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RESEARCH ARTICLE

Identification of Rice Mutants with Altered Grain Alkali Digestion Trait

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ABSTRACT Gelatinization temperature (GT) is an important component of eating and cooking quality (ECQ) of rice. While direct measurement of GT is cumbersome, the alkali spreading value (ASV) test is a robust method commonly used to rapidly identify different GT types. In this study, we employed a modified ASV assay to screen a population of chemically-induced rice (cv. Kitaake) mutants (n = 405). Two mutant families, KDS-1623B and KDS-1824B, with significantly lower ASV (higher GT type) than wild type Kitaake (low GT type) were isolated. A nonsynonymous homozygous mutation in the isoamylase-type starch debranching enzyme gene *ISA1* was identified in KDS-1623B. The mutation (G2709T) is predicted to change a valine at position 354 to a leucine in the α-amylase catalytic domain of ISA1. This result is consistent with the shrunken endosperm exhibited by KDS-1623B grains and the replacement of starch with phytoglycogen in *isa1* (*sugary-1*) mutants. The altered ASV trait in KDS-1824B appears to be controlled by a single recessive mutation; however, the causal genetic lesion remains to be determined. These mutants will be useful resources for elucidating the complex nature of starch metabolism and its influence on ECQ of rice.

Keywords Induced mutation, Alkali spreading value, Eating and cooking quality, Targeted exon capture, Next-generation sequencing, Rice (*Oryza sativa* L.)

INTRODUCTION

Rice (*Oryza sativa* L.) is unique among the major cereals as almost the entire crop is used directly for human consumption in the form of whole milled kernels. As such, the eating and cooking qualities (ECQs) of rice grains, which consist mainly of starch (~90%), are critical to consumers. Rice starch is comprised of amylopectin and amylose, extensively branched and long unbranched polysaccharides, whose chemical and physical properties are the major determinants of ECQs (Umemoto *et al*. 2008; Tian *et al*. 2009). While apparent amylose content (AAC) is the most important factor affecting ECQ of rice grains (Umemoto *et al*. 2008; Tian *et al*. 2009), differences in ECQ observed among varieties with similar AAC point towards the role of other starch properties (Pang *et al*. 2016).

Pasting viscosity, gel consistency (GC), and gelatinization temperature (GT) are examples of other significant factors affecting ECQ of milled rice grains (Pang *et al*. 2016; Wang *et al*. 2019). GT is the critical temperature at which about 90% of rice starch gelatinizes or transforms from a semicrystalline structure to a gel-like, edible form (Waters *et al*. 2006; Gao *et al*. 2011). GT influences cooking time and the texture of cooked and cool cooked rice, making it an important ECQ factor (Waters *et al*. 2006). While GT can be determined directly using differential scanning calorimetry (Umemoto *et al*. 2008), it is

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typically estimated using the alkali spreading or digestion value (ASV, ADV) which is determined by the degree of dispersal or disintegration of whole milled rice grains incubated in a dilute alkali solution (Little *et al*. 1958). Rice grains that have low, intermediate, and high GT exhibit complete disintegration, partial dispersal, and very little or no change in morphology. The ASV test has been widely used in rice breeding programs due to the ease and simplicity of the assay compared to direct determination of GT (Tian *et al*. 2005).

Using the ASV assay, the involvement of the rice starch biosynthesis genes in controlling GT was established with the genetic mapping of a major gene, *ALK*, to the same locus on chromosome 6 as the starch synthase IIa (*SSIIa*, also known as *SSII-3*) gene, which affects amylopection structure (Umemoto *et al*. 2002) followed by the confirmation of this as the *ALK* gene by map-based cloning (Gao *et al*. 2003). Further characterization of the *ALK* gene alleles from rice varieties of various GT types revealed a number of functional single nucleotide polymorphisms, which could be used to classify varieties (Umemoto *et al*. 2004; Nakamura *et al*. 2005; Umemoto and Aoki 2005; Bao *et al*. 2006; Waters *et al*. 2006; Yu *et al*. 2010). In addition to the *SSIIa/ALK* gene, about twenty QTLs affecting GT, have been mapped onto chromosomes of 1, 2, 5, 6, 7, 8, 10, 11 and 12 (Wang *et al*. 2019).

