Aphid Resistance in Florist's Chrysanthemum (Chrysanthemum morifolium Ramat.) Induced by Sea Anemone Equistatin Overexpression

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Full Length Research Paper

Aphid resistance in florist's chrysanthemum (*Chrysanthemum morifolium* Ramat.) induced by sea anemone equistatin overexpression

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Florist's chrysanthemum (*Chrysanthemum morifolium* Ramat.) belongs to the Asteraceae family and represents the second most important floricultural crop in the world. Most genotypes are sensitive to aphids and infestations can lower quality and cause transmission of viruses. The protease inhibitor Sea Anemone Equistatin (SAE) carries three domains responsible for the inhibition of both cysteine and aspartic proteases. Artificial diet bioassays showed that SAE is readily toxic when ingested by the pea aphid, *Acyrthosiphon pisum*, and the cotton aphid, *Aphis gossypii*. We transformed chrysanthemum genotype 1581 by *Agrobacterium tumefaciens*-mediated transformation with the SAE gene under the control of the chrysanthemum RbcS promoter to induce aphid resistance. Non-choice leaf disk and whole plant bioassays were carried out to analyze deleterious effects of SAE on population growth and survival of both *Myzus persicae* and *A. gossypii*. After 7 days, *M. persicae* populations on specific transgenic lines were up to 69% smaller relative to control populations in a whole plant bioassay. The mortality of cotton aphids was 11% on control lines and up to 32% on transgenic lines after 5 days. The results show that SAE may be a promising agent for the control of some aphid species in transgenic plants.

Key words: *Chrysanthemum morifolium*, aphid resistance, RbcS promoter, sea anemone equistatin, agrobacterium transformation.

INTRODUCTION

Cultivated chrysanthemum (*Chrysanthemum morifolium* Ramat), also classified as *Dendranthema × grandiflora* (Anderson, 1987), belong to the Asteraceae family (Salinger, 1991). Commercially, it is known as florist's

chrysanthemum or autumn queen and predominantly sold as cut flowers in many countries of the world (Erler and Siegmund, 1986). After rose, it is globally the most important floricultural crop (Visser et al., 2007). The wide spectrum of colors and shapes, excellent vase life, and their ability to produce desired grades and types at any-time during the year promoted their economic impor-tance. Control of the main insect pests, various aphids (chrysanthemum aphid *Macrosiphoniella sanborni* Gilette, peach aphid *Myzus persicae* Sulzer, pea aphid *Acyrthosiphon pisum* Harris, and cotton aphid *Aphis gossypii* Glover), thrips (mainly *Frankliniella occidentalis* Pergande) and spider mites (*Tetranychus urticae* Koch) on vegetative and flowering chrysanthemum is a critical problem. Insects often develop resistance against fre-quently used pesticides, many pesticides are banned in important production areas, and there is a high cost to the application of pesticides (Visser et al., 2007). Although, desirable traits have been introduced using classical breeding, there are severe limitations to this technique in a hexaploid species like (cultivated) chrysanthemum. As an alternative, traits have been intro-duced by means of *Agrobacterium*-mediated transforma-tion (Fukai et al., 1995; Jaime and Teixeira, 2005; Mao et al., 2011) including practical characteristics relating to biotic resistance against aphids, beet army worm, botrytis and tomato spotted wilt virus (TSWV) (Kim et al., 2011; Visser et al., 2007).

Compared to conventional breeding, genetic engi-neering offers the advantage that a single gene can be inserted in the background of an established cultivar, and it is possible to stack multiple genes for various traits into a single cultivar (Visser et al., 2007). The search for plant compounds that are suitable for genetically based aphid control has resulted in a wide variety of candidate phytochemicals. Methyl ketones, sesquiterpene carbo-xylic acids and acyl-glucose esters are examples of compounds from trichomes with toxic properties against aphids (Goffreda et al., 1990). Some plant lectins or agglutinins are toxic to sap-sucking insects (Rahbé et al., 1995). *Galanthus nivalis* agglutinin (GNA) (Stoger et al., 1999; Nagadhara et al., 2003), *Pinellia ternate* agglutinin (PTA) (Yao et al., 2003), concanavalin A (ConA) (Gatehouse et al., 1999) and a lectin from *Amaranthus caudatus* (ACA) (Rahbé et al., 1995) are examples of compounds that significantly inhibit the population development of aphids. Wu et al. (2006) overexpressed the *aca* gene (*A. caudatus* agglutinin) in cotton plants under the control of a phloem-specific promoter. Bio-assays using cotton aphid showed that most transgenic plants significantly inhibited population growth of aphids. Aharoni et al. (2003) reported that leaves of transgenic plants of Arabidopsis constitutively expressing a dual linalool/nerolidol synthase in the plastids (FaNES1) pro-duced linalool and its glycosylated and hydroxylated derivatives. In dual-choice assays with *M. persicae*, the

