Theor Appl Genet (2010) 120:1501–1510
DOI 10.1007/s00122-010-1271-3

ORIGINAL PAPER

Potential gene flow of two herbicide-tolerant transgenes from oilseed rape to wild *B. juncea* var. *gracilis*

Xiaoling Song · Zhou Wang · Jiao Zuo · Chaohe Huangfu · Sheng Qiang

Received: 19 December 2007 / Accepted: 19 January 2010 / Published online: 12 February 2010

Abstract Four successive reciprocal backcrosses between F_1 (obtained from wild *Brassica juncea* as maternal plants and transgenic glyphosate- or glufosinate-tolerant oilseed rape, *B. napus*, as paternal plants) or subsequent herbicide-tolerant backcross progenies and wild *B. juncea* were achieved by hand pollination to assess potential transgene flow. The third and forth reciprocal backcrosses produced a number of seeds per silique similar to that of self-pollinated wild *B. juncea*, except in plants with glufosinate-tolerant backcross progeny used as maternal plants and wild *B. juncea* as paternal plants, which produced fewer seeds per silique than did self-pollinated wild *B. juncea*. Germination percentages of reciprocal backcross progenies were high and equivalent to those of wild *B. juncea*. The herbicide-tolerant first reciprocal backcross progenies produced fewer siliques per plant than did wild *B. juncea*, but the herbicide-tolerant second or third reciprocal backcross progenies did not differ from the wild *B. juncea* in siliques per plant. The herbicide-tolerant second and third reciprocal backcross progenies produced an amount of seeds per silique similar to that of wild *B. juncea* except for with the glufosinate-tolerant first and second backcross progeny used as maternal plants and wild *B. juncea* as paternal plants. In the presence of herbicide selection pressure, inheritance of the glyphosate-tolerant transgene was stable across the second and third backcross generation, whereas the glufosinate-tolerant transgene was maintained, despite a lack of stabilized introgression. The occurrence of fertile, transgenic weed-like plants after only three crosses (F_1, first backcross, second backcross) suggests a potential rapid spread of transgenes from oilseed rape into its wild relative wild *B. juncea*. Transgene flow from glyphosate-tolerant oilseed rape might be easier than that from glufosinate-tolerant oilseed rape to wild *B. juncea*. The original insertion site of the transgene could affect introgression.

Communicated by A. Bervillé.

X. Song · Z. Wang · J. Zuo · C. Huangfu · S. Qiang (✉)
Weed Research Laboratory,
Nanjing Agricultural University, Nanjing 210095, China
e-mail: wrl@njau.edu.cn

Introduction

One of the concerns about releasing genetically modified herbicide-tolerant (GMHT) crops is that herbicide-tolerant (HT) transgenes may escape from GMHT crops to their weedy relatives through pollen flow. If this happens, weeds with the HT trait might produce new problems for weed control (Dale 1992; Kling 1996; Chèvre et al. 1997; Chapman and Burke 2006). Therefore, predicting potential gene flow from GMHT crop to weed species before GMHT crops are released into the environment is necessary.

Many researchers have reported that transgenic oilseed rape may hybridize with many of its related wild plants spontaneously (Jørgensen and Anderson 1994, Jørgensen et al. 1996; Bing et al. 1996; Darmency and Fleury 2000; Rieger et al. 2001; Warwich et al. 2003; Pu et al. 2005; Song et al. 2007) or by hand pollination (Lefol et al. 1997; Metz et al. 1997). In some cases, special techniques, such as embryo rescues or flower culture are required to produce the hybrids (Kerlan et al. 1992; Metz et al. 1995). Furthermore, gene exchange could occur between *Brassica napus* and *B. rapa* (Hauser et al. 1998; Snow et al. 1999; Hansen et al. 2001, 2003; Ammitzbøll et al. 2005).

In China, more than 0.6 million tons of transgenic oilseed rape seeds are imported for edible oils every year, account for 60% of the total imported oilseed rape seeds.

The seeds may escape along transportation routes (Lu et al. 2005; Yoshimura et al. 2006; Aono et al. 2006; Kawata et al. 2009). Furthermore, transgenic oilseed rape has been under development locally and some varieties could be released within the next few years if they are approved by Chinese regulatory agencies (Guan and Li 1997; Pu et al. 2003; Wang et al. 2005; Guan 2005). Therefore, assessing the potential risks of gene flow from GMHT oilseed rape to weed species in China is needed.

Mustard [*Brassica juncea* (L.)] is cultivated in Asia, the United States and Canada, especially for oil and mustard production. Wild *B. juncea* is found as a weed or ruderal in fields, empty lots and roadsides in China and has become a major weed of cropping systems across western China, where it is a vigorous competitor for water, light and nutrients. It seriously infests wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), oilseed rape (*Brassica napus* L.), horse bean (*Vicia faba* L.), pea (*Pisum sativum* L.) and some autumn crops (Guan 1996; Guo et al. 1998; Pu et al. 2003). In recent years, wild *B. juncea* has extended eastward along the Changjiang River valley. The flowering time of wild *B. juncea* overlaps for a long period with that of oilseed rape (Pu 2003; Lu et al. 2005); thus gene flow from GMHT oilseed rape to wild *B. juncea* may pose an agricultural risk in China.

Pollen-mediated gene flow depends on sexual compatibility, sympatry, flowering synchrony between donor and recipient plants and pollen transport. The extent of sexual compatibility between pairs of overlapping species is an important factor that controls the natural hybridization between species (Dale 1992; Scheffler and Dale 1994; Lègère 2005; Chapman and Burke 2006; FitzJohn et al. 2007). Oilseed rape (AACC) and wild *B. juncea* (AABB) share the A-genome. Because Roy (1984) crossed *B. napus* × *B. juncea* to create a recombinant line, carrying a *B. juncea* blackleg resistance gene for the first time, spontaneous hybridization of conventional and GMHT oilseed rape with *B. juncea* has been reported (Bing et al. 1991; Frello et al. 1995; Bing et al. 1996; Jørgensen et al. 1998; Pu et al. 2005; Song et al. 2007). F_1 hybrids have been produced easily by hand-crossing wild *B. juncea* as maternal plants and conventional or GMHT oilseed rape as paternal plants (Song and Qiang 2003; Song et al.2007). The HT transgene is transmitted relatively easily to the F_1 hybrids and retains activity when GMHT oilseed rape is used as paternal plants. However, the sexual fertility of the F_1 hybrid with the transgene is low (Song et al. 2007).

Several subsequent steps must be completed for efficient gene flow: (1) production of fertile and fit offspring from the hybrids (2) gene transmission during successive generations, (3) effective gene introgression through recombination between genomes and (4) maintenance of the transgene in the natural populations (Chèvre et al. 2007). Until now, few studies have considered these subsequent steps for gene flow between transgenic oilseed rape and *B. juncea*. In our study, we crossed transgenic glyphosate- and glufosinate-tolerant *B. napus* plants with wild *B. juncea* under controlled conditions to investigate whether the HT backcross progenies could be obtained and whether the HT backcross progenies could be as fit as wild *B. juncea* in terms of germination, growth and fertility under optimal conditions.

