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ORIGINAL RESEARCH

Assessment of Gene Flow from Genetically Modified Anthracnose-Resistant Chili Pepper (*Capsicum annuum* L.) to a Conventional Crop

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Abstract We conducted a 2-year field assessment of the gene flow from genetically modified (GM) chili pepper (*Capsicum annuum* L.), containing the *PepEST* (pepper esterase) gene, to a non-GM control line "WT512" and two commercial hybrid cultivars, "Manidda" and "Cheongpung Myeongwol (CM)." After seeds were collected from the pollen-recipient non-GM plants, hybrids between them and the GM peppers were screened by a hygromycin assay. PCR with the targeting *hpt* gene was performed to confirm the presence of transgenes in hygromycin-resistant seedlings. Out of 7,071 "WT512" seeds and 6,854 "Manidda" seeds collected in 2006, eight and 12 hybrids, respectively, were detected. In 2007, 33 hybrids from 3,456 "WT512" seeds and 50 hybrids from 3,457 "CM" seeds were found. The highest frequency of gene flow, 6.19%, was observed in that 2007 trial. These results suggest that a limited isolation distance would be sufficient to prevent gene flow from GM to conventionally bred chili peppers.

Keywords *Capsicum annuum* · Chili pepper · Gene flow · Genetically modified (GM) crop

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Anthracnose is caused by several species of *Colletotrichum*, including *Colletotrichum gloeosporioides*, *Colletotrichum capsici*, and *Colletotrichum coccodes*. It is considered one of the most destructive diseases affecting chili peppers (*Capsicum annuum* L.), reducing annual fruit yields in Korea by about 13% (Yoon et al. 2004).

Expression of an esterase gene isolated from chili pepper is involved in anthracnose resistance by ripe fruits (Kim et al. 2001; Ko et al. 2005). Kim et al. (2006) have now obtained genetically modified (GM) plants resistant to anthracnose disease by introducing and over-expressing *PepEST* (pepper esterase) gene.

Although GM crops are currently not allowed into Korean cultivation, this is expected in the near future based on the pace of their development. Because of the abundance of such research, it would be valuable to investigate gene flow from GM to non-GM chili peppers in order to devise guidelines for field-testing and assessing environmental risks, which are mandatory prior to GM commercialization.

Mexico and northern Central America are considered the center of diversity for chili pepper (Heiser and Smith 1953; Pickersgill 1997; Perry et al. 2007). No compatible wild relatives exist in Korea, and *C. annuum* is the only species of *Capsicum* cultivated there. Although chili pepper is a partially self-pollinated crop (OECD 2006), high outcrossing rates often occur among cultivars (Campodonico 1983; Tanksley 1984). A gene flow study conducted by Kim et al. (2009) reported frequencies as high as 17.89% from GM chili pepper containing a coat protein gene (from cucumber

Table 1 Monthly rainfall, mean air temperature, humidity, and wind speed during the 2006 and 2007 growing seasons, recorded at the Cheongju Weather Station, 8 km from the study area

Months	Rainfall (mm)		Air temperature (°C)		Humidity (%)		Wind speed (m/s)	
	2006	2007	2006	2007	2006	2007	2006	2007
May	119.4	145.5	18.9	18.9	57.9	56.8	1.8	1.9
June	115.5	81.2	22.9	23.1	64.7	60.5	1.7	1.8
July	508.0	273.2	23.8	24.7	80.2	74.0	1.6	1.7
August	52.5	385.5	26.9	26.6	69.8	76.6	1.4	1.6
September	18.4	391.4	20.2	21.7	63.5	80.4	1.4	1.7
October	21.3	43.5	17.3	15.4	64.7	68.1	1.1	1.3

mosaic virus) to conventional chili pepper at the closest distance. Our research objective here was to evaluate the frequencies of gene flow from anthracnose-resistant GM chili pepper to its non-GM control line and commercial hybrid cultivars in the field.

