Dramatic reduction of crop-to-crop gene flow within a short distance from transgenic rice fields

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Summary

• Genetically modified (GM) rice with enhanced agronomic traits and pharmaceutical uses are ready for widespread adoption. Little is known about isolation requirements for achieving stringent transgene confinement in rice. To investigate the extent of pollen-mediated crop-to-crop transgene flow, we conducted a field experiment with four plot-size treatments of adjacent GM and nonGM rice (*Oryza sativa*) in China.
• Three insect-resistant GM rice (*Bt/CpTI*) and nonGM isogenic lines were used in the study. The hygromycin-resistance transgene (*hpt*) marker was used to screen seeds from the nonGM rice rows at different distance intervals from GM rice plots.
• Based on the examination of > 2.1 million germinated seeds, we found a dramatic reduction in transgene frequencies with increasing distance from the GM crop, ranging from *c.* 0.28% at 0.2 m to < 0.01% at 6.2 m. In addition, different plot size did not significantly affect the frequencies of gene flow.
• In conclusion, pollen-mediated crop-to-crop transgene flow in rice can be maintained at negligible levels with short spatial isolation. The model can also be applied to other crops with self- and wind-pollination.

Key words: cross-pollination, gene flow, hygromycin resistance, isolation distance, *Oryza sativa*, transgenic rice.

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Introduction

Rice (*Oryza sativa*) provides a staple food for nearly half of the global population and is the third most important crop in China (FAO, 2004). The output of rice production in China declined by 16% between 1999 and 2003 (Jia, 2004), while the nation's population increased by *c.* 3% (National Bureau of Statistics of China, 2003) during this interval, raising serious concerns about food security. To meet the demands of national rice production, China has invested heavily in the research and development of genetically modified rice (Jia, 2004). Rice is one of the first crop species for which transgenic methods have been applied to develop high-yielding, high-quality stress-tolerant varieties. Sequencing of the entire rice genome (Yu *et al.*, 2002; International Rice Genome Sequencing Project, 2005) opens new opportunities for genetic improvement owing to ongoing advances in functional genomic and DNA-marker-assisted selection. To date, many genetically modified (GM) rice varieties have been developed worldwide; in China, some of these varieties have been grown in farm-scale pre-production trials, which is the last step before commercialization (Xiong, 2004; Huang *et al.*, 2005).

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Plans for the environmental release and commercial production of GM rice raise questions related to biosafety, labeling, and consumer acceptance. As with all GM crops, information about gene flow, defined here as the dispersal of crop genes via seeds and pollen, is a crucial component of assessments that influence biosafety policies and trade (Huang *et al.*, 2005; Lu & Snow, 2005). Depending on the transgene product, further study may be needed to guard against unwanted effects on human health, animal health, or the environment. Here, we focus on pollen-mediated, crop-to-crop gene flow because remarkably little is known about this process in rice (Messeguer, 2003).

Most rice cultivars worldwide are highly self-pollinating (Khush, 1993), with the exception of male-sterile rice lines that are used for hybrid seed production. To maintain phenotypically distinct rice varieties, seed producers typically rely on pollen isolation distances of only *c.* 2 m between adjacent conventional varieties and *c.* 10 m for hybrid seed production involving male-sterile lines (Khush, 1993). However, these standard practices are not designed to completely prevent gene flow between nearby varieties. Extremely low frequencies of cross-pollination between GM rice and nonGM rice varieties (e.g. 0.01%) are easily detected in nonGM rice seeds using DNA-based markers, and in some cases the adventitious presence of GM products causes problems with consumer acceptance. Thus, pollen-mediated gene flow may alter the deployment of GM and nonGM rice in a given region, perhaps leading to legal disputes over regional or international trade. Moreover, for transgenic traits such as herbicide resistance and insect resistance, it may be desirable to avoid cross-pollination between GM rice and weedy rice (the same species as the crop) or wild rice species (e.g. *O. rufipogon*), both of which are known to hybridize with the crop (Song *et al.*, 2003; Chen *et al.*, 2004; Lu & Snow, 2005). When farmers exchange seeds or save part of their crop for replanting, transgenes could disperse further by means of both pollen and seeds.

