Particle Bombardment-mediated Transformation of *Ornithogalum dubium* for Ornithogalum Mosaic Virus Resistance

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**Abstract**

*Ornithogalum* mosaic virus (OrMV) causes flower deformation and deterioration of planting stocks in species and hybrids of *Ornithogalum* and *Lachenalia*. No resistance to viral infection by OrMV is known, making utilization of transformation technologies the natural choice for the introduction of virus resistance. Transformation with viral coat protein (CP) and replicase genes has been shown to confer resistance to viral infection in many plant species. Liquid-grown cell clusters of *O. dubium* were bombarded with gold particles coated with a plasmid carrying *nptII* gene, conferring kanamycin resistance, GUS reporter gene, and either CP gene or the viral replicase (NIb) target genes under the control of either polyubiquitin (UBQ3) or the strawberry vein-banding virus deleted (∆SVB) promoters. Following prolonged selection in a liquid medium supplemented with 80 mg/l kanamycin in darkness, the cultures were transferred to regeneration medium in the light, where hundreds of putative transgenic plantlets developed. Most of the regenerated plants were GUS-positive. PCR analysis indicated the presence of GUS reporter gene and *nptII* selectable gene, and either the CP or replicase transgenes. Transgenic plants are being propagated vegetatively before being challenged with virus infection to confirm their state of resistance.

**INTRODUCTION**

Genetic transformation of cells and tissues mediated by bombardment with tungsten or gold particles carrying target genes is the preferred method for the introduction of foreign genes into monocotyledonous plants, which are generally recalcitrant to the infection and subsequent transformation by *Agrobacterium* (Vain et al., 1995; Finer et al., 1999). This method was employed for the transformation of several monocotyledonous ornamental crops such as tulip (Wilminks et al., 1992), gladiolus (Kamo et al., 1995), lily (Langeveld, 1995; Van der Leede-Plegt et al., 1997; Watad et al., 1998; Lipsky et al., 2000), and is widely used for many other crops. It also proved successful in the transformation of *Ornithogalum* (De Villiers et al., 2000).

Viral genes are capable of conferring resistance to viral infection when incorporated into the plant genome. Following the discovery in Beachy’s laboratory on the coat protein-mediated resistance (see Beachy et al., 1990; Fitchen and Beachy, 1993; and references therein for review of the early work), there have been hundreds of publications on CP and other viral genes that confer resistance to viral infection or spread. The gene for the virus replicase is also known to induce a strong protection against viral infection in transgenic plants (Anderson et al., 1992; Carr and Zaitlin, 1993; Baulcombe, 1994).

The orange-yellow colored *Ornithogalum* (*O. dubium*) has been established in the last decade as a commercially important flower bulb. Israeli growers enjoy a near-monopoly as suppliers of bulbs, cut flowers and flowering pot plants to the flower markets in both the USA and Europe. The commercial potential of *Ornithogalum* is severely...
hampered by its susceptibility to Ornithogalum Mosaic Virus (OrMV). The virus is a monopartite single stranded RNA potyvirus (Burger and Von Wechmar, 1989) which infects Ornithogalum and Lachenalia. It is readily transmitted by several species of aphids and by mechanical means causing mosaic symptoms in the leaves, twisted stems, flower stunting and deformation (Smith and Brierly, 1944). The virus spread is very rapid causing deterioration of the plants. The growers are forced to replace the entire planting stock every two years even after starting with virus-free bulbs.

Since no naturally occurring resistance to OrMV is known, molecular breeding through transformation with viral genes is an obvious choice. We report an efficient particle bombardment-mediated transformation of O. dubium with viral genes and the recovery of transgenic plants in an attempt to obtain resistance to virus infection.

MATERIALS AND METHODS

Plant Material and the Establishment of Cell Cultures

*O. dubium* cell cultures were started from calli derived from basal parts of axenic plantlets of a selected clone from our breeding lines (ATD) grown on an agar-solidified MS medium (Murashige and Skoog, 1962) supplemented with 0.1 mg/l NAA, 2 mg/l BA and 3% sucrose (medium 206, Cohen et al., 2004). Highly-competent cell clusters, suitable for bombardment, developed within 9 weeks following the transfer of the initial calli onto a liquid 206 medium. Cell clusters were subcultured to a fresh medium every two weeks.

