Genetic Modified Chrysanthemum (*Dendranthemum x gradiflora*) Plants Carrying the Sweet Potato Sporamin Gene via Petal Explants Transformation Showing Resistance to *Spodoptera litura*

Yuhsin Chen[1], Kuei-Fang Pai[1], Chi-Chu Tsai[2], Sheng-Chung Huang[1]

ABSTRACT

A spormin gene, trypsin inhibitor A (TIA), was introduced into chrysanthemum (*Dendranthemum gradiflora*) cv. Ah-Lai Feng. Increase the duration of pre-culture of petal (ray florets) up to 7 days increased the percentage successful regeneration. Two lines confirmed the integration of TIA gene into chromosome showed resistance to *Spodoptera litura*. Both lines were vegetatively propagated with good stability.

Key words: Genetic transformation, chrysanthemum, sporamin, proteinase inhibitor, petal, *Spodoptera litura*

Introduction

Chrysanthemum is one of the most important horticultural crops on the world flower market. However, modern chrysanthemum cultivars are often susceptible to many insects and pathogens (Robinson and Firoozabady, 1993). The control of insect pests is often dependent on the use of chemical insecticides during field cultivation, as the incompatibility barriers and high ploid levels have restrained traditional breeding of insects and pathogen resistance in chrysanthemum. Recent progress in plant genetic transformation technology has made it possible to introduce specific traits into an agronomic superior cultivar without altering ornamental characteristics that in sexual crosses may occur. Many protocols have been proposed to develop transgenic chrysanthemum (Courtney-Gutterson *et al.*, 1993; Fukai *et al.*, 1995; Sherman *et al.*, 1998).

[1] Taichung District Agriculture Improvement Station, Chang Hwa, 515, Taiwan.
[2] Kaohsiung District Agriculture Improvement Station, Ping-Tung, 900, Taiwan.

In the development of insect-resistant transgenic crops, proteinase inhibitors such as α-amylase inhibitor, trypsin inhibitor, are also beginning to receive more attention (Hilder *et al*., 1990, 1993; Lin, 1993; Yeh *et al*., 1997) because these plant derived inhibitors are part of the natural plant defense system against insect predation (Green *et al*., 1971; Lin, 1993). It is demonstrated when foreign plant proteinase inhibitors genes were introduced into tabacco, transgenic tabacco plants expressing the proteinase inhibitor proteins at relatively high levels were resistant to tabacco insect pests (Johnson *et al*., 1989; Boulter *et al*., 1991; Gatehouse *et al*., 1991). The introduction of sweet potato sporamin gene, a homologue to Kunitz type trypsin inhibitor, into tabacco was also shown to be resistant to *Spodotera litura* when it was expressed (Yeh, 1997).

In this study, we describe the introduction of the gene encoding a sporamin into chrysanthemum. To provide effective protection of transgenic chrysanthemum against insect pests, the constitutive active 35S promoter was used to derive the expression of the TIA (Trypsin inhibitor type A) gene in transgenic chrysanthemum transformants.

Materials and Methods

Plant materials

The single pink chrysanthemum (*Dendranthema x grandiflorum* Tzvel.) variety, Ah-Lai Feng, was employed in this study. It is widely cultivated in summer crop for its stability in floral initiation and differentiation even at supra-optimal temperatures. Mother plants of Ah-Lai Feng were grown in a green house and allow natural flowering. Ray florets were cut from the mother plants, surface sterilized with 1% sodium hypochloride for 5 min and rinsed 3 times with sterilized distilled water. Ray florets explants were placed on MS medium in a Petri dish, containing 0.1 mg/l NAA, 1mg/l BAP, 3% sucrose with pH 5.7 and solidified with 0.8% w/v Agar (SR medium). Culture were incubated at 25℃ with a light intensity of 25 μ mol/m^2/s provided by cool white fluorescent tubes for 16 h/day.

