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Article

Genome-Wide Identification of Candidate Genes Associated with *β*-glucan Traits in a Hulled and Hulless Barley (*Hordeum vulgare* L.) Population

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Abstract: Barley grain is a valuable source of *β*-glucan, which is an important component of dietary fiber with significant human health benefits. Although the genetic basis of *β*-glucan biosynthesis has been widely studied, a genome-wide association study (GWAS) is still required for a scan of the candidate genes related to the complex quantitative trait based on the high-quality barley reference genome. In this study, a GWAS was conducted using a population composed of 87 barley landraces (39 hulled and 48 hulless, *β*-glucan from 2.07% to 6.56%) with 191,098 nucleotide polymorphisms (SNPs) markers to cover the chromosomes with the highest density. The population was divided into four sub-populations (POP1~POP4), and the *β*-glucan content in POP2 was significantly higher than that in other groups, in which most of the hulless barley landraces are from Qinghai-Tibet Plateau in China. Among seven SNP markers identified by GWAS, two (SNP2 and SNP3) of them showed positive correlation to *β*-glucan trait and the remaining five (SNP1, SNP4, SNP5, SNP6 and SNP7) showed the negative relationship. Two candidate genes linked to SNP7, *HORVU7Hr1G000320* and *HORVU7Hr1G000040*, belong to the nucleotide triphosphate hydrolase superfamily which is probable to affect the activities of *β*-glucan synthase. Another candidate gene associated with SNP1, *HORVU1Hr1G000010*, is possibly involved in sugar response. In conclusion, our results provide new insights into the genetic basis of *β*-glucan accumulation in barley grains, and the discovery of new SNP markers distributed in each chromosome and the associated candidate genes will be valuable for the breeding of functional barley varieties with high *β*-glucan.

Keywords: GWAS; *β*-glucan; SNP; barley; grains

1 Introduction

Barley (*Hordeum vulgare* L.) is the fourth most-cultivated cereal crop and commonly used for brewing and animal feeds. Currently, barley has been recognized as a functional food due to the high level of *β*-glucan in grains [1,2]. The consumption of barley products riching in *β*-glucans could be benefit for lowering glycemic response, plasma cholesterol and food intake [3]. Barley *β*-glucans are linear, unbranched

polysaccharides consisting of mixed-linkage (1→3), (1→4)-*β*-D-glucopyranose units (hereafter termed as *β*-glucans) and exist in the cell wall of grains as one of the major components [4]. In barley seeds, the *β*-glucan content ranges from 2% to 7%, which was far higher than that of rice, corn and oat [5,6]. Given the promising prospects of barley *β*-glucan in functional food development and healthy product consumption, more attention has been paid on the understanding of the molecular regulation mechanism of *β*-glucan accumulation in barley [7].

The quantitative trait locus (QTL) mapping strategy is used to identify the genomic loci or related genes contributing to quantitative traits [8]. As to the traits related to *β*-glucan, Han et al. [9] identified several major QTLs that contributed to the *β*-glucan traits using a population composed of 150 double haploid (DH) lines from 'Steptoe' × 'Morex' hybridization. A similar study was carried out in barley by Molina et al. [10]. In addition, Igartua et al. [11] mapped a major QTL for *β*-glucan trait in chr7 by using a recombinant inbred line population produced by 'Derkado' × 'B83' hybridization. Kim et al. [12] identified several QTLs for *β*-glucan traits on chr7 based on an entire single-grain population obtained from 'Yonezawa Mochi' × 'Neulssalbori' hybridization. The identified QTLs related to *β*-glucans in barley or highland barley populations/mutants are useful for determining new candidate genes associated with *β*-glucan accumulation.

