



Gene flow from herbicide resistant genetically modified rice to conventional rice (*Oryza sativa* L.) cultivars

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Abstract

Rice (*Oryza sativa* L.) is an important feeding crop in Asia, and utilization of genetically modified (GM) rice is highly demanding. For co-existence of GM rice and non-GM rice, the proper confinement measures should be provided. Thus, we surveyed gene flow from herbicide resistant GM rice to the conventional rice cultivars in the field tests. Gene flow frequency decreased with increasing distance between the pollen donor and recipients and did not exceed more than 1% even at the nearest distance. In single recipient model plot, a maximum gene flow frequency was observed at the shortest distance and hybrid was detected up to 12 m from the pollen donor. The direction of gene was coincided with the dominant wind direction. Gene flow assessment to multiple recipient plots was conducted under the high raining season by chance, and abrupt decline of gene flow frequency and maximum distance were resulted. According to the survey results, current regulation for isolation distance is reasonable for environmental safety or for general crop production. However, we suggest an alternative measure for GM rice cultivation that should be supplemented to overcome the out of estimation and in the environment asking higher security levels.

Key words: GM rice, isolation distance, *Oryza sativa* L., wind direction

INTRODUCTION

Many genetically modified (GM) crops harboring transgenes have been developed using molecular techniques and are already grown in worldwide for commercial purpose (James 2014). Rice is the staple food to most of the Asian people. Because of increase of human population and gap in supply, and threats to crop yields due to climate changes, the need for GM rice for food supply is increasing day by day. Many GM rice that are taking advantages of nutritional value, resistance to biotic/abiotic stress, or resistance to herbicides have been developed

(Lu and Snow 2005, Lu and Yang 2009), however, commercial release of GM rice is still in a debate for the environmental safety.

Prevention of gene escape is one of the most important concerns in environmental safety especially for GM crops. Gene escape could occur through pollen mediated gene flow, volunteers by seed dispersal, or failures in the safety management of GM crops during storage or transport. Escape of transgenes could disrupt the agronomical environment, local and international trade, and ultimate-

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ly cause huge losses in terms of economics and ecology, too (Messeguer 2003, Vermij 2006).

Investigations of gene flow conducted in various GM crop to wild/weedy plants and to non-GM counterpart have commonly shown that pollen-mediated gene flow is reduced markedly by increasing the distance between the pollen donor and receiver and the dominant wind direction affects the gene flow frequency (Arias and Rieseberg 1994, Ellstrand et al. 1999, Messeguer et al. 2004, Breckling et al. 2011, Rieben et al. 2011). Thus, an appropriate isolation distance is considered as the primary measure for confinement (Ellstrand et al. 1999, Messeguer et al. 2004). In addition, pollen-mediated gene flow is influenced by the biological factors of each crop species, local climate and geographical aspects, the scale of plots, and the cultivation system (Walklate et al. 2004, Gustafson et al. 2005, Beckie et al. 2012). Prediction models of gene flow have been made reflecting various factors, assisted by results obtained from field tests (Walklate et al. 2004, Gustafson et al. 2005, Yao et al. 2008, Rong et al. 2010). To support the development of proper guidelines for GM rice confinement, studies on gene flow from GM-rice to wild/weedy rice and to conventional rice cultivars have already been conducted (Messeguer et al. 2001, Zhang et al. 2003, Chen et al. 2004, Messeguer et al. 2004, Rong et al. 2005, Rong et al. 2007, Lu and Yang 2009, Olguin et al. 2009, Chun et al. 2011, Rao et al. 2012, Serrat et al. 2013). Outcrossing rate of cultivated rice (*Oryza sativa* L.) is, however, relatively low in compared to other crops, such as maize or *Brassicaceae*, because its floral structure prefers self-pollination and the distance of gene flow is short because the pollen is heavy and has short life time (Yoshida 1981, Messeguer et al. 2001).

Beyond ecological risks through contamination to the wild/weed rice, regulation of coexistence of GM crop and non-GM crop is an issue to focus (Devos et al. 2009). Contamination of GM crop to non-GM crop might cause serious economic problems and complement of regulations to prevent this problem (Jia et al. 2007, Rong et al. 2007, Davison 2010). In addition, when we consider the cultural habits in Asia, where the small scale farms are neighbor each other with high density, obtaining enough isolation distance may not easily be available. Also when the GM fields are surrounded by different cultivars, estimation of gene flow would be more complex. Differences in rice varieties might cause differences in gene flow properties by the differences in sexual compatibility or interaction with environmental factors (Rong et al. 2005, Devos et al. 2009), and this point has not been investigated sufficiently (Messeguer 2003, Lu and Snow 2005, Rong et al. 2007).

