Theor Appl Genet (2008) 117:701–709
DOI 10.1007/s00122-008-0811-6

ORIGINAL PAPER

Quantitative trait loci for grain fructan concentration in wheat (*Triticum aestivum* L.)

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Received: 22 March 2008 / Accepted: 21 May 2008 / Published online: 7 June 2008

Abstract Fructans (fructo-oligosaccharides) are prebiotics that are thought to selectively promote the growth of colonic bifidobacteria, thereby improving human gut health. Fructans are present in the grain of wheat, a staple food crop. In the research reported here, we aimed to detect and map loci affecting grain fructan concentration in wheat using a doubled-haploid population derived from a cross between a high-fructan breeding line, Berkut, and a low-fructan cultivar, Krichauff. Fructan concentration was measured in grain samples grown at two locations in Australia and one in Kazakhstan. Fructan concentration varied widely within the population, ranging from 0.6 to 2.6% of grain dry weight, and was quite repeatable, with broad-sense heritability estimated as 0.71. With a linkage map of 528 molecular markers, quantitative trait loci (QTLs) were detected on chromosomes 2B, 3B, 5A, 6D and 7A. Of these, the QTLs on chromosomes 6D and 7A had the largest effects, explaining 17 and 27% of the total phenotypic variance, respectively, both with the favourable (high-fructan concentration) alleles contributed from Berkut. These chromosome regions had similar effects in another mapping population, Sokoll/Krichauff, with the favourable alleles contributed from Sokoll. It is concluded that grain fructan concentration of wheat can be improved by breeding and that molecular markers could be used to select effectively for favourable alleles in two regions of the wheat genome.

Communicated by Y. Xu.

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Introduction

Fructans (fructo-oligosaccharides) are non-digestible carbohydrates with potentially beneficial effects on human health (Tungland and Meyer 2002; Ritsema and Smeekens 2003). Because humans lack the enzymes to break down fructans, food fructans escape digestion in the small intestine and selectively stimulate the growth of beneficial bifidobacteria in the colon (Gibson et al. 1995). This favourable fermentation reduces the risk of development of colonic disorders, such as constipation and hemorrhoids (Jenkins et al. 1999) and infection by pathogenic gut bacteria (Roberfroid 2002). It also increases gut ability to absorb more nutrients from diets, particularly calcium and iron (Scholz-Ahrens et al. 2001; Coudray et al. 2003; Raschka and Daniel 2005; Yeung et al. 2005; Lobo et al. 2006), thereby improving the mineralization of bone and reducing the risk of iron deficiency anaemia, which is prevalent in the developing world (Welch and Graham 2004). In addition, a high-fructan diet can improve the health of patients with diabetes (Ayman et al. 2004) and reduce the risk of colonic cancers (Jacobsen et al. 2006).

Fructans are naturally present in various cereal grains including wheat (*Triticum aestivum* L.) (White and Secor 1953; Henry and Saini 1989; Schnyder et al. 1993). As wheat is a major staple food, increasing its fructan level could increase fructan intake for a large number of people. Significant genotypic variation has been reported for fructan concentrations in wheat grain (Huynh et al. 2007), but information on the inheritance of this trait is still lacking. To our knowledge, there have been no reports of genetic mapping for grain fructan accumulation in wheat or other cereals. Quantitative trait loci (QTLs) with major effects on the fructan level of vegetative tissues have been detected in barley (Hayes et al. 1993), onion (McCallum et al. 2006) and perennial ryegrass (Turner et al. 2006). Further, Yang et al. (2007a) reported QTLs for water-soluble carbohydrates in wheat stems. Given that stem water-soluble carbohydrates consist mainly of fructans and sucrose and can serve as a source for grain development and fructan synthesis in the grain (Ruuska et al. 2006), genes that affect water-soluble carbohydrate content might also affect grain fructan accumulation. However, fructan concentration in wheat grain can be complicated by source-sink relationships, dilution effects and fructan degradation during grain development (Schnyder et al. 1993; Nardi et al. 2003). In this study, we aimed to detect and map QTLs affecting grain fructan concentration in a wheat population derived from a cross between low- and high-fructan parents.

