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The potato *Lhca3.St.1* promoter confers high and stable transgene expression in chrysanthemum, in contrast to CaMV-based promoters

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Abstract

The enhanced cauliflower mosaic virus 35S (dCaMV) promoter and the potato *Lhca3.St.1* promoter were evaluated for their expression abilities in chrysanthemum. The promoters were fused to the β-glucuronidase (GUS) reporter gene with and without flanking matrix-associated regions (MARs). They were transferred into chrysanthemum via *Agrobacterium*-mediated transformation. The quantitative evaluation of GUS activity in a total of 127 independently derived transformants established that in chrysanthemum the *Lhca3.St.1* promoter was 175 fold more active in the leaves than the dCaMV promoter was. The latter was as poor in expression as the single CaMV promoter. The use of such CaMV-based promoters in the genetic engineering of chrysanthemum should be discouraged when high levels of transgene expression are desired. No clear influence of the presence of MARs was observed on the variability of GUS gene expression, in contrast to earlier studies in tobacco. This may indicate a possible plant species dependent activity of MAR elements. *Lhca3.St.1* promoter-driven GUS activity was relatively higher in the stem of chrysanthemum and proved stable over extensive time periods. Therefore this potato promoter is attractive to obtain high expression levels in chrysanthemum.

Introduction

Chrysanthemum (*Dendranthema grandiflora* Tzvel.) is the second largest cut-flower crop after rose (*Rosa hybrida*). Commercially grown plants of chrysanthemum are propagated by cuttings, while mother plants are maintained *in vitro*. Genetic engineering of chrysanthemum is attractive, as conventional breeding has not been able to find breeding lines with suitable gene pools for traits such as resistance to pests and longer vase life, partly due to the outbreeding autohexaploid genetics of chrysanthemum coupled to a large genome size (Rout and Das 1997; van Wordragen et al. 1991).

In recent years, several transformation protocols have become available for chrysanthemum (Robinson and Firoozabady 1993; Rout and Das 1997). Transgene expression studies in chrysanthemum have focussed on the use of various cauliflower mosaic 35S (CaMV) promoter variants to drive the transgene (de Jong et al. 1995; Lowe et al. 1993; Urban et al. 1994), (see also discussion), often with β-glucuronidase (GUS) as a reporter gene (Lazo et al. 1991). Previous results have established that transgenic chrysanthemum plants with a single CaMV-GUS construct show bright blue coloration upon over night X-gluc staining, but GUS expression is very low to undetectable when quantified by fluorometry (de Jong et al. 1994, 1995; Fukai et al. 1995). This is also the case when large numbers of independent transformants are generated and analysed. To improve transgene expression levels in chrysanthemum, we have evaluated two putatively stronger promoters. The first was the doubled cauliflower mosaic virus 35S (dCaMV) promoter. This promoter has a duplication of the upstream enhancer sequence (Odell et al. 1985) and is a stronger

variant of the single CaMV promoter (de Jong et al. 1995; Ledger et al. 1991; Lowe et al. 1993; Urban et al. 1994). The potato *Lhca3.St.1* promoter was the second promoter evaluated. Both in tobacco and in potato this promoter shows considerably higher levels of GUS activity than the dCaMV promoter (Nap et al. 1993). The *Lhca3.St.1* promoter is active in leaves, stems and other green parts of the plant and its activity is light dependent (Nap et al. 1993). To accurately characterise promoter activity, it is important to remove position effects that are, among other factors, due to the random place of integration of the transgene. The placement of a matrix-associated region (MAR) on either side of transgenes can reduce such position effects in plants (Mlynárová et al. 1994, 1995, 1996). This reduction is thought to be due to the creation of independent transcription domains of the T-DNA, irrespective of their position in the recipient genome. In this paper we present an evaluation of both dCaMV and *Lhca3.St.1* promoter-GUS constructs with and without flanking MAR elements in transgenic chrysanthemum. The chicken lysozyme A element was used in the T-DNA configurations as MAR element, since it has been previously shown to be highly effective in the reduction of position effects in tobacco (Mlynárová et al. 1994, 1995, 1996).

