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

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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

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-mediated transformation can be an efficient method for soybean transformation, and is often genotype-independent (Arango 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 (Vacheret 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; Stum 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 remodeling proteins to these sites (Meyer, 2000; Wang and Waterhouse, 2002). Co-suppression 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 fluorescent 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 (Ala81b) DNA, (13-I (1.4 kb)), with a synthetic DNA encoding four continuous methionines. pHVS contains DNA encoding four continuous methionines. pHVS contains the hygromycin phosphotransferase coding region, (sGFP(S65T) (0.8 kb)), under regulatory control of 35Spro in the flanking region of the 13-I 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; Hadfi et al., 1996; El-Shemy et al., 2004). Soybean plants, cv. Jack, were grown in soil in a glass-house controlled at 25°C. Soybean embryogenic tissues were initiated at 25°C under cool white fluorescent light (23/1 light phase was prolonged to 7 min at 72°C; 1 min at 55°C; 1 min at 94°C) and subjected to 30 cycles as follows: 1 min at 55°C; 1 min at 94°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'-ATCTCCTCGACAAGCCCTTC3-3' (35S promoter) and 5'-GGTTCGTCGATCCAGTGGTT-3' (hpt). The primer set for 13-I was designed for amplification of a 1403 bp fragment; sequences are 5'-TTCAAGACGAGACACGCC-3' and 5'-CTGATGCATC.
A TCA TCTGAGG-3'. That for sGFP(S65T) was designed for amplification of a 708 bp fragment; sequences are 5'-AAGG T ACC GGA TCCCCCCTCAGAA-3' and 5'-AAGAGCTCCGA TCT AG T A ACA T AGA TGACACC -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, SacI, 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-mercaptoethanol. 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).
Results

Embryogenic cells induced from a soybean cultivar, Jack, were transformed by micro-particle 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 FNLS3S3 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-I 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 ascertain 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-I 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-I 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-I 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 may be 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.

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

<table>
<thead>
<tr>
<th>Construct</th>
<th>pHVS</th>
<th>pHV</th>
</tr>
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<tbody>
<tr>
<td>Regenerated plants</td>
<td>115</td>
<td>95</td>
</tr>
<tr>
<td>hpt-positive by PCR</td>
<td>75</td>
<td>29</td>
</tr>
<tr>
<td>Transgenic plants</td>
<td>42 (22%/20%)</td>
<td>29</td>
</tr>
<tr>
<td>Absence of glycinin</td>
<td>22 (8%/14%)</td>
<td>21</td>
</tr>
<tr>
<td>Glycinin expression level %</td>
<td>47.6</td>
<td>31</td>
</tr>
<tr>
<td>Ratio of 11S gene</td>
<td>52.4 (36.4%/70%)</td>
<td>72.4</td>
</tr>
</tbody>
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a Transforms giving hpt, sGFP, and V3-I bands by PCR analysis
b Transgenic soybean expressing sGFP
c Transgenic soybean not expressing sGFP

Fig. 2. Detection of foreign genes in transgenic soybean plants by PCR: hpt (A), V3-I (B) and sGFP (C) genes. M: molecular standards of 1 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-I and sGFP genes, respectively.
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.

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; Wippersieg et al., 2002) to subcellular...
modified glycinin soybean was frequently observed on the introduction of the 1990). Suppression of endogenous glycinin in the transformed plants produced seeds lacking all subunits of glycinin (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 phenomenon, 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).

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