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A DISSERTATION FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

**Potential Gene Flow from Genetically
Modified Oilseed Rape (*Brassica
napus*) to Its Relatives**

BY

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AUGUST, 2015

MAJOR IN CROP SCIENCE AND BIOTECHNOLOGY

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GENERAL ABSTRACT

Potential Gene Flow from Genetically Modified Oilseed Rape (*Brassica napus*) to Its Relatives

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Both greenhouse and field studies to evaluate gene flow from GM *Brassica napus* to its relatives were conducted in the Experimental Farm Station of Seoul National University, Suwon, Korea. The greenhouse study was conducted for setting up basic experimental conditions and then field studies were conducted to quantify the potential gene flow from GM *Brassica napus* to its relatives under different pollination conditions. Simple sequence repeats (SSR) and herbicide resistance (glufosinate-ammonium) markers were used for confirming F1 hybrids resulted from outcrossing between GM *B. napus* and its relatives. The experimental data clearly demonstrated the possibility of gene flow from GM *B. napus* to its relatives such as *B. napus* and *B. juncea* in the field condition, but showed no gene flow to *Raphanus sativus*. Under simulated favorite pollination by synchronizing flowering time and placing honeybee hive, the potential gene flow from GM *B.*

napus to its relatives was evaluated. To MS relatives, it was estimated to be 32.48% and 21.95% to MS *B. napus* and MS *B. juncea*, respectively, at 2 m distance, and decreased with increasing distance, reaching 0.3% and 0.25%, respectively, at 128 m distance. In contrast, to male fertile (MF) relatives, the potential gene flow was estimate to be 2.33% and 0.076% to MF *B. napus* and MF *B. juncea*, respectively, at 2 m distance and decreased to 0.007% for MF *B. napus* at 75 m and 0.025% for MF *B. juncea* at 16 m distance. Therefore, the gene flow rates to MS relatives at 2 m distance were 16 times for *B. napus* and 288 times for *B. juncea* greater than the gene flow rate to FM relatives, suggesting big difference between the maximum potential gene flow and the practical gene flow.

The three-parameter log-logistic model well described gene flows against distance, indicating that the suitability of the equations for prediction of gene flow rate at a specified distance. Isolation distances between GM *B. napus* and its GM relatives were then recommended based on the model and the tolerable threshold (0.01%) made by EU. The isolation distance for less than 0.01% gene flow from GM *B. napus* was esimted to be 2710 m and 254 m for MS *B. napus* and *B. juncea*, respectively, and 122.5 m and 23.7 m for MF *B. napus* and *B. juncea*, respectively. Our studies would provide informative reference values and scientific basis for risk assessment of gene flow from GM *B. napus* to its relatives under Korean climatic condition. The modeled equation will also provide scientific evidence for the determination of isolation distance and the regulation of GM crops cultivation.

Keywords: *Brassica napus*, *Brassica juncea*, gene flow, genetically modified, modeling, pollination, *Raphanus sativus*, risk assessment

Student Number: 2012-30757

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GENERAL INTRODUCTION

Genetically modified (GM) crops are dominated in four crop species such as maize (*Zea mays*), soybean (*Glycine max*), cotton (*Gossypium hirsutum*), and oilseed rape (*Brassica napus*) with two traits, insect resistance and herbicide tolerance (James 2005). These crops have demonstrated great advantage in crop production as well as environmental benefits resulted from decreased pesticide use. However, with dramatic increase in GM crops cultivation, environmental impacts on natural and agricultural ecosystems have raised public concerns, particularly with emphasis on gene dispersal from GM crops to non-GM crops and relative species. One of the main concerns over agricultural releases of GM crops is the escape of the transgenes into the natural ecosystem through outcrossing with their wild or weedy relatives (Warwick et al. 2003; Snow 2002), which transfer the traits (herbicide, insect or drought tolerance) to relatives, resulting in increased fitness and consequential invasiveness of the relatives. *Brassica napus* is one of the most important oil crops in the world. It is currently the third most important oil plant after soybean and oil palm and its global production continues to increase. It is an allotetraploid (AACC, $2n=38$) and has many wild or weedy relatives such as Indian *B. juncea* (AABB, $2n=36$), *B. rapa*, (AA, $2n=20$), *B. niga* (BB, $2n=16$), *B. carinata* (BBCC, $2n=34$), *B. oleracea* (CC, $2n=18$), *Raphanus sativus* (RR, $2n=18$), *R. raphanistrum* (RrRr, $2n=18$), *Erucastrum gallicum* ($2n=30$), *Hirshfeldia incana* ($2n=18$), and *Sinapis arvensis* ($2n=18$) persisting in or near the areas of *B. napus* cultivation. This close species relationship between diploid and allotetraploid *Brassica* species contributes to the ease interspecies out-crossing occurred in certain cross combinations. In both natural and controlled conditions, many events of outcrossing among *Brassica* species have been reported (Bing et al. 1991; Halfhill et al. 2002; Hansen et al. 2001; Jenkins et al. 2001; Warwick et al. 2003; Wilkinson et al. 2003).

Several factors affect outcrossing among the *Brassica* spp., such as plant genotype, direction of cross, types and presence of pollinators, degree of synchrony of flowering period, local climatic conditions, etc. As *B. napus* is both entomophilous and anemophilous plant, such factors, in particular, pollinators and wind significantly affect outcrossing (Alford 1978; Williams et al. 1987). For an open pollinated male fertile *B. napus*, the seed production was resulted from three factors, self pollination, wind pollination by neighbouring plants, and insect pollination primarily by bees such as honeybee and bumblebee (Alford 1978). As bees directly take part in pollen transfer and pollination, factors affecting the honeybee's behavior such as the density of flowers, types of plants, distance from the beehive, wind direction, weather condition, etc., would indirectly influence the pollination. In addition, the degree of synchrony of flowering period between pollen donor and recipient is also a key factor for determining the outcrossing (Bing et al. 1996; Landbo et al. 1996). An increase of synchronized flowering period between them would potentially increase the outcrossing rate as there are more opportunities for recipient flowers to accept foreign pollens for outcrossing. In contrast, lack of synchrony of geographic distribution or in flowering periods, the outcrossing will drastically reduce or may not occur (Tsuda et al. 2012).

To date, the GM *B. napus* has commercially cultivated in USA, Canada, Australia, and Chile but not cultivated in European countries such as United Kingdom, Germany, and France, and Asian countries such as China, Korea, and Japan. Numerous studies have been conducted worldwide for accumulating knowledge and better understanding of gene flow. Based on findings from many studies, governmental regulations have implemented for GM *B. napus* cultivation such as isolation distance, crops rotation, buffer zone, etc. However, due to different cropping system between North America and Asian countries, the research interests in gene flow are different. In North America and Australia, mono cropping in large-scale farms is very common for *B. napus*, soybean, and maize. Large-scale mono cropping does not raise much concern on gene flow between GM crop and non-GM relatives. Surrounding natural ecosystem is much far away from the crop area, so

probability of gene flow from GM crop to its non-GM relatives in the crop area, and wild and weed relatives in the surrounding natural or agro-ecosystem is not much high. Thus, more studies were focused on the distance of pollen dispersal and gene flow occurrence. Previous studies reported that the outcrossing could occur at a considerable rate and distance from pollen source, ranging from 0.07-1.6% from 1.5-800 m (Beckie 2001; Staniland et al. 2000). In a large-scale field investigation in Australia, the pollen even can flow beyond 2000 m (Rieger et al. 2002). In addition, studies conducted in two different sites, California and Georgia in USA, showed difference in outcrossing rate at the same distance from the pollen donor, indicating that the gene flow is affected by the local climatic and eologic conditions (Morris et al. 1994).

Unlike the large-scale crop cultivation in North America and Australia, co-existence of *B. napus* with its relative species in diverse cropping systems in Asia is quite common, where farmers generally manage small-scale fields and rotate different crops by each year, posing a high risk of outcrossing between *B. napus* and its relatives such as *B. rapa*, *B. oleracea*, *B. niga*, or *R. sativus*, etc. Therefore, studies in this regard have been conducted in neighboring Asian countries under both artificial and field conditions. In China, the glyphosate resistant hybrids (*B. juncea* × *B. napus*) obtained by hand-crossing were assessed and showed the increased fitness (Huangfu et al. 2011; Song et al. 2010). A gene flow risk assessment conducted in a field condition of Japan showed the outcrossing (*B. juncea* × *B. napus*) could occur at 17 m with the rate of 0.03% (Tsuda et al. 2012), indicating considerably high outcrossing between *B. napus* and its relatives. Spilled GM *B. napus* seeds have been detected at major ports and along the motorway in Japan, some of them found with resistance to multiple herbicides (Aono et al. 2005). Korea imports annually around 8 million tons of GM crops every year for edible oil processing and animals feeding. Although the previous monitoring work did not detect any spilled GM seeds in Korea (Lee et al. 2007), unintentionally spilled GM seeds may occur. Countries growing GM crops have more experience of gene flow study and may accumulate many gene flow data

under different conditions. However, due to the difference in cropping system between North America and Asian countries, the experiences from North America and Australia, in some senses, are not much suitable for Asian countries, particularly those which grow diverse crops in the immediate vicinity each other in a small farm. Therefore, we should have our own case study for gene flow risk assessment in a specific local cropping system and climatic condition. In present studies, gene flow assessments were made under favorite conditions by providing abundant pollinator insects (honeybee) and long duration of synchrony of flowering period between pollen donor and recipient. We quantified gene flow in field conditions and modeled the maximum potential gene flow from *B. napus* to its relatives under different environmental (greenhouse and field) and pollination (with and without honeybee) conditions (open and wind).

GENERAL OBJECTIVES

The aims of this study were to evaluate and model the potential gene flow from genetically modified (GM) *Brassica napus* to its relatives under Korean climatic condition. The studies had the following specific objectives for each chapter:

Chapter I

To quantify the maximum potential gene flow from *B. napus* to its male sterile (MS) relatives both under greenhouse and field conditions.

Chapter II

To quantify the potential gene flow from GM *B. napus* to its male fertile (MF) relatives under Korean field condition and to determine a proper isolation distance between GM *B. napus* and its relatives.

Chapter III

To evaluate the maximum potential gene flow from GM *B. napus* to its male sterile (MS) relatives under open and wind pollination conditions and to develop gene flow simulation models for predicting gene flow from GM *B. napus* to its relatives.

LITERATURE REVIEW

Genetically modified crops and gene flow

Genetically modified organisms (GMOs), are plants created through the gene splicing techniques of biotechnology (also called genetic engineering, or GE).

Gene flow means the movement of genes from one individual plant to another individual within the same species or between different species or genus. In population genetics, gene flow (also known as gene migration) is the transfer alleles or genes from one population to another. Gene flow usually occurs via cross-pollination or movement of seeds.

Taxonomy of *Brassica* species

The taxonomy and genetics of the *Brassica* species are complex (Fig. 1). The *Brassica* genus includes crops and weed species. The U's Triangle (U, 1935) demonstrates how three of the *Brassica* species (denoted by the letters AABB, AACC, and BBCC) were derived from three ancestral genomes (AA, BB, or CC) (Fig. 1).

- *B. rapa* (AA, $2n=2x=20$; turnip, Chinese cabbage)
- *B. nigra* (BB, $2n=2x=16$; black mustard)
- *B. oleracea* (CC, $2n=2x=18$; cabbage, broccoli, Brussels sprouts, cauliflower)
- *B. juncea* (AABB, $2n=4x=36$; brown mustard)
- *B. napus* (AACC, $2n=4x=38$; oilseed rape, rutabaga)
- *B. carinata* (BBCC, $2n=4x=34$; Ethiopian mustard)

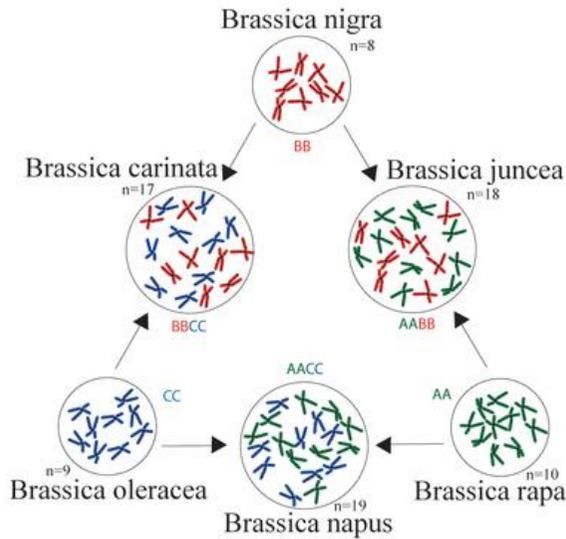


Fig. 1. Triangle of U (1935) showing the genetic relationships between the six species of the *Brassica* genus. Numbers indicate haploid chromosome set; letters designate genomes.

B. napus is an allotetraploid (AACC, $2n=38$) and has many wild or weedy relatives such as *B. juncea* (AABB, $2n=36$), *B. rapa* (AA, $2n=20$), *R. sativus* (RR, $2n=18$), *R. raphanistrum* (RrRr, $2n=18$), *Erucastrum gallicum* ($2n=30$), *Hirshfeldia incana* ($2n=18$), and *Sinapis arvensis* ($2n=18$) persisting in or near areas of cultivation (Table 1). This close species relationship between diploid and allotetraploid *Brassica* species contributes to the ease with which interspecies crossing occurs in certain cross combinations.

Table 1. Wild relatives and allies of *Brassica napus*

Species	Common name	Ploidy level	Genome	Life form and weediness
<i>B. rapa</i> (including Chinese cabbage, mizu-ma)	Field mustard, bird rape, turnip	Diploid ($2n=2x=20$)	A	Annual or biennial
<i>B. nigra</i>	Black mustard	Diploid ($2n=2x=16$)	B	Annual; weedy
<i>B. oleracea</i> (including broccoli, cauliflower, kale)	Cabbage (wild and cultivated)	Diploid ($2n=2x=18$)	C	Biennial or perennial
<i>B. juncea</i>	Brown mustard, indian mustard	Allotetraploid ($2n=4x=36$)	AB	Annual, derived from <i>B. nigra</i> x <i>B. rapa</i>
<i>B. napus</i> (including Siberian kale, swede rape)	<i>B. napus</i> , canola, rutabaga	Allotetraploid ($2n=4x=38$)	AC	Annual or biennial, derived from <i>B. rapa</i> x <i>B. oleracea</i>
<i>B. carinata</i>	Ethiopian mustard	($2n=4x=34$)	BC	Annual; derived from <i>B. nigra</i> x <i>B. oleracea</i>
<i>Raphanus raphanistrum</i> L.	Wild <i>R. sativus</i>	Diploid ($2n=18$)	Rr	
<i>Raphanus sativus</i> L.	Garden <i>R. sativus</i>	Diploid ($2n=18$)	R	Annual or biennial; weedy
<i>Hirschfeldia incana</i>	Buchan weed, hoary mustard	Diploid ($2n=14$)	Ad	Annual or biennial; weedy
<i>Sinapis arvensis</i> L.	charlock	Diploid ($2n=18$)	Sar	Annual; crop weed

Outcrossing events between *B. napus* and its Relatives

Gene flow from *B. napus* to *Brassica* spp.

Outcrossing to B. rapa (field mustard)

B. napus (AACC, $2n=4x=38$) and *B. rapa* (AA, $2n=2x=20$) have a common set of chromosomes, which facilitates interspecific hybridization and gene flow between these two species. Numerous studies have indicated a high potential for hybridization between *B. napus* and *B. rapa*. Spontaneous hybridization and backcrossing between *B. napus* and *B. rapa* in the field has been reported in several countries, including Denmark (Landbo et al. 1996; Hansen et al. 2001), New Zealand (Jenkins et al. 2001), United States (Halfhill et al. 2002), Canada (Warwick et al. 2003; Yoshimura et al. 2006), and United Kingdom (Allainguillaume et al. 2006; Wilkinson et al. 2003). Frequency of hybrids depends on parental genotypes, direction of crosses, experimental design and population sizes (Bing et al. 1991; Jørgensen and Andersen, 1994; Jørgensen et al. 1998; Halfhill et al. 2002; Hansen et al. 2003; Hauser et al. 1997; Pallett et al. 2006; Scott and Wilkinson, 1998).

For instance, frequency of hybrids is highest where single *B. rapa* plants are present in *B. napus* fields (Jørgensen et al. 1996), as *B. rapa* is self-incompatible and an obligate outcrosser. Hansen et al. (2003) reported that the introgression process between *B. napus* and *B. rapa* primarily progressed with *B. rapa* as the maternal plant, and that both recombination between the A and C genomes and exchange of chloroplasts between *B. napus* and *B. rapa* appear to have occurred. An earlier study in UK, Scott and Wilkinson (1998) documented that *B. rapa* populations growing outside of *B. napus* fields showed low levels of hybrids (0.4-15.0%). However, Warwick et al. (2003) reported a higher naturally occurring herbicide (glyphosate) tolerance gene transfer rate (approximately 13.6%) from *B. napus* to *B. rapa* in the commercial fields in Canada. Similarly, in the United States, under a higher (600:1) and a lower (180:1) *B. napus*-*B. rapa* ratio, hybridization frequency from transgenic *B. napus* to *B. rapa* determined at approximately 10% and 2%, respectively (Halfhill et al. 2002).

The F1 hybrids, triploid (AAC, $2n=29$), crossed between *B. napus* and *B. rapa* are viable and present rather high levels of fertility, albeit with greatly varying amounts of seed production. Where natural interspecific hybrids have occurred,

they have reduced fertility and seed set compared with the parents (Jørgensen and Andersen, 1994). On average, less than 2% of all hybrid seedlings survived (Scott and Wilkinson, 1998). This low hybrid survival rate reduces the rate at which introgression of transgenes into *B. rapa* can occur (Jørgensen et al. 1996; Sweet et al. 1999). When interspecific hybrids are present, spontaneous backcrossing takes place at very low frequency (Hauser et al. 1998). Hauser et al. (2001) reported that hybrids and backcross offspring were produced mainly by a few of the *B. rapa* female plants, indicating that the degree of hybridization and backcrossing depended on the presence of certain *B. rapa* genotypes.

Norris and Sweet (2001) noted that while *B. rapa* seed banks showed evidence of introgression, there was no evidence of introgression in samples taken from mature *B. rapa* plants in the field, indicating that there may be selection pressure against backcrossed individuals. The gene flow rate to *B. rapa* via introgression will depend greatly on the selection pressure exerted on the herbicide tolerant gene (Scott and Wilkinson 1998; Sweet et al. 1999; Snow and Jørgensen, 1999).

Introgression into *B. rapa* is more likely to occur if the transgene in *B. napus* is located on the A genome which is common to both species. Hansen et al. (2003) further concluded that transgenes could be transferred at a high outcrossing rate from *B. napus* to *B. rapa*, regardless of transgene position in the nuclear or plastid DNA. There is no evidence that the presence of an introgressed herbicide tolerant gene in *B. rapa* has increased its fitness (Snow and Jørgensen, 1999; Sweet et al. 1999), indicating that *B. rapa* resulting from the introgression of the gene can be controlled by alternative herbicides.

Outcrossing to B. juncea (Indian mustard)

B. juncea is widely cultivated and naturalized worldwide, especially for oil production, and frequently found as a weed or a ruderal component of roadsides and waste places (Liu et al. 2010; Song et al. 2010). *B. juncea* (AABB, 2n=36) share a common set of chromosomes with *B. napus*, enhancing the probability of interspecific hybridization and gene flow. Researches on hybridization and gene

flow between *B. napus* and *B. juncea* are less compared to *B. napus/B. rapa* case. However, successful hybrids in controlled crosses and spontaneous occurrence of interspecific hybrids in the field have been reported in Canada, Denmark and China (Bing et al. 1991, 1996; Frello et al. 1995; Jørgensen et al. 1998; Di et al. 2009; Liu et al. 2010). The relatively high compatibility between *B. napus* and wild *B. juncea* raises the biosafety concern of transgene introgression.

Depending on the proportions of *B. napus* and *B. juncea* plants, up to 3% of hybrids from *B. juncea* have been found, although less hybrids are formed with *B. napus* as the female (Bing et al. 1991; Jørgensen et al. 1996, 1998). In field co-cultivation experiments with *B. napus* and *B. juncea*, Bing et al. (1996) found five interspecific hybrids out of 469 plants when *B. napus* was the female and three out of 990 plants when *B. juncea* was the female. Interspecific hybrids have reduced fertility and low seed set compared with the parents. However, the hybrids were able to survive in the field and produce a small amount of seeds. Introgression of herbicide tolerant genes from GM *B. napus* to *B. juncea* observed both in controlled crosses and in open field (Di et al. 2009; Liu et al. 2010; Song et al. 2010; Warwick 2007). Warwick (2007) reported on field experiments where gene flow from herbicide resistant *B. napus* to neighbouring fields of *B. juncea* was measured and found to be 0.245% at the field border and 0.005% at 200 m.

Based on fitness of the hybrids and backcrosses by hand pollination, Song et al. (2010) found that glyphosate- or glufosinate-tolerance genes of GM *B. napus* could be transmitted to wild *B. juncea* when wild *B. juncea* was used as the maternal or paternal plants, and the possibility for transmission may be greater with glyphosate-tolerant than glufosinate-tolerant *B. napus* to wild *B. juncea*. Similarly, Hangfu et al. (2011) detected a higher transfer ratio of glyphosate- tolerant *B. napus* specific *loci* and the variation of populations in fitness-related parameters in F₁ hybrids. Fitness test from Di et al. (2009) showed that the F₁ hybrids formed between transgenic (GT, linked GFP and Bt *CryIAc* cassettes) *B. napus* and wild *B. juncea* revealed maternal effects, high vegetative fitness and seed dormancy compared with the parents, indicating that increased the probability of the survival of hybrids after

the occurrence of gene flow. Recently, Liu et al. (2010) reported that herbicide (chlorsulfuron, ALS inhibitor) resistant BC₁ were not different of their susceptible counterparts for plant weight, seed weight and seed number, but most of them exhibited *B. napus* morphology and larger flowers than the susceptible BC₁, which displayed additional genetic variability that allowed for further adaptation of the plants and propagated the herbicide-resistance gene. Therefore, current evidences that the introgressed herbicide tolerant gene from *B. napus* to *B. juncea* could certainly enhanced weediness of *B. juncea* in arable fields or spread as a weed relative to conventional, non-GM *B. juncea*.

Gene flow from *B. napus* to *B. vegetables*

Gene flow from *B. napus* to *B. napus* vegetables (CC, 2n=18, e.g. rutabaga, Siberian kale, swedes) and to *B. rapa* vegetables (e.g. Chinese cabbage, Pak choi, turnip) is theoretically possible, due to sharing a common set of chromosomes. However, *B. napus* and *B. rapa* vegetables are generally harvested prior to flowering and seed development, unless being used as a seed production. So a lack of physical proximity and synchrony of flowering means the probability of outcrossing occurrence with *B. napus* is extremely unlikely. Lately, Ford et al. (2006) reported the first spontaneous gene flow from *B. napus* to wild *B. oleracea* in the UK, and used flow cytometry and crop-specific microsatellite markers to identify one triploid F₁ hybrid, together with nine diploid and two near triploid introgressants.

Gene flow from *B. napus* to wild or weedy species

*Outcrossing to *R. raphanistrum* (wild radish)*

Spontaneous gene flow between *B. napus* and *R. raphanistrum* (RrRr, 2n=18) in the field has been reported in Australia, Canada, Denmark, and France at very low frequencies (Ammitzboll and Jørgensen 2006; Baranger et al. 1995; Chevre et al. 2000, 2003; Darmency et al. 1995, 1998; Rieger et al. 2001; Warwick et al. 2003).

The frequency of outcrossing varies, depending on several factors, among them the *B. napus* variety (male-sterile or fertile) and genotype used, local environmental conditions, the ratio of parents, and the breeding system (Baranger et al. 1995; Chevre et al. 2003; Ammitzboll and Jørgensen 2006). Hybridization is more likely to occur between *B. napus* and *R. raphanistrum* when *B. napus* is the maternal parent (Rieger et al. 1999). No hybrids were detected among 25,000 seedlings grown from seed collected from imidazolinone-resistant *R. raphanistrum* plant (Rieger et al. 2001). However, two hybrids were obtained from more than 52-million *B. napus*, and both hybrids were characterized as amphidiploids (AACCRrRr, 2n=56) and fertile. The frequency of hybridization into *B. napus* using male fertile *B. napus* was 4×10^{-8} . Chevre et al. (2000) reported that only one herbicide-tolerant hybrid was characterized among the 189,084 seedlings between *B. napus* and *R. raphanistrum* under normal agronomic conditions, and the frequency of hybridization was assessed to range from 10^{-7} to 10^{-5} . In a Canada study, no hybrids were detected in the greenhouse experiments (1,534 seedlings), the GFP (green fluorescence protein marker) experiment (4,059 seedlings) or in commercial fields (22,114 seedlings) in Quebec and Alberta. But a single herbicide (glyphosate) resistant *R. raphanistrum* × *B. napus* F₁ hybrid was detected in 32,821 seedlings from the herbicide resistant *B. napus* field experiment. The hybrid had a genomic structure consistent with the fusion of an unreduced gamete of *R. raphanistrum* and a reduced gamete of *B. napus* (RrRrAC, 2n=56), both *R. raphanistrum* and *B. napus*-specific AFLP markers, and had <1% pollen viability (Warwick et al. 2003). In USA, Halfhill et al. (2002) reported that no hybrids were detected among the 19,274 seedlings between the GFP (green fluorescence protein) *B. napus* and *R. raphanistrum* at a high crop-weed ratio (600:1) under field conditions. In Denmark, Ammitzboll and Jørgensen (2006) analyzed spontaneous hybridization between a transgenic male sterile line of *B. napus* and, as pollen donors, three European populations of *R. raphanistrum*. The authors reported that the frequency of confirmed hybrids differed significantly among populations of *R. raphanistrum*. In the cross with a French population, all offspring were hybrids; in

the cross with a Swiss population, 53% of the offspring were hybrids; and in the cross with a Danish population, only 2% of the offspring were found to be hybrids.

Although pollen germination and ovule fertilization vary considerably within populations, post-zygotic barriers to hybridization are generally minor. F₁ hybrids are mainly allotriploids and show very low (<2 seeds/plant) fertility (Baranger et al. 1995; Darmency et al. 1998). F₁ hybrids commonly show decreased fitness in terms of reduced seedling emergence, a significant emergence delay, and a lower survival rate than both parents. Despite this, a small number of highly fertile F₁ hybrids have been reported by Salisbury (2002).

Outcrossing to R. sativas (radish)

The first report on spontaneous hybridization between a transgenic male sterile *B. napus* line and a variety of cultivated *R. sativus* was reported by Ammitzbohl and Jørgensen (2006). All offspring were found to be hybrids with low pollen fertility (0-15%).

Outcrossing to Sinapis arvensis (charlock) (SarSar, 2n=18)

A recent study on outcrossing between *B. napus* and another problematic weed, charlock, found very low rates of hybrid formation from both hand pollination experiments and field trials. In most of cases, charlock was served as the maternal plants. Moyes et al. (2002) got sexual hybrids when charlock was the maternal parent under greenhouse condition but failed to detect a hybrid crossed between charlock and *B. napus* plants in a field experiment.

The reciprocal cross with charlock as female parent gave only one hybrid out of 1127 hand pollinations (a rate of 0.0015% of the potential seed set). In a field experiment under natural condition in France, male-sterile *B. napus* has been shown to produce up to 0.18 hybrid seeds per plant (Chèvre et al. 1996). Artificial hybridizations using ovary culture and embryo rescue produce up to 1 seed per 100 pollinated flowers, but the F₁ plants were weak and highly or completely sterile compared to its parental plants (Bing et al. 1996). F₂ or backcross seed production

is extremely rare, and no gene introgression has been detected thus far (Bing et al. 1996; Rieger et al. 1999; Warwick et al. 2003).

Factors affecting gene flow

Genotype and zygotic barriers

The genotypes of the pollen donor and recipient are one of the important factors affecting the outcrossing rates. The use of different herbicide resistant plant varieties, such as with glyphosate and with glufosinate resistance as a transgenic marker system or other conventional herbicide resistant varieties, demonstrates that different marker systems have different genetic backgrounds, which can influence the flowering time, the pollen quantity, longevity and selfing rate (Hüsken and Dietz-Pfeilstetter 2007). The differences in reciprocal outcrossing rates using different transgenic varieties in controlled crosses or in open field indicate that the different transgenic varieties show the different transgene introgression levels (Rieger et al. 2002; Simpson et al. 1999; Song et al. 2010) under the same experimental and environmental condition. Furthermore, different types of transgenic HR can show different patterns of inheritance. In the case of homozygous glyphosate resistant and glufosinate resistant lines all of the pollen carries the HR gene. However, the hybrids derives from hemizygous HR parents show a reduced HR rates (Beckie et al. 2003). For instance, the hemizygous female and the homozygous male parent of the glufosinate resistant hybrid *B. napus* system contain the *bar* HR gene at two different loci. As a result, the hybrid produces transgenic and non-transgenic pollen in a ratio of 5:3, leading to a reduced amount of transgenic pollen as compared to homozygous herbicide resistant lines. Gene dosage effects have been demonstrated in several cases by comparing hemizygous and homozygous transgenic plants, with homozygous having higher transgene expression levels (Richards et al. 2003; Tang et al. 2003).

In plants, cross-incompatibility after pollination is caused by two types of fertilization barriers (Hadley and Openshaw 1980; MacNair 1989), pre- and post-

zygosity. Pre-zygosity occurs before fertilization and results mainly from pollen-pistil interactions, whereas post-zygosity happens when the development of young zygotes is arrested.

Direction of the cross

Hybridization is more likely to occur in one direction rather than the other. In general, when MS crop varieties are used, hybrids from crosses in which the crop plant is the female are again much more likely to occur than the reverse. This has been observed, for example, in *B. napus* (Darmency et al. 1998; Chèvre et al. 2000). Hybrids resulting from crosses of MS *B. napus* with its wild relatives (e.g., *R. raphanistrum*) are usually partially sterile. However, hybrid fertility can be restored by backcrossing with the wild relative, which would then result in GM traits moving towards wild relatives that act as the female plant. This example illustrates the fact that the direction of hybridization does not necessarily allow for conclusions about the possible containment of GM traits, as backcrossing can reverse this effect within only a few generation (Andersson and de Vicente 2010).

Shape, orientation, and size of pollen source and recipient field

Isolation distance is one means to ensure seed purity. Some outcrossing studies investigated the effectiveness of zones for reducing gene flow compared to the use of non-transgenic buffer areas. When crops are isolated by open ground or low growing crops, it appears that the first rows of the recipient field intercept a high proportion of foreign pollen due to the low convarietal pollen load of the field margin.

Pollinators

The concentration of pollen collected decreased rapidly with distance from the pollen source. McCartney and Lacey (1991) reported similar results and concluded that windborne pollen was unlikely to play a significant role in long distance cross-pollination of *B. napus*. Insects, especially honeybees (*Apis mellifera* L.) and

bumblebees (*Bombus terrestris* L.), collect nectar and pollen from *B. napus* plants, and pollen is transferred as the bees move from plant to plant. As honeybees can travel one to two kilometers from the hive, it is possible that pollen could be transferred far beyond the borders of an experimental plot. However, studies indicate that honeybees tend to visit plants that are located in a small area as near to the hive as possible, and often forage only on plant species per foraging trip, although there are exceptions (Gary 1975; Martin and McGregor 1973; Ribbands 1953). Bumblebees exhibit similar behavior, but are more likely to visit more than one plant species during a foraging trip (Alford 1978).

Wind

The extent of pollen flow in *B. napus* is strongly relied on local environment and climatic conditions (e.g. wind speed and direction) as well as its pollen characteristics. *B. napus* pollen is relatively large (32-33 μm), heavy and sticky, with viability estimates ranging from 1 to 5 days under natural conditions (Treu and Emberlin 2000). Mesquida et al. (1982) detected the majority *B. napus* pollen at 32 m from pollen source, but noted that the concentration of pollen collected decreased rapidly with the distance from the pollen source. Some studies have indicated that viable pollen can be found 1.5 km from the pollen source (Timmons et al. 1995). In discontinuous pollen-dispersal experiments, outcrossing rate was estimated to be 0.0156 % and 0.0038 % at 200 m and 400 m, respectively (Scheffler et al. 1995), whereas in a continuous pollen dispersal experiment, the outcrossing rate decreased dramatically to 0.02 % at 12 m and was only 0.00033 % at 47 m from the central pollen source (Scheffler et al. 1993).

Methods for detecting gene flow

The detection for hybrids usually was advocated (Poppy and Wilkinson 2005) a two-stage process: 1) the preliminary screens for putative hybrid plants identification; and 2) the confirmation assay for the putative hybrids confirmed as using a more accurate assay.

Preliminary screens (biology-based methods) for hybrids

Morphology

Morphological character analysis, plant phenotypes including seed color, weight, size, is an obvious possibility for detection of interspecific hybrids, provided that morphological differences distinguish the parental species and the hybrids share some of the characteristics of both species. However, in some cases, hybrids may appear indistinguishable from one parent or exhibit widely variable phenotypes that range between those of its parents. For instance, in controlled crosses between *B. tournefortii* and *B. rapa*, the F₁ hybrids were intermediate to their parents for most the morphological traits but a few characters were inherited selectively from the maternal or the paternal parent (Choudhary and Joshi 2001). This skewed distribution of phenotypic traits has also been observed following hybridization in other genera, for example in interspecific hybrids of *Cucumis* (Chen et al. 2004).

When using phenotype as a preliminary screen, ideally plants should be grown as a cohort and examined regularly. A small number of qualitative features are generally preferable to the more time-consuming process of collecting several complex measurements. In this way large numbers of plants can be examined in a relatively short time frame. For example, Scott and Wilkinson (1998) used leaf pruinosity colour and hairness to screen through 13, 000 seedlings collected from *B. rapa* plants growing next to fields of *B. napus*, they found just 46 hybrids.

Therefore, morphological identification of hybrids should not be used in isolation but should be combined with other methods (related methods reviewed below) for hybrid identification as there are often few morphological characters differing between taxa, and the genetic background of these characters is usually complicated, unknown and modulated by the environment (Rieseberg and Wendel 1993).

Sterility

Hybrids may show a decreased fertility compared to their parental genotypes. Seed production or seed viability can be a good indicator of hybridity as seed production per flower is often reduced. Reduced pollen fertility is of less value, largely because of the need for microscopic examination, although it can be a useful additional indicator for plants showing other signs of hybrid status (reduced seed set, intermediate morphology). Pollen viability can be evaluated by different viability stains or more simply by observations on pollen size and shape to identify the frequency of misshapen grains. Methods for estimating pollen fertility are described in most textbooks on staining procedures in biology. Jørgensen and Andersen (1994), Hauser et al. (2003) reported on male and female fertility in hybrids between *B. napus* and *B. rapa*.

Herbicide bioassay

The most widely grown GM herbicide tolerant plants are tolerant to the herbicides glyphosate (EPSPS inhibitor) or glufosinate (GS inhibitor) but imidazolinone (ALS inhibitor) as well other mode of action herbicide tolerant GM plants have also been developed. Successful hybrids between GM and non-GM plant would exhibit specific herbicide resistance attributing to introgression novel trait. Herbicide bioassay has been widely used for preliminary detection of hybrids, which can be carried out large amount of selection of introgression hybrids under glasshouse and field conditions. This method involves testing germination of seeds on herbicide- incorporating medium or filter paper, or spraying the herbicide on seedlings and assessing herbicidal efficacy by visual assessments of mortality or plant vigor. Seeds or seedlings that test positive for the presence of the herbicide GM tolerant trait will germinate and develop normally, whereas those that growth depress or do not develop normally will be non-GM. However, In order to exclude seeds or seedlings which had survived due to not enough absorption or contact of herbicide, a further herbicide application and followed analysis are still needed to be performed with confirmation of particular trait in selected survival individuals.

Mikkelsen et al. (1996) detected spontaneous transfer of a herbicide tolerance gene from *B. napus* to *B. rapa* by Basta spraying, and Hall et al. (2000) showed transfer of multiple herbicide resistance genes to *B. napus* volunteers in spray tests. In *B. napus*, Rieger et al. (2002) showed the intraspecific dispersal at the landscape level of a gene-encoding tolerance to an ALS inhibitor spraying by the offspring; the herbicide-tolerant *B. napus* was produced by traditional breeding and not through genetic modification. Pfeilstetter et al. (2000) compared different screening tests using the Basta spray test, the drop test, ELISA (enzyme-linked immunosorbent assay) screening and PCR amplification, and found good correspondence between the results from the different types of tests.

Confirmation assays for hybrids

Protein-based methods

When dealing with gene flow from transgenic plants, the transgene product can be identified in the recipient. The methods can be based on antibodies that are specific against the new proteins that are produced in the plants. Presently, commercial methods are available for *Bt* toxin and for herbicide tolerance. These lateral flow strip tests are cheap and can be used on site for detection but not quantification. However, as a result of operator performance, false negative seem to be frequent (Fagan 2004). The ELISA is more sensitive and can in principle be used for quantification. Stave (1999) described the quantitative ELISA detection of Roundup Ready® soybean. Nevertheless, these tests are useful tools for preliminary hybrid screens.

DNA -based methods

Random amplified polymorphic DNA (RAPD)

RAPD method was the first PCR-based approach to be widely used for hybrid detection. The method used arbitrarily selected primer sequences to generate

multiple products by PCR. RAPD analysis is fast, cheap and usually produces sufficient polymorphisms to allow for the identification of most hybrid types. However, the amplicons in the multiple band profiles are anonymous and usually inherited in a dominant fashion, which can limit its usefulness for hybrid verification. Nevertheless, this technique has proved useful for hybrid confirmation in the past. For instance, Jørgensen and Andersen (1994) revealed spontaneous hybridization between *B. napus* and *B. rapa* using RAPD markers, and Isoda et al. (2000) detected spontaneous interspecific hybridization in *Abies* by way of RAPD markers.

Amplified fragment length polymorphism (AFLP)

AFLP is a powerful PCR-based DNA fingerprinting technique for hybrid detection. It is based on selective amplification of a subset of restriction fragments from a digest of DNA, with subsequent visualization of the PCR products on a gel. Usually the method generated very complex amplicon profiles comprising 50-100 products. As with RAPD analysis, the markers in these complex profiles are usually inherited in a dominant fashion. However, their complexity and reliability offers huge advantages for confirming the identity of hybrids and introgressants, potentially allowing designation of cultivar of origin and semi-quantification of the extent of introgression. There are several studies where AFLP method has been used for detecting hybrids between cultivated crops and wild species. For instance, Hansen et al. (2001 and 2003) used AFLP to confirm interspecific hybrids and introgressed progeny arising from gene flow between *B. napus* and *B. rapa*.

Simple sequence repeats (SSRs)

Simple sequence repeats (SSRs) are stretches of DNA that consist of tandem arrays of 2-8 base motifs. SSRs are abundant in all investigated eukaryotic genomes, and lead to a high level of intraspecific polymorphism for the number of repeat motifs within an array. Length variability within an SSR can be visualized by PCR analysis using primers that are specific to the flanking sequences of the SSR-

locus, followed by high-resolution electrophoresis. However, the main disadvantages are that that sequence information is required to design the flanking species or species complex, which involves cloning and sequencing. It is usually desirable to use several SSR loci to confirm hybrid identify, and the cost and time of analysis can be reduced if several SSR analyses are performed together. This is known as multiplexing and has been used widely, for instance in the study of *B. napus* (Tommasini et al. 2003) and bean (Masi et al. 2003).

Real-time PCR

Real-time PCR is a very sensitive quantification of a specific sequence, which has proved very useful, powerful and widely been used for identification of interspecific hybrids from GM crops to wild or weedy species. This method is based on the quantification of fluorescent reporter molecular that increase in proportion to the amount of PCR product in the reaction.

Case studies of GM *B. napus* in other countries

Research in GM *B. napus* cultivation countries

GM *B. napus* has commercially cultivated in USA, Canada, and Australia (Table 2). Many studies on gene flow from GM *B. napus* to conventional *B. napus* have been carried out under field condition. Outcrossing rate was found to dramatically decrease when increase the distance from pollen donor. A Canadian Study (Staniland et al. 2000) revealed that outcrossing rate was 1.6% at 1.5 m from the pollen donor and sharply decreased to 0.03% when the distance increased to 31.25 m. Even the distance increases to 800 m, the outcrossing also can occur, the rate approximately 0.07% (Beckie 2001). Rieger et al. (2002) showed the pollen flow can move beyond 2000 m with large field sizes in Australia, and the tendency of pollen movement did not obey a leptokurtic or exponential decline. Moreover, studies conducted in two different sites, California and Georgia in USA (Morris et al. 1994), indicated that the outcrossing rate is variable even at the same distance

from the pollen donor. In California, the outcrossing rate was 2.0% when close to pollen donor and 0.4% at the 4.6 m from pollen donor. In Georgia, the outcrossing rate was 3.5% and 0.7% at the same distance compared with the California study. These studies suggest that the outcrossing rate is importantly affected by the local climatic condition.

Due to large acreage of cultivation *B. napus*, there is less worry on gene flow from GM-*B. napus* to its relatives. However, several studies have been conducted on this concern. Warwick et al. (2003) assessed the probability of gene flow from transgenic *B. napus* to its four wild relatives: *B. rapa*, *R. raphanistrum*, *S. arvensis*, and *E. gallicum* (Willd.) O.E. Schulz in greenhouse and/or field experiments in Canada, and found the probability of hybridization between *B. napus* and *B. rapa* was considerably high (approximately 13.6%), and the probability of hybridization with *R. raphanistrum* ($< 2.5 \times 10^{-5}$), *S. arvensis* L. (no hybrids detected), or *E. gallicum* (Willd.) O.E. Schulz (no hybrids detected) is very low. The similar hybridization experiment between *B. napus* and *R. raphanistrum* was also conducted reciprocally in the Australian field (Rieger et al. 2001). They found that no hybrids were detected amongst seedlings grown from seed collected from *R. raphanistrum* plants, but the amphidiploids (AACCRrRr, $2n=56$) and fertile hybrids were obtained with the hybridization frequency of 4×10^{-8} when *B. napus* served as maternal plants, indicating that the probability of hybridization with *R. raphanistrum* is very low.

Research in Non-GM *B. napus* cultivation countries

Up to now, GM *B. napus* is not grown commercially in western European countries such as UK, Germany, and France, Asian countries such as China, Korea, Japan. However, researches on assessing GM *B. napus* gene flow have been carried out for a long time (Table 2). Especially in UK, so many studies have been conducted over the distance dispersal of pollen, the outcrossing rate between *B. napus*, and gene flow from *B. napus* to its relatives. Paul et al. (1995) showed that in the mixed population the outcrossing rate was ranged from 3% to 12%. Another study conducted by Simpson (2000) showed that the outcrossing rate was 1.0% at

1.5 m and 0.05% at 91.5 m from the pollen donor. When the distance increased beyond 400 m, the outcrossing also can occur (Ramsay et al. 2003). The two studies conducted by Ramsay (2003) indicated that the environmental effect on outcrossing rate. In Germany, Krato and Petersen (2012) assessed the outcrossing rate was 2.1% at the 2 m and 0.05% when the distance increases to 45 m from the pollen donor under field condition. In China, Cai et al. (2008) reported that the outcrossing rate was found 1.2% at the distance of 1.4 m from the pollen donor and increased to 0.06% when the distance beyond 2000 m. In France, several studies also assessed the outcrossing rate under France climatic condition. Champolivier et al. (1999) reported that the outcrossing rate was 4.0% in the mixed population and decreased to 0.6% at 30 m. The outcrossing also can happen even the distance increasing to 800 m (Darmency and Renard 1992). Thus, when comparing all of the outcrossing cases studies on *B. napus*, we can find that the specific local region study is necessary due to the different cultivation environmental condition in different countries.

It is unlike the large-scale farm production and comparatively mono cropping system in North America and Australia. In European and Asian countries, co-existence of *B. napus* with its relative species such as Indian mustard, field mustard, and other weeds species is very common, where farmers generally have small land and cultivate different crops by each year, thus having a high risk of hybridization risk between transgenic varieties and wild or weedy relatives. Therefore, many studies in this regard were conducted in these countries. In France, Denmark, and UK, hybridization experiments between *B. napus* and *B. rapa*, *B. oleracea*, *R. raphanistrum*, or *R. sativus* in controlled greenhouse or field condition have been tried because of the extensively distribution of wild species, and the hybrids were obtained and confirmed. In China, the herbicide resistant hybrids (*B. juncea* × *B. napus*) obtained by mean of artificial crossing (hand pollination) were assessed and proven the increased fitness (Huangfu et al. 2011; Song et al. 2010). A designed field transgene flow experiment from *B. napus* to *B. juncea* showed that the outcrossing even occurred beyond 17 m with the outcrossing rate of 0.03% (Tsuda

et al. 2012). All of the results from there suggests that the considerable higher possibility of hybridization between *B. napus* and its relatives. Therefore, in case the GM *B. napus* is released in those countries, more attentions must be focusing on that topic. At the same time, gene flow studies from GM *B. napus* to its relatives should be continued to carry out in related countries. In Japan and Korea, where the GM *B. napus* is not cultivated commercially, however, several GM *B. napus* plants have been detected at major ports and along roadsides, some of them with resistance to multiple herbicides (Aono et al. 2005). From this point, the monitoring work must be done before a specific transgene is being planted in a country.

Table 2. Summaries of some representative studies on gene flow of *B. napus* in GM and non-GM *B. napus* cultivation countries

Pollen donor	Transgene trait	Recipient	Pollen dispersal distance (m)	% outcrossing (max value)	Detection method	Country	References
GM <i>B. napus</i> cultivation countries studies on gene flow							
<i>B. napus</i>	erucic acid	<i>B. napus</i>	0	21.8	erucic acid content	Canada	Rakow & Woods 1987
<i>B. napus</i>	bromoxynil resistance	<i>B. napus</i>	0.4, 0.8, 1.2	9.5, 5.6, 3.9	herbicide tolerance	Canada	Cuthbert & McVetty 2001
<i>B. napus</i>	bromoxynil resistance	<i>B. napus</i>	1.5, 4, 11.5, 21.5, 31.5	1.6, 0.7, 0.3, 0.2, 0.03	herbicide tolerance	Canada	Staniland et al. 2000
<i>B. napus</i>	glyphosate resistance	<i>B. napus</i>	20, 50, 100	1.5, 0.4, 0.4	herbicide tolerance	Canada	Downey 1999
<i>B. napus</i>	glyphosate resistance	<i>B. napus</i>	47, 137, 366	2.1, 1.1, 0.6	herbicide tolerance	Canada	Stringam & Downey 1982
<i>B. napus</i>	glyphosate resistance	<i>B. napus</i>	0	7.0	herbicide tolerance	Canada	Warwick et al. 2003
<i>B. napus</i>	glyphosate resistance	<i>B. napus</i>	0, 50, 100, 200, 800	1.4, 0.2, 0.15, 0.2, 0.07	herbicide tolerance / PCR	Canada	Beckie et al. 2003
<i>B. napus</i>	kanamycin resistance	<i>B. napus</i>	0, 0.3, 0.6, 3, 4.6	2.0, 1.0, 0.8, 0.8, 0.4	kanamycin analog G418 tolerance	USA (California)	Morris et al. 1994
<i>B. napus</i>	kanamycin resistance	<i>B. napus</i>	0, 0.3, 0.6, 3, 4.6	3.5, 1.5, 1.2, 0.7, 0.7	kanamycin analog G418 tolerance	USA (Georgia)	Morris et al. 1994
<i>B. napus</i>	glyphosate resistance	<i>B. napus</i>	0, 7.5	6.3, 0.5	herbicide tolerance	USA	Brown et al. 1996

<i>B. napus</i>	imidazolinone resistance	<i>B. napus</i>	500, 1000, 2000, 5000	0.15, 0.1, 0.2, 0	herbicide tolerance	Australia	Rieger et al. 2002
Non-GM <i>B. napus</i> cultivation countries studies on gene flow							
<i>B. napus</i>	-	<i>B. napus</i>	1, 16, 32	0.1, 0.001, 0.001	-	Hungary	Pauk et al. 1995
<i>B. napus</i>	-	<i>B. napus</i>	100, 150	0.05-0.07, 0	-	Germany	Gotz and Ammer 2000
<i>B. napus</i>	imidazolinone resistance	<i>B. napus</i>	2, 45	1.3, 0.05	herbicide tolerance/ PCR	German	Krato and Petersen 2012
<i>B. napus</i>	glufosinate resistance	<i>B. napus</i>	1.4, 4.5, 11.5, 200	1.19,0.11, 0.04, 0.006	herbicide tolerance/ PCR	China	Cai et al. 2008
<i>B. napus</i>	glyphosate resistance	<i>B. napus</i>	1, 5, 10, 50	0.16, 0.059, 0, 0	herbicide tolerance/ PCR	China	Di et al. 2009
<i>B. napus</i>	glufosinate resistance	<i>B. napus</i>	0.5, 5, 10, 50	2.50, 0.34, 0.20, 0.004	herbicide tolerance/ PCR	China	Zhao et al. 2013
<i>B. napus</i>	asulam resistance	<i>B. napus</i>	1.4, 4.5, 11.5, 2000	1.19,0.11, 0.04, 0.006	herbicide tolerance/PCR	UK	Paul et al. 1995
<i>B. napus</i>	-	<i>B. napus</i>	0 (mixed plants)	3-12	-	UK	Sweet et al. 1999
<i>B. napus</i>	-	<i>B. napus</i>	4, 8, 20, 34, 56	2, 0.33, 0.16, 0.16, 0.11	-	UK	Simpson et al. 1999
<i>B. napus</i>	-	<i>B. napus</i>	6, 30, 42, 50	0.05, 0.05, 0.33, 0.16	-	UK	Simpson et al. 1999
<i>B. napus</i>	glufosinate resistance	<i>B. napus</i>	1, 3, 12, 47	1.5, 0.4, 0.02, 0.00033	herbicide tolerance	UK	Scheffler et al. 1993
<i>B. napus</i>	-	<i>B. napus</i>	1.5, 11.5, 26.5, 51.5, 91.5	1.0, 0.5, 0.15, 0.1, 0.05	-	UK	Manasse & Kareiva 1991

<i>B. napus</i>	glufosinate resistance	<i>B. napus</i>	2, 50, 150	0.76, 0.04, 0.02	herbicide tolerance	UK	Weekes et al. 2005
<i>B. napus</i>	-	<i>B. napus</i>	2, 50, 100	*0.5, 0.02, 0.01	-	UK	Norris 2001
<i>B. napus</i>	-	<i>B. napus</i>	5, 25, 40, 50, 100, 200	1.2, 0.25, 0.65, 0.1, 0.5, 0.2	-	UK	Norris 2002
<i>B. napus</i>	glufosinate resistance	<i>B. napus</i>	5, 25, 50, 100, 250	3.3, 0.7, 0.4, 0.25, <0.1	herbicide tolerance	UK	Scheffler et al. 1995
<i>B. napus</i>	kanamycin resistance	<i>B. napus</i>	200, 400	0.016, 0.004	PCR	UK	Ramsay et al. 2003
<i>B. napus</i>	kanamycin resistance	<i>B. napus</i>	0, 10, 50, 225, 550, 800	*0.12, 0.04, 0.02, 0.02, 0.001, 0.03	PCR	UK	Ramsay et al. 2003
<i>B. napus</i> ♀	Seed set on male-sterile bait plants	<i>B. napus</i> ♀	0, 10, 50, 225, 550, 800	*0.58, 0.31, 0.33, 0.21, 0.1, 0.02	No. of seed sets	UK	Simpson et al. 1999
<i>B. napus</i> ♀	Seed set on male-sterile bait plants	<i>B. napus</i> ♀	6, 20, 42, 54, 150	21, 0.16, 0.33, 0.11, 0.22	No. of seed sets	UK	Simpson 2002
<i>B. napus</i> ♀	Seed set on male-sterile bait plants	<i>B. napus</i> ♀	100, 200, 400	0.13, 0.03, 0.06	No. of seed sets	UK	Thompson et al. 1999

※ estimated gene flow rate

- information not available

♀ using male sterile varieties or emasculated “bait” plants (petals and stamens removed)

Recommended isolation distances

According to experimental field studies measuring pollen dispersal and outcrossing rates, various requirements of thresholds and recommended isolation distances have been established in conventional *B. napus* production as showed in Table 3. These can also serve as guidelines for recommended separation distances from GM *B. napus* to minimize gene escape through pollen flow.

