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GENETIC TRANSFORMATION AND HYBRIDIZATION

Sequential transformation to pyramid two Bt genes in vegetable Indian mustard (*Brassica juncea* L.) and its potential for control of diamondback moth larvae

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Abstract Vegetable Indian mustard (*Brassica juncea* cv. "Green Wave") plants that control *Plutella xylostella* (diamondback moth) (DBM) were produced by introduction of one or two *Bacillus thuringiensis* (Bt) genes. A *cry1Ac* Bt gene associated with the *npt*II gene for kanamycin selection or a *cry1C* Bt gene with the *hpt* gene for hygromycin selection was introduced individually through *Agrobacterium*-mediated transformation of seedling explants. A *cry1C* line was then transformed with the *cry1Ac* gene to produce pyramided *cry1Ac* + *cry1C* plants. Sixteen *cry1C*, five *cry1Ac*, and six *cry1Ac* + *cry1C* plants were produced. PCR and Southern analyses confirmed the presence of the *cry1C*, *cry1Ac* or pyramided *cry1Ac* + *cry1C* genes in the Indian mustard genome. ELISA analysis showed that production of Bt proteins varied greatly among individual transgenic plants, ranging from undetectable to over 1,000 ng Bt/mg total soluble protein. The levels of the Bt proteins were correlated with the effectiveness of control of diamondback moth (DBM) larvae. Insect bioassays indicated that both the *cry1C* and *cry1Ac* plants were toxic to susceptible DBM. The *cry1C* plants also controlled Cry1A-resistant DBM while *cry1Ac* plants controlled Cry1C-resistant DBM, and the pyramided *cry1Ac* + *cry1C* plants effectively controlled all three types of DBM. These Bt-transgenic plants could be used either for direct control of DBM and other lepidopteran insect pests or for tests of "dead-end" trap crops as protection of high value non-transgenic crucifer vegetables such as cabbage.

Keywords *Bacillus thuringiensis* · *Brassica juncea* · Cry1Ac and Cry1C proteins · Diamondback moth · Insect resistance management · *Plutella xylostella* · Trap crops

Abbreviations

Bt	*Bacillus thuringiensis*
DBM	Diamondback moth
ELISA	Enzyme-linked immunosorbent assay
TSP	Total soluble protein

Introduction

Bacillus thuringiensis (Bt) genes for control of insect pests have been transferred into a wide variety of crop plants, but only Bt corn and cotton are grown commercially on a large scale (James 2006). Two other Bt crops that have undergone extensive field testing and are likely to be released in the near future are rice (James 2006) and eggplant (Medakker and Vijayaraghavan 2007), but they have not received final regulatory approval in China and India, respectively. Most other potential Bt crops have not yet

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been deployed because of complex regulatory/biosafety/consumer acceptance issues.

Besides the commercial value of Bt corn and cotton, Bt plants have served as excellent research tools. For example, Bt broccoli plants have been used in empirical tests of various resistance management strategies designed to delay the evolution of resistant insects (Zhao et al. 2003, 2005). Bt Brassicas are particularly suitable for such work because of the availability of resistant populations of diamondback moth (DBM), the most destructive insect pest of crucifers worldwide and the only insect that has developed high levels of resistance to insecticidal Bt proteins in the field (Tabashnik et al. 2003). The results from these studies have provided information relevant to management of the commercial Bt crops.

An additional possible use of Bt plants is to combine transgenic technology with the biocontrol strategy of trap cropping. Trap crop strategies offer the potential to minimize or eliminate the use of insecticides and preserve natural enemies that control pests, while maintaining or increasing crop quality and yield (Hokkanen 1991; Shelton and Badenes-Perez 2006). The concept is simple: intercept the insect pest with a plant that is more attractive to it than the main crop. A strong host plant preference of a pest for the trap crop is a key requirement for the success of this strategy.

Two cruciferous plants, collards (*Brassica oleracea* ssp. *acephala*) and Indian mustard (*Brassica juncea*), have been proposed and tested for use as trap crops for protection of cabbage from DBM (Srinivasan and Moorthy 1991; Luther et al. 1996; Bender et al. 1999; Charleston and Kfir 2000; Mitchell et al. 2000). However, neither collards nor Indian mustard are toxic to the larvae that hatch after oviposition, so this approach may actually lead to an increase in the pest populations (Shelton et al. 2007). “Dead-end” trap crops, on which larvae do not survive, could overcome this problem (Shelton and Nault 2004). Such dead-end trap crops could be obtained either via the intrinistic characteristics of a plant species, e.g. the weed yellow rocket [*Barbarea vulgaris* (R. Br.) var. *arcuata*] (Badenes-Perez et al. 2005), or created by introduction of an insecticidal transgene, such as a Bt gene (Cao et al. 2005).