FaNES1-expressing lines significantly repelled the aphids. Similarly, the expression of heterologous pro-tease inhibitors in plants has been used as an approach to induce plant resistance to insects (Ryan, 1990). Protease inhibitors (PIs) are proteins that form complexes with gut proteases and inhibit their proteolytic activity. Plants utilize PIs for defense to moderate the adverse effects from attacking herbivores or pathogens (Christou et al., 2006; Ferry et al., 2006; Zavala et al., 2008; Jongsma and Beekwilder, 2011). Many host plant-derived inhibitors are unsuitable; however, due to the fact that many insects have evolved resistance (Jongsma and Beekwilder 2011). Extensive screening of non-host plant-derived inhibitors has resulted in novel candidate proteins such as SAE that demonstrated promising levels of resistance *in vitro* and *in vivo* against various insect pests including aphids (Gruden et al., 1998; Outchkourov et al., 2004a, 2004b; Ceci et al., 2003).

In contrast to leaf-eating insect pests, aphids are exclu-sively feeding on plant phloem-sap, which usually dis-plays a high free/bound amino acid ratio. Consequently, they are thought not to rely on extensive protein digestion for their nitrogen supply. Nevertheless, a significant number of PIs was shown to promote deleterious effects on different aphid species (Rahbé et al., 1995; Casaretto and Corcuera, 1998; Quillien et al., 1998; Ceci et al., 2003). Also, a modified Oryza cystatin-I (Oc-IDD86) and a chicken egg white cystatin were shown to reduce the growth and survival of nymphs of *M. persicae* in artificial diet assays (Cowgill et al., 2002). The cystatin from rice, OC-I, caused growth reductions of up to 40% and reduced fecundity in pea aphid (*A. pisum*), cotton aphid (*A. gossypii*) and peach potato aphid (*M. persicae*) when fed at levels up to 0.25 mg ml^{-1} (Rahbé et al., 2003a). Serine PIs also show insecticidal effects towards aphids. For example, a systematic study of isoforms of Bowman-Birk type PIs from pea seeds showed varying anti-metabolic effects, including significant mortality, to pea aphid, which were associated with inhibitory activity towards pea aphid chymotrypsin (Rahbé et al., 2003b). Ceci et al. (2003) could identify a novel MTI-2 anti-chymotrypsin inhibitor, Chy8, with highest affinity for bovine chymotrypsin that was highly toxic to nymphs of the pea aphid *A. pisum*, and moderately toxic to nymphs of *A. gossypii* and *M. persicae*.

Equistatin is a protease inhibitor from the sea anemone *Actinia equina* that consists of three thyroglobulin type I domains. The first N-terminal domain acts as a cysteine protease inhibitor, and the second as an aspartic protease inhibitor, while the function of the third domain is not yet known (Strukelj et al., 2000). The protein was found to be highly active against the gut proteases of Colorado potato beetle (CPB; *Leptinotarsa decemlineata* Say), and on artificial diets, the protein induced high mortality of CPB larvae (Gruden et al., 1998). In potato leaves, accumulation of only partly functional equistatin

up to levels of 7% of total soluble protein was achieved (Outchkourov et al., 2003b). Co-expression of cystatins promoted the toxic effects against western flower thrips (Outchkourov et al., 2004a, 2004b). In this study, we examined the deleterious effects of purified equistatin on population growth and mortality of pea, peach and cotton aphids using artificial diet bioassays, and then tested peach and cotton aphid resistance in chrysanthemum plants overexpressing sea anemone equistatin in both leaf disk and whole plant bioassays.

MATERIALS AND METHODS

Plant material and insects

Sterile florist's chrysanthemum cultivars 1581 (Plant Research International) were propagated on MS (Murashige and Skoog, 1962) medium containing 3% sucrose and 0.8% agar (w/v). Internode explants were used for transformation. For artificial diet assays, pea, cotton and peach aphids were obtained from established colonies at Biologie Fonctionelle Insects & Interactions (Lyon, France) maintained on *Vicia faba*. For plant bioassays, peach and cotton aphids were obtained from established colonies on Chinese cabbage at the Departments of Entomology and Biointeractions (Wageningen University and Research Center, The Netherlands). Transgenic plants were grown in the greenhouse with supplementary high-pressure sodium light under 16/8 h light/dark rhythm and temperature regime of 21/18°C, and used for molecular and biochemical analyses as well as bioassays.

