Agrobacterium-mediated gene transfer to chrysanthemum

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Proefschrift
ter verkrijging van de graad van doctor
in de landbouw- en milieuwetenschappen,
op gezag van de rector magnificus,
dr. H. C. van der Plas,
in het openbaar te verdedigen
op dinsdag 19 november 1991
des namiddags te vier uur in de aula
van de Landbouwuniversiteit te Wageningen.

STELLINGEN

1) Het 'leaf-disk' systeem voor het transformeren van planten met behulp van *Agrobacterium*, blijkt voornamelijk efficient in combinatie met modelgewassen en is niet zo algemeen toepasbaar als vaak wordt verondersteld.

Horsch, R.B. et al. (1985) Science 227; 1229-1231.
Dit proefschrift

2) Het onduidelijk omschrijven of soms zelfs geheel niet vermelden van gebruikte plasmiden en bacteriestammen, is een ernstige belemmering voor het reproduceren van onderzoeks resultaten door derden.

Purdy, L.H. and Dickstein, E.R. (1989) Plant Disease 73; 638-639.
Schmidt, R. and Willmitzer, L. (1988) Plant Cell Reports 7; 583-586.
Chee, P.P. (1990) Plant Cell Reports 9; 245-248.

3) Het gebruik van de frequentie van tumorvorming en de grootte van tumoren als maat voor de transformatie-efficientie van *Agrobacterium* is onjuist.

Charest, P.J. et al. (1989) Plant Cell Reports 8; 303-306.
Clapham, D. et al. (1990) Theor.Appl.Genet. 79; 654-656.
Delzer, B.W. et al. (1990) Crop Sci. 30; 320-322.

4) Het gebruik van ß-glucuronidase assays als bewijs voor genoverdracht, ondanks achtergrondactiviteit, terwijl opinedetectie als analyse methode wordt afgewezen, op grond van de aanwezigheid van endogene produkten, is meten met twee maten.

Ahokas, H. (1989) Theor. Appl. Genet. 77; 469-472.

5) GUS-assays, als indicator voor de weefsel- en celspecifieke expressie van een promoter, zijn alleen betrouwbaar als het expressiepatroon bevestigd wordt door *in situ* localisaties aan het corresponderende mRNA.

Koltunow, A.M. et al. (1990) The Plant Cell 2; 1201-1224

6) Aangezien apo-lipoproteine B niet uitwisselbaar is tussen verschillende lipoproteine fracties, is de aanwezigheid van dit eiwit op de lipidenemulsie, als gevolg van incubatie van de emulsie met VLDL-vrij serum, waarschijnlijk een artefact.

Mamö et al. (1991) Biochimica et Biophysica Acta 1081; 241-245

7) Daar de eindprodukten van genetische manipulatie en kruisingsveredeling principieel gelijk zijn hadden de 'Ziedende Bintjes' net zo goed, en met minder schade voor de maatschappij, zichzelf om kunnen ploegen.

8) Het zelfstandig verzorgen van patienten door leerling verpleegkundigen, zoals dat gebruikelijk is in ziekenhuizen waar Integrerende Verpleging wordt toegepast, is niet verantwoord.

9) De huidige problemen in de sociale zekerheid zijn op te lossen door het invoeren van een ministelsel.

10) De superioriteit als huisdier van katten boven honden blijkt alleen al uit het feit dat men bij katten nooit de behoefte heeft gevoeld het basismodel te veranderen.

Stellingen, behorende bij het proefschrift 'Agrobacterium-mediated gene transfer to chrysanthemum' door Monique F. van Wordragen, in het openbaar te verdedigen op dinsdag 19 november, te Wageningen.

"De ware ontdekkingsreis bestaat niet uit het zoeken van nieuwe vergezichten, maar in het krijgen van nieuwe ogen."

Marcel Proust

Aan mijn ouders,
voor Edwin.

CONTENTS

CHAPTER 1

Outline of this thesis

Aim of the research

The cutflower chrysanthemum (*Dendranthema grandiflora* Tzvel.) is one of the economically most important ornamental crops in the Netherlands. Like most ornamental crops, culture of chrysanthemum is threatened by a wide range of diseases and pests like Japanese Rust, leafspot disease, nematodes, aphids and insects like leafminers and the larvae of species like *Spodoptera exigua* and *S. littoralis* (Hill, 1987). To cope with this problem growers use large amounts of chemical controlling agents. Preventive controlling regimes, which imply spraying with a mixture of controlling agents several times a year, are routinely applied (Berends, 1988). The use of vast amounts of pesticides effectively control diseases and pests in chrysanthemum culture. (Hill, 1987; Duyvensteyn et al. 1990). However, the extensive use of pesticides and insecticides is no longer acceptable, because of the environmental pollution and the dangers for human and animal health. Many pesticides are already legally forbidden or will be in the near future. Dutch governmental policy, as indicated in the National Environmental Policy Plan, NMP (Ministerie van V.R.O.M., 1989), is aimed at a substantial reduction of the use of chemical controlling agents by 1995. New pesticides or insecticides will only be allowed if they are less detrimental to the environment than the agents they will replace. Therefore it is extremely important that great effort is put in both conventional and molecular breeding for resistance in ornamentals. The study presented here has originated from this notion.

Breeding for resistance

In conventional breeding the introduction of new traits is managed by crossing cultivated varieties within the species or with related wild species that possess the desired trait, followed by numerous, and often time consuming, back crossings with the cultivated genotype, to regain the characteristics of the crop. Conventional breeding for resistance in ornamental crops has not been developed much, because breeders have

concentrated mainly on flower color, flower morphology and traits like longer vase life (Dons et al., 1991). Therefore, rapid development of resistant genotypes by conventional breeding is not to be expected. This is especially true for chrysanthemum, because no resistances are known in closely related species. If a desired trait, is only present in far related species the breeder has to deal with crossing barriers. Crossing barriers can be overcome by techniques like ovarium culture or embryo rescue (Van Tuyl et al. 1991), but even then the resulting progeny is often not fertile. In those cases in which conventional breeding is problematic or impossible, the application of molecular techniques might be an alternative.

The rapid progress that has been made over the past 15 years in molecular and cellular biology has lead to the development of several techniques aimed at the introduction of one precisely known gene into the genome of a single plant cell and the subsequent regeneration of that cell into a mature plant (Uchimiya et al., 1989; Potrykus, 1990). The most obvious advantage is that the recipient plant does not loose its characteristics and only gains one property. This means that usually back crossings will not be necessary, which will be a substantial saving of time. Another advantage is that gene transfer is no longer limited to related species. Genetic engineering offers the opportunity to introduce resistances, bypassing all existing crossing barriers. It is now even possible to introduce genes from outside the plant kingdom. In fact most of the genes, so far introduced, originate from prokaryote species, because they are more accessible to molecular genetics. Genes from viruses and bacteria have been used successfully in protecting plants from viral or insect attack (Lawson et al., 1990; Vaeck et al. 1987) and the isolation of new resistance genes is frequently reported (Höfte et al., 1987; Hilder et al., 1987; Ward and Ellar, 1988; Hilder, 1989). Of course, the application of these genetic modification techniques brings along its own problems. The integration of a gene in the plant DNA is a random process, therefore it is not possible to predict the level of expression of the new gene. Because the underlying mechanisms in the plant involved in genetic transformation are often not known in detail, most of the protocols have been worked out using trial and error procedures. This also means that for each new plant species that has to be genetically engineered the basic procedures of regeneration and transformation have to be developed anew.

Research on genetic engineering in vegetable crops is far ahead of that in ornamentals. The only ornamental crop that can be routinely genetically modified until now is petunia. Petunia belongs to the solanaceous species, which are in general easy to transform and are therefore often used as model species in plant molecular genetics studies (Willmitzer, 1988; Van Der Krol et al., 1990). Thus far, there are no examples of ornamental crops that have been genetically engineered in order to breed for resistance.

Introduction of insect resistance in chrysanthemum.

The research project 'Resistance to insects in Chrysanthemum by introduction of toxin genes from *Bacillus thuringiensis*' has started in 1987 as a co-operation project of the Institute for Horticultural Plant breeding (IVT), the Research Institute Ital (in 1990 both institutes merged with the Foundation for Agricultural Plant Breeding (SVP) to form the Centre for Plant Breeding Research (CPO)), and the Research Institute for Plant Protection (IPO), and was partly financed by the Innovation Fund for Plant Breeding (InPla). The aim of this project was to introduce insect resistance in *Dendranthema grandiflora*, by using the toxin genes from the entomopathogenic bacterium *Bacillus thuringiensis* (Bt). This toxin is harmless for vertebrate species and has been used for many years as a biological controlling agent (Krieg, 1986). The advantages of having the plant producing the toxin itself above spraying it on the crop are obvious. First the toxin is only present at the site where it is needed, namely in the crop plant and not in the soil or on surrounding vegetation. This can even be refined by using gene expression regulators that only activate the introduced gene when the plant is attacked by insects. Secondly the toxin reaches not only those insects that feed on the surface of the leaves but also the ones that feed in (leafminers) or under (Spodoptera) the leaf. Furthermore, the introduction of the gene spares the grower the labour and the costs involved in spraying the insecticide.
Chrysanthemum was chosen as a model species for several reasons. As pointed out above, the crop is economically important and is threatened by many pest insects.

Moreover, for several years research was performed on *in vitro* culture and regeneration of chrysanthemum, and application of biotechnology in ornamental crops was (and is) far behind on vegetable crops. Last but not least, less difficulties were expected with the release of a genetically modified ornamental crop, compared to a food crop. The research partners IVT and Ital each focussed on one of the two main issues, that constituted the project.

At the IVT research concentrated on the development of a genetic modification system for chrysanthemum. To select the most efficient way to introduce genes into plants a distinction can be made between monocotyledonous and dicotyledonous plants. For ornamentals this means roughly a division into bulb flowers and non-bulb species. This separation is based on regeneration possibilities and on the limitations of the genetic modification technique based on the natural gene transferring capacity of the plant pathogenic soil bacterium *Agrobacterium tumefaciens*. This bacterium is able to transfer foreign genes to the genome of plants and to stably integrate them in the plant DNA (for a detailed description see chapter 7). This process is very efficient in many dicots, but is extremely difficult to apply on monocots. As chrysanthemum belongs to the dicotyledonous plant species, we chose to develop an *Agrobacterium*-mediated gene transfer protocol. This part of the research project is described in the present thesis.

At the Research Institute Ital the molecular cloning and characterization of Bt genes was performed. For efficient control of the target insect it is necessary to carefully select the *B. thuringiensis* gene that is to be used. To be able to use the bacterial gene in plants it has to be modified and plant regulatory sequences have to be added to replace the bacterial regulation signals. Furthermore, domain-function relations in the toxin gene were studied. This part of the research project will be described by Guy Honée in a thesis, which is in preparation.

This thesis

The research described in this thesis deals with the *Agrobacterium* mediated transformation of chrysanthemum and the expression of Bt.-toxin genes in transformed

tissue. In chapter 2 the screening of several chrysanthemum cultivars and a range of *Agrobacterium* strains in order to find an efficient combination is described. As shown in chapter 3, the selected cultivar 'Parliament' was used in transformation experiments with *A. rhizogenes*. From these experiments it became clear that tumour induction is not always a good measure for transformation efficiency. This is confirmed in chapter 4 for *A. tumefaciens* transformation of chrysanthemum, representing a good example of the problems that can arise because of the limited knowledge on the gene transfer proces. The reporter gene GUSintron that is used in the research described in chapters 4 and 5 enabled us to gain a lot of information on early transformation events. Chapter 4 describes the use of the gene to select efficient cultivar/bacterial strain combinations, circumventing the use of tumour induction efficiency as a measure. The GUSintron gene was also used as an easy reporter gene to evaluate the effects of alterations in the transformation/regeneration protocol (chapter 5). These changes were necessary, because it was shown that in the standard procedure, the bacterial infection caused a severe reduction of the regeneration capacity of chrysanthemum genotypes. While the optimization of the method was still in progress Bt-toxin genes were introduced into chrysanthemum tissue using tumorigenic *Agrobacterium* strains. Chapter 6 describes the high level of resistance against feeding of the test insect, the tobacco bud worm (*Heliothis virescens*), that was achieved using this method. Finally, in chapter 7 a survey is given of crops that have been used in transformation research between 1987 and 1991. Both transformation with and without successful regeneration into transgenic plants were taken into account, resulting in a complete overview of the state of the art in transforming recalcitrant crops. From this and from results described in this thesis, some general rules could be deduced, which may be useful for future research on transformation of other plant species.

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CHAPTER 2

Genetic transformation of Chrysanthemum using wild type *Agrobacterium* strains; strain and cultivar specificity

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(Plant Cell Reports (1991) 9: 505-508)

Summary

To develop a procedure for *Agrobacterium*-mediated transformation of chrysanthemum we studied the transformation efficiency of commonly used *A. tumefaciens* strains on 14 chrysanthemum genotypes by comparing tumour size and frequency. One genotype was analyzed in detail using 14 strains of both *A. tumefaciens* and *A. rhizogenes*. Only a few genotype/strain combinations resulted in significant tumour formation. Especially 0-type strains were highly efficient. An 0-type strain was used to transfer genes for neomycine phosphotransferase (NPT II) and ß-glucuronidase (GUS) to a susceptible cultivar. Transfer of the GUS gene was confirmed by using the Polymerase Chain Reaction (PCR).

Introduction

The aim of our research is the genetic engineering of the cutflower chrysanthemum, which is globally one of the most important ornamental crops. Genetic transformation of dicotyledonous plants is still most efficiently achieved by using the natural gene transfer system of *Agrobacterium*. The susceptibility of chrysanthemum to *Agrobacterium* has already been demonstrated (Miller 1975; DeCleene and DeLey 1976), but there is no information on genetic variation in susceptibility among chrysanthemum cultivars and within a cultivar for various *Agrobacterium* strains. This is of interest because both the *Agrobacterium* strain and the cultivar used might influence transformation efficiency in recalcitrant species (Puonti-Kaerlas et al. 1989). Recently, the supervirulent strain A281 was found to be very useful for transforming recalcitrant crops (Fillipone and Lurquin 1989; Raineri et al. 1990). Strain A281 has a very broad host range and induces tumours that appear faster and are larger than tumours induced by other strains (Hood et al. 1986). The transformation efficiency of poplar was enhanced 14 times with strains harbouring the essential vir genes of A281 (Pythoud et al. 1987). We have included A281 in our experiments because of these supervirulent characteristics. The aim of the

investigation presented here was: to study genetic variation in tumour formation; to analyse the susceptibility of a sensitive cultivar for 14 *Agrobacterium* strains and to investigate whether tumours are stably transformed. For this latter purpose the transfer and expression of the NPT II and GUS genes was studied.

Material and Methods

Plant material. For greenhouse experiments 6 week old cuttings of chrysanthemum (*Dendranthema grandiflora*, Tzvel.) grown under long day conditions were used. For leaf explant transformations, four week old, sterile cuttings of cultivar (cv.) 'Parliament' were used, grown on MS medium (Murashige and Skoog 1962) containing 3 % w/v sucrose and 0.5 μM IAA, pH 5.8, solidified with 0.8 % w/v Purified Oxoid agar.

Bacterial strains: *Agrobacterium* strains were obtained from the Phabagen collection (Utrecht, the Netherlands) and from Dr. P. Hooykaas (Leiden, the Netherlands). All strains used are tumorigenic, except strain LBA4404, which is the disarmed derivative of Ach5 (Hoekema et al. 1983). Strain A281 has the chromosomal background of C58 and the Ti-plasmid of BO542 (Sciaky et al. 1978). Part of the vir region of pTiBO542, responsible for the supervirulent properties has been cloned in a cosmid vector, pTVK291 (Komari et al. 1986; Jin et al. 1987). Both A281 and A281(pTVK291) were used. The binary vector pCPO1 was derived from pPCV708 (Koncz and Schell 1986) and harbours the NPT II gene driven by the nos promoter. Insertion of GUS in pCPO1 behind the TR-2' promoter resulted in the vector pCPO1-G. Both vectors were introduced in A281(pTVK291) by conjugation according to Rogers et al. (1988). Transconjugants were selected on kanamycin and carbenicillin (both 50 mg/l). All strains were cultured in Luria Broth: 10 g/l trypton, 5 g/l yeast-extract, 5 g/l NaCl, 1 g/l glucose, pH7.0.

Greenhouse infection. Plants were wounded at an internodium and infected at the wounded spot with *Agrobacterium*, using a sterile toothpick. At least 4 plants were used for each strain. Effects were measured by looking at the number of tumour forming plants and tumour size.

Leaf explant transformation. Leaf explants were incubated for 5 min in an *Agrobacterium* suspension diluted to a concentration of about 5 x 10^8 cells/ml. Explants were blotted dry and placed upside down on hormone free MS medium, pH 5.7, containing 3% w/v sucrose and 0.7% w/v purified Oxoid agar. After two days explants were transferred to medium containing 250 mg/l cefotaxime and 400 mg/l vancomycine and after six days to medium containing half of the antibiotic concentration and if required 100 mg/l kanamycin. Tumour tissue was subcultured on the same hormone free medium.

Determination of transgene expression. GUS activity of transformed cells was determined using a fluorimetrical assay (Jefferson et al. 1987). To measure NPT II activity a dot blot assay was used, based on the protocols described by Platt and Yang (1987) and by McDonnel et al. (1987). Specific activity, bound to phosphocellulose P81 was determined by measuring the amount of label per spot in a liquid scintillation analyzer. Opine assays were performed according to Petit et al. (1983). Protein amounts in extracts were determined by using the Biorad kit.

DNA-analysis. DNA was isolated from tumour tissue following the procedure of Koes et al. (1989). To remove the excess of poly-saccharides an extra purification step was required. 1 M NaCl was added and the solution was incubated for 15 min at -20° C, followed by centrifugation at 10.000 *g* for 15 min and precipitation of the DNA from the supernatant. The presence of the GUS gene in tumours was demonstrated by using PCR (Mullis et al. 1986). Amplitaq polymerase was purchased from Perkin Elmer Cetus. The amplification protocol was: 1 min melting at 92 °C, 1 min annealing at 63 °C and 2 min elongation at 72 °C for 30 cycles. The oligonucleotide primers were 5'-CTG TAG AAA CCC CAA CCC GTG-3' and 5'-CAT TAC GCT GCG ATG GAT CCC-3', yielding a fragment of 514 basepairs.

Table 1. Virulence of 3 *Agrobacterium* strains on 14 genotypes of chrysanthemum.

	size classes														
Genotype	LBA4404					Ach5					C58				
	0	1	2	3	4	0	1	2	3	4	0	1	2	3	4
Parliament	4	-	-	-	-	-	-	4	-	-	2	2	-	-	-
P.Lane	3	1	-	-	-	2	2	-	-	-	3	1	-	-	-
D.Flamenco	4	-	-	-	-	2	2	-	-	-	3	-	-	-	1
Refour	4	-	-	-	-	3	-	-	-	-	4	-	-	-	-
Daymark	3	1	-	-	-	4	-	-	-	-	-	4	-	-	-
S.Cassa	4	-	-	-	-	3	1	-	-	-	2	2	-	-	-
Greenpeas	3	1	-	-	-	-	4	-	-	-	-	4	-	-	-
L.Bijoux	3	1	-	-	-	3	1	-	-	-	-	2	2	-	-
Cottonball	4	-	-	-	-	4	-	-	-	-	-	4	-	-	-
D.P.Pompom	1	3	-	-	-	-	1	3	-	-	-	-	3	-	-
Album	3	-	-	-	-	4	-	-	-	-	1	2	1	-	-
Topper	2	2	-	-	-	3	1	-	-	-	2	2	-	-	-
Carroussel	2	2	-	-	-	4	-	-	-	-	-	2	2	-	-
Guilderland	3	1	-	-	-	4	-	-	-	-	4	-	-	-	-

Italicized genotypes were tested in a separate experiment. Tumours were divided into five classes; 0=0-1 mm, 1=1-3 mm, 2=3-5 mm, 3=5-7, 4=larger than 7 mm. Underlined means significantly different from control.

Results

Genotype effect

Fourteen cultivars were infected with two wild type bacterial strains; C58 (LBA201), a nopaline strain, and Ach5 (LBA1), an octopine strain. LBA4404 was used as a control. After 4 weeks the effect of infection was scored. The susceptibility to Ach5 and C58 was highly genotype dependent (table 1). The wound reaction also varied between genotypes and in some cases was rather strong, which interfered with detection of tumour formation. Those genotype/strain combinations in which all four plants showed larger tumours than the controls were considered to be significantly susceptible to *Agrobacterium*. Three genotype strain combinations resulted in a significant tumour induction.

Table 2. Susceptibility of *D. grandiflora* cv. 'Parliament' to 14 *Agrobacterium* strains.

Bacterial strain		opine type	size classes 0	1	2	3	4	5	
B6	(LBA1)	o	2	4	-	-	-	-	A
15955	(LBA8250)	o	6	-	-	-	-	-	A
T37	(LBA8370)	n	-	5	1	-	-	-	B
ID135	(LBA8150)	n	-	6	-	-	-	-	B
C58	(LBA201)	n	-	5	1	-	-	-	B
398	(LBA8180)	0	-	-	-	-	-	6	C
BO542	(LBA8120)	0	-	-	-	1	4	1	C
-	(LBA8490)	c	-	5	1	-	-	-	B
223	(LBA9220)	n	-	6	-	-	-	-	B
1855	(LBA9402)	a	-	4	2	-	-	-	B
8196	(LBA9365)	m	1	5	-	-	-	-	A
EU6	(LBA9000)	n	4	2	-	-	-	-	A
1771	(LBA9070)	0	-	-	-	1	5	-	C
CHR3	-		5	1	-	-	-	-	A
Control			6	-	-	-	-	-	A

Strains are referred to by their original names, LBA numbers are given between brackets. Six tumour classes were distinguished; 0=0-1 mm, 1=1-3 mm, 2=3-4 mm, 3=5-7 mm, 4=8-10 mm, 5=larger than 10 mm. Underlined means significant reaction. A, B and C are significantly different groups. Control plants were inoculated with water. Abbreviations: o=octopine, n=nopaline, a=agropine, m=mannopine, c=cucumopine, 0=not octopine or nopaline.

Effect of bacterial strain

To analyse the susceptibility of cv. 'Parliament', a broad range of bacterial strains was tested covering all common opine types all biotypes and *A. tumefaciens* as well as *A. rhizogenes* strains. 'Parliament' was chosen because regeneration protocols were available, which made it attractive for future transformation experiments (Broertjes and Lock 1985). Six weeks after inoculation the effects of infection were examined. The reaction was regarded to be significant only when there was no tumour size overlap with the control plants. Strain CHR3, which is an *Agrobacterium* strain isolated from chrysanthemum tumours (J.Tempé, pers. comm.) is not virulent on cv. 'Parliament'. Nine strains induced a reaction that differed significantly from the control (table 2). In this experiment infection with C58 resulted in small tumours, whereas in the first infection assay C58 did not induce tumours on cv. 'Parliament'. This can be explained by the fact that tumour formation in the second experiment was scored 2 weeks later. Strains 398, 1771 and BO542, that significantly induce the largest tumours (group C in table 2) all belong to the 0-type.

Tumour induction in vitro.

To analyse the expression of transferred marker genes, leaf explant transformation experiments were performed. Infections were carried out with 4 strains: A281, A281(pTVK291), A281(pTVK291+pCPO1) and A281(pTVK291+pCPO1-G). Previous experiments had shown that tumour growth is completely inhibited on medium containing 50 mg/l kanamycin. On non selective, hormone free medium 85%-100% of the explants produced hormone autotrophic callus, which was already visible eight to ten days after infection. The extra vir region on the cosmid pTVK291 did not significantly enhance tumour formation. Three weeks old callus induced on non selective medium by all 4 strains, was subcultured under selective and nonselective conditions. For each treatment 144 independent callus lines were used. Kanamycin (100 mg/l) completely inhibited growth of wild type tumours (fig.1). Half of the tumours induced by bacteria containing binary plasmids were resistant to kanamycin, indicating 50% cotransfer of the two T-DNA's. The tumours have now been subcultured for more than a year on selective medium.

Analysis of transformants; use of PCR.

Eight calli, derived after transformation of cultivar 'Parliament' with strain A281(pTVK291+CPO1-G) were analysed in more detail. The calli were maintained on selective medium. Callus of each transformant was used in NPT II, GUS and opine

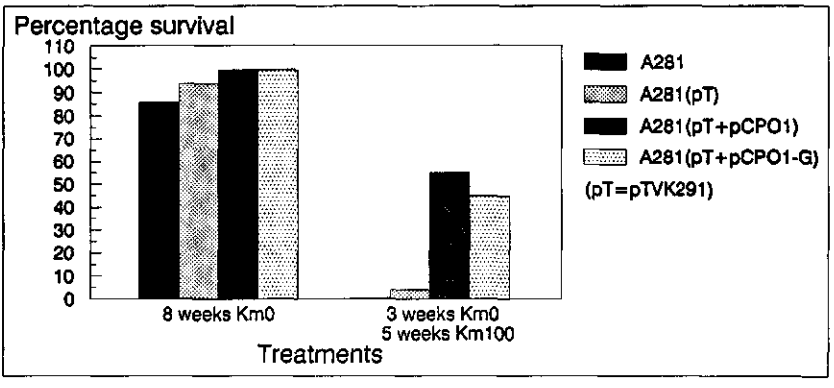

Figure 1.
Survival of tumours induced on non selective medium, 5 weeks after transfer to selective or fresh non selective medium. Figures are based on 144 pieces of callus for each treatment.

Table 3. Analysis of 8 independent transformed calli

Callus No.	GUS activity		NPT II activity	
	mean	sd	mean	sd
G3	386.0	30.0	40	9
G4	0.0	-	n.d.	-
G7	0.0	-	276	190
G8	144.0	30.0	51	17
G9	0.0	-	894	290
G15	466.0	17.0	227	139
G16	12.7	6.0	84	71
G17	148.3	30.0	98	69

The results represent the mean of 4 determinations. GUS activity is in μmol MU/min/mg protein, NPT II activity is in cpm/μg protein; sd = standard deviation.

assays. In all calli NPT II activity as well as opines could be detected (table 3, fig.2). In G9 and G15, only mannopine was found and agropine was absent.

Although the GUS and NPT II gene are closely linked on the same T-DNA not all of the analysed calli exhibited GUS activity. The level of expression of the NPT II and GUS genes varied considerably between the calli (Table 3). No correlation between relative GUS and NPT II activities was observed. DNA-analysis using Southern blotting methods was difficult because of the large genome of *D. grandiflora* (25 pg DNA/cell, determined flowcytometrically). Therefore the presence of transferred genes in the calli was demonstrated using the PCR technique. The GUS gene was selected as target DNA because it is the gene located closest to the left T-DNA border. In 6 out of 8 calli the presence of the GUS gene could be demonstrated (fig.3). No positive signals were found for tumours G7 and G9, which were also negative in the GUS assay.

Discussion

The efficiency of *Agrobacterium* mediated gene transfer to chrysanthemum strongly depends both on genotype and bacterial strain. Out of 14 genotypes of

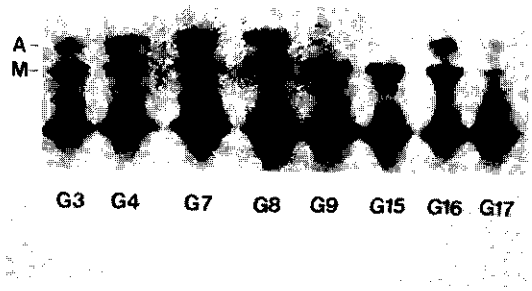

Figure 2.
Agropine/mannopine assay on 8 independently transformed tumours (Abbr.: A=agropine, M=mannopine).

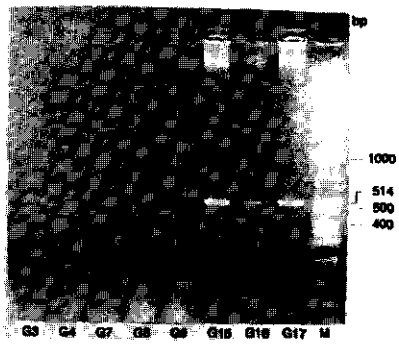

Figure 3.
Polymerase chain reaction analysis of 8 independently transformed tumours. Amplification of a 514 bp fragment of the ß-glucuronidase gene.

chrysanthemum only 4 were found to be significantly susceptible to infection with Ach5 and C58. Nine other strains, including the supervirulent strain BO542, showed significant virulence on 'Parliament'. Remarkably a correlation between opine-type and virulence was found. The strains significantly inducing the largest tumours all belonged to the 0-type. Unlike a correlation between nopaline type strains and virulence (Byrne et al. 1987), a relation between 0-type strains and host range has not been reported before. For nopaline strains the presence of the *tzs* locus can explain the enhanced virulence (Akiyoshi et al. 1985) but the biological basis for a correlation between 0-type strains and virulence is not clear. The results of the infection assays stress the fact that it is extremely important to select both cultivar and bacterial strain with great care if recalcitrant plant species are involved.In contrast to results with *Nicotiana glauca* (Jin et al. 1987), the presence of an extra vir-region on cosmid pTVK291 did not enhance the virulence of A281 towards chrysanthemum. Using A281(pTVK291) containing a binary plasmid, harbouring the NPT II and GUS genes, it was shown that genes can be stably integrated and expressed in chrysanthemum. Though closely linked on the same T-DNA no relation was found between relative enzyme activities of NPT II and GUS. This may be

caused by different regulation of the promoters or by a position effect. Therefore enzyme activity of a reporter gene is not a measure of the degree of expression of cotransferred genes.

