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Genetic variation and association mapping of silica concentration in rice hulls using a germplasm collection

R. Bryant · A. Proctor · M. Hawkridge · A. Jackson · K. Yeater · P. Counce · W. Yan · A. McClung · R. Fjellstrom

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Abstract An association analysis on the genetic variability for silica concentration in rice hulls was performed using a "Mini-Core" set of 174 accessions representative of the germplasm diversity found in the USDA world collection of rice. Hull silica concentration was determined in replicated trials conducted in two southern states in the USA and was analyzed for its association with 164 genome-wide DNA markers. Among the accessions, the average silica concentration ranged from 120 to 251 mg g^{-1}. Ample variation was seen within each of the five sub-populations of rice, as well as the 14 geographic regions that the accessions originated from. There was also an effect due to location and accession × location (G × E) interaction demonstrating the importance of assessing silica concentration across multiple environments. Twelve markers on ten chromosomes were significantly associated with hull silica concentration. Six markers (RM5644, RM5371, RM1335, RM283, RM263, and RM178) corroborated quantitative trait locus for silica concentration identified in other mapping studies. Our results provide germplasm and genetic markers that will assist breeding efforts to develop cultivars that have either high or low hull silica concentration. High silica hulls are good raw material for silica based industrial compounds, while low silica hulls are more biodegradable.

Keywords Silica · Association mapping · Germplasm · Grain quality · *Oryza sativa*

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R. Bryant (✉) · A. Jackson · W. Yan · A. McClung · R. Fjellstrom
USDA-ARS, Dale Bumpers National Rice Research Center, 2890 Hwy 130 E, Stuttgart, AR 72160, USA
e-mail: rolfe.bryant@ars.usda.gov

A. Proctor
Department of Food Science, University of Arkansas, Fayetteville, AR 72701, USA

M. Hawkridge
Department of Physics, University of Arkansas, Fayetteville, AR 72701, USA

K. Yeater
USDA-ARS, Southern Plains Area, College Station, TX 77840, USA

P. Counce
University of Arkansas Rice Research and Extension Center, Stuttgart, AR 72160, USA

Introduction

Rice (*Oryza sativa* L.) is a silicon (Si) accumulating plant and, although Si is not an essential nutrient, it plays an important role in the growth and health of rice plants. Silicified cells are found in the epidermis of leaf blades and in the vascular tissues of the leaf sheath and stem (Ma and Takahashi 2002). Silica is found in varying concentrations throughout the plant and can make up to 10% of the shoot dry weight. Dai et al. (2005) reported that the silica concentration of two parental lines used in their mapping population had roughly twice the silica concentration in the hull than was present in the stem. Lombi et al. (2009) observed that the majority of the silica in the rice seed was deposited in the palea and lemma making up the hull.

Numerous studies have shown that Si in rice plant tissue increases along with an increase of Si application to soil

(Datnoff et al. 2000; Kim et al. 2002; Rodrigues et al. 2004). In the 1930s and 1940s, Japanese researchers indicated that Si was effective in controlling some rice diseases (Takahashi 1995). Others have shown that an increase in plant Si significantly decreased the severity of diseases (Marschner 1995; Kim et al. 2002; Rodrigues et al. 2004). Silicon has been reported to increase yield up to 48%, reduce grain discoloration, control pests, and reduce the need for fertilizer (Correa-Victoria et al. 2001; Ishiguro 2001; Prabbu et al. 2001).

Genes responsible for silica uptake and transport have been identified in rice. Those genes regulate the total silica concentration in the rice plant as well as the distribution of silica concentration in specific tissues. Using conventional linkage mapping and mutant screening techniques, Ma and Yamaji (2006) identified the rice gene *Low silicon rice1* (*Lsi1*). Lsi1 protein is a member of the aquaporin family and regulates silica concentration in the plant by functioning as an active silica influx transporter for silica uptake in root cells from the surrounding environment. Ma et al. (2007) later identified *Lsi2*, which regulates silica concentration in the plant by functioning as a silica efflux transporter that moves silica from the root cells into the stele. Using a homology search approach Yamaji et al. (2008) identified *Lsi6*, which also codes for a silica influx transporter and is a member of the aquaporin family. Lsi6 protein is active in the distribution of silica in the sheath and leaves of rice and functions by moving silica (silicic acid) out of the xylem into the surrounding xylem parenchyma cells.

While prior studies have examined silica concentration from a genetic perspective using conventional linkage mapping populations, mutant screening, and homology search techniques, there has been no association mapping study conducted to elucidate the genetic control of silica concentration in rice. Unlike linkage mapping studies, association mapping analyses, which take advantage of a whole genome scan approach, do not require prior knowledge of the genetic variation or the number of genes controlling the trait being analyzed. Association mapping can also be advantageous in its ability to examine hundreds, or even thousands, of varieties for marker-trait associations rather than being limited to the genetic constraints imposed when only two varieties are used in the creation of a mapping population (Zhu et al. 2008). This in turn can increase the probability of finding a novel gene or quantitative trait locus (QTL) that may not be present in the two parents of a conventional mapping population. Association mapping studies involving a wide range of markers and population sizes have been successful in identifying QTL for various traits (Sneller et al. 2009; Yu et al. 2009). In addition, association mapping can provide the fundamental knowledge necessary for developing conventional mapping populations desired for fine mapping specific QTLs or for direct use in breeding programs via marker assisted selection.

One of the issues confronting rice farmers and rice mills is how to dispose of the large amounts of abrasive, low nutritional value, high silica content rice straw and hulls. The US production of rough rice for 2010–2011 is estimated to be 11.4 billion kg (Childs and Baldwin 2010). Therefore, approximately 2.3 billion kg of hulls and 4.6 billion kg of straw will potentially be produced. Due to the high silica concentration, low density, high chemical stability, and low nutritional value of straw and hulls, the cost of transporting and disposal of these co-products is a major problem for the US rice industry. With the exception of California, most of the rice straw is burned in the field to facilitate its decomposition. However, burning rice straw is becoming less of an option due to environmental concerns. Some mills burn rice hulls to produce steam or electricity, which in turn makes disposal of the rice hull ash difficult due to the high silica concentration (70–95%) (Marshall 2004). Unused hulls and ash are usually taken to a landfill where they remain for years due to their chemical stability.

In an effort to reduce plant accumulation of Si, Ma et al. (2002) developed a rice mutant defective in Si uptake and found that the leaf blades were droopy, lodging increased, and photosynthesis decreased. Another approach for reducing the amount of hulls and ash going to the landfill is to use the silica for value-added products. Rice hulls have been used in such products as: particle board, poultry bedding, brick making, package cushioning, and absorbents. Due to the high silicon concentration, rice hulls and ash can also be used as raw materials in the production of silicon-based industrial materials with high economic value. These include silicon carbide, silica, silicon nitride, silicon tetrachloride, pure silicon, and zeolite (Sun and Gong 2001). As the silica in rice hulls is amorphous, it can be extracted at lower temperatures than silica derived from conventional manufacturing techniques, therefore, reducing the cost of production (Kalapathy et al. 2002).

Understanding the genetic control of silica concentration in rice will facilitate the development of new varieties with high silica concentration that will bring value to what is otherwise considered a waste product. For this to occur it is necessary to know the genetic variation of silica in rice hulls and to identify possible QTLs associated with silica concentration that can be used in breeding. The USDA world collection of rice contains over 18,000 accessions from 115 countries (Bockelman et al. 2003). In order to improve the efficiency of phenotypic and genotypic characterization, a representative Core subset (~1790 accessions) was developed using a stratified random sampling of the whole collection (Yan et al. 2007). From the Core subset, a "Mini-Core" collection (217 accessions) was

selected using PowerCore software. Analysis of 26 phenotypic traits and 70 SSR markers proved that the Mini-Core represents the genetic diversity in the whole collection (Agrama et al. 2009). This Mini-Core collection includes representatives of all five sub-populations of rice; *indica* (IND), *aus* (AUS), *temperate japonica* (TEJ), *tropical japonica* (TRJ), and *aromatic* (ARO) as well as *wild rice* accessions (Li et al. 2010). The international origination of the Mini-Core accessions from 76 countries results in one of the most diversified collections in rice, which is valuable for mining genes of interest.

