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Transgenic Plants of Easter Lily (*Lilium longiflorum*) with Phosphinothricin Resistance

Byung Joon Ahn[1*], Young Hee Joung[2], Kathryn K. Kamo[3]

[1]College of Bioresources Science, Dankook University, Choenan 330-714, Korea; [2]Plant Cell Biotechnology Lab, KRIBB, Taejon 305-333, Korea; [3]Floral & Nursery Plants Research Unit, USDA, US National Arboretum, Beltsville, MD 20705-2350, U.S.A.

Abstract

Transient *uidA* expression was used to optimize parameters required for biolistic transformation of suspension cells of Easter lily, *Lilium longiflorum*. Maximum *uidA* expression occurred following bombardment with gold particles as compared to tungsten. A 3 hr pre-treatment of suspension cells with 0.125 M osmoticum resulted in a 1.5X increase in *uidA* expression. A helium pressure of 1550 psi combined with a particle travelling distance of 6 cm resulted in maximum *uidA* expression as compared to either 1100, 1200, or 1800 psi. Transient transformation resulted in up to 493 *uidA* expressing cells/Petri plate. For stable transformation suspension cells of *Lilium longiflorum*, were co-bombarded with plasmid DNA containing cucumber mosaic virus (CMV) replicase under the rice actin (*Act1*) promoter and either the *bar* or PAT genes under the cauliflower mosaic virus (CaMV 35S) promoter. Ten regenerated plants contained the transgene as analyzed by PCR, and two of the ten plants were confirmed to contain the transgene by Southern hybridization. The two transgenic plants were independent transformants, one containing the *bar* gene and the other both the CMV replicase and *bar* genes. Plants were sprayed at the rosette stage and found to be resistant to 1000 mg/L of phosphinothricin (Trade name-Ignite) indicating expression of the *bar* gene throughout the leaves when *bar* was under control of the CaMV 35S promoter.

Key words: CMV replicase, *bar* gene, PAT gene, biolistics, bulb crops

* Corresponding author, E-mail: bjahn@dankook.ac.kr
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Introduction

Lilies are an important floral crop produced either as pot plants or for cut flowers. In the United States the wholesale value of Easter lilies, *Lilium longiflorum*, sold as pot plants was $35,273,000 in 2000 (USDA Statistics, 2000).

Lily plants are typically multiplied vegetatively by bulbs resulting in transmission of viruses to the next generation of plants. Lilies infected with cucumber mosaic virus (CMV) showed chlorotic yellow spots or stripes in the leaves, leaf-curling, vein clearing, petal-curling and sometimes a color breaking pattern in the flowers (Derks, 1995; Kim et al. 1996).

Genetic engineering offers the potential for developing lily cultivars with virus resistance that has not been achieved by conventional breeding of this increasingly important floral crop. There have been only two reports (Langeveld et al. 1997; Watad et al. 1998) on the development of transgenic lilies, *Lilium longiflorum*, that contained either a reporter gene, *uidA*, or the coat protein gene of lily symptomless virus, and the selectable marker gene, *bar*. These transgenic lily plants were grown *in vitro* and not evaluated for resistance to either lily symptomless virus or phosphinothricin following transplanting to soil. This is the first report on the development of transgenic lily, *Lilium longiflorum*, plants grown to flowering and demonstrated to be resistant to phosphinothricin by spraying plants growing in the soil, and represents an initial report on incorporation of a CMV replicase gene (Anderson et al. 1992) into lilies for virus resistance.

Materials and Methods

Plant culture

Callus was initiated from bulb scales of a *Lilium longiflorum* Thunb., hybrid seedling ('Nellie White' x 'Ace') cultured on Murashige and Skoog's (MS) basal salts medium (Murashige and Skoog, 1962) supplemented with 3% sucrose, 0.2% phytagel (Sigma Chemical Co., St. Louis, MO) and the following in mg/L: glycine, 1.0; thiamine, 1.0; pyridoxine, 0.5; nicotinic acid, 0.5, and 9.1 μM 3,6-dichloro-2-methoxybenzoic acid (dicamba), pH 5.8. All media were autoclaved at 121℃/18 psi. Suspension cells were initiated from the callus and cultured in 20 mL/flask of the same medium as for callus induction, except that Phytagel was omitted. Callus and suspension cells were grown in the dark at 25℃. Suspension cells were transferred every four weeks and shaken on a gyratory shaker at 120 rpm and 25℃ in the dark.

