Crop Breeding and Applied Biotechnology 5:259-264, 2005
Brazilian Society of Plant Breeding. Printed in Brazil

Transformation of *Lilium longiflorum* via particle bombardment and generation of herbicide-resistant plants

Vagner Augusto Benedito[1,2*], Bernadette C E van Kronenburg-van der Ven[1], Jaap M van Tuyl[1], Gerco C Angenent[1], and Frans A Krens[1]

Received 2 May 2005

Accepted 16 June 2005

ABSTRACT - *This study is a contribution to the improvement of lily (*Lilium longiflorum *Thunb.) transformation procedures. A vector carrying the* Arabidopsis *SUPERMAN gene driven by the petunia flower-specific FLORAL BINDING PROTEIN 1 promoter and the resistance gene* phosphinothricin acetyltransferase *against herbicide bialaphos under the CaMV35S promoter was used to transform bulblet slices by particle bombardment. Our aims were to improve the transformation parameters for lily transformation in order to enhance the efficiency and to create novel phenotypes in lily flowers using transcription factors originating from dicot plants. We managed to obtain transgenic lines expressing* in vitro *resistance to bialaphos. The transgenic plants were transferred to the greenhouse, grown and monitored for two flowering seasons. Flowers derived from these plants appeared normal and indistinguishable from wild-type flowers.*

Key words: ABCDE model, floral development, homeotic gene, lily, transcription factor.

INTRODUCTION

Lily species (*Lilium* spp.) are highly appreciated for their aesthetic value as cut flowers or pot plants and have been among the most important ornamental species worldwide for a long time.

Numerous lily breeding programs aim at the establishment of novel phenotypes with higher market values. Until now these programs are based mainly on segregation and selection of natural variants and interspecific hybridisation by wide crosses. Although the search for rapid and inexpensive techniques for massive *in vitro* propagation is still going on, tissue culture is already well established for lily (see Aswath et al. 2001) and is used to assist interspecific hybridisation, especially with embryo rescue, and to propagate commercial clones.

Despite the importance of lily as ornamental species, genetic manipulation at a molecular level has proven to be difficult and reports on lily transformation are scarce. Few reports showed transformed products in lily species so far, most of them by means of particle bombardment such as the DNA delivery system and at a low efficiency (van der Leede-Plegt et al. 1997, Watad et al. 1998, Zeng et al. 2001, Lipsky et al. 2002).

[1] Plant Research International, P. O. box 16, 6700 AA Wageningen, The Netherlands

[2] Current address: Center for Nuclear Energy in Agriculture, University of Sao Paulo - C. P. 96, 13.400-970 Piracicaba, SP, Brasil. *E-mail: benedito@cena.usp.br

Langeveld et al. (1995) were the first to indicate that *Agrobacterium* was able to transform lily cells, although they could not regenerate a transgenic plant by this method. Only more recently, Hoshi et al. (2004) reported on the first workable transformation method using this biological delivery system.

An optimised transformation protocol for lily and the availability of molecular tools such as genes and promoters may contribute to speed up breeding programs in this species.

SUPERMAN (SUP) is a zinc-finger transcription factor involved in flower development of *Arabidopsis*, defining the boundaries between the third and the fourth whorls by limiting cell expansion and proliferation (Bowman et al. 1992, Sakai et al. 1995, Kater et al. 2000). Ectopic expression of SUP controlled by constitutive promoters in heterologous species such as tobacco (Bereterbide et al. 2001), petunia (Kater et al. 2000) and rice (Nandi et al. 2000) has been shown to lead to dwarf plants.

FLORAL BINDING PROTEIN 1 (FBP1) is a flower-specific gene of petunia *(Petunia* x *hybrida*) which is involved in the ABCDE model for flower development. It is a B class gene, expressed in petals and stamens as shown in petunia flower development (Angenent et al. 1993). Expression of SUP under the control of the FBP1 promoter resulted in reduced petal and stamen growth in petunia and tobacco (Kater et al. 2000). We were aiming at new floral phenotypes in lily using this construct cassette.

Here we report the transformation of *Lilium longiflorum* by the particle bombardment method using a vector containing the *SUP* gene from *Arabidopsis* driven by the petunia FBP1 promoter and the bialaphos resistance gene PAT under the constitutive *CaMV35S* promoter. Transgenic plants showed *in vitro* resistance to herbicide in the culture media and were transferred to a greenhouse for flower morphology evaluation. They did not show alterations when compared to wild-type plants.