Binary plasmid and bacterial strain

A full-length (0.93kb) cDNA fragment, TIA (trypsin inhibitor A), encoding sporamin was introduced downstream of the 35S promoter to the binary vector pBI121. The TIA::pBI121 construct was named spTi also carries a NPT II selective maker gene programmed by the nopaline synthase promoter (Yeh *et al*. 1997, Figure 1), was then transferred into the disarmed *Agrobacterium tumefaciens* strain LBA 4404 for subsequent transformation.

Plant transformation and regeneration

Explants from petals (approx. 10 mm^2 petal disks) were pre-cultured for 0-7 days before inoculation. Plant transformation was carried out according to Fukai *et al*. (1995) with some

modification. Briefly, petal explants pre-cultured for 0, 1, 2 or 7 days were dipped in 100 ml liquid SR medium supplemented with 1 ml of bacteria suspension (*A. tumefaciens* strain LBA 4404 in LB medium, O.D. 1.0) and 100 μM acetosyringone for 30 min. The explants were then blotted dried on 2 sheets of filter paper, and co-cultured in SR medium for 2 days. Following the 2 days co-cultivation, explants were transferred to a fresh selection medium (S medium), based on the SR medium supplemented with 250 mg/l cefotaxime, 100 mg/l kanamycin. The explants were then transferred to a fresh medium every 3 weeks thereafter until 12 weeks after inoculation.

DNA analysis

DNA was extracted from young leaves of greenhouse-grown putatively transformed plants and the control following the protocols of Wagner *et al.* (1987). The presence of GUS and TIA genes were demonstrated by using PCR with the following primers: 5' atgtta cgtcctgtag aaacc 3' and 5'-tcatt gtttg cctcc ctgct gc-3', 5'-aattaaacat cattacctct tc-3' and 5'-ggaga attaa aacaa acaca g-3', respectively. Amplitaq polymerase was employed and the amplification protocol was: 1 min melting at 94 ℃, 1 min annealing at 50 ℃ and 2 min elongation at 72 ^{0}C for 30 cycles, yielding a fragment of 0.93 and 1.8 kb for TIA and GUS gene, respectively.

DNA integration into chromosome was confirmed by Southern analysis. The TIA fragment, excised with *Xba* I/*Bam* HI from the spTi plasmid following cultivation of *A. tumefaciens* strain LBA 4404 in LB medium, was 3' end labeled with Digoxgenin's dig-dUTP (Roche Molecular Biochemicals) as probe. Sample DNA (20 μg) was double digested 18h with *Xba* I/*Bam* HI and electrophoresis 18 h with 18V through a 0.8% SeaKaem LE agarose gel and transferred to a 0.45mm positively charged nylon membrane (Roche Molecular Biochemicals). The detection of probes on the blot employed the Chemiluminescent methods. The procedures for labeling and detection of probes followed the instruction provided by the manufacturer (Roche Molecular Biochemicals).

Insect Bioassays.

Young, vigorous greenhouse-grown plants were prepared for *Spodoptera litura* bio-assays. Fifteen 3rd larvae were placed on young mature leaves of a plant as one replicate. Four replications each line were incubated in a constant temperature room at 25 ± 2℃ and under 25 μ Em^{-2}s^{-1} fluorescent light. Mortality and body weight of the larvae were determined after 72 h. Percentage mortality data was arcsin transformed for analysis while presented in percentage. Mean comparisons were done using Fisher's least significant differences (α =0.05) with the software INSTAT developed by the University of Reading, UK.

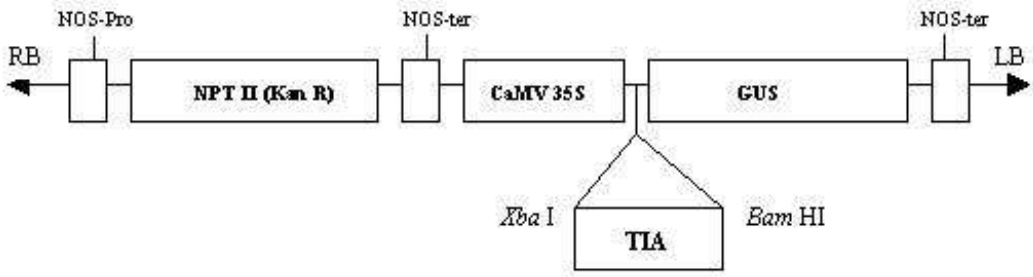

Fig. 1. The full length sporamin cDNA (TIA) was inserted to PBI121 vector via restriction site *Xba* I/*Bam* HI programmed by the constitutive CaMV 35S promoter.

