LILY REGENERATIVE CALLUS AND CELL CULTURES FOR TRANSFORMATION

A. Tribulato
Institute of Horticulture and Floriculture
University of Catania
Via Valdisavoia, 5-95123-Catania
Italy

P.C. Remotti and H.J.M. Löffler
Centre for Plant Breeding and Reproduction Research (CPRO-DLO)
Department of Ornamental Crops
P.O.Box 16, 6700 AA, Wageneingen
The Netherlands

Abstract

Callus cultures were established from different lily types (Easter lily and Oriental hybrids). Pistils and flower pedicels were incubated on MS-medium supplemented with 2,4-D, NAA, dicamba or picloram in various concentrations. 'Star Gazer' and 'Snow Queen' formed callus easily on medium with dicamba picloram or. Friable calli clumps of 'Snow Queen' were suspended in liquid medium. The resulting suspension consisted of small cell aggregates rather than loose cells and the presence of embryo like structures (ELS) was noticed. On solidified media, with and without plant growth regulators, germination of ELS was checked. Plant regeneration occurred on NAA and hormone-free media. Gene transfer experiments with the particle deliver system were carried out in order to compare the activity of CaMV 35S, rice Actine and maize Ubiquitin promoters in suspension callus and cell cultures of Easter lily 'Snow Queen'. To this goal constructs harbouring the gus reporter gene were used. β-glucuronidase expression was studied and the three promoters were found to be active in suspension cultured calli clumps and cells.

Key words: *Lilium longiflorum*, somatic embryogenesis, dicamba, particle gun, DNA, GUS gene expression.

1. Introduction.

The successful application of various techniques for plant breeding are based on the possibility to clone plant material To this purpose, as for in vitro selection, protoplasts fusion and gene transfer experiments, regenerative callus and cell suspension cultures are used world-wide. Cell suspension cultures are generally initiated from compact (defined as type I) or friable callus, of which friable callus (defined as type II) is generally considered to be the most suitable for cells release and induction of fast growing cell lines when suspended in liquid media. Plant regeneration occurs from cell suspension cultures via somatic embryogenesis, embryo like structures germination or organogenesis. When cell suspension cultures are established from type II callus, regeneration via somatic embryogenesis is most likely to occur. Somatic embryogenesis has the advantage of the probable single cell origin for regenerated plants and the high regeneration rate even from long-term cultures. In literature, for genetic transformation of callus or cell suspension cultures, somatic embryogenesis is described as a suitable

method for regeneration of transformants (Kamo *et al.*, 1995). When protocols for callus induction and plant regeneration are defined, the assessment of all the parameters which influence the successes of transformation techniques are necessary. Most techniques for gene transfer are based on *Agrobacterium tumefaciens* mediated transformation, eletctroporation, electrophoresis (Griesbach, 1994) or particle deliver system. The latter method is the most suitable for monocotyledons like lily. Particle deliver system was successfully used for transformation of a number of monocot flower bulbs as tulip via flower stem sections (Wilmink *et al.*, 1996), lily via pollen transformation and following cross pollination (Van der Leede-Plegt *et al.*, 1992) and gladiolus via embryogenic cell suspension and cormel slices (Kamo *et al.*, 1995). In the present paper we describe a protocol for the establishment of a regenerative cell suspension in which the occurrence of embryo like structures is shown. Particle bombardment was applied to the cell suspensions to check the activity of rice *Actine* CaMV 35S and maize *Ubiquitin* promoters aiming at future stable transformation. The expression of the *gus* reporter gene was studied to evaluate the differerent activity of the three promoters in suspension cultured calli clumps and cells.

2. Materials and Methods

2.1 Establishment of callus and cell suspension cultures

Callus cultures were induced from sliced flower pedicels and inner flower organs from greenhouse grown plants of *Lilium longiflorum* 'Snow Queen', 'Gelria' and 'White American' and the Oriental Hybrid 'Star Gazer'. Flower pedicels were sterilized in 80% alcohol solution for 2 minutes followed by 20 minutes in a 2% sodium hypochlorite solution, rinsed three times in distilled sterile water, sliced and placed on callus induction media. Flowers in pre-anthesis were submerged in 80% alcohol solution for a few seconds and flamed for surface sterilisation. All inner flower organs were incubated for callus induction culture. Explants were incubated in the dark at 25°C. MS salts medium with vitamins was supplemented with 30 μM adenine sulphate, 3 μM thiamin-HCl, 3% sucrose, 0.3% Gelrite. Various auxins in the following concentrations were used:5 μM and 25 μM NAA, 2 μM and 10 μM 2,4-D, Dicamba and Picloram.

