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Outcrossed cottonseed and adventitious *Bt* plants in Arizona refuges

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Outcrossing of non-*Bt* cotton (*Gossypium hirsutum* (L.)) in refuges by transgenic *Bt* cultivars could reduce the efficacy of refuges for delaying resistance in seed-feeding pests. Based on reports that outcrossing decreased as distance from *Bt* cotton increased in small-scale studies, we hypothesized that increasing refuge width or distance from *Bt* fields would reduce outcrossing. In a large-scale study in Arizona, we quantified *Bt* seed in refuges of experimental and commercial fields, comparing outcrossing between in-field (narrow) and external (wide) refuges and among rows of refuges at various distances from *Bt* fields. Some refuges, including those in tightly controlled experimental plots, contained up to 8% adventitious *Bt* plants. Some, but not all, *Bt* plants likely resulted from *Bt* seed in the non-*Bt* seed bags. We did not detect a difference in outcrossing between in-field and external refuges. However, statistical power was low because outcrossing was low (< 0.4% of seeds) in both treatments. Higher outcrossing levels (≤ 4.6% of seeds) were observed in the studies measuring outcrossing at various distances from *Bt* fields, yet outcrossing did not decrease as the distance from *Bt* fields increased. We hypothesize that *Bt* plants in refuges cross-pollinated surrounding non-*Bt* plants, overshadowing the expected association between distance from *Bt* fields and outcrossing.

Keywords: *Bt* cotton / gene flow / outcrossing / adventitious plants / refuge

INTRODUCTION

Refuges of non-*Bt* host plants in or near transgenic *Bacillus thuringiensis* (*Bt*) crop fields are widely used for delaying pest resistance to *Bt* crops (Carrière et al., 2005; Matten and Reynolds, 2003). Refuges provide an environment where *Bt*-susceptible insects can proliferate. These susceptible insects can then mate with rarely occurring resistant insects emerging from *Bt* crops, thereby reducing the abundance of individuals that are homozygous for resistance (Carrière et al., 2004a, b; Gould, 1998; Tabashnik and Carrière, 2007; Tabashnik et al., 2004).

Gene flow from *Bt* fields into crop refuges results in seeds that produce *Bt* toxin in the refuges. This, in turn, decreases the effective size of refuges for seed-feeding insect pests (Chilcutt and Tabashnik, 2004). Such gene flow could threaten non-*Bt* cotton refuges that are used to delay resistance to *Bt* cotton in pink bollworm (*Pectinophora gossypiella* (Saunders)), a pest that eats cotton seeds. Cotton primarily self-pollinates (Free, 1970), but can also be outcrossed by insects, most notably honey bees and bumble bees (McGregor, 1959).

Studies with experimental plots show that outcrossing of non-*Bt* cotton by neighboring *Bt* cotton fields dramatically declines as distance into non-*Bt* fields increases (Llewellyn and Fitt, 1996; Llewellyn et al., 2007; Umbeck et al., 1991; Zhang et al., 2005). Thus, we hypothesized that outcrossing would be higher in in-field refuges than in external refuges. In-field refuges are narrow strips (often single rows) of non-*Bt* cotton plants in *Bt* cotton fields, whereas external refuges are large blocks of non-*Bt* cotton in or near *Bt* cotton fields (Carrière et al., 2005). Narrow refuges should have higher outcrossing than wide refuges, because a higher proportion of non-*Bt* plants are adjacent to *Bt* cotton. We also hypothesized that outcrossing would decrease in external refuges as the distance between the refuge and *Bt* cotton increased. Barren zones of 200–400 m between cotton fields have been used to limit outcrossing of seed cotton and experimental cotton varieties by other varieties (Hutmacher and Vargas, 2006; United States Environmental Protection Agency, 2006; California Crop Improvement Association, 2007). However, cotton growers often plant external refuges within 10–50 m of *Bt* cotton fields.

In this study, rates of *Bt* outcrossing were measured in bolls from refuges of experimental field plots and

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12 commercial fields surveyed in 2004. Three studies were conducted to investigate (1) whether outcrossing was more frequent in in-field refuges than in external refuges of experimental plots, (2) whether outcrossing decreased in experimental plots as distance into external refuges increased, and (3) whether outcrossing decreased in external refuges of commercial fields as distance between refuges and *Bt* fields increased. An important assumption behind these predictions was that refuges contained only non-*Bt* cotton plants. To test this assumption, we analyzed both seeds and maternal plant tissues for *Bt* toxin, allowing us to differentiate between outcrossed seeds from non-*Bt* plants and seeds from adventitious *Bt* plants. In response to encountering adventitious *Bt* plants, follow-up investigations were performed to identify potential sources.