Results

Effects of pre-culture days on the regeneration of chrysanthemum

Only young fully expanded petals were employed for genetic transformation as it was found the regeneration rate of explants decreased when petals are aged (data not shown). The regeneration capacity was correlated with the duration of pre-culture days. The generation of callus on explants for pre-cultured 0, 1, 2 and 7 treatments was 0%, 2.5%, 17.9 % and 68.5%, respectively (Table 1). The results indicated that establishment of callus before genetic transformation is beneficial to the successful regeneration of explants in subsequent operation. Shoots formation was also increased with the increase of days of pre-culture (0%, 2.5%, 7.1% and 48.6%, Table 1). No phenotypic variation was observed after cultivation and flowering.

Table 1. Effects of pre-culture duration on the regeneration of chrysanthemum after 12 weeks culture on selection medium.

Pre-culture days	0	1	2	7
Callus formation (%)	0	2.5	17.9	68.6
Shoot formation (%)	0	2.5	7.1	48.6

Confirmation of transformation

The confirmation of transformation was screened by PCR amplification (data not shown) initially then confirmed by Southern blotting. Among the putatively transformed plants, two lines showed both GUS and TIA gene positive in PCR were selected for subsequent assay. Electrophoresis (Fig. 2A) and Southern blots (Fig. 2B) of the control and two lines of putative transformants were shown.

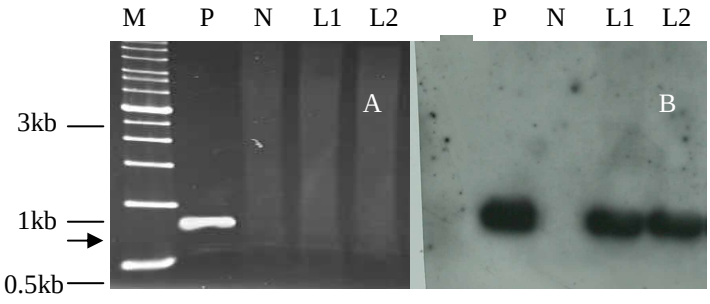

Fig. 2. Electrophoresis (A) and Southern blots (2B) of the control and two lines of putative transformants. Genomic DNA of the control was double digested with *Xba* I/*Bam* HI and electrophoresis through a 0.8% agarose gel. M: 1kb molecular marker. P: plasmid DNA. N: Not transformed plants. L1, L2: putative transformed lines. Arrow indicates the 0.93 kb TIA fragment.

Insect bioassay

Larvae fed on the transformants were observed trying to escape. The body weight of larvae fed on the transformants was significantly lower than the control (Table 2), especially the line1-4. The survival of both genetic transformed lines was also considerably lower than the control, although it was not significant (P<0.1).

Table 2. The body weight and mortality of 3rd larvae of *Spodoptera litura* after feeding 3 days on chrysanthemum plants.

Lines	Body wt. (mg)	Mortality (%)
Wild type	57±9 b	11.7±5.5 a
L8-24	48±5 ab	26.7±14.9 a
L1-4	41±6 a	23.3±14.5 a

Discussion

On the genetic transformation of chrysanthemum, it has been reported successful using leaf, stem explants but not petal explants as reported here, probably due to that petal explants were reported as a source of variation (Malaure, 1991a, b). However, we found that the petal was more advantageous over leaf explants for its easier sterilization and the phenotype can be identified easily. Also, generation of variants is an important purpose in breeding programs thus it should not limit the use of petal explants. The regenerants showed no variation in floral characteristics compared to the control, however.