For wind-pollinating crops, including rice, the likelihood and extent of pollen-mediated gene flow are considerably affected by factors such as reproductive characteristics of pollen donors and receivers (e.g. flowering synchronization and pollen dispersal), experimental plot size, isolation distance, and climatic conditions (Walklate *et al.*, 2004; Gustafson *et al.*, 2005). Rice pollen is capable of dispersing at least 100 m from its source (Song *et al.*, 2004), but the extent of cross-pollination among crops is poorly understood (Messeguer, 2003; Lu & Snow, 2005). Therefore, field assessments of transgene flow from GM rice to nonGM rice are an essential first step for a thorough evaluation of gene flow and its potential consequences.

To determine the crop-to-crop gene flow in GM rice in relation to the factors concerned, we designed a field experiment to examine the frequencies of pollen-mediated gene flow under controlled field conditions, involving three insect-resistant GM rice lines and their nonGM isogenic counterparts. The objectives of this study were to answer the following questions:

- What are the effective isolation distances that can minimize gene flow from GM to nonGM rice varieties in the fields?
- Are there significant experimental plot size effects on frequencies of rice gene flow?
- Can different rice varieties significantly affect frequencies of rice gene flow?

The answer to these questions will provide much-needed information for designing field trials and minimizing unwanted cross-pollination among rice fields, and for addressing similar issues for other crops with self- and wind-pollination.

Materials and Methods

Plant materials

The experiment included three transgenic insect-resistant rice (*Oryza sativa* L.) lines – KeFeng6 (labeled as MSR+), IIYouKeFeng6 (HY1+) and 21SKeFeng6 (HY2+) – as well as their nontransgenic counterparts Minghui-86 (labeled as MSR–), IIYouming-86 (HY1–) and Liangyou-2186 (HY2–) (Rong *et al.*, 2005; Chen *et al.*, 2006). The transgenic lines, which were used as pollen donors, contained two tightly linked and single-inserted transgenes for insect resistance, *Bt* gene (*cryIAc*) from *Bacillus thuringiensis* and cowpea trypsin inhibitor (*CpTI*) gene modified from *Vigna unguiculata*, having a constitutive ActID promoter (for *CpTI*) and a constitutive ubiquitin (for *Bt*) promoter, respectively (Chen *et al.*, 2006). These two transgenes (*Bt*/*CpTI*) were linked to a selectable marker gene (*hpt*) for hygromycin resistance, driven by a CaMV 35S promoter (Chen *et al.*, 2006). The GM KeFeng6 was a conventional rice line (restorer), hence, all plants of this line were homozygous. The GM IIYouKeFeng6 and 21SKeFeng6 were hybrid rice lines, consequently, all plants were heterozygous (Rong *et al.*, 2005). The nontransgenic varieties, which were used as receivers of transgenic pollen, were a male-sterile restorer variety (Minghui-86) and two hybrid varieties (IIYouming-86 and Liangyou-2186) that are widely used in rice production in China. The transgenic and nontransgenic rice pairs were isogenic lines, and each pair (MSR+ vs MSR–, HY1+ vs HY1– or HY2+ vs HY2–) had the same timing of growth and flowering, which guaranteed maximum opportunities for transgene flow. All genetic lines were produced by Fujian Province Key Laboratory of Genetic Engineering for Agriculture, Fujian Academy of Agricultural Sciences, Fujian Province in China. The transgenic rice lines received permission from the National Biosafety Committee of China for pre-release production tests under controlled field conditions.

Field experimental design

The experiment was conducted in June–October 2004 at an experimental area with restricted access in Shaxian County, Fujian Province, China. This area was approved by the National

Biosafety Committee of China for growing transgenic rice for environmental biosafety assessments. Four plots (treatments) of different sizes (Fig. 1) were used to detect gene flow from each of the three transgenic rice lines to their nontransgenic counterparts, for a total of 12 plots. For treatments A, B and C, the areas of GM subplots were 400 m^2, 200 m^2 and 100 m^2, respectively, and the adjacent nonGM subplots were 640 m^2. For treatment D, the GM subplot was a long rectangle of 380 m^2 and the nonGM area was a square of 1216 m^2. Thus, effects of the size of the GM donor area can be compared directly for treatments A–C, which had the same rectangular area of recipient plants, while treatment D had nearly twice as much area of recipient plants. The 12 plots were randomly assigned to positions within the field, with at least 30 m between neighboring plots to minimize gene flow from outside the plot.

