AGROBACTERIUM – MEDIATED GENETIC TRANSFORMATION OF THE RESURRECTION PLANT HABERLEA RHODOPENSIS FRIV.

G. PETROVA* and D. DJILIANOV
AgroBioInstitute, BG – 1164 Sofia, Bulgaria

Abstract


Resurrection plants are widely used models for desiccation tolerance studies. Several genes have been successfully isolated from these species. In an attempt to study their role, these genes have been successfully transferred in other model plants. Since resurrection plants are as a rule, polyploids, they are pure targets for mutational studies. In this respect, the establishment of efficient and repeatable transformation system will contribute significantly for the elucidation of stress tolerance. In this study we describe for the first time, a procedure for Agrobacterium tumefaciens – mediated genetic transformation of the resurrection plant Haberlea rhodopensis Friv. For in vitro regeneration of Haberlea, we used liquid WPM media. It enables us to achieve direct regeneration and transformation system, which is an alternative to the callus-based transformation, used in other resurrection plants. The A. tumefaciens strain LBA4404 harbouring the plasmid pCAMBIA 1305.1 which contains the gus gene as a reporter gene and hpt II gene as a selectable marker gene was used. The initial experiments were conducted in order to establish the suitable concentration of cefotaxime for the elimination of Agrobacterium from cultures, as well as the optimal concentration of hygromycin for the selection of transformed plants. It was found that the highest concentration of cefotaxime that protocorms of H. rhodopensis could tolerate is 500 mg.l⁻¹ and they are inhibited at 0.75 mg.l⁻¹ hygromycin. Transformation was confirmed by histochemical GUS assay and 35S- / NOS- PCR analysis. The percentage of GUS activity was 3% and the optimal co-cultivation time was 60 minutes.

Key words: Agrobacterium, Haberlea rhodopensis, regeneration, transformation

Introduction

The Gesneriacae are relatively large family comprising over 3200 species in 150-160 genera (Weber, 2004; Weber and Skog, 2007), widespread predominantly in tropical and subtropical regions of Eastern Asia, Indonesia, and South and Central America. There are a few outliers in Europe, with five species in three genera: Ramonda myconi (L) Rchb., R. nathaliae Pančić & Petrović, R. serbica Pančić, Haberlea rhodopensis Friv., and Jancaea heldreichii Boiss (Thompson, 2005).

Haberlea rhodopensis is an endemic resurrection plant of the Balkan Peninsula, and occurs in Central (Balkan Mts.) and Southern (Rhodope Mts.) Bulgaria, as well as in Greece (north-eastern Pangeon Mts. and Falakron Mts.) (Strid, 1991).

The climatic changes and global warming drastically reduce plant productivity (Boyer, 1982). To overcome these challenges stress tolerant crops should be developed and cultivated (Khush, 1999). In this respect, genetic transformation of plants is considered among the most appropriate approaches used to confirm the putative involvement of genes of interest in plant stress tolerance (Djilianov et al., 2009).

Due to their extreme desiccation tolerance, the so-called resurrection plants are widely used as model for molecular, physiological and metabolic studies with the final goal to elucidate the mechanisms of tolerance and to try to transfer these important traits to crop plants (Djilianov et al., 2011; Dinakar et al., 2012; Georgieva et al., 2012).

So far, within the group of resurrection plants, successful protocols for genetic transformation have been established.
for only three species: Ramonda myconi, Craterostigma plantagineum and Lindernia brevidens (Furini et al., 1994; Toldi et al., 2002; Toth et al., 2006; Smith-Espinoza et al., 2007). Most of them are based on callus induction and regeneration of putative transformants.

In this study, we propose an alternative to callus-based transformation, comprising direct regeneration in liquid media and subsequent Agrobacterium – mediated transformation of Haberlea rhodopensis, without a callus stage.

Materials and Methods

In vitro cultivation

In vitro cultures of Haberlea were initiated according to Petrova et al. (2010). Fresh young leaves of Haberlea were used as explant sources in order to initiate in vitro culture. Surface sterilization with 70% ethanol was performed for 30 sec, followed by treatment with 0.75% HgCl₂ for 6 min. The explants were washed three times with sterile dH₂O for removing the residual of HgCl₂ and then were germinated on basic WPM (Woody Plant Medium, Lloyd and McCown, 1980). Plantlets were grown at 25°C under photoperiod of 16 h light (approximately 4500 lx)/8 h dark regime. The plants were subcultured every 4 weeks.

Bacterial strain and plasmid vector

The A. tumefaciens strain LBA 4404, which harbours the plasmid pCAMBIA 1305.1 was used for establishment of the transformation protocol. The plasmid pCAMBIA contains β-glucoronidase (GUS) and hygromycin resistance (hpt II) genes. Both genes were expressed under the control of CaMV 35S promoter.