To date, only one high confidence candidate gene family controlling *β*-glucan metabolism has been identified. Burton et al. [13] speculated that the expression of cellulase (*HvCsl*) gene family is the main factor affecting the seed *β*-glucan content, especially for the *HvCslF6* gene. Considering the importance of *HvCslF6* in regulating seeds *β*-glucan content, many researchers tried to develop the molecular markers (i.e., functional markers) linked to *HvCslF6* gene with the aim of accelerating the breeding to achieve a higher *β*-glucan content. For example, Taketa et al. [14] analyzed the full-length of *HvCslF6* genomic DNA sequences between 29 barley varieties, and sequence BLAST results identified 30 polymorphic sites based on the complete cDNA sequence of the *HvCslF6* gene (*cv.* Morex, GenBank accession: EU267181). In addition, Cory et al. [15] cloned a series of *HvCslF6* genes from barley varieties with different *β*-glucan contents via the chromosome-walking method, and identified four SNPs located in exon regions of *HvCslF6*.

Although QTL mapping aid in the identification of genomic loci or candidate genes contributing to targeted phenotype, several inherent disadvantages of this approach remain, interfering with the contributive effects. First, QTL mapping requires a specific mapping population constructed from two parents, and the differences in allelic diversity can only be calculated from the parental hybrids [16]. However, GWAS (genome-wide association studies) based on natural populations are an alternative strategy to QTL mapping. By exploring the relationship between marker(s) and trait(s), GWAS can allow the degree of LD (linkage disequilibrium) between markers associated with functional polymorphisms across diverse germplasm to be investigated with high-resolution genetic mapping, more time saving and more efficient on the identification of allele numbers [17]. Many studies have confirmed that GWAS can be used as a powerful tool to evaluate the large population of crop germplasms and accelerate the process of crop breeding [18–21].

The genome-wide association studies based on SNPs associated with barley *β*-glucan traits have been applied in several studies [22–26]. It provided a new idea for the understanding of *β*-glucan accumulation in barley. As the complete genomic sequence of barley was renewed and available in 2017 [27], up to now, it has not been reported on the GWAS using high density of SNPs based on the fined genome sequences, which will improve the precise on the location of the confidence interval of QTL position, and discover the interests underlying important functional genes cannot be identified in previous studies.

To further explore the new loci underlying *β*-glucan accumulation in barley, we collected 87 barley landraces from different cultivated zones in China, and the *β*-glucan contents in grains were determined. The whole genomic sequences of the barley population were screened against the renewed barley genome

reference released in 2017 on RRGS platform, and a high density of SNPs distributed in the each chromosome was constructed. The genetic structure of the population was analyzed and sub-populations was evaluated. Finally, GWAS for β-glucan traits was conducted in FarmCPU model executed in rMVP R package. Our study discovered seven new SNPs markers and the associated candidate genes related to barley β-glucan traits, which will be valuable for the future breeding of β-glucan improvement.

2 Materials and Methods

2.1 Plant Materials

Eighty-seven (87) barley (*Hordeum vulgare* L.) landraces were collected from the main barley cultivated zones in China (i.e., 6 from Gansu, 9 from Jiangsu, 36 from Shanghai, 11 from Sichuan, 6 from Xizang, 7 from Yunnan, 12 from Zhejiang) and grown in the farm of Shanghai Academy of Agricultural Sciences, 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 out in the growing season of 2018–2019. The seeds from each genotype were harvested and dried for the quantification of β-glucan in grains.

2.2 β-Glucan Measurement

The content of β-glucan in the grains from 87 barley landraces was measured according to the manual of the Megazyme kit (Cat. No. K-BGLU) from BIOSTEST Co., Ltd. (China). First, the barley seeds were crushed with a grinder, followed by the weighing of 0.5 g samples into a 1.5 ml PE tube. Then, 1.0 ml aqueous ethanol (50% v/v) and 5.0 ml sodium phosphate buffer (20 mM, pH 6.5) were added to each tube, followed by stirring with a whirlpool mixer and incubation in a boiling water bath for 2 mins. Next, 0.2 mL lichenase (10 U) was added to each tube, followed by incubation at 40°C for 1 hour, and centrifugation at 1000 g for 10 min. Then, 0.1 mL of the supernatant liquid was carefully transferred to three tubes, followed by the addition of 0.1 mL sodium acetate buffer (50 mm, pH 4.0) to one of the test tubes (reaction blank) and 0.1 mL β-glucosidase (0.2 U) to the two remaining tubes (reaction) and incubation at 40°C for 15 mins. Finally, 3.0 mL GOPOD reagent was added to each tube, followed by incubation at 40°C for 20 mins. The absorbance values of each reaction tube (EA) and reaction blank tube (EBI) were determined at 510 nm in an ultraviolet spectrophotometer (UV-2401PC).

