Genetic characterization and QTL mapping for grain fructan in wheat (*Triticum aestivum* L.)

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By

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Abbreviations

CIMMYT : International Maize and Wheat Improvement Center
DArT : Diversity Array Technology™
DH : Doubled Haploid
HPLC : High-Performance Liquid Chromatography
MAS : Marker-Assisted Selection
MPBCRC : Molecular Plant Breeding Cooperative Research Centre
QTL : Quantitative Trait Loci
RIL : Recombinant-Inbred Lines
SARDI : South Australian Research and Development Institute
SSR : Simple Sequence Repeats

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Abstract

Fructans are polysaccharides that are made up mainly of fructose. They are non-digestible carbohydrates and act as prebiotics to selectively promote the growth of colonic bifidobacteria, thereby improving human gut health. Fructans are present in the grain of wheat (*Triticum aestivum* L.), a staple food crop. Until now, there has been no research on genetic improvement of the concentration of fructans in wheat grain, partly because it has been difficult to accurately measure. One aim of this research project was to develop a simple and effective method to measure the fructan concentration in wheat grain. This was achieved by modifying a method that involves extraction of fructans from wheat grain followed by enzymatic hydrolysis to break down fructans into monosaccharides and quantification by anion-exchange liquid chromatography coupled with pulsed amperometric detection. The modified procedure is reliable and allows the handling of large numbers of flour samples at a relatively low cost, and can therefore be useful for assessing large numbers of wheat breeding lines. Using this method, grain samples taken from a diverse set of 117 wheat cultivars and breeding lines, including parents of mapping populations, were analysed for grain fructan concentration. There was significant genotypic variation among these materials, with grain fructan concentration ranging from 0.3 to 2.3% of grain dry weight. There was no evidence of strong genotype-by-environment interaction; the fructan concentrations of the same genotypes were positively correlated over different environments in Australia. Genetic mapping was carried out to detect and map loci affecting grain fructan concentration in wheat using a doubled haploid population derived from a cross between Berkut (high fructan) and Krichauff (low fructan). Grain samples were obtained from two field sites in South Australia and one in Kazakhstan. Fructan concentration varied widely within the population (0.6-2.6% of grain dry weight), with heritability estimated as $h^2 = 0.71$. A linkage map of 528 molecular markers covering 21 wheat chromosomes was used for locating quantitative trait loci (QTL). Genetic mapping identified two major QTLs on chromosomes 6D and 7A, with the (high fructan concentration) alleles contributed from Berkut, contributing to a 30-40% increase in wheat grain fructan compared to the Krichauff alleles. Effects of these chromosome regions were validated in additional environments and in another mapping population, Sokoll/Krichauff, with the favourable alleles contributed from Sokoll. The

major QTL on chromosome 7A was in the same region with a reported fructosyltransferase orthologue (*AB029888*), while the major QTL on chromosome 6D seemed to be co-located with a reported gene encoding for a fructan-degrading enzyme 1-exohydrolase (*1-FEHw2*). 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.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

Bao Lam Huynh
September, 2008

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Chapter 1

General Introduction

In recent years, trends in food science have emphasized the use of functional foods to promote good health (Milner, 1999; Weststrate et al., 2002). These foods often contain biologically active components that can improve functions and reduce the risk of disease. Fructans (fructo-oligosaccharides) are non-digestible carbohydrates with potentially beneficial effects on human health (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 can be incorporated in food as functional additives, but this commercial source can be expensive and therefore would not be available for most people, especially the poor in developing countries. On the other hand, fructans are naturally present in various vegetables and cereal grains including wheat (*Triticum aestivum* L.) (Van Loo et al., 1995). As wheat is a major staple food, increasing its fructan level could increase fructan intake for a large number of people. This effort may be achieved through plant breeding or genetic manipulations, so-called biofortification (Graham et al., 2001). Understanding the genetics and environmental influences on wheat grain fructan content could benefit breeding programs. To date, there has been no information on these effects, although wheat grain fructan has undergone a long history of research.

The occurrence of fructans in wheat grain was first discovered by White and Secor (1953). They observed two homologous series of oligosaccharides from grain extract using chromatographic paper, but structures of these polymers remained unknown. Four years later, Montgomery and Smith (1957) reported the complex structure of one fraction of the oligosaccharide series using methylation; the glucofructan, now known as fructans, composed of branched beta-(1-2) and beta-(2-6) linkages between fructose units and a glucose moiety. The structures were confirmed and studied further by Nilsson and Dahlqvist (1986), who also described the degrees of polymerisation of wheat fructans. Other research since then has focused on wheat fructan stability (Nilsson et al., 1988), measurement (Henry and Saini, 1989) and physiology of accumulation (Schnyder et al., 1993). The contribution of wheat to fructan intake in humans was surveyed by Van Loo et al. (1995) and Moshfegh et al. (1999), showing that wheat is the main carrier of fructans in Western diets. Increasing the fructan level in wheat by breeding would contribute considerably to fructan consumption in countries where wheat is an important food. Breeding relies on availability of sufficient genetic variation, and can make use of understanding of the trait inheritance. No information on either of these has been reported up to now.

The genetics of wheat grain fructan is not expected to be simple, due to the complex physiological pathways of fructan biosynthesis, which involve concerted actions of various fructosyltransferases (Vijn and Smeekins 1999). Further, 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). With advances in modern biotechnology, it should be possible to study the inheritance of such a complex trait at the molecular level. Molecular markers can be used to dissect quantitative traits into discrete quantitative trait loci (QTLs) that can be targeted in breeding.

The main aim of this study was to detect and map QTLs affecting grain fructan concentration in wheat. Prior to this activity, genetic variation was surveyed in order to obtain an indication of the potential for improvement through breeding, and to identify suitable genetic materials for mapping. To enable convenient assessment of larger numbers of genetic materials for the variation and mapping studies, a simplified method for grain fructan measurement was also developed. As outlined in Table 1, this thesis will review relevant literature (Chapter 2), report on and discuss experimental methods and results

(Chapters 3, 4 and 5) and provide a general discussion (Chapter 6). Two final chapters will state the main conclusions of the research (Chapter 7) and its contributions to knowledge (Chapter 8).

Table 1 Thesis structure.

Chapter	Content
Chapter 1	General introduction
Chapter 2	Review general literature on biochemistry and physiological functions of fructans, effective dosages of fructan intake, and methodologies to improve the level of fructans in food crops with particular reference to applications of molecular markers in genetic studies and breeding.
Chapter 3	Develop a simplified fructan assay for use in further chapters. Its convenience in genetic studies and breeding will be discussed.
Chapter 4	Survey genetic variation in grain fructan levels among wheat cultivars/lines grown across environments. Parental lines of each of available mapping population are also assessed for differences in grain fructan levels, from which suitable populations can be selected for further genetic mapping.
Chapter 5	Map quantitative trait loci affecting grain fructan concentration using DH mapping populations identified from the previous chapter. QTL validation and mapping of candidate genes are also reported.
Chapter 6	Discuss results achieved from this study and their future applications. Suggestions on follow-up studies are also discussed.
Chapter 7	State the main conclusions of the research
Chapter 8	State the contributions of the research to knowledge

Some of the information included in Chapters 3, 4 and 5 has already been published in two journal articles:

> Huynh BL, Palmer L, Mather DE, Wallwork H, Graham RD, Welch RM, Stangoulis JCR (2008) Genotypic variation in wheat grain fructan content revealed by a simplified HPLC method. Journal of Cereal Science 48:369-378
>
> Huynh BL, Wallwork H, Stangoulis JCR, Graham RD, Willsmore KL, Olson S, Mather DE (2008) Quantitative trait loci for grain fructan concentration in wheat (*Triticum aestivum* L.). Theoretical and Applied Genetics 117:701-709

For the work presented in these publications, I planned the research, developed an improved fructan assay, grew a glasshouse experiment, assayed grain fructan concentration, assayed 12 polymorphic DNA markers to improve an existing genetic map, performed statistical analyses, QTL mapping and validation, and wrote the manuscripts.

My co-authors' contributions were as follows:

My supervisors Diane Mather, Hugh Wallwork, Robin Graham and James Stangoulis gave advice and suggestions throughout the project, and contributed towards editing the manuscripts. Hugh Wallwork also developed mapping populations and provided me with access to grain from field experiments in South Australia.

Lachlan Palmer assisted in operating and maintaining the Dionex HPLC system that I used for analysis of grain fructan.

Ross Welch gave technical advice for the development of the improved fructan assay, and contributed towards editing the manuscript.

Kerrie Willsmore and Steve Olson assayed most of the DNA markers for a linkage map of the Berkut/Krichauff population.

In integrating the information from these publications into this thesis, I edited the text and restructured the content to provide a logical and coherent thesis. Introductory sections were strengthened to justify the aims of the research in each chapter, and in some cases shortened to avoid repetition of information presented in previous chapters of the thesis. New information was also added in as follows:

1. An additional test of fructan recovery in which bacterial levans were analysed using the simplified fructan assay (Chapter 3). This gave more evidence on activity of inulinase towards complete digestion of beta-(2-6) linkages in wheat grain fructans.

2. A survey of variation for grain fructan concentration using 98 lines of cultivated hexaploid wheat of diverse origin (Chapter 4).

3. More detailed analysis of data to compare the grain fructan concentrations of parents of mapping populations (Chapter 4), using linear contrasts within pairs of parents.

4. Variation in yield and grain weight and their correlations with grain fructan concentrations (Chapter 4).

5. Investigation of the effects of two major QTLs on yield and grain weight from multi-site experiments (Chapter 5).

6. Mapping of a candidate gene (Chapter 5).

Chapter 2

Literature Review

2.1 Introduction

Fructans are polysaccharides that are made up mainly of fructose. The fructose units are joined together by either β(1-2) or β(2-6) glycosidic bonds. Inulin, a fructan type with mostly linear β(1-2) linkages, has documented health benefits, acting as a prebiotic (Roberfroid and Delzenne, 1998; Tungland and Meyer, 2002; Gibson et al., 2004) and is used as a functional food supplement. Wheat (*Triticum aestivum* L.) grain contains branched fructans with both β(1-2) and β(2-6) linkages (Montgomery and Smith, 1957; Nilsson and Dahlqvist, 1986). However, it is not known whether such fructans also exert beneficial health effects, or to what extent there is genetic variation in wheat grain fructan levels that could provide a basis for increasing the contribution that wheat makes to fructan consumption for humans through natural daily diets. This review will cover aspects of fructan biochemistry and measurement, physiological functions of fructans, fructan consumption by humans, and possibilities of improving fructan levels in food crops, especially wheat. Relevant methodologies to achieve this aim will also be reviewed.

2.2 Fructan biochemistry

Terminology and chemical structure

Historically, fructans have had several names. The first discoverer of plant fructans called them a "peculiar substance" (Rose, 1804). Later, they were known as inulin (Thomson, 1818), glucofructan (Montgomery and Smith, 1957), fructosan (Edelman and Jefford, 1968) or fructan (Henry and Derbyshire, 1979). Descriptions of fructan structures and their terminology have been frequently updated in reviews by French and Waterhouse (1993), Vijn and Smeekens (1999) and Ritsema and Smeekens (2003).

As generally defined, fructans are polymers of fructose, in which the fructose units are linked to each other by either β(1-2) or β(2-6) glycosidic bonds and may also be attached to the glucose moiety of a sucrose molecule (French and Waterhouse, 1993; Vijn and Smeekens, 1999). This results in different types of fructans with various structures and degrees of polymerisation (DP, i.e., the number of hexose units per fructan molecule).

Inulins are composed mainly of linear β(1-2) polymers of fructose with a terminal glucose residue. This type of fructans is typically found in the Asteraceae, such as chicory (*Cichorium intybus* L.) and Jerusalem artichoke (*Helianthus tuberosus* L.), with DP up to 40 (Bonnett et al., 1994).

Inulin neoseries have two linear β(1-2)-linked fructosyl chains, one attached to the fructosyl residue of the sucrose, the other attached to the glucosyl residue. This type of fructan is found in plants of the Liliaceae such as onion (*Allium cepa* L.) (Shiomi, 1989).

Levans are composed mainly of linear β(2-6) polymers of fructose with a terminal glucose residue. Levan can be found in some grasses such as cocksfoot or orchardgrass (*Dactylis glomerata* L.) with DP up to 20 (Bonnett et al., 1997). Levan is also produced by some bacteria with DP up to 100,000 (Stivala and Khorramian, 1982; Geel-Schutten et al., 1999).

Levan neoseries have two linear β(2-6)-linked fructosyl chains, one attached to the fructosyl residue of the sucrose, the other attached to the glucosyl residue. Neo-series levans are found in a few plants of the Poaceae such as oat (*Avena sativa* L.) (Livingstone, 1993).

Fructofuranosyl-only oligosaccharides contain fructose units only. They are found in roots of chicory and Jerusalem artichoke with DP up to 18 (Ernst et al., 1996); they are thought to be products of inulin hydrolysis that are formed during inulin mobilisation.

Mixed levans have a combination of β(1-2) and β(1-6) linkages. This type of fructan is found in vegetative tissues of most plant species of the Poaceae, such as wheat, barley (*Hordeum vulgare* L.) and rye (*Secale cereale* L.) (Bancal et al., 1992; Bonnett et al., 1997). Mixed levans are also found in wheat grain, with DP up to 20 (Montgomery and Smith, 1957; Nilsson and Dahlqvist, 1986).

Fructan biosynthesis

Historically, fructan synthesis was first elucidated in bacteria (Dedonder, 1966). Bacterial fructans are mainly levans. They are produced under the action of levansucrase or β(2-6)-fructan:D-glucose 1-fructosyltransferase, which mediates the transfer of a fructose unit from sucrose to a fructosyl receptor, resulting in polymers with thousands of fructosyl units. Levan-producing bacteria are found in a large range of taxa, especially lactic acid bacteria (e.g., *Lactococcus*, *Streptococcus* and *Lactobacillus*) (Geel-Schutten et al., 1999; Van Hijum et al., 2004; Shih et al., 2005).

Fructan synthesis in plants was first elucidated by Edelman and Jefford (1968) using Jerusalem artichoke as a model. Inulin-type fructans are synthesised in cell vacuoles by the action of two key enzymes, sucrose:sucrose 1-fructosyltransferase (*1-SST*) and fructan-fructan 1-fructosyltransferase (*1-FFT*). The enzyme *1-FFT* catalyses the transfer of a fructose unit from sucrose to another sucrose molecule, forming trisaccharide 1-kestose, and the enzyme *1-FFT* mediates the elongation of fructan polymers by transferring a fructose unit from one fructan molecule to another (Edelman and Jefford 1968; Luscher et al. 1996; Koops and Jonker 1996).

The production of inulin neoseries in liliaceous species is catalysed by the action of fructan:fructan 6G-fructosyltransferase (*6G-FFT*) and *1-FFT* (Shiomi, 1989; Vijn et al., 1997; Ueno et al., 2005). The enzyme *6G-FFT* mediates the formation of trisaccharide neo-kestose by transferring a fructose unit from 1-kestose to carbon 6 of the glucose moiety of sucrose. Subsequently, the fructose chains on either end of the glucose moiety can elongate under the action of *1-FFT* to form inulin neoseries. Vijn and Smeekens (1999) proposed that neo-kestose also serves as a substrate for enzyme sucrose:sucrose 6-fructosyltransferase (*6-FST*) (Duchateau et al., 1995) to produce levan neoseries.

Fructofuranosyl-only oligosaccharides are thought to be synthesized by the action of *1-FFT*. Incubation of chicory inulin with fructose and purified *1-FFT in vitro* has been found to yield a series of oligofructoses similar to those found *in vivo*, indicating that fructose (not glucose) acts as the acceptor for enzyme *1-FFT*, leading to the formation of fructosyl-only polymers with different chain lengths (Van den Ende et al., 1996).

The biosynthesis of mixed levans in grasses and cereals involves three enzymes, *1-SST*, *1-FFT* and *6-FST*. As proposed by Vijn and Smeekens (1999), the enzyme *6-FST* first mediates the formation of trisaccharide 6-kestose by transferring a fructose unit from sucrose to another sucrose molecule, and then catalyses the formation of bifurcose from 1-kestose. Bifurcose and 6-kestose then serve as substrates for the action of *6-SFT* and *1-FFT*, which mediate the elongation of fructan chains containing both β(1-2) and β(1-6) fructosyl linkages.

Genes encoding different fructosyltransferases have been studied based on cDNA extracted from various plant species. The *l-FFT* genes have been identified in Jerusalem artichoke (Van der Meer et al., 1998), globe thistle (*Echinops ritro* L.) (Van den Ende et al., 2006), onion (Vijn et al., 1998), perennial ryegrass (*Lolium perenne* L.) (Lidgett et al., 2002; Lasseur et al., 2006; Chalmers et al., 2003; Chalmers et al., 2005) and wheat (Kawakami and Yoshida, 2005). Genes encoding *l-SST* have been found in chicory (De Halleux and van Cutsem, 1997), Jerusalem artichoke (Van der Meer et al., 1998), fescue (*Festuca arundinacea* L.) (Luscher et al., 2000), perennial ryegrass (Chalmers et al., 2003), barley (Nagaraj et al., 2004) and wheat (Kawakami and Yoshida, 2002). Genes encoding *6-FST* have been identified in perennial ryegrass (Chalmers et al., 2005), bluegrass (*Poa secunda* L.) (Wei et al., 2002), wheatgrass (*Agropyron cristatum* L.) (Wei and Chatterton, 2001), barley (Sprenger et al., 1995; Nagaraj et al., 2004) and wheat (Kawakami and Yoshida, 2002; Kawakami and Yoshida, 2005). Genes encoding 6G-FFT have been found in perennial ryegrass (Lasseur et al., 2006) and onion (Vijn et al., 1997). There is gene similarity between plant species. For example, orthologues of the perennial ryegrass *l-FFT* and *l-SST* genes (Lidgett et al., 2002) have also been found in wheat (Francki et al., 2006).

Fructan degradation

Levan can be degraded by bacterial endo-and exo-levanases (Uchiyama, 1993). The exo-levanase removes a terminal fructosyl unit of a levan molecule, while the endo-levanase cuts the levan chain in between. In plants, fructans are broken down by the action of fructan exo-hydrolases (*FEHs*), which remove fructose units at the end of the fructan chains (Ritsema and Smeekens, 2003). They include 1-exohydrolase (*1-FEH*), which breaks down β(1-2) linkages, and 6-exohydrolase (*6-FEH*), which targets β(2-6) linkages. Chicory and other inulin-producing plants are known to contain *1-FEH* (Van den Ende et

al., 2000), while wheat produces both *1-FEH* and *6-FEH* (Van den Ende et al., 2003a; Van Riet et al., 2006). Internal bonds of fructan molecules can also be hydrolysed by endo-inulinases, which have been found in fungi (Uhm et al., 1998; Ohta et al., 1998a; Wang et al., 2003) and bacteria (Kang and Kim, 1999; Bruyn et al., 1992) but not in plants. Low-DP fructans can also be degraded by invertases, which are present in most species of higher plants (Cairns, 1993).

Genes encoding fructan exo-hydrolases have been reported for several plant species. The *1-FEH* genes have been cloned from chicory (Van den Ende et al., 2000; Van den Ende et al., 2001) and wheat (Van den Ende et al., 2003a). The 6-*FEH* genes have been isolated from sugar beet (*Beta vulgaris* L.) (Van den Ende et al., 2003b), *Arabidopsis thaliana* L. (De Coninck et al., 2005) and wheat (Van Riet et al., 2006).

2.3 Fructan measurement

Like other carbohydrates, fructans can be measured using the colorimetric reagent anthrone (Yemm and Willis, 1954). This method involves heating a plant extract with a mixture of anthrone and sulphuric acid, and estimating the total quantity of carbohydrates based on colour intensity. Due to its simplicity, the anthrone method has been commonly used in fructan determination in vegetative tissues of various species such as perennial ryegrass (Smouter and Simpson, 1991; Turner et al., 2001; Turner et al., 2006), barley (Wang and Tillberg, 1996) and wheat (Wardlaw and Willenbrink, 1994; Yang et al., 2004).

Acid hydrolysis has also been commonly used to measure total fructans (e.g., Jenkins et al., 2002). Acid can digest fructans into monosaccharides, which are then measured by either spectrophotometry or high performance liquid chromatography (HPLC); subsequently, fructans are estimated based on hexose equivalents. This approach has been applied to fructan measurement in vegetative tissues of wheat (Jenkins et al., 2002; Goggin and Setter, 2004; Ruuska et al., 2006).

Near-infrared reflectance spectroscopy (NIR) has also been used as a simple non-destructive method for estimating fructan concentration in vegetative tissues of various species, including wheat (Batten et al., 1993; McGrath et al., 1997), bentgrass (*Agrostis palustris* L.) (Narra et al., 2005) and fescue (*Festuca arundinacea* L.) Shewmaker et al., 2006). This approach requires accurate calibration based on the relationship between

known concentrations of fructans in standard samples and their light reflectance values. Therefore, NIR relies on a separate method that provides an accurate measurement of fructan levels in standard samples so that they can serve as standard values for establishing NIR calibration curves.

The anthrone, acid hydrolysis and NIR methods are all suitable for fructan measurement in plant vegetative tissues. In these tissues, fructans are the major component of soluble carbohydrates. Thus, analytical errors caused by the action of acid, anthrone or light reflectance on other carbohydrates would not be serious.

For cereal grains and many food products, however, fructans are present in small quantities, along with large amounts of starch, sugars and other carbohydrates. Fructan analyses therefore involve multiple digestions and measurements to minimise interference from these carbohydrates. In most existing methods, amyloglucosidase is used to remove starch interference, and inulinases break down fructans into glucose and fructose, which are then quantitatively measured by either spectrophotometry (e.g., Official AOAC Method 999.03; Andersen and Sorensen, 2000; Steegmans et al., 2004) or anion-exchange chromatography (e.g., Official AOAC Method 997.08; Quemener et al., 1994; Corradini et al., 2004). However, the inulinases break down not only fructans but also other carbohydrates such as sucrose and galacto-oligosaccharides including raffinose, stachyose and verbascose (Andersen and Sorensen, 2000). This can cause overestimation of fructan concentration. Raffinose is naturally present in various cereal grains including wheat, while stachyose and verbascose occur in seed of Leguminosae species (Kuo et al., 1988). Interference from these galacto-oligosaccharides has not been considered in most existing methods, except the latest version of the Megazyme fructan-assay kit (Megazyme K-FRUC), a commercialized protocol based on the AOAC Method 999.03 (McCleary et al., 2000). Nonetheless, this method is rather laborious and expensive due to high enzyme usage and the implementation of consecutive enzymatic hydrolyses and measurements of sugars. A procedure described by Quemener et al. (1994) appears more straightforward in that it enables different hydrolytic analyses to be performed in parallel, yet it still has the disadvantage in that interference of galacto-oligosaccharides is not excluded. It would therefore be useful to incorporate all analytical advantages from different existing methods into one improved procedure so that it can be conveniently used for fructan measurement in wheat grain and other cereal-based food.

2.3 Roles of fructans

Physiological functions in plants

Like starch, fructans play a role as reserve carbohydrates for the regrowth of plants. However, while starch is stored mainly in storage organs, fructans are present in many other plant tissues, such as leaf bases and roots, and they are then used for the regrowth (Morvan-Bertrand et al., 2001) or supporting the tolerance of plants against adverse environmental conditions. Fructans are known to be involved in the prevention of membrane damage by interacting with membrane lipids (Hincha et al., 2002), contributing to the integrity and normal functioning of plant cells under stressful conditions. Transgenic tobacco with an expressed levansucrase gene exhibited tolerance to drought (Pilon-Smits et al., 1995) and low temperature (Konstantinova et al., 2002).The breakdown of fructans during water deficit stress increases the concentration of low molecular weight carbohydrates (short-chain oligofructoses and reducing sugars), leading to a decline in osmotic potential, contributing to maintenance of cell turgor under drought stress (Virgona et al., 1991; Wardlaw and Willenbrink, 2000). Similarly, degradation of fructans results in increased concentration of monosaccharides, contributing to the osmotic driving force that favours flower expansion (Vergauwen et al., 2000).

In cereals, fructans can be temporarily accumulated in the stem and leaf sheath during vegetative growth and are then hydrolysed into sucrose and monosaccharides and transported to the developing grain. This remobilization is thought to contribute significantly to final grain yield, especially when crops are subject to adverse conditions (e.g., freezing and light/water deficit) in which photosynthetic assimilation is limited (Foulkes et al., 2002; Setter et al., 1998; Yang et al., 2004; Ruuska et al., 2006). On the other hand, fructan synthesis in the grain may also enhance grain development. As proposed by Pollock (1986), the formation of fructans reduces the concentration of sucrose in filial cells of grain, preventing sugar-induced feedback inhibition of photosynthesis and thereby maintaining the rate of sucrose loading into developing grains. Rapid grain development, which is contributed from remobilisation of stem fructans and synthesis of grain fructans, can also result in more fructans deposited in wheat endosperm (Schnyder et al., 1993), increasing fructan levels of milled products.

