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High fructan barley lines produced by selective breeding may alter β-glucan and amylopectin molecular structure

Shishanthi Jayarathna [a,*], Yunkai Jin [b], Gleb Dotsenko [a,1], Mingliang Fei [b,c,d], Mariette Andersson [e], Annica A.M. Andersson [a], Chuanxin Sun [b], Roger Andersson [a]

[a] Department of Molecular Sciences, BioCenter, Swedish University of Agricultural Sciences, P.O. Box 7015, SE-750 07 Uppsala, Sweden
[b] Department of Plant Biology, BioCenter, Swedish University of Agricultural Sciences, P.O. Box 7080, SE-750 07 Uppsala, Sweden
[c] Key Laboratory of Crop Epigenetic Regulation and Development in Hunan Province, Hunan Agricultural University, Changsha 410128, China
[d] Key Laboratory of Education Department of Hunan Province on Plant Genetics and Molecular Biology, College of Bioscience and Biotechnology, Hunan Agricultural University, Changsha 410128, China
[e] Department of Plant Breeding, Swedish University of Agricultural Sciences, P.O. Box 190, SE-234 22 Lomma, Sweden

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ABSTRACT

Six cross-bred barley lines developed by a breeding strategy with the target to enhance the fructan synthesis activity and reduce the fructan hydrolysis activity were analyzed together with their parental lines, and a reference line (Gustav) to determine whether the breeding strategy also affected the content and molecular structure of amylopectin and β-glucan. The highest fructan and β-glucan content achieved in the novel barley lines was 8.6 % and 12 %, respectively (12.3-fold and 3.2-fold higher than in Gustav). The lines with low fructan synthesis activity had higher starch content, smaller building blocks in amylopectin, and smaller structural units of β-glucans than the lines with high-fructan synthesis activity. Correlation analysis confirmed that low starch content was associated with high amylose, fructan, and β-glucan content, and larger building blocks in amylopectin.

1. Introduction

Barley (*Hordeum vulgare*) was one of the first domesticated crops, and the main component of barley grain is starch. It is also rich in dietary fiber and attracting growing interest as a healthy food (Baik & Ullrich, 2008; Sullivan, Arendt, & Gallagher, 2013). There are two major components in barley dietary fiber, mixed linkage (1 → 3, 1 → 4)-β-D-glucan (β-glucan) and arabinoxylan. The β-glucan content typically ranges from 3 % to 7 % (Oscarsson, Andersson, Salomonsson, & Åman, 1996) and the molecular structure of the β-glucan plays a vital role in determining its functionality (Du, Meenu, Liu, & Xu, 2019). The arabinoxylan content in barley typically varies from 4 % to 11 % (Oscarsson et al., 1996). Cellulose, fructan, and lignin are among the minor components of barley dietary fiber.

Since the main component of barley endosperm is starch, the quality of barley-based foods could be affected by the quality of the starch (Zhu, 2017). The molecular structure, composition, and amylose:amylopectin ratio in starch play a vital role in determining the quality of the starch for food and non-food applications. In general, barley starch contains 20–25 % amylose and 70–75 % amylopectin (Morrison, Milligan, & Azudin, 1984), although the amylose content depends on the method of determination. Barley amylose is a linear molecule with molecular weight 1.03×10^5 g/mol, compared with 1.15×10^6 g/mol for highly branched amylopectin (Bello-Pérez, Rodríguez-Ambriz, Agama-Acevedo, & Sanchez-Rivera, 2009). The properties of starch have been shown to be affected by amylopectin molecular structure and amylose: amylopectin ratio (e.g., Källman et al., 2015; Vamadevan & Bertoft, 2015; Vamadevan & Bertoft, 2018; Zhao, Hofvander, Andersson, & Andersson, 2023; Zhu, 2018; Zhu & Liu, 2020).

A building block (BB) backbone model is currently used to describe the distribution of chains in amylopectin molecules (Bertoft, 2017; Tetlow & Bertoft, 2020). BBs, which are the basic structural units of amylopectin, are tightly branched and distributed along the long chains of amylopectin. The BBs are made up of approximately 2–11 chains,

* Corresponding author.
E-mail addresses: shishanthi.jayarathna@slu.se (S. Jayarathna), yunkai.jin@slu.se (Y. Jin), mariette.andersson@slu.se (M. Andersson), annica.andersson@slu.se (A.A.M. Andersson), Chuanxin.Sun@slu.se (C. Sun), roger.andersson@slu.se (R. Andersson).
[1] Present address: Laboratory of Evolutionary Genomics, Vavilov Institute of General Genetics, Russian Academy of Sciences, Moscow, Russia.

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with the number of chains increasing proportionally with increasing BB size (Bertoft, Koch, & Åman, 2012). BBs contribute directly in determining the physical properties of starch such as gelatinisation and retrogradation properties (Källman et al., 2015; Zhao et al., 2023). Therefore, information regarding BBs contributes to determining the end uses of particular starch types and the knowledge can be utilized by plant breeders to tailor starches that are, for example, more stable for freeze-thaw cycles.

Barley is one of the most genetically diverse cereals, which provides ample opportunities for breeders to identify and produce novel varieties for specific end uses (Baik & Ullrich, 2008). For example, when barley is bred for human consumption, high β-glucan content in grain is preferred since high β-glucan is favorable to reduce blood cholesterol and risk of colorectal cancer (Kerckhoffs, Hornstra, & Mensink, 2003). Therefore, improving grain β-glucan content has become one of the foci in barley breeding programs (e.g., Ehrenbergerová et al., 2008; Steele et al., 2013). High fructan content is an advantage in edible dietary fiber, since fructan and fructooligosaccharides are standard prebiotics with beneficial health effects and considered as a low calorie healthy food and feed ingredient (Bosscher, 2009; Ritsema & Smeekens, 2003; Roberfroid, 2007; Roberfroid et al., 2010). Fructans also play a role in protecting plants against stress factors such as drought and freezing (Benkeblia, 2022; Livingston, Hincha, & Heyer, 2009).

