a selection marker was used to develop an efficient transformation protocol and avoid the co-suppression of the target gene.

Materials and Methods

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

Initiation and proliferation of embryogenic cultures. Transformation and regeneration systems for soybean were optimized according to methods described elsewhere (Finer and Nagasawa 1988; Sato *et al*., 1993, Hadi *et al*., 1996; El-Shemy *et al*., 2004). Soybean plants, cv. Jack, were grown in soil in a glass-house controlled at 25ºC 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, 40 mg/L 2,4-D, and 0.2% Gelrite (Wako Pure Chemical Industries, Osaka, Japan) (pH7.0). Embryogenic tissues were initiated at 25ºC 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).

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 20 mg/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) 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 15 mg/L hygromycin B (Roche Diagnostics). 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 30 mg/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).

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) 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) in the corresponding buffer. Reaction were hot-started (9 min at 94ºC) and subjected to 30 cycles as follows: 30 s at 94ºC; 1 min at 55ºC; and 1 min at 72ºC. The last extension phase was prolonged to 7 min at 72ºC. The primer set for *hpt* was designed for amplification of a 560 bp fragment; sequences are 5'-ATCCTTCGCAAGACCCTTCCT-3' (35S promoter) and 5'-GGTG TCGTCCATCACAGTTTG-3' (*hpt*). The primer set for *V3-1* was designed for amplification of a 1403 bp fragment; sequences are 5'-TTCAGTTCCAGAGAGCAGCAGCCT-3' and 5'-CTGATGCATC

