Gene flow from genetically modified herbicide-resistant rapeseed to cruciferous weeds*

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Abstract The sexual compatibility between genetically modified (GM) glyphosate-resistant rapeseed variety Q3 (*Brassica napus* L.) and 5 cruciferous weeds is studied through the observation of fluorescence microscopy and cross-fertility after manual pollination. The results indicated that Q3 (as male) was highly incompatible with *Thlaspi arvense* L., *Capsella bursa-pastoris* (L.) Medic, *Cardamine hirsuta* L. and *Rorippa palustris* (L.) Besser (as female). Fluorescence microscopic observation showed that growing of pollen tubes terminated on the stigma surface or at the upper 1/3 part of the style. However, *B. juncea* × Q3 was compatible, and the compatibility index was 1.65. Under the neighboring growth and natural pollination conditions, the rates of gene flow from Q3 to *T. arvense*, *C. bursa-pastoris*, *C. hirsute* and *R. palustris* were all 0, while it was 0.86% for *B. juncea*. These results indicate that there is difference in the rate of gene flow between GM rapeseed and cruciferous wild weeds, and frequency of gene flow is highly correlated with sexual compatibility.

Keywords: herbicide-resistant rapeseed, cruciferous weeds, aniline blue fluorescence, interspecific hybridization, sexual compatibility, gene flow.

Genetically modified herbicide-resistant crops are being increasingly produced worldwide[1]. A major concern about these releases is that transgene might flow from genetically modified crops (GMC) to their wild relatives through pollen drift, which may cause certain ecological risks. Prior to commercialization of GMC, the assessment of the potential gene flow to the relatives is very necessary[2,3].

Oilseed rape is one of the most important oil crops in the world; it belongs to the family of Cruciferae, genus *Brassica*. *Thlaspi arvense* L., *Capsella bursa-pastoris* (L.) Medic, *Cardamine hirsuta* L., *Rorippa palustris* (L.) Besser and *Brassica. juncea* L. are the common cruciferous wild weeds growing in the rapeseed fields in China[4,5]. These weeds and oilseed rape are all cross-pollinated plants, and the major method of pollen dispersal is anemophily and entomophlily. Due to herbicide-resistant gene escape to the compatible weeds through pollen drift, special concern is given to the rapeseed[6]. Up to date, most studies on gene flow between genetically modified (GM) rapeseed and its relatives focused on the turnip (*B. rapa* L.), the wild radish (*Raphanus raphanistrum* L.), *Sinapis arvensis* L., *Sinapis alba* L., etc.[7—12]. But detection of gene flow from herbicide-resistant rapeseed to *T. arvense*, *C. bursa-pastoris*, *C. hirsute* and *R. palustris* has been rarely reported. Because the rate of gene flow from GMC to its relatives is directly related to the sexual compatibility between them[13], in this work, we studied the sexual compatibility between glyphosate-resistant GM rapeseed variety Q3 (as male) and 5 cruciferous wild weeds (as female) through the observation of fluorescence microscopy and cross-fertility after manual pollination. The rate of gene flow from Q3 to these weeds under the neighboring growth and natural pollination conditions was also investigated so as to provide evidence for the evaluation of the ecological safety of growing GM herbicide-resistant rapeseed.

1 Materials and methods

1.1 Materials

GM glyphosate-resistant rapeseed variety Q3

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(*B. napus*) which had *GOX* and *CP4-EPSPS* genes conferring resistance to glyphosate from Canadian variety "Quest" was used as a pollen donor. The pollen receptors included 5 cruciferous weeds, i.e. *T. arvense*, *C. bursa-pastoris*, *C. hirsuta*, *R. palustris* and *B. juncea*. The Roundup™ (Monsanto, USA) was used for screening and identifying the individual progenies tolerant to glyphosate.

