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Increasing Transient and Subsequent Stable Transgene Expression in Chrysanthemum Following Optimization of Particle Bombardment and *Agro*infection Parameters

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Abstract

Transient and stable transgene expression was maximized in *in vitro* and greenhouse-derived chrysanthemum 'Lineker' and 'Shuhou-no-chikara' stem explants. In *Agro*infection, *Agrobacterium tumefaciens* LBA4404 - with either pBI121 or pKT2 - or AGL0 (with pKT3) were utilized, while in particle bombardment pSKGN1 was utilized. Transformation efficiency was affected by both the gene introduction method and its experimental parameters and the origin and developmental state of the explant. There was a decrease in GUS focal points with a simultaneous increase in the number of blue staining areas for both 'Lineker' and 'Shuhou-no-chikara' in both *Agro*infection (pKT2 and pKT3) and particle bombardment (pSKGN1) over time (0, 24, 48, 72 h and 1, 2 and 4 weeks). GUS transformation efficiency was 6.6, 26.89, 25.0 and 11.84% for *Agro*infection, bombardment, sonication and *Agro*listics, respectively for 'Lineker', and 0, 2.17, 0 and 7.69%, respectively for 'Shuhou-no-chikara', the highest level of stable GUS transgene expression occurring in the venation.

Key words: *Agro*infection, *Agro*listics, biolistics, *Dendranthema* × *grandiflora*, sonication.

Abbreviations

BSA, blue staining area; GFP, GUS focal point; SAAT, sonication assisted *Agrobacterium*-mediated transformation.

Introduction

Chrysanthemum, *Dendranthema* × *grandiflora* (Ramat.) Kitamura is the most important cut flower crop in Japan (economically and culturally-speaking), and one of the most popular cut flower crops and pot-plants in the world. Due to this importance, the introduction of useful traits by recombinant DNA technology has profound economic consequences on global floricultural markets. The use of selector and marker genes to initially study the transient transgene expression is an important first step to the attainment of stable genetic transformants, as it allows one to visualize and quantify marker gene expression, prior to its substitution by a gene of interest.

Reports on the successful genetic transformation of chrysanthemum remain scarce, and are spontaneous rather than routine. Even though transformation studies on chrysanthemum exist, only a single report in which *Dendranthema* transformants were obtained by particle bombardment exists (Yepes *et al.*, 1995), another by *Agrobacterium rhizogenes* (van Wordragen *et al.*, 1992) while all others are by *A. tumefaciens* (Firoozababy *et al.*, 1991; Ledger *et al.*, 1991; Renou *et al.*, 1993; Chasan, 1994; Courtney-Gutterson *et al.*, 1994; de Jong *et al.*, 1994; Pavingerová *et al.*, 1994; Urban *et al.*, 1994; Benetka and Pavingerová, 1995; de Jong *et al.*, 1995; Fukai *et al.*, 1995; Boase *et al.*, 1998a, 1998b; Fu *et al.*, 1998; Sherman *et al.*, 1998; Shinoyama *et al.*, 1998; Young *et al.*, 1998; Takatsu *et al.*, 1999; Tosca *et al.*, 2000). The utility of most of these transformation systems is reduced however by their genotype-specificity, and low transformation efficiencies, with regeneration and transformation capacities in various *Dendranthema* cultivars being inversely related (de Jong *et al.*, 1993). As a result of these limitations, few transgenic chrysanthemum plants containing applied genes have been obtained: resistance to tomato spotted wilt virus (Urban *et al.*, 1994; Yepes *et al.*, 1995); transgenic plants expressing *bt* toxin, *rol*C, *chs* and *afp* genes (Dolgov *et al.*, 1997) as well as

resistance to *Botrytis cinerea* (Takatsu *et al.*, 1999). The genetic transformation of cultivars such as 'Shuhou-no-chikara' has been avoided due to their low shoot regeneration capacities, despite their economic importance. The scarcity and genotype-dependence of transgenic chrysanthemum and the continuing existence of many problems and limitations in its transformation prompted us to optimize the transgene expression of spray ('Lineker') and standard ('Shuhou-no-chikara') chrysanthemums, the ultimate objective to seek cultivar-independent protocols to obtain genetic transformants and a high transformation efficiency.

Materials and Methods

Plant material: in vitro and greenhouse culture conditions

In vitro 'Lineker' and 'Shuhou-no-chikara' chrysanthemum cultivars, the former one a spray-type while the latter a disbud-type, were maintained in the greenhouse under long-day conditions, and used as initial explant material. *In vitro* shoot and plantlet cultures were maintained under a 16 h photoperiod (40 μmol m^{-2} s^{-1}) at 25 ℃ on Hyponex® (soluble fertilizer, N:P:K = 6.5:6:19; 3 g l^{-1}) medium containing 20 g l^{-1} sucrose.

Explant preparation

In vitro plantlet stem internodes were cut longitudinally into 3-5 mm long and 1-2 mm thick semi-cylindrical explants. In greenhouse plants, the terminal 10 cm of plants were harvested. Stems were surface-sterilized with a 1% NaOCl solution (1% active chlorine) for 15 min followed by three rinses with sterile distilled water, and internode tissue was sliced into 3 mm thick explants. *In vitro*-derived explants were placed cut surface down on optimized *in vitro* shoot induction medium (MSs: MS + benzyladenine (BA) 2 mg l^{-1} + α-napthalene acetic acid (NAA) 0.5 mg l^{-1} + 40 g l^{-1} sucrose; Fukai *et al.*, 1987) while greenhouse-derived explants were placed abaxial surface down onto optimized greenhouse explant shoot induction medium (MS + 1 mg l^{-1} BA + 1 mg l^{-1} NAA + 40 g l^{-1} sucrose). Following a 7-day pre-culture period with a 16 h photoperiod (40 μmol m^{-2} s^{-1}) at 25 ℃, *in vitro* or greenhouse explants were subjected to either particle bombardment or *Agro*infection, as described in the following sections.

