Expression of Viral Resistance in Transformed Petunia Plants Regenerated in Vitro

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Abstract

Vegetatively propagated trailing petunia is sensitive to virus infection. Attempts were made to develop a transformation protocol to introduce genes for virus resistance. Leaf segments from in vitro plants of two Cascadias varieties were cultured in vitro. Plants were regenerated from meristematic cell clusters that developed on segments cultured on a selected medium containing BA and NAA. Transformation was carried out with Agrobacterium tumefaciens strains EHA105 and LBA4404. EHA105 carrying the plasmids pME504, pGA492PVY or pGA492PVYM and LBA4404 carrying pCMV N/B23, were used as a vector system for transformation. The plasmid pME504 carried the β-Glucuronidase (GUS) as a reporter gene and pGA492PVY carried the last three cistrons of PVY (Protease-Replicase-Coat protein). The pGA492PVYM plasmid contained the same genes as described above with one distinction, the gene encoded the replicase had a mutation. The presence of acetosyringone during the incubation stage of the leaf segments increased the transformation efficiency. A total of 55 transgenic plants belonging to 55 independent lines transformed with a defective CMV replicase gene were challenged with CMV. Using the ELISA assay, two of the lines were found to be resistant to the virus while all the rest were susceptible. In addition, a total of 22 transgenic plants belonging to 22 independent lines transformed with the plasmid pGA492PVY were also challenged by PVY. Using ELISA assay, two lines were found to have a delay in symptom development. A total of 23 transgenic plants transformed with the plasmid pGA492PVYM were also challenged by PVY. Using ELISA assay, only one line was found to be resistant to the virus. Southern blot analysis of resistant and susceptible transgenic plants showed the presence of the insert. The implication of these results is discussed.

INTRODUCTION

The Cascadias petunia varieties are highly demanded as garden bedding and flowering pot plant. As they are vegetatively propagated they are highly susceptible to viral infection (Sikron et al., 1999; Lesemann, 1996). Plant tissue culture methods can be used for either virus elimination by shoots meristem culture or for the introduction of viral resistance by transformation. Viral genes such as coat protein and replicase, or any part from the virus genome, can be used to obtain resistant plants using genetic transformation in tissue culture (Anderson et al., 1992; Fitchen and Beachy 1993). Tissue culture methods have been used for plant regeneration and transformation in petunia (Michalczuk and Michalczuk, 2000; Yamada et al., 2001). Various plant organs, leaves, stems or flower parts were used as an efficient source of explants for regeneration. Transformation in petunia using Agrobacterium tumefaciens was reported previously, however with a relatively low number of resistant lines expressing the introduced genes (Esposito et al., 2000). In the present work an efficient tissue culture regeneration protocol and expression of resistance...
to CMV and PVY in commercially vegetatively propagated petunia is described.

**MATERIALS AND METHODS**

Virus free mother plants from the Cascadis petunia cvs. Doublon Blue Sky (DBS) were established in vitro using shoots collected from mature plants. The plants were grown in a greenhouse with an average temperature of 18°C±2 in the winter and 20-25°C±2 in the summer.

The culture medium contained inorganic salt minerals (Murashige and Skoog, 1962) supplemented with 15g/l sucrose, 40mg/l Thiamine-HCL and 1mg/l FeNaEDTA. The pH was adjusted to 5.8 before the addition of 8g/l agar. All cultures were incubated for 30 days at 23-25°C and 16h light and 8h dark photoperiod. Light was provided by cool white fluorescent tubes at 30-35µmol*m⁻²*sec⁻¹. Adventitious shoot regeneration was recorded at the end of the incubation period. The effect of growth hormone level, light and explant position on shoot regeneration were tested. Seven explants with the dorsal side and seven with the ventral side were placed on the medium in each Petri dish. Half of the plates were placed in the dark for 1 week and then transferred to the light, the other half were placed in the light during the whole experiment period.

Two *A. tumefaciens* strains were used to transform DBS: LBA4404 and EHA105. Three binary plasmids were introduced to the EHA105 strain: the pME504 plasmid carrying the reporter gene GUS, the pGA492PVY plasmid carrying three genes from PVY protease, replicase and coat protein. The pGA492PVYm plasmid carrying the same three genes as described above had a mutation in the replicase gene. The strain LBA4404 contained the pCMVN/B23 plasmid harboring a defective CMV replicase gene. *A. tumefaciens* used for plant inoculation was grown on selection LB medium (Sambrook et al., 1989). For transformation experiments, isolated colonies of bacteria were taken from selection plates and were grown at 28°C overnight in 10ml liquid LB until an optical density of 0.6 at 600nm was reached. Leaf explants from in vitro shoots were isolated and incubated with the bacterial suspension in MS medium with 200mg/l Kanamycin and 500 mg/l Carbenicillin or 500mg/l Cefotaxime and were subcultured every 2-3 weeks for shoots regeneration. Regenerated shoots were transferred to hormone free medium for 3-4 weeks. Expression of the introduced genes in transformed plants was tested by GUS activity. Expression of the introduced GUS gene in the transformed shoots was performed by incubating leaf tissue in X-GLUC solution overnight at 37°C (Jefferson et al., 1988). The presence of introduced genes was also tested by PCR analysis. To perform PCR analysis genomic DNA was extracted from leaf tissue of putative transformants (Dellaporta et al., 1983). The DNA was PCR amplified using two primers specific gene.