Artificial diet bioassays

The effects of purified recombinant equistatin was tested against the pea aphid (*A. pisum*), peach aphid (*M. persicae*) and cotton aphid (*A. gossypii*) to determine the toxicological indices of recombinant SAE towards these aphids according to the methods described by Rahbé et al. (2003a). Aphids were weighed on a Setaram (Lyon, FRA) analytical microbalance at the nearest microgram, and bioassays were carried out as fully described previously (Rahbé and Febvay, 1993). Recombinant equistatin was produced and purified as described by Outchkourov et al. (2002). The protein sample was diluted in a stock solution of the aphid diet and water was added to reach the desired concentration. A control diet was prepared in the same way using the buffer of the protein sample.

Transformation and regeneration

Internodes of chrysanthemum cultivar 1581 were pre-cultured on regeneration medium (MS supplemented with 1.0 mg l^{-1} BAP and 0.1 mg l^{-1} IAA) for 2 days. The highly virulent *Agrobacterium tumefaciens* strain AGL-0 with the binary vector pBINPLUS and Rubisco small subunit promoter driving the native equistatin (EIM) gene (pUCRBC-EIM) was used in this experiment (Outchkourov et al., 2003b). A single colony was cultured in 5 ml liquid LB containing 50 mg l^{-1} Kanamycin and Rifampicin and grown at 28°C on a shaker overnight. The culture was diluted 1/100 in fresh LB medium with the same antibiotics and was used for transformation after overnight culture at 28°C. Internodes with a length of 3 mm were cut by scalpel and kept on regeneration medium for 2 days.

Pre-cultured internodes were then collected in 30 ml liquid MS and

then 0.6 ml Agrobacterium culture with 30 µl acetosyringone (0.1 M) were added and incubated for 30 min. Explants were then first transferred to sterile filter paper for a few minutes to remove excess bacteria and then co-cultured on solid regeneration medium with 100 µM acetosyringone in darkness at 25°C for 2 days. After co-culture, the explants were transferred to selection medium (regeneration medium containing 400 mg l^{-1} vancomycine, 250 mg l^{-1} cefotaxime and 30 mg l^{-1} kanamycin) and incubated under light for selection. All explants were transferred to fresh selection medium every 21 days and maintained for 65 days after inoculation.

Regenerating shoots were induced from Agrobacterium-inoculated explants 2 weeks after applying antibiotic selection, and reached a final transformation frequency of 8.75%. Green regenerated shoots were transferred to rooting medium (½ MS supplemented with 200 mg l^{-1} vancomycin and 125 mg l^{-1} cefotaxime). Rooted plants were transferred to the greenhouse after being hardened and *Agrobacterium*-free test. Transformation frequency was calculated as the number of PCR-positive greenhouse plants divided by the number of inoculated explants.

DNA and RNA analysis

Genomic DNA was isolated from young leaves as described by Pereira and Aarts (1998) and was used primarily for PCR screening. Primers with product size of 100 bp were designed to identify the EIM sequence (EIM-Forward: GATGTTCGTGCCAGAGTGTT; EIM-Reverse: TCGGAACCTGGAACCTTTAC) using website http://www.genscript.com for use in both PCR and qRT-PCR analysis. A volume of 4 µl DNA was used for PCR, adding 0.5 µl superTaq polymerase and 2.5 µl of 10× buffer, 1 µl of 10 mM specific forward and reverse primers, 0.25 µl of 10 mM dNTP and water to a final volume of 25 µl. Amplification was performed in the GeneAmp PCR system at the following conditions: 94°C, 5 min, 35 cycles of 94°C, 30 s and 55°C, 30 s; 72°C, 20 s; then finally 72°C, 7 min with a drop to 4°C). Positive lines were analyzed by qRT-PCR to determine the level of gene expression. Total RNA was extracted by the TriPure^{TM} small sample method. cDNA synthesis was done using the TaqMan^{TM} Reverse Transcription Reagents. Reverse transcription was performed in the GeneAmp PCR system at the following conditions: 25°C for 10 min; 48°C, 30 min; 95°C, 5 min. A volume of 1 µl of cDNA (2 µg) was used for qPCR, with 10 µl BIO-RAD iQ^{TM} SYBR$^®$ Green Supermix, 2 µl of 3 µM specific forward and reverse primers, and a volume of 20 µl water. The housekeeping gene actin was used as reference gene (Actin-Forward: CCTCTTAATCCTAAGGCTAATCAG; Actin-Reverse: CCAGGAATCCAGCACAATACC). Amplification and real-time measurements were performed in the iCycler iQ5 (Bio-Rad, USA) (95°C, 3 min, 40 cycles of 95°C, 10 s and 60°C, 30 s; 95°C, 1 min; 60°C, 1 min).