In 3 out of 8 analysed kanamycin resistant calli no GUS activity was observed. This could be caused by imperfect transfer of the T-DNA. T-DNA single strand synthesis starts from the right border (Zambryski et al. 1989), which means that the left part of the T-DNA can be incomplete. This is in line with the observation that with the PCR technique, which proved to be very useful to detect single copy genes in large plant genomes, the GUS gene was only found in 1 out of the 3 GUS negative tumours. The lack of GUS activity in tumour G4 must be caused by absence of transcription. Selection for a correctly expressed NPT II gene does not necessarily mean that complete T-DNA's are transferred. Therefore it would be better to use vectors on which the selection marker is located close to the left hand border, so that selection for kanamycin resistance is also selection for presence of a full length T-DNA.

Acknowledgement

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C H A P T E R 3

Agrobacterium rhizogenes mediated induction of untransformed roots and callus in chrysanthemum

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and Hans J.M. Dons.

(Submitted for publication)

Summary

The agropine type *Agrobacterium rhizogenes* strain LBA9402 induced callus and roots on stems of greenhouse grown plants and on leaf disks of *in vitro* grown plantlets of chrysanthemum (*Dendranthema grandiflora* Tzvel.). In this callus and roots no opines were detected, nor were any of the other features of the 'hairy root' syndrome observed. Experiments aimed to identify the nature of the tumour-like growth revealed that induction was correlated with the presence of the TR-DNA on the Ri-plasmid. Root induction was probably the result of auxin synthesis following transient expression of *iaaM* and *iaaH* genes, present on the TR-DNA. The chrysanthemum cultivar used, cv. 'Parliament', showed a high auxin sensitivity compared to tobacco. Analysis of early transformation events using the GUSintron reporter gene revealed that low efficient gene transfer and transient gene expression took place, but most probably without stable integration of the T-DNA in the plant genome. The results presented here stress the fact that callus formation or root induction as measures for transformation efficiency should be used with caution.

Introduction

Agrobacterium rhizogenes is the causative agent of the hairy root syndrome, the proliferation of highly branched roots, and affects a wide range of plant species (De Cleene and De Ley, 1981). The induced roots have an altered phenotype, with specific features such as phytohormone independent growth, extensive branching and plagiotropism (Gelvin, 1990). The underlying mechanism is the transfer of several genes to the plant genome, mediated by the bacterium. These genes, the root loci or *rol* genes, are located on a piece of DNA, the T(ransfer)-DNA, that is part of a large plasmid, the root inducing or Ri-plasmid. The products of the *rol*-genes probably alter the sensitivity of plant cells to auxin (Gelvin, 1990). In addition to this, some *A. rhizogenes* strains are able to transfer a second piece of DNA, carrying the *iaaM* and

iaaH genes, coding for enzymes involved in auxin biosynthesis. The piece of DNA carrying the rol genes is called TL-DNA whereas the other part, harbouring the *iaa* genes, is known as TR-DNA. Analogous to the *A. tumefaciens* system artificial T-DNA can be transferred to the plant using *A. rhizogenes*, either in addition to its own T-DNA or by using disarmed strains (Vilaine and Casse-Delbart, 1987; Ottaviani et al., 1990). Gene transfer mediated by *A. rhizogenes* can be advantageous compared to gene transfer by *A. tumefaciens*, because *A. rhizogenes* strains are often more virulent than *A. tumefaciens* strains. It is also relatively simple to regenerate shoots from transformed 'hairy' roots (Noda et al., 1987; Ottaviani et al., 1990), which is valuable when direct regeneration of shoots from transformed cells is not possible.

As a part of our effort to develop a genetic transformation protocol for chrysanthemum we have tested the virulence of several *A. rhizogenes* strains *in vivo* (Chapter 2: Van Wordragen et al., 1991), which revealed that both cucumopine and agropine strains induced tumours on *Dendranthema grandiflora* cultivar 'Parliament'. In this study the tumour proliferation after infection with the agropine strain LBA9402 was further analysed. Surprisingly, the often large tumours and the induced roots never contained opines, nor could any of the other characteristics of the hairy root disease be detected. To be able to answer the question whether the induced 'tumours' are the result of gene transfer or not, we investigated root induction on leaf disks from *in vitro* grown plants.

Material and methods

Plant material. Plants from the chrysanthemum (*Dendranthema grandiflora*) cultivar 'Parliament' were used both in greenhouse experiments and *in vitro*. *Nicotiana tabacum* 'Petit Havana SR1' was used in *in vitro* experiments only. Plants were grown as described earlier (Chapter 2: Van Wordragen et al., 1991).

Bacterial strains. The *A. rhizogenes* strain LBA9402, carrying the plasmid pRi1855, is an agropine strain (Table 1). Its T-DNA consists of two parts; TL, containing the rol loci and TR harbouring the genes for agropine/ mannopine synthesis and the *iaa* genes, involved in auxin biosynthesis in the transformed plant cell (Vilaine and Casse-Delbart, 1987). LBA8490 is a cucumopine strain. The T-DNA of pRi8490 is homeologous to TL-DNA of pRi1855, and TR-DNA is missing (Filetici et al., 1987).
The plasmids pE110 and pH3018 were constructed by Vilaine and Casse-Delbart (1987) and contain the complete TR- and TL-region of the agropine type Ri-plasmid respectively. The plasmids were both introduced into LBA4404 by conjugation as described by Rogers et al. (1988). pE110 was also introduced into LBA8490. Transconjugants were selected on 50 mg/l rifampicine and 1.5 mg/l

Table 1. Agrobacterium strains used, and some of their characteristics.

name	Ri-plasmid	sec. plasmid[1]	T-DNA[2]	reporter gene[3]
LBA9402	pRi1855	-	TL + TR	agr/man
LBA9402(pGUSi)	pRi1855	p35SGUSintron	TL + TR + Ta	agr/man/GUS/neo
LBA8490	pRi8490	-	TL	cuc
LBA8490(pE110)	pRi8490	pE110	TL + TR	cuc/agr/man
LBA4404	pAL4404	-	-	-
LBA4404(pE110)	pAL4404	pE110	TR	agr/man
LBA4404(pH3018)	pAL4404	pH3018	TL	-

1) sec.plasmid = secondary plasmid; 2) TL= left T-DNA (or homologous to left T-DNA), TR = right T-DNA, Ta= artificial T-DNA; 3) agr = agropine, man = mannopine, cuc = cucumopine, GUS = ß-glucuronidase, neo = neomycin phosphotransferase II.

tetracycline, resulting in the strains LBA4404(pE110), LBA4404(pH3018) and LBA8490(pE110). The binary vector p35SGUSintron (Vancanneyt et al., 1990) was introduced into LBA9402 using the same conjugation protocol. The transconjugant strain LBA9402(pGUSi) was selected on 50 mg/l rifampicine and 50 mg/l kanamycin. Restriction analysis was used to confirm the transconjugant nature of the strains. Bacterial strains were maintained as glycerol stocks at -80° C. All strains were cultured at 28 °C in MYA broth (0.8 % mannitol, 0.05 % casamino acids, 0.2 % $(NH_4)_2SO_4$, 0.5 % yeast extract, 0.5 % NaCl) except for LBA9402, which was cultured in YMB (1.0 % mannitol, 0.05 % K_2HPO_4, 0.01 % NaCl, 0.04 % yeast extract, 0.02 % $MgSO_4.7H_2O$)

Greenhouse infection. Two days before infection 10 μl of an overnight grown liquid *Agrobacterium* culture was spread on solid culture plates and grown at 28°C. Greenhouse grown plants were wounded with an sterile toothpick at the 6th internode from the top. The wounded spots were inoculated using a toothpick, dipped in a bacterium colony. For each strain eight plants were infected. The infected site was protected against desiccation with parafilm tape. After two days the parafilm was replaced by wetted rockwool and covered with plastic (Van der Mark et al., 1990). Six weeks after infection the effects were measured by counting the number of tumour forming plants and by estimating the tumour size. As a control plants were inoculated with the disarmed *A. tumefaciens* strain LBA4404.

Explant transformation. Leaf explants (diameter 7 mm) were punched out of the upper 4 or 5 leaves of *in vitro* grown plantlets. stem explants were prepared from the upper three internodes, that were longitudinally cut into two halves. Infection and cocultivation of the explants was carried out as described before (Chapter 2: Van Wordragen et al., 1991). Four to six weeks after infection, 'tumour' tissue was taken from the explants and subcultured on the same medium.

Determination of auxin sensitivity. Leaf explants from tobacco and chrysanthemum were placed on MS culture medium (Murashige and Skoog, 1962), supplemented with 30 g/l sucrose and containing 0, 2.5, 5, 10, 20 or 50 μM indole acetic acid (IAA). Each treatment consisted of 4 petridishes with 6 explants each. After two days the explants were transferred to hormone free MS medium containing 200 mg/l vancomycin and 125 mg/l cefotaxim, to mimic culture conditions after *Agrobacterium* infection. The number of roots per explant was determined after four weeks.

Regeneration of adventitious shoots on internodes. Internode explants were infected with LBA4404 or LBA9402 and placed with the wounded site on culture medium either without phytohormones, with 4 μM benzyl adenine (BA), or with 4 μM BA and 0.5 μM IAA. Non-infected explants were used as a control. Each treatment consisted of 2 petridishes with 6 explants each. After 2 days explants were transferred to medium with 200 mg/l vancomycin and 125 mg/l cefotaxim to suppress bacterial growth. Regeneration of adventitious shoots was scored after 6 weeks.

Determination of GUS-activity. ß-Glucuronidase (GUS) activity of plant cells, transformed with LBA9402(pGUSi), was determined by using the histochemical X-Gluc assay, as described by Jefferson et al. (1987) with one adjustment. The use of a phosphate buffer of pH 7.5 instead of pH 7.0 was necessary to prevent endogenous enzyme activity (Chapter 2: Van Wordragen et al., 1991). The assay consisted of 4 petridishes containing 6 explants each. Half of the explants (derived from all 4 dishes) were examined 6 days after infection, and the other half was stained at day 21.

Determination of opines. Opine assays were performed using paper chromatography. Agropine and mannopine were detected using silver staining as described by Petit et al. (1983). Cucumopine was detected using the Pauly reagent as described by Savka (1990).

Results

Tumour induction in vivo

Greenhouse grown plants from the cultivar 'Parliament' were infected with the oncogenic *A. rhizogenes* strain LBA9402. On the site of infection a large proliferation of undifferentiated tissue and the development of roots was observed (Fig.1A). The noninfectious strain LBA4404, which was used as a control, caused no reaction of the plant except occasionally a minor development of wound callus at the site of infection. The size of tumours on LBA9402 infected plants was enlarged compared to previous

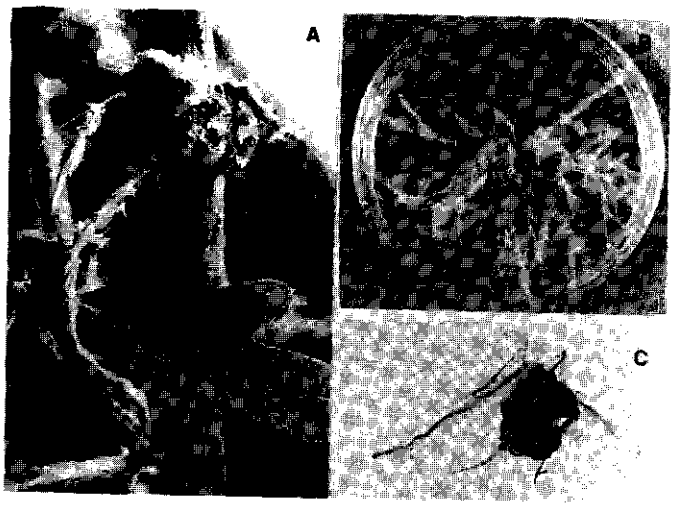

Figure 1.
Tumorous tissue induced by *A. rhizogenes* LBA9402. A) Stem tumour on 'Parliament' *in vivo*. B) Hairy roots originating from tobacco SR1 leaf explants. C) Leaf disk tumour on 'Parliament' *in vitro*.

Table 2. Root and callus induction on leaf explants of chrysanthemum and tobacco upon infection with various *Agrobacterium* strains.

	SR1			Parliament		
	roots[1]	callus[2]	opines[3]	roots[1]	callus[2]	opines[3]
LBA4404	0	-	nd	0	-	nd
LBA4404(pE110)	0	++	A/M	3	+	none
LBA4404(pH3018)	13	+	none	0	-	nd
LBA8490	23	+	C	0	-	nd
LBA8490(pE110)	30	+	A/M/C	0	-	nd
LBA9402	>100	+	A/M	25	+++	none

1) The figures represent the mean nr. of roots and callus per petridish, containing 6 explants; 2) - = no callus, +, ++ and +++ = increasing amounts of callus; 3) A = agropine, M = mannopine, C = cucumopine, none = no opines detected, nd = not determined.

assays (Chapter 2: Van Wordragen et al., 1991), by protection of the infected wound with wetted rockwool. Galls varied in diameter from 6 to 12 mm, measured from the stem. On each tumour adventitious roots developed. A mean number of 5 $\pm$ 2 roots was calculated over eight infected plants. Tissues from six independent tumours and from ten roots derived from these plants were assayed for the presence of opines. Neither agropine nor mannopine was found in any of the tissues. To enable further analysis of this phenomenon several *in vitro* transformation experiments were carried out.

In vitro infection with various A. rhizogenes strains; effect of TL and TR DNA

Leaf explants of tobacco SR1 and the chrysanthemum cv. 'Parliament' were infected with the strains LBA9402, LBA8490, LBA8490(pE110), LBA4404, LBA4404(pE110) and LBA4404(pH3018). The disarmed control strain LBA4404 did not induce roots or substantial amounts of callus on leaf explants of either tobacco or chrysanthemum (Table 2).

Upon infection with the various strains of *A. rhizogenes*, explants from tobacco SR1 formed neoplastic outgrowths, as expected. Infection with the TL-DNA containing strains LBA9402, LBA8490, LBA8490(pE110) and LBA4404(pH3018) resulted in the formation of large numbers of highly branching, fast growing roots, which were easily

recognized as 'hairy roots'. Occasionally some callus was formed on the edge of the leaf explant at sites where many roots originated. The hairy roots grew vigorously on hormone free culture medium with a growth rate of approximately 4 cm per week (Fig 1B). Opine assays revealed the presence of agropine and mannopine in most of the tested roots, originating from LBA9402 infection and cucumopine in most roots induced by strain LBA8490. In roots induced by LBA8490(pE110), in which the Ri-plasmid of the cucumopine strain is combined with the TR-DNA of the agropine strain, either one or both opine types were detected. As expected, roots induced by strain LBA4404(pH3018), harbouring TL-DNA only, did not contain opines as the opine synthetase genes are located on the TR-DNA. No roots were induced after infection of tobacco with LBA4404(pE110). This strain contains the TR-DNA, which is known to be unable to induce hairy roots in tobacco (Vilaine and Casse-Delbart, 1987). Instead some callus was formed, which contained agropine and mannopine.

The results obtained with leaf disks of chrysanthemum strongly contrast those obtained with tobacco (Table 2). Chrysanthemum explants produced adventitious roots only upon infection with the strains LBA9402 and LBA4404(pE110), both harbouring TR-DNA. Leaf explants infected with LBA8490, LBA8490(pE110) or LBA4404(pH3018) produced no roots or callus and became necrotic within two weeks. Infection with LBA9402 also resulted in a strong proliferation of callus tissue, very similar to the tumour-like growth induced *in vivo* (Fig.1C). Subculture of callus and roots on hormone free medium, however, was not possible. After four to five weeks the callus stopped growing and excised roots first grew at a rate of less then 0.5 cm per week but died within three weeks. The phenotype of the roots and their growth properties on hormone free medium resembled those of normal roots.

More than a hundred calli and roots induced on chrysanthemum leaf disks by LBA9402 infection, originating from twelve experiments, were assayed for the presence of agropine and mannopine. None of them contained detectable amounts of opines. Infection of chrysanthemum leaf disks with LBA4404(pE110), harbouring TR-DNA alone, also resulted in the induction of phenotypically normal roots. These roots resembled the roots induced by LBA9402, but they appeared in smaller numbers. Agropine and mannopine were not detectable in these roots.

The role of auxin in root induction.

The observed root induction on chrysanthemum explants upon LBA9402 infection could be attributed to the stimulating effect of auxin, synthesized either by the bacteria or by the plant cell. To investigate this hypothesis, leaf explants of 'Parliament' and tobacco SR1 were cultured on a range of auxin concentrations, and the number of induced roots was scored. Root induction on leaf explants of chrysanthemum occurred at high rates and appeared even at the lowest concentration of auxin, whereas tobacco leaf disks only formed a low number of roots at the highest auxin concentration used (Fig.2). The induced roots of both species were phenotypically normal. No callus was induced on tobacco explants, not even at the highest auxin concentration, whereas on 'Parliament' callus was formed at auxin concentrations of 10 μM and higher. This compact, green callus resembled the callus induced upon infection of 'Parliament' leaf explants with LBA9402, but the ratio of callus to roots was inversed, as upon culture on auxin little callus and many roots were formed.

To determine an auxin-like effect of LBA9402 on chrysanthemum, internode explants of 'Parliament' were infected with LBA9402, and shoot regeneration was studied in the

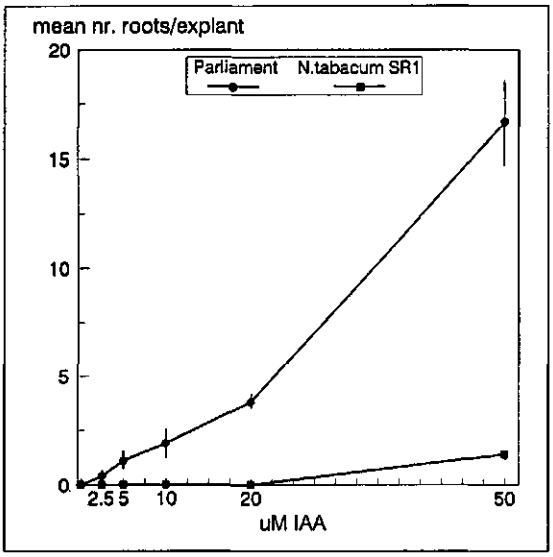

Figure 2.
The effect of auxin on root formation on leaf explants of chrysanthemum cv. 'Parliament' and tobacco SR1. Figures are the mean number of roots induced per explant, measured over twelve leaf explants. Vertical lines represent the standard deviation.

Table 3. Shoot and root induction on internode explants of chrysanthemum cv. 'Parliament'

strain	+BA,+IAA[1]		+BA,-IAA		-BA,-IAA	
	roots	shoots	roots	shoots	roots	shoots
uninfected	0	135	0	10	0	0
LBA4404	0	30	0	0	0	0
LBA9402	0	39	0	29	25	0

The figures represent the mean number of roots and shoots per petridish, containing 6 explants.
1) BA=4 μM benzyl adenine, IAA=0.5 μM indole acetic acid.

presence or absence of exogenous auxin. This should reveal whether LBA9402 could compensate for auxin in the culture medium (Table 3). Uninfected explants placed on regeneration medium, supplemented with both auxin and cytokinin, produced numerous adventitious shoots all over the surface of the internode. Shoot regeneration was reduced upon infection with both wild type and disarmed *Agrobacteria*. On medium with BA but without IAA shoot regeneration on uninfected explants and explants infected with the disarmed LBA4404 was severely inhibited, only rarely shoots were formed at the basal end of the explant. In contrast, LBA9402 infected explants formed shoots to the same extent as on culture medium with IAA, suggesting complementation for the externally applied auxin. On hormone free medium no shoots were induced, but explants infected with LBA9402 showed root induction. The addition of BA inhibited this root induction by LBA9402 infection.

Detection of early transformation events using the GUSintron reporter gene.

Early transformation events were detected by using an intron containing GUS gene. Using this gene, bacterial GUS activity, which hampers detection of transformed plant tissue when the uninterrupted GUS gene is used, was eliminated.

Leaf explants from tobacco SR1 and 'Parliament' were infected with LBA9402 or with LBA9402(pGUSi) and glucuronidase activity was determined after 6 or 21 days (Table 4). Both on chrysanthemum and on tobacco, less roots were formed if the strain carrying the binary vector was used compared to infection with LBA9402. In tobacco, many blue spots were observed at day 6 and 45% of the roots, formed after 21 days

Table 4. Early detection of transformation in tobacco and chrysanthemum leaf explants by determining expression of the GUSintron gene.

	SR1				'Parliament'			
	LBA9402		LBA9402 (GUSint)		LBA9402		LBA9402 (GUSint)	
	spots	% pos.	spots	% pos	spots	% pos.	spots	% pos.
day 6	0	0	173	92	0	0	11	42
day 21	0	0	114	83	0	0	0	0

Each figure represents the result of twelve explants. Both the total number of blue spots and the percentage of positive explants are shown (100 % = 12).

stained blue, mainly at the roottip and in the vascular tissues. In 'Parliament' only occasionally a transformation event at day 6 was detected and none after 21 days. The few blue spots were found mainly in callus tissue that started to develop. No blue spots were found in root primordia at day 6 nor in roots at day 21. These results suggest that gene transfer to 'Parliament' by LBA9402 is a rare and transient process.

Discussion

Infection of the chrysanthemum cultivar 'Parliament' with the *A. rhizogenes* strain LBA9402 resulted, both *in vivo* and *in vitro*, in the proliferation of large amounts of callus and some roots. This tissue did not show hormone autotrophic growth and did not contain opines. However, the seeming tumour induction on chrysanthemum could not be ascribed to a wound or stress response, because infection with the disarmed strain LBA4404 did not result in any callus or root proliferation. In contrast to this LBA9402 induced large numbers of hormone autotrophic, agropine and mannopine containing, roots on explants of tobacco.

Transformation of chrysanthemum using another *A. rhizogenes* strain, and *Agrobacterium* strains carrying only TL- or TR-DNA revealed that the phenomenon was correlated with the presence of TR-DNA in the infecting bacterial strain. TR-DNA harbours the

opine and auxin synthesis genes, whereas the *rol*-loci, responsible for the hairy root syndrome are located on TL-DNA (White et al., 1985; Gelvin, 1990). The fact that opines were never detected in callus and roots after infection with *A. rhizogenes* strains contrasts with the distinct expression of opine synthesis genes in chrysanthemum tissue induced by the *A. tumefaciens* strain A281 (Chapter 2: Van Wordragen et al., 1991). This indicates that transferred agropine and mannopine synthetase genes are not subject to inactivation and can be expressed in chrysanthemum. Thus, the lack of opines in LBA9402 induced callus and roots on chrysanthemum suggests that this tissue does not result from stable integration of T-DNA, whereas tumours induced by A281 do originate from stable gene transfer. However, genes present on the TR-DNA are involved and it is tempting to postulate that the observed neoplastic growth is a result of unstable expression of the auxin synthesis genes. Evidence for an auxin-like effect of LBA9402 infection was provided by the capability of LBA9402 to compensate for IAA during shoot regeneration. The inhibition of *A. rhizogenes* induced root formation by cytokinin also contributes to the evidence for the hypothesis that TL-DNA is not involved, because Spena et al. (1987) showed that root induction by the rol genes is not inhibited by externally applied cytokinin.

Results with auxin application showed a very high sensitivity of 'Parliament' to externally applied auxin, compared to tobacco. A low concentration of auxin in the culture medium for only two days was sufficient to induce roots on leaf explants of 'Parliament' and therefore, temporary TR-DNA expression might be enough to induce the proliferation of roots and callus.

Branca et al. (1991) have found that high auxin concentrations favour the induction of callus over roots. In chrysanthemum mainly callus is induced upon LBA9402 infection and only a few roots, and therefore, the pronounced sensitivity to auxin possibly reflects a high endogenic auxin level. Such a high endogenous auxin level might also explain the absence of TL-DNA transfer in chrysanthemum. Expression of the *rol* genes, located on TL-DNA, would result in an even enhanced auxin sensitivity of the plant cell (Shen et al., 1990). Such a change might very well be lethal in the case of the apparently very narrowly tuned internal hormone balance of 'Parliament'. Therefore, we assume that TL and TR DNA transfer both occur, but TL transformants will either

die or inactivate the transferred DNA, leaving only TR transformants to cause the observed effects. A similar conclusion was reached by Prinsen et al. (1990) to explain the consistent inactivity of the auxin synthesis gene, *iaaM*, transferred to Asparagus by oncogenic *A. tumefaciens* strains.

The relatively low number of roots induced by LBA4404(pE110) and the lack of roots induced by LBA8490(pE110), both strains harbouring the TR-DNA, might be due to their chromosomal background, which differs from LBA9402.

In conclusion, it is likely that root proliferation on chrysanthemum explants after infection with *A. rhizogenes* results from the temporary expression of the *iaa* genes on the TR-DNA, resulting in an enhanced auxin level in the plant cells. Considering the high sensitivity of the cv. 'Parliament' to auxin, even a minor increase of the auxin level can result in the induction of root primordia. Moreover, the transient nature of *iaa* gene expression is in agreement with the transient expression of the GUS gene in chrysanthemum tissue after transformation with LBA9402 containing the GUSintron gene.

One of the first steps in the development of an *Agrobacterium* mediated gene transfer protocol for recalcitrant crops is often the screening of a range of genotypes and *Agrobacterium* strains by infection with wild type strains. This procedure is applied for rapid selection of efficient genotype/bacterial strain combinations. (Byrne et al., 1987; Stomp et al., 1990; Chapter 2: Van Wordragen et al., 1991). In some studies only tumour size and frequency are used as a selection criterium (Armstead and Webb, 1987; Clapham et al., 1990). The results presented in this paper show that this approach can easily lead to false conclusions, because tumour induction is the result of several factors such as T-DNA transfer, T-DNA expression and hormone sensitivity of the plant. Thus, tumour size or root induction is not always directly proportional to efficiency of gene transfer. In our opinion tumour size and frequency should not be used as a measure for transformation efficiency, to avoid misleading results; gene transfer must at least be confirmed by opine assays and preferably by DNA analysis.

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C H A P T E R 4

Rapid screening for host-bacterium interactions in *Agrobacterium*-mediated gene transfer to chrysanthemum, by using the gus-intron gene

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(Submitted for publication)

Summary

The susceptibility to gene transfer of seven genotypes of chrysanthemum (*Dendranthema grandiflora*), upon infection of leaf explants with wild-type *Agrobacterium* strains, were compared in a search for highly efficient cultivar/bacterial strain combinations. Large differences between genotypes were found, but for all genotypes the supervirulent strain A281 was more efficient than the non supervirulent strain Ach5. Early transformation events could be monitored by using an intron containing ß-glucuronidase (GUS) gene. It was found that expression of the GUS gene in chrysanthemum is detectable several days later than in tobacco. There was no clear correlation between size and frequency of tumour formation following infection with wild-type strains of *Agrobacterium* and transformation efficiency, as determined with the GUS-intron gene. The GUS-intron gene proved useful in the location of preferential gene transfer sites. Cells or cell clusters expressing GUS were mostly found near the edge of the leaf explant.

Introduction

The cutflower chrysanthemum (*Dendranthema grandiflora* Tzvel., previously known as *Chrysanthemum morifolium* Ramat.) is an economically important ornamental crop. As in many ornamentals, conventional cross breeding of chrysanthemum is seriously hampered by the high ploidy level. The development of molecular breeding strategies for chrysanthemum would therefore be very useful. Of all known gene transfer methods, the *Agrobacterium tumefaciens* mediated transformation is the most efficient way to introduce foreign genes into dicotyledonous plants. Nevertheless, the careful selection of both bacterial strain and plant genotype is reported to be essential for successful transformation of recalcitrant crops (Davis et al., 1991; Dandekar and Martin, 1988; Vahala et al., 1989). A major problem in establishing efficient *Agrobacterium* mediated gene transfer is the fact that the effects of changes in the

protocol are only noticeable after several weeks of selection. If the efficiency of the used protocol is very low the effect of changes may not be distinguished at all. This makes the development and optimization of protocols for recalcitrant species a time consuming and inefficient process. The widely used reporter gene ß-glucuronidase (GUS), on the binary vector pBI121, was primarily constructed to locate transformed tissue (Jefferson et al., 1987). However, although the gene is directed by the eukaryotic CaMV-35S promotor it is also transcribed by *Agrobacteria*. This makes early detection of transformation, within 1 or 2 weeks after infection, impossible because no distinction can be made between bacterial and plant GUS activity. Recently two approaches have been adopted to solve this problem. Janssen and Gardner (1989) used a GUS gene that lacks a bacterial ribosome binding site and Vancanneyt et al. (1990) constructed a GUS gene that contains an intron derived from the potato gene LS1. As prokaryotes are not able to splice introns out of RNA, bacterial GUS activity is efficiently prevented. The usefulness of the construct is illustrated by the recent development of a transformation protocol for sunflower using the GUS-intron gene (Schrammeijer et al., 1990). We made use of the intron containing GUS gene to investigate the efficiency of initial gene transfer in leaf explants of chrysanthemum and to find out if transformation occurs in the same regions as regeneration. The construct enabled us to study early gene transfer events (within 1 or 2 weeks after infection), without interference of bacterial GUS activity. Soon after infection, part of the visualized gene expression will represent unstable gene transfer, but we assumed that the rate at which stable integration in the genome occurred, would be proportional to the initial incidence of gene transfer. We also wanted to know whether there is a correlation between tumour proliferation and gene transfer efficiency. Tumours are the result of two factors other than transformation efficiency; expression of transferred genes and sensitivity of the plant to a surplus of phytohormones, which in turn is related to the internal hormone status.

