

# EXPRESSION OF POTATO MULTICYSTATIN IN FLORETS OF CHRYSANTHEMUM AND ASSESSMENT OF RESISTANCE TO WESTERN FLOWER THRIPS, *FRANKLINIELLA OCCIDENTALIS*

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## **Abstract**

The gene of the 85 kDa cysteine protease inhibitor (CPI) potato multicystatin (PMC), was PCR-cloned and expressed under the control of the *UEP1* promoter in chrysanthemum (*Dendranthema grandiflora*). Over 30 independent transgenic plant lines were analysed for PMC expression in florets by immunoassay and a selection of those for papain inhibitor activity (PIA). A significant correlation between PIA activity and PMC expression levels was established demonstrating that the highest expressers raised the concentration of PIA to 0.28 pmol/μg from an endogenous background level of 0.15 pmol/μg. On this basis, the PMC gene was estimated to be expressed at a level of 0.13% of total protein. Some of the transgenic lines exhibited up to 5-fold higher levels of PIA (0.82 pmol/μg protein). This did not correlate with the immunological data, however, and may be the result of frequently occurring somaclonal variation. A non-choice bioassay of 7-10 days on whole flowers was carried out to study the effect of PMC on the fecundity of Western Flower Thrips in terms of the number of larvae produced. No correlation between the reduction in the oviposition rate and PMC expression could be established, which may be due to the relatively low expression level of PMC in chrysanthemum. The transgenic lines with the highest levels of endogenous PIA also had the lowest thrips reproduction rates, but further experiments are required to exclude artefacts caused by the aberrant phenotype.

## **1. Introduction**

Chrysanthemum, *Dendranthema grandiflora*, is world-wide the second largest cutflower crop grown next to roses. Transgenic approaches are required for improving chrysanthemum traits such as crop protection, enhanced vase life, novel colour and fragrance, as it is difficult to combine these traits in parental lines used for conventional breeding. Chrysanthemum can be efficiently regenerated (Annadana *et al.*, 2000) and there are several reports on transformation using *Agrobacterium tumefaciens* (De Jong *et al.*, 1995). The Cauliflower Mosaic Virus 35S (CaMV35S) promoter with one or two enhancers confers very low levels of transgene expression in chrysanthemum (Annadana *et al.*, submitted for publication). This is a drawback for the application of protease inhibitors (PIs) to target insect pests, as PIs are required to be expressed at 0.5-1% of total plant protein to be effective. Recently, the homologous *UEP1* promoter was cloned, which confers 50-fold higher levels of expression as compared to the CaMV 35S based promoters (Annadana *et al.*, submitted for publication). This *UEP1* promoter was also identified as the best among several other heterologous promoters tested for expression in

petal tissues of chrysanthemum florets (Annadana *et al.*, submitted for publication).

Western Flower Thrips (WFT) is a sucking insect, polyphagous, small sized with a secretive, thigmotactic habit of remaining in inner whorls of flowers and opening buds. WFT incidence results in direct damage of pierced and emptied cells resulting in loss of photosynthesis and stunted growth (Harrewijn *et al.*, 1996) and in indirect damage by tospovirus transmission (Wijkamp *et al.*, 1995). WFT has developed into the most prevalent insect pest predated on vegetable and ornamental crops since 1983 in greenhouses in The Netherlands (Mantel and Van der Vrie, 1988). Damage caused by WFT is reported on more than 240 species of plants belonging to 62 different families (Loomans *et al.*, 1995) comprised of cut flower, vegetable, field, fruit and plantation crops. WFT currently is the most predominant pest on greenhouse grown chrysanthemum, where it generally prefers the flower and reaches high rates of reproduction (Oetting, 1991; van Dijken *et al.*, 1995). The overall cost incurred on thrips in 1996 in the Netherlands was 2.4 million Euros of which 1 million Euros were spent on chemical control (Roosjen *et al.*, 1998). The thigmotactic behaviour of WFT and rapid development of resistance has made chemical control arduous and decreasingly effective. Biological control does not present a sufficient alternative and is an economical hazard as also the presence of beneficial insects on the marketed product reduces its value (Loomans, personal communication).