Effect of antibiotic concentration on plant growth

For determining the effect of antibiotic concentration on growth of H. rhodopensis, hygromycin and cefotaxime were added to the sterilized regeneration medium at different concentrations (0, 0.75, 1.0, 1.5, 2.0, 2.5, 5.0 mg.l⁻¹ hygromycin and 100, 200, 300, 400, 500 mg.l⁻¹ cefotaxime). Plants were cultured at 25°C under photoperiod of 16 h light. After four weeks of culturing, the effectiveness of antibiotics was evaluated.

Effect of cefotaxime on growth of A. tumefaciens LBA 4404 (pCAMBIA 1305.1)

A. tumefaciens strain LBA4404 (pCAMBIA 1305.1) was grown in YEB liquid medium supplemented with kanamycin (50 mg.l⁻¹) and cefotaxime with different concentrations (50, 100, 150, 200, 250, 300, 350 mg.l⁻¹). Bacterial cultures were grown on a shaker (200 rpm) at 28°C for 24 h. The absorbance of bacterial suspension was measured at 550 nm.

Agrobacterium mediated in vitro transformation

50 ml of liquid YEB medium (5 g beef extract, 1 g of yeast extract, 5 g bactopeptone, and 5 g of sucrose per liter, pH 7.2) supplemented with 50 mg.l⁻¹ kanamycin and 100 mg.l⁻¹ rifampicin was poured into 250 ml Erlenmeyer flasks to propagate Agrobacterium – mediated transformation. Bacterial cultures were kept in dark regime at 28°C overnight on a shaker at 200 rpm. Healthy-white and dense bacterial cultures were pelleted by centrifugation (10 min, 8000 rpm). These cultures were then re-suspended in 40 ml liquid WPM medium supplemented with 50 μM acetylsyringone. The explants were prepared with a scalpel while submerged in the leaf suspension, which was placed into Petri dishes (10 cm in diameter, each) containing 10 ml liquid WPM medium. Subsequently, 1 ml suspension of Agrobacterium was added into each Petri dish. The infection was carried out during a 60 min gentle shaking in dim light at 22°C. The proliferation medium also contains mixture of antioxidants (0.15 mg.l⁻¹ ascorbic acid and 0.1 mg.l⁻¹ citric acid). After co-cultivation, the infected leaf suspensions were subcultured for 2 weeks on liquid WPM medium by addition of the antioxidant mixture, cefotaxime (500 mg.l⁻¹) and hygromycin (0.75 mg.l⁻¹). Further, in order to obtain shoot clusters, five rounds of subcultivation on identical medium were performed. At each subcultivation cycle, the concentration of cefotaxime was reduced by 100 mg.l⁻¹. The plants, rooted in liquid WPM medium were transplanted to maturity according to the regime described for in vitro cultured plants.

β-glucoronidase (GUS) activity assay

The histochemical assay for GUS gene expression was performed according to the method described by Jefferson (1987). Plants were immersed in X-gluc (5-bromo-4-chloro-3-indol glucuronide) solution and then were incubated overnight at 37°C. After staining, the plant material was treated with 70% ethanol to remove chlorophyll before the observation step.

PCR analysis

Total DNA was extracted from the in vitro-grown plants and the putative transgenic plants according to the procedure described by Dellaporta et al. (1983). The amplification was performed on a GeneAmp® PCR System (Applied Biosystems, Foster City, CA, USA). The final volume of each PCR mixture was 25 μl: 1 x PCR buffer (Fermentas, Vilnius, Lithuania), 25 ng DNA, 1 μM of each primer, 100 μM of each dNTP and 1U DNA polymerase (Fermentas, Vilnius, Lithuania).

The primer sequences for PCR-reactions were as follows: 35S: (F): 5’-GCTCCTACAATGCCCATCA-3’;

(R): 5’-GATAGTGGGATTGTCGTA-3’
NOS (F): 5’-GAATCCTGTTGCCGGTCTTG-3’;
(R): 5’-TTATCCTAGTTTGCGCGCTA-3’

PCR reactions were started with a denaturation step at 94°C, for 4 min, followed by 35 cycles with the following parameters: 94°C for 1 min, 55°C for 1 min, and 72°C for 3 min. The program was terminated by extension at 72°C for 7 min. All amplification products were checked on 1.0% agarose gels.

Statistical analysis of transformation frequency

Student’s t-tests were performed using MS Excel 2000 (Microsoft Corporation, Seattle, USA). Differences between results are described as being significant where \( P \leq 0.05 \), and not significant where \( P > 0.05 \).

Results and Discussion

It is well known, that during the establishment of systems for regeneration and genetic transformation, all resurrection plants possessed an extreme sensitivity due to the various types of physiological stress (Furini et al., 1994; Toldi et al., 2002; Toth et al., 2006; Smith-Espinoza et al., 2007).

