TOWARDS PROTEASE INHIBITOR MEDIATED RESISTANCE TO WESTERN FLOWER THRIPS IN CHRYSANTHEMUM

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Proefschrift

Ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
prof.dr.ir L. Speelman
in het openbaar te verdedigen
op vrijdag 7 december 2001
des morgens te elf uur in de Aula

The investigations described in this thesis were carried out at Plant Research International, Wageningen University and Research Centre.

CIP-DATA KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Seetharam Annadana (Annadana S.)

Towards protease inhibitor mediated resistance to western flower thrips in chrysanthemum

Thesis Wageningen University and Research Centre – with references- with summaries in English and Dutch. Plant Research International, P.O. Box 16, 6700 AA Wageningen, The Netherlands.

ISBN 90-5808-550-3

Bibliographic Abstract:

Dendranthema grandiflora (chrysanthemum) is a cutflower grown across the world, with an acreage similar to roses. *Frankliniella occidentalis* (Western flower thrips, WFT) is a major pest against a large number of crops, including chrysanthemum, in both field and glasshouse cultivation. Aiming at WFT resistance in chrysanthemum by a transgene-mediated approach, the prerequisite of an efficient regeneration system was first established. Subsequently, promoters for high level transgene expression were identified. The predominant group of proteases active in WFT was identified as the class of cysteine proteases, and the recombinant cysteine protease inhibitors potato cystatin (PC) and equistatin (EI) were shown *in vitro* to achieve over 90% inhibition of WFT protease activity. PC and EI ingested by adult WFT on a pollen diet for a period of five days resulted in 50% reduction in the oviposition rate. Effects on adult mortality were not observed during this brief period. An eight domain cysteine protease inhibitor potato multicystatin (PMC) was expressed in chrysanthemum in an attempt to endow it with WFT resistance. Low expression levels ranging at maximum 0.1-0.13% of total protein were generated. Hence, no correlation with thrips resistance could be established with these lines. However, the results in this thesis provide evidence of potentially significant effects of cysteine protease inhibitors on WFT oviposition rate. This would have a strong effect on restricting WFT population build up. The concept of enhancing cysteine protease inhibitor activity by various approaches in the different parts of chrysanthemum to combat WFT is discussed in relation to the existing alternatives.

Propositions (Stellingen)

1. Alternately use of the variation in hormone levels endogenously and exogenously is an effective tool to improve shoot regeneration in vitro in chrysanthemum (this thesis).

2. Light qualities with transmission absorption maxima at 575 nm & 480 nm produce more shoots and propagative nodes in chrysanthemum for a given set of in vitro culture conditions (this thesis).

3. Conventionally used CaMV based promoters are in-effective in chrysanthemum, but the improved performance of other promoters still remains several fold lower compared to transgenes in tobacco (this thesis).

4. Cysteine proteases are dominant in thrips, and their inhibitors effectively reduce oviposition rates in adult females, but their effects on larvae, egg hatch are still unknown (this thesis).

5. Use of a combination of protease inhibitors, either targeting the different specificities within a family or targeting proteases of different families may prove to be more effective than a single gene approach.

6. What you spend years building may be destroyed in hours but build anyway says Karma Yoga, and so in science what we spend years finding may be useless overnight but let us keep finding anyway.

7. Not everything that is faced can be changed, but nothing can be changed until it is faced. - Lewis Mumford

8. He is happy whose circumstances suit his temper, but he is most excellent who can suit his temper to any circumstances.

9. **ABCD** are the salient features of the daily life at **P**lant **R**esearch **I**nternational, **A**genda; **B**irthday; **C**offee break; **D**iscussion.

Propositions (Stellingen) associated with the Ph.D thesis of Seetharam Annadana:

Towards protease inhibitor mediated resistance to western flower thrips in chrysanthemum.

Wageningen, The Netherlands

28th September, 2001

Preface

This thesis was carried out within the framework of the sandwich PhD between Wageningen University and Research Centre and University of Agricultural Sciences, Bangalore, India. Additional financial aid from CSIR, India and ongoing projects at Plant Research International BV came in as timely support in order to continue the research work both in Bangalore and Plant Research International BV respectively. I am grateful to these organisations for making this thesis possible. This thesis contains results that were obtained during the past four years of my study entitled "Towards Protease Inhibitor Mediated Resistance To Western Flower Thrips In Chrysanthemum". It would have been impossible to cherish the dream of having a PhD and make it come true without the help of several individuals both in India and in the Netherlands. It is my great pleasure to take this opportunity to thank them all.

I have to first thank both my promoter Prof. E. Jacobsen and Co-Promoter
Dr. M. Udaya Kumar for the trust they rested in me since the beginning of the project. Both understood the situations well during the course of work and have dealt with patience irrespective of their busy schedules. Both showed interest and desire in bringing out the scientist in me to work and dwell in the world of science. There is no doubt that without their help I would not have been able to bring this thesis to its present state.

Dr. M. Ramanna was the person who persuaded me to take up PhD and helped me in filing the initial papers for a sandwich PhD. I thank him for his support and encouragement.

I wish to express my gratitude to my three supervisors on different aspects conducted in this study. Firstly Dr. Jan de Jong was responsible to first accept me as a PhD student to work in his ongoing projects on Chrysanthemum. He was extremely supportive both technically and socially especially during the start of the project when most of the work on regeneration was initiated. He was also responsible to lead me into molecular work and introduced me to my two other supervisors who took over from him. His family members are also to me mentioned, as I have become a part of their family. Secondly the bridge from tissue culture work to hard core molecular biology was created strongly in the characteristic style of Dr. JPH NAP. Starting from the basics of mini prepping DNA to some of the thumb rules of bio-molecular work was thought to me by him. The most important part of his supervision was in training me to write and think analytically with the results. Lastly but never the least
Dr. Jongsma has been supervising most of my work in the last 3 years and looking into publishing of my results and giving the exact shape to this thesis. He has been extremely kind by providing financial support in times of need. His contribution to this thesis is maximum in terms of concept, technique and quality of science brought out. He has been very kind, polite and very friendly as a supervisor. It has been a pleasure working with him and would like to continue to do as long as possible. It is clear that the whole concept of proteinase inhibitors was conceived by Dr. Jongsma which has taken a predominant part of this thesis. Along with Dr. Jongsma his family members have to be mentioned as they have been in regular touch with me and my work in progress. Technically, scientifically, financially maximum credit goes to

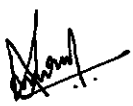
Dr. Jongsma for his time, concept and contribution towards the creation of this project and the outcome of this thesis. The proof of concept on the effects of cysteine protease inhibitors on Thysanoptera highlighted in this thesis is a significant contribution, and the credit for the same, entirely goes to Dr. Jongsma.

Postdocs, technicians, PhD and graduate students at Plant Research International made life easier and interesting. They have been kind at times of need, provided their technical expertise and helped me in difficult situations. I wish to thank Jules, Anja, Boris, Nikolay, Mieke, Bert, Petra, Jos, Annelies, Wim and the rest of the lab members. The foreign population at Plant Research International was my social asset and motivation. I wish to recollect names like Raffa, Asaph, Jeroen, Diana, Olga, Silvia, Nayelli, Ela, Raj, Pradeep, Mr. & Mrs. Ramulu.

Dutch friends have been constant admirers and supporters of me outside the institute. They have been part of my life in Wageningen and have palyed a significant role in my academic success here. Marjo, Ruben, Evert Fokkema, Roy, Kwa, Edwin van der Vossen are some of the many names I can get into my memory instantly. Jan, Nia, Marjolein, Francis, Steven my Dutch family from Hoek van Holland have been caring starting from day 1 with a cycle as a present which has been the most essential part of my movement in Wageningen. I have spent several Christmas vacations in their wonderful home out of home. Mr. R.P. Geerlofs another family friend also has been in regular touch and enquiring my well doing. Friends from India who have been a support of their own kind are Kaja, Shalini, Anand, Vasanthi Gopi, Kulku, Savitha, Bhoja, Gani, Purshi, Rama, Dhanu, Madhura, among the many. I have to thank Mr. Suresh of Floral Graphics for the excellent work he has done in given colour and life to this printed version of my hard labour.

There are two individuals close to my heart but due to presentation reasons they come at the near end of this text. Smt. Vishalakshi Iyer and Shri. A. Seshadri Iyer my mother and father who have been the guiding light motivation and zeal behind me. They have supported me financially, mentally, and socially which has made me what I am today. If there is some qualities and traits I have gained than the credit has to go to them for the sacrifices they have made to give the best of what ever they had for their children. I am what I am only because of their support and blessings. My elder brother Shri. A.S. Ram Mohan and his family have been constantly supporting me and my work and also looked into the family affairs in my absence, without which I could never have worked in peace.

Smt. Veena Annadana the one who stole my heart before I had heard of Wageningen, the one who also waited in patience and trust to wed me during the course of this PhD comes last but is never the least. She has borne the stress, failures and success of my work with utmost patience, care, and love. I am lucky to have such a wonderful person as my better half. In the meanwhile Samarth Annadana right now winks as I come towards the end of my PhD starting a new chapter of my life as father.



Seetharam Annadana
Wageningen 28th September 2001

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Chapter-1

General Introduction

1.1 History

Chrysanthemum has been cultivated since early times. In ancient literature of the orient, chrysanthemum is described as elegant creations of god. The present day chrysanthemum has undergone several centuries of selection and breeding, from the wild daisy as what they first appeared in Eastern Asia. The Chinese have been credited for the initial remodelling of the weed into a jewel. The Japanese added diversity to the flower type, to give more choice to the buyer. The French in Europe were the first to honour chrysanthemum and appreciate its possibilities in European gardens. As the worldwide statistics attest, chrysanthemum is one of the few crops that are grown in large areas, in greenhouses all year around. This is possible due to the achievements of developing year-round chrysanthemum.

1.2 Commercial Importance

The total turnover of chrysanthemums in 1995 alone at the Dutch auction halls was over Dfl 600 million. At the auction, chrysanthemums are primarily grouped under three categories namely the standard type, the spray type and the pot chrysanthemums. In the Netherlands during 1995, there were more than 70 cultivars for standard type, 500 cultivars for spray types and more than 30 cultivars for pot chrysanthemum in cultivation both indoor and outdoor. Next to roses, chrysanthemum is rated second as a cut flower and fourth as a pot plant on turnover basis. The area under greenhouses for chrysanthemum cultivation is also rated second next to roses. In 1995, the total area under greenhouse for chrysanthemum was 772 hectares and may still increase in the future (Dutch flower council).

1.3 Chrysanthemum The Crop Of Interest In India And Globally

The enormous diversity in shape, colour and form of chrysanthemum available on the market today hardly reveals the origin of this early group. All chrysanthemums still do have the same basic floral structure, which is grouped under the genus *Dendranthema*. Currently, classification based on embryo sac development, cypsela anatomy and morphology, plant habit, and phytochemical properties divides chrysanthemums into five major genera. *Argyranthemum, Chrysanthemum, Dendranthema, Leucanthemum and Tanacetum.* The genus chrysanthemum in the strictest sense contains only three species of which the most common is Chrysanthemum *morifolium, which* is now called *Dendranthema grandiflora* (Anderson 1987). In India chrysanthemum is an important flower crop for irrigated farms near big cities. The crop is grown in the open fields and mainly propagated from seed. It is the second largest flower crop cultivated next to *Polyanthus (Polyanthus tuberosum)* . Chrysanthemum is one of the oldest field-grown flowers after Jasmine in Southern India. The use of chrysanthemum in India is different as compared to western countries. It is not harvested with the entire stem as cut flower, instead only the pedicel is cut, and the flower is used in this form for worship, or made into garlands, which are most popular in Indian temples. The two colours of chrysanthemum popularly grown are white and yellow both indeed being ordained colours for Indians. In the recent past, other colours of

chrysanthemum, also have been released for commercial cultivation from the Indian Institute of Horticultural Research, Bangalore.

1.4 Current Status Of Chrysanthemum Breeding And Its Predominant Pests

There are problems with the cultivars presently grown, implying they can still be improved for some desired characters. A longer season of bloom, greater frost resistance, increased winter hardiness, are only a few examples of the many aims, plant breeders are currently working on. Commercial breeders are always engaged in extensive breeding programs trying to achieve one of these several aims. Breeding for fragrance is now a new novelty, growers look for to beat the competition. Removing the bad fragrance in chrysanthemum and introducing a pleasant scented redolence is still difficult. For the future, this is a very potential character for breeding, as an increasing number of scented flowers, including scented roses are now being introduced as novelties. It is now clear after all these years of breeding chrysanthemum, with the possibility of having new colours and shapes, breeders have reached a point of saturation. Breeders indeed have achieved a lot in diverse colours, shapes, yield potential and cropping season of chrysanthemum. However with all the effort in the last decade in breeding, there is still one major problem with chrysanthemum cultivation. The aspect of pest and diseases, which has been remained as a problem, not easy to solve. Chrysanthemum are susceptible to diseases such as mildew, rust, leaf spot, bacterial blight, mosaic and stunt. There are also a number of pests that attack chrysanthemum, namely thrips, beet army worm, several bugs, beetles and nematodes.

The negative side effects of the chemicals used to control these diseases and pests on human beings, is now a well-established fact. The chemicals are hazardous not only to the health of humans, but also to the health of the environment. Hazardous chemicals bring about drastic changes in the biotic and abiotic components of the environment. With the aim of protecting the health of humans and the environment from such chemicals, alternative modes of pest and disease control have to be considered. It has therefore become essential for developing plants that have an in borne resistance to pest and disease by virtue of which the use of such hazardous chemicals could be reduced.

From practical training and experience it is concluded that thrips and *Sphodoptera* (beet army worm) are the two principal pests in the greenhouses used for the cultivation of chrysanthemum in the Netherlands, and in the open air cultivation of chrysanthemum in India. Damage caused by thrips does not generally reduce production, but results in scarring and deformation of flowers, which drastically reduce the aesthetic and commercial value of chrysanthemum. Beet army worm on the contrary feeds on the leaves of chrysanthemum, and can reduce flower yield substantially. Hence a lot of breeding effort has gone into screening for lines, showing resistance or tolerance to these major pests.

To the bad luck of plant breeders, there are no such exclusive chrysanthemum species or lines resistant to these major pests. Even if such resistant genotypes were identified, it would be difficult to introduce genes for resistance from them into the present day hexaploid cultivars because of the polysomic inheritance. As there are no major genes for resistance to insects in chrysanthemum, the only alternative left is to introduce resistance genes from other sources, like genes from plants of other genus or from microbes.

1.5 Molecular Breeding Of Chrysanthemum

The genes for resistance against these major pests have been cloned, and are accessible at different institutes all over the world. The problem, however, is that some of these genes are not from plants and hence introducing them into chrysanthemum will have to be achieved with the aid of some genetic engineering techniques. To introduce alien genes into plants, the *Agrobacterium* mediated gene transfer system is a convenient method. This is now successfully utilised for introducing alien genes into several plant species. *Agrobacterium* is host explicit and its host range has been mainly restricted to dicots until very recently. Since chrysanthemums is a dicot an *Agrobacterium* mediated gene transfer protocol is suitable.

The use of *Agrobacterium* for genetic transformation has been applied to chrysanthemum for over a decade in many institutes in Europe and the United States of America (de Jong et al.,1993; Song et al., 1991; Bush et al., 1992a, 1992b; Ledger et al.,1991). Chrysanthemums have been cocultivated with many strains of *Agrobacterium* carrying different plasmids. The *Agrobacterium* strain AGL0 has been found to be most efficient in T-DNA transfer into chrysanthemum as compared to other strains like LB4404 (de Jong et al., 1994). The plasmids within the *Agrobacterium* are also alterable. After testing different plasmids, it has been shown that the most effective plasmid for chrysanthemum is pMOG410 (de Jong et al.,1994). There has been a lot of fundamental work in the last five years on the selection of the *Agrobacterium* strains, (Bush et al., 1992b, Becker et al., 1992), choice of plasmids (Hood, E.E., et al., 1993) and regeneration methods suitable for transforming chrysanthemum.

1.6 Steps In "Thrips Resistance In Chrysanthemum By Transgenic Approaches"

- The first aim was oriented towards developing regeneration techniques for a large number of cultivars as an efficient technique was unavailable.

- The second aim was at improving the levels of transgene expression in chrysanthemum and identify suitable promoters for expressing transgene in different tissue of the plant.

- Last but not he least Identify and apply Proteinase inhibitors as an effective tool against flower thrips which is a major pest on chrysanthemum and other cutflowers in greenhouses globally.

1.7 A Reasons For Low Expression And Modes For Improving Expression

To use transgenic plants in commercial cultivation there needs to be an effective level of expression of the introduced transgene. In circumstances of purely fundamental investigation, mere expression is a commendable result, but on the contrary practical research with transgenes of interest expects more. Currently lab results need to reach growers and meet their demands hence an effort towards stabilised and high performing transgene expression system in chrysanthemum is interesting. Unstable transgenic plants can introduce serious problems in yield potential, quality of product and also may pave the way for disease epidemic or pest attack. For this reason, it is important, to have high level of stable transgene expression in the transgenic plant. The promoter is one of the many vital

factors responsible for the differential expression of genes in different tissues. All the cells of the plant contain all genes but only a few are switched on in any particular tissue or at any particular time. This tissue and time explicit activity of the genes is well established, and promoters happen to be one of the most important factors responsible for it. The promoter employed to drive transgenes in chrysanthemum in most cases has been CaMV (35S) promoter and we find in chrysanthemum that it is not as active as in Tobacco and potato.

Promoter methylation and progressive transgene inactivation in *Arabidopsis* has been studied in detail (Kilby et al.,1992). The reduction in transgene expression in green house grown transgenic chrysanthemum, as compared to in vitro grown plants, may indeed be due to methylation. Therefore, to analyse the greenhouse grown transgenic chrysanthemum with methylation specific restriction enzymes was productive to perceive the behaviour of transgenes in chrysanthemum. Recent results indicate that the promoter is not methylated (personal communication Hans Sansbrink). Therefore promoter methylation may not be the cause for low expression of transgene in chrysanthemum. For that reason, to study the level of transgene expression driven by different promoters was desirable. Other promoters conferring higher levels of transgene expression as compared to the CaMV promoter were identified and presented in this thesis.

Transgene inactivation has been a topic of intense interest in the recent past. There are a few mechanisms responsible for transgene inactivation. Occurrence of multiple transgene integration, transgene methylation, co-supression, chromatin restructuring among others bring about transgene inactivation (Finnegan et al.,1994). With the hypothesis to overcome such problems it is considered appropriate to use MAR sequences (matrix associated regions). These sequences are hypothesised to usher extension in the level of transgene expression and reduction of expression variability, associated with transgene. It is hypothesised that MAR elements could overcome the transcriptional blocks, but may not influence post transcriptional interference.

1.7 B Thrips *Frankinella occidentalis* A Major Pest In Greenhouses

Frankliniella occidentalis, Thrips has become the most predominant insect present on may vegetable and ornamental crops since 1983 in the greenhouses in The Netherlands (Mantel and De Vrie, 1988). Thrips is been reported to live on at least 240 species of plants belonging to 62 different families (Loomans et al., 1995). Thrips can cause yield losses like in cotton (Johnson et al., 1996), tea (Rattan et al., 1996), onion (Srivastava et al., 1996), chilli and sweet pepper (Kumar NKK, 1995) etc. Thrips can induce direct damage by piercing and sucking leading to emptied cells (Harrewijn et al., 1996) or indirectly by acting as vectors for tospoviruses (Wijkamp et al., 1995). Thrips is a sucking pest, polyphagous, small size and secretive habit makes it difficult to be controlled by chemicals. The association of greenhouse growers in The Netherlands have decided to have no insecticide usage in the greenhouses starting 2010. Considering the importance we identified proteinase inhibitors as a interesting new candidate to investigate on its potential to metabolically effect the insect. In case of thrips a reduction in the population by affecting the egg laying ability in adults may be an interesting approach contrary to other biocides leading to complete mortality of the insect. The field population of F. occidentalis are bisexual but females often dominate, occur in larger numbers and live longer than males

(Lewis 1973). They measure 1.3 to 1.4 mm about .3 to .4 mm longer than males (Looman et al., 1996). Such being the case aiming at reduction in fecundity appears a viable strategy to combat the thrips menace, which is the aim of this investigation.

1.7 C Proteinase Inhibitors As An Approach To Combat Thrips Menace

Proteinase inhibitors inhibit the gut proteases of insects and result in stunted growth (Broadway and Duffey, 1986; Johnston et al., 1993; Oppert et al., 1993; Burgess et al., 1994; Orr et al., 1994). The inhibition of proteases will have to be at large and many require multiple proteinase inhibitors to act on all major gut proteases (Jongsma et al., 1994, 1995; Bolter and Jongsma 1995). The gut proteases can be classified into 4 classes based on the amino acid residue or metal ion responsible at the binding site of the proteinase for hydrolysis of the peptide bonds. The 4 classes are serine; cysteine; aspartic and metallo proteases and the inhibitors that bind them are know as serine proteinase inhibitors etc. We have characterised and partially purified the gut proteases of thrips which is identified as cysteine proteases dominantly. Similarly they have observed cysteine proteases as dominant also in the alfalfa weevil which was effectively inhibited by cysteine proteinase inhibitors like E-64, pHMB and leupeptin at 0.1% concentration leading to reduced growth and fecundity (Cowgill et al., 1999).

1.8 Layout Of The Thesis

- For a competent transformation experiment it is imperative that a well-authenticated plant regeneration protocol in-vitro is first established. This is the first step before transforming a plant. The first component of this thesis is designed at developing diversified regeneration protocols for chrysanthemums described in chapter 2. As the existing regeneration protocol was cultivar specific, (de Jong et al, 1995) it was essential to have different regeneration protocols for new elite cultivars of chrysanthemums. A new, leaf explant based protocol having a separate induction protocol followed by a regeneration protocol was developed. Chapter two deals with the different regeneration protocols developed for the elite cultivars obtained specially for this research project from five chrysanthemum breeders.

- The physiological effect of light transmission absorption spectra in the tissue culture containers and their influence on the quality and quantity of harvestable shoots is the second aspect investigated in this thesis. A useful conclusion has been drawn on the most optimum light absorption peak for regenerating chrysanthemums in vitro. This is presented in chapter 3.

- The next aspect was screening two different promoters for their activity in chrysanthemum leaf. The dCaMV (double 35S) promoter and the Lhca3.St.1 promoter from potato were tested. The study consisted of comparing the activity of dCaMV and the Lhca3.St.1 promoters with and without MAR (matrix associated regions) flanking based on the expression of the GUS reporter gene. This is presented in chapter 4.

- The fourth aspect studied in this thesis is the cloning, sequencing and characterising a homologous promoter from chrysanthemum for conferring high levels of

transgene expression in the ray florets of chrysanthemum. This is the part of chrysanthemum which is severely damaged in the greenhouse by the thrips *Frankinella occidentalis*. In addition the activity of several heterologous promoters from petunia, *Arabidopsis* and potato were compared with the homologous promoter for expression in the ray florets, presented in Chapter 5.

- Chapter 6 presents the cloning of the potato cysteine proteinase inhibitor (PI) and its recombinant expression in yeast. The recombinant protein was checked with westerns and activity assays by binding to papain.

- Two recombinant proteins Equistatin and potato cystatin were tested for their activity against thrips in a bioassay. Results directed us towards identifying a PI which would be effective against thrips, which also would be the likely candidate for expression in ray florets of chrysanthemum. This forms the highlight and focus of chapter 7.

- In chapter eight we discuss the expression of potato multicystatin in ray florets of chrysanthemum. We characterise expression levels in ray florets and also perform activity assays (papain binding) with floral extracts. The high expressing ray florets were used for a bioassay against thrips and the results are presented in this chapter.

- Discussion is presented in chapter nine followed by a short summary.

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Chapter- 2

Response of stem explants to screening and explant source as a basis for methodical advancing of regeneration protocols for chrysanthemum

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Published in Plant Cell Tissue and Organ Culture (2000), 62: 47-55

Abstract

Logical advancing of regeneration protocols as an end result of response to screening and use of alternate explants is reported. Stem explants screened on medium 1 (MS with 1 mgl^{-1} BAP and 0.1 mgl^{-1} IAA) produced 4 responses and were classified as Group 1: more than 1.6 shoots per explant; Group 2: less than 1.6 shoots per explant; Group 3: only callus and Group 4: no response. To regenerate groups 2, 3 and 4, specific media were developed (media 2 a&b, media 3 a&b and media 4 a-i). Media 2 & 4 had changes in hormonal combination as compared to medium 1, while medium 3 had additional changes in vitamins. Nineteen out of the 37 cvs. tested were regenerated on stem explants based on the response to screening technique. Cultivars recalcitrant on stem explants even after exposure to modified media were reinvestigated on leaf. High concentration hormone pulse as induction (medium 5) followed by a modified regeneration medium (medium 6d) ended in successfully regeneration of leaf explants. Four out of the 14 cvs. recalcitrant on stem were successfully regenerated using leaf explants. Response based advancing of regeneration protocols in addition to use of alternate explants for recalcitrant cultivars resulted in efficient regeneration of 23 of the 37 cvs on assessment.

Key Words: chrysanthemum, *Dendranthema*, explant-phenotype, hormones, induction, regeneration and response.

Abbreviations- BAP-Benzylaminopurine; cvs.- cultivars GA3-gibbrelic acid; IAA-Indole acetic acid; MES-2 (N-morpholino) ethanesulfonic acid; NAA- Naphthylene acetic acid;

Introduction

Regeneration has been reported for several cvs. of chrysanthemum of the species *Dendranthema grandiflora* (Anderson, 1987). Successful regeneration has been reported using different explants like flower receptacle (Hill, 1968), shoot tips (Earle & Langhans, 1974), pedicel (Roest & Bokelmann, 1975), petal segments (Bush et al., 1976) and leaf (Slusarkiewicz et al., 1981; Bhattacharya et al., 1990; Kaul et al, 1990).

A drawback of most protocols is their cultivar specificity, as an effect of which regeneration of a new cultivar is by trial and error. The problem is further complicated as most chrysanthemums are hexaploids and are developed via mutation breeding techniques. Regeneration depends on expression of endogenous hormone genes which could be affected by these mutations like in cotton (Chen et al., 1996). Mutations can bring about change in hormone concentrations or and changes in sensitivity to hormones (Reid & Davies, 1987). Strong interaction between nucleus and cytoplasm in influencing regeneration in sunflower has been observed (Nestares et al., 1998). Thus regeneration is a result of the combined effect of endogenous hormone gene expression and responsiveness to external stimuli.