MATERIAL AND METHODS

Plant material and tissue culture

Inner bulblet scales from *Lilium longiflorum* Thunb. cv. Snow Queen established under *in vitro* conditions were sliced in transversal 1 mm thick and 3-5 mm long segments. Sixty explants per Petri dish were placed on callus induction medium (CIM, 1/2 strength MS salts with vitamins [Murashige and Skoog 1962], 3% sucrose, 0.1 mg L^{-1} BAP and 1 mg L^{-1} picloram) for one week in the dark at 24 °C. Before bombardment, these explants were transferred to fresh CIM and concentrated in the centre of the dish in a 5cm-diameter shooting target area.

Just after bombardment, explants were spread again and kept on the same medium for an additional week at the same pre-bombardment conditions. After this period, explants were transferred to CIM supplemented with the selective agent (herbicide Basta® 2 mg L^{-1} supplemented prior to media sterilisation) for two weeks. The explants were then transferred to regeneration medium (full strength MS medium with vitamins, 3% sucrose and 0.1 mg L^{-1} NAA) supplemented with the selection agent and kept in the dark for one week and then transferred to dimmed light conditions (under light with a white towel covering the dishes, ca. 30 μmol m^{-2} s^{-1}) for one week. After that, they were exposed to direct light (ca. 70 μmol m^{-2} s^{-1}).

Subculture was carried out every 4 weeks on the same medium until the regenerated plants with bulblets and leaves were recovered and put on propagation medium (1/2 strength MS salts and vitamins with 5% sucrose) supplemented with the selection agent.

Transformation vector and bombardment procedures

The FBP1::SUP cassette was inserted in the pBluescript KS+ plasmid harbouring the bialaphos resistance *phosphinothricin acetyltransferase* (PAT) gene under the constitutive CaMV35S promoter for selection. This vector was referred to as pB-SUP and the final size of this construct was 6.8kb. As a control for the transformation events, a 7.4-kb plasmid (pPG5) harbouring the β-glucuronidase (GUS) marker gene under the CaMV35S promoter was used for transient expression analysis and transformation efficiency measurement. DNA was purified by columns using the Plasmid Maxi Kit (QIAGEN, Hilden, Germany).

The transformation experiment used about 900 explants divided up among 15 dishes. The bombardment was carried out with the Biolistics PDS 1000/He system (Sanford et al. 1993) using 1.0 μm gold particles (Bio-Rad, Veenendaal, the Netherlands) as a carrier. Three milligrams of particles were coated with 5 μg plasmid DNA (in a 60 μL solution) and 7.5 μL was used for each bombardment. Experiments were set up with 1 shooting at 1100 psi (treatment 1x1100) onto about 400 explants or 3 shootings at 1800 psi (3x1800) onto around 500 explants using a target distance of 9 cm. Additionally, 50 explants per treatment were bombarded with the control plasmid pPG5.

Selection and identification of transgenic plants

Plants were kept on propagation medium supplemented with 2 mg L^{-1} Basta® to select plants constitutively expressing the PAT gene. The regenerated

plants were tested for the presence of the transgenes by PCR. Additionally to the Basta® resistance, the presence of PAT was identified by a PCR product amplification using the oligonucleotides 5'-GAT TAG GCC AGC TAC AGC AGC-3' and 5'-CCT TGG AGG AGC TGG CAA CTC-3' as forward and reverse primers, respectively, at an annealing temperature of 65°C. The presence of SUP in the genome was recognised by the amplification of a 613-bp fragment using the gene-specific primers 5'-ATG GAG AGA TCA AAC AGC-3' (forward) and 5'-TTA AGC GAA ACC CAA ACG-3' (reverse) at 55 °C for the annealing step. The genomic lily DNA was extracted through the method described by van Heusden et al. (2000) and 500 ng were used in the PCR reaction.

Explants bombarded with pPG5 were assayed for transient β-glucuronidase activity 5 days after the bombardment using standard conditions (Jefferson et al. 1987).

Transgenic plants were kept *in vitro* until their bulblets were 2-3 cm diameter. Then they were vernalized and transferred to soil in greenhouse conditions, where they were observed for two flowering seasons under standard environmental conditions for greenhouse flower production.

Assessing transgene expression

The floral meristems and leaves derived from transgenic lily plants grown under greenhouse conditions were harvested. They were immediately frozen in liquid nitrogen and kept in –80°C for further analysis. Total RNA was extracted by the method of Zhou et al. (1999). Northern blot, probe synthesis and purification, and hybridisation procedures were as described elsewhere.