Under suboptimal conditions, \( H. \) rhodopensis secretes polyphenols into culture media, which is associated with stress and tissue necrosis. These negative effects are paralleled with moderate reaction to positive effects, which is difficult to be explained, but this reaction probably could be related to the slow growth of plant in nature (Djilianov et al., 2005; Toldi et al., 2010).

\( H. \) rhodopensis does not require high concentrations of nutrients during the tissue cultivation. The high salt containing rich mediums are toxic for \( Haberlea \) explants cultured in vitro (Djilianov et al., 2005).

Previously, our group developed an effective method for in vitro micropropagation and regeneration of \( H. \) rhodopensis, which could serve as a basis for the establishment of its \( Agrobacterium \)-mediated genetic transformation (Djilianov et al., 2005).

The initial step in establishment of new procedures for \( A. \) tumefaciens mediated genetic transformation in plants is to test the effects of antibiotics used either as suppressers of the \( Agrobacterium \) overgrowth, or as selective agents on the in vitro morphogenesis of the transformed plant.

Effect of antibiotics on the plant and bacterial growth

The lowest dose of hygromycin, which inhibited protocorms growth, was 0.75 mg.l\(^{-1}\) (Figure 1). All of protocorms turned brown color after transfer to selective medium. The highest dose of cefotaxime that yielded surviving protocorm was 500 mg.l\(^{-1}\) (Figure 2). The growth of \( A. \) tumefaciens was inhibited at 50 mg.l\(^{-1}\) cefotaxime (\( OD_{550} = 0.030 \)), (Figure 3).

\[ \text{Fig. 1. Effect of hygromycin on growth of} \ H. \ rhodopensis \]

\[ \text{Fig. 2. Effect of cefotaxime on growth of} \ H. \ rhodopensis \]

\[ \text{Fig. 3. Effect of cefotaxime on growth of} \ A. \ tumefaciens \]

LBA 4404 (pCAMBA 1305.1)
Impact of physical and biochemical treatment on the establishment of transformation

According to Tóth et al. (2006), the impacts of physical (microwounding) and biochemical treatments (WPM medium, liquid medium supplemented with acetosyringone) have a primary importance for the successful Agrobacterium mediated transformation of R. myconi. They observed that more microwounding is necessary for the enhancement of the bacterial penetration.

Our results showed that the microwounding also has a key importance and it is essential for the successful genetic transformation of H. rhodopensis (Figure 4A). There was no transformation when leaves were gently punched with a sharp scalpel tip (four to five holes per leaf), as well as when Haberlea explants were prepared by conventional way (excising 5–7 mm × 5–7 mm leaf segments by cutting off the edges of leaf blades). We found that transgenic plants could only be recovered when applying a leaf suspension of Haberlea supplemented with acetosyringone as a target for transformation.

Non-lethal selection strategy

Most of the antibiotics, which are used as selective agents, and inhibitors of bacterial growth could depress plant regeneration (Oreifig et al., 2004). The survival rates of plants should be as high as possible in the presence of antibiotics and on the other hand, the optimal concentration should suppress, but not inhibit morphogenesis on transformed plants.

The fact that in our case the transgenic plants remained green at 0.75 mg.l⁻¹ hygromycin and showed morphologically normal phenotype, while non-transgenic regenerants have showed retarded growth, is an evidence for the sublethal concentrations of the applied selective agent (Figure 4B).

Selection of transgenic plants

The results from the β-glucuronidase activity assay, as well as the DNA integration of transformed plants (proved by PCR) confirmed the successful transformation (Table 1). Blue staining was observed after 30–40 days of co-cultivation. The highest number of blue spots was observed from plants co-cultivated in Agrobacterium for 60 min (Figure 5). All PCR positive amplification products were of the expected size of 195 bp for 35S promoter (Figure 6A) and 180 bp for NOS terminator (Figure 6B). The non-transformed control plants did not show any of the expected band sizes.

Conclusion

Based on the obtained results, it might be concluded that our attempts to establish the regeneration system in liquid medium without callus resulted in a hopeful method for genetic transformation of model resurrection plant H. rhodopensis. The low percent of transgenic plants (Table 1) may be explained by the side effects of the antibiotics that are used as selective agents or inhibitors of bacterial growth,
Table 1
The impacts of explant type, microwounding and biochemical enhancements of gene delivery on the efficiency of transformation

<table>
<thead>
<tr>
<th>Rate of Regeneration</th>
<th>PCR positive</th>
<th>GUS positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rate, %a</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>72.3±1.4</td>
<td>25.7±1.8</td>
<td>3.0±0.3</td>
</tr>
</tbody>
</table>

*Experiments were repeated three times by using 100 explants/treatment in each repetition (± S.E).

References


