



Stable *Bacillus thuringiensis* transgene introgression from *Brassica napus* to wild mustard *B. juncea*



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ABSTRACT

Transgenic canola (*Brassica napus*) with a *Bacillus thuringiensis cry1Ac* gene and a green fluorescent protein (*GFP*) marker gene was used in hybridization experiments with wild *Brassica juncea*. Hybrid F₁ and successive five backcross generations were obtained.

The pod-set frequency on backcrossed *B. juncea* plants was over 66%, which suggested relatively high crossing compatibility between the hybrids and wild species. The seed setting in BC₁ was the least of all generations tested, and then increased at the BC₂ generation for which the thousand-seed weight was the highest of all generations. Seed size in backcrossed generations eventually approached that of the wild parent. The plants in all backcrossed generations were consistent with the expected 1:1 segregation ratio of the transgenes. The Bt Cry1Ac protein concentrations at bolting and flowering stages was higher compared to the 4–5-leaf and pod-formation stages. Nonetheless, the Bt toxin in the fifth backcrossing generation (BC₅) was sufficient to kill both polyphagous (*Helicoverpa armigera*) and oligophagous (*Plutella xylostella*) Lepidoptera.

As a consequence, the subsequent generations harboring the transgene from F₁ to BC₅ could have selection advantage against insect pests. The result is useful in understanding gene flow from transgenic crops and the followed transgene introgression into wild.

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1. Introduction

Gene flow from transgenic plants to wild closely related species has raised concern recently. Since transgenic crops were released in 1996, the global area of transgenic crops has been increasing rapidly, over 90% of which are cultivated in developing countries including China [1]. The transgene introgression from transgenic crops to their wild relatives is unavoidable in some species. Transgene introgression is of concern because the crop–wild plant hybrids might be conferred with a selection advantage to increase their performance, which could result in negative ecological consequences to natural ecosystems [2–4]. Gene flow or gene introgression is a natural process, however, transgenic plants add complexity of introgression of potentially novel traits [5], thus its process and consequence should be closely investigated. For annual crops and wild plants, it might take several years before the

transgene would be established in a wild plant population. A herbicide resistant gene had persisted over 6-year in the absence of herbicide selection pressure [6]. The hybrids F₁ and involved backcrossing generations BC₁, BC₂, etc. can exchange genes with wild species which allowed the transgene coexist last for a long time [7].

The genus *Brassica* has 159 species, including a number of wild species that are of great importance to the economy [8]. Most transgenic *Brassica* gene flow research has focused on the most successful cross between transgenic oilseed rape *Brassica napus* (2n = 38, genomic composition AACC) and its wild relatives *Brassica rapa* (2n = 20, AA), a widely distributed weed in the farming system in Europe and America, since the hybridization can spontaneously happen and the generations can backcross to *B. rapa* easily in the wild conditions [3,9–11]. *B. napus* can outcross with related species and pollen can disperse over long distances by wind and insects [12]. However, limited information is known about the consequence of introgression between *B. napus* and *B. juncea*, while their relatively high hybridization rate ranks immediately after the one between *B. napus* and *B. rapa* [2]. Wild *B. juncea* (2n = 36, AABB) is a weedy species widely found along the roadsides or abandoned lots in China that has spread eastwards along the Yangtze River.

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The weed is a vigorous competitor for water, light and nutrients, thus affecting crop production [13]. The flowering phenology of *B. juncea* overlaps that of *B. napus* [14]. Sexual compatibility is of great importance affecting the probability of hybridization occurring, but the fitness of crop–wild hybrids defines the persistent existence of the transgenic hybrids [15]. There is urgent need to investigate the hybridization between the two species and the transgene introgression in their successive backcrossing generations.