We measured (1) seeds per silique and the percentage of pollinated flowers developing into siliques by backcrossing wild *B. juncea* and HT F_1 or subsequent backcross progeny; (2) germination percentage, siliques per plant and seeds per silique of backcrossed progeny under self-pollination as a measure of fitness; and (3) ratio of herbicide tolerance and sensitivity in different backcross progeny. We aimed to determine (1) whether the transgene for HT from *B. napus* could be transmitted and incorporated into the genome of wild *B. juncea* by means of backcrossing and selection; and (2) the relative fitness of the tolerant backcross progenies when compared with wild *B. juncea* in terms of germination, siliques per plant and seeds per silique. Although our study did not investigate the evolution of hybrid genome structures during the successive generations that follow the formation of interspecific *B. juncea* × *B. napus*, the results may be helpful in assessing potential introgression from GMHT oilseed rape to wild *B. juncea.*

Materials and methods

Materials

The glyphosate-tolerant oilseed rape (DS-Roughrider, Roundup Ready, event RT73) and glufosinate-tolerant oilseed rape (Swallow, Liberty Link, event HCN92) (*Brassica napus* L.; genome, AACC; diploid chromosome number, 2n = 38) were obtained from Canada. RT 73 and HCN92 were produced from spring-type variety *B. napus* cv. Westar and *B. napus* cv. Topas, respectively. RT 73 was homozygous for a single DNA insertion consisting of the T-DNA containing one complete copy of the *cp4 epsps* gene and a complete copy of the *gox* gene and their respective regulatory sequences. HCN92 was homozygous for one insertion site and contained two linked copies of the *pat* gene (http://www.agbios.com/dbase.php). For both events, whether the transgene locus was located on chromosomes of A- or of the C-genome was not known. Seeds of wild *B. juncea* were collected from Jiangpu, Nanjing City, China.

F_1 interspecific hybrids were obtained by hand pollination, with wild *B. juncea* (genome, AABB; diploid chromosome number, $2n = 36$) used as the maternal plants and

glyphosate- or glufosinate-tolerant oilseed rape as the paternal plants in 2002 (Song et al. 2007).

The percentage of pollinated flowers developing into silique and seeds per silique by backcrosses

The study was conducted from 2003 to 2006. A schematic presentation of the crosses is given in Fig. 1, whereby *m* and *p* denote backcross (BC) progeny obtained with wild *B. juncea* used as maternal or paternal plants, respectively. *R* and *L* denote transgenic oilseed rape, F_1 hybrids and BC progeny with glyphosate- or glufosinate-tolerant genes, respectively.

Backcrosses were performed by hand pollination from March to May in separate greenhouses to prevent any pollen spread by insects and air flow. All flowers used as maternal plants were bagged for 3 days before pollination, and were wiped gently with small cotton balls soaked in 70% (v/v) ethanol to prevent uncontrolled cross-pollination before hand pollination. Mature flower buds of maternal plants were emasculated before anthesis and pollinated by rubbing a couple of anthers of paternal plants on the stigma surface. Pollen donors were randomly assigned to maternal plants. Surplus flowers on maternal plants were removed. Flowers were enclosed in pollination bags immediately after hand pollination. All crossed plants were placed in the greenhouse in a random manner and watered as needed. A minimum of 10 maternal plants and 10 paternal plants were used for each replicate, with four replicates for each backcross. More than 10 flowers were crossed per maternal plant, and the number of pollinated flowers per maternal plants was recorded. Self-pollination of wild *B. juncea* by hand served as a control. Siliques were carefully collected at maturity from maternal plants. The number of siliques and the fully developed seeds per silique were counted. The percentage of pollinated flowers developing into silique and seeds per silique were calculated as the number of siliques/pollinated flowers and the number of full seeds/the number of siliques.

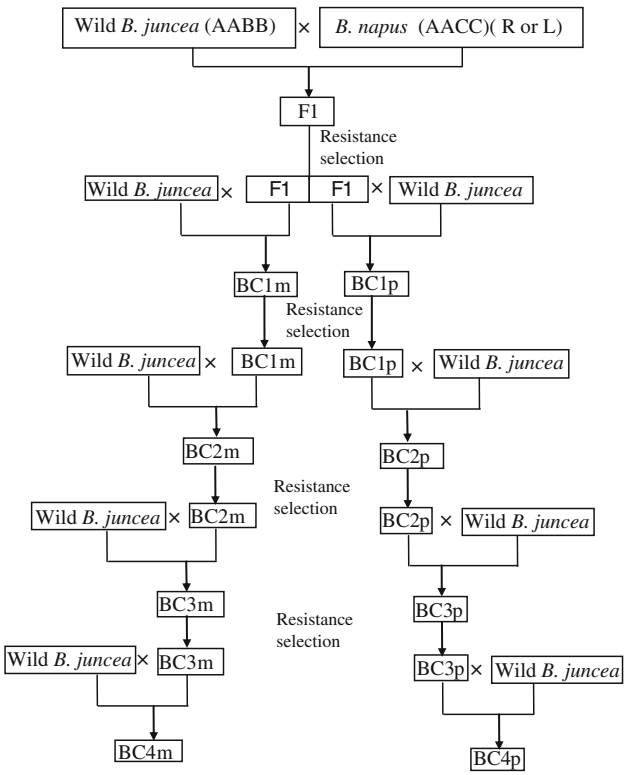

Fig. 1 Crossing scheme of hybridization and backcrossing between wild *Brassica juncea* and transgenic herbicide-tolerant oilseed rape. Combinations involved in this study are indicated as maternal plants × paternal plants. Populations in front of × are always maternal plants, and populations in the back of × are always paternal plants. *m* backcross progeny obtained with wild *B. juncea* as maternal plants. *p* Backcross progeny obtained with wild *B. juncea* as paternal plants, *R* transgenic oilseed rape, F_1 and backcross progeny with glyphosate-tolerant gene, *L* transgenic oilseed rape, F_1 and backcross progeny with glufosinate-tolerant gene, F_1 denotes hybrids between wild *B. juncea* as maternal plants and glyphosate- or glufosinate- tolerant oilseed rape as paternal plants. *BC* backcross generation

Selection for tolerance to herbicide, growth and siliques per plant, seeds per silique of backcross progeny under self-pollination

All harvested seeds were cleaned and stored at 4°C until use. The BC progeny seeds from the same parent were mixed and then sown directly in plastic pots (6-cm diameter) containing growth media (mixture of garden soil and peat at 1:1 (v:v) for the duration of the studies. Each pot contained an individual seed. Plants were grown under natural light at 21–24°C day and 18–21°C night temperatures, respectively, and watered as needed. A total of 120 pots were randomly selected for calculating germination percentage for each type of BC progeny (4 replicates, 30 pots for each replicate). Emerged plants were counted after 14 days of sowing, and germination percentage was calculated.

