Gene Flow between *Gossypium hirsutum* L. and *Gossypium barbadense* L. is Asymmetric

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**ABSTRACT**

As both Pima cotton (*Gossypium barbadense* L.) and Acala (Upland; *G. hirsutum* L.) are grown in the southwestern United States, understanding gene flow within and among these species is important to maintain genetic purity. In small scale and commercial fields, gene flow decreased exponentially from 1.44% at 1 m to less than 0.50% at 10 m in Acala cotton. Corresponding values for Pima cotton were 0.19% at 3 m and 0% at 10 m or beyond. When interspecies outcrossing occurred, Pima cotton was preferentially pollinated by Acala cotton. Gene flow was nondetectable in 7.5 m borders. Asymmetric pollination between Acala and Pima cotton was also observed in samples collected in borders located 6 m from the pollen source with no detectable gene flow beyond 7.5 m. At a given distance, in commercial scale Pima fields, gene flow was tenfold less than reported in Acala fields with gene flow not exceeding 0.43% beyond 10 m. No gene flow was detected beyond 800 m (0.5 mile). This study indicates that isolation distances can be shorter in Pima cotton than in Acala cotton to maintain the same level of genetic purity. Comparison with historical evidence suggests that there has been a shift in the relative gene flow among species in modern cultivars of Pima cotton.
In the United States, biotech cultivars are not separated from conventional crops once the introduced trait has been approved by government agencies for commercial release. Pollen-mediated gene flow (referred to here as gene flow) or other sources of adventitious presence (e.g., seed contamination or mechanical mixtures) can therefore pose problems for export of cottonseed into countries where the biotech trait may not be approved or deregulated. Although California grows only 6.5% of U.S. cotton, exports from California constituted 11% of U.S. cotton lint (G. hirsutum and G. barbadense) and 9% of the seed (California Department of Food and Agriculture, 2009). Cotton generally is considered to be a self-pollinating crop but is sometimes cross-pollinated, and the majority of cultivars are a mixture of closely related pure lines. Cotton flowers are visited by honeybees, bumblebees, and Melissodes bees. Studies document that provision of honeybees can increase both seed and lint yield of cotton via improved pollination, and outcrossing rates are affected by bee activity (McGregor, 1976).

Even as Upland cotton acreage declines in California and shifts toward higher value long-staple types such as Pima, the state is becoming increasingly attractive as a site for seed production. Companies marketing cotton seed in the southeastern U.S. are looking to California as a location to produce high yields of high quality seed of elite Upland varieties. Growing both Acala and Pima cotton in close proximity requires isolation standards to assure genetic purity for certified seed production for different markets (California Crop Improvement Association, 2008). Isolation distances required for certification impact the area available for seed production. Current standards in California for Pima cotton follow those based on Acala cotton with 200 m (660 ft) between varieties within each species and 400 m (1320 ft) between Acala and Pima cotton for foundation seed (California Crop Improvement Association, 2008). These distances are reduced to 6 to 30 m (20 to 100 ft) between varieties within a species and 200 m (660 ft) between species for certified seed. There is an increasing need to understand the gene flow potential and impacts on adequacy of isolation standards between these two species and among modern Pima cultivars to maintain high-quality seed and cotton production. We therefore assessed gene flow under California conditions within and between Acala and Pima cotton to define pragmatic certification standards that ensure seed quality but are not excessive, allowing maximum utilization of available cropping area for seed production.

**MATERIALS AND METHODS**

Gene flow was assessed in 14 commercial fields in the San Joaquin Valley in 2006 and 2007 and in one field experiment in 2007 at the University of California Kearney Research & Extension Center, Parlier, CA. For the purposes of this discussion, “conventional” varieties refers to commercially available cotton cultivars that do not have transgenic insect-resistance or herbicide-resistance traits, while transgenic varieties discussed within this paper will in all cases be “Roundup-Ready” (RR) varieties that incorporate resistance to the glyphosate herbicide.