Transgenic oilseed rape *B. napus* was produced to harbor a green fluorescent protein (GFP) gene and a *Bacillus thuringiensis* insecticidal crystal endotoxin-coding gene (*Bt Cry1Ac*), each under the control of separate cauliflower mosaic virus 35S promoters [5]. Green fluorescent protein (GFP) is a useful real-time indicator of gene flow and can be assayed without damaging plants and could facilitate the monitoring of transgene movement and transgenic plants in the field [16,17]. *Bacillus thuringiensis* Cry1Ac protein is toxic to certain caterpillar species. Although *Bt Brassica* crop plants have not been commercially released, the crop and its wild relatives have become a very useful model to assess the ecological risks of *Bt* crops as an experimental system for risk assessment [13,18]. To assess whether the transgene can be transferred into the wild weeds, the hybridization frequency between two related species, the survival rate and the reproduction frequency of the subsequent generations should be also taken into account [9].

In this study, we aimed to characterize transgene introgression, segregation, and expression in backcrossed generations. The insecticidal efficacy against two herbivore species was studied. These results will contribute to the environmental risk assessment and assist in biosafety management.

2. Materials and methods

2.1. Plant materials

Ten plant types were included: *B. napus* ‘Westar’ and its transgenic line that was transformed with GFP and *Bacillus thuringiensis* crystal endotoxin transgene regulated by CaMV 35S promoters (labeled as GT1) [5], wild brown mustard (*B. juncea*), their hybrids (F_1) formed on *B. juncea* with transgenic pollen from GT1, first backcross generation (BC_1) that was formed by pollinating of wild *B. juncea* with pollen of transgenic hybrids, second backcross generation (BC_2), third backcross generation (BC_3), fourth backcross generation (BC_4) and fifth backcross generation (BC_5) (Fig. 1). All backcrossed generations were generated using wild mustard as the maternal parent.

The study was conducted from 2006 to 2013. GT1 was one of the nine transgenic events of GFP/*Bt B. napus* produced by Halfhill et al. [5]. Wild brown mustard accessions were provided by Nanjing Agriculture University [17]. First hybrid generation (F_1) was obtained by hand-pollination, with *B. juncea* as the maternal parent

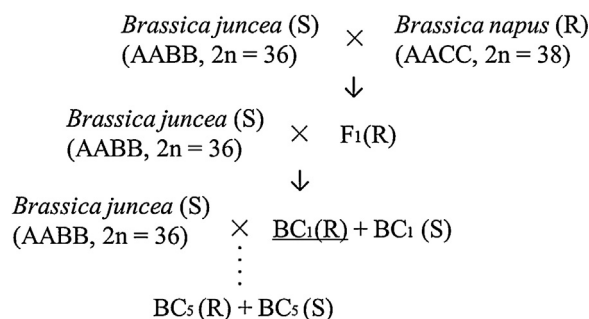


Fig. 1. Crossing scheme. *Bacillus thuringiensis* (*Bt*)-transgenic inter-specific hybrid plants used as paternal parents were backcrossed to *Brassica juncea*. R and S represent resistant and susceptible plants.

and *B. napus* as the paternal parent. All F_1 plants were transgenic as determined by a GFP fluorescence meter (Opti-Science, Inc., Hudson, NH, USA) [17,19]. The F_1 plants served as pollen donors and were backcrossed with *B. juncea* to generate the BC_1 generation. The BC_1 plants passing the GFP fluorescence screen were used as pollen donors and backcrossed to *B. juncea* to produce BC_2 . Another three rounds of backcrosses were made producing successive BC_3 to BC_5 generations for further study. The plants were kept in the greenhouse at 15–23 °C. All crosses were conducted by hand pollination in separate greenhouses, in which pollen contamination via insects and air flow was prevented. Female flowers were emasculated before anthesis and pollinated by the paternal pollen. More than 30 flowers on each maternal plant were emasculated and pollinated. At least 10 plants of each donor and receipt plants were used in each crossing or backcrossing. Seeds harvested from the maternal plants were stored in separate envelopes at 4 °C until use. The same generation of seeds was evenly mixed before sowing to generate the next backcross generation.