Particle bombardment

A series of 6 trials were conducted to select optimal particle bombardment conditions, and then utilized as the routine particle bombardment conditions, in an additive manner. Pre-cultured *in vitro*-derived stem explants were subjected to two rounds of particle bombardment with or without (control) novel plasmid pSKGN1 [nos-L-*npt*II (wild); 35S-LEI-uidA; **Fig. 1A**; Kirin Breweries, Inc.]-coated

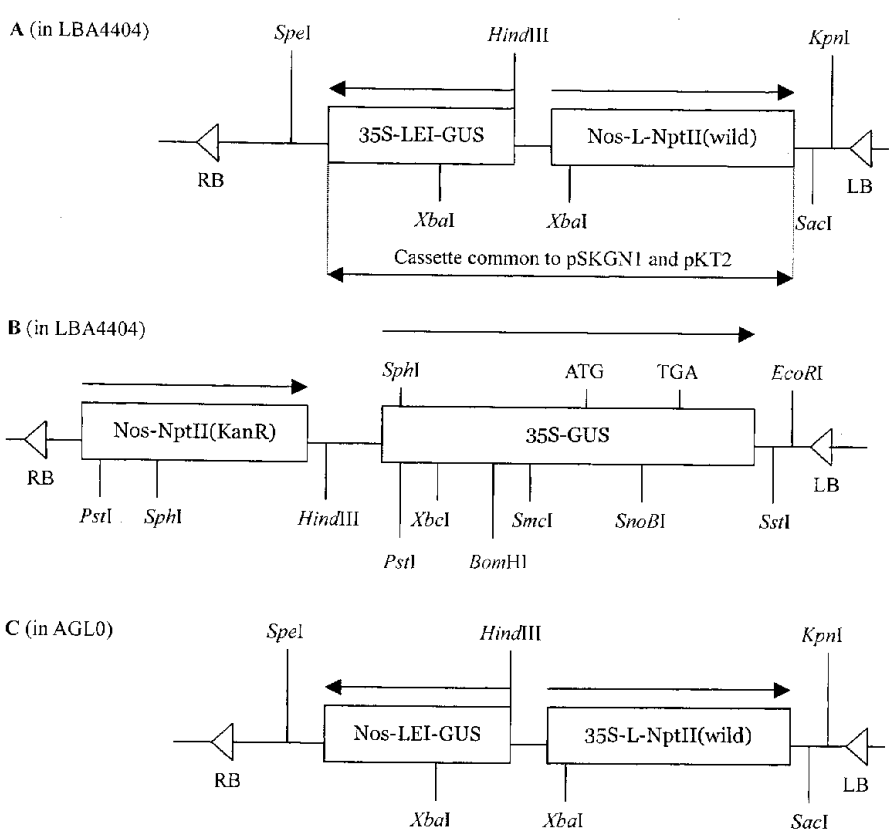

Fig.1 Derailed structure of pKT2 or pSKGN1 (A), pBI121 (B) and pKT3 (C).

1 μm gold particles (microcarriers) using the Bio-Rad Biolistic PDS-1000/He® particle delivery system (rupture pressure 1100 p.s.i.; target distance 6 cm;~0.8 μg plasmid DNA/500 μg Au microcarriers), following optimization of bombardment conditions, and according to manufacturer's conditions, when placed within a 3 cm central zone of the petri dish. Bombarded explants were placed immediately onto selective medium (MSs + kanamycin 10 mg l^{-1}) and subcultured every two weeks.

Optimization of bombardment parameters

Explants were subjected to particle bombardment utilizing optimized conditions defined above, except for those being tested.

Trial 1: Effect of number of shots (one versus two)
Trial 2: Effect of rupture disk pressure (900, 1100, or 1350 p.s.i.)
Trial 3: Effect of target distance (3, 6 or 9 cm)
Trial 4: Effect of explant source (*in vitro* or greenhouse)
Trial 5: Effect of explant source orientation (intact or cut surface down on the medium)
Trial 6: Effect of explant pre-culture period (0, 1, 3, 5, 7 or 9 days)

Agrobacterium-mediated transformation

Another series of six trials were conducted to select optimal *Agro*infection conditions [measured by maximized transgene (*uid*A) activity] and were then utilized as the routine conditions, in an additive manner. Transformation experiments were performed using two different *A. tumefaciens* LBA4404, one carrying pBI121 (nos-*npt*II; 35S-*uid*A; Vancanneyt *et al.*, 1990; **Fig. 1B**), the other carrying pKT2 [nos-L-*npt*II (wild); 35S-LEI-*uid*A; Kirin Breweries, Inc.; **Fig. 1A**] and an *A. tumefaciens* AGL0 (**Fig. 1C**), carrying pKT3 (35S-L-*npt*II; nos-LEI-*uid*A; Kirin Breweries, Inc.). *A. tumefaciens* LBA4404 and AGL0 strains were cultured in 20 ml Luria Broth medium for 16-20 h at 27 ℃ . Hereafter, 1 ml of broth culture was centrifuged and then resuspended in 1 ml 10 mM glucose supplemented with 100 mM acetosyringone and adjusted to an OD_{540}=0.4-0.5. Precultured (7 days) explants were placed on filter paper overlying non-selective medium, and bacteria were applied at 10 μl or 20 μl per explant and co-cultured for 3 or 4 days for *in vitro* or greenhouse material, respectively. Following co-cultivation explants were placed on MSs with 10 mg l^{-1} kanamycin and 250 mg l^{-1} cefotaxime (Claforan®) for 1 week, then transferred onto fresh selective medium supplemented with 10 mg l^{-1} kanamycin and 125 mg l^{-1} cefotaxime bi-monthly.

In the case of Sonication Assisted *Agrobacterium*-mediated Transformation (SAAT), explants were pre-cultured on appropriate medium for 24-36 h, then placed in 1.5 ml eppendorf tubes containing 1 ml 10 mM glucose + 100 mM acetosyringone. Tubes were placed at 27 ℃ in a styrofoam float at the center of a bath sonicator (Iuchi® Sonicator, Japan) at 60 Hz for 0-20 min. Following sonication, explants were all blot-dried on sterilized filter paper, and placed on non-selective MSs for a 2 day co-culture period with *Agrobacterium* at an OD_{540} = 0.4-0.5. *Agro*listics involved the application of particle bombardment, followed by *Agro*infection, in this order, and utilizing the optimized conditions specified above.

Explants from any *Agro*infection treatment were plated (n = 60) onto Luria Broth medium with the same selection pressure (10 mg l^{-1} kanamycin + 250 mg l^{-1} cefotaxime) in both the light (16 h photoperiod at 100 μmol m^{-2} s^{-1}) and the dark at 25 ℃ , with explant survival measured at t = 0, 24, 48 and 72 h, 1, 2 and 4 weeks following the gene introduction method.