Materials and methods

Grain materials

The population used here consisted of 154 doubled-haploid (DH) lines derived from a cross between an inbred line Berkut (pedigree: Irena/Babax//Pastor), developed by the International Maize and Wheat Improvement Center (CIMMYT), Mexico and the cultivar Krichauff (pedigree: Wariquam//Kloka/Pitic62/3/Warimek/Halberd/4/3Ag3Arona), released in 1997 by the University of Adelaide, Australia. Berkut has higher grain fructan concentration than Krichauff (Huynh et al. 2007). Grain samples of each Berkut/Krichauff DH line were obtained from two field sites in Australia and one in Kazakhstan. In Australia, the population was grown at Rosedale, South Australia (34.55°S, 138.83°E) during the 2005 and 2006 winter growing seasons. In 2005, plants in the experiment suffered from moisture stress and some lines were heavily infected with stripe rust caused by *Puccinia striiformis f.*sp. *tritici*. Foliar fungicide treatments were applied late to minimise the effects of the disease. In 2006, the experiment was irrigated using overhead sprinklers and fungicides were used to prevent fungal infection. In these experiments, each DH or parental line was sown in one 2 m-long 2-row plot, with a different randomisation applied at each site. In Kazakhstan, the population was grown at Koshy (50.55°N, 71.25°E) in summer 2006 with each line and parent sown in a 1 m × 1 m plot. No irrigation was applied at this field site and the plants were not subject to any severe biotic stresses. At maturity, grain was harvested mechanically and one representative 20-g sample of grain from each plot was used for analysis of fructan concentration.

Fructan assay

Grain fructan concentration was measured by enzymatic hydrolysis followed by high-performance liquid chromatography (HPLC) using procedures described by Huynh et al. (2007). Whole-grain samples were ground into fine powder and fructans were extracted with boiling water in 15-mL tubes. For each grain sample, two aliquots of extract were hydrolysed separately in 1.5-mL Eppendorf tubes, using different enzyme mixtures. Both mixtures contained amyloglucosidase and α-galactosidase to hydrolyse starch and raffinose, respectively, to remove major sources of interference and error in fructan determination. One of the mixtures also contained inulinase, to hydrolyse fructans into glucose and fructose. The carbohydrate hydrolysates were then measured on a Dionex ICS-3000 Ion Chromatography system (Dionex Corporation, Sunnyvale, USA) equipped with an eluent generator, a CarboPac PA20 guard column (3 × 30 mm) coupled to a CarboPac PA20 analytical column (3 × 150 mm) and a Dionex ED40 electrochemical detector working in pulsed amperometric detection mode (PAD) using a disposable gold (Au) working electrode and a combination pH-Ag/AgCl reference electrode. For details of the HPLC conditions see Huynh et al. (2007). Fructan concentrations were calculated based on differences in glucose and fructose concentrations between the two analyses.

Analysis of variance (ANOVA) was performed with computer software GenStat, Version 8.0. Factors for the ANOVA model were doubled-haploid line and block, with each of the three field environments considered as block. Variance components attributable to variation among lines (VG) and residual variation (VE) were derived and used to estimate broad-sense heritability for grain fructan concentration (VG/(VG + VE)).

Genetic mapping

DNA was extracted from leaves of 2-week-old seedlings of Berkut, Krichauff and each DH line using the method described by Bansal et al. (2008). A total of 1,071 simple sequence repeat (SSRs) markers was assessed for polymorphism between Berkut and Krichauff, and 216 polymorphic

markers were used to genotype the DH lines. Most of these SSRs were assayed using the Multiplex-Ready PCR Technology as described by Hayden et al. (2007). In addition, genotyping with Diversity Array Technology® (DArT) was performed by Triticarte Pty Ltd (Australia) using the method described by Akbari et al. (2006). Map construction was performed using MapManager QTXb20 for Windows (Manly et al. 2001), RECORD (Van Os et al. 2005) and alignment of linkage groups with previously constructed wheat linkage maps.

