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Whole genome association mapping identifies naked grain locus *NUD* as determinant of β-glucan content in barley

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Abstract

ß-glucan content in barley grain is an important component in determination of seed quality and end use. While high ß-glucan content is considered beneficial for human food, it is undesirable in malting barley and feed. Association of ß-glucan content in grain of 89 barley accessions of Latvian origin including 22 hulless accessions with 1273 high quality genome wide Single Nucleotide Polymorphism markers was tested. P-values were calculated using mixed linear model combining Q matrix and K matrix by computer program *Tassel*. P-values were adjusted using Bonferroni correction. Significant associations were identified for three markers 1_0673 (114.58 cM), 2_0880 (116.68 cM) and 1_1445 (116.68 cM) on chromosome 7H bin 7. Recessive mutation in the *NUD* locus also located on chromosome 7H bin 7, which determines hulless barley phenotype and has been previously suspected to affect ß-glucan content, was also genotyped and included in the association analysis. A significant marker – trait association was found identifying *NUD* gene as a major source of variation for ß-glucan content in our association mapping panel.

Key words: association mapping, barley, ß-glucan content, hulless barley, *NUD* gene.

Introduction

ß-glucan ((1-3, 1-4)-ß-D-glucan) is a cell wall polysaccharide. In barley it is mainly found in endosperm and subaleurone layer. Amount of ß-glucan in the barley grain determines its end use. Grain with elevated levels of ß-glucan content is favorable for inclusion in human diet as it has the potential to reduce serum cholesterol level (Ames, Rhymer, 2008). Increased content of ß-glucan reduces grain digestibility and therefore is not recommended for feed (McNab, Smithard, 1992). Grain with elevated levels of ß-glucan content is also not recommended for the malt production as it reduces the quality (Wang et al., 2004).

Amount of ß-glucan in barley may differ in accessions which vary in major morphological traits. ß-glucan content in grain is higher in accessions with hulless grain, short awns and waxy endosperm (Fastnaught et al., 1996). Each of these traits is controlled by single genes *nud* (hulless grain), *lks2* (short awns), *waxy* (waxy endosperm) respectively (Swanston, 1995). Barley with different spike morphology (two-row and six-row) does not differ in ß-glucan content (Fastnaught et al., 1996). Recently, the *NUD* gene was cloned, and molecular marker became available for genotyping of hulless barley varieties carrying the *nud* allele (Taketa et al., 2008).

ß-glucan content in grain may be influenced by environmental conditions, such as cropping system and growing season (Ehrenbergerová et al., 2003). Higher ß-glucan content is observed in low-moisture, hot years (Fastnaught et al., 1996; Ehrenbergerová et al., 2008).

Previously, several quantitative trait loci (QTL) for ß-glucan content have been mapped in four bi-parental populations. In Steptoe × Morex (Han et al., 1995) QTLs were mapped on chromosomes 1H and on two locations on 2H, in Beka × Logan (Molina-Cano et al., 2007) QTLs were found on chromosome 1H, 5H and 7H, in CDC Bold × TR251 (Li et al., 2008) – chro-

mosomes 2H, 3H, 5H and 7H, and in Yonezawa Mochi × Neulssalbori (Kim et al., 2004) on two locations on chromosome 7H.