Biolistics

Callus cells were transformed using the PDS- 1000/He system (BioRad, Richmond, CA) using gold particles (1 μM) (Sanford et al. 1991; 1993). The target distance was 6 cm, and 43 plates of suspension cells were co-bombarded at 1200 psi with psan101 (Sanford Scientific, Inc., Waterloo, NY) that consists of the CMV replicase gene (M. Zaitlin, Cornell University, Ithaca, NY) under the *Act1* promoter (McElroy et al. 1990) and with either pDM302 (Cao et al. 1992) or p35SAc (P. Eckes, Hoechst Roussell Co., Somervillle, NY) that contain the *bar* or PAT genes, respectively, under the CaMV 35S promoter. Both the *bar* and PAT genes code for phosphinothricin acetyltransferase and confer resistance to phosphinothricin (PPT). Suspension cells had been placed in liquid MS basal salts medium supplemented with 9.1 μM dicamba and 0.125 M osmoticum (1:1 mannitol: sorbitol) 3 hr prior to bombardment.

Transient gus expression

The method of Jefferson et al. (1987) was used for histochemical staining of cells 48 hr following bombardment. The substrate 5-bromo-3-chloro- 4-indolyl-D-glucuronic acid was added directly to the filter paper on which the suspension cells were cultured. Blue spots indicative of *uidA* expression were counted after 16 hr incubation of the cells with substrate at 37℃. Cells bombarded with pUC8 were the negative control. Cells were bombarded at 1200 psi, 6 cm particle travelling distance, with pDM343 (received from D. McElroy, Cornell University) that contains the *bar-uidA* fusion gene under the *Act1* promoter to test effect of osmoticum pre-treatment, helium pressure, and either gold or tungsten on transient *uidA* expression. One plate of cells was bombarded for each treatment, and this was replicated three times.

Selection for putative transformants

One week following bombardment the suspension cells were placed on either solidified or in liquid MS basal salts medium supplemented with 9.1 μM dicamba and 2 mg/L PPT (Hoechst Roussell). There were 21 Petri plates co-bombarded with p35SAc and psan101 of which 5 plates were selected on solidified medium and 16 plates of cells were placed in 16 flasks containing liquid medium for selection. Of the 22 plates co-bombarded with pDM302 and psan101, 6 plates were selected on solidified medium and 16 plates placed in liquid medium for selection. Every three weeks the callus or suspension cells were transferred to fresh solid or liquid medium, respectively, supplemented with 2 mg/L PPT and grown in the dark for 3-4 months. Regenerating callus and plants were grown on solidified MS basal salts medium supplemented with 1 mg/L PPT and grown under a 16 hr light photoperiod at 75 $\mu E/m^2/sec$ using cool white fluorescent light bulbs at 25℃.

DNA analysis

DNA for both PCR and DNA blots was isolated from leaves of *in vitro*-grown plants using the Quiagen DNeasy Plant Mini Kit (Valencia, CA) according to the manufacturer's instructions. The *bar* gene was amplified using the primer sequence: 5'-gtc aac ttc cgt acc gag ccg cag -3' and 5'-cat gcc agt tcc cgt gct tga ag-3' and microcycler conditions: 95℃ for 5 min (one cycle), 94℃ for 30 sec, 62℃ for 1 min, 72℃ for 1 min (35 cycles), 72℃ for 10 min. The CMV replicase gene and nos terminator were amplified using the primer sequence: 5'-tcg tca cca tgg tga ccc gag-3' and 5'-cat cgc aag acc ggc aac agg-3' and microcycler conditions: 95℃ for 5 min (one cycle), 94℃ 30 sec, 62℃ 1 min, 72℃ 1 min (35 cycles), 72℃ 10 min.

The DNA for Southern hybridization with the *bar* gene was digested with *Eco* RI followed by electrophoresis on an 0.8 % agarose gel in TBE (89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA, pH 8) buffer. Following electrophoresis the DNA was transferred to Nytran membrane

(Schleicher and Schuell, Keene, NY) (Maniatis et al. 1982). The *bar* gene probe used for hybridization was a 670 bp PCR-amplified DNA product that amplified between the *bar* gene and *nos* terminator using the primers: 5'-gtc aac ttc cgt acc gag ccg cag-3' and 5'-cat cgc aag acc ggc aac agg-3' with the same PCR conditions as above for the *bar* gene. The genomic DNA used for detecting the CMV replicase gene was digested with *Xho* I and *Sac* I, and the probe was a 1.3 kb PCR-amplified CMV replicase gene product that was amplified using the primers: 5'-gtc taa gtt tga taa gtc tca agg-3' and 5'-tgg tct cct ttt gga ggc ccc ac-3' with the same PCR conditions as for the CMV replicase and *nos* terminator. Both probes were labeled using the DIG-dUTP (PCR DIG Probe Synthesis kit, Boehringer Mannheim Biochemica). Hybridization and detection of the digoxigenin-labeled nucleic acid was performed using the DIG Easy Hyb and DIG Nucleic Acid Detection kits (Boehringer Mannheim Biochemicals).