Direct organogenesis protocol used for *Agrobacterial* transformation (De Jong et al., 1994) was used as medium for screening stem explants. Subsequently altering the inorganic and organic constituents paved the way for development of new protocols. To regenerate cvs. recalcitrant on stem we tried leaf explants, successfully used in green bean (Franklin et al., 1993), tomato (Van Roekel et al., 1993), apple (De Bondt, 1996), begonia (Kiyokawa et al., 1996), Kiwi (Yamakawa & Chen, 1996) and chrysanthemum (Slusarkiewicz et al., 1981;

Bhattacharya et al., 1990; and Kaul et al., 1990). The principle behind changing the explant is to make use of the differences in levels of endogenous hormones which effect regeneration like in poplar (Sasamoto et al., 1995).

We focussed our aims in the current investigation on 1) providing a methodical tissue culture technique for a large number of cvs., 2) use of response to screening as a basis in addition to 3) use of alternate explants (leaf) for cvs. recalcitrant on stem.

Materials and Methods

Experimental layout

Every experiment had four replications in four petridishes with ten explants per dish. The data analysed is presented in tables as number of shoots per explant. Cultivars producing 1.6 shoots per explant were considered good based on our experience and requirement for efficient transformation.

Plant material & surface sterilisation

Experiments were based on 37 cvs. obtained from breeders, listed in Table 2. Material was grown in the greenhouse under standard conditions (Machin & Scopes, 1978). Stem explants were obtained from the first two internodes of three week old shoot tips while leaf explants from the third and fourth position. The shoots tips, and leaves, were surface sterilised in 1 % (W/V) hypochlorite for 20 minutes and rinsed in sterilised water thrice for 15 minutes.

Stem explants

Subsequent to the surface sterilisation, the shoot tips were sliced across their length to obtain slices of 2-4 mm. Slices were placed distal side down on medium1 and transferred to climate rooms maintained at 25°C with a 16-h photoperiod. The dishes were covered with a white cloth to keep the light level at 4 $\mu molm^{-2}s^{-1}$.

Leaf explants

The leaves were cut 0.5 cm away from the midrib on either side, followed by a slicing of the midrib resulting in two strips. The strips were cut across their length to produce explants of 0.5 cm^2. This ensured a high degree of uniformity of the explant with four cut-edges, with a sliced midrib on one side. Explants were placed abaxial side down on medium 5 and transferred to the climate room.

Media

The screening of stem explants was on medium 1 (de Jong et al., 1994, 1995) composed of: MS (Murashige and Skoog, 1962) salts + vitamins, 1 mgl^{-1} BAP, 0.1 mgl^{-1} IAA, 30gl^{-1} sucrose and 7gl^{-1} plant tissue culture agar. The screening of leaf explants was on medium 5 (Gutterson et al. 1994, MS salts, B5 vitamins, 2 mgl^{-1} BAP, 1 mgl^{-1} NAA, 7 gl^{-1} tissue

culture agar, 30 gl^{-1} sucrose and 3 mM MES). Based on the response, medium 5 was limited to induce the leaf explants. Further regeneration after induction was on medium 6d, (Table 6). A summary of the media used are presented in Table 1 and the movement of cultivars from one medium to another are described in Fig. 1.

Table 1. Different media tested for different groups of stem and leaf explants of chrysanthemum. All media contain MS salts, 30 g/l sucrose and 7g/l plant tissue culture agar. Hormones mentioned are mg per litre. These were the modifications developed for each group with the aim of regenerating them.

Media	Media number	Vitamins	Auxin mgl^{-1}	Cytokinin & other components mgl^{-1}
Group 1	1	MS	IAA 0.1	BAP 1
Group 2	2a	MS	IAA 0.1	BAP 0.1
Group 2	2b	MS	IAA 0.1	BAP 2.0
Group 3	3a	B5	IAA 0.1	Kinetin 1 Coconut water 10W/V
Group 3	3b	B5	IAA 0.1	Kinetin 1 GA 3 10
Group 4	4a	MS	NAA 1	BAP 1
Ind.media (leaf)	5	B5	NAA 1	BAP 2 3 mM MES buffer
Reg.med (leaf)	6d	B5	-	BAP 0.25 3 mM MES buffer

Key:
Ind.media: Induction media for four days
Reg.med: Regeneration media.

Regeneration and phenotype of cultured explants.

Stem explants

Shoot primordia were counted, 13 days after the initiation of culture, and recorded as number of shoot primordia per explant. After 21 days, the explants were transferred to fresh medium. At the end of 42 days the microshoots were harvested and recorded as number of harvestable shoots per explant. Based on the response of the stem explants to medium 1 (number of harvestable shoots, callus formation or none) cvs. were classified into four groups (Table-2).

Modification of regeneration protocols for stem explants

Group 2

To regenerate the cvs. that produced less than 1.6 shoots per explant (Group 2), only the BAP concentrations in the medium 1 (1 mgl^{-1}) were either lowered to 0.1 mgl^{-1} or increased to 2 mgl^{-1} (media 2 a&b). Recalcitrant cultivars that failed on the media 2 a&b were retested using leaf explants.

Response of explant to screening	Group	Media	Decision I	Result of decision I	Decision II	Result of decision II
>1.6 shoots (stem ex)	**1**	**1**	Ready for transformation	Table 2	-	-
<1.6 shoots (stem ex)	**2**	**2a&b**	To medium with lower and higher BAP than **medium 1**	Three out of the 12 cultivars tested produced 1.6 or more shoots, **Table 3**	Test leaf explant for cultivars that fail to regenerate on **media 2 a&b**	No cultivar was regenerated successfully
Only Callus (stem ex)	**3**	**3a&b**	Callus induced on **medium 1** and transferred to shoot induction media	Three out of the nine cultivars tested produced 1.6 or more shoots	Test leaf explant for cultivars that fail to regenerate on **media 3a&b**	Three out of the five cultivars tested produced 1.6 shoots or more, **Table 7**
No response (stem ex)	**4**	**4 a-i**	Test NAA and BAP in 9 different combinations, **Table 4**	One out of the three cultivars tested produced more than 1.6 shoots	Test leaf explant for cultivars that fail to regenerate on **media 4 a-i**	The only cultivar tested did produce 1.6 shoots, **Table 7**
Lot of shoot primordia only (leaf ex) on medium 5	**All inert cvs. of groups 2 - 4**	**5&6d**	Transfer to a **medium 6d**	Produced the maximum number of shoots in this combination, **Table 6**	-	Table 7

Fig 1. Flow chart of the mode of advancing of several regeneration protocols based on the stem explant (stem ex) response to screening and shift to leaf explants (leaf ex) for recalcitrant cultivars. The number of harvestable shoots are per explant obtained as an average value of four replications, with each replication consisting of 10 explants.

Group 3

The cvs. that produced only callus (group 3), were callused on medium 1 for three weeks and transferred to medium 3a (Lemieux et al., 1991, MS salts, B5 vitamins, 1 mgl^{-1} kinetin and 10% W/V coconut water). At the end of three weeks on medium 3a explants were transferred to medium 3b (composed exactly like 3a but coconut water was replaced by 10 mgl^{-1} GA). Cultivars recalcitrant to media 3 a&b were retested using leaf explants.

Group 4

The cvs. which had no response in the screening (Group 4), were tested on media 4 a to i (MS organic and inorganic, with NAA and BAP in nine different combinations, Table 4). Cultivars recalcitrant on media 4 a to i were retested using leaf explants.

Developing a regeneration protocol using leaf explants

Leaf explants of cv. 1581 were induced on medium 5 (Gutterson et al., 1994), for 4, 8 or 16 days, and a statistical programme was run to study its influence (Table 6). Following induction, explants were transferred to nine different regeneration media (MS inorganic, B5

organic with IAA and BAP in 9 different combinations, 6 a to i). The best combination of induction and regeneration (Table 6) was used to test 14 of the 18 cvs. totally recalcitrant on stem explants (Table 7).

The screening of cultivars on stem explants and based on their response their grouping and further re testing on different media and different explants are summarised in a flow chart (Fig. 1).

Results

Grouping based on response of stem explants to screening

There was considerable variation in response to screening on stem explants from the 37 cvs. Based on the response they were classified into four groups (Table 2). In 12 cvs. (32.4 %) there were 1.6 or more shoots per explant and hence classified as Group 1. There were 13 cvs. (35.1 %) which produced less than 1.6 shoots per explant and had no callus so were classified as Group 2. In nine cvs. (24.3 %), only callus was formed so this formed Group 3 while three cvs. (8.2 %) were completely inert which formed Group 4.

Regeneration of Group 2

The response of Group 2 cvs. to BAP concentrations and a statistical analysis for the same is presented in Table 3. There was successful regeneration in XW 109, Sheba and Tigerrag on medium 2a (BAP concentration 0.1 mgl^{-1}). BAP at 0.1 mgl^{-1} concentration is highly significant (P = <.001), for seven cvs. over 1.0 mgl^{-1}, while for three cvs. over 2.0 mgl^{-1}. However for three cvs. 1.0 mgl^{-1} BAP concentration is still significant over 2.0 mgl^{-1}.

Regeneration of Group 3 and 4

Three out of nine cvs. of Group 3 tested (Moneymaker, Tourmalin and Tr. 2) produced 1.6 or more harvestable shoots per explant. One out of the three cvs. of Group 4 (CBA 2053) produced 2.3 harvestable shoots per explant on medium 4a (Table 4) and the rest remained recalcitrant. In Table 5 all the cultivars regenerated using stem explants with their respective media are summarised. Response of leaf explants of cv. 1581 to medium 5 produced only primordia like structures with no further response hence they were transferred to regeneration media (6 a to I, Table 6).

The results were assessed 42 days after the initiation of the culture, using the number of harvestable shoots developed in each of the treatment. Induction on medium 5 for four days was slightly better than eight, which was as good as 16. Four days of induction was best for explants regenerating on media numbers 6d, 6e and 6g. Eight days of induction did best on regenerating media 6f, 6h and 6i. The 16-day induction period did best on regeneration media 6a, b & c. However the highest number of harvestable microshoots was recorded for 4 days of induction followed by transfer to a regeneration medium 6d (0.0 mgl^{-1} IAA and 0.25 mgl^{-1} BAP, Table 6). Hence this combination (media 5 & 6d) was selected for testing of cvs. recalcitrant on stem explants. The variate is number of shoots with LSD at 1.7

Table-2-Characterisation and classification of 37 cultivars based on the response to regeneration in initial screening on media number 1. The numbers of harvestable shoots are per explant obtained as an average value of four replications, with each replication consisting of 10 explants. Based on the phenotype of the explant the cultivars were easily grouped for further investigation.

Cultivar	Primor	Shoot	Callus	Concl	Cultivar	Primor	Shoot	Concl	Callus
Cleo	50	4.6	-	Group 1	Roubiax	4	2	Group 2	-
Rush	40	3.6	-	Group 1	Sheba	2	1	Group 2	-
Kris	22	2.8	-	Group 1	Alencon	2	0.7	Group 2	-
CBA 1403	28	2.6	-	Group 1	Granada	2	0.7	Group 2	-
Albert Hein	24	2.3	-	Group 1	Napoli	2	0.5	Group 2	-
Venezia	23	2.1	-	Group 1	Le mans	30	0	Group 2	-
Toulouse	23	2.1	-	Group 1	Mike	0	0	Group 3	+++
Tr. 1	22	2.0	-	Group 1	Hugo	0	0	Group 3	+++
Mayfair	20	1.8	-	Group 1	Money maker	4	0	Group 3	+++
Lineker	20	1.6	-	Group 1	Tr. 2	0	0	Group 3	+++
Tornato	18	1.6	-	Group 1	Tr. 5	0	0	Group 3	+++
Biarritz	20	1.6	-	Group 1	Rubakest	0	0	Group 3	+++
Cocarde	10	8	-	Group 2	Petra	0	0	Group 3	++
Tigerrag	7	5	-	Group 2	Funshine	0	0	Group 3	+
David	6	4.5	-	Group 2	Poso doble	0	0	Group 3	+
Stallion	6	4	-	Group 2	CBA 2053	0	0	Group 4	Nr
XW 109	4	4	-	Group 2	Rainbow	0	0	Group 4	Nr
Tr . 3	4	3	-	Group 2	Tr. 4	0	0	Group 4	Nr
Tourmalin	3	2	-	Group 2					

Key :
Primor- The number of primordia per petridish.
Shoot- The number of harvestable shoots per petridish.
Callus- (- completely absent, + little, ++ moderate, +++excessive).
Nr- No response. (cvs. not producing shoots, callus or primordia) .

Group:
Group 1- More than 16 harvestable shoots (2 cm) per petridish.
Group 2- Less than 16 harvestable shoots per petridish.
Group 3- Producing only callus.
Group 4- Has no response.

Regeneration of cultivars recalcitrant on stem explant

The combination of media 5 & 6d was extended to 14 recalcitrant cvs. from Group 2, 3 and 4. Successful regeneration was obtained in 4 of the 14 cvs. tested (Rainbow, TR-5, Hugo and Mike). Three other cvs. Poso Doble, TR-2 and Stallion also responded, but produced less than 1.6 shoots per explant. The remaining seven cvs. produced five to eight primordia only without further development. The list of cvs. and their response is mentioned in Table 7. No cultivar from Group 2 was successfully regenerated using leaf explants.

Table 3. The response of 13 cultivars of Group 2 tested on two different concentrations of BAP (0.1 and 2.0 mg/l), with 1.0 mg/l as control. The numbers of harvestable shoots are per explant obtained as an average value of four replications, with each replication consisting of 10 explants. A statistical analysis indicating the probability for the response within each cultivar for the media 1, 2a and 2b is also presented.

Cultivar	Shoot 0.1 BAP Media 2a	Shoot 1.0 BAP Media 1	Shoot 2.0 BAP Media 2b	0.1 vs 1.0	0.1 vs 2.0	1.0 vs 2.0
David	0	.45	0	***		***
Stallion	.5	.4	.4	*	**	***
Xw 109	1.6	.4	.2	***	***	**
Tr. 2	0	0	.25		**	**
Tr. 3	0	.3	0	***		***
Lemans	0	0	.3		*	*
Sheba	1.6	.1	0	***	***	*
Granada	0	.075	.15	***	*	*
Roubijax	0	.2	.15	**	*	
Tigerrag	2.5	.5	.11	***	***	
Cocarde	0	.8	.4	***		**
Tourmalin	0	.2	.9	*	**	

* P<0.05
** P<0.01
*** P<0.001Leaf explants

Table 4. The nine different combinations of NAA and BAP tested along with MS salts and vitamins on stem explants of Group 4 cultivars.

Media	NAA mgl^{-1}	BAP Mgl^{-1}
4a	1	1
4b	1	2
4c	1	4
4d	2	1
4e	2	2
4f	2	4
4g	4	1
4h	4	2
4i	4	4

Table 5. Number of shoots regenerated using stem explants, of cultivars from the four different groups. Modifications tested for regenerating each group (media 2a, 3a &b and 4a). The number of harvestable shoots are per explant obtained as an average value of four replications, with each replication consisting of 10 explants

Cultivar	Media	Group	No. Of Shoot	Cultivar	Media	Group	No. Of Shoot
Cleo	1	1	4.6	Tornato	1	1	1.6
Rush	1	1	3.6	Lineker	1	1	1.6
Kris	1	1	2.8	Tigerrag	2a	2	2.5
CBA 1403	1	1	2.6	Sheba	2a	2	1.6
Albert Hein	1	1	2.3	Xw 109	2a	2	1.6
Toulouse	1	1	2.1	Tr. 2	3 a&b	3	2.5
Venezia	1	1	2.1	Tourmalin	3 a&b	3	1.6
Tr. 1	1	1	2.0	Money m	3 a&b	3	1.6
Mayfair	1	1	1.8	CBA 2053	4a	4	2.5
Biarritz	1	1	1.6				

Table 6. The influence of induction period (days on media 5) and media after induction for regeneration of leaf explants of Cv. 1581. Based on the data the protocol of 4 days induction on media 5 and regeneration on a media 6d was selected. . The numbers of harvestable shoots are per explant obtained as an average value of four replications, with each replication consisting of 10 explants.

Media number	Media BAP X IAA		Induction period 4 days	8 days	16 days
6a	0.0 mg/l	0.0 mg/l	0.3	0.1	0.4
6b	0.0 mg/l	0.25 mg/l	0.8	0.1	0.4
6c	0.0 mg/l	0.50 mg/l	0.25	0.2	0.5
6d	0.25mg/l	0.0mg/l	2.5	2.1	1.6
6e	0.25mg/l	0.25mg/l	0.6	0.3	0.2
6f	0.25mg/l	0.50mg/l	0.3	0.4	0.3
6g	0.50mg/l	0.0 mg/l	1.9	1.1	1.6
6h	0.50mg/l	0.25mg/l	0.5	0.5	0.4
6i	0.50mg/l	0.50mg/l	0.4	0.7	0.2
	Mean		0.7	0.6	0.6

Table 7. Shoot regeneration from leaf explants of cultivars recalcitrant on stem explants (from groups 2 to 4). Among the 14 cultivars tested, four showed successful regeneration when induced on medium 5 and regenerated on medium 6d. The number of harvestable shoots are per explant obtained as an average value of four replications, with each replication consisting of 10 explants

Varieties	Group	shoots	Varieties	Group	shoots	Varieties	Group	shoots
Rainbow	4	1.6	TR-2	3	1.0	Roubiax	2	0
TR-5	3	1.7	Stallion	2	.7	David	2	0
Hugo	3	1.6	Lemans	2	0	Granada	2	0
Mike	3	1.8	Alencon	2	0	TR-3	2	0
Paso doble	3	1.3	Cocarde	2	0			

Discussion

Using modifications of protocols used by Gutterson et al., (1994), De Jong et al., (1995) and Lemieux et al., (1991), we have developed a protocol flowchart (Fig.1) leading to regeneration in 23 of the 37 cvs. The modifications investigated were based on the response of the explants and not trial and error. The hormone concentrations ranged from equimolar amounts of auxin and cytokinin (Kaul et al., 1990) to the use of a 2 fold molar excess of cytokinin (Bush et al., 1976; Earle et al., 1974; Slusarkiewicz et al., 1981; Ledger et al., 1993; Lowe et al., 1993; Urban et al., 1994). Cultivar requirements in chrysanthemum for regeneration appeared quite stringent, which has been previously reported (Kaul et al., 1990; Lowe et al., 1993; Sauvadet et al., 1990).

Potential of varying the concentration of BAP to influence the shoot number is evident from the regeneration of Group 2 cvs. It is interesting to note that the reduction in BAP concentration in medium 2a induced a better response on three cultivars. Such responses in improved regeneration upon lowering of cytokinins has been observed in brassica (Block et al., 1989). It is possible the endogenous levels of cytokinins in these three cultivars are so high that upon placing on a media with low BAP the conditions suitable for regeneration are created. However the reasons for differences in endogenous cytokinin levels and their differential response to regeneration is still unclear. We have efficient assays for measuring endogenous levels of cytokinin oxidases but not endogenous cytokinin levels. The low levels of cytokinins in addition make it more difficult to evolve and standardise such protocols.

Initially callusing stem explants on medium 1 and later transferring them to media 3 a&b provided a method to regenerate Group 3. The media 3 a&b composed of different vitamins and hormones could have induced differentiation in the calli. Different sources of cytokinin (BAP, coconut water and kinetin) could generate varied response like protein synthetic capacity, growth status (Gaudino & Pikaard, 1997) and thus influence organogenesis. The switch from BAP to Kinetin and finally to a medium with GA3 did induce organogenesis in our calli. Kinetin is a non aromatic cytokinin as compared to BAP which may have had a different response on the explants. GA3 is a know agent in inducing organogenesis in calli including long term calli of citrus (Chakravarty & Goswami, 1999).

Stronger auxin (heat stable NAA) could induce better de-differentiation signifying their potential in inducing regeneration of otherwise recalcitrant explants. Group 4 failed to have any response in the screening on medium 1, but by providing a stronger auxin it was possible to regenerate some of them. Varying auxin source, concentration and time of exposure would be combinations worth testing on recalcitrant explants. Stronger auxins induce higher levels of polyamines (Maatar & Hunault, 1997) which could directly or indirectly affect the regeneration process.

The results on leaf explant based regeneration implied the role of hormones, especially auxin in regeneration. By pulsing high concentrations of auxin (medium 5) for different time periods and subsequently transferring to different hormone combinations, it was possible to regenerate genotypes recalcitrant on stem explants. The cvs. from Groups 3 & 4 produced direct organogenesis on leaf explants upon strong auxin pulsing which was not so

for cultivars from Group 2. For a given genotype the effect of a certain set of hormone and media combinations may have varying influences based on the explant.

A methodology for developing regeneration protocols based on response of the explant, proved to be successful. Material recalcitrant for a given explant can be regenerated using alternate explants and media combinations. Regeneration could be further influenced by the quality of light incident on the cultures.

Acknowledgement

We very much appreciate the co-operation of the chrysanthemum breeders : CBA, Delta Stek, Dekker, Fides and Goldstock. They provided with rooted cuttings of the elite cvs. on which our experiments were based.

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Chapter- 3

Light quality (spectral distribution and transmission wavelength maxima), influences regeneration efficiency and microshoot quality in chrysanthemum

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Submitted to HortScience

Summary

Influence of light quality (intensity, spectral distribution and transmission wavelength maxima) on regeneration efficiency and microshoot quality is described. We present a prospective potential to modify regeneration efficiency and microshoot quality by varying the quality of incident light. Light quality was evaluated by means of three tinted (blue, yellow & red) and one transparent culture container (control) all receiving light in the range of 400-700 nm. Primordia, number of microshoot, shoot and internodal length were significantly higher in yellow containers. Whereas the number of nodes and leaf length was significantly higher in blue containers but with lowest green coloration. On the other hand significantly higher green coloration of shoots were observed only in control containers. Induction in light / dark and regeneration on two BAP levels had no consequence, indicative of light quality as the inimitable attribute influencing regeneration efficiency and microshoot quality in chrysanthemum *Dendranthema grandiflora*.

Key words: Chrysanthemum, *Dendranthema grandiflora*, light quality, microshoot quality, regeneration efficiency, spectral distribution and transmission wavelength maxima.

Introduction

Failure to regenerate adventitious shoots is a shortcoming dealt with by changing culture media, temperature, tissue source, genotype, and hormone concentration. Incident light quality in addendum can to a large extent influence morphogenesis and quality of microshoots in numerous crop species. Varying light quality to alter regeneration efficiency (number of microshoots and nodes) and microshoot quality (length of shoot, internode and leaf and green coloration of leaves) is not well examined, as a result it turned out to be the focal point of our study.

Red light (655 ± 20 nm) enhanced initiation of somatic embryogenesis in date palm compared with white or blue (420 ± 12 nm) light (Calero et al., 1990). Callus regeneration of *Actinidia deliciosa* showed regeneration only in red light (Muelo and Morini, 1990). Maximum induction of new growth occurred in triticales irrespective of whether blue or red light was used (Matveenko et al., 1991). Likewise in chrysanthemum we were interested in ascertaining the influence of light quality, on regeneration efficiency and microshoot quality. We intended to identify explicit traits of light quality for modifying exclusive traits in microshoot quality and improving regeneration efficiency.

Materials and methods

Three experiments were conducted, each specifically aimed to study the influence of induction (light and dark), BAP levels and light quality on microshoot quality and regeneration efficiency, whose results are compiled in Tables 1 to 4. Light quality was varied using three tinted polystyrene containers (Sigma phytatrays II) and a transparent control. The three tints were blue, yellow and red (Fig. 1), with 400-500, 550-610 and 610-690 nm spectral distribution with 475, 580 and 660 nm transmission wavelength maxima respectively. The transparent container acting as control permitted white light with a spectral range from 400-700 nm. Light intensity in the climate room was measured using a lux meter as 55 $\mu Molm^{-2}s^{-1}$, however in the containers was 4.4 $\mu Molm^{-2}s^{-1}$ (blue),

15.4 $\mu Molm^{-2}s^{-1}$ (yellow), 2.6 $\mu Molm^{-2}s^{-1}$ (red) and 53.5 $\mu Molm^{-2}s^{-1}$ (control). Four containers were used per tint with 10 explants per container.

Surface sterilised greenhouse grown leaves of cv. 1581 were cut 0.5 cm away on either sides of the midrib, which was subsequently sliced resulting in two strips. Each strip was sliced perpendicular to its length ending in uniform square explants on their abaxial surface on induction medium. The containers were placed in a climate room maintained at 25°C with a 16h photoperiod.

The induction medium was composed of MS inorganic, B5 organic, 2 mgl^{-1} BAP, 1 mgl^{-1} NAA, 7 gl^{-1} Tissue culture Agar, 30 gl^{-1} sucrose and 3mM MES (Gutterson et al., 1994). Explants were induced in dark or in four different qualities of light and transferred to media composed as above but lacking NAA and differing in BAP levels (0.25 mgl^{-1} and 0.5 mgl^{-1}, Seetharam et al., 2000, in press).

Periodically on 3, 7, 10, 13 and 21 days of incubation, the number of primordia were counted and transferred to fresh containers on 21st day. On the 42nd day the number of microshoots and nodes, length of harvestable shoot, internode, leaf and green coloration of the shoots (estimation by eye) were recorded and statistically analysed.

Results and discussion

ANOVA designated light quality as a variable with significant influence on microshoot quality and regeneration efficiency. Other variables, induction in light was faintly superior over dark and two levels of BAP (0.25 & 0.5 mgl^{-1}) had no consequence (Table 1 & Fig. 3). Interactions amid induction, BAP levels and light quality were non significant hence pooled data illustrated main effects. (Table 2).

