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Preserving Rice Quality: Fine-Mapping and Introgressing a Fissure Resistance Locus

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Preserving Rice Quality:
Fine-Mapping and Introgressing a Fissure Resistance Locus

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ABSTRACT

Rice (*Oryza sativa L*.) kernel fissuring is a major concern of both rice producers and millers. Fissures are small cracks in rice kernels that increase breakage of kernels when milled, and decrease the value of processed rice. This study employed molecular gene tagging methods to fine-map a fissure resistance (FR) locus found in 'Cybonnet', a semidwarf tropical *japonica* cultivar, as well as transfer this trait to rice genotypes of taller, non-semidwarf plant height that are better adapted to some rice production systems. Three QTLs for FR were previously reported; the FR locus with strongest effect resides near the semidwarf *sd-1* locus on the long arm of chromosome 1, explaining associations observed between increased FR and reduced plant height. This study began with F_2 progeny from a cross between a U.S. inbred breeding line with non-semidwarf (*Sd-1/Sd-1*) plant height and poor milling yields, and Cybonnet, which is semidwarf (*sd-1/sd-1*) and noted for having improved milling quality due to increased FR. Simple sequence repeat (SSR) molecular markers were used to select 11 F_2 progeny plants that retained at least one copy of the *Sd-1* allele, but also contained evidence of genetic recombination in the region of chromosome 1, known to contain *Sd-1* and *qFIS1-2*, so that the positon of *qFIS1*-2 relative to *Sd-1* could be determined more precisely, and so that FR allele could be recombined with the *Sd-1* allele. Three of the 11 selected plants were also homozygous at the two known FR QTLs that are not closely linked to *sd-1;* another four plants were homozygous at one but not both of the two additional FR loci. The $F_{2:3}$ progeny generated were genotyped prior to being phenotyped; only individuals homozygous for the new

recombination underwent extensive evaluation for FR. Progeny from three of 11 populations have been phenotyped. Marker-trait linkages observed in the first two populations indicated that *qFIS1-2* resides distal to RM1068. Research efforts were then focused on just those populations whose recombination points were distal to RM1068 (i.e., at a base pair location higher 1:38439184). Results from the three populations observed to date indicate that the *qFIS1-2* locus resides distal to RM1068 at 1:38439184 but anterior to RM3482 at 1:39720039, or approximately 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 linked to, but not pleiotropic with, *sd-1* and thus can be recombined with *Sd-1* during introgression breeding to increase the FR of rice cultivars having non-semidwarf stature.

ACKNOWLEDGMENTS

I would like to express my humble gratitude to all who were involved with this project. Chiefly, I would like to thank to my Major advisor Dr. Karen Moldenhauer for her patience and her flexibility, as well as her advice and guidance through my graduate program. I would also like to extend my warmest gratitude to Dr. Shannon Pinson for her contribution of preliminary research and methodology. Her research on fissuring was the catalyst for my project, allowing me to build on her finding. I would also like to thank Dr. Terry Siebenmorgen for his generosity in permitting me to use his equipment in the University of Arkansas Rice Processing Program Pilot Plant. Additionally, I appreciated Dr. Esten Mason's insight and advice as my on-campus advisor. I am grateful that he included me in his lab and wheat breeding operation, which gave me a second lens by which to understand row crop breeding. I would also like to include a fellow committee member Dr. Richard Norman for his support and cooperation throughout my graduate studies. Finally, I would like to thank my family both my mother and father, Jackie and Joe Sater for supporting me and encouraging me to never stop learning.

DEDICATION

I dedicate this work to John and Laura, Hannah and Sarah. Always remember the value of hard work and education, but never let that stop you from pursuing the wild adventures that inhabit your imagination.

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NOMECLATURE

cM - Centimorgan

h – Hours

HRY – Head rice yield

FR - Fissure resistance

FS – Fissure susceptible

Mbp – Mega base pairs

MC – Moisture content

QTL – Quantitative trait locus

RH – Relative humidity

RIL – Recombinant inbred lines

Sd-1 – Wild type, non-semidwarf, or standard plant height allele at the *sd-1* locus for the GA 20-oxidase gene

sd-1 – Mutant recessive allele for semidwarfism at the *sd-1* locus for the GA 20-oxidase gene

I. INTRODUCTION

Rice (*Oryza sativa*) is one of the most important crops on the planet. The carbohydrates from rice comprise 20% of all calories consumed by humans on a daily global basis (2004). The majority of rice is grown and consumed in Asia. Nevertheless, rice is the dietary staple of many developing countries, where it supplies more calories per unit of currency than most other food sources. Stable rice prices help ensure that the working poor and farmers are not subject to the poverty traps that result from food shortages or spikes in food prices (Dawe and Peter Timmer, 2012). Thus, production of rice at affordable prices enables some economic mobility for many of the most impoverished citizens of the world.

In 2009 in the United States alone, rice production represented a three billion dollar industry (Richardson and Outlaw, 2010). However, rice is grown in highly varied conditions from small independent farms to thousand-acre family conglomerates on six continents. In most Asian countries the production scale is small with farms averaging less than two hectares (five acres) (Hossain and Narciso, 2004). For the majority of these farms the most important objective is to produce enough to feed one's own family; while in the United States, Australia, Europe and some parts of South America farming occurs on a much larger-scale (Hossain and Narciso, 2004). The primary objective of these large-scale farms is to produce as much high-yielding, good quality rice as possible to sell to millers and distributors who will subsequently mill the grain and or trade on the world commodity market. Approximately 40% of long-grain rice produced in the U.S. is exported to other countries (Childs, 2012). In order to maximize

returns it is important to maintain the integrity of American rice because quality determines the value of rice on the world market (Childs, 2013). While rice is regarded as a commodity, the country of origin factors into the price and competitiveness of all internationally-traded rice. Rice from the United States has been hailed for its quality and subsequently fetches a higher price on the export market than rice from many Asian countries, specifically Vietnam (2011). However, in recent years complaints regarding the quality of American rice have arisen from countries that have purchased un-milled, or paddy rice, according to the USA Rice Federation (2015). These complaints with regards to quality could have a negative impact on the demand, or the competitive pricing, of U.S. rice on the world market. Thus, it is essential that U.S. rice farmers have the tools and technology needed to produce high quality rice.

In the United States, there are six major rice producing states; Arkansas, California, Louisiana, Mississippi, Missouri, and Texas. Rice has been adapted to different climates, but production of rice is still restricted to areas of the country with tropical summer weather, with the exception of Northern California where low humidity and nighttime temperatures render it temperate-climate status in spite of its very high daytime temperatures. Farmers in Southern states primarily grow long-grain rice in the Mississippi Delta, where summer rains and daily fluctuations in humidity are common. Fluctuations in atmospheric moisture present a problem when rice is maturing on the panicle and can ultimately diminish the quality of rice before and during the milling process.

Some cultivars of long-grain rice are believed to have a genetically-inherited mechanism, which functionally prevent them from fissuring or cracking when exposed to

fluctuations in humidity, ultimately resulting in fewer broken kernels after milling (Jodari and Linscombe, 1996). Identifying the genes that are responsible for this phenotypically superior trait would be of great interest to rice breeders who could then utilize marker-assisted selection (MAS). In order to build confidence in the use of linked markers for breeding purposes, markers reported as linked to a desired trait must first be validated in additional environments and genetic backgrounds.

A long-term goal is to develop breeding lines with fissure resistance combined with other kernel qualities, and with high agronomic yield. This study seeks to verify the existence and genomic location of a previously-identified fissure resistance quantitative trait locus (QTL) and separate this trait from the semidwarfism allele which it has been linked to in U.S. Rice cultivars (Pinson et al., 2013; Pinson et al., 2012). Because other kernel qualities, such as kernel shape, can influence the likelihood of fissuring (Jindal and Siebenmorgen, 1994; Wadsworth et al., 1982), grain shape was monitored in the present study.

Objectives

1. To develop a segregating population with recombination in the targeted FR QTL, *qFIS1-2* region in order to validate, and fine-map, the *qFIS1-2* region.
2. Obtain an FR recombinant line with *Sd-1* for non-semidwarf plant height for breeding purposes.
3. Identify whether differences in kernel dimensions are also associated with recombination of markers in the *qFIS1-2* region and whether fissure resistance correlates to any kernel dimensional traits.

II. LITERTURE REVIEW

Origin of Domestication

Most cultivated rice belongs to the species *Oryza sativa*, which is a true grass. African rice, *Oryza glaberrima*, was subject to a separate domestication event(Siebenmorgen et al., 2003). According to Molina et al. (2011), *Oryza sativa* domestication occurred in a tropical, southern region of China approximately 10,000 years ago. However other researchers contend that the two large subspecies of *Oryza sativa*, *indica* and *japonica*, underwent separate domestication events (Sweeney and McCouch, 2007). These two primary subspecies compose most of the cultivated rice around the world. *Indica* rice is the more widely grown. It is generally cultivated in tropical environments, primarily southern Asia. *Japonica* rice is more adapted to growing in more temperate-climates such as those in the highland northern regions of China, Japan, Korea, and the United States. As grouped, the *japonica* rices are considered more cold tolerant than *indica* rices

While *indica* rice is more widely grown, *japonica* rice plays an important role in the world market. *Indica* and *japonica* cultivars differ, as a groups, in many physical traits including kernel dimensions. *Indica* grains are generally long and thin. *Japonica* rice tends to be rounder and wider than *indica* kernels. Most short and medium grain cultivars belong to the *japonica* subspecies. Exceptions in each direction are not uncommon, however, with several *indica* lines produce short, wide kernels, and several *japonica* cultivars produce long slender kernels. The

kernel dimensions may ultimately affect milling quality of the grain. Studies by Lu and Siebenmorgen (1995); Siebenmorgen and Qin (2005) showed that length and width did not affect overall kernel strength under milling pressure, but thickness of the kernel did contribute to fewer broken kernels upon milling.

In general, the short and medium grain *japonica* rice cultivars have lower amylose than the long-grain *indica* cultivars (Webb et al., 1985). Grain shape and amylose content are controlled by different genes and all combinations of shape and amylose content exist and are created by breeders. However, because the globally recognized market classes, as well as consumer expectation, link shape with a specific cooked texture driven by amylose content, breeders predominantly release cultivars that fit the standard market classes. Variability for amylose content in *Indica* and *japonica* cultivars has been attributed to mutations in the *Waxy* gene that facilitates the packing of starches (Chen et al., 2010; Yamanaka et al., 2004). Rice with lower amylose content tends to have a stickier texture when cooked (Heong, 2005). Different cuisine around the world utilizes the types and textures of rice that have been locally adapted. In most of Western Asia, such as the regions of India, Pakistan and Nepal, long-grain, light (fluffy), non-clingy (or non-sticky) rice, such as Basmati, is common, whereas sticky rice is preferred in North Eastern Asian countries like Japan and Korea. The U.S. consumer tends to prefer rice that separates easily into individual kernels, similar to that of rice grown in the North East Asian countries mentioned above. However, there is a strong market for the softer, stickier cooking medium grain rice in the U.S. as well.

Although rice cultivars grown in the United States primarily have *japonica* progenitors, within the country there are two distinct types of rice grown. Long-grain tropical *japonica* rice has dominated the rice growing landscape of the Southern Delta since the early 1950's with the release of 'Bluebonnet' and 'Bluebonnet 50' from the Texas Breeding Program (Moldenhauer et al., 2004). Conversely, short and medium grain temperate *japonica* cultivars have been traditionally produced in California where water and night temperatures are cooler. According to Webb et al. (1985) long-grain rice produced in the United States tends to have a dry fluffy texture after cooking. This is because most long-grain rice cultivars in the United States have been selected to have intermediate amylose content. Short and medium grain cultivars of rice in the United States are noted for their sticky texture, a quality more similar to the rice eaten in Japan and Korea.

These texture differences also differentiate long-grain and medium grain marketability. Long-grain rice, with high to intermediate amylose content, can be cooked directly, frozen, parboiled and used in canned foods or soups (Singh et al., 2000). Medium grains, with intermediate to low amylose content, are preferred by cereal and baby food companies, as well as brewers (Webb et al., 1985). Texture and cooking quality are large factors in consumer preference. Thus, long-grain rice from the United States is purchased by countries that are familiar with or prefer the dry fluffy texture, while the U.S. medium grains are used in cereal production or exported to locations that prefer softer cooked rice texture.