Cell suspension cultures were initiated form friable callus grown from pistils or flower pedicels of *L. longiflorum* 'Snow Queen' on medium supplemented with 2 μM Dicamba. The callus was transferred monthly to fresh medium. When friable callus segments occurred, small calli clumps were transferred to the same medium without Gelrite and placed on a gyratory shaker at 100 rpm, 25°C and dark condition. Released cells of the suspension culture were sampled every two weeks for microscopic observation by staining with FDA. Culture medium was completely refreshed every two weeks by separating the more heavy calli and cells from the debris and dead cells by centrifugation at 500 rpm for 5 min at 25°C. In this cultures, embryo like structures formation occurred either from free floating cells or on the clumps. Embryo like structures were transferred from liquid culture medium to filter paper on 0.3% Gelrite solidified media with different hormones for germination test (Table 2.).

2.2 Particle bombardment

Suspension cultured cells and calli clumps were placed in 6-cm petri dishes in such a way that the surface of the plate was completely covered. Suspension cultured calli clumps and cells were kept wet by covering them with filter paper soaked in liquid callusing medium. Three different plasmids harbouring the *gus* reporter gene were tested: pCal1GC with CaMV 35S promoter, pAct1-F with rice *Actin* promoter and pAHC25 with maize *Ubiquitin* promoter (Christensen *et al.*, 1992). In the three plasmids the promoters were in combination with the maize *adh1* intron and with NOS termination signals (Wilmink *et al.*1992). M10 tungsten particles (average diameter 0.75 μm) coated with DNA (Klein *et al.*, 1987), were delivered into the tissues using a DuPont Particle Deliver System by helium pressure (1100 Psi). Bombarded tissues were incubated at 24° C in the dark. After two days *gus* gene expression was evaluated. Explants were incubated in a solution of 0.5 M $NaPO_4$ (pH 7.5), 2% NaN_3, 10% Triton, 0.5 M EDTA, 0.5 M ferrocyanide, 0.5 M ferricyanide containing 20 mg/ml X-gluc and kept over night at 37°C. After staining, explants were rinsed in 50% and 75% ethanol for 30 min and kept in 96% ethanol, to count the blue spots.

3. Results

3.1 Callus induction and cells suspension culture

The tested cultivars reacted differently upon the auxin treatments. Bulblet formation occurred on all the explants (Table 1). Roots were formed on media containing 25μM NAA from pistils and ovary slices of 'Snow Queen' and 'White American', respectively. Two different types of callus were observed: type I was compact and originated from all the explants and friable (type II) callus was obtained two months after culture onset only from flower pedicels or pistils of 'Snow Queen' and 'Star Gazer' cultured on media with 2 μM Dicamba or Picloram. After three months of subculture, a few grams of friable callus of 'Snow Queen' were transferred to liquid medium for cell suspension initiation. The suspension was initially composed of bigger cell aggregates that released cells continuously. Cultures grew rapidly, doubling in volume every two weeks. Six weeks after culture onset, the big cell aggregates were removed because of the continuous formation of roots and shoots. Thereafter, the cell suspension, which remained undifferentiated, was composed only of small cell clumps and loose cells floating in the medium. Two different cell types were observed by microscopic observation of cell suspensions stained with FDA. One type consisted of large, vacuolated, elongated cells that hardly showed cell division while the second type consisted of smaller, round, with an apparent nucleus that underwent rapid cell division and containing starch grains. A few weeks after removal of the bigger clumps, embryo like structures (ELS) formation were observed attached to the clumps or floating freely in the medium. When placed on filter paper on solidified MS-V and MS-N media, the ELS germinated readily forming complete plantlets (Table 2). The presence of auxins induced callus formation from ELS. From this callus, plants regeneration occurred through organogenesis. Multiple shoots formation was observed only when ELS were placed on MS-B medium (Table 2).

Table 1: Response of explants of different lily genotypes to auxins in different concentrations

Explant type		Flower pedicels				Stamen				Pistil				Ovary slice			
Auxin	Cultivar	SQ	Gl	WA	SG	SQ	Gl	WA	SG	SQ	Gl	WA	SG	SQ	Gl	WA	SG
NAA	25 μM	B	-	B	-	-	-	-	-	B,R	-	B	-	B	-	B,R	-
NAA	5 μM	-	-	-	-	-	B,C	-	B	C	B,C	-	-	-	-	-	B
2,4-D	10 μM	B	-	B,C	-	-	-	-	-	-	-	-	-	-	-	-	-
2,4-D	2 μM	-	-	B,C	-	-	-	-	-	-	-	-	-	-	B,C	-	-
Dicamba	10 μM	-	-	-	-	-	C	-	-	F	-	-	-	-	-	-	-
Dicamba	2 μM	B	-	-	F	-	-	-	-	-	-	-	-	-	-	-	-
Picloram	10 μM	-	-	-	-	-	C	-	-	F	-	-	-	-	-	-	-
Picloram	2 μM	B	-	-	F	-	-	-	-	-	-	-	-	-	-	-	-

SQ- Snow Queen; Gl- Gelria; WA- White American; SG- Star Gazer
B- bulblets; R- roots, F- friable callus, C- compact callus, - no reaction

Table 2 - Response of embryo like structures placed on MS media containing vitamines (MS-V), dicamba (MS-D), NAA (MS-N) and BA (MS-B). (n=22)