In barley carbon allocation between starch and fructan is regulated via sugar signaling in barley (SUSIBA) transcription system where SUSIBA2 is a transcription factor which induces starch synthesis, and SUSIBA1 is a negative transcription factor which inhibits fructan synthesis. The presence of SUSIBA2 and SUSIBA1 forms a carbon competing system in barley that could be employed for the barley breeding (Jin et al., 2017). In a recent study, a cross-breeding strategy was applied to produce barley lines with enhanced fructan content by upregulating fructan synthesis activity and downregulating fructan hydrolysis activity (Fei et al., 2022). During breeding, the SUSIBA transcription system (Jin et al., 2017) was used as a molecular marker for progeny screening as described by Fei et al. (2022). In parallel with artificial screening for high-fructan lines, starch content was modified based on the function of the SUSIBA system in regulating carbon allocation (Fei et al., 2022; Jin et al., 2017; Sun et al., 2003). Moreover, an interesting correlation between fructan and β-glucan in high fructan barley lines was already revealed by Fei et al. (2022) (with Pearson correlation coefficient (r) of0.9121), showing the possibility of producing barley lines with simultaneous high fructan and β-glucan.

In the present study, starch and β-glucan from the high-fructan barley lines and their parents were chemically characterized, to test the hypothesis that the function of the SUSIBA system in regulating carbon allocation affects the content, composition, and molecular structure of starch and β-glucan, in addition to the fructan content.

2. Materials and methods

2.1. Development of cross-bred barley lines

Barley plants were cultivated in phytotrons, with conditions of 9 h light (300 μmol m^{-2} s^{-1}) at 12 °C, 15 h darkness at 8 °C, 60 % relative humidity for the first 4 weeks, and then to 16 h light (400 μmol m^{-2} s^{-1}) at 20 °C, 8 h darkness at 12 °C, and 70 % relative humidity until maturation. Barley crossing was performed according to Poehlman and Sleper (2013) and as explained by Fei et al. (2022). In brief, SW 28708 (line 224, Lantmännen, Sweden) was used as the maternal to cross with KVL 1113 (line 199, Royal Veterinary and Agricultural University, Denmark) and SLU 7 (line 155, Swedish University of Agricultural Sciences), and KVL 1113 was used as the maternal to cross with SW 49368 (line 235, Lantmännen, Sweden). All the parental lines were genetically homozygous. The flat seed phenotype was correlated with high fructan content, so in the selection process flat seed phenotype was used as a screening marker of high-fructan grain to increase the speed of screening high fructan lines (Fei et al., 2022). After three-generations of inbreeding the F_4 progeny of barley lines with unique flat seeds (Fig. S1) were screened and used in further analyses unless otherwise stated.

The selection of parental lines was based on the fructan content of the developing barley grains at 9 day after flowering (daf), 22 daf and at maturity respectively and the fructan level reduction between 22 daf and maturity (Table 1 and Fei et al. (2022). As measured by Fei et al. (2022), lines 155 and 199 accumulated high amount of fructan during the developmental stages of 9 daf to maturity and reach >3 % at mature stage, while lines 224 and 235 accumulated low level of fructan than 155 and 199 at mature stage. The fructan level decrease between 22 daf and maturity were highest in 199 (decreased by 12.0 %), followed by 155 (decreased by 7.8 %). Lines 224 and 235 reported the lowest fructan level (decreased by <2.0 %) between 22 daf and maturity (Fei et al., 2022). Based on the fructan accumulation and fructan level reduction between 22 daf and maturity, different combinations of parental lines were crossed with each other to combine the properties of a high rate of fructan synthesis and a low rate of fructan hydrolysis as explained by (Fei et al., 2022).

Fructan synthesis and hydrolysis activity were defined based on fructan content analysis using the F3 progenies, as described by Fei et al. (2022). Five grains from middle positions of each spike were collected from ten individual plants of each line where three plants were randomly selected to present the fructan levels. Grain samples from the same plants were collected at 15:00 h at developmental stages 9 daf, 22 daf, and maturity. As fructan synthesis in barley generally occurs at an early stage of development, the fructan content at 9 daf in experimental barley lines compared with a reference line (Gustav) was used to represent fructan synthesis activity. Lines with significantly higher fructan content than Gustav (20.7 ± 0.8 %) were classified as having high fructan synthesis activity, while all the other lines, together with Gustav, were classified as lines with low fructan synthesis activity (Table 1).

Fructan hydrolysis generally occurs at the later stage of barley grain development, and therefore the hydrolysis rate between 22 daf and maturity was used to define hydrolysis activity. Lines with hydrolysis rate higher than 50 % were defined as having high hydrolysis activity, while all other lines were defined as having low hydrolysis activity (Table 1).

The samples obtained were categorized into two main groups based on fructan synthesis activity as (1): samples with high fructan synthesis activity (group A) and (2): samples with low fructan synthesis activity (group B) (Table 1).

2.2. Starch extraction

A laboratory-scale barley starch extraction procedure was developed based on the method described by Källman et al. (2015) with the following three modifications: i) Barley grains were milled using an ultra-centrifugal mill of type ZM 200 (Retsch GmbH, Germany) at a speed of 1800 min^{-1}; ii) barley whole flour (3 g) was steeped in 15 mL 0.02 M HCl, the pH was adjusted to between 2.5 and 3.0, and the mixture stirred overnight before neutralizing and mixing with an Ultra-Turrax; and iii) 0.05 M Tris-HCl buffer (pH 7.8, containing 0.25 % $NaHSO_3$ and 0.02 % sodium azide) and proteinase K (from *Parengyodontium album* (*Tritirachium album*); *E*-PRKMB, EC 3.4.21.62, specific activity >40 U/mg protein (on urea-denatured hemoglobin) at pH 7.5 and 37 °C) were used.

2.3. β-Glucan and fructan content

β-Glucan content was determined using the mixed-linkage β-glucan kit (K-BGLU, Megazyme, Bray, Ireland) according to McCleary and Codd (1991). Fructan content was determined using the fructan kit (K-FRUC, Megazyme, Bray, Ireland) according to McCleary, Murphy, and Mugford (1997).

Table 1
Grain fructan content (%, DM basis) of barley lines of F_3 progenies at different development stages, their fructan hydrolysis rate between 22 daf and maturity and the groups based on fructan synthesis activity (A = high, B = low) together with the grain fructan and β-glucan content (%, DM basis) of 10 experimental barley lines (samples 1–10) and a reference barley variety (Gustav, sample 11) of F_4 progenies (mean ± standard deviation). Values within columns with different superscript letters differ significantly (ANOVA, α = 0.05). Some of the data presented in the table has previously reported by Fei et al. (2022)*. Standard deviations are modified for sample standard deviation.

