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Development of insect-resistant transgenic cauliflower plants expressing the trypsin inhibitor gene isolated from local sweet potato

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Abstract *Agrobacterium*-mediated transformation was used to introduce a trypsin inhibitor gene into Taiwan cauliflower (*Brassica oleracea* var. *botrytis* L.) cultivars. The TI gene was isolated from a well-adapted Taiwan sweet potato cultivar and was expected to be especially effective in combating local pests. In vitro regeneration studies indicated that 4-day-old cauliflower seedling hypocotyl segments, pretreated with 2,4-dichlorophenoxyacetic acid for 3 days and incubated on a silver-ion-containing shoot induction medium, gave regeneration rates greater than 95%. Optimum transformation conditions were determined. G418 selection at 15 mg/l was initiated 1 week after cocultivation, and the dose was doubled 1 week later. Over 100 putative transgenic plants were produced. Transgenic status was confirmed by in vitro TI activity, and Southern and Western hybridization assays. The transgenic plants demonstrated in planta resistance to local insects to which the control plants were vulnerable.

Key words Cauliflower · *Brassica oleracea* var. *botrytis* L. · Trypsin inhibitor · Insect resistance · *Agrobacterium* mediated transformation

Abbreviations *BA* N^6-Benzyladenine · *CI medium* Callus-inducing medium · *2,4-D* 2,4-Dichlorophenoxyacetic acid · *IAA* Indole-3-acetic acid · *NPTII* Neomycin phosphotransferase II · *SI medium* Short induction medium · *TI* Trypsin inhibitor

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Introduction

Annual world production of cauliflower was over 12.7 million metric tons in 1996, with China leading with more than 30% of world production. One of the major problems in cauliflower cultivation is insect pests. Massive quantities of synthetic insecticides are used, giving rise to major concerns about food safety and environmental pollution in addition to the high chemical and labor costs.

The development of genetic engineering protocols for cauliflower with marker genes have been reported by David and Tempe (1988), Srivastava et al. (1988), De Block et al. (1989), and Eimert and Siegemund (1992) using *Agrobacterium*-mediated infection. The first case of transferring agronomically useful traits into cauliflower involved insertion of the capsid gene and the antisense gene VI of cauliflower mosaic virus via *Agrobacterium* (Passelegue and Kerlan 1996). In this work, while the transcription of the transgenes was detected in all transgenic plants, translation of the capsid protein was not detected.

Our work involves transferring the gene that encodes a trypsin inhibitor (TI), a subgroup of protease inhibitor, into Taiwan cauliflower cultivars to combat insect pests. Protease inhibitors are part of the natural plant defense system against insect predation. They are present in the edible parts of numerous crop species, e.g., TI makes up over 80% of the soluble proteins in the storage roots of Taiwan sweet potato cultivars (Lin 1996) and actinidin makes up to 60% of the soluble proteins of the kiwi fruit tissue (Praekelt et al. 1988). Moreover, they are capable of controlling a wide spectrum of insect pests (Hilder et al. 1990). Since the gene we used was isolated from the storage root of a well-adapted local sweet potato cultivar (Yeh et al. 1997), we expected that it would be especially effective in combating local insect pests. The sweet potato, of New World origin, is well-established in China (over 86% of world production) and is spreading as a wild plant in Taiwan.

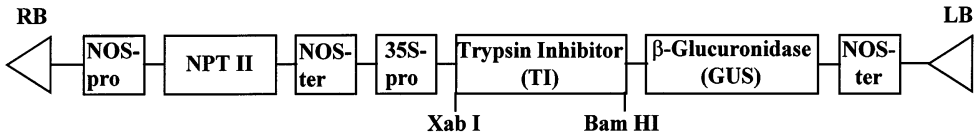

Fig. 1 Construct of pBI121/TI vector used in cauliflower transformation (TI 0.66 kb, pBI121 13 kb, pBI121/TI 13.66 kb)

Materials and methods

Plant materials and in vitro conditions

Three key Taiwanese cauliflower (*Brassica oleracea* var. *botrytis* L.) cultivars, Known You Early no. 2, Snow Lady, and Beauty Lady, were used. Seeds were disinfected in 70% ethanol for 10 s, followed by 0.5% sodium hypochlorite for 50 min (with sonication during the initial 20 min). B5 medium (Gamborg et al. 1968) with 3% sucrose was used as the basal medium, and for solid medium, 0.8% Bactoagar was used. The incubation conditions for germination and in vitro culturing, unless stated otherwise, were 25°C and 16-h photoperiod of approximately 28 μE m^{-2} s^{-1}. Four-day-old germinated seedlings were cut into explant sections: 5 mm for hypocotyl and cotyledon petiole, 2 mm^2 for cotyledon.