Cry1 Bt genes have been introduced into several *Brassica* species, conferring resistance to major lepidopteran pests including DBM (Earle et al. 2004). One potential Brassica trap crop, collards, has been transformed with *cry1Ac* and *cry1C* genes (Cao et al. 2005). A variety of *B. juncea* transformants have been produced and tested for their possible agricultural value (e.g., Mehra et al. 2000; Prasad et al. 2000; Banuelos et al. 2005; Mondal et al. 2007) but none carried Bt genes. The only report of transgenic insect resistance in *B. juncea* involves control of a non-lepidopteran insect, the mustard aphid (*Lipaphis erysimi* Kalt.), via a cDNA encoding wheat germ agglutinin (Kanrar et al. 2002). To date, all the *B. juncea* materials used in transgenic studies have involved seed mustards, rather than cultivars used as vegetables.

Although Bt *B. juncea* could have commercial potential, the primary focus of our study was possible use of Bt *B. juncea* as a dead end trap crop for protection of cabbage or other *Brassica* vegetables against DBM. As a first step toward that goal, this paper reports introduction of single or multiple *cry1* Bt genes into the vegetable Indian mustard cultivar “Green Wave” and effective control of Bt-resistant and susceptible DBM larvae by the transgenic plants. Ovipositional preference of DBM for “Green Wave” over cabbage was also demonstrated. Potential use of the transgenic materials as a dead end trap crop is discussed.

Materials and methods

Plasmid vectors

Two binary vectors were used. The vector pMON10517, which contains the *35S 5′/cry1Ac/nos 3′* associated with the *nptII* gene for kanamycin selection, was provided by Monsanto Co. (Metz et al. 1995). The vector pNS6 carrying the *35S 5′/cry1C/35S 3′* associated with the *hpt* gene for hygromycin selection was provided by Strizhov et al. (1996).

Plant transformation

Seeds of *B. juncea* (L.) Czern “Green Wave” (Johnny’s Selected Seeds, Albion, ME) were surface sterilized with 70% ethanol for 5 min and then 10% Clorox for 10 min, washed, and then germinated on MS medium (Murashige and Skoog 1962) with no phytohormones. All cultures were maintained at 25°C with a 16-h photoperiod. Hypocotyl and petiole explants were excised from 5–7 day old seedlings and pre-cultured for 2 days on MS regeneration medium containing MS salts, 100 mg/l *m*-inositol, 1 mg/l thiamine HCl, 2 mg/l BA, 0.5 mg/l NAA, and 3% sucrose. This medium was routinely used except in experiments testing effects of plant growth regulators on regeneration of transgenic Indian mustard. In those experiments, BA (2 mg/l) was used alone or in combination with 0.01 mg/l 2,4-D, 0.5 mg/l NAA, or 0.1 mg/l IAA, respectively. Pre-cultured explants were infected and co-cultivated with *Agrobacterium tumefaciens* ABI cells for 2–3 days. Then, explants were transferred onto and cultured on the MS regeneration medium containing Timentin (300 mg/l; SmithKline Beecham) for 7 days to eliminate *Agrobacterium* cells. Subsequently, explants were transferred to the

same medium supplemented with Timentin (300 mg/l) and kanamycin (25 mg/l) or hygromycin (10 mg/l) to screen for kanamycin-resistant or hygromycin-resistant plants. Explants were subcultured on this selective medium every 2 weeks. Regenerated shoots were further subcultured once or twice on the same medium before transfer to rooting medium containing MS salts plus appropriate antibiotics without phytohormones (Cao and Earle 2003). The rooted plantlets were then transplanted into soil and covered with a plastic bag. Healthy and normally growing plants were transferred to a greenhouse with 80% relative humidity, 25°C, and photoperiod of 16/8 (light/dark).

Sequential transformation was used to produce plants carrying both Bt genes. After surface sterilization, T_1 progeny seeds of a *cry1C* line carrying one insert of the T-DNA were germinated on MS medium containing 35 mg/l of hygromycin. Hypocotyl and petiole explants were excised from 5-day-old hygromycin-resistant seedlings, pre-cultured on MS regeneration medium for 2 days, and then co-cultivated with *Agrobacterium* cells harboring pMON10517 carrying a *cry1Ac* gene. Subsequently, the procedures described in previous section were used to select kanamycin-resistant plants on MS regeneration medium containing kanamycin (25 mg/l).