QTL mapping was performed with QTLNetwork 2.0 (Yang et al. 2008) using mixed linear composite interval mapping (Yang et al. 2007b). The general model incorporated fixed terms for the main additive effects of QTL and the additive–additive epistatic effects of pairs of QTL and random terms for environmental effects, additive-environmental interaction effects and additive–additive–environmental interaction effects. The analysis involved several consecutive steps. Candidate marker intervals were selected using the method described by Piepho and Gauch (2001). The selected intervals were used as co-factors in composite interval mapping (Zeng 1994) to conduct a one-dimensional (1D) scan of the genome to search for putative QTLs. Next, a two-dimensional (2D) genome scan was performed to search for epistatic interactions between QTLs. In each step, significance testing was based on the *F*-test using Henderson method III (Searle et al. 1992), with thresholds corresponding to probability levels of 0.10 (for candidate interval selection) and 0.01 (for detection of QTL and epistasis) defined using 10,000 permutations. A multiple-QTL model was selected by subjecting significant peaks from the *F*-statistic profiles to stepwise selection involving iterative forward and backward selection steps. QTL and epistatic effects were estimated using a Bayesian method via 20,000-cycle Gibbs sampling (Wang et al. 1994).

QTL validation

Effects of two fructan QTLs that were detected in all three of the above-mentioned environments were further examined using grain samples from experiments in which the Berkut/Krichauff population was grown in 2006 at Booleroo (32.52°S, 138.21°E), Minnipa (32.51°S, 135.09°E) and Roseworthy (34.31°S, 138.44°E), South Australia. Each experiment involved a randomised complete block design with two blocks. The 154 DH lines were classified into four marker-allele classes according to their genotypes at *barc54-6D* and *gwm681-7A*: Berkut at both loci (BB), Krichauff at *barc54-6D* and Berkut at *gwm681-7A* (KB), Berkut at *barc54-6D* and Krichauff at *gwm681-7A* (BK) and Krichauff at both loci (KK). For each block from each location, one bulk of grain was formed for each marker-allele class, using an equal quantity (2 g) of grain from each line within the class. The grain bulks were ground into fine powder, well mixed and analysed for fructan concentration as described above. For data analysis, each experiment was considered as a 2 × 2 factorial of two marker loci and two parental alleles in a randomised complete block design with two blocks; REML variance components analysis was performed in which main marker and marker–marker interaction effects were fixed and environment effects random.

The same two genomic regions were also examined in a population derived from a cross between Sokoll (pedigree: Pastor/3/Altar84/*Ae. squarrosa*(Taus)//Opata, a breeding line from CIMMYT) and Krichauff. The markers *barc54-6D* and *gwm681-7A* were assayed on each of the 150 DH lines of this population, and the lines were classified into four marker-allele classes (SS, KS, SK and KK) according to their genotypes at the two marker loci. Grain samples of Sokoll, Krichauff and the 150 DH lines were obtained from a field experiment conducted at Rosedale, South Australia in 2006 and 2007. For each year, one bulk of grain was formed for each marker-allele class, using 2 g of grain from each line within the marker-allele class. The grain bulks were ground into fine powder, well mixed and analysed for fructan concentration as described above. For data analysis, the experiment was considered as a 2 × 2 factorial of two marker loci and two parental alleles in a randomised complete block design with two blocks, with each of the two years considered as a block.

Results

Genotypic variation in grain fructan

The concentration of grain fructans varied widely within the Berkut/Krichauff population, ranging from 0.7 to 2.0% (percentage of grain dry weight) at Rosedale in 2005, from 1.0 to 2.6% at Rosedale in 2006 and from 1.0 to 2.2% at Koshy, with significant ($P < 0.001$) positive correlations among the three environments (Fig. 1). Berkut had higher fructan concentrations than Krichauff in all environments. Transgressive segregation was observed, and broad-sense heritability was estimated as 0.71.