At the end of each growth cycle, the number of pods set on each maternal plant was recorded, seed number per pod and thousand-seed weight were also calculated. We defined the pod setting rate as the percentage of pollinated flowers developing into pods on one plant [14]. The selfed *B. juncea* were used as the controls. The pollination experiments were conducted from 2009 to 2012. As the number of pollinated flowers per maternal plant varied during the hand-crossing, total seed production per plant was not reported here.

2.2. Segregation ratio

The GFP screen is a convenient and accurate tool for in vivo *Bt* transgene monitoring [17,19]. A GFP fluorescence meter (Opti-Science Inc.) was used to screen the presence of transgenes. The upper most expanded plant leaves were screened for GFP fluorescence and repeated three times at the 4–5 leaf stage at four weeks after seed germination. Transgenic plants were selected by GFP fluorescence among seedlings originating from seeds harvested from *B. juncea* that were used for hybridization and backcrossing (Fig. 1). Both hybrids and backcrossed plants were screened for the presence of transgene for three times during the years of 2012 and 2013. At least 50 seedlings were screened each time. The presence of transgenes was confirmed by PCR with specific primers for GFP and *Bt* transgenes [5] [Supplementary Information, Fig. S1].

2.3. ELISA test of *Bt* protein concentration

Seeds for the seven plant types (GT1, F_1 , BC_1 to BC_5) were sown in March, 2012 in an experimental field of the Institute of Botany, Chinese Academy of Sciences, Beijing, China (E116°12', N39°59'). Distance between plants was kept at 45 cm to avoid competition during the growth season. Eight leaf samples of GFP fluorescence positive plants were randomly taken from the seven plants types at each four development stages: four-five-leaf stage, bolting stage, flowering stage and pod formation stage, respectively. *Bt Cry1Ac* protein concentration of hybrid and backcross generations of transgenic oilseed rape was obtained using Agdia ELISA kits (Elkhart, IN, USA).

The maternal *B. juncea* was used as the negative control and GT1 served as the positive control. Fresh leaves were harvested into a centrifuge tube and homogenized in 400 μ L PBST buffer, then centrifuged for 10 min at 18,000 \times g at 4 °C. The supernatant of approximately 300 μ L was transferred into a new tube, which was kept at 4 °C for further analysis. The concentrations were expressed as ng g⁻¹ leaf fresh weight (FW). The samples were screened with a 620 nm filter and absorbency (OD) was recorded with a Labsystems Multiskan RCmicroplate reader (Labsystems, Helsinki, Finland).

By measuring the optical density values of the Bt Cry1Ac protein standards in the ELISA kits and the samples, Bt protein concentration of plants were calculated using Genesis software (Labsystems). The concentrations were recorded as nanograms per gram fresh leaf weight (FW) and were used to compare the differences among plant types at the four growing stages. Each sample was tested at two concentrations to prevent dilution effects on the quantification and the results were averaged.

2.4. Insect experiments

Five plant types were included in bioassays: non-transgenic oilseed rape (Westar), transgenic GT1, wild brown mustard *B. juncea*, resistant backcross BC₅ and susceptible non-transgenic BC₅ plants. The Lepidopteran larvae of diamondback moth (*Plutella xylostella*) and cotton bollworm (*Helicoverpa armigera*) were employed; the biotypes were susceptible to Bt Cry1Ac protein. Insect eggs were kindly provided by Prof. Qin Qi-lian from the Institute of Zoology, Chinese Academy of Sciences and hatched in plastic cylinders (10 cm diameter, 10 cm height) in the insectary of the Institute of Botany, Chinese Academy of Sciences. The hatched larvae were fed with leaf lettuce under controlled temperature conditions at 25 ± 2 °C with a light circle of 16:8 h (16 h light, 8 h dark). After a week when the larvae were at second-instar, each of the five plant types was assayed with 10 repeats for each of the two insect species. For this purpose, 10 larvae of the same size of each insect species were picked with a hair bush and transferred into each of 10 new plastic cylinders cultured with leaves from the assayed plant type for seven days. The leaves were taken from healthy individuals of the five plant types at the 6–7-leaf stage and replaced daily. GFP fluorescence was measured to ensure the presence of transgenes in the leaves that were fed to larvae. In this GFP fluorescence measurement, *B. napus* 'Westar' was used as negative control. The result showed that the resistant backcross BC₅ plants had similar GFP fluorescence to transgenic GT1 while the wild *B. juncea* and susceptible non-transgenic BC₅ plants had slight GFP fluorescence [Supplementary Information, Table S1]. Insect survival was recorded every 24 h.