In 2012 the top importers of U.S. rice were: Mexico, Japan, Haiti, Canada, Venezuela, and Turkey (Childs, 2012; Hoddle et al., 2003). U.S. rice has traditionally been acclaimed for its

reliable milling, and cooking quality because of low chalk and stable amylose content. Generally, the price of U.S. rice on the world commodity market has not experienced the extreme price fluctuations that have been common in other countries which intensively regulate and restrict their national rice sales. Some of the quality improvements that have been made can be attributed to superior cultivars, 'Cypress' (Linscombe et al., 1993) and, to some extent, 'Cybonnet' (Gibbons et al., 2006), which are known to have less breakage upon milling even when exposed to stressful environmental conditions (Bautista et al., 2009b; Cooper et al., 2008).

Breeding for Desirable Characteristics

Rice breeding focuses principally on the development of good quality, high yielding cultivars with some disease resistance and or tolerance. Most disease resistance traits can be classified as qualitative traits (Yano and Sasaki, 1997). In cases where a resistance gene's location is known, the presence or absences of the quantitative disease resistance allele can be assessed by using genetic markers in the early generations of a breeding population. However, traits like yield and grain quality are much less heritable and are more dependent on the environment. These quantitative traits are controlled by multiple genes, with small individual effects (Holland, 2007). The combination of many high quality alleles leads to superior cultivars such as Cypress. The vast majority of these rice quality genes have not yet been identified (Yano and Sasaki, 1997).

While high yield is considered a vital trait to breeders, good rice quality is also crucial. Rice quality however, is a multidimensional trait. The aspects of quality include; cooking texture and taste, kernel size and uniformity, head rice yield (HRY - which is a percentage derived from the weight of whole white rice kernels compared to the original weight of the rough rice), translucency of kernels (low chalk), percent of kernels without damage from disease or insect pests, and purity (number of weed seeds mixed into the rice, including red rice).

As previously mentioned, disease commonly affects kernels and diminishes the quality of rice. The most common diseases responsible for kernel damage include rice blast (*Magnaporthe oryzae*), sheath blight (*Rhizoctonia solani khün*), kernel smut (*Tilletia barclayana (Bref.) Sacc. & Sydinsacc*), and false smut (*Ustilaginoidea virens (Cooke) Takah*) (Siebenmorgen et al., 2003). These diseases can inhibit grain-fill, resulting in an empty hull or less-developed lighter-weight kernel. They may also lead to smaller deformed kernels, or cause spotting, or discoloration of the kernel itself, an undesirable consequence (Siebenmorgen et al., 2011).

Other quality factors considered are the color, shape and translucency of the individual kernels. Translucency is controlled by some established genes, but it is highly subject to environmental effects, which occur while the starches are developing in the kernel (Bautista et al., 2009b; Zhou et al., 2009). There is evidence that rice quality is diminished due to high nighttime temperatures during grain-fill, which can cause chalkiness (Cooper et al., 2008). Kernel chalk is the result of loosely packed starch, which also makes the kernel physically weaker. Thus, years with high nighttime temperatures have also been correlated with greater

percentages of broken kernels that ultimately reduce milling quality and the profits made by the producer (Ambardekar et al., 2011; Cooper et al., 2006; Counce et al., 2005).

Rice Processing and Grading

Rice in the United States is graded upon the criteria of overall quality and kernel dimensions. The length-to-width ratio of the grain dimensions is used to define short, less than 1.9:1, medium between 2.0:1 to 2.9:1, and long-grain rice, equal to or greater than 3.0:1 for milled rice (2014). In total, there are seven different classes of milled rice defined by the USDA; long-grain, medium-grain, short-grain, mixed, second head, screenings, and brewers (USDA, 2009). The first four classes of milled rice mentioned have greater percentages of unbroken and undamaged kernels and are also separated on the basis of kernel dimensions. They command higher prices on the rice market than the last three classes of rice. The last three classes mentioned second head, screening and brewers contain more broken kernels.

Rice harvested in the United States is milled to remove the hull, the outside high-silica content coat consisting of the lemma and palea, which accounts for roughly 20% of the rough rice mass (Prahlad Jat, 2008). Most rice is then subsequently milled to white rice by removing the bran, which includes the pericarp, seed coat, nuclleus, aleurone layers and embryo, which accounts for 8-10% of the rough rice mass (Frank and Jennifer, 2004; Juliano and Bechtel, 1985). The resulting milled kernels are sorted into head rice and brokens, where brokens are considered to be any kernel less than 75% of the length of an unbroken rice kernel (USDA,

2009), and with the whole (unbroken) milled kernels being known as head rice (HR) defined as being at least 75% of the length of an unbroken rice kernel. Head rice yield is derived from the mass of head rice divided by the original rough rice mass. Most of the rice that breaks during the milling process, known as “brokens”, is separated from HR and sold in one of the lower grades, like second heads, screenings or brewer’s rice. These lesser grades of rice are sold for as little as 60% of the price of HR (personal communication Dr. Terry Siebenmorgen, 2015).

The percentage of HR obtained from rough rice is dependent on both genetic and environmental factors. One such factor is as high relative humidity (RH) when the kernel has filled and grain is drying down to a lower moisture content (MC). Additional, environmental factors which can affect HR are high nighttime temperatures during grain development and the starch packing stage, harvest conditions, including kernel maturity and moisture content at harvest, and post-harvest conditions and processing. Furthermore, the kernels on a panicle do not all mature at the same time. The moisture content and kernel maturity at harvest are dependent on the kernel’s location on the panicle (Kunze, 2009; Li et al., 2003; Nelson et al., 2011). These qualities influence the kernel’s likelihood of fissuring, which increases the propensity to break while under the physical pressure of milling.

Rice Kernel Fissuring

Fissuring, as previously mentioned, is the development of hairline fractures within a rice kernel (Kunze, 2009; Lan and Kunze, 1996; Pinson et al., 2012). Fissures in rice that appear

before milling account for a significant proportion of the rice that breaks during milling, decreasing HRY (Kunze, 2009; Pinson et al., 2012).

In past centuries it was assumed that fissures were caused by drying rice in the sun (Kunze, 2009). Farmers referred to this phenomenon as “sun-checking.” Inquiry into the mechanism behind cracking in the twentieth century, however, revealed that fissures are not necessarily caused by drying in the sun, but rather the fluctuation in humidity that the rice experiences between the transition from day to night, or caused by precipitation (Jodari and Linscombe, 1996; Kunze, 2009; Lan and Kunze, 1996). After each kernel has filled, it begins to dry out, or lose moisture which decreases kernel moisture content. However, at night when the temperature is lower and the relative humidity (RH) is higher the drying rice kernel can reabsorb atmospheric moisture (Lan and Kunze, 1996). This process of reabsorbing moisture and then drying out during the day, also called rewetting, or rehydration, makes rice more susceptible to fissuring because of the physical stress generated by the moisture gradient within the kernel. The moisture gradient is the change in moisture concentration between cells from the exterior layers of the kernel to the interior layers. When the outer cells of the kernel expand because of the moisture that has been absorbed they create hydrostress on the interior layers of the kernel. When the interior layers of the kernel fall below a certain threshold of moisture, estimated to be roughly 15% wet basis, the kernel becomes increasingly susceptible to fissuring (Bautista and Siebenmorgen, 2008; Kunze, 2009; Thompson and Mutters, 2006). This is because the chemical configuration of the kernel becomes less flexible or pliable as it dries down and more brittle (Prahlad Jat, 2008). Kernels that are drying in the field are

particularly susceptible to rewetting after a rain or heavy dew because of the immediate soaking effect on the kernel (Siebenmorgen et al., 1998).

In order to achieve the greatest HRY, long-grain rice is harvested at approximately 18 or 19 to 21 or 22%, MC (Bautista et al., 2009a; Bautista and Siebenmorgen, 2008; Kocher et al., 1990; Siebenmorgen et al., 2007; Siebenmorgen and Lanning, 2014). The ideal MC for harvest varies distinctly among cultivars and regions. In California, where most farmers produce medium-grain rice, the recommended harvest MC is above 21%, or roughly between 22-24% MC (Siebenmorgen and Lanning, 2014). According to both Lu and Siebenmorgen, and Siebenmorgen and Lanning (1995, 2014), HRY decreases significantly when rice is harvested bellow 14-15% MC.

In reality it is difficult to harvest rice at the ideal MC because of the wide range of individual kernel MCs on a single panicle (Bautista and Siebenmorgen, 2008). There are also many tillers that compose one rice plant, and tillers on the same plant are not necessarily in the same physiological stages (Counce et al., 1996). The introduction of hybrid cultivars to the rice market in the U.S. has caused farmers to use seeding rates that are less than those used for traditional inbred lines and contributes to more tillers per plant. Tillers emerge at different times. Because tillers are at different physiological stages, some tillers may reach maturity before other tillers because grain development is dependent on the date of anthesis. On a single panicle, anthesis may vary as much as ten days with the top of the panicle filling and drying before the bottom kernels mature (Julia and Dingkuhn, 2012). In a study by Kocher et al. (1990), the distribution of kernel MCs for the cultivar ‘Katy’ (Moldenhauer et al., 1990) was

found to be skewed in which most kernels had MC less than the average MC. The same skewed distribution for kernel MC at harvest was observed in a more recent study in southern China (Li et al., 2003). Other biological and environmental factors can also influence kernel development (Chen et al., 2012). These findings further complicate the rationale by which farmers must harvest their field, and once rice has been harvested it typically must be dried to a lower MC. The ideal MC for milling and storage purposes is below 14%, between 12-13% (Fan et al., 2000). Rice stored at a greater MC is more vulnerable to fungal infection (Siebenmorgen et al., 2003; Trigo-Stockli and Pedersen, 1994).

Rapid drying of grain can also cause fissuring (Kunze, 2009). In the U.S., to decrease the MC of rough rice, it is dried in industrial-sized dryers. Different methods of drying exist, but they all share a similar function; circulating warm, dry, air through the bulk of the rough rice. The paradox of drying rice relates to the heated air, which creates a moisture gradient as moisture rapidly evacuates from the exterior of the kernel (Li et al., 1998). As the grain is exposed to the dry air, the moisture from the interior layers migrate to the exterior to resolve the gradient. (Cnossen and Siebenmorgen, 2000; Kunze, 1983; Kunze, 2009). The physical tension that exists as a result of the moisture gradient between the inner layers of the kernel and outer layers can result in a fissure that appears a few hours after drying (Cnossen et al., 2003; Kunze, 1983; Schluterman and Siebenmorgen, 2007). Drying-induced fissures are most problematic when grain is dried quickly at high temperatures (Abud-Archila et al., 2000; Kunze, 2009).

Grain Shape

Grain shape, as previously mentioned, can affect the milling process as well as the drying process. This is because kernel dimensions influence the rate at which the kernel either gains or loses moisture content. Kernel dimensions affect milling in because the ratio of length to width to thickness can affect HRY. Mills primarily differentiate and set milling standards according to three rice types: long, medium and short grain rice, but this does not account for the slight cultivar to cultivar differences in kernel shape. As an example the brown rice of the long-grain cultivar 'Saber' (McClung et al., 2004) is cited as being 6.57 mm long, 1.96 mm wide and 1.68 mm thick, whereas, the brown rice of long-grain cultivar 'Lemont' (Bollich et al., 1985) has an average length of 7.60 and width of 2.28 mm. Aspects other than kernel dimensions that also contribute to grain shape and can be measured. For instance, kernel curvature, straightness, and twistedness. These are all physical properties of an individual grain that can affect milling. Physical deformities in kernel may result from disease such as straighthead or other factors in the environment during grain fill. Although, there are some cultivars that are notably more prone to kernel shape deviations such as the "parrot beak shape." These physical irregularities in kernel shape also contribute to kernel brakeage during milling or lower overall HRY.