1.2 Sexual compatibility analysis

1.2.1 Germination of Q3 pollen on receptor stigma In September 2003, seeds of the pollen donor and receptors were sown in the greenhouse of Yangzhou University. All pollen receptor plants were grown in 50 cm × 45 cm pots, 30 pots for each variety. Before sowing, the receptors' seeds were treated with GA_3 to break dormancy using the method of Pu[14]. Artificial sterilization was conducted during flowering season, and the pistil left was bagged immediately. Manual pollination of each receptor plant using GM rapeseed pollen was carried out on the following day. The receptors were also self-pollinated as control. The 20—25 pistils of each combination were sampled at 0.5, 1, 4, 8, 12, 18, 24, 48 and 72 h after pollination, fixed in fixation solution of FAA (90 mL 70% ethanol, 5 mL acetum and 5 mL formalin) immediately, and stored below 4℃. The germination of pollen grain on the stigma of the receptors was observed with a fluorescence microscope (Leica DMLB) at an ultraviolet wavelength of 355—425 nm according to the aniline blue fluorescence (ABF) method[15].

1.2.2 Observation of cross-fertility after manual pollination Manual pollination using GM rapeseed pollen was carried out for those sterilized receptors. Totally 100—200 flowers were pollinated for each combination. Pod and seed settings were counted, and the compatibility index (the number of full seed settings/pollinated flower) was calculated.

1.3 Gene flow from Q3 under natural pollination conditions

1.3.1 Cultivation Experiment was conducted in an isolated open field on the Central Experimental Farm, Huai'an, Jiangsu Province. During 2003—2004, the GM glyphosate-resistant rapeseed variety Q3 plants were grown in pairs with each of the five pollen receptors at the row ratio of 1∶1. The plot area for each pair was 20 m^2. Randomized block design with 3 replicates was used in the experiment. Q3 plants were planted around the plots. Seedlings were transplanted to ensure 18 plants/m^2. Seeds of the receptors were treated to break dormancy before sowing. The cultivation and management were locally recommended. Time of beginning and end of flowering were recorded for each plant. All plants pollinated naturally. Each receptor variety was harvested separately for each plot after maturity.

1.3.2 Screening of the herbicide-resistant plants Seeds of each plot were sampled and sown under the field condition in order to screen and identify the individual progenies that showed glyphosate-tolerance in the following autumn. The preceding crop was spring maize. The experimental field included 15 25 m × 4 m plots without spontaneous seedlings of crucifers. Seedlings at 4—5 leaves stage were sprayed with 0.2% glyphosate at 750 kg/ha. Both dead and survived seedlings were counted 15 days later after being sprayed, and the rate of gene flow (the number of glyphosate-resistant plants/ total number of tested plants) through pollen drift from Q3 was calculated. Prior to spraying the herbicide, 200 plants of each receptor were tagged randomly, and leaves of 0.2—0.4 g for each plant were sampled in the 2.0 mL graduated centrifuge tubes, stored under −20℃.

1.3.3 Glyphosate-resistant gene detected by PCR All surviving and sensitive plants were detected by PCR analysis of the herbicide-resistant gene. Primers of *GOX* and *CP4-EPSPS* genes were synthesized according to the sequences published by Chen et al. and Pan et al.[16,17]. DNA was extracted from the leaves with sodium dodecyl sulfate (SDS) extraction method[18]. The PCR mixture (20 μL) contained 0.2 mmol/L dNTPs, 2 mmol/L $MgCl_2$, 0.10 μmol/L primer (Shenergy Biocolor, Shanghai, China), 40 ng template DNA, and 1 unit of *Taq* polymerase. The PCR amplification was performed with the program as follows: 4 min at 95℃, 30 cycles of 1 min at 94℃, 40 s at 59℃, and 40 s at 72℃, and a final extension of 10 min at 72℃. The PCR products were analyzed by electrophoresis using 1.5% agarose gel in Tris-acetate (TAE) buffer, photographed under ultraviolet light with Genegenius.