Sample	Genealogy	Fructan content at 9 daf (%, DM basis) of F_3 progenies	Fructan synthesis activity	Fructan synthesis activity group	Fructan content at 22 daf (%, DM basis) of F3 progenies	Fructan content at maturity (%, DM basis) of F3 progenies	Fructan hydrolysis rate (%)	Fructan hydrolysis activity	Fructan content of at maturity (%, DM basis) of F4 progenies	β-Glucan content at maturity (%, DM basis) of F4 progenies
1	#155	30.3* ± 2.2^{a}	High	A	12.0*	4.2*	65.2	High	3.8 ± 0.2^{c}	9.6 ± 0.2^{c}
2	#199	32.2* ± 1.3^{a}	High	A	15.6*	3.9*	74.9	High	3.4 ± 0.1^{d}	9.3 ± 0.3^{c}
3	#224	21.3* ± 0.9^{b}	Low	B	3.9*	2.5*	35.2	Low	2.2 ± 0.1^{e}	7.5 ± 0.1^{d}
4	#235	21.2* ± 0.8^{b}	Low	B	3.7*	1.9*	47.7	Low	1.0 ± 0.1^{f}	6.6 ± 0.2^{e}
5	♀#224×♂#155, flat	36.0* ± 3.4^{a}	High	A	14.3*	11.8*	17.7	Low	8.6 ± 0.1^{a}	11.5 ± 0.1^{a}
6	♀#224×♂#199	31.5* ± 2.0^{a}	High	A	14.3*	11.1*	22.7	Low	8.1 ± 0.2^{b}	12.0 ± 0.0^{a}
7	♀#199×♂#155	34.4 ± 1.0^{a}	High	A	12.9	5.3	58.5	High	2.1 ± 0.1^{e}	10.6 ± 0.1^{b}
8	♀#155×♂#199	30.9 ± 0.9^{a}	High	A	18.2	6.0	66.9	High	2.0 ± 0.1^{e}	10.9 ± 0.0^{b}
9	♀#224×♂#155, round	21.5 ± 1.0^{b}	Low	B	7.7	2.5	67.0	High	0.8 ± 0.0^{f}	7.1 ± 0.1^{d,e}
10	♀#199×♂#235	25.5 ± 2.8*^{a}	High	A	11.6*	6.1*	47.5	Low	3.3 ± 0.1^{d}	10.8 ± 0.1^{b}
11	249 (Gustav)	20.7 ± 0.8^{b}	Low	B	8.9	1.8	80.2	High	0.7 ± 0.1^{f}	3.8 ± 0.0^{f}

2.4. β-Glucan structural composition

β-Glucan structural composition was determined by lichenase digestion followed by high performance anion-exchange chromatograph/pulsed amperometric detection (HPAEC-PAD) analysis, as described by Andersson et al. (2004). HPAEC-PAD was performed with a CarboPac PA-100 column eluted at 1 mL/min with eluent A (0.15 M NaOH) and eluent B (0.15 M NaOH and 0.5 M NaOAc) according to the following program: 0–20 min: 15–28 % eluent B; 20–35 min: 28–50 % B; 35–45 min: 50 % B; 45–50 min: 50–15 % B (return to the start mixture); and 50–65 min: 15 % B. Electrode pulse potential and duration were as follows: E1 = 0.1 V, 0.4 s; E2 = −2.0 V, 0.02 s; E3 = 0.6 V, 0.01 s; E4 = −0.1 V, 0.06 s. Signals were integrated over 0.2 s (0.2 to 0.4 s).

2.5. Starch content and amylose content

The starch content in barley whole flour was determined according to an existing method (Åman, Westerlund, & Theander, 1994) with the slight modifications that barley whole flour (20 mg), 50 μL thermostable α-amylase from *Bacillus licheniformis* (EC 3.2.1.1, 3000 U/mL, Megazyme, Wicklow, Ireland), 100 μL of 10-fold diluted amyloglucosidase from *Aspergillus niger* (EC 3.2.1.3, 3260 U/mL soluble starch, Megazyme, Wicklow, Ireland) in acetate buffer, and 3 mL of GOPOD reagent (Megazyme, Wicklow, Ireland) were used. The absorbance was measured at 510 nm against a reagent blank. A two-point calibration curve was developed using 1 mg/mL and 0.5 mg/mL glucose standards incubated with a 3 mL GOPOD reagent, to calculate the glucose concentration of the samples.

The amylose content in barley whole flour was analyzed by a colorimetric method according to Chrastil (1987) with the following slight modifications: Barley whole flour (30 mg) was solubilized according to Morrison and Laignelet (1983) in 3 mL UDMSO (0.6 M urea in 90 % DMSO) added on two occasions, and incubated in a 100 °C water bath for 30 min with occasional mixing. Then 100 μL of sample were transferred to each of two Eppendorf tubes and 200 μL and 700 μL of 99.5 % ethanol were added and mixed on two occasions. The tubes were left to stand in an ice bath for 30 min, centrifuged at 10500 ×*g* for 15 min and the pellet was washed with 1.8 mL 95 % ethanol and redissolved in 100 μL UDMSO at 100 °C for 15 min. The samples were transferred to new tubes by washing with 3 × 1 mL of 0.5 % Trichloroacetic acid (TCA) and an additional 2 mL of 0.5 % TCA were added. Two reaction blanks containing 5 mL of TCA were included from this step onwards. Then 50 μL 0.01 N I_2-KI solution (1.27 g I_2 and 3 g KI per L) were added, the tube contents were mixed, and the tubes were placed in a 25 °C water bath for 30 min. The absorbance was read against water at 620 nm. The amylose content was determined using a standard curve with defined amylose content and values are reported as average of two replicates based on total starch content (DM basis).

2.6. Production of BBs

Production and characterization of BBs were performed according to the method developed and described by Zhao, Andersson, and Andersson (2021). For BB block distribution analysis, the BBs were prepared by hydrolyzing whole starch using β-amylase (*E*-BARBL, Megazyme, Wicklow, Ireland) and α-amylose (E-BAASS, Megazyme, Wicklow, Ireland).

First, whole starch samples were subjected to β-amylolysis by β-amylase to remove the linear chains of amylose and amylopectin external chains and to produce β-limit dextrins (β-LD) s. The β -amylase was twice de-salted through PD-10 desalting columns (Sephadex, Amersham Pharmacia Biotech AB, Uppsala, Sweden) using sodium acetate (NaOAc) buffer before using.

The β-LDs produced were then hydrolyzed with α-amylase. Extensive α-amylosis yielded α-limit dextrins (α-LD) s. The isolated α-LDs were again treated with β-amylase to ensure no external chains remained in the resulting BBs. The enzymes were then denatured by heating in a boiling water bath and the BBs were isolated by, filtering through a membrane filter (0.45 μm) (Zhao et al., 2021).