Health benefits of fructans for humans

Unlike plants, humans lack the enzymes to break down fructans. Food fructans therefore escape human digestion and become a carbohydrate source that selectively promotes the growth of beneficial bifidobacteria in the colon. This prebiotic effect is well documented for inulin-type fructans (Gibson et al., 1995; Jenkins et al., 1999; Gibson et al., 2004) and has been extended to other fructan types including branched fructans and levans (Dal Bello et al., 2001; Kilian et al., 2002). The effect may vary among different fructan types. For example, branched fructans have been found to have the best prebiotic effect *in vitro*, followed by inulin and pure levan (Probert and Gibson, 2002).

Favourable effects of fructans on gut microbial activity provide a number of health benefits. Dominance of beneficial bifidobacteria limits the development of gut pathogens, reducing the risk of colonic diseases (Buddington et al., 1996; Kleessen et al., 2007). Biomass and stool bulking are also increased as a result of fructan fermentation (Roberfroid et al., 1993; Gibson et al., 1995; Kleessen et al., 1997). Because fructans are not readily digested in the small intestinal tract, their intake can help control the blood sugar level and thus reduce the insulin requirement in both healthy and diabetic people (Rumessen et al., 1990; Ayman et al., 2004). Levan supplementation can be beneficial for the suppression of high-fat diet induced obesity, due to positive activation of lipid metabolism and suppression of lipogenesis in the liver (Kang et al., 2006). In addition, the colonic fermentation of inulin and other non-digestible oligosaccharides also results in the production of short-chain fatty acids, including acetate, propionate and butyrate (Baghurst et al., 1996; Van Loo et al., 1999), which have a positive effect on systemic lipid metabolism and colonic health. For example, butyrate plays a role in nourishing the colonic mucosa and in the prevention of colonic cancers by promoting cell differentiation, cell-cycle arrest and apoptosis of transformed colonocytes, inhibiting the enzyme histone deacetylase and decreasing the transformation of primary to secondary bile acids as a result of colonic acidification (Wong et al., 2006; Nguyen et al., 2006). A feeding study by Jacobsen et al. (2006) on rats exposed to azoxymethane, a colon carcinogenic agent, showed that the risk of colonic cancers was reduced as a result of high inulin intake. Supplements of levan-type fructans have also been reported to reduce the development of tumor cell lines *in vitro* (Yoon et al., 2004).

Fructan intake can also enhance nutrient availability and absorption in the gut. In a nutritional study using a rat model, Delzenne et al. (1995) demonstrated that the retention of Ca, Mg, Fe, Zn and Cu was increased in rats fed with inulin compared to those fed with normal diets. This positive effect on mineral absorption was confirmed in a separate study in which Fe-deficient anaemic rats recovered after feeding with inulin (Ohta et al., 1995). Inulin consumption has also been found to enhance the absorption of Ca in humans and animals, thereby improving the mineralization of bone (Abrams et al., 2005; Lobo et al., 2006).

Increased mineral absorption may be attributable to changes in the colonic environment as a result of fructan intake. Fermentation of fructans reduces the pH in the colon, thereby increasing the solubility of luminal calcium and facilitating the reduction of insoluble Fe^{3+} to soluble Fe^{2+}, as explained by Scholz-Ahrens et al. (2001) and Yeung et al. (2005). Another mechanism could be the role of fructans in modifying intestinal mucins and their Fe-binding proteins (Meslin et al., 1999) and Ca-binding proteins (Ohta et al., 1998b) that serve as transporters for Ca and Fe absorption. Increased dietary intake of fructans therefore has the potential to address problems of calcium and iron deficiencies in women and children in the developing world.

2.4. Fructan consumption in humans

Sources of fructan intake

Fructans have been incorporated into various commercial products, such as Beneo™ inulin, Beneo™ oligofructose, Raftilose® P95 and Raftiline® HP (Orafti, Belgium) and Actilight® (Beghin Meiji, France). These products are obtained from fructan-rich plants, such as chicory and Jerusalem artichoke using conventional manufacturing techniques, including extraction with water, purification, isolation and drying (Roberfroid and Delzenne, 1998; Tungland and Meyer, 2002; Kleessen et al., 2007). The extracted inulin can also be partially hydrolysed by endo-inulinases into low-DP fructans which can serve as functional sweeteners, providing carbohydrate sources suitable for diabetics (Park et al., 1998; Zhengyu et al., 2005). Neosugar, another commercial fructan product, comprises short-chain inulin which is synthesized from sucrose by incubating with yeast fructosyltransferases (Oku et al., 1984). Due to the cost of processing and distribution,

commercial fructans can be expensive and are therefore not be available for the majority of people, especially the poor in developing countries.

Natural fructans from everyday food are a less expensive source that is within the reach of most people. Fructans are naturally present in a range of plant-derived food sources, including cereal grains (e.g., barley, wheat and oat), vegetables (e.g., onion, garlic, lettuce, asparagus, leek and dandelion) and fruits (e.g., banana and plum) (Hendry and Wallace, 1993; Van Loo et al., 1995). These plant sources vary in the fructan content. According to Van Loo et al. (1995), chicory root is the most concentrated source of fructans (42%), followed by Jerusalem artichoke (18%), dandelion greens (14%), garlic (13%), leek (7%), globe artichoke (5%), onions (4%), asparagus (2.5%), wheat (2.5%), barley (0.8%), rye (0.7%) and banana (0.5%). Although cereal grains have low fructan concentrations, they can provide the highest total intake of fructans for humans because they are staple foods. 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 (5%). Therefore, any increase in wheat fructan concentration could contribute considerably to fructan consumption in countries where wheat is an important food.

Effective and safe dosages

A number of studies have been undertaken *in vivo* to investigate the effect of fructan supplementation at various dosages. Low intakes of inulin (2.5 g per day) have been shown to significantly modify the gut environment by reducing colonic pH (Bouhnik et al., 1999). Intakes at 4 or 5 g inulin per day can significantly increase bifidobacteria growth (Buddington et al., 1996; Bouhnik et al., 1999; Rao, 2001; Kolida et al., 2007). High doses of fructan have no toxic effects but may cause some intestinal discomfort (e.g., increased flatulence and osmotic pressure) in sensitive people (Coussement, 1999), such as patients with lactose malabsorption (Teuri et al., 1999).

2.5 Genetic improvement of fructan in food crops

Genetic engineering

In plants that lack endogenous fructan metabolism, genetic transformation has been used to make them produce fructans in their edible parts. For example, bacterial genes encoding for levansucrase were successfully transformed into maize and potato, allowing them to accumulate levan-type fructans in their storage organs (Van der Meer et al., 1994; Caimi et al., 1996). Recently, genes encoding *1-SST* and *1-FFT* from Jerusalem artichoke have been transformed into maize and potato, allowing them to produce inulin-type fructans in their kernels and tubers (Stoop et al., 2007). In plants that can synthesize fructans, genetic transformation has also been applied in order to diversify their fructan component. For example, introduction of onion *6G-FFT* into chicory plants, which produces linear inulin, resulted in mixed fructans including both linear and branched fructose polymers as found in barley (Vijn et al., 1997). This could make the transgenic fructans healthier (i.e., more pronounced prebiotic effects) than linear fructans (Probert and Gibson, 2002).

Although genetic transformation can produce new plant sources for human fructan intake, this approach has encountered some problems. As reviewed by Cairns (2003), the levels of fructans measured in the storage tissues of transgenic plants have been universally low. This may be due to the limitation of substrates (e.g., sucrose), or to degradation by enzymes, such as *FEHs* in sugar beet (Van den Ende et al., 2003b) and invertases with *FEH* activity in rice (Ji et al., 2007). Future genetic transformation for high fructan might need also to target genes for fructan degradation by somehow reducing or eliminating their functions. However, this action could be detrimental or even lethal for plants. Invertases and *FEHs* are ubiquitous in higher plants. Invertases are required for sucrose metabolism, while *FEHs* are known to help plants tolerate water deficit (Yang et al., 2004) and freezing (Gaudet et al., 1999; Van den Ende et al., 2005) and may have protective functions in pathogen-defense mechanisms (Van den Ende et al., 2003b). Furthermore, genetic transformation may cause developmental aberrations, such as those found for potato transformed with bacterial levansucrase genes (Caimi et al., 1997; Gerrits et al., 2001). Thus, it is uncertain whether genetic engineering will be a successful strategy for biofortification of crop plants with fructans.

Plant breeding

Fructan levels in storage organs of fructan-producing crops may be improved by breeding. In principle, breeding for a trait requires sufficient genetic variation (Knight, 1979). Considerable genetic variation in fructan concentration has been observed in some vegetables such as garlic (Hong et al., 1997), onion (McCallum et al., 2006) and Jerusalem artichoke (Kocsis et al., 2007), and cereal grains such as oat (Aman, 1987) and rye (Hansen et al., 2003), indicating that breeding for high fructan in these food crops is possible. However, no such results have been reported for wheat. Nardi et al. (2003) reported grain fructan variation for bread wheat, durum wheat, rye, barley and triticale, but they included only one genotype of each species. Other research in wheat has mainly focussed on fructan chemistry and physiology of accumulation (e.g., White and Secor, 1953; Montgomery and Smith, 1957; Dahlqvist and Nilsson, 1984; Nilsson and Dahlqvist, 1986; Schnyder et al., 1993).

Understanding the inheritance of a trait can contribute to the efficiency and effectiveness of a breeding program. For example, genetic loci with major effects have been found to affect the content of fructans in onion bulb, facilitating the selection for this trait using molecular markers (McCallum et al., 2006). There have been no such reports on the inheritance of fructan accumulation in the storage organs of other food crops. Most research has focussed on fructans in the vegetative tissue of plants, such as barley (Hayes et al., 1993) and perennial ryegrass (Turner et al., 2006). Further, Yang et al. (2007b) have reported loci with major effects on the accumulation of water-soluble carbohydrates in wheat stems. Because water-soluble stem 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 in stems might also affect fructan levels in grain. However, fructan accumulation in the grain can be complicated by source-sink relationships and by dilution effects during grain development (Schnyder et al., 1993). In addition, most synthesized fructans are lost late in grain development (Nardi et al., 2003; Schnyder et al., 1993), indicating that other factors such as fructan-degrading enzymes contribute to the final fructan level in wheat mature grains. With molecular markers, it may be possible to dissect this complex trait into quantitative trait loci (QTLs) that can be used in breeding for high fructan wheat.

2.6 QTL mapping: methodology and application

Genetic markers

Genetic markers are useful tools for genetic studies. They may be near genes of interest and thus tend to be inherited together. While localising the genes can be a challenge, the genetic markers are easily tracked since they involve simple assays capable of detecting DNA polymorphisms at specific locations in the genome. Detection may be done at the level of DNA itself (molecular markers), the gene product (protein markers), biochemical processes or the plant phenotype (morphological markers). Polymorphic marker loci can be mapped relative to one another based on observed frequencies of recombinants (e.g., Botstein et al., 1980). In some cases, marker loci can be assigned to positions on physical maps (e.g., Sourdille et al., 2004). These lead to development of marker linkage maps that can be used to detect and map quantitative trait loci or genes across plant genomes.

Molecular marker technologies enable detection of DNA polymorphisms in different ways. Restriction enzymes can be used to cut DNA at specific sequence motifs (Meselson and Yuan, 1968), yielding fragments of different lengths, so-called restriction fragment length polymorphism (RFLP). Polymerase chain reaction (PCR) has been used to amplify specific DNA fragments (Mullis and Faloona, 1987); enabling assays for marker types including random amplified polymorphic DNA (RAPD) (Williams et al., 1990), amplified fragment length polymorphism (AFLP) (Vos et al., 1995) and simple sequence repeats (SSR) (Lagercrantz et al., 1993). DNA fragments differing in length can be separated on gel or capillary electrophoresis and then visualised using radioisotopes, silver stain or florescent tags. Sequencing or hybridisation technologies can also be used to detect DNA polymorphism at the single nucleotide level, the so-called single nucleotide polymorphism (SNP) (Landegren et al., 1998). Diversity Array Technology™ (DArT), which combines the use of restriction enzymes, DNA hybridisation and microarray technologies (Jaccoud et al., 2001), enables highly parallel detection of DNA polymorphisms throughout the genomes of plant species including wheat (Akbari et al., 2006).

Construction of molecular marker maps in wheat initially relied mainly on RFLP markers, then mainly on SSR markers and now often the use of both SSR and DArT markers. The generation of RFLP markers is rather laborious and requires large amounts of genomic DNA, while SSR marker makes use of the PCR technology, which can be automated and

requires minute amounts of genomic DNA. Further, SSR loci are highly polymorphic, abundant and well dispersed in the genome (Tautz and Renz, 1984; Lapitan, 1992). Primers for wheat SSR markers are internationally available, many of which have been physically mapped to wheat chromosomes (Sourdille et al., 2004) and thus serve as reference or anchor markers for linkage map construction. High-throughput methods to detect SSR loci have now been established, including that described by Hayden et al. (2008) which enables large numbers of SSRs to be assayed simultaneously and economically. Likewise, the generation of DArT markers is genetically and economically efficient. This technology can track hundreds to thousands of genomic loci in parallel and thus also contribute to a rapid generation of saturated marker maps for linkage analyses (Akbari et al., 2006). While SNPs promise the most abundant and polymorphic class of genetic markers (Landegren et al., 1998), their use in wheat is still limited, due to the difficulty of discovering genome-specific SNPs in polyploid species.

Many molecular marker linkage maps have been constructed for wheat using data from populations of doubled haploid (DH) lines (e.g., Kammholz et al., 2001; Chalmers et al., 2001; Williams et al., 2002, 2003) or recombinant inbred lines (RIL) (e.g., Garland et al., 1999; Hazen et al., 2002; Somers et al., 2004). A wheat DH population can be obtained from the F_1 generation from a cross between two inbred lines. Haploids derived from wheat F_1 plants, by either anther culture or wide crossing between wheat and maize, are subject to chromosome doubling with cochicine, resulting in lines that are completely homozygous (Zamani et al., 2000; Daniel et al., 2005). RIL populations are produced by crossing two inbred lines followed by repeated selfing or sibling mating to create a set of new inbred lines. Both DH and RIL populations are particularly convenient for QTL mapping. The homozygous lines can be propagated eternally and thus phenotyped repeatedly within and between environments, reducing residual effects.

QTL mapping methods

Mapping QTLs involves a systematic search for linkage disequilibrium between QTLs and genetic markers. This association analysis therefore requires both marker information and phenotypic values measured for each individual in the mapping population. QTL mapping can also involve more sophisticated analyses due to the fact that genes controlling the quantitative trait may also interact with each other and with environments (Mackay, 2001).

Different methods of QTL mapping have been developed with increasing levels of complexity. The first level, single marker analysis (SM), involves tests of association between trait values and the genotypes at each marker locus (Edwards et el., 1987). Since these tests consider each marker locus separately, the relative position of QTL on a marker linkage map cannot be established. The second level of QTL mapping, simple interval mapping (SIM), was first developed by Lander and Botstein (1989). It involves a test of association between trait values and positions within marker intervals along the marker linkage map. Using SIM, however, the identification of QTLs can be misleading due to possible interference of other markers. Zeng (1994) addressed this problem by proposing a method called composite interval mapping (CIM), in which markers outside the test interval (i.e., background markers) serve as covariates. The computerization for CIM is rather heavy due to the simultaneous fitting of parameters for both target intervals and their background markers. Tinker and Mather (1995a) tackled the issue by introducing a simplified composite interval mapping (sCIM) approach. This approach also enabled the analysis of QTL x environment interactions using large sets of data from multiple environments. To investigate QTL x QTL interactions, Kao et al. (1999) introduced a method so-called multiple interval mapping (MIM) that could fit multiple putative QTLs simultaneously to reveal their individual effects as well as their epistatic interactions. However, MIM cannot detect epistatic QTLs with no individual effects. This limitation was then surmounted by the use of the algorithm-adapted DIRECT developed by Ljungberg et al. (2004).

Along with the methodology development, various computer programs have also been written to perform the sophisticated algorithms of SM, SIM, CIM, sCIM and MIM, such as MQTL (Tinker and Mather, 1995b; performing SIM and sCIM), QGENE (Nelson, 1997; performing SM and SIM), QTL Cartographer (Wang et al., 2007; performing SM, SIM, CIM and MIM). However, none of these has integrated all functions of QTL methodologies in one mapping system. Recently, Yang et al. (2008) has introduced the program QTLNetwork that enables detection of multiple QTLs, their interactions with each other and with the environments using a full QTL model (Yang et al., 2007a). This approach has been applied to elucidating the complex genetic architecture of wheat stem carbohydrates (Yang et al., 2007b), wheat coleoptile growth (Rebetzke et al., 2007), wheat plant height (Zhang et al., 2008a), nutritional quality of cotton (Song and Zhang, 2007) and grain yield of rice (Liu et al., 2008).

Applications of QTL information

Once a QTL for a trait is detected in a given cross, marker-assisted selection (MAS) may be performed in which desirable offspring are selected based on the presence of the favourable allele at one or more markers near that QTL. With MAS, breeders can impose early selection by screening the DNA of juvenile plants. Early identification of plants carrying favourable alleles can speed up the breeding process, and early elimination of plants carrying unfavourable alleles can reduce space and resources necessary for performance trials.

MAS is particularly useful for backcross breeding because it can enable accurate selection for the gene from the donor parent and accelerate recovery of the recurrent parent genome (Frisch et al., 1999; Miklas, 2007; Garzon et al., 2008). For example, successful gene introgression using marker-assisted backcrossing has been done for one or two genes conferring pathogen resistance in rice (Huang et al., 1997), barley (Jefferies et al., 2003), tomato (Barone et al., 2005), soybean (Maroof et al., 2008) and wheat (Zhou et al., 2003; Datta et al., 2006). Recently, Kuchel et al. (2007) reported a successful application of MAS dealing with multiple genes and multiple traits at a time (disease resistance, plant types and end-use quality). The strategy combined the use of MAS with restricted backcrossing and DH technology, thereby reducing selection intervals and increasing selection accuracy.

Identification of QTLs can also provide a framework for fine mapping to identify 'perfect' markers or gene-based markers that are directly useful for MAS. Fine mapping of major QTLs have been reported for important traits, such as barley seed dormancy (Han et al., 1999), tomato fruit size (Frary et al., 2000), barley malting quality (Gao et al., 2004), soybean seed protein (Nichols et al., 2006), rice grain length (Wan et al., 2006), soybean insect resistance (Zhu et al., 2006), wheat grain protein content (Distelfeld et al., 2006) and rice grain yield (Xie et al., 2008). Fine mapping also enables positional cloning and functional analysis of candidate genes under detected QTLs, providing understanding of molecular mechanisms controlling quantitative variation of the trait. Such an application would be important for grain fructan research, because genes controlling grain fructan accumulation have not been isolated. Even though genes involved in fructan metabolism have been identified for wheat (Kawakami and Yoshida, 2002; Van den Ende et al., 2003a; Kawakami and Yoshida, 2005; Van Riet et al., 2006; Francki et al., 2006), it is still not

known whether they cause differential accumulation of grain fructans. With QTL information, it would be possible to target polymorphic genes that are directly useful for marker assisted breeding.

2.7 Conclusions and implications for the thesis

The health benefits of fructans have been widely documented, mostly based on evidence from studies in which inulin-type fructans were used as functional additives. Fructans from wheat and some other food crops may exert more pronounced effects due to their diverse chemical structures. Efforts have been made to increase fructan concentrations in plant-sourced foods that are consumed in daily diets. Genetic engineering could make non-fructan crops produce fructans, but this action may negatively impact on plant health and productivity. One alternative is to breed fructan-producing crops for increased fructan levels. Wheat is of particular interest in this respect, as it is a major carrier of fructans in human diets. Breeding for high fructan relies on availability of sufficient genetic variation, and can make use of an understanding of trait inheritance. However, no information on either of these has been reported for wheat grain fructan to date, suggesting the need for research. Limitations in grain fructan measurement technology can make the research difficult, thus raising the need for further analytical improvement. Genetic studies would be favoured by advances in molecular marker technologies and QTL mapping methods. Combined with candidate-gene information, it would be possible to study the genetic control of quantitative variation (if any) in wheat grain fructan accumulation, providing genetic markers for use in marker-assisted breeding.

Chapter 3

Measurement of Fructan Concentration in Wheat Grain [1]

Abstract

Fructans are non-digestible carbohydrates with potential health benefits. They are naturally present in the grain of various cereals, including wheat, and may be suitable targets for biofortification by plant breeding or genetic manipulation. Understanding the genetic control of grain fructan accumulation could benefit breeding programs aiming to increase grain fructan content, but to date there is no information available. Studies that include surveys of genotypic variation and genetic mapping require an efficient fructan measurement to assess large numbers of breeding lines. Current methods are intricate and expensive and can be inaccurate for cereal grain due to interference from starch and galacto-oligosaccharides. The aim of the research reported in this chapter was to develop an improved analytical procedure to measure wheat grain fructan concentration. The method involves extracting fructans from wheat grain with boiling water followed by enzymatic hydrolyses to remove major sources of interference and to break down fructans into monosaccharides that can then be quantitatively measured by anion-exchange liquid chromatography coupled with pulsed amperometric detection. The modified procedure is reliable and allows the handling of large numbers of flour samples at a low cost. It could therefore be useful for assessing large numbers of wheat breeding lines.

[1] This chapter contains information that is also published in: Huynh BL, Palmer L, Mather DE, Wallwork H, Graham RD, Welch RM, Stangoulis JCR (2008) Genotypic variation in wheat grain fructan content revealed by a simplified HPLC method. Journal of Cereal Science 48:369–378

3.1 Introduction

Fructans (fructo-oligosaccharides) have been used as functional food supplements due to their beneficial health attributes, including prebiotic effects (Gibson et al., 1995; Buddington et al., 1996; Probert and Gibson, 2002), increased nutrient absorption (Delzenne et al., 1995; Abrams et al., 2005), controlled blood sugar and lipid (Jackson et al., 1999; Ayman et al., 2004), and reduced colonic cancers (Yoon et al., 2004; Jacobsen et al., 2006). These compounds also occur in a range of plant-sourced foods, including cereal grains (e.g., barley, wheat and oats), vegetables (e.g., onion, garlic, lettuce, asparagus, leek and dandelion) and fruits (e.g., banana and plum) (Hendry and Wallace, 1993; Van Loo et al., 1995) and thus provide an alternative source of fructan intake for humans. Among these fructan-producing crops, wheat is the main carrier of fructans in Western diets, based on surveys of Van Loo et al. (1995) and Moshfegh et al. (1999). Any genetic improvement for wheat fructan could contribute considerably to fructan consumption in countries where wheat is a staple main food (e.g., South Asian countries including Pakistan and India).

There has been little research on genetic improvement of grain fructan in wheat and other cereals, partly because grain fructan level is difficult to measure accurately. Genetic studies such as variation surveys and mapping experiments require an efficient fructan measurement method in order to assess large numbers of wheat breeding lines.

Fructans have various chain lengths and structures, so their total content is normally measured through enzymatic hydrolysis to break down fructans into monosaccharides (glucose and fructose) and subsequent analysis by either spectrophotometry (McCleary et al., 2000; Official AOAC Method 999.03; Andersen and Sorensen, 2000) or HPLC (Quemener et al., 1994; Official AOAC Method 997.08; Corradini et al., 2004). With samples from cereal grains, analytical errors can occur during fructan analysis due to interference of starch and free sugars, such as glucose, fructose, sucrose and galacto-oligosaccharides including raffinose, stachyose and verbascose, which are naturally present in various cereal grains (Kuo et al., 1988; Henry and Saini, 1989). Handling this source of error requires multiple analyses in order to differentiate the fructose and glucose components of fructans from those of other carbohydrates, making fructan analysis intricate, expensive and potentially inaccurate.

The AOAC Method 997.08 (Official Methods of Analysis, 2000) has been recommended for the determination of fructans in various food products. This method involves three separate HPLC analyses, including a direct analysis of fructan extract, an analysis of the extract after amyloglucosidase treatment and an analysis of the extract after inulinase treatment (Fig. 1). Because the inulinase used in the third analysis digests not only fructans but also galacto-oligosaccharides into fructose (Andersen and Sorensen, 2000), use of the AOAC Method 997.08 may lead to overestimation of the fructan concentration in cereal grain. This issue was addressed in the development of the latest version of the Megazyme fructan-assay kit (Megazyme K-FRUC), a commercialized protocol based on the AOAC Method 999.03 (McCleary et al., 2000). In this protocol, the interference of galacto-oligosaccharides is eliminated early in the procedure using α-galactosidase. Subsequent analyses involve hydrolyses of starch and sucrose, removal of reducing sugars by borohydride, and inulinase treatment to hydrolyse fructans into glucose and fructose, which are then measured by spectrophotometry (Fig. 1). Both AOAC 997.08 and AOAC 999.03, however, are generally laborious and expensive due to high enzyme usage and the implementation of consecutive enzymatic hydrolyses and measurements of sugars. In contrast, a procedure described by Quemener et al. (1994) appears more straightforward in that it enables different hydrolytic analyses to be performed in parallel (Fig. 1), which is more convenient for the handling of large numbers of samples. Nonetheless, this method still has the drawback in that galacto-oligosaccharides interference is not excluded from the analysis. The aim of the research in this chapter is to develop an improved procedure for fructan measurement (Fig. 1), which is based on those analytical procedures described in Quemener et al. (1994), the AOAC method 997.08 and the Megazyme fructan-assay kit but modified to eliminate interference from galacto-oligosaccharides and to handle larger numbers of wheat grain samples, thereby reducing cost.