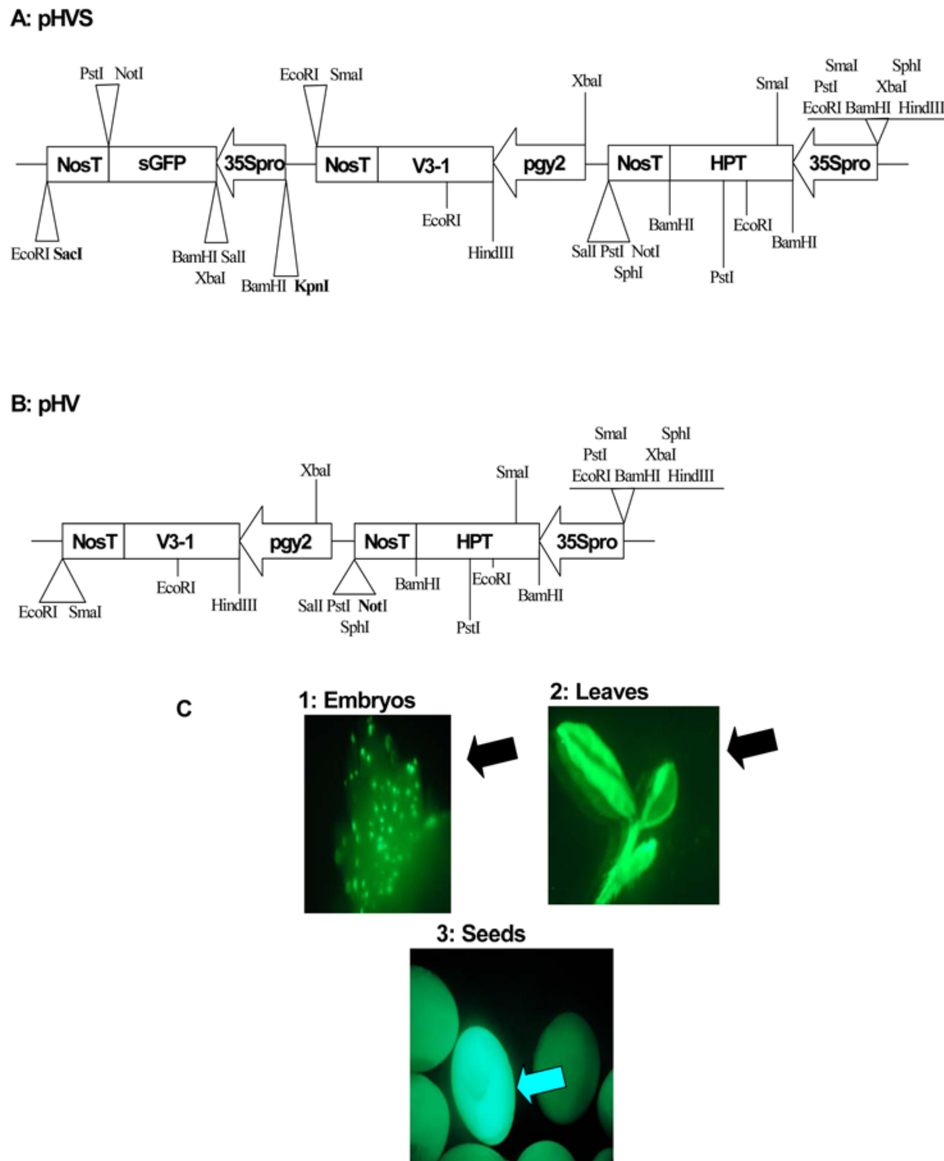

Fig. 1. Structures of plasmid constructs for soybean transformation. **A**, pHVS contains a modified glycinin gene, *V3-1*, between *hpt* as a selectable gene and *sGFP(S65T)* as a reporter gene. **B**, pHV contains *V3-1* flanked by *hpt* as a selectable gene. Restriction sites are indicated. **C**, Visualization of *sGFP(S65T)* expression in transformed soybean plantlets. Embryos (**1**), leaves (**2**) and Seeds (**3**).

ATCATCTGAGG-3'. That for *sGFP(S65T)* was designed for amplification of a 708 bp fragment; sequences are 5'-AAGGTACC GGATCCCCCCTCAGAA-3' and 5'-AAGAGCTCCGATCTAGTA ACATAGATGACACC -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). 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) 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 gel containing 12% (w/v) acrylamide and 0.2% (w/v) bis-acrylamide, and were stained with Bio-Safe CBB G-250 stain (Bio-Rad).

Table 1. Transformation and gene co-suppression efficiency in soybean by using two constructs

Construct	pHVS	pHV
Regenerated plants	115	95
hpt-positive by PCR	75	29
Transgenic plants[a]	42 (22[b]/20[c])	29
Absence of glycinin	22 (8[b]/14[c])	21
Glycinin expression level %	47.6	31
Ratio of 11S gene Co-suppression %	52.4 (36.4[b]/70[c])	72.4

[a]Transformats giving *hpt*, *sGFP*, and *V3-1* bands by PCR analysis
[b]Transgenic soybean expressing *sGFP*
[c]Transgenic soybean not expressing *sGFP*

Results

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. 1C).

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 115 regenerated plants obtained from the introduction of pHVS, 75 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 95 regenerated plants obtained from the introduction of pHV yielded the 0.5 kbp *hpt* fragment in PCR analysis (Table 1). PCR analysis for *V3-1* and/or *sGFP(S65T)* genes was conducted in the soybean plants that yielded a *hpt* band to confirm the presence of all transgene cassettes (Fig. 2). The expression of *sGFP(S65T)* was detected in about 52 % of the *hpt*-positive soybeans engineered using pHVS (Fig. 1C).

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. 2), was digested with *SacI*, and hybridized with one of the three probes for *hpt*, *V3-1* and *sGFP(S65T)* (Fig. 3). 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. 3A). 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. 3B). 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. All four transgenic soybeans with pHVS gave novel bands hybridized with the *sGFP(S65T)* probe, though the two plants in lanes 3 and 4 only exhibited GFP expression (Fig. 3C). The individual plants exhibited different banding patterns, confirming that they resulted from different events.