In this study, we screened a *temperate japonica* (cv. Kitaake) M_2 mutant population (n = 405) derived from chemical seed mutagenesis using an ASV assay to identify lines exhibiting altered alkali spreading/digestion trait in milled rice grains. Four M_2 mutant families derived from three independently mutagenized M_1 plants (KDS-1623B, 1824B, 1835B and 1835C) were identified which showed significantly lower ASV than wild type Kitaake. Two of the mutant lines (KDS-1623B and 1824B) appeared to be homozygous (fixed) for the mutant phenotype in the M_3 generation and were subjected to further characterization. While both mutations were determined to be recessive, the brown rice grains of the KDS-1623B mutant were significantly smaller and thinner than wild type and appeared similar to the previously described *sugary-1* phenotype (Nakamura *et al*. 1997; Kubo *et al*. 1999; Kawagoe *et al*. 2005; Kubo *et al*. 2005). Using an in-solution target enrichment approach in conjunction with next-generation sequencing, a single nonsynonymous mutation, a G→T transversion at nucleotide 2709, was found in *ISA1*, an isoamylase-type starch debranching enzyme involved in biosynthesis of amylopectin, which was consistent with the *sugary-1* appearance of the KDS-1623B grains and their resistance to alkali digestion (Kubo *et al*. 1999, 2005; Chao *et al*. 2019).

MATERIALS AND METHODS

Plant materials, alkali digestion assay, and grain phenotyping

Screening for rice mutants with altered alkali spreading value (ASV) or alkali digestion trait was performed using a population derived from sodium azide seed mutagenesis of the *temperate japonica* variety Kitaake (Monson-Miller *et al*. 2012). A total of 405 M_2 families, representing 235 independently mutagenized M_1 plants, were screened using a modified alkali digestion assay. In brief, ten M_3 seeds from each family were manually husked and milled for 15 seconds in a laboratory grain polisher (Pearlest, Kett US, Villa Park, CA, USA). For initial screening, three milled grains from each line were placed in a 5 mL (35 mm × 10 mm) plastic petri dish with 4 mL of freshly prepared 1.7% potassium hydroxide [KOH]. Grains were incubated at 20℃ for 23 hours before visual evaluation as described by Little *et al*. (1958). The California long grain rice variety A-202, intermediate gelatinization temperature (GT) type, and wild type Kitaake, low GT type, were included as controls. Lines exhibiting possible altered ASV in the initial screen were re-tested in the same manner to confirm phenotypes. Representative samples of brown rice grains (n = 20) from two Kitaake, KDS-1623B, and KDS-1824B plants grown under standard greenhouse conditions (Orchard Park greenhouse complex, Department of Plant Sciences, University of California, Davis) were prepared by manual husking and the grain length, width, and weight were measured. Length and width were determined using a VIBE QM3 Rice Analyzer (Burlingame, CA, USA). Individual grains were weighed, and the means and standard deviations were calculated, and one-way analysis of variance (ANOVA)

and Bonferroni-corrected posthoc t-tests were performed using MS Excel 2016.

Exon capture, sequencing, and data analysis

Exon capture was performed using the MYbaits® platform (MYcroarray, Ann Arbor, MI, USA). A capture reagent consisting of 19,748 custom biotinylated RNA probes (i.e. baits) with about 2.85X tiling density was designed and generated by MYcroarray (now Arbor Biosciences) from a set of 321 rice genes that were selected to cover various biosynthetic pathways and gene families of interest to our research program (Kim and Tai 2019). The gene set included starch biosynthesis genes (Kharabian-Masouleh *et al*. 2011), glutathione transferases (Jain *et al*. 2010), phytic acid biosynthesis genes (Kim and Tai 2014), microtubule cytoskeleton genes (Guo *et al*. 2009), ATP-binding cassette (ABC) transporter genes (Nguyen *et al*. 2014), and *Glossy1*-like (*GL1*-like) genes (Islam *et al*. 2009). Exon capture and sequencing was performed on three wild type controls (Nipponbare, Kitaake, and Sabine) and nine mutants (Kim and Tai 2019). Of the mutants, four were wet leaf/glossy (i.e., *wax crystal-sparseless leaf*, *wsl*) mutants (Tai 2015; Kim and Tai 2019) and five grain quality mutants in the Kitaake (four including KDS-1623B) and Nipponbare (one) backgrounds. As described previously, DNA samples were extracted from one month-old seedlings of M_4 generation mutants and wild-type lines, quantified, and one μg of genomic DNA from each sample was sheared by sonication to an average fragment size of 300 bp. Genomic libraries were constructed using a KAPA HyperPlus Kit (KAPA Biosystems, Wilmington, MA, USA) and equal amounts of the 12 libraries were pooled and subjected to in-solution target enrichment using the MYbaits® kit.

