Genome-wide identification of candidate genes associated with β -glucan trait in a hulled and hulless barley (*Hordeum vulgare* L.) population

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Research article

Keywords: GWAS, β -glucan content, hulled and hulless barley, SNP, nucleotide triphosphates hydrolases superfamily, plant sugar response gene

Posted Date: March 13th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-17112/v1>

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Version of Record: A version of this preprint was published at Phyton on January 1st, 2020. See the published version at <https://doi.org/10.32604/phyton.2020.011728>.

Abstract

Background: To date, excavating the genomic sites based on the whole barley assembled genome sequences associated with β -glucan trait remains blank, resulting in omission of several genetic loci or existence of false positive.

Results: Here, of 87 barley resources (39 hulled and 48 hulless) collected from China, the β -glucan content ranged from 2.07% to 6.56%. GWAS (genome-wide association study) was carried out using 191,098 SNP markers by RRGs (Reduced-Representation Genome Sequencing) platform in FarmCPU model executed in rMVP R package. Four subpopulations (POP1–POP4) were identified. GWAS showed one SNP in chromosome 7 (chr7), negatively mapped and for two genes *HORVU7Hr1G000320*, *HORVU7Hr1G000040*, which are from nucleotide triphosphates hydrolases superfamily; previous study had proved the superfamily could inhibit β -glucan synthase activities. GWAS also showed that five SNPs for β -glucan trait were separately mapped in chr2, chr3, chr4, chr5 and chr6 non-coding regions and one SNP mapped for *HORVU1Hr1G000010* in chr1, which is for plant sugar response.

Conclusions: Through the highest density of SNP markers to date, this study identifies several SNP genetic loci with high degree of confidence for β -glucan trait, and has a certain value for β -glucan regulatory mechanism research in future.

Introduction

Barley is the world's fourth largest planted crop and is one of the model crop plants in genetics and molecular biology research (Purugganan and Fuller, 2009; Dawson et al., 2015). The consumption with those barley genotypes that are rich in β -glucan component could be benefit for the lower plasma cholesterol, blood sugar control, between immunomodulatory (Skau-Mikkelsen et al., 2014); the β -glucan was consistent by continuous β -(1,4)-glycosidic bonds and is the grain cell wall's main component (Li et al., 2008). In barley seeds, the β -glucan content distributes from 2–7%, which was far higher than that of rice, corn, wheat and oat (Collins et al., 2010; Andersson et al., 2008). Considering the barley β -glucan has a special broad prospect for meeting the requirements of optimizing the people's dietary structure (Skau-Mikkelsen et al., 2014; Agostini et al., 2015), the molecular regulation mechanism of higher β -glucan accumulation in barley needs to keep groping, such as the genomic regulatory sites identification.

Previous research has verified the classical QTL (Quantitative Trait Locus) mapping strategy is suitable for identifying genomic loci for quantitative traits in several model cereals (Paterson et al., 2005). Of the β -glucan trait, Han et al. (2004) identified several major QTL loci that contributed to β -glucan trait assigned to chr2 and chr1 using a 150 lines double haploid (DH) population by a hybrid combination of 'Steptoe' $\times$ 'Morex'. A similar work was carried out also based on the DH population by Molina et al. (2007). What's more, Igartua et al. (2014) mapped a major QTL for β -glucan trait in chr7 near centromere region in a recombinant inbred line population produced by 'Derkado' $\times$ 'B83' hybridization. Kim et al. (2011) identified several QTLs for β -glucan trait on chr7 in an entire single-grain population obtained from

'Yonezawa Mochi' × 'Neulssalbori' hybridization. Above all, these β -glucan related genes/QTLs mapped through the study of barley or highland barley populations/mutants could be benefit for new determining the β -glucan-associated candidate genes.