Construction of Transformation Vectors

Four different gene constructs were built for transformation purposes. These included two target genes: the coat protein gene (CP) of the Israeli isolate of OrMV (Zeidan et al., 1998), and the virus replicase gene (*Nib*). These were placed under the control of one of the two constitutive promoters: the *Arabidopsis* polyubiquitin promoter (UBQ3) and the strawberry vein-banding virus deleted promoter (ΔSVB, accession no. AF331666), and cloned into a pCAMBIA2301 vector that has nptII gene, conferring kanamycin resistance, and a GUS reporter gene (both under control of 35S promoter). In the construction of the chimeric gene standard procedures of DNA restriction, subcloning and propagation in *E. coli* were employed. Firstly, a NOS terminator was PCR cloned into pCAMBIA2301 KpnI-EcoRI sites to create pCAMBIA-2301-NOS. The SVB deleted promoter (ΔSVB) and the *Arabidopsis* UBQ3 promoter were then PCR cloned into the HindIII-BamHI sites of pCAMBIA2301-NOS to create pCAMBIA2301-ΔSVB-NOS and pCAMBIA2301-UBQ3-NOS. The Israeli OrMV-CP and the South African OrMV *Nib* genes were then PCR cloned into the BamHI site or BamHI-KpnI sites of either pCaMBIA2301-ΔSVB-NOS or pCaMBIA2301-UBQ3-NOS, respectively to create pCaMBIA2301-ΔSVB-CP or *Nib* and pCaMBIA2301-UBQ3-CP or *Nib*. Sense orientation of the CP gene was verified by PCR and restriction analysis. All other constructs were verified by restriction analysis. The restriction maps of the four constructs are outlined in Fig. 1.

Microprojectile Bombardment

A particle inflow gun (PIG) was built following the model of Finer et al. (1992) with adaptation according to Gray et al. (1994). The PIG was used to propel 1.5-3 mm gold particles coated with the appropriate constructs. The plasmid DNA for the particle bombardment was isolated from *E. coli* strain DH5α, purified using plasmid Midi-Kit (Qiagen Inc., Valencia, CA, USA) and concentrated to 1 µg/µl prior to bombardment. The ratio of 1 µg DNA to 0.7 mg gold particles in 9 µl ethanol was used. Samples of 2-3 g cell clusters were used for each bombardment and every sample was bombarded twice.

GUS Histochemical Staining

GUS reaction was assessed using histochemical staining following the
improvement suggested by Kosugi et al. (1990) for the assay developed by Jefferson (1987). The transient GUS expression was assayed 48 hours after bombardment. The blue spots were counted in tissue samples and calculated per g fresh weight. Cell samples or plant tissues were also subjected to GUS assays during each developmental stage. No GUS activity was evident in either non-bombarded plant tissue, or in tissue bombarded with gold particles not carrying the plasmid.

Selection on Kanamycin

Two days following bombardment with plasmid DNA the bombarded cells were recultured onto liquid proliferation medium 206 supplemented with 80 mg/l kanamycin for prolonged selection in shake flasks in darkness. The cultured cells were subcultured into a fresh medium at 1-2 week intervals. The *O. dubium* cells were kept on the proliferation medium for 6 months for the development of clusters of transformed cells prior to the transfer to the regeneration medium in the light.

Regeneration of Transgenic Plants

Following the prolonged selection in the proliferation medium the dark-grown cell cultures were transferred to shoot regeneration medium in the light (16 hours photoperiod). Plantlet regeneration in *Ornithogalum* was induced on MS medium devoid of any growth substances, with 3% sucrose and 50 mg/l kanamycin. Upon regeneration, plants were transferred to agar-solidified MS medium containing 50 mg/l kanamycin for continued plant growth and development.

PCR Amplification

Leaves were collected from shoots surviving on medium with 50 mg/l kanamycin and control shoots regenerated from non-bombarded explants. DNA was isolated from the leaves according to the procedure of Fulton et al. (1995).

Approximately 500 ng of DNA was used as the template for PCR. Amplification of the nptII gene utilized the upstream primer 5’ - GCC CCT GAT GCT CTT CGT CCA GAT C - 3’ and the downstream primer 5’ - TCG GCT ATG ACT GGG CAC AAC AGA C - 3’ resulting in amplification of a 434 bp fragment. The PCR reaction was performed in 30 µl reaction volume with 1x buffer (JMR Holding, London), 1.5 mM MgCl2, 0.2 mM dNTP mix plus 7.5% DMSO, 0.25 µM of each primer and 0.75 units of Taq DNA polymerase. The reaction was 2 min at 95°C, followed by 35 cycles of 60 sec at 94°C, 45 sec at 57°C, and 90 sec at 72°C with a final extension at 72°C for 5 min. For amplification of the GUS (uidA) gene the forward primer 5’ - GAC GCC CTG TGG GCA TTC AGT CTG G - 3’ and the reverse primer 5’ - GTG TAG AGC ATT ACG CTG GCA TGG A - 3’ were used, resulting in amplification of 487 bp fragment. For the amplification of OrMV-CP gene the forward primer 5’ - CG GGA TCC ATG GGC AAA ACG AGA TAC ATT TCA G - 3’ and the reverse primer 5’ - GG GGT ACC TCA CAT ATT AAC ACC AAG TAG TGA ATG - 3’ were used, resulting in amplification of 900 bp - the entire length of the cloned gene. For amplification of the replicase (N1b) gene the forward primer 5’ - GGA AGG GTA CAA CAA CGT GCT CAG - 3’ and the reverse primer 5’ - GCT ATC GAA CTG TGA GCC GTC AGC - 3’ were used to amplify a 490 bp fragment. The PCR reaction was performed in 30 µl reaction volume as above. PCR products were separated on 1% agarose gels and visualized with ethidium bromide.