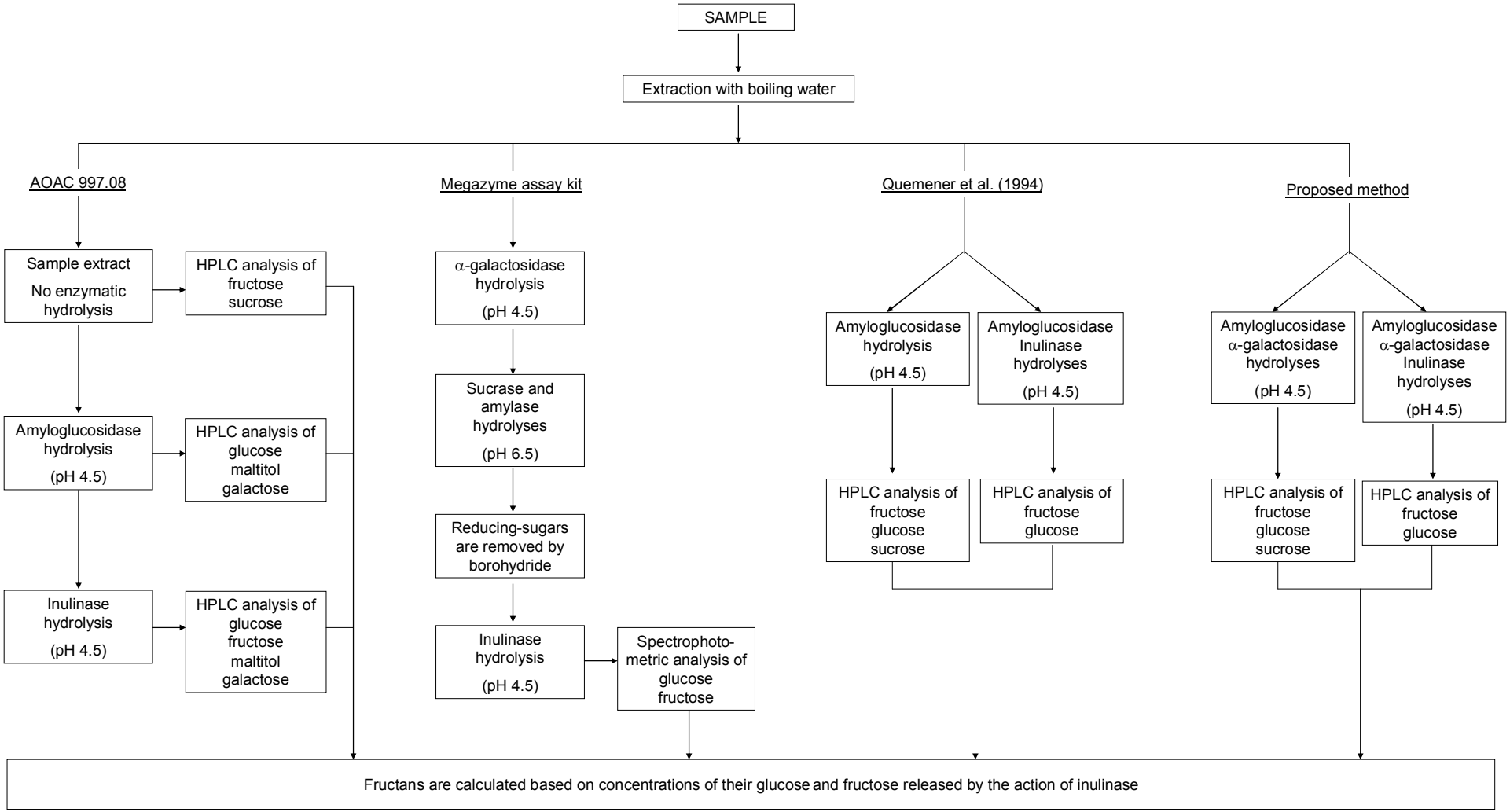


Figure 1 Comparison of four analytical procedures for fructan measurement: AOAC Method 997.08, AOAC Method 999.03 as implemented in a Megazyme fructan-assay kit, a method presented by Quemener et al. (1994) and the method proposed in this chapter.

3.2 Materials and Methods

3.2.1 Fructan analysis

The principle

Fructans must be extracted from wheat flour with boiling water, because high temperature can enhance fructan solubility and denature enzymes in the grain that may interfere with the analysis. Two aliquots of extract are hydrolysed separately, using different enzyme mixtures (A and B). These mixtures both contain amyloglucosidase and α-galactosidase. One of them (mixture B) also contains inulinase. Amyloglucosidase will hydrolyse starch into glucose, α-galactosidase will hydrolyse galacto-oligosaccharides into galactose and sucrose, and inulinase (when present) will further hydrolyse the sucrose and fructans into glucose and fructose. The hydrolysates are then analysed using high-performance anion-exchange liquid chromatography coupled with pulsed amperometric detection. Fructans are then calculated from their glucose and fructose concentrations (i.e., the difference in sugar concentrations between the two analyses), the sucrose concentration from the mixture without inulinase and the weights of wheat flour and boiling water. This principle is generally similar to that described by Quemener et al. (1994) except that α-galactosidase is used in both analyses to eliminate raffinose interference (Fig. 1). In addition, the preparation of enzymes and other reagents was modified based on AOAC method 997.08 (Official Methods of Analysis, 2000) and Megazyme fructan assay kit (Megazyme, Ireland) (McCleary et al., 2000) to allow specific and complete digestion of fructans and other sugar polymers.

Apparatus

Carbohydrate measurement was conducted on a Dionex ICS-3000 HPLC system equipped with an eluent generator, a CarboPac PA20 guard column (3 x 30mm) coupled to a CarboPac PA20 analytical column (3 x 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. Other instruments included a 4-digit balance, a shaking incubator (Ratek, model OM15), a portable Eppendorf thermomixer (Thermomixer comfort, Eppendorf), a tube rotator mixer

(Barloworld Scientific, model SB2), centrifuges for 15mL-tubes (Hettick Zentrifugen, model Universal 32) and 1.5mL tubes (Hettick Zentrifugen, model Mikro 20) and micropipettes (20, 200 and 1000µL). The micropipettes were calibrated by mass, which allows volumetric-to-mass conversions during fructan analysis.

Preparation of reagents

Lyophilized amyloglucosidase (Sigma A7420), α-galactosidase (Megazyme E-AGLAN) and inulinase (Megazyme E-FRMXPD) were prepared at 20 units/mL in acetate buffer solution, pH 4.5, and stored at -20 ^{o}C. One enzyme unit (U) is the amount of that enzyme for hydrolysing 1 mg of its substrate (AOAC Method 997.08). The pH-4.5 acetate buffer was made by diluting 28 mL of 0.2M acetic acid and 22 mL 0.2M Na acetate to 100 mL using high purity deionized water (18.2 MΩ cm^{-1} resistivity) (AOAC Method 997.08). Prior to analysis, stock enzyme solutions were combined into two enzyme mixtures. Enzyme mixture A had a 1:1:1 volumetric ratio of amyloglucosidase, α-galactosidase and buffer solution. Enzyme mixture B had a 1:1:1 volumetric ratio of amyloglucosidase, α-galactosidase and inulinase in buffer solution. As a result, these mixtures contained 1 U/150 µl for each enzyme, which was adequate for enzymatic hydrolysis of one sample described in the "*Enzymatic hydrolysis*" section.

Sugar standards (glucose, fructose, sucrose and rhamnose) were of analytical-grade quality (Sigma-Aldrich Pty. Ltd.). Working-standard solutions of each sugar were prepared at 1, 10, 100 and 500 µM by diluting with deionized water (containing 0.05% sodium azide to prevent microbial growth), and an internal rhamnose standard. The final concentration of rhamnose was 100 µM in all working-standard solutions.

Extracting fructans

For each sample analysed, approximately 150 mg of wheat flour was weighed (M_S) and extracted with boiling water (>85^{o}C) in a 15 mL centrifuge tube for 5 min using a tube rotator mixer and then in a shaking incubator (80^{o}C, 250 rpm) for 15 min to optimize fructan solubility, starch gelatinization and solution homogeneity. By this means, one extraction was sufficient to fully extract fructans from wheat flour as revealed from

multiple-extraction checks. To prevent formation of a thick glue-like consistency within the mixture of wheat flour and hot water, 0.5 mL of cold water was added to the flour and vortexed to create a suspension before hot water was added (approximately 14 mL). The tube was then cooled to room temperature and the amount of water (M_W) was calculated by taking the difference between tube weights before and after adding water. The extract was centrifuged at 9000 rpm for 10 min in the 15-mL tube and a further 13,000 rpm for 15 min in a 1.5-mL Eppendorf tube (containing 1 mL of supernatant). The supernatant was then transferred into a new Eppendorf tube for further analyses. This extraction step can be done for a large number of samples. The supernatants in the 15-mL tubes may be kept frozen at -20 ^{o}C for days but before analysis need to be re-heated at 80 ^{o}C in a shaking incubator to dissolve any precipitated fructans that may form during storage before analysis.

Enzymatic hydrolysis

One 50-µl aliquot of extract was injected into each of two 1.5-mL Eppendorf tubes, one containing 150 µl of enzyme mixture A and one containing 150 µl of enzyme mixture B. The tubes were covered, incubated at 60 ^{o}C and mildly shaken for 30 min using a shaking incubator or portable Eppendorf thermomixers. The hydrolysate solutions were then cooled to room temperature and diluted to 1 mL (i.e. dilution factor = 20) by adding 700 µL of deionized water containing 0.05% sodium azide and 100 µL of internal standard rhamnose (1 mM). This resulted in a final rhamnose concentration of 100 µM in each diluting solution. The solutions were centrifuged at 13,000 rpm for 15 min and supernatants transferred to 1-mL vials for HPLC analysis. Two blank (water) samples were also included in analyses A and B in order to control for sugars present in enzyme solutions during HPLC analysis. This enzymatic hydrolysis step can be performed for a large number of samples before analysis, and the hydrolysates may be kept frozen at -20 ^{o}C until further analysed.

HPLC analysis

The hydrolysates (glucose, fructose and sucrose) were separated as anions with KOH eluent (Table 2) through a CarboPac PA20 guard column (3 x 30mm) coupled to a

CarboPac PA20 analytical column (3 x 150 mm) and detected by pulsed amperometry on a disposable gold (Au) working electrode programmed as shown in Table 3. The eluent flow rate was 0.42 mL min^{-1}, and the column temperature was 30°C. Chromatographic signals were analysed by the computer software Chromeleon (version 6.70), with glucose, fructose and sucrose determined based on their standards with reference to the internal standard and the blank samples. Analysis A (without inulinase) gave the amounts of total glucose (from soluble starch and free glucose), sucrose (released from galacto-oligosaccharides by α-galactosidase plus free sucrose) and free fructose (Fig. 2B). Analysis B (with inulinase) gave the amounts of total glucose and fructose, which were equal to their amounts in analysis A plus those cleaved from sucrose and fructans (Fig. 2C).

Table 2 Eluent profile for separating carbohydrates using an eluent generator.

Time (min)	KOH (mM)	Comment
-20.00[a]	100	Column wash
-17.00[a]	10	Equilibration
0.00	10	Injection, acquisition start
20.00	10	Acquisition end

[a] Negative time indicates time prior to sample injection

Table 3 Waveform for carbohydrate analysis using an electrochemical detector.

Time (s)	Potential (V)	Integration
0.00	+0.1	
0.20	+0.1	Begin
0.40	+0.1	End
0.41	-2.0	
0.42	-2.0	
0.43	+0.6	
0.44	-0.1	
0.50	-0.1	

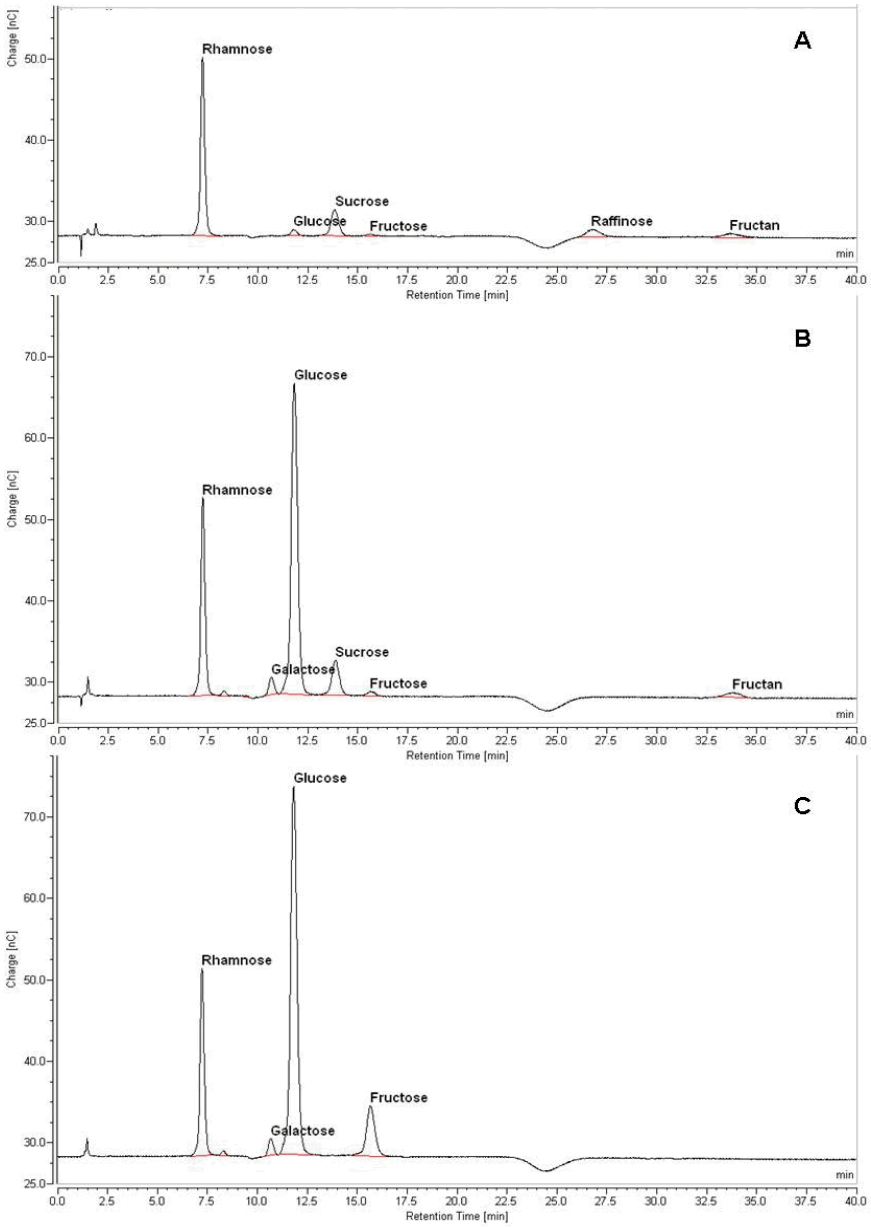


Figure 2 HPLC profiles of a wheat sample (cv. Gandum Zaapharoni) from different analyses: **(A)** Direct analysis (no enzyme treatment): rhamnose (internal standard), free sugars (glucose, sucrose, fructose and raffinose) and a trace of fructans; **(B)** Analysis A (digested by amyloglucosidase and α-galactosidase): rhamnose (internal standard), galactose (from raffinose), glucose (from soluble starch and free glucose), sucrose (from raffinose and free sucrose), free fructose and a trace of fructans; **(C)** Analysis B (digested by amyloglucosidase, α-galactosidase and inulinase): rhamnose (internal standard), galactose (from raffinose), and glucose and fructose, which were equal to their amounts in analysis A plus those cleaved from sucrose (in analysis A) and fructans.

Fructan concentration calculation

Fructan concentration was calculated from glucose and fructose components cleaved by the action of inulinase. Concentrations of glucose and fructose from fructans were determined using the following equations.

$$G_{Fructans}(\%) = \frac{180.16\, M_W (G_B - G_A - S)\, DF}{1000\, M_S}$$

$$F_{Fructans}(\%) = \frac{180.16\, M_W (F_B - F_A - S)\, DF}{1000\, M_S}$$

Where,

180.16 = molecular weight of glucose or fructose,

M_W = amount of water in the initial extract (g),

M_S = sample weight (mg),

DF = Dilution factor of the hydrolysates, in this case DF = 20

G_A = glucose concentration from analysis A (µM),

G_B = glucose concentration from analysis B (µM),

F_A = fructose concentration from analysis A (µM),

F_B = fructose concentration from analysis B (µM), and

S = Sucrose concentration from analysis A (µM). This sucrose includes free sucrose in the flour and those released from galacto-oligosaccharides. Under the action of inulinase, the sucrose produced the same molarity of glucose and fructose that were subtracted in the equations.

The fructan concentration was then obtained by the following equation (AOAC Method 997.08).

$$\text{Fructans}(\%) = k(G_{Fructans} + F_{Fructans})$$

Where, $k = \frac{180 + 162(n-1)}{180n}$; n = average degree of polymerisation = $\frac{F_{Fructans}}{G_{Fructans}} + 1$

3.2.2 Method validation

Recovery of fructans was measured in triplicate using pure fructan samples (Sigma I2255) at seven levels (1, 2.5, 5, 10, 50, 100 and 150 mg per extraction) and by spiking fructan into wheat reference flour at three levels (approximately 0.5, 5 and 10% of the flour weight) in 10 replications. Recovery of spiked fructans was calculated using the following expression.

$$\text{Recovery}(\%) = \frac{1}{10}\sum_{i=1}^{10} \frac{\text{Flour weight}_i \text{ x (total fructan concentration}_i \text{ - A)}}{\text{Spiked fructan weight}} \text{ x } 100$$

Where, A is the fructan concentration of straight wheat flour (no spiked fructans). Simple linear regression analysis was used to describe the recovery response of external fructans.

The precision of the method was assessed by analysing a wheat flour and fructan control flour (28.8% fructan), provided in the Megazyme fructan assay kit (Megazyme, Ireland), in 10 replications. The upper and lower limits of the 95% confidence interval were estimated using the following expression.

$$\text{Mean} \pm \frac{\text{Student t variable } (t_{0.95}) \text{ x Standard deviation}}{\sqrt{\text{number of determinations}}}$$

The relative standard deviation (standard deviation x 100 / mean x 100%) was computed for ten replicates of each fructan-spiked sample and reference flour to provide an indicator of repeatability.

An additional test was performed in which bacterial levan (Sigma L8647) was analysed using the proposed method. This fructan has very high DP (molecular mass 16.9 x 10^6), branching and comprising mainly β(2-6) linkages (Stivala and Khorramian, 1982). To facilitate complete digestion of this high DP fructan, the analysis was also performed using higher dose of inulinase (3 U) and longer incubation time (100 min). Samples of two wheat lines (cv. Berkut and cv. Krichauff) were also concurrently analysed in order to compare their fructan levels between the two hydrolytic conditions.

3.2.3 Measuring galacto-oligosaccharides interference

The interference of galacto-oligosaccharides (e.g., raffinose, stachyose and verbascose) was examined by comparing fructan values between the proposed procedure and an existing method where galacto-oligosaccharides are not considered (Quemener et al., 1994). In this method, the fructan concentration was also determined based on analyses of two aliquots of the extract using different enzyme mixtures (amyloglucosidase vs. amyloglucosidase + inulinase) but not containing α-galactosidase. The materials were a wheat sample (cv. Berkut) and its raffinose-spiked flour (approximately 10% of the flour weight). The extent of galacto-oligosaccharide interference was also examined using seed samples from some other species known to have higher galacto-oligosaccharide contents than wheat (Kuo et al., 1988). They included barley (*Hordeum vulgare* L., cv. Sloop), triticale (X *Triticosecale* Wittmack, cv. Credit), faba bean (*Vicia faba* L., cv. Fiesta) and field pea (*Pisum sativum* L., cv. Kaspa). These materials were obtained from seed banks of the South Australian Research and Development Institute and the University of Adelaide. Seed galacto-oligosaccharide contents for these materials were also measured in hot-water extract using HPLC conditions described previously (Tables 2 and 3) but the acquisition time was increased up to 40 minutes. The concentration of each sugar was quantified based on standards (raffinose, stachyose and verbascose) containing 10, 25 and 50 μM of that sugar and also containing rhamnose (100 μM) as an internal standard.

3.3 Results and discussion

Recovery of pure fructans at multiple concentrations showed a linear recovery response ($R^2 = 0.9997$) with up to 99.5% recovery (Fig. 3), indicating that the method could be used to measure a large range of fructan concentrations (i.e., up to 100% or 150 mg fructans per extraction). Acceptable recoveries (98-100%) were also found for fructans spiked into wheat flour (Table 4). The relative standard deviation for fructan measurement was small, ranging between 0.7 and 7.7% in replicated analyses of reference flours and fructan-spiked samples (Table 4 and 5). Thus, the quantification of fructan concentration was both accurate and precise.

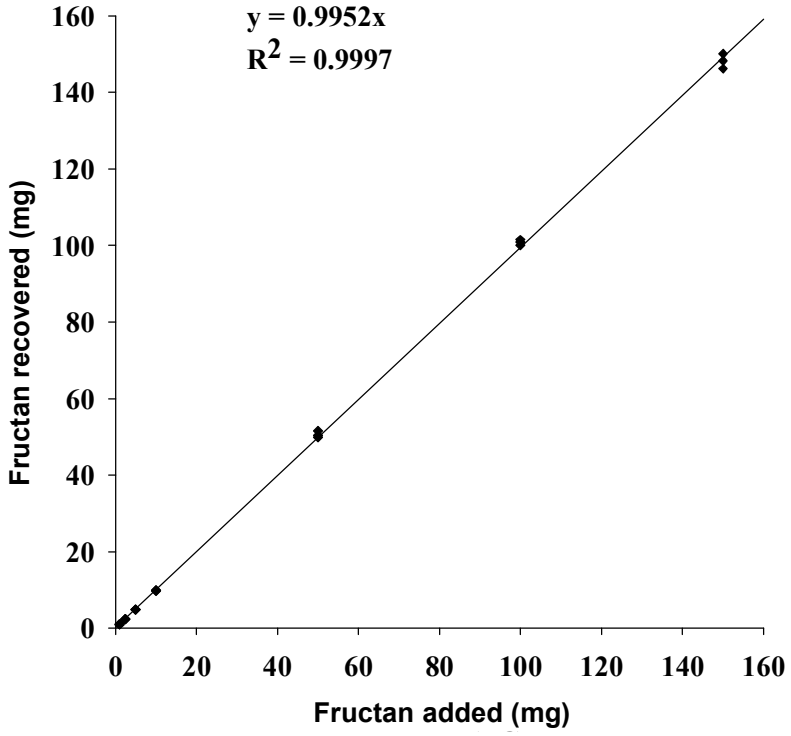


Figure 3 Recovery of fructans using the proposed method. Pure fructan samples (Sigma I2255) were prepared in triplicate at 1, 2.5, 5, 10, 50, 100 and 150mg. The straight line represents 99.52% recovery.

Table 4 Recovery of spiked fructans using the proposed method.

Fructan-spiked flour	Mean [and 95% confidence limits] for total fructans (%)	Relative standard deviation (%)	Recovery (%)
Spike A (0.72 mg fructans)	1.69 [1.61, 1.77]	7.7	98.5
Spike B (7.20 mg fructans)	5.63 [5.57, 5.69]	1.8	97.8
Spike C (14.4 mg fructans)	10.4 [10.29, 10.51]	1.8	99.6

Table 5 Results of the determination of fructans in reference flours.

Reference flours	Mean [and 95% confidence limits] for total fructans (%)	Relative standard deviation (%)
Megazyme fructan control flour	28.9 [28.79, 29.01]	0.7
Wheat reference flour (whole-grain)	1.11 [1.07, 1.15]	6.3

Analysis of bacterial levan gave 30% recovery using the proposed method (Fig. 4A). This observed low recovery is probably due to the high DP of levan molecules, which may require more enzyme activity and incubation time to be fully digested. Indeed, increasing inulinase dosage (3 units) and incubation time (90 min) almost digested levan (91%, Fig. 4A). However, applying this hydrolytic condition did not increase the estimate of fructan concentration of wheat (Fig. 4B), indicating that long-DP fructans like bacterial levan may not occur in wheat grain. The mixed-levan with shorter DP in wheat grain (Nilsson and Dahlqvist, 1986) should be fully digested using the hydrolytic conditions described in the proposed method.