Bacterium and plasmid

Agrobacterium tumefaciens LB4404::pBI121/TI was used to transform seedling explants. Plasmid pBI121/TI (Fig. 1) was constructed by inserting the TI structural gene (Yeh et al. 1997) between the 35S promotor and the GUS structural gene of pBI121. The plasmid was introduced into LB4404 via microprojectile bombardment. A single colony of bacterium was transferred into liquid bacterial medium (YEP medium containing 100 mg/l kanamycin) and shaken at 240 rpm under 28°C for 2 days. The suspensions were then mixed with sterile glycerol (1:1 vol/vol) and stored at –80°C until used. Eighteen hours before inoculation, the bacterium suspension was mixed with the bacterial medium (1:25 vol/vol) and shake-incubated in the above conditions. Acetosyringone was added to the suspension after a 10-h incubation at a concentration of 50 μM. By the end of 18 h of incubation the suspension reached an OD_{600} reading of 0.8 with approximately 3×10^8 cells/ml. The bacterial medium in the suspension was then centrifuged and replaced with an equal volume of inoculation medium [liquid CI medium (see below)+10 mM D-glucose] before use.

Stage 1: developing an efficient in vitro regeneration system

Initially three explant types were tested for their regeneration capacities in basal medium with 0.2 mg/l indole-3-acetic acid (IAA) and 0.5, 1.0, 2.0, or 4.0 mg/l N^6-benzyladenine (BA). Hypocotyl explants were used in all the subsequent experiments including the testing of desirable BA concentration from a gradient ranging from 0.5 to 5.0 mg/l at 0.5 mg/l increments with a fixed amount of 0.2 mg/l IAA. The ability to pretreat explants on a callus-inducing (CI) medium [basal medium with 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 mg/l kinetin] and supplementing the shoot induction medium with silver ion (14.7, 29.4, 58.8, 86.2, and 117.6 μM as silver thiosulfate; prepared by mixing 1:3 molar concentration solutions of silver nitrate and sodium thiosulfate and added to culture medium via filter sterilization) to increase shoot-bud regeneration percentages was also tested. Basal medium, supplemented with 0.2 mg/l IAA, 1.0 or 5.0 mg/l BA and 29.4 μM silver ion, was used as the standard shoot induction (SI) medium in later experiments.

Stage 2: defining the *Agrobacterium* transformation parameters

Since neomycin phosphotransferase II (NPTII) was the selectable marker gene in our system, explants were first tested for their responses in SI medium containing various concentrations of the selective agents: kanamycin (50, 25, 12.5, and 6.25 mg/l) or G418 (30, 15, 7.5, and 3.25 mg/l). In transformation experiments, the 150 mg/l carbenicillin-containing SI medium with no selective agent was designated as SI-1 medium; supplemented with 25 mg/l kanamycin or 15 mg/l G418 it was designated SI-2 medium; with 50 mg/l kanamycin or 30 mg/l G418 as SI-3 medium.

The CI-medium-pretreated hypocotyl explants were inoculated with 10 ml bacteria-containing inoculation medium. Bacterial inoculation concentrations tested were 1, 10 and 100× dilutions. Dishes were incubated in darkness for 1 h at 10, 15, 20, 25, or 30°C. After blotting dry with sterile filter papers the explants were incubated on solid CI medium for 72 h, 25°C, dark cocultivation. The cocultivated explants were washed with 1.5% D-mannitol with 1500 mg/l car-

A Regimes flowchart

Week	a	b	c	d	e	f
1	H	L	L	N	N	N
⇓	⇓	⇓	⇓	⇓	⇓	⇓
2	H	H	L	H	L	N
⇓	⇓	⇓	⇓	⇓	⇓	⇓
3	H	H	H	H	H	H
⇓	⇓	⇓	⇓	⇓	⇓	⇓
4	H	H	H	H	H	H