Optimization of Agroinfection parameters

Explants were *Agro*infected utilizing optimized conditions defined above, except for those being tested.

Trial A: Effect of explant source (*in vitro* or greenhouse)
Trial B: Effect of explant orientation (intact or cut surface down on the medium)
Trial C: Effect of pre-culture period (0, 1, 3, 5, 7 or 9 days)
Trial D: Effect of co-cultivation period (0, 1, 2, 3, 4 or 5 days)
Trial E: Effect of light conditions (light or dark)
Trial F: Effect of acetosyringone (100 mM or absence)

GUS testing and histological analyses

GUS expression was measured following incubation overnight at 37 ℃ in a histochemical GUS assay (Jefferson *et al.*, 1987). Following incubation, explants were fixed and bleached in 70% EtOH and the position and intensity of GUS expression was recorded. GUS expression was measured as the number of GUS focal points (GFPs) or percentage surface blue-staining areas (BSAs) at t = 0, 24, 48 and 72 h, 1, 2 and 4 weeks following the gene introduction method. The explant survival was also checked at the same time intervals. Explants from all treatments were observed under light and scanning electron microspcopy to observe shoot formation as well as any histological changes arising from

the treatments. GUS expression was measured in old (basal), middle-aged (mid-positioned) and young (terminal) leaf tissue (3 leaves from each point) from 8 cm *in vitro* shoots (~45 and 60 days-old for 'Lineker' and 'Shuhou-no-chikara', respectively). These shoots were harvested from explants in any treatment once 2-3 or 4-5 nodes developed in 'Lineker' or 'Shuhou-no-chikara' (shorter internode length), respectively, and were placed on selective rooting medium (Hyponex® + 20 g l^{-1} sucrose + 10 mg l^{-1} kanamycin + 125 mg l^{-1} cefotaxime, the latter only in *Agro*infection). The percentage and position of GUS transgene expression was recorded, while transformation efficiency was calculated as the percentage of GUS and PCR positive plants per explant.

DNA extraction and PCR analyses

DNA was extracted according to the CTAB method (Murray and Thompson, 1980), and the presence of the *uid*A and *nptII* genes was confirmed using PCR. PCR experiments were carried out in 25 μl (final volume) and performed with TaKaRa® Taq polymerase with 0.5 mg genomic DNA samples. The synthetic oligonucleotide primer sequences GUS-1 5′-CTGTAGAAACCCCAACCCGTG 3′ and B-2 5′-GCTGTGCGTAATTACCTGACCTAA CC 3′ amplify a 21 bp fragment containing a portion of the GUS coding region while the NP-1 5′-GAGAGGCTATTCGGCTATGA-3′ and NP-2 5′-GATGCTCTTCGTCCAGATCA -3′ sequences amplify a 20 bp fragment containing a portion of the *nptII* coding region. PCR reactions were run using the TaKaRa® PCR Reagent Kit and were performed according to standard procedures in a Perkin Elmer GeneAmp PCR System 2400® thermocycler. Amplification conditions were 94 ℃ for 5 min, then 50 cycles of: 94 ℃ for 1 min, 50 ℃ for 2 min and 72 ℃ for 2 min, then finally for 72 ℃ for 10 min with a drop to 4 ℃ .

Statistical analyses

Any treatment had n = 60 (*in vitro* or greenhouse explants). Experiments were organized according to a complete randomized block design with three blocks (n = 20 per treatment). Data was analysed for significance (P = 0.05) by ANOVA with the mean separation by Duncan's New Multiple Range Test (DMRT).

Results

Bombardment trials (with pSKGN1) - Table 1

Two shots resulted in significantly higher GFPs per explant than one shot, in both 'Lineker' and 'Shuhou-no-chikara' (**Fig. 2A, D**). Two shots increased explant mortality in 'Lineker' only. A 1100 p.s.i. rupture pressure gave significantly higher GFP values than at either 900 or 1350 p.s.i. in 'Lineker', while at 900 p.s.i. 'Shuhou-no-chikara' demonstrated highest GFP values, although all three rupture pressures were not significantly different. An increase in rupture pressure resulted in a decrease in explant survival in both cultivars. A 6 cm target distance was ideal for 'Lineker', and either a 3 or 6 cm rendered high GFPs in 'Shuhou-no-chikara'. The shorter the target distance, the greater the negative impact on explant survival in both 'Lineker' and 'Shuhou-no-chikara'. *In vitro* explant material is noticeably superior to greenhouse material with 1.27 or 0.63 GFPs per explant forming in *in vitro* 'Lineker' and 'Shuhou-no-chikara', respectively versus 0.15 and 0.20 for greenhouse explants. The orientation of the explant was not significantly different when placed either adaxial or abaxial cut surface down on the medium, although large differences could be observed in shoot regeneration capacity (data not shown). Neither explant source nor orientation affected the explant survival in both 'Lineker' and 'Shuhou-no-chikara'. Pre-culture period was not important for determining stable transgene expression in 'Lineker', but a maximal GFP could be achieved with a 7 day pre-culture period in 'Shuhou-no-chikara', resulting too in increased escape formation (**Table 1**). Shortening the pre-culture period resulted in increasing explant mortality in both cultivars. *Agro*listics improved the stable transgene expression by increasing both the number of GFPs and BSAs at an early developmental stage (**Fig. 2G**).

Agroinfection trials (with pKT2) - Table 1

In vitro 'Lineker' and 'Shuhou-no-chikara' explants showed significantly higher levels of GFPs and BSAs than greenhouse explants (**Fig. 2B, C, E, H**), the latter having a lower explant survival than the former. Unlike in bombardment, placing the cut surface down on the medium of either 'Lineker' or 'Shuhou-no-chikara' explants resulted in significantly higher GFPs and BSAs than explants placed cut surface up, while explant survival was not significantly affected. A range of 3-9 days or 1-9 days pre-culture period for 'Lineker' and 'Shuhou-no-chikara', respectively, produced similar amounts of GFPs, with an increase in pre-culture period resulting in an increase in BSAs for 'Shuhou-no-chikara' only. Increasing pre-culture period resulted in increased explant survival, in both 'Lineker' and 'Shuhou-no-chikara', but also in increased escape formation (**Fig. 2J, K**). The longer

