The Green Fence of Chinese Hibiscus (*Hibiscus rosa-sinensis* L.) Prevents Pollen Dispersal of Transgenic Rice (*Oryza sativa*)

Ching-Shan Tseng¹,², Min-Tze Wu¹, Hung-Chang Huang¹, and Yann-Rong Lin²

(¹Division of Biotechnology, Taiwan Agricultural Research Institute, Wufeng, Taichung 41362, Taiwan; ²Department of Agronomy, National Taiwan University, Taipei 10617, Taiwan)

Abstract: Transgene escape mediated by pollen dispersal is one of the preeminent concerns about genetically modified crops, including rice. In this study, the rice pollen donor of non-glutinous *Oryza sativa* ssp. *japonica* cv. ‘Tainung 67’ [TNG 67] had a greater potential of pollen flow, which was shown by the greater quantity, germination rate, and viability of pollen, than the other rice pollen donor of transgenic AAN. The pollen-mediated gene flow was detected by the frequency of outcrossed seeds in a “checker-board pattern” and alternating row arrangement of rice pollen donor (TNG 67 or AAN) and pollen recipient (glutinous rice ‘TNG 73’) in the fields. We conducted field experiments to assess rice pollen dispersal with or without a “green” fence of Chinese hibiscus, *Hibiscus rosa-sinensis* L., of approximately 2 m in height and 0.6 m in thickness. Without a green fence, the outcrossing rate of TNG 73 seeds decreased with increasing distance from the pollen donor, from 1.68% at 1 m to 0.01% at 35 m, with no outcrossing beyond 40 m. The outcrossing rate varied with the direction of pollen donor, from 0.05% in the northeastern direction to 0.78% in the southern direction, which was caused by prevailing wind direction. With the green fence, no seeds of TNG 73 were outcrossed. Since a buffer zone of at least 40 m is needed to prevent outcrossing of rice by pollen dispersal in an open field, planting of Chinese hibiscus around the rice field as a green fence would be an effective measure for preventing transgene escape mediated by pollen flow.

Key words: Green fence, *Hibiscus rosa-sinensis*, *Oryza sativa*, Pollen barrier, Pollen flow, Transgenic rice.

Rice is one of the major food crops in the world and provides up to 23% of calories consumed by humans (Fisher et al., 2000). Asia produces 90% of the world’s rice, yet current production does not meet the demand; projected increases in population growth indicate that rice production needs to be increased up to 40% (Khush, 2005). Rice is the leading crop in Taiwan, with an estimated production area of 420,000 ha, 51% of the total cultivation area, in 2008 (COA, 2008). Gene transformation by biotechnology is now being used to develop rice with defense against various stresses caused by the global climate change. Significant progress has been made in recent years in genetic engineering in rice for resistance to diseases (Bishun et al., 2008), insects (Huang et al., 2005; Saha et al., 2006; Zhang et al., 2008), herbicides (Kawahigashi et al., 2007; Kumar et al., 2008), drought, and salt (Majee et al., 2004; Hu et al., 2006). Moreover, rice has been transformed with genes conferring the traits of biofortification, such as high contents of protein, iron, and β-carotene (Gura, 1999; Hasler, 2000; Ye et al., 2000; Krishnan et al., 2003; Paine et al., 2005; Sivaprakash et al., 2006). In Taiwan, genetically modified (GM) rice with high contents of lactoferrin (Tsay et al., 2001), phytase (Hong et al., 2004), and amylomaltase (Chiang et al., 2005) have been developed.

Pollen dispersal is one of the primary concerns in commercialization of transgenic rice. Transgene escape to cultivated crops and wild relatives by pollen flow, which can contaminate non-GM crops and lead to resistance in wild relatives, is a major economic and environmental biosafety concern (Lu and Snow, 2005). The outcrossing rate of cultivated rice is in general low, less than 1%, because of self-pollination. Nevertheless, pollen-mediated gene flow from cultivated rice (*Oryza sativa* L.) to weedy rice (red rice, *O. sativa* f. *spontanea*), wild rice (*O. nivara, O. rufipogon*), and wild relative barnyard grass (*Echinochloa crusgalli*) under field conditions (Messegue et al., 2001, 2004; Song et al., 2003, 2004, 2009; Chen et al., 2004; Wang et al., 2006; Gao et al., 2009; Endo et al., 2009) has been reported. The outcrossing rate between pollen donor and recipient plants was 11–18% when they were 0–1 m apart, and the maximum distance of gene dispersal by pollen...
reported was 43.2 m (Song et al., 2003, 2004; Lu et al., 2003; Chen et al., 2004). Field experiments and simulated transgene flow models revealed the frequency of outcrossing rate negatively correlated with distance (Wang et al., 2006; Yao et al., 2008). The velocity and direction of wind are important vectors for pollen dispersal (Devos et al., 2005). The gene flow in populations downstream of prevailing wind was 6.47–26.4%, and that in populations upstream was only 0.39–3.03% (Yuan et al., 2007). Moreover, gene flow of 0.01% from transgenic rice to O. rufipogon was found at a distance of 250 m because of the high wind speed in Sanya, China (Wang et al., 2006). Distance and wind have a great impact on outcrossing in fields and were the 2 critical parameters in models predicting outcrossing rate between GM and non-GM corn (Song et al., 2004; Ivanovska et al., 2009).

Transgene escape is a serious biosafety issue and measures must be developed to prevent contamination of non-GM crops before transgenic rice can be widely used for commercial production. Isolation by time (time of flowering) and space (distance, buffer zone, and “green” fences) can reduce or prevent transgene escape. Long overlapped flowering time increases the probability of cross fertilization (Devos et al., 2005; Olguin et al., 2009). Wheat with asynchronous flowering time showed two-to-four fold reduced gene flow frequency (Willenborg et al., 2010). However, varied flowering time in rice might have disadvantages for cropping systems and field management, as well as poor production and grain quality. The isolation distance was suggested to be up to 110 m in rice (Song et al., 2004). Growing non-transgenic crops as a buffer zone might be useful to prevent gene flow from transgenic crops. Nevertheless, most studies indicate that the buffer zone is only partially effective in preventing pollen transmission from transgenic to non-transgenic plants (Damgaard and Kjellsson, 2005). To grow tall plants surrounding the field can form biological windbreaks which serve as pollen barriers for confining pollen flow and reducing cross fertilization consequently (Devos et al., 2005; Prescher et al., 2010). With tall annual plants, such as sorghum sudangrass and corn, used as a green fence, the maximum distances and frequency of pollen could be reduced to half (Aritt et al., 2007; Peñas et al., 2007). Field isolation by growing more bushy plants as a green fence to reduce the wind effect on pollen dispersal might be another method to prevent pollen flow of rice, but information is lacking.

In Taiwan, 2 native wild relatives of Asian cultivated rice, O. rufipogon and O. nivara, have become extinct in the field. The major concern in releasing transgenic rice is pollen-mediated crop-to-crop gene flow, which might cause problems of consumer acceptance and legal disputes over local or international rice trade. The release of phytase-transformed rice in Taiwan was put on hold by the Taiwan government because of lack of information concerning the risk assessment of pollen dispersal of transgenic rice. Some GM rice lines are currently under assessment for biosafety in the isolated fields at Taiwan Agriculture Research Institute (TARI, 24°01’ N, 120°41’ E), Taichung, Taiwan, specifically approved by the Taiwanese government. The rice variety, capability of pollen hybridization, pollen source size, distance between pollen donor and recipient, and climate conditions (wind and humidity) are factors contributing to pollen-mediated gene flow (Shivrain et al., 2007; Wang et al., 2006). In this study, we aimed to estimate the outcrossing rate caused by pollen-mediated gene flow in open fields by 2 different field designs: “checker-board pattern” and alternating row arrangement of pollen donor rice, including transgenic rice and recipient plants. The airborne pollen densities were different between these two different designs because one pollen recipient plant was surrounded by 4 pollen donor plants in the checker-board pattern but only 2 pollen donor plants in the alternating row arrangement. An isolated paddy-field site at TARI was approved by the government for assessing the biosafety of GM crops in Taiwan in May 2007. Some fields at this site were separated by Chinese hibiscus, Hibiscus rosa-sinensis L., used as a green fence. Chinese hibiscus, a perenniel evergreen shrub, is well adapted to subtropical climates and is grown year-round in Taiwan. Plants of Chinese hibiscus grow rapidly to a height of 2–3 m and have multiple branches and form a dense fence within 1 year (Fig. 1). Since the majority of rice grains were detected at the height of 1.0–1.5 m and relatively few at 2 m (Song et al., 2004), we trimmed the plants to a height of 2 m. Since the width of ridges surrounding the paddy rice fields in Taiwan is 0.6 m, we trimmed the plants to 0.6-m thickness and examined the effectiveness of this green fence in preventing pollen dispersal from phytase-transformed rice to non-transformed rice.
Materials and Methods

1. Plant materials
We used 2 non-transgenic popular rice cultivars (*Oryza sativa* ssp. *japonica* cv. ‘Tainung 67’ [TNG 67]) and (*O. sativa* ssp. *japonica* cv. ‘Tainung 73’ [TNG 73]) and one phytase-transformed rice line AAN (*O. sativa* ssp. *japonica*). The transgenic phytase line AAN, containing the phytase gene *SrPf6* of *Selenomonas ruminantium* and thus producing high activity of recombinant phytase, was produced from the non-transgenic variety TNG 67 (Cheng et al., 1999; Hong et al., 2004). Non-glutinous rice, TNG 67 and AAN, were the pollen donors, and glutinous rice TNG 73 was the pollen recipient.

2. Investigation of pollen characters
We used pollen quantity and pollen activity to investigate the potential of pollen flow. Pollen quantity was estimated as follows. The panicles of TNG 67 and AAN were pulled out before anthesis before 0800, then immersed in distilled water in flasks and kept at 32°C in an oven for 30−60 min until anthesis. Then, 6 anthers were removed and put into a 1.5-mL Eppendorf tube containing 1 mL distilled water. The pollen was evenly distributed in water after being thoroughly squashed and vigorously vortexed. In total, 10 µL pollen solution was dropped onto a slide, and pollen quantity of each sample was counted 4 times with 3 replications under a microscope. The pollen quantity of one anther was estimated as total number of pollen grains × 100/6.

The viability of pollen was assessed by *in vitro* germination and fluorochromatic reaction method (FCR). The medium for *in vitro* germination followed Shivanna and Rangaswamy (1992) with minor modifications. The liquid medium was composed of 15% sucrose, 20 mg L⁻¹ boric acid, and 600 mg L⁻¹ calcium nitrate. Before anthesis, anthers were added to the liquid medium on a slide, broken by forceps to release pollen, then covered with a cover glass and incubated at 28 ± 1°C for 60 min. When the length of pollen tubes was equal to or greater than one half of the pollen diameter, the pollen was considered as germinated. Germination was quantified as percentage of germinated grains from 500 grains with 3 replications.

The FCR procedure followed Heslop-Harrison and Heslop-Harrison (1970) with slight modifications. Fluorescein diacete (FDA) was dissolved in acetone (2 mg mL⁻¹) and 30% (w/v) sucrose was added until white precipitation appeared. Pollen was placed on a slide and 1 or 2 drops of the turbid FDA solution was added for 15 min. Pollen viability was estimated as percentage fluorescent grains of 500 pollen grains observed under a fluorescence microscope (Nikon Eclipse E6000) with 3 replications (fluorochromatic reaction, FCR).

3. Field experiments
To detect seeds outcrossed by pollen flow, we used non-glutinous rice TNG 67 or AAN as the pollen donor and glutinous rice TNG 73 as the pollen recipient. All rice seedlings at the 4−5 leaf stage were transplanted to fields. To synchronize the flowering period, we transplanted the seedlings of the pollen recipient TNG 73 1 week after TNG 67 and AAN. The row spacing was 25 cm and the within-row plant spacing 25 cm for all fields. The same cultural practices and field maintenance were used throughout the entire 2 growing seasons.

(1) Assessment of pollen flow
We used 2 field experimental designs, the checker-board pattern and alternating row arrangement, to assess pollen-mediated gene flow in open fields in the first crop season, 2007. In the checker-board pattern, one pollen donor plant of TNG 67 or AAN was transplanted next to a pollen...
recipient TNG 73 plant. Therefore, 1 pollen recipient plant was surrounded by 4 pollen donor plants and vice versa (Fig. 2a). In the alternating row arrangement, 1 row of pollen donor plants, TNG 67 or AAN, was transplanted next to the row of pollen recipient plants and vice versa. For both the checker-board pattern and alternating row arrangement, the distance between hills of pollen donor and recipient plants was only 25 cm. Therefore, the panicles of adjacent plants overlapped, and pollen escaping from the donor plants easily hybridized with the recipient plants. Each plot comprised 10 rows, with 10 plants in each row. We used 3 replications of each plot of the checker-board pattern and alternating row arrangement. The rice flowering period was from May 10 to 17 in AAN, from May 11 to 17 in TNG 67, and from May 9 to 17 in TNG 73. An overlap of flowering periods of at least 7 days allowed for outcrossing of recipient plants in the fields. All seeds of pollen recipient TNG 73 in each plot were harvested at maturity, individually and pooled together, from which we randomly selected 6,000 seeds which were divided into 3 bags with three replications for estimating frequency of outcrossed seeds.

(2) Pollen flow with and without a green fence

In the first cropping season of 2008, we examined the dispersal of rice pollen from TNG 67 in an open field without a green fence and in 4 confined fields from AAN with a green fence of Chinese hibiscus. The open field, 100×100 m, was at TARI. Rice seedlings of the pollen donor TNG 67 were transplanted in the center of the field in a block of 10×10 m. Rice seedlings of the pollen recipient TNG 73 were transplanted in the same field in 8 directions: north, northeast, east, southeast, south, southwest, west, and northwest (Fig. 3a). For each direction, 12 plots of TNG 73 were located at 1, 2, 3, 5, 10, 15, 20, 25, 30, 35, 40, and 45 m away from the block of the pollen donor TNG 67, and each plot contained 30 plants. The rice flowering period was from May 7 to 19 in TNG 67, May 10 to 18 in AAN, and May 8 to 18 in TNG 73. An overlap of flowering periods of at least 9 days allowed for outcrossing of recipient plants in the fields. At maturity, seeds of pollen recipient TNG 73 were collected from the 12 plots in each direction. A total of 6,000 seeds from each plot were randomly divided into 3 bags and examined for frequency of outcrossing with donor pollen TNG 67.

The 4 confined fields with a green fence were each 40×50 m (L×W) and were surrounded by 1-year-old Chinese hibiscus plants, trimmed to a height of 2.0-m and thickness of 0.6-m (Fig. 1, Fig. 3b). The spaces between each field and adjacent green fence and between two adjacent fields were 5 m, and these spaces were weeded by spraying herbicide during seedling stages and by hand until harvest. Seedlings of the pollen donor AAN were transplanted into fields I and III, and seedlings of the pollen recipient TNG 73 were transplanted into fields II and IV (Fig. 3b). Each field of TNG 73 was divided into 9 even sections, and 30 mature plants from each section were randomly selected for seed harvest. In total, 6,000 seeds from the 30 plants were mixed, air-dried, and divided into 3 bags of 2,000 seeds per bag. Seeds in each bag were then examined to estimate rates of outcrossing of TNG 73 seeds with pollen from the donor AAN.
In the open plots of the checker-board pattern, the mean frequency of outcrossed seeds was significantly lower from AAN (5.91%) than TNG 67 (7.13%) (Fig. 4). The same phenomenon was observed with the alternating row arrangement, for a significantly lower frequency of outcrossed seeds from AAN (1.53%) than TNG 67 (2.8%) (Fig. 4). The lower capability of pollen flow of AAN might account for the lower frequency of outcrossed seeds (Table 1, Fig. 4). The mean frequency of outcrossed seeds was higher with the checker-board pattern (6.52%) than the alternating row arrangement (2.17%) (Fig. 4). Thus, the high density of pollen donor plants invoked a high frequency of outcrossed seeds resulted by pollen-mediated gene flow.

4. **Estimation of gene flow by frequency of outcrossed seeds**

Outcross of glutinous rice TNG 73 by pollen from donor plants of non-glutinous rice TNG 67 or AAN can be easily detected by grain appearance, whereas grains of outcrossed seeds of TNG 73/TNG 67 or TNG 73/AAN would be translucent. Seeds were dried to 14% moisture content at 45°C, dehulled by use of a Satake rice sheller (Satake Engineering Co., Tokyo) and examined for grain appearance. For further confirmation of outcrossing, each translucent seed was cut into 2 halves, and the cut surface was stained with a drop of iodine solution for 1 min. The endosperm colors were brown and purple black in selfed and outcrossed seeds of TNG 73, respectively. We used the following formula for calculation: frequency of outcrossed seeds (%) = (number of TNG 73/TNG 67 or TNG 73/AAN seeds) / total number of examined seeds × 100%.

5. **Weather data**

Data on wind speed and direction were collected at 0800, 0900, 1000 and 1100 during the entire rice flowering period at the TARI weather station near the experimental fields.

**Results**

1. **Pollen characters of pollen donors, TNG 67 and AAN**

   Quantity, germination rate, and viability of pollen are 3 important factors accounting for different capabilities of pollen flow of different varieties. The mean pollen quantity per anther and pollen viability were lower, but not significantly, in the phytase-transformed line AAN than in the non-transgenic variety TNG 67 as revealed by FCR analysis (Table 1). Nevertheless, the germination rate was significantly lower in AAN (37.5 ± 0.01%) than in TNG 67 (49.76 ± 0.03%) (Table 1). Overall, TNG 67 had greater capability of pollen flow than AAN.

2. **Frequency of outcrossed seeds in a checker-board pattern and alternating row arrangement**

   In the open plots of the checker-board pattern, the mean frequency of outcrossed seeds was significantly lower from AAN (5.91%) than TNG 67 (7.13%) (Fig. 4). The same phenomenon was observed with the alternating row arrangement, for a significantly lower frequency of outcrossed seeds from AAN (1.53%) than TNG 67 (2.8%) (Fig. 4). The lower capability of pollen flow of AAN might account for the lower frequency of outcrossed seeds (Table 1, Fig. 4). The mean frequency of outcrossed seeds was higher with the checker-board pattern (6.52%) than the alternating row arrangement (2.17%) (Fig. 4). Thus, the high density of pollen donor plants invoked a high frequency of outcrossed seeds resulted by pollen-mediated gene flow.

3. **Gene flow estimated in the open field**

   In the open field without green fences, the frequency of outcrossed seeds varied with the distance and direction of the pollen donor TNG 67 to the pollen recipient TNG 73. Pollen dispersal leading to outcrossing was negatively associated with distance. Outcrossed seeds were found from all 8 directions within a 3-m distance. The mean frequency of outcrossed seeds was 1.68%, 0.74% and 0.61% at 1, 2 and 3 m, respectively (Table 2). The highest outcross rate (4.45%) was found at plots located 1 m to the south. For distances between 5 and 35 m, the frequency of outcrossed seeds and number of directions with outcrossed seeds decreased with increasing distance. Outcrossed TNG 73 seeds were detected in 6 directions in 5-m plots and only 1 direction in 30- and 35-m plots, and the mean outcrossed TNG 73 seeds ranged from 0.33% to 0.01% (Table 2). No outcrossed TNG 73 seeds were detected in any direction farther than 40 m.

   The frequency of outcrossed TNG 73 seeds ranged from 0.05% to 0.78% among the 8 different directions (Table 2). The frequency of outcrossed seeds was significantly higher.

Table 1. The production quantity, germination rate, and viability of pollen generated by the 2 pollen donors, transgenic AAN or *Oryza sativa* ssp. japonica cv. ‘Tainung 67’ (TNG 67).

| Pollen donor | Pollen number/anther | Germination rate (%) | Viability (%) |
|--------------|----------------------|----------------------|---------------|
| AAN          | 57290±2484.6*        | 37.51±0.01*          | 73.18±0.02*   |
| TNG 67       | 64600±3242.8*        | 49.76±0.03*          | 76.95±0.01*   |

*Mean±SE (n=3). Values with the different letters are significantly different at level of p < 0.05 according to t-test.*

---

Fig. 4. The frequency of outcrossed seeds detected in the 2 different field plot designs, checker-board pattern (left) and alternating row arrangement (right). AAN and TNG 67 indicate the hybrid grain of TNG 73 derived from AAN and TNG 67, respectively. Error bars represent standard error of three replications. * indicates significantly difference at level of p < 0.05 according to t-test.
Tseng et al. — Chinese Hibiscus "Green" Fence Prevents Rice Pollen Dispersal

In all plots to the south and southeast within 35 m. The furthest plot of outcrossed seeds was found to the south at 35 m, which was twice as far as the most-distant plots to the east and southwest at 15 m. Nevertheless, relatively fewer outcrossed seeds were observed from the plots to the northeast, northwest, and north, and no outcrossed seeds were detected from plots to the northeast and north at 5 m (Table 2). In the open field, the frequency of outcrossed TNG 73 seeds, caused by pollen flow of TNG 67 from the center plot, differed with the direction. Weather data collected from the TARI weather station near the open field showed that the prevailing wind direction was northwest for 30 hr, northeast for 5 hr, and west for 4 hr, with a minimum and maximum wind speed of 1.7 and 9.0 m/s, respectively, during the 11 days of the rice flowering period (Table 3). The frequency of outcrossed TNG 73 seeds was high in plots 1 m away from the donor plots to the south (4.45%) and southeast (3.92%), respectively, which was related to the prevailing direction of northwest wind. In addition, the outcrossing frequency was 0.05% and 0.07% in plots 30- and 35-m away in the downwind direction of the south and southeast, respectively (Table 2). Outcrossed seeds were found in plots up to 35 m downwind of the prevailing direction (Tables 2, 3). However, with 5 m between TNG 73 and TNG 67, we detected no pollen outcrossing of TNG 73 in plants located in the upwind directions of the north and northeast.

4. Effect of the green fence of Chinese hibiscus on gene flow

As the open field experiment demonstrated, the frequency of outcrossed seeds was heavily influenced by wind speed and direction. Thus, a green fence surrounding the fields might diminish the effect of wind on pollen dispersal. With Chinese hibiscus at a height of 2-m and thickness of 0.6-m as a green fence to block the wind effect on pollen dispersal (Figs. 1, 3b), among the 108,000 seeds of TNG 73 collected, we found none outcrossed with pollen from AAN, regardless of the distance between recipient plants and donor plants. Thus, Chinese hibiscus as a green fence effectively prevented TNG 73 from outcrossing with pollen from AAN in neighboring fields.

Discussion

1. Potential pollen flow associated with pollen characters

The reasons for the diverse pollen flow frequencies of different rice varieties include pollen quantity from pollen
donors, hybridization ability of pollen recipients, and pollen competition between different species (Song et al., 2004). AAN is a phytase-transformed line derived from the elite *japonica* cultivar TNG 67 (Hong et al., 2004). AAN and TNG 67 do not differ in agronomic traits or pollen production (Tseng et al., 2008), as we found: AAN produced fewer pollen grains per anther than did TNG 67, but the differences were not significant. The 2 varieties did not differ in pollen viability revealed by FCR test, neither. However, the germination rate was lower in AAN (37.51±0.01%) than TNG 67 (49.76±0.03%) (Table 1) because the phytase gene SrPf6 of AAN is driven by an α-amylase promoter, αAmy8, for high expression in endosperm (Hong et al., 2004). The overexpression of α-amylase regulating starch metabolism in endosperm might influence the seed germination of AAN. Transformed genes might influence the expression of non-target genes near the gene insertion site of the genome or somatic mutation arising during callus formation, thus leading to aberrant expression of some unknown genes. A transgenic line APU, generated by transformation of the amylolullanase gene from the bacterium *Thermoanaerobacter ethanolicus* into TNG 67, showed increased susceptibility to brown spot caused by *Bipolaris oryza* (Ting et al., 2008). Because of its lower germination rate, AAN showed less pollen-mediated gene flow as detected by outcrossed seeds in both checker-board pattern and alternating row arrangement in the open field (Table 1, Fig. 4).

2. Pollen flow associated with pollen density and distance of pollen source

When transgenic rice and non-transgenic rice are cultivated near the same location and had overlapped flowering time, a high frequency of transgene escape mediated by pollen flow would occur under a high density of transgenic pollen. In the checker-board pattern, 4 donor plants of both AAN and TNG 67 provided twice more airborne pollen density than in alternating row arrangement to recipient plants of TNG 73. The increased pollen density in the checker-board pattern contributed to threefold pollen-mediated gene flow, as evidenced by outcrossed seeds (Fig. 4).

Model simulation revealed that pollen density decreased in a simple exponential pattern with increasing distance to the pollen source (Rong et al., 2010). A high frequency of gene flow (11–18%) was detected within a 1-m distance from the *bar* transgenic rice to *O. rufipogon*, and the frequency greatly decreased with distance (Wang et al., 2006). Examination of our seed samples collected from recipient plots of TNG 73 in all 8 directions in the open field experiment without the green fence showed that the frequency of pollen outcrossing was negatively related to distance of the pollen donor plants TNG 67, especially when donor plants were planted at 15 m or further. In TNG 73 grown at 35 m from the donor plants to the south, the gene flow was 0.05% (Table 2), which suggests that 35 m is still an unsafe zone for preventing gene flow of rice pollen. Nevertheless, no outcrossed seeds were observed at 40 m in any of the 8 directions (Table 2). This result was consistent with other reports on the dispersal of rice pollen with the distance (Song et al., 2003 2004; Lu et al., 2003; Chen et al., 2004) and suggested that a buffer zone of at least 40 m is necessary to cultivate transgenic rice in commercial fields.

3. The effectiveness of a green fence with Chinese hibiscus in preventing pollen-mediated gene flow

Because rice is a wind-pollinated crop, wind speed and direction during the flowering period are important factors affecting pollen dispersal leading to transgene escape by pollen-mediated gene flow. The frequency of transgene escape to *O. rufipogon* was much higher in Sanya, China, than in Guanzhou, China (2.25–11.24% vs. 0.9–3.9%) within 1–10 m because the maximum speed of wind was much higher in Sanya. In addition, the furthest rice pollen dispersal distance, 250 m, was reported in Sanya (Wang et al., 2006). During the 11-day flowering period in our open field experiment, the prevailing northwest wind promoted pollen dispersal in plots to the south and southeast, which showed the highest mean frequencies of outcrossed seeds (0.73% and 0.78%) and in the plot located at 35 m. However, the frequency of outcrossed seeds was low in plots upstream of the prevailing wind, northeast (0.05%) and northwest (0.08%), within 5 m (Table 2, 3). These data suggest that pollen dispersal is more prevalent downwind, and thus wind direction influencing pollen distribution is a concern in preventing pollen contamination of GM crops. In one study of gene flow in compass sectors, approximately 10-fold cumulative gene flow was found in the 4 sectors downstream of the prevailing wind (90–96%), but only 4–10% of this flow occurred in the 4 sectors upstream of the prevailing wind (Yuan et al. 2007). The evidence of rice pollen-mediated gene flow revealed in this study was in accordance with maize pollen-mediated gene flow on the multi-year field experiments at several locations (Devos et al., 2005). Therefore, speed and direction of wind are crucial vectors mediating pollen dispersal. Minimizing the effect of wind on pollen dispersal would reduce transgene escape and alleviate problems concerning the biosafety and contamination of non-GM crops.

Growing tall plants as a green fence may be effective in minimizing the risk of pollen-mediated gene flow of transgenic crops in commercial fields (Devos et al., 2005; Arritt et al., 2007; Prescher et al., 2010). Peñas et al. (2007) reported the dispersal of rice pollen was reduced from 0.056% to 0.034% by using corn as a green fence. Tall vegetation such as sugarcane was suggested to confine
pollen dispersal because the majority of rice pollen grains flowed below the height of 1.5 m (Song et al., 2004). The different life cycles of tall crops such as corn and sugarcane cause problems in rice field management, and those crops having a main stalk with few or without tillers do not form a green fence dense enough to prevent pollen flow effectively. The green fence of Chinese hibiscus with a tall height (2-m tall) and dense canopy (0.6-m thick) might be effective to reduce wind speed and thus prevent pollen dispersal from field-to-field. None of the examined 108,000 TNG 73 seeds was contaminated by the transgenic line AAN when the green fence of hibiscus was used. However, bushy-dense plants such as Chinese hibiscus might be especially effective in preventing pollen dispersal, as indicated by the complete absence of outcrossed TNG 73 seeds in this study. Perennial Chinese hibiscus provides an additional advantage in that it can be easily trimmed, and an effective height and thickness is easy to maintain (Fig. 1).

The potential risk of gene flow in the environment is a major concern confining the production of transgenic rice in commercial fields. In open field conditions, the isolation method or buffer zones by keeping a distance might be ineffective in preventing pollen-mediated gene flow of transgenic rice because of the complication of weather conditions such as wind direction and velocity (Tables 2, 3). However, our study revealed that planting Chinese hibiscus as a green fence surrounding rice fields (2-m tall, 0.6-m thick) effectively prevented outcrossing of rice by transgenic rice from neighboring fields. Chinese hibiscus, an evergreen shrub, is well adapted to subtropical climate and would be attractive as a green fence to encompassing crops such as potatoes, broccoli, and rapeseed could be grown with such a fence. The effectiveness of Chinese hibiscus as a green fence plant preventing pollen-mediated gene flow of these crops warrants further investigation in subtropical regions.

Acknowledgements

The study was funded by the National Science Council, Taiwan (NSC96-2317-B-055-009). Dr. H.C. Huang is Chair Professor at the Taiwan Agriculture Research Institute, under the National Science Council Project NSC98-2811-P-055-001.

References

Arritt, R.W., Astini, J., Clark, C.A., Westgabe, J.M.E. and Goggi, A.S. 2007. Biological windbreaks for pollen confinement. The 3rd International Conference on Coexistence between Genetically Modified (GM) and Non-GM Based Agricultural Supply Chains. Seville, Spain, 20-21 November 2007. 131-134.

Bishun, D.P., Sanjay, J. and Bharat, B.C. 2008. Transgenic indica rice expressing Mirabilis jalapa antimicrobial protein (Mj-AMP2) shows enhanced resistance to the rice blast fungus Magnaporthe oryzae. *Plant Sci.* 175: 364-371.

Cao, Q.J., Xia, H., Yang, X. and Lu, B.R. 2009. Performance of hybrids between weedy rice and insect-resistant transgenic rice under field experiments: implication for environmental biosafety assessment. *J. Integr. Plant Biol.* 51: 1138-1148.

Chen, L.J., Lee, D.S., Song, Z.P., Suh, H.S. and Lu, B.R. 2004. Gene flow from cultivated rice (*Oryza sativa*) to its weedy and wild relatives. *Ann. Bot.* 93: 67-73.

Cheng, K.J., Sellnger, L.B., Yanke, L.J., Bae, H.D., Zhou, L. and Forsberg, C.W. 1999. Phytases of ruminal microorganisms. US Patent No. 5,939,303.

Chiang, C.M., Yeh, F.S., Tseng, T.H., Wang, C.S., Lu, H.S., Shaw, J.F. and Yu, S.M. 2005. Expression of a biofunctional and thermostable amylopululanase in transgenic rice seeds leads to starch autohydrolysis and altered composition of starch. *Mol. Breed.* 15: 125-143.

Council of Agriculture. 2008. Annual Report of Rice Improvement. http://www.afa.gov.tw/Public/GrainStatistics/20094216525334.pdf.

Damgaard, C. and Kjellsson, G. 2005. Gene flow of oilseed rape (*Brassica napus*) according to isolation distance and buffer zone. *Agric. Ecosyst. Environ.* 108: 291-301.

Devos, Y., Reheul, D. and de Schrijver, A. 2005. The co-existence between transgenic and non-transgenic maize in the European Union: a focus on pollen flow and cross-fertilization. *Environ. Biosafety Res.* 4: 71-87.

Endo, J., Sato, H., Yamaguchi, M., Kataoka, T., Nakagomi, K., Ito, T. and Mori, K. 2009. Estimate of outcrossing rates in a rice plant (*Oryza sativa L.*) under field conditions using a purple grain rice cultivar, Okunomurasaki. *Breeding Sci.* 59: 195-202.

Fisher, K.S., Barton J., Khush, G.S., Leung, H. and Cantrell, R. 2000. Genomics and Agriculture. Collaborations in rice. *Science* 290: 279-280.

Gura, T. 1999. Biotechnology. New genes boost rice nutrients. Science 285: 994-995.

Hasler, C.M. 2000. The changing face of functional foods. *J. Am. Coll. Nutr.* 19: 4998-5068.

Heslop-Harrison, J. and Heslop-Harrison, Y. 1970. Evaluation of pollen viability by induced fluorescence; intracellular hydrolysis of fluorescein diacetate. *Stain Technol.* 45: 115-120.

Hong, C.Y., Chen, K.J., Liu, L.F., Tseng, T.H., Wang, C.S. and Yu, S.M. 2004. Production of two highly active bacterial phytases with broad pH optima in germinating transgenic rice seeds. *Transgenic Res.* 13: 29-39.

Hu, H., Dai, M., Yao, J., Xiao, B., Li, X., Zhang, Q. and Xiong, L. 2006. Overexpressing a NAM, ATAF, and CUC (NAC) transcription factor enhances drought resistance and salt tolerance in rice. *Proc. Natl. Acad. Sci. U. S. A.* 103: 12987-12992.

Huang, J., Hu, R., Rozelle, R. and Pray, C. 2005. Insect-resistance GM rice in farmers’ field: assessing productivity and health effects in China. *Science* 308: 688-690.

Ivanovska, A., Todorovski, L., Debeljak, M. and Džeroski, S. 2009. Modelling the outcrossing between genetically modified and conventional maize with equation discovery. *Ecological Modelling* 220: 1063-1072.

Kawahigashi, H., Hirose, S., Ohkawa, H. and Ohkawa, Y. 2007. Herbicide resistance of transgenic rice plants expressing human CYP1A1. *Biotechnol. Adv.* 25: 75-84.
Prescher, S., Schiemann, J. and Hüsken, A. 2010. Study of maize fields

Majee, M., Maitra, S., Ghose, K., Pattnaik, S., Chatterjee, A., Hait, N.,

Messeguer, J., Fogher, C., Guiderdoni, E., Marfà, V., Català, M.M.,

Lu, B.A., Song, Z.P. and Chen, J.K. 2003. Can transgenic rice cause

Kumar, V., Bellinder, R.R., Brainard, D.C., Malik, R.K. and Gupta, R.K. 2008. Risks of herbicide-resistant rice in India: A review. Crop Prot. 27: 320-329.

Lu, B.A., Song, Z.P. and Chen, J.K. 2004. A field study of pollen-mediated gene flow from genetically modified rice and its environmental consequences. BioScience 55: 669-678.

Majee, M., Maitra, S., Ghose, K., Pattnaik, S., Chatterjee, A., Hait, N., Das, K.P. and Majumder, A.L. 2004. A novel salt-tolerant L-myoinositol 1-phosphate synthase from Porteresia coarctata Tateoka, a halophytic wild rice: Molecular cloning, bacterial overexpression, characterization and functional introgression into tobacco conferring salt-tolerance phenotype. J. Biol. Chem. 279: 28539-28552.

Messeguer, J., Fogher, C., Guiderdoni, E., Marfà, V., Català, M.M., Baldi, G. and Melé, E. 2001. Field assessment of gene flow from transgenic to cultivated rice (Oryza sativa L.) using a herbicide gene as tracer marker. Theor. Appl. Genet. 103: 1101-1109.

Messeguer, J., Marfà, V., Català, M.M., Guiderdoni, E. and Melé, E. 2004. A field study of pollen-mediated gene flow from Mediterranean GM rice to conventional rice and the red rice weed. Mol. Biol. 13: 103-112.

Olguín, E.R., Arrieta-Espinoza, G., Lobo, J.A. and Espinoza-Esquível, A.M. 2009. Assessment of gene flow from a herbicide-resistant indica rice (Oryza sativa L.) to the Costa Rican weedy rice (Oryza sativa) in Tropical America: factors affecting hybridization rates and characterization of F1 hybrids. Transgenic Res. 18: 633-647.

Paine, J.A., Shipston, C.A., Chaggar, S., Howells, R.M., Kennedy, M.J., Vernon, G., Wright, S.Y., Hinchliffe, E., Adams, J.L., Silverstone, A.L. and Drake, R. 2005. Improving the nutritional value of Golden Rice through increased pro-vitamin A content. Nat. Biotechnol. 23: 482-487.

Peñas, G., Català, M., Melé, E., Llorach, T., Pla, E. and Meseguer, J. 2007. Effects of field size and physical barriers on GM-pollen mediated gene flow in rice. The 3rd International Conference on Coexistence between Genetically Modified (GM) and Non-GM Based Agricultural Supply Chains. Seville, Spain, 20-21 November 2007. 237-238.

Prescher, S., Schiemann, J. and Häusken, A. 2010. Study of maize fields and their surroundings in European regions regarding the suitability for coexistence of different maize cultivars. In B. Breckling and R. Verhoeven eds., Implication of GM-Crop Cultivation at Large Spatial Scales. Peter Lang, Frankfurt, German. 84-88.

Rong, J., Song, Z., de Jong, T.J., Zhang, X., Sun, S., Xu, X., Xia, H., Liu, B. and Lu, B.R. 2010. Modelling pollen-mediated gene flow in rice: risk assessment and management of transgene escape. Plant Biotechnol. J. 8: 452-464.

Saha, P., Majumder, P., Dutta, I., Ray, T., Roy, S.C. and Das, S. 2006. Transgenic rice expressing Allium sativum leaf lectin with enhanced resistance against sap-sucking insect pests. Planta 223: 1329-1343.

Shivanna, K.R. and Rangaswamy, N.S. 1992. Pollen biology: a laboratory manual. Springer, Berlin Heidelberg New York. 9-20.

Shivrain, V.K., Burgos, N.R., Anders, M.M., Rajguru, S.N., Moore, J. and Sales, M.A. 2007. Gene flow between Clearfield™ rice and red rice. Crop Prot. 26: 349-356.

Sivaprakash, K.R., Krishnan, S., Datta, S.K. and Parida, A.K. 2006. Tissue-specific histochemical localization of iron and ferritin gene expression in transgenic indica rice Pusa Basmati (Oryza sativa L.). J. Genet. 85: 157-160.

Song, X., Liu, L., Wang, Z., and Qiang, S. 2009. Potential gene flow from transgenic rice (Oryza sativa L.) to different weedy rice (Oryza sativa f. spontanea) accessions based on reproductive compatibility. Pest Manag. Sci. 65: 862-869.

Song, Z.P., Lu, B.R., Zhu, Y.G. and Chen, J.K. 2003. Gene flow from cultivated rice to wild species Oryza rufipogon under experimental field conditions. New Phytop. 157: 657-665.

Song, Z.P., Lu, B.R. and Chen, J.K. 2004. Pollen flow of cultivated rice measured under experimental conditions. Biodivers. Conserv. 13: 579-590.

Ting, M.Y., Shih, H.D. and Lin, C.Y. 2008. Increased susceptibility of rice following insertion of amylopullulanase gene, to brown spot caused by Bipolaris oryzae. J. Phytopathology 156: 530-533.

Tsay, J.Y., Chen, R.B., Chang, J.C., Liao, C.H., Yang, H.Y., Wang, S.R. and Chen, L.J. 2001. Production of porcine lactoferrin in transgenic rice. The 3rd Cross-strait Symposium on Plant Molecular Biology and Biotechnology. Hong Kong, 5-11 August 2001. 18.

Tseng, C.S., Wu, M.T., Huang, H.C., Lai, M.H. and Chern, C.G. 2008. Field assessment of agronomic performance and biosafety of phytase-gene transformed rice. J. Taiwan Agric. Res. 57: 175-182*

Wang, F., Yuan, Q.H., Shi, L., Qian, Q., Liu, W.G., Kuang, B.G., Zeng, D.L., Liao, Y.L., Gao, B. and Jia, S.R. 2006. A large-scale field study of transgene flow from cultivated rice (Oryza sativa) to common wild rice (O. rufipogon) and barnyard grass (Echinochloa crus-galli). Plant Biotechnol. J. 4: 667-676.

Willenborg, C.J., Brulé-Babel, A.L. and Van Acker, R.C. 2010. Identification of a hybridization window that facilitates sizeable reductions of pollen-mediated gene flow in spring wheat. Transgenic Res. 19: 449-460.

Yao, K., Hu, N., Chen, W., Li, R., Yuan, Q., Wang, F., Qian, Q. and Jia, S. 2008. Establishment of a rice transgene flow model for predicting maximum distances of gene flow in southern China. New Phytop. 180: 217-228.

Ye, X., Al-Babili, S., Klöti, A., Zhang, J., Lucca, P., Beyer, P. and Potrykus, I. 2000. Engineering the provitamin A (beta-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. Science 287: 303-305.

Yuan, Q.H., Shi, L., Wang, F., Cao, B., Qian, Q., Lei, X.M., Liao, Y.L., Liu, W.G., Cheng, L. and Jia, S.R. 2007. Investigation of rice transgene flow in grassy sectors by using male sterile line as a pollen detector. Theor. Appl. Genet. 115: 549-560.

Zhang, Q.J., LU, C.G., Xia, S.J., Zong, S.Y., Qi, Q.M., Yu, D.R. and Sun, Y.H. 2008. Obtaining transgenic rice plants harboring sbk and sk insecticidal genes. Mol. Plant Biol. 6: 49-52.

* In Chinese with English abstract.