2 Results

2.1 Germination of Q3 pollen

In *T. arvense*, *C. bursa-pastoris* and *C. hir-*

sute, the number of adhesive pollen grains was extremely small and the pollen germinated slowly after pollination with Q3 pollen. The pollen tubes were short and coiled on stigma surface. No pollen tube penetrated into style and ovary even 48—72 h later. The growing of pollen tube was restrained on stigma surface (Fig. 1(a), (b)). In contrast, as to the self-pollination of *T. arvense*, *C. bursa-pastoris* and *C. hirsute*, some pollen tubes penetrated into ovule via micropyle 24—48 h after pollination.

The germination of Q3 pollen on *R. palustris* stigma showed some difference. There were 41—83 adhesive pollen grains on the stigma of *R. palustris* 18 h after pollination with Q3 pollen, and most of them germinated. It was observed that 11—16 pollen tubes penetrated into the upper 1/3 part of the style via papilla cells (Fig. 1(c)), other pollen tubes coiled on stigma surface. Some of papillary cells that touched with the pollen tubes generated callose. Between 48 and 72 h after pollination, there was no pollen tube penetrating into style bottom and ovary. The growing of pollen tube was restrained at the upper 1/3 part of the style. On the contrary, some pollen tubes penetrated into *R. palustris* ovule via micropyle 48 h after self-pollination.

However, the germination of Q3 pollen on the stigma surface of *B. juncea* showed more significant difference. The number of adhesive pollen grains was big and the pollen germinated fast. 18 h after pollination with Q3 pollen, 11—20 pollen tubes penetrated into the middle of ovary; 24 h later, 1—5 pollen tubes were found to penetrate into ovule via micropyle (Fig. 1(d)).