RESULTS

Adventitious plants

Our assumption that refuges contained only non-*Bt* cotton plants was refuted in all three experiments. Adventitious *Bt* plants, as indicated by presence of the *Bt* toxin Cry1Ac in the pericarp (fruit wall) of bolls, were particularly abundant in the experimental plots at Marana Agricultural Center. Of bolls sampled from Marana Agricultural Center in the experiment comparing in-field and external refuges (n = 160 bolls), 7.5% were from adventitious *Bt* plants. Similarly, in the experiment comparing outcrossing among rows of external refuges at Marana Agricultural Center (n = 160 bolls), 8.1% of sampled bolls were from adventitious *Bt* plants. In commercial field refuges, 1 of 20 bolls were from adventitious *Bt* plants in four of the 12 sampled fields (4 bolls/240 bolls = 1.7%).

In 67% of bolls from adventitious plants in experimental plots and 50% of bolls from adventitious plants in commercial fields, all tested seeds contained Cry1Ac. The remaining 33% and 50% of such bolls from experimental plots and commercial fields, respectively, had both *Bt* and non-*Bt* seeds. Plants with both seed types had a mean of 78% *Bt* seeds (95% confidence interval, 70 to 86%).

Potential sources of adventitious *Bt* plants included volunteers from previous years' *Bt* crops, unintentional seed mixing during planting, or the seed bag. While all of these sources may have contributed to adventitious plants in commercial field refuges, we can rule out seed mixing during planting as a source in our experimental plots. When planting the experimental fields, we did not open seed bags until the time of planting and thoroughly cleaned hoppers when switching between *Bt* and non-*Bt* cotton seed. To examine the possibility that

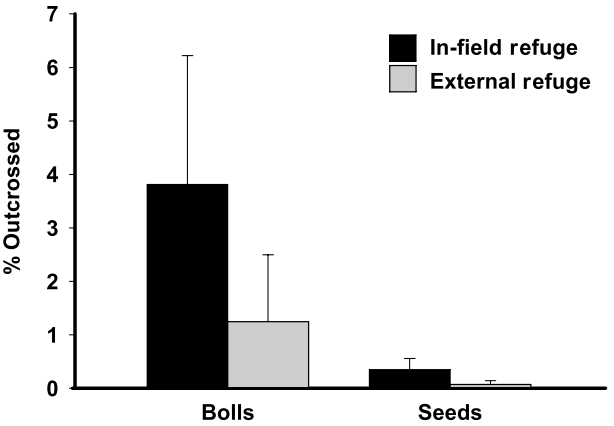

Figure 1. Outcrossing in external and in-field refuges. Mean outcrossing (± SE) is shown for bolls and seeds. Outcrossed bolls are those containing at least one *Bt*-outcrossed seed. Each treatment was replicated in two non-adjacent field blocks and in two time periods of boll set (20–26 July, 2004 and 28 July to 11 August, 2004). Eighty bolls were sampled from each treatment (40 per block).

Bt plants were volunteers, we conducted ELISA tests for glyphosate resistance, because glyphosate-resistant seed was not planted in our experimental plots prior to 2004. All tested seeds and maternal tissue from adventitious bolls tested positive for glyphosate resistance, indicating that either adventitious *Bt* plants originated from seed in the 2004 planting, or that adventitious glyphosate-resistant plants entered fields in previous years, resulting in volunteer plants with both transgenic traits.

Refuge configurations and outcrossing

Bolls from adventitious *Bt* plants were not used in analyses of outcrossing. Contrary to our first hypothesis, in-field and external refuges did not differ significantly in the percentage of outcrossed seeds (coefficient = 0.0028, t_4 = 1.2, P = 0.28), or outcrossed bolls (bolls containing at least one *Bt*-outcrossed seed, coefficient = 0.026, t_4 = 0.91, P = 0.41), after accounting for the effects of field block and pollination time period (Fig. 1). Only four outcrossed bolls, containing 4.5%, 5.0%, 10% and 50% outcrossed seeds, were identified in the experiment comparing the two refuge types. Three of these bolls occurred in in-field refuge plots and one occurred in an external refuge plot. Accordingly, we had little statistical power to detect significantly higher outcrossing in in-field refuges. The percentage of outcrossed bolls (P = 0.44) and outcrossed seeds (P = 0.85) did not differ between blocks, after accounting for refuge type. Similarly, no difference was observed between the two pollination time periods

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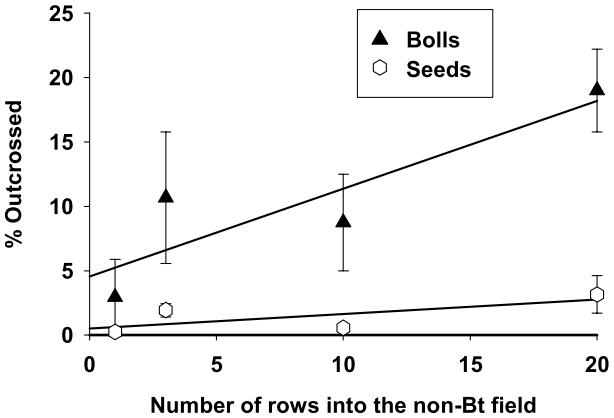

Figure 2. Mean outcrossed bolls and seeds (± SE) in experimental external refuge plots as a function of number of rows into the non-*Bt* plots. Outcrossed bolls are those containing at least one *Bt*-outcrossed seed. A value of one on the *x*-axis represents the first row of non-*Bt* cotton, adjacent to *Bt*. The distance between the external refuge and the adjacent *Bt* plot was 4 m for Block 1 and 2 m for Block 2.

