Genetic Analysis of (1-3), (1-4)-β-D-Glucans in Barley, Azhul × Falcon Cross

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Abstract: β-glucans are non-starchy polysaccharides composed of (1-3), (1-4) mixed linked glucose polymers. They are located mainly in the cell walls of the endosperm and the aleurone layer of different cereal grains, specially oat and barleys. Recently, several studies have revealed the benefits of including β-glucans into the diet to the human health, such as cholesterol diminution in blood and reduction of risk of cancer, coronary heart disease and diabetes. Six generations (P1, P2, F1, F2, BC1 and BC2), from cross Azhul × Falcon were used for generation mean and variance analyses for estimating genetic effects and variances and also for determining the number of genes (QTLs) governing β-glucan content. In addition, bulked segregant analysis (BSA) was used to identify RAPD markers associated with β-glucan in barley. RAPD markers linked to β-glucan was identified in two DNA pools (high and low β-glucan), which were established using selected F2 individuals. The analyses of gene actions indicated that a large part of the total genetic variation observed for β-glucan content was in the form of dominance genetic effects. In addition, duplicate type of epistasis observed in the expression of this trait. Estimating gene number by different formulae showed that several QTL were involved in the genetic control of this trait. In bulked segregant analysis two separate RAPD fragment (S39-150 b.p and WE013-1750 b.p) was found among the 3968 amplified bands that was related to the β-glucan content. Hence, these fragments can be utilized in a marker-assisted selection (MAS) programme in barley. Efforts are underway to convert the two RAPD primers into SCAR markers and subsequently map them.

Key words: β-glucan · Barley · Generation mean and variance analyses · QTLs · Bulked segregant analysis · RAPDs

INTRODUCTION

β-glucans are a kind of non-starchy polysaccharides (formed by the linkage of glucose units via β-(1-3) (approximately 30%) and β-(1-4)-glucosidic linkages) that can be found in several kinds of cereals, such as barley, oat or rye in concentrations from 2 to 12% in dry basis. In barley (Hordeum vulgare L.), β-glucans are minor components (up to 6-7%), located majoritary in the aleurone and endosperm cellwalls of the grain [1].

The physiological benefits due to β-glucan seem to stem from their effect on lipid metabolism and postprandial glucose metabolism. Several recent studies, in both hypercholesterolemic [2] and healthy [3] subjects, found that the daily consumption of 5 g of β-glucan significantly decreased serum total and LDL cholesterol. Davidson et al. [4] found that only a daily consumption of 3.6 g β-glucan was needed to produce the same significant effects. The same relationship also has been reported to occur between β-glucan and postprandial glucose and insulin responses in both diabetic and healthy subjects. Biorklund et al. [5] found that 5 g of β-glucan significantly decreased postprandial glucose and insulin levels in healthy adults. Tappy et al. [6] reported the same results in adult subjects diagnosed with type two diabetes who consumed 4.0, 6.0 or 8.4 g of β-glucan. On 21 January 1997, FDA (Food and Drug Administration, USA) has included β-glucan in its list of products that contribute to lower the cholesterol level in blood, pointing out how to label the products that contain barley to remark its positive effect on health [7, 8].

On the other hand, for barley breeders aiming to improve malting quality, reduction of β-glucan has been a consistent target as high levels have deleterious effects in malting. During malting, they may reduce the rate of endosperm modification [9], by forming a barrier to the enzymes responsible for protein solubilisation and starch breakdown. In addition, if high levels of β-glucan persist
into the final malt and are solubilised during hot water extraction, they cause increase in wort viscosity which may lead to filtration problems [10].

For a successful breeding programme, the availability of genetic variability and knowledge of gene action to improve each quantitative trait such as β-glucan are essential, otherwise choice of breeding methods used may not result in appreciable improvement. Different biometrical techniques viz., biparental cross [11, 12], diallele and partial diallele cross [13] and line × tester cross [14] have been developed which provide information about additive and dominance genetic variances and fail to produce information about epistasis variance because their procedures are based on certain genetical assumptions including absence of non-allelic interactions [15-17]. Some other biometrical tools viz. generation mean analysis [18, 19], triallele and quadrallele analysis [20, 21] and triple test cross [22] provide information about all three components of genetic variance i.e., additive, dominance and epistatic variances. Among these methods, generation mean analysis is a simple but useful technique for estimating gene effects for a polygenic trait, its greatest merit lying in the ability to estimate epistatic gene effects such as additive × additive [i], additive × dominance [j] and dominance × dominance [l] effects.