Constitutive transcription for RNA loading control in northern blots was assessed through the expression of the *glyceraldehyde-3-phosphate dehydrogenase* (GAPDH) gene, which participates in the glycolysis pathway. A 620-bp fragment of the lily *GAPDH* was amplified by RT-PCR using the degenerate primers (5'-GTK GAR TCN ACY GGY GTC TTC ACT-3' and 5'-GTR TGR AGT TGM CAN GAR ACA TC for forward and reverse annealing, respectively), cloned in pGEM-T-easy vector (Promega, Madison, WI), sequenced and used as probe.

RESULTS

Slices of *in vitro* cultivated bulblet scales from *Lilium longiflorum* were placed for one week on callus induction medium and bombarded with a plasmid harbouring the CaMV35S::PAT and the FBP1::SUP cassettes. Quantification of the transformation events and transient gene expression were tracked by parallel bombardments using a plasmid with the CaMV35S::GUS.

About 900 explants were exposed to the SUP-containing plasmid under the petunia flower-specific promoter FBP1. These dishes were divided into 2 treatments: one shot at 1100psi (1x1100) or three consecutive shots at 1800psi (3x1800). Main differences between the bombardment protocol described by Watad et al. (1998) and the protocol we used here are visualised in Table 1.

Table 1. Differences between the method for *Lilium longiflorum* transformation via particle bombardment as described by Watad et al. (1998) and the protocol used in this report

Parameters	Watad et al. (1998)	this report
Bombarded tissue	Embryogenic callus	Bulblet scale slices
plasmid purification	Cesium Chloride	Column (Qiagen)
DNA carrier	Tungsten	Gold
DNA amount/ bombardment	2 μg	~ 0.8 μg
Target distance	6 and 9 cm	9 cm
Shooting times and pressure	1x900, 1x1100 and 1x1500psi	1x1100 and 3x1800psi
Selection agent (Basta)	Added after sterilization	Added prior to sterilization
Transfer to light	2 days after bombardment	4 weeks after bombardment
Transgenic plant recovery	19 plants/1800 calli	3 clones/900 bulblet slices

Transient GUS expression was measured by counting the number of blue spots per explant 5 days after bombardment. The number of transient events observed in the 1x1100 treatment was twice as high as in the 3x1800 (Figure 1).

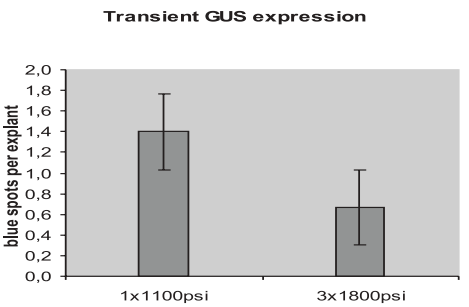

Figure 1. Transient GUS expression 5 days after bombardment. A single bombardment at 1100psi (1x1100psi) resulted in twice the number of transient expression events compared to the treatment with 3 shots at 1800psi (3x1800psi). Vertical bars show standard error

The construct containing the FBP1::SUP cassette could not be accessed for transient expression because it does not contain a reporter gene. Explants were maintained in darkness for 4 weeks and then transferred to dimmed light conditions (ca. 30 μE s^{-1} m^{-2}) for an additional week before they were exposed to direct light (ca. 60 μE s^{-1} m^{-2} with a photoperiod of 16 h). Addition of selective agent to the culture medium began one week after bombardment and was kept up throughout the subsequent steps.

From among the bombarded 900 explants, 3 Basta®-resistant plants were recovered. Despite the higher efficiency of the 1x1100 treatment for transient expression, these plants originated from the 3x1800 treatment.

Plants were tested for genome integration of PAT and SUP genes. Attempts to perform Southern analysis of the transgenic lily genomes were made but no conclusive results could be obtained. Alternatively, the presence of these genes was assessed by PCR. All three regenerated plants grown on 2mg L^{-1} Basta® were positive for the presence of the PAT gene, in accordance with their observed resistance. Two of them, clones 1 and 2, were positive for SUP (Figure 2). Importantly, clones 1 and 2 were derived from the same bombardment dish. Final evidence for their individual transgenic character requires Southern analysis; however, since this technique is not applicable for lily, this question remains unanswered. All clones showed a normal phenotype during the vegetative phase, although clone number three showed a slightly weaker phenotype, with fewer and thinner roots under *in vitro* conditions.