Also, the candidate genes controlling β -glucan metabolism have been investigated. Burton et al. (2006) speculated that the expression of cellulase (HvCsl) gene family is the main factor affecting the seeds β -glucan content, especially for the HvCslF6 gene. Considering the importance of HvCslF6 in regulating seeds β -glucan content, many researchers tried to develop linked molecular markers (i.e. functional markers) by focusing on the the HvCslF6 gene aimed to accelerate the breeding, utilization of target β -glucan trait. Of these studies, Taketa et al. (2012) used full-length primers to analyze the HvCslF6 genome sequences in 29 barley varieties, and sequences blast results identified 30 polymorphic sites based on temple cDNA sequence of HvCslF6 gene (cv. Morex, GenBank accession: EU267181). In addition, Cory et al. (2012) cloned a series of HvCslF6 gene sequences from barley varieties with different β -glucan content by chromosome-walking method, with identifying four SNPs located in HvCslF6 exon regions.

Although QTL mapping assists to identify of genomic loci for quantitative traits, there was still existing several inherent disadvantages, which would result in interfering the contributive effects. First of all, QTL mapping requires a specific mapping population constructed by two parents, which can only calculate the different allelic diversity from the parent hybrids (Yang et al., 2012). However, GWAS (Genome-wide association analysis) based on nature populations is an alternative strategy comparing to QTL mapping. Through exploring the relationship between marker(s) and trait(s), GWAS could investigate the degree of LD (linkage disequilibrium) between markers associated with the functional polymorphisms across diverse germplasm resources, with high-resolution gentic mapping, reduced research time, and greater allele numbers (Lee et al., 2017). Many researches have confirmed that GWAS helps breeders use potentially powerful and rapid breeding programs to screen large germplasms and complete crop improvement (Zhou et al., 2016; Maniatis et al., 2007; Shi et al., 2017; Godoy et al., 2018).

So far, genome-wide association analysis based on SNPs associated with β -glucan trait has been studied in several studies (Shu and Rasmussen., 2014; Chutimanitsakun et al., 2013; Mohammadi et al., 2014; Houston et al., 2014; Gyawali et al., 2017). The GWAS analysis method provided a new idea for the discovery and utilization of β -glucan trait functional candidate genes and their associated SNPs in barley. However, the latest whole genome sequences of barley was available in 2017 (Mascher et al., 2017), to date, the β -glucan, an important agronomic trait in barley, has not been reported by genome-wide association analysis using a large number of SNPs based on the whole genome sequences, which could restrict the confidence interval of QTL position, it is inevitable that some important functional genes cannot be identified.

To further detailedly understand the genomic sites underlying contributes to β -glucan trait for breeding of excellent barley varieties, in this study, we firstly calculated the β -glucan content in 87 barley natural resources; Secondly, the whole genome sequences of the barley population was sequenced based on

RRGS platform with obtaining a large number of SNPs distributed in the whole genome, as well as investigating the evolution event, genetic structure of the population; Finally, GWAS for β -glucan trait was carried out in FarmCPU model executed in rMVP R package. This study would lay a good foundation for the further development and utilization of barley candidate gene resources associated with β -glucan trait.

Materials And Methods

Plant materials

87 barley genotypes (*H. vulgare* L.) with various genetic backgrounds from China was obtained and bred in Shanghai Agricultural Academy (Shanghai, P.R. China, East longitude 120°52'-122°12', North latitude 30°40'-31°53'). A field experiment in a randomized complete block design with three replications was carried in the growing season 2018–2019 in Huacao Agricultural Farm, which is the property of Shanghai Agricultural Academy, and professors of academy are allowed to use the land for research work. Furthermore, we confirmed that the field studies did not involve endangered or protected species. The seeds from each genotype were collected for the analysis of β -glucan.