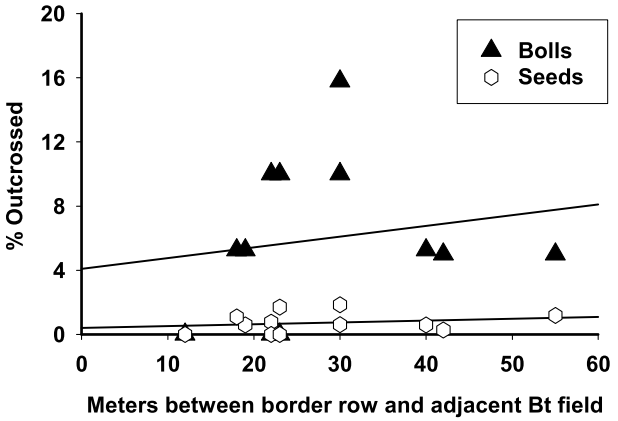

Figure 3. Percentage of outcrossed bolls and seeds in border rows of commercial external refuges as a function of distance between the non-*Bt* field and the adjacent *Bt* cotton field. Outcrossed bolls are those containing at least one *Bt*-outcrossed seed. Twelve refuges located throughout Arizona were surveyed.

in the percentage of outcrossed bolls ($P = 0.41$) or outcrossed seeds ($P = 0.28$).

Contrary to our second hypothesis, the abundance of *Bt*-outcrossed bolls in external refuge plots increased by 0.68% for each one row increase in distance from the refuge edge, after accounting for field block (Fig. 2: $t_5 = 4.00$, $P = 0.010$). There was no significant association between distance into the plots and occurrence of adventitious plants ($t_5 = 0.06$, $P = 0.96$, after accounting for field block), suggesting that increased outcrossing in the center of plots was not due to a higher incidence of adventitious plants there. The percentage of outcrossed bolls was 7.5% greater in Block 2 than in Block 1, after accounting for distance into the refuge ($t_5 = 2.96$, $P = 0.032$). In contrast to the percentage of outcrossed bolls, the percentage of outcrossed seeds was not significantly affected by distance into the refuge, after accounting for field block (slope = 0.0011, $t_5 = 1.89$, $P = 0.12$), although a trend of increased outcrossing with distance into the refuge was observed (Fig. 2). The percentage of outcrossed seeds did not significantly differ between refuge blocks, after accounting for distance into the refuge ($P = 0.27$). Outcrossed bolls contained an average of 14.1% outcrossed seeds (95% C.I. = 8.7% to 19.5%).

Contrary to our third hypothesis, distance from the nearest *Bt* cotton field did not affect either the percentage of outcrossed bolls (Fig. 3: slope = 0.00067, $t_{10} = 0.54$, $P = 0.60$) or the percentage of outcrossed seeds (Fig. 3: slope = 0.00011, $t_{10} = 0.71$, $P = 0.49$) in border rows of commercial field refuges. Outcrossed cotton bolls from commercial fields contained an average of 11.8% outcrossed seeds (95% CI = 7.3% to 16.3%). Across all three experiments, individual bolls contained an average of 28 total seeds (95% C.I. = 27.4 to 28.5 seeds).

Seed bags as a potential source of adventitious plants

In 2006, we detected *Bt* seed in three of 11 sampled non-*Bt* cotton seed bags, each at a rate of 1% of seeds (3 seeds/1100 seeds = 0.27%). Two pairs of the seed bags resulted from identical seed lots, meaning that they were from a similar field origin. These bags did not contain *Bt* seed. Therefore, of the nine represented seed lots, three lots contained *Bt* seed at the 1% rate. Of the three contaminated seed bags, two were glyphosate-resistant cotton varieties.

DISCUSSION

In the three field studies, outcrossing in refuges did not decrease as distance from rows of *Bt* cotton increased. In the experiment comparing outcrossing of in-field *versus* external refuges, however, a lack of statistical power may have prevented us from detecting a small effect. Unexpectedly, adventitious *Bt* plants were identified in refuges in addition to outcrossed seed in non-*Bt* plants. We hypothesize that these adventitious *Bt* plants cross-pollinated with non-*Bt* plants in the refuges. This, in turn, may have exerted an overriding influence on outcrossing

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rates and patterns throughout the refuges. Our results do not necessarily contradict findings from previous studies, where outcrossing decreased as distance from *Bt* cotton increased. Rather, our results indicate that the expected outcrossing patterns may not be as marked when refuges contain adventitious *Bt* plants.

Refuge configurations and outcrossing

We had predicted that outcrossing rates in the middle of external refuges would be particularly low, resulting in lower outcrossing rates in external (wide) refuges relative to in-field (narrow) refuges. Instead, in the first experiment, low and similar outcrossing levels were observed in in-field and external refuges in the experimental fields (Fig. 1). In the second experiment, outcrossing levels in external refuge plots were similar or higher in middle rows compared to edge rows (Fig. 2).