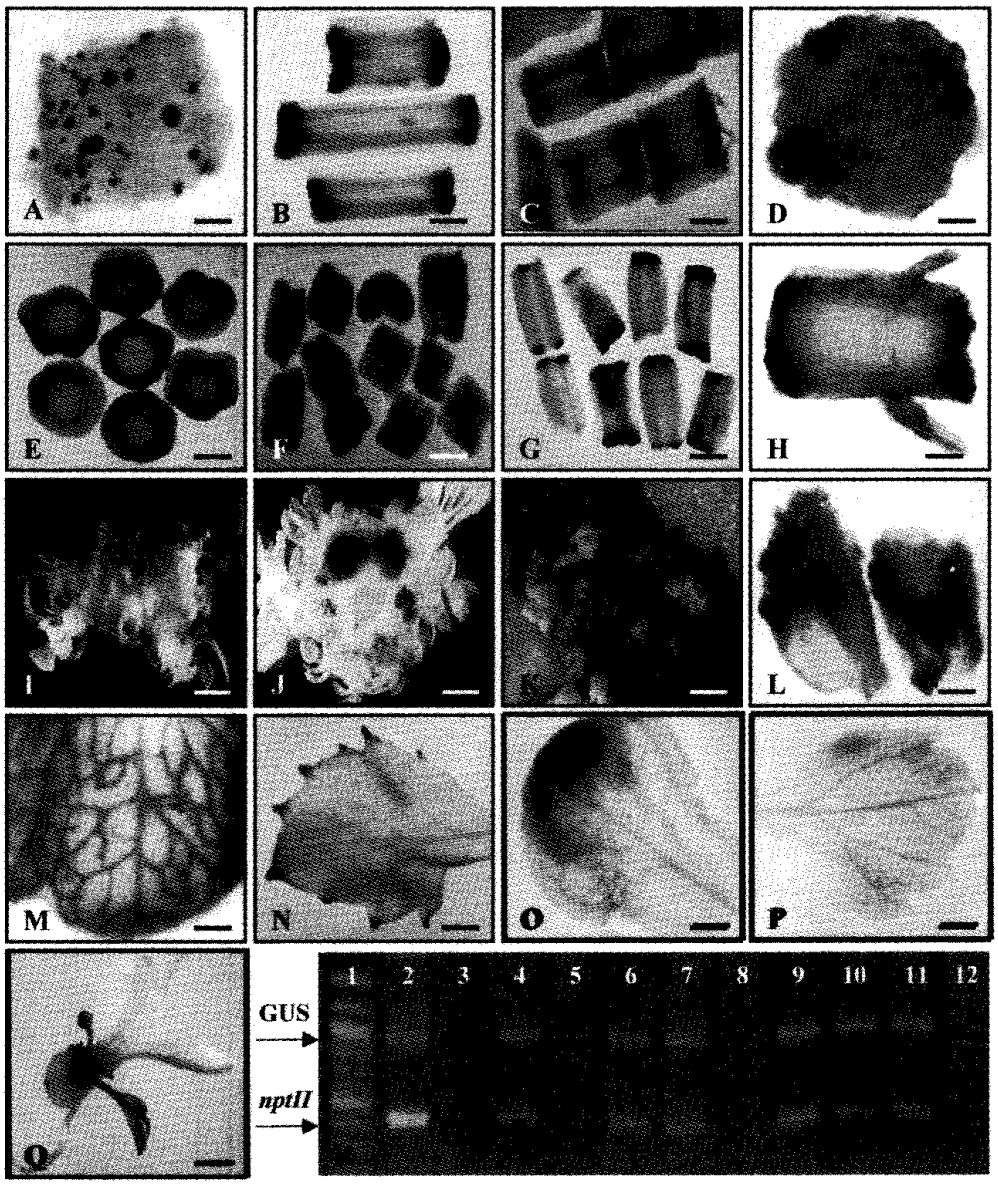

Fig. 2 Transient GUS expression in *in vitro* stem explant 72 h (A, GFPs) and 1 month (D, BSAs) after particle bombardment. *Agro*infection with pKT2 results in weak transient transgene expression (B) localized at cut surfaces only (72 h after *Agro*infection), while the use of pKT3 gives rise to stronger expression in *in vitro* (C) and young greenhouse (E) stem explants. Sonication followed by *Agro*infection with pKT2 results in widespread and uniform GUS transient transgene expression (F) but also in increased explant mortality. *Agro*listics of internode (G) and node (H) stem explants results in both GFPs and BSAs 72 h following treatment. Chimeric transgenic shoot formation after particle bombardment (I) and *Agro*infection (J) with pKT2, while putative transgenic shoots arise from GUS-positive (i.e. transformed) cut surfaces while non-transformed tissue dies (control and bleached shoots, K). The use of AGL0 (pKT3) results in a greater percentage GUS transient transgene expression (L) and explant survival on kanamycin-supplemented medium. Predominant GUS expression in venation (M), shoot tip (N), leaf edge (O) and epidermis (P) of old *in vitro* plantlet leaves. Putative 'Lineker' transformant (Q) grown on 30 mg l^{-1} kanamycin MSs containing both *gus* and *nptII* gene constructs. PCR analysis (R) of GUS transient and stable gene expression. Lane 1: Size marker; Lane 2: purified pSKGN1from LBA4404, *GUS* = 954 bp, upper band, *nptII* = 438 bp, lower band; Lane 3: Negative control, *in vitro* 'Lineker'. Transient transgene expression and stable transgene expression at t = 72 h (Lane 4) and 2 months (Lane 5) after *Agro*infected (pBI121) *in vitro* 'Lineker'; t = 72 h (Lane 6) and 1 month (Lane 7) for particle bombarded (pSKGN1) *in vitro* 'Lineker'. Negative *in vitro* 'Shuhou-no-chikara' control plant (Lane 8). Transient transgene expression and stable transgene expression at t = 72 h (Lane 9) and 2 months (Lane 10) for *Agro*infected (pKT2) *in vitro* 'Shuhou-no-chikara'; t = 72 h (Lane 11) and 2 months (Lane 12) for particle bombarded (pSKGN1) *in vitro* 'Shuhou-no-chikara'. Scale bars: 50 μm (A-H, M-P), 200 μm (I-L) and 400 μm (Q).

