When rice (Oryza sativa L.) is milled to remove the hull and bran layer, the resulting white rice is graded according to physical properties. A key component determining the grade or economic value for a consignment of rice is its milling quality, which is defined by the proportion (in mass) of rice that remains intact (unbroken) after milling. After rice is milled, the broken kernels (defined as having grain length less than three-fourths the sample average) are separated from the unbroken rice (also known as having an intact kernel or fissure resistance).

Fine Mapping of qFIS1-2, a Major QTL for Kernel Fissure Resistance in Rice


ABSTRACT
Rice (Oryza sativa L.) kernel fissuring causes breakage during milling and thereby decreases the value of rice. This study employed molecular gene-tagging methods to fine map a fissure resistance (FR) locus in ‘Cybonnet’, a semidwarf tropical japonica cultivar, as well as to transfer this trait to rice genotypes of non-semidwarf stature that are better adapted to some rice production systems. Three quantitative trait loci (QTLs) for FR were previously reported, with the FR locus linked with the semidwarf sd-1 locus on chromosome 1 having the strongest effect. For fine mapping, F₂ progeny were produced from a cross between US breeding line RU1201108, having non-semidwarf height (Sd-1/Sd-1) and poor milling quality, and Cybonnet (sd-1/sd-1), having semidwarf height and containing multiple FR loci. Simple sequence repeat (SSR) markers were used to select F₂ plants that both retained at least one copy of the Sd-1 allele and showed evidence of genetic recombination in the region of chromosome 1 known to contain Sd-1 and qFIS1-2, a QTL for FR. Progeny from three F₂ individuals with desirable recombination at the qFIS1-2 were phenotyped for kernel FR. Marker-trait linkages observed in the three populations indicated that the qFIS1-2 locus resides on chromosome 1 distal to RM1068 at 38.44 Mb, but anterior to RM3482 at 39.72 Mb, placing it ~6 to 10 cM distal to sd-1 on chromosome 1. The recombination documented in this study verifies that the previously identified qFIS1-2 is not pleiotropic with nor reliant on sd-1 and can be recombined with Sd-1 to increase the FR of non-semidwarf rice cultivars.

Abbreviations: ABC, ATP-binding cassette; CYBT, Cybonnet, the male parent of the present cross progeny; FR, fissure resistance; FS, fissure susceptibility; LOD, logarithm of odds; MAS, marker-assisted selection; PCR, polymerase chain reaction; QTL, quantitative trait locus; RH, relative humidity; RREC, Rice Research and Extension Center; RU12, breeding line RU1201108 female parent of the present cross progeny; Sd-1, wild-type, non-semidwarf, or standard plant height allele at the GA 20-oxidase gene locus; sd-1, mutant recessive allele for semidwarfism at the sd-1 GA 20-oxidase gene locus; SNP, single-nucleotide polymorphism; SSR, simple sequence repeat.
as head rice) and typically sold to markets such as rice flour and pet food ingredients, where they generally command just 50 to 60% of the value of head rice (Siebenmorgen et al., 2008). A key factor contributing to kernel breakage during milling is prior fissuring (Siebenmorgen et al., 2005; Zhang et al., 2005; Pinson et al., 2009). Kernel fissures are stress fractures that develop in either the inner or outer layers of the kernel endosperm, rendering the kernel more susceptible to breakage. Studies have shown that kernel fissuring can be caused by rapid adsorption of moisture into a rice kernel after the starchy endosperm has dried to a sufficiently low level (Kunze and Choudhury, 1972; Siebenmorgen et al., 1998, 2009; Zhang et al., 2005; Kunze, 2008). Evaluation of rice samples from five cultivars at initial kernel moisture contents ranging from 9 to 17% determined that kernel fissuring from rapid moisture adsorption increases when kernels dry to ≤15% kernel moisture content (Mukhopadhyay and Siebenmorgen, 2013). Rapid moisture adsorption occurs during heavy dew or rainfall events, or in environments with high relative humidity (RH; e.g., 90–100% RH) (Kunze, 2008; Bautista et al., 2009; Siebenmorgen et al., 2009). Such environments cause the starch granules in the outer layers of the dry kernels to rapidly absorb moisture and swell. The expanded outer layers produce compressive forces at the kernel surface that, in turn, produce interior, tensile forces along the primary axis of the kernel. If the tensile forces exceed the kernel tensile strength, a hairline crack or fissure forms (Kunze and Choudhury, 1972; Kunze, 2008; Siebenmorgen et al., 2009). Moisture adsorption fissuring can happen before or after harvesting; when it occurs prior to harvest, it is also known as field fissuring.

