Genetic Architecture of Grain Chalk in Rice and Interactions with a Low Phytic Acid Locus

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Abbreviations: cM, centimorgans; MAS, marker-assisted selection; Mb, megabase pairs in reference to physical location along a chromosome; QTL, quantitative trait locus; SNP, single nucleotide polymorphism; SSR, simple sequence repeat.

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ABSTRACT

Grain quality characteristics have a major impact on the value of the harvested rice crop. In addition to grain dimensions which determine rice grain market classes, translucent milled kernels are also important for assuring the highest grain quality and crop value. Over the last several years, there has been increasing concern by the rice industry regarding prevalence of chalky grains which are resulting in a loss of some markets. This study was conducted to identify genetic markers associated with grain chalk that could be used by breeders to develop new varieties that have translucent grains across different environments. A mapping population developed from KBNT-1-1, a translucent, low phytic acid (LPA) mutant derived from the US long grain variety Kaybonnet, crossed with Zhe733, a chalky, long grain variety developed in China, was evaluated in replicated trials conducted across two years and two planting dates that differed by one month. The progeny were evaluated for days to flowering in the field. Grain was harvested at maturity and brown or milled rice were used to determine grain length, width, and percent chalk using an image analysis system. The 187 F₁₂ progeny were evaluated using 174 genome-wide microsatellite markers and one SNP marker, and QTL analysis was performed. A large effect QTL was co-located with the LPA gene, and the mutated KBNT-1-1 LPA allele was associated with increased chalk suggesting that low phytic acid may be one cause of chalk in this cross. Nine additional QTL were detected with the KBNT-1-1 allele associated with reduced chalk and one with increased chalk. Overlaps were identified between chalk QTL, candidate genes for chalk, and QTL for other traits such as grain width. Measures of night temperature and humidity experienced during grain filling were positively correlated with increased chalk. Year and planting time were also shown to significantly affect chalk. However there was no

23 interaction with the pattern of QTL detected and any of the environmental differences,
24 indicating that these QTL are robust across diverse growing conditions.
25

26 INTRODUCTION

27 The degree of chalkiness in rice affects grain quality and milling yield. Chalk negatively
28 impacts cooking quality characteristics, such as texture and palatability which subsequently
29 reduces the market value of rice (Chun et al., 2009; Lisle et al., 2000). The presence of chalk also
30 negatively impacts milling yield by increasing the number of broken kernels, which in turn
31 reduces the proportion of whole kernels (Khush et al., 1979). The frequent breakage during
32 milling of chalky rice is caused by less densely packed starch granules in the chalky areas and
33 less hardness than the translucent areas of the grain (Del Rosario et al., 1968). Broken grains
34 sell for a lower price than unbroken (head rice); over the last ten years the world market price
35 of broken rice has fluctuated between 35 to almost 50% less than that of head rice (Childs,
36 2016). Reduced milling yield can be especially problematic with long grain rice varieties as
37 their greater length to width ratio makes them more susceptible to grain structure
38 inconsistencies caused by chalk (Wan et al., 2005). In the USA, over 70% of the rice grown in
39 2015 consisted of long grain varieties (Childs, 2016) and thus, it becomes critical to understand
40 and manage grain chalk that can significantly reduce milling yield.

41 Chalk is influenced by the environment and genetics. Higher temperatures, and
42 particularly higher night air temperatures (NTAT) during grain filling, significantly increase chalk
43 (Ambardekar et al., 2011; Counce et al., 2005; Lanning et al., 2011). There is a positive
44 quadratic relationship between the 95th percentiles of the NTAT frequencies (NT_{95}) occurring
45 during the grain filling stage and the degree of chalk. The amount of chalk is also strongly
46 affected by variety and there is a differential response to increased NT_{95} between varieties

(Ambardekar et al., 2011). High quality rice varieties have been developed to have low chalk, an important factor in grain grading. However, when genetically diverse lines are used as parents in rice hybrids to increase yield through heterosis, or when exotic varieties are used as donors to introgress alleles for improved stress resistance or agronomic traits, there is a potential to increase chalk and reduce grain quality. Thus, it is critical to understand the genetic factors that affect chalk to be able to make full use of rice genetic diversity for variety development while sustaining grain quality in competitive commercial markets.

Chalk in rice occurs as an opaque white discoloration in the endosperm or as localized patches in the center, back or throughout the grain (Ashida et al., 2009). Chalky endosperm contains amyloplasts with a more rounded appearance and a disordered cellular structure than in translucent endosperm. Inside chalky grains the amyloplasts contain small loosely packed starch granules with greater airspace between them (Chun et al., 2009; Lisle et al., 2000). The airspaces hinder the transmission of light resulting in the characteristic opaque chalky appearance, as well as creating mechanical weaknesses within the cellular structure of the rice grain, which in turn may increase the incidence of breakage during milling (Kepiro et al., 2008).

Multiple environmental and genetic factors influence the biochemical pathways involved in the deposition of storage components during the source/sink process of grainfill, which in turn affects chalk formation in the developing grain. Li et al. (2014) recently cloned *Chalk5*, which encodes a vacuolar H⁺-translocating pyrophosphatase (V-PPase). Over expression of *Chalk5* is believed to contribute to chalk formation by disturbing pH homeostasis of the endomembrane trafficking system and interfering with the biogenesis of protein bodies during endosperm development. This in turn leads to the formation of airspaces around starch

granules and protein bodies (Li et al., 2014). Other studies have implicated amylose, starch biosynthesis and starch structure as components of chalk formation. Patindol and Wang (2003) performed an analysis of the starch content of chalky kernels and found a greater abundance of amylopectin with long branch chains, and a decrease in overall amylose content. A microarray study performed by Yamakawa et al. (2007) exposed developing rice grains to high temperatures during the milky stage. They found high temperatures caused down regulation of starch branching enzymes (particularly *branching enzyme IIb*), granule-bound starch synthase I and cytosolic pyruvate orthophosphate dikinase while enzymes involved in starch degradation such as α -amylase and heat shock proteins were up regulated.

A review paper by Sreenivasulu et al. (2015) reports over 140 quantitative trait loci (QTL) related to chalkiness have been identified spanning all 12 chromosomes in rice. A wide range of genes involved with starch formation and storage protein regulation have been implicated in grain chalk. Biparental mapping populations have identified QTL responsible for chalk at or near genes such as *Waxy*, *starch synthase III A*, *pyruvate orthophosphate dikinase*, *UDP glucose pyrophosphatase*, *cell wall invertase*, and *H⁺-translocating pyrophosphatases* (Fujita et al., 2007; Kang et al., 2005; Li et al., 2014; Peng et al., 2014; Wang et al., 2008; Woo et al., 2008). Sreenivasulu et al. (2015) point out that QTL studies combined with transcriptome analysis suggest that many high temperature response pathways involved with starch and storage protein metabolism during grain development are co-located with chalk QTL. Storage proteins levels have also been shown to be influenced by higher temperatures during different stages of seed development, which in turn may contribute to the formation of chalk (Li et al., 2011).

Our study involved examining a biparental mapping population consisting of F12-F13 recombinant inbred lines (RIL's) of the Kaybonnet *low phytic acid* mutant, KBNT-1-1, (henceforth referred to as KBNT *lpa*) X Zhe733. The KBNT *lpa* parent was derived from 3632 gamma irradiated M2 lines and the resulting *lpa1-1* mutant is a single gene recessive non-lethal mutation (Larson et al., 2000; Rutger et al., 2004). KBNT *lpa* is from the *tropical japonica* sub-population of *Oryza sativa*, which contains resistance to rice blast (*Magnaportha grisea*) isolates IB-1, IB-49, IC-17, and IG-1 and has translucent grain. Zhe733 is a chalky, high yielding *indica* variety that is resistant to straight head disorder, rice water weevil and some rice blast races (IE1K and IB-33) (Lee et al., 2009; Liu et al., 2008; Yan et al., 2005). The KBNT *lpa* x Zhe733 is a registered mapping population (Rutger and Tai, 2005) which has previously been used for mapping water weevil resistance, disease resistance, and straighthead (Yan et al., 2005). It was chosen for this study as there is a large difference in chalk concentration between the parents (KBNT *lpa* is mean of 3.15% chalk and Zhe733 with an is mean of 25.37% chalk) and because both parents are long grain varieties and thus, grain shape is not a confounding factor (Wan et al. 2005).

