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ORIGINAL ARTICLE

Vacuum infiltration transformation of non-heading Chinese cabbage (*Brassica rapa* L. ssp. *chinensis*) with the *pinII* gene and bioassay for diamondback moth resistance

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Abstract Non-heading Chinese cabbage (*Brassica rapa* L. ssp. *chinensis*) is a popular vegetable in Asian countries. The diamondback moth (DBM), *Plutella xylostella* (L.), an insect with worldwide distribution, is a main pest of Brassicaceae crops and causes enormous crop losses. Transfer of the anti-insect gene into the plant genome by transgenic technology and subsequent breeding of insect-resistant varieties will be an effective approach to reducing the damage caused by this pest. We have produced transgenic non-heading Chinese cabbage plants expressing the potato proteinase inhibitor II gene (*pinII*) and tested the pest resistance of these transgenic plants. Non-heading Chinese cabbages grown for 45 days on which buds had formed were used as experimental materials for *Agrobacterium*-mediated vacuum infiltration transformation. Forty-one resistant plants were selected from 1166 g of seed harvested from the infiltrated plants based on the resistance of the young seedlings to the herbicide Basta. The transgenic traits were further confirmed by the Chlorophenol red test, PCR, and genomic Southern blotting. The results showed that the *bar* and *pinII* genes were co-integrated into the resistant plant genome. A bioassay of insect resistance in the second generation of individual lines of the transgenic plants showed that DBM larvae fed on transgenic leaves were severely stunted and had a higher mortality than those fed on the wild-type leaves.

Keywords Non-heading Chinese cabbage · Vacuum infiltration transformation · *pinII* gene · Insect resistance

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Introduction

Non-heading Chinese cabbage (*Brassica rapa* L. ssp. *chinensis*), a leafy vegetable, is popular in Asian countries. The diamondback moth (DBM), *Plutella xylostella* (L.), is a major pest of Brassicaceae crops and causes crop damage worldwide amounting to as much as one billion dollars annually (Talekar and Shelton 1993). The synthetic insecticides currently used to control the pest have raised public concerns about food safety and environmental pollution (Jiang et al. 2008). Moreover, insects have become resistant to these synthetic chemicals (Shelton et al. 1993). Conventional breeding methods for improving Chinese cabbage with high levels of durable resistance to this pest are confronted with the problems of a shortage of germplasm and inefficiency. The most recent techniques in plant genetic engineering have opened a new avenue for crop improvement. The introduction of an anti-insect gene into the non-heading Chinese cabbage genome and the

subsequent breeding of insect-resistant varieties by transgenic technology represents an effective approach to solving these serious problems (Yang et al. 2002).

The *Agrobacterium*-mediated transformation of *Brassica* vegetables has been reported to be successful by the leaf-disc culture method (Bhattacharya et al. 2002; Park et al. 2005; Zhang et al. 2006). However, the transformation frequency in these studies was low, especially in Chinese cabbage, which is regarded as recalcitrant plant due to its low frequency of regeneration and transformation and high genotype dependency. Extensive efforts have been made to establish tissue culture-independent transgenic approaches. The first non-tissue culture-based in planta transformation procedure was described in *Arabidopsis thaliana* and involves the use of germinating seeds (Feldmann and Marks 1987). This method is simple and convenient, and it can overcome the limitation of genotypes as well as avoid possible somaclonal variations associated with tissue culture-based transformation and regeneration processes. A vacuum infiltration transformation technique was subsequently developed, also in *A. thaliana* (Bechtold et al. 1993). It has been first successfully adapted to non-heading cabbage (Cao et al. 2000; Liu et al. 1998), thereby providing an alternative way for cabbage genetic transformation.

