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Genome-wide Association Study of Resistant Starch (RS) Phenotypes in a Barley Variety Collection

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Supporting Information

ABSTRACT: Barley is primarily grown for feed and malt, but in some regions of the world it is also considered to be a staple food. Some barley types such as high-amylose barley have also gained importance as health-promoting foods. Starch that is not readily digested in the upper mammalian gastrointestinal system, or resistant starch (RS), is considered to be valuable because it prevents some diet-related diseases such as colon cancer. RS was quantified in a diverse collection of 209 spring barley varieties released in Europe during the past 100 years. The RS content varied from <1% to >15% in the collection, with 13 varieties having high RS content (>11%) and 15 varieties below 1%. Combined with genome-wide association scanning (GWAS), SNP markers and candidate genes controlling the RS content in grains were identified. This identified 40 SNP markers with a LOD score above 2, located on chromosomes 2H, 3H, 5H, and 6H, respectively. Among these SNPs, 10 genes with a known role in starch biosynthesis were associated on the basis of synteny conservation to the rice genome. The β-glucan content was quantified in 61 varieties selected to represent extreme as well as medium RS values. The β-glucan amount in the 15 varieties with RS <1% ranged from 1.7 to 3.2%, ranged from 1.76 to 2.54% in the 13 varieties with RS >11%, and ranged from 1.95 to 2.82% for those with 1% < RS < 11%. No statistically significant correlation between RS content and β-glucan content was found. This association analysis of commercial varieties revealed a large variation in RS content and identified a number of SNP markers that can be explored for selection and further dissection of the pathway and control of RS phenotype.

KEYWORDS: *Hordeum vulgare, glycemic index, synteny, resistant starch, β-glucan, waxy, high-amylose*

INTRODUCTION

Cereals, the staple foods for humans, provide energy and many nutrients, and a high consumption of cereals may decrease the risk of chronic diseases.[1] In cereals, carbohydrates account for approximately 75% of the dry weight; starch itself contributes up to 70% of the seed weight,[2] and it provides the largest supply of calories. Most of the starch in cereal products is digested rapidly in the upper gastrointestinal track except for a variable portion that remains undigested, which is defined as resistant starch (RS).[3] Resistant starch is classified into four types based on the sources:[4] RS_1 is physically inaccessible or indigestible starch present in the food matrix, RS_2 is starch in raw food maintaining the natural granular form, RS_3 is crystalline and formed in starchy food during processing including the retrogradation of starch granules, and RS_4 comprises all types of chemically modified starches. Food with high RS content is beneficial for human health because it prevents diseases such as colon cancer, diabetes, obesity, osteoporosis, and cardiovascular diseases.[4] In addition, RS has potential application in the development of high-quality food products such as bread, pasta, and dumplings.

In barley grain, starch ranges from 62 to 77% of grain dry weight and contains both highly branched amylopectin and linear amylose, accompanied by nonstarch polyglucans (NSP) including pentosans and arabinoxylans. Normal barley contains about 25−27% amylose, waxy barley contains <5% amylose, and the increased amylose types contain >35% amylose. In contrast to amylopectin, amylose contributes toward a low glycemic index (GI) and also promotes bowel health.[5] High-amylose mutants have been found with increased RS content in barley,[6] wheat,[7] maize,[8] and rice.[9] Consumption of a diet combining barley NSP produced by extrusion and RS derived from high-amylose starch shows beneficial effects in rats.[10] Screening or enhancing RS content of barley might further improve the health-promoting effect of barley, a food that is considered to be healthy due to its high content of β-glucan. A negative correlation between amylose and β-glucan has been reported,[11] which could make it difficult to aim for a new barley variety improved in both RS and β-glucan because high RS always results from high amylose content.[12]

Besides amylose content,[13] both starch granule size[14] and starch chain structure[5] affect the RS content in barley. In addition, cell wall structure might affect the RS_2 type by determining the accessibility of amylase. There is still a paucity of information about the genetic and biochemical mechanisms controlling RS. Starch synthase SSIIa and starch branching enzyme SBEIIb genes might be responsible for the increased RS in barley[6] and maize[15] due to the increased amylose and modified amylopectin structure. Using a transgenic approach,

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inhibition of the starch branching enzymes SBEI and SBEIIb in *indica* rice increased the RS content from 0 to 14.6% due to an amylose content increased from 27.2 to 64.8%.[16] Although one resistant starch rice mutant induced by γ rays was reported,[17] no further information about the gene(s) responsible for this mutation is available.[18]

Genome-wide association (GWAS), also named genome-wide linkage disequilibrium (LD) scan, is a method to test for significant association between nucleotide sequence and trait expressions within a large population of unrelated individuals.[19] The technique was first used in human disease genetics and, due to its success, later applied in maize[20] and rice[21] to screen for genetic variations underlying diverse complex traits using a large number of cultivars. The level of LD differs among plant species and populations chosen for the analysis and determines the strategy used in GWAS. In a situation with low LD, typical for out-breeding species such as maize, already within a single gene, association between a sequence polymorphism and a trait expression can be lost. This enables a very precise identification of the nucleotide sequence causing the change in trait expression; on the other hand, a genome-wide search for association is practically impossible due to the large number of necessary markers. However, in species with high LD such as the in-breeding plant barley in which LD can stretch for several centimorgans, the resolution of the technique is slow, but it does enable GWAS studies.[22] The whole genome sequence of barley remains to be assembled and annotated, but a high-density single nucleotide polymorphism (SNP) map has been developed[23] and has shown its potential in digging out new genes for genomic assistant breeding and the development of new powerful molecular markers. Whole genome association scans have been carried out within barley elite germplasm[24] and led to successful reports on determining genetic factors influencing complex traits in barley.[25−27]

Here we present a genome-wide association study in an elite collection of European spring barley varieties to identify the genes responsible for RS content in barley. Phenotyping 209 commercial varieties revealed a large variation in RS content, and the GWAS identified a number of SNP markers that can be explored for selection and further dissection of the biosynthetic pathway and control of the RS phenotype. This provides some insights into the genetics of the formation of RS and delivers markers for crop improvement and the breeding of high RS content barley varieties.