Incorporating low phytic acid mutations into rice and other crops can be beneficial for both health and environmental reasons. Phytic acid binds much of the available phosphorus in seeds into an insoluble form that cannot be digested by humans and other monogastric animals. In food products phytic acid has a two-fold negative effect on the environment. The insoluble phosphorus is passed by humans and agricultural animals leading to high phosphorus contents in sewage/waste and contributes to eutrophication of lakes, rivers and drinking water. The resulting insoluble phosphorus in manure is unavailable to agricultural crops until it is

broken down by soil microorganisms requiring the additional use of non-renewable inorganic phosphorus fertilizers, which in turn compounds excess phosphorus run off into waterways (Brinch-Pedersen et al., 2002). While a few health benefits have been attributed to phytic acid, the negative aspects of phytic acid in the diet out-weigh the benefits (Raboy, 2009; Schlemmer et al., 2009). Phytic acid is a strong chelator and can bind iron, zinc, calcium, magnesium and manganese into insoluble salts in human and animal digestive systems also. This in turn increases the risk of vitamin and micronutrient deficiencies, especially in developing countries where dietary choices are limited (Raboy, 2007).

In seeds phytic acid, also known as myo-inositol-1,2,3,4,5,6-hexakisphosphate, serves as the main storage form of phosphorus and may contain approximately 75% of the total phosphorus in the seed (Raboy, 2009). Phytic acid forms salt complexes with many nutrients that can later be released in the presence of phytases to provide nutrition to the developing embryo during seed germination. The nearest known homolog to the rice *lpa1-1* is a 2-phosphoglycerate kinase found in hyperthermophilic methanogens (Kim et al., 2008; Lehmacher and Hensel, 1994). The exact mechanism by which the *lpa1-1* gene functions is currently unknown. However, Raboy (2009) suggests that the protein encoded by *Lpa1* may function similarly to 2-phosphoglycerate kinase by creating an intermediate compound for phytic acid synthesis as well as producing a second compound that protects phytic acid intermediary compounds from degradation.

In the current study, our aims were to identify the QTL that control chalk within long grain rice, and to determine the effect of the *low phytic acid 1-1* allele on chalk (if any). This population was genotyped and phenotyped for chalk and potential interacting agronomic traits,

135 and QTL analysis was performed. The long term objectives are to understand the genetic and
136 biochemical components that cause chalk, and to obtain molecular markers to be used for
137 selection of low chalk grains in rice breeding.

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MATERIALS AND METHODS

Seed Source and growing conditions

In 2013, RIL's of KBNT *lpa* x Zhe733 F12 seed were obtained from the USDA Genetic Stocks Oryza (GSOR) collection (<https://www.ars.usda.gov/Main/docs.htm?docid=14200>) that had been produced in 2004 in Stuttgart, AR. The software MapPop V 1.0 (Vision et al., 2000) and existing molecular data (Rutger and Tai, 2005) were used to select 202 diverse RIL's for planting. To account for environmental variables, particularly differences in temperatures during grainfill that affect chalk, seed was planted on two planting dates a month apart, April 15th 2013 and May 15th 2013, in Stuttgart, Arkansas. Three replications were used for each planting date and plants were grown in a randomized complete-block design. F13 seed from RIL's grown in 2013 was used for planting the following year. In 2014, the same experimental design was used at the Stuttgart, Arkansas location with two planting dates (April 21st 2014 and May 21st 2014).

Seed was planted in single rows approximately 0.27 m² with a Hege 1000 plot planter (Wintersteiger Inc., Salt lake City, UT). The parents KBNT *lpa* and Zhe733 were grown as checks within each replication in the randomized complete-block design. The field was bordered by two rows of rice to prevent edge effects. In both years, prior to planting 56 kg phosphorus ha⁻¹, 101 kg potassium ha⁻¹, and 11 kg zinc ha⁻¹ were applied according to soil test recommendations. To reduce the possibility of lodging, only 56 kg nitrogen ha⁻¹ was applied just prior to permanent flood. A pre-emergent herbicide was applied prior to planting and subsequent treatments of herbicide or pesticide applications were used as necessary throughout the

growing season. The lines were harvested individually as their seed reached maturity when the full panicle appeared ripened and straw color, at 18-20% moisture.

Field and lab measurements

Heading Days (days to 50% heading from emergence), Days to Harvest, and Grainfill (Harvest-Heading days) were determined for all plots. Hand-harvested plots were threshed (Bill's Welding, Pullman, WA), the rice was dried to approximately 12% moisture with a forced air drier at 28 C and then stored at 4 C and 50% relative humidity prior to further analysis. The grain was aspirated (Air Blast Seed Cleaner, Almaco, Nevada, IA) and 100 g of rough rice was sampled prior to milling. Following the 2013 field season, the rough rice was milled and broken grains separated (PAZ-1 DTA, Zaccaria USA, Anna, TX) to determine percent total milled rice and percent whole milled rice. Using samples of the original 2004 produced seed and that from the 2013 and 2014 harvests, approximately 10g of rough rice were de-hulled to produce brown rice (Satake Rice Machine, Satake Engineering Co., Ltd, Tokyo, Japan). Percent chalk and grain dimensions were calculated using WinSEEDLE Pro V.2007E (Regents Instruments Inc., Canada) and an Epson Perfection V700 photo scanner (Epson America Inc., Long Beach, CA) using approximately 140 whole milled grain or brown rice kernels.

Temperature and weather data were collected (hourly) on site by the USDA-ARS weather station (www.ars.usda.gov/Main/Docs.htm?docid=23563). We calculated the daily averages for air temperature, night time air temperature (NTAT), relative humidity, and rainfall over a 20 day grainfill period following heading for each plot. We considered those temperatures that occur between the hours of 8PM and 6AM to be NTATs. Degree-day-10

(DD10) thermal units over the course of each day were calculated according to methods used by Ambardekar et al. (2011) and averaged across the 20 days following heading for each plot. Frequencies of the NTATs during a 20 day interval following heading were used to calculate the 95th percentiles of NTAT frequency (NT₉₅) for each plot (Ambardekar et al., 2011). All weather calculations were made using the R statistical language (R Core Team, 2016).

Statistical analyses were performed using JMP® 12 Software (SAS Institute Inc., 2015) to assess the effects of weather on chalk and to calculate Least-Squares Means (LSmeans) of percent chalk of each RIL to use in subsequent QTL analysis. Percent chalk was used as response vector in a linear model. The RIL (genotype) was used as a fixed effect. Weather data from within a 20 day interval after heading were also used as fixed effects including: average air temperature, average NTAT, NT₉₅, DD10, average relative humidity, and total rainfall. The planting year and planting date were used as random effects. The mixed model partitions the model into fixed and random effects. The year and planting date are restricted maximum likelihood (REML) estimators that are free of fixed effects. Subsequently, to calculate LSmeans of each RIL, percent chalk was again used as response vector in a linear model that included only the significant fixed effects of the previous model (NT₉₅, average NTAT, and average relative humidity). LSmeans of percent chalk were also calculated for each RIL without the weather related fixed effects for the purpose of comparison in the QTL analysis. Correlations between traits were determined using the pairwise method (SAS Institute Inc, 2015).

Data from one hundred and sixteen SSR markers had been gathered on the KBNT *lpa* x Zhe733 F₁₀ population during prior studies as described in Liu et al. (2008) and Lee et al. (2009). RIL lines were advanced to the F₁₂ generation and 10 high polymorphic information content

(PIC) value markers were used to verify that F12 lines were consistent with the molecular data in the F₁₀ population and no out crossing had occurred. An additional 56 SSR markers and two SNP markers were used on the F₁₂ RIL population. The markers used for this study and their corresponding linkage map positions are shown in Supplemental Table 1. PCR conditions were performed as described in Costanzo et al. (2011). An ABI Prism 3730 DNA analyzer was used to identify the amplified fragments and data was analyzed using GeneMapper V 3.7 software (Life Technologies Corp., 2004). One hundred and eighty five RIL's that had phenotypic data for both years and genotype data consistent with F10 lines were selected for mapping.