Table 1 Bombardment and *Agro*infection trials: effect of different treatments on GUS transient transgene expression at t=72 h

n = 60	Trial	Lineker (LIN)			Shuhou-no-chikara (SNC)		
Bombardment (pSKGN1)							
		GFP	BSA ∇	ES %*	GFP	BSA ∇	ES %*
Number of shots	**1**						
1		0.84 ± 0.31 **b**	0	100	0.97 ± 0.32 **b**	0	100
2		2.00 ± 0.74 **a**	0	83	1.34 ± 0.46 **a**	0	100
Rupture disk pressure (p.s.i.)	**2**						
900		0.11 ± 0.05 **b**	0	100	0.26 ± 0.14 **a**	0	100
1100		0.63 ± 0.20 **a**	0	92	0.23 ± 0.09 **a**	0	100
1350		0.21 ± 0.09 **b**	0	68	0.18 ± 0.06 **a**	0	77
Target distance (cm)	**3**						
3		0.13 ± 0.07 **b**	0	63	0.14 ± 0.08 **ab**	0	72
6		0.68 ± 0.17 **a**	0	93	0.38 ± 0.15 **a**	0	98
9		0.12 ± 0.06 **b**	0	100	0.09 ± 0.04 **b**	0	100
Explant source	**4**						
In vitro		1.27 ± 0.53 **a**	0	100	0.63 ± 0.26 **a**	0	100
Greenhouse		0.15 ± 0.08 **b**	0	100	0.20 ± 0.09 **b**	0	100
Explant orientation	**5**						
Cut surface down on medium		2.07 ± 0.59 **a**	0	100	1.02 ± 0.36 **a**	0	100
Intact surface down on medium		2.11 ± 0.74 **a**	0	100	0.99 ± 0.40 **a**	0	100
Pre-culture period (days)	**6**						
0		2.02 ± 0.61 **a**	0	43	1.03 ± 0.36 **ab**	0	73
1		1.95 ± 0.59 **a**	0	47	1.05 ± 0.48 **ab**	0	75
3		2.03 ± 0.63 **a**	0	68	0.97 ± 0.43 **b**	0	92
5		1.98 ± 0.59 **a**	0	98	1.18 ± 0.43 **ab**	0	97
7		2.10 ± 0.71 **a**	0	100	1.20 ± 0.52 **a**	0	100
9		1.97 ± 0.58 **a**	0	100	1.05 ± 0.38 **ab**	0	100
***Agro*infection (pKT2)**							
Explant source	**A**						
In vitro		0.17 ± 0.09 **a**	7	95	0.09 ± 0.05 **a**	10	97
Greenhouse		0.07 ± 0.04 **b**	5	83	0.01 ± 0.02 **b**	0	87
Explant orientation	**B**						
Cut surface down on medium		0.21 ± 0.10 **a**	5	97	0.16 ± 0.09 **a**	3	100
Intact surface down on medium		0.06 ± 0.04 **b**	3	95	0.01 ± 0.02 **b**	2	98
Pre-culture period (days)	**C**						
0		0.22 ± 0.10 **b**	5	35	0.22 ± 0.08 **b**	0	54
1		0.25 ± 0.10 **b**	5	43	0.27 ± 0.08 **ab**	0	88
3		0.30 ± 0.11 **ab**	7	77	0.30 ± 0.12 **ab**	0	92
5		0.40 ± 0.13 **a**	5	93	0.47 ± 0.14 **a**	3	93
7		0.48 ± 0.11 **a**	7	98	0.47 ± 0.12 **a**	7	97
9		0.33 ± 0.12 **ab**	5	98	0.37 ± 0.13 **ab**	5	97
Co-cultivation period (days)	**D**						
0		0 **b**	0	98	0 **b**	0	100
1		0 **b**	0	98	0 **b**	0	100
2		0.05 ± 0.04 **b**	12	92	0 **b**	0	93
3		0.10 ± 0.06 **ab**	13	83	0.08 ± 0.04 **ab**	3	88
4		0.23 ± 0.10 **a**	27	65	0.07 ± 0.04 **ab**	12	73
5		0.28 ± 0.11 **a**	23	43	0.17 ± 0.09 **a**	10	57
Light condition	**E**						
Light		0.37 ± 0.14 **a**	22	97	0.21 ± 0.09 **a**	17	100
Dark		0.17 ± 0.09 **b**	8	88	0.09 ± 0.04 **b**	5	95
Acetosyringone 100mM	**F**						
Yes		0.52 ± 0.16 **a**	18	98	0.15 ± 0.07 **a**	7	98
No		0.25 ± 0.10 **b**	7	97	0 **b**	0	100

GFP = GUS Focal Point; BSA = Blue Staining Area; ES = Explant survival; *= 30 days after treatment; ∇ = % explants with BSAs; different letters within a column for any one treatment are significantly different ($P < 0.05$) using Duncan's Multiple Range Test.

the co-cultivation period the greater the GFPs and BSAs in both cultivars, with an inverse, sharp decline in explant survival. The presence of light and of acetosyringone was beneficial to the number of GFPs, BSAs and to explant survival in both 'Lineker' and 'Shuhou-no-chikara'. Sonication with either pKT2 or pKT3 plasmid types resulted in increased GFPs and BSAs (**Fig. 2F**), but also in

increased explant mortality over 5 min SAAT.

Application of optimized bombardment and Agroinfection parameters

The parameters that were defined to give the greatest stable transgene expression in both 'Lineker' and 'Shuhou-no-chikara' (bombardment and *Agro*infection) were applied to observe the transition of the transgene expression of different plasmid constructs over time (**Fig. 3**). *Agro*infection with pBI121 gave the highest BSA and GFP values for both 'Lineker' and 'Shuhou-no-chikara', followed by pKT3, then pKT2 (**Fig. 3A-D**). High transient GUS expression by pBI121 was primarily due to *Agro*infection, since this plasmid is intronless. While the number of GFPs decreased to zero in all three plasmid types and in both 'Lineker' and 'Shuhou-no-chikara', the BSA levels decreased to a low level in 'Lineker', and to an even lower level (1%) in 'Shuhou-no-chikara'. In bombardment, pKT2 was significantly inferior to any *Agro*infection treatment in both %GFPs and %BSAs per explant in both 'Lineker' and 'Shuhou-no-chikara' (**Fig. 3E-H**) and like *Agro*infection, the levels of GFPs decreased to zero, but the BSA level remained higher (5%) after 1 month in culture on selective