MATERIALS AND METHODS

Germplasm and Genotyping. Spring barley varieties obtained from the *Hordeum vulgare* Core Collection were used. All 209 varieties analyzed were hulled and normal barleys; among them, 35 were 6-row types and the rest were 2-row types. All material was grown at SCRI, now JHI Dundee, Scotland (UK), in 2010. Grains were ground using a Culatti grinder DFH 48 and passed through 1 mm sieves. Before the analyses, the barley flour was dried at 60 °C for 48 h. All varieties had been genotyped at TraitGenetics, GmbH, Gatersleben, Germany.

RS and β-Glucan Quantification. RS content was measured according to the methods of AACC 32−42 with minor modification. Briefly, 100 ± 1 mg of flour was weighed into a 14 mL tubes with caps, digested with 600 U of pepsin (P700, Sigma) at 37 °C for 1 h, and then treated with 10 mg/mL pancreatic α-amylase (A3176, Sigma) in sodium maleate buffer (pH 6.0) containing 3 U/mL amyloglucosidase (A10115, Sigma) for 16 h at 37 °C; then 4 mL of 95% ethanol was added to stop the reaction. The residues were collected by centrifugation and washed twice with 50% ethanol and resuspended in distilled water, and an equivalent volume of 4 M KOH was added; the mixture was agitated for 20 min in an ice bath. After hydrolysis by 330 UI amyloglucosidase, glucose content was measured with a GOPOD Kit (K-Gluc, Megazyme), and RS was calculated according to the equation RS (% w/w) = $A_1/A_0 \times 0.9 \times 0.1 \times 10.3/0.1 \times 100/W$, where A_1 and A_0 are the absorbance of sample and glucose standard, respectively, at 510 nm, and W is the weight of samples. β-Glucan content was measured according to the assay for barley with a Megazyme mixed-linkage β-glucan kit (K-BGLU) and calculated according to the equation β-glucan (% w/w) = $\Delta A \times F/W \times 27$, where ΔA is the absorbance after β-glucosidase treatment (reaction) minus reaction blank absorbance, F is a factor for the conversion of absorbance values to micrograms of glucose, and W is the weight of sample.

Statistical Analysis. *Heritability of RS.* RS summary statistics including frequency and the normal P−P plot, as well as the variance and broad-sense heritability of RS, were analyzed with SPSS 15.0 (SPSS Inc., Chicago, IL, USA). The heritability was calculated according to the formula $h^2 = \sigma_g^2/(\sigma_g^2 + \sigma_e^2/n)$, where σ_g^2 is the genotypic variance, σ_e^2 is the environmental variance, and n is the number of replications.

Population Structure. The genetic structure of association population was analyzed using Structure 2.3.3, both with a panel of 6810 SNPs panel and with a subset of about 461 SNPs spread over the whole genome with >2 cm spacing.[28] The number of groups k was estimated using the admixture model with correlated allele frequencies modeled with a burn-in of 500 000 cycles followed by 500 000 cycles with duplicate runs between k = 1 and 12; each returned matrices (Q) of fractional subpopulation membership for each cultivar. Each accession was assigned to a group according to its maximum ancestry index assessed by Structure for the following analysis. Principal component analysis (PCA) conducted with Stat 10.0 (StataCorp, 2007) was used to infer the population structure in the barley samples. The first three principal components were used to visualize the dispersion of the samples.

Linkage Disequilibrium Analysis. LD analysis was conducted with TASSEL 3.0.9 (http://www.maizegenetics.net/tassel) with the entire 6810 SNP panel, and the significance of LD was determined by 1000 permutations for each pair. The LD for each pair of loci was assessed within the population and also the subpopulation because LD could be seriously affected by a small population. The number of marker pairs with LD probability values less than the threshold of 0.001 was counted, and LD decay was considered when r^2 = 0.2. The genetic distance (cm) between SNP marker pairs was based on Barley Integrated Map 08/05/11 (http://www.harvest-web.org).

Model Comparison of Association Mapping. The generalized and mixed model in TASSEL[29] was used to identify markers associated with traits. Besides the naïve model without correction for population structure, correction with Q model (Q matrix inferred ancestry from Structure), PCA statistical models (the same dimension as Q), K model (K kinship matrix), and also Q+K and P+K were applied in the general linear and mixed linear regression models as recommended by Yu et al.;[30] phenotypic data were also incorporated into the process to compare the model based on Bayesian information criterion (BIC)[31] and on the P−P plot. Kinship between two individuals was estimated as pairwise correlation based on standardized genotypes with SpageDi 1.3,[32] and all negative values were set to zero according to the method of Yu et al.[30]

The selected model was then used to map SNP markers associated with RS significantly ($P < 0.01$) with TASSEL 3.0. Allelic effects of a particular SNP locus were compared in the Mixed procedure. The −Log(P) values were plotted using SPSS 15.0 (SPSS Inc.) to visualize where significant markers were located across the genome. Significant markers were declared at the P = 0.01 threshold.

Synteny to Rice and Brachypodium Genomes. The barley SNPs defining the QTL were located using genome zipper[33] and Strudel,[34] and the syntenic chromosomal region within five genes in rice was searched for likely candidate genes using the genome browser in Gramene (http://www.gramene.org). Probabilistic functional gene networks of candidate genes in starch metabolite were integrated on the basis of the data from Ricenet (http://www.functionalnet.org) and RiceCyc and BrachCyc (http://pathway.gramene.org).