Southern analysis was performed with genomic DNA extracted from PCR positive transformants and from plants derived from transformants by vegetative propagation. Total DNA was extracted as described above and digested with Eco RV. The samples were separated by capillary blotting and were hybridized to a 35S probe.

Inoculated Petunia lines were analyzed for the presence of CMV-Fny and PVY. The presence of CMV was detected by indirect ELISA using a specific antiserum prepared against CMV-Fny. PVY was detected by direct ELISA using a specific antiserum prepared against CP-PVY.

Statistical analysis was performed on 3-6 replicate plates containing about 14 explants per plate, and experiments were repeated at least twice. Analysis of means, standards deviation and variance were carried out by JUMP software. The differences between treatments were tested by Tukey-Kramer test (Zar, 1984).

**RESULTS AND DISCUSSION**

**Effect of Growth Hormones, Explant Orientation and Light on Regeneration**

Trials in developing an efficient regeneration system in DBS showed that the highest regeneration occurred when the explants were placed in the light on MS medium
containing 1BA+0.1NAA mg/l (Fig. 1). The regeneration reached 80% with ±18 shoots per culture. Explant position, either dorsal or ventral, had a minor effect on regeneration.

**Effect of Acetosyringone and Light During co-cultivation on Transformation**

The addition of 200µM acetosyringone during the incubation stage increased transformation efficiency. In the case of EHA105 strain the increment in efficiency was 51%, from 14% up to 21% transformed lines. In the case of LBA4404 strain the increment in efficiency was 100%, from 6.4% to 12.8% transformed lines. Transformation efficiency increased in the presence of acetosyringone probably as a result of increased virulence as was shown also in bean transformation (Yamada et al., 2001) When the co-cultivation of the leaf explants with *A. tumefaciens* was under light, the transformation of DBS was abolished. A dark period during the co-cultivation enhanced the number of regenerated shoots, as was observed also by Michalczuk and Michalczuk (2000). Putative transformants were confirmed by PCR analysis or GUS expression.

**ELISA Assay**

A total of 275 transgenic plants belonging to 55 independent lines transformed with a defective CMV replicase gene were infected with CMV. Using ELISA assay, two resistant lines were found (3.6%), all other lines (96.4%) were susceptible (Fig. 2).

Anderson et al (1992) transformed tobacco lines with the same plasmid construct and obtained higher yield of resistant lines (11.1%). A total of 110 transgenic plants belonging to 22 independent lines transformed with the plasmids pGA492PVY were infected with PVY. One resistant line (4.5%) and one line showing delayed symptoms were found (4.5%). In addition out of 23 transformed lines with the plasmid pGA492PVYm which were infected with the virus PVY, two lines showed a delay of symptoms development (8.6%) while all the rest were found to be susceptible to the virus (Fig. 3).

**Southern Blot Analysis**

Transgenic plant analysis by southern blot showed that both the susceptible and the resistant plants contained the target genes (Fig. 4). In our system no correlation was found between gene copy number and the resistance level.

The results of the present study provide an efficient system for petunia plant regeneration in vitro. Despite the fact that both resistant and susceptible transgenic lines showed the presence of the insert, it appears that there is no guarantee that a particular transformed plant will be resistant to the virus.

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**Literature Cited**


**Figures**

![Fig. 1. Shoot development from petunia leaf explants placed on MS medium containing 1BA+0.1NAA mg/l in the light after 40 days culture.](image1)

![Fig. 2. ELISA assay in transgenic lines infected with CMV. Each line is represented by a different number. ■ 10 days after inoculation □ 20 days after inoculation](image2)
Fig. 3. ELISA assay in transgenic lines infected with PVY. Each line is represented by a different number.
■ 10 days after inoculation  □ 20 days after inoculation

Fig. 4. Southern blot analysis of DNA extracted from transgenic petunia plants digested with ECORV. Lane 1-genomic DNA from control plants. Lane 2-6-genomic DNA from transgenic lines containing genes from the PVY(P1-5) genome. Lane 7-11-genomic DNA from transgenic lines with a defective replicase gene from CMV(C1-5) genome.
Resistant (+) Sensitive (-) Delayed (±)