2.5. Statistical analysis

Analysis of variance (ANOVA) was used to compare the pod-set rate, seed number per pod and thousand-seed weight in each backcross generation using SPSS 17.0 software (SPSS Inc., 2009). Statistical significance level was set at $P < 0.05$. The comparison of means was conducted using the Duncan's multiple range test. The Chi-squared test was performed to test if the segregation rate of transgene introgression in hybrids and the backcrosses from BC₁ to BC₅ followed the expected 1:1 ratio. Confidence limits were set at 95%. ANOVA was also used to study the difference of Bt Cry1Ac protein concentration among plant generations at those four growth stages in the field. The survival rate of larvae fed with different leaf samples were analyzed using one-way ANOVA in the SPSS system.

3. Results

3.1. Pod and seed production for hybrids and backcrossed generations

The pod-set frequency of backcross generations ranged from 66% to 89%, in which there were significant differences among generations ($F_{6,195} = 7.262, P < 0.01$). The results showed a high pod-set rate in hybrid F₁, whereas the lowest rate was found at the BC₁ generation. The pod and seed apparently recovered at the BC₂ generation (Table 1). The seed number per pod ($F_{6,181} = 75.609, P < 0.01$) data had the same trend as the pod-set data, again, with the introgression bottleneck at the BC₁ generation. In contrast, the thousand-seed weight data ($F_{6,88} = 60.734, P < 0.01$) showed the reverse pattern. F₁ seeds were smallest whereas BC₂ seeds were the heaviest. It was found that the thousand-seed weight increased from BC₁ to BC₃ and decreased from BC₄ and changed to similar weight to the seeds of wild maternal plants.

3.2. Segregation ratio and Bt Cry1Ac protein in hybrid and backcrossed progenies

As expected, all the generations could successfully harbor the transgenes (Table 2). The segregation between the presence and absence of GFP fluorescence in the backcrossed progenies did not deviate from the ratio of 1:1. As a whole, the result suggested the introgression of Bt gene into the wild *B. juncea* genome followed the Mendelian genetic model.

Bt Cry1Ac protein (ng g⁻¹ FW) showed significant differences among the four growth stages ($F_{3,228} = 185.236, P < 0.01$) and the seven generations ($F_{6,228} = 12.379, P < 0.01$; Fig. 2). No significant interaction was found between growth stage and plant generation. At 4–5-leaf stage, the Bt concentration in BC₄ was highest, whereas the differences between generations were not significant [Supplementary Information, Table S2]. Among all four stages in all GFP fluorescence positive plants, Bt Cry1Ac protein concentration per unit leaf fresh weight (FW) at bolting stage was highest, while the level of Bt Cry1Ac protein ranked second at the flowering stage and followed by the 4–5-leaf stage and the pod formation stage, respectively, except in the BC₁ generation, where the Bt concentration at flowering stage was higher than that of the bolting stage and in BC₂, where no difference was found between the two stages. The Bt protein synthesis level in GT1 at bolting and flowering stage were all higher than others. A gradual upward trend was found at the pod formation stage among various generations, in which BC₄ and BC₅ had the higher level of Bt Cry1Ac than others. The highest value of Bt concentration decreased from GT1 to BC₅ generations whereas the lowest value showed a rising trend (Fig. 2). The variation in Bt Cry1Ac protein concentration among various growth stages decreased from GT1 to its introgressed progenies and reached stable transgene expression in BC₅ generation. The negative control (wild brown mustard) had no measurable Bt protein in any growth stage.