RESULTS AND DISCUSSION

RS and β-Glucan in the Barley Collection. Among the 209 barley cultivars, 35 cultivars were 6-row and the rest were 2-row types. RS content showed left-skewed distribution (Figure 1A) and varied from 0.07 to 15.48% in all analyzed cultivars, 13 varieties having high RS content (>11%) and 15 varieties below 1% RS content (Figure 1B). The distribution of RS showed no relationship to geographic origin (data not shown) or row type; the average RS contents in 2-row and 6-row barley were 5.61 and 5.22% with CVs of 0.61 and 0.64%, respectively, which were consistent with the variances in the whole panels. The broad-sense heredity of RS content in the barley samples was 77.96%. The high variation and relatively even distribution of RS content (Figure 1A) among the complete barley collections or the 2- and 6-row barley cultivars made it possible to carry out the GWAS. The average RS content was higher than that reported by Mikulikova et al.[35] (average RS content of 2.51% in spring barley). However, those authors studied RS_3 that formed after degradation, and we measured RS_2 that exists in raw food. For RS_2, Emani et al.[36] found values about 17% in normal barley. The relatively high heredity of RS content could be expected as RS content mostly depends on the amylose/amylopectin ratio. Also, in rice and potato, it has been found that starch digestibility was mainly affected by genotypic variation.[37,38]

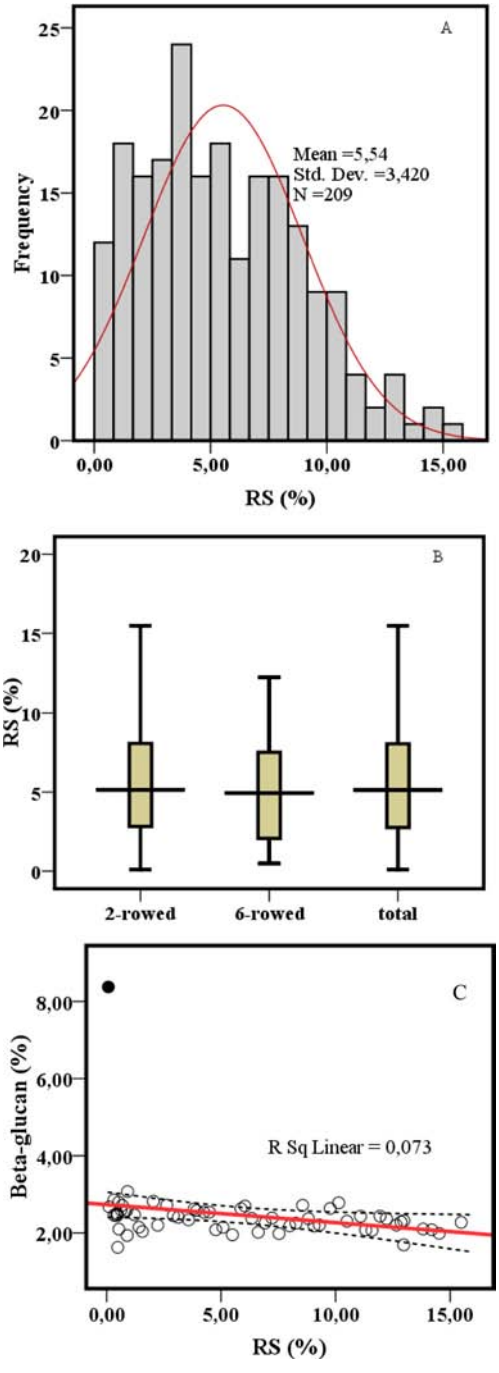

Figure 1. Distribution of RS in the complete population and relationship to β-glucan content in selected 61 varieties: (A) histogram of the RS content overlaid with the resulting normal distributions; (B) box plot of RS content for 2-row types, 6-row types, and the complete population of spring barley lines; (C) correlation between RS content and β-glucan content in varieties with extreme (13 varieties higher than 11% and 16 varieties lower than 1%) and medium RS contents (32 varieties) (mutant Risø17 is shown as a solid circle).

β-Glucan was quantified in 61 varieties including varieties with extremely low, exstremely high, and medium RS contents (Supporting Information, Table 1S). The 13 varieties with high RS (>11%) showed a β-glucan content of 1.76−2.54% and the 15 with RS <1% had a β-glucan content ranging from 1.7 to 3.2%, which could indicate a negative correlation between RS and β-glucan content; however, this correlation was not statistically significant ($r = -0.27$, $p = 0.053$). The isoamylase mutant Risø 17[39] had 0.08% RS and 8.38% β-glucan and was thus completely different from all of the barley varieties in the collection (Figure 1C).

β-Glucan is the principal NSP of the barley endosperm cell wall, whereas arabinoxylan or pentosans are the major NSP in most other cereals and the cell walls of barley grain tissues other than the endosperm. β-Glucan shows potential effects in reducing glycemic index (GI) and serum cholesterol levels, flattening the postprandial blood glucose levels and insulin rises.[40] Whereas the ability of β-glucan to lower the GI was dependent on barley starch type, the GI-increasing effect by waxy starch could counteract the GI-reducing action by β-glucan.[41] The β-glucan content was higher in waxy- and increased-amylose genotypes and positively affected the slowly digested starch,[5] whereas Huang et al.[11] and Lee et al.[42] found that there were negative correlations between amylose and β-glucan content. More precisely, they actually claimed a negative correlation between RS_3 and β-glucan, and in the present study RS_2 (and not RS_3) was measured, thus leading to a different but not necessarily conflicting conclusion.