2.3 SNP Molecular Markers Acquisition

The genomic DNA extraction kit (Cat. No. B518261) from Shanghai Sangon Biotech Co., Ltd. (China) was used to extract DNA from the fresh leaves of 87 barley landraces. The purity and integrity of the DNA were examined in a 1.5% agarose gel, and the concentrations and quality were examined with a Nanodrop$^{@}$ 2000c spectrophotometer (Thermo Scientific Co., Ltd., Barrington, IL, USA). The RRGS method [28] was used to develop SNP molecular markers for the 87 qualified barley genomic DNA samples using the recently released barley genome sequence as the reference genome [27]. The restriction endonuclease digestion step involved the combination of the MseI/TaqI restriction endonuclease, and the obtained fragment sizes ranged from 500 bp to 600 bp.

Following the digestion step, the ends of the enzyme-digested fragments were modified with T4 ligase (Takara Biotechnology [Dalian] Co., Ltd.) for joining and the addition of barcodes. The magnetic beads (MBs) (Thermo Scientific Co., Ltd.) were used to recover fragments, and the recovered fragments were amplified by PCR using PrimeSTAR HS DNA Polymerase (Takara Biotechnology [Dalian] Co., Ltd). The concentration of PCR products was determined using Qubit (Thermo Scientific Co., Ltd), and the final concentration of products should be more than 5 ng/uL. Finally, the samples were subjected to paired-end sequencing on the Illumina Hiseq 2500 platform.

The clean reads were obtained after filtering of the raw reads referred to the quality control processing by Qi et al. [28]. Those clean reads with the highest depth in each restriction fragment were selected as the

template sequence for analyzing by using the BWA software [29], followed by aligning the sequenced clean reads against the reference of barley genome [27] for obtaining the location attribution information. Finally, SNPs were identified through using GATK software [30] with high confidence at the 2 X depth, 0 deletion rate and 5% minor allele frequencies (MAF).

2.4 Population Structure Analysis

The NJ (neighbor-joining) tree was constructed according to the Nei-Gojobori method using the MEGA 2.0 program (the bootstrap value was set as 1,000) based on the SNP markers identified above. The population substructure of the 87 barley landraces was analyzed by using Admixture mapping software, and the cluster number (K value) of the population was assumed to be 1~10. The clustering results were cross-verified, and the optimal number of groups was determined according to the valley value of the cross-validation (CV) error rate [31]. PCA (principal components analysis) was conducted with the rMVP R package (https://github.com/XiaoleiLiuBio/rMVP).

2.5 Genome-Wide Association Study

The SNP distribution on the seven chromosomes was determined with the rMVP R package. The Bonferroni multiple correction method based on the FarmCPU model executed in the rMVP R package was applied for mapping the trait-associated SNPs according to the screening threshold at the 0.05 level. The Quantile-Quantile scatter map was constructed by using GGplot2 and QQman software, and the threshold for Manhattan (Manhattan) mapping was set at the −Log (0.01/marker numbers) level for significant SNP loci that were screened. 'R' represents the effects of SNPs associated with the target traits, where the greater the absolute value, the greater the effects that are represented; '−' indicates negative regulation, while '+' indicates positive regulation. Gene models were selected within the range of 5 Mb upstream/downstream of the trait-associated SNPs, with reference to the EnsemblPlants website (https://plants.ensembl.org/index.html).

