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Gene transferability from transgenic *Brassica napus* L. to various subspecies and varieties of *Brassica rapa*

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Abstract Gene transferability from transgenic rapeseed to various subspecies and varieties of *Brassica rapa* was assessed in this study. Artificial crossability was studied in 118 cultivars of 7 *B. rapa* subspecies and varieties with the transgenic rapeseed GT73 (*Brassica napus*) as the pollen donor. On average 5.7 seeds were obtained per pollination, with a range from 0.05 to 19.4. The heading type of *B. rapa* L. showed significantly higher crossability than non-heading types of *B. rapa*. The spontaneous outcrossing rate between *B. rapa* (female) and the transgenic rapeseed Ms8 × Rf3 (*B. napus*) (male) ranged from 0.039 to 0.406%, with an average of 0.19%. The fertilization process and the development of the hybrid seeds as shown by fluorescent staining techniques indicated that the number of adhered pollens on the stigma was reduced by 80%, the number of pollen tubes in the style was reduced by 2/3 and the fertilization time was delayed by over 20 h when pollinated with the transgenic rapeseed Ms8 × Rf3 in comparison with the bud self-pollination of *B. rapa* as control. About 10–70% of the interspecific hybrid embryos were aborted in the course of development. Some seeds looked cracked in mature pods, which showed germination abilities lower than 10%. The spontaneous outcrossing rates were much lower than the artificial crossability, and their survival fitness of the interspecific hybrid was very low, indicating that it should be possible to keep the adventitious presence of the off-plants under the allowed threshold, if proper measures are taken.

Keywords Transgenic *Brassica napus* · *Brassica rapa* subspecies · Crossability · Outcrossing rate · Fertilization process · Germination

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Introduction

Brassica rapa L. (AA, $2n = 20$) is a collection of *Brassica* species consisting of the A genome. Due to natural evolution and artificial selection, *B. rapa* comprises many subspecies and cultivar groups, including those for swollen edible roots (*B. rapa* L. ssp. *rapifera*), stems (*B. rapa* L. ssp. *chinensis* var. *utilis*, *B. rapa* L. ssp. *chinensis* var. *parachinensis*,

B. rapa L. ssp. *chinensis* var. *multiceps*), leaves (*B. rapa* L. ssp. *pekinensis*, *B. rapa* L. ssp. *chinensis*, *B. rapa* L. ssp. *chinensis* var. *rosularis*), flower buds (*B. rapa* L. ssp. *chinensis* var. *purpurea*) and oil-rich seeds (*B. rapa* L. ssp. *chinensis* var. *oleifera*). The *B. rapa* variety derived for its leaves is the most diversified, consisting of various heading and non-heading groups. As one of the biodiversity centers of *B. rapa* in the world, China has abundant resources of *B. rapa* all over the country. *B. rapa* have been cultivated there for thousands of years as very important vegetables and feeds and as an oil source.

With the commercialization of transgenic rapeseed in the world, the problems concerning the transgene dispersal from *B. napus* to *B. rapa* are brought much into focus. It has long been known that natural interspecific crossing can and does occur among the oilseed *Brassica* species (i.e., *B. napus*, *B. rapa* and *B. juncea*) (Bing et al. 1996; Jorgensen and Andersen 1994; Frello et al. 1995) and in some countries a weedy form of *B. rapa* occurs. Both pre- and postzygotic barriers, e.g., temporal divergence, gametic and zygotic incompatibility, hybrid inviability and sterility have the potential to reduce gene flow between species (Levin 1978). If the barriers are incomplete, first and later generation hybrids may be formed and function as a bridge for gene transfer. Different alleles may flow from one species into another at different rates, depending on rates of dispersal, the strength of selection, breeding system, and linkage to selected loci (Christiansen et al. 1995).

The use of transgenic rapeseed in production requires a careful consideration of the possible risks related to the unintended spread of transgenes into new habitats. Management measures for reducing the gene flow from transgenic populations are needed in order to prevent possible unwanted effects of transgenes on ecosystems (Ellstrand and Hoffman 1990; Raybould and Gray 1993, 1994; Snow and Palma 1997). Consequently, it is important to quantify the gene flow and to try to establish strategies to control or minimize it, while accounting for the possible ecological effect of the newly introduced genes, whether advantageous or disadvantageous.

Although many studies have investigated crossability between *B. napus* and *B. rapa*, many of them have been done with wild *B. rapa* (Jorgensen and Andersen 1994; Halfhill et al. 2001, 2002; Hansen et al. 2001; Snow et al. 1999) or oilseed rape (Li et al. 2006; Metz et al. 1997; Zhao et al. 2005), and very few studies have been done on the gene flow from transgenic rapeseed to various subspecies and varieties of *B. rapa*. The aim of this study was to quantify the gene transferability from *B. napus* to various subspecies and varieties of *B. rapa* and to estimate the germination ability of the interspecific hybrids.

Materials and methods

Plant materials

Glyphosate-tolerant transgenic *Brassica napus* c.v. GT73 was provided by Monsanto Company. Phosphinothricin-tolerant transgenic *B. napus* c.v. Ms8 × Rf3 was provided by Byer Crop Science Company. One hundred eighteen cultivars of *B. rapa* were maintained by Kerun Vegetable Research Institute of Tianjin, including 60 cultivars of *B. rapa* L. ssp. *pekinensis* Olsson, 33 cultivars of *B. rapa* L. ssp. *chinensis* var. *chinensis* Kitam, 4 cultivars of *B. rapa* L. ssp. *chinensis* var. *purpurea* Mao, 10 cultivars of *B. rapa* L. ssp. *chinensis* var. *parachinensis* Tsen et Lee, 6 cultivars of *B. rapa* L. ssp. *chinensis* var. *rosularis* Tsen et Lee, 3 cultivars of *B. rapa* L. ssp. *chinensis* var. *oleifera* and 2 cultivars of *B. rapa* L. ssp. *raifera* Matzg (Table 1). The representative morphologies of the various subspecies and varieties are shown in Fig. 1.

