BREEDING FOR WHEAT GRAIN FRUCTANS

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Lynn Dorothy Veenstra

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BREEDING FOR WHEAT GRAIN FRUCTAN CONTENT

Lynn Dorothy Veenstra, Ph. D.

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Wheat (*Triticum aestivum* L.) is a widely consumed staple crop and essential component of a healthy whole-grain diet. One component of wheat, fructans, is known to serve physiological roles in the plant and confer health benefits to humans. Fructans serve as reserve carbohydrates and osmotic regulators against stresses (i.e., drought, cold temperatures, and salinity) that affect grain yield in the wheat plant. For humans, fructans are prebiotics that promote growth of healthy gut bacteria, aid in immune support, reduce colon cancer incidence, and support bone health. Given the roles of fructans in plant and human physiology, breeding for wheat grain fructan content would produce climate resilient, nutritionally improved varieties

Breeding for nutritional traits is a long-term, resource intensive process. Genomic Selection (GS) could help accelerate the breeding process while decreasing phenotyping requirements. This work focused on key issues surrounding the implementation of GS for wheat grain fructan content. First, the existence of genotype-by-environment (GxE) interactions and impacts of interactions on genomic selection (GS) was examined for grain fructan content. Second, the underlying genetic architecture of wheat grain fructan content was examined to determine the feasibility of implementing genomic selection (GS). Lastly, two GS selection methodologies were compared in terms of realized gain from selection for grain fructan content, and impacts on inbreeding, genetic variance, and agronomic characteristics. The key

messages of these studies are 1) GxE interactions do exist for wheat grain fructan content, but their impacts on GS prediction accuracies is small, 2) the underlying genetic architecture of wheat grain fructan content supports the use of GS methodologies, 3) GS for wheat grain fructan content is a feasible, effective selection methodology which produces significant gains from selection, 4) the use of Optimum Contribution Selection (OCS) to control for long term inbreeding does effectively control inbreeding and better conserve genetic variance relative to GBLUP selection populations. Breeding programs implementing GS for wheat grain fructan content should focus their efforts on designing breeding pipelines to capitalize on varying maturity of crossing materials, controlling inbreeding within populations, updating training populations often, and evaluation of materials over multiple years of field trials.

BIOGRAPHICAL SKETCH

Lynn Dorothy Veenstra was born in 1990 in Des Moines, Iowa, as the first child to Molly and Bob Veenstra. Lynn grew up in a family of plant lovers, including her mother and grandmother who were avid gardeners. Lynn's grandmother, Polly, was actively involved in the creation of the Des Moines Botanical Center, a place where Lynn would often visit as a child. In addition to trips to the Botanical Center, Lynn's favorite childhood activities included swimming, reading, helping her mother in the garden, baking bread with her Grandpa Dick, and weekly visits to see her Grandma Norena, where she would also tend to her own gladiola patch during the summer.

At age 4, Lynn was enrolled in the Downtown School and discovered her love of learning. Lynn would go on to attend Callanan Middle School, Roosevelt High School, and Central Academy. During her sophomore year, Lynn took AP Biology with Mrs. Stroope where she first was exposed to the concepts of introductory genetics. When Lynn was trying to decide where to go to college and what to study, she received a call from the Iowa State University Agronomy department. She visited and immediately fell in love with the idea of combining her interests in genetics with her love of plants. Lynn moved to Ames, Iowa, in August 2008 to choose her adventure at Iowa State.

By May 2012, Lynn had spent a summer in Peru with the International Potato Center as a Borlaug Scholar, learned how rewarding 70 hour weeks of corn pollination could be with Pioneer Hi-Bred, and survived weeks of 100+ degree summer weather

in Oklahoma while interning at the Noble Foundation as a Summer Research Scholar. Lynn had also fulfilled the requirements for Bachelor of Science Degrees in Agronomy and Genetics, and accepted an offer of admission to the Plant Breeding and Genetics Graduate Program at Cornell University. In July 2012, she packed up her life and drove halfway across the country to Ithaca, New York, to pursue her PhD in Plant Breeding.

During her time at Cornell, Lynn served as Synapsis President, taught an introductory course in genetics through the Cornell Prison Education Program, volunteered with the Tompkins County Beautification Brigade, discovered hiking, and spent countless hours working on her PhD project. Following her time at Cornell, Lynn hopes to apply her education, skills, and love of plants and genetics to the field of plant breeding to help solve some of the world's most imminent needs.

To Dr. Richard M. Moore and Henry R. Veenstra

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[1]Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the USDA. USDA is an equal opportunity provider and employer.

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CHAPTER 1

INTRODUCTION

Rationale and significance

Bread wheat (*Triticum aestivum* L.) is a widely consumed staple crop and essential component of a healthy whole-grain diet. Increases in climate variability have placed new emphasis on the need for resilient wheat varieties. Alongside demands for increased resiliency, consumer interest in healthier, more functional foods is growing. The identification of potential breeding targets to create climate resilient, nutritionally improved wheat varieties is of particular interest for meeting consumer demands.

One potential breeding target for creating climate resilient, nutritionally improved wheat varieties is fructans. Fructans are naturally occurring plant polymers composed of fructose molecules. Approximately 15% of flowering plant species contain fructans, including wheat. In plants, fructans serve as carbon stores and as a potential form of protection against water deficit, cold temperatures, and high salinity. In addition to serving valuable roles in plant growth and development, the characteristics of fructans have potentially beneficial effects on human health through the promotion of gut health. Given the key roles fructans serve in plant and human physiology, fructans are a potential breeding target for developing climate resilient, nutritious wheat varieties.

While genotypic variance for wheat grain fructan content exists, the breeding of nutritional traits such as fructans is a lengthy, resource intensive process. With the rapid decreases in cost of genotyping, marker assisted selection technologies are gaining traction within the plant breeding community. One marker assisted selection method, Genomic Selection (GS), has the potential to increase rates of genetic gain in crop plants while allowing for reduced phenotyping requirements. GS has been widely studied in simulations and holds great potential for areas such as nutritional breeding; however, few empirical studies have examined the implementation of GS for nutritional breeding.

Objectives

This work addresses four major topics relevant for the implementation of GS for grain fructan content in wheat.

1. The existence and impact of genotype-by-environment interactions on grain fructan content and prediction for grain fructan content.
2. The underlying genetic architecture of wheat grain fructan content.
3. Realized gain from GS for wheat grain fructan content for two selection methodologies.
4. The impact of GS selection methodologies on genetic variance and inbreeding.

Dissertation Organization

The second chapter provides a comprehensive review addressing the current state of knowledge of wheat fructans and highlights key areas of future research with the ultimate goal of providing a potential target for nutritional wheat breeding. The third chapter focuses on GxE interactions for wheat grain fructan content and the impacts prediction accuracies for GS breeding methodologies. The fourth chapter is an evaluation of realized gain from GS for wheat grain fructan content between two selection methodologies. The fifth and final chapter provides a summary and highlights the overall conclusions of the dissertation.

CHAPTER 2

WHEAT FRUCTANS: A POTENTIAL BREEDING TARGET FOR NUTRITIONALLY IMPROVED, CLIMATE RESILIANT VARIETIES[2]

Abstract

Wheat (*Triticum aestivum* L.) is a widely consumed staple crop and essential component of a healthy whole-grain diet. One component of wheat, fructans, is known to serve physiological roles in the plant and confer health benefits to humans. Fructans serve as reserve carbohydrates and osmotic regulators against stresses (i.e., drought, cold temperatures, and salinity) that affect grain yield in the wheat plant. For humans, fructans are prebiotics that promote growth of healthy gut bacteria, aid in immune support, reduce colon cancer incidence, and support bone health. While cereals are the main source of fructans in the American diet, fructans are found in many other plant foods. Variation in wheat grain fructan content has been observed with several widely accepted fructan quantification techniques. Given the observed variation in grain fructan content and potential physiological benefits of fructans for wheat plants and consumers, fructans are a potential breeding target for developing climate-resilient, nutritionally improved wheat varieties.

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Abbreviations

1-FEH, exo-inulinase; 1-FFT, fructan:fructan 1-fructosyltransferase; 1-K, 1-kestose; 1-SST, sucrose:sucrose 1-fructosyltransferase; 6-FEH, levanase; 6G-FFT, fructan:fructan 6G-fructosyltransferase; 6G-K, 6G-kestose; 6-K, 6-kestose; 6-SFT, sucrose:fructan 6-fructosyltransferase; DP, degrees of polymerization; DP_{av}, average degrees of polymerization; FEH, fructan exohydrolases; G × E, genotype-by-environment; GE, genetic engineering; GS, genomic selection; IBS, irritable bowel syndrome; LC-MS, liquid chromatography–mass spectrometry; MAS, marker-assisted selection; mRNA, messenger RNA; NCGS, non-celiac gluten sensitivity; QTLs, quantitative trait loci; SCFA, short chain fatty acid

Introduction

Fructans are carbohydrate polymers composed primarily of β-linked fructose molecules found in algae, bacteria, fungi, and plants. Fructans are present in foods such as artichoke (Cynara scolymus L.), banana (Musa acuminate Colla), broccoli (Brassica oleracea L.), garlic (Allium sativum L.), leek bulb (Allium ampeloprasum L.), melon [Citrullus lanatus (Thunb.) Matsum. & Nakai, Cucumis melo L.], onions (Allium cepa L.), white peach [Prunus persica (L.) Batsch], rye (Secale cereal L.), and wheat (Triticum aestivum L.) (Nelson and Smith, 1986; Roberfroid, 2005; Muir et al., 2007; Fedewa and Rao, 2014). The linkages in fructan molecules allow for passage through the upper gastrointestinal system of humans without undergoing digestion and entrance into the large intestine, where molecules are subjected to microbial fermentation. Fructan consumption confers health benefits to humans by promoting gut health and providing immune support (Gibson et al., 1995; Rao, 1999; Roberfroid, 2005; Lomax and Calder, 2008). Additionally, positive effects of fructan consumption on mineral bioavailability have been observed in adolescents and postmenopausal women (Coudray et al., 1997; Abrams et al., 2005, 2007). The health outcomes of

fructan consumption have been extensively studied and reviewed in previous literature (Roberfroid et al., 2010; Di Bartolomeo et al., 2013; Peshev and Van Den Ende, 2014). Despite the wide diversity of fructan-containing foods, wheat is the primary source of fructans in the American diet due to the volume of wheat products consumed (Van Loo et al., 1995).

Wheat is an allohexaploid containing three genomes (A, B, and D). Wheat domestication began approximately 10,000 yr ago, with modern-day bread wheat (AABBDD genome) arising ~8000 yr ago around Armenia and the Caspian Sea from hybridization of Aegilops tauschii Host (DD genome) and Triticum turgidum L. (AABB genome) (Kihara, 1944; Dvorak et al., 1998; Dubcovsky and Dvorak, 2007). Wheat is a member of the Poales order and is known to contain several classes of fructans in vegetative tissues, roots, and grain (Kafi et al., 2003). Fructans in wheat plants are reserve carbohydrates that act as osmoregulators involved in plant stress tolerance under conditions of limited water availability, cold temperatures, and excess salinity (Hendry, 1993; Pilon-Smits et al., 1995; Kafi et al., 2003; Joudi et al., 2012). The various physiological functions supported by fructans suggest that wheat grain fructan content may be influenced by environment (Karppinen and Liukkonen, 2000; Huynh and Wallwork, 2008; Haskå et al., 2008).

Quantification of fructans in wheat grain has shown variation in content (0.7–2.9 g 100 g^{-1} dry wt.), and in wheat, fructan content is moderately heritable (Huynh et al., 2008). The observed variation, moderate heritability, and potential positive physiological effects of fructans suggest that increasing fructan content could be a useful target for wheat breeding programs. Though previous reviews of fructans have

been recently published addressing the current knowledge (Vijn and Smeekens, 1999; Verspreet et al., 2015a), this review focuses specifically on wheat fructans and provides novel insights regarding the value of fructans in developing climate-resilient, nutritionally improved wheat varieties. This review addresses the current state of knowledge of wheat fructans and highlights key areas of future research with the ultimate goal of providing a potential target for nutritional wheat breeding.

Fructan Structure

Fructans are fructose-rich carbohydrate polymers derived from sucrose. More specifically, fructans are oligo- and polysaccharides in which fructose-fructosyl osidic linkages constitute the majority of osidic bonds (Roberfroid and Delzenne, 1998). Fructans are known to exist in algae, bacteria, fungi, and plants. The degree of polymerization (DP) is much higher for bacterial fructans (≤100,000) than for plant fructans (≤150) (Kaur and Gupta, 2002). Although smaller than bacterial fructans, plant fructans exhibit varying DP, glucose content, and bond composition. The large amount of structural diversity exhibited by plant fructans has resulted in the categorization of fructans by observed β linkage composition into five main classes (inulin, levan/phlein, graminan, neo-inulin, and neo-levan) (Vijn and Smeekens, 1999; Ritsema and Smeekens, 2003; Harrison et al., 2012; Di Bartolomeo et al., 2013; Van den Ende, 2013).

Inulin is a predominantly linear fructan containing β(2-1) linkages and is found in many plants, including wheat, onions, bananas, garlic, and Asterales order plants (Niness, 1999; Vijn and Smeekens, 1999). Within the Asterales order, chicory and

Jerusalem artichoke are known to contain high amounts of inulin (Ritsema and Smeekens, 2003).

Linear fructans containing β(2-6) linkages are referred to as levan and phlein. Levans are typically high-DP molecules from bacteria, whereas phleins are lower-DP molecules from plants including grasses in the Poales order (Waterhouse and Chatterton, 1993; Chatterton and Harrison, 1997; Wei and Chatterton, 2002; Ritsema and Smeekens, 2003; Roberfroid, 2007; Haskå et al., 2008). Despite the distinct origins of levan and phlein, the term levan is often used in place of phlein in the plant literature. This paper will observe the distinct origin and use the term phlein when referring to β(2-6)-linked fructans found in plants.

Graminan-type fructans are branched polymers containing a mixture of β(2-1) and β(2-6) linkages. Graminans are widely found in plant species belonging to the Poales order, including wheat and barley (*Hordeum vulgare* L.) (Carpita et al., 1989; Bonnett et al., 1997). Inulin, levan, phlein, and basic graminan polymers may or may not contain terminal glucose molecules. Fructans with a DP less <10, regardless of bond composition, are considered to be fructooligosaccharides (Roberfroid, 2005).

Neo-inulin and neo-levan fructans are complex fructose polymers with an internal glucose residue containing predominantly β(2-1) and β(2-6) linkages, respectively (Nilsson et al., 1986; Van den Ende, 2013; Verspreet et al., 2013a, 2013b). Neo-inulins are found in Liliaceae plant species, whereas neo-levans are found in a few Poales order species (e.g., oat) (Shiomi, 1989; Livingston et al., 1993). Neo-inulin and neo-levan fructans are also referred to as neoseries fructans throughout the literature (Vijn and Smeekens, 1999).

Structurally diverse fructans exist in many monocot, eudicot, and dicot species; however, not every plant species contains all classes of fructans. Fructans in the wheat stem and vegetative tissue primarily contain β(2-6) linkages, whereas fructans in the grain are a heterogeneous mixture of structures with average DP (DP_{av}) of five units and a maximum DP of 15 to 19 units (Nilsson et al., 1986; Bancal et al., 1992; Bancal et al., 1993; Bonnett and Simpson, 1995; Fretzdorff and Welge, 2003b; Van den Ende et al., 2003; Ruuska and Rebetzke, 2006; Haskå et al., 2008). Fructan structures known to exist in wheat vegetative tissues and grains include inulins, graminans, and phleins (Nilsson et al., 1986; Spollen and Nelson, 1988; Hendry, 1993; Verspreet et al., 2013a). Fructan neoseries are also exclusively present in the wheat grain (Verspreet et al., 2013a, 2015b).

Metabolism of Fructans

The metabolism of fructans is generally understood, with fructans in all organisms arising from sucrose, the main substrate for fructan synthesis (Vijn and Smeekens, 1999). Within plants, fructans are synthesized and subsequently stored within the cell vacuole (Frehner et al., 1984; Wiemken et al., 1986; Darwen and John, 1989).

To accomplish the synthesis of structurally diverse fructans in plants, multiple enzymes are needed (Fig. 2.1). De novo synthesis of fructan structures begins with the

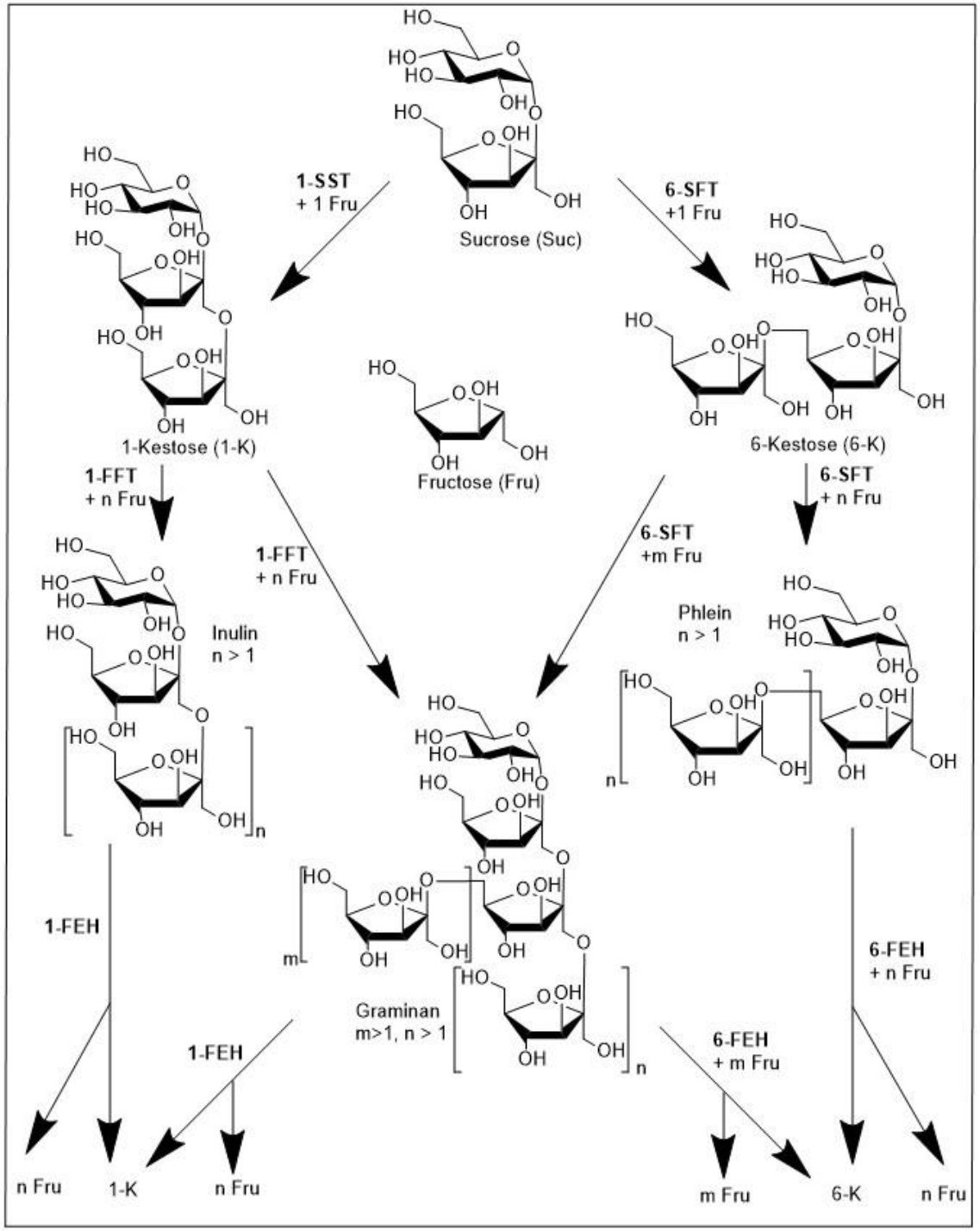

Figure 2.1: Fructan metabolism in plants. 1-SST, sucrose:sucrose 1-fructosyltransferase; 6-SFT, sucrose:fructan 6-fructosyltransferase; 1-FFT, fructan:fructan 1-fructosyltransferase; 1-FEH, exo-inulinase; 6-FEH, levanase. formation of 1-kestose (1-kestotriose, 1-K) (Ritsema and Smeekens, 2003).