PCR and Southern analysis

DNA isolation from fresh leaf tissues and polymerase chain reaction (PCR) analysis of genomic DNA were done as described previously (Cao et al. 1999). Primers specific to *cry1Ac* or *cry1C* gene were used for PCR. The sequences of the *cry1Ac* specific primers are 5′ primer: 5′-CAACTAG GTCAGGGTGTC-3′, 3′ primer: 5′-AGCGCATCTGTTA GGCTC-3′. Sequences of the *cry1C* specific primers are 5′ primer: 5′-GGAGAAAGATGGGGATTG-3′, 3′ primer: 5′-AACTCGTGCATCCCTACT-3′. For Southern blot analysis, plant DNA was digested with *Bam*HI, electrophoresed on a 1% agarose gel, and transferred onto nylon membrane. DNA gel blot hybridization was performed as described by Cao et al. (2005) with a digoxigenin-dUTP DNA labeling and detection starter kit II from Roche Molecular Biochemicals (Cat. No. 1 858 61).

Protein extraction and ELISA

Total soluble protein was extracted from fresh leaf tissues of *cry1Ac, cry1C, cry1Ac + cry1C*, or non-transgenic plants with 8–10 leaves as previously described (Cao et al. 2002) with the isolation kits from Envirologix Co. (Portland, ME). The protein concentration was determined with Bio-Rad Protein Assay Kit II (Catalog No. 5000-0066) following its micro assay procedure. Protein dilution and ELISA were performed according to the manufacturer's instructions with the Cry1C or Cry1Ab/Cry1Ac plate kits from Envirologix. The OD values of the Bt proteins were determined by a microplate reader set at 450 nm. The amount of Cry1C or Cry1Ac protein was calculated from standard concentrations obtained with Cry1C or Cry1Ac (Envirologix) and expressed as ng Bt protein/mg total soluble protein (TSP).

Segregation analysis

In order to determine the segregation of the *cry1C* gene in the transgenic plants, seeds were collected from self-pollinated T_0 plants. A random sample of 80–170 seeds from individual T_0 plants were plated in Magenta boxes of the selective MS medium containing 1% sucrose, 25 mg/l hygromycin, and 2.2 g/l Gelrite (Sigma) in the light. Following germination, the percentage of seedlings with development of true leaves and normal roots was determined.

Insect bioassays

Second instar larvae of susceptible, Cry1A-resistant (Cry1A^R), and Cry1C-resistant (Cry1C^R) DBM were used for insect bioassays. At the time of the bioassays, the Cry1A^R population was over 280,000-fold resistant to Cry1Ac protoxin (J-Z Zhao, unpublished data), but was susceptible to Cry1C-expressing broccoli (Cao et al. 1999). The Cry1C^R strain was over 500-fold resistant to Cry1C protoxin but was susceptible to Cry1Ac-expressing broccoli (Zhao et al. 2001). The DBM colonies were kept in an environmental chamber at 27 ± 1°C, 35 ± 2% RH, and photoperiod of 16 h/8 h (light/dark). Prior to bioassays, eggs were hatched and larvae reared on an artificial diet (Shelton et al. 1991).

A leaf from *cry1Ac*, *cry1C*, or *cry1Ac + cry1C* Indian mustard plants or untransformed plants was placed in a baby food jar containing 1% Phytagar. Five susceptible, Cry1A^R, or Cry1C^R larvae were placed on the surface of the leaf and maintained at 25°C under a 16/8h light/dark regime. All insect bioassays were performed in triplicate. Leaf damage (estimated visually) and insect mortality were scored after 5 days, although observations of the bioassays were made earlier as well.

Tests of ovipositional preference

Ovipositional tests were done using a two-choice method comparing Indian mustard ("Green Wave") and yellow

rocket [*Barbarea* (R. Br.) var. *arcuata*] to cabbage (*B. oleracea*, ssp. *capitata*, variety "Huron") or cabbage to cabbage (check), using three replicates of each choice test. A single leaf of each plant type was placed into a 50 ml flask filled with water and the lip of the flask was sealed with Parafilm. A flask with the test leaf was placed into a 1 m^3 chamber along with a flask of a cabbage leaf. Care was taken to use only leaves of a similar size. Newly emerged DBM adults (3 females and 3 males) were introduced into each chamber. Moths were allowed to mate and lay eggs for 24 h after which the eggs were counted. Each leaf was placed with its flask into a smaller chamber for 7 days at which time the number of surviving larvae was counted.