The use of PIs in transgenic plants is an alternative to the present control methods. Insect gut proteases may be inhibited with appropriate PIs, resulting in reduced protein turn-over leading to stunted growth and increased mortality (Jongsma *et al.*, 1996). Cysteine proteases (CP) were shown to be predominant in total thrips extracts, and the cysteine protease inhibitor (CPI) potato cystatin (one of the domains of PMC) resulted in 55% reduction in oviposition rate (OR) after 5 days of exposure to the inhibitor incorporated in an artificial diet (Annadana *et al.*, submitted for publication). PMC is an 85 kDa CPI that can bind eight papain molecules simultaneously, and is naturally found in the sub-pherlogen layer in potato as natural cuboidal crystals (Rodis and Hoff, 1984). The PMC gene is wound inducible (Walsh *et al.*, 1993) and was shown earlier *in vivo* to be active against Western corn rootworm (Orr *et al.*, 1994).

The aim of the investigation was to generate transgenic chrysanthemum resistant to WFT. To achieve this aim PMC was expressed in petal tissues of ray florets of chrysanthemum under the control of the *UEP1* promoter and flowers were tested for their effects on WFT.

## **2. Materials and methods**

### 2.1. Oligonucleotides used for cloning the PMC gene

PMC-DN2: *CCCCCATGGCATAACGTAAAATTATTCGCAGTGATGGCAATCGTA*

PMC-UP2: *CCCCCGCGGCCGCCACACCAACATAAAAGTAGTTTC*

### 2.2. Preparation of constructs and plant transformation

Genomic DNA from potato cv. Superior was isolated using the Nucleon kit from Amersham Life Science. Two hundred ng of genomic DNA was used as template for a PCR reaction with primers PMC-DN2 and PMC-UP2 designed using the published sequence (Waldron *et al.*, 1993). For the PCR reactions Clontech's advantage genomic PCR kit was used which provided two polymerases (Tth and a 5'-3' proof reading polymerase) as a cocktail allowing longer fragment amplification (Barnes, 1994; Cheng *et al.*, 1994). The cycle parameters for the PCR reaction were 35 cycles (94° C for 1 minute, 55° C for 1 minute and 72° C for 6 minutes). After 35 cycles an additional extension time of 20 minutes at 72° C was provided. After A-tailing, the PCR product was cloned into the pGEM-T [Promega] vector and checked by restriction enzyme analysis. The PMC gene was obtained as an *NcoI-NotI* fragment and subcloned into pUCAP (van Engelen *et*

*al.*, 1995) which harbours the *UEP1* promoter (Annadana *et al.*, submitted for publication) as a *SalI-NcoI* fragment. Subsequently, the entire fragment containing the *UEP1* promoter with the PMC gene was obtained as an *AscI-SacI* fragment and subcloned into the binary vector pBINPLUS (van Engelen *et al.*, 1995) in front of the NOS terminator. This construct was transformed into *Agrobacterium* strain Agl0 and subsequently to chrysanthemum cv. 1581, as described by De Jong *et al.*, (1995).

### 2.3. Immuno- and inhibitor assay of PMC expression

Ten ray florets from a single flower were harvested, transferred into 1.5 ml centrifuge tubes, and ground in 200  $\mu$ l of extraction buffer (50 mM Tris-HCl pH 5, 50mM EDTA, PI cocktail, 1 tablet/10 ml [Complete Mini, Roche]). The tubes were centrifuged at 4° C for 15 minutes, the supernatant was transferred to a fresh tube and the total protein was quantified (Bradford, 1976). A protein dotblot (Nitrocellulose membrane 0.2  $\mu$ m) was prepared with 3  $\mu$ l of supernatant and 97  $\mu$ l of 40 mM CAPS, pH 11, as loading buffer for the BioRad TransBlot apparatus. The samples on the blot were immunoassayed using IgG raised in rabbit against Gluthation S-Transferase-potato cystatin (PC) fusion protein. The secondary antibody was horseradish peroxidase conjugated to sheep-anti-rabbit IgG (H+L) [JacksonImmuno Research, USA]. A reference curve was established using recombinant PC produced in *Pichia pastoris* (Annadana *et al.*, submitted for publication). The quantitative data were obtained by exposing the blots with substrate for horseradish peroxidase in a Lumi-Imager [BioRad].

