Oriental Lily Hybrids Engineered to Resist Aphid Attack

F.A. Krens[a], T.R. Menzel, Chang Liu, D.C.T. Dees and B.C.E. van Kronenburg
Wageningen UR Plant Breeding
P.O. Box 16, 6700 AA Wageningen
The Netherlands

Keywords: *Agrobacterium tumefaciens*, transformation, marker-free, regeneration, insect resistance

Abstract

Establishing in vitro bulb scale cultures of lily cultivars followed by callusing and regeneration after gene transfer was found to be not very successful in our hands, except for 'Snow Queen'. Identifying a more generally applicable system to generate callus with the ability to regenerate and amenable to *Agrobacterium*-mediated gene transfer was the goal of the research described here. Callus was induced on style and filament explants of 26 cultivars of lily. The cultivars were chosen from the hybrid groups longiflorum, asiatics and orientals of the genus *Lilium* but also interspecific hybrids were represented. Most cultivars were diploids but some were of triploid level and one was tetraploid. In general once callus was induced, it could relatively easily be maintained and propagated for further use. Regeneration was observed on both callus types from all cultivars tested with an efficiency ranging from 40 to 100%. Gene transfer as demonstrated by positive reporter gene *uid*A activity was found in all cultivars tested. Transgenic plants could be obtained in the first series of transformations and the applicability of a marker-free system was proven in lily. The protocol can now be used for the introduction of genes aiming at conferring resistance to aphids.

INTRODUCTION

Improvement of crops is generally achieved by breeding. In order to be successful in breeding programmes several requirements need to be met, such as the availability of genetic variation in the germplasm of the species, the absence of crossing barriers, fast generation cycles allowing multiple crosses within a year and self-compatibility in order to reduce linkage drag by back-crosses. In many bulbous, monocotyledonous ornamental crops these conditions are not ideally present and success depends on the skills and patience of the breeders. An alternative is provided by the technology of genetic modification. In this way, well-characterized genes can be introduced directly into existing elite cultivars, maintaining their good quality traits and adding desired new traits. Crossing barriers and long generation times are avoided, as is the negative effect of linkage drag. Prerequisites are a gene transfer protocol for the crop of interest and genes coding for the desired traits.

Insects such as aphids are the major animal vectors for the spread of viruses in lily cultivation. Viral infections in lilies lead to a decrease in bulb and flower quality provoking a significant negative impact on the economic value of the crop. Pyrethrins and mineral oil are the main chemicals used to fight viral infestations mediated by aphids. The availability of insect-resistant cultivars would provide means to reduce the use of chemicals and to allow a more sustainable cultivation. Proteinase inhibitors have been used successfully in engineering insect resistance against feeding or sucking insects, such as aphids and thrips, after introduction of the appropriate genes. Genes coding for volatile repellents were also found to be effective by deterring insects. In a dual approach, a gene coding for a proteinase inhibitor will be combined with a gene coding for a monoterpenoid repellent in our marker-free binary vector, pMF2, and used for gene transfer to lilies.

[a] frans.krens@wur.nl

MATERIALS AND METHODS

Plant Material

A large number of cultivars, representing several hybrid groups within the genus *Lilium*, e.g. longiflorums, asiatics and orientals, but also interspecific hybrids varying in ploidy level, such as OTs, LAs and LOs were used (Table 1).

Flowering stems, carrying still closed flower buds were taken. The flower buds were freed from the stems, briefly dipped in 70% (v/v) ethanol and quickly flamed. Subsequently, the buds were opened aseptically by forceps and scalpel and the carpels and stamens were taken out. After removal of the stigmas and anthers respectively, the remaining styles and filaments were cut in 1 cm pieces and placed on callus induction medium.

Callus Induction and Regeneration

The callus induction medium (CIM) consisted of MS salts and vitamins (Murashige and Skoog, 1962) supplemented with 30 g/L sucrose, 3 g/L gelrite and 8.3 µM picloram, pH 5.8. Regeneration ability was tested by transferring calli to MS medium with 30 g/L sucrose, 3 g/L gelrite, 0.4 µM picloram and 0.044 µM 6-benzyladenine (BA), pH 5.8. 25 Pieces of callus were placed on regeneration medium (RM) per dish and 4 dishes were prepared for each cultivar. Scoring for regeneration was done after 5 weeks on RM.

Transformation and Selection

Transformation was essentially done as described by Hoshi et al. (2004) with minor modifications. Wounding was achieved by cutting and chopping the calli with a scalpel. Selection was on RM supplemented with 250 mg/L cefotaxime and 150 mg/L timentin for agrobacterial knockout and with 15 mg/L hygromycin for selection of putative transgenic callus and regenerants.