Proteinase inhibitor II (PIN II) is a serine peptidase inhibitor that usually possesses reactive sites towards both trypsin and chymotrypsin (Sin and Chye 2004). It may have great potential in crop protection because the major digestive endoproteases in the insect gut are the serine proteases trypsin and chymotrypsin (Lawrence and Koundal 2002; Mosolov and Valueva 2008). The anti-insect spectrum of Pin II is broad, and this inhibitor can kill or stunt the growth of both lepidopteran and coleopteran insects (Fan and Wu 2005; Lawrence and Koundal 2002). Some transgenic plants producing protease inhibitors showed significant insect resistance in the form of increased mortality and developmental inhibition on larvae fed on transgenic leaves (Abdeen et al. 2005; Duan et al. 1996; Maheswaran et al. 2007).

Here we report our work on the effective vacuum infiltration transformation of non-heading Chinese cabbage expressing the proteinase inhibitor II gene from potato (*pinII*) and the bioassay of insect resistance of the transgenic plants.

Materials and methods

Plant material

The plant material used in this experiment was non-heading Chinese cabbage '49 Caixin', a very early-maturing cultivar originating from southern China. The seeds, provided by the Beijing Vegetable Research Center, were periodically sown in pots in the greenhouse. After about 45 days, the flowering plants were 50–60 cm high and could be placed easily in a vacuum infiltration chamber. Plants for transformation were divided into two types: plants with blooms (over 10% of flower buds were opened) and plants without blooms (plant with inflorescences but no open flower buds).

Bacterial strain and plasmid

Agrobacterium tumefaciens C58 carrying the plasmid pBBBasta-*pinII* (Fig. 1) constructed in our laboratory was used in the experiment. The pBBBasta vector was provided by Dr. Robaglia, CEA-Cadarache, France. The vector contained the *bar* gene as a selectable marker, which encodes the phosphinothricin acetyltransferase protein that detoxifies the PPT herbicide (phosphinothricin, the active ingredient of herbicides such as Basta). The *pinII* gene with potato wound-inducible promoter and terminator was provided by professor Ray Wu of Cornell University (New York, USA). The plasmid pBBBasta-*pinII* was introduced into *Agrobacterium* strain C58 by the freeze–thaw method, as described by Chen et al. (1994).

Plant transformation and selection

Preparation of Agrobacterium

Agrobacterium C58/pBBBasta-*pinII* stock suspension was inoculated into 50 mL liquid Luria broth (LB) medium containing 100 mg/L kanamycin and cultured at 28°C in an incubator shaker at 185 rpm overnight. The suspension was then diluted 50-fold into LB medium containing 75 mg/L kanamycin for large-scale culture until the OD_{600} was >1.0. The bacterial cells were then harvested by centrifugation at 5,000 rpm for 15 min and resuspended in an equal volume of the infiltration medium [MS basal medium salts (Murashige and Skoog 1962), 0.18 pmol/L 6-benzyladenine (6-BA), 5% sucrose, 0.02% Silwet L-77 (Osi Specialties, A Witco Co, Endicott, NY), pH 5.7] for plant transformation.

Vacuum infiltration transformation and cultivation management

Vacuum infiltration transformation and cultivation management were carried out according to a protocol modified from Cao et al. (2000). Infiltration was performed using a 20-L cubage desiccator containing the *Agrobacterium* suspension. Plants of non-heading Chinese cabbage were uprooted, washed clean, and then placed in the desiccator

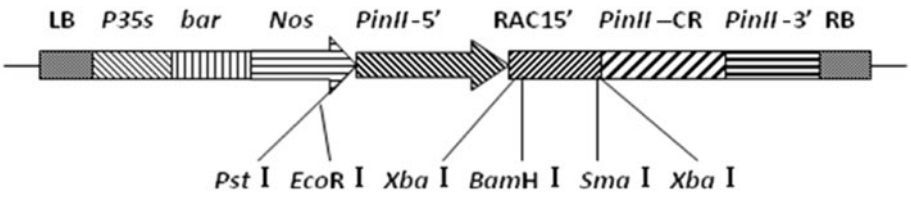

Fig. 1 The map of plasmid pBBBasta-*pinII*. *pinII*-CR *pinII* (proteinase inhibitor II) gene, *pinII* 5′, *pinII* 3′ promoter and terminator, respectively, *RAC15′* intron of rice *act1* gene, *LB*, *RB* left and right border, respectively, *bar* selectable marker encoding phosphinothricin acetyltransferase (PPT) protein, *nos* nopaline synthase terminator, *p35S* cauliflower mosaic virus promoter. Restriction enzymes are shown *under the map*

