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Cloning of the chrysanthemum *UEP*1 promoter and comparative expression in florets and leaves of *Dendranthema grandiflora*

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Abstract

To attain high transgene expression in petal tissue of ray florets of chrysanthemum an endogenous *ubiquitin extension protein* (*UEP*1) promoter was cloned and tested with the *β-glucuronidase* (GUS) reporter gene. Expression levels were compared with four heterologous promoters: *chalcone synthase* (*chs-A*) and *zinc finger transcription factor* (*EPF*2-5) from petunia, *eceriferum* (*CER*6) from *Arabidopsis* and *multicystatin* (*PMC*) from potato. The comparison of the expression levels of the different constructs in ray florets, disc florets, and leaves is presented. The highest mean expression in petal tissue of ray and disc florets was conferred by the *UEP*1 promoter, followed by *CER*6 and *EPF*2-5. The *UEP*1 promoter in ray florets confers over 50-fold enhancement in expression as compared to CaMV 35S-based promoters.

Introduction

Chrysanthemum *Dendranthema grandiflora* (Anderson, 1987) is the second largest cut flower produced next to roses (*Rosa hybrida*). Insects cause direct and indirect damage on plants resulting in reduced flower quality and lower marketable value. Two important pests of chrysanthemum are beet armyworm, *Spodoptera exigua* (Lepidoptera: noctuidae) (Cuijpers, 1994) and western flower thrips, *Frankliniella occidentalis* (Thysanoptera) (De Jager et al., 1995). The prospect of achieving insect resistance through genetic engineering has received attention in the recent past. The use of Bt toxins (Dolgov et al., 1995) and proteinase inhibitors could be a method of developing insect resistant chrysanthemums, but requires high level of expression in leaves and flowers.

Studies on transgene expression in chrysanthemums have focused on the 35S-CaMV (cauliflower mosaic virus) promoter (Ledger et al., 1991; Renou et al., 1993; De Jong et al., 1995). However, with the 35S-CaMV promoter only low levels of expression were observed by us and other groups (Van Wordragen et al., 1993; Boase et al., 1998; Sherman et al., 1998). The enhanced d35S-CaMV promoter fused to GUS showed bright blue coloration in histochemical assays, but the quantitative β-glucuronidase (GUS) measurements indicated low expression in all tissues (Annadana et al., in press). The western flower thrips, *Franklinella occidentalis*, damages the petal tissue of ray florets in chrysanthemums. In order to develop resistance to thrips in transgenic plants the identification of a promoter conferring high expression levels in floral tissues was required. Recently, the light harvesting cab promoter *Lhca3.St.*1 was identified as a strong promoter for expression in chrysanthemum leaves and stems (Annadana et al., in press), but the expression in ray florets was 6-fold lower than in leaf tissue.

Chrysanthemum flowers consist of ray and disc florets. Ray florets are the long florets, which radiate

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from the centre of the bloom. They contain only the female sexual organs, with predominantly petal type of tissue. The disc florets, in the centre of the bloom forming the central pin cushion, have both male and female organs, but have an extremely reduced amount of petal tissue. Information on promoters conferring high expression in petal tissue of ray florets of chrysanthemum is not available. An approach was taken in which, known heterologous promoters from genes expressed or expected to be expressed in flowers of other plants were used in addition to the *de novo* isolation of a promoter from an abundantly expressed endogenous gene of chrysanthemum such as ubiquitin. Known heterologous promoters derived from genes expressed in flowers are genes associated with flavonoid biosynthesis in petunia such as the chalcone synthase gene *chs-A* (van der Meer et al., 1990), and transcription factors in the corolla of petunia, such as the zinc finger transcription factor *EPF*2-5 (Takatsuji et al., 1994). Petals are rich in wax, so the promoter of the *Arabidopsis eceriferum* gene *CER*6 involved in wax biosynthesis (Pereira, unpublished) would potentially also express in petals. For applications against insects, wound-inducible promoters would offer strong localised gene expression at the site of the wound. The wound-inducible promoter of potato multicystatin, *PMC* (Walsh et al., 1993) was selected on the basis of its presumed role in defence against insects. Ubiquitin proteins are involved in diverse fundamental cellular events such as vascular differentiation, programed cell death, controlling cell cycle progression, response to stress and determining steady state levels of proteins. The choice for ubiquitin genes as candidates for providing a potentially strong homologous promoter was based on DNA microarray data demonstrating that ubiquitin genes in *Arabidopsis* were among the most abundant messengers in most plant organs including flowers (Ruan et al., 1998).