Greenhouse growth

In vitro-grown plants were transplanted to 4 cm pots filled with a growing medium (ProMix- BX, Stamford, Conn) and grown in a greenhouse maintained at 23℃/21℃, day/night. During the day time, high intensity discharge lamps were used to provide about 300 $\mu E/m^2/sec$ of supplementary lighting. Six plants at the rosette stage of growth were sprayed with each concentration of PPT for each transgenic plant line.

Results and Discussion

Transient *uidA* expression

The conditions that resulted in maximum transient *uidA* expression in Easter lily cells were determined for stable transformation experiments. Suspension cells cultured 3 hr in MS basal salts medium supplemented with 9.1 μM dicamba and 0.125 M mannitol/ sorbitol showed 1.5X more cells that expressed *uidA* as compared with cells that had not been pre-treated 3 hr with an osmoticum (Figure 1 & 2). Maximum *uidA* expression by suspension cells occurred following bombardment with 1550 psi combined with a 6 cm particle travelling distance (Figure 3). A lower pressure, 1200 psi, was used to bombard cells for stable transformation so that cells would remain viable. There were 493 blue spots/Petri plate using 1 μM gold particles as compared to 314 spots/plate with M10 tungsten for bombardment. These experiments showed that an osmoticum pre-treatment of 0.125 M, a helium pressure of 1550 psi with gold particles and a particle travelling distance of 6 cm were

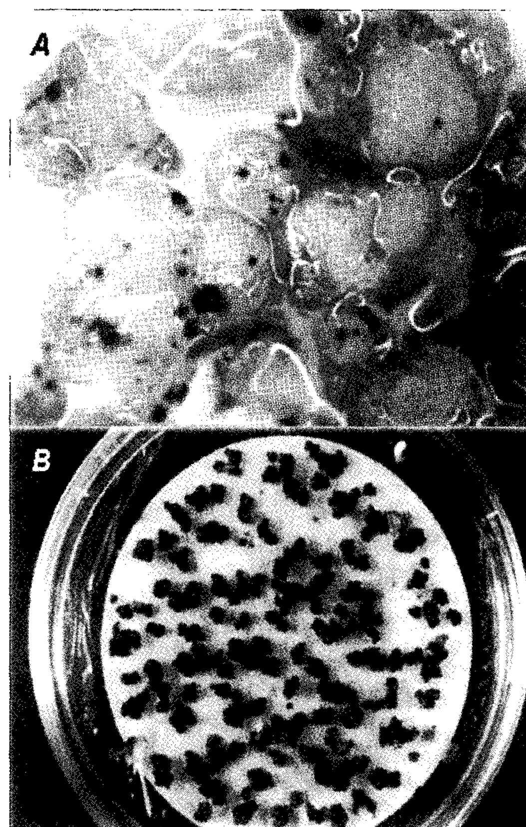

Figure 1. (A) Transient gus gene expression of embryogenic cell clumps of *Lilium longiflorum* after bombardment with a GUS expression vector (pDM343), (B) and a selection culture of lily cell clumps co-transformed with bar and CMV replicase genes on phosphinothricin containing MS medium.

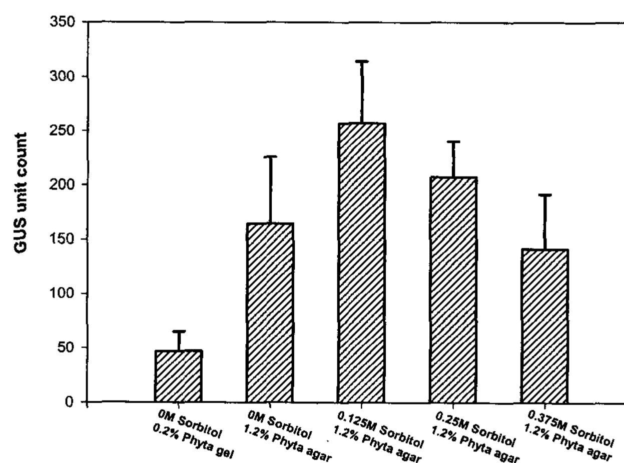

Figure 2. Effect of osmotic conditions on transient GUS expression of bombarded embryogenic cells of *Lilium longiflorum.* Data indicates means and bars represents standard errors.