Results

Production of Indian mustard plants carrying a single Bt gene

The effects of three different auxins in combination with 2 mg/l of the cytokinin 6-benzylaminopurine (BA) on regeneration of transgenic *B. juncea* plants were evaluated in two transformation experiments using pNS_6 carrying a *cry1C* gene associated with *hpt* (Table 1). Treatment with BA alone gave no recovery of transgenic plants on medium containing 10 mg/l hygromycin. All combinations of BA with an auxin (NAA, IAA, or 2,4-D) allowed regeneration of hygromycin-resistant plants, with variable transformation frequency. The combination of BA with NAA gave the best results in both experiments and was therefore used in all subsequent transformation experiments.

About 3 weeks after explants were placed on regeneration medium containing 10 mg/l hygromycin, green calli emerged from the cut ends of some explants inoculated with *Agrobacterium* harboring pNS_6, but not from non-inoculated controls. Shoots subsequently regenerated from some of the green calli. In the first two subcultures on regeneration medium containing 25 mg/l kanamycin, explants co-cultivated with *Agrobacterium* harboring pMON10517 carrying the *cry1Ac* gene associated with *nptII* for kanamycin selection produced calli with green, white, or purple shoots. The majority of the initially green shoots turned white or purple and became dead after further subcultures; only a few remained green and produced additional green shoots on the medium with kanamycin. Green shoots selected from hygromycin- or kanamycin-containing regeneration medium were transferred to the rooting medium containing 10 mg/l hygromycin or 25 mg/l kanamycin. Roots started to develop from all shoots 3–4 weeks after the transfer.

Table 1 Effects of growth regulator combinations on *Agrobacterium*-mediated transformation of *Brassica juncea* cv. "Green Wave" with pNS_6 carrying the *cry1C Bacillus thuringiensis* gene and the *hpt* gene for resistance to hygromycin

Growth regulator combination	No of explants in experiment		No. of hyg^{Res} plants		Transformation efficiency[a] (%)
	I	II	I	II	
BA (2 mg/l)	176	152	0	0	0
BA (2 mg/l) + 2,4-D (0.01 mg/l)	170	144	3	1	1
BA (2 mg/l) + NAA (0.5 mg/l)	196	135	5	4	3
BA (2 mg/l) + IAA (0.1 mg/l)	182	156	2	1	1

[a] Aggregate transformation efficiency in the two experiments I and II

A total of 16 *cry1C* and 5 *cry1Ac* Indian mustard plants were recovered through *Agrobacterium*-mediated transformation. Primary transformants transferred to the greenhouse showed normal morphology and flowering characteristics. All were fertile, and thousands of seeds were obtained from individual *cry1Ac*, *cry1C*, or *cry1Ac* + *cry1C* T_0 plants.

PCR and Southern blot analyses

Initial screening of putative transformants obtained by selection with hygromycin and/or kanamycin was done through PCR analysis using primers specific for *cry1Ac* or *cry1C* gene. The expected 1.1 kb band representing the *cry1C* fragment was amplified in samples from the hygromycin-resistant plants and from the pNS6 plasmid, while the expected 1.3 kb band representing the *cry1Ac* fragment was seen in samples from the kanamycin-resistant plants and the pMON10517 plasmid (Fig. 1). Neither band was seen in non-transformed control samples. These results presented initial evidence that the *cry1C* or *cry1Ac* gene was present in all of the tested hygromycin- or kanamycin-resistant plants, respectively.

Southern blot analysis was done for further confirmation of hygromycin-resistant plants. DNA was isolated from young leaf tissues of individual transgenic plants, digested with *Bam*HI, and hybridized with probes from the PCR products of the *cry1C* gene. DNA from non-transformed plants, used as a negative control, showed no hybridization signals to the probe used. On the other hand, the majority of transgenic plants analyzed gave the expected 1.9 kb band (Fig. 2). Two transgenic plants showed high molecular weight bands, indicating that a rearrangement of inserted T-DNA had been occurred when the T-DNA was inserted into the plant genome.

