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ORIGINAL PAPER

Investigation of rice transgene flow in compass sectors by using male sterile line as a pollen detector

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Abstract Rice is the most important staple food in the world. The rapid development of transgenic rice and its future commercialization have raised concerns regarding transgene flow and its potential environmental risk. It is known that rice is a self-pollinated crop; the outcrossing rate between common cultivars is generally less than 1%. In order to improve the detection sensitivity of rice transgene flow, a male sterile (ms) line BoA with a high outcrossing rate was used as a pollen detector in this study. A concentric circle design was adopted, in which the transgenic rice B2 containing *bar* gene as a pollen donor was planted in the center circle and the recipient BoA was planted in eight compass sectors. The frequency of transgene flow in compass sectors was analyzed by continuous sampling to generate cumulative data. The results of two years with sound reproducibility demonstrated that the rice gene flow was closely associated with the wind direction. According to the mean frequency of transgene flow, the eight sectors can be divided into two groups: a higher frequency group downstream of the prevailing wind (DPW) with a mean frequency ranging from 6.47 to 26.24%, and a lower frequency group lateral to or upstream of the prevailing wind (UPW) with a mean frequency of 0.39 to 3.03%. On the basis of the cumulative data, 90–96% of the cumulative gene flow events occurred in the four DPW sectors, while it was 4–10% in the four UPW sectors. By using these systematic data, simulation models and isograms of transgene flow in the eight compass sectors were calculated and drawn, respectively.

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Introduction

The high adoption rate of transgenic crops in the recent 11 years reflects that biotechnology is the fastest growing technology among all agricultural technologies to date. The cultivation area of transgenic crops has increased by 60-fold, from 1.70 million hectares in 1996 to 102 million hectares in 2006 worldwide (James 2006). The adoption of transgenic crops has offered substantial economic, health, social and environmental (reduction of pesticide use) benefits, particularly for resource-poor farmers from developing countries, whose increased incomes from biotech crops contributed to the alleviation of poverty.

However, concerns have also been raised whether transgenic crops may cause any potential risk to the ecological environment. Hence science-based risk assessment of transgenic crops is needed to provide sufficient scientific data and corresponding safety measures.

Rice is one of the most important cereal crops and over 50% of the people in the world rely on it as a staple food (FAO 2002). Achievements have been made in the development of transgenic rice resistant to insects, diseases, and tolerant to herbicides, drought and salt (Ajisaka et al. 1993; Yahiro et al. 1993; Matsuda 1998; Huang et al. 2002b; Jia and Peng 2002; Jia 2004; Lu and Snow 2005). Some of them are under field-testing that might be approved for commercial production in the near future (Jia 2004). The potential environmental risk associated with rice transgene flow is one of the concerns (Messeguer et al. 2001, 2004; Song et al. 2002, 2003, 2004a, b; Chen et al. 2004; Rong et al. 2004, 2005; Lu and Snow 2005).

Currently, the Asian countries and China in particular, are the largest producers of the hybrid rice. Most of the farmers in China and other Asian countries only have small pieces of land generally less than half a hectare; thus, different rice cultivars may grow side-by-side to form a mosaic structure. In addition, the growth duration and flowering time of different varieties used in a given region are usually similar due to the limitation of the growing season. If transgenic rice is approved for commercialization, the GM and non-GM rice may co-exist as a mosaic and cross each other. Therefore, it is urgently needed to study the transgene flow to male sterile (ms) lines of hybrid rice so as to provide detailed reference data for setting proper isolation distances in hybrid rice seed production. Unfortunately, there is no report on the transgene flow to hybrid rice and its ms lines except for one of our papers (Jia et al. 2007), in which the maximum distance of transgene flow to hybrid rice and its ms lines under the prevailing wind direction was determined. Nevertheless, the relationship between the gene flow frequency and the wind direction has not yet been reported. Therefore, a further elucidation of the gene flow frequency associated with the wind direction is necessary.

In the previous rice transgene flow studies, a circular or a rectangular field experimental design was usually adopted. In the rectangular design, the pollen donor and recipients are planted side by side and the recipients are planted at various distances to the pollen donor. This design may save the land and labor, by which a relatively small experiment plot can provide data on the maximal frequency and maximal distance of gene flow for determination of a proper, safe isolation distance. In the circular design, on the other hand, the pollen donor is located at the center, while the recipients are planted at various distances from the donor to form concentric circles. This design would be more informative for providing gene flow frequencies in compass directions.

Song et al. (2002, 2003) and Chen et al. (2004) conducted a rectangular field experiment to investigate gene flow from a rice cultivar Minghui 63 to common wild rice (*Oryza rufipogon* L.) and weedy rice. They found the gene flow frequency to *O. rufipogon* and weedy rice ranged 1.21–2.94% and 0.011–0.046%, respectively. The maximum distance of gene flow to common wild rice was 43.2 m. Rong et al. (2004) reported the gene flow frequency between the common rice cultivar Huangkenuo and Shanyou-63 ranged from 0.04 to 0.18%. Messeguer et al. (2001) carried out field experiments in the paddy fields of two main Mediterranean rice-growing areas in Spain and Italy, and a gene flow frequency slightly lower than 0.1% was detected in a normal side-by-side plot design. Similar results were obtained in a circular plot when recipient plants were placed at 1 m distance from the transgenic central nucleus. A strong asymmetric distribution of the gene flow was detected among the circle and the highest value (0.53%) was recorded following the direction of the dominant wind. Messeguer et al. (2004) further investigated the transgene flow to the common rice variety Senia and the red rice in compass directions. They found that with a wind velocity of 0.51–1.13 m/s in the prevailing wind direction, the mean gene flow frequency in all directions was 0.086% to the rice variety Senia and 0.036% to the red rice.

By adopting either a circular or a rectangular design, in the previous studies, the size of the experiment plot was small (less than 300 m^2). The radius of the pollen donor in the circular design was less than 3 m, and only a small number of recipient plants (several hundreds) were planted along the eight radial lines at 1, 2, 5 and 10 m away from the pollen donor, while there were no recipient plants planted within the compass sectors (Messeguer et al. 2001, 2004). Due to the small number of recipient plants used and the non-continuous sampling (only the seeds from the recipient plants grown at certain distances were harvested for further analysis), it was therefore difficult to generate large data for assessment of the rice gene flow. More importantly, the recipient used was the common rice cultivar with a low outcrossing rate usually less than 1% (Song et al. 2002, 2003; Chen et al. 2004; Rong et al. 2004, 2005; Messeguer et al. 2001, 2004), so that it is not possible to differentiate the variations in gene flow frequency between the compass directions associated with the wind direction. In order to improve detection sensitivity, it is necessary to use a recipient with a higher outcrossing rate so as to amplify and differentiate gene flow frequency in the various compass directions. Based on the above consideration, a circular design combining three measures was used in this study to detect gene flow frequency in the compass sectors: (1) a male sterile (ms) line BoA with a

high outcrossing rate was used as recipient; (2) a large number of recipient plants were grown in the eight compass sectors, instead of being grown along the eight radial lines; (3) a method of continuous sampling at different distances from the pollen donor was adopted for generating cumulative data.