Herbicide resistance is the highest target trait in GM crop development. A GM rice event (CPPO06) harboring tolerance to the protoporphyrinogen oxidase (protoporphyrinogen oxidase) (protoporphyrinogen oxidase) (protoporphyrinogen oxidase)-inhibiting herbicide had been developed by introduction of *Protox* gene of *Myxococcus xanthus* (Li et al. 2003, Lee et al. 2007). Here we present the results of gene flow from GM rice, CPPO06, to non-GM conventional rice lines to describe the gene flow dependency on distance and wind direction, and discussion about influence of biological factors and environment factors on gene flow.

MATERIALS AND METHODS

Plant materials

A herbicide-tolerant GM rice line (CPPO06, T5 generation) was used as a pollen donor and four conventional rice cultivars (Dongjin, Chuchung, Onnuri, and Ilpum) were used as pollen receivers to produce hybrids. CPPO06 was developed from Dongjin to contain a single copy of *Protox* gene obtained from a soil bacterium, *M. xanthus*, and provided tolerance to peroxidizing herbicides (Lee et al. 2007). This gene, controlled by the *Ubi* promoter and *nos* terminator, can be used as a selection marker by itself. Chuchung, Onnuri, and Ilpum were chosen because of popular cultivars in different regions of Korea and have different growth properties. CPPO06 and Dongjin were provided by Prof. K. Back in Chonnam National University, Korea. Chuchung, Onnuri, and Ilpum seeds were obtained from the National Institute of Crop Science, Korea.

Field trials

Field trials were conducted for two growing seasons (2009 and 2012) in a confined field located at the Korea Research Institute of Bioscience and Biotechnology, Cheongju-Si, Chungcheongbuk-do, Republic of Korea (36°43'04" N, 127°26'06" E, elevation: 35 m). In 2009, forty day-old pollen donor (CPPO06) and pollen recipient (Dongjin) seedlings were transplanted into a 25 m square field plot to examine gene flow across various distances from the pollen donor (Fig. 1). CPPO06 seedlings were placed in the center as a circle (5 m diameter) by hand, while the outer area was filled with Dongjin from the 0.5 m periphery of the GM plot using a rice-transplanting machine. The planting distance was 30 × 15 cm and 2-3 seedlings were planted in a spot.

In the year 2012 trial, the plot was designed to study gene flow for four different cultivars at the same place.

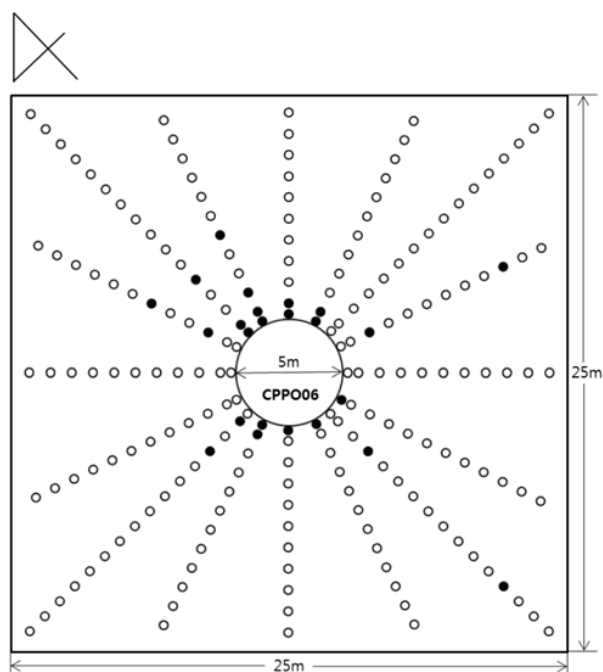


Fig. 1. Model of experimental field design in 2009 trial to test gene flow at different distance from CPPO06 as pollen donor to Dongjin as pollen recipient. The open circles are the sampling points, and filled circles are points that the hybrids were detected. The empty space is also filled with rice in 30 x 15 cm interval.