Two SNP markers were designed for this study at or near the KBNT *lpa* mutation using a three primer SNP detection method similar to that described in Hayashi et al. (2004). In this method a mismatch is made in the third base pair from the 3' end of the forward primer and the final base pair of the primer corresponds to the SNP. Two forward primers specific to their corresponding SNP are used with a shared fluorophore labeled reverse primer. An additional 2bp, matching the target sequence, is added on the 5' end of one of the forward primers to allow for a detectable size difference in the amplified product. The SNP ID2015477 is found on chromosome two at 34.48 Mb and was amplified with the forward primers CTTGCTTGCGAAGTTGTGG (KBNT *lpa* specific), TTGCTTGCTTGCGAAGTTGTGA (Zhe733 specific), and a shared reverse primer ATAATACGAGCGGCGGAGC. The functional nucleotide polymorphism responsible for the KBNT *lpa* mutation is a C to T substitution and occurs on chromosome 2 at 35.17 Mb. The KBNT *lpa* specific forward primer is AGCAAGAAGAAAGTCAGCCTTATA, wild type forward is CAAGAAGAAAGTCAGCCTTATG, and the shared reverse is CAAGACCCAAACAACCATCG.

A genetic linkage map was calculated using the Kosambi mapping function of MapDisto V 1.7.5 Beta 4 (Lorieux, 2012). QTL analysis was performed using R/qtl (Broman et al., 2003). Phenotypes and genotypes loaded with cross type set to “riself”. Interval mapping and composite interval mapping (CIM) were done using the commands “scanone” and “cim” respectively using the “EM” algorithm. The logarithm (base 10) of odds (LOD) threshold was determined using 1000 permutations. A QTL model was constructed using all loci exceeding the LOD threshold. This model was used with the “addqtl” command to identify additional QTL given the model. The “scantwo” command was used to perform a two-dimensional genome scan with a two-QTL model. A QTL model was then built including all significant loci detected by CIM, “addqtl”, and the 2D QTL scan. The “addint” command was used to test for QTL interactions. All significant interactions were then added to the QTL model. Drop-one-term regression analysis was performed using “fitqtl” to select significant terms and interactions. The “fitqtl” command was then used to calculate percent of variation explained and estimated effects of each QTL and each QTL interaction term, and percent of the variance explained by the full model.

RESULTS

The population of 187 individuals was genotyped with 174 SSR markers and 2 SNP markers with 5.2 percent missing data (Supp. Table 1, Supp. Figure 1). The marker allele calls were 44.9% homozygous for KBNT *lpa*, 54.2 % homozygous for Zhe733, and 0.9% heterozygous. The heterozygous loci were treated as missing data in the QTL analysis. The physical distance to genetic map distance is approximately 0.2 to 0.3 cM per megabase across entire chromosomes,

but this is highly variable depending on the genomic region. Phenotypically, the RIL population had a mean percent chalk (brown) of 18.69% (12.16 SD), mean heading date (days to flowering) of 77.09 days (8.48 SD), mean kernel length of 6.77 mm (0.55 SD), and mean kernel width of 2.40 mm (0.24 SD).

Chalk measurements for 2013 were taken on both brown and milled white rice harvested that year. There was concern that chalky grains would break up during milling resulting in an under-estimate of percent grain chalk on the milled rice. However percent chalk using milled rice was highly correlated with that of brown rice ($r=0.96$, $p<0.0001$). In addition, grain dimensions traits were highly correlated between the two; grain length, $r=0.92$, grain width $r=0.90$, length:width $r=0.93$, $p<0.0001$). Moreover, a simple linear regression model demonstrated that percent chalk in brown rice was an adequate predictor of percent chalk in milled rice ($r^2=0.92$) (Supp. Figure 2). Thus, brown rice was deemed suitable for chalk and grain dimension measurements and only brown rice measurements were determined for the 2014 growing season. Whole grain milling percent (percent head rice) was determined in the 2013 study and was not strongly correlated with any of the milled grain dimension traits ($r<0.2$), but was correlated with brown rice width ($r=-0.48$, $p<0.0001$). Whole grain milling percent was more strongly correlated with percent chalk in brown rice ($r=-0.56$, $p<0.0001$) than with percent chalk in milled rice ($r=-0.52$, $p<0.0001$).

Chalk phenotypic data were collected over two years with two planting dates per year. The RILs were considered fixed effects in the analysis of variance and accounted for 99% of the variance in the model for percent grain chalk (data not shown). Of the remaining 1% variation in the model, year explained 19% whereas planting date explained 5% of the variation (Supp.

Table 2). Thus, the wide distribution of the population for chalk was far greater than the environmental effect (Figure 1).

Temperature is known to affect chalk development during grain filling (Ambardekar et al. 2011, Lisle et al. 2000, Lanning et al. 2010, Counce et al., 2005). Thus, the effects of weather were examined on a per-plot basis within a window of 20 days following heading, during grain development. A significant effect was observed for NT95, average night temperature, and average relative humidity (Table 1). However, average temperature, DD10, and total rainfall were not significant. When only significant fixed effects were included in the model, increased NT95 and average night temperature significantly increased the percent chalk ($p < 0.0001$) with a lesser positive effect from average relative humidity ($p < 0.037$) (Table 2). Unadjusted LSmeans and LSmeans with adjustments for NT95, night temperature, and humidity were calculated for each RIL and parent line for use in QTL mapping (Supp. Table 3). The RILs ranged from 2.43 to 52.61% chalk, whereas the parental means were 3.15% for KBNT *lpa* and 25.37% for Zhe 733. The distribution of percent chalk LSmeans (adjusted for environmental predictors) in the RILs indicates substantial transgressive variation for higher chalk with 51 out of 187 progeny (26.8%) exceeding the percent chalk of Zhe733, the higher chalk parent, while only two lines had lower chalk than KBNT *lpa*, the lower chalk parent (Figure 1). The two lines that were lower in LSmean chalk were not significantly lower than KBNT *lpa*.

Composite interval mapping, using combined data across both years and all planting dates and adjusted for weather data, shows five distinct loci with LOD scores exceeding the 0.05 threshold as determined by 1000 permutations (Figure 2, Table 3). Supp. Figure 3 shows the QTL analysis done separately by year and by planting date. Supp. Figure 4 shows the QTL

analysis done separately for weather adjusted and non-adjusted chalk. Similar patterns of QTL peaks between adjusted and non-adjusted for weather and across planting dates and years suggests that, although environmental factors influence the levels of chalk, the genetic loci that affect chalk are consistent across environments (i.e., no gene x environment interaction).

Scanning for additional QTL in the context of the multiple QTL model revealed five additional QTL with LOD > 1.5 (Table 4). A two dimensional QTL scan (scantwo) revealed the same five additional QTL with evidence of conditional interactions (Supp. Table 4, Supp. Figure 5). Testing all possible interactions by adding one pairwise interaction at a time with the addint procedure detected three significant interactions (Supp. Table 5), two of which were shared interactions identified by scantwo. Table 5 shows the significance, percent of variation explained, and estimated effects of all of the significant chalk QTL. All nine QTL and the two QTL interactions in the model are significant ($p < 0.031$) and the full model explains 63.2% of the variation for chalk (Supp. Table 6). At the preponderance of the loci, the Zhe 733 allele increased chalkiness (indicated by positive estimated effect values), however at the qBCHK2 locus (2@148.0), the allele from Zhe 733 results in a 5.29% decrease in chalk, possibly because it associated with the wild-type (non-mutant form) of the phytic acid allele (Table 5).

Furthermore, although the parents differed in heading by 20 d, there was no relationship of days to heading and grain chalk nor was there any overlap in these QTL (Figure 6).