The objectives of this study were to (1) determine the growth stage and location of maximum silica deposition in the rice grain, (2) determine the genetic variability for silica concentration in the Mini-Core which is a representative subset of the USDA rice world collection; (3) determine the relationship of silica concentration with subpopulation structure and geographic region of accession origin; and (4) determine the genetic markers associated with rice hull silica concentration using linkage disequilibrium mapping techniques.

Experimental

Materials (silica concentration in the rice grain)

Seeds of the cultivar "Wells" (PI 612439, http://www.ars-grin.gov/cgi-bin/npgs/acc/display.pl?1588913) and its recessive mutant for brittle culm, designated "Wells *brittle*" (GSOR 6, http://www.ars-grin.gov/cgi-bin/npgs/acc/display.pl?1651509), were planted in the greenhouse at the Dale Bumpers National Rice Research Center, Stuttgart, Arkansas (AR). Nomenclature (V1–V13, R0–R9) developed by Counce et al. (2000) was used to designate growth stages for plant sampling. At vegetative growth stage V2, twenty plants were transplanted into individual vented 2-liter pots filled with finely ground silt loam soil and moistened with water. The soil was kept moist and the plants were fertilized once a week with a commercial soluble fertilizer solution (15% N, 30% P_2O5, 15% K_2O, 0.02% B, 0.07% Cu, 0.15% chelated Fe, 0.05% chelated Mn, 0.0005% Mo and 0.06% water soluble Zn). Pots were arranged in a randomized complete block, split plot design experiment. Main plot treatments were the reproductive stages of development (R3, R4, R5, R6, R7 and R8) and subplot treatments were cultivar (Wells and Wells *brittle*). Pots within a subplot replication were rotated twice weekly to minimize position effect within the greenhouse. Beginning at vegetative growth stage V11 each plant was monitored daily for reproductive growth stage development. When a sufficient number of seeds of a designated growth stage were present on the plant, the panicles were harvested and the individual grains were removed and grouped according to reproductive growth stages R3 through R8. Silica concentrations were determined on the whole grains at each developmental stage. Because of the variation in development of grains along the length of the panicle and among tillers of the same plant, and in order to obtain enough sample for each reproductive stage, grains were harvested from plants in different pots and at different times within a subplot replication and bulked together. Since the weights of the seeds are different at each developmental stage (Fig. 1), the number of seeds needed for 0.2 g varied. Therefore, the data was reported on per grain basis, instead of per gram basis, derived by dividing the concentration by the number of seeds needed to make 0.2 g.

Wells and Wells *brittle* were also grown in field plots at University of Arkansas Rice Research and Extension Center near Stuttgart, AR in 2002. Rice seeds were sown in rows 19 cm apart, 500 seeds m^2. The soil was a DeWitt silt Loam (Typic Albaqualfs). Rice was flooded at the V4 through V5 stages of development. Standard fertilization and weed control practices were used. Rice was drained for harvest 14 days after 50% heading. The rice plants were harvested by hand at R8, threshed, and aspirated. After drying overnight, rice grain for these analyses were allowed to equilibrate to approximately 12% moisture. Two aliquots of R8 seeds (0.2 g) from each replication (4) of each cultivar were analyzed for their silica concentration with and without the hulls and after milling to 10% (removal of bran layer) degree of milling.

Materials (Mini-Core)

One hundred and seventy-four accessions selected from the Mini-Core that originated from 14 global regions (Agrama et al. 2009, 2010) and representing six genetic groups (IND n = 54, AUS n = 37, TEJ n = 32, TRJ n = 26, ARO n = 5, and Admix n = 20) were grown in field plots near the Dale Bumpers National Rice Research Center, Stuttgart, AR and the Rice Research Unit (RRU), Beaumont, Texas (TX) in 2009. The accessions were selected based on those that would mature and set seed at both locations. Seed originating from a single plant selection of each accession was used for sowing. A randomized complete block design was used with two replications per location. The samples from each plot were harvested by hand, threshed and cleaned using standard field equipment (ALMACO, Nevada, Iowa), and dried to approximately 12% moisture using a forced air drier. Rough rice samples from the plots were dehulled with a Satake Rice Machine (Satake Engineering Co., LTD., Ueno Taito-Ku, Tokyo) and after drying at 80°C for 2 h, the hulls (~3 g) were stored in 50 ml polypropylene tubes (Cat. # 05-539-5, Fisher Scientific, Houston, TX) at room temperature (22°C) until analyzed.

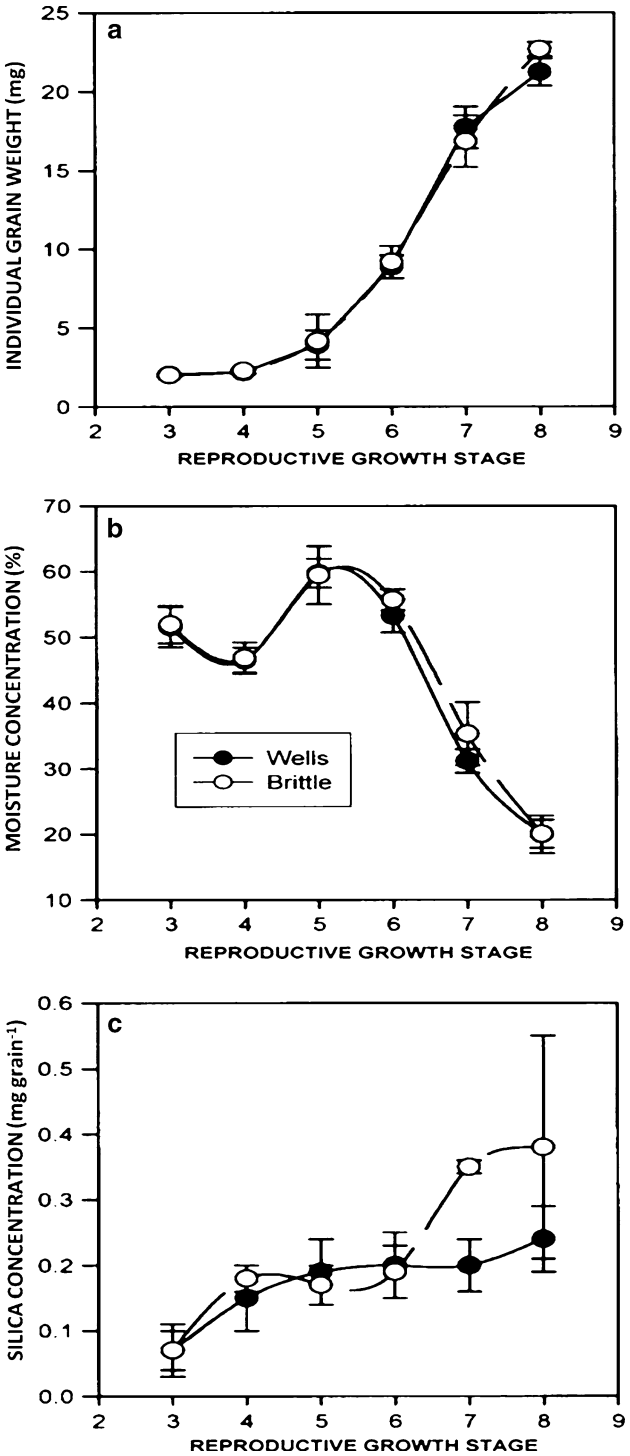

Fig. 1 The weight (**a**), percent moisture (**b**), and silica concentration of the grains of Wells and Wells *brittle* during their developmental stages from R3 to R8