Explants response Media	Regeneration [%]	Callus formation and organogenesis [%]	Multiple-shoot formation [%]	Callus formation [%]	No reaction [%]
MS-V[2]	78	-	-	-	22
MS-D[3]	-	100	-	-	-
MS-N[4]	55	25	-	10	10
MS-B	-	21	62	4	13

[1]- MS (basal salts incl.vit.) + 30 μM adenine sulphate, 3 μM thiamin HCl, 30 gl^{-1} sucrose.
[2]- MS (basal salts incl.vit.) + 2 μM Dicamba, 30 μM adenine sulphate, 3 μM thiamin HCl, 30 gl^{-1} sucrose.
[3]- MS (basal salts incl.vit.) + 0.5 μM NAA, 60 g l^{-1} sucrose.
[4]- MS (basal salts incl.vit.) + 0.4 μM BA, 10 g l^{-1} glucose, 35.4 gl^{-1} mannitol.

3.2 Particle bombardment

According to the histochemical GUS assay the three promoters tested expressed activity in all the samples treated. The *gus* gene was expressed with a higher number of blue spots under the control of rice *Actine* and CaMV 35S promoters than under the control of maize *Ubiquitin* promoter (Table 3). Because of the high variance due to the method itself and the different reaction of all the samples, no significant differences were found when analysis of the variance was applied to the results.

Table 3: Absolute and mean (*) values representing activity of *Act*, CAMV 35S and *Ubi* promotors in cell suspensions of *Lilium longiflorum* 'Snow Queen'. Values for *gus* gene activity are expressed as number of blue spots per plate (n=5).

Repetitions	I	II
Promotors		
Actin	101 (20)	252 (50)
CaMV 35S	286 (57)	154 (30)
Ubiquitin	62 (12)	48 (9)

4. Discussion

Two types of callus were formed from lily explants. A strong interaction among the explants, genotypes and culture conditions was reported. Friable callus was obtained from flower pedicels and pistils, and among these two explants the flower pedicels appeared to be more suitable since type II callus occurred in two different genotypes (Table 1).

Genotype effect was noticed on the callus formation. This effect was not due to to the botanical species or hybrid group but it was related to the genotype itself. Friable callus, in fact, was obtained from *L. longiflorum* 'Snow Queen' and the Oriental Hybrid 'Star Gazer' which are two genotypes belonging to two different groups and no type II callus formation was obtained from *L. longiflorum* 'Gelria' and 'White American'.

The effect of the hormones showed that with low concentrations of dicamba and picloram friable callus occurs form flower pedicels and pistils of 'Snow Queen' and 'Star Gazer' while with 2,4-D and NAA organogenesis occurred in all the genotypes and explants. Previous of dicamba, in comparison to other auxins, described it as a suitable growth regulator for friable callus formation and induction of somatic embryogenesis in maize (Furini *et al.*, 1994, 1995).

Two cell types were observed floating in the medium. One type was small round and rich of cytoplasm in which the presence of starch was noticed while the other was big with large vacuolated cells. The round small cells tended to organise into cell clusters forming round globular structures which can be considered ELS. When kept in the same culture conditions these newly formed ELS underwent callus formation, in the continuous presence of the auxin. This callus released both big and small embryogenic-like cells and the occurrence of ELS around it was noticed. Similar cell characteristics for embryogenic cell suspensions have been described (Remotti, 1995). The presence of starch is often related with embryogenic cells and is generally considered to be an indicator of development towards somatic embryos (Schwendiman *et al.* 1988).The bigger type of cells were found floating in the culture medium and making part of the callus but hardly any cell division activity was noticed.

From the present results we can conclude that flower pedicels are suitable explants for friable callus formation when cultured on either dicamba or picloram containing media in low concentrations (2 μM). The success of friable callus production is also determined by the genotypes. When regeneration of ELS was tested, the typical

behaviour of somatic embryo germination was noticed. Plant regeneration occurred when ELS were placed on filter paper on MS hormone-free medium with first a small heary root followed by a single cotyledon-like shoot. In the near future tissue microsections will be made to confirm two different meristematic areas in the ELS.

The described cell suspension appeared to be suitable for transformation via particle gun. The *gus* gene was expressed in fact in loose cells, calli clumps and ELS. Aiming at transformation, the expression of the *gus* gene in both loose cells and calli clumps is promising for selecting the transformed cells on selective agents and to regenerate plants via ELS germination. In contrast to previous reports, in which CaMV 35S showed less activity in lily leaves compared to rice *Act* and maize *Ubi* (Wilmink *et al.*, 1995), in the present work better expression of the *gus* gene was scored under the control of CaMV35S and rice *Actine* promoters than maize *Ubiquitin* promoter after particle bombardment of cell suspension cultures. CaMV 35S was previously found to be not active in *Lilium longiflorum* pollen (van der Leede-Plegt *et al.,* 1992) but from the present data we can conclude that both *Act* and 35S showed to be suitable for transformation of this *L. longiflorum* cell suspension.

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