Statistical Prediction of Population Structure. Four subgroups were obtained by Structure 2.3.3 based on log probability Ln $P(D)$ and the first derivative of log probability $L'(K)$. One of the groups (pop4) included the 6-row barley lines, whereas the other three included the 2-row lines. The genetic structure of 209 barley accessions was explored by combining the results of Structure and PCA. The two-dimensional scatter plots involving all accessions displayed two main subpopulations by PC1 accounting for 15.54% variance (Figure 2A), dividing the varieties into the plants in the Structure groups pop4 (the 6-row barley) and the other Structure populations, whereas PC2 and PC3 revealed the other groups resulting from the Structure analysis (Figure 2B). Thus, the result of four groups in the core barley germplasm was confirmed. A similar population structure had also been found by Pasam et al.[27] The Q values from Structure for $K = 4$

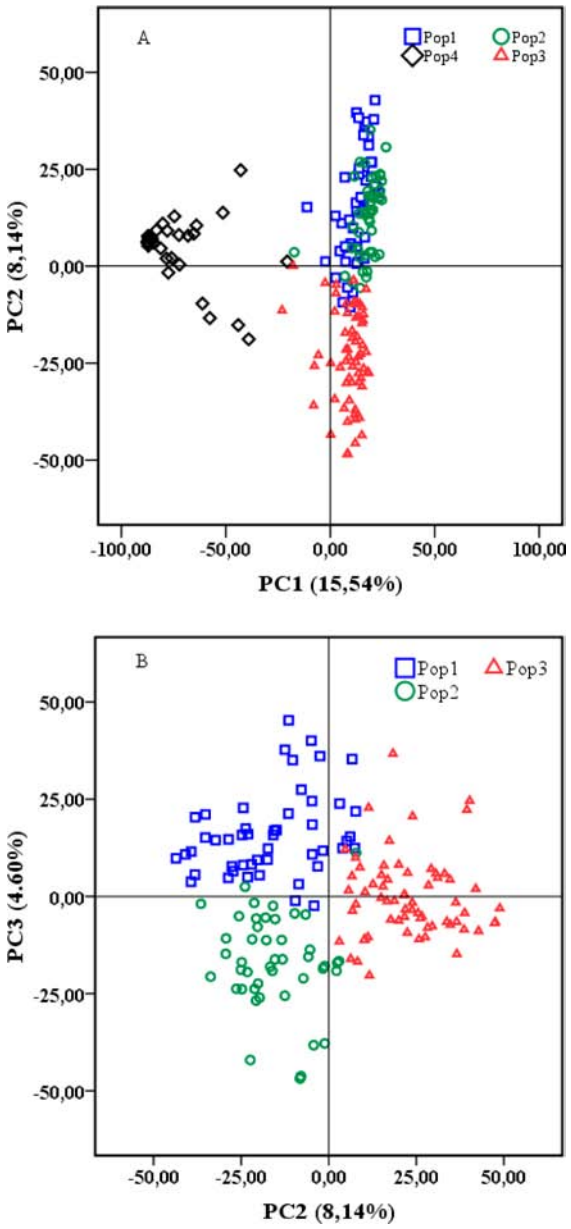

Figure 2. Population structure obtained by STRUCTURE 2.3.0 explored by PCA plot with the first two PCs within all core germplasms (A) and with PC2 and PC3 for only 2-row barley genotypes (B).

were then used as cofactors for the linear or mixed model in the following association analysis.

Although there were four subgroups identified according to different methods, the biggest differences existed primarily between 6-row and 2-row type barleys, which is in accordance with the results of other experiments.[26] Within the 2-row or 6-row type panel, the individuals were closely related to each other, presumably because they all originated from Europe, and for 2-row barley, the breeding programs aim mainly at malting, thus quality-restricting genetic diversity.

Linkage Disequilibrium Analysis. From almost 9000 SNP analyzed in this experiment, 6810 showed polymorphism in the selected population. SNPs with a minor allele frequency (MAF) of <0.1 and no accurate location were excluded from subsequent LD analyses, resulting in a reduced set of 5325 informative assays. Genome-wide LD structure in the 209 barley varieties showed high LD values across a wide range of the genome with 43.6% of the significant pairwise marker associations ($P < 0.001$) being interchromosomal. When analyzed within the four groups identified by Structure, the proportion of significant interchromosome marker-pair association reduced to <18% in pop1, pop2, and pop3, and only 4.3% in pop4, whereas the significant LD between linked marker pairs remained similar (Table 1). The lower portion of

Table 1. Linkage Disequilibrium (LD) Pairwise Pattern of 6810 SNP Markers with All Samples and within 2-Row Barley Genotypes

group	marker pairs with significant LD ($P < 0.001$) (%)	mean of LD in marker pairs with significance (R^2)	marker pairs with linkage ($r^2 > 0.2$) (%)	mean of LD in marker pairs with linkage (R^2)
pop4 (6-row)	4.3	0.841	23.21	0.536
pop3	17.92	0.62	25.24	0.598
pop2	17.75	0.631	23.14	0.558
pop1	17.5	0.614	21.87	0.558
whole	43.59	0.325	26.46	0.515

significant LD in pop4 might be due to a small group size with only 26 accessions. The extent of LD in the analyzed barley population was around 6 cM on average and ranged from 3 to 15 cm within the chromosome. Those findings are consistent with the previous reports that average LD ($R^2 = 0.2$) decayed over a distance of 5−10 cM.[27]

The reduction of the interchromosomal marker LDs showed the importance of considering the population structure as the higher LD in the whole panels was mostly due to the influence of population structure. Population structure can result in biased allele effects and inflate the LD, which result in spurious correlations in association mapping.[43] The fact that LD stretched from 3 to 15 cM in this population showed that the marker density of 5.3 markers per centimeter was sufficient.

Marker-Trait Association and Model Comparison for Association Analysis. A single marker association (naïve model) test was first carried out to scan for markers showing significant association with RS variation in the 209 barley samples. Five structure corrections, *K*, *Q*, PCA, *Q*+*K*, and PCA +*K*, were applied to separate the genuine associations from background noise in the genome-wide mapping with all samples.