Since β-glucan content is a quantitatively inherited trait, controlled by several genetic loci [23], identification of molecular markers associated with a major locus contributing to β-glucan content would be useful for indirect selection for β-glucan content [24]. Thus, an alternative would be to identify molecular markers associated with β-glucan content and to use these markers to indirectly select for this trait [25]. Molecular marker analysis allows to identify genome segments contributing to the genetic variance of a trait and thus to select superior genotypes at these loci, without uncertainties regarding the genotype, due to environment interaction and experimental error. However, in most instances, identifying genetic markers associated with important genes or traits requires screening of a relatively large number of individuals in the population [26]. For this reason we chose to use bulked segregant analysis (BSA) technique [27], since comparing bulk samples is more convenient than evaluating many individuals in different populations [28]. Among the several DNA based techniques, random amplified polymorphic DNA (RAPD) [29, 30], gained importance due to its simplicity, efficiency and non requirement of sequence information [31, 32]. RAPD provides virtually limitless set of descriptors with which to compare individual plants and among the population.

In the present study, the inheritance of (1-3), (1-4)-β-D-glucans content has been studied through generation mean and variance analyses to choose an efficient breeding method for the improvement of this trait. Other main objective of this investigation was to determine whether different polymorphic RAPD markers could be identified in barley bulks made up based on β-glucan content.

**MATERIAL AND METHODS**

**Plant Materials:** Plant material under study included two spring hulless barley genotypes, Azhul (Six-Rowed, originating from USDA-ARS/University of Arizona in 1993, with high β-glucan content) and Falcon (Six-Rowed, originating from Alberta Agriculture in 1993, with low β-glucan content).

**Chemical Analysis:** Mature grains were milled and passed through 0.5 mm sieve using Ultracentrifugal Mill (ZM 100 Retsch, Germany). The level of β-D-glucan was determined using Mixed-linkage β-glucan assay kit (Megazyme, Ireland) based on the method published by McCleary and Codd [33]. This method is accepted by the AOAC (Method 995.16) and the AACC (Method 32-23) [34].

**Generation Mean and Variance Analyses:** Generation mean analysis was carried out on the six basic generations the P1 and P2 (parent cultivars), the F1 and F2 (first and second filial generations) and the BC1 and BC2 (first and second back crosses) of the parental cultivars, Azhul × Falcon. We used the parents of the respective crosses as the male parent and the F1 generation as the female parent and effect back crosses to produce the BC1 (F1 back crossed to P1) and BC2 (F1 back crossed to P2) generations and the F2 hybrids were selfed to obtain F3 seeds. All these generations were produced during two cropping seasons and, as such, all the six generations had to be grown together during the same cropping season (2009-2010) in a randomized block design with three replications at Moghan region. At maturity 15 guarded plants from each of the parents and F1, 20 plants from each of the backcross populations and 120 plants from each of the F2 populations were selected at random to record the data on individual plant basis.

Data were subjected to analysis of variance according to Steel and Torrie [35] to find significant differences among generations for the recorded data. Generation mean analysis was performed using the Mather and Jinks method [36]. In this method, mean of each character is expressed as follows:
\[ Y = m + \alpha[d] + \beta[h] + \alpha^2[i] + 2\alpha\beta[j] + \beta^2[l] \]

Where:
- \( Y \) : mean of one generation
- \([m]\) : mean of all generations
- \([d]\) : sum of additive effects
- \([h]\) : sum of dominance effects
- \([i]\) : sum of additive \(\times\) additive interaction
- \([j]\) : sum of additive \(\times\) dominance interaction
- \([l]\) : sum of dominance \(\times\) dominance interaction and \(\alpha, \beta, \alpha', 2\alpha\beta\) and \(\beta^2\) are the coefficients of genetic parameters. The genetic model that best fits the data was found by the mean of joint scaling test \([36]\) and the accuracy of the models was verified by chi-square (\(\chi^2\)) test. Components within each model were evaluated for significance by t-test. The type of epistasis was determined only when dominance \([h]\) and dominance \(\times\) dominance \([l]\) effects were significant. When these effects had the same sign, the effects were complementary while different signs indicated duplicate epistasis \([37]\).