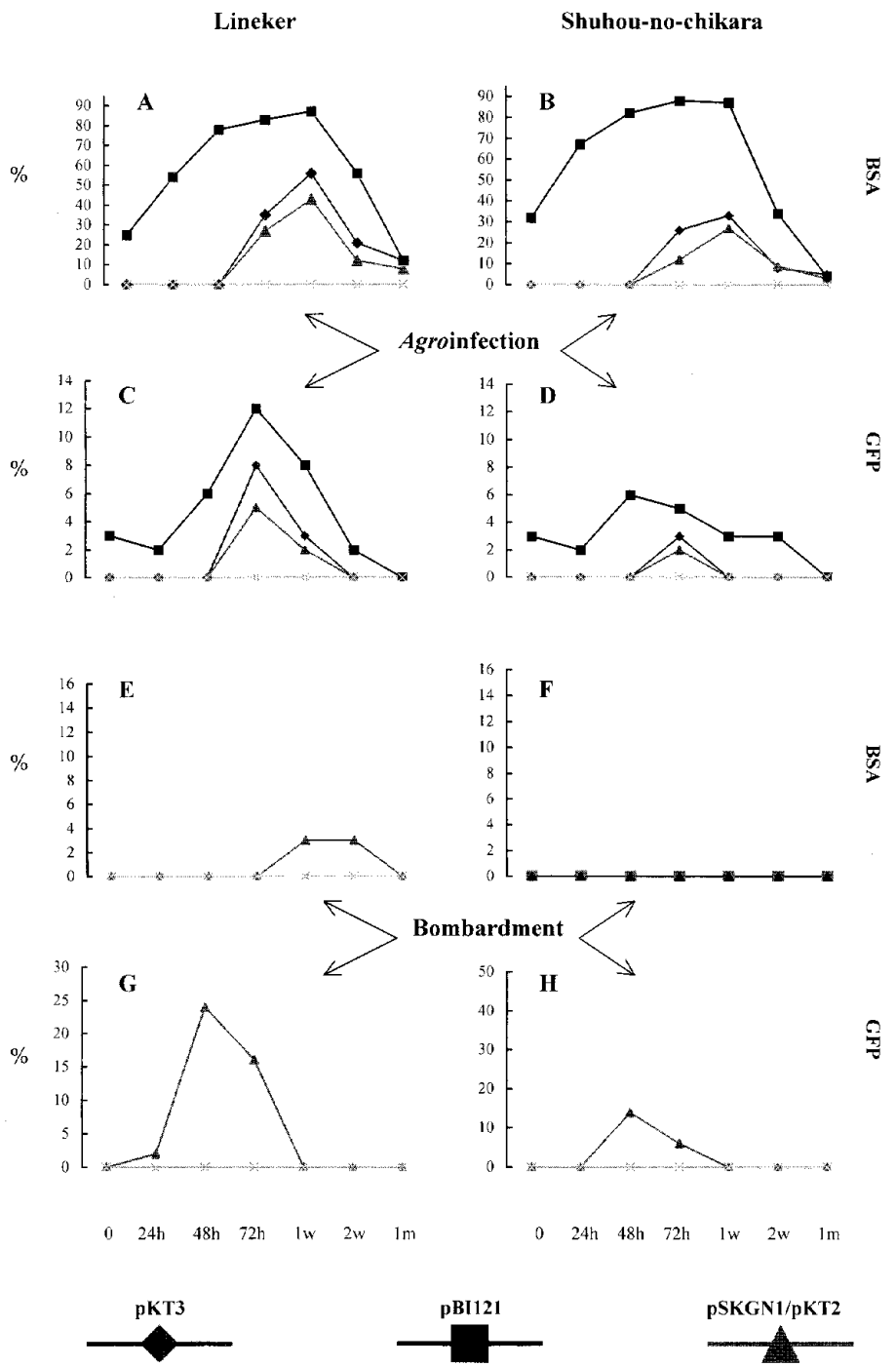

Fig. 3 Effect of *Agro*infection (A – D) or particle bombardment (E – H) on the occurrence of GFPs or BSAs in 'Lineker' and 'Shuhou – no – chikara' chrysanthemums.

medium for 'Lineker' only.

Resulting stable transgene expression

Independent of the cultivar, plasmid type and gene introduction method, a higher level of stable GUS expression could be observed in older, basal leaves than in either young or middle-aged leaves, and an increasing gradient of GUS expression with an increase in leaf age was clearly evident (**Table 2**). No controls exhibited any visible GUS expression. In both chrysanthemum cultivars, the highest level of GUS expression was found to occur in the venation (veins and mid-ribs) of leaves (**Fig. 2M**). Intermediate levels of GUS expression could be observed in the leaf tips and leaf edges of transgenic plants (**Fig. 2N**), while the lowest expression was in the epidermis and inter-veins (**Fig. 2O, P**).

Transformation efficiencies

Transformation efficiency is strongly dependent on the initial level of transient transgene expression. Excessive *Agro*infection and sonication, however lead to an increased transient transgene expression with a paralleled decrease in shoot regeneration and increased explant mortality (**Table 1**), with the overall outcome being a decreased transformation efficiency. Transformation efficiency values (GUS transformation efficiency : PCR transformation efficiency; **Fig. 2R**) showed that in 'Lineker', bombardment (26.89 : 20.63) > SAAT (25 : 15.79) > *Agro*listics (16.67 : 11.84) > *Agro*infection (6.6 : 5.0), while in 'Shuhou-no-chikara', *Agro*listics (7.69 : 0) > bombardment (2.17 : 0) > SAAT (0 : 0) = *Agro*infection (0 : 0).

Discussion

Transgenic *in vitro* chrysanthemum plantlets (GUS and PCR positive) were obtained for both 'Lineker' and 'Shuhou-no-chikara', albeit with different transformation efficiencies, and resulted from optimized protocols for maximizing transient transgene expression in *Agro*infection and biolistics, coupled to an optimized shoot regeneration system.