The recommended isolation distances required for GM *B. napus* trials worldwide range from 50-400 m with a threshold requirement less than 0.9% (Table 3). 50 m isolation distance is recommended for GM trials in the UK, with 200 m isolation recommended for organic *B. napus* (SCIMAC 1999). For certified seeds production, recommended isolation distances of 100-200 m are generally considered sufficient to limit outcrossing and maintain seed purity (Scheffler et al. 1995; USDA 2008). Such isolation distances are not intended to completely prevent outcrossing, but to reduce it to an acceptable level (Scheffler et al. 1995). Current recommended isolation distances required for foundation seeds (99.9% purity) are 400 m and 500 m in Canada and OECD, respectively, with a threshold requirement less than 0.1% (Table 3). In USA, 400 m isolation distance is recommended the required distance for foundation seeds requirements with a threshold requirement less than 0.05% (USDA 2008).

Canadian government regulations stipulate 200 m large isolation zone for GM *B. napus* studies, or 10 m wide non-GM border of synchronously flowering around the studies area (Staniland et al. 2000). An isolation distance of 400 m is required for GM trials in France, Belgium, and Sweden. Australian GM trial requirements including a 400 m isolation distance and a 15 m non-GM buffer zone (Salisbury 2002). In China, a minimum isolation distance of 300 m, rather than 1000 m according to the Implementation Regulations on Safety Assessment of Agricultural GMOs of China (MOA 2002), is recommended as a reasonable distance to ensure a tolerable threshold of pollen flow (less than 0.01%) in GM *B. napus* field trial in China (Zhao et al. 2013).

Based on these data, the separation distances established for seed production by regulatory authorities generally seem to be insufficient to minimize seed contamination considering the long-distance pollen flow outcrossing events (Cai et al. 2008; Paul et al. 1995; Rieger et al. 2002; Zhao et al. 2013). Therefore, to obtain seed purity levels that comply with stipulated thresholds of, for example, 0.05% impurities or less, separation distances of at least 800 m would be required (Andersson and de Vicente 2010).

Table 3. Recommended isolation distances for *B. napus* to meet different thresholds

Threshold ^a	Minimum isolation distance (m)	Country	Reference	Comment
<1%	1.5	UK	Ingram 2000	recommended separation distance between GM and non-GM <i>B. napus</i> , for fields > 2 ha
<0.9%	30	France	CETIOM 2000	based on the survey of field level outcrossing
	50	UK	SCIMAC 1999	recommended separation distance between GM and conventional non-GM <i>B. napus</i>
	200	UK	SCIMAC 1999	recommended separation distance between GM and organic non-GM <i>B. napus</i> and for certified seed production
<0.5%	10	UK	Ingram 2000	recommended separation distance between GM and non-GM <i>B. napus</i> , for fields > 2 ha
	120	France	CETIOM 2000	based on the survey of field level outcrossing
<0.25%	100	USA	USDA 2008	certified seed requirements
	100	Canada	-	certified seed requirements
<0.1%	100	UK	Ingram 2000	recommended separation distance between GM and non-GM <i>B. napus</i> , for fields > 2 ha
	400	France	CETIOM 2000	based on the survey of field level outcrossing
	400	Canada	-	foundation seed requirements
	500	OECD	OECD 2008	foundation seed requirements
	400	USA	USDA 2008	foundation seed requirements
<0.05%	400	USA	USDA 2008	foundation seed requirements
<0.01%	800	Canada	CSGA 2009	certified seed requirements

Modeling of gene flow

It is necessary to establish a mathematical model to predict gene flow and validate the relevant factors affecting gene flow under various conditions. There are two main methods, mechanistic and empirical, for modeling the gene flow at a landscape level (Klein et al. 2003, 2006; Gustafson et al. 2005; Snäll et al. 2007). The mechanistic modeling incorporates physical and biological factors that influence gene flow, and provides insights into the process of gene flow (Klein et al. 2003; Snäll et al. 2007). This modeling method can reveal the general pattern of contemporary gene under influences of various factors. Therefore, it can potentially predict the gene flow under diverse conditions (Snäll et al. 2007). However, mechanistic models are usually mathematically complex and computationally expensive, and often contain many parameters that are difficult to measure in natural conditions (Klein et al. 2003; Snäll et al. 2007). Empirical modeling ignores the details of the dispersal process and often simulates gene flow by applying regression analysis to achieve an empirical function that fits the experimental gene flow data (Klein et al. 2003; Gustafson et al. 2005; Snäll et al. 2007). The empirical method can be easily performed in practice, but cannot be extended to a wider range of environmental conditions (Klein et al. 2003; Snäll et al. 2007). To some extent, although this empirical model is difficult to describe gene flow at various environment conditions, it exhibits a flexible and practical experience for a certain purpose to predict gene flow.

Up to now, several models such as quadratic, log-logistic models, exponential decay, inverse power-law model, rational function, etc., have been developed for prediction of gene flow at a specified distance (Simpson et al. 2006; Stallings et al. 1995; Weeks et al. 2005; Yuan et al. 2007; Zhao. et al. 2013). Although some model showed a good fit to the models, there is a lack of biological meaning for explanation of the estimated parameters. Therefore, the biological meaningful model was more suitable for interpreting the parameters with biological meaning.

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CHAPTER I

Quantifying maximum potential gene flow from *Brassica napus* to its male sterile relatives under greenhouse and field conditions

ABSTRACT

Gene flow from *Brassica napus* to its male sterile (MS) relatives, MS *B. napus*, MS *B. juncea*, and MS *Raphanus sativus* was evaluated in the greenhouse and field conditions. Vernalized plants were planted in plastic pots and placed at various distances up to 24 m and 40 m in the greenhouse and the field, respectively. *B. napus* was used as the pollen donor and its MS relatives as a pollen recipient. In the greenhouse test as a model case study, bumble bee was used as an insect pollinator, while honeybee was used in the field test. No. of flowers and pods set were assessed to calculate pod setting rate, which was then further calibrated. The calibration factor was determined using molecular marker to determine the proportion of pollen donor allele genes introgressed into pollen recipients. In the greenhouse condition, the gene flow from *B. napus* to MS *B. napus* were 20.13% at 3 m distance and reached down to 1.96% at 24 m, decreasing with distance from pollen donor. For MS *B. juncea*, the gene flow was 1.19% at 3 m and decreased down to 0.06% at 24 m. In the field condition, the gene flow rates from *B. napus* to its MS relatives were much greater than those values obtained in the greenhouse condition. The gene flow

to MS *B. napus* was 37.38% at 4 m and decreased down to 10.71% at 40 m, providing greater gene flow in the field condition than in the greenhouse condition. For *B. juncea*, it was 34.59% at 4 m and decreased to 1.97% at 40 m. No gene flow was detected from *B. napus* to *R. sativus* in both greenhouse and field conditions. Even though gene flow rate was relatively lower in the greenhouse study than the field study, the gene flow estimated in the greenhouse condition still provide scientifically comparable and valuable estimation of gene flow and is still viable. The platform for gene flow study in the greenhouse can be used for risk assessment of gene flow where field study is unavailable.

Keywords: *Brassica napus*, *Brassica juncea*, gene flow, pollinator, *Raphanus sativus*, male sterile

INTRODUCTION

With the rapid development of transgenic biotechnology, more and more genetically modified (GM) crops have been released into the agricultural ecosystem. Based on a global scale of biotech crops report, the cultivation area has increased from 1.7 million ha in 1996 to 160 million ha in 2011 (James 2011). Such advantages as improvement of crops production and eco-friendly environment (decreased herbicide applications) have demonstrated by adoption of those crops (Beckie et al. 2006). However, with dramatically increasing acreage of GM crops cultivation worldwide, those associated with unexpected ecological change and negative effects have raised public concerns about the gene dispersal to non-GM crops and relative species (Ellstrand et al. 1999; Firbank and Forcella 2000; Wilkinson et al. 2003). One of the main concerns over these agricultural releases of GM crops is the escape of the transgenes into the natural ecosystem through outcrossing with their wild or weedy relatives (Dunwell 2002; Warwick et al. 2003; Snow 2002).

Brassica napus (AACC, $2n=38$) is currently the third most important oil crop after soybean and oil palm and its global production continues to increase rapidly. GM *B. napus* with such traits as resistant to diseases, tolerant to herbicides, drought, and other stress has been developed and benefited human in crop production. Due to having many wild or weedy relative such as *B. juncea* (AABB, $2n=36$), *B. rapa* (AA, $2n=20$), *B. niga* (BB, $2n=16$), *B. oleracea* (CC, $2n=18$), *R. sativus* (RR, $2n=18$), *R. raphanistrum* (RrRr, $2n=18$), etc., persisting in or near areas of cultivation. Potential gene flow to its relatives as one of the main concerns has been raised.

Asian countries and Korea in particular, co-existence of *B. napus* with its relatives in the diverse cropping system is quite common, where farmers generally manage small-scale fields and form a mosaic agricultural structure. In one cropping season, different *B. napus* varieties with its relatives may grow side-by-side, and

synchrony of flowering period may happen during cultivation, those pose a high potential outcrossing between *B. napus* and its relatives. In case the GM *B. napus* is approved for commercial cultivation, GM *B. napus* and its relatives may co-exist and -cultivate.

Pollinator, bumblebee (*Bombus* spp.) and honeybee (*Apis mellifera*) were introduced for pollination in greenhouse and field studies (Cresswell 1994; Mesa et al. 2013; Pierre et al. 2010). Bumblebee are regarded as an important and effective pollinator for commercial crops pollination. Especially in greenhouse crops pollination, the use of bumblebee is very common practice. Because of its high environmental adaptability (such as poor phototaxis, low and high temperature resistance) and lower degree of evolution, it would be more concentrated on pollinating (Picard-Nizou et al. 1995). By contrast, honeybee, due to their great number, their social life and their ability to pollinate a broad variety of different flowers, are commonly used for effective pollination under field conditions (Durán et al. 2010; Sabbahi et al. 2005).

To date, some studies on gene flow have been reported in Asian countries such as China and Japan, the data indicated a very region-based characteristic which was closely associated with experimental condition and regional climate (Bing et al. 1996; Cai et al. 2008; Chèvre et al. 2003; Jørgensen et al. 1996; Tsuda et al. 2012; Warwick et al. 2003; Weekes et al. 2005; Zhao et al. 2013). However, in Korea there is no report on this regard. Thus, it is necessary to conduct the Korean climate-based assessment to evaluate the potential gene flow from *B. napus* to its relatives and provide sufficient scientific basis for gene flow risk assessment and GM safety management. In the previous gene flow studies, a circular or a rectangular experimental design was usually used (Hüsken and Dietz-Pfeilstetter 2007). In the circular design, the pollen donor is spaced at the center, whereas the pollen recipients are planted at various distances from the pollen donor. This design may be suitable for providing information on gene flow within two species in compass directions. In the rectangular design, the pollen donor is located on one side and pollen recipients are planted at various distances from the pollen donor. Taken into

account the diverse agricultural structure with *B. napus* co-cultivated with its relatives, in the present study, we adopted the rectangular design by planting three relatives at various distances from *B. napus* pollen donor to simulate a mosaic agricultural structure.

It is worth pointing out that our concern is to assess the potential gene flow from *B. napus* to its relatives at a specified distance. Potential gene flow is different from “over-estimate gene flow”, which is the scientific-based approach for assessing the potential gene flow risk with showing the upper threshold. In order to achieve this aim to obtain the upper threshold, using male sterile recipients, entire synchrony of flowering period between donor and recipient, and presence and abundance of pollinators were adopted. With consideration of no previous experience on gene flow risk assessment in Korea, the objectives of this studies were i) to determine optimal environmental conditions by a greenhouse evaluation as a case study; ii) on the basis of the greenhouse experience, to quantify the maximum potential gene flow from *B. napus* to its MS relatives under Korean field climatic condition by artificially introducing pollinator and overlapping flowering periods between pollen donor and recipients.

MATERIALS AND METHODS

Plant materials

Two varieties of *B. napus* cv. Tammi and Tamla (AACC, 2n=38) were used as the pollen donor, and the male sterile (MS) plants, including MS *B. napus*, two varieties of MS *B. juncea*-9 and -10 (AABB, 2n=36), and MS *R. sativus* (RR, 2n=18) were used as the pollen recipients, respectively. All seeds were obtained from National Academy of Agricultural Science, Korea. The seeds were sowed and cultivated in greenhouse of the experimental farm station of Seoul National University, Korea. The seedlings at two-true leaf growth stage were stored in a cold chamber (4°C) for vernalization treatment at National Academy of Agricultural Science, Korea.

Experimental design

In this study, both greenhouse and field experiments were conducted under the authorization GM field of the experimental farm station of Seoul National University, Suwon, Korea (authorization number: RDA-AB-2011-014) from June to October in 2012. Firstly, the controlled greenhouse study was performed as a model case to determine the fundamental conditions for quantifying potential gene flow, and then in an open field, the maximum potential gene flow from *B. napus* to its MS relatives was evaluated. Pollinator, bumblebee (*Bombus* spp.) and honeybee (*Apis mellifera*) were respectively introduced in greenhouse and field to facilitate gene flow from *B. napus* to its MS relatives.

Greenhouse study

Vernalised seedlings were transplanted and cultivated in pots with a mix reconstructed soil (commercial horticultural soil: normal soil = 1:1). Fifty pollen donor plants (25 plants for each variety of Tammi and Tamla) were placed at one side in greenhouse (Size of greenhouse: 24 m × 6 m). Prior to flowering, MS pollen recipients were accordingly arranged at the various distances of 3, 6, 12, 18, and 24

m from the pollen donor, and each MS pollen recipient line at each distance consists of 2 MS *B. napus*, 5 MS *R. sativus*, 3 MS *B. juncea-9*, and 4 MS *B. juncea-10* (Fig. 2A). A bumblebee hive with approximately 250 populations was placed at 12 m from pollen donor. During the flowering period, regular observations such as the phenology of plants flowering, the number and pollinating behavior of bees (investigated everyday at 8:30-9:00) in each plot were made. Total number of flowers exposed and pods formed were counted for each inflorescence and each individual recipient. Pods setting rate was calculated by comparing total number of pods set and total number of flowers for each individual recipient. The fertilizer application, insecticide spray, and irrigation management were carried out regularly.

Field study

The procedures for seedlings transplanting, cultivating, and transferring in field prior to flowering were described as in greenhouse study. Taking into account the greenhouse study and field scale constraints, distances from the pollen donor for field study were adjusted to 4, 8, 16, 24, 32, and 40 m. One hundred pollen donor plants in total (50 plants for each variety of Tammi and Tamla) were planted at one side in field. Each MS recipient line consists of 5 MS *B. napus* plants, 7 MS *R. sativus* plants, 4 MS *B. juncea-9* plants, and 4 MS *B. juncea-10* plants and was accordingly planted at those distances (Fig. 2B). A honeybee hive with approximately 10,000 populations was placed at 20 m from pollen donor during flowering and honeybee's visit frequency was investigated everyday at 10:00-11:00 am except raining day. Regular observations and plant management were made as described in greenhouse study. Total number of flowers exposed and pods formed were also counted for each inflorescence and each individual recipient for determination of pods setting rate.

Determination of gene flow rate

Both in greenhouse and field studies, pods formed on MS recipients could be

considered the result of pollination from pollen donor. But there was the possibility for MS plants to recover the fertility and produce seeds by selfing. In order to check the proportion of selfed seeds among the progenies, PCR analysis was performed by detecting presence or absence of pollen donor allele introgression in randomly selected pollen recipient seeds. Thus, the gene flow rate was calculated accordingly, with the pods setting rate estimate for each species at each distance being multiplied by calibration factor based on PCR analysis.

Primer screening

Three out of 99-pairs of primers (source from Izzah et al. 2013) were screened for identifying the progenies of MS plants by selfing or outcrossing with pollen donor (Table 4).

Table 4. Selected primers for identifying hybrids among the progenies of MS recipients

Species	Markers	Primers application	Primer sequences (5'→3')
<i>B. juncea</i>	BnGMS539	Positive control	F: CATCACTCAATCCAAGACCT R: AGAACCTGAAACAAACGATG
	BoKAH45TR	Non-hybrids or hybrids	F: ATTATGACGCCTGGTTTTA R: ATTGGTTAGAAGTTATGGGAAC
<i>R. sativus</i>	COS0842	Non-hybrids or hybrids	F: TGGGCTGCCTTGAGAACA R: AGATGCTGAACTTGAATCCACTG

PCR analysis

The genomic DNA was extracted by following the minor modified CTAB method (Doyle and Doyle 1990) and standardized to a final concentration of 20 ng μL^{-1} . DNA amplification was performed in a reaction volume of 10 μL containing 20 ng of template DNA (0.5 μL), 10 \times PCR reaction buffer (Sigma Co., USA) (200 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM MgCl_2) (1 μL), 2.5 μM of dNTPs (Sigma

Co., USA) (0.8 μ L), 5 μ M each of the forward and reverse primers (Sigma Co., USA) (0.4 μ L), 2 units of *Taq* DNA polymerase (0.5 μ L), and sterile distilled water (6.8 μ L). The PCR was conducted in a T100TM 96-Well Thermal cycler (Bio-Rad Laboratories, Singapore), under the following conditions: 94°C for 4 min, followed by 35 cycles (94°C for 30 s, 54°C for 30 s, and 72°C for 30 s), and finally 72°C for 10 min for pollen donor allele gene introgression in progenies of *B. juncea*. For pollen donor allele gene introgression in progenies of *R. sativus*, the conditions were 94°C for 5 min, followed by 35 cycles (94°C for 30 s, 55°C for 30 s, and 72°C for 30 s), and finally 72°C for 5 min . The expected PCR product size was around 180-200, 269, and 220-230 bp for primer 8, primer 11, and primer COS0842, respectively. The PCR products were analyzed by electrophoresis on 1.2% (w/v) agarose gel containing ethidium bromide (Et-Br) in 0.5 \times TAE buffer. The gel was then visualized and the images were photographed using Digital Gel Documentation System-200 (the Alpha Innotech Corporation, USA). Samples with the introgressed allele gene DNA fragment were confirmed as the hybrids outcrossed with pollen donor.

Statistical analysis

Both greenhouse and field studies, each individual recipient at each distance was considered as replication and arranged completely randomized in each plot. The full data set was subjected to analysis of variance (ANOVA) using Genstat5 (Genstat Committee, 1997). The pods setting rate for each species at each distance was mean of replications of each individual rate. Calibration factor was calculated by determination of the proportion of progenies with introgressed pollen donor allele gene among the total progenies tested. The gene flow rate was finally confirmed by pods setting rate multiplied by calibration factor.

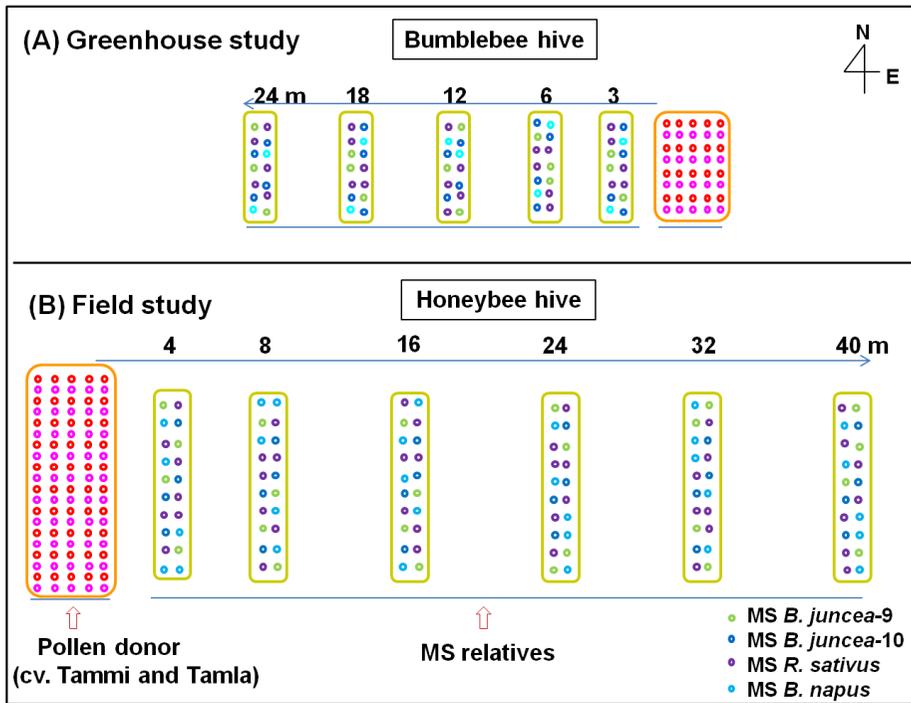


Fig. 2. Experimental design (A, greenhouse mode case study; B, field study) for evaluation of gene flow from *B. napus* to its MS relatives in 2012, Suwon, Korea. The *B. napus* cv. Tammi and Tamla was used as pollen donor, and its MS relatives were used as pollen recipients. The locations of the beehives are shown.

RESULTS

Gene flow in the greenhouse study

Synchrony of flowering period

In order to maximize synchrony of flowering periods between pollen donor and recipients, two varieties of pollen donors (*B. napus* cv. Tammi and Tamla) with different flowering timings were planted. During the flowering period, biologically important dates of experimental plants, such as dates of 1st flower blooming, peak blooming, and end of blooming, were recorded (Table 5). *B. napus* cv. Tammi has an earlier flowering time (approximately 1 week) than *B. napus* cv. Tamla, and the later flowering time of *B. napus* cv. Tamla guaranteed the maximal synchrony of flowering with pollen recipients. The date of peak blooming for both pollen donor and recipient was around middle of July (Table 5). A significant flowering synchronization between pollen donor and recipients was achieved with the 26, 30, 30, and 31 days of flowering period overlapped for MS *B. napus*, MS *B. juncea-9*, MS *B. juncea-9*, and MS *R. sativus* with the pollen donor, respectively. Therefore, the periods of flowering synchronization are long enough for evaluation gene flow from *B. napus* to its relatives.

Bumblebee visit

As *B. napus* cv. Tammi and Tamla are only pollen sources and produced many flowers, a relatively larger number of bumblebees gathered in this area with a mean visit frequency of 0.62 (plant⁻¹ min⁻¹) (Fig. 3). Moreover, the bumblebee visit differed significantly from the species of pollen recipients and the distance from the pollen donor. The closest pollen recipient from the pollen donor showed the most frequent bumblebee visit with approximately 0.6, 0.1, and 0.2 plant⁻¹ min⁻¹ on MS *B. napus*, MS *B. juncea*, and MS *R. sativus* plants, respectively. The visit frequency decreased with increasing the distance from the pollen donor, indicating that

bumblebees gather nectar from flowers located near their hive. These data also showed that flowers of *B. napus* were more attractive to bumblebee compared with flowers of *B. juncea* and *R. sativus* although they grew at the same location and broomed simultaneously (Fig. 3). Interestingly, bumblebee visited the flowers of *R. sativus* more frequently than those of *B. juncea* (Fig. 3), which might be due to the difference in flower size, *R. sativus* flower is larger than that of *B. juncea*, and pollinating preference of bumblebee.

Pods setting rate

MS plants were used as the tracing marker, so pods setting on these recipients can be considered as the result from gene flow from pollen donor plants. No significant difference was found in the number of flowers of each MS recipient at various distances ($P < 0.05$). The average number of flowers plant⁻¹ was 1580.8±32.5, 2235.2.4±36.7, and 2034.8±55.1 for each MS *B. napus*, *B. juncea*, and *R. sativus*, respectively. The average number of pods plant⁻¹ for these three species decreased with increasing the distance from the pollen donor (data not shown). Pod setting rate of MS *B. napus* was 24.25% at 3 m, 10.91% at 6 m, and sharply decreased down to 2.36% at 24 m. Pod setting rates of MS *B. juncea*-9 and -10 were 0.298% and 1.294% at 3 m, 0.099% and 0.967% at 6 m, and decreased down to 0.029% and 0.147% at 12 m, respectively. No pods were set at 18 m for MS *B. juncea*-9, while the pod setting of MS *B. juncea*-10 was 0.055% even at 24 m, the longest distance. As for MS *R. sativus*, a few pods formed in the plants, but no seeds were found in the pods, indicating that the low possibility of gene flow from *B. napus* to *R. sativus* (Table 6).

Determination of calibration factor and gene flow rate

Calibration factor and gene flow rate were summarized in Table 6. The calibration factor was determined using molecular marker to check the proportion of seeds with introgressed *B. napus* allele gene among the randomly selected MS seeds (Fig. 4). The gene flow rate was calculated by multiplied pods setting rate by

calibration factor (Table 6). The gene flow rate between *B. napus* under controlled greenhouse condition was estimated ranging from 1.96-20.13% at 3-24 m. In the case of *B. juncea*, it was varied with the varieties. For *B. juncea-9*, it was estimate of 0.298% at 3 m, 0.099% at 6 m, 0.029% at 12 m, and not detected beyond 18 m from the pollen donor, respectively. By contrast, for *B. juncea-10*, it was estimate of 1.19% at 3 m, 0.903% at 6m, 0.147% at 12 m, 0.085% at 18 m, and 0.055% at 24 m, respectively (Table 6). With regard to *R. sativus*, no gene flow was detected in the present greenhouse condition with entire synchrony of flowering period and presence of bumblebee.

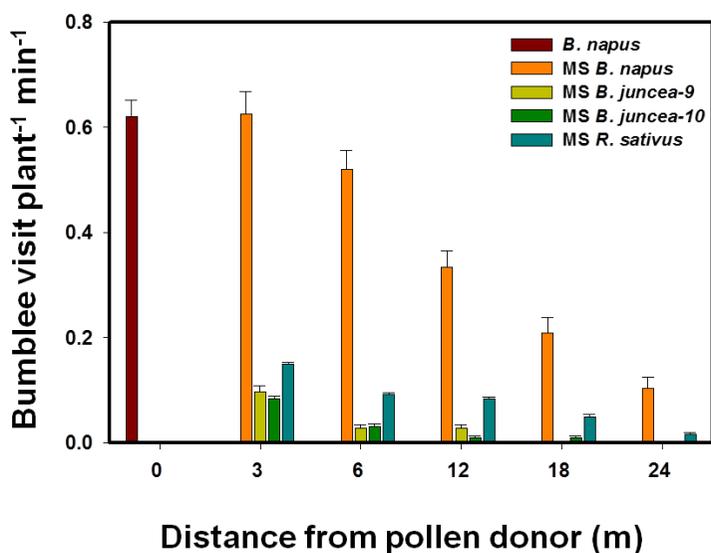


Fig. 3. Bumblebee visit frequency ($\text{plant}^{-1} \text{min}^{-1}$) ($\pm\text{SE}$) at various distances (m) from pollen donor during the flowering periods in the greenhouse in 2012.



Fig. 4. PCR confirmation using MS *B. juncea-9*, *-10* and MS *R. sativus* genomic DNA. (A), the detection of the presence of tested *B. juncea-9* and *-10* genomic DNA using positive control primer BnGMS539; (B), the confirmation of hybrid seeds using primer BoKAH45TR for *B. juncea-9* and *-10*. (C), the confirmation of hybrid seeds using primer COS0842 for *R. sativus*. Bn, *B. napus* cv. Tammi; Bj, *B. juncea-9* and *-10*; Rs, *R. sativus*, Bn+Rs, DNA mixtures of *B. napus* and *R. sativus*; (-) Con: negative control, amplified by PCR without DNA sample.

Table 5. Biological dates for a specific reproductive growth stage of experimental plants in the greenhouse in 2012

Experimental plants		1 st flowering	Peak flowering	End of flowering	Flowering Duration (days)	Synchrony of flowering (days)
Pollen donor	Tammi	28 Jun	12 Jul	26 Jul	29	-
	Tamla	5 July	20/Jul	4 Aug	30	-
Total flowering duration		-	-	-	38	-
Pollen recipients	<i>B. napus</i>	3 July	19 Jul	28 Jul	26	26
	<i>B. juncea-9</i>	27 Jun	12 Jul	27 Jul	31	30
	<i>B. juncea-10</i>	29 Jun	15 Jul	28 Jul	30	30
	<i>R. sativus</i>	2 Jul	13 Jul	1 Aug	31	31

Table 6. Summary of pods setting rate (PSR), calibration factor (CF), and gene flow rate (GFR) in the greenhouse in 2012

Distance (m)	<i>B. napus</i>			<i>B. juncea-9</i>			<i>B. juncea-10</i>			<i>R. sativus</i>		
	PSR (%) a	CF (%) ^b	GFR (%) ^c	PSR (%)	CF (%)	GFR (%)	PSR (%)	CF (%)	GFR (%)	PSR (%)	CF (%)	GFR (%)
3	24.25 ^a	0.83	20.13	0.30	1.00	0.30 ^b	1.29	0.92	1.19	0.05	-	0.00
6	10.91	0.83	9.06	0.10	1.00	0.10	0.97	0.93	0.90	0.04	-	0.00
12	6.77	0.83	5.62	0.03	1.00	0.03	0.15	1.00	0.15	0.02	-	0.00
18	4.51	0.83	3.74	0.00	-	0.00	0.09	1.00	0.09	0.04	-	0.00
24	2.36	0.83	1.96	0.00	-	0.00	0.06	1.00	0.06	0.00	-	0.00
LSD _{0.05}	3.73	NC ^d	2.12	0.03	NC	0.03	0.32	0.93	NC	0.01	NC	NS ^e

^a Pods setting rate (PSR) calculated by dividing no. of pods by that of no. of flowers for each plant of MS *B. napus*, MS *B. juncea-9*, MS *B. juncea-10*, and MS *R. sativus* at various distance from pollen donor.

^b Calibration factor (CF) for *B. napus* calculated based on the field trial in 2014; for *B. juncea* and *R. sativus*, the CF calculated by dividing no. of tested seeds with introgressed *B. napus* allele gene by that of total no. of tested seeds.

^c Gene flow rate (GFR) calculated by multiplied PSR by CF.

^d NC, not calculated.

^e NS, not significant.

Gene flow in the field study

Synchrony of flowering period

For field study, a 29-overlapped-day, 28-overlapped-day, and 27-overlapped-day for MS *B. napus*, MS *B. juncea-9*, and MS *B. juncea-10*, respectively, were synchronized with pollen donor (Table 7). For MS *R. sativus*, a relatively longer flowering period was observed compared with other recipients and pollen donors, therefore, the experiment was immediately terminated as soon as pollen donor end of flowering, and accordingly, unsynchronized flowers were eliminated, finally resulting in a 31-overlapped-day with pollen donors.

Honeybees visit

As observed in greenhouse, a larger number of honeybee gathering was found near pollen donor area, and consistently decreased with increasing the distance from pollen donor in the three species. The number of honeybee visit also differed from the species of pollen recipients as bumblebee in greenhouse. *B. napus* showed the most attractive to honeybee compared to other two species (Fig. 5). For example, at 4 m it was approximate $0.85 \text{ plant}^{-1} \text{ min}^{-1}$ for *B. napus*, more frequent than that of $0.45 \text{ plant}^{-1} \text{ min}^{-1}$ for *B. juncea* and $0.32 \text{ plant}^{-1} \text{ min}^{-1}$ for *R. sativus*. Similar patterns of flowers visit were found at other distances as well. In addition, the difference of honeybee visit was also observed in the two varieties of *B. juncea* (Fig. 5).

Pods setting rate

No significant difference ($P < 0.05$) was found in average number of flowers plant^{-1} for each MS recipient of *B. napus*, *B. juncea*, and *R. sativus* at various distances (1312.8 ± 13.7 , 1428.3 ± 21.3 , and 1041.2 ± 17.8 , respectively), but the average number of pods plant^{-1} were decreasing as increasing the distance from the pollen donors for those three species, respectively (data not shown). For pods setting rate of MS *B. napus*, it was 45.0% at 4 m, 35.7% at 16 m, 31.8% at 24 m, and decreased to 12.9%

at 40 m from the pollen donor (Table 8). For MS *B. juncea*, the pods setting rate was differed from the varieties. It was 14.13% vs. 35.12% for MS *B. juncea*-9 and -10 at 4 m, 6.34% vs. 19.79% at 16 m, and decreased to 1.97% vs. 3.63% at 40 m, respectively (Table 8). In the case of MS *R. sativus*, it was 15.9% at 4 m, 8.4% at 16 m, decreasing to 6.1% at 24 m, and 1.3% at 40 m (Table 8).

Determination of calibration factor and gene flow rate

The calibration factors based on PCR analysis for the three species were summarized in Table 8. The gene flow rate obtained from field study was very different from greenhouse study, indicating that the environmental effect on gene flow (Table 8). Based on the Table 8, for MS *B. napus*, the gene flow rate was estimated ranging from 10.71-37.35% at 4-40 m. For the two varieties of *B. juncea*, the gene flow rates ranged from 1.97-13.78% for MS *B. juncea*-9 and 3.63-34.59% for MS *B. juncea*-10 at 4-40 m, respectively (Table 8). Data from the PCR analysis showed that all tested MS *R. sativus* progenies were not hybrids (Table 8), indicating that no gene flow occurred from *B. napus* to *R. sativus* in the present experimental condition.

Table 7. Biological dates for a specific reproductive growth stage of experimental plants in the field in 2012

Experimental plants		1 st flowering	Peak flowering	End of flowering	Flowering Duration (days)	Synchrony of flowering (days)
Pollen donor	Tammi	23 Aug	17 Sep	22 Sep	30	-
	Tamla	1 Sep	25 Sep	29 Sep	29	-
Total flowering duration		-	-	-	68	-
Pollen recipients	<i>B. napus</i>	24 Aug	23 Sep	22 Sep	29	29
	<i>B. juncea-9</i>	23 Aug	18 Sep	20 Sep	28	28
	<i>B. juncea-10</i>	27 Aug	20 Sep	23 Sep	27	27
	<i>R. sativus</i>	30 Aug	21 Sep	29 Sep	31	31

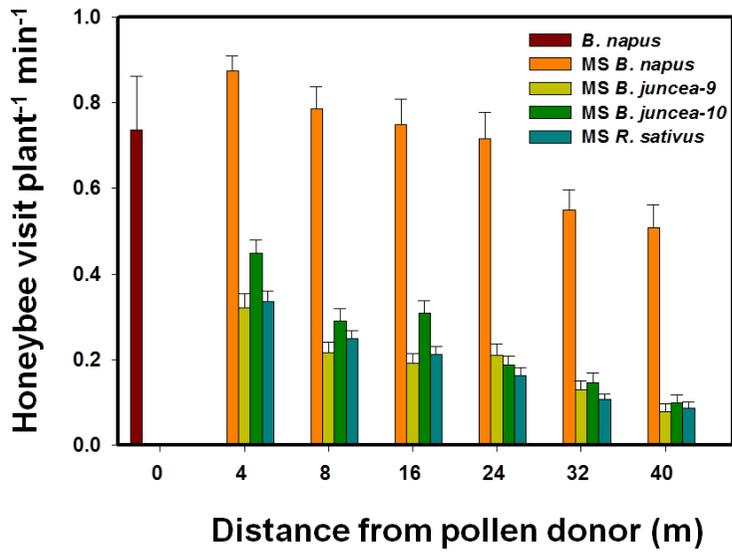


Fig. 5. Honeybee visit frequency ($\text{plant}^{-1} \text{min}^{-1}$) ($\pm\text{SE}$) at various distances (m) from pollen donor during the flowering periods in the field in 2012.

Table 8. Summary of pods setting rate (PSR), calibration factor (CF), and gene flow rate (GFR) in the field in 2012

Distance (m)	<i>B. napus</i>			<i>B. juncea-9</i>			<i>B. juncea-10</i>			<i>R. sativus</i>		
	PSR (%) ^a	CF (%) ^b	GFR (%) ^c	PSR (%)	CF (%)	GFR (%)	PSR (%)	CF (%)	GFR (%)	PSR (%)	CF (%)	GFR (%)
4	45.0 ^a	0.83	37.35	14.13	0.98	13.78	35.12	0.99	34.59	15.90	0.00	0.00
8	39.6	0.83	32.87	9.03	0.90	8.13	25.30	1.00	25.30	13.10	0.00	0.00
16	35.7	0.83	29.61	6.34	1.00	6.34	19.79	1.00	19.79	8.40	0.00	0.00
24	31.8	0.83	26.39	4.78	1.00	4.78	12.16	1.00	12.16	6.10	0.00	0.00
32	26.5	0.83	22.00	3.59	0.90	3.23	6.36	1.00	6.36	4.40	0.00	0.00
40	12.9	0.83	10.71	1.97	1.00	1.97	3.63	1.00	3.63	1.30	0.00	0.00
LSD _{0.05}	9.37	NC ^d	8.76	2.32	NC	3.45	4.13	NC	9.78	2.42	NC	NS ^e

^a Pods setting rate (PSR) calculated by dividing no. of pods by that of no. of flowers for each plant of MS *B. napus*, MS *B. juncea-9*, MS *B. juncea-10*, and MS *R. sativus* at various distance from pollen donor.

^b Calibration factor (CF) for *B. napus* calculated based on the field trial in 2014; for *B. juncea* and *R. sativus*, the CF calculated by dividing no. of tested seeds with introgressed *B. napus* allele gene by that of total no. of tested seeds.

^c Gene flow rate (GFR) calculated by multiplied PSR by CF.

^d NC, not calculated.

^e NS, not significant.

DISCUSSION

Greenhouse model case study

To our knowledge, up to date there is no study on *B. napus* gene flow to its relatives in Korea. The basic knowledge regarding gene flow risk assessment such as the potential distance of gene flow and the possibility of gene flow among *Brassica* family for Korean case is not available. Moreover, the affecting factors associated with gene flow including the degree of synchrony of flowering period, the influence of types of pollinators as well as surrounding environment are also needed to reveal for better understanding gene flow. In the previous field studies conducted in other countries, the gene flow rate estimated against the distance was significantly influenced by several factors. Therefore, it is difficult to decide proper planting distance between pollen donor and recipient, population size under a specific climatic or geographic condition. In addition, since the GM *B. napus* has not been approved for commercial cultivation in Korea, a field study involving GM *B. napus* is almost impossible. With the consideration of all these, the greenhouse study as a model case was conducted with bumblebee as a pollinator to accumulate the basic knowledge for Korean case and set up initial experimental conditions. The greenhouse study revealed that the maximum distance allowing gene flow from *B. napus* to MS *B. napus* and *B. juncea* was farther than 24 m, where the rates of gene flow from *B. napus* were 2.36% (24 m) and 0.06% (24 m) to MS *B. napus* and MS *B. juncea*, respectively. However, the distance varied with the varieties of *B. juncea*. In the case of MS *B. juncea-9*, the maximum distance was only 12 m with the gene flow rate of 0.03%, and no gene flow was detected at farther than 18 m, while with regard to MS *B. juncea-10*, it was 24 m with the gene flow rate of 0.06%. This difference might be resulted from the difference in attractiveness of their flowers to bumblebee (Fig. 3). No gene flow was detected in *R. sativus* regardless of distance from pollen donor, indicating the extreme low possibility of gene flow from *B. napus* to *R. sativus* in the natural ecosystem.

Potential gene flow under the field condition

In the previous studies, the evaluations were generally conducted under natural condition and the common recipients were used. The gene flow rate obtained from those studies was quite approaching the actual values because of mimicking a similar natural field condition. However, due to the common recipients used in those studies with showing a lower gene flow rate (<3%), to some extent, it is not easy to differentiate the variance in gene flow with increasing the distance from pollen donor in some cases. More importantly, it is impossible to estimate the potential gene flow rate. Potential gene flow is different from “over-estimate gene flow”, which is the scientific-based approach for assessing the potential gene flow risk with showing the upper threshold.

In order to obtain the potential gene flow rate from *B. napus* to its relatives, MS recipients as the ideal material were used for achieving a higher gene flow rate. Moreover, two varieties of *B. napus* with different flowering timings were performed as pollen donor for providing an entire synchrony of flowering period with MS recipients. Meanwhile, honeybee was introduced as the pollinator during the flowering period to facilitate gene flow. By doing so, the potential gene flow rate from *B. napus* to MS *B. napus* was estimated ranging from 10.71-37.35% at 4-40 m from the pollen donor. The values were much higher than the previous studies (for common recipient used, the rates ranging from 0.5-1.3% at 2-45 m, Krato and Petersen 2012; 0.04-2.5% at 0.5-50 m, Zhao et al. 2013; for MS recipient used, 0.33-0.58% at 0-50 m, Simpson et al. 1999; 0.11-21.0% at 6-54 m, Simpson 1999). For MS *B. juncea*, the potential gene flow rate was estimated ranging from 1.97-34.59% at 4-40 m from the pollen donor. It is the first to use the MS *B. juncea* as ideal material for assessing the potential gene flow rate and the values obtained in the study were much higher than the Japanese field evaluation using common *B. juncea* (gene flow rate ranging from 0.03-1.62% at 0-17.5 m) (Tsuda et al. 2012). No gene flow was detected from *B. napus* to *R. sativus* in our study. Although the pod setting rate was estimated, the PCR analysis demonstrated that the pods were not outcrossed by *B. napus*, indicating the recovery of male sterile of *R. sativus*. For

the decreasing pattern of pods setting rate associated with distance, honeybee visit frequency was hypothesized to interpret that phenomena. As more frequent honeybee visit observed near the pollen donor with 0.32 plant min⁻¹ at 4 m and was much higher than other distances (0.21 at 16 m and 0.08 plant min⁻¹ at 40 m) (Fig. 4). Inevitably, such more frequent visit would promote the pollination between male sterile recovered and male sterile unrecovered *R. sativus*, resulting in honeybee visit frequency related pod setting rate.

Here, we'd like to highlight the potential application of greenhouse evaluation as a model case study for those countries without previous experience on gene flow assessment or having the restrictions on GM crops field evaluation. Because the greenhouse was a manually controlled environment, the factors (or condition) could be decided (or controlled) on the basis of the experimental purposes. With excluding the influence of natural environment (climatic factors), the single effect of pollinator associated with pollen dispersal would be estimated. Moreover, there is no need to worry about the pollen escape to outside of greenhouse.

In summary, this is the first study on gene flow risk assessment from *B. napus* to its MS relatives under Korean climatic environment. The greenhouse model case study was conducted for determining basic experimental conditions. Accordingly, potential gene flow from *B. napus* to its MS relatives under field condition was further evaluated. The greenhouse model case study proved to be a flexible and controllable ways to determine experimental conditions and reveal the relationship among the factors on affecting gene flow. The field evaluation using MS recipients with artificially providing favorite pollination condition showed the maximum potential gene flow rate from *B. napus* to its MS relatives. According to the experimental result, the potential gene flow rate to its MS relatives ranging from 12.9-45.0% for MS *B. napus* and 1.97-34.59% for MS *B. juncea* at 4-40 m, respectively, and no gene flow was detected from *B. napus* to *R. sativus*. The experimental data provides informative reference values and scientific basis for risk assessment on gene flow under Korean climatic condition, and also would help decision-making on proper isolation distance for GM safety management.

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CHAPTER II

Quantifying gene flow from genetically modified *Brassica napus* to its male fertile relatives under field condition

ABSTRACT

Gene flow from genetically modified (GM) plants to compatible relatives is one of the most concerns for growing of GM crops. In this study, a potential gene flow from GM *Brassica napus* to non-GM *B. napus* as well as its relatives, *B. juncea* and *Raphanus sativus*, was evaluated in the authorized GM trial field located in Suwon, Korea in 2013 and 2014. Vernalized plants planted in pots were transferred to the GM field with a range of distances up to 95 m and 128 m in 2013 and 2014, respectively, from GM *B. napus* to its relatives. To provide a favorable condition for gene flow, entire synchrony of flowering periods between pollen donor and recipient was achieved by planting GM *B. napus* at three different times and one honeybee (*Apis mellifera*) hive was placed in the immediate vicinity of the field to provide sufficient number of honeybees. Herbicide resistance (glufosinate-ammonium) and simple sequence repeats (SSR) markers were used to confirm F1 hybrids resulted from outcrossing between GM *B. napus* and its relatives. The gene flow rate from GM *B. napus* to non-GM *B. napus* was 2.33% at 2 m distance and decreased with increasing distance, reaching 0.007% at 75 m distance with no gene flow observed farther than 85 m. In the case of non-GM *B. juncea*, it was 0.076% at 2 m and reached 0.025% at 16 m distance with no gene flow observed beyond 24 m.

No gene flow was observed from GM *B. napus* to non-GM *R. sativus*, suggesting that gene flow risk is very low or does not exist. The log-logistic model well described gene flow against distance for both *B. napus* and *B. juncea*. The model estimated that the isolation distances for 0.01% as a tolerable threshold were 122.5 m and 23.7 m for *B. napus* and *B. juncea*, respectively. Therefore, our findings suggest that gene flow risks from GM *B. napus* to non-GM *B. napus* and non-GM *B. juncea* exist although gene flow rate is low in the field condition. The data and model presented in this study may be useful and reference for gene flow risk management and future study.

Keywords: *Brassica juncea*, *Brassica napus*, gene flow, genetically modified, log-logistic model, *Raphanus sativus*, synchrony of flowering

INTRODUCTION

Brassica napus (AACC, $2n=38$) is a global important oil crop having many wild or weedy relatives co-existing in agricultural ecosystems. Achievements have been made in the development of GM *B. napus* for high oil content, resistance to drought or insects, tolerant to herbicides, novel fatty acid, and an increase of bioactive compounds. The cultivation of GM *B. napus* has increased over the last two decades, especially in North America and Austria. The acreage of GM *B. napus* was amount 8.0 million hectares in the world, occupying 26% of the total acreage of *B. napus* (James 2011). Although the advantages for adoption of GM *B. napus* has demonstrated such as substantial yield increase and environmental benefits (reduction of herbicide use) (Beckie et al. 2006), concerns have also been raised on potential risk to ecological environment through outcrossing (gene flow) with its relatives (Ellstrand et al. 1999; Firband and Forcella 2000; Warwick et al. 2003; Snow 2002). Thus, science-based risk assessment on this regard is imperative to accumulate knowledge and provide informative data to guide the agricultural practice.

Generally, the Asian countries have the diverse cropping system with many crops co-existence including *B. napus* and its relatives. Most of the farmers only have small pieces of land with the land-scale less than half a hectare. Hence, a mosaic agricultural cropping structure was quite common with different crops growing side-by-side. Currently, in Korea, the GM *B. napus* is not approved for commercial production, however, import of the GM crops has been conducting for animal feeding or edible oil processing and dramatically increased during past five years. The spilled GM *B. napus* seeds have been found around the Incheon port, indicating that the potential risk of gene flow from spilled GM *B. napus* to its relatives (Lee et al. 2007). The previous studies on evaluation of gene flow showed a very region-based characteristic which was closely associated with experimental condition and regional climate (Beckie 2001; Bing et al. 1996; Cai et al. 2008; Chèvre et al. 2003;

Jørgensen et al. 1996; Krato and Petersen 2012; Rieger et al. 2002; Warwick et al. 2003; Weekes et al. 2005).

The isolation distance required for GM trial quite varies widely, in the case of *B. napus*, although nations-wide, it ranges from 50-400 m (EU 2003; Salisbury 2002; SCIMAC 1999; Staniland et al. 2000). Recently, a Chinese study recommended that 300 m, rather 1000 m, is a reasonable distance to ensure a tolerable threshold of gene flow less than 0.01% (Zhao et al. 2013). Up to now, there is no regulation on isolation distance for GM trial in Korea. A longer isolation distances increase both costs and the difficulty of implementing regulations, which together substantially restrict GM crops development. In contrast, insufficient isolation distances have potential health and environmental risks. Therefore, a proper isolation distance must be required to guarantee the food and environmental biosafety of GM crops, and the market acceptability of products.

To our knowledge, there is no report on *B. napus* gene flow study except our previous study in 2012 in Korea. In that study, a greenhouse model case study was firstly conducted to determine the experimental conditions. With those experience and knowledge accumulated, a field evaluation of maximum potential gene flow was further conducted using male sterile (MS) relatives as pollen recipients with artificial synchrony of flowering period between pollen donor and recipient and introduction of pollinator. The experimental results showed the distance of *B. napus* pollen flow to its male sterile (MS) relatives was farther than 40 m with a higher gene flow rate under Korean field climatic condition. Due to MS lines used as the pollen recipient with representing the maximum potential gene flow, it is necessary to evaluate the actual potential gene flow using male fertile (MF) pollen recipient to provide more realistic and informative data based on Korean climatic condition. However, owing to the restrictions of field scale and regulation of use (management) of GM seeds, a large scale of field evaluation is almost impossible to conduct in Korea. Meanwhile, with consideration of the mosaic agricultural structure with co-existence of *B. napus* and its relatives growing side-by-side, a discontinuous design with planting pollen donor at one side and locating its several

recipients at various distances from pollen donor is more practical and feasible.

In order to facilitate gene flow from GM *B. napus* to its relatives to provide the potential gene flow for risk assessment, an entire synchrony of flowering period between GM pollen donor and recipient was artificially achieved by three-time's GM *B. napus* transplanting. Also, honeybee was introduced as pollinator during the flowering period. Therefore, the objective in the present study were i) to evaluate the potential gene flow from GM *B. napus* to its MF relatives in Korean field climatic condition and ii) to determine a proper isolation distance between GM *B. napus* and its relatives.

MATERIALS AND METHODS

Plant materials

Genetically modified (GM) *B. napus* (AACC, 2n=38), homozygous and containing the *bar* gene (Phosphinothricin acetyltransferase) which is a dominant gene conferring resistance to the herbicide glufosinate-ammonium (trade name Basta[®]), was used as the pollen donor. Non-GM *B. napus* cv. Tammi (AACC, 2n=38), *B. juncea* (AABB, 2n=36), and *R. sativus* (RR, 2n=18) were used as the pollen recipients. All seeds were obtained from National Academy of Agricultural Science, Korea, and sowed in the greenhouse of the experimental farm station of Seoul National University, Suwon, Korea. The vernalization treatment was made under natural condition during the winter season (approximately 1.5 months) when the growth stage of seedlings reached at the two-true leaf stages.

Field design

Field studies were performed under the authorization field of the experimental farm Station of Seoul National University, Suwon, Korea (authorization number: RDA-AB-2011-014), in 2013 and 2014 from March to July. The vernalised experimental plants were transplanted in the GMO isolated field prior to flowering. A discontinuous design with planting pollen donor at one side and locating its three relatives at three independent directions at various distances was adopted (Fig. 6). In 2013, ten distances of 4, 8, 16, 24, 32, 45, 65, 75, 85, and 95 m (ten recipients at each distance for each species) and in 2014 seven distances of 2, 4, 8, 16, 32, 64, and 128 m (six recipients at each distance for each species) from the GM pollen donor were included, respectively.

In order to ensure maximum synchrony of flowering periods between pollen donor and recipient, three different times of GM *B. napus* transplanting were made in both years based on a previous study in 2012. Therefore, a total of 450 GM *B. napus* in 2013 (15 m × 8 m, with plant spacing of 50 × 50 cm, 9 plants m⁻²) and 256

GM *B. napus* in 2014 (8 m × 8 m, same planting spacing as in 2013) were employed. In addition, the apical dominance of GM *B. napus* removed when they were beginning to flower for increasing the numbers of tillers and prolonging the flowering duration. Biological important dates such as 1st flower blooming, peak-flowering, and end of flowering for donor and recipients plants were recorded accordingly.