Variance components (additive, dominance and environmental) were estimated as described by Kearsey and Pooni \([37]\), using the following equations:

\[
\begin{align*}
D &= 4VF_2 - 2(VBC_1 + VBC_2) \\
H &= 4(VBC_1 + VBC_2 - VF_2 - E) \\
F &= VBC_1 - VBC_2 \\
E &= (VP_1 + VP_2 + 2VF_1)/4
\end{align*}
\]

Where:
- \( D \) : Additive variance
- \( H \) : Dominance variance
- \( F \) : Correlation between \( D \) and \( H \) over all loci
- \( E \) : Environmental component of variance

Broad-sense and narrow-sense heritabilities values calculated using the following formula:

\[
\begin{align*}
\hat{h}_{b,s}^2 &= \{(VF_2 - (VP_1 + VP_2 + 2VF_1)/4)/VF_2\} \quad [36] \\
\hat{h}_{n,s}^2 &= \{(VF_2 - (VF_1 + VP_2)\}/VF_2\} \quad [38] \\
\hat{h}_{b,s}^2 &= \{(VF_2 - (VP_1 + VP_2 + VF_1)/3)/VF_2\} \quad [39] \\
\hat{h}_{b,s}^2 &= \{(VF_2 - (VP_1 + VP_2 + VF_1)\}/VF_2\} \quad [40] \\
\hat{h}_{n,s}^2 &= \{(VF_2 - (VP_1 + VP_2)/2)/VF_2\} \quad [39] \\
\hat{h}_{n,s}^2 &= \{(VF_2 - (VBC_1 + VBC_2)/2)/VF_2\} \quad [40]
\end{align*}
\]

Response to selection was estimated with 5% selection intensity (\(i\)) (selection differential, \(K = 2.06\)) as:

\[ Gs = i \times \hat{h}_{n,s}^2 \times \sqrt{VF_2} \]

Gene number was calculated using the following formula:

Formula number 1:

\[ n = \left( \frac{\bar{P}_1 - \bar{P}_2}{2} \right)^2 /[8(VF_2 - VF_1)] \quad [41] \]

Formula number 2:

\[ n = \left( \frac{\bar{P}_1 - \bar{P}_2}{2} \right)^2 /[8(VF_2 - VBC_1 - VBC_2)] \quad [42] \]

Formula number 3:

\[ n = \left( \frac{\bar{P}_1 - \bar{P}_2}{2} \right)^2 /[8(VF_2 - VE)] \quad [41] \]

Formula number 4:

\[ n = \left( \frac{\bar{P}_1 - \bar{P}_2}{2} \right)^2 /[8(VBC_1 + VBC_2 - VE)] \quad [42] \]

**Bulked segregant analysis with RAPDs:** Young leaf materials from parents and \(F_2\) individuals were extracted for genomic DNA, according to the method described by Dellaporta et al. \([43]\). Bulked segregant analysis (BSA) technique \([27]\) with RAPD markers was used to identify markers associated with \(\beta\)-glucan content in the population Azhul \(\times\) Falcon. Four RAPD reactions were performed with each primer pair on the DNA extracted from the following groups of barley plants: 1- the parent with high \(\beta\)-glucan content (Azhul), 2- the parent with low \(\beta\)-glucan content (Falcon), 3- the pooled DNA of eight \(F_2\) plants with high \(\beta\)-glucan content and 4- the pooled DNA of seven \(F_2\) plants with low \(\beta\)-glucan content.