The density of plants in each plot was similar to small-scale rice production in China. In each plot, rice plants were individually transplanted with distances of 0.2 m between the GM and nonGM subplots, 0.2 m between rows, and 0.2 m between hills within rows. To obtain the maximum gene flow, the subplots were arranged along the prevalent wind direction (north-east to south-west), with transgenic pollen donors grown up-wind relative to nonGM plants. To achieve the synchronous flowering, seeds of transgenic rice and its nontransgenic counterparts were sown in a seedbed on the same day (17 June, 2004). Rice seedlings were transplanted into the experimental plots 34–36 d after the seeds were sown.

Seed harvest

At seed maturity, only seeds from the nontransgenic subplots were harvested. Entire rows of nontransgenic rice plants were harvested at distance intervals of 0.2 m, 0.4 m, 0.6 m, 0.8 m, 1.0 m, 1.4 m, 1.8 m, 2.2 m, 2.6 m, 3.0 m, 3.8 m, 4.6 m, 5.4 m, 6.2 m, 8.2 m, 10.2 m, 12.2 m, 16.2 m, 20.2 m, 24.2 m, 28.2 m and 32.0 m from the transgenic rice subplots. All harvested seeds from each row were mixed, and *c.* 3000–53 000 seeds from each row were randomly selected for the identification of transgenic hybrids.

Identification of transgenic seedlings

After being stored at room temperature for *c.* 3 months to break seed dormancy, the harvested seeds were soaked in fresh water for 2 d and placed in a seed germination chamber without hygromycin B at 37°C for 1–2 d. The germinated seeds were cultured in Petri dishes containing 0.5× Murashige and Skoog (MS) liquid medium with macronutrients and micronutrients, in addition to 50 μg ml^{-1} hygromycin B (Roche Diagnostics (Shanghai) Ltd, Shanghai, China) for *c.* 5 d in an illuminated growth chamber at 25–27°C. The surviving individuals were considered to be transgenic plants containing the hygromycin-resistance gene. This was confirmed for > 10% of the surviving seedlings using polymerase chain reaction (PCR) identification with a specific primer pair (forward TACACAGCCATCGGTCCAGA; reverse TAGGAGGGCGTGGATATGTC) designed for the hygromycin-

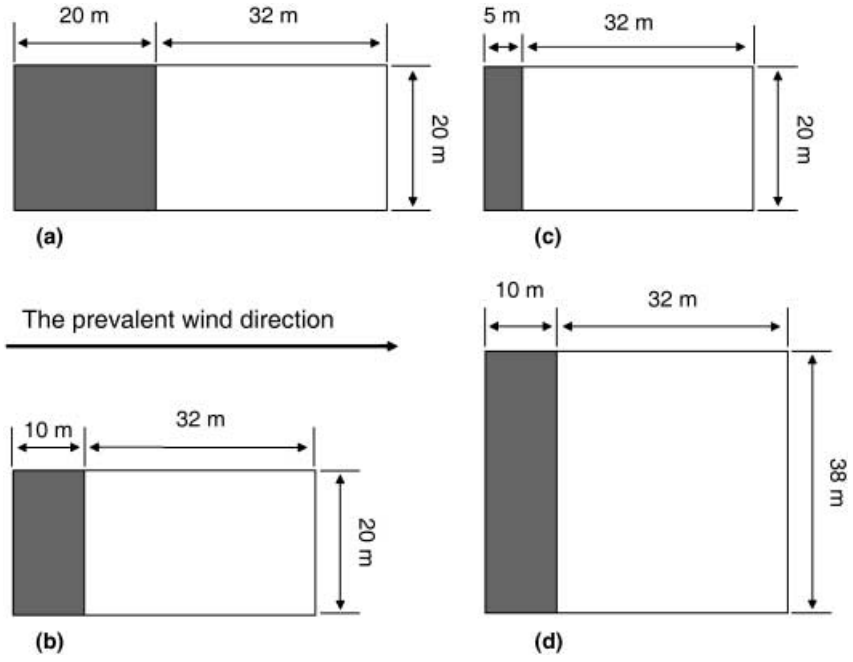

Fig. 1 Diagram of the experimental design with four sizes of plots (treatments a–d) for investigating transgene flow to non-transgenic plants for each of three pairs of rice (*Oryza sativa*) lines. Shading, subplots with genetically modified (GM) rice plants; clear, subplots with nonGM rice plants. To quantify transgene flow, seeds produced on nonGM plants were examined at 16 distances from the edge of the GM subplots (0.2 m, 0.4 m, 0.6 m, 0.8 m, 1.0 m, 1.4 m, 1.8 m, 2.2 m, 2.6 m, 3.0 m, 3.8 m, 4.6 m, 5.4 m, 6.2 m, 8.2 m and 10.2 m) and screened for transgenes.