β -glucan assessment

β -glucan measured was according to the manual of Megazyme kit produced by BIOTEST Co., Ltd (China, cat. no. K-BGLU). Firstly, the barley seeds were crushed by a grinder and followed by weighing 0.5 g samples in a 1.5 ml PE tube. Then, add 1.0 ml aqueous ethanol (50% v/v) and 5.0 ml sodium phosphate buffer (20 mM, pH 6.5) to each tube, with stirring in a whirlpool mixer and incubating in boiling water bath for 2 min, followed by cooling the tubes to 40 °C. Secondly, each tube was then added 0.2 mL lichenase (10 U) followed by incubating at 40 °C for 1 hour followed by adding distilled water and adjusting the solution volume of each tube to 30.0 mL, with centrifugating for 1000 g and 10 min. Thirdly, the 0.1 mL supernatant liquid was carefully transferred into three tubes followed by adding 0.1 mL sodium acetate buffer (50 mM, pH 4.0) to one of the test tubes (reaction blank), and adding 0.1 mL β -glucosidase (0.2 U) to the left two tubes (reaction) followed by incubating at 40 °C for 15 min. Lastly, the 3.0 mL GOPOD reagent was added to each tube followed by incubating at 40 °C for 20 min. The absorbance values of each reaction tube (EA) and reaction blank tube (EBI) were determined at 510 nm by Ultraviolet spectrophotometer (UV-2401PC).

SNP molecular markers acquisition

The genomic DNA extraction kit produced by Shanghai Shenggong Bioengineering Co., Ltd. (China, cat. no. B518261) was used to extract DNA from the fresh leaves of 87 barley varieties. The DNA purity and integrity of DNA were tested by 1.5% agarose gel and the concentration and quality were tested by Nanodrop® 2000c Spectrophotometer (Thermo Scientific Co., Ltd, Barrington, IL, USA). The RRGS method procedures (Qi et al., 2018) were used to develop SNP molecular markers for 87 qualified barley genomic DNA, with using the recent published barley genome sequence as the reference genome (Mascher et al.,

2017). For the step of restriction endonuclease digestion, it was prediction by the combination of MseI/TaqI restriction endonuclease, and the fragment sizes obtained rang from 500 to 600 bp.

Followed by the digestion step, the ends of the enzyme-digested fragments were modified by T4 ligase (Takara Biotechnology [Dalian] Co., Ltd) for adding joint and barcode. After using the magnetic beads (MBs) (Thermo Scientific Co., Ltd) to recover fragments, the recovered fragments were amplified by PCR using PrimeSTAR HS DNA Polymerase (Takara Biotechnology [Dalian] Co., Ltd) and using Qubit (Thermo Scientific Co., Ltd) to determine the concentration of PCR products, with the final concentration should be more than 5 ng/ul. Finally, the samples were sequenced using the Illumina Hiseq 2500 plantform with paired-end reads.

The clean reads was obtained by data quality control from raw reads referred to the analysis procedures (Qi et al., 2018). Those clean reads with the highest depth in each restriction fragment were selected as the temple sequence for analyzing by using the BWA software (Li et al., 2009), followed by aligning the sequenced clean reads against the reference barley genome (Maher et al., 2017) for obtaining the location attribution information. Finally, SNPs were indentified through using the GATK software (Mckenna et al., 2010) with high evidence at the 2 X depth, 0 deletion rate and 5% minor allele frequencies (MAF).

Population evolution, structure and PCA analysis

The NJ (Neighbor-joining) tree was constructed according to the Nei-Gojobori method by using the MEGA 2.0 program (Bootstrap value was set as 1,000) based on the SNP markers identified above. The population sub-structure of the 87 barley varieties was analyzed by using the Admixture mapping software, and the cluster number (K value) of the population was assumed to 1–10. The clustering results are cross-verified, and the optimal number of groups is determined according to the valley value of the cross-validation (CV) error rate (Alexander et al., 2009). The PCA (principal components analysis) was carried out by rMVP R package (<https://github.com/XiaoleiLiuBio/rMVP>).

Association analysis and functional annotation of trait-associated SNPs

The SNP density in seven chromosomes was constructed by the rMVP R package. The Bonferroni Multiple Correction method based on FarmCPU model excuted in rMVP R package was applied for mapping the trait-associated SNPs by the screening threshold at 0.05 level. The Quantile-Quantile scatter map was constructed by GGplot2 and QQman software, and the threshold set for Manhattan (Manhattan) mapping was at the -Log (0.01/marker numbers) level for significant SNP loci screened. 'R' represents the effects of SNP associated with target trait and the greater the absolute value, the more effects represented; '-' means negative regulation while '+' means positive regulation. Gene models annotation of SNPs associated were set at the range of 5 Mb in upstream/downstream of the trait-associated SNPs, which was referred to the EnsemblPlants website (<https://plants.ensembl.org/index.html>).