Materials and methods

Oligonucleotides used for cloning and sequencing

Primer UBDN1:	*AARATHCARGAYAARGAR*
Primer POLY-T:	*CCCGGATCCTCTAGAGC GGCCGCTTTTTTTTTTTTT TTTTT*
Primer UBGSP1:	*GCAAACTATTCAAATTGA CTATATAAAGC*
Primer UBQP1:	*CCCCCCAGATCTGAGCT CCCATGGAATCTAGAATTT TGG ACCACGGTGGGTACGG*
Primer PMCDN1:	*CCCCCCGGCGCGCCAGA TGATTTCGAAGATTTAAGG GTATA*
Primer PMCP:	*CCCCCCAGATCTGAGCTC CCATGGAATCTAGAATCAC TGCGAATAATTAATTTAATT AAG*
Primer PETCHS-DN:	*GGGGAATTCAAGCTTACT GGTGTGATTCTTGAATC*
Primer PETCHS-UP:	*GTCGAGCTCAGATCTGGG CCATGGTTTTTCTAGAAAA AAGTTTGGTATTT*

All oligonucleotides were obtained from Eurogentec (Seraing, Belgium).

Cloning and sequencing the homologous UEP1 *promoter*

Ubiquitin genes from seven plant species (sunflower X57004, parsley X64344, *Arabidopsis thaliana* X12853, *Glycine max* D26092, pea X17020, tomato X58253, and tobacco Y09107) were aligned and a conserved region of 49 aminoacids was observed. The degenerate primer UBDN1 was designed based on the 5′part of the conserved region. First strand cDNA was generated by reverse transcriptase using the POLY-T primer on poly A^+RNA isolated from ray florets in bloom before anthesis. A PCR fragment was generated using UBDN1 and POLY-T on first strand cDNA and used as a probe on a small cDNA library cloned in the SK^+ plasmid vector (250 clones, made using the ClonTech cDNA synthesis kit). The one hybridising cDNA clone was sequenced and based on 5′-sequence, the primer UBGSP1 and UBQP1 were designed to walk upstream towards the promoter, by nested PCR using the Genome Walker kit (ClonTech). The genomic DNA was digested with *Eco*RV, *Sca*I, *Dra*I, *Pvu*II, and *Ssp*I, ligated to adapters, and used as a template for PCR with Adapter specific primer 1 provided in the Genome Walker kit and the UBGSP1. The products generated were used as a template for nested PCR using adapter primer 2 and UBQP1. The longest promoter fragment (2.1 kb) from the library produced by digestion with *Pvu*II was cloned into vector pGEM-T (after A-tailing) sequenced (Figure 1) and named *UEP*1. The *UEP*1 promoter was digested with *Sal*I-*Xba*I and ligated to