Materials and Methods

Plant Materials

Derived from inbred line "WT512," GM chili pepper (*C. annuum* L.) resistant to anthracnose disease was developed through *Agrobacterium tumefaciens*-mediated transformation (Kim et al. 2006). Plants contain *PepEST* under the control of the cauliflower mosaic virus (CaMV) 35 S promoter, the *nos* terminator, and the hygromycin phosphotransferase (*hpt*) gene for hygromycin selection. Seeds of this GM pepper, its control line "WT512," and two commercial hybrid cultivars—"Manidda" and "Cheongpung Myeongwol (CM)"—were used.

Field Trials

Trials were conducted over 2 years in an isolated field at the Korea Research Institute of Bioscience and Biotechnology (KRIBB), Cheongwon-gun, Chungcheongbuk-do, Republic of Korea (36°43′04″ N, 127°26′07″ E, elevation 37 m). Total rainfall in the 2006 and 2007 growing seasons was 835.1 and 1,320.3 mm, respectively (Table 1; Korea Meteorological Administration 2009). Mean air temperature was 15.4–26.9°C, and mean humidity was 56.8–80.4%. Wind speed was higher in May–July than in August–October.

In May 2006, we established a 5×5 m central plot and two 5×20 m plots of four rows mulched with black plastic films (Fig. 1). On 18 May, 7-week-old seedlings were transplanted. These included: (1) 40 GM chili peppers at 50-cm spacings in the four rows of the central plot and (2) untransformed seedlings at 50-cm spacings in the east and west plots, with each plot containing 80 "Manidda" in rows a and b and 80 "WT512" in rows c and d. The closest plants to the central plot were 0.5 m away; the farthest, 20.0 m. Both GM and non-GM peppers were cultivated according to conventional practice. Insecticides were sprayed to control aphids and Oriental tobacco budworm from June to August.

The 2007 field trial, established in May, was conducted in plots designed for investigating nontarget effects and disease susceptibility of GM chili peppers. On 14 May, we established nine 7×8 m plots, six rows each (Fig. 2) that contained 7-week-old GM and non-GM "WT512" and "CM" seedlings. In each plot, 114 seedlings were planted 40 cm apart in rows a to f. Fungicides and insecticides were sprayed once in June to control *Phytophthora* blight and aphids, respectively.

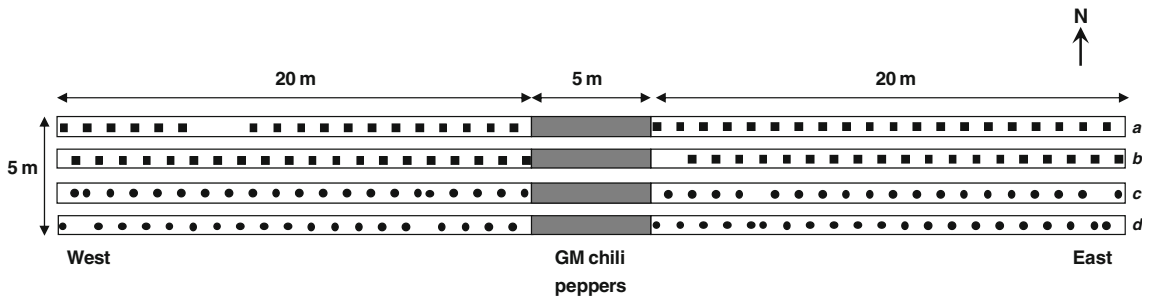

Fig. 1 Experimental design of 2006 field trial. GM chili peppers were planted in central plot. Non-GM control "WT512" and commercial cultivar "Manidda" were planted in four rows. *Filled circles* ("WT512") and *squares* ("Manidda") indicate plants from which fruits were collected for screening hybrids