Results from the third experiment failed to demonstrate utility of small barren zones (≤ 55 m) for limiting gene flow (Fig. 3). In a similar study, Llewellyn et al. (2007) reported that barren zones of 100 m or less were ineffective at limiting gene flow between transgenic and non-transgenic cotton fields. Furthermore, barren zones of 200–400 m have been recommended for limiting gene flow in cotton (California Crop Improvement Association, 2007; Hutmacher and Vargas, 2006; United States Environmental Protection Agency, 2006).

In experimental plots, outcrossing rates were lower in the experiment comparing in-field and external refuges (four total outcrossed bolls out of 160 tested bolls, Fig. 1) than in the experiment comparing outcrossing among rows of external refuges (15 outcrossed bolls out of 160 tested bolls, Fig. 2). This variation may be attributable to the different boll sampling procedures used. Bolls were collected at the end of the season in the experiment comparing rows in external refuges, whereas flowers were tagged during a fixed set of days in the experiment comparing in-field and external refuges. Thus, outcrossing may have been underestimated in the latter experiment if we did not tag bolls on days when the highest outcrossing occurred.

While bees are the only well-documented outcrossing agent of cotton, it is possible that cotton plants could be cross-pollinated by physical contact between flowers of adjacent plants, or through transportation of pollen on tractors. Similarly, we note that pollen could have been inadvertently transported among flowers during our field monitoring, although monitoring only occurred in the in-field *versus* external refuge study, which was also the study from which the fewest outcrossed bolls were reported (Fig. 1).

Insect pests were uncommon in bolls from the experimental field plots, but pink bollworm were occasionally identified in bolls from commercial field refuges. Because pink bollworm are highly susceptible to *Bt* cotton yet readily destroy non-*Bt* cotton seeds, the presence of these pests could have increased the relative frequency of *Bt* seed in the bolls sampled in this study.

Outcrossing and distance from *Bt* cotton

Outcrossing rates reached a maximum of 4.6% of seeds in rows of experimental plots and 1.9% of seeds in border rows of commercial fields (Figs. 1–3). These values are within the range observed in similar studies (Llewellyn and Fitt, 1996; Llewellyn et al. 2007; Umbeck et al., 1991; Zhang et al., 2005). However, the result that outcrossing did not decrease in external refuge plots as distance from the refuge edge increased (Fig. 2) is unique. Similar studies measured outcrossing in rows of non-*Bt* cotton surrounding *Bt* cotton test plots. All of these studies reported a decrease in outcrossing as the number of rows away from the *Bt* test plots increased. In Mississippi, up to 5.7% of seeds were outcrossed in non-*Bt* cotton rows adjacent to a 136 m × 30 m test plot of *Bt* cotton, and outcrossing decreased to < 1% of seeds at a distance of 7 m from the *Bt* plants (Umbeck et al., 1991). In China, rows of non-*Bt* cotton plants adjacent to a 6 m^2 plot of *Bt* cotton were outcrossed at rates up to 8.2% of seeds. Outcrossing decreased to zero at a distance of 50 m from the *Bt* plants (Zhang et al., 2005). In Australia, up to 0.9% of seeds were outcrossed in rows of non-*Bt* cotton adjacent to a block of ~ 3000 *Bt* cotton plants, with average rates declining to < 0.03% at 16 m from the *Bt* plants (Llewellyn and Fitt, 1996).

Because non-*Bt* cotton surrounded *Bt* cotton test plots in all of these studies, the non-*Bt* rows adjacent to *Bt* plots that had maximum cross-pollination were somewhat central in the field configuration (Llewellyn and Fitt, 1996; Umbeck et al., 1991; Zhang et al., 2005). Thus, they would not have been subjected to an edge effect. However, in our field plots, non-*Bt* plots were adjacent to *Bt* plots, and there were 2–4 m gaps between the plots. We noted an edge effect in our experimental plots, as edge rows contained noticeably smaller plants. If the small plants in edge rows were less attractive to pollinators than larger plants inside the field, different rates of pollination by insects could have caused the positive association between distance into external refuges and outcrossing rates (Fig. 2). After excluding data from border rows and accounting for block, no significant association occurred between distance into the field and the outcrossing rate of bolls (t_3 = 2.42, P = 0.095) or seeds (t_3 = 0.96, P = 0.41).

Most outcrossing studies conducted with transgenic crops other than cotton also showed a decline in outcrossing with increasing distance into non-transgenic fields

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Table 1. Presence of adventitious *Bt* transgenes in Arizona cotton.