2.7. High performance size exclusion chromatography (HPSEC) for BB analysis

The BBs were analyzed using HPSEC and the HPSEC setting was as described by Zhao et al. (2021). In brief, the HPSEC system was equipped with a refractive index (RI) detector (Wyatt Technology Corp., Santa Barbara, CA) which measures the concentration of the elutes, and two serially connected OHpak SB-802.5 HQ columns with a guard column (Shodex, Showa Denko KK, Miniato, Japan) which have been kept at 35 °C. The fragments were eluted using 0.1 M $NaNO_3$, containing 0.02 % NaN_3 with a flow rate of 0.5 mL/min. Data were analyzed using ASTRA software (version 4.70.07, Wyatt Technology Corp., Santa Barbara, CA).

The results were presented as the mean of two replicates, and the sample blank was subtracted to eliminate the enzyme and buffer peaks

in the elution profiles. The chromatograms were normalized for the peak area between 13 and 17.5 mL elution volume and divided into 9 buckets for further analysis.

2.8. HPAEC-PAD for BB analysis

An HPAEC instrumentation (Series 4500i, Dionex Corp., Sunnyvale, CA, USA) equipped with a BioLC gradient pump and a pulsed amperometric detector (PAD) was used in this study. The HPAEC-PAD setting was as described by Zhao et al. (2021). In brief, a Carbopac PA-100 (4 × 250 mm) analytical column (Dionex, Sunnyvale USA) equipped with a guard column was used for separation. Elution was performed at 25 °C, at a flow rate of 1 mL/min, with an injection volume of 25 μL, using 0.15 M NaOH (eluent A) and 0.50 M NaOAc +0.15 M NaOH (eluent B) with the following gradient: 0–15 min: 15–28 % eluent B; 15–45 min: 28–55 % B; 45–55 min: 55 % B; and 55–60 min: 55–15 % B (return to the start mixture). The PAD response of BBs was calculated as relative peak area.

2.9. Statistical analyses

Differences in measured parameters were studied by One-way analysis of variance (ANOVA). Tukey pairwise comparisons and Dunnett's test were performed using Minitab 18 (State College, PA, USA). Principal component analysis (PCA) was carried out using Simca 14.0 (Umetrics, Umeå, Sweden). Spearman's rank correlation coefficient analysis was performed using Minitab 18 (State College, PA, USA).

3. Results and discussion

3.1. Fructan synthesis activity of the barley lines

Based on the fructan synthesis activity of the F_3 progenies, the barley lines were divided into two groups, defined as having high fructan synthesis activity with an average fructan content of 31.5 ± 3.6 % at 9 daf (group A) and low fructan synthesis activity, with an average fructan content of 21.2 ± 0.8 % at 9 daf (group B) (Table 1).

3.2. Fructan and β-glucan content of mature grain

Fructan and β-glucan content in mature grain of F_4 progenies differed ($p < 0.05$) between the two groups. Group A lines had a higher grain fructan content (4.5 ± 2.6 % on average) than group B lines (1.2 ± 0.6 % on average). The average grain β-glucan content of group A lines was 10.7 ± 0.9 % and that of group B lines was 6.3 ± 1.6 %. Individual values of fructan and β-glucan content in grains of F4 generation are presented in Table 1 and used for further analysis and interpretation of the data. The maximum fructan content was 8.6 % (sample 5), which was 12-fold higher than in Gustav. All experimental barley lines (samples 1–10) demonstrated at least 1.5-fold higher β-glucan content than Gustav. In general, the β-glucan content in barley varies between 3 and 11 % (Loskutov & Khlestkina, 2021) and the maximum β-glucan content obtained in the current study was 12.0 % (sample 6), closer to upper limit reported by Loskutov and Khlestkina (2021), which was nearly 3-fold higher than in Gustav.

An interesting correlation between β-glucan and fructan content was observed. Higher β-glucan content was accompanied by higher fructan content, which demonstrated an exponential increase (Fig. 1). One possible explanation for this is that processes of β-glucan and fructan biosynthesis are orchestrated (or at least upregulated) by the same mechanism.

3.3. β-Glucan structural composition

Molecular features of β-glucan affect its solubility and rheological properties (Skendi, Biliaderis, Lazaridou, & Izydorczyk, 2003). The molecular structure of β-glucan was investigated using lichenase

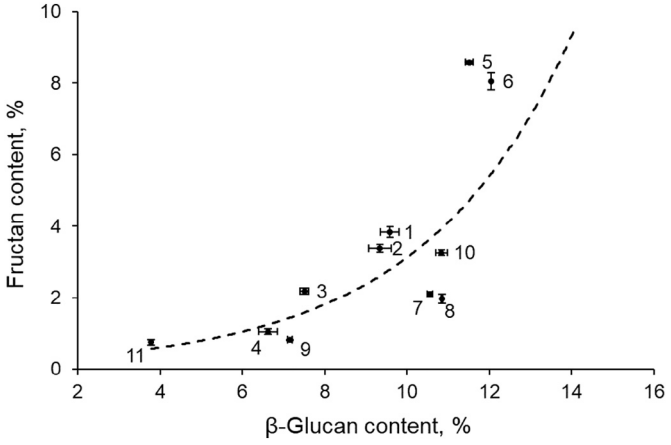

Fig. 1. Relationship between β-glucan content and fructan content in grain from the experimental barley lines (1−10) and the reference variety (11). Mean ($n = 2$), bars indicate standard deviation. For sample descriptions, see Table 1.

analysis. Lichenase (*endo*-β-1,3-1,4-glucanase; EC 3.2.1.73) specifically cleaves the β-(1 → 4)-linkage of the 3G1 → 4G1 units of β-Glucan, yielding oligosaccharides containing a single β-(1 → 3)-linkage adjacent to the reducing end (Izydorczyk & Dexter, 2008). As β-glucan consists mainly of cellotriosyl and cellotetraosyl units, trisaccharide (Degree of polymerization (DP)3), and tetrasaccharide (DP4) are the main products of β-glucan hydrolysis by lichenase (Izydorczyk & Dexter, 2008; Skendi et al., 2003; Stevenson & Inglett, 2009).