The environmental conditions that cause rewetting and fissuring in the field are not easily controlled. Therefore, one solution to prevent loss of rice quality as a result of fissuring is for rice breeders and producers to select and plant cultivars that are genetically less susceptible to kernel fissuring. Previous studies have shown that kernel fissuring occurs at higher rates in some cultivars than in others when exposed to the same environmental triggers (Waggoner et al., 2003; Zhang et al., 2005; Kunze and Choudhury, 1972; Kunze, 2008; Siebenmorgen et al., 2009). Moisture adsorption fissuring can happen before or after harvesting; when it occurs prior to harvest, it is also known as field fissuring.

The physical locations of the SSRs were determined using the Os-Nipponbare-Reference-IRGSP-1.0 rice genome assembly (Kawahara et al., 2013), with a logarithm of odds (LOD) peak nearest to RM6292 (39.2 Mb) (Pinson et al., 2013). The LOD peak predicted qFIS1-2 to be closely linked but distal to the semidwarf gene sd-1, which, at 38.4 Mb, was within the QTL region. Both the Cypress and Cybonnet genomes possess the semidwarf allele (sd-1) (Linscombe et al., 1993; Gibbons et al., 2006). Thus, in both these FR lines, the alleles for FR and semi-dwarfism are genetically linked on chromosome 1, as also observed by Pinson et al. (2012).

This experiment sought to use recombination in additional cross progenies to validate and more precisely map the location of the qFIS1-2 locus. Taller rice cultivars are desirable for certain production environments; therefore, a second objective was to identify one or more recombinant progeny in which the wild-type or non-semidwarf allele (Sd-1) was linked with FR. This study also investigated if segregation of qFIS2-1 was associated with a difference in any kernel dimensions, because slight differences in kernel dimensions have been reported to affect kernel FR (Jindal and Siebenmorgen, 1994; Waggoner et al., 2003; Zhang et al., 2005).
Populations 36 and 37 were all of tall height, whereas Population 67 segregated visibly for tall and semidwarf height.

Seed was collected from each F$_3$ plant as it reached the 18 to 20% moisture content stage of maturity. Visual assessment (per Pinson et al., 2012, 2013) was used to judge when each plant was ready for harvest; plots were monitored thrice weekly until all plants were harvested. Five panicles were collected per F$_3$ plant, dried gently using room temperature air (~22°C) for 3 d, and then stored together at room temperature to allow grain moisture to equilibrate between samples. Careful harvesting and drying of grains minimized the development of fissures in the grains due to field fissuring or postharvest handling. More than 300 panicles per each parent genotype, plus three check cultivars (Cypress and ‘Saber’ as FR lines, ‘Lemont’ as a fissure-susceptible [FS] line), were similarly harvested, dried, and stored.

Genotyping of F$_3$ Progeny

Leaf samples from each F$_3$ plant were harvested prior to heading and stored at −80°C until DNA extractions were performed. A high-throughput alkali extraction method (Xin et al., 2003) was used to extract DNA from the frozen F$_3$ leaf samples. Extracted DNA was stored at −20°C until used for PCR amplification. Each F$_3$ population was genotyped for RM1339 plus two or more markers for each side of their specific recombination points to determine the F$_3$ haplotypes (Fig. 1).

Genotypic data were used to eliminate from further study the F$_3$ plants that were heterozygous for markers in the qFIS1-2 and qFIS8 in this experiment but was most closely linked with RM23000 (Pinson et al., 2013).
Preliminary Trials to Optimize the Laboratory Fissure Induction Procedure

The kernel fissuring phenotypes of the F$_3$ progeny were determined using a laboratory fissure-induction method shown capable of supporting effective breeding selections for FR as early as the F$_2$ generation (Pinson et al., 2012) and subsequently used to discover the three FR QTLs (Pinson et al., 2013). Relative ratings for FR are obtained by comparing percentages of fissured kernels in small samples (~50 kernels) of mature, dried rice seed exposed to fissure-inducing humid air (100% RH) conditions in the laboratory. The humidity conditions were created by suspending seed samples in individual tulle bags over water in sealed boxes placed in a 45°C chamber (Fig. 2). Samples of paddy rice (hulls intact) were exposed to humidity to induce fissures then dried overnight at room temperature before being mechanically dehusked (twice through a Satake THU-35A, Satake Engineering Company) and evaluated visually for percentage of kernels fissured. Kernels that broke during the dehusking process were considered to have fissured. Exposing seed samples to dry heat (45°C) for a period of time immediately prior to the humidity treatment enhanced the differences observed between FR and FS genotypes (Pinson et al., 2012, 2013). However, the optimum amount of preheating was variable over both year and location of seed production. The published procedure recommends using seed from FR and FS check cultivars grown in the test environment to evaluate multiple lengths of preheat (0–8 h) and humidity treatments (8–16 h) to identify the optimum combination where the FR and FS parents and check cultivars from that harvest condition exhibit the greatest difference in fissuring percentages.