Materials and methods

Plant material for transformation

Stem explants of chrysanthemum cultivar 1581 were obtained from plants grown in the greenhouse under standard conditions (Machin and Scopes 1978), which were raised from cuttings, three weeks after pruning. The first two internodal regions from the tip of the shoot were utilised to obtain the explants for transformation. Stems were surface sterilised in 1% hypochlorite with four drops of Tween 20 for twenty minutes, followed by three rinses in sterile water to remove traces of hypochlorite. Stem segments of about 2–4 mm were sliced along the length of the stem and used as explants for transformation.

Media and Agrobacterium inoculum

The explants were placed in Petridishes containing CHR 04 medium (de Jong et al. 1995) overlaid by a Whatmann filter (9-cm diameter). The explants were precultured for 2 hours prior to cocultivation. A growing culture of the *Agrobacterium tumefaciens* strain AGLO (Lazo et al. 1991) harbouring the binary plasmid of interest was generated by inoculation of 20 ml liquid broth medium (10 g/l trypton, 5 g/l yeast extract, 5 g/l NaCl, 1 g/l glucose) supplemented with 50 mg/l rifampicin and 50 mg/l kanamycin in a 50 ml flask and incubated on a rotary shaker in the dark at 28 °C for 16 hours. A 100 μl aliquot of this overnight culture was transferred to 20 ml of fresh liquid broth medium in a 50 ml flask and incubated for four hours under similar conditions. Cultures between 0.7 to 1.0 optical density at 540 nm were centrifuged and the pellet was resuspended in 1 ml of liquid CHR 04 medium supplemented with 100 μM acetosyringone.

Transformation and selection

A 20 μl aliquot of the resuspended *Agrobacterium* culture was pipetted on top of the surface of each explant. The explants were incubated for 48 hours at 25 °C under cool white fluorescent lamps and a 12-h photoperiod. After 48 hours the explants were transferred to fresh CHR 04 medium containing 100 mg/l of Timentin to prevent *Agrobacterium* overgrowth and 25 mg/l kanamycin for selection of transgenic shoots. After about 6 weeks the first batch of transgenic shoots (10 mm long) was harvested. These shoots were transferred to glass jars containing chrysanthemum rooting media (half strength MS salts, vitamins, 30 g/l sucrose, 7 g/l MC29 agar, 100 mg/l Timentin, 125 mg/l cefotaxime, 25 mg/l kanamycin) and cultured at 25 °C in a 12-h photoperiod. The remaining explants were transferred to fresh CHR 04 media containing 100 mg/l Timentin, 125 mg/l cefotaxime and 25 mg/l kanamycin. After another six weeks incubation, a second batch of shoots was harvested and incubated as above. Rooted plants were transferred to the greenhouse.

Plant transformation vectors

The binary vectors evaluated for GUS activity in chrysanthemum have been described previously (Mlynárová et al. 1994, 1995). They carry different T-DNAs, designated NCG, ANCGA, NLG and ANLGA as shorthand for the DNA elements present in the T-DNA. All constructs are based on pBIN19 and carry a kanamycin resistance gene (NPTII) under control of the nopaline synthase (NOS) promoter (shorthand: N) and a β-glucuronidase (GUS) gene either under control of the potato *Lhca3.St.1* promoter