Table 1
Characteristics (mean ± SE) of seed production set on the maternal wild *Brassica juncea* during hybridization and backcrossing.

Seed type	No. of plants	Pod-set frequency	Seed number per pod	Thousand-seed weight
F ₁	23	89.0 ± 3.4 ^a	9.8 ± 0.5 ^b	0.97 ± 0.03 ^c
BC ₁	43	66.1 ± 2.5 ^c	2.8 ± 0.2 ^d	1.17 ± 0.07 ^b
BC ₂	37	68.6 ± 2.1 ^{bc}	6.7 ± 0.4 ^c	1.86 ± 0.03 ^a
BC ₃	51	70.4 ± 2.6 ^{bc}	9.7 ± 0.4 ^b	1.31 ± 0.04 ^b
BC ₄	22	79.8 ± 2.5 ^{ab}	10.7 ± 0.4 ^b	0.89 ± 0.08 ^{cd}
BC ₅	16	69.8 ± 3.3 ^{bc}	7.4 ± 0.5 ^c	0.72 ± 0.02 ^d
<i>B. juncea</i>	4	82.7 ± 2.5 ^a	20 ± 1.1 ^a	0.77 ± 0.03 ^d

Significant differences between seed types are indicated by different letters (Duncan's multiple range test, $P < 0.05$).

Table 2
Segregated ratio in hybrid and backcrossed generations.

Plant generation	Expected ratio	Replicate 1		Replicate 2		Replicate 3		Overall
		R:S	χ^2	R:S	χ^2	R:S	χ^2	
F ₁	1:0	86:0	NS	101:0	NS	103:0	NS	NS
BC ₁	1:1	58:52	0.227 ^{NS}	46:48	0.09 ^{NS}	47:41	0.28 ^{NS}	0.27 ^{NS}
BC ₂	1:1	28:31	0.27 ^{NS}	65:70	0.26 ^{NS}	66:71	0.26 ^{NS}	0.59 ^{NS}
BC ₃	1:1	57:49	0.46 ^{NS}	59:40	3.27 ^{NS}	30:25	0.29 ^{NS}	3.69 ^{NS}
BC ₄	1:1	66:73	0.46 ^{NS}	50:45	0.16 ^{NS}	148:154	0.16 ^{NS}	0.15 ^{NS}
BC ₅	1:1	64:69	0.27 ^{NS}	143:147	0.08 ^{NS}	513:484	0.78 ^{NS}	0.25 ^{NS}

NS, not significant, $P > 0.05$.

R and S represent resistant and susceptible plants, respectively.

3.3. Effects of backcrossed generation on insects

The survival rate of second instar larvae of cotton bollworm that fed on the five plant types differed significantly ($F_{4,350} = 590.996$, $P < 0.01$) during the seven-day bioassay. The larvae that fed on transgenic *B. napus* GT1 and transgenic BC₅ died quickly (at day 3) and exhibited no differences after the third day. The results clearly showed that the non-transgenic plants without the Bt gene were not toxic to the insects. The survival rate of insects that fed on non-transgenic BC₅ was not different from the non-transgenic *B. napus* 'Westar' and wild *B. juncea*, yet the other two plant types GT1 and transgenic BC₅ all reduced the survival of caterpillars significantly during the bioassay experiment.