Figure 2. PCR amplification of inserted genes. PAT gene fragment was amplified in the three recovered clones, whereas the SUP gene was only present in two of the clones. Positive control (+) was carried out with the plasmid used for transformation, and wild-type genome of *Lilium longiflorum* was used as a negative control (-) of PCR reaction

Each regenerated clone was first propagated *in vitro*, vernalized for at least 90 days at 4 °C and then transferred to greenhouse conditions for acclimation in order to induce flowering. These plants were monitored for two growing seasons. The clone that showed absence of *SUP* (clone three) flowered normally in both seasons, whereas plants derived from the other two clones flowered aberrantly in the first year, stopping floral bud development at early stages, but produced normal flowers during the second season.

SUP and PAT expression was examined by northern analysis of leaves and floral meristems. The preliminary transcription profile is shown in Figure 3. Northern blot analysis using RNA derived from leaves of bialaphos resistant plants shows expression of PAT and absence of its transcription in a non-transgenic plant (Figure 3a). A transcriptional evaluation of the floral meristem of clone 2 collected in the first flowering season show SUP expression which is not observed in non-transgenic floral meristems (Figure 3b), indicating that the dicot FBP1 promoter had actively coordinated SUP expression in clone 2. Transcription of the constitutive *glyceraldehyde-3-phosphate dehydrogenase* (GAPDH) gene was used for comparison of RNA loading in the northern blots.

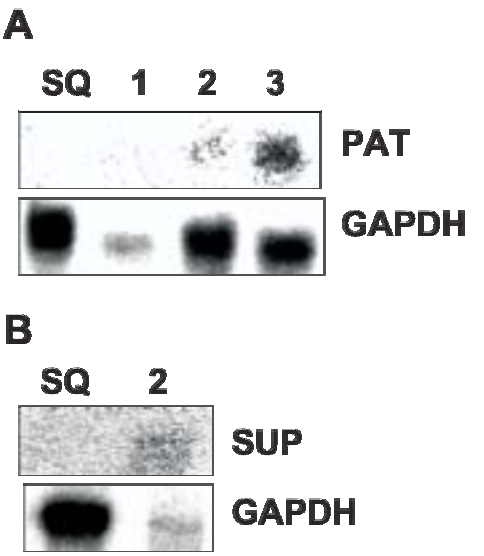

Figure 3. Northern analysis of gene expression in transgenic plants. (**A**) Total RNA isolated from leaves derived from transgenic clones (1, 2 and 3) and a non-transgenic plant (SQ) hybridised with the PAT probe. (**B**) Total RNA from floral meristems of clone 2 and from a non-transgenic plant was hybridised with a SUP fragment. Loading control is shown by hybridisation with the constitutive GAPDH gene visualised in the lower panels

Acknowledgements

The authors would like to express their appreciation to Jurriaan Mes for his efforts during the early stages of this work and to Santie de Villiers for valuable suggestions and discussions. This research was supported by CNPq, the Science and Technology Development Organ of the Brazilian Government (200851/98-5 GDE) and by the Dutch Ministry of Agriculture, Nature Management and Fisheries (DWK 364).

Transformação de *Lilium longiflorum* via bombardeamento de partículas e produção de plantas resistentes a herbicida

RESUMO - *Este trabalho é uma contribuição para melhorar a metodologia de transformação de lírio (*Lilium longiflorum *Thunb.). Um vetor com o gene SUPERMAN de* Arabidopsis *dirigido pelo promotor FLORAL FLORAL BINDING PROTEIN 1, além do cassete com o gene fosfinotricina acetiltransferase de resistência ao herbicida bialafós com o promotor CaMV35S, foi usado para transformar secções de bulbilhos por bombardeamento de partículas. Os objetivos foram melhorar os parâmetros de transformação para a transformação de lírio no intuito de atingir maior eficiência, bem como criar novos fenótipos florais de lírio usando-se fatores de transcrição originários de espécies dicotiledôneas. Foi possível obter plantas transgênicas expressando resistência* in vitro *ao bialafós. As plantas transgênicas foram transferidas para casa de vegetação, cultivadas e monitoradas por duas estações de florescimento. As flores derivadas dessas plantas mostraram-se com fenótipo normal e indistinguível das flores de tipo selvagem.*

Palavras-chave: modelo ABCDE, desenvolvimento floral, gene homeótico, lírio, fator de transcrição.

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