Sucrose:sucrose 1-fructosyltransferase (1-SST) catalyzes the transfer of one fructose molecule between two sucrose molecules to form 1-K and glucose (Edelman and Jefford, 1968; Kawakami and Yoshida, 2002). While 1-K is the predominant molecule for de novo fructan synthesis, the process can also start with formation of 6-kestose (6-kestotriose, 6-K) through fructosyl transfer between sucrose molecules via sucrose:fructan 6-fructosyltransferase (6-SFT) action (Duchateau et al., 1995; Sprenger and Bortlik, 1995).

The five main classes of fructans all arise from extension of 1-K or 6-K oligomers into fructan polymers. Inulins are formed through fructan:fructan 1-fructosyltransferase (1-FFT)-catalyzed fructosyl transfer from 1-K to a previously formed fructan molecule or sucrose with the subsequent formation of β(2-1) linkages (Jeong and Housley, 1992; Kawakami and Yoshida, 2005). Graminans are formed through the joint action of 1-SST and 6-SFT. The molecule 6-SFT catalyzes transfer of fructose from sucrose to sucrose, 1-K, or another fructan and formation of β(2-6) linkages (Duchateau et al., 1995; Sprenger and Bortlik, 1995). While 6-SFT has several possible fructosyl receivers, the predominant products are bifurcose (1,6-kestotetraose) and larger fructans (Sprenger and Bortlik, 1995). The proportion of 6-K produced through 6-SFT fructosyl transfer is small relative to the other products (Sprenger and Bortlik, 1995). When sucrose is the only substrate available, phleins are the product of 6-SFT-catalyzed transfer of fructose from one sucrose to another sucrose, 6-K, or fructan (Duchateau et al., 1995). Fructan neoseries are formed through fructan:fructan 6G-fructosyltransferase (6G-FFT)-catalyzed transfer of fructose from 1-K to the glucose moiety of sucrose to form a 6G-kestose (6G-K)

(Shiomi, 1982, 1989; Wiemken et al., 1995; Vijn et al., 1997). Further elongation of 6G-K by 1-FFT and 6-SFT leads to the formation of neo-inulin and neo-levan (Vijn and Smeekens, 1999).

Fructan degradation is catalyzed by fructan exohydrolases (FEH). Exo-inulinase (1-FEH) and levanase (6-FEH) are known to exist in fructan containing plants and act to hydrolyze β(2-1) and β(2-6) linkages, respectively (Van den Ende et al., 2004). Fructan exohydrolases catalyze the removal and release of terminal fructose molecules from fructan polymers (Henson and Livingston, 1996; Marx et al., 1997a, 1997b). Fructose molecules released through FEH action are available for other carbon metabolism. Endo-inulinase enzymes also catalyze fructan degradation, but through hydrolysis of internal β(2-1) linkages in the polymer to yield inulotriose, tetraoses, or pentaoses. Endo-inulinases are known to exist in bacteria, yeasts, and filamentous fungi; however, no record of endo-inulinases in plants exists (Liu et al., 2013).

The ability of fructosyltransferase enzymes to hydrolyze sucrose for the purposes of fructan synthesis appears similar to sucrose hydrolyzing enzymes known as invertases. The functional similarities between fructosyltransferase and invertase enzymes have led to the discovery that the two enzymes have a high degree of identity in amino acid sequence (Vijn and Smeekens, 1999; Wei and Chatterton, 2001). It is believed that fructosyltransferase enzymes evolved from acid or vacuolar invertase enzymes through a few mutational changes (Vijn and Smeekens, 1999). Fructan exohydrolase enzymes are also believed to have evolved from invertases. Unlike their anabolic counterparts, these catabolic enzymes are most closely related to cell-wall

invertases, although the enzymes are functionally different (Van den Ende et al., 2002, 2004; De Coninck et al., 2005). The strong degree of functional similarity between fructosyltransferase and invertase enzymes may affect the fructan metabolism pathway. Under high sucrose concentrations in vitro, invertases have been shown to exhibit some level of 1-SST activity and produce 1-K and 6-K molecules (Cairns and Ashton, 1991). If sucrose is the only substrate available for 6-SFT, the enzyme will act as an invertase and hydrolyze sucrose into glucose and fructose (Vijn and Smeekens, 1999).

Induction and modulation of fructan metabolism in plants has not been fully elucidated; however, it is known that sucrose plays an important role in these processes. Modulation of fructan synthesis is influenced by the presence of light and availability of sucrose in the cell (Vijn and Smeekens, 1999). Light indirectly modulates sucrose availability in the cell through processes related to photosynthesis and carbon metabolism (Vijn and Smeekens, 1999). Once sucrose levels surpass a concentration threshold, sucrose signaling is believed to initiate the signal transduction chain, which results in an increase in fructosyltransferase gene expression and fructan synthesis through the activity of 1-SST and 6-SFT (Martinez-Noël et al., 2001; Nagaraj et al., 2001; Pollock et al., 2003; Xue et al., 2013). Calcium, protein kinases, and type-2A protein phosphatase activity are essential components of the sucrose signal transduction pathway for inducing fructan synthesis (Martínez-Noël et al., 2006, 2007, 2009). While this transduction pathway with respect to fructan synthesis is not fully understood, emerging theories of connections between modulation of fructan metabolism and plant hormones have been established on the basis of crosstalk

between the sugar signaling processes and plant hormone networks (Valluru, 2015). In addition to indirect modulation through gene expression and sucrose signaling, degradation of fructans is also directly modulated by the inhibition of many FEH enzymes by sucrose (Van den Ende et al., 2002; Verhaest et al., 2007). Given that invertases hydrolyze sucrose molecules, the enzymatic activity of invertases may also influence the activity of fructan metabolism enzymes through the control of sucrose concentrations within cells.

Three enzymes involved in fructan metabolism are believed to play a role in determination of fructan DP. In barley, 1-SST has been suggested to act as a pacemaker enzyme in controlling flow of carbon into fructan synthesis (Khoshro et al., 2014). The pacemaker function of 1-SST likely controls rates of fructan synthesis and may control fructan DP. Fructan patterns in Asteraceae species have been linked to species-specific 1-FFT and 1-FEH activities (Hellwege et al., 1998; Itaya et al., 2007). Factors related to the physiological and metabolic state of the cell, such as substrate availability and energy status, may also influence the DP of fructans produced.

Several enzymes involved in fructan metabolism have been isolated from wheat. The fructan synthesis enzymes 1-SST, 6-SFT, and 1-FFT have been isolated from vegetative tissues in wheat (Kawakami and Yoshida, 2002; Gao et al., 2010). Numerous FEH enzymes have been identified in wheat stems, including 1-FEH and 6-FEH (Van den Ende et al., 2003; Van Riet et al., 2006, 2008). In addition to a wheat 6-FEH known to act on bacterial levan, phlein, and 6-Ketose in vitro, two 6-FEH isozymes exhibiting substrate specificity for 6-K from fructans have also been identified and cloned from cold-hardened wheat crown tissue (Van Riet et al., 2006).

Thus far, the activities of all 6-FEH enzymes isolated from wheat are not directly modulated by the presence of sucrose. Further research is needed to fully understand the role of sucrose-insensitive 6-FEH isozymes in wheat plants. Given the large number of fructan metabolism enzymes present in wheat, it is reasonable to assume that fructan synthesis and degradation occurs similarly to the processes described above. In vitro assays of wheat kernel extracts confirm the presence and activity of all fructan synthetic enzymes previously found in wheat vegetative tissues (Verspreet et al., 2013a). Additionally, the in vitro assays indicated the presence of 6G-FFT and FEH enzymes in wheat kernels (Verspreet et al., 2013a, 2015b).

Fructan Quantification

Early fructan quantification techniques provided measurements of total fructan content through spectrophotometric quantification of fructose and glucose. With the advancement of liquid chromatography and mass spectrometry technologies, it has become possible to identify and quantify different types of fructans.

For all fructan quantification techniques, the first step is extraction of carbohydrates from the sample. Wheat samples are ground with mills, coffee grinders, homogenizers, or liquid nitrogen and are dissolved in hot water or hot ethanol solutions to extract fructans and degrade endogenous enzymes (Jenkins et al., 2002; Haskå et al., 2008; Verspreet et al., 2012). Dissolution of fructans in hot water or ethanol allows for enhanced solubility of fructans (ethanol- and water-soluble), and gelatinization of starch which occurs at 80°C (Charley, 1998; Huynh et al., 2008). To extract higher DP fructans, an additional water extraction step may be required if the

initial dissolution was performed with ethanol (Haskå et al., 2008). Filtration or centrifugation of the fructan extract aids in further removal of gelatinized starch (Huynh et al., 2008). Supernatant from the carbohydrate extraction process can be collected and further analyzed using fructan measurement techniques. Currently, there are two widely accepted methods available for use in quantification of fructans.

The first approved fructan quantification method is the enzymatic–spectrophotometric method (AOAC 999.03) (McCleary et al., 2000). This method quantifies fructan content of samples through the colorimetric measurement of reducing sugars resulting from the enzymatic digestion of fructans (i.e., inulin and graminan) by inulinases (McCleary and Blakeney, 1999). With relatively basic lab equipment, this method can provide a quantification of total fructan content but does not provide information on the DP or types of fructan structures found in samples.

The second approved fructan quantification method is the ion-exchange chromatographic method (AOAC 997.08) (Hoebregs, 1997). Sugars released by enzymatic digestion of fructans by inulinases are determined by various ion exchange chromatography technologies. The adaptation of Huynh et al. (2008) facilitated a larger number of samples to be measured and allowed for the calculation of the DP_{av} of fructans contained in a sample under the assumption that every fructan molecule contains a terminal glucose molecule. Verspreet et al. (2012) further optimized the method of fructan hydrolysis with a mild acid treatment in place of inulinase hydrolysis. The acid hydrolysis of fructans allowed for more accurate calculation of fructan DP_{av} by determining the true amount of glucose released from fructan molecules in samples (Verspreet et al., 2012).

Wheat contains several carbohydrates such as starch, sucrose, and various galacto-oligosaccharides that can cause overestimation of fructan content if not controlled in samples (Kuo et al., 1988; Henry and Saini, 1989). Each quantification method controls for the presence of interfering sugars differently. In the spectrophotometric method, a mixture of α-galactosidase, sucrase, and amylase enzymes are added to samples to facilitate degradation of raffinose, sucrose, and starch found in wheat prior to fructan hydrolysis (Andersen and Sorensen, 1999; McCleary et al., 2000; Huynh et al., 2008). All reducing sugars present in the sample after digestion of potentially interfering sugars are reduced to sugar alcohols that will not interact with the color complex used to quantify reducing sugars in the colorimetric reaction (McCleary et al., 2000). In the ion-exchange chromatographic method, initial chromatograms containing quantification information for the sugars released from the digestion of starch, raffinose, and maltodextrins are produced for each sample prior to fructan hydrolysis (Hoebregs, 1997; Verspreet et al., 2012). Fructan content measured in the ion-exchange method is then determined by subtracting the amount of fructose and glucose present after fructan hydrolysis from the initial chromatogram measurements (Hoebregs, 1997).

Most recently, protocols for liquid chromatography–mass spectrometry (LC-MS) have been developed (Verspreet et al., 2014; Liu and Rochfort, 2015). The LC-MS technology has allowed for the identification of fructan structures in developing and mature wheat grains (Verspreet et al., 2015b). The use of LC-MS will greatly improve understanding of fructan structures within plants and can be used in the future to differentiate wheat varieties on the basis of kernel fructan composition.

The quantification of fructans can be accomplished by either the spectrophotometric or ion-exchange chromatography method. While both are widely accepted, the choice of methodology depends on the type of information sought on fructans present in samples, sample type, project timeline, equipment accessibility, and materials budget. Regardless of methodology, it is essential that interfering sugars be taken into account when determining fructan content. Further advancement of the field of carbohydrate chemistry will increase the capacity to obtain information on plant fructans.

Fructan Functions in Wheat

In wheat, fructans are reserve carbohydrates that are involved in many physiological functions. Fructans are initially synthesized from sucrose in vacuoles of wheat vegetative tissue cells through the action of the fructan synthetic enzymes 1-FFT, 1-SST, and 6-SFT (Fig. 2.2) (Schnyder et al., 1993; Vijn and Smeekens, 1999). In particular, graminan-type fructans are the predominant storage carbohydrates in these tissues (Van den Ende et al., 2005). Fructan molecules remain in cell vacuoles until the component sugars are needed elsewhere within the wheat plant. Since fructose is not believed to be phloem mobile, the constituent groups of fructans must be transported through the phloem in a more mobile form. After anthesis, stored fructan molecules in stems and leaf sheaths are hydrolyzed by FEH enzymes to free sugars such as glucose and fructose, which are subsequently converted into sucrose by sucrose phosphate synthase and sucrose 6-phosphate phosphatase (Fig. 2.2) (Schnyder et al., 1993; Joudi et al., 2012). The resulting sucrose is transported between

vegetative tissues and developing kernels via the phloem (Fig. 2.2) (Fisher and Gifford, 1986; Komor, 2000). Once in the developing kernel, phloem-transported sucrose molecules are reincorporated into fructan molecules (Schnyder et al., 1993). Fructan concentration within the developing and maturing wheat kernel is known to change through the three kernel development stages.

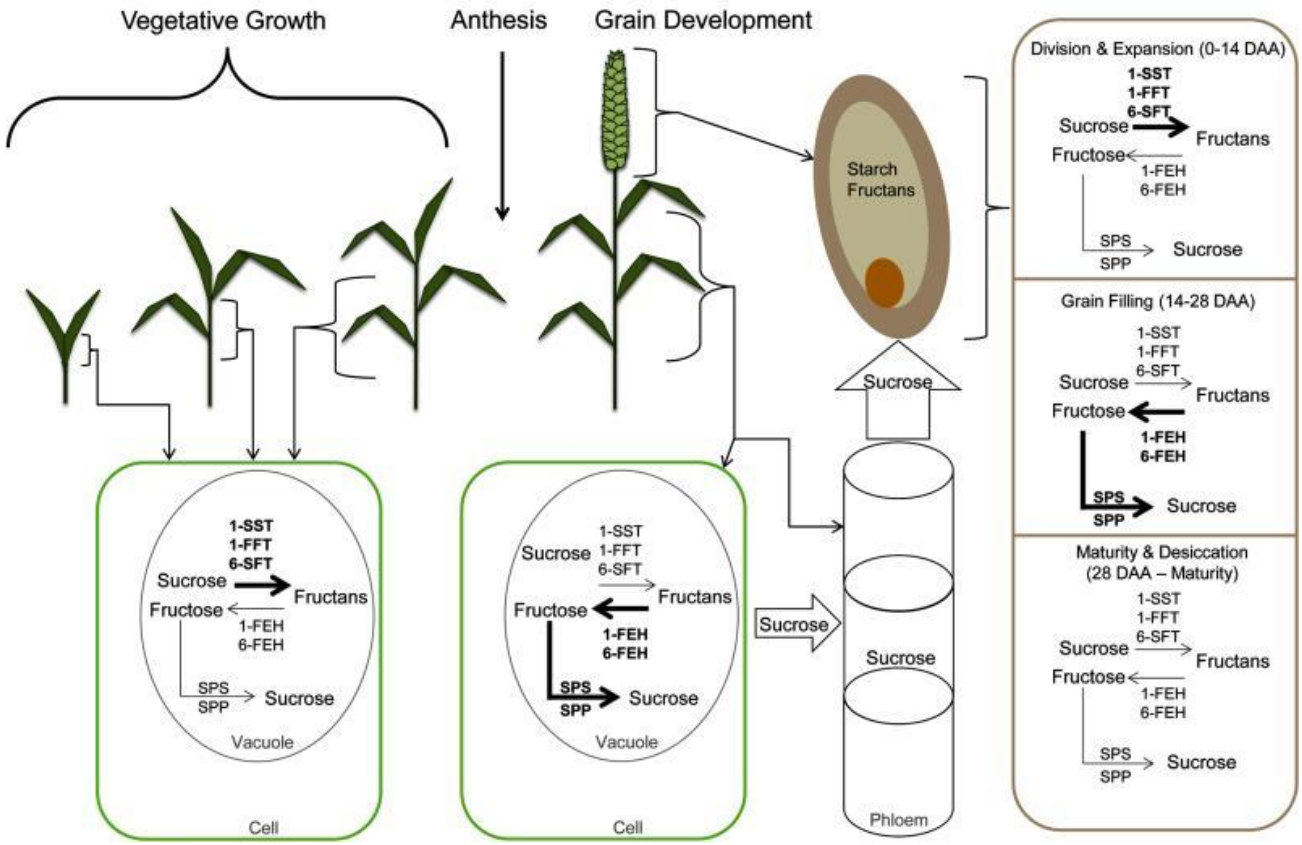

Figure 2.2: Fructan storage, metabolism, and transport through the life stages of wheat plants. 1-SST, sucrose:sucrose 1-fructosyltransferase; 6-SFT, sucrose:fructan 6-fructosyltransferase; 1-FFT, fructan:fructan 1-fructosyltransferase; 1-FEH, exo-inulinase; 6-FEH, levanase; DAA, days after anthesis; SPS, sucrose phosphate synthase; SPP, sucrose 6-phosphate phosphatase.

