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Outcrossing Potential between U.S. Blackhull Red Rice and *Indica* Rice Cultivars

David R. Gealy, Nilda R. Burgos, Kathleen M. Yeater, and Aaron K. Jackson*

Weedy red rice is a major weed pest of rice in the southern United States. Outcrossing between red rice and commercial *tropical japonica* rice cultivars has resulted in new weed biotypes that further hinder the effectiveness of weed management. In recent years, *indica* rice has been used increasingly as a germplasm source for breeding and for reduced-input systems in the United States, but little is known about its outcrossing potential with U.S. weedy red rice biotypes. In a 2-yr study, simple sequence repeat marker analysis was used to show that blackhull (BH) red rice (PI 653424) outcrossing to four, late-maturing *indica* cultivars averaged 0.0086% and ranged from 0.002% for 'TeQing' to 0.0173% for '4484' (PI 615022). Rates of outcrossing to a *tropical japonica* cultivar standard, 'Kaybonnet' (0.032%), were substantially greater than for the *indica* cultivars. These differences in outcrossing were due largely to synchronization of flowering times between rice and red rice, with Kaybonnet and TeQing exhibiting the greatest and least synchronization, respectively. Outcrossing rates also may have been affected by rice–red rice flower density differences within the rice plots. Outcrossing from cultivated rice to the red rice (as pollen recipient), which was taller than all rice cultivars, was undetectable in these studies, and environmental conditions (e.g., temperature, humidity, solar radiation, and rainfall) were not strongly correlated with the outcrossing rates observed. Grain yields of the original BH red rice line were greatest in the Kaybonnet plots, demonstrating that the *indica* cultivars were superior competitors against this weed. Collectively, these results suggest that red rice biotypes that flower synchronously with rice cultivars are a potential source of pollen for outcrossing and gene flow in rice fields in the southern United States.

Nomenclature: Red rice, *Oryza sativa* L.; rice, *Oryza sativa* L.; *indica* rice cultivars 'TeQing', '4484'; *tropical japonica* rice cultivar 'Kaybonnet'.

Key words: Outcrossing, gene flow, crop–weed hybridization.

Weedy red rice presents a major obstacle to rice production in the southern United States because of its aggressive competition with rice growth, contamination of harvested rice grain with its unwanted red-colored pericarp, persistence from shattering and dormancy, and natural outcrossing with cultivated rice (Delouche et al. 2007). The U.S. red rice populations are genetically diverse and can be divided phenotypically and evolutionarily into two major groups: strawhull (SH; awnless) and blackhull (BH; awned) (Gealy et al. 2009, 2012; Londo and Schaal 2007; Reagon et al. 2010; Shivrain et al. 2010a).

For more than a decade, southern U.S. farmers have used herbicide-resistant (HR) rice production systems, in which true-breeding *tropical japonica* cultivars or *indica*/*japonica* rice hybrids are grown to improve control of grass weeds (Burgos et al. 2008; Norsworthy et al. 2013). Presently, an estimated 33 to 70% of the rice acreage in the southern United States is planted with imazethapyr-resistant cultivars (Ziska et al. 2015). However, red rice has remained a widespread and challenging problem in rice fields in spite of the effectiveness of these systems, and outcrossing between red rice and HR rice can occur when prescribed herbicides fail to kill red rice, prevent its flowering, or both (Burgos et al. 2008, 2014; Shivrain et al. 2007, 2009a,b,c; Zhang et al. 2003, 2006; Ziska et al. 2015).

Southern U.S. rice cropping systems and breeding programs have traditionally produced *tropical japonica* long grain and medium grain cultivars, but *indica* rice, which is better adapted to tropical environmental conditions, has been used increasingly as a source for high yield, pest tolerance, and weed suppression in U.S. rice germplasm improvement and breeding programs and in low-input production

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* First and fourth authors: Research Plant Physiologist and Geneticist, Dale Bumpers National Rice Research Center, U.S. Department of Agriculture, Agricultural Research Service, 2890 Hwy. 130 East, Stuttgart, AR 72160; second author: Associate Professor, Department of Plant, Soil, and Environmental Sciences, University of Arkansas, Fayetteville, AR 72701; third author: Statistician, U.S. Department of Agriculture, Agricultural Research Service, Southern Plains Area, College Station, TX 77840. Current address of third author: U.S. Department of Agriculture, Agricultural Research Service, Plains Area, Fort Collins, CO 80526. Corresponding author's E-mail: david.gealy@ars.usda.gov

systems (Gealy et al. 2013a,b; Marchetti et al. 1998; Yan and McClung 2010). *Indica* rice and U.S. red rice also appear to be better adapted to projected changes in global climate (Ziska and McClung 2008; Ziska et al. 2010, 2012). *Indica* cultivars such as 'Minghui-63' (MG-63), which are commonly used as parents in hybrid rice systems because of their strong outcrossing and hybridization potential, have been investigated in gene flow studies with weedy and wild rice in Asia (Chen et al. 2004; Rong et al. 2007, 2012). Cultivated *indica* rice has been closely linked evolutionarily with U.S. weedy red rice biotypes (Londo and Schaal 2007; Reagon et al. 2010), which may improve the potential for outcrossing and gene flow between these groups.