The papain inhibitor activity of PMC and native CPIs in petal tissue of rayflorets of chrysanthemum cv. 1581 were determined by titration of papain. The florets were ground in 50 mM MES buffer, pH 6.5, centrifuged at 4 °C and the protein in the supernatant was quantified (Bradford, 1976). Equal volumes of control and transgenic ray floret extracts were incubated for thirty minutes with 14 nM papain, (pre-titrated against E-64 to determine actual protease activity). The inhibition of papain was determined using zoyloxycarbonyl-Phe-Arg-*p*NA (Z-Phe-Arg-*p*NA) as substrate [Bachem AG]. The *p*-NA release was measured at 18-second intervals for 30 consecutive readings at 405 nm in a microtiter plate reader. The initial part of the titration curve up to 50% inhibition was used to calculate the potential volume of extract required for complete papain inhibition (2.8 pmol). Values thus obtained were converted to pmol papain inhibitory activity per microgram total soluble protein in the extracts.

### 2.4. Whole flower bioassays with WFT

Whole flowers were assayed by detaching single flowers and placing them in a 50 ml Erlenmeyer flask filled with water and closed with parafilm. The flasks with flowers were placed in plastic bioassay cages made of transparent polystyrene bottles. A hole of 5-cm diameter was made on one side of the cage and sealed with nylon gauze of 120- $\mu$ m mesh to permit air circulation. Ten adult females were anaesthetised with CO<sub>2</sub> and placed on the centre of the flower. The bottles were closed by stretching a piece of parafilm across the opening. The cages were maintained in controlled climate chambers maintained at 25°C temperature, 60% r.h. and 16:8 light:dark period. After 7 or 10 days the flowers were analysed for the numbers of thrips present on them as described by De Jager *et al.*, (1995). The experiments were conducted in four replications.

## **3. Results**

### 3.1. Cloning of the PMC gene and generation of transgenic plants

The PCR on genomic DNA of cv. Superior with primers PMC-DN2 and PMC-UP2 yielded a 3517 bp fragment which was A-tailed and cloned into the vector pGEM-T. The fragment showed the expected band sizes when digested with *EcoRI* and *HindIII*.

Sequencing of the 5' and 3' ends confirmed identity of the gene with the published sequence, but the gene was not fully sequenced. The PMC gene was sub-cloned into the binary vector pBINPLUS under the control of the homologous *UEP1* promoter and *Nopaline synthase* terminator and named p*UEP1*-PMC. In total 35 transgenic plants were generated with p*UEP1*-PMC. The plants were transferred from the greenhouse with long day conditions (16 h : 8 h light:dark) to a special growth chamber with short day conditions (18°C and 8 h :16 h light:dark period), maintained free of insects, predators and insecticide. The transgenic plants flowered under the short day conditions, but it was observed that two lines (T50-12 and T50-31) developed more slowly. As all cuttings were transferred from long day to short day at the same time, these two lines remained short and also produced fewer flowers. The flowers of T50-12 were darker in colour (purple), which gradually turned to pale purple as the flower developed. The ray florets from control flowers were light purple turning white in full bloom. The ray florets in flowers from T50-31 were reduced in size as compared to control. No variations were observed in leaf size, leaf colour or leaf shape in any other transgenic lines.

### 3.2. PMC expression and activity assays

A protein dot blot of three microliter of ray floret extract was analysed by immunoassay for 35 different transgenic lines and one control. The available reference protein was a single domain potato cystatin, which was homologous, but not identical to the 8 domains of PMC. Thus, the differences between the two proteins did not allow an evaluation of the absolute expression levels using the immuno assay. The relative expression levels of PMC ranged between 0.1 to 2.2-units/ $\mu\text{g}$  total protein. In order to obtain independent information on the PMC expression level 13 of the 35 lines total were selected based on the dot blots (high, medium and low expressers). Data are presented for these 13 lines plus a control (Table 1). Three lines had expression levels of 1.0-units/ $\mu\text{g}$  total protein and above, four lines between 0.5 to 1.0 units/ $\mu\text{g}$  total protein while the remaining six lines had 0 – 0.4 units/ $\mu\text{g}$  total protein expression. In total eleven out of 31 lines were found to have more than 0.1 units/ $\mu\text{g}$  total protein PMC expression.