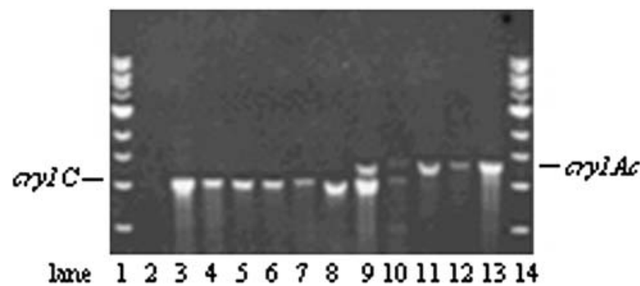

Fig. 1 PCR analysis of genomic DNA from *cry1C*, *cry1Ac,* or *cry1Ac* + *cry1C Brassica juncea* "Green Wave" plants. PCR products in *lanes 3–8* were obtained with the *cry1C*-specific primers; those in *lanes 11–13* with *cry1Ac*-specific primers; those in *lanes 2*, *9*, and *10* with both *cry1C*- and *cry1Ac*-specific primers simultaneously. *Lane 1* and *lane 14*: 1 kb molecular weight marker; *lane 2*: DNA from non-transformed Indian mustard; *lane 3*: pNS_6 carrying the *cry1C* gene; *lane 13*: pMON10517 carrying the *cry1Ac* gene. *Lanes 4–8* are from hygromycin-resistant plants; *lanes 11* and *12* from kanamycin-resistant plants; *lanes 9* and *10* from kanamycin-resistant *cry1C* plants sequentially transformed with the *nptII* gene

Production of Bt proteins and control of diamondback moth larvae

Production of Bt proteins in the transgenic Indian mustard plants was quantified by enzyme-linked immunosorbent assay (ELISA). The levels of Cry1Ac and Cry1C proteins produced varied greatly among independent transformants, ranging from undetectable (<25 ng/mg TSP) to over 0.1% of TSP. The levels of Cry1Ac or Cry1C protein in the transgenic plants were correlated with the plants' effectiveness in control of DBM (Table 2). After 5 days of feeding, five DBM larvae were able to consume an entire leaf from non-transformed control plants. There was no larval mortality, and the larvae increased in size. Four *cry1C* plants and one *cry1Ac* plant that produced no detectable Cry1C or Cry1Ac protein, respectively, showed no control of susceptible or resistant DBM larvae. These transgenic plants were comparable to non-transgenic control plants with severe leaf defoliation and no mortality.

In contrast, four *cry1C* plants that produced high levels of Cry1C protein (>1,000 ng/mg TSP) showed complete resistance to susceptible and $Cry1A^R$ DBM with 100% mortality and no visible leaf damage in the detached leaf assay (Table 2). Similarly, leaves from *cry1Ac* line pMON-1, which produced a high level of Cry1Ac protein (>1,000 ng/mg TSP), were highly toxic to susceptible or $Cry1C^R$ larvae (data not shown). The larvae stopped feeding, and all were dead within 48 h with little or no defoliation. The *cry1C* plants with low levels of Cry1C protein suffered up to 25% defoliation by susceptible DBM and up to 20% by $Cry1A^R$ DBM (Table 2). Most of the larvae were dead by the end of the detached leaf assay, and growth of the remaining larvae was severely inhibited.

Inheritance of the *cry1C* gene in transgenic plants

The inheritance of the *hpt* gene associated with the *cry1C* gene in the T_1 progeny from each T_0 plant was determined by evaluating the germination and growth of seedlings on medium supplemented with 25 mg/l hygromycin (Table 3). The hygromycin level was increased over that used for initial selection so that hygromycin-resistant seedlings could be clearly distinguished from the hygromycin-sensitive ones. Five of the 11 T_0 plants tested showed 3:1 segregation for resistance to hygromycin in their progeny, suggesting that the *hpt* transgene was integrated into a single locus. Two lines showed a 15:1 ratio, while the rest fit a ratio of 63:1, indicative of two or three unlinked loci, respectively.

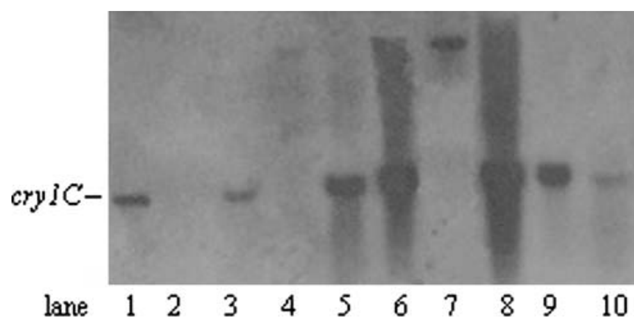

Fig. 2 DNA gel blot analysis of *cry1C Brassica juncea* plants. Ten micrograms of genomic DNA used for each lane were digested with *Bam*HI and probed with a non-radioactive DIG-labeled 1.1 kb PCR product of the *cry1C* gene. *Lane 1*: a 1.9 kb *Bam*HI fragment covering the entire *cry1C* gene isolated from plasmid pNS_6; *lane 2*: non- transformed "Green Wave" control; *lanes 3–10*: independent transgenic *cry1C* plants