Materials and methods

Materials

Transgenic pollen donor

The transgenic line of *japonica* rice B2 containing *bar* gene with herbicide Basta® resistance was used as a pollen donor. The B2 line was a sibling of L201. The only difference between them is that the B2 has a longer growth duration than the L201. The generation, molecular characterization and genetic analysis of the L201 were previously described (Jia et al. 2007; Wang et al. 2006). The L201 and the B2 were confirmed to be homozygous at the *bar* gene locus and genetically stable after 14 generations of selection and maintenance.

Pollen recipient

An ms line BoA with almost 100% pollen sterility (no seed setting by self-pollination) was used as a recipient. Currently, a series of hybrid rice varieties of BoYou derived from crossing BoA as a female parent with various restorer lines has been widely adopted in southern China and the annual planting area in Guangdong, Guangxi, Fujian, Jiangxi and Hainan provinces has exceeded 1 million hectares (Huang et al. 2002a). Therefore, the BoA used in this study is considered to be a good representative.

Non-transgenic pollen competitor

Because the ms line does not produce its own viable pollen, the gene flow frequency to the ms line will theoretically be 100% due to transgene escape when there is no non-transgenic pollen competition. Generally, during the ms line multiplication or hybrid rice seed production, a maintainer line or a restorer line with normal pollen fertility is planted in parallel with an ms line at a certain proportion to provide sufficient pollen for fertilization. These lines actually provide non-transgenic pollen to compete with transgenic pollen. To simulate an actual situation in the ms line multiplication and hybrid rice seed production, a non-transgenic rice variety Texianzhan25 was used as the pollen competitor and planted along the eight radial lines of the concentric circles.

Experimental design

The field experiment was conducted in Sanya, Hainan province, from 2003 to 2004. A diagrammatic presentation of the experimental design is shown in Fig. 1. The total area of the experiment plot was 2,827 m^2.

(1) *Pollen donor B2* A circular area of 78.5 m^2 with a radius of 5 m was located at the center of the field (Fig. 1).

(2) *Recipient BoA* The concentric circle of the recipient was equally divided into eight compass sectors (N, NE, E, SE, S, SW, W, NW) with a radius of 25 m and the recipient BoA was planted in each sector (Fig. 1). The 0–19 m (2003) or 0–18 m (2004) was used as a sampling zone with an area of 224 m^2 (2003) and 205 m^2 (2004) in each compass sector, respectively. The rows of recipient plants grown in the area of 20–25 m (2003) or 19–25 m (2004) served as the protective rows to eliminate the border effect and to protect the sampling plot from accidental damage by animals. With the plant spacing of 17 × 20 cm, the total number of plants in each sector was estimated to be 6,700 (2003) and 6,500 (2004).

(3) *Pollen competitor Texianzhan25* Two rows of pollen competitors were planted at each radial line in eight compass directions (Fig. 1).

The plant spacing of the pollen donor, recipient and competitor was all 17 × 20 cm. Cultivation practices such as irrigation, fertilizer and pest management were regularly conducted, except that no glufosinate ammonium herbicides were used for weed control in the experiments.

Adjustment of flowering period

To ensure synchronization of the flowering period of the pollen donor, recipient and competitor, a preliminary study was conducted in 2002, in which the pollen donor B2 was sown three times, i.e. on January 20, 25 and 30 according to the previous data. It was found that the flowering period of B2 sown on January 30 overlapped with that of the recipient BoA. Hence, B2 was sown on 30 January 2003 and on 1 February 2004. To increase the number of tillers and prolong the flowering duration of B2, a growth regulator paclobutrazol [(2*RS*,3*RS*)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1H-1,2,4-trizol-1-yl)penten-3-ol] was sprayed at a concentration of 300 ppm 10 days after B2 transplanting. By these means, the flowering period of B2 and BoA was essentially synchronized (Table 1). The seeding time of Texianzhan25 (12 and 13 January) was 11–13 days earlier than that of BoA, so as to synchronize the flowering period with B2 (Table 1).

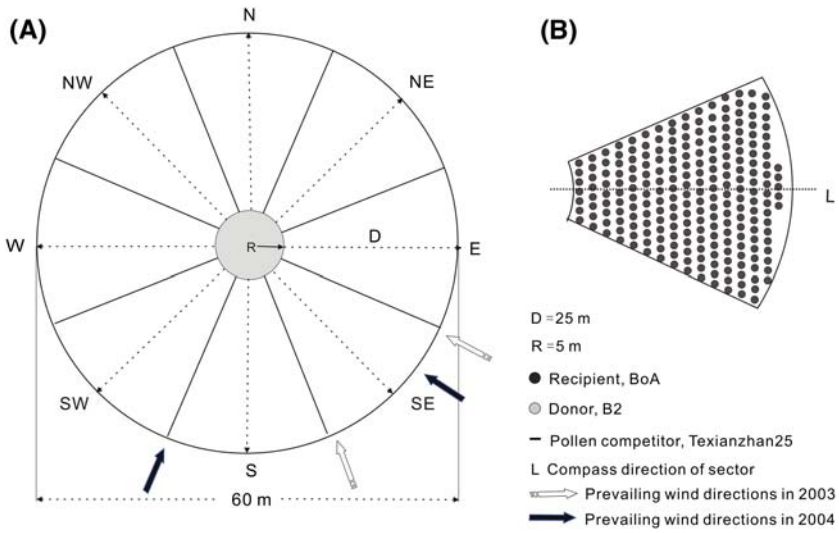

Fig. 1 Diagrammatic presentation of the experimental design. A 78.5 m^2 of circular donor B2 plot with radius 5 m was planted at the center of field (**a**). The radius of concentric circle of the recipient BoA was 25 m. The concentric circle of recipient was equally divided into eight sectors separated by two rows of pollen competitor Texianzhan25 that was grown as linear line at eight compass directions. One sector of recipient is shown in **b**. The sector central line corresponds the compass direction L of the sector. The plant spacing was 17 × 20 cm

Scoring wind direction and speed

The rice flowering time is generally from 8:30 to 15:00 each day. During the 13–14 days of the flowering period, the wind directions and speeds at 1.5 m height above the ground were scored three times at 10:00 am, 12:30 pm and 3:00 pm every day by using a portable aerovane. The mean value and the frequency of wind direction and speed were calculated based on every three readings scored each day during the whole flowering period. The wind direction occurring with the higher frequency was defined as the prevailing wind direction.