While conducting these treatment optimization studies using seed from parental and check cultivars stored indoors at the RREC laboratory (per Pinson et al., 2012, 2013), it was noted that fissure induction rates between trials were sensitive to weather, with trials that began 1 d after the occurrence of rain producing fewer fissures than trials conducted in drier periods. This indicated that variable indoor RH at the RREC was causing day-to-day fluctuations in the initial kernel moistures, introducing confounding nongenetic variability into the FR studies. To ensure consistent initial kernel moistures between runs, seed samples were preconditioned for a minimum of 1 wk at 25 ± 1°C and 55 ± 5% RH in a controlled environmental control chamber (ESPEC EPL-4H, ESPEC North America). Using check cultivar seed and an individual kernel moisture meter (CTR-500E, Shizuoka Seiki Company), it was determined that conditioning seed samples in this manner resulted in an average kernel moisture content of 11.5%. Due to time and space limitations, seed samples from each of the three study populations were conditioned separately in a single ESPEC chamber and stored in sealed Ziploc Double Zipper bags until fissure induction treatment. The fissure induction step is limited to batches of 47 seed samples; each sealed bag contained a single batch of seed samples consisting of 33 to 35 F$_2$:3 samples, three samples of each parent line, and two samples of each of the three additional check cultivars. Three completely randomized analytical replicates of seed from each F$_3$ plant per population were prepared and bagged in this manner.

Each population required 2 wk to evaluate three analytical seed replications from each F$_3$ plant. Three days before initiating fissure induction treatment of each of the three F$_2$:3 populations, seed samples from parent and check cultivars preconditioned.
and sealed at the same time as the population test samples were evaluated to identify the optimal fissure induction conditions for that set of seed. Optimal fissure induction conditions for Populations 36 and 37 were determined to be 4 h preheating at 45°C dry heat, followed by 16 h humidity chamber treatment. Population 67 was not analyzed until 1 mo after completing analyses of Populations 36 and 37, at which time optimum fissure induction conditions were determined to be 7 h preheat, followed by 16 h humidity.

All fissure inductions were followed by a 24- to 48-h dry-down period before being mechanically dehusked and visually inspected for fissures. Redrying of the samples allowed the kernels to contract, making fissures more visible (Pinson et al., 2012). The published procedure (Pinson et al., 2012, 2013) recommends that 60 to 65 kernels sample−1 undergo the fissure induction and drying process, at which time subsets of precisely 50 kernels are counted for dehusking. Kernels that remain intact (unbroken) after dehusking are visually examined and determined to be either fissured or un fissured. The number of kernels that break during dehusking is determined by subtraction and then added to the number of visibly fissured but unbroken kernels before calculating the percentage of fissured kernels per sample.

Phenotyping F3 Progeny for Fissure Resistance

Following the Pinson et al. (2012, 2013) phenotyping procedure, only kernels hand threshed from the top third of carefully harvested panicles were used. Careful selection of kernels from carefully selected panicles ensured that the studied kernels were neither immature nor overmature (risking prefissuring). Once kernels were separated from the five panicles per F3 plant by hand, they were mixed well and allocated into three subsamples of 1.6 g, comprising 60 to 65 kernels. Subsamples were placed in tulle mesh bags (Fig. 2) arranged in a randomized order for analytical replications, conditioned in the ESPEC environmental control chamber, sealed to preserve moisture content, and then stored and evaluated for relative fissuring rates, as described in the section above.

Phenotypic evaluations for FR were analyzed individually for each F3 population, evaluating all three analytical replications of a single population before evaluating any seed samples from the next F2,3 population. Fissure inductions were limited to 47 samples box−1, whereas >100 F2,3 individuals were evaluated for each of the three populations. Individuals in a single replication per Population 36 or 67 were randomly assigned into three treatment boxes; Population 37, being larger, was divided into four boxes per analytical replication. Each box contained three samples of seed from each of the two parents plus two samples of each of the three check cultivars to allow statistical examination of between-box variation. This would occur if boxes were improperly sealed or might occur from sample exposure to variable RH of the facility while being placed into bags after conditioning, or while being removed from the Ziploc bags and placed into treatment baskets.