Different predictors were called by different models (Supporting Information, Table 2S) and, as expected, structure correction decreased the number of associations due to the exclusion of false positives.[20] Nevertheless, this process may also reduce the power to detect true positives,[41] and it was therefore important to find a model optimally balanced. Therefore, the relative performance of each association mapping model was evaluated on the basis of the BIC and the fitting plot (observed against expected probability, PP plot). Among six models, the *Q* model showed the smallest BIC value and also the best fitting in the PP plot (Figure 3). The naïve with the smaller BIC value showed a poorer PP plot fitting. The model that used the kinship table had a higher BIC and in addition a skewed PP plot. Therefore, only the significant associations called by *Q* model were considered. Nevertheless, the significant associations obtained with the other models are also shown in Table 2S of the Supporting Information.

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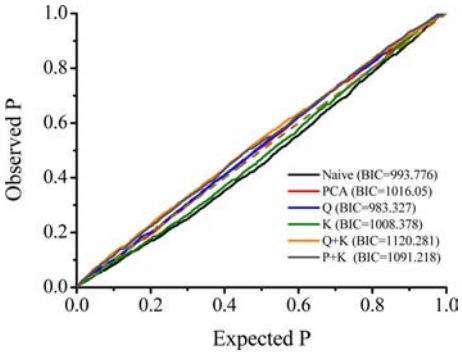

Figure 3. Plots of observed versus expected *P* value for naïve, kinship (*K*), *Q*, PCA, *Q* installed with kinship (*Q*+*K*), and PCA installed with kinship (PCA+*K*) models. The Bayesian information criterion (BIC) is also presented.

The choice of the *Q* model as the optimal model in our case did not agree with some other studies on cross-pollinated plants and humans,[30,31] where the relatedness among accessions in a population was quite complex because of the mating style. Bradbury et al.[44] found that kinship-based correction performed as well as or better than other models with barley CAP genotypes. In contrast, Li et al.[45] found that kinship did not improve the model for association mapping of rice's traits, and Shao et al.[46] found also that the *Q*+*K* model performed similarly to the *Q* model alone in a rice panel. These contradicting findings showed the importance of optimizing the model used for the specific cases under analysis; a single correction model might not be the best solution to fit all cases.

Mapping Results and Allelic Effects. To determine whether the inclusion of both 6-row and 2-row barleys had an effect on the detection of associations, the LD analysis was carried out for both the whole panel and the panel of 2-row types alone. Although the results varied slightly between these two approaches, the most significant associations were located in the same regions on chromosomes 2H, 3H, 5H, and 6H (Figure 4).

With the selected *Q* model, 40 SNPs were identified having significant marker-trait associations at $-\mathrm{Log}(P) > 2$ ($P < 0.01$) level. The most significant associations were detected in the

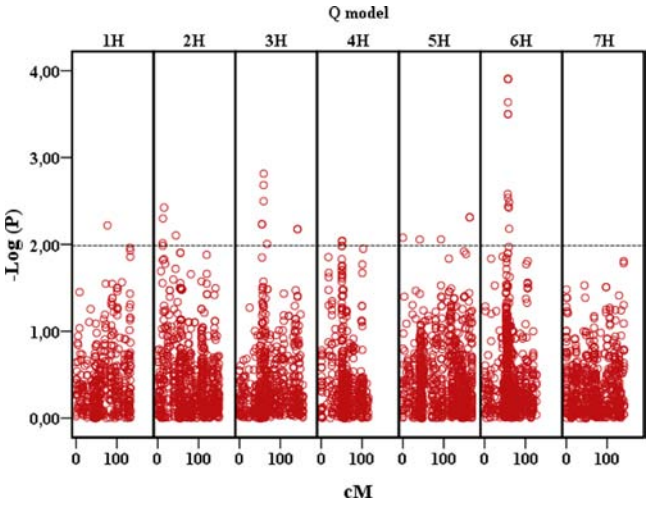

Figure 4. Associations across the whole barley genome called by *Q* model.

regions located around 12−16, 55−76, 93.4, and 56−61 cM on chromosomes 2H, 3H, 5H, and 6H, respectively (Table 2). The possible QTL regions mapped here were close to the QTLs for starch content, which were mapped on chromosomes 3H, 5H, and 6H around the regions at 51.73−55.57, 110.26, and 71.08 cM, respectively, reported by Pasam et al.[27] Wang et al.[47] also found a major QTL on chromosome 3H around a similar position controlling starch pasting properties.

Individual SNPs accounted for only a small portion of phenotypic variation (low marker R^2), and the highest *P* value was 0.000124 before Bonferroni correction. The lowest trait mean and highest effect were observed in the allele with the lowest frequency (BOPA2_12_10803, allele C) (Table 2). The *P* values were not as high as found in other studies,[25,26] but comparable with those found by Pasam et al.[27] Whereas the pilot GWAS studies as proof-of-concept studies were often carried out on less complex traits, GWAS has its greatest advantage in rather complex traits where the effect of single genes might be less important.[48] The remaining heritability not explained by the found QTLs may be explained by other factors such as rare variants, epistasis, epigenetics, and genotype−environment interactions, but may also just imply that complex traits truly were affected by thousands of variants of small effect.[49] Integrating GWAS and linkage mapping with biparental population might be useful to isolate the major genes, and targeted resequencing efforts paired with large-scale genotyping will increase estimates of complex trait heritability explained by known loci.[50]

Gene Discovery Using Synteny to Rice and Brachypodium Genomes. A search of genes that might be functionally related to resistant starch for SNPs with detected associations was carried out by taking advantage of the synteny between barley and the sequenced rice and *Brachypodium* genomes, respectively. Orthologous genes and genes located close to the orthologues of the SNPs (<5 genes apart from SNP in question), which could be related to carbohydrate metabolites, were considered as putative associated genes (Table 3). SCRI_RS_127140 on 3H was orthologous to rice gene LOC_Os01g52260 encoding a putative serine acetyltransferase, whereas a putative starch synthase gene LOC_Os01g52250 is just next to it. Another significant association at 93.4 cm on 5H (SCRI_RS_147762) was orthologous to a putative glycosyl hydrolase family 29 gene (GH29) (LOC_Os09g34920). Furthermore, one association resulting from the naïve model at the same position (SCRI_RS_159430) tagged an orthologoue to the rice gene LOC_Os09g34214, which codes for a protein containing a UDP-glucosyl transferase domain. A further three adjacent genes were also found to code for UDP-glucosyl transferases (Table 3).