The commercial scale fields sampled for this study are located in the heart of the cotton growing area in California’s San Joaquin Valley, and the surrounding area is essentially a cotton monoculture managed conventionally for insect control. Commercial conventional Pima fields neighboring glyphosate-tolerant RR Pima cotton fields were sampled at distances between 3 m and 1625 m (1 mile) from the herbicide-resistant pollen source. At maturity, cotton bolls were harvested from lower, middle, and upper locations on plants. Samples were collected at 3, 30, 200, 400, 800, and 1625 m away from the pollen source field. In three fields, samples were taken at the nearest edge of the conventional field (90–170 m). To increase relative sample size, data for these samples were averaged and plotted as a single data point at 140 m. The nearest RR cotton (other than the intended pollen source) was greater than 1.5 km away for all fields. Samples were also taken from the source fields as positive controls. Three fields were sampled in 2006 and 11 in 2007. Due to certified seed isolation requirements (minimum 200 m between species), no commercial fields were identified as suitable for assessing gene flow between Pima and Acala types.

To measure gene flow between species, an experiment was designed and conducted at the Kearney Research and Extension Center to measure all combinations of gene flow between Pima and Acala cotton up to a distance of 60 m. The Kearney Center is a mixed cropping area including bee pollinated crops such as orchards, melon, and alfalfa seed. Honeybees will forage the food source nearest their hive with the maximum reward and avoid collecting pollen or nectar from sources where competition from different colonies is high (Gary et al., 1972; Visscher and Seeley, 1982). Bee hives were placed adjacent to cotton test plots to maximize the potential for outcrossing. The Kearney Center therefore represents a worst case environment to investigate insect-mediated pollen flow, such as might occur with a cotton field adjacent to a bee-pollinated crop in California.

A block of RR Pima cotton (Phytoen-805RF, plot size 150 m²) and RR Acala cotton (Phytoen 725RF, 150 m²) were hand planted to prevent cross contamination. Adjacent strips (10 × 60 m) of herbicide-susceptible Pima (‘Pima 800’) and Acala (‘Phytoen 72’) were planted to the north and the south of the herbicide-resistant blocks. To test the effect of borders, a 15 m-wide strip of herbicide-susceptible Pima was planted to the east and a strip of herbicide-susceptible Acala was planted to the west. These border strips were 6 m of open space away from the pollen source (Fig. 1). At Kearney, preplant herbicides Treflan (Dow AgroSciences LLC, Indianapolis, IN) (trifluralin) and Dual (Syngenta Crop Protection, Inc., Greensboro, NC) (metolachlor) were applied for weed control with no insecticides applied throughout the growing season. Roundup Weathermax (Monsanto Co., St. Louis, MO) (glyphosate, rate 1.63 L ha⁻¹) was applied to the herbicide-tolerant plots to ensure purity of stands. Four bee hives were placed at a single corner of the experiment at the beginning of the flowering and pollination period to ensure a high level of pollinators to simulate a worst case scenario for gene flow. Samples were taken at 1, 3, 10, 30, and 60 m from the pollen source to the north and south and at the closest edge (6 m), middle (8 m), and far edge (15 m)
Winnipeg, MB, Canada). Regression analysis was performed on the sample data and best fit curve determined.

RESULTS

Gene Flow Among Commercial Pima Fields

Samples were taken from three fields in 2006 and 11 fields in 2007. Leaf samples from up to 10 surviving plants were assayed for the EPSPS protein using immune test strips. All samples tested positive, indicating no false positives escaped from the herbicide bioassays. Analysis of variance indicated no significant differences \( (p = 0.05) \) among environments (data not shown). From 22,000 to 32,000 seedlings were assayed for each distance except at 3 m where 14,643 and at 90 to 170 m where 2000 to 2,400 seedlings were assayed. Lower numbers of seedlings were sampled for these distances as they represent the shortest distance between neighboring fields. These distances also represent non-crop space between the pollen source and sampling area, whereas distances from 200 m and higher represent within-crop gene flow. The highest levels of gene flow (1.06% in a single field) was detected at 3 m in commercial fields and decreased with distance to nondetectable levels at 200, 400, and 1625 m. At 800 m, 0.04% (1 out of 2,250 plants) was detected at a single location (Fig. 3a and 3b, Table 1). When averaged across environments gene flow ranged from 0.23% at 3 m to nondetectable beyond 170 m, except a single seed from 26,850 plants (0.004%) at 800 m. The gene flow data for each evaluation plot was averaged over replicates and regressed over distance using the inverse function with a best fit line of \( y = 0.8287x + 0.0024 \) and \( R^2 = 0.26 \) (Fig. 4). A hyperbolic curve was derived from the above equation by dividing the slope (0.83) by each distance and displayed.
Gene Flow Between Species

