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Full Length Research Paper

Several methods to detect the inheritance and resistance to the Diamondback Moth in transgenic Chinese cabbage

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A positive selection system was developed for Agrobacterium-mediated trasnsformation of using hypocotyl segments of aseptic seedlings of chinese cabbage as explants, regenerated plants with kanamycin resistance were obtained. The transformed plants with *CryIA*(*c*) (Bt) gene were confirmed by Southern blotting analysis, indicating the integration of the transgene into the cabbage genome. Majority of the transgenic plants had only a single copy of the inserted *CryIA*(*c*) gene. Leaf section bioassays showed that resistance against larvae of diamondback moth in *CryIA*(*c*) transgenic cabbage was significantly enhanced. The inheritance patterns of the transgene in T_1 offspring of transgenic cabbage were investigated using PCR analysis and kanamycin resistance test on young seedling leaves.

Key words: Cabbage, Bt transgenic, insect resistance Agrobacterium-mediated trasnsformation.

INTRODUCTION

Recent advances in Cabbage(Brassica olerecea var. capitata L.) transformation have predominantly utilized antibiotic or herbicide selection systems (Aldemita and Hodges, 1996; Burkhardt et al., 1997). However, the potential consequences of antibiotic- or herbicide-resistant genes on the environment, especially in vegetable and corps, which were suffering from huge yield and quality loss caused by insect pest. Chemical applications have imposed serious pollutions and considerable costs. Traditional breeding strategy could not get much progress as it was time-consuming and labor-intensive (Xie, 1999; Khus and Bar, 1991). Rapidly developed genetic engineering has paved the way for efficient crop improvement. Previous study has proven that the Bt gene was toxic to lepidopteran insects including diamondback moth and *P. rapae* (Mao et al., 1996; Cao et al., 2001). The Bt gene has already been applied to confer significant insect resistance in transgenic cotton and tobacco.

Brassica vegetables are known as recalcitrant plants for genetic transformation, though certain progress has been made in obtaining transgenic plants via *Agrobacterium tumefaciecin* transformation method (Metz et al., 1995; Mao et al., 1996; Sparrow et al., 2004). The present study aims at establishing regeneration and transformation systems for cabbage and transforming Bt gene into cabbage to generate a transgenic plant with improved insect resistance, thus providing elite breeding germplasm.

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MATERIALS AND METHODS

Plant material

The cabbage varieties used in this study were purchased from a local market in Wuhan, China; the varieties are Zhonggan No.8 (ZG), Niuxin (NX) and Zaoqiu (ZQ).

Plasmid construction and bacteria strain

The plasmid was constructed by our laboratory and the essential structure is shown in Figure 1. The *A. tumefaciecin* LBA4404 harbouring plasmid pBI-Bt was stored in our laboratory.

Culture media

The LB (10 g/l bacto-tryptone, 5 g/l bacto-yeast extract and 10 g/l NaCl) medium was used for *Agrobacterium* culture. The tissue culture media for cabbage transformation are presented : SM (MS

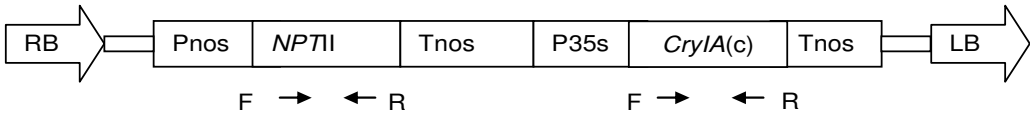

Figure 1. The T-DNA region of pBI-Bt plasmid. RB, right border; LB, left border; Pnos, Nopaline synthase gene promoter; *NPT*II, neomycin phosphotransferase gene; Tnos, Nopaline synthase terminator; P35s, cauliflower mosaic virus 35S promoter; *CryIA*(*c*), insecticidal *Bacillus thuringiensis CryIA*(*c*) gene; F, forward primer; R, reverse primer.

Table 1. Compositions of plant tissue culture media for cabbage genetic transformation.

Media	Compositions
SM	MS + 3.0 mg/l BA + 0.05 mg/l NAA + 7.4 g/l agar, pH 5.8
SMS	SM + 10 mg/l Km + 200 mg/l Cef + 7.4 g/l agar, pH5.8
RM	1/2 MS +0.1 mg/l IAA + 3% Sucrose + 7.4 g/l agar, pH 5.8
MS_0	Liquid MS, pH 5.8

MS, Murashige and Skoog medium; BA, 6-Benzyl-aminopurine; NAA, Alpha-naphthaleneacetic acid; Km, kanamycin; Cef, Cefotaxime; IAA, Indole-3-acetic acid.