B Effects of selection regimes

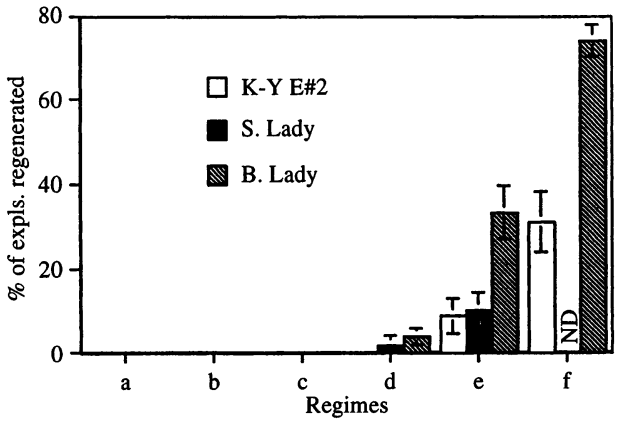

Fig. 2A, B Effects of timing and concentration of antibiotic selective regimes on resistant cauliflower plant regeneration following cocultivation with pBI121/TI vector containing *Agrobacterium*. **A** Flow chart of antibiotic selection regimes [*1–4* week after cocultivation; selective media: *N* no selection, using SI-1 medium (SI medium+150 mg/l carbenicillin), *L* low-level selection, using SI-2 medium (SI-1+25 mg/l kanamycin or 15 mg/l G418), *H* high-level selection, using SI-3 medium (SI-1+50 mg/l kanamycin or 30 mg/l G418]. **B** Percentages of explants of Known You Early no. 2, Snow Lady, and Beauty Lady producing putative transgenic plants under different G418 selection regimes (*vertical bar* standard error, *ND* not determined)

benicillin, blotted dry and placed in solid SI-3 medium to induce regeneration.

Timing for antibiotic selection was determined by transferring explants into SI-1, SI-2, and SI-3 media in various combination regimes (Fig. 2A) during the 4-week periods following cocultivation.

Stage 3: confirmation of the TI transgenic status of the resultant plants

Some of the putative transgenic plants derived from the selection regime "e" (Fig. 2A) were used for in vitro TI activity, Southern blotting and immunoblot (Western blotting) assays.

Protein extraction

Soluble proteins were extracted from 0.3 g of fresh leaf of each of ten putative transgenic and two control plants by grinding in liquid nitrogen with 3× vol of extraction buffer containing 30 mM Tris-HCl (pH 7), 1% PVPP, and 1% vitamin C. The soluble-protein-containing supernatant obtained after centrifugation at 12 000 rpm for 40 min was stored at –70°C for in vitro TI activity and immunoblot assays. The protein concentration of the supernatant was determined with Bio-Rad Protein Assay Kit II following its microassay procedure.

In vitro TI activity assay

The TI activity assay was based on the Geiger and Fritz (1984) cell-free trypsin assay method with Bz-L-Arg-4NA [N^a-benzoxyl-1-arginine-4-nitroanilide hydrochloride (Merck)] as substrate. Trypsin hydrolyzes the substrate and forms 4-nitroaniline which absorbs light at OD_{405}. TI activities were measured by the reduction in the above OD_{405} readings due to the presence of TI over the TI-free control.

Genomic DNA isolation

DNA was isolated from fresh leaf tissues as described by Dellaporta et al. (1984). Primers used in PCR were the flanking sequences of the TI cDNA with 5′CATGAAAGCCCTCACACTG3′ at the 5′ end and 5′CATTACACATCGGTAGGTTTG3′ at the 3′end. PCR products and biotinylated probe of TI cDNA [prepared according to the Feinberg and Vogelstein (1983) protocol] were used in Southern hybridization using the NEBlot Phototope Kit and its procedure from New England Biolabs (Beverly, Mass.).

Immunoblotting

Appropriate quantities of protein were fractionated by SDS-PAGE following the Laemmli (1970) procedure, and using Mini Trans-Blot Cell (Bio-Rad, Hercules, Calif.) transferred to nitrocellulose. The TI polypeptide was detected using a rabbit anti-TI serum and a goat-rabbit IgG coupled to horseradish peroxidase as secondary antibody.

In planta bioassays

Small-scale insect-feeding trials were carried out. Leaves of two in vitro cloned plants of the transgenic Snow Lady plant no. 1296 1-1 were used to feed the first-instar larvae of the common *Brassica* mandible Lepidopteran pests, *Spodoptera litura* and *Plutella xylostella*, in closed containers. The remaining confirmed transgenic plants were cloned in vitro and used in an open infestation test carried out in a greenhouse with opened windows.