In both years, an approximate 10,000 population of honeybee (*Apis mellifera*) colony was introduced in field during flowering period. Bee behaviors, including flowers visit frequency and bees cluster habit, were recorded during the flowering period. The location of the honeybee hive was shown in Fig. 6. The fertilizer application, insecticide spray, and irrigation management were conducted regularly.

rates of glufosinate-ammonium on the survivals of GM *B. napus*, non-GM *B. napus* cv. Tammi, non-GM *B. juncea*, and non-GM *R. sativus* in the greenhouse of the experimental farm station of Seoul National University, Suwon, Korea in 2013. All species seeds were sowed and grown in the 200-well multi-pots (hole size: 2.5 cm × 2.5 cm × Height 3 cm). At the one-true-leaf stage, the seedlings were sprayed with a range of glufosinate-ammonium (Basta[®], SL, 1.2 g ai L⁻¹, Bayer CropScience Ltd., Korea) doses (0, 0.15, 0.3, 0.6, 1.2, 2.4, 4.8, and 9.6 g ai L⁻¹) using a compressor pressurized belt-driven sprayer (R & D Sprayer, USA) equipped with an 8002E flat-fan nozzle (Spraying System Co., USA) to deliver a spray volume of 600 L ha⁻¹. The survivals of each species were counted 14 d after herbicide treatment. The experiments were arranged in a completely randomized design with three replications and repeated two times. Based on the dose-response test (Fig. 7), the dose of 0.6, 1.2, and 2.4 g ai L⁻¹ was determined as the appropriate doses for screening resistant hybrids of progenies of non-GM *B. juncea*, non-GM *B. napus* cv. Tammi, and non-GM *R. sativus* for both years, respectively.

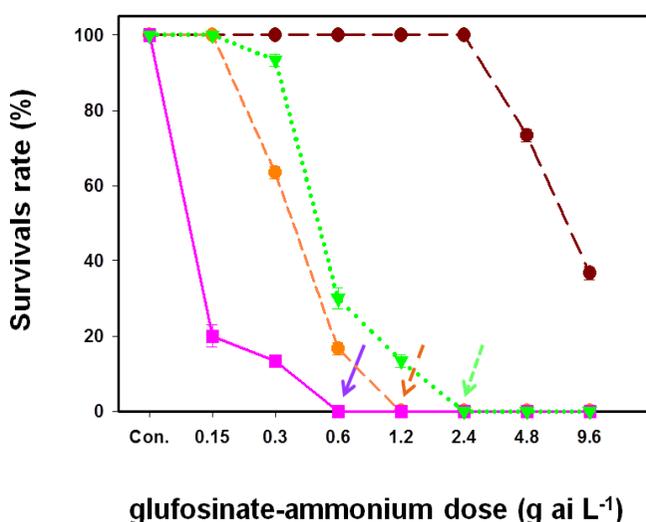


Fig. 7. The survival rate of GM *B. napus* (●), non-GM *B. napus* cv. Tammi, *B. juncea* (■), and non-GM *R. sativus* (▼) at glufosinate-ammonium (recommended dose of 1.2 g ai L⁻¹) tolerance/sensitivity test. The arrows indicate the selected dose

for herbicide resistance screening for each species.

Greenhouse screening of hybrids in progeny

The pods of each recipient plant at various distances were harvested separately in both years, and shelled, dried, counted (seed counter, USA), and stored at 4°C before herbicide screening test. Plants waste was treated as the conditions laid down by the Korean regulatory authorities. Greenhouse screening of hybrids was carried out from September to December for each year. Approximately 15% obtained *B. napus* and *B. juncea* (approximately 15,000 seedlings tested for each distance of each species), and 100% *R. sativus* (approximately 1,000 seedlings tested for each distance) progenies seeds were tested for each year. The number of progenies seedlings was counted prior to herbicide treatment. Based on the evaluation of glufosinate-ammonium tolerance test, glufosinate-ammonium was applied to those seedlings at one-true-leaf stage at the dose of 0.6, 1.2, and 2.4 g ai L⁻¹ for progenies of *B. juncea*, *B. napus* cv. Tammi, and *R. sativus*, respectively, using a compressor pressurized belt-driven sprayer as described before. Parental plants of *B. juncea*, *B. napus* cv. Tammi, *R. sativus*, and GM *B. napus* were always used as control. The survivals were counted 14 d after herbicide treatment. The survivals derived from glufosinate-ammonium treatment were considered as suspected hybrids outcrossed between GM *B. napus* and its relatives.

PCR analysis

In order to check whether the false-positive survivals were present in those suspected individuals due to insufficient absorption of the herbicide, a subsequent polymerase chain reaction (PCR) confirmation was conducted to detect the *bar-specific* gene introgression. Genomic DNA was extracted by following the minor modified cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle 1990) and standardized to a final concentration of 20 ng μL⁻¹. Two sets of primers were employed. One set containing three pairs of primers served as positive primers

was used to check whether the DNA extraction and PCR were successful for each sample and the other set was specific for the *bar-specific* gene which encodes phosphinothricin acetyltransferase, conferring resistance to the herbicide glufosinate-ammonium (Table 9). The PCR amplification was performed twice to ensure their reproducibility in a total volume of 20 μ L containing 40 ng of template DNA (2 μ L), 10 \times PCR reaction buffer (Sigma Co., USA) (200 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM MgCl₂) (2 μ L), 2.5 μ M of dNTPs (Sigma Co., USA) (1.6 μ L), 10 μ M each of the forward and reverse primers (Sigma Co., USA) (0.8 μ L), 2 units of *Taq* DNA polymerase (1 μ L), and sterile distilled water (12.6 μ L) in a T100™ 96-Well Thermal cycler (Bio-Rad Laboratories, Singapore) which was programmed to include 95°C for 2 min, followed by 30 cycles (95°C for 30 s, 64°C for 30 s, and 72°C for 30 s), and finally 72°C for 5 min. The PCR products were analyzed by electrophoresis on 1.2% (w/v) agarose gel containing ethidium bromide (Et-Br) in 0.5 \times TAE buffer and visualized. The images were photographed using Digital Gel Documentation System-200 (the Alpha Innotech Corporation, USA). We used the non-GM *B. napus* cv. Tammi, non-GM *B. juncea*, and non-GM *R. sativus* as negative control and GM *B. napus* as positive control. Seedlings with the detected *bar-specific* gene DNA fragment were confirmed as true hybrids.

Table 9. Positive and *bar-specific* gene detecting primers used for PCR analysis

Primer	Sequences (5'→3')	Application
BoKAH45TR	F: ATTATGACGCCTGGTTTTA R: ATTGGTTAGAAGTTATGGGAAC	Positive control (non-GM <i>B. napus</i>)
BnGMS539	F: CATCACTCAATCCAAGACCT R: AGAACCTGAAACAAACGATG	Positive control (non-GM <i>B. juncea</i>)
COS0842	F: TGGGCTGCCTTGAGAACA R: AGATGCTGAACTTGAATCCACTG	Positive control (non-GM <i>R. sativus</i>)
<i>bar</i> gene detection	F: CCGTGCCACCGAGGCGGACAT R: TCAAATCTCGGTGACGGGCAGGACC	Distinguishing (non-hybrids or hybrids)

Statistical analysis

In both years, each individual recipient at various distances was harvested separately and considered as replications. Gene flow rate was finalized with survival rate being multiplied by hybrid rate and analyzed separately for each year to describe the variation in gene flow across the two experimental years. Survival rate was calculated by dividing number of survivals after herbicide spray by that of total number of seedlings tested (herbicide resistance screening test). Hybrid rate was defined the proportion of survivals with *bar-specific* gene introgression in those survivals individuals (PCR analysis with those suspected survivals). Gene flow rate was expressed as following formula:

$$\text{Gene flow rate (\%)} = \frac{\text{Number of survivals}}{\text{Total number of seedlings tested}} \times \text{hybrid rate} \times 100\%$$

In order to estimate potential gene flow from GM *B. napus* to its non-GM relatives at a specific distance, the relatively higher gene flow rates at same distance within two years were used to fit the three-parameter biological meaningful log-logistic model as below:

$$Y = \frac{Y_0}{1 + \left(\frac{X}{D_{50}}\right)^b}$$

In this formula, Y denotes an estimate of gene flow rate (%) against distance, Y_0 denotes the maximum gene flow rate when the GM pollen donor and pollen recipient were adjacently planted, X is the distance from the GM pollen donor, b is the slope of the curve, and D_{50} is the distance where the gene flow declines by 50%. A lack of fit was checked for fitted regression model. R^2 and residual mean square were used to indicate the goodness of fit for the regressions models. All data was analyzed using R 3.2.0.

RESULTS

Synchrony of flowering period

In order to ensure synchrony of flowering period between pollen donor and pollen recipient, three different times of GM pollen donor transplanting and apical dominance removal for GM *B. napus* were conducted. Flowering phenology including 1st flower blooming, peak-flowering, and end of flowering for pollen donor and recipients plants were recorded (Table 10). In 2013, for GM pollen donor, the duration of flowering was 26, 28, and 27 d for the three different times' transplanting, respectively, resulting a total of 44 d flowering duration. For non-GM pollen recipients, the flowering duration was 29, 31, and 48 d for those recipients with overlapped flowering periods of 29, 31, and 32 d with GM pollen donor, respectively (Table 10). In 2014, a total of 54 d flowering durations was achieved by the three different times' GM *B. napus* transplanting and was completely overlapped with pollen recipients with the overlapped flowering periods of 38, 43, and 49 d for non-GM *B. napus*, non-GM *B. juncea*, and non-GM *R. sativus*, respectively (Table 10). All data presented here demonstrated the flowering period between pollen donor and recipient was essentially overlapped.

Honeybee visit

Honeybee (*Apis mellifera*) was introduced as the pollinator for facilitating gene flow between pollen donor and pollen recipient. In each year, during the flowering period, honeybee visit frequency was investigated every day (except raining days) at 10:00-11:00 am, and was described as $\text{plant}^{-1} \text{min}^{-1}$. The Fig. 8 demonstrated the honeybee visit frequency varied significantly ($P < 0.05$) by increasing distance from pollen donor and among the recipient species. With increasing the distance from the GM pollen donor, the honeybee visit frequency ($\text{plant}^{-1} \text{min}^{-1}$) was decreased accordingly. As showed in Fig. 8, in 2013, the closest pollen recipients at 4 m has the most honeybee visit frequency approximately 1.14, 0.87, and 1.04 $\text{plant}^{-1} \text{min}^{-1}$

in non-GM *B. napus*, non-GM *B. juncea*, and non-GM *R. sativus* compared with other distances, and decreased to 0.33, 0.28, and 0.35 plant⁻¹ min⁻¹ at farthest distance of 95 m, respectively (Fig. 8a). A similar decay pattern in visit frequency was also found in 2014 (Fig. 8b). Moreover, in both years, a relatively higher density of honeybees gathering observed near the GM pollen donor area with a mean visit frequency of 1.69 in 2013 (Fig. 8a) and 1.51 plant⁻¹ min⁻¹ in 2014 (Fig. 8b). Overall, the honeybee visit frequency (plant⁻¹ min⁻¹) in recipients in 2013 was lower than that of in 2014, with a mean visit frequency in those three recipients ranging from 0.32-1.01 plant⁻¹ min⁻¹ at 4-95 m in 2013 and 0.41-1.15 plant⁻¹ min⁻¹ at 2-128 m in 2014, respectively. Additionally, when compared the honeybee visit frequency at the same distance in each species, it was differed notably ($P < 0.05$). For instance, in 2013, at 4 m from the GM pollen donor, the honeybee visit frequency was 1.13, 0.87, and 1.04 (plant⁻¹ min⁻¹) in non-GM *B. napus*, non-GM *B. juncea*, and non-GM *R. sativus*, respectively, and 1.00, 0.73, and 0.91 in those of three species at 8 m. In 2014, the honeybee visit frequency was 1.09, 0.69, and 1.66 (plant⁻¹ min⁻¹) at 2 m and 0.90, 0.64, and 1.57 at 4 m in those of three species (Fig. 8). Among the three species, the flower of non-GM *R. sativus* showed the most attractive to honeybee in both years, followed by non-GM *B. napus* and *B. juncea*.

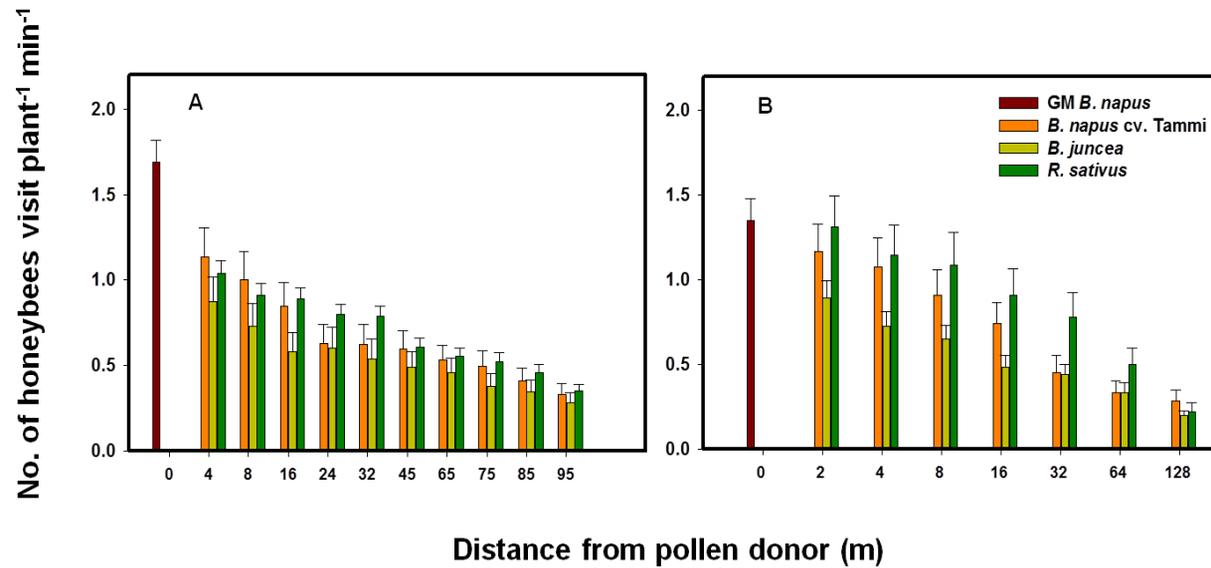


Fig. 8. Honeybee visit frequency ($\text{plant}^{-1} \text{min}^{-1}$) at various distances (m) from GM pollen donor during the flowering periods in both years of 2013 (A) and 2014 (B).

Table 10. Biological dates for a specific reproductive growth stage of pollen donor (GM *B. napus*) and pollen recipients (*B. napus*, *B. juncea*, and *R. sativus*) in both years of 2013 and 2014

Years	Experimental plants	1 st blooming	Peak-flowering	End of flowering	Flowering Duration (days)	Synchrony of flowering periods (days)
2013	Pollen donor: Three times GM <i>B. napus</i> transplanting					
	1 st time: Mar 29	May 2	May 12	May 28	26	-
	2 nd time: Apr 12	May 10	May 21	June 8	28	-
	3 rd time: Apr 26	May 18	May 28	June 15	27	-
	Total flowering duration	-	-	-	44	
	Pollen recipient: non-GM MF plants (transplanting date: Mar 29)					
	<i>B. napus</i> :	May 4	May 13	June 2	29	29
	<i>B. juncea</i> :	May 2	May 20	June 4	31	31
	<i>R. sativus</i> :	May 7	May 24	June 25	48	32
	2014	Pollen donor: Three times GM <i>B. napus</i> transplanting				
1 st time: Mar 4		Mar 27	Apr 19	May 7	41	-
2 nd time: Mar 11		Apr 1	Apr 26	May 14	44	-
3 rd time: Mar 18		Apr 6	May 2	May 19	43	-
Total flowering duration		-	-	-	54	
Pollen recipient: non-GM MF plants (transplanting date: Mar 4)						
<i>B. napus</i> : Mar 4		Mar 30	Apr 22	May 6	38	38
<i>B. juncea</i> : Mar 4		Apr 3	Apr 25	May 16	43	43
<i>R. sativus</i> : Mar 4		Apr 1	Apr 25	May 19	49	49

Survival rate and determination of hybrid rate

Survival rate was calculated by dividing the number of survivals in herbicide resistance screening test by that of total number of progenies seedlings tested. Those survivals were further subjected to PCR analysis for determination of hybrid rate by detection the proportion of survivals with GM *bar-specific* gene introgression in all survivals tested. For PCR analysis, two sets of primers were employed (Table 9). Under the positive primers of BoKAH45TR (specific to *B. napus*), BnGMS539 (specific to *B. juncea*), COS0842 (specific to *R. sativus*), all DNAs from survival were presented (data not shown). Under the *bar-specific* primer, the tested DNA from those survivals not showing the *bar-specific* DNA fragment were considered as the false-positive hybrids (Fig. 9). For instance, 9 and 7 out of 10 suspected survivals of *B. napus* and *B. juncea* with showing *bar-specific* band were confirmed to be positive hybrids, respectively. In the case of *R. sativus*, all suspected survivals were false-positive hybrids (Fig. 9). The summarized survival rate and hybrid rate for both 2013 and 2014 were showed in Table 11 and 12.

In 2013, for non-GM *B. napus* cv. Tammi progenies, the survival rate ranged from 0-1.993% at 4-95 m with the highest survival rate of 1.993% at 4 m and persistently decreasing to 0.007% at 75 m, and no survivals were detected at 85 m. The calibrate factor for those survivals ranged from 66.67-100% (Table 11). For non-GM *B. juncea* progenies, the survival rate was 0.056% at 4 m, 0.042% at 8 m, and 0.025% at 16 m, and no survivals were detected beyond 24 m. The corresponding hybrid rate for those survivals was 100% at 4 m, 66.67% at 8 m, and 33.33% at 16 m (Table 11). With regard to non-GM *R. sativus* progenies, the survival rate ranged from 0.278-0.978% with highest survival rate of 0.978% at 45 m and lowest rate of 0.278% at 75 m, but the PCR analysis showed all survivals were false-positive ones (Table 11).

In 2014, the survival rate for non-GM *B. napus* cv. Tammi progenies ranged from 0-2.394% at 2-128 m with the highest survival rate of 2.394% at 2 m, 1.255% at 4 m, 0.637% at 8 m, decreasing to 0.077% at 64 m, and no survivals were detected at 128 m. The hybrid rate for those survivals ranged from 92.5-100%

(Table 12). In the case of non-GM *B. juncea* progenies, the survival rate was 0.085% at 2 m, 0.034% at 4 m, 0.009% at 8 m, and 0.008% at 16 m, and no survivals were detected beyond 32 m. The PCR analysis showed 1 out of 10 at 2 m and one survival at 16 m without having the *bar-specific* DNA fragment, resulting from a hybrid rate ranging from 0-100% (Table 12). For non-GM *R. sativus* progenies, only two survivals were detected from all tested seedlings for various distances with the survival rate of 0.105% at 2 m and 0.113% at 4 m, respectively, but the PCR analysis on the two survivals demonstrated they were false-positive survivals (Table 12).

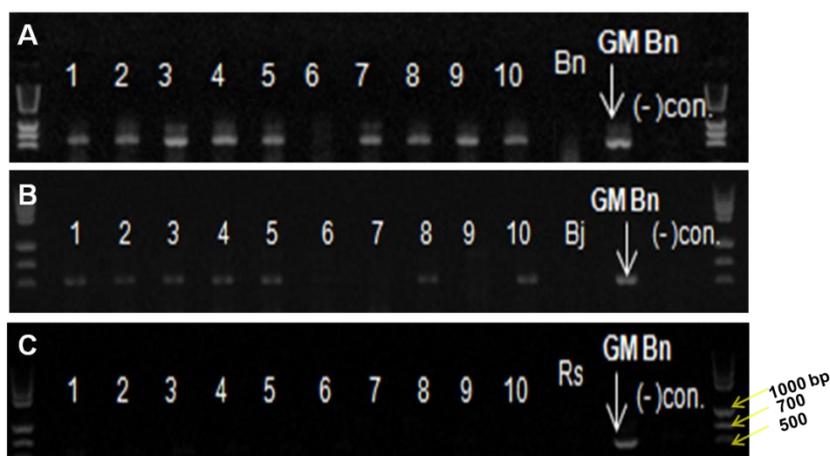


Fig. 9. PCR confirmation using survivals of *B. napus* cv. Tammi (A), *B. juncea* (B), and *R. sativus* (C) genomic DNA after glufosinate-ammonium application. 1-10, suspected survivals after glufosinate-ammonium application. Bn, *B. napus* cv. Tammi; Bj, *B. juncea*; Rs, *R. sativus*; GM Bn, glufosinate-ammonium resistant *B. napus*; (-) Con: negative control, amplified by PCR without DNA sample.

Table 11. Summary of survival rate (SR) and hybrid rate (HR) of non-GM pollen progenies (*B. napus*, *B. juncea*, and *R. sativus*) at various distances from the GM pollen donor in 2013

Distance (m)	<i>B. napus</i>		<i>B. juncea</i>		<i>R. sativus</i>	
	SR (%) ^a	HR (%) ^b	SR (%) ^a	HR (%) ^b	SR (%) ^a	HR (%) ^b
4	1.993	98.51	0.056	100.00	0.721	0.000
8	1.072	98.33	0.042	66.67	0.457	0.000
16	0.472	97.75	0.025	33.33	0.957	0.000
24	0.229	95.00	0.000	-	0.672	0.000
32	0.113	94.44	0.000	-	0.393	0.000
45	0.080	83.33	0.000	-	0.978	0.000
65	0.019	66.67	0.000	-	0.784	0.000
75	0.007	100.00	0.000	-	0.278	0.000
85	0.000	-	0.000	-	0.393	0.000
95	0.000	-	0.000	-	0.500	0.000
LSD _{0.05}	0.53	12.1	0.03	18.3	0.06	0

Survival rate (SR) and hybrid rate (HR) were determined by herbicide resistance screening test and PCR analysis, respectively.

^a Data in column of survivals rate means number of survivals after herbicide resistance screening test/total number of seedlings tested for various distances recipients of non-GM *B. napus*, non-GM *B. juncea*, and non-GM *R. sativus*

^b Data in column of hybrid rate means number of tested survivals with GM *B. napus bar-specific* DNA fragment/total number of survivals for various distances recipients of non-GM *B. napus*, non-GM *B. juncea*, and non-GM *R. sativus*

Table 12. Summary of survival rate (SR) and hybrid rate (HR) for non-GM pollen recipients (*B. napus*, *B. juncea*, and *R. sativus*) at various distances from the GM pollen donor in 2014

Distance (m)	<i>B. napus</i>		<i>B. juncea</i>		<i>R. sativus</i>	
	SR (%) ^a	HR (%) ^b	SR (%) ^a	HR (%) ^b	SR (%) ^a	HR (%) ^b
2	2.394	97.60	0.085	90.00	0.105	0.000
4	1.256	96.90	0.034	100.00	0.113	0.000
8	0.637	98.33	0.009	100.00	0.000	-
16	0.342	92.50	0.008	0.00	0.000	-
32	0.138	93.33	0.000	-	0.000	-
64	0.077	100.00	0.000	-	0.000	-
128	0.000	-	0.000	-	0.000	-
LSD _{0.05}	0.12	7.3	0.01	9.2	0.04	0

Survival rate (SR) and hybrid rate (HR) were determined by herbicide resistance screening test and PCR analysis, respectively.

^a Data in column of survivals rate means number of survivals after herbicide resistance screening test/total number of seedlings tested for various distances recipients of non-GM *B. napus*, non-GM *B. juncea*, and non-GM *R. sativus*

^b Data in column of hybrid rate means number of tested survivals with GM *B. napus bar-specific* DNA fragment/total number of survivals for various distances recipients of non-GM *B. napus*, non-GM *B. juncea*, and non-GM *R. sativus*

Gene flow rate from GM *B. napus* to its relatives

non-GM *B. napus* (♀) × GM *B. napus* (♂)

The confirmed gene flow rates from GM *B. napus* to its relatives in 2013 and 2014 were summarized in Table 13. For non-GM *B. napus* recipient, in 2013, the gene flow rate ranged from 0-1.961% at distance of 4-95m, with the highest rate of 1.961% at 4m, 1.059% at 8 m, 0.459% at 16 m, constantly decreasing to 0.007% at 75 m, and no gene flow was detected beyond 85 m. In 2014, it was 2.330% at 2 m, 1.217% at 4 m, 0.624% at 8 m, 0.317% at 16 m, 0.125% at 32 m, and 0.066% at 64 m, and no gene flow was detected at 128 m (Table 13). Overall, the gene flow rate estimated at the same distance in 2013 was higher than those estimated in 2014, with the values of 1.961% vs. 1.217% at 4 m, 1.059% vs. 0.624% at 8 m, and 0.459% vs. 0.317% at 16 m, respectively.

non-GM *B. juncea* (♀) × GM *B. napus* (♂)

For non-GM *B. juncea* recipient, in 2013, the gene flow rate ranged from 0-0.056% at distance of 4-95m, with the highest rate of 0.056% at 4 m, 0.042% at 8 m, and 0.025% at 16 m, and no gene flow was detected beyond 24 m. In contrast, in 2014, it was 0.076% at 2 m, 0.034% at 4 m, and 0.008% at 8 m, and no gene flow was detected beyond 16 m (Table 13). A relatively higher gene flow values was also observed in 2013 compared to 2014.

non-GM *R. sativus* (♀) × GM *B. napus* (♂)

With regard to gene flow to non-GM *R. sativus*, regardless of the distances from GM *B. napus*, no gene flow was detected in both years (Table 13).

Table 13. The confirmed gene flow rate (%) for non-GM pollen recipients (*B. napus*, *B. juncea*, and *R. sativus*) at various distances from GM pollen donor in 2013 and 2014

Distance (m)	<i>B. napus</i>		<i>B. juncea</i>		<i>R. sativus</i>	
	2013	2014	2013	2014	2013	2014
2	- ^a	2.330	-	0.076	-	0.000
4	1.961	1.217	0.056	0.034	0.000	0.000
8	1.059	0.624	0.042	0.008	0.000	0.000
16	0.459	0.317	0.025	0.000	0.000	0.000
24	0.216	-	0.000	-	0.000	-
32	0.107	0.125	0.000	0.000	0.000	0.000
45	0.068	-	0.000	-	0.000	-
64	-	0.066	-	0.000	-	0.000
65	0.013	-	0.000	-	0.000	-
75	0.007	-	0.000	-	0.000	-
85	0.000	-	0.000	-	0.000	-
95	0.000	-	0.000	-	0.000	-
128	-	0.000	-	0.000	-	0.000
LSD _{0.05}	0.034	0.089	0.036	0.025	NS ^b	NS

The confirmed gene flow rate calculated by multiplied survivals rate by hybrid rate for various distances recipients of non-GM *B. napus*, non-GM *B. juncea*, and non-GM *R. sativus*

^a Short dash lines mean the distance not included in the corresponding year ^b Not significant at $P=0.05$

Estimation of potential gene flow

The significant difference in gene flow from GM *B. napus* to its relatives of non-GM *B. napus* and *B. juncea* was found between 2013 and 2014 years ($P < 0.05$). To estimate potential gene flow, the relatively higher gene flow rates of non-GM *B. napus* and *B. juncea* within two years respectively selected were fitted to the biological meaningful log-logistic model.

There was no evidence of lack of fit of the log-logistic model ($P = 0.8685$ for non-GM *B. napus* and 0.9996 for non-GM *B. juncea*). The smaller RMS values (0.03 for non-GM *B. napus* and 0.001 for non-GM *B. juncea*) and R^2 (0.963 for non-GM *B. napus* and 0.313 for non-GM *B. juncea*), indicating that the three-parameter log-logistic model provided an acceptable fit to the gene flow data both for non-GM *B. napus* and non-GM *B. juncea*. Based on the predictable model, the maximum potential gene flow rate was 2.57% (and 0.08%) when GM *B. napus* and non-GM *B. napus* (and non-GM *B. juncea*) were planted adjacently. The D_{50} value indicating that the distance required for 50% of gene flow rate reduction compared to the estimated gene flow rate was 6.94 m for non-GM *B. napus* and 8.98 m for non-GM *B. juncea*, respectively (Fig. 10; Table 14).

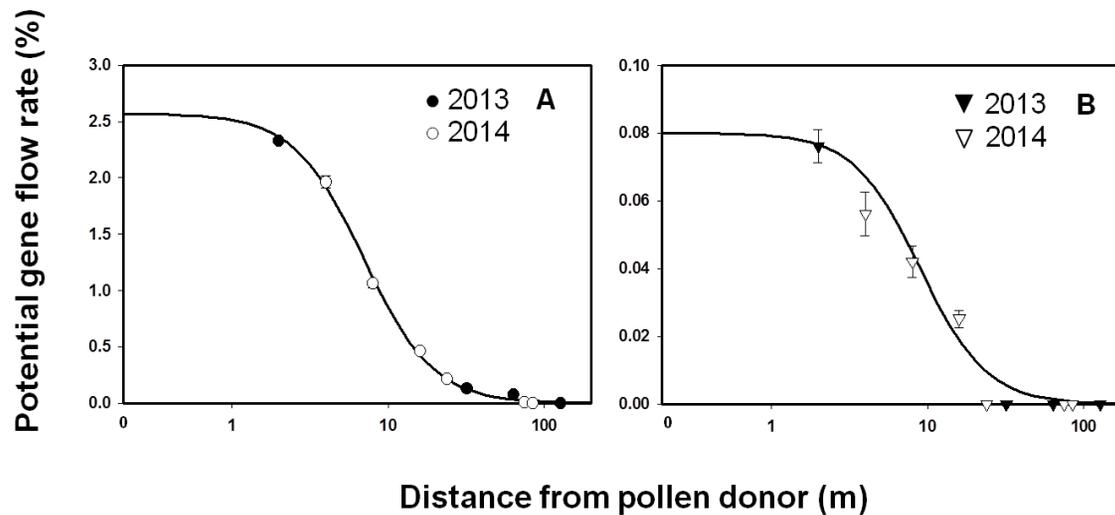


Fig. 10. The estimated equations for prediction of potential gene flow rate for non-GM *B. napus* (A, $Y = \frac{2.57}{1+(\frac{X}{6.94})^{1.95}}$, lack of fit, $P=0.8685$) and non-GM *B. juncea* (B, $Y = \frac{0.08}{1+(\frac{X}{8.98})^{2.06}}$, lack of fit, $P=0.9996$) using higher gene flow rate within each year.

Table 14. Estimated parameters for log-logistic analysis of the gene flow rate for recipients of non-GM *B. napus* and *B. juncea* under field condition

Species	Parameters ^a			DF	RMS	R ²	P values
	Y ₀	b	D ₅₀ (95% CI)				
<i>B. napus</i>	2.57	1.95	6.94 (5.7-9.2)	81	0.03	0.963	< 0.0001
<i>B. juncea</i>	0.08	2.06	8.98 (6.25-9.7)	81	0.001	0.313	< 0.0001

^a Y₀, the maximum gene flow rate when distance between pollen donor and recipient is 0; b, slope of the log-logistic curve; D₅₀, the distance required for 50% of gene flow rate reduction compared to the maximum gene flow rate
 DF, degree of freedom; RMS, residual mean square; R², coefficient of determination

DISCUSSION

A two-year study of potential risk assessment on gene flow from GM *B. napus* to its relatives was conducted in Korea. In order to quantify the potential gene flow, a favorite condition was stimulated including entire synchrony of flowering periods between GM pollen donor and recipient and introduction of pollinator. Herbicide resistance and simple sequence repeat (SSR) markers were employed to detect the hybrids among the progenies. The studies demonstrated that the considerable GM *B. napus* gene flow rate to non-GM *B. napus* and non-GM *B. juncea* at a specified distance under simulated favorite condition, but no gene flow occurred to non-GM *R. sativus*. Based on the experimental data, a simulation model was developed for prediction of gene flow from GM pollen donor to its relatives at a specific distance from GM pollen donor.

Numerous studies on outcrossing events and gene flow of GM *B. napus* with its relatives have been carried out world-wide including GM *B. napus* cultivated countries, such as Canada, USA, and Australia, and non-GM *B. napus* cultivated countries, such as European and Asian countries (Beckie et al. 2003; Cai et al. 2008; Krato and Petersen 2012; Landbo et al. 1996; Morris et al. 1994; Rieger et al. 2002; Warwick et al. 2003; Weekes et al. 2005). *B. napus* displayed the extensive intraspecific and interspecific crossability with its relatives such as *B. rapa*, *B. juncea*, *R. raphanistrum*, etc., both in artificial means and under field condition. In our studies, the outcrossing result between non-GM *B. juncea* (♀) × GM *B. napus* (♂) was also agreement with the previous outcrossing event. But in the present simulated conditions, we did not detect hybrids between non-GM *R. sativus* (♀) × GM *B. juncea* (♂) in two years studies, although some hybrids have been reported using artificial means such as ovule culture and embryo rescue methods (Ellerstrom 1978; Paulmann and Robbelen 1988; Takeshita et al. 1980). Up to now, only one existing study on this regard without hand pollination or embryo rescue method was reported by using a transgenic male sterile *B. napus* as recipient (Ammitzboll and

Jørgensen 2006).

Gene flow was importantly influenced by several factors including experimental design (e.g., the shape, orientation and size of pollen donor and recipient field), the distance from pollen donor, the insect movement and types, local climatic environment condition as well as the genotype and zygoty of donors (Becker et al. 1992; Hüsken and Dietz-Pfeilstetter 2007; Scheffler et al. 1993; Tang et al. 2003). The previous studies showed a decrease of pattern on gene flow with increasing the distance from pollen donor. Staniland et al. (2000) revealed that gene flow rate was 1.6% at 1.5 m, 0.7% at 4 m, 0.3% at 11.5 m, and sharply decreased to 0.03% at 31.25 m from the pollen donor by using bromoxynil resistance marker. The similar decrease patterns were also seen in other studies (Cai et al. 2008; Krato and Petersen 2012; Paul et al. 1995; Scheffler et al. 1993). Not only within a field scale, but the gene flow also can occur at a considerable distance. A rate of 0.07% at 800 m from the GM pollen donor was reported by Beckie (2003), and an investigation over one-third of Australia covering a range of environments showed that pollen-mediated gene flow can occur beyond 2000 m (Rieger et al. 2002). Moreover, two sites of American studies, California and Georgia, indicated that gene flow was greatly affected by the local climatic condition (Ramsay 2003).

non-GM *B. napus* (♀) × GM *B. napus* (♂)

In our field experiments in both years, with presence and abundance of honeybee and entire synchrony of flowering with non-GM *B. napus*, the gene flow rate ranged from 0.007-1.961% at 4-75 m in 2013, and 0.066-2.330% at 2-128 m in 2014. The gene flow rates were (2.330% at 2 m in 2014 and 1.961% at 4 m in 2013) much higher than rates estimated at the similar distance from pollen donor in other studies, 0.74% at 2 m in China (Zhao et al. 2013), 0.41% at 3 m in UK (Scheffler et al. 1993), 0.7% at 4 m in Canada (Staniland et al. 2000), 0.4% at 4.6 m in USA (Morris et al. 1994). In other distances in our experiment, the estimated gene flow rate are also relatively higher in comparison with values estimated in other studies (Di et al. 2009; Krato and Petersen 2012; Scheffler et al. 1993), the reason for that

could be ascribed to more honeybee visit frequency and longer overlapped flowering periods between GM pollen donor and pollen recipient. However, unlike other studies reported gene flow occurred at far distance from pollen donor, in our experiment we did not detect gene flow at the distance beyond 85 m, which might be associated with the difference in sizes of the GM pollen donors and pollen recipients as well as discontinuous design in our studies.

non-GM *B. juncea* (♀) × GM *B. napus* (♂)

Lots of studies have been conducted on outcrossing events between GM *B. napus* and non-GM mustard, few on outcrossing rate in association with distances from pollen donor. A recent Japanese study on this regard revealed a decreased gene flow rate in non-GM *B. juncea* with increasing the distance from pollen donor (GM *B. napus*) (Tsuda et al. 2012). As concluded before, synchrony of flowering between pollen donor and recipient is the key factor to determine the gene flow occurring, a lack of synchrony of geographic distribution or in flowering periods, the outcrossing either drastic reduction or no occurred. Therefore, if flowering periods overlapped, the possibilities of gene flow would be increased greatly (Jorgensen and Andersen 1994; Landbo et al. 1996). In a two-year' gene flow studies of *B. juncea* (♀) × *B. napus* (♂) conducted in Japan, 55 hybrids were detected with an entire synchrony of flowering (34 days) by artificial supply of GM donor plants in 2010 in comparison with none of hybrids detected with asynchrony of flowering (19 days) under natural flowering condition in 2009 (Tsuda et al. 2012), indicating that gene flow is significantly associated with the degree of periods of synchronous flowering.

In our experiment, an entire synchrony of flowering of 31 (in 2013) and 43 days (in 2014) between *B. juncea* and *B. napus* was achieved by transplanting of three different times' GM *B. napus*, resulting from a gene flow rate ranging from 0.025-0.056% at 4-16 m and 0.008-0.076% at 2-8 m in 2013 and 2014, respectively. The gene flow rate was 0.076% at 2 m (in 2014) and 0.056% at 4 m (in 2013) from the GM *B. napus* compared with 0.049% at 1 m and 0.037% at 5 m reported in a

Japanese study (Tsuda et al. 2012). The higher gene flow rate estimated in our experiment is closely related to the higher honeybee visit frequency during flowering periods and entire synchrony of flowering between pollen donor and pollen recipient. Similar to the previous study, a decreased pattern of gene flow rate with increasing the distance from GM pollen donor was also found in our studies. For instance, in 2013, the gene flow rate was 0.056% at 4 m, 0.042% at 8 m, and decreased to 0.025% at 16 m, and in 2014, it was 0.076% at 2 m, 0.034% at 4 m, and 0.008% at 8 m. In addition, in our studies, no hybrids were detected at the distance of >24 m from the GM pollen donor in both years (Table 13), which was similar to the previous estimate of 20 m (Tsuda et al. 2012). Moreover, despite at the presence and abundance of honeybee pollination and entire synchrony of flowering between donor and recipient, our results showed that gene flow rate from GM *B. napus* to non-GM *B. juncea* under field condition was still low, which would be related with the high self-compatibility of *B. juncea* (Ohsawa and Namai 1987). However, due to the possibility of outcrossing existing between these two species, the further study for fitness of hybrids still needs to be continued along with the long-term investigation of agricultural fate of hybrids.

non-GM *R. sativus* (♀) × GM *B. napus* (♂)

In two-year's studies, no hybrids were detected between non-GM *R. sativus* (♀) × GM *B. napus* (♂) under the artificially stimulated conditions, indicating that the possibility of GM *B. napus* gene flow to non-GM *R. sativus* was extreme low. Moreover, because *R. sativus* is generally harvested prior to flowering and seed development, unless being used for seeds production, such a lack of synchrony of flowering would make the gene flow event extremely unlikely under natural agricultural condition. However, in rare event that such hybrid occurred using a genetically male sterile *B. napus* as recipient, although they were proved to be highly sterile. By this token, more and further outcrossing studies should be conducted at different crosses directions and combinations between *B. napus* and a range of *R. sativus* cultivars.

In summary, this is the first field study of risk assessment on GM *B. napus* gene flow to its relatives in Korea. This study provides useful baseline information for potential risk assessment on gene flow between GM *B. napus* and its relatives and decision-making for proper isolation distance in Korea. The experimental data presented here clearly indicated the possibility of gene flow from GM *B. napus* to non-GM *B. napus* under simulated favorite field condition. It was worth pointing out that gene flow and pollen dispersal distance varied in different regions due to the local climatic and varietal differences. In the present study with presence of honeybee and entire synchrony of flowering between GM *B. napus* and non-GM *B. napus*, gene flow rate was much higher than rates estimated in other studies at the similar distance. Therefore, we could expect a considerable higher gene flow rate if stimulating more suitable condition under field environment. Nowadays, GM *B. napus* is not commercially cultivated in Korea and other Asian countries. However, more knowledge are needed to accumulate for better understanding gene flow under natural environment, outcrossing events under different experimental condition, consequence of gene, long-term gene flow monitoring, and gene flow mitigation as well as management.

Gene flow between *B. napus* and *B. juncea* could possibly occur both under natural field and optimal simulated conditions. Although gene flow rate has been proved very low, the possibility of occurrence has raised the biosafety concern of growing GM *B. napus* with outcrossing with *B. juncea*. *B. juncea* is widely distributed in Korea and its flowering periods is completely synchronized with *B. napus*, and natural occurrence of gene flow could be predicted in case GM *B. napus* growing adjacently with *B. juncea*. In our experiment, we did not detect gene flow at the distance >24 m. However, we believe that the natural spontaneous gene flow between GM *B. napus* and *B. juncea* could be occurred at a considerable distance under optimal conditions, such as the abundance of pollinators, maximum synchrony of flowering, suitable climatic environments etc. Therefore, large-scale and long distance field evaluation of gene flow should be conducted. Because crossed hybrids between GM *B. napus* and *B. juncea* have been reported showing

higher fertility and inherited herbicide resistance (Di et al. 2009; Song et al. 2010), the further studies on fitness test of hybrids and inheritance behavior of gene in backcrossed generation should be continued as well.

Gene flow between GM *B. napus* and *R. sativus* under natural field condition is practically impossible. No hybrids were detected despite at the presence and abundant of honeybee during the flowering periods and entire synchrony of flowering. Up to now, only one paper was reported successful natural outcrossing between these two species with a genetically modified male sterile *B. napus* as the maternal plant, but the hybrids would be highly sterile. Since a lack of synchrony of flowering between them as well, therefore, there is no much concern on gene flow between GM oilseed and *R. sativus*. However, the further studies should be continued to investigate in more detail on gene flow under various simulated natural condition, different cross directions, and different combinations between *B. napus* and a various *R. sativus* cultivars.

The isolation distance required for GM trial quite varies widely, in the case of *B. napus*, although nations-wide, it ranges from 50-400 m. In United Kingdom, a 50 m isolation distance is recommended for GM cultivation, with a 200 m isolation distance recommended for organic crops. In addition, an isolation distance of 50 m is officially required for growing crops with high levels of erucic acid in UK (Salisbury 2002; SCIMAC 1999). In Canada, a 200 m isolation distance for GM canola trials is stipulate (Staniland et al. 2000). An isolation distance of 400 m is required for GM trials in France, Belgium and Sweden (EC 2002). Australian GM trial requirements include a 400 m isolation distance and a 15 m non-GM buffer zone (Salisbury 2002). In China, a recent study recommended that 300 m, rather than 1000 m, is a reasonable isolation distance for GM trials (Zhao et al. 2013). Up to now, there is no regulation on isolation distance for GM trial in Korea. If a 0.1% tolerance threshold of gene flow rate is considered to be acceptable, then an isolation distance between GM *B. napus* and non-GM *B. napus* (e.g. 35 m) is acceptable for based on our study, which is similar to the previous study in China of 25 m (Zhao et al. 2013) and shorter than UK's regulation of 50 m (Salisbury 2002;

SCIMAC 1999). The tolerance threshold of a 0.01% gene flow rate is chosen on the basis of the lowest threshold value for labeling of GM in food and feed in the world (EU 2003). By this taken, keeping the gene flow rate below 0.01% should ensure both seed purity and the market acceptability of products. Thus, according to the equation in our study, an isolation distance of 122.5 m is recommended for preventing gene flow from GM *B. napus* and non-GM *B. napus*. When the distance is farther than 500 m, the gene flow rate is lower than 0.001%. In the case of the isolation distance for *B. juncea*, although there is no specific regulation worldwide, based on our study, a 24 m isolation is sufficient to prevent gene flow rate exceed the tolerance threshold of 0.01%, and a 60 m isolation distance could prevent the gene flow rate lower than 0.001%. However, for *R. sativus*, our results demonstrated that the extreme low possibility for outcrossing with *B. napus*.

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CHAPTER III

Modeling of maximum potential gene flow from genetically modified *Brassica napus* to its male sterile relatives under open and wind pollination conditions

ABSTRACT

A field experiment was conducted to evaluate gene flow from genetically modified (GM) *Brassica napus* to its non-GM male sterile (MS) recipients, *B. napus*, *B. juncea*, and *Raphanus sativas* under simulated open and wind pollination conditions in 2014. The three non-GM MS relatives were planted at three independent directions at various distances of 2, 4, 8, 16, 32, 64, and 128 m from the GM *B. napus* pollen donor. The honeybee was introduced as the pollinator during the flowering period. At each distance, a half of non-GM MS relative plants were covered with the insect-preventing net to simulate wind pollination condition and the other half were not covered with the net to simulate open pollination condition. Pods setting rate was initially determined by counting the number of flowers and pods. Herbicide (glufosinate-ammonium) and polymerase chain reaction (PCR) marker were used to confirm the F1 hybrids resulted from outcrossing between GM *B. napus* and its MS relatives. Gene flow rate from GM *B. napus* to MS *B. napus* were 32.48% and 14.69% at 2 m and, decreased with increasing distance down to 0.3% and 0.26% at 128 m distance under open and

wind pollination conditions, respectively. Gene flow rates to MS *B. juncea* were 21.95% and 6.62% at 2 m and, 0.24% and 0.16% at 128 m distance under open and wind pollination conditions, respectively. The honeybee-mediated gene flow rates estimated by comparing the difference in gene flow between open and wind pollination conditions were 19.01% at 2 m and 0.03% at 128 m distance to MS *B. napus*, and 15.36% at 2 m and 0.06% at 128 m distance to MS *B. juncea*. Comparison of mathematical models to describe the relationship between gene flow rate and distance between GM *B. napus* and its relative showed that the log-logistic model is mathematically comparable to the double decay exponential model and provides better biological meaning. The model simulation recommended 2710 m and 254 m for MS *B. napus* and *B. juncea*, respectively, for 0.01% of gene flow as the tolerable threshold (isolation distance). No gene flow from GM *B. napus* to MS *R. sativus* was observed, suggesting no or low gene flow risk. Our results thus conclude that potential maximum gene flow rates from GM *B. napus* to MS *Brassica* species are much higher than those to male fertile (MF) *Brassica* species. The MS pollen recipients are useful to estimate potential gene flow from GM crops to their relatives.

Keywords: *Brassica juncea*, *Brassica napus*, gene flow, isolation distance, log-logistic model, open pollination, *Raphanus sativus*, wind pollination

INTRODUCTION

Over the past three decades, the global scale of cultivation GM crops has increased from 1.7 million ha in 1996 to 160 million ha in 2011 (James 2011). Rapid development and commercialization of genetically modified (GM) crops have more and more raised public concerns on unexpected ecological change and environmental effects (Beckie et al. 2006; Chèvre et al. 2003; Fagan 2004). One of the main concerns is the transgenes escape through outcrossing with their wild or weedy relatives, which has been the restriction for adaption of GM crops (Ellstrand et al. 1999; Firband and Forcella 2000; Warwick et al. 2003; Snow 2002). GM *B. napus* is such the case. The studies have shown that *B. napus* (AACC, 2n=38) could outcross with its many relatives such as *B. juncea* (AABB, 2n=36), *B. rapa* (AA, 2n=20), *B. niga* (BB, 2n=16), *B. oleracea* (CC, 2n=18), as well as *R. raphanistrum* (RrRr, 2n=18), etc. In addition, the pollen of GM oilseed rape could flow ranging from several to thousands meters based on the different climatic environment and agricultural structures (Beckie 2001; Bing et al. 1996; Cai et al. 2008; Scheffler et al. 1993, 1995; Chèvre et al. 2003; Krato and Petersen 2012; Rieger et al. 2002; Warwick et al. 2003; Weekes et al. 2005).

In GM *B. napus* cultivated countries such as United State, Canada, and Australia, the large-scale gene flow studies revealed that the outcrossing could occur at a considerable distance approximately 800 m with the outcrossing rate of 0.07% (Beckie 2001). Rieger et al. (2002) reported the pollen flow even could move beyond 2000 m with large field sizes in Australia. Moreover, studies conducted in two different sites, California and Georgia in United State (Morris et al. 1994), showed that gene flow was importantly affected by the local climatic environment. However, unlike the large farm scale agricultural system in North America and Australia, in Asian countries, co-existence of oilseed rape with its relative species is very common, where farmers generally managed small land and cultivated different crops side-by-side by each year. Overlapped flowering periods between *B. napus*

and its relatives or weedy species observed in many place, which poses a high risk of gene flow between them. In Japan, a field evaluation of gene flow from GM *B. napus* to *B. juncea* showed that the gene flow could occur at 17.5 m with the rate of 0.03% (Tsuda et al. 2012). The difference in overlapped flowering periods between GM *B. napus* and *B. juncea* resulted in a different gene flow rate (Tsuda et al. 2012). In comparison with our studies in 2013 in Korea, with artificially stimulated favorite conditions for facilitating gene flow, the potential gene flow rate from GM *B. napus* to *B. juncea* ranged from 0.008-0.076% at 2-16 m and from GM *B. napus* to non-GM *B. napus* ranged from 0.007-2.330% at 2-75 m, respectively. All data presented here showed that gene flow was very region- and climate -based, indicating that the necessity for conducting gene flow risk assessment under a specified climatic condition.

It is necessary and essential to establish a mathematical model to predict gene flow and reveal the relevant factors affecting gene flow under various conditions. Generally, there are two main approaches used for modeling gene flow (Klein et al. 2003; Snall et al. 2007). The first is mechanistic modeling that incorporates various biological factors affecting gene flow such as pollinators, pollen density, etc., and it can provide the detailed process of gene flow and reveal the pattern of gene flow under various factors (Klein et al. 2003; Gustafson et al. 2005; Snall et al. 2007). However, mechanistic models are usually mathematically complex containing many parameters and needed many precise data to perform the simulations, in some case those parameters are difficult to measure in natural conditions (Klein et al. 2003; Snall et al. 2007). The second approach is empirical modeling, which ignores the details of the gene flow process and establishes the equations by fitting the experimental data to existed regression model (Klein et al. 2003; Gustafson et al. 2005; Snall et al. 2007). The empirical method can be easily performed in practice, and usually contains few parameters. To some extent, although this empirical model is difficult to describe gene flow at various environment conditions, it exhibits a flexible and practical experience for a certain purpose to predict gene flow.

Some studies agree that bees carrying pollen travel a short distance within *B.*

napus field, whereas wind is responsible for long-distance pollen flow (Cresswell et al. 2004; Williams et al. 1987). However, regarding the relative contribution of wind and bees to gene flow and how they affect gene flow, these are still unknown. Thus, in the present study, by simulating the different pollination conditions, we evaluated the gene flow affected by wind and bees. In addition, taken into account the practical Asian agricultural structure (with co-existence of *B. napus* and its relatives), a discontinuous design was performed by locating GM pollen donor at one side and planting pollen recipients at various distances from pollen donor to simulate a mosaic agricultural structure. In addition, in order to improve the detection accuracy and sensitivity, a male sterile (MS) line was used as pollen recipient to amplify and differentiate gene flow rate resulting from wind and (/or) bees associated with distance increasing from the pollen donor.

Therefore, the objectives of this study were to i) evaluate the maximum potential gene flow from GM *B. napus* to its MS relatives under open and wind pollination conditions and accordingly, estimate honeybee-mediated gene flow by comparing the difference between the two pollination conditions. To this end, the insect preventing-net treatment, with or without honeybee, was conducted to simulate the open and wind pollination conditions; ii) by fitting the obtained experimental data to log-logistic model, to establish gene flow equation for prediction of gene flow rate against distance.

MATERIALS AND METHODS

Plant materials

The genetically modified (GM) and non-genetically modified male sterile (non-GM MS) plants materials were used in this study. The GM *B. napus* (obtained from National Academy of Agricultural Science, Korea) homozygous and having the *bar* gene (Phosphinothricin acetyltransferase) which confers resistance to the herbicide glufosinate-ammonium (trade name Basta[®]), was used as the pollen donor and non-GM MS plants including *B. napus*, *B. juncea*, and *R. sativas* were used as the pollen recipients. The non-GM MS recipients can flower normally but failed to produce pollen. All seeds were sowed and cultivated in greenhouse of the experimental farm station of Seoul National University, Suwon, Korea. The vernalization treatment was performed under natural condition during the winter season (approximately 1.5 months) when the growth stage of seedlings was at two-true leaf stages.

