Communication

Transient Gene Expression in Protoplasts of Phaseolus vulgaris Isolated from a Cell Suspension Culture

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ABSTRACT

A cell suspension culture of Phaseolus vulgaris cv Negro jamapa was established. Protoplasts isolated from this culture are suitable for transient gene expression studies after DNA transfer by either electroporation or treatment with polyethylene glycol. The optimized conditions for both methods were established, taking into consideration expression and cell viability. We have investigated two features of posttranscriptional gene regulation. We demonstrate that inclusion of the RNA leader sequence, omega (Ω), derived from tobacco mosaic virus, and a dicot intron in the leader region together enhance gene expression in this legume species about 10-fold.

Several methods have been developed to transfer DNA into plant protoplasts. In the legume family, both electroporation and PEG gene transfer (18) have been successfully used for transient expression and to stably transform protoplasts from soybean (3) and moth bean (14). In the case of Phaseolus vulgaris, no stable transformation and regeneration method is available, and the level of transient gene expression reported by Dron et al. (8) is very low. In this paper we report the establishment of a rapidly growing suspension culture of P. vulgaris as well as the conditions required for a high level of transient gene expression using constructs containing the CaMV35S promoter.

Because one of our objectives is to obtain a high level of expression in beans, we have tested the effect of including sequences reported to stimulate gene expression in other species. For instance, introns have been shown to increase the levels of gene expression in both mammalian cell cultures (1) and in plant cells (2). Several reports have corroborated this finding using different introns from monocot genes; however, there are few reports on this 'intron enhancement' phenomenon in dicot species. Dean et al. (6) analyzed two differentially expressed rbcS genes of petunia in transgenic tobacco plants. They demonstrated that removal of all three intron sequences from the highly expressed gene resulted in a fivefold decrease in steady state mRNA levels. The intron enhancement effect appears to be mediated by posttranscriptional steps rather than the presence of a transcriptional enhancer within the intron sequences, because all introns tested provide some enhancement (2).

Gallie et al. (9) have shown that the 67 bp leader sequence, Ω, derived from TMV, stimulates gene expression in animals, plants and bacteria. The proposed mechanism is that Ω promotes preferential translation. Similarly, the leader sequence of brome mosaic virus (12) acts as a translational enhancer. We have tested the impact of introns and the Ω leader on gene expression. We show that even though the use of a monocot intron did not increase expression, inclusion of a dicot intron and the Ω leader sequence enhanced expression up to 10-fold in P. vulgaris.

MATERIALS AND METHODS

Protoplast Isolation

Protoplasts from Phaseolus vulgaris cv Negro jamapa were isolated 3 d after subculture from cells harvested by centrifugation and resuspended in an equal volume of an enzyme solution prepared by dissolving 1% (w/v) cellulase (CELF, Worthington Biochemical), 0.2% (w/v) pectinase (Pectolyase Y23, Seishin Pharmaceutical Co.), 0.5% (w/v) BSA (Sigma), and 0.05% (v/v) β-mercaptoethanol in a buffer containing 50 mm CaCl2, 12 mm sodium acetate, and 450 mm mannitol (isolation buffer). After 60 to 90 min of digestion on a rotary shaker (60 rpm), the enzyme-protoplast mixture was filtered successively through a tea strainer, a 200 μm mesh screen, and a 60 μm mesh screen; the protoplasts were pelleted by centrifugation at about 500 rpm for 5 min and washed once with the isolation buffer.