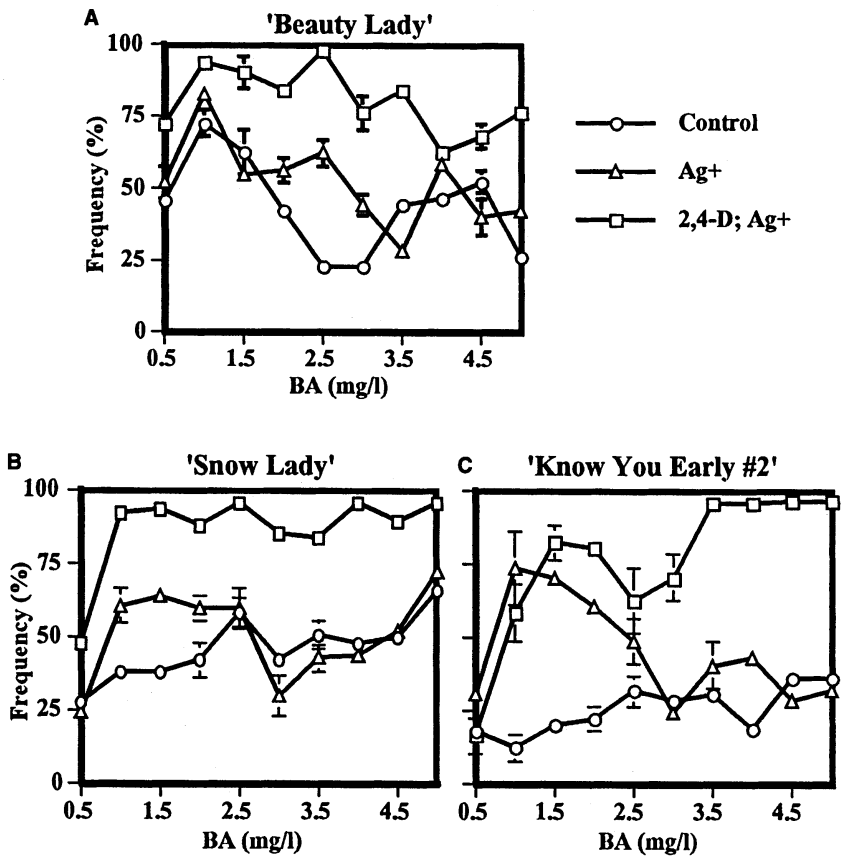

Fig. 3A–C Effects of 3 days 2,4-D preincubation and addition of ethylene inhibitor (29.4 μM silver thiosulfate) in regeneration medium on adventitious bud formation of cauliflower hypocotyl explants (*vertical bar* standard error, some of which were smaller than the symbols)

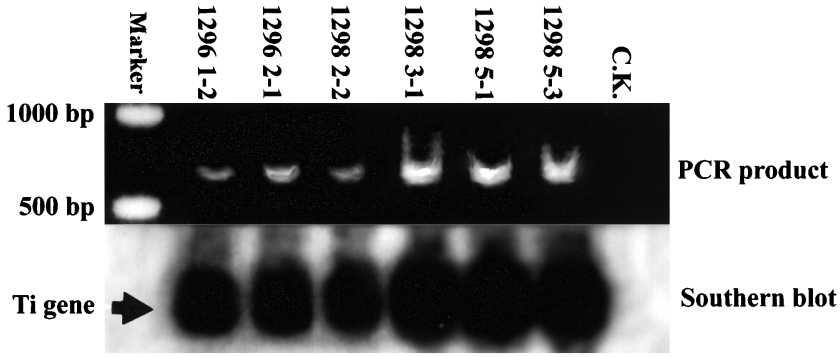

Fig. 4 PCR and Southern blot analysis of the selected G418-resistant regenerant (R_0) cauliflower plants (*1296* Snow Lady, *1298* Beauty Lady, *C. K.* control)