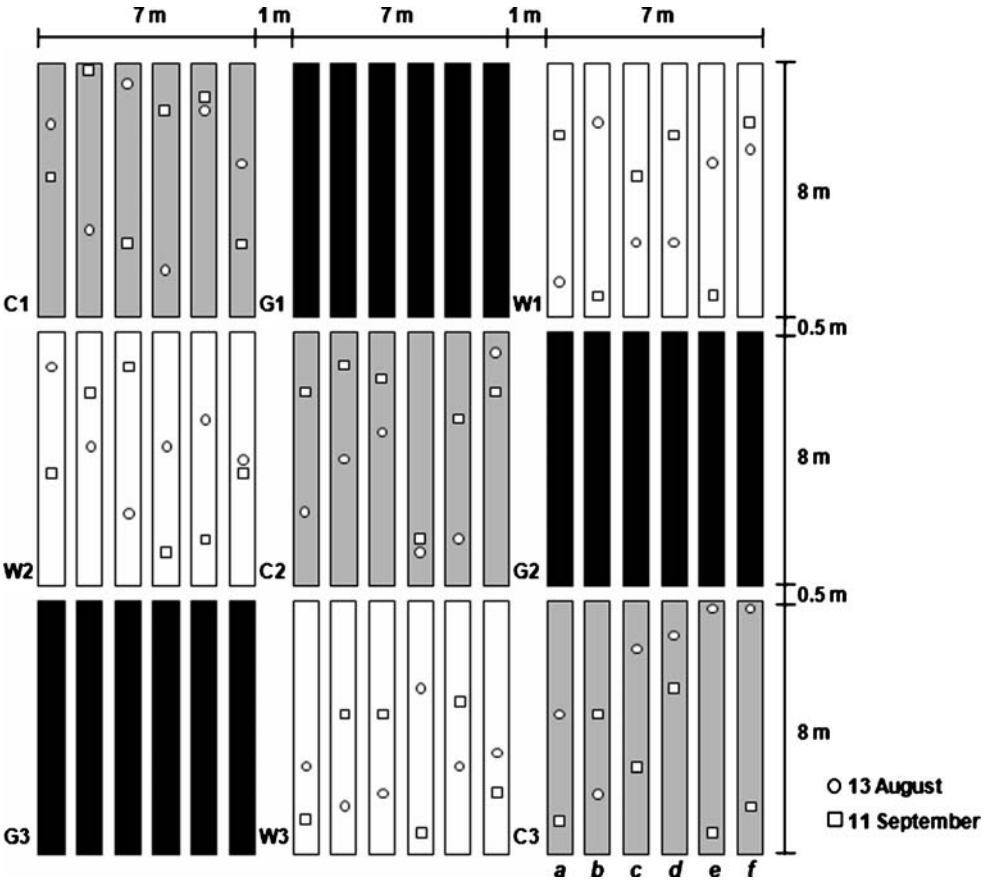

Fig. 2 Experimental design of 2007 field trial. GM chili peppers were planted in G1–G3 plots. Non-GM control "WT512" and commercial cultivar "CM" were planted in W1–W3 and C1–C3 plots. *Circles* and *squares* indicate plants from which fruits were collected for screening hybrids in August and September, respectively

Seed Sampling

In 2006, we harvested the ripened red fruits of "WT512" and "Manidda" on 11 August and 5 September. Ten per plant were collected at increasing distance (0.5 to 20.0 m) from the GM center plot. These were oven-dried at 60°C for 2 days before their seeds were separated and stored at room temperature (RT).

In 2007, the ripened red fruits of "WT512" and "CM" were harvested on 13 August and 11 September. Ten were collected from a plant randomly selected in each row within the "WT512" and "CM" plots. They were oven-dried at 40°C for 7 days, and their seeds were then separated and stored at RT.