Thickness

The third dimension, thickness, affects how quickly or slowly rice loses or gains moisture. Thickness is thought to contributes the most to water dissipation (Fan et al., 2000). Kernel thickness is the dimension which has been most associated with fissuring and HRY (Sun and Siebenmorgen, 1993). This is because water must diffuse in and out of the kernel from the outer layer of cells. When water is absorbed by thicker kernels it must diffuse through more cell layers in order to reach the inner layers, before moisture equilibration can be achieved in the kernels. Thus these thicker kernels experience great disparities in moisture content when the environment is either extracting or introducing moisture into the kernel. When kernels were grouped by thickness smaller ranges of MC variation were observed reducing the broken kernels (Sun and Siebenmorgen, 1993). Thicker kernels have a greater moisture gradient, and the result of this exacerbated moisture gradient has been shown to have lower HRY (Jindal and Siebenmorgen, 1994). Additionally, thinner (1.6 mm) kernels often break when milled under the same conditions as other kernels (Wadsworth et al., 1982).

Medium grains, which have a smaller length to width ratio than long-grains, and generally wider kernels than long-grains, have been shown to undergo greater decreases in HRY when conditions for drying are set more aggressively (with higher heat and more airflow) (Lloyd and Siebenmorgen, 1999).

Length

Variation between kernel length within a single cultivar was not been highly correlated with HRY as observed by Lu and Siebenmorgen (1995). However, kernel length was found to be associated with HRY in a study by Yadav and Jindal (2001). Longer kernels have a greater distance over which fissures can develop. It has been proposed by Waggoner et al. (2003) that longer kernels may fissure less from the moisture gradient because pressure from the exterior layers of the kernel is dispersed over the whole distance of the length of the kernel. A shorter, wider kernel is rounder and focuses more pressure towards the center of the kernel, where longer kernel focuses pressure across the lateral access of the kernel (Figure 1).

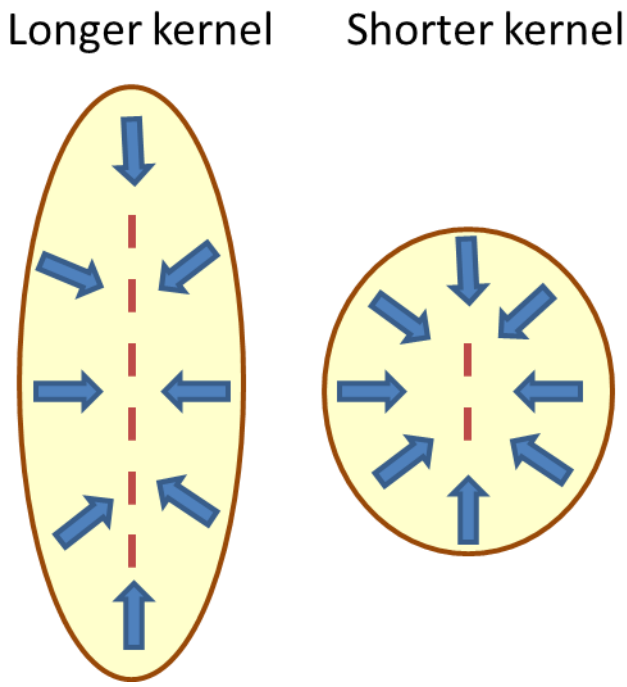

Figure 1. Depicted is the difference between long and short kernels and how shorter kernels experience more stress as a result of outer kernel swelling. The lateral access is pictured with the dotted line. In the longer kernel the pressure from the outer layers of the kernel is more spread out along the access. However, in the shorter kernel the pressure from outer layers is more concentrated towards the center of the kernel which has been hypothesized to predispose the kernel to fissuring as demonstrated by (Waggoner et al., 2003).

Rice Genetics

Rice, specifically *Oryza sativa*, is a diploid organism with twelve chromosome pairs. Results of the first entirely sequenced *de novo* rice genome sequencing projects were released in 2002. A single cultivar of both the *japonica* and *indica* subspecies were sequenced (Goff et al., 2002; Yu et al., 2002). These collaborative efforts marked the first completed sequencing projects of all food crops. The relatively brief time it took to prepare the first two rice genome draft sequences was possible because the rice genome is the shortest of all the cereal grains: 466 mega basepairs (Mbp) in *indica* with 46,022 to 55,615 genes, and 420 Mbp in *japonica* with 32,000 to 50,000 genes (Goff et al., 2002; Yu et al., 2002). Prior to the sequencing of the entire rice genome, multiple biparental linkage maps had been constructed by Cornell University and other institutes (Causse et al., 1994; Harushima et al., 1998; Singh et al., 1996). Researchers are now able to reference specific areas of the genome based on these completed published maps. Because of the relative simplicity of the rice genome and its importance to global food security, rice has been used as a model organism for other crop plant systems and genetics research.

One of the most well documented rice genes corresponds to the semidwarf trait, which enabled the Green Revolution in the 1960s and 1970s. This semidwarf allele of the gene *sd-1* was introduced to the germplasm in the U.S. from IR-8 whose *sd-1* allele originated from the cultivar Dee-Gee-Woo-Gen. The allele is the result of a mutation that creates a defective 20-oxidase gibberellic acid biosynthetic enzyme which, in turn, causes a deficiency of active gibberellic acid in the elongating stem (Causse et al., 1994; Kim et al., 2009). The resulting

phenotype of this recessive allele is easily selectable in rice because it causes visibly shortened plant stature, and it has been mapped at 38.7 Mbp on chromosome 1 where molecular markers for its presence are well established (Kim et al., 2009; Spielmeyer et al., 2002). Not all U.S. cultivars are semidwarf. One primary advantage of semidwarf plant height is lodging resistance, which can be mitigated by stiff straw strength in some U.S. cultivars. Arkansas, in particular, has developed many non-semidwarf cultivars. These cultivars do not have the *sd-1* mutation, and instead generally have stiff straw strength to prevent yield loss due to lodging (Kim et al., 2009). Consequentially, Arkansas rice acreage is only about half semidwarf cultivars (Wilson, 2012).

Cypress, Parent of Cybonnet

Cypress is a semidwarf, tropical *japonica* cultivar grown in the Southern United States with stable as well as high HRY potential (Nelson et al., 2011; Oard et al., 2010; Venu et al., 2011), which is ostensibly the result of having a genetic mechanism for fissure resistance (FR) trait (Pinson et al., 2012). The specific mechanism that facilitates FR in Cypress is a function of the hull, which has been reported to be less permeable or more watertight or than in other cultivars, protecting the grain from external fluctuations in RH (Pinson, 2004; Waggoner et al., 2003). Because Cypress has been determined empirically to have stable high milling quality, it has been used as a parent in crosses that have produced 'Trenasse' (Linscombe et al., 2006) and Cybonnet according to Pinson et al. (2012). Of these two released cultivars, only Cybonnet is thought to have conserved the FR trait (Pinson et al., 2012). While breeders would like to utilize

the FR of Cypress, now also in Cybonnet, the FR trait is difficult to phenotype, and thus difficult to select. Without a way to select for FR, it has not been incorporated into modern cultivars which thus tend to have variable HRY dependent on grain MC. Therefore, even though Cypress and Cybonnet have stably high HRY, in recent years they have been replaced by newer cultivars, often hybrids, which have higher yield potential, but one study has showed they have lower HRY (Blanche et al., 2009).

Both Cypress and Cybonnet are homozygous for the recessive *sd-1* allele. While semidwarf cultivars are common in most of the United States, Arkansas producers still opt to grow taller cultivars of rice on much of their acreage (Kim et al., 2009).

Quantitative Trait Loci

Quantitative trait locus (QTL) analysis is a component of genetics research wherein regions on a chromosome are identified as containing genes contributing to a phenotypic trait in a given species. QTL analysis is used to compare populations of individuals with varying phenotypes in conjunction with their genotypes. Genotypic data is acquired using molecular markers such as single nucleotide polymorphisms (SNPs) and simple sequence repeats (SSRs) spaced throughout the genome in order to detect regions of heterogeneity between individuals in a population. These regions of genetic variation are then regressed against each individual's phenotype to establish the logarithm in base 10 of odds (LOD). The LOD is a function of likelihood or probability that a genetic locus is mathematically correlated to a particular

phenotypic trait. Identifying a significant QTL is the first step in determining the genes that contribute to any given quantitative trait.

Rice Quality QTL

One method for discovering QTLs in the rice genome is to use mapping populations created by crossing two pure-breeding parents and studying the varied recombination in the selfed progeny. When the progeny have been selfed or inbred until they are predominantly homozygous, the mapping populations are referred to as recombinant inbred lines (RILs). The later, more homozygous and thus more replicable generations ($F_5 – F_8$) of RILs are usually used to identify genetic linkage to explain phenotypic variation. A study of progeny from a cross involving an *O. glaberrima* and *sativa* parent line (V20A) suggested that by backcrossing V20A with *glaberrima* rice they were able to introgress favorable quality traits, from the *glaberrima* parent such as longer grain length and higher protein. As previously discussed most grain quality traits are quantitative, and thus are controlled by multiple genes. However, identifying key genetic loci that contribute a significant portion of variation for a particular trait has the potential to incrementally increase the trait of interest.

In other previous population studies, probing for HRY and QTLs affecting rice fissure resistance (FR QTLs), crosses were made between Cypress X 'Panda' (Kepiro et al., 2008), 'L-204' X 01Y110 (Nelson et al., 2012), Cypress X RT0034 (Nelson et al., 2011) and Cypress X 'LaGrue' (Moldenhauer et al., 1994; Nelson et al., 2011; Pinson et al., 2012). In all of these

studies one parent used in the cross had been determined empirically to have stably high HRY or FR, and the other parent had variable HRY, identified as fissure susceptible (FS). For most of the populations evaluated for FR, Cypress was the FR parent. In each of the studies the two parents were used as checks or controls, and the segregating progeny or RILs were phenotyped for the grain quality traits of interest. The phenotypes of the individuals were regressed against the different markers used to characterize the progeny genotypes. When a region tagged by one or more particular markers is found to be highly associated with high HRY, or a low rate of fissuring, it is considered a positive QTL. The researchers use the genotype of the checks to see which parent contributed genetic material at that locus.

Because HRY is a quantitative trait, breeding two lines with different average HRYs, Cypress and LaGrue, resulted in a population with a normal distribution for HRY, as demonstrated by Oard et al. (2010). Within the population, progeny exhibiting transgressive phenotypes were present on both ends of the distribution (i.e., for more and less fissuring). This study clearly demonstrated the quantitative nature of HRY.

Fissuring and HRY are inversely correlated because a greater percentage of fissured kernels results in low HRY, or a low percentage of fissured kernels results in a high HRY (Cnossen et al., 2003). In the study conducted by Nelson et al. (2011) numerous quality traits were assessed in two RIL populations, MY1 (Cypress X RT0034) and MY2 (Cypress X LaGrue) in order to locate QTLs. Specifically, these researchers were looking for the QTLs for HRY and other traits believed or known to contribute to HRY, including the percentage of fissured kernels. While they did not identify any QTLs for fissuring per se, four QTLs for HRY were

discovered in the MY2 population, one of which is located on chromosome 1 succeeding the *sd-1* region.

As previously stated, HRY is directly affected by the percentage of fissured kernels (Cnossen et al., 2003), but there are a number of other factors, or confounding variables, that can affect HRY such as kernel moisture content at harvest, percent of immature or thin kernels, chalkiness, drying method and milling pressure (Nelson et al., 2012; Nelson et al., 2011; Pinson, 2013). While these factors directly influence HRY, it is difficult to distinguish which factor or factors have the greatest effect and to quantify how much interaction occurs among factors. Conversely, studying a smaller or specific component of HRY, such as fissure resistance under rewetting conditions, permits the analysis of the specific genetic conditions that enable cultivars such as Cypress and Cybonnet to express FR.

In a study that followed Nelson et al. (2011) performed by Pinson et al. (2012) involving early-generation (F_2 and $F_{2:3}$) Cypress X LaGrue Progeny, transgressive segregation for the FR trait, which is a critical factor that contributes to high HRY, was not observed at a significant level. Additionally, in a sample of the 10% most FR individuals, markers flanking the *sd-1* gene were found to be homozygous for Cypress alleles. This is one of the same regions that was identified as containing a QTL for high HRY originating from Cypress in the MY2 (Cypress X LaGrue) population studied by Nelson et al. (2011).