Positions of chalk loci in megabases (Mbp) are presented relative to positions of previously reported QTLs and candidate genes in Figure 3 (Liu et al., 2011; Wan et al., 2005), and relative to the QTL positions of other grain quality and agronomic traits measured in this population in Supp. Figure 5.

DISCUSSION

The RIL population derived from KBNT *lpa* x Zhe733 showed clear differences between lines in percent chalk (Table 1, Sup. Table 3) indicating that chalk is largely controlled by genetics. There was transgressive variation in percent chalk, but only in the direction of increased chalk (greater than the higher chalk parent, Zhe 733). There were no lines with significantly lower chalk than the low chalk KBNT *lpa* parent. The transgressive variation suggests that epistasis or additive effects at multiple recombined loci can act together to substantially increase chalkiness. This may be a consequence of the inter-subspecific cross between a tropical *japonica* (KBNT *lpa*) and an *indica* (Zhe733) line. Because these results suggest that the level of chalk in populations derived from wide crosses has the potential to be very high in some lines, such populations would need to be screened for chalk when using such material in breeding programs.

QTL for chalk were detected in the KBNT *lpa* x Zhe733 RIL population that together explain the majority (63.2%) of the variation for the trait. Five significant QTL were found by CIM (Table 3). Additionally, five smaller QTL that improve the predictive power of the model were found by two-dimensional QTL and multiple-QTL model scans (Table 5). Out of the ten QTL, the only loci where the Zhe733 allele reduced chalk were near the *lpa1-1* gene and on chromosome 12 near marker RM270. The *lpa1-1* mutant allele could itself be a cause of increased chalk and the non-mutant form of KBNT would not contain the allele that increases chalk. If so, Zhe733 has little to offer in its potential to contribute alleles that would further reduce chalk in an already low chalk *tropical japonica* line. This also helps to explain the transgressive variation only occurring in the direction of higher chalk. Even though this

mapping population was derived from two long grain cultivars, there was variation in grain width of KBNT *lpa* was 2.09 mm and Zhe 733 was 2.62 mm with the RILs having 2.40 mm (0.24 SD). Of the ten chalk QTL identified, four were associated with grain width or grain length:width (Supp. Figure 6). Fitzgerald et al. (2009) have indicated that grain width is associated with grain chalk. These results suggest that even small differences in grain width are related to chalk development. Three of the other chalk QTL were co-located with total or whole grain milling yields demonstrating the interrelationship of these traits.

QTL with marginally significant effects were supported by additional lines of evidence. The QTL qBCHK5 co-locates (Figure 3) with a cloned gene that has a known chalky phenotype (Li et al., 2014). The QTL qBCHK1-2 and qBCHK12 co-locate with *Sar1a* and *Sar1b* which have an associated phenotype of floury shrunken seed and an increased level of glutelin precursor in the endosperm (Tian et al., 2013). Five of the nine chalk QTL overlapped with previously reported QTLs related to chalk (Figure 3).

The chalk QTL on chromosome 2 co-locates with the *lpa1-1* gene. The mutant *lpa1-1* may be causal or a closely linked gene has an allele from KBNT that has a large (increasing) effect on percent chalk. To differentiate between the two possibilities, a new population could be generated using the non-mutant KBNT line to be crossed with Zhe733. The exact mechanisms by which low phytic acid may increase chalk are not known. Landoni et al. (2013) reported the *lpa* gene impacted lignin and protein content as well as the size and structure of starch granules in maize. This suggests that *lpa* in rice could impact how densely the starch granules in the rice endosperm are packed and influence the appearance of chalk. Given the importance of phosphorus in a multitude of enzymatic reactions and compounds that could

contribute to chalk formation, it is reasonable that a gene responsible for regulating the availability of inorganic phosphorus, such as *lpa1-1*, could play a role in influencing chalk formation. The KBNT lpa line has the mutated lpa allele that was associated with increased chalk however it contains alleles for low chalk at nine additional QTL that additively explain the low chalk phenotype of this line. The mutated KBNT-1-1 LPA allele is a unique allele that occurred in a Kaybonnet genetic background that may be associated with an increase in chalk relative to the non-mutant allele. However, currently there is no evidence that natural variation in phytic acid levels occurring in other breeding lines are correlated with chalkiness.

Along with genetics, the environment, particularly the weather during the grain filling stage, affects chalkiness. In this study we found that (in the two weeks following heading) the night temperature and NT₉₅ are positively correlated with percent chalk. This is in agreement with previous reports (Ambardekar et al., 2011; Counce et al., 2005; Lanning et al., 2011; Li et al., 2011). Average relative humidity was also positively correlated with chalk, although the magnitude of the effect was weak (Table 2). Correcting for weather data during grain filling did not substantially change the QTL that were detected or their relative LOD scores (Supp. Figure 4). This suggests that the loci will have a similar effect across environments and that the markers are an effective selection tool.

To meet the challenge of breeding high yielding and high quality rice for a future with potential climate change and limited resources, it will be important to understand the genetics that govern grain quality traits such as chalk (Sreenivasulu et al., 2015). Warmer temperatures increase chalk directly, and along with higher CO₂ levels, may indirectly increase other biotic and abiotic stresses that will exacerbate problems with chalk (Fitzgerald et al., 2009; Lyman et

al., 2013). Limited water resources could necessitate changes in production methods or shifts in growing regions that could impact chalk as well. The incorporation of new genetic diversity into elite rice breeding lines through wide crosses could provide the needed variation to breed for the adaptation to environmental or limited resource challenges. However use of new genetic material has a strong potential of negatively impacting grain quality traits such as chalk. Also, hybrids often rely on intra-subspecific variation for heterosis. Molecular markers for chalk could help to eliminate alleles for high chalk in breeding programs through MAS and allow for greater use of the full range of genetic diversity available in rice germplasm.

REFERENCES

- Ambardekar, A.A., Siebenmorgen, T.J., Counce, P.A., Lanning, S.B., Mauromoustakos, A., 2011. Impact of field-scale nighttime air temperatures during kernel development on rice milling quality. *F. Crop. Res.* 122, 179–185. doi:10.1016/j.fcr.2011.03.012
- Ashida, K., Iida, S., Yasui, T., 2009. Morphological, physical, and chemical properties of grain and flour from chalky rice mutants. *Cereal Chem.* 86, 225–231.
- Brinch-Pedersen, H., Sørensen, L.D., Holm, P.B., 2002. Engineering crop plants: Getting a handle on phosphate. *Trends Plant Sci.* 7, 118–125. doi:10.1016/S1360-1385(01)02222-1
- Broman, K.W., Wu, H., Sen, S., Churchill, G.A., 2003. R/qtl: QTL mapping in experimental crosses. *Bioinformatics* 19, 889–890. doi:10.1093/bioinformatics/btg112
- Childs, N., 2016. Rice Yearbook 2016 [WWW Document]. USDA ERS. URL <http://www.ers.usda.gov/data-products/rice-yearbook.aspx>
- Chun, A., Song, J., Kim, K.-J., Lee, H.-J., 2009. Quality of head and chalky rice and deterioration of eating quality by chalky rice. *J. Crop Sci. Biotech* 12, 239–244. doi:10.1007/s12892-009-0142-4
- Counce, P.A., Bryant, R.J., Bergman, C.J., Bautista, R.C., Wang, Y., Siebenmorgen, T.J., Moldenhauer, K.A.K., Meullenet, J.C., 2005. Rice milling quality, grain dimensions, and starch branching as affected by high night temperatures. *Cereal Chem.* 82, 645–648.
- Del Rosario, A.R., Briones, V.P., Vidal, A.J., 1968. Composition and endosperm structure of developing and mature rice kernel. *Cereal Chem.* 45, 225–235.
- Fitzgerald, M.A., McCouch, S.R., Hall, R.D., 2009. Not just a grain of rice: the quest for quality.