Outcrossing between the red rice biotypes and the *tropical japonica* rice cultivars that are commonly produced in the southern United States has been well documented (Shivrain et al. 2007, 2009a,c; Zhang et al. 2006). In genetic marker analysis of recently discovered imazethapyr-resistant HR red rice populations in Arkansas, BH red rice biotypes were shown to be the source of weedy introgressions in 42 of 48 HR accessions tested (Burgos et al. 2014), suggesting that the BH types may be more prone to outcrossing and gene flow in the United States than are the SH types.

Although *indica* rice germplasm has been increasingly used in the United States, its outcrossing and gene flow dynamics with weedy red rice have not been investigated in southern U.S. agro-ecosystems. The objectives of this study were to determine the outcrossing potential and competitive interaction between a BH red rice biotype and four, high-yielding *indica* cultivars and to discern potential environmental influences on the outcrossing process.

Materials and Methods

Outcrossing Plots. A single red rice biotype (TX4, awned blackhull type; PI 653424) and five rice cultivars were selected based on previous field trials in which this red rice biotype and the five rice cultivars exhibited significant overlap in flowering dates (Gealy et al. 2006; Gealy, unpublished data). TX4 flowers relatively earlier than most Arkansas BH biotypes, and all of the awnless SH types that were considered for this study typically complete flowering much earlier than any of the *indica* cultivars studied.

Indica rice cultivars were 'TeQing', 'Rondo', '4484', and '4593' (PI 536047, PI 657830, PI 615022, and PI 615031, respectively). Rondo was genetically mutated to be shorter in height than its parent, 4484 (Yan and McClung 2010). 'Kaybonnet' was included as a *tropical japonica* commercial standard known to flower synchronously and be cross-fertile with TX4 (Gealy et al. 2006). Outcrossing plots, consisting of a single row of TX4 red rice plants flanked by four rows of a rice cultivar on each side, in plots 5.2 m long with 17.8-cm-wide rows (total plot width of 1.6 m), were established at Stuttgart, AR, on April 30, 2008, and April 29, 2009. The experimental design was a randomized complete block with four replications. All rice cultivars were drill-seeded at a rate of 104 kg ha^{-1}. Thirty-seven seeds of TX4 red rice were hand-planted at equidistant spacing into an empty furrow (middle row, row 5) created by the rice drill. This produced ≥ 25 red rice plants that were potentially available for outcrossing with the rice cultivars. To minimize the potential for cross-pollination between the rice or red rice plants growing in adjacent plots or in different irrigation bays (blocks), each plot was isolated from neighboring plots by an *Oryza*-free border of ≥ 3 m on the north and south and ≥ 5 m on the east and west. Volunteer rice and red rice plants growing in or near plots were hand-removed before the onset of heading.

Nitrogen fertilizer was applied at 56 kg ha^{-1} as urea immediately before application of the permanent flood. Clomazone plus quinclorac, propanil plus halosulfuron [Permit, Gowan Company, Yuma, AZ, http://www.gowanco.com/], and bensulfuron were applied POST at recommended rates to control unwanted weeds.

The following rice and red rice plant data were recorded for each plot. Rice emergence date (date of 50% rice seedling emergence; the emergence date was the same for rice and red rice); rice plant density (average number from five and six 0.5-m sections of row in 2008 and 2009, respectively [no./m of row]; seedlings were counted at about the three-leaf stage before permanent flood); red rice plant density (total number in row 5).

At the beginning of the flowering period, the percentage of red rice and rice plants that had flowered within each plot were determined every 2 to 3 d on the basis of visual estimates (Gealy et al. 2013a). From these estimates we obtained the following data from direct observation or extrapolation. Rice heading initiation (HDi; date of first complete panicle emergence in plot); date that rice plants reached 25, 50, 75, and 100% heading (HD25, HD50, HD75, and HD100, respectively; these dates were extrapolated from graphs of our heading estimates); date that red rice plants reached 25, 50, 75, and 100% heading (RHD25, RHD50, RHD75, and RHD100, respectively; these dates were extrapolated from graphs of our visual heading estimates); rice and red rice maximum plant height (distance to tip of panicle [cm]; average of five plants); rice panicle density (average number of panicles in 1-m-long sections from rows 2 and 8); red rice panicle density (average number of panicles in two, 1-m-long sections of row).

At grain filling, all red rice panicles in each plot were enclosed in bags (46 cm wide by 91 cm long; apertured, breathable, polyolefin fabric; Delstar Technologies Inc., Middletown, DE) to capture all of the seed that shattered from the panicles. Bags were secured to the stalks using plastic cable ties. At maturity, the rice in rows 4 and 6 (rice$_{4,6}$) was harvested and composited. The rice in rows 2 and 8 (rice$_{2,8}$) was composited separately so that the respective yields and outcrossing rates with red rice for rice$_{2,8}$ and rice$_{4,6}$ could be evaluated independently. The grain yield of rice in rows 2 and 8 (yield$_{2,8}$) was considered to be independent of the influence of the red rice in row 5 and thus represented the "weed-free" yield for these rice cultivars under the conditions of the test. The yield of rice in rows 4 and 6 (yield$_{4,6}$) was affected by the red rice present in row 5. The stalks of rice$_{2,8}$, rice$_{4,6}$, and red rice from each plot were bulked separately, and their panicles were threshed using a stationary "Vogel-type" thresher. Grain yields (kg ha^{-1}; adjusted to 12% moisture) were determined as described in Gealy et al. (2013b). Rice and red rice grain was stored at 4 C and 20% relative humidity until it was planted in observation plots the following year.