Silica concentration

Silica was determined using the molybdenum yellow method described by Saito et al. (2005). Dried hulls (section "Materials (Mini-Core)") or grains (section "Materials (silica concentration in the rice grain)") (0.2 g) were placed in 15 ml polypropylene tubes and 10 ml of hydrofluoric acid (HF) solution (1.5 M hydrofluoric acid–0.6 M hydrochloric acid) was added. The samples were allowed to sit at room temperature (22°C) for 1 h swirling every 15 min. The solution from the hulls was diluted 1:1 with deionized water, and 100 µl of the diluted solution was place into a 15 ml centrifuge tube. To neutralize the acid and obtain the desired pH, 6 ml of 0.5 M boric acid and 2 ml of working molybdenum solution was added to each tube. After vortexing and allowing to sit at room temperature for 3 min., 1 ml of 0.5 M citric acid and 900 µl of water was added to each sample (total = 10 ml), vortexed and allowed to sit at room temperature for 10 min. The developed color was read at 400 nm using a Cary 50 spectrophotometer (Varian Australia Pty. Ltd., Mulgrave, Victoria, Australia) and converted to silica concentration according to Saito et al. (2005). Silica concentrations were expressed on a dry weight basis. All tubes were made of polypropylene and reagent blanks ran on each batch to ensure they contained no detectable silica. A reagent blank and standards were run with each set and each sample was run in duplicate.

X-ray diffraction

The structural analysis of the silica in the hull before, during, and after extraction was determined using x-ray diffraction (XRD) by adapting the method of Proctor (1990). A long grain rice cultivar, "Rondo" (Yan and McClung 2010), was planted in a field plot at the Dale Bumpers National Rice Research Center, Stuttgart, AR in 2009 and bulk harvested at maturity. After drying the rough rice to 12% moisture, grains were dehulled and the hulls dried as described above. The dried hulls (0.2 g) were extracted for 0, 15, 30, 45, and 60 min with HF solution (see above). After each extraction time the hulls were rinsed with water and dried at 130°C for 2 h. The processed hulls were characterized by x-ray diffraction using a Philips PW1830 model powder diffractometer (Philips, Almelo, The Netherlands) with Cu–Kα radiation (1.5419Å), a diffracted beam monochromator, ¼° divergence and 0.45 mm receiving slits. The silica concentration was determined semi-quantitatively by measuring the area under the associated peak at 22°, less the smooth continuous background determined using the PANalytical X'Pert HighScore software package (v. 2.2.5, PANalytical B.V., Almelo, The Netherlands).

Genotyping

One hundred and fifty-six SSR markers (marker sequence and genomic location information available from Gramene,

http://www.gramene.org), two indel markers (RID 12, Sweeney et al. 2006; and Pi-ta, Wang et al. 2010), and six SNP markers (*Waxy* EX1 G/T, EX6, and EX10, Chen et al. 2010; and 3 ALK genes, SNPs, Bao et al. 2006) distributed across the rice genome at approximately every 10 cM were used to genotype 174 accessions from the Mini-Core collection. Markers sequences for AL606682-1, AP5652-1, AP5652-2, con673, and LJSSR1 are referenced in Li et al. 2011. The physical location (Mb) of the markers in the rice genome are shown in Fig. 4.

DNA from the leaf tissue of each Mini-Core accession was extracted using two methods, a CTAB method as described by Hulbert and Bennetzen (1991) and a rapid DNA extraction method described by Xin et al. (2003). PCR marker amplifications were performed in 25 µl volumes containing 20 ng of template DNA, and final concentrations of: 10 mM Tris–HCl at pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 300 nM each primer, and 1 unit of Taq DNA polymerase (Promega, Madison, WI, USA). For PCR with template DNA prepared using the rapid extraction method, bovine serum albumin and PVP40 were added at 0.1 and 1% concentrations relative to the final reaction volume, respectively. For each marker, the primer was labeled with a fluorescent dye (6FAM, NED or Hex; Applied Biosystems, Foster City, CA, USA or Integrated DNA Technologies, Coralville, IA, USA).

PCRs were performed in MJ Research thermal cyclers (Bio-Rad, Hercules, CA, USA). PCR amplification and analysis of SSR's markers were performed as described in Costanzo et al. 2011.

Association mapping and genetic analysis

Ancestry relationship and sub-population structure among the Mini-Core accessions have been previously described (Agrama et al. 2010; Li et al. 2010) and, in part, verified again in this study. The software Structure 2.3.3 (Pritchard et al. 2000; Falush et al. 2003) was run with a burn in of 50,000 and run length of 80,000 to confirm population structure. Eight independent replications using sub-population numbers ranging from one to eight were run with the options of admixture (Admix) being present and correlated allele frequencies selected. Structure results were interpreted using the Evanno et al. (2005) method that examines the ad hoc statistic ΔK, which measures the rate of change in the log-likelihood value and provides an estimate of the population number. Principal components analysis (PCA) was performed with Nei's genetic distance (Nei 1973) using GenAlEx 6.3 (Peakall and Smouse 2006) with 164 markers as an alternative to the program Structure to determine the population sub-structure (*Q*) of the lines. PowerMarker 3.25 (Liu and Muse 2005) was used to determine polymorphism information concentration (PIC values) of the markers and allele frequency. Rare genotypes occurring less than two percent of the time were removed from the data set prior to association mapping analysis. Frequency based distance was generated using the Rogers 1972 distance option and the subsequent results were used to create a UPGMA tree in PowerMarker. Mega 4 (Tamura et al. 2007) was used to graphically display the UPGMA tree. Kinship values were determined using SPAGeDI 1.3 (Hardy and Vekemans 2002) with the Loiselle et al. (1995) option.

Tassel 2.1 (http://www.maizegenetics.net/) was used to run association mapping. Two mapping models were compared; the mixed linear model (MLM) as described in Yu et al. (2006), which accounts for both population (Q) and kinship (K), and the general linear model, which accounts for Q only. Silica concentration was averaged across the two field replications at each location. The means of each location were analyzed both independently (AR and TX locations) and then averaged together to examine the overall effect (combined locations).

Statistical analysis of silica concentration

The silica concentration was analyzed with the GLIMMIX procedure in SAS (Version 9.2, SAS Institute, Cary, NC). The statistical model followed the experimental design of randomized complete block, with all effects considered as random (accessions, locations, and blocks). Within the GLIMMIX procedure, best linear unbiased predictors (BLUPs) and predicted standard errors were calculated using the SOLUTIONS option. Broad sense heritability estimates were calculated using the method of Singh et al. (1993).

Results and discussion

Silica location in rice kernels

Figure 1 shows the grain weight, moisture concentration, and silica concentration of Wells *Brittle* and Wells at each developmental stage, as defined by Counce et al. (2000). Figure 1a, b show the individual grain weight (mg) and moisture concentration (%) of Wells *brittle* and Wells, respectively at each developmental stage. Wells *brittle* and Wells both gained weight at the same rate and the moisture profiles for each were also the same. The grain weight at R3 (panicle exertion) was 2 mg $grain^{-1}$ and the weight increased slightly till R4 (anthesis). From R4 to R8, the grain weight dramatically increased to 23 mg $grain^{-1}$ due to grain filling. The grain moisture concentration decreased during anthesis from 52% at R3 to 47% at R4 and then increased to 59% at R5 (caryopsis elongation) (Fig. 1b). Then, the moisture started steadily decreasing in the grain from R5 to

R8 (grain dry-down) (Fig. 1b). During the R5 growth stage and early R6 (grain filling), the caryopsis is expanding and filling with starch, however, the contents are liquid or soft dough, and thus, have high moisture. The starch hardens during mid- and late-R6 as the moisture content begins to decrease and the grain continues to mature.