QTLs and epistatic interactions

A total of eight QTLs with two pairs of epistatic interactions were found for grain fructan concentration (Table 1, Fig. 2). Among them, *QGfc.aww-2B.1*, *QGfc.aww-2B.2*, *QGfc.aww-3B.1*, *QGfc.aww-6D.2* and *QGfc.aww-7A.1* had additive effects. Only *QGfc.aww-2B.1* exhibited significant interaction with environments. At that locus, the allele from Berkut had a greater positive effect ($P < 0.05$)

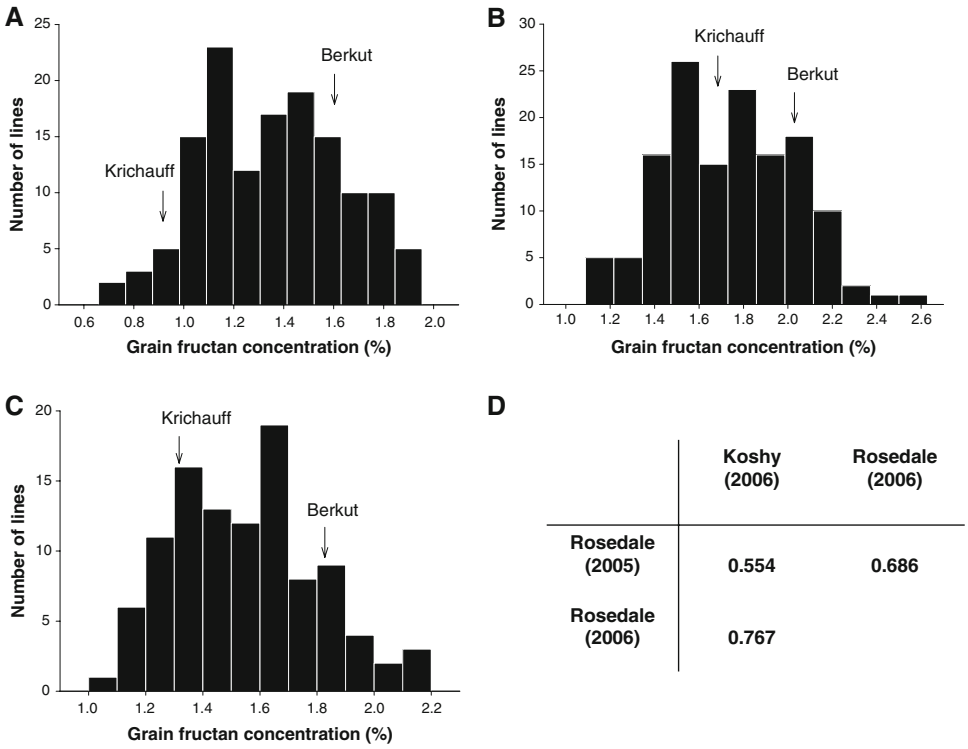

Fig. 1 Variation in grain fructan concentration within the Berkut/Krichauff population grown at Rosedale, Australia in 2005 (A) and 2006 (B) and at Koshy, Kazakhstan in 2006 (C); and the phenotypic correlations among the three field environments (D)

Table 1 QTLs and epistasis for grain fructan concentration (% of dry weight) measured on the Berkut/Krichauff population grown in three field environments (e_1: Rosedale 2005, e_2: Rosedale 2006 and e_3: Koshy 2006)

QTL	Nearest marker	Position (cM)	Support interval (cM)	*F*-value[a]	Additive main effect[b]		Interaction with environment[b]		
					a	R^{2c} (%)	ae_1	ae_2	ae_3
QGfc.aww-2B.1	*barc91-2B*	89.6	84.5–95.2	13.5	0.057***	4	0.031*	−0.003	−0.029
QGfc.aww-2B.2	*wPt-7161-2B*	208.7	183.4–220.7	9.5	0.058***	4	0.002	0.001	–0.003
QGfc.aww-3B.1	*gwm802-3B*	82.9	80.2–90.9	8.4	−0.043***	2	−0.007	−0.008	0.015
QGfc.aww-6D.2	*barc54-6D*	58.6	52.6–66.6	32.2	0.122***	17	0.000	0.000	0.000
QGfc.aww–7A.1	*gwm681-7A*	5.4	2.2–8.2	54.8	0.152***	27	–0.017	0.015	0.001