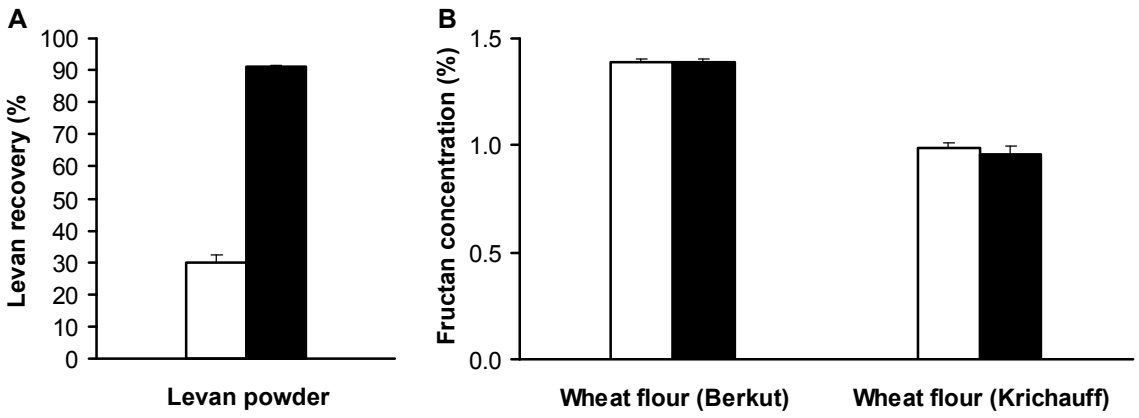


Figure 4 (**A**) Recovery of pure levan (Sigma L8647) and (**B**) fructan concentrations of wheat grain (cv. Berkut and cv. Krichauff) measured by the proposed method applying different digesting conditions: (□) 1 unit of inulinase for 30 min and (■) 3 units of inulinase for 90 min. Error bars represent standard errors of the mean of three determinations.

The modified procedure has several advantages that might contribute to its accuracy and precision. Firstly, the use of α-galactosidase in both digestions eliminates interference from galacto-oligosaccharides, a major source of error for grain fructan analysis as reported by Andersen and Sorensen (2000). Direct analyses of grain extracts showed that galacto-oligosaccharides were present mainly as raffinose in grains of wheat, barley and triticale and also as stachyose and verbascose in peas and bean seeds, ranging between 0.3 and 6.5% of grain dry weight (Table 6). This is in agreement with reports by Kuo et al. (1988) and Henry and Saini (1989) that raffinose is a major galacto-oligosaccharide in grains of wheat and other cereals. Without using α-galactosidase, raffinose could have been partly digested into reducing sugars (mainly fructose and some glucose) under the action of inulinase (Andersen and Sorensen, 2000), causing an overestimation of the fructan concentration. Indeed, a comparison in fructan values between addition and exclusion of α-galactosidase during the enzymatic hydrolytic step clearly showed this difference. Estimated fructan concentrations were higher in analyses where interference from galacto-oligosacharides was not excluded (Table 6), even for seeds of faba bean and field pea, which are not fructan-producing crops. This analytical issue is also mentioned in the instructions for the Megazyme fructan assay kit, in which α-galactosidase pre-treatment is highly recommended for samples containing galacto-oligosaccharides. Use of the modified method can also minimize the interference of co-extracted "starch glucose" from starch in grains of wheat and other cereals. Starch gelatinizes during hot-water extraction at 80 ^{o}C (Charley, 1998), so it is mostly removed by centrifugation. Interference from any residual starch in solution (less than 5% of the grain weight) can then be prevented simply by using amyloglucosidase in both digestions as done for galacto-oligosaccharides using α-galactosidase. Finally, in the method proposed here, fructans are extracted and digested in small closed tubes, preventing analytical errors due to contamination and evaporation, and allowing for simultaneous handling of a large number of samples at once with reduced labour and low enzyme usage. However, the long retention time (40 min, Table 2 of this chapter) can limit the number of samples to be analysed on HPLC (12 samples per day). One way to increase the throughput is to analyse samples simultaneously on more than one HPLC or on a single HPLC system equipped with multiple columns and detectors.

Table 6 Galacto-oligosaccharide concentrationsa in wheat (straight flour and galacto-oligosaccharide-spiked flour) and in other plant species and estimated concentrations of fructan in these samples with and without the removal of interference from galacto-oligosaccharides.

Flour sample	Galacto-oligosaccharides (%)			Fructans (%)	
	Raffinose	Stachyose	Verbascose	Interference from galacto-oligosaccharides excluded	No exclusion of interference from galacto-oligosaccharides
Wheat (*Triticum aestivum* L.) cv. Berkut	0.30 ± 0.02	Not detected	Not detected	2.13 ± 0.05	2.30 ± 0.02
Wheat (Berkut) spiked with 10% raffinose	9.69 ± 0.10	Not detected	Not detected	1.98 ± 0.03	5.34 ± 0.05
Barley (*Hordeum vulgare* L.) cv. Sloop	0.59 ± 0.03	Not detected	Not detected	1.65 ± 0.04	1.79 ± 0.02
Triticale (X *Triticosecale* Wittmack) cv. Credit	0.51 ± 0.06	Not detected	Not detected	2.21 ± 0.07	2.46 ± 0.07
Faba bean (*Vicia faba* L.) cv. Fiesta	0.38 ± 0.06	0.91 ± 0.01	2.72 ± 0.05	Not detected	1.12 ± 0.10
Field pea (*Pisum sativum* L.) cv. Kaspa	1.43 ± 0.04	2.71 ± 0.07	2.31 ± 0.05	Not detected	1.47 ± 0.06

[a] Means ± standard error of three determinations.

3.4 Conclusion

Fructan concentration in wheat grains can be accurately and precisely quantified using the method proposed in this study. Galacto-oligosaccharides, a source of error in fructan determination, were removed during fructan analysis. The modified method is convenient in that it allows the handling of a large number of samples all at once with at low cost due to reduced labour and low enzyme usage. Therefore, it could be used for mass screening of wheat samples in further genetic studies on wheat grain fructan. Due to its analytical convenience, the proposed method could also be extended to other plant materials, potentially including grain samples from food crops and cereals other than wheat.

Chapter 4

Survey of Genotypic Variation in Wheat Grain Fructan Concentration [4]

Abstract

Nineteen lines of cultivated hexaploid wheat (*Triticum aestivum* L.) that have been used as parents of doubled haploid mapping populations were assessed for variation in grain fructan concentration, using grain samples that had been grown together at Rosedale, Maitland and Brinkworth (South Australia) in 2006 and in a glasshouse experiment conducted at the Waite Campus of the University of Adelaide. There was significant genotypic variation in grain fructan concentration, ranging from 0.7 to 1.6% in the glasshouse study and from 1.5 to 2.3% in the field. In addition, a wider set of 98 wheat lines of diverse origin was also surveyed, using grain samples that had been grown together at Narrabri (New South Wales, Australia) in 2006; grain fructan concentration ranged from 0.3 to 2%. For the 19 mapping parents, broad-sense heritability was estimated as h^2 = 0.94 and 0.64 for glasshouse and field screenings, respectively. There was no evidence of strong genotype-by-environment interaction; the fructan concentrations of field-grown grain samples were positively correlated (r = 0.83, P < 0.001) with those of glasshouse-grown samples of the same lines. There was also no evidence of negative impact of increased grain fructan on grain size and yield; grain fructan concentration was not significantly correlated with grain size in both glasshouse and the field but had a positive correlation (r = 0.59, P < 0.01) with grain yield of the glasshouse-grown plants. For several of the mapping populations, the two parents consistently differed in grain fructan concentration in both glasshouse and field conditions, namely (1) Berkut was higher than Krichauff, (2) Sokoll was higher than Krichauff, (3) Molineux was higher than Trident and (4) Tasman was higher than Sunco. It should therefore be reasonable to use these mapping populations to investigate the genetic control of variation for grain fructan accumulation in wheat.

[4] This chapter contains information that is also published in: Huynh BL, Palmer L, Mather DE, Wallwork H, Graham RD, Welch RM, Stangoulis JCR (2008) Genotypic variation in wheat grain fructan content revealed by a simplified HPLC method. Journal of Cereal Science 48:369–378

4.1 Introduction

Biofortification is defined as the enrichment of the nutritional value of staple crops (Graham et al., 2001) and is a relatively new agricultural strategy to improve human nutrition (Bouis, 2002; Nestel et al., 2006; Graham et al., 2007). Fructans naturally occur in wheat grain and may confer health benefits by acting as prebiotics. If there is sufficient genetic variation in the level of fructans in wheat grain, it may be possible to biofortify wheat through plant breeding. While considerable genetic variation has been observed for fructan levels in some crops such as garlic (Hong et al., 1997), onion (McCallum et al., 2006), Jerusalem artichoke (Kocsis et al., 2007), oat (Åman, 1987) and rye (Hansen et al., 2003), little is known about the variation for wheat grain fructan. Nardi et al. (2003) reported grain fructan variation for bread wheat, durum wheat, rye, barley and triticale, but they included only one variety of each species and the fructan was measured when the grain was not completely mature. Other reports are rather fragmentary based on grain fructan levels measured from limited numbers of wheat materials which were not all grown together. For example, MacLeod and Preece (1953) demonstrated the presence of 1% (of flour dry weight) fructan in a sample of commercial wheat flour, while Henry and Saini (1989) reported 0.8% fructan in the grain of one wheat variety. Schnyder et al. (1993) also investigated one wheat variety by following the change in fructan level during grain development and found that fructan concentration reached 7% of dry weight at ten days after anthesis but decreased to 1.6% at maturity. Given that fructan level may be affected by growing conditions (Kiniry, 1993, Yang et al., 2004; Ruuska et al., 2006) or storage conditions (Pollock and Cairns, 1991; Merry et al., 1995; Noureddine and Norio, 2006), it cannot be certain whether differences among these reported fructan levels are due to genetic or environmental causes.

The initial aim of the research reported in this chapter was to survey the variation in grain fructan concentration among cultivated hexaploid wheat lines, which were grown together in field or glasshouse experiments. The second aim was to evaluate the parents of wheat mapping populations and determine if there were differences in grain fructan level between the two parents of each population, in order to select a suitable population for use in further mapping studies. The relationship between wheat grain fructan level and important traits was also examined.

4.2 Materials and Methods

4.2.1 Evaluation of parents of wheat mapping populations

Nineteen wheat cultivars and breeding lines were used in this study. They are parents of ten DH mapping populations: the Trident/Molineux population (Williams et al., 2003), the Sunco/Tasman population (Chalmers et al., 2001), the Cranbrook/Halberd population (Chalmers et al., 2001), the Tammin/Excalibur population (Williams et al., 2002), the Cascades/RAC875-2 population (Genc et al., 2008), the Kukri/Janz population (Kammholz et al., 2001), a Chara/VN870R population developed by Dr Russell Eastwood (Australian Grain Technologies), and Stylet/Westonia, Berkut/Krichauff and Sokoll/Krichauff populations developed by Dr Hugh Wallwork (SARDI). Most of the parental lines were developed in Australia, except for Berkut and Sokoll, which are new breeding lines developed at the International Maize and Wheat Improvement Center (CIMMYT) in Mexico. Seeds for sowing were obtained from the seed bank of SARDI.

Glasshouse experiment

The 19 parental lines were grown in a controlled glasshouse environment using a standard University of California potting mix (Barker et al., 1998). In this potting mix, sand collected at Waikerie (South Australia) was washed, sterilized at 100 ^{o}C and mixed with peat moss, lime and Osmocote® Exact® Mini (Scotts International BV, The Netherlands), a slow-release fertilizer that contains essential nutrients for plant needs. Pure seed of each line was pre-germinated for 2 days at 20 °C in the dark on filter paper in glass Petri dishes moistened with 4 mL of distilled water. Ten germinated seeds of each line were transplanted into each of three pots containing 8 kg of the UC mix. The pots were arranged in a randomised complete block design with three blocks on three benches side by side (Fig. 5). Thinning was carried out two weeks after transplantation in order to leave five healthy and uniform plants per pot; all of which survived to harvest. The experiment was managed to avoid water stress, leaf diseases and insect infestation on plants. At harvest, mature grain from the five plants of each pot (experimental unit) was bulked, thoroughly mixed and weighed.

Figure 5 Glasshouse screening of 19 wheat parental lines.

Samples from field experiments

Grain samples of the 19 parental lines were obtained from field experiments grown by SARDI at Rosedale (34.55 °S, 138.83 °E), Maitland (34.37 °S, 137.67 °E) and Brinkworth (33.41 °S, 138.24 °E), in South Australia during the 2006 winter growing season. At Rosedale, each line was sown in a 2m-long 2-row plot. At the other two sites, each line was sown in a 6m-long 4-row plot. The sowing rate was approximately 200 seeds/m^2. Different randomisations were used at each site. For analysis, the overall experiment was treated as a randomized complete block design with three blocks, with each of the three sites considered as a block. Nutrients were supplied at sowing as 100 kg/ha of the 18N:20P fertilizer. The experiments were managed to exclude leaf diseases and broadleaf weeds. The plants were subject to natural drought during the 2006 growing season, with the total regional rainfall less than 200 mm (Bureau of Meteorology of Australia), except at Rosedale, where irrigation was applied at anthesis using overhead sprinklers. Grain was harvested when mature by mechanical harvester.

Measurements of fructan concentration and other traits

Samples of whole grain (approximately 20 g from each experimental unit) were collected, ground into fine powder using an IKA A11 grinder, mixed well and oven dried at 85 ^{o}C for 24 h prior to fructan analysis. Grain fructan concentration was measured using the method described previously (Chapter 3 of this thesis; Huynh et al., 2008).

Grains were counted using a seed counter (CONTADOR, Pfeuffer GmbH) and the 500-grain weight was measured. For the glasshouse experiment, grain yield (g/plant) was calculated by dividing the grain dry weight from each experimental unit (pot) by the number of plants per pot (5 plants).

Statistical analyses

Analysis of variance (ANOVA) was performed with the computer software GenStat version 8. Factors for the ANOVA model were lines and block, with each of the three replications (for the glasshouse screening) or each of the field locations (for the field screening) considered as a block. Trait repeatability was estimated based on variance components attributable to variation among lines (VG) and residual variation (VE) (h^2 = VG / (VG + VE)). Fructan concentrations of two parents of each DH population were compared using linear contrasts. Simple linear correlation analysis was used to investigate association between fructan levels of glasshouse-grown and field-grown samples of the same lines and between grain fructan and other traits.

4.2.2 Survey of wheat cultivars from international collections

The extent of genotypic variation in wheat grain fructan was also examined using a wider range of materials, including materials that are not closely related to modern cultivars. Ninety-eight wheat lines of diverse origin (Table 7) were analysed for grain fructan concentration. Grain samples were obtained from Professor Robert Henry, Southern Cross University, Australia. They came from field experiments grown in 2006 at Narrabri (30.34

°S, 149.76 °E) in New South Wales and Biloela (24.38 °S, 150.52 °E) and Toowoomba (27.58 °S, 151.93 °E) in Queensland, Australia.

Grain samples coming from Narrabri were initially assessed for variation in grain fructan concentration. The eight lines with the lowest fructan concentration and the eight lines with the highest fructan concentration were then selected and analysed using grain samples coming from the other two environments, Biloela and Toowoomba. Fructan concentration was measured using the method described previously (Chapter 3 of this thesis; Huynh et al., 2008). Grains were counted using a seed counter (CONTADOR, Pfeuffer GmbH) and the 100-grain weight was measured.

For the lines for which fructan concentration was assessed for grain samples from three field environments, ANOVA was performed with the computer software GenStat, Version 8.0. Factors for the ANOVA model were line and block, with each of the three field environments considered as block. The two groups of lines with lowest and highest fructan concentrations were compared using linear contrasts. Simple linear correlation analysis was used to investigate association between grain fructan level and grain weight.

Table 7 Wheat materials for grain fructan survey.

Country	Name of lines
Afghanistan	H 1160, H 1287, H 501, H 865
Algeria	Mehon Denias, Rogue In Ble Du Oasis
Argentina	La Prevision
Australia	Allora, Arnhem, Banks, Batavia, Bowerbird, Drysdale, Ellison, Gabo, Gluyas Early, H45, Hermitage, Insignia, Katyil, Machete, Olympic, Qalbis, Qal 2000, Rees, Warren, Wylah, Yitpi
Brazil	Frontana, Veranopolis
Bulgaria	Varna 6
Chile	Pumafen
China	Kefeng, Yangmai 3
Croatia	Dalmatia 2

(Table 7 continued)

Ecuador	Altar
Egypt	Giza 139
Ethiopia	Beladi 42
Former Soviet Union	Surhak Jubilejnyj
Greece	Crete 11, D.E.S. 0043, D.E.S. 0111, Salonica 17
India	India 259, Kiran, Punjab 7, W139a, W143, W145, W216
Iran	AMC 106, AMC 125, AMC 136, Iran 158 , Persia 80
Iraq	AMC 83
Israel	Sion
Japan	Akadaruma, Sapporo
Kenya	Bounty
Lebanon	Beyrouth 1
Mexico	Lerma Rojo, Saturno
Morocco	Morocco 16, Morocco 21, Morocco 59
Nepal	NW19A, NW25A, NW91A, NW96A, NW99A
New Zealand	Kopara 73
Pakistan	India 211, Pakistan C273, VIR 45738, W38, W43A, W45B, W49A, W58, W63, W75
Portugal	Portugal 102
Romania	Moldova
Spain	Salamanca 10, Seville 22
Switzerland	Relin
Syria	AMC 53, AMC 61, Kandahary
Tunisia	Tunis 24
Turkey	AMC 512, Asure Bugday, Karizik, Smyrna 13, Smyrna 2
United States	Clubhead
Venezuela	Pelada

4.3 Results and discussion

4.3.1 Genetic variation in grain fructan concentration

Significant ($P < 0.001$) variation in the grain fructan concentration was also observed among the parental wheat materials (Fig. 6). In the glasshouse study, the fructan concentrations ranged between 0.7 and 1.6% of grain dry weight. In the field, the fructan concentrations ranged between 1.5 and 2.3% of grain dry weight, and were positively correlated with those from the glasshouse experiments ($r = 0.83$, $P < 0.001$). Westonia consistently had the lowest fructan level, while Sokoll, Halberd and Cranbrook had the highest. For four of the mapping populations, the two parents significantly ($P < 0.05$) differed in grain fructan concentration in both glasshouse and field environments: Berkut was higher than Krichauff, Sokoll was higher than Krichauff, Molineux was higher than Trident, and Tasman was higher than Sunco (Fig. 6). Further assessment of segregating populations from these parents should be useful for understanding the genetic control of variation for the trait.

The lower fructan level observed in the grain from glasshouse-grown plants may have been due to effects of plant growth conditions on the availability of tissue carbon for fructan biosynthesis. It is known that, for example, water-soluble carbohydrate accumulation is decreased by shading (Kiniry, 1993) but increased in conditions where vegetative growth is limited (van Herwaarden et al., 2003; Ruuska et al., 2006). Under glasshouse conditions, the light intensity would be lower than in the field and sufficiency of nutrient and water supply would favour vegetative growth.

Postharvest factors may also affect the fructan level of stored grain or grain products, due to possible effects of storage conditions or storage duration on the activity of micro-organisms or fructan-degrading enzymes (Pollock and Cairns, 1991; Merry et al., 1995; Noureddine and Norio, 2006). Such factors are unlikely to have confounded these results, given that all wheat lines were grown together, grain samples were not subject to long-term storage, and the results were highly repeatable as observed for the parents of mapping populations (repeatability of 0.94 and 0.64 for the glasshouse and the field, respectively).

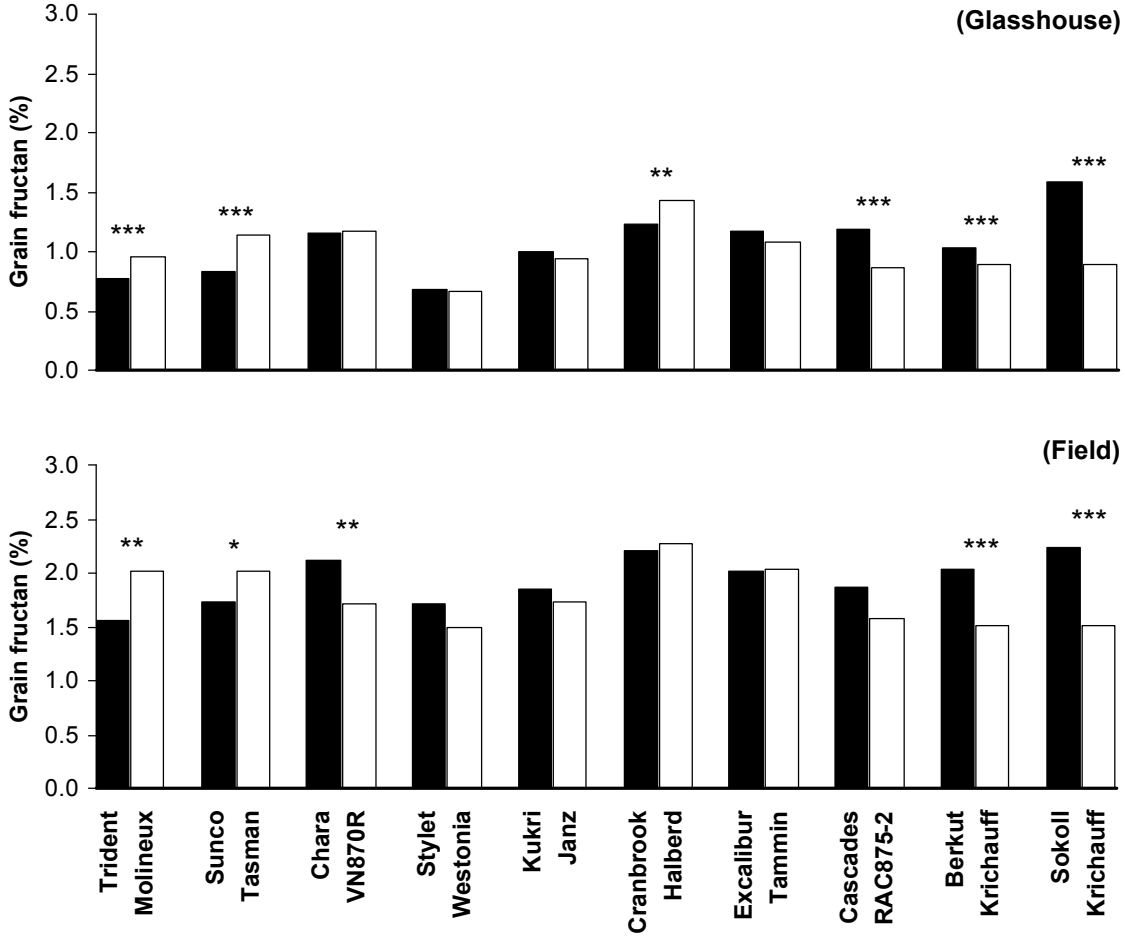


Figure 6 Mean grain fructan concentrations (% of dry weight) of parental wheat lines grown in the glasshouse (above) and in the field (below) of South Australia in 2006; two parental lines of each DH mapping population are grouped together and compared using linear contrasts.

Grain fructan concentration also varied widely among 98 wheat lines grown at Narrabri, ranging from 0.3 to 2% of grain dry weight (Fig. 7). W75 had the highest fructan level, while NW25A had the lowest. Fructan analyses of 16 lines with extreme grain fructan concentrations showed that differences among them were quite repeatable across three field environments (Fig. 8), with significant ($P < 0.001$) difference between the two groups. However, none of these lines had higher fructan levels than modern elite materials including some mapping parents (Fig. 6) and CIMMYT breeding lines (Huynh et al., 2008). It is possible that the change of growing environment might have affected the fructan level of those international cultivars, especially those sensitive to day length and/or vernalisation. Other factors such as plant height, maturity or yield may also influence grain fructan accumulation by affecting the mobilisation of carbohydrates into grain as a source for fructan biosynthesis, which would be worthwhile to be further investigated.

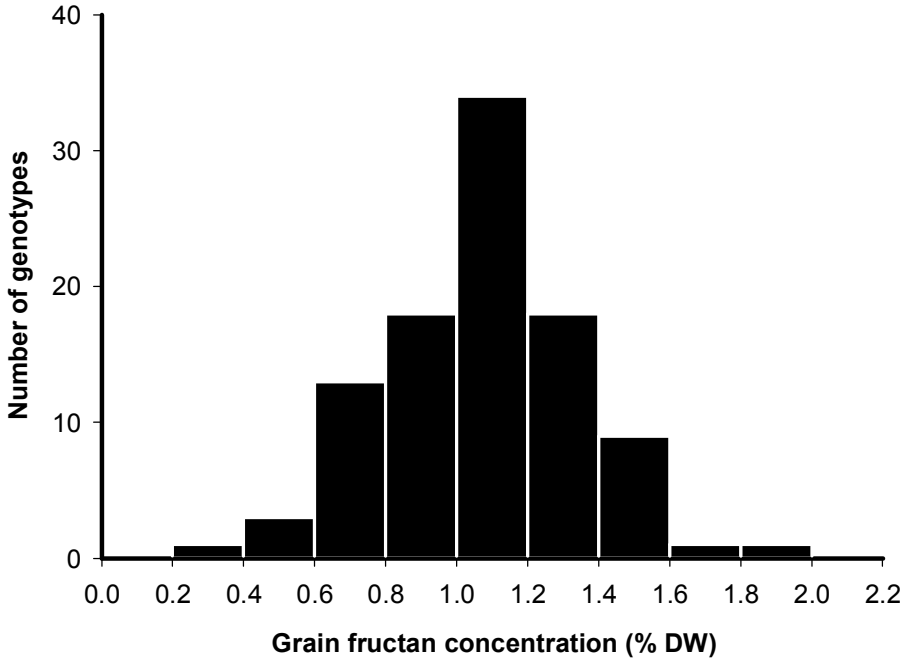


Figure 7 Variation in grain fructan concentration among 98 bread wheat lines grown at Narrabri (New South Wales, Australia) in 2006.