In our study, we found that the regeneration of petal explants was considerably influenced by their physiological age and cultivars in chrysanthemum. Compared with

reported chrysanthemum transformation protocols, the efficiency of petal explants was no less than other protocols reported (Fukai *et al*., 1995; Sherman *et al*., 1998) and sterilization was better controlled. However, in order to obtain a higher percentage successful transformation, the formation of callus is needed before inoculation. Pre-culture for 7 days show the highest rate of success transformation (Fig. 3A). Longer duration of pre-culture, however, formed shoot and restraint the genetic transformation process.

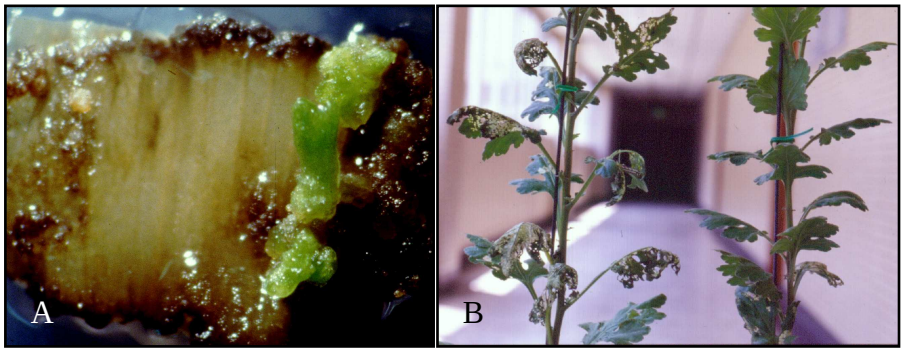

Fig. 3. (A) Petal explants pre-cultured 7 days showing highest regeneration rate; (B) The line 1-4 showed pest resistance (right) than the control (left).

In insect bioassays, chrysanthemum lines confirmed integration of TIA gene showed varied effects on mortality of larvae. Among the several lines regenerated, the line 1-4 showed the best effects of pest resistance (Fig. 3B). The varied effects of pest resistance was likely due to differential expression of the inserted gene, caused by high copy number of the same gene, position effects and co-suppression (Hilder *et al*., 1993). The expression of proteinase inhibitors reported in the study has been proposed for the purpose of pest management. The TI protein confers resistant to *Spodoptera litura* in chrysanthemum indicates that the natural mechanism of plant resistance may be of great value in an IPM program as another pest control strategy option.

Acknowledgements

We greatly thank Prof. K. W. Yeh of National Taiwan University, for providing the spTi plasmid and *A. tumefaciens* strain LBA 4404 for transformation. This project is sponsored by the Council of Agriculture, Taiwan.

References

1. Boulter, D., G. A. Edward, A. M. R. Gatehouse, J. A. Gatehouse, and V. A. Hilder. 1991. Additive protective effects of different plant-derive insect resistant genes in transgenic tobaco plants. Crop Protection 9:351-354.