Method of continuous sampling

To obtain systematic and cumulative data, all seeds at different distances in each compass sector were harvested through continuous sampling at the mature stage. The circumference of the pollen donor was used as a starting point and the sector central line corresponds to the compass direction L of the sector (Fig. 1). Two rows of BoA nearest to the pollen donor (0–0.4 m) were marked as 0 m, 0.6–1.4 m (5 rows) as 1 m, 1.6–2.4 m (5 rows) as 2 m, 2.6–3.4 m (5 rows) as 3 m, and so on, up to 19 m (2003) or 18 m (2004). Each sampling zone was equally divided into three segments as three replicates and the seeds from the recipient plants at different distances in each replicate were individually harvested. The seeds were then sown in a seedling nursery for further testing of Basta^R resistance at seedling stage. The total seeds harvested from all the eight compass sectors weighed 245 kg (2003) and 114 kg (2004), respectively. The major factors that resulted in the big difference of total seed weight harvested in 2003 and 2004 were the different sizes of the experimental plot (1,730 m^2 in 2003 and 1,583 m^2 in 2004) used, and the plants of both pollen donor and recipient in 2003 grew more vigorously than that in 2004 due to the favorable weather conditions in 2003.

Table 1 Flowering period of the pollen donor (B2), recipient (BoA) and pollen competitor (Texianzhan25)

Year	Rice variety or line	Sowing date	Flowering period
2003	B2	Jan 30	April 2–15
	BoA	Jan 23	April 2–11
	Texianzhan25	Jan 12	April 2–9
2004	B2	Feb 1	April 12–24
	BoA	Jan 26	April 13–25
	Texianzhan25	Jan 13	April 9–20

Test of Basta^R resistance and calculation of transgene flow frequency

The seeds in the three replicates harvested from different distances in each compass sector were individually sown in a seedling nursery. The total number of seedlings was estimated by using the seed weight and seed germination rate. Starting from the three-leaf stage, the herbicide Basta® was sprayed three times (500, 500 and 800 ppm, respectively) at an interval of 7 days, and the number of surviving green seedlings was counted. The seedlings containing no *bar* gene became yellowish and wilted, while those containing the *bar* gene kept green, suggesting that the survivors were derived from seeds subjected to the transgene flow. In our previous experiment, the results of PCR analysis on the Basta^R survivors after three times of Basta® spray indicated that there were no false-positive plants found (Wang et al. 2006; Jia et al. 2007), demonstrating that the judgment based on the Basta^R phenotype was reliable.

Table 2 Wind direction and speed during rice flowering period

Year	Flowering period	Wind direction	Frequency	Maximum wind speed (m/s)	Minimum wind speed (m/s)	Average wind 2speed (m/s)
2003	April 2–15	SSE (140°–180°)	35	4.2	0.5	2.3
		SEE (100°–140°)	14	3.8	0.8	2.2
		NEE (60°–90°)	5	3.0	0.6	1.7
		NNE (30°)	1	2.1	1.8	1.9
2004	April 12–24	SEE (91°–134°)	20	3.8	0.0	1.4
		SSW (191°–225°)	15	3.1	0.0	1.2
		SSE (143°–174°)	9	2.5	0.0	1.5
		NEE (78°–89°)	5	2.8	0.0	0.9
		SWW (226°–270°)	3	3.4	0.5	1.7
		NWW (304°)	1	1.2	0.5	0.8
		NNE (23°)	1	2.0	1.1	1.6

The gene flow frequency at different distances in each compass sector was calculated by using the following formula:

$$\text{Gene flow frequency (\%)} = \text{Number of survived green seedlings/Total number of seedlings tested} \times 100\%$$

The cumulative gene flow events in compass sectors

Each Basta^R-resistant seedling was considered as an independent transgene flow event. Through continuous sampling, the cumulative transgene flow events to different distances (0–18 m in 2003 and 2004) in eight sectors were calculated for comparison of the results of the 2 years. Finally the distance (m) at which the cumulative gene flow events reached 60 to 90% in each sector was determined.

Statistical analysis

The statistical analysis (ANOVA) of transgene flow frequency at 0–19 m (2003) and 0–18 m (2004) was conducted by using SPSS software (SPSS Inc.). Student's *t* test was performed to analyze significant differences in frequency at the level of $P_{0.01}$. For the establishment of a simulation model, the curve depicting the reduction of gene flow frequency with distance increase was plotted and analyzed by using Microsoft Excel.

Drawing isograms of transgene flow frequency in compass directions

Based on the established simulation model, the distance (m) at which the gene flow reached a frequency of 5, 10, 20, 30, 40, 50, 60 and 70% in compass directions was calculated, respectively. Then a database containing the data of gene flow frequency, direction and distance was created. Finally, isolines in compass directions were drawn by using Microsoft Excel.

Results

Wind direction and speed during rice flowering period

The wind direction and speed were scored three times a day in the flowering period of the pollen donor and recipient. As shown in Table 2, the prevailing wind direction was SSE and SEE with the highest frequency in the flowering period of 2 to 15 April in 2003. The average wind speed ranged from 2.2 to 2.3 m/s and the maximal wind speed was 3.8 to 4.2 m/s. The prevailing wind direction was SEE and SSW during the flowering period of 12 to 24 April in 2004 with average wind speed 1.2–1.4 m/s and maximal wind speed 3.1–3.8 m/s (Table 2).

Overlap of the flowering period of the pollen donor, recipient and competitor

Since the growth duration of the pollen donor B2, recipient BoA and competitor Texianzhan25 in Sanya is quite different, the sowing date was individually adjusted so as to ensure synchronization of the flowering period. As a result, the flowering period of pollen donor, recipient and competitor was essentially overlapped (Table 1).