Association mapping has been proposed as an alternative method to bi-parental mapping to locate genes of interest in the genome. Association mapping tests for marker – trait association in a population of varieties, thus, potentially sampling much higher diversity of traits. Whole genome association (WGA) mapping analyzes genome wide marker data set for association with a given trait. A prerequisite for WGA mapping is availability of molecular marker systems that allow for efficient high throughput genotyping of a large number of individuals at a large number of loci. Notwithstanding their biallelic nature, single nucleotide polymorphisms (SNPs) are considered as particularly useful as molecular markers, due to their abundance and amenability to high throughput analyses (Kruglyak, 1997). Several re-sequencing efforts, e.g. by Rostoks et al. (2005), and expressed sequence tag data from different barley varieties identified a large amount of barley SNPs allowing to establish a high throughput genotyping platform for barley using Illumina Golden Gate technology (Rostoks et al., 2006) and to develop a high density SNP map of barley (Close et al., 2009). For association mapping to be successful, several factors need to be taken into account. First, the extent of linkage equilibrium in a set of germplasm must be known and the marker density must be adjusted accordingly. While in outbreeding species such as maize, the LD decays within less than 1500 bp (Remington et al., 2001), in local populations of inbreeding plant species, e.g. *Arabidopsis thaliana*, the LD can persist to a centimorgan distance (Nordborg et al., 2002). In cultivated barley LD extends to 1–2 cM depending on a set of germplasm (Caldwell et al., 2006; Rostoks et al., 2006), thus, a relatively modest number of SNPs may be required for WGA mapping. Second, population structure is an important factor in association mapping because it can lead to type I and type II errors. To remove confounding effects in association mapping, it is suggested to use mixed linear model in which information on population structure (Q-matrix) and differences in genetic relatedness (kinship or K-matrix) is included (Zhao et al., 2007). Several methods have been developed to reduce possibility of finding false marker-trait associations (type I error) which arise in multiple testing, of which the most conservative is Bonferroni correction (Balding, 2006). Here we report whole genome association mapping of ß-glucan content QTL in a set of Latvian barley varieties and breeding lines that were genotyped with 1536 genome-wide SNPs and a marker for naked caryopses (*nud*) gene. Highly stringent mapping procedure identified a QTL on chromosome 7H that coincided with the mutation in barley *NUD* gene.

Materials and methods

Plant material and field trials. Ninety five Latvian spring barley accessions were studied including 22 hulless two-row accessions and 73 hulled accessions, which included 67 two-row and six six-row accessions. All six-row accessions were excluded from the further analysis because they appeared to be genetically distinct from two-row accessions (data not shown). Accessions were grown in State Priekuļi Plant Breeding Institute (SPPBI) and State Stende Cereal Breeding Institute (SSCBI) for three seasons in 2007–2009, arranged by a randomized complete block design grown in three replicates in 2 m^2 field plots. Plants were grown in sod-podzolic or sod-podzolic gley soil, sandy loam at SPPB, and in sod-podzolic soil at SSCBI with fertilization (N 80 kg ha^{-1}, P 40–53 kg ha^{-1}, K 67 kg ha^{-1}). Both locations (SPPBI – 57°19′ N, 25°20′ E, 115 meters above sea level; SSCBI – 57°12′ N, 22°33′ E, 78 meters above sea level) differ markedly in environmental and agronomic conditions. Total received precipitation during growing season was 291.3, 230.0 and 327.8 mm at SPPBI and 281.6, 292.6, and 352.4 mm at SSCBI in years 2007, 2008 and 2009 respectively, and there was large variation in the amount of precipitation in each location and each season. Average temperature during growing season was 14.69, 14.08 and 14.19°C at SPPBI and 14.44, 13.25 and 13.68°C at SSCBI in years 2007, 2008 and 2009 respectively.

ß-glucan content in grain was determined using Near Infrared Transmittance grain analyzer "Infratec 1241" ("Foss", Denmark) which was calibrated using grain in which ß-glucan content was determined using commercial kit provided by "Megazyme" (catalogue number K-BGLU; Ireland).

Genotyping. DNA for genotyping was extracted from leaves of a single plant using "DNeasy Plant Min Kit" ("Qiagen", Germany). Illumina high-throughput genotyping was done as described (Rostoks et al., 2006). Barley oligo pooled assay, BOPA1, contains 1536 SNP selected from pilot assays (Close et al., 2009). Data was controlled for presence of excessive number of heterozygous loci, missing data points and potential null alleles as previously described (Rostoks et al., 2006). Barley consensus linkage map based on the SNP markers (Close et al., 2009) was used throughout the study. Mutation in the *NUD* gene was genotyped according to protocol developed by Taketa et al. (2008).

Statistical analysis and association mapping. Means, standard deviations and analysis of variance of ß-glucan data were calculated using *Microsoft Excel* ("Microsoft", USA). Results of *Anova* were combined over locations and years to get components of variance and to calculate the broad-sense heritability (%) (Gordon et al., 1972).

Association analysis was conducted using *Tassel* software (Bradbury et al., 2007) by implementing either general linear model (GLM) with or without structure (Q) matrix or mixed linear model (MLM) in which both structure (Q) and kinship (K) matrices were included. The default run parameters were used with convergence criterion set at 1.0 x 10^{-4} and the maximum number of iterations set at 200. Markers with minor allele frequency (MAF) less than 10% were removed from analysis.