Primordia emerged exclusively from the cut edges of the explant (Fig. 2) on day 7, 10 and 13 in yellow, blue and red/control containers respectively (Table 3). Number of primordia per explant in blue and yellow were ~18 (Table 3) while in red and control were ~9. Number of microshoots per explant in blue, yellow, red and control containers were 3.9, 4.0, 1.9 and 2.0 respectively (Table 4). Shoots from blue, yellow, red and control containers had 19, 06, 06 and 10 nodes respectively (Table 4, Fig 3). Prunus produced higher number of nodes at 475 nm than red (660 nm), far-red and white light (Muelo & Thomas, 1997) and also in *Azorina vidalii* (Silva & Debergh, 1997). Quality of light influenced number of primordia but not it's genetic engineering and auxiliary development demonstrating importance of light quality only during primordia production. Production of shoots in blue and yellow containers doubled the numbers present in red and control. Enhancing the number of primordia and number of harvestable shoots by shifting only the quality of light influenced regeneration efficiency. Higher number of nodes should be inferred as higher number of propagules per shoot, by which we can alternatively boost multiplication rate and regeneration efficiency. The internodal length in blue, yellow, red and control containers were 03, 12, 06 and 06 mm (Table 4, Fig. 3). Between 400-550 nm the internodal length is shortest, but is longest between 550-610 nm and a value in-between is observed from 610-690 nm (red) and control. The microshoots length in yellow and blue containers were 100% and 71% longer (07 & 06 cm) than in red (3,5 cm, Table 4, Fig. 3).

TABLE 1. *Induction in light and dark, two levels of BAP and four light quality parameters were the variables tested. ANOVA indicates significant influence of light quality on the number of microshoots (NM), number of nodes (NN), shoot length (SL), internodal length (IL) leaf length (LL) and green coloration of leaf (GC). Induction and BAP, and the interactions between the variables do not have any significant influence.*

	NM	NN	SL	IL	LL	GC
Source of Variation	F.pr	F.pr	F.pr	F.pr	F.pr	F.pr
Induction	0.342	0.460	0.275	0.644	1.000	0.012
BAP levels	0.680	0.564	0.781	1.000	0.384	0.357
Quality of light	<0.001	<.001	<0.001	<0.001	<.0.001	<.001
Induction X BAP	0.342	0.460	0.176	1.000	0.384	0.042
Induction X light	0.694	0.605	0.983	0.585	0.939	0.207
BAP X light	0.944	0.317	0.939	0.929	0.939	0.463
Ind X BAP X Qu of l	0.911	0.384	0.867	0.929	0.755	0.207

TABLE 2. *Influence of light and dark induction, different levels of BAP (0.25 mg/l and 0.5 mg/l) on the different parameters are presented. Standard deviations are mentioned in parenthesis. Means are based on 16 replications of 10 explants each.*

Treatment	Number of primordia/ explant (0.50)	Number of shoots/ explant (0.05)	Number of nodes/ shoot (0.95)	Length of shoot (cm) (0.05)	Length I. node (mm) (0.82)	Length of leaf (mm) (0.50)	Green colour Of shoots
Induction in light	10	2	11	3.5	7	7	100 %
Induction in dark	9	1.9	9	3.4	5	6	100 %
Regene. media 1 0.25 mg/l	9	2	9	3.5	6	7	100%
Regene. media 2 0.5 mg/l	9	2	10	3.4	6	7	100%

TABLE 3. *The influence of light quality on the timing and the number of primordia per explant are presented. The Standard deviation is 5.08. Means are based on 16 replications of 10 explants per replication.*

Light	Intensity in μ Mol	Day-3	Day-7	Day-10	Day-13	Day-21 (Total)
Blue	4.4	0	0	4.8	10.9	17.8
Yellow	15.4	0	5.9	10.9	15.2	17.5
Red	2.6	0	0	0	4.8	9.3
Control	53.6	0	0	0	5	8.7

Similarly yellow light influenced shoot length in chrysanthemum (Mortensen & Stromme, 1987) and rice (Gomosta & Vergara, 1988). In red containers (610-690 nm) the microshoot length was consistent with control, which is contrasting to rice (Haraki, 1977) and *Argyanthemum* (Jatzowski & Zimmer, 1994), wherein red light caused elongation. Subject

to characteristics desired, one may possibly produce longer shoots with lesser nodes and leaves in chrysanthemum by culturing in yellow containers or vice versa by culturing in blue containers. Leaves from the blue, yellow, red and control containers were 15, 05, 04 and 08 mm long (Table 4, Fig. 3). Likewise in *Arabidopsis* leaf area and petiole length were influenced by blue light irradiance (Eskins, 1992). Greenness of leaves (determined by eye) in control was 100 %, while in blue, yellow and red containers were 50, 75 and 90 % green. None of the shoots in the tinted containers appeared greener than in control (Table 4 & Fig.3). White light with complete spectral range from 400 to 700 nm appears optimum for maximum chlorophyll synthesis. Diminished green coloration at 475 nm is a phenomenon also observed in rice (Li & Pan, 1995). This variation in greenness may be due to variation in levels of light harvesting *Cab* transcripts. Transcript levels of single light harvesting complex (*Lhc*) were determined in etiolated cress (*Lepidium sativum*) and maximum transcript was observed at 660 nm, followed by far red and 475 nm (Brunner & Rudiger, 1995). The etiolation observed at 475 nm and 580 nm may be due 3 reasons; firstly due to lack of transcripts activated exclusively at 660 nm.

TABLE 4. *Number and quality of shoots regenerated on leaf explants of chrysanthemum cultured under different light quality. Standard deviations are mentioned in parenthesis). Means are based on a total of 16 replications of 10 explants per replication tested in three independent experiments.*

Light	Number of shoots / explant (1.15)	Number of Nodes / Shoot (6.13)	Length of harvest. shoot (cm) (1.77)	Length of Internode (mm) (3.77)	Length of Leaves (mm) (4.96)	Degree of Green Coloration (21.74)
Blue- (475 nm)	3.9	19	06	03	15	50 %
Yellow- (580 nm)	4.0	06	07	12	05	75 %
Red- (660 nm)	1.9	06	3.5	06	04	90 %
Control	2.0	10	3.5	06	08	100 %

Secondly red and white lights are indispensable for protochlorophyllide reduction to chlorophyllide, which is subsequently esterfied to yield chlorophyll (Apel, 1984). Thirdly wavelength of 638 nm is absorbed by protochlorophyllide itself through its reduction to chlorophyllide (Griffiths, 1991). Features observed in the tinted containers can also be ascribed to the exclusive activation of phytochromes under that light quality. Accumulation of a particular phytochrome may augment or hinder the activity of other phytochromes. Consequently a known phenotype for a specific quality of light may well be due to overproduction, complete absence or alterations in the ratio between distinct phytochromes. There is no association between intensity and microshoot quality but there is with the spectral distribution and transmission wavelength maxima. As transmission wavelength maxima shifts from 660 to 475 nm the number of shoots, shoot length, leaf length increases but green coloration decreases. We propose spectral distribution and transmission wavelength maxima have a classic role in microshoot quality over light intensity.

Fig :1 Layout and design

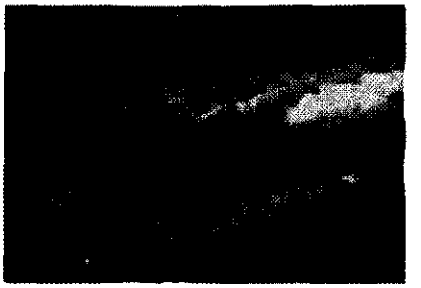

Fig :2 Primordia in a yellow container

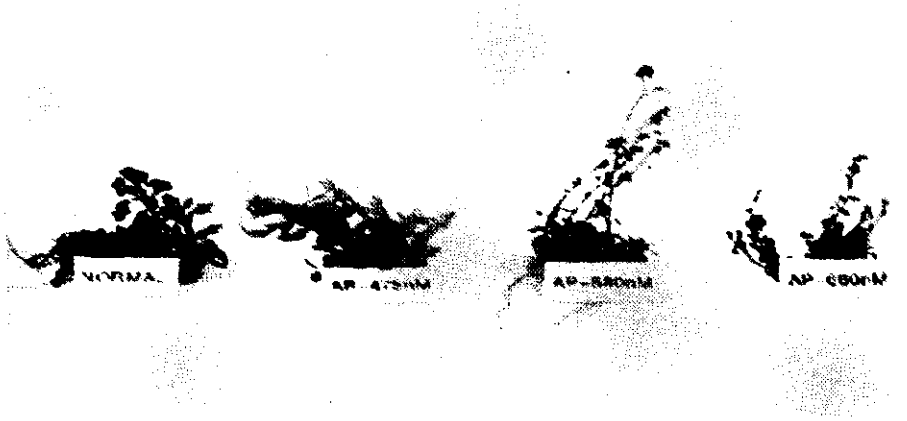

Fig. 3 Leaf explant with microshoots from the three different TINTED containers and control. The variation in shoot length, internodal length, leaf length and green coloration is clearly visible from this photograph.

We advocate use of light with transmission wavelength maxima at 580 nm to turn out more shoots in shorter period to enhance regeneration efficiency. Those desiring to produce more nodes and broader leaves may perhaps try transmission wavelength maxima at 475 nm.

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Chapter- 4

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

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Accepted for publication in Molecular Breeding

Abstract

The enhanced cauliflower mosaic virus 35S (dCaMV) promoter and the potato *Lhca.3.St.1* promoter were evaluated for their expression abilities in chrysanthemum. The promoters were fused to the ß-glucuronidase reporter gene with and without flanking matrix-associated regions (MARs) and were introduced into chrysanthemum via *Agrobacterium tumefaciens* AGL0. A quantitative evaluation of a total of 127 active transformants established that in chrysanthemum the *Lhca.3.St.1* promoter was over 175 fold more active in the leaves than the dCaMV promoter. This large difference was not apparent from accompanying histochemical assays. The dCaMV promoter was as silent as the previously evaluated single CaMV promoter. The use of CaMV-based promoters in the genetic engineering of chrysanthemum should be discouraged if 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, indicating a possible plant dependent activity. This may indicate a possible plant dependent activity of MAR activity. *Lhca3.St.1* promoter-driven GUS activity was highest 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.

Key words: *Dendranthema*, 35 S, high expression, MARs, molecular breeding, stable expression

Introduction

Chrysanthemum (*Dendranthema grandiflora* Tzvel.) is the second largest cut-flower crop next to rose (*Rosa hybrida*). Commercial material is 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 auto hexaploid genetics of chrysanthemum coupled to a large genome size [32, 38].

In recent years, several transformation protocols have become available for chrysanthemum [31, 32]. Transgene expression studies in chrysanthemum have focussed on the use of the cauliflower mosaic 35S (CaMV) promoter to drive the transgene [11, 21, 37, see also discussion], often with the gene for bacterial ß-glucuronidase gene (GUS) as reporter [18]. In our experience, transgenic chrysanthemum carrying a CaMV-GUS construct shows bright blue coloration upon over night X-gluc staining [10,11,15], but according to GUS quantification by fluorometry the expression is very low to undetectable. Also in case of the generation and analysis of large numbers of independent transformants. To improve transgene expression levels in chrysanthemum, we have evaluated two putatively stronger promoters viz.; doubled 35S the dCaMV (28) and the potato *Lhca3St.1 promoter, which conferred higher levels of expression as compared to dCaMV (27).* The doubled cauliflower mosaic virus 35S (dCaMV) promoter is a stronger variant of the CaMV promoter, due to the duplication of the upstream enhancer sequence [28]. 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 does [27]. The *Lhca3.St.1* promoter is active in leaves, stems and other green parts of the plant and its activity is light dependent. 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 matrix-associated region (MAR) element on either side of transgenes can reduce such position effects in plants [23-25]. It is hypothesised that this reduction is due to the creation of independent transcriptional domains of the T-DNA, irrespective of their position in the recipient genome. We here present the evaluation of both the dCaMV and the *Lhca3.St.1* promoter with and without flanking MAR elements (chicken lysozyme A element) in the same configurations show effective in tobacco (23-25). As MAR element, the chicken lysozyme A element is used in the configurations previously shown to be highly effective in tobacco [23-25].

Materials and methods

Plant material for transformation

Stem explants of chrysanthemum cultivar 1581 were obtained from greenhouse grown plants under standard condition [22], 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. Stem segments of about 2-4 mm thickness were sliced along the width of the stem, after sterilising the stems in 1% hypochlorite plus four drops of Tween 20 for twenty minutes. Surface-sterilised explants were rinsed in sterile water three times to remove traces of hypochlorite.

Media and *Agrobacterium* inoculum

The explants were placed in petridishes containing CHR 04 medium [11] overlaid by a Whatmann filter (9 cm, grade 40). The explants were precultured for 2 hours prior to co-cultivation. A growing culture of the *Agrobacterium tumefaciens* strain AGL0 [18] 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. 100 µl 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

Of the resuspended *Agrobacterium* culture, 20 µl was pipetted on top of the surface of each explant. The explants were cultured for 48 hours at 25° C and a 12-h photoperiod. After 48 hours the explants were transferred to fresh CHR 04 medium containing 100 mg/l of timentin to kill *Agrobacterium* and 25 mg/l kanamycin for selection of transgenic shoots. After about 6 weeks the first set of transgenic shoots (10 mm long) were harvested. The harvested shoots (harvest 1) 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) and cultured at 25° C and a 12-h photoperiod. The remaining explants were transferred to fresh CHR 04 media containing timentin, cefotaxime and kanamycin. This process was repeated twice in a period of six weeks. At the

end of twelve weeks was the second harvest of shoots. Rooted plants were transferred to the greenhouse.

Plant transformation vectors

The binary vectors evaluated for GUS activity in chrysanthemum have been described previously [23, 24] and carry different T-DNAs, here designated NCG, ANCGA, NLG and ANLGA as shorthand for the A elements and genes present on the T-DNA. The T-DNA configurations used are outlined in Figure 1.

Determination of GUS activity

Quantitative GUS measurements were performed essentially as described previously [23], using a Fluoroskan II microtiterplate reader (Titertek, Finland). In some cases (see results) the sensitivity of the standard assay was increased. Samples were harvested as nine 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. Histochemical GUS assays were conducted as described previously [39].

Results

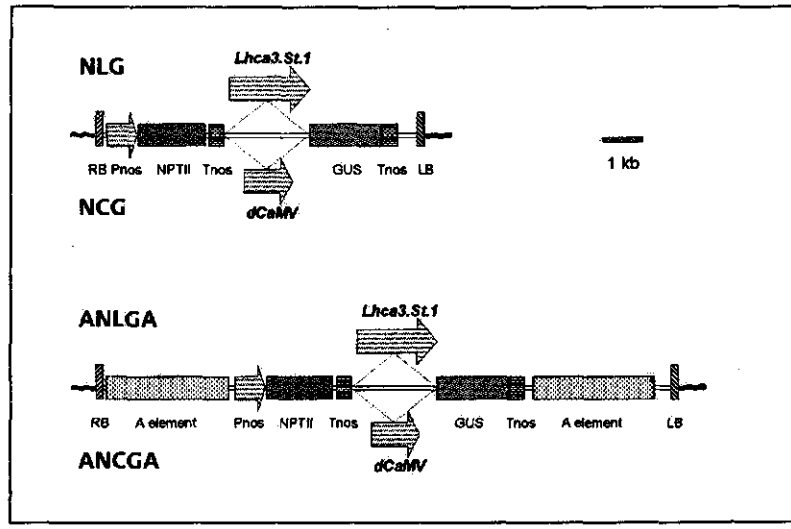

Figure 1. Schematic representation of the T-DNA regions of the four constructs used in this study. RB, LB, right and left T-DNA borders respectively; A element, 3 kb chicken lysozyme A element; Pnos, nopaline synthase promoter; NPT II, neomycin phosphotransferase II coding region; Tnos, nopaline synthase 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.

Generation of populations of GUS-containing transgenic chrysanthemum

The four T-DNA vectors outlined in Figure 1 were introduced in cultivar 1581 and a minimum of 30 plants per construct rooting in the presence of 25 mg/l kanamycin were generated. The two dCaMV promoter-containing constructs tended to be more difficult in getting rooting transgenic plants. Overall 195 transgenic plants were generated and checked for GUS expression by the fluorometric assay in young leaves of greenhouse grown plants, wherein the activity of dCaMV- GUS activity was low. GUS activity was assayed in young leaves of greenhouse-grown plants. Notably the GUS activity in the dCaMV promoter-carrying 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 accurately, the sensitivity of the assay was lowered to about 20 pmol per min per microgram of protein. For this, 50 µl plant extract was assayed for considerably longer incubation times (up to 24 hrs) and an end point measurement after addition of Na_2CO_3 was taken. With this improved sensitivity of the assay, 9 plants from the NCG and also 9 plants from the ANCGA population showed still no detectable activity. For the NLG and ANLGA populations, a total of 48 plants showed no detectable GUS activity. All 66 plants showing no expression were considered to have not received the GUS gene. They were discarded from further analysis. 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. In Figure 2, the size-sorted GUS activities in leaves of individual transformants are given after natural logarithmic transformation. In Figure 2A the two non-MAR-containing populations NCG and NLG are shown; in Figure 2B the two MAR-containing populations ANCGA and ANLGA.

Comparative analyses of populations of GUS-containing transgenic chrysanthemum

The descriptive statistics of the four populations are given in Table 1. As shown previously [26], a proper analysis of GUS activities in populations of plants requires a logarithmic transformation. The average activities in these populations are here in the text 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 indeed: 6.42 pmol MU per microgram protein per hour for NCG and 11.6 pmol MU per microgram protein per hour for ANCGA. Table 2 shows the results of the comparative statistical tests performed. There are no highly influential outliers in this population (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, the difference is just 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), but the MAR-containing ANCGA population has three-fold more plants with an activity of more than 45 pmol MU per microgram protein per hour. Averaged over all 55 dCaMV-carrying plants, the overall activity of GUS driven by the dCaMV promoter in chrysanthemum

yields a poor 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 expression data are apparently severely skewed in these populations of plants.

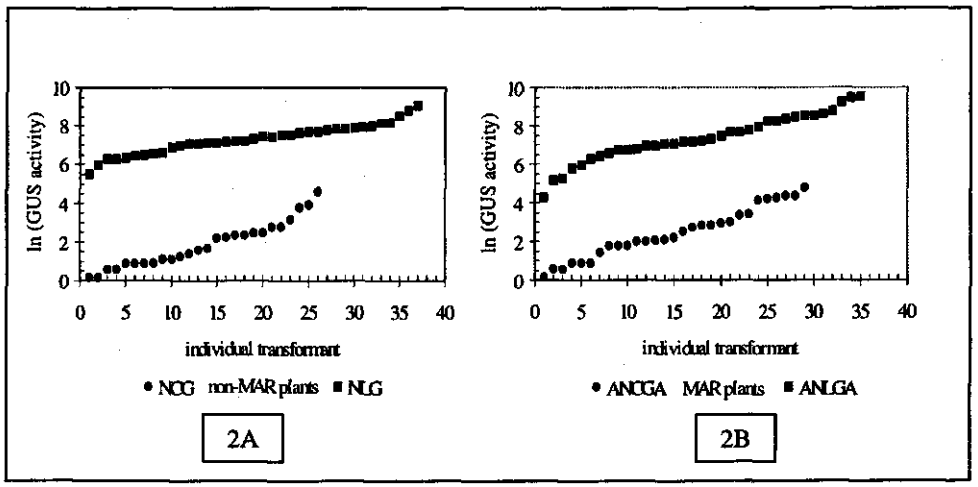

Figure 2. GUS activities in the four populations of transgenic chrysanthemum. *2A*, populations carrying the non-MAR constructs NCG with the dCaMV promoter driving the GUS gene and NLG with the *Lhca3.St.1* promoter driving the GUS gene; *2B*, populations carrying the MAR-containing constructs ANCGA 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.

Table 1. Descriptive statistics of the four chrysanthemum populations.

Plant population	**NCG**	**ANCGA**	**NLG**	**ANLGA**
Promoter driving GUS	dCaMV	dCaMV	*Lhca3.St.1*	*Lhca3.St.1*
Presence of A element	-	+	-	+
No. plants generated	35	38	64	58
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 (%)	63.5	53.1	10.7	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]Backtransformed mean on the natural logarithmic scale in pmol per hour per microgram protein

Table 2. Comparative statistics of the chrysanthemum populations in this study

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 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 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.

In contrast, the potato *Lhca3.St.1* promoter-GUS configuration does give appreciable GUS activities of 1510 pmol MU per microgram per hour in case of the NLG population and 1572 for the ANLGA population. As is the case for the two-dCaMV promoter-carrying populations, the difference in mean is not significant (Table 2, t-test). In contrast, there is now a large difference between the highest active plants in the populations: 8542 for NLG plants and 13721 for an ANGLA transformant. 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 over 175 fold more than the average activity given by the dCaMV promoter. Also 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. The coefficient of variation, which allows comparisons of variability independent of scale, is about 4-fold lower for the populations carrying the *Lhca3.St.1* promoter-GUS fusion. 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 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 dCaMV promoter-carrying transformants were analysed for GUS activity in other parts of the plant. Analysed were stem, pedicel and ray florets. In none of the dCaMV-GUS carrying chrysanthemum plants analysed a GUS activity considerably higher than in the leaf was observed (data not shown). In contrast, five randomly chosen chrysanthemums ANLGA transformants analysed for GUS activity in the same tissues showed high activity in all above ground plant parts. In Figure 3, the GUS activities in these plants are plotted and in Table 3, the quantitative data are summarised. Overall, the GUS activity in the stem is relatively the highest and the GUS activity in the pinkish white-coloured ray floret is the relatively lowest. The *Lhca3.St.1* promoter is active in mature pollen of transgenic *Lhca3.St.1*-GUS carrying chrysanthemum (data not shown), as is the case in tobacco [6].

Table 3. Distribution of GUS activities over different tissues and 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.

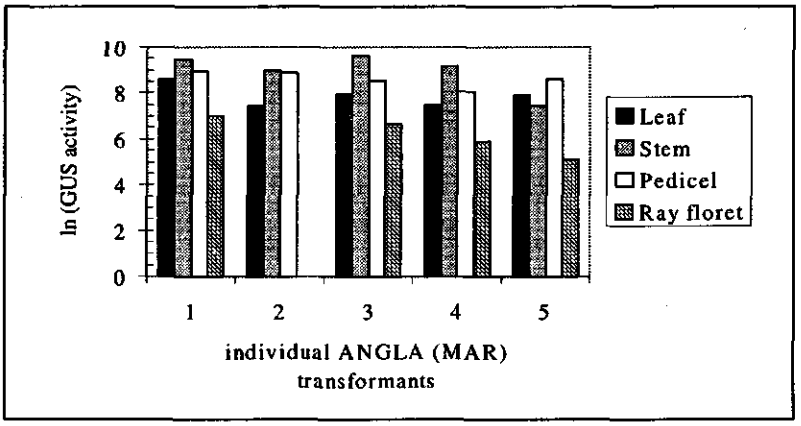

Figure 3. GUS activity in different organs of chrysanthemum of five individual transformants carrying the ANLGA construct. The natural logarithm (ln) of the GUS activity in the different tissues is given in pmol MU per microgram protein per hour.

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 [22]. After a period of 21 months, leaves were again analysed for GUS activity and compared to the activity in the leaves of the young, three-month-old plants. In Figure 4, the GUS activities of the three-month and the 21-month-old plants are plotted. 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 there is no large difference in stability in time of GUS gene expression between plants from the NLG and from the ANLGA population. GUS gene expression driven by the *Lhca3.St.1* promoter is stable upon vegetative propagation and growth.

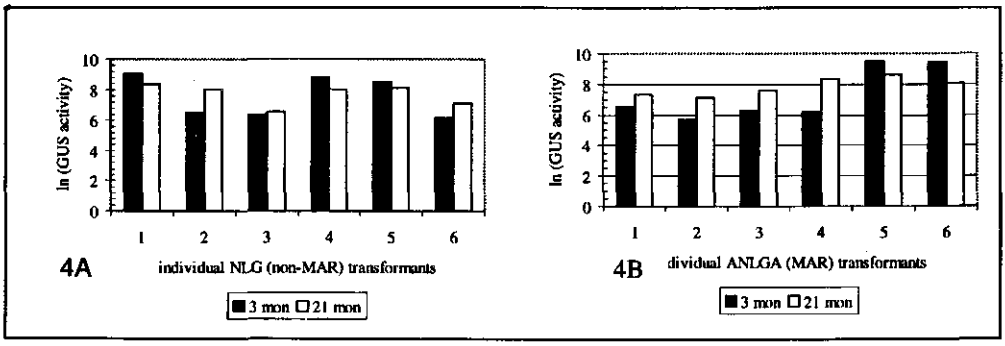

Figure 4. Stability of GUS activity over a period of 21 months in selected chrysanthemum transformants. 4A, plants from the non-MAR NLG population; 4B, plants from the MAR ANLGA population. The natural logarithm (ln) of the GUS activity of each individual transformant at 3 months (3 mon) in black and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

Discussion

Low activity of the dCaMV promoter in chrysanthemum

Based on the quantitative analyses of 55 plants, the results presented here show that the enhanced cauliflower promoter (dCaMV) yield no appreciable transgene (GUS) expression levels in any of the tissue of chrysanthemum analysed. Histochemical assays of (d)CaMV-GUS transformed chrysanthemum do show cells and tissue patches that turn blue upon incubation with X-gluc [10,11,15, our unpublished data]. However, the fluorometric assays demonstrate that this histochemical staining reflects either very low and/or very localised GUS activity. Apparently, a promoter characterisation should not be performed on the basis of histochemical staining alone. The average GUS activity in leaves of dCaMV-GUS carrying chrysanthemum transformants is about 400-fold lower than the activity of the same promoter-GUS constructs in tobacco [24]. The activity of the dCaMV promoter is comparable to that of the non-enhanced CaMV promoter, so in this case the influence of doubling the CaMV enhancer sequence has been minimal.

The reason for the apparent poor behaviour of CaMV-based promoters in chrysanthemum is at present unclear. An experimental difference between the tobacco transformation with the same T-DNA vectors here introduced into chrysanthemum, is the use of the more virulent *Agrobacterium tumefaciens* strain AGL0 [18]. In case of tobacco, we used strain LBA4404, but in our laboratory and others [37] this strain proved too inefficient for chrysanthemum transformation, possibly due to the T-DNA vectors or configurations [4]. As a general rule in plant transformation, about 10-20% of a population of transgenic plants show low expression due to position effects and/or some form of gene silencing. Possibly the AGL0 strain has here yielded much higher percentages of such low expressing plants. This could be, for example, by generating predominantly multicopy and complex transgene configurations in chrysanthemum that are subject to some form of gene silencing. The 8 plants from a population of 73 (10%) showing detectable activity in our standard semi-high-throughput GUS assay may represent the transformants with intact or simple integration. However, our results with the *Lhca3.St.1* promoter-GUS fusion in chrysanthemum (see below) show that in that case not all plants are silent/silenced. This

would indicate that strain AGL0 is unlikely to result in silenced loci only. This argument does not fully exclude the possibility that the dCaMV-GUS fusion maybe particularly prone to gene silencing in hexaploid chrysanthemum, or a combination of factors. Chrysanthemum has the genome size 9000 Mb (2, tobacco X 6 and *Arabidopsis* X 70) hence routine determination of transgene copies and a detailed genetic analysis to support the above conclusions has been rather impossible. To show that all or most dCaMV-GUS transformants carry complex loci would require detailed copy number and transgene configuration determinations in all plants generated. The large genome size of chrysanthemum, of about 9000 Mb [2]/ 25 pg/cell [38], 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. A next possibility to explain the poor behaviour of CaMV-based promoters is that the (d)CaMV promoter is not silenced but transcriptionally silent in chrysanthemum. This plant species may lack the necessary transcription factors for efficient (d)CaMV promoter activation.