Screening for Hybrids

Resistance to hygromycin (30 mg L^{-1}) was tested to screen for hybrid seedlings. In 2006, the samples collected in August and September were mixed before hygromycin

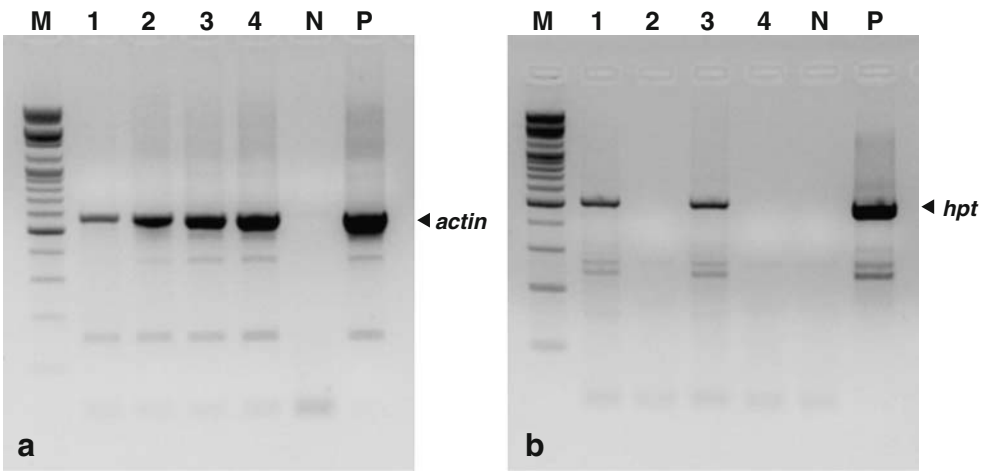

Fig. 3 Representative agarose gel electrophoresis patterns of PCR products from DNA of hygromycin-resistant hybrids obtained from 2006 field trial. **a** Amplification of *actin*; **b** amplification of *hpt*. *M* 100-bp DNA ladder; *lane 1* hybrid of GM/non-GM ("WT512"); *lane 2* "WT512"; *lane 3* hybrid of GM/non-GM ("Manidda"); *lane 4* "Manidda"; *N* negative control (no DNA); *P* positive control (GM chili pepper)

Table 2 Gene flow from GM chili peppers to non-GM control ("WT512") and a commercial hybrid cultivar ("Manidda") at increasing distances in 2006

Distance from GM chili pepper plot (m)	"WT512"		"Manidda"		Pooled data
	East	West	East	West	
0.5	2/56 (3.57)	2/88 (2.27)	1/98 (1.02)	0/86	5/328 (1.52)
1.0	0/70	1/94 (1.06)	ND	0/89	1/253 (0.40)
1.5	1/98 (1.02)	0/97	1/98 (1.02)	2/87 (2.30)	4/380 (1.05)
2.0	0/90	0/95	0/90	0/96	0/371
2.5	0/90	0/91	0/91	3/95 (3.16)	3/367 (0.82)
3.0	0/94	0/96	0/96	1/100 (1.00)	1/386 (0.26)
3.5	0/96	ND	0/67	0/92	0/255
4.0	0/51	1/90 (1.11)	0/87	1/97 (1.03)	2/325 (0.62)
4.5	0/98	ND	0/92	0/91	0/281
5.0	1/96 (1.04)	0/96	0/94	1/89 (1.12)	2/375 (0.53)
5.5	0/92	0/99	0/92	0/95	0/378
6.0	0/98	0/97	ND	0/100	0/295
6.5	0/89	0/77	0/89	0/95	0/350
7.0	0/78	0/98	0/86	1/98 (1.02)	1/360 (0.28)
7.5	0/93	0/91	0/94	0/93	0/371
8.0	0/97	0/90	0/90	0/94	0/371
8.5	0/84	0/96	0/91	0/93	0/364
9.0	0/98	0/71	0/94	0/100	0/363
9.5	0/94	0/84	0/91	0/94	0/363
10.0	0/99	0/82	0/78	0/99	0/358
10.5	0/95	0/96	0/91	0/90	0/372
11.0	0/95	0/89	0/93	0/53	0/330
11.5	0/96	0/90	0/87	0/96	0/369
12.0	0/81	0/97	0/93	0/99	0/370
12.5	0/96	0/84	ND	0/89	0/269
13.0	0/91	0/83	0/99	0/95	0/368
13.5	0/88	0/95	0/93	0/85	0/361
14.0	0/96	0/92	0/96	ND	0/284
14.5	0/91	0/95	0/96	0/93	0/375
15.0	0/91	0/88	0/90	0/94	0/363
15.5	0/91	0/85	0/90	0/95	0/361
16.0	0/93	0/92	0/61	0/65	0/311
16.5	0/91	0/77	0/92	0/93	0/353
17.0	0/96	0/97	0/76	0/84	0/353
17.5	0/98	0/93	0/85	0/90	0/366
18.0	0/96	0/94	1/92 (1.09)	0/92	1/374 (0.27)
18.5	0/98	0/93	0/93	0/94	0/378
19.0	0/95	0/91	0/91	0/91	0/368
19.5	0/94	0/82	0/98	0/59	0/333
20.0	0/99	0/94	0/88	0/92	0/373
Total	4/3632 (0.11)	4/3439 (0.12)	3/3322 (0.09)	9/3532 (0.26)	20/13925 (0.01)