resistance gene. As expected, all of the surviving seedlings that were screened had the DNA fragment from this transgene. The details of these procedures are described in our previous report (Rong *et al.*, 2005).

Determination of transgene flow

For each row, *c.* 2400–52 000 germinated seeds were examined, and the frequency of transgene flow (F_{gf}) was calculated as the number of transgenic seedlings divided by the total number of seedlings examined. The gene flow frequencies dramatically reduced to nearly zero beyond 6 m, even though a great number of seed samples (> 10 000–50 000 per row) were examined. Therefore, for practical and statistical reasons, seed samples from the rows beyond 10 m were not examined. More than 2.1 million seedlings were examined in the entire study.

Data analysis

Statistical analysis was performed using the package of R (R Development Core Team, 2005). A logistic regression model was applied to test for significant effects of distance, rice pair, plot size (treatment), and their interactions on transgene flow frequencies. A penalized maximum likelihood model was used for the logistic regression analysis (Firth, 1993; Heinze & Schemper, 2002; Heinze & Ploner, 2003), using the brlr software package of R (R Development Core Team, 2005). Differences were considered significant at $P < 0.05$. According to the analysis of deviance results based on the logistic regression model, pairs of rice lines (X_1), plot size (X_2), their interaction (X_1X_2), and distance (X_3) were selected for the logistic regression model: $\text{logit}(P) = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_1 X_2 + \beta_4 X_3$. These analyses were carried out using the four plot-size treatments (A–D), as well as using just A–C, which differed only in the size of the transgenic subplot. Very similar results were obtained in the two sets of analyses, so we have reported the results from treatments A–D in Table 2.

Transgene flow frequency (F_{gf}) was used to estimate the transgene flow probability (P) and distance (X_3) was treated as a discrete variable. The well-represented model was used to estimate the transgene flow frequencies with given variables (e.g. rice pairs, plot size and distance). To illustrate the dynamics of transgene flow frequency with increasing distance, logistic regression curves were drawn with transgene flow frequency as the *y*-axis and distance from pollen donor as the *x*-axis for the three rice pairs and the four plot size treatments (plots A–D, Fig. 2). Odds ratios (OR) were used to compare the transgene flow frequencies of different rice pairs and distances.

Results

We observed extremely low frequencies of transgene flow at the various distance intervals in the four plot size treatments (Table 1). Maximum frequencies of transgene flow were detected at the distance of 0.2 m for the three rice pairs (i.e. 0.28% from the GM rice line HY2+, 0.06% from line MSR+, and 0.04% from line HY1+, respectively). At this short distance, the nonGM plants were so close to the GM plants that cross-pollination could occur from physical contact as well as pollen transfer by air currents. At greater distances from the GM rice subplots, transgene flow frequencies declined dramatically, although variation occurred at certain distance intervals (e.g. 0.4 m, 2.6 m, and 5.4 m; Table 1). When the distance increased to 6.2 m and beyond, the observed transgene flow frequencies were extremely low (< 0.01%) in all treatments. A total of seven transgenic seeds were found at 10.2 m, which was the maximum distance examined, and the frequency of transgenic seeds at this distance was 0.0021%.