Good stand establishment and synchronous flowering among the four varieties were achieved at the Kearney field experiment. Ample bee activity was observed during flowering. Two-factor (direction and distance) ANOVA for gene flow revealed no effect of direction on gene flow in our experiment and that gene flow was significantly different among distances for both Pima and Acala (data not shown). Therefore, data from both directions were combined at each distance with 3470 to 4600 seedlings being assayed per sample. After two herbicide applications, surviving plants were allowed to flower and classified as being pollinated by Acala or Pima. Data sets were split into four categories, Pima–Pima, Pima–Acala, Acala–Pima, and Acala–Acala, based on results of flowers of survivors (spotted, faint spot, and no spot; Fig. 2) and analyzed separately. Survivors from Acala cotton with Acala flowers were classified as being pollinated by Acala and those with hybrid flowers were pollinated by Pima. Similarly, Pima survivors with Pima flowers were pollinated from Pima and Pima samples with hybrid flowers were pollinated from Acala. There were no Acala survivors with Pima flowers or Pima survivors with Acala flowers, indicating high purity of the original seed lots.

Total gene flow decreased from 1.48% at 1 m to less than 0.50% at 10 m with no detectable gene flow at 60 m in Acala cotton (Fig. 5). Except for 0.04 and 0.03% at 1 and 3 m,
respectively, all Acala survivors were pollinated by Acala cotton. Pima cotton had a total gene flow of 0.44% at 1 m and 0.25% at 3 m with no gene flow detected from 10 to 60 m (Fig. 5). In contrast to the Acala results, Pima cotton had a high degree of pollination from Acala cotton, resulting in hybrids.

Gene flow data were collected from Acala and Pima borders situated 6 m from the pollen sources with open space between the border and the pollen source. Total gene flow in borders was similar for Acala and Pima cotton with 0.25–0.27% at the edge of the border, approximately one half that at 7.5 m, and nondetectable at 15 m into the border (Fig. 2). Except for 0.05% of seedlings in the Pima sampled at the edge of the border, all pollinations were from Acala cotton, resulting in the majority of Pima survivors producing hybrid flowers (Fig. 2 and 6, Supplementary Table S2). Leaf samples from up to 10 surviving plants were assayed for the EPSPS protein using immune test strips. All of the samples tested positive, indicating no false positives from the herbicide bioassays.

**DISCUSSION**

**Gene Flow within Pima**

Although pollen-mediated gene flow is well characterized in Upland cotton (Llewellyn et al., 2007; Van Deynze et al., 2005), to our knowledge there are no published reports of gene flow within Pima cotton, perhaps due to the lack of good genetic markers. The availability of herbicide tolerance as a marker allows large numbers of progeny to be conveniently tested for outcrossing and gene flow. In the present study, gene flow in Pima decreased exponentially with increasing distance from the pollen source with the highest detected levels at 3 m being 0.19% in a small scale experiment (Fig. 5, Supplementary Table S1). This value is confounded as Acala pollen was present as well to compete with Pima pollinations onto Pima. As honeybees were provided to the small scale field trial, these values may overestimate gene flow in commercial fields where pollinators are not as abundant (Van Deynze et al., 2005).
fields, gene flow did not exceed 0.04% at 30 m and reached nondetectable levels at 200 m. For the field experiments, the detection sensitivity was 1 in 26,000 seeds sampled per distance (0.004%) or 0.012% with 95% confidence.

Border rows have been used to mitigate gene flow in transgenic studies but also to maintain genetic purity on a field scale. The data in this study indicate that borders of 15 m can effectively mitigate gene flow to nondetectable levels (sensitivity of 0.15% with 95% confidence) in both Pima and Acala cotton. This is consistent with studies in Australia in which 20-m buffer zones of Upland cotton fields were sampled in 2 yr, and only one field out of twelve had detectable (0.04%) gene flow (Llewellyn et al., 2007). As the majority of gene flow detected in Pima border rows was from Acala, gene flow within Pima may be even more effectively controlled by borders.

