Gene flow from transgenic oilseed rape (*Brassica napus* L.) to cruciferous weeds under mentor pollen inducement*

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Abstract The alien gene flow between genetically modified glyphosate-resistant rapeseed variety Q3 (*Brassica napus* L.) and four cruciferous weeds was studied under mentor pollen inducement. The results showed that when *Thlaspi arvense* L., *Capsella bursa-pastoris* (L.) Medic, *Cardamine hirsuta* L. and *Rorippa palustris* (L.) Besser were pollinated with mentor pollen, the mixed Q3 and the weed, pollen grains aggregated largely and germinated quickly, and the numbers of pollen tubes penetrating into the style and the ovary were greatly increased as compared with corresponding self-pollination groups. Twenty four to forty eight hours after pollination, several pollen tubes were observed to penetrate into the ovule via micropyle in each mentor combination. However, when the mentor progenies were analyzed by PCR, all of them showed negative for the Q3 herbicide-resistant gene. Collectively, these results indicated that crossing between *T. arvense*, *C. bursa-pastoris*, *C. hirsuta*, *R. palustris* (as female) and Q3 (as male) was highly incompatible and the herbicide-resistant gene could not flow from Q3 to these four weeds.

Keywords: herbicide-resistant rapeseed, cruciferous weeds, mentor pollination, aniline blue fluorescence, gene flow.

Transgenic technology has advanced rapidly and is being widely used for the crop improvement in recent years[1]. In spite of the great economic benefits to humankind, genetically modified crops (GMC) have raised some bio-safety issues. One major concern is that transgene might flow from GMC to its relatives through pollen drift, which may cause certain ecological risks[2—6]. Therefore, the research on the risk of gene flow between GMC and its relatives is of great scientific importance.

Oilseed rape, which belongs to genus *Brassica* and the family of Cruciferae, is one of the most important oil crops in the world. *Thlaspi arvense* L., *Capsella bursa-pastoris* (L.) Medic, *Cardamine hirsuta* L. and *Rorippa palustris* (L.) Besser are four common cruciferous wild weeds growing in the rapeseed fields[7,8]. These weeds and oilseed rape are either cross-pollinated or often cross-pollinated plants, and the major method of pollen dispersal is through anemophily and entomoplily. The transgene escape of the GM rapeseed to the compatible weeds through pollen drift has raised special concerns. Up to date, most studies on gene flow between GM rapeseed and its relatives have been focused on the turnip (*B. rapa* L.), the wild radish (*Raphanus raphanistrum* L.), the wild mustard (*Sinapis arvensis* L.), the white mustard (*Sinapis alba* L.), etc.[9—15]. But detection of gene flow from GM rapeseed to *T. arvense*, *C. bursa-pastoris*, *C. hirsute* and *R. palustris* has rarely been reported.

Our previous studies demonstrated that *T. arvense*, *C. bursa-pastoris*, *C. hirsute* and *R. palustris* (all as female) were incompatible with glyphosate-resistant rapeseed variety Q3 (*B. napus*, as male) because pollen tube growth terminated on the stigma surface or at the upper 1/3 part of the style. This implied that the herbicide-resistant gene of Q3 might not flow to these four weeds[16]. Since these weeds all grow together with rapeseed, there must be a mixture of the weed pollen and the rapeseed pollen in the air. Theoretically, the pollen of GM rapeseed could germinate on the stigma of the weed and the pollen tubes could penetrate into the ovary and the ovule under mentor pollen inducement, which is a pollination method of using the mixture of com-

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patible and incompatible pollen. With pollen-stigma interaction, the compatible pollen may induce stigma to accept incompatible pollen[17,18].

In this paper, the experimental results of alien gene flow between glyphosate-resistant rapeseed variety Q3 (*B. napus*) and four cruciferous weeds under mentor pollination are reported. This study will provide a method for the evaluation of the ecological safety of growing GM herbicide-resistant rapeseed.