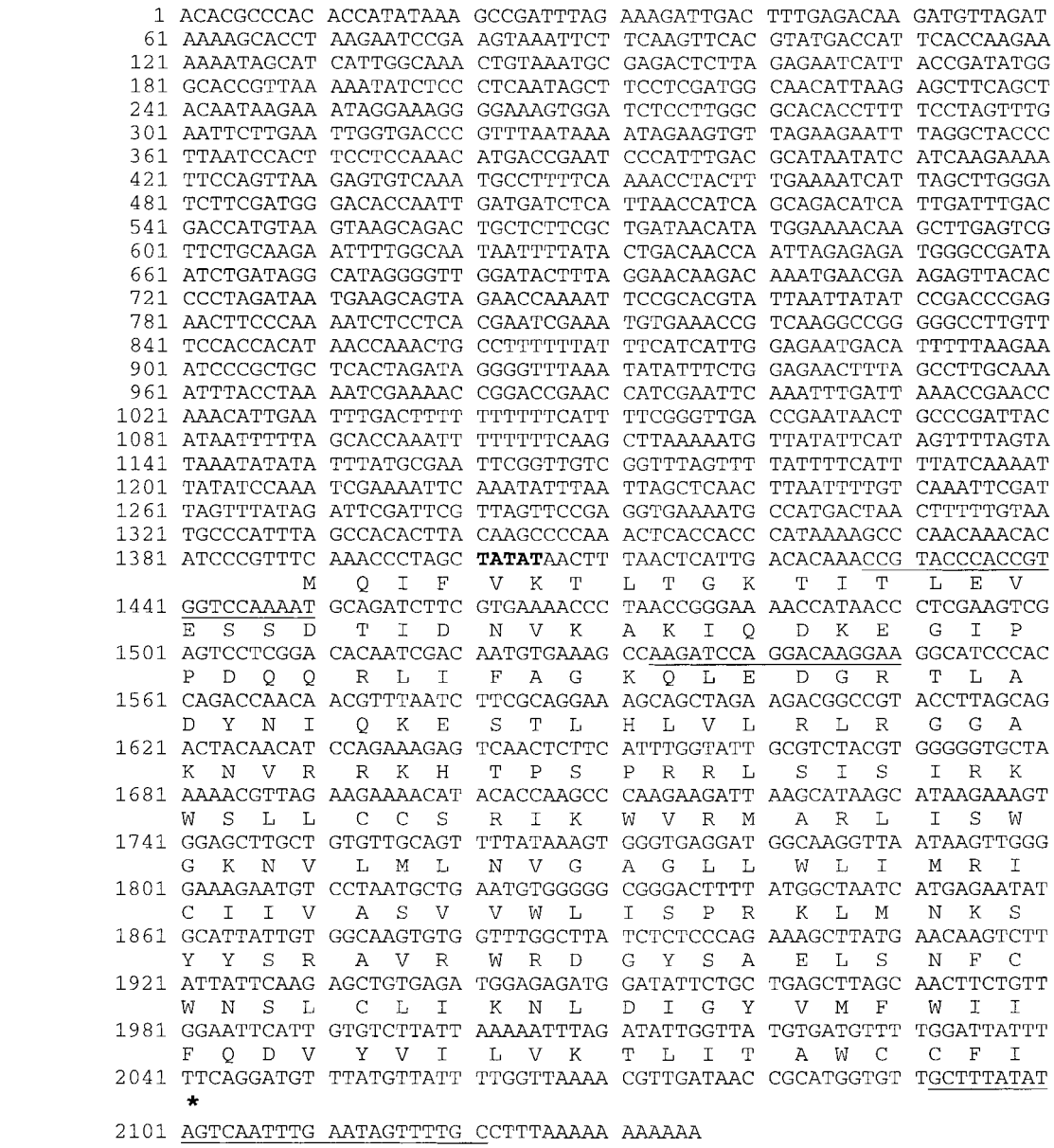

Figure 1. The 1448 bp *UEP*1 promoter (1-1448) of the chrysanthemum ubiquitin extension protein (1449-2102). The amino acid sequence of the ubiquitin monomer extends from Met-1 to Gly-76 (1449-1676) and the extension protein from Ala-77 to Ile-141 (1677-2102) is given in one letter code above the centre of each codon. The extension protein starting at Gly-76 is also the site for the ubiquitin-specific proteases, which cleave the extension protein and release the active ubiquitin monomer. The primers designed for cloning the promoter namely the UBDN1 (1533-1550), UBGSP1 (2092-2122), and UBQP1 (1428-1450) are underlined. The putative TATA box 48 bp upstream of Met-1 is indicated in bold.

the *Xba*I-*Sph*I β-glucuronidase (GUS) fragment with NOS terminator from pMOG410 (Hood et al., 1993), into a pUCAP vector (van Engelen et al., 1995) digested with *Sal*I-*Sph*I. The entire expression cassette was then isolated as *Sal*I-*Pac*I and ligated into the *Sal*I-*Pac*I digested binary vector pBINPLUS (van Engelen et al., 1995), resulting in p*UEP*1-GUS (Figure 2). All DNA manipulations were carried out using *E. coli* strain XL-1 blue. p*UEP*1-GUS was checked for promoter activity in transient assays with gladioli calli by particle bombardment as described by Wilmink et al. (1992). p*UEP*1-GUS was finally electroporated to electro-competent *Agrobacterium* AGL0 (de Jong et al., 1995).