Values are the number of PCR-positive seedlings/number of germinated seeds examined. Data in parentheses are gene flow frequencies (%).
ND no data

screening. However, for the 2007 samples, hybrids were screened from each August and September sample. Two autoclaved filter papers (no. 1; Advantec) were placed in a disposable 9-cm Petri dish. From each sample, 100 seeds were sterilized with a 5% sodium hypochlorite solution for 10 min, then washed five times with autoclaved distilled water for 5 min. They were then plated on the filter papers, and 8 mL of a 1/2 MS liquid medium (Murashige and Skoog 1962) containing 30 mg L^{-1} of hygromycin solution was added. In each batch, GM and "WT512" seeds were also prepared as controls.

All Petri dishes were incubated in a growth chamber (28°C, 70% relative humidity, and 16-h photoperiod) for 2 weeks, and the total number of germinated seedlings was counted. Seedlings with poor root and root hair growth compared with the GM controls were considered hygromycin-sensitive. Cotyledon and hypocotyl samples collected from hygromycin-resistant seedlings were crushed on PlantSaver™ FTA cards (Whatman, USA) to contain the plant DNA. They were then stored at RT.

Polymerase chain reaction (PCR) was performed with these carded samples in December of 2007 to detect hybrids. Forward primer Hygro-546F (GTG TCG TCC ATC ACA GTT T) and reverse primer Hygro-3R (GAA AAA GCC TGA ACT CAC C) were designed to amplify *hpt* (543 bp). Actin F (TGG ACT CTG GTG ATG GTG TC) and Actin R (CCT CCA ATC CAA ACA CTG TA) were designed to amplify the 560-bp *actin* gene. PCR utilized a final volume of 50 μL containing two disks from the FTA card, 0.5 μL of a 10 mM dNTP mixture, 0.5 μL of Taq DNA polymerase, 5 μL of 10× Taq buffer, and 2 μL of 10 pmol of each primer. Conditions for amplification included an initial denaturation at 95°C for 5 min; then 38 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 30 s, and extension at 72°C for 1 min; followed by a final extension at 72°C for 5 min.