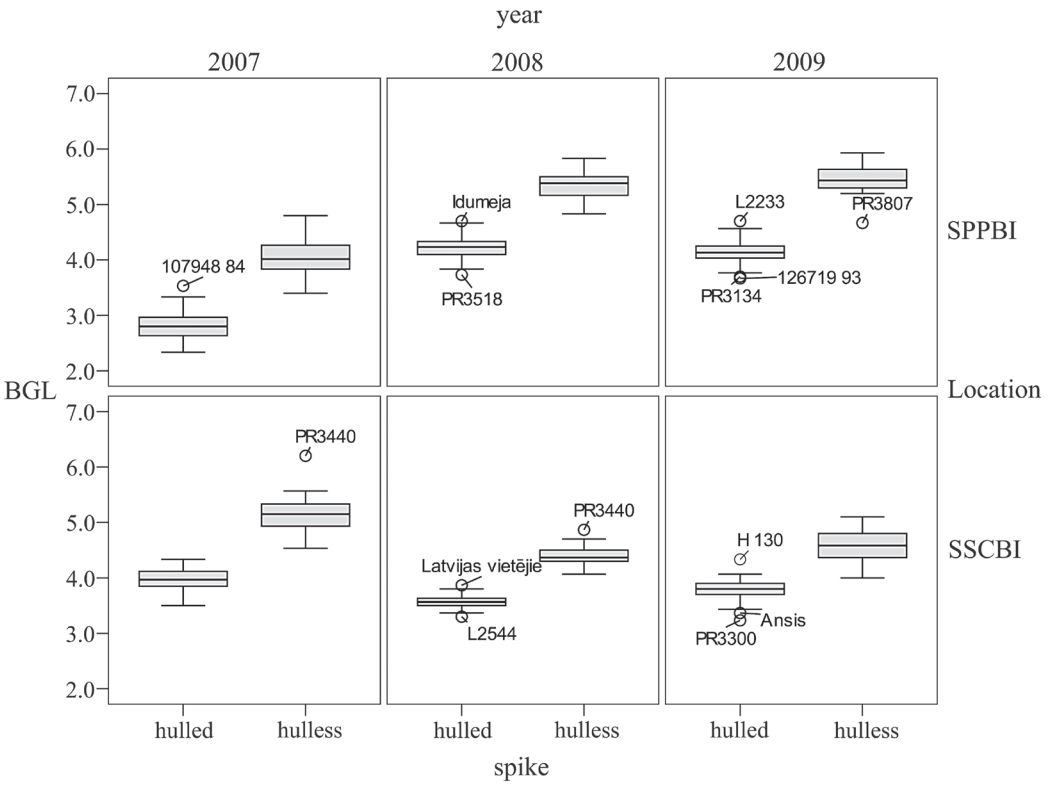
The Q matrix was estimated using software *Structure* (Pritchard et al., 2000). Thirty eight SNPs (five to six per chromosome) were selected based on criteria that they are at least 10 cM apart and have MAF > 0.33. Hypothesis of 1 to 20 clusters (K) was tested using burn-in of 100000 and run length of 200000, and admixture model in 15 iterations. Method developed by Evanno et al. (2005) was used to assign accessions to the most probable number of clusters. Largest value of an ad hoc statistic ΔK was used as an indicator for the true number of clusters. ΔK is based on the rate of change in the log probability of data between successive K values.

Pair-wise relatedness coefficient – "kinship" (K matrix) was estimated either with *SPAGeDi* software (Hardy, Vekemans, 2002) or *Tassel* software (Bradbury et al., 2007) and hereafter is referred to as $K_{spagedi}$ and K_{tassel} respectively.

Bonferroni correction was used to correct for multiple testing, and experiment-wise significance level was set at $p < 0.05$. The number of independent tests was determined in software *Haploview* (Barrett et al., 2005) using SNP tagger application. The criterion for independency was set at $r^2 < 0.8$.