2.6 Data Statistics and Analysis

Data statistics and charting were performed in Microsoft Office Excel 2010. Univariate significant ANOVA and the box plot construction were performed with SPSS 24.0 software. '*' represents the significant level ($P < 0.05$), and '**' represents the highly significant level ($P < 0.01$).

3 Results

3.1 Quantification of β-Glucans and Identification of SNP Markers

In the collected population, the β-glucan content ranged from 2.07% to 6.56%, with the mean of 4.09% and the standard deviation of 0.77. The Shapiro-Wilk value used to detect a normal distribution was 0.097 (>0.05), which indicated that the presence of the β-glucan trait in this population was in accordance with the normal distribution (Fig. 1). A total of 22,826 M reads were obtained, with an average of 3.2 Gb per sample, and the sequencing Q30 was 92.6% and GC percent was 46.9%. By bioinformatics analysis, 191,098 SNP markers were identified for the GWAS. The 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, respectively (Fig. 2).

3.2 Construction of Population Structure

The population structure of 87 barley landraces was analyzed using 191,098 SNP markers. According to the analysis with STRUCTURE software, the most confident and the highest likelihood number of subpopulations was obtained to be K = 4, with the lowest 'CV error' (i.e., POP4) (Fig. 3A). POP1 consists of 20 accessions, whereas POP2, POP3, and POP4 were composed of 31, 21 and 15 accessions, respectively (Fig. 4A). The β-glucan content in POP2 was the highest (4.83 ± 0.82%), which was