When plants reached the 4- to 5-leaf stage after 3–4 weeks, 30 individuals of each type of BC progeny were selected at random and labeled. Leaves were separately collected and stored in liquid N_2 for PCR analysis. All plants were sprayed with 41% (a.i.) glyphosate isopropylammonium SL (Roundup Ultra; Monsanto, St. Louis, MO, USA) at 1,000 g a.i. ha^{-1} or 20% (a.i.) glufosinate-ammonium SL (Zhejiang Chem., Zhejiang, China) at 1,000 g a.i.ha^{-1}. Plants were treated with the use of an indoor track sprayer (Shixia Sprayer, Taizhou, China) equipped with a flat fan nozzle delivering 100 L ha^{-1} at 300 kPa. At least, 30 individuals of wild *B. juncea* and transgenic oilseed rape were used as controls for every spray.

Plants were allowed to grow for at least 15 days after herbicide application to visually determine glyphosate and

glufosinate susceptibility. Herbicide was applied a second time to surviving plants by the use of the same procedure. All dead plants were counted. The surviving plants were transplanted into pots (25 cm in diameter) containing the same growth media as described previously. Each pot contained a single plant. All plants were put in the greenhouse and cultured by usual methods. Some of the plants were used to observe growth, determine siliques per plant and seeds per silique. The inflorescences of these plants were bagged before anthesis and allowed to self-pollinate. Wild *B. juncea* plants were used as a control. Plants were harvested at maturity, and the number of siliques per plant and number of fully developed seeds within siliques were counted. For each type of BC progeny, 40 individuals were counted (10 plants for each replicate and 4 replicates). For each plant, 50 siliques were randomly selected on the bottom of the main branch for counting. Seeds per siliques were calculated as the number of full seeds/the number of siliques.

PCR analysis of backcross progeny

Thirty individuals selected randomly from every type of BC progeny underwent PCR analysis as described by Chen et al. (1999) and Pan et al. (2001) before herbicide application. Wild *B. juncea* and transgenic oilseed rape were used as controls. The primer sequences are in Table 1. The testing results were recorded. After applying herbicide twice, we compared the PCR test results with that of herbicide application to confirm that dead plants did not have the tolerant gene and surviving plants did. In addition, we selected surviving plants of every type of BC progeny randomly for PCR testing.

The primer pairs for PCR analysis were designed on the basis of the sequence of the *pat* gene included in the transgenic oilseed rape Swallow or of the *cp4 EPSPS* gene included in the transgenic oilseed rape DS-Roughrider. Each PCR reaction involved 50 µl of reaction mixture containing 5 µl 10× *Ex Taq* buffer, 4 µl $MgCl_2$ (25 mM), 4 µl dNTP mixture (2.5 mM each), 2 µl each primer (10 µM), 0.5 µl *TaKaRa Ex Taq* DNA polymerase (5 U/µl) [TaKaRa Biotechnology (Dalian, China)] and 20 ng of genomic DNA. PCR amplifications were performed on a Whatman Biometra TGRADIENT Thermocycler at 94°C for 5 min for initial denaturation, then 35 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 2 min and a final extension at 72°C for 10 min for the *bar* gene and 94°C for 5 min for initial denaturation, then 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min and a final extension at 72°C for 10 min for the *EPSP* gene. Amplified DNA products were separated on 2% agarose gels at 80 V for 3 h, in 1× TBE buffer, stained with ethidium bromide and visualized under UV light.

Table 1 Sequence of primers used for PCR detection of *EPSPS* and *bar* gene

Detected gene	Orientation	Primer sequence(5′–3′)
EPSPS	Forward primer	AAGGCATTCATTCCCATTTG
	Reverse primer	TAACATCTTCACCTTCCAAAAG
bar	Forward primer	GCACCATCGTCAACCACTAC
	Reverse primer	GCCAGAAACCCACGTCAT

Data analysis

Data were analyzed by ANOVA with the use of SAS V 9.1 (SAS Inst., Cary, NC). Statistical significance was set at $P < 0.05$ for analyzing differences in seeds per silique and the percentage of pollinated flowers developing into siliques among reciprocal backcrosses (obtained from wild *B. juncea* and herbicide-tolerant F_1 or subsequent BC generation) and self-pollination of wild *B. juncea* in the same year, as well as differences in germination percentages, siliques per plant and seeds per silique among the same BC generations and self-pollinated wild *B. juncea*. The analysis of means involved the use of the Duncan's multiple range test. Phenotypic segregation ratios were tested for goodness of fit by χ^2 test.

Results

Seeds per silique by backcross

Despite the use of glyphosate- or glufosinate-tolerant oilseed rape, the first and second reciprocal backcrosses exhibited similar number of seeds per silique: low at the first backcross and increased at the second (Table 2). The second reciprocal backcrosses produced more than four times the number of seeds in each silique than their respective first backcross, but still less than the self-pollinated wild *B. juncea*. The first and second backcrosses produced more seeds per silique with wild *B. juncea* used as maternal plants than as paternal plants. Seeds per silique of the third and fourth reciprocal backcrosses between wild *B. juncea* and BCR progeny or backcrosses with wild *B. jucnea* used as maternal plants and BCL progeny as paternal plants increased to a similar number as that of wild *B. juncea*. However, the third and fourth backcrosses with BCL progeny used as maternal plants and wild *B. juncea* as paternal plants did not result in as many seeds per silique as wild *B. juncea*. Although the proportions of pollinated flowers developing into silique in the first backcross were lower than that in self-pollinated wild *B. juncea*, the values in the second, third and fourth backcrosses were similar to that in self-pollinated wild *B. juncea*. The higher proportion of

Table 2 Means (±SE) of seed set (no. of full seeds/siliques), percentage of pollinated flowers developing into silique of different backcrosses between wild *B. juncea* and F_1 or backcross progenies with herbicide-resistant transgene in the hand-backcrossing experiment