Epistatic interaction	Nearest marker	Position (cM)	Support interval (cM)	*F*-value	*aa*	R^2 (%)	aae_1	aae_2	aae_3
QGfc.aww-2B.1 and *QGfc.aww-5A.1*	*barc91-2B* and *cfa2155-5A*	89.6 and 184	(84.5–95.2) and (180.0–184.0)	8.0	0.034***	1	0.009	−0.019	0.011
QGfc.aww-2D.1 and *QGfc.aww-6D.1*	*gwm296-2D* and *cfd13-6D*	19.3 and 27.8	(4.6–33.3) and (22.2–35.6)	10.7	0.062***	5	–0.012	0.011	0.001

[a] *F*-statistics of the peaks, with significance thresholds ($P = 0.01$) are 7.9 and 6.8 for QTLs and epistatic interactions, respectively

[b] A positive effect indicates that the allele from Berkut contributes to higher grain fructan levels, while a negative effect indicates that the allele from Krichauff contributes to higher grain fructan levels

[c] Percentage of phenotypic variation explained

*, **, *** Significantly different from zero at $P = 0.05$, 0.01 and 0.001, respectively

in Rosedale in 2005 than in the other environments (Table1). The QTLs with the largest additive effects were at *QGfc.aww-6D.2* and at *QGfc.aww-7A.1*, explaining 17 and 27% of the total phenotypic variation, respectively. Other QTLs had smaller additive effects, each explaining only 2 or 4% of the total phenotypic variation. At all QTLs except for *QGfc.aww-3B.1,* the favourable alleles came from Berkut. Epistatic interactions explained approximately 6% of the phenotypic variation, and were similar in all environments. Of the QTLs involved in epistatic interactions, only *QGfc.aww-2B.1* had a significant individual effect. In a model including all of these QTLs, 42% of the