GT73 and Ms8 × Rf3 are two representative transgenic rapeseed events, the cultivars from which have occupied over 90% of the cultivation area of the total transgenic rapeseed commercialized so far. One hundred eighteen cultivars varieties of *B. rapa* represent almost all of the types of the cultivated *B. rapa* in China.

Interspecific crossability analysis

Experiments were conducted in the greenhouse. The interspecific crossability analysis was made with *B. napus* c.v. GT73 as the pollen donor and 118 cultivars of *B. rapa* as the seed parents, respectively by means of artificial emasculation and crossing (Table 1). For each cross combination, about 100–200 flowers from 10 separate plants were crossed. The pod number and seed number per pod

Table 1 Crossability of transgenic rapeseed with cultivars from various subspecies of *B. rapa* L

No.	Cultivars	No. of pollinated flowers	No. of seeds	Crossability index	No.	Cultivars	No. of pollinated flowers	No. of seeds	Crossability index
A1	Gongguan 2	177	1832	10.35	B1	Zhonggengbai	162	434	2.68
A2	Mengbai 1	177	448	2.53	B2	Kangreqingcai	151	1094	7.25
A3	Shennongchaojibaicai	183	1800	9.84	B3	Baiyewuyuemanqingcai	185	1033	5.58
A4	Qingluwang	169	1088	6.44	B4	Kangre 605(Shanghai)	180	488	2.71
A5	Huangya 14	161	1062	6.6	B5	Aiqiqing	157	304	1.94
A6	Xinxiangmiaobao 23	166	1240	7.47	B6	Shanghaiqing	169	1416	8.38
A7	Yuqingbaicai	173	1389	8.03	B7	Suzhouqing	184	435	2.36
A8	Luxing 80	173	2776	16.05	B8	Aijiaohuang(Nanjing)	201	1088	5.41
A9	Xiayang 50	157	984	6.27	B9	Gaogengbai	198	2328	11.76
A10	Jingxia 2	161	1376	8.55	B10	Aijiaosuzhouqing	152	147	0.97
A11	Xiaoza 50	167	984	5.89	B11	Aijiaohuang(Hubei)	165	632	3.83
A12	XiaoZa 60	170	459	2.7	B12	Aikangqing	165	824	4.99
A13	Qingdao 83-1	254	1874	7.38	B13	Jiangyibaiguan	84	664	7.9
A14	Benjingxin 3	191	1294	6.77	B14	Xinbaixuegongzhuaijiaoxiaobaicai	186	1960	10.54
A15	Juhongxin	170	1436	8.45	B15	Xiangtanshuyaoaijiaobai	183	1016	5.55
A16	Hongkang 1	163	1416	8.69	B16	Kangre 605(Jiangxi)	180	808	4.49
A17	Fengkang 78	172	1304	7.58	B17	Chaojibawangqing	169	798	4.72
A18	Xibai 4	177	1770	10	B18	Japanese Jingxuanqingjiangbaicai	183	762	4.16
A19	Xibai 5	172	1048	6.09	B19	Huangguanqingjianbaicai	169	1120	6.63
A20	87 Chun 34	167	2808	16.81	B20	Datouqingjiangbaicai	169	722	4.27
A21	Degao 8	167	3240	19.4	B21	Hongkong Zhongjiaoheiyebai	185	1116	6.03
A22	Jiaoyanxiaqiuwang	163	1076	6.6	B22	Quanxingaizhuangqing	145	838	5.78
A23	Luxing 58	167	604	3.62	B23	Siyueman	240	2184	9.1
A24	Liangqing	182	2230	12.25	B24	Luqiu 91-1	171	380	2.22
A25	Qiangshi	164	2392	14.59	B25	Jinglu 2	195	464	2.38
A26	Beijingxin 5	198	768	3.88	B26	Zaoshenghuahing	164	410	2.5
A27	Xibai 3	183	1197	6.54	B27	Huaguan	162	788	4.86
A28	Chunquan	181	728	4.02	B28	Huawang	175	936	5.35
A29	Chunqiuyu	159	2448	15.4	B29	Xiawang	163	230	1.41
A30	CR Huangxin	186	1472	7.91	B30	Xiadi	168	664	3.95
A31	Yangchun	169	2680	15.86	B31	Gaohuaqinggengbaicai 128	188	582	3.1
A32	Jinguan	164	1354	8.26	B32	Zaoshengjinpin 21	176	1096	6.23

Table 1 continued

No.	Cultivars	No. of pollinated flowers	No. of seeds	Crossability index	No.	Cultivars	No. of pollinated flowers	No. of seeds	Crossability index
A33	Shengchun	165	2128	12.9	B33	Guangyeheibaicai	169	1352	8
A34	Xizangbaicai	170	900	5.29	C1	Tezao50	168	940	5.6
A35	Kuaichun	162	2032	12.54	C2	Jiuyuexian	165	1524	9.24
A36	Chunxiamingjia	187	1824	9.75	C3	Shiyuehong	161	1012	6.29
A37	Lujian 70	190	1912	10.06	C4	Hongza 60	165	982	5.95
A38	Kemengxiaqiuwang	179	683	3.82	D1	Zaobai 30	196	704	3.59
A39	Chunyuehuang	147	1280	8.71	D2	Zaotai 50	159	684	4.3
A40	Qiuzhenbai 6	194	2230	11.49	D3	Zaoshubaicaitai	168	2176	12.95
A41	Zhenbai 1	144	1472	10.22	D4	Xiangtai 1	194	1752	9.03
A42	Zaoshu 5	174	742	4.26	D5	Sijiubaigengcaixin 19	144	404	2.81
A43	Qiangchun	170	749	4.41	D6	Baishayouqingcaixin 45	153	360	2.35
A44	Qingchun	176	1168	6.64	D7	Baishayouqingtiancaixin 50	170	1110	6.53
A45	Chunxiabawang	161	1032	6.41	D8	Chihua 4	185	254	1.37
A46	Qiulu 55	177	222	1.25	D9	Techunyouqingtiancaixin 31	162	652	4.02
A47	Qiulu 60	139	400	2.88	D10	Sijiuhuangcaixin	166	1040	6.27
A48	Qiulu 75	150	712	4.75	E1	Shanghaitacai	172	680	3.95
A49	Jinqiu 1	165	456	2.76	E2	Lulingwutacai	179	1026	5.73
A50	Jinbai 56	158	728	4.61	E3	Lulingheixinwu	178	1992	11.19
A51	Jinxia 2	148	1550	10.47	E4	Xuelian	180	1312	7.29
A52	Jinxia 3	175	932	5.33	E5	Huangxinwq	174	1784	10.25
A53	Zhongmaowawacai	166	8	0.05	E6	Wutacai	276	1058	3.83
A54	Jingxiawawacai	166	1376	8.29	F1	Wuenzhoupancai	173	60	0.35
A55	Jingchunwawacai	170	1196	7.04	F2	Manqing	162	974	6.01
A56	Dafengtexuanxiaobaicai	192	656	3.42	G1	0069	162	956	5.9
A57	Japanese Zaoshengxiaobaicai	90	104	1.16	G2	0046	145	836	5.77
A58	Jingxuanhuangjinxiaobaicai	157	2160	13.76	G3	0179	167	510	3.05
A59	Huonanfeng 1	174	1768	10.16					
A60	Shiubaicai	179	1464	8.18					