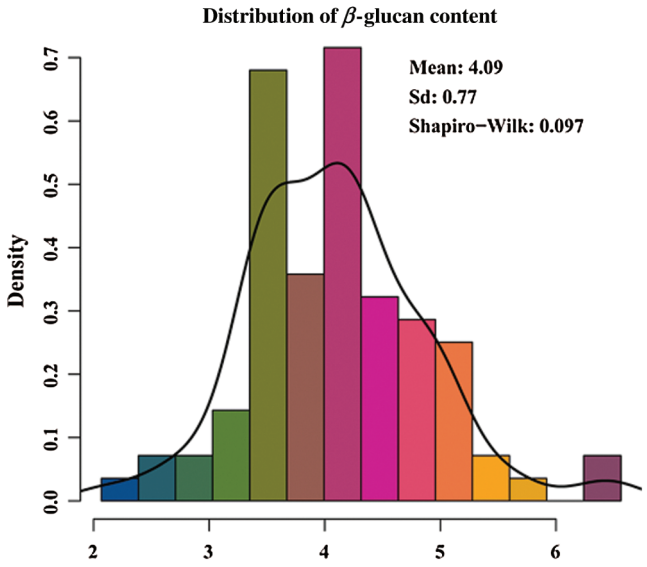

Figure 1: Distribution of β-glucan contents in grains from 87 barley landraces

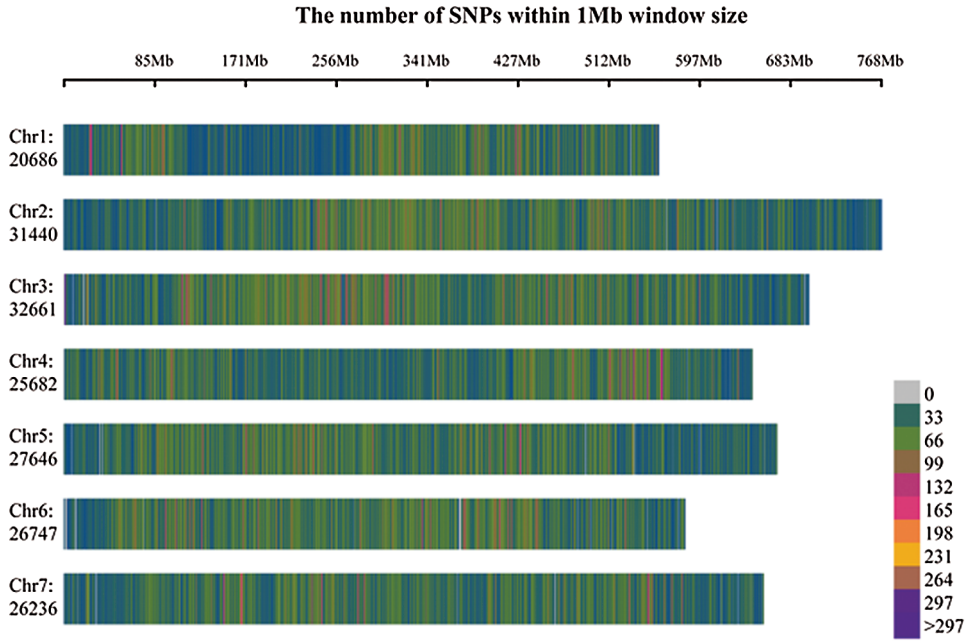

Figure 2: Distribution of SNPs located in seven chromosomes within 1 Mb window size

significantly higher than that of POP1 (3.98 ± 0.39%), POP3 (4.02 ± 0.76%) and POP4 (3.53 ± 0.58%) (Fig. 3B), in which 18 of 21 hulless barley landraces are from Qinghai-Tibet Plateau in China. Phylogenetic tree and PCA analysis also confirmed the four subpopulations (Figs. 4A and 4B). All of the barley accessions in POP4 were hulless (Fig. 4B), and POP4 showed the lowest average β-glucan content among the four POPs, which was highly significant lower than those of POP2 and POP1 ($P < 0.01$), and significantly lower than that of POP3 ($P < 0.05$) (Fig. 3B).

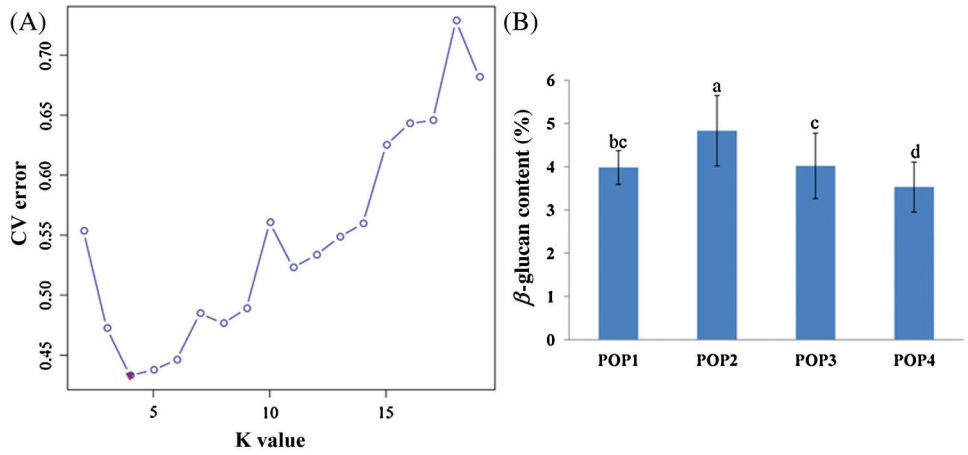

Figure 3: (A) Evaluation of the population structure of 87 barley landraces based on 191,098 SNP markers; (B) comparison of seed *β*-glucan contents between four sub-populations