DNA Transfer

The isolated protoplasts were resuspended in electroporation buffer (10 mm Hepes, 130 mm potassium chloride, 10 mm sodium chloride, 4 mm calcium chloride, and 0.2 mm mannitol [pH 7.2]) at a concentration of about 1 × 106 protoplasts per mL. Electroporation (X-Cell 450 Electroporation System from Promega Biotech) was performed by mixing...
0.8 mL of the protoplast suspension, 20 μg of plasmid DNA, and 50 to 100 μg denatured salmon sperm DNA as a carrier. The electrical pulse was delivered from a 1550 μF capacitor charged to the desired voltage for various times as specified for each experiment; the gap between the electrodes was 0.4 cm. Following electroporation, the protoplasts were kept at 4°C for 10 min and then diluted at room temperature with 4 mL of protoplast growth medium containing SH salts (7), 0.5 M mannitol, and 20% conditioned medium. To transform bean protoplasts with polyethylene glycol, the method of Shillito et al. (18) was used with PEG 4000 (Sigma) in a buffer consisting of 0.1 M Ca(NO₃)₂ and 0.4 M mannitol. After incubation at room temperature for 5 min (unless indicated), the protoplasts were diluted carefully with the protoplast growth medium, spun down at 500×g for 5 min, and resuspended in 4 mL of the protoplast growth medium. After transformation the protoplasts were incubated in the dark at 25°C for 24 h prior to analysis of reporter gene expression.

**Assays**

**Viability**

Viability was tested using two methods: methylene blue staining (40 mg/mL in 0.5 M mannitol) and fluorescein diacetate/propidium iodide staining. All counting and photographic procedures were completed within 10 min of staining.

**LUC**

The protoplasts were collected by centrifugation (500×g for 5 min) and resuspended in 400 μL extraction buffer containing 100 mM potassium phosphate (pH 7.8), 1 mM EDTA, and 7 mM β-mercaptoethanol. The protoplasts were disrupted by sonication. Of the extract, 5 to 10 μL were mixed with 200 μL of assay buffer (25 mM Tricine [pH 7.8], 15 mM MgCl₂, 5 mM ATP, 7 mM β-mercaptoethanol, and 0.25 mg/mL BSA). The photons emitted in 10 s were counted in a luminometer (Analytical Luminescence Laboratories, model Monolight 2001). The reaction was started by injecting 100 μL of 0.25 mM luciferin (Analytical Luminescence Laboratories) solution.

**GUS**

The fluorometric assay described by Jefferson (11) was used to determine GUS activity. Fluorescence was measured by excitation at 365 nm and emission at 455 nm in a TKO 100 DNA minifuorometer (Hoefer Scientific Instruments).

**Protein Assay**

Protein content was determined using a commercial kit (Bio-Rad Protein Assay).

**RESULTS AND DISCUSSION**

*P. vulgaris* cv Negro jamapa was grown in the greenhouse. Immature cotyledons were harvested 12 to 15 d postanthesis and used to initiate the cultures. Two media were tested for their ability to induce and maintain callus cultures: a Gamborg’s B5 medium (10) supplemented with 2 mg/L of 2,4-D and an SH medium as modified by Dixon and Fuller (7) supplemented with 2 mg/L pCA, 0.4 mg/L 2,4-D, and 1 mg/L kinetin. Callus formation was observed within 1 week of placing immature cotyledons on SH medium in approximately 55% of the cultured cotyledons. When the immature cotyledons were placed on B5, callus formation occurred at a very low frequency (1–2% of the cultured cotyledons), and the calli formed degenerated after 2 to 4 weeks. After 6 to 8 weeks, calli maintained on SH medium developed into a more friable callus consisting of two cell types: (a) very elongated and (b) small, densely cytoplasmic cells.

Pieces of the most friable calli (1–2 g) were transferred to SH medium in order to establish a cell suspension culture. Initially, the suspension contained the same mixed population of cells. To select for a more homogeneous population consisting mainly of small cells, the suspension was filtered through a 500 μm mesh screen when subcultured. After several subcultures, the cell suspension was more homogeneous for the size of the cell clusters, in which about 65% of the cells were small and densely cytoplasmic. The suspension was then subcultured every 5 d with a 1:3 (inoculum:fresh SH medium) dilution. This suspension culture was used for protoplast isolation.