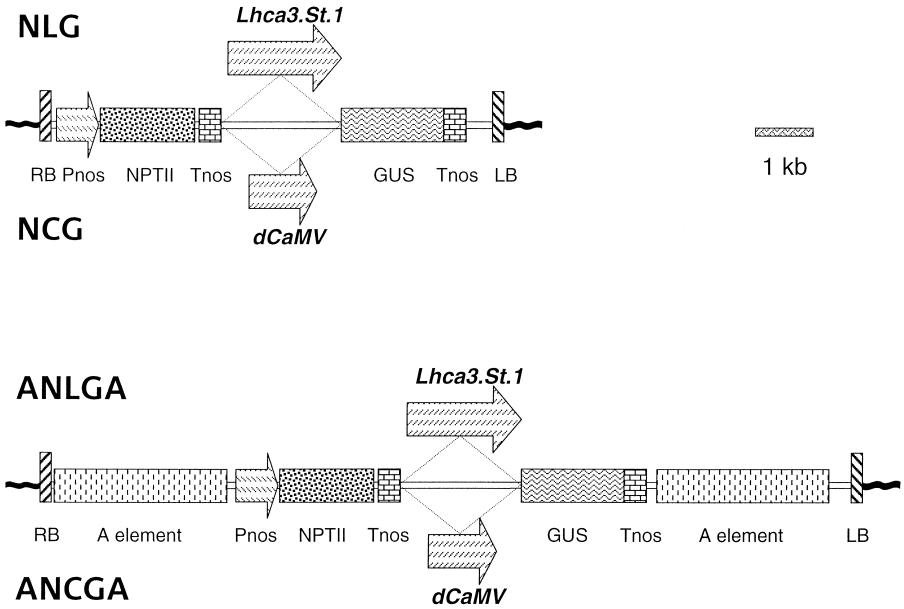

Figure 1. Schematic representation of the T-DNA regions of the four constructs used in this study. RB, LB, right and left T-DNA borders respectively; A element, 3 kb chicken lysozyme A element; Pnos, nopaline synthase promoter; NPT II, neomycin phosphotransferase II coding region; Tnos, nopaline synthase terminator; *Lhca3.St.1*, promoter from the gene encoding potato apoprotein 2 of the light-harvesting complex of photosystem I; dCaMV, doubled CaMV 35S promoter; GUS, β-glucuronidase coding region.

(Nap et al. 1993) (shorthand: LG) or of a doubled CaMV 35S (dCaMV) promoter (shorthand: CG). Another version of both constructs was also used, in which the 3 kb chicken lysozyme A element was inserted inside each T-DNA border (shorthand: A). The structures of the T-DNAs used for chrysanthemum transformation are illustrated in Figure 1.

Determination of GUS activity

Quantitative GUS measurements were performed essentially as described previously (Mlynárová et al. 1994), using a Fluoroskan II microtiterplate reader (Titertek, Finland). In some cases, the sensitivity of the standard assay was increased. Samples were harvested as 9 mm-diameter discs, taken at a common position from a leaf to maintain similarity between samples and minimise sampling variation. GUS activity was also determined in ray florets, stems and pedicels.

Results

Generation of populations of GUS-containing transgenic chrysanthemum

The four T-DNA vectors outlined in Figure 1 were transformed into chrysanthemum cultivar 1581 and a minimum of 30 independently derived plants per construct were generated that rooted in the presence of 25 mg/l kanamycin. Regenerants derived from the two dCaMV promoter-containing constructs exhibited difficulty to initiate roots in the presence of kanamycin. In total 193 transgenic plants were analysed for GUS gene expression by a fluorometric assay. GUS activity was assayed in young leaves of about 3-month-old greenhouse-grown plants. Notably the GUS activity in plants of the dCaMV promoter-carrying NCG and ANCGA populations was very low. Our standard semi-high-throughput GUS assay uses an incubation of 2–5 μl plant extract (with 1–3 μg of soluble protein) in a time of 1 hr without the use of Na_2CO_3. This assay has a detection limit of about 0.75 pmol per minute per microgram of soluble protein. With that standard assay, only two out of 35 plants (5%) in the NCG population and six out of 38 plants (15%) in the ANCGA population showed detectable activity. To measure very low activities more