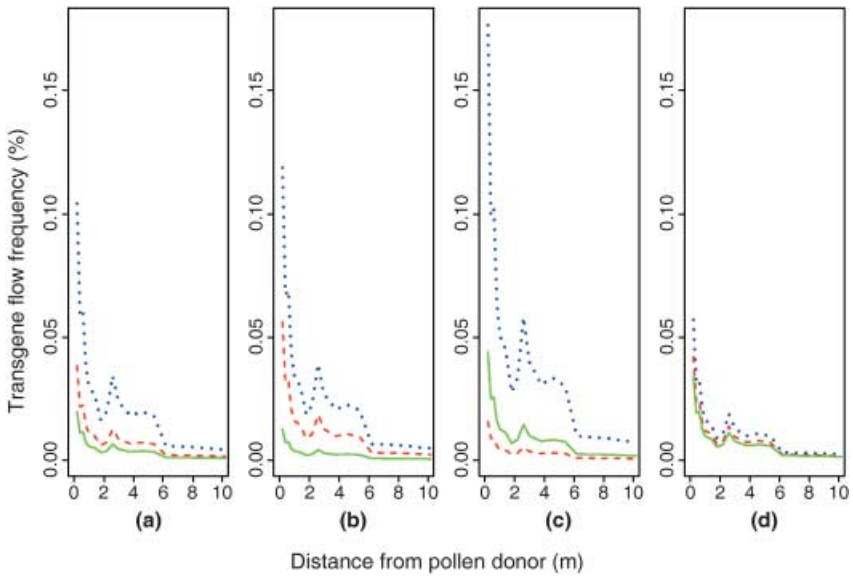

Fig. 2 Logistic regression curves showing the reduction of transgene flow frequencies from genetically modified (GM) rice (*Oryza sativa*) lines to their nonGM counterparts at various distances from the edge of the GM subplots. Areas of the four GM : nonGM subplots (in m^2) are 400 : 640 (treatment a), 200 : 640 (treatment b), 100 : 640 (treatment c), and 380 : 1216 (treatment d). The three GM and nonGM rice pairs: dotted line, HY2+ vs HY2–; dashed line, MSR+ vs MSR–; solid line, HY1+ vs HY1–.

Table 1 Transgene flow frequencies detected in rows of nongenetically modified rice (*Oryza sativa*) at different distances from the GM pollen donor subplot, for three pairs of rice lines