It is important to note the potential linkage between *sd-1* and loci associated with FR in the genotype of Cypress. Stated differently, one or more genes controlling the FR inherited from Cypress appeared to be linked with the semidwarf gene. This hypothesis was further

corroborated by the contrasting high proportion of LaGrue alleles present in the most FS individuals in the F_2 and F_3 populations (Pinson et al., 2012). Further inquiry by Pinson et al. (2013) into the underlying genetic mechanisms of FR detected a QTL in the region of the long arm of chromosome 1 known to contain the *sd-1* gene. Additionally, Pinson et al. (2013) detected association between FR and two other genomic regions, one on the short arm of chromosome 1, another on chromosome 8. These three loci were identified in two populations, early generation Cypress x LaGrue progeny, and a set of Cybonnet x Saber RILs. Future research is necessary to fully understand the location of the FR trait and its proximity to *sd-1*, and what, if any, epistatic and additive effects these 3 loci impose.

Fissure Resistance Phenotyping

Studying a phenotypic trait such as FR is difficult on multiple levels. First, one must establish multiple environments in order to find reliable QTLs as opposed to false QTLs, which may result from using too few locations, years, or replications. This is because there are gerat variations in the percentage of kernels that fissure between years and locations in the same inbred line or cultivar. This variation that occurs from year to year or location to location is considered environmental error. Additionally, there may also be error due to genotype by environment interaction (G X E interaction). G X E interaction can be identified by inconsistencies between the performances of the same genotype in different environments, such as a genotype that outperforms other genotypes in some, but not all environments.

Sample-to-sample variability also confounds FR estimates. Finally, the phenotyping process used to induce fissuring is labor intensive. The method used by Pinson et al. (2012, 2013) required standard drying of grain carefully harvested at optimum maturity, followed by several months of storage. Then 50-kernel rice samples collected from the mature, but not over-mature (potentially pre-fissured) tips of carefully harvested panicles (avoiding immature kernels from panicle bottoms). Next panicles were subjected to dry heat at 45°C for 0 - 7 hours before being exposed to high humidity (95-100% RH) in a growth chamber for 16 hours to induce repeatable levels of rewetting of the kernels. Lastly, the grains were dried for 24, 48, and 72 hours, although the length of this final drying time was not found to impact the rate of kernel fissuring. During the Pinson et al. studies (2012, 2013) it was determined that grains of the same cultivars, but grown in different years or locations, did not fissure the same when exposed to identical laboratory fissure-inducing regimes. It appears that some seed-production conditions cause grains to be more, or less, susceptible to fissuring when rewetted. The result being that the fissure evaluation technique must be adjusted and optimized for each grain harvest. Otherwise the fissure-induction system may be too harsh, causing all grain samples to become highly fissured, negating the ability to detect a phenotypic difference between the FR and FS check cultivars. Conversely, if the fissure-induction system is too gentle for a particular harvest of grains, then neither the FS nor FR check cultivars fissure significantly. To enhance ability to detect genotypic differences, (Pinson et al. 2012) tested multiple preheat and humidity exposure times in order to identify those that maximized the ability to detect differences between the FR and FS check cultivars for each replication of grain-harvest.

Once grain had undergone fissure induction and dried it was subjected to dehusking and each kernel evaluated for fissuring. Each sample of 50 grains was run though the dehusker twice. Because the sample contained only fully mature, undispersed kernels and not immature or insect damaged ones, it was reasonable to consider all broken kernels to have fissured. The remaining whole kernels were examined visually to identify any fissures that existed even if they were not severe enough to cause the kernel to split under the pressure of the dehusker. Use of polarized light at this stage makes the fissures more detectable by the naked eye. The total number of whole kernels without fissures were divided by the number of kernels present in the initial sample (50) yielding the percentage of unfissured kernels present in the sample. Genotypes with more unfissured grains were ranked as more FR than genotypes with many fissured kernels. Recognizing that grain quality is a determinate factor in the market price of rice, opportunity exists to study the functional and genetic mechanisms of fissuring that cause a reduction in HRY. Once the location of the genetic loci responsible for FR are determined and molecularly tagged, present and future plant breeders can use MAS or genotypic selection technologies to select improved cultivars for overall grain quality, the ultimate objective of the related breeding program.

III. PRESERVING RICE QUALITY: FINE-MAPPING AND INTROGRESSING A FISSURE RESISTANCE LOCUS

Shorter version of abstract - 50 words

This study used laboratory induced kernel fissuring to fine-map a fissuring QTL on the long arm of chromosome 1. The fissure resistance (FR) conveyed by the single QTL was found to be significant. In addition, linkage with the nearby *sd-1* allele for semi-dwarfism was broken, with some progeny containing FR and standard height *Sd-1* alleles.

ABSTRACT

Rice (*Oryza sativa L*.) kernel fissuring is a major concern of both rice producers and millers as it increases breakage during milling and decreases the value of processed rice. This study employed molecular gene tagging methods to fine-map a fissure resistance (FR) locus found in 'Cybonnet', a semidwarf tropical *japonica* cultivar, as well as transfer this trait to rice genotypes of non-semidwarf stature better adapted to some rice production systems. Three QTLs for FR have been previously reported, with the FR locus linked with the semidwarf *sd-1* locus on the long arm of chromosome 1 having the strongest reported effect. For fine mapping, F_2 progeny were developed from a cross between U.S. breeding line STG08L-42-005 with non-semidwarf height (*Sd-1/Sd-1*) and poor milling yield, and Cybonnet (*sd-1/sd-1*) with semidwarf height and containing the FR locus. Simple sequence repeat (SSR) markers were used to select F_2 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*. In total 11 plants were screened which included three plants homozygous at two known FR QTLs not closely linked to *sd-1* and four plants homozygous at one but not both of these FR loci. The $F_{2:3}$ progeny were genotyped prior to being phenotyped with only individuals homozygous for the new recombination evaluated for FR. Progeny from three of 11 populations were phenotyped. Marker-trait linkages observed in the first two populations indicated that *qFIS1-2* resides distal to RM1068. Research efforts were then focused on just those populations whose recombination points were distal to RM1068 (i.e., at a base pair location higher than 1:38439184). Results from the three populations indicate that the *qFIS1-2* locus resides distal to RM1068 at 1:38439184, but anterior to RM3482 at 1:39720039, or approximately 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 linked to, but not pleiotropic with, *sd-1* and thus can be recombined with *Sd-1* during introgression breeding to increase the FR of non-semidwarf rice cultivars.

INTRODUCTION

Rice is an economically important crop in the state of Arkansas, and Arkansas growers accounted for 51% of total rice production in the United States and 59% of all long-grain rice production in 2015 (Childs, 2015). Rice is traded globally as a commodity crop. When rice is

processed in mills it is graded according to physical qualities. Higher quality classes of rice are sold for higher prices, increasing the profit for farmers as well as mills. Because U.S. rice has a reputation for superior grain quality, it is sold globally at prices much higher than rice from other rice exporting countries. Currently, long-grain milled rice from the U.S. sells for 140% the price of premium Thai rice and 154% Vietnamese rice (Childs, 2015).

Once rice has been harvested it is dried to roughly 12.5% moisture content (MC) before being taken to a mill for processing (Lloyd and Siebenmorgen, 1999). Drying to a specific MC is important because MC can affect the ease of milling the outer layer of the kernel (Lanning and Siebenmorgen, 2011; Lloyd and Siebenmorgen, 1999; Siebenmorgen and Lanning, 2014). The first phase of milling is the removal of the hull, the outside papery layer that protects the kernel. Then the bran, the outer fatty brown layer, is polished away yielding the final product, white rice. White rice is then sorted into head rice (HR) and broken rice. A broken kernel is defined as being less than ¾ the length of an intact kernel (USDA, 2009). Sorting kernels into head and broken rice is necessary because rice consumers prefer intact kernels. The majority of broken kernels are separated and sold as lower grades and subsequently can be priced as much as 60% lower than the price of HR (USDA ERS 2015). After the process of milling paddy rice into head rice a calculation is performed to obtain the head rice yield (HRY). HRY is the percentage of whole white kernels/paddy rice in total weight and is not necessarily correlated to overall rice yield (Gravois, 1998). The HRY varies among farms and years, and for different cultivars of rice. Low HRY results from a high proportion of kernels breaking during milling and this constitutes a financial loss for both farmers and millers.

There are several reasons why kernels break during milling. Common causes include:

1. Chalkiness, caused by the loose packing of starch during grain fill which subsequently weakens the kernel (Bautista et al., 2009b; Lanning et al., 2011).
2. Disease or insect damage, which can interfere with kernel development and grain filling and cause the kernel to be misshapen, weak, and in the case of insect damage pecky.
3. Kernel dimensions, length, width and thickness are the physical dimensions which can influence the breaking force of the kernel. Thin kernels in particular have been associated with lower HRY values (Jindal and Siebenmorgen, 1994).
4. Fissuring, which is a stress fracture that develops in either the inner or outer layers of the kernel. Fissured kernels are highly susceptible to breaking when exposed to outside force or pressure (Kunze, 2009; Siebenmorgen et al., 2009; Zhang et al., 2005) such as that experienced during the milling process.

Investigation into kernel fissuring has revealed multiple environments which cause kernels to be vulnerable to fissuring, and shows that the critical time points are primarily during in-field dry down prior to harvest, and post-harvest drying (Kunze, 2009). Kernel fissuring is caused by rapid diffusion of moisture into or out of the kernel after the starchy endosperm has dried and hardened to a point where it is no longer pliable. Rapid moisture diffusion is caused by environments where the relative humidity (RH) is very high or very low (90-100%, very low <10%) (Siebenmorgen et al., 2009). The absorption of water by the outer layer of cells in the kernel is called rewetting (Kunze and Choudhury, 1972; Kunze, 2009), and can be caused by diurnal shifts in RH. During the day RH is low which causes gradual drying or moisture loss

within the kernel, then an overnight increase in RH causes the outer layers of the kernel to reabsorb moisture and swell (Lan and Kunze, 1996). When the outer layers of the kernel swell they exert an inward directed pressure on the interior layers of the kernel. In immature kernels, the endosperm is still wet and pliable, but in more mature kernels, the endosperm is drier and brittle, making them more susceptible to fissuring. When the tension from the swelling outer layers exceeds the kernel's tensile strength a hairline crack, or fissure, forms (Kunze and Choudhury, 1972; Kunze, 2009; Lan and Kunze, 1996; Prahlad Jat, 2008).

Field fissuring in rice generally occurs when average kernel MC falls below 15% wet basis (Bautista and Siebenmorgen, 2008; Siebenmorgen et al., 1998). Ideally farmers harvest before this threshold. The advised range for long-grain rice harvest in Arkansas is 19-21% MC (Bautista and Siebenmorgen, 2008; Kocher et al., 1990; Siebenmorgen and Lanning, 2014). However, even if a farmer harvests within this MC window not every kernel will be at this ideal harvest MC because kernels at the top of the panicle are more mature than kernels at the bottom of the same panicle. Rice panicles range from 18.57 to 34.23 cm in length (Soni et al., 2014) and flower over four to 10 days from tip to the bottom (Julia and Dingkuhn, 2012). Additional differences in anthesis dates occur between late and early flowering tillers of individual rice plants. Among different plants in a single field there can be a 10 to 14 d difference between plant anthesis (Yoshida, 1981). Thus, even though farmers may successfully harvest a field of rice at 18-21% average MC, some kernels will be below 15% MC, the threshold at which fissuring after rewetting becomes likely.

In order to maintain high standards of rice quality and high HRY it is important that kernel fissure rates be kept to a minimum. The environmental conditions which cause rewetting and fissuring in the field are not easily controlled, therefore, the solution to preventing loss of rice quality as a result of fissuring is for rice breeders and farmers to select and plant cultivars of rice which are less susceptible to 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 (Jodari and Linscombe, 1996; Pinson, 2004). These findings suggest that genetic differences between cultivars account for their different phenotypes; genes that could be exploited by breeders.