A third difference between the inactive dCaMV-GUS and the active *Lhca3.St.1*-GUS constructs evaluated here is the 5' leader sequence of the *Lhca3.St.1*-GUS-derived messenger. Such 5' leader sequences have been shown to be able to raise expression levels considerably [12, 16]. Notably the leader of the petunia chlorophyll a/b binding protein (Cab22L) was shown to be able to modulate GUS activity levels in transgenic tobacco dramatically [12]. The potato *Lhca3.St.1* gene is likely to have a similar leader which by cloning is added to the GUS gene in the ANLGA and NLG T-DNAs [23]. Although the difference in GUS activity between the dCaMV-GUS- carrying and the Lhca3.St.1-GUS-carrying populations in tobacco was only 2-5 fold [23, 24, and 27], possibly transgenic chrysanthemum is much more sensitive to the presence of such RNA stabilising leader sequences. This could mean that the problem of the low GUS activity is on the level of the stability of the GUS messenger, rather than on the level of promoter activation or gene silencing. Experiments with a CaMV promoter-*Lhca3.St.1* leader-GUS fusion would be required to resolve this point.

Assessment of the behaviour of CaMV promoters in transgenic chrysanthemum

The poor behaviour of CaMV-based promoters in our populations of transgenic chrysanthemum prompted us to carry out a critical review of all papers reporting on transgene expression in chrysanthemum. Although the GUS (or GUS-intron) gene was used frequently, most of the assays performed have been histochemical. In the few papers in which quantitative GUS data driven by CaMV promoters are put forward in the literature, activities have a quite wide range. In one case activity is zero [21], other cases report 0.06-9.6 pmol per hour per microgram protein [37; published as 10-160 pmol/min/mg protein] or 1.5-13.8 pmol/hr/ug protein [35; published as 25-238 pmol/min/mg protein). These activities are all at the lower end of the 1.2-119 pmol per hour per microgram protein range observed in our dCaMV populations. In contrast, two other publications give 660-4020 pmol per hour per mg fresh weight [1, 29; published as 11-67 pmol MU/min/mg fresh weight]. In our hands, 1 mg chrysanthemum leaf tissue is equivalent to about 20 microgram (data not shown). If so, the latter activity would be equivalent to 35-201 pmol per hour per microgram protein. This is a 2-fold (based on the maximum) to 10-fold (based on the geometric mean) higher activity than we find. In addition, in callus, activities of 0.014 - 219.6 pmol per hour per microgram protein [38, 40; published as 12.7 - 466 pmol

MU/min/mg protein and 0.11 - 3.66 pmol/min/microgram, respectively], were reported, which are in the same somewhat higher range. With other CaMV-promoter driven genes, only the leafy gene showed a faint signal on a Northern [33]. In other cases, the phenotype reported could be due to low expression levels, such as in case of Bt-mediated insect resistance [13] or aminoglycoside antibiotic selection [7, 30]. With other genes, no effect was discernible [3], or the observed phenotype was due to antisense or gene silencing [8, 14]. In case of the TSWV N gene, poly A RNA Northern analysis was required to detect the expression [34], again suggesting very low steady state levels of N mRNA. However, all TSVW N gene transformants that showed resistance may have been subject to co- or sense suppression phenomena and as a result of that will show low steady state expression levels. The same authors report without showing any data that the level of CaMV-GUS gene expression in chrysanthemum is about 10-fold lower than the activity of the same constructs in tobacco [34]. Of particular interest is the recent use of the 4x enhanced CaMV promoter with the rice chitinase RCC2 gene to confer resistance to gray mold (*Botrytis cinerea*) in chrysanthemum [36]. In this case a positive ELISA is reported and the ELISA readings correlate reasonably well with fungal resistance. However, the highest reading reported for a sensitive 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 [36]. This indicates that relatively very small differences in amount of chitinase determine resistance. Overall, indeed there seem to be problems with the high expression of CaMV promoter-driven genes in chrysanthemum.

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

In contrast to the low activity of the (d)CaMV promoter, the potato *Lhca3.St.1* promoter gives much higher activities in chrysanthemum, about 175-fold higher activity than the dCaMV promoter does. The similar comparison in tobacco resulted in a difference of 2-5 fold [26, 27, 29]. Expression is highest in stems, but still appreciable in all other above-ground tissues evaluated (Fig. 3). The *Lhca3.St.1* potato promoter is therefore a suitable promoter to obtain much higher expression levels in the above ground parts of the chrysanthemum plant. The average GUS activity, expressed per amount of soluble protein, that is conferred by the *Lhca3.St.1* promoter in transgenic chrysanthemum is about four-fold lower than the average GUS activity found for the same promoter-GUS constructs evaluated in tobacco [24, 25].

The non-MAR (NLG) and MAR (ANLGA) chrysanthemum populations do not differ significantly in mean, but the MAR-containing population ANGLA shows a higher variance (Table 1). This is in marked contrast to the data generated previously for tobacco [23] and potato (unpublished data). In these cases, addition of the same chicken lysozyme MAR elements reduced position effects significantly. 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 role of the MAR element may also be the result of the 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 clearly present in the control population. The two chrysanthemum populations analysed here may differ in such characteristics. Any activity of the MAR element could be confounded by large differences in copy numbers or transgene configurations between the NLG and the ANLGA populations. Again, only

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 in stability of GUS gene expression. This indicates that once a *Lhca3.St.1*-GUS fusion has settled actively in the chrysanthemum genome, it remains stably active. As in tobacco [25], also in chrysanthemum the GUS activity driven by the *Lhca3.St.1* promoter shows a much lower variability around its mean than the GUS activity driven by the dCaMV promoter. The *Lhca3.St.1* promoter apparently has some kind of intrinsic stability associated with it.

Future molecular breeding of chrysanthemum

In summary, the results presented here establish that CaMV-based promoters in the configuration used in this study are less suitable for obtaining high transgene expression levels in chrysanthemum. Various other reports on CaMV promoter-driven transgene expression in chrysanthemum could be interpreted in the same way. Although determining the precise reason for this characteristic of CaMV-derived promoters in chrysanthemum will require more experiments and more detailed molecular analyses, for now 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 is expressing almost 200-fold better in the above-ground parts of chrysanthemum. Although it remains to be seen whether endogenous promoters could yield again higher expression levels [32], for most applications in the molecular breeding of chrysanthemum, the activity of this heterologous potato promoter may well be sufficient. This potato promoter is therefore a suitable candidate to use for improvement of chrysanthemum by genetic engineering for traits like insect resistance using biocides (*Bt*, proteinase inhibitors and terpenes). based on -high- expression levels. It will, for example, be useful to use the *Lhca3.St.1* promoter to express genes targeted against insect pests. This would be a relatively straightforward way to confer resistance against pests and diseases that cause severe damage in the aboveground parts of the chrysanthemum plant, such as the beet army worm (*Spodoptera exigua)* or the western flower thrips (*Frankliniella occidentalis*).

Acknowledgements

The authors wish to thank the Plant Research International greenhouse team for excellent plant care; Wim Rademaker for help with transformation and GUS assays; Han Sasbrink and Herma Koehorst-van Putten for assistance and attempts to establish a routine chrysanthemum southern analysis protocol. SA was supported by a Wageningen UR/Bangalore sandwich PhD program, LM by a fellowship from the Dutch Organisation for Scientific Research (NWO) and JPN by program subsidy 347 from the Dutch Ministry of Agriculture, Nature Management and Fisheries.

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Chapter- 5

Cloning of the chrysanthemum *UEP1* promoter and comparative expression in florets and leaves of *Dendranthema grandiflora*

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Key words: chrysanthemum, florets, petal, genetic engineering, GUS,
molecular breeding, transgene expression, ubiquitin extension protein

Accepted for publication in Transgenic Research

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 levels 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, De Jong *et al.* 1995, Renou *et al.* 1993). However, with the 35S-CaMV promoter only low levels of expression were observed by us and other groups (Van Wordragen *et al.*, 1993; Sherman *et al.*, 1998; Boase 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.

2. Materials and methods

2.1. Oligonucleotides used for cloning and sequencing

Primer UBDN1: *AARATHCARGAYAARGAR*
Primer POLY-T: *CCCGGATCCTCTAGAGCGGCCGCTTTTTTTTTTTTTTTT*
Primer UBGSP1: *GCAAACTATTCAAATTGACTATATAAAGC*

Primer UBQP1: *CCCCCCAGATCTGAGCTCCCATGGAATCTAGAATTTTGG ACCACGGTGGGTACGG*
Primer PMCDN1: *CCCCCCGGCGCGCCAGATGATTTCGAAGATTTAAGGGTATA*
Primer PMCP: *CCCCCCAGATCTGAGCTCCCATGGAATCTAGAATCACTG CGAATAATTAATTTAATTAAG*
Primer PETCHS-DN: *GGGGAATTCAAGCTTACTGGTGTGATTCTTGAATC*
Primer PETCHS-UP: *GTCGAGCTCAGATCTGGGCCATGGTTTTTCTAGAAAAAA GTTTGGTATTT*

All oligonucleotides were obtained from Eurogentec (Seraing, Belgium)

2.2 Cloning and sequencing the homologous *UEP*1 promoter

Ubiquitin genes from seven plant species (sunflower X57004, parsley X64344, *Arabidopsis thaliana* X12853, *Glycine max* D26092, pea X17020, tomato X58253 & 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 (Fig. 1) and named *UEP*1. The *UEP*1 promoter was digested with *Sal*I-*Xba*I and ligated to the *Xba*I-*Sph*I ß-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 (Fig. 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).

2.3 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 (Fig.2). The construct p*EPF*2-5-GUS (Fig.2) was, digested with *EcoRI-SalI* 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 ß-glucuronidase (GUS) fragment with NOS terminator fragment from pMOG410. Subsequently the entire expression cassette was obtained as a *SalI-Hind*III fragment from pGEM-T, cloned into the binary vector pBINPLUS, and named p*PMC*P-GUS (Fig.2). All plant constructs were electroporated to AGL0.

2.4 Plant Transformation

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

2.5 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 petridishes (243 x 243 x 18 mm from Nunc Denmark). A drop of MeJa was put on a filter paper placed inside the petridish sealed with parafilm. The petridish was incubated at 25°C for 24 hours. Treatments with α- and γ-linolenic acid were conducted as described by Farmer and Ryan (1992). After 24 hours induced tissues were analysed for GUS expression as described in section 2.6.

2.6 GUS activity assays

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

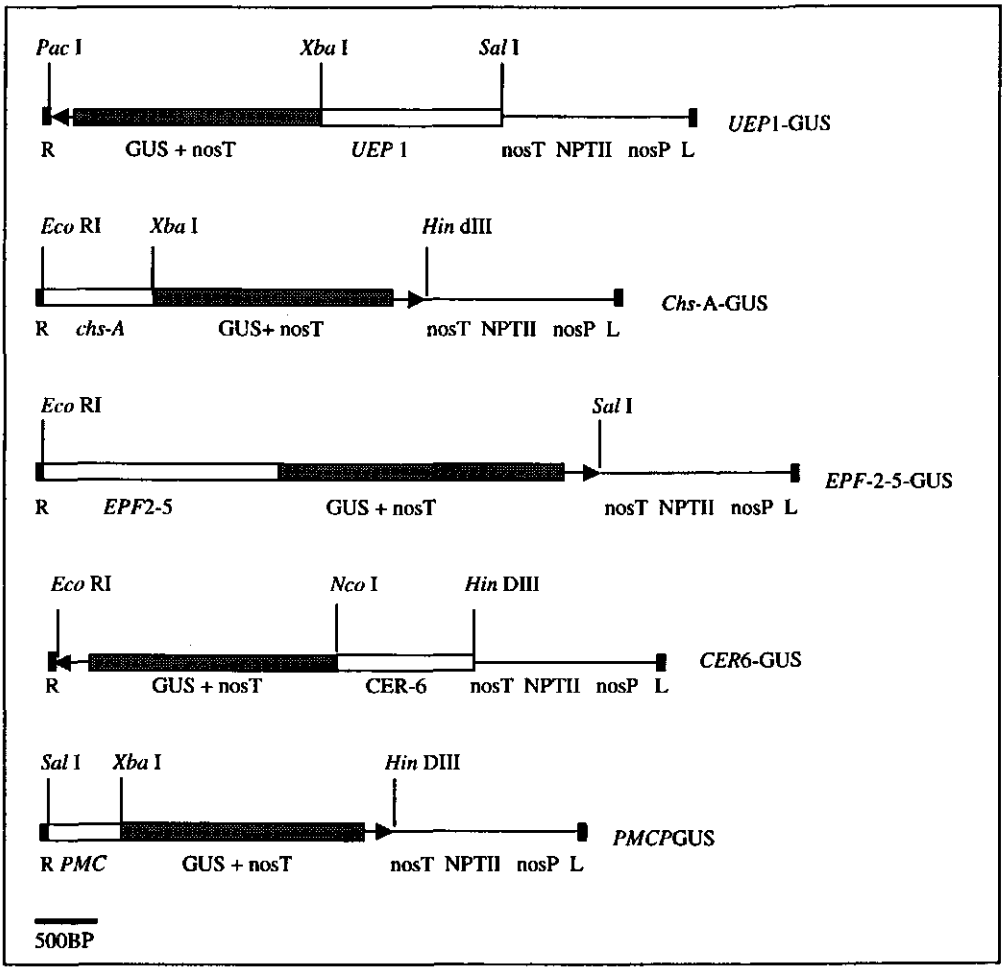

Figure.2 Schematic representation of the T-DNA regions of the five constructs used in this investigation. R, L, right and left T-DNA borders respectively; GUS + nosT, is the β-glucuronidase gene with intron with *Nopaline synthase* terminator, nosP, nopaline synthase promoter; NPT II, neomycin phosphotransferase 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.

3. Results

3.1 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 a probe for a Northern blot with total RNA from ray florets and leaf. A three fold higher signal was observed in ray florets compared to leaves, demonstrating higher expression in the ray florets (not presented). 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 3 small fragments (< 500 bp) generated on the library of

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   1 ACACGCCCAC ACCATATAAA GCCGATTTAG AAAGATTGAC TTTGAGACAA GATGTTAGAT
  61 AAAAGCACCT AAGAATCCGA AGTAAATTCT TCAAGTTCAC GTATGACCAT TCACCAAGAA
 121 AAAATAGCAT CATTGGCAAA CTGTAAATGC GAGACTCTTA GAGAATCATT ACCGATATGG
 181 GCACCGTTAA AAATATCTCC CTCAATAGCT TCCTCGATGG CAACATTAAG AGCTTCAGCT
 241 ACAATAAGAA ATAGGAAAGG GGAAAGTGGA TCTCCTTGGC GCACACCTTT TCCTAGTTTG
 301 AATTCTTGAA TTGGTGACCC GTTTAATAAA ATAGAAGTGT TAGAAGAATT TAGGCTACCC
 361 TTAATCCACT TCCTCCAAAC ATGACCGAAT CCCATTTGAC GCATAATATC ATCAAGAAAA
 421 TTCCAGTTAA GAGTGTCAAA TGCCTTTTCA AAACCTACTT TGAAAATCAT TAGCTTGGGA
 481 TCTTCGATGG GACACCAATT GATGATCTCA TTAACCATCA GCAGACATCA TTGATTTGAC
 541 GACCATGTAA GTAAGCAGAC TGCTCTTCGC TGATAACATA TGGAAAACAA GCTTGAGTCG
 601 TTCTGCAAGA ATTTTGGCAA TAATTTTATA CTGACAACCA ATTAGAGAGA TGGGCCGATA
 661 ATCTGATAGG CATAGGGGTT GGATACTTTA GGAACAAGAC AAATGAACGA AGAGTTACAC
 721 CCCTAGATAA TGAAGCAGTA GAACCAAAAT TCCGCACGTA TTAATTATAT CCGACCCGAG
 781 AACTTCCCAA AATCTCCTCA CGAATCGAAA TGTGAAACCG TCAAGGCCGG GGGCCTTGTT
 841 TCCACCACAT AACCAAACTG CCTTTTTTAT TTCATCATTG GAGAATGACA TTTTTAAGAA
 901 ATCCCGCTGC TCACTAGATA GGGGTTTAAA TATATTTCTG GAGAACTTTA GCCTTGCAAA
 961 ATTTACCTAA AATCGAAAAC CGGACCGAAC CATCGAATTC AAATTTGATT AAACCGAACC
1021 AAACATTGAA TTTGACTTTT TTTTTTCATT TTCGGGTTGA CCGAATAACT GCCCGATTAC
1081 ATAATTTTTA GCACCAAATT TTTTTTCAAG CTTAAAAATG TTATATTCAT AGTTTTAGTA
1141 TAAATATATA TTTATGCGAA TTCGGTTGTC GGTTTAGTTT TATTTTCATT TTATCAAAAT
1201 TATATCCAAA TCGAAAATTC AAATATTTAA TTAGCTCAAC TTAATTTTGT CAAATTCGAT
1261 TAGTTTATAG ATTCGATTCG TTAGTTCCGA GGTGAAAATG CCATGACTAA CTTTTTGTAA
1321 TGCCCATTTA GCCACACTTA CAAGCCCCAA ACTCACCACC CATAAAAGCC CAACAAACAC
1381 ATCCCGTTTC AAACCCTAGC TATATAACTT TAACTCATTG ACACAAACCG TACCCACCGT
          M   Q   I   F   V   K   T   L   T   G   K   T   I   T   L   E   V
1441 GGTCCAAAAT GCAGATCTTC GTGAAAACCC TAACCGGGAA AACCATAACC CTCGAAGTCG
     E   S   S   D   T   I   D   N   V   K   A   K   I   Q   D   K   E   G   I   P
1501 AGTCCTCGGA CACAATCGAC AATGTGAAAG CCAAGATCCA GGACAAGGAA GGCATCCCAC
     P   D   Q   Q   R   L   I   F   A   G   K   Q   L   E   D   G   R   T   L   A
1561 CAGACCAACA ACGTTTAATC TTCGCAGGAA AGCAGCTAGA AGACGGCCGT ACCTTAGCAG
     D   Y   N   I   Q   K   E   S   T   L   H   L   V   L   R   L   R   G   G
1621 ACTACAACAT CCAGAAAGAG TCAACTCTTC ATTTGGTATT GCGTCTACGT GGGGGT
```

Figure.1 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.

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 (Fig.1).

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

Totally five constructs viz.; p*UEP1*-GUS, p*chs*-A-GUS, p*EPF*2-5-GUS, p*CER*6-GUS and p*PMC*P-GUS (Fig. 2) were generated, and transformed to chrysanthemum using *Agrobacterium tumefaciens*. Per construct at least 18 plants 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 ^{10}log, as a proper comparison of GUS expression levels requires logarithmic transformation (Fig. 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 to 4-fold higher than the rest. The level of variation in GUS expression levels between independent transformants was remarkably low for the p*UEP1*-GUS and p*EPF*2-5-GUS populations as compared to the rest (Fig. 3).

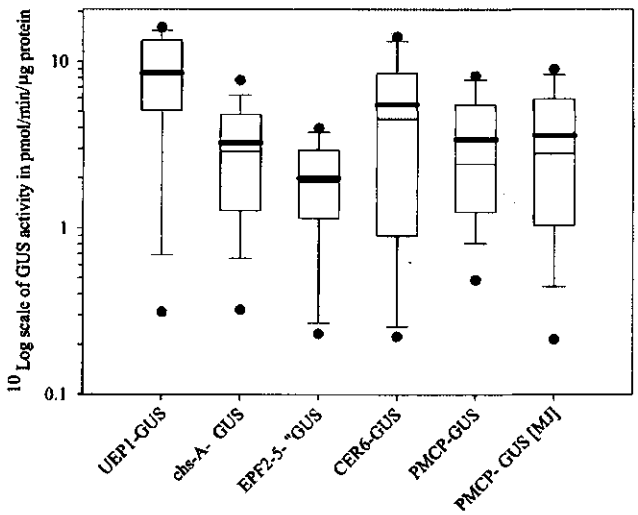

Figure. 3 The activity of GUS in the entire population in the ray florets of chrysanthemum for the respective constructs. The black dots on top and bottom of the box represent the 5th and 95th percentile respectively. The thin and bold lines in the box represent the median and mean respectively. The 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.

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 to 8-fold higher than the other populations. The 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 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 *PMC*P by either α-or γ- linolenic acid (data not presented).

3.1 GUS activity in the ray florets

Per construct at least 18 plants were analysed wherein significant differences were observed in levels of GUS activity, conferred by the different constructs (Fig. 1). The GUS activity was expressed as picomoles (pmol) per minute per microgram protein and converted to ^{10}log as a proper GUS analysis requires logarithmic transformation (Nap *et al.*, 1993). The highest activity was conferred by the homologous *UEP1* promoter, with a mean of 8.5 pmol/min/µg protein, significantly higher than the other promoters tested (Table 1). The variation with reference to the mean was minimal for the *UEP1*-GUS and *EPF*2-5-GUS populations as compared to other constructs (Fig. 3). The LSD (least significant difference)

for the constructs were 2.1 pmol/min/µg protein and based on this significance constructs are grouped in Table 1. Histochemical staining showed most of the GUS activity in the petal tissues of the ray florets (not presented).

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

Construct	No.P [1]	Ray florets			Disc florets			Leaves		
		Max	Min	Mean [2]	Max	Min	Mean [2]	Max	Min	Mean [2]
UEP1-GUS	22	16.5	0.2	8.5^{a}	7	0.7	2.9^{bc}	1.4	0.6	0.9^{bc}
Chs-A-GUS	20	8.5	0.2	3.2^{bc}	3.1	0.2	1.2^{c}	0.3	0.2	0.2^{d}
EPF2-5-GUS	19	4.0	.2	2.0^{c}	3.4	0.7	1.7^{c}	1.0	0.3	0.6^{c}
CER6-GUS	18	14.5	.2	5.5^{b}	3	0.6	1.6^{c}	1.6	0.3	0.7^{bc}
PMCP-GUS	20	8.3	0.2	3.4^{bc}	.5	.1	$.28^{d}$	1.5	0.3	$.9^{bc}$
PMCP-GUS (MJ)	20	9.1	.21	3.6^{bc}	.49	.19	$.34^{d}$	1.4	0.6	1^{b}

[1] No.P is number of transgenic plants

[2] The whole population is used to arrive at the mean for the ray florets. The top six 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.

4. Discussion

4.1 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.

4.2 Activity of the *UEP*1 promoter and the heterologous promoters

Thus far few genes have been cloned from ray florets of any composite plant species (Williams *et al.*, 1999, Helariutta *et al.*, 1993). The newly cloned chrysanthemum promoter of the *UEP1* 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 two- to ten-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. The histochemical analysis of the *UEP1* 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 presented). 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 lowered 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 and 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 recorded, 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 be represent the complete promoter fragment as a result of which the cis-acting elements necessary for wound induction may be absent.

4.3 Comparison of the *UEP*1 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 reports low expression in the range of 0.1 to 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 (Sherman et al, 1998; Boase et al., 1998). Recently, 35S CaMV promoters with 2 enhancers and the AMV untranslated leader sequence did not significantly improve the low expression levels (Annadana et al., in press). We now observe 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 can 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.

Acknowledgement

Dr. Ingrid Van den Meer and Dr. Hiroshi Takatsuji are acknowledged for providing the *Chs*-A promoter and the p*EPF*2-5-GUS construct used in this study. In addition Anneke Balkema is acknowledged for the statistical analysis she performed.

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Chapter- 6

Cloning, functional expression in *Pichia pastoris*, and purification of potato cystatin and potato multicystatin

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To be resubmitted to Protein Expression and Purification

Abstract

In tubers and leaves of potato, *Solanum tuberosum,* cysteine protease inhibitors are thought to play roles in the defence against herbivores and in regulating physiological processes like senescence and cell death. The cDNAs for two such inhibitors, potato multicystatin (PMC) with 8 cystatin domains and potato cystatin (PC) with a single domain, were cloned and expressed in the yeast *Pichia pastoris.* PC yielded up to 80 mg/L of active protein in the culture media. It was purified to homogeneity in a one step ion exchange procedure, and inhibited papain with a K_i of 0.1 nM. Cloning of the PMC cDNA was successful despite apparent toxicity for *E.coli* and high frequency of recombination events in RecA$^-$ strains of *E.coli.* In yeast the expression of the cloned full length PMC gene was poor compared to that of the single domain.

Key words: multicystatin, cystatin, cloning, potato, *Pichia pastoris*

Abbreviations: BMG; buffered minimal glycerol, BMMY; buffered methanol-complex medium, CAPS, kDa; kilo dalton, PC; potato cystatin, PCR; polymerase chain reaction, PMC; potato multicystatin, OD; optical density, RT; room temperature, TE; TRIS-EDTA, YNB; yeast nitrogen base.

Introduction

The sub-phellogen layer of potato tubers contains the 85 kDa cysteine protease inhibitor potato multicystatin (PMC), a predominantly cuboidal, and occasionally prismoidal, crystalline protein (1). The purified protein from tubers is an effective inhibitor of cysteine proteases of western corn rootworm (*Diabrotica virgifera*), and suppresses larval growth (2). The PMC gene is wound-inducible (3) and is assumed to play a role in defence against insect pests, but possibly also in regulating sprouting of potato tubers (D. Michaud, personal communication), leaf senescence and plant cell death. The PMC protein stays crystalline at pH 6.0 and above and is soluble at an acidic pH. PMC can bind to eight papain molecules simultaneously and is stable over a pH range of 3.5 to 10, and a temperature of 60°C for ten minutes (1). Upon incubation with serine proteases PMC is cleaved into 10, 20 and 32 kDa fragments, yet retaining its full papain-binding activity (4). This suggests that expression of a single 10 kDa domain of potato multicystatin (PC) may yield an active and stable cysteine protease inhibitor similar to the cystatins found in other plant species which normally contain only a single domain (5,6).

For insect bioassay studies, the use of purified recombinant protein is preferred for the higher degree of accuracy in predicting the gene activity in transgenic plants. Yeasts are suitable hosts for production of heterologous proteins as they can perform post-translational modifications, folding and processing of eukaryotic proteins. The methylotrophic yeast *P. pastoris* has in the past decade increasingly been used for this purpose due to its ability to generate high yields of foreign proteins (7-8).

To obtain enough material of pure protein for insect feeding trials we expressed the eight and the single domain cystatins in the yeast Pichia pastoris. To investigate the functionality of the obtained proteins we have performed inhibition studies towards papain. Our study shows that a functional single domain of PC can be expressed in *Pichia pastoris* at high levels.

Materials and Methods

Oligonucleotides

All oligonucleotides were obtained from Eurogentec (Seraing, Belgium)