Out of a large number of regenerable *D. grandiflora* genotypes we made a selection of seven genotypes. Two wild-type *Agrobacterium* strains, Ach5 and A281, were used to determine the susceptibility of these genotypes to infection. A281 efficiently induces large tumours *in vivo* and *in vitro* on the chrysanthemum genotype 'Parliament'

(Chapter 2: Van Wordragen et al., 1991). The strain has also proved useful in transforming other recalcitrant crops such as goosefoot (Komari, 1990) and rice (Raineri et al., 1990). A281 is called supervirulent, because it is a broad host range strain and induces tumours that appear faster and are larger than tumours induced by other strains (Guyon et al., 1980; Hood et al., 1986). The results presented show that this supervirulent strain will also be very useful in the development of a transformation system for chrysanthemum. The importance of discrimination between tumour induction and transformation is stressed.

Material and methods

Plant material: Sterile cuttings of the chrysanthemum genotypes 'Recital', 'Greta Verhagen', 'Calgary', 'Toon Hermans' and 'Parliament', and the selection lines 1275 and 1610 were grown on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 3 % w/v sucrose and 0,5 μM indole-3-acetic acid (IAA), pH 5.8, solidified with 0.8 % w/v Purified Oxoid agar. Sterile cuttings of *Nicotiana tabacum* 'Petit Havana SR1' were grown on the same medium but with 2 % sucrose and without hormones. Four week old plantlets were used in transformation experiments.

Bacterial Strains: Strains were derived from the oncogenic *A. tumefaciens* strains A281 and Ach5. Two plasmids were introduced into these wild-type strains. pBI121 (Jefferson et al., 1987) harbours the neomycin phosphotransferase (NPTII) gene and the GUS gene, directed by the nos and CaMV 35S promoter respectively. The construct p35SGUSintron (Vancanneyt et al., 1990) is a BIN19 derived vector carrying an intron containing GUS gene under the control of the CaMV 35S-promoter. The plasmids were introduced by conjugation according to the protocol described by Rogers et al. (1988), resulting in the strains A281(pBI121), A281(p35SGUSint) and Ach5(p35SGUSint) (Table 1). The disarmed *A. tumefaciens* strain LBA4404 (Hoekema et al., 1983) was used as a control. All strains were cultured on Luria Broth (10 g/l trypton, 5 g/l yeast extract, 5 g/l NaCl, 1 g/l glucose, pH 7.0) with the antibiotics listed in table 1.

Transformation protocol: The upper surfaces of leaves from four week old sterile plantlets were slightly wounded with a soft sterile brush. Explants with a diameter of 7 mm were punched out of the leaves and were incubated for 5 minutes in an overnight culture of *Agrobacterium* diluted with liquid MS30 medium to a density of 5 x 10^8 cells/ml. The explants were blotted dry and placed upside down on hormone free MS medium, pH 5.7, containing 3% sucrose and 0,7% purified Oxoid agar. Six leaf explants were placed on each 9 cm Petri dish. After 2 days explants were transferred to fresh medium containing 250 mg/l cefotaxime and 400 mg/l vancomycine. Four days later the explants were transferred to fresh medium containing half of the antibiotic concentration.

Determination of opines: Four weeks after infection tumours were assayed for opine content. Agropine/ mannopine assays were performed using paper chromatography as described by Petit et al. (1983). Octopine synthetase assays were performed as described by Reynaerts et al. (1988).

Determination of GUS activity: ß-Glucuronidase in bacteria was determined in overnight cultures, resuspended in phosphate buffer (50 mM, pH 7.5) containing 1 mM 5-bromo,4-chloro,3-indolyl-ß-D-glucuronide (X-Gluc). After an incubation of 48 h. at 37 °C the appearance of a blue colour was

indicative of GUS activity. ß-glucuronidase activity of transformed plant cells was determined by using the histochemical assay, described by Jefferson et al. (1987), with one adjustment. To avoid hydrolysis of the substrate X-Gluc. by endogenous enzyme activity it was essential to use a phosphate buffer of pH 7.5 instead of pH 7.0. For microscopical observations explants were bleached and fixed in ethanol and cleared in a saturated solution of chloralhydrate in lactic acid. In all assays at least twelve explants were used for each treatment.

Results

Tumour induction in vitro

Leaf explants from seven chrysanthemum genotypes and from *N. tabacum* SR1 were infected with the *A. tumefaciens* octopine strain Ach5 and the supervirulent agropine strain A281. Almost all leaf explants developed callus which was able to grow in the absence of phytohormones (Table 2). In control experiments using the disarmed derivative of Ach5, LBA4404, no callus formation was observed and the leaf disks showed necrosis after 3 weeks. The amount of callus formed was estimated visually and the results are shown in table 2. Both strains were highly tumorigenic on *N. tabacum* SR1; the tumours grew fast and contained octopine after Ach5 transformation or agropine and mannopine after A281 transformation. Tumour formation in chrysanthemum was less extensive than in tobacco and highly genotype dependent.

Table 1. *Agrobacterium tumefaciens* strains.

Name	Virulence plasmid	T-DNA plasmid	Transgenes	Antibiotics in mg/l
A281	pTiBO542	-	ags, mas	R 100
A281(pBI121)	pTiBO542	pBI121	ags, mas, NPTII, GUS	R 100, K 50
A281(p35SGUSint)	pTiBO542	p35SGUSint	ags, mas, NPTII, GUSi	R 100, K 50
Ach5	pTiAch5	-	ocs, ags	R 100
Ach5(p35SGUSint)	pTiAch5	p35SGUSint	ocs, ags NPTII, GUSi	R 100, K 50
LBA4404	pAL4404	-	-	R 100

Abbreviations: transgenes = transferred genes besides oncogenes, ags = agropine synthetase, mas = mannopine synthetase, ocs = octopine synthetase, GUS = ß-glucuronidase, NPTII = neomycinephosphotransferase II, R = rifampicin, K = kanamycin.

Table 2: Callus induction after infection with three *A. tumefaciens* strains.

genotype	Ach5		Oct.	A281		Agr.	LBA4404	
	%pos.	cal.		%pos.	cal.	Man.	%pos.	cal.
Recital	100	4	-	92	4	+	0	0
1275	100	3	+	83	4	+	0	0
G.Verhagen	83	3	-	100	3	+	0	0
1610	92	2	+	100	4	+	0	0
Calgary	100	1	+	83	2	+	0	0
T.Hermans	67	1	-	100	1	+	0	0
Parliament	50	3	-	92	4	+	0	0
Tobacco SR1	100	4	+	100	5	+	0	0

Figures represent the mean of two separate experiments. The amount of callus (cal.) was scored 3 weeks after infection by using an arbitrary scale from 0 (no callus) to 5 (large amount of callus). Only callus forming explants (% positive) were taken into account. Each figure represents the mean from 4 Petri dishes with 6 explants each. Abbreviations: Oct=octopine, Agr.=agropine, Man.=mannopine, '-'=no opine, '+'=opines detected.

Tumour proliferation following infection with the supervirulent strain was more efficient than infection with Ach5. Both chrysanthemum and tobacco tumours, induced by Ach5,were more compact than those induced by A281. All the tumours induced by A281 contained agropine and mannopine (Fig. 1), indicating that genes from the T-DNA were expressed and that stable transformation had occured. In Ach5 induced tumours on chrysanthemum octopine was only detected occasionally and in low amounts.

Determination of background GUS expression by bacteria

To detect transformation soon after infection the plasmids pBI121 and p35SGUSintron were introduced into the wild-type *Agrobacterium* strain A281. The vector p35SGUSintron was also introduced into strain Ach5. The expression of the GUS gene in the resulting A281 strains was determined. The suspension with A281(pBI121) turned blue within 1 h., showing that the non-interrupted GUS gene is expressed. In contrast, the suspension with A281(p35SGUSint) showed no blue colouring at all, even after 48 h. incubation. Furthermore histochemical assays were carried out on leaf explants of genotype 'Parliament' 6 days after infection with A281(pBI121) and A281(p35SGUSint). Explants transformed with A281(pBI121) showed a large amount of blue precipitate

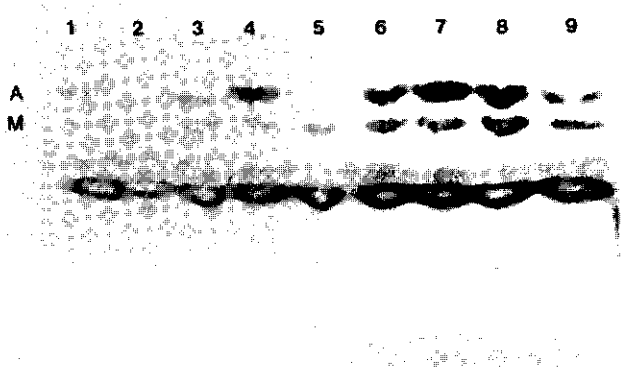


Figure 1.
Detection of agropine and mannopine in A281 induced tumours of seven chrysanthemum genotypes. Lanes: 1=tobacco SR1, 2=untransformed chrysanthemum tissue, 3='Recital', 4=1275, 5='G.Verhagen', 6=1610, 7='Calgary', 8='T.Hermans', 9='Parliament'. Abbreviations: A=agropine, M=mannopine.

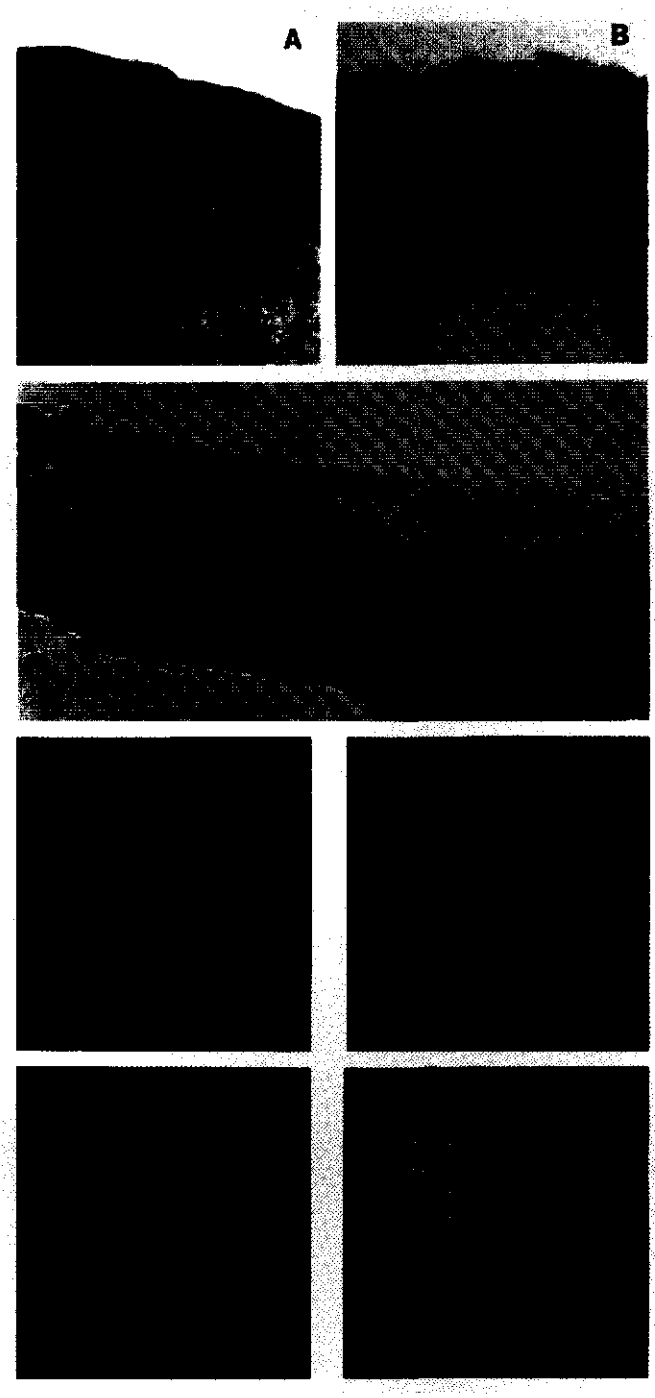
distributed all over the explant, including intercellular staining and blue leaf hairs, which had to be ascribed to bacterial GUS activity (Fig. 2A). It was not possible to distinguish between GUS expression in the transformed cells and bacterial GUS activity. If the intron containing GUS gene was used, only distinct blue spots and no intercellular coloration or blue leaf hairs were observed (Fig. 2B). This leads to the conclusion that the intron is spliced out correctly in *D. grandiflora* and that bacterial GUS activity is effectively prevented, by the presence of intron sequences.

Effect of bacterial strain and genotype

Leaf explants of seven chrysanthemum genotypes and tobacco SR1 were infected with A281(p35SGUSint) and Ach5(p35SGUSint) and the number of blue spots, indicative for gene transfer, was determined 5 days after infection using the histochemical GUS assay. Each separate spot, either cell or cell cluster, was assumed to represent one independent gene transfer event.

Figure 2:
X-Gluc assay on leaf discs of chrysanthemum 5 days after infection

A) Infection of 'Parliament' with A281(pBI121)
B) Infection of 'Parliament' with A281(p35SGUSint)
C) Cross section through a leafdisk of genotype 1610.
D) Effect of genotype on the efficiency of gene transfer. 'Calgary' x A281(p35SGUSint)
E) Effect of genotype on the efficiency of gene transfer. 1610 x A281(p35SGUSint)
F) Effect of bacterial strain on the efficiency of gene transfer 1275 x Ach5(p35SGUSint)
G) Effect of bacterial strain on the efficiency of gene transfer 1275 x A281(p35SGUSint)


A
B

Strong effects of both genotype and bacterial strain were observed (Fig. 2D-G and Fig. 3). Some chrysanthemum genotypes were at least as susceptible to *Agrobacterium* mediated gene transfer as tobacco. The supervirulent strain A281 was very effective giving rise to 80-100% positive explants, for all genotypes. The mean number of blue spots per explant varied considerably between genotypes (compare Fig. 2D and Fig. 2E), but the number of blue spots per explant was always higher after A281 infection. This was most obvious in the highly sensitive genotypes 'Recital' and 1275 (Fig. 2F and Fig. 2G). Control infections with tobacco SR1 also showed this effect of supervirulence.

Location of transformation

The location of the blue spots was similar for all genotypes. Spots occurred mostly near the edge of the explant, preferably close to the basal cut site of a major vein (Fig. 2B and Fig. 2F). The same region is the characteristic site for regeneration of adventitious shoots in all seven genotypes. When the number of spots per explant was very high, blue spots could be found all over the explant, sometimes covering large zones of the explant (Fig. 2G). Both single blue cells and blue cell clusters were found regardless of the time passed since infection. Intensity of the blue staining varied from pale blue to deep indigo. The intensity of the blue colour was not related to cell type, time or size of the blue spot, and is probably a combined effect of expression and location, on the surface or inside the callus mass, of the transformed cell. Cross sections through leaf explants revealed that blue cells could be found in every cell layer except the epidermis and vein tissue (Fig. 2C). Blue spots were never found after infection with the disarmed strain LBA4404, provided that a phosphate buffer of pH 7.5 was used. At pH 7.0 endogenous hydrolytic activity was occasionally found at the guttation points of the leaves.

Monitoring GUS expression in time

To study the development of GUS expression after infection leaf explants from genotypes 1610 and 'Calgary' and from tobacco SR1 were infected with A281(p35SGUSint) and sampled every 2 or 3 days. Two days after infection there was already a substantial number of blue spots in explants from tobacco, but none in

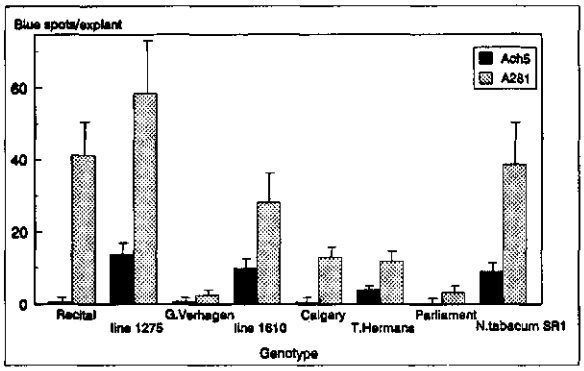


Figure 3.
Transformation efficiency of seven chrysanthemum genotypes and tobacco SR1, with A281(p35SGUSint) and Ach5(p35SGUSint). Values are the mean of 24 observations. Standard deviation is indicated by the error bars above the columns.

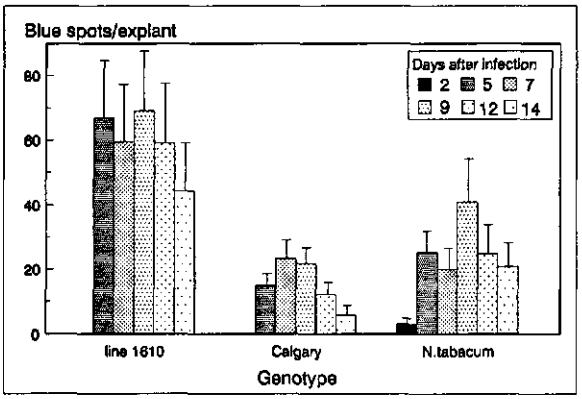


Figure 4.
Transformation of two chrysanthemum genotypes and tobacco SR1 with A281(p35SGUSint). Monitoring GUS-activity in time. Values are the mean of twelve observations. Standard deviation is indicated by the error bars above the columns.

explants from the two chrysanthemum genotypes (Fig. 4). The first blue spots could be detected 3 days (in 1610) and 4 days (in 'Calgary') after infection. Five days after infection the number of transformation events reached a peak level. In some explants of 1610 the number of spots made it impossible to distinguish between them, making it difficult to count the number of blue spots per explant (compare Fig. 2G). In these explants the number of spots was taken to be 100, the number of spots at which accurate counting was no longer possible. Though this obviously led to an underestimation of the values at 5, 7 and 9 days after infection, it was necessary to enable statistical analysis of the results. The number of blue spots tended to decrease at later times after infection for both chrysanthemum genotypes, but not for tobacco. For genotype 1610, statistical analysis was hampered by the underestimation of the values as described, but the decrease was statistically significant for 'Calgary'.

Discussion

Both by tumour formation and by GUS-intron screening it was demonstrated that in *Agrobacterium*-mediated transformation of chrysanthemum the bacterial strain as well as the plant genotype are important for the efficiency of gene transfer. The commonly used octopine strain Ach5 as well as the supervirulent strain A281 were able to induce phytohormone independent tumours on seven selected chrysanthemum genotypes, showing that chrysanthemum is highly sensitive to crown gall formation. A281 induced tumours always contained detectable amounts of agropine and mannopine, but only three genotypes formed octopine producing tumours upon Ach5 infection, whereas all seven genotypes produced autonomously growing callus. This indicates that in four cases a part of the Ach5 T-DNA, was not transferred or inactivated after transformation. Genetic transformation was confirmed in histochemical assays, in which expression of the GUS-intron gene, detectable as blue stained spots in the explant, was indicative for gene transfer. Histochemical analysis revealed that gene transfer could be achieved using the strain Ach5 but the efficiency was elevated by using the supervirulent *Agrobacterium* strain A281. After A281 infection the transformation efficiency in some genotypes was as high as in tobacco. The genotype had a large effect on the efficiency of transformation, but despite this variation A281 was always more virulent than Ach5. With the supervirulent strain blue spots were induced at the same rate as in tobacco. In most chrysanthemum genotypes gene transfer mediated by Ach5 was 4 to 5 times less efficient compared to A281, but in 'Recital' there was a twentyfold difference. This indicates that the virulence of the bacteria is a critical factor superimposed on the transformation potential of the plant. Comparison of the quantitative results of the histochemical assays using the GUS-intron gene with the results of tumour formation (Table 2) revealed that tumour formation is not always a good indicator of transformation efficiency. For instance, A281 as well as Ach5 induce tumours on the genotype 'Parliament' but histochemical assays showed little or no gene transfer events. However, tumour induction and blue spots are the outcome of different processes, namely the transfer of wild-type T-DNA and the transfer of 'artificial' T-DNA located on a binary plasmid. Therefore the discrepancy between tumour induction and number

of blue spots might be due to a very low number of co-transformations, or to preferential transfer of the wild-type T-DNA. Another explanation, which fits the genotype dependent nature of the phenomenon, is that tumour induction in this genotype might be enhanced by high phytohormone sensitivity. In assays measuring the effect on leaf explants of a short pulse of auxin in the culture medium, 'Parliament' was much more sensitive to externally applied IAA than other genotypes (data not shown). Therefore only a few transformed cells, or even transiently expressing cells, might be enough to induce tumour proliferation in 'Parliament'. Previously 'Parliament' was assumed to be highly susceptible to *Agrobacterium* infection (Chapter 2: Van Wordragen et al., 1991). The results presented here suggest that this might be true for the disease symptoms, but not for the underlying gene transfer process.

The location of the blue spots seems to indicate that the susceptibility for T-DNA transfer is different in various sectors in an explant. Cells close to the cut basal end of a major vein and near the wounded edge are most susceptible to transformation. This is partly due to the fact that these sites are wounded and will have high concentrations of wound induced substances. However, an additional factor has to be involved, because cells at the centre of the leaf explants were rarely transformed, though the upper surface of the leaf was also wounded. The large number of blue spots near the basal end of the veins might indicate that a transport dependent factor is involved as well.

Regeneration of adventitious shoots also occurs mainly on the edge of the leaf explants near the major veins. This is advantageous for the future development of a protocol aimed at the creation of transgenic shoots, using disarmed *Agrobacterium* strains. It suggests that cells that are competent of regeneration have some features in common with cells that are suitable for transformation. This characteristic might be the capability to dedifferentiate and start dividing shortly after wounding.

In some explants the formation of large GUS expressing zones could be observed. This phenomenon was also found in petunia (Janssen and Gardner, 1989), where it was ascribed to localized high gene transfer frequencies and not to cell-to-cell transport of transcription/translation products of the GUS gene. We favour the same explanation, because in chrysanthemum many spots can be found where the blue precipitate is limited to one cell, and there is no evidence that the enzyme is exported to non

transformed neighboring cells. The decrease in the number of spots per explant later on might be due to the death of stably transformed cells, to the decline of transient expression or to the joining of separately transformed cells into clusters.

The CaMV-35S promoter directed transcription of the linked GUS-intron gene in virtually all types of tissue in chrysanthemum leaf explants, though expression of GUS started slow compared to tobacco. If this also holds for the nos promoter, directing the expression of the NPT II gene, it indicates that the moment at which selection for transformed cells is applied should be postponed. Preliminary experiments in which explants from a limited number of cultivars were placed on selective medium at several times after infection with disarmed *A. tumefaciens* strains, seem to confirm this hypothesis.

In conclusion, we have demonstrated that it is possible to transform several chrysanthemum genotypes with high efficiency using a normal and a supervirulent strain and that foreign genes can effectively be expressed. The results stress the usefulness of the intron containing ß-glucuronidase gene. In a relative short period of time it is possible to obtain information about transformation efficiency, location of transformation events, effect of selection on the number of gene transfer events, which can be of great importance for the development of a transformation protocol. At the moment we are developing a transformation/regeneration protocol for chrysanthemum using the *A. tumefaciens* strains EHA101 and LBA4404. These strains are the disarmed derivatives of A281 and Ach5, respectively (Hood et al., 1986; Hoekema et al., 1983) and therefore they offer the opportunity to obtain regenerated transformed shoots directly from the explant. According to the results presented here the supervirulent strain EHA101 would be particularly useful, because of the highly efficient gene transfer rates that can be reached with A281 in chrysanthemum.

Acknowledgements

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CHAPTER 5

Restoring adventitious shoot formation on chrysanthemum leaf explants following cocultivation with *Agrobacterium tumefaciens*

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(Submitted for publication)

Summary

Explants from leaves of *in vitro* grown chrysanthemum cultivars regenerated adventitious shoots without a callus phase. Additional wounding of the explants by brushing increased regeneration of shoots in some genotypes. However, in explants which were cocultivated with *Agrobacterium tumefaciens* brushing had a strongly adverse effect on shoot formation. This negative effect of brushing could be overcome by delaying the infection with *Agrobacterium* for 8 days. Delayed infection did alter the location of transformed sites, not the number of transformation events. Although transformation occured at numerous sites along the edge of explants, no transgenic shoots were found. The failure to recover transgenic shoots from transformed leaf explants may be explained by the inability of single transformed cells to develop into shoots amongst dying non transformed tissue. Induction of a callus phase to create a larger mass of transformed cells prior to regeneration is suggested.

Introduction

Chrysanthemum (*Dendranthema grandiflora Tzvel.*) is host to many insects, feeding on leaves and flowers and frequent applications of insecticides are required to limit the damage. Plant resistance to insects would prevent losses and reduce the need for insecticidal control. Since it is not possible to introduce insect resistance into existing chrysanthemum genotypes by conventional breeding, it appears of great interest to study whether insect resistance can be obtained by molecular mehods. For that reason we want to develop a procedure for the introduction of the insecticidal toxin gene of *Bacillus thuringiensis* in chrysanthemum via *Agrobacterium* mediated transformation.
Application of *Agrobacterium* mediated gene transfer requires the regeneration of plants from transformed somatic cells. Methods to produce shoots from somatic tissues of chrysanthemum have been well documented. Adventitious shoots may be formed directly, without an intermediate callus phase, on explants of leaves (Chin-Yi Lu et al.,

1990, Kaul et al., 1990), stems (Kaul et al., 1990) and pedicels (Roest and Bokelmann, 1975). Using different media and cultivars, indirect regeneration via callus, has also been reported. Hill (1968), Miyazaki et al., (1979) and Bhattacharya et al., (1990) raised shoots from callus induced on stem explants. Petal explants may form green callus along the edges from which eventually meristematic areas develop (Bush et al., 1976). Furthermore, shoot tips have given rise to regenerable callus (Huitema et al., 1989; Earle and Langhans, 1974). Shoot formation through a callus phase enhances somaclonal variation, which results in mutated plants. If these are unwanted, like in gene transfer studies, where the genotype needs to remain otherwise unchanged, callus should be avoided. Leaf explants, which regenerate fast and directly, were therefore chosen as starting material for the development of an *Agrobacterium* mediated transformation protocol.

As in alfalfa (Hernandez-Fernandez et al., 1989), tomato (Koornneef et al., 1987) and cucumber (Nadolska-Orczyk, 1989), regeneration of chrysanthemum is genotype dependent. Regeneration protocols developed for chrysanthemum are particularly adapted to responsive genotype(s) selected for study, but frequently fail for other, less responsive, genotypes (Fukai et al., 1987). We developed an alternative medium to suit the need of the cultivar 'Parliament' and used this medium and two other documented media (Roest and Bokelmann,1975; Fukai and Oe, 1986) in a search for cultivars, that efficiently produced adventitious shoots on these media.

Following cocultivation with *Agrobacterium tumefaciens*, however, we observed that the regeneration of otherwise well regenerating cultivars was significantly reduced.

Both *Agrobacterium* mediated transformation and adventitious shoot formation are triggered by wounding the plant. Therefore, the degree and timing of the wounding are expected to be important factors in the gene transfer and regeneration proces. Experiments are reported in which the effect of wounding on the frequency of transformation, the location of the gene transfer sites and the number of shoots regenerated is studied.

Material and methods

Plant material. Plant material was maintained *in vitro* by subculturing shoot tips at 5 week intervals onto CHR 01 medium. Shoots were grown at 25°C at 0.7 W m^{-2} provided by cool white fluorescent light for 16 h per day. Explants of 7 mm were cut from the youngest three fully grown leaves. In some experiments leaves were wounded by brushing prior to *Agrobacterium* infection. Care was taken not to punch through the midrib. The explants were blotted dry and placed upside down on the media. Twenty explants, equally divided over four Petri dishes were observed per treatment. The number of shoots was counted weekly.

Plant media. All media used for the culture of plant tissue were MS basal media (Murashige and Skoog, 1962), supplemented with 30 g/l sucrose and 7 g/l purified oxoid agar. The pH was adjusted to 5.8. IAA, when used, was added after autoclaving. The following growth regulators were added:
CHR 01 : 0.1 mg/l IAA
CHR 02 : 4.0 mg/l NAA, 1.0 mg/l BA
CHR 03 : 0.1 mg/l IAA, 1.0 mg/l BA (Roest and Bokelmann, 1975)
CHR 04 : 0.5 mg/l NAA, 2.0 mg/l BA (Fukai and Oë, 1986)
Silvernitrate, when used, was added after autoclaving

Transformation. Agrobacterium tumefaciens strains were grown overnight in liquid Luria Broth (10 g/l trypton, 5 g/l yeast extract, 5 g/l NaCl, 1 g/l glucose, pH 7.0) supplemented with 50 mg/l rifampicin and, if the binary vector p35SGUSintron (Vancanneyt et al., 1990) was used with 50 mg/l kanamycin as well. Leaf explants were incubated for 5 min in an *Agrobacterium* suspension diluted with liquid MS30 medium to a concentration of about 5 x 10^8 cells/ml. Explants were blotted dry and placed on plant culture medium. After two days cocultivation the explants were transferred to fresh media with 250 mg/l cefotaxime and 400 mg/l vancomycin to eliminate the bacteria. After four days this medium was replaced by one containing half the antibiotic concentrations.
The *Agrobacterium tumefaciens* strains used were LBA4404, a disarmed derivative of the octopine strain Ach5 (Hoekema et al., 1983), and LBA4404(p35SGUSint) carrying the binary vector p35SGUSintron, which transfers ß-Glucuronidase and Neomycin phosphotransferase II (Vancanneyt et al., 1990).