Kernel Dimension Analysis

Kernel shape has been reported to affect FR (Jindal and Siebenmorgen, 1994; Waggoner et al., 2003; Zhang et al., 2005) and was thus determined among the F2,3 progeny along with FR. Grain length, width, and thickness were measured on the kernels that remained un broken after the percentage fissuring determinations described above. Combining intact seed across the three subsamples per F2,3 plant provided 50 to 150 kernels per plant for dimension analysis. A digital imaging system (SeedCount SC5000 Rice Analyser, Next Instruments) was used to determine kernel dimensions. This system evaluated kernel length and thickness on approximately half of each seed sample and evaluated length and width on the remaining kernels (Supplemental Fig. S1).

Statistics

Statistical analyses of fissure result were performed using JMP 10 (SAS Institute, 2012). Variance of fissuring rates between boxes was evaluated two ways. First, an ANOVA of the multiple samples per parent and check cultivar that were contained in each box was conducted. Second, Student’s t-tests were used to compare the means and variances of the data from F3 test samples per box. Box-to-box variance proved negligible using both methods, and all following analyses were conducted using averages calculated across the three analytical replicates per population.

As associations between FR and the various phenotypic traits was detected using marker allele data to classify the F3 progeny per each of the populations into those homozygous for CYBT alleles and those homozygous for RUI2 alleles in the recombinant region (Fig. 1), then using Student’s t-tests to compare the trait data from the two marker-defined progeny classes. A significant difference in fissuring rates between the two genotypic classes would indicate that qFIS1-2 resides within the chromosomal region segregating among the progeny of that population (e.g., RM5501–RM1361 in Population 67; Fig. 1). In contrast, a lack of significant difference between genotypic classes would indicate that qFIS1-2 resides in a genet-ically fixed portion of the chromosome (e.g., R.M3482–R.M14 in Population 67; Fig. 1). Data from the two parents of the cross, CYBT and RUI2, were also compared.

Associations between FR and the various kernel dimensions were similarly compared between the marker-defined progeny groups using Student’s t-tests. Regression analyses were then conducted to evaluate if any of the kernel dimensions were associated with FR independent of the marker-defined subgrouping. For these regression analyses, the grain shape traits were used as the independent variables, and the fissure rates were considered the dependent variables.

RESULTS AND DISCUSSION

Analysis of Box-to-Box Variance Using Repeated Check Samples

The ANOVA of the multiple samples of each parent and check cultivar per fissure induction box indicated that fissuring rates were consistent (all p-values > 0.05) between boxes within each of the three population studies, both within and across the three analytical replications per population. Furthermore, fissuring rates of the parent and check genotypes did not differ between the Population 36 and Population 37 studies but were notably higher within the
study of Population 67 (Table 1). Similarly, comparison of data from F₃ samples per box indicated no significant box-to-box differences in fissuring rates within any of the three populations studied (all $p$-values $> 0.1$), but an increased rate of fissuring of all samples among Population 67 was again detected (Table 1). Higher rates of fissuring of both control and F₃ progeny samples within Population 67 were likely due to the longer (7 vs. 4 h) preheat exposure time used in the phenotypic evaluation of that population.

The difference in fissuring rates between the two parental lines was small but consistent, with CYBT fissuring less than RU12 across the three population studies (Table 1). The standard deviations among the parental fissuring data indicated high sample-to-sample variation for fissuring rates, even within nonsegregating genotypes, in all three populations studied. Though it was most evident during analysis of the Population 67 data, analysis of parental data indicated a higher than normal risk of type II error in these fissuring analyses. A threshold of $\alpha = 0.1$ was therefore applied to the means comparisons among F₃ fissuring data, described below.