Gene flow in compass sectors

The prevailing wind direction was SSE and SEE in 2003 with mean and maximal wind speed 2.2–2.3 and 3.8–4.2 m/s, respectively (Table 2). The transgene flow frequencies from 0 to 19 m in the eight compass sectors

Table 3 Gene flow frequency (%) at different distances in eight compass sectors in 2003

Distances (m)	Compass sectors							
	NW	N	W	NE	SW	S	E	SE
0	73.08 ± 15.53	86.73 ± 7.99	53.60 ± 4.86	41.41 ± 1.56	26.40 ± 1.85	11.16 ± 0.86	5.83 ± 1.81	4.86 ± 0.52
1	64.82 ± 8.52	62.60 ± 18.70	40.55 ± 4.71	21.89 ± 0.74	7.73 ± 0.10	3.27 ± 0.29	1.34 ± 0.08	1.22 ± 0.06
2	52.70 ± 2.94	38.08 ± 1.70	28.61 ± 2.09	13.26 ± 0.64	5.83 ± 0.54	2.06 ± 0.57	0.44 ± 0.21	0.56 ± 0.12
3	36.54 ± 1.00	31.32 ± 4.23	23.07 ± 1.71	10.29 ± 0.66	3.00 ± 0.45	1.69 ± 0.57	0.18 ± 0.24	0.35 ± 0.09
4	36.01 ± 5.05	25.52 ± 3.41	16.42 ± 2.20	8.46 ± 0.51	2.60 ± 0.12	0.95 ± 0.14	0.09 ± 0.08	0.13 ± 0.07
5	33.29 ± 2.64	22.81 ± 2.92	15.45 ± 1.77	5.76 ± 0.31	1.48 ± 0.19	0.44 ± 0.10	0.05 ± 0.03	0.12 ± 0.06
6	28.78 ± 1.10	17.57 ± 1.45	11.89 ± 1.74	5.16 ± 0.38	1.29 ± 0.18	0.42 ± 0.07	0.06 ± 0.02	0.10 ± 0.06
7	26.22 ± 2.90	15.82 ± 2.99	8.00 ± 0.53	4.17 ± 0.87	1.10 ± 0.02	0.45 ± 0.13	0.05 ± 0.05	0.06 ± 0.00
8	25.17 ± 3.09	15.42 ± 1.62	8.33 ± 0.89	2.94 ± 0.32	1.03 ± 0.09	0.41 ± 0.10	0.05 ± 0.06	0.08 ± 0.03
9	22.44 ± 0.78	13.26 ± 0.23	7.46 ± 0.56	2.08 ± 0.07	0.94 ± 0.04	0.32 ± 0.07	0.02 ± 0.04	0.06 ± 0.02
10	19.96 ± 1.47	11.84 ± 1.07	6.32 ± 1.13	1.68 ± 0.21	0.69 ± 0.08	0.25 ± 0.03	0.03 ± 0.03	0.05 ± 0.04
11	16.92 ± 2.31	10.35 ± 1.16	5.42 ± 1.61	1.63 ± 0.17	0.73 ± 0.10	0.18 ± 0.05	0.01 ± 0.02	0.05 ± 0.01
12	14.96 ± 2.28	9.45 ± 0.52	6.10 ± 1.70	2.46 ± 0.64	0.68 ± 0.15	0.22 ± 0.08	0.01 ± 0.00	0.02 ± 0.02
13	14.28 ± 1.60	8.53 ± 0.73	4.93 ± 0.55	2.02 ± 0.57	0.72 ± 0.34	0.21 ± 0.06	0.01 ± 0.00	0.03 ± 0.01
14	12.08 ± 0.77	7.96 ± 0.64	3.45 ± 0.36	1.52 ± 0.51	0.38 ± 0.09	0.17 ± 0.02	0.00	0.03 ± 0.02
15	11.29 ± 1.24	6.01 ± 0.59	3.63 ± 0.62	1.09 ± 0.22	0.21 ± 0.08	0.15 ± 0.05	0.00	0.02 ± 0.01
16	9.63 ± 0.62	5.08 ± 0.77	2.74 ± 0.24	1.09 ± 0.27	0.35 ± 0.11	0.14 ± 0.09	0.00	0.01 ± 0.01
17	9.80 ± 1.14	6.01 ± 0.56	2.77 ± 0.10	1.07 ± 0.11	0.28 ± 0.01	0.12 ± 0.06	0.00	0.01 ± 0.00
18	8.90 ± 0.79	6.49 ± 1.03	2.38 ± 0.23	0.81 ± 0.32	0.26 ± 0.06	0.12 ± 0.02	0.00	0.02 ± 0.01
19	7.86 ± 0.90	5.74 ± 0.65	3.15 ± 0.26	0.71 ± 0.24	0.28 ± 0.03	0.18 ± 0.04	0.00	0.03 ± 0.01
Mean*	26.24^{A}	20.33 ^{B}	12.71 ^{C}	6.47 ^{D}	2.80 ^{E}	1.15 ^{E}	0.41 ^{E}	0.39 ^{E}
Average gene flow frequency (%) in all eight sectors				8.81				

* Figures followed by same letter are not significantly different at the level of $P_{0.01}$

were listed in Table 3. The maximal gene flow frequency was 86.73% and the frequency decreased with the increase in the distance from the pollen donor. The cumulative mean frequency from 0 to 19 m in the compass sectors was NW 26.24%, N 20.33%, W 12.71%, NE 6.47%, SW 2.80%, S 1.15%, E 0.41%, and SE 0.39%, respectively. Obviously, a higher transgene flow frequency was observed in the NW, N, W and NE sectors that were downstream of the prevailing or sub-dominant wind (we refer this as DPW in the following text) and the maximal frequency appeared in the NW sector. A relatively low frequency was observed in the SW, S, E and SE sectors that were lateral to or upstream of the prevailing wind direction (UPW) (Table 3, Fig. 2). The average gene flow frequency in all eight sectors was 8.81% in 2003.

The prevailing wind direction was SEE and SSW in 2004, with the mean and maximal wind speed 1.2–1.4 and 3.1–3.8 m/s, respectively (Table 2), which was lower than that in 2003. The transgene flow frequencies from 0 to 18 m in the eight compass sectors were shown in Table 4 with the maximal frequency being 67.14%. The cumulative mean frequency from 0 to 18 m was as follows: NW 20.52%, N 15.97%, NE 12.01%, W 10.73%, SW 3.03%, E 2.26%, S 1.57%, and SE 1.02%. A relatively high frequency was observed in the NW, N, NE and W sectors situated at DPW and the maximal frequency appeared in the NW sector. A relatively low frequency was observed in the SW, SE, S and E sectors located at UPW. As shown in Fig. 3, the transgene flow frequencies could be divided into two groups: a higher frequency group and a lower frequency group that distributed on the curve chart without any overlap or intersection. The average gene flow frequency in all the eight sectors was 8.39% in 2004.