The protoplast isolation and purification procedures described in the “Materials and Methods” yield clean populations of uninucleated protoplasts; these protoplasts have a dense cytoplasm, vary in size from 40 to 60 μm, and are viable based on differential staining. Three concentrations of mannitol (0.3, 0.45, and 0.6 M) were tested to optimize the osmolarity of isolation buffer. Both yield and viability were optimal when the osmolarity of the isolation buffer was adjusted with 0.45 M mannitol (data not shown). Because both protoplast isolation efficiency and the level of transient gene expression are affected by the culture growth phase, for all experiments the protoplasts were isolated while in exponential growth (3 d after subculture). This time was chosen because protoplast yield was maximal compared to older growth phases where fewer cells became digested.

Optimal transformation conditions with constructs containing the CaMV 35S promoter coupled to the LUC or GUS genes (Fig. 1), were established for both PEG- and electroporation-mediated transformation. In the case of electroporation, two parameters, field intensity and pulse length, were optimized. To optimize field intensity, the input voltage was varied keeping the pulse length constant at 12 ms (Fig. 2). This pulse length was chosen because it has been shown to be optimal for maize protoplasts isolated from maize or carrot suspension cultures (J De Wet, V Walbot, manuscript submitted for publication). The LUC and GUS activities were maximal between 450 V/cm and 525 V/cm (Fig. 2, A and B). At higher voltages, both activities decreased because fewer protoplasts remained alive. A second parameter that can alter electroporation efficiency and transient expression is the discharge time applied to the protoplasts (pulse length). As shown in Figure 2, C and D, both reporter genes activities are maximal when the electric pulse is applied for 50 ms; longer pulses result in less activity. Protoplast viability was also monitored during this experiment (Fig. 2). The optimized pulse length for both expression and viability is between 12 and 20 ms (Fig. 2, C and D).

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Figure 1. Plasmids used in this study. All the plasmids listed were constructed in the pUC18 cloning vector. The features of the plasmids are shown in boxes as follows: 3S corresponds to the −363 to +1 CaMV 3S promoter fragment; LUC is the gene coding for the firefly luciferase (5); nos 3' is the polyadenylation signal of the nopaline synthetase gene; the black box is the TMV Ω sequence (9); Adhl intron 1 is from the maize Alcohol dehydrogenase-1 gene (2); intron is IVS2 from the ST-LS gene (19); GUS is the gene encoding β-glucuronidase (11). Major restriction sites are shown in pJD300; only new restriction sites are shown in the other plasmids. The restriction sites are abbreviated as BamHI, B; HindIII, H3; KpnI, K; Ncol, N; PstI, P; SalI, S; Sall, Sa; SnaBI, Sn.

Figure 2. Voltage and pulse length optimization. In A (GUS) and B (LUC), specific activity and the percent cell viability is plotted relative to voltage during a 12 ms electroporation. For pulse length optimization, a 450 V/cm field was used. In C (GUS) and D (LUC), the percent viability is plotted relative to a variable pulse given in ms. In A and C, the GUS plasmid was pCaG and in B and D, the LUC plasmid was pJD301; the data are the average of four different experiments. A and C, GUS specific activity given in nmol/min/μg of protein (□•◇); the endogenous GUS activity of the protoplasts was negligible. B and D, Luciferase specific activity is given in 10⁶ photons/10 s/μg protein (□•◇); the control background of approximately 50 photons was subtracted from each assayed value. The percent viability (♦♦♦) represents the percentage of living protoplasts compared to the control sample before electroporation.
Table I. Transient Expression of P. vulgaris Obtained Using Two Transformation Methods

<table>
<thead>
<tr>
<th>Transformation Method</th>
<th>Plasmid</th>
<th>LUC/GUS* in Experiment No.</th>
<th>Increment Relative to pJD300</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Electroporation</td>
<td>pJD300</td>
<td>$1.5 \times 10^4$</td>
<td>$2.2 \times 10^4$</td>
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<tr>
<td>PEG</td>
<td>pJD300</td>
<td>$2.7 \times 10^4$</td>
<td>$4.3 \times 10^4$</td>
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</table>

* Expression is given as LUC specific activity corrected by GUS specific activity. LUC specific activity is the photons per 10 s per µg of protein; GUS specific activity is nanomoles of MU per min per µg of protein. Fold increase is an average of the difference in expression found between the two methods.