```
PMC-DN:   CCCCCATGGCATACGTAAAATTATTCGCAGTGATGGCAATCGTA
PMC-UP:   CCCCCCGCGGCCGCCACACCAACATAAAAGTAGTTTC
PC-DN:    GTATCTCTCGAGAAAAGAGAGAGGCTGAAGCTGCAATCGTAGGGGGC
PC-UP:    CCCATGCGGCCGCCTACTTTGTAGCATCACCAACAAGTTTAAATTCTTG
```

Cloning the PMC and PC cDNAs

Total RNA was isolated from fresh potato peels (cv. Superior) according to the hot phenol method (9). Subsequently mRNA was purified from the total and first strand cDNA was synthesised by using a PolyA-Tract Series 9600 mRNA Isolation and cDNA Synthesis System Kit (Promega). The protocol for the above kit was modified by extending the duration of first strand synthesis at 37°C from 30 minutes to two hours in order to ensure the synthesis of long length cDNA (~2.3 kb for PMC). The published genomic DNA sequence of the PMC gene (10) was used to design primers for PCR. The prepared first strand cDNA was treated with a cocktail of RNases at 37°C for 1 hour and the reaction was terminated by holding the mixture at 95°C for five minutes. A fraction of 2-4 µl of this mixture was taken as a template for the PCR reaction using Advantage Genomic PCR Kit (Clontech). The following cycling parameters were used 94°C: 1 min., 60°C: 30 sec., 72°C: 4 min., for 31 cycles with an additional extension time of 20 minutes at 72°C. To prevent annealing to the highly homologous repeated internal cystatin domains of the PMC-gene, the primers PMC-DN and PMC-UP were designed using the non-coding regions of the PMC-gene (10) as well for better specificity (Fig. 1). On the basis of the published PMC gene information, primers PC-DN and PC-UP were designed to clone only the last domain of the PMC gene of 303 bp. The 303 bp fragment was amplified using the conditions mentioned earlier, except that the time for extension was reduced to one minute instead of four minutes. The PMC cDNA fragment was cloned directly into the *Pichia* expression vector pPIC9 by digesting the vector and the 2276 bp PCR product with *Not*I and *Sna*BI, ligating them overnight at 14°C and transforming the ligated mixture into *E. coli* XL1 blue. The PC cDNA fragment was first A-tailed using standard methods (11), cloned into pGEM-T and sequenced. Subsequently, the PC cDNA and the pPIC9 vector were digested with *Xho*I and *Not*I, ligated and transformed. The PMC and PC pPIC9 vectors were linearized with *Sal*I and used to electroporate to competent GS115 cells of *Pichia pastoris* (Invitrogen). Histidine positive transformants were selected on minimal MD (minimal dextrose) plates.

Expression of PMC and PC

Ten single colonies per construct were inoculated in 10 ml of BMG medium (Buffered Minimal Glycerol, 100mM Potassium phosphate pH 6.0, 1.34% YNB, 450 µg/L biotin and 1% glycerol). Cultures were grown overnight at 30 °C on a plate agitator at 250 rpm and the OD_{600} was checked after 16 hours of culturing. The ten clones had an OD_{600} of 1.28 to 1.50. Cells were pelleted (3500 rpm for 5 min) and re-suspended to OD_{600} = 1.0 in 10 ml BMMY (Buffered Methanol-complex Medium, 1% yeast extract, 2 % peptone, 100 mM Potassium phosphate, pH 6.0, 1. 34 % YNB, 4 x 10^{-5} % biotin and 0.5% methanol.) and cultured for 6 days. Every 24 hours methanol (100%) was freshly added to a final concentration of 0.5%. At the end of day 6 the PMC cultures only were dialysed against pH 5 at 4°C, overnight to dissolve the crystals formed if any. Expression levels were determined by immunoblot using 50 µl of supernatant with 150 µl blotting buffer (12).

Screening for high expressor and protein molecule size

A dotblot was prepared with 50 µl of supernatant in 100 µl loading buffer (100 mM CAPS + 1% SDS) per well on the Bio Rad Trans Blot Nitro-cellulose membrane (0.2 µm). The membrane was used for a dotblot western with the primary antibody being rabbit IgG raised against Gluthation S-Transferase-PC fusion protein. The secondary antibody was sheep-anti-rabbit IgG (H+L) conjugated to horseradish peroxidase (Jackson Immuno Research, USA). Purified potato multicystatin from tubers of known concentration, was used for the reference curve in the immuno dotblot. The highest expresser identified by dotblot was checked for size, by running on an SDS gel, blotting and following western blot procedures (Fig. 4). The highest expressing clone was used for scaling up of the culture.

Scaling up of the expression of the PC and the 8 domain PMC

Scaling up expression of PMC and PC was taken up in a BioFlo 3000 bench-top fermentor (New Brunswick Scientific Co. Inc., USA). Methanol concentration in the medium was monitored by a methanol concentration monitor and controller, MC168 (PTI Instruments, Inc., USA) under software control of NB208 Windows while fermentation itself was monitored by AFS-BioCommand based software (New Brunswick Scientific Co. Inc.). The inoculum was grown overnight in a shake flask containing 150 ml of rich medium with glycerol. The fermentation vessel, containing 1.5 l of basal salts medium was brought to the set temperature of 30°C and the pH of 5.0 (13). The dissolved oxygen set point was 30%. The medium in the vessel was inoculated with the entire o/n culture and the process was run as presented earlier (13), and stopped after 70h, 239 OD_{600} and PC was purified from the medium as stated below.

PMC was also tried for scaling up with conditions as mentioned above and in addition a fermentation was tested at pH 3.

FPLC purification of PC

The cultures were centrifuged at 4°C, 3500*g* and the supernatant was sterilised by passing through a 0.45 µm filter. The filtered supernatant was diluted 1:2 with equilibration buffer (20mM citrate pH 2.8) for ion exchange chromatography and the pH was reduced to 2.8.

The filtrate (500 ml of 1:2 diluted) was loaded on a 5 ml SP HI-trap column (AP Biotech), previously equilibrated with the same buffer, at a flow rate of 1 ml/min. The column was then washed with five volumes of equilibration buffer. The adsorbed PC protein was eluted with a linear 0-1 M NaCl gradient in equilibration buffer. The column float trough was monitored at 280 nm. A peak was observed at approx. 400 mM NaCl, pooled (only from the peak) and sterilised by passing on a 0.22 µm filter.

Determination of the apparent equilibrium dissociation constant

The apparent equilibrium dissociation constant (K_i) of PC for papain was determined as follows. PC (0-34 µl) was added to 130-96 µl of assay buffer (50 mM MES pH 6.5 with 5 mM cysteine and 0.1 mg/ml BSA fraction V) and 20 µl of 150 nM papain (2x Crystallised, Sigma, USA, titrated with E-64) to a final volume of 150 µl. After 30 min pre-incubation at room temperature the amount of uncomplexed papain was measured by adding 50 µl of substrate (0.9 mg/ml Z-Phe-Arg-pNA (Bachem, Switzerland) in methanol diluted 20x in assaybuffer) and spectrophotometrically recording the change in OD_{405} (14) on a benchtop microtiterplate reader (BioRad). The residual protease activity was plotted vs. the volume of the inhibitor. The data sets were fitted to the equation $[I^\circ] = (1 - a) / a * K_i + [E^\circ] * (1 - a)$, where I° represents the initial concentration of the inhibitor, E° the initial concentration of the enzyme, and a the fraction of the free enzyme (13). A nonlinear regression analysis program was used for determination of K_i and the initial concentration of the inhibitor (Sigmaplot 5.0).

Results and Discussion

Cloning of PMC

The cDNA of the cysteine protease inhibitor PMC from potato, *Solanum tuberosum* cv. Superior, was cloned by PCR into the pPIC9 vector and transformed into yeast *Pichia pastoris* for large scale production of PMC protein with the aim of conducting bioassays with insects. PMC is an interesting protein as it binds to eight papain molecules simultaneously (1). The 2276 bp cDNA fragment of PMC proved to be difficult both to amplify and clone, though the primers were stringently designed to allow only amplification of the entire coding region. Figure 1 shows that as the first two amino acids are unique to the first domain of PMC only the last 6 nucleotides at the 3' end of primer PMC-DN correspond to sequences reccurring in several internal domains of the PMC gene. None of the PMC-UP nucleotides were overlapping with the other domains. Nevertheless, despite the use of hot starts, the PCR reaction always generated a ladder of products which was a multiple of 300 nucleotides (300, 600, 900, 1200 etc.). This may be explained by priming of incomplete amplification products on internal domains which could not be avoided by the prolonged extension times. The full length product was cut out of gel and transformed to several different standard cloning vectors. The cloning of the 2.3 kb PMC cDNA fragment, however, always resulted in a small 303 bp product in all the vectors tested (pGEM-T, pSK^+ and pPCRSCRIPT)). When sequenced, the 303 bp product was identified as a recombination product of domain 1 and 8. This problem in cloning the full length gene is probably due to a combination of high toxicity and high homology of the 8 domains, because the same gene with introns could be cloned without a problem in these same vectors. Apparently, a strong selection to reduce the toxicity resulted in recombination

despite the use of RecA⁻ *E.coli* strains like XL1-blue. It is unclear why the non-directional cloning procedures with the pGEM-T vector still resulted in the same phenomena, considering that the 50% chance for an inverse orientation in this vector would not be expected to result in a toxic product. Also, the addition of 2% glucose to the selective medium (as a repressor of Lac promoter activity), (15) did not result in the cloning of the entire fragment. In the end, the direct cloning into the yeast expression vector, pPIC9, in the same *E.coli* background reduced the recombination in this vector to a great extent. Plasmid isolations from single colonies would typically yield a mix of plasmids containing both 8-domain and 4 domain genes. Apparently, the recombination occurred during the growth of the colony and subsequent culturing, but in a less frequent and drastic manner in this vector background.

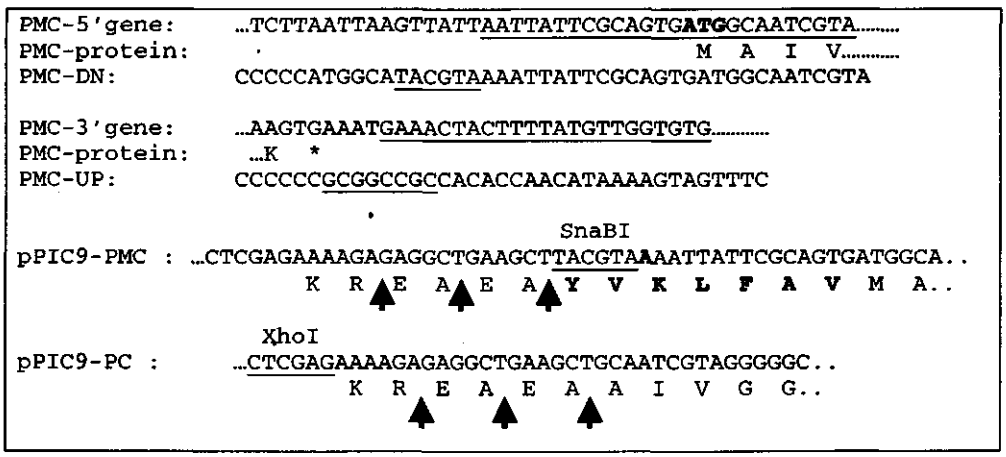

Fig. 1. Diagram depicting the original PMC gene (5'and 3' ends) and the way the PMC and PC coding regions were cloned into pPIC9. The primers PMC-DN and -UP were designed to avoid annealing to all 8 homologous domains by making use of non-coding 5'- and 3'- regions of the gene. This led to 7 additional amino acids (in bold) on the N-terminal after removal of the MF-alpha secretion signal and the Glu-Ala repeat by Kex2 and Ste13 peptidases, respectively (arrows). The nucleotides underlined in the 5' and 3 ' gene sequence indicate the primers (PMC-DN and PMC-UP) used for cloning, while the underlined sequences in the primers are the restriction sites used for cloning.

Cloning of PC

A 309 bp PC cDNA fragment obtained by PCR was cloned into pGEM-T. The sequence of this cDNA fragment is presented in Fig. 2. The sequence of PC did not show complete identity to any of the eight domains of PMC (Fig. 3). The PC cDNA fragment that was cloned is, therefore, expected to be a domain of one of the other 4 to 6 cystatin genes in potato (8). PC was expressed in *Pichia pastoris* by cloning the 309 bp PCR product into the secretion vector pPIC9.

```
      K R E A E A A I V G G I I N V P F P N N P E
GAGCTCAAAAGAGAGGCTGAAGCTGCAATCGTAGGGGGCATTATCAATGTTCCATTCCCAAACAACCCCGAG
 F Q D L A C F A V Q D Y N K K E N A H L E F V E
TTCCAAGATCTTGCTTGTTTTGCTGTTCAAGATTATAATAAGAAAGAGAATGCTCATTTGGAGTTTGTAGAA
 N L N V K E Q V V A G M M Y Y I T L V A T D A
AATTTGAATGTGAAAGAACAAGTTGTTGCTGGAATGATGTACTATATAACACTTGTGGCAACTGATGCT
 R K K E I Y E T K I L M K E W E N F K E V Q E
AGAAAGAAGGAAATATATGAGACCAAGATTTTGATGAAGGAATGGGAGAATTTCAAGGAAGTTCAAGAA
 F K L V G D A T K  *
TTCAAGCTTGTTGGTGATGCTACAAAGTAGGCGGCCGC
```

Fig. 2. The 303 bp sequence of the PC cDNA cloned by PCR from potato cv. Superior. The primers PC-DN and PC-UP are underlined. In bold at the N-terminus are 6 amino acids of the MF-alpha secretion signal removed during secretion into the medium, and therefore, not present in the purified PC.

```
AIVGGIINVPFPYNPEFQDLVCFAVQDYNKKENAHLEFVENLNV    (PC 1 to 44)
-----LVD---ENKV--D--AR-------Q-NDSS---KKV---     (DOMAIN 1A)
TMP---V---N-N-E-AR-I------Q-----------------     (DOMAIN 2A)
-KL---TD----N--------AR-I-V------V-----------     (DOMAIN 3A)
-KT-------N-NS------AR--------NTQ-------------    (DOMAIN 4A)
KKL--FTE----NS------TR---HQ---DQ--------------    (DOMAIN 5A)
-KL----------N-------AR-----------------------    (DOMAIN 6A)
--I--FTD----N-------AR---------------Y-------    (DOMAIN 7A)
-KP----I----NS------AR-----F-----G----------     (DOMAIN 8A)