Thirteen lines quantified for PMC expression by dotblot plus line T50-13 and a control line were tested for the quantitative level of papain inhibitor activity. The papain inhibitor activity was determined by incubating different dilutions of ray floret extracts with papain. The PIA (papain inhibitory activity) in the transgenic plants (0.10 to 0.82-pmol/ $\mu\text{g}$  total protein) ranged from slightly below to almost 6-fold above the level found in the control plant. A correlation graph was plotted of PMC expression (units/ $\mu\text{g}$  protein) vs. PIA (pmol/ $\mu\text{g}$  protein) for each tested plant line (Figure 1). It was found that while plant T50-12 and T50-20 had the by far highest PIA level, their expression of PMC was only intermediate. This suggests that this high inhibitor level is not derived from PMC, but from variation in the expression of endogenous inhibitor genes. In the case of T50-12 the potential somaclonal effects of regeneration on endogenous gene expression was also visible in its aberrant slow growing phenotype. If these two outliers are excluded from the correlation graph, a trendline with a similar slope can be produced in which the  $R^2$  value improves from 0.13 to 0.67. The improved trendline intercepts the y-axis at 0.15 pmol/ $\mu\text{g}$  suggesting that this value represents the average endogenous concentration of inhibitor. The highest expresser at around 2.2 units PMC/ $\mu\text{g}$  total protein inhibits papain with 0.28 pmol/ $\mu\text{g}$  protein suggesting that PMC contributes at maximum about 0.13 pmol/ $\mu\text{g}$  inhibition units to the endogenous level. Each of the eight cystatin domains of PMC contribute to the inhibition of papain so that a molecular weight of 10 kD can be assumed for each inhibitor unit of PMC. The percentage of total protein of PMC as per the measured activity would then be estimated to be at maximum 0.13% of total protein in the case of the highest expressing plant.

### 3.3. Whole flower assays for resistance to WFT

In total 12 PMC-expressing plant lines and one control were tested for resistance against WFT. Measurement of the effect on reproduction in whole flowers was chosen as the most easily measured parameter. Four high expressers with PMC at 1.0-2.2 units/ $\mu\text{g}$  protein (T50-12, 15, 17 and 31), three medium expressers with PMC at 0.5-1.0 units/ $\mu\text{g}$  protein (T50-3, 19 and 20) and four low expressers with PMC at 0-0.4 units/ $\mu\text{g}$  protein (T50-1, 06, 14, 24) and one line with PMC not accurately determined (T50-13) were tested by inoculating with 10 adult WFT. After incubations of 10 and 7 days (repeated experiment), larvae and adults were collected in alcohol and counted. Thus, the number of eggs/offspring produced during the first 7 or 4 days (incubation time minus the time it takes for eggs to hatch) on the flower could be analysed. The average number of larvae produced on detached control flowers (256 for the 10 day experiment and 174 for the 7 day experiment) was taken as an oviposition rate (OR) of 100 %. Table 1 shows all the individual data that were collected on the plants. In Figure 2A and 2B the correlation of the number of offspring with either PMC expression or PIA levels is given. In this experiment, there was clearly no correlation at all between oviposition rate and PMC expression. This could be explained by the relatively low level of expression achieved in these plants. The calculated maximum expression of PMC of 0.13% of total protein does not exceed the necessary threshold of at least 0.5%. The downward trend in the trendline of PIA vs OR is not significant either and is fully dependent on plant T50-12 with 49% reduction in OR. Analysis by ANOVA results in an LSD of 34 %, which results in significant differences with the control only for transgenic plant T50-12. The cause of the low reproduction may be related to the high endogenous inhibitor level, but could be equally well explained by the aberrant phenotype. The phenotype resulted in more rapid ageing of the flowers and one of the four replicates had much lower larval counts than the other three. If the value of this replicate were excluded, then the reductions in OR are in the range of 8-30% of the control only.