The results were analyzed using the IQ5 Optical System Software and $2^{-\delta\delta Ct}$ CT Method (Livak and Schmittgen, 2001).

Western-blot analysis

A piece of a fully expanded young leaf (200 to 300 mg) was placed in a 1.5 ml eppendorf tube and ground in liquid nitrogen to a fine powder. The powder was re-suspended in 300 µl of extraction buffer [100 mM Tris-HCl, pH 7.6, 25 mM sodium-diethyldithiocarbamate, 50 mM EDTA, 10% (w/v) polyvinylpolypyrrolidone (PVPP), and 1 tablet 25 ml^{-1} protease inhibitors cocktail complete (Prod. Nr. 04693159001, Roche Diagnostics)], and this crude extract was twice centrifuged for 5 min at 14,000 rpm and 4°C. Each time, the supernatant was replaced into a new tube. SDS-PAGE was performed using a 15% (w/v) precast resolving gel
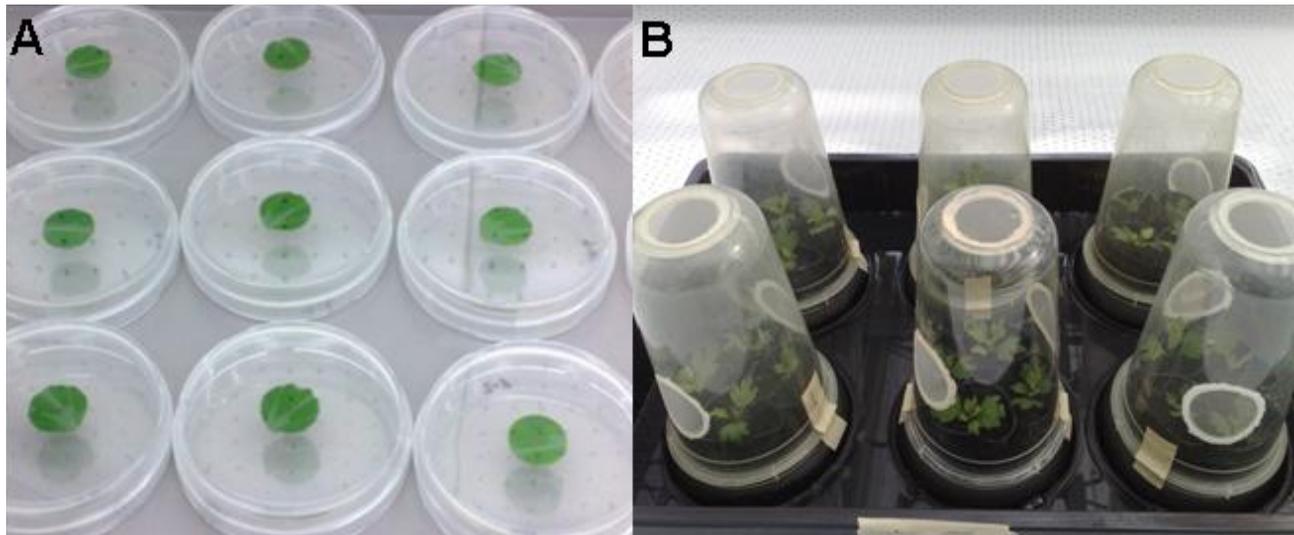
Figure 1. Non-choice leaf disk bioassay A), Whole plant bioassay B).

(Bio-Rad Laboratories) on a mini-Protean II slab cell apparatus (Bio-Rad Laboratories). The gels were run according to the manufacturer's instructions. The separated protein samples from the SDS-PAGE gels were transferred to Trans-Blot (Bio-Rad Laboratories) nitrocellulose membranes using the mini-Protean II electro-transfer apparatus (Bio-Rad Laboratories). Subsequently, the membranes were blocked in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% (v/v) Tween 20 containing 2% (w/v) non-fat milk powder for 1 h and then incubated with rabbit anti-EI antibodies (Eurogentec, Seraing, Belgium). The blots were subsequently washed and incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase (Jackson Immunoresearch Laboratories, West Grove, PA).

The membranes were visualized with Lumi-Light western blotting substrate and scanned in the Lumi-Imager F1 under the control of Lumi-Analist software (Roche Diagnostics, Mannheim, Germany).