Based on the data obtained the samples were divided into two groups (the same groupings as for fructan synthesis activity) according to their pattern of β-glucan molecular structure. The sample group A (samples 1, 2, 5, 6, 7, 8, 10) displayed a higher proportion of longer structural units, with a normalized average relative peak area of 24.0 ± 1.5 %, compared to barley lines belonging to group B (samples 3, 4, 9, and 11), which had a normalized average relative peak area of 14.0 ± 1.2 % ($p < 0.05$). However, the sample group B (samples 3, 4, 9, and 11), displayed a higher proportion of DP3 and DP4 structural units, with a normalized average relative peak area of 85.9 ± 1.2 %, compared to barley lines belonging to group A (samples 1, 2, 5, 6, 7, 8, 10), which had a normalized average relative peak area of 76 ± 1.5 % ($p < 0.05$). It should be noted that barley lines of group A had high fructan synthesis activity, while barley lines of group B had low fructan synthesis activity.

The ratio of cellotriosyl to cellotetraosyl units (DP3/DP4) was found to be similar in all samples (DP3 / DP4 = 1.65) (Supplementary Table S1), which is common for barley cultivars sharing the same genotypic and environmental background (Izydorczyk & Dexter, 2008). The DP3/DP4 ratio obtained was slightly lower than reported previously for barley grain, e.g., 1.8–2.2 (Izydorczyk, Macri, & MacGregor, 1998) and 1.8–2.4 (Lazaridou, Chornick, Biliaderis, & Izydorczyk, 2008).

There was an interesting correlation between β-glucan molecular structure and β-glucan content. As the proportion of β-glucan increased, proportions of DP3 and DP4 structural units decreased, while the proportions of longer structural units increased (Figs. 2, Fig. S2). Therefore, as it follows from the data obtained, upregulation of β-glucan biosynthesis resulted in production of β-glucan with lower ratios of DP3 and DP4 structural units, but with higher ratios of longer structural units. Although the genes associated with β-glucan synthesis have been identified, the method by which genetic regulation of β-glucan accumulation in barley grains occurs, is not yet known (Geng et al., 2021).

3.4. Starch and amylose content

A low starch content (mean 38.7 ± 3.5 %) was found to be associated with group A samples with high fructan synthesis activity ($p < 0.05$). A high starch content (mean 49.9 ± 3.9 %) was associated with group B

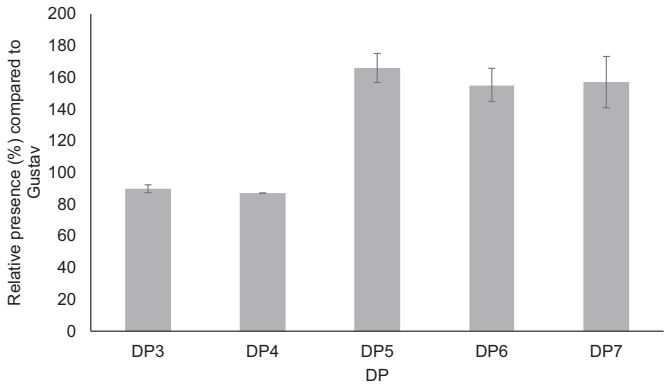

Fig. 2. Relative presence (%) of β-glucan structural units of barley grain samples of experimental line 6 (♀#224 × ♂#199) with high β-glucan content (12 %) compared to Gustav (100 %) with low β-glucan content (3.8 %, reference variety 11). Bars indicate standard deviation of two replicates.

samples with low fructan synthesis activity ($p < 0.05$). As reported in subsection 3.2, Group B samples with high starch content of 49.9 % had low fructan and β-glucan content than group A samples ($p < 0.05$). The negative relationship between starch and β-glucan content is in agreement with findings by Munck, Møller, Jacobsen, and Søndergaard (2004) who reported lower starch content in high-lysine barley mutants with elevated β-glucan content. Shimbata et al. (2011) reported high fructan content in sweet wheat lacking two enzymes involved in starch synthesis (GBSS1 and SSIIa). According to Shimbata et al. (2011), decreased starch synthesis and high abundance of sucrose may be the reason for higher fructan content, since sucrose is the substrate for fructan.

Amylose content, as analyzed by colorimetric assay, varied between the samples, with the highest (39.5 %) and lowest (1.8 %) in samples 2 and 3, respectively (Fig. 3). The amylose content was <10 % in samples 3, 4, 6, and 9, which were therefore considered to be waxy lines.

In the present study, we speculated that because of the constant carbon source, a low level of β-glucan and fructan apparently led to allocation of more carbon to starch synthesis.

3.5. BB distribution

The distribution of BBs was studied using HPSEC and HPAEC. In the HPSEC chromatogram (Fig. 4), the BB distributions were divided into nine buckets (B1–B9) for further analysis. Buckets B1–B6 contained branched BBs from hydrolysis of the amylopectin β-LD, while buckets B7, B8, and B9 contained linear dextrins produced during BB preparation. PCA identified two major clusters associated with the BB distribution (Fig. 5). These were group A, with high fructan synthesis activity (dashed ellipse in Fig. 5a), and group B, with low fructan synthesis activity (solid ellipse in Fig.5a). From the data derived from peak area of different buckets (B1–B9) of HPSEC BB distribution, variations in the BB distribution between these groups are shown in Table 2. Group A was associated with a higher proportion of larger BBs (B1 = 20.0 ± 1.8, B2 = 107.7 ± 4.2, B3 = 107.6 ± 2.1) and lower proportion of smaller BBs (B5 = 256.8 ± 4.7, B6 = 301.0 ± 7.2), while group B lines had a lower proportion of B1–B3 (B1 = 10.6 ± 2.4, B2 = 83.4 ± 4.2, B3 = 98.1 ± 1.7) and higher proportion of B5 and B6 (B5 = 268.1 ± 3.8, B6 = 323.0 ±7.9) ($p < 0.05$). There was no difference in abundance of medium-size BBs (B4) between the groups (Table 2). Hence for BBs derived from the amylopectin fraction (B1–B6), there was a good balance between distribution as affected by fructan synthesis activity or suppression of starch synthesis, with high fructan synthesis (suppressed starch synthesis) being associated with larger BBs and vice versa. For the buckets that contained the linear dextrins (B7–B9), higher proportions, based on refractive index (Fig. 4) were found for samples belonging to group A.

The results of HPAEC analysis complemented those of HPSEC analysis of BB distribution, with higher resolution. Categorization of BBs into groups (G2–6) was performed according to Bertoft, Källman, Koch, Andersson, and Åman (2011) and Zhao et al. (2021), but with slight modifications (Fig. S3). However, resolving the peaks separately for G5 and G6 was not possible and therefore BBs belonging to G5 and G6 were treated together as G5 + G6. Group G1 was not considered, since it mostly contained linear dextrins (glucose, maltose, maltotriose) produced during BB preparation.

In agreement with the HPSEC results, PCA revealed two categories based on the groups of BBs (Fig. S4). One cluster consisted of samples in group A and the other of samples in group B.