Figure 1c shows the silica concentration (mg grain^{-1}) in the seeds of Wells *brittle* and Wells at each developmental stage. Unlike the moisture and grain weight, Wells *brittle* and Wells did not have the same silica profile. Both lines had <0.1 mg grain^{-1} silica at the R3 and up to ~0.2 mg grain^{-1} at the R5. There was very little increase in silica for Wells after R5. However, the silica concentration for Wells *brittle* went from 0.2 mg grain^{-1} at R6 to 0.4 mg grain^{-1} at R8, while it only increased to 0.25 mg grain^{-1} for Wells. Therefore, the accumulation of silica appears to be different for some genotypes after the R6 growth stage.

Figure 2 shows the silica concentration (mg grain^{-1}) of rough, brown (the bran still on but without the hull), and milled (without the bran and hull) rice grains of Wells and Wells *brittle* at the R8 developmental stage. The majority of silica (75%) appeared to be in the hull. For Wells *brittle* and Wells the rough rice contained 0.40 and 0.33 mg grain^{-1}, brown rice contained 0.08 and 0.01 mg grain^{-1}, and milled rice contained 0.02 and 0.01 mg grain^{-1}, respectively. Therefore, analysis of rice hulls at the R7 (physiological maturity) or later can be used to differentiate maximum silica accumulation in the grain among genotypes. The difference in silica concentration seen at R8 in the two sample sets (Figs. 1, 2) could be due to the environment in which they were grown. The first set was grown in the greenhouse and the second set was grown in the field.

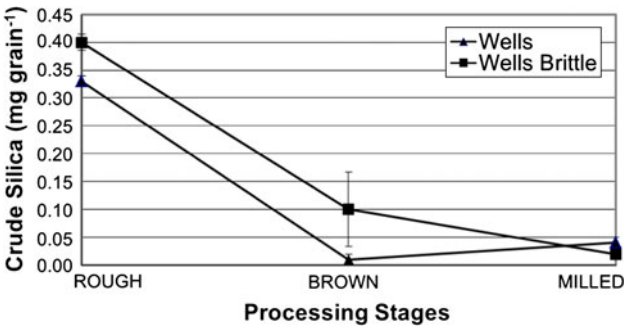

Fig. 2 Silica concentration of rough, brown and milled grains of Wells and Wells *brittle* at different processing stages

X-ray diffraction analysis

XRD showed that, after digesting for 1 h, peaks were seen at $2\theta = 15.0$, 22.0 and 34.5° (Fig. 3). Although the peaks at $2\theta = 15.0$ and 34.5° remained approximately the same, the area under the peak at $2\theta = 22.0°$ did not show significant reduction with extraction time up to 30 min. Indeed, the area under the peak at $2\theta = 22.0°$ increased with extraction time in some cases. The $2\theta = 22.0°$ peak is characteristic of disordered opal CT, α-cristobalite, which is indicative of amorphous silica (Proctor 1990). The area under the curve was found not to be representative of the concentration of

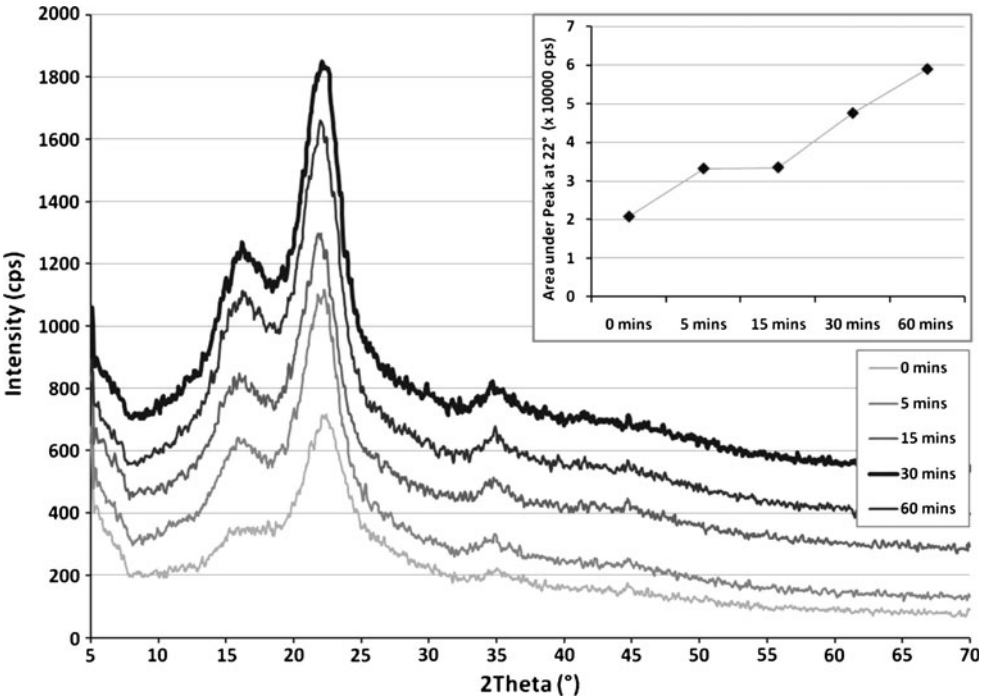

Fig. 3 X-ray Diffraction patterns of residual rice hulls obtained following digestion with HF for time periods of 0, 5, 15, 30 and 60 min, respectively. The intensities of the datasets are offset by arbitrary values to make viewing easier. The *inset* shows the integrated peak area above background for the peak at $2\theta = 22°$, which, for this series, increases with digestion time

Table 1 Rice hull silica concentration of 174 accessions from the Mini-Core, their global region of origin, and sub-population designation

ACNO[a]	Region[a]	Group[b]	Silica[c] (mg g^{-1})	SD[d]
2,169	Central America	TRJ	197.76	3.27
7,404	China	IND	204.43	14.28
8,913	Central America	TRJ	208.78	29.47
11,030	North America	TRJ	217.48	15.56
12,492	China	IND	185.90	18.90
37,215	South America	TRJ	224.65	20.54
54,344	Southeast Asia	TRJ	223.12	35.85
67,153	Southern Asia	IND	209.64	18.67
127,076	Southern Asia	AUS	182.32	49.08
154,435	China	Admix(TRJ-TEJ)	190.74	10.91
154,531	North Pacific	TEJ	163.13	0.28
155,990	Oceania	TRJ	216.48	18.34
157,385	North Pacific	TEJ	185.83	18.27
160,530	China	IND	203.61	30.89
160,700	China	IND	204.60	0.50
161,567	South America	TRJ	237.30	4.59
162,113	North Pacific	TEJ	188.23	3.40
168,946	Western Europe	TEJ	217.41	11.05
177,224	Mideast	TEJ	166.75	15.36
182,254	Mideast	TEJ	166.72	13.44
184,506	North Pacific	TEJ	198.87	20.16
189,460	Western Europe	TEJ	185.66	44.04
198,134	Southeast Asia	TRJ	184.21	1.60
199,553	Central America	TRJ	243.21	2.77
202,864	Central America	IND	240.61	0.05
214,077	Central America	IND	241.43	16.19
215,478	Western Europe	TEJ	221.80	17.26
215,970	China	Admix(AUS-IND-TEJ-TRJ)	214.59	7.19
220,214	Central America	TRJ	209.05	14.04
223,612	South America	Admix(TEJ-TRJ)	233.59	21.09
226,204	North Pacific	TEJ	205.83	38.96
240,638	Southern Asia	AUS	237.17	30.17
245,071	China	Admix(TRJ-TEJ-ARO)	169.69	5.31
256,340	Southern Asia	ARO	228.21	16.78
263,813	South America	IND	175.96	4.01
264,242	Central America	IND	226.59	18.26
265,110	Eastern Europe	TEJ	171.39	41.17
266,122	Eastern Europe	TEJ	167.36	27.84
267,996	Western Europe	TEJ	200.34	3.94
268,003	Western Europe	TEJ	247.48	13.39
277,414	Southern Asia	ARO	171.70	20.44
277,415	Southern Asia	AUS	196.98	19.06
281,914	Southeast Asia	IND	213.37	6.47
282,173	Eastern Europe	TEJ	186.57	12.81
282,768	Africa	TRJ	217.36	13.60
282,769	Africa	TRJ	216.95	14.01
291,430	Central Asia	TEJ	162.75	48.94
291,539	Western Europe	TEJ	195.35	7.53
291,608	South America	TRJ	214.00	0.53