Production of Indian mustard plants carrying both *cry1Ac* and *cry1C* Bt genes

We used a sequential transformation strategy to produce plants that carried both the *cry1C* and the *cry1Ac* gene. T_1 seeds from *cry1C* plant H4, which produced a high level of Cry1C protein and had a 3:1 segregation ratio for hygromycin resistance (Tables 2, 3), were germinated on medium containing 35 mg/l hygromycin. This high level of hygromycin was used to ensure that all seedlings providing explants for the sequential transformation contained the *cry1C* gene associated with the *hpt* gene. Hypocotyls and petioles of seedlings that grew actively on the germination medium were used as explant sources for *Agrobacterium*-mediated transformation with pMON10517 carrying the *cry1Ac* gene. A total of six *cry1Ac* + *cry1C* plants were recovered from 466 explants with a transformation

Table 2 Production of Cry1C protein and control of diamondback moth larvae by *B. juncea* plants carrying the *cry1C* gene

Line	Level of Cry1C protein	Cry1C protein (ng/mg TSP)	Susceptible larvae		Cry1A^R larvae	
			Defoliation (%)	Mortality (%)	Defoliation (%)	Mortality (%)
Control[a]	ND	–	80–100	0	80–100	0–20
H1, H2, H3, H5	ND	–	80–100	0–20	70–95	0–20
H6, H8, H9	Low	139–450	0–25	80–100	0–20	60–100
H4, H10, H11, H12	High	1,350–1,467	0	100	0–2	100

TSP Total soluble protein

[a] Non-transformed "Green Wave"

Table 3 Segregation of selfed progeny of *cry1C B. juncea* plants for resistance to hygromycin

Plant (T_0)	Resistant (R) progeny	Susceptible (S) progeny	Ratio (R:S)	Ratio tested	χ^2 value	*P* value
H1	109	33	3.3:1	3:1	0.235	0.628
H2	97	31	3.1:1	3:1	0.417	0.838
H3	115	41	2.8:1	3:1	0.137	0.712
H4	130	32	4.1:1	3:1	2.378	0.123
H5	119	41	2.9:1	3:1	0.033	0.855
H6	162	4	40.5:1	63:1	0.775	0.379
H8	167	3	55.7:1	63:1	0.045	0.832
H9	147	3	49:1	63:1	0.187	0.666
H10	130	7	18.6:1	15:1	0.304	0.581
H11	131	3	43.7:1	63:1	0.398	0.528
H12	76	4	19:1	15:1	0.213	0.644

efficiency of a little over 1%. PCR analysis of T_0 *cry1Ac* + *cry1C* plants showed the presence of both the 1.1 kb *cry1C* and the 1.3 kb *cry1Ac* bands (Fig. 1).

Production of Bt proteins and insect control in the *cry1Ac* + *cry 1C* plants

All *cry1Ac* + *cry1C* Indian mustard plants produced more than 0.1% Cry1C protein/TSP, comparable to the high level of production of Cry1C protein in the original H4 *cry1C* line (Table 4). However, production of Cry1Ac protein varied greatly among individual *cry1Ac* + *cry1C* plants, ranging from undetectable to over 0.1% Cry1Ac protein/TSP.

The detached leaf assay showed that leaves from all *cry1Ac* + *cry1C* lines as well as the H4 line consistently exhibited high toxicity to susceptible and Cry1A^R DBM larvae (Table 4). On the other hand, the *cry1Ac* + *cry1C* lines showed differences in control of Cry1C^R DBM larvae, correlated with levels of Cry1Ac protein produced (Table 4). Leaves from *cry1Ac* + *cry1C* lines QH-P1 and QH-P3, with >700 ng Cry1Ac protein/mg TSP, showed little or no feeding damage and caused 100% mortality of the Cry1C^R DBM larvae within 2–3 days. On leaves of transgenic lines QH-P2 and QH-P4 with the production of 474 and 408 ng Cry1Ac protein/mg TSP, respectively, only small holes from early feeding were detected in some cases. All larvae feeding on these plants were dead by day 4 after infestation. Line QH-P5, with a low level of Cry1Ac protein, suffered moderate defoliation (5–25%) and only showed partial lethality to the Cry1C^R larvae. However, the growth of the surviving larvae was severely inhibited. In contrast, leaves from *cry1Ac* + *cry1C* line QH-P6, which produced little or no detectable Cry1Ac protein, suffered severe leaf damage and allowed not only larval survival, but also vigorous growth.