Sequencing of the captured libraries was performed using the Illumina HiSeq2500 (3% of a lane; SR50 run) and HiSeq4000 (5% of a lane; PE150 run) platforms. Candidate mutations were detected using the Mutation and Polymorphism Survey tool with parameter 10 threads, minimum of 6 libraries, minimum coverage of 20, maximum coverage of 2000 (Henry *et al*. 2014). Protein effect was determined based on the *Oryza sativa* ssp. *japonica* cv. Nipponbare pseudomolecules (MSU version 7.0) using Geneious v9.1.5 (www.geneious.com; Kearse *et al*. 2012). Novelty of the mutations was based on a search of a 32 Mb single nucleotide polymorphism (SNP) dataset from the IRRI 3,000 Rice Genomes Project sequence information without any threshold (Alexandrov *et al*. 2014; Mansueto *et al*. 2017). Information on protein families and transmembrane regions was predicted using Pfam 31.0 (http://pfam.xfam.org) and TMHMM (Krogh *et al*. 2001), respectively, and implemented by the Rice Genome Annotation Project server (http://rice.plantbiology.msu.edu).

Validation of KDS-1623B mutation and segregation analysis

The putative candidate mutation identified by exon capture and next-generation sequencing were validated by Sanger sequencing of PCR products spanning those mutations. Sanger sequencing was also used to confirm the F_1 of crosses made between mutants (M_4 generation) and with wild type Kitaake. Given the relationship of the *sugary-1* grain type and the altered ASV, the KDS-1623B/Kitaake F_2 population was scored by visual evaluation of the brown rice morphology. For genotyping of the mapping population, genomic DNA samples were extracted from the F_2 seedlings using a DNeasy®96 Plant Kit. The DNAs were subjected to PCR with primers (5′-AGTTGATGCCCTG CCATGAA-3′ and 5′-TCCCTGTAGGCACAAACACC-3′) which amplified a 1,085 bp containing the *ISA1* SNP generated in the KDS-1623B mutant. PCR reactions and conditions used for amplifying DNA fragments for sequencing were as previously described (Kim and Tai 2014). PCR products were purified using the Agencourt Ampure® XP magnetic beads (Beckman Coulter Genomics, Danvers, MA, USA) and Sanger sequencing was performed by the College of Biological Sciences UCDNA Sequencing Facility at UC Davis. Sequence data alignment and analysis were performed using Geneious v9.1.5. Segregation ratios were subjected to Pearson's χ^2 test for goodness-of-fit.

Preliminary genetic analysis of KDS-1824B mutant

Reciprocal crosses between the KDS-1824B mutant and the wild type progenitor variety Kitaake were performed to examine the mode of inheritance of its altered alkali digestion trait. Approximately 100 F_2 seeds from a single F_1 plant of each cross were husked using a laboratory rice

sheller (TR200; Kett US, Villa Park, CA, USA) and then milled using a Pearlest grain polisher for 1 minute. Single milled F_2 grains (n = 48) for each F1 were placed in individual wells of a 24 well culture plate (MP Biomedicals LLC, Solon, OH, USA) and each well was filled with 1 mL of freshly prepared 1.7% KOH solution. Plates were incubated at 30℃ for 23 hours before visual evaluation (Supplementary Fig. S1). The segregation ratio of wild type to mutant ASV was subjected to Pearson's χ^2 test for goodness-of-fit to the single recessive gene mode of inheritance.