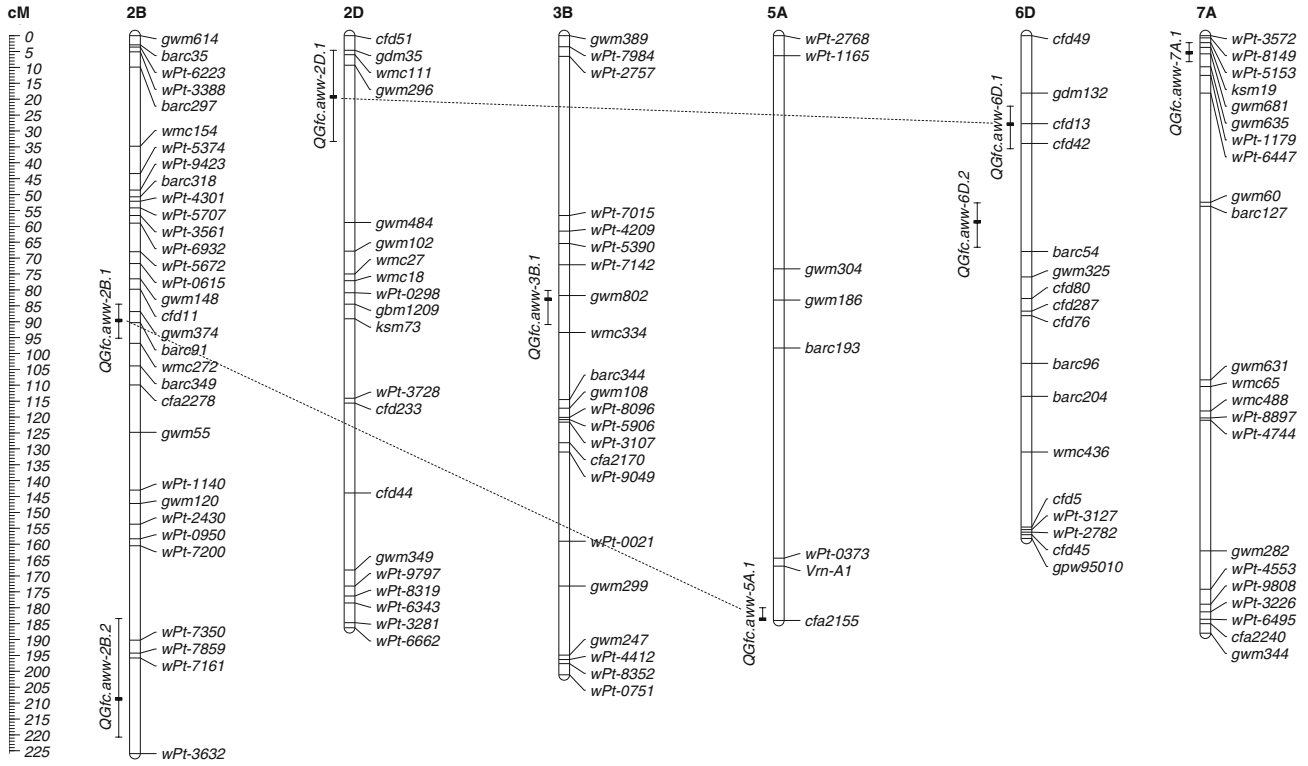

Fig. 2 Chromosome locations of regions associated with grain fructan concentration in the Berkut/Krichauff double-haploid population. Dashed lines show epistatic interactions between QTLs

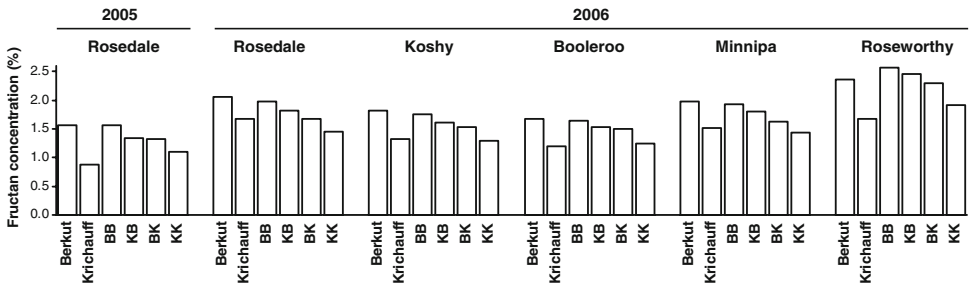

Fig. 3 Mean grain fructan concentrations for Berkut, Krichauff and four genotypic classes of Berkut/Krichauff doubled-haploid lines: BB (with Berkut alleles at markers *barc54-6D* and *gwm681-7A*), BK (Berkut at *barc54-6D*; Krichauff at *gwm681-7A*), KB (Krichauff at *barc54-6D*; Berkut at *gwm681-7A*) and KK (Krichauff at both marker loci). Values shown for Rosedale and Koshy are mean values from individual lines. Values shown for Booleroo, Minnipa and Roseworthy are based on assessment of grain samples bulked within genotypic classes

phenotypic variation was explained by QTLs and their epistatic interactions, and 31% by variation among experimental environments. The predicted fructan concentration was 2.08% for lines carrying the most favourable combination of alleles, and 1.02% for lines carrying the least favourable combination of alleles.

QTL validation

The markers nearest to the two QTLs with the largest effects are *barc54-6D* (near *QGfc.aww-6D.2*) and *gwm681-7A* (near *QGfc.aww-7A.1*). In all environments, lines that are homozygous for Berkut alleles at both of these loci had higher grain fructan concentration than lines that are homozygous for Krichauff alleles (Fig. 3). These two loci interacted significantly ($P < 0.05$) with each other, but not with environments. The two marker loci had similar effects in the Sokoll/Krichauff mapping population grown at Rosedale in 2006 and 2007. In that population, the high-fructan alleles were contributed from Sokoll (Fig. 4) and there was significant ($P < 0.01$) interaction between the two loci.

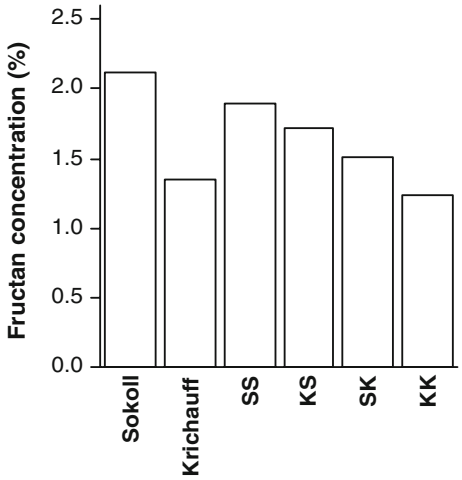

Fig. 4 Mean grain fructan concentrations for Sokoll, Krichauff and bulk grain samples representing four classes of Sokoll/Krichauff doubled-haploid lines: SS (with Sokoll alleles at markers *barc54-6D* and *gwm681-7A*), SK (Sokoll at *barc54-6D*; Krichauff at *gwm681-7A*), KS (Krichauff at *barc54-6D*; Sokoll at *gwm681-7A*) and KK (Krichauff at both marker loci), all grown at Rosedale, SA, Australia in 2006 and 2007