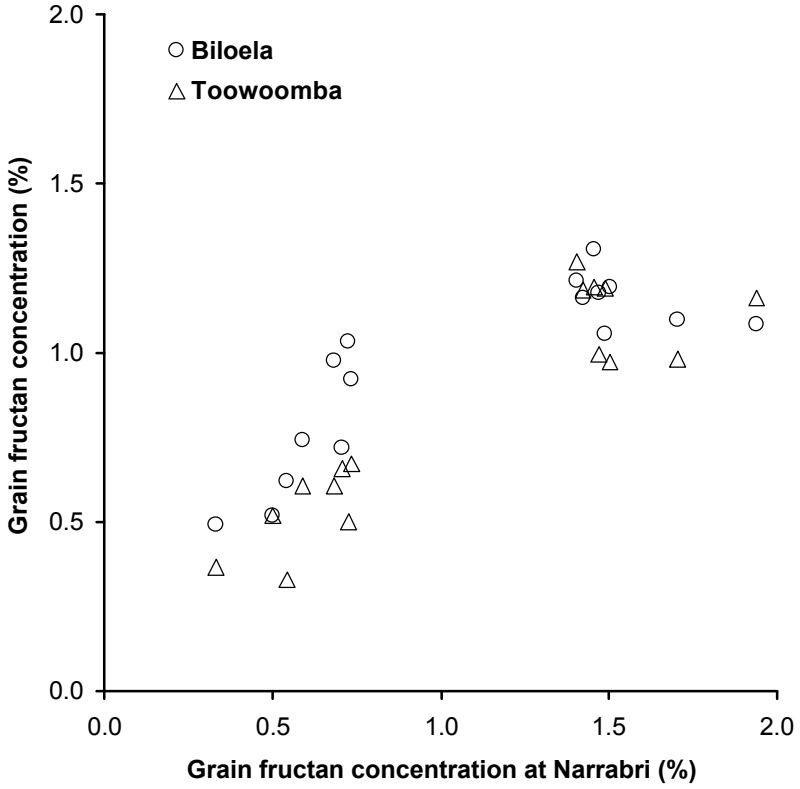


Figure 8 Grain fructan concentrations of wheat grain grown at Biloela and Toowoomba QLD in 2006 for 16 wheat lines selected for multiple-site fructan evaluation based on the fructan concentration (high or low) of grain grown at Narrabri NSW in 2006.

Considering the high repeatability observed here for the wheat breeding materials, 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; the fructan concentrations of field-grown grain samples were positively correlated with those of glasshouse-grown samples of the same lines, and differences between high- and low-fructan lines were consistent across field environments. Even though the presence of fructans in wheat grain was discovered more than 50 years ago (White and Secor, 1953), there are no previous reports on the potential for increasing grain fructan concentration in wheat. Most studies have focussed on characterisation of fructan structure (Montgomery and Smith, 1957; Nilsson and Dahlqvist, 1986), measurement (Dahlqvist and Nilsson, 1984) or physiology of accumulation (Schnyder et

al., 1993) using limited numbers of wheat materials which were not all grown together. Several studies have compared grain fructan level among wheat and other cereals (MacLeod and Preece, 1953; Henry and Saini, 1989; Nardi et al., 2003), but they also included only one variety for each species, and therefore did not provide any information on genetic variation for grain fructan within wheat. Other research has largely focused on wheat stem fructan (Willenbrink et al., 1998; Yang et al., 2004; Ruuska et al., 2006) or grain fructan of other cereals such as oat (Åman, 1987) and rye (Hansen et al., 2003). To the author's knowledge, this is the first report on genetic variation in wheat grain fructan in which different wheat cultivars and breeding lines were grown together and investigated.

4.3.2 Variation in grain weight and yield and their correlations with grain fructan

Significant ($P < 0.001$) variation in grain weight was also observed among the parental lines (Table 8). In the glasshouse study, the 500-grain weight ranged between 16 and 24 g. In the field, the 500-grain weight was lower, ranging between 13 and 19 g, and were positively correlated with those from the field screening ($r = 0.63$, $P < 0.01$). It is therefore possible that the larger grain size might cause a dilution of grain fructan concentrations, contributing to a reduction in fructan levels of the glasshouse-grown plants compared to the field-grown plants (Fig. 6). However, there were no significant correlations between grain fructan concentration and the 500-grain weight of the parental lines grown in the same environments. There was also no significant correlation between these two traits measured in the set of 98 wheat lines grown at Narrabri. This implies that selection for high grain fructan level may not have a negative impact on grain size, which is an economically important trait in wheat breeding since larger grains can confer higher milling yield (Marshall et al., 1986; Wiersma et al., 2001). Likewise, there was also no evidence of negative impacts of grain fructan level on yield as measured in the glasshouse-grown plants. In this study, the total grain weight produced from each plant significantly ($P < 0.05$) varied between lines, ranging from 8.3 to 14.5 g (Table 8), and positively correlated with grain fructan concentration ($r = 0.59$, $P < 0.01$) (Fig. 9). However, it is not expected that the amount of grain harvested from each plant grown in the glasshouse is an indicator of yield in the field. Further investigation would therefore be needed to examine the relationship between grain fructan and yield in the field environment.

Table 8 Mean grain size and/or yield of parental wheat lines grown in the glasshouse and the field (South Australia, 2006).

Line	500-grain weight (g)		Yield (g/plant)
	Glasshouse	Field	Glasshouse
Berkut	22.42	15.27	11.91
Cascades	21.83	16.20	11.28
Chara	17.22	15.33	9.57
Cranbrook	22.30	14.67	11.39
Excalibur	20.94	15.20	12.01
Halberd	21.86	15.70	14.52
Janz	19.35	13.60	9.16
Krichauff	18.63	14.03	11.07
Kukri	19.56	15.13	8.87
Molineux	17.33	13.33	12.27
RAC875-2	23.53	19.43	8.34
Sokoll	23.85	16.37	12.48
Stylet	16.41	14.77	10.78
Sunco	17.93	13.90	8.51
Tammin	21.61	16.07	9.79
Tasman	16.86	15.53	10.26
Trident	17.85	14.30	11.66
VN870R	19.02	14.07	9.29
Westonia	22.33	15.77	10.12
l.s.d. ($P = 0.05$)	2.16	2.13	3.01

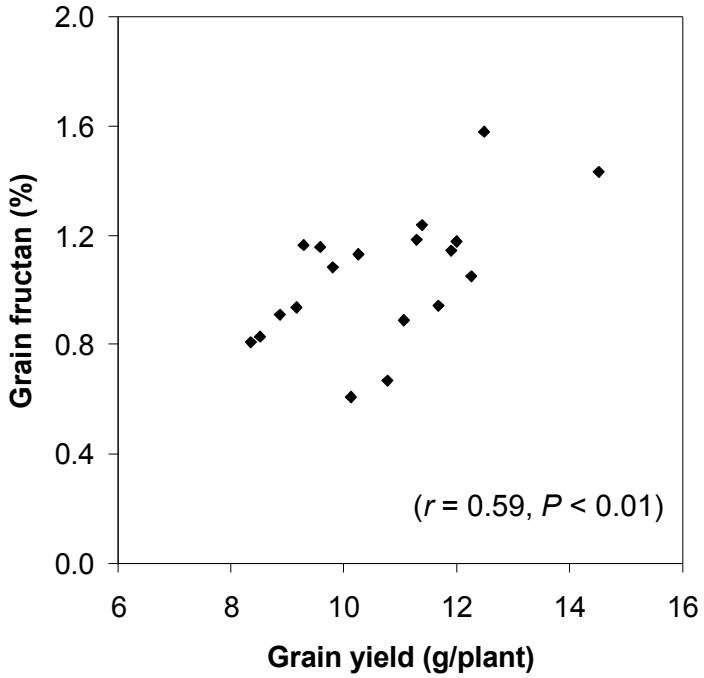


Figure 9 Association between grain fructan concentration and grain yield measured in the 19 parental lines grown in a glasshouse experiment.

4.4 Conclusion

Grain fructan concentration varied widely among wheat cultivars and breeding lines and was highly repeatable, indicating a potential to improve this trait by breeding. There was no evidence of negative association of grain fructan level with grain yield or grain weight. Further investigation is needed to understand the genetic control of variation in grain fructan accumulation as well as its relationship with other traits. Such studies could employ the genetic-mapping approach using the DH wheat populations whose parents significantly differed in their grain fructan concentrations as reported in this study.

Chapter 5

Mapping Loci Affecting Wheat Grain Fructan Concentration [5]

Abstract

A DH population derived from a cross between a high-fructan breeding line, Berkut, and a low-fructan cultivar, Krichauff, was used to map loci affecting grain fructan concentration in wheat (*Triticum aestivum* L.). 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. The major QTL on chromosome 7A was in the same region with a reported fructosyltransferase orthologue (*AB029888*). 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.

[5] This chapter contains information that is also published in: Huynh BL, Wallwork H, Stangoulis JCR, Graham RD, Willsmore KL, Olson S, Mather DE (2008) Quantitative trait loci for grain fructan concentration in wheat (*Triticum aestivum* L.). Theoretical and Applied Genetics 117:701–709

5.1 Introduction

Significant genotypic variation has been observed for fructan concentration in wheat grain (Chapter 4 of this thesis), indicating the potential to improve this trait by breeding. Understanding the genetic control for this variation would benefit breeding programs, but this information is still lacking. There have been no reports of genetic mapping for grain fructan accumulation in wheat or other cereals. Quantitative trait loci 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. (2007) 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 (Setter et al., 1998; Wardlaw and Willenbrink, 2000; Ruuska et al., 2006), genes that affect water-soluble carbohydrate content might also affect grain fructan accumulation. Fructan concentration in wheat grain could also be affected by source-sink relationships, dilution effects and fructan degradation during grain development (Schnyder et al., 1993; Nardi et al., 2003).

The aim of the research reported in this chapter was to detect and map loci affecting grain fructan concentration in a wheat population derived from a cross between low- and high-fructan parents.

5.2 Materials and Methods

5.2.1 Genetic material

The population used in the QTL mapping experiment consisted of 154 DH lines derived from a cross between an inbred line Berkut (pedigree: Irena/Babax//Pastor, developed by CIMMYT) and the cultivar Krichauff (pedigree: Wariquam//Kloka/Pitic62/3/Warimek/ Halberd/4/3Ag3Aroona, released in 1997 by the University of Adelaide, Australia). The population used for marker validation consisted of 150 DH lines derived from a cross between an inbred line Sokoll (pedigree: Pastor/3/Altar84/*Ae. squarrosa*(Taus)//Opata, developed by CIMMYT) and Krichauff. Both populations were obtained from Dr Hugh Wallwork (SARDI). Both Berkut and Sokoll have higher grain fructan concentration than Krichauff (Chapter 4 of this thesis).

5.2.2 Field screening

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 that disease. In 2006, the experiment was irrigated using overhead sprinklers and fungicides were used to prevent fungal infection (Fig. 10). In these experiments, each DH or parental line was sown in one 2m-long 2-row plot. 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 1m x 1m plot. No irrigation was applied at this field site and the plants were not subject to any severe biotic stresses. The Rosedale site was a seed increase block in which the lines were arranged in the same order in both years. At Kazakhstan, the lines were arranged in a different (randomized) order. For analysis, the overall experiment was treated as a randomized complete block experiment with three blocks, with each of the sites considered as a block. Grain was mechanically harvested when mature. Representative samples of whole grain (20 g for each plot) were ground into fine powder using an IKA A11 grinder, well mixed and oven dried at 85 ^{o}C for 24 h prior to fructan analysis. Grain fructan concentration was measured using the method described previously (Chapter 3 of this thesis; Huynh et al., 2008).

Analysis of variance (ANOVA) was performed with the computer software GenStat, Version 8.0. Factors for the ANOVA model were DH line and 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)).

Figure 10 The Berkut/Krichauff population grown at Rosedale, South Australia in 2006.

5.2.3 Genetic mapping

DNA samples and a genetic map of 206 SSR markers for the Berkut/Krichauff population were obtained from Kerrie Willsmore, SARDI (Appendix 2). The genetic map was not complete in that not all linkage groups had been aligned with previously constructed wheat linkage maps. Initial QTL mapping was performed with Windows QTL Cartographer V2.5 (Wang et al. 2005) using the composite interval mapping method (Zeng, 1994). Two regions on chromosomes 6D and 7A were detected that had consistent effects on grain fructan level across three field environments (Appendix 3). The region on 7A was flanked by closely linked markers ('*gwm635*' and '*ksm19*', 6 cM), whereas the 6D region was flanked by markers with long genetic distance ('*barc54*' and '*cfd13*', 45 cM). To increase genome coverage, more SSR markers were then added to this chromosome of the Berkut/Krichauff map.

A total of 47 new SSR markers that had previously been assigned to wheat chromosome 6D (Sourdille et al., 2004; Somers et al., 2004; Song et al., 2005; Hayden et al., 2006) were assessed for polymorphism between Berkut and Krichauff. Twelve polymorphic markers were used for genotyping the DH lines. The SSRs were assayed using the Multiplex-Ready PCR Technology as described by Hayden et al. (2007).

Subsequently, the population was genotyped by Triticarte Pty Ltd (Australia) with Diversity Array Technology® (DArT) using the method by Akbari et al. (2006). This provided data on 312 additional markers. These data were obtained, along with a new version of the genetic map, which had been constructed by Kerrie Willsmore (SARDI) and Gregory Lott (University of Adelaide). The new SSR markers were assigned to this map using MapManager QTXb20 for Windows (Manly et al., 2001) and RECORD (Van Os et al., 2005). The improved genetic map is attached in Appendix 4 of this thesis.

QTL mapping was performed with QTLNetwork 2.0 (Yang *et al.*, 2008) using mixed linear composite interval mapping (Yang *et al.*, 2007). The general model implemented by QTLNetwork 2.0 incorporates 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 involves several consecutive steps. Candidate marker intervals are selected using the method described by Piepho and Gauch (2001). The selected intervals are then 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 is performed to search for epistatic interactions between QTLs. In each of these steps, significance testing is based on the *F*-test using Henderson method III (Searle et al., 1992), with significance thresholds defined by permutation. A multiple-QTL model is selected by subjecting significant peaks from the *F*-statistic profiles to stepwise selection involving iterative forward and backward selection steps. QTL and epistatic effects are estimated using a Bayesian method via Gibbs sampling (Wang *et al.*, 1994). Here, these methods were applied using a significance level of 0.10 for the selection of candidate intervals, a more stringent significance level (0.01) in further selection steps to avoid false positives, and 20,000 cycles of Gibbs sampling.

5.3.4 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. Each genotype was sown in a 3.2 m^2 plot. The sowing rate was approximately 200 seeds/m^2. The 154 DH lines were classified into four marker-allele classes according to their genotypes *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 g*wm681-7A* (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 x 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 the Sokoll/Krichauff population. DNA was extracted from leaves of 2-3 week old seedlings of Sokoll, Krichauff and each of 150 DH lines using a freeze dry extraction method (Fox et al., 2003). Two pieces from a 2-3 week old seedling of leaf (approximately 45 mm in length) were collected and placed into a 1.1 mL microtube and freeze-dried overnight. The dried leaves were crushed by adding two stainless steel ball bearings in each microtube and grinding in the Qiagen grinder (Retsch mill, Type MM 300) for 5 min at a frequency of 25 cycles per second. Six hundred µl of pre-warmed (65 ^oC) extraction buffer (0.1 M Tris-HCl, 0.05 M EDTA, 1.25% SDS, pH 8.0) was added in each tube. The solution was incubated at 65 ^oC for 30 min and then cooled down to room temperature before adding 300 µl of 6 M ammonium acetate, which had been stored at 4 ^oC. The solution was vigorously shaken and then left to stand for 15 min in the fridge before being centrifuged at 5000 rpm for 15 min. The supernatant (600 µl) was collected into a new collection microtube containing 360 µl of iso-propanol. The solution was mixed thoroughly, left on the bench for 5 min and

centrifuged at 5000 rpm for 15 min to precipitate the DNA. The pellet was washed with 500 µl of 70% ethanol and centrifuged at 5000 rpm for 15 min. The supernatant was discarded before re-suspension of the pellet overnight in 400 µl of milli-Q water in a refrigerator. This solution was then centrifuged at 5000 rpm for 20 min and 100 µl of the supernatant transferred into a 96 well PCR plate. DNA was quantified using Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies).

The markers *barc54-6D* and g*wm681-7A* 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 x 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.

5.3.5 Investigation of the effect of grain-fructan loci on other traits

The markers *barc54-6D* and g*wm681-7A* were examined whether they had any effects on grain yield and grain weight. Data of grain yield and 1000-grain weight of the Berkut/Krichauff population were obtained from four experiments grown by SARDI in 2007 at Booleroo, Minnipa, Roseworthy and Balaklava (34.08 °S, 138.25 °E), South Australia. Each experiment involved a randomised complete block design with two blocks. Each genotype was sown in a 3.2 m^2 plot. The sowing rate was approximately 200 seeds /m^2. REML variance components analysis was performed in which main marker and marker-marker interaction effects were fixed and DH lines random.

5.3.6 Candidate-gene mapping

Based on the QTL mapping results, combined with information published by Francki et al. (2006), a fructosyltransferase orthologue (*AB029888*, Lidgett et al., 2002) was selected for further investigation as a candidate gene with possible effects on grain fructan content.

Four pairs of reverse/forward primers (Table 9) were kindly provided by Dr Michael Francki (Department of Agriculture and Food, Western Australia). These primers were used to amplify portions of *AB029888* from Berkut, Sokoll and Krichauff. Subsequently, one of these primer pairs (*AB029888-15F* and *AB029888-19R*, Table 9), for which a polymorphism was observed between Krichauff and the other two parental lines, was used to assay each of the Berkut/Krichauff DH lines.

PCR was performed in 12.5 µL reaction volumes containing 100 ng genomic DNA, 1.5 mM $MgCl_2$, 0.25 µM of each primer, 200 µM dNTPs, 1 x PCR reaction buffer and 0.5 U Taq polymerase (QIAGEN Pty Ltd, Australia). The thermal cycling profile involved 3 min at 94 °C followed by 10 cycles of 94 °C for 1 min, 65 °C for 1 min (decreasing 1 °C per cycle), and 72 °C for 2 min, 25 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min, and final extension at 72 °C for 5 min. PCR amplification products were separated by electrophoresis for 2 hours at 80 V on 1.5% agarose gels. Gels were stained with ethidium bromide and visualised under ultraviolet light.

A polymorphism detected using primers *AB029888-15F* and *AB029888-19R* was added to the Berkut/Krichauff linkage map using MapManager QTXb20 for Windows (Manly et al., 2001) and RECORD (Van Os et al., 2005).

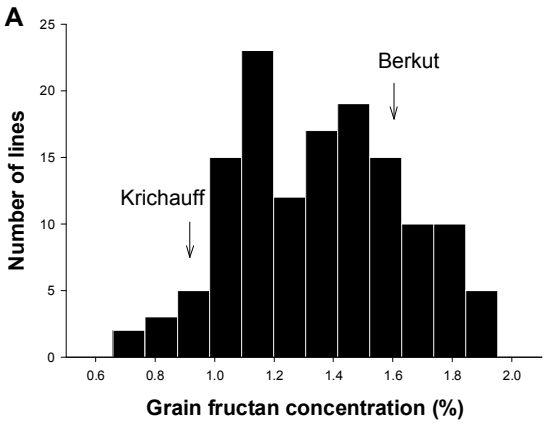
Table 9 Primers used for amplifying portions of the *AB029888* sequence in wheat.

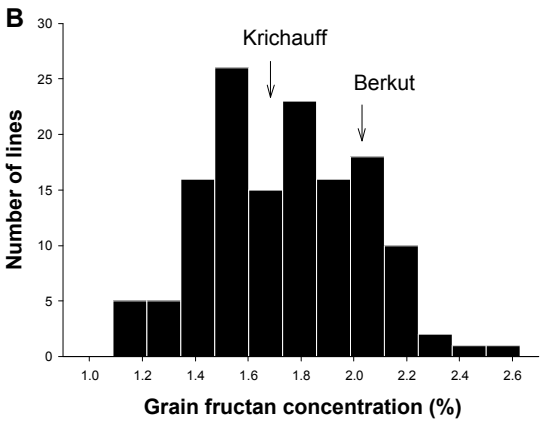
Name of primer	Type of primer	Sequence 5'——3'
AB029888-6	Forward	GATGTTAGCTACAACTGCAC
AB029888-8	Reverse	TGCCATTTTCATTCCCCAAC
AB029888-11	Forward	ACCATTTCCAGCCGGACAAG
AB029888-16	Reverse	TCGTCGCTGCTCTCCTTGAG
AB029888-15	Forward	AGCGACATGTACAACTCGAC
AB029888-19	Reverse	CGAGGCCCTTGGACACATAG
AB029888-20	Forward	GAGTTACCTCGGGATCATCG
AB029888-21	Reverse	CGTTGGGATCGTTCTGGTAG

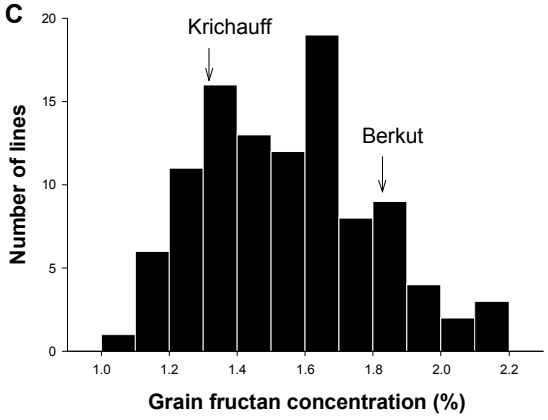
5.3 Results and discussion

5.3.1 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 (Fig. 11A), from 1.0 to 2.6% at Rosedale in 2006 (Fig. 11B) and from 1.0 to 2.2% at Koshy (Fig. 11C), with significant ($P < 0.001$) positive correlations among the three environments (Fig. 11D). Berkut had higher fructan concentrations than Krichauff in all environments. Transgressive segregation was observed, indicating that this trait might be under complex genetic control.







D

	Koshy (2006)	Rosedale (2006)
Rosedale (2005)	0.554	0.686
Rosedale (2006)	0.767	

Figure 11 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**).

Broad-sense heritability was estimated as 0.71. This high heritability was consistent with the high repeatability reported for other wheat materials in Chapter 4 of this thesis. There was no evidence of strong genotype-by-environment interaction for the trait; the fructan concentrations of the same genotypes were positively correlated among three contrasting field environments in Australia and Kazakhstan (Fig. 11D). Based on these observations, it seems likely that grain fructan concentration could be improved effectively using phenotypic selection.

5.3.2 Inheritance of grain fructan concentration

QTLs and epistatic interactions

Eight QTLs, involving two pairs of epistatic interactions, were found for grain fructan concentration (Table 10 and Fig. 12). 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 ($P < 0.05$) in Rosedale in 2005 than in the other environments (Table 10). 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 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.

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 Smeekins, 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 the 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 that have previously been detected by Yang et al. (2007) 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.

Table 10 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. *, **, *** Significantly different from zero at $P = 0.05$, 0.01 and 0.001, respectively.

[c] Percentage of phenotypic variation explained.

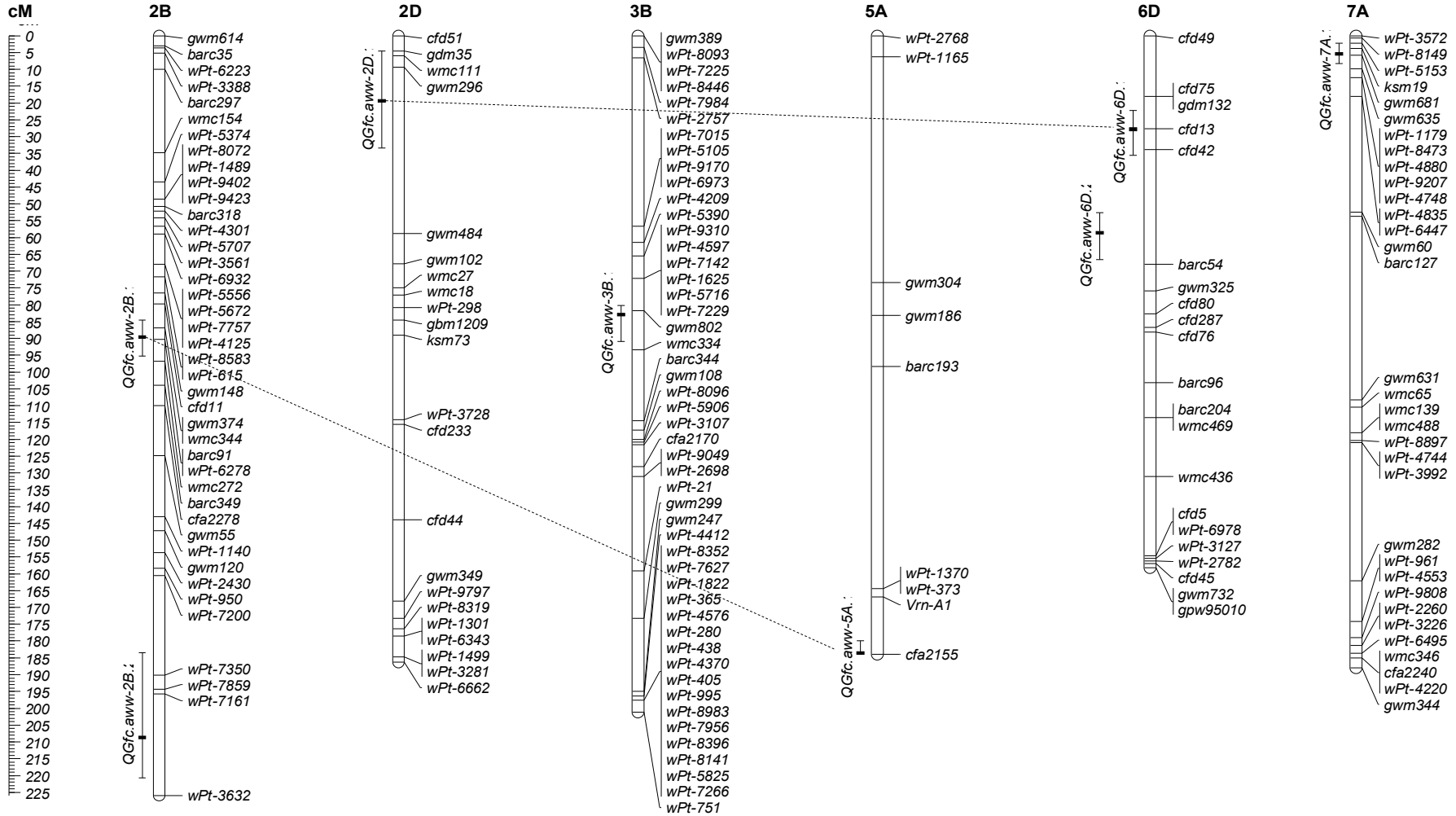


Figure 12 Chromosome locations of regions associated with grain fructan concentration in the Berkut/Krichauff double-haploid population. Dashed lines show epistatic interactions between QTLs.