Source of samples (*n*)	Total bolls or seeds tested	Bolls or seeds with *Bt* toxin (%)	Adventitious bolls hemizygous for *Bt* transgene (%)
Experimental plots (2)	320 bolls	7.8	33 (4/12)[a]
Commercial fields (12)	240 bolls	1.7	50 (2/4)
Seed bags (11)	1100 seeds	0.27[b]	NA[c]

[a] From a subsample of 12 out of 25 adventitious *Bt* plants from which individual seeds were tested.
[b] 1% in each of three bags. Percentages between 0 and 1 could not be detected for individual bags at the sample size used (100 seeds per bag).
[c] Not applicable. We could not distinguish between seeds hemizygous and homozygous for *Bt* toxin production with the qualitative ELISA test.

(*e.g.*, Chilcutt and Tabashnik, 2004; Ma et al., 2004; Pla et al., 2006; Watrud et al., 2004). However, in a survey of 63 conventional oilseed rape fields in Australia, Rieger et al. (2002) found similar outcrossing levels throughout fields of conventional oilseed rape when the conventional fields were separated from herbicide-resistant cultivars by ≤ 100 m. They proposed that "sporadic pollen movement" by insects could have contributed to the unexpected outcrossing patterns.

Origin of adventitious plants

Adventitious plants occurred in both experimental refuge plots and commercial field refuges (Tab. 1). The discovery of *Bt* seeds in non-*Bt* seed bags suggests that some of the adventitious plants resulted from contamination of the seed bag. The proportion of adventitious plants in commercial fields in 2004 was higher than the proportion of *Bt* seeds in non-*Bt* seed bags in 2006 (Tab. 1). Although these contamination rates are not directly comparable to each other because they represent different years and seed origins, this discrepancy seems to suggest that other sources, such as volunteer plants or seed mixing at planting, contributed to contamination in commercial fields. In experimental plots where planting errors could be ruled out, contamination of the seed bag was the most parsimonious explanation for the observed glyphosate-resistant *Bt* plants, because glyphosate-resistant cotton was not planted in the plots until 2004. However, our results from seed bag testing in 2006 suggest that 7–8% *Bt* contamination in seed bags is atypically high (Tab. 1). An alternative, albeit more complex, hypothesis is that adventitious glyphosate-resistant cotton plants entered plots in previous years *via* seed bag contamination or outcrossing. Because *Bt* cotton was grown in the area of plots in previous years, such an introgression could have yielded glyphosate-resistant *Bt* volunteer plants in subsequent generations. Either scenario illustrates the potential for adventitious *Bt* cotton plants in non-*Bt* fields.

Other studies have identified adventitious transgenes in the seed supply but, to our knowledge, this is the first study to detect them in cotton. Mellon and Rissler (2004) reported transgene presence in seed bags of conventional oilseed rape, corn, and soybeans ranging from less than 0.05% to greater than 1% of seeds. In Canada, Friesen et al. (2003) found adventitious herbicide-resistant oilseed rape seed in conventional oilseed rape seed lots at rates of 3–5% for one cultivar and less than 1% for nine other cultivars.

Gaines et al. (2007) identified adventitious herbicide-resistant wheat seed (non-transgenic) in bags of non-resistant wheat seed and proposed that "seed-mediated" gene flow (*i.e.*, volunteer plants or human error), rather than pollen-mediated gene flow, was responsible. This hypothesis was based on their discovery that only 7% of adventitious wheat seeds were hemizygous for the transgene, a percentage too low to suggest that outcrossing between varieties was the major source of gene flow (Gaines et al., 2007).

Similarly, we used zygosity of adventitious plants to gain insight on the source of adventitious cotton plants in this study. Production of *Bt* toxins is dominantly inherited in cotton (Heuberger et al., 2008; Sachs et al., 1998; Zhang et al., 2000). Moreover, *Bt* cotton varieties marketed in the United States are homozygous for the *Bt* gene (Adamczyk and Meredith, 2006; Jayaraman, 2005). Thus, the production of mature non-*Bt* seeds by plants that produce *Bt* in their maternal tissues demonstrates hemizygosity, indicating that outcrossing between *Bt* and non-*Bt* plants occurred in previous generations. Of the adventitious plants from which individual seeds were tested, 33% in experimental plots and 50% in commercial fields contained both *Bt* and non-*Bt* seeds (Tab. 1). Plants with both seed types had a mean of 78% *Bt* seeds (95% confidence interval, 70 to 86%), which fits the expected 3:1 ratio of *Bt* expression in self-pollinating *Bt*-hemizygous plants (Zhang et al., 2000). Therefore, many of the adventitious plants resulted from outcrossing between *Bt* and non-*Bt* cotton in previous generations.