Ovipositional preference

Studies of ovipositional preference showed that DBM laid 10-fold more eggs on Indian mustard leaves than on cabbage (Table 5). The survival of larvae hatched from the eggs laid on Indian mustard was also higher than on cabbage. In contrast, yellow rocket showed high ovipositional preference with no larval survival (Table 5).

Discussion

Work on transformation of *B. juncea* has focused on oilseed cultivars. In almost all cases, auxin in combination with cytokinins was required to promote the regeneration of transgenic plants (Mehra et al. 2000; Kanrar et al. 2002; Paul and Sikdar 1999). Little attention has been devoted to tissue culture and transformation of *B. juncea* vegetable crops. Guo et al. (2005) has reported the effects of plant hormones on shoot regeneration from cotyledon and leaf segments of stem mustard (*B. juncea* var. *tsatsai*), in which NAA in combination with various cytokinins increased frequency of shoot regeneration. To our knowledge, this paper is the first report of the successful recovery of

Table 4 Production of Bt protein and control of diamondback moth larvae by *cry1C* + *cry1Ac B. juncea* plants

Lines	Cry1C protein (ng/ mg TSP)	Cry1A protein (ng/ mg TSP)	Susceptible larvae		Cry1A[R] larvae		Cry1C[R] larvae	
			Defoliation (%)	Mortality (%)	Defoliation (%)	Mortality (%)	Defoliation (%)	Mortality (%)
Control[a]	–	–	100	0	100	0	100	0
H4	1,303	–	0	100	0	100	100	0
QH-P1	1,234	1074	0	100	0	100	0	100
QH-P2	1,284	474	0	100	0	100	0–5	100
QH-P3	1,248	718	0	100	0	100	0	100
QH-P4	1,230	408	0	100	0	100	0–5	100
QH-P5	1,357	117	0	100	0	100	5–25	80–100
QH-P6	1,452	ND	0	100	0	100	100	0

The lines were produced by transformation of seedling explants of cry1C line H4 with pMON10517

TSP Total soluble protein

[a] Non-transformed "Green Wave"

transgenic plants from a commercial *B. juncea* vegetable variety. Our results provide additional evidence that auxins are required in combination with cytokinins to achieve regeneration of transgenic *B. juncea* plants. The transformation efficiency (about 1%) was similar to the efficiency (0.5–1.5%) recently reported for oilseed *B. juncea* (Mondal et al. 2007). Transformation efficiency was similar in the initial transformation and in the addition of the second transgene (*cry1Ac*) to explants already carrying a *cry1C* gene.

Significant advances in multiple gene manipulations have been made in recent years (Halpin 2005). Two or more genes can be combined or sequentially introduced into a plant by either by sexual crosses or by sequential transformation techniques. In broccoli and collard transformations, we used hypocotyls and petioles from germinating seedlings as the initial targets for *Agrobacterium*-mediated transfer of *cry1Ac* or *cry1C* Bt genes (Cao et al. 1999, 2005). Large numbers of seeds were required to generate sufficient seedling explants. Moreover, recovery of seeds from our broccoli and collard plants carrying a single Bt gene was difficult and/or slow because of self-incompatibility (broccoli) or biannual characteristics (collard). For that reason, we used sexual crosses between single gene plants to produce pyramided *cry1Ac* + *cry1C* broccoli or collard plants (Cao et al. 2002, 2005). However, in the current study with Indian mustard, which is a self-compatible annual, large numbers of seeds were easily recovered from the *cry1Ac* and *cry1C* primary transformants. We therefore were able to make use of sequential transformation to add a *cry1Ac* gene to a *cry1C* Indian mustard plant.

Table 5 Ovipositional preference and larval survivorship of susceptible *Plutella xylostella* in Indian mustard, yellow rocket and cabbage

Plant material	Ovipositional ratio[a]	Larval survivorship[b]
Cabbage/cabbage (check)	1.1a	65a
Yellow rocket/cabbage	9.36b	0b
Indian mustard/cabbage	11.1c	79c

Means within a column followed by different letters are significantly different, $P \leq 0.05$ (Fisher's Protected LSD, SAS Institute, 1999)

[a] Number of eggs laid on the leaf of the test plant, compared to the number of eggs laid on the cabbage leaf in the same chamber for each replicate

[b] Percent of *P. xylostella* larvae that survived from the eggs laid on each leaf