and immersed in the *Agrobacterium* suspension. A vacuum pump linked to the desiccator provided the vacuum treatment (about 10^4 Pa) for 5 min. The treated plants were then removed from the desiccator and transplanted into the greenhouse. They were covered with plastic film for 7 days to recover and then uncovered. Flowering plants were hand pollinated. Seeds were separately harvested according to the silique-setting position (upper siliques and lower siliques), and the quantity was weighed. Upper siliques were considered to be the uppermost six or seven siliques on silique-setting branches of each treated plant; lower siliques were the lowermost six or seven siliques on silique-setting branches of each treated plant.

Seedling screening for PPT resistance

Seeds were sown according to batch in a tray containing nutrient soil. Seedlings at the cotyledon stage were sprayed with 750 mg/L Basta solution (Hoechst AG, Frankfurt, Germany); this was followed by a second spraying of Basta solution (approx. 300 mg/L PPT) at the two to three true leaf stage. The number of surviving PPT-resistant plants was recorded, and resistant plants were transplanted to the soil in pots for further growth and seed setting.

Chlorophenol red assay

The Chlorophenol red (CR) assay was carried out as described by Kramer et al. (1993) on the PPT-resistant plants. The assay solution contained 1/10× Gamborg's salt mixture (Sigma Chemical, St. Louis, MO), 50 mg/L CR, 15 μmol/L BA, 0.5 μmol/L naphthaleneacetic acid (NAA), and 5 mg/L PPT. The pH of the solution was adjusted to 6.0 at which pH the assay solution was red. Leaf pieces removed from resistant and non-transformed plants (control), respectively, were incubated in plates with assay solution in a growth chamber at 21°C under a 16/8-h (day/night) photoperiod. The color of the assay solution was recorded after 1–2 days of incubation. The color change of the CR assay solution is pH-dependent, being red colored at pH ≥6.0 and orange/yellow at pH <6.0. Those leaf tissues resistant to PPT cause acidification of the medium, leading to the yellowing of the assay solution, while those sensitive to PPT cause medium alkalescence, leading to the red/purple coloration of the solution.

Molecular analysis of the resistant plants

Total genomic DNA was isolated from young leaves of each PPT resistant plant using the cetyltrimethylammonium bromide (CTAB) method (Fulton et al. 1995). PCR analyses were carried out by amplifying the *bar* gene and *pinII* gene using the following sets of oligonucleotide primers: *bar* 5′-AAC TTC CGT ACC GAG CCG CA-3′ (forward) and 5′-ATG CCA GTT CCC GTG CTT GA-3′ (reverse); *pin II* 5′-GGA TGT TCT ACA AGG AAG T T-3′ (forward) and 5′-GAT GGA CAA GTC TAG GGT C-3′ (reverse). The non-transformed plant was the negative control, and the pBBBasta-*pinII* plasmid DNA was the positive control. The cycling schedule for the PCR amplifications was 94°C for 5 min; 34 cycles of 94°C for 40 s, 56°C for 30 s, and 72°C for 1 min 30 s, with a final extension at 72°C for 7 min.

For the Southern blot analysis, plant genomic DNA of resistant and control plants was digested with *Eco*RI, which cut the T-DNA at a unique restriction site. Digested genomic DNA was fractionated on a 0.8% agarose gel and transferred to Hybond N^+ nylon membranes (Amersham Pharmacia, Amersham, UK). The *bar* and *pin II* genes were used as the probes and labeled with digoxigenin (DIG). Probe labeling, hybridization, membrane washing, and signal detection were performed according to the manufacturer's instructions for the DIG-labeling and detection kit (DIG High Prime DNA Labeling and Detection Starter kit I; Roche Diagnostics, Indianapolis, IN).