Plant bioassays

Non-choice leaf disk and whole plant bioassays were carried out to test the effects of equistatin expression on population development and mortality. Adult aphids of both species were collected with a fine brush from a rearing on Chinese cabbage. For non-choice bioassays, a single 2 cm leaf disk from mature green leaves of transgenic or wild type chrysanthemum plants were placed abaxial side up on a thin layer of 1% water-agar in a Petri dish with 9 cm diameter (Figure 1A). Five separate Petri dishes were prepared as replicates for each transgenic line examined. Five aphids were released in the lid of a Petri dish and, after closing, the Petri dishes were incubated upside down at 20°C under long-day conditions (16 h of light/8 h of dark) with a RH of 70%. The numbers of aphids on each leaf disk were recorded at a range of time points after the start of the experiment. For whole plant bioassays, young plants were used in cages containing four replicates for each line (Figure 1B). Each plant was inoculated with ten aphids and the number of aphids on each plant was counted after 1 week. All data were analyzed by SAS v.7.0. Analysis of variance (ANOVA) was used to compare different lines and the significant difference of the means was calculated using Duncan's multiple range test (P = 0.05).

RESULTS

Artificial diet bioassays

To test the activity of equistatin artificial diet, bioassays were carried out against pea, peach and cotton aphids, which are known to colonize chrysanthemum plants. Artificial diet bioassays showed that SAE is readily toxic when ingested by the pea aphid, *A. pisum* [IC50 ≈ 150 µg ml^{-1} (10 µM); LC50 ≈ 190 µg ml^{-1} (13 µM)], and the cotton aphid *A. gossypii* [IC50 ≈ 870 µg ml^{-1} (58 µM); LC50 ≈ 203 µg ml^{-1} (14 µM)], while it showed only moderate growth inhibition of the peach aphid *M. persicae* (Figure 2). Equistatin at a concentration of 482 µg ml^{-1} caused 75 to 80% mortality of pea and cotton aphid (Figure 3), but there was no significant effect on survival of peach aphid (not shown).

Molecular and biochemical analysis of transgenic plants

The result of PCR for some transgenic lines is shown in Figure 4. Subsequently, gene expression levels were analyzed by qRT-PCR using the same primers as used in PCR. Based on the number of PCR cycles, the relative transcript levels were estimated to be in the range of the native Rubisco small subunit gene (data not shown). The expression level of the SAE gene ranged in 7 independent transformants 10-fold, and was highest in line 15-4 (Tables 1 and 2). The expression of equistatin protein was demonstrated by western blot of SDS-PAGE gels of extracted leaf protein. Four bands were assigned to SAE as they were absent in the control. There was no
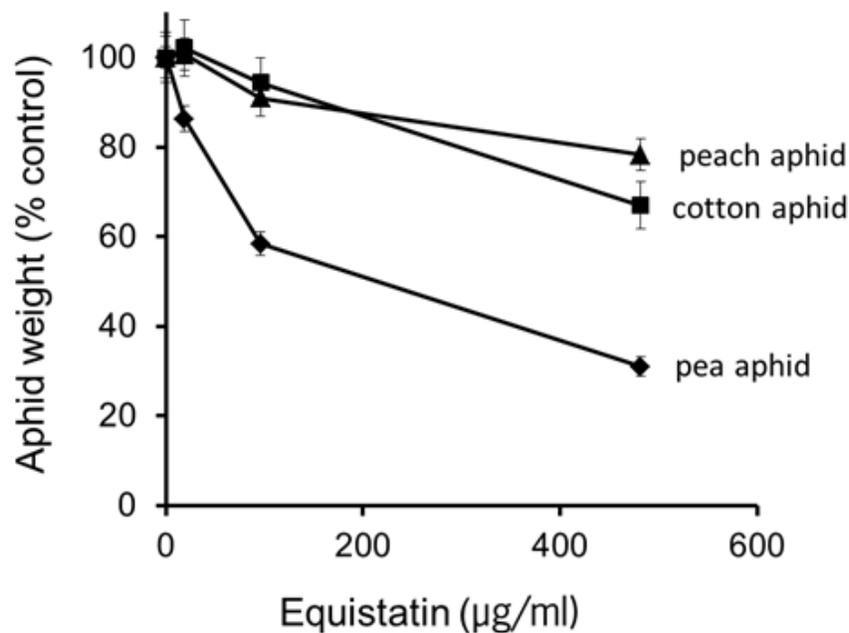
Figure 2. The effects of recombinant SAE protein on the growth of pea, peach and cotton aphids in artificial diet experiments.
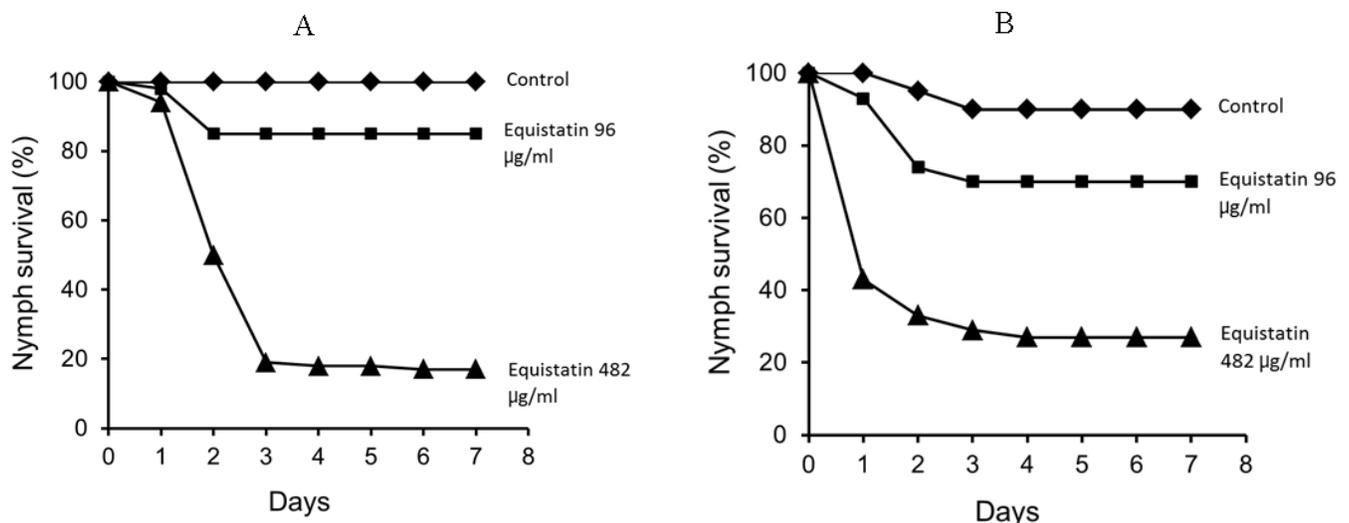