Susceptibility to kernel fissuring is difficult to phenotype. It must be done postharvest, and is not utilized by breeding programs across the Southern U.S. Identification of molecular markers linked to FR loci would allow breeders to identify FR progeny using marker assisted selection (MAS). The premier Southern U.S. cultivar credited with FR is Cypress (Oard et al., 2010; Nelson et al., 2011; Nelson et al., 2012; Pinson et al., 2012, 2013). Cypress is also the maternal parent of the cultivar Cybonnet which exhibits FR qualities as well (Gibbons et al., 2006; Pinson et al., 2013). These two long-grain tropical *japonica* cultivars have been the donor parent in several populations used to map FR QTLs using marker trait linkage. Pinson et al. (2013) identified three FR QTLs in two populations; one derived from Cypress, the other from Cybonnet, with all identified FR alleles originating from Cypress or Cybonnet. Because Cybonnet is believed to have inherited its FR from Cypress, the genetic mechanism for the FR phenotype is likely conserved (Blanche et al., 2009; Pinson et al., 2013).

Among the three FR QTLs identified by Pinson et al. (2013), *qFIS1-2* had the highest probability and phenotypic effect. Within the Cypress X LaGrue $F_{2:3}$ population, the QTL was found on the long arm of chromosome 1 between 36.6 and 40.7 Mbp on the Gramene Annotated Nipponbare Sequence 2009, with a LOD peak nearest to RM6292 (39.2 Mbp) (Pinson et al., 2013). This predicted *qFIS1-2* to be closely linked, but distal (nearer to the telomere), to the semidwarf gene *sd-1* which is located at 38.4 Mbp (Gramene Annotated Nipponbare Sequence, 2009). Both the Cypress and Cybonnet genomes have the mutant semidwarf allele (*sd-1*) (Linscombe et al., 1993; Gibbons et al., 2006). Thus, in both these FR lines, the FR QTL and the allele for semidwarfism are genetically linked on chromosome 1, which was also observed in Pinson et al. (2012)

This experiment sought to use additional cross progeny to validate and more precisely map the location of the *qFIS1-2* locus. Taller rice cultivars are desirable for certain production conditions; therefore, a second objective was to identify one or more recombinant progeny in which the wildtype or non-semidwarf allele (*Sd-1*) was now linked with FR. This study also investigated if integration of *qFIS2-1* was associated with difference in any grain dimensions because slight differences in grain dimensions have been found to affect kernel FR.

MATERIALS AND METHODS

Germplasm Utilized

A cross was made in 2011 between the advanced Arkansas breeding line STG08L-42-005 (hereafter called RU12) and Cybonnet (CBNT) with the female parent, RU12, selected for several desired agronomic properties in a line of standard height (*Sd-1*). Cybonnet was selected as the FR parent and used as the male due to ease of crossing. Both of the parents have cytoplasm derived from 'L-202' making cytoplasmic effects irrelevant. The F_2 progeny were grown as 135 individual plants in summer of 2012 in research field plots at the University of Arkansas Rice Research and Extension Center (UAR RREC), Stuttgart, AR.

Genotyping and Selecting F_2 Progeny

Leaf tissue samples collected from of each F_2 individual in the vegetative stage were frozen and processed for total genomic DNA using a modified CTAB/chloroform method (Williams and Ronald, 1994). Twelve PCR-based simple sequence repeats (SSRs) markers (Fig. 2) were chosen to saturate the *qFIS1-2* QTL region reported previously by Pinson et al. (2013). Six additional SSRs were used to molecularly tag the other two FR QTL regions reported by Pinson et al. (2013). Among the 135-F_2 progeny, 11 were determined to have favorable recombination in the region of the long arm of chromosome 1 flanked by RM5501 and RM529 reported to contain both *sd-1* and *qFIS1-2* (Fig. 3). New recombination in this region among F_2 plants was identified by homozygosity of marker alleles at one end of the QTL region with heterozygous markers at the other. Allelic composition at RM1339, known to be closely linked with *sd-1* (Sharma et al., 2009) was used to verify that selected F_2 individuals contained at least one *Sd-1*

allele. Homozygosity at one or both of the two other known FR QTLs (determined using linked markers, Fig. 2) was also considered in the selection of 11 F_2 plants to continue to the F_3 generation, though marker monomorphism prevented precise monitoring of the QTL region on chromosome 8.

In the summer of 2013, each F_3 plant from the 11 $F_{2:3}$ populations selected for having genetic recombination between RM1339 and RM529 was numbered individually using flags during the late stages of vegetative growth. Tissue samples were harvested from each plant. Harvested tissue samples were stored at -80° C until DNA extraction was performed using a rapid high-throughput method (Xin et al., 2003). Extracted DNA was stored at 4°C until it was used for PRC amplification

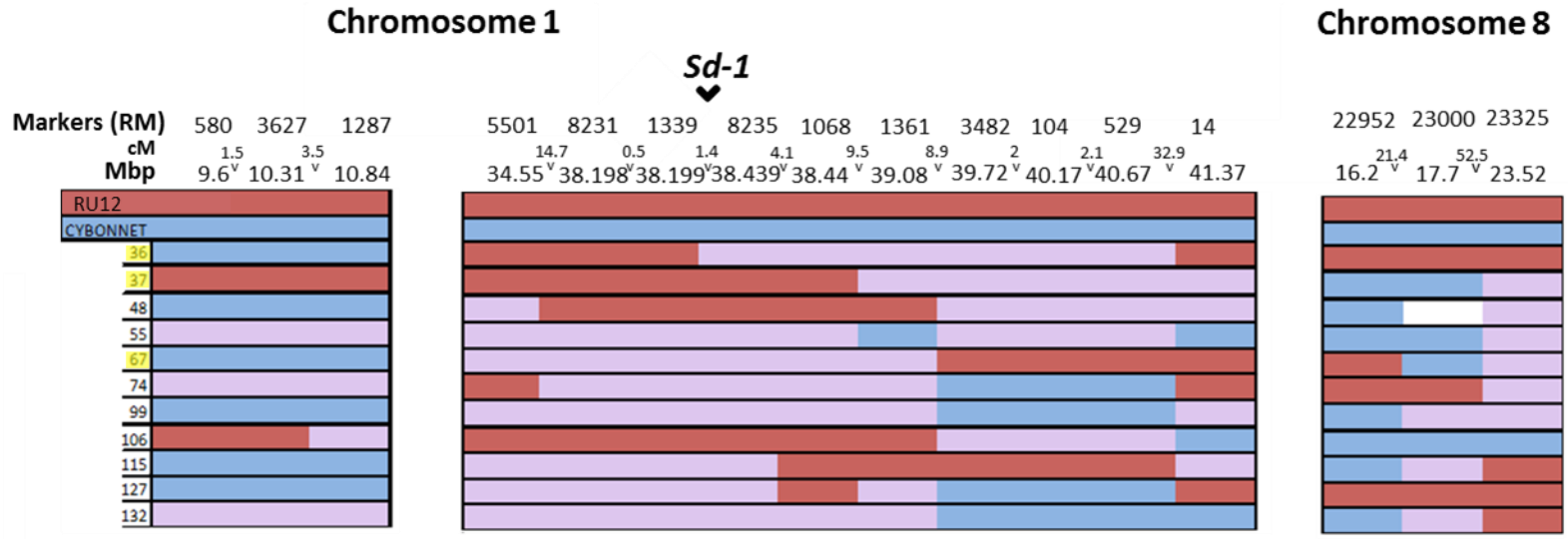

Figure 2. The genotypes of 11 F_2 plants found to contain recombination in or near the *qFIS1-2* QTL region, as needed for fine-mapping of this QTL, were determined using the indicated SSR markers, whose names all begin with RM. Red regions are homozygous for maternal, RU12, marker alleles. Blue regions are homozygous for the paternal, CBNT marker alleles. Purple regions are heterozygous. This map was constructed using the 18 SSRs in the three separate genomic regions depicting the 3 FR QTL regions identified by Pinson et al. (2013). The yellow-highlighted F_2 numbers are the three F_3 populations that were eventually genotyped and phenotyped. Mbp locations are per the Gramene Annotated Nipponbare Sequence (2009); The Haldane cM distances were calculated using the recombination rates observed in the present CBNT/RU12 F_2 population.

$F_{2:3}$ Plant Growth and Harvest

With the goal of obtaining a minimum of 150 $F_{2:3}$ plants per population for further analysis (expected to segregate 1:2:1 for recombinant haplotypes), 10 g (approximately 350 kernels) of F_3 seed from each of the 11 selected F_2 plants was drill seeded on May 20, 2013 using a Hege 90 grain drill (Antedes, Beauvais, France) to sparsely plant the 10 g of seed over 33.4 m^2 field-plot area (six 18.3 m rows per plot separated by 30.5 cm); or approximately one seed per 30.5 cm x 30.5 cm area. Plants found growing less than 15 cm apart at the second tiller stage were physically separated by transplanting one plant to less densely planted areas of the same plot. Field plots of the two parental lines were also grown, being seeded May 6, 2013. Each $F_{2:3}$ plant was individually numbered and marked using flags at panicle initiation. Leaf samples from each plant were harvested and stored at -80° C until DNA extractions were performed (described later).

Visual ratings for semidwarf versus non-semidwarf height per F_3 plant were performed during heading, with date of heading per plant recorded at the same time. Marker data (RM1339) indicated that three of the 11 selected F_2 plants (F_2 # 36, 37, 48) were homozygous for *Sd-1;* which was verified by visual $F_{2:3}$ observation. Segregation for plant height verified also that seven of the eight remaining populations of $F_{2:3}$ plants were heterozygous *Sd-1/sd-1*. Population 106 was omitted from the study because all $F_{2:3}$ had semidwarf height.

Seed harvest was conducted three times a week so that seed was collected from each plant as panicles reached the 18-20% MC stage of maturity. Visual assessment was used to

judge when each plant's panicles met these criteria (per Pinson et al., 2012, 2013). Plants which were too immature to harvest were evaluated again two to three days later. Five panicles were collected from each visually tall $F_{2:3}$ plant, dried gently using room temperature air (approximately 22° C) for 3 days, then stored at room temperatures to allow grain moisture to equilibrate between samples. More than 300 parental panicles were also harvested from each parental line immediately upon maturity for use as a bulk supply of control seed in the fissure phenotyping assay.

Genotyping F_3

A rapid 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 4°C until used for PCR amplification. The markers evaluated per F_3 population were specific to the recombination point(s) within each of the three $F_{2:3}$ populations (Fig. 2 and 3). Marker-trait linkages among the $F_{2:3}$ progeny were determined by allocating the $F_{2:3}$ progeny per population into two marker classes: 1) those containing the marker allele from CBNT versus 2) those containing the RU12 allele in the chromosomal region that was heterozygous in the recombinant F_2 parent (Fig. 2 and 3). These homozygous recombinant type individuals were identified in each of the three populations (Fig. 2). Only these homogenous recombinant haplotypes were phenotyped and used to determine marker-trait linkages. The sites of recombination were specific for each population.

$F_{2:3}$ Phenotyped Population

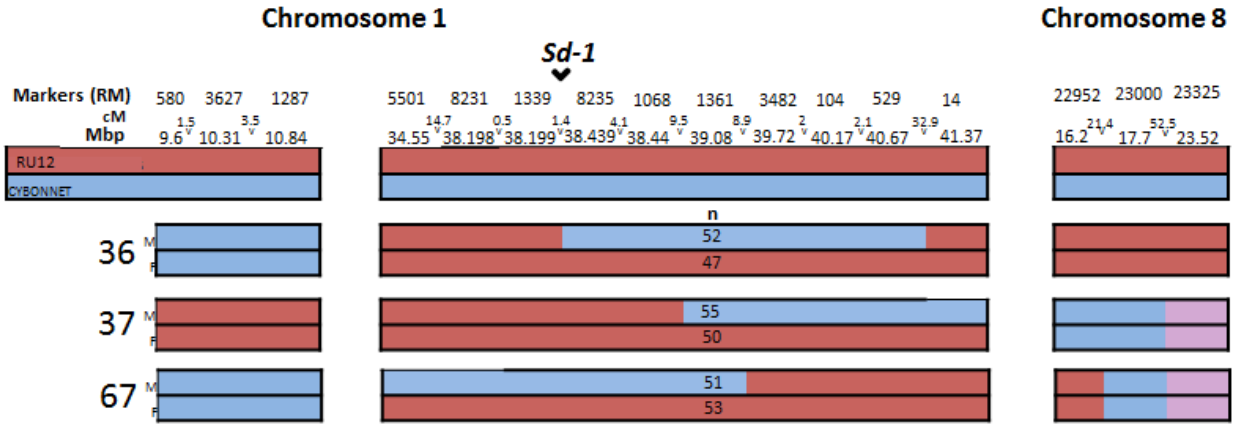

Figure 3. Marker alleles from the two parental lines are presented (upper left) color coded to serve as a reference for determining the origin of genetic material in the recombinant cross progeny. Red represents RU12 marker alleles, blue represents paternal alleles from CBNT, and violet represents heterozygous alleles. Each $F_{2:3}$ population was selected for haplotype homozygosity, as shown above, before being phenotyped for fissuring response. Numbers on the diagrammed haplotypes indicate the number of F_3 plants per haplotype that was phenotyped. The Mbp numbers shown are per the Gramene Annotated Nipponbare Sequence (2009); cM values were calculated by MapDisto 2.0 based on recombination frequency in the present in the F_2 generation.