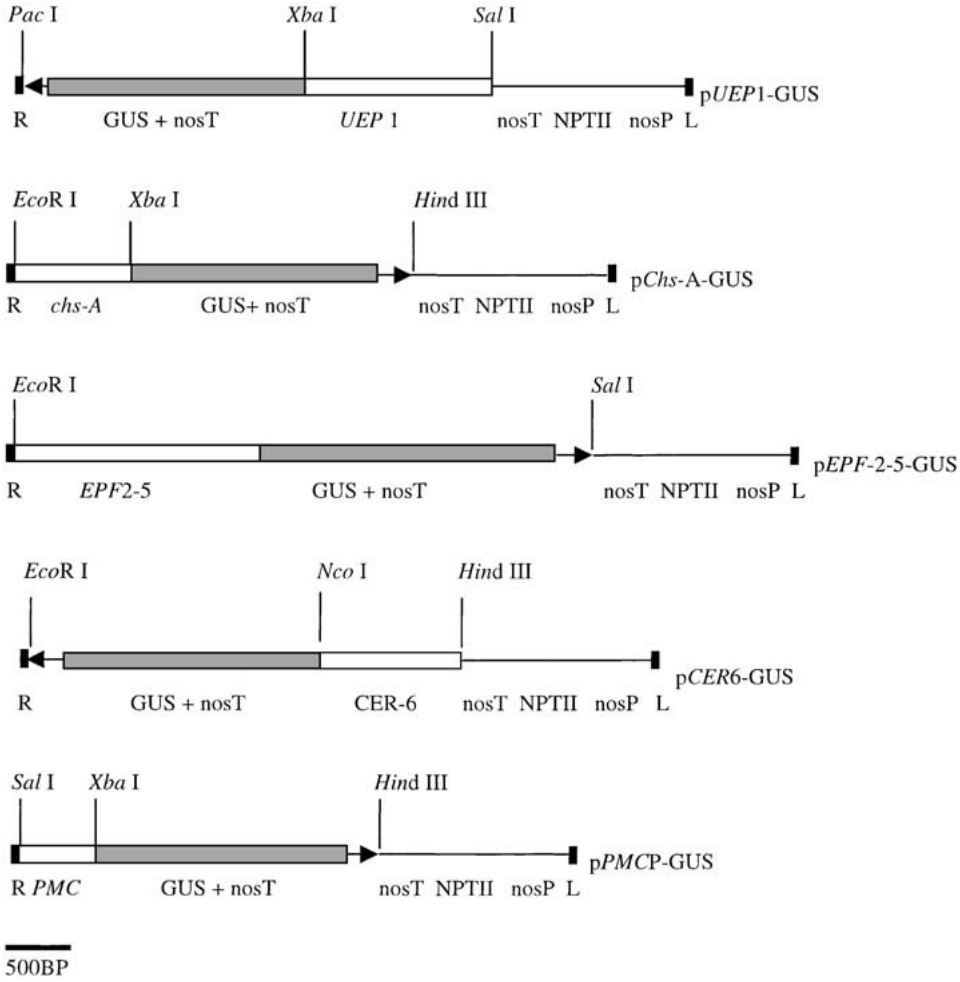

Figure 2. Schematic representation of the T-DNA regions of the five constructs used in this investigation. R, L, right and left T-DNA borders, respectively; GUS + nosT, is the β-glucuronidase gene with intron with *Nopaline synthase* terminator; nosP, nopaline synthase promoter; NPT II, neomycin phosphotransferase II coding region; UEP1, ubiquitin extension protein promoter; *chs*-A, *Chalcone synthase* promoter; *EPF*2-5, Zinc finger transcription factor promoter; *CER*6, Eceriferum 6 promoter; *PMC*, potato multicystatin promoter.

Preparation of GUS-constructs with heterologous promoters

The *chs-A* promoter and p*EPF*2-5-GUS construct were obtained from Dr. I. Van der Meer (Plant Research International, NL) and Dr. Takatsuji (National Institute of Agrobiological Resources, Japan), respectively. The *chs*-A promoter was cloned into the pBINPLUS by PCR using primers PETCHSDN and PETCHSUP, digestion with *Eco*RI-*Xba*I, and ligated with an *Xba*I-*Hin*dIII 2.2 kb GUS fragment with NOS terminator obtained from pMOG410 into binary vector pBINPLUS digested with *Eco*RI-*Hin*dIII. This construct was named p*chs-A*-GUS (Figure 2). The construct p*EPF*2-5-GUS (Figure 2) was digested with *Eco*RI-*Sal*I from the pBI vector and transformed to pBINPLUS. The p*CER*6-GUS construct was already provided in pBINPLUS (Pereira unpublished). The 670 bp *PMC* promoter (*PMC*P) was obtained by PCR on genomic DNA of potato cv Superior using primers PMCDN1 and *PMC*P based on the published sequence (Waldron et al., 1993). The PCR fragment was A-tailed and cloned into pGEM-T and sequenced. The *PMC*P-containing pGEMT was digested with *Xba*I-*Sph*I, and ligated with a 2.2 kb *Xba*I-*Sph*I β-glucuronidase (GUS) fragment with NOS terminator fragment from pMOG410. Subsequently the entire expression cassette was obtained as a *Sal*I-*Hin*dIII fragment from pGEM-T, cloned into the binary vector pBINPLUS, and named p*PMC*P-GUS (Figure 2). All plant constructs were electroporated to AGL0.

Plant transformation

Stem explants of chrysanthemum cv. 1581 were obtained from 3-week old cuttings raised in the greenhouse. The explants were surface sterilised, transformed, and selected as described by de Jong et al. (1995).

Induction of the PMC *wound-inducible promoter*

Plants harboring the *PMC*P-GUS construct were induced with methyl jasmonate (MeJa), α- and γ-linolenic acid. The fourth leaf from the top of the plant was placed in a microfuge tube containing 0.4% water agar. The tubes with leaves were placed in square petri dishes (243 × 243 × 18 mm from Nunc Denmark). A drop of MeJa was put on a filter paper placed inside the petri dish sealed with parafilm. The petri dish was incubated at 25°C for 24 h. Treatments with α- and γ-linolenic acid were conducted as described by Farmer and Ryan (1992). After 24 h induced tissues were analysed for GUS expression as described.

GUS activity assays

GUS measurements were performed as described by Mlynarova et al. (1994) for which 10 ray florets were used per flower per plant. For the top six expressers, 30 disc florets, and 9-mm-diameter leaf discs were also analysed. The ray and disc florets were stained with X-gluc for histochemical assays (Jefferson et al., 1987).