The PEG transformation was performed according to Shil-\textit{lito et al.} (18) with some modifications (see "Materials and Methods"). Both transient expression and viability were measured during different incubation times of the protoplast-DNA mixture in the PEG solution. An incubation time of 5 min was the best compromise between expression and viability (data not shown). A comparison was made of the two transformation methods, carried out with the same protoplasts and plasmid DNA preparation and using optimal conditions for each. In our hands, the optimized PEG transformation could give a higher level of transient gene expression (Table I), but was inherently more variable, therefore, subsequent tests were done with electroporation.

In our attempt to increase transient gene expression in beans, we examined the effects of including sequences affecting posttranscriptional steps in gene expression. The 5'-untranslated leader of TMV as well as monocot and dicot introns were inserted into the untranslated leader of the LUC gene. The analysis was done using a basic plasmid construct, pJD300, that contains the LUC gene coupled to the nos 3'polyadenylation signal from the Ti plasmid of \textit{Agrobacterium tumefaciens} (Fig. 1). The reporter gene is expressed from the CaMV 35S promoter. The CaMV fragment used in this experiment extends from –363 to +1 relative to the transcription initiation site. The level of transient gene expression was determined using the optimal electroporation conditions outlined above. Supercoiled DNA was introduced into duplicate protoplast samples in each experiment and assayed for LUC activity after 18 to 24 h of incubation at 25°C (see "Materials and Methods"). Relative expression levels in transient assays show substantial variability between separate experiments because of differences between cell preparations (20); in bean protoplasts this variability can be up to 25-fold. Because of this variability in protoplast response, an internal control was introduced by coelectroporating a second plasmid carrying the GUS reporter gene. The internal control used was the pCaG plasmid (Fig. 1) that contains the ß-glucuronidase gene driven by the CaMV 35S promoter. Table II shows that, when the LUC expression levels of the pJD300 plasmid from four independent experiments were normalized with the GUS activity, variability between experiments was reduced to about 2.5-fold.

Inclusion of the TMV ß sequence (pJD301), which has been reported to enhance translation (9), increased expression in beans about fourfold compared with pJD300 (Table II). This enhancement is comparable to that reported for tobacco and agrees with the hypothesis that the TMV ß sequence functions in all dicots.

Enhancement of transient gene expression by the inclusion of various introns in the transcriptional unit has been reported for several monocots (2). Specifically, the maize \textit{Adh1} intron 1 enhances expression when this sequence is placed in the untranslated leader of a reporter gene (2). We were interested to find out if this phenomenon also occurs in bean protoplasts. We used plasmid pJD313 for this purpose; this plasmid contains the TMV ß sequence and the maize \textit{Adh1} intron 1 inserted upstream of the LUC gene (Fig. 1). The level of LUC transient gene expression was determined in two different experiments. As shown in Table II, the presence of this monocot intron provides no enhancement; rather, LUC expression in bean protoplasts is reduced compared to pJD301, a similar plasmid that lacks the intron sequence. We hypothesize that the maize intron is inefficiently spliced in beans and other dicots, hence intron enhancement would be prevented. In support of this idea, \textit{Keith and Chua} (13), found that intron 6 of the maize \textit{Adh1} was inefficiently spliced in transgenic tobacco plants. Similarly, \textit{Adh1} intron 1 is inefficiently spliced in tobacco and carrot protoplasts (J De Wet, V Walbot, personal communication).