	Transgene flow frequencies detected (%)[1]															
	Rice pair					Rice pair					Rice pair					
	MSR+ and MSR–					HY1+ and HY1–					HY2+ and HY2–					
Distance (m)	A	B	C	D	Subtotal	A	B	C	D	Subtotal	A	B	C	D	Subtotal	Total
0.2	0.0386 (2589)	0.0299 (3342)	0.0296 (3375)	**0.0566** (3531)	0.0387 (12837)	0.0238 (8398)	0.0128 (7780)	**0.0356** (8428)	0.0350 (8568)	0.0268 (33174)	0.0280 (3574)	0.0843 (3558)	**0.2827** (2476)	0.0600 (3333)	0.1138 (12941)	0.0598 (58952)
0.4	0.0258 (3870)	0.0000 (3462)	0.0000 (3762)	0.0394 (2535)	0.0163 (13629)	0.0112 (8881)	0.0000 (8678)	0.0231 (8670)	0.0110 (9065)	0.0113 (35294)	0.0658 (4560)	0.1357 (3684)	0.0901 (3331)	0.0000 (3531)	0.0729 (15106)	0.0335 (64029)
0.6	0.0294 (3400)	0.0319 (3136)	0.0507 (3944)	0.0285 (3508)	0.0351 (13988)	0.0328 (9155)	0.0124 (8062)	0.0000 (9072)	0.0106 (9432)	0.0140 (35721)	0.0227 (4398)	0.1002 (2994)	0.0954 (3144)	0.0000 (3892)	0.0546 (14428)	0.0346) (64137
0.8	–[2]	0.0267 (3740)	0.0000 (3400)	0.0350 (2860)	0.0206 (10000)	0.0105 (9503)	0.0107 (9308)	0.0000 (9496)	0.0000 (9492)	0.0053 (37799)	0.0891 (4491)	0.0240 (4160)	0.0578 (3457)	0.0000 (3988)	0.0427 (16096)	0.0229 (63895)
1.0	0.0248 (4040)	0.0000 (3396)	0.0000 (4132)	0.0000 (3132)	0.0062 (14700)	0.0000 (9987)	0.0000 (9844)	0.0100 (9968)	0.0000 (10076)	0.0025 (39875)	0.0711 (4217)	0.0238 (4192)	0.0883 (3398)	0.0000 (4160)	0.0458 (15967)	0.0182 (70542)
1.4	0.0000 (4424)	0.0225 (4440)	0.0000 (4428)	0.0219 (4568)	0.0111 (17860)	0.0000 (10753)	0.0000 (10356)	0.0092 (10832)	0.0000 (10725)	0.0023 (42666)	0.0452 (4421)	0.0446 (4484)	0.0241 (4149)	0.0211 (4732)	0.0338 (17786)	0.0157 (78312)
1.8	0.0000 (3852)	0.0294 (3396)	0.0000 (4296)	0.0000 (5116)	0.0074 (16660)	0.0000 (11659)	0.0000 (11484)	0.0087 (11535)	0.0000 (11859)	0.0022 (46537)	0.0000 (5527)	0.0000 (4972)	0.0760 (5263)	0.0000 (4968)	0.0190 (20730)	0.0095 (83927)
2.2	0.0000 (5660)	0.0000 (4820)	0.0000 (5250)	0.0000 (5250)	0.0000 (20980)	0.0078 (12754)	0.0000 (12580)	0.0158 (12640)	0.0000 (12217)	0.0059 (50191)	0.0305 (6560)	0.0186 (5360)	0.0207 (4838)	0.0190 (5250)	0.0222 (22008)	0.0094 (93179)
2.6	0.0000 (5585)	0.0000 (5845)	0.0000 (5945)	0.0000 (5220)	0.0000 (22595)	0.0000 (13583)	0.0076 (13068)	0.0149 (13417)	0.0148 (13556)	0.0093 (53624)	0.0181 (5510)	0.0169 (5905)	0.1365 (5127)	0.0164 (6095)	0.0470 (22637)	0.0188 (98856)
3.0	0.0189 (5283)	0.0352 (5682)	0.0000 (5877)	0.0188 (5313)	0.0182 (22155)	0.0068 (14669)	0.0000 (13898)	0.0138 (14509)	0.0067 (14945)	0.0068 (58021)	0.0152 (6589)	0.0000 (6357)	0.0174 (5762)	0.0157 (6363)	0.0121 (25071)	0.0124 (105247)
3.8	0.0185 (5400)	0.0000 (6962)	0.0000 (6923)	0.0000 (6482)	0.0046 (25767)	0.0059 (17044)	0.0059 (17029)	0.0118 (16982)	0.0172 (17437)	0.0102 (68492)	0.0136 (7341)	0.0000 (7718)	0.0144 (6956)	0.0000 (7742)	0.0070 (29757)	0.0073 (124016)
4.6	0.0000 (7072)	0.0298 (6708)	0.0000 (8320)	0.0128 (7840)	0.0106 (29940)	0.0000 (19719)	0.0051 (19702)	0.0051 (19500)	0.0048 (20655)	0.0038 (79576)	0.0000 (8559)	0.0225 (8884)	0.0222 (9004)	0.0318 (9436)	0.0191 (35883)	0.0112 (145399)
5.4	0.0098 (10221)	0.0000 (9372)	0.0000 (10206)	0.0000 (8217)	0.0024 (38016)	0.0000 (22837)	0.0000 (21949)	0.0087 (23075)	0.0125 (23928)	0.0053 (91789)	0.0192 (10443)	0.0374 (10692)	0.0098 (10191)	0.0090 (11148)	0.0188 (42474)	0.0088 (172279)
6.2	0.0000 (12350)	0.0000 (9373)	0.0000 (11423)	0.0000 (11010)	0.0000 (44156)	0.0000 (26396)	0.0000 (26058)	0.0037 (26760)	0.0036 (27580)	0.0018 (106794)	0.0081 (12311)	0.0000 (12783)	0.0000 (11989)	0.0149 (13381)	0.0058 (50464)	0.0025 (201414)
8.2	0.0000 (19259)	0.0066 (15035)	0.0000 (18245)	0.0000 (15552)	0.0016 (68091)	0.0000 (37304)	0.0000 (35157)	0.0027 (37277)	0.0025 (40374)	0.0013 (150112)	0.0054 (18421)	0.0104 (19323)	0.0000 (18278)	0.0049 (20242)	0.0052 (76264)	0.0027 (294467)
10.2	0.0000 (25340)	0.0054 (18331)	0.0000 (27109)	0.0000 (22741)	0.0014 (93521)	0.0000 (51517)	0.0000 (47536)	0.0038 (52035)	0.0020 (50642)	0.0014 (201730)	0.0073 (27519)	0.0069 (29018)	0.0000 (27171)	0.0000 (30902)	0.0036 (114610)	0.0021 (409861)

See Fig. 1 for an explanation of plot size treatments A–D (numbers in bold type indicate the maximum transgene flow frequencies in each rice pair).
[1]Numbers in parentheses indicate the number of germinated seeds examined.
[2]Missing value.