Table 1 continued

ACNO[a]	Region[a]	Group[b]	Silica[c] (mg g^{-1})	SD[d]
321,183	South Pacific	IND	196.21	34.32
346,827	South America	TEJ	219.17	9.25
352,687	North Pacific	Admix(AUS-TEJ-ARO)	175.48	25.23
353,722	Southern Asia	AUS	251.35	12.66
353,723	Southern Asia	AUS	238.79	7.33
373,232	Southeast Asia	Admix(TRJ-TEJ-ARO)	162.99	10.94
373,249	Southeast Asia	Admix(TRJ-TEJ-ARO)	167.77	0.11
373,335	Southern Asia	IND	201.20	23.33
373,340	Southern Asia	AUS	202.86	43.75
373,341	Southern Asia	AUS	212.00	38.61
373,347	Southern Asia	AUS	194.85	32.03
373,403	Southern Asia	AUS	220.67	14.71
373,536	Southern Asia	IND	229.84	3.63
373,761	Oceania	TEJ	194.09	5.96
373,771	Oceania	TRJ	246.63	9.08
373,781	Oceania	TRJ	219.19	35.40
373,786	Oceania	TRJ	245.12	31.47
373,795	Southern Asia	IND	190.66	13.62
373,816	South Pacific	TRJ	248.34	20.69
373,820	South Pacific	TRJ	192.91	38.60
373,899	Eastern Europe	TRJ	225.42	7.82
373,939	Southern Asia	TEJ	170.59	15.96
385,697	Southern Asia	AUS	193.07	37.38
385,826	Southern Asia	AUS	201.19	20.20
388,304	North Pacific	IND	171.92	33.91
388,427	South America	TEJ	192.29	11.26
388,436	South America	AUS	208.92	22.11
389,037	China	IND	208.51	34.77
389,069	China	IND	196.76	36.55
389,234	Southeast Asia	IND	181.69	20.16
389,267	Southeast Asia	IND	192.72	12.37
389,863	Southern Asia	AUS	216.58	19.67
389,876	South Pacific	TRJ	219.19	0.54
389,923	China	IND	214.18	21.56
389,960	Southeast Asia	IND	188.21	55.25
391,214	Western Europe	IND	193.72	30.31
391,218	South America	IND	218.18	39.63
391,264	Southern Asia	IND	232.26	32.52
392,605	Southern Asia	IND	203.76	19.65
392,630	Africa	AUS	196.72	33.80
392,637	Africa	AUS	230.99	8.57
392,677	Southern Asia	AUS	174.12	34.30
392,694	Southeast Asia	AUS	192.16	38.35
392,768	Southeast Asia	AUS	214.67	22.81
392,813	South America	IND	196.70	33.84
393,070	Southeast Asia	IND	229.92	40.52
393,112	Southern Asia	AUS	184.40	20.93
393,180	Africa	IND	202.72	23.66
393,292	Southern Asia	IND	175.93	17.12

Table 1 continued

ACNO[a]	Region[a]	Group[b]	Silica[c] (mg g^{-1})	SD[d]
399,748	South Pacific	IND	211.44	39.58
400,398	China	IND	185.49	15.53
400,607	China	IND	179.92	13.65
400,672	Western Europe	Admix(TRJ-IND-AUS)	186.44	16.73
400,771	Africa	Admix(IND-AUS)	186.15	14.85
400,780	Africa	Admix(IND-AUS)	175.90	41.23
402,673	Southern Asia	Admix(IND-AUS)	185.14	12.24
402,689	Southern Asia	AUS	203.99	41.03
402,789	Oceania	TEJ	181.74	40.71
402,794	Western Europe	TEJ	204.85	16.09
402,983	South Pacific	AUS	209.56	35.80
403,082	Southern Asia	AUS	196.84	29.60
403,114	Southern Asia	AUS	198.16	32.59
403,214	Southern Asia	AUS	193.05	21.00
403,289	Southeast Asia	IND	203.75	35.01
403,422	Africa	IND	170.04	18.84
403,469	Africa	AUS	193.06	19.47
406,073	South America	ARO	187.67	23.62
408,406	South Pacific	IND	174.90	3.26
412,790	Southern Asia	AUS	223.33	19.64
412,811	Southern Asia	Admix(AUS-IND)	181.32	54.46
417,820	South Pacific	IND	184.11	4.41
418,207	Africa	AUS	227.49	27.76
420,960	South America	IND	173.24	1.79
430,254	Central America	IND	218.46	13.84
430,339	Oceania	AUS	213.55	23.16
430,387	Africa	TRJ	218.70	50.23
430,397	Africa	TRJ	225.06	35.24
430,740	Southern Asia	IND	181.95	8.98
430,909	Southern Asia	Admix(ARO-IND-AUS)	120.47	21.40
430,936	Southern Asia	Admix(AUS-ARO-IND)	225.95	21.26
430,979	Mideast	Admix(IND-AUS)	222.96	8.35
431,092	Mideast	AUS	195.27	18.89
431,128	Southern Asia	Admix(IND-TRJ)	233.75	19.37
431,172	South America	AUS	235.71	38.34
431,201	Central Asia	IND	204.96	35.62
431,204	Central Asia	AUS	186.05	45.54
431,210	Central Asia	IND	188.76	1.27
431,499	Southeast Asia	IND	200.72	4.51
433,799	Africa	AUS	189.33	14.84
434,614	Southern Asia	Admix(TEJ-ARO-TRJ)	166.30	18.91
434,632	Africa	TRJ	221.15	12.38
439,024	Southern Asia	AUS	210.91	29.90
439,669	Central Asia	ARO	197.04	35.02
439,674	Eastern Europe	TEJ	178.61	23.91
439,683	Eastern Europe	TEJ	147.36	4.47
464,597	South Pacific	IND	166.14	5.33
493,131	Southern Asia	IND	203.59	4.84
494,105	North America	TEJ	198.96	26.32

Table 1 continued

ACNO[a]	Region[a]	Group[b]	Silica[c] (mg g^{-1})	SD[d]
497,682	South Pacific	IND	171.50	27.64
514,663	North Pacific	TEJ	207.77	8.13
549,224	Southern Asia	Admix(TEJ-TRJ-ARO)	175.67	5.12
549,253	Southern Asia	AUS	231.86	3.40
561,734	North America	TRJ	190.49	24.70
584,555	Southern Asia	Admix(TEJ-ARO-TRJ)	169.22	17.37
584,564	Mideast	AUS	197.20	43.00
584,566	Southern Asia	AUS	234.79	46.89
584,620	Central Asia	AUS	197.72	41.02
584,624	Central Asia	TEJ	181.18	16.69
584,625	Central Asia	ARO	204.88	10.06
584,629	Central Asia	TEJ	187.26	21.05
584,632	Eastern Europe	TEJ	181.26	13.56
585,042	South America	TRJ	208.67	32.58
596,818	Southern Asia	Admix(TEJ-TRJ-ARO)	180.79	1.41
596,902	Southeast Asia	IND	195.57	41.59
596,941	Southern Asia	IND	165.19	25.58
596,990	South Pacific	IND	184.03	42.63
602,606	Eastern Europe	TEJ	175.87	35.60
602,637	Africa	TRJ	223.87	36.10
602,654	Central America	IND	210.11	24.35
608,431	Southeast Asia	IND	195.17	15.49
614,989	China	IND	216.80	37.37
615,022	China	IND	187.53	36.27
615,033	China	IND	211.26	54.79
615,192	China	IND	212.86	21.33
615,198	China	TEJ	189.35	46.74
Mean of Beaumont\Stuttgart			200.39	28.97
Beaumont, TX Mean			187.28	25.60
Stuttgart, AR Mean			213.50	26.12