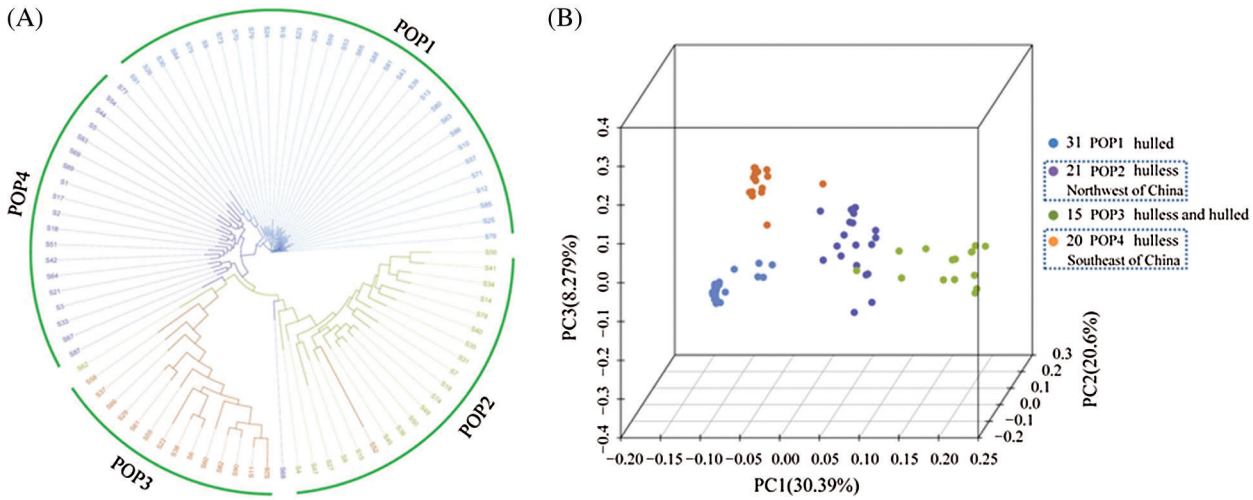

Figure 4: (A) Phylogenetic tree analysis and (B) PCA analysis based on 191,098 SNP markers for 87 barley landraces

3.3 Genome-Wide Association Analysis

In the Manhattan-plot, each point in the graph represents one SNP locus, and the ($-\log_{10}{}^{P\text{-value}}$) results for all 191,098 SNP loci are represented by the Y-coordinate. Through the use of the FarmCPU model in the GWAS, a total of 7 SNP loci were found to be significantly associated with the *β*-glucan trait; each chromosome included only one SNP locus, and all of the loci were located at the distal ends of each chromosome (Fig. 5). In addition, the QQ diagram of the GWAS results showed that the same seven genomic sites (i.e., SNPs) were valid (Fig. 6). The points in the circle in the QQ-plot diagram are potential candidate genomic sites significantly related to the *β*-glucan trait, which were divided into the 'Observed *P*-value' and the 'Expected *P*-value' groups. Those genomic sites (i.e., loci) for which the 'Observed *P*-value' exceeding the 'Expected *P*-value' were indicated to be significantly associated with the *β*-glucan trait (Fig. 6).

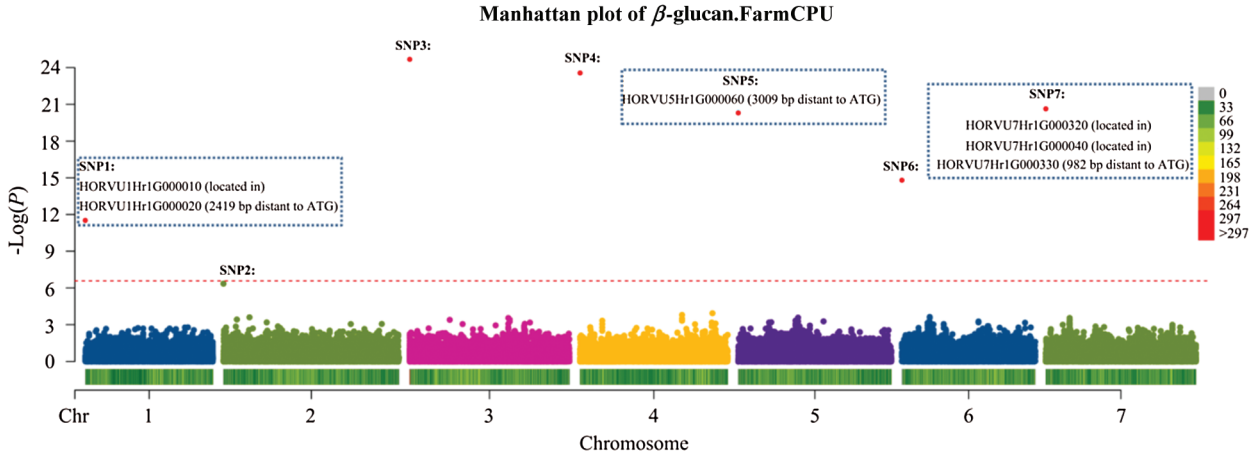

Figure 5: Manhattan plot for the genome-wide association mapping of the β-glucan trait using the FarmCPU model executed in the rMVP R package. The horizontal line is the threshold at $P < 4.33 \times 10^{-7}$