Table 2 An analysis of deviance table presenting degrees of freedom (df), deviance, likelihood ratio test statistic (LRT) and probability values (Pr (χ) computed using Wald inference) for effects of distance, rice (*Oryza sativa*) pairs (genetically modified (GM) and nonGM), treatment (plot size), and their interactions on transgene flow frequencies

Source	df	Deviance	LRT	Pr (χ)
Distance	15	145.880	43.872	0.000115
Rice Pair	2	111.250	9.242	0.009842
Treatment (= plot size)	3	108.793	6.785	0.079064
Distance × Rice pair	30	123.285	21.277	0.879021
Distance × Treatment	45	138.993	36.985	0.796604
Rice pair × Treatment	6	122.598	20.590	0.002173

The statistical analysis was conducted using the logistic regression model.

To test for significant effects of distance, rice pairs, plot sizes (treatments) and their interactions on transgene flow frequencies, we conducted logistic regression model analyses (Table 2). In addition to the obvious and expected effect of distance, considerable differences were seen among rice pairs ($P = 0.0098$), with the greatest level of gene flow in variety HY2. Distance did not interact significantly with the rice pair ($P = 0.88$) or plot size ($P = 0.80$), indicating that the effect of isolation distance on transgene flow frequency was independent of rice variety and plot size.

The effect of plot size on transgene frequencies was marginally significant ($P = 0.079$) and the interaction between plot size and rice pair was highly significant ($P = 0.002$). This interaction could be partly attributable to transgene frequencies for line MSR, which were lower in treatment C than in treatments A, B and D (Fig. 2). Treatment D was unusual because the area of nonGM rice was almost twice the size of the nonGM in treatments A–C (Fig. 1). The difference in the area of nonGM plants may have resulted in less frequent pollen from outside the nonGM area in treatment D. However, when we removed treatment D from the analysis of deviance, this did not change the overall results, yielding a plot size effect with a significance level of $P = 0.076$ and an interaction effect between plot size and rice pair of $P = 0.011$. Thus, the small effect of GM subplot size is not considered to be a main effect, although an interaction with rice pair was detected, suggesting that patterns of transgene flow can be more complex than one might assume.

We also constructed curves using the logistic regression model to estimate transgene frequencies at different distance intervals from the GM crop (Fig. 2). The model curves closely follow the experimental data (deviance = 164.93, penalized deviance = 99.78, residual df = 164, deviance/residual df ≈ 1). Based on the model, transgene frequencies of the three GM rice lines were very low and the maximum frequencies (< 0.3%, $P < 0.05$) occurred at the distance of 0.2 m (ranging from 0.013 to 0.18%). The transgene frequency in rice pair HY2 was always the highest among the three rice pairs in different treatments ($P < 0.05$). The decrease of transgene flow frequencies showed an odd peak at 2.6 m (ranging from 0.004 to 0.058%). Transgene frequencies were nearly zero (0.0007–0.0097%) at 6.2 m, representing only 5.5% of the highest transgene flow frequencies found at 0.2 m, based on the regression model. This pattern was consistent in the four treatments for the three rice pairs.

Discussion

This study demonstrated extremely low frequencies of rice transgene flow to nonGM plants that were only 0.2 m from the GM pollen sources, based on both observations (maximum 0.28%) and model analyses (max. 0.18%) for the distance of 0.2 m. These findings are consistent with our previous reports (Rong *et al.*, 2004; Rong *et al.*, 2005) and others (Messeguer *et al.*, 2001; Messeguer, 2003), where very low frequencies of gene flow were observed between intermixed plants (< 0.9%) (Messeguer *et al.*, 2001; Rong *et al.*, 2004; Rong *et al.*, 2005). We previously estimated gene flow frequencies between intermixed plantings of traditional and hybrid rice varieties grown in small experimental plots in Yunnan Province, China (Rong *et al.*, 2004). Using molecular markers (simple sequence repeat (SSR)), we found very low and asymmetric gene flow between traditional and hybrid varieties, with an average frequency of 0.04% off-types in the traditional rice and 0.18% off-types in the hybrid rice. We conducted further field experiments to quantify transgene flow from insect-resistance GM rice lines to nonGM rice varieties growing intermixed within the small plots (64 m^2), where the rice materials were the same as those used in this study (Rong *et al.*, 2005). We found that even in the extreme case of close spacing and a high proportion of GM plants, the greatest frequency of transgenic seeds on the nonGM rice was < 0.9% (Rong *et al.*, 2005). Within large fields of nonGM rice plants, most outcrossing is likely to involve other nonGM plants growing nearby rather than distant pollen sources. Therefore, the frequency of transgene flow from the GM rice to nonGM varieties in other fields is expected to be much lower than 0.9% because cultivated rice is mainly self-pollinated with a very low outcrossing rate.