Environmental data at the field site were recorded from planting to harvest using weather data loggers (University of Arkansas East Station; 34.464°N, 91.401°W). These data can be accessed online (http://www.ars.usda.gov/Main/Docs.htm?docid=23563), along with daily measurements of air temperature (minimum, maximum, average) and relative humidity (minimum, maximum, average), total rainfall (inches), total solar radiation (megajoules, MJ), and average wind speed (miles per hour, mph). Calendar dates were converted to Julian days for all manipulations of environmental data. The following variables were calculated from these environmental data: growing degree day heat units using 10 C as the base temperature were calculated as described by Shivrain et al. (2010b). For relative humidity (RH), a cumulative average for each daily high and low was calculated. The mean of those two daily averages was used as an overall daily relative humidity value, which was used to investigate potential trends in the weather patterns. The daily average wind speed was collected by the weather station, and a cumulative average wind speed (WS) was calculated for each day and used to investigate potential trends in the weather patterns. The daily accumulation of rainfall (Rain) and solar radiation (Solar) was recorded.

Observation Plots and DNA Marker Analysis. Seed collected in the 2008 and 2009 outcrossing plots from rice$_{2,8}$, rice$_{4,6,}$ and red rice were subsequently subsampled and planted in field observation/screening plots in April 27, 2009, and April 20, 2010, respectively. Plots were nine rows wide and 12 m long. Crop management was similar to that in the outcrossing plots, except that nitrogen fertilizer was applied at 34 kg ha^{-1}. Before N fertilizer application, the number of seedlings in 6 and 12 randomly selected 0.5-m lengths of row was recorded from the observation plots in 2009 and 2010, respectively. In 2009, an average of 4,000 to 4,500 seedlings per replicate was present in observation plots. In 2010, there were 3,100 to 3,300 seedlings per replicate.

As plants in the observation plots approached the flowering stage, plots were inspected periodically to identify putative hybrids, which are characteristically taller, more robust, and have longer flag leaves than their parents (Shivrain et al. 2007). Thus, plots that had been sown with rice and red rice seed were inspected for plants matching these expected hybrid phenotypes and marked for subsequent leaf sampling. Green leaf tissue was collected from these plants as well as from parental rice and red rice plants growing in the outcrossing plots nearby. DNA was extracted using the method described by Bryant et al. (2011). Genotypic analysis was performed using five molecular markers. These were the InDel, Rid 12 (a specific marker for red pericarp; Sweeny et al. 2006) and the rice simple sequence repeat primer pairs, RM234, RM253, RM232, and RM5, which are known to be polymorphic between red rice and selected cultivars (Gealy et al. 2012). Polymerase chain reaction run conditions and separation of amplified products by capillary electrophoresis (ABI Prism 3730 DNA Analyzer) were as described by Bryant et al. (2011). Putative hybrids were identified on the basis of their phenotypes at heading. Those that were found to be heterozygous for the five selected marker loci and

Table 1. Outcrossing (OC) rates between red rice and rice in a 2-yr study, with red rice serving as the pollen donor.[a–c]

Rice cultivar	Outcrossing rate		
	$\text{Rows}_{2,8}$ ($OC_{2,8}$)	$\text{Rows}_{4,6}$ ($OC_{4,6}$)	Average $\text{rows}_{2,8}$ and $\text{rows}_{4,6}$ (OC)
	% × 10^{-4}		
4484	181 ab	170 ab	173 b
Rondo (1693)	73 ab	138 ab	103 bc
4593	98 ab	0 b	48 bc
Kaybonnet	354 a	298 a	323 a
TeQing	0 b	44 ab	20 c
	$P = 0.017$	$P = 0.057$	$P = 0.0016$

[a] Plants were grown in the field in a standard drill-seeded, flooded rice production system at Stuttgart, AR. Outcrossing plots were established in 2008 and 2009, and the hybrid phenotypes produced in these plots were subsequently identified in observation plots grown in 2009 and 2010, respectively.

[b] Values in table are arithmetic means over 2 yr. Means within columns followed by the same letter are not significantly different according to Tukey's honest significant difference test at $P \leq 0.05$. Note that any means separation data or contrasts shown in this table were generated for least square means from the statistical model; not these arithmetic means. The following single degree of freedom contrast was also significant. OC: (Rondo, 4484) > (4593, TeQing), $P = 0.0044$.

[c] Outcrossing values refer only to pollen transfer from red rice to rice (red rice serving as the pollen donor). Outcrossing resulting from pollen transfer from rice to red rice was not detected in any plot.

consistent with the alleles found in the designated rice and red rice parents were considered to be true hybrids. Outcrossing rates for $\text{rice}_{2,8}$ and $\text{rice}_{4,6}$, designated as $OC_{2,8}$ and $OC_{4,6}$, respectively, were calculated relative to the total number of plant seedlings emerged in the respective observation plots. Outcrossing rate was defined as 100 × (no. of true F_1 hybrids in the observation plot)/(no. of seedlings in observation plot). The average rice outcrossing rate per plot (OC) was the weighted average of $OC_{2,8}$ and $OC_{4,6}$.