Years	Cross combination	SS	PPFS (%)
2003	Wild *B. juncea* × wild *B. juncea*	15.75 ± 0.37a	99.48 ± 0.42a
	Wild *B. juncea* × F_{1R}	2.26 ± 0.20b	83.84 ± 0.61b
	F_{1R} × wild *B. juncea*	0.87 ± 0.04c	84.32 ± 0.75b
	Wild *B. juncea* × F_1L	2.00 ± 0.06b	84.80 ± 0.58b
	F_{1L} × wild *B. juncea*	0.84 ± 0.05c	84.05 ± 1.15b
2004	Wild *B. juncea* × wild *B. juncea*	15.98 ± 0.24a	99.03 ± 0.74a
	Wild *B. juncea* × BC1mR	10.65 ± 0.27b	99.13 ± 0.34a
	BC1pR × wild *B. juncea*	3.87 ± 0.18d	99.04 ± 0.73a
	wild *B. juncea* × BC1mL	9.63 ± 0.10c	98.59 ± 0.60a
	BC1pL × wild *B. juncea*	3.89 ± 0.37d	98.17 ± 1.07a
2005	Wild *B. juncea* × wild *B. juncea*	15.84 ± 0.16a	99.28 ± 0.73a
	Wild *B. juncea* × BC2mR	16.16 ± 0.11a	98.72 ± 0.86a
	BC2pR × wild *B. juncea*	15.26 ± 0.66a	98.39 ± 0.99a
	Wild *B. juncea* × BC2mL	15.45 ± 0.19a	99.00 ± 0.60a
	BC2pL × wild *B. juncea*	3.96 ± 0.26b	99.19 ± 0.82a
2006	Wild *B. juncea* × wild *B. juncea*	15.99 ± 0.17a	98.98 ± 0.77a
	Wild *B. juncea* × BC3mR	15.65 ± 0.27a	99.28 ± 0.73a
	BC3pR × wild *B. juncea*	15.30 ± 0.22a	99.20 ± 0.33a
	Wild *B.juncea* × BC3mL	16.20 ± 0.80a	98.80 ± 0.97a
	BC3pL × wild *B. juncea*	9.74 ± 0.67b	99.23 ± 0.77a

Values followed by the same letters in the same year in each column are not significantly different at the 5% probability level

SS seed set, *PPFS* the percent of pollinated flowers developing into silique (%), *BC* backcross generation, *m* backcross progeny obtained with wild *B. juncea* as maternal plants, *p* backcross progeny obtained with wild *B. juncea* as paternal plants, *R* backcross generations with glyphosate-tolerant gene, *L* backcross generations with glufosinate-tolerant gene, F_1 denotes hybrids between wild *B. juncea* as maternal plants and glyphosate or glufosinate-tolerant oilseed rape as paternal plants

pollinated flowers developing into silique by backcrossing ensured pollination efficiency (Table 2).

Growth, siliques per plant, and seeds per silique of backcross progeny under self-pollination

The germination percentages of all BC progenies were above 95%, and were similar to that of wild *B. juncea* (Table 3). All plants of BC progenies grew and bloomed vigorously. No plants died from transplantation to harvest. The plants of BC1m progenies exhibited morphologic traits of wild *B. juncea*, whereas BC1p progenies exhibited morphologic traits intermediate to those of both parents. The plants had fewer trichomes on their leaves than did wild *B. juncea* and had large glaucous leaves resembling transgenic oilseed rape. The plants of reciprocal BC2 and BC3 progenies exhibited traits of wild *B. juncea*.

Table 3 Germination percentage and number of silique per plant (mean ± SE) of reciprocal backcross progenies of wild *Brassica juncea* and F_1 (obtained from wild *B. juncea* as maternal plants and transgenic herbicide-tolerant oilseed rape as paternal plants) or subsequent backcross progenies

Year	Backcross progeny	Germination percentage	Number of silique per plant
2004	Wild *B. juncea*	97.50 ± 1.59a	253.25 ± 41.05a
	BC1mR	95.00 ± 2.15a	101.75 ± 29.32b
	BC1pR	95.00 ± 2.15a	113.50 ± 24.13b
	BC1mL	95.84 ± 0.84a	129.00 ± 30.58b
	BC1pL	93.33 ± 1.36 a	109.25 ± 25.40b
2005	Wild *B. juncea*	97.78 ± 0.96a	220.14 ± 33.21a
	BC2mR	95.83 ± 1.60a	216.20 ± 27.10a
	BC2pR	96.67 ± 1.36a	236.62 ± 30.78a
	BC2mL	96.67 ± 1.36a	227.74 ± 31.45a
	BC2pL	96.67 ± 2.35a	243.20 ± 31.14a
2006	Wild *B. juncea*	97.50 ± 0.83a	253.30 ± 37.70a
	BC3mR	95.00 ± 2.15a	225.36 ± 31.30a
	BC3pR	95.84 ± 4.19a	246.18 ± 22.23a
	BC3mL	94.17 ± 2.10a	239.54 ± 34.15a
	BC3pL	95.83 ± 1.59a	247.10 ± 30.92a

m backcross progeny obtained with wild *B. juncea* as maternal plants, *p* backcross progeny obtained with wild *B. juncea* as paternal plants, *R* transgenic oilseed rape, F_1 and backcross generations with glyphosate-tolerant gene, *L* transgenic oilseed rape, F_1 and backcross generations with glufosinate-tolerant gene, *BC* backcross generation

The reciprocal BC1 progenies produced fewer siliques per plant than did wild *B. juncea.* Reciprocal BC2 or BC3 progenies and wild *B. juncea* showed no difference in siliques per plant (Table 3). Reciprocal BC2R and BC3R progenies or BC2mL and BC3mL progenies produced as many seeds per silique as did self-pollinated wild *B. juncea.* The number of seeds per silique for BC2m and BC3m progenies of both trangenes was six times, and BC2pR and BC3pR were 10 times that of their respective BC1 progenies (Fig. 2). However, BC2pL and BC3pL progenies produced nearly two and three times, respectively, the number of seeds per silique as did BC1pL, which produced much fewer seeds per silique than did wild *B. juncea* (Fig. 2).

Tolerance of backcross progeny to herbicide

None of the wild *B. juncea* plants as control survived the herbicide spray, but all plants of transgenic oilseed rape survived. Table 4 gives the number and percentage of glyphosate- and glufosinate-tolerant plants in BC progenies. One quarter of BC1m and 60% of BC1p plants expressed