Results

Variation in ß-glucan content. ß-glucan content in the set of 89 two-row barley accessions had a bimodal distribution in all environments except for SPPBI07 which showed a positively skewed distribution. When bimodal distribution was separated in two unimodal distributions, each referred to either hulled or hulless accessions. ß-glucan content was significantly higher ($p = 0.05$) in hulless barley genotypes compared to hulled barley genotypes for each season and location and across seasons and locations. The average ß-glucan content in individual accessions across all seasons and locations ranged from 4.41 to 5.35 in hulless barley and 3.52 to 4.06 in hulled barley. The average content in all hulless accessions was higher is SSCBI in 2007 but lower in 2008 and 2009 compared to SPPBI; the same applies to hulled accessions (Fig. 1).



Notes. Box indicates upper and lower quartile with the horizontal line indicating median, while whiskers indicate 5th and 95th percentile. Outliers are indicated with open circle.

Figure 1. ß-glucan (BGL) content of hulled and hulless barley accessions in State Priekuli Plant Breeding Institute (SPPBI) and State Stende Crop Breeding Institute (SSCBI) in seasons 2007 to 2009

Analysis of variance of ß-glucan content showed that in a pooled set of accessions genotype, season and season × location had the largest effect on ß-glucan content (Table 1), indicating that environmental factors may have a large effect on expression of quantitative traits. Analysis of variance of ß-glucan content showed that interaction of season × location had the largest effect on ß-glucan content, followed by season and location in both hulled and hulless barley. The estimated broad-sense heritability was 0.97 in hulless accessions, 0.37 in hulled accessions and 0.93 in pooled dataset.

Table 1. Sum of squares of the analysis of variance of ß-glucan content

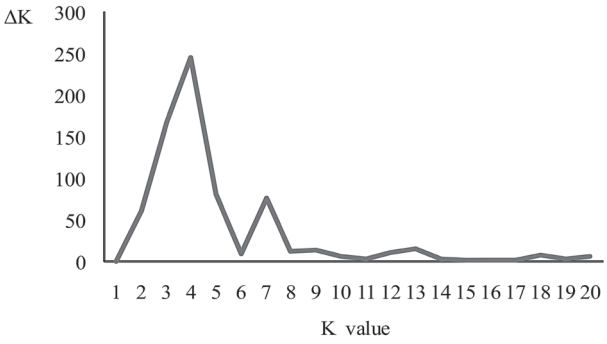
Source of variation	Hulless		Hulled		Total	
	d.f.	sum of squares	d.f	sum of squares	d.f	sum of squares
accession	21	20.849*	66	18.374*	88	393.533*
season	2	12.896*	2	81.502*	2	91.976*
location	1	5.555*	1	1.087*	1	0.071
accession x season	42	3.444*	132	10.688*	176	16.554*
season x location	2	88.227*	2	193.818*	2	279.736*
accession x location	21	2.807*	66	4.997*	88	14.374*
accession x location x season	42	4.953*	132	10.592*	176	17.854*
replicate	2	0.028	2	0.736*	2	0.674
residual	262	9.946	802	20.210	1066	30.246

* – indicate significance at the probability level 0.001

Correlations of ß-glucan content of accessions of pooled dataset among environments were positive and significant and ranged from 0.83 to 0.93. Correlations of ß-glucan content of hulless varieties ranged from 0.31 to 0.86 and all but SPPBI07/ SSCBI07, SPPBI07/ SPPBI08, SPPBI07/ SPPBI09 were significant. Correlations of ß-glucan content of hulled varieties ranged from 0.18 to 0.49 and all but SPPBI07/ SSCBI08, SPPBI07/ SPPBI08 were significant.

Molecular markers and genetic structure. Ninety five Latvian barley varieties and breeding lines were genotyped at 1536 loci using Illumina Golden Gate technology. The resulting 145,920 genotypes were controlled for low quality calls, excessive number of heterozygous loci and putative null alleles (data not shown). After quality control, 1368 (89%) genotype calls remained. Of these loci, 1273 have been positioned on barley consensus map (Close et al., 2009). Detailed analysis of the genetic diversity in Latvian barley and the extent of LD will be reported elsewhere. The genotype data of the mapped loci with MAF > 0.1 were used for association mapping.