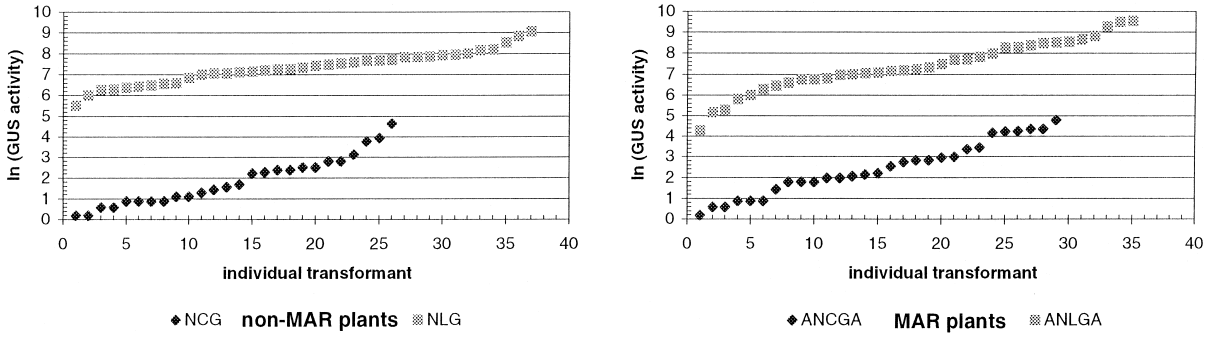

Figure 2. GUS activities in the four populations of transgenic chrysanthemum. *2A*, populations carrying the non-MAR constructs NCG with the dCaMV promoter driving the GUS gene and NLG with the *Lhca3.St.1* promoter driving the GUS gene; *2B*, populations carrying the MAR-containing constructs ANCGA and ANLGA. The natural logarithm (ln) of the GUS activity of each individual transformant in pmol MU per microgram protein per hour is sorted according to size.

accurately, the sensitivity of the assay was lowered to about 20 fmol per min per microgram of protein. For this, 50 μl plant extract was assayed for considerably longer incubation times (up to 24 hrs). Also, an end point measurement after addition of 100 μl Na_2CO_3 was taken. With this improved sensitivity of the assay, 26 plants (74%) from the NCG population and 29 plants (76%) from the ANCGA population showed detectable GUS activity, whereas 9 plants from each population still did not show any activity. For the NLG and ANLGA populations, a total of 48 plants (40%) did not show detectable GUS activity. The 66 chrysanthemum transformants showing no GUS activity were discarded from further analysis, because for each construct sufficient plants were identified with detectable activity. To prevent analytical problems with negative natural logarithms, GUS activity was expressed as pmol methylumbelliferone (MU) per microgram protein per hour, rather than per minute. The size-sorted GUS activities in leaves of individual transformants from the four populations of transgenic chrysanthemum plants are presented after natural logarithmic transformation (Figure 2).

Comparative analyses of populations of GUS-containing transgenic chrysanthemum

The descriptive statistics of the four populations are given in Table 1. As shown previously (Nap et al. 1993), a proper statistical analysis of GUS activities in populations of plants requires a logarithmic transformation. To provide meaningful comparisons, the average activities in these populations are compared with the use of the geometric (i.e. backtransformed natural logarithmic) mean. The quantitative data in Table 1 show that the dCaMV promoter gives very low activities of GUS in chrysanthemum; 6.42 pmol MU per microgram protein per hour for NCG and 11.6 pmol MU per microgram protein per hour for ANCGA. Averaged over all 55 dCaMV-carrying plants, the overall activity of GUS driven by the dCaMV promoter in chrysanthemum yields only 8.75 pmol MU per microgram protein per hour. Given the range of GUS activities (1.2–119.4 pmol MU per microgram protein per hour), the GUS activity data are severely skewed in these populations.

Table 2 shows the results of the comparative statistical tests performed. There are no highly influential outliers in these populations following logarithmic transformation (analyses not shown). The variances exhibited by these two populations are not significantly different, despite the presence of the MAR elements in the ANLGA transformation vector (Table 2; F-test). Although the ANCGA plants have somewhat (1.8-fold) higher average activity than the NCG plants (Table 1), the difference is not significant (Table 2, t-test). The difference between the highest active transformant in both populations is marginal (102.6 for NCG and 119.4 for ANCGA on the scale of measurement). In contrast, the potato *Lhca3.St.1* promoter-GUS configuration results in average GUS activities of 1510 pmol MU per microgram per hour in the NLG population and 1572 for the ANLGA population. The variances (on the ln scale) differ significantly (Table 2, F-test). The MAR-containing ANLGA population exhibits an almost 2.5-fold higher variance, but the difference in mean is not significant (Table 2, t-test). Averaged over all 72 plants, the *Lhca3.St.1*-driven GUS activity in chrysanthemum is 1535 pmol MU per microgram protein per hour. This is 175 fold more than the average activity given by the dCaMV promoter. The coefficient of variation

Table 1. Descriptive statistics of the GUS activity in the four chrysanthemum populations.