For the semi-quantitative reverse transcription (RT)-PCR analysis of *pinII* expression, the total RNA was extracted from the leaves of transgenic lines and the non-transformed plant using TRIzol reagent (Invitrogen, Beijing, China) and treated with RNase-free DNaseI (Invitrogen, Carlsbad, CA) to eliminate contaminating DNA. The first-strand cDNA was synthesized from 2 μg of total RNA in a total volume of 20 μL using the M-MLV reverse transcriptase (Invitrogen). A 600-bp *pinII* cDNA

fragment was amplified using the following *pinII* primers: TCACCCCAAAATTAAAAGAA (forward) and reverse: GTCTAGGGTCACATTGCAGGG (reverse). The cycling schedule for PCR amplifications was 94°C for 5 min; 25 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 7 min. The elongation factor 1 α (*EF1α*) gene was used as an internal control.

Insect feeding bioassay

The DBM (*Plutella xylostella* L.) was provided by the College of Plant Protection, China Agricultural University. Leaves of comparable physiological ages from the transgenic and control plants were used for the insect feeding assay. The leaves were dipped in 0.1% NaClO for 5 min, rinsed well with sterilized water, and then blotted dry using tissue paper. These leaves were placed on moistened sterile filter paper in 9-cm-diameter petri dishes. Freshly hatched larvae were used for a feeding assay. Ten larvae of DBM per dish, with five replications, were inoculated on the leaves. The leaves were replaced with fresh ones every 2 days. Mortality and developmental status of larvae were observed and recorded. Pupae were weighed and maintained until the emergence of adults.

Data are presented as the mean and their standard deviation (SD). To evaluate the differences among the transgenic plants studied, we used one-way analysis of variance (ANOVA) to analyze all data. Probability values of $P < 0.05$ were considered to be statistically significant.

Results

The screening of Basta-resistant plants

Four batches of about 190 plants in total were treated by the vacuum infiltration transformation process. Forty-one PPT-resistant plants were obtained from the seeds harvested from the first three batches (142 plants). No resistant plant was found from the fourth batch of treated plants because most of these plants died in the greenhouse in the late growth phase due to high temperature and attacks by pests. As can be seen in Table 1, the transformation frequency of treated plants (TFP) and the transformation frequency of harvested seeds (TFS) of the plants without blooms were 28.9 and 0.088‰, respectively; in comparison, the TFP and TFS of the plants with blooms were 29.5 and 0.088‰, respectively. Therefore, there were no significant difference in the transformation frequency between plants with or without blooms. The average TFS was approximately 0.1‰, which is consistent with the TFS reported by Cao et al. (2000). A subsequent analysis showed that the silique-setting position had a significant effect on the TFS, with the TFS from lower siliques (0.160‰) being threefold higher than that from upper siliques (0.055‰). This result indicates the importance of obtaining sufficient seed setting in the early period after plant infiltration transformation.

Chlorophenol red assay

A total of four surviving Basta-resistant independent plants were assayed. The color changes of the CR assay solution were in accordance to the principle of leaf tissues resistant to PPT causing acidification of the medium (yellow assay solution) and those sensitive to PPT causing medium alkalescence (red/purple coloration of the solution), thereby verifying the authenticity of the herbicide Basta resistance of these plants (Fig. 2).

Molecular analysis of transgene integration

The results of the PCR analyses indicated that both the 0.4-kb *bar* gene fragment (Fig. 3a) and the 1.5-kb *pinII* gene fragment (Fig. 3b) were present in the DNA of positive control and PPT-resistant plants but were absent in the

Table 1 Transformation frequency according to different developmental stages and different positions of siliques

Items	Number of treated T_0 plants	Number of seeds harvested	Number of resistant T_1 plants	TFP[a] (%)	TFS[b] (‰)
Growth stage					
Plants with blooms	85	28,4017	25	29.5	0.088
Plants without blooms	57	18,2499	16	28.1	0.088
Silique position					
Upper siliques		32,5970	18		0.055
Lower siliques		14,0546	23		0.160

TFP transformation frequency of treated plants, *TFS* transformation frequency of harvested seed, *PPT* phosphinothricin

[a] TFP = number of PPT-resistant T_1 plants/number of treated T_0 plants

[b] TFS = number of PPT-resistant T_1 plants/number of harvested seeds

DNA of negative control plants. This finding confirmed the integration of *pinII* and *bar* genes into the plant genome.