For the 2007 samples, a duplex PCR method was used. Forward primer Hygro-546F (GTG TCG TCC ATC ACA GTT T) and reverse primer Hygro-3R (GAA AAA GCC TGA ACT CAC C) were designed to amplify *hpt* while, 850U (GGT GAA CCT TGG AAC GAA TG) and 1184L (GAG CAG TCT AAT GCA CAA AGC) were designed to detect *lipocalin* (334 bp) as an internal PCR-positive control (Jeong, unpublished data). The primers were synthesized by Bioneer (Daejeon, Korea). Duplex PCR was performed with a final volume of 50 μL containing two disks from the FTA card, 0.5 μL of a 10 mM dNTP mixture, 0.3 μL of Taq DNA polymerase, 2.5 μL of 10× Taq buffer, and 2 μL of 10 pmol of each primer. PCR conditions included an initial denaturation at 95°C for 5 min; then 38 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 30 s, and extension at 72°C for 1 min; followed by a final extension at 72°C for 5 min.

To determine the presence of false negatives (i.e., death of GM and non-GM hybrids), PCR was performed for 60 hygromycin-susceptible seedlings. Because no false negatives were found, this screening was conducted throughout the experiment. Gene flow frequencies were calculated as the percentages of transgene-detected hybrids per number of germinated seedlings.

Results and Discussion

From seeds collected in the 2006 field trials, 13,925 seeds germinated. Our hygromycin assay and PCR targeting *hpt* gene confirmed 20 hybrids between GM and non-GM chili peppers (Fig. 3).

Gene flow frequencies from the anthracnose-resistant GM chili pepper to its non-GM control line ("WT512") in 2006 were very low. Only eight hybrids were obtained from 7,071 germinated seedlings, and the highest frequency was 3.57% at the closest distance (0.5 m), with hybrids found at only up to 5.0 m in the east plot (Table 2). Frequencies were also low between GM and the commercial "Manidda," with 12 hybrids out of 6,854 seedlings tested (Table 2). These were located at up to 18.0 m in the east plot. The highest frequency was 3.16%, at 2.5 m in the west plot.

From seeds collected in 2007, 6,913 seeds germinated. PCR showed 33 hybrids between GM and "WT512" and 50 between GM and "CM" (Fig. 4).

Gene flow between GM and "WT512" in 2007 was greater in samples collected in August than September (Table 3). The highest frequency was 6.19% in the August

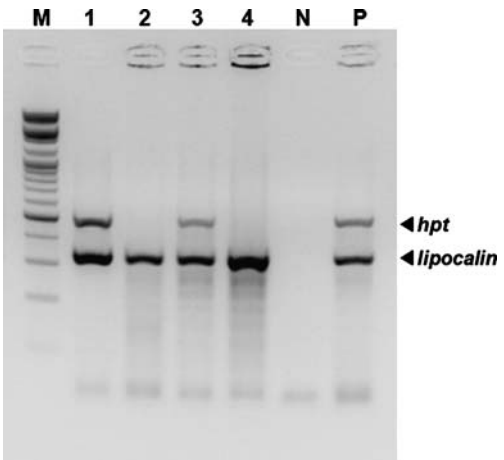

Fig. 4 Representative agarose gel electrophoresis patterns of PCR products from DNA of hygromycin-resistant hybrids obtained from 2007 field trial. *M* 100-bp DNA ladder; *lane 1* hybrid of GM/non-GM ("WT512"); *lane 2* "WT512"; *lane 3* hybrid of GM/non-GM ("CM"); *lane 4* "CM"; *N* negative control (no DNA); *P* positive control (GM chili pepper)

Table 3 Gene flow from GM chili peppers to non-GM control ("WT512") and a commercial hybrid cultivar ("CM") in 2007