Plant population	NCG	ANCGA	NLG	ANLGA
Promoter driving GUS	dCaMV	dCaMV	*Lhca3.St.1*	*Lhca3.St.1*
Present of A element	–	+	–	+
No. plants generated	35	38	63	57
No. plants without activity[a]	9	9	26	22
No. plants in analysis	26	29	37	35
Natural logarithmic scale[b]				
Mean	1.86	2.45	7.32	7.36
Median	1.63	2.20	7.32	7.22
Variance	1.40	1.69	0.62	1.51
CV (%)	63.6	53.1	10.8	16.7
Scale of measurement				
Geometric mean[c]	6.42	11.6	1510	1572
Maximum	102.6	119.4	8542	13721
Overall geometric mean	8.75		1535	

[a]Plants without detectable activity in the enhanced sensitivity assay
[b]Based on GUS activities in pmol per hour per microgram protein
[c]Mean on the natural logarithmic scale backtransformed to pmol per hour per microgram protein

Table 2. Comparative statistics of the GUS activity in the four chrysanthemum populations.

Parameter tested	Mean					Variance	
Statistical test used			t-test[a]			F-test[b]	
	Test statistic	t	df[c]	P[d]	F	df	P
Combination of populations							
NCG	ANCGA	1.73	53	0.089 (ns)	0.83	53	0.32 (ns)
NLG	ANLGA	0.18	57	0.86 (ns)	0.41	70	0.008 (**)
NCG	NLG	20.5	40	< 0.001 (***)	2.27	61	0.024 (*)
ANCGA	ANLGA	15.4	58	< 0.001 (***)	1.12	62	0.38 (ns)
dCaMV[e]	*Lhca3.St.1*[f]	24.2	102	< 0.001 (***)	1.55	124	0.08 (ns)

[a]Student t-test. Depending on the outcome of the corresponding F-test a t-test assuming equal variances or a t-test assuming unequal variances was performed.
[b]F-test for homogeneity of variances.
[c]Degrees of freedom for the statistical test used.
[d]Two-tailed P-value; the P-labeled column gives the P value and between brackets the assessment of the probability value in terms of significance; ns, not significant (at P = 0.05), ***, significant at P < 0.001, **, significant at P < 0.01, *, significant at P < 0.05.
[e]Combined NCG and ANCGA populations.
[f]Combined NLG and ANLGA populations.

(Table 1, CV), which allows comparisons of variability independent of scale, is about 4-fold lower for the populations carrying the *Lhca3.St.1* promoter-GUS fusion compared to the populations with the dCaMV promoter-GUS fusions. This shows that the much lower activity of the dCaMV promoter-GUS fusion in chrysanthemum is also more spread out relative to its mean than the *Lhca3.St.1* promoter-driven GUS activity.

Distribution and stability of GUS activities in transgenic chrysanthemum

Given the very low activity of the dCaMV promoter in transgenic chrysanthemum leaves, several randomly chosen NCG/ANCGA transformants were analysed for GUS activity in stem, pedicel and ray floret. In all plants analysed, the GUS activity in these organs was not markedly higher than the low activity in the leaf (data not shown). Five randomly chosen ANLGA transformants analysed for GUS activity in

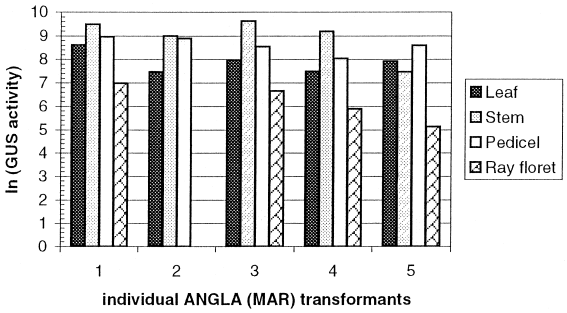

Figure 3. GUS activity in different organs of chrysanthemum of five individual transformants carrying the ANLGA construct. The natural logarithm (ln) of the GUS activity in the different tissues is given in pmol MU per microgram protein per hour.