RESULTS

Identification mutants with altered alkali digestion

In order to identify rice mutants with altered grain quality phenotypes, a modified alkali digestion assay (i.e. alkali spreading value [ASV] test) was employed to screen a population of Kitaake rice mutants derived from sodium azide mutagenesis. Initial screening was performed by evaluating the digestion of three milled M_3 grains from each of 405 M_2 families, representing 235 independently mutagenized M_1 plants. Preliminary evaluation of Kitaake, the progenitor wild type variety of the mutant population, revealed similar ASV (low gelatinization temperature [GT] type) at both 20℃ and 30℃ and the initial screening was performed at the lower temperature for convenience. Grain appearance and digestion was visually rated as described by Little *et al*. (1958). Under the conditions of the modified alkali digestion test, Kitaake was typically rated as low GT type (ASV score between 6 and 7) and the intermediate GT type California long grain variety A-202 was rated as high/high-intermediate (ASV score of 2 and above but below 3), likely due to the lower temperature at which the screen was conducted.

Preliminary evaluation resulted in the identification of 42 M_2 families with putative altered alkali digestion compared to Kitaake based on the ASV score and differences in grain appearance. Of these 42 families, the milled grains of 32 families exhibited heterogeneous digestion phenotypes while those of 10 families were uniform in their appearance after the digestion with 1.7% KOH. Among the 10 families, two were rated as exhibiting a higher ASV than Kitaake (ASV = 7) and were derived from the same M_1 individual (KDS-1578A and KDS-1578C). The remaining eight lines appeared to have lower ASV ratings and less disintegration than Kitaake. Of these lines, two were most clearly distinct from Kitaake. KDS-1623B milled grains exhibiting no apparent change in shape (ASV = 2) and KDS-1824B milled grains exhibiting a high-intermediate/intermediate GT type (ASV = 3.7) (Fig. 1). A phenotype similar to KDS-1824B was observed among two lines exhibiting heterogeneous digestion, KDS-1835B and KDS-1835C, which are derived from the same M_1 individual (Supplementary Fig. S2). These results were consistent with homozygous mutations in KDS-1623B and KDS-1824B and heterozygous mutations in the sibling M_2 lines KDS-1835B and KDS-1835C underlying the altered alkali digestion trait observed in these lines. Following confirmation of the initial observed phenotypes for these mutant lines by testing additional M_3 milled grains, KDS-1623B and KDS-1824B were selected for further genetic analysis due to the likelihood that the underlying mutations in these lines were homozygous.

Seed morphology and grain weight of low ASV mutants

Visual evaluation of representative brown rice grains from Kitaake and the KDS-1623B and KDS-1824B mutants indicated that the grains from the two mutants are

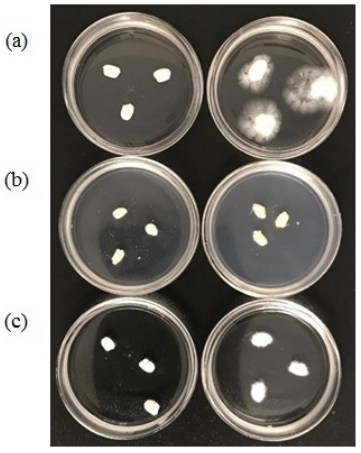

Fig. 1. Modified alkali spreading value (ASV) assay. (a) Kitaake (b) KDS-1623B (c) KDS-1824B. Petri dishes on the right contain water and petri dishes on the left contain 4 mL 1.7% KOH. Milled grains were incubated at 20℃ for 23 hours.

smaller than the wild type (Fig. 2). For each genotype, twenty randomly selected seeds from two representative plants (n = 40) were dehulled and grain widths, lengths, and weights were measured and the means and standard deviations (SD) determined (Table 1). Statistically significant differences were detected among the group means for each of the traits using one-way ANOVA ($P < 0.01$). Posthoc t-tests (Bonferroni-corrected $P < 0.003$) indicated that Kitaake and KDS-1824B grain widths were significantly different from KDS-1623B but not from each other. With regard to grain length and weight, all three genotypes were significantly different from each other. The KDS-1623B mutant grain weighed less than half that of Kitaake and had the same shrunken appearance as the *sugary-1* mutant (Kubo *et al*. 1999, 2005). In these mutants, the endosperm starch is completely replaced by phytoglycogen resulting in a characteristic lack of iodine staining of *sugary-1* grains (Kubo *et al*. 1999; Kawagoe *et al*. 2005; Kubo *et al*. 2005). This effect was confirmed in the KDS-1623B grain whereas iodine staining of the KDS-1824B grain was comparable to Kitaake (T. Tai, data not shown).