The PCR reaction mixture consisted of 20-50ng genomic DNA, 1×PCR buffer, 2.0 mM MgCl\(_2\), 100 iM of each dNTP, 0.1 iM primer and 1 unit of Taq polymerase in a 25L volume. The amplification protocol was 94°C for 4 min to pre-denature, followed by 45 cycles of 94°C for 1 min, 36°C for 1 min and 72°C for 1 min, with a final extension at 72°C for 10 min. Amplification products were fractionated on 1.4% agarose gel. Gels were photographed under UV light with Polaroid 667 films. Reproducibility of the RAPD analytical procedure was investigated with repeated analysis of samples. Only those bands which showed consistent amplification were chosen for use in this study.
Table 1: Estimates of different genetic parameters for β-glucan content in cross Azhul × Falcon

<table>
<thead>
<tr>
<th>Genetic parameters</th>
<th>Generation mean analysis</th>
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</thead>
<tbody>
<tr>
<td>Mean [m]</td>
<td>6.43±0.167**</td>
</tr>
<tr>
<td>Additive effects [d]</td>
<td>2.48±0.167**</td>
</tr>
<tr>
<td>Dominance effects [h]</td>
<td>6.22±0.868**</td>
</tr>
<tr>
<td>Additive × additive effects [i]</td>
<td></td>
</tr>
<tr>
<td>Additive × dominance effects [j]</td>
<td>-6.12±1.24**</td>
</tr>
<tr>
<td>Dominance × dominance effects [l]</td>
<td>-3.53±0.91**</td>
</tr>
<tr>
<td>Chi-square [χ²]</td>
<td>0.841**</td>
</tr>
</tbody>
</table>

Generation variance analysis

| Additive variance [D]                       | 0.59                    |
| Dominance variance [H]                     | 1.01                    |
| Correlation between D and H over all loci [F]| 0.13                    |
| Environmental variance [E]                 | 0.14                    |
| Average degree of dominance [(H/D)¹/²]     | 1.31                    |
| Genetic advance [Gs]                       | 8.39                    |

Heritabilities

| Broad sense heritability [h²n]             | 0.79                    |
| Broad sense heritability [h²e]             | 0.82                    |
| Broad sense heritability [h²d]             | 0.80                    |
| Broad sense heritability [h²h]             | 0.80                    |
| Mean [h²]                                 | 0.81                    |
| Narrow sense heritability [h²n]            | 0.42                    |

Number of segregating genes (QTLs)

| Number of QTLs (Formula number 1)          | 5.81                    |
| Number of QTLs (Formula number 2)          | 7.77                    |
| Number of QTLs (Formula number 3)          | 5.63                    |
| Number of QTLs (Formula number 4)          | 3.84                    |
| Mean                                       | 5.76                    |

ns, * and ** : Non significant, significant at 5% and 1% of probability levels, respectively

RESULT AND DISCUSSION

Generation Mean and Variance Analyses: Analysis of variance for (1−3), (1−4)-β-D-glucans revealed significant difference among generations (data not shown). We were therefore, allowed to go ahead to study heredity and to analyze generation means.

The estimates of additive, dominance and epistasis effects, additive, dominance and environment components of variance and broad and narrow sense heritabilities are presented in Table 1. The results of generation mean analysis revealed that five parameter model [m, d, h, j, l] is adequate, the χ² being non-significant. We can, therefore, interpret the β-glucan in terms of the additive, dominance and digenic non-allelic interactions including additive × dominance and dominance × dominance effects. The gene effects [h] and [l] took opposite sign indicating presence of duplicate dominance epistasis in the inheritance of this character [44].

The estimates of genetic variance components showed that, although both additive and dominance variances were important, the greater ratio of (H/D)¹/² from unity indicated over dominance gene action for this trait. Also, narrow sense heritability estimate (0.42) was lower than broad sense heritability (0.81) suggested that the inheritance of this trait is complex and polygenic.

The estimated numbers of QTLs controlling β-glucan using different formulae are presented in Table 1. Although the estimates of different formulae may be subjected to their inherent assumptions, it is seen that estimated numbers, were close to each other. Based on these formulae, it appears that almost 4 to 8 genes are segregating in the Azhul × Falcon cross. These types of analyses estimate the maximum number of genes; however, the genes controlling quantitative traits could be linked and could, therefore, segregate as a group or effective factor [37]. If this were true for the present study, the formulae would have estimated the number of effective factors and the number of individual genes would have been greater.