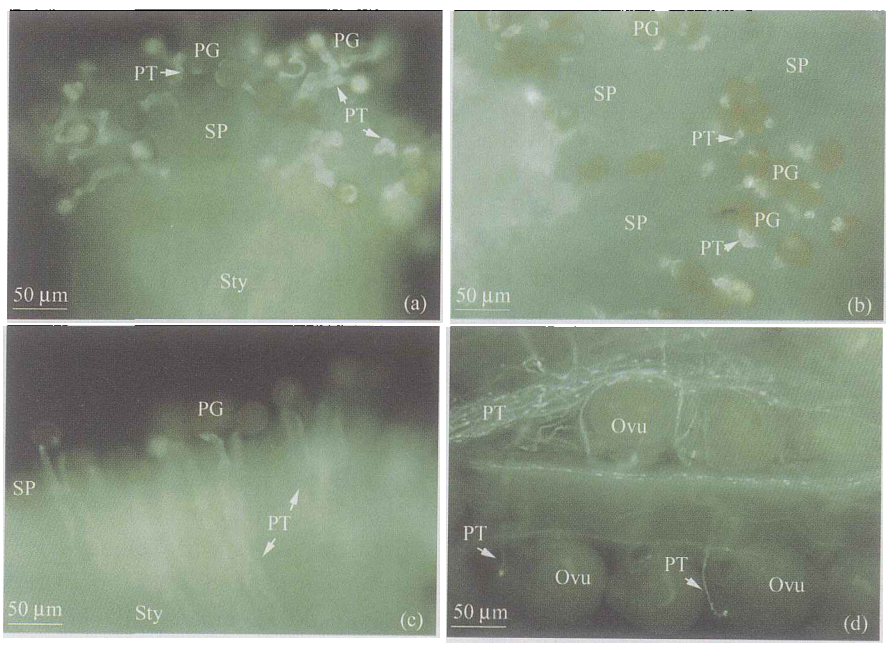

Fig. 1. The germination and growth of Q3 pollen grain on the stigma of the receptors using ABF method. (a) The stigma and style of *T. arvense* 12 h after pollination with Q3 pollen, the arrows showing cross-incompatibility and the pollen tubes coiled on stigma surface; (b) the stigma of *C. hirsuta* 18 h after pollination with Q3 pollen, the arrows showing cross-incompatibility and the pollen tubes coiled on stigma surface; (c) part of *R. palustris* stigma 18 h after pollination with Q3 pollen, the arrows showing cross-incompatibility and a few of pollen tubes penetrate into style via papillary cell, terminated at the upper 1/3 part of the style; (d) part of *B. juncea* ovary 24 h after pollination with Q3 pollen, the arrows showing pollen tubes penetrate into ovule through micropyle. PT, pollen tube; Sty, style; Ovu, ovule; SP, stigma papilla; PG, pollen grain.

In conclusion, *T. arvense*, *C. bursa-pastoris*, *C. hirsute* and *R. palustris* (as female) were found to be incompatible with Q3 (as male) for growing of pollen tube terminated at the upper 1/3 part of the style of *R. palustris* and on the stigma surface of the other 3 weeds. *B. juncea* × Q3 was compatible. This implied that there was a potential risk of gene flow from Q3 to *B. juncea*. As far as *T. arvense*, *C. bursa-pastoris*, *C. hirsuta* and *R. palustris* were concerned, the possibility of gene flow to them was very small.

2.2 Cross-fertility after manual pollination

After pollination with Q3 pollen, the percentage

of pod settings of *B. juncea* was 63.00%, 2.62 seeds were found per pod, and the compatibility index was 1.65. While the percentages of pod settings of *T. arvense*, *C. bursa-pastoris*, *C. hirsute* and *R. palustris* were very low, varying from 3.03% to 10.50%, and no seeds were obtained (Table 1).

Table 1. The percentage of pod settings and compatibility indices in the crosses of Q3 and the pollen receptor plants

Cross combinations	No. of pollinated flowers	No. of pods	Percentage of pod settings (%)	Full seeds	No. of seeds/pod	Compatibility indices
T. arvense × Q3	200	9	4.50	0	0	0
C. bursa-pastoris × Q3	200	21	10.50	0	0	0
C. hirsuta × Q3	150	5	3.33	0	0	0
R. palustris × Q3	165	5	3.03	0	0	0
B. juncea × Q3	100	63	63.00	165	2.62	1.65

This was consistent with the results of fluorescence microscopic observation. It indicated that the herbicide-resistant gene could not flow from Q3 to *T. arvense*, *C. bursa-pastoris*, *C. hirsuta* and *R. palustris*, while the possibility of gene flow to *B. juncea* was high.

2.3 Flowering synchronization

The synchronization of flowering between Q3 and the pollen receptors is listed in Table 2, which shows that there are long overlaps (17—25 days) in the blooming stage between Q3 and five receptor plants, indicating that Q3 pollen is very likely to drift onto the stigma of the 5-receptor plants under normal cultivation conditions.

Table 2. Synchronization of flowering between Q3 and the pollen receptor plants

Cultivars	Beginning of flowering (month/day)	End of flowering (month/day)	Days of flowering	Synchronization days of flowering
Q3	3/23	4/17	25	—[a)]
T. arvense	3/19	4/18	30	25
C. bursa-pastoris	3/13	4/22	40	25
C. hirsuta	3/19	4/14	26	22
R. palustris	3/31	4/22	22	17
B. juncea	3/28	4/21	24	20

a) Indicates a blank

2.4 The frequency of herbicide-resistant gene flow

The results of the screening of glyphosate-resistant plants by natural crossing in the open field are presented in Table 3. The table shows that the rate of gene flow from Q3 to *B. juncea* is 0.86%, and there is no gene flow to *T. arvense*, *C. bursa-pastoris*, *C. hirsute* and *R. palustris*.