Effect of different parameters on transgene expression

An extended explant pre-culture period from 0 days to 8 days almost tripled the observed number of blue spots in LBA4404 *Agro*infected *D. grandiflora* '1610' leaf disks (de Jong *et al.*, 1990). Transient GUS activity could be observed as blue spots on the cut edge of leaf disks when there was no pre-culture but, following a 8 day pre-culture period, as many blue spots in callus cells could be observed, apparently derived from the epidermis

Table 2 Absolute percentage GUS+ tissue localization in 1-3 month-old *in vitro* chrysanthemum plantlets

Treatment	Cv	Leaf age			Leaf localization					
		Y	M	O	lt	v	mr	le	iv	e
A-pBI121	LIN	65	50	65	3.3	13.9	10.6	1.7	1.1	2.8
A-pBI121	SNC	0	0	100	33.3	-	-	-	-	-
A-pSKGN1	LIN	14	14	86	15.9	11.1	20.6	15.9	4.8	15.9
B-pKT2	LIN	20	27	90	3.8	8.7	1.6	2.0	0.4	2.8
B-pKT2	SNC	0	0	100	-	11.1	11.1	-	-	11.1
SAAT-pBI121*	LIN	6	47	88	11.1	2.0	0.7	9.2	0.7	7.8
SAAT-pBI121*	SNC	0	33	67	11.1	-	-	3.7	-	-
SAAT-pSKGN1*	LIN	8	21	96	4.2	0.5	0.9	8.8	0.9	7.9
AB-Nopl+pBI121	LIN	12	24	100	8.5	5.9	5.9	11.1	5.9	8.5
AB-Nopl+pBI121	SNC	0	100	100	11.1	11.1	-	-	-	-
AB-Nopl+pSKGN1	LIN	0	20	100	5.9	4.4	4.4	6.7	4.4	10.4
AB-Nopl+pSKGN1	SNC	0	0	100	18.5	37.0	18.5	22.2	-	-
AB-pKT2+pBI121	LIN	10	23	87	5.2	3.0	5.2	6.7	1.5	4.8
AB-pKT2+pBI121	SNC	17	42	100	8.3	1.9	6.5	0.9	-	0.9
AB-pKT2+pSKGN1	LIN	11	33	78	6.2	7.4	7.4	7.4	4.9	8.6
AB-pKT2+pSKGN1	SNC	33	0	67	7.4	3.7	7.4	3.7	3.7	3.7

Treatments: C = Control, A = *Agro*infection, B = Particle Bombardment, AB = *Agro*listics, SAAT = Sonication Assisted *Agrobacterium*-mediated Transformation; Nopl = No plasmid. Cultivars: LIN = 'Lineker', SNC = 'Shuhou-no-chikara'. Leaf age: Y = young; M = middle-aged; O = old. Leaf position: lt = leaf tip; v = vein; mr = mid-rib; le = leaf edge; iv = inter-vein; e = epidermis.

(**Fig. 2A**). Pre-culture period had little effect on the number of GFPs and BSAs in both 'Lineker' and 'Shuhou no-chikara' when particle bombardment was used, but these increased in both cultivars when explants were *Agro*infected. An increase in pre-culture period positively affected explant survival in both cultivars both with particle bombardment and *Agro*infection. An increase in pre-culture period also resulted in increased escape formation in both particle bombardment (**Fig. 2I**) and *Agro*infection (**Fig. 2J**). The application of a gene introduction method early in the developmental process, that is with a low pre-culture period would increase explant mortality, but increase transient transgene expression and stable transgene expression in either particle bombardment (**Fig. 2D**) or *Agro*infection (**Fig. 2L**).

Transient expression was shown to be strain-dependent (**Table 1**), independent of the *D. grandiflora* cultivar used, similar to studies in which greatest transgene expression occurred when wild type CHRY5 and A281 were used (Urban *et al.*, 1994). *In vitro*-derived leaf explants had higher transient GUS gene expression levels than greenhouse-derived leaves, independent of the *D. grandiflora* cultivar and *A. tumefaciens* strain (Boase *et al.*, 1998b). This could be similarly observed in both 'Lineker' and 'Shuhou-no-chikara' stem explants (**Table 1**), but only with the application of *Agro*infection (**Fig. 2B, C, H**), *Agro*listics (**Fig. 2G**) or SAAT (**Fig. 2F**). Greenhouse explants (**Fig. 2E**) had lower transient transgene expression than their *in vitro* counterparts (**Table 1**).

The osmotic pretreatment of bombarded maize cells with 0.2 M sorbitol and 0.2 M mannitol resulted in a 2.7-fold and 6.8-fold increase in transient and stable transgene expression, respectively (Vain *et al.*, 1993). "Starvation" (i.e. carbon source, plant growth regulator or MS-nutrient free media) experiments conducted on 'Lineker' and 'Shuhou-no-chikara' showed that both low transient transgene expression and explant survival occurred in explants placed on any medium following particle bombardment or *Agro*infection (data not shown).

Transformation inefficiencies can be partly overcome by the addition of acetosyringone to induce the expression of *vir* genes (Stachel *et al.*, 1985). The presence of acetosyringone significantly increased the GUS transient transgene expression (GFPs) in both 'Lineker' and 'Shuhou-no-chikara' *in vitro* and greenhouse explants (**Table 1**). Light also gave a similar genotype- and explant source-independent response.

Electrotransfection was shown to cause 50% mortality in chrysanthemum axillary shoots, while about 25% showed transient GUS expression (Burchi *et al.*, 1995). Over 5 min sonication in 'Lineker' and 'Shuhou-no-chikara' caused exponential explant mortality, but increased GUS transient transgene expression and BSAs (data not shown).

Temporal changes in transgene expression

For stable transformation, it is necessary for the T-DNA to be incorporated into the host DNA, and T-DNA that is not integrated is gradually lost and inactivated. This phenomenon is evident in the GUS transgene expression in the intron-containing plasmids (pSKGN1, pKT2 and pKT3) where such a gradual loss can be seen by a decrease in the number of GFPs up to 72 h and a total loss of GFPs by a maximum of 1 week following a gene introduction method (**Fig. 3**). The amount of *Agro*infection of explants was reduced to 2% after 2 weeks of culture on selective MS medium, indicating that the GUS-positive and PCR-positive BSAs were as a result of stable transformation and transgene integration, and not as a result of *Agro*infection. Experiments with *Agro*infection of eleven *D. grandiflora* cultivars with LBA4404 showed that there was no significant reduction in the number of GFPs over two weeks, indicating a possible stable integration and subsequent expression of the T-DNA (de Jong *et al.*, 1990). Large differences were observed between cultivars with some leaf explants exhibiting as little as 0 and as many as 26 GFPs per leaf disk when measured 6 days after infection. LBA4404 (pKIWI110) infected *D. morifolium* and *D. indicum* showed that 83% of leaf explants showed GUS activity 48 h after infection (Ledger *et al.*, 1991).