Sampling months	Rows	"WT512" plots			"CM" plots		
		W1	W2	W3	C1	C2	C3
August	a	1/99 (1.01)	0/97	2/92 (2.17)	0/96	6/97 (6.19)	0/98
	b	6/97 (6.19)	5/98 (5.10)	0/97	1/95 (1.05)	0/91	0/98
	c	2/100 (2.00)	1/99 (1.01)	2/100 (2.00)	1/94 (1.06)	1/96 (1.04)	1/99 (1.01)
	d	0/97	2/96 (2.08)	0/95	2/100 (2.00)	1/98 (1.02)	2/96 (2.08)
	e	1/97 (1.03)	0/93	0/100	5/98 (5.10)	1/97 (1.03)	4/96 (4.17)
	f	1/97 (1.03)	2/97 (2.06)	0/95	6/98 (6.12)	5/94 (5.32)	3/95 (3.16)
	Total	11/587 (1.87)	10/580 (1.72)	4/579 (0.69)	15/581 (2.58)	14/573 (2.44)	10/582 (1.72)
September	a	0/97	0/91	0/94	0/98	2/99 (2.02)	0/98
	b	0/97	0/98	0/97	1/90 (1.11)	0/92	0/95
	c	0/95	0/92	1/96 (1.04)	3/97 (3.09)	0/94	0/95
	d	0/94	2/97 (2.06)	0/93	0/94	1/98 (1.02)	1/97 (1.03)
	e	0/96	2/97 (2.06)	0/90	1/97 (1.03)	0/92	0/94
	f	0/94	3/94 (3.19)	0/98	1/99 (1.01)	1/96 (1.04)	0/96
	Total	0/573	7/569 (1.23)	1/568 (0.18)	6/575 (1.04)	4/571 (0.70)	1/575 (0.17)

Values are the number of PCR-positive seedlings/number of germinated seeds examined. Data in parentheses are gene flow frequencies (%).

W1 plot. That rate was also found between GM and "CM" (Table 3). Among the "WT512" and "CM" plots, overall flows were greatest in W2 (1.48%) and C1 (1.82%), respectively.

Although chili pepper is partially self-pollinated, high outcrossing rates can occur among cultivars. Rates as high as 54.9% and 91% have been calculated among those in Mexico (Campodonico 1983) and in the USA (Tanksley