Table 3. Distribution of GUS activities over different organs of ANLGA-carrying chrysanthemum transformants.

Tissue	Leaf	Stem	Pedicel	Ray floret
No. plants analysed	5	5	5	4
Natural logarithmic scale[a]				
Mean	7.89	8.95	8.61	6.16
Variance	0.22	0.75	0.13	0.69
Median	7.92	9.19	8.59	6.27
Scale of measurement				
Geometric mean	2670	7708	5486	473
% activity of leaf	100	290	210	17

[a]Activities as in Table 1.

the same organs showed similar high activity in all aerial plant parts (Figure 3), GUS activity in the stem being relatively higher and that in the pinkish white-coloured ray floret relatively lower (Table 3). The *Lhca3.St.1* promoter is also active in mature pollen of transgenic *Lhca3.St.1*-GUS carrying chrysanthemum (data not shown).

Stability of transgene expression during growth and propagation is of particular interest for a vegetatively propagated crop as chrysanthemum. Therefore, six transformants of both the NLG and the ANLGA population were chosen and maintained as parental stocks under standard conditions in the greenhouse (Machin and Scopes 1978). After a period of 21 months of cutting, leaves were again analysed for GUS activity and compared to the activity in the leaves of the same plants when three months old (Figure 4). The correlation between the activity of three-month-old plants and the 21-month-old plants is 0.67 for the plants from the NLG population, 0.76 for the ANLGA population and 0.68 for all twelve transformants combined. This establishes that GUS gene expression driven by the *Lhca3.St.1* promoter is stable upon vegetative propagation and growth.

Discussion

Low activity of the dCaMV promoter in chrysanthemum

The detailed quantitative analysis of GUS activity in 55 transgenic chrysanthemum plants clearly established that an enhanced cauliflower promoter (dCaMV) does not result in appreciable activity in any tissue analysed. Tobacco plants carrying the same promoter-GUS construct have an average GUS activity in leaves that is about 400-fold higher than the activity in chrysanthemum leaves (data from Mlynárová et al. (1995)). This indicates that it is the promoter/plant combination that is the cause of the low expression level. Previously, histochemical assays of CaMV-GUS transformed chrysanthemum allowed the identification of cells and tissue patches that turn blue upon incubation with X-gluc (de Jong et al. 1994, 1995; Fukai et al. 1995), (our unpublished data). However, more quantitative assays demonstrated that this histochemical staining reflects either very low and/or very localised GUS activity. The same is observed for the dCaMV promoter-GUS plants analysed in this study (data not shown). The influence of doubling the CaMV enhancer sequence has apparently been minimal in case of chrysanthemum. A promoter characterisation using the GUS reporter gene in transgenic plants should not be performed on the basis of histochemical staining alone.

The precise reason for the poor behaviour of the dCaMV promoter in chrysanthemum is unclear. With the *Lhca3.St.1* promoter-GUS constructs, GUS-active chrysanthemum plants are easily obtained. This indicates that it is the promoter driving the GUS gene that is the cause of the poor expression. A minor difference between the poorly active dCaMV-GUS and the highly active *Lhca3.St.1*-GUS constructs in chrysanthemum is the 5′-leader sequence of the GUS-derived messenger. Such a leader may influence mRNA stability (De Loose et al. 1995; Harpster et al. 1988). The leader of the petunia chlorophyll a/b binding protein (Cab22L) was shown to be able to modulate GUS activity levels in transgenic tobacco (De Loose et al. 1995). The potato *Lhca3.St.1* gene has a similar leader, only part of which is present in the GUS mRNA of NLG and ANLGA plants (Mlynárová et al.