Discussion

Trait inheritance

Based on the observation of considerable phenotypic variation for grain fructan concentration in the Berkut/Krichauff population, it seemed that this trait might be under complex genetic control. With the mixed-model QTL analysis approach used here, it was possible to detect multiple QTLs, their interactions with each other and their environments. We used a significance level of 0.10 for the selection of candidate intervals, and a more stringent significance level (0.01) for further selection steps to avoid false positives. Multiple QTLs were detected and some of them exhibited epistatic interactions. At some loci, the favourable alleles came from Berkut, while at others they came from Krichauff, providing a genetic explanation (transgressive segregation) for the presence of lines with more extreme grain fructan concentration than either parent. Although the environment had an influence on grain fructan concentration (accounting for 31% of the phenotypic variance), its interactions with QTLs were not significant. Among the QTLs detected, two (*QGfc.aww-6D.2* and *QGfc.aww-7A.1)* had major effects. The favourable alleles at these loci may have been inherited from Pastor, a parent of both Berkut and Sokoll; Pastor is also the recurrent parent of other new CIMMYT breeding lines that also have high grain fructan concentration (Huynh et al. 2007). In the QTL mapping experiment, the two major QTLs had independent effects on grain fructan level, but they exhibited some interactions in both validation tests. In both cases, the effect of having favourable alleles at both loci was less than the sum of the effects of having favourable alleles at either of the two loci (Figs. 3, 4). Considering this apparent partial duplication of effects, combined with the larger effects of *QGfc.aww-7A.1* (Table 1; Figs. 3, 4), and the detection of minor epistatic QTL (*QGfc.aww-6D.1*) near *QGfc.aww-6D.2* (Fig. 2), *QGfc.aww-7A.1* would be the logical initial target for selection to increase grain fructan concentration.

The detection of multiple QTLs and of epistatic interactions among QTLs for grain fructan concentration is consistent with complex physiological models of fructan accumulation in plants. Fructan molecules with different structures can be produced by the concerted action of different fructosyltransferases (Vijn and Smeekens 1999), and fructans can be enzymatically degraded (Henson and Livingstone 1996; Kawakami et al. 2005). Further, fructan accumulation in cereal grains can be complicated by source-sink relationships and by dilution effects during grain development (Schnyder et al. 1993). The loci detected here may contribute to physiological mechanisms that enhance grain fructan accumulation. They may affect carbohydrate accumulation in vegetative parts of the plant, influencing the source of substrates for fructan synthesis in the grain. In fact, two of the minor QTLs detected here seem to be co-located with QTLs detected by Yang et al. (2007a) for stem carbohydrate traits. *QGfc.aww-2D.1* is near *QSwscf.cgb-2D.1* (for water-soluble stem carbohydrates at the flowering stage) and *QGfc.aww-3B.1* is near *QAesec.cgb-3B.1* (for accumulation efficiency of water-soluble stem carbohydrates). Loci detected here may also help maintain a normal flow of photosynthates into the grain by affecting the rate of grain sucrose loading and thereby increasing fructan synthesis, lowering sucrose concentration and preventing sugar-induced feedback inhibition of photosynthesis (Pollock 1986). On the other hand, given that most synthesized fructans are lost late in grain development (Schnyder et al. 1993; Nardi et al. 2003), it is possible that these QTLs act by interfering with fructan degradation.

Investigation of candidate genes involved in fructan synthesis or degradation might help explain the functions of the QTLs detected in this study. Possible candidate genes include a fructan exohydrolase gene on chromosome 6D (Zhang et al. 2008) and a fructosyltransferase orthologue on chromosome 7A (Francki et al. 2006), which seem to be co-located with the major QTLs detected here. Further functional analysis could be conducted through physiological investigation of genotypes carrying different allelic combinations at the major QTLs.