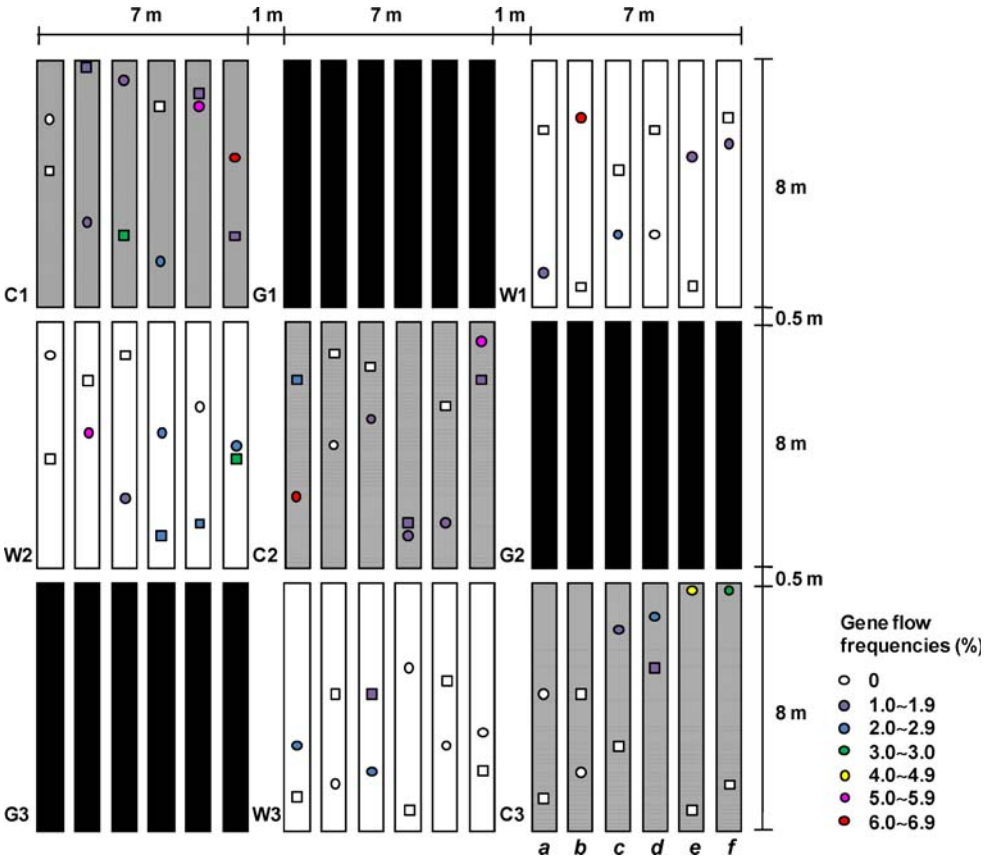

Fig. 5 Frequencies of gene flow from GM chili peppers to non-GM control ("WT512") and commercial cultivar ("CM") in 2007 study. *Colored circles* and *squares* indicate different levels of frequencies for fruits collected for screening hybrids in August and September, respectively

1984), respectively. Activity by insect pollinators is one cause for these values. However, compared with those earlier reports of natural cross-pollination, we found considerably lower frequencies of gene flow. In 2006, that flow mostly occurred within 5 m of the pollen-donor plot; only two hybrids were found at 7.0 m and 18.0 m away (Table 2). This result is consistent with that from Kim et al. (2009), who showed that gene flow from CMV-resistant GM chili pepper to conventional peppers mostly happens within 5 m when pollinating plots are close to a donor plot.

Our pollen-donor plots were enlarged from 40 plants in 2006 to 342 plants in 2007. Although gene flow frequency was much greater in the second year, the maximum value was only 6.19% (Table 3). It was difficult with the 2007 experimental design to estimate the distance from our GM plot where hybridization could occur. Nevertheless, hybrids between GM and non-GM crops generally were found from those non-GM peppers planted close to the GM plots (Fig. 5). Therefore, a minimum isolation distance of 20 m would be sufficient for the field-testing of GM chili peppers in Korea, as was previously suggested in the guidelines for a confined field trial of GM crops in Canada (CFIA 2000).

Greater gene flow was determined with seeds collected in August than in September in 2007, indicating more pollinator activity in June. Ripening fruits collected in September might have been pollinated 2 months earlier, in July. A large amount of rain that month could have deterred this activity. Because wind or rain is not directly associated with the cross-pollination of chili peppers (Bosland 1993; Free 1993; Delaplane and Mayer 2000), wind speed may not be an important environmental factor contributing to gene flow from those GM to non-GM plants. Bumble bees, honeybees, and thrips are potentially effective pollinators of both chili and sweet peppers (Rabinowitch et al. 1993; Shipp et al. 1994; Saxena et al. 1996). Among them, thrips and honeybees were observed in our fields, although their abundances were not quantified. Such pollinators might have been more attracted to the hybrid cultivars, as evidenced by growth that was more vigorous than from the inbred line ("WT512"). Consequently, the frequencies of gene flow from GM to hybrid cultivars were greater than those from the GM to its non-GM control inbred line.

We conducted only two yearlong trials in a restricted and isolated field; tests with multiple locations would give more information on gene flow from GM plants. However, chili peppers are partially self-pollinating and their outcrossing has been associated with insect pollinators (OECD 2006). These especially include honeybees, which are distributed widely and whose activities may not be significantly different in various regions of Korea. In fact, the results of single-field trials have been reported for gene flow of GM cotton (Umbeck et al. 1991), potato (McPartlan and Dale 1994), soybean (Yoshimura et al. 2006), and watermelon (Kim et al. 2008), all of which rely on insect pollination. Moreover, data from two field trials conducted at the same site and over 2 years have been presented elsewhere (Llewellyn and Fitt 1996; Kim et al. 2009).

Here, we showed that the gene flow frequencies of GM chili pepper resistant to anthracnose were not greater than outcrossing rates among standard cultivars. Such gene escape, therefore, would be prevented with a limited isolation distance.

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