To test gene flow from CPPO06 to conventional rice cultivars, CPPO06 and four rice cultivars of pollen recipients were grown in a greenhouse for 40 days and transplanted into a circular field plot (23 m diameter). Seedlings of CPPO06 were placed in the innermost circle (GM plot, 5 m diameter), while the outer area was sectorized into eight rectangles to plant pollen recipients at a 9-m distance. Four different cultivars (Ilpum, Onnuri, Chuchung, and Dongjin) were planted sequentially as four rows outward to the each 8-direction by hand and Dongjin was planted by the side as a guard row. The planting distance was 30 cm between each cultivar and 15 cm between the same cultivar and one seedling was planted in a spot.

Flowering time and weather information

Weather information during the flowering period at the Cheongju (9 km distance from the KRIBB confined field site) was obtained from the website of National Climate Data Service System (<http://sts.kma.go.kr/jsp/home/contents/main/main.do>) (data not shown). The most frequent wind direction (MFWD) during the overlapped flowering period was obtained by an analysis program of climate data on the website. The wind direction for every

hour during the flower opening time in 2009 was analyzed using the OriginPro 8.1 data analysis software (Originlab, Northampton, MA, USA) using a wind rose plot. Flowering periods of each cultivar in 2009 and 2012 were listed in Table 1.

Screening for hybrids

In October 2009, we harvested panicles of pollen recipient from two plants in the 16 direction at 0.5 m from the periphery of the GM and at 1 m intervals to the boundary of the plot (maximum 14 m). In October 2012, panicles of four conventional rice cultivars were harvested at distances of 0.5, 1, 3, 5, 7, and 9 m from the GM center in eight directions. Ripened seeds from individual plant were tallied by an automatic seed counter (Aidex Co., Nagoya, Japan). Before hybrid selection, 50 to 100 seeds were taken to determine the germination rate of at least three replicates. The total number of germinated seeds was corrected by multiplying seed numbers per plant by the seed germination rate of each cultivar.

Approximately 500 seeds were placed in a plastic flat tray (30 × 60 × 3 cm) containing commercial agricultural soil (Punong Co., Kyoungju, Korea) in the greenhouse. Trays were watered as needed. When plants reached the three-leaf stage after 7 weeks, a protox-inhibiting herbicide, oxyfluorfen Goal_2XL (Kyungnong Co., Seoul, Korea) was applied with a dose equivalent to 480 g/ha a.i. using a garden sprayer. After 2 weeks, the surviving seedlings were considered to be herbicide-resistant.

Hybrids that resistant to herbicide were confirmed by PCR amplification of the fragment of *Protox* genes. Primers were designed to detect *protox* (436 bp) and another set of primers was used to detect the *actin* gene (194 bp)

Table 1. Flowering period of GM and four conventional rice cultivars, and frequency of precipitation during overlapping flowering period

Year	Cultivar	First heading ^a	Last heading	Precipitation frequency ^b
2009	CPPO06	26 Aug.	1 Sep.	
	Dongjin	19 Aug.	31 Aug.	2/6
2012	CPPO06	22 Aug.	27 Aug.	
	Dongjin	19 Aug.	24 Aug.	3/3
	Chuchung	21 Aug.	29 Aug.	4/6
	Onnuri	17 Aug.	26 Aug.	4/5
	Ilpum	17 Aug.	24 Aug.	3/3

^a'First heading' and 'Last heading' are around 10% and 80%, respectively, panicles were exerted out from the stems in the field.

^bNumber of precipitated days/number of overlapped flowering days.

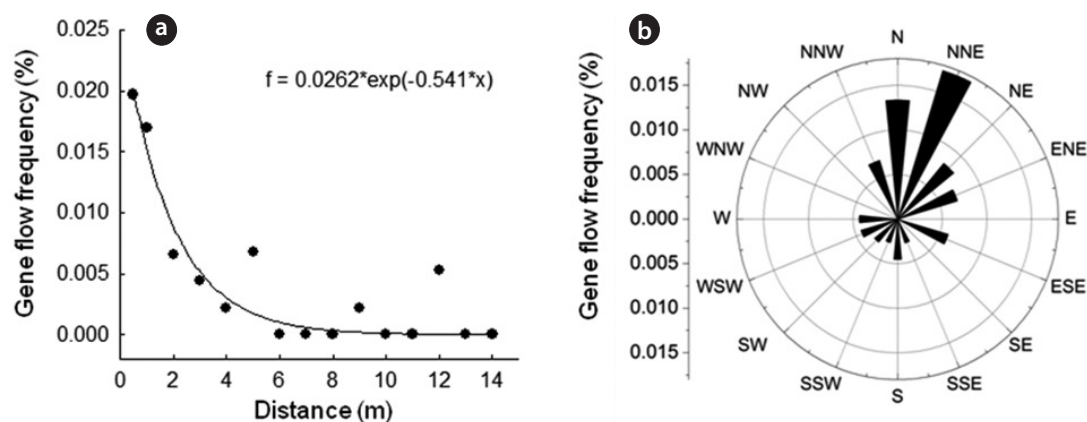


Fig. 2. Gene flow frequency at different distance (a) and direction (b) from pollen donor CPPO06 to the Dongjin.