ß-Glucuronidase assay. Explants were histochemically assayed for ß-glucuronidase activity (GUS) according to Jefferson et al.(1987) except for using a phosphate buffer with a pH 7.0 instead of 7.5 to avoid endogenic hydrolysis of the substrate X-Gluc. Chlorophyll was removed from stained explants with ethanol. Further clarification, when necessary, was done in a saturated solution of chloralhydrate in lactic acid. Per treatment at least 12 explants were assayed.

Results

Cultivar effects on regeneration.

At the outset of our study mainly the cultivar Parliament was used. Leaf explants of this cultivar were incubated on CHR 02 for two days to initiate cell division and subsequently transferred to CHR 01 for shoot development. Regeneration was greatly enhanced (from 1.4 to 4.6 shoots per explant; $p < 0.01$) by slightly stabbing the leaves with a paint brush prior to cutting explants. To prevent desiccation, leaves were

brushed and cut in CHR 02. Regeneration of explants not cut in liquid medium was poor.

Using this method for preparing explants, a large number of cultivars was tested for regeneration both on the medium sequence CHR 02/CHR 01, developed for 'Parliament', and on medium CHR 03, a general medium designed for pedicel explants (Roest and Bokelman, 1975). The results are presented in figure 1. A considerable number of cultivars form shoots on both media, but most of them produce only very few shoots. Some genotypes, like Parliament, performed better on CHR 02/CHR 01, while others, like line 1610, regenerated better on CHR 03 medium. Seven genotypes, Recital, line 1275, Calgary, Toon Hermans, Greta Verhagen, line 1610 and Parliament, that efficiently produce adventitious shoots on one or both media were selected. Those seven cultivars, together with 'Vincent', a cultivar that produces 3.1 shoot/explant on CHR 03, but was not tested for the medium sequence CHR 01/CHR 02, were used in further regeneration and transformation experiments.

Shoot formation.

On both regeneration media, primordia became visible along the cut surface of the leaf explants within 2 weeks days after incubation. From that moment on the number increased rapidly, as shown in Fig.2. About half of the primordia developed into shoots, defined as plantlets with visible internodes. The first shoots were observed 24 days after cutting and their number increased until about 6 weeks after incubation, when they could be excised for rooting.

A thin layer of friable callus cells formed along the cut edges but did not proliferate further. The primordia did not originate from the callus, but emerged directly from the explant tissue, along the edge of the leaf disk (Fig. 3). Larger clusters of non regenerating callus were sometimes formed at the basal end of the cut veins.

Brushing gave wounds over the whole surface of the leaf explant, but had no effect on the location of the adventitious shoots. Both in brushed and unbrushed explants, the shoots arose along the cut surfaces, frequently close to basal ends of cut veins.

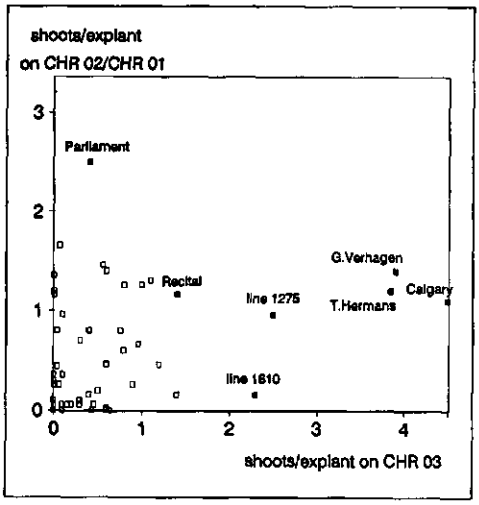

Figure 1.
Regeneration capacity of 60 cultivars of chrysanthemum for 2 regeneration media. The mean number of shoots/explant obtained on CHR03 is plotted against the mean number of shoots regenerated on CHR02/CHR01. Each mark represents one genotype.

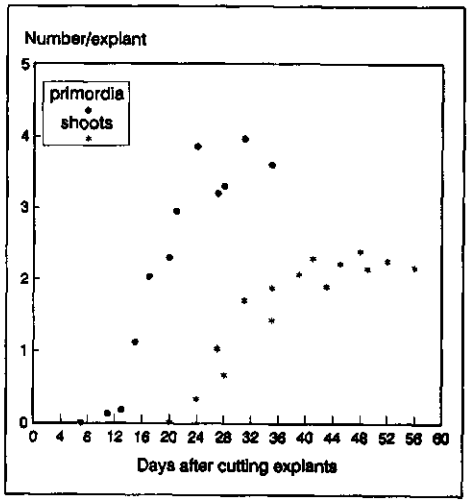

Figure 2.
Number of shoot primordia and shoots formed on 7 mm leaf explants of chrysanthemum on medium CHR03. Data are collected from 3 separate experiments. Each dot represents the mean over 6 cultivars with 20 explants per cultivar. The cultivars were Recital, 1275, Greta Verhagen, 1610, Calgary and Toon Hermans.

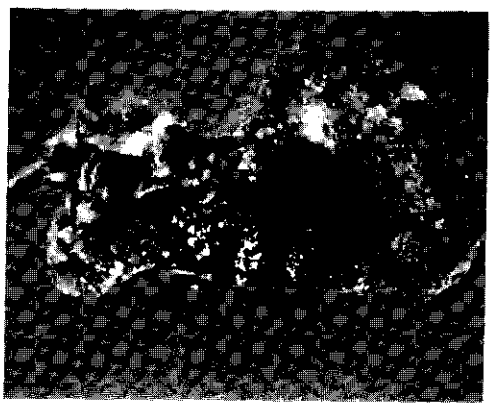

Figure 3.
Regeneration of adventitious shoots from leaf explants of chrysanthemum.

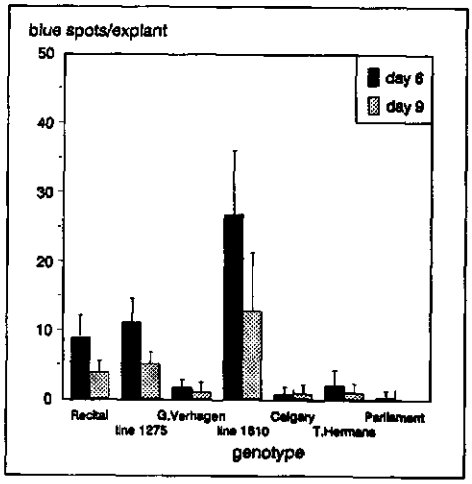

Figure 4.
Mean number of blue spots per leaf explant of 7 cultivars of chrysanthemum and tobacco SR1, 6 and 9 days after infection with LBA4404(p35SGUSint).

Table 1. The number of shoots formed per leaf explant 6 weeks after infection with *Agrobacterium tumefaciens* LBA4404 compared to the controls with and without antibiotics.

Experiment	Medium	Number of shoots		
		- Agrobacterium		+ Agrobacterium
		- antibiotics	+ antibiotics	+ antibiotics
1	CHR 02/CHR 01		3.40a	0.52b
2	CHR 04		4.75a	0.00b
3	CHR 03	1.98a	1.58b	0.70c
4	CHR 03	1.97a	1.94a	0.91b
5	CHR 03	2.24a	1.13ab	0.36c

The data are from the cultivar Parliament in experiments 1 and 2 and averaged over the cultivars Recital, 1275, Greta Verhagen, 1610, Calgary and Toon Hermans in experiments 3 and 4, while 11 additional cultivars were used in experiment 5. Mean separation in lines at 5% probability. Values indicated by different letters (a, b or c) differ significantly.

Transformation

To measure the susceptibility of 7 different genotypes for transformation by *Agrobacterium*, leaf explants were cocultivated with LBA4404(p35SGUSint), that can deliver the reporter gene ß-glucuronidase to plant cells. The number of distinct blue spots per leaf explant, detectable 6 days after infection, was used as an indicator for the efficiency of gene transfer. However, the number of blue spots did not remain constant, but appeared to be decreased when GUS-assays were performed 9 days after infection (Fig. 4). Large variation among cultivars is apparent in the number of blue spots. The breeding line 1610 was the most susceptible genotype, whereas Parliament was the least susceptible. No GUS expression was observed in explants not cocultivated with *Agrobacterium*.

Regeneration after cocultivation with Agrobacterium tumefaciens.

If leaf explants of the cultivar Parliament were cocultivated with the disarmed strain LBA4404 for two days, the number of shoots formed decreased considerably compared to controls with antibiotics (Table 1).

Other selected, well regenerating genotypes were subsequently screened for capacity to

regenerate after infection with *Agrobacterium* and a decline in number of adventitious shoots was observed in all cultivars tested.

Controls, with and without antibiotics added to the media, showed that the reduction in number of shoots is mainly due to cocultivation with *Agrobacterium*, while a smaller part can be attributed to the negative effects of the antibiotics added after cocultivation.

The effect of brushing and cocultivation with *Agrobacterium tumefaciens* on shoot formation and transformation of leaf explants was analyzed (Table 2). Cocultivation significantly reduced the number of shoots formed on brushed explants, except in breeding line 1610, where the reduction is not significant. Brushing itself, without cocultivation had minor effects on regeneration, except again for the positive stimulus in line 1610. Gene transfer efficiency, as measured by the number of blue loci, was not affected by brushing. Despite the usual large variation observed in number of blue spots among explants of identical treatments, genotype remains the overriding factor in the determination of the number of blue spots. Although blue spots were occasionally observed in the center of the explants, the majority of the spots were located along the cut edge, irrespective of brushing.

Table 2. The number of shoots formed per leaf explant on three cultivars of chrysanthemum.

	- Agrobacterium shoots		+ LBA4404(p35SGUSint) shoots		blue spots	
brushing	-	+	-	+	-	+
Recital	1.25a	0.95a	1.85b	1.05a	0.0	1.0
1610	0.20a	0.95b	0.60ab	0.15a	16.3	11.9
Calgary	3.60b	3.25b	2.25b	0.60a	1.8	0.0

The explants were cut from sterile grown leaves either brushed (+) or not (-) prior to cocultivation with *Agrobacterium tumefaciens*. The number of blue spots per leaf explant was determined 6 days after infection. Mean separation in lines for shoots produced at 5% probability. Values indicated by different letters (a or b) differ significantly.

Table 3. The number of shoots per leaf explant of three cultivars of chrysanthemum without (-) or with (+) cocultivation with *Agrobacterium*, averaged over 6 growth regulator combinations.

cultivar	LBA4404(p35SGUSint)	
	-	+
Recital	0.75	0.56
1610	0.49	0.21
Vincent	2.24	2.77

Table 3, compiled from an experiment in which 20 unbrushed explants per cultivar were incubated on MS30 medium with 6 growth regulator concentrations (BA 0.1 or 1.0 mg/l with IAA 0.1, 1.0 or 10 mg/l), confirms that regeneration is not significantly reduced when unbrushed explants are used for cocultivation.

Postponed infection.

The deleterious effect of cocultivation with *Agrobacterium* on regeneration could also be fully prevented by separating explant preparation (cutting and brushing) from cocultivation. Leaf explants infected with *Agrobacterium* 4 days after excision and brushing showed a partly restored regeneration, while delaying infection for eight days fully prevented the deleterious effect of *Agrobacterium* on regeneration.

Fig. 5 illustrates the positive effect of culture prior to infection with *Agrobacterium tumefaciens* on the number of primordia and shoots produced. The variation among different cultivars are illustrated in Fig. 6. Only two cultivars, Calgary and Toon Hermans, retained an adequate regeneration capacity after cocultivation without delayed infection. Preculturing explants for 8 days prior to infection restored regeneration for all cultivars and raised the level to that of the not infected controls. Breeding line 1610 was earlier shown to be the cultivar with the largest number of blue spots in GUS assays and this line was used to test the effect of postponed infection on the frequency of transformation events. Immediate infection resulted in 14 spots per explant, while delaying infection for 4 or 8 days gave 12 and 37 spots per explant

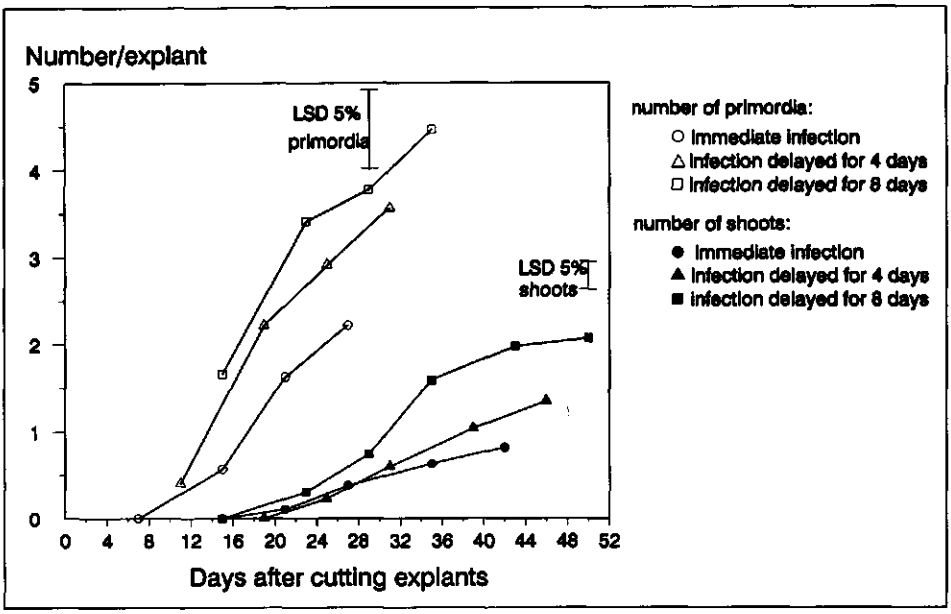

Figure 5.
Number of primordia and adventitious shoots formed per leaf explant cocultivated with *Agrobacterium tumefaciens* for two days immediately after cutting explants or with a delay of 4 to 8 days. The data are means over the cultivars Recital, 1275, Greta Verhagen, 1610, Calgary and Toon Hermans.

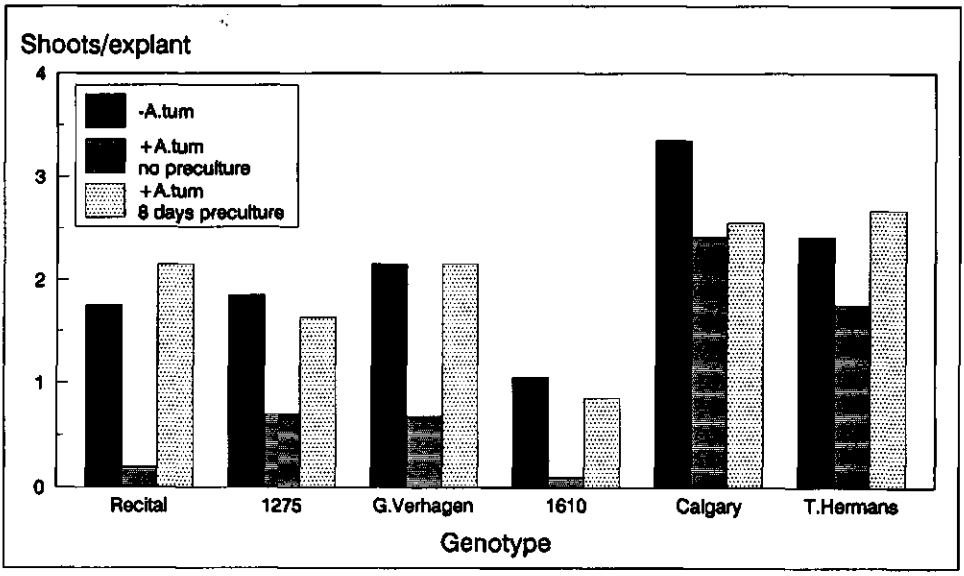

Figure 6.
The effect of immediate and delayed infection with *Agrobacterium tumefaciens* on the number of shoots produced in 6 cultivars of chrysanthemum.

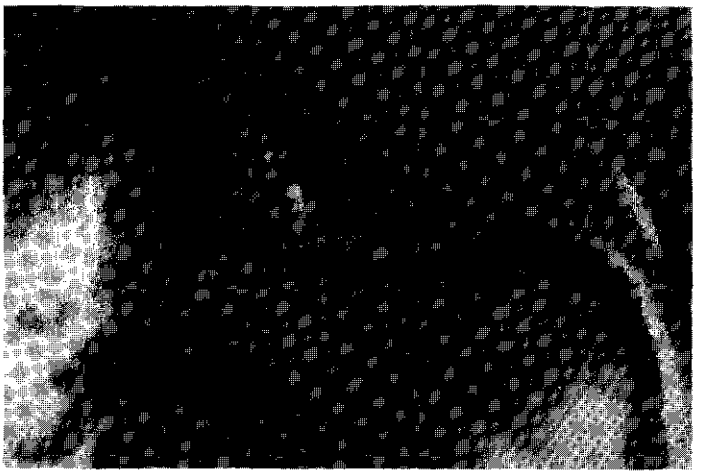

Figure 7.
Location of blue spots in a leaf explant of line 1610, 5 days after infection with LBA4404(p35SGUSint). Infection was postponed for eight days.

respectively. On delayed cocultivation the transformed cells were still to be found along the cut edge, with a preference for the loose callus cells formed in the vicinity of basal vein ends (Fig. 7).

In order to find out whether rewounding of precultured explants would stimulate regeneration or transformation, brushed leaf explants were precultured for 8 days and then brushed again immediately prior to cocultivation. Renewed wounding had no effect on regeneration of cocultivated explants (Table 4), but in the non-infected treatment

Table 4. The mean number of shoots and blue spots per leaf explant of 3 cultivars of chrysanthemum.

cultivar	- Agrobacterium		+ Agrobacterium			
	shoots		shoots		blue spots	
	brushing		brushing		brushing	
	-	+	-	+	-	+
Recital	1.60a	1.75a	2.05ab	2.85b	0.1	0.1
1610	1.15c	0.35ab	0.15a	0.33ab	25.1	50.9
Calgary	5.00c	2.85b	2.35ab	1.75a	0.5	0.0

Explants were precultured for 8 days and then brushed (+) or not (-) prior to cocultivation with *A. tumefaciens* for 2 days. Values indicated by different letters (a, b or c) differ significantly.

Table 5, Mean number of shoots formed on leaf explants of three chrysanthemum cultivars incubated on regeneration medium CHR 03 supplemented with silvernitrate in 4 concentrations.

	- Agrobacterium	+ Agrobacterium				
$AgNO_3$ (mg/l)	0.0	0.0	2.5	5.0	10.0	20.0
Cultivar						
Recital	0.55	0.85	0.20	0.15	0.50	0.80
1275	0.45	0.45	1.55	0.30	0.75	1.20
T.Hermans	3.25	3.05	2.70	1.85	2.50	2.40

the regeneration of Calgary and line 1610 was significantly reduced by brushing. Additional brushing did increase the number of blue spots observed in the readily transformable line 1610, while in Recital and Calgary the transformation frequency was not higher after additional wounding of callusing explants.

Silvernitrate.

As the inhibition of regeneration after cocultivation with *Agrobacterium* might be related to a response to stress, inhibitors of the stress response might have a positive effect on the regeneration. To test this, silvernitrate, which is an inhibitor of the ethylene response, was added to the regeneration medium, in concentrations ranging from 0 to 20 mg/l. The number of shoots produced, was, however, not affected by addition of $AgNO_3$ in any of the concentrations used (Table 5).

Discussion

Leaf explants taken from *in vitro* grown plantlets of chrysanthemum regenerate well along the cut surfaces. Additional wounding of the surface of the leaves by brushing significantly increased the number of regenerated shoots in some genotypes. However, if this regeneration procedure was used in an *Agrobacterium*-mediated transformation

protocol the number of adventitious shoots was markedly reduced. It was shown that additional wounding had an adverse effect on the regeneration of explants, cocultivated with *Agrobacterium tumefaciens*. Therefore, brushing of leaf explants should be omitted in transformation experiments. Extensive wounding lowered the transformation and regeneration frequency in potato as well (De Block, 1988). An explanation could be that extensive wounding leads to excessive colonization of leaf explants by *Agrobacterium tumefaciens*, which may be detrimental to regeneration. Limiting the ports of entry to the leaf margins, by omitting brushing, may prevent entire colonization of the explants during cocultivation.

The stress imposed on the chrysanthemum explants, by infection with *Agrobacterium*, seems not associated with ethylene action, as $AgNO_3$, which blocks ethylene action by binding to ethylene receptor sites, had no effect on regeneration. This is in contrast with the results of De Block et al. (1989) in *Brassica*, where addition of AgNO3 restores regeneration of shoots from hypocotyl explants, under selective conditions.

Histochemical analysis showed that most gene transfer sites are located along the cut margins with a minority in the center region. This is also true for brushed explants, suggesting a factor that opposes gene transfer to cells in the center of the explant. Transport processes, either direct or indirect, probably play a role, as blue spots are most frequently observed near the basal end of cut veins. Internal transport of growth regulators may stimulate cell divisions at this site, thus creating cells competent for transformation (Firoozabady and Galbraith, 1984)

If wounding the whole surface of the leaf is a prerequisite for regeneration, an alternative to prevent inhibition of regeneration is delaying cocultivation for 4 to 8 days. Using this method shoot formation is initiated by wounding, but explants are allowed to recover before infection with *Agrobacterium tumefaciens*. In this way, cocultivation is less detrimental to the regeneration capacity. Delayed infection has successfully been applied in Flax (McHughen et al., 1989) where a preculture period of 9 to 12 days yielded the highest number of transgenic plants. The flax explants were stripped of their epidermis after preculture and immediately prior to infection, probably to solicit a wound response. In chrysanthemums renewed wounding after 8 days preculture is not

necessary to obtain gene transfer, but it may be required to direct the sites of gene transfer. GUS activity after preculture, without rewounding, was mainly observed in a thin layer of unorganized tissue covering the explant. However, these callus cells do not participate in regeneration of shoots, which makes the production of transgenic shoots using preculture, without rewounding, highly improbable.

Our effort to regenerate transformed shoots has not been successful, despite numerous attempts with and without wounding of the leaf explants and with and without delayed infection. The failure to obtain transgenic shoots does not seem to be due to wrong targeting of transformation. Blue spots are observed throughout the tissue along the cut surface, which is the area where shoots originate from.

Recently, successful transformation of chrysanthemum was reported from stem and leaf explants. The reason for success with stem tissue (Lemieux et al., 1990) could be the difference in regeneration associated with explant type. Regeneration from stem explants is indirect, via a callus phase, while on leaf tissue shoots emerge directly from the explant, without an intermediate callus. Regeneration of single transformed cells may be facilitated by the presence of a larger body of transformed tissue. Transformation using leaf explants (Ledger et al., 1991) was succesful with one *Dendranthema indicum* genotype, that regenerated proliferously, while regeneration in six *D. morifolium* genotypes, that were tested in the same study, was poor. Our results indicate that many of the present day chrysanthemum cultivars show inefficient regeneration. As regeneration capacity is of crucial importance for succesful transformation, regeneration procedures need to be further improved, taking into account the effects of bacterial infection and antibiotics on shoot production. Our finding, that deleterious effects on regeneration of cocultivation with *Agrobacterium* can be prevented by limiting wounding of the explant or delaying infection, might enlarge the range of transformable chrysanthemum genotypes.

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C H A P T E R 6

Insect resistant chrysanthemum tissue by introduction of a *Bacillus thuringiensis* crystal protein gene

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(Submitted for publication)

Summary

A 3' truncated crystal protein gene, cryIA(b), derived from *Bacillus thuringiensis* (Bt) subspecies *aizawai* 7.21 was constructed. The Bt gene was inserted into a binary plasmid also carrying the neomycin phosphotransferase II (NPT II) gene and the ß-glucuronidase (GUS) reporter gene, and introduced in the oncogenic *Agrobacterium tumefaciens* strain A281, harbouring the Ti-plasmid pTiBO542. This strain was used to transform leaf explants of chrysanthemum (*Dendranthema grandiflora*) cultivar 'Parliament'. The resulting tumours were kanamycin resistant, exhibited ß-glucuronidase activity and produced agropine and mannopine. In most tumours all simultaneously transferred genes were expressed, due to selection for the presence of both T-DNA's, but no correlation was found between the level of expression of the various genes. The presence of the truncated cryIA(b) gene, as well as the GUS and NPT II gene, in the plant DNA was confirmed by using the polymerase chain reaction (PCR). Expression of the NPT II gene and the truncated cryIA(b) gene was confirmed using a coupled reverse transcription and PCR assay (RT-PCR). A bioassay was developed in which larvae were fed with tumourous chrysanthemum tissue, to detect the effect of the transferred toxin gene on larval development. Using this bioassay with 2nd instar larvae of *Heliothis virescens* (tobacco bud worm) seventeen tumour lines were tested. Several of these lines proved to be strongly inhibitory to larval growth, whereas tumours without the 3'-truncated cryIA(b) gene were not.

Introduction

The cutflower chrysanthemum (*Dendranthema grandiflora*) is the second most important ornamental crop in the Netherlands. Chrysanthemum culture suffers from a wide variety of pest insects (Hill, 1987). Plants that show even minor visible insect damage are not allowed at the auctions, the so-called zero-tolerance. Therefore culture of chrysanthemum requires the preventive use of insecticides. As chemical insecticides are

environmentally polluting, their use has to be diminished. The aim of our research is to develop an alternative for the use of chemical insecticides in chrysanthemum culture. Breeding for resistance in ornamentals has, until recently, been given a low priority compared to breeding for traits such as flower color and morphology (Dons et al., 1991). Moreover, the high ploidy level of *D. grandiflora* (hexaploid) hampers conventional cross breeding for resistance. Molecular resistance breeding, that has already proved to be successful in vegetable crops, might be a good alternative. This technique allows the introduction of resistance genes originating from outside the plant kingdom, like the crystal protein genes from *Bacillus thuringiensis* (Bt), encoding proteins with insecticidal activity. Several research groups have already successfully generated insect resistant plants by the introduction of Bt toxin genes. Vaeck et al. (1987) were the first to transfer a Bt gene to tobacco and Fischhoff et al. (1988) succeeded in the transformation of tomato with the same gene. Complete resistance against pest insects was found in some of these transgenic plants, but it also became clear that the Bt-genes are generally poorly expressed in plant cells. This can partly be ascribed to the occurrence of putative poly-adenylation signals and to preferential codon use, which is different for plants and bacteria (Perlak et al., 1991). To reach our goal, the development of an alternative for the use of chemical insecticides in chrysanthemum culture, a high resistance to pest insects is required. Therefore, in view of the expression difficulties described above, it is of great importance to investigate the expression and biological activity of Bt genes in chrysanthemum tissue.

The group of the lepidopteran specific crystal protein genes of *Bacillus thuringiensis* (CryI) encompasses all genes that have thusfar been used in plant transformation, including the gene described in this paper. These genes all code for a protoxin, with a molecular mass of 130,000 - 140,000 Da., that is processed by proteases in the larval midgut, resulting in the release of a toxic fragment of Mr. 60,000. The now active toxin binds to receptors on the midgut epithelial cells and thereby disturbs the ion permeability of the cell membrane. This results in cell swelling and eventually cell lysis, causing larval death. (for a review see Höfte and Whitely (1989)). The toxic fragment is localized on the N-terminal half of the crystal protein. Deletion studies of several

crystal protein genes confirmed that the C-terminal half is not required for toxicity of the protein (Adang et al., 1985; Höfte et al., 1988). In this study a 3'-truncated toxin gene, encoding a protein of MR 70,000 is used. The truncated gene encompasses the toxic fragment of cryIA(b), which is highly toxic against larvae of *Heliothis virescens*. Transformation of chrysanthemum was carried out using the supervirulent *A. tumefaciens* strain A281. This tumour forming strain was used, because yet no well-established methods are available for the production of transgenic plants. The strain A281 has been proven to be able to transform the chrysanthemum cultivar 'Parliament' (Chapter 2: Van Wordragen et al., 1991; Chapter 4). In view of our primary research goal, to investigate if Bt-expression in chrysanthemum can be sufficiently high to induce insect resistance, the use of this strain has additional advantages. The induced tumours grow very rapid, thus permitting fast and easy screening of, if necessary, large numbers of transformants. The results can subsequently be used to design transformation experiments using disarmed strains.

Material and methods

Vector construction: DNA restriction, isolation of plasmid DNA's, ligation and other DNA manipulations were performed according to Sambrook et al. (1989). The plasmid pPCV708 was kindly provided by C. Koncz (Max Planck Inst., Köln)

Bacterial strains: Transformation was performed using the oncogenic *A. tumefaciens* strain A281. This strain carries the Ti-plasmid pTiBO542 in a C58 chromosomal background (Hood et al., 1986). Binary plasmids were introduced by conjugation according to the protocol described by Rogers et al. (1988). All strains were cultured at 28 °C, on Luria Broth (10 g/l trypton, 5 g/l yeast extract, 5 g/l NaCl, 1 g/l glucose, pH 7.0) with rifampicine (50 mg/l). After introduction of a binary plasmid, carbenicillin (50 mg/l) was used as a selective antibiotic.