The first phase of kernel development is known as the cell division and expansion or kernel enlargement phase (Jenner et al., 1991; Shewry et al., 2012). This

phase lasts ~2 wk postanthesis and is a time of rapid water deposition into the kernel (Sofield et al., 1977; Pepler et al., 2006; Verspreet et al., 2013a). Fructan accumulation mainly occurs during this phase, with the majority of high-DP fructans being deposited by 5 d after anthesis in the outer pericarp (Schnyder et al., 1993; Verspreet et al., 2013a). More than 5d postanthesis, fructan accumulation predominantly occurs in the endosperm, inner pericarp and testa (Schnyder et al., 1993). During this phase, fructans can represent up to one third of the dry weight in immature kernels under normal, nonstress growing conditions (Nardi and Calcagno, 2003; Paradiso et al., 2008). In vitro assays of developing wheat kernel extracts have indicated high levels of enzymatic activity for 1-SST and 6-SFT and moderate 1-FFT activity (Verspreet et al., 2013a). In addition to fructosyltransferase activity, acid invertases may actively hydrolyze sucrose in developing wheat kernels to further reduce sucrose concentrations and promote fructan synthesis in developing kernels (Verspreet et al., 2013a). High rates of fructan synthesis and accumulation keep sucrose concentrations low to maintain a high glucose:sucrose ratio while controlling osmotic flow into the developing kernel (Schnyder et al., 1993; Verspreet et al., 2013a).

Approximately 2 to 3 wk postanthesis, kernel fructan concentration and DP begin to decrease (De Gara et al., 2003; Verspreet et al., 2013a). This timing corresponds with the rapid accumulation of dry matter and starch anabolism observed during the second phase of kernel development. A spike in sucrose concentration in the developing kernel also marks the transition from endosperm cell proliferation to starch accumulation (Sabelli and Larkins, 2009). In vitro assays have shown a rapid decrease in enzymatic activity for fructan synthetic enzymes and an increase in

activity of fructan degradative enzymes (Verspreet et al., 2013a). During this phase, the majority of fructan degradation occurs in maternal grain parts, particularly the outer pericarp (Schnyder et al., 1993). The observed shifts in enzymatic activity correspond with the observed decrease in wheat kernel fructan concentration and increase in sucrose concentrations.

Fructan concentration and structures in maturing wheat kernels remain unchanged during the third phase of development (Verspreet et al., 2013a). The only notable change occurring during the maturation and desiccation phase of wheat kernels is the accumulation of raffinose, which is believed to confer desiccation tolerance to the kernel by preventing sugar crystallization (Chen and Burris, 1990; Blackig et al., 1996; Taji et al., 2002; Verspreet et al., 2013a).

Despite the observed decrease in kernel fructan content during kernel development and maturation, fructans are still present at detectable levels in the mature seed (0.7–2.9 g 100 g^{-1} dry wt.) (Huynh et al., 2008). The stability of fructans within mature wheat kernels is currently unknown; however, there are several pieces of evidence suggesting that fructans are fairly stable. The in vitro activity of fructan hydrolyzing enzymes in mature wheat kernel extracts has been observed to be low (Verspreet et al., 2013a), yet messenger RNA (mRNA) for fructan-hydrolyzing enzymes has been detected in mature durum wheat kernels (*Triticum durum* Desf.) (Cimini et al., 2015). Although further research is needed to confirm the presence of FEH mRNAs in mature wheat kernels, the presence of FEH-encoding mRNA in mature durum kernels suggests that fructans contained in mature wheat kernels may provide energy during early stages of wheat seedling germination (Cimini et al.,

2015). Despite the in vitro evidence suggesting relative stability of fructans in mature wheat kernels, it is still possible that fructans may be subjected to postharvest degradation. Postharvest factors such as storage conditions, seed dormancy, time, and possible presence of microorganisms and FEH mRNAs may have a long-term impact on fructan stability in mature kernels (Pollock and Cairns, 1991; Noureddine and Norio, 2006; Merry et al., 2008; Huynh et al., 2008).

The primary role of fructans in wheat vegetative tissues is to serve as a reserve carbohydrate. The exact reason why the reserve carbohydrate of choice for wheat is fructans, and not starch, is not entirely understood; however, it is theorized that fructans serve as a form of osmotic control mechanism in times of limited water availability (Hendry, 1993). According to the fossil record, fructan-containing plants flourished during eras when water availability was low (Hendry, 1993). Under normal conditions, a steady supply of carbohydrates for grain filling are provided by green tissue photoassimilate and remobilization of stored reserves in vegetative tissues (Ehdaie et al., 2006). Under water-stress conditions, particularly drought, remobilization of fructans from vegetative tissues to developing wheat kernels is extremely important due to limited availability of photosynthate (Joudi et al., 2012). Fructans located in wheat stems are among the most commonly stored carbohydrates that subsequently contribute to wheat grain development through sugar remobilization (Kühbauch and Thome, 1989). Depending on environment and genotype, 6 to 100% of wheat grain yield can be attributed to stem reserve carbohydrates (Borrell, 1993; Blum et al., 1994). When drought stress is present, proportionately more stem reserve

carbohydrates are remobilized to developing wheat grains (Palta et al., 1994; Yang et al., 2000).

In addition to drought stress, fructan molecules serve as osmoregulators under cold and salt stress (Pilon-Smits et al., 1995). Alterations in soluble sugar composition are often associated with increased cold hardiness in a variety of plant species, including wheat (Kawakami and Yoshida, 2002; Van den Ende et al., 2003; Gibson, 2005). Upregulation of enzymes involved in fructan synthesis (1-FFT, 1 SST, and 6-SFT) results in fructan accumulation in various wheat tissues (Bancal et al., 1992; Kawakami and Yoshida, 2005). Prolonged cold exposure results in the accumulation of inulin-type fructans, with fructans composing 10% or more of winter wheat crown tissue weight (Suzuki and Nass, 1988; Yoshida et al., 1998). At low temperatures, the induction of fructan synthesis is believed to be indirectly stimulated by increasing sucrose concentrations due to lower carbon use (Pollock, 1990). In addition to cold tolerance, fructan accumulation also has been observed to confer resistance to snow mold [caused by *Microdochium nivale* (Fr.:Fr.) Samuels & I. C. Hallett, *Typhula incarnata* Lasch. ex Fr., or *Typhula ishikariensis* S. Imai] in regions where it poses a threat to plant viability (Yukawa and Watanabe, 1991; Yoshida et al., 1998). Notable fructan accumulation has also been observed in seedlings, leaves, roots, and maturing seeds of salt-tolerant wheat varieties (Kerepesi and Galiba, 2000; Kafi et al., 2003).

Cellular membranes likely experience significant stress on exposure to low temperatures or osmotic stress. Fructan polymers are believed to help maintain membrane integrity and stability, particularly in times of thermic stress and drought, through insertion of a portion of the fructan polysaccharide into the polar surfaces of

membranes (Livingston et al., 2009). The DP of fructans in the membranes of cold-tolerant cereals can range greatly in vivo; thus, it is hypothesized that a mixture of higher- and lower-DP fructans may provide optimal membrane stability (Suzuki and Nass, 1988; Valluru and Van den Ende, 2008; Livingston et al., 2009). Although fructans are synthesized and stored in the vacuole, fructans and FEHs have been located in the apoplast (Van den Ende et al., 2005). The origin of fructans and FEHs present in the apoplast and plasma membrane is unknown, although it is hypothesized that vacuolar fructans and FEHs are transported from the vacuole to the apoplast via exocytosis (Etxeberria et al., 2005; Van den Ende et al., 2005; Valluru and Van den Ende, 2008).

Given that fructans exhibit multiple physiological roles in wheat, it is reasonable to question whether environmental factors influence observed wheat grain fructan content. Growing conditions can vary greatly among outdoor locations, as well as indoor glasshouses and outdoors. Compared with outdoor growth, wheat kernels from plants in glasshouses have been found to contain lower concentrations of grain fructan content (1.5–2.3 vs. 0.7–1.6%, respectively) (Huynh et al., 2008). Vegetative growth conditions in glasshouses are often not resource limited, thereby reducing the necessity for extensive fructan remobilization into developing grains (van Herwaarden et al., 2003; Ruuska and Rebetzke, 2006). Accumulation of water-soluble carbohydrates is decreased by shading, and the lower levels of light exposure in glasshouses may reduce levels of water-soluble carbohydrates in the developing plants, thereby explaining the lower grain fructan content for wheat varieties grown in glasshouses (Kiniry, 1993). Despite the lower observed grain fructan content indoors,

grain fructan contents for varieties grown in glasshouses and outdoors are positively correlated (r = 0.83) (Huynh et al., 2008). Grain fructan content of field-grown wheat has been examined in several studies (Table 2.1), with total dry matter content ranging between 0.7 and 2.9% (Huynh et al., 2008). Various factors such as seeding date and agronomic management practices can affect fructan accumulation in wheat (D'Egidio et al., 1997; Gaudet et al., 2001). Fructan metabolism also varies with environmental conditions such as soil type, growing season temperature, and rainfall or irrigation (D'Egidio et al., 1997).

Table 2.1: Grain fructan content for field-grown wheat reported in the literature.

Fructan Range (g/100g)	Average Fructan Content	Number of Genotypes	All Genotypes Grown Together	Location	Source
0.7-2.9	-	62	No	Australia	(Huynh et al., 2008)
1.5-2.3	-	19	Yes	Australia	(Huynh et al., 2008)
0.84-1.85	1.28	129	Yes	Hungary	(Andersson et al., 2013)
2.2-2.3	-	2	-	Sweden	(Haskå et al., 2008)
0.9-1.8	1.4	5	-	-	(Fretzdorff and Welge, 2003a)

It is clearly established that environmental factors such as temperature, light exposure, and resource availability influence wheat grain fructan content. If the effects of environmental factors on wheat grain fructan content vary among the wheat genotypes grown in multiple environments, a genotype-by-environment (G × E) interaction is said to exist. Strong G × E interactions for cereal grain fructan content

have been detected (Karppinen and Myllymäki, 2003; Haskå et al., 2008), but not consistently (Huynh et al., 2008).

Health Benefits of Fructans

Fructans consumed in the diet are believed to support a variety of health benefits in humans. The absence of human enzymes capable of hydrolyzing β linkages allows β-linked dietary fructans to pass through the upper gastrointestinal tract without undergoing digestion. Upon arrival in the large intestine, undigested or incompletely digested fructans are fermented by specific bacterial species located in the gut. *Bifidobacteria* and *Lactobacillus* selectively ferment and use fructans to support bacterial growth and proliferation in the large intestine (Gibson et al., 1995; Crittenden and Playne, 1996; Kaplan and Hutkins, 2000; Roberfroid, 2005). *Bifidobacterium* and *Lactobacillus* species are also capable of producing antibacterial substances that can inhibit the growth and survival of pathogens (Gibson and Wang, 1994). Of the five classes of fructans, inulins and their impacts on health have been the most widely studied. Due to the nondigestible nature of inulin molecules and the stimulatory effects inulins exhibit on *Bifidobacteria* and *Lactobacillus*, microorganisms that are considered to be health promoting and beneficial for the host, inulin-type fructans are classified as prebiotics (Gibson et al., 1995; Rao, 1999; Roberfroid, 2005; Macfarlane et al., 2006). Inulins are also widely considered as dietary fiber (Roberfroid, 2005). The prebiotic and fibrous nature of fructans further promotes gut health through the promotion of regularity, particularly through increased stool frequency (Gibson and Roberfroid, 1995; Kleessen et al., 1997; Roberfroid, 2007).

In addition to promotion of healthy bacteria and suppression of harmful bacteria and pathogens, inulin-type fructans are believed to play a role in supporting host immune function by providing substrates for gut bacteria short chain fatty acid (SCFA) production in the gut (Gibson et al., 1995). The resulting SCFAs function in immune signaling and acidification of the gut to kill pathogens (Gibson and Wang, 1994; Blaut, 2002; Brown et al., 2003; Le Poul et al., 2003; Nilsson et al., 2003; Sanderson, 2007). A comprehensive review of inulin-type fructans and host immune function can be found in Lomax and Calder (2008). Additionally, fructan consumption and resulting SCFA production is suggested to reduce colon carcinogenesis through observed reductions in tumor incidence and formation in animals and human cell lines (Hidaka et al., 1990; Rowland et al., 1998; Pool-Zobel et al., 2002; Pool-Zobel, 2005). A key function of inulin-type fructans and SCFAs in counteracting colon carcinogenesis is their ability to induce apoptosis of colonic cells carrying DNA mutations (Di Bartolomeo et al., 2013).

Inulin-type fructans confer physiological effects on the host beyond promotion of gut health. Inulin-type fructan consumption increases calcium bioavailability in adolescents and postmenopausal women (Coudray et al., 1997; Abrams et al., 2005, 2007). Increased calcium availability resulting from dietary inulins supports bone health, particularly in human adolescents (as reviewed in Bosscher et al., 2006). Preliminary research in murine models also suggests that inulin-type fructans may increase bioavailability of copper, iron, magnesium, and zinc (Raschka and Daniel, 2005; Coudray et al., 2006; Lobo et al., 2009); however, these impacts have not been observed in humans (Coudray et al., 1997). Dietary inulins have been shown to

promote satiety hormone production (Cani et al., 2009) and weight loss in obese individuals (Parnell and Reimer, 2009).

While many of these health impacts are observed throughout the body, inulin-type fructan consumption likely indirectly modulates many of these observed outcomes through improvement of overall gut health. Many studies examining fructan function have specifically studied the health effects of inulin; however, in vitro evidence suggests that all types of fructans, particularly inulin and graminan types, exhibit similar effects in humans (Roberfroid, 2005; Jenkins et al., 2011). Further research is needed to determine if all types of fructans support most of the aforementioned health benefits in vivo.

Fructan consumption has been shown to confer a wide range of health benefits to humans. Within the gut, intestinal microflora rapidly ferment fructans and produce SCFAs, hydrogen gas, carbon dioxide, and occasionally methane as byproducts (Stone-Dorshow and Levitt, 1987; Rumessen and Gudmand-Høyer, 1998). Intestinal regularity and flatulence resulting from consumption of fructans and other types of dietary fiber is considered healthy; however, the boundary between wanted and unwanted fructan-stimulated gastrointestinal effects is unclear and likely varies among individuals. The point at which fructan consumption produces unwanted gastrointestinal discomfort often corresponds with the line between healthy bowel activity and irritable bowel syndrome (IBS) (Macfarlane et al., 2006). Several studies have suggested that ingestion of fructans is associated with dose-dependent gastrointestinal symptoms when added to diets (Cummings and Macfarlane, 2002). Accordingly, high rates of fructan consumption are strongly discouraged for

individuals who suffer from IBS. It remains to be seen if benefits resulting from stimulation of *Bifidobacterium* growth in the gut associated with low levels of fructan consumption would outweigh the gastrointestinal discomfort associated with fructan consumption (Roberfroid et al., 2010). The connection between fructan consumption and IBS has also led to suggestions that non-celiac gluten sensitivity (NCGS) and its associated symptoms of gastrointestinal discomfort may be caused by fructans, not gluten proteins, as higher levels of fructans are often present in gluten-containing cereals and cereal products (Biesiekierski et al., 2013). Further examination of the underlying causes of NCGS is needed to fully determine if wheat fructans or gluten are the causative agents in NCGS (Biesiekierski and Iven, 2015).

Fructans in Food Products

Fructans are present in many fruits (banana, melons, and peaches), vegetables (artichoke, broccoli, garlic, and onions), and cereals (rye and wheat) (Nelson and Smith, 1986; Roberfroid, 2005; Muir et al., 2007; Fedewa and Rao, 2014). In addition to whole foods, fructans are also used as additives in many processed food products to increase fiber content or decrease caloric density through replacement of fat or sugar (Barclay et al., 2010). The average daily fructan consumption is estimated to be 1 to 4 g in the United States and 3 to 11 g in Europe (Van Loo et al., 1995). While fructans are present in a wide range of foods and food products, the main source of fructans in the American diet is wheat (Van Loo et al., 1995).

Though wheat is lower in fructan content than other foods, the abundance of wheat in American diets results in wheat providing ~70% of dietary fructans (Van

Loo et al., 1995; Moshfegh et al., 1999). Wheat grains and flour serve as key ingredients in many food products such as baked goods, frozen meals, and sauces. The addition of wheat ingredients to these products contributes to the total daily fructan consumption; however, the reported amounts of fructans in raw wheat ingredients may not accurately reflect the amount remaining in processed foods (Table 2.2). Fructan stability through processing is primarily affected by two main factors, yeast-mediated fermentation and heat exposure.

Table 2.2: Fructan content of wheat-based processed food products.

Product	**Location**	**Fructan (g/100g)**	**Source**
Bread (White)	Australia	0.68	(Biesiekierski et al., 2011)
Bread (White)	United Kingdom	0.86	(Whelan et al., 2011)
Bread (Whole grain)	Australia	0.69	(Biesiekierski et al., 2011)
Breakfast Cereal (Whole grain)	-	0.8-3.2	(Shepherd and Gibson, 2006)
Crackers	-	0.8-3.4	(Shepherd and Gibson, 2006)
Pasta (Wheat)	Australia	0.34	(Biesiekierski et al., 2011)
Pretzels	Australia	1.4	(Biesiekierski et al., 2011)

The addition of yeast to wheat bread products results in products with lower fructan levels relative to the original wheat flour and air-leavened, nonyeasted wheat bread (Verspreet et al., 2013b). Yeast invertase has been shown to hydrolyze fructans present in the dough during fermentation (Nilsson et al., 1987). The amount of fructan degradation during fermentation depends on fructan DP and the strain of yeast used. Yeast has a preference for lower-DP fructans and likely first degrades the smallest fructan molecules during the bread production process (Nilsson et al., 1987). Use of yeast strains with lower yeast invertase activity have been shown to reduce fructan degradation in bread production (Verspreet et al., 2013b). Observed decreases in fructan concentration in yeast-leavened breads range from 50 to 78% depending on wheat genotype, fructan composition, and yeast strain (Nilsson et al., 1987; Verspreet et al., 2013b). In addition to degradation of fructans in baked yeast-leavened wheat products, yeast-mediated fermentation in beer brewing reduced >90% of fructans initially present in raw ingredients (Krahl et al., 2009).