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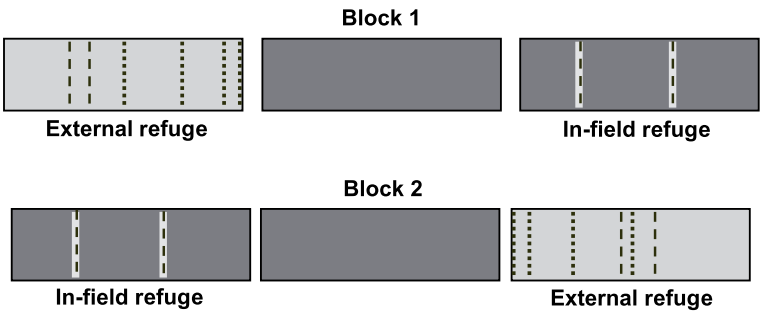

Figure 4. Experimental plots in 2004 at the University of Arizona experimental farm in Marana, Arizona. Non-*Bt* cotton rows are represented by light gray, while *Bt* cotton rows are represented by dark gray. Block 1 was NW of Block 2, and the blocks were separated by ca. 200 m in the east-west direction. Dashed lines indicate rows sampled in the first experiment, which compared outcrossing in two randomly sampled external refuge rows per plot *versus* the in-field refuge rows. Dotted lines indicate transects that were sampled in the second experiment, which compared outcrossing among various rows in external refuge plots.

Such outcrossing could have occurred between non-*Bt* cotton seed production fields and nearby *Bt* fields, between non-*Bt* and *Bt* cotton plants in the same seed production field if adventitious plants were present, or between volunteer plants and surrounding plants.

The presence of adventitious *Bt* seed in bags of non-*Bt* cotton seed could negatively impact cotton seed producers, as maintaining true varieties is a priority for the seed industry. This could also be an issue for growers of organic or otherwise non-transgenic cotton. However, we note that only one of the three contaminated seed bags identified in this study was non-transgenic, as the other contaminated bags were glyphosate-resistant cotton varieties.

Implications for pest management

Bt-outcrossed cotton seeds and adventitious *Bt* plants could decrease effective refuge size for pests such as pink bollworm that eat cotton seeds. Bolls produced by adventitious *Bt* plants in the refuges may pose a greater threat than outcrossed bolls, as they produce significantly more *Bt* seeds than outcrossed bolls.

To our knowledge, this is the first study to report the frequency of outcrossed cotton bolls in addition to total outcrossed cotton seeds. Such data are critical for understanding pest exposure to *Bt* toxin, as pests encounter one cotton boll at a time in the field. Results from computer simulations based on hypothetical data predict a rapid increase in pink bollworm resistance to *Bt* cotton when ≥ 35% of plants are adventitious *Bt* plants or when outcrossed bolls favor survival of insects with a single resistance allele (*i.e.*, heterozygotes) over homozygous susceptible insects (Heuberger et al., 2008). However, simulations incorporating empirical data from this study and from pink bollworm survival bioassays indicate that observed levels of *Bt* seed in Arizona refuges should have only minor effects on resistance evolution in pink bollworm (Heuberger et al., 2008).

MATERIALS AND METHODS

Experimental refuge plots

In April 2004, two blocks of cotton separated by approximately 200 m were planted at the University of Arizona Marana Agricultural Center (Fig. 4). Each block had the following plots: (1) 38 rows of *Bt* plus two embedded non-*Bt* rows (in-field refuge), (2) 40 rows of non-*Bt* (external refuge), and (3) 40-row *Bt* plots adjacent to the 40-row non-*Bt* plots (Fig. 4). For in-field refuge plots, non-*Bt* rows were located at rows 10 and 27 for Block 1, and 11 and 26 for Block 2 (Fig. 4). These rows were selected to allow use of a single seed hopper from the four-row planter for all non-*Bt* rows. Individual plots (each 40 m × 183 m) were separated by 4 m in Block 1 and 2 m in Block 2. Seed hoppers and delivery mechanisms of the planter were thoroughly cleaned before the onset of planting and when switching between *Bt* and non-*Bt* seed. Plots were seeded at 12.1 kg.ha^{-1}, with row spacing of 1 m. Seed bags were unopened prior to planting. Blocks were bordered by unpaved roads (ca. 20 m wide) or 2–4 m fallow spaces, with commercial *Bt* cotton on the other side of roads or fallow spaces.

We planted commonly used varieties of *Bt* and non-*Bt* cotton (specific varieties not disclosed to protect the privacy of seed producers). Both varieties were genetically engineered for tolerance to glyphosate herbicides. In previous years, only non-glyphosate-resistant cotton varieties were planted in the areas of experimental blocks. To control weeds as well as volunteer cotton from previous years' crops, Roundup WeatherMAX® (glyphosate,

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2.3 L.ha^{-1}) was applied on 19 May for Block 2 and 20 May for Block 1, followed by Roundup UltraMAX® (glyphosate, 1.6 L.ha^{-1}) on 27 May for Block 2 and 29 May for Block 1. Glyphosate can be used to control non-resistant varieties of volunteer cotton (Roberts et al., 2002). On 2 August, Cotton-Pro® (2.0 L.ha^{-1}) and AIM® (73 mL.ha^{-1}) herbicides were applied to control morning glory. A single insecticide application was applied on 21 August, when the field was aerially sprayed with Orthene® (1.1 kg.ha^{-1}) in response to an outbreak of *Lygus hesperus*. The plots were chemically defoliated before bolls were collected.

Two experiments were performed in the refuge plots: one comparing outcrossing between in-field and external refuges, and the other comparing outcrossing among rows in external refuges. In both experiments, bolls were not collected from the 10 m borders at the ends of rows to reduce edge effects.