In dicots, inclusion of intron sequences in expression constructs has yielded contradictory results. For example, \textit{Dean et al.} (6) reported that constructs containing the petunia \textit{rbcS} gene with three introns were expressed fivefold higher than the cDNA versions in transgenic tobacco plants. In this case, however, the enhancement effect was not associated with a particular intron. In contrast, \textit{Kuhlmeier et al.} (16) found similar expression levels from the genomic and a cDNA version of a pea \textit{rbcS} gene in transgenic tobacco plants. To test whether a dicot intron could enhance LUC expression, we obtained the second intron (IVS2) of the ST-LS1 gene

Table II. Effect of TMV ß and Intron Sequences in the Level of LUC Transient Gene Expression

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>LUC/GUS* in Experiment No.</th>
<th>Increment Relative to pJD300</th>
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<td>pJD301</td>
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<td>$7.5 \times 10^4$</td>
</tr>
<tr>
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<td>$2.5 \times 10^4$</td>
</tr>
<tr>
<td>pJD1</td>
<td>$7.8 \times 10^4$</td>
<td></td>
</tr>
<tr>
<td>pJD2</td>
<td>$1.0 \times 10^4$</td>
<td>$1.3 \times 10^4$</td>
</tr>
</tbody>
</table>

* LUC specific activity is given in photons per 10 s per µg of protein; GUS specific activity is the nanomoles of MU per min per µg of protein.
from Solanum tuberosum to test if it could enhance the expression level of the LUC gene. The IVS2 represents a typical plant intron in being small, in having a high A+T content and in having conserved splice junctions (19). This intron was selected because it has been extensively characterized and because it has been modified by introducing useful restriction sites at both the 5' and 3' splice junctions (19). We made gene constructs in which the IVS2 and 38 bases of 5'-flanking chloramphenicol acetyltransferase sequences were inserted into the SaI site upstream of the LUC gene in pJD301 to create plasmid pID1 (Fig. 1). After splicing, pID1 should yield an mRNA with 118 bases of untranslated leader. In plasmid pID2, 25 bases of the chloramphenicol acetyltransferase flanking sequences were removed leaving a 93 base untranslated leader.

These intron-containing constructs were transformed into bean protoplasts and LUC expression was analyzed (Table II). Both plasmids stimulated LUC expression 1.5- to 2.5-fold compared to similar constructs without IVS2 (pJD301 versus pID1 and pID2). This demonstrates that inclusion of the IVS2 increases transient gene expression in dicot cells. We did find a difference between expression levels of pID1 and pID2. These plasmids are identical except in the untranslated leader region: pID1 has 25 extra bases of CAT + polylinker sequence. These extra bases in pID1 reduced the intron-enhancing effect. Our result likely reflects a difference in the splicing efficiency of the two transcripts, because the construction of pID2 changed conserved sequences at the 5' exon splice boundary (17). Kozak (15) has shown that stem-loop regions in the leader can reduce expression; however, no stable secondary structure is expected in either the pID1 or pID2 leaders.

Successful application of standard genetic engineering procedures to P. vulgaris has been limited by the lack of an efficient transformation method and the inability to reproducibly regenerate plants. Although regeneration of transformed plants is required for studies on tissue-specific gene expression or for the introduction of new traits for agricultural benefit, the possibility of introducing DNA into bean protoplasts to measure transient gene expression allows analysis of factors affecting expression in a homologous system. Several P. vulgaris tissue culture lines have been established (4, 7); however, none has been successfully used for transformation. In the present work, we report the establishment of a suspension culture of P. vulgaris. Protoplasts from this culture are capable of a high level of transient gene expression, equivalent to other dicots such as tobacco and carrot. This culture provides a constant source of protoplasts compared to isolation methods using plant tissues. We have generated a new vector that gives high transient gene expression in beans. We propose that this vector, pID2, will be helpful for the study of the expression of homologous genes in P. vulgaris, especially those with low promoter expression. In the future, we plan to analyze the transcripts from this vector and, using additional introns, to study the splicing efficiency and splicing requirements in bean cells.

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LITERATURE CITED

13. Keith B, Chua N-H (1986) Monocot and dicot pre-mRNAs are processed with different efficiencies in transgenic tobacco. EMBO J 5: 2419-2425