1 Materials and methods

1.1 Materials

The pollen donor of GM glyphosate-resistant rapeseed variety Q3 (*B. napus*) bearing the *GOX* and *CP4-EPSPS* genes that confer resistance to glyphosate was a descendant from the Canadian variety "Quest"[19]. The pollen receptors, which included four cruciferous wild weeds (*T. arvense*, *C. bursa-pastoris*, *C. hirsuta* and *R. palustris*) were kindly provided by Jiangsu Academy of Agricultural Sciences, China.

1.2 Germination of the mixed pollen on the receptor stigma

Seeds of pollen donor and receptors were sown in the greenhouse of Yangzhou University in 2003. All pollen receptor plants were grown in 50 cm × 45 cm pots. Before sowing, the receptors' seeds were treated with GA_3 to break dormancy using the method of Pu[20]. Artificial sterilization was conducted during flowering season, and the pistil left was bagged immediately. Manual pollination of each receptor plant with the mentor pollen, the mixture of Q3 pollen and the receptor's own pollen, was carried out on the following day. The receptors were also self-pollinated as a control. After 0.5, 2, 4, 8, 12, 18, 24 and 48 h of pollination, 20—25 pistils of each combination were collected, fixed in fixation solution of FAA immediately, and stored below 4 ℃. The germination of the mixture pollen 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 of Hu[21].

1.3 Observation of cross-fertility after mentor pollination

T. arvense, *C. bursa-pastoris*, *C. hirsuta* and *R. palustris* were pollinated using the mentor pollen (the mixture of Q3 pollen and the receptor's own pollen) inducement. One hundred to two hundred flowers were pollinated for each combination. Pod and seed setting were also investigated at a later growth stage.

1.4 Detection of glyphosate-resistant gene by PCR

The seeds of mentor plants were sown in 2004. At the three-leaf stage, all mentor progenies were analyzed by PCR to test for the presence or the absence of the transgenes *GOX* and *CP4-EPSPS* as we previously reported[16]. Seedlings at 4-5-leaf stage were sprayed with the 0.2% glyphosate at 750 kg/ha[22]. The survived seedlings were counted 15 days later.

2 Results

2.1 Germination of the mixed pollen

2.1.1 Adhesion of pollen grains onto the stigma surface

The adhesion of pollen grains onto the stigma surface was examined. In *R. palustris*, on average, 111.2 pollen grains were observed on the stigma 2 h after pollination with mentor pollen, and some of them germinated (Fig. 1(a)). However, there were only 26.3 pollen grains on the stigma 2 h after self-pollination, and none of them germinated (Fig. 1(b)). Four to forty-eight hours after mentor pollination, the number of pollen grains adhered to the *R. palustris* stigma reached 115.3—133.4; but 4—48 h after self-pollination, the number of adhered pollen grains was only 31.2—47.5.

The germination of the mixed pollen on *T. arvense*, *C. bursa-pastoris* and *C. hirsuta* stigma was similar to that on *R. palustris*, and the number of adhered pollen grains on the stigma was very large and the pollen germinated quickly when compared with the self-pollinated weeds (Table 1).

2.1.2 The number of pollen tubes penetrating into style and ovary

The number of pollen tubes penetrating into the style and the ovary was also investigated and the results are listed in Tables 2 and 3. From the tables, it can be seen that 2—48 h after mentor pollination the number of pollen tubes penetrating into the style and the ovary of *T. arvense*, *C. bursa-pastoris*, *C.*

hirsuta and *R. palustris* was higher than that of the corresponding self-pollination group. For instance, 4 h after pollination, 13.6 pollen tubes penetrated into *R. palustris* style, and 3.2 of them penetrated into the ovary in the mentor pollination group (Fig. 1(c)), whereas, no pollen tube was found in the corresponding self-pollination group (Fig. 1(d)). Eighteen hours after the pollination with mentor pollen on *C. hirsuta* stigma, the number of pollen tubes penetrating into the ovary reached 34.5 when compared with 10.1 in its self-pollination combination (Fig. 1(e)). In addition, 24—48 h after mentor pollination, several pollen tubes were found to penetrate into ovule via micropyle in the mentor pollination groups (Fig. 1(f)).