Note: A1–A60: *Brassica rapa* L. ssp. *pekinensis* Olsson; B1–B33: *Brassica rapa* L. ssp. *chinensis* var. *chinensis* Kitam; C1–C4: *B. rapa* L. ssp. *chinensis* var. *purpurea* Mao; D1–D10: *B. rapa* L. ssp. *chinensis* var. *parachinensis* Tsen et Lee; E1–E6: *B. rapa* L. ssp. *chinensis* var. *rosularis* Tsen et Lee; F1–F2: *B. rapa* L. ssp. *raifera* Matzg; G1–G3: *B. rapa* L. ssp. *chinensis* var. *oleifera*. The representative morphologies of the various subspecies and varieties are shown in Fig. 1

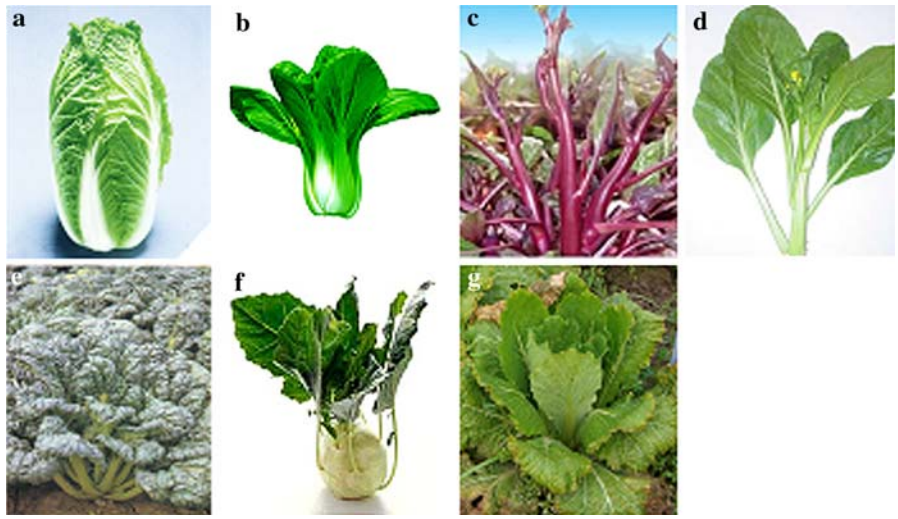

Fig. 1 Representative morphologies of various subspecies and varieties of *B. rapa*. **a** *Brassica rapa* L. ssp. *pekinensis* Olsson; **b** *Brassica rapa* L. ssp. *chinensis* var. *chinensis* Kitam; **c** *B. rapa* L. ssp. *chinensis* var. *purpurea* Mao; **d** *B. rapa* L. ssp. *chinensis* var. *parachinensis* Tsen et Lee; **e** *B. rapa* L. ssp. *chinensis* var. *rosularis* Tsen et Lee; **f** *B. rapa* L. ssp. *raifera* Matzg; **g** *B. rapa* L. ssp. *chinensis* var. *oleifera*

were investigated after harvest. The crossability index was calculated using the formula:

Crossability index = Number of full seeds obtained/Number of flowers pollinated

Seed germination test

The germination rate of the F_1 hybrid seeds from 34 cross-combinations was measured with two replications with *B. napus* c.v. Zhongyou 821 as a control to provide the normal germination rate estimate. Germination was conducted in a 90 mm Petri dish padded with a moistened filter paper disc, on which 100 seeds were placed evenly, in a growth chamber at a temperature of 23°C, humidity of 90% and day length of 12 h (3000–6000 lux). Germinated seeds were counted after 7 days to estimate the germination rate as described in Lu et al. (2001).

Seed morphology observation

The F_1 hybrid seeds of transgenic rapeseed and the parents were observed and photographed under Olympus stereromicroscope SZX12.

Spontaneous outcrossing rate analysis

Experiments were conducted at the Hanchuan Experimental Station of the Inspection, Detection and Testing Center of Transgenic Crops Environmental Biosafety of the Ministry of Agriculture (Wuhan). The spontaneous outcrossing rate was assessed using *B. rapa* c.v. Wutacai, c.v. Wuyuemanqingcai and c.v. Shiyuehong as the seed parents and transgenic *B. napus* c.v. Ms8 × Rf3 as the pollen donor. *B. napus* c.v. Zhongyou 821(CK) was used as the control of the seed parents. The experiments were carried out in a randomized block design with 3 replicates. Each plot contained two rows of transgenic rapeseed and two rows of a seed parent. The space between rows and plants was 35 × 25 cm and each row was 6 m long. To synchronize the flowering time, the seed parents were sown at two times, each at an interval of one row every 15 days. All plants were pollinated naturally. After maturation, the seeds were harvested and then sown again to estimate the outcrossing rate.