QTL validation

The markers nearest to the two QTLs with the largest effects are *barc54-6D* (near *QGfc.aww-6D.2*) and g*wm681-7A* (near *QGfc.aww-7A.1*) (Table 11 and Fig. 12). The validation study showed that 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. 13). These two loci interacted significantly ($P < 0.05$) with each other.

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. 14) and there was significant ($P < 0.01$) interaction between the two loci.

In both validation tests, 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 (Fig. 13 and 14). Considering this apparent partial duplication of effects, combined with the larger effects of *QGfc.aww-7A.1* (Table 11 and Fig. 13 and 14), and the detection of minor epistatic QTL (*QGfc.aww-6D.1*) near *QGfc.aww-6D.2* (Fig. 12), *QGfc.aww-7A.1* would be the logical initial target for selection to increase grain fructan concentration.

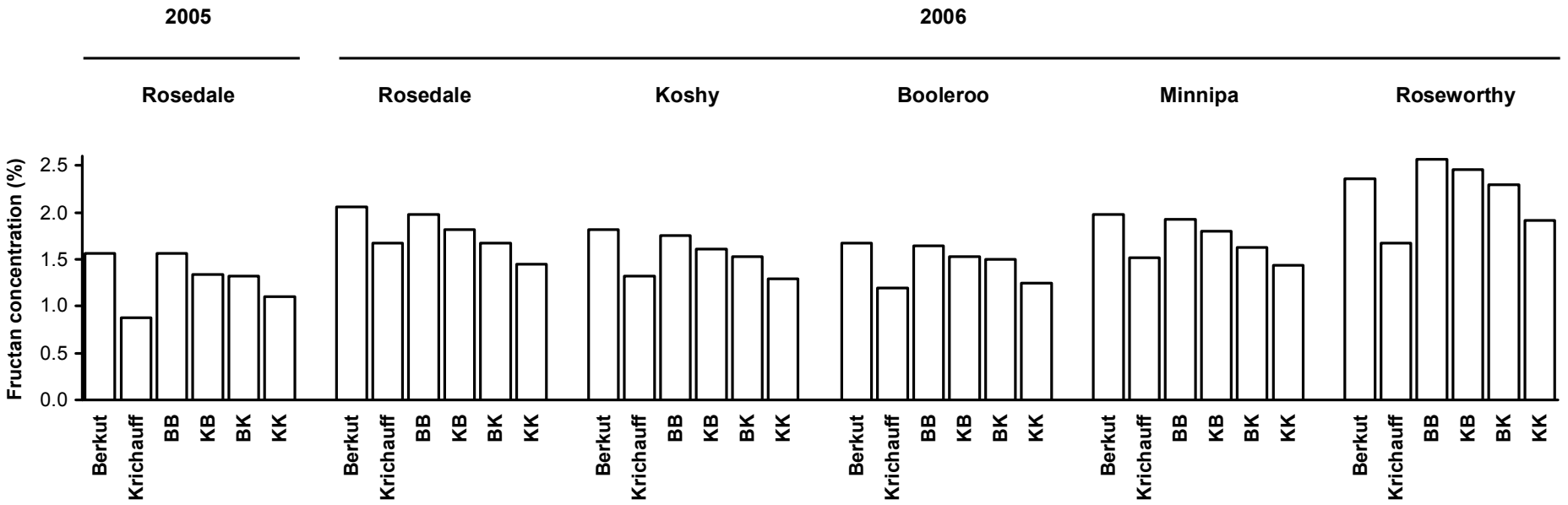


Figure 13 Mean grain fructan concentrations for Berkut, Krichauff and four genotypic classes of Berkut/Krichauff doubled haploid lines: BB (with Berkut alleles at markers *barc54-6D* and *gwm681-7A*), BK (Berkut at *barc54-6D*; Krichauff at *gwm681-7A*), KB (Krichauff at *barc54-6D*; Berkut at *gwm681-7A*) 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.

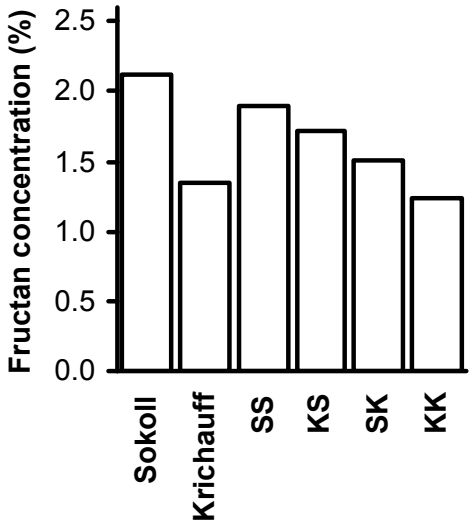


Figure 14 Mean grain fructan concentrations for Sokoll, Krichauff and bulk grain samples representing four classes of Sokoll /Krichauff doubled haploid lines: SS (with Sokoll alleles at markers *barc54-6D* and *gwm681-7A*), SK (Sokoll at *barc54-6D*; Krichauff at *gwm681-7A*), KS (Krichauff at *barc54-6D*; Sokoll at *gwm681-7A*) and KK (Krichauff at both marker loci), all grown at Rosedale, SA, Australia in 2006 and 2007.

Relationship between grain-fructan loci and important traits

There was no significant interaction between *barc54-6D* and g*wm681-7A* in the grain yield of the Berkut/Krichauff DH lines grown at Roseworthy, Booleroo, Minnipa and Balaklava, South Australia in 2007. Effects of each marker on yield were calculated and presented in Table 11. Although grain yield widely varied among the environments, mean values for lines homozygous for the Berkut or Krichauff alleles at each marker locus were not significantly different. Consistent results were also found for many other environments (Howard Eagles, personal communication), indicating that using these markers to select for increased grain fructan concentration do not affect grain yield.

Table 11 Effects on grain yield (kg/ha) of *barc54-6D* and *gwm681-7A* at the four field sites (Balaklava, Roseworthy, Booleroo and Minnipa), South Australia in 2007. Effects were calculated individually for each marker in REML with the markers fixed and doubled-haploids random.

Parental allele	*barc054-6D*	*gwm681-7A*
	Roseworthy	
Berkut allele	1883	1925
Berkut allele	1918	1870
Standard error of difference	65	66
	Booleroo	
Berkut allele	1437	1396
Berkut allele	1398	1445
Standard error of difference	28	28
	Minnipa	
Berkut allele	561	577
Berkut allele	577	558
Standard error of difference	22	22
	Balaklava	
Berkut allele	632	635
Berkut allele	658	654
Standard error of difference	33	33

For 1000-grain weight, there was also no significant interaction between *barc54-6D* and g*wm681-7A*. The marker *barc54-6D* had a significant ($P < 0.05$) positive effect. Lines that are homozygous for the Berkut allele at *barc54-6D* had higher 1000-grain weight than lines that are homozygous for the Krichauff allele (Fig. 15). This locus may act by affecting the rate of grain sucrose loading and thereby increasing both grain weight and fructan synthesis.

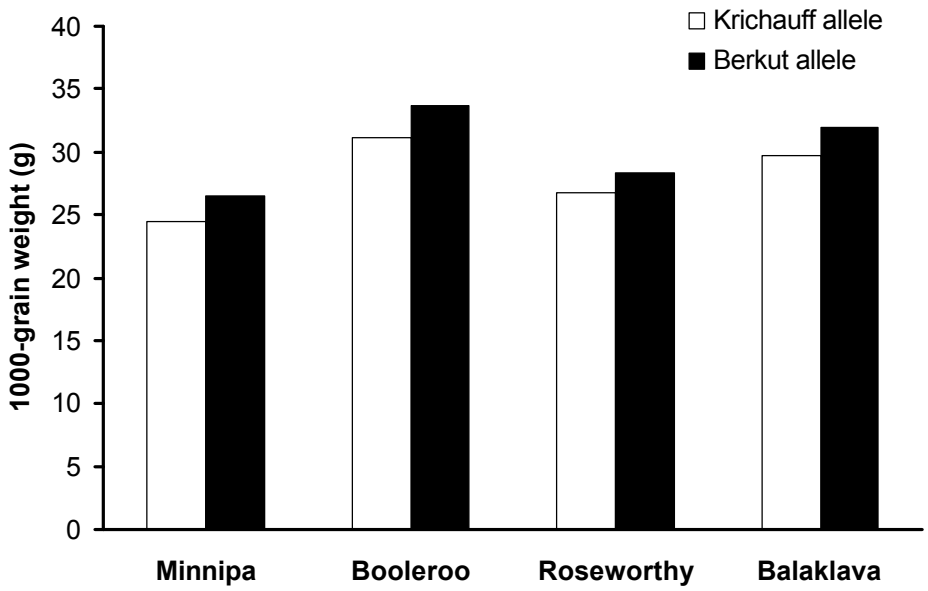


Figure 15 Significant ($P < 0.05$) association between the marker *barc54-6D* and the 1000-grain weight measured in the Berkut/Krichauff population grown at Minnipa, Booleroo, Roseworthy and Balaklava, South Australia in 2007. Data are mean 1000-grain weight for DH lines carrying either Berkut or Krichauff alleles.

Candidate gene controlling grain fructan accumulation

The major QTL *QGfc.aww-7A.1* detected on chromosome 7A (Fig. 12) is in the same region in which Francki et al. (2006) reported the fructosyltransferase orthologue (*AB029888*) based on deletion bin mapping. With primers *AB029888-15F* and *AB029888-19R*, polymorphic DNA fragments of *AB029888* were amplified, with an approximately 100 bp difference (Fig. 16). Krichauff had a fragment of approximately 1.7 kb, while

Berkut and Sokoll had longer fragments, approximately 1.8 kb (Fig. 16). Mapping of this polymorphism confirmed that *AB029888* coincides with the peak of major QTL *QGfc.aww-7A.1* on chromosome 7A of the Berkut/Krichauff genetic map (Fig. 16). With the co-dominant inheritance, this marker is directly useful for marker-assisted selection for this QTL.

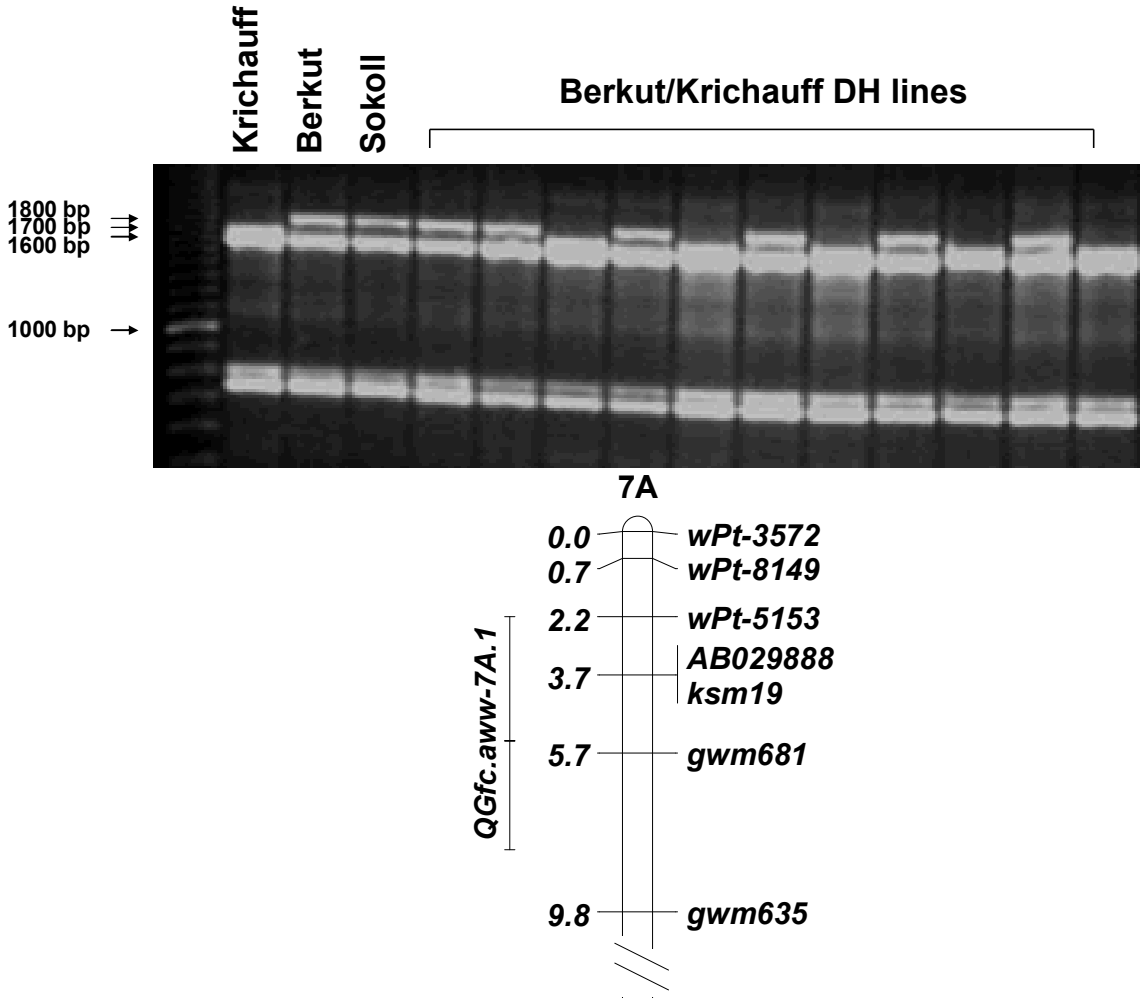


Figure 16 DNA fragments of Krichauff, Berkut, Sokoll and DH lines amplified by primers *AB029888-15F* and *AB029888-19R*. The polymorphism (approximately 1.7 kb and 1.8 kb) was mapped to grain-fructan QTL *QGfc.aww-7A.1* on chromosome 7A.

5.4 Conclusion

Grain fructan concentration varied widely within the Berkut/Krichauff population and was quite repeatable across environments. This variation was largely controlled by two major

QTLs on chromosomes 6D and 7A. Their effects were validated in the Berkut/Krichauff population grown in additional environments and in the Sokoll/Krichauff population. The QTL on 7A coincides with a fructosyltransferase orthologue *AB029888*. It should therefore be possible to improve fructan concentration in wheat grain by breeding, using both phenotypic and marker-assisted selection whilst not negatively affecting important traits including yield and grain weight.

Chapter 6

General Discussion

Although more than 50 years have passed since fructans in wheat grain were first discovered, genetic research on wheat grain fructans appears to be lacking. Previous studies have largely focused on fructan chemistry, the physiology of fructan accumulation, or the comparison of fructan levels between wheat and other cereals, with no focus on the genetic improvement of this trait within wheat. There is no information on the inheritance of fructan accumulation in grain of wheat or other cereals. In contrast, progress has been made in understanding the genetics of fructan accumulation in vegetative tissues of wheat (e.g., Ruuska et al., 2006; Zhang et al., 2008; Xue et al., 2008), barley (Hayes et al., 1993) and perennial ryegrass (e.g., Turner et al., 2006). A reason for this imbalance is that it is more straightforward to measure fructan levels in the vegetative tissue than in the grain. In the vegetative tissue, fructans are the major component of soluble carbohydrates, so their total content can be simply quantified using the colorimetric reagent anthrone, acid hydrolysis or near-infrared reflectance spectroscopy. In wheat grain, however, fructans are present in a small quantity, along with large amounts of starch, galacto-oligosaccharides, sucrose and monosaccharides. Fructan measurement in grain samples therefore requires multiple enzymatic hydrolyses to minimise interference, making previous methods laborious and expensive. Furthermore, methods that do not consider interference of wheat raffinose are likely to be inaccurate. These analytical issues could affect genetic research by making it impractical to accurately assess grain fructan concentration in large numbers of grain samples, such as those required for surveys of genotypic variation and for genetic mapping.

6.1 Fructan measurement

With the simplified method for fructan analysis developed here, grain fructan concentration can be assayed on large numbers of samples. Starch and raffinose, the major sources of interference, are eliminated during fructan analysis. In total, 674 grain samples, representing 421 genetically different lines from hexaploid wheat germplasm, parental

materials and mapping populations, were analysed for grain fructan concentrations. Among these, 48 samples were bulks of grain from DH lines carrying contrasting fructan QTL combinations and the other grain samples were from individual lines. Based on fructan analyses of all wheat materials, grain fructan concentration ranged from 0.3 to 2.6% (of grain dry weight). The largest variation was observed for the germplasm set, with grain fructan concentration ranging from 0.3 to 2.0% (Fig. 7 in Chapter 4), but the highest fructan levels were observed in several Australian cultivars (Chara, Cranbrook and Halberd), new CIMMYT breeding lines (Berkut, Sokoll) and some DH lines of the Berkut/Krichauff population (Fig. 6 in Chapter 4, and Fig. 11 in Chapter 5). These materials could therefore directly contribute to fructan consumption, or they may be crossed with existing cultivars which are inherently low in grain fructan.

With the range of fructan levels reported here, wheat contains much less fructan than some other plant-source foods, such as 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 is a staple food and can be the largest source of fructan intake for humans. Indeed, a survey by Moshfegh et al. (1999) showed that wheat contributed 70% of fructan in American diets, followed by onions (25%). Therefore, any increase or decrease in grain fructan levels within commercial wheat could largely affect the fructan consumption in countries where wheat is an important part of the diet. For example, with the differential observed here for grain fructan levels within wheat, the highest fructan sample could provide nine times more fructan intake than the lowest fructan sample (i.e., 2.6% *versus* 0.3% of grain dry weight). Considering the generally low fructan levels (on average 1%) observed in the germplasm set of internationally-grown hexaploid wheat (Fig. 7 in Chapter 4, and Appendix 1), any genetic improvement of fructan level towards a new commercial wheat would contribute considerably to fructan consumption in wheat-producing countries.

6.2 Breeding potential

Based on the large variation and the high repeatability observed among the wheat breeding materials, it seems likely that grain fructan concentration can be improved effectively by breeding 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 different environments

(Fig. 6, 8 in Chapter 4, and Fig. 11 in Chapter 5). However, obtaining grain fructan data can be difficult due to the cost of fructan analysis and the need for growing plants until harvest. It would be more efficient to select for high grain fructan based on other traits that are easier to measure and genetically correlated with grain fructan. Since vegetative-tissue fructan concentrations may be simply measured during the plant growing stage, it would be worthwhile to examine whether this trait can be used as an indicator for grain fructan concentration, for the purposes of indirect selection.

Generally, conventional plant breeding that relies on phenotypic selection can be time consuming, as segregating generations and breeding lines need to be grown until certain stages for fructan analysis. On the other hand, applying marker-assisted selection can speed up the breeding process by allowing breeders to select for high fructan lines by screening the DNA of young plants; unsuitable plants can also be excluded early from the breeding program without the need to grow them in lengthy performance trials.

The mapping of two major QTLs (*QGfc.aww-6D.2* and *QGfc.aww-7A.1*) affecting grain fructan concentration in wheat could provide breeders with markers to select for high fructan lines. Based on the QTL mapping result, *QGfc.aww-6D.2* and *QGfc.aww-7A.1* explained 17% and 27% of the total phenotypic variation, respectively. Berkut or Sokoll alleles at these loci conferred higher grain fructan than Krichauff alleles by a factor of 0.4 and 0.5, respectively (Fig. 13 and 14 in Chapter 5). Using marker-assisted backcrossing, Berkut or Sokoll alleles could be introgressed into Krichauff, an Australian adapted variety but inherently low in grain fructan level (around 1.5% of grain dry weight). Markers to be used include the candidate gene *AB029888* (primers *AB029888-15F* and *AB029888-19R*, Fig. 16 in Chapter 5) at *QGfc.aww-7A.1* and/or SSR markers flanking each QTL. Size polymorphism of flanking markers distinguishing between Krichauff and the donors (Berkut, Sokoll) was described in Figures 17 and 18. It is noted that Berkut and Sokoll had the same allele size at *barc54-6D* or *ksm19-7A*, so they may be identical by descent. 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., 2008). However, at the other two markers *cfd42-6D* and *gwm681-7A*, Berkut and Sokoll had different allele sizes (Fig. 17 and 18), implying that recombination might occur between the markers and fructan genes. Otherwise, each of these markers might link to different favourable alleles in Berkut and Sokoll, given these lines showed consistent differentials in grain fructan across environments (i.e., Sokoll was

higher than Berkut). Further validation would be needed, but at this stage *barc54-6D* and *ksm19-7A* could be useful for MAS, because *barc54-6D* is nearest to *QGfc.aww-6D.2* (Fig. 12 in chapter 5), while *ksm19-7A* co-segregated with the candidate gene *AB029888* under *QGfc.aww-7A.1* (Fig. 16 in Chapter 5).