DNA samples from non-transformed (control) and transgenic plants were digested with *Eco*RI and subjected to Southern blot hybridization. The DIG-labeled 1.5-kb *pinII* gene fragment and the 0.4-kb *bar* gene fragment were used as probes, respectively. No hybridization was detected in the negative control DNA (Fig. 3c, d, lane N). Resistant plants 2-3, 3-2, and 3-18 produced multiple *bar* gene hybridization fragments, and resistant plants 2-24 and 3-16 produced two *bar* gene hybridization fragments. Resistant plants 2-11, 2-13, 3-4, and 3-11 showed a single *bar* gene hybridization fragment, indicating the insertion of a single copy (Fig. 3c) in the plant genome. The hybridization results of *pinII* gene probe showed that all DNA samples from the resistant plants had hybridization signals (Fig. 3d, lanes 1–4), which proved the existence of the foreign *pinII* gene in the genomes of Basta-resistant plants. Plant 2-13 had one hybridization band of the *bar* gene, which is consistent with the result in the *pinII* gene hybridization experiment indicating the insertion of only one copy of the *bar–pinII* gene (Fig. 3c, d, lane 3).

Fig. 2 Chlorophenol red assay on a number of T_2 progeny of the transgenic lines. Wells containing a darker assay solution (*top row*; bright red-coloration) indicate PPT sensitivity, while those containing a lighter colored assay solution (*bottom two rows*; yellow/orange-coloration) indicate resistance. *First row* Wild-type leaves (control), *second* and *third rows* leaves from plants 2-3, 2-11, 2-13, and 2-24

The results of semi-quantitative RT-PCR showed that the *EF1α* gene expression level was similar between transgenic and non-transformed plants (Fig. 4a). However, transgenic plants showed different *pinII* gene expression levels, although there was no detectable DNA band in the PCR product of non-transformed plants (Fig. 4b). Since there are two introns in the *pinII* gene, the PCR product is 1200 bp with the genomic DNA as the template. In conclusion, the *pinII* gene was correctly transcribed in the five transgenic lines.

Insect feeding tests

The insect resistance assay was conducted on four independent transgenic lines. The mortality of DBM larvae fed on transgenic leaves was higher in each instar stage and in the pupal period than that of larvae fed on control leaves, with the difference being significant ($P < 0.05$) in terms of total mortality (Table 2). Larvae fed on transgenic leaves did not develop synchronously (mostly slower) with those fed on the control leaves (Fig. 5). The pupation rates, eclosion rates, and pupa weight of larvae fed on leaves from the four transgenic lines were all lower than those of larvae fed on wild-type leaves. The leaf damage level of transgenic line 2-24 was significantly less than that of the wild-type, indicating that transgenic line 2-24 had better insect resistance (Fig. 5b). These experimental results suggest that the larvae fed on the *pinII* transgenic plants suffered an obvious growth inhibition and in some cases mortality.

Discussion

In planta transformation was first described in *A. thaliana* using germinating seeds (Feldmann and Marks 1987).Vacuum infiltration, also an in planta transformation method,