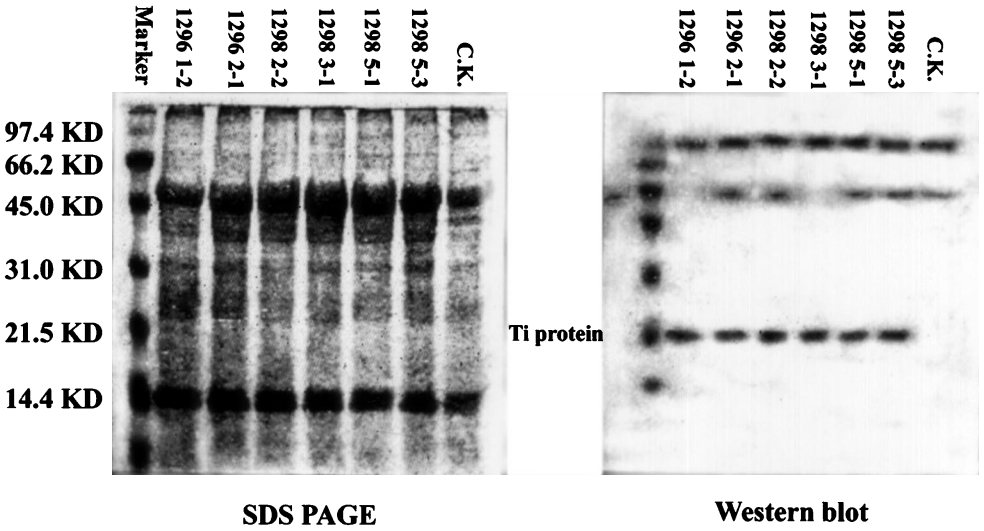

Fig. 5 SDS-PAGE and Western blot analysis of TI protein from the selected G418-resistant regenerant (R_0) cauliflower plants (*1296* Snow Lady, *1298* Beauty Lady, *C. K.* control)

Results and discussion

Stage 1: developing an efficient in vitro regeneration system

Among the three explant types tested, the hypocotyl explants of all the cultivars expressed the highest adventitious bud regeneration capacity (data not shown) after 3 weeks incubation, primarily at the cut ends adjacent to the apical meristems. The regeneration capacity was weaker on petiole explants and few, if any, buds appeared on the cotyledon explants. The optimum BA concentration for shoot bud regeneration for Known You Early no. 2 and Snow Lady was 5.0 mg/l and for Beauty Lady 1.0 mg/l. At these BA concentrations, the percentage of explants regenerating for Beauty Lady, Snow Lady and Known You Early no. 2 were 74, 68, and 37, respectively (Fig. 3, Controls).

A significant breakthrough was achieved when the explants were (a) pretreated with CI medium for 3 days, followed by (b) supplementing the regeneration medium with an ethylene inhibitor, 29.4 μM silver ion. The regeneration percentages of all three cultivars, even the weakly regenerating Known You Early no. 2, exceeded 95% (Fig. 3). All silver ion concentrations tested showed a similar degree of stimulation (data not shown). This work is the first successful case of applying an ethylene inhibitor to in vitro cauliflower regeneration. The stimulating effects of the silver ion and other ethylene inhibitors on in vitro regeneration of *Brassica* have been documented in recent years (Chi et al. 1990; Palmer 1992; Burnett et al. 1994). Added proof of ethylene regulating in vitro shoot regeneration was provided by constructing transgenic *Brassica* plants with an antisense ACC oxidase gene (Pua and Lee 1995).

Table 1 In vitro TI activity assay of G418-resistant regenerant (R_0) cauliflower plants and the non-resistant control (*CK*) plants (*Sample 1296* Snow Lady, *1298* Beauty Lady, *1-1*, *1-2*, etc. putative transgenic plants). Values are the average of three readings ±SD. The lower the OD value the stronger the suppression of trypsin by TI; thus, the lower the OD value, the higher the TI level. Values followed by different letters are significantly different at the 5% level

Sample		OD_{405}	Sample		OD_{405}
1296	1-1	$1.256^{b} \pm 0.061$	1298	2-2	$1.101^{d} \pm 0.037$
	1-2	$1.120^{c} \pm 0.022$		3-1	$1.175^{c} \pm 0.007$
	1-3	$1.265^{b} \pm 0.041$		3-2	$1.127^{b} \pm 0.051$
	2-1	$0.943^{d} \pm 0.010$		3-3	$1.184^{bc} \pm 0.009$
	CK	$1.387^{a} \pm 0.016$		5-1	$1.074^{d} \pm 0.014$
				5-3	$0.831^{e} \pm 0.031$
				CK	$1.358^{a} \pm 0.003$
	LSD = 0.0643			LSD = 0.0649	