KEQVVAGMMYYITLVATDARKKEIYETIKLVKEWENFKEVQEFKL (PC 45 to 89)
-Q-I---I-----FE--EGGN-KE--A---LRK--DL-KVVG---    (DOMAIN 1B)
-------I------A---DAG-KKIYKAKI-----D--K-V----    (DOMAIN 2B)
-Q-------------A-I--G--K------W-----D--K-V----    (DOMAIN 3B)
---L-S--------A----GN-KE--A--W-----D--K-ID--L    (DOMAIN 4B)
-K------L----FA---GG--K------W--V-----K-V----    (DOMAIN 5B)
---L----L-------I--G--K---A--N--------K-I----    (DOMAIN 6B)
---L----I----------G--K---A--N-----D--K-V----    (DOMAIN 7B)
---------------A-------------------------------  (DOAMIN 8B)
```

Fig. 3. Alignment of the 8 domains of PMC at the amino acid level indicating a minor variation between the 8 domains. None of the 8 domains are identical to the cloned domain of PC but greatest homology is with the last domain 8 of PMC. The first 44 amino acids here are designated with A and the last 45 amino acids with B.

Expression of recombinant PC and PMC in Pichia pastoris

Ten different colonies of PMC and PC were grown overnight and induced with methanol for expression. This resulted in stable and biologically active protein in the case of PC. Between clones there was no variation in the expression level of PC. Out of the 10 clones tested 7 expressed PC at a similar level and clone-3 was chosen for scaling up. For PMC variable expression levels of PMC were observed among the twelve positive clones tested. With dot blots it was determined that 2 were high, 5 were medium and 3 were low expressors, while 2 clones had no detectable expression. The PMC produced in all yeast cultures was similar in size (85 kD) to PMC extracted from potato tuber peel (Fig. 4),

indicating that in yeast unlike in *E.coli,* the gene was stably expressed and maintained in the selected yeast cultures.

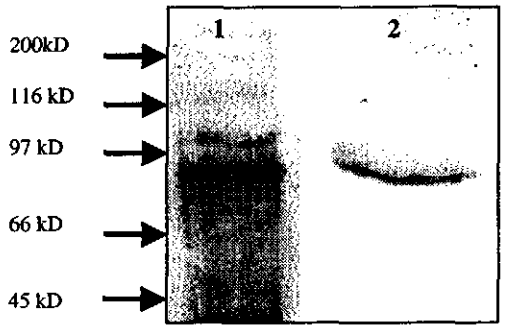

Fig 4. The protein size marker and the size, as represented in the figure with arrows are indicated. Lane 1 is 20 ng of purified PMC from potato peel (cv. Superior) in 50 mM sodium phosphate at pH 6, denatured with SDS and than loaded onto a 10% SDS-PAGE gel. There are actually 2 bands very close to each other indicating 2 gene products. Lane 2 is 50 µl of Pichia supernatant obtained after acidification overnight and spinning down from 10 ml cultures. The primary antibody was Cystatin fused to GST raised in rabbit. The secondary antibody was HRP conjugated to sheep anti-rabbit.

Scaling up of PMC and PC in P. pastoris

Several media and pH conditions in the fermentor were tested for scaling up of the selected PMC high expressing clone. However, production of high levels of secreted PMC sufficient for purification could not be achieved. The amount of PMC present in the pellet was several fold higher than in the supernatant in the scale up cultures indicating problems in secretion of the large PMC protein. Though cellular expression in the cytoplasm could be an alternative, it was not tried in this investigation. The expression may also have been affected by the presence of the *Sna*BI site used for cloning the gene. Recently, it was found that the SnaBI site is identical to an mRNA efficiency element and may cause strong 10-fold reductions of gene expression in Pichia pastoris using the pPIC9 vector (16).

Production of PC was done in 1.5 l fermentation medium in an oxygenated bench top fermentor. The supernatant was purified by cation exchange using an FPLC (Fig. 5) set up from which finally 160 mg of PC was recovered at a concentration of 0.2 mg/ml. By titrating recombinant PC against the cysteine protease papain, its K_i (apparent equilibrium dissociation constant) was found to be 0.1 nM (Fig. 6).

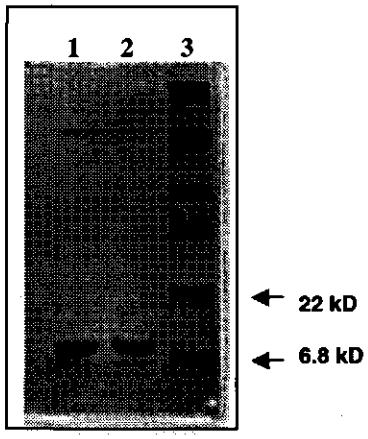

Fig.5. In lane 1, 2 µl of PC from 60 ml (0.5 mg/ml), from a second repeated purification is loaded. In lane 2, 2 µl from 300 ml (0.4 mg/ml), from the first purification with SP Sepharose high performance is loaded. The protein size marker is loaded in lane 3 and the relevant sizes are indicated with arrows.

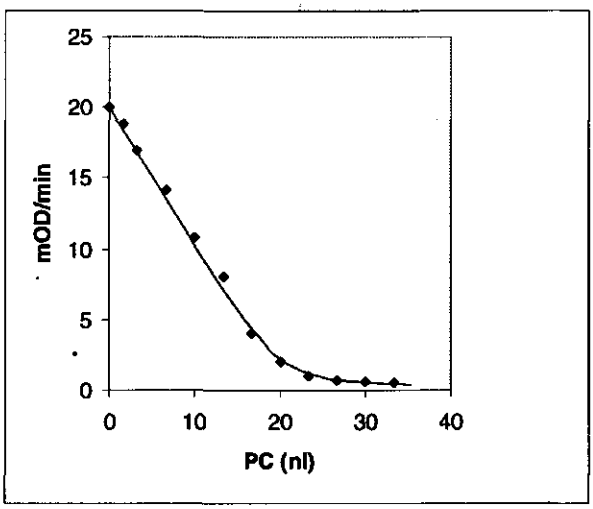

Fig. 6 Graph showing the titration of papain (64 ng) with recombinant PC (nanolitre). Papain was previously titrated with E-64 to determine the active concentration.

Conclusion

This is the first report on the production of a plant cystatin in *Pichia pastoris*. Phytocystatins from rice, chestnut, papaya and corn have so far been exclusively produced in *E.coli* (20, 21, 22). It is shown that yeast provides a good alternative expression system for these inhibitors. Expression levels were rather low with the PMC gene, but the gene could at least be maintained in the yeast background unlike the case for *E.coli*. The efficient

production of PC will enable the further study of this protein for its potential effects against proteases from plants and insects.

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Chapter- 7

Cysteine proteinase inhibitors reduce fecundity in western flower thrips; *Frankliniella occidentalis*"

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Submitted to Journal of Insect Physiology

Abstract

Proteolytic activity in whole insect extracts of the western flower thrips, *Frankliniella occidentalis*, was found to belong predominantly to the class of cysteine proteases. The pH optimum of the general proteolytic activity was determined to be 3.5, which is low when compared to other insects using cysteine proteases for protein digestion. The proteinaceous cysteine protease inhibitors chicken cystatin, potato cystatin and sea anemone equistatin inhibited more than 90% of *in vitro* protease activity. To test *in vivo* the biological effect of such inhibitors on the oviposition rate of western flower thrips, recombinant potato cystatin and equistatin were fed to adult females. A gradual reduction in oviposition rate to about 45% was observed when reared on these PIs for a period of 5 days, with no increase in mortality. These results are discussed in the light of the application of protease inhibitors in transgenic plants to control this insect pest.

Introduction

The Western flower thrips (WFT), *Frankliniella occidentalis* (Thysanoptera) is the most prevalent insect pest in greenhouses on vegetable and ornamental crops since 1983 in the Netherlands (Mantel and De Vrie, 1988). WFT is highly polyphagous as it is reported to live on at least 240 species of 62 different families of plants (Loomans *et al.*, 1995). Economic damage is not limited to greenhouses as WFT also cause yield loss in field crops like onion, cotton, chilli, and capsicum; in fruit crops like citrus, apple, and strawberry; in plantation crops like tea and in many more. The small size and the secretive (thigmotactic) habit of hiding in the inner whorls of flowers and buds makes chemical control of WFT arduous. 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). Several protocols exist for testing WFT resistance on plants or plant tissues, like: measurement of (silver) damage, population dynamics (Dijken *et al.*, 1995), reproduction (Soria & Mollema 1995) and egg production (Brouwer *et al.*, 1996). Bioassays to test pure proteins and compounds are not available. Hence, we modified the assay developed by (Brouwer *et al.*, 1996), and tested metabolic inhibitors like protease inhibitors (PIs) on oviposition rate of WFT. Oviposition rate is a suitable parameter to assess metabolic inhibitors like protease inhibitors (PIs) on adults because this process relies heavily on the conversion of plant protein into yolk protein.

Proteases are comprised of 4 dominant classes (cysteine; serine; aspartic and metallo proteases) based on the amino acid or metal ion involved in the catalytic hydrolysis of peptide bonds. The classes of proteases found in the gut and stomach of insects varies strongly between species. Proteases are usually inhibited by proteinaceous PIs through tight reversible binding to the substrate binding site itself or to subsites, in both cases causing sterical hindrance for the enzymatic conversion of normal peptide substrates. PIs are named after the protease they inhibit, and, hence, there are serine PIs, cysteine PIs and so on. Effective inhibition may require multiple PIs to act on all major gut proteases. Ingestion of effective PIs may result in stunted growth, lowered oviposition rate and increased mortality of insects due to lowered release of amino acids from food protein (Jongsma and Bolter, 1997). However, insects may also overcome the effect of PIs by proteolytic inactivation of

PIs (Michaud, 1997; Girard *et al.*, 1998b) or by the induction of an inhibitor-insensitive complement of proteases (Jongsma and Bolter, 1997).

Our interest was in identifying PIs antagonistic to WFT. The characterisation of the dominant gut proteases and the pH optima for their activity would allow the selection of effective PIs against the gut proteases. This study describes the partial characterisation of WFT proteolytic activity and identification of PIs active *in vitro*. Specific cysteine PIs were tested *in vivo* against adult female WFT to observe effects on oviposition rate. The response of WFT to ingestion of PIs and the potential of expressing cysteine PIs in plants to manage this major insect pest is discussed.

Materials and Methods

Abbreviations

$CdCl_2$, Cadmium chloride; E-64, trans-epoxy succinyl-L-leucylamido-(4-guanidino)-butane; PI, protease inhibitor; PI1, (Calbiochem), potato proteinase inhibitor 1; PI2 potato proteinase inhibitor 2; PMSF, phenylmethylsulfonyl fluoride; SBBI, soybean Browman Birk inhibitor; STI, soybean trypsin inhibitor; PC, potato cystatin; EI, Equistatin; WFT, western flower thrips; FITC, fluorescein isothiocyanate.

Materials

Unless otherwise noted all chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo).

Characterisation of WFT proteases.

Adult WFT feeding on chrysanthemum (*Dendranthema grandiflora*) cv. Sunny Casa were collected from the rearing chamber. The small size of WFT did not permit the separation of the guts from the carcass. Hence, whole insects were homogenised in a plastic potter fitting an Eppendorf tube containing 300 µl water. The pH optimum for the proteolytic activity in the total WFT extract was determined exactly as described by Jongsma *et al.*, (1996) using [^{14}C] methemoglobin (NEN, Du Pont de Nemours) (Fig. 1). For inhibition assays, 200 µl of this extract was diluted with 800 µl of 200 mM β-alanine-HCL pH 3.5 buffer. The extract was tested against the inhibitors (Table 1) as described by Jongsma *et al.*, (1996).

A second series of inhibition assays was carried out using FITC-labeled methemoglobin as described by Twining (1984). Potato cystatin (PC, Annadana *et al.*, in review), equistatin (EI, Rogelj *et al.*, 2000) and PI2 (Beekwilder *et al.*, 2000) were recombinantly produced while chicken cystatin (Zerovnik, *et al.*, 1990) was purified from native source.

Bioassay for testing oviposition rate in WFT

Bioassay cages were prepared from transparent perspex tubes 3 cm in length with a 3.5 cm diameter. The bottom of the tube was closed with nylon gauze of 120 µm mesh size. The top was sealed with 2 sheets of stretched parafilm, with a liquid water sandwich of 300 µl in between. The effect of the pure proteins (PIs), was tested in a bioassay using these

bioassay cages by using protein solutions in the liquid sandwich. The experiment was conducted with six replications for all PI treatments and with nine replications for the control. The cages with 20 adult female WFT were incubated in climate controlled chambers maintained at 25°C temperature, 60 % relative humidity and 16 hours of light. Pollen of mixed plant origins (health product from grocery store), compacted into grains of 1-2 mm diameter, were used as a food source for WFT in our bioassay. Moisture is critical to rehydrate the otherwise dry pollen grains. Only rehydrated pollen have the correct physical consistency for WFT feeding. Hence, bioassay cages were placed gauze side down in a tray, on top of holes of $1cm^2$, permitting the diffusion of moisture. A piece of parafilm with 4 pollen grains on top was placed under the gauze side of the cage (next to the hole). In this way the pollen were directly accessible to the WFT through the gauze and easily replaced without disturbing the insects. PIs were diluted into diluted FPLC buffer (1.5 mM Na-citrate 36 mM NaCl, pH 5), which also served as the control solution. All eggs were deposited inside the PI/FPLC buffer sandwich. On a daily basis eggs were counted and adult mortality was scored. Subsequently, the sandwich and pollen were refreshed daily after CO_2 anaesthesis of the insects.

Results

Characterisation of WFT proteases

A pH profile of protease activity was tested in a range of pH 2 to 12. The protease activity in extracts of total adult WFT was found to be maximal at pH 3.5 with very low activity levels at pH values greater than 5 (Fig. 1). Proteases of adult females when analysed separately from the mixture of males and females had a similar pH optimum (data not shown). At pH 3.5 the protease activity was characterised with non-proteinaceous inhibitors of cysteine, serine, metallo and aspartic protease inhibitors respectively (Table 1). Greater than 80% inhibition was observed with E-64, chymostatin and leupeptin, but less than 10% inhibition with benzamidin, PI1, SBBI and STI suggesting a predominance of cysteine proteases in total WFT extracts. Elastatinal and potato carboxypeptidase inhibitor, a serine and a metallo protease inhibitor respectively showed 20 % inhibition of proteolytic activity.

Four proteinaceous PIs and one chemical inhibitor (E64) were subsequently tested for their inhibition of the total WFT proteolytic activity, using FITC-labeled methemoglobin in a fluorescence assay. The cysteine PIs chicken cystatin, E-64, PC and EI showed inhibition in the range of 90-96 % whereas PI2, a serine PI showed 20 % inhibition of total WFT proteolytic activity (Fig. 2).

Bioassay to test effect on recombinant PC and EI on oviposition rate

In total more than six bioassays were conducted to study the effect of PC and EI on the oviposition rate of WFT. Results of one such bioassay is presented, which had similar results as was observed in the other bioassays conducted. The bioassay was performed in three phases, first three days of adaptation on control diet (phase I), followed by five days of exposure to PIs (phase II) and finally three days to recover from exposure to PIs by returning to control diet (phase III). The average number of eggs laid in the control on day -1 and 0 of phase I was 2.50 ± 0.18 eggs/female. The average number of eggs by each

bioassay cage during day -1 and 0 of phase I was taken as 100 % against which egg counts of phase II and III were compared for all treatments.

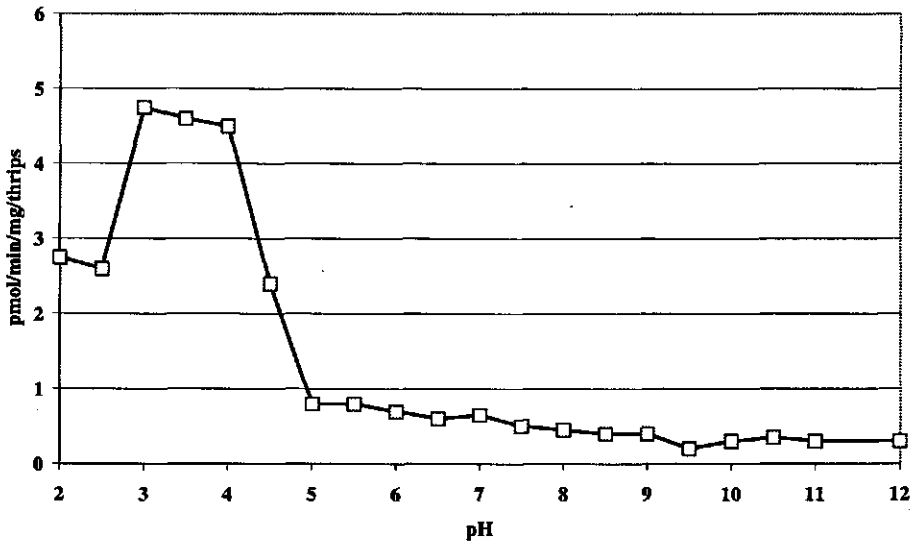

Fig. 1 pH profile of the proteolytic activity of whole WFT extract assayed using [^{14}C]methemoglobin as a substrate.

TABLE 1. Effect of inhibitors on [^{14}C]methemoglobin hydrolysis by proteases in total extract of *Frankliniella occidentalis*

Inhibited class	Inhibitor*	Concentration (μM)	Crude extract (% control)
Serine proteases	Aprotinin	5	89
	Benzamidin	1	92
	Elastatinal	5	80
	PMSF	5000	87
	PI1	6	93
	SBBI	12	95
	STI	2.5	90
Serine/cysteine proteases	Antipain	100	29
	Chymostatin	100	13
	Leupeptin	1000	29
Cysteine proteases	E-64	10	13
Aspartic proteases	Pepstatin	10	84
Metallo proteases	Potato carboxypeptidase inhibitor	5	80
	$CdCl_2$	5000	89

* See methods for abbreviations

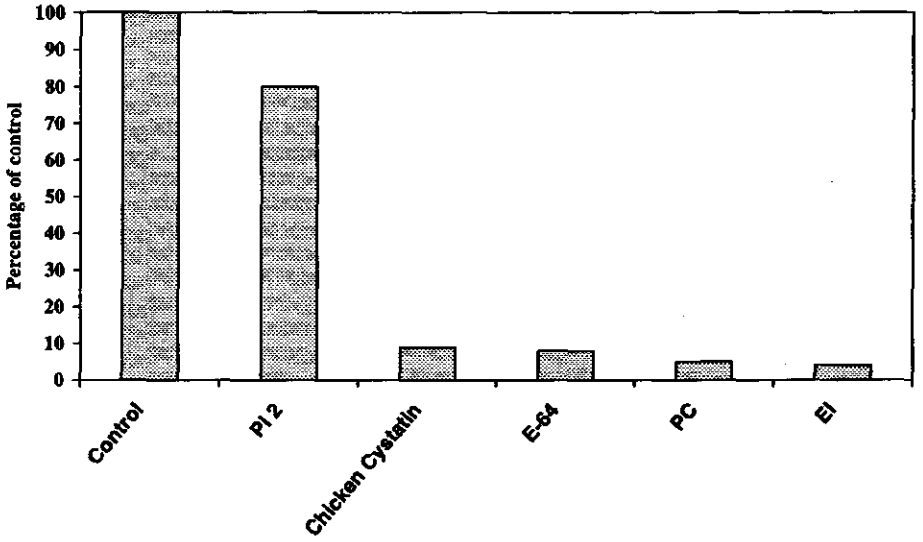

Fig. 2 Inhibition of WFT proteolytic activity by proteinaceous PIs. Whole WFT extracts were assayed for protease activity using FITC-labeled methemoglobin as a substrate.

During phase I when all cages were on control diet there was no significant change between the cages in oviposition rate on day -1 and zero, indicating good adaptation of the WFT to the conditions of the bioassay.

Two recombinant PIs, PC and EI were used in phase II of the bioassay at 30 μM concentration (PC = 300 ppm, and EI = 600 ppm) to assess the effects of PI ingestion on oviposition rate of WFT. On day 1 a drastic reduction in oviposition rate, was observed for all three PI treatments (Fig. 3). PC resulted in 38 %, in EI 28 % and PC+EI in 45 % reduction in oviposition rate respectively. All treatments recovered on the second day of phase II resulting in 27.5 %, 17 % and 21 % of reduction in oviposition rates respectively (Fig. 3). During the remaining three days a steady downward trend was observed which possibly would have continued if the inhibitor diet would not have been replaced by control diet. On day 5 the last day of phase II PC, EI and PC+EI had oviposition rates of 51 %, 66 % and 52 % relative to their original day 0 rates. Relative to the control on day 5, oviposition rates were 44%, 57% and 45% respectively (Fig. 3, Table 2).

In phase III (days 6-8) the insects were returned to control diet of pollen and buffer. The oviposition rate was found to increase at a similar rate at which the decrease was observed in phase II (Fig. 3).

The relative egg count and LSD at the end of phase I, II and III are presented in Table 2. ANOVA clearly indicates the significance of PIs feeding and reduction in oviposition rate over control at end of phase II (***) and phase III (**). PC appeared to be more effective than EI and there was no synergistic effect with PC+EI at 30 μM.

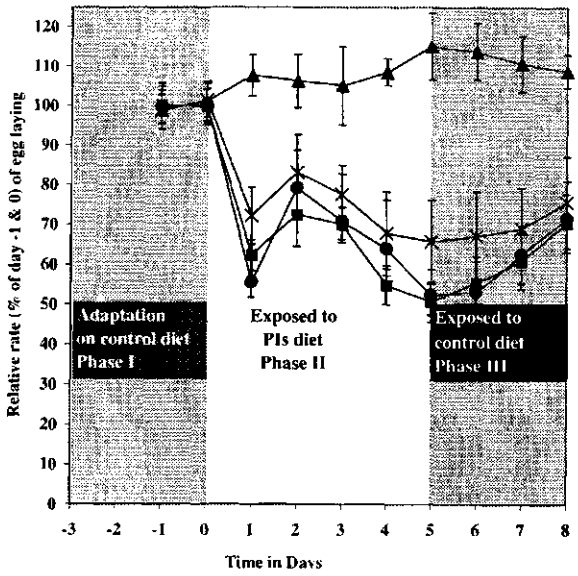

Fig. 3 Influence of dietary PIs on oviposition rate rates of adult WFT. Graph indicates the oviposition rate rates of WFT reared on pollen grains as food source exposed to 30µM PC (■), 30µM EI (×), and a combination of both PC+EI at 30µM each (●) vs. a control CON (▲). The points of the graph are generated based on average of 6 values for the treatments and an average of 9 values for CON. All values are represented as a percent of the average number of eggs laid on day -1 and 0 with ± standard error bars. The 3 phases viz.; adaptation, exposure to PIs and recovery are indicated on the graph with phase I and III are shaded.

TABLE 2. The effect of PIs on oviposition rates in WFT.

Treatment	Concentration	Day 0 % [1]	Day 5 % [1]	Day 8 % [1]	Day 8 % survival [2]
-	-	101^{a} (100)	115^{a} (100)	109^{a} (100)	94^{a}
PC	30 µM	99^{a} (98)	51^{b} (44)	70^{b} (64)	96^{a}
EI	30 µM	101^{a} (100)	66^{b} (57)	76^{b} (70)	97^{a}
PC + EI	30 µM + 30 µM	100^{a} (99)	52^{b} (45)	71^{b} (65)	97^{a}

[1] The relative number of eggs for days 0 (end of phase I, adaptation to control diet), 5 (end of phase II, exposure to PI diet) and 8 (end of phase III, recovery on control diet) are presented as a % of Day –1 and 0 for PI treatments with 6 replications and control with 9 replications. Between brackets is the percentage relative to the control of that same day (corrects for interday variation). The LSD for days 0, 5 and 8 are 17 %, 33 % and 28 % respectively The treatments are grouped based on significance with values denoted with different letters differing significantly.

2 The survival is presented as a % of the number alive on day -1.

Discussion

The objective of this investigation was to evaluate the potential of PIs to control WFT. First, the predominant group of proteases in whole WFT extract was determined, with the assumption that in such an extract the gut proteases will dominate. The dominant group of proteases were of the cysteine class with an activity optimum of pH 3.5. Cysteine PIs in bioassays were found to reduce the oviposition rate of WFT by more than 50% in five days.

There is only one earlier study conducted by Thomas *et al.*, (1994), which reported effects of PIs on WFT. In that study it was found that the expression of a serpin elastase inhibitor from *Manduca sexta*, in transgenic alfalfa resulted in delayed WFT damage. However, there was no study of the characterisation of WFT proteases or the effects of PIs on *in vitro* protease activity or adult oviposition rate.

In this report it is shown that cysteine proteases are predominant in whole WFT extract and that elastase-like activity is minor. The protease activity has a pH optimum of 3.5, which is low, when compared to the pH optimum of gut cysteine proteases of other insects like *Psylloides chrysocephala* with a pH optimum of 6-6.5 (Girard *et al.*, 1998a), and *Phaedon cochleariae* and *Ceutorhynchus assimilis* with a pH optimum of 5 (Girard *et al.*, 1998b). Along with cysteine proteases as a dominant class of proteases, some insects like *Leptinotarsa decemlineata* (Wolfson and Murdock, 1987; Gruden *et al.*, 1998) and *Diabrotica undecimpunctata* (Edmonds *et al.*, 1996) in addition have aspartic proteases. In WFT the aspartic protease inhibitor pepstatin showed 16% inhibition of proteolytic activity in total extract. This suggests that in WFT the contribution of aspartic proteases to total gut proteolytic activity may be limited despite the low pH optimum. The inhibition by elastase inhibitors like elastatinal and PI2 is unexpected, because at pH 3.5 the serine proteases would be expected to be inactive, while at alkaline pH no secondary peak of activity is observed.

WFT were subsequently fed with proteinaceous PIs at concentrations of 30 μM (0.03-0.06%), which were in the range of concentrations that can be found in plant leaves. The exposure to PC and PC+EI for a limited period of five days resulted in a 55 % reduction in oviposition rate relative to the control. This level of reduction might have gone down further if the exposure to PIs were extended. A reduction in oviposition rate by more than 50 % can have important effects on the exponentially growing population of WFT in plant cultivations. Yano *et al.*, (1989) reported a 75 % reduction in whitefly population on tomato, as a result of a 50 % reduction in oviposition rate, in a simulation model over a 80-day period. A mathematical model taking into account the relevant life history parameters of WFT on chrysanthemum in greenhouses (18 day egg-egg, 3 eggs/female) predicts 92% reduction in the density of the population relative to control, at the end of a ninety day period, as a consequence of 50 % reduction in oviposition rate. The reduction in the density is enhanced by 6%, from 92% to 98% if 50% reduction in ovisposition rate coupled with 50 % delay in larval development (Jongsma, unpublished data).

Kirk reported that egg laying in thrips species in general comes to a complete stop within 2 to 3 days after being deprived of adequate nutrition, (Kirk, 1985). In our case only the production of free amino acids from food protein was blocked. This resulted in an increasing reduction of the oviposition rate by more than 50% after 5 days, without affecting adult mortality. The absence of effects on adult mortality is expected because the adults have a very low protein requirement beyond what they need for the production of eggs. The relatively slow effect on oviposition rate suggests, that there was probably still some background protease activity providing dietary protein. The steady decrease suggests that this activity can only support a relatively low oviposition rate for which we did not yet discover the bottom level. The slow rate of recovery in oviposition in phase III, suggests that the insects need to restore their reserves first before they can produce eggs at the original rate. Thus, we conclude that our results suggest that PIs may more strongly affect

oviposition rates if the exposure to PIs is extended beyond 5 days and that additional inhibitors like elastase or carboxypeptidase inhibitors may be required to more effectively inhibit the gut activity.

Similar to our results with PC and EI on WFT, Spates & Harris (1994) also showed reduction in oviposition rate in *Stomoxys calcitrans* upon feeding with PIs. In this case serine protease inhibitor, soybean trypsin inhibitor at 45 µM concentration (1000 ppm) reduced oviposition rate by over 70 %. Interestingly, they also showed reduced egg hatch, which is functionally equivalent to a further lowered production of eggs, but which we did not investigate. The larger part of the literature on the effects of PIs on insects actually concentrates on the effects on the development of larvae rather than on oviposition and hatching rates of adults and eggs. This follows from the fact that unlike the adult, the larva requires protein for its own growth and development. PIs have thus been reported to cause delay in larval development, to cause stunted growth and to increase larval mortality (Jongsma & Bolter, 1997). If we expect PC and EI to also affect egg hatch, growth, development and survival very strong effects on population development can be expected.

Future investigations on the potential of PIs to control thrips are best carried out using transgenic plants. Transgenic plants will allow a proper assessment of the effectiveness of agents like PIs which are not immediately toxic to the pest insect. Considering the positive results reported by Thomas *et al.* (1994) it will be worthwhile to produce transgenic plants which target both elastase as well as cysteine protease activities in WFT.

Acknowledgement

Mrs. A. Balkema-Boomstra is acknowledged for her assistance in providing batches of reared WFT, organising the growth chambers and running the statistical analysis for our results. The subsidy from EU by project FAIR6-CT98-4239 that contributed to the results is duly acknowledged.

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Chapter- 8

Effects of potato multicystatin expressed in florets of chrysanthemum on western flower thrips *Frankliniella occidentalis*

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Key words: chrysanthemum, florets, genetic engineering, GUS, molecular breeding, transgene expression, ubiquitin extension protein

To be submitted to Acta Horticulturae

Abstract

The gene of the 85 kDa cysteine protease inhibitor (CPI) potato multicystatin (PMC), was PCR-cloned and expressed under the control of the *UEP*1 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 WFT 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

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 find 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.*, 2001a in press). 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 *UEP*1 promoter was cloned, which confers 50-fold higher levels of expression as compared to the CaMV 35S based promoters (Annadana *et al.*, 2001b in press). This *UEP*1 promoter was also identified as the best among several other heterologous promoters tested for expression in petal tissues of chrysanthemum florets (Annadana *et al.*, 2001b, in press).

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 predating on vegetable and ornamental crops since 1983 in greenhouses in The Netherlands (Mantel and De 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 5.2 million guilders of which 2 million guilders 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.*, 2001c, in review). PMC is an 85 kDa CPI that can bind eight papain molecules simultaneously, and is naturally found in the sub-phellogen layer in potato as natural cuboidal crystals (Rodis & 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 *UEP*1 promoter and flowers were tested for their effects on WFT.

Materials and Methods

2.1 Oligonucleotides used for cloning the PMC gene

PMC-DN2: *CCCCCATGGCATACGTAAAATTATTCGCAGTGATGGCAATCGTA*
PMC-UP2: *CCCCCCGCGGCCGCCACACCAACATAAAAGTAGTTTC*

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. The PCR product was cloned into the pGEM-T vector (after A-tailing) and checked by restriction enzyme analysis. The PMC gene was obtained as an *Nco*I-*Not*I fragment and subcloned into pUCAP (van Engelen *et al.*, 1995) which was harbouring the *UEP*1 promoter (Annadana *et al.*, 2001b) as a *Sal*I-*Nco*I fragment. Subsequently the entire fragment containing the *UEP*1 promoter with the PMC gene was

obtained as an *Asc*I-*Sac*I fragment and subcloned into the binary vector pBINPLUS (van Engelen *et al.*, 1995) which harboured the NOS terminator obtained by PCR using primers on pMOG410 (Hood *et al.*, 1993) as *Sac*I-*Eco*RI fragment. This construct was transformed into 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 µl of extraction buffer (50 mM Tris-HCl pH 5, 50mM EDTA, PI cocktail [Complete Mini, Roche]). The tubes were centrifuged at 4° C for 15 minutes, the supernatant was taken to a fresh tube and the total protein was quantified (Bradford, 1976). A protein dotblot (Nitrocellulose membrane 0.2 µm) was prepared with 3 µl of supernatant and 97 µl 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.*, 2001c). 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). 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 bioassay cages made of transparent polystyrene bottles. A hole of 5-cm diameter was made on one side of the bottle and sealed with nylon gauze of 120-µm mesh to permit air circulation. Ten adult females were anaesthetised with CO_2 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.

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 sizes of smaller bands when digested with *Eco*RI and *Hin*dIII. 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 *UEP*1 promoter and *Nopaline synthase* terminator and named p*UEP*1-PMC. In total 35 transgenic plants were generated with p*UEP*1-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 08 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. There were no observable variations in leaf size, leaf colour or leaf shape in any of the 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/µg 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 13 lines plus control (Table 1) of which 3 lines had expression levels of 1.0-units/µg total protein and above, four lines between 0.5 to 1.0 units/ug total protein while the remaining six lines had 0 – 0.4 units/µg total protein expression. In total eleven out of 31 lines were found to have more than 0.1 units/µg 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/µg 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/µg protein) vs. PIA (pmol/µg 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.

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

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

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/µg suggesting that this value represents the average endogenous concentration of inhibitor. The highest expressor at around 2.2 units PMC/µg total protein inhibits papain with 0.28 pmol/µg protein suggesting that PMC contributes at maximum about 0.13 pmol/µg 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.

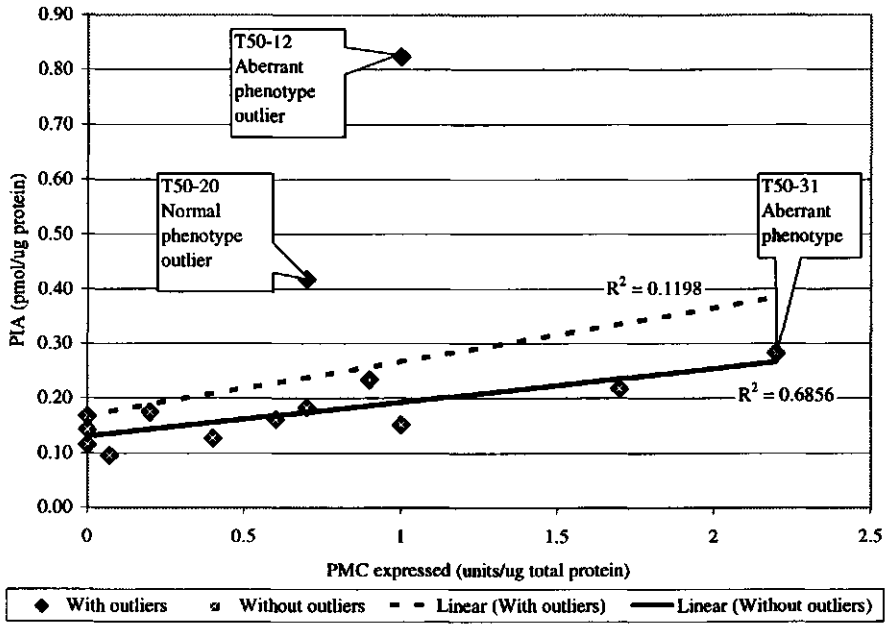