Statistical Analyses. Statistical analyses were performed using PROC GLIMMIX in SAS 9.4 (SAS/STAT 13.1) and JMP 11 (SAS Institute). In all statistical models, year and block effect were considered random, and cultivar was a fixed effect. The LSMEANS option was used for means separation, with the Tukey–Kramer P value adjustment for all multiple comparisons among the cultivars. Also, single degree of freedom orthogonal contrasts were performed, where appropriate, between selected cultivars or cultivar groups within linear model fitting in SAS JMP 11. Pearson pairwise correlations among environmental, agronomic, and outcrossing variables were performed using the Multivariate procedure in JMP.

Results and Discussion

Outcrossing was expressed as the percentage of true hybrid plants relative to the number of seedlings emerged in each growout plot. The $OC_{2,8}$ and $OC_{4,6}$ values were statistically similar for each cultivar; thus, the average OC are presented for the remainder of the manuscript (Table 1).

OC in rice and red rice plants typically decreases rapidly with separation distance. For instance, OC fell from 0.08% at 0.2 m to undetectable at 2.4 m in Italy (Messeguer et al. 2001). In Spain, OC fell from ~ 0.1% at ≤ 1 m, to 0.01% at 5 m and undetectable at > 10 m (Messeguer et al. 2001, 2004; Shivrain et al. 2007). Similar decreases in OC have been observed between insect-resistant (IR) transgenic rice and nonresistant rice, decreasing from 0.28% at 0.2 m to 0.01% at 6.2 m (Rong et al. 2007). This trend was not apparent in our $OC_{2,8}$ vs. $OC_{4,6}$ data, apparently because the maximum distance between the red rice row and the rice analyzed in this test ($\text{rice}_{2,8}$) was only ~ 0.53 m.

With rice serving as the female (pollen recipient), OC was greatest for Kaybonnet (0.032%) followed by 4484, Rondo, 4593, and TeQing (0.0020%) (Table 1). OC of all *indica* cultivars averaged 0.0086%. Overall, the OC rates between red rice and the *indica* rice cultivars used in this study were in the lower range of those reported between red rice and *tropical japonica* cultivars in this region (Gealy 2005; Magalhaes et al. 2001; Shivrain et al. 2007, 2009a,c; Zhang et al. 2003, 2006), but maximum OC rates ranging from 0 (undetectable) to > 3% have been reported from field tests conducted under a broad range of environments and experimental layouts (Delouche et al. 2007; Gealy 2005). In a 2-yr study, Zhang et at. (2006) found that SH and BH red rice in 24 field locations in Louisiana outcrossed with both true-breeding

Table 2. Relationship between rice and red rice heading dates in a 2-yr study of red rice outcrossing.[a,b]

Rice cultivar	Rice–red rice difference in time span from emergence to 50% heading: Degree days (C)	Rice–red rice difference in time span from emergence to 50% heading: d	Ratio of (rice and red rice heading overlap) to (rice heading span) (d/d, decimal %)
4484	70.6 a	4.6 a	0.79 a
Rondo (1693)	89.4 a	5.6 a	0.63 ab
4593	102.2 a	6.2 a	0.58 ab
Kaybonnet	−22.6 b	−1.0 b	0.88 a
TeQing	107.2 a	6.9 a	0.33 b

[a] Plants were grown in the field in a standard drill-seeded, flooded rice production system at Stuttgart, AR. Outcrossing plots were established in 2008 and 2009, and the hybrid phenotypes produced in these plots were subsequently identified in observation plots grown in 2009 and 2010, respectively.

[b] Values in table are least square means over 2 yr. Means within columns followed by the same letter are not significantly different according to Tukey's honest significant difference test at $P \leq 0.05$. The following are selected or additional significant single degree of freedom contrasts. Rice–red rice difference in growing degree days from emergence to 50% heading: (TeQing, 4593) > (4484, Rondo), $P = 0.0118$. Rice–red rice difference in days from emergence to 50% heading: (TeQing, 4593) > (4484, Rondo), $P = 0.0139$; Kaybonnet (KBNT) > (4484, Rondo), $P < 0.0001$; KBNT > (4484), $P < 0.0001$; TeQing > (4593, 4484, Rondo), $P = 0.0388$; TeQing > 4484, $P = 0.0070$. (Rice and red rice heading overlap) : (rice heading span) ratio: KBNT > (4484, Rondo, 4593), $P = 0.0405$.

and HR hybrid rice, averaging 0.43% with a maximum of 3.2%.

Using transgenic insecticide-resistant rice lines from *indica*-based hybrid breeding systems as a pollen source, OC rates to non-IR rice was ~ 0.28% at 0.2 m in one study (Rong et al. 2007), and 0.0815% in a related study in which a pollen source area of 1 m^2 was imbedded in the center of a 3 by 3-m non-IR plot area (Rong et al. 2012). Brownbeard rice and the *indica* cultivar, MG-63 exhibited substantial overlap in flowering and outcrossing in China (Lu et al. 2003). OC rates between MG-63 and brownbeard rice (*Oryza rufipogon* Griffiths) were also substantially greater than those reported in the present study, averaging 2.2% at 0.5 m and up to 1.5% at 5 to 43 m (Song et al. 2003). OC rates as high as 18% have been documented between transgenic rice and wild rice (Wang et al. 2006).

Outcrossing from rice cultivars (as pollen source) to red rice (as pollen recipient) was undetectable in our studies. Similar results have been observed in red rice × *tropical japonica* outcrossing tests in which OC rates were substantially lower or undetectable when red rice was the pollen recipient. In Louisiana, OC from rice to red rice, which was ≥ 18 cm taller than the rice, was undetectable (Zhang et al. 2003). In contrast, Noldin et al. (2002) found OC from rice to BH red rice was greater than from BH to rice; however, OC with BH red rice was less than for SH red rice.