Three of the $F_{2:3}$ populations were genotyped: 36, 37, and 67 (Fig. 2, and Fig. 3). Populations 36 and 37, were the first to be examined for FR. Population 36 contained recombination between RM1339 and RM8235, while the recombination in population 37 was more distal, between RM1068 and RM1361. Plants with homozygosity in these regions were selected from both populations. They also both inherited two copies of the wild type allele for height which means both populations were of non-semidwarf height (Fig. 2 and Fig. 3). In population 36, 52 plants were identified as homozygous for the CBNT alleles in the recombinant region from RM8235 through RM529, and 47 were identified as having Ru12 alleles in this

region. In population 37, 55 Ru12 recombinant and 50 CBNT recombinant plants were identified and phenotyped (Fig. 3).

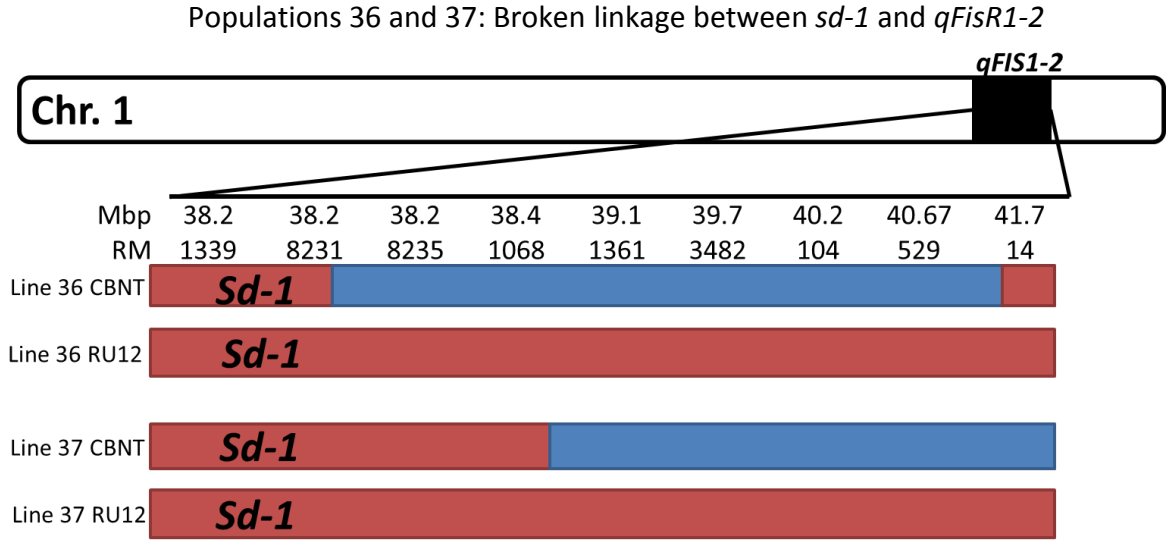

Figure 4. Intervals of the two homozygous recombinant types of individuals chosen for phenotyping in the two populations which inherited both wild type (non-semidwarf height) alleles from the maternal parent. Marker physical locations (Mbp) are per the Gramene Annotated Nipponbare Sequence (2009).

Establishing and Optimizing Laboratory Fissure Induction Conditions in Arkansas

The procedure for evaluating fissuring rates used in this this experiment was developed elsewhere by Pinson et al. (2012). A preliminary study to validate local facilities for this procedure was performed. The FR and FS cultivars used for preliminary testing of the equipment and facilities in Arkansas were: Saber (FR), LaGrue (FS), Lemont (FS), Cypress (FR), Cybonnet (FR) and RU12 (FS) all harvested at optimum maturity in the same year (2013) from Stuttgart, AR research plots.

Mechanical dehusking was conducted with a Satake THU-35A (Satake Engineering Co. Ltd., Penrith, Australia), in which the rubber rollers were set as wide as reasonable to dehusk each sample without inflicting excessive force (i.e., check samples were 90-100% dehusked in a single pass, 98 – 100% dehusked in two passes). This measure minimized breakage due to the rollers. Preliminary comparison of fissuring rates in paired hand-dehusked and machine dehusked samples showed that machine dehusking did not alter incidence of fissures as long as the kernels that broke during the machine dehusking were included in the count of fissured kernels. This was true even when kernels were passed through the dehusker twice. Visual observation for fissures were then conducted only on kernels remaining whole after dehusking. Double-dehusking of the samples was employed as a labor saving tool designed to both fully dehusk the seed samples and to break as many fissured kernels as possible, negating the need to visually identify them as fissured.

Exposing rice kernels to dry heat for a period of time before exposure to fissure inducing humidity can increase the differential response shown by FR and FS germplasm (Pinson et al., 2012, 2013). Without preheating, FS lines may fissure too little to distinguish them from FR and moderately FR lines. If the preheat treatment is too severe, however, then moderately FR lines appear as FS. Because the optimum amount of preheating is reportedly variable over both year and location of seed production (Pinson et al, 2012, 2013), the published procedure recommends using FR and FS check cultivars grown in the test environment to evaluate multiple lengths of preheat and humidity treatments to identify those where the FR and FS check cultivars from that harvest condition exhibit the greatest difference in fissuring

percentages (and ideally separated by 30 percentage points). Preliminary evaluation of the two parents and seed from a subset of 25 $F_{2:3}$ progeny for this study included 2, 4, 5, 6, 7 h preheat times, followed by a 16 h humidity period and revealed that wide variability (and fairly consistent ranking) existed among $F_{2:3}$ progeny even though the parental lines were not themselves widely divergent for FR under any of the tested laboratory conditions (the parental lines differed by as much as 15 or as little as 2 percentage points). To provide further reference for fissure rates within the present study, Cypress (FR), Saber (FR), and Lemont (FS) were added to all further fissure induction trials. Seed for these check cultivars had been grown in plots at the UAR RREC, Stuttgart, AR the same summer as the $F_{2:3}$ study progeny.

With such small differences observed for FR between the parents, it was decided to test and enact additional measures for removing non-genetic variance from the present study. During the preliminary study of parental lines (stored indoors at the UAR RREC laboratory). It was noted that fissure-induction rates between trials was sensitive to weather; with trials that began one day 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 non-genetic variability into the FR studies. Since evaluation of the test samples would require a minimal period of one month to accomplish, it was decided to identify and enact procedures to better standardize the initial kernel MC at the beginning of each run. Paired subsamples of seed were placed into environments of varying levels of temperatures and RH (Table 1). This pre-treatment conditioning was initiated to target a particular starting kernel MC that could maximize

differences between the two phenotypically similar parents. The ESPEC chamber (EPL-4H, ESPEC North America, INC. Hudsonville, Michigan) was eventually selected because it provided the most reliable control over RH and temperature. The machine was set at 25 ± 1° C and 55 ± 5% RH, conditioning the seed samples to an average kernel MC of 11.5%. Individual kernel moisture content was measured by an individual kernel moisture meter model CTR-500E (Shizuoka Seiki Co., Tokyo, Japan). Seed samples from each of the three study populations were conditioned separately using the same equipment and settings. Once conditioned, samples were removed from the chamber and placed in a Ziploc® bag and transported to the USDA Dale Bumpers National Rice Research Center, in Stuttgart AR, where they underwent fissure induction treatment. To minimize moisture loss during later sample handling, the samples were arranged into Ziploc® bags based on their preassigned boxes per replication.

Before each population underwent fissure induction treatment, seed samples from each parent and check cultivars preconditioned at the same time as the test samples were evaluated to identify the fissure-induction conditions that best distinguished FR from FS. Optimal conditions for populations 36 and 37 were determined to be 4 hours preheat at 45°C dry heat followed by 16 h humidity chamber treatment. Fissure inductions were all followed by a 24 hour dry down period before being mechanically dehusked and visually inspected for fissures. Population 67 progeny were slated for further study after results from populations 36 and 37 were analyzed for marker-trait linkages. Optimum fissure induction conditions after this lengthier period of storage proved to be 7 h of preheating followed by 16 h humidity.

Table 1. Five environments were tested for conditioning seed prior to fissure induction treatment. The purpose of these environments was to bring the seed to a moisture content equilibrium which would induce an ideal level of fissuring for evaluating differences between FR seed and FS seed. Reliability and consistency of conditions within each environment was also taken into account to choose the best environment for conditioning sample in.

Categories	Laboratory refrigerator USDA DBNRRC		Cold storage room #184, USDA DBNRRC		Laboratory L-21NE, USDA DBNRRC		AARC†		ESPEC ‡	
Avg. RH §	98%		45%		40%		30%		55%	
Temperature C	4		10		18		22		25	
Predicted MC ¶	>13%		11%		11.9%		<9%		11.5%	
Fissure rates parents	CBNT	RU12	CBNT	RU12	CBNT	RU12	CBNT	RU12	CBNT	RU12
Control										
0 h. preheat	8.7%	7.4%					16%	31.3%		
1 h.					31.3%	31.6%				
2 h.	7%	8.7%					73%	66%		
3 h.					42%	40.3%			41.4%	44%
4 h.	15%	26%	33%	48.7%			94%	95.3%		
5 h.					64%	79.3%			73%	75%
6 h.			83%	76.7%						

†Rejected when found to have variable RH after days with precipitation.

‡ Environmental control test chamber, model EPL-4H by ESPEC North America, INC. Hudsonville, Michigan.

§ Averaged over time. In some conditions severe fluctuations occurred such as increases in RH after a rain storm.

¶ (Ondier et al., 2012)

Fissure Phenotyping

Following the Pinson et al. (2012, 2013) phenotyping procedure, only kernels which had been hand threshed using only the top third of the panicle were used. This ensured that the kernels to be studied were neither over mature (risking pre-fissuring), nor immature.

Once kernels were separated from the panicles by hand, they were mixed well and allocated into three replications of 1.6 g subsamples, equivalent to 60-65 kernels, per F_3 plant. The subsamples were placed in tulle mesh bags (Fig. 5 A and B) rearranged per replication randomized order, then conditioned in the ESPEC chamber. For fissure induction treatment, the bags were placed into stainless steel woven wire boxes and heated in a chamber containing air of less than 20% RH and 45° C for a period of 4-7 h determined from the above-mentioned optimization studies. Immediately following heat treatment samples were placed in a 45° C growth chamber inside a sealed plastic box which contained water to generate an environment of 95-100% RH. Samples remained inside the humid environment for 16 h (per the optimization studies), then were air dried for 24-48 h before mechanical dehusking. The redrying of the samples was to allow the kernels to contract in size making fissures more visible (Pinson et al., 2012). After kernels were dried, a set of 50 kernels was counted from each tulle bag and dehusked as described in the preliminary study section, with kernels that broke during dehusking considered as fissured (Fig. 5 C). Broken kernels were not counted directly, but were

determined by subtracting the counted number of unbroken dehusked kernels from the original number per sample (50 kernels).