Breeding implications

Considering the high heritability estimated here for the Berkut/Krichauff population and the consistent variation

observed among other materials by Huynh et al. (2007), it seems likely that grain fructan concentration can be improved effectively using phenotypic selection. This is also supported by the fact that there was no evidence of strong genotype-by-environment interaction for the trait; the fructan concentrations of the same lines were positively correlated among three contrasting field environments in Australia and Kazakhstan (Fig. 1). Further, the mapping of two major loci affecting grain fructan concentration in wheat could allow breeders to use molecular markers to select for high-fructan genotypes. Thus, both phenotypic and marker-assisted selection could lead to the development of higher fructan wheat, providing a means of improving nutrient bioavailability for humans. Poor bioavailability can cause nutrient malnutrition, especially iron and zinc deficiencies which are prevalent in developing countries (Welch and Graham 2004; Meng et al. 2005). This problem is compounded by the presence of inhibitory factors, "anti-nutrients" such as phytic acid and certain tannins, in foods from plant sources. These factors reduce the absorption of iron and zinc into human blood. In contrast, enhancing factors or "promoters", such as prebiotics, long-chain fatty acids and vitamin C, can improve bioavailability but are not present at adequate levels in cereal-sourced food (Graham et al. 2001). Breeding for bioavailability therefore involves not only increasing the content of iron and zinc but also ensuring a minimum level of anti-nutrients and an optimum level of promoters. Breeding for increased iron and zinc can be difficult, due to soil nutrient variation and strong genotype-by-environment interactions (Oury et al. 2006; Ortiz-Monasterio et al. 2007). Reducing the level of anti-nutrients like phytates may have disadvantages in that some of them may confer other benefits for plant and human health (Marschner 2002; Welch 2002). Therefore, breeding for prebiotics like fructans may be the most effective option.

The fructan levels reported here and previously for wheat (Henry and Saini 1989; Van Loo et al. 1995; Huynh et al. 2007) are lower than those in some other plant-source foods, such as chicory root (42%), garlic (28%), onion (18%), Jerusalem artichoke (18%), dandelion greens (14%) and leek (7%) (Van Loo et al. 1995). However, wheat could provide the greatest fructan intake for humans because it is a major staple food. For example, a survey by Moshfegh et al. (1999) showed that wheat contributed 70% of fructans in American diets, followed by onions (25%), banana, garlic and others. Therefore, any genetic improvement for wheat fructan could contribute considerably to fructan consumption in countries where wheat is the main food.

The health benefits of fructans have been well documented based on nutritional studies in which fructans were used as food supplements (e.g., Buddington et al. 1996; Jackson et al. 1999; Probert and Gibson 2002; Abrams et al. 2005; Kang et al. 2006; Van de Wiele et al. 2007). In terms of effective dosages, fructan supplementation at 4 g per day exerted significant prebiotic effects (Buddington et al. 1996), while higher doses had no toxic effect but might cause some intestinal discomfort in sensitive people (Coussement 1999). Additional research may be needed to confirm whether similar effects can be derived from a natural daily diet including products derived from wheat with inherently high fructan levels. To avoid confounding effects from other factors that could vary among wheat cultivars with contrasting fructan levels, such research could employ bulks of grain from lines carrying contrasting fructan QTL combinations, as was done for the validation component of this research. By this means, large amounts of wheat flour can be produced with varying fructan concentrations but equal amounts of essential nutrients and other factors (anti-nutrients and promoters) with potential to interact with the fructan effect in the gut.

Acknowledgments Scholarship funding awarded to the first author by the University of Adelaide (International Postgraduate Research Scholarship) and the Molecular Plant Breeding Cooperative Research Centre is gratefully acknowledged. We thank the HarvestPlus Challenge Program and Waite Analytical Services for access to laboratory equipment, Mr. Jim Lewis and Mr. Bakhyt Nurzhanuly for providing grain samples, Mr. Lachlan Palmer, Dr. Genet Mekuria, Ms. Thao Nguyen, Mr. Greg Lott and Dr. Matthew Hayden for advice and assistance.

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