Data statistic and analysis

Data statistics and charting were used by Windows. Excel 2010. Univariate significant ANOVA and the box plot were constructed by SPSS 24.0 software. '*' represents the significant level ($P < 0.05$), and '**' represents the high significant level ($P < 0.01$).

Results

β -glucan trait and SNP density determination

In the collected population, the content of β -glucan was from 2.07–6.56%, the average content was 4.09% and the standard deviation was 0.77. The Shapiro-Wilk value used to detect normal distribution was 0.097, which was over 0.05 and indicated the content of β -glucan trait in this population was in accordance with the normal distribution (Fig. 1). A total of 22,826 Mb reads data were obtained in this project, with an average of 3.2 Gb, sequencing Q30 was 92.6% and GC content was 46.9%. Through bioinformatics analysis, 191,098 SNP markers were totally identified for genome-wide association analysis. Separately, the individual number of SNPs distributed in seven barley chromosomes (i.e. chr1, chr2, chr3, chr4, chr5, chr6, chr7) was 20,686, 31,440, 32,661, 25,682, 27,646, 26,747, and 26,236 (Fig. 2).

Population structure, PCA and evolution analysis

The population structure of 87 *H. vulgare* accessions was analyzed using 191,098 SNP markers. Through using the STRUCTURE software, the most evident and highest likelihood number for subpopulations was observed as $K = 4$ and its 'CV error' was the least (i.e. POP4) (Fig. 3A). POP1 included 20 genotypes, whereas POP2, POP3, and POP4 respectively contained 31, 21 and 15 genotypes (Fig. 4A). The β -glucan content of barley accessions in POP2 was the highest ($4.83 \pm 0.82\%$), which was significantly higher than that of POP1 ($3.98 \pm 0.39\%$), POP3 ($4.02 \pm 0.76\%$) and POP4 ($3.53 \pm 0.58\%$) (Fig. 3B). Population evolution and PCA analysis also confirmed the four endogenetic subpopulations (Fig. 4A-B). Whereas all of the barley accessions in POP4 were also hulless (Fig. 4B), their average β -glucan content was the lowest of the four POPs, which was high significantly lower than that of POP2, POP1 ($P < 0.01$), as well as significantly lower than that of POP3 ($P < 0.05$) (Fig. 3B).

Association mapping analysis

In Manhattan-plot, each point on the graph represents one SNP locus, the $(-\log_{10} P\text{-value})$ for all 191,098 SNP loci formed the Y-coordinate. Through the FarmCPU model in GWAS analysis, totally 7 SNP loci were significantly associated with β -glucan trait; one chromosome obtained one SNP locus and all of them were located on the distal end of each chromosome (Fig. 5). In addition, the QQ diagram of GWAS analysis also showed the same seven genomic sites (i.e., SNPs) are eligible (Fig. 6). The points in the circle of the QQ-plot diagram were potential candidate genomic sites significantly related to β -glucan trait, which were divided into the 'Observation P-value' and the 'Expected P-value'. Those genomic sites (i.e.

loci) with the 'Observed P-value' exceeded the 'Expected P-value' means they were significantly associated with β -glucan trait (Fig. 6).