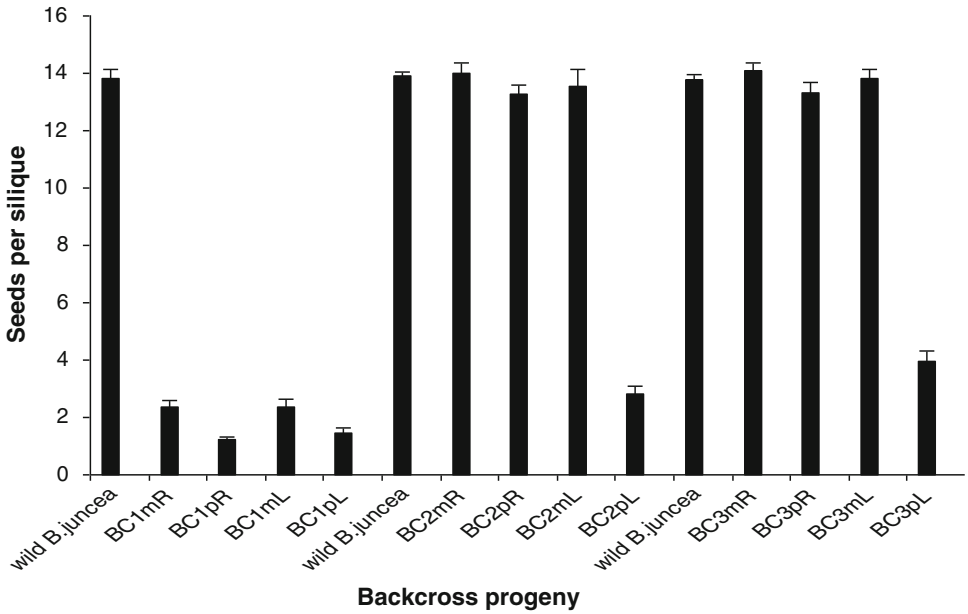

Fig. 2 Seeds per silique (mean ± SE) of reciprocal backcross progenies between wild *Brassica juncea* and F_1 (obtained from wild *B. juncea* as maternal plants and transgenic herbicide-tolerant oilseed rape as paternal plants) or subsequent backcross progenies *m* backcross progeny obtained with wild *B. juncea* as maternal plants, *p* backcross progeny obtained with wild *B. juncea* as paternal plants. *R* transgenic oilseed rape, F_1 and backcross generations with glyphosate-tolerant gene, *L* transgenic oilseed rape, F_1 and backcross generations with glufosinate-tolerant gene, *BC* backcross generation

Table 4 Number and percentage of glyphosate or glufosinate-tolerant plants in backcross progenies (BCn) after spraying with 1,000 g a.i.ha^{-1} glyphosate or glufosinate

Backcross progeny	Observed		Percentage of surviving plants (%)	χ^2 Value under the 1:1 expected ratio
	Number of dead plants	Number of surviving plants		
BC1mR	660	230	25.84	206.7876[a]
BC2mR	110	105	48.84	0.0744
BC3mR	120	108	47.37	0.5307
BC1pR	141	215	60.39	14.9691[a]
BC2pR	90	102	53.13	0.6302
BC3pR	143	145	50.35	0.0035
BC1mL	407	133	24.63	138.0167[a]
BC2 mL	422	201	32.26	77.6886[a]
BC3 mL	317	193	37.84	29.6647[a]
BC1pL	147	219	59.84	13.7732[a]
BC2pL	57	114	66.67	18.3392[a]
BC3pL	91	58	38.93	6.8725[a]

m backcross progeny obtained with wild *B. juncea* as maternal plants, *p* backcross progeny obtained with wild *B. juncea* as paternal plants, *R* backcross generations with glyphosate-tolerant gene, *L* backcross generations with glufosinate-tolerant gene, *BC* backcross generation

[a] Significant deviation ($P = 0.05$) from expected ratio

glyphosate tolerance. In the subsequent reciprocal BC2 and BC3 progenies, 50% of plants were glyphosate tolerant. Glyphosate tolerance segregation seemed to be biased from the 1:1 normal Mendelian segregation in BC1 plants, but was normal in BC2 and BC3, which indicated integration of the glyphosate-tolerance gene into the wild *B. juncea* genome. However glufosinate tolerance segregations were highly variable, from 25 to 67%. The glufosinate-tolerant transgene was maintained despite a lack of stabilized introgression.

PCR analysis of backcross progeny

Molecular data from PCR analysis confirmed that dead plants did not have the tolerance gene and living plants did. All the surviving backcross progenies tested by PCR contained the glufosinate- or glyphosate-tolerance gene, as demonstrated by the presence of the specific DNA fragments of 429 and 527 bp, respectively.

Discussion

Several researches have reported that hybridization between *B. juncea* and oilseed rape (*B. napus*) is successful in controlled crosses and spontaneous in the field (Frello et al. 1995; Bing et al. 1996; Jørgensen et al. 1998). Our results showed that glyphosate- or glufosinate-tolerance genes of transgenic oilseed rape could be transmitted to wild *B. juncea* when wild *B juncea* was used as the maternal

or paternal plants. However, the possibility of gene flow was greater with wild *B. juncea* used as maternal than paternal plants, because the number of seeds per silique by the first and second backcrosses was higher with wild *B. juncea* used as maternal plants than paternal plants. *B. juncea* seemed to function most often as maternal plants in the transmission process. This finding is similar to the results by Hansen et al. (2003) in a study of *B. rapa* and transgenic oilseed rape. Moreover, the possibility for transmission may be greater with glyphosate-tolerant than glufosinate-tolerant oilseed rape to wild *B. juncea* because of more seeds per silique by the third and fourth backcrosses, with BC progeny having glyphosate-tolerant rather than glufosinate-tolerant genes used as maternal plants and by self-pollination of BC2pR and BC3pR than BC2pL and BC3pL.

The limited seeds per silique of the first backcross with wild *B. juncea* used as maternal plants and the first and second backcrosses with wild *B. juncea* used as paternal plants could be a major limitation for transgene introgression from GMHT to wild *B. juncea*.

BC generations were able to grow vigorously. The reciprocal BC2 and BC3 progenies produced as many siliques per plant as did wild *B. juncea*, and reciprocal BC2R, BC3R, BC2mL and BC3mL progeny produced as many seeds per silique as did wild *B. juncea*. This finding indicates that, in theory, a hybrid plant with an HT gene capable of surviving under field condition could be produced.

The probability of transgene establishment into another species depends highly on the fitness of the F_1 between a crop and a wild species and subsequent generations. Therefore, the fitness of the hybrids and backcrosses must be measured for assessing the potential introgression from GMHT oilseed rape to wild relatives (Jenczewski et al. 2003). The fitness of inter- and intraspecific plant hybrids depends on the parental genotype (Mercer et al. 2007), the testing environment (Hauser et al. 2003; Vacher et al. 2004; Campbell and Snow 2007), and their interaction (Campbell and Waser 2001; Whitney et al. 2006). Fitness costs, if they exist, associated with novel characteristics depend on the mutant allele and on the environmental conditions where plants carrying the novel trait grow. Complex variations in resource allocation can modify the performance of individual plants carrying mutations when grown under competitive conditions. Fitness costs should be compared throughout the life cycle (from germination to germination), in different environments, under competitive conditions and in the field where possible (Neve 2007). The bottom line in relation to risk analysis and fitness is the persistence of the hybrids in the field under nonselective conditions (in the absence of the herbicide). Guèritaine et al. (2002)examined the ability of plants to emerge, develop and reproduce within an oilseed rape field (under competition) and on the field border (without competition). Warwick et al. (2008) presented interesting results of weedy *B. rapa* carrying a glyphosate-resistant transgene from *B. napus,* with resistant hybrids persisting over a 6 years period in the absence of selection by glyphosate, despite a fitness cost associated with hybridization. We tested only the proportion of germination, siliques per plant and seeds per silique of BC progeny under optimal conditions. Therefore, more reliable information about the fitness should be obtained by conducting proper studies, especially under competition conditions in the field.