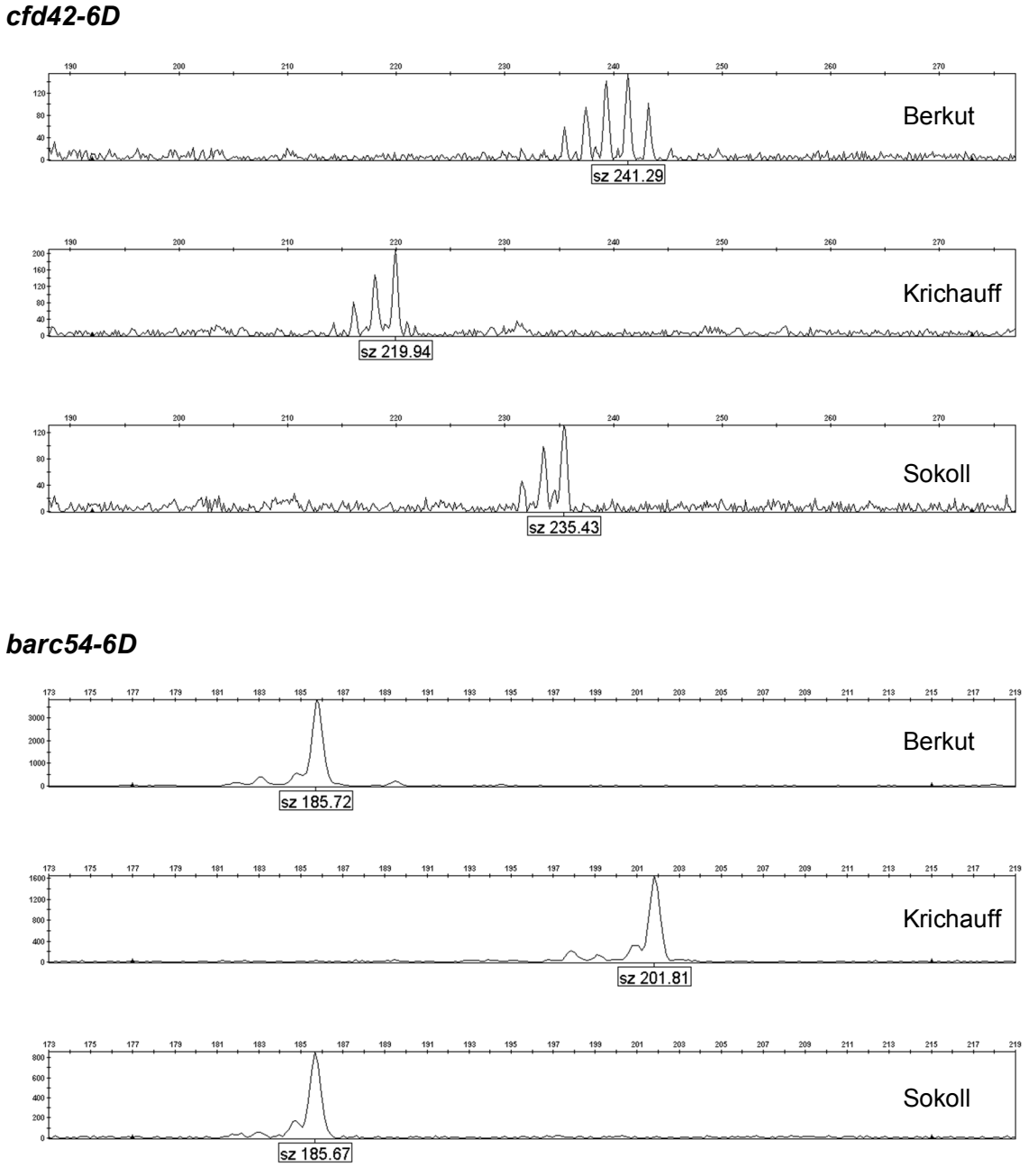
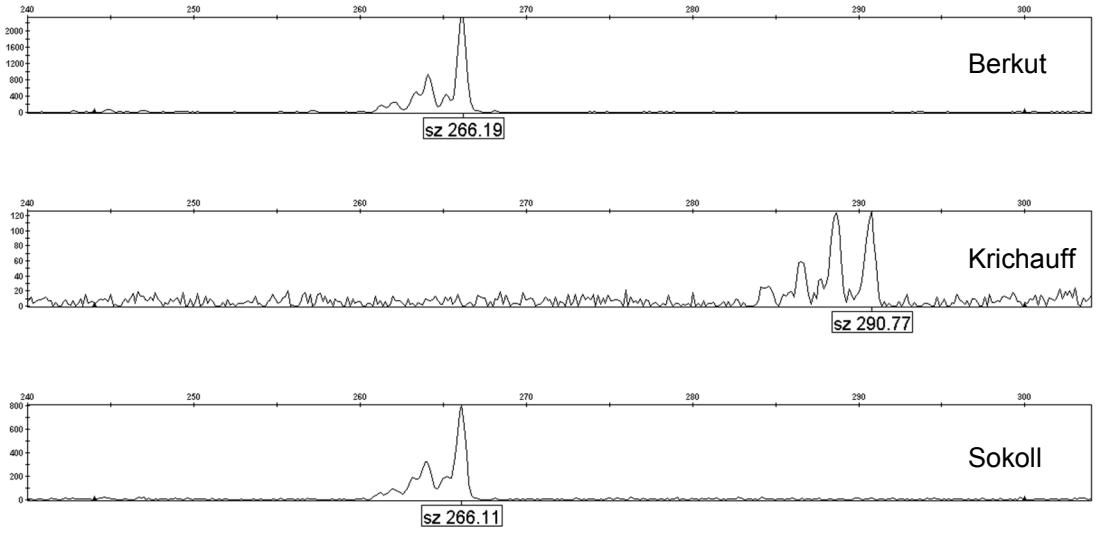


Figure 17 Size polymorphism of fluorescent-labelled SSR markers flanking the QTL *QGfc.aww-6D.2* which distinguished between Berkut, Krichauff and Sokoll.

ksm19-7A



gwm681-7A

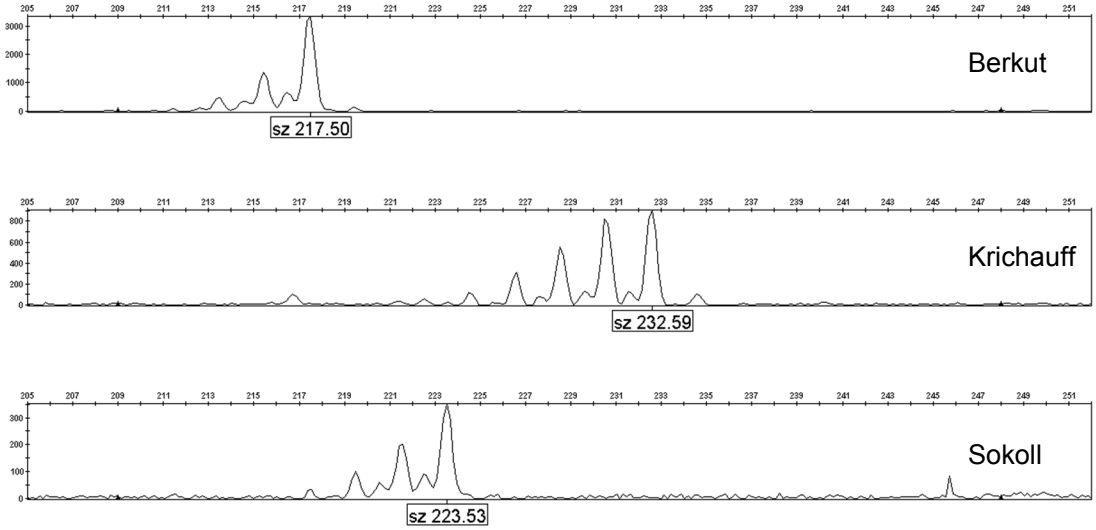


Figure 18 Size polymorphism of fluorescent-labelled SSR markers flanking the QTL *QGfc.aww-7A.1* which distinguished between Berkut, Krichauff and Sokoll.

6.3 Nutritional contribution

Krichauff is a broadly adapted and high yielding wheat variety in South Australia. However, this variety has low grain fructan concentration across environments as observed from this study (1.5% or less). With the expected genetic gain contributed from both QTLs (Fig. 13 and 14 in Chapter 5), a backcross derivative of Krichauff could contribute up to a

40-50% increase in fructan consumption in markets consuming wheat produced in South Australia.

Targeting the two QTLs in MAS also has the potential to increase the fructan level of international wheat cultivars, which were generally low in grain fructan (1% on average) as observed from a survey in this study (Fig. 7 in Chapter 4, and Appendix 1). This would be particularly useful in breeding programs developing wheat for developing countries where nutrient malnutrition is prevalent. Fructan intake selectively promotes the growth of beneficial bifidobacteria in the colon; the fermentation subsequently modifies the colon environment by lowering the pH and thereby facilitating nutrient solubility and absorption (bioavailability) (Delzenne et al., 1995; Ohta et al., 1995; Abrams et al., 2005; Lobo et al., 2006). Breeding for high fructan wheat could therefore be the most feasible way to improve bioavailability. Other options appear more difficult. For example, breeding for increased grain iron can be challenging due to soil nutrient variation and strong genotype-by-environment interactions (Oury et al., 2006; Ortiz-Monasterio et al., 2007). Breeding for reduced levels of anti-nutrients like phytates may have disadvantages in that some of them may confer other benefits for plant and human health (Marschner, 1995; Welch, 2002).

Based on the large variation in wheat grain fructan observed here, the consumption of higher fructan wheat is expected to increase health benefits. Fructans in wheat are composed of branched β(1-2) and β(2-6) linkages (Nilsson and Dahlqvist, 1986), which can exert more pronounced prebiotic effect than inulin-type fructans (Probert and Gibson, 2002). A rate study using inulin-type fructans showed that fructan intake at 2.5 g/day for 7 days significantly modified the gut environment by reducing colonic pH (Bouhnik et al., 1999). This inulin supplement is equivalent to the consumption of 125 g wheat of 2% fructan or less, due to the more pronounced effect of branched fructans. As humans consume food on a concentration basis, they would not prefer to eat double the amount of lower fructan wheat (e.g., 1%) to obtain the effective fructan level. Also, eating a lower quantity of (high fructan) wheat also reduces the consumption of starch, thereby keeping blood glucose at safe levels, especially in patients with diabetes. Since fructans may also interact with other nutritional factors in wheat grain, including macro- and micro nutrients, anti-nutrients and promoter substances (Graham et al., 2001), it would be useful to also investigate the relative contribution of low and high fructan wheat on absorption of

essential nutrients. 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. For example, two bulks of grain can be formed for several DH lines with extreme fructan concentrations in the Berkut/Krichauff or Sokoll/Krichauff populations, using an equal quantity of grain from each line within the group. By this means, large amounts of wheat flour can be produced with contrasting 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.

Prior to clinical trials, feeding studies may be conducted using animal models. For example, populations of mouse recombinant-inbred lines developed by Mott and Flint (2002) may be suitably used. Animals in each recombinant-inbred line are genetically similar, which helps minimise the residual effect. In addition, using the genetic map of the mice population would enable genetic mapping of loci affecting bioavailability and other health attributes derived from high fructan wheat. A proposed strategy could involve feeding the mouse RIL population with two treatments (i.e., two grain bulks of contrasting fructan concentrations). The animals would be measured for prebiotic effects (e.g., bifidobacteria growth), bioavailability (e.g., micro-and macro nutrients, blood cells and body weight) and other traits (e.g., blood glucose level). Quantitative trait loci for these traits will be mapped on the mouse genetic map (Mott and Flint, 2002). Differential effects of two feeding treatments (low and high grain fructan) could be assessed based on trait performance or QTLs if detected. Putative response genes in the mouse to the presence of fructans in the diet and to induced probiotic microorganisms in the gut can be identified from the bioinformatics database developed for the population, and knowledge of the sequenced mouse genome.

6.4 Candidate genes

The QTL detected on chromosome 7A (*QGfc.aww-7A.1*) is in the same region in which Francki et al. (2006) reported a fructosyltransferase orthologue (*AB029888*) in the Chinese Spring genome. Since *AB029888* contains functional domains similar to those in a perennial ryegrass fructosyltransferase (Lidgett et al., 2002; Francki et al., 2006) it may

also play a role in fructan metabolism in wheat. As observed here, Berkut and Sokoll had longer amplified fragments than Krichauff (around 100 bp differentials, Fig. 16 in Chapter 5). Combined with the higher fructan of Berkut and Sokoll, it is possible that the longer fragment amplified from these lines may be caused by duplication within functional domains (exons) thereby making the gene more functional. Otherwise it may be due to length polymorphism in the introns of the wheat gene encoding for the *AB029888* full-length cDNA. Further sequencing of this fragment and adjacent regions from Krichauff, Berkut and Sokoll would identify the sequence differences underlying this polymorphism among these parental lines. Alternatively, investigation of allelic-specific expression for *AB029888* in Krichauff, Berkut and Sokoll and their F_1 progeny could provide insights into whether *AB029888* is the causal gene for *QGfc.aww-7A.1*.

Further, the QTL detected on chromosome 6D (*QGfc.aww-6D.2*) seems to be located in the same region in which Zhang et al. (2008) reported a gene encoding for a fructan exohydrolase (*1-FEHw2*). This enzyme is postulated to be important not only during the period of fructan degradation but also as a putative β-(2,1)-trimmer during the period of active fructan biosynthesis in wheat (Van den Ende et al., 2003a). Re-sequencing of this candidate gene from Krichauff, Berkut, Sokoll or other materials with varying fructan levels may help identify the sequence differences underlying their phenotypic variation. Alternatively, gene expression measured in Krichauff, Berkut and Sokoll and their F_1 progeny could provide insights into whether the *1-FEH* gene is the causal gene for *QGfc.aww-6D.2*.

It may also be useful to track possible allelic variation for these candidate genes from species other than hexaploid wheat, potentially including lines from other *Triticum* species or other cereals such as rye (*Secale cereale* L.) which has higher grain fructan compared to wheat (MacLeod and Preece, 1953; Henry and Saini, 1989; Nardi et al., 2003). This could help discover more sources of favourable (high fructan concentration) alleles for use in marker-assisted backcrossing as well as in functional research on why the genes conferred differential fructan accumulation in the grain of wheat and other cereals. The information would contribute to a better understanding of the molecular mechanism underlying grain fructan accumulation that could lead to opportunities for a further increase of grain fructan content through genetic engineering.

6.5 Fructan retention

Grain materials used in this study were not subject to long-term storage, except for the grain from the Berkut/Krichauff mapping population grown at Rosedale in 2005, which had been stored for one year. In addition, grain materials obtained from overseas (Kazakhstan) and other Australian states (New South Wales and Queensland) might also have been stored for a while before delivery to Adelaide for fructan analysis. During storage, it is possible that fructans may be degraded by the activity of micro-organisms or fructan-degrading enzymes (Pollock and Cairns, 1991; Merry et al., 1995; Noureddine and Norio, 2006). However, positive correlation among environments indicated that there did not seem to have been any serious differential degradation (i.e., differences in fructan degradation among lines). Long-term storage may affect the stability of fructans, as it may incur breakdown of seed dormancy, thereby activating fructan-degrading enzymes to hydrolyse grain fructans. Likewise, post-harvest processing may also affect fructan retention due to increased moisture or other conditions which may favour the activity of fructan-degrading enzymes or bacterial growth. It would therefore be worthwhile to examine whether there is differential degradation among wheat breeding materials subject to long-term storage or processing to final products. Such information could lead to opportunities to optimise fructan retention in stored grain or end-used products via wheat breeding or technical improvement of storage/processing conditions.

In summary, fructans are prebiotics, with potential benefits on human and animal gut health as revealed from the literature. Based on this study, the level of fructans in wheat grain could be improved by breeding, using both phenotypic and marker-assisted selection, whilst not negatively affecting other important traits (yield and grain weight). The simplified analytical method developed could be adopted in wheat breeding for mass screening of grain fructan concentration. Wheat materials of varying fructan concentrations identified from this study are now available for use in further nutritional research. Opportunities for further increase of grain fructan content would be achieved via further investigations of the candidate genes of major QTLs, while retention studies would help maintain the level of wheat fructans in stored grain or end-use products.

Chapter 7

Conclusions

The main conclusions of the research reported in this thesis are:

Analytical improvement for grain fructan analysis

The simplified method developed in this study accurately and precisely measures fructan concentration in wheat grain. Interference of starch, raffinose and other carbohydrates is removed during fructan analysis. The method is convenient for mass screening of wheat breeding lines.

Genotypic variation in wheat grain fructan

Fructan concentration in wheat grain varies widely and is highly repeatable. Fructan concentrations of the same lines were positively correlated among different environments. There is no evidence of negative associations between grain fructan concentration and important traits (yield and grain weight). Thus, grain fructan concentration can be improved by breeding.

Inheritance of grain fructan concentration

Genetic control of variation in wheat grain fructan is complex. Based on genetic mapping using the Berkut/Krichauff population, grain fructan concentration is affected by eight QTLs and two epistatic interactions, most of which do not interact with environments. Two QTLs with major effects are *QGfc.aww-6D.2* on chromosome 6D and *QGfc.aww-7A.1* on chromosome 7A. Berkut carries favourable alleles at both QTLs.

Validation of QTLs with major effects

Two major QTLs (*QGfc.aww-6D.2* and *QGfc.aww-7A.1*) had effects in additional environments and in another population (the Sokoll/Krichauff population). Sokoll also carries favourable alleles at both QTLs. There are no negative associations between favourable alleles and important traits (yield and grain weight). Thus, these QTLs can be targeted in marker-assisted breeding to increase grain fructan content.

Candidate gene for the major QTL *QGfc.aww-7A.1*

The QTL *QGfc.aww-7A.1* is in the same region as a reported fructosyltransferase orthologue (*AB029888*). Primers *AB029888-15F* and *AB029888-19R* amplify co-dominant polymorphic DNA. Berkut and Sokoll have longer fragments than Krichauff. Thus, this marker is directly useful for marker-assisted selection for this QTL.

Chapter 8

Contributions to Knowledge

This research was focused on finding information for a new strategy of genetic biofortification of wheat with increased fructan content, contributing to fructan intake for humans from daily diet. The research involved development of an improved method to determine the amount of fructans in wheat grain and use of this method to survey the extent of genetic variation in fructan levels, and to map and detect quantitative trait loci affecting the trait. This thesis presents the following new contributions to knowledge:

1. An improved procedure for grain fructan measurement (Chapter 3). This procedure incorporates different advantages from existing methods, with modifications to overcome their limitations, especially for assessment of large numbers of wheat breeding lines. The preparation of enzymes and reagents was based on the AOAC 997.08 and AOAC 999.03 methods to allow specific and complete digestion of fructans and other sugar polymers. The implementation of multiple hydrolyses in parallel was based on the method described by Quemener et al. (1994). The use of α-galactosidase to remove interference of raffinose in wheat grain was based on the Megazyme fructan-assay kit (Megazyme K-FRUC). One important modification in the simplified procedure is that fructans are extracted and digested in small closed tubes, preventing analytical errors caused by contamination and evaporation, and allowing for simultaneous handling of a large number of samples with reduced labour and low enzyme usage.

2. Information on genetic variation in wheat grain fructan (Chapter 4), demonstrating that the variation and repeatability are both sufficient to support the increase of grain fructan levels by breeding. This is the first report of an investigation of this variation among different wheat cultivars and breeding lines that had been grown together. The large variation and the high repeatability of wheat grain fructan concentration indicate that it is possible to increase wheat grain fructan levels by breeding.

3. Information on QTLs affecting grain fructan concentration in wheat (Chapter 5), including two major QTLs and several minor QTLs and epistatic interactions.

4. Addition of 12 SSR markers and one candidate gene to the new Berkut/Krichauff genetic map (Chapter 5).

5. Detection of a polymorphism within the fructosyltransferase orthologue (*AB029888*), and confirmation that this polymorphism coincides in position with the major QTL *QGfc.aww-7A.1*, This is the first candidate gene reported for grain fructan in wheat, and it provides a potentially gene-based marker that could be used for marker-assisted selection. Although this gene orthologue was studied by Francki et al. (2006), they did not report any polymorphisms or consider any possible effect on accumulation of grain fructans.

6. Presentation and use of a novel QTL validation approach in which validation phenotyping was conducted on grain bulks formed using lines that had been selected based on their genotypes at markers linked to the QTLs to be validated. This permitted validation of the two major QTLs across environments and populations. This approach will be used in future feeding trials to investigate nutritional and health effects of grain fructan differences. By using this means, large amounts of wheat flour can be produced with contrasting fructan concentrations but equal amounts of other factors (nutrients, anti-nutrients and promoters), reducing their confounding with fructan effects in the gut.

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Appendix

Appendix 1: Grain fructan concentration (% of dry weight) and 100-grain weight of 98 hexaploid wheat lines grown at Narrabri (New South Wales, Australia) in 2006.

Name of line	Aus number	Origin	Grain fructan (%)	100-grain weight (g)
Akadaruma	21846	Japan	1.11	2.02
Allora	10398	Australia	1.22	3.26
Altar	20775	Ecuador	0.75	2.52
AMC 106	19143	Iran	1.16	3.36
AMC 125	19217	Iran	1.09	3.13
AMC 136	19156	Iran	1.09	3.57
AMC 512	19193	Turkey	1.00	3.60
AMC 53	19111	Syria	0.96	3.30
AMC 61	19114	Syria	0.93	3.33
AMC 83	19133	Iraq	1.27	3.23
Arnhem	25607	Australia	1.23	2.77
Asure Bugday	17696	Turkey	0.79	3.23
Banks	20599	Australia	1.33	2.82
Batavia	25271	Australia	1.52	2.67
Beladi 42	13085	Ethiopia	1.28	3.04
Beyrouth 1	4203	Lebanon	1.22	3.04
Bounty	11996	Kenya	1.41	2.82
Bowerbird	30434	Australia	0.68	2.54
Clubhead	130	United States	1.07	3.13
Crete 11	4287	Greece	1.04	3.30
D.E.S. 0043	4374	Greece	1.47	3.11
D.E.S. 0111	4384	Greece	1.17	2.98
Dalmatia 2	4342	Croatia	1.04	3.33
Drysdale	30498	Australia	0.98	3.26
Ellison	33371	Australia	0.88	2.89
Frontana	2451	Brazil	1.01	3.00
Gabo	246	Australia	1.35	3.23
Giza 139	12957	Egypt	0.71	2.84
Gluyas Early	172	Australia	1.37	3.60
H 1160	14455	Afghanistan	1.17	3.33

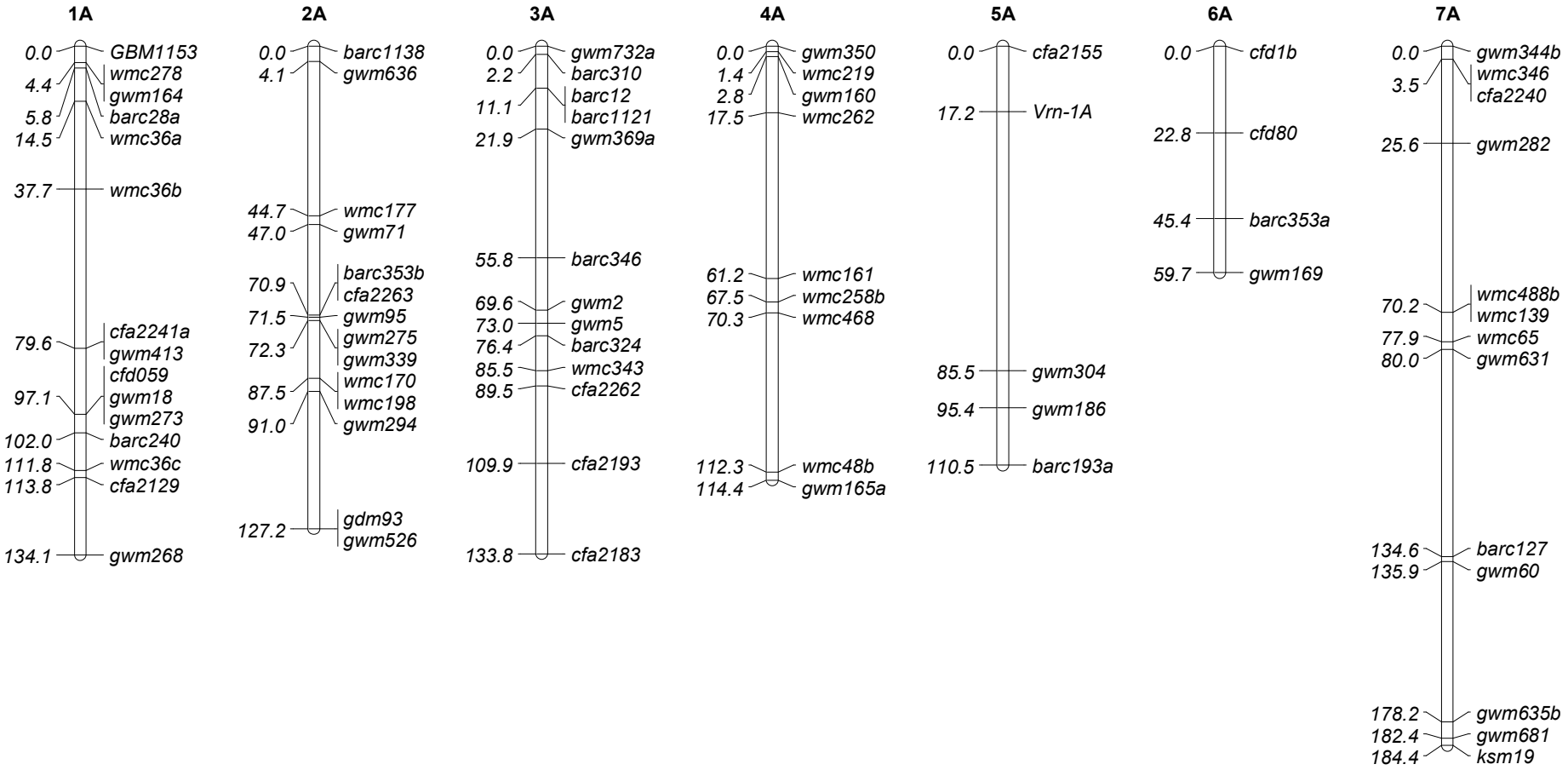
(Appendix 1 continued)

H 1287	14546	Afghanistan	0.96	3.33
H 501	13373	Afghanistan	0.72	2.52
H 865	13568	Afghanistan	1.12	2.83
H45	29488	Australia	0.90	2.71
Hermitage	321	Australia	1.24	2.84
India 211	15330	Pakistan	1.03	2.79
India 259	4838	India	0.73	3.10
Insignia	2642	Australia	1.16	2.83
Iran 158	17860	Iran	0.84	2.71
Kandahary	16132	Syria	0.92	3.20
Karizik	8270	Turkey	1.06	3.11
Katyil	21229	Australia	1.17	2.72
Kefeng	13971	China	1.31	2.61
Kiran	16304	India	1.18	3.19
Kopara 73	17017	New Zealand	0.77	3.14
La Prevision	2836	Argentina	1.12	2.74
Lerma Rojo	473	Mexico	1.42	3.48
Machete	23038	Australia	1.00	2.88
Mehon Denias	13212	Algeria	1.01	3.64
Moldova	22146	Romania	1.26	2.98
Morocco 16	5006	Morocco	0.98	2.86
Morocco 21	5015	Morocco	1.46	3.53
Morocco 59	5080	Morocco	0.99	3.37
NW19A	14978	Nepal	0.54	2.07
NW25A	14981	Nepal	0.33	1.63
NW91A	15020	Nepal	0.62	2.59
NW96A	15025	Nepal	0.50	2.04
NW99A	15028	Nepal	1.15	2.21
Olympic	3118	Australia	1.13	2.63
Pakistan C273	760	Pakistan	0.59	3.41
Pelada	7449	Venezuela	0.91	3.03
Persia 80	5251	Iran	1.13	2.31
Portugal 102	5416	Portugal	1.08	3.10
Pumafen	20969	Chile	1.23	2.99