## **4. Discussion**

The PMC gene, coding for an 85 kDa cysteine protease inhibitor protein was cloned and expressed in chrysanthemum under the control of the homologous *UEP1* promoter. Thirty-five independent transgenic lines were generated and the expression of PMC, papain inhibitor activity (PIA) and the oviposition rate (OR) of WFT, when exposed to transgenic flowers, was recorded in 12 plant lines (Table 1). Correlation graphs were generated for PMC expression vs. PIA, for OR vs. PMC, and finally for OR vs. PIA (Fig.'s 1, 2A and 2B). ANOVA of the data indicated no significant reduction in OR except for a single aberrant line T50-12. The lack of effects are tentatively explained by the relatively low expression (0.13% of total protein) of PMC in chrysanthemum relative to what is required to obtain thrips resistance.

The relatively low expression level of 0.13% using the strong *UEP1* promoter is unfortunate considering that no alternative promoters stronger than the *UEP1* promoter are currently available for expression of heterologous proteins in florets of chrysanthemum. However, it may be that the low expression level is associated with properties of the PMC gene. Transformation with a different cysteine protease inhibitor gene may circumvent those problems and achieve much higher expression levels using the same *UEP1* promoter. On the other hand, the GUS gene with the dCaMV35S promoter expresses 60 pmol/min/ $\mu\text{g}$  protein in leaves of tobacco and is also known to be able to drive protein expression to levels of around 1% of total protein. The *UEP1* promoter however only achieves a GUS expression level of about 8.5 pmol/min/ $\mu\text{g}$  protein in petals of chrysanthemum (Annadana *et al.*, submitted for publication) which is about 8-fold less and would confirm the level of 0.13% that we observe with PMC using this promoter. Levels of 45 pmol/min/ $\mu\text{g}$  protein are achieved in chrysanthemum by the *Lhca3.St.1* promoter but only in the leaves. This would predict that using the *Lhca3.St.1*

promoter levels of PMC protein in the toxic range of 1% could be expressed in the leaves of chrysanthemum. In retrospect, expression of PMC in leaves may be more relevant to the practical aspects of WFT than expression in flowers alone, despite the fact that WFT prefer rayflorets and thrive on them in contrast to leaves. However, WFT tends to reside in greenhouses that are largely filled with plants which are budding but not yet flowering. Just before bloom the plants are normally harvested and taken to the market preventing WFT to complete a generation on the petals. This implies that the most relevant reproductive phase, harming the grower, is most likely to take place mainly on the green parts of chrysanthemum and not on the flowers itself.

An alternative explanation for the apparent low expression level may be the highly unusual nature of the protein to spontaneously form cubical crystals in plant cells of the sub-phellogen layer in potato (Rodis and Hoff, 1984). In a heterologous expression host like chrysanthemum, this is not known to occur as well. If crystals are formed, however, the methodology for determining the PIA may not have been suitable, as the inhibitor activity determination was conducted in alkaline medium, while PMC is soluble only in acidic medium. In that case the actual levels of PMC that were achieved in chrysanthemum may have been underestimated. If the expression levels were severely underestimated (the dotblots, with an inadequate reference protein, suggested 10-fold higher levels) than also the question could be raised why no correlation of PMC expression with OR was observed. This raises another issue related to the crystalline nature of PMC: Would the crystals be ingested by WFT during their regular feeding habit of sucking plant sap through their stylets. If the crystalline particles are too big to enter through the stylets, this could explain the lack of correlation with potentially high expressing PMC plants. Therefore, further detailed analysis of the plants showing high expression in dot blots but low PIA is necessary (T50-15, 20 and 31), to confirm the true PMC expression level and the ability of thrips to ingest potential crystals.