Fructan content of wheat products may also be affected by heat. Reductions in fructan content of 10 to 20% have been observed during the baking (210°C for 60 min) of yeast-leavened wheat bread (Fretzdorff and Welge, 2003a), and fructan content for wheat pasta was found to decrease by 50% when cooked in hot water (Gélinas et al., 2015). Fructan molecules with a higher DP are more resistant to degradation during the baking process (Praznik et al., 2002). Additionally, shorter baking times for wheat bread may result in a reduction of fructan loss (Verspreet et al., 2013b). Studies comparing fructan degradation in bread production have concluded that yeast has a more significant role in fructan degradation than heat exposure.

Whole wheat grains are becoming increasingly popular among consumers and are often consumed in various dishes. The impacts of cooking whole wheat grains on components such as fructans has not been widely studied, but the presence of the seed coat on whole grains may decrease the observed leaching effect seen with fructans in pasta and maintain a fairly high concentration of fructans in the grains.

Breeding Prospects

Wheat fructans exhibit many desirable physiological impacts on humans and plants. Increasing fructans in wheat vegetative tissues and grains will help plants withstand environmental stresses such as cold temperatures, salinity, and drought and aid in preventing yield losses under such conditions. Increased wheat grain fructan content will result in higher fructan wheat flour and may confer early vigor to young wheat seedlings by providing non-starch carbohydrate stores during germination and initial growth stages. Products made with high-fructan wheat flour, even with exposure to yeast fermentation and heat, will likely contain higher levels of fructans that will further increase fructan-mediated health benefits for consumers. Quantification has shown that genotypic variation and moderate heritability (h^2 = 0.64–0.94) exist for wheat grain fructans (Huynh et al., 2008). The variation, moderate heritability, and beneficial impacts of fructans allow wheat grain fructan content to be a desirable breeding target for development of improved wheat varieties.

Implementing selection for higher grain fructan content in wheat breeding programs is possible with several different breeding methodologies. Phenotypic selection for fructans is possible, as content can be quantified with any

abovementioned method; however, measuring fructan content is a destructive process that is time and labor intensive. Development of a higher-throughput phenotyping method would greatly reduce time and effort required for phenotyping wheat grain fructan content. Using marker-assisted selection (MAS) to select for fructan quantitative trait loci (QTLs) is a cost-efficient breeding method that can be easily implemented. Quantitative trait loci for wheat grain fructans have been detected on chromosomes 2B, 3B, 5A, 6D, and 7A (Huynh and Wallwork, 2008). The QTLs located on chromosomes 6D and 7A explain the largest amounts of phenotypic variance observed (17 and 27%, respectively) (Huynh and Wallwork, 2008). Prior to implementing MAS, it is recommended that populations be screened for previously discovered fructan QTLs, as expression and effects of QTLs are not consistently found in all genetic backgrounds.

Newer breeding methods such as genomic selection (GS) or genetic engineering (GE) are also possible options in breeding for wheat grain fructans. Genomic selection will decrease phenotyping costs and requirements, accelerate breeding cycles, facilitate collection of genotypes for breeding materials, and allow for selection on the entire genome (Heffner et al., 2009). Prior to implementing GS, it is recommended that training population design be optimized to fully realize potential gains from selection (Lorenz et al., 2011; Isidro et al., 2015). Using GE to modulate fructan metabolism during plant growth and grain development in wheat is another breeding option (as reviewed in Verspreet et al., 2015a). Changing the balance of enzyme activity to promote upregulation of fructan synthetic enzymes (1-SST, 1-SFT, 6-SFT, and 6G-FFT) and downregulation of fructan hydrolytic enzymes (1-FEH and

6-FEH) in grain would likely result in the most rapid increases in wheat grain fructan content. While using GE for increasing wheat grain content could be an efficient method, other traditional plant breeding methods would likely result in comparable genetic gain over time.

Incorporating fructans as a breeding target into wheat breeding programs does come with potential challenges. Phenotyping costs and equipment access will largely determine the end goals of fructan breeding. Total fructan content can be selected using the spectrophotometric methodology, whereas more advanced technology such as LC-MS is needed to directly breed for targeted grain fructan composition. With further advancement of high-throughput phenotyping technologies, breeders will be able to incorporate goals of increasing wheat kernel fructan DP. Given that low-DP fructans (DP < 5) currently comprise a substantial proportion (>50%) of wheat kernels (Dahlqvist and Nilsson, 1984), breeding for increased DP will result in wheat fructans that can better withstand yeast-mediated fermentation. While the existence of linkage drag or pleiotropy associated with genes controlling fructan accumulation is poorly understood, it is possible that selection for increased wheat grain fructan content may result in unintentional selection for other traits associated with fructan accumulation. Wheat varieties with increased grain fructan content will need to be evaluated to determine if unintentional selection will result in any potential tradeoffs in agronomic qualities, yield, or other plant characteristics. The presence of G × E will also affect observed wheat grain phenotypes and influence the effectiveness of selection methodologies. Further research on the effects of G × E on wheat grain fructans will allow for strategic design of fructan breeding and evaluation protocols to fully realize

potential gains from selection. Despite these challenges, fructans are still a desirable breeding target for development of high-yielding, climate-resilient wheat varieties.

Conclusion

Wheat fructans are a structurally diverse set of oligomers that fulfill essential roles in plant and human health. Quantification of fructans is easily accomplished, although further advancement of quantification technologies will provide information on complete fructan profiles in wheat tissues and grains. Given the multiple physiological roles served by fructans in wheat, and their impact on grain yield, developing breeding targets for increased fructan content will align well with preexisting breeding goals for yield. Developing wheat varieties with higher fructan levels may result in more resilient wheat plants with greater adaptation to cold temperatures, droughts, and highly saline soils.

While processed food products already contain fructans from wheat ingredients, increasing total fructans and fructan DP in wheat grains through breeding will result in high-fructan varieties that facilitate the development of fructan-rich processed food. An increase in fructan content in whole wheat grains and processed foods will likely result in increased daily consumption of fructans, further stimulating the abovementioned health benefits. The modulation of gut health through fructan consumption will become more important as the connections between gut microbiome composition and human health are further elucidated. Wheat grain fructans will not be the sole solution to the obesity epidemic; however, these molecules hold great potential for maintaining a healthy world population.

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CHAPTER 3

INFLUENCE OF GENOTYPE AND ENVIRONMENT ON WHEAT GRAIN FRUCTAN CONTENT[3]

Abstract

Fructans are naturally occurring plant polymers composed of fructose molecules. Approximately 15% of flowering plant species contain fructans, including wheat. In plants, fructans serve as carbon stores and as a potential form of protection against water deficit. In addition to serving valuable roles in plant growth and development, the characteristics of fructans have potentially beneficial effects on human health. Genotypic variation for inulin content, a specific type of fructan, ranges from 0.4 to 2% in wheat seeds. The purpose of this study was to examine the effects of genotype and environment on in winter wheat grain fructan content. Total grain fructan content was determined for 288 winter wheat genotypes grown over two years with three locations per year. The factors significantly influencing the observed variation in wheat grain fructan were genotype, environment, and genotype x environment interactions. This study found significant genotype x environment interactions for wheat grain fructan content; however, the contributions to overall variation were small. The results of this study will be useful for implementing recurrent genomic selection in winter wheat and guiding future decisions regarding

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breeding methodologies for total fructan content in wheat. A greater understanding of the effects of genotype and environment on fructan content will have implications for breeders seeking to develop nutritionally improved, climate resilient wheat varieties.

Abbreviations

ANOVA: Analysis of Variance; AMMI: Additive Main Effects and Multiplicative Analysis; C: Caldwell; FDR: False discovery rate; G: Genotype; GxE: Genotype-by-Environment; GBS: Genotyping-by-Sequencing; GEBV: Genomic estimated breeding value; GS: Genomic selection; GWAS: Genome wide association study; E: Environment; H: Helfer; H^2: Broad sense heritability; h^2: Narrow sense heritability; IPC: Interaction Principal Component; K: Ketola; MET: Multi-Environment Trial; PCR: Polymerase Chain Reaction; QTL: Quantitative trait locus; SNPs: Single Nucleotide Polymorphisms; SS: sum of squares

Introduction

Wheat (*Triticum aestivum* L.) is a widely consumed staple crop that provides essential calories and nutrients in diets worldwide. The rising demand for climate resilient, nutritionally improved crops is beginning to drive breeding goals in several crops. One component of wheat, fructans, is of particular interest with respect to developing nutritionally improved, climate resilient wheat varieties.

Fructans are carbohydrate polymers composed primarily of β-linked fructose molecules found in approximately 15% of plant species worldwide, including wheat (Hendry, 1993). Fructans serve many essential functions in plant and human physiology. Plant fructans act as osmoregulators that confer cold, drought and salt tolerance (as reviewed in Hendry, 1993; Joudi et al., 2012; Kafi, Stewart, & Borland, 2003; Pilon-Smits et al., 1995). Human consumption of fructans, particularly inulin-

type fructans, stimulates growth of healthy gut bacteria and supports overall gut health (as reviewed in Di Bartolomeo, Startek, & Van den Ende, 2013; Peshev & Van Den Ende, 2014; Roberfroid et al., 2010). Wheat serves as the primary source of fructans in the American diet on account of the volume of wheat based products consumed (van Loo, Coussement, de Leenheer, Hoebregs, & Smits, 1995).

Wheat grain fructan content has not been an area of focus for wheat breeding programs though genotypic variation for wheat grain fructan content is known to exist (Huynh, Palmer, & Mather, 2008). Grain fructan content may be influenced by environment as fructans exhibit various physiological roles within plants, especially in times of osmotic stress (Haskå, Nyman, & Andersson, 2008; Huynh & Wallwork, 2008; Karppinen & Liukkonen, 2000). Given the importance of fructans in plant growth responses to environmental conditions and the existence of genotypic variation, an important consideration in breeding for wheat grain fructan content is the existence and extent of genotype-by-environment (GxE) interactions. GxE has been observed for wheat grain fructan content (Haskå et al., 2008), but not consistently (Huynh et al., 2008).

Gaining a better understanding of GxE interactions for grain fructan content can help effectively guide breeding efforts for developing high fructan varieties while allowing for proper resource allocation within breeding programs. If there is a large effect of GxE, breeding for wheat grain fructan content will require extensive allocation of resources for testing and phenotyping of materials in multi-environment trials (METs) to ensure the development of robust high fructan varieties. If GxE effects are minimal, the development of high fructan varieties can be accomplished

with marker based breeding technologies such as marker assisted selection (MAS) or genomic selection (GS) in the greenhouse or in a small number of field locations. Utilizing GS to breed for wheat grain fructan content is of particular interest due to the reduced phenotyping costs and increased gain per unit time for selection (as reviewed in Veenstra, Jannink, & Sorrells, 2017).

The development of nutritionally improved, climate resilient wheat varieties can be accomplished by breeding for increased wheat grain fructan content. It is crucial to understand how GxE interactions impact traits of interest as these interactions are difficult to control and can hamper breeding efforts. To investigate the nature and magnitude of GxE interactions for wheat grain fructan content, a multi-year, multi-location field experiment containing 288 winter wheat genotypes was performed. The objectives of this multi-year, multi-location study were to estimate GxE effects on wheat grain fructan content, to determine the magnitude of GxE relative to other factors contributing to phenotypic variation, and to assess the feasibility of utilizing GS for grain fructan content in the genotypes evaluated in this study.

Materials and Methods

Germplasm

The Cornell Small Grains Breeding Program (Ithaca, NY) actively maintains a Master collection of 1290 soft winter wheat lines consisting of elite, F_5-derived advanced breeding lines and varieties. A subset of 288 lines from the Master collection were randomly selected to be grown in a multi-location, multi-year trial to

evaluate grain fructan content.

Genotypic Data

All lines were genotyped using genotyping-by-sequencing (GBS) as described by Poland et al. (2012) using the P384A adaptor set. In brief, following DNA co-digestion by *PstI* and *MspI* restriction enzymes, barcoded adaptors were ligated to genotype samples and resulting fragments were amplified by polymerase chain reaction (PCR) to create libraries. The resulting libraries were pooled by plate to 95-plex and sequenced on a single lane of Illumina HiSeq2000. Detailed protocols for the GBS methodology can be found in Poland et al. (2012) and the latest updates on the GBS approaches and protocols can be found on the website (http://www.wheatgenetics.org/research/genotyping-by-sequencing).

The sequence reads were processed to remove sequencing errors and identify single nucleotide polymorphisms (SNPs) with TASSEL-GBS (Glaubitz et al., 2014) and the IWGSC RefSeq v1.0 reference genome. The resulting SNPs for the entire collection of 1290 lines were filtered for marker presence and individual coverage by markers. After applying filters to retain markers with presence over 30%, a minor allele frequency above 1%, and heterozygosity under 10%, a total of 14,890 SNPs were obtained. Individuals missing over 70% of the 14,890 filtered SNPs were subsequently removed. Imputation with the simple marker mean was performed for remaining missing data points.

Field Evaluations

The 288 randomly selected lines were grown in three locations in 2014 and 2015. All trials were located on Cornell University Research Farms (Caldwell, Helfer and Ketola fields) near Ithaca, New York. The environments at each location were unique due to locally variable weather patterns and soil types. Monthly weather data for the Ithaca, New York area was obtained from annual summaries published on the Northeast Regional Climate Center (NRCC) website (http://www.nrcc.cornell.edu/wxstation/ithaca/ithaca.html).

One biological replicate of the 288 lines was planted in an unreplicated α-lattice design in each environment. Each entry within the trial was planted as a 1 meter headrow. Once physiologically mature, each headrow within the field was hand harvested and resulting grain was bulked.

Phenotypes

Phenotypic data for plant height and heading date (Julian days) were collected for the 288 lines in each environment. Total grain fructan content for each line within an environment was measured from bulked field seed in the laboratory using the enzymatic/spectrophotometric method (AOAC 999.03) (McCleary, Murphy, & Mugford, 2000).

Briefly, 3g of seed were ground in a Geno/Grinder® 2000 and 1.0g of resulting flour was dissolved in 80C water for 15 minutes. The volume of the dissolved solution was adjusted to 100mL and supernatant produced from centrifugation of solution

aliquots was stored for later analysis. All ground flour and dissolved samples were stored at -20C to minimize endogenous enzymatic degradation of fructans.

All dissolved samples were analyzed with an enzymatic assay kit (K-FRUC, MegaZyme International). The enzymatic assay protocol was adapted (downscaled to 1/5th) for 96 well plates (Newell et al., 2014) to increase throughput and reduce phenotyping costs. Sample absorbance was measured with a SpectraMax® Plus 384 Microplate Reader and total fructan content was calculated (Equation 1). To control for variation between plates, fructan content for each plate was adjusted by the controls on each plate.

Fructan (% w/w as is): $= \Delta_A \times F \times V/W \times 2.48$ (1)

Δ_A = sample absorbance – sample blank absorbance (both read against the reagent blank)
F = factor to convert absorbance values to μg of D-fructose
V = volume (mL) of extractant used (i.e. 50 or 100 mL)
W = weight (mg) of sample extracted
2.48 = unit conversion factor

Data Set

After filtering of genotypic data, 13 of the 288 lines lacked sufficient genotype data and were removed from the dataset. In total, the resulting data set contained a total of 1,650 data points for total wheat grain fructan content representing the 275 winter wheat lines grown in six environments. For environments examined, the locations were C for Caldwell, H for Helfer, and K for Ketola and the years were 2014 and 2015. Additionally, genotypic data consisting of 14,890 SNPs were available for each of the 275 lines analyzed. For each genotype, the mean, median and variance in

fructan content were computed. Pearson correlations were computed between the observed variance and genotype mean and median.

Kinship Determination

The additive genetic relationship matrix for the 275 lines was computed from the mean imputed SNPs in R (Version 3.2.2) with the A.mat function in rrBLUP (Endelman, 2011). This additive genetic relationship matrix (K) was utilized in further analyses requiring kinship information.

Heritability Estimates

The heritability of total grain fructan content was computed with variance estimates from ASREML-R (Equations 2-4; Butler, Cullis, Gilmour, & Gogel, 2009).

$$y = \mu + \mathbf{X}\beta + \mathbf{Z}g + \varepsilon \quad (2)$$

y: Vector of fructan phenotypes
X: Incidence matrix for environments
β: Vector of fixed environmental effects
Z: Incidence matrix for genotypes
g: Vector of random genotype effects
ε: Vector of residuals
$g \sim N(0, \sigma_g^2\mathbf{I})$ or $N(0, \sigma_a^2\mathbf{K})$
I: Identity matrix
K: Additive genetic covariance matrix

Broad sense heritability was computed per equation 3 and narrow sense computed per equation 4.

$$H^2 = \sigma_g^2 / \sigma_g^2 + \sigma_e^2 \quad (3)$$

$$h^2 = \sigma_a^2 / \sigma_a^2 + \sigma_e^2 \quad (4)$$

σ_g^2: Genetic variance
σ_a^2: Additive genetic variance
σ_e^2: Environmental variance

AMMI

In order to accurately assess the significance of additive and non-additive effects and their contributions to observed phenotypes in this study, an AMMI bilinear model (Equation 5) was fit on the data set described above. The model was fit with the Bilinear package in R (available at https://github.com/nsantantonio/Bilinear). To ensure optimal model diagnosis, the Bilinear package utilized parametric bootstrapping (Forkman & Piepho, 2014) to determine the appropriate number of significant multiplicative terms.

$$Y_{ge} = \mu + \alpha_g + \beta_e + \Sigma_n \lambda_n \gamma_{gn} \delta_{en} + \rho_{ge} \quad (5)$$

Y_{ge}: Fructan content of genotype *g* in environment *e*
μ: grand mean
α_g: genotype deviation from grand mean
β_e: environment deviation from grand mean
λ_n: singular value for interaction principal component $(IPC)_n$
γ_{gn}: eigenvector value for genotype *g* and interaction principal component *n*
δ_{en}: eigenvector value for environment *e* and interaction principal component *n*
ρ_{ge}: residual

To determine the proportion of total phenotypic variance explained by each model term, the sum of squares (SS) for each model term was divided by the total SS from the AMMI ANOVA.

Interaction scores for every genotype-environment combination observed in this study were computed per equation 6 and utilized to construct AMMI plots and biplots.

$$IPC_n = \lambda^{0.5}{}_n \gamma_{gn} * \lambda^{0.5}{}_n \delta_{en} \quad (6)$$

$\lambda^{0.5}{}_n$ = Singular value for interaction principal component $(IPC)_n$ scaled to 0.5
γ_{gn} = eigenvector value for genotype *g* and interaction principal component *n*
δ_{en} = eigenvector value for environment *e* and interaction principal component *n*

* λ_n is commonly scaled to 0.5 to enable direct estimation of interaction (Gauch, 1992, p.85).