+ 3.0 mg/l BA + 0.05 mg/l NAA + 7.4 g/l agar, pH 5.8); SMS(SM + 10 mg/l Km + 200 mg/l Cef + 7.4 g/l agar, pH5.8); RM (1/2 MS +0.1 mg/l IAA + 3% Sucrose + 7.4 g/l agar, pH 5.8); MS0 (Liquid MS, pH 5.8)

Cabbage transformation procedure

Agrobacterium-mediated cabbage transformation

Sterile hypocotyls from 6 - 7 day old cabbage seedlings were cut into segments and inoculated onto an SM medium. 24 segments were placed on each Petri dish and incubated at 25 ± 2℃ for 2 days with a photoperiod of 16 h.

The hypocotyl segments subjected to a pre-culture were then transferred onto an MS_0 in a new Petri dish, followed by incubation with *Agrobacterium* (OD_{600} = 0.5) with occasional gentle shaking for 5 min. The hypocotyls were then blotted dry on a sterile filter paper and returned to preculture media for co-cultivation. After co-cultivation for 2 days on SM, the hypocotyl segments were transferred to the selective culture medium (SMS) for shoot regeneration. After a biweekly subculture on the selective culture for 1 month, kanamycin resistance shoots regenerated from hypocotyls and shoots, 2 cm in length, were cut off and transferred to a rooting media (RM). When the roots grew by 2 - 3 cm in length, the lid was slightly taken off and water added to prevent bacteria development. The plantlets were acclimated gently for 2 - 3 days, followed by transplantation to a greenhouse.

Molecular analysis

Genomic DNA extraction was carried out as reported previously by Fulton et al. (1995). PCR analysis was performed with the Bt and *NPT*II primers respectively: Bt-FW: 5′- CCGACCATGGACAACA ACC-3′, Bt-RV: 5′-GCTGATGAACGATGCTGGAC-3′, NPTII-FW: 5′-AGACAATCGGCTGCTCTGAT-3′ and NPTII-RV: 5′- TCATTTCGA ACCCCAGAGTC-3′. Primers were synthesized by Shanghai Shenggong Biotechnology Company (Shanghai, China).

Southern hybridization was carried out as described by Sambrook et al. (1989). Genomic DNA (15 µg) was incubated with *Hin*dIII at 37℃ overnight, and transferred on to a nylon membrane and hybridized with Bt gene labeled with ^{32}P-dCTP.

Genetic analysis

T_0 transgenic plants were self-pollinated to harvest seeds. Kanamycin screening and PCR detection were employed to characterize the transgenic progeny. *NPT*II gene specific primers were used for PCR analysis. Leaves color was investigated 5 days after kanamycin spraying (10 mg/l). Yellow leaves indicated kanamycin sensitive plants, while green leaves indicated resistant ones.

Insect bioassay

In vivo insect bioassay was designed to test insect resistance of transgenic cabbage. 5 young leaves from each transgenic plant were placed in a 9 cm diameter Petri dish, each containing 10 larvae with identical weight and incubated under 25 - 28℃ for 72 h. Then larvae mortality and weight were scored. The experiment was repeated 3 times. When the control mortality exceeded 20%, the experiment was considered invalid and consequently, repeated. The corrected larva mortality (CM) was calculated by 2 formulae:

$$M(\%) = \frac{Nd}{Nd + Ns} \times 100\%$$

and

$$CM(\%) = \frac{Ms - Mc}{100 - Mc} \times 100\%,$$

where *M* (%) = larva mortality (%), *CM* (%) = corrected larva mortality (%), *Nd* = death number of larvae, *Ns* = survival number of larvae, *Ms* = sample mortality of larvae, and *Mc* = control mortality of larvae.

RESULTS

Transgenic plant regeneration

During genetic transformation of cabbage with *CryIA*(*c*),

Table 2. Effect of different genotypes on transformation frequency in cabbage.

Genotype	No. of explants	No. of Km resistant shoots
ZG	510	26 (5.1%)
NX	211	15 (7.1%)
ZQ	416	0

the resistant shoot regeneration frequency was highest in ZG (5.1%). The shoot differentiation frequency and plantlet rooting are shown in Table 2, respectively. Resistant shoots frequency was only 1.7% in NX, while no resistant shoots were obtained on ZQ. This indicated that genotype has much influence on genetic transformation. Thus, it appears necessary to test and screen the genotypes for optimizing the transformation procedure. After a biweekly subculture on selective culture for 1 month, only few hypocotyls could re-generate kanamycin resistant shoots, while the rest hypo-cotyls developed well.

Confirmation of transformation at the molecular level

To confirm the presence of the transgenic chinese cabbage, DNA samples of a total of 56 putative T0 transgenic plants were obtained from ZG and NX. PCR analysis with *CryIA*(*c*) specific primers indicated that 43 plants could produce the same bands as plasmid (pBI-Bt) (Figure 2, upper panel). PCR analysis with *NPT*II specific primers showed that 31 plants produced the target fragment (740 bp), indicating the integration of *CryIA*(*c*) into 31 cabbage plants (Figure 2, bottom panel).