Results

Isolation of the homologous promoter and confirming its expression and activity

A ubiquitin probe of 250 bp was generated by reverse transcriptase PCR on total RNA from ray florets. This probe was used to identify an abundantly expressed ubiquitin cDNA clone in the ray florets, in a small ray florets cDNA library of 250 clones. One of the clones gave a strong signal. This cDNA clone 155 was used as a probe for a northern blot with total RNA from ray florets and leaf. A 3-fold higher signal was observed in ray florets compared to leaves, demonstrating higher expression in the ray florets (not shown). 5′Race did not yield any longer transcripts. The *UEP*1 promoter plus cDNA fragment of 2.1 kb in addition to three smaller fragments (<600 bp) were generated by PCR on the *Pvu*II library of fragments. There were also three small fragments (<500 bp) generated on the library of fragments generated with *Sca*I (data not presented). As the cDNA part was 650 bp, the smaller fragments generated on the *Pvu*II and *Sca*I library were discarded. The 2.1 kb *UEP*1 promoter fragment was found to contain a promoter region of 1448 bp followed by a 650 bp coding region matching with the original cDNA sequence (Figure 1).

GUS activity conferred by the different promoters in the florets and leaves

Five constructs viz.; p*UEP*1-GUS, p*chs*-A-GUS, p*EPF*2-5-GUS, p*CER*6-GUS, and p*PMC*P-GUS (Figure 2) were generated, and transformed into chrysanthemum using *Agrobacterium tumefaciens*. At least 18 plants per construct were analysed for expression levels of GUS. The expression of GUS in the ray florets, measured in picomoles per minute per microgram protein (pmol/min/μg protein) was converted to $\log_{10}$, as a proper comparison of GUS expression levels requires logarithmic transformation (Figure 3, Nap et al., 1993). The homologous p*UEP*1-GUS population, with a mean GUS activity of 8.5 pmol/min/μg protein was 1.5–4-fold higher than the rest. The level of variation in GUS expression levels between independent transformants was remarkably low for the p*UEP*1-GUS and p*EPF*2-5-GUS populations as compared to the rest (Figure 3).

For each construct the top six expressers for ray florets were selected for additional analysis of the expression levels in the disc florets and leaves (Table 1). In the disc florets the highest level of expression was observed in the p*UEP*1-GUS population with a mean of 2.9 pmol/min/μg protein which was 2–8-fold higher than the other populations. Expression in the leaf was highest in both the p*UEP*1-GUS and p*PMC*P-GUS populations (0.9 pmol/min/μg protein) and upto 4-fold higher than the remaining constructs.

Cystatin genes in potato and tomato are induced by MeJa (Akers & Hoff, 1980). The application of MeJa on the population of p*PMC*P-GUS resulted in an induction in the range of 5–25% which was small and not significant (Table 1). Normally upon induction there is a several-fold increase in the protein level. Induction with alpha- and gamma-linolenic acid

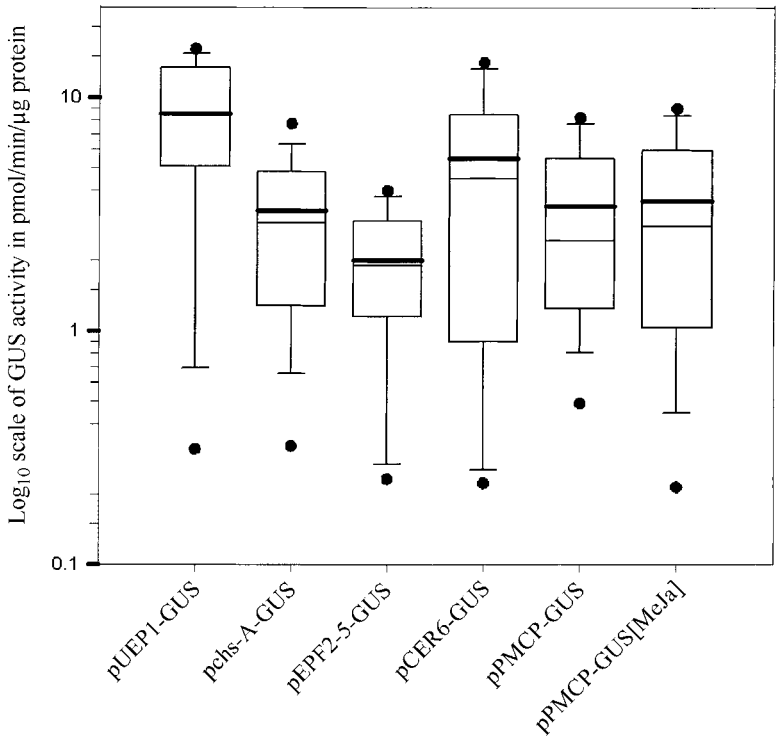

Figure 3. GUS activity in the entire population in the ray florets of chrysanthemum. Black dots on top and bottom of the box represent the 5th and 95th percentile, respectively. The thin and bold lines in the box represent the median and mean, respectively. The top and bottom line of the box represent the 25th and 75th percentile for the population. Based on these data it is possible to see the variation of expression in the population, which is minimal for *UEP*1 and *EPF*2-5 promoters and maximum for *CER*6.