Table 1. The number of pollen grains on the stigma with different types of pollination

Combination	Hours after pollination							
	0.5	2	4	8	12	18	24	48
T. arvense (mentor pollination)	5.2	34.5	39.8	53.3	58.6	65.2	66.3	65.1
T. arvense (self-pollination)	0.3	15.2	23.4	40.3	43.7	48.3	50.2	48.2
C. bursa-pastoris (mentor pollination)	15.4	21.8	49.1	57.3	57.8	66.0	69.2	67.5
C. bursa-pastoris (self-pollination)	0	2.5	5.4	15.2	30.4	35.2	37.8	34.1
C. hirsuta (mentor pollination)	4.5	12.9	24.8	35.2	46.8	65.2	64.9	63.5
C. hirsuta (self-pollination)	0	1.9	7.6	16.5	23.9	33.8	35.4	34.2
R. palustris (mentor pollination)	25.8	111.2	115.3	118.0	125.3	128.2	132.2	133.4
R. palustris (self-pollination)	0.8	26.3	31.2	33.1	36.0	37.2	47.1	47.5

Table 2. The number of pollen tubes penetrating into the style with different types of pollination

Combination	Hours after pollination							
	0.5	2	4	8	12	18	24	48
T. arvense (mentor pollination)	0	9.7	20.1	23.2	25.9	32.5	38.5	37.1
T. arvense (self-pollination)	0	1.5	9.1	12.3	18.2	24.1	28.2	30.0
C. bursa-pastoris (mentor pollination)	0	14.5	40.8	43.5	47.5	53.0	55.5	51.6
C. bursa-pastoris (self-pollination)	0	0	0	0.5	23.8	25.9	28.0	24.1
C. hirsuta (mentor pollination)	0	0	7.2	15.2	33.5	45.2	47.9	48.6
C. hirsuta (self-pollination)	0	0	0	4.2	11.6	23.4	25.6	26.9
R. palustris (mentor pollination)	0	0	13.6	26.2	32.8	34.2	36.1	34.8
R. palustris (self-pollination)	0	0	0	17.5	19.4	22.1	26.2	26.1

Table 3. The number of pollen tubes penetrating into the ovary with different types of pollination

Combination	Hours after pollination							
	0.5	2	4	8	12	18	24	48
T. arvense (mentor pollination)	0	0	9.5	16.7	18.8	26.5	30.2	29.1
T. arvense (self-pollination)	0	0	0.2	0.8	5.8	14.3	17.2	20.8
C. bursa-pastoris (mentor pollination)	0	9.3	22.1	28.0	35.5	38.4	41.2	42.0
C. bursa-pastoris (self-pollination)	0	0	0	0	13.5	17.0	19.5	17.2
C. hirsuta (mentor pollination)	0	0	0	2.1	14.3	34.5	36.8	35.1
C. hirsuta (self-pollination)	0	0	0	0	2.6	10.1	12.5	16.9
R. palustris (mentor pollination)	0	0	3.2	6.8	9.2	11.2	16.5	24.5
R. palustris (self-pollination)	0	0	0	0	3.1	4.6	11.1	15.8