[a] Accession number and region as designated in Agrama et al. Natural Science 2:247–291 (2010)

[b] Aromatic (ARO), Aus (AUS), Indica (IND), Temperate Japonica (TEJ), Tropical Japonica (TRJ), Admixture (Admix, genetically interbred)

[c] Silica means were calculated using 2 technical reps, ×2 field reps. ×2 locations

[d] SD = standard deviation of 2 technical reps, ×2 field reps. ×2 locations

extractable silica. Since the extraction process will remove components other than silica, the increase in the $2\theta = 22.0°$ peak may be due to the difference in the relative concentration of the remaining silica to the undissolved material. Saito et al. (2005) found that the aforementioned method had a high correlation to the gravimetric method. They speculated that the slight increase in silica concentration seen in the gravimetric method may be due to incomplete dehydration or impurities. XRD shows that it may be due to a small amount of silica that is not extractable by HF. However, the amount of silica remaining is negligible and, therefore, the HF extraction can be used as an alternative to the gravimetric method.

Hull silica concentration analyses

Silica concentration of the Mini-Core averaged 200 mg g^{-1}, and ranged from 120 mg g^{-1} ± 21.40 for ACNO 430909, an Admixture of *aus* (AUS), *indica* (IND) and *aromatic* (ARO) sub-population genotypes, to 251 mg g^{-1} ± 12.66 for ACNO 353722, an AUS sub-population accession, both from Southern Asia (Table 1). The non-Admix sub-population accession with the lowest silica concentration was ACNO 439683, a TEJ from Eastern Europe, having a silica concentration of 147 mg g^{-1} ± 4.47. Wide variation in silica concentration was observed in all genetic populations. The means of the TRJ accessions (219 mg g^{-1}) and AUS accessions (208 mg g^{-1}) were greater than the overall mean of the entire Mini-Core (200 mg g^{-1}), whereas, other groups (TEJ, ARO, IND and Admix) had mean values less than the average of the Mini-Core. All the accessions native to Central America region (n = 9) except ACNO 2169, a TRJ, had higher silica concentration than the Mini-Core average, while the silica concentrations of accessions native to the Mideast (n = 5), Eastern Europe (n = 8), Central Asia (n = 9), and North America (n = 3) were below the Mini-Core average with a few exceptions. Some 25% of the Mini-Core was represented by accessions from Southern Asia

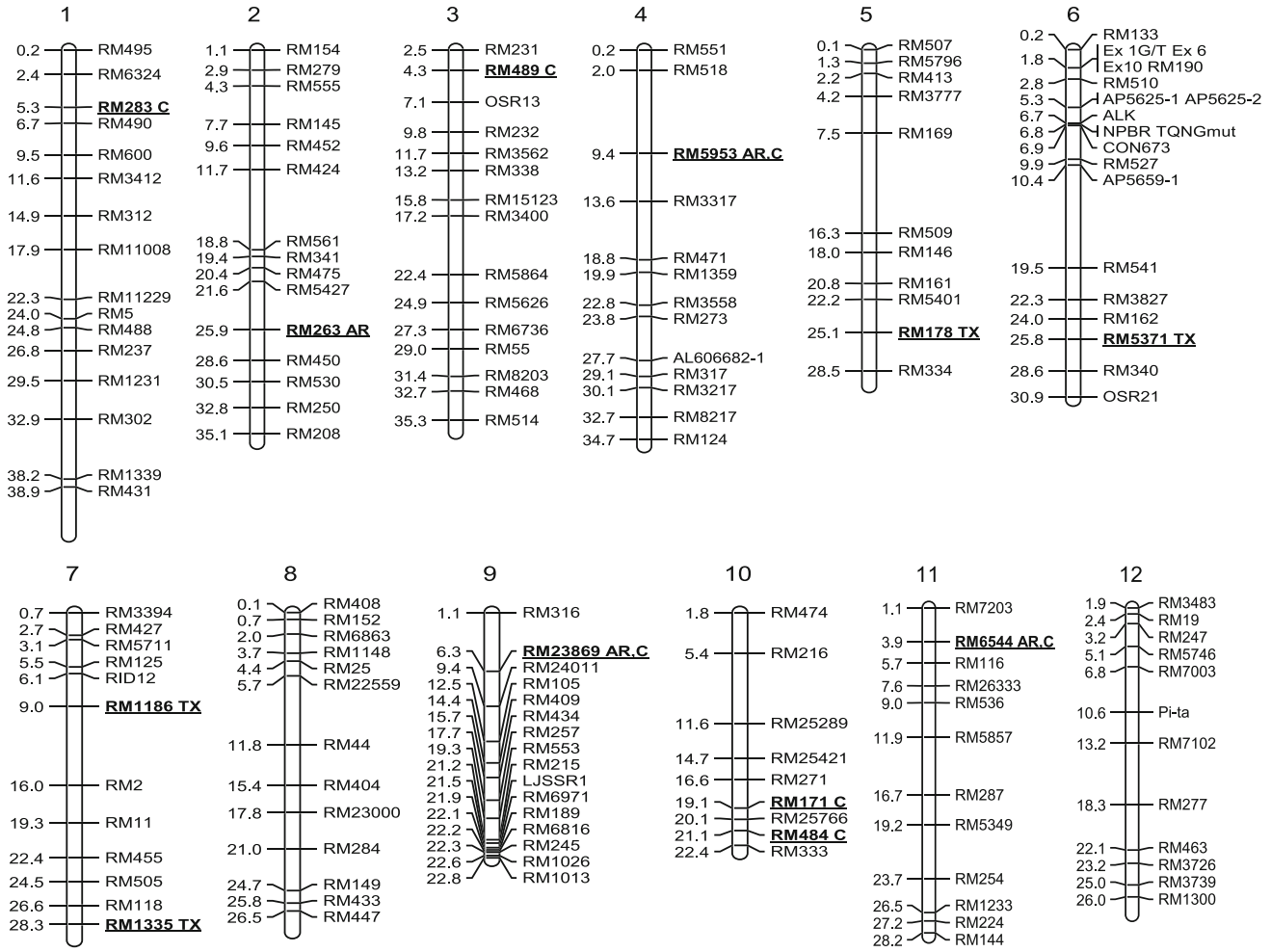

Fig. 4 Physical map (Mb) of the markers used for association mapping. *Underlined bold faced markers* denoted by AR (Arkansas), TX (Texas), or C (combined analysis) were significantly associated with silica concentration in rice hulls at $p \leq 0.01$ for their respective location

(n = 44) which exhibited the widest range of silica concentration, 120–251 mg $g^{-1} \pm 5.17$. The wide variation in silica concentration in rice hulls among the Mini-Core accessions, genetic populations, and geographic regions of the world demonstrates that there is considerable germplasm variability for breeders to utilize for modifying silica concentration in the development of new cultivars.

The variance due to genetics (i.e. accessions) accounted for 32.4% of the total variation for silica concentration, indicating that this trait has a moderately high level of heritability (48%). The silica concentration of samples grown in TX were lower than those grown in AR, with means of 187 mg $g^{-1} \pm 25.60$ and 213 mg $g^{-1} \pm 26.12$, respectively, with environment (i.e. location) accounting for 32.2% of the silica concentration variation. The cause of the location effect is most likely due to differences in soil composition or temperature during the growing season. However, the genetic × environment effect accounted for 25.6% of the total variation, highlighting the importance of considering GxE interactions when selecting accessions for breeding. Some accessions were identified as being stable across environments. Accessions ACNO 549253 and ACNO 373536, an AUS and IND from Southern Asia, respectively, were identified as having consistently high silica concentrations (228–236 and 220–237 mg g^{-1}), respectively, whereas, accessions ACNO 154531, a TEJ from North Pacific, was consistently low (156–169 mg g^{-1}) (Fig. 4).