Field design

The study was performed under the authorization field of the experimental farm station of Seoul National University, Suwon, Korea (authorization number: RDA-AB-2011-014) from March to July in 2014. The seedlings' transplanting and cultivation as described in Chapter II. A discontinuous design with planting GM pollen donor at one side and locating non-GM MS recipient at various distances from GM pollen donor was adopted for mimicking the mosaic-like agricultural structure. Both pollen donor and recipients plants were planted in field on 27th March. Three different growth stages of GM *B. napus* pollen donor in total 256 plants (planting area: 8 m × 8 m, with 50 cm plant spacing and row spacing, 9 plants m⁻²) were employed to ensure maximum synchronization of flowering period with non-GM MS recipients. Three non-GM MS recipients including *B. napus*, *B. juncea*, and *R. sativas* were planted at three independent directions at the various distances of 2, 4, 8, 16, 32, 64, and 128 m from the GM pollen donor (Fig. 11). Each

pollen recipient line at the each distance consists of 6 plants (planting in one row with 50 cm spacing). Prior to flowering, half of the recipients (3 plants) at each distance was treated by insects-preventing nets for only allowing wind pollination, but others being pollinated both by insects and wind. An approximately 10,000 population of honeybee colony was introduced during flowering period from 3th April. Flowering phenology and honeybee visit were investigated as described in Chapter II. Fertilizer application, insecticide spray, and irrigation management were conducted accordingly.

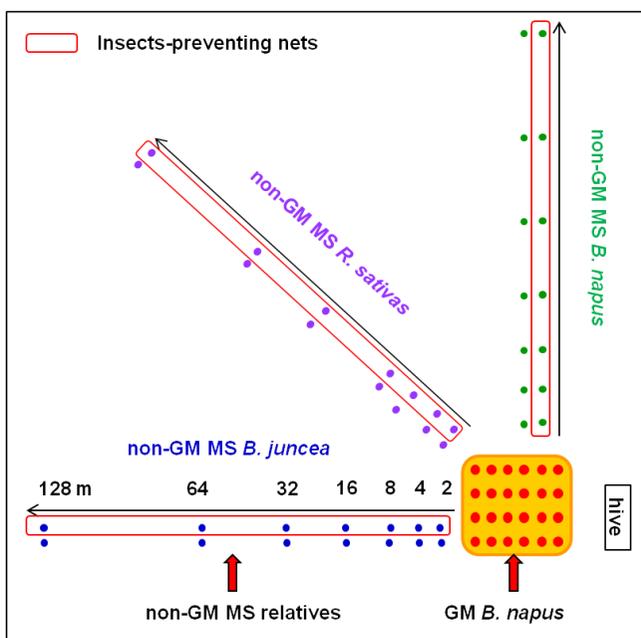


Fig. 11. Experimental field design for evaluation of gene flow from GM *B. napus* to its non-GM MS relatives in Suwon, Korea. The GM *B. napus* and non-GM MS relatives (*B. napus*, *R. sativas*, and *B. juncea*) were used as pollen donor and recipient, respectively. The recipient plants were placed at various distances of 2, 4, 8, 16, 32, 64, and 128 m. Half of recipient plants at each distance was treated by the insects-preventing nets prior to flowering. The location of the honeybee hive is shown.

Identification of hybrids

At maturity, all pods were hand-harvested from all recipients plants at various distances from GM pollen donor as illustrated in Fig. 11. All harvested pods were shelled, dried, counted (seed counter, USA), and stored at 4°C before herbicide screening test. The herbicide resistance screening test was conducted in 200-well multi-pots in the greenhouse of the experimental farm station of Seoul National University, Suwon, Korea from September to November in 2014. The glufosinate-ammonium was applied to seedlings of progenies of *B. napus*, *B. juncea*, and *R. sativas* at the one true-leaf stage at the pre-determined dose of 1.2 g, 0.6 g, and 2.4 g ai L⁻¹, respectively (see Chapter II), by using a compressor pressurized belt-driven sprayer (R & D Sprayer, USA) equipped with an 8002E flat-fan nozzle (Spraying System Co., USA) to deliver a spray volume of 600 L ha⁻¹. The number of tested seedlings for all species was counted prior to herbicide treatment. The survivals were counted 14 d after herbicide treatment. The survivals with resistance to the herbicide glufosinate-ammonium were considered as the hybrids that were outcrossed between GM *B. napus* and its relatives. A subsequent PCR confirmation was further performed to check the proportion of hybrids with *bar-specific* DNA fragment among total survivals. To comply with the conditions laid down by the Korean government, all the vegetative and seeds not used for further analysis were burned.

PCR analysis

The young leaf samples were collected from those survived 2-week-old seedlings after glufosinate-ammonium screening, immediately treated with liquid nitrogen, and stored at -80°C freezer. Genomic DNA was extracted by following the minor modified cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle 1990) and standardized to a final concentration of 20 ng µL⁻¹. PCR analysis was described as in Chapter II. Seedlings with the detected *bar-specific* DNA fragments were confirmed as true hybrids.

Statistical analysis

Each individual recipient at various distances was harvested separately and considered as replications. Gene flow rate for each species and each distance was calculated separately for both open (pollinated both by honeybee and winds) and wind (pollinated only by wind) pollination conditions. Honeybee-mediated gene flow was estimated by comparing the difference between open and wind pollination. The formula for calculating gene flow rate was showed as below:

$$\text{Gene flow rate (\%)} = \text{pods setting rate} \times \text{calibration factors rate} \times 100\%$$

The pods setting rate was calculated by dividing number of pods set by that of number of flowers. The calibration factors rate were determined by herbicide screening and PCR analysis. Herbicide screening with randomly selected seeds from all progenies was to check the proportion of number of survivals in total number of seedlings tested and further calibrated using PCR analysis by detection of the proportion of survivals with *bar-specific* DNA fragment in those survivals from herbicide screening test. Pearson's correlation coefficients were computed within the parameters (such as distance, number of flowers, number of pods, pods setting rate, honeybee visit, etc.) to determine the possible correlation among them with the use of statistical software IBM SPSS for windows (version 22.0, SPSS Inc., Chicago, USA). Various regressions models including linear, inverse function, exponential decay, and log-logistic models were tested to fit the gene flow data against distance using statistical software R 3.2.0.

RESULTS

Synchrony of flowering

The GM *B. napus* was transplanted three different times on 4th, 11th, and 18th May for maximum synchrony of flowering periods with non-GM MS pollen recipients (non-GM MS *B. napus*, non-GM MS *B. juncea*, and non-GM MS *R. sativas*). Biological important dates such as 1st blooming, peak-flowering, and end of flowering for both pollen donor and recipients were summarized in Table 15. In the case of GM pollen donor, the duration of flowering was 41, 44, and 43 d for the three different times' transplanting, respectively, resulting a total 54 d flowering duration. For the non-GM MS pollen recipients, the duration of flowerings were 32, 38, and 38 d for *B. napus*, *B. juncea*, and *R. sativas*, respectively, those were completely synchronized with GM pollen donor (54 d) (Table 15).

Honeybee visit

Honeybee (*Apis mellifera*) was performed as the pollinator between GM *B. napus* and its relatives in the present gene flow study. During the flowering period, honeybee pollinating behavior was investigated every day (except raining days) at 10:00-11:00 am, and the visit frequency was described by $\text{plant}^{-1} \text{min}^{-1}$. Based on the data summarized in Fig. 12, a relatively higher density of honeybee gathering (approximately $1.35 \text{ plant}^{-1} \text{min}^{-1}$) observed near the GM pollen donor area. In addition, it also revealed that the honeybee visit frequency ($\text{plant}^{-1} \text{min}^{-1}$) in those three species was decreasing with increasing the distance from GM pollen donor accordingly (Fig. 12). For instance, for non-GM MS *B. napus*, the number of honeybee visit frequency ($\text{plant}^{-1} \text{min}^{-1}$) was 1.17 at 2 m, 1.08 at 4 m, 0.91 at 8 m, 0.74 at 16 m, and decreased to 0.29 at 128 m. In the case of non-GM MS *B. juncea*, it was 0.89 at 2 m, 0.48 at 16 m, and decreased to 0.20 at 128 m. For non-GM MS *B. juncea*, it was 0.89 at 2 m, 0.73 m at 4 m, 0.65 at 8 m, 0.48 at 16 m, and

decreased to 0.19 ($\text{plant}^{-1} \text{min}^{-1}$) at 128 m. The same pattern of honeybee visit also observed in non-GM MS *R. sativas* (Fig. 12). Additionally, when compared the number of honeybee visit frequency at the same distance in each species, it was differed notably (Fig. 12). For example, at 2 m from the GM pollen donor, it was 1.17, 0.89, and 1.31 ($\text{plant}^{-1} \text{min}^{-1}$) in non-GM MS *B. napus*, non-GM MS *B. juncea*, and non-GM MS *R. sativas*, respectively, and 1.08, 0.73, and 1.14 at 4 m, and 0.91, 0.65, and 1.09 at 8 m in those of three species. Among the three species, the flower of non-GM MS *R. sativas* showed the most attractive to honeybee, followed by non-GM MS *B. napus* and non-GM MS *B. juncea*.

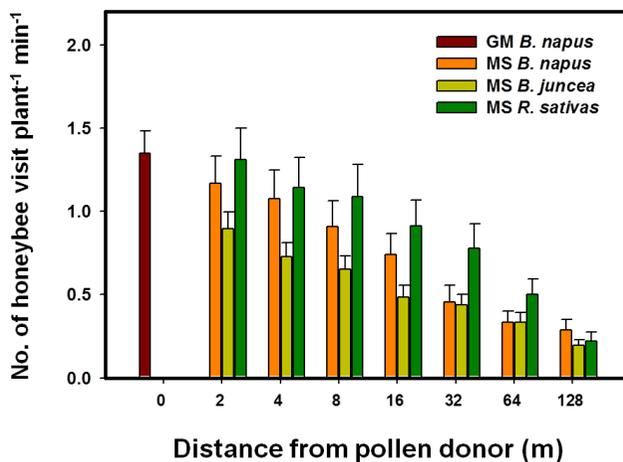


Fig. 12. Honeybee visit frequency ($\text{plant}^{-1} \text{min}^{-1}$) at various distances (m) from GM pollen donor during the flowering periods in 2014.

Table 15. Biological dates for a specific reproductive growth stage of pollen donor (GM *B. napus*) and pollen recipients (non-GM MS *B. napus*, non-GM MS *B. juncea*, and non-GM MS *R. sativas*) in 2014

Experimental plants	1 st blooming	Peak-flowering	End of flowering	Flowering duration (days)	Synchrony of flowering periods (days)
Pollen donor: Three times GM <i>B. napus</i> transplanting					
1 st time: Mar 4	Mar 27	Apr 19	May 7	41	-
2 nd time: Mar 11	Apr 1	Apr 26	May 14	44	-
3 rd time: Mar 18	Apr 6	May 2	May 19	43	-
Total flowering duration	-	-	-	54	
Pollen recipient: non-GM MS plants (transplanting date: Mar 4)					
<i>B. napus</i>	Apr 4	Apr 20	May 6	32	32
<i>B. juncea</i>	Apr 8	Apr 24	May 16	38	38
<i>R. sativas</i>	Apr 11	May 4	May 19	38	38

Determination of pods setting rate

Pods setting rate for non-GM MS *B. napus*, *B. juncea*, and *R. sativas* under open and wind pollination conditions were summarized in Table 16-18. According to the data, no significant difference was found in number of flowers in same species among the various distances ($P=0.05$), however, number of pods per plant for these three species was significantly decreased when increasing the distance from the GM pollen donor ($P=0.05$), resulting in a constantly decreased pods setting rate both under open and wind pollination conditions (Table 16-18). For non-GM MS *B. napus*, the pods setting rates were ranged from 0.7-38.9% under open pollination condition and 0.7-21.5% under wind pollination condition, 0.6-31.0% and 0.7-21.5% for non-GM MS *B. juncea*, and 0.6-13.4% and 0.7-4.4% for non-GM MS *R. sativas*, respectively.

Moreover, pods setting rates obtained under open pollination condition were higher than those values under wind pollination condition at the corresponding distance (Table 16-18). For instance, the pods setting rate for non-GM MS *B. napus* was 38.9% at 2 m, 34.5% at 4 m, 25.2% at 8 m, 18.2% at 16 m, and decreased down to 10.4% at 32 m under open pollination condition. Correspondingly, it was 21.5% at 2 m, 14.6% at 4 m, 10.1% at 8 m, 6.6% at 16 m, and 3.4% at 32 m under wind pollination condition (Table 16). In the case of non-GM MS *B. juncea*, the pods setting rate was 31.0% at 2 m, 21.5% at 8 m, 11.3% at 16 m, and 4.7% at 32 m under open pollination condition. Correspondingly, under wind pollination condition it was 10.8% at 2 m, 6.6% at 8 m, 4.8% at 16 m, and 3.0% at 32 m (Table 17). With regard to non-GM MS *R. sativas*, the similar pattern was showed as non-GM MS *B. napus* and *B. juncea* (Table 18). However, when the distance is farther than 32 m, the pods setting rate between open pollination and wind pollination condition in these three species did not show the notable difference ($P<0.05$). The rate under open pollination and wind pollination condition were 2.6 vs. 2.1% at 64 m and 0.7 vs. 0.7% at 128 m in non-GM MS *B. napus*, 2.4 vs. 1.8% at 64 m and 0.6 vs. 0.5% at 128 m in non-GM MS *B. juncea*, 0.7 vs. 0.6% at 64 m and 0.6 vs. 0.7% at 128 m in non-GM MS *R. sativas*, respectively (Table 16-18).

Table 16. Number of flowers, pods, and pods setting rate per plant for non-GM MS *B. napus* under open pollination and wind pollination condition

Distance (m)	Open pollination ^a			Wind pollination ^b		
	No. of flowers/plant	No. of pods/plant	Pods setting rate (%) ^c	No. of flowers/plant	No. of pods/plant	Pods setting rate (%)
2	1477	573	38.9	1321	281	21.5
4	1258	433	34.5	1455	211	14.6
8	1465	369	25.2	1406	144	10.1
16	1417	255	18.2	1143	76	6.6
32	1386	134	10.4	1649	55	3.4
64	1525	37	2.6	1483	31	2.1
128	1342	10	0.7	1438	10	0.7
LSD _{0.05}	387	43	5.1	360	26	2.4

^a Open pollination means pollination caused by both pollinators and wind

^b Wind pollination means by setting pollinators preventing-net only allowing wind pollination

^c Pods setting rate calculated by number of pods per plant/ no. of flowers per plant × 100%

Table 17. No. of flowers, pods, and pods setting rate per plant for non-GM MS *B. juncea* under open pollination and wind pollination condition

Distance (m)	Open pollination ^a			Wind pollination ^b		
	No. of flowers/plant	No. of pods/plant	Pods setting rate (%) ^c	No. of flowers/plant	No. of pods/plant	Pods setting rate (%)
2	1576	487	31.0	1687	182	10.8
4	1489	416	28.0	1622	141	8.7
8	1537	330	21.5	1627	107	6.6
16	1579	177	11.3	1599	77	4.8
32	1498	71	4.7	1593	47	3.0
64	1565	37	2.4	1614	28	1.8
128	1573	10	0.6	1589	8	0.5
LSD _{0.05}	140	28	2.5	138	18	1.1

^a Open pollination means pollination caused by both pollinators and wind

^b Wind pollination means by setting pollinators preventing-net only allowing wind pollination

^c Pods setting rate calculated by number of pods per plant/ no. of flowers per plant × 100%

Table 18. No. of flowers, pods, and pods setting rate per plant for non-GM MS *R. sativas* under open pollination and wind pollination condition

Distance (m)	Open pollination ^a			Wind pollination ^b		
	No. of flowers/plant	No. of pods/plant	Pods setting rate (%) ^c	No. of flowers/plant	No. of pods/plant	Pods setting rate (%)
2	1826	245	13.4	1875	83	4.4
4	1723	223	13.1	1888	64	3.4
8	1780	162	9.2	1751	41	2.4
16	1862	88	4.7	1779	25	1.4
32	1871	35	1.9	1840	18	1.0
64	1766	12	0.7	1635	9	0.6
128	1697	10	0.6	1668	9	0.7
LSD _{0.05}	183	19	1.7	198	11	0.6

^a Open pollination means pollination caused by both pollinators and wind

^b Wind pollination means by setting pollinators preventing-net only allowing wind pollination

^c Pods setting rate calculated by number of pods per plant/ no. of flowers per plant × 100%

Correlations between the parameters

Pearson correlation analysis was performed to calculate the possible relationship among the parameters of distance, number of pods, number of flowers, number of bees visit, and pods setting rate for the three species (Table 19). The results demonstrated that the number of pods, number of honeybee visit, or distance significantly correlated with pods setting rate, which means the three parameters greatly influence on gene flow ($P=0.05$ or 0.01). Number of pods and pods setting rate positively and significantly correlated with number of honeybee visit, indicating that the effect of pollinator on pollen transfer between pollen donor and recipient ($P=0.01$). No significant correlation was found between number of flowers and other parameters since it was negatively correlated with others ($P=0.05$ or 0.01).

Determination of calibration factors

For determination of calibration factors, herbicide screening test with randomly seeds selected from the progenies and a subsequent PCR confirmation with survivals from the herbicide screening test were performed. Based on survivals after herbicide screening test and PCR confirmation with those survivals by detection of GM *bar-specific* DNA fragment, the calibration factors for survivals and hybrids rates were calculated and shown in Tables 6-8. In herbicide screening test, approximately 10% of seeds in total for each species at each distance was tested. The survival rates for non-GM MS *B. napus* progenies ranged from 41.67-85.09% for open pollination condition and 36.11-83.53% for wind pollination condition at 2-128 m from GM pollen donor, respectively, and the rates for hybrid rate confirmed by PCR ranged from 96.67-100% and 75-100% for open and wind pollination conditions, respectively (Table 20). In the case of survival rate for non-GM MS *B. juncea* progenies, in herbicide screening test, it ranged from 41.67-84.57% and 45.45-81.45% for open pollination and wind pollination conditions, respectively, and hybrid rate confirmed by PCR ranged from 66.67-100% and

66.67-91.67% at 2-128 m from GM pollen donor (Table 21). For non-GM MS *R. sativas* progenies, only two survivals were screened, but both of them were proved to be false hybrids (Table 22). Overall, the survival rate for both recipients of non-GM MS *B. napus* and non-GM MS *B. juncea* near GM poll donor showed a significant greater than those of locating in a farther distance regardless of under open pollination or wind condition ($P<0.05$) (Table 20 and 21).

Table 19. Coefficients of the Pearson correlation among the parameters

Species	Parameters	Distance	No. of flowers	No. of bees	No. of pods	Pods setting rate	Gene flow rate
<i>B. napus</i>	Distance	1.000					
	Flowers no.	-0.082	1.000				
	Bees no.	-0.831*	-0.115	1.000			
	Pods no.	-0.823*	-0.043	0.991**	1.000		
	Pods setting rate	-0.832*	-0.138	0.995**	0.993**	1.000	
	Gene flow rate	-0.828*	-0.145	0.995**	0.991**	0.995**	1.000
<i>B. juncea</i>	Distance	1.000					
	Flowers no.	0.342	1.000				
	Bees no.	-0.862*	-0.202	1.000			
	Pods no.	-0.780*	-0.169	0.973**	1.000		
	Pods setting rate	-0.781*	-0.199	0.970**	0.999**	1.000	
	Gene flow rate	-0.763*	-0.230	0.950**	0.992**	0.995**	1.000
<i>R. sativas</i>	Distance	1.000					
	Flowers no.	-0.531	1.000				
	Bees no.	-0.950**	0.388	1.000			
	Pods no.	-0.761*	0.017	0.913**	1.000		
	Pods setting rate	-0.754*	-0.017	0.904**	0.999**	1.000	
	Gene flow rate	nc ^a	nc	nc	nc	nc	1.000

*correlation is significant at $p=0.05$, **correlation is significant at $p=0.01$ (two-tailed)

^a nc, not computed because gene flow rate estimated for *R. sativas* is 0

Table 20. Determination of calibration factors in non-GM MS *B. napus* progenies by herbicide screening test and PCR confirmation

Distance (m)	Open pollination ^a		Wind pollination ^b	
	Survival rate (%) ^c	Hybrid rate (%) ^d	Survival rate (%)	Hybrid rate (%)
2	83.34	100.00	81.93	83.50
4	85.05	100.00	82.16	86.31
8	84.75	100.00	80.52	100.00
16	82.78	96.67	79.78	91.67
32	77.87	100.00	83.53	100.00
64	76.52	100.00	76.59	75.00
128	41.67	100.00	36.11	100.00
LSD _{0.05}	8.2	3.8	6.2	22.2

^a Open pollination means pollination caused by both pollinators and wind

^b Wind pollination means by setting pollinators preventing-net only allowing wind pollination

^c Survival rate by herbicide screening test, calculated by dividing no. of survivals by total no. of seedlings tested

^d Hybrid rate by PCR confirmation, calculated by dividing no. of survivals with GM *B. napus bar-specific* bands by total no. of sampled survivals

Table 21. Summary of calibration factors in non-GM MS *B. juncea* progenies by herbicide screening test and PCR confirmation

Distance (m)	Open pollination ^a		Wind pollination ^b	
	Survival rate (%) ^c	Hybrid rate (%) ^d	Survival rate (%)	Hybrid rate (%)
2	84.57	84.49	81.45	76.39
4	81.38	94.19	78.57	87.78
8	84.28	95.83	78.91	91.67
16	78.55	83.33	77.78	85.00
32	78.47	66.67	80.41	91.67
64	66.39	75.00	72.94	88.89
128	41.67	100.00	45.45	66.67
LSD _{0.05}	8.0	16.4	9.7	28.9

^a Open pollination means pollination caused by both pollinators and wind

^b Wind pollination means by setting pollinators preventing-net only allowing wind pollination

^c Survival rate by herbicide screening test, calculated by dividing no. of survivals by total no. of seedlings tested

^d Hybrid rate by PCR confirmation, calculated by dividing no. of survivals with GM *B. napus bar-specific* bands by total no. of sampled survivals

Table 22. Summary of calibration factors in non-GM MS *R. sativas* progenies by herbicide screening test and PCR confirmation

Distance (m)	Open pollination ^a		Wind pollination ^b	
	Survival rate (%) ^c	Hybrid rate (%) ^d	Survival rate (%)	Hybrid rate (%)
2	0.18	0.00	0.00	0.00
4	0.00	0.00	0.00	0.00
8	0.00	0.00	0.00	0.00
16	0.45	0.00	0.00	0.00
32	0.00	0.00	0.00	0.00
64	0.00	0.00	0.00	0.00
128	0.00	0.00	0.00	0.00
LSD _{0.05}	0.6	NS	NS	NS

^a Open pollination means pollination caused by both pollinators and wind

^b Wind pollination means by setting pollinators preventing-net only allowing wind pollination

^c Survival rate by herbicide screening test, calculated by dividing no. of survivals by total no. of seedlings tested

^d Hybrid rate by PCR confirmation, calculated by dividing no. of survivals with GM *B. napus bar-specific* bands by total no. of sampled survivals

Gene flow rate from GM *B. napus* to its non-GM MS relatives

Finalized gene flow rate from GM *B. napus* to its non-GM MS recipients was summarized in table 23. Overall the date, a higher gene flow rate occurred with non-GM MS *B. napus* in comparison of non-GM MS *B. juncea* at the corresponding distance (Table 23). Gene flow rate showed a decreasing pattern with increasing the distance from the GM pollen donor in both species, regardless of open or wind pollination condition (Table 23). In the case of non-GM MS *B. napus* under open pollination condition, gene flow rate was 32.48%, 29.43%, 21.39%, 14.71%, and 8.21% at 2, 4, 8, 16, and 32 m, and greater than those rates of 14.69%, 10.42%, 8.14%, 4.77%, and 2.85% at the corresponding distances under wind pollination condition, respectively (Table 23). With regard to non-GM MS *B. juncea*, under open pollination condition it was 21.95%, 21.40%, 17.48%, 7.19%, and 2.44% at 2, 4, 8, 16, and 32 m, and in comparison of 6.62%, 6.05%, 4.72%, 3.22%, and 2.17% at the corresponding distances under wind pollination condition, respectively (Table 23). However, for gene flow rate in both species at 64 and 128 m, there was no significant difference between open pollination and wind pollination conditions ($P < 0.05$), with the values of 1.98% vs. 1.21% at 64 m and 0.30% vs. 0.26% at 128 m in *B. napus*, and 1.20% vs. 1.14% and 0.24% vs. 0.16% in *B. juncea*, respectively (Table 23). No matter what the distance was, the outcrossing did not occur between GM *B. napus* and non-GM MS *R. sativas* (Table 23).

Estimate of honeybee-mediated gene flow

The difference between open and wind pollination indicates that honeybee-mediated gene flow. In the case of gene flow rate from GM *B. napus* to non-GM MS *B. napus*, the estimate of honeybee-mediated gene flow rate was 17.78%, 19.01%, 13.24%, 9.94% at 2 m, 4 m, 8 m, 16 m, and decreasing to 0.03% at 128 m. As for non-GM MS *B. juncea*, the estimate of rate was 15.53%, 15.36%, 12.76%, 3.97% at 2 m, 4 m, 8 m, 16 m, and decreasing to 0.06% at 128 m (Table 23). There was no significant difference ($P < 0.05$) in honeybee-mediated gene flow at the farther distance as showed in Table 9, 0.77% (64 m) vs. 0.33% (128 m) for non-GM

MS *B. napus* and 0.03% (64 m) vs 0.03% (128 m) for non-GM MS *B. juncea*, respectively. The pattern of the estimated values for honeybee-mediated gene flow rate was closely associated with honeybee visit frequency, and decreased with increasing the distance from the GM pollen donor (Fig. 12; Table 23).

Table 23. Gene flow rate (%) from GM *B. napus* to its non-GM MS recipients (*B. napus*, *B. juncea*, and *R. sativas*) under open pollination (OP) and wind pollination (WP) conditions

Distance (m)	<i>B. napus</i>			<i>B. juncea</i>			<i>R. sativas</i>		
	OP (%)	WP (%)	(OP-WP) ^a	OP (%)	WP (%)	(OP-WP)	OP (%)	WP (%)	(OP-WP)
2	32.48	14.69	17.78	21.95	6.62	15.33	0.00	0.00	0.00
4	29.43	10.42	19.01	21.40	6.05	15.36	0.00	0.00	0.00
8	21.39	8.14	13.24	17.48	4.72	12.76	0.00	0.00	0.00
16	14.71	4.77	9.94	7.19	3.22	3.97	0.00	0.00	0.00
32	8.21	2.85	5.36	2.44	2.17	0.27	0.00	0.00	0.00
64	1.98	1.21	0.77	1.20	1.14	0.06	0.00	0.00	0.00
128	0.30	0.26	0.03	0.24	0.16	0.06	0.00	0.00	0.00
LSD _{0.05}	5.5	2.5	6.5	2.4	1.5	2.7	NS ^b	NS	NS

Gene flow rate calculated by pods setting rate × calibration factors

^a OP-WP, represents the contribution of honeybee to pollination

^b NS, not significant at $P=0.05$

Modeling of gene flow under different pollination conditions

Various regressions containing log-logistic, linear, inverse first order function, rational function, and double exponential decay models were performed to fit the gene flow data (Table 24 and 25). R^2 and RMS values were used to indicate the goodness of fit between gene flow data and those models. In the case of non-GM MS *B. napus*, the results suggested that the three-parameter log-logistic and four-parameter double exponential decay models were better suitable for data fitting than the other three models. As summarized in Table 24, the log-logistic and double exponential decay models have the similar smaller RMS values (approximately 8.5) and greater R^2 (>0.95) compared to other three models. The R^2 and RMS values estimated for other three models were 0.655 and 56.1 (linear model), 0.738 and 42.7 (inverse first order function), and 0.953 and 18.13 (rational function), respectively. With regard to non-GM MS *B. juncea*, log-logistic ($R^2=0.981$; RMS=1.81) and double exponential decay models ($R^2=0.965$; RMS=3.58) also showed the best goodness of fit among the tested models when comparing R^2 and RMS values with other models (Table 25). In the previous studies, double exponential decay model has been proposed for description of the gene flow rate against distance, but there is a lack of biological meaning for explanation of the estimated parameters. Therefore, the biological meaningful log-logistic model was considered as the most suitable model to fit the gene flow data.

As shown in Table 26, the log-logistic model provided a good fit to the data both for non-GM MS *B. napus* (Fig 13A, P values of lack of fit= 0.965) and non-GM *B. juncea* (Fig 13B, P values of lack of fit= 0.989) under different pollination conditions. The estimated honeybee-mediated gene flow was also well described by the log-logistic model with $R^2=0.892$ and RMS=12.0 for non-GM MS *B. napus* and $R^2=0.964$ and RMS=2.0 for non-GM MS *B. juncea*, respectively (Table 26). In addition, the estimated D_{50} values, the distance for 50% of gene flow rate reduction under open pollination condition (12.1 m for non-GM MS *B. napus* and 12.6 m for non-GM MS *B. juncea*), were farther than those of estimated under wind pollination condition (7.7 m for non-GM MS *B. napus* and 8.9 m for non-GM MS *B. juncea*),

indicating the contribution of honeybee-mediated gene flow. Moreover, the D_{50} estimated for honeybee pollination is greater than that of estimated for wind pollination in both species.

Table 24. Various models tested to fit the gene flow data from GM *B. napus* to non-GM *B. napus* against distances under open pollination condition

Models tested ^a		Parameters				DF	RMS	R ²	P values
Log-logistic	$y = y_0 / (1 + (x/D_{50})^b)$	$y_0 = 35.1$ (2.7) ^b	$D_{50} = 12.1$ (1.7)	$b = 1.40$ (0.2)		18	8.8	0.951	< 0.0001
Linear	$y = y_0 + ax$	$y_0 = 23.9$ (2.2)	$a = -0.23$ (0.04)			19	56.1	0.655	< 0.0001
Inverse first order function	$y = y_0 + a/x$	$y_0 = 6.60$ (1.9)	$a = 62.8$ (8.6)			19	42.7	0.738	< 0.0001
Rational function	$y = (a + bx)/(c + x)$	$a = 475.3$ (92.3)	$b = -4.0$ (2.0)	$c = 12.2$ (3.2)		18	18.13	0.953	< 0.0001
Double exponential decay	$y = ae^{-bx} + ce^{-dx}$	$a = 13.9$ (13.2)	$b = 0.17$ (0.2)	$c = 24.8$ (15.5)	$d = 0.04$ (0.02)	17	8.4	0.954	< 0.0001

^a For log-logistic model: y_0 , the maximum gene flow rate when distance between pollen donor and recipient is 0; b , slope of the log-logistic curve; D_{50} , the distance required for 50% of gene flow rate reduction compared to the maximum gene flow rate; For linear model: y_0 , the maximum gene flow rate when distance between pollen donor and recipient is 0; a , the coefficient of determination of the curve; For inverse first order function: y_0 , constant, a , the coefficient of determination of the curve; For rational function: a , c , constant; b , the coefficient of determination of the curve; For double exponential decay model: a , c , constant; b , d , the coefficient of determination of the curve.

^b The values in parentheses are the standard error of parameters estimated for models
DF, degree of freedom; RMS, residual mean square; R², coefficient of determination

Table 25. Various models tested to fit the gene flow data from GM *B. napus* to non-GM *B. juncea* against distances under open pollination condition

Models tested ^a		Parameters				DF	RMS	R ²	P values
Log-logistic	$y = y_0 / (1 + (x/D_{50})^b)$	$y_0 = 22.4$ (0.7) ^b	$D_{50} = 12.6$ (0.7)	$b = 2.6$ (0.3)	18	1.8	0.981	< 0.0001	
Linear	$y = y_0 + ax$	$y_0 = 16.1$ (1.8)	$a = -0.16$ (0.03)		19	38.8	0.572	< 0.0001	
Inverse first order function	$y = y_0 + a/x$	$y_0 = 3.75$ (1.5)	$a = 46.0$ (6.8)		19	26.4	0.709	< 0.0001	
Rational function	$y = (a + bx)/(c + x)$	$a = 298.9$ (64.4)	$b = -3.5$ (1.5)	$c = 10.3$ (2.9)	18	5.3	0.945	< 0.0001	
Double exponential decay	$y = ae^{-bx} + ce^{-dx}$	$a = 14.2$ (3.7)	$b = 0.07$ (0.1)	$c = 12.7$ (4.3)	$d = 0.07$ (0.1)	17	3.6	0.965	< 0.0001

^a For log-logistic model: y_0 , the maximum gene flow rate when distance between pollen donor and recipient is 0; b , slope of the log-logistic curve; D_{50} , the distance required for 50% of gene flow rate reduction compared to the maximum gene flow rate; For linear model: y_0 , the maximum gene flow rate when distance between pollen donor and recipient is 0; a , the coefficient of determination of the curve; For inverse first order function: y_0 , constant, a , the coefficient of determination of the curve; For rational function: a , c , constant; b , the coefficient of determination of the curve; For double exponential decay model: a , c , constant; b , d , the coefficient of determination of the curve.

^b The values in parentheses are the standard error of parameters estimated for models
DF, degree of freedom; RMS, residual mean square; R², coefficient of determination

Table 26. Estimated parameters for log-logistic analysis of the gene flow rate for both recipients of non-GM MS *B. napus* and non-GM MS *B. juncea* under different types of pollination

Species	Types of pollination	C (%)	b	D ₅₀ (m)	DF	RMS	R ²	P values
<i>B. napus</i>	Open pollination	35.1 (2.7)	1.4 (0.2)	12.1 (1.7)	18	8.5	0.951	<0.0001
	Wind pollination	16.4 (1.2)	1.3 (0.2)	7.7 (1.3)	18	1.9	0.932	<0.0001
	Honeybee pollination	19.0 (2.3)	1.7 (0.6)	16.2 (4.3)	18	12.0	0.892	<0.0001
<i>B. juncea</i>	Open pollination	22.4 (0.7)	2.6 (0.3)	12.6 (0.7)	18	1.8	0.981	<0.0001
	Wind pollination	8.9 (1.0)	0.9 (0.3)	8.9 (2.5)	18	0.7	0.900	<0.0001
	Honeybee pollination	15.5 (0.6)	3.8 (0.7)	12.1 (0.8)	18	2.0	0.964	<0.0001

C, the maximum gene flow rate predicted by log-logistic curve; SE, standard error; b, slope of the log-logistic curve; D₅₀, the distance required for 50% of gene flow rate reduction compared to the maximum gene flow rate; R², coefficient of determination; RMS, root mean square; df, degree of freedom

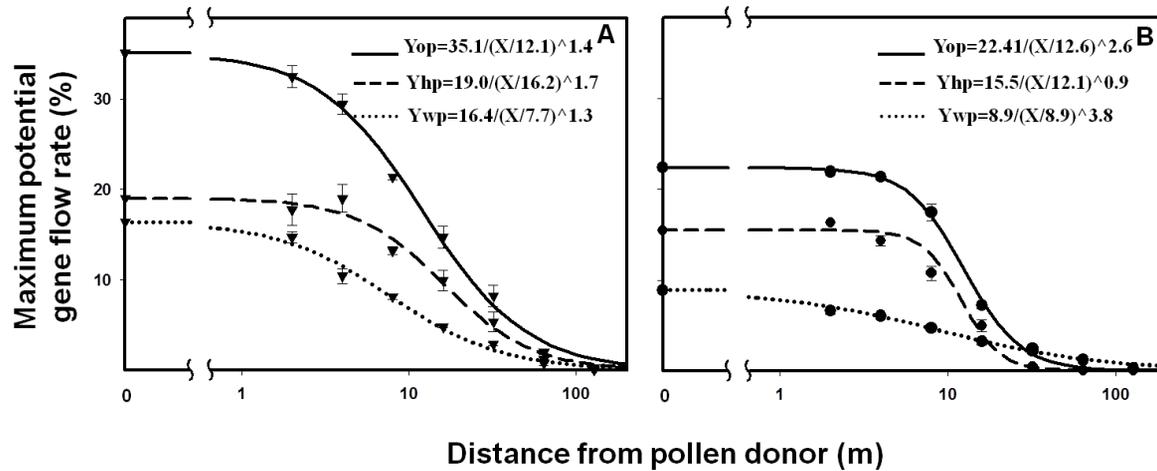


Fig. 13. The log-logistic model equations for prediction of gene flow rate against distance for non-GM MS *B. napus* (A, lack of fit, $P=0.965$) and non-GM MS *B. juncea* (B, lack of fit, $P=0.989$). The modeled equations of Y_{op} , Y_{hp} , and Y_{wp} represent the gene flow rate estimated under open pollination, honeybee-mediated pollination, and under wind pollination conditions, respectively.

DISCUSSION

In this study, a discontinuous design for estimating gene flow from GM *B. napus* to its MS relatives was performed under Korean climatic condition. Non-GM MS lines as recipients, presence and abundance of pollinators- honeybee, and entire synchrony of flowering period between pollen donor and recipients were involved in order to quantify the possibility of interspecific outcrossing and the maximum potential gene flow rate. The Pearson correlation analysis demonstrated that the gene flow rate was significantly correlated with honeybee visit frequency. Recipients treated with or without insect-preventing nets revealed the difference in gene flow between wind and pollinator pollination. On the basis of the finally confirmed gene flow data calibrated by herbicide screening and PCR confirmation test, a biological meaningful log-logistic equation was established for prediction of the gene flow rate at a specified distance from the pollen donor, which could be further used as a reference for determination of safe isolated distance when GM *B. napus* is cultivated.

Outcrossing

Numerous studies have reported the outcrossing events within *Brassica* family such as *B. napus* (AACC, 2n=38), *B. rapa* (AA, 2n=20), and *B. juncea* (AABB, 2n=36) both by artificial means and under natural field condition, due to these species sharing a common set of chromosomes and therefore facilitating interspecific outcrossing among them (Bing et al. 1996; Hansen et al. 2001; Jørgensen et al. 1996; Warwick et al. 2003). The outcrossing events also occurred between *Brassica* species and other cruciferous species such as *R. raphanistrum* (Darmency et al. 1998; Chèvre et al. 2000; Rieger et al. 2001), *R. sativas* (Ammitzboll and Jørgensen 2006; Gupta 1997; Huang et al. 2002), and *Sinapis arvensis* (Chèvre et al. 1996; Lefol et al. 1996), etc.

In our studies three species of recipient involved including non-GM MS *B. napus*, non-GM MS *B. juncea*, and non-GM MS *R. sativas*. The results are

agreement with the previous outcrossing events between *B. napus* and *B. juncea* even occurred at a considerable distance from the pollen donor (non-GM MS *B. juncea* (♀) × GM *B. napus* (♂)) (Tsuda et al. 2012). However, in the case of outcrossing between *B. napus* and *R. sativas*, even in the favorable condition with entire synchrony of flowering period and presence and abundant of honeybee, there was none of hybrid detected outcrossed between them (non-GM MS *R. sativas* (♀) × GM *B. napus* (♂)). The existing report on successfully outcrossing between *B. napus* and *R. sativas* was published by Ammitzboll and Jørgensen (2006) when a GM MS *B. napus* served as pollen recipient (GM MS *B. napus* (♀) × *R. sativas* (♂)), which might be hypothesized that the parental genotypes and direction of crosses result in the difference in comparison with our outcrossing trials (Bing et al. 1999; Jørgensen and Andersen 1994; Jørgensen et al. 1998; Halfhill et al. 2002; Scott and Wilkinson 1998). But one point is very clear that the probability of outcrossing between these two species is extreme low.

Factors affecting gene flow

Gene flow rate is affected by intrinsic and external factors, such as genotype, direction of cross, experimental design, local climatic conditions, etc (Bing et al. 1999; ; Jørgensen et al. 1998; Halfhill et al. 2002; Warwick et al. 2003). In the present studies, in order to simulate the gene flow under favorable conditions and reveal the relationship between factors and gene flow, several measures are adopted containing using MS recipients, introducing pollinators, and completely overlapping flowering period.

In the previous studies pollen recipients used were non-GM MF plants, as these plants could produce fertile pollen and compete with GM pollen donor, resulting in a lower gene flow rate, less than 2% even at mixing area, which is difficult to differentiate the difference between the factors. In the present studies, we used the MS line as recipients to achieve a higher outcrossing rate as it does not produce viable pollen but only accept the foreign GM pollen for outcrossing. By this means, an average of gene flow rate ranged from 0.26-32.48% obtained for non-GM MS *B.*

napus and 0.16-21.95% for non-GM MS *B. juncea* at 2-128 m, respectively (Table 23). These rates enabled to differentiate the difference between the factors on affecting gene flow rate.

The degree of synchrony of flowering period between pollen donor and recipient is also a key factor for determining the gene flow rate (Bing et al. 1991, 1996; Landbo et al. 1996). An increase of overlapped flowering days between pollen donor and recipients may potentially increase the outcrossing rate as there are more opportunities for exposed recipient flowers to accept the foreign pollen for outcrossing. In contrast, lack of synchrony of geographic distribution or in flowering periods, the outcrossing either drastic reduction or no occurred. Tsuda et al. (2012) reported none of hybrids were detected when a 19 days synchrony of flowering period was achieved between *B. juncea* and *B. napus* under natural flowering condition in 2009. However, when the synchrony of flowering period was increased to 34 days by artificially means in 2010, a total of 55 hybrids was detected. In our studies, according to the three different times of GM *B. napus* pollen donor transplanting, the flowering periods was entirely synchronized with non-GM MS recipients (Table 15).

Honeybee has been considered to be the most prominent and efficient pollinators for the rape crop (Duran et al. 2010; Williams et al. 1987). For an open pollinated male fertile *B. napus*, the seed production results from three factors, self pollination, wind pollination by neighbouring plants, and insect pollination primarily by honeybee. As honeybee directly takes part in pollen transfer and pollination, any factors affecting the honeybee's behavior such as the density of flowers, types of plants, distance from the beehive, wind direction, weather condition, etc., would indirectly influence the pollination. In field observations, the number of honeybee visit and honeybee's activity were associated with weather conditions with a small number in a cloudy day and none in a raining day. Pods setting rate was significantly correlated with the honeybee visit frequency ($\text{plant}^{-1} \text{min}^{-1}$) in those three species based on the Pearson's correlation analysis ($P= 0.01$) (Table 19). In addition, the honeybee visit frequency showed a negative and significant correlation with distance increasing from the pollen donor ($P= 0.05$) (Table 19). A relatively

higher density of honeybee visit found near the GM pollen donor area in those three species because of the higher density of flowers in this area, and decreased with increasing the distance from GM pollen donor accordingly (Fig. 12).

Determination of calibration factors

Based on the previous studies, in some cases due to the recovery of fertility in MS plants, it also can produce pods by selfing, which certainly would over-estimate the outcrossing rate. In order to improve the accuracy of gene flow rate estimation, a herbicide resistance screening test and following the PCR confirmation were performed in our studies for detection of the true hybrids among the progenies. Herbicide screening test supplied a mass screening method for detecting hybrids in progenies. According to a herbicide dose-response test on plants survival rate, a single optimal dose was determined, which can discriminate between true hybrids outcrossed from parental plants and false hybrids selfed from maternal plant. In our studies, doses of 1.2 g (recommended dose), 0.6 g, and 2.4 g ai L⁻¹ glufosinate-ammonium treatment of one-true-leaf stage seedlings of *B. napus*, *B. juncea*, and *R. sativas* for detection of hybrids were determined, respectively. The survivals were counted after 14 days after glufosinate-ammonium treatment. The screening gave the survival rates ranging from 36.11-85.05%, 41.67-84.57%, and 0.18-0.45% for the progenies of non-GM MS *B. napus*, *B. juncea*, and *R. sativas*, respectively (Table 20-22). In order to detect whether there were negative survivals existed in herbicide resistance screening test due to insufficient absorption of the herbicide or intrinsic tolerance, a subsequent polymerase chain reaction (PCR) confirmation was performed to detect the *bar-specific* band in those survivals. According to the PCR confirmation, more accurate calibration factors were determined with ranging from 75-100% and 66.7-100% for non-GM MS *B. napus* and non-GM MS *B. juncea*, respectively. The two survivals of *R. sativas* from herbicide screening test were proved to be false-positive hybrids (Table 20-22). This demonstrates the necessity of herbicide screening together with molecular level detection for finally determination of true hybrids.

Modeling of gene flow under open and wind pollination conditions

In the present studies, a discontinuous design was conducted to estimate the gene flow from a small pollen source as spilled GM *B. napus* to its relatives under open and wind pollination conditions. By introducing honeybee and covering the recipients with or without the insect-preventing nets, we could set up two different pollination conditions. Honeybee-mediated pollination could be estimated using the difference between them. In order to improve the accuracy of simulation model, both herbicide resistance and PCR confirmation markers were used to confirm F1 hybrids resulted from gene flow from GM *B. napus* to its relatives. By non-linear regression analyses to fit the log-logistic model to the gene flow rate data, we could get a simulation model for predicting gene flow from GM *B. napus* to a MS relatives at a specified distance (Fig. 13). The previous studies on modeling used different models such as single or double exponential decay equations (Yuan et al. 2007), linear function equation (Pla et al. 2006). These models provided considerable determination coefficients, but they do not provide sufficient biological meanings. In contrast, the log-logistic model adapted in this study have biological meaning in its parameters, such as the estimated maximum gene flow (y_0), the slope (b) around the 50% of y_0 reduction (D_{50}).

Our study was not designed to cover various wind directions, thus the gene flow estimated in this study might be influenced by wind direction and speed. Nonetheless, the model might be more suitable for predicting gene flow events where fields are small and uni-directionally arranged. Because the MS lines were used as pollen recipients in these studies under favorite conditions with abundant presence of pollinators and entire synchrony of flowering periods, the gene flow rates values presented here are the maximum potential rates. It is worth pointing out that the maximum potential gene flow rate may provide a valuable reference for gene flow risk assessment. With these maximum potential values, the proper isolation distance for risk management can be determined. Using the model and

based on the tolerable threshold (0.01%) made by EU (2003), the isolation distance for less than 0.01% gene flow from GM *B. napus* was estimated to be 2710 m and 254 m for MS *B. napus* and *B. juncea*, respectively. These figures are more than 20 times and 10 times greater than the isolation distances established in Chapter II, where pollen recipients were male fertile (MF) relatives (122.5 and 23.7 m for non-GM *B. napus* and *B. juncea*, respectively).

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GENERAL CONCLUSION

Gene flow from GM crops to non-GM crops or wild relatives takes place naturally when GM crops are grown. This is exactly true for Northern American and Australia with mono cropping systems, and for European and Asian countries with diverse cropping systems. Both greenhouse and field studies showed that the possibility of gene flow from *B. napus* to its relatives. Greenhouse as a model case study provided a flexible and controllable ways for setting up basic experimental conditions for evaluating gene flow from GM crops to their relatives. The field evaluation using MS recipients revealed that the pollen of *B. napus* could transfer farther than 128 m to fertilize MS *B. napus* and MS *B. juncea*. For MF recipients, the farthest distance was 75 m for *B. napus* and 16 m for *B. juncea*, respectively. Using the log-logistic model and parameter estimates, with gene flow rate less than 0.01% stipulated by EU (2002) as a tolerable threshold, the isolation distance between GM *B. napus* and MF recipient was estimated to be 122.5 m and 23.7 m for *B. napus* and *B. juncea*, respectively. In the case of MS recipients it was estimated to be 2710 m and 254 m for *B. napus* and *B. juncea*, respectively.

The experimental data in our study provides informative reference values and scientific basis for risk assessment of gene flow from GM *B. napus* to its relatives under Korean climatic condition. It seems that more studies and knowledge are needed to accumulate and strengthen, which is necessary for better understanding of different factors affecting gene flow and simulations to assess the practical gene flow in the agricultural ecosystem, and thus to provide scientific approaches for gene flow risk assessment and systemic management of GM crops. For the future studies on gene flow, more attention should be paid to more long-term consequence of gene flow and its consequential hybrids in various agricultural conditions and the possibility of outcrossing under different cross directions and combinations.

ABSTRACT IN KOREAN

형질전환 유채와 근연종간의 유전자이동 가능성

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본 연구는 형질전환 유채(*Brassica napus*)와 그 근연종간의 유전자 이동 가능성을 정량화하기 위해, 온실 및 포장에서 실험을 수행하였다. 형질전환 유채와 근연종간의 이격거리에 따른 유전자이동 가능성을 예측하기 위해 Log-istic 모델을 사용하였으며, 이를 바탕으로, 격리거리(Isolation distance)를 예측하였다. 유채와 그 근연종간의 교잡종을 찾기 위한 방법으로 SSRs (Simple sequence repeats) 마커와 제초제 저항성 (glufosinate-ammonium) 마커를 사용하였다. 3년간의 포장실험에서 화분공여자(pollen donor)로서 형질전환 유채는 화분 수용체(pollen recipient)인 유채와 갓(*B. juncea*)으로의 유전자 이동에 따른 교잡종이 형성됨을 보여주었으나, 무(*Raphanus sativus*)와는 이격 거리에 상관없이 유전자 이동에 따른 교잡가능성이 없음을 보여주었다. 화분 수용체로서 웅성불임(MS) 유채 및 웅성불임 갓으로의 유전자이동 가능성은 개화기가 동일하고 수분매개체가 충분히 존재할 때, 가장 가까운 2 m 이격거리와 가장 먼 128 m 에서 각각 37.35%와 0.30% 및 34.59%와 0.24%씩의 유전자 이동에 따른 교잡이 발생하였다. 화분 수용체로서 웅성가임(MF) 유채로의 유전자이동에 따른 교잡은 가장 가까운 2 m에서 2.33%가 발생하였으며, 75 m 이격거리에서 0.007%

가 발생하였으며, 웅성가입 갓으로는 가장 가까운 2 m에서 0.076%, 16 m에서는 0.025%가 발생하였으며 이 이상의 이격거리에서는 유전자이동이 없었다. 상관분석결과 벌의 방문수가 유전자이동 가능성과 가장 유의적인 상관관계를 보였다.

유전자변형 유채에서 근연종으로의 유전자 이동에 있어 매개충인 꿀벌과 바람의 영향을 비교하기 위해 웅성불임 근연종을 화분수용체로, 방충망 설치 유무를 달리하여 포장평가를 실시한 후 꿀벌에 의해 매개된 유전자이동성을 계산한 결과 꿀벌에 의해 웅성불임 유채로의 유전자이동성은 이격거리 2 m에서 19.01%, 이격거리 128 m에서 0.03%이었고, 웅성불임 갓으로는 2 m에서 15.36%, 128 m에서 0.06%이었다. 이는 꿀벌이 유전자이동성에서 중요한 부분을 차지하고 있음을 의미하며, 아울러 바람에 의한 유전자 이동도 매우 유의적으로 높음을 의미한다. 본 실험에서 얻은 유전자이동성과 이격거리간의 관계를 log-logistic 모델을 이용하여 설명할 수 있었으며, 이 모델을 이용하여 특정거리에서 유전자이동 가능성을 예측할 수 있었다.

이상의 결과는 한국의 환경조건에서 형질전환 유채로부터 근연종인 유채와 갓으로의 유전자 이동성을 정량적으로 제시하였으며 이를 통해 형질전환 유채에서 근연종으로의 유전자이동 위험성이 높음을 시사한다. 본 연구에서 제시된 모델은 향후 형질전환 유채로부터 근연종으로의 유전자이동의 위험성의 예측은 물론 위험관리를 위한 안전격리거리의 결정에도 활용될 것으로 기대된다.

주요어: 유채, 갓, 유전자 이동, 형질전환, 모델링, 수분, 무, 위험성평가

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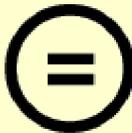
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A DISSERTATION FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

**Potential Gene Flow from Genetically
Modified Oilseed Rape (*Brassica
napus*) to Its Relatives**

BY

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AUGUST, 2015

MAJOR IN CROP SCIENCE AND BIOTECHNOLOGY

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GENERAL ABSTRACT

Potential Gene Flow from Genetically Modified Oilseed Rape (*Brassica napus*) to Its Relatives

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Both greenhouse and field studies to evaluate gene flow from GM *Brassica napus* to its relatives were conducted in the Experimental Farm Station of Seoul National University, Suwon, Korea. The greenhouse study was conducted for setting up basic experimental conditions and then field studies were conducted to quantify the potential gene flow from GM *Brassica napus* to its relatives under different pollination conditions. Simple sequence repeats (SSR) and herbicide resistance (glufosinate-ammonium) markers were used for confirming F1 hybrids resulted from outcrossing between GM *B. napus* and its relatives. The experimental data clearly demonstrated the possibility of gene flow from GM *B. napus* to its relatives such as *B. napus* and *B. juncea* in the field condition, but showed no gene flow to *Raphanus sativus*. Under simulated favorite pollination by synchronizing flowering time and placing honeybee hive, the potential gene flow from GM *B.*

napus to its relatives was evaluated. To MS relatives, it was estimated to be 32.48% and 21.95% to MS *B. napus* and MS *B. juncea*, respectively, at 2 m distance, and decreased with increasing distance, reaching 0.3% and 0.25%, respectively, at 128 m distance. In contrast, to male fertile (MF) relatives, the potential gene flow was estimate to be 2.33% and 0.076% to MF *B. napus* and MF *B. juncea*, respectively, at 2 m distance and decreased to 0.007% for MF *B. napus* at 75 m and 0.025% for MF *B. juncea* at 16 m distance. Therefore, the gene flow rates to MS relatives at 2 m distance were 16 times for *B. napus* and 288 times for *B. juncea* greater than the gene flow rate to FM relatives, suggesting big difference between the maximum potential gene flow and the practical gene flow.