Table 1. GUS activity (pmol/min/µg protein) conferred by different promoters in the ray florets, disc florets, and leaves of *Dendranthema grandiflora*

Construct	#[1]	Ray florets			Disc florets			Leaves		
		Max[2]	Min[2]	Mean[3]	Max[2]	Min[2]	Mean[3]	Max[2]	Min[2]	Mean [3]
P*UEP1-GUS*	22	16.5	0.2	8.5^{a}	7.0	0.7	2.9^{a}	1.4	0.6	0.9^{ab}
P*Chs*-A-GUS	20	8.5	0.2	3.2^{bc}	3.1	0.2	1.2^{bc}	0.3	0.2	0.2^{c}
P*EPF2–5*-GUS	19	4.0	0.2	2.0^{c}	3.4	0.7	1.7^{ab}	1.0	0.3	0.6^{b}
P*CER6*-GUS	18	14.5	0.2	5.5^{b}	3	0.6	1.6^{ab}	1.6	0.3	0.7^{ab}
P*PMCP-GUS*	20	8.3	0.2	3.4^{bc}	0.5	0.1	0.28^{c}	1.5	0.3	0.9^{ab}
P*PMCP*-GUS (MeJa)	20	9.1	0.21	3.6^{bc}	0.49	0.19	0.34^{c}	1.4	0.6	1^{a}

[1]Number of transgenic plants.
[2]The lowest (Min) and highest (Max) expression level in the population of plants.
[3]The whole population is used to arrive at the mean for the ray florets. The top six expressing ray florets expressers were used to detect expression in the disc florets and leaves. The LSD for ray, disc florets, and leaf are 2.3, 1.5, and 0.4 pmol/min/µg protein, respectively. The values denoted with different letters differ significantly from each other.

was tested as some insect/pathogen-inducible promoters, not induced by MeJa, do get induced by either of these acids (C. Girard, personal communication). However, there was no observable induction of *PMCP* by either α- or γ-linolenic acid (data not shown).

Discussion

Aim and approach

Our aim was to identify a strong promoter for expression in the petal tissue of ray florets. Such promoters could be used for crop protection or to improve

flower quality traits. Ruan et al. (1999) who monitored expression profiles of 1400 genes using cDNA microarrays of *Arabidopsis*, found that ubiquitin was the dominant transcript in all tissues (root, leaf, flower bud, and open flowers). On this basis a small cDNA library from ray florets of chrysanthemum was checked for ubiquitin clones and a ubiquitin extension protein cDNA (*UEP*1) was identified. The *UEP*1 promoter was cloned by PCR, tested using the β glucuronidase reporter gene and compared with other heterologous promoters expressing the same reporter gene. The *UEP*1 promoter was found to be the most active compared to any of the other promoters tested in both ray and disc florets.

Activity of the UEP1 *promoter and the heterologous promoters*

Thus far few genes have been cloned from ray florets of any composite plant species (Helariutta et al., 1993; Williams et al., 1999). The newly cloned chrysanthemum promoter of the *UEP*1 gene confers the highest levels of expression in the petal tissues of ray florets (8.5 pmol/min/μg protein) while 3-fold and 9-fold lower expression levels are observed in the disc florets (2.9 pmol/min/μg protein) and leaves (0.9 pmol/min/μg protein), respectively. Comparison of the homologous *UEP*1 promoter to the heterologous promoters from other plants and genes showed higher expression in ray and disc florets with the UEP1 promoter in a range of 2–10-fold, depending on the promoter and tissue. The difference in expression levels between the constructs was maximal in the disc florets and minimal in the ray florets. Histochemical analysis of the *UEP*1 promoter indicated that activity in ray florets was limited to petal tissues and did not extend into the tube of the petal and the sexual whorls of the floret. In the disc florets it was limited to the reduced petal structure and developing pollen, while in developed pollen we were unable to observe staining, which may have been due to reduced penetration of substrate through the hard exine of the pollen (data not shown). Thus, the promoter appears to have high activity in petal and pollen tissue, but low activity in other tissues of the florets and the vegetative structures of chrysanthemum, such as leaves. The variability in expression for the *UEP*1-GUS and *EPF*2-5-GUS populations was minimal in comparison to the other plant populations suggesting less susceptibility of the construct to position effects.