Figure 1 Correlation graph of PMC expression vs the papain inhibitory activity (PIA) in chrysanthemum ray florets. The correlation coefficient R^2 increases from 0.11 to 0.68 when two outliers (T-50-12 & 20) are not considered for the calculation of R^2. Two aberrant phenotypes resulting from somaclonal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

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 expresser with PMC at 1.0-2.2 units/µg protein (T50-12, 15, 17 & 31), three medium expresser with PMC at 0.5-1.0 units/µg protein (T50-3, 19 & 20) and four low expressers with PMC at 0-0.4 units/µg 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 or 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 after 10 days and 174 after 7 days) was taken as an oviposition rate (OR) of 100 %. Table 1 shows all the individual data that were collected on the plants and in Figure 2A and 2B the correlation of the number of offspring with either PMC expression or PIA levels is given. There was clearly no correlation at all between oviposition rate with PMC expression in this experiment. This could be explained by the relatively low level of expression achieved in these plants. The calculated maximum expression of PMC of 0.15% 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 LSD of 34 %, which results in significant differences with the control for only this plant T50-12. The cause of the low reproduction may be related to the high endogenous inhibitor level, but could be equally

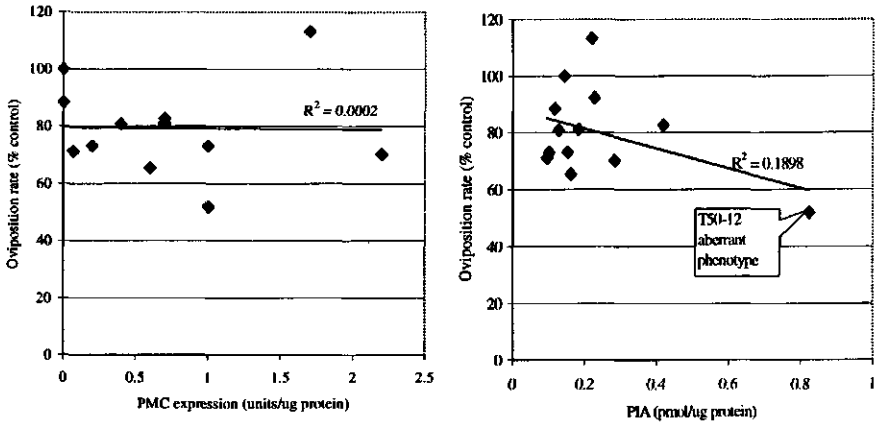

Fig. 2. Correlation graphs of the oviposition rates (OR) versus PMC expression or Papain inhibitor activity (PIA) with the linear correlation coefficients and the R^2 value.

well explained by the aberrant phenotype. The phenotype resulted in more rapid ageing of the flowers and at least one of the four replicates had much lower larval counts than the other three. If this value were excluded than the reductions in OR are in the range of 8-30% of control only.

Discussion

The PMC gene of 3517 bp, coding for an 85 kDa cysteine protease inhibitor protein was cloned and expressed in chrysanthemum under the control of the homologous *UEP*1 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 & 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 *UEP*1 promoter is unfortunate considering that no alternative promoters stronger than the *UEP*1 promoter are currently available for expression of heterologous proteins in florets of chrysanthemum. It may be that the low expression level is associated with properties of the PMC gene, however. Transformation with a different cysteine protease inhibitor gene may circumvent those problems and achieve much higher expression levels using the same *UEP*1 promoter. On the other hand, by rule of thumb the GUS gene with the dCaMV35S promoter expresses

60 pmol/min/µg 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 *UEP*1 promoter however only achieves a GUS expression level of about 8.5 pmol/min/µg protein in petals of chrysanthemum 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/µg 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 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 & Hoff, 1984). In a heterologous expression host like chrysanthemum, this is not certain 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 mouth parts 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., 2001c). 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 pestcide 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 ecologically sound alternative for integrated WFT management.

Acknowledgements

We wish to acknowledge the services of Van Nieuwburg J., and Wiegers G.L., in conducting the bioassays with WFT and Dr. Devendra for the statistical analysis he performed on the data. We also acknowledge the scientific inputs provided by Beekwilder J., Schipper B. and Outchkourov N. during the course of the investigations.

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GENERAL DISCUSSION

Chapter- 9

General Discussion

Aim and introduction

Dendranthema grandiflora or chrysanthemum, as it is better known, is after roses worldwide the second largest cutflower crop. *Frankliniella occidentalis,* commonly known as western flower thrips (WFT) is its most threatened pest both under cover and in field cultivation. With the aim of developing transgene-mediated WFT resistance in chrysanthemum, efficient regeneration techniques were developed, along with the identification of effective promoters which confer high levels of expression in chrysanthemum. Recombinant cysteine protease inhibitors effective against WFT were identified based on the inhibition of the predominant group of proteases *in vitro* and reduction in oviposition rate (OR) *in vivo.* Potato multicystatin (PMC) an eight domain cysteine protease inhibitor was expressed in chrysanthemum in an attempt to endow chrysanthemum with thrips resistance. Plants with levels of expression ranging at maximum 0.1-0.13% of total protein were generated. A correlation with thrips resistance could not be established at these relatively low levels of expression, but the results in this thesis provide evidence that with higher expression levels the control of thrips is feasible.

Chrysanthemum history and status

Chrysanthemum is one of the few cutflowers which has been widely grown across the globe, finding different uses like floral arrangements, individual flowers in garlands and detached ray florets stuck on walls and paper for decoration etc. It is a unique cutflower in many different shapes, on the basis of which they are classified as single, spidery, intermediate, anemone-centred, incurved, reflexed, pompon and other types. The origin of this interesting cutflower is a little obscure, but it is known to be in cultivation for over 3000 years. The earliest records of chrysanthemum date back to 500 B.C. in Chinese scriptures, and it was described in the ninth moon by Confucius as the "chrysanthemum with it's yellow glory". France introduced chrysanthemum from China into Europe in 1789, reached the Kew garden in UK and was mentioned in the *Botanical Magazine* in 1796. Seeds were produced in Europe for the first time in 1827. Only by the middle of the nineteenth century chrysanthemum was also introduced to the USA, when cultivar William Penn was shown at the Pennsylvania Horticultural Society in 1841 (Smith, 1975). Since then chrysanthemums have undergone a huge rise in popularity leading to their present economic status as the second largest selling cutflower next to roses.

Chrysanthemum is grown in greenhouses with commercial material being vegetatively multiplied and supplied as cuttings or plugs. The production of chrysanthemum under cover today is threatened by insect pests like leafminers, aphids, and western flower thrips (WFT) (van Dijken et al., 1992; Guldemond et al., 1994; Robb, 1989). WFT is the most predominant, mainly because chemical control of this species is difficult, due to their secretive habits and because of rapid development of resistance to chemicals (Broadsgaard, 1994). Biological control is mostly not effective enough. In chrysanthemum the flowers are

preferred for feeding (De Jager, 1995), wherein the ray florets are most preferred (Dijken et al., 1995).

The western flower thrips *Frankliniella occidentalis*

The western flower thrips (WFT) *Frankliniella occidentalis* belongs to the insect order Thysanoptera, sub-order Terebrantia and family Thripidae. It is a haplo-diploid; females are diploid and males are haploid. Fertilised females produce large numbers of females from fertilised eggs and a few males, while unfertilised females produce only males (arrhenotoky). The life cycle consists of six stages comprising of an egg, 2 larval, 2 pupal and an adult stage. The eggs are laid in parenchymatous tissue of the host plant, in a pocket created by the ovipositor (Brodsgaard, 1989).

Host range and pest status

WFT was till 1980 a local pest limited to the western USA (Brodsgaard, 1991). Since then it has become a major problem world-wide on many crops and wild plants. Its polyphagous nature is extraordinary as it can feed and propagate on at least 240 species from 62 different families of plants (Loomans et al., 1995). The rapid world-wide spread has mainly been attributed as an effect of the practice of long distance trade in glasshouse plants.

WFT is extremely difficult to control using any single mechanism and causes damage to a wide range of cash crops like cotton and tea, vegetable crops like onion, tomato and cucumber, fruit crops like pear, apple and orange, and practically all cutflowers. WFT generally feed by sucking plant juices by way of tissue penetration causing discoloration, resulting in reduced market quality (Gullan & Cranston, 2000). Additionally WFT also cause indirect damage as transmission vectors for tospoviruses (Bunyaviridae, Wijkamp et al., 1993) wherein larvae acquire and multiply virus while adults only transmit (Sakimura, 1962). In the absence of visual perception WFT tend to prefer the fragrance of open buds rather than fully open flowers (Smits et al., 2000). On the contrary, visually, the fully open chrysanthemum is preferred over the open bud, but the attraction for colour also depends on other local conditions and stimuli. In the presence of only chrysanthemum flowers, the colour of the flower had no influence, but the plant stage influenced the feeding pattern (Dijken et al., 1993).

A high variability in Western flower thrips populations has been reported in their response to natural resistance in chrysanthemum (de Kogel, 1997), insecticides (Immaraju et al., 1992; Brodsgaard, 1989; 1991; Mcdonald, 1995), and virus transmission (Van de Wetering et al., 1996). Some of the reasons for WFT to be so virulent are attributed to its unique characteristics: arrhenotoky of sexual differentiation, a polyphagous feeding habit, opportunistic herbivory, thigmotactic behaviour and tendency for cannibalism (Lewis, 1973). In the years 1988-90 Dutch growers spent 39.5 million euro per year on crop protection in the specialized horticultural segment. This was close to 30% of the total crop protection costs in the Netherlands (Brouwer et al., 1994). Cost of pestcides used by the

Dutch to protect ornamental and cutflower crops during that period was 25 million euro, which was the highest in the European Union for that category of crops (Brouwer et al., 1994). WFT control in 1996 in the Netherlands cost 2.4 million euro (Roosjen et al., 1998) indicating the importance of this pest and the need to control it.

WFT control: chemical strategies

Chemical control is the simplest and most widely used, but development of resistance by WFT to insecticides is a major problem. Insecticide compounds effective against thrips as systemic insecticides are acephate, heptenophos and imidocloprid while dichlorvos, fenitrothion, fipronil, and malathion are effective as contact insecticides (Lewis, 1999). Insecticides also have the problem that they tend to be hazardous for human health leading to allergies and other skin and lung problems. Indiscriminate and irrational use of broad-spectrum insecticides has lead to the development of resistance in WFT (Loomans et al., 1995, Helyer and Brobyn, 1992). Income from chrysanthemum per square meter per year is 41 euro, while the cost of controlling WFT on chrysanthemum is 0.19 euro/m^2/year. Only 0.5% of revenue is, therefore, lost this way (Agrimonitor, 1997). The total area of greenhouse with chrysanthemum is approximately 750 hectares with nearly 1,425,000 euro spent on chemical control of WFT alone (Agrimonitor, 1997).

WFT control: biological strategies

Arthropod predators and parasitoids are well known natural enemies of Western flower thrips and can be effective in warmer climates where they occur naturally (Loomans et al., 1995). Biological control of WFT can be achieved in greenhouses by introduction of insect parasitoids or predators (Table 1). Compared to the number of predators available for *Thrips tabaci*, availability of predators for WFT, is less since WFT is relatively a newer pest. Predator insects belonging to Anthocoridae are considered to be effective while those like Phytoseiidae and Nabidae have failed to yield expected results (Loomans, et al., 1995). There have been few attempts in the past to use hymenopterous parasitoids for biological control and release them seasonally to inoculate WFT without much encouraging results. Negative side effects like hyperparasitism, attack on non-target and beneficial hosts are potential risks. Parasitoid cultivation and maintenance is also a cumbersome process as their environmental adaptation, seasonal synchronisation, reproductive capacity and foraging behaviour etc., have to be well studied and set-up before exploiting them successfully (Loomans et al., 1995). Ideal parasitoids showing a potential for controlling WFT in a greenhouse system have been a rarity. Overall the use of predators and parasitoids has not been successful, because a zero tolerance is expected on WFT count on flowers for export. Using biological control it has not been possible to bring WFT populations at the levels of tolerance required for export.

WFT control: host plant resistance through breeding

The third strategy is enhancement of the innate host plant resistance in chrysanthemum. Host plant resistance can be defined as the genetically inherited qualities that result in a plant of one cultivar or species being less damaged than a susceptible plant, which lacks these qualities (Smith, 1989).

Table 1. The list of predators and parasitoids known to feed on *Frankliniella occidentalis* along with the natural or experimental host plant on which they are found.

Predator	**Crop on which it is found / used**	**Parasitoids**
Anthocoris nemorum	Ornamentals *Rose, carnation, cacti, alfalfa, mustard*	*Ceranisus menes*
Orius albidipennis	Cucumber, pepper, vegetables *Alfalfa, wild mustard, swept*	*C. americensis*
O. insidiosus	Cucumber, pepper, ornamentals *Weeds*	*C. lepidotus*
O. laevigatus	pepper, vegetables *Xanthocephalum microcephalum*	Eucharitidae species
O. limbatus	Vegetables	*C. russelli*
O. majusculus	Cucumber, pepper, vegetables	*C. Vinctus*
O. niger	Pepper	*Goetheana shakespearei*
O. tristicolor	Cucumber, pepper, vegetables & *	*Megaphragma longiciliatum*
Nabis alternatus	alfalfa, field crops	
N. americofernus	bean, alfalfa, field crops	
Geocoris articolor	alfalfa, field crops	
G. pallens	alfalfa, field crops & *	
D. tamaninii	Cucumber	
Amblyseius barkeri	Cucumber, ornamentals	
A. degenerans	Pepper	
Neoseiulus cucumeris	Cucumber, pepper, vegetables, ornamentals	
Anystis agilis	-	
Hypoaspis aculifer	-	
Crytomorpha desjardinsi	-	

The list of plants on which the parisitoids are used or found is mentioned in italics to distinguish them from host plants of the predators. * refers to the cotton crop as host. This table has been compiled after reviewing the chapters by Riudavets on predators and Loomans and van Lenteren on parasitoids in Loomans et al., (1996).

Fig. 1 broadly depicts the resistance mechanisms that may be working in plants, wherein one mechanism or several mechanisms may act in tandem at any given time on one or several insect species. Well established protocols exist for the measurement of the level of resistance in chrysanthemum against WFT (De Jager, 1995; De Kogel, 1997). De Jager (1995) reported that the magnitude of resistance exhibited by chrysanthemum against Western flower thrips is highly variable. The chrysanthemum cultivars analysed for several parameters with either fold difference or values for resistant (R) and susceptible (S) in parenthesis are; number of WFT surviving on chrysanthemum with (2-fold) and without flowers (3-fold), silver damage (R=8.4, S=413mm^2) growth damage (R=0, S=12 leaves) oviposition (R=2.2, S=66.9) rate, larval growth (2-fold), and longevity (5-fold), survival (4-fold), De Jager (1995). The chrysanthemum lines resistant to WFT were also resistant to leaf miners (44% explained variance) and aphids. Thus, the resistance to WFT is not a specific phenomenon, but is possibly a general herbivore resistance.

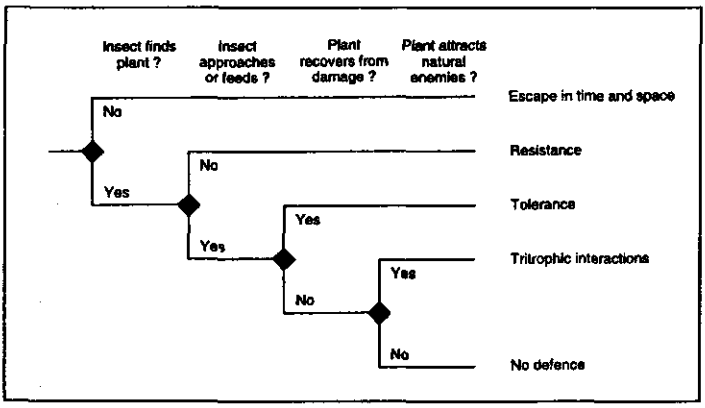

Fig.1 Different mechanisms of resistance that can be evolved in the plant presented in a schematic view. Any one or more of these could be working at the same time, or at different times, in response to different stimuli. In nature the mechanism of resistance would be more complex and highly cross linked.

De Jager (1995) found that individual leaf sap fractions had no negative effect, but that the combinations did have a suppressing effect on Western flower thrips. Improved host plant resistance in chrysanthemum as a measure to control Western flower thrips has certain practical disadvantages. First of all, the traits impart only a partial resistance on the plant, and the factors responsible for those traits are not clearly identified and quantified in the plant. Thus, the breeder does not obtain the full benefit of reduced input costs (0.5%) and increased income from a higher quality product from the deployment of these traits. Secondly, breeding for host plant resistance against insects and especially thrips is very costly due to the elaborate procedures required to carry out and evaluate insect assays on whole plants. These costs are hard to recover from the sales years later, because of the very large number of different flower varieties that are marketed. Each variety on its own only represents a small market value. Breeding will also be extra difficult if the resistance is a multigene trait in a complex genetic background like that of hexaploid chrysanthemum.

Regeneration of Chrysanthemum

To develop WFT resistance using a transgene approach, the pre-requisite is an efficient regeneration system for *Agrobacterium*-mediated gene transfer. Chrysanthemum has been regenerated using different explants like flower receptacle shoot tips, pedicel, petal segments and leaf (references in chapter 3). However these protocols are cultivar specific, as a result of which regeneration of a new cultivar is by trial and error which can be a draw back to genetically engineer a large number of cultivars.

Thirty five chrysanthemum cultivars were tested for regeneration using stem explants on a direct organogenesis protocol used for Agrobacterium-mediated gene transfer (De Jong et al., 1994). The cultivars were grouped into four groups based on the kind of growth put forth (response) on the stem explants namely Group 1: more than 1.6 shoots per explant, Group 2: less than 1.6 shoots per explant, Group 3: No shoots but only callus and lastly Group 4: no response. Hormone source and concentration were modified and alternate explants were tested based on the performance of cultivars to initial screening (grouping 2-4). The initial screening provided indirect information on the endogenous hormone levels which was the criterion for the direction of change. The principle behind this approach was exposure of explants to different sources and concentrations of hormones or use of different explants to make use of the differences in endogenous hormone levels to affect regeneration. Five different groups of media (Table 1, 4 & 6 of chapter 2) totalling 23 different combinations were tested in total. By systematically following this approach it was possible to successfully regenerate 23 of the 35 lines tested while standard protocols only allowed regeneration of 11 lines.

In addition to developing regeneration protocols, the quality of light incident on the cultures was tested in view of enhancing the regeneration efficiency (number of shoots or regenerative nodes per explant). Light of different qualities was evaluated by means of three tinted (blue, yellow & red) and one transparent culture container (control) all receiving light in the range of 400-700 nm. The coloured containers had different spectral distribution, transmission wavelength maxima and intensity of light inside them (chapter 3). The coloured containers produced 2-fold more and longer shoots, and 2-fold more multipliable nodes over the control containers. The number of microshoots and nodes were enhanced in the specific coloured containers, (yellow and blue) which is a measure of regeneration efficiency for a given medium and culture condition. In addition to enhancing the regeneration efficiency other changes like length of internode, leaves and colour of leaves were significantly different over control.

From this study it is not clear what factors in the explant or microshoot are influenced by the quality of light. Specific phytochromes may get activated under specific light quality, which in turn can enhance or reduce or influence the activity of other phytochromes, leading to the differences observed. The reduction in green coloration may be due to reduced light harvesting complex transcripts in the absence of light at 680 nm (chapter 3). However the study clearly presents the potential of light quality to significantly influence regeneration efficiency and other quality parameters of the regenerated microshoot. Light having transmission wavelength maxima at 475 nm and at 580 nm is recommended to produce higher number of nodes and shoots starting from chrysanthemum leaf explants.

Transgene expression in green and floral parts of Chrysanthemum

After having developed a systematic approach for regenerating chrysanthemum, the focus shifted towards the improvement of transgene expression in chrysanthemum, which had a unique problem. Various cauliflower mosaic 35S (CaMV) promoter variants used in chrysanthemum to drive different transgenes are listed in Table 3. The single CaMV-GUS construct showed bright blue coloration upon overnight X-gluc staining, but fluorometric analysis showed very low to undetectable expression. This is a problem not commonly observed in other plant species, which had to be solved to genetically engineer chrysanthemum with protease inhibitors. Thus, with the aim of improving transgene expression levels in green parts of chrysanthemum, two putatively stronger promoters, the doubled cauliflower mosaic virus 35S (dCaMV) promoter and the potato *Lhca3.St.1* promoter, were tested.

In contrast to the low activity of the dCaMV promoter, it was observed that the potato *Lhca3.St.1* promoter gave up to 175-fold higher activities in chrysanthemum, while the same two promoters tested in tobacco resulted only in a 2-5 fold difference (Mlynarova et al., 1994, 1995 & 1996, chapter 4). Average GUS activity in chrysanthemum leaves is 44 pmol MU/min/microgram protein under the control of the *Lhca3.St.1* promoter, while the same promoter-GUS construct in tobacco was 4-fold higher (Mlynarova et al., 1994). Expression in chrysanthemum is somewhat higher in stems, but lowest in the ray florets (7.8 pmol MU/min/microgram protein). The *Lhca3.St.1* potato promoter is, therefore, a suitable promoter to obtain high expression levels in the green parts of chrysanthemum.

It remains un-explained why the *Lhca3.St.1* promoter performs 4-fold higher in tobacco over chrysanthemum. This phenomenon is not specific to this foreign promoter as it is also observed with the chrysanthemum derived *Rbcs* promoter (Outchkourov et al, submitted). Secondly, it is also unclear why the CaMV based promoters are not active in chrysanthemum. Possibly, the necessary transcription factors commonly found in other plant species are present in lower concentrations, have diverged from ancestral specificities or are simply absent in chrysanthemum, resulting in the observed weak activity.

WFT predominantly damages the ray florets. Hence, a promoter conferring high levels of transgene expression in the ray florets would be useful. Known heterologous promoters like, the chalcone synthase gene *chs-A* (van der Meer *et al.*, 1990), the zinc finger transcription factor *EPF*2-5 (Takatsuji *et al.*, 1994), promoter of the *Arabidopsis* eceriferum gene *CER6* involved in wax biosynthesis (Pereira, unpublished) and the wound-inducible promoter of potato multicystatin, *PMC* (Walsh *et al.*, 1993) were selected. Ubiquitin genes were chosen to contain potentially strong homologous promoters based on DNA microarray data which demonstrated that ubiquitin genes in *Arabidopsis* are among the most abundant messengers in most plant organs including flowers (Ruan *et al.*, 1998). Based on the activity of the GUS reporter gene the homologous ubiquitin extension protein (*UEP1*) promoter conferred significantly higher GUS expression (8.5 pmol/min/µg protein) over the heterologous promoters tested (2-5.5 pmol/min/µg protein), and was comparable to the activity of *Lhca3.St.1* in the florets (7.8 pmol/min/µg protein). The activity of the *UEP1* promoter was, however, 3-fold lower in the disc florets and 9-fold lower in the leaves. The activity of *UEP*1 promoter was observed only histochemically in the pollen grains, hence, there is no quantitative expression data. As the *UEP1* promoter confers significantly higher

levels of transgene expression in the petal tissue of the ray florets over the heterologous promoters they can be used to express genes to alter flower colour, vase life, fragrance etc. In this study the *UEP1* promoter was used to express the potato multicystatin gene in chrysanthemum. Thus, two promoters were identified one heterologous from potato and the other homologous from chrysanthemum whose activity are comparable in the ray florets. However, the *Lhca3.St.1* promoter confers GUS at 44 pmol/min/µg protein in the leaves, which is 50-fold higher than the *UEP1* promoter (0.9 pmol/min/µg protein), thus giving each of them unique potential for different applications.

Table 2. Overview of transgene expression studies in chrysanthemum using CaMV-derived promoter sequences. The number in the reference column refers to the references mentioned in chapter 4.

CaMV promotera	Gene usedb	Expression assay	Assay results	Reference
1x	GUS (i)c	Histochemistry	Blue spots	4,5,9,10,19,21, 39
			Blue tissue	10,11,15,20
		Fluorometry	Activity (callus)	38,39,40
			Activity (leaf)	1,29,35,37
			No activity	21
	TSWV N	ELISA	No detection	34, 37
		Western	No detection	34, 37
		Northern (total)	No detection	34
		Northern (poly A)	Positive	34
		Virus (TSWV)	Resistance	34
	HYG	Selection	Shoot formation	30
	Leafy	Northern	Detection	33
		Plant phenotype	Changed flowering time	33
	Leaf colour	Northern	No detection	3
		Plant phenotype	No change	3
	CHS (as)	Flower phenotype	Changed colour	14
	Bt toxin	Insect (*Tetranychus*)	Resistance	13
1x + CMV-L	TSWV N	ELISA	No detection	41, 42
1x + cab22-L	NPTII	Root formation	Rooting transformants	7,8
	CHS (s,as)	Flower phenotype	Changed colour	7,8
		RNAse protection	No RNA	8
2x	GUS	Histochemistry	Blue tissue	This study
		Fluorometry	Activity	This study
2x +AIMV-L	TSWV N	Virus (TSWV)	Resistance	42
4x	Chitinase	ELISA	Positive	36
		Fungus (*Botrytis*)	Resistance	36

a. The CaMV strain from which the 35S promoter was isolated is not taken into account here. The number (1x, 2x, 4x) refers to the number of CaMV enhancers used. CMV-L, cucumber mosaic virus leader; cab22-L, Petunia cab22L leader; AIMV-L, alfalfa mosaic virus leader.

b. GUS, β-glucuronidase; TSWV, tomato spotted wilt virus; HYG, hygromycin resistance gene; CHS, chalcon synthase; Bt, *Bacillus thuringiensis*; NPTII, kanamycin resistance gene; as, antisense orientation; s, sense orientation.

c. No distinction was made between the bacterial GUS gene without (GUS) and with an intron (GUSi).

Accurate characterisation of promoter activity has to be position effect independent. In order to remove position effects, resulting from the random place of integration of the transgene, the effect of flanking matrix-associated region (MAR) on either side of

transgenes was followed (Mlynarova et al., 1994, 1995 & 1996, chapter 4). There was no difference in variance between the populations (different to the results presented by Mlynarova et al., 1994) generated with and without MAR flanking. These results imply that in chrysanthemum the chicken lysozyme MAR element is not effective and the action of a MAR element may depend on the host organism.

Characterisation of WFT proteases *in vitro*

To select effective protease inhibitors against insect herbivores, it is essential to first determine the predominant protease activity and the pH optimum in the insect gut. On that basis proteinaceous protease inhibitors can be selected in a second step for optimal inhibition *in vitro*. In a third step, this is followed by studying effects on the insect in bioassays *in vivo*.

To study the effects of protease inhibitors on WFT proteases, the whole Western flower thrips were ground (seperating guts is not practical due to size), with the presumption that the gut proteases would be dominant also in whole WFT extract. Over 90% of the WFT protease activity was inhibited when they were exposed to chemical and proteinaceous cysteine protease inhibitors (chapter 6). Thus, cysteine proteases were dominant in the whole WFT extract, and exhibited a relatively low pH activity optimum of 3.5. This pH optimum is low compared to other insects, which also utilise cysteine proteases for protein digestion in the gut.

Production of cysteine protease inhibitors in the yeast *Pichia pastoris*

The results of in vitro experiments are no guarantee of effectiveness in vivo. This is due to the fact that many insects are known to be able to adapt to protease inhibitors in their diet by inducing the synthesis of proteases which are insensitive to the inhibitors. To test *in vivo* effects substantial quantities of purified protein are required. Purifying the protein from its natural source has its limitations in terms of the heterogeneity of such samples, making a link with a specific candidate inhibitor gene rather dubious. These problems can be overcome by producing the recombinant proteins in microbial hosts. The yeast *Pichia pastoris* is a preferred expression system for protease inhibitors due to its high yield of protein secreted into the medium; which is relatively free of other proteins.

Potato cystatin (PC) and potato multicystatin (PMC) were expressed in *Pichia pastoris*. PC was successfully produced in large quantities, purified by FPLC, and the apparent equilibrium dissociation constant was determined to be 0.6 nM. However, there was no success in large scale production of secreted PMC in spite of trying several media combinations and conditions for fermentation. Most of the little PMC that was expressed was retained in the pellet. Hence, we did not further purify. The reasons for lack of efficient secretion of this large 86 kDa protein is unclear. The cellular expression of PMC in the cytoplasm could be an alternative, to improve expression, which was not tried in this investigation. It is to be still clarified if PMC is also forming crystals in *Pichia* as it does in