Analysis of the abundance of each group of BB revealed that small BBs from G2 were present in high abundance in samples in group B, while medium (G3) and larger (G4, G5 + 6) BBs were more abundant in

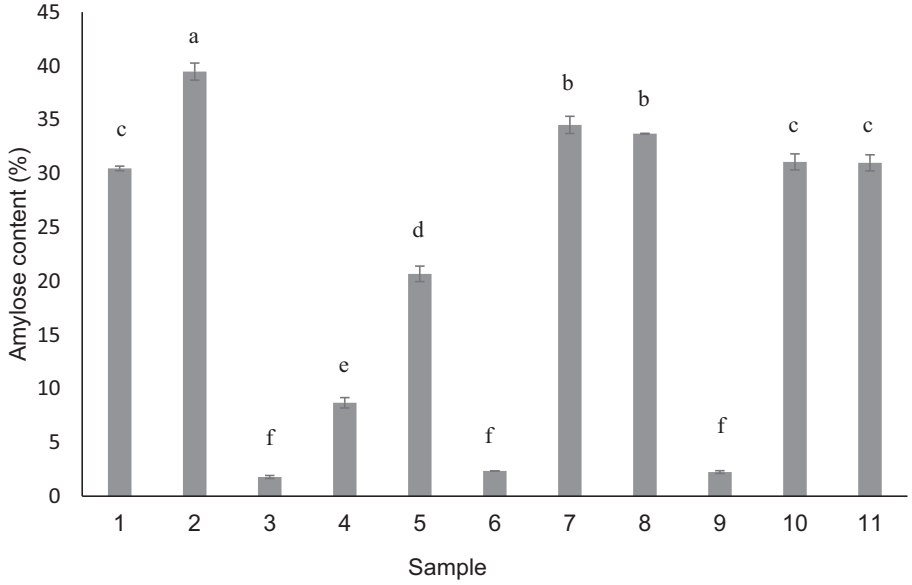

Fig. 3. Amylose content (% of starch content) in the experimental barley lines (samples 1–10) and the reference barley variety Gustav (sample 11). Different letters on bars indicate statistically significant differences. For sample descriptions, see Table 1.

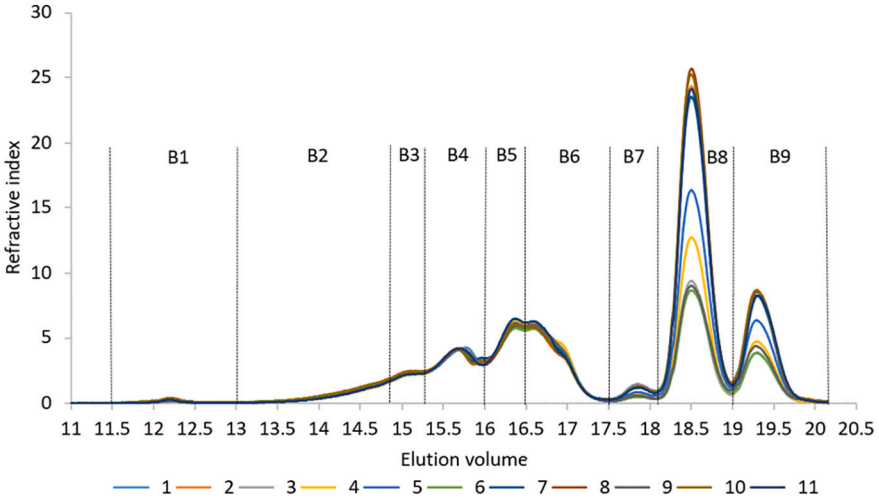

Fig. 4. Building block distribution in starch from the experimental barley lines (samples 1–10) after normalization for the peak area between 13 and 17.5 mL, as determined by HPSEC. The variety Gustav (sample 11) is included for reference. The distribution was bucketed as: B1: elution volume 11.49–12.99 mL, B2: 13.00–14.79 mL, B3: 14.80–15.27 mL, B4: 15.28–15.94 mL, B5: 15.95–16.49 mL, B6: 16.50–17.46 mL, B7: 17.50–18.08 mL, B8: 18.09–18.99 mL, B9: 19.00–20.15 mL. Refractive index signal is proportional to the concentration (weight/volume). 1–11 refer to different genotypes used in the study and for descriptions, see Table 1.

samples in group A ($p < 0.05$) (Supplementary Table S2). These observations are in agreement to results from HPSEC analysis.

3.6. Correlations of BB to other parameters

Correlations between BB size and other parameters (content of amylose, starch, β-Glucan, fructan) were investigated using PCA (Fig. 6) and Spearman's rank correlation coefficient analysis (Table 3). A clear grouping of samples based on the different parameters was determined, where samples in group B (cluster within solid ellipse in Fig. 6) were associated with small BBs and high starch content, while samples belonging to group A (within dashed ellipse) were associated with medium and large BBs and a high content β glucan, and fructan. These associations with each bucket of BBs (as determined by HPSEC analysis) were further analyzed based on Spearman's rank correlation coefficients (Table 3).

Interestingly, starch synthesis showed a prominent relationship to BB size, with low starch content associated with a higher proportion of larger BBs, and vice versa. It is known that regulation of starch synthesis is mediated to a large extent by sugar signaling in plants and numerous studies have found that genes related to starch synthesis, i.e., ADP-glucose pyrophosphorylase, granule-bound starch synthase (*GBSSI*), and branching enzymes (*SBEs*), are regulated by sugars (Nakata & Okita, 1995; Wang, Yeh, & Tsai, 2001). According to a recent review by Tetlow and Bertoft (2020), BBs are the major structural components of amylopectin. The main enzymes involved in determining the structure of the BBs are soluble starch synthases (SSSI and/or SSSII) and SBEII isoforms (Tetlow & Bertoft, 2020). Hence, SUSIBA transcription factor-based selective breeding could affect one or all of those enzymes or their combined complex, thereby affecting the structure and/or size of the BBs. These results suggest that SUSIBA transcription factor-based cross-breeding can allow breeding programs to achieve novel types of starch with tailored structure at the BB level. It has been reported previously that genetic background has a direct link to BB size in barley starch (Källman et al., 2015; Zhao et al., 2021). However, to the best of our knowledge, this study is the first to report an effect on starch structure at BB level as affected by SUSIBA-based sugar regulation for fructan synthesis.