Functional annotation of trait-associated SNPs

Of the 7 SNPs identified by GWAS analysis, SNP2 (i.e., SNP was located on chr2, $R = +0.06$, $P\text{-value} = 4.33\text{E-}07$) and SNP3 ($R = +0.15$, $P\text{-value} = 2.10\text{E-}25$) had the positive association relationship with β -glucan trait, while SNP1 ($R = -0.13$, $P\text{-value} = 3.00\text{E-}12$), SNP4 ($R = -0.27$, $P\text{-value} = 2.73\text{E-}24$), SNP5 ($R = -0.14$, $P\text{-value} = 5.04\text{E-}21$), SNP6 ($R = -0.14$, $P\text{-value} = 1.56\text{E-}15$) and SNP7 ($R = -0.21$, $P\text{-value} = 2.33\text{E-}21$) had the negative association relationship (Fig. 5). Moreover, in the 5 Mb up-/or downstream regions round of 7 SNPs, SNP1 was associated with two gene models (HORVU1Hr1G000010, HORVU1Hr1G000020), one association event was located in gene region and the other association event was located in 5' upstream region (2419 bp distant to gene's ATG position); SNP5 was associated with one gene model (HORVU5Hr1G000060), and the SNP was 3009 bp distant to gene's ATG position; While SNP7 (effects = -0.21 , $P\text{-value} = 2.33\text{E-}21$) was associated with three gene models (HORVU7Hr1G000320, HORVU7Hr1G000040, HORVU7Hr1G000330), of the three association events, two events were located in two gene regions (Fig. 5), these two genes (HORVU7Hr1G000320, HORVU7Hr1G000040) are homologous to the rice gene Os10g0370100, which was one member of the nucleoside triphosphate hydrolases according to the RGAP annotation result (Rice Genome Annotation Project), the left one association event was 982 bp distant to gene's ATG position. Of the seven SNPs, the SNP1, at the position 44,687 in chr1, this SNP was located in the region of HORVU1Hr1G000010, which is the homologous gene of SIS3 (AT3G47990).

Discussion

In this study, genome-wide association analysis (GWAS) was used to identify the barley whole genome-wide genetic sites associated with β -glucan trait by using 191,098 SNPs markers (i.e., the largest amount of SNP markers by far). Through using FarmCPU model executed in rMVP R package, with the PCA parameters as the covariable, which could effectively control false positives (Stich et al., 2009; Park et al., 2019), seven highly significant SNPs ($P < 4.33 \times 10^{-7}$) were clearly and intuitively identified in Manhattan plot diagram, also, the association results were meanwhile confirmed by QQ-plot diagram.

The high content of β -glucan in barley is one of the important standards for its edible value, and it is meaningful to apply the strategy of molecular marker-assisted breeding in the traditional breeding. Here, we focused on the rapid identification of highly accurate variation markers (SNPs) in a population of barley natural germplasm resources by using the RRGs method. First of all, for GWAS applied in a species, the genome LD levels determine the density of markers needed. Crop barley was suitable for GWAS, as comparing to the outbred species, the LD levels in barley (inbred species) are higher and attenuate slower (Plagnol et al., 2006). For example, as a cross-pollinating plant, the LD attenuation of maize was significantly faster than that of *Arabidopsis thaliana*, rice and other crops (Tenaillon et al.,

2001). In our study, the seven SNPs identified separately in seven chromosomes didn't have any significant LD relationships.

The GWAS technology based on RRGs had been applied in several crop traits analysis (Ma et al., 2016; Xu et al., 2014). However, as far as we know, in the important crop barley, the GWAS study by using SNPs developed from the whole genome sequences related to the β -glucan trait remains unknown, even though β -glucan has been confirmed to be recognized as a very important food health ingredient. Only some genetic map-based association analysis based on parental narrow resources (Han et al., 2004; Molina et al., 2007; Igartua et al., 2014), as well as some GWAS analysis based on limited number of SNPs markers (Shu and Rasmussen., 2014; Chutimanitsakun et al., 2013; Mohammadi et al., 2014) were carried out. In this study, through using the RRGs technology, we identified 7 high significantly SNP markers from 191,098 SNP markers, each has an effect on the β -glucan trait. In addition, of the barley population analyzed, a mix of hulled and hulless barley accessions was selected. It is generally believed that the quality of functional nutritional components of naked(i.e., hulless) barley is higher than that of hulled barley, including β -glucan trait (Oscarsson et al., 1997), our results confirmed that the content of β -glucan in naked barley of POP2 (hulless) was higher than of POP1 (hulled) and POP3 (hulless and hulled). Also, we found that, of the POP2 (hulless) barley (from northwest China), β -glucan content was higher than that of POP4 (from southeast of China) and the content of β -glucan in POP4 was also the least in four POPs, we concluded that the β -glucan trait was also affected by environmental effects, as several previous studies showed (Zhang et al., 2001; Yalcin et al., 2007).