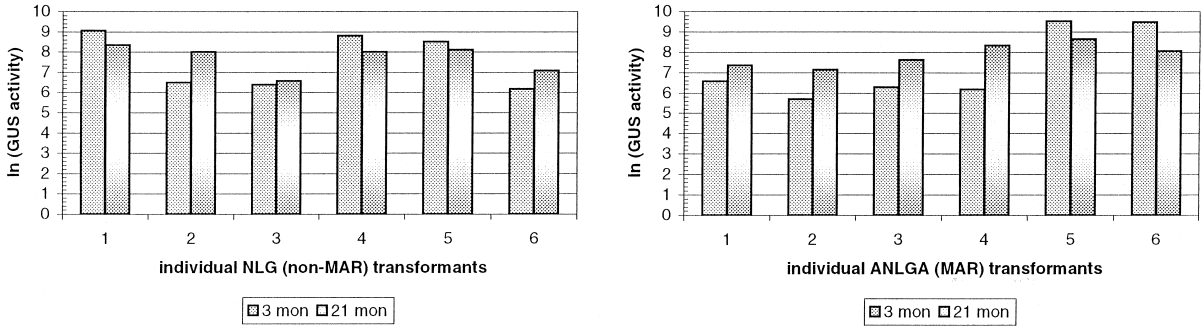

Figure 4. Stability of GUS activity over a period of 21 months in selected chrysanthemum transformants. *4A*, plants from the non-MAR NLG population; *4B*, plants from the MAR ANLGA population. The natural logarithm (ln) of the GUS activity of each individual transformant at 3 months and 18 months is plotted in pmol MU per microgram protein per hour.

1994). The 175-fold difference in activity between *Lhca3.St.1*-GUS and dCaMV-GUS chrysanthemum plants is too high to be related to only mRNA stability, especially given the much smaller difference of the activities of the same promoter-GUS constructs in tobacco (a 2–5 fold difference (Mlynárová et al. 1994, 1995, 1996)). The high GUS activity in *Lhca3.St.1* promoter-GUS plants also rules out the possibilities that the GUS enzyme is for unknown biochemical reasons not a good marker for chrysanthemum, or that the virulent *Agrobacterium* strain AGLO used for transformation (see also Urban et al. (1994)) is generating only complex and therefore silenced transgene loci in chrysanthemum. Therefore, the low activity must be related to the dCaMV promoter itself. Possibly chrysanthemum lacks transcription factors necessary for efficient dCaMV promoter activation. At present there is no reason to hypothesise that the dCaMV-GUS fusion is particularly prone to gene silencing in hexaploid chrysanthemum. The large genome size of chrysanthemum, of about 9000 Mb/cell or 25 pg/cell (Bennett and Smith 1976; van Wordragen et al. 1991), i.e. about 6 times tobacco and 70 times *Arabidopsis*, has unfortunately been severely prohibitive for a routine determination of transgene copy numbers and transgene configurations in our laboratory.

Results on transgene expression with CaMV-derived promoters in chrysanthemum are often reported in the literature as histochemical assays without quantitative data (Boase et al. 1998; Burchi et al. 1995; de Jong et al. 1993, 1994, 1995; Fukai et al. 1995; Ledger et al. 1991; Lemieux et al. 1990; Lowe et al. 1993; van Wordragen et al. 1992). In the papers in which quantitative GUS data driven by CaMV promoters are put forward, the reported activities are in the same order of low magnitude as reported here (Benetka and Pavingerová 1995; Lowe et al. 1993; Pavingerová et al. 1994; Sherman et al. 1998; Urban et al. 1994; van Wordragen et al. 1991, 1992, 1993). Furthermore, for other genes transformed into chrysanthemum under the control of CaMV-derived promoters, the RNA, protein or a phenotype is poorly detectable (Shao et al. 1999; Sherman et al. 1998; Urban et al. 1994), due to low expression levels and/or gene silencing (Courtney-Gutterson et al. 1993, 1994; Dolgov et al. 1995, 1997; Renou et al. 1993; Takatsu et al. 1999). An example of such low expression levels is the use of the 4× enhanced CaMV promoter driving the rice chitinase RCC2 gene to confer resistance to gray mould (*Botrytis cinerea*) in chrysanthemum (Takatsu et al. 1999). In this paper a positive ELISA is shown and the ELISA readings correlate reasonably well with fungal resistance. However, the highest reading reported for a non-resistant transformant is 1.04 (relative to the untransformed control calibrated to 1.0) and the lowest reading reported for a resistant transformant was 1.18 (Takatsu et al. 1999). This indicates that relatively very small differences in the amount of chitinase may determine resistance. Overall, a critical review of the results on transgene expression in chrysanthemum confirms that indeed there are problems with obtaining high expression levels with CaMV promoter-driven genes in chrysanthemum. Similar problems could occur in other plant species.