its native state in potato (Rodis and Hoff, 1984). Such crystals may get aggregated in the cytoplasm, and, hence, may not be secreted. PMC produced in *Pichia* was checked for size by western blot. This indicated that protein of the correct size of 85 kDa was produced, which implied the gene was maintained and stable. The *Sna*BI site used for the cloning may have affected the expression of PMC, as recently it was found that the *Sna*BI site is identical to an mRNA efficiency element and may cause strong 10-fold reductions of gene expression in Pichia pastoris using the pPIC9 vector (Outchkourov et al., in press). PMC expression could be tested without using the *Sna*BI site for cloning.a Alternatively, expression in the cytoplasm or the use of other strains of *Pichia* and other cloning vectors could be investigated.

Effects of cysteine protease inhibitors on WFT *in vivo*

Dietary protein ingested by adult thrips mainly serves to support the production of eggs as the adult insect is full grown already. Limiting the availability of protein by adding protease inhibitors (PIs) or reducing the protein content in a diet will, therefore, directly affect the number of viable eggs that can be produced. In case of larvae dietary protein serves mainly to support rapid larval growth. Limiting the available protein with protease inhibitors affects larval growth and may result in death of the larvae if the inhibition is strong enough.

Effects of cysteine PIs have been studied on other insects like oryzacystatin I on *Perillus bioculatus* (Ashouri et al., 1998), E-64, pHMB, cystatin, leupeptin on *Hypera postica*, E-64 and equistatin on *Leptinotarsa decemlineata* (references chapter 7). There are no earlier reports, however, on testing of cysteine PIs against WFT and other members of the order of Thysanoptera, or the characterisation of dominant proteases present in WFT. In this study cysteine PIs, PC and EI were tested for their effects separately and in combination on adult WFT in a bioassay cages made of transparent perspex tubes. The bottom of these cages were sealed with nylon gauze while the top was sealed with 2 sheets of stretched parafilm, with a liquid water sandwich in-between. PC and EI introduced into the liquid sandwich at 30 µM concentration, in the range of concentrations found in plant leaves (1.5-3% of total leaf protein), were provided to WFT for a period of five days. Both PC and PC+EI in combination reduced the oviposition rate by 50% compared to control.

PC and EI were exposed to WFT for only 5 days, while the trend of reduction in oviposition rate was downward from day 3. If PC and EI were exposed for a longer period, there may have been a further decrease in egg production. Kirk (1985) reported that egg production by adult thrips would stop after 2-3 days of deprivation of nutrition. At the end of PI feeding, day-5 in the experiment, egg production was reduced by 50% relative to control, suggesting that five days were insufficient to fully deplete WFT from its protein reserves. This indicates that there may be a threshold level of protease activity.

The 50% reduction in oviposition rate achieved by exposure of WFT to PC and PC+EI could have a stronger effect on the size of the final WFT population after a certain period of time. As a consequence of a 50% reduction in oviposition rate, for example, a 92% reduction in the population of WFT, after a 90-day period, is predicted by a mathematical

model based on the relevant life history parameters of WFT on chrysanthemum in greenhouses (18-day egg-egg, 3 eggs/female). Similar mathematical models for whitefly by Yano et al., (1989) predicted 75% reduction in population, over an 80-day period, resulting as a consequence of 50% reduction in oviposition rate. In addition to reducing the oviposition rate, PIs can also affect egg hatch which is functionally equivalent to further reducing the oviposition rate like shown in *Stomoxys calcitrans* by Spates & Harris (1994). However such effects of PC and EI on WFT, in addition to their effects on the different larval stages is yet to be investigated.

EI has been shown to have an aspartic PI domain in addition to the cysteine PI domain (Strukelj et al., 2000). The combination of PC and EI did not result in further reduction in oviposition rate as compared to the reduction with PC alone. However in other insects having cysteine proteases as a dominant class of proteases, like *Leptinotarsa decemlineata* (Wolfson and Murdock, 1987; Gruden *et al.*, 1998) and *Diabrotica undecimpunctata* (Edmonds *et al.*, 1996) in addition have aspartic proteases (references chapter 7). In WFT the aspartic protease inhibitor pepstatin showed 16% inhibition of proteolytic activity in total extract. This suggests that in WFT the contribution of aspartic proteases to total gut proteolytic activity may be limited despite the low pH optimum, which may be an explanation for the lack of additional effects with EI on WFT. Nevertheless serine and metallo PIs have not been tested in combination with PC, hence the possibility of generating a synergistic effect by combing inhibitors with PC is still possible. Considering the positive results reported by Thomas *et al.* (1994), and based on the serine PI elastatinal inhibiting 20% of WFT proteases *in vitro*, it may be worthwhile to test elastase and cysteine protease inhibitors in combination against WFT. In addition, the combination of cysteine PIs of different subclasses, with unique specificities from plant and non-plant sources may result in further improvements of the effects on WFT.

Thus the *invivo* effects of the tested PIs provide a first proof of concept that cysteine protease inhibitors may be used to control the population of WFT (chapter 7). On this basis transgene-mediated resistance to WFT may be designed (chapter 8).

Transgenic chrysanthemum expressing potato multicystatin

In vitro and *in vivo* studies on artificial diets indicated that the cysteine protease inhibitors (PC and EI) were effective in reducing the oviposition rate of adult WFT. With the aim of extending these results to chrysanthemum plants the next step was to express related genes in transgenic chrysanthemum.

The homologous *UEP*1 promoter characterised in chapter 5 was used to drive in chrysanthemum the expression of a 3.5 kb PMC gene to generate an 85 kDa potato multicystatin protein. Transgenic lines were analysed by immuno-dotblot. However, as the protein used for the standard curve was the single domain PC while the 8 domain PMC was expressed in chrysanthemum, reliable quantitation of the absolute levels was not possible. The highest expression of PMC was estimated at 2.2 units/µg protein which conferred 0.28

pmol/µg protein of papain inhibitory activity (PIA). After subtraction of the background activity (0.15 pmol/µg protein) the expression was estimated to be 0.13% of total protein. This was rather low as protease inhibitors should be 0.5-1.0% of the total protein to effectively inhibit insect gut proteases of WFT in this case. On the artificial diets WFT were exposed to PC and EI at 30 µM concentration, which is equivalent to 0.03-0.06 % (w/v). Assuming the protein content in plant leaves to be 2%, than it is necessary to obtain a transgene expression level in the range of 1.5-3.0% of total protein. As a consequence of low PMC expression (based on the analysis hitherto) driven by the *UEP1* promoter, we observe 10-20 fold lower levels of expression than required. Hence, no correlation between oviposition rate and PMC expression or PIA is observed. It will be necessary to generate plants with higher levels of transgene expression to obtain resistance. By increasing the level of expression one would be able to significantly reduce the WFT population on chrysanthemum based on the proof of concept. By changing the promoter driving the PMC gene we not only enhance the expression by 5-fold but also spatially change expression to leaf. As leaves of chrysanthemum in the greenhouse are exposed to WFT for a longer duration over the flowers, the building up of the population will normally be on leaves. Expression of PMC at 5-fold higher levels in the leaves of chrysanthemum, may extend the proof of concept provided in this thesis on the use of cysteine inhibitors to control WFT.

Alternatively the cysteine protease inhibitory activity (CPIA) can be enhanced by identifying the natural variation for CPIA among the breeding lines of chrysanthemum, based on PIA. We observe 0.15 pmol MU/min/µg protein of PIA in the control ray florets and leaves of chrysanthemums, which is relatively high as compared to similar tissues from other plants. When this variation is not available than the CPIA can be enhanced by mutation breeding to achieve the same

Thoughts for the future

Though difference in regeneration efficiency related to quality of light incident is shown, it still remains to be seen how microshoots from the different coloured boxes would perform in the greenhouse, under standard light conditions. In addition it remains to be seen if light quality has any influence on the generation of somaclonal variants and transformation efficiency. Studies on these aspects would be interesting and also broaden the influences of light quality.

It will be relevant to study the effect of PC and EI also on the different larval stages of WFT and on egg hatch. Bioassays with pure protein on different larval stages are not practical, hence these proteins have to be tested in transgenic plants. To further improve the effects of PC observed on WFT, testing a combination of PIs of different classes and PIs within the cysteine PI family with different protease specificites may generate stronger effects than by using a single inhibitor alone.

The use of metabolic inhibitors like PIs for insect control is a relatively new area. In the present work both problems of gene expression in chrysanthemum and identification of effective inhibitors needed to be solved. This has laid the groundwork to achieve resistance to WFT. Currently, at Plant Research International new improved inhibitor genes and

improved promoters are applied in parallel work building on what is reported here. The first results indicate that effective control of thrips (>80% reduction after 14 days on whole plant non choice assays) using PIs is indeed feasible in transgenic plants. This implies that an interesting future lies ahead in implanting these traits in cultivars, establishing the level of control in the greenhouse, and finding ways to help breeders and growers to bring such a transgenic product to the market. This will take years more to achieve, but the benefits to society will be a product which can be produced in an environmentally friendly way with no compromise on quality.

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Summary

Chrysanthemum *Dendranthema grandiflora* is the second largest cutflower grown all over the globe. Though the exact origin of chrysanthemum is not clear, the earliest mention of chrysanthemum in ancient scriptures dates back to 3000 BC. Chinese take the credit of identifying the wild daisy and developing in into a cultivated cutflower which has a global status. Japanese have added tremendous diversity to chrysanthemum, making it one of the few cutflowers with a large variation in flower shape and bearing habit. French were the first to honour chrysanthemum in Europe in 1789 and almost after 50 years in 1941 it was first showcased in the USA. Commercial material is supplied as shoot tip cuttings, while mother plants are maintained *invitro*. Till very recently most of the commercial breeding activities have been limited to mutation breeding and breeding between selections from different parts of the world. A spectrum of colours are available in this flower which is one of the hardiest flowers in the vase. However there are some major problems in the commercial cultivation of this crop in the greenhouses all over the world, mainly attributed to the attack of insect pests. Among the insect pests *Frankliniella occidentalis* commonly known as Western flower thrips (WFT), due to its origin, is the most predominant pest. WFT is a major pest not limited to chrysanthemum but the entire cutflower industry in general, and other crops thus finding itself at top position in resistance breeding activities.

WFT has developed as such a major pest due to several characteristics of the insect like it's polyphagous nature (feeds on over 240 species), opportunistic herbivory, cannibalism, arrhenotokic sexual differentiation, thigmotactic behaviour, resistance to chemicals etc. In crops multiplied by seed protecting the crop for 60-days after sowing is now possible by new seed chemical treatments like Goucho (Bayer), however these treatments are not very useful in plants like chrysanthemum where the material is multiplied vegetatively. There have been efforts in three directions to protect plants from WFT menace namely; chemical control, biological control and host plant resistance with all three mechanisms currently in use for chrysanthemums. As discussed the three approaches have their advantages and disadvantages and hence chrysanthemum breeders are in the look out for better technologies. Currently new laws are framed in many parts of the world to reduce use of chemicals in greenhouse production of flowers, and in The Netherlands where a large part of the flower production is under protection, the new laws aims at no chemical usage from the year 2010. As a result of drawbacks in the present approaches to control WFT and in addition the new laws have brought about a new vigour in identifying and developing alternative mechanisms for WFT resistance.

There are not many reports of developing resistance to WFT by a transgene-mediated approach, especially so based on the use of protease inhibitors (PIs). PIs is a relatively new area in insect resistance as compared to toxic proteins like *Bt* (*Bacillus thurigiensis*) which has established itself as a transgenic approach against major *lepidopteran* pests like cotton bollworm. PIs have a different mode of action and are required in much higher concentrations (1% of total protein) to effectively inhibit gut proteases and confer resistance against insect pests.

With the aim of developing transgene-mediated WFT resistance in chrysanthemum, the prerequisites like regeneration of chrysanthemum, and identification of suitable promoters was worked out. A large number of chrysanthemum cultivars are grown for the market

hence transforming them is faster and efficient over backcrossing several cultivars with a resistant parental line. As regeneration of chrysanthemum so far has been by a trial and error approach, a technique of systematically regenerating a large number of chrysanthemum cultivars was developed. Based on the response of explants to a specific medium, several new combinations of varied source and concentration of hormones exogeniously (media) and endogenously (explant source) were tested. Twenty three of the 37 cultivars tested were regenerated in a manner suitable for *Agrobacterial* transformation. The second prerequisite was to identify suitable promoters for transgene expression in chrysanthemum, which had a unique problem wherein the CaMV based promoters conferred very low or undetectable levels of transgene expression. Though the reasons for such low expression by CaMV based promoters, widely used for transgene expression is not clear, two other promoters were identified which significantly improved expression. Firstly the heterologous *Lhca3.St.1* promoter from potato enhanced the transgene expression in the leaves by over 175-fold as compared to the CaMV and the dCaMV promoters. Secondly a homologous promoter *UEP*1 was cloned from chrysanthemum and found to confer over 50-fold higher levels of transgene expression in the petal tissue of the ray florets of chrysanthemum. The *Lhca3.St.1* and the *UEP1* promoter with their unique expression levels in the different parts of chrysanthemum will find a number of applications in genetically engineering chrysanthemum.

To identify PIs effective against WFT, the proteases dominant in WFT were characterised and cysteine proteases were found to be dominant with a pH optimum at 3.5, reported in this thesis for the first time. Based on the characterisation, effective proteinaceous PIs were identified by *invitro* inhibition assays namely potato cystatin (PC) and equistatin (EI) which inhibited over 90% of WFT proteases. PC and EI were recombinantly produced in yeast *Pichia* pastoris in large quantities, enabling the tests to study the effects of PC and EI on live adult WFT bioassays. WFT when exposed to PC and EI for a period of five days in a bioassay on pollen diet showed 50% reduction in oviposition rate. During this period no mortality was observed in adult WFT, however the effects of PC and EI on the larval stages of WFT is yet to be studied. Fifty percent reduction in oviposition rate drastically confines population build up of WFT to 8% of control in a simple mathematical model using all growth and reproduction parameters of WFT. Cysteine protease inhibitors effectively reduce oviposition rate in WFT, resulting in drastic reduction in the size of population is the proof of concept presented by this thesis which should be a landmark in developing WFT resistance.

The proof of concept was extended to chrysanthemum by using the *UEP1* promoter to express potato multicystatin a cysteine PI which simultaneously binds to 8 papain molecules. The levels of papain inhibitory activity obtained by this approach was limited to only 0.13% of total protein, which was rather low, to have an significant effect on adult WFT. Efforts in improving the expression of PMC to the range of 0.5-1% and other approaches to increase the cysteine protease inhibitory activity in pollen and petals of the chrysanthemum flowers will evolve eco-friendly techniques to combat WFT.

Samenvatting

De chrysant *Dendranthema grandiflora* is de op één na grootste snybloem ter wereld. Alhoewel de exacte oorsprong van de chrysant niet duidelijk is, dateren de vroegste verwijzingen in oude geschriften naar 3000 jaar voor Christus. De chinezen wordt toegeschreven dat zij het wilde bloemetje voor het eerst identificeerden en geleidelijk ontwikkelden tot een gecultiveerde snijbloem met een wereldnaam. De Japanners hebben de enorme diversiteit aan de chrysant toegevoegd, waardoor het één van de weinige snijbloemen is met een grote variatie in bloemvorm en bloemdracht. De fransen waren de eersten om de chrysant in Europa te verwelkomen in 1789 en meer dan 50 jaar later in 1941 werd hij voor het eerst tentoongesteld in de VS. Commercieel materiaal wordt geleverd als scheutstekken, terwijl de moederplanten *in vitro* in stand gehouden worden. Tot voor kort waren de meeste commerciele veredelingsactiviteiten beperkt tot mutatieveredeling en kruisingsveredeling met selecties verkregen uit verschillende delen van de wereld. Een breed spectrum van kleuren is beschikbaar bij deze bloem die ook kan bogen op een uitzonderlijk lang vaasleven. Er zijn echter wereldwijd een paar belangrijke problemen die gepaard gaan met de commerciele kweek van dit gewas in kassen, en die vooral betrekking hebben op insektenplagen. Van deze plaaginsekten is *Frankliniella occidentalis*, ook bekend als Californische trips (WFT), het meest problematisch. Problemen met WFT beperken zich niet tot chrysant, maar bestaan in brede zin in de gehele snijbloemen industrie, en in andere gewassen. Hierdoor heeft resistentie tegen WFT prioriteit in veel resistentieveredelingswerk.

WFT heeft zich tot zo'n belangrijke plaag kunnen ontwikkelen door een aantal karakteristieken zoals polyfagie (meer dan 240 waardplanten), opportunistisch voedingsgedrag, kannibalisme, arrhenotokische sexuele differentiatie, thigmotactische gedrag, resistentie tegen pesticiden, etc.. In zaadvermeerderde gewassen is het nu mogelijk het gewas gedurende 60 dagen te beschermen met nieuwe systemische pesticiden zoals Gaucho (Bayer) die aan de zaadcoating worden toegevoegd. Deze behandelingen zijn echter niet van toepassing op planten als chrysant waar het materiaal vegetatief vermeerderd wordt. Er zijn in drie richtingen pogingen ondernomen om planten tegen WFT te beschermen, namelijk: chemische middelen, biologische middelen, waardplantresistentie. Alle drie deze mechanismen worden op dit moment in de praktijk toegepast. De drie benaderingen hebben echter voor- en nadelen waardoor chrysantenveredelaars in de praktijk uitzien naar betere technologieen. Tegenwoordig worden in veel delen van de wereld steeds meer nieuwe wetten van kracht die beogen het gebruik van chemische middelen in bloemenkassen te verminderen. In Nederland, waar een groot deel van de bloemenproductie onder glas gebeurt, beogen de nieuwe wetten het gebruik van chemische middelen volledig uit te bannen in het jaar 2010. Ten gevolge van de beperkingen van de huidige alternatieven om WFT te bestrijden en de komst van de nieuwe wettelijke beperkingen, wordt er extra intensief gezocht naar het identificeren en ontwikkelen van alternatieve mechanismen van WFT resistentie.

Er zijn niet veel publikaties over het ontwikkelen van WFT-resistentie via een transgene benadering, zeker niet waar die benadering gebaseerd is op protease remmers (PIs). De toepassing van PIs voor insektenresistentie is een relatief nieuw terrein van insektenresistentie vergeleken met toxische eiwitten zoals Bt (*Bacillus thuringiensis*) toxines, die zich inmiddels bewezen hebben als een succesvolle transgene benadering voor

resistentie tegen belangrijke lepidoptere plagen zoals *Helicoverpa armigera* op katoen. PIs hebben een ander werkingsmechanisme en zijn pas in veel hogere concentraties werkzaam (1% van het totaal eiwit) omdat ze dan pas effectief alle darmproteases kunnen remmen en aldus resistentie bewerkstelligen.

Met de doelstelling om transgene WFT resistentie in chrysant te bereiken, werden in dit proefschrift de voorwaarden uitgewerkt zoals regeneratie van chrysant, en identificatie van geschikte promoters. Een groot aantal verschillende chrysantencultivars worden voor de markt gekweekt, waardoor genetische transformatie een snellere en efficientere methode is dan terugkruising van verschillende cultivars met een resistente ouderlijn. Aangezien regeneratie van chrysant tot nu vooral een proces van "trial and error" was, werd een systematische techniek van het regenereren van een groot aantal explantaten opgezet. Gebaseerd op de reactie van verschillende explantaten op een specifiek medium, werden nieuwe combinaties van verschillende typen en concentraties van exogene (media) en endogene (explantaattype) hormonen uitgetest. 23 van de 37 geteste cultivars konden op deze wijze geregenereerd worden op een wijze die zich leende voor *Agrobacterium* transformatie. De tweede voorwaarde was het identificeren van geschikte promoters voor de expressie van heterologe genen in chrysant, omdat de veelgebruikte CaMV gebaseerde promoter alleen zeer lage expressieniveaus te zien gaf. Alhoewel de oorzaak van de lage expressie onduidelijk is gebleven, werden niettemin twee promotoren geidentificeerd die een significant verhoogde expressie te zien gaven. In het eerste geval verbeterde de heterologe *Lhca3.St1* promoter uit aardappel de genexpressie in blad met meer dan een factor 175 vergeleken met de CaMV en dCaMV promoters. Ten tweede werd een homologe promoter *UEP1* uit chrysant gecloneerd die in de petalen van chrysantenbloemen een 50-voudig hogere expressie te zien gaf vgl. met de CaMV promoter. De *Lhca3.St.1* en de *UEP1* promoter met hun eigen weefselspecifieke expressiepatroon zullen bij genetisch modificatie van chrysant verschillende toepassingen kunnen vinden.

Ten einde PIs te identificeren die effectief tegen WFT zouden kunnen zijn, werd de dominante protease activiteit bepaald. Cysteine proteases bleken dominant te zijn met een pH optimum van 3.5. Op basis van *in vitro* remmingsproeven werden effectieve protease remmers geidentificeerd, nl. aardappel cystatine (PC) en equistatine (EI) die de protease activiteit van trips met meer dan 90% konden remmen. PC en EI werden geproduceerd als recombinante eiwitten in de gist *Pichia pastoris* in hoeveelheden die het toelieten om *in vivo* bioassays te doen met volwassen tripsvrouwtjes. Onder invloed van PC en EI produceerden de volwassen vrouwtjes na 5 dagen op een pollendieet 50% minder eieren. Gedurende deze periode nam de mortaliteit ten gevolge van de remmers niet toe. De effecten van PC en EI op de larvale stadia van WFT moeten in de toekomst nog bestudeerd worden. Vijftig procent vermindering van de vruchtbaarheid heeft theoretisch een sterk beperkende invloed op de populatiegroei van WFT tot 8% van de controle na drie maanden op een gewas. Dit wordt voorspeld door een mathematisch model waarin alle groei en reproductie parameters van WFT verwerkt zijn. Cysteine protease remmers kunnen dus de vruchtbaarheid van WFT sterk reduceren, resulterend in een drastische reductie in de grootte van de populatie. Dit is het concept dat gepresenteerd wordt in dit proefschrift en dat een belangrijke stap vormt in het ontwikkelen van WFT resistentie.

Het concept werd uitgetest in chrysant met behulp van de UEP1 promoter en aardappel multicystatine (PMC). PMC is een cysteine protease remmer die in staat is om gelijktijdig 8

papaine moleculen te binden. Het niveau van expressie in chrysant dat gehaald werd was slechts 0.13% van het totaaleiwit, wat te laag is om een significant op volwassen thrips vrouwtjes te hebben. Het zal nodig zijn om de expressie van PMC of soortgelijke protease remmers in de buurt te brengen van 0.5-1.0%. Wat dat betreft is het ook interessant om te selecteren op verhoogde endogene cysteine protease remmers in de bladeren, bloemen en pollen. Uiteindelijk zal dit kunnen leiden tot een eco-vriendelijke techniek om WFT onder controle te houden

Curriculum Vitae

Seetharam Annadana was born in Bangalore city, in the state of Karnataka, Southern India on 16th of September 1968. He studied his primary school in Carmel Convent and later joined The Valley School, from Jiddu Krishnamurthy Foundation. In 1985 after passing the ICSE examinations, he joined the National College of Jayanagar to purse his Pre-University study in science taking Physics, Chemistry, Mathematics and Biology as specialisation. In 1989 he sought admission at the College of Agriculture, Raichur, University of Agricultural Sciences Dharwar. He underwent the BSc (Agriculture) technical degree program for four years and specialised in Plant Breeding. He tested different agronomic practices for optimised rain-fed cotton as his topic for his research experience project. He worked on the standardisation of mutation breeding protocols for linseed as his major research project at the end of the program. He represented the University twice in both the south zone and national level youth festivals and showed keen interest in theatre and Karnatic classical music. He was awarded three gold medals for having obtained the highest academic grade point average for the batch 1989-1993 of BSc (Agriculture) from the College of Agriculture Raichur by the University.

From 1993 to 1995 he joined his father Shri. A. Seshadri Iyer, a leading Advisor for Hi-Tech Horticulture in establishing 100% export oriented cut flower production units in Karnataka. During this period he was also trained for over 6 months in one of the leading tissue culture labs to get acquainted with all the basic techniques of plant propagation and regeneration. He also underwent a certified training program on tissue culture of Orchids and Anthuriums at the IIHR, Hessarghatta, Bangalore. During this period he helped in the preparation of feasibility reports; on cutflower projects, production of coco pith from coir waste and setting of mineral water projects at TEDMAG a turnkey project consultation firm. He was involved in the building of greenhouses and tissue culture labs as well during this period.

From August 1995 to January 1997 he joined the MSc Biotechnology program of Wageningen University, Wageningen, The Netherlands, as a self-paying student with the support of his parents. He specialised in plant and microbial production and showed interest in the biotechnology of cut flower crops. For a short while he worked on Alstromeria at the Department of Plant Breeding and than went on to CPRO.DLO, the institute specialising on cut flower research. It is here that he was introduced to Dr. Jan de Jong who introduced chrysanthemums to Seetharam Annadana. He was trained in tissue culture of chrysanthemum, transformation of chrysanthemum and to some extent on the production of chrysanthemums in the greenhouses by Wim Rademaker. He also tested some MADS box gene promoters and expression of some of the MADS box genes like *fBP*14, *Apetala* and *CAL* for expression in chrysanthemum. Chrysanthemum being a determinate flower, he did see some intermediate in-determinate like flowers with buds in the middle of a chrysanthemum in full bloom. This work was done in co-operation with G. Angenent and O. Shulga. The MSc work was supervised by Dr. Jan De Jong with the official supervisor from the University being Dr. M. Ramanna of Department of Plant Breeding.

In 1997 developing a regeneration protocol for chrysanthemum was on a trial and error basis, while obtaining high levels of expression in chrysanthemum was still a major

bottleneck. The training and expertise gained during the period of work for MSc thesis helped him in understanding and arriving at solutions to some of these problems. The expertise gained here was now sufficient to start on a PhD project which to a very great extent had to be application oriented due to the commercial background of Seetharam Annadana. During October 96 to March 1997 start up training in bio-molecular techniques was provided by Dr. J.P.H. NAP.

From April 1997 onwards he registered as a PhD student at the Department of Plant Breeding but worked under the supervision of Dr. M.A. Jongsma at Plant Research International (CPRO.DLO). The research work was conducted with the cluster Biocides in the business unit Cell Cybernetics. The results of his work lasting just over 4 years is presented in this thesis.

In addition to conducting research work for his PhD, Seetharam Annadana has been involved in developing markets and acquiring clients for Wageningen UR, its institutions and some of the commercial biotechnology companies based in Wageningen. He is currently the Co-ordinator India for Wageningen UR and KeyGene Genetics. His major interest for the future will be bringing novel technologies in to crops important for Indian Agriculture through use of molecular markers and genetic engineering.