It was demonstrated that glyphosate-resistant gene of Q3 was not likely to escape to *T. arvense*, *C. bursa-pastoris*, *C. hirsute* and *R. palustris*, but it could escape to *B. juncea* and made it produce resistance under natural pollination. This is consistent with the results assessed by the sexual compatibility, also showing that frequency of gene flow is highly related to sexual compatibility.

2.5 Herbicide-resistant plants examined by PCR

The true transgenic identity of the plants contaminated by gene flow has been further examined by PCR using primers derived from the respective herbicide-resistant gene. The results showed that a 398-bp target *CP4* DNA band and a 450-bp target *GOX* DNA band from every resistant plant of *B. juncea* were presented after PCR amplification, nevertheless, no target DNA segment band from the sensitive

Table 3. The frequency of *CP4* + *GOX* gene flow from Q3 to the pollen receptor plants

Cultivars	Total No. of tested plants			No. of glyphosate-resistant plants			Frequency of gene flow (%)			
	I	II	III	I	II	III	I	II	III	Average
T. arvense	11040	12580	12360	0	0	0	0	0	0	0
C. bursa-pastoris	10840	12020	9660	0	0	0	0	0	0	0
C. hirsuta	9820	11040	10180	0	0	0	0	0	0	0
R. palustris	10120	9760	9840	0	0	0	0	0	0	0
B. juncea	14360	15480	16020	122	116	159	0.85	0.75	0.99	0.86

Three replicates were adopted in this experiment

plants of the 5 weeds (Fig. 2(a), (b)). These findings proved that the glyphosate-resistant plants acquired in the open field had carried *CP4* + *GOX* gene, while the sensitive plants did not. This also clearly confirmed the high reliability of the above data.

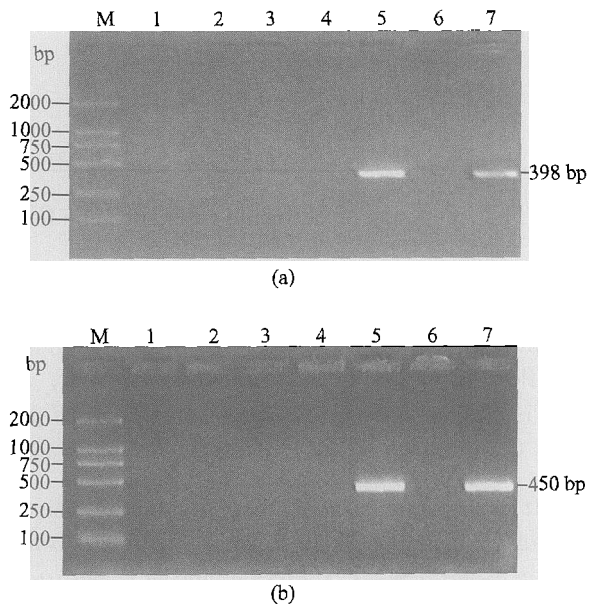

Fig. 2. Electrophoretic profile of PCR products amplified with *CP4* and *GOX* primers. M, DNA Marker; 1. sensitive plant of *T. arvense*; 2. sensitive plant of *C. bursa-pastoris*; 3. sensitive plant of *C. hirsute*; 4. sensitive plant of *R. palustris*; 5. glyphosate-resistant plant of *B. juncea*; 6. sensitive plant of *B. juncea*; 7. Q3.

3 Discussion

For herbicide-resistant gene escape to occur and make the relatives produce resistance, three conditions need to be met: (i) spatially, GMC and its relatives should be sympatrically distributed, i.e. grow in the same vicinity; (ii) temporally, the flowering time of them should overlap; and (iii) biologically, they must have a crossability to some extent[2]. Though all these factors are essential for gene flow, the crossability is of the greatest importance. In this study, the five cruciferous weeds are all often found growing with oilseed rape, there were long overlaps (17—25 days) in blooming stage between Q3 and these weeds. Q3 (as male) was found to be highly incompatible with *T. arvense*, *C. bursa-pastoris*, *C. hirsute* and *R. palustris* (as female) and compatible with *B. juncea* (as female) to some extent. Under the neighboring growth and natural pollination conditions, the rates of gene flow from Q3 to *T. arvense*, *C. bursa-pastoris*, *C. hirsuta* and *R. palustris* were 0 respectively, while *B. juncea* was 0.86%. The frequency of gene flow was highly relevant to sexual compatibility.