The three-parameter log-logistic model well described gene flows against distance, indicating that the suitability of the equations for prediction of gene flow rate at a specified distance. Isolation distances between GM *B. napus* and its GM relatives were then recommended based on the model and the tolerable threshold (0.01%) made by EU. The isolation distance for less than 0.01% gene flow from GM *B. napus* was esimted to be 2710 m and 254 m for MS *B. napus* and *B. juncea*, respectively, and 122.5 m and 23.7 m for MF *B. napus* and *B. juncea*, respectively. Our studies would provide informative reference values and scientific basis for risk assessment of gene flow from GM *B. napus* to its relatives under Korean climatic condition. The modeled equation will also provide scientific evidence for the determination of isolation distance and the regulation of GM crops cultivation.

Keywords: *Brassica napus*, *Brassica juncea*, gene flow, genetically modified, modeling, pollination, *Raphanus sativus*, risk assessment

Student Number: 2012-30757

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GENERAL INTRODUCTION

Genetically modified (GM) crops are dominated in four crop species such as maize (*Zea mays*), soybean (*Glycine max*), cotton (*Gossypium hirsutum*), and oilseed rape (*Brassica napus*) with two traits, insect resistance and herbicide tolerance (James 2005). These crops have demonstrated great advantage in crop production as well as environmental benefits resulted from decreased pesticide use. However, with dramatic increase in GM crops cultivation, environmental impacts on natural and agricultural ecosystems have raised public concerns, particularly with emphasis on gene dispersal from GM crops to non-GM crops and relative species. One of the main concerns over agricultural releases of GM crops is the escape of the transgenes into the natural ecosystem through outcrossing with their wild or weedy relatives (Warwick et al. 2003; Snow 2002), which transfer the traits (herbicide, insect or drought tolerance) to relatives, resulting in increased fitness and consequential invasiveness of the relatives. *Brassica napus* is one of the most important oil crops in the world. It is currently the third most important oil plant after soybean and oil palm and its global production continues to increase. It is an allotetraploid (AACC, $2n=38$) and has many wild or weedy relatives such as Indian *B. juncea* (AABB, $2n=36$), *B. rapa*, (AA, $2n=20$), *B. niga* (BB, $2n=16$), *B. carinata* (BBCC, $2n=34$), *B. oleracea* (CC, $2n=18$), *Raphanus sativus* (RR, $2n=18$), *R. raphanistrum* (RrRr, $2n=18$), *Erucastrum gallicum* ($2n=30$), *Hirshfeldia incana* ($2n=18$), and *Sinapis arvensis* ($2n=18$) persisting in or near the areas of *B. napus* cultivation. This close species relationship between diploid and allotetraploid *Brassica* species contributes to the ease interspecies out-crossing occurred in certain cross combinations. In both natural and controlled conditions, many events of outcrossing among *Brassica* species have been reported (Bing et al. 1991; Halfhill et al. 2002; Hansen et al. 2001; Jenkins et al. 2001; Warwick et al. 2003; Wilkinson et al. 2003).

Several factors affect outcrossing among the *Brassica* spp., such as plant genotype, direction of cross, types and presence of pollinators, degree of synchrony of flowering period, local climatic conditions, etc. As *B. napus* is both entomophilous and anemophilous plant, such factors, in particular, pollinators and wind significantly affect outcrossing (Alford 1978; Williams et al. 1987). For an open pollinated male fertile *B. napus*, the seed production was resulted from three factors, self pollination, wind pollination by neighbouring plants, and insect pollination primarily by bees such as honeybee and bumblebee (Alford 1978). As bees directly take part in pollen transfer and pollination, factors affecting the honeybee's behavior such as the density of flowers, types of plants, distance from the beehive, wind direction, weather condition, etc., would indirectly influence the pollination. In addition, the degree of synchrony of flowering period between pollen donor and recipient is also a key factor for determining the outcrossing (Bing et al. 1996; Landbo et al. 1996). An increase of synchronized flowering period between them would potentially increase the outcrossing rate as there are more opportunities for recipient flowers to accept foreign pollens for outcrossing. In contrast, lack of synchrony of geographic distribution or in flowering periods, the outcrossing will drastically reduce or may not occur (Tsuda et al. 2012).

To date, the GM *B. napus* has commercially cultivated in USA, Canada, Australia, and Chile but not cultivated in European countries such as United Kingdom, Germany, and France, and Asian countries such as China, Korea, and Japan. Numerous studies have been conducted worldwide for accumulating knowledge and better understanding of gene flow. Based on findings from many studies, governmental regulations have implemented for GM *B. napus* cultivation such as isolation distance, crops rotation, buffer zone, etc. However, due to different cropping system between North America and Asian countries, the research interests in gene flow are different. In North America and Australia, mono cropping in large-scale farms is very common for *B. napus*, soybean, and maize. Large-scale mono cropping does not raise much concern on gene flow between GM crop and non-GM relatives. Surrounding natural ecosystem is much far away from the crop area, so

probability of gene flow from GM crop to its non-GM relatives in the crop area, and wild and weed relatives in the surrounding natural or agro-ecosystem is not much high. Thus, more studies were focused on the distance of pollen dispersal and gene flow occurrence. Previous studies reported that the outcrossing could occur at a considerable rate and distance from pollen source, ranging from 0.07-1.6% from 1.5-800 m (Beckie 2001; Staniland et al. 2000). In a large-scale field investigation in Australia, the pollen even can flow beyond 2000 m (Rieger et al. 2002). In addition, studies conducted in two different sites, California and Georgia in USA, showed difference in outcrossing rate at the same distance from the pollen donor, indicating that the gene flow is affected by the local climatic and eologic conditions (Morris et al. 1994).

Unlike the large-scale crop cultivation in North America and Australia, co-existence of *B. napus* with its relative species in diverse cropping systems in Asia is quite common, where farmers generally manage small-scale fields and rotate different crops by each year, posing a high risk of outcrossing between *B. napus* and its relatives such as *B. rapa*, *B. oleracea*, *B. niga*, or *R. sativus*, etc. Therefore, studies in this regard have been conducted in neighboring Asian countries under both artificial and field conditions. In China, the glyphosate resistant hybrids (*B. juncea* × *B. napus*) obtained by hand-crossing were assessed and showed the increased fitness (Huangfu et al. 2011; Song et al. 2010). A gene flow risk assessment conducted in a field condition of Japan showed the outcrossing (*B. juncea* × *B. napus*) could occur at 17 m with the rate of 0.03% (Tsuda et al. 2012), indicating considerably high outcrossing between *B. napus* and its relatives. Spilled GM *B. napus* seeds have been detected at major ports and along the motorway in Japan, some of them found with resistance to multiple herbicides (Aono et al. 2005). Korea imports annually around 8 million tons of GM crops every year for edible oil processing and animals feeding. Although the previous monitoring work did not detect any spilled GM seeds in Korea (Lee et al. 2007), unintentionally spilled GM seeds may occur. Countries growing GM crops have more experience of gene flow study and may accumulate many gene flow data

under different conditions. However, due to the difference in cropping system between North America and Asian countries, the experiences from North America and Australia, in some senses, are not much suitable for Asian countries, particularly those which grow diverse crops in the immediate vicinity each other in a small farm. Therefore, we should have our own case study for gene flow risk assessment in a specific local cropping system and climatic condition. In present studies, gene flow assessments were made under favorite conditions by providing abundant pollinator insects (honeybee) and long duration of synchrony of flowering period between pollen donor and recipient. We quantified gene flow in field conditions and modeled the maximum potential gene flow from *B. napus* to its relatives under different environmental (greenhouse and field) and pollination (with and without honeybee) conditions (open and wind).

GENERAL OBJECTIVES

The aims of this study were to evaluate and model the potential gene flow from genetically modified (GM) *Brassica napus* to its relatives under Korean climatic condition. The studies had the following specific objectives for each chapter:

Chapter I

To quantify the maximum potential gene flow from *B. napus* to its male sterile (MS) relatives both under greenhouse and field conditions.

Chapter II

To quantify the potential gene flow from GM *B. napus* to its male fertile (MF) relatives under Korean field condition and to determine a proper isolation distance between GM *B. napus* and its relatives.

Chapter III

To evaluate the maximum potential gene flow from GM *B. napus* to its male sterile (MS) relatives under open and wind pollination conditions and to develop gene flow simulation models for predicting gene flow from GM *B. napus* to its relatives.

LITERATURE REVIEW

Genetically modified crops and gene flow

Genetically modified organisms (GMOs), are plants created through the gene splicing techniques of biotechnology (also called genetic engineering, or GE).

Gene flow means the movement of genes from one individual plant to another individual within the same species or between different species or genus. In population genetics, gene flow (also known as gene migration) is the transfer alleles or genes from one population to another. Gene flow usually occurs via cross-pollination or movement of seeds.

Taxonomy of *Brassica* species

The taxonomy and genetics of the *Brassica* species are complex (Fig. 1). The *Brassica* genus includes crops and weed species. The U's Triangle (U, 1935) demonstrates how three of the *Brassica* species (denoted by the letters AABB, AACC, and BBCC) were derived from three ancestral genomes (AA, BB, or CC) (Fig. 1).

- *B. rapa* (AA, $2n=2x=20$; turnip, Chinese cabbage)
- *B. nigra* (BB, $2n=2x=16$; black mustard)
- *B. oleracea* (CC, $2n=2x=18$; cabbage, broccoli, Brussels sprouts, cauliflower)
- *B. juncea* (AABB, $2n=4x=36$; brown mustard)
- *B. napus* (AACC, $2n=4x=38$; oilseed rape, rutabaga)
- *B. carinata* (BBCC, $2n=4x=34$; Ethiopian mustard)

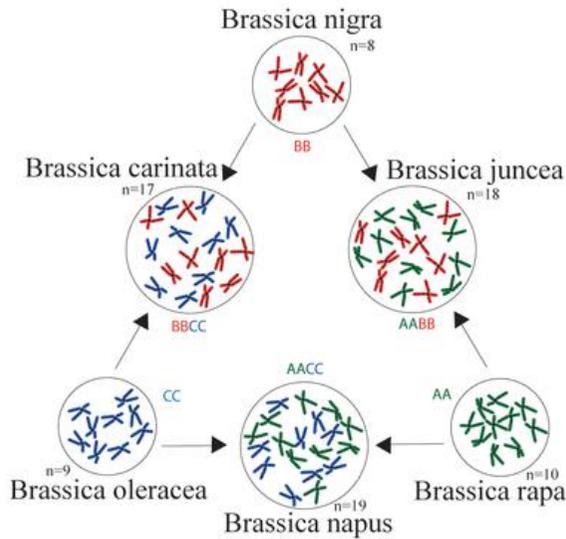


Fig. 1. Triangle of U (1935) showing the genetic relationships between the six species of the *Brassica* genus. Numbers indicate haploid chromosome set; letters designate genomes.

B. napus is an allotetraploid (AACC, $2n=38$) and has many wild or weedy relatives such as *B. juncea* (AABB, $2n=36$), *B. rapa* (AA, $2n=20$), *R. sativus* (RR, $2n=18$), *R. raphanistrum* (RrRr, $2n=18$), *Erucastrum gallicum* ($2n=30$), *Hirshfeldia incana* ($2n=18$), and *Sinapis arvensis* ($2n=18$) persisting in or near areas of cultivation (Table 1). This close species relationship between diploid and allotetraploid *Brassica* species contributes to the ease with which interspecies crossing occurs in certain cross combinations.

Table 1. Wild relatives and allies of *Brassica napus*

Species	Common name	Ploidy level	Genome	Life form and weediness
<i>B. rapa</i> (including Chinese cabbage, mizu-ma)	Field mustard, bird rape, turnip	Diploid (2n=2x=20)	A	Annual or biennial
<i>B. nigra</i>	Black mustard	Diploid (2n=2x=16)	B	Annual; weedy
<i>B. oleracea</i> (including broccoli, cauliflower, kale)	Cabbage (wild and cultivated)	Diploid (2n=2x=18)	C	Biennial or perennial
<i>B. juncea</i>	Brown mustard, indian mustard	Allotetraploid (2n=4x=36)	AB	Annual, derived from <i>B. nigra</i> x <i>B. rapa</i>
<i>B. napus</i> (including Siberian kale, swede rape)	<i>B. napus</i> , canola, rutabaga	Allotetraploid (2n=4x=38)	AC	Annual or biennial, derived from <i>B. rapa</i> x <i>B. oleracea</i>
<i>B. carinata</i>	Ethiopian mustard	(2n=4x=34)	BC	Annual; derived from <i>B. nigra</i> x <i>B. oleracea</i>
<i>Raphanus raphanistrum</i> L.	Wild <i>R. sativus</i>	Diploid (2n=18)	Rr	
<i>Raphanus sativus</i> L.	Garden <i>R. sativus</i>	Diploid (2n=18)	R	Annual or biennial; weedy
<i>Hirschfeldia incana</i>	Buchan weed, hoary mustard	Diploid (2n=14)	Ad	Annual or biennial; weedy
<i>Sinapis arvensis</i> L.	charlock	Diploid (2n=18)	Sar	Annual; crop weed

Outcrossing events between *B. napus* and its Relatives

Gene flow from *B. napus* to *Brassica* spp.

Outcrossing to B. rapa (field mustard)

B. napus (AACC, $2n=4x=38$) and *B. rapa* (AA, $2n=2x=20$) have a common set of chromosomes, which facilitates interspecific hybridization and gene flow between these two species. Numerous studies have indicated a high potential for hybridization between *B. napus* and *B. rapa*. Spontaneous hybridization and backcrossing between *B. napus* and *B. rapa* in the field has been reported in several countries, including Denmark (Landbo et al. 1996; Hansen et al. 2001), New Zealand (Jenkins et al. 2001), United States (Halfhill et al. 2002), Canada (Warwick et al. 2003; Yoshimura et al. 2006), and United Kingdom (Allainguillaume et al. 2006; Wilkinson et al. 2003). Frequency of hybrids depends on parental genotypes, direction of crosses, experimental design and population sizes (Bing et al. 1991; Jørgensen and Andersen, 1994; Jørgensen et al. 1998; Halfhill et al. 2002; Hansen et al. 2003; Hauser et al. 1997; Pallett et al. 2006; Scott and Wilkinson, 1998).

For instance, frequency of hybrids is highest where single *B. rapa* plants are present in *B. napus* fields (Jørgensen et al. 1996), as *B. rapa* is self-incompatible and an obligate outcrosser. Hansen et al. (2003) reported that the introgression process between *B. napus* and *B. rapa* primarily progressed with *B. rapa* as the maternal plant, and that both recombination between the A and C genomes and exchange of chloroplasts between *B. napus* and *B. rapa* appear to have occurred. An earlier study in UK, Scott and Wilkinson (1998) documented that *B. rapa* populations growing outside of *B. napus* fields showed low levels of hybrids (0.4-15.0%). However, Warwick et al. (2003) reported a higher naturally occurring herbicide (glyphosate) tolerance gene transfer rate (approximately 13.6%) from *B. napus* to *B. rapa* in the commercial fields in Canada. Similarly, in the United States, under a higher (600:1) and a lower (180:1) *B. napus*-*B. rapa* ratio, hybridization frequency from transgenic *B. napus* to *B. rapa* determined at approximately 10% and 2%, respectively (Halfhill et al. 2002).

The F1 hybrids, triploid (AAC, $2n=29$), crossed between *B. napus* and *B. rapa* are viable and present rather high levels of fertility, albeit with greatly varying amounts of seed production. Where natural interspecific hybrids have occurred,

they have reduced fertility and seed set compared with the parents (Jørgensen and Andersen, 1994). On average, less than 2% of all hybrid seedlings survived (Scott and Wilkinson, 1998). This low hybrid survival rate reduces the rate at which introgression of transgenes into *B. rapa* can occur (Jørgensen et al. 1996; Sweet et al. 1999). When interspecific hybrids are present, spontaneous backcrossing takes place at very low frequency (Hauser et al. 1998). Hauser et al. (2001) reported that hybrids and backcross offspring were produced mainly by a few of the *B. rapa* female plants, indicating that the degree of hybridization and backcrossing depended on the presence of certain *B. rapa* genotypes.

Norris and Sweet (2001) noted that while *B. rapa* seed banks showed evidence of introgression, there was no evidence of introgression in samples taken from mature *B. rapa* plants in the field, indicating that there may be selection pressure against backcrossed individuals. The gene flow rate to *B. rapa* via introgression will depend greatly on the selection pressure exerted on the herbicide tolerant gene (Scott and Wilkinson 1998; Sweet et al. 1999; Snow and Jørgensen, 1999).

Introgression into *B. rapa* is more likely to occur if the transgene in *B. napus* is located on the A genome which is common to both species. Hansen et al. (2003) further concluded that transgenes could be transferred at a high outcrossing rate from *B. napus* to *B. rapa*, regardless of transgene position in the nuclear or plastid DNA. There is no evidence that the presence of an introgressed herbicide tolerant gene in *B. rapa* has increased its fitness (Snow and Jørgensen, 1999; Sweet et al. 1999), indicating that *B. rapa* resulting from the introgression of the gene can be controlled by alternative herbicides.

Outcrossing to B. juncea (Indian mustard)

B. juncea is widely cultivated and naturalized worldwide, especially for oil production, and frequently found as a weed or a ruderal component of roadsides and waste places (Liu et al. 2010; Song et al. 2010). *B. juncea* (AABB, 2n=36) share a common set of chromosomes with *B. napus*, enhancing the probability of interspecific hybridization and gene flow. Researches on hybridization and gene

flow between *B. napus* and *B. juncea* are less compared to *B. napus/B. rapa* case. However, successful hybrids in controlled crosses and spontaneous occurrence of interspecific hybrids in the field have been reported in Canada, Denmark and China (Bing et al. 1991, 1996; Frello et al. 1995; Jørgensen et al. 1998; Di et al. 2009; Liu et al. 2010). The relatively high compatibility between *B. napus* and wild *B. juncea* raises the biosafety concern of transgene introgression.

Depending on the proportions of *B. napus* and *B. juncea* plants, up to 3% of hybrids from *B. juncea* have been found, although less hybrids are formed with *B. napus* as the female (Bing et al. 1991; Jørgensen et al. 1996, 1998). In field co-cultivation experiments with *B. napus* and *B. juncea*, Bing et al. (1996) found five interspecific hybrids out of 469 plants when *B. napus* was the female and three out of 990 plants when *B. juncea* was the female. Interspecific hybrids have reduced fertility and low seed set compared with the parents. However, the hybrids were able to survive in the field and produce a small amount of seeds. Introgression of herbicide tolerant genes from GM *B. napus* to *B. juncea* observed both in controlled crosses and in open field (Di et al. 2009; Liu et al. 2010; Song et al. 2010; Warwick 2007). Warwick (2007) reported on field experiments where gene flow from herbicide resistant *B. napus* to neighbouring fields of *B. juncea* was measured and found to be 0.245% at the field border and 0.005% at 200 m.

Based on fitness of the hybrids and backcrosses by hand pollination, Song et al. (2010) found that glyphosate- or glufosinate-tolerance genes of GM *B. napus* could be transmitted to wild *B. juncea* when wild *B. juncea* was used as the maternal or paternal plants, and the possibility for transmission may be greater with glyphosate-tolerant than glufosinate-tolerant *B. napus* to wild *B. juncea*. Similarly, Hangfu et al. (2011) detected a higher transfer ratio of glyphosate- tolerant *B. napus* specific *loci* and the variation of populations in fitness-related parameters in F₁ hybrids. Fitness test from Di et al. (2009) showed that the F₁ hybrids formed between transgenic (GT, linked GFP and Bt *CryIAc* cassettes) *B. napus* and wild *B. juncea* revealed maternal effects, high vegetative fitness and seed dormancy compared with the parents, indicating that increased the probability of the survival of hybrids after

the occurrence of gene flow. Recently, Liu et al. (2010) reported that herbicide (chlorsulfuron, ALS inhibitor) resistant BC₁ were not different of their susceptible counterparts for plant weight, seed weight and seed number, but most of them exhibited *B. napus* morphology and larger flowers than the susceptible BC₁, which displayed additional genetic variability that allowed for further adaptation of the plants and propagated the herbicide-resistance gene. Therefore, current evidences that the introgressed herbicide tolerant gene from *B. napus* to *B. juncea* could certainly enhanced weediness of *B. juncea* in arable fields or spread as a weed relative to conventional, non-GM *B. juncea*.

Gene flow from *B. napus* to *B. vegetables*

Gene flow from *B. napus* to *B. napus* vegetables (CC, 2n=18, e.g. rutabaga, Siberian kale, swedes) and to *B. rapa* vegetables (e.g. Chinese cabbage, Pak choi, turnip) is theoretically possible, due to sharing a common set of chromosomes. However, *B. napus* and *B. rapa* vegetables are generally harvested prior to flowering and seed development, unless being used as a seed production. So a lack of physical proximity and synchrony of flowering means the probability of outcrossing occurrence with *B. napus* is extremely unlikely. Lately, Ford et al. (2006) reported the first spontaneous gene flow from *B. napus* to wild *B. oleracea* in the UK, and used flow cytometry and crop-specific microsatellite markers to identify one triploid F₁ hybrid, together with nine diploid and two near triploid introgressants.

Gene flow from *B. napus* to wild or weedy species

*Outcrossing to *R. raphanistrum* (wild radish)*

Spontaneous gene flow between *B. napus* and *R. raphanistrum* (RrRr, 2n=18) in the field has been reported in Australia, Canada, Denmark, and France at very low frequencies (Ammitzboll and Jørgensen 2006; Baranger et al. 1995; Chevre et al. 2000, 2003; Darmency et al. 1995, 1998; Rieger et al. 2001; Warwick et al. 2003).

The frequency of outcrossing varies, depending on several factors, among them the *B. napus* variety (male-sterile or fertile) and genotype used, local environmental conditions, the ratio of parents, and the breeding system (Baranger et al. 1995; Chevre et al. 2003; Ammitzboll and Jørgensen 2006). Hybridization is more likely to occur between *B. napus* and *R. raphanistrum* when *B. napus* is the maternal parent (Rieger et al. 1999). No hybrids were detected among 25,000 seedlings grown from seed collected from imidazolinone-resistant *R. raphanistrum* plant (Rieger et al. 2001). However, two hybrids were obtained from more than 52-million *B. napus*, and both hybrids were characterized as amphidiploids (AACCRrRr, 2n=56) and fertile. The frequency of hybridization into *B. napus* using male fertile *B. napus* was 4×10^{-8} . Chevre et al. (2000) reported that only one herbicide-tolerant hybrid was characterized among the 189,084 seedlings between *B. napus* and *R. raphanistrum* under normal agronomic conditions, and the frequency of hybridization was assessed to range from 10^{-7} to 10^{-5} . In a Canada study, no hybrids were detected in the greenhouse experiments (1,534 seedlings), the GFP (green fluorescence protein marker) experiment (4,059 seedlings) or in commercial fields (22,114 seedlings) in Quebec and Alberta. But a single herbicide (glyphosate) resistant *R. raphanistrum* × *B. napus* F₁ hybrid was detected in 32,821 seedlings from the herbicide resistant *B. napus* field experiment. The hybrid had a genomic structure consistent with the fusion of an unreduced gamete of *R. raphanistrum* and a reduced gamete of *B. napus* (RrRrAC, 2n=56), both *R. raphanistrum* and *B. napus*-specific AFLP markers, and had <1% pollen viability (Warwick et al. 2003). In USA, Halfhill et al. (2002) reported that no hybrids were detected among the 19,274 seedlings between the GFP (green fluorescence protein) *B. napus* and *R. raphanistrum* at a high crop-weed ratio (600:1) under field conditions. In Denmark, Ammitzboll and Jørgensen (2006) analyzed spontaneous hybridization between a transgenic male sterile line of *B. napus* and, as pollen donors, three European populations of *R. raphanistrum*. The authors reported that the frequency of confirmed hybrids differed significantly among populations of *R. raphanistrum*. In the cross with a French population, all offspring were hybrids; in

the cross with a Swiss population, 53% of the offspring were hybrids; and in the cross with a Danish population, only 2% of the offspring were found to be hybrids.

Although pollen germination and ovule fertilization vary considerably within populations, post-zygotic barriers to hybridization are generally minor. F₁ hybrids are mainly allotriploids and show very low (<2 seeds/plant) fertility (Baranger et al. 1995; Darmency et al. 1998). F₁ hybrids commonly show decreased fitness in terms of reduced seedling emergence, a significant emergence delay, and a lower survival rate than both parents. Despite this, a small number of highly fertile F₁ hybrids have been reported by Salisbury (2002).

Outcrossing to R. sativas (radish)

The first report on spontaneous hybridization between a transgenic male sterile *B. napus* line and a variety of cultivated *R. sativus* was reported by Ammitzbohl and Jørgensen (2006). All offspring were found to be hybrids with low pollen fertility (0-15%).

Outcrossing to Sinapis arvensis (charlock) (SarSar, 2n=18)

A recent study on outcrossing between *B. napus* and another problematic weed, charlock, found very low rates of hybrid formation from both hand pollination experiments and field trials. In most of cases, charlock was served as the maternal plants. Moyes et al. (2002) got sexual hybrids when charlock was the maternal parent under greenhouse condition but failed to detect a hybrid crossed between charlock and *B. napus* plants in a field experiment.

The reciprocal cross with charlock as female parent gave only one hybrid out of 1127 hand pollinations (a rate of 0.0015% of the potential seed set). In a field experiment under natural condition in France, male-sterile *B. napus* has been shown to produce up to 0.18 hybrid seeds per plant (Chèvre et al. 1996). Artificial hybridizations using ovary culture and embryo rescue produce up to 1 seed per 100 pollinated flowers, but the F₁ plants were weak and highly or completely sterile compared to its parental plants (Bing et al. 1996). F₂ or backcross seed production

is extremely rare, and no gene introgression has been detected thus far (Bing et al. 1996; Rieger et al. 1999; Warwick et al. 2003).

Factors affecting gene flow

Genotype and zygotic barriers

The genotypes of the pollen donor and recipient are one of the important factors affecting the outcrossing rates. The use of different herbicide resistant plant varieties, such as with glyphosate and with glufosinate resistance as a transgenic marker system or other conventional herbicide resistant varieties, demonstrates that different marker systems have different genetic backgrounds, which can influence the flowering time, the pollen quantity, longevity and selfing rate (Hüsken and Dietz-Pfeilstetter 2007). The differences in reciprocal outcrossing rates using different transgenic varieties in controlled crosses or in open field indicate that the different transgenic varieties show the different transgene introgression levels (Rieger et al. 2002; Simpson et al. 1999; Song et al. 2010) under the same experimental and environmental condition. Furthermore, different types of transgenic HR can show different patterns of inheritance. In the case of homozygous glyphosate resistant and glufosinate resistant lines all of the pollen carries the HR gene. However, the hybrids derives from hemizygous HR parents show a reduced HR rates (Beckie et al. 2003). For instance, the hemizygous female and the homozygous male parent of the glufosinate resistant hybrid *B. napus* system contain the *bar* HR gene at two different loci. As a result, the hybrid produces transgenic and non-transgenic pollen in a ratio of 5:3, leading to a reduced amount of transgenic pollen as compared to homozygous herbicide resistant lines. Gene dosage effects have been demonstrated in several cases by comparing hemizygous and homozygous transgenic plants, with homozygous having higher transgene expression levels (Richards et al. 2003; Tang et al. 2003).

In plants, cross-incompatibility after pollination is caused by two types of fertilization barriers (Hadley and Openshaw 1980; MacNair 1989), pre- and post-

zygosity. Pre-zygosity occurs before fertilization and results mainly from pollen-pistil interactions, whereas post-zygosity happens when the development of young zygotes is arrested.

Direction of the cross

Hybridization is more likely to occur in one direction rather than the other. In general, when MS crop varieties are used, hybrids from crosses in which the crop plant is the female are again much more likely to occur than the reverse. This has been observed, for example, in *B. napus* (Darmency et al. 1998; Chèvre et al. 2000). Hybrids resulting from crosses of MS *B. napus* with its wild relatives (e.g., *R. raphanistrum*) are usually partially sterile. However, hybrid fertility can be restored by backcrossing with the wild relative, which would then result in GM traits moving towards wild relatives that act as the female plant. This example illustrates the fact that the direction of hybridization does not necessarily allow for conclusions about the possible containment of GM traits, as backcrossing can reverse this effect within only a few generation (Andersson and de Vicente 2010).

Shape, orientation, and size of pollen source and recipient field

Isolation distance is one means to ensure seed purity. Some outcrossing studies investigated the effectiveness of zones for reducing gene flow compared to the use of non-transgenic buffer areas. When crops are isolated by open ground or low growing crops, it appears that the first rows of the recipient field intercept a high proportion of foreign pollen due to the low convarietal pollen load of the field margin.

Pollinators

The concentration of pollen collected decreased rapidly with distance from the pollen source. McCartney and Lacey (1991) reported similar results and concluded that windborne pollen was unlikely to play a significant role in long distance cross-pollination of *B. napus*. Insects, especially honeybees (*Apis mellifera* L.) and

bumblebees (*Bombus terrestris* L.), collect nectar and pollen from *B. napus* plants, and pollen is transferred as the bees move from plant to plant. As honeybees can travel one to two kilometers from the hive, it is possible that pollen could be transferred far beyond the borders of an experimental plot. However, studies indicate that honeybees tend to visit plants that are located in a small area as near to the hive as possible, and often forage only on plant species per foraging trip, although there are exceptions (Gary 1975; Martin and McGregor 1973; Ribbands 1953). Bumblebees exhibit similar behavior, but are more likely to visit more than one plant species during a foraging trip (Alford 1978).

Wind

The extent of pollen flow in *B. napus* is strongly relied on local environment and climatic conditions (e.g. wind speed and direction) as well as its pollen characteristics. *B. napus* pollen is relatively large (32-33 μm), heavy and sticky, with viability estimates ranging from 1 to 5 days under natural conditions (Treu and Emberlin 2000). Mesquida et al. (1982) detected the majority *B. napus* pollen at 32 m from pollen source, but noted that the concentration of pollen collected decreased rapidly with the distance from the pollen source. Some studies have indicated that viable pollen can be found 1.5 km from the pollen source (Timmons et al. 1995). In discontinuous pollen-dispersal experiments, outcrossing rate was estimated to be 0.0156 % and 0.0038 % at 200 m and 400 m, respectively (Scheffler et al. 1995), whereas in a continuous pollen dispersal experiment, the outcrossing rate decreased dramatically to 0.02 % at 12 m and was only 0.00033 % at 47 m from the central pollen source (Scheffler et al. 1993).

Methods for detecting gene flow

The detection for hybrids usually was advocated (Poppy and Wilkinson 2005) a two-stage process: 1) the preliminary screens for putative hybrid plants identification; and 2) the confirmation assay for the putative hybrids confirmed as using a more accurate assay.

Preliminary screens (biology-based methods) for hybrids

Morphology

Morphological character analysis, plant phenotypes including seed color, weight, size, is an obvious possibility for detection of interspecific hybrids, provided that morphological differences distinguish the parental species and the hybrids share some of the characteristics of both species. However, in some cases, hybrids may appear indistinguishable from one parent or exhibit widely variable phenotypes that range between those of its parents. For instance, in controlled crosses between *B. tournefortii* and *B. rapa*, the F₁ hybrids were intermediate to their parents for most the morphological traits but a few characters were inherited selectively from the maternal or the paternal parent (Choudhary and Joshi 2001). This skewed distribution of phenotypic traits has also been observed following hybridization in other genera, for example in interspecific hybrids of *Cucumis* (Chen et al. 2004).

When using phenotype as a preliminary screen, ideally plants should be grown as a cohort and examined regularly. A small number of qualitative features are generally preferable to the more time-consuming process of collecting several complex measurements. In this way large numbers of plants can be examined in a relatively short time frame. For example, Scott and Wilkinson (1998) used leaf pruinosity colour and hairness to screen through 13, 000 seedlings collected from *B. rapa* plants growing next to fields of *B. napus*, they found just 46 hybrids.

Therefore, morphological identification of hybrids should not be used in isolation but should be combined with other methods (related methods reviewed below) for hybrid identification as there are often few morphological characters differing between taxa, and the genetic background of these characters is usually complicated, unknown and modulated by the environment (Rieseberg and Wendel 1993).

Sterility

Hybrids may show a decreased fertility compared to their parental genotypes. Seed production or seed viability can be a good indicator of hybridity as seed production per flower is often reduced. Reduced pollen fertility is of less value, largely because of the need for microscopic examination, although it can be a useful additional indicator for plants showing other signs of hybrid status (reduced seed set, intermediate morphology). Pollen viability can be evaluated by different viability stains or more simply by observations on pollen size and shape to identify the frequency of misshapen grains. Methods for estimating pollen fertility are described in most textbooks on staining procedures in biology. Jørgensen and Andersen (1994), Hauser et al. (2003) reported on male and female fertility in hybrids between *B. napus* and *B. rapa*.

Herbicide bioassay

The most widely grown GM herbicide tolerant plants are tolerant to the herbicides glyphosate (EPSPS inhibitor) or glufosinate (GS inhibitor) but imidazolinone (ALS inhibitor) as well other mode of action herbicide tolerant GM plants have also been developed. Successful hybrids between GM and non-GM plant would exhibit specific herbicide resistance attributing to introgression novel trait. Herbicide bioassay has been widely used for preliminary detection of hybrids, which can be carried out large amount of selection of introgression hybrids under glasshouse and field conditions. This method involves testing germination of seeds on herbicide- incorporating medium or filter paper, or spraying the herbicide on seedlings and assessing herbicidal efficacy by visual assessments of mortality or plant vigor. Seeds or seedlings that test positive for the presence of the herbicide GM tolerant trait will germinate and develop normally, whereas those that growth depress or do not develop normally will be non-GM. However, In order to exclude seeds or seedlings which had survived due to not enough absorption or contact of herbicide, a further herbicide application and followed analysis are still needed to be performed with confirmation of particular trait in selected survival individuals.

Mikkelsen et al. (1996) detected spontaneous transfer of a herbicide tolerance gene from *B. napus* to *B. rapa* by Basta spraying, and Hall et al. (2000) showed transfer of multiple herbicide resistance genes to *B. napus* volunteers in spray tests. In *B. napus*, Rieger et al. (2002) showed the intraspecific dispersal at the landscape level of a gene-encoding tolerance to an ALS inhibitor spraying by the offspring; the herbicide-tolerant *B. napus* was produced by traditional breeding and not through genetic modification. Pfeilstetter et al. (2000) compared different screening tests using the Basta spray test, the drop test, ELISA (enzyme-linked immunosorbent assay) screening and PCR amplification, and found good correspondence between the results from the different types of tests.

Confirmation assays for hybrids

Protein-based methods

When dealing with gene flow from transgenic plants, the transgene product can be identified in the recipient. The methods can be based on antibodies that are specific against the new proteins that are produced in the plants. Presently, commercial methods are available for *Bt* toxin and for herbicide tolerance. These lateral flow strip tests are cheap and can be used on site for detection but not quantification. However, as a result of operator performance, false negative seem to be frequent (Fagan 2004). The ELISA is more sensitive and can in principle be used for quantification. Stave (1999) described the quantitative ELISA detection of Roundup Ready® soybean. Nevertheless, these tests are useful tools for preliminary hybrid screens.

DNA -based methods

Random amplified polymorphic DNA (RAPD)

RAPD method was the first PCR-based approach to be widely used for hybrid detection. The method used arbitrarily selected primer sequences to generate

multiple products by PCR. RAPD analysis is fast, cheap and usually produces sufficient polymorphisms to allow for the identification of most hybrid types. However, the amplicons in the multiple band profiles are anonymous and usually inherited in a dominant fashion, which can limit its usefulness for hybrid verification. Nevertheless, this technique has proved useful for hybrid confirmation in the past. For instance, Jørgensen and Andersen (1994) revealed spontaneous hybridization between *B. napus* and *B. rapa* using RAPD markers, and Isoda et al. (2000) detected spontaneous interspecific hybridization in *Abies* by way of RAPD markers.

Amplified fragment length polymorphism (AFLP)

AFLP is a powerful PCR-based DNA fingerprinting technique for hybrid detection. It is based on selective amplification of a subset of restriction fragments from a digest of DNA, with subsequent visualization of the PCR products on a gel. Usually the method generated very complex amplicon profiles comprising 50-100 products. As with RAPD analysis, the markers in these complex profiles are usually inherited in a dominant fashion. However, their complexity and reliability offers huge advantages for confirming the identity of hybrids and introgressants, potentially allowing designation of cultivar of origin and semi-quantification of the extent of introgression. There are several studies where AFLP method has been used for detecting hybrids between cultivated crops and wild species. For instance, Hansen et al. (2001 and 2003) used AFLP to confirm interspecific hybrids and introgressed progeny arising from gene flow between *B. napus* and *B. rapa*.

Simple sequence repeats (SSRs)

Simple sequence repeats (SSRs) are stretches of DNA that consist of tandem arrays of 2-8 base motifs. SSRs are abundant in all investigated eukaryotic genomes, and lead to a high level of intraspecific polymorphism for the number of repeat motifs within an array. Length variability within an SSR can be visualized by PCR analysis using primers that are specific to the flanking sequences of the SSR-

locus, followed by high-resolution electrophoresis. However, the main disadvantages are that that sequence information is required to design the flanking species or species complex, which involves cloning and sequencing. It is usually desirable to use several SSR loci to confirm hybrid identify, and the cost and time of analysis can be reduced if several SSR analyses are performed together. This is known as multiplexing and has been used widely, for instance in the study of *B. napus* (Tommasini et al. 2003) and bean (Masi et al. 2003).

Real-time PCR

Real-time PCR is a very sensitive quantification of a specific sequence, which has proved very useful, powerful and widely been used for identification of interspecific hybrids from GM crops to wild or weedy species. This method is based on the quantification of fluorescent reporter molecular that increase in proportion to the amount of PCR product in the reaction.

Case studies of GM *B. napus* in other countries

Research in GM *B. napus* cultivation countries

GM *B. napus* has commercially cultivated in USA, Canada, and Australia (Table 2). Many studies on gene flow from GM *B. napus* to conventional *B. napus* have been carried out under field condition. Outcrossing rate was found to dramatically decrease when increase the distance from pollen donor. A Canadian Study (Staniland et al. 2000) revealed that outcrossing rate was 1.6% at 1.5 m from the pollen donor and sharply decreased to 0.03% when the distance increased to 31.25 m. Even the distance increases to 800 m, the outcrossing also can occur, the rate approximately 0.07% (Beckie 2001). Rieger et al. (2002) showed the pollen flow can move beyond 2000 m with large field sizes in Australia, and the tendency of pollen movement did not obey a leptokurtic or exponential decline. Moreover, studies conducted in two different sites, California and Georgia in USA (Morris et al. 1994), indicated that the outcrossing rate is variable even at the same distance

from the pollen donor. In California, the outcrossing rate was 2.0% when close to pollen donor and 0.4% at the 4.6 m from pollen donor. In Georgia, the outcrossing rate was 3.5% and 0.7% at the same distance compared with the California study. These studies suggest that the outcrossing rate is importantly affected by the local climatic condition.

Due to large acreage of cultivation *B. napus*, there is less worry on gene flow from GM-*B. napus* to its relatives. However, several studies have been conducted on this concern. Warwick et al. (2003) assessed the probability of gene flow from transgenic *B. napus* to its four wild relatives: *B. rapa*, *R. raphanistrum*, *S. arvensis*, and *E. gallicum* (Willd.) O.E. Schulz in greenhouse and/or field experiments in Canada, and found the probability of hybridization between *B. napus* and *B. rapa* was considerably high (approximately 13.6%), and the probability of hybridization with *R. raphanistrum* ($< 2.5 \times 10^{-5}$), *S. arvensis* L. (no hybrids detected), or *E. gallicum* (Willd.) O.E. Schulz (no hybrids detected) is very low. The similar hybridization experiment between *B. napus* and *R. raphanistrum* was also conducted reciprocally in the Australian field (Rieger et al. 2001). They found that no hybrids were detected amongst seedlings grown from seed collected from *R. raphanistrum* plants, but the amphidiploids (AACCRrRr, $2n=56$) and fertile hybrids were obtained with the hybridization frequency of 4×10^{-8} when *B. napus* served as maternal plants, indicating that the probability of hybridization with *R. raphanistrum* is very low.

Research in Non-GM *B. napus* cultivation countries

Up to now, GM *B. napus* is not grown commercially in western European countries such as UK, Germany, and France, Asian countries such as China, Korea, Japan. However, researches on assessing GM *B. napus* gene flow have been carried out for a long time (Table 2). Especially in UK, so many studies have been conducted over the distance dispersal of pollen, the outcrossing rate between *B. napus*, and gene flow from *B. napus* to its relatives. Paul et al. (1995) showed that in the mixed population the outcrossing rate was ranged from 3% to 12%. Another study conducted by Simpson (2000) showed that the outcrossing rate was 1.0% at

1.5 m and 0.05% at 91.5 m from the pollen donor. When the distance increased beyond 400 m, the outcrossing also can occur (Ramsay et al. 2003). The two studies conducted by Ramsay (2003) indicated that the environmental effect on outcrossing rate. In Germany, Krato and Petersen (2012) assessed the outcrossing rate was 2.1% at the 2 m and 0.05% when the distance increases to 45 m from the pollen donor under field condition. In China, Cai et al. (2008) reported that the outcrossing rate was found 1.2% at the distance of 1.4 m from the pollen donor and increased to 0.06% when the distance beyond 2000 m. In France, several studies also assessed the outcrossing rate under France climatic condition. Champolivier et al. (1999) reported that the outcrossing rate was 4.0% in the mixed population and decreased to 0.6% at 30 m. The outcrossing also can happen even the distance increasing to 800 m (Darmency and Renard 1992). Thus, when comparing all of the outcrossing cases studies on *B. napus*, we can find that the specific local region study is necessary due to the different cultivation environmental condition in different countries.

It is unlike the large-scale farm production and comparatively mono cropping system in North America and Australia. In European and Asian countries, co-existence of *B. napus* with its relative species such as Indian mustard, field mustard, and other weeds species is very common, where farmers generally have small land and cultivate different crops by each year, thus having a high risk of hybridization risk between transgenic varieties and wild or weedy relatives. Therefore, many studies in this regard were conducted in these countries. In France, Denmark, and UK, hybridization experiments between *B. napus* and *B. rapa*, *B. oleracea*, *R. raphanistrum*, or *R. sativus* in controlled greenhouse or field condition have been tried because of the extensively distribution of wild species, and the hybrids were obtained and confirmed. In China, the herbicide resistant hybrids (*B. juncea* × *B. napus*) obtained by mean of artificial crossing (hand pollination) were assessed and proven the increased fitness (Huangfu et al. 2011; Song et al. 2010). A designed field transgene flow experiment from *B. napus* to *B. juncea* showed that the outcrossing even occurred beyond 17 m with the outcrossing rate of 0.03% (Tsuda

et al. 2012). All of the results from there suggests that the considerable higher possibility of hybridization between *B. napus* and its relatives. Therefore, in case the GM *B. napus* is released in those countries, more attentions must be focusing on that topic. At the same time, gene flow studies from GM *B. napus* to its relatives should be continued to carry out in related countries. In Japan and Korea, where the GM *B. napus* is not cultivated commercially, however, several GM *B. napus* plants have been detected at major ports and along roadsides, some of them with resistance to multiple herbicides (Aono et al. 2005). From this point, the monitoring work must be done before a specific transgene is being planted in a country.

Table 2. Summaries of some representative studies on gene flow of *B. napus* in GM and non-GM *B. napus* cultivation countries

Pollen donor	Transgene trait	Recipient	Pollen dispersal distance (m)	% outcrossing (max value)	Detection method	Country	References
GM <i>B. napus</i> cultivation countries studies on gene flow							
<i>B. napus</i>	erucic acid	<i>B. napus</i>	0	21.8	erucic acid content	Canada	Rakow & Woods 1987
<i>B. napus</i>	bromoxynil resistance	<i>B. napus</i>	0.4, 0.8, 1.2	9.5, 5.6, 3.9	herbicide tolerance	Canada	Cuthbert & McVetty 2001
<i>B. napus</i>	bromoxynil resistance	<i>B. napus</i>	1.5, 4, 11.5, 21.5, 31.5	1.6, 0.7, 0.3, 0.2, 0.03	herbicide tolerance	Canada	Staniland et al. 2000
<i>B. napus</i>	glyphosate resistance	<i>B. napus</i>	20, 50, 100	1.5, 0.4, 0.4	herbicide tolerance	Canada	Downey 1999
<i>B. napus</i>	glyphosate resistance	<i>B. napus</i>	47, 137, 366	2.1, 1.1, 0.6	herbicide tolerance	Canada	Stringam & Downey 1982
<i>B. napus</i>	glyphosate resistance	<i>B. napus</i>	0	7.0	herbicide tolerance	Canada	Warwick et al. 2003
<i>B. napus</i>	glyphosate resistance	<i>B. napus</i>	0, 50, 100, 200, 800	1.4, 0.2, 0.15, 0.2, 0.07	herbicide tolerance / PCR	Canada	Beckie et al. 2003
<i>B. napus</i>	kanamycin resistance	<i>B. napus</i>	0, 0.3, 0.6, 3, 4.6	2.0, 1.0, 0.8, 0.8, 0.4	kanamycin analog G418 tolerance	USA (California)	Morris et al. 1994
<i>B. napus</i>	kanamycin resistance	<i>B. napus</i>	0, 0.3, 0.6, 3, 4.6	3.5, 1.5, 1.2, 0.7, 0.7	kanamycin analog G418 tolerance	USA (Georgia)	Morris et al. 1994
<i>B. napus</i>	glyphosate resistance	<i>B. napus</i>	0, 7.5	6.3, 0.5	herbicide tolerance	USA	Brown et al. 1996

<i>B. napus</i>	imidazolinone resistance	<i>B. napus</i>	500, 1000, 2000, 5000	0.15, 0.1, 0.2, 0	herbicide tolerance	Australia	Rieger et al. 2002
Non-GM <i>B. napus</i> cultivation countries studies on gene flow							
<i>B. napus</i>	-	<i>B. napus</i>	1, 16, 32	0.1, 0.001, 0.001	-	Hungary	Pauk et al. 1995
<i>B. napus</i>	-	<i>B. napus</i>	100, 150	0.05-0.07, 0	-	Germany	Gotz and Ammer 2000
<i>B. napus</i>	imidazolinone resistance	<i>B. napus</i>	2, 45	1.3, 0.05	herbicide tolerance/ PCR	German	Krato and Petersen 2012
<i>B. napus</i>	glufosinate resistance	<i>B. napus</i>	1.4, 4.5, 11.5, 200	1.19, 0.11, 0.04, 0.006	herbicide tolerance/ PCR	China	Cai et al. 2008
<i>B. napus</i>	glyphosate resistance	<i>B. napus</i>	1, 5, 10, 50	0.16, 0.059, 0, 0	herbicide tolerance/ PCR	China	Di et al. 2009
<i>B. napus</i>	glufosinate resistance	<i>B. napus</i>	0.5, 5, 10, 50	2.50, 0.34, 0.20, 0.004	herbicide tolerance/ PCR	China	Zhao et al. 2013
<i>B. napus</i>	asulam resistance	<i>B. napus</i>	1.4, 4.5, 11.5, 2000	1.19, 0.11, 0.04, 0.006	herbicide tolerance/PCR	UK	Paul et al. 1995
<i>B. napus</i>	-	<i>B. napus</i>	0 (mixed plants)	3-12	-	UK	Sweet et al. 1999
<i>B. napus</i>	-	<i>B. napus</i>	4, 8, 20, 34, 56	2, 0.33, 0.16, 0.16, 0.11	-	UK	Simpson et al. 1999
<i>B. napus</i>	-	<i>B. napus</i>	6, 30, 42, 50	0.05, 0.05, 0.33, 0.16	-	UK	Simpson et al. 1999
<i>B. napus</i>	glufosinate resistance	<i>B. napus</i>	1, 3, 12, 47	1.5, 0.4, 0.02, 0.00033	herbicide tolerance	UK	Scheffler et al. 1993
<i>B. napus</i>	-	<i>B. napus</i>	1.5, 11.5, 26.5, 51.5, 91.5	1.0, 0.5, 0.15, 0.1, 0.05	-	UK	Manasse & Kareiva 1991

<i>B. napus</i>	glufosinate resistance	<i>B. napus</i>	2, 50, 150	0.76, 0.04, 0.02	herbicide tolerance	UK	Weekes et al. 2005
<i>B. napus</i>	-	<i>B. napus</i>	2, 50, 100	*0.5, 0.02, 0.01	-	UK	Norris 2001
<i>B. napus</i>	-	<i>B. napus</i>	5, 25, 40, 50, 100, 200	1.2, 0.25, 0.65, 0.1, 0.5, 0.2	-	UK	Norris 2002
<i>B. napus</i>	glufosinate resistance	<i>B. napus</i>	5, 25, 50, 100, 250	3.3, 0.7, 0.4, 0.25, <0.1	herbicide tolerance	UK	Scheffler et al. 1995
<i>B. napus</i>	kanamycin resistance	<i>B. napus</i>	200, 400	0.016, 0.004	PCR	UK	Ramsay et al. 2003
<i>B. napus</i>	kanamycin resistance	<i>B. napus</i>	0, 10, 50, 225, 550, 800	*0.12, 0.04, 0.02, 0.02, 0.001, 0.03	PCR	UK	Ramsay et al. 2003
<i>B. napus</i> ♀	Seed set on male-sterile bait plants	<i>B. napus</i> ♀	0, 10, 50, 225, 550, 800	*0.58, 0.31, 0.33, 0.21, 0.1, 0.02	No. of seed sets	UK	Simpson et al. 1999
<i>B. napus</i> ♀	Seed set on male-sterile bait plants	<i>B. napus</i> ♀	6, 20, 42, 54, 150	21, 0.16, 0.33, 0.11, 0.22	No. of seed sets	UK	Simpson 2002
<i>B. napus</i> ♀	Seed set on male-sterile bait plants	<i>B. napus</i> ♀	100, 200, 400	0.13, 0.03, 0.06	No. of seed sets	UK	Thompson et al. 1999

※ estimated gene flow rate

- information not available

♀ using male sterile varieties or emasculated “bait” plants (petals and stamens removed)

Recommended isolation distances

According to experimental field studies measuring pollen dispersal and outcrossing rates, various requirements of thresholds and recommended isolation distances have been established in conventional *B. napus* production as showed in Table 3. These can also serve as guidelines for recommended separation distances from GM *B. napus* to minimize gene escape through pollen flow.

The recommended isolation distances required for GM *B. napus* trials worldwide range from 50-400 m with a threshold requirement less than 0.9% (Table 3). 50 m isolation distance is recommended for GM trials in the UK, with 200 m isolation recommended for organic *B. napus* (SCIMAC 1999). For certified seeds production, recommended isolation distances of 100-200 m are generally considered sufficient to limit outcrossing and maintain seed purity (Scheffler et al. 1995; USDA 2008). Such isolation distances are not intended to completely prevent outcrossing, but to reduce it to an acceptable level (Scheffler et al. 1995). Current recommended isolation distances required for foundation seeds (99.9% purity) are 400 m and 500 m in Canada and OECD, respectively, with a threshold requirement less than 0.1% (Table 3). In USA, 400 m isolation distance is recommended the required distance for foundation seeds requirements with a threshold requirement less than 0.05% (USDA 2008).