The bacterial strain used was AGL0 (Lazo et el., 1991) carrying as binary vectors, pCAMBIA1301 (CAMBIA, Australia) or pRCNG (Schaart et al., 2004). The last vector allows selectable marker excision after induction of recombinase activity leading to subsequent GUS expression. Activation of the recombinase in lily was achieved by subjecting small scales from transgenic lily plantlets to an overnight treatment with 25 µM dexamethasone (DEX). Subsequent regeneration was done on RM supplemented with 250 mg/L fluorocytosine (FC) in order to select for successful recombination of the T-DNA.

Characterization of Putative Transgenic Plants

1. GUS Histochemical Staining. GUS histochemical staining was done according to Jefferson et al. (1987).

2. PCR. DNA was isolated from plantlets according to Doyle and Doyle (1987) with 2% (w/v) polyvinylpyrrolidone-10 in the DNA extraction buffer. Molecular characterization was done by performing PCRs on the *hpt* gene (hygromycin selectable marker) and the *uid*A gene (GUS reporter gene). The primers used were *hpt* Forward TGGGGAGTTTAGCGAGAGCCTGAC; *hpt* Reverse GCGCGTCTGCTGCTCCATAC AAG and *uid*A Forward TACACCACGCCGAACACCTG; *uid*A Reverse CCGCA TCTTCATGACGACCA. The amplification protocol consisted of 5 min. 94°C, 35×30 s 94°C, 30 s 55°C, 1 min. 72°C, followed by 10 min. at 72°C. Amplified fragments were visualized by EtBr staining after gel electrophoresis on 1.5% (w/v) agarose.

RESULTS AND DISCUSSION

Callus Induction and Regeneration

Callus induction on styles and filaments on the described CIM proved to be generally very efficient, although a few cultivars were found to perform slowly in the

induction of callus and also slowly in subsequent growth of the generated callus (See Table 1, category LL). From three cultivars, i.e. 'Manissa', 'White Heaven' and 'Yellow Diamond', no callus could be obtained in the first attempt, but the latter two proved successful in a second attempt albeit in the LL category. All calli of both types, once established, could be propagated and maintained on CIM, until further use in regeneration or transformation experiments. Most material was originally harvested from filament explants because the number of explants that could be obtained from styles was less. A lily flower contains 1 style and 6 anthers.

Table 2 shows the percentages of calli showing bulblet or shoot formation for the cultivars that were put on RM. Regeneration ability ranged from 40 to 100% and was observed on calli derived from styles as well as from filaments.

Production of Transgenic Lily Plants

In a first series of experiments, calli from the cultivars 'White Fox', 'Barbados' and 'Marrero' were compared to the standard system of our institute consisting of callus derived from in vitro bulb scales of cultivar 'Snow Queen' (Benedito et al., 2005). *Agrobacterium* strains AGL0(pCAMBIA1301) and AGL0(pRCNG) were used for inoculation. Table 3 shows that putative transgenic plants were obtained with both strains and on all cultivars, however, differences in efficiency were obvious and not all combinations were successful. Transgenic plants obtained after transformation using the vector pRCNG do not stain positively in a GUS assay because the *uid*A coding region is not combined with a promoter (Schaart et al., 2004). Recombination and excision of the selectable markers will lead to a 35S promoter – *uid*A gene combination that will result in GUS positive staining. After the induction of recombinase activity by a DEX treatment and subsequent regeneration on FC-containing medium of transgenic 'White Fox' and 'Snow Queen' bulb explants generated by AGL0(pRCNG), GUS positive plantlets were obtained, proving the applicability of the marker-free system based on recombination in lily.

In a second series of experiments a selection of seven cultivars was used in transformation. Three dishes carrying 25 growing callus pieces each were taken from each cultivar and from filament and style origin both. Inoculation was with AGL0(pCAMBIA1301); chopping in the agrobacterial suspension resulted in 10 dishes with approximately 40 callus pieces from each type per dish per cultivar. After the seven day co-cultivation period half of the number of calli was placed on selection medium and half was used for GUS staining. The results as presented in Table 4 showed that gene transfer is possible in all cultivars and in both callus types. Four weeks later, GUS positive staining was reproduced in new material after selection. Selection is continued.

CONCLUSIONS

Establishing in vitro bulb scale cultures of lily cultivars followed by callusing and regeneration after gene transfer was found to be less successful in our hands, except for 'Snow Queen'. Generating callus with the ability to regenerate and amenable to *Agrobacterium*-mediated gene transfer could be done reproducibly in many cultivars representing several sections of the genus *Lilium* and interspecific hybrids using flower parts such as filaments and styles. This opens up possibilities for introducing genes-of-interest directly in elite cultivars that can also be used for further breeding. This work is continued with the introduction of genes aiming at conferring aphid resistance and thus preventing spread and negative effects of viral diseases.