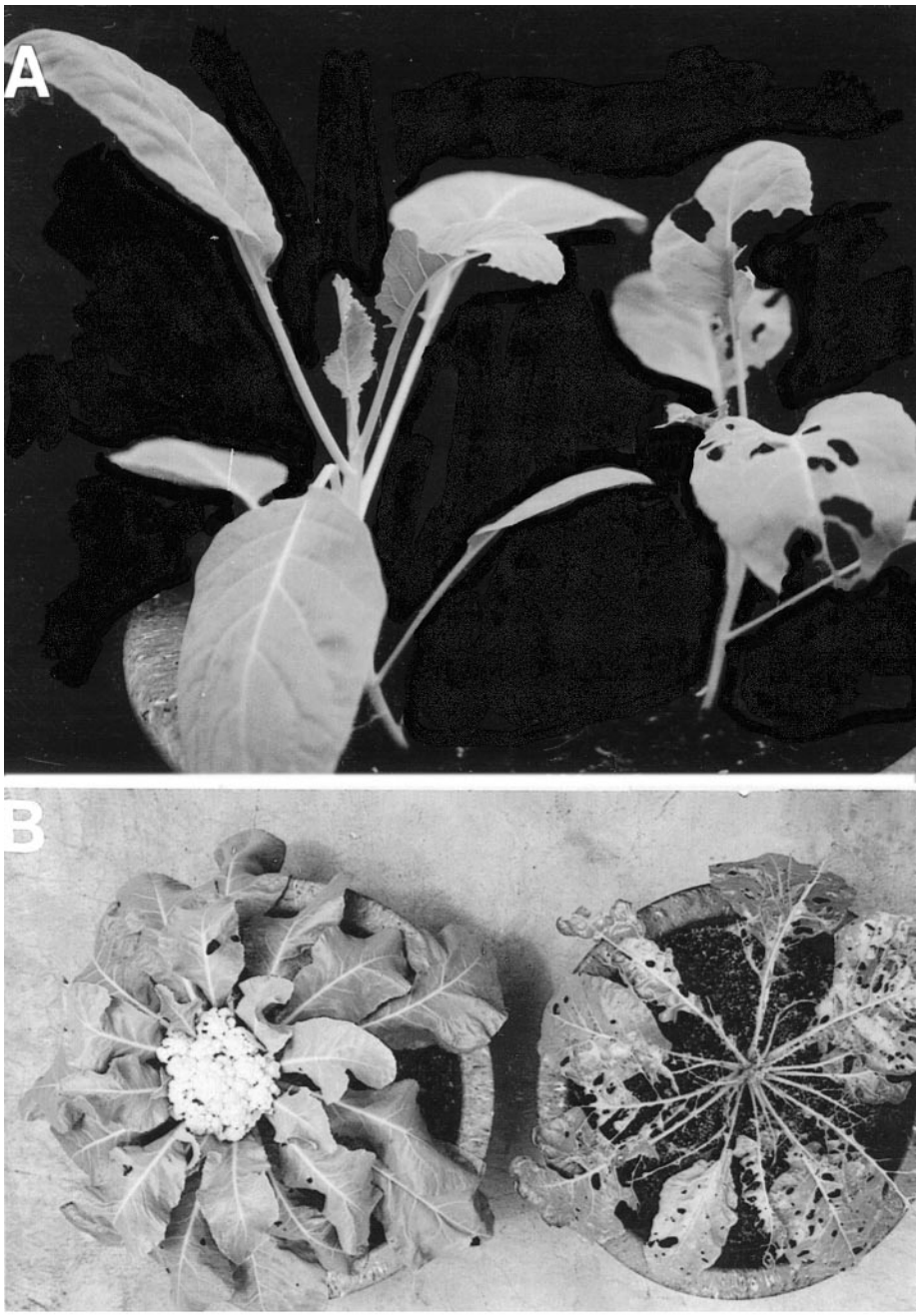

Fig. 6A, B In planta open infestation test carried out in a greenhouse with open windows (*left* TI transgenic cauliflower plants in vitro cloned from the R_0 plants, *right* control plants). The insects were identified as *Pieris conidia* (**A**) and *Plutella xylostella* (**B**)

Stage 2: defining the *Agrobacterium* transformation parameters

The optimum inoculation conditions determined (data not shown) were 100× dilution of bacterium suspension inoculated at 20–25°C. All three cauliflower cultivars had similar antibiotic sensitivities (data not shown). In media containing the higher two concentrations of either antibiotic, the regenerated buds, when produced, were chlorotic and the explants gradually turned white or brown. At least 2% of the explants in the lower two antibiotic concentrations regenerated green buds. Therefore, the higher concentrations of 25–50 mg/l kanamycin or 15–30 mg/l G418 were used to exert selective pressures in subsequent transformation experiments.