Figure 3. The effects of recombinant SAE protein on survival of pea (A) and cotton (B) aphids in artificial diet experiments.

obvious correlation of the western blot results with the qRT-PCR based ranking of expression levels. If we took samples at the end of the day, our gels did not reveal any protein (Figure 5).

Plant bioassay

To test the effectiveness of SAE gene expression in controlling aphid populations, various bioassays on leaf disks and whole plants were carried out with both peach and cotton aphids. In non-choice leaf disk bioassays with peach aphids, like in the artificial diet, no increase in mortality was observed, but the growth of the peach aphid population was strongly inhibited from a 120% increase on wild type chrysanthemum plants to only 8% on line 15-4 with the highest expression level. Intermediate expression levels demonstrated growth reductions that correlated well with the expression level as measured by qRT-
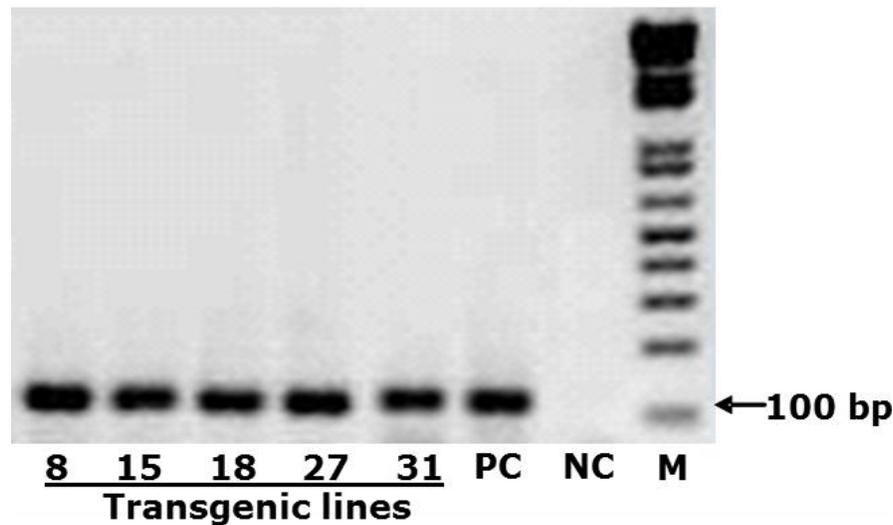
Figure 4. PCR analysis for the presence of the SAE gene in Agrobacterium negative transgenic chrysanthemum plants from the greenhouse. M: size marker, PC: positive control, NC: negative control.