It is important to point out that transgene flow frequencies reduced dramatically with the increase in distance intervals from GM rice in this experiment, and the rice pairs or plot size did not significantly affect the above trends. The density of GM rice pollen decreases significantly with distance from its source (Song *et al.*, 2003; Song *et al.*, 2004), and the proportion of nonGM pollen is expected to increase accordingly around the pollen receiver, which resulted in the sharp drops in transgene flow frequencies within short distance intervals. Based on these results, we suggest that isolation distance should be a very effective way to minimize crop-to-crop gene flow in the rice fields. This is particularly the case to significantly reduce

transgene escape to wild and weedy rice populations that occur in the vicinity of GM rice fields (Song *et al.*, 2003; Chen *et al.*, 2004). This conclusion is in a good agreement with Walklate *et al.* (2004) who proposed that isolation distance was the most effective way to regulate gene flow for self-pollinating crops based on a pollen-mediated gene flow model. However, for weedy rice populations that are commonly present within rice fields, transgene escape through gene flow from GM rice to weedy rice coexisting in the same fields is unavoidable. Therefore, the thorough assessment of environmental risks caused by the crop-to-weedy gene flow should be performed.

The experiment also showed that transgene flow frequencies were not obviously influenced by the sizes of GM rice subplots. The results may have contributed to the low outcrossing rate of rice. A similar conclusion is also suggested by Gustafson *et al.* (2005) based on the regression model of pollen-mediated gene flow in wheat, where very small size effects on frequencies of gene flow (< 0.1%) were predicted. Our results further showed that frequencies of gene flow were significantly affected by rice pairs, indicating that different rice varieties may have different gene flow frequencies. This might be caused by differences in reproductive characteristics of rice varieties such as outcrossing rates and pollen dispersal behaviors. Nevertheless, based on our earlier report (Rong *et al.*, 2004; Rong *et al.*, 2005) and results from this study, we believe that the density of GM pollen grains in air currents relative to the density of local nonGM pollen is more important than the GM field size and GM varieties in terms of transgene flow frequencies. Small nonGM rice fields that are completely surrounded by GM pollen sources are expected to receive more GM pollen than those in the present study, but most of the transgenic seeds in these fields are expected to occur within *c.* 6 m of the GM crop's perimeter, and overall frequencies should be very low.

In conclusion, pollen-mediated crop-to-crop transgene flow in rice can be maintained at negligible levels with sufficient spatial isolation, although seed dispersal still represents a major pathway for gene flow. Our study confirms that isolation by distance is a very effective way to confine pollen from GM rice, and we show that distances of only a few meters can be expected to dramatically reduce transgene frequencies in surrounding rice fields. Such a low frequency of crop-to-crop transgene flow should rarely cause problems with seed purity in neighboring rice fields, although crop-to-wild and crop-to-weedy gene flow may arouse more complex biosafety concerns in rice. The very large sample sizes (> 2.1 million seeds examined) in our study provide well-supported evidence for effective prevention of transgene 'contamination' (< 0.01%) by pollen. Future studies should test for similar results using a wider range of donor and recipient field sizes, other rice varieties, windier environments, and greater distances from transgenic crop fields, but we expect that our general findings will hold true. The low frequency of crop-to-crop gene flow in rice may be associated with the nature of its mating system, with self- and wind-pollination and a very low outcrossing rate. This model, based on pollen-mediated gene flow in rice, may also apply to other crops with self- and wind-pollination such as wheat, barley, and millet.

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