Plant material: Sterile cuttings of the chrysanthemum cultivar 'Parliament' were grown on Murashige and Skoog (MS) medium (1962) containing 3 % sucrose and 0.5 μM IAA, pH 5.8, solidified with 0.8 % Purified Oxoid agar. Four weeks old plantlets were used in transformation experiments.

Transformation protocol: The leaf disk transformation of chrysanthemum cv. 'Parliament' was carried out as described before (Chapter 2: Van Wordragen et al., 1991). Kanamycin resistant tumour tissue was taken from the explants 6 weeks after infection and placed on hormone free MS medium. Tumours were subcultured every four weeks. After two subculture steps the tissue was assayed for the presence of bacteria by grounding a part of it in liquid LB medium and incubating the homogenate on LB culture plates at 28 °C for one week. If the tumours were free of bacteria, samples of the tissue were used to determine NPT II, GUS and opine synthetase activities.

Determination of opines: Agropine/ mannopine assays were performed using paper chromatography as described by Petit et al. (1983).

Determination of GUS-activity: ß-Glucuronidase activity of transformed cells was determined using the fluorimetrical assay, as described by Jefferson (1987). Protein amounts in extracts were determined by using the kit from Biorad Laboratories based on the Bradford method (1976).

Determination of NPT-II activity: A dot blot assay was used, based on the protocols described by Platt and Yang (1987) and by McDonnel et al. (1987). Callus tissue, 100-200 mg, was extracted in 100 μl buffer (62.5 mM Tris.HCl (pH 6.8), 10 % glycerol, 0.1 % SDS, 5 % ß-mercaptoethanol). Of the supernatant 40 μl was added to 110 μl assay buffer (67 mM Tris-maleate (pH 7.1), 42 mM MgCl2, 400 mM NH_4Cl_2, 100 μg/ml kanamycin) and 20 μl τ-^{32}P-ATP (1 mCi/ml, 2000 Ci/mmol). The mixture was incubated for 2.5 h. at room temperature and then applied to the wells of a vacuum dot blot device, loaded with a sheet of nitrocellulose covering a sheet of phosphocellulose (P81). The slots were rinsed three times with sterile water, whereupon the membranes were removed and washed for several hours in sterile water at 60 °C. NPT II activity, bound to phosphocellulose and aspecific kinase activity, bound to nitrocellulose was determined by autoradiography and by measuring the amount of label per spot in a liquid scintillation analyzer.

DNA-analysis: DNA was isolated from tumour tissue following a procedure that has been described previously (Chapter 2: Van Wordragen et al., 1991). The presence of the transferred GUS, NPT II and toxin genes was demonstrated by using the polymerase chain reaction (PCR) technique, developed by Mullis et al.(1986). The Amplitaq polymerase from Perkin Elmer Cetus was used. The protocol used for all genes was 30 cycles of: 1 minute melting at 92 °C, 1 minute annealing at 63 °C and 2 minutes elongation at 72 °C The primers were for the GUS gene: 5'-CTG TAG AAA CCC CAA CCC GTG-3' and 5'-CAT TAC GCT GCG ATG GAT CCC-3' resulting in a amplified fragment of 514 bp; for the Bt toxin gene: 5'-GTG GGA AGC AGA TCC TAC TAA TCC-3' and 5'-CCA TCA AAT GTG GAC TCC TAA TAC-3' resulting in an amplified fragment of 544 bp; for the NPT II gene: 5'-CAA GAT GGA TTG CAC GCA GGT TC-3' and 5'-TCC AGA TCA TCC TGA TCG ACA AG-3' resulting in an amplified fragment of 465 bp.

RNA-analysis: Total RNA was isolated by grinding 3 gr. callus tissue in liquid nitrogen and suspending the ground tissue in 2.5 ml. of extraction buffer (0.1 M Tris.HCL, pH 8.5, 0.1 M NaCl, 0.02 M EDTA and 1% Sarkosyl). The suspension was extracted twice with phenol/chloroform/iso-amylalcohol (24:24:1) and the RNA was isolated by subsequent precipitations with 0.8 vol. isopropanol, 4 M LiCl and 2 vols. ethanol. To remove traces of DNA the resolved RNA was treated with DNAse. An amount of 2 μg of this RNA was used as a template in a coupled reversed transcription-PCR assay, which has been described before (Sambrook et al. 1989). The same toxin gene primers were used as in the DNA analysis described above. The downstream primer was added in the reverse transcription reaction, both primers were added just before PCR. To exclude amplification of contaminating DNA, control reactions were performed, consisting of RNAse T1 treated samples.

Bioassay: Eggs from *Heliothis virescens* were presterilized by incubating them for four hours in 4% formaldehyde vapour two days before hatching. Larvae were fed for two days with synthetic medium consisting of 160 g/l Polenta maize flour, 80 g/l wheat germ, 80 g/l yeast extract, 8 g/l ascorbic acid, 2 g/l sorbic acid, 1 g/l p-OH benzoic acid, 0.1 g/l streptomycin and 30 g/l agar. Larvae were transferred to a well of a 24-wells plate, each well containing a piece of callus of approximately 0.5 cm^3 placed on 1 ml wateragar (15 g/l agar, 2 g/l sorbic acid) to prevent desiccation. For every independent transformant 16 larvae were used. As a control, larvae were placed on water agar without callus. After five days larvae were weighed and the mean weight per transformant was determined.

Statistical analysis: The variability in larval weight was not constant but increased as the mean weight increased. To achieve a better fit in the statistical model and a constant variance, natural logarithm transformation was applied on the figures. Thereupon regression analysis was performed.

Results

Construction of the vectors pCPO1-Gs and pCPO1-GsT.

The vector pCPO1 is a derivative of pPCV708 (Koncz et al., 1990), in which the CaMV 35S promoter and pAG7 terminator sequences are replaced by the CaMV 35S promoter and CaMV 35S terminator sequences of pRT103 (Töpfer et al., 1987). The vector pCPO1 carries four eukaryotic expression cassettes, one of which is occupied by the NPT II gene (Fig. 1). In the unique BglII site of the vector a 1.9 kb BamH1-BglII fragment was cloned, containing the ß-glucuronidase coding region. This fragment was obtained from the donor vector pBI101.2 (Jefferson et al., 1987) in which a BglII site was created by linker insertion in the unique SacI site, located at the 3' end of the ß-glucuronidase coding region. In the resulting vector pCPO1-Gs, transcription of the ß-glucuronidase gene is under the control of the 35S promoter. The cryIA(b) gene was isolated from *Bacillus thuringiensis* subsp. *aizawai* 7.21 and truncated from the 3' end

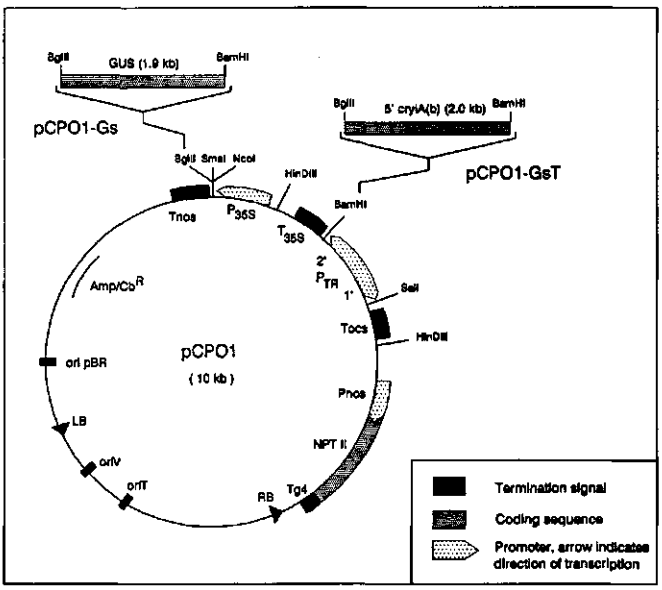

Figure 1.
Schematic representation of the plasmid vector pCPO1 and the construction of vectors pCPO1-Gs and pCPO1-GsT. Between the the right border (RB) and left border (LB) direct repeats four eukaryotic expression cassettes, consisting of promoter (P) and terminator (T) signals, are located. The Neomycin Phosphotransferase gene (NPT II) is driven by the nopaline synthase promoter (nos), while the ß-Glucuronidase (GUS) and truncated cryIA(b) gene are controlled by the CaMV35S and TR2' promoter respectively.

Table 1. Expression of reporter genes in kanamycin resistant tumours of chrysanthemum.

nr. transformant	GUS pMol MU/min/μg	NPT II cpm/μg	mas	ags
1011	1.25	11	+	-
1012	0.22	114	-	-
1015	0.24	27	-	-
1022	3.66	13	+	+
1025	1.75	55	+	+
1031	2.61	70	+	+
1111	0.14	280	+	-
1121	2.45	152	+	+
1161	0.15	133	+	-
1171	0.95	62	+	+
1181	0.11	166	+	+
1191	0.12	62	+	+
1202	0.44	55	-	-
1203	0.41	19	+	+
2231	2.69	10	+	+
2232	2.32	12	+	-
2233	0.68	33	+	-
1061	**0.82**	**29**	**+**	**+**
1063	**3.23**	**73**	**+**	**-**
1102	**2.23**	**73**	**+**	**+**
control	0.25	2	-	-

The figures represent the mean of two separate assays, and represent the specific activity. Bold printed lines are data from non-Bt tumours, induced by A281(pCPO1-Gs).Agropine/mannopine assays were performed only once. In the control experiment, the assay was performed on an extract of non-transformed 'Parliament' tissue. Abbreviations: GUS = ß-Glucuronidase activity, NPT II = neomycine phosphotransferase II activity, mas = mannopine synthetase activity, ags = agropine synthetase activity.

as described previously (Honée et al., 1990). This fragment encodes a protein with a calculated molecular weight of 72,185 Da, that exhibits full toxicity. The vector pCPO1-GsT was constructed by inserting a 2.0 kb BamH1-BglII fragment, containing the 3' truncated cryIA(b) gene, in the unique BamH1 site of pCPO1-Gs. The truncated cryIA(b) gene is thereby placed under the control of the TR 2' promoter. Both vectors were introduced into the oncogenic strain A281, by triparental mating.

Transformation of cv. 'Parliament'.

Leaf explants of *D.grandiflora* cv. 'Parliament' were infected with the strains A281(pCPO1-Gs) and A281(pCPO1-GsT). Of the explants 27 % developed kanamycin

resistant, hormone autotrophic callus. From the induced kanamycin resistant tumours 20 rapidly growing calli were subcultured. The calli all originated from different leaf explants, to ensure their independent establishment. Three of them (nrs. 1061, 1063 and 1102) originated from infection with A281(pCPO1-Gs) and were ment to be used as a control in the bioassays, the other 17 were induced after A281(pCPO1-GsT) infection. As expected, infection with the control *A. tumefaciens* strain A281, only harbouring the Ti-plasmid, did not result in the induction of kanamycin resistant tumours.

After two subcultures tumours were no longer infected with bacteria, which allowed the determination of the expression of the transferred agropine/mannopine synthetase, located on the Ti-plasmid and the reporter genes NPT II and GUS, located on the binary plasmid. As selection for the presence of both T-DNA's was applied during the procedure, it was not surprising that expression of all reporter genes was found in the majority of the tumours (Table 1). Although all tumours grew phytohormone autotrophic, indicating the transfer of the T-DNA of the Ti-plasmid, mannopine and agropine were not always present. In 5 tumours only mannopine, which is a precursor

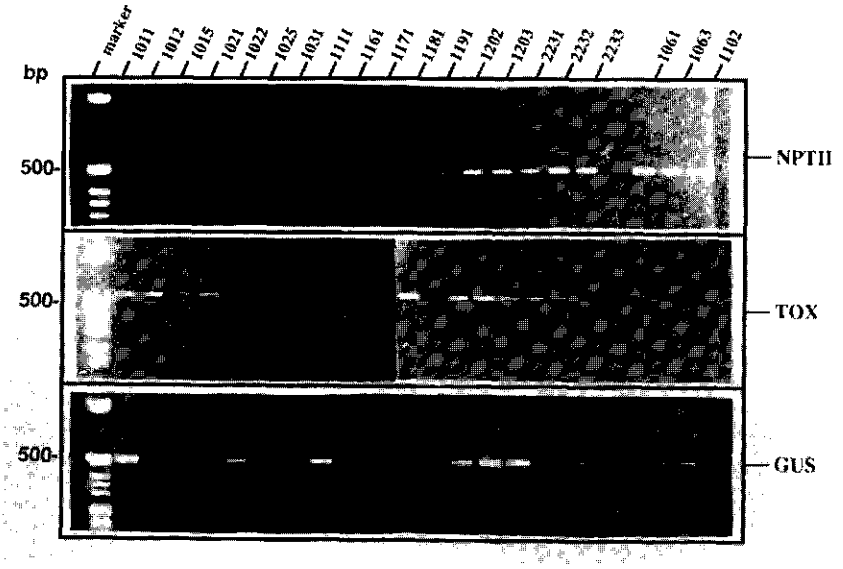

Figure 2.
PCR analysis of 20 chrysanthemum tumours. Lanes 2-18 contain DNA derived from the tumours induced by A281(pCPO1-GsT). The last three lanes contain DNA from the control tumours induced by strain A281(pCPO1-Gs). The three panels show the result with the primers for the NPTII gene, the cryIA(b) gene (TOX) and the GUS gene respectivcly.

of agropine, was detected. In all cases T-DNA of the binary plasmid was also transferred, since all tumours expressed the NPT II gene. In 14 tumours ß-glucuronidase activity was detected as well. The figures in table 1 show a large variation in the activity measured for both enzymes, also there is no correlation between the levels of expression of GUS and NPT II.

DNA analysis.

To confirm the presence of the transferred genes from the binary T-DNA in the chrysanthemum genome, DNA analysis was performed using PCR. DNA derived from all twenty tumour lines was tested, using the three primer pairs for the NPT II, GUS and cryIA(b) genes respectively. If all three primer pairs yielded a fragment of the proper size it was assumed that at least one full length T-DNA copy was present. This analysis revealed that 13 out of the 17 A281(pCPO1-GsT) induced tumours, and all three A281(pCPO1-Gs) induced tumours contained at least one complete copy of the binary T-DNA (Fig. 2). The tumour lines 1111, 1161 and 1191, did not contain the

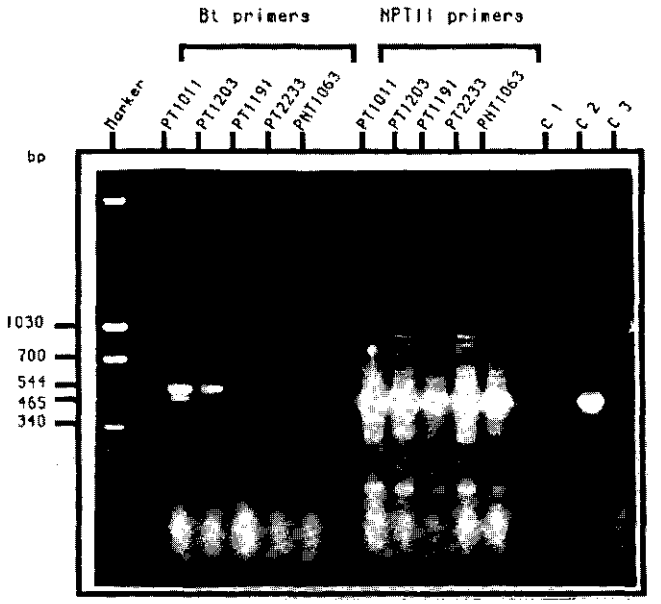

Figure 3.
RNA analysis of five callus lines, by RT-PCR. The five callus lines were assayed for the presence of cryIA(b) (Bt-primers) and NPT II (NPT II primers) RNA. Controls were: addition of RNAse before reverse transcription (C1), addition of RNAse after reverse transcription (C2) and omission of reverse transcriptase (C3).

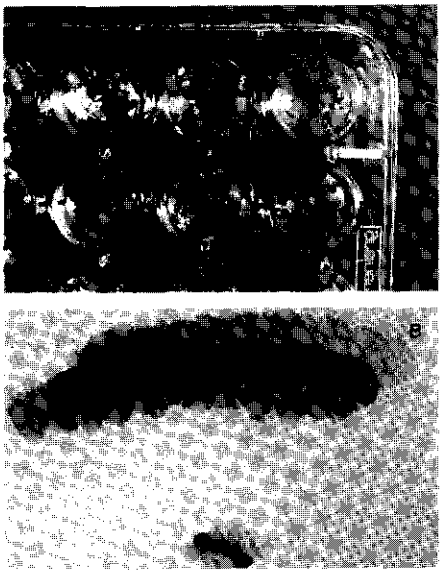

Figure 4.
A) Bioassay. Part of a 24-wells plate after 5 days incubation at 28 °C with larvae of H.virescens. Each well contains a different tumour line.
B) Largest difference between a normally grown larva (upper), fed with control tumour tissue and a larva fed with cryIA(b) containingtumour tissue, after 5 days incubation at 28 °C.

GUS gene nor the toxin gene. Line 1015 did contain the toxin gene, but lacked the GUS gene. RNA analysis revealed the presence of transcription products of the NPTII gene in all tumour lines tested. Expression of the cryIA(b) gene was shown in callus lines 1011, 1203 and 2233, but was absent in line 1191 and the control line 1063, that do not contain the Bt gene gene (Fig. 3). Two other lines, in which the cryIA(b) gene was detected at DNA level, showed no expression of the BT-gene in the RT-PCR assay (data not shown). Expression of the NPT II gene was observed in all tested lines.

Bioassays

The transferred cryIA(b) gene codes for a protein that is highly toxic against larvae of *Heliothis virescens* (tobacco bud worm) which therefore was used as a test insect.
Though *H. virescens* can easily be cultured on artificial food and leaves we first had to test if the insect showed normal growth on a diet of callus tissue. Therefore, 2nd instar larvae were placed on leaves and on A281 induced tumours of chrysanthemum cv. 'Parliament'. After 5 days there was no difference in the mean weights of the two groups of larvae and on both diets normal growth properties were observed.
Thereupon, the twenty Km-resistant calli, described above, were used in two separate bioassays to test the biological activity of the Bt toxin. After the five days incubation period the effect of the toxin gene was clearly distinguishable. The amount of callus left in the wells varied reciprocally with the size of the larvae (Fig. 4A). All surviving larvae

of the sixteen that were fed with one tumour line were weighed and their mean weight was determined. Mortality was, in this case, not a good measure for toxicity, because even in the control experiment with larvae that had not been fed at all during the period of 5 days, not all larvae had died.

From figure 5, which represents the mean weight of the surviving larvae in each of two bioassays, it is clear that the two assays match very well. There was a substantial variation in the weight of the larvae, which had been fed with the same transformed tissue (Fig. 6), but this did not interfere with the statistical analysis. Ten out of the seventeen A281(pCPO1-GsT) induced transformants, caused a significant growth inhibition on *H. virescens* larvae ($p < 0.001$), compared to larvae fed on the three tumours induced by A281(pCPO1-Gs). Moreover, the larvae that had been fed with Bt-tumours differed as a group significantly from the control group. Growth inhibition induced by toxic calli, varied between 35% and 100% and two tumour lines (1171 in both assays and 1011 in assay B) inhibited the development of the larvae completely. Extreme differences in size of larvae fed with control callus (1061, 1063, 1102) and larvae fed with the most toxic callus lines were found. Normally developed larvae could reach a length of 18 mm and a weight of 16.0 mg, whereas larvae fed with e.g. tumourline 1171 could measure only 2 mm and weigh 0.2 mg (Fig. 4B). The three callus lines, in which the toxin gene could not be detected by PCR (1111, 1161 and 1191), were not toxic in the bioassay.

Comparing the results of the growth inhibition, representing the expression of the toxin gene (Fig. 5) with the expression of the GUS gene and the NPT II gene (Table 2) clearly shows, that there is no correlation between the expression of the three genes, located on the same T-DNA.

Discussion

We have reported here the successful introduction and expression of a *Bacillus thuringiensis* derived crystal protein gene in chrysanthemum tissue. The introduction of

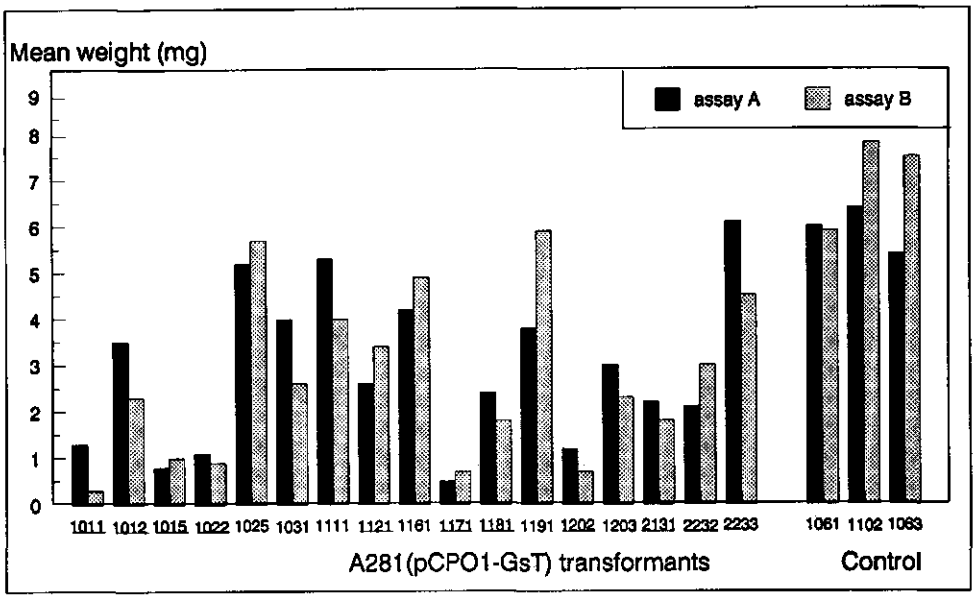

Figure 5.
Mean larval weight from two separate bioassays (A and B) after 5 days feeding on 20 independent chrysanthemum tumour lines. Each bar represents the mean weight of the surviving individuals out of a group of 16 larvae, fed with the same tumour line. Seventeen tumour lines were derived from transformation with A281(pCPO1-GsT), harbouring the cryIA(b) gene. Tumour lines 1061, 1063 and 1102 are controls, induced by A281(pCPO1-Gs), which lacks the cryIA(b) gene. Underlined numbers are significantly different from the controls. ($p < 0.001$).

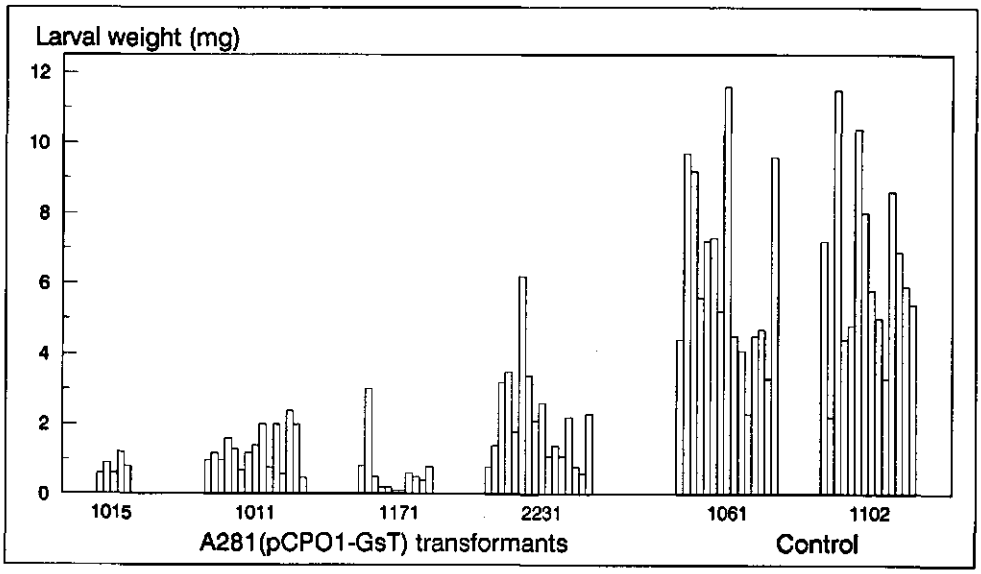

Figure 6.
Variation in weight among individual larvae fed on tissue from the same tumour line. The results shown are from larvae fed on four different tumour lines containing cryIA(b) and two control tissues, respectively. Larval weights are derived from bioassay A (Fig. 5). Only surviving larvae were taken into account.

Bt toxin genes is one of the most effective applications of molecular biology in resistance breeding. High levels of insect resistance have been reached in such diverse species as tobacco (Vaeck et al., 1987), tomato (Fischhoff et al., 1987) and Populus (McCown et al., 1991). However, expression of Bt genes in plants is often difficult and requires the screening of large numbers of transgenic plants. Alternatively the Bt gene can be altered to meet the plants preferential codon usage and to remove putative poly-adenylation signals (Perlak et al., 1991). Recently, this was done for cotton, resulting in enhanced numbers of transgenic plants exhibiting high insect resistance (Perlak et al., 1990).

In view of the above, the results described here, showing that in chrysanthemum a high level of insect resistance can be achieved in a large percentage of the transformants, using a truncated, but otherwise unmodified, cryIA(b) gene, are remarkable. Out of no more then 17 independent transformants, 10 were significantly toxic against *H. virescens* larvae. The tissue samples of the most toxic tumour lines show no visible signs of feeding after 5 days, and the larvae concerned show the same growth characteristics as larvae that have been starved. Therefore, the conclusion seems to be justified, that in chrysanthemum not only inhibition of larval growth is accomplished, but also cessation of feeding.

These results were obtained in tumourous tissue, in which the TR-2' promoter drives the Bt-gene. This is a wound inducible promoter that has been reported to cause constitutive expression at a high level of the coupled gene in callus (Harpster et al., 1988). Several reports have shown the stimulatory effect of auxin on the TR-2' promoter (Teeri et al., 1989; Saito et al., 1991). Since the oncogenic *A. tumefaciens* strain A281 was used as transformation vector, the *iaa* genes of the Ti-plasmid, coding for auxin biosynthesis enzymes, were transferred together with the TR-2'-cryIA(b) construct. Expression of the *iaa* genes will thus enhance the expression of the toxin gene and this probably generates the high percentage of toxic tumour lines found. In contrast to the assumed high expression it was not always possible to detect transcription products of the toxin gene. The same phenomenon was reported previously by Vaeck et al. (1987) for fully insect resistant tobacco, and is probably due to instability of mRNA. Furthermore, very low amounts of mRNA (0.0001 % of total

mRNA) are reported to be sufficient for complete protection of the plant (Vaeck et al., 1987).

Another factor explaining the lack of cryIA(b) transcription products in previously toxic calli, might be the fact that tumour lines were subcultured several times between bioassays and RNA analysis. Selection for NPTII expression was applied, but selection for cryIA(b) expression is not possible. Therefore, in some lines gene expression could have been changed or the gene could have been lost during subculture.

The introduction and proper expression of the oncogenes could be deduced from the vigorous hormone autotrophic growth of the calli. Besides this, the expression of five, simultaneously introduced genes in chrysanthemum was analysed; GUS, NPT II, cryIA(b), ags and mas. There was no correlation at all between the expression levels of the genes, not even between genes, originating from the same T-DNA. All tumours possessed and expressed the NPT II gene, and presumably the oncogenes, because these genes were selected for. Most tumours contained at least one full copy of the binary T-DNA. Incomplete copy's, that were found in some tumours, always lacked genes located on the left site of the T-DNA, due to the fact that the selection marker, the NPT II gene was situated closest to the right border. Moreover T-strand transfer is assumed to proceed from the right border to the left border (Zambryski, 1988), so incomplete copies are most likely to lack lefthand sequences.

In conclusion, we have demonstrated that it is possible to direct the proper expression of foreign genes in chrysanthemum tissue by 5 different promoters. The results presented here show that it is possible to obtain a high level of insect resistance in chrysanthemum after introduction of crystal protein genes from *Bacillus thuringiensis*. Though this result was obtained in tumours, where the enhanced level of auxin stimulated the expression of the chimeric TR-2'-cryIA(b) gene, it is encouraging for the possibility of future application of Bt-toxin genes in chrysanthemum to reduce the use of insecticides.