Diamondback moth larvae that fed on five plant types also had various survival rates ($F_{4,245} = 327.967$, $P < 0.01$). Transgenic GT1 and BC₅ plants also had similar effects on diamondback moth larvae as for cotton bollworm larvae and the survival rate of insects decreased rapidly after the first three days because of the highly lethal toxicity (Fig. 3B). The larvae that fed on non-transgenic *B. napus* 'Westar' and wild brown mustard *B. juncea* presented similar trend of survival, while the survival rate on GT1, transgenic

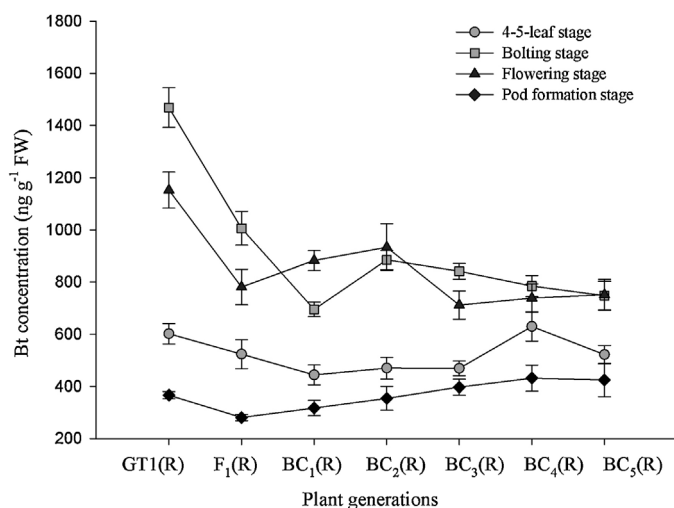


Fig. 2. Dynamics of *Bacillus thuringiensis* Cry1Ac protein concentration (ng g^{-1} FW) at four developmental stages in transgenic oilseed rape *Brassica napus* (GT1), hybrid (F₁) (R) and the first backcross (BC₁) (R) to the fifth backcross (BC₅) (R) generations. FW: abbreviation for fresh weight of leaf. F₁(R) and BC₁(R) to BC₅(R) are GFP fluorescent positive plants that screened by a GFP meter and confirmed by DNA polymerase chain reaction (PCR) using transgenes specific primers [Supplementary Information, Fig. S1]. Four developmental stages include the four-five-leaf stage, the bolting stage, the flowering stage and the pod formation stage. The difference of Bt Cry 1Ac protein is significant among various generations and between different growth stages (Duncan's multiple range test, $P < 0.05$) [Supplementary Information, Table S2].

and non-transgenic BC₅ generations differed from each other significantly. The results indicated that the Bt Cry1Ac protein in the BC₅ generation was able to kill insects within three or four days. The transgenic BC₅ plants that inherited the *Bt Cry1Ac* transgene were insecticidal to the insects at the same level as the transgenic paternal GT1 plants. Non-transgenic BC₅ progenies were not toxic to the insects, which was similar to wild brown mustard and the non-transgenic *B. napus* 'Westar'.