Table 1. Population development of peach aphids (*Myzus persicae*) in no choice leaf disk and whole plant bioassays of chrysanthemum 1581 transformed with the SAE gene.

Line	SAE gene expression	No choice leaf disk bioassay (5 nymphs, 5 days, n = 5)		Whole plant bioassay (10 aphids, 7 days, n = 4)	
	qRT-PCR (%)	Average number	% increase	Average number	% increase
15-4	100.0[a]	5.4 (±0.24)[b]	8[b]	27.7 (±9.41)[b]	177[b]
31-1	71.0[a]	Nt	Nt	20.7 (±3.97)[b]	107[b]
27-2	33.7[b]	6.0 (±0.77)[b]	20[b]	36.2 (±6.91)[ab]	262[ab]
18-4	33.7[b]	Nt	Nt	50.2 (±12.81)[ab]	402[ab]
8-2	11.2[c]	8.4 (±1.53)[ab]	68[ab]	36.7 (±9.52)[ab]	267[ab]
23-3	10.5[c]	8.6 (±1.29)[ab]	72[ab]	40.7 (±13.71)[ab]	307[ab]
WT*	0.0[d]	11.0 (±2.32)[a]	120[a]	67.0 (±12.46)[a]	570[a]

* Wild type plant (negative control); Nt, Not tested. Different letters in the same column represent significant differences by Duncan's multiple range test (P = 0.05).

Table 2. Population growth and mortality of cotton aphids (*Aphis gossypii*) in non-choice leaf disk bioassays of chrysanthemum 1581 transformed with the SAE gene.

Lines	SAE gene expression	No choice leaf disk bioassay (5 nymphs, 5 days, n = 5)		
	qRT-PCR (%)	Mean of population (total)	Mean of dead aphids	Mean % mortality (dead/total per replicate)
15-4	100[a]	15.6 (±3.7)[a]	4.4 (±0.51)[a]	32 (±6.3)[a]
31-1	71.0[a]	17.0 (±4.5)[a]	2.8 (±0.37)[bc]	27 (±13)[ab]
27-2	33.7[b]	17.8 (±3.7)[a]	2.6 (±0.68)[bc]	18 (±5.0)[bc]
18-4	33.7[b]	18.4 (±1.5)[a]	2.8 (±0.2)[bc]	16 (±2.1)[bc]
8-2	11.2[c]	14.6 (±1.2)[a]	3.0 (±0.55)[abc]	22 (±5.0)[abc]
23-3	10.5[c]	13.8 (±1.6)[a]	3.4 (±0.4)[ab]	26 (±4.0)[ab]
WT*	0.0[d]	17.6 (±3.0)[a]	1.6 (±0.51)[c]	11 (±3.0)[c]

* Wild type plant (negative control); Nt, Not tested; A. U. arbitrary unit. Different letters in the same column represent significant differences by Duncan's multiple range test (P = 0.05). % Mortality is calculated per replicate (n = 5) and the average is given.
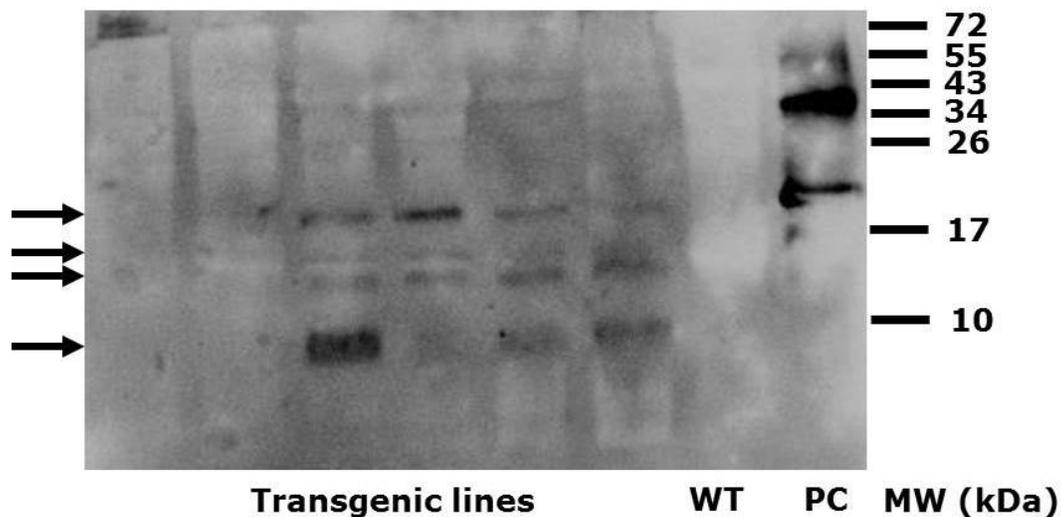
Figure 5. Western-blot analysis of the equistatin protein accumulated in chrysanthemum plants. PC: Positive control protein, produced in *E. coli*. WT: wild type plant (negative control), M: size marker.