ACKNOWLEDGEMENTS

This work was funded by the Dutch Commodity Board for Horticulture, grants nos. PT11053 and PT12966. The lily cultivars were made available by the Dutch breeding companies Gebr. Vletter & Den Haan, Rijnsburg, Mak Breeding, Wieringerwerf, De Jong Lilies, Andijk, Van Zanten Flowerbulbs, Hillegom, Marklily, Hillegom and World Breeding, Breezand.

Literature Cited

Benedito, V.A., van Kronenburg-van der Ven, B.C.E., van Tuyl, J.M., Angenent, G.C. and Krens, F.A. 2005. Crop Breed. Appl. Biotechnol. 5:259-264.

Doyle, J.J. and Doyle, J.L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf material. Phytochem. Bull. 19:11-15.

Hoshi, Y., Kondo, M., Mori, S., Adachi, Y., Nkano, M. and Kobayashi H. 2004. Production of transgenic lily plants by *Agrobacterium*-mediated transformation. Plant Cell Rep. 22:359-364.

Jefferson, R.A. 1987. Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol. Biol. Rep. 5:387-405.

Lazo, G.R., Stein, P.A. and Ludwig, R.A. 1991. A DNA transformation-competent *Arabidopsis* genomic library in *Agrobacterium*. Nat. Biotechnol. 9:963-967.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.

Schaart, J.G., Krens, F.A., Pelgrom, K.T.B. and Rouwendal, G.J.A. 2004. Effective production of marker-free transgenic strawberry plants using inducible R/Rs site specific recombination and a bifunctional selectable marker gene. Plant Biotechnol. J. 2:233-240.

Tables

Table 1. List of lily cultivars and responses for callus induction on filament and style explants.

Cultivar	Type	Genome + ploidy	Induction/ growth rate	Cultivar	Type	Genome + ploidy	Induction/ growth rate
Barbados	O	OO	H/H	Regata	A	AA	I/I
Brindisi	LA	ALA	L/L	Robina	OT	OOT	I/I
Burlesca	O	OO	I/H	Santander	O	OO	H/H
Cherbourg	O	OO	I/H	Sheila	O	OO	I/H
Gracia	O	OO	H/H	Snow Queen	L	LL	n.t.
Lake Carey	O	OO	H/H	Sorbonne	O	OO	I/H
Legend	O	OO	H/inf.	Topwhite	O	OOOO	L/I
Lesotho	OT	OOT	L/I	White Express	O	OO	L/L
Lexus	O	OO	I/I	White Fox	L	LL	H/H
Manissa	OT	OOT	0	White Heaven	L	LL	L/L
Marrero	O	OO	I/I	White Triumph	LO	LLO	L/L
Montezuma	O	OO	H/H	Yellow Diamond	LA	ALA	L/L
Paradero	O	OO	I/H	Yelloween	OT	OT	L/I

O=Oriental; A=Asiatic; L=longiflorum; T=Trumpet; H=High; I=Intermediate; L=low; inf.=infected.

Table 2. Regeneration response of the two callus types of the cultivars tested.

Cultivar	Regeneration filament type (%)	Regeneration style type (%)
Barbados	76	40
Burlesca	66	72
Cherbourg	40	72
Gracia	68	74
Lake Carey	57	79
Legend	62	64
Lexus	72	n.d.
Montezuma	84	60
Paradero	40	68
Robina	61	40
Santander	100	74
Sheila	73	90
Topwhite	66	68
White Express	74	n.d.
White Triumph	n.d.	80
Yelloween	62	n.d.

n.d.: Growth is slow and no sufficient amount of callus was available; cultivars not listed, ibid.

Table 3. Efficiency of *Agrobacterium*-mediated transformation in four lily cultivars of filament-derived callus, first series.

Cultivar	Construct	Number of GM shoots	GUS staining	PCR gus	PCR hpt
Barbados	pCambia1301	3	+	+	+
	pRCNG	0			
Marrero	pCambia1301	1	n.d.	n.d.	n.d.
	pRCNG	0			
White Fox	pCambia	6	+	+	+
	pRCNG	6	+/-*	n.d.	n.d.
Snow Queen	pCambia1301	14	+	+	+
	pRCNG	8	+/-*	+	+

*= with pRCNG GUS-staining can only be observed after recombination has occurred.

Table 4. Results of GUS staining after the co-cultivation period of 7 days, second series.

Cultivar	No. of calli stained; filaments/styles		%-age of GUS + calli (no. of spots); filament	%-age of GUS + calli (no. of spots); style
Barbados	200	200	3.5 (31)	0.5 (1)
Gracia	100	100	3 (7)	7 (24)
Lake Carey	200	200	5 (23)	11.5 (79)
Santander	200	150	2 (11)	4.6 (20)
Sheila	100	100	10 (17)	6 (8)
Sorbonne	200	200	1 (5)	1 (2)
Yelloween	200	150	5.5 (44)	1.3 (10)