A. rhizogenes transformation experiments in *D. grandiflora* 'Parliament', with tobacco as an external control, showed that 42% of leaf explants expressed the GUS and *iaa* genes 6 days after inoculation, but 0% at 21 days, indicating the transient and non-integrated nature of the transgene expression (van Wordragen *et al.*, 1992a). Tobacco in the same study, however, showed an increase in the percentage of GUS-expressing explants from 6 to 21 days. Separate experiments by the same group on seven *D. grandiflora* cultivars indicated that the use of A281 was far superior to Ach5, and both GUS and opines were detected at earliest 2 days and 5 days after infection for tobacco and chrysanthemum, respectively, indicating only the transient nature of transgene expression (van Wordragen *et al.*, 1992b). Intron-containing plasmids (pSKGN1 /pKT2 and pKT3) had an increase in transient

transgene expression from 0-72 h (observed by the GFP number) and higher stable GUS expression levels increasing from 1 week after gene introduction method (measured by the BSAs), the former plasmid type with lower GFP and BSA levels than the latter (**Fig. 3**).

Localization of transgene expression

Transient transgene expression has been shown to range from distinct 10-cell loci to diffuse zones occupying up to half of the original explant (Ledger *et al.*, 1991). These zones tended to be localized near veins but others could be observed on the cut edge. A low number of GFPs per explant, concentrated at cut surfaces, were obtained in seven *D. grandiflora* cultivars (except for cv. '1601') when transformed with LBA4404 (de Jong *et al.*, 1993). GUS transient transgene expression could be detected in callus from stem explants 14-18 h after inoculation with several wild type (A6, 181, Ach5, C58 and T37) and disarmed (C58, LBA4404 and GV2260) *A. tumefaciens* and *A. rhizogenes* (R1000) strains, and despite shoots forming, none were stably transformed (Lowe *et al.*, 1993). The use of another *A. tumefaciens* strain AGL0 resulted in small or large GUS spots, depending on the plasmid used, and these were visible only on cut surfaces (de Jong et al, 1994). 'Lineker' and 'Shuhou-no-chikara' showed a genotype-independent response in stable transgene expression (**Table 2**), but localization was strongly *Agrobacterium* plasmid dependent. Similar localization in petunia (Janssen and Gardner, 1990) may indicate a zone of actively dividing cells that are in the correct phase of cell division to enhance transformation (Chriqui *et al.*, 1988).

Increased tumor formation was synonymous with transformation in *D. grandiflora* 'Parliament' *Agro*infected by tumorigenic (C58 and Ach5), supervirulent (A281) and non-tumorigenic (LBA4404) strains (van Wordragen *et al.*, 1991). GUS activity in pine cotyledons was lost 12 days following particle bombardment with a 35S promoter construct (Rey *et al.*, 1996). The higher GUS transient transgene expression detected in 'Lineker' and 'Shuhou-no-chikara' *in vitro* explants as opposed to greenhouse explants (**Table 1**) may be explained by the fact that there are more cells undergoing cell division and proliferation in young tissue. It has been shown that for stable GUS gene expression to occur both cell division (M-phase) and DNA duplication (S-phase) are required, that is, stable transformation is cell cycle dependent (Villemont *et al.*, 1997). Arias and Sayre (1993) have attributed the low levels of transient transgene expression to high levels of DNase activity in receptor cells.

Resultant stable transgene expression and transformation efficiency

Predominant GUS gene expression occurred in vascular tissue (**Fig. 2M**), but expression could be detected in almost all tissue types, at varying degrees (**Table 2, Fig. 2N-P**). Since vascular layers have young cells and active cell division, the metabolic activity would be high, resulting in greater amounts of GUS. In both apple and tomatillo, the phloem stained GUS-positive, but not the xylem (Assad-García *et al.*, 1992; Ko *et al.*, 1998), due to a higher density of actively dividing cells in the former. GUS expression was detected at higher levels in older, basal leaves but could also be detected at high levels in middle-aged and young tissues (**Table 2**).

Transformation efficiency was cultivar-dependent, with 'Lineker' being more prone to transformation using any gene introduction method than 'Shuhou-no-chikara'. Despite a high transient transgene expression being observed in 'Shuhou-no-chikara' explants following any gene introduction method, due to poor regeneration and high explant mortality, the number of transformants was low in 'Shuhou-no-chikara', although the transient transgene expression and GUS transformation efficiency levels of 'Shuhou-no-chikara' and 'Lineker' were comparable, independent of the gene introduction method. Transformation efficiencies in different *Agrobacterium*-mediated transformation studies of *D. grandiflora* range from <0.1% for 'Moneymaker' to a high of 0.6% in '1581'(Ledger *et al.*, 1991; Renou *et al.*, 1993; Courtney-Gutterson *et al.*, 1994; Pavingerová *et al.*, 1994). Transformation efficiencies could be improved from 0.1% to 0.5% in 'Iridon' (Urban *et al.*, 1994). By modifying the regeneration procedure, high transformation efficiencies were obtained in 'Hekla' and 'Polaris' (Sherman *et al.*, 1998), with 4.1% and 1.7%, respectively while a 2.5-4.6% transformation efficiency for four cultivars were obtained in a biolistic-mediated transformation procedure (Yepes *et al.*, 1995). Improvement of transformation efficiencies has been attributed to a four-step plant regeneration procedure coupled with *Agro*infection: callus induction, shoot primordia, shoot elongation and rooting (Sherman *et al.*, 1998). The transformation efficiency values presented in this study for 'Lineker' and 'Shuhou-no-chikara' fall into the range of 2-27% (GUS positive) and 0-21% (PCR positive; **Fig. 2R**, lanes 6,7,11,12) for bombardment related treatments, and in the range of 7-25% (GUS posi-

tive) and 0-16% (PCR positive; **Fig. 2R,** lanes 4,5,9,10) for *Agro*infection related treatments. PCR screening of *D. grandiflora* explants exposed to *Agro*infection reveals that the GUS gene was expressed in 100%, 83% and 75%, respectively, of transformed 'Polaris', 'Hekla' and 'Iridon' plants (Sherman *et al.*, 1998), in which 23 and 3 PCR positive transgenics were recovered from 275 and 59 GUS positive shoots in *in vitro* and greenhouse treatments respectively, translating into a 8.4% and 5.1% transformation efficiency, respectively.

In conclusion, by modifying and optimizing numerous *Agro*infection and/or biolistic parameters in an additive manner has allowed for the increase in transformation efficiency of chrysanthemum genotypes that were previously thought to be recalcitrant to transformation.

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