Canadian government regulations stipulate 200 m large isolation zone for GM *B. napus* studies, or 10 m wide non-GM border of synchronously flowering around the studies area (Staniland et al. 2000). An isolation distance of 400 m is required for GM trials in France, Belgium, and Sweden. Australian GM trial requirements including a 400 m isolation distance and a 15 m non-GM buffer zone (Salisbury 2002). In China, a minimum isolation distance of 300 m, rather than 1000 m according to the Implementation Regulations on Safety Assessment of Agricultural GMOs of China (MOA 2002), is recommended as a reasonable distance to ensure a tolerable threshold of pollen flow (less than 0.01%) in GM *B. napus* field trial in China (Zhao et al. 2013).

Based on these data, the separation distances established for seed production by regulatory authorities generally seem to be insufficient to minimize seed contamination considering the long-distance pollen flow outcrossing events (Cai et al. 2008; Paul et al. 1995; Rieger et al. 2002; Zhao et al. 2013). Therefore, to obtain seed purity levels that comply with stipulated thresholds of, for example, 0.05% impurities or less, separation distances of at least 800 m would be required (Andersson and de Vicente 2010).

Table 3. Recommended isolation distances for *B. napus* to meet different thresholds

Threshold ^a	Minimum isolation distance (m)	Country	Reference	Comment
<1%	1.5	UK	Ingram 2000	recommended separation distance between GM and non-GM <i>B. napus</i> , for fields > 2 ha
<0.9%	30	France	CETIOM 2000	based on the survey of field level outcrossing
	50	UK	SCIMAC 1999	recommended separation distance between GM and conventional non-GM <i>B. napus</i>
	200	UK	SCIMAC 1999	recommended separation distance between GM and organic non-GM <i>B. napus</i> and for certified seed production
<0.5%	10	UK	Ingram 2000	recommended separation distance between GM and non-GM <i>B. napus</i> , for fields > 2 ha
	120	France	CETIOM 2000	based on the survey of field level outcrossing
<0.25%	100	USA	USDA 2008	certified seed requirements
	100	Canada	-	certified seed requirements
<0.1%	100	UK	Ingram 2000	recommended separation distance between GM and non-GM <i>B. napus</i> , for fields > 2 ha
	400	France	CETIOM 2000	based on the survey of field level outcrossing
	400	Canada	-	foundation seed requirements
	500	OECD	OECD 2008	foundation seed requirements
	400	USA	USDA 2008	foundation seed requirements
<0.05%	400	USA	USDA 2008	foundation seed requirements
<0.01%	800	Canada	CSGA 2009	certified seed requirements

Modeling of gene flow

It is necessary to establish a mathematical model to predict gene flow and validate the relevant factors affecting gene flow under various conditions. There are two main methods, mechanistic and empirical, for modeling the gene flow at a landscape level (Klein et al. 2003, 2006; Gustafson et al. 2005; Snäll et al. 2007). The mechanistic modeling incorporates physical and biological factors that influence gene flow, and provides insights into the process of gene flow (Klein et al. 2003; Snäll et al. 2007). This modeling method can reveal the general pattern of contemporary gene under influences of various factors. Therefore, it can potentially predict the gene flow under diverse conditions (Snäll et al. 2007). However, mechanistic models are usually mathematically complex and computationally expensive, and often contain many parameters that are difficult to measure in natural conditions (Klein et al. 2003; Snäll et al. 2007). Empirical modeling ignores the details of the dispersal process and often simulates gene flow by applying regression analysis to achieve an empirical function that fits the experimental gene flow data (Klein et al. 2003; Gustafson et al. 2005; Snäll et al. 2007). The empirical method can be easily performed in practice, but cannot be extended to a wider range of environmental conditions (Klein et al. 2003; Snäll et al. 2007). To some extent, although this empirical model is difficult to describe gene flow at various environment conditions, it exhibits a flexible and practical experience for a certain purpose to predict gene flow.

Up to now, several models such as quadratic, log-logistic models, exponential decay, inverse power-law model, rational function, etc., have been developed for prediction of gene flow at a specified distance (Simpson et al. 2006; Stallings et al. 1995; Weeks et al. 2005; Yuan et al. 2007; Zhao. et al. 2013). Although some model showed a good fit to the models, there is a lack of biological meaning for explanation of the estimated parameters. Therefore, the biological meaningful model was more suitable for interpreting the parameters with biological meaning.

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CHAPTER I

Quantifying maximum potential gene flow from *Brassica napus* to its male sterile relatives under greenhouse and field conditions

ABSTRACT

Gene flow from *Brassica napus* to its male sterile (MS) relatives, MS *B. napus*, MS *B. juncea*, and MS *Raphanus sativus* was evaluated in the greenhouse and field conditions. Vernalized plants were planted in plastic pots and placed at various distances up to 24 m and 40 m in the greenhouse and the field, respectively. *B. napus* was used as the pollen donor and its MS relatives as a pollen recipient. In the greenhouse test as a model case study, bumble bee was used as an insect pollinator, while honeybee was used in the field test. No. of flowers and pods set were assessed to calculate pod setting rate, which was then further calibrated. The calibration factor was determined using a molecular marker to determine the proportion of pollen donor allele genes introgressed into pollen recipients. In the greenhouse condition, the gene flow from *B. napus* to MS *B. napus* were 20.13% at 3 m distance and reached down to 1.96% at 24 m, decreasing with distance from pollen donor. For MS *B. juncea*, the gene flow was 1.19% at 3 m and decreased down to 0.06% at 24 m. In the field condition, the gene flow rates from *B. napus* to its MS relatives were much greater than those values obtained in the greenhouse condition. The gene flow

to MS *B. napus* was 37.38% at 4 m and decreased down to 10.71% at 40 m, providing greater gene flow in the field condition than in the greenhouse condition. For *B. juncea*, it was 34.59% at 4 m and decreased to 1.97% at 40 m. No gene flow was detected from *B. napus* to *R. sativus* in both greenhouse and field conditions. Even though gene flow rate was relatively lower in the greenhouse study than the field study, the gene flow estimated in the greenhouse condition still provide scientifically comparable and valuable estimation of gene flow and is still viable. The platform for gene flow study in the greenhouse can be used for risk assessment of gene flow where field study is unavailable.

Keywords: *Brassica napus*, *Brassica juncea*, gene flow, pollinator, *Raphanus sativus*, male sterile

INTRODUCTION

With the rapid development of transgenic biotechnology, more and more genetically modified (GM) crops have been released into the agricultural ecosystem. Based on a global scale of biotech crops report, the cultivation area has increased from 1.7 million ha in 1996 to 160 million ha in 2011 (James 2011). Such advantages as improvement of crops production and eco-friendly environment (decreased herbicide applications) have demonstrated by adoption of those crops (Beckie et al. 2006). However, with dramatically increasing acreage of GM crops cultivation worldwide, those associated with unexpected ecological change and negative effects have raised public concerns about the gene dispersal to non-GM crops and relative species (Ellstrand et al. 1999; Firbank and Forcella 2000; Wilkinson et al. 2003). One of the main concerns over these agricultural releases of GM crops is the escape of the transgenes into the natural ecosystem through outcrossing with their wild or weedy relatives (Dunwell 2002; Warwick et al. 2003; Snow 2002).

Brassica napus (AACC, $2n=38$) is currently the third most important oil crop after soybean and oil palm and its global production continues to increase rapidly. GM *B. napus* with such traits as resistant to diseases, tolerant to herbicides, drought, and other stress has been developed and benefited human in crop production. Due to having many wild or weedy relative such as *B. juncea* (AABB, $2n=36$), *B. rapa* (AA, $2n=20$), *B. niga* (BB, $2n=16$), *B. oleracea* (CC, $2n=18$), *R. sativus* (RR, $2n=18$), *R. raphanistrum* (RrRr, $2n=18$), etc., persisting in or near areas of cultivation. Potential gene flow to its relatives as one of the main concerns has been raised.

Asian countries and Korea in particular, co-existence of *B. napus* with its relatives in the diverse cropping system is quite common, where farmers generally manage small-scale fields and form a mosaic agricultural structure. In one cropping season, different *B. napus* varieties with its relatives may grow side-by-side, and

synchrony of flowering period may happen during cultivation, those pose a high potential outcrossing between *B. napus* and its relatives. In case the GM *B. napus* is approved for commercial cultivation, GM *B. napus* and its relatives may co-exist and -cultivate.

Pollinator, bumblebee (*Bombus* spp.) and honeybee (*Apis mellifera*) were introduced for pollination in greenhouse and field studies (Cresswell 1994; Mesa et al. 2013; Pierre et al. 2010). Bumblebee are regarded as an important and effective pollinator for commercial crops pollination. Especially in greenhouse crops pollination, the use of bumblebee is very common practice. Because of its high environmental adaptability (such as poor phototaxis, low and high temperature resistance) and lower degree of evolution, it would be more concentrated on pollinating (Picard-Nizou et al. 1995). By contrast, honeybee, due to their great number, their social life and their ability to pollinate a broad variety of different flowers, are commonly used for effective pollination under field conditions (Durán et al. 2010; Sabbahi et al. 2005).

To date, some studies on gene flow have been reported in Asian countries such as China and Japan, the data indicated a very region-based characteristic which was closely associated with experimental condition and regional climate (Bing et al. 1996; Cai et al. 2008; Chèvre et al. 2003; Jørgensen et al. 1996; Tsuda et al. 2012; Warwick et al. 2003; Weekes et al. 2005; Zhao et al. 2013). However, in Korea there is no report on this regard. Thus, it is necessary to conduct the Korean climate-based assessment to evaluate the potential gene flow from *B. napus* to its relatives and provide sufficient scientific basis for gene flow risk assessment and GM safety management. In the previous gene flow studies, a circular or a rectangular experimental design was usually used (Hüsken and Dietz-Pfeilstetter 2007). In the circular design, the pollen donor is spaced at the center, whereas the pollen recipients are planted at various distances from the pollen donor. This design may be suitable for providing information on gene flow within two species in compass directions. In the rectangular design, the pollen donor is located on one side and pollen recipients are planted at various distances from the pollen donor. Taken into

account the diverse agricultural structure with *B. napus* co-cultivated with its relatives, in the present study, we adopted the rectangular design by planting three relatives at various distances from *B. napus* pollen donor to simulate a mosaic agricultural structure.

It is worth pointing out that our concern is to assess the potential gene flow from *B. napus* to its relatives at a specified distance. Potential gene flow is different from “over-estimate gene flow”, which is the scientific-based approach for assessing the potential gene flow risk with showing the upper threshold. In order to achieve this aim to obtain the upper threshold, using male sterile recipients, entire synchrony of flowering period between donor and recipient, and presence and abundance of pollinators were adopted. With consideration of no previous experience on gene flow risk assessment in Korea, the objectives of this studies were i) to determine optimal environmental conditions by a greenhouse evaluation as a case study; ii) on the basis of the greenhouse experience, to quantify the maximum potential gene flow from *B. napus* to its MS relatives under Korean field climatic condition by artificially introducing pollinator and overlapping flowering periods between pollen donor and recipients.

MATERIALS AND METHODS

Plant materials

Two varieties of *B. napus* cv. Tammi and Tamla (AACC, 2n=38) were used as the pollen donor, and the male sterile (MS) plants, including MS *B. napus*, two varieties of MS *B. juncea*-9 and -10 (AABB, 2n=36), and MS *R. sativus* (RR, 2n=18) were used as the pollen recipients, respectively. All seeds were obtained from National Academy of Agricultural Science, Korea. The seeds were sowed and cultivated in greenhouse of the experimental farm station of Seoul National University, Korea. The seedlings at two-true leaf growth stage were stored in a cold chamber (4°C) for vernalization treatment at National Academy of Agricultural Science, Korea.

Experimental design

In this study, both greenhouse and field experiments were conducted under the authorization GM field of the experimental farm station of Seoul National University, Suwon, Korea (authorization number: RDA-AB-2011-014) from June to October in 2012. Firstly, the controlled greenhouse study was performed as a model case to determine the fundamental conditions for quantifying potential gene flow, and then in an open field, the maximum potential gene flow from *B. napus* to its MS relatives was evaluated. Pollinator, bumblebee (*Bombus* spp.) and honeybee (*Apis mellifera*) were respectively introduced in greenhouse and field to facilitate gene flow from *B. napus* to its MS relatives.

Greenhouse study

Vernalised seedlings were transplanted and cultivated in pots with a mix reconstructed soil (commercial horticultural soil: normal soil = 1:1). Fifty pollen donor plants (25 plants for each variety of Tammi and Tamla) were placed at one side in greenhouse (Size of greenhouse: 24 m × 6 m). Prior to flowering, MS pollen recipients were accordingly arranged at the various distances of 3, 6, 12, 18, and 24

m from the pollen donor, and each MS pollen recipient line at each distance consists of 2 MS *B. napus*, 5 MS *R. sativus*, 3 MS *B. juncea-9*, and 4 MS *B. juncea-10* (Fig. 2A). A bumblebee hive with approximately 250 populations was placed at 12 m from pollen donor. During the flowering period, regular observations such as the phenology of plants flowering, the number and pollinating behavior of bees (investigated everyday at 8:30-9:00) in each plot were made. Total number of flowers exposed and pods formed were counted for each inflorescence and each individual recipient. Pods setting rate was calculated by comparing total number of pods set and total number of flowers for each individual recipient. The fertilizer application, insecticide spray, and irrigation management were carried out regularly.

Field study

The procedures for seedlings transplanting, cultivating, and transferring in field prior to flowering were described as in greenhouse study. Taking into account the greenhouse study and field scale constraints, distances from the pollen donor for field study were adjusted to 4, 8, 16, 24, 32, and 40 m. One hundred pollen donor plants in total (50 plants for each variety of Tammi and Tamla) were planted at one side in field. Each MS recipient line consists of 5 MS *B. napus* plants, 7 MS *R. sativus* plants, 4 MS *B. juncea-9* plants, and 4 MS *B. juncea-10* plants and was accordingly planted at those distances (Fig. 2B). A honeybee hive with approximately 10,000 populations was placed at 20 m from pollen donor during flowering and honeybee's visit frequency was investigated everyday at 10:00-11:00 am except raining day. Regular observations and plant management were made as described in greenhouse study. Total number of flowers exposed and pods formed were also counted for each inflorescence and each individual recipient for determination of pods setting rate.

Determination of gene flow rate

Both in greenhouse and field studies, pods formed on MS recipients could be

considered the result of pollination from pollen donor. But there was the possibility for MS plants to recover the fertility and produce seeds by selfing. In order to check the proportion of selfed seeds among the progenies, PCR analysis was performed by detecting presence or absence of pollen donor allele introgression in randomly selected pollen recipient seeds. Thus, the gene flow rate was calculated accordingly, with the pods setting rate estimate for each species at each distance being multiplied by calibration factor based on PCR analysis.

Primer screening

Three out of 99-pairs of primers (source from Izzah et al. 2013) were screened for identifying the progenies of MS plants by selfing or outcrossing with pollen donor (Table 4).

Table 4. Selected primers for identifying hybrids among the progenies of MS recipients

Species	Markers	Primers application	Primer sequences (5'→3')
<i>B. juncea</i>	BnGMS539	Positive control	F: CATCACTCAATCCAAGACCT R: AGAACCTGAAACAAACGATG
	BoKAH45TR	Non-hybrids or hybrids	F: ATTATGACGCCTGGTTTTA R: ATTGGTTAGAAGTTATGGGAAC
<i>R. sativus</i>	COS0842	Non-hybrids or hybrids	F: TGGGCTGCCTTGAGAACA R: AGATGCTGAACTTGAATCCACTG

PCR analysis

The genomic DNA was extracted by following the minor modified CTAB method (Doyle and Doyle 1990) and standardized to a final concentration of 20 ng μL^{-1} . DNA amplification was performed in a reaction volume of 10 μL containing 20 ng of template DNA (0.5 μL), 10 \times PCR reaction buffer (Sigma Co., USA) (200 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM MgCl_2) (1 μL), 2.5 μM of dNTPs (Sigma

Co., USA) (0.8 μ L), 5 μ M each of the forward and reverse primers (Sigma Co., USA) (0.4 μ L), 2 units of *Taq* DNA polymerase (0.5 μ L), and sterile distilled water (6.8 μ L). The PCR was conducted in a T100TM 96-Well Thermal cycler (Bio-Rad Laboratories, Singapore), under the following conditions: 94°C for 4 min, followed by 35 cycles (94°C for 30 s, 54°C for 30 s, and 72°C for 30 s), and finally 72°C for 10 min for pollen donor allele gene introgression in progenies of *B. juncea*. For pollen donor allele gene introgression in progenies of *R. sativus*, the conditions were 94°C for 5 min, followed by 35 cycles (94°C for 30 s, 55°C for 30 s, and 72°C for 30 s), and finally 72°C for 5 min . The expected PCR product size was around 180-200, 269, and 220-230 bp for primer 8, primer 11, and primer COS0842, respectively. The PCR products were analyzed by electrophoresis on 1.2% (w/v) agarose gel containing ethidium bromide (Et-Br) in 0.5 \times TAE buffer. The gel was then visualized and the images were photographed using Digital Gel Documentation System-200 (the Alpha Innotech Corporation, USA). Samples with the introgressed allele gene DNA fragment were confirmed as the hybrids outcrossed with pollen donor.

Statistical analysis

Both greenhouse and field studies, each individual recipient at each distance was considered as replication and arranged completely randomized in each plot. The full data set was subjected to analysis of variance (ANOVA) using Genstat5 (Genstat Committee, 1997). The pods setting rate for each species at each distance was mean of replications of each individual rate. Calibration factor was calculated by determination of the proportion of progenies with introgressed pollen donor allele gene among the total progenies tested. The gene flow rate was finally confirmed by pods setting rate multiplied by calibration factor.

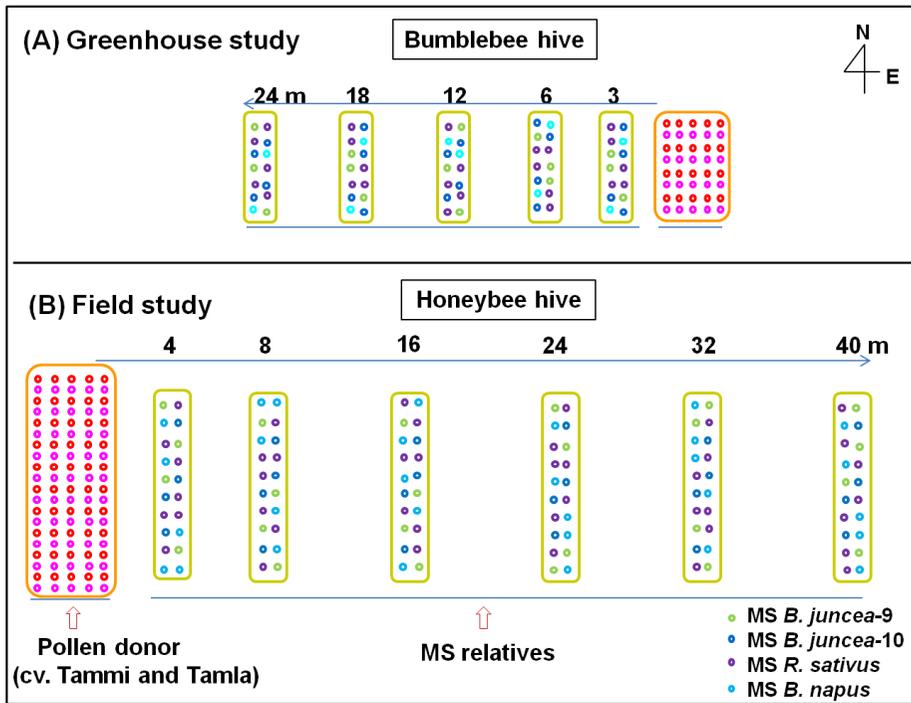


Fig. 2. Experimental design (A, greenhouse mode case study; B, field study) for evaluation of gene flow from *B. napus* to its MS relatives in 2012, Suwon, Korea. The *B. napus* cv. Tammi and Tamla was used as pollen donor, and its MS relatives were used as pollen recipients. The locations of the beehives are shown.

RESULTS

Gene flow in the greenhouse study

Synchrony of flowering period

In order to maximize synchrony of flowering periods between pollen donor and recipients, two varieties of pollen donors (*B. napus* cv. Tammi and Tamla) with different flowering timings were planted. During the flowering period, biologically important dates of experimental plants, such as dates of 1st flower blooming, peak blooming, and end of blooming, were recorded (Table 5). *B. napus* cv. Tammi has an earlier flowering time (approximately 1 week) than *B. napus* cv. Tamla, and the later flowering time of *B. napus* cv. Tamla guaranteed the maximal synchrony of flowering with pollen recipients. The date of peak blooming for both pollen donor and recipient was around middle of July (Table 5). A significant flowering synchronization between pollen donor and recipients was achieved with the 26, 30, 30, and 31 days of flowering period overlapped for MS *B. napus*, MS *B. juncea-9*, MS *B. juncea-9*, and MS *R. sativus* with the pollen donor, respectively. Therefore, the periods of flowering synchronization are long enough for evaluation gene flow from *B. napus* to its relatives.

Bumblebee visit

As *B. napus* cv. Tammi and Tamla are only pollen sources and produced many flowers, a relatively larger number of bumblebees gathered in this area with a mean visit frequency of 0.62 (plant⁻¹ min⁻¹) (Fig. 3). Moreover, the bumblebee visit differed significantly from the species of pollen recipients and the distance from the pollen donor. The closest pollen recipient from the pollen donor showed the most frequent bumblebee visit with approximately 0.6, 0.1, and 0.2 plant⁻¹ min⁻¹ on MS *B. napus*, MS *B. juncea*, and MS *R. sativus* plants, respectively. The visit frequency decreased with increasing the distance from the pollen donor, indicating that

bumblebees gather nectar from flowers located near their hive. These data also showed that flowers of *B. napus* were more attractive to bumblebee compared with flowers of *B. juncea* and *R. sativus* although they grew at the same location and broomed simultaneously (Fig. 3). Interestingly, bumblebee visited the flowers of *R. sativus* more frequently than those of *B. juncea* (Fig. 3), which might be due to the difference in flower size, *R. sativus* flower is larger than that of *B. juncea*, and pollinating preference of bumblebee.

Pods setting rate

MS plants were used as the tracing marker, so pods setting on these recipients can be considered as the result from gene flow from pollen donor plants. No significant difference was found in the number of flowers of each MS recipient at various distances ($P < 0.05$). The average number of flowers plant⁻¹ was 1580.8±32.5, 2235.2.4±36.7, and 2034.8±55.1 for each MS *B. napus*, *B. juncea*, and *R. sativus*, respectively. The average number of pods plant⁻¹ for these three species decreased with increasing the distance from the pollen donor (data not shown). Pod setting rate of MS *B. napus* was 24.25% at 3 m, 10.91% at 6 m, and sharply decreased down to 2.36% at 24 m. Pod setting rates of MS *B. juncea*-9 and -10 were 0.298% and 1.294% at 3 m, 0.099% and 0.967% at 6 m, and decreased down to 0.029% and 0.147% at 12 m, respectively. No pods were set at 18 m for MS *B. juncea*-9, while the pod setting of MS *B. juncea*-10 was 0.055% even at 24 m, the longest distance. As for MS *R. sativus*, a few pods formed in the plants, but no seeds were found in the pods, indicating that the low possibility of gene flow from *B. napus* to *R. sativus* (Table 6).

Determination of calibration factor and gene flow rate

Calibration factor and gene flow rate were summarized in Table 6. The calibration factor was determined using molecular marker to check the proportion of seeds with introgressed *B. napus* allele gene among the randomly selected MS seeds (Fig. 4). The gene flow rate was calculated by multiplied pods setting rate by

calibration factor (Table 6). The gene flow rate between *B. napus* under controlled greenhouse condition was estimated ranging from 1.96-20.13% at 3-24 m. In the case of *B. juncea*, it was varied with the varieties. For *B. juncea-9*, it was estimate of 0.298% at 3 m, 0.099% at 6 m, 0.029% at 12 m, and not detected beyond 18 m from the pollen donor, respectively. By contrast, for *B. juncea-10*, it was estimate of 1.19% at 3 m, 0.903% at 6m, 0.147% at 12 m, 0.085% at 18 m, and 0.055% at 24 m, respectively (Table 6). With regard to *R. sativus*, no gene flow was detected in the present greenhouse condition with entire synchrony of flowering period and presence of bumblebee.

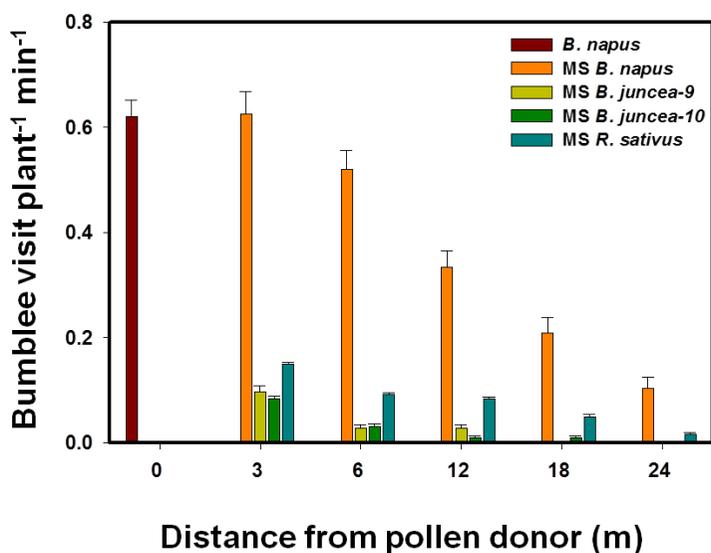


Fig. 3. Bumblebee visit frequency ($\text{plant}^{-1} \text{min}^{-1}$) ($\pm\text{SE}$) at various distances (m) from pollen donor during the flowering periods in the greenhouse in 2012.



Fig. 4. PCR confirmation using MS *B. juncea-9*, *-10* and MS *R. sativus* genomic DNA. (A), the detection of the presence of tested *B. juncea-9* and *-10* genomic DNA using positive control primer BnGMS539; (B), the confirmation of hybrid seeds using primer BoKAH45TR for *B. juncea-9* and *-10*. (C), the confirmation of hybrid seeds using primer COS0842 for *R. sativus*. Bn, *B. napus* cv. Tammi; Bj, *B. juncea-9* and *-10*; Rs, *R. sativus*, Bn+Rs, DNA mixtures of *B. napus* and *R. sativus*; (-) Con: negative control, amplified by PCR without DNA sample.

Table 5. Biological dates for a specific reproductive growth stage of experimental plants in the greenhouse in 2012

Experimental plants		1 st flowering	Peak flowering	End of flowering	Flowering Duration (days)	Synchrony of flowering (days)
Pollen donor	Tammi	28 Jun	12 Jul	26 Jul	29	-
	Tamla	5 July	20/Jul	4 Aug	30	-
Total flowering duration		-	-	-	38	-
Pollen recipients	<i>B. napus</i>	3 July	19 Jul	28 Jul	26	26
	<i>B. juncea-9</i>	27 Jun	12 Jul	27 Jul	31	30
	<i>B. juncea-10</i>	29 Jun	15 Jul	28 Jul	30	30
	<i>R. sativus</i>	2 Jul	13 Jul	1 Aug	31	31

Table 6. Summary of pods setting rate (PSR), calibration factor (CF), and gene flow rate (GFR) in the greenhouse in 2012

Distance (m)	<i>B. napus</i>			<i>B. juncea-9</i>			<i>B. juncea-10</i>			<i>R. sativus</i>		
	PSR (%) ^a	CF (%) ^b	GFR (%) ^c	PSR (%)	CF (%)	GFR (%)	PSR (%)	CF (%)	GFR (%)	PSR (%)	CF (%)	GFR (%)
3	24.25 ^a	0.83	20.13	0.30	1.00	0.30 ^b	1.29	0.92	1.19	0.05	-	0.00
6	10.91	0.83	9.06	0.10	1.00	0.10	0.97	0.93	0.90	0.04	-	0.00
12	6.77	0.83	5.62	0.03	1.00	0.03	0.15	1.00	0.15	0.02	-	0.00
18	4.51	0.83	3.74	0.00	-	0.00	0.09	1.00	0.09	0.04	-	0.00
24	2.36	0.83	1.96	0.00	-	0.00	0.06	1.00	0.06	0.00	-	0.00
LSD _{0.05}	3.73	NC ^d	2.12	0.03	NC	0.03	0.32	0.93	NC	0.01	NC	NS ^e

^a Pods setting rate (PSR) calculated by dividing no. of pods by that of no. of flowers for each plant of MS *B. napus*, MS *B. juncea-9*, MS *B. juncea-10*, and MS *R. sativus* at various distance from pollen donor.

^b Calibration factor (CF) for *B. napus* calculated based on the field trial in 2014; for *B. juncea* and *R. sativus*, the CF calculated by dividing no. of tested seeds with introgressed *B. napus* allele gene by that of total no. of tested seeds.

^c Gene flow rate (GFR) calculated by multiplied PSR by CF.

^d NC, not calculated.

^e NS, not significant.

Gene flow in the field study

Synchrony of flowering period

For field study, a 29-overlapped-day, 28-overlapped-day, and 27-overlapped-day for MS *B. napus*, MS *B. juncea-9*, and MS *B. juncea-10*, respectively, were synchronized with pollen donor (Table 7). For MS *R. sativus*, a relatively longer flowering period was observed compared with other recipients and pollen donors, therefore, the experiment was immediately terminated as soon as pollen donor end of flowering, and accordingly, unsynchronized flowers were eliminated, finally resulting in a 31-overlapped-day with pollen donors.

Honeybees visit

As observed in greenhouse, a larger number of honeybee gathering was found near pollen donor area, and consistently decreased with increasing the distance from pollen donor in the three species. The number of honeybee visit also differed from the species of pollen recipients as bumblebee in greenhouse. *B. napus* showed the most attractive to honeybee compared to other two species (Fig. 5). For example, at 4 m it was approximate $0.85 \text{ plant}^{-1} \text{ min}^{-1}$ for *B. napus*, more frequent than that of $0.45 \text{ plant}^{-1} \text{ min}^{-1}$ for *B. juncea* and $0.32 \text{ plant}^{-1} \text{ min}^{-1}$ for *R. sativus*. Similar patterns of flowers visit were found at other distances as well. In addition, the difference of honeybee visit was also observed in the two varieties of *B. juncea* (Fig. 5).

Pods setting rate

No significant difference ($P < 0.05$) was found in average number of flowers plant^{-1} for each MS recipient of *B. napus*, *B. juncea*, and *R. sativus* at various distances (1312.8 ± 13.7 , 1428.3 ± 21.3 , and 1041.2 ± 17.8 , respectively), but the average number of pods plant^{-1} were decreasing as increasing the distance from the pollen donors for those three species, respectively (data not shown). For pods setting rate of MS *B. napus*, it was 45.0% at 4 m, 35.7% at 16 m, 31.8% at 24 m, and decreased to 12.9%

at 40 m from the pollen donor (Table 8). For MS *B. juncea*, the pods setting rate was differed from the varieties. It was 14.13% vs. 35.12% for MS *B. juncea*-9 and -10 at 4 m, 6.34% vs. 19.79% at 16 m, and decreased to 1.97% vs. 3.63% at 40 m, respectively (Table 8). In the case of MS *R. sativus*, it was 15.9% at 4 m, 8.4% at 16 m, decreasing to 6.1% at 24 m, and 1.3% at 40 m (Table 8).

Determination of calibration factor and gene flow rate

The calibration factors based on PCR analysis for the three species were summarized in Table 8. The gene flow rate obtained from field study was very different from greenhouse study, indicating that the environmental effect on gene flow (Table 8). Based on the Table 8, for MS *B. napus*, the gene flow rate was estimated ranging from 10.71-37.35% at 4-40 m. For the two varieties of *B. juncea*, the gene flow rates ranged from 1.97-13.78% for MS *B. juncea*-9 and 3.63-34.59% for MS *B. juncea*-10 at 4-40 m, respectively (Table 8). Data from the PCR analysis showed that all tested MS *R. sativus* progenies were not hybrids (Table 8), indicating that no gene flow occurred from *B. napus* to *R. sativus* in the present experimental condition.

Table 7. Biological dates for a specific reproductive growth stage of experimental plants in the field in 2012

Experimental plants		1 st flowering	Peak flowering	End of flowering	Flowering Duration (days)	Synchrony of flowering (days)
Pollen donor	Tammi	23 Aug	17 Sep	22 Sep	30	-
	Tamla	1 Sep	25 Sep	29 Sep	29	-
Total flowering duration		-	-	-	68	-
Pollen recipients	<i>B. napus</i>	24 Aug	23 Sep	22 Sep	29	29
	<i>B. juncea-9</i>	23 Aug	18 Sep	20 Sep	28	28
	<i>B. juncea-10</i>	27 Aug	20 Sep	23 Sep	27	27
	<i>R. sativus</i>	30 Aug	21 Sep	29 Sep	31	31

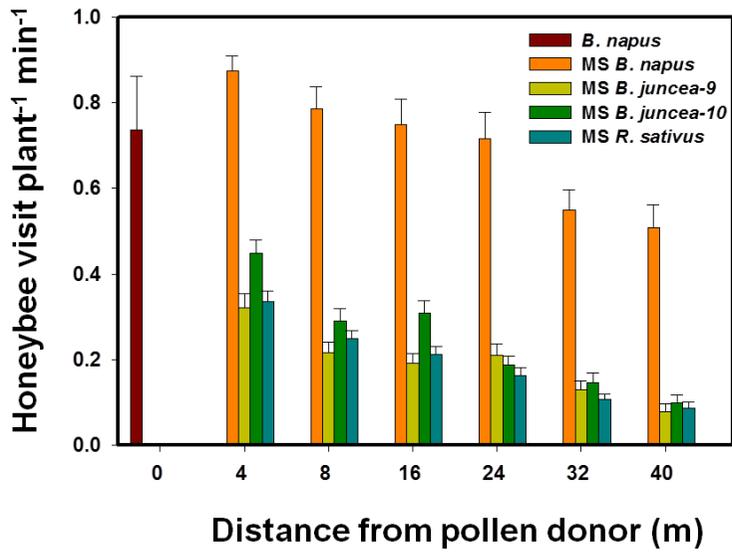


Fig. 5. Honeybee visit frequency ($\text{plant}^{-1} \text{min}^{-1}$) ($\pm\text{SE}$) at various distances (m) from pollen donor during the flowering periods in the field in 2012.

Table 8. Summary of pods setting rate (PSR), calibration factor (CF), and gene flow rate (GFR) in the field in 2012

Distance (m)	<i>B. napus</i>			<i>B. juncea-9</i>			<i>B. juncea-10</i>			<i>R. sativus</i>		
	PSR (%) ^a	CF (%) ^b	GFR (%) ^c	PSR (%)	CF (%)	GFR (%)	PSR (%)	CF (%)	GFR (%)	PSR (%)	CF (%)	GFR (%)
4	45.0 ^a	0.83	37.35	14.13	0.98	13.78	35.12	0.99	34.59	15.90	0.00	0.00
8	39.6	0.83	32.87	9.03	0.90	8.13	25.30	1.00	25.30	13.10	0.00	0.00
16	35.7	0.83	29.61	6.34	1.00	6.34	19.79	1.00	19.79	8.40	0.00	0.00
24	31.8	0.83	26.39	4.78	1.00	4.78	12.16	1.00	12.16	6.10	0.00	0.00
32	26.5	0.83	22.00	3.59	0.90	3.23	6.36	1.00	6.36	4.40	0.00	0.00
40	12.9	0.83	10.71	1.97	1.00	1.97	3.63	1.00	3.63	1.30	0.00	0.00
LSD _{0.05}	9.37	NC ^d	8.76	2.32	NC	3.45	4.13	NC	9.78	2.42	NC	NS ^e

^a Pods setting rate (PSR) calculated by dividing no. of pods by that of no. of flowers for each plant of MS *B. napus*, MS *B. juncea-9*, MS *B. juncea-10*, and MS *R. sativus* at various distance from pollen donor.

^b Calibration factor (CF) for *B. napus* calculated based on the field trial in 2014; for *B. juncea* and *R. sativus*, the CF calculated by dividing no. of tested seeds with introgressed *B. napus* allele gene by that of total no. of tested seeds.

^c Gene flow rate (GFR) calculated by multiplied PSR by CF.

^d NC, not calculated.

^e NS, not significant.

DISCUSSION

Greenhouse model case study

To our knowledge, up to date there is no study on *B. napus* gene flow to its relatives in Korea. The basic knowledge regarding gene flow risk assessment such as the potential distance of gene flow and the possibility of gene flow among *Brassica* family for Korean case is not available. Moreover, the affecting factors associated with gene flow including the degree of synchrony of flowering period, the influence of types of pollinators as well as surrounding environment are also needed to reveal for better understanding gene flow. In the previous field studies conducted in other countries, the gene flow rate estimated against the distance was significantly influenced by several factors. Therefore, it is difficult to decide proper planting distance between pollen donor and recipient, population size under a specific climatic or geographic condition. In addition, since the GM *B. napus* has not been approved for commercial cultivation in Korea, a field study involving GM *B. napus* is almost impossible. With the consideration of all these, the greenhouse study as a model case was conducted with bumblebee as a pollinator to accumulate the basic knowledge for Korean case and set up initial experimental conditions. The greenhouse study revealed that the maximum distance allowing gene flow from *B. napus* to MS *B. napus* and *B. juncea* was farther than 24 m, where the rates of gene flow from *B. napus* were 2.36% (24 m) and 0.06% (24 m) to MS *B. napus* and MS *B. juncea*, respectively. However, the distance varied with the varieties of *B. juncea*. In the case of MS *B. juncea*-9, the maximum distance was only 12 m with the gene flow rate of 0.03%, and no gene flow was detected at farther than 18 m, while with regard to MS *B. juncea*-10, it was 24 m with the gene flow rate of 0.06%. This difference might be resulted from the difference in attractiveness of their flowers to bumblebee (Fig. 3). No gene flow was detected in *R. sativus* regardless of distance from pollen donor, indicating the extreme low possibility of gene flow from *B. napus* to *R. sativus* in the natural ecosystem.

Potential gene flow under the field condition

In the previous studies, the evaluations were generally conducted under natural condition and the common recipients were used. The gene flow rate obtained from those studies was quite approaching the actual values because of mimicking a similar natural field condition. However, due to the common recipients used in those studies with showing a lower gene flow rate (<3%), to some extent, it is not easy to differentiate the variance in gene flow with increasing the distance from pollen donor in some cases. More importantly, it is impossible to estimate the potential gene flow rate. Potential gene flow is different from “over-estimate gene flow”, which is the scientific-based approach for assessing the potential gene flow risk with showing the upper threshold.

In order to obtain the potential gene flow rate from *B. napus* to its relatives, MS recipients as the ideal material were used for achieving a higher gene flow rate. Moreover, two varieties of *B. napus* with different flowering timings were performed as pollen donor for providing an entire synchrony of flowering period with MS recipients. Meanwhile, honeybee was introduced as the pollinator during the flowering period to facilitate gene flow. By doing so, the potential gene flow rate from *B. napus* to MS *B. napus* was estimated ranging from 10.71-37.35% at 4-40 m from the pollen donor. The values were much higher than the previous studies (for common recipient used, the rates ranging from 0.5-1.3% at 2-45 m, Krato and Petersen 2012; 0.04-2.5% at 0.5-50 m, Zhao et al. 2013; for MS recipient used, 0.33-0.58% at 0-50 m, Simpson et al. 1999; 0.11-21.0% at 6-54 m, Simpson 1999). For MS *B. juncea*, the potential gene flow rate was estimated ranging from 1.97-34.59% at 4-40 m from the pollen donor. It is the first to use the MS *B. juncea* as ideal material for assessing the potential gene flow rate and the values obtained in the study were much higher than the Japanese field evaluation using common *B. juncea* (gene flow rate ranging from 0.03-1.62% at 0-17.5 m) (Tsuda et al. 2012). No gene flow was detected from *B. napus* to *R. sativus* in our study. Although the pod setting rate was estimated, the PCR analysis demonstrated that the pods were not outcrossed by *B. napus*, indicating the recovery of male sterile of *R. sativus*. For

the decreasing pattern of pods setting rate associated with distance, honeybee visit frequency was hypothesized to interpret that phenomena. As more frequent honeybee visit observed near the pollen donor with 0.32 plant min⁻¹ at 4 m and was much higher than other distances (0.21 at 16 m and 0.08 plant min⁻¹ at 40 m) (Fig. 4). Inevitably, such more frequent visit would promote the pollination between male sterile recovered and male sterile unrecovered *R. sativus*, resulting in honeybee visit frequency related pod setting rate.

Here, we'd like to highlight the potential application of greenhouse evaluation as a model case study for those countries without previous experience on gene flow assessment or having the restrictions on GM crops field evaluation. Because the greenhouse was a manually controlled environment, the factors (or condition) could be decided (or controlled) on the basis of the experimental purposes. With excluding the influence of natural environment (climatic factors), the single effect of pollinator associated with pollen dispersal would be estimated. Moreover, there is no need to worry about the pollen escape to outside of greenhouse.

In summary, this is the first study on gene flow risk assessment from *B. napus* to its MS relatives under Korean climatic environment. The greenhouse model case study was conducted for determining basic experimental conditions. Accordingly, potential gene flow from *B. napus* to its MS relatives under field condition was further evaluated. The greenhouse model case study proved to be a flexible and controllable ways to determine experimental conditions and reveal the relationship among the factors on affecting gene flow. The field evaluation using MS recipients with artificially providing favorite pollination condition showed the maximum potential gene flow rate from *B. napus* to its MS relatives. According to the experimental result, the potential gene flow rate to its MS relatives ranging from 12.9-45.0% for MS *B. napus* and 1.97-34.59% for MS *B. juncea* at 4-40 m, respectively, and no gene flow was detected from *B. napus* to *R. sativus*. The experimental data provides informative reference values and scientific basis for risk assessment on gene flow under Korean climatic condition, and also would help decision-making on proper isolation distance for GM safety management.

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CHAPTER II

Quantifying gene flow from genetically modified *Brassica napus* to its male fertile relatives under field condition

ABSTRACT

Gene flow from genetically modified (GM) plants to compatible relatives is one of the most concerns for growing of GM crops. In this study, a potential gene flow from GM *Brassica napus* to non-GM *B. napus* as well as its relatives, *B. juncea* and *Raphanus sativus*, was evaluated in the authorized GM trial field located in Suwon, Korea in 2013 and 2014. Vernalized plants planted in pots were transferred to the GM field with a range of distances up to 95 m and 128 m in 2013 and 2014, respectively, from GM *B. napus* to its relatives. To provide a favorable condition for gene flow, entire synchrony of flowering periods between pollen donor and recipient was achieved by planting GM *B. napus* at three different times and one honeybee (*Apis mellifera*) hive was placed in the immediate vicinity of the field to provide sufficient number of honeybees. Herbicide resistance (glufosinate-ammonium) and simple sequence repeats (SSR) markers were used to confirm F1 hybrids resulted from outcrossing between GM *B. napus* and its relatives. The gene flow rate from GM *B. napus* to non-GM *B. napus* was 2.33% at 2 m distance and decreased with increasing distance, reaching 0.007% at 75 m distance with no gene flow observed farther than 85 m. In the case of non-GM *B. juncea*, it was 0.076% at 2 m and reached 0.025% at 16 m distance with no gene flow observed beyond 24 m.

No gene flow was observed from GM *B. napus* to non-GM *R. sativus*, suggesting that gene flow risk is very low or does not exist. The log-logistic model well described gene flow against distance for both *B. napus* and *B. juncea*. The model estimated that the isolation distances for 0.01% as a tolerable threshold were 122.5 m and 23.7 m for *B. napus* and *B. juncea*, respectively. Therefore, our findings suggest that gene flow risks from GM *B. napus* to non-GM *B. napus* and non-GM *B. juncea* exist although gene flow rate is low in the field condition. The data and model presented in this study may be useful and reference for gene flow risk management and future study.

Keywords: *Brassica juncea*, *Brassica napus*, gene flow, genetically modified, log-logistic model, *Raphanus sativus*, synchrony of flowering

INTRODUCTION

Brassica napus (AACC, $2n=38$) is a global important oil crop having many wild or weedy relatives co-existing in agricultural ecosystems. Achievements have been made in the development of GM *B. napus* for high oil content, resistance to drought or insects, tolerant to herbicides, novel fatty acid, and an increase of bioactive compounds. The cultivation of GM *B. napus* has increased over the last two decades, especially in North America and Austria. The acreage of GM *B. napus* was amount 8.0 million hectares in the world, occupying 26% of the total acreage of *B. napus* (James 2011). Although the advantages for adoption of GM *B. napus* has demonstrated such as substantial yield increase and environmental benefits (reduction of herbicide use) (Beckie et al. 2006), concerns have also been raised on potential risk to ecological environment through outcrossing (gene flow) with its relatives (Ellstrand et al. 1999; Firband and Forcella 2000; Warwick et al. 2003; Snow 2002). Thus, science-based risk assessment on this regard is imperative to accumulate knowledge and provide informative data to guide the agricultural practice.

Generally, the Asian countries have the diverse cropping system with many crops co-existence including *B. napus* and its relatives. Most of the farmers only have small pieces of land with the land-scale less than half a hectare. Hence, a mosaic agricultural cropping structure was quite common with different crops growing side-by-side. Currently, in Korea, the GM *B. napus* is not approved for commercial production, however, import of the GM crops has been conducting for animal feeding or edible oil processing and dramatically increased during past five years. The spilled GM *B. napus* seeds have been found around the Incheon port, indicating that the potential risk of gene flow from spilled GM *B. napus* to its relatives (Lee et al. 2007). The previous studies on evaluation of gene flow showed a very region-based characteristic which was closely associated with experimental condition and regional climate (Beckie 2001; Bing et al. 1996; Cai et al. 2008; Chèvre et al. 2003;

Jørgensen et al. 1996; Krato and Petersen 2012; Rieger et al. 2002; Warwick et al. 2003; Weekes et al. 2005).

The isolation distance required for GM trial quite varies widely, in the case of *B. napus*, although nations-wide, it ranges from 50-400 m (EU 2003; Salisbury 2002; SCIMAC 1999; Staniland et al. 2000). Recently, a Chinese study recommended that 300 m, rather 1000 m, is a reasonable distance to ensure a tolerable threshold of gene flow less than 0.01% (Zhao et al. 2013). Up to now, there is no regulation on isolation distance for GM trial in Korea. A longer isolation distances increase both costs and the difficulty of implementing regulations, which together substantially restrict GM crops development. In contrast, insufficient isolation distances have potential health and environmental risks. Therefore, a proper isolation distance must be required to guarantee the food and environmental biosafety of GM crops, and the market acceptability of products.

To our knowledge, there is no report on *B. napus* gene flow study except our previous study in 2012 in Korea. In that study, a greenhouse model case study was firstly conducted to determine the experimental conditions. With those experience and knowledge accumulated, a field evaluation of maximum potential gene flow was further conducted using male sterile (MS) relatives as pollen recipients with artificial synchrony of flowering period between pollen donor and recipient and introduction of pollinator. The experimental results showed the distance of *B. napus* pollen flow to its male sterile (MS) relatives was farther than 40 m with a higher gene flow rate under Korean field climatic condition. Due to MS lines used as the pollen recipient with representing the maximum potential gene flow, it is necessary to evaluate the actual potential gene flow using male fertile (MF) pollen recipient to provide more realistic and informative data based on Korean climatic condition. However, owing to the restrictions of field scale and regulation of use (management) of GM seeds, a large scale of field evaluation is almost impossible to conduct in Korea. Meanwhile, with consideration of the mosaic agricultural structure with co-existence of *B. napus* and its relatives growing side-by-side, a discontinuous design with planting pollen donor at one side and locating its several

recipients at various distances from pollen donor is more practical and feasible.

In order to facilitate gene flow from GM *B. napus* to its relatives to provide the potential gene flow for risk assessment, an entire synchrony of flowering period between GM pollen donor and recipient was artificially achieved by three-time's GM *B. napus* transplanting. Also, honeybee was introduced as pollinator during the flowering period. Therefore, the objective in the present study were i) to evaluate the potential gene flow from GM *B. napus* to its MF relatives in Korean field climatic condition and ii) to determine a proper isolation distance between GM *B. napus* and its relatives.

MATERIALS AND METHODS

Plant materials

Genetically modified (GM) *B. napus* (AACC, 2n=38), homozygous and containing the *bar* gene (Phosphinothricin acetyltransferase) which is a dominant gene conferring resistance to the herbicide glufosinate-ammonium (trade name Basta[®]), was used as the pollen donor. Non-GM *B. napus* cv. Tammi (AACC, 2n=38), *B. juncea* (AABB, 2n=36), and *R. sativus* (RR, 2n=18) were used as the pollen recipients. All seeds were obtained from National Academy of Agricultural Science, Korea, and sowed in the greenhouse of the experimental farm station of Seoul National University, Suwon, Korea. The vernalization treatment was made under natural condition during the winter season (approximately 1.5 months) when the growth stage of seedlings reached at the two-true leaf stages.

Field design

Field studies were performed under the authorization field of the experimental farm Station of Seoul National University, Suwon, Korea (authorization number: RDA-AB-2011-014), in 2013 and 2014 from March to July. The vernalised experimental plants were transplanted in the GMO isolated field prior to flowering. A discontinuous design with planting pollen donor at one side and locating its three relatives at three independent directions at various distances was adopted (Fig. 6). In 2013, ten distances of 4, 8, 16, 24, 32, 45, 65, 75, 85, and 95 m (ten recipients at each distance for each species) and in 2014 seven distances of 2, 4, 8, 16, 32, 64, and 128 m (six recipients at each distance for each species) from the GM pollen donor were included, respectively.

In order to ensure maximum synchrony of flowering periods between pollen donor and recipient, three different times of GM *B. napus* transplanting were made in both years based on a previous study in 2012. Therefore, a total of 450 GM *B. napus* in 2013 (15 m × 8 m, with plant spacing of 50 × 50 cm, 9 plants m⁻²) and 256

GM *B. napus* in 2014 (8 m × 8 m, same planting spacing as in 2013) were employed. In addition, the apical dominance of GM *B. napus* removed when they were beginning to flower for increasing the numbers of tillers and prolonging the flowering duration. Biological important dates such as 1st flower blooming, peak-flowering, and end of flowering for donor and recipients plants were recorded accordingly.

In both years, an approximate 10,000 population of honeybee (*Apis mellifera*) colony was introduced in field during flowering period. Bee behaviors, including flowers visit frequency and bees cluster habit, were recorded during the flowering period. The location of the honeybee hive was shown in Fig. 6. The fertilizer application, insecticide spray, and irrigation management were conducted regularly.

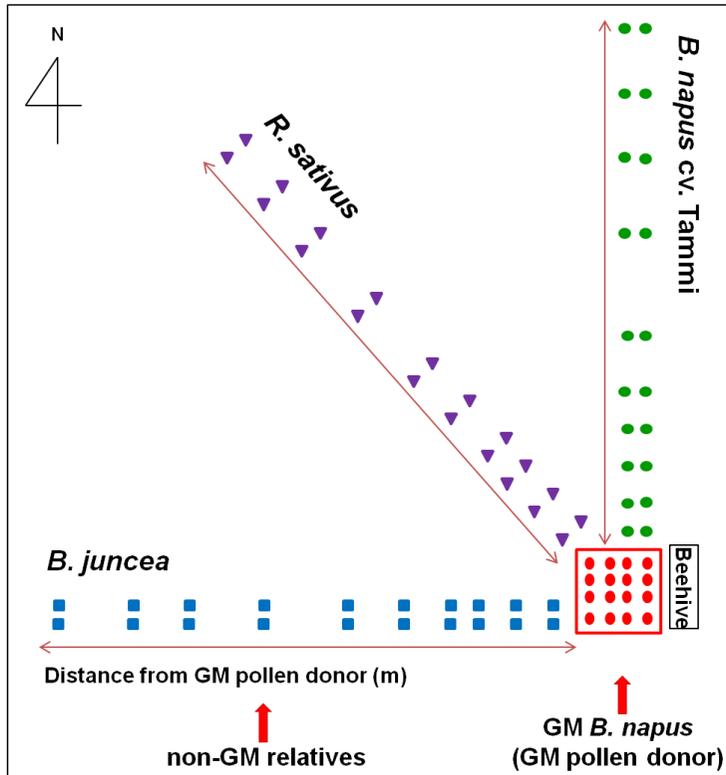


Fig. 6. Experimental field design for evaluation of gene flow from genetically modified (GM) *B. napus* to its non-GM relatives in Suwon, Korea, in 2013 and 2014. The GM *B. napus* was used as pollen donor, and the non-GM *B. napus* cv. Tammi, *R. sativus*, and *B. juncea* were used as pollen recipients in both years. The recipient plants were placed at three independent directions at the distances of 4, 8, 16, 24, 32, 45, 65, 75, 85, and 95 m in 2013, and 2, 4, 8, 16, 32, 64, and 128 m in 2014, respectively. The location of the honeybee hive is shown.