The fertilization of plant often begins with the pollen falling on the stigma, followed by germination of pollen and pollen tubes growing through the stylar canal or transmitting tissue of style, and then penetrating into ovule and female gametophyte, which ends the fertilization[19]. Only when the parents are closely relative can they succeed in fertilizing. In this study, pollen donor Q3 (*B. napus*) had AACC genome, pollen receptor *B. juncea* had AABB genome, both donor and receptor plants contained AA genome. Therefore, crossing between *B. juncea* and Q3 expressed a certain extent of compatibility. The results of fluorescence microscopic observation, manual hybridization and natural pollination in this study all justified this statement. It is also in agreement with the others' findings[20].

The intergeneric hybridization between rapeseed and wild weeds often shows cross-incompatibility. This kind of incompatibility is determined by the rejection reaction between the protein in the pollen wall and the protein pellicle on the stigma papilla surface[21], which causes the germination of pollen or growing of pollen tube restrains on a certain part of pistil or the gametes fail to mate[22—24]. In this study, the germination of Q3 pollen on *T. arvense*, *C. bursa-pastoris*, *C. hirsuta* and *R. palustris* stigma surface was observed by fluorescence microscopy. The results showed that pollen tubes of Q3 coiled on stigma surface of *T. arvense*, *C. bursa-pastoris* and *C. hirsuta*, growing of pollen tube was restrained on stigma. While some pollen tubes of Q3 coiled on *R. palustris* stigma, the others penetrated into the style, and the growing of pollen tube was restrained at the upper 1/3 part of the style. It indicated that the herbicide-resistant gene of Q3 could not flow to *T. arvense*, *C. bursa-pastoris*, *C. hirsuta* and *R. palustris*.

Rapeseeds are widely cultivated in China and most of them are *B. napus*. *B. juncea* is a cruciferous weed widely found in our country. The results of this study indicated that the compatibility of *B. juncea* × Q3 was high; the frequency of gene flow from Q3 reached 0.86% under natural pollination conditions. Therefore, we should pay more attention to the gene flow between GM rapeseed and wild weeds in the large-scale growth of transgenic rape-

seed. In addition, further studies are expected to be given on the issues of the genetic transfer capacity of herbicide-resistant gene in contaminated progenies, the sexual compatibility and gene flow between herbicide-resistant rapeseed and other cruciferous wild weeds, such as *Lepidium apetalum* Willd., *Malcolmia africana* (L.) R. Br., *Draba nemorosa* L., *Nasturtium officinale* L. and *Erysimum cheiranthoides* L.