On chromosome 6H several significant predictions were located within 56−61 cM; all rice orthologues of these predictions were located on rice chromosome 2 from 20989673 to 26404857 bp. Within this region, <5 genes away from the orthologous, 3 genes (LOC_Os02g35020, LOC_Os02g41520, LOC_Os02g42280) might be involved in carbohydrate metabolism. Although the orthologous rice genes of the associated SNPs on chromosome 2H are located on different rice chromosomes, each is located close to a gene participating in carbohydrate metabolism (Table 3). The syntenic genes to *Brachypodium* belonged to the same gene ontology as those of rice (Table 3), and most also code for the glycosyl hydrolyase and glycosyl transferase families.

Table 2. SNPs Associated with the Highest Possibility Called by Q4 Model and the Allelic Effect, Analyzed with Only the Effect Alleles Have Lower Frequency

SNP	Ch	position (cm)	*P* value	R^{2a} (%)	allele	percentage	trait mean (%)	effect	Se
SCRI_RS_231806	2H	12.11	0.0097	3.6	C	74.2	5.34		
					T	25.8	6.33	0.928	0.338
SCRI_RS_155957	2H	12.75	0.00503	4.22	A	70.6	5.62		
					C	28.9	5.54	0.858	0.33
SCRI_RS_66401	2H	15.44	0.00377	5.35	A	41.2	5.24	0.678	0.251
					G	58.8	5.98		
SCRI_RS_127140	3H	59.14	0.00153	3.69	T	51.1	6.25		
					G	48.9	1.21	−0.712	0.252
SCRI_RS_207408	3H	67.63	0.00989	5.01	T	82.9	5.78		
					C	17.1	4.36	−0.776	0.274
SCRI_RS_147762	5H	93.40	0.00875	4.83	T	67.2	6.04		
					G	32.8	4.66	−0.718	0.27
SCRI_RS_159430	5H	93.40	0.00225	7.15	T	68.1	5.08		
					G	31.9	6.71	0.817	0.267
BOPA2_12_30144	6H	56.66	0.00227	7.74	A	79	5.62		
					C	21	4.1	−0.991	0.304
SCRI_RS_114613	6H	57.22	0.000231	6.85	T	65.6	6.09		
					G	34.4	4.66	−0.819	0.277
SCRI_RS_140091	6H	57.22	0.000124	7.74	A	66.7	6.11		
					G	33.3	4.57	−0.858	0.276
SCRI_RS_153707	6H	57.22	0.000316	6.85	A	66.7	6.06		
					G	32.3	4.62	−0.8	0.278
SCRI_RS_174583	6H	57.22	0.000124	7.74	T	77.7	6.11		
					G	33.3	4.57	−0.858	0.276
SCRI_RS_187697	6H	57.22	0.000316	4.49	A	66.7	6.06		
					G	32.3	4.62	−0.8	0.278
SCRI_RS_222944	6H	57.22	0.000124	4.62	G	66.7	6.11		
					T	33.3	4.57	−0.858	0.276
BOPA2_12_10803	6H	59.63	0.00378	4.00	A	94.1	5.77		
					C	5.9	2.76	−1.531	0.532
BOPA2_12_10758	6H	60.2	0.00328	3.6	A	77.4	6.01		
					C	22.6	4.18	−0.917	0.296
BOPA2_12_31444	6H	60.2	0.0066	4.22	G	78.3	6.01		
					A	21.7	4.27	−0.87	0.303

[a] R^2 indicates the variations explained by the markers.

Association genetics was successfully used to map yield, yield stability QTL, resistance, and other traits of agronomic relevance in European spring barley using AFLP markers.[51] In this experiment two of the rice gene orthologues of the associated SNPs contribute directly to the carbohydrate metabolism; all other candidate genes were adjacent to other orthologous. These findings can be expected to result from a relatively low resolution due to the high LD found in this population. Nevertheless, these associations can be used to develop markers and are a starting point for the isolation of these genes. Better resolution can be found in barley populations with higher genetic variability as landraces or wild barley.[25]

Putative Functions of Candidate Genes. Genes obtained by the association scan described above were considered to be candidate genes for controlling RS content, and the encoded enzymes located in the carbohydrate metabolic pathway (Figure 5) almost all participated in regulating starch