Rate of herbicide resistance plants

The outcrossing rate was estimated by the survival rate of the plants after treatment with the herbicide BASTA. The experiments were carried out with a randomized block design with 4 genotypes and 3 replications (Table 3). Each plot was 10 m^2 in size with an established plant density of about 200–300/m^2. Seedlings at the 4–5 leaf stage were sprayed with 1:200 diluted 13.5% BASTA herbicide. The second spray was conducted 4 days later. Both dead and survived seedlings were counted 4–7 days later, after the second spraying. The spontaneous outcrossing rate was calculated using the formula:

Outcrossing rate = 100 × Number of herbicide-tolerant plants/total number of plants treated.

Hybridity confirmation by PCR

The plants that survived herbicide treatment were analyzed by PCR in order to prevent the false positive results. About 1.0 g of leaves from each plant was selected for DNA extraction using the improved 1% sodium dodecyl sulfate (SDS) method. Primers of the *bar* gene were designed according to the coding region of the sequence of GenBank (Accession NO. AY582737) as BarF: ATCGGATCCATGAGCCCA GAACGACGCCC, and BarR: ATCAAGCTTCAGA TTTCGGTGACGGGCA, producing a 544 bp fragment after amplification. The 20 μl PCR mixture contained 0.2 mmol/l dNTPs, 2 mmol/l $MgCl_2$, 1× Buffer, 0.15 mmol/l primers, 30 ng template DNA, and 1 unit of Taq polymerase. The PCR amplification was performed in PE9700 with the program as follows: 4 min at 95°C, 35 cycles of 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C, and a final extension step of 7 min at 72°C. The PCR products were analyzed by electrophoresis using a 1% agarose gel.

Pollen germination and fertilization

The pollen germination and fertilization processes of *B. napus* on *B. rapa* stigma were observed with the self-pollinated *B. rapa* as a control by aniline blue fluorescence (ABF) staining under a microscope according to the protocol of Hu (1994). *B. rapa* ssp. *rosularis* Tsen et Lee(Wutacai), *B. rapa* ssp. *chinensis* Kitam (Wuyuemanqingcai) and *B. rapa* ssp. *oleifera* (0069) were crossed separately as female parents with the phosphinothricin-tolerant *B. napus* c.v. Ms8 × Rf3. The flowers sampled at 1, 3, 5, 8, 27, 31 and 48 h after pollination were rinsed, fixed and stored in 70% alcohol after 1 h fixation in Carnoy's Fluid to assess the pollen germination and fertilization.

Results

Crossability test

In total 20,225 flowers of 118 *B. rapa* varieties were emasculated and pollinated with GT73. On average, 171.4 flower buds were pollinated for each variety and 5.7 seeds were obtained from one pollination. The crossability varied largely from 0.05 to 19.4 seeds per pollination, depending on different seed parents, with 5 seed parents bearing over 15 seeds, accounting for 4.2% of the total cross combinations, 22 parents bearing more than 10 seeds, for 18.6%, and 20 parents bearing fewer than 3 seeds, for 17% (Table 1). Degao 8, a heading type of *B. rapa*, showed the highest crossability (19.4 seeds/flower) and Zhongmaowawacai, a non-heading type, exhibited the lowest crossability (0.05 seeds/flower). Luxing 80, 87 Chun 34, Yangchun, Chunqiuyu and Qiangshi also showed relatively high crossability (>14 seeds/pod), whereas the varieties Wenzhoupancai, Aijiaosuzhouqing, Japanese Zaoshengxiaobaicai, Qiulu 55, Chihua 4 and Xiawangaiqiqing produced fewer than 2 seeds per pollination (Table 1). On average, *B. rapa* L. ssp. *pekinensis* showed the highest crossability (7.86), followed by *B. rapa* L. ssp. *chinensis* var. *rosularis* (7.04), *B. rapa* L. ssp. *chinensis* var. *purpurea* (6.77), *B. rapa* L. ssp. *chinensis* var. *parachinensis* (5.32), *B. rapa* L. ssp. *chinensis* var. *chinensis* (5.06), *B. rapa* L. ssp. *chinensis* var. *oleifera* (4.91) and *B. rapa* L. ssp. *raifera* (3.18) (Table 2). A groupwise *t*-test showed

Table 2 Average crossability of different subspecies and varieties of *B. rapa*

Type of *B. rapa*	Number of combinations	Mean (seeds per flower)	Standard deviation
A: *B. rapa* L. ssp. *pekinensis* Olsson	60	7.86	4.17
E: *B. rapa* L. ssp. *chinensis* var. *rosularis* Tsen et Lee	6	7.04	3.14
C: *B. rapa* L. *ssp. chinensis* var. *purpurea* Mao	4	6.77	1.67
D: *B. rapa* L. ssp. *chinensis* var. *parachinensis* Tsen et Lee	10	5.32	3.51
B: *B. rapa* L. ssp. *chinensis* var. *chinensis* Kitam	33	5.06	2.61
G: *B. rapa* L. ssp. *chinensis* var. *oleifera*	3	4.91	1.61
F: *B. rapa* L. ssp. *raifera* Matz	2	3.18	4.01
Total	118	5.73	

Note: A is heading type, others belong to non-heading types of *B. rapa* as shown in Fig. 1

that the difference between the heading and the non-heading types of *B. rapa* L. was highly significant ($P = 0.00022$), and no significant differences were found among the non-heading types of *B. rapa*.

Spontaneous outcrossing rate

The spontaneous outcrossing rate was measured with three *B. rapa* varieties. The highest outcrossing rate was 0.406%, found in Wutacai (*B. rapa* L. ssp. *chinensis* var. *rosularis* Tsen et Lee), followed by 0.144% in Wuyuemanqingcai (*B. rapa* L. ssp. *chinensis* var. *chinensis* Kitam) and 0.039% in Shiyuehong (*B. rapa* L. ssp. *chinensis* var. *purpurea* Mao) (Table 3). A groupwise t-test showed that the difference between Wutacai and Zhongyou 821 (CK) was not significant ($P = 0.468$) but the differences between Wutacai and Wuyuemanqingcai or Shiyuehong were significant ($P = 0.045$ and $P = 0.002$). However, differences between Wuyuemanqingcai and Shiyuehong were not significant ($P = 0.231$). This was consistent with the results of the artificial crossability, suggesting that the stronger the crossability, the higher was the spontaneous outcrossing rate. The frequency of gene flow to Wutacai was higher than that of Wuyuemanqingcai and Shiyuehong.