Although pyramiding two or more identical or similar sequences of genes, promoters, or enhancers into a single plant genome could result in gene silencing, a number of studies including our own, have indicated that this does not always occur (Greenplate et al. 2000; Cao et al. 2002). ELISA analysis of *cry1Ac* + *cry1C* broccoli plants produced by sexual crosses showed that Cry1Ac and Cry1C proteins were produced in the hybrids and in their F1 progeny at levels comparable to the original single gene parental lines (Cao et al. 2002). Silencing of the *cry1Ac* and *cry1C* genes was not observed even though both genes were controlled by a 35S CaMV promoter. In the current study using the same constructs, the *cry1Ac* gene was introduced into a highly expressing *cry1C* Indian mustard plant via sequential transformation. All *cry1Ac* + *cry1C* plants maintained the original high level of production of the Cry1C protein, although a wide range of production of Cry1Ac protein was observed. Taken together, these results provide additional support for the concept that two closely related Bt genes can be pyramided into a single cultivar.

Pyramiding two or more insect resistance genes in the same plant provides an effective way to inhibit pest

adaptation, even with a smaller and more economically feasible refuge size. Computer simulation models have suggested that deployment of two insect resistance genes pyramided in a single cultivar was in general more durable than the sequential deployment of cultivars with single insect resistance traits (Roush 1998). A greenhouse trial with Bt-transgenic broccoli plants provided direct evidence that adaptation of insect populations to two Bt genes pyramided in a single cultivar is slower than if single Bt genes are deployed either sequentially or in a mixture (Zhao et al. 2003). The *cry1Ac* + *cry1C* Indian mustard plants that we produced by sequential transformation controlled not only susceptible but also Cry1A- and Cry1C-resistant DBM and are expected to delay the evolution of Bt-resistant DBM populations, like the pyramided broccoli lines (Zhao et al. 2003).

An effective trap crop must be preferred for insect pest oviposition. We found that DBM have a strong ovipositional preference for Indian mustard over cabbage (ovipositional ratio >11). This is higher than the ovipositional ratio (7.6–7.9) for glossy leaf collards, another suggested trap crop for crucifer vegetables (Cao et al. 2005). The tests of DBM ovipositional preference for Indian mustard and collards over cabbage reported in this paper and in Cao et al. (2005), respectively, were done simultaneously and so are directly comparable. However, the value of Indian mustard as a conventional trap crop is limited by the high survival of hatched larvae on its leaves. In contrast, yellow rocket shows both high ovipositional preference and high mortality of DBM larvae. In spite of these favorable features, yellow rocket suffers from several problems as a "dead end trap crop". It is a small weedy plant with limited biomass, for which agronomic practices have not been developed. In addition, it is susceptible to at least one other lepidopteran pest species, imported cabbageworm, *Pieris rapae* L. (unpublished data). For these reasons, creation of Bt-transgenic Indian mustard as a potential "dead end trap crop" seemed worthwhile. A vegetable cultivar was selected as the target for transformation because its extensive leaf area provides a large target for oviposition. Our oviposition preference tests were done with non-transgenic "Green Wave" plants, but previous work has shown that DBM oviposition on *Brassica* leaves is not affected by the presence of a *Cry1* gene (Tang et al. 1999; Cao et al. 2005; Shelton et al. 2007). We therefore expect DBM to show the same high oviposition preference on Bt *B. juncea* as seen in Table 5.

Availability of the single gene or pyramided *cry1Ac* + *cry1C* Indian mustard plants permits tests of the efficacy of these plants as a dead end trap crops to protect cabbage or other crucifers against DBM and other lepidopteran insect pests. Initial studies with an Indian mustard line carrying the *cry1C* gene suggest that this can indeed be an effective approach (Shelton et al. 2007). Moreover Bt Indian mustard could also be useful for direct control of lepidopteran pests, if deployed as a commercial crop.

When transgenic plants are intended for field applications, either as commercial crops or as trap crops, gene flow via pollen or seeds is a major concern. In the case of Bt-collards, another possible dead end trap crop, the biennial nature of the crop is a possible solution to this problem. No flowers or seeds are produced without vernalization, and the first year crop could be removed from the field. In contrast, Indian mustard is an annual with a high production of seeds, so gene flow is a significant issue. An effective control would be use of cytoplasmic male sterile (CMS) Bt plants that produce no pollen. Such plants could be obtained by backcrossing the Bt plants to CMS *B. juncea* lines or perhaps by transgenic CMS mechanisms (e.g. Mariani et al. 1990).

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