Synchronization of red rice flowering differed greatly among the rice cultivars. As evidenced by the difference in degree days from emergence to 50% heading for rice cultivars and red rice (Table 2), Kaybonnet was the most highly synchronized with red rice, having a difference of −22.6 degree days (negative value indicates rice headed before red rice), 4484 and Rondo were intermediate (average difference of 80 degree days), and 4593 and TeQing were least synchronized (average difference of 105 degree days). When expressing rice–red rice heading differences in terms of days, results among cultivars were similar to the degree day estimates. Kaybonnet heading averaged 1 d earlier, and 4484 and Rondo, and 4593 and TeQing averaged 5.1 and 6.5 d later, respectively, than red rice (Table 2). When expressing flowering synchronization of rice and red rice in terms of the days of heading overlap for rice and red rice as a fraction of the total time span for rice heading, the results followed a trend generally similar to that expressed above, indicating that the extent of heading overlap was greatest for Kaybonnet; intermediate for 4484, Rondo, and 4593; and least for TeQing (Table 2). Although this correlation between outcrossing and heading synchrony is commonly observed (Gealy, unpublished data), it differs somewhat from earlier work in Arkansas, which found that heading synchrony with rice ($R^2 = 0.01$) influenced OC less than red rice biotype (Shivrain et al. 2009c). Shivrain et al. (2010b) found that BH red rice headed ~ 30 heat units later than SH red rice. Some SH biotypes head substantially earlier and nearly synchronously with commercial HR cultivars (Shivrain et al. 2007, 2009c).

OC rate was negatively correlated with the rice–red rice differential in degree days to 50% heading ($r = -0.471$), and when expressed in terms of the cultivar means, these differentials were Kaybonnet < 4484 < Rondo < 4593 < TeQing ($r = -0.9452$)

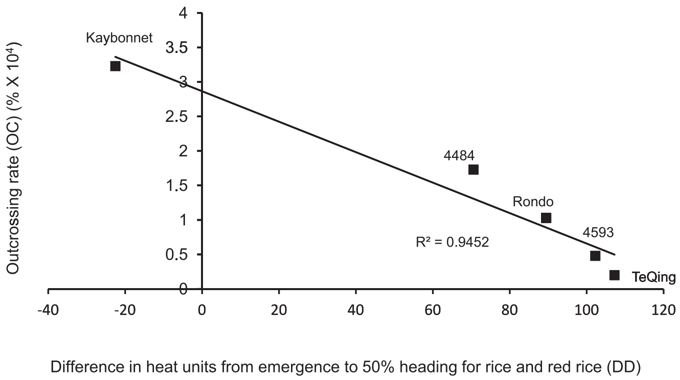

Figure 1. Correlation of outcrossing rate (OC) with the difference in heat units (DD) from emergence to 50% heading for rice and red rice. Data points are cultivar means over 2 yr. Negative heat units indicate that rice reached 50% heading before red rice.

(Figure 1). Results were similar when the above data were expressed in terms of days instead of degree days (data not shown). Conversely, OC rate was positively correlated ($r = 0.978$) with the percentage of heading overlap relative to rice cultivars (as shown in Table 2), supporting the expectation that the greater the period of heading overlap relative to the total rice flowering period, the greater the OC frequency.

Earlier research in controlled studies with wild rice demonstrated that OC rates increased with earlier arrival of pollen (Song et al. 2002). This suggested that outcrossing from red rice to rice might increase in the present study if red rice heading slightly preceded rice heading. However, such trends were not discernible in our study. In related red rice outcrossing research with HR cultivars planted at four dates in April and May, CLXL8 hybrid rice exhibited greater flowering overlap with red rice and higher OC rates compared with CL161, but overall, flowering synchrony was not highly correlated with OC (Shivrain et al. 2009c).

Relationships between red rice yield and rice yields from individual rows within each plot can provide useful insights into potential interactions between the crop and weed. $\text{Yield}_{2,8}$ represents the yield potential of each cultivar assuming negligible influence from red rice in row 5. $\text{Yield}_{2,8}$ of *indica* cultivars averaged 7,489 kg ha^{-1}, which was 43% greater than Kaybonnet, and among the *indica* cultivars, TeQing and Rondo yielded the most and least, respectively (Table 3).

$\text{Yield}_{4,6}$ was greater than $\text{yield}_{2,8}$ for the three *indica* cultivars—4484, Rondo, and 4593—indicating that the red rice in row 5, which was planted less densely than rice, was less competitive than adjacent rows of these rice cultivars. $\text{Yield}_{4,6}$ was less than $\text{yield}_{2,8}$ for Kaybonnet and TeQing, indicating that these cultivars were less competitive than the red rice. These relative differences in rice yields according to row position within the plots were due to the interactive effects of red rice on each cultivar.

The innate competitiveness of each rice cultivar against TX4 red rice is reflected in the red rice yield (yield_{R}). Yield_{R} was typically greatest in the lowest yielding and least competitive cultivar, Kaybonnet (Table 3). In related research, a diverse group of red rice biotypes reduced yields of CL161 and CLXL8 rice from 14 to 45% and 35 to 86%, respectively (Shivrain et al. 2009b).