Software *Structure* was used to test highest probability of number of clusters (K) among accessions. Highest value of ΔK was observed with four clusters (Fig. 2) suggesting that the accessions most likely segregate into four groups. The probabilities of each accession belonging to each group were used as a Q matrix for association analysis in *Tassel* software (Bradbury et al., 2007) implementing mixed linear model.



Notes. ΔK is expressed as a mean of the absolute values of ratio of change of the likelihood function with respect to K. The height of the ΔK is the indicator of the strength of the signal detected by *Structure*.

Figure 2. Detection of number of clusters (K) by estimation of ΔK over 15 runs for each K value with software *Structure*

The cumulative distribution of p-values of each model of association mapping was compared (Fig. 3). The model that was most strongly skewed towards significance as indicated by larger fraction of low p-values was MLM + Q + $K_{spagedi}$, followed by GLM, and GLM + Q, and finally the least skewed one was MLM + Q + K_{tassel}. Therefore model MLM + Q + K_{tassel} was used for association mapping to limit the possibility of false-positives.

Because of high effect of season, location and interactions of season × location on ß-glucan content,

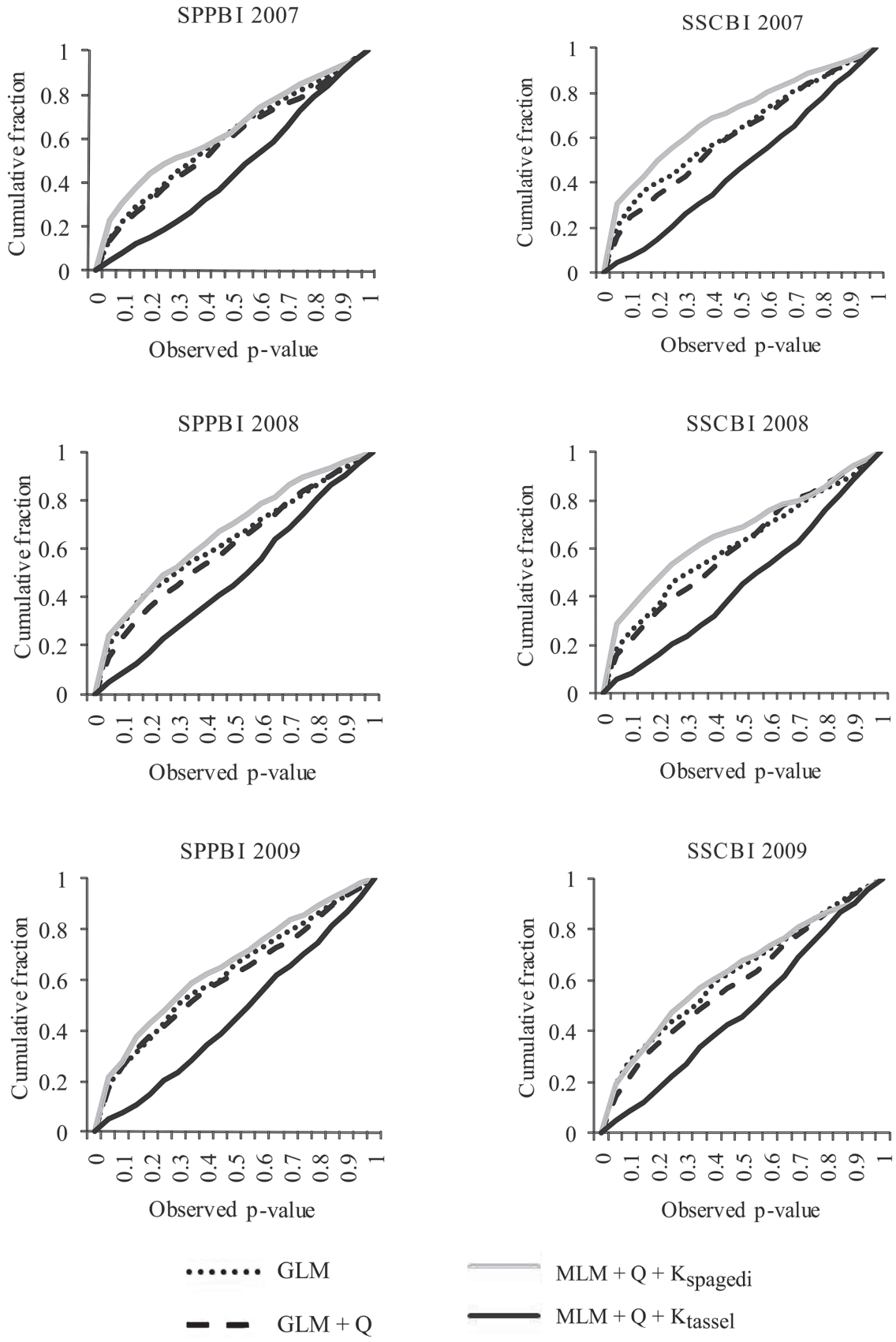
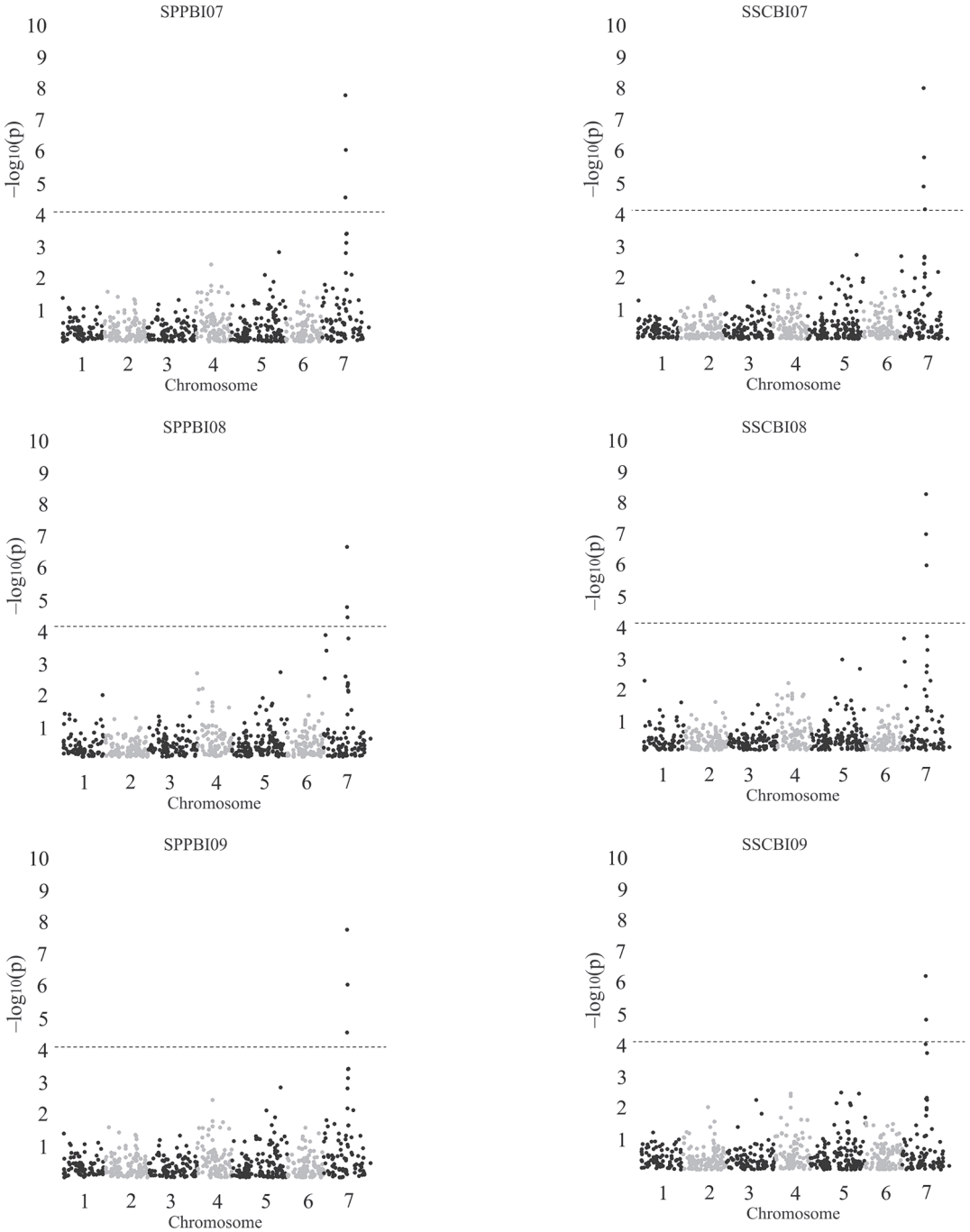