All hybrid plants were confirmed by PCR assay. The results showed that a target DNA band with over 500 bps was detected from all tolerant plants and the positive control Ms8 × Rf3 but not from the sensitive plants after PCR amplification (Fig. 2), indicating that herbicide-tolerant plants contain the *bar* gene from the spontaneous outcrossing.

Fig. 2 Identification of resistant plants by PCR. *M* DNA Marker; *1* transgenic rapeseed Ms8Rf3; *2* no template control; *3* negative control; *4–6* resistant plants of Wutacai; *7–9* sensitive plants of Wutacai; *10–12* resistant plants of Wuyuemanqingcai; *13–15* sensitive plants of Wuyuemanqingcai; *16–18* resistant plants of Shiyuehong; *19–21* sensitive plants of Shiyuehong

Pollen germination and fertilization

When *B. rapa* L. ssp. *chinensis* var. *rosularis* (Wutacai) was pollinated with transgenic rapeseed, about 8–15 adhesive pollens were observed on the stigma 1 h after pollination (HAP; Fig. 3b). The number increased to 30–50 at 3 HAP, with a few pollen tubes found in stigma. At 8 HAP, the beam of pollen tubes penetrated into the style, and the pollen tubes reached approximately the upper 1/3 of the style (Fig. 3d). At 27 HAP, the pollen tubes penetrated into the lower part of the style, with 10–20 pollen tubes in each style (Fig. 3f). At 48 HAP, some pollen tubes penetrated into the ovule via the micropyle and they began to fertilize, whereas bright yellow-green pollen tubes were seen on the micropyle (Fig. 3h). In the self-pollinated control, 50–100 adhesive pollens were observed on the stigma for an hour after self-pollination (Fig. 3c), Three hours later, the number of adhesive pollens increased to 200–300 and many germinated and penetrated into papilla cells. Eight hours later, more than 50 beams of pollen tubes penetrated into half the style (Fig. 3e). Twenty-seven hours after pollination, the pollen tubes had penetrated into the ovule via the micropyle and began to fertilize (Fig. 3g). Forty-eight hours later, it was clear that the bundled pollen tubes had

Table 3 Spontaneous outcrossing rate of transgenic rapeseed and *B. rapa*

Cultivars	Total number of tested plants			Number of herbicide-resistant plants			Outcrossing rate (%)			Mean	Standard error	Difference with Wutacai *t*-test(*P*)
	I	II	III	I	II	III	I	II	III			
Wutacai	13483	26164	16971	64	79	75	0.475	0.302	0.442	0.406	0.092	
Wuyuemanqingcai	11980	22300	8096	32	34	1	0.267	0.153	0.012	0.144	0.128	0.045
Shiyuehong	18740	14673	16509	4	7	8	0.021	0.048	0.049	0.039	0.015	0.002
ZY821	13080	27068	23160	72	262	53	0.551	0.968	0.229	0.583	0.371	0.468
Mean							0.254	0.167	0.168	0.196	0.189	

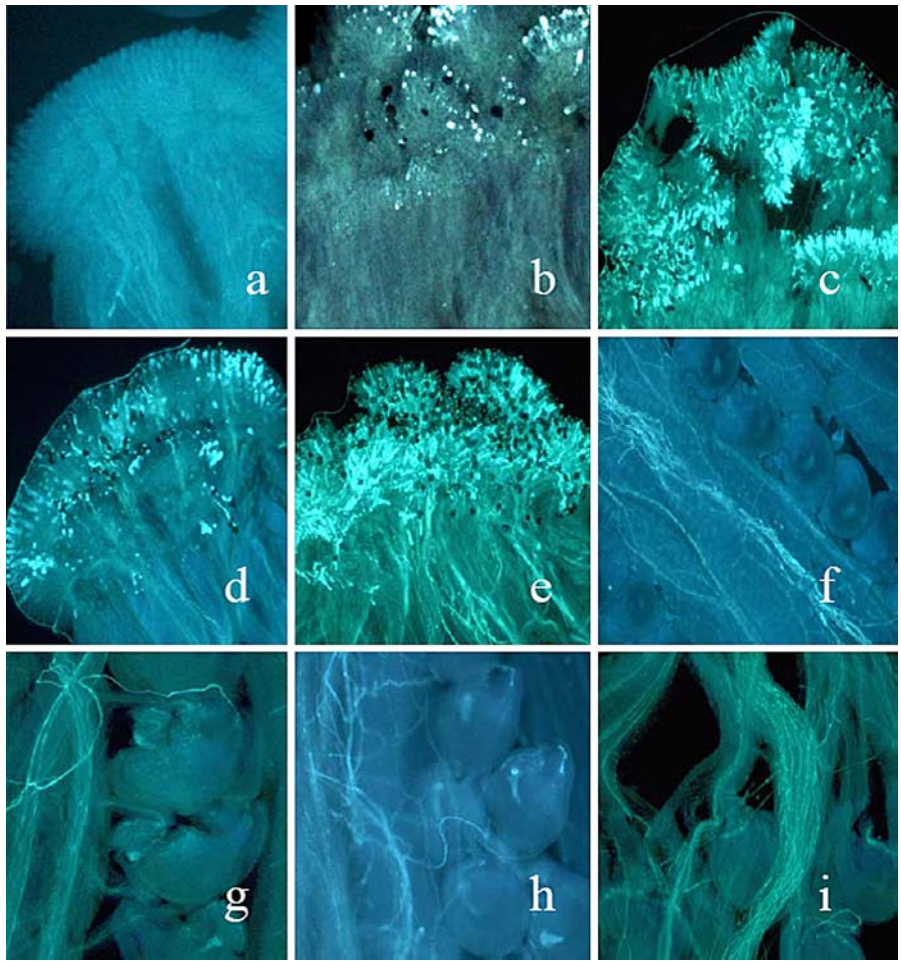

Fig. 3 **a** No pollination of *B. rapa*; **b** *B. rapa* pollinated with pollens of Ms8 × Rf3 at 1 HAP; **c** Self-pollination of *B. rapa* at 1 HAP; **d** *B. rapa* pollinated with pollens of Ms8 × Rf3 at 8 HAP; **e** Self-pollination of *B. rapa* at 8 HAP; **f** *B. rapa* pollinated with pollens of Ms8 × Rf3 at 27 HAP; **g** Self-pollination of *B. rapa* at 27 HAP; **h** *B. rapa* pollinated with pollens of Ms8 × Rf3 at 48 HAP; **i** Self-pollination of *B. rapa* at 48 HAP

penetrated into the ovule and fertilized completely (Fig. 3i).