**Kernel Fissuring Rates among Three F₃ Progeny Populations**

In all three populations evaluated, the F₃ progeny group selected as being homozygous for CYBT markers in the recombinant QTL region were more FR (exhibited less fissuring) than the F₂ subgroup selected as containing RU12 marker alleles (Fig. 3). Population 36 was evaluated first because the recombination event in this population included a long segment from CYBT, making this population more likely than others to segregate for the entire qFIS1-2 QTL, rather than a partial gene or gene cluster. No segregation for height was detected among the field-grown F₃ plants, which were all tall or non-semidwarf in height. The fixation of Sd-1 was expected from the fixation of the RU12 allele at RM1339 (Fig. 1). The Population 36 F₃ progeny that were molecularly homozygous for CYBT alleles from RM8235 through RM529 (Fig. 1) exhibited detectably lower rates of fissuring than 47 F₃ progeny containing RU12 alleles in this chromosomal region ($\alpha = 0.029$; Table 2, Fig. 3). Pinson et al. (2013) reported significant association between FR from chromosomal region (a region slightly greater than 47 F₃ through RM529 (Fig. 1) exhibited detectable lower rates of fissuring than the F₃ progeny containing CYBT versus RU12 marker alleles (Fig. 3, Table 2) determined that qFIS1-2 is anterior to RM3482.

When data from all three recombinant populations were considered together, they indicated that qFIS1-2 resides in the portion of chromosome 1 segregating (not fixed) in all three populations, which is the 1.3-Mb region between RM1068 (at 38.4 Mb) and RM3482 (at 39.7 Mb). This places qFIS1-2 into a region nearly half the length reported previously, where qFIS1-2 was located in the 2.5-Mb region between RM8231 and RM529 (Pinson et al., 2013).

**Kernel Length, Width, and Thickness**

Kernels from parental lines were found to have similar width (2.37 mm) and thickness (2.06 mm) but to differ in

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**Table 1. Differences in parental fissuring rates on the basis of multiple samples (minimum 27) per parent analyzed in each population study.**

<table>
<thead>
<tr>
<th></th>
<th>Population 36</th>
<th>Population 37</th>
<th>Population 67</th>
</tr>
</thead>
<tbody>
<tr>
<td>F₃ progeny range, min.–max. (%)†</td>
<td>0–39</td>
<td>3.33–65.33</td>
<td>7.29–72.17</td>
</tr>
<tr>
<td>Cybonnet (fissure resistant) parent mean (%) ± SD</td>
<td>23 ± 10.6</td>
<td>19.8 ± 14.11</td>
<td>48.7 ± 14.22</td>
</tr>
<tr>
<td>RU1201108 (fissure susceptible) parent mean (%) ± SD</td>
<td>37.3 ± 12.51</td>
<td>39 ± 23.32</td>
<td>56.7 ± 17.97</td>
</tr>
<tr>
<td>SE of parental means (%)</td>
<td>6.70</td>
<td>8.10</td>
<td>7.80</td>
</tr>
<tr>
<td>Difference between parents (%)</td>
<td>14.30</td>
<td>19.20</td>
<td>8.00</td>
</tr>
<tr>
<td>Means comparison $p$-value‡</td>
<td>0.029</td>
<td>0.014</td>
<td>0.161</td>
</tr>
</tbody>
</table>

† Averaged across three analytical replicates per F₃ plant.
‡ From one-tailed $t$ test with equal variance assumed.
length. Cybonnet produced kernels 0.2 mm shorter than those from RU12 (7.33 vs. 7.51 mm in average length, respectively; \( p < 0.0001 \)). Among the F_3 progeny selected and grouped on the basis of homozygosity for either the CYBT or RU12 marker alleles on the qFIS1-2 QTL region (Fig. 1), only kernel length showed a consistent difference due to marker genotype, with progeny containing CYBT alleles having shorter average kernel length in all three populations (Table 2). For Populations 36 and 67, selection for CYBT markers was also associated with a slight increase in kernel width, whereas kernel thickness did not appear affected. In contrast, selection for CYBT markers among the Population 67 progeny decreased both width and thickness. Lack of consistency for shift in width and thickness among the CYBT and RU12 selected progeny between the populations indicates the observed trait differences were due to genes located in chromosomal regions that were segregating in some populations, but not all three. For example, the decrease in thickness that was associated with CYBT alleles only in Population 37

Table 2. Comparison of rice kernel fissuring rates and grain dimension traits between the two F_3 progeny groups divergently selected as homozygous for ‘Cybonnet’ (CYBT) or RU1201108 (RU12) marker alleles in the recombinant region for three F_2:3 populations (Fig. 1).