Genomic Prediction

To further examine the impacts of GxE on wheat grain fructan content, genomic predictability of wheat grain fructan content was evaluated. True genetic values (G) for wheat grain fructan content were set equal to observed genotype means. The true GxE values (G + GxE) were set equal to values obtained for each observation from the AMMI model (Y_{ge}, Equation 5).

To predict genomic performance within environment, a 5-fold cross-validation was performed 100 times to fit the additive genetic model and general GxE model (Equations 2, 7) in ASREML-R. Genomic prediction accuracies were computed as the correlation between genetic estimated breeding value (GEBVs) and the corresponding true values.

$$y = \mu + \mathbf{X}\beta + \mathbf{Z}g + \mathbf{W}h + \varepsilon \quad (7)$$

y: Vector of fructan phenotypes
X: Incidence matrix for environments
β: Vector of fixed environmental effects
Z: Incidence matrix for genotypes
g: Vector of random genotype effects
W: Incidence matrix for genotype within environment
h: Vector of genotype by environment random effects
ε: Vector of residuals
$g \sim N(0, \sigma_a^2\mathbf{K})$
$h \sim N(0, \sigma_{gxe}^2\mathbf{I} \otimes \mathbf{K})$
I: Identity matrix
K: Additive genetic covariance matrix

Genome Scan

A genome-wide association study (GWAS) was performed on the observed phenotypes with the GWAS function in rrBLUP to examine the underlying genetic structure of wheat grain fructan content. The original set of 14,890 SNPs was further filtered to remove redundant markers and markers with minor allele frequencies above 5%. For markers with over 90% similarity, the marker with least missing data was retained. After filtering, 2,929 SNPs were retained for the analysis. Within the analysis, original phenotypes were examined, trials were treated as fixed effects, and population structure was controlled for with the inclusion of one principal component and the mean imputed K matrix. The significance threshold applied for the GWAS was a 10% false discovery rate (FDR; Storey and Tibshirani, 2003). Regions surrounding significant SNPs were subsequently analyzed with an open reading frame to search for proteins related to fructan metabolism.

Results

Initial Data Visualization and Heritability Estimates

The observed wheat grain fructan content in this study ranged from 0.43 to 1.62g/100g (Figure 3.1). Fructan content was normally distributed with an overall mean of 0.949g/100g and standard deviation of 0.172g/100g. The observed fructan content by environment varied with the Caldwell 2014 environment exhibiting the highest values (Figure 3.2).

Examination of fructan content by genotype (Figure 3.3) showed a wide range of means and variances across the 275 observed genotypes. The Pearson correlation between genotype variance and means or median was 0.195 and 0.201, respectively.

The broad and narrow sense heritability estimates for wheat grain fructan content in this study were 0.598 and 0.523, respectively.

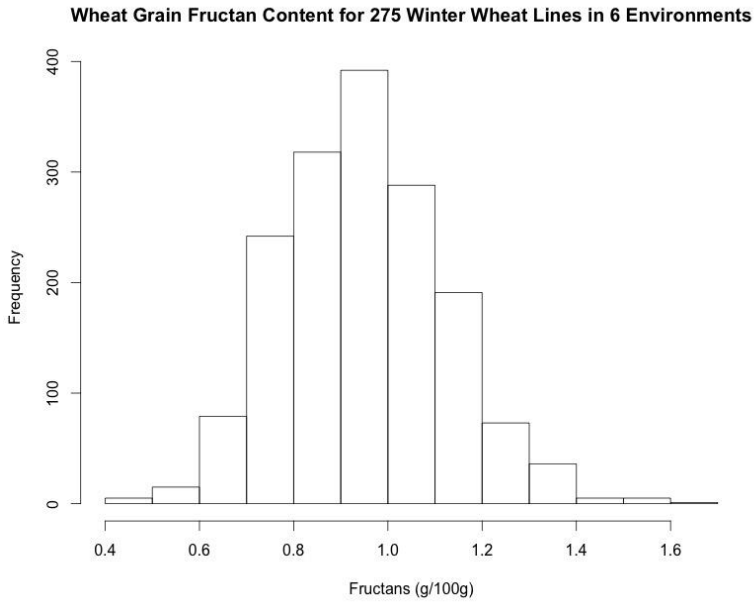

Figure 3.1: Total wheat grain fructan content for 275 winter wheat genotypes grown across six environments.

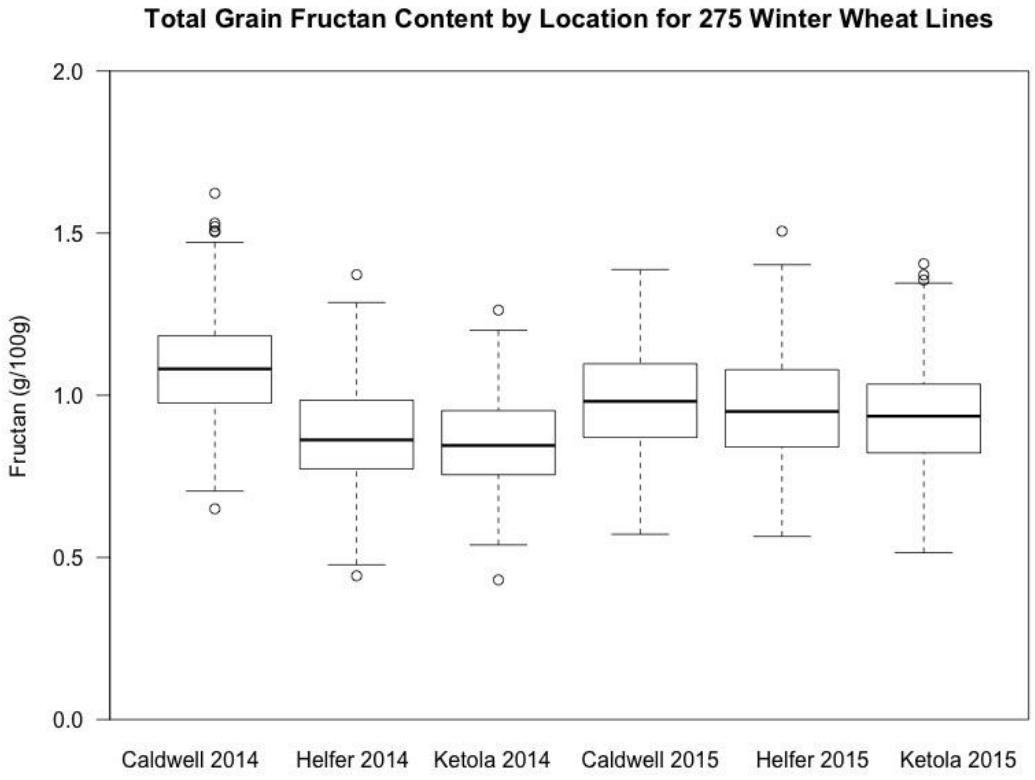

Figure 3.2: Total fructan content for 275 winter wheat lines by environment.

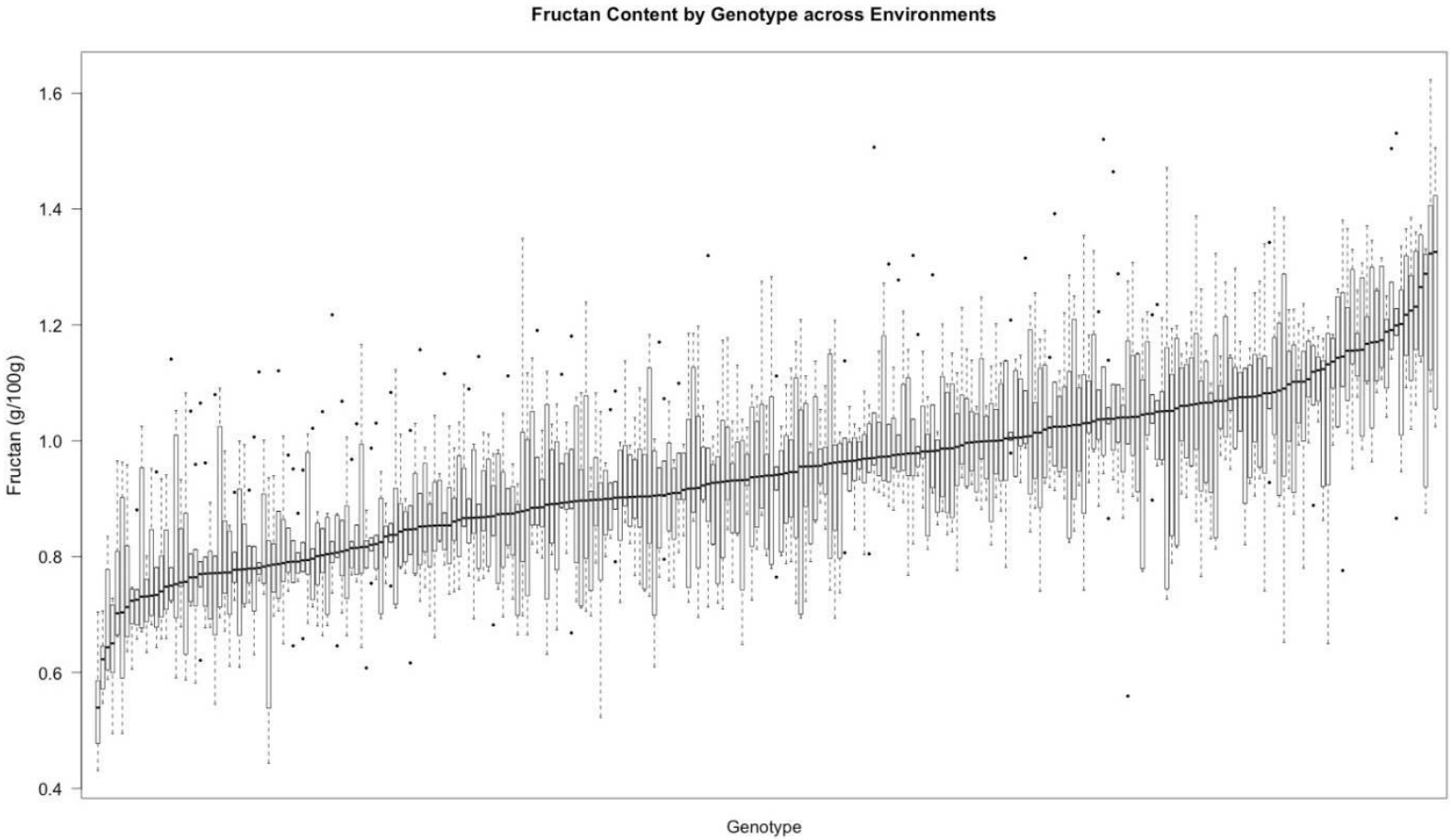

Figure 3.3: Total fructan content across six environments for 275 winter wheat lines.

AMMI analyses on fructan content, plant height, and heading date found significant GxE for all traits (Tables 3.1-3.3). The AMMI2 model fit on the data set indicated two multiplicative terms best captured the observed GxE for wheat grain fructan content (Table 3.1). When assuming genotypic independence, genotype and environment explained 53.6% and 19.4% (Table 3.4) of the observed phenotypic variance, respectively. The total phenotypic variance explained by all multiplicative model terms was 24.5%. IPC1 captured 27.1% of the non-additive variance and 9.37% of the total phenotypic variance, whereas, IPC2 captured 24.9% and 7.71%, respectively.

Table 3.1: ANOVA table for grain fructan content

AMMI Analysis of Variance - Grain Fructan Content

	Df	SS	MS	p-value	
Environment	5	9.44	1.89	< 1e-05	***
Genotype	274	26.1	0.0952	< 1e-05	***
PC1	278	4.56	0.0164	< 1e-05	***
PC2	276	3.75	0.0136	< 1e-05	***
PC3	274	1.94	0.0071	0.0590	
PC4	272	1.65	0.0061	0.0707	
Residuals	270	1.24	0.0046		
Total	1559	48.7	2.03		

Significance Codes: 0.05 * 0.01 ** 0.001 ***

The significant GxE detected for plant height explained 17.6% of the observed variation. The largest GxE effects were observed for heading date with IPC1 explaining 4.15% of the observed variation.

Table 3.2: ANOVA table for plant height

AMMI Analysis of Variance - Height

	Df	SS	MS	p-value	
Environment	5	15056	3011	< 1e-05	***
Genotype	274	103020	376	< 1e-05	***
PC1	278	17516	63	< 1e-05	***
PC2	276	12082	43.8	< 1e-05	***
PC3	274	8176	29.8	0.119	
PC4	272	6785	24.9	0.410	
Residuals	270	5767	21.4		
Total	1649	168402	3570		

Significance Codes: 0.05 * 0.01 ** 0.001 ***

Table 3.3: ANOVA table for plant heading date

AMMI Analysis of Variance - Heading Date

	Df	SS	MS	p-value	
Environment	5	17803	3561	< 1e-05	***
Genotype	274	7600	27.7	< 1e-05	***
PC1	278	628	2.3	< 1e-05	***
PC2	276	513	1.86	3.80e-4	***
PC3	274	426	1.55	1.05e-3	
PC4	272	328	1.20	1.56e-2	*
Residuals	270	231	0.85		
Total	1649	27528.6795			

Significance Codes: 0.05 * 0.01 ** 0.001 ***

Table 3.4: Percent of variance for each trait explained by sum of squares (SS) for each factor from the AMMI ANOVA

Factor	Fructan Content	Height	Heading Date
Environment	19.4	8.9	64.7
Genotype (Independent)	53.6	61.2	27.6
Total GxE	24.5	26.5	6.88
PC1 (Included in Total GxE)	9.37	10.4	2.28
PC2 (Included in Total GxE)	7.71	7.17	1.86
Residuals	2.56	3.42	0.837

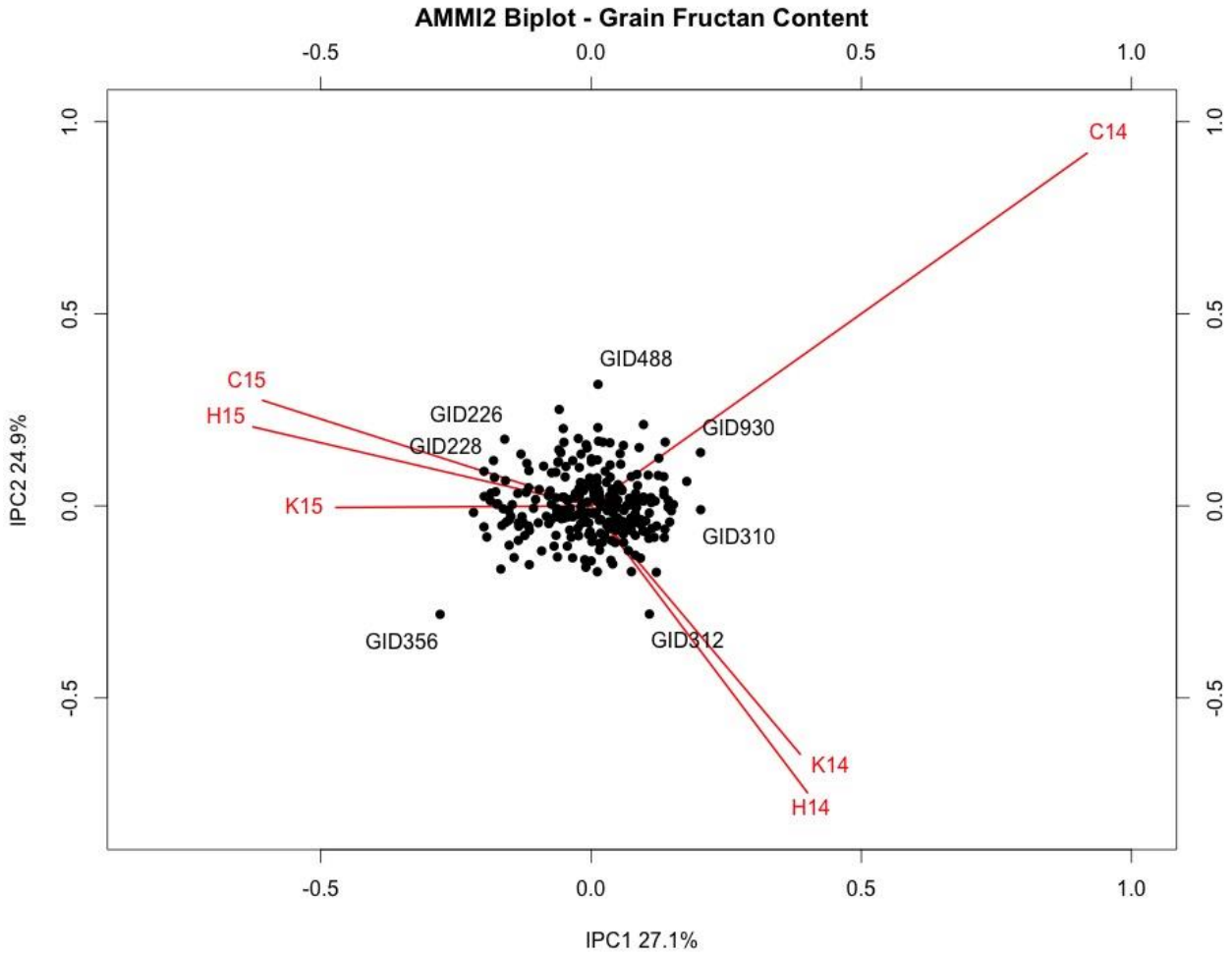

Figure 3.4: AMMI2 biplot for grain fructan content comparing IPC1 and IPC2 scores for genotypes (black dots) and environments (red lines).

The AMMI2 biplot (Figure 3.4) showed a clear division of environments by year along IPC1 and separation between 2014 locations along IPC2. IPC2 did not clearly separate the 2015 environments as they all cluster in the upper left quadrant of the biplot.

The length of the spoke connecting the observed environment to the origin in the AMMI2 biplot indicates the relative strength of observed interaction for that environment. The longest spoke in the AMMI2 biplot indicates Caldwell 2014 exhibited the largest interaction effect for grain fructan content.

The influence of GxE interactions on genotypes can be inferred based on the distance of genotypes from the origin of the AMMI2 biplot. The genotypes GID356, was most strongly influenced by GxE interactions as indicated by its notable distance from the origin. The strong GxE for GID 356 was likely partly due to variation in kernel size and presence of shriveled kernels across the six environments.