Similar results were also found in *B. rapa* L. ssp. *chinensis* var. *chinensis* Kitam (Wuyuemanqingcai) and *B. rapa* L. ssp. *chinensis* var. *oleifera* (0069), which were pollinated with transgenic *B. napus*. There were 8–15 adhesive pollens on the stigma of *B. rapa* for 1 HAP and 30–50 adhesive pollens 3–8 HAP with a few pollens germinating. A large amount of callose was observed on the surface of the papillary cells that touched the pollen tubes. At 27 HAP, a few pollen tubes were seen penetrating into the bottom of the styles, whereas numerous pollen tubes penetrated into the ovary and began to fertilize the eggs in the control. Up to 48 HAP, a few pollen tubes were still seen in ovaries and were begining to fertilize the eggs.

The above observation indicated that the number of pollens on the stigma was reduced by 80%, the number of pollen tubes in the style were reduced by 2/3, and the fertilization time was delayed by over 20 h when pollinated with the transgenic *B. napus* in comparison with the bud self-pollination of *B. rapa* as a control.

Seed morphology

The seeds of *B. napus* c.v. GT73 were large and black, whereas the seeds of *B. rapa* observed in this experiment were generally small and red (Fig. 4). When *B. rapa* was pollinated with GT73, the F_1 hybrids tended to have an intermediate size, red seed coat and high frequency of cracked seeds (Fig. 4b, e–g), which was not found in the F_1 hybrids when the resynthesized *B. napus* was used as the male parent (data not cited). The rate of the cracked seeds varied with cross-combinations from 15 to 70%, generally about 30%.

Seed germination ability

The seeds collected from the *B. rapa* plants pollinated with transgenic *B. napus* germinated poorly (Fig. 5; Table 4). The germination rate of the seeds without discarding the cracked seeds averaged 19.7%, with a range from 1 to 70%. *B. rapa* L. ssp. *pekinensis* Olsson showed the highest germination ability, with an average rate of 41.7% and *B. rapa* L. ssp. *raifera* Matzg exhibited the lowest, with an average rate of 3%. *B. rapa* L. ssp. *chinensis* var. *rosularis* Tsen et Lee, *B. rapa* L. ssp. *chinensis* var. *oleifera*, *B. rapa* L. ssp. *chinensis* var. *parachinensis* Tsen et Lee, *B. rapa* L. ssp. *chinensis* var. *purpurea* Mao and *B. rapa* L. ssp. *chinensis* var. *chinensis* Kitam showed a medium germination rate ranging from 5 to 17.7%. Once

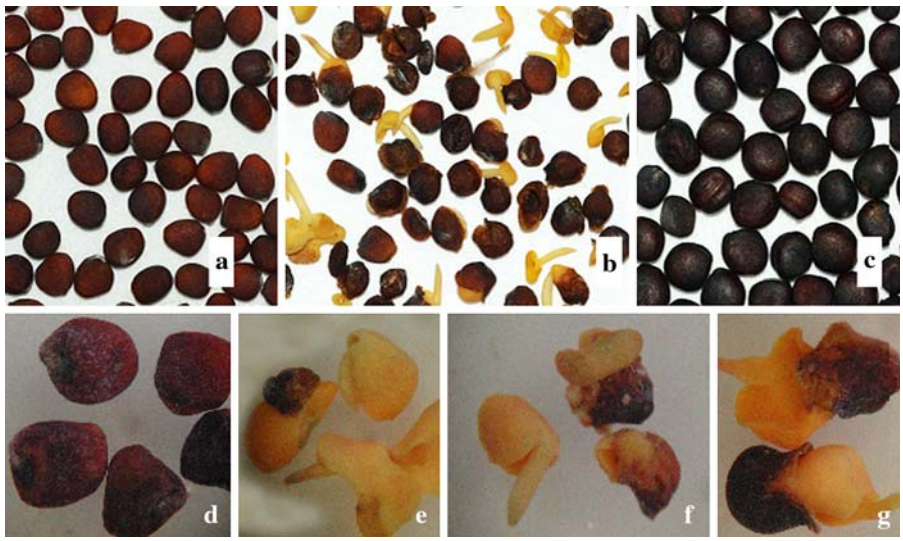

Fig. 4 Interspecific hybrid seeds and their parents. **a** Seeds of female parent *B. rapa*; **b** Seeds of F_1 hybrid; **c** Seeds of transgenic *B. napus* as pollen donor; **d** Full seeds of F_1 hybrid; **e–g** Seeds of F_1 hybrid showing various degree of cracked seeds