Fig. 2. Seed and brown rice of wild type and low ASV mutants. (a) Mature seeds and brown rice grains of Kitaake (right), KDS-1623B (center), and KDS-1824B (left). (b) Iodine staining of brown rice cross sections of Kitaake (top), KDS-1623B (middle) and KDS-1824B (bottom). Bar: 5 mm.

Exon capture and sequencing

As we reported previously, an in-solution target enrichment and next generation sequencing approach was employed using the MYbaits® platform in order to examine the utility of targeted exon capture and sequencing for identifying candidate mutations (Kim and Tai 2019). Custom biotinylated RNA probes were designed from 321 genes of interest including sixteen involved in starch biosynthesis (Kharabian-Masouleh *et al*. 2011). Based on the appearance of the seed and the altered alkali digestion trait, the KDS-1623B was among five grain quality mutants that were subjected to this approach (Kim and Tai 2019). Results of the targeted sequencing strategy for the KDS-1623B mutant and the wild-type progenitor cultivar Kitaake are shown in Table 2. Approximately 103 million sequenc-

Table 1. Dehulled grain width, length, and weight of wild type and low ASV mutants[z].

Accession	Width (mm)	Length (mm)	Weight (mg)
Kitaake	3.13 ± 0.12[a]	5.08 ± 0.19[a]	22.58 ± 1.57[a]
KDS-1623B	2.75 ± 0.16[b]	4.74 ± 0.20[b]	9.58 ± 0.66[b]
KDS-1824B	3.15 ± 0.19[a]	4.47 ± 0.19[c]	19.02 ± 1.50[c]

[z]Values shown are means ± SD of 40 seeds. Significant differences between mean values for each trait are indicated by different letters (Bonferroni-corrected posthoc t-test, $P < 0.003$).

Table 2. Homozygous nonsynonymous mutation detected in KDS-1623B by target enrichment and next generation sequencing.

Accession	Reads[z] (10^6)	Coverage[y]	Gene	Locus ID[x]	Mutation[w]	Effect[v]
Kitaake	102.84	65.11	-	-	-	-
KDS-1623B	103.47	65.51	*ISA1*	LOC_Os08g40930	G2709T	V354L
			OsABCB22	LOC_Os08g45030	C1213T	A321V
			OsGSTU50	LOC_Os10g38740	G1103A	E149K

[z]Total number of aligned reads on target.
[y]Coverage on target (i.e., number of times target region covered by sequencing).
[x]Locus identification from *Oryza sativa* ssp. *japonica* cv. Nipponbare pseudomolecules MSU version 7.0 (http://rice.plantbiology.msu.edu).
[w]Nucleotide base change and position in the genomic DNA from the start codon.
[v]Predicted amino acid change and position in the protein.

ing reads were "on target" (i.e., covering the baits used for enrichment) for both KDS-1623B and Kitaake, representing about 65X coverage of the coding regions (i.e. exons) of the genes from which the baits were designed.

Single nonsynonymous homozygous point mutations were detected in three genes in the KDS-1623B mutant (Table 2). One of these genes is *ISA1* (LOC _Os08g40930), which encodes an isoamylase-type starch debranching enzyme (Kubo *et al*. 1999; Kawagoe *et al*. 2005; Kubo *et al*. 2005; Chao *et al*. 2019). This mutation was validated by Sanger sequencing of the original DNA (M_4 generation) used for exon capture and DNA from a M_5 generation mutant. The *ISA1* mutation detected in KDS-1623B, a transversion from G to T at position 2709 in the gene, is predicted to result in the substitution of a valine at position 354 with a leucine in the α-amylase catalytic domain of the protein (Fig. 3). The mutation was not found in any of the naturally-occurring alleles of *ISA1* in the 3,000 Rice Genomes Project database (Alexandrov *et al*. 2014).