Determination of glufosinate-ammonium dose for herbicide screening

In order to determinate the most appropriate condition for hybrids screening, dose-response experiments were conducted to evaluate the effects of increasing

rates of glufosinate-ammonium on the survivals of GM *B. napus*, non-GM *B. napus* cv. Tammi, non-GM *B. juncea*, and non-GM *R. sativus* in the greenhouse of the experimental farm station of Seoul National University, Suwon, Korea in 2013. All species seeds were sowed and grown in the 200-well multi-pots (hole size: 2.5 cm × 2.5 cm × Height 3 cm). At the one-true-leaf stage, the seedlings were sprayed with a range of glufosinate-ammonium (Basta[®], SL, 1.2 g ai L⁻¹, Bayer CropScience Ltd., Korea) doses (0, 0.15, 0.3, 0.6, 1.2, 2.4, 4.8, and 9.6 g ai L⁻¹) using a compressor pressurized belt-driven sprayer (R & D Sprayer, USA) equipped with an 8002E flat-fan nozzle (Spraying System Co., USA) to deliver a spray volume of 600 L ha⁻¹. The survivals of each species were counted 14 d after herbicide treatment. The experiments were arranged in a completely randomized design with three replications and repeated two times. Based on the dose-response test (Fig. 7), the dose of 0.6, 1.2, and 2.4 g ai L⁻¹ was determined as the appropriate doses for screening resistant hybrids of progenies of non-GM *B. juncea*, non-GM *B. napus* cv. Tammi, and non-GM *R. sativus* for both years, respectively.

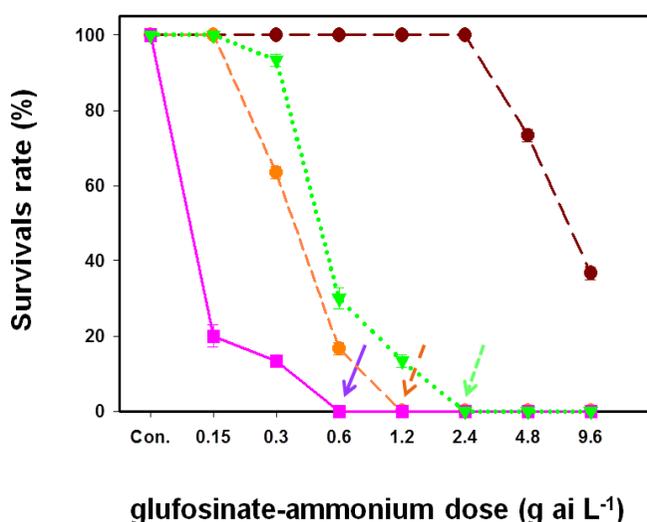


Fig. 7. The survival rate of GM *B. napus* (●), non-GM *B. napus* cv. Tammi, *B. juncea* (■), and non-GM *R. sativus* (▼) at glufosinate-ammonium (recommended dose of 1.2 g ai L⁻¹) tolerance/sensitivity test. The arrows indicate the selected dose

for herbicide resistance screening for each species.

Greenhouse screening of hybrids in progeny

The pods of each recipient plant at various distances were harvested separately in both years, and shelled, dried, counted (seed counter, USA), and stored at 4°C before herbicide screening test. Plants waste was treated as the conditions laid down by the Korean regulatory authorities. Greenhouse screening of hybrids was carried out from September to December for each year. Approximately 15% obtained *B. napus* and *B. juncea* (approximately 15,000 seedlings tested for each distance of each species), and 100% *R. sativus* (approximately 1,000 seedlings tested for each distance) progenies seeds were tested for each year. The number of progenies seedlings was counted prior to herbicide treatment. Based on the evaluation of glufosinate-ammonium tolerance test, glufosinate-ammonium was applied to those seedlings at one-true-leaf stage at the dose of 0.6, 1.2, and 2.4 g ai L⁻¹ for progenies of *B. juncea*, *B. napus* cv. Tammi, and *R. sativus*, respectively, using a compressor pressurized belt-driven sprayer as described before. Parental plants of *B. juncea*, *B. napus* cv. Tammi, *R. sativus*, and GM *B. napus* were always used as control. The survivals were counted 14 d after herbicide treatment. The survivals derived from glufosinate-ammonium treatment were considered as suspected hybrids outcrossed between GM *B. napus* and its relatives.

PCR analysis

In order to check whether the false-positive survivals were present in those suspected individuals due to insufficient absorption of the herbicide, a subsequent polymerase chain reaction (PCR) confirmation was conducted to detect the *bar-specific* gene introgression. Genomic DNA was extracted by following the minor modified cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle 1990) and standardized to a final concentration of 20 ng μL⁻¹. Two sets of primers were employed. One set containing three pairs of primers served as positive primers

was used to check whether the DNA extraction and PCR were successful for each sample and the other set was specific for the *bar-specific* gene which encodes phosphinothricin acetyltransferase, conferring resistance to the herbicide glufosinate-ammonium (Table 9). The PCR amplification was performed twice to ensure their reproducibility in a total volume of 20 μ L containing 40 ng of template DNA (2 μ L), 10 \times PCR reaction buffer (Sigma Co., USA) (200 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM MgCl₂) (2 μ L), 2.5 μ M of dNTPs (Sigma Co., USA) (1.6 μ L), 10 μ M each of the forward and reverse primers (Sigma Co., USA) (0.8 μ L), 2 units of *Taq* DNA polymerase (1 μ L), and sterile distilled water (12.6 μ L) in a T100™ 96-Well Thermal cycler (Bio-Rad Laboratories, Singapore) which was programmed to include 95°C for 2 min, followed by 30 cycles (95°C for 30 s, 64°C for 30 s, and 72°C for 30 s), and finally 72°C for 5 min. The PCR products were analyzed by electrophoresis on 1.2% (w/v) agarose gel containing ethidium bromide (Et-Br) in 0.5 \times TAE buffer and visualized. The images were photographed using Digital Gel Documentation System-200 (the Alpha Innotech Corporation, USA). We used the non-GM *B. napus* cv. Tammi, non-GM *B. juncea*, and non-GM *R. sativus* as negative control and GM *B. napus* as positive control. Seedlings with the detected *bar-specific* gene DNA fragment were confirmed as true hybrids.

Table 9. Positive and *bar-specific* gene detecting primers used for PCR analysis

Primer	Sequences (5'→3')	Application
BoKAH45TR	F: ATTATGACGCCTGGTTTTA R: ATTGGTTAGAAGTTATGGGAAC	Positive control (non-GM <i>B. napus</i>)
BnGMS539	F: CATCACTCAATCCAAGACCT R: AGAACCTGAAACAAACGATG	Positive control (non-GM <i>B. juncea</i>)
COS0842	F: TGGGCTGCCTTGAGAACA R: AGATGCTGAACTTGAATCCACTG	Positive control (non-GM <i>R. sativus</i>)
<i>bar</i> gene detection	F: CCGTGCCACCGAGGCGGACAT R: TCAAATCTCGGTGACGGGCAGGACC	Distinguishing (non-hybrids or hybrids)

Statistical analysis

In both years, each individual recipient at various distances was harvested separately and considered as replications. Gene flow rate was finalized with survival rate being multiplied by hybrid rate and analyzed separately for each year to describe the variation in gene flow across the two experimental years. Survival rate was calculated by dividing number of survivals after herbicide spray by that of total number of seedlings tested (herbicide resistance screening test). Hybrid rate was defined the proportion of survivals with *bar-specific* gene introgression in those survivals individuals (PCR analysis with those suspected survivals). Gene flow rate was expressed as following formula:

$$\text{Gene flow rate (\%)} = \frac{\text{Number of survivals}}{\text{Total number of seedlings tested}} \times \text{hybrid rate} \times 100\%$$

In order to estimate potential gene flow from GM *B. napus* to its non-GM relatives at a specific distance, the relatively higher gene flow rates at same distance within two years were used to fit the three-parameter biological meaningful log-logistic model as below:

$$Y = \frac{Y_0}{1 + \left(\frac{X}{D_{50}}\right)^b}$$

In this formula, Y denotes an estimate of gene flow rate (%) against distance, Y_0 denotes the maximum gene flow rate when the GM pollen donor and pollen recipient were adjacently planted, X is the distance from the GM pollen donor, b is the slope of the curve, and D_{50} is the distance where the gene flow declines by 50%. A lack of fit was checked for fitted regression model. R^2 and residual mean square were used to indicate the goodness of fit for the regressions models. All data was analyzed using R 3.2.0.

RESULTS

Synchrony of flowering period

In order to ensure synchrony of flowering period between pollen donor and pollen recipient, three different times of GM pollen donor transplanting and apical dominance removal for GM *B. napus* were conducted. Flowering phenology including 1st flower blooming, peak-flowering, and end of flowering for pollen donor and recipients plants were recorded (Table 10). In 2013, for GM pollen donor, the duration of flowering was 26, 28, and 27 d for the three different times' transplanting, respectively, resulting a total of 44 d flowering duration. For non-GM pollen recipients, the flowering duration was 29, 31, and 48 d for those recipients with overlapped flowering periods of 29, 31, and 32 d with GM pollen donor, respectively (Table 10). In 2014, a total of 54 d flowering durations was achieved by the three different times' GM *B. napus* transplanting and was completely overlapped with pollen recipients with the overlapped flowering periods of 38, 43, and 49 d for non-GM *B. napus*, non-GM *B. juncea*, and non-GM *R. sativus*, respectively (Table 10). All data presented here demonstrated the flowering period between pollen donor and recipient was essentially overlapped.

Honeybee visit

Honeybee (*Apis mellifera*) was introduced as the pollinator for facilitating gene flow between pollen donor and pollen recipient. In each year, during the flowering period, honeybee visit frequency was investigated every day (except raining days) at 10:00-11:00 am, and was described as $\text{plant}^{-1} \text{min}^{-1}$. The Fig. 8 demonstrated the honeybee visit frequency varied significantly ($P < 0.05$) by increasing distance from pollen donor and among the recipient species. With increasing the distance from the GM pollen donor, the honeybee visit frequency ($\text{plant}^{-1} \text{min}^{-1}$) was decreased accordingly. As showed in Fig. 8, in 2013, the closest pollen recipients at 4 m has the most honeybee visit frequency approximately 1.14, 0.87, and 1.04 $\text{plant}^{-1} \text{min}^{-1}$

in non-GM *B. napus*, non-GM *B. juncea*, and non-GM *R. sativus* compared with other distances, and decreased to 0.33, 0.28, and 0.35 plant⁻¹ min⁻¹ at farthest distance of 95 m, respectively (Fig. 8a). A similar decay pattern in visit frequency was also found in 2014 (Fig. 8b). Moreover, in both years, a relatively higher density of honeybees gathering observed near the GM pollen donor area with a mean visit frequency of 1.69 in 2013 (Fig. 8a) and 1.51 plant⁻¹ min⁻¹ in 2014 (Fig. 8b). Overall, the honeybee visit frequency (plant⁻¹ min⁻¹) in recipients in 2013 was lower than that of in 2014, with a mean visit frequency in those three recipients ranging from 0.32-1.01 plant⁻¹ min⁻¹ at 4-95 m in 2013 and 0.41-1.15 plant⁻¹ min⁻¹ at 2-128 m in 2014, respectively. Additionally, when compared the honeybee visit frequency at the same distance in each species, it was differed notably ($P < 0.05$). For instance, in 2013, at 4 m from the GM pollen donor, the honeybee visit frequency was 1.13, 0.87, and 1.04 (plant⁻¹ min⁻¹) in non-GM *B. napus*, non-GM *B. juncea*, and non-GM *R. sativus*, respectively, and 1.00, 0.73, and 0.91 in those of three species at 8 m. In 2014, the honeybee visit frequency was 1.09, 0.69, and 1.66 (plant⁻¹ min⁻¹) at 2 m and 0.90, 0.64, and 1.57 at 4 m in those of three species (Fig. 8). Among the three species, the flower of non-GM *R. sativus* showed the most attractive to honeybee in both years, followed by non-GM *B. napus* and *B. juncea*.

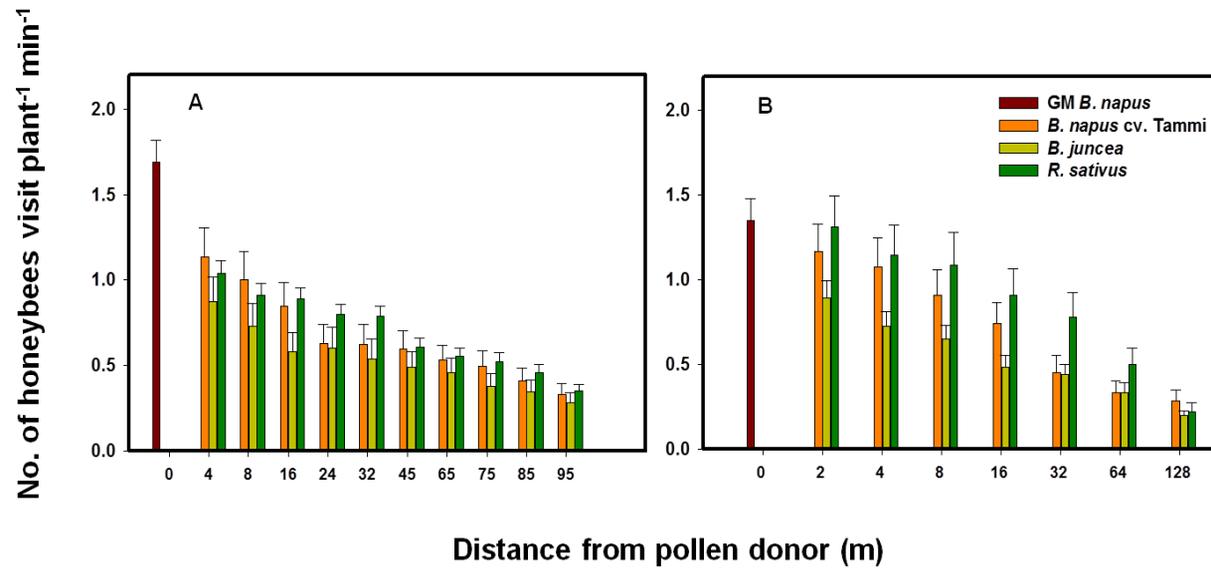


Fig. 8. Honeybee visit frequency ($\text{plant}^{-1} \text{min}^{-1}$) at various distances (m) from GM pollen donor during the flowering periods in both years of 2013 (A) and 2014 (B).

Table 10. Biological dates for a specific reproductive growth stage of pollen donor (GM *B. napus*) and pollen recipients (*B. napus*, *B. juncea*, and *R. sativus*) in both years of 2013 and 2014

Years	Experimental plants	1 st blooming	Peak-flowering	End of flowering	Flowering Duration (days)	Synchrony of flowering periods (days)
2013	Pollen donor: Three times GM <i>B. napus</i> transplanting					
	1 st time: Mar 29	May 2	May 12	May 28	26	-
	2 nd time: Apr 12	May 10	May 21	June 8	28	-
	3 rd time: Apr 26	May 18	May 28	June 15	27	-
	Total flowering duration	-	-	-	44	
	Pollen recipient: non-GM MF plants (transplanting date: Mar 29)					
	<i>B. napus</i> :	May 4	May 13	June 2	29	29
	<i>B. juncea</i> :	May 2	May 20	June 4	31	31
	<i>R. sativus</i> :	May 7	May 24	June 25	48	32
	2014	Pollen donor: Three times GM <i>B. napus</i> transplanting				
1 st time: Mar 4		Mar 27	Apr 19	May 7	41	-
2 nd time: Mar 11		Apr 1	Apr 26	May 14	44	-
3 rd time: Mar 18		Apr 6	May 2	May 19	43	-
Total flowering duration		-	-	-	54	
Pollen recipient: non-GM MF plants (transplanting date: Mar 4)						
<i>B. napus</i> : Mar 4		Mar 30	Apr 22	May 6	38	38
<i>B. juncea</i> : Mar 4		Apr 3	Apr 25	May 16	43	43
<i>R. sativus</i> : Mar 4		Apr 1	Apr 25	May 19	49	49

Survival rate and determination of hybrid rate

Survival rate was calculated by dividing the number of survivals in herbicide resistance screening test by that of total number of progenies seedlings tested. Those survivals were further subjected to PCR analysis for determination of hybrid rate by detection the proportion of survivals with GM *bar-specific* gene introgression in all survivals tested. For PCR analysis, two sets of primers were employed (Table 9). Under the positive primers of BoKAH45TR (specific to *B. napus*), BnGMS539 (specific to *B. juncea*), COS0842 (specific to *R. sativus*), all DNAs from survival were presented (data not shown). Under the *bar-specific* primer, the tested DNA from those survivals not showing the *bar-specific* DNA fragment were considered as the false-positive hybrids (Fig. 9). For instance, 9 and 7 out of 10 suspected survivals of *B. napus* and *B. juncea* with showing *bar-specific* band were confirmed to be positive hybrids, respectively. In the case of *R. sativus*, all suspected survivals were false-positive hybrids (Fig. 9). The summarized survival rate and hybrid rate for both 2013 and 2014 were showed in Table 11 and 12.

In 2013, for non-GM *B. napus* cv. Tammi progenies, the survival rate ranged from 0-1.993% at 4-95 m with the highest survival rate of 1.993% at 4 m and persistently decreasing to 0.007% at 75 m, and no survivals were detected at 85 m. The calibrate factor for those survivals ranged from 66.67-100% (Table 11). For non-GM *B. juncea* progenies, the survival rate was 0.056% at 4 m, 0.042% at 8 m, and 0.025% at 16 m, and no survivals were detected beyond 24 m. The corresponding hybrid rate for those survivals was 100% at 4 m, 66.67% at 8 m, and 33.33% at 16 m (Table 11). With regard to non-GM *R. sativus* progenies, the survival rate ranged from 0.278-0.978% with highest survival rate of 0.978% at 45 m and lowest rate of 0.278% at 75 m, but the PCR analysis showed all survivals were false-positive ones (Table 11).

In 2014, the survival rate for non-GM *B. napus* cv. Tammi progenies ranged from 0-2.394% at 2-128 m with the highest survival rate of 2.394% at 2 m, 1.255% at 4 m, 0.637% at 8 m, decreasing to 0.077% at 64 m, and no survivals were detected at 128 m. The hybrid rate for those survivals ranged from 92.5-100%

(Table 12). In the case of non-GM *B. juncea* progenies, the survival rate was 0.085% at 2 m, 0.034% at 4 m, 0.009% at 8 m, and 0.008% at 16 m, and no survivals were detected beyond 32 m. The PCR analysis showed 1 out of 10 at 2 m and one survival at 16 m without having the *bar-specific* DNA fragment, resulting from a hybrid rate ranging from 0-100% (Table 12). For non-GM *R. sativus* progenies, only two survivals were detected from all tested seedlings for various distances with the survival rate of 0.105% at 2 m and 0.113% at 4 m, respectively, but the PCR analysis on the two survivals demonstrated they were false-positive survivals (Table 12).

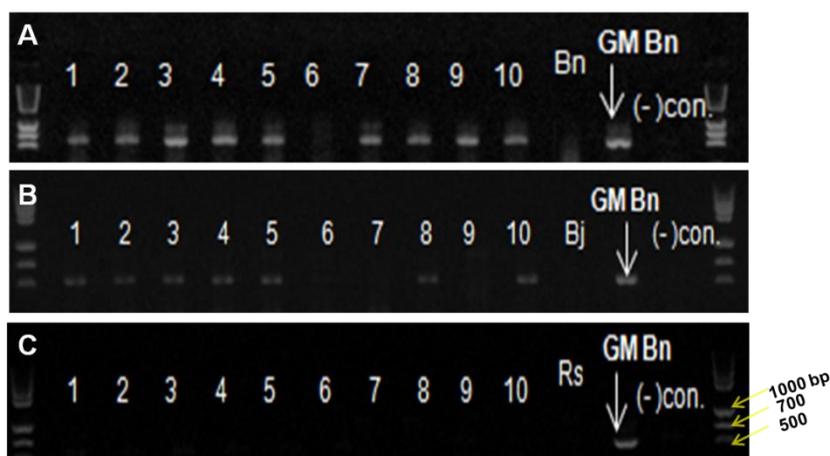


Fig. 9. PCR confirmation using survivals of *B. napus* cv. Tammi (A), *B. juncea* (B), and *R. sativus* (C) genomic DNA after glufosinate-ammonium application. 1-10, suspected survivals after glufosinate-ammonium application. Bn, *B. napus* cv. Tammi; Bj, *B. juncea*; Rs, *R. sativus*; GM Bn, glufosinate-ammonium resistant *B. napus*; (-) Con: negative control, amplified by PCR without DNA sample.

Table 11. Summary of survival rate (SR) and hybrid rate (HR) of non-GM pollen progenies (*B. napus*, *B. juncea*, and *R. sativus*) at various distances from the GM pollen donor in 2013

Distance (m)	<i>B. napus</i>		<i>B. juncea</i>		<i>R. sativus</i>	
	SR (%) ^a	HR (%) ^b	SR (%) ^a	HR (%) ^b	SR (%) ^a	HR (%) ^b
4	1.993	98.51	0.056	100.00	0.721	0.000
8	1.072	98.33	0.042	66.67	0.457	0.000
16	0.472	97.75	0.025	33.33	0.957	0.000
24	0.229	95.00	0.000	-	0.672	0.000
32	0.113	94.44	0.000	-	0.393	0.000
45	0.080	83.33	0.000	-	0.978	0.000
65	0.019	66.67	0.000	-	0.784	0.000
75	0.007	100.00	0.000	-	0.278	0.000
85	0.000	-	0.000	-	0.393	0.000
95	0.000	-	0.000	-	0.500	0.000
LSD _{0.05}	0.53	12.1	0.03	18.3	0.06	0

Survival rate (SR) and hybrid rate (HR) were determined by herbicide resistance screening test and PCR analysis, respectively.

^a Data in column of survivals rate means number of survivals after herbicide resistance screening test/total number of seedlings tested for various distances recipients of non-GM *B. napus*, non-GM *B. juncea*, and non-GM *R. sativus*

^b Data in column of hybrid rate means number of tested survivals with GM *B. napus bar-specific* DNA fragment/total number of survivals for various distances recipients of non-GM *B. napus*, non-GM *B. juncea*, and non-GM *R. sativus*

Table 12. Summary of survival rate (SR) and hybrid rate (HR) for non-GM pollen recipients (*B. napus*, *B. juncea*, and *R. sativus*) at various distances from the GM pollen donor in 2014

Distance (m)	<i>B. napus</i>		<i>B. juncea</i>		<i>R. sativus</i>	
	SR (%) ^a	HR (%) ^b	SR (%) ^a	HR (%) ^b	SR (%) ^a	HR (%) ^b
2	2.394	97.60	0.085	90.00	0.105	0.000
4	1.256	96.90	0.034	100.00	0.113	0.000
8	0.637	98.33	0.009	100.00	0.000	-
16	0.342	92.50	0.008	0.00	0.000	-
32	0.138	93.33	0.000	-	0.000	-
64	0.077	100.00	0.000	-	0.000	-
128	0.000	-	0.000	-	0.000	-
LSD _{0.05}	0.12	7.3	0.01	9.2	0.04	0

Survival rate (SR) and hybrid rate (HR) were determined by herbicide resistance screening test and PCR analysis, respectively.

^a Data in column of survivals rate means number of survivals after herbicide resistance screening test/total number of seedlings tested for various distances recipients of non-GM *B. napus*, non-GM *B. juncea*, and non-GM *R. sativus*

^b Data in column of hybrid rate means number of tested survivals with GM *B. napus bar-specific* DNA fragment/total number of survivals for various distances recipients of non-GM *B. napus*, non-GM *B. juncea*, and non-GM *R. sativus*

Gene flow rate from GM *B. napus* to its relatives

non-GM *B. napus* (♀) × GM *B. napus* (♂)

The confirmed gene flow rates from GM *B. napus* to its relatives in 2013 and 2014 were summarized in Table 13. For non-GM *B. napus* recipient, in 2013, the gene flow rate ranged from 0-1.961% at distance of 4-95m, with the highest rate of 1.961% at 4m, 1.059% at 8 m, 0.459% at 16 m, constantly decreasing to 0.007% at 75 m, and no gene flow was detected beyond 85 m. In 2014, it was 2.330% at 2 m, 1.217% at 4 m, 0.624% at 8 m, 0.317% at 16 m, 0.125% at 32 m, and 0.066% at 64 m, and no gene flow was detected at 128 m (Table 13). Overall, the gene flow rate estimated at the same distance in 2013 was higher than those estimated in 2014, with the values of 1.961% vs. 1.217% at 4 m, 1.059% vs. 0.624% at 8 m, and 0.459% vs. 0.317% at 16 m, respectively.

non-GM *B. juncea* (♀) × GM *B. napus* (♂)

For non-GM *B. juncea* recipient, in 2013, the gene flow rate ranged from 0-0.056% at distance of 4-95m, with the highest rate of 0.056% at 4 m, 0.042% at 8 m, and 0.025% at 16 m, and no gene flow was detected beyond 24 m. In contrast, in 2014, it was 0.076% at 2 m, 0.034% at 4 m, and 0.008% at 8 m, and no gene flow was detected beyond 16 m (Table 13). A relatively higher gene flow values was also observed in 2013 compared to 2014.

non-GM *R. sativus* (♀) × GM *B. napus* (♂)

With regard to gene flow to non-GM *R. sativus*, regardless of the distances from GM *B. napus*, no gene flow was detected in both years (Table 13).

Table 13. The confirmed gene flow rate (%) for non-GM pollen recipients (*B. napus*, *B. juncea*, and *R. sativus*) at various distances from GM pollen donor in 2013 and 2014

Distance (m)	<i>B. napus</i>		<i>B. juncea</i>		<i>R. sativus</i>	
	2013	2014	2013	2014	2013	2014
2	- ^a	2.330	-	0.076	-	0.000
4	1.961	1.217	0.056	0.034	0.000	0.000
8	1.059	0.624	0.042	0.008	0.000	0.000
16	0.459	0.317	0.025	0.000	0.000	0.000
24	0.216	-	0.000	-	0.000	-
32	0.107	0.125	0.000	0.000	0.000	0.000
45	0.068	-	0.000	-	0.000	-
64	-	0.066	-	0.000	-	0.000
65	0.013	-	0.000	-	0.000	-
75	0.007	-	0.000	-	0.000	-
85	0.000	-	0.000	-	0.000	-
95	0.000	-	0.000	-	0.000	-
128	-	0.000	-	0.000	-	0.000
LSD _{0.05}	0.034	0.089	0.036	0.025	NS ^b	NS

The confirmed gene flow rate calculated by multiplied survivals rate by hybrid rate for various distances recipients of non-GM *B. napus*, non-GM *B. juncea*, and non-GM *R. sativus*

^a Short dash lines mean the distance not included in the corresponding year ^b Not significant at $P=0.05$

Estimation of potential gene flow

The significant difference in gene flow from GM *B. napus* to its relatives of non-GM *B. napus* and *B. juncea* was found between 2013 and 2014 years ($P < 0.05$). To estimate potential gene flow, the relatively higher gene flow rates of non-GM *B. napus* and *B. juncea* within two years respectively selected were fitted to the biological meaningful log-logistic model.

There was no evidence of lack of fit of the log-logistic model ($P = 0.8685$ for non-GM *B. napus* and 0.9996 for non-GM *B. juncea*). The smaller RMS values (0.03 for non-GM *B. napus* and 0.001 for non-GM *B. juncea*) and R^2 (0.963 for non-GM *B. napus* and 0.313 for non-GM *B. juncea*), indicating that the three-parameter log-logistic model provided an acceptable fit to the gene flow data both for non-GM *B. napus* and non-GM *B. juncea*. Based on the predictable model, the maximum potential gene flow rate was 2.57% (and 0.08%) when GM *B. napus* and non-GM *B. napus* (and non-GM *B. juncea*) were planted adjacently. The D_{50} value indicating that the distance required for 50% of gene flow rate reduction compared to the estimated gene flow rate was 6.94 m for non-GM *B. napus* and 8.98 m for non-GM *B. juncea*, respectively (Fig. 10; Table 14).

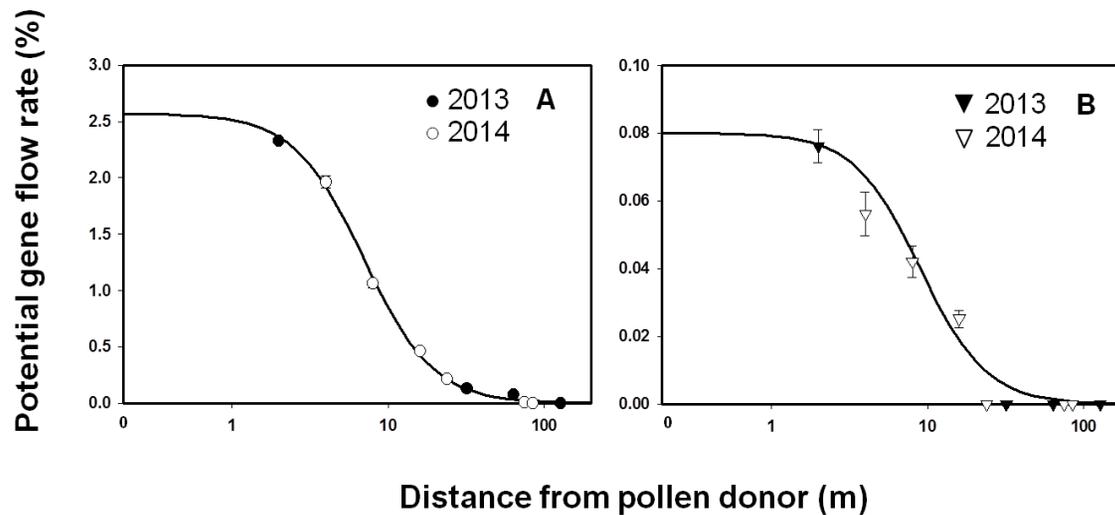


Fig. 10. The estimated equations for prediction of potential gene flow rate for non-GM *B. napus* (A, $Y = \frac{2.57}{1+(\frac{X}{6.94})^{1.95}}$, lack of fit, $P=0.8685$) and non-GM *B. juncea* (B, $Y = \frac{0.08}{1+(\frac{X}{8.98})^{2.06}}$, lack of fit, $P=0.9996$) using higher gene flow rate within each year.

Table 14. Estimated parameters for log-logistic analysis of the gene flow rate for recipients of non-GM *B. napus* and *B. juncea* under field condition

Species	Parameters ^a			DF	RMS	R ²	P values
	Y ₀	b	D ₅₀ (95% CI)				
<i>B. napus</i>	2.57	1.95	6.94 (5.7-9.2)	81	0.03	0.963	< 0.0001
<i>B. juncea</i>	0.08	2.06	8.98 (6.25-9.7)	81	0.001	0.313	< 0.0001

^a Y₀, the maximum gene flow rate when distance between pollen donor and recipient is 0; b, slope of the log-logistic curve; D₅₀, the distance required for 50% of gene flow rate reduction compared to the maximum gene flow rate
 DF, degree of freedom; RMS, residual mean square; R², coefficient of determination

DISCUSSION

A two-year study of potential risk assessment on gene flow from GM *B. napus* to its relatives was conducted in Korea. In order to quantify the potential gene flow, a favorite condition was stimulated including entire synchrony of flowering periods between GM pollen donor and recipient and introduction of pollinator. Herbicide resistance and simple sequence repeat (SSR) markers were employed to detect the hybrids among the progenies. The studies demonstrated that the considerable GM *B. napus* gene flow rate to non-GM *B. napus* and non-GM *B. juncea* at a specified distance under simulated favorite condition, but no gene flow occurred to non-GM *R. sativus*. Based on the experimental data, a simulation model was developed for prediction of gene flow from GM pollen donor to its relatives at a specific distance from GM pollen donor.

Numerous studies on outcrossing events and gene flow of GM *B. napus* with its relatives have been carried out world-wide including GM *B. napus* cultivated countries, such as Canada, USA, and Australia, and non-GM *B. napus* cultivated countries, such as European and Asian countries (Beckie et al. 2003; Cai et al. 2008; Krato and Petersen 2012; Landbo et al. 1996; Morris et al. 1994; Rieger et al. 2002; Warwick et al. 2003; Weekes et al. 2005). *B. napus* displayed the extensive intraspecific and interspecific crossability with its relatives such as *B. rapa*, *B. juncea*, *R. raphanistrum*, etc., both in artificial means and under field condition. In our studies, the outcrossing result between non-GM *B. juncea* (♀) × GM *B. napus* (♂) was also agreement with the previous outcrossing event. But in the present simulated conditions, we did not detect hybrids between non-GM *R. sativus* (♀) × GM *B. juncea* (♂) in two years studies, although some hybrids have been reported using artificial means such as ovule culture and embryo rescue methods (Ellerstrom 1978; Paulmann and Robbelen 1988; Takeshita et al. 1980). Up to now, only one existing study on this regard without hand pollination or embryo rescue method was reported by using a transgenic male sterile *B. napus* as recipient (Ammitzboll and

Jørgensen 2006).

Gene flow was importantly influenced by several factors including experimental design (e.g., the shape, orientation and size of pollen donor and recipient field), the distance from pollen donor, the insect movement and types, local climatic environment condition as well as the genotype and zygosity of donors (Becker et al. 1992; Hüsken and Dietz-Pfeilstetter 2007; Scheffler et al. 1993; Tang et al. 2003). The previous studies showed a decrease of pattern on gene flow with increasing the distance from pollen donor. Staniland et al. (2000) revealed that gene flow rate was 1.6% at 1.5 m, 0.7% at 4 m, 0.3% at 11.5 m, and sharply decreased to 0.03% at 31.25 m from the pollen donor by using bromoxynil resistance marker. The similar decrease patterns were also seen in other studies (Cai et al. 2008; Krato and Petersen 2012; Paul et al. 1995; Scheffler et al. 1993). Not only within a field scale, but the gene flow also can occur at a considerable distance. A rate of 0.07% at 800 m from the GM pollen donor was reported by Beckie (2003), and an investigation over one-third of Australia covering a range of environments showed that pollen-mediated gene flow can occur beyond 2000 m (Rieger et al. 2002). Moreover, two sites of American studies, California and Georgia, indicated that gene flow was greatly affected by the local climatic condition (Ramsay 2003).

non-GM *B. napus* (♀) × GM *B. napus* (♂)

In our field experiments in both years, with presence and abundance of honeybee and entire synchrony of flowering with non-GM *B. napus*, the gene flow rate ranged from 0.007-1.961% at 4-75 m in 2013, and 0.066-2.330% at 2-128 m in 2014. The gene flow rates were (2.330% at 2 m in 2014 and 1.961% at 4 m in 2013) much higher than rates estimated at the similar distance from pollen donor in other studies, 0.74% at 2 m in China (Zhao et al. 2013), 0.41% at 3 m in UK (Scheffler et al. 1993), 0.7% at 4 m in Canada (Staniland et al. 2000), 0.4% at 4.6 m in USA (Morris et al. 1994). In other distances in our experiment, the estimated gene flow rate are also relatively higher in comparison with values estimated in other studies (Di et al. 2009; Krato and Petersen 2012; Scheffler et al. 1993), the reason for that

could be ascribed to more honeybee visit frequency and longer overlapped flowering periods between GM pollen donor and pollen recipient. However, unlike other studies reported gene flow occurred at far distance from pollen donor, in our experiment we did not detect gene flow at the distance beyond 85 m, which might be associated with the difference in sizes of the GM pollen donors and pollen recipients as well as discontinuous design in our studies.

non-GM *B. juncea* (♀) × GM *B. napus* (♂)

Lots of studies have been conducted on outcrossing events between GM *B. napus* and non-GM mustard, few on outcrossing rate in association with distances from pollen donor. A recent Japanese study on this regard revealed a decreased gene flow rate in non-GM *B. juncea* with increasing the distance from pollen donor (GM *B. napus*) (Tsuda et al. 2012). As concluded before, synchrony of flowering between pollen donor and recipient is the key factor to determine the gene flow occurring, a lack of synchrony of geographic distribution or in flowering periods, the outcrossing either drastic reduction or no occurred. Therefore, if flowering periods overlapped, the possibilities of gene flow would be increased greatly (Jorgensen and Andersen 1994; Landbo et al. 1996). In a two-year' gene flow studies of *B. juncea* (♀) × *B. napus* (♂) conducted in Japan, 55 hybrids were detected with an entire synchrony of flowering (34 days) by artificial supply of GM donor plants in 2010 in comparison with none of hybrids detected with asynchrony of flowering (19 days) under natural flowering condition in 2009 (Tsuda et al. 2012), indicating that gene flow is significantly associated with the degree of periods of synchronous flowering.

In our experiment, an entire synchrony of flowering of 31 (in 2013) and 43 days (in 2014) between *B. juncea* and *B. napus* was achieved by transplanting of three different times' GM *B. napus*, resulting from a gene flow rate ranging from 0.025-0.056% at 4-16 m and 0.008-0.076% at 2-8 m in 2013 and 2014, respectively. The gene flow rate was 0.076% at 2 m (in 2014) and 0.056% at 4 m (in 2013) from the GM *B. napus* compared with 0.049% at 1 m and 0.037% at 5 m reported in a

Japanese study (Tsuda et al. 2012). The higher gene flow rate estimated in our experiment is closely related to the higher honeybee visit frequency during flowering periods and entire synchrony of flowering between pollen donor and pollen recipient. Similar to the previous study, a decreased pattern of gene flow rate with increasing the distance from GM pollen donor was also found in our studies. For instance, in 2013, the gene flow rate was 0.056% at 4 m, 0.042% at 8 m, and decreased to 0.025% at 16 m, and in 2014, it was 0.076% at 2 m, 0.034% at 4 m, and 0.008% at 8 m. In addition, in our studies, no hybrids were detected at the distance of >24 m from the GM pollen donor in both years (Table 13), which was similar to the previous estimate of 20 m (Tsuda et al. 2012). Moreover, despite at the presence and abundance of honeybee pollination and entire synchrony of flowering between donor and recipient, our results showed that gene flow rate from GM *B. napus* to non-GM *B. juncea* under field condition was still low, which would be related with the high self-compatibility of *B. juncea* (Ohsawa and Namai 1987). However, due to the possibility of outcrossing existing between these two species, the further study for fitness of hybrids still needs to be continued along with the long-term investigation of agricultural fate of hybrids.

non-GM *R. sativus* (♀) × GM *B. napus* (♂)

In two-year's studies, no hybrids were detected between non-GM *R. sativus* (♀) × GM *B. napus* (♂) under the artificially stimulated conditions, indicating that the possibility of GM *B. napus* gene flow to non-GM *R. sativus* was extreme low. Moreover, because *R. sativus* is generally harvested prior to flowering and seed development, unless being used for seeds production, such a lack of synchrony of flowering would make the gene flow event extremely unlikely under natural agricultural condition. However, in rare event that such hybrid occurred using a genetically male sterile *B. napus* as recipient, although they were proved to be highly sterile. By this token, more and further outcrossing studies should be conducted at different crosses directions and combinations between *B. napus* and a range of *R. sativus* cultivars.

In summary, this is the first field study of risk assessment on GM *B. napus* gene flow to its relatives in Korea. This study provides useful baseline information for potential risk assessment on gene flow between GM *B. napus* and its relatives and decision-making for proper isolation distance in Korea. The experimental data presented here clearly indicated the possibility of gene flow from GM *B. napus* to non-GM *B. napus* under simulated favorite field condition. It was worth pointing out that gene flow and pollen dispersal distance varied in different regions due to the local climatic and varietal differences. In the present study with presence of honeybee and entire synchrony of flowering between GM *B. napus* and non-GM *B. napus*, gene flow rate was much higher than rates estimated in other studies at the similar distance. Therefore, we could expect a considerable higher gene flow rate if stimulating more suitable condition under field environment. Nowadays, GM *B. napus* is not commercially cultivated in Korea and other Asian countries. However, more knowledge are needed to accumulate for better understanding gene flow under natural environment, outcrossing events under different experimental condition, consequence of gene, long-term gene flow monitoring, and gene flow mitigation as well as management.

Gene flow between *B. napus* and *B. juncea* could possibly occur both under natural field and optimal simulated conditions. Although gene flow rate has been proved very low, the possibility of occurrence has raised the biosafety concern of growing GM *B. napus* with outcrossing with *B. juncea*. *B. juncea* is widely distributed in Korea and its flowering periods is completely synchronized with *B. napus*, and natural occurrence of gene flow could be predicted in case GM *B. napus* growing adjacently with *B. juncea*. In our experiment, we did not detect gene flow at the distance >24 m. However, we believe that the natural spontaneous gene flow between GM *B. napus* and *B. juncea* could be occurred at a considerable distance under optimal conditions, such as the abundance of pollinators, maximum synchrony of flowering, suitable climatic environments etc. Therefore, large-scale and long distance field evaluation of gene flow should be conducted. Because crossed hybrids between GM *B. napus* and *B. juncea* have been reported showing

higher fertility and inherited herbicide resistance (Di et al. 2009; Song et al. 2010), the further studies on fitness test of hybrids and inheritance behavior of gene in backcrossed generation should be continued as well.

Gene flow between GM *B. napus* and *R. sativus* under natural field condition is practically impossible. No hybrids were detected despite at the presence and abundant of honeybee during the flowering periods and entire synchrony of flowering. Up to now, only one paper was reported successful natural outcrossing between these two species with a genetically modified male sterile *B. napus* as the maternal plant, but the hybrids would be highly sterile. Since a lack of synchrony of flowering between them as well, therefore, there is no much concern on gene flow between GM oilseed and *R. sativus*. However, the further studies should be continued to investigate in more detail on gene flow under various simulated natural condition, different cross directions, and different combinations between *B. napus* and a various *R. sativus* cultivars.

The isolation distance required for GM trial quite varies widely, in the case of *B. napus*, although nations-wide, it ranges from 50-400 m. In United Kingdom, a 50 m isolation distance is recommended for GM cultivation, with a 200 m isolation distance recommended for organic crops. In addition, an isolation distance of 50 m is officially required for growing crops with high levels of erucic acid in UK (Salisbury 2002; SCIMAC 1999). In Canada, a 200 m isolation distance for GM canola trials is stipulate (Staniland et al. 2000). An isolation distance of 400 m is required for GM trials in France, Belgium and Sweden (EC 2002). Australian GM trial requirements include a 400 m isolation distance and a 15 m non-GM buffer zone (Salisbury 2002). In China, a recent study recommended that 300 m, rather than 1000 m, is a reasonable isolation distance for GM trials (Zhao et al. 2013). Up to now, there is no regulation on isolation distance for GM trial in Korea. If a 0.1% tolerance threshold of gene flow rate is considered to be acceptable, then an isolation distance between GM *B. napus* and non-GM *B. napus* (e.g. 35 m) is acceptable for based on our study, which is similar to the previous study in China of 25 m (Zhao et al. 2013) and shorter than UK's regulation of 50 m (Salisbury 2002;

SCIMAC 1999). The tolerance threshold of a 0.01% gene flow rate is chosen on the basis of the lowest threshold value for labeling of GM in food and feed in the world (EU 2003). By this taken, keeping the gene flow rate below 0.01% should ensure both seed purity and the market acceptability of products. Thus, according to the equation in our study, an isolation distance of 122.5 m is recommended for preventing gene flow from GM *B. napus* and non-GM *B. napus*. When the distance is farther than 500 m, the gene flow rate is lower than 0.001%. In the case of the isolation distance for *B. juncea*, although there is no specific regulation worldwide, based on our study, a 24 m isolation is sufficient to prevent gene flow rate exceed the tolerance threshold of 0.01%, and a 60 m isolation distance could prevent the gene flow rate lower than 0.001%. However, for *R. sativus*, our results demonstrated that the extreme low possibility for outcrossing with *B. napus*.

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CHAPTER III

Modeling of maximum potential gene flow from genetically modified *Brassica napus* to its male sterile relatives under open and wind pollination conditions

ABSTRACT

A field experiment was conducted to evaluate gene flow from genetically modified (GM) *Brassica napus* to its non-GM male sterile (MS) recipients, *B. napus*, *B. juncea*, and *Raphanus sativas* under simulated open and wind pollination conditions in 2014. The three non-GM MS relatives were planted at three independent directions at various distances of 2, 4, 8, 16, 32, 64, and 128 m from the GM *B. napus* pollen donor. The honeybee was introduced as the pollinator during the flowering period. At each distance, a half of non-GM MS relative plants were covered with the insect-preventing net to simulate wind pollination condition and the other half were not covered with the net to simulate open pollination condition. Pods setting rate was initially determined by counting the number of flowers and pods. Herbicide (glufosinate-ammonium) and polymerase chain reaction (PCR) marker were used to confirm the F1 hybrids resulted from outcrossing between GM *B. napus* and its MS relatives. Gene flow rate from GM *B. napus* to MS *B. napus* were 32.48% and 14.69% at 2 m and, decreased with increasing distance down to 0.3% and 0.26% at 128 m distance under open and

wind pollination conditions, respectively. Gene flow rates to MS *B. juncea* were 21.95% and 6.62% at 2 m and, 0.24% and 0.16% at 128 m distance under open and wind pollination conditions, respectively. The honeybee-mediated gene flow rates estimated by comparing the difference in gene flow between open and wind pollination conditions were 19.01% at 2 m and 0.03% at 128 m distance to MS *B. napus*, and 15.36% at 2 m and 0.06% at 128 m distance to MS *B. juncea*. Comparison of mathematical models to describe the relationship between gene flow rate and distance between GM *B. napus* and its relative showed that the log-logistic model is mathematically comparable to the double decay exponential model and provides better biological meaning. The model simulation recommended 2710 m and 254 m for MS *B. napus* and *B. juncea*, respectively, for 0.01% of gene flow as the tolerable threshold (isolation distance). No gene flow from GM *B. napus* to MS *R. sativus* was observed, suggesting no or low gene flow risk. Our results thus conclude that potential maximum gene flow rates from GM *B. napus* to MS *Brassica* species are much higher than those to male fertile (MF) *Brassica* species. The MS pollen recipients are useful to estimate potential gene flow from GM crops to their relatives.

Keywords: *Brassica juncea*, *Brassica napus*, gene flow, isolation distance, log-logistic model, open pollination, *Raphanus sativus*, wind pollination

INTRODUCTION

Over the past three decades, the global scale of cultivation GM crops has increased from 1.7 million ha in 1996 to 160 million ha in 2011 (James 2011). Rapid development and commercialization of genetically modified (GM) crops have more and more raised public concerns on unexpected ecological change and environmental effects (Beckie et al. 2006; Chèvre et al. 2003; Fagan 2004). One of the main concerns is the transgenes escape through outcrossing with their wild or weedy relatives, which has been the restriction for adaption of GM crops (Ellstrand et al. 1999; Firband and Forcella 2000; Warwick et al. 2003; Snow 2002). GM *B. napus* is such the case. The studies have shown that *B. napus* (AACC, 2n=38) could outcross with its many relatives such as *B. juncea* (AABB, 2n=36), *B. rapa* (AA, 2n=20), *B. niga* (BB, 2n=16), *B. oleracea* (CC, 2n=18), as well as *R. raphanistrum* (RrRr, 2n=18), etc. In addition, the pollen of GM oilseed rape could flow ranging from several to thousands meters based on the different climatic environment and agricultural structures (Beckie 2001; Bing et al. 1996; Cai et al. 2008; Scheffler et al. 1993, 1995; Chèvre et al. 2003; Krato and Petersen 2012; Rieger et al. 2002; Warwick et al. 2003; Weekes et al. 2005).

In GM *B. napus* cultivated countries such as United State, Canada, and Australia, the large-scale gene flow studies revealed that the outcrossing could occur at a considerable distance approximately 800 m with the outcrossing rate of 0.07% (Beckie 2001). Rieger et al. (2002) reported the pollen flow even could move beyond 2000 m with large field sizes in Australia. Moreover, studies conducted in two different sites, California and Georgia in United State (Morris et al. 1994), showed that gene flow was importantly affected by the local climatic environment. However, unlike the large farm scale agricultural system in North America and Australia, in Asian countries, co-existence of oilseed rape with its relative species is very common, where farmers generally managed small land and cultivated different crops side-by-side by each year. Overlapped flowering periods between *B. napus*

and its relatives or weedy species observed in many place, which poses a high risk of gene flow between them. In Japan, a field evaluation of gene flow from GM *B. napus* to *B. juncea* showed that the gene flow could occur at 17.5 m with the rate of 0.03% (Tsuda et al. 2012). The difference in overlapped flowering periods between GM *B. napus* and *B. juncea* resulted in a different gene flow rate (Tsuda et al. 2012). In comparison with our studies in 2013 in Korea, with artificially stimulated favorite conditions for facilitating gene flow, the potential gene flow rate from GM *B. napus* to *B. juncea* ranged from 0.008-0.076% at 2-16 m and from GM *B. napus* to non-GM *B. napus* ranged from 0.007-2.330% at 2-75 m, respectively. All data presented here showed that gene flow was very region- and climate -based, indicating that the necessity for conducting gene flow risk assessment under a specified climatic condition.

It is necessary and essential to establish a mathematical model to predict gene flow and reveal the relevant factors affecting gene flow under various conditions. Generally, there are two main approaches used for modeling gene flow (Klein et al. 2003; Snall et al. 2007). The first is mechanistic modeling that incorporates various biological factors affecting gene flow such as pollinators, pollen density, etc., and it can provide the detailed process of gene flow and reveal the pattern of gene flow under various factors (Klein et al. 2003; Gustafson et al. 2005; Snall et al. 2007). However, mechanistic models are usually mathematically complex containing many parameters and needed many precise data to perform the simulations, in some case those parameters are difficult to measure in natural conditions (Klein et al. 2003; Snall et al. 2007). The second approach is empirical modeling, which ignores the details of the gene flow process and establishes the equations by fitting the experimental data to existed regression model (Klein et al. 2003; Gustafson et al. 2005; Snall et al. 2007). The empirical method can be easily performed in practice, and usually contains few parameters. To some extent, although this empirical model is difficult to describe gene flow at various environment conditions, it exhibits a flexible and practical experience for a certain purpose to predict gene flow.

Some studies agree that bees carrying pollen travel a short distance within *B.*

napus field, whereas wind is responsible for long-distance pollen flow (Cresswell et al. 2004; Williams et al. 1987). However, regarding the relative contribution of wind and bees to gene flow and how they affect gene flow, these are still unknown. Thus, in the present study, by simulating the different pollination conditions, we evaluated the gene flow affected by wind and bees. In addition, taken into account the practical Asian agricultural structure (with co-existence of *B. napus* and its relatives), a discontinuous design was performed by locating GM pollen donor at one side and planting pollen recipients at various distances from pollen donor to simulate a mosaic agricultural structure. In addition, in order to improve the detection accuracy and sensitivity, a male sterile (MS) line was used as pollen recipient to amplify and differentiate gene flow rate resulting from wind and (/or) bees associated with distance increasing from the pollen donor.

Therefore, the objectives of this study were to i) evaluate the maximum potential gene flow from GM *B. napus* to its MS relatives under open and wind pollination conditions and accordingly, estimate honeybee-mediated gene flow by comparing the difference between the two pollination conditions. To this end, the insect preventing-net treatment, with or without honeybee, was conducted to simulate the open and wind pollination conditions; ii) by fitting the obtained experimental data to log-logistic model, to establish gene flow equation for prediction of gene flow rate against distance.