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>CYBT</th>
<th>RU12</th>
<th>CYBT</th>
<th>RU12</th>
<th>CYBT</th>
<th>RU12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rates of kernel fissuring</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean per F_3 marker class (%)</td>
<td>7.55</td>
<td>10.52</td>
<td>25.17</td>
<td>29.38</td>
<td>37.81</td>
<td>41.82</td>
</tr>
<tr>
<td>SD (%)</td>
<td>7.62</td>
<td>7.74</td>
<td>14.65</td>
<td>13.19</td>
<td>12.38</td>
<td>14.22</td>
</tr>
<tr>
<td>SE mean</td>
<td>0.011</td>
<td>0.011</td>
<td>0.021</td>
<td>0.018</td>
<td>0.019</td>
<td>0.022</td>
</tr>
<tr>
<td>Difference between marker classes (%)</td>
<td>2.97</td>
<td>4.21</td>
<td>4.01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Means comparison p-value</td>
<td>0.029</td>
<td>0.064</td>
<td>0.086</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kernel length</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean per F_3 marker class (mm)</td>
<td>7.23</td>
<td>7.12</td>
<td>7.35</td>
<td>7.50</td>
<td>7.62</td>
<td>7.47</td>
</tr>
<tr>
<td>SE mean</td>
<td>0.006</td>
<td>0.006</td>
<td>0.026</td>
<td>0.040</td>
<td>0.040</td>
<td>0.003</td>
</tr>
<tr>
<td>Means comparison p-value</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kernel width</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean per F_3 marker class (mm)</td>
<td>2.37</td>
<td>2.34</td>
<td>2.36</td>
<td>2.37</td>
<td>2.32</td>
<td>2.30</td>
</tr>
<tr>
<td>SE mean</td>
<td>0.009</td>
<td>0.008</td>
<td>0.053</td>
<td>0.008</td>
<td>0.006</td>
<td></td>
</tr>
<tr>
<td>Means comparison p-value</td>
<td>0.003</td>
<td>0.008</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kernel thickness</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean per F_3 marker class (mm)</td>
<td>2.02</td>
<td>2.01</td>
<td>2.02</td>
<td>2.04</td>
<td>2.05</td>
<td>2.04</td>
</tr>
<tr>
<td>SE mean</td>
<td>0.026</td>
<td>0.006</td>
<td>0.006</td>
<td>0.008</td>
<td>0.008</td>
<td>0.259</td>
</tr>
<tr>
<td>Means comparison p-value</td>
<td>0.395</td>
<td>&lt;0.0001</td>
<td></td>
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</tr>
</tbody>
</table>
may be due to a gene near RM14, on the telomere of chromosome 1, where Populations 36 and 67 were both already fixed for RU12 alleles. Conversely, the observation of association between kernel width and CYBT alleles in Populations 36 and 67, paired with a lack of association observed in Population 37, would be consistent with a kernel width gene residing between RM1339 and R.M1068, where Population 37 was fixed for RU12 alleles but Populations 36 and 67 continued to segregate.

Previous research suggested that longer kernels may be better suited to resist fissuring because their longer length allows for pressure to be dispersed along a longer internal axis within the kernel instead of concentrated on a short internal axis, as would happen within a rounder short-grain rice kernel (Waggoner et al., 2003). Association between round kernel shape and increased fissuring rates was also reported by Jindal and Siebenmorgen (1994). Our observation of decreased kernel length (rounder shape) and decreased fissuring among progeny containing CYBT markers was therefore opposite of what would have been predicted if kernel shape was causing the shift in FR. Regression analysis of length, thickness, and width against fissuring rates was used to test for association between each kernel dimension and FR among all phenotyped F₃ progeny, irrespective of their marker allele subgrouping (Table 3). When viewed in this way, kernel length was not associated with FR in any of the three populations, indicating that kernel length, which was affected by additional genes segregating in the unmarked background of these F₃ progeny, did not significantly affect kernel fissuring rates. The differences detected for both kernel length and FR after marker selection in all three populations (Table 3) were therefore more likely due to linkage between qFIS1-2 and an allele for reduced kernel length in the CYBT parent, rather than kernel length itself determining the FR trait.

### Candidate Genes

Pinson et al. (2013) reported a LOD peak for qFIS1-2 between RM1361 and RM104 (chromosome 1:39080910…40168103). By demonstrating that qFIS1-2 resides anterior to RM3482, the QTL was hereby mapped more precisely to the 0.64-Mb region between RM1339 and R.M3482. The Michigan State University Rice Genome Annotation Project Rice Genome Browser (MSU7) (http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice/#search) (Kawahara et al., 2013) lists 102 gene loci between RM1361 and RM3482 and annotates them with predicted gene functions on the basis of sequence similarity to genes of known function or by the presence of functional domains. The data above showing that qFIS1-2 does not affect FR by affecting kernel shape, and studies that indicate that the hull, bran, and endosperm attributes can all contribute to FR (Waggoner et al., 2003), comprise what is known about this gene. Little is known about how qFIS1-2 functions, which impedes use of gene annotations to determine which genes in this region are most likely candidates for qFIS1-2.