At the whole genome scale, the probability of crop gene transfer depends on the level of genetic and structural homology between the genomes of crop and wild plants. More frequent introgression is expected when crops and their wild relatives share high homology (Jenczewski et al. 2003). *B. napus* ($2n = 38$, AACC) and wild *B. juncea* ($2n = 36$, AABB) are allotetraploids possessing the AA-genome from *B. rapa*. The position of transgenes on the A chromosomes of *B. napus* may be transferred relatively easily to wild *B. juncea* and the theoretical segregation ratio of tolerant and sensitive BC progeny should not deviate from the normal 1:1 Mendelian segregation. F_1 hybrids between *B. napus* and wild *B. juncea* should have an allotetraploid AABC-genome of $2n = 37$. However, BC progeny should have a varying chromosome number of 20 (2A) + 8 (B) + 0–8 (B) + 0–9 (C) because of aneuploid and the C-genome are likely being lost during meiosis with increased number of backcrosses. The reciprocal BC1 progeny likely deviated from the expected 1:1 segregation; the subsequent reciprocal BC2 and BC3 generations seem to show a normal 1:1 Mendelian segregation with glyphosate selection. For this reason, we predicted that there was a high probability that glyphosate-resistant gene was located on A-genome. The transmission frequency of aneuploid gametes as females is higher than that of gametes as males (Ahloowalia 1971). This finding may explain the lower proportion of BC1mR plants showing glyphosate tolerance (25%) than that of BC1pR plants (60%).

However, the proportion of glufosinate-tolerant progeny varied greatly, and significantly deviated from Mendelian segregation. Although the location of *pat* gene in glufosinate-tolerant oilseed rape is unknown, the gene is likely located on the A-genome than on the C-genome. The C chromosomes have no homologous partners during meiosis. Because of irregular transmission of C chromosomes to the gametes, a transgene located on the C-genome of *B. napus* should be transmitted at a low frequency in the gametes and be lost after one or a few generations. Metz et al. (1997) indicated that the frequency of the C chromosome transgenic plants in the BC 1 generation was 26% and was stabilized at about 10% in subsequent BC2 to BC4 generations with transfer of the glufosinate-tolerance transgene from *B. napus* to *B. rapa*. However, we found

25–67% reciprocal of BC1 to BC4 progenies expressing glufosinate tolerance.

Partial homology between the A and C chromosomes has been revealed by studies involving marker analysis, sequence-level genome comparison, genomic in situ hybridization (GISH) and fluorescence in situ hybridization (FISH) techniques (Hosaka et al. 1990; Kerlan et al. 1993; Frello et al. 1995; Yang et al. 2006; Town et al. 2006; Hasterok et al. 2005; Ge and Li 2007). This incidentally may trigger homologous recombination between the chromosomes A of these genomes and cause non-Mendelian segregation. Hansen et al. (2003) demonstrated that introgression can lead to incorporation of the *B. napus* C-genome DNA into the *B. rapa* (AA, $2n = 18$) genome. Leflon et al. (2006) also demonstrated that gene transfer between A and C chromosomes occur in triploid hybrids of *B. napus* and *B. rapa*. In addition, because of the structural polymorphism of chromosomes within *B. napus* and wild *B. juncea,* gene silencing and heterogeneous instability may cause unstable inheritance (Tomiuk et al. 2000; Al Mouemar and Darmency 2004). Several chromosomal anomalies have been previously demonstrated in *Brassica* hybrids (Guèritaine et al. 2002; Benabdelmouna et al. 2003; Al Mouemar and Darmency 2004).

Chèvre et al. (1998) studied the transmission rate of different oilseed rape loci from glufosinate-resistant oilseed rape to wild radish and found that among the 17 oilseed rape loci studied, including the *bar* gene, the proportion of transmission in BC1 varied according to the locus. The specific integration position of transgenes may cause different transmission frequency. The differences in transmission of glyphosate- and glufosinate-tolerant genes in BC progenies may be due to the specific integration position of the transgene or uncertain cross between the chromosomes of genomes.

We did not estimate the chromosome numbers of BC plants nor did we test crossovers between A, B and C chromosome of backcross-generation genomes. The introgression for glyphosate tolerance gene may be deduced from the observed genetic stability, but is not showed for the glufosinate tolerance gene because of abnormal segregation. Therefore, further studies of the evolution of genome structures of backcross generations and the inheritance behavior of the transgene in backcrossed generation by chromosome-specific (simple sequence repeats) markers and molecular cytogenetics by GISH and FISH techniques will offer direct evidence for efficient gene flow.

Previous research examined transgene expression in different BC generations and showed the examined transgenes to be stably expressed, regardless of genetic background, cytoplasmic origin and environmental conditions (Zhu et al. 2004; Ammitzbøll et al. 2005). Stable expression of transgenes that are introgressed to wild relatives allows for reliable and straightforward risk assessment and post-release monitoring. Therefore, examining the transgene expression in different BC generations in different environments is of interest.

HT wild *B. juncea* populations might be developed if GMHT oilseed rape is released in China. In an agricultural field, the immediate problem caused by HT wild *B. juncea* is that it can no longer be controlled by the herbicides. The introgression of either the glyphosate or glufosinate tolerance gene to wild *B. juncea* may cause much serious agricultural effects. The two wild *B. juncea* populations have evolved glyphosate tolerance in China (Huangfu et al. 2007). Therefore, the evolved tolerance to glyphosate, coupled with the glufosinate tolerance, could be detrimental in terms of weed control. Nevertheless, release of either glyphosate or glufosinate-tolerant oilseed rape should depend on a thorough risk assessment, especially in areas infested with wild *B. juncea* in China.

Acknowledgments This research was financially supported by the National Basic Research and Development Program (2007CB109202) and the National Natural Science Foundation of China (30400059). We express our deep appreciation to Dr. Robert E. Blackshaw, Suzanne Warwick, Yong Woong Kwon and Tae Jin Yang for their valuable comments on this manuscript.