Due to the presence of over dominance type of gene action and the presence of duplicate dominant epistasis in the expression of this trait, selection of this trait in early generation will be difficult. As selection based on progeny performance exploits only additive component of
genetic variances for this trait bi-parental mating followed by recurrent selection or diallel selective mating, which allows intermating among the selected segregates in the different cycles, would be useful to recover superior homozygote in later generations. Huan et al. [45] have also reported similar type of gene action for this trait in barley. However, Powell et al. [46], Holthaus et al. [47], Cervantes-Martinez et al. [48] and Eshghi and Akhundova [49] showed that β-glucan is controlled by a simple additive genetic system in barley and oat.

In conclusion, although the results of this experiment may be applicable only to the germplasm used herein, the identification of dominance and epistatic effects suggest that additional research is necessary.

**Bulked Segregant Analysis:** Of the two hundred eighty one RAPD primers screened, two RAPD primers S39 (5’ CAAACGTCGG 3’) and WE013 (5’ CCCGTCAGCA 3’) showed consistent banding pattern after repeated amplifications. RAPD primer WE013 amplified a 1750 b.p band (Figure 1) in the parent with high β-glucan content (Azhul) and eight individuals of the high β-glucan content bulk, whereas no band was observed in the other parent (Falcon) and seven individuals of the low β-glucan content bulk at this region. The reason for the one individual in both the bulks not amplifying the required band could be due to β-glucan content being a quantitative trait and this particular locus being one of them or possibly due to selection error. This proved that WE013 was a positive marker for high β-glucan content. On the contrary, RAPD primer S39 amplified a 150 b.p band (Figure 2) in the parent with low β-glucan content (Falcon) and seven individuals of the low β-glucan content bulk, but absent in the parent with high β-glucan content (Azhul) and all the individuals of the high β-glucan bulk. Thus S39 was used as a marker to selection of plants with low β-glucan content. In addition to the above genotypes, these RAPD primers were screened on several other diverse genotypes and the results were consistent with their respective (1–3), (1–4)-β-D-glucan contents (Figure 3). The reproducible amplification of these fragments in repeated amplifications suggests that they are associated with a major gene(s) that contributes to enhanced β-glucan content. Hence, both S39 and WE013 can be utilized in a marker-assisted selection (MAS) programme for β-glucan content in barley. Efforts are underway to convert the two RAPD primers into SCAR markers and subsequently map them. In separate studies, Yu et al. [50] and Eshghi and Akhundova [51] also, reported the correlation between S39 primer and β-glucan content in hulless barley genotypes.

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Fig. 1: Results of RAPD amplification based on the use of primer WE013 in barley. 1: DNA marker, 2: Azhul, 3: Falcon, 4: bulk with high β-glucan content, 5: bulk with low β-glucan content. 6-13: F2 individuals with high β-glucan content, 14-20: F2 individuals with low β-glucan content. RAPD marker is indicated with an arrow (only relevant part of the representative gel is shown)

Fig. 2: Results of RAPD amplification based on the use of primer S39 in barley. 1: DNA marker, 2: Falcon, 3: Azhul, 4: bulk with low β-glucan content, 5: bulk with high β-glucan content. 6-12: F2 individuals with low β-glucan F2 content, 13-20: F2 individuals with high β-glucan content. RAPD marker is indicated with an arrow (only relevant part of the representative gel is shown)
Fig. 3: RAPD analysis of barley genotypes with primer S39. 1-DNA marker, 2- IAC- 8501/31, 3- IAC-8501/12, 4- IAC-8612/421, 5- M351, 6- Niran, 7- CDC Bold, 8- CDC 92-05-06-48, 9- CDC Alamo, 10- CDC 92-55-06-54, 11- SR93135, 12- Merkur, 13: Orbit, 14: Heda, 15: Zlatan, 16: Atlas. RAPD marker is indicated with an arrow (only relevant part of the representative gel is shown).

The results also demonstrate that a BSA strategy may be useful even for the identification of markers for quantitative traits, such as β-glucan content. Recently, similar approaches have been used to identify markers associated with other quantitatively inherited traits, namely disease resistance in tomato [52], economically important traits in Pinus [53], anther culture response in potato [54], water-stress tolerance in barley [55] and salt tolerance in Wheat [56].

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