The difference between the yield of red rice in row 5 and the yield of rice in rows 4 and 6 ($\Delta\text{yield}_{\text{r,rr}}$) can provide insights into the competitive interaction between TX4 red rice and the individual rice cultivars, as well as the relative levels of rice and weed seed production (Table 3). The $\Delta\text{yield}_{\text{r,rr}}$ was greater for *indica* cultivars (a positive value) than for Kaybonnet (a negative value), indicating that seed production of Kaybonnet relative to red rice was severely limited compared with the *indica* cultivars. It is possible that the stresses resulting from these competitive interactions with Kaybonnet may also have influenced OC rates directly or indirectly.

To further explore potential yield-related influences on rice–red rice outcrossing, we undertook an analysis of the differences in flower densities of red rice and rice within each plot. Differences in flower density can be inferred from $\Delta\text{yield}_{\text{r,rr}}$ in the following way. Because seeds are formed from fertile, filled flowers, grain yield is strongly correlated with the number of such flowers. Thus, we estimated fertile flower densities from grain yields of rice and red rice after accounting for the differences in their respective 100-seed weights (Table 4). The $\Delta\text{yield}_{\text{r,rr}}$ can provide insights into the dynamics of outcrossing and the potential direction of net pollen flow (i.e., red rice to rice vs. rice to red rice). For instance, we would expect that massively greater densities of red rice flowers compared with rice will increase the likelihood of outcrossing to any individual rice flower because more red rice flowers would be competing to pollinate relatively fewer rice flowers. A practical demonstration of this can sometimes be observed in outcrossing estimates from commercial farm fields. Some of the highest OC between HR rice (as pollen source) and red rice (e.g., $> 1.0\%$) have been reported in HR rice fields that contained somewhat

Table 3. Rice and red rice grain yields in a 2-yr study of red rice outcrossing.[a,b]

	Grain yield				
Rice cultivar	Rice rows 2,8	Rice rows 4,6	Rice − rice difference (rows 2,8 − rows 4,6)	Red rice row 5	Rice − red rice difference (rice rows 4,6 − red rice row 5)
	kg ha^{-1}				
4484	7,328 ab	8,472 a	−1144 c	3,927 a	4,326 a
Rondo (1693)	6,701 b	7,401 a	−700 bc	3,713 a	3,874 a
4593	7,384 ab	8,212 a	−828 bc	3,629 a	4,289 a
Kaybonnet	5,247 c	5,002 b	245 ab	6,811 b	−1,079 b
TeQing	8,542 a	7,920 a	622 a	4,708 a	3,345 a

[a] Plants were grown in the field in a standard drill-seeded, flooded rice production system at Stuttgart, AR. Outcrossing plots were established in 2008 and 2009, and the hybrid phenotypes produced in these plots were subsequently identified in observation plots grown in 2009 and 2010, respectively.

[b] Values in table are least square means over 2 yr. Means within columns followed by the same letter are not significantly different according to Tukey's honest significant difference test at $P \leq 0.05$. The following are selected or additional significant single degree of freedom contrasts. Rice yield in rows 4,6: (4484, 4593) > (Rondo, TeQing), $P = 0.0408$. Red rice yield: TeQing > (4484, Rondo, 4593), $P = 0.0239$.

light infestations of red rice surrounded by high densities of monoculture rice (Zhang et al. 2006).

Red rice outcrossing is typically maximized at a separation distance of ≤ 0.5 to 1 m between the pollen donor and the pollen-receiving plants (Gealy 2005; Messeguer et al. 2001, 2004; Shivrain et al. 2007). Thus, the relative numbers of total fertile flowers on red rice plants and rice plants (as determined by averaging $rice_{4,6}$ and $rice_{2,8}$ plants) in each plot might be expected to influence OC between red rice and different rice cultivars. The difference in rice–red rice total flower densities ($\Delta flower_{r,rr}$) averaged 2.18×10^5 $plot^{-1}$ for the *indica* cultivars and 1.74×10^5 $plot^{-1}$ for Kaybonnet. When considering only the middle three rows (red rice and $rice_{4,6}$) of the plot, the differences in rice–red rice flower densities (no. ha^{-1}) were even more pronounced in favor of the *indica* cultivars, which averaged $+1.62 \times 10^8$ ha^{-1} compared with -0.034×10^8 ha^{-1} for Kaybonnet (detailed data not shown). These data indicate that the number of red rice flowers available to contribute pollen to the limited number of Kaybonnet flowers in our plots was relatively greater than the number available to fertilize the *indica* cultivars (Table 4).

These data suggest that rice–red rice flower density differentials for Kaybonnet and *indica* cultivars might increase OC rates with Kaybonnet as the pollen recipient because the ratio of rice to red rice flowers was relatively lower for Kaybonnet than for the *indica* cultivars.

In addition to their correlation with flowering synchrony demonstrated above, OC rates were also negatively correlated with differentials in flower densities between rice and red rice, whether expressed per area for the middle rows only or per the entire plot ($r = -0.487$, $P = 0.0014$ and $r = -0.5049$, $P = 0.0009$, respectively; detailed data not shown). Thus, it is possible that flower density differentials may also have influenced the differences among OC rates that we observed in these studies.