PCR (Table 1). Also, in whole plant bioassays, transgenic lines 15-4 and 31-1 with the highest expression levels had 58 to 69% lower aphid population (Table 1). Cotton aphids were tested only in a non-choice bioassay on leaf discs. Similar to the artificial diet bioassays, these insects responded much more sensitively to the SAE protein product. The mortality increased significantly from 11% in control lines to 27 to 32% on transgenic lines 15-4 and 31-1 (Table 2).

DISCUSSION

Aphids are worldwide important pests and virus vectors. The search for a chemical basis of genetic plant resistance against aphids has resulted in a range of compounds and proteins with antibiotic and/or repellent/ deterrent activities. These mainly feature various lectins, serine and cysteine protease inhibitors, acyl sugar esters and some mono- and sesquiterpenes (Wu et al., 2006; Rahbé et al., 1995, 2003ab; Stoger et al., 1999; Nagadhara et al., 2003; Yao et al., 2003; Gatehouse et al., 1999; Goffreda et al., 1990; Aharoni et al., 2003). In the present study, we demonstrate that the cysteine and aspartic protease inhibitor sea anemone equistatin (SAE) is a highly potent insecticidal protein against a range of different aphids that are commonly found on chrysanthemum. The pea aphid and cotton aphid have LC_{50}s of 190 to 203 µg ml^{-1}, while the peach aphid was suffering from significant growth reductions on artificial diets containing SAE protein in that concentration range. Previously, SAE has been expressed in potato at a maximum expression level of 3 to 7% of total protein, when the SAE protein was carrying a KDEL signal at its C-terminus (Outchkourov et al., 2004a). If we can assume

that plant leaves contain 15 mg gFW^{-1} of protein, then an expression level of 1% roughly represents 150 µg ml^{-1}, which is in the range of the required LC50 concentrations for pea and cotton aphid. Earlier experiments with Arabidopsis had shown that when SAE carried a KDEL signal; however, that resistance to aphids was not observed, presumably as a result of different targeting, leading to differential exposure to the protein (results not shown). Experiments with chrysanthemum were, therefore, limited to SAE without KDEL tag, which presumably secretes SAE by default into the apoplast. In potato that construct yielded an average expression level of 0.36% of total protein based on the strong RbcS1 rubisco small subunit promoter from chrysanthemum (Outchkourov et al., 2002), but the strong degradation of SAE in transgenic chrysanthemum as seen in the western blots did not allow us to make similar assessments of the actual protein expression level.

Alternative to protein blots expression levels could also be ranked on the basis of mRNA levels, and in this way correlations could be established between the expression levels and the growth reduction of peach aphid and mortality of cotton aphid. The growth reductions on plants for peach aphid were much higher than observed on artificial diet. The reasons for this are difficult to answer, but might be related to the fact that artificial diets are protein free, only containing free amino acids. This may create a lesser dependence on proteolytic activity on those diets compared to plant phloem sap. As reported for potato by Outchkourov et al. (2003b), also in this study, two thirds of the SAE protein appeared to be processed into several distinct bands in the early morning, and in the late afternoon, no detectable signal remained (data not shown). It is not known for chrysanthemum how

the first steps of this degradation affect the bioactivity of the SAE. In principle, the separate domains may still be active as was demonstrated in potato by affinity purification of the degradation products using a papain column. The distinct bands are target for further proteolysis; however, as afternoon and night levels were no longer detectable. Potential ways of preventing this degradation are co-expression of additional cysteine protease inhibitors which target the plant proteases. That method was shown to work well for potato and may work for chrysanthemum as well (Outchkourov et al., 2004b).

The SAE gene is potentially useful for the genetic engineering of enhanced aphid and thrips resistance in chrysanthemum and other crops. Chrysanthemum is mostly grown in greenhouses, which makes our tests on whole plants representative of commercial conditions. Nevertheless, tests were done on small plants only and in closed containers, so that a long term study on the effects of this gene on the population build up on these plants remains to be done. Furthermore, aphids tend to accumulate most on plants that are forming flower buds. Analysis of these stages will therefore need to be done in a follow up study as well. In that case, it will also be relevant to study whether SAE changes probing behaviour and thus could affect the transmission of viruses.

ACKNOWLEDGEMENTS

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