Activity conferred by the Lhca3.St.1 promoter with and without flanking MAR elements

In contrast to the low activity of the dCaMV promoter, the potato *Lhca3.St.1* promoter gives much higher activities in chrysanthemum. The activity in leaves is about 175 fold higher than conferred by the dCaMV promoter. The similar comparison in tobacco

resulted in a difference of only 2–5 fold between the two promoters (Mlynárová et al. 1994, 1995, 1996). Average GUS activity in chrysanthemum leaves is 1535 pmol MU per microgram protein per hour. Activity of this promoter-GUS construct in tobacco was about four-fold higher [data from Mlynárová et al. (1994)). Expression in chrysanthemum is somewhat higher in stems, but appreciable in all other aerial tissues evaluated (Figure 3). The GUS activity observed in chrysanthemum pollen is consistent with results in tobacco (data not shown, Conner et al. (1999)). The *Lhca3.St.1* potato promoter is therefore a suitable promoter to obtain much higher expression levels in the aerial parts of the chrysanthemum plant.

The non-MAR (NLG) and MAR (ANLGA) chrysanthemum populations do not differ significantly in mean, but the MAR-containing population ANLGA shows a higher variance (Table 1). This is in marked contrast to the data generated previously for tobacco (Mlynárová et al. 1994) and potato (unpublished data). In these cases, addition of the same chicken lysozyme MAR elements reduced position effects significantly, up to 20 fold. These results could imply that in chrysanthemum the chicken lysozyme MAR element is not effective at all. The action of a MAR element may depend on the host organism. However, the conflicting results in the presence of the MAR element may also be due to the required statistical analysis. In tobacco, the addition of the MAR elements generated preferably single-copy plants and revealed a copy number dependence of gene expression not present in the control population (Mlynárová et al. 1994). The chrysanthemum populations analysed here may differ in copy number distribution. Extensive Southern blot analyses would be able to resolve this point. The individual NLG and ANLGA plants analysed for the stability of GUS gene expression in time after vegetative growth and propagation do not differ appreciably in GUS activity. This indicates that once expression of a *Lhca3.St.1*-GUS fusion is observed upon integration in the chrysanthemum genome, it activity remains stable. Similar to tobacco (Mlynárová et al. 1996), the GUS activity driven by the *Lhca3.St.1* promoter in chrysanthemum shows a much lower variability around its mean compared with GUS activity driven by the dCaMV promoter. This indicates that the *Lhca3.St.1* promoter has an intrinsic stability associated with its activity.

Future prospects for the molecular breeding of chrysanthemum

The results presented here have established that CaMV-based promoters are not suitable for obtaining an high level of transgene expression in chrysanthemum. It will be interesting to see whether the same applies to other plant species, for example species from the *Compositae* family. Determination of the precise reason for the low activity of CaMV-derived promoters in chrysanthemum will require more detailed molecular analyses and more experiments. For now, however, it can be concluded that the use of CaMV-based promoters in the molecular breeding of chrysanthemum should be discouraged. In contrast, the potato *Lhca3.St.1* promoter results in high GUS gene expression levels in the aerial parts of chrysanthemum. Possibly endogenous promoters of chrysanthemum could yield again higher expression levels (Rout and Das 1997). However, for most applications in the molecular breeding of chrysanthemum, the gene expression level given by this heterologous promoter may well be sufficient. For example, the use of this potato promoter to express genes targeting insect pests would be a relatively straightforward way to confer resistance to pests such as the beet army worm (*Spodoptera exigua*) or the western flower thrips (*Frankliniella occidentalis*) that cause severe damage in the aerial parts of the chrysanthemum plant.

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