Outcrossing rates reported for Upland (including Acala) cotton vary depending on the location but remain relatively low even at short distances from neighboring fields in commercial settings. Generally, gene flow remains below 1% at distances beyond 10 m but can be detected at very low levels (<0.05%) at distances up to 1625 m (1 mile) (Llewellyn et al., 2007; Van Deynze et al., 2005). On a field scale, gene flow fell to nondetectable levels at 55 m in Arizona and to 0.25% at 3.7 m in Mississippi (Berkey et al., 2003). Similarly, studies summarizing data from Arizona, Arkansas, Mississippi, and North Carolina (>15,000 samples) showed that gene flow decreased exponentially with increasing distance from the pollen source and was below 1% beyond 10 m at all locations, although it was detected at 20 m (Kareiva et al., 1994).

In the present study and a similar study on Acala cotton in California (Van Deynze et al., 2005), gene flow was 10-fold greater in Acala than in Pima cotton (compare Fig. 3 and Fig. 1 from Van Deynze et al. [2005]). The actual cross-pollination in cotton is usually underestimated, as the majority of cross-pollinations are from plants of the same cultivar and cultivars tend to be mostly homozygous, thus not detectable using genetic markers. In a controlled study, Kearney (1923) interplanted Pima and Acala cottons and allowed only a single flower at similar height to open at a time. By collecting seed from only the Pima and Acala flowers facing each other and counting hybrids, he estimated that the real level of outcrossing was lower in Pima (12%) than in Acala (28%), which is consistent with our findings.

Studies on behavior of honeybees indicate that they forage in small areas, sequentially visiting nearby flowers with the same reward for pollen and nectar, especially when pollen and nectar are abundant. Furthermore, honeybees will work a specific crop and even specific varieties if they differ (Free, 1993). Although studies between male-sterile and male-fertile rows indicate that honeybees may preferentially visit flowers within a row vs. among rows (Lederhouse et al., 1972), there is no indication that this is the case for cotton (Free, 1993). In a study on Acala cotton, there was no detectable difference in gene flow in four directions when samples were taken at 1 to 30 m from a pollen source (Van Deynze et al., 2005). Consequently, the row direction in the current research, (sampling points were perpendicular to rows) should have not biased results.

Relative size of source and sink can affect gene flow. In our studies, pollen source was often (but not always) much smaller than the sink. Nine of 14 of the commercial sites had a 2 to 3 ha source vs. 130 ha sink whereas the rest were of similar source and sink sizes (100–250 ha). Gene flow should be proportional to the size of the relative pollen load if everything else were equal. A large sink:source ratio may reduce gene flow in borders and commercial fields (Brubaker et al., 1993; Llewellyn et al., 2007).

Figure 6. Gene flow in borders within and among cotton species. Border was separated by 6 m (20 ft) of open space from the pollen source. Zero represents the closest edge to the pollen source, and other distances are measured from that point. Hybrid refers to interspecific gene flow.
Acala and Pima Interactions

As both Acala and Pima cottons are grown in areas such as Arizona and California, it is also important to understand their potential for intercrossing. Our data indicate that Acala cotton is a more effective pollen source for both Acala and Pima cottons (Fig. 4 and 5). This may be a result of pollinator preferences, flower structure, synchrony, and pollen viability and quantity. As the four lines selected for these studies had synchronous flowering, cotton has indeterminate flowering and we collected seed samples from bottom to top of plants in two samplings; asynchronous flowering can be ruled out as a source of variation. Even in the second sampling late in the season (7 Nov. 2007) both Acala and Pima flowers were present. Cotton flowers develop along fruiting branches that extend out from one or more main stems, with flowering progressing sequentially from the bottom to the top of the plant and out the fruiting branches. The cream colored (Acala cotton) or pale yellow flowers (Pima) open in the morning shortly after dawn, turn pink in the afternoon, and close at night, never to reopen. The stigma is receptive only until early afternoon. The flowers are self-fertile and pollen grains are large and coated with a viscous material that causes them to adhere to each other and prevents them from being carried by wind (McGregor, 1976). Pima flowers have a deeper and more closed corolla compared to Upland cottons, which would further limit any movement of pollen by wind (Fig. 2). There is a difference in pollen release and flower opening between species. *Gossypium barbadense* pollen is released early just as flowers are opening, whereas Upland pollen is not available to pollinators until the flower is much more open. Although the stigmatic surface is receptive along the style in both species, foreign pollen is accessible to only the extrastaminal surfaces of the stigma in Pima due to the tight whorl of stamens. The staminal arrangement in Acala allows access to the whole stigma (Kearney, 1923). Contrary to our results, this suggests that Pima pollen would be available sooner than Upland pollen and could preferentially pollinate Upland cotton, as found in studies of Upland and wild *G. barbadense* populations (Brubaker et al., 1993) and early Pima cultivars (Kearney, 1923). Kearney concluded that the earlier relative availability of pollen from Pima and the longer, later flowering in Pima relative to Acala were the primary reasons for higher outcrossing from Pima to Acala rather than the reverse.