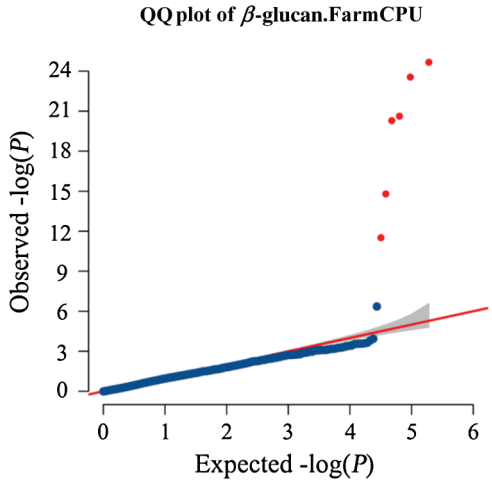

Figure 6: QQ-plot diagram of candidate genomic sites for the β-glucan trait using the 'Observed *P*-value' and the 'Expected *P*-value'

3.4 Associations of Targeted SNPs with Candidate Genes

Among the 7 SNPs identified by GWAS, SNP2 (i.e., SNP was located on chr2, R = +0.06, *P*-value = 4.33E-07) and SNP3 (R = +0.15, *P*-value = 2.10E-25) showed a positive association relationship with the β-glucan trait, while SNP1 (R = −0.13, *P*-value = 3.00E-12), SNP4 (R = −0.27, *P*-value = 2.73E-24), SNP5 (R = −0.14, *P*-value = 5.04E-21), SNP6 (R = −0.14, *P*-value = 1.56E-15) and SNP7 (R = −0.21, *P*-value = 2.33E-21) presented a negative association (Fig. 5). Moreover, in the 5 Mb up-/downstream regions of the 7 SNPs, SNP1 was associated with two gene models (*HORVU1Hr1G000010*, *HORVU1Hr1G000020*), where one association event was located in the gene region and the other association event was located in the 5' upstream region (2419 bp from the ATG position in the gene). SNP5 was associated with one gene model (*HORVU5Hr1G000060*), and the SNP was 3009 bp from the ATG position in the gene. SNP7 (effects = −0.21, *P*-value = 2.33E-21) was associated with three gene models (*HORVU7Hr1G000320*, *HORVU7Hr1G000040*, *HORVU7Hr1G000330*), where two of the three association events were located in two gene regions (Fig. 5); these two genes (*HORVU7Hr1G000320*,

HORVU7Hr1G000040) are homologous to the rice gene *Os10g0370100*, which is a member of the nucleoside triphosphate hydrolases according to the RGAP annotation result (Rice Genome Annotation Project), and the remaining association event (SNP7 for *HORVU7Hr1G000330*) was 982 bp from the ATG position in the gene. Among the seven SNPs, the SNP1, at the position 44,687 on chr1, was located in the region of *HORVU1Hr1G000010*, which is the homologous gene of *SIS3* (*AT3G47990*).

4 Discussion

In this study, GWAS was used to identify the loci associated with β-glucan traits with 191,098 SNP markers to cover the whole barley genome with the highest density so far. By using the FarmCPU model executed in the rMVP R package and fitted with the PCA parameters as the covariates, it effectively reduced the false positives [32,33]. Manhattan-plots clearly showed that seven SNPs were significantly ($P < 4.33 \times 10^{-7}$) associated with β-glucan traits. In addition, the detected SNPs were also confirmed by the QQ-plots.