ANOVA and Student's *t* test (Tables 3, 4) demonstrated that the gene flow frequency was significantly higher in the sectors that located at DPW than in those at UPW, indicating close association with the wind direction. In 2003, the gene flow frequency was significantly higher in the sectors (NW, N, W and NE) at DPW than in those (SW, S, E and SE) at UPW ($P < 0.01$). There was no significant difference in the frequency between the SW, S, E and SE sectors (lower frequency group), while there were significant differences between the NW, N, W and NE sectors (higher frequency group) ($P < 0.01$). Similarly, in 2004, the gene flow frequency was significantly higher in the sectors (NW, N, W and NE) at DPW than that in the sectors

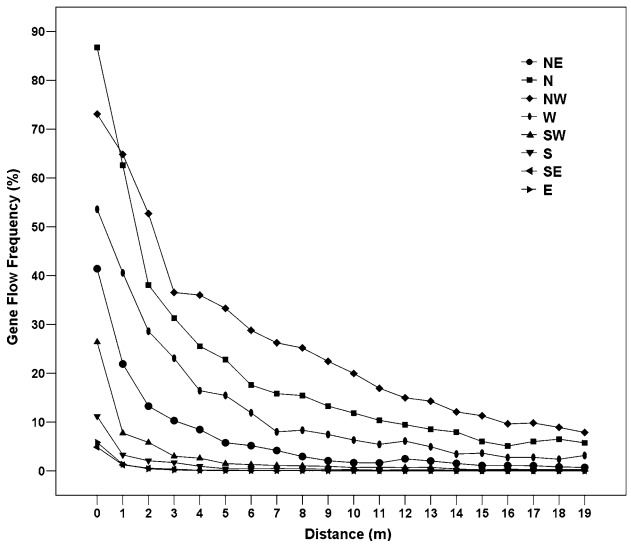

Fig. 2 Curve of transgene flow frequency (%) at different distances in eight compass sectors (2003)

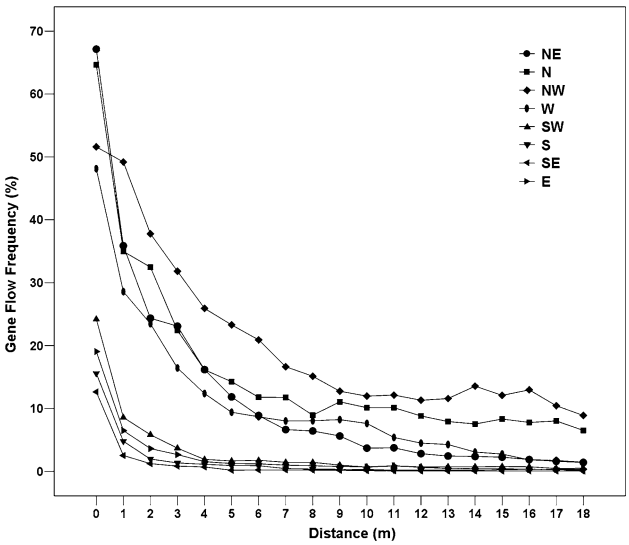

Fig. 3 Curve of transgene flow frequency (%) at different distances in eight compass sectors (2004)

Table 4 Gene flow frequency (%) at different distances in eight compass sectors in 2004

Distances (m)	Compass sectors							
	NW	N	NE	W	SW	E	S	SE
0	51.60 ± 4.32	64.64 ± 14.15	67.14 ± 12.88	48.13 ± 6.08	24.20 ± 2.95	19.05 ± 4.36	15.57 ± 3.00	12.63 ± 1.34
1	49.20 ± 1.67	34.98 ± 7.23	35.87 ± 3.92	28.57 ± 2.99	8.56 ± 0.34	6.48 ± 0.44	4.77 ± 1.22	2.52 ± 0.27
2	37.78 ± 0.92	32.47 ± 3.03	24.35 ± 3.00	23.47 ± 2.29	5.82 ± 0.55	3.62 ± 0.30	1.95 ± 0.21	1.21 ± 0.49
3	31.82 ± 1.20	22.39 ± 4.02	23.08 ± 0.75	16.43 ± 1.84	3.68 ± 0.40	2.66 ± 0.14	1.33 ± 0.17	0.82 ± 0.19
4	25.91 ± 3.12	16.14 ± 1.77	16.17 ± 2.74	12.37 ± 1.57	1.89 ± 0.20	1.53 ± 0.06	1.11 ± 0.17	0.69 ± 0.58
5	23.29 ± 4.70	14.25 ± 2.20	11.84 ± 1.32	9.39 ± 1.03	1.65 ± 0.14	1.23 ± 0.26	0.92 ± 0.21	0.17 ± 0.07
6	20.90 ± 6.09	11.79 ± 1.30	8.86 ± 1.10	8.67 ± 0.83	1.75 ± 0.04	1.19 ± 0.25	0.88 ± 0.03	0.22 ± 0.12
7	16.64 ± 1.69	11.75 ± 0.59	6.63 ± 0.63	8.01 ± 0.59	1.43 ± 0.22	0.98 ± 0.11	0.44 ± 0.04	0.23 ± 0.13
8	15.12 ± 0.74	8.92 ± 1.25	6.42 ± 1.07	8.04 ± 0.89	1.42 ± 0.35	0.88 ± 0.17	0.36 ± 0.05	0.19 ± 0.09
9	12.74 ± 0.12	11.05 ± 0.67	5.61 ± 1.18	8.23 ± 0.51	0.99 ± 0.27	0.77 ± 0.10	0.32 ± 0.04	0.15 ± 0.05
10	11.96 ± 1.57	10.12 ± 0.64	3.69 ± 1.03	7.61 ± 0.89	0.67 ± 0.06	0.71 ± 0.07	0.29 ± 0.04	0.11 ± 0.04
11	12.13 ± 1.25	10.12 ± 1.03	3.74 ± 0.42	5.39 ± 0.39	0.84 ± 0.18	0.88 ± 0.20	0.20 ± 0.03	0.09 ± 0.03
12	11.31 ± 0.38	8.81 ± 1.59	2.81 ± 0.72	4.49 ± 0.52	0.71 ± 0.10	0.63 ± 0.03	0.21 ± 0.03	0.08 ± 0.01
13	11.57 ± 1.02	7.92 ± 1.69	2.44 ± 0.62	4.26 ± 0.56	0.71 ± 0.08	0.47 ± 0.03	0.22 ± 0.02	0.06 ± 0.03
14	13.55 ± 1.83	7.53 ± 0.46	2.36 ± 0.69	3.07 ± 0.84	0.71 ± 0.07	0.41 ± 0.05	0.21 ± 0.04	0.05 ± 0.02
15	12.08 ± 1.95	8.32 ± 1.61	2.25 ± 0.69	2.75 ± 0.11	0.75 ± 0.11	0.42 ± 0.02	0.26 ± 0.12	0.06 ± 0.02
16	12.96 ± 1.10	7.78 ± 0.81	1.87 ± 0.12	1.82 ± 0.22	0.72 ± 0.33	0.30 ± 0.05	0.33 ± 0.13	0.07 ± 0.02
17	10.43 ± 1.19	8.01 ± 0.76	1.59 ± 0.14	1.73 ± 0.12	0.45 ± 0.04	0.33 ± 0.08	0.28 ± 0.12	0.06 ± 0.02
18	8.87 ± 0.47	6.50 ± 0.40	1.41 ± 0.33	1.45 ± 0.15	0.50 ± 0.13	0.32 ± 0.02	0.15 ± 0.06	0.05 ± 0.03
Mean*	20.52 ^{A}	15.97 ^{B}	12.01 ^{C}	10.73 ^{C}	3.03 ^{D}	2.26 ^{DE}	1.57 ^{DE}	1.02 ^{E}
Average gene flow frequency (%) in all eight sectors				8.39				