MATERIALS AND METHODS

Plant materials

The genetically modified (GM) and non-genetically modified male sterile (non-GM MS) plants materials were used in this study. The GM *B. napus* (obtained from National Academy of Agricultural Science, Korea) homozygous and having the *bar* gene (Phosphinothricin acetyltransferase) which confers resistance to the herbicide glufosinate-ammonium (trade name Basta®), was used as the pollen donor and non-GM MS plants including *B. napus*, *B. juncea*, and *R. sativas* were used as the pollen recipients. The non-GM MS recipients can flower normally but failed to produce pollen. All seeds were sowed and cultivated in greenhouse of the experimental farm station of Seoul National University, Suwon, Korea. The vernalization treatment was performed under natural condition during the winter season (approximately 1.5 months) when the growth stage of seedlings was at two-true leaf stages.

Field design

The study was performed under the authorization field of the experimental farm station of Seoul National University, Suwon, Korea (authorization number: RDA-AB-2011-014) from March to July in 2014. The seedlings' transplanting and cultivation as described in Chapter II. A discontinuous design with planting GM pollen donor at one side and locating non-GM MS recipient at various distances from GM pollen donor was adopted for mimicking the mosaic-like agricultural structure. Both pollen donor and recipients plants were planted in field on 27th March. Three different growth stages of GM *B. napus* pollen donor in total 256 plants (planting area: 8 m × 8 m, with 50 cm plant spacing and row spacing, 9 plants m⁻²) were employed to ensure maximum synchronization of flowering period with non-GM MS recipients. Three non-GM MS recipients including *B. napus*, *B. juncea*, and *R. sativas* were planted at three independent directions at the various distances of 2, 4, 8, 16, 32, 64, and 128 m from the GM pollen donor (Fig. 11). Each

pollen recipient line at the each distance consists of 6 plants (planting in one row with 50 cm spacing). Prior to flowering, half of the recipients (3 plants) at each distance was treated by insects-preventing nets for only allowing wind pollination, but others being pollinated both by insects and wind. An approximately 10,000 population of honeybee colony was introduced during flowering period from 3th April. Flowering phenology and honeybee visit were investigated as described in Chapter II. Fertilizer application, insecticide spray, and irrigation management were conducted accordingly.

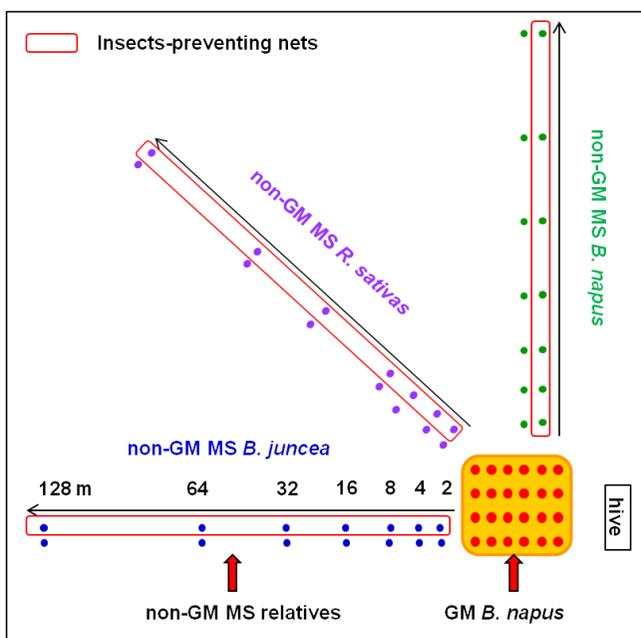


Fig. 11. Experimental field design for evaluation of gene flow from GM *B. napus* to its non-GM MS relatives in Suwon, Korea. The GM *B. napus* and non-GM MS relatives (*B. napus*, *R. sativas*, and *B. juncea*) were used as pollen donor and recipient, respectively. The recipient plants were placed at various distances of 2, 4, 8, 16, 32, 64, and 128 m. Half of recipient plants at each distance was treated by the insects-preventing nets prior to flowering. The location of the honeybee hive is shown.

Identification of hybrids

At maturity, all pods were hand-harvested from all recipients plants at various distances from GM pollen donor as illustrated in Fig. 11. All harvested pods were shelled, dried, counted (seed counter, USA), and stored at 4°C before herbicide screening test. The herbicide resistance screening test was conducted in 200-well multi-pots in the greenhouse of the experimental farm station of Seoul National University, Suwon, Korea from September to November in 2014. The glufosinate-ammonium was applied to seedlings of progenies of *B. napus*, *B. juncea*, and *R. sativas* at the one true-leaf stage at the pre-determined dose of 1.2 g, 0.6 g, and 2.4 g ai L⁻¹, respectively (see Chapter II), by using a compressor pressurized belt-driven sprayer (R & D Sprayer, USA) equipped with an 8002E flat-fan nozzle (Spraying System Co., USA) to deliver a spray volume of 600 L ha⁻¹. The number of tested seedlings for all species was counted prior to herbicide treatment. The survivals were counted 14 d after herbicide treatment. The survivals with resistance to the herbicide glufosinate-ammonium were considered as the hybrids that were outcrossed between GM *B. napus* and its relatives. A subsequent PCR confirmation was further performed to check the proportion of hybrids with *bar-specific* DNA fragment among total survivals. To comply with the conditions laid down by the Korean government, all the vegetative and seeds not used for further analysis were burned.

PCR analysis

The young leaf samples were collected from those survived 2-week-old seedlings after glufosinate-ammonium screening, immediately treated with liquid nitrogen, and stored at -80°C freezer. Genomic DNA was extracted by following the minor modified cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle 1990) and standardized to a final concentration of 20 ng μL⁻¹. PCR analysis was described as in Chapter II. Seedlings with the detected *bar-specific* DNA fragments were confirmed as true hybrids.

Statistical analysis

Each individual recipient at various distances was harvested separately and considered as replications. Gene flow rate for each species and each distance was calculated separately for both open (pollinated both by honeybee and winds) and wind (pollinated only by wind) pollination conditions. Honeybee-mediated gene flow was estimated by comparing the difference between open and wind pollination. The formula for calculating gene flow rate was showed as below:

$$\text{Gene flow rate (\%)} = \text{pods setting rate} \times \text{calibration factors rate} \times 100\%$$

The pods setting rate was calculated by dividing number of pods set by that of number of flowers. The calibration factors rate were determined by herbicide screening and PCR analysis. Herbicide screening with randomly selected seeds from all progenies was to check the proportion of number of survivals in total number of seedlings tested and further calibrated using PCR analysis by detection of the proportion of survivals with *bar-specific* DNA fragment in those survivals from herbicide screening test. Pearson's correlation coefficients were computed within the parameters (such as distance, number of flowers, number of pods, pods setting rate, honeybee visit, etc.) to determine the possible correlation among them with the use of statistical software IBM SPSS for windows (version 22.0, SPSS Inc., Chicago, USA). Various regressions models including linear, inverse function, exponential decay, and log-logistic models were tested to fit the gene flow data against distance using statistical software R 3.2.0.

RESULTS

Synchrony of flowering

The GM *B. napus* was transplanted three different times on 4th, 11th, and 18th May for maximum synchrony of flowering periods with non-GM MS pollen recipients (non-GM MS *B. napus*, non-GM MS *B. juncea*, and non-GM MS *R. sativas*). Biological important dates such as 1st blooming, peak-flowering, and end of flowering for both pollen donor and recipients were summarized in Table 15. In the case of GM pollen donor, the duration of flowering was 41, 44, and 43 d for the three different times' transplanting, respectively, resulting a total 54 d flowering duration. For the non-GM MS pollen recipients, the duration of flowerings were 32, 38, and 38 d for *B. napus*, *B. juncea*, and *R. sativas*, respectively, those were completely synchronized with GM pollen donor (54 d) (Table 15).

Honeybee visit

Honeybee (*Apis mellifera*) was performed as the pollinator between GM *B. napus* and its relatives in the present gene flow study. During the flowering period, honeybee pollinating behavior was investigated every day (except raining days) at 10:00-11:00 am, and the visit frequency was described by $\text{plant}^{-1} \text{min}^{-1}$. Based on the data summarized in Fig. 12, a relatively higher density of honeybee gathering (approximately $1.35 \text{ plant}^{-1} \text{min}^{-1}$) observed near the GM pollen donor area. In addition, it also revealed that the honeybee visit frequency ($\text{plant}^{-1} \text{min}^{-1}$) in those three species was decreasing with increasing the distance from GM pollen donor accordingly (Fig. 12). For instance, for non-GM MS *B. napus*, the number of honeybee visit frequency ($\text{plant}^{-1} \text{min}^{-1}$) was 1.17 at 2 m, 1.08 at 4 m, 0.91 at 8 m, 0.74 at 16 m, and decreased to 0.29 at 128 m. In the case of non-GM MS *B. juncea*, it was 0.89 at 2 m, 0.48 at 16 m, and decreased to 0.20 at 128 m. For non-GM MS *B. juncea*, it was 0.89 at 2 m, 0.73 m at 4 m, 0.65 at 8 m, 0.48 at 16 m, and

decreased to 0.19 ($\text{plant}^{-1} \text{min}^{-1}$) at 128 m. The same pattern of honeybee visit also observed in non-GM MS *R. sativas* (Fig. 12). Additionally, when compared the number of honeybee visit frequency at the same distance in each species, it was differed notably (Fig. 12). For example, at 2 m from the GM pollen donor, it was 1.17, 0.89, and 1.31 ($\text{plant}^{-1} \text{min}^{-1}$) in non-GM MS *B. napus*, non-GM MS *B. juncea*, and non-GM MS *R. sativas*, respectively, and 1.08, 0.73, and 1.14 at 4 m, and 0.91, 0.65, and 1.09 at 8 m in those of three species. Among the three species, the flower of non-GM MS *R. sativas* showed the most attractive to honeybee, followed by non-GM MS *B. napus* and non-GM MS *B. juncea*.

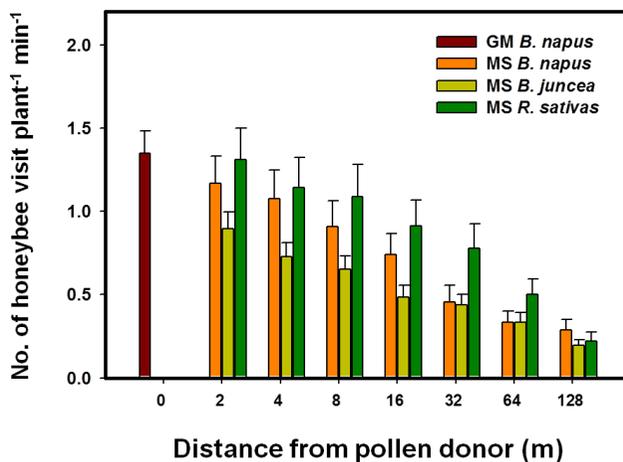


Fig. 12. Honeybee visit frequency ($\text{plant}^{-1} \text{min}^{-1}$) at various distances (m) from GM pollen donor during the flowering periods in 2014.

Table 15. Biological dates for a specific reproductive growth stage of pollen donor (GM *B. napus*) and pollen recipients (non-GM MS *B. napus*, non-GM MS *B. juncea*, and non-GM MS *R. sativas*) in 2014

Experimental plants	1 st blooming	Peak-flowering	End of flowering	Flowering duration (days)	Synchrony of flowering periods (days)
Pollen donor: Three times GM <i>B. napus</i> transplanting					
1 st time: Mar 4	Mar 27	Apr 19	May 7	41	-
2 nd time: Mar 11	Apr 1	Apr 26	May 14	44	-
3 rd time: Mar 18	Apr 6	May 2	May 19	43	-
Total flowering duration	-	-	-	54	
Pollen recipient: non-GM MS plants (transplanting date: Mar 4)					
<i>B. napus</i>	Apr 4	Apr 20	May 6	32	32
<i>B. juncea</i>	Apr 8	Apr 24	May 16	38	38
<i>R. sativas</i>	Apr 11	May 4	May 19	38	38

Determination of pods setting rate

Pods setting rate for non-GM MS *B. napus*, *B. juncea*, and *R. sativas* under open and wind pollination conditions were summarized in Table 16-18. According to the data, no significant difference was found in number of flowers in same species among the various distances ($P=0.05$), however, number of pods per plant for these three species was significantly decreased when increasing the distance from the GM pollen donor ($P=0.05$), resulting in a constantly decreased pods setting rate both under open and wind pollination conditions (Table 16-18). For non-GM MS *B. napus*, the pods setting rates were ranged from 0.7-38.9% under open pollination condition and 0.7-21.5% under wind pollination condition, 0.6-31.0% and 0.7-21.5% for non-GM MS *B. juncea*, and 0.6-13.4% and 0.7-4.4% for non-GM MS *R. sativas*, respectively.

Moreover, pods setting rates obtained under open pollination condition were higher than those values under wind pollination condition at the corresponding distance (Table 16-18). For instance, the pods setting rate for non-GM MS *B. napus* was 38.9% at 2 m, 34.5% at 4 m, 25.2% at 8 m, 18.2% at 16 m, and decreased down to 10.4% at 32 m under open pollination condition. Correspondingly, it was 21.5% at 2 m, 14.6% at 4 m, 10.1% at 8 m, 6.6% at 16 m, and 3.4% at 32 m under wind pollination condition (Table 16). In the case of non-GM MS *B. juncea*, the pods setting rate was 31.0% at 2 m, 21.5% at 8 m, 11.3% at 16 m, and 4.7% at 32 m under open pollination condition. Correspondingly, under wind pollination condition it was 10.8% at 2 m, 6.6% at 8 m, 4.8% at 16 m, and 3.0% at 32 m (Table 17). With regard to non-GM MS *R. sativas*, the similar pattern was showed as non-GM MS *B. napus* and *B. juncea* (Table 18). However, when the distance is farther than 32 m, the pods setting rate between open pollination and wind pollination condition in these three species did not show the notable difference ($P<0.05$). The rate under open pollination and wind pollination condition were 2.6 vs. 2.1% at 64 m and 0.7 vs. 0.7% at 128 m in non-GM MS *B. napus*, 2.4 vs. 1.8% at 64 m and 0.6 vs. 0.5% at 128 m in non-GM MS *B. juncea*, 0.7 vs. 0.6% at 64 m and 0.6 vs. 0.7% at 128 m in non-GM MS *R. sativas*, respectively (Table 16-18).

Table 16. Number of flowers, pods, and pods setting rate per plant for non-GM MS *B. napus* under open pollination and wind pollination condition

Distance (m)	Open pollination ^a			Wind pollination ^b		
	No. of flowers/plant	No. of pods/plant	Pods setting rate (%) ^c	No. of flowers/plant	No. of pods/plant	Pods setting rate (%)
2	1477	573	38.9	1321	281	21.5
4	1258	433	34.5	1455	211	14.6
8	1465	369	25.2	1406	144	10.1
16	1417	255	18.2	1143	76	6.6
32	1386	134	10.4	1649	55	3.4
64	1525	37	2.6	1483	31	2.1
128	1342	10	0.7	1438	10	0.7
LSD _{0.05}	387	43	5.1	360	26	2.4

^a Open pollination means pollination caused by both pollinators and wind

^b Wind pollination means by setting pollinators preventing-net only allowing wind pollination

^c Pods setting rate calculated by number of pods per plant/ no. of flowers per plant × 100%

Table 17. No. of flowers, pods, and pods setting rate per plant for non-GM MS *B. juncea* under open pollination and wind pollination condition

Distance (m)	Open pollination ^a			Wind pollination ^b		
	No. of flowers/plant	No. of pods/plant	Pods setting rate (%) ^c	No. of flowers/plant	No. of pods/plant	Pods setting rate (%)
2	1576	487	31.0	1687	182	10.8
4	1489	416	28.0	1622	141	8.7
8	1537	330	21.5	1627	107	6.6
16	1579	177	11.3	1599	77	4.8
32	1498	71	4.7	1593	47	3.0
64	1565	37	2.4	1614	28	1.8
128	1573	10	0.6	1589	8	0.5
LSD _{0.05}	140	28	2.5	138	18	1.1

^a Open pollination means pollination caused by both pollinators and wind

^b Wind pollination means by setting pollinators preventing-net only allowing wind pollination

^c Pods setting rate calculated by number of pods per plant/ no. of flowers per plant × 100%

Table 18. No. of flowers, pods, and pods setting rate per plant for non-GM MS *R. sativas* under open pollination and wind pollination condition

Distance (m)	Open pollination ^a			Wind pollination ^b		
	No. of flowers/plant	No. of pods/plant	Pods setting rate (%) ^c	No. of flowers/plant	No. of pods/plant	Pods setting rate (%)
2	1826	245	13.4	1875	83	4.4
4	1723	223	13.1	1888	64	3.4
8	1780	162	9.2	1751	41	2.4
16	1862	88	4.7	1779	25	1.4
32	1871	35	1.9	1840	18	1.0
64	1766	12	0.7	1635	9	0.6
128	1697	10	0.6	1668	9	0.7
LSD _{0.05}	183	19	1.7	198	11	0.6

^a Open pollination means pollination caused by both pollinators and wind

^b Wind pollination means by setting pollinators preventing-net only allowing wind pollination

^c Pods setting rate calculated by number of pods per plant/ no. of flowers per plant × 100%

Correlations between the parameters

Pearson correlation analysis was performed to calculate the possible relationship among the parameters of distance, number of pods, number of flowers, number of bees visit, and pods setting rate for the three species (Table 19). The results demonstrated that the number of pods, number of honeybee visit, or distance significantly correlated with pods setting rate, which means the three parameters greatly influence on gene flow ($P=0.05$ or 0.01). Number of pods and pods setting rate positively and significantly correlated with number of honeybee visit, indicating that the effect of pollinator on pollen transfer between pollen donor and recipient ($P=0.01$). No significant correlation was found between number of flowers and other parameters since it was negatively correlated with others ($P=0.05$ or 0.01).

Determination of calibration factors

For determination of calibration factors, herbicide screening test with randomly seeds selected from the progenies and a subsequent PCR confirmation with survivals from the herbicide screening test were performed. Based on survivals after herbicide screening test and PCR confirmation with those survivals by detection of GM *bar-specific* DNA fragment, the calibration factors for survivals and hybrids rates were calculated and shown in Tables 6-8. In herbicide screening test, approximately 10% of seeds in total for each species at each distance was tested. The survival rates for non-GM MS *B. napus* progenies ranged from 41.67-85.09% for open pollination condition and 36.11-83.53% for wind pollination condition at 2-128 m from GM pollen donor, respectively, and the rates for hybrid rate confirmed by PCR ranged from 96.67-100% and 75-100% for open and wind pollination conditions, respectively (Table 20). In the case of survival rate for non-GM MS *B. juncea* progenies, in herbicide screening test, it ranged from 41.67-84.57% and 45.45-81.45% for open pollination and wind pollination conditions, respectively, and hybrid rate confirmed by PCR ranged from 66.67-100% and

66.67-91.67% at 2-128 m from GM pollen donor (Table 21). For non-GM MS *R. sativas* progenies, only two survivals were screened, but both of them were proved to be false hybrids (Table 22). Overall, the survival rate for both recipients of non-GM MS *B. napus* and non-GM MS *B. juncea* near GM poll donor showed a significant greater than those of locating in a farther distance regardless of under open pollination or wind condition ($P<0.05$) (Table 20 and 21).

Table 19. Coefficients of the Pearson correlation among the parameters

Species	Parameters	Distance	No. of flowers	No. of bees	No. of pods	Pods setting rate	Gene flow rate
<i>B. napus</i>	Distance	1.000					
	Flowers no.	-0.082	1.000				
	Bees no.	-0.831*	-0.115	1.000			
	Pods no.	-0.823*	-0.043	0.991**	1.000		
	Pods setting rate	-0.832*	-0.138	0.995**	0.993**	1.000	
	Gene flow rate	-0.828*	-0.145	0.995**	0.991**	0.995**	1.000
<i>B. juncea</i>	Distance	1.000					
	Flowers no.	0.342	1.000				
	Bees no.	-0.862*	-0.202	1.000			
	Pods no.	-0.780*	-0.169	0.973**	1.000		
	Pods setting rate	-0.781*	-0.199	0.970**	0.999**	1.000	
	Gene flow rate	-0.763*	-0.230	0.950**	0.992**	0.995**	1.000
<i>R. sativas</i>	Distance	1.000					
	Flowers no.	-0.531	1.000				
	Bees no.	-0.950**	0.388	1.000			
	Pods no.	-0.761*	0.017	0.913**	1.000		
	Pods setting rate	-0.754*	-0.017	0.904**	0.999**	1.000	
	Gene flow rate	nc ^a	nc	nc	nc	nc	1.000

*correlation is significant at $p=0.05$, **correlation is significant at $p=0.01$ (two-tailed)

^a nc, not computed because gene flow rate estimated for *R. sativas* is 0

Table 20. Determination of calibration factors in non-GM MS *B. napus* progenies by herbicide screening test and PCR confirmation

Distance (m)	Open pollination ^a		Wind pollination ^b	
	Survival rate (%) ^c	Hybrid rate (%) ^d	Survival rate (%)	Hybrid rate (%)
2	83.34	100.00	81.93	83.50
4	85.05	100.00	82.16	86.31
8	84.75	100.00	80.52	100.00
16	82.78	96.67	79.78	91.67
32	77.87	100.00	83.53	100.00
64	76.52	100.00	76.59	75.00
128	41.67	100.00	36.11	100.00
LSD _{0.05}	8.2	3.8	6.2	22.2

^a Open pollination means pollination caused by both pollinators and wind

^b Wind pollination means by setting pollinators preventing-net only allowing wind pollination

^c Survival rate by herbicide screening test, calculated by dividing no. of survivals by total no. of seedlings tested

^d Hybrid rate by PCR confirmation, calculated by dividing no. of survivals with GM *B. napus bar-specific* bands by total no. of sampled survivals

Table 21. Summary of calibration factors in non-GM MS *B. juncea* progenies by herbicide screening test and PCR confirmation

Distance (m)	Open pollination ^a		Wind pollination ^b	
	Survival rate (%) ^c	Hybrid rate (%) ^d	Survival rate (%)	Hybrid rate (%)
2	84.57	84.49	81.45	76.39
4	81.38	94.19	78.57	87.78
8	84.28	95.83	78.91	91.67
16	78.55	83.33	77.78	85.00
32	78.47	66.67	80.41	91.67
64	66.39	75.00	72.94	88.89
128	41.67	100.00	45.45	66.67
LSD _{0.05}	8.0	16.4	9.7	28.9

^a Open pollination means pollination caused by both pollinators and wind

^b Wind pollination means by setting pollinators preventing-net only allowing wind pollination

^c Survival rate by herbicide screening test, calculated by dividing no. of survivals by total no. of seedlings tested

^d Hybrid rate by PCR confirmation, calculated by dividing no. of survivals with GM *B. napus bar-specific* bands by total no. of sampled survivals

Table 22. Summary of calibration factors in non-GM MS *R. sativas* progenies by herbicide screening test and PCR confirmation

Distance (m)	Open pollination ^a		Wind pollination ^b	
	Survival rate (%) ^c	Hybrid rate (%) ^d	Survival rate (%)	Hybrid rate (%)
2	0.18	0.00	0.00	0.00
4	0.00	0.00	0.00	0.00
8	0.00	0.00	0.00	0.00
16	0.45	0.00	0.00	0.00
32	0.00	0.00	0.00	0.00
64	0.00	0.00	0.00	0.00
128	0.00	0.00	0.00	0.00
LSD _{0.05}	0.6	NS	NS	NS

^a Open pollination means pollination caused by both pollinators and wind

^b Wind pollination means by setting pollinators preventing-net only allowing wind pollination

^c Survival rate by herbicide screening test, calculated by dividing no. of survivals by total no. of seedlings tested

^d Hybrid rate by PCR confirmation, calculated by dividing no. of survivals with GM *B. napus bar-specific* bands by total no. of sampled survivals

Gene flow rate from GM *B. napus* to its non-GM MS relatives

Finalized gene flow rate from GM *B. napus* to its non-GM MS recipients was summarized in table 23. Overall the date, a higher gene flow rate occurred with non-GM MS *B. napus* in comparison of non-GM MS *B. juncea* at the corresponding distance (Table 23). Gene flow rate showed a decreasing pattern with increasing the distance from the GM pollen donor in both species, regardless of open or wind pollination condition (Table 23). In the case of non-GM MS *B. napus* under open pollination condition, gene flow rate was 32.48%, 29.43%, 21.39%, 14.71%, and 8.21% at 2, 4, 8, 16, and 32 m, and greater than those rates of 14.69%, 10.42%, 8.14%, 4.77%, and 2.85% at the corresponding distances under wind pollination condition, respectively (Table 23). With regard to non-GM MS *B. juncea*, under open pollination condition it was 21.95%, 21.40%, 17.48%, 7.19%, and 2.44% at 2, 4, 8, 16, and 32 m, and in comparison of 6.62%, 6.05%, 4.72%, 3.22%, and 2.17% at the corresponding distances under wind pollination condition, respectively (Table 23). However, for gene flow rate in both species at 64 and 128 m, there was no significant difference between open pollination and wind pollination conditions ($P < 0.05$), with the values of 1.98% vs. 1.21% at 64 m and 0.30% vs. 0.26% at 128 m in *B. napus*, and 1.20% vs. 1.14% and 0.24% vs. 0.16% in *B. juncea*, respectively (Table 23). No matter what the distance was, the outcrossing did not occur between GM *B. napus* and non-GM MS *R. sativas* (Table 23).

Estimate of honeybee-mediated gene flow

The difference between open and wind pollination indicates that honeybee-mediated gene flow. In the case of gene flow rate from GM *B. napus* to non-GM MS *B. napus*, the estimate of honeybee-mediated gene flow rate was 17.78%, 19.01%, 13.24%, 9.94% at 2 m, 4 m, 8 m, 16 m, and decreasing to 0.03% at 128 m. As for non-GM MS *B. juncea*, the estimate of rate was 15.53%, 15.36%, 12.76%, 3.97% at 2 m, 4 m, 8 m, 16 m, and decreasing to 0.06% at 128 m (Table 23). There was no significant difference ($P < 0.05$) in honeybee-mediated gene flow at the farther distance as showed in Table 9, 0.77% (64 m) vs. 0.33% (128 m) for non-GM

MS *B. napus* and 0.03% (64 m) vs 0.03% (128 m) for non-GM MS *B. juncea*, respectively. The pattern of the estimated values for honeybee-mediated gene flow rate was closely associated with honeybee visit frequency, and decreased with increasing the distance from the GM pollen donor (Fig. 12; Table 23).

Table 23. Gene flow rate (%) from GM *B. napus* to its non-GM MS recipients (*B. napus*, *B. juncea*, and *R. sativas*) under open pollination (OP) and wind pollination (WP) conditions

Distance (m)	<i>B. napus</i>			<i>B. juncea</i>			<i>R. sativas</i>		
	OP (%)	WP (%)	(OP-WP) ^a	OP (%)	WP (%)	(OP-WP)	OP (%)	WP (%)	(OP-WP)
2	32.48	14.69	17.78	21.95	6.62	15.33	0.00	0.00	0.00
4	29.43	10.42	19.01	21.40	6.05	15.36	0.00	0.00	0.00
8	21.39	8.14	13.24	17.48	4.72	12.76	0.00	0.00	0.00
16	14.71	4.77	9.94	7.19	3.22	3.97	0.00	0.00	0.00
32	8.21	2.85	5.36	2.44	2.17	0.27	0.00	0.00	0.00
64	1.98	1.21	0.77	1.20	1.14	0.06	0.00	0.00	0.00
128	0.30	0.26	0.03	0.24	0.16	0.06	0.00	0.00	0.00
LSD _{0.05}	5.5	2.5	6.5	2.4	1.5	2.7	NS ^b	NS	NS

Gene flow rate calculated by pods setting rate × calibration factors

^a OP-WP, represents the contribution of honeybee to pollination

^b NS, not significant at $P=0.05$

Modeling of gene flow under different pollination conditions

Various regressions containing log-logistic, linear, inverse first order function, rational function, and double exponential decay models were performed to fit the gene flow data (Table 24 and 25). R^2 and RMS values were used to indicate the goodness of fit between gene flow data and those models. In the case of non-GM MS *B. napus*, the results suggested that the three-parameter log-logistic and four-parameter double exponential decay models were better suitable for data fitting than the other three models. As summarized in Table 24, the log-logistic and double exponential decay models have the similar smaller RMS values (approximately 8.5) and greater R^2 (>0.95) compared to other three models. The R^2 and RMS values estimated for other three models were 0.655 and 56.1 (linear model), 0.738 and 42.7 (inverse first order function), and 0.953 and 18.13 (rational function), respectively. With regard to non-GM MS *B. juncea*, log-logistic ($R^2=0.981$; RMS=1.81) and double exponential decay models ($R^2=0.965$; RMS=3.58) also showed the best goodness of fit among the tested models when comparing R^2 and RMS values with other models (Table 25). In the previous studies, double exponential decay model has been proposed for description of the gene flow rate against distance, but there is a lack of biological meaning for explanation of the estimated parameters. Therefore, the biological meaningful log-logistic model was considered as the most suitable model to fit the gene flow data.

As shown in Table 26, the log-logistic model provided a good fit to the data both for non-GM MS *B. napus* (Fig 13A, P values of lack of fit= 0.965) and non-GM *B. juncea* (Fig 13B, P values of lack of fit= 0.989) under different pollination conditions. The estimated honeybee-mediated gene flow was also well described by the log-logistic model with $R^2=0.892$ and RMS=12.0 for non-GM MS *B. napus* and $R^2=0.964$ and RMS=2.0 for non-GM MS *B. juncea*, respectively (Table 26). In addition, the estimated D_{50} values, the distance for 50% of gene flow rate reduction under open pollination condition (12.1 m for non-GM MS *B. napus* and 12.6 m for non-GM MS *B. juncea*), were farther than those of estimated under wind pollination condition (7.7 m for non-GM MS *B. napus* and 8.9 m for non-GM MS *B. juncea*),

indicating the contribution of honeybee-mediated gene flow. Moreover, the D_{50} estimated for honeybee pollination is greater than that of estimated for wind pollination in both species.

Table 24. Various models tested to fit the gene flow data from GM *B. napus* to non-GM *B. napus* against distances under open pollination condition

Models tested ^a		Parameters				DF	RMS	R ²	P values
Log-logistic	$y = y_0 / (1 + (x/D_{50})^b)$	y ₀ = 35.1 (2.7) ^b	D ₅₀ = 12.1 (1.7)	b= 1.40 (0.2)		18	8.8	0.951	< 0.0001
Linear	$y = y_0 + ax$	y ₀ = 23.9 (2.2)	a= -0.23 (0.04)			19	56.1	0.655	< 0.0001
Inverse first order function	$y = y_0 + a/x$	y ₀ = 6.60 (1.9)	a= 62.8 (8.6)			19	42.7	0.738	< 0.0001
Rational function	$y = (a+bx)/(c+x)$	a= 475.3 (92.3)	b= -4.0 (2.0)	c= 12.2 (3.2)		18	18.13	0.953	< 0.0001
Double exponential decay	$y = ae^{-bx} + ce^{-dx}$	a= 13.9 (13.2)	b= 0.17 (0.2)	c= 24.8 (15.5)	d= 0.04 (0.02)	17	8.4	0.954	< 0.0001

^a For log-logistic model: y₀, the maximum gene flow rate when distance between pollen donor and recipient is 0; b, slope of the log-logistic curve; D₅₀, the distance required for 50% of gene flow rate reduction compared to the maximum gene flow rate; For linear model: y₀, the maximum gene flow rate when distance between pollen donor and recipient is 0; a, the coefficient of determination of the curve; For inverse first order function: y₀, constant, a, the coefficient of determination of the curve; For rational function: a, c, constant; b, the coefficient of determination of the curve; For double exponential decay model: a, c, constant; b, d, the coefficient of determination of the curve.

^b The values in parentheses are the standard error of parameters estimated for models
DF, degree of freedom; RMS, residual mean square; R², coefficient of determination

Table 25. Various models tested to fit the gene flow data from GM *B. napus* to non-GM *B. juncea* against distances under open pollination condition

Models tested ^a		Parameters				DF	RMS	R ²	P values
Log-logistic	$y = y_0 / (1 + (x/D_{50})^b)$	y ₀ = 22.4 (0.7) ^b	D ₅₀ = 12.6 (0.7)	b= 2.6 (0.3)	18	1.8	0.981	< 0.0001	
Linear	$y = y_0 + ax$	y ₀ = 16.1 (1.8)	a= -0.16 (0.03)		19	38.8	0.572	< 0.0001	
Inverse first order function	$y = y_0 + a/x$	y ₀ = 3.75 (1.5)	a= 46.0 (6.8)		19	26.4	0.709	< 0.0001	
Rational function	$y = (a + bx)/(c + x)$	a= 298.9 (64.4)	b= -3.5 (1.5)	c= 10.3 (2.9)	18	5.3	0.945	< 0.0001	
Double exponential decay	$y = ae^{(-bx)} + ce^{(-dx)}$	a= 14.2 (3.7)	b= 0.07 (0.1)	c= 12.7 (4.3)	d= 0.07 (0.1)	17	3.6	0.965	< 0.0001

^a For log-logistic model: y₀, the maximum gene flow rate when distance between pollen donor and recipient is 0; b, slope of the log-logistic curve; D₅₀, the distance required for 50% of gene flow rate reduction compared to the maximum gene flow rate; For linear model: y₀, the maximum gene flow rate when distance between pollen donor and recipient is 0; a, the coefficient of determination of the curve; For inverse first order function: y₀, constant, a, the coefficient of determination of the curve; For rational function: a, c, constant; b, the coefficient of determination of the curve; For double exponential decay model: a, c, constant; b, d, the coefficient of determination of the curve.

^b The values in parentheses are the standard error of parameters estimated for models
DF, degree of freedom; RMS, residual mean square; R², coefficient of determination

Table 26. Estimated parameters for log-logistic analysis of the gene flow rate for both recipients of non-GM MS *B. napus* and non-GM MS *B. juncea* under different types of pollination

Species	Types of pollination	C (%)	b	D ₅₀ (m)	DF	RMS	R ²	P values
<i>B. napus</i>	Open pollination	35.1 (2.7)	1.4 (0.2)	12.1 (1.7)	18	8.5	0.951	<0.0001
	Wind pollination	16.4 (1.2)	1.3 (0.2)	7.7 (1.3)	18	1.9	0.932	<0.0001
	Honeybee pollination	19.0 (2.3)	1.7 (0.6)	16.2 (4.3)	18	12.0	0.892	<0.0001
<i>B. juncea</i>	Open pollination	22.4 (0.7)	2.6 (0.3)	12.6 (0.7)	18	1.8	0.981	<0.0001
	Wind pollination	8.9 (1.0)	0.9 (0.3)	8.9 (2.5)	18	0.7	0.900	<0.0001
	Honeybee pollination	15.5 (0.6)	3.8 (0.7)	12.1 (0.8)	18	2.0	0.964	<0.0001

C, the maximum gene flow rate predicted by log-logistic curve; SE, standard error; b, slope of the log-logistic curve; D₅₀, the distance required for 50% of gene flow rate reduction compared to the maximum gene flow rate; R², coefficient of determination; RMS, root mean square; df, degree of freedom

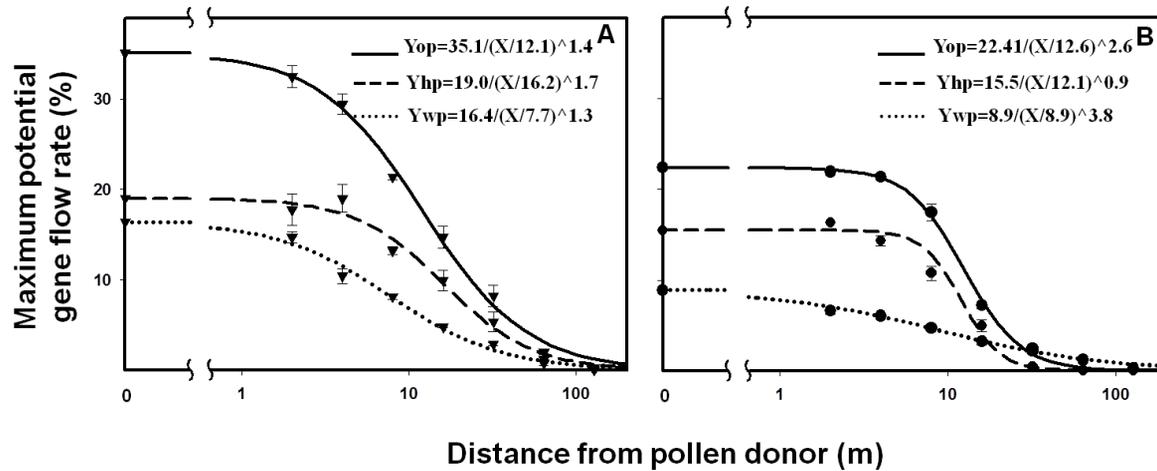


Fig. 13. The log-logistic model equations for prediction of gene flow rate against distance for non-GM MS *B. napus* (A, lack of fit, $P= 0.965$) and non-GM MS *B. juncea* (B, lack of fit, $P= 0.989$). The modeled equations of Y_{op} , Y_{hp} , and Y_{wp} represent the gene flow rate estimated under open pollination, honeybee-mediated pollination, and under wind pollination conditions, respectively.

DISCUSSION

In this study, a discontinuous design for estimating gene flow from GM *B. napus* to its MS relatives was performed under Korean climatic condition. Non-GM MS lines as recipients, presence and abundance of pollinators- honeybee, and entire synchrony of flowering period between pollen donor and recipients were involved in order to quantify the possibility of interspecific outcrossing and the maximum potential gene flow rate. The Pearson correlation analysis demonstrated that the gene flow rate was significantly correlated with honeybee visit frequency. Recipients treated with or without insect-preventing nets revealed the difference in gene flow between wind and pollinator pollination. On the basis of the finally confirmed gene flow data calibrated by herbicide screening and PCR confirmation test, a biological meaningful log-logistic equation was established for prediction of the gene flow rate at a specified distance from the pollen donor, which could be further used as a reference for determination of safe isolated distance when GM *B. napus* is cultivated.

Outcrossing

Numerous studies have reported the outcrossing events within *Brassica* family such as *B. napus* (AACC, 2n=38), *B. rapa* (AA, 2n=20), and *B. juncea* (AABB, 2n=36) both by artificial means and under natural field condition, due to these species sharing a common set of chromosomes and therefore facilitating interspecific outcrossing among them (Bing et al. 1996; Hansen et al. 2001; Jørgensen et al. 1996; Warwick et al. 2003). The outcrossing events also occurred between *Brassica* species and other cruciferous species such as *R. raphanistrum* (Darmency et al. 1998; Chèvre et al. 2000; Rieger et al. 2001), *R. sativas* (Ammitzboll and Jørgensen 2006; Gupta 1997; Huang et al. 2002), and *Sinapis arvensis* (Chèvre et al. 1996; Lefol et al. 1996), etc.

In our studies three species of recipient involved including non-GM MS *B. napus*, non-GM MS *B. juncea*, and non-GM MS *R. sativas*. The results are

agreement with the previous outcrossing events between *B. napus* and *B. juncea* even occurred at a considerable distance from the pollen donor (non-GM MS *B. juncea* (♀) × GM *B. napus* (♂)) (Tsuda et al. 2012). However, in the case of outcrossing between *B. napus* and *R. sativas*, even in the favorable condition with entire synchrony of flowering period and presence and abundant of honeybee, there was none of hybrid detected outcrossed between them (non-GM MS *R. sativas* (♀) × GM *B. napus* (♂)). The existing report on successfully outcrossing between *B. napus* and *R. sativas* was published by Ammitzboll and Jørgensen (2006) when a GM MS *B. napus* served as pollen recipient (GM MS *B. napus* (♀) × *R. sativas* (♂)), which might be hypothesized that the parental genotypes and direction of crosses result in the difference in comparison with our outcrossing trials (Bing et al. 1999; Jørgensen and Andersen 1994; Jørgensen et al. 1998; Halfhill et al. 2002; Scott and Wilkinson 1998). But one point is very clear that the probability of outcrossing between these two species is extreme low.

Factors affecting gene flow

Gene flow rate is affected by intrinsic and external factors, such as genotype, direction of cross, experimental design, local climatic conditions, etc (Bing et al. 1999; ; Jørgensen et al. 1998; Halfhill et al. 2002; Warwick et al. 2003). In the present studies, in order to simulate the gene flow under favorable conditions and reveal the relationship between factors and gene flow, several measures are adopted containing using MS recipients, introducing pollinators, and completely overlapping flowering period.

In the previous studies pollen recipients used were non-GM MF plants, as these plants could produce fertile pollen and compete with GM pollen donor, resulting in a lower gene flow rate, less than 2% even at mixing area, which is difficult to differentiate the difference between the factors. In the present studies, we used the MS line as recipients to achieve a higher outcrossing rate as it does not produce viable pollen but only accept the foreign GM pollen for outcrossing. By this means, an average of gene flow rate ranged from 0.26-32.48% obtained for non-GM MS *B.*

napus and 0.16-21.95% for non-GM MS *B. juncea* at 2-128 m, respectively (Table 23). These rates enabled to differentiate the difference between the factors on affecting gene flow rate.

The degree of synchrony of flowering period between pollen donor and recipient is also a key factor for determining the gene flow rate (Bing et al. 1991, 1996; Landbo et al. 1996). An increase of overlapped flowering days between pollen donor and recipients may potentially increase the outcrossing rate as there are more opportunities for exposed recipient flowers to accept the foreign pollen for outcrossing. In contrast, lack of synchrony of geographic distribution or in flowering periods, the outcrossing either drastic reduction or no occurred. Tsuda et al. (2012) reported none of hybrids were detected when a 19 days synchrony of flowering period was achieved between *B. juncea* and *B. napus* under natural flowering condition in 2009. However, when the synchrony of flowering period was increased to 34 days by artificially means in 2010, a total of 55 hybrids was detected. In our studies, according to the three different times of GM *B. napus* pollen donor transplanting, the flowering periods was entirely synchronized with non-GM MS recipients (Table 15).

Honeybee has been considered to be the most prominent and efficient pollinators for the rape crop (Duran et al. 2010; Williams et al. 1987). For an open pollinated male fertile *B. napus*, the seed production results from three factors, self pollination, wind pollination by neighbouring plants, and insect pollination primarily by honeybee. As honeybee directly takes part in pollen transfer and pollination, any factors affecting the honeybee's behavior such as the density of flowers, types of plants, distance from the beehive, wind direction, weather condition, etc., would indirectly influence the pollination. In field observations, the number of honeybee visit and honeybee's activity were associated with weather conditions with a small number in a cloudy day and none in a raining day. Pods setting rate was significantly correlated with the honeybee visit frequency ($\text{plant}^{-1} \text{min}^{-1}$) in those three species based on the Pearson's correlation analysis ($P= 0.01$) (Table 19). In addition, the honeybee visit frequency showed a negative and significant correlation with distance increasing from the pollen donor ($P= 0.05$) (Table 19). A relatively

higher density of honeybee visit found near the GM pollen donor area in those three species because of the higher density of flowers in this area, and decreased with increasing the distance from GM pollen donor accordingly (Fig. 12).

Determination of calibration factors

Based on the previous studies, in some cases due to the recovery of fertility in MS plants, it also can produce pods by selfing, which certainly would over-estimate the outcrossing rate. In order to improve the accuracy of gene flow rate estimation, a herbicide resistance screening test and following the PCR confirmation were performed in our studies for detection of the true hybrids among the progenies. Herbicide screening test supplied a mass screening method for detecting hybrids in progenies. According to a herbicide dose-response test on plants survival rate, a single optimal dose was determined, which can discriminate between true hybrids outcrossed from parental plants and false hybrids selfed from maternal plant. In our studies, doses of 1.2 g (recommended dose), 0.6 g, and 2.4 g ai L⁻¹ glufosinate-ammonium treatment of one-true-leaf stage seedlings of *B. napus*, *B. juncea*, and *R. sativas* for detection of hybrids were determined, respectively. The survivals were counted after 14 days after glufosinate-ammonium treatment. The screening gave the survival rates ranging from 36.11-85.05%, 41.67-84.57%, and 0.18-0.45% for the progenies of non-GM MS *B. napus*, *B. juncea*, and *R. sativas*, respectively (Table 20-22). In order to detect whether there were negative survivals existed in herbicide resistance screening test due to insufficient absorption of the herbicide or intrinsic tolerance, a subsequent polymerase chain reaction (PCR) confirmation was performed to detect the *bar-specific* band in those survivals. According to the PCR confirmation, more accurate calibration factors were determined with ranging from 75-100% and 66.7-100% for non-GM MS *B. napus* and non-GM MS *B. juncea*, respectively. The two survivals of *R. sativas* from herbicide screening test were proved to be false-positive hybrids (Table 20-22). This demonstrates the necessity of herbicide screening together with molecular level detection for finally determination of true hybrids.

Modeling of gene flow under open and wind pollination conditions

In the present studies, a discontinuous design was conducted to estimate the gene flow from a small pollen source as spilled GM *B. napus* to its relatives under open and wind pollination conditions. By introducing honeybee and covering the recipients with or without the insect-preventing nets, we could set up two different pollination conditions. Honeybee-mediated pollination could be estimated using the difference between them. In order to improve the accuracy of simulation model, both herbicide resistance and PCR confirmation markers were used to confirm F1 hybrids resulted from gene flow from GM *B. napus* to its relatives. By non-linear regression analyses to fit the log-logistic model to the gene flow rate data, we could get a simulation model for predicting gene flow from GM *B. napus* to a MS relatives at a specified distance (Fig. 13). The previous studies on modeling used different models such as single or double exponential decay equations (Yuan et al. 2007), linear function equation (Pla et al. 2006). These models provided considerable determination coefficients, but they do not provide sufficient biological meanings. In contrast, the log-logistic model adapted in this study have biological meaning in its parameters, such as the estimated maximum gene flow (y_0), the slope (b) around the 50% of y_0 reduction (D_{50}).

Our study was not designed to cover various wind directions, thus the gene flow estimated in this study might be influenced by wind direction and speed. Nonetheless, the model might be more suitable for predicting gene flow events where fields are small and uni-directionally arranged. Because the MS lines were used as pollen recipients in these studies under favorite conditions with abundant presence of pollinators and entire synchrony of flowering periods, the gene flow rates values presented here are the maximum potential rates. It is worth pointing out that the maximum potential gene flow rate may provide a valuable reference for gene flow risk assessment. With these maximum potential values, the proper isolation distance for risk management can be determined. Using the model and

based on the tolerable threshold (0.01%) made by EU (2003), the isolation distance for less than 0.01% gene flow from GM *B. napus* was estimated to be 2710 m and 254 m for MS *B. napus* and *B. juncea*, respectively. These figures are more than 20 times and 10 times greater than the isolation distances established in Chapter II, where pollen recipients were male fertile (MF) relatives (122.5 and 23.7 m for non-GM *B. napus* and *B. juncea*, respectively).

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GENERAL CONCLUSION

Gene flow from GM crops to non-GM crops or wild relatives takes place naturally when GM crops are grown. This is exactly true for Northern American and Australia with mono cropping systems, and for European and Asian countries with diverse cropping systems. Both greenhouse and field studies showed that the possibility of gene flow from *B. napus* to its relatives. Greenhouse as a model case study provided a flexible and controllable ways for setting up basic experimental conditions for evaluating gene flow from GM crops to their relatives. The field evaluation using MS recipients revealed that the pollen of *B. napus* could transfer farther than 128 m to fertilize MS *B. napus* and MS *B. juncea*. For MF recipients, the farthest distance was 75 m for *B. napus* and 16 m for *B. juncea*, respectively. Using the log-logistic model and parameter estimates, with gene flow rate less than 0.01% stipulated by EU (2002) as a tolerable threshold, the isolation distance between GM *B. napus* and MF recipient was estimated to be 122.5 m and 23.7 m for *B. napus* and *B. juncea*, respectively. In the case of MS recipients it was estimated to be 2710 m and 254 m for *B. napus* and *B. juncea*, respectively.

The experimental data in our study provides informative reference values and scientific basis for risk assessment of gene flow from GM *B. napus* to its relatives under Korean climatic condition. It seems that more studies and knowledge are needed to accumulate and strengthen, which is necessary for better understanding of different factors affecting gene flow and simulations to assess the practical gene flow in the agricultural ecosystem, and thus to provide scientific approaches for gene flow risk assessment and systemic management of GM crops. For the future studies on gene flow, more attention should be paid to more long-term consequence of gene flow and its consequential hybrids in various agricultural conditions and the possibility of outcrossing under different cross directions and combinations.

ABSTRACT IN KOREAN

형질전환 유채와 근연종간의 유전자이동 가능성

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본 연구는 형질전환 유채(*Brassica napus*)와 그 근연종간의 유전자 이동 가능성을 정량화하기 위해, 온실 및 포장에서 실험을 수행하였다. 형질전환 유채와 근연종간의 이격거리에 따른 유전자이동 가능성을 예측하기 위해 Log-istic 모델을 사용하였으며, 이를 바탕으로, 격리거리(Isolation distance)를 예측하였다. 유채와 그 근연종간의 교잡종을 찾기 위한 방법으로 SSRs (Simple sequence repeats) 마커와 제초제 저항성 (glufosinate-ammonium) 마커를 사용하였다. 3년간의 포장실험에서 화분공여자(pollen donor)로서 형질전환 유채는 화분 수용체(pollen recipient)인 유채와 갓(*B. juncea*)으로의 유전자 이동에 따른 교잡종이 형성됨을 보여주었으나, 무(*Raphanus sativus*)와는 이격 거리에 상관없이 유전자 이동에 따른 교잡가능성이 없음을 보여주었다. 화분 수용체로서 웅성불임(MS) 유채 및 웅성불임 갓으로의 유전자이동 가능성은 개화기가 동일하고 수분매개체가 충분히 존재할 때, 가장 가까운 2 m 이격거리와 가장 먼 128 m 에서 각각 37.35%와 0.30% 및 34.59%와 0.24%씩의 유전자 이동에 따른 교잡이 발생하였다. 화분 수용체로서 웅성가임(MF) 유채로의 유전자이동에 따른 교잡은 가장 가까운 2 m에서 2.33%가 발생하였으며, 75 m 이격거리에서 0.007%

가 발생하였으며, 웅성가입 갓으로는 가장 가까운 2 m에서 0.076%, 16 m에서는 0.025%가 발생하였으며 이 이상의 이격거리에서는 유전자이동이 없었다. 상관분석결과 벌의 방문수가 유전자이동 가능성과 가장 유의적인 상관관계를 보였다.

유전자변형 유채에서 근연종으로의 유전자 이동에 있어 매개충인 꿀벌과 바람의 영향을 비교하기 위해 웅성불임 근연종을 화분수용체로, 방충망 설치 유무를 달리하여 포장평가를 실시한 후 꿀벌에 의해 매개된 유전자이동성을 계산한 결과 꿀벌에 의해 웅성불임 유채로의 유전자이동성은 이격거리 2 m에서 19.01%, 이격거리 128 m에서 0.03%이었고, 웅성불임 갓으로는 2 m에서 15.36%, 128 m에서 0.06%이었다. 이는 꿀벌이 유전자이동성에서 중요한 부분을 차지하고 있음을 의미하며, 아울러 바람에 의한 유전자 이동도 매우 유의적으로 높음을 의미한다. 본 실험에서 얻은 유전자이동성과 이격거리간의 관계를 log-logistic 모델을 이용하여 설명할 수 있었으며, 이 모델을 이용하여 특정거리에서 유전자이동 가능성을 예측할 수 있었다.

이상의 결과는 한국의 환경조건에서 형질전환 유채로부터 근연종인 유채와 갓으로의 유전자 이동성을 정량적으로 제시하였으며 이를 통해 형질전환 유채에서 근연종으로의 유전자이동 위험성이 높음을 시사한다. 본 연구에서 제시된 모델은 향후 형질전환 유채로부터 근연종으로의 유전자이동의 위험성의 예측은 물론 위험관리를 위한 안전격리거리의 결정에도 활용될 것으로 기대된다.

주요어: 유채, 갓, 유전자 이동, 형질전환, 모델링, 수분, 무, 위험성평가

학번: 2012-30757