Interestingly, there was a strong association between amylose content and B7, B8, and B9 buckets considered to represent maltotriose, maltose, and glucose, respectively (Table 3). In the method used for isolating BBs, β-LDs are produced from branched amylose during the first β-amylolysis, and continuing α-amylolysis converts β-LD to α-LD. This α-LD is converted into glucose, maltose, and maltotriose during the second β-amylolysis (Zhao et al., 2021). As reviewed by Bertoft (2017), the linear part of amylose is completely hydrolyzed into maltose during the first β-amylolysis and the branched component of amylose partly forms maltose and β-LD. During α-amylolysis, further linear dextrins are produced by cleaving the internal chain segments between the BBs (Zhao et al., 2021). Considering the steps associated with production of BBs from amylose, the origin of B7 and B8 can be linked to both the linear and branched fraction of the amylose component. Although branches from the amylose fraction account for only 1–2 % of total branches in normal starch (Zhu, Bertoft, & Seetharaman, 2013), around 10–70 % of amylose molecules (depending on the botanical source) are branched and contain 5–20 chains (Kong, 2020). The strong positive association of B7, B8, and B9 with amylose in the present study reveals the connection between the selective breeding technique applied and the amylose component in barley starch. Studies focused in understanding amylose fine structure by approaches such as model fitting of chain length distributions has become a recent research interest to better understand the starch biosynthesis-structure-property relations (e.g., Li, Yu, Dhital, Gidley, & Gilbert, 2019; Yu et al., 2019). Hence, future work to study the fine structure of amylose as affected by SUSIBA activity would be advantageous for the knowledge platform which connects starch biosynthesis-structure-property relations, since our results indicate a possible correlation of amylose fine structure as affected by SUSIBA activity.

The correlations between β-glucan content and different categories of BBs opposed to the correlations observed for starch content and different categories of BBs. This observation is supported by the fact that β-glucan content and starch content had a negative relationship. Correlations between the different categories of BBs and fructan content were observed for B1, B2 and B5 of the BB distribution groups as determined by HPSEC analysis.

4. Conclusions

A recent study adopted a new strategy for cross-breeding of barley based on SUSIBA transcription factor to generate barley lines with high fructan content. The present study revealed that these high fructan lines also had elevated content of β-glucan with decreased proportions of DP3 and DP4 structural units and increased proportions of longer structural units. Starch content and the molecular structure of amylopectin were also altered in the high-fructan lines. Upregulation of fructan synthesis is likely to suppress starch synthesis and is furthermore associated with larger building blocks in amylopectin. These results provide insights to plant breeders regarding *in planta* modification of starch and glucan to fit with the intended end use. Further detailed characterization of amylose fine structure would improve understanding of the effect of

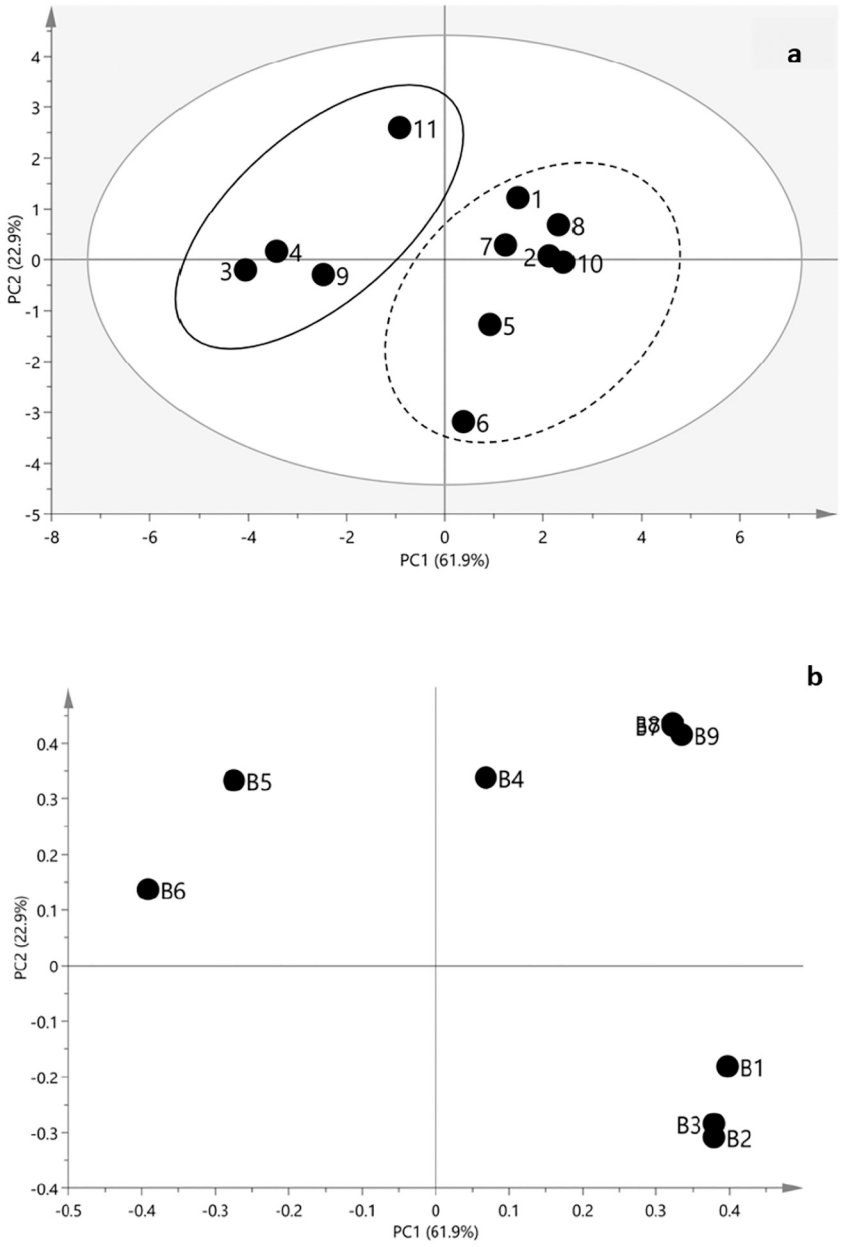

Fig. 5. Principal component analysis (a) score plot and (b) loading plot of building block distribution in starch based on HPSEC results. Each dot represents a sample. The buckets of building block distribution (B1–B9) are as shown in Fig. 5. Clusters within the dashed ellipse belong to group A (lines with high fructan synthesis activity) and those within the solid ellipse to group B (lines with low fructan synthesis activity). For sample descriptions, see Table 1.