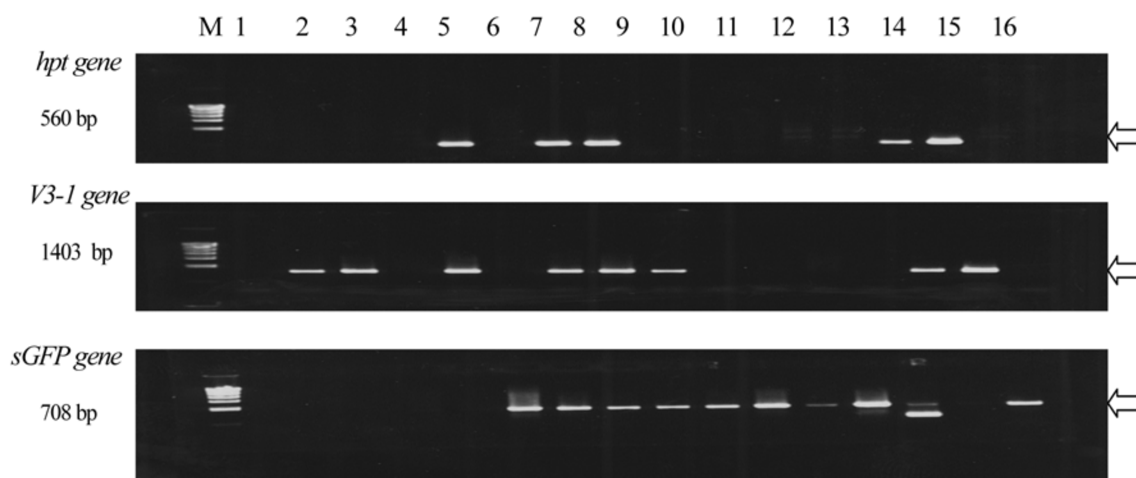

Fig. 2. Detection of foreign genes in transgenic soybean plants by PCR: *hpt* (A), *V3-1* (B) and *sGFP* (C) genes. M: molecular standards of l DNA digested with *HindIII*; Lane 1: non- transformed soybean plant; Lanes 2-16: transformed soybean plants. Arrowheads expected 560 bp, 1403 bp and 708 bp fragments of *hpt*, *V3-1* and *sGFP* genes, respectively.

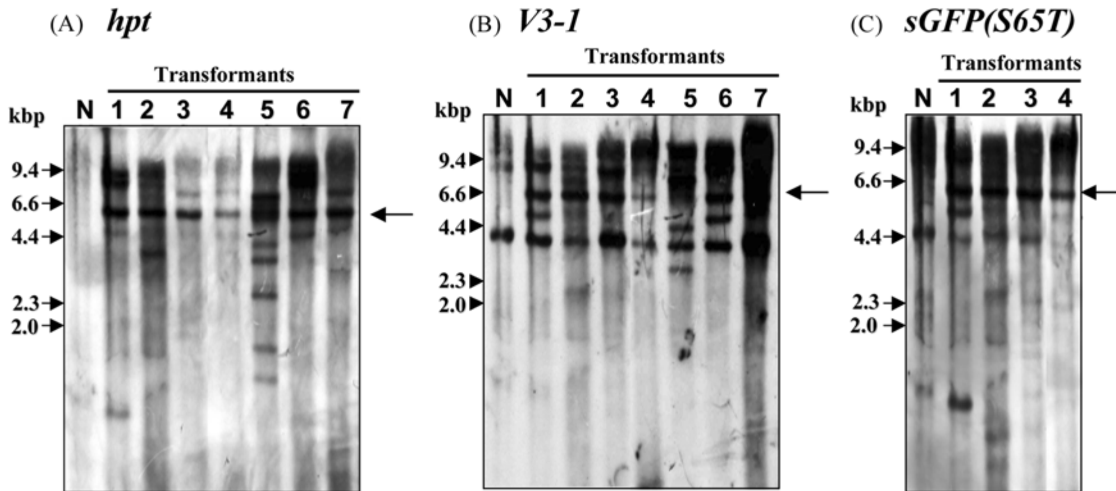

Fig. 3. Southern blot analysis of soybeans transformed with pHVS and pHV. Total DNA was digested with *Sac*I and loaded in lane N (untransformed control), 1-4 (transformed soybeans with pHVS), and 5-7 (transformed soybeans with pHV). Separated DNA was transferred to a nylon membrane and hybridized with each probe containing the coding region of *hpt* (A), *V3-1* (B), and *sGFP(S65T)* (C). Arrowheads expected gene bands.

The accumulation of glycinin was confirmed by SDS-PAGE analysis of the globulin fraction extracted from transgenic seeds (Fig. 4). 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 nontransformants (Fig. 4). 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 co-suppression (Table 1, Fig. 4). Western blot analysis with antibodies against glycinin confirmed the complete lack of glycinin in seeds of some transformants (data not shown). Out of a total of 29 transgenic plants obtained from the introduction of pHV, 21 plants lacked an accumulation of endogenous glycinin (Table 1). On the other hand, 8 out of 22 transgenic plants with GFP expression lacked glycinin. The ratio of gene co-suppression was lower in transformants engineered with the pHVS construct and selected based on GFP expression than transgenic soybean engineered with the pHV construct.