Molecular sub-structure of Mini-Core collection

Structural analysis indicated that the Mini-Core collection could be classified into five sub-populations, consistent with the known structural studies in rice (Garris et al. 2005; Agrama et al. 2010). Only five ARO accessions were present in this study, and they formed a separate cluster in both the UPGMA tree and PCA. Twenty accessions were classified as admixtures, as was previously reported by Agrama et al. (2010), indicating that these accessions are a result of intermating between sub-populations. The PCA of marker allele variation explained 73.25% of the total

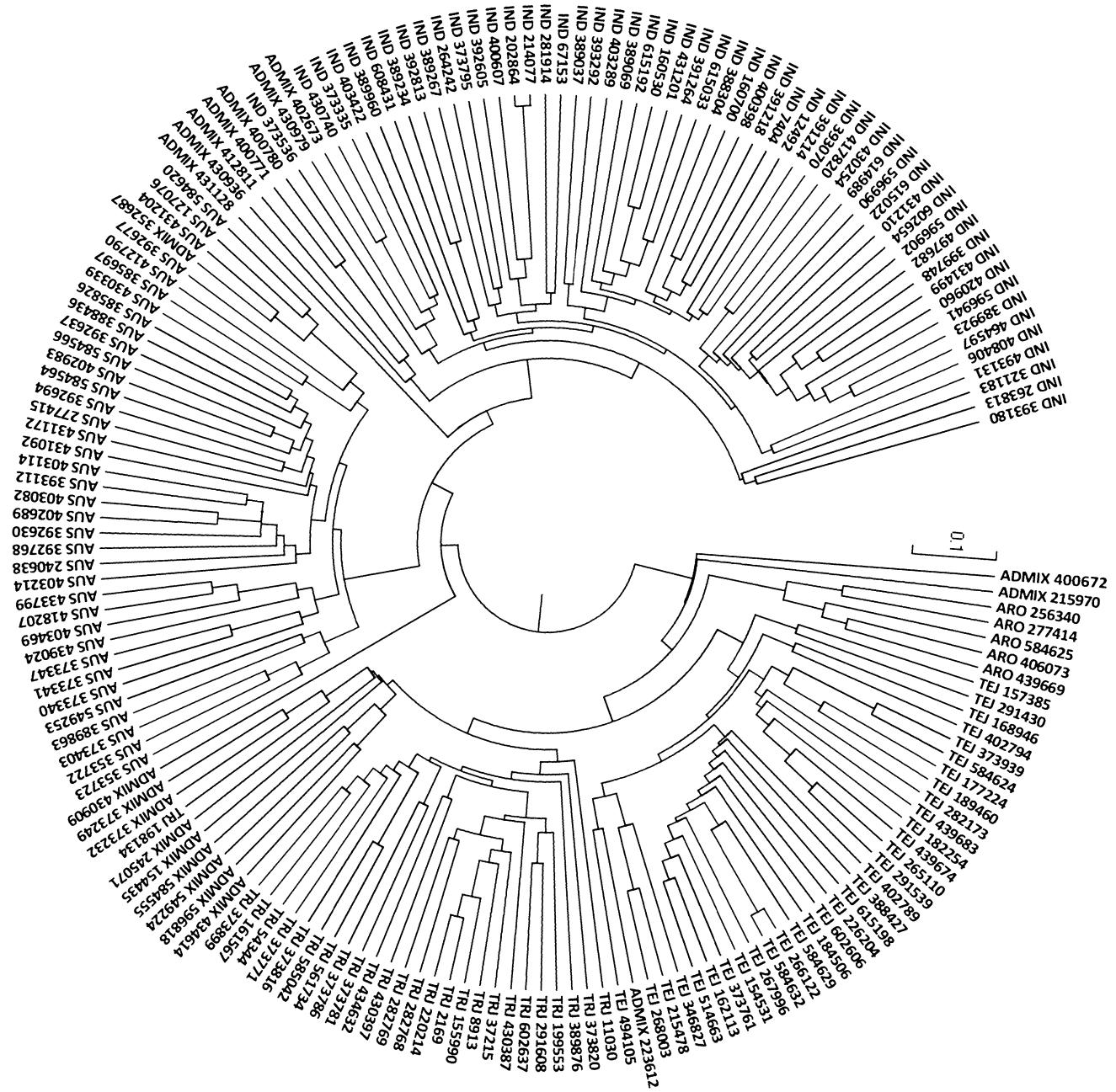

Fig. 5 UPGMA tree of 174 lines genotyped with 164 molecular markers and clustered using Roger's genetic distance. Five rice sub-populations are represented in individual clusters with the small group of *aromatics* (five lines) forming their own distinct cluster. *Lines* are labeled with their corresponding sub-population and ACNO number. Sub-populations are *aromatic* (ARO), *aus* (AUS), *indica* (IND), *temperate japonica* (TEJ), *tropical japonica* (TRJ), and admixtures (ADMIX)

genotypic variation in the Mini-core and confirmed the five main sub-populations identified by the Structure analysis. The UPGMA tree (Fig. 5) based on Roger's genetic distance provided another way to visualize the genetic similarities of the accessions and showed similar results with both Structure and PCA. This information is useful to breeders for designing crosses to change silica concentration while maximizing/minimizing genetic variability in other genomic regions.

Association mapping

Association mapping results of the mean silica concentration for the AR and TX samples were compared using both the GLM and MLM method of Tassel 2.1. The MLM method controls Type I and Type II error rates by accounting for both population structures (Q) and kinship (K) (Yu et al. 2006), while GLM only takes population structure into account. In this study the MLM method

Table 2 MLM association mapping results for Stuttgart, AR, Beaumont, TX, and the combined location study

Location	Locus	Chr.	Position (Mb)	*p* value	$-\log_{10}P$	Major allele (bp)	Effect	# of observed genotypes
Stuttgart	RM23869	9	6.3	2.30E−04	3.64	182	−14.6	37
	RM6544[ab]	11	3.9	3.10E−03	2.51	165	20.2	11
						170	−17.9	47
	RM5953	4	9.4	3.30E−03	2.48	111	15.3	35
	RM263[b]	2	25.9	5.20E−03	2.28	176	−22.8	6
						158	22.0	30
Beaumont	RM1335[a]	7	28.3	3.70E−04	3.44	173	22.8	7
						163	−22.3	10
						165	21.1	6
						171	−19.1	13
	RM5371[bc]	6	25.8	6.30E−04	3.2	170	−18.6	7
	RM1186	7	9	1.40E−03	2.85	120	−17.5	18
	RM178[a]	5	25.1	4.40E−03	2.36	114	−23.6	72
Combined	RM5953	4	9.4	8.60E−04	3.07	111	14.6	35
	RM23869	9	6.3	2.30E−03	2.64	182	−9.9	37
	RM283[a]	1	4.9	5.10E−03	2.29	150	−19.7	5
	RM6544[ab]	11	3.9	6.80E−03	2.17	165	14.9	11
	RM489	3	4.3	7.90E−03	2.1	269	15.2	25
	RM171	10	19.1	8.40E−03	2.08	328	−18.8	42
	RM484	10	21.1	8.80E−03	2.06	290	−22.4	4

Marker-trait associations with *p* values less than 0.01 are shown along with chromosomal location (Chr.) and position (in Mb). Marker effects are shown for the major allele (alleles having the greatest effect on silica content) for each loci. Effects are shown as either a negative value, indicating the presence of the allele was associated with decreased silica content, or a positive value if the presence of the allele was associated with increased silica content. The number of observed genotypes is the numbers of times the allele occurred in a homozygous state in the 174 lines tested

[a] Markers that occur within 1.5 Mb or less of previously identified silica QTL's

[b] Markers that occur within 1.5 Mb or less of previously identified additive-by-additive QTL regions

[c] Markers that occur within 1.5 Mb or less of previously identified arsenic QTL's

provided better control of the error rates, thus only results from the MLM are reported. Results of the MLM showed significant silica concentration associations ($p \leq 0.01$) for four markers from AR of which three were detected in the combined analysis, four markers from TX of which none were detected in either the AR or combined analysis, and seven markers from the combined study including three markers seen in AR and four markers unique to the combined analysis (Table 2; Fig. 4). Marker effects were generated for the major alleles (alleles having the greatest effect on increasing or decreasing the silica concentration) of significant markers (Table 2). Rare alleles making up 2% or less of the number of observations were discarded from analysis. In this study, all of the markers in which ten or fewer of the major alleles had been observed in a homozygous condition were either validated by previous QTL mapping studies, or in the case of RM484, by nearby markers.