Visual Fissure Detection

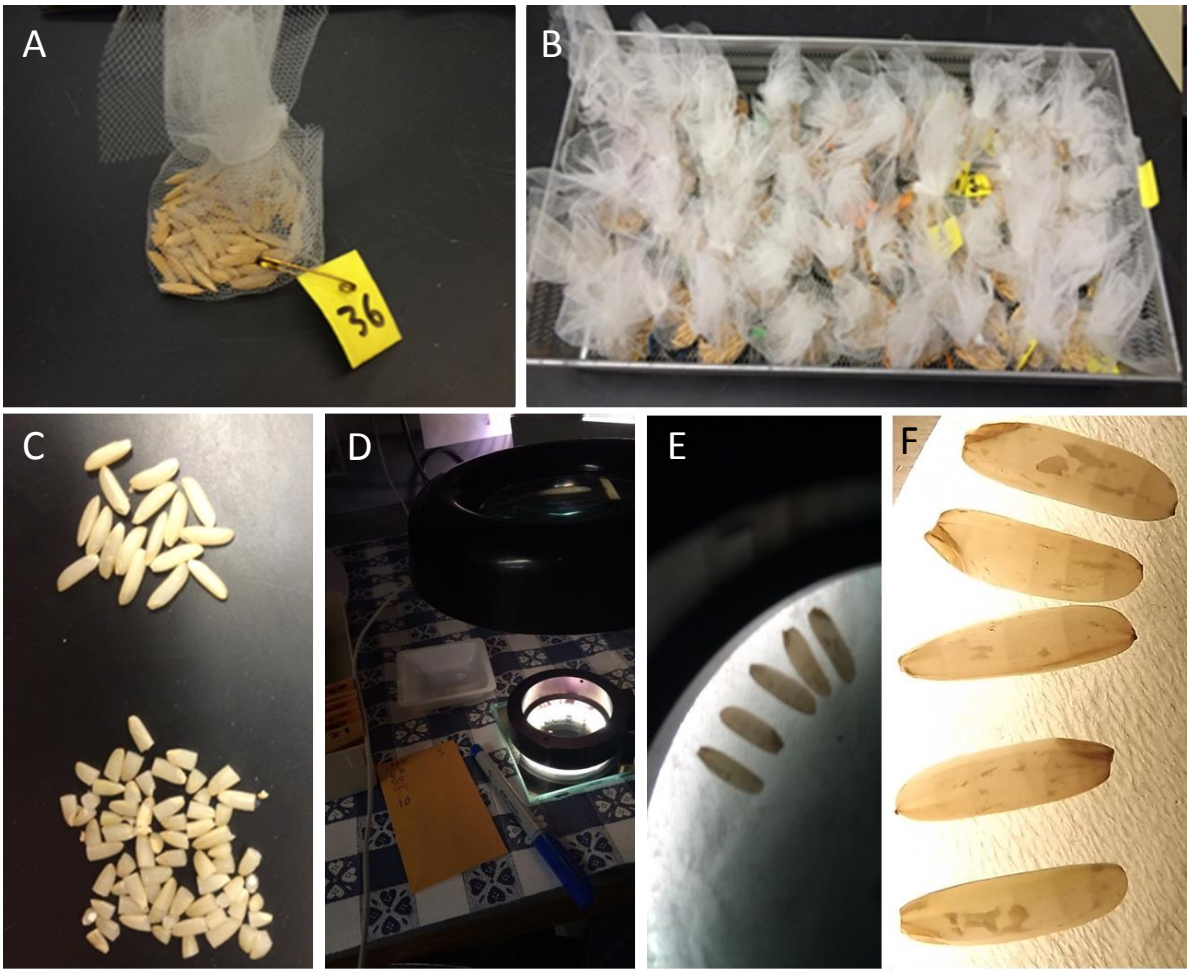

Figure 5. Seed samples were placed into prelabeled tulle mesh bags (A) and conditioned at 25 ^{O}C 55% RH for 2 weeks in an ESPEC chamber. Forty samples were then placed into a stainless steel mesh basket (B) for preheating and humidity exposure in batches. After air drying, tulle bags were opened and 50 kernel samples were counted, dehulled, and divided into whole versus broken kernels (C). Broken kernels were considered fissured kernels; whole kernels required additional observation using polarized light to accentuate the hairline cracks in fissured kernels (D, E, and F).

The method of fissure detection required the use of light to illuminate the hairline cracks within the grain (Fig. 5 D, E and F). Kernels were placed on a mirror inside a round lamp where beams of light illuminated translucent kernels. Fissured kernels generally had a crack which ran through the longitudinal axis of the kernel and had one visibly darker end and one lighter end caused by light diffraction though the dividing fissure (Fig. 5 F). The number of unbroken kernels per sample that were visibly fissured kernels were counted. The combined total of fissured kernels and broken kernels was obtained to generate the percentage of fissured kernels for each individual sample. Phenotypic evaluations for fissure resistance were analyzed individually for each population. Three replications of seed per $F_{2:3}$ plant were evaluated. Each replication was too large to fit into a single stainless steel basket to undergo fissure-inductions simultaneously. Thus, individuals in a single replication per population were randomly assigned into 3 treatment boxes. Each box contained multiple samples of seed from the three check cultivars plus each parent to allow statistical examination of between-box variation as might occur if boxes were improperly sealed or 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 impact FR (Waggoner et al., 2003) and was thus monitored among the $F_{2:3}$ progeny along with FR. Grain length, width, and thickness were measured on the kernels that remained unbroken after the fissure-induction and dehusking described above. Combining seed across the three replications per $F_{2:3}$ plant provided 50 to 150 kernels per $F_{2:3}$ plant for dimension analysis. Kernel dimensions were determined from digital images collected and analyzed using SeedCount SC5000 Rice Analyser (Next Instruments Pty Ltd, Sydney, Australia) property of Riceland Foods Inc. (Fig. 6). This system evaluated kernel length and thickness on approximately half of each seed sample, and evaluated length and width on the remaining kernels (Fig. 6).

Kernel Dimension Analysis

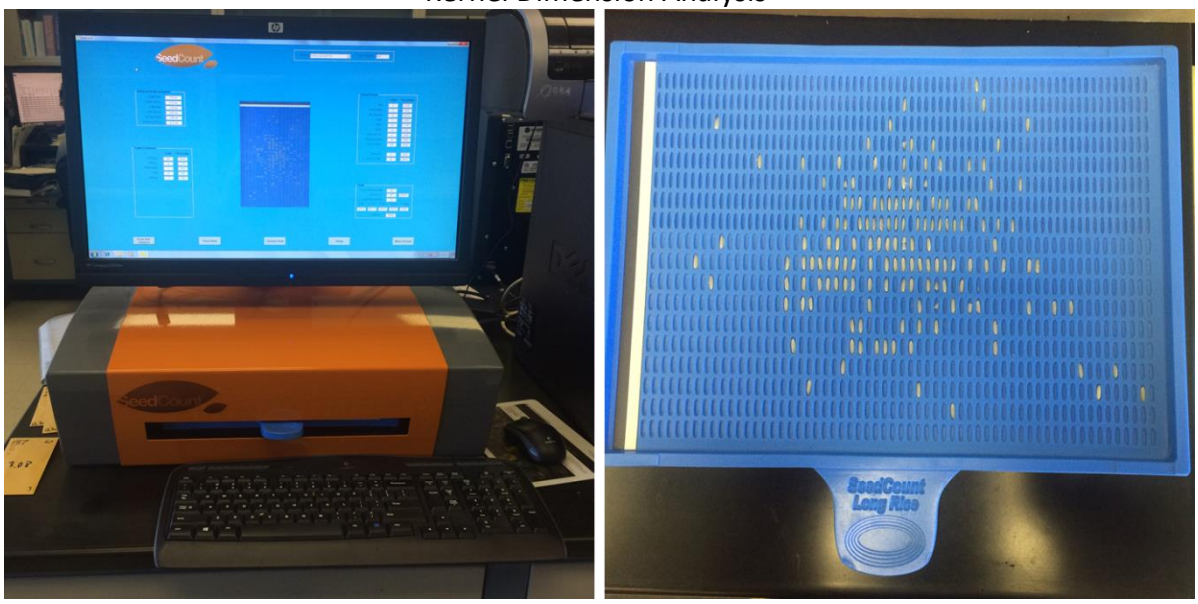

Figure 6. Shows the SeedCount terminal setup as well as the long-grain tray used to visually scan and calculate kernel dimensions for each individual kernel sample. The blue tray in the picture to the right in the figure has individual slots which are shaped to accommodate long-grain rice. The tray is divided in half horizontally with the top half allowing grains to align into the slots in a manner that allows kernel thickness to be measured while the slots in the bottom half the tray allow the grains to lay flat, facilitating the profiled image to reflect grain width. The SeedCount image analysis software uses a comparison of each kernel's length with average

sample length to remove broken kernels (as defined as < ¾ the sample average length) from the dimension calculations.

Statistics

Statistical analyses of fissure results were performed using *JMP® 10*. (Cary, NC: SAS Institute Inc.). Variance between boxes for fissuring rates was evaluated two ways. Initially, an ANOVA of the multiple parental and check cultivar samples that were repeated in each box was conducted, followed by Student's T tests which were used to compare the means and variances of the F_3 test sample data in each box. Linkage between marker alleles and the various traits was detected using marker allele data to classify the F_3 progeny per each of the populations into maternal and paternal genotypic classes (Fig. 2 and 3), and trait data from the two genotypic classes were significantly different. Data from the two cross parents, CBNT and RU12, were also compared. A significant difference in fissuring rates between the two genotypic classes would indicate that *qFIS1-2* resides within the molecularly segregating chromosomal region (Fig. 3 and 4) used to determine the two genotypic classes. In contrast a lack of significant difference would indicate that *qFIS1-2* resides in a genetically fixed portion of the chromosome, causing case both marker-defined genotypic classes to be equally resistant or equally susceptible to fissuring, depending on their allelotypes at the other two FR QTLs as well as at *qFIS1-2*. Likewise, grain dimensions were compared between the genotype classes using Student's T tests. A regression analysis using the continuous metric of each kernel dimension was used as the independent variable in a regression analysis and the fissure rates were

considered to be the dependent variable. Regression analysis was used to determine if any of the kernel dimensions was associated with FR independent of the genetic recombination at *qFIS1-2*.

Table 2. An ANOVA comparison of the fissure rates among the 5 check cultivars (RU12, CBNT, Cypress, Lemont, and Saber) in all boxes per population. None of the populations had significant ($\alpha \leq 0.05$) box-to-box variation based on check cultivars.

Population	36	37	67
P value	0.9169	0.0784	0.8427

RESULTS AND DISCUSSION

Parental Fissuring Rates

The two parents chosen for a bi-parental cross generally have very different levels of expression of the trait being studied (Holland, 2007). The difference in fissuring seen between the parents was small, but consistent with CBNT fissuring less than RU12 across the three population studies (Table 3) The difference between the parents was wider for the first two populations (36 and 37) than for the later population (67), which was exposed to a longer (7 h) preheat time, explaining the increase in fissuring for both parents in the population 67 fissure-induction boxes.

Table 3. Differences between the parents' fissuring rates averaged across the multiple samples per parent included in each fissure-induction box. P value is the result of a one tail T test where equal variance was assumed.

Populations	36	37	67
CBNT (FR) fissure rate	23%	19.8%	48.7%
RU12 (FS) fissure rate	37.3%	39%	56.7%
Difference FS-FR	14.3%	19.2%	8.0%

Standard error of sample	6.7%	8.1%	7.8%
P value one tail T test	0.0294*	0.0145*	0.1613

Kernel Fissuring Among the Three F_3 Progeny Populations

Population 36 F_3 progeny were evaluated first because the recombination event in this population included a long segment from the parent CBNT and thus was expected to segregate for the entire *qFIS1-2* QTL rather than a partial gene or gene cluster. This population was verified molecularly and visually as being homozygous for *Sd-1.* Therefore, detection of segregation of FR and FS among population 36 progeny would allow this study to 1) confirm expression of FR in taller, *Sd-1* progeny, and 2) confirm the ability to detect the small phenotypic differences one anticipates from segregation of one of multiple loci affecting a trait. Indeed, lower rates of fissuring (increased FR) ($\alpha = 0.1$) were detected among the progeny homozygous for CBNT alleles from RM8235 through RM529 than among the population 36 F3 progeny that contained RU12 alleles in this chromosomal region (Fig. 3 and 4). Pinson et al. (2013) reported significant association between FR from *qFIS1-2* and SSRs from RM5501 through RM529, with a LOD peak between RM1361 and RM104. Population 36 results confirmed that *qFIS1-2* is distal to RM1339 and *Sd-1*, but anterior to RM14.