The lines with the highest fructan content across environments were GID502 and GID120. The genotype GID502 exhibited the highest fructan content in 2015 as indicated by the three triangles on the lower left side of the PC1 axis in figure 3.5. In contrast, GID120 exhibited the highest fructan content in 2014 environments even with the large separation of environments within 2014.

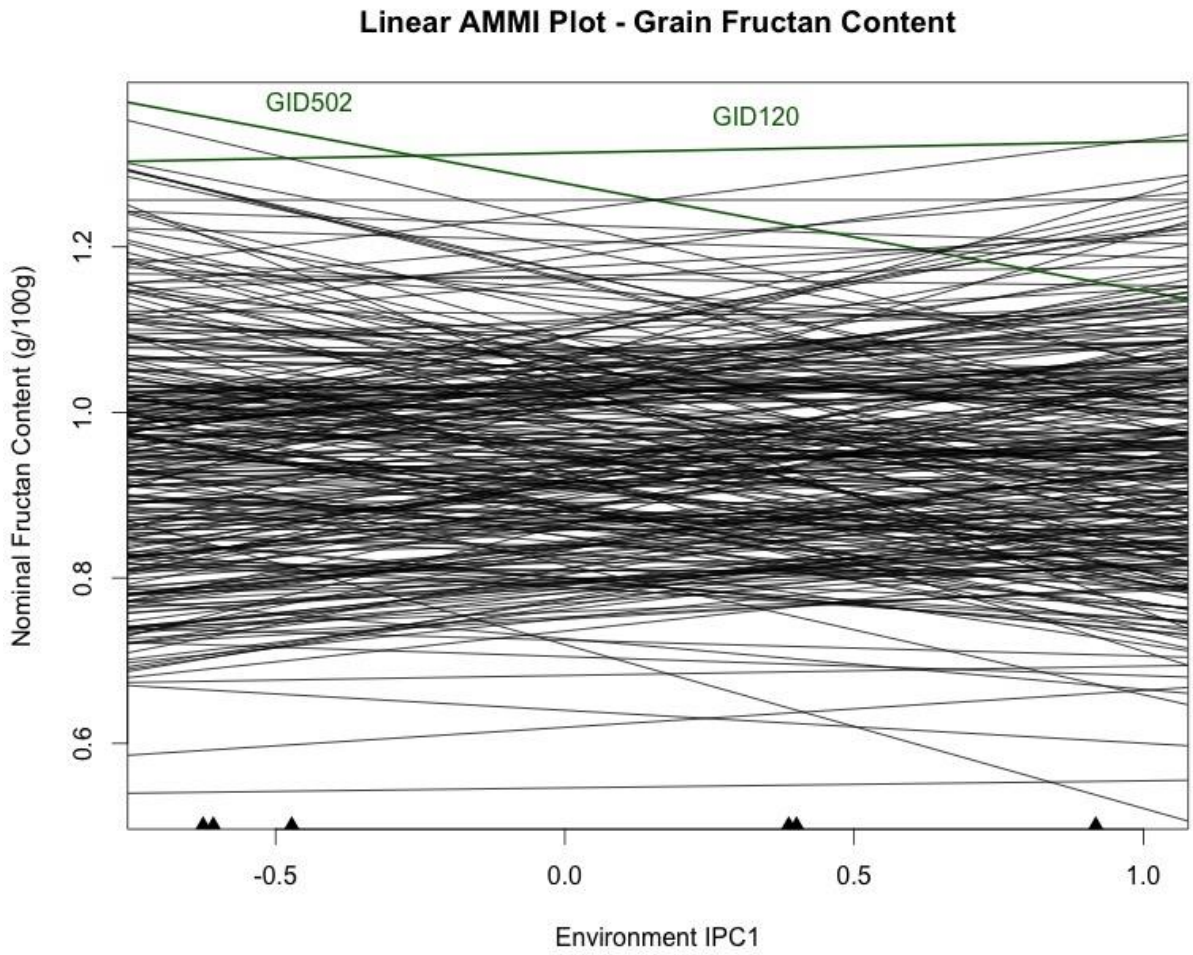

Figure 3.5: Linear AMMI plot of 275 winter wheat genotypes. Nominal fructan content represents the average environment. Environments are denoted by small triangles along the X axis. The lines with the highest grain fructan content in 2014 and 2015 environments are visible at the top of the figure.

Genomic Prediction

The correlations between observed phenotypes and AMMI model values (G + GxE) across environments were fairly high (Table 3.5). Correlations between genetic values (observed genotypic means) and AMMI model fit values were notably lower for all 2014 environments (Table 3.6).

Table 3.5: Correlations between observed phenotypes and AMMI model values for genotypes within environments

C14	C15	H14	H15	K14	K15
0.999	0.919	0.936	0.944	0.907	0.897

Table 3.6: Correlations between genetic values (G) and AMMI model values (G + GxE) for genotypes within environments

C14	C15	H14	H15	K14	K15
0.731	0.921	0.851	0.922	0.878	0.955

The mean genomic prediction accuracy for main genetic effect with the additive genetic model was 0.518. When GxE effects were included in the prediction, the genomic prediction accuracies for wheat grain fructan content over all 2014 environments averaged 0.093 lower than the additive genetic accuracy (Table 3.7).

Table 3.7: Genomic prediction accuracies for wheat grain fructan content within environment

	C14	C15	H14	H15	K14	K15
G + GxE	0.311	0.528	0.430	0.543	0.422	0.546
G	0.461	0.515	0.499	0.527	0.481	0.521

Genome Scan

The GWAS performed for wheat grain fructan content found two significant quantitative trait loci (QTL) explaining 0.63% and 0.03% of the observed phenotypic variation on chromosome arms 3AS and 5AS, respectively (Figures 3.6, 3.7). No other QTL detected in this analysis passed the significance threshold of a 10% FDR.

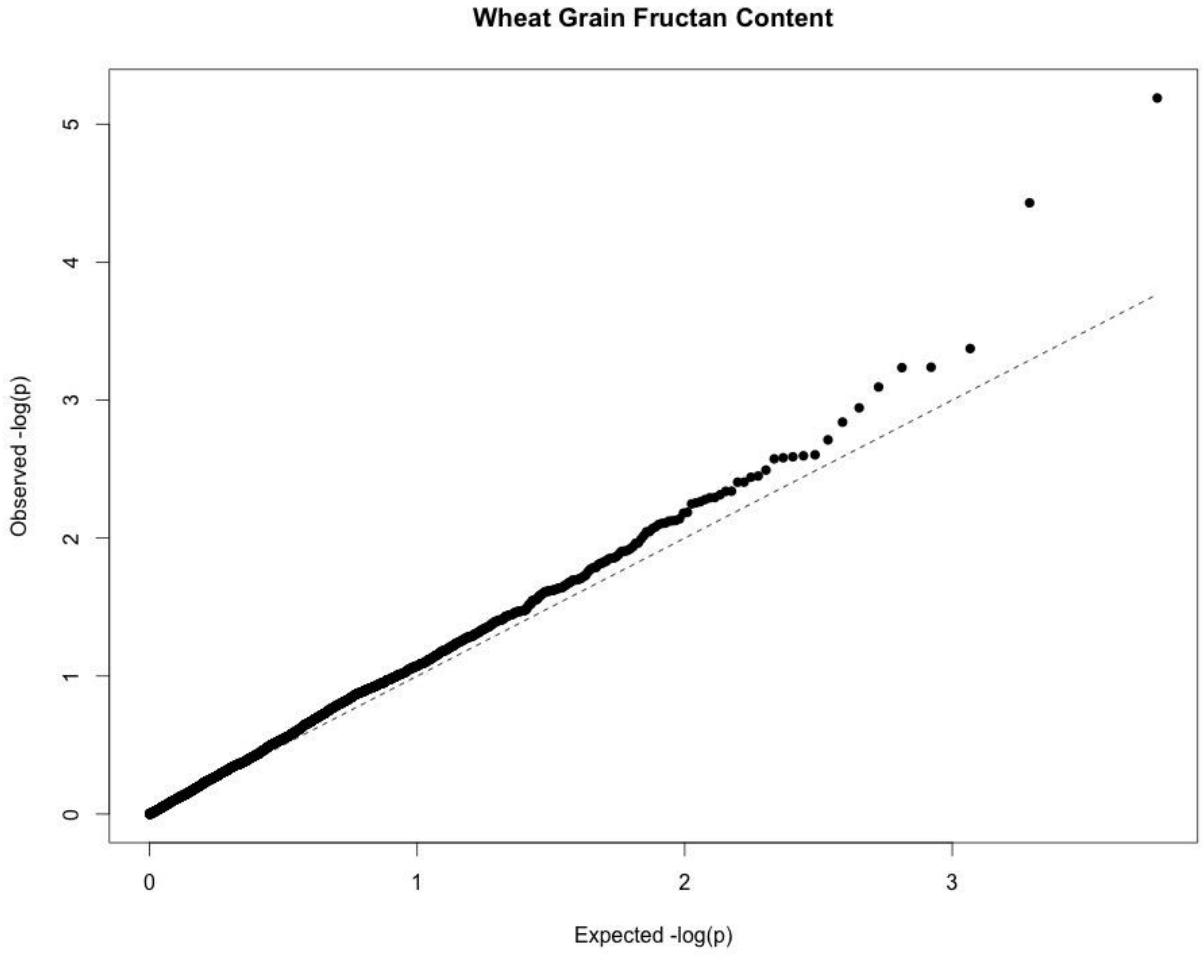

Figure 3.6: QQ-Plot for wheat grain fructan content

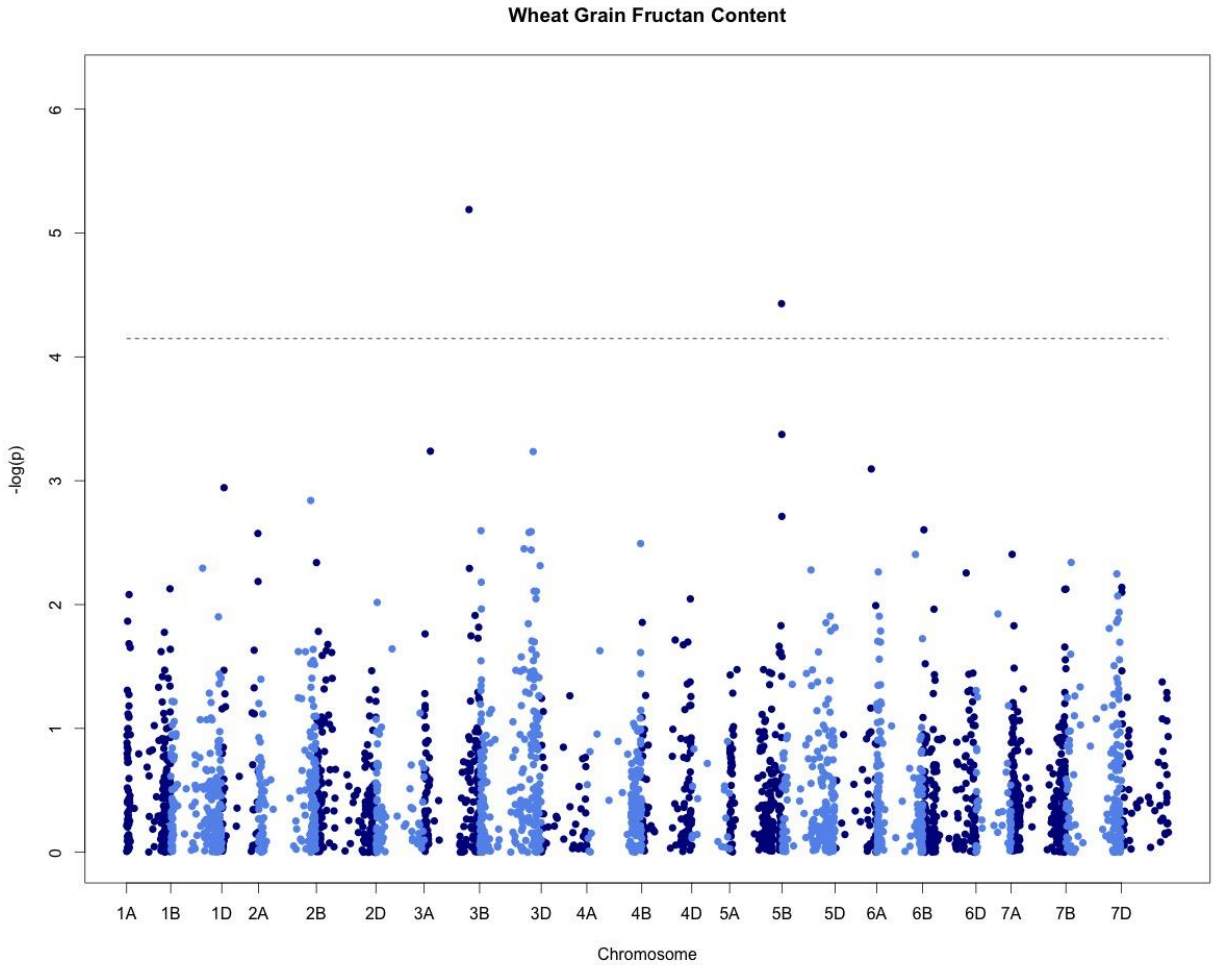

Figure 3.7: Manhattan plot for wheat grain fructan content. The dashed line corresponds to a significance threshold of a 10% false discovery rate.

Discussion

Data Visualization and Heritability Estimates

The range of grain fructan content observed in this study was slightly lower than in previously reported literature 0.4307-1.6232g/100g vs. 0.7-2.9g/100g) (Huynh et al., 2008). The overall mean was also lower the previously reported average fructan content of 1.0g/100g for wheat flour (MacLeod and Preece, 1954). Given that wheat grain fructan content is influenced by genotype and environment, it is not unexpected to observe deviations from previously reported values.

The genotypes examined in this study exhibited a wide range of fructan content across environments. The correlations between genotype means and medians and the observed variances were weakly positive (0.20). It can be concluded there was no clear relationship between the average grain fructan content and observed variability in content for the wheat genotypes examined in this study.

The narrow sense heritability estimate for wheat grain fructan content in the study was lower than previously reported estimates (0.523 vs. 0.64) (Huynh et al., 2008). For this study, heritability was calculated on a plot basis. Though Huynh et al. (2008) did not report the basis of heritability calculations, the calculation of an entry mean heritability may explain the difference in heritability estimates. Due to lack of replication within environment, entry mean heritability could not be computed for this study. Additionally, the difference in heritability estimates between this study and previously reported results may be partly due to environmental growing conditions as Huynh et al. (2008) reported narrow sense heritabilities of 0.94 and 0.64 for the

glasshouse and field, respectively. The proportion of phenotypic variance attributable to environment was likely higher for this study relative to previous studies.

AMMI Analysis

In this MET study, statistically significant GxE interactions for wheat grain fructan, plant height and heading date were detected. Further investigation of the GxE interactions with AMMI models determined that two statistically significant terms represented the observed GxE interactions for wheat grain fructan. Genotype explained a majority of the observed phenotypic variance (53.6%) with GxE interactions and environment accounting for 24.5% and 19.4% of the observed variance, respectively. Though no previous studies have examined the contribution of different factors to variation observed for wheat grain fructan content, the results observed in this study confirm genotypic and environmental variance does exist for wheat grain fructan content.

The overall climate for all environments was similar over the two growing seasons (Tables 3.8- 3.9). Given the similarity in climate, unmeasured factors other than climate may be responsible for the split in years detected by IPC1for wheat grain fructan content. The environments in the 2014 growing season were slightly wetter with 102% of normal precipitation received over the growing season compared to 94% received in the same time period for 2015.

Table 3.8: Temperature (°F) data for the 2014 and 2015 growing seasons in Ithaca, New York. Normal temperature records are 30 year averages (1981-2010).

	2013-2014		2014-2015		Normal
	Monthly Mean	Departure from Normal	Monthly Mean	Departure from Normal	Monthly Mean
October	51.5	2.8	51.4	2.7	48.7
November	35.7	-3.9	35.3	-4.3	39.6
December	27.7	-1	31.7	3	28.7
January	17.7	-5.6	16.6	-6.7	23.3
February	18.4	-6.9	10.1	-15.2	25.3
March	24.5	-8.1	24.7	-7.9	32.6
April	43.4	-1.3	42.8	-1.9	44.7
May	56.4	1	61.6	6.2	55.4
June	65.1	0.5	63.7	-0.9	64.6
July	67.8	-1	67.3	-1.5	68.8
Season Mean	40.82	-2.35	40.52	-2.65	43.17

Table 3.9: Precipitation (in) data for the 2014 and 2015 growing seasons in Ithaca, New York. Normal precipitation records are 30 year averages (1981-2010).

	2013-2014			2014-2015			Normal
	Monthly Sum	Departure from Normal	Percent of Normal	Monthly Sum	Departure from Normal	Percent of Normal	Monthly Sum
October	2.65	-0.77	77%	3	-0.42	88%	3.41
November	3.63	0.47	115%	2.36	-0.8	75%	3.16
December	2.26	-0.14	94%	2.36	-0.04	98%	2.4
January	1.63	-0.45	78%	0.97	-1.11	47%	2.08
February	1.97	-0.01	99%	1.94	-0.04	98%	1.98
March	3.04	0.4	115%	1.33	-1.31	50%	2.64
April	2.44	-0.85	74%	3.6	0.31	109%	3.29
May	4.44	1.25	139%	2.69	-0.5	84%	3.19
June	5.14	1.15	129%	6.59	2.6	165%	3.99
July	3.85	0.02	101%	4.93	1.1	129%	3.83
Season Total	31.05	1.07	104%	29.77	-0.21	99%	29.97

The separation of environments within 2014 by IPC2 is likely due to the difference in fructan content observed between Caldwell 2014 and other 2014 environments.

To account for non-independence between genotypes examined in this study, an AMMI model accounting for genotype relationships was fit on residuals of an additive genetic model containing GxE and error variances (equation 2). The results of the residual-fitted AMMI did not differ from the aforementioned AMMI analysis assuming genotypic independence.

Prediction

The observed prediction accuracy of 0.52 for main genetic effect with the additive genetic model suggests genomic prediction is feasible for wheat grain fructan content. The inclusion of GxE values resulted in prediction accuracies for 2015 environments that surpassed observed additive genetic model accuracy. This pattern was not observed for the genomic predictions within 2014 environments as the inclusion of GxE values resulted in lower observed prediction accuracies ranging from 0.31 to 0.43. The prediction accuracy of main effects within the GxE model was improved with the omission of GxE terms; however, the observed accuracies did not surpass 0.52. It is unclear why the decrease in within environment predictability was observed for 2014 environments with the GxE model. Prediction with the AMMI model accounting for genetic relatedness exhibited minimal impact on accuracies. Overall, genomic prediction of main effects had a relatively high accuracy.