The proof of concept that inhibition of cysteine proteases can significantly influence the oviposition rate of WFT has been shown earlier (Annadana *et al.*, submitted for publication). This concept can be extended to plants using either transgenic approaches as presented in this report or by alternate mechanisms to enhance cysteine protease inhibitor activity in leaves, florets and pollen of chrysanthemum. Chrysanthemum leaves and ray florets have a significant level of native cysteine protease inhibitor activity, for which variation exists between cultivars (Jongsma, unpublished results). Host plant resistance to WFT is known to vary with different cultivars and it will be interesting to see if a correlation can be established between partially resistant cultivars and cysteine protease inhibitor activity. This inhibitor activity can be measured by recording papain inhibition in leaf or floral extracts, which can be a quick screening technique to select suitable genotypes for breeding WFT resistance. Alternatively, somaclonal variants could be induced in chrysanthemum for enhancing the expression of cysteine protease inhibitors in leaves, florets and pollen. Such methods of enhancing transgenic or endogenous protease inhibitor expression to confer WFT resistance may be an alternative to the present day chemical control measures. The need for alternative solutions has become very high as new laws in The Netherlands will come into force from 2010, insisting on no pesticide usage in the greenhouses. Enhancing cysteine protease inhibitor activity by traditional or biotechnological means in chrysanthemum would be ideal to combat WFT. Hence more research in this direction can result in an approach to control WFT in a way which is friendly to the environment.

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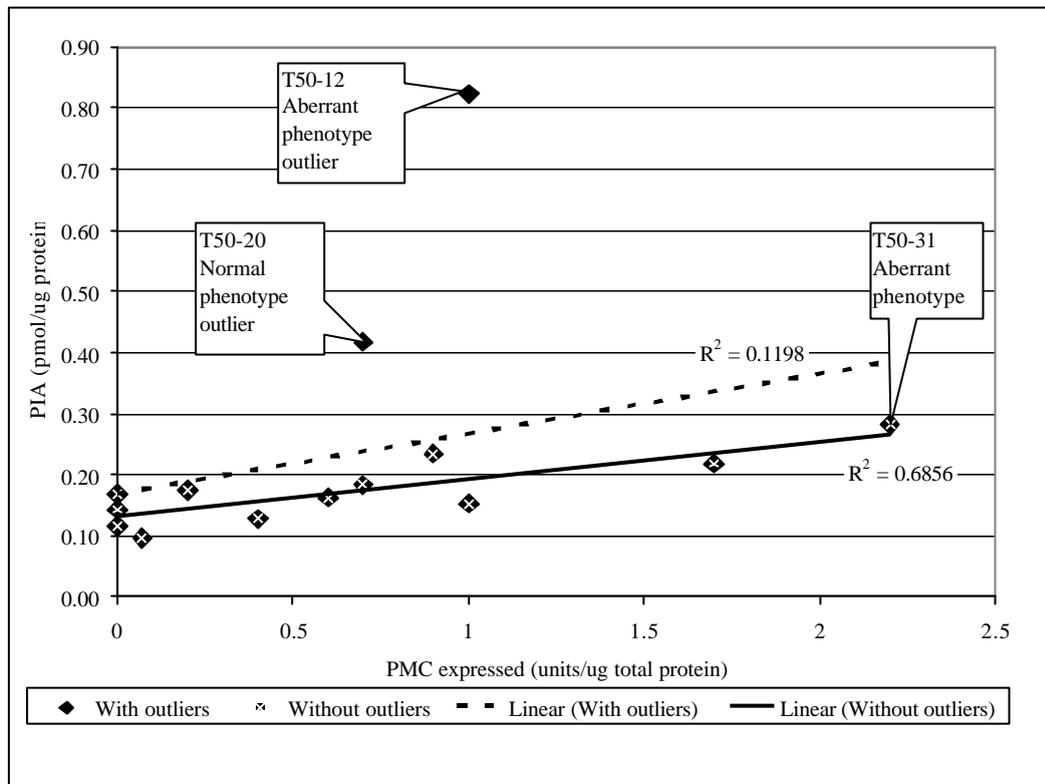


Figure. 1. Correlation graph of PMC expression vs. the papain inhibitory activity (PIA) in chrysanthemum ray florets. The correlation coefficient  $R^2$  increases from 0.11 to 0.68 when two outliers (T-50-12 & 20) are not considered for the calculation of  $R^2$ . Two aberrant phenotypes resulting from somaclonal variation are indicated. The two outliers are considered to be a different expression of somaclonal variation.