The *CER*6 genes associated with the wax biosynthesis pathway are known to be expressed in flowers (Hannoufa et al., 1996). It was found that the GUS activity in the ray florets conferred by the *CER*6 promoter was the highest among the heterologous promoters tested for expression. However, a potential drawback of this promoter is that, the variability in expression was highest as compared to the other populations. Also the fact that its activity is limited to the L1 layer of the epidermis in *Arabidopsis* may limit its applications (Pereira, unpublished data). It would, for example, be less suitable for the expression of certain antagonists of thrips as these insects also suck sap from below the L1 layer of chrysanthemum tissues.

The PMC promoter was selected as the gene is reported to be wound inducible (Walsh & Strickland, 1993). In our hands the PMC promoter showed about 4-fold higher activity in ray florets compared to leaves which was unexpected as these genes are mainly known to be expressed in the leaf and tuber (Walsh & Strickland, 1993). Wound induction could potentially further enhance those levels and hence, the induction with MeJa and similar inducers like α- and γ-linolenic acid were tested. No induction was observed, however. The *PMC* promoter sequence analysed for motifs in the PLACE program (http://www.dna.affrc.go.jp/htdocs/PLACE/signalscan.html), recognised several motifs and the TATA box but did not recognise any known motifs for wound response. The 600 bp *PMC* promoter may, therefore, not represent the complete promoter fragment as a result of which the *cis*-acting elements necessary for wound induction may be absent.

Comparison of the UEP1 *promoter with 35S CaMV and* Lhca3.St.1 *promoter*

The *UEP*1 promoter is an alternative to the 35S CaMV based promoters in chrysanthemum. GUS expression data in chrysanthemum driven by the 35S CaMV promoter show low expression in the range of 0.1–0.2 pmol/min/μg protein (Annadana et al., 2000, in press). This low expression is not limited to GUS, but was also observed with other transgenes (Boase et al., 1998; Sherman et al., 1998). Recently, 35S CaMV promoters with two enhancers and the AMV untranslated leader sequence did not significantly improve the low expression levels (Annadana et al., in press). We now observed more than 50-fold enhancement in GUS expression by *UEP*1 over dCaMV based promoters in the petal tissues of ray florets of chrysanthemum.

The *Lhca3.St.*1 promoter from potato has a similar expression level in petals (7.8 pmol/min/μg protein), but over 6-fold higher expression levels in the leaves (44.8 pmol/min/μg protein, Annadana et al., in press). This is in contrast to the *UEP*1 promoter with 9-fold lower expression levels in the leaves (0.9 pmol/min/μg protein), resulting in an effective 50-fold expression difference in the leaves comparing the *UEP*1 promoter to the *Lhca3.St.*1 promoter. This may have distinct advantages for some applications involving flower quality traits. For crop protection it may be relevant that the *UEP*1 promoter is likely not light-dependent in expression like *Lhca3.St.*1 and may react differently to stress.

The expression patterns of all constructs demonstrate highest levels in the petal tissues of the ray florets, suggesting that a good selection of promoters for high activity in petal tissue of ray florets was made. The homologous *UEP*1 promoter is better than the selected group of heterologous promoters for conferring high levels of transgene expression in petal tissue. The data on the comparison of the promoters in chrysanthemum may have relevance to the engineering of the corolla from other cut flowers as well. The *UEP*1 promoter has the potential to strongly express transgenes, with limited variation in expression in the petal tissues, which can be applied to improve floral quality (vase life, colour, fragrance, resistance) in flowers.

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References

Ackers CP and Hoff JE (1980) Simultaneous formation of chymopapain inhibitor activity and cubical crystals in tomato leaves. *Can J Bot* **58**: 1000–1003.

Anderson NO (1987) Reclassification of the genus *Chrysanthemum* L. *Hort Sci* **22**: 313.

Annadana S, Mlynarova L, Udayakumar M, de Jong J and Nap JPH (2002) The potato *Lhca3.St.*1 promoter confers high and stable transgene expression in chrysanthemum, in contrast to CaMV-based promoters. *Mol Breed* **8**: 335–344.

Boase MR, Bradley JM and Borst NK (1998) Genetic transformation by *Agrobacterium tumefaciens* of florists chrysanthemum (*Dendranthema X Grandiflorium*) cultivar Peach Margaret. *Vitro Cell Dev Biol Plant* **34**: 46–51.

Cuijpers TAMM (1994) SPOD-X GH, recently registered in the Netherlands as a viral insecticide for control of beet army worm *Spodoptera exigua* in cutflowers and potted plants in greenhouses. Mededelingen Faculteit Landbouwkundige en Toegepaste Biologische Wetenschappen Universiteit Gent **59**: 393–403.

De Jager CM, Butot RPT, Klinkhamer PGL and Van Der Meijden E (1995) Chemical characteristics of chrysanthemum cause resistance to *Frankliniella occidentalis* (Thysanoptera: Thripidae). *J Econ Entomol* **88**: 1746–1753.