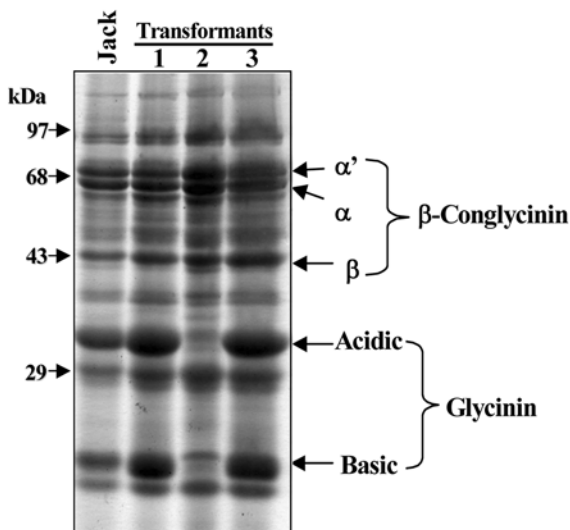

Fig. 4. SDS-PAGE analysis of components of seed storage proteins in transformed soybean. Globulin fractions were isolated from Jack (untransformed control) and transformants, and 25 μg of each fraction was fractionated by SDS-PAGE and then stained with CBB.

Discussion

Particle bombardment can be a very efficient method of transformation and is often independent of genotype. However, protocols to produce transgenic soybean are still laborious and genotype dependent. The β-glucuronidase (GUS) system is widely used to optimize transformation protocols. However, it is not suitable for following the time course of gene expression in living cells or as a means of rapidly screening for primary transformants, because the assay of GUS activity requires exogenous substrate for histochemical visualization, and generally involves destructive testing (Jefferson 1987). The green fluorescent protein (GFP) of jellyfish (*Aequorea victoria*) has significant advantages over other reporter genes such as GUS, because GFP expression can be detected in living cells without any substrates. GFP has grown in popularity as a reporter gene in biology and biotechnology research. Its characteristic fluorescence makes it useful for a variety of studies ranging from the regulation of chimeric gene cassettes (Barz *et al*., 2002; Wippersteg *et al*., 2002) to subcellular

localization of fusion proteins (Morin *et al*., 2001). The potential of GFP appears to be 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). This increased potential is especially pertinent to plants, since it is often desirable to quantify gene expression on the plant and in the field.

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. 1 C). 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). Silencing was first observed with regard to 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. More than 50% of the transgenic plants produced seeds lacking all subunits of glycinin (Table 1). Particle bombardment mediated transformation system is prone to result in the integration of multiple copies and rearrangement of transgenes. 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), 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. 3). It is suggested that the transgene causes the effects of gene co-suppression. There is some predictability to silencing. Counterintuitively, 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. 4).

In conclusion, two different constructs with or without the *sGFP(S65T)* gene for soybean transformation were used to consider the transformation efficiency and stable gene expression of the target gene. The selection based on hygromycin and the expression of *sGFP(S65T)* perfectly endorsed the integration of the target gene into the soybean genome, and resulted in a high frequency of the accumulation of the modified glycinin *V3-1* compared with the results for the construct without *sGFP(S65T)* (Table 1). More than 52% of transgenic plants engineered using pHVS showed an absence of glycinin, while only 36.4% of the transgenic plants lacked glycinin after the selection based on GFP expression. 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). Therefore, *sGFP(S65T)* can be effectively used to select the transformants expressing all the gene cassettes (El-Shemy *et al*., 2004). The transgenic plants expressing *sGFP(S65T)* grew and reproduced normally, and the GFP expression was inherited without any abnormalities. Therefore, using *sGFP(S65T)* as a reporter gene was reduced the gene co-suppression in transgenic soybean and future studies should be pursued to understand the regulate gene expression by *sGFP(S65T)*.

Acknowledgments The authors thank Yasuo Niwa, School of Food and Nutritional Science, University of Shizuoka, for providing the *sGFP(S65T)* gene. We are grateful to Yumi Nakamoto, Yumi Naganuma and Midori Yokoyama for technical assistance. Hany A. El-Shemy (Cairo Univ., Fac. of Agric., Egypt) was supported by a postdoctoral fellowship from the Japanese Science and Technology Corporation. Mutasim Khalafalla (NCR, Sudan) was supported by a postdoctoral fellowship from the Japan Society for the Promotion of Science.

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