2. Bush, S. R., E. D. Earle, and R. W. Langhans. 1976. Plantlets from petal segments, petal epidermis and shoot tips of the periclinal chimera, Chrysanthemum morifolium <<Indianapolis>>. Amer. J. Bot. 63:729-737.
3. Courtney-Gutterson N., E. Firoozabady, C. Lemieux, J. Nicholas, A. Morgan, K. Robinson, A. Otten and M. Akerboom. 1993. Peoduction of genetically enfgineerer color-medified chrysanthemum plants carrying a homologous chalcone synthase gene and their field performance. Acta Horticulturae 336. 57-62.
4. Fukai, S., J. De Jong, and W. Rademaker. 1995. Agrobecterium-mediated genetic transformation of chrysanthemum. Acta Horticulturae 392: 147-152.
5. Gatehouse, A. M. R., D. S. Howe, J. E. Flemming, V. A. Hilder, and J. A. Hatehouse. 1991. Biochemical basis of insect resistance in winged bean (*Psophocarpus tetragonolobus*) seeds. J. Sci. Food Agric. 55:63-74.
6. Green, T. R. and C. A. Ryan. 1971. Wound-induced proteinase inhibitor on plant leaves: a possible defense mechanism aainst insects. Science 175: 776-777.
7. Hattori, T., T. Nakagawa, M. Maeshima, K. Nakamura, and T. Asahi. 1985. Molecular cloning and nucleotide sequence of cDNA for sporamin, the major soluble protein of sweet potato tuberous roots. Plant Mol. Biol. 5:313-320.
8. Hilder, V. A., A. M. R. Gatehouse, and D. Boulter. 1990. In genetic engineering of crop plants. Ed. G. Lycett and D. Grierson. pp. 51-66. London, Butterworths.
9. Hilder, V. A., A. M. R. Gatehouse, and D. Boulter. 1993. Transgenic plants conferring insect tolerance: Protease inhibitor approach. Transgenic plants. Vol. 1. Engineering and utilization. Ed. Kung, S. pp.317-335. Academic Press, New York.
10. Johnson, R., J. Narvaez, G. An, and C. Ryan. 1989. Expression of proteinase inhibitors I and II in transgenic tobacco plant: Effects on natural defense against *Manduca Sexta* larvae. Proc. Natl. Acad. Sci. USA 86:9871-9875.
11. Lin, Y. H. 1993. Trypsin inhibitors of sweet potato: review and prospect. Recent Adv. Botany 13; 179-185.
12. Lin, Y. H. and H. H. Chu. 1988. Endopeptidases of sprouts and resting roots of sweet potato (*Ipomoea batatas* (L.) Lam. cv. Tainong 57). J. Chinese Biochem. Soc. 18: 18-28.
13. Malaure, R. S., G. Barclay, J. B. Power, and M. R. Davey. 1991a. The production of novel plants from florets of *Chrysanthemum morifolium* using tissue culture. 1. Shoot regeneration from ray florets and somaclonal variation exhibited by the regenerated plants. J. Plant. Physiol. 139: 8-13.

14. Malaure, R. S., G. Barclay, J. B. Power, and M. R. Davey. 1991b. The production of novel plants from florets of *Chrysanthemum morifolium* using tissue culture. 2. Securing natural mutations (sports). J. Plant. Physiol. 139: 14-18.
15. Robinson, K. E. P. and E. Firoozabady. 1993. Transformation of floriculture crops. Scientia Horticulturae. 55: 83-89.
16. Sherman, J. M., J. W. Moyer, and M. E. Daub. 1998. A regeneration and Agrobacterium-mediated transformation system for genetically diverse Chrysanthemum cultivars. J. Amer. Soc. Hort. Sci. 123: 189-194.
17. Van Wordragen M. F., De Jong, J., Huitema, H. B. M., and Dons, H. J. M. 1991. Genetic transformation of chrysanthemum using wild type *Agrobacterium* strains; strain and cultivar specificity. Plant Cell Report 9: 505-508.
18. Wagner D. B., Furnier G. R., Saghai-Maroof M. A., Williams S. M., Dancik B. P., Allard R. W. 1987. Chloroplast DNA polymorphisms in lodgepole and jack pines and their hybrids. Proc Natl Acad Sci USA 84:2097-2100.
19. Xu, D., Q. Hue, D. McElroy, Y. Mawal, V. A. Hilder and R. Wu. 1996. Constitutive expression of a cowpea trypsin inhibitor gene, CpTi, in transgenic rice plants confers resistance to two major insect pests. Molecular Breeding 2:167-173.
20. Yeh, K. W., M. I. Lin, S. J. Tuan, Y. M. Chen, C.Y. Lin and S. S. Kao. 1997. Sweet potato (*Ipomoea batatas*) tyypsin inhibitors expressed in transgenic tobacco plants confer resistance against *Spodoptera litura*. Plant Cell Rep 16: 696-699.