De Jong J, Rademaker W and Ohishi K (1995) *Agrobacterium*-mediated transformation of chrysanthemum. *Plant Tissue Cult Biotechnol* **1**: 38–42.

Dolgov SV, Mityshkina TU, Rukavtsova EB, Buryanov YI, Vainstein A and Weiss D (1995) Production of transgenic plants of *Chrysanthemum morifolium* Ramat with the *Bacillus thuringiensis* delta endotoxin. *Acta Hort* **420**: 46–47.

Farmer EE and Ryan CA (1992) Octadecanoid precursors of jasmonic acid activate the synthesis of wound-inducible proteinase inhibitors. *Plant Cell* **4**: 129–134.

Hannoufa A, Negruk V, Eisner G and Lemieux B (1996) The CER3 gene of *Arabidopsis thaliana* is expressed in leaves, stems, roots, flowers and apical meristems. *Plant J* **10**: 459–467.

Helariutta Y, Elomaa P, Kotilainen M, Seppanen P and Teeri TH (1993) Cloning of cDNA coding for dihydroflavonol-4-reductase (DFR) and characterisation of dfr expression in the corollas of *Gerbera hybrida* var. Regina (Compositae). *Plant Mol Biol* **22**: 183–193.

Hood EE, Gelvin SB, Melchers LS and Hoekema A (1993) New *Agrobacterium* helper plasmids for gene transfer to plants. *Transgenic Res* **2**: 208–218.

Jefferson RA, Kavanagh TA and Bevan MW (1987) GUS fusion: *β-glucuronidase* as a sensitive and versatile gene fusion marker in higher plants. *EMBO J* **6**: 3901–3907.

Ledger SE, Deroles SC and Given NK (1991) Regeneration and *Agrobacterium* mediated transformation of chrysanthemum. *Plant Cell Rep* **10**: 195–199.

Mlynarova L, Loonen A, Heldens J, Jansen RC, Keizer P, Stiekema WJ and Nap JP (1994) Reduced position effect in mature transgenic plants conferred by the chicken lysozyme matrix-associated region. *Plant Cell* **l6**: 417–426.

Nap JP, Keizer P and Jansen R (1993) First generation transgenic plants and statistics. *Plant Mol Biol Rep* **11**: 156–164.

Renou JPP, Brochard P and Jalouzout R (1993) Recovery of transgenic chrysanthemum *Dendranthema grandiflora* after hygromycin resistance selection. *Plant Sci* **89**: 185–187.

Ruan Y, Gilmore J and Conner T (1998) Towards *Arabidopsis* genome analysis: monitoring expression profiles of 1400 genes using cDNA microarrays. *Plant J* **15**: 821–833.

Sherman JM, Moyer JW and Daub ME (1998) Tomato Spotted Wilt Virus resistance in chrysanthemum expressing the viral nucleocapsid gene. *Plant Dis* **82**: 407–414.

Takatsuji H, Nakamura N and Katsumoto Y (1994) A new family of Zinc finger proteins in petunia: structure, DNA sequence recognition and floral organ-specific expression. *Plant Cell* **6**: 947–958.

Van der Meer I, Spelt CE, Mol NM and Stuitje AR (1990) Promoter analysis of the chalcone synthase (*chs-A*) gene of *Petunia hybrida*: a 67 bp promoter region directs flower-specific expression. *Plant Mol Biol* **15**: 95–109.

Van Engelen FA, Molthoff JW, Conner AJ, Nap JP, Pereira A and Stiekema WJ (1995) PBINPLUS: an improved plant transformation vector based on pBIN19. *Transgen Res* **4**: 288–290.

Van Wordragen MF, Honée G and Dons HJM (1993) Insect-resistant chrysanthemum calluses by introduction of a *Bacillus thuringiensis* crystal protein gene. *Transgen Res* **2**: 170–180.

Waldron C, Lynette M, Wegrich P, Merlo AO and Walsh TA (1993) Characterisation of a genomic sequence coding for potato multicystatin, an eight domain cysteine proteinase inhibitor. *Plant Mol Biol* **23**: 801–812.

Walsh TA and Strickland JA (1993) Proteolysis of the 85-kilodalton crystalline cysteine proteinase inhibitor from potato releases functional cystatin domains. *Plant Physiol* **103**: 1227–1234.

Williams CA, Harborne JB, Geiger H and Hoult JRS (1999) The flavonoids of *Tanacetum parthenium* and *T. vulgare* and their anti-inflammatory properties. *Phytochemistry* **51**: 417–423.

Wilmink A, Vanden Ven B and Dons H (1992) Expression of the GUS gene in the monocot tulip after introduction by particle bombardment *Agrobacterium*. *Plant Cell Rep* **11**: 76–80.