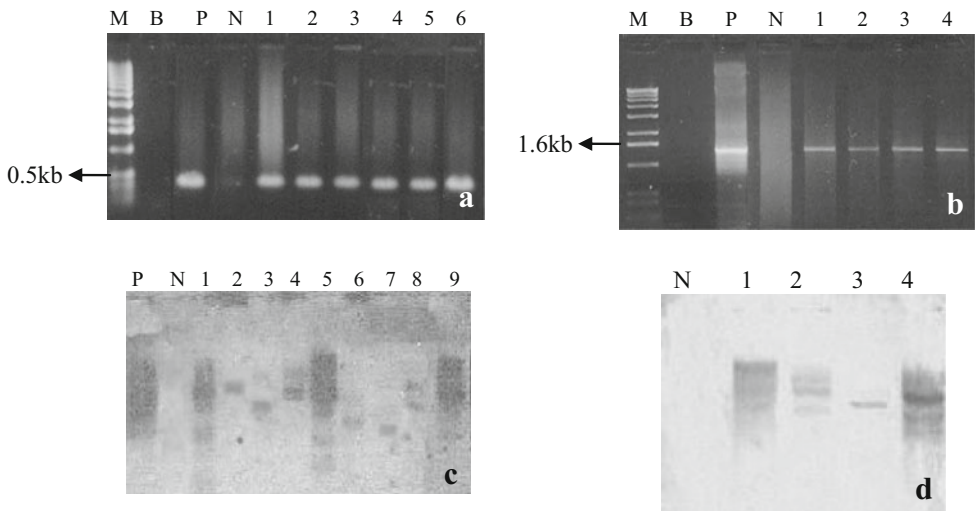

Fig. 3 PCR detection of the target gene and genomic DNA Southern blotting of the *bar* and *pinII* genes. **a** PCR of *bar* gene in plant genome, **b** PCR of *pinII* gene in plant genome, **c** genomic DNA Southern blotting using Dig-labeled *bar* as the probe; **d** genomic DNA Southern blotting using digoxigenin (DIG)-labeled *pinII* as the probe. *M* DNA ladder, *B* blank with water, *P* plasmid as positive control, *N* non-transformed plant, *lanes 1–9* transgenic lines 2-3, 2-11, 2-13, 2-24, 3-2, 3-4, 3-11, 3-16, 3-18, respectively

was subsequently developed, also in *A. thaliana* (Bechtold et al. 1993). Non-heading Chinese cabbage was the first plant of economic value to be successfully transformed by vacuum infiltration (Cao et al. 2000). Cao et al. (2003) reported that the TFS was 0.1‰, and a similar result was obtained in our experiment, which confirmed the reproducibility of this technique. We also evaluated the TFP, which was about 30% in our experiments, although it has been reported to vary considerable in different experiments (3.5–80.0%); however, it was positively related with the number of seeds harvested although the TFS itself is variable (Cao et al. 2003). These results indicate that both good recovery and good seed setting of the infiltrated plants provide a significant advantage for transgenic seed production. The TFS was higher in the lower siliques, suggesting that the transformation events happened more frequently during the short period directly after infiltration than during the longer period of plant growth after infiltration. This may be correlated with the vigor and number of the *Agrobacterium* cells infiltrated into the plant (Xu et al. 2005).

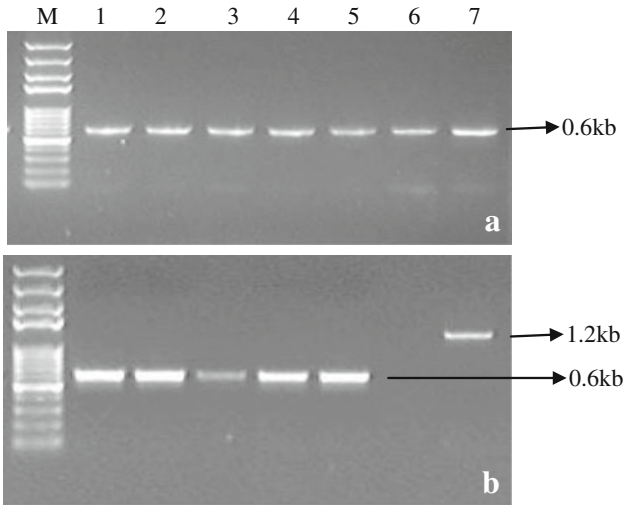

Fig. 4 Semi-quantitative reverse transcription (RT)-PCR analysis of *pinII* gene expression in transgenic plants. **a** RT-PCR analysis of the internal reference *EF1α* gene, **b** RT-PCR analysis of the *pinII* gene. *M* DNA ladder, *lanes 1–5* transgenic lines cDNA of 2-3, 2-11, 2-13, 2-24, 3-2 and 3-4, respectively; *lane 6* non-transformed plant cDNA, *lane 7* transgenic line 2-24 genomic DNA