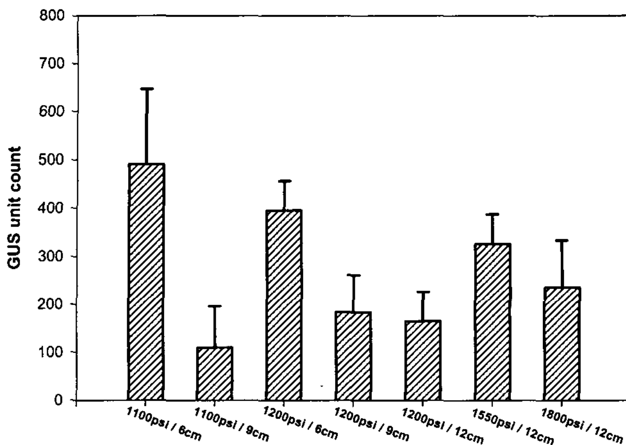

Figure 3. Effect of helium pressure and particle flying distance on transient GUS expression of bombarded embryogenic cells of *Lilium longiflorum*. Data indicates means and bars represents standard errors.

optimal for transient *uidA* expression in suspension cells of Easter lily. Watad et al. (1998) reported similar optimal conditions, 1100 psi and 6 cm target distance, for bombardment of Easter lily callus cells although data on the number of *uidA* expressing cells was not presented. Our results showed a 1.5X increase in transient *uidA* expression as compared to Watad et al. (1998) who reported no effect by pre-culturing callus on osmoticum. Larger, 3X, increases in transient *uidA* expression have been reported following pre-treatment in osmoticum prior to bombardment of tobacco and maize suspension cells (Vain et al. 1993).

Recovery of transformed plants

Southern hybridization confirmed the presence of the *bar* gene in two of the 10 plants that appeared to contain either the *bar* or PAT genes by PCR analysis (Figure 4). Genomic DNA was cut with *Eco* RI to release the *bar* gene and *nos*

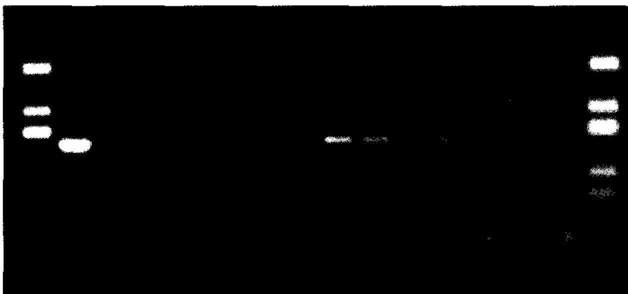

Figure 4. PCR analysis of bar gene in transformed *Lilium longiflorum* plants. Lane 1: markers, DdeI-cut pUC18 DNA; 2: pBY505-RCH1 positive control; 3: Untransformed control plant; 4-17: putatively transformed lilies.

terminator insert consisting of 0.9 kb (Figure 5A). One of the two plants containing the *bar* gene also contained the CMV replicase gene (Figure 5B). The genomic DNA was cut with *Xho* I and *Sac* I to release 1.3 kb of the 3 kb CMV replicase gene insert.

Almost all of the lily cells that we had cultured in liquid medium with 2 mg/L PPT formed numerous roots rather than shoots as desired. The two transformed lily plants were recovered from selection on solid medium, not liquid medium.

Transgenic plants in the greenhouse

Plants of both transformed lines survived spraying with 1000 mg/L PPT as compared to non-transgenic lily plants that died at 500 mg/L PPT (Figure 6A). The ability of the transformed plants to survive spraying with PPT indicates expression of the *bar* gene throughout the plant under control of the CaMV 35S promoter. The transgenic plants grew to maturity (Figure 6B).

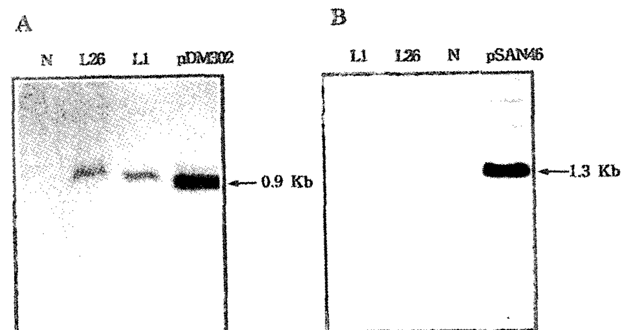

Figure 5. DNA blot of genomic DNA isolated from leaves of Easter lily grown *in vitro*. (A) Genomic DNA was digested with *Eco* RI and hybridized with a probe of 670 bp from a PCR-amplified fragment of the *bar* gene. The 0.9 kb band (arrow) is shown following digestion of pDM302, transgenic lines L26 and L1 but not in the non-transformed plant (N). (B) Genomic DNA was digested with *Xho* I and *Sac* I and hybridized with a 1.3 kb probe resulting from amplification of the CMV replicase gene. The 1.3 kb band (arrow) was shown for psan 101 and transgenic L1 but not for both L26 and the non-transformed plant (N) lines.

Figure 6. Transgenic Easter lily plants. (A) Transgenic plants tolerant to 1000 mg/L of phophinothricin treatment. (B) Transgenic plants blooming in the greenhouse.

In conclusion, these results showed preliminary results on production of transgenic Easter lily plants for virus resistance by using phosphinothricin for selection of transformants at both the tissue culture and whole plant level.

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