Table 2
Variation in peak area for the different buckets (B1–B9) from the HPSEC data, this estimate building block distribution for barley lines in sample group A (high fructan synthesis activity) and group B (low fructan synthesis activity). Values within columns with different superscript letters differ significantly (ANOVA, $\alpha = 0.05$). Buckets are as described in Fig. 4. For sample descriptions, see Table 1.

Sample group	Bucket								
	B1	B2	B3	B4	B5	B6	B7	B8	B9
A	$20.0^{a} \pm 1.8$	$107.7^{a} \pm 4.2$	$107.6^{a} \pm 2.1$	$226.2^{a} \pm 3.5$	$256.8^{b} \pm 4.7$	$301.0^{b} \pm 7.2$	$45.5^{a} \pm 12.6$	$863.9^{a} \pm 250.6$	$324.3^{a} \pm 74.3$
B	$10.6^{b} \pm 2.4$	$83.4^{b} \pm 4.2$	$98.1^{b} \pm 1.7$	$226.8^{a} \pm 3.1$	$268.1^{a} \pm 3.8$	$323.0^{a} \pm 7.9$	$30.2^{b} \pm 11.1$	$575.3^{b} \pm 263.4$	$231.9^{b} \pm 79.3$

upregulating fructan synthesis on starch synthesis.

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CRediT authorship contribution statement

Shishanthi Jayarathna: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Visualization, Writing – original draft, Writing – review & editing. **Yunkai Jin:** Methodology, Formal analysis, Writing – original draft, Writing – review & editing. **Gleb**

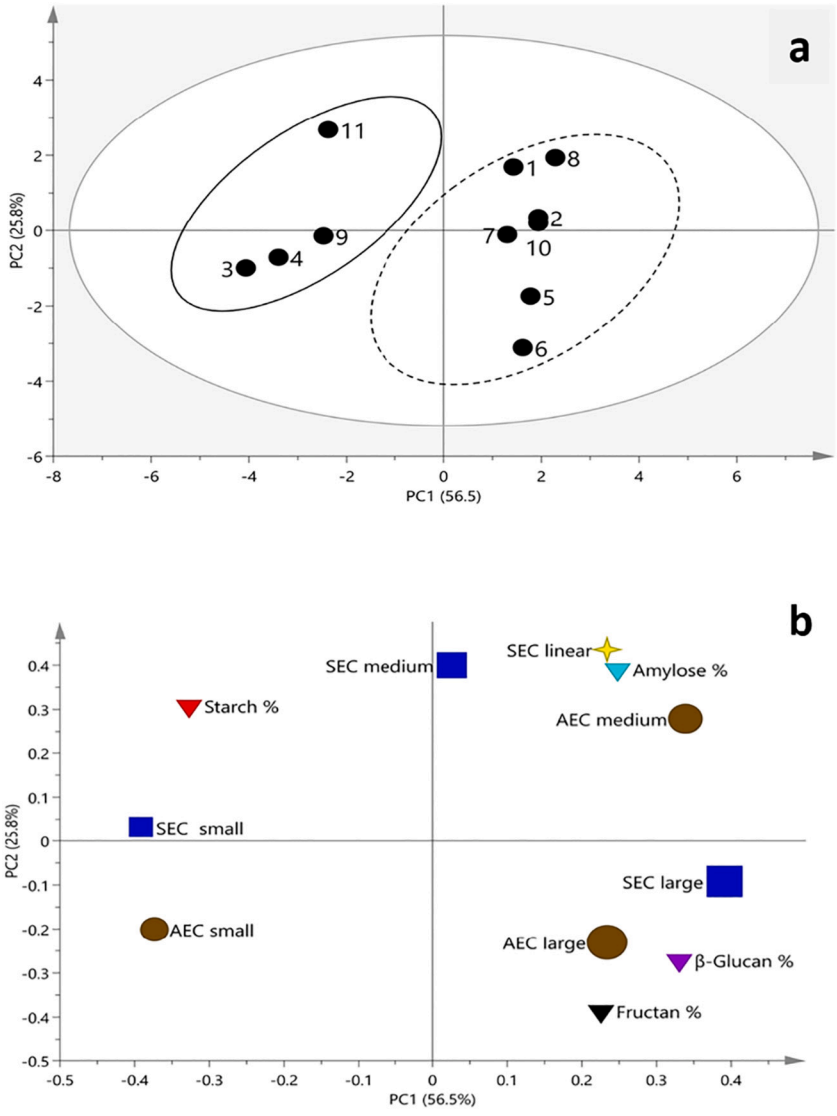

Fig. 6. Principal component analysis (a) score plot and (b) loading plot for bucket distribution of building blocks (BBs) as determined by HPSEC and HPAEC, amylose content, starch content, β glucan content, and fructan content. The BB distribution buckets based on HPSEC analysis are as described in Fig. 5, while those based on HPAEC are as follows: building blocks of group G2 (AEC small), building blocks of G2 and G3 (AEC medium), and building blocks of G5 + G6 (AEC large). Clusters within the dashed ellipse belong to group A (barley lines with high fructan synthesis activity) and those with the solid ellipse to group B (lines with low fructan synthesis activity). For sample descriptions, see Table 1.

Table 3
Spearman's rank correlation coefficient for relationships between buckets B1–B9 of building block distribution (see Fig. 5) and starch, amylose, β-glucan, and fructan content.

Bucket	Starch	Amylose	β-glucan	Fructan
B1	−0.79**	0.52	0.64*	0.62*
B2	−0.90***	0.37	0.85**	0.66*
B3	−0.84**	0.57	0.72*	0.54
B4	0.10	0.04	0.10	−0.04
B5	0.80**	−0.15	−0.88***	−0.71*
B6	0.75**	−0.73*	−0.64*	−0.48
B7	−0.17	0.8**	0.05	0.13
B8	−0.08	0.78**	0.11	−0.05
B9	−0.21	0.79**	0.19	0.1

* Indicates statistical significance at $p < 0.05$.
** Indicates statistical significance at $p < 0.01$.
*** Indicates statistical significance at $p < 0.001$.

Dotsenko: Methodology, Formal analysis, Investigation, Data curation, Visualization, Writing – original draft, Writing – review & editing. **Mingliang Fei:** Methodology, Formal analysis, Investigation, Data curation, Writing – review & editing. **Mariette Andersson:** Writing – review & editing. **Annica A.M. Andersson:** Methodology, Investigation, Data curation, Writing – review & editing. **Chuanxin Sun:** Conceptualization, Supervision, Project administration, Funding acquisition. **Roger Andersson:** Conceptualization, Supervision, Project administration, Funding acquisition, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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