Acknowledgements

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C H A P T E R 7

Agrobacterium tumefaciens mediated transformation of recalcitrant crops; a review

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(In press, Plant Mol. Biol. Reporter)

Introduction

The rapid progress in the field of plant molecular biology has enabled the development of several techniques for precise and controlled addition of genes to the genome of a plant cell. Regeneration of such genetically modified cells results in transgenic plants with new traits and the application of these procedures for crop improvement is a major step forward in plant breeding. An obvious advantage of genetic modification compared to conventional breeding is the reduced need for back crossings, since the plant genome is only limitedly changed. Even more important is the possibility of bypassing the crossing barriers, which permits the use of genes from outside the plant species or even the plant kingdom. Furthermore the identity of the introduced gene is precisely known, though the integration into the genome can occur at many different sites, nearly randomly. These advantages enable interference with the plants biochemical processes in a well-considered way. Biochemical pathways can be altered or extended, as has been demonstrated for pathways affecting flower color (Van der Krol et al. 1988), male fertility (Mariani et al. 1990), fruit ripening (Smith et al. 1990) or nutritional value (Yang et al. 1989). It is possible to introduce completely new features into plants, like resistance to insects or viruses, as long as they are monogenic or oligogenic. Chemical processes as well as gene expression and regulation can be studied in detail by introducing minor modifications. However, an important limitation of gene transfer to plants is that virtually all genetic modification techniques are only routinely applicable on a few model plant species and can not yet be used for major horticultural and agricultural crops. This is a significant drawback for the application of these techniques in plant breeding.

Techniques that have been developed to mediate gene transfer to plants are extensively reviewed by Potrykus (1990). From the large number of innovative and inventive strategies that have been developed, there are only a few methods which are used successfully in a number of plant species. These are: direct gene transfer, in which naked DNA is introduced into protoplasts via PEG or electroporation; micro injection, in which naked DNA is inserted mechanically into protoplasts or intact cells; biolistics,

in which DNA is introduced into intact cells or plant tissue through bombardment with DNA coated particles and *Agrobacterium*-mediated gene transfer, in which the DNA is transferred to plant cells using the natural gene transfer capacity of the soil bacterium *Agrobacterium*.

A prerequisite for the production of transformed plants is the availability of a method to regenerate a complete plant from the altered cell. This holds for virtually all genetic manipulation systems. Especially in monocots this is a severe problem because here regeneration from protoplasts or tissue explants is extremely difficult. In many dicots complete and healthy plants can be regenerated from various types of tissue, including protoplasts, either with or without a callus phase. However, there is increasing support

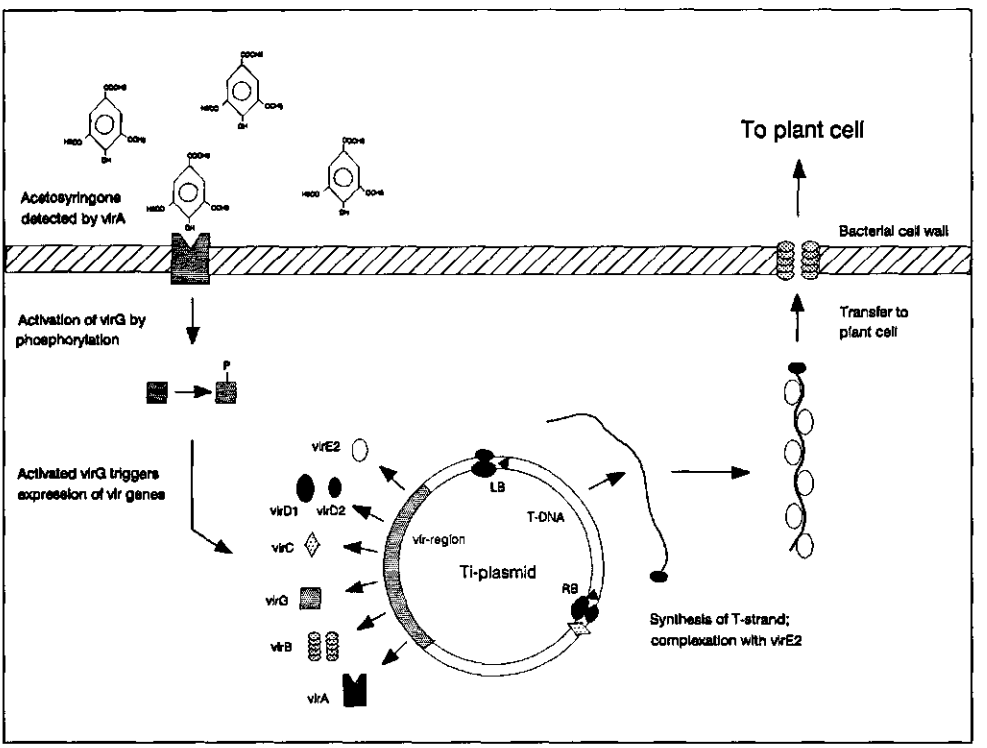

Figure 1.
Agrobacterium gene transfer mechanism. Signal molecules excreted by the plant cell are recognized by the bacterium and trigger expression of vir-genes. Vir-products accomplish synthesis of a single stranded T-DNA copy. The T-strand, covered by protective virE proteins, is transported to the plant cell, through a channel in the bacterial cell wall.

for the hypothesis that not all cells are omnipotent, but that a few cells or cell types in each tissue have the capacity to regenerate into a adventitious shoot. This results in an additional problem in plant transformation as gene transfer has to be directed to those cells that have regeneration capacity, which is, in view of the random nature of most gene transfer techniques, not very easy.

In this paper the state of the art in *Agrobacterium*-mediated transformation of non-model plant species will be reviewed, focusing on literature published during the years 1987-1991 (for an earlier review see Klee et al., 1987). Only reports published in the generally known, english written scientific journals, are reviewed. To enhance the reliability of the tables, the so-called 'grey' literature (proceedings from congresses and poster abstracts) and unpublished claims from industry are not taken into account. Also, model species e.g. *Nicotiana tabacum* and *Petunia hybrida*, for which routine transformation protocols have been established, are not taken into consideration. Other species of the Solanaceae, that are not commonly used as model systems, will be discussed, because they include some important crops. The review will concentrate on transformation by *A.tumefaciens*. The state of the art in transformation using *A.rhizogenes* has recently been reviewed by Tepfer (1990) and will be described only occasionally. Finally an attempt will be made to identify, out of the gathered experiences with a diversity of crops, some general rules for the development of a transformation protocol.

Agrobacterium gene transfer mechanism

Agrobacterium is a plant pathogenic soil bacterium that infects wounded plant cells, which leads to the induction of tumorous growth on the infected spot. There are two tumorigenic species of *Agrobacterium*; *A.tumefaciens*, that induces crown galls and *A.rhizogenes*, that induces the hairy root disease, an uncontrolled proliferation of highly branched roots with many root hairs (Gelvin, 1990). The transformation into tumour cells is accomplished by the transfer of a piece of DNA, the T-DNA, from the

bacterium to the plant cell. The T-DNA contains genes for phytohormone biosynthetic pathways or genes that enhance the sensitivity of the plant to phytohormones. Furthermore some T-DNA genes code for enzymes that are involved in the synthesis of certain amino acid derivatives, called opines, that are used by the bacteria as carbon and nitrogen source. Various strains direct the synthesis of different opines such as octopine, nopaline, mannopine, agropine, cucumopine and succinamopine. This has led to a classification of *Agrobacterium* strains based on opine type. The transferred T-DNA becomes integrated in the plant genome and is expressed by the plant transcription and translation apparatus, leading to a disturbance of the internal hormone balance, which results in uncontrolled growth of transformed, opine producing cells. Both *Agrobacterium* species carry a large plasmid, named tumour inducing (Ti) or root inducing (Ri) plasmid respectively, on which the T-DNA and the genes involved in gene transfer and virulence, the *vir*-region, are located (Zambryski et al., 1989). Some virulence functions, especially the attachment of the bacterium to the plant cell, are provided by *vir*-genes on the chromosome, the chv genes. The plasmid *vir* region includes at least 6 loci, named *vir*A, *vir*B, *vir*C, *vir*D, *vir*E and *vir*G, each consisting of 1 to 11 open reading frames. Some *Agrobacterium* strains have an additional *vir*F locus and most nopaline strains possess a transzeatine synthetase (*tzs*) gene that is located near the *vir* region. The function of several *vir* genes is known (for an extensive review see Zambryski et al. 1989; Howard and Citovsky, 1990), which enabled the formulation of a model for the gene transfer mechanism of *Agrobacterium* (Fig.1).

VirA products are membrane receptor proteins which can detect aromatic signal molecules, such as acetosyringone, excreted by wounded plant tissue. The occupied *vir*A receptor, activates via phosphorylation, the product of the constitutively expressed *vir*G. Activated *Vir*G proteins bind to the promoters of all *vir* genes resulting in transcription and translation. *Vir*C proteins bind to an overdrive sequence upstream of the right T-DNA border. VirD provides the endonuclease/polymerase functions, enhanced by *vir*C, that are necessary to make single strands nicks at the border sequences and to synthesize a single stranded T-DNA copy. VirE proteins, which have ss-DNA binding capacity, cover the naked T-DNA strand and the complex is transported through a membrane channel, composed of the products of 11 different *vir*B products. The route

of the T-DNA through the plant cell wall, cell membrane and nuclear envelope to the final integration in the plant nuclear DNA remains a black box. There is some evidence that *vir*E and sequences just inside the borders are involved in integration (Howard and Citovsky, 1990; Matsumoto et al., 1990), but literature on this subject is not very consistent.

Application

Modification of the Ti-plasmid

The genes of the *vir* region are sufficient to transfer any piece of DNA that is located on any plasmid in the bacterium if flanked by border sequences. This led to one of the major steps in plant genetic manipulation, the development of a binary gene transfer system, in which the T-DNA is separated from the *vir* region and located on a small plasmid, that can also be propagated in *E.coli*. These small T-DNA plasmids are easy

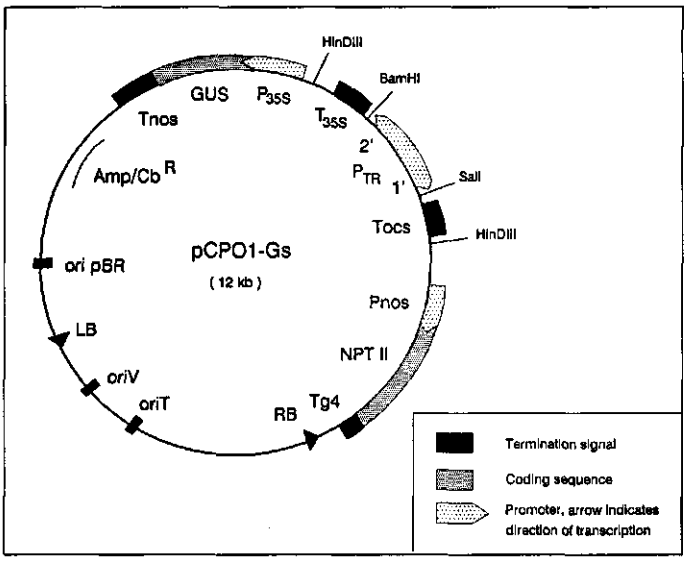

Figure 2.
Example of a binary vector, harbouring, between the left border (LB) and right border (RB) sequences, a selective marker gene, neomycin phosphotransferase II (NPT II) and ß-Glucuronidase (GUS), a reporter gene. Both genes are flanked by eukaryotic expression signal sequences; the nopaline synthase promoter (P_{nos}) and the g4 terminator (T_{g4}) for NPT II and the CaMV 35S promoter (P_{35S}) and the nopaline synthase terminator (T_{nos}) for GUS. Two additional genes, which can be inserted in the SalI and BamHI sites, will be driven by the bidirectional TR promoter (P_{TR} 1' and 2') and termination sequences are derived from the CaMV 35S gene (T_{35S}) and the octopine synthase gene (T_{ocs}) respectively.

to manipulate and the oncogenes can be replaced by interesting genes. The plasmid can be reintroduced in an *Agrobacterium* strain harbouring a disarmed (T-DNA deprived) *vir*-plasmid, where it can stay separate from the *vir*-plasmid (binary system) or integrate in the *vir*-plasmid via homologous recombination (cointegrate system) (Hooykaas, 1989). Both plasmid types have proven to be versatile tools in transformation and are commonly used, though a preference for binary vectors is noticeable (Table 2). A T-DNA plasmid harbours, between the border repeats, a selection gene and the gene to be studied (often a reporter gene) flanked by eukaryotic expression signals (Fig.2). The selection gene usually codes for an enzyme that confers resistance to antibiotics, such as kanamycin and hygromycin, that are lethal to untransformed plant cells. Only cells expressing the transferred gene survive the selection and can be regenerated into mature plants. Reporter genes facilitate visual detection of the expression of the transferred gene either in situ or in plant extracts. The ß-glucuronidase gene, derived from *E.coli*, is one of the most widely used reporter genes (Jefferson et al., 1987). The enzyme, which is not present endogenously in most plant species can easily and sensitively be detected either in situ, as a blue precipitate or in extracts by quantitative fluorimetric or colorimetric assays. Transfer of T-DNA to plant cells first gives rise to transient expression of the introduced genes. At a much lower frequency genes become stably integrated in the plant genome and regeneration of such transformed cells leads to transgenic plants. Application of Agrobacterium infection in an existing regeneration protocol has been successful for some species. Well known is the so-called leaf disk transformation procedure (Horsch et al., 1985) in which transformed shoots regenerate directly from the wounded surface of leaf explants. However, in our experience with a number of recalcitrant crops, like chrysanthemum, it is often more efficient to invert this procedure and adapt the regeneration protocol to the transformation procedure.

A survey

Since the development of *Agrobacterium*-mediated transformation for model plant species like *Nicotiana tabacum*, much effort has been put in the application of the technique to crop species, as it would be a useful supplement to the classical cross breeding. Almost a decade has past since the creation of the first transgenic tobacco

plant, but routine transformation procedures have been established for just a few plant species. The data reported in the literature on this subject are summarized in table 1, concerning transformation with tumorigenic strains, and table 2, focusing on transformation with disarmed strains. In both tables essential information on the transformation protocol, such as the *Agrobacterium* strain used, is given. Several important topics concerning the procedure are discussed in more detail in the following paragraphs.

Table 1 and table 2 show that for more then sixty species successful gene transfer, either with or without regeneration into transformed plants, has been claimed. Unfortunately, adequate molecular evidence is sometimes lacking and usually there is no confirmation from other research groups. Regeneration of transformed plants, confirmed by molecular evidence, is reported for 27 species. However, if the criterium is used that the new gene should be inherited by progeny, it becomes clear that only fifteen species are proven to be stably transformed, and five of them belong to the Solanaceae family (see the regeneration columns in tables 1 and 2). Only for *Arabidopsis thaliana, Brassica napus, Linum usitatissimum* and *Lycopersicon esculentum* inheritable gene transfer was reported from more than one lab.

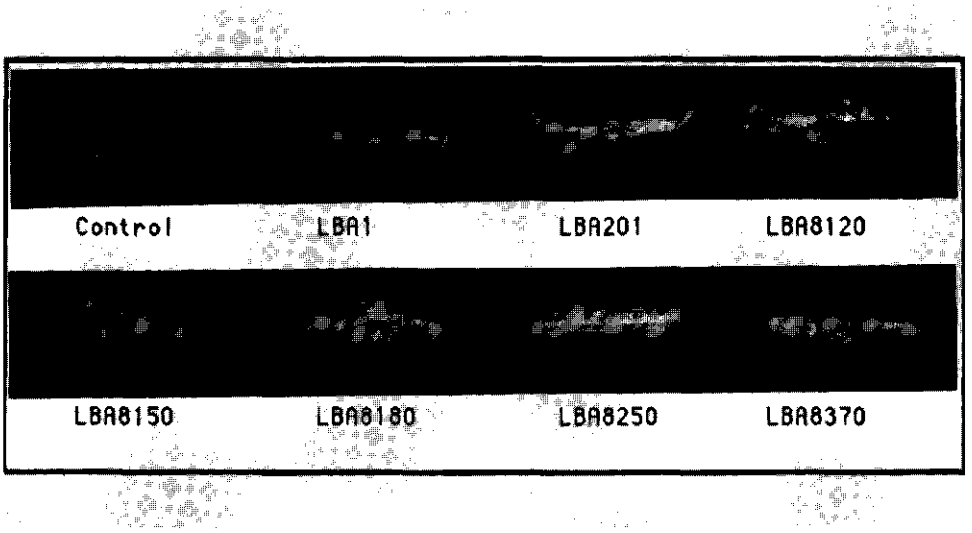

Figure 3.
Differential response of the chrysanthemum cultivar 'Parliament' upon infection of stems with several oncogenic *Agrobacterium* strains.

Table 1: Transformation using oncogenic *Agrobacterium tumefaciens* strains.

Species	Common name	Strain	Vir.	Binary	Explant	Reg.	Reference
Abies procera	fir	>5 STRAINS			seedling	no	Morris et al., 1989
Allium cepa	onion	>5 STRAINS			bulb	no	Dommisse et al., 1990
Alnus incana	speckled alder	ACH5	pTiAch5	-	stem	no	Mackay et al., 1988
Alnus incana	speckled alder	C58	pTiC58	-	stem	no	Mackay et al., 1988
Alnus glutinosa	black alder	ACH5	pTiAch5	-	stem	no	Mackay et al., 1988
Alnus glutinosa	black alder	C58	pTiC58	-	stem	no	Mackay et al., 1988
Beta vulgaris	sugar beet	LBA4001	pTiAch5	-	stem/seedling	no	Krens et al., 1988
Beta vulgaris	sugar beet	LBA1501	pTiB6::tn1831	-	stem/seedling	no	Krens et al., 1988
Betula papyrifera	paper birch	ACH5	pTiAch5	-	stem	no	Mackay et al., 1988
Betula papyrifera	paper birch	C58	pTiC58	-	stem	no	Mackay et al., 1988
Brassica campestris	field mustard	C58	pTiC58	-	stem	no	Ohlsson & Eriksson, 1988
Brassica campestris	field mustard	B6S3	pTiB6S3	-	stem	no	Ohlsson & Eriksson, 1988
Brassica juncea	brown mustard	>5 STRAINS			stem	no	Charest et al., 1989
Brassica napus	oilseed rape	C58	pTiC58C1	-	stem	no	Ohlsson & Eriksson, 1988
Brassica napus	oilseed rape	A281	pTiBO542	pCGN767	hypocotyl	yes+prog	Radke et al., 1988
Brassica napus	oilseed rape	>5 STRAINS			stem	no	Charest et al., 1989
Brassica oleracea	cauliflower	C58	pTiC58	-	stem	no	Ohlsson & Eriksson, 1988
Brassica oleracea	cauliflower	B6S3	pTiB6S3	-	stem	no	Ohlsson & Eriksson, 1988
Brassica oleracea	cauliflower	C58C1	pLGVTi23neo	-	leaf	yes	Srivastava et al., 1988
Carica papaya	papaya	GV3111	pTiB6S3::pMON200	-	leaf/stem/petiole	no	Pang & Sanford, 1988
Chenopodium quinoa	goosefoot	A281	pTiBO542	pTOK162::pTOK171	suspension culture	no	Komari, 1990
Dendranthema grandiflora	chrysanthemum	>5 STRAINS			stem	no	Wordragen et al., 1991
Dendranthema grandiflora	chrysanthemum	A281	pTiBO542/pTVK291	-	leaf	no	Wordragen et al., 1991
Dioscorea bulbifera	yam	C58	pTiC58	-	bulbil	no	Schafer et al., 1987
Fragaria vesca	strawberry	>5 STRAINS			runner	no	Uratsu et al., 1991
Fragrea fragrans	tembusu tree	A208	pTiT37	-	stem	yes	Loh & Rao, 1987
Glycine max	soybean	C58	pTiC58	-	single cell	no	Baldes et al., 1987
Glycine max	soybean	C58	pTi201	-	single cell	no	Baldes et al., 1987
Glycine max	soybean	C58	pTiC58::pMON200-pfdA2		single cell	no	Baldes et al., 1987
Glycine max	soybean	C58	pTiC58	-	cotyledon	no	Delzer et al., 1990
Glycine max	soybean	A208	pTiT37	-	cotyledon	no	Delzer et al., 1990
Glycine max	soybean	A281	pTiBO542	-	cotyledon	no	Delzer et al., 1990

Juglans regia	walnut	K12x562E	pTiA6::pCGN562	-	stem	no	Dandekar & Martin, 1988
Juglans regia	walnut	K12x167	pTiA6::pCGN167	-	somatic embryo	no	McGranahan et al., 1988
Juglans regia	walnut	K12x562E	pTiA6::pCGN562	-	somatic embryo	no	McGranahan et al., 1988
Lactuca sativa	lettuce	ACH5	pTiAch5	-	seedling	no	Michelmore et al., 1987
Lactuca sativa	lettuce	C58	pTiC58	-	seedling	no	Michelmore et al., 1987
Lactuca sativa	lettuce	GV3111	pTiB6S3::pMON200	-	seedling	no	Michelmore et al., 1987
Libocedrus decurrens	incense cedar	C58	pTiC58	-	seedling	no	Stomp et al., 1990
Libocedrus decurrens	incense cedar	M2-73	pTiM2-73	-	seedling	no	Stomp et al., 1990
Lotus corniculatus	birds foot trefoil	C58	pTiC58	-	seedling	no	Armstead & Webb, 1987
Lycopersicon esculentum	tomato	A281	pTiBO542	pTOK119	callus	no	Komari, 1989
Lycopersicon esculentum	tomato	A66	pTiA66	-	leaf	yes	Davis et al., 1991
Lycopersicon esculentum	tomato	A281	pTiBO542	-	leaf	yes	Davis et al., 1991
Medicago sativa	alfalfa	GV3101	pGV3850(?)	pGV2206	leaf	yes	Kuchuk et al., 1990
Medicago varia	alfalfa	A281	pTiBO542	pVW130	leaf/petiole	yes	Chabaud et al., 1988
Medicago varia	alfalfa	LBA4404	pAL4404	pVW130	leaf/petiole	yes	Chabaud et al., 1988
Nicotiana edwardsonii	tobacco	A281	pTiBO542	pSJ62/pSJ65	leaf	yes	Kiernan et al., 1989
Nicotiana glauca	shrub tobacco	A281	pTiBO542	pTOK119	callus	no	Komari, 1989
Nicotiana glutinosa	tobacco	A208	pTiT37	pTOK119	callus	no	Komari, 1989
Nicotiana glutinosa	tobacco	A281	pTiBO542	pTOK119	callus	no	Komari, 1989
Nicotiana glutinosa	tobacco	A348	pTiA6	pTOK119	callus	no	Komari, 1989
Nicotiana plumbagenifolia	tobacco	A208	pTiT37	pTOK119	callus	no	Komari, 1989
Nicotiana plumbagenifolia	tobacco	A281	pTiBO542	pTOK119	callus	no	Komari, 1989
Oryza sativa	rice	A281	pTiBO542	-	embryo	no	Raineri et al., 1990
Oryza sativa	rice	A856	pTiAg162	-	embryo	no	Raineri et al., 1990
Pharbitis nil	morning glory	A281	pTiBO542	pBI121	stem	no	Araki et al., 1989
Phaseolus vulgaris	dry bean	A208	pTiT37	pGA482	seedling	no	McClean et al., 1991
Phaseolus vulgaris	dry bean	A281	pTiBO542	pGA482	seedling	no	McClean et al., 1991
Phaseolus vulgaris	dry bean	LBA4001	pTiACH5	pGA482	seedling	no	McClean et al., 1991
Picea abies	Norway spruce	A281	pTiBO542	-	seedling	no	Clapham et al., 1990
Picea abies	Norway spruce	A281	pTiBO542	-	seedling	no	Hood et al., 1990
Picea engelmanii	Engelman spruce	>5 STRAINS			stem	no	Ellis et al., 1989
Picea glauca	white spruce	>5 STRAINS			stem	no	Ellis et al., 1989
Picea sitchensis	Sitka spruce	>5 STRAINS			stem	no	Ellis et al., 1989
Pimpinella anisum	anise	A281	pTiBO542	pTOK119	callus	no	Komari, 1989
Pinus eldarica	Afghan pine	U3	pU3	-	seedling	no	Stomp et al., 1990
Pinus elliottii	slash pine	A281	pTiBO542	-	seedling	no	Stomp et al., 1990
Pinus elliottii	slash pine	BO542	pTiBO542	-	seedling	no	Stomp et al., 1990

Pinus elliottii	slash pine	U3	pTiU3	-	seedling	no	Stomp et al., 1990
Pinus jeffreyi	Jeffrey pine	C58	pTiC58	-	seedling	no	Stomp et al., 1990
Pinus lambertiana	sugar pine	B0542	pTiBO542	pEND4K	stem	no	Loopstra et al., 1990
Pinus lambertiana	sugar pine	A281	pTiBO542	-	stem	no	Loopstra et al., 1990
Pinus lambertiana	sugar pine	B0542	pTiBO542	-	seedling	no	Stomp et al., 1990
Pinus lambertiana	sugar pine	A281	pTiBO542	-	seedling	no	Stomp et al., 1990
Pinus lambertiana	sugar pine	C58	pTiC58	-	seedling	no	Stomp et al., 1990
Pinus lambertiana	sugar pine	M2-73	pTiM2-73	-	seedling	no	Stomp et al., 1990
Pinus lambertiana	sugar pine	U3	pTiU3	-	seedling	no	Stomp et al., 1990
Pinus ponderosa	Ponderosa pine	>5 STRAINS			seedling	no	Morris et al., 1989
Pinus ponderosa	Ponderosa pine	M2-73	pTiM2-73	-	seedling	no	Stomp et al., 1990
Pinus ponderosa	Ponderosa pine	U3	pTiU3	-	seedling	no	Stomp et al., 1990
Pinus radiata	Monterey pine	A281	pTiBO542	-	seedling	no	Stomp et al., 1990
Pinus radiata	Monterey pine	BO542	pTiBO542	-	seedling	no	Stomp et al., 1990
Pinus radiata	Monterey pine	M2-73	pTiM2-73	-	seedling	no	Stomp et al., 1990
Pinus radiata	Monterey pine	U3	pTiU3	-	seedling	no	Stomp et al., 1990
Pinus sylvestris	Scots pine	BO542	pTiBO542	-	seedling	no	Stomp et al., 1990
Pinus sylvestris	Scots pine	C58	pTiC58	-	seedling	no	Stomp et al., 1990
Pinus sylvestris	Scots pine	M2-73	pTiM2-73	-	seedling	no	Stomp et al., 1990
Pinus taeda	Loblolly pine	A281	pTiBO542	-	seedling	no	Stomp et al., 1990
Pinus taeda	Loblolly pine	BO542	pTiBO542	-	seedling	no	Stomp et al., 1990
Pinus taeda	Loblolly pine	U3	pTiU3	-	seedling	no	Stomp et al., 1990
Pinus virginiana	Virginia pine	BO542	pTiBO542	-	seedling	no	Stomp et al., 1990
Pinus virginiana	Virginia pine	M2-73	pTiM2-73	-	seedling	no	Stomp et al., 1990
Pinus virginiana	Virginia pine	U3	pTiU3	-	seedling	no	Stomp et al., 1990
Pisum sativum	pea	A281	pTiBO542	-	stem	no	Hobbs et al., 1989
Pisum sativum	pea	C58	pTiC58	-	stem	no	Hobbs et al., 1989
Pisum sativum	pea	ACH5	pTiAch5	-	stem	no	Hobbs et al., 1989
Pisum sativum	pea	A281	pTiBO542	-	stem	no	Puonti-Kaerlas et al., 1989
Pisum sativum	pea	C58	pTiC58	-	stem	no	Puonti-Kaerlas et al., 1989
Pisum sativum	pea	B6S3	pTiB6S3	-	stem	no	Puonti-Kaerlas et al., 1989
Pisum sativum	pea	GV3101	pGV3304/pGV2298	-	stem	no	Puonti-Kaerlas et al., 1989
Prunus persica	peach	A348	pTiA6-tms328::Tn5	-	stem	no	Hammerschlag et al., 1989
Pseudotsuga menziesii	Douglas fir	K12x562E	pTiA6::pCGN562	-	shoot/seedling	no	Dandekar et al., 1987
Pseudotsuga menziesii	Douglas fir	K12x167	pTiA6::pCGN167	-	shoot/seedling	no	Dandekar et al., 1987
Pseudotsuga menziesii	Douglas fir	>5 STRAINS			stem	no	Ellis et al., 1989
Pseudotsuga menziesii	Douglas fir	>5 STRAINS			seedling	no	Morris et al., 1989

Pseudotsuga menziesii	Douglas fir	A281	pTiBO542	-	seedling	no	Stomp et al., 1990
Pseudotsuga menziesii	Douglas fir	BO542	pTiBO542	-	seedling	no	Stomp et al., 1990
Salix schwerinii	willow	C58	pTiC58	-	stem	no	Vahala et al., 1989
Salix schwerinii	willow	GV3101	pGV3851/pGV3304/pGV2215/pGV2298		stem	no	Vahala et al., 1989
Salix schwerinii	willow	B6	pTiB6	-	stem	no	Vahala et al., 1989
Salix schwerinii	willow	B6S3	pTiB6S3	-	stem	no	Vahala et al., 1989
Salix viminalis	willow	C58	pTiC58	-	stem	no	Vahala et al., 1989
Salix viminalis	willow	GV3101	pGV3851/pGV3304/pGV2215/pGV2298		stem	no	Vahala et al., 1989
Salix viminalis	willow	B6	pTiB6	-	stem	no	Vahala et al., 1989
Salix viminalis	willow	B6S3	pTiB6S3	-	stem	no	Vahala et al., 1989
Sinapis alba	white mustard	C58	pTiC58	-	stem	no	Ohlsson & Eriksson, 1988
Sinapis alba	white mustard	B6S3	pTiB6S3	-	stem	no	Ohlsson & Eriksson, 1988
Solanum melongena	eggplant	A281	pTiBO542	pGA472	cotyledon/leaf	yes	Fillipone & Lurquin, 1989
Solanum melongena	eggplant	A208	pTiT37	pTOK119	callus	no	Komari, 1989
Solanum melongena	eggplant	A281	pTiBO542	pTOK119	callus	no	Komari, 1989
Solanum melongena	eggplant	A348	pTiA6	pTOK119	callus	no	Komari, 1989
Solanum tuberosum	potato	A281	pTiBO542	pTOK119	callus	no	Komari, 1989
Stylosanthes guianensis	pencil flower	A281	pTiBO542	-	leaf	no	Manners, 1987
Stylosanthes guianensis	pencil flower	C58	pTiC58	-	leaf	no	Manners, 1987
Stylosanthes hamata	pencil flower	A281	pTiBO542	-	leaf	no	Manners, 1987
Stylosanthes hamata	pencil flower	C58	pTiC58	-	leaf	no	Manners, 1987
Stylosanthes humilis	Townsville stylo	A281	pTiBO542	pGA472/pBIN6	leaf	no	Manners, 1987
Stylosanthes humilis	Townsville stylo	C58	pTiC58	-	leaf	no	Manners, 1987
Theobroma cacao	cacao	CHRY9	?	-	stem	no	Purdy & Dickstein, 1989
Tsuga heterophylla	hemlock fir	>5 STRAINS			seedling	no	Morris et al., 1989
Vigna unguiculata	cowpea	LBA1010	pTiB6	-	stem	no	Garcia et al., 1986
Vigna unguiculata	cowpea	LBA958	pTiC58	-	stem	no	Garcia et al., 1986
Vitis vinifera	grapevine	5805	pTiBO542	GA474-68/pBIN19	stem/apices	no	Baribault et al., 1990
Vitis vinifera	grapevine	AG162	?	GA474-68/pBIN19	stem/apices	yes	Baribault et al., 1990
Vitis vinifera	grapevine	K12	K12::pCGN167	-	stem/apices	no	Baribault et al., 1990

Bold plant names are monocotyledonous species. Abbreviations: Vir.: vir plasmid or cointegrate plasmid carrying both vir-region and T-DNA; Binary: T-DNA plasmid in binary system; Explant: explant source or type of tissue used in infection; Reg.:regeneration of transformed shoots; Prog: transgenic progeny.