as an internal PCR positive control, as listed in Table 2 (Bioneer Co., Daejeon, Korea). Genomic DNA sample was obtained from 100-200 mg leaf tissue of survived seedlings using a FastDNA™ kit (Qbiogene, Carlsbad, CA, USA). PCR was performed in a volume of 50 μ L containing 50 ng of genomic DNA, 0.2 μ M dNTP mixture, 1.5 U of Taq DNA polymerase (Bioneer Co.), 5 μ L of 10 \times Taq buffer, and each primer (0.28 μ M). PCR amplification was performed with the following program: initial denaturation at 94°C for 3 min, 34 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 50 s, plus a final extension at 72°C for 5 min. Gene flow frequency

was obtained as percentage of the number of hybrids per total number of germinated seeds.

RESULTS

In 2009, to assay gene flow frequency, hybrid detection was performed with seeds that were collected at different distances, up to 14 m, in 16 directions. We observed the maximum gene flow frequency at the shortest distance (0.5 m) as 0.0161%, and it decreased exponentially by increase of distance from the pollen donor (Fig. 2a). Gene

Table 2. Primer sets used to detect transgenic F1 hybrid and positive control

Primer set	Name	Sequence (5' to 3')	Amplified size (bp)
Protox specific	Ptx-rb-F	AGCGCGCAAACCTAGGATAAA	436
	Ptx-rb-gDNA-R	GTCCAAGACCCACCCATCTC	
Positive control	Actin-F	CCCTCTTTTCATCGGTATGGA	194
	Actin-R	TTGATCTTCATGCTGCTTGG	

Table 3. Frequency of gene flow (%) in four conventional rice cultivars from GM rice at different distances in 2012 trial

Cultivar	Distance from GM rice plot						Mean gene flow frequency ^a
	0.5 m	1 m	3 m	5 m	7 m	9 m	
Dongjin	0.0788 (6348) ^b	0.0761 (2629)	0.0000 (3363)	0.0000 (2959)	0.0000 (2623)	0.0000 (3355)	0.0329 (21277)
Chuchung	0.0000 (7126)	0.0380 (5258)	0.0000 (4701)	0.0000 (4087)	0.0000 (3639)	0.0000 (4211)	0.0069 (29021)
Onnuri	0.1421 (6333)	0.0533 (3749)	0.0568 (3522)	0.0000 (3901)	0.0000 (2782)	0.0000 (3343)	0.0550 (23630)
Ilpum	0.0000 (9072)	0.0000 (3797)	0.0000 (4522)	0.0000 (5324)	0.0000 (3482)	0.0000 (6004)	0.0000 (32201)

^aMean gene flow frequency^a was obtained by dividing the number of hybrids by the total tested seed numbers.

^bNumbers in parentheses are the total number of germinated seeds.

flow frequency arrived plateau after around 6 m (Fig. 2a), but hybrids were detected up to 12 m from the pollen donor (Fig. 1).

We analyzed whether gene flow is dependent on wind direction or not. Gene flow frequency was biased to the north-northeast and north (Fig. 2b), while the most far distance of gene flow was detected at the south (Fig. 1). At first we compared with the MFWD of the overlapping flowering period, which was north-northeast (data not shown), but it was the opposite direction to dominant gene flow. Wind frequency was reanalyzed by selection of data during rice flower opening time (from 8 am to 2 pm), because rice flower open and close in the morning (Yoshida 1981, An 2010). Then south and south-southwest were ranked as main wind directions in sequence (data not shown), and these coincide with the dominant gene flow frequency directions. Wind frequency from north-northeast was followed as third direction, and when we narrowed to the date 26 to 28, August, wind from north-northeast was not dominant anymore (data not shown).