In-field versus *external refuges*

Samples were collected from the two non-*Bt* rows of each in-field refuge plots and two randomly selected rows from each external refuge plot (avoiding the 10 border rows on each side). The random rows chosen were 11 and 14 from the external refuge plot in Block 1, and 18 and 23 from the external refuge plot in Block 2 (Fig. 4). During peak flowering, flowers in selected rows were tagged with the date, to obtain bolls representing a range of pollination dates. Because flowers were systematically sampled at flagged sites spaced ca. 4 m apart in the rows, multiple flowers may have occasionally been tagged on the same plant on different dates. Flowers were marked with paper labels fastened with string to the base of petioles (DeGrandi-Hoffman and Morales, 1989), and were handled carefully to avoid enhancing self-pollination. Tagging was performed between 20 July and 11 August, on five dates for Block 1 and seven dates for Block 2.

We collected 46.6% of tagged bolls at the end of the season on 13–15 October. The remaining 53.4% of bolls were presumed to have shed, as is typical of cotton fields (University of California, 1996). Collected bolls were freeze-dried and sorted by field row. Within rows, they were sorted by flowering date into two time periods: 20 July to 26 July and 28 July to 11 August. For each row, 10 cotton bolls from each time period were analyzed for *Bt* toxin using enzyme-linked immunosorbent assay (ELISA) as described below. Although multiple bolls may have been tagged occasionally on the same plant, the high number of bolls that shed from plants and the small number of tagged bolls that were tested with ELISA greatly reduced the probability that multiple bolls were tested from the same plant. Therefore, the number of bolls that resulted from adventitious *Bt* plants can be used to estimate the overall percentage of adventitious *Bt* plants. In all, 160 bolls containing 4276 seeds were tested.

Rows of various distances from the external refuge edge

On 23 October, end-of-season samples were collected in the external refuge plots. For each external refuge plot, bolls were collected from the border row closest to the adjacent *Bt* plot, and rows 3, 10, and 20 from the border (Fig. 4). One hundred mature bolls were sampled per row, with equal numbers from the top, middle, and lower thirds of the cotton plants to control for temporal variation in outcrossing (Llewellyn et al., 2007; Umbeck et al., 1991). In this experiment, no more than one boll was collected from any plant. Bolls were freeze-dried and 20 per row were analyzed for Cry1Ac toxin with ELISA, as described below. In all, 160 bolls containing 4811 seeds were tested.

Commercial fields

To compare outcrossing in external refuges at various distances from *Bt* fields, we sampled mature bolls from 12 external refuges of commercial cotton fields that were located throughout the cotton growing regions of Arizona. All of these refuges were adjacent to Bollgard® *Bt* cotton fields, and bolls were only collected from the refuge row nearest to the *Bt* field. The space between refuges and *Bt* fields comprised unpaved roads, irrigation ditches, and fallow land at the edge of fields. One hundred bolls were collected per field, equally representing high, middle, and low fruiting branches on the plants. In this experiment, no more than one boll was collected from any plant. We measured the distance between the sampled row and the adjacent *Bt* field for each refuge. Bolls were freeze-dried and 20 per refuge were analyzed with ELISA, as described below. In all, 240 bolls containing 6526 seeds were tested.

ELISA analyses

Cry1Ac in cotton bolls

Commercially available ELISA strips (ImmunoStrips™, Agdia, Elkhart, IN) were used to detect the *Bt* toxin Cry1Ac in cotton seeds and in the pericarp of bolls from all three field experiments. This was a qualitative ELISA analysis. ELISA is an economical method for detecting Cry1Ac in cotton and has been used for this purpose in many studies (Abel and Adamczyk, 2004; Adamczyk et al., 2001; Anklam et al., 2002; Kranthi et al., 2005; Sims and Berberich, 1996).

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From each boll, we first pooled and tested subsamples of all cotton seeds. Seeds were cut in half using wire strippers, and half of each seed was wrapped in aluminum foil and archived at room temperature. Kernels of all retained seed halves from the boll were removed from the seed coats and, as a group, were crushed with a hammer between pieces of wax paper. The resulting powder was transferred to a 25 mL scintillation vial and diluted at a 1:10 ratio with SEB4 sample extraction buffer (Agdia, Elkhart, IN), homogenized, extracted for 2 h at room temperature, and tested with an ELISA strip according to the manufacturer's protocol. For positive controls, we used composite seed pools, each containing 44 halves of non-*Bt* seeds grown in the greenhouse plus half of a *Bt* seed collected from the experimental field. Pools of 45 non-*Bt* seed halves obtained from greenhouse-grown cotton plants served as negative controls. We used 45 seeds in composite controls because sampled bolls had 45 seeds or fewer. Only seeds weighing over 10 mg provided sufficient material for ELISA, and we excluded 5.9% of seeds because they had severely underdeveloped kernels.