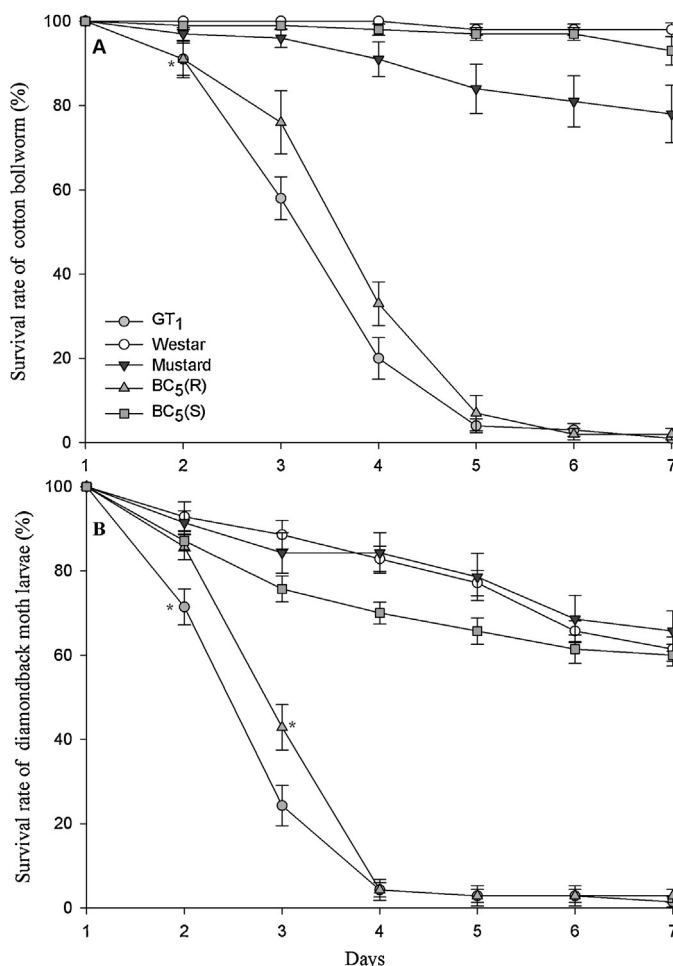


Fig. 3. Survival rate of insects fed on different plant types through one week feeding. Survival rate of cotton bollworm (*Helicoverpa armigera*) (A) and diamondback moth larvae (*Plutella xylostella*) (B) fed on five plant types: transgenic *Brassica napus* GT1, non-transgenic *B. napus* Westar, wild *B. juncea*, transgenic backcross BC₅ and non-transgenic backcross BC₅. The asterisk (*) represents the initial date when survival of caterpillars between plant types became significant (Duncan's multiple range test, $P < 0.05$).

4. Discussion

Gene flow from transgenic plants to wild species is an inevitable phenomenon that mainly depends on the fitness of first and later generation backcrosses, which may enable transgene introgression and full expression of the transgenic trait [9,20]. *B. juncea* seemed to function most often as maternal plants in crosses with *B. napus* [14]. Metz et al. (1997) suggested that there would be higher seed yield on hybrids formed between *B. napus* and *B. rapa* backcrossed with the maternal plants *B. rapa* as the female parents than selfing of inter-specific hybrids. We report here the seed characters in hybrids formed between transgenic *B. napus* and wild *B. juncea* and successive backcrossed progenies with maternal plants.

Both the pod-set rate and the number of seeds per pod in BC₁ were all the lowest among previous studied transgenic generations, which is probably the result of low F₁ pollen fertility [9,21,22] and consistent with the results in previous reports [15,23]. These two characteristics increased from BC₂, which could suggest the increased pollen fertility from BC₁ [24]. The seed number per pod could be associated with pod length, which depends to a great extent on the genetic background [25], however, seed number per pod increased from F₁ until a significant decrease in BC₅. Those changes in seed characteristics could result from the interaction between paternal and maternal plant genomes or variation between years when backcrossed generations were obtained in different years. Thousand-seed weight could be affected by environmental factors such as plant density, source capacity and the supply of nutrients and would not be exclusively inherited from the parental plants [26]. However, as generations passed, seed size approached that of its wild parent at BC₅ generation, which is consistent with the similarity in other morphology, e.g. leaf shape, plant size, seed color, etc. [Supplementary Information, Figs. S2 and S3]. Further study should investigate the similarity in their genetic components as well as their field performance. The transgene may spread by introgression from crops into the wild species via pollen [14]. However, the limited pod setting rate, lower seeds per plant as well per pod in BC₁ could be a major limitation for the gene flow to the wild species that might slow down the process of transgene dispersal.

For transgenic Bt/GFP *B. napus* and its hybrid progeny with wild *B. juncea*, a close correlation was observed between the expression of the Bt insecticidal toxin and GFP fluorescence in plants [19]. GFP-positive backcrossed progenies were insecticidal in the bioassays in the current study system, which contained both Bt and GFP transgenes. With the aid of presence of GFP fluorescence, the segregation of transgene in backcrossed progenies followed an expected ratio of 1:1. Metz et al. posited that transgene located on chromosomes of the C-genome would reduce the chance of gene transfer from *B. napus* to *B. rapa* [27]. It was suggested that the introgression of the transgene might be unpredictable for the transgenic events as random recombination between A and C chromosomes after inter-genomic chromosomal crossover could occurred [28]. However, the segregation ratio 1:1 confirmed the transgene is likely located on the A-genome rather than on the C-genome in the GT1 event. The result showed that the transgene in GT1 could be easily transferred and stably inherited in subsequent generations.