412 Trends Plant Sci. 14, 133–139. doi:10.1016/j.tplants.2008.12.004

413 Fujita, N., Yoshida, M., Kondo, T., Saito, K., Utsumi, Y., Tokunaga, T., Nishi, A., Satoh, H.,
 414 Park, J.H., Jane, J.L., Miyao, A., Hirochika, H., Nakamura, Y., 2007. Characterization of
 415 SSIIIa-deficient mutants of rice: the function of SSIIIa and pleiotropic effects by SSIIIa
 416 deficiency in the rice endosperm. Plant Physiol 144, 2009–2023.
 417 doi:10.1104/pp.107.102533

418 Hayashi, K., Hashimoto, N., Daigen, M., Ashikawa, I., 2004. Development of PCR-based SNP
 419 markers for rice blast resistance genes at the Piz locus. Theor. Appl. Genet. 108, 1212–
 420 1220. doi:10.1007/s00122-003-1553-0

421 Kang, H.G., Park, S., Matsuoka, M., An, G., 2005. White-core endosperm floury endosperm-4 in
 422 rice is generated by knockout mutations in the C4-type pyruvate orthophosphate dikinase
 423 gene (OsPPDKB). Plant J. 42, 901–911. doi:10.1111/j.1365-313X.2005.02423.x

424 Kepiro, J.L., McClung, A.M., Chen, M.H., Yeater, K.M., Fjellstrom, R.G., 2008. Mapping QTLs
 425 for milling yield and grain characteristics in a tropical japonica long grain cross. J. Cereal
 426 Sci. 48, 477–485. doi:10.1016/j.jcs.2007.12.001

427 Khush, G.S., Paule, C.M., dela Cruz, N.M., 1979. Rice grain quality evaluation and improvement
 428 at IRRI, in: In: Proceedings of Workshop in Chemical Aspects of Rice Grain Quality. Int.
 429 Rice Res. Inst., Los Baños, Laguna, Philippines. International Rice Research Institute, Los
 430 Baños, Laguna, Philippines, p. 22–31.

431 Kim, S.I., Andaya, C.B., Goyal, S.S., Tai, T.H., 2008. The rice OsLpa1 gene encodes a novel
 432 protein involved in phytic acid metabolism. Theor. Appl. Genet. 117, 769–779.

doi:10.1007/s00122-008-0818-z

Landoni, M., Cerino Badone, F., Haman, N., Schiraldi, A., Fessas, D., Cesari, V., Toschi, I., Cremona, R., Delogu, C., Villa, D., Cassani, E., Pilu, R., 2013. Low phytic acid 1 mutation in maize modifies density, starch properties, cations, and fiber contents in the seed. *J. Agric. Food Chem.* 61, 4622–4630. doi:10.1021/jf400259h

Lanning, S.B., Siebenmorgen, T.J., Counce, P.A., Ambardekar, A.A., Mauromoustakos, A., 2011. Extreme nighttime air temperatures in 2010 impact rice chalkiness and milling quality. *F. Crop. Res.* 124, 132–136. doi:10.1016/j.fcr.2011.06.012

Larson, S.R., Rutger, J.N., Young, K.A., Raboy, V., 2000. Isolation and Genetic Mapping of a Non-Lethal Rice (*Oryza sativa* L.) low phytic acid 1 Mutation. *Crop Sci.* 40, 1397–1405. doi:10.2135/cropsci2000.4051397x

Lee, S., Wamishe, Y.A., Jia, Y., Liu, G., Jia, M.H., 2009. Identification of two major resistance genes against race IE-1k of *Magnaporthe oryzae* in the indica rice cultivar Zhe733. *Mol. Breed.* 24, 127–134. doi:10.1007/s11032-009-9276-9

Lehmacher, A., Hensel, R., 1994. Cloning, sequencing and expression of the gene encoding 2-phosphoglycerate kinase from *Methanothermobacter thermophilus*. *Mol. Gen. Genet.* 242, 163–168.

Li, H., Chen, Z., Hu, M., Wang, Z., Hua, H., Yin, C., Zeng, H., 2011. Different effects of night versus day high temperature on rice quality and accumulation profiling of rice grain proteins during grain filling. *Plant Cell Rep.* 30, 1641–1659. doi:10.1007/s00299-011-1074-2

Li, Y., Fan, C., Xing, Y., Yun, P., Luo, L., Yan, B., Peng, B., Xie, W., Wang, G., Li, X., Xiao, J.,

454 Xu, C., He, Y., 2014. Chalk5 encodes a vacuolar H(+)-translocating pyrophosphatase
 455 influencing grain chalkiness in rice. *Nat. Genet.* 46, 398–404. doi:10.1038/ng.2923
 456 Life Technologies Corp., 2004. GeneMapper, Version 3.7.
 457 Lisle, A.J., Martin, M., Fitzgerald, M.A., 2000. Chalky and translucent rice grains differ in starch
 458 composition and structure and cooking properties. *Cereal Chem.* 77, 627–632.
 459 doi:10.1094/CCHEM.2000.77.5.627
 460 Liu, G., Bernhardt, J.L., Jia, M.H., Wamishe, Y.A., Jia, Y., 2008. Molecular characterization of
 461 the recombinant inbred line population derived from a japonica-indica rice cross. *Euphytica*
 462 159, 73–82. doi:10.1007/s10681-007-9459-6
 463 Liu, X., Wan, X., Ma, X., Wan, J., 2011. Dissecting the genetic basis for the effect of rice
 464 chalkiness, amylose content, protein content, and rapid viscosity analyzer profile
 465 characteristics on the eating quality of cooked rice using the chromosome segment
 466 substitution line population across ei. *Genome* 54, 64–80. doi:10.1139/G10-070
 467 Lorieux, M., 2012. MapDisto: Fast and efficient computation of genetic linkage maps. *Mol.*
 468 *Breed.* 30, 1231–1235. doi:10.1007/s11032-012-9706-y
 469 Lyman, N.B., Jagadish, K.S. V, Nalley, L.L., Dixon, B.L., Siebenmorgen, T., 2013. Neglecting
 470 rice milling yield and quality underestimates economic losses from high-temperature stress.
 471 *PLoS One* 8. doi:10.1371/journal.pone.0072157
 472 Patindol, J., Wang, Y.J., 2003. Fine structures and physicochemical properties of starches from
 473 chalky and translucent rice kernels. *J. Agric. Food Chem.* 51, 2777–2784.
 474 doi:10.1021/jf026101t

475 Peng, C., Wang, Y., Liu, F., Ren, Y., Zhou, K., Lv, J., Zheng, M., Zhao, S., Zhang, L., Wang, C.,
 476 Jiang, L., Zhang, X., Guo, X., Bao, Y., Wan, J., 2014. FLOURY ENDOSPERM6 encodes a
 477 CBM48 domain-containing protein involved in compound granule formation and starch
 478 synthesis in rice endosperm. *Plant J.* 77, 917–930. doi:10.1111/tpj.12444

479 Raboy, V., 2009. Approaches and challenges to engineering seed phytate and total phosphorus.
 480 *Plant Sci.* 177, 281–296. doi:10.1016/j.plantsci.2009.06.012

481 Raboy, V., 2007. The ABCs of low-phytate crops. *Nat. Biotechnol.* 25, 874–875.
 482 doi:10.1038/nbt0807-874

483 R Core Team, 2016. R: A language and environment for statistical computing.

484 Rutger, J.N., Raboy, V., Moldenhauer, K.A.K., Bryant, R.J., Lee, F.M., Gibbons, J., 2004.
 485 Registration of KBNT lpa1-1 Low Phytic Acid Germplasm of Rice. *Crop Sci.* 44, 363.