In a comprehensive study of gene flow between *G. barbadense* and *G. hirsutum*, it was found that gene flow, as well as introgression of nuclear and cytoplasmic genomes, was asymmetric and that it depended on the genetic background and coexistence of the populations (Brubaker et al., 1993). They reported that although introgression of cytoplasmic and nuclear genes is bidirectional, introgression of *G. hirsutum* alleles into *G. barbadense* occurred only in accessions or improved cultivars that have had directed crossing through breeding, namely Pima cotton. Introgression of *G. barbadense* alleles was relatively common in *G. hirsutum* wild accessions in areas of historical sympathy but is rare in modern cultivars. Furthermore, Kearney (1923) reported that although there was no selective fertilization by pollen within and among flowers or cultivars within Pima cotton, Acala pollen had a selective advantage over Pima pollen to fertilize Pima. He also reported that although Pima pollen is available earlier than Acala in the day, pollen viability in both species is low in the early morning and peaks in the midday. Our results using modern cultivars showed that gene flow preferentially occurs from Upland to Pima cotton. The above evidence suggests that the difference between previous studies and ours may due to a shift in the genome of modern Pima that have an increasing amount of Upland (*Acala*) DNA. Modern varieties of Pima are routinely crossed with Upland to introgress desirable traits. This is particularly true in the current study as the herbicide resistance gene in Pima was introgressed from Upland varieties through breeding, introducing additional Upland DNA. We propose that the merging of Pima and Upland germplasm, the selective advantage of Acala pollen to fertilize Pima flowers, and specifically the synchronous flowering of the varieties in our study has shifted the relative introgression of Acala and Pima alleles. Although beyond the scope of this paper, molecular-genetic analyses using DNA markers between species may identify specific genomic areas of introgression and recombination associated with interspecific crosses.

Implications of Gene Flow in Cotton

The impact of gene flow must be considered to develop management strategies to maintain genetic purity. One must consider the impact based on fiber quality, lint yield, resistance to abiotic and biotic stresses, and economics. Although current standards to maintain genetic purity vary, groups such as the Association of Official Seed Certifying Agencies (Association of Official Seed Certifying Agencies, 2008) and Organization of Economic Cooperation and Development (Organization of Economic Cooperation and Development, 2010) maintain standards that must be met during seed production for sale as pedigreed seed. These isolation standards are based on decades of experience and have been adequate to meet seed market needs for the cotton industry. Nonetheless, improved accuracy of gene flow data can guide producers to meet lower or higher standards. Our data suggest that isolation distances can be shorter in Pima cotton than in Acala cotton to maintain the same level of genetic purity. On a final note, pollen-mediated gene flow is not the only factor to consider to maintain genetic purity. Seed admixtures can be a large source of gene flow. In studies of several crops, Chapman and Burke (2006) concluded that it is natural selection of favorable
alleles, not pollen-mediated gene flow, that governs the spread of genes in natural or wild populations. This would be especially true for mostly self-pollinated crops with low pollen-mediated gene flow such as cotton.

CONCLUSIONS

We have measured pollen-mediated gene flow within Pima cotton and between it and Acala cotton. Pollen mediated gene flow decreases exponentially with distance. In commercial fields, at a given distance, it is 5- to 10-fold less in Pima cotton than in Acala. The rate of gene flow among Acala and Pima cotton fields is asymmetric. At a given distance, Acala cotton will pollinate Pima more often than Pima pollinates Acala. These differences have implications in seed production practices required to produce seed with high genetic purity. Comparison with historical evidence suggests that there has been a shift in the relative gene flow among species in modern cultivars of Pima cotton.

Supplemental Information Available

Supplemental information associated with this manuscript is available free of charge online at http://www.crops.org/publications/cs.

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