Outcrossed non-*Bt* plants and adventitious *Bt* plants, both of which contained seed pools with Cry1Ac, were identified in refuges. To differentiate between the two, we tested the pericarp (fruit wall) of each boll, which is composed of maternal tissue. Of bolls with Cry1Ac-positive seed pools, those with Cry1Ac-negative pericarps indicated non-*Bt* parent plants with some outcrossed seeds, while those with Cry1Ac-positive pericarps indicated adventitious *Bt* parent plants. Pericarp samples were ground with a ceramic mortar and pestle. For each boll, 50 mg of ground sample was mixed with 1 mL buffer in a 1.5 mL microcentrifuge tube, and extracted with a plastic pestle. Samples were vortex-homogenized and kept at room temperature for 4 h. Samples were tested with ImmunoStrips™ according to the manufacturer's protocol for seed and leaf testing.

The accuracy of pericarp tests for detecting Cry1Ac was demonstrated with 30 positive and 30 negative controls run alongside samples. Pericarp samples collected from experimental plots of *Bt* cotton were used as positive controls, and pericarp samples from non-*Bt* cotton grown in the greenhouse were used as negative controls. One false negative result was produced by a positive control, probably due to insufficient extraction, indicating a failure rate of 3.3%. In further evidence that we accurately distinguished between outcrossed and adventitious bolls, the two boll types segregated out by seed composition. All bolls with Cry1Ac-negative pericarps (outcrossed) yielded 50% or fewer *Bt* seeds in subsequent testing (method described below), whereas bolls with Cry1Ac-positive pericarps (adventitious) contained 70–100% *Bt* seeds (100% *Bt* seeds if homozygous, ~70–80% *Bt* seeds if hemizygous; see Discussion).

To estimate the number of outcrossed seeds in outcrossed bolls, seeds from outcrossed bolls were individually tested in sets of 10 until at least one Cry1Ac-producing seed was encountered. Seeds were tested with ImmunoStrips™ according to the manufacturer's protocol. The number of outcrossed seeds in a boll was estimated by multiplying the number of seeds in the boll by the percentage of outcrossed seeds in the subsample. Individual seed tests were similarly conducted for the bolls from adventitious *Bt* plants identified in commercial fields and in the in-field *versus* external refuges experiment. Non-*Bt* cotton seeds from the greenhouse were used as negative controls and *Bt* seeds grown in the experimental field were used as positive controls.

Glyphosate resistance in adventitious plants

Because we did not observe planting of the commercial fields that we sampled at season's end, we could not rule out on-farm mixing of *Bt* seed at planting or volunteer plants as sources of adventitious *Bt* plants in those fields. However, at the Marana Agricultural Center, this could be done based on our planting methods. Extensive precautions were taken to prevent seed mixing at planting, ruling that out as a potential source. Similarly, emergence of plants as volunteer *Bt* cotton was deemed unlikely, as volunteer cotton was targeted with two over-the-top glyphosate applications. However, to further examine this possibility, we tested maternal tissue and seeds from 10 adventitious *Bt* plants from experimental plots for the Roundup Ready® enzyme using ImmunoStrips™ for Roundup Ready (Agdia, Elkhart, IN). Because 2004 was the first year when Roundup Ready® varieties were planted in our experimental plots, the absence of the glyphosate resistance enzyme in samples would indicate that plants were re-seeded from previous years' crops.

Cry1Ac in non-Bt cotton seed bags

To examine the possibility that commercial bags of non-*Bt* cotton seed contained *Bt* transgenes, potentially resulting in the adventitious plants observed in 2004, we screened bags of non-*Bt* cotton seed for *Bt* toxin in 2006. Seed samples were taken from previously unopened bags purchased by cotton farms throughout Arizona. Seed samples from eleven bags of seed that represented nine unique seed lots were collected. Five bags (four lots) were from glyphosate-resistant cotton varieties. From each bag, 100 seeds were tested for Cry1Ac with ImmunoStrips™ using the method described previously for seed pool testing. Seeds were pooled in groups of no more than 25, and were then tested individually for Cry1Ac according to the manufacturer's protocol when pools tested positive.

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Statistical analyses

Two response variables were examined in all three experiments: percentage of outcrossed seeds and percentage of outcrossed bolls. The percentage of outcrossed seeds was estimated for each experimental unit (see below) as the percentage of Cry1Ac-producing seeds from all non-*Bt* bolls. The percentage of outcrossed bolls was calculated for each experimental unit as the percentage of all tested non-*Bt* bolls that contained at least one *Bt*-outcrossed seed. Two regression analyses were conducted per experiment, one for each response variable (JMP, Version 5, SAS Institute Inc., Cary, NC, 1989–2004).

For the experiment comparing in-field and external refuges, the two sampled rows in each plot were combined for each time period, such that plot/time period served as the experimental unit. Refuge type, time period of pollination, and field block were included as explanatory variables. For the experiment comparing outcrossing among rows of the experimental external refuge plots, rows were used as the experimental unit. Number of rows into the external refuge was an explanatory variable, as was field block. For the commercial fields, the distance between the sampled border row and the neighboring *Bt* field served as the only explanatory variable, and individual fields were the experimental unit.

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