References

Ahloowalia BS (1971) Frequency, origin, and survival of aneuploids in tetraploid ryegrass. Genetica 42:129–138

Al Mouemar A, Darmency H (2004) Lack of stable inheritance of introgressed transgene from oilseed rape in wild radish. Environ Biosaf Res 3:209–214

Ammitzbøll H, Mikkelsen TN, Jørgensen RB (2005) Transgene expression and fitness of hybrids between GM oilseed rape and *Brassica rapa*. Environ Biosaf Res 4:3–12

Aono M, Wakiyama S, Nagatsu M, Nakajima N, Tamaoki M, Kubo A, Saji H (2006) Detection of feral transgenic oilseed rape with multiple-herbicide resistance in Japan. Environ Biosaf Res 5:77–87

Benabdelmouna A, Guèritaine G, Abirached-Darmency M, Darmency H (2003) Genome discrimination in progeny of interspecific hybrids between *Brassica napus* and *Raphanus raphanistrum*. Genome 46:469–472

Bing DJ, Downey RK, Rakow GFW (1991) Potential of gene transfer among oilseed *Brassica* and their weedy relatives. In: Proceedings of GCIRC 8th international rapeseed congress, Saskatoon, Canada, pp 1022–1027

Bing DJ, Downey RK, Rakow GFW (1996) Hybridization among *Brassica napus, B. rapa* and *B. juncea* and their two weedy relatives *B. nigra* and *Sinapis arvensis* under open pollination conditions in the field. Plant Breed 115:470–473

Campbell LG, Snow AA (2007) Competition alters life history and increase the relative fecundity of crop-wild radish hybrids (*Raphanus* spp.). New Phytol 173:648–660

Campbell DR, Waser NM (2001) Genotype-by-environment interaction and the fitness of plant hybrids in the wild. Evolution 55:669–676

Chapman MA, Burke JM (2006) Letting the gene out of the bottle: the population genetics of genetically modified crops. New Phytol 170:429–443

Chen LH, Wang XW, Zhang WJ, Zhang XD, Hu DF, Liu GT (1999) Transformation of common wheat (*Triticum aestivum* L.) with herbicide-resistant *EPSP*s gene. Acta Genet Sin 26:239–243

Chèvre AM, Eber F, Baranger A, Renard M (1997) Gene flow from transgenic crops. Nature 389:924

Chèvre AM, Eber F, Baranger A, Hureau G, Barret P, Picault H, Renard M (1998) Characterization of backcross generations obtained under field conditions from oilseed rape-wild radish F_1 interspecific hybrids: an assessment of transgene dispersal. Theor Appl Genet 97:90–98

Chèvre AM, Adamczyk K, Eber F, Huteau V, Coriton O, Letanneur JC, Laredo C, Jenczewski E, Monod H (2007) Modelling gene flow between oilseed rape and wild radish. I. Evolution of chromosome structure. Theor Appl Genet 114:209–221

Dale PJ (1992) Spread of engineered genes to wild relatives. Plant Physiol 100:13–15

Darmency H, Fleury A (2000) Mating system in *Hirschfeldia incana* and hybridization to oilseed rape. Weed Res 40:231–238

Agbios GM database (2008) AGBIOS Canadian Company. http://www.agbios.com/dbase.php. Accessed 22 Sept 2008

FitzJohn RG, Armstrong TT, Newstrom-Lloyd LE, Wilton AD, Cochrane M (2007) Hybridisation within *Brassica* and allied genera: evaluation of potential for transgene escape. Euphytica 158:209–230

Frello SK, Hansen R, Jensen J, Jørgensen RB (1995) Inheritance of rapeseed (*Brassica napus*)-specific RAPD markers and a transgene in the cross *B. juncea* × (*B. juncea* × *B. napus*). Theor Appl Genet 91:236–241

Ge HX, Li ZY (2007) Intra- and intergenomic homology of B-genome chromosomes in trigenomic combinations of the cultivated *Brassica* species revealed by GISH analysis. Chromosome Res 15:849–861

Guan CY (1996) Comparative studies of inheritable character in Xinjiang wild rape and *Sinapis arvensis* L. Acta Agro Sin 22:214–219

Guan CY (2005) Transgenic breeding of oilseed rape. Chin J Oil Crop Sci 27:97–103

Guan CY, Li X (1997) Research and application of transgenic oilseed rape. J Cell Biol 19:18–23

Guèritaine G, Sester M, Eber F, Chèvre AM, Darmency H (2002) Fitness of backcross six of hybrids between transgenic oilseed rape (*Brassica napus*) and wild radish (*Raphanus raphanistrum*). Mol Ecol 11:1419–1426

Guo QY, Tu HL, Qiu XL, Xin Y (1998) Research on occurrence pattern and control technology of wild mustard. Sci Tech Qinghai Agric For 4:38–41

Hansen LB, Siegismund HR, Jørgensen RB (2001) Introgression between oilseed rape (*Brassica napus* L.) and its weedy relative *B. rapa* L. in a natural population. Genet Resour Crop Evol 48:621–627

Hansen LB, Siegismund HR, Jørgensen RB (2003) Progressive introgression between *Brassica napus* (oilseed rape) and *B. rapa*. Heredity 91:276–283

Hasterok R, Wolny E, Kulak S, Zdziechiewicz A, Maluszynska J, Heneen WK (2005) Molecular cytogenetic analysis of *Brassica rapa–Brassica oleracea* var. *alboglabra* monosomic addition lines. Theor Appl Genet 111:196–205

Hauser TP, Jørgensen RB, Ø stergard H (1998) Fitness of backcross and F_2 hybrids between weedy *Brassica rapa* and oilseed rape (*B.* napus). Heredity 81:436–443

Hauser TP, Damgaard C, Jørgensen RB (2003) Frequency-dependent fitness of hybrids between oilseed rape (*Brassica napus*) and weedy *B. rapa* (Brassicaceae). Am J Bot 90:571–578

Hosaka K, Kianian SF, McGrath JM, Quiros CF (1990) Development and chromosomal localization of genome-specific DNA markers of *Brassica* and the evolution of amphidiploids and $n = 9$ diploid species. Genome 33(1):131–142

Huangfu CH, Song XL, Sheng Qiang, Zhang HJ (2007) Response of wild *Brassica juncea* populations to glyphosate. Pest Manage Sci 63:1133–1140

Jenczewski E, Ronfort J, Chèvre AM (2003) Crop-to-wild gene flow, introgression and possible fitness effects of transgenes. Environ Biosaf Res 2:9–24

Jørgensen RB, Anderson B (1994) Spontaneous hybridization between oilseed rape (*Brassica napus*) and weedy *B. campestris* (Brassicaceae): a risk of growing genetically modified oilseed rape. Am J Bot 81:1620–1626

Jørgensen RB, Anderson B, Landbo L, Mikkelsen TR (1996) Spontaneous hybridization between oilseed rape (*Brassica napus*) and weedy relatives. Acta Hortic 407:193–200

Jørgensen RB, Andersen B, Hauser TP, Lanbo L, Mikkelsen TR, Østergård H (1998) Introgression of crop genes from oilseed rape (*Brassica napus*) to related wild species—an avenue for the escape of engineered genes. Acta Hortic 459:211–217

Kawata M, Murakami K, Ishikawa T (2009) Dispersal and persistence of genetically modified oilseed rape around Japanese harbors. Environ Sci Pollut Res 16(2):120–126

Kerlan MC, Chèver AM, Eber F, Renard M (1992) Risk assessment of outcrossing of transgenic rapeseed related species: interspecific hybrid production under optimal condition with emphasis on pollination and fertilization. Euphytica 62:145–153