RESULTS AND DISCUSSION

The system presented here starts with the development of highly competent cell lines as evident from the large number of transient blue GUS expressing foci presented in Fig. 2b. The number of cells expressing transient GUS in the cell clumps following the bombardment with all four constructs was ca 1300/g FW. The prolonged 6-months’ selection on kanamycin allows the development of transgenic meristematic centers (Fig. 2d) and the gradual elimination of non-transgenic cells. This ensures the regeneration of hundreds of solid transgenic plants upon transfer to the light. An attempt to transfer the
cell clumps to the regeneration medium in the light after only two months resulted in the regeneration of very few plants. GUS expression was evident both in the cell clumps and in the regenerants (Fig. 2f-g). *Ornithogalum* cultures were mostly organogenic and the regenerated plantlets had high rate of hyperhydricity (vitrification). These plantlets developed into normal-looking plants after the transfer to an agar-solidified MS medium (Fig. 2h).

The putative transgenic plants of *O. dubium* were analyzed by PCR for the presence of the transgenes (Fig. 3 and 4). The presence of the *nptII* gene was confirmed in all of the analyzed plants, as was expected since the plants were maintained on high levels of kanamycin through all stages of development. In fact, the majority of the plants showed co-transformation of both the reporter gene (GUS), the selectable marker (*nptII*), and the target gene (CP or *Nlb*). In some plants, however, the presence of GUS was not confirmed indicating a possibility for genetic recombination (Fig. 3).

Currently, transgenic plants produced following bombardment with each one of the four constructs (Fig. 1) are growing in the greenhouse. The plants are being propagated and the propagules will be challenged with virus using either mechanical inoculation or via aphid transmission. The clonal propagation of the transgenic plants is needed since the efficiency of the virus transmission from infected to healthy *O. dubium* was found to be only 90%, as reported by Zeidan et al. (1998). We expect that independent transgenic *Ornithogalum* plants carrying the same transgene show variable expression of the transgene and, hence, phenotypic variation, as was reported in many other species in which attempts were made to induce resistance through transformation with viral genes. This is often attributed to the position of the transgene incorporation in the plant genome and/or gene silencing at the transcriptional or translational level. In order to obtain transgenic plants carrying target genes with optimal levels of phenotypic expression, it is imperative that sufficient numbers of independent transgenic plants are produced, from which the appropriate clones could be selected. Using the method described here we obtained hundreds of solid transgenic plants. It is now possible to use this method for the transgenic breeding of *Ornithogalum* with other valuable genes.

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**Literature Cited**


Fig. 1. Map and restriction sites of the promoter-target-gene inserts cloned into the pCAMBIA2301 vector for transformation of *Ornithogalum dubium*. 
Fig. 2. Genetic transformation of *O. dubium*: 

a: Cell clusters of *Ornithogalum* grown in liquid medium 206 in darkness prior to the bombardment. 

b: Transient GUS expression in competent cells bombarded with pUBQ3genGUS. 

c: Stable expression of the reporter gene in cells grown in selection medium containing 80 mg/l kanamycin for 54 days. 

d: The development of organized transgenic meristematic centers in the liquid medium after prolonged selection. 

e: Plantlet regeneration in light-grown cultures in liquid MS medium containing 80 mg/l kanamycin. 

f: GUS expression in regenerating cell clumps from the culture in e. 

g: GUS expression in transgenic plantlets regenerated in the liquid cultures. The plantlet on the right is a non-transgenic control. 

h: Transgenic *Ornithogalum* plants on solid MS with 50 mg/l kanamycin.
Fig. 3. PCR analysis for the reporter gene (GUS), the selectable marker (nptII), and the target CP gene in putative *Ornithogalum dubium* transformants. Lanes marked with M present 1 kb DNA ladder. Lane Co is an untransformed control and lane P is the plasmid vector. The plants in lanes 2 and 8 show the presence of the nptII and the CP gene but not that of the GUS reporter gene.

Fig. 4. PCR analysis for the target replicase (*NiB*) gene in putative *Ornithogalum dubium* transformants. Lanes marked with M present 1 kb DNA ladder. Lane Co is an untransformed control and lane P is the plasmid vector. The target gene is missing from the plant in lane 15.