Genome Scan

To further evaluate the feasibility of MAS vs. GS, the underlying structure of genetic control of grain fructan content was examined with a genome scan on observed phenotypes. The two significant QTL detected in this study were located on chromosome 3A and 5A. The inclusion of the significant SNPs as a covariate in the GWAS model did not result in the detection of additional significant SNPs. An open reading frame search of the 150mb region surrounding the significant QTL on chromosome 3A and 5A found no known proteins involved in fructan metabolism.

QTL associated with wheat grain fructan content in a double-haploid mapping population have been reported on chromosomes 2B, 3B, 5A, 6D, and 7A (Huynh and Wallwork, 2008). Although the results of this GWAS do not fully align with previously reported findings, the lack of significant QTL that could be used in MAS for wheat grain fructan content suggests GS is an appropriate selection methodology for wheat grain fructan content.

Presence/Extent of GxE

From the perspective of breeding for increased wheat grain fructan content, GxE does influence grain fructan content. Although GxE can exhibit negative impacts on genomic prediction accuracy for grain fructan content, the lowest observed accuracy for this study was 0.311 for the Caldwell 2014 environment. The moderately high heritability ($h^2 = 0.52$), moderate prediction accuracies and phenotypic variation explained by the main genotypic effect suggest GS can be utilized to breed for increased wheat grain fructan.

Conclusion

For this study of 275 winter wheat lines grown in six environments, genotype-by-environment interactions were significant for wheat grain fructan content, plant height and heading date. Despite the statistical significance of observed GxE interactions, the genotype and environment main effects explained a large proportion of the observed phenotypic variance for grain fructan content (73%), plant height (70%), and heading date (92%).

Fitting of an AMMI2 model indicated two multiplicative factors represented the dimensionality of the GxE interactions for wheat grain fructan content. The first multiplicative factor detected a clear separation of GxE interactions based on year effects with the second multiplicative factor separating environments within a year.

Because the observed GxE explained a small proportion of the total variance, genomic prediction accuracy of grain fructan content did not improve with the addition of GxE effects in the model. Genomic prediction on the main effect, without consideration of GxE, resulted in prediction accuracies that justify the use of genomic selection. This finding was further corroborated by the GWAS results which detected no large effect QTL controlling wheat grain fructan content in the population examined.

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CHAPTER 4

RECURRENT GENOMIC SELECTION FOR WHEAT GRAIN FRUCTANS[4]

Abstract

Fructans are carbohydrates found in many plants, including wheat (*Triticum aestivum* L.), which serve physiological roles in both plants and humans. Wheat grain fructans are a potential breeding target for developing climate resilient, nutritionally improved wheat varieties. Genomic selection (GS) could facilitate the rapid development of high fructan wheat varieties while decreasing phenotyping requirements; however, few empirical studies have examined GS for nutritional breeding. While GS can accelerate gain from selection, the rapid loss of genetic variation and increases in inbreeding may limit the potential for long term gains. The objectives of this study were to (i) determine realized gain from GS for wheat grain fructan content with genomic BLUP (GBLUP) and Optimized Contribution Selection (OCS) methods, (ii) determine if gains agree with theoretical expectations, and (iii) compare impacts of selection on inbreeding, genetic variance and indirect selection on agronomic characteristics. Over 2 years, two cycles of GS were performed with GBLUP and inbreeding constrained OCS selection. A diverse TP was used and the TP was updated following each cycle of selection. GS with GBLUP and OCS led to a 25 ± 14% and 31 ± 6.2% increase in wheat grain fructan content,

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respectively. Constrained selection populations retained greater genetic variance and reached lower inbreeding levels relative to GBLUP populations. The selection for wheat grain fructan content was not associated with a change plant height but heading date was significantly earlier (~ 0.5 days) in constrained selection populations. This shows that GS can be used for developing varieties with increased wheat grain fructan content and further research is needed for controlling rates of inbreeding.

Abbreviations

BLUP: Best linear unbiased prediction; C_0: Cycle-zero; C_1: Cycle-one; C_{1S}: Cycle-one subset; C_2: Cycle-two; C_{2S}: Cycle-two subset; GBLUP: genomic BLUP; GEBV: Genomic estimated breeding value; GBS: Genotyping-by-sequencing; GS: genomic selection; GxE: Genotype-by-Environment; h^2: Narrow sense heritability; H^2: Broad sense heritability; LOOCV: Leave-one-out cross validation; OCS: Optimum-contribution selection; RGS: recurrent genomic selection; SNP: Single Nucleotide Polymorphism; TP: Training Population; TBV: true breeding value.

Introduction

Fructans are naturally occurring plant polymers composed of fructose molecules found in approximately 15% of flowering plant species, including wheat. Fructans confer stress tolerance by acting as omsoregulators in times of cold, drought and excess salinity (as reviewed in Hendry, 1993; Pilon-Smits et al., 1995; Kafi et al., 2003; Joudi et al., 2012). Fructan consumption, particularly inulin-type fructans, stimulates growth of healthy gut bacteria and supports overall gut health in hosts (Roberfroid et al., 2010; as reviewed in Di Bartolomeo et al., 2013; Peshev and Van Den Ende, 2014). The important physiological roles of fructans allow them to be a

desirable breeding target for developing nutritionally improved, climate resilient wheat varieties (as reviewed in Veenstra et al., 2017).

The development of nutritionally improved wheat varieties often requires extensive resources. High-throughput phenotyping methods for measuring wheat grain fructan content have been developed (Li et al., 2017). The use of genomic selection (GS) in breeding for wheat grain fructan content would shorten the selection cycle and reduce phenotyping requirements.

Genomic selection (GS) (as reviewed in Heffner et al., 2009; Lorenz et al., 2011) utilizes phenotype and genotype data from a relevant population to train a statistical model and predict breeding values of genotyped selection candidates based on markers alone. Selecting candidates based on predicted breeding values allows for crossing of selected individuals before phenotyping. For crops with long growth cycles, such as winter wheat, young plants can be genotyped and selected for crossing with GS prior to flowering.

While GS has the ability to accelerate the breeding cycle, conservation of genetic variance and management of inbreeding levels are necessary for optimizing long term gain from selection. High rates of inbreeding per breeding cycle have been observed with GS in simulations (Lin et al., 2016) and empirical studies (Rutkoski et al., 2015). Several methods to control the rate of inbreeding have been proposed, including optimum-contribution selection (OCS; Meuwissen, 1997). OCS methodologies were originally proposed as a way to maximize genetic gain while controlling the rates of inbreeding in animal breeding. Though the use of OCS methodologies may result in a small reduction of short term genetic gain, simulations

suggest long term gains are comparable with non-constrained selection (Henryon et al., 2015).

The empirical application of GS for nutritional traits in crops has not been widely examined. Furthermore, no empirical studies have employed OCS within the GS framework in plants. Applying GS for nutritional traits has great potential in many crops; however, the rapid rates of inbreeding and loss of genetic variance may limit long term gain from selection. A recurrent genomic selection (RGS) scheme for increasing grain fructan content in winter wheat was implemented to evaluate the feasibility of utilizing GS for nutritional breeding. The objectives of this study were to (i) determine realized gain from GS for wheat grain fructan content with two selection methods, (ii) determine if gains agree with theoretical expectations, and (iii) compare impacts of selection on inbreeding, genetic variance and correlated changes in agronomic characteristics.

Materials & Methods

Germplasm

All germplasm utilized in this study was obtained from the Cornell Small Grains Master collection. The Master collection is an association mapping population consisting of 1290 elite, F_5-derived advanced soft winter wheat breeding lines and varieties.

Training Population

The population used for GS model training consisted of 284 lines. Within the 284 lines, 14 lines were breeding population founders and the remaining 270 lines were Master collection lines were selected with the mean coefficient of determination methodology (Rincent et al., 2012) to serve as a representative sample of the genetic diversity in the larger collection.

Validation Population

The cycle-zero population, C_0, was founded with 14 elite lines selected from the Master collection based on perceived diversity and seed availability. To generate the C_0 population, the founders were randomly intermated for two generations by hand pollination to produce F_1 and double cross F_1 progenies. All F_1 and double cross F1 progenies were confirmed with simple-sequence repeat genotyping. The double cross F_1 families were subsequently self-pollinated to increase seed thereby resulting in double cross F_2 seed representing 64 families. Ten double cross F_2 individuals from each family were sampled to create a C_0 population of 640 individuals. To replicate the selection schemes, the 640 individuals were randomly split by family into two replicate populations of 320 consisting of 32 families each.

Breeding Scheme

Two GS methods were used for increasing wheat grain fructan content. The first selection method, genomic BLUP (GBLUP), determined the genomic estimated breeding values (GEBVs) for all selection candidates with the top 12 individuals

selected for intermating. The second method, constrained selection, optimized the contribution of selected individuals in the next generation by applying Optimum Contribution Selection (OCS) to maximize genetic gain and control inbreeding in the following generation. G-BLUP assumes equal contributions of all selected individuals to future progeny, while constrained selection determines the optimal contribution of each selection candidate to the progeny of the next generation to control inbreeding.

Each replicate of C_0 underwent two cycles of GS with GBLUP or constrained selection thereby creating four selection populations (Figure 3.1). For the first GS cycle, C_0 individuals were genotyped, and their GEBVs were predicted with the training population (TP) of 284 lines from the Master population. The best twelve C_0 individuals were selected using GBLUP and intermated to produce progenies with approximately equal parental contribution. For constrained selection, the selected individuals were intermated to produce progeny containing optimal contributions of selected parental lines.

Following cycle-zero, the genotypes and phenotypes of bulked parental material for each population were added to the TP. For cycle-one subset (C_{1S}), a random subset of selection candidates with the highest GEBVs in the C_1 selection populations were selected and intermated. For cycle-two (C_2), selection candidates in the C_1 selection populations were selected and S_1 seed of individuals from C_1 were intermated to create C_2 populations consisting of S_1 families. For cycle-two subset (C_{2S}), an updated TP with genotypes and phenotypes of parental material from C_1 to was used to select candidates for crossing from C_{1S} populations. As with previous cycles, the top 12 individuals were selected with GBLUP and the number of

individuals selected with the constrained selection for C_{1s}, C_2 and C_{2S} varied according to the optimized contribution results. The resulting C_{2S} progeny seed was increased for one generation.

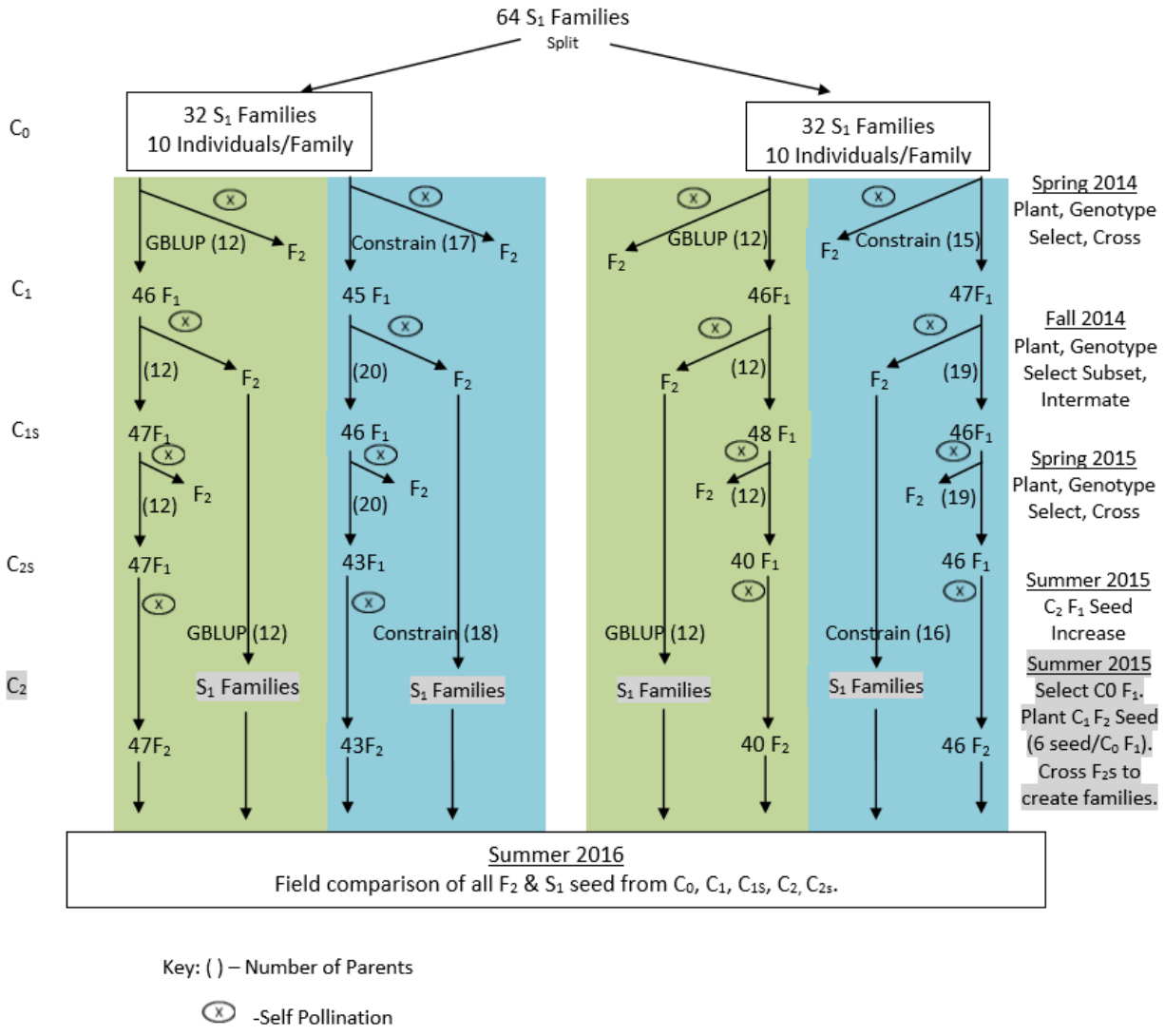

Figure 4.1: Genomic selection (GS) schemes. C_0 is the cycle zero population; C_1 is the cycle 1 population; C_{1S} is the cycle 1 population which underwent subset selection; C_{2S} is the cycle 2 population obtained from subset selection of the cycle 1 population; C_2 is the population of S_1 families obtained from crossing F_2 seed from the cycle 1 population; Constrain is GS with previously imposed constraints on inbreeding; GBLUP is GS with the GBLUP method.

The selection intensity and number of selected individual for each selection cycle within the four programs can be found in table 4.1. Selection intensities were calculated per equation 1 with GEBVs originating from estimates based on the initial training populations at the time of selection. All genotyped material was directly selected on and crossing of selected individuals was performed in the greenhouse

Table 4.1: Selection intensities and number of individuals planted and selected. Rep: replicate; *i*: selection intensity.

Population	Rep	# Candidates	# Selected	*i*
C_0 GBLUP	1	320	12	2.022
	2	320	12	1.899
C_0 Const.	1	320	17	1.851
	2	320	15	1.781
C_1 GBLUP	1	46	12	1.425
	2	46	12	1.169
C_1 Const.	1	45	21	1.344
	2	47	22	0.943
C_{1S} GBLUP	1	46	12	-1.834
	2	46	12	-1.547
C_{1S} Const.	1	45	18	-1.583
	2	47	16	-1.028
C_{2S} GBLUP	1	47	12	1.219
	2	48	12	0.956
C_{2S} Const.	1	46	20	1.298
	2	46	19	0.826

$$i = \frac{\mu_s - \mu}{\sigma} \text{ where } \mu_s = \Sigma\, c_j^e GEBV_j \quad (1)$$

i: Selection Intensity
μ_s: Mean GEBV of selected individuals
μ: Mean GEBV of selection population
σ: Standard deviation of fructan content for selection population
c_j^e: Realized contribution of individual j
$GEBV_j$: BLUP estimated breeding values of individual j
j = Selected individual (1…n)

Genotypic Data

Genotypic data for all populations, with the exception of C_2 populations, was generated with genotyping-by-sequencing (GBS; Elshire et al., 2011) according to the protocol described by Poland et al. (2012). The GBS data for the TP and breeding populations (C_0, C_1, C_{1S}) were processed through different SNP calling pipelines with UNEAK (Lu et al., 2013). To assimilate the GBS markers from separate pipelines, markers with matching SNP tags and variable positions within tags were retained from both genotypic data sets. The populations were filtered separately (MAF > 5%, marker presence > 50%, individual presence > 20%, heterozygosity < 20% for TP) and expectation maximization (EM) imputation was performed in the respective populations with the A.mat function in the R package rrBLUP (Endelman, 2011). After merging the processed SNP calls, the resulting 8,903 markers were utilized for selections.

In order to infer inbreeding to its fullest potential across selection cycles, raw sequence reads from previous GBS genotyping were utilized in combination with TASSEL-GBS (Glaubitz et al., 2014) and the IWGSC RefSeq v1.0 reference genome to recall SNPs which align to the reference genome for the TP, C_0, C_1, C_{1S} and C_{2S} populations. The resulting SNPs for all RGS materials were filtered for marker presence and individual coverage by markers. After applying filters to retain markers with presence over 30%, a minor allele frequency above 1%, and heterozygosity under 10%, a total of 12,599 SNPs were obtained. Individuals missing over 70% of the 12,599 filtered SNPs were subsequently removed. Imputation with the EM imputation algorithm was performed for remaining missing data points with rrBLUP.

Phenotypic Data

Fructan Quantification: Phenotypic data for total grain fructan content was measured from bulked seed in the laboratory using the enzymatic/spectrophotometric method (AOAC 999.03) (McCleary et al., 2000). Briefly, 3g of seed were ground in a Geno/Grinder® 2000 and 1.0g of resulting flour was dissolved in 80C water for 15 minutes. The volume of the dissolved solution was adjusted to 100mL and supernatant produced from centrifugation of solution aliquots was stored for later analysis. All ground flour and dissolved samples were stored at -20C to minimize endogenous enzymatic degradation of fructans.

All dissolved samples were analyzed with an enzymatic assay kit (K-FRUC, MegaZyme International). The enzymatic assay protocol was adapted (downscaled to 1/5th) for 96 well plates (Newell et al., 2014) to increase throughput and reduce phenotyping costs. Sample absorbance was measured with a SpectraMax® Plus 384 Microplate Reader and total fructan content was calculated (Equation 2). To control for variation between plates, fructan content for each plate was adjusted by the controls on each plate.