Table 3. Synteny to Rice and Brachypodium Genes[a]

marker	Ch	position (cm)	−Log(P)	orthologous to rice genes	putative function	orthologous to bachypodium	putative function
SCRI_RS_231806	2H	12.11	2.01	LOC_Os03g20290	aspartic proteinase nepenthesin-1 precursor	Bradi3g61060	aspartic proteinase nepenthesin-1 precursor
				LOC_Os03g20300	**glucose-6-phosphate 1-dehydrogenase, chloroplast precursor,**		
SCRI_RS_155957	2H	12.75	2.3	LOC_Os09g36000	expressed protein	Bradi5g03840	expressed protein
				LOC_Os09g35970	**6-phosphogluconolactonase, putative**	**Bradi3g21020**	**UDP-glucoronosyl and UDP-glucosyl transferase domain containing protein**
SCRI_RS_66401	2H	15.44	2.42	LOC_Os10g05980	POEI13, pollen Ole e I allergen and extensin family protein precursor	Bradi3g21000	POEI11, pollen Ole e I allergen and extensin family protein
SCRI_RS_127140	3H	59.14	2.82	LOC_Os01g52260	serine acetyltransferase protein	Bradi2g48430	serine acetyltransferase
				LOC_Os01g52250	**starch synthase**	**Bradi2g48457**	**glycosyl hydrolyase family 3 protein**
SCRI_RS_207408	3H	67.63	2.01	LOC_Os01g56180	expressed protein	Bradi2g51010	expressed protein
				LOC_Os01g56130	CSLC1, cellulose synthase-like family C, expressed	Bradi2g50980	arabinasnase
SCRI_RS_147762	5H	93.40	2.06	LOC_Os09g34920	glycosyl hydrolase family 29	Bradi4g35500	glycosyl hydrolase family 29
SCRI_RS_159430	5H	93.40	2.65	LOC_Os09g34214	UDP-glucoronosyl and UDP-glucosyl transferase domain containing protein	Bradi4g35350	UDP-glucoronosyl and UDP-glucosyl transferase domain containing protein
				LOC_Os09g34230	**UDP-glucoronosyl/UDP-glucosyl transferase, putative**	**Bradi4g35342**	**UDP-glucoronosyl and UDP-glucosyl transferase domain containing protein**
				LOC_Os09g34250	**UDP-glucoronosyl and UDP-glucosyl transferase domain containing protein**	**Bradi4g35356**	**UDP-glucoronosyl and UDP-glucosyl transferase domain containing protein**
				LOC_Os09g34270	**UDP-glucoronosyl and UDP-glucosyl transferase domain containing protein**		
SCRI_RS_187697	6H	57.22	3.5	LOC_Os02g34990	ACT domain containing protein	Bradi3g20207	ACT domain containing protein
				LOC_Os02g35020	**glycosyl transferase**		
BOPA2_12_30144	6H	56.66	2.58	LOC_Os02g41580	CAMK_CAMK_like.14, CAMK includes calcium/ calmodulin-depedent protein kinases	Bradi3g49220	CAMK_CAMK_like.14, CAMK includes calcium/ calmodulin-depedent protein kinases
				LOC_Os02g41520	**glycosyl transferase 8 domain containing protein**	**Bradi3g49197**	**glycosyl transferase 8 domain containing protein**
						Bradi3g49460	***β*-galactosidase precursor**
SCRI_RS_182180	6H	57.22	2.54	LOC_Os02g42200	transcription factor-related	Bradi3g49467	transcription factor-related
				LOC_Oso2g42280	**UDP-glucoronosyl/UDP-glucosyl transferase family protein, putative**	**Bradi3g49560**	**UDP-glucoronosyl and UDP-glucosyl transferase domain containing protein**
SCRI_RS_222944	6H	57.22	3.91	LOC_Os02g42300	expressed protein	Bradi3g49580	expressed protein

[a]Genes orthologous to the associated SNPs and the genes apart from the orthologues less than 5 genes that might participate in carbohydrate metabolism are shown in boldface, and putative functions are also listed.

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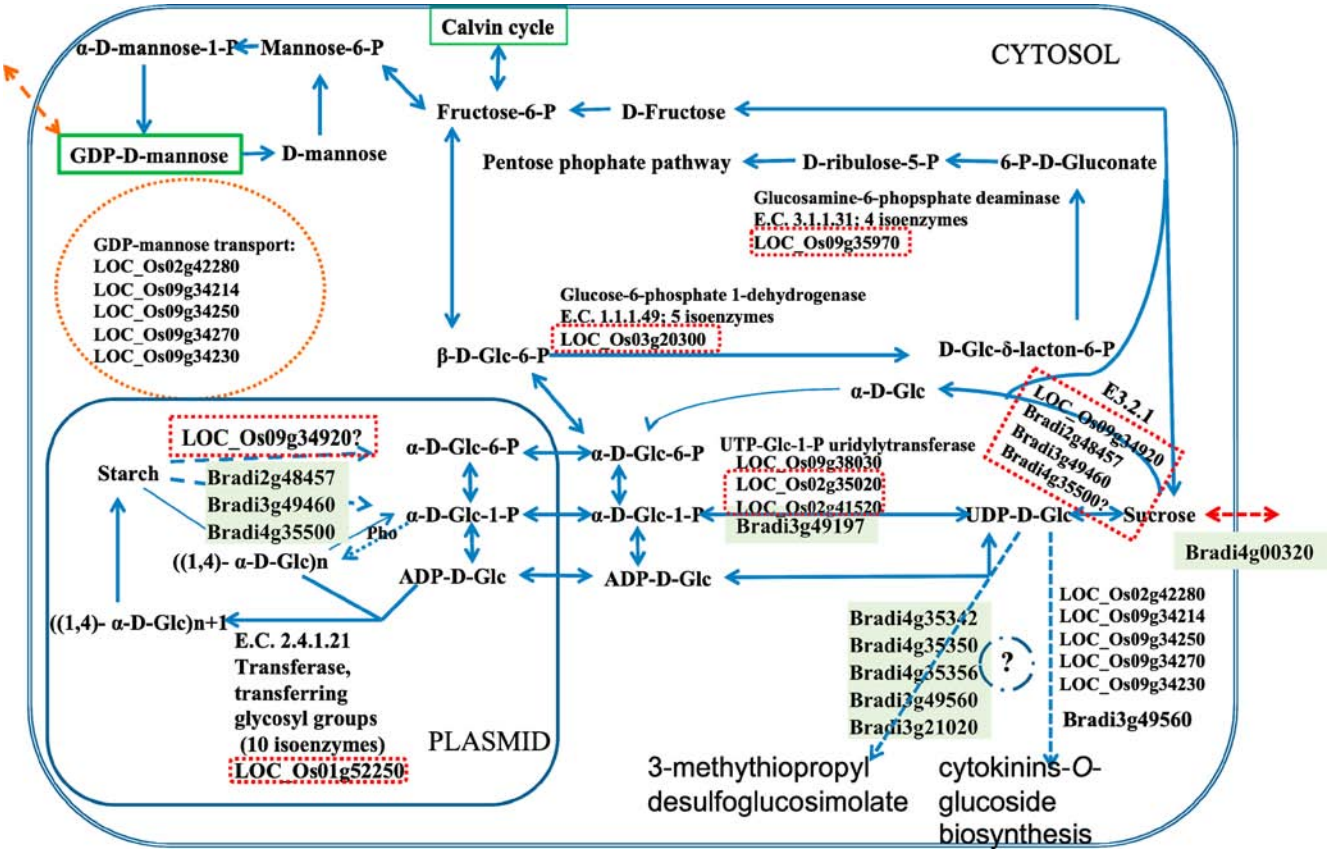