Population 37 was then evaluated and a significant difference between the two recombinant progeny haplotypes (Fig. 3 and 4) indicated that the location of *qFIS1-2* is yet more

distal to the recombination point in this population which was located between RM1068 at 38.4 Mbp and RM1361 at 39.1 Mbp.

When FR was found associated with the paternal alleles at markers distal to both the recombination points in populations 36 and 37, it was decided to focus further effort on populations whose recombination points were distal to that in population 37. Thus population 67 was selected from the remaining 9 F3 populations for further research. The recombination point in population 67 was between RM1361 and RM3482, dissecting the LOD peak region reported by Pinson et al. (2013). Association between FR and the CBNT alleles segregating among the Progeny 67 progeny determined that *qFIS1-2* is anterior to RM3482.

In all three populations, the F_3 progeny containing marker alleles from RU12 (FS) fissured significantly more than the progeny that contained marker alleles from the CBNT (FR) parent (Table 4 and Fig. 3), indicating that *qFIS1-2* resides in the portion of the chromosome that was segregating (not fixed) in all three populations. The results from populations 36, 37 and 67 taken together indicate that *qFIS1-2* resides in the 1.3 Mbp region between RM1068 (at 38.4 Mbp) and RM3482 (at 39.7 Mbp). While RM1068 is anterior to RM1361, the anterior flank of the LOD peak reported by Pinson et al. (2013), placement of *qFIS1-2* anterior to RM3482 reduces the distal flank of the QTL region from 40.2 Mbp (RM104) to 39.7 Mbp (RM3482). These data map *qFIS1-2* more precisely than previously reported, with Pinson et al., (2013) reporting the LOD peak between RM8278 (36.6 Mbp) and RM529 (at 40.7 Mbp). This study reports the region, between 38.4 and 39.7 Mbps where the search for a candidate gene should be pursued

Fissure Rates for the 3 $F_{2:3}$ Populations

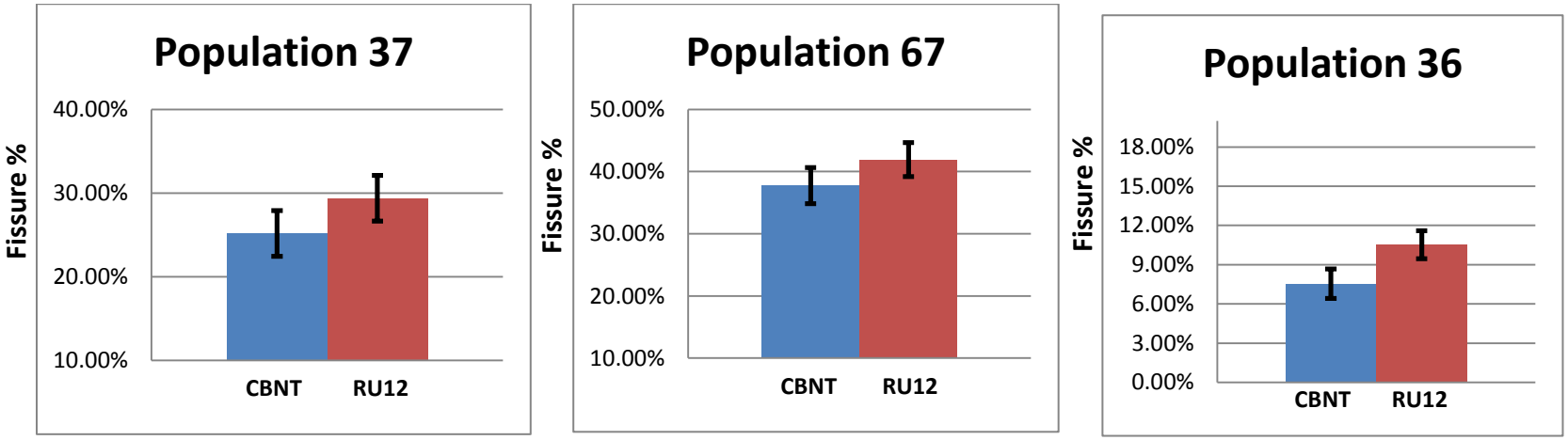

Figure 7. One tailed T-tests were used to compare fissuring rates between the progeny containing RU12 versus CBNT marker alleles in the segregating regions. In all populations RU12 recombinant types fissured at higher rates than CBNT recombinant types. The three populations had different recombination points, but in all three populations the RU12 recombinant types contained alleles from the FS parent in the region between Mbps 38.4 - 39.7 (Mbps locations per SSR from Gramene Annotated Nipponbare Sequence (2009)).

Table 4. Mean rates of fissuring for each marker-defined subset of progeny per population. For each population the difference in the rate of fissuring between CBNT and RU12 recombinant were observed (T test p-values indicate significance of differences detected).

Populations	Genotype	Number	Fissure rate	Std Dev	Std Err Mean	Fissure rate difference	Populations
36	F	47	10.52%	7.74%	0.01129	2.97%	0.0293*
	M	51	7.55%	7.62%	0.01067		
37	F	53	29.38%	13.19%	0.01812	4.21%	0.0637*
	M	51	25.17%	14.65%	0.02051		
67	F	43	41.82%	14.22%	0.02168	4.01%	0.0855*
	M	41	37.81%	12.38%	0.01934		

Kernel Dimensions

The same two marker-progeny classes per population compared for fissuring rates were also evaluated to see if they differed significantly for one or more of the grain kernel dimensions: length, width, and thickness. Significant differences (two-tailed T test, $\alpha = 0.05$) were found for kernel length in the three observed populations (Table 5). In each population, the marker alleles associated with longer kernel length came from RU12, the parent that had longer kernels (Table 6) as well as FS.

In population 37, recombinant progeny with RU12 alleles were also significantly thicker than those with CBNT alleles (Table 5). However, no difference for thickness was seen between the two recombinant haplotypes of populations 36 and 67, nor between CBNT and RU12. In contrast, in population 36 only (Table 5) CBNT recombinants had wider kernel length than the RU12 recombinant progeny. Again, none of the other populations nor the parents, CBNT and RU12, were found to have significant differences in kernel width.

Regression analysis on length, thickness, and width against fissuring rates was used to test for association between each grain dimension and FR irrespective of marker allele subgrouping. FR and grain measurements were found associated in only one population, that of population 37, where kernels that were both thicker and wider were associated with higher

rates of fissuring (Table 7). This association between thickness and increased susceptibility to fissuring is in support of an earlier report (Jindal and Siebenmorgen, 1994).

Kernel length varied between CBNT recombinants and RU12 recombinants, suggesting a gene affecting grain length was linked to *qFIS1-2.* Previous work has hypothesized 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 kernel (Waggoner et al., 2003). However the results of this study indicated that the recombinant individuals with CBNT markers were both FR and of shorter grain length than the RU12 recombinant progeny, contrary to the relationship predicted by Waggoner et al. (2003). Additionally, between the two parents the FR CBNT was significantly shorter than the FS RU12. However, further evaluation of the relationship between kernel length and rates of fissuring using regression analysis across all the measured recombinant progeny combined did not detect association between kernel length and rate of fissuring, suggesting the presence of other loci with even stronger effect on kernel length with without association with FR. Thus, the fact that the FR CBNT haplotypes had shorter average kernel length t than the FS RU12 haplotypes, but appears to be due to linkage between alleles for shorter length and FR rather than shorter kernel length causing a decrease in FR.

Table 5. Populations 36, 37, and 67 descriptive statistics of the kernel dimensions for each recombinant genotype.

Populations and Dimension	Genotype	Sample Size	Mean mm	Standard Error	Prob < t
36 Length	RU12	46	7.2287	0.00634	<0.0001**
	CBNT	49	7.12102		
36 Thickness	RU12	46	2.01478	0.02616	0.3946
	CBNT	49	2.0202		
36 Width	RU12	46	2.33717	0.008989	0.0003*
	CBNT	49	2.37082		
37 Length	RU12	52	7.49731	0.02618	<0.0001**
	CBNT	51	7.34765		
37 Thickness	RU12	52	2.04481	0.00555	<0.0001**
	CBNT	51	2.02176		
37 Width	RU12	52	2.37077	0.00751	0.0533
	CBNT	51	2.35608		
67 Length	RU12	42	7.61524	0.04021	0.0003*
	CBNT	38	7.47053		
67 Thickness	RU12	42	2.03857	0.00751	0.2591
	CBNT	38	2.04711		
67 Width	RU12	42	2.2981	0.008368	0.0063*
	CBNT	38	2.32158		

Table 6. Means comparison between the two parents, CBNT and Ru12 used as checks within population 36.

Dimension	Genotype	Sample Size	Mean mm	Standard Error	Prob < t
Length	RU12	285	7.51	0.027	<0.0001**
	CBNT	279	7.33		
Thickness	RU12	285	2.06	0.008	1.0000
	CBNT	279	2.06		
Width	RU12	285	2.37	0.008	0.1908
	CBNT	279	2.38		

Table 7. Regression analysis for each population where fissure rate was compared to the length, thickness, and width per population to see if these physical dimensions influenced kernel fissuring.

Category	R^2	F	Linear fit
Population 36			
Length	0.008	0.36	
Thickness	0.031	0.08	
Width	0.001	0.33	
Population 37			
Length	0.012	0.27	
Thickness	0.053	0.02*	fissure % = -1.901787 + 1.0693789*Thickness
Width	0.093	0.002***	fissure % = -2.349908 + 1.1096252*Width
Population 67			
Length	0.002	0.72	
Thickness	0.019	0.26	
Width	0.014	0.35	

Implications on U.S. Germplasm

Marker assisted selection has several advantages to breeders over traditional phenotypic selection. Marker assisted selection can be conducted much earlier in the plant growth cycle for the FR trait than phenotypic selection, including before plant heading, thus saving breeders the time and resources required for labor intensive phenotypic evaluation. Introgressing the single *qFIS1-2* locus into a breeding line is not expected to result in strong FR. Overall, FR is a low heritability trait. Pinson et al. (2013) reported a 12 percentage point improvement in FR in progeny that had inherited two copies of *qFIS1-2*. In this experiment where bulked seed from molecularly selected homozygous $F_{2:3}$ plants of a breeding cross were evaluated, the average decrease in the rate of fissuring from this single introgressed QTL was just 3.7 percentage points, smaller than that predicted by Pinson et al. (2013). However, the increased HRY obtained by decreasing fissuring even a few percentage points would increase

productivity and profitability for farmers, and would improve the quality of U.S. rice being shipped as paddy rice and milled abroad.

Breaking the Linkage Between *sd-1* and *qFIS1-2*

In populations 36 and 37, linkage between the semidwarf *sd-1* allele and the FR was broken, with the FR trait being found within *Sd-1/Sd-1* F_3 progeny. Breaking the linkage between these two traits provides concrete evidence that previous detection of association between FR and short plant height Pinson et al. (2012) was due to genetic linkage rather than plant architecture or other pleiotropic explanation.

The ultimate finding of this research is that individuals that had inherited the CBNT marker alleles between 38.44–39.72 Mbp had measurably lower rates of fissuring than individuals which inherited alleles from a RU12 the FS parent. This region of the genome is distal to (nearer the telomere) than the *sd-1* gene and in two of these populations all of the progeny inherited the non-semidwarf or the wild type *Sd-1* allele. The FR donor parent CBNT has semidwarf alleles at this locus. Thus, two of the populations studied broke the linkage between *sd-1* and *qFIS1-2*, and successfully recombined *qFIS1-2* with *Sd-1*. Additionally, in populations 36 and 67 marker data also indicated that both populations inherited the FR CBNT allele for *qFIS1-1* reported by Pinson et al. (2013) to be on the short arm of chromosome 1. Thus these populations are useful as FR donors to breeding programs that target development

of improved *Sd-1* cultivars. This study also effectively narrowed the interval in which to seek candidate genes for the FR QTL, *qFIS1-2,* initially discovered by Pinson et al. (2013).

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