Kerlan MC, Chèver AM, Eber F (1993) Interspecific hybrids between a transgenic rapeseed (*Brassica napus*) and related species: cytogenetical characterization and detection of the transgene. Genome 36:1099–1106

Kling J (1996) Could transgenic super crops one day breed super weeds? Science 274:180–181

Leflon M, Eber F, Letanneur JC, Chelysheva L, Canton O, Huteau V, Ryder CD, Barker G, Jenczewski E, Chèvre AM (2006) Pairing and recombination at meiosis of *Brasscia rapa* (AA) × *Brassica napus* (AACC) hybrids. Theor Appl Genet 113:1467–1480

Lefol E, Sèguin-Swartz G, Downey RK (1997) Sexual hybridisation in crosses of cultivated *Brassica* species with the crucifers *Erucatrum gallicum* and *Raphanus raphanistrum*: potential for gene introgression. Euphytica 95:127–139

Lègère A (2005) Risks and consequences of gene flow from herbicide-resistant crops: canola (*Brassica napus* L.) as a case study. Pest Manage Sci 61:292–300

Lu CM, Xiao L, Wu YH (2005) Ecological risk assessment of transgenic rapeseed in China. J Agric Biotechnol 13:267–275

Mercer KL, Andow DA, Wyse DL, Shaw RG (2007) Stress and domestication traits increase the relative fitness of crop-wild hybrids in sunflower. Ecol Lett 10:383–393

Metz PLJ, Nap JP, Stiekema WJ (1995) Hybridization of radish (*Raphanus sativus* L.) and oilseed rape (*Brassica napus* L.) through a flower-culture method. Euphytica 83:159–168

Metz PLJ, Jacobsen E, Nap JP, Pereira A, Stiekema WJ (1997) The impact on biosafety of the phosphinothricin-tolerant transgene in inter-specific *B. rapa* × *B. napus* hybrids and their successive backcrosses. Theor Appl Genet 95:442–450

Neve P (2007) Challenges for herbicide resistance evolution and management: 50 years after Harper. Weed Res 47:365–369

Pan LW, Chen JH, Shen YF, Hu YQ, Tao J, Han W (2001) Detection comparison of CP4 EPSP gene from genetically modified roundup ready rape seed and soybean. Lett Biotechnol 12:175–207

Pu HM (2003) Transgenetic herbicide-tolerant oilseed rape and its ecological security. Chin J Oil Crop Sci 25:89–92

Pu HM, Gao JQ, Qi CK, Zhang JF, Chen XJ, Fu SZ (2003) Inheritance of herbicide resistance in *Brassica napus* and its utilization. Jiangsu J Agric Sci 19:81–86

Pu HM, Qi CK, Zhang JF, Fu SZ, Gao JQ, Chen XJ, Zhao XX (2005) The studies on gene flow from GM herbicide-tolerant rapeseed to cruciferous weeds. Acta Ecol Sin 25:910–916

Rieger MA, Potter TD, Preston C, Powles SB (2001) Hybridization between *Brassica napus* L. and *Raphanus raphanistrum* L. under agronomic field conditions. Theor Appl Genet 103:555–560

Roy NN (1984) Interspecific transfer of *Brassica juncea*-type high blackleg resistance to *Brassica napus*. Euphytica 33:295–303

Scheffler, Dale (1994) Opportunities for gene transfer from transgenic oilseed rape (*Brassica napus*) to related species. Transgenic Res 3:263–278

Snow AA, Anderson B, Jørgensen RB (1999) Costs of transgenic herbicide resistance introgressed from *Brassica napus* into weedy *B. rapa*. Mol Ecol 8:605–615

Song XL, Qiang S (2003) Sexual compatibility of three species of oilseed rape(*Brassica* Spp.) with wild rapes (*B. juncea* var. *gracilis* Tsen et Lee) and the fitness of F_1—potential for gene transfer. Chin J Appl Environ Biol 9:357–361

Song XL, Huangpfu CH, Qiang S (2007) Gene flow from transgenic glufosinate- or glyphosate-tolerant oilseed rape to wild rape. J Plant Ecol 31:729–737

Tomiuk J, Hauser Tp, Jørgensen RB (2000) A- or C-chromosomes, does it matter for the transfer of transgenes from *Brassica napus*. Theor Appl Genet 100:750–754

Town CD, Cheung F, Maiti R, Crabtree J, Haas BJ, Wortman JR, Hine EE, Althoff R, Arbogast TS, Tallon LJ, Vigouroux M, Trick M, Bancroft L (2006) Comparative genomics of *Brassica oleracea* and *Arabidopsis thaliana* reveal gene loss, fragmentation, and dispersal after polyploidy. Plant Cell 18:1348–1359

Vacher C, Weis AE, Hermann D, Kossler T, Young C, Hochberg M (2004) Impact of ecological factors on the initial invasion of Bt transgenes into wild populations of birdseed rape (*Brassica rapa*). Theor Appl Genet 109:806–814

Wang XF, Wang HZ, Liu GH, Hu ZL, Zhen YB (2005) Transgenic hybrid parents in *Brassica napus* transformed with bivalent genes for resistance to *Sclerotinia sclerotiorum*. Chin Bull Bot 22:292–301

Warwich SI, Simard MJ, Lègère A, Beckie HJ, Braun L, Zhu B, Mason P, Sègain-swartz G, Stewart CN (2003) Hybridization between transgenic *Brassica napus* L. and its wild relatives: *Brassica rapa* L., *Raphanus raphanistrum* L., *Sinapis arvensis* L., and *Erucastrum gallicum* (Wild.) O. F. Schulz. Theor Appl Genet 107:528–539

Warwick SI, Legere A, Sinard MJ, James T (2008) Do escaped transgenes persist in nature? The case of an herbicide resistance transgene in a weedy *Brassica rapa* population. Mol Ecol 17:1387–1395

Whitney KD, Randell RA, Rieseberg LH (2006) Adaptive introgression of herbivore resistance traits in weedy sunflower *Helianthus annuus*. Am Nat 167:794–807

Yang TJ, Kim JS, Kwon SJ, Lim KB, Choi BS, Kim JA, Jin M, Park JY, Lim MH, Kim H, Lim YP, Kang JJ, Hong JH, Kim CB, Bhak J, Bancroft I, Park BS (2006) Sequence-level analysis of the diploidization process in the triplicated flowering locus C region of *Brassica rapa*. Plant Cell 18:1339–1347

Yoshimura Y, Beckie HJ, Matsuo K (2006) Transgenic oilseed rape along transportation routes and port of Vancouver in western Canada. Environ Biosaf Res 5:67–75

Zhu B, Lawrence JR, Warwick SI, Mason P, Braun L, Halfhill MD, Stewart CN (2004) Stable *Bacillus thuringiensis* (Bt) toxin content in interspecific F_1 and backcross populations of wild *Brassica rapa* after Bt gene transfer. Mol Ecol 13:237–241