Figure 5. Possible functions of candidate genes orthologous to rice and *Brachypodium* in starch metabolism pathway. Genes in dashed or shaded rectangles and circles were the candidate genes.

biosynthesis directly or indirectly through different pathways (Figure 5). The starch synthase IV gene LOC_Os01g52250 participates directly in the starch biosynthesis and acts on synthesizing 1,4-glucan (amylose) from ADP-glucose (Figure 5). LOC_Os02g35020 and LOC_Os02g41520 located close to the orthologoues of SCRI_RS_187697 and BOPA2_12_30144 on chromosome 6H, respectively, belong to the nucleotide-diphosphosugar transferase superfamily, and the resulting proteins putatively contribute, similarly to LOC_Os09g38030, to the sucrose synthesis and degradation.

The orthologous gene LOC_Os09g34214 of SCRI_RS_159430 on chromosome 5H and its adjacent three genes all belong to the UDP-glycosyltransferase/glycogen phosphorylase superfamily (EC 2.4.1), which putatively contributes to the downstream regulation of UDP-glucose. The possible functions of these genes are transferring glycosyl from ADP-glucose such as starch synthase or transferring glycosyl units from glucose-1-P to α-1,4-D-glucan chains such as starch phosphorylase, so they might also have a function to form polyglucans such as amylose or participate in sucrose metabolism. Furthermore, according to their gene function ontology, they have putative functions as mannose transporters and could regulate RS formation through two mechanisms: (1) GDP-mannose is transported from the extracellular space to the cytosol to synthesize cell wall. Cell wall without breakdown can detain α-amylase penetration and reduce/hinder starch digestion. (2) GDP-mannose is involved in the mannose cycle and regulates the concentration of fructose-6-phosphate, which plays a role in starch and sucrose metabolite.

The syntenic genes of two associated SNPs located on chromosome 2H located on different rice chromosomes, whereas LOC_Os03g20300, adjacent to the orthologues of SNPs and LOC_Os09g35970, located two genes apart from the orthologues. Both are involved in the pentose phosphate pathway (oxidative branch) (Figure 5) and might act on starch synthesis by regulating the concentration of β-D-glucose-phosphate.

The syntenic genes in *Brachypodium* encoding UDP-glycosyl transferase proteins (Table 3) are believed to belong to the downstream pathways of UDP-glucose such as the synthesis of 3-methylthiopropyl-desulfoglucosimolate according to BrachyCyc (Figure 5). However, they might also function by catalyzing sucrose or polyglucans biosynthesis from UDP-D-glucose like other genes within the same family and participate in all pathways involving those reactions such as sucrose or starch synthesis. The candidate genes identified by synteny either to rice or to *Brachypodium* mostly belong to the glycosyl transferase and hydrolase families.

The activity of cytosolic AGPase is higher than that of plastidial AGPase in barley, accounting for 85% of the total activities; there might be alternate pathways of the flux of carbon to starch in barley endosperm.[52] UDP-glucose in cytosol derives majorly from sucrose degradation and can be transformed into ADP-glucose directly or through 1-P-Glc indirectly. Starch synthesis and cytokinin biosynthesis might be coregulated;[53] the candidate genes responsible for sucrose metabolism and UDP-glucose metabolism obtained in our GWAS might regulate RS metabolism through altering the concentration of ADP-glucose.

Although the expression of two SSIV genes was observed in rice endosperm during grain filling,[54] not much is known about the contributions of starch synthase IV to glucan chain extension.[55] It might be required for the initiation of starch granules,[56] whereas future identification and characterization are needed to establish the exact role of SSIV. As RS also is a certain kind of starch, the factors affecting starch synthesis should have influences on RS. Down-regulation of SSIIa and SBEIIb genes in barley,[6] maize,[15] and rice[16] can result in increased amylose and/or modified amylopectin structure, which then results in high RS. Furthermore, the changed activities of these enzymes also affected other starch biosynthetic enzymes, which might also play a role in RS. Due to the complex interaction among different enzymes and network regulation among different pathway branches, many factors besides the enzymes participating in the pathways might also be involved in starch biosynthesis; a single step of the pathway can alter the metabolite network as a whole.[53]

With the help of GWAS, we identified 40 SNP markers on chromosomes 2H, 3H, 5H, and 6H, respectively, and obtained 10 candidate genes by synteny that might participate in the metabolism of resistant starch. The associated SNPs on chromosome 5H map to genes directly participating in starch metabolism, and additional associated SNPs are located close to candidate genes related to starch metabolism; the candidate genes regulated the metabolism of resistant starch through different ways. These results show the feasibility of GWAS in cereal crops to effectively identify genetic regions of interesting traits.

■ ASSOCIATED CONTENT

Supporting Information

Additional tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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