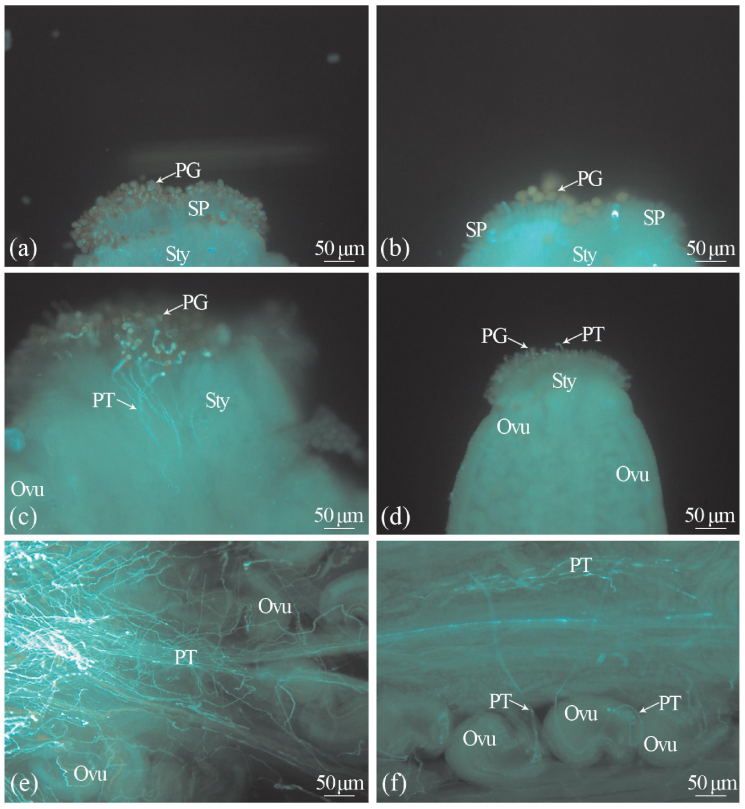

Fig. 1. The germination of the mixed pollen on the stigma of the receptor weeds using ABF method. (a) After mentor pollination for 2 h, many pollen grains adhered onto the stigma of *R. palustris*; (b) after self-pollination for 2 h, a few pollen grains adhered onto the stigma of *R. palustris*; (c) after mentor pollination for 4 h, some pollen tubes penetrated into the style and the ovary of *R. palustris*; (d) after self-pollination for 4 h, no pollen tube penetrated into the style and ovary of *R. palustris*; (e) after mentor pollination for 18 h, a number of pollen tubes penetrated into the ovary of *C. hirsuta* (f) after mentor pollination for 48 h, some pollen tubes penetrated into the ovule of *R. palustris* via micropyle. PG, pollen grain; SP, stigma papilla; Sty, style; PT, pollen tube; Ovu, ovule.

The above observations indicated that when *T. arvense*, *C. bursa-pastoris*, *C. hirsute* and *R. palustris* were pollinated with the mentor pollen, pollen grains adhered in large quantity and germinated quickly as compared to their corresponding self-pollination combination. In addition, the number of pollen tubes penetrating into the style and the ovary greatly increased. However, we could not determine whether the pollen tubes penetrating into the ovule were from Q3 or from the weeds by the observations of fluorescence microscopy only. Therefore, the following experiments were carried out.

2.2 Cross-fertility after mentor pollination

The results of cross-fertility of the four weeds after mentor pollination are presented in Table 4. As shown, when *T. arvense*, *C. bursa-pastoris*, *C. hirsute* and *R. palustris* were pollinated with the mixed pollen, the percentages of pod settings reached 40.51%—63.50%, and the number of seeds per pod reached 3.03—4.04.

Table 4. Pod and seed settings of the four weeds under mentor pollen inducement

Combination	No. of pollinated flowers	No. of pods	Pod settings (%)	No. of seed settings	Seeds per pod
T. arvense (mentor pollination)	200	127	63.5	407	3.2
C. bursa-pastoris (mentor pollination)	195	79	40.51	283	3.58
C. hirsuta (mentor pollination)	115	59	51.3	179	3.03
R. palustris (mentor pollination)	150	73	48.67	295	4.04

2.3 Glyphosate-resistant gene detection of mentor progenies

PCR analysis did not identify glyphosate-resistant gene product from 293 mentor progenies of *T. arvense*, 204 mentor progenies of *C. bursa-pastoris*, 109 mentor progenies of *C. hirsuta* and 185 mentor progenies of *R. palustris*. On the other hand, the positive control of Q3 plant produced a 398-bp band that correlated to the size of the *CP4* gene and a 450-bp band that correlated to the *GOX* gene. In addition, after spraying with glyphosate, no seedlings survived. These results indicated that the stigma of these weeds was selective in choosing their own pollen for fertilization rather than other pollens.