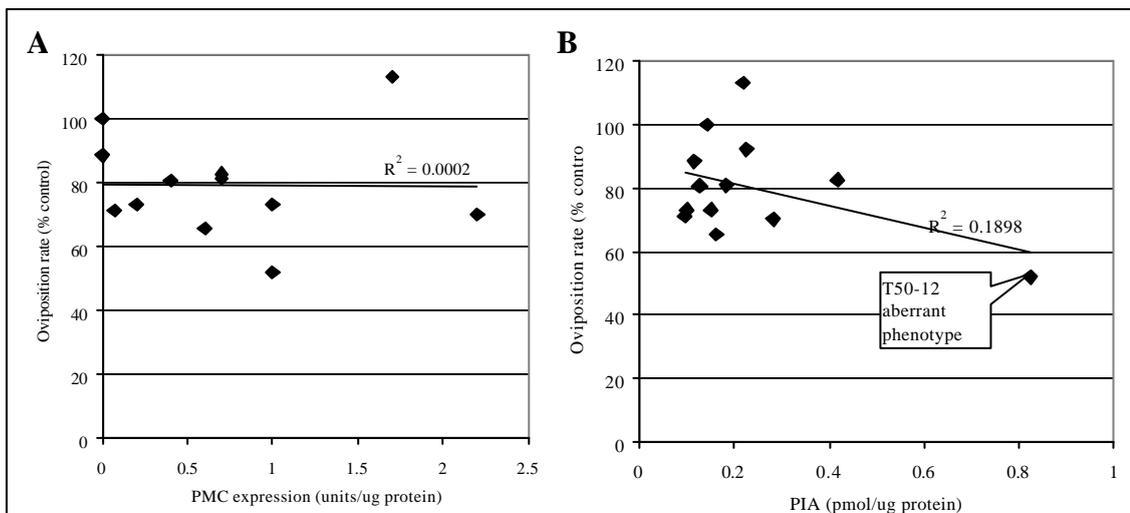


Figure 2. Correlation graphs of the oviposition rates (OR) versus PMC expression (A) or Papain inhibitor activity (PIA) (B) with linear correlation coefficients and  $R^2$  value.

Table 1. Analysis of PMC expression, protease inhibitory activity (PIA) and the reduction in oviposition rate (OR) of WFT for transgenic chrysanthemum lines harbouring the construct *UEP1-PMC*.

Plant line	PMC (units/ $\mu$ g total protein)	PIA (pmol/ $\mu$ g total protein)	OR (% control) exp 1 (10 days) with $\pm$ error	OR (% control) exp 2 (7 days) with $\pm$ error	Phenotype
50-1	0.2	0.10	73 $\pm$ 38 a	-	Normal
50-3	0.6	0.16	65 $\pm$ 16 b	-	Normal
50-6	0.07	0.10	71 $\pm$ 58 a	-	Normal
50-9	0.2	0.17	-	-	Normal
50-10	0.9	0.23	-	-	Normal
50-12	1.0	<b>0.82</b>	51 $\pm$ 34 b	-	<b>Aberrant, Outlier</b>
50-13	-	0.22	92 $\pm$ 10 a	-	Normal
50-14	0.4	0.13	80 $\pm$ 15 a	-	Normal
50-15	1.7	0.22	-	113 $\pm$ 13 a	Normal
50-17	1.0	0.15	73 $\pm$ 29 a	-	Normal
50-19	0.7	0.18	-	81 $\pm$ 15 a	Normal
50-20	0.7	<b>0.42</b>	82 $\pm$ 25 a	-	<b>Outlier</b>
50-24	0	0.12	88 $\pm$ 14 a	-	Normal
50-30	0	0.17	-	-	Normal
50-31	2.2	0.28	-	70 $\pm$ 24 a	<b>Aberrant</b>
con	0	0.14	100 $\pm$ 22 a	100 $\pm$ 24 a	Normal

Plant line T50-13 was not accurately quantified for PMC expression hence the value is missing.

PMC: Potato multicystatin

PIA: Papain inhibitory activity

OR: Oviposition rate

The LSD in OR was calculated to be 34% based on the ANOVA of the data. Plants are grouped based on significance, with plants represented by the same alphabet having no significant difference. Only two plants show significant reduction in OR, of which one is aberrant phenotype but with high PIA (T50-12) and the other is a normal phenotype without any significantly enhanced PIA over control (T50-03).