Rong et al. (2012) modeled the influence of pollen density gradients on outcrossing and demonstrated such a "dilution effect" of pollen source to recipient. In their tests, a 64-m^2 pollen recipient area surrounded by pollen source plants (totaling 512 m^2) on all sides resulted in an average 40% lower OC rate to recipient plants compared with a 1-m^2 pollen recipient area surrounded by pollen source plants totaling 8 m^2. In simplistic terms, all of the potential pollen recipient plants were ≤ 1 m from the closest pollen source plants (i.e., highest OC potential) in the 1-m^2 design. By contrast, less than half of pollen recipient plants were in similar, close proximity to the source plants in the 64-m^2 design. Thus, their results appear to be consistent with our observation that increased OC into the rice plants occurred with a relative decrease in rice : RR flower density. Rong et al. (2007) also showed that different pairs of transgenic and nontransgenic hybrids and a male-sterile restorer line that were well synchronized for flowering frequency sometimes produce significantly different OC% rates, and interestingly, the male-sterile line did not exhibit the highest OC rate.

As expected, the trends in panicle density for rice, red rice, and their differentials were similar to those for grain yield (Table 3), with red rice densities being greater and rice–red rice differentials being less in Kaybonnet than for *indica* cultivars

Table 4. Estimated rice and red rice flower densities in a 2-yr study of red rice outcrossing.[a–c]

Rice cultivar	Total flowers per plot		
	Rice	Red rice	Difference: rice − red rice
	No. $\text{plot}^{-1} \times 10^5$		
4484	2.34 b	0.142 a	2.14 ab
Rondo (1693)	2.09 bc	0.132 a	1.87 b
4593	2.20 bc	0.129 a	1.99 b
Kaybonnet	1.82 c	0.268 a	1.74 b
TeQing	2.85 a	0.178 a	2.70 a

[a] Plants were grown in the field in a standard drill-seeded, flooded rice production system at Stuttgart, AR. Outcrossing plots were established in 2008 and 2009, and the hybrid phenotypes produced in these plots were subsequently identified in observation plots grown in 2009 and 2010, respectively.

[b] Values in table are least square means over 2 yr. Means within columns followed by the same letter are not significantly different according to Tukey's honest significant difference test at $P \leq 0.05$. The following are selected or additional significant single degree of freedom contrasts. (Total rice flowers) − (total red rice flowers per plot) difference: Kaybonnet < (all *indica* cultivars), $P = 0.0360$; TeQing > (4484, Rondo, 4593), $P = 0.0008$; TeQing > (4484, Rondo), $P = 0.0015$.

[c] Flower densities were calculated on the basis of grain yields, 100-seed weights of red rice and each cultivar, and dimensions of the outcrossing plots, drill rows, or both. See Materials and Methods.

(Table 5). Red rice panicle density ($P = 0.0344$) and seedling density ($P = 0.0304$), and rice 25% heading date ($P = 0.0110$) were significant covariates for OC rate (detailed data not shown). This statistical information is consistent with the major competitive advantage of red rice against Kaybonnet, which coincidentally, was highly synchronized with red rice for heading date.

Consistent with the results for grain yield and panicle density, the tallest red rice plants and the greatest height differentials between red rice and rice were found in plots of Kaybonnet rice (Table 6). Logically, the smaller height differentials with *indica* cultivars (i.e., closer vertical proximity to red rice flowers) might be expected to facilitate greater outcrossing than with Kaybonnet. Reduced outcrossing rates from shorter to taller plants compared with the reverse direction have been observed in other *Oryza* OC tests (Chen et al. 2004; Messeguer et al. 2004; Noldin et al. 2002; Zhang et al. 2003). Overall, our data did not generally follow this pattern. Although Kaybonnet's inferior competitive ability resulted in a large height differential with red rice, this did not appreciably overcome the advantage to the OC rate that was imparted by synchronized heading. By contrast, there was a small tendency toward greater red rice outcrossing with 4484 than with Rondo, its mutant progeny, which was shorter and had less height differential with red rice (Tables 1 and 6). However, any potential red rice outcrossing effect of height appeared to be far outweighed by the synchronization of heading.

Among the environmental data analyzed, only daily wind speed ($r = -0.3086$, $P = 0.0527$) and low RH ($r = -0.2989$, $P = 0.0610$) from initial rice heading date to HD100 were slightly correlated with OC (detailed data not shown).

Most environmental factors that correlated with OC appeared to derive this relationship from a strong association with Kaybonnet traits. Thus, to remove the dominant influence of Kaybonnet, a separate correlation analysis was conducted using only the four *indica* cultivars. Again, only wind speed during the heading period was potentially associated with OC rates in the environments of both years. OC rate was moderately negatively correlated with wind speed ($r = -0.433$, $P = 0.01339$) (detailed data not shown). Messeguer et al. (2004) showed previously that most outcrossing between *Oryza* plants occurred in the direction of the prevailing wind. In our tests, greater wind speeds were associated with lower OC rates, which conceivably could have increased desiccation of stigma or pollen (therefore reducing their viability) or decreased the concentration of pollen grains available to fertilize nearby plants. Other environmental variables were slightly negatively correlated with OC rate. These included low RH from HDi to HD100 ($r = -0.305$; $P = 0.0893$); Solar from HDi to HD100 ($r = -0.207$); and Rain from HDi to HD100 ($r = -0.207$) (detailed data not shown). Comparatively lower wind and humidity during the flowering period of 2009 may have facilitated overall greater red rice–*indica* OC rates in that year.