Fortunately, Cypress and LaGrue, the FR and FS parents of the mapping population where segregation first mapped qFIS1-2 to the long arm of chromosome 1 (Pinson et al., 2013), were among the 104 elite rice cultivars resequenced by Duitama et al. (2015b), with data made publicly available (Duitama et al., 2015a). This sequence data revealed 489 single-nucleotide polymorphism (SNP) and insertion or deletion differences between Cypress and LaGrue in the region between RM1361 and RM3482 using a custom SNP genome browser track provided by Ricebase (http://ricebase.org) (Edwards et al., 2016). When the sequence polymorphism data were compared with the MSU7 gene annotations using Ricebase SNP effect prediction, 329 of the polymorphisms were found to reside in intergenic regions, making them less likely as candidates underlying qFIS1-2. The remaining 160 polymorphisms were contained in 53 of the 102 annotated genes between RM1361 and RM3482. Cypress and LaGrue have common ancestors. Many of the 49 annotated genes not containing SNPs were clustered in two regions, which suggests that these DNA segments may be identical by descent.

Also among the 104 sequenced elite cultivars were four lines having Cypress as a progenitor, ‘Cocodrie’, ‘CL152’, ‘CL261’, and ‘Mermentau’ (Linscombe et al., 2000; Oard et al, 2014a, 2014b, 2014c). Like CYBT, these lines are also expected to have inherited some alleles from Cypress but are not considered FR, suggesting that they did not inherit qFIS1-2. Thus, any sequence differences associated with qFIS1-2 would be expected to be polymorphic also between Cypress and these four progeny lines. Of the 53 annotated genes with SNPs between Cypress and LaGrue, only three were also polymorphic between Cypress and one or more of the four FS Cypress-derived cultivars. These three annotated genes, LOC_Os01g67330, LOC_Os01g67331, and one or more of the four FS Cypress-derived cultivars.

### Table 3. Regression Analyses indicated that Kernel Length was not significantly associated (F-value > 0.05) with Kernel Fissuring Rates among the F₃ Progeny from any of the Three Populations studied. Kernel Width and Thickness were Associated Only among Population 37 Progeny where they had Relatively Small Effect, with Each Dimension explaining <10% of the Total Variance observed for Fissuring Rates.

<table>
<thead>
<tr>
<th></th>
<th>Population 36</th>
<th></th>
<th>Population 37</th>
<th></th>
<th>Population 67</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R²</td>
<td>F-value</td>
<td>R²</td>
<td>F-value</td>
<td>R²</td>
<td>F-value</td>
</tr>
<tr>
<td>Kernel length</td>
<td>0.008</td>
<td>0.36</td>
<td>0.012</td>
<td>0.27</td>
<td>0.002</td>
<td>0.72</td>
</tr>
<tr>
<td>Kernel width</td>
<td>0.031</td>
<td>0.09</td>
<td>0.053</td>
<td>0.02</td>
<td>0.019</td>
<td>0.26</td>
</tr>
<tr>
<td>Kernel thickness</td>
<td>0.001</td>
<td>0.33</td>
<td>0.093</td>
<td>0.002</td>
<td>0.014</td>
<td>0.35</td>
</tr>
</tbody>
</table>
LOC_Os01g67580, and LOC_Os01g67980, were thus identified as the most likely candidate genes for qFIS1-2.

Cypress contained a unique SNP variant at chromosome 1:39085334, which is in an intron within the 5' untranslated region of LOC_Os01g67330. Thus, it may modify the expression of this putative nucleotide-sugar transporter family protein gene. Nucleotide–sugar transporters affect cell wall biosynthesis (Zhang et al., 2011), and altered cell wall formation in the hull (lemma and palea), seed coat, and/or endosperm could conceivably result in altered kernel FR.