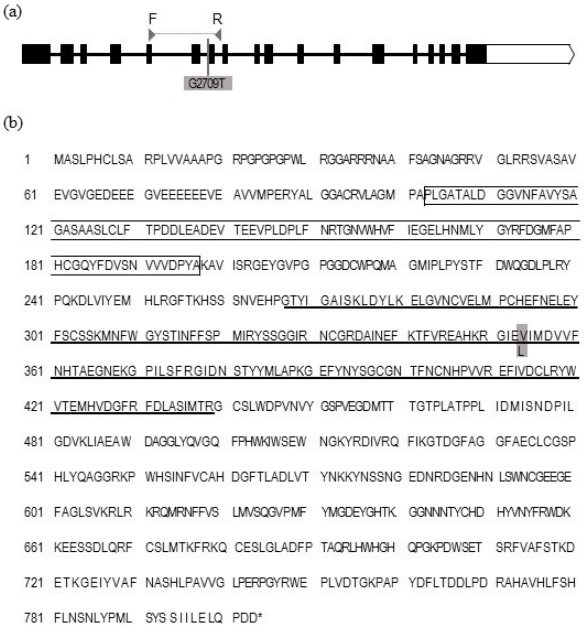

Fig. 3. Mutation in the *ISA1* (LOC_Os08g40930) gene (a) and corresponding protein sequence (b). Gene model (5′ → 3′) showing location of SNP mutation from KDS-1623B in the 7th exon with gray line and box; 3' untranslated regions indicated by open box; exons by filled box; introns by lines between boxes; 1,085 bp amplified region for sequencing shown in gray lines with forward (F) and reverse (R) primers above the gene model. Protein sequence change from V (Valine) to L (Leucine) in KDS-1623B with gray box; Carbohydrate-binding module (CBM) 48 (Isoamylase N-terminal domain) in closed rectangle; α-amylase catalytic domain indicated with black underline.

Genetic analysis of low ASV rice mutants

The inheritance of the low ASV mutant phenotypes was examined by performing crosses between the mutants and the wild-type progenitor Kitaake. In the case of crosses with KDS-1623B, M_3 generation mutants were used as the female parents only because of their poor fertility. F_1 seeds were obtained from crosses in which the KDS-1623B parent was sterile or low fertility. True F_1 hybrids were confirmed by sequencing of the KDS-1623B mutant SNP. An F_2 population (n = 122) from one F_1, which was derived from a cross involving a sterile KDS-1623B maternal parent, was grown for genotyping by Sanger sequencing and to produce F_3 sseeds for phenotypic evaluation. Of the 122 F_2 plants, 85 produced seeds and 37 were sterile. These observed segregation ratios did not significantly deviate from those expected for a single gene recessive mutation conferring sterility (χ^2 = 1.847, df = 1, P = 0.174, not significant at $P \leq 0.01$). Visual evaluation of brown rice grains (n ≈ 20) of those plants for which F_3 seeds were available resulted in the identification of 35 lines with all normal (wild type ASV) grains, 44 lines segregating for the *sugary-1* (low ASV) grains, and 6 lines with all *sugary-1* (low ASV) grains. Sanger sequencing was performed to genotype the *ISA1* mutant SNP in all 122 of the F_2. Among the fertile plants, the SNP alleles segregated completely in agreement with the brown rice phenotypes (i.e. all 6 *sugary-1* mutants had homozygous mutant T alleles for the SNP in *ISA1*, all 35 normal lines had homozygous wild-type G alleles, and all 44 lines with segregating grain phenotypes were heterozygous for the SNP). Of the 37 sterile F_2 plants, 19 were homozygous for the wild-type G allele, 17 were heterozygous, and one was homozygous for the mutant T allele. The segregation ratios of the *ISA1* SNP marker were significantly distorted (χ^2 = 36.21, df = 2, $P <$ 0.00001, significant at $P \leq 0.01$).