* Figures followed by same letter are not significantly different at the level of $P_{0.01}$

(SW, S, E and SE) at UPW ($P < 0.01$). The only difference in 2004 was that there was no significant difference in the frequency between the NE and W sectors, while there was a significant difference between the SE and SW sectors ($P < 0.01$). The statistical analysis of the results of the two years indicated that the eight sectors could be divided into two groups: a higher frequency group at the DPW direction and a lower frequency group at the UPW direction. Despite

some differences in the wind direction and speed over the two years, the results were reproducible with the same tendency (Tables 3, 4).

On the basis of the total number of seedlings with Basta® resistance (Basta^R) in each sector, the eight sectors could also be divided into a DPW group (NW, N, W and NE) and a UPW group (SW, S, E and SE). Basta^R seedlings ranged from 33,296 to 214,562 in 2003 and 36,300 to 70,895 in 2004 in the former group, while it was 1,220–10,466 in 2003 and 2,023–8,675 in 2004 in the later group. There was one order of magnitude difference in the number of Basta^R seedlings between the two groups (Table 5). The ratio of Basta^R seedlings in each sector to the total number of Basta^R seedlings in all eight sectors ranged from 7.1–46.0% (2003) and 16.2–31.7% (2004) in the higher frequency group, while it was 0.3–2.2% (2003) and 0.9–3.9% (2004) in the lower frequency group. When the data of the four sectors in each group were accumulated by year, the ratio was 90.2–96.3% in the higher frequency group and 3.7–9.8% in the lower frequency group (Table 5).

The sharp cut-off point of gene flow frequency in compass sectors

As shown in Figs. 2 and 3, the maximal gene flow frequency was observed at 0 m. The frequencies in all the sectors sharply decreased with the increase in distance from the pollen donor. There were significant differences in the sharp cut-off point between the lower frequency group (at 1–2 m) and the higher frequency group (at 5–10 m or even longer). The descending rate of curves in the higher frequency group was relatively slow. The sharp cut-off point was more pronounced in 2004 than that in 2003, possibly due to a lower wind speed in 2004. The results indicated that the sharp cut-off point and the curve shape were associated with both wind direction and speed (Figs. 2, 3).

Table 5 The number and percentage of Basta^R seedlings in each sector in 2003 and 2004

Year	Sector	No. of Basta^R seedlings	Percentage (%)	% Basta^R in four sectors
2003	NW	214,562	46.0	96.3
	N	133,931	28.7	
	W	67,534	14.5	
	NE	33,296	7.1	
	SW	10,466	2.2	3.7
	S	3,795	0.8	
	E	1,641	0.4	
	SE	1,220	0.3	
	Total	466,445	100.0	
2004	NW	70,895	31.7	90.2
	N	56,133	25.1	
	NE	38,397	17.2	
	W	36,300	16.2	
	SW	8,675	3.9	9.8
	E	7,467	3.3	
	S	3,677	1.6	
	SE	2,023	0.9	
	Total	223,567	100.0	

The percentage in each sector was calculated by: the number of Basta^R seedlings in each sector divided by the total number of Basta^R seedlings in all eight sectors

Simulation models of gene flow in compass sectors

The gene flow frequency at different distances in the compass sectors was analyzed for the establishment of a simulation model by using Microsoft Excel. The simulation model for each sector was formulated as: $y = ax^{-b}$ (Table 6).

Where: x represents the distance from the center of the pollen donor ($x > 5$, radius of pollen donor is 5 m), Y represents gene flow frequency (%), a and b are the constants with interaction affecting the decline speed of gene flow frequency. If the absolute value of a or b was bigger, the decline speed of gene flow frequency would be larger. The absolute value of a and b depends on the wind speed and the degree of turbulence; a mainly influence the gene flow frequency at closer distances while b mainly influences the frequency at longer distances. Results showed a high determination coefficient (R^2) ranging from 0.8855 to 0.9940 ($P < 0.01$), indicating the degree of simulation was relatively high. The optimal simulation model $y = 3435.00x^{-2.48}$ was obtained for the NE sector with a determination coefficient of 0.9940 in 2004.

Isograms of gene flow frequency (%) in compass directions

The isograms of the gene flow frequency were drawn on the basis of the simulation models obtained in 2003 and

Table 6 Simulative formula of rice gene flow and its determination coefficient (R^2) in eight compass sectors

Sectors	2003		2004	
	$y = ax^{-b}$	R^2	$y = ax^{-b}$	R^2
N	$y = 1226.80x^{-1.73}$	0.9842	$y = 342.50x^{-1.29}$	0.9113
NE	$y = 1630.10x^{-2.42}$	0.9764	$y = 3435.00x^{-2.48}$	0.9940
E	$y = 7293.20x^{-4.86}$	0.9344	$y = 382.10x^{-2.32}$	0.9418
SE	$y = 285.50x^{-3.18}$	0.9181	$y = 738.00x^{-3.20}$	0.9228
S	$y = 304.10x^{-2.56}$	0.9290	$y = 339.60x^{-2.51}$	0.8855
SW	$y = 951.10x^{-2.64}$	0.9520	$y = 401.20x^{-2.21}$	0.9188
W	$y = 1358.00x^{-1.98}$	0.9852	$y = 1287.60x^{-2.05}$	0.9489
NW	$y = 827.90x^{-1.42}$	0.9802	$y = 323.60x^{-1.14}$	0.9390

2004 (Fig. 4). The results indicated that the isolines in the various compass directions were significantly different. As most of the transgene flow events occurred closer to the 0 m zone in the sectors at UPW, the isolines in the UPW direction could not be plotted that resulted in a discontinuous shape. The isolines were distributed sparsely and asymmetrically in the sectors at DPW distant from the pollen donor. The longer the distance from the pollen donor, the sparser the line distribution. Figure 4 clearly indicates that the shape of the isograms was very similar in 2003 and 2004.