Within LOC_Os01g67580, Cypress and LaGrue had three SNP differences, a synonymous variant in an exon on chromosome 1:39278883, and two neighboring SNPs in a splice region at chromosome 1:39276841.39276842. The four Cypress-derived varieties also contained the Cypress variant at the exon SNP, making it a less likely candidate for qFIS1-2 than the two SNPs in the splice region, where Cypress was unique among the 104 resequenced cultivars as being GG as opposed to AA. Unfortunately, the resequencing study had poor read–depth coverage at the two splice sites, with 12 of the 104 cultivars having zero calls, 17 with one call, and another eight with two calls. Cypress and LaGrue both had read–depth coverage of two, Cocodrie and Mermentau were among the lines with no calls, but read depth (and therefore confidence in the sequence data) were higher for CL152 and CL261. The available sequence data did reveal polymorphisms in this gene between Cypress and LaGrue, and also between Cypress and two of its FS progeny, CL152 and CL261. LOC_Os01g67580 is annotated as a putative multidrug resistance-associated protein by MSU7 and as an ATP-binding cassette (ABC) transporter by the Rice Annotation Project Database (Sakai et al., 2013). It belongs to the multidrug resistance–associated protein subfamily of ABC transporters involved in the sequestration of toxic metabolites into the vacuole (Klein et al., 2006). It is possible that this gene affects FR by alleviating oxidative stress induced by chemicals, heat, or other environmental factors. High temperatures during grain filling are known to affect starch synthesis and decrease rice milling quality (Nagata et al., 2004; Counce et al., 2005; Lanning et al., 2011; Hayashi et al., 2015).

Cypress contains a unique exon missense SNP variant at chromosome 1:39502029 within LOC_Os01g67980. This locus is annotated as a putative cysteine proteinase EP-B1 precursor gene by MSU7 and by the Rice Annotation Project Database as the known gene REP-1. The REP-1 protein is a key enzyme in the breakdown of glutelin (the major seed storage protein in the rice endosperm) during germination (Kato and Mtamikawa, 1996). REP-1 expression in the aleurone layer is induced by gibberellic acid and inhibited by abscisic acid (Shintani et al., 1997). Cysteine proteinases, also known as thiol proteases, regulate protein turnover and thus play a role in effectively every aspect of plant physiology and development (Grudkowski and Zagdański, 2004; Salas et al., 2008). Cysteine proteinases are significant to seed germination, leaf and flower senescence, programmed cell death, resistance to biotic and abiotic stress, and seed storage protein mobilization, accumulation, and catabolism. Thus, altered levels of cysteine proteinase due to a missense mutation in this precursor gene could affect kernel FR in a number of ways, through hull, bran, or endosperm attributes.

Further study would be required to determine the gene, physiological, and biochemical mechanisms underlying qFIS1-2. The current identification of candidate genes with putative gene functions provides new testable hypotheses. Rather than relying solely on recombination and map–based cloning, this new information allows future gene identification studies to include analysis of gene function, gene expression, and/or gene editing.

Implications to Rice Breeding

The ultimate finding of this research is that individuals that had inherited the CYBT marker alleles between 38.44 and 39.72 Mb had measurably lower rates of fissuring than individuals that inherited alleles from RU12, the FS parent. This region of the genome is distal to (near to the telomere than) the sd-1 gene, and in two of these populations (36 and 37), all of the progeny inherited the non-semidwarf or the wild-type Sd-1 allele. Thus, within Populations 36 and 37, the linkage between sd-1 and qFIS1-2 was broken, with qFIS1-2 now conjoined with Sd-1. Segregation for FR among these tall (homozygous Sd-1) progeny documents for the first time that the FR imparted by qFIS1-2 does not require the altered plant architecture associated with sd-1 to impart measurable impact on kernel FR. Though still segregating for many genes, progeny from Populations 36 and 37 can be useful as FR donors to breeding programs that target development of improved Sd-1 cultivars. The fine mapping effort also provides breeders with PCR-based markers more closely and confidently linked with qFIS1-2 than those previously reported (Pinson et al., 2013).

Marker-assisted selection (MAS) has several advantages for breeders over traditional phenotypic selection. While phenotypic selection for FR cannot occur until after flowering and seed maturity, MAS can be conducted in the seedling stage, allowing breeders to select and intercross plants, speeding the process of breeding the FR trait into improved cultivars and bypassing the labor required for phenotypic evaluation. The increased yield of intact milled kernels obtained by decreasing fissuring even a few percentage points will increase productivity and profitability for both producers and millers (Siebenmorgen et al., 2008) and improve the quality of US rice being shipped abroad as paddy rice.
Supplemental Material Available
Supplemental material for this article is available online.

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References
Duitama, J., A. Silva, Y. Sanabria, D.F. Cruz, C. Quintero, C. Ballen et al. 2015b. Data from: Whole genome sequencing of elite rice cultivars as a comprehensive information resource for marker assisted selection. Dryad Digital Repository. doi:10.5061/dryad.8hg32