To further genetically characterize the KDS-1824B mutant, reciprocal crosses were made to Kitaake. F_2 seeds

(n = 48) were randomly selected from a representative F_1 plant from each cross and the dehulled grains were phenotyped using the ASV assay resulting in the identification of 35 wild type (high ASV) and 13 mutant (low ASV) grains from the Kitaake/KDS-1824B F_1 (segregation ratio of 2.7:1) and 36 wild type and 12 mutant from the KDS-1824B/Kitaake F_1 (segregation ratio of 3:1). The segregation ratios observed from both populations were consistent with a single gene recessive mutation conferring the altered alkali digestion trait observed in KDS-1824B (χ^2 = 0.111, df = 1, P = 0.739, not significant at $P \leq 0.01$; $\chi^2 = 0$, df = 1, P = 1, not significant at $P \leq 0.01$).

DISCUSSION

ECQ of rice is governed primarily by AAC and other physico-chemical properties of starch such as GC and GT (Pang *et al*. 2016; Wang *et al*. 2019). GT directly reflects the energy and time needed for cooking rice grains (Tian *et al*. 2005) and is related to the digestibility and taste of cooked rice (Kim and Kim 2016). Since the direct measurement of GT is not trivial, the alkali spreading value (ASV) as determined by the dispersal or disintegration and spread of milled rice grains in dilute alkali solution is commonly used instead as it is a much simpler and rapid way to evaluate rice GT type (Little *et al*. 1958; Tian *et al*. 2005). The ASV assay was employed to identify the first major gene (*ALK*) controlling GT in rice (He *et al*. 1999; Umemoto *et al*. 2002; Gao *et al*. 2003) as well as numerous GT QTLs (Wang *et al*. 2019). Most *japonica* varieties like Kitaake have lower GT due to three SNPs in the *SSIIa* (*ALK*) gene that significantly reduce SSIIa activity, leading to more amylopectin short chains with degree of polymerization (DP) ≤ 12 which are only able to form short double helical structures that do not require as much energy to transform (Miura *et al*. 2018).

In this study, we used a modified ASV assay to evaluate milled grains from M_2 mutants derived from the low GT type rice variety Kitaake. Three M_2 families exhibiting low ASV scores (i.e. higher GT type) compared to wild type Kitaake were identified, two of which appeared to be fixed (i.e. homozygous) for the mutant phenotype based on the uniform appearance and response of the milled grains to the dilute alkali solution (KDS-1623B and KDS-1824B). Exposure to the 1.7% KOH had almost no effect on the KDS-1623B grains which exhibited shrunken endosperms characteristic of the *sugary-1* mutant (Kubo *et al*. 1999). In *sugary-1* mutants the endosperm starch is replaced by phytoglycogen due to reduced activity of the isoamylase-type starch debranching enzyme encoded by *ISA1* (Kubo *et al*. 1999; Kawagoe *et al*. 2005). This lack of starch is consistent with the low ASV phenotype of KDS-1623B. Identification of a missense mutation in the catalytic domain of ISA1 by targeted exon capture and sequencing and the complete association of this mutation with the shrunken endosperm/low ASV phenotype in and F_2 mapping population (KDS-1623B/Kitaake) strongly supports the causal relationship of this mutation and the altered alkali digestion trait. Interestingly, a severe segregation distortion was observed in the F_2 population, which was derived from a cross in which the maternal KDS-1623B parent was found to be sterile. The segregation distortion suggests that a closely linked mutation affects viability.

Milled grains of the KDS-1824B mutant exhibited ASV ratings corresponding to high intermediate/intermediate GT type. While the same average width as Kitaake, KDS-1824B brown rice grains were shorter and weighed about 13% less. Initial genetic analysis indicates that a single recessive gene mutation underlies the altered alkali digestion trait observed in this mutant and F_2 populations are being grown for genotyping and phenotyping of F_3 seeds. While the targeted exon capture and sequencing approach was successful in identifying the likely causal mutation in KDS-1623B, the KDS-1824B mutant was not included in this pilot experiment due to sample limitations (Kim and Tai 2019). Given the limited number of starch-related target genes included in the capture reagent, a mutation mapping approach (Abe *et al*. 2012) may be a more robust strategy considering the large number of QTLs identified for GT (Kim and Kim 2016; Wang *et al*. 2019).

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