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References

1 James C. Preview: Global status of commercialized transgenic crops: 2003. International Service for the Acquisition of Agri-Biotech Applications, Briefs No. 30-2003. Ithaca, New York.
2 Lu B. R., Song Z. P. and Chen J. K. Can transgenic rice cause ecological risks through transgene escape. Progress in Natural Science, 2003, 13(1): 17—24.
3 Qian Y. Q., Tian Y. and Wei W. Ecological risk assessment of transgenic plants. Acta Phytoecologica Sinica (in Chinese), 1998, 22(4): 289—299.
4 Wang Z. R., Xin M. Y., Ma D. H. et al. Farmland weeds in China. In: A Collection of Coloured Illustrative Plates. Beijing: China Agriculture Press (in Chinese), 1990, 96—112.
5 Ma C. Z., Liu B., Xu J. et al. Distinguishing and Controls of Field Weeds. Beijing: China Agricultural Press (in Chinese), 1999, 136—151.
6 Pu H. M. Review of genetically modified rapeseed with herbicide-resistance and ecologic safety. Chinese Journal of Oil Crop Sciences (in Chinese), 2003, 25(2): 89—93.
7 Halfhill M. D., Millwood R. J., Raymer P. L. et al. Bt transgenic oilseed rape hybridization with its weedy relative *Brassica rapa*. Environ. Biosafety Res., 2002, 1: 19—28.
8 Hansen L. B., Siegismund H. R. and Jorgenson R. B. Introgression between oilseed rape (*Brassica napus* L.) and its weedy relative *B. rapa* L. in a natural population. Genet. Resour. Crop Evol., 2001, 48: 621—627.
9 Warwick S. I., Simard M. J., Legere A. et al. Hybridization between transgenic *Brassica napus* L. and its wild relatives: *Brassica rapa* L., *Raphanus raphanistrum* L., *Sinapis arvensis* L., and *Erucastrum gallicum* (Willd.) O. E. Schulz. Theor. Appl. Genet., 2003, 107: 528—539.
10 Moyes C. L., Lilley J. M., Casais C. A. et al. Barriers to gene flow from oilseed rape (*Brassica napus*) into populations of *Sinapis arvensis*. Mol. Ecol., 2002, 11: 103—112.
11 Rieger M. A., Potter T. D., Preston C. et al. Hybridization between *Brassica napus* L. and *Raphanus raphanistrum* L. under agronomic field conditions. Theor. Appl. Genet., 2001, 103: 555—560.
12 Chevre A. M., Eber F., Darmency H. et al. Assessment of interspecific hybridization between transgenic oilseed rape and wild radish under agronomic conditions. Theor. Appl. Genet., 2000, 100: 1233—1239.
13 Dale P J. Spread of engineered genes to wild relatives. Plant Physiol., 1992, 100: 13—15.
14 Pu H. M. and Gao J. Q. Study on dormancy release of Cruciferous weed seeds. Weed Science (in Chinese), 2003, 1: 9—11.
15 Hu S. Y. Method of preparation of slides used to examine the pollen germination on the stigma and pollen tube growth in the style. Chinese Bulletin of Botany (in Chinese), 1994, 11: 58—60.
16 Chen J. H., Pan L. W., Shen Y. F. et al. Detection method of glyphosate oxidoreductase gene from genetically modified Roundup-Ready rapeseed. Chinese Journal of Oil Crop Sciences (in Chinese), 2001, 23(2): 63—67.
17 Pan L. W., Chen J. H., Shen Y. F. et al. Detection comparison of *CP4-EPSPS* gene from genetically modified Roundup-Ready rapeseed and soybean. Letters in Biotechnology (in Chinese), 2001, 12(3): 175—177.
18 Li J., Shen B. Z., Han J. X. et al. An effective procedure for extracting total DNA in rape. Journal of Huazhong Agricultural University (in Chinese), 1994, 13(5): 521—523.
19 Hu S. Y. and Yang H. Y. Biology of Angiosperm Fertilization (in Chinese). Beijing: Science Press, 2002, 128—130.
20 Bing D. J., Downey R. K. and Rakow G. F. W. Hybridizations 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, 1996, 115: 470—473.
21 Hu S. Y. Embryology of Angiosperm Plants. Beijing: People Education Press (in Chinese), 1983, 128—130.
22 Meng J. L., Liu D. F., Luo P. et al. Genetics of Plant Reproduction (in Chinese). Beijing: Science Press, 1995, 300—301.
23 Johri B. M. Experimental Embryology of Vascular Plants (in Chinese). Shanghai: Press of East China Normal University, 1986, 127—139.
24 Meng J. L. Studies on pollen-pistil interaction between *Brassica napus* and its relative species and genus. Acta Agronomica Sinica (in Chinese), 1990, 16(1): 19—25.