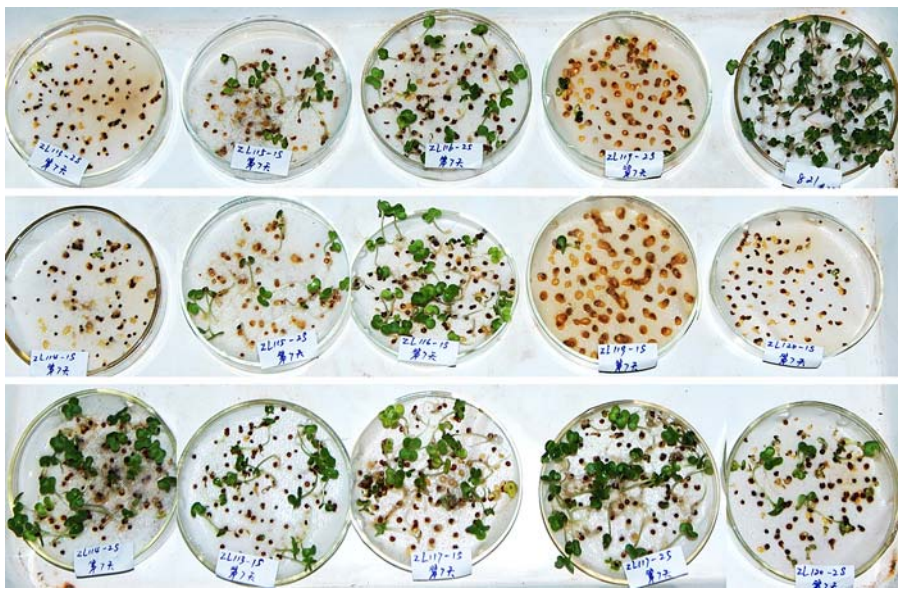

Fig. 5 Germination of hybrid seeds collected from *B. rapa* pollinated with transgenic *B. napus* in contrast to the check *B. napus* c.v. Zhongyou 821 at the right upper corner of the first row

Table 4 Germination rate of F_1 hybrid seeds of *B. rapa* crossed with transgenic rapeseed

Cultivars	Number of seeds	Germination rate discarding cracked seeds (%)	Germination rate including cracked seeds (%)	Cultivars	Number of seeds	Germination rate discarding cracked seeds (%)	Germination rate including cracked seeds (%)
A18	200	55.0	22.0	D1	200	64.0	1.5
A19	200	51.0	25.5	D3	200	98.0	14.5
A20	200	74.0	63.5	D4	200	81.0	21.5
A21	200	83.5	70.0	D6	200	61.0	1.5
A22	200	82.0	44.0	D7	200	90.0	5.0
A23	200	48.5	7.5	D9	200	60.0	1.5
A24	200	93.0	43.5	D10	200	82.0	3.0
A25	200	85.0	66.5	E1	200	63.0	7.5
A27	200	68.0	9.0	E2	200	82.0	12.0
A29	200	88.5	65.0	E3	200	78.0	18.0
B30	200	66.0	6.0	E4	200	83.0	22.5
B31	200	36.0	1.0	E5	200	97.0	28.5
B32	200	78.0	2.5	F2	200	75.0	3.0
B33	200	90.0	11.5	Mean of 10 cultivars of A	–	72.9	41.7
C1	200	53.0	6.5	Mean of 4 cultivars of B	–	67.5	5.3
C2	200	64.0	23.0	Mean of 4 cultivars of C	–	67.8	11.4
C3	200	70.0	6.0	Mean of 7 cultivars of D	–	76.6	6.9
C4	200	84.0	10.0	Mean of 5 cultivars of E	–	80.6	17.7
G1	200	67.0	8.0	Mean of 1 cultivars of F	–	75.0	3.0
G2	200	76.0	36.0	Mean of 3 cultivars of G	–	65.0	15.5
G3	200	52.0	2.5	Total Mean	–	72.9	19.7
				CK(821)	200	80.5	80.5

discarding the cracked seeds, the germination rate of the hybrids increased conspicuously to an average of 72.9%, ranging from 36 to 98%, which did not differ statistically from the rate of control *B. napus* c.v. Zhongyou 821 ($P = 0.587$). The highest germination rate (98%) was found in the Zaoshubaicaitai hybrid and the lowest (36%) in the Gaohuaqinggengbaicai hybrid. The results indicated that the cracked seeds germinate poorly. On the other hand, the germinated interspecific hybrids appeared to grow faster and showed significant heterosis compared with the control Zhongyou 821 (Fig. 5). Different repetitions of the same genotype were consistent in the germination rate, indicating that the germination rate was highly inherited.

Discussion

Numerous studies have investigated the gene flow of transgenic rapeseed to wild weed *B. rapa* and oilseed rape *B. rapa* (Paul et al. 1995; Baranger et al. 1995; Mikkelsen et al. 1996; Rieger et al. 1999; Messeguer 2003; Brown 1995), but few studies have been conducted on various subspecies or varieties of *B. rapa*. In this study, we investigated the interspecific crossability of 118 cultivars from 7 *B. rapa* subspecies and varieties, and showed that all of the *B. rapa* varieties could bear seeds by artificial pollination with transgenic *B. napus*. Different varieties can exhibit different levels of crossability and outcrossing under the same experimental and

environmental conditions (Tables 2, 3). The average crossability of *B. rapa* by the transgenic *B. napus* was 5.7 seeds per flower, with a range from 0.05 to 19.4, which was about 1/3 of the self-pollination. On the whole, the heading types of *B. rapa* showed a higher crossability than the non-heading types of *B. rapa*.

The variation of crossability among the different genotypes of *B. rapa* may derive from different factors: (1) Adhesion of pollens to the stigma; (2) Penetration of pollen tubes into the style; and (3) Abortion of fertilized embryos during development. Compared with the flower-bud self-pollination of *B. rapa*, the amount of pollen adhesion on the stigma and the number of pollen tubes in the style were significantly reduced. Furthermore, the amount of callose deposited in papillose cells increased with the pollination of *B. napus* on *B. rapa* (Fig. 3), indicative of reproductive barriers before fertilization, which was in agreement with the reports of Meng (1990). Many studies also reported that there were similar fertilization barriers with self-incompatibility during the self-pollination of *B. rapa*. Although most varieties of *B. rapa* used in this experiment were self-incompatible, they did not show such heavy self-incompatibility because we made the crosses with the flower-buds and the self-incompatibility had not yet developed.