Markers identified in the association mapping study differed between the AR and TX locations ($p \leq 0.01$). When coupled with the variance associated with the genetic × environment interaction (25.3%), this highlights the importance of environmental factors on silica uptake. The amount of silica present in the soil, the presence of other elements and/or nutrients, the amount of light, and temperature are all factors known to affect silica concentrations in the plant (Ma and Takahashi 2002; Ma et al. 2002). It is conceivable that different genes and/or gene pathways influenced by different environmental variables are involved in regulating the silica concentration of the plant. In order to account for possible environment effects, and identify where silica concentration is significantly associated with markers in both environments, data of silica concentrations from the AR and TX locations were combined and a third association mapping analysis was conducted.

In addition to the known silica transporter genes *Lsi1* (chromosome 2 at 29.83 Mb), *Lsi2* (chromosome 3 at 1.11 Mb), and *Lsi6* (chromosome 6 at 6.65 Mb), other studies have reported numerous QTLs involved with silica

concentration. The mapping study performed by Wu et al. (2006) found a QTL for silica concentration in rice sheaths and leaves spanning a large region on chromosome 7 from 16.9 to 27.1 Mb, which is near RM1335 (chromosome 7, 28.3 Mb), the marker with the highest significance in the TX study. Similar conventional mapping studies by Dai et al. (2005, 2008) identified QTLs for rice hull silica concentration within 1.5 Mb of the marker-trait associations identified in our association mapping studies. They reported a QTL on chromosome 1 between 5.6 and 5.8 Mb which is in close proximity to the marker RM283 (chromosome 1 at 4.9 Mb) from our combined study. Another silica concentration QTL on chromosome 5 from 25 to 25.6 Mb reported by Dai et al. (2005) positionally overlaps with RM178 (chromosome 5 at 25.1 Mb) from our TX study. The major allele of RM178 (114 bp) is present in over 40% of the lines tested and had a strong negative effect on silica concentration, lending further support for its involvement with silica concentration in the hull. RM 263 (chromosome 2 at 25.9 Mb) from the AR study, RM6544 (chromosome 11 at 3.9 Mb) from the AR and combined analyses, and RM5371 (chromosome 6 at 25.8 Mb) from the TX study all occur within a 1.5 Mb region that Dai et al. (2005) had previously identified as having additive by additive QTL effects.

Additionally, RM5371 which has a major allele with a negative effect (-18.6 mg g^{-1}) on hull silica concentration, falls within a QTL region identified for arsenic concentration in rice grains (Zhang et al. 2007). Silica uptake is known to be involved with both arsenic and phosphorus uptake in rice (Guo et al. 2007). High arsenic concentration in rice grain is associated with health concerns in certain areas of the world. Understanding the relationship between arsenic and silica deposition in the grain could become an important step in overcoming high arsenic concentration in the grain. The marker trait-association for silica concentration in the hulls found in this study coupled with the arsenic concentration QTL found by Zhang et al. 2007, suggests that the region near RM5371 could be promising for further study.

Marker RM5953, which was not significant at the TX location, was the most significant marker (p value = 0.00086) in the combined analysis and was significant in the AR study. The major allele of RM5953 (111 bp) had a 14.5 and 15.3 mg g^{-1} marker effect in the combined and the AR study, respectively, showing a strong positive effect for increased silica concentration associated with this allele. RM23869 was the second most significant marker-trait association in the combined analysis and the most significant marker-trait association in the AR study ($p = 0.00023$). The major allele (182 bp) was well supported by 37 homozygous lines and had a negative effect on silica concentration in both locations. RM6544 was another marker that was detected in both the combined analysis and AR study, having a major allele (165 bp) with positive effect.

Four markers, RM283, RM489, RM171, and RM484 were unique to the combined analysis. RM283, a marker less than 1 Mb away from a previously identified silica QTL region on chromosome 1 (Dai et al. 2005), had a major allele with a large negative effect on silica concentration (-19.7 mg g^{-1}), but was found homozygous in only 5 accessions. RM489 on chromosome 3 had a major allele with 25 homozygous observations and a positive effect on silica concentration of 15.2 mg g^{-1}. Two other markers of interest in the combined study were RM171 (chromosome 10 at 19.1 Mb) and RM484 (chromosome 10 at 21.1 Mb). Both RM171 and RM484 flank the rice *OsNIP3;1* gene (chromosome 10 at 19.7 Mb). *OsNIP3;1*, like the previously identified rice silica transport genes *Lsi1* and *Lsi6,* is a member of the aquaporin protein superfamily. *OsNIP3;1* is expressed in the roots, inflorescence and seeds of rice (Liu et al. 2009). Mitani et al. (2008) expressed *OsNIP3;1* in *Xenopus* oocytes and found it to function as a transporter although it was incapable of transporting silicic acid in their system. Evidence that *OsNip3;1* plays a role in silica accumulation in grain hulls has yet to be reported. The major alleles of RM171 and RM484 both had strong negative effects on silica concentration, -18.7 and -22.4 mg g^{-1}, respectively (Table 2). While the major allele of RM484 had only four homozygous observations, RM171 had 42 homozygous observations as well as a strong negative effect. These two markers occur in close proximity to each other, within 2 Mb, lending support to a potential QTL being present in this region. The marker-trait associations provided by this association mapping study should provide a starting point to look for additional silica concentration controlling genes or QTLs as well as to develop rice cultivars with high or low hull silica concentration.

Conclusions

The silica concentrations present in rice hulls varied across all five sub-populations of rice. Eighty-five percent of TRJ accessions had higher silica concentrations than the Mini-Core mean, indicating that TRJ is a rich genepool for increasing rice silica concentration. Seventy-five percent of the USA rice production is for the long grain market, with essentially all of this germplasm derived from TRJ. Agronomically acceptable rice cultivars with high silica concentration would increase the value of the hulls as they could be utilized for the production of silica based industrial compounds. Silica concentration also varied among accessions from different geographic regions. Most of the

accessions from Central America had higher silica concentrations while those from the Mideast and Eastern Europe had lower silica concentrations as compared to the Mini-Core mean. Accessions from these regions may offer germplasm that could be used to develop rice varieties that extract less silica from the soil. Decreased silica concentrations would aid in the biodegradation of waste hulls and straw however, negative effects on disease resistance or lodging susceptibility would have to be tested. Environment effects were an important factor in the association mapping analyses, highlighting the importance of GxE interactions and the necessity of having multiple test sites for silica evaluation. In our study four significant marker-trait associations were found at both the AR and the TX locations and seven marker-trait associations were found in the combined analysis of AR and TX. Five of the marker-trait associations found in this study are within 1.5 Mb of reported QTLs for silica concentrations from linkage mapping studies, and one marker-trait association (RM5371 on chromosome 6 at 25.83 Mb) overlaps with a QTL involved in grain arsenic concentration as well as silica concentration. Association mapping of the diverse germplasm in the USDA rice Mini-Core collection was an effective method for identifying new genetic markers and validating previously reported marker regions associated with silica concentration.

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