OC rates were highly variable in these studies, and average OC tended to be highest in 2009. In previous research, relative humidity during flowering has been correlated with OC rates between rice and red rice. With hybrid rice CLXL8, OC averaged 0.12% and 0.38% when RH from midmorning to noon was

Table 5. Rice and red rice panicle densities in a 2-yr study of red rice outcrossing.

Rice cultivar	Rice	Red rice	Difference: rice − red rice
	No. panicles m^{-1}		
4484	71.3 a	58.7 a	12.6 a
Rondo (1693)	70.4 a	56.6 a	13.8 a
4593	64.9 a	57.5 a	7.4 a
Kaybonnet	57.6 a	73.7 a	−16.1 b
TeQing	61.5 a	64.2 a	−2.8 ab

[a] Plants were grown in the field in a standard drill-seeded, flooded rice production system at Stuttgart, AR. Outcrossing plots were established in 2008 and 2009, and the hybrid phenotypes produced in these plots were subsequently identified in observation plots grown in 2009 and 2010, respectively.

[b] Values in table are least square means over 2 yr. Means within columns followed by the same letter are not significantly different according to Tukey's honest significant difference test at $P \leq 0.05$. The following are selected or additional significant single degree of freedom contrasts. Red rice panicle density: Kaybonnet (KBNT) > (all *indica* cultivars), $P = 0.0053$; KBNT > (4484, Rondo, 4593), $P = 0.0029$; (KBNT, TeQing) > (4484, Rondo, 4593), $P = 0.0069$. Difference in rice–red rice panicle density: TeQing < (all other *indica* cultivars), $P = 0.0250$; KBNT < TeQing, $P = 0.0777$.

< 54% and ≥ 54%, respectively (Shivrain et al. 2009c). Their study also found that a minimum evening air temperature of > 24 C was associated with increased OC rates in CL161, and minimum air temperatures from midmorning to noon of < 24 C, in combination with high RH, were associated with increased OC rates in CLXL8. However, pollen characteristics of the rice hybrid and inbred lines could not be ruled out as contributing factors. Because of the limited number of cultivar × red rice biotype combinations evaluated in the present study, it is unclear whether any of these environmental factors affected the OC rates.

Average temperatures during the growing season were higher in 2008 than in 2009, resulting in faster accumulation of degree days in 2008 than in 2009 (~ 16.6 vs. ~ 15.4 degree days/$d_{[\text{emergence to HD50}]}$). In 2009, there were also fewer sunny days in combination with record high rainfall amounts (data not shown). Collectively, these conditions apparently affected the overall growth or health of rice plants, or both, resulting in an average reduction in rice yield of ~ 30% in 2009 compared with 2008 (data not shown). Interestingly, average red rice yields were similar in both years, suggesting that red rice was able to exploit the limited solar radiation in 2009 at the expense of the shorter rice plants.

In a recent study of hybrid sterility genes that potentially could help block gene flow between U.S. red rice and rice cultivars, researchers found that the probability of partial hybrid sterility was slightly higher for BH than SH red rice crossing with *japonica* cultivars, but given the distribution of hybrid sterility alleles in these plant types, hybrid sterility was not likely to limit gene flow between rice and weedy red rice (Craig et al. 2014).

Greater overlap in flowering time has been shown between *tropical japonica* and *indica* rice and blackhull red rice than with SH red rice (Reagon et al., 2011; Thurber et al. 2014). Thurber et al. (2014) found that BH red rice and U.S. rice both possess nonfunctional alleles of *Hd1* (a gene controlling

Table 6. Rice and red rice maximum plant heights in a 2-yr study of red rice outcrossing.[a,b]

	Maximum plant height		
Rice cultivar	Rice	Red rice	Difference: red rice − rice
	cm		
4484	98.5 a	132 ab	32.7 b
Rondo (1693)	90.0 a	127 b	35.8 b
4593	94.3 a	129 b	33.9 b
Kaybonnet	97.0 a	140 a	45.45 a
TeQing	96.6 a	130 ab	33.8 b

[a] Plants were grown in the field in a standard drill-seeded, flooded rice production system at Stuttgart, AR. Outcrossing plots were established in 2008 and 2009, and the hybrid phenotypes produced in these plots were subsequently identified in observation plots grown in 2009 and 2010, respectively.

[b] Values in table are least square means over 2 yr. Means within columns followed by the same letter are not significantly different according to Tukey's honest significant difference test at $P \leq 0.05$.

flowering time in rice), which generally results in day length insensitivity in these groups. They suggested that these nonfunctional alleles might facilitate more simultaneous flowering and gene flow between BH red rice and rice. Burgos et al. (2014) suggested that this phenomenon might account for the relatively greater BH red rice contribution to HR weedy red rice reported from Arkansas rice fields.

Controlled crossing performed with a commercial HR *tropical japonica* inbred cultivar and 12 U.S. weedy red rice types revealed no major barriers to hybridization, with seed set ranging from 70 to 94% for 10 of the 12 types and all of the BH types (Shivrain et al. 2008). In a companion experiment in the field in which the red rice flowering overlapped with rice, outcrossing rates were as high as 0.25% (Shivrain et al. 2008).

Our research showed that *indica* rice cultivars can outcross with U.S. red rice at rates within the ranges previously reported for *indica* cultivars in Asia and U.S. *tropical japonica* cultivars. Flowering synchrony with red rice appeared to play the dominant role in determining the rates of outcrossing observed in these tests; however, rice–red rice flower density differentials may also have influenced outcrossing among the rice cultivars.

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