In 2012, experimental plan was to observe gene flow pattern to the conventional rice lines which have difference in growth properties at the same time. Differences in growth properties include sexual compatibility, growth vigor, response to the temperature, humidity, wind, and so on. The gene flow frequency into Dongjin at 0.5 m was 0.0788% in this year, higher than that in 2009 (0.0197%) and into Onnuri was 0.1421%, which was even higher than Dongjin in either year. Interestingly the maximum distance of gene flow was only 3 m in four cultivars, and no hybrid was detected even at the closest (0.5 m) distance (Table 3) in Ilpum line. Evident directional gene flow influenced by wind was detected as much as in 2009.

DISCUSSION

In 2012, the experiment was designed to test gene flow to various cultivars. Differences in growth properties might respond to different degree to environment and result in alteration in gene flow pattern. However in this year, gene flow showed unusual pattern; instead exponential decay, abrupt decay or blocking of gene flow, subsequently resulting limit in data points to analyze, were observed. Thus it was difficult to get convincing ideas about the variation among the cultivars. We hypothesized two possibilities for this result. First one is the frequent precipitation during the flowering period (data not shown) which could affect on the cross-pollination. When it rains, the rice flower does not open and achieve

self-pollination within the closed flower (Yoshida 1981). Additionally, frequent raining might lower rice yield and make below the detection level of gene flow frequency. In second, the plot design could be inappropriate to test the gene flow in two aspects. In 2012, one seedling was planted instead of 2-3 seedlings for one data point making reduction of number of recipients. Also the spaces between the 8 segments were remained as empty and it might affect on the cross-pollination by unknown reason at the moment. Wind could be turbulent without hindrance by plants. The wind would increase pollen dissipation and fail cross-pollination.

Meanwhile, gene flow frequency was highest in Onnuri among the four cultivars. We exclude the overlap of flowering period as a factor, because Ilpum flowered similar period with Onnuri but no gene flow was recorded. Because Onnuri did not show higher gene flow frequency in the other field test (data not shown), we excluded cross-compatibility neither as a reason. We want to add another result to notice. Interestingly, although the overall gene flow frequency was low, it was higher within the close distance (3 m) than in 2009. High humidity and low temperature during the precipitation at this time might have made pollen dispersion difficult, and resulting in a high local gene flow frequency as mentioned in Song et al. (2004) and Sofiev et al. (2013). Most of all, it needs to be confirmed to have significance, but we suggest that in unfavorable condition, the degree of effects of various factors on gene flow could be twisted and change the pattern of gene flow. Although in our study the effect resulted negatively in gene flow frequency, when ones consider the global climate change and agricultural environment can be changed anytime, alternative provisions should be prepared.

In many countries, the isolation distance for GM crop application refers to a regulations for pure seed production in conventional breeding indications: for example, 3 m in Korea and the USA, 3-5 m at International Rice Research Institute (IRRI), and 30 m in Japan. Many gene flow studies from GM to non-GM rice had conducted with different cultivars, regions, and plot designs, where they observed exponential decrease by distance and were less than 1% gene flow frequency (Messeguer et al. 2001, Messeguer et al. 2004, Rong et al. 2005, Rong et al. 2007) and our findings were consistent to those results. Thus, 3 m for the isolation distance for rice looks reasonable as 'least safety distance'.

However, it is curious about making simple regulation for GM rice growth. Because, it is not difficult to find experimental results of hybrid detection beyond 3 m even

though it is very low rate (<0.1%); 0.0029% at 12 m in our study, 0.0125% at 10 m in Messeguer et al. (2004) and <0.01% at 6.2 m in Rong et al. (2007) and theoretically rice pollen was capable to disperse at least 100 m from its source (Song et al. 2004). The farm lands could be surrounded by environments asking different security levels. When the neighbor lands are destined to GM-free organic farm, to seed production, confined field experiment which testing the environmental safety for commercial release, or habitat of wild/weedy rice, the isolation distance should be increased to higher level (Yao et al. 2008, Davison 2010).

Increase of isolation distance could make gene flow frequency to below the level of regulation and also is an effective way, but it does not mean complete prevention. In addition, in the areas where the small scale farms located each other in close distance, farmers want to reduce the waste of farming area used for isolation distance. Alternative measures, such as planting the GM rice to avoid overlap of flowering period, or pollen barrier, or extensive monitoring have been suggested (Weber et al. 2007, Prabhu 2009). Although pollen mediated gene flow has similar properties between crops, the practical alternative measures for GM rice has not been investigated in enough.

We found that gene flow frequency decreased exponentially by increase of distance and dependent on the main wind direction at the time of flowering. In addition, we suggest that alternative measure for GM rice cultivation should be supplemented to overcome the out of estimation caused by the difference in local environment, global climate change, and plant growth properties.

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