Figure 3. Cumulative fractions of observed p-values of marker – trait associations of four statistical models

marker – trait associations were determined for each environment separately. Three significant marker – trait associations exceeding the threshold for Bonferroni correction were found with p-values < 0.05 in all six environments for markers 1_0673, (114.58 cM), 2_0880 (116.68 cM), 1_1445 (116.68 cM) on chromosome 7H (Fig. 4).

nud locus responsible for hulless phenotype has been mapped previously to chromosome 7H bin 7 (Taketa et al., 2004), the region that contains the markers identified in the association scan, and hulless barley has been previously shown to contain higher amount of ß-glucan (Fastnaught et al., 1996). Therefore all accessions were genotyped at *nud* locus with indel marker developed by Taketa et al. (2008) and results were used for repetitive marker – trait association mapping. Significant association was found with uncorrected p-value ranging from 6.34×10^{-16} to 5.01×10^{-38} in separate environments.



Notes. Year and location are indicated above the graphs. Dashed line represents significance threshold for Bonferroni correction. SNPs from all seven barley chromosome are in linear order on X axis.

Figure 4. Whole genome association scan results showing $-\log_{10}(p)$ for marker associations with ß-glucan content in barley grain in separate environments

Once the 22 hulless accessions were removed from mapping population, the association mapping in 67 hulled genotypes gave no statistically significant marker-trait associations with any of the statistical models (data not shown).

Discussion

Whole genome association mapping approach was used to identify quantitative trait loci for ß-glucan content in Latvian barley germplasm. As multiple testing involves forming of spurious associations, several steps were taken to avoid false positive results. The set of markers employed in the study were selected based on criteria of MAF > 0.1, as recommended for plant populations with complex pedigrees to avoid spurious associations caused by increased relatedness between individuals sharing rare alleles. The stringent Bonferroni correction, the most conservative of the correction methods (Balding, 2006), was used to set the threshold for genome-wide significance. The high correlation of closely spaced adjacent markers was taken into consideration and the number of independent tests was calculated based on r^2 values of markers, setting the criterion for independency to < 0.8. Because high environment and genotype × environment interaction may influence phenotype, multilocational and multiyear trials were carried out to avoid false associations caused by environmental effect. QTLs were declared significant, only if they were significant in all six environments after Bonferroni correction.

Confounding by population structure can be successfully reduced by application of methods that separate true from spurious associations; particularly promising is mixed model approach (Zhao et al., 2007). In this study, association mapping was carried out using three models – general linear model, general linear model with Q, and mixed linear model with Q and K. Because determination of kinship matrix can have a major effect on outcome and reliability of association mapping (Zhao et al., 2007; Ehrenreich et al., 2009), two approaches to create kinship matrix were tested. In the first case kinship matrix was created in SPAGeDi ($K_{spagedi}$), while in the second case in *Tassel* (K_{tassel}). The cumulative distributions of p-values of each model were compared to find the curve which is least skewed towards significance, i.e. shows the most gradual rise of the cumulative fraction of p-values, to reduce the possibility to claim false-positive results. In this study the least skewed model was MLM with Q and K_{tassel} and, therefore it was used for association mapping.

Three SNPs significantly associated with ß-glucan content on chromosome 7H bin 7 were identified, 1_0673, (114.58 cM), 2_0880 (116.68 cM), 1_1445 (116.68 cM). In line with our results, Li et al. (2008) mapped a grain ß-glucan concentration QTL to chromosome 7H bin 7 in CDC Bold × TR251 population. In the same region two genes with impact on ß-glucan content have been mapped before – a recessive gene for ß-glucanless (*bgl*) grain (Tonooka et al., 2009) and the naked caryopsis (*nud*) gene (Taketa et al., 2004). Hulless barley has been previously shown to have higher ß-glucan content (Fastnaught et al., 1996). To test whether *nud* locus itself has an influence on ß-glucan content in barley, the *nud* allele was genotyped in all barley accessions and included in the association analysis. The correlation between *nud* locus and ß-glucan content was identified and it was more significant than association of the three SNP markers with ß-glucan content. Thus, *nud* locus was shown to affect ß-glucan content in genome wide association scan. However, it was not clear whether it is the only locus that affected ß-glucan content in our mapping population, because the highly significant marker-trait associations at *nud* locus could be masking other minor QTL. Therefore we conducted association mapping in 67 hulled genotypes. No significant marker-trait associations were detected by any of the statistical models. The number of accessions used for mapping may be too low to identify minor QTLs considering the comparatively low variation in ß-glucan content among the hulled accessions.