Our result confirmed the trend of Bt *Cry1Ac* expression that increased during the vegetative growth stages until bolting and flowering stages and decreased at the pod formation stage [18]. The decreased Bt concentration of leaves in the pod formation could be the result of leaf senescence and decreased synthesis of total protein [29]. At bolting stage, the dynamic trend of Bt concentration from F₁ to BC₃ is consistent with Zhu et al. (2004) who used the GT1 and *B. rapa* as parental plants to obtain the successive backcross generations [30]. Lower variation in the Bt protein

expression was found among various growth stages in BC₅, which suggested that the transgenes could persist and be stably expressed in the backcross generations.

Ours is the first report of Bt-transgene-mediated insect resistance in an advanced (BC₅) generation of introgressed transgenes in a wild plant genetic background. Diamondback moth (*P. xylostella*) is the pest specific to a number of cruciferous plants and may cause economic damage around the world [31]. Cotton bollworm (*H. armigera*) is one of the most serious and common insect pests of cotton, vegetables and other crops in Asia [32]. Insecticidal rape-seed transformed with a Bt transgene was shown to be insecticidal to lepidopterans including the diamondback moth *Plutella xylostella* [29] and the cotton earworm [18]. Tang et al. observed that the survival of 2nd instar of *P. xylostella* that fed on transgenic broccoli expressing *Cry1Ac* toxin of *Bacillus thuringiensis* exhibited a significant decline within 72 h feeding [33]. The lowest *Cry1Ac* level in transgenic Bt *B. napus* was enough to result in a high mortality of the susceptible *P. xylostella* larvae in three days of feeding [29]. Within the seven days feeding in our study, the survivorship of insects feeding on the plants with Bt transgene decreased significantly, while survivorship on other three plant types without the transgenes (wild *B. juncea*, Westar and non-transgenic BC₅) seemed to be unaffected. The same generations (transgenic BC₅ and non-transgenic BC₅) revealed contrasted effects on the insects due to the segregation of Bt transgene when crossing with wild *B. juncea*. The mortality of cotton bollworm larvae feeding on the wild *B. juncea* was different than the non-transgenic *B. napus* 'Westar' plant, which could be due to that *H. armigera* is not accustomed to digest the metabolic substance of the wild crucifers although it is an euryphagous herbivore that could feed on various plant species among different families [34]. In contrast, diamondback moth is an oligophagous insect that specializes on feeding on a few cruciferous plant species such as members of the genus *Brassica*; no difference was found between the mustard *B. juncea* and the non-transgenic Westar.

The transgenic *B. napus* GT1, which were homozygous, had higher Bt gene expression and toxicity to insects compared with backcrossed hybrids, which were hemizygous for the transgenes. Since transgene expression is additive in this system [35], these results are not surprising. Nonetheless, the transgenes were passed on to advanced backcross generations that were lethal to insects. Once F₁ hybrids and backcrossed generations are formed, together with Bt-oilseed rape, they might increase the exposure of target and non-target insects, which might increase the risk of resistance evolving in insects. The Bt transgene introgression mediated by pollen from transgenic plants to non-transgenic plants could be detrimental in terms of insect control both for targets and non-targets [36].

Using hybrid and backcrosses between *B. napus* and *B. juncea* as a model system, our study suggested that transgene Bt could persist and be stably expressed in the wild conditions once the transgenic oilseed rape were released, especially in the areas where wild *B. juncea* simultaneously occurs. Risk assessment should be conducted and aspects of ecological safety should be evaluated before deliberate release of transgenic insect resistant plants into environment, taking into consideration the possibility of transgene introgression and its effect on target and non-target organisms.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.plantsci.2014.06.018>.

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