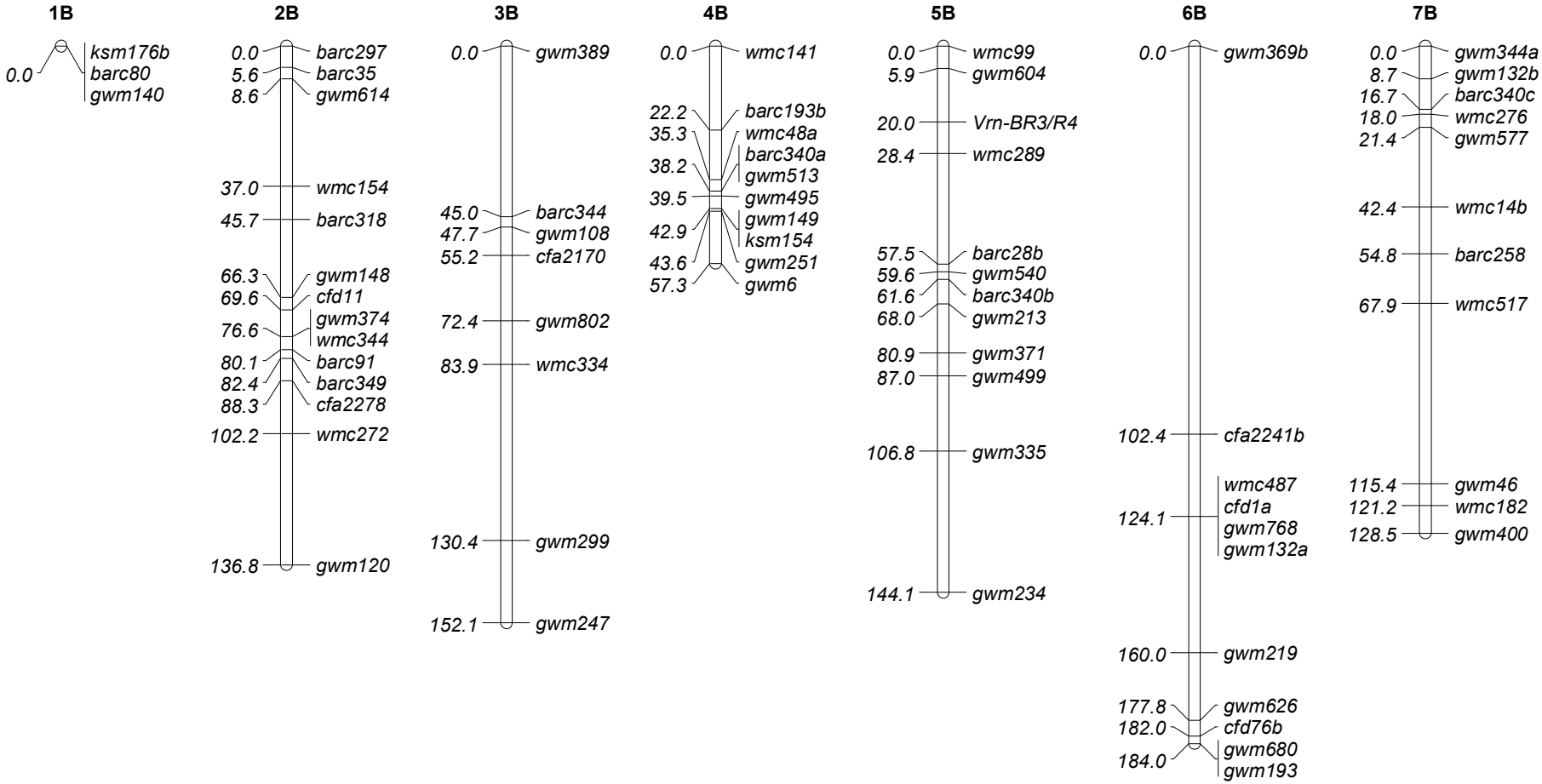
(Appendix 1 continued)

Punjab 7	879	India	1.05	2.70
QAL 2000	30782	Australia	1.08	2.54
QALBIS	33372	Australia	0.82	2.61
Rees	33373	Australia	0.75	2.82
Relin	12440	Switzerland	1.19	2.48
Rogue In Ble Du Oasis	15265	Algeria	0.93	2.42
Salamanca 10	12091	Spain	1.44	2.42
Salonica 17	5627	Greece	0.68	3.45
Sapporo	7359	Japan	1.49	2.48
Saturno	24431	Mexico	0.78	3.37
Seville 22	5639	Spain	1.19	2.95
Sion	19639	Israel	1.13	3.21
Smyrna 13	5667	Turkey	0.96	3.36
Smyrna 2	5646	Turkey	1.17	3.24
Surhak Jubilejnyj	16175	Former S Union	0.78	4.29
Tunis 24	13160	Tunisia	1.19	3.33
Varna 6	1539	Bulgaria	1.45	3.33
Veranopolis	19533	Brazil	0.90	2.86
Vir 45738	19389	Pakistan	0.79	2.22
W139A	19254	India	1.71	2.95
W143	19258	India	1.38	3.16
W145	19260	India	1.50	2.77
W216	19310	India	1.39	2.30
W38	17767	Pakistan	1.31	2.95
W43A	17774	Pakistan	1.17	2.16
W45B	17780	Pakistan	1.25	1.96
W49A	17785	Pakistan	1.09	2.46
W58	17796	Pakistan	1.32	2.63
W63	17802	Pakistan	0.99	2.45
W75	17814	Pakistan	1.94	2.60
Warren	1637	Australia	1.18	2.94
Wylah	29486	Australia	1.01	2.58
Yangmai 3	23893	China	1.23	3.40
Yitpi	30492	Australia	0.86	2.72

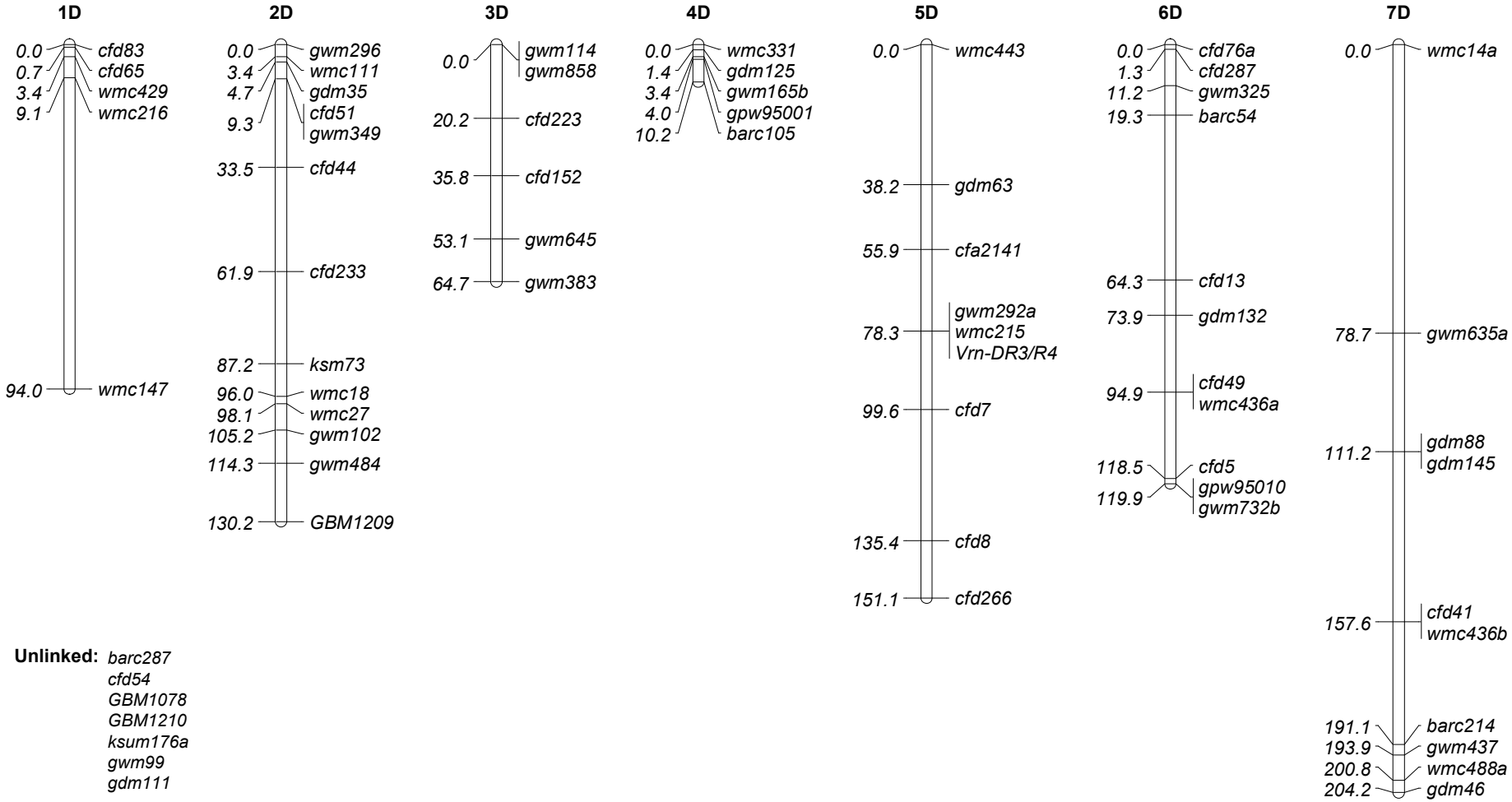
Appendix 2: A preliminary linkage map of 206 SSR markers for the Berkut/Krichauff mapping population obtained from SARDI, with not all linkage groups aligned with previously constructed wheat linkage maps.



(Appendix 2 continued)

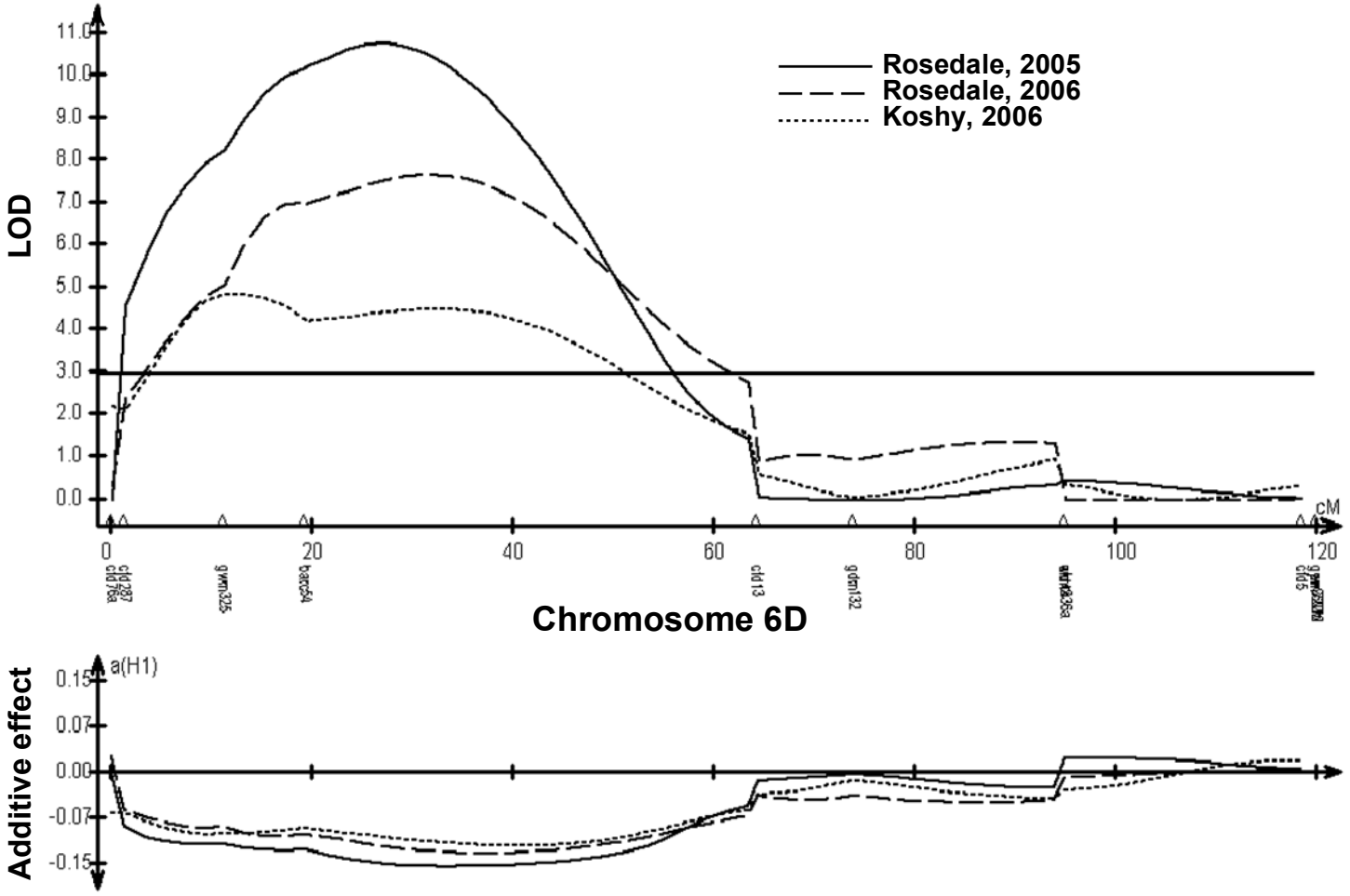


(Appendix 2 continued)

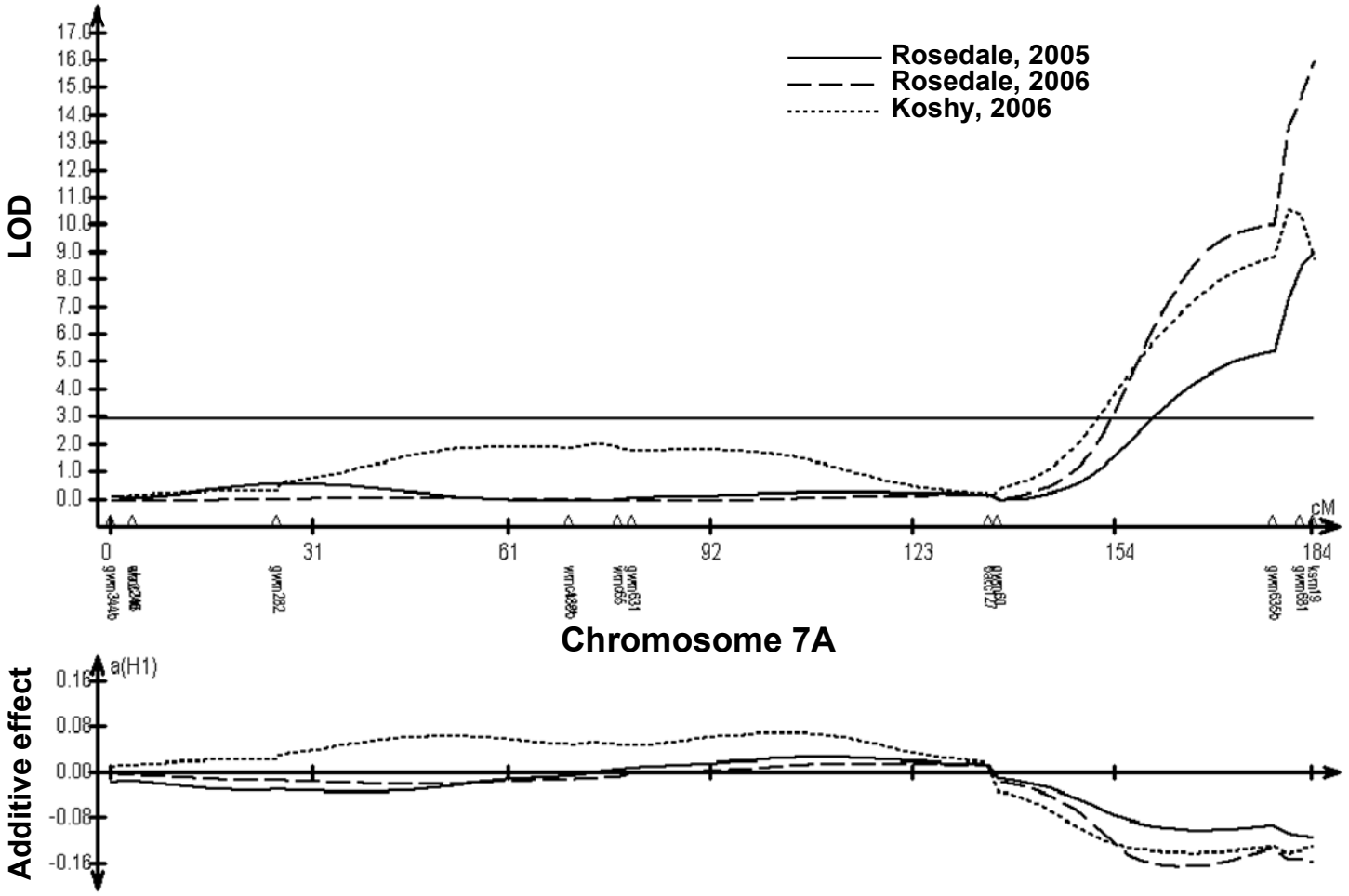


Unlinked: *barc287*
cfd54
GBM1078
GBM1210
ksum176a
gwm99
gdm111

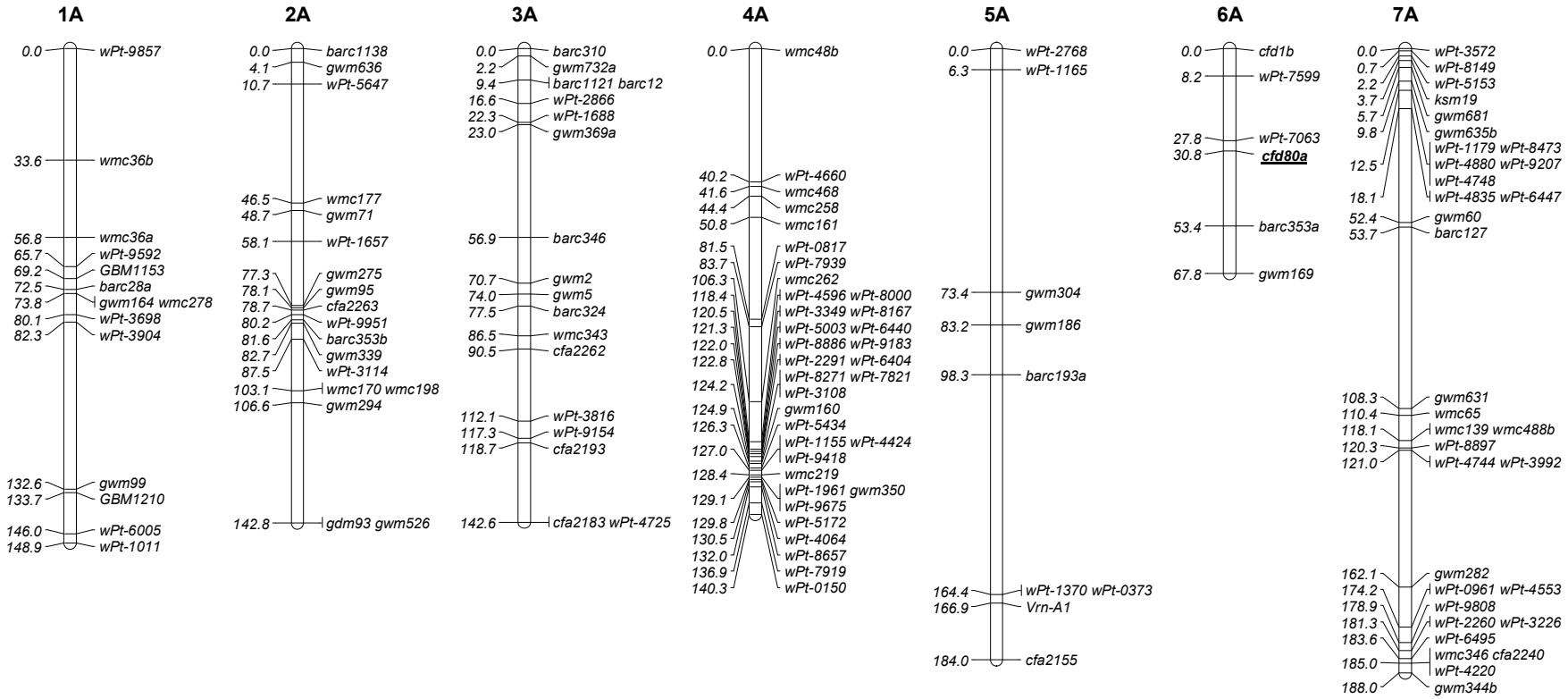
Appendix 3: Initial QTL analysis based on the preliminary linkage map, with grain fructan measured on the Berkut/Krichauff population grown in three field environments (Rosedale in 2005 and 2006, Koshy in 2006). Composite interval mapping was performed with QTL Cartographer V2.5.



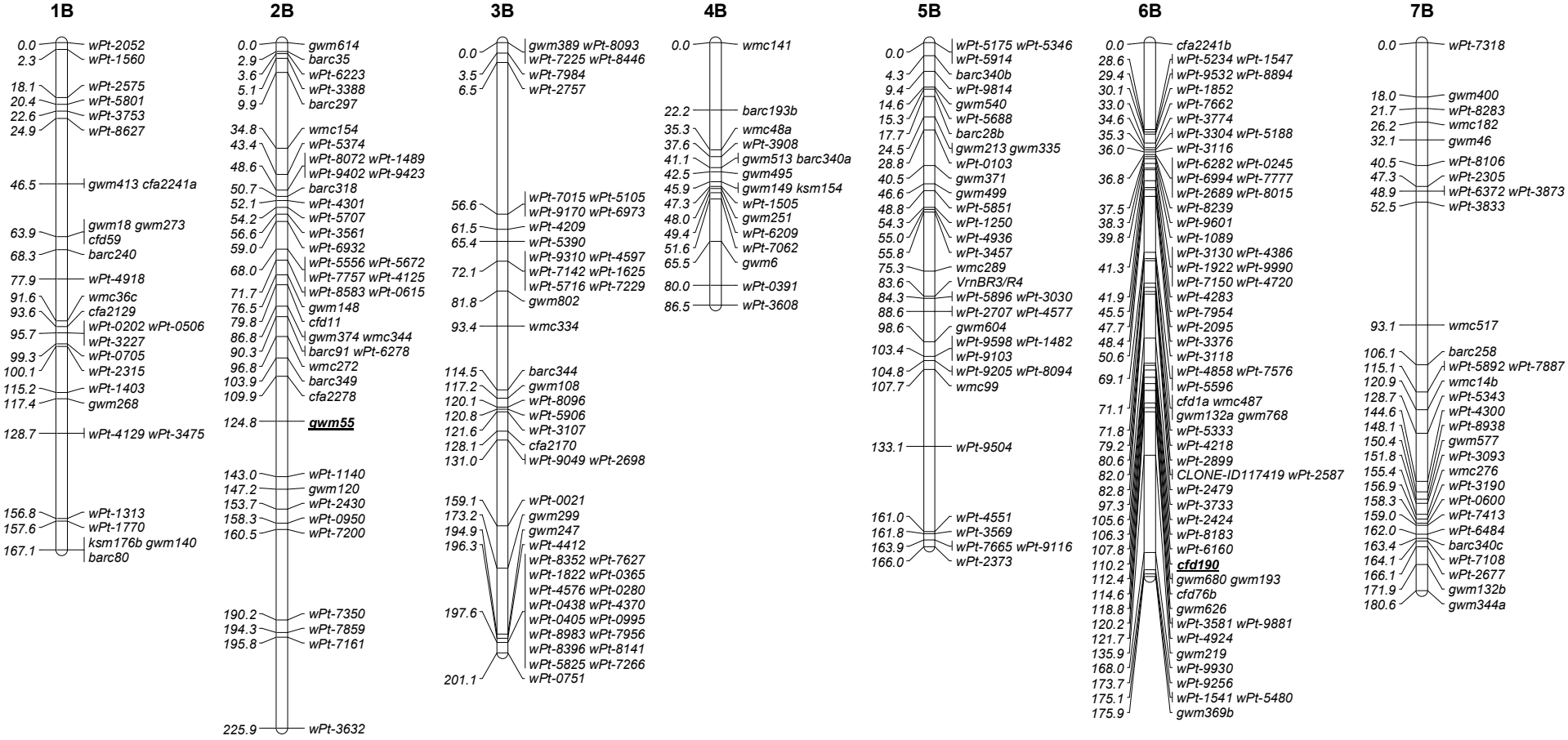
(Appendix 3 continued)



Appendix 4: The improved linkage map of the Berkut/Krichauff mapping population, with the addition of 12 new SSR (in bold and underlined font) and 312 DArT markers and linkage groups aligned with previously constructed wheat linkage maps.



(Appendix 4 continued)



(Appendix 4 continued)