486 Rutger, J.N., Tai, T.H., 2005. Registration of K/Z mapping population of rice. *Crop Sci.* 45,
 487 2671–2672.

488 SAS Institute Inc., 2015. JMP®, Version 12.

489 Schlemmer, U., Frølich, W., Prieto, R.M., Grases, F., 2009. Phytate in foods and significance for
 490 humans: Food sources, intake, processing, bioavailability, protective role and analysis. *Mol.*
 491 *Nutr. Food Res.* 53, 330–375. doi:10.1002/mnfr.200900099

492 Sreenivasulu, N., Butardo, V.M., Misra, G., Cuevas, R.P., Anacleto, R., Kishor, P.B.K., 2015.
 493 Designing climate-resilient rice with ideal grain quality suited for high-temperature stress. *J.*
 494 *Exp. Bot.* 66, 1737–1748. doi:10.1093/jxb/eru544

495 Tian, L., Dai, L.L., Yin, Z.J., Fukuda, M., Kumamaru, T., Dong, X.B., Xu, X.P., Qu, L.Q., 2013.
 496 Small GTPase Sar1 is crucial for proglutelin and α -globulin export from the endoplasmic
 497 reticulum in rice endosperm. *J. Exp. Bot.* 64, 2831–2845. doi:10.1093/jxb/ert128
 498 Vision, T.J., Brown, D.G., Shmoys, D.B., Durrett, R.T., Tanksley, S.D., 2000. Selective
 499 mapping: A strategy for optimizing the construction of high- density linkage maps. *Genetics*
 500 155, 407–420.
 501 Wan, X.Y., Wan, J.M., Weng, J.F., Jiang, L., Bi, J.C., Wang, C.M., Zhai, H.Q., 2005. Stability of
 502 QTLs for rice grain dimension and endosperm chalkiness characteristics across eight
 503 environments. *Theor. Appl. Genet.* 110, 1334–1346. doi:10.1007/s00122-005-1976-x
 504 Wang, E., Wang, J., Zhu, X., Hao, W., Wang, L., Li, Q., Zhang, L., He, W., Lu, B., Lin, H., Ma,
 505 H., Zhang, G., He, Z., 2008. Control of rice grain-filling and yield by a gene with a potential
 506 signature of domestication. *Nat. Genet.* 40, 1370–4. doi:10.1038/ng.220
 507 Woo, M.O., Ham, T.H., Ji, H.S., Choi, M.S., Jiang, W., Chu, S.H., Piao, R., Chin, J.H., Kim,
 508 J.A., Park, B.S., Seo, H.S., Jwa, N.S., McCouch, S.R., Koh, H.J., 2008. Inactivation of the
 509 UGPase1 gene causes genic male sterility and endosperm chalkiness in rice (*Oryza sativa*
 510 L.). *Plant J.* 54, 190–204. doi:10.1111/j.1365-313X.2008.03405.x
 511 Yamakawa, H., Hirose, T., Kuroda, M., Yamaguchi, T., 2007. Comprehensive expression
 512 profiling of rice grain filling-related genes under high temperature using DNA microarray.
 513 *Plant Physiol.* 144, 258–277.
 514 Yan, W., Dilday, R.H., Tai, T.H., Gibbons, J.W., McNew, R.W., Rutger, J.N., 2005. Differential
 515 response of rice germplasm to straighthead induced by arsenic. *Crop Sci.* 45, 1223–1228.

516 doi:10.2135/cropsci2004.0348

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Figure legends

Figure 1. Histogram showing the distribution of percent chalk (LSmeans adjusted for environmental predictors) in the KBNT *lpa* x Zhe733 RILs.

Figure 2. Composite interval mapping (solid line) and standard interval mapping (dotted line) for chalk QTL. Significant LOD thresholds (at $p < 0.05$) based on 1000 permutations are shown as a horizontal line of the style corresponding to the mapping method.

Figure 3. Chromosome map showing positions (in Mbp) of chalk QTL found in the current study (labeled with prefix qBCHK), previously reported chalk QTL and candidate genes. QTL where the TeQing parent allele reduced chalk are blue and those that increased chalk are red.

Figure 1.

Figure 2.

Figure 3.

Table 1. Linear model fixed effect tests for genotype and environmental predictors of chalk.

Effect	F Ratio	Prob > F
Line	145.51	<.0001
NT95	42.18	<.0001
Avg. night temp. C	19.48	<.0001
Avg. temp. C	0.64	0.424
DD10	0.85	0.357
Avg. rel. humidity	27.67	<.0001
Total rainfall cm	0.67	0.414

Table 2. Linear model parameter estimates for significant fixed effects for environmental predictors of chalk.

Effect	Estimate	Std Error	t Ratio	Prob> t
NT95	1.034	0.193	5.36	<.0001
Avg. night temp. C	1.356	0.206	6.58	<.0001
Avg. rel. humidity	0.090	0.043	2.09	0.0367

Table 3. Nearest markers to significant ($p < 0.05$) chalk QTL peaks and estimated cM positions.

Nearest Marker	Chr.	cM	LOD	P-value
RM5496	1	31.42	7.17	0.001
RM486	1	106.42	5.47	0.005
ID2015477	2	148.00	14.65	0.001
RM335	4	2.72	5.29	0.005
RM303	4	104.00	3.97	0.048

Table 4. Additional chalk QTL with LOD > 1.5 in the context of a multiple QTL model (addqtl).

Nearest Marker	Chr.	cM	LOD
RM10149	1	1.42	1.88
RM437	5	32	2.13
RM105	9	33	2.78
RM229	11	69	2.92
RM270	12	68	1.54

Table 5. Estimated effects of all significant chalk QTL and pairs of QTL in the model. The effect is the change in phenotype conferred by the Zhe733 allele.

QTL	df	Type III SS	LOD	% var	F value	Pvalue (F)	Est. effect	SE	t
1@1.4	1	434	1.84	1.70	8.06	0.005	1.78	0.63	2.82
1@31.4	3	2205.6	8.59	8.65	13.66	4.82E-08	2.37	0.73	3.27
1@106.4	1	1516.7	6.09	5.95	28.18	3.34E-07	2.82	0.57	4.91
2@148.0	2	6661.8	21.82	26.13	61.89	<2E-016	-5.29	0.58	-9.18
4@2.0	2	1866.9	7.38	7.32	17.34	1.35E-07	2.80	0.57	4.91
4@104.0	1	1115.4	4.57	4.37	20.73	9.3E-06	2.60	0.59	4.41
5@32.0	1	277.6	1.19	1.09	5.16	0.024	1.43	0.61	2.34
9@33.0	1	528.7	2.23	2.07	9.82	0.002	1.34	0.59	2.27
11@69.0	1	362.1	1.54	1.42	6.73	0.010	1.84	0.59	3.13
12@68.0	1	411.3	1.75	1.61	7.64	0.006	-1.67	0.63	-2.65
1@31.4:2@148.0	1	432.3	1.83	1.70	8.03	0.005	-1.57	0.59	-2.67
1@31.4:4@2.0	1	255.6	1.09	1.00	4.75	0.031	0.83	0.58	1.43
Intercept							17.79	0.62	28.76

Supplementary Table 1. Markers used for genotyping the Kbnt *lpa* x Zhe733 RIL population including primer sequences, MSU7 pseudomolecule assembly coordinates, and estimated genetic map positions.

[See attachment]

Supplementary Table 2. REML variance component estimates for random effects of year and planting date.

Random Effect	Var Ratio	Var Component	Std Error	95% Lower	95% Upper	Percent of Total
Year	0.251	2.817	4.581	-6.161	11.794	19.032
Planting	0.066	0.746	0.771	-0.765	2.257	5.04
Residual		11.237	0.348	10.585	11.951	75.93
Total		14.799	4.596	8.734	30.387	100

Supplementary Table 3. LSmeans of each line for chalk and chalk adjusted for weather with RILs sorted from low to high percent chalk.