Previous attempts to identify QTL for ß-glucan content in barley grain have used mapping in bi-parental populations. Even though QTLs have been mapped on six out of seven barley chromosomes, only in one case QTLs from different mapping populations have been mapped to the same region of the chromosome, i.e. bins 11–12 on chromosome 7H (Kim et al., 2004; Li et al., 2008). The QTL that was found on chromosome 7H bin 7 in the present study is consistent with a previous study of Li et al. (2008) that located a QTL on chromosome 7H bin 7 in bi-parental mapping population of CDC Bold × TR251 and with study of Tonooka et al. (2009), who found a ß-glucanless gene in the same region.

Despite the limited number of accessions used for the association mapping and the conservative approach employed, our genome wide association study identified *NUD* locus as a major source of variation in ß-glucan content in barley grain. Thus, our results are in line with Zhao et al. (2007), who noted that small and highly structured datasets can be used to find polymorphisms with a major effect on the trait. The enlargement of the set of accessions could lead to discovery of the loci with a smaller impact on the trait.

Conclusions

1. Major QTL for ß-glucan content has been mapped to chromosome 7H bin 7 using genome wide association mapping approach in a barley population consisting of hulled and hulless accessions in the region, where the *nud* gene has been mapped before.

2. *nud* mutation showed highly significant association with ß-glucan content suggesting it as a major factor affecting ß-glucan content in the set of barley germplasm under study.

3. Association mapping data showed good agreement with the previously mapped ß-glucan QTL in bi-parental population CDC Bold × TR251 on chromosome 7H.

4. In order to map other minor QTLs for ß-glucan content, it may be necessary to enlarge the mapping population, particularly with hulled accessions showing wider range of variation in ß-glucan content.

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Genomo asociacijų genolapio sudarymas, identifikuojant *NUD* lokusą – β gliukanų kiekio miežiuose determinantą

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Santrauka

Nustatant miežių grūdų kokybę ir paskirtį, svarbus elementas yra ß gliukanų kiekis. Nors didelis kiekis ß gliukanų laikomas naudingu žmonių maiste, jis yra nepageidaujamas salykliniuose miežiuose ir pašaruose. Tyrinėta 89 latviškos kilmės miežių genotipų ß gliukanų kiekio asociacija grūduose, taip pat ir 22 belukščiuose genotipuose, naudojant 1273 aukštos kokybės viso genomo vieno nukleotido polimorfizmo (SNP) žymenis. *P* vertės buvo apskaičiuotos kompiuterine programa *Tassel*, taikant mišrų linijinį modelį, jungiantį Q ir K matricas. *P* vertės koreguotos taikant *Bonferroni* korekciją. Chromosomos 7H dalyje bin 7 nustatytos trijų žymenų 1_0673 (114.58 cM), 2_0880 (116.68 cM) ir 1_1445 (116.68 cM) esminės asociacijos. Recesyvinė mutacija *NUD* lokuse taip pat buvo lokalizuota chromosomos 7H dalyje bin 7, kuri lemia belukščių miežių fenotipą ir, kaip buvo teigiama, veikia ß gliukanų kiekį, taip pat buvo genotipuota ir įtraukta į asociacijos analizę. Nustatyta svarbi žymens ir požymio asociacija, tyrinėtų genotipų rinkinyje identifikuojanti *NUD* geną kaip pagrindinį ß gliukanų kiekio variacijos šaltinį.

Reikšminiai žodžiai: asociacijų genolapio sudarymas, miežiai, ß gliukanų kiekis, belukščiai miežiai, *NUD* genas.