The cumulative gene flow events in compass sectors

The cumulative gene flow frequency calculated on the basis of the data obtained through continuous sampling would be more informative than the one calculated on the basis of the simulation models. Results in Table 7 indicated that in the sectors located at UPW, the cumulative gene flow events amounted to 60% of the total number of events within 0 to 5.2 m, while it was 5.4 to 8.8 m in the sectors located at DPW. The distance at which the cumulative gene flow events reached 90% was 2.2 to 13.6 m in the sectors at UPW and 13.2 to 16.2 m in the sectors at DPW. Roughly, 60 and 90% of the cumulative gene flow events occurred within 0–14 m in the sectors upstream of the prevailing wind, while it was approximately 5–16 m in the sectors downstream of the prevailing wind.

Discussion

In this study, a concentric design combining three measures was adopted, i.e. the ms line with a higher rate of outcrossing was used as a recipient, the recipient plants were planted in eight sectors, and continuous sampling was taken. The results demonstrated that rice gene flow was closely associated with the wind direction. According to the data obtained, the eight sectors could be divided into two groups: i.e. a higher frequency group (sectors downstream of the prevailing wind) and a lower frequency group (sectors lateral to or upstream of the prevailing wind). The results generated in 2 years showed sound reproducibility. Meanwhile, simulation models and isograms of rice gene flow were developed and drawn, respectively. These data could serve as a reference for determination of safe isolation distances in different directions from transgenic rice.

Recipient and gene flow

In the previous studies on rice gene flow, the recipients used were common rice cultivars, red rice or wild rice (*O. rufipogon*). As the gene flow frequency is relatively low, particularly to common cultivars that is usually less than 1% (Messeguer et al. 2001, 2004; Song et al. 2002, 2003; Lu et al. 2003; Chen et al. 2004; Rong et al. 2004, 2005), it is difficult to differentiate the differences in gene flow frequency between the compass directions associated with the wind direction. In the present study, the ms line BoA with a high rate of outcrossing was used as a recipient in the light of the following two considerations: (1) given that Asian countries, China in particular, are the largest producers of hybrid rice, and that the transgenic rice has advanced nearly to the commercialization stage (Jia et al. 2004), a study of the transgene flow to the ms lines of hybrid rice is urgently needed. (2) Rice is an autogamous crop in nature. However, as the ms line does not produce viable pollen, but has a higher rate of stigma exertion, it is easy to accept foreign pollen for outcrossing (IRRI 2004; Yuan 1995). In this respect, the ms line is allogamous in nature and is an ideal material for the investigation of rice gene flow as a pollen detector to amplify differences in

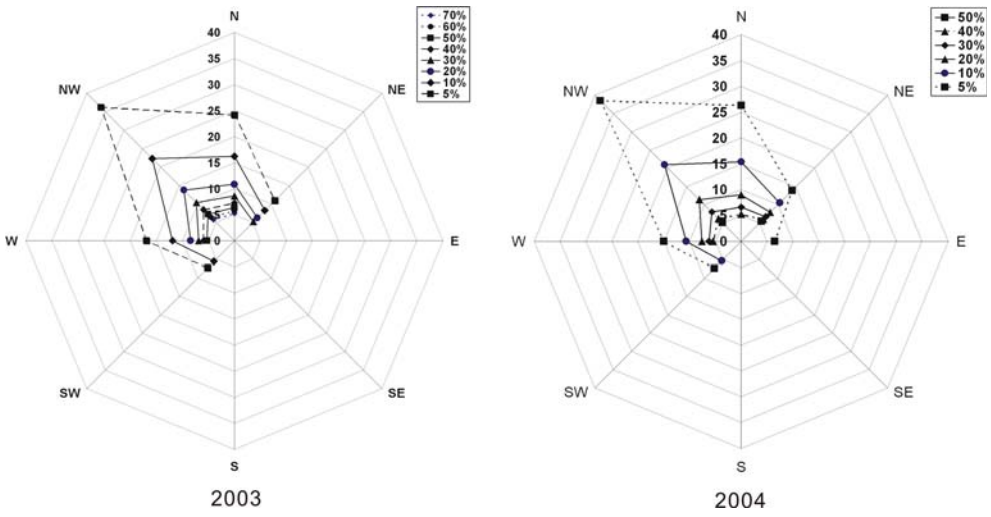

Fig. 4 Isolines of rice gene flow frequency (%) in compass directions in 2003 and 2004. Each line located at a distance of 40–0 m from outer to center circle represents 5, 10, 20, 30, 40, 50, 60 and 70% (in 2003), and 5, 10, 20, 30, 40 and 50 (in 2004) of the gene flow frequency, respectively

Table 7 The distances (m) where 60 to 90% of cumulative gene flow events occurred in eight compass sectors

Year	Cumulative gene flow events (%)	Sectors and distances from edge of donor (m)							
		NW	N	NE	W	SW	E	S	SE
2003	60.0	8.8	7.9	5.7	7.7	4.5	0.0	2.0	0.0
	70.0	10.7	9.8	7.2	9.7	6.7	0.0	3.8	0.7
	80.0	13.0	12.1	10.1	11.9	9.2	0.5	7.1	2.3
	90.0	15.8	15.3	13.2	14.8	12.5	2.2	11.7	7.2
2004	60.0	8.7	8.6	5.4	6.0	5.2	4.1	1.2	0.1
	70.0	11.5	11.2	7.1	8.0	7.0	6.2	3.6	0.9
	80.0	14.4	14.3	9.7	10.6	9.7	8.7	6.0	2.7
	90.0	16.2	16.1	13.2	13.7	13.6	11.9	10.9	6.9

gene flow frequency between the compass directions so as to reveal the association of gene flow with wind direction. Messeguer et al. (2004) conducted a circular field experiment to detect gene flow frequency of transgenic rice to the common cultivar Senia and the red rice in the compass directions. The maximal frequency of gene flow from transgenic to non-transgenic rice was 0.106% and the mean frequency in all compass directions was 0.086%. In our study, the maximal gene flow frequency was 86.73 and 67.14%, and the mean frequency in all eight sectors was 8.81 and 8.39% in 2003 and 2004, respectively (Tables 3, 4). The figure was much higher than that reported by Messeguer et al. (2001, 2004), demonstrating that the ms line had a significantly amplifying effect.