Line	Least Sq Mean	Std Error	Adjusted Least Sq Mean	Adjusted Std Error
GSOR100171	2.43	1.97	2.35	1.59
GSOR100137	2.49	1.97	2.48	1.59
GSOR100099	3.30	1.97	3.24	1.59
GSOR100083	3.32	1.97	3.38	1.59
GSOR100043	3.42	1.97	3.73	1.59
GSOR100169	3.48	1.97	3.37	1.59
GSOR100341	3.74	1.97	3.16	1.59
GSOR100298	3.76	1.97	3.61	1.59
GSOR100295	3.97	1.97	4.11	1.59
GSOR100329	4.16	1.97	3.79	1.59
GSOR100241	4.17	1.97	4.42	1.59
GSOR100159	4.29	1.97	4.04	1.59
GSOR100069	4.46	1.97	4.50	1.59
GSOR100317	4.65	1.97	3.96	1.59
GSOR100032	4.73	1.97	4.06	1.59
GSOR100067	4.93	1.97	5.07	1.59
GSOR100085	5.00	1.97	4.44	1.59
GSOR100237	5.01	1.97	5.14	1.59
GSOR100092	5.40	1.97	5.48	1.59
GSOR100312	5.40	1.99	6.01	1.62
GSOR100059	5.49	1.97	4.82	1.59
GSOR100168	5.80	1.97	6.26	1.59
GSOR100068	5.83	1.97	6.05	1.59
GSOR100337	5.86	1.97	5.81	1.59
GSOR100319	6.14	1.97	6.34	1.59
GSOR100030	6.19	1.97	5.98	1.59
GSOR100270	6.28	1.97	6.17	1.59
GSOR100154	6.48	1.97	5.95	1.59
GSOR100140	6.50	1.99	6.82	1.62
GSOR100231	6.84	1.97	6.96	1.59
GSOR100273	6.85	1.97	6.81	1.59
GSOR100123	6.94	3.88	7.80	3.58
GSOR100324	6.94	1.97	6.78	1.59
GSOR100118	6.97	1.99	6.75	1.62
GSOR100125	6.97	1.99	6.82	1.62

GSOR100094	7.21	1.97	7.56	1.59
GSOR100251	7.24	1.97	7.29	1.59
GSOR100242	7.27	1.97	7.51	1.59
GSOR100039	7.28	1.97	7.44	1.59
GSOR100147	7.39	1.97	7.55	1.59
GSOR100185	7.51	1.97	7.00	1.59
GSOR100208	7.53	1.97	7.09	1.59
GSOR100248	7.87	1.97	7.40	1.59
GSOR100274	8.05	1.97	7.41	1.59
GSOR100234	8.27	1.97	8.37	1.59
GSOR100139	8.34	1.97	8.44	1.59
GSOR100347	8.47	1.99	8.96	1.62
GSOR100041	8.94	1.97	9.16	1.59
GSOR100296	9.12	1.97	8.55	1.59
GSOR100316	9.27	1.97	10.34	1.59
GSOR100290	9.38	1.97	9.30	1.59
GSOR100115	9.48	1.97	9.26	1.59
GSOR100267	9.53	1.97	10.04	1.59
GSOR100283	9.61	1.97	9.54	1.59
GSOR100038	9.67	1.97	9.87	1.59
GSOR100177	9.82	1.97	9.97	1.59
GSOR100082	9.87	1.97	9.62	1.59
GSOR100145	10.06	1.97	10.20	1.59
GSOR100093	10.64	1.97	10.72	1.59
GSOR100074	10.84	1.97	10.74	1.59
GSOR100116	11.15	1.97	11.95	1.59
GSOR100284	11.35	1.97	11.61	1.59
GSOR100339	11.41	1.97	12.21	1.59
GSOR100353	11.79	1.97	12.61	1.59
GSOR100042	12.25	1.97	12.15	1.59
GSOR100176	12.28	1.97	12.26	1.59
GSOR100089	12.50	1.97	12.37	1.59
GSOR100344	12.72	1.97	12.92	1.59
GSOR100048	12.76	1.97	12.33	1.59
GSOR100007	12.85	1.97	12.64	1.59
GSOR100167	12.97	1.97	12.81	1.59
GSOR100180	13.17	1.97	13.91	1.59
GSOR100111	13.35	1.97	13.97	1.59
GSOR100299	13.47	1.97	13.54	1.59
GSOR100128	13.62	1.97	13.62	1.59
GSOR100330	13.74	1.97	13.45	1.59

GSOR100292	13.97	1.97	13.72	1.59
GSOR100120	13.98	1.97	14.05	1.59
GSOR100260	14.05	1.97	14.06	1.59
GSOR100309	14.15	1.97	15.02	1.59
GSOR100285	14.30	1.97	14.20	1.59
GSOR100306	14.33	1.97	13.73	1.59
GSOR100025	14.36	1.97	14.07	1.59
GSOR100304	14.55	1.97	14.63	1.59
GSOR100301	14.68	1.97	15.04	1.59
GSOR100152	14.76	1.97	14.81	1.59
GSOR100340	14.77	1.97	15.02	1.59
GSOR100143	14.83	1.97	15.10	1.59
GSOR100350	15.16	1.97	15.32	1.59
GSOR100117	15.46	1.97	14.75	1.59
GSOR100227	15.49	1.97	14.97	1.59
GSOR100333	15.52	1.97	15.21	1.59
GSOR100293	15.57	1.97	15.92	1.59
GSOR100294	16.05	1.97	15.95	1.60
GSOR100050	16.21	1.97	16.68	1.59
GSOR100228	16.28	1.97	16.31	1.59
GSOR100017	16.42	1.97	15.73	1.59
GSOR100282	16.47	1.97	16.43	1.59
GSOR100210	16.87	1.97	16.90	1.59
GSOR100012	16.91	1.97	16.70	1.59
GSOR100277	17.05	1.97	16.74	1.59
GSOR100151	17.09	1.97	17.29	1.59
GSOR100160	17.38	1.97	16.62	1.59
GSOR100031	17.43	1.97	17.59	1.59
GSOR100003	17.93	1.97	17.74	1.59
GSOR100320	18.16	1.97	17.99	1.59
GSOR100252	18.48	1.97	18.27	1.59
GSOR100336	18.57	1.97	18.55	1.59
GSOR100289	19.07	1.99	19.14	1.62
GSOR100193	19.12	1.97	19.33	1.59
GSOR100044	20.36	1.97	19.98	1.59
GSOR100129	20.56	1.97	20.87	1.59
GSOR100146	20.88	1.97	21.51	1.59
GSOR100195	21.11	1.97	21.02	1.59
GSOR100264	21.21	1.97	21.02	1.59
GSOR100352	21.34	1.97	20.58	1.59
GSOR100066	21.38	1.97	21.25	1.59

GSOR100063	21.62	1.97	21.73	1.59
GSOR100351	21.76	1.97	22.78	1.59
GSOR100016	21.92	1.97	21.83	1.59
GSOR100105	22.25	1.97	22.22	1.59
GSOR100191	22.47	1.97	22.71	1.59
GSOR100008	22.57	1.97	22.18	1.59
GSOR100109	22.79	1.97	22.64	1.59
GSOR100057	23.02	1.97	23.50	1.59
GSOR100342	23.02	1.97	23.01	1.59
GSOR100291	23.07	1.97	22.84	1.59
GSOR100302	23.11	1.97	23.64	1.59
GSOR100328	23.18	1.97	23.95	1.59
GSOR100165	23.19	1.97	24.06	1.59
GSOR100028	23.33	1.97	23.45	1.59
GSOR100112	23.62	1.97	23.75	1.59
GSOR100308	23.63	1.97	24.48	1.59
GSOR100332	23.64	1.97	23.51	1.59
GSOR100287	23.81	1.97	24.00	1.59
GSOR100196	24.08	1.97	24.31	1.59
GSOR100226	24.16	1.97	24.93	1.59
GSOR100307	24.46	1.97	24.09	1.59
GSOR100149	25.36	1.97	24.77	1.59
GSOR100077	25.69	1.97	25.49	1.59
GSOR100014	25.75	1.97	25.79	1.59
GSOR100253	25.80	1.97	25.88	1.59
GSOR100141	25.98	1.97	26.00	1.59
GSOR100053	26.03	1.97	25.78	1.59
GSOR100064	26.53	1.97	26.81	1.59
GSOR100072	26.62	1.97	27.73	1.59
GSOR100163	26.73	1.97	26.43	1.59
GSOR100058	26.92	1.97	26.90	1.59
GSOR100121	27.05	1.97	27.05	1.59
GSOR100326	27.67	1.97	27.14	1.59
GSOR100187	27.80	1.97	27.76	1.59
GSOR100236	27.97	1.97	27.80	1.59
GSOR100303	28.03	1.97	28.80	1.59
GSOR100311	28.08	1.97	28.04	1.59
GSOR100325	28.16	1.97	28.36	1.59
GSOR100247	28.36	1.97	28.12	1.59
GSOR100004	29.03	1.97	29.08	1.59
GSOR100182	29.10	1.97	28.57	1.59