Fructan (% w/w as is): = Δ_A x F x V/W x 2.48 (2)

Δ_A: sample absorbance – sample blank absorbance (both read against the reagent blank)

F : factor to convert absorbance values to μg of D-fructose

V: volume (mL) of extractant used (i.e. 50 or 100 mL)

W: weight (mg) of sample extracted

2.48: unit conversion factor

GS Model Training

All lines for the initial TP were grown in one location (Caldwell Farm, Cornell University, Ithaca, New York) in summer 2013 to minimize non-genotype variation. Phenotypes for updating TPs as breeding programs diverged were measured on bulked S_1 greenhouse seed from selected individuals (Figure 4.2).

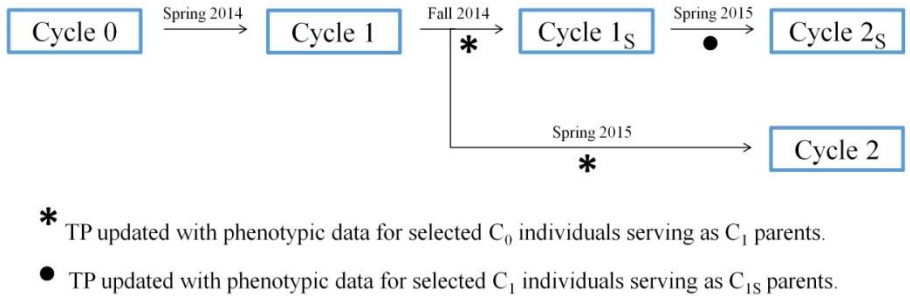

Figure 4.2: Training population updates. The asterisk represents the point at which phenotypes for selected C_0 individuals serving as C_1 parents were added to the training population for the respected selection method and replicate. The solid circle represents updates of the respective populations with phenotypes of selected C_1 individuals serving as C_{1S} parents.

Realized Gain Field Validation

The C_0, C_1, and C_2 populations were evaluated in 1m head rows of S_1 progeny in two locations (Caldwell and Ketola) near Ithaca, New York, during the 2016 growing season to estimate cycle means, genetic variances, and genetic correlations. Due to the large size of the C_0 populations, selected individuals and a random selection of 36 unselected individuals from each replicate population were planted in the field validation experiment. A random incomplete block design containing cycle populations, TP, and checks was utilized. Plant height and heading date were collected

on each line evaluated in the trial. Fructan content was measured on bulked field seed in fall 2016. Monthly weather data for the Ithaca, New York area was obtained from annual summaries published on the Northeast Regional Climate Center (NRCC) website (http://www.nrcc.cornell.edu/wxstation/ithaca/ithaca.html).

Statistical Models for Selection

Genomic Selection with GBLUP: Genomic selection with the GBLUP model (Equation 3) was implemented with rrBLUP (Endelman, 2011). Selection candidate breeding values from GBLUP are equivalent to breeding values from ridge regression BLUP (Hayes et al., 2009). The additive genetic relationship was calculated with the A.mat function in rrBLUP (Endelman, 2011).

$$y = \mu + \mathbf{X}\beta + \mathbf{Z}g + \varepsilon \quad (3)$$

y: Vector of fructan phenotypes
μ: Mean
$\mathbf{X}$: Incidence matrix for environments
β: Vector of fixed environmental effects
$\mathbf{Z}$: Incidence matrix for genotypes
g: Vector of random genotype effects
ε: Vector of residuals
$g \sim N(0, \sigma_a^2\mathbf{K})$
$\varepsilon \sim N(0, \sigma_e^2)$
$\mathbf{K}$: Additive genetic covariance matrix

Genomic Selection with Constrained Selection: Genomic selection with the constrained method was performed in two stages. First, genetic values for individuals

were estimated with GBLUP (Equation 3). The resulting GEBVs, additive genetic relationship matrix, and a list of top 12 selection candidates were subsequently utilized in the OCS method proposed by Meuwissen (1997) to maximize genetic gain and control inbreeding in the following generation.

In brief, expected gain from selection of the top 12 individuals with GBLUP selection is computed from selection candidate GEBVs assuming equal contribution of the selection candidates to progeny in the next generation (Equation 4). The baseline level of estimated inbreeding for GBLUP selection is set equal to the average coancestry of selection candidates (Equation 5). LaGrangian multipliers are then utilized to obtain the optimal contribution of selected candidates for which expected genetic gain is equal to GBLUP selection with estimated inbreeding levels equivalent to 50% of the baseline level.

$$G_{t+1} = c_t'EBV_t \quad (4)$$

G_{t+1}: Genetic level of the next generation

c_t: Vector of genetic contributions of the selected candidates to generation t+1

EBV_t: Vector of BLUP estimated breeding values of the candidates of selection in generation t

$$\overline{C}_{t+1}=c_t'A_tc_t/2 \quad (5)$$

$\overline{C}_{t+1}$: Average coancestry between selected candidates

A_t: Matrix of additive genetic relationships among selection candidates in generation t

Realized Gain Calculations

Wheat Grain Fructan Content: Adjusted population means for each replicate of C_0, C_1, C_{1S}, C_2 and C_{2S}, were calculated as the sum of population effects and mean

($p + \mu$, Equation 6). Realized gains were calculated by subtracting from population means their respective C_0 population means. Percentage gain for each population was calculated as realized gain divided by the corresponding C_0 population mean. Paired two-tailed *t*-tests were used to test differences in realized gain per cycle between GBLUP and OCS methods. Paired two-tailed *t*-tests were also used to test between realized and expected gains for each GS method within each cycle. One-sided, one-sample *t*-tests were used to test the significance of realized genetic gains between cycles for each method and overall genetic gain relative to C_0.

$$y_{ijlk} = \mu + \beta_i + r_j + p_k + \varepsilon_{ijk} \quad (6)$$

y_{ijlk}: Fructan phenotype
μ: Mean
β_i: Fixed environment effect (i = 1,2)
r_j: Random effect for blocks (j = 1,…24)
p_k: Fixed population effect (k = 1,…18)
ε_{ijk}: Residual
$q_l \sim N(0, \sigma_q^2)$
$\varepsilon \sim N(0, \sigma_e^2)$

$$y_{ijlk} = \mu + \beta_i + g_j + r_k + \varepsilon_{ijk} \quad (7)$$

y_{ijlk}: Fructan phenotype
μ: Mean
β_i: Fixed environment effect (i = 1,2)
g_j: Random genotype effect (j = 1,…970)
r_k: Random effect for blocks (k = 1,…24)
ε_{ijk}: Residual
$g_j \sim N(0, \sigma_g^2)$
$r_k \sim N(0, \sigma_r^2)$
$\varepsilon \sim N(0, \sigma_e^2)$

Genetic values for individuals across environments were calculated with the mixed model in Equation 7. Genetic values of individuals within each environment were obtained by fitting the mixed model in Equation 7 for each environment with environment effects removed.

Narrow sense heritability was calculated for genotyped lines per equation 8. Variance estimates for narrow sense heritability calculations were obtained by fitting the mixed model in equation 9.

$$h^2 = \sigma_a^2 / \sigma_a^2 + \sigma_e^2 \quad (8)$$

σ_a^2: Additive genetic variance
σ_e^2: Environmental variance

$$y_{ijlk} = \mu + \beta_i + g_j + r_k + \varepsilon_{ijk} \quad (9)$$

y_{ijk}: Fructan phenotype
μ: Mean
β_i: Fixed environment effect (i = 1,2)
g_j: Random genotype effect (j = 1,…970)
r_k: Random effect for blocks in environment (k = 1,…24)
ε_{ijk}: Residual
$g_j \sim N(0, \sigma_a^2\mathbf{K})$
$r_k \sim N(0, \sigma_r^2)$
$\varepsilon \sim N(0, \sigma_\varepsilon^2)$
K: Marker based relationship matrix

Genetic correlations between field environments were calculated based on genetic values for each environment. Additionally, the genetic correlations between greenhouse and each field environment were computed.

Expected Gain Calculations: Expected gain in wheat grain fructan content from GS for each population was calculated with equation 10. The selection intensity for each population was calculated per equation 1. To estimate genetic variances for grain fructan content, non-genetic effects were removed (equation 11) with population-specific models fit (equation 12) on the residuals (y_i') from the non-genetic model. Estimates of genetic variance from the validation trial, σ_g^2, were used to approximate the additive genetic variance, σ_a^2, for cycle 2 populations.

$$R = ir\sigma_A \quad (10)$$

R: Response to selection
i: Selection intensity
r: Selection accuracy
σ_A: Square root of additive genetic variance of true breeding value (TBV)

$$Y_{ij} = \mu + \beta_i + r_j + \varepsilon_{ij} \quad (11)$$

y_{ij}: Fructan phenotype
μ: Mean
β_i: Fixed environment effect (i = 1,2)
r_j: Random effect for blocks (j = 1,…24)
ε_{ij}: Residual
$r_j \sim N(0, \sigma_r^2)$
$q_k \sim N(0, \sigma_q^2)$
$\varepsilon \sim N(0, \sigma_e^2)$

$$y_i' = \mu + g_j + \varepsilon_l \quad (12)$$

y_i': Fructan phenotype

μ: Mean

g_i: Random genotype effect ($i = 1,\ldots n$)

ε_j: Residual

$\underline{g_i} \sim N(0, \sigma_a^2\mathbf{K})$ [Cycles 0, 1, 1S, 2S]

$\underline{g_i} \sim N(0, \sigma_g^2)$ [Cycle 2]

$\varepsilon_j \sim N(0, \sigma_e^2)$

GS Accuracies

The realized accuracy of the GS models utilized in this study were computed as the Pearson correlation between the GEBV and TBV) (Falconer and Mackay, 1996). For realized accuracy calculations, the GEBVs were obtained from 2013 field and greenhouse materials and TBVs were obtained from 2016 field trials. To further confirm realized accuracies, the correlations between ranks of individual GEBV and TBV values were examined for each population. Additionally, leave-one-out cross validation (LOOCV) was performed per equation 9 for each population on 2016 phenotypes.

Correlated Agronomic Responses

To calculate correlated response to selection on grain fructan content for plant height and heading date, adjusted population means were estimated with Equation 6 ($p + \mu$) where y was the corresponding phenotype of plant height or plant heading date. Realized gain and percentage gain was calculated from population mean estimates. Realized gains were calculated by subtracting all population means by their respective

C_0 population means. Percentage gain for each population was calculated as realized gain divided by the corresponding C_0 population mean. Paired two-tailed *t*-tests were used to test differences in gain per cycle between the GS methods. One-sided, one-sample *t*-tests were used to test if genetic gains were significant. One-sided *t*-tests were used to test if heading dates for GBLUP populations were significantly greater than constrained population heading dates within each cycle.

Genetic values for individuals across environments for plant height and heading date were calculated with the mixed model in Equation 7Pearson correlations between genetic values for fructan content and agronomic traits were computed.

Inbreeding Assessment

To assess the impacts of GS on inbreeding, the level of observed inbreeding was compared to the expected level of inbreeding based on recalled SNPs and pedigrees. The expected level of inbreeding for each population was set equal to the average coancestry of selected individuals which served as parental material for the population (per Equation 5). Observed levels of inbreeding (f) based on markers for each population were estimated by computing the mean of the off-diagonal elements of the marker-based relationship matrix. The mean level of observed inbreeding for individuals within each population based on pedigrees was computed with the R package pedigreemm (Bates and Vazquez, 2014).

Paired two-tailed *t*-tests were used to determine if mean observed inbreeding levels differed significantly from expected levels for populations in each cycle. Paired one-tailed *t*-tests were used to determine if mean observed inbreeding levels for OCS

populations were significantly lower than GBLUP populations within each cycle. The inbreeding values utilized in the *t*-tests were mean inbreeding values for populations inferred from markers or pedigrees as described above.

Genetic Variance Assessment

To test the significance of difference of genetic variances between selection populations, populations were grouped by selection method and cycle, and assigned indicator variables. An analysis was performed on the genetic effects of the groups (y_i' per equation 12) allowing heterogenous genetic variance for the groups of entries: Cycle 0, Cycle 1 GBLUP, Cycle 1 constrained, Cycle 1S GBLUP, Cycle 1S constrained, Cycle 2 GBLUP, Cycle 2 constrained, Cycle 2S GBLUP, Cycle 2S constrained, and TP/Checks. Subsequent analyses were performed where groups within the same cycle (e.g., Cycle 1 GBLUP and Cycle 1 Constrained) were placed in the same group, thereby forcing them to have the same estimate of genetic variance. To determine the significance of difference of variance, a likelihood ratio test was performed. Under the likelihood ratio test, the difference between the -2 REML Log Likelihood values for the full and reduced models were computed and p-values were calculated based on the chi-square distribution (Saxton, 2004) with one degree of freedom because there were ten parameters in the first test and nine in the second test. All analyses for assessing genetic variance were performed in SAS PROC MIXED (SAS/STAT User's Guide, 2011).

Subset Selection

In order to determine if subsets of individuals selected in C_{1S} were random, C_1 GEBVs for each population were sampled 1,000 times to obtain estimates of mean and standard deviation of GEBVs for the population. For GBLUP, twelve GEBVs were randomly sampled. For constrained selection, twelve GEBVs were randomly selected and further optimized with the Meuwissen OCS method. Two-tailed *t*-tests were performed for each population to determine if there was a significant difference in means between the random samples and selected C_{1S} individuals.

Results

Realized Gain Trial

Observed wheat grain fructan content differed between selection populations (Figure 4.3). The observed fructan content for Caldwell and Ketola environments averaged 1.46g/100g (±0.3) and 1.39g/100g (±0.28), respectively. Narrow sense heritability across environments was 0.71. Estimated narrow sense heritabilities for Caldwell and Ketola were 0.63 and 0.69, respectively. Estimates of genetic values were consistent across environments with a correlation of 0.83. Correlation of genetic values between greenhouse and field locations ranged from 0.29 at Caldwell to 0.28 at Ketola. Additionally, correlation of genetic values between 2013 and 2016 field environments was 0.51. Adjusted population means are summarized in table 4.2.

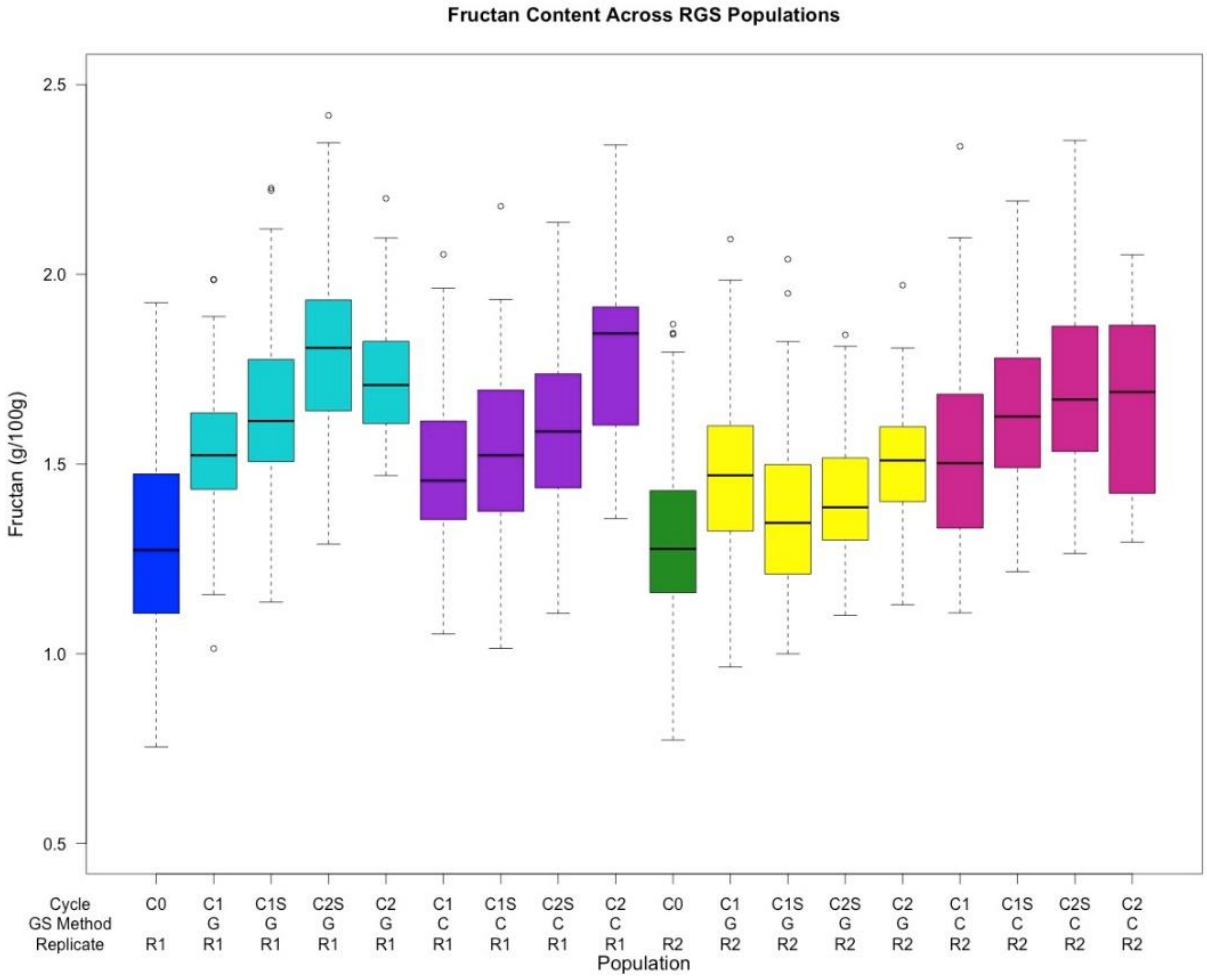

Figure 4.3: Observed fructan content of RGS populations.

Table 4.2: Means for grain fructan content, plant height, heading date and level of inbreeding for each population and each selection method.
[+]C0, cycle 0; G-BLUP, genomic BLUP selection; Const., constrained genomic BLUP selection; C1, cycle 1; C1S, cycle 1 subset; C2, cycle 2; C2S, cycle 2 subset; Rep., replicate.
*Families.

Population[+]		Number of Individuals Evaluated	Mean Fructan Content (g/100g)	Mean Height (cm)	Mean Heading Date (Julian Days)	f (Markers)	f (Pedigree)
C_0	Rep. 1	58	1.320	92.2	151.1	0.052	0.000
	Rep. 2	54	1.327	88.1	151.5	0.038	0.000
	Mean	56	1.324 ± 0.004	90.2 ± