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Original Paper

Efficacy of a special screened greenhouse covered by duplex fine mesh in reducing maize outcrossing

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Abstract Gene flow via pollen dispersal leading to the escape of transgenes is a potential concern associated with the introduction of transgenic plants. Therefore, it is necessary to clarify the relationship between pollen dispersal and outcrossing rate for strict biosafety management during risk assessment. Maize (*Zea mays*) is one of the crops most at risk for gene leakage via pollen flow into the environment. Here, we report the results of a cross-pollination field investigation using maize varieties showing a xenia effect with tricolor kernels, which allowed us to designate a pollen donor with natural outcrossing and one with reduced outcrossing (via a screened greenhouse) in the same experimental field at the same time. Although a previous study showed that a special screened greenhouse covered by 1-mm single fine mesh may be effective in reducing outcrossing in maize, we used 1-mm duplex fine mesh to reduce further the possibility of outcrossing. We report how a special screened greenhouse covered by 1-mm duplex fine mesh reduced pollen dispersal, and affected the outcrossing rate of non-genetically modified (GM) yellow maize in the greenhouse and white maize outside the greenhouse, compared to natural outcrossing.

Key words: Genetically modified, 1-mm duplex fine mesh, outcrossing, special screened greenhouse, Type 2 use, *Zea mays*.

Advances in plant biotechnology have enabled the transfer of genes from various types of organisms into plants, resulting in the production of genetically modified (GM) varieties expressing new traits of agronomic, scientific, or medical interest. However, most of these lines are still at the stage in which they are grown in experimental incubators or closed greenhouses in Japan, because of concerns about the hybridization of transgenic plants with wild relatives and the successive introgression of transgenic traits into the gene pools of wild plant populations (Ellstrand and Hoffman 1990). However, it is important that the regulation of risk should not become the risk of regulation, and the most appropriate baseline for comparison when performing risk assessments of GM crops is the effect of plants developed by traditional breeding (Conner et al. 2003).

Gene flow is very likely to occur through pollen dispersal because reproductive organs are intended to create gene movement, and the crossing of transgenic plants with wild plants is therefore difficult to control. A risk assessment using a special screened greenhouse for Type 2 use covered with 1-mm fine mesh (as specified by biosafety regulations of the Japanese government) by a domestic institute is required for GM plants of Type 2 use in Japan. It is important to determine the possible distance of gene flow via pollen escape from such a screened greenhouse during risk assessment, and, thus, the management area around the greenhouse.

Maize is predominantly a wind-pollinated species and is monoecious, with separate male and female flowers at different locations on the same plant (Poethig 1982). Moreover, maize shows the xenia effect: pollen from the male parent genetically affects fruit development or seed color. Thus, it is advantageous to study maize outcrossing rates using two varieties with different kernel colors, without using GM maize.

In our previous studies of risk assessments of GM plants, we reported the importance of verifying previously determined information, such as pollen size and pollination form (Watanabe et al. 2006a). We also performed a cross-pollination field experiment using maize varieties with two different-colored kernels using a special screened greenhouse covered by 1-mm single mesh (Watanabe et al. 2006b). Our results suggested that this greenhouse was effective in reducing pollen dispersal compared to natural outcrossing results from the literature (Watanabe et al. 2006b). However, because we did not use a natural outcrossing control in the

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Abbreviations: GM, genetically modified; N, north; E, east; S, south; W, west.
This article can be found at http://www.jspcmb.jp/

previous study, we did not directly estimate how much the outcrossing rate was reduced.

Here, we show the results of a pollen-dispersal experiment performed using three varieties of non-GM maize: a pollen recipient, a pollen donor for natural outcrossing, and a pollen donor grown within a special screened greenhouse for reduced outcrossing. Our field experiment was designed to create a high possibility of outcrossing between the donor and recipient maize, to determine a threshold for the greatest risk of genetic contamination in the case of a physical contaminant. The aim of this study was to estimate how a special screened greenhouse for Type 2 use covered by a fine, duplex 1-mm mesh would reduce pollen dispersal of non-GM maize compared to natural outcrossing.

Materials and methods

Plant materials

Three commercial varieties of corn with different grain colors were selected. *Z. mays* L. cv. Kimochitoumorokoshi with yellow grains (yellow maize) and cv. Kuromochitoumorokoshi with black grains (black maize) were used as pollen donors; *Z. mays* L. cv. Shiromochtoumorokoshi with white grains (white maize) was used as the pollen recipient. The xenia phenomenon was used to determine the percentage of outcrossing, as shown by the presence of yellow or black grains on the female ears of the recipient.

Field design

The experiment was performed in a 40×37-m experimental field in Tsukuba, Ibaraki Prefecture, Japan. Adequate nutrition, as defined by standard prescriptions, was supplied to the field. To ensure congruence between male pollen release from the black and yellow maize and silk receptivity on the white maize, yellow and black maize were sown on 25 and 30 April and 5 May 2005. The yellow and black maize were sown in adjoining 6×7.6-m plots at the center of the experimental field (Figure 1A).

A pipe-frame greenhouse without a cover (W : D : maximum H = 6.3 : 7.65 : 2.8 m) was constructed over the area where the yellow maize was sown. The greenhouse was covered by 1-mm nylon fine mesh both inside and outside the pipe frame, just before the tassels at the top of maize plants opened, to reduce pollen dispersal from the yellow maize. The area surrounding the greenhouse was sown with white maize on 30 April 2005. All black, yellow, and white maize plants were sown in rows at intervals of approximately 0.75 m, with a distance of approximately 0.3 m between individual plants.

The ratio of pollen donors to pollen recipients can significantly affect the outcrossing ratio in gene-flow research. Here, we used 200 black, 200 yellow, and approximately 6000 white maize plants. The 200 pollen donors were considered sufficient for biosafety assessment; this was also the maximum number of plants that would fit into the 6.0×7.6-m plot, which corresponds to the standard size of the special screened greenhouses used in Japan. Therefore, the number of pollen donors was chosen to present a high risk of outcrossing.

Data monitoring

Meteorological data, including air temperature, luminous intensity, precipitation, and wind speed and direction at the experimental field, were measured using a Vantage Pro weather station (Davis Instruments Corp., Haywood, CA, USA) during the flowering period. Temperature and humidity data were also collected inside the greenhouse (instruments from Hioki Co., Ueda, Japan) during the flowering period.

We measured the height of all yellow maize plants and approximately 800 white maize plants on 11 August 2006. The flowering condition of both male and female flowers was determined once a week after flowering began at the beginning of July 2005 until flowering ceased. For both male and female flowers, the flowering rates (%) were calculated as the number of flowering plants/the total number of plants examined×100.

Sampling and analysis

On 1 July 2005, just before the tassels opened, the entire greenhouse was covered by duplex 1-mm nylon fine mesh. On 11 August 2005, all yellow and black maize fruits were harvested and approximately 400 white maize fruits were sampled in 16 different directions from the greenhouse to the edge of the experimental field (Figure 1A). After sampling, the number of yellow and black kernels and the total number of kernels were counted on each white maize fruit to calculate the outcrossing rate using the following formula:

$$\text{Outcrossing rate (\%)} = \frac{\text{number of black or yellow kernels}}{\text{row number} \times \text{line number of fruit}} \times 100.$$

Supplemental experiment to check natural outcrossing rates

A supplemental experiment was performed in a 3.4×8-m experimental field at the University of Tsukuba to check the natural outcrossing rates between white maize and black or yellow maize under typical field conditions (Figure 1B). From the results of the flowering investigation in the main experiment, to ensure congruence between male pollen release from the black and yellow maize and silk receptivity on the white maize, white maize was sown on 9 August, black maize was sown on 9, 11, and 13 August, and yellow maize was sown on 11, 13, and 15 August 2005. We used 22 plants each of black, yellow, and white maize.

The flowering condition of both male and female flowers was determined about once a week after flowering started at the end of September 2005 until flowering finished at the end of October. On 20 November 2005, after measuring the height of all plants, all maize fruits were harvested. After the number of yellow and black kernels and the total number of kernels were counted on each white maize fruit, the outcrossing rate was calculated using the formula described above.

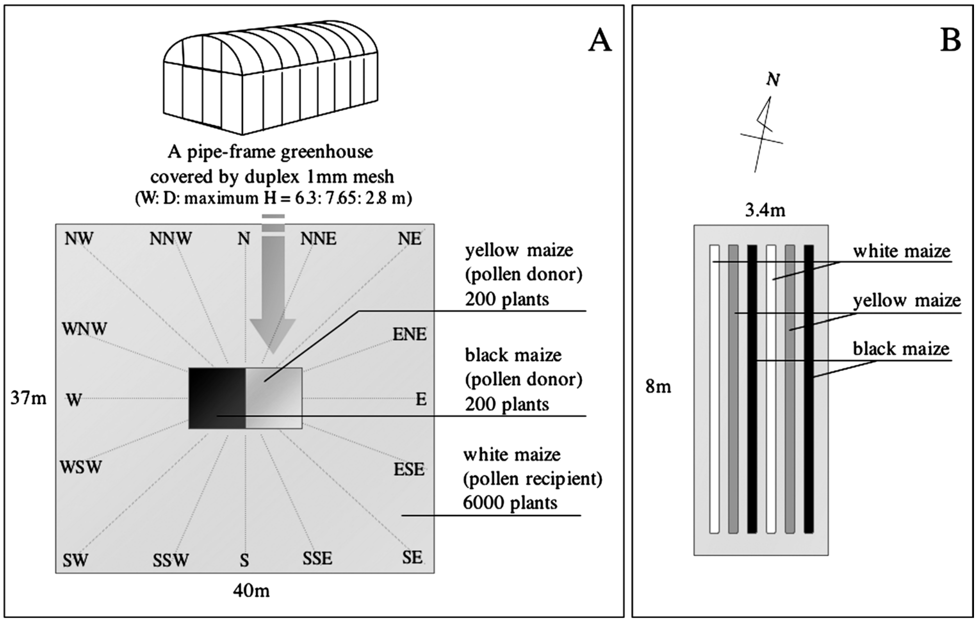

Figure 1. (A) Experimental field design of the 40×37-m maize field. A 6.0×7.6-m plot with 200 black maize plants served as the control pollen donor population, and a 6.0×7.6-m plot with 200 yellow maize plants served as the treatment pollen donor population; these two central plots were located within a field containing 6000 white maize pollen recipient plants of the same variety. Radial lines spreading in 16 directions indicate sampling transects. A pipe-frame greenhouse over the yellow maize plot was covered with 1-mm duplex fine mesh just before the flowering period began at the end of June 2005. (B) Experimental design of the 3.4×8-m maize field to examine natural cross-pollination among the three maize cultivars. Twenty-two plants were sown in each row.

Results

Weather conditions during the flowering period

The average maximum temperature in July 2005 was 27.9°C at the Mito Meteorological Observatory in Tsukuba, 5 km southeast of the experimental field; this was similar to the average maximum July temperature of 28.3°C from 1990 to 2000. The total precipitation of 184 mm in July 2005 was much greater than the average of 139 mm in July from 1990 to 2000. The July precipitation and maximum temperature data from Tsukuba were also similar to those recorded by our observational equipment at the experimental field (Figure 2A). In July 2005, the rain frequency was concentrated in early July (Figure 2A).

Wind direction changed daily, with an average wind speed of about 1.7 m/s during the flowering period. The maximum wind speed in the N, E, S, and W directions in 10-min intervals was measured on 22 days (1–22 July; data were not collected after 22 July because of equipment malfunctions). Winds were mainly from the E and N from 20:00 to 08:00; winds came from the S around 06:00, and winds from the S and E strengthened until around 16:00 (Figure 2B).

Plant growth conditions

Black, yellow, and white maize plants grew to an average height of 2.32±0.31 m, 2.39±0.23 m, and 2.47±0.36 m, respectively, suggesting little height difference in the pollen release points among the three maize cultivars. Black, yellow, and white maize plants had tassel lengths of approximately 30 cm. Most maize plants had two 20–25-cm-long fruits per plant. Almost all ears were successfully fertilized. The ears were located at the mid-height of plants.

Timing of flowering

Male and female flowers on all cultivars began to flower at the beginning of July and finished flowering by the end of July (Figure 3). The male flowering rate of black maize increased earlier than that of both yellow and white maize; the male flowering rate of yellow maize increased secondarily (Figure 3). The female flowering rate of white maize increased last among all maize cultivars but caught up with that of male yellow maize (Figure 3). Consequently, the flowering timing of male yellow maize flowers and female white maize flowers was synchronized best, and may have resulted in a greater opportunity for outcrossing than in black maize. Although maize pollen viability ranges from 3 h to 9 days, depending on the environment conditions, with cool temperatures and high relative humidity allowing for longer survival time (Glover 2002), the daytime relative humidity values in the experimental field during the flowering period were approximately 50–90%, field conditions may not have affected pollen longevity.

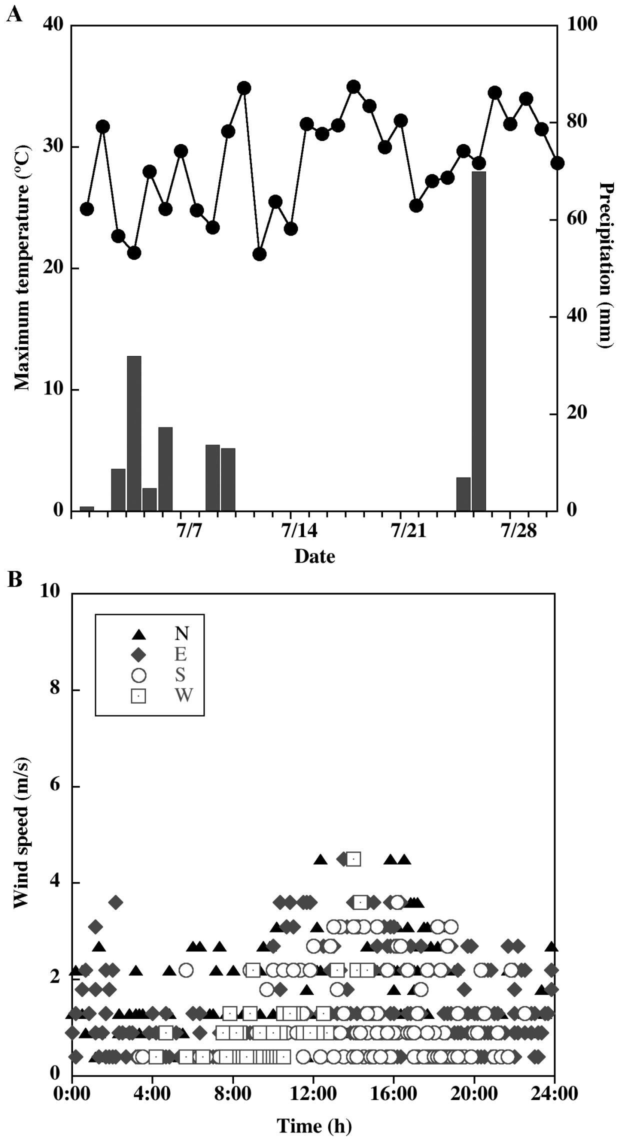

Figure 2. Meteorological conditions in the experimental field during the flowering period. (A) Daily maximum temperature (circles) and precipitation (bars) recorded during the flowering period. (B) Representative data of diurnal changes in wind direction during the flowering period. The data were collected at the four cardinal directions at 10-min intervals on 22 days (1–22 July 2005) during the flowering period.

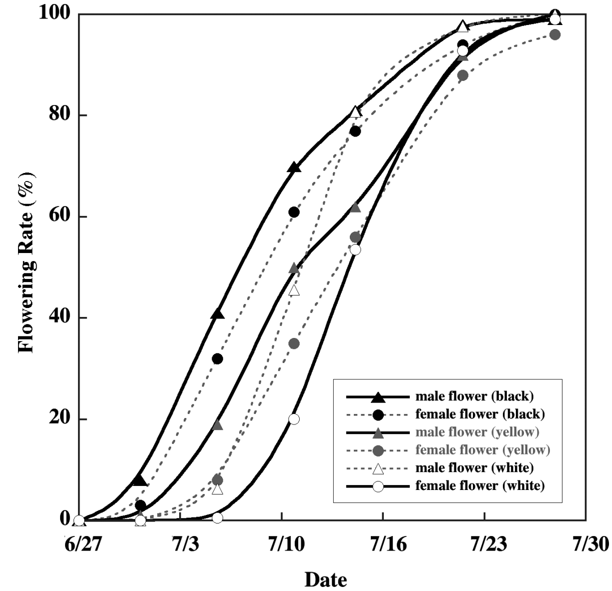

Figure 3. Flowering rates of black, yellow, and white maize plants during the flowering period. For both male and female flowers, the flowering rates (%) were calculated as the number of flowering plants/the total number of plants examined×100.

Outcrossing rate

Of the sampled white maize, 78% did not outcross with either black or yellow maize. The outcrossing rate decreased with increasing distance between black or yellow maize and white maize (Figure 4B). The outcrossing rate between black and white maize decreased sharply when white maize plants were beyond 5 m from the black maize (Figure 4B). A 1.0% outcrossing rate between yellow and white maize was observed 9.2 m from the yellow maize and a 1.0% outcrossing rate between black and white maize was observed 11.9 m from the black maize (Figure 4B). In total, 139 yellow kernels and 594 black kernels were counted on the sampled white maize. Therefore, the duplex 1-mm fine mesh reduced yellow maize outcrossing with white maize by 76.6%. Higher outcrossing rates occurred at a greater distance mainly to the N and W, which were downwind from the pollen donor plants (Figure 4C). For black kernels, 77.9% of outcrossing with sampled white maize occurred <5 m from the pollen donor and 96.6% of outcrossing occurred <10 m from the pollen donor; for yellow kernels, 85.6% of outcrossing with sampled white maize occurred <5 m from the pollen donor and 97.1% of outcrossing occurred <10 m from the pollen donor (Figure 4D).

Supplemental natural outcrossing experiment

The total precipitation in October 2005, including 17 rainy days, was 197 mm. Despite the staggering of planting times to obtain congruous timing of flowering among the tricolor maize species, the male flowering rate of black maize increased earlier than that of both yellow and white maize, resulting in flowering patterns similar to the main experiment. Thus, the timing of flowering of male yellow maize flowers and female white maize flowers was synchronized best (Figure 5A). All maize yielded satisfactory products at harvest and there was no significant difference for fertility between main experiment and supplemental experiment. The mean numbers of black and yellow kernels were counted on each white maize fruit (Figure 5B). Cross-pollination between black and white maize and yellow and white maize was similar under typical field conditions without the special screened greenhouse.

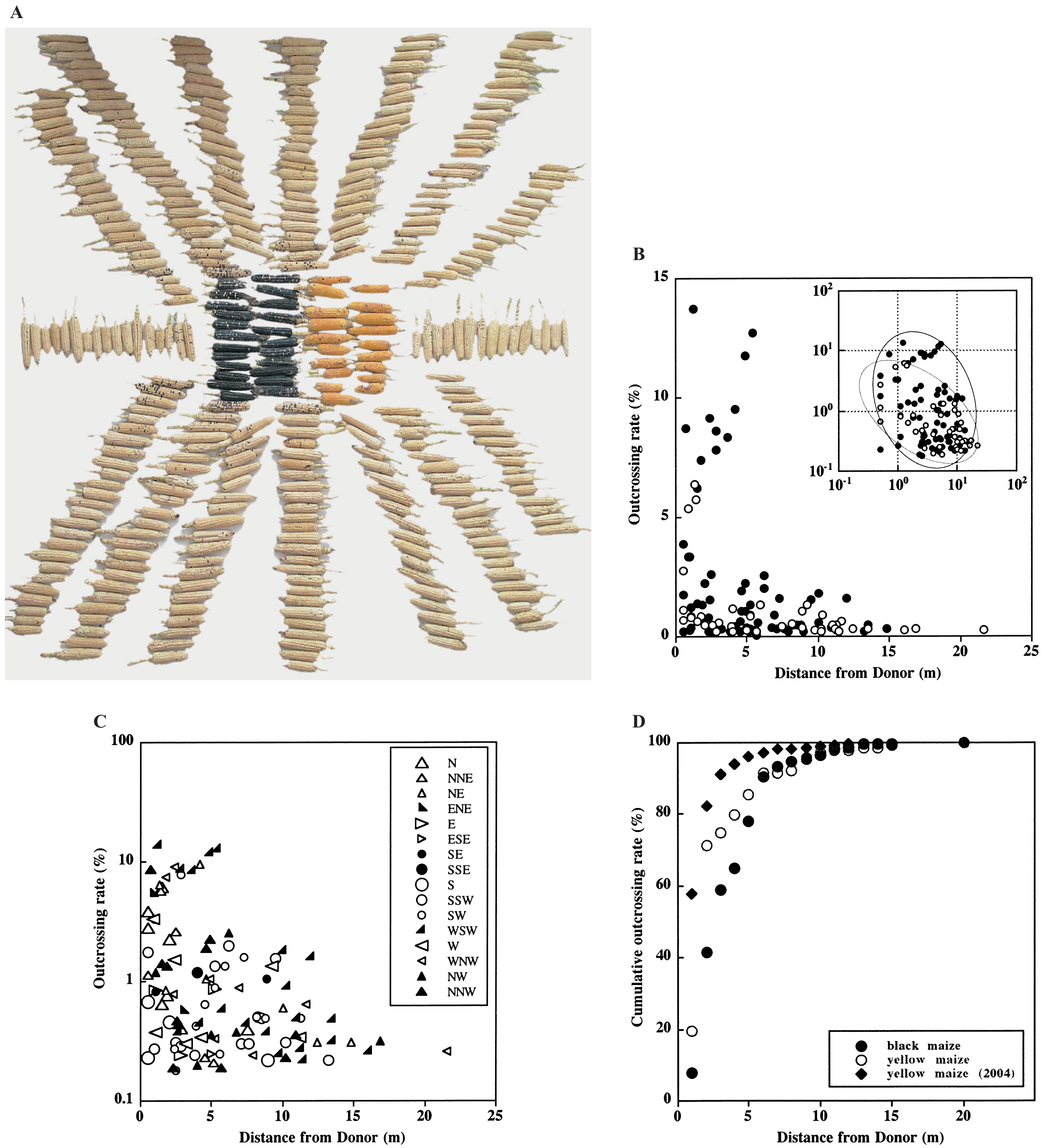

Figure 4. Outcrossing between black and white maize and yellow and white maize. (A) The white maize harvest arranged according to location in the experimental field. The black and yellow maize samples in the center were from the pollen donors. (B) Relationship between the cross-pollination rate and distance from the pollen donor. The closed circles show the outcrossing between black and white maize and the open circles show the outcrossing between white and yellow maize. Data plotted on the linear scale suggest a definable dispersal-length scale, whereas data plotted on the log–log scale (inset) suggest a long tail of dispersal by atmospheric turbulence. (C) Relationships among the cross-pollination rate, distance from the pollen donor, and wind direction on a linear–log plot. (D) Relationship between the distance from the pollen donor and cumulative outcrossing rate (%). The 2004 data are from Watanabe et al. (2006b).

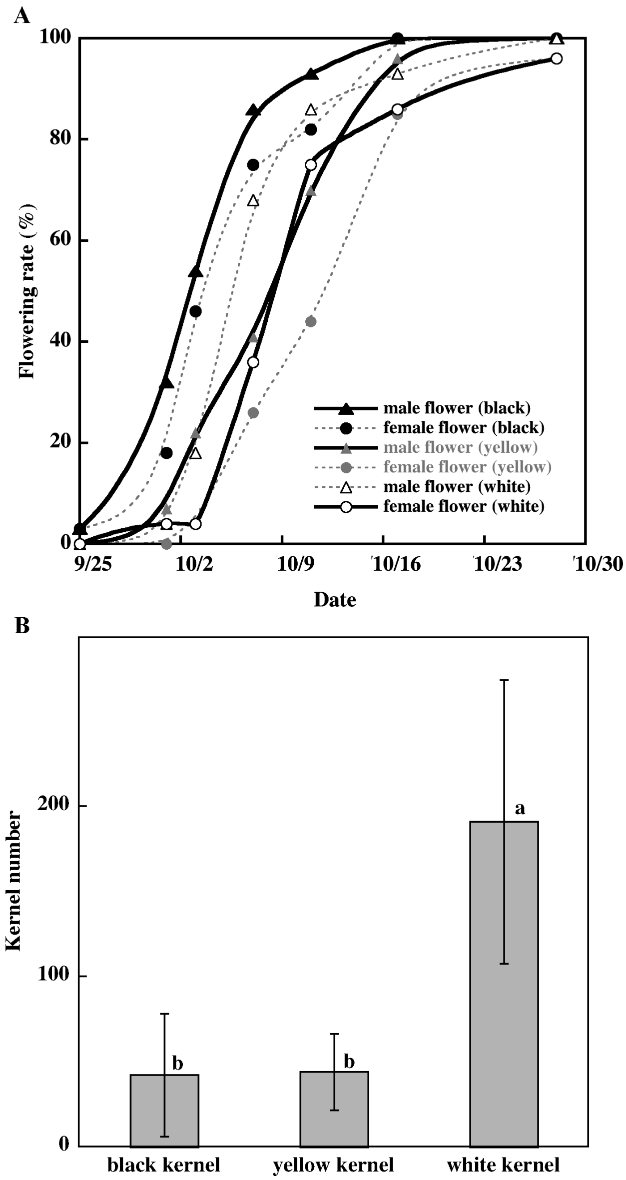

Figure 5. Results of the supplemental experiment to examine natural outcrossing rates between black and white maize and yellow and white maize. (A) Flowering rate of black, yellow, and white maize plants during the flowering period. For both male and female flowers, the flowering rates (%) were calculated as the number of flowering plants/the total number of plants examined×100. (B) Number of outcrossed kernels of each color on fruit of white maize. Bars indicate the means; error bars denote standard deviations (n=22). Significant differences among all paired combinations were determined using Mann–Whitney *U*-tests with a Bonferroni correction at $p=0.01$; different lowercase letters indicate significant differences.

Discussion

Because of the comparatively mild weather conditions, which continued throughout the flowering period, the highest air temperature difference outside and inside the special screened greenhouse covered by duplex 1-mm mesh was about 3–5°C at 100 Klux (data not shown). Despite the fact that the highest temperature inside the greenhouse sometimes reached >35°C, the yellow maize grew similarly to the black and white maize outside the greenhouse, and all maize yielded satisfactory products at harvest (Figure 4A).

We previously reported micrometeorological patterns in daily changes in wind direction in the experimental field during the flowering period in 2004 (Watanabe et al. 2006b); similar, but slightly different patterns were found in 2005 (Figure 2B). Although the wind mainly blew from the S during the day in July 2004, in July 2005, strengthening winds mainly blew from the S and E from midmorning until around 16:00 (Figure 2B). A higher rate of outcrossing occurred to the W and N at a greater distance from the pollen donors because the majority of daily pollen release usually occurs from midmorning to midday (Ogden et al. 1969, Jarosz et al. 2003; Figure 4C). The results of experiments in both 2004 and 2005 suggest that knowing the micrometeorological patterns would aid in predicting the main direction of pollen dispersal.

This study was conducted in an approximately 0.15-ha field, with a maximum distance of 28 m between pollen recipient and donor plants. We confirmed that cross-pollination between black and white maize was similar to that between yellow and white maize under typical field conditions without the special screened greenhouse (Figure 5B). The 1-mm duplex fine mesh reduced the outcrossing rate by 76.7%. However, considering the higher synchronicity in flowering between the yellow and white maize than between the black and white maize, the outcrossing rate may be even lower. A 1.0% outcrossing rate occurred at 9.2 m from the yellow maize and at 11.9 m from the black maize (Figure 4B). On a log–log plot of the same data (inset, Figure 4B), the positional difference between the black and yellow maize (solid and dotted ellipse, respectively) showed that the 1-mm duplex fine mesh was mainly effective in reducing the outcrossing rate at close range from the pollen donor. The cumulative outcrossing rate between yellow and white maize was higher than that between black and white maize up to 6 m from the pollen donors, but was similar for yellow and black maize at greater distances (Figure 4D).

Numerous studies have measured actual gene flow using the percentage of outcrossing per fruit (Jones and Brooks 1950, Jemison and Vayda 2001, Eastham and Sweet 2002, Loos et al. 2003). Jones & Brooks (1950) measured the percentage of outcrossing between large blocks of donor and receptor crops over a period of three years at a maximum distance of 500 m. The mean hybridization directly adjacent to the crop measured 25.4%, falling to 1.6% at 200 m and 0.2% at 500 m. Another study using Roundup-Ready corn and conventional hybrid corn reported that mean cross-pollination of 1% was observed between 40 m and 110 m from the pollen source of Roundup-Ready corn (Jemison and Vayda). Compared to these studies, the outcrossing rate between black and white maize was quite low because of the difference in cultivar, amount of initial pollen source and the unsuitable weather conditions for outcrossing. This shows the difficulty in making parallel estimates of gene flow under different treatments.

We performed a similar study in 2004 using a special screened greenhouse covered by 1-mm single fine mesh and the bicolor corn varieties of *Z. mays* L. cv. Honey Bantam (Watanabe et al. 2006b). A 1.0% outcrossing rate between yellow maize and white maize occurred at 6.3 m (Watanabe et al. 2006b), which was a shorter distance than that estimated in 2005. However, because the mean number of kernels on sampled fruit of white maize was 576±85 in 2004 and 344±115 in 2005, the difference in the denominator directly affected the outcrossing rate in percentage per fruit. If we correct for the differences in denominators, the 1.0% outcrossing rate between yellow and white maize would fall to within 1.5 m in 2005. There was a higher cumulative outcrossing rate at all distances from the pollen donor in 2004 than in 2005 (Figure 4D); thus, it was difficult to determine the contribution of various effects, such as variety, flowering synchrony, and weather conditions, to reduce the outcrossing rate in addition to the effect of duplex versus single fine mesh. Another possible effect on outcrossing was created by the field design, in which the black and yellow maize pollen donors were adjacent to each other. The high outcrossing rates close to the pollen donors were masked where the pollen donors were adjacent within a space of 6.3×7.65 m.

Most risk assessments involving the use of special screened greenhouses may be applied to molecular breeding in crops. Thus, early crop-wild hybrid fitness and the intrinsic costs and benefits of transgenes are important parameters that must be accounted for when considering gene establishment. Crop-to-weed gene flow appears to be highly idiosyncratic and may be crop-specific and, to some extent, variety-specific, site-specific, or even season-specific (Raybould and Gray 1993).

Jenczewski et al. (2003) reviewed the degree of cross-compatibility between cultivated plants and their wild relatives, such as in *Zea* (Doebley 1990), *Oryza* (Langevin et al. 1990), *Triticum* (Seefeldt et al. 1998), *Medicago* (Jenczewski et al. 1999), and *Cucurbita* (Wilson 1990). They concluded that crops are usually cross-compatible with their direct progenitors, and that the probability of crop–wild plant hybridization decreases with increasing genetic and phenotypic divergence (Jenczewski et al. 2003). This knowledge aids in the determination of which wild plants require attention during the period of risk assessment in a special screened greenhouse. Jenczewski et al. (2003) suggested that it would be very difficult to prevent gene dispersion entirely, and even our present data are not sufficient to draw definite conclusions about the associated risks.

We determined the effect of a special screened greenhouse covered by 1-mm duplex fine mesh on outcrossing compared to natural outcrossing in a wind-pollinated plant with a high potential for long-distance pollen release using a high-risk (maximum) number of plants. Field measurements of outcrossing levels at various distances from a pollen source depend on local conditions, such as topography, rainfall, and wind speed and direction (Aylor et al. 2003). Thus, to compensate for the different conditions in field experiments, the establishment of a model to simulate a special screened greenhouse is desirable.

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References

Aylor DE, Schultes NP, Shields EJ (2003) An aerobiological framework for assessing cross-pollination in maize. *Agric For Meteorol* 119: 111–129

Conner AJ, Glare TR, Nap JP (2003) The release of genetically modified crops into the environment: Part II. Overview of ecological risk assessment. *Plant J* 33: 19–46

Doebley JF (1990) Molecular evidence for gene flow among *Zea* species. *BioScience* 40: 443–448

Eastham K, Sweet J (2002) Genetically modified organisms (GMOs): the significance of gene flow through pollen transfer. *Environmental Issues Report 28*. European Environmental Agency, Copenhagen, pp 38–42

Ellstrand NC, Hoffman CA (1990) Hybridization as an avenue of escape for engineered genes. *BioScience* 40: 438–442

Glover J (2002) *Gene flow study: implications for GM crop release in Australia*. Bureau of Rural Sciences, Canberra, Australia, pp iv–vi

Jarosz N, Loubet B, Durand B, McCartney HA, Foueillassar X, Huber L (2003) Field measurements of airborne concentration and deposition rate of maize pollen (*Zea mays* L.) downwind of an experimental field plot. *Agricul Forest Meteorol* 119: 37–51

Jemison JM, Vayda ME (2001) Cross pollination from genetically engineered corn: wind transport and seed source. *AgBioForum* 4: 87–92

Jenczewski E, Prosperi JM, Ronfort J (1999) Evidence for gene flow between wild and cultivated *Medicago sativa* (Leguminosae) based on allozyme and quantitative traits. *Am J Bot* 86: 677–687

Jenczewski E, Ronfort J, Chevre AM (2003) Crop-to-wild gene flow, introgression and possible fitness of transgenes. *Environ Biosafety Res* 2: 9–24

Jones MD, Brooks JS (1950) Effectiveness of distance and border rows in preventing outcrossing in corn. *Oklahoma Agricultural Experiment Station*, *Technical Bulletin* No. T-38: 1–18

Langevin C, Godelle B, Reboud X, Gouyon PH (1990) The incidence and effects of hybridization between cultivated rice and its related weed red rice (*Oryza sativa* L.). *Evolution* 44: 1000–1008

Loos C, Seppelt R, Meier-Bethke S, Schiemann J, Richter O (2003) Spatially explicit modelling of transgenic maize pollen dispersal and cross-pollination. *J Theor Biol* 225: 241–255

Ogden EC, Hayes JV, Raynor GS (1969) Diurnal patterns of pollen emission in *Ambrosia*, *Phleum*, *Zea*, and *Ricinus*. *Am J Bot* 56: 16–21

Poethig RS (1982) Maize—the plant and its parts. In: Sheridan WF (Ed) *Maize for Biological Research*. University of North Dakota Press, Grand Forks, ND, USA, pp 9–18

Raybould AF, Gray AJ (1993) Genetically modified crops and hybridization with wild relatives: a UK perspective. *J Appl Ecol* 30: 199–219

Seefeldt SS, Zemettra RS, Young FL, Jones SS (1998) Production of herbicide-resistant jointed goatgrass (*Aegilops chlindrica*)× wheat (*Triticum aestivum*) hybrids in the field by natural hybridization. *Weed Sci* 46: 632–634

Watanabe S, Sano T, Kamada H, Ezura H (2006a) Reducing gene flow from pollen dispersal of genetically modified plants in special screened greenhouses. *Plant Biotechnol* 23: 129–135

Watanabe S, Kamada H, Ezura H (2006b) Effect of a special screened greenhouse covered by fine mesh on maize outcrossing. *Plant Biotechnol* 23: 309–316

Wilson HD (1990) Gene flow in squash species. *BioScience* 40: 449–455