The insect resistance characteristics of some transgenic non-heading Chinese cabbage were tested in the insect feeding experiment. The mortality rate of *Plutella xylostella* L. larvae fed on transgenic leaves was significantly higher than that of larvae fed on control leaves, and the larval development of the former were more asynchronous. In most of the dishes, the consumed portion of the transgenic leaves was smaller than that of control leaves (we did not measure the exact areas), possible due to the higher mortality and the stunted development of the feeding

Table 2 Influence of the proteinase inhibitor II gene (*pinII*) in transgenic Chinese non-heading cabbage on the growth and development of diamondback moth

Transgenic lines[a]	Mortality (%)					Total mortality (%)	Pupation ratio (%)	Eclosion ratio (%)	Pupa weight (mg)
	Instar1	Instar2	Instar3	Instar4	Pupal period				
2-3	9.80	2.17	11.11	12.5	28.57	49.02 ± 2.48 c	87.50 ± 2.60 a	71.43 ± 3.97 a	4.85 ± 0.30 a,b
2-11	13.73	22.73	2.33	11.9	8.11	33.33 ± 2.88 b	88.10 ± 3.01 a,b	91.89 ± 4.56 b	4.82 ± 0.28 a,b
2-13	7.50	24.32	10.71	16.00	33.33	52.00 ± 2.82 c	84.00 ± 4.20 a	66.67 ± 2.27 a	4.34 ± 0.25 a
2-24	12.00	20.45	14.29	10.00	33.33	64.00 ± 3.35 d	90.00 ± 4.17 a,b	66.67 ± 4.04 a	4.93 ± 0.99 b
Control	4.00	2.08	2.13	4.35	6.82	18.23 ± 1.68 a	95.65 ± 4.61 b	93.18 ± 2.57 b	5.15 ± 0.28 b

The data are presented as the mean or mean ± standard deviation. In the same column, means followed by the same letters are not significantly different, while those followed by different letters are statistically significant different at $P < 0.05$

[a] 2-3, 2-11, 2-13, and 2-24 are independent transgenic lines

Fig. 5 Survey of resistance for *Plutella xylostella* L. **a** Wild-type plant, **b** transformed plant (transgenic line 2-24)

larvae. However, we were unable to obtain any impression of complementary consumption of the feeding larvae in this case. This was partially due to the sensitivity of the *Plutella xylostella* L. larvae to the PINII protein, as in the case of a mustard trypsin inhibitor (De Leo et al. 2001). There were certain differences in mortality rates among the different transgenic lines. One possible explanation is that the insert locus and copy number of *pinII* gene were different in different transgenic lines (Fig. 3d), resulting in the different *pinII* gene expression levels in the transgenic lines (Fig. 4b) and leading to different levels of insect resistance, as is the case in Bt transgenic Chinese cabbage (Xiang et al. 2000). There has also been a strong correlation between pupal weight and reproductive fitness reported for *Epiphyas postvittana*, with lighter pupae resulting in smaller adults with reduced reproductive fitness (Danthanarayana 1975). The light pupae that developed from the larvae fed with the *pinII* transgenic Chinese cabbage are likely to show reduced reproductive fitness, which will impact the next generation by reducing the population size.

Since proteinase inhibitors are common components of plant protein in many kinds of plants, the introduction of the proteinase inhibitor gene (*pinII*) into new crops can be regarded as a relatively safe strategy for insect control from the environment safety standpoint. The pH value was about 8.5 in the gut of *Plutella xylostella* larvae, and the activity peak of mustard trypsin inhibitor MTI-2 was at pH 9.0 (De Leo et al. 2001). The human stomach has a pH value of <4, and the optimum environment for pepsin activity is about pH 2.0. We observed this big difference between the insect and human digestive system, but cannot yet report the potential effect of PINII protein on human body. Consequently, further research on transgenic Chinese cabbage is required before its adoption for commercial crop improvement.

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