GSOR100015	29.38	1.97	29.59	1.59
GSOR100113	29.47	1.97	29.49	1.59
GSOR100033	30.98	1.97	30.44	1.59
GSOR100022	31.11	1.97	31.00	1.59
GSOR100233	31.86	1.97	31.12	1.59
GSOR100323	32.32	1.97	32.13	1.59
GSOR100045	32.83	1.97	32.69	1.59
GSOR100202	35.34	1.97	35.25	1.59
GSOR100173	35.45	1.97	35.34	1.59
GSOR100245	35.91	1.97	35.97	1.59
GSOR100023	36.48	1.97	36.79	1.59
GSOR100103	36.53	1.97	36.10	1.59
GSOR100055	36.86	1.97	36.82	1.59
GSOR100201	36.97	1.97	36.66	1.59
GSOR100062	37.04	1.97	36.86	1.59
GSOR100199	37.95	1.97	37.79	1.59
GSOR100192	38.12	1.97	37.93	1.59
GSOR100204	38.28	1.97	38.12	1.59
GSOR100060	38.52	1.97	38.58	1.59
GSOR100104	38.78	1.97	38.78	1.59
GSOR100235	39.44	1.97	39.40	1.59
GSOR100086	39.91	1.97	39.86	1.59
GSOR100114	39.97	1.97	40.08	1.59
GSOR100278	40.54	1.97	41.02	1.59
GSOR100049	42.97	1.97	43.00	1.59
GSOR100026	43.62	1.97	43.67	1.59
GSOR100020	44.38	1.97	43.96	1.59
GSOR100271	44.57	1.97	44.75	1.59
GSOR100155	45.84	1.97	45.73	1.59
GSOR100013	47.72	1.97	47.26	1.59
GSOR100343	48.66	1.97	48.89	1.59
GSOR100065	52.61	1.99	52.64	1.62
KBNT_lpa1-1	3.15	1.97	2.96	1.59
Zhe733	25.37	1.97	24.58	1.61

Supplementary Table 4. Positions (cM) for pairs of chalk QTL identified in a two-dimensional genome scan with a two-QTL model (scantwo).

Position 1 (cM)	Position 2 (cM)	LOD	P-value
1@6.42	<u>1@41.4</u>	10.2	< 0.001
<u>1@31.42</u>	<u>2@150</u>	18.65	< 0.001
<u>1@31.42</u>	<u>4@5</u>	9.83	< 0.001
<u>1@31.42</u>	<u>5@30</u>	7.49	0.003
<u>2@150</u>	<u>4@5</u>	16.08	< 0.001
<u>2@150</u>	<u>5@35</u>	13.07	< 0.001
<u>2@150</u>	<u>9@45</u>	12.9	< 0.001
<u>2@150</u>	<u>11@70</u>	14.05	< 0.001
<u>4@5</u>	4@105	6.89	0.009

Supplementary Table 5. Tests of all possible pairwise interactions, added one at a time to the multiple QTL model (addint). Significance levels of 0.01, 0.05, and 0.1 are indicated with the symbols “***”, “*”, and “.” respectively.

QTL pair	df	Type III SS	LOD	% var	F value	Pvalue (Chi2)	Pvalue (F)	
1@1.4:1@31.4	1	39.69	0.16	0.16	0.69	0.39	0.41	
1@1.4:1@106.4	1	38.71	0.16	0.15	0.68	0.40	0.41	
1@1.4:2@148.0	1	27.60	0.11	0.11	0.48	0.47	0.49	
1@1.4:4@2.0	1	40.01	0.16	0.16	0.70	0.39	0.40	
1@1.4:4@104.0	1	-9.58	-0.04	-0.04	-0.17	1.00	1.00	
1@1.4:5@32.0	1	118.87	0.48	0.47	2.09	0.14	0.15	
1@1.4:9@33.0	1	33.51	0.14	0.13	0.59	0.43	0.45	
1@1.4:11@69.0	1	179.15	0.73	0.70	3.17	0.07	0.08	.
1@1.4:12@68.0	1	-16.13	-0.07	-0.06	-0.28	1.00	1.00	
1@31.4:1@106.4	1	32.78	0.13	0.13	0.57	0.44	0.45	
1@31.4:2@148.0	1	435.42	1.80	1.71	7.92	0.00	0.01	***
1@31.4:4@2.0	1	258.71	1.06	1.01	4.62	0.03	0.03	*
1@31.4:4@104.0	1	36.05	0.15	0.14	0.63	0.41	0.43	
1@31.4:5@32.0	1	64.07	0.26	0.25	1.12	0.27	0.29	
1@31.4:9@33.0	1	52.54	0.21	0.21	0.92	0.32	0.34	
1@31.4:11@69.0	1	35.33	0.14	0.14	0.62	0.42	0.43	
1@31.4:12@68.0	1	-28.01	-0.11	-0.11	-0.49	1.00	1.00	
1@106.4:2@148.0	1	87.13	0.35	0.34	1.53	0.20	0.22	
1@106.4:4@2.0	1	116.88	0.47	0.46	2.06	0.14	0.15	
1@106.4:4@104.0	1	36.03	0.15	0.14	0.63	0.41	0.43	

1@106.4:5@32.0	1	20.98	0.08	0.08	0.37	0.53	0.55
1@106.4:9@33.0	1	6.61	0.03	0.03	0.12	0.73	0.73
1@106.4:11@69.0	1	122.26	0.50	0.48	2.15	0.13	0.14
1@106.4:12@68.0	1	37.69	0.15	0.15	0.66	0.40	0.42
2@148.0:4@2.0	1	148.40	0.60	0.58	2.62	0.10	0.11
2@148.0:4@104.0	1	101.69	0.41	0.40	1.79	0.17	0.18
2@148.0:5@32.0	1	3.81	0.02	0.01	0.07	0.79	0.80
2@148.0:9@33.0	1	16.24	0.07	0.06	0.28	0.58	0.60
2@148.0:11@69.0	1	209.77	0.86	0.82	3.73	0.05	0.06 .
2@148.0:12@68.0	1	90.09	0.37	0.35	1.58	0.20	0.21
4@2.0:4@104.0	1	9.79	0.04	0.04	0.17	0.67	0.68
4@2.0:5@32.0	1	26.79	0.11	0.11	0.47	0.48	0.50
4@2.0:9@33.0	1	196.61	0.80	0.77	3.49	0.06	0.06 .
4@2.0:11@69.0	1	76.81	0.31	0.30	1.35	0.23	0.25
4@2.0:12@68.0	1	123.81	0.50	0.49	2.18	0.13	0.14
4@104.0:5@32.0	1	-3.59	-0.01	-0.01	-0.06	1.00	1.00
4@104.0:9@33.0	1	147.58	0.60	0.58	2.61	0.10	0.11
4@104.0:11@69.0	1	24.58	0.10	0.10	0.43	0.50	0.51
4@104.0:12@68.0	1	168.67	0.69	0.66	2.99	0.08	0.09 .
5@32.0:9@33.0	1	4.16	0.02	0.02	0.07	0.78	0.79
5@32.0:11@69.0	1	256.85	1.05	1.01	4.59	0.03	0.03 *
5@32.0:12@68.0	1	112.03	0.45	0.44	1.97	0.15	0.16
9@33.0:11@69.0	1	116.16	0.47	0.46	2.05	0.14	0.15
9@33.0:12@68.0	1	55.02	0.22	0.22	0.96	0.31	0.33
11@69.0:12@68.0	1	107.93	0.44	0.42	1.90	0.16	0.17

Supplemental Table 6. Fit of the full chalk multiple QTL model including 10 loci and two interactions.

	df	SS	MS	LOD	% var	Pvalue (Chi2)	Pvalue (F)
Model	12	16135.14	1344.60	40.68	63.28	< 0.001	< 0.001
Error	174	9364.68	53.82				
Total	186	25499.82					