Southern blot analysis

The PCR amplified *CryIA*(*c*) fragment was purified with Sephodex and used for probes. Genomic DNA from transgenic plants and control were extracted and digested with *Hin*dIII, transferred on to a nylon membrane and hybridized with *CryIA*(*c*) probe (Figure 3). As shown in Figure 3, the untransformed control exhibited no signal, while most of the PCR positive transgenic plants showed strong hybridization signal. Most of the transgenic plants harbored 1 copy transgene, some had two loci and a few other plants had multiple copies. This result indicates the successful integration of *CryIA*(*c*) into the cabbage genome.

Insect bioassay

Five leaves are randomly selected from each T_0 cabbage transformant and used for preliminary *in vivo* bioassay of

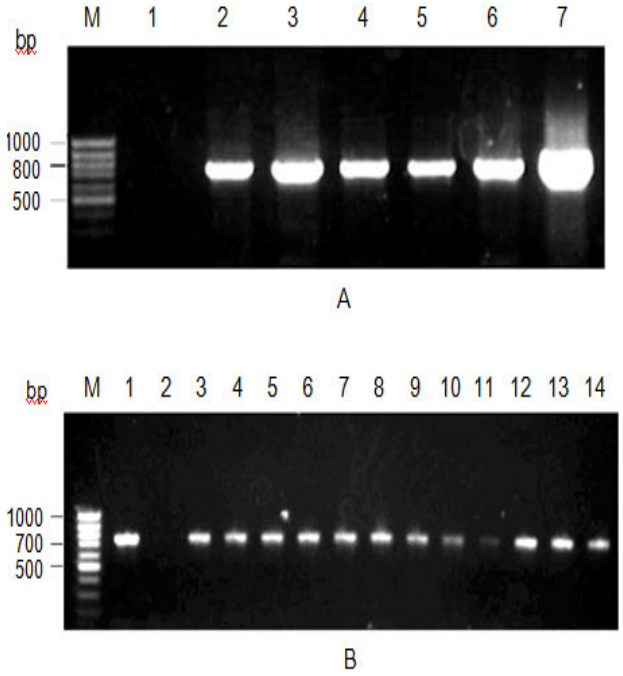

Figure 2. PCR analysis of putative transgenic cabbage plants. **A.** PCR amplification with *CryIA*(*c*) primers of putative transgenic plants. Lane M: Marker (100 bp ladder). 1, negative control (CK); 2–6, the transgenic plants; 7, positive control (pBI-Bt); **B.** PCR amplification with *NPT*II primers of some putative transgenic plants. M, Marker (100 bp ladder); 1, positive control (pBI-Bt); 2, negative control (CK); 3–14, the putative transgenic plants.

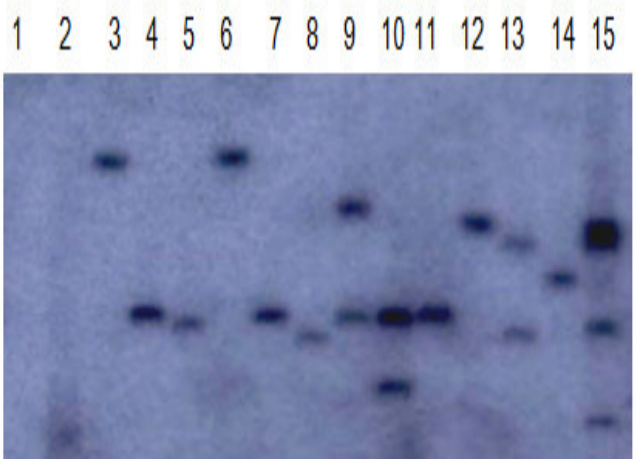

Figure 3. Southern blotting analysis on transgenic cabbage plants with *CryIA*(*c*) probe. 1, negative control (CK); 2–15, transgenic cabbage plants.

diamondback moth resistance (Table 3). As could be drawn from Table 3, many transgenic lines did not show much resistance to diamondback moth, while a few others exhibited improved resistance. Among these

Table 3. Bioassay of *in vitro* leaves from transgenic cabbages of ZG for evaluating resistance to diamondback moth larva.