The high content of β-glucan in barley is one of the important features for health benefits, and the molecular marker-assisted strategies will accelerate the processing of genetic improvement. Moreover, barley is a self-pollinating crop and more suitable for the application of the GWAS strategy. Compared to the cross-pollinating species, linkage disequilibrium (LD) levels in self-pollinating species are higher and the decay is slower [34]. For example, as a cross-pollinating plant, the LD decay in maize is significantly faster than that in *Arabidopsis thaliana*, rice and other crops [35]. In our study, high quality and high density of SNPs markers (SNPs) were obtained between the collected barley landraces by using the reduced-representation genome sequencing (RRGS) method. Seven SNPs markers associated with β-glucan traits were identified on seven chromosomes respectively and there are no significant LD relationships between them.

GWAS based on RRGS has been applied in several crops for identification of loci or candidate genes associated with the traits [36,37]. However, to our knowledge, it has not been reported in barley to perform a GWAS using high density of SNPs developed from RRGS for a population to study the β-glucan traits. Only genetic map-based association analyses based on limited parental resources [9–11], and GWAS based on a limited number of SNP markers [22–24] have been reported previously. In this study, we generated 191,098 SNP markers from RRGS for 87 barley landraces, and identified seven SNPs markers high significantly with the β-glucan traits. In addition, in the tested barley population, both hulled and hulless barley accessions were picked up randomly. It is generally believed that the accumulation of some functional components such as β-glucan in hulless (i.e., naked) barley is higher than that in hulled barley [38]. Our results indicated that the content of β-glucan in POP2 composed of hulless barley was higher than that in POP1 (hulled) or POP3 (mixture of hulless and hulled types). Additionally, we found that the β-glucan content in the POP2 (hulless) barley (from northwest China, and the most from the Qinghai-Tibet Plateau) was higher than that in POP4 (from southeast China), and the latter was the least among the four POPs. It was suggested that the β-glucan trait was affected by the origin of cultivated zones, which is supported by several studies previously reported [39,40].

Based on the SNPs developed from RRGS, we successfully identified some new candidate genes associated with the β-glucan traits by GWAS tool. It was the first time to employ GWAS in sea beet (*Beta vulgaris* L.) to identify *bolting gene B*, which determines the growth habit of bolting [41]. The GWAS presents a powerful tool to link the genotypic data and phenotypic traits across a population, with the advantages of high throughput and high efficiency to identify the candidate loci or genes. In this study, we identified the two candidate loci associated to β-glucan traits in barley. The locus identified with SNP1 is linked to a homologous gene (*HORVU1Hr1G000010*) of *SIS3* gene (*AT3G47990*) in *Arabidopsis*, which is involved in the sugar response [42], and it awaits further study whether it is implicated in the biosynthesis of β-glucan. The locus identified with SNP7 is linked to two homologous

genes (*HORVU7Hr1G000320* and *HORVU7Hr1G000040*) of *Os10g0370100* in rice, which is annotated as the member of nucleoside triphosphate hydrolases. BillonGrand et al. [43] reported that nucleotide triphosphates (GTP and ATP) inhibit the activities of 1,3-*β*-glucan synthase. It was suggested that the two homologous genes probably affect the activities of *β*-glucan synthase. In addition, the SNPs (SNP1, SNP5 and SNP7) located at the upstream of the *HORVU1Hr1G000020*, *HORVU5Hr1G000060* and *HORVU7Hr1G000330* revealed that they are possible *cis*-regulation regions of regulating those genes through the open chromosome sites, which has become a hot spot in recent years [44].

In conclusion, GWAS provides informative contents to aid understanding of the genetic basis for the complex quantitative, economic *β*-glucan traits in barley. High density of SNPs markers were constructed by RRGS across 87 hulled and hulless barley landraces based on the renewed complete barley genome reference. Seven SNPs markers associated *β*-glucan traits were identified on seven chromosome respectively, and some candidate genes linked to those SNPs markers are worthy of further investigation.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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