Based on the SNPs developed from whole barley genome sequences from GWAS analysis, here, we finally quickly identify functional genes associated with β -glucan trait. Previously, the genome-wide association analysis technique was used for the first time in plant sugarbeet (*Beta vulgaris* L.), with the help of this technique, the bolting gene B was identified, which determines the bolting purification of sugarbeet (Hansen et al., 2001). The method of GWAS analysis has the advantages of high throughput, high efficiency, no candidate genes involved, no need to construct any assumptions, no dependence on mapping populations, etc, which have helped identify many loci/or alleles associated with traits in the face of a wide range of germplasm resources. In this study, the gene SIS3(AT3G47990) we identified, which is the homologous gene involved in plant sugar response in *Arabidopsis* (Huang et al., 2010), and it's a good candidate gene for further transgene confirmation; What'more, BillonGrand et al. (1997) had proved the nucleotide triphosphates could inhibit β -glucan synthase activities, considering the SNP7 had a high negative association with β -glucan trait (i.e., negative to HORVU7Hr1G000320, HORVU7Hr1G000040), and we concluded that SNP7 might be a positively active genomic site for β -glucan synthesis. In addition, the SNPs in the upstream of genes HORVU1Hr1G000020, HORVU5Hr1G000060 and HORVU7Hr1G000330 may be through the way of cis- for regulating those genes, for example, through the open chromosome sites, which was a hot research topic in these years (Lu et al., 2017).

Here, GWAS shows promising features that could help elucidate the genetic basis of the qualitative and economic β -glucan trait in barley. This is the first study to investigate the candidates genes behind the H.

vulgare seed β -glucan trait by whole genome association mapping analysis since the latest barley genome sequences was available in 2017. Our results could broaden the scope of functional genes identification on seed β -glucan in *H. vulgare*.

Abbreviations

GWAS

Genome-wide association study; RRGS:Reduced-representation genome sequencing; SNP:Single nucleotide polymorphism; QTL:Quantitative trait locus; DH:Double haploid; LD:Linkage disequilibrium; PCA:Principal components analysis.

Declarations

Acknowledgments

The authors are grateful to Shanghai Agrobiological Gene Center (SAGC) for their generous provision of landraces and improved varieties used in this study.

Authors' contributions

LC, LR and HJ conceived and designed the study. LY and LJ assembled the panel and participated in genotyping. GG and ZL assembled the RNA-seq data. ZL and HT prepared the manuscript and carried out the data analysis. ZL, LY and LC revised and edited the manuscript and approved the final version of the manuscript.

Funding

This work was financially supported by the Key Technology R & D Project of Shanghai Agriculture-developed with Science & Technology Program, China (Grant No. 2018(1-2)); The earmarked fund for China Agriculture Research System (CARS-05-01A-02); The Natural Science Foundation of Shanghai, China (Grant No. 19ZR1417000).

Availability of data and materials

The data sets supporting the results of this article are included in this manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

β -glucan content of 87 genotypes of *H. vulgare* L.

Figure 2

The density of SNPs was distributed in seven chromosomes within 1Mb window size.

Figure 3

(A) Evaluation of population structure of 87 *H.vulgare* accessions based on 191,098 SNP markers; (B) Comparison among different subpopulation of *H.vulgare* on the basis of seed β -glucan content.

Figure 4

(A) Population evolution and (B) PCA analysis of 87 genotypes of *H. vulgare* L. based on 191,098 SNP markers.

Figure 5

Manhattan plot of genome-wide association mapping of β -glucan using FarmCPU model excuted in rMVP R package. The horizontal line is the $P < 4.33 \times 10^{-7}$ cutoff.

Figure 6

QQ-plot diagram of candidate genomic sites of β -glucan using 'Observation P-value' and the 'Expected P-value'.