Among the various antibiotic selection regimes (Fig. 2), the regime "f" produced the highest percentage of antibiotic-resistant buds. Regime "e" had the next highest percentages. The in vitro quantitative TI protein activity analysis performed later indicated (data not shown) that the average TI content of the putative transgenic plants from regime "f" was considerably lower than that from regimes "d" and "e" which were comparable to each other. This suggested that many plantlets from regime "f" might be escapes or chimeras. Thus, this regime could not be relied on. There was no green-bud regeneration when antibiotic selective pressure

Table 2 In planta feeding bioassays using two clonal plantlets of a TI transgenic cauliflower Snow Lady plant 1296 1-1 and non-transgenic control (*CK*) plants. The experiment was carried out once without replication

Insect species	Host plant	Larvae survived (%)	Larvae reaching pupa stage (%)	Larvae reaching adult stage (%)
Spodoptera litura	1-1	56.7	22.7	13.2
	CK	86.7	63.6	49.1
Plutella xylostella	1-1	60.0	36.0	21.6
	CK	100.0	100.0	100.0

began right after cocultivation in regimes "a", "b", and "c". The above results indicated that the buds of cauliflower, even the NPTII-gene-containing ones, were more sensitive to the selective agents at the early stages of development. They became progressively more tolerant to the selective antibiotics, even without the resistant gene. Thus, the lack of selective agent during the first week after cocultivation, followed by progressively increasing the selective pressure, i.e. regime "e", provided the optimum conditions for selecting NPTII-containing transgenic buds.

Data from the same experiment also indicated that G418 selection resulted in a higher number of regenerated buds than that of kanamycin. In regime "e", for example, the percentages of explants regenerating buds from Known You Early no. 2, Snow Lady, and Beauty Lady were 8.7, 10.3, and 33.3, respectively, from G418 selection (see Fig. 2B), while from kanamycin selection the percentages were 0, 2.0, and 0, respectively (data not shown). Thus, G418 selection provided a higher probability of obtaining putative transgenic plants.

Stage 3: confirmation of the TI transgenic status of the resultant plants

Over 100 G418/kanamycin-resistant plantlets were obtained from stage 2 experiments. The functional integrity of the TI gene products in these plants was demonstrated by an in vitro TI activity assay (Table 1). All the tested putative transgenic plants showed significantly higher TI protein content than control plants.

The genomic DNAs from all tested plants produced 0.66-kb DNA segments following PCR, while that of the control plant did not (Fig. 4). Southern hybridization (Fig. 4) with TI cDNA probe confirmed that these 0.66-kb DNA segments were TI gene sequences. We recognize that the Southern procedure we performed could not preclude the possibility of DNA contamination from *Agrobacterium* which might still be present on the tested regenerant (R_0) plants. Nevertheless, the combination of results from the in vitro TI activity assay, immunoblotting, and in planta bioassay indicated that functional TI protein indeed had been translated in the tissues of these R_0 plants. A progeny test is currently underway which will verify inheritance of the TI transgene.

The TI polypeptide was detected as a 24-kD band (Fig. 5) which was present in all six transformant plants tested, while the control plant did not give such a band. The expression of the TI genes at both transcriptional and translational levels was, thus, verified in the transgenic plants. The in vitro TI activity assays performed above (Table 1) indicated that those translational products were functionally active in vitro as well.

A high degree of insect protection was evident in our bioassays (Table 2, Fig. 6). These results demonstrated that the TI proteins produced in the transgenic cauliflower plants were functionally active in planta. High expression of protease inhibitor genes in transgenic plants have previously been reported to have enhanced insect resistance potentials (Hilder et al. 1990; Thomas et al. 1995; Duan et al. 1996; Xu et al. 1996). Our insect bioassays, although at small scales initially, also provided evidence that the introduction of a protease inhibitor gene into transgenic crop plants can be efficacious.

Our protocol developed for the effective genetic engineering of Taiwan cauliflower can be used as a practical starting point for the transformation of other cauliflower cultivars. Analysis of the resultant R_1 and R_2 generation plants as well as a larger scale field test of the clonal R_0 plants are currently in progress.

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