In theory, if there is no non-transgenic pollen competitor, the transgene flow frequency to the ms line will be 100%. To simulate the actual conditions in hybrid rice production, a non-transgenic pollen competitor was used in this study. In this case, the maximal gene flow frequency was 86.73 and 67.14% in 2003 and 2004, respectively, instead of 100%. Although the maximal gene flow frequency in 2003 was higher than that in 2004, the average frequency in all eight sectors was very close (8.81 vs. 8.39%) (Tables 3, 4), indicating that a similar strength of competition was provided in 2003 and 2004. It should be pointed out that when there is no pollen competition and the experimental plot is strictly isolated, then the transgene flow frequency to the ms line could be estimated directly on the basis of the seed-setting rate.

Relationship between gene flow frequency and wind direction

Rice is a wind-pollinated crop. In this study, the mean gene flow frequency differed significantly between compass sectors, which were closely related to the wind direction and the location of the sectors. In general, the eight sectors can be divided into a higher frequency group in sectors of NE, N, NW and W at DPW direction, and a lower frequency group in sectors of SW, E, S and SE located at UPW direction. The major differences between the two are as follows: (1) the mean frequency of transgene flow ranged from 6.47 to 26.24% in the higher frequency group, while it was 0.39–3.03% in the lower frequency group (Tables 3, 4). (2) The ratio of the number of Basta^R seedlings in each sector out of the total number of Basta^R seedlings in all eight sectors was shown in Table 5. It was evident that 90–96% of the cumulative gene flow events occurred in the four sectors located at DPW direction and only 4–10% occurred in the four sectors at UPW direction. (3) The sharp cut-off point of gene flow frequency was 1–2 m in the lower frequency group, whereas it was 5–10 m in the higher frequency group (Figs. 2, 3). These data elucidated quantitatively the relationship between the wind direction and the transgene flow frequency.

Simulation models of transgene flow in compass sectors

In order to establish simulation models of rice transgene flow in the compass sectors, we adopted a concentric circular design. The pollen donor with a radius of 5 m and area of 78.5 m^2 was located at the center circle. The recipient BoA was planted in eight compass sectors with an area of 205–224 m^2 each. Based on the plant spacing 17 × 20 cm, the total number of plants in each sector was approximately 6,500–6,700. All seeds from the recipient plants in eight sectors were harvested, and the Basta^R seedlings in each sector were counted after three times of Basta® spray. On the basis of the systematic data obtained, the simulation model in each sector was developed (Table 6). Regarding the methodology, we have dramatically increased the number of recipient plants, which were planted in eight compass sectors, instead of being planted along the eight radial lines, so that the accuracy of the simulation model could be improved.

Previous studies on gene flow of different crops demonstrated that the transgene flow frequency or outcrossing rate is a function of distance. Frequently, the curve shape

was negative exponential, i.e. the gene flow frequency decreased sharply when distance increased (Scheffler et al. 1993; Wang et al. 1997; Hall et al. 2000; Beckie et al. 2003; Meagher et al. 2003; Damgaard et al. 2005; Gustafson et al. 2005; Funk et al. 2006a, b; Jia et al. 2007; Staniland et al. 2006). In this study, the curves of simulation model in eight sectors were all exponential with the determination coefficient ranging from 0.8855 to 0.9940 ($P < 0.01$). Out of which the simulation model $y = 3435.00x^{-2.48}$ for the NE sector at DPW in 2004 had a determination coefficient 0.9940. In our study applying rectangular design conducted in Sanya, 2004, we detected that the maximum distance of transgene flow to the same recipient BoA downstream of the prevailing wind was 300 m with a frequency of 0.001% (unpublished data). When verified by using $y = 3435.00x^{-2.48}$, the gene flow frequency y at 300 m is 0.002%, the maximal distance x was 380 m with a frequency y being 0.001%, indicating there was still an approximate 27% difference between the observed and the predicted figure. It might be due to some factors that were not taken into consideration in the process of model establishment. One is pollen viability usually within several minutes of shedding from the anther. Other meteorological parameters such as temperature, humidity and radition are not considered. In practice, the wind direction changes frequently, which limits the application of the simulation model unless there is a prevailing wind direction during the rice flowering period.

Isolines and sharp cut-off points of gene flow frequency in compass sectors

Isolines of gene flow frequency in different directions were shown in Fig. 4. Since gene flow frequency was closely related to the wind direction and speed, the location of isolines varied from one to another. Isolines appeared discontinuously in UPW sectors while it distributed sparsely and asymmetrically in distant areas from the transgenic pollen donor at DPW direction. The shape of isolines obtained in 2003 and 2004 was very similar (Fig. 4).

Noteworthy, we investigated the rice transgene flow to six recipients (including common rice cultivars, hybrid rice and ms lines) in Guangzhou in 2002 and found that the gene flow frequency sharply cut-off (one order of magnitude) at 1–2 m away from the pollen donor in the case of maximal wind speed 2.2 m/s during the flowering period. We referred this as a sharp cut-off point (Jia et al. 2007). However, the sharp cut-off point extended to approximately 10 m at the Sanya experiment in 2003 where the maximal wind speed was 4.8 m/s (unpublished data). Such difference might be due to a higher wind speed in Sanya than that in Guangzhou. Interestingly, as shown in Figs. 2 and 3, the sharp cut-off point differed obviously between the lower frequency group (within 1–2 m) and the higher frequency group (extended to 5–10 m). These data further support the conclusion that the rice gene flow is associated with the wind direction and speed.

Conclusions

The study of maximal frequency and maximal distance of transgene flow to the same species or related wild species is a prerequisite for the environmental risk assessment, which may provide data not only for scientific evaluation, but also for determination of safe isolation distance in commercial production. Simultaneously, the study of gene flow frequency at different directions will provide more basic information for revealing the overall picture of gene flow and establishing simulation models. In the sense of agriculture practice, these data may also help to assess the environmental impact of bi-directional gene flow at different directions between transgenic and non-transgenic rice within a co-existence system after large-scale commercialization of transgenic rice. It is particularly important in Asian countries where most farmers generally only have small pieces of land. In this case, the plantation of transgenic and non-transgenic rice will form a mosaic pattern at close spaces. As demonstrated by this study, the gene flow frequency was significantly lower in the directions lateral to or upstream of the prevailing wind than that in the prevailing or sub-dominant wind direction. This finding with detailed data coincides with the general practice in hybrid rice seed production and ms line multiplication, in which the field is chosen at upstream of the prevailing wind for decreasing or avoiding the influence of pollen dispersal on seed purity. It is well known that the gene flow is affected by many biological and environmental factors, such as the size of pollen donor and recipient, the degree of synchronization of flowering period, pollen viability, outcrossing ability of recipient, wind speed and direction, temperature, humidity, pollination insects, topography and geomorphy, and physical barriers, etc. In order to determine proper isolation distance and apply relevant agricultural practice in a given region, these factors must be studied in detail taking into account the given donor and recipient under specific local conditions.

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