Under natural pollination, the outcrossing rate between *B. rapa* and the transgenic *B. napus* ranged from 0.039 to 0.406%, with an average of 0.19%, which was about 1/3 of the rate of the control (Table 3). Scott and Wilkinson (1998) found that the spontaneous outcrossing rate of *B. napus* and *B. rapa* ranged from 0.4 to 1.5%, which was closer to our results in this experiment. However, other researchers reported that the spontaneous outcrossing rate of *B. napus* and *B. rapa* was as high as 9–93%, with the condition that a *B. rapa* plant was surrounded by a large number of *B. napus* plants (Jorgensen et al. 1996). In addition to the genotypic difference of crossability, the spontaneous outcrossing rate is also affected by flowering synchrony, the height of parental plants, the topography and weather factors (Scheffler et al. 1995; Staniland et al. 2001). In addition, in the case of homozygous glyphosate and glufosinate resistant plant lines, all pollen carries the herbicide resistance gene. By contrast, in studies of cross-fertilization from glufosinate-resistant hybrids, the amount of transgenic pollen was lower, resulting in only about 5/8 of the outcrossing frequencies of homozygous herbicide-resistant lines.

If interspecific fertilization does take place, the F_1 hybrid embryos and plants often suffer from malfunction and low survival and fertility upon reproduction (Stebbins 1958). The interspecific embryos were aborted and stopped during their development to seeds at various stages (Fig. 4). Only some of the embryos developed to seeds with normal germination ability (Table 4; Fig. 5). Usually a pistil of *B. rapa* has about 30 ovules and, on average, 1/6 of the ovules develop into normal seeds after pollination with *B. napus* c.v. GT73, which means that about 5/6 of the ovules were aborted in various stages of the development.

A high frequency of broken or germinated seeds was found in pods in all 118 interspecific hybrids, whereas few were found in hybrids when the resynthesized *B. napus* was used as the male parent, indicating that the frequency of broken seeds in pods was highly related to the genotype of the pollen donor. On the other hand, hybrids derived from different female parents showed significant differences in the frequency of broken seeds in the pods. For example, with the same transgenic rapeseed GT73 as the male parent hybrids from Gongguan2, Yuqingbaicai and Luxing80 showed relatively low frequency of broken seeds (<15%), whereas most of the *B. rapa* varieties as females showed frequencies higher than 40%. Broken seeds observed in this experiment may have significant ecological and agronomical impacts due to the reduced fitness of the seeds of interspecific hybrids and the reduced quality of seeds of *B. rapa* vegetables.

Broken or germinated seeds were also reported by other researchers in a similar experiment (Hauser and Ostergard 1999) and was called precocious germination in the pod. According to our observation, however, the broken seeds in pods resulted not only from precocious germination but also from the abortion of the fertilized embryos at various developmental stages. According to previously published work (Black 1991), in immature seeds, the high abscisic acid (ABA) levels prevent precocious germination, whereas desiccation does the same in dry seeds. This indicates that ABA and low water potentials control seed germination. The seed-specific immunomodulation resulted in the switch from seed maturation to germination (Phillips et al. 1997).

Therefore, the precocious germination found in this experiment may result from the wrong timing of the switch from the seed maturation to a germination. In general, seeds of *B. rapa* are smaller than seeds of *B. napus*. The hybrids look like *B. rapa* female parents in seed size and seed color (Fig. 4). Because the hybrid embryos come from the fertilization of both parents, whereas the pods and the seed coat comprised maternal tissue coming from the *B. rapa* female parent, the disharmonization of the developing embryos in a limited space of seed coat and pod may be partly responsible for the high frequency of broken seeds found in this experiment.

So far, few reports exist on the germination rate and morphological features of the hybrid seeds between *B. rapa* and *B. napus*. We found that the germination rate of the full seeds of the interspecific hybrids ranged from 36% to 98%, with an average of 72.9%, which a little lower than the rate of normal seeds. By contrast, the cracked seeds showed a much lower germination ability.

The interspecific hybrid between *B. rapa* and *B. napus* is a sesquidiploid, with an AAC genome composition and a chromosome number of $2n = 29$. The F_1 hybrid plants grew well and bore an average of 4 seeds per pod in open pollination (Lu et al. 2002). A mean of 0.1–2.0 seeds per flower were set in the backcrosses of the interspecific hybrids to *B. rapa* (Lu et al. 2002). Most backcross progenies were aneuploid (Lu et al. 2001), and their survival fitness was about one-tenth of the normal fitness. The backcross progeny was relatively weak in growth and very low in pollen and seed fertility, with only a few exceptions that were close to the normal rates (Lu and Kato 2001; Hauser et al. 1998a, b). If there was no pressure of positive selection, the frequency of the exogenous gene in the population of *B. rapa* would gradually decline and disappear (Lu et al. 2002). However, if foreign genes immigrated continuously, especially under favorable selection pressure, there should be a greater possibility that the foreign genes will be integrated into *B. rapa* (Lu et al. 2002). The spread of transgenic plants to conventional populations depends on the traits introduced by genetic modification and on the fitness advantage of the genotypes carrying the transgene. Therefore, careful estimation of the relative fitness of transgenic plants in conventional populations is needed to assess the risk of transgene spread.

Some practical measures suggested to control gene flow are isolation distances and local and aerial restrictions and barriers (Eastham and Sweet 2002). It appears that the use of predominantly self-pollinating, male sterile, or cleistogamous cultivars as a biological containment strategy will also reduce gene flow. Our data indicated that such genotypes of *B. rapa* as the cultivar Zhongmaowawacai tend to be very low in crossability with *B. napus* in favor of reduction of gene flow. On the other hand about 4% of the *B. rapa* cultivars showed high crossiblity with *B. napus*, being over 15 seeds per pollination. For those *B. rapa* cultivars more strict measures should be taken in order to prevent the pollen contamination. This is particularly important for vegetable seed production, because the pollen contamination may result in high frequency of cracked seeds.

In summary, the gene transferability from *B. napus* to various *B. rapa* subspecies (or varieties) were documented. The interspecific crossability varied with heading and non-heading varieties and with different cultivars. The natural outcrossing rates were much lower than the artificial crossability, indicating that it should be possible to keep the adventitious presence of the off-plants under the allowed threshold, if proper measures are taken.

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