In conclusion, crossing between *T. arvense*, *C. bursa-pastoris*, *C. hirsuta*, *R. palustris* (all female) and Q3 (male) was highly incompatible, and the sexual-incompatibility between them could not be broken even after pollination with the mixed pollen. It further indicated that the herbicide-resistant gene could not flow from Q3 to these weeds.

3 Discussion

Whether the herbicide-resistant gene can flow from GM rapeseed to the wild weeds and confer resistance to the weed mainly depends on the sexual compatibility between these species[23]. 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[17, 18], which causes the germination of the pollen or the growth of pollen tube restrains on a certain part of the pistil or the failure of the gametes to mate[24, 25]. With pollen-stigma interaction, the incompatible pollen may enter into stigma under the inducement of the compatible pollen, which is called the mentor pollen or the recognition pollen. The mentor pollination method may overcome the incompatibility barriers using the mixture of the incompatible pollen and the inactivated compatible pollen, whose proteins in the pollen wall remain untouched. Since the recognition reaction between the pollen and the stigma is based on the proteins in pollen wall, incompatible pollen may be accepted by the stigma under the "cheat" of the proteins from the compatible pollen wall[17, 18]. Several such experiments had been conducted successfully in some plants.

T. arvense, *C. bursa-pastoris*, *C. hirsuta* and *R. palustris* are the common cruciferous wild weeds growing in the rapeseed fields, and they are all cross-pollinated or often cross-pollinated. These weeds all grow together with rapeseed, so there must be a mixture of the weed pollen and the rapeseed pollen in the air. Based on the mentor pollination theory, the weed pollen (compatible) may induce stigma to accept the rapeseed Q3 pollen (incompatible) when the mixed pollen falls on the stigma of the weed. In this study, *T. arvense*, *C. bursa-pastoris*, *C. hirsuta* and *R. palustris* were pollinated with the mixed pollen to simulate the natural conditions. By the fluorescence microscopic observation, we found that pollen grains germinated quickly and some pollen tubes penetrated into the ovule via micropyle. However, identification by PCR showed that all mentor progenies were negative with the Q3 herbicide-resistant gene. This indicated that there was a high level of cross-incompatibility between Q3 and these weeds, and the weeds preferred their own pollen to fertilize even after pollination with the mentor pollen. It further demonstrated that the alien glyphosate-resistant gene of Q3 could not flow to these weeds.

In this work, the mentor pollen failed to break the sexual incompatibility between transgenic rapeseed and the weeds, but accelerated the pollen germination and increased the fecundity. For instance, our previous studies have shown that the percentages of pod settings of *T. arvense*, *C. bursa-pastoris*, *C. hirsute* and *R. palustris* were only 3.03%—10.50% after pollination with Q3 pollen, and no seeds were obtained[16]; in contrast, the percentages of pod settings significantly increased when they were pollinated with the mentor pollen, varying from 40.51% to 63.50%, and 3.03—4.04 seeds per pod were detected. This is consistent with the report that the mentor pollen not only provides identifiable substance, but also provides stimulating substance for pollen and accelerates the fruit growth[26].

In order to simulate the natural conditions, the mentor pollen used in this study was the mixture of Q3 pollen and weed pollen. Because the weed pollen was not removed, we could not distinguish whether the pollen belongs to Q3 or the weed in the fluorescent microscopy study. In our further study, we will treat the mentor pollen with UV radiation or repeated freezing and thawing to investigate the gene flow from GM rapeseed to the wild weeds.

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