Transgenic lines	Corrected mortality	Weight of larva (g)	Leaf damage degree
ZG-CK	0.00	0.0078	++++
ZG-1	40.00	0.0052**	+
ZG-2	13.33	0.0069*	++
ZG-3	26.67	0.0063*	+++
ZG-5	29.67	0.0067*	+++
ZG-6	26.67	0.0058**	+
NX-CK	0.00	0.0076	++++
NX-1	16.67	0.0073	+++
NX-2	40.00	0.0050**	+
NX-3	0.00	0.0072	++++
NX-4	40.00	0.0055**	++
NX-5	29.67	0.0065*	++
NX-6	29.67	0.0064*	++++
NX-7	13.33	0.0072	++++
Initial larvas weight		0.0014	

Duncan's test (LSR); * -significance at $P<0.05$ level; ** -extreme significance at $P<0.01$ level; + indicates leaf damage degree; several "+" indicate serious leaf damage.

Figure 4. Insect resistance bioassay of *CryIA*(*c*) transgenic cabbage. A, Leaf of wild type cabbage; B, Leaf of transgenic cabbage.

transgenic plants (ZG-1, ZG-2, ZG-5, ZG-6, NX-1, NX-2, NX-4, NX-5, NX-6 and NX-7), larva weight increased much slowly and the larvae exhibited inaction and anorexia; although corrected mortality was not very high (Figure 4). The cabbage leaves were not much damaged, showing an improved insect resistance. As for transgenic lines ZG-3 and NX-3, the larvae weight and development status was similar to untransformed control.

Genetic segregation in transgenic progeny

Among transgenic cabbage lines, 2 transgenic lines with high insect resistance, ZG-1 and ZG-6, were selected and self pollinated. Kanamycin spray screening and PCR analysis was used to investigate exogenous gene segregations. As could be drawn from Table 4, progeny segregation followed Mendel's fashion with a ratio of 3:1 by applying χ^2 test. These results showed that these 2 transgenic lines contained a single copy transgene, which was stably inheritable. PCR analysis with *NPT*II specific primers further confirmed the kanamycin spray results (Table 5).

DISCUSSION

Laboratory selction increased resistance to CryIA(c) in the LBA4404 strain harbouring plasmid pBI-Bt. The present study established an efficient plant regeneration and genetic transformation procedure for cabbage, and successfully transformed insecticidal *Bacillus thuringiensis*

Table 4. Resistance segregation in two T_1 generations of transgenic cabbages tested by kanamycin spray.

T_1 generations	Kanamycin resistance plants	Kanamycin sensitive plants	χ^2 (3:1)
ZG-1	9	5	$0.081<\chi^2_{0.05}$
ZG-6	16	10	$1.633<\chi^2_{0.05}$
Total	25	15	$2.810<\chi^2_{0.05}$

$\chi^2_{0.05}$ =3.84 (df = 1).

Table 5. Segregation of *NPT*II gene in two T_1 generations of transgenic cabbages tested by PCR.

T_1 generations	PCR positive plants	PCR negative plants	χ^2 (3:1)
ZG-1	15	8	$0.710<\chi^2_{0.05}$
ZG-6	24	11	$0.467<\chi^2_{0.05}$
Total	39	19	$1.471<\chi^2_{0.05}$

$\chi^2_{0.05}$ =3.84 (df = 1)

thuringiensis CryIA(c) gene into cabbage genome. Like the previous research, more transgenic plants were obtained and transformation frequency was much higher. In addition, transgenic cabbage plants were systemically analyzed on both molecular and insect bio-assay levels, indicating improved resistance to diamond-back moth.

In vivo insect bioassay showed various resistance levels among different transgenic lines; as also observed in previous research works (Li et al., 1995; Cao et al., 1999). The various resistance levels are probably associated with exogenous gene integration sites or gene modification (Matzke et al., 1994). Southern blot analysis also demonstrated the random insertion of *CryIA*(*c*). Although some transgenic lines did not cause high mortality to diamondback moth, the larvae exhibited inaction, anorexia and low weight, and cabbage leaves were rarely damaged indicating an improved insect resistance. Besides selection of individual plants with high insect resistance, crossing between different transgenic plants to increase transgene copy number might serve to improve resistance level. In addition, combining different Bt genes is also an effective way for engineering insect resistance (Cao et al., 1999; 2002).

Genetic engineering for crop improvement entails appropriate transformation frequency, whose influencing factors are various. In this study, we found that the genotypes had the most impact on cabbage transformation frequency. Obvious differences existed in the 3 cabbage varieties. Thus suitable genotypes should be screened for cabbage transformation.

In a previous report, Mao et al. (1996) demonstrated that exogenous gene could be inherited in T_2 generations in Mendel's fashion by insect bioassay, kanamycin screening and molecular hybridization. The present study showed that *NPT*II and *CryIA*(*c*) were stably inherited and expressed in T_1 generations through same method. χ^2 test indicated *NPT*II was inherited as a single gene in Mendel's fashion. *CryIA*(*c*) transgenic cabbage would provide an alternative breeding strategy for engineering insect resistance.

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