Table 2: Transformation using disarmed *Agrobacterium tumefaciens* strains.

Species	Common name	Strain	Vir.	Binary	Explant	Reg.	Reference
Apium graveolens	celery	C58(?)	pTiC58::pMON200	-	petiole	yes+prog	Catlin et al., 1988
Arabidopsis thaliana	mouse ear cress	C58C1	pGV3850::pAK1003	-	leaf	yes+prog	Sheikoleslam & Weeks, 1987
Arabidopsis thaliana	mouse ear cress	C58C1	pGV3850	Hygro(?)	leaf/cotyledon	yes+prog	Schmidt & Willmitzer, 1988
Arabidopsis thaliana	mouse ear cress	C58C1	pGSFR1161	-	root	yes+prog	Valvekens et al., 1988
Arabidopsis thaliana	mouse ear cress	C58C1	pGV2260	pCLH88	root	yes+prog	Valvekens et al., 1988
Beta vulgaris	sugar beet	LBA4404	pAL4404	pBIN19CAT/pBI121	shoot base	yes	Lindsey & Gallois, 1990
Brassica campestris	field mustard	GV3101(?)	pGV3850::2103neo	-	protoplasts	no	Ohlsson & Eriksson, 1988
Brassica napus	oilseed rape	A208	pTiT37SE::pMON200	-	stem	yes+prog	Fry et al., 1987
Brassica napus	oilseed rape	A208	pTiT37SE	pMON505	stem	yes+prog	Fry et al., 1987
Brassica napus	oilseed rape	A208	pTiT37SE	pMON809	stem	yes+prog	Pua et al., 1987
Brassica napus	oilseed rape	EHA101	pEHA101	pCGN767	hypocotyl	yes+prog	Radke et al., 1988
Brassica napus	oilseed rape	C58C1	pMP90	pGSFR780A	hypocotyl	yes+prog	De Block et al., 1989
Brassica napus	oilseed rape	GV3850	pGV3850	pCV730	microspore/proembryos	yes	Pechan, 1989
Brassica napus	oilseed rape	LBA4404	pAL4404	pBIN19	microspore/embryo	yes	Swanson & Erickson, 1989
Brassica napus	oilseed rape	C58-3	pTiC58dis	pJIT73	petiole	yes	Boulter et al., 1990
Brassica napus	oilseed rape	LBA4404	pAL4404	pJIT73	petiole	yes	Boulter et al., 1990
Brassica napus	oilseed rape	GV3111	pB6S3SE::pMON316	-	stem epidermis	yes+prog	Misra, 1990
Brassica napus	oilseed rape	C58C1	pGV3850::1103neo	-	single cell	yes+prog	Thomzik & Hain, 1990
Brassica oleracea	cauliflower	C58C1	pMP90	pGSFR780A	hypocotyl	yes+prog	De Block et al., 1989
Cucumis melo	musk melon	LBA4404	pAL4404	pCIB10	cotyledon	yes+prog	Fang & Grumet, 1990
Cucumis sativus	cucumber	C58Z707	?	pGA482	cotyledon	yes+prog	Chee, 1990
Daucus carota	carrot	C58C1	pGV3850::1101	-	embryogenic suspension	yes	Scott & Draper, 1987
Daucus carota	carrot	LBA4404	pAL4404	pRGUSII/pBI101/pBI121	hypocotyl	no	Thomas et al., 1989
Fragaria x Anannassa	strawberry	LBA4404	pAL4404	pBIN6/pSS1	leaf/petiole	yes+prog	James et al., 1990
Fragaria x Anannassa	strawberry	C58C1	pMP90	pBI121	leaf	yes	Nehra et al., 1990
Glycine max	soybean	A208	pTiT37SE::pMON9749	-	cotyledon	yes+prog	Hinchee et al., 1988
Glycine max	soybean	C58Z707	?	pGA482G	germinating seed	yes+prog	Chee et al., 1989
Gossypium hirsutum	cotton	LBA4404	pAL4404	pH575	cotyledon	yes	Firoozabady et al., 1987
Gossypium hirsutum	cotton	1592	pTi15955::pH592	-	cotyledon	yes	Firoozabady et al., 1987
Helianthus annuus	sunflower	LBA288	pDAL715/pDAL936	-	hypocotyl	yes+prog	Everett et al., 1987
Helianthus annuus	sunflower	EHA101	pEHA101	pMOG131	apical meristem	yes	Schrammeyer et al., 1990
Helianthus annuus	sunflower	LBA4404	pAL4404	pGUSint	apical meristem	yes	Schrammeyer et al., 1990
Juglans regia	walnut	K61	pTiA6dis	pCGN200/pCGN594	somatic embryo	yes	McGranahan et al., 1988
Juglans regia	walnut	EHA101	pEHA101	pCGN7001/pCGN7314	somatic embryo	yes	McGranahan et al., 1990
Kalanchoe lacinata	kalanchoe	A208	pTiT37SE	pROA93	leaf	yes	Jia et al., 1989
Kalanchoe lacinata	kalanchoe	GV3111	pTiB6S3SE	pROA93	leaf	yes	Jia et al., 1989
Kalanchoe lacinata	kalanchoe	EHA101	pEHA101	pROA93	leaf	yes	Jia et al., 1989

Lactuca sativa	lettuce	GV3111	pTiB6S3SE::pMON200	-	cotyledon	yes+prog	Michelmore et al., 1987
Linum usitatissimum	flax	C58C1	pGV3850::1103neo	-	hypocotyl	yes	Basiran et al., 1987
Linum usitatissimum	flax	A208	pTiT37SE::pMON570	-	hypocotyl	yes+prog	Jordan & McHughen, 1988
Linum usitatissimum	flax	C58C1	pGV3850::?	-	hypocotyl	yes+prog	McHughen, 1989
Linum usitatissimum	flax	A208	pTiT37::pMON894	-	hypocotyl	yes+prog	McHughen, 1989
Lycopersicon esculentum	tomato	LBA4404	pPMG85-587	-	cotyledon	yes+prog	Fillatti et al., 1987
Malus pumila	apple	LBA4404	pAL4404	pBIN6	leaf	yes	James et al., 1989
Medicago sativa	alfalfa	C58C1	pMP90	pGSFR780A/pGSFR761A	stem/petiole	yes	D'Halluin et al., 1990
Nicotiana clevelandii	tobacco	LBA4404	pAL4404	pGA492	leaf	yes+prog	Tavazza et al., 1988
Nicotiana edwardsonii	tobacco	C58C1	pGV3850	pKB27	leaf	yes	Kiernan et al., 1989
Nicotiana glutinosa	tobacco	PC2760	pAL4404	pTOK119	callus	no	Komari, 1989
Nicotiana plumbagenifolia	tobacco	PC2760	pAL4404	pTOK119	callus	no	Komari, 1989
Oryza sativa	rice	LBA4404	pAL4404	pKIWI1105	embryo	no	Raineri et al., 1990
Pisum sativum	pea	GV3101	pGV3850::pCAP212/pGV3851::pLD1		stem/leaf/epicotyl	no	Puonti-Kaerlas et al., 1989
Pisum sativum	pea	C58C1	pGV2260	p35SGUSintron	epicotyl/node	yes	De Kathen & Jacobsen, 1990
Pisum sativum	pea	GV3101	pGV2260::pGV1503	-	shoot culture/epicotyl	yes	Puonti-K et al., 1990
Pisum sativum	pea	EHA101	pEHA101	pBI1042	stem	no	Lulsdorf et al., 1991
Phaseolus vulgaris	dry bean	C58Z707	?	pGA482	cotyledon/node	no	McClean et al., 1991
Populus alba x P.tremula	aspen	C58C1	pMP90	pGSFR780A/pGSFR781A pTHW40/pTVE2	stem	yes	De Block, 1990
Populus trichocarpa x P.deltoides	poplar	C58C1	pMP90	pGSFR780A/pGSFR781A pTHW40/pTVE2	stem	yes	De Block, 1990
Ribes nigrum	black current	LBA4404	pAL4404	pBI121.X	internode	yes	Graham & McNicol, 1991
Rubus spp.	berry	LBA4404	pAL4404	pBI121/pBIN19	leaf/stem	yes	Graham et al., 1990
Solanum melongena	eggplant	GV3101	pGV3850	pBCAT1	leaf	yes+prog	Rotino & Gleddie, 1990
Solanum tuberosum	potato	C58C1	pMP90	pGSFR760A/pGSFR780A	leaf	yes	De Block, 1988
Solanum tuberosum	potato	C58C1	pGSFR1161	-	leaf	yes	De Block, 1988
Solanum tuberosum	potato	C58C1	pGFSR1280	-	leaf	yes	De Block, 1988
Solanum tuberosum	potato	LBA4404	pAL4404	pBIN6	tuber	yes	Sheerman & Bevan, 1988
Solanum tuberosum	potato	LBA4404	pAL4404	pGA492	leaf	yes	Tavazza et al., 1988
Solanum tuberosum	potato	PC2760	pAL4404	pBI121	microtuber	yes	Ishida et al., 1989
Solanum tuberosum	potato	LBA4404	pAL4404	pVU1011	leaf/stem	yes	Visser et al., 1989
Solanum tuberosum	potato	LBA4404	pAL4404	pBI121/pPS20A-G	leaf	yes	Wenzler et al., 1989
Stylosanthes humilis	Townsville stylo	GV3101	pGV3850::1103neo	-	leaf	yes+prog	Manners, 1988
Vicia narbonensis	vetch	C58C1	pGV3850hpt	-	epicotyl/shoot tip	no	Pickardt et al., 1991
Vigna aconitifolia	moth bean	C58C1	pGV3850::1103neo	-	protoplasts	no	Eapen et al., 1987
Vigna unguiculata	cowpea	GV3101	pGV3850::1103neo	-	leaf	no	Garcia et al., 1986
Vitis vinifera	grapevine	GV3101	pGV3850::1103neo	-	cell suspension	no	Baribault et al., 1989
Vitis vinifera	grapevine	EHA101	pEHA101	GA474-68	cell suspension	no	Baribault et al., 1989
Vitis vinifera	grapevine	LBA4404	pAL4404	pBI121	shoot apices	yes	Baribault et al., 1990
Vitis vinifera	grapevine	GV3101	pGV3850::1103neo	-	shoot apices	yes	Baribault et al., 1990

Bold plant names are monocotyledonous species. Abbreviations: see legend Table 1.

Development of a transformation protocol

The *Agrobacterium*-mediated transformation procedure has turned out to be not so generally applicable as was expected in the early days of the development of this gene transfer method. Not only are monocots hard to transform, but dicotyledonous crop species are also recalcitrant and the development of successful and efficient transformation protocols often requires several years (Baribault et al. 1989/1990; McGranahan et al. 1988/1990). Therefore it is of great importance to gather the experience of researchers in this field, who, though concerned with different plant species, often have to deal with the same problems. In the following paragraphs some of the general appearing obstacles are discussed and, if possible, the best way to deal with them is given.

Effect of bacterial strain and plant genotype.

Plant genotype dependency

Most *Agrobacterium* strains appearing in table 1 and table 2 are referred to as 'wide host range strain'. Though these strains are pathogenic for a wide range of plants species, this term is confusing, because the virulence of these *Agrobacterium* strains, within one species, can be genotype dependent. In our opinion, the genotype effect might be partly caused by various responses to stress. The stress reaction of plants involves the excretion of phenolic compounds, which direct the bacterium to the wounded plant cells and switches on the *vir* genes. Consequently, differential stress response may lead directly to differential virulence. As transformation efficiency can be genotype dependent, it is necessary to test more than one genotype or cultivar. In some species, like lettuce (Michelmore et al., 1987), Arabidopsis (Schmidt and Willmitzer, 1988) and potato (Wenzler et al., 1989), the effect of the genotype is much larger than the effect of the bacterial strain used. In other species the bacterial effect overrules the genotype effect, as in tomato (Davis et al., 1991), pea (Puonti-Kaerlas et al., 1989) and Brassica species (Charest et al., 1989; Ohlsson and Eriksson, 1988). In most species however, both plant genotype and bacterial strain affect the efficiency of gene transfer (e.g. walnut (Dandekar and Martin, 1988), willow (Vahala et al., 1989) and chrysanthemum (Van Wordragen et al., 1991)).

Screening for virulence

Often the development of an *Agrobacterium* mediated transformation procedure for a plant species starts with the screening of several wild type *Agrobacterium* strains to determine their virulence, the efficiency of gene transfer, on the species involved. This is a reasonable procedure, because practice has shown that there are large differences in virulence between the commonly used strains (Fig.3). Unfortunately, in some cases transformation efficiency is measured just by the size and frequency of tumours (Armstead and Webb, 1987; Charest et al., 1989; Clapham et al., 1990; Delzer et al., 1990). This can easily lead to misleading results, especially when several genotypes are used, because tumour induction and tumour growth not only depend on gene transfer efficiency, but also on the hormonal status of the plant. Therefore the expression of introduced reporter genes should be analysed. This can be done by an assay on the presence of opines, but a more certain and quantitative way to detect gene transfer is to make use of the reporter gene GUS, coding for ß-glucuronidase (Jefferson et al., 1987). The GUS reporter gene has recently been improved by the insertion of an intron (Vancanneyt et al., 1990). This GUSintron gene, which can only be expressed in the plant cell, enables the detection of single transformed cells, within several days after infection (Schrammeijer et al., 1990; Chapter 4: Van Wordragen et al., submitted).

The screening may be narrowed down to *Agrobacterium* strains for which disarmed derivatives have been developed, namely Ach5, C58, A281, B6S3, T37, A6 (Charest et al., 1989; Delzer et al., 1990; Komari, 1989; Puonti-Kaerlas et al., 1989; Vahala et al., 1989). These strains include all opine types and together their host ranges cover all species, listed in table 1 and table 2, for which gene transfer has been reported. Therefore, it is not necessary to extend such a screening by using more than these six *A.tumefaciens* strains, as was done by Morris et al.(1989) for fir, by Stomp et al. (1990) for pine species and by Dommisse et al. (1990) for onion. In our

Table 3. A representative selection of *Agrobacterium tumefaciens* strains and their characteristics.

Name	chromosomal background	Ti-plasmid	opine type	disarmed derivative	disarmed vir-plasmid
Ach5	Ach5	pTiAch5	octopine	LBA4404	pAL4404
C58	C58	pTiC58	nopaline	GV3850	pGV3850
A281	C58	pTiBO542	succinamopine/ agropine	EHA101	pEHA101

opinion, an even smaller selection of three strains (table 3), that cover the *A.tumefaciens* opine types octopine, nopaline and succinamopine/agropine, are in most cases enough for the purpose. Both the wild type strains and their disarmed derivatives can be used in combination with a T-DNA plasmid with suitable marker genes, e.g. the binary vector p35SGUSintron. Apart from the labour involved in screening many strains, these strains will need to be cured before they can be used in a transformation/regeneration procedure. Just a few reports appeared in which regeneration of transgenic plants was obtained using oncogenic strains (Table 1) and in *Brassica napus* these plants were shown to be fertile (Radke et al., 1988). A different, but not very commonly applied procedure is the use of shooter mutants. These semi-oncogenic *Agrobacterium* strains lack an active auxin locus at the T-DNA (Ooms et al., 1981) and induce an enhanced endogenous cytokinin level in transformed tissue, which results in some species in tumours with a 'shooty' phenotype. These strains are sometimes used to transform recalcitrant species, which are difficult to regenerate (Steffen et al., 1986; Krens et al., 1988). The main disadvantage using tumorigenic strains is that the introduction of hormone genes often results in the regeneration of aberrant and non-fertile plants.

Supervirulence.

Several reports indicate that the chromosomal background of the strain is less important than the origin of the *vir*-region on the Ti-plasmid. In pea, transformation using the B6 *vir* region is less efficient compared to transformation using the C58 *vir* region. This is also true if the B6 Ti-plasmid is placed in a C58 background (De Kathen and Jacobsen, 1990). The *vir* region of pTiBO542, present in the hypervirulent strain A281, is able to confer the supervirulent properties of A281 to strains with different chromosomal backgrounds. The supervirulent strain A281 and its disarmed derivative EHA101 (Hood et al., 1986) are more virulent than other strains in many species (e.g. alfalfa (Chabaud et al., 1988), pea (Hobbs et al., 1989; Lulsdorf et al., 1991; Puonti-Kaerlas et al., 1989), Stylosanthus (Manners, 1987), walnut (McGranahan et al., 1990)). This is particularly true for Solanaceae (Komari, 1989). In goosefoot (Komari, 1990) and poplar (Pythoud et al., 1987), the *vir*G and *vir*B loci of the pTiBO542 *vir* region, present on an additional plasmid next to the Ti-plasmid, are essential for transformation. In other species however the supervirulent strain is not better than other strains or even worse, as in kalanchoe (Jia et al., 1989).

The basic mechanisms behind differences in virulence are not very clear. In case of the supervirulent pTiBO542 plasmid the supervirulent properties were found to be correlated with the *vir*G and 3' *vir*B loci (Jin et al., 1987). Therefore, supervirulence is probably results from enhanced transcription of the *vir* genes, leading to a more efficient transport of the T-strand through the bacterial cell wall.

Phytohormone biosynthetic pathway genes

Another factor that explains some of the differences in virulence is the presence of the tzs (trans zeatine synthetase) gene on the Ti plasmid. This gene, which is not essential for tumour induction, is present in nopaline strains, but not in octopine strains. The gene product is involved in the biosynthesis of transzeatine, a cytokinin. This phytohormone is excreted by the bacterium and probably stimulates dedifferentiation and cell division on the site of infection, which enhances the susceptibility of the plant cell for transformation (Binns and Tomashow, 1988). The presence of this gene on the Ti-plasmid might explain the fact that nopaline strains are often more virulent than octopine strains (soybean (Byrne et al., 1987), pea (Hobbs et al., 1989; Puonti-Kaerlas et al., 1989), oilseed rape (Charest et al., 1989), lettuce (Michelmore et al., 1987), mustard (Ohlsson and Eriksson, 1988)), though the reversed situation is also found e.g. in Alnus and Betula species (Mackay et al., 1988).

A comparable phenomenon is found in *A.rhizogenes* strains. The agropine strains harbour two T-DNA's; TL and TR DNA. The TL-DNA contains the *rol* loci, responsible for the hairy root disease. This T-DNA is homologous to the single T-DNA of other *A.rhizogenes* strains. The TR-DNA carries the *tms* genes, involved in auxin biosynthesis, that are also found on the T-DNA of *A.tumefaciens* strains (Zambryski et al., 1989). These *tms* genes are not involved in the induction of hairy roots, but they are responsible for an extended host range of agropine strains compared to other *A.rhizogenes* strains (Cardarelli et al., 1987).

These two examples, *tms* genes in *A.rhizogenes* and the *tzs* gene in *A.tumefaciens* stress the importance of the hormonal status of the plant, during the transformation process. Differences in the tuning of the internal phytohormone balance might explain the genotype effect. This is supported by the fact that in some papers a different reaction upon *Agrobacterium* infection of in vivo and in vitro grown plants from the same genotype is reported (Hobbs et al., 1989). In our laboratory we found that for several recalcitrant plant species (e.g. chrysanthemum) gene

transfer is more efficient with greenhouse grown material than with *in vitro* grown plants.

Effect of developmental stage

Considering the assumed influence of internal hormone balance mentioned before, it is not surprising that many researchers have reported a large effect of the type and age of the tissue on gene transfer efficiency, measured either by tumour frequency or by number of transgenic shoots (Armstead and Webb, 1987; Dandekar et al., 1988; De Kathen and Jacobsen, 1990; James et al., 1990; McClean et al., 1991; McGranahan et al., 1990; Pang and Sanford, 1988 and Visser et al., 1989). In most cases meristematic tissue with actively dividing cells is found to be the most susceptible starting material. Seedling explants or embryos are often effectively used with both oncogenic (oilseed rape, soybean, walnut, lettuce, cedar, rice, spruce, pine, fir and eggplant; Table 1) and disarmed strains (arabidopsis, oilseed rape, cauliflower, musk melon, cucumber, carrot, soybean, cotton, sunflower, walnut, lettuce, flax, tomato and rice; Table 2). In other cases shoot tips, apices, microspores, cells suspensions or other rapidly dividing tissue is used. Leaves from young plantlets are a better explant source compared to older leaves (Schmidt and Willmitzer, 1988) and young potato tubers do produce more transgenic shoots than old tubers (Sheerman and Bevan, 1988). To our knowledge only two reports describe the opposite phenomenon; old leaves of papaya were more efficiently transformed than younger leaves or cotyledons (Pang and Sanford, 1988) and in tomato older leaves and cotyledons were slightly more susceptible than younger leaves (Davis et al., 1991). In both cases oncogenic strains were used.

Therefore a general prerequisite seems to be that cells should be used that are not yet fully differentiated, or that are easily able to dedifferentiate, and can be initiated to start cell division. In general, such a condition is also favorable for regeneration of adventitious shoots, needed to obtain transgenic plants. Despite this, regeneration and transformation often occur in different cell types (Colby et al., 1991).

The successful use of mature leaves as explant source is almost exclusively restricted to model plants like Solanaceae, kalanchoë and arabidopsis. These species are all highly susceptible to *Agrobacterium* mediated gene transfer; optimization of the protocol will have no detectable influence on a transformation rate, which is already optimal. The well-known leaf disk protocol,

developed by Horsch et al. (1985) for tobacco, petunia and tomato, is therefore in our opinion not the best choice for the transformation of a (recalcitrant) crop species.

Other factors influencing transformation efficiency

Several other factors, not concerning the plant or the bacteria, have been found to influence transformation efficiency. Most of these factors probably have more influence on the regeneration of shoots from transformed cells, than on the gene transfer process itself. In this paragraph only those factors will be discussed, that are regularly mentioned to be beneficial for transformation. Such factors might be important to take into consideration when developing a transformation protocol.

Induction of vir-genes

The agrobacterial virulence genes, located on the Ti-plasmid, are activated by certain phenolic compounds that are excreted by wounded plants cell (Fig. 1). Methods applied to enhance transformation efficiency are often based on this knowledge. The most commonly used enhancers are culture on feeder layers or the addition of phenolic compounds, usually acetosyringone, to the culture medium. Feeder layers or nursing plates are layers of cells derived from a cell suspension of a readily transformable plant species like tobacco, petunia or potato. The explants are usually placed on this cell layer, separated from it by a filter paper, one or more days prior to infection and throughout the cocultivation period. The beneficial effect on the transformation rate is probably largely due to the excretion by the nursing cells of phenolic compounds that efficiently induce the bacterial *vir*-region. However, it is not unlikely that other excreted compounds, influencing dedifferentiation and regeneration of explant cells or diminishing the negative effects of wounding and infection stress, also play a role.

Induction of the *vir*-region can also be achieved by adding acetosyringone to the cocultivation medium or to the bacterial culture (Stachel et al., 1985). In many reports acetosyringone is used without first investigating its influence on transformation efficiency. Acetosyringone is only useful if the plant does not produce one of the many phenolic compounds that can be recognized by the bacterium (Ashby et al., 1988), which is seldom among dicots, or if the cocultivation period is too short to allow induction, chemotaxis, attachment and gene transfer. Although acetosyringone is generally used irrespective of its promotive effect, in some cases it might be

deleterious for the transformation procedure. Some authors, who did investigate the effect of phenolic compounds, report a negative effect of acetosyringone or feeder layers on the number of transgenic shoots (De Kathen and Jacobsen, 1990; Catlin et al., 1988; Godwin et al., 1991). In other cases acetosyringone merely reduces the genotype effect (Delzer et al., 1990) and sometimes there is no effect at all (James et al., 1990; Lindsey and Gallois, 1990). Other components in the medium and environmental factors are also known to affect expression of the *vir* genes. Sucrose in the culture medium, an acidic pH and culture below 28 °C are essential for expression of *vir*D and *vir*G (Alt-Mörbe et al., 1989). The importance of a precise tuning of pH was also shown by Godwin et al. (1991), who found that shifts of 0.3 pH units can have a pronounced effect on the transformation efficiency in several plant species.

Reduction of stress

Other elements that may influence the final number of transformed shoots are factors that reduce the effects of stress. Medium supplements like AgNO3 are inhibitors of the ethylene response, that triggers many stress reactions. De Block (1988, De Block et al., 1989) found this compound essential in transformation of *Brassica napus*, *B.oleracea* and less responsive potato genotypes. A preculture period, prior to infection is often effectively applied (McHughen et al., 1989; Schmidt and Willmitzer, 1988; Tavazza et al., 1988); this will probably reduce wounding stress. The preculture period is presumably partly responsible for the positive effect of feeder layers, which usually require a culture period on the nurse plate before infection.

Infection stress can be reduced by carefully establishing the incubation and cocultivation time (Chabaud et al., 1988; Fang and Grumet, 1990; Fillatti et al., 1987; Jia et al., 1989; Tavazza et al., 1988) and the concentration of bacteria used (Davis et al., 1991; Fillatti et al., 1987; Michelmore et al., 1987). The latter factor may also be important because of the growth phase of the bacteria at the time of infection. Again this is not a general rule, because in transformation of sunflower the cocultivation time is not important (Schrammeijer et al., 1990) and in potato the bacterial concentration has no effect on the transformation efficiency (Tavazza et al., 1988).

Another stress-related problem in the development of transgenic plants is the finding in chrysanthemum, that leaf disk regeneration is seriously hampered by cocultivation with the bacteria (Chapter 5: De Jong et al., in preparation). This inhibition of regeneration might be

caused by the cumulation of wounding and infection stress. A comparable mechanism is probably responsible for the low transformation efficiencies in flax (McHughen et al., 1989). Finally, the way the selection for transformed cells is performed might influence the regeneration of transgenic shoots. Usually the neomycin phosphotransferase (NPT II) gene is (co)transferred to the plant cells to enable selection for transformed cells by the addition of the antibiotic kanamycin to the culture medium. Untransformed tissue will bleach and die, and only cells expressing the NPT II gene will survive. However, even if the cells are expressing neomycin phosphotransferase, kanamycin is often reported to inhibit regeneration of transformed shoots (Everett et al., 1987; Graham and McNicol et al., 1990), caused by the death of all surrounding untransformed tissue. A delay before kanamycin is added to the medium, during which the cells may recover from infection, start to divide and produce a sufficient amount of NPT II, is often beneficial (Boulter et al., 1990; Chabaud et al., 1988; Thomzik and Hain, 1990; Visser et al., 1989). Several authors report that the use of an alternative selective agent, usually hygromycin, leads to better results compared to kanamycin selection (Lulsdorf et al., 1991; Puonti-Kaerlas et al., 1990), though the opposite is also found (D'Halluin et al., 1990).

Concluding remarks

Despite the fact that transformation of recalcitrant crops is a hot research topic, only limited progress has been made the past four years. Just a few species can routinely be transformed and still little knowledge on underlying processes in the plant cell is available, which hampers the identification of bottle-necks in transformation and subsequent regeneration.

The most obvious conclusion from the large number of reports that have been published the past four years, is that not many general rules can be given on the development of an *Agrobacterium* mediated transformation protocol for recalcitrant crop species. However, it is possible to recommend some main lines for the development of a transformation/regeneration procedure in a specific plant species.

First of all it is important to carefully select both plant genotype and bacterial strain. Usually it will not be necessary to screen more than three *A. tumefaciens* strains, namely Ach5, C58 and A281. These strains are commonly used representives of three major opine types: octopine,

nopaline and agropine/succinamopine, and disarmed derivatives of these strains are available. The most virulent strains are often A281 and nopaline type strains, like C58, due to the presence of hypervirulent *vir*-genes and the *tzs*-locus, respectively.

Transformation should not be measured just by tumour size, but must be confirmed by opine assays or DNA analysis. The plant genotype to be chosen should easily regenerate adventitious shoots from various explants, as it is convenient to have a variety of explant types from which the best transformable explant type can subsequently be selected. Regeneration protocols should be established in the presence of bacteria and antibiotics. Usually, leaf explants are poor starting material for transformation.

The number of transgenic shoots or calli is often a very unreliable measure for the determination of gene transfer efficiency, as usually very few, if any, transformants are obtained, before optimization of the procedure. This hampers statistical analysis of the effect of changes in the procedure. Furthermore, the essential establishment of the effects of acetosyringone, feeder layers, length of cocultivation period, selection etc. will then be an elaborate and time consuming process. Therefore, the use of the GUSintron reporter gene (Vancanneyt et al., 1990) is strongly recommended for the first steps in the development of a transformation protocol. The effects on the gene transfer efficiency of plant genotype, bacterial strain, selection and other changes in the protocol can then be visualized within several days after infection. Although transient expression is visualized too, we have found this gene a very useful tool to obtain a lot of information in a relatively short period of time (Van Wordragen et al., submitted).

The GUSintron gene can also be used to select for suitable explant sources. The best results are often found with seedling explants, hypocotyls or cotyledons, embryos or shoot apices. Transformed cells will not appear equally distributed over the whole explant. The GUSintron gene can be used to locate the cells on which the regeneration protocol should be directed.

Very little is known about the plant-bound factors that are essential for gene transfer. The fate of the T-DNA in the plant cell is still a black box. This means that optimization of a transformation protocol remains largely a proces of trial and error. Experience obtained with other plant species is only of limited value, as even genotypes of the same species can react very different. When more transformation protocols will be established, the essential factors

involved may become clear. More research should be aimed at the unraveling of the processes involved on the plant site. This knowledge will enable or facilitate the transformation of recalcitrant species like woody crops and monocots.

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CHAPTER 8

Summary / Samenvatting

Summary

Genetic manipulation of plants is a technique that enables us to add to the plant genome, in a precise and well controlled manner, one or a few new genes, coding for desirable traits. In contrast to this, the conventional method for the introduction of new properties in plants, by cross breeding, is a random process in which two complete genomes are mixed and the desired phenotype has to be regained by repeated back crossing with the cultivated parent line. Despite these differences, both procedures basically accomplish the same; the addition of new inheritable characteristics to the genome of a plant. If both are available, the choice between molecular or conventional breeding for the introduction of a trait is often determined by the unique advantages and disadvantages of the techniques. Though the development of protocols is very laborious, genetic manipulation is in principal faster than cross breeding, because of the reduced need for back crossings. On the other hand, cross breeding is still very successful in introducing traits, which can only be recognized by phenotypic expression. Genetic modification requires precise knowledge of the gene involved, and as this knowledge is still very limited, only a few genes are available. The most important advantage of genetic modification is the fact that it is not hampered by crossing barriers. Therefore, the technique opens the possibility to introduce genes even from outside the plant kingdom into crops. This offers new opportunities to develop crop genotypes, resistant to pests and diseases, that formerly could only be controlled by the (often excessive) use of chemical pesticides and insecticides. Therefore, much research effort has been put in the development of genetic modification protocols for a wide range of plants. Examples of genes that have been successfully applied in this respect are: viral coat protein genes, which confer resistance to various viral diseases, proteinase inhibitor genes and *Bacillus thuringiensis* toxin genes, which both confer resistance to feeding by a wide variety of pest insects. Other options, which are less interesting from an environmental point of view, but important for growers, are e.g. the introduction of new flower colours and elevation of food value by directing the synthesis of nutritious proteins.

The aim of the research described in this thesis, was the development of a genetic

manipulation protocol for the ornamental crop chrysanthemum, employing the natural gene transfer capacity of the soil bacterium *Agrobacterium tumefaciens*, and the introduction of insect resistance genes derived from the insecticidal bacterium *Bacillus thuringiensis* (Bt). Though, until now, no Bt crystal proteins are known that are specifically toxic against the major pests in chrysanthemum culture (e.g. thrips, leaf miner and red spider mite), some minor pests, e.g. the Florida moth (*Spodoptera exigua*) are within reach. Moreover the development of genetic modification protocols for an ornamental crop in itself is important, in view of the large arrearage compared to applied biotechnology in vegetable crops (chapter 7).

All genetic manipulation protocols, including the *Agrobacterium* mediated transformation, must fulfill two conditions: it should be possible to stably introduce a new gene in a plant cell and to regenerate a complete plant from that single altered cell. The genotype 'Parliament' that we chose as starting material seemed to meet both prerequisites. Gene transfer by several *Agrobacterium* strains was demonstrated by tumour induction in vivo and in vitro (chapter 2) and several direct regeneration protocols starting from diverse types of tissue had already been developed.
A difficulty was the fact that induced tumourous outgrowths were sometimes not really tumours, or were the result of a gene transfer process, with a very low efficiency (chapter 3, chapter 4). It appeared that tumour-like tissue resulted even from a slight disturbance of the apparently very narrowly tuned hormonal status of 'Parliament'. Thus, very few gene transfer events, which needed not to be stable, were sufficient to induce cell proliferations. These findings were done by utilizing a newly developed reporter gene, the intron containing ß-glucuronidase gene. This gene allowed the rapid analysis of transformation events, shortly after infection. Previously this type of analysis was done by counting the number of transformed shoots or calli that were formed, a time consuming process and moreover a process that is virtually useless when the transformation efficiency is very low, as was the case in transformation of 'Parliament'. The new reporter gene allowed the screening of a range of chrysanthemum genotypes and the investigation of the effects of changes in the protocol and of the use of different *Agrobacterium* strains (chapter 4, chapter 5).

This work resulted in the selection of a few readily transformable genotypes and the preferential use of the supervirulent *Agrobacterium* strain A281 or its disarmed derivative EHA101. This part of the research is still being continued at the Centre for Plant Breeding and Reproduction Research (CPRO).

A second problem we met was the fact that the efficient regeneration of adventitious shoots in 'Parliament' was severely or even completely inhibited by infection with *Agrobacterium*. A recent publication, by Ledger et al., in which a different chrysanthemum variety was transformed, also stressed the importance of highly efficent regeneration for succesful *Agrobacterium*-mediated gene transfer. Further investigations revealed that inhibition of regeneration due to infection was a general problem in chrysanthemum. However, the phenomenon turned out to be partly genotype dependent, which enabled us to select for less sensitive cultivars. Also, procedures were developed to diminish the repressive effect of infection on the regeneration (chapter 5). It was recognized that the detrimental effect on regeneration was caused by the superimposement of infection stress and wound stress. Therefore, adaptations of the procedure were aimed at the reduction of stress, either by omitting brushing of the leaf explants prior to cocultivation, or by separating explant excision and infection in time, by preculturing the explants for eight days before infection. the results of this research suggest that the inhibitory effect of *Agrobacterium* infection might be partly responsible for low transformation efficiencies obtained in recalcitrant crops. However, since the control for regeneration often consists of uninfected explants instead of explants infected with disarmed strains, this phenomenon might have escaped attention in many studies.

In the course of our studies a successful transformation protocol for chrysanthemum was reported by Dr. C. Lemieux, at DNAP, California. The protocol has been reported on congresses, but is not yet published in literature. Though detailed information is unfortunately not available, which hampers comparison of their procedure with ours, it did become clear that the explant source might be of crucial importance. In the procedure described in this thesis we used leaf explants, taken from in vitro grown plants. Regeneration of shoots is very efficient and shoots develop directly from the

explants, without intermediate callus production. From the results of Lemieux, it was apparent that a callus phase before regeneration was essential. This is best achieved if explants from greenhouse grown plants are used. This option is now being explored at the CPRO.

Expression of transgenes in plants is influenced by many factors. This also holds for Bt toxin genes, for which recent analyses in several plant species have revealed poor expression of the protein. This might be caused by the presence of poly-adenylation signals and other plant regulatory sequences within the coding sequence, leading to mRNA instability and reduced translation efficiency. This latter phenomenon may be deteriorated by the bacterial codon use, which is different from the preferential codon use in plants. This information, necessitated the investigation of the level of expression and biological activity of Bt genes in chrysanthemum, even though a transformation/ regeneration protocol was not yet available. To explore the attainability of insect resistance in chrysanthemum by the introduction of Bt genes, we introduced the cryIA(b) gene in *Agrobacterium* induced tumours. In that way it was possible to analyse the expression and translation of the foreign gene in chrysanthemum cells, and moreover to assess the insect resistance of the transgenic tissue.
A bioassay was developed for larvae of the tobacco budworm, *Heliothis virescens*, with which the effect of feeding with tumourous chrysanthemum tissue on the growth and development of larvae could be measured accurately. In view of the expression problems pointed out above, the bioassays were remarkably successful. In some tumour lines a complete resistance to feeding by larvae of *Heliothis virescens* (tobacco budworm) was reached (chapter 6). Several other lines showed intermediate growth inhibition of the larvae and some lines were not resistant at all. These results indicate that it will eventually be possible to introduce insect resistance in chrysanthemum by utilizing the toxin genes from *Bacillus thuringiensis*.

Samenvatting

Genetische manipulatie van planten is een techniek die het mogelijk maakt, om op een nauwkeurige en controleerbare manier, één of enkele genen te introduceren in het DNA van een plant. Op deze manier kan een gewenste eigenschap worden toegevoegd aan het erfelijke materiaal van een plant. De klassieke methode om cultuur planten aan te passen aan de wensen van de kweker, de kruisings veredeling, is een willekeuriger proces. Hierbij worden twee complete sets erflijke informatie gemengd, waarna door terugkruisingen met de gecultiveerde ouder de gewenste nieuwe plant geselecteerd moet worden. Ondanks dit verschil wordt met beide technieken in essentie hetzelfde doel nagestreefd, het erfelijk materiaal van een cultuurplant wordt verrijkt met een nieuwe eigenschap. De keuze voor de ene of de andere techniek, als ze allebei beschikbaar zijn, wordt in hoge mate bepaald door hun specifieke voor- en nadelen. Zo is genetische manipulatie, ondanks het feit dat de techniek, wat cultuurgewassen betreft, nog in de kinderschoenen staat, vaak veel sneller dan de conventionele methode, omdat de langdurige terugkruisings programma's vaak overbodig zijn. Aan de andere kant heeft kruisingsveredeling veel goede resultaten geboekt met het inkruisen van eigenschappen waarvan alleen de uiterlijke kenmerken bekend zijn. Genetische modificatie vereist exacte kennis van het gen dat overgedragen moet worden en dit soort kennis is nog steeds erg beperkt, zodat er ook nog maar weinig genen beschikbaar zijn. Het belangrijkste voordeel van genetische manipulatie boven kruisingsveredeling is echter dat er voor de nieuwe techniek geen kruisingsbarrieres bestaan, wat de mogelijkheid geeft om eigenschappen zelfs van buiten het plantenrijk te introduceren. Hiermee wordt een enorm potentieel aan resistentie genen ontsloten, waardoor de mogelijkheden om planterassen te creëren, die niet meer vatbaar zijn voor virussen en insecten, verruimd worden. Op dit moment worden veel van die plagen nog steeds bestreden door bespuitingen met schrikbarend grote hoeveelheden chemische insecticiden en pesticiden. Om deze reden wordt er overal ter wereld veel onderzoeksinspanning gestoken in het ontwikkelen van genetische modificatie methodes voor een grote verscheidenheid aan planterassen. Voorbeelden van genen, die in dit verband al met succes gebruikt zijn, zijn: genen voor viraal mantel eiwit, die resistentie

verlenen tegen diverse virus ziektes, genen voor proteinase remmers en toxine genen uit *Bacillus thuringiensis*, die beiden bescherming geven tegen vraat door een groot aantal verschillende insecten. Minder interessant uit milieu oogpunt, maar wel belangrijk voor telers zijn de opties om nieuwe bloemkleuren in te brengen en om de voedingswaarde van bijvoorbeeld veevoedergewassen te verhogen door het laten aanmaken van nieuwe eiwitten met een hoge voedingswaarde.

Het doel van het onderzoek, dat in dit proefschrift wordt beschreven, is het ontwikkelen van een protocol voor de genetische manipulatie van het siergewas chrysant. Daarbij wordt gebruik gemaakt van *Agrobacterium tumefaciens*, een grondbacterie, die van nature de capaciteit heeft om genen over te dragen naar planten. In de natuur resulteert dit in tumorvorming op de plant, voor laboratorium toepassingen zijn de tumor verwekkende eigenschappen meestal verwijderd. Uiteindelijk zou een gen uit de bacterie *Bacillus thuringiensis* (Bt), dat de plant resistent kan maken tegen insektenvraat, overgedragen moeten worden naar chrysant. Hoewel er, tot nog toe, nog geen Bt kristal eiwitten bekend zijn die specifiek werkzaam zijn tegen één van de belangrijkste plagen in chrysant (bijv. trips, mineervlieg en spinmijt) kunnen sommige andere plaaginsecten wel bestreden worden. Bovenal is chrysant als modelgewas gekozen, omdat het ontwikkelen van genetische transformatie protocollen op zich voor siergewassen heel belangrijk is, gezien de grote achterstand op het terrein van de biotechnologie, in vergelijking met groentegewassen (hoofdstuk 7).

In elk genetisch manipulatie protocol, dus ook transformatie met behulp van Agrobacterium, zijn twee noodzakelijke processen te onderscheiden. Ten eerste moet het nieuwe gen ingebracht worden in een plantecel en ten tweede moet er een methode zijn om uit die ene veranderde cel weer een complete plant te laten groeien, de regeneratie. Met de door ons gekozen chrysantecultivar 'Parliament', leek dat allebei mogelijk. Genoverdracht door *Agrobacterium* werd aangetoond door tumor vorming na infectie met de bacterie (hoofdstuk 2). Regeneratie protocollen, gebaseerd op verschillende typen weefsel, zoals blad, stengel en bloemsteeltjes, waren al ontwikkeld. Toch bleek al snel dat het opzetten van een transformatie systeem voor chrysant niet eenvoudig zou zijn.

Een eerste obstakel was het feit dat het op chrysant geinduceerde woekerweefsel, niet of maar voor een heel klein deel uit veranderde cellen bestond (hoofstuk 3, hoofdstuk 4). Op tumoren lijkend weefsel ontwikkelde zich al na minieme verstoringen van het, blijkbaar zeer wankele, interne hormoon evenwicht van 'Parliament'. Daardoor was het mogelijk dat heel weinig transformatie gebeurtenissen, waarbij de genoverdracht niet eens stabiel hoefde te zijn, al voldoende waren om cellen tot woekeren aan te zetten. Deze ontdekking werd mogelijk gemaakt door het gebruik van een nieuw ontwikkeld zogenaamd 'reporter' gen; het onderbroken ß-glucuronidase gen. Met dit gen konden de transformatie gebeurtenissen in een stukje planteweefsel zeer snel en kort na infectie geanalyseerd worden. Voorheen werden dit soort analyses gedaan door het aantal getransformeerde scheutjes of calli te tellen dat onstond. Een tijdrovende procedure, die bovendien zo goed als onbruikbaar is als de tranformatie efficientie erg laag is, zoals by 'Parliament'. Het nieuwe reportergen maakte het mogelijk om een hele reeks chrysantecultivars te bestuderen en om het effect van kleine veranderingen in het protocol en van het gebruik van verschillende *Agrobacterium* stammen te onderzoeken (hoofdstuk 4, hoofdstuk 5). Dit werk resulteerde in de selectie van een aantal makkelijk te transformeren cultivars, waar 'Parliament' achteraf niet toe bleek te behoren. Bovendien kon een voorkeur worden uitgesproken voor het gebruik van de supervirulente *Agrobacterium tumefaciens* stam A281 of de daarvan afgeleide, niet-tumorigene EHA101. Met deze combinatie van cultivars en bacteriestammen wordt het onderzoek nog steeds voortgezet op het Centrum voor Planteveredelings en Reproduktie Onderzoek (CPRO-DLO).

Een andere moeilijkheid, was het feit dat de efficiente regeneratie van scheutjes uit losse cellen bij 'Parliament', sterk geremd werd door infectie met *Agrobacterium* (hoofdstuk 5). Een recente publicatie van Ledger et al., waarin transformatie van een verwante chrysantesoort wordt gemeld, benadrukt nog eens het belang van een goede regeneratie voor het succesvol tranformeren van chrysant met *Agrobacterium.* Nader onderzoek leerde dat, hoewel het verminderde regeneratie vermogen na infectie een algemeen probleem was in chrysant, er een duidelijk cultivareffect was. Dit maakte het mogelijk om minder gevoelige cultivars te selecteren. Daarnaast werden er procedures

ontwikkeld die het remmende effect op de regeneratie nog verder verminderden (hoofdstuk 5). Het werd duidelijk dat het nadelige effect op de regeneratie te wijten was aan een opeenstapeling van verwondingsstress en infectiestress. Daarom waren de aanpassingen in het protocol erop gericht om de stress te verminderen door minder verwonding van de blaadjes of door de het scheiden van de stressveroorzakers in de tijd. De resultaten van dit onderzoek doen vermoeden dat dit negatieve effect op de regeneratie ook bij andere gewassen (mede)verantwoordelijk kan zijn voor de lage transformatiepercentages, die behaald worden. In veel studies ontsnapt dit feit aan de aandacht, doordat als contrôle op de regeneratie experimenten uitgevoerd worden met ongeïnfecteerd weefsel in plaats van met weefsel, dat is geïnfecteerd met een avirulente bacteriestam.

In de loop van het project werd successvolle transformatie van chrysant gerapporteerd door Dr. C. Lemieux, van DNAP in Californië. Van de methode werd melding gemaakt op congressen, maar het protocol is nog niet gepubliceerd in de wetenschappelijke literatuur. Hoewel het daardoor moeilijk was om ons protocol te vergelijken met de Californische methode, lijkt het er op dat de oorsprong van het uitgangsmateriaal van belang is. Op explantaten van steriel opgekweekte planten, die gebruikt werden in de in dit proefschrift beschreven procedure, worden scheuten op een heel efficiente manier direct vanuit het explantaat geregenereerd, zonder intermediaire callus fase. Uit de resultaten van Lemieux bleek, dat een callus fase vóór de regeneratie van scheuten essentieel was. Callusinductie verloopt het best op explantaten afkomstig van kasplanten. Het effect van het gebruik van kasplanten wordt momenteel bestudeerd op het CPRO.

De expressie van overgedragen genen wordt door veel factoren beinvloed. Dit gaat ook op voor toxinegenen van *Bacillus thuringiensis*, waarvoor recent onderzoek in verschillende plantesoorten heeft uitgewezen dat de expressie van het toxische eiwit bijzonder laag is. Waarschijnlijk wordt dit veroorzaakt door de aanwezigheid van poly-adenyleringssignalen en ander regulatoire sequenties binnen het coderende gedeelte, wat resulteert in instabiliteit van het mRNA en slechte translatie. Dit laatste wordt

waarschijnlijk nog verergerd door het bacteriële codongebruik, dat afwijkt van het preferentiële codongebruik in planten. Deze informatie maakte onderzoek naar het expressieniveau en de biologische activiteit van Bt genen in chrysant noodzakelijk. Om een uitspraak te kunnen doen over de haalbaarheid van insecteresistentie in chrysant via introductie van Bt-genen, werd besloten om het gen in te brengen in chrysantetumoren. Op deze manier was het mogelijk, om de expressie en vertaling van het vreemde gen in een chrysantecel te bestuderen en vooral, om vast te stellen of het nieuwe gen inderdaad leidt tot de ontwikkeling van insecteresistentie.

Daarvoor werd een bioassay ontwikkeld voor rupsen van een tabaks mot, *Heliothis virescens*, waarmee het mogelijk was om het effect van het eten van chrysantetumoren op de groei en ontwikkeling van de larven accuraat te meten. Gezien de hierboven geschetste expressieproblemen, was de uitkomst van deze bioassays een onverwacht groot succes. In sommige transformanten werd een volledige resistentie tegen vraat door het testinsect, de rups van een tabaksmotje *(Heliothis virescens)* gevonden (hoofdstuk 6). Verschillende andere lijnen leken een gedeeltelijke weerstand te hebben, terwijl er ook waren, die helemaal niet resistent bleken. Zo'n patroon weerspiegelt de mate waarin het gen tot expressie komt in de verschillende transformanten. Deze resultaten geven aan dat het uiteindelijk mogelijk moet zijn om met behulp van Bt genen volledig insecteresistente chrysanten te creëren.

Nawoord

Errare humanum est. Toen ik in het begin van dit jaar serieus met schrijven begon dacht ik niet dat dit proefschrift ooit af zou komen, maar toch leest u nu in het eindprodukt. Na bestudering van de inhoud van dit boekje zal het duidelijk zijn dat, hoewel alleen mijn naam op de omslag staat, het niet tot stand had kunnen komen zonder de hulp van vele anderen, waarvan ik een aantal met name wil noemen.

Het onderzoeksproject is tot stand gekomen dankzij de financiële steun van de Stichting Innovatiefonds Plantenveredeling (InPla). Een bijdrage in de drukkosten van het proefschrift wordt geleverd door het LEB-fonds.

In de eerste plaats heb ik van het begin af aan veel gehad aan de ruime weefselkweek ervaring van het vaste chrysantenteam van het IVT/CPRO, Jan de Jong en Wim Rademaker, sinds kort versterkt met Jan van den Berg. Vooral de intensievere samenwerking in de laatste fase van het project, is heel vruchtbaar geweest.
Wat samenwerking betreft, mogen ook de mensen van de 'andere kant' van het project, Guy Honée en Bert Visser, niet onvermeld blijven. Hoewel de beide onderdelen van het project niet zo nauw verweven konden worden als de bedoeling was, is het contact altijd heel goed geweest en waren we voortdurend op de hoogte van elkaars resultaten. De hulp van Bert Visser en Elly Munsterman, bij het opzetten van de bioassays, was van grote waarde voor mij.

De uitwisseling van gegevens en onderzoeksresultaten in het Italse Promovendi-clubje van Lous van Vloten-Doting heb ik altijd heel stimulerend gevonden, niet in het minst door de levendige belangstelling van Lous voor zowel het onderzoek als de onderzoeker.

Het regelmatige overleg met mijn copromoter Hans Dons, wiens kritische blik soms wel eens een beetje te scherp was naar mijn zin, voorkwam vele fouten in de opzet van proeven en zijn inzicht en brede ervaring zijn een grote steun geweest bij het

interpreteren van resultaten. Ook in de laatste schrijffase bleef Hans heel betrokken bij de voortgang en kon ik er altijd van op aan dat stukken ook inderdaad op de afgesproken datum gecorrigeerd waren.

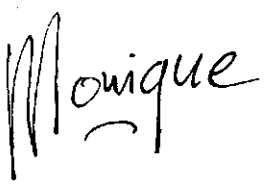
Ook mijn promoter Ab van Kammen heeft van het begin af aan grote belangstelling voor het project getoond, waarbij zijn betrokkenheid niet geremd werd door het feit dat het onderzoek niet in zijn eigen vakgroep plaatsvond. Zijn adviezen en herhaalde oproepen tot meer samenwerking zijn vaak bepalend geweest voor de richting van het onderzoek.

Van groot belang voor elk promotie onderzoek zijn de studenten die eraan meewerken. In mijn geval waren dat vier onvolprezen jongemannen, die stuk voor stuk een grote inzet en interesse voor het onderzoek toonden. Gilles Jonker beet het spits af met onderzoek naar het effect van een alternatief selectief agens en andere cultivars. Pieter Ouwerkerk, die net als Gilles uit het verre westen kwam, namelijk van de Vrije Universiteit te Amsterdam, is een vol jaar gebleven. In die periode heeft hij een groot deel van de gegevens uit hoofdstuk 3 verzameld. Wat korter, maar toch nog langer dan hij van plan was, is Martin Schornagel gebleven. In die relatief korte periode van enkele maanden heeft hij een enorme hoeveelheid werk verzet. De gegevens die hij boven tafel bracht worden beschreven in hoofdstuk 4 en 5. Ook de resultaten die Arjan van der Bij behaalde, zowel tijdens zijn afstudeervak voor de Internationale Agrarische Hogeschool Lahrenstein, als in een maand vakantiewerk, zijn belangrijk geweest, vooral voor het inzicht in de expressie van verschillende promoters in chrysant.

Produktief onderzoek kan alleen maar plaatsvinden in een vruchtbare, stimulerende omgeving en daarvoor stonden de collega's van het CPO garant. Speciale vermelding verdienen mijn beide paranimfen Ingrid Hoek, mijn kamergenoot, en Anne-Claire van Altvorst, een mede-transformator. Het enige bezwaar van hun aanwezigheid was dat het weleens te gezellig werd, wat niet bevordelijk was voor het doorworstelen van die stapel literatuur. Alle andere mensen van de oude sector Biotechnologie en de hoofdafdeling Ontwikkelingsbiologie, waarvan er veel in de loop van die drieëneenhalf

jaar goede vrienden zijn geworden, wil ik bedanken voor hun gezelligheid en saamhorigheid. Hoewel we nog steeds geen vaste kroegmiddag hadden, kun je toch bepaald niet stellen dat we gebrek hebben gehad aan borrels, labuitjes en andere festviteiten!

Tenslotte moet ik het thuisfront nog noemen. Mijn zus Barbara wil ik bedanken voor het aandragen van een actueel onderwerp voor een stelling. Mijn broer Peter-Paul is aan het begin van mijn promotieonderzoek naar de U.S.A. vertrokken en zijn bijdrage bestaat dan ook slechts uit het organiseren van een weekendje Grand Canyon na het UCLA congres in Park City, Utah.
Dit proefschrift is niet voor niets opgedragen aan Anke en Jacques, mijn ouders, en aan Edwin, al tien jaar mijn steun en toeverlaat. Mijn ouders hebben vanaf de lagere school mijn leergierigheid gestimuleerd. Zonder hun warme belangstelling en (ook financiële) steun was het niet mogelijk geweest om mijn studie en promotie-onderzoek af te ronden, en dat terwijl ze eigenlijk niet geloofden dat er een droge boterham te verdienen viel in de Biologie. De steun van Edwin gaat natuurlijk niet zo ver terug maar is daarom niet minder essentiëel geweest. Elke promovendus kan erover meepraten, dat in de regelmatig voorkomende tot-over-je-oren-in-het-werk periodes, het niet altijd mogelijk is om eerlijk 50% van de huishoudelijke beslommeringen op je te nemen. Een begrijpende partner is dan onontbeerlijk, niet alleen voor de praktische zaken, maar vooral voor de morele steun en het aanbieden van een schouder om af en toe op uit te huilen. Edwin, jij hebt ondanks je eigen, soms hectisch drukke, baan altijd klaar gestaan als ik je nodig had. Zonder jou had ik het niet gered!



Curriculum vitae

Monica Francisca van Wordragen werd geboren op 29 april 1964 in Haarlem. In 1982 behaalde zij het diploma 'ongedeeld V.W.O.' aan het Dom Helder Camara College te Haarlem. In datzelfde jaar werd gestart met de studie Biologie aan de Rijks Universiteit Leiden. Aldus behorende tot de eerste lichting twee-fase structuur studenten, kon na de propedeuse, waarvan het diploma werd behaald in 1983, direct begonnen worden met de doctoraal fase. Als specialisatie richting koos zij Moleculaire Biologie. De praktijkpunten werden behaald in twee stages van 9 maanden. De eerste, in 1985/1986, was bij de vakgroep 'Plantevirussen' van de subfaculteit Scheikunde, in samenwerking met de Biologie vakgroep 'Moleculaire basis van celdifferentiatie bij planten', onder directe begeleiding van Dr. Ir. C. van Dun. Dit onderzoek behelsde het transformeren, met behulp van *Agrobacterium*, van tabak, met genen van het Alfalfa Mosaic Virus, met als uiteindelijk doel het introduceren van virusresistentie. De tweede stage periode, in 1986/1987, werd doorgebracht op de afdeling 'Research and Development' van Gist brocades te Delft. Onder leiding van Dr. Ir. W. van der Wilden, hoofd van de onderzoeksgroep 'Celbiochemie', werd de inductie van heatshock eiwitten in gist bestudeerd. In augustus 1987 werd de studie Biologie met het behalen van het doctoraal diploma afgerond. Op 15 juli van dat jaar werd zij aangesteld als Onderzoeker bij het Instituut voor de Veredeling van Tuinbouwgewassen te Wageningen, later het DLO-Centrum voor Plantenveredelings- en Reproduktie-Onderzoek (CPRO-DLO). Tot 1 januari 1991 werd daar het onderzoek verricht, dat beschreven is in dit proefschrift. Sinds 1 mei 1991 is Monique van Wordragen werkzaam bij de vakgroep Moleculaire Biologie van de Landbouw Universiteit Wageningen. Daar werkt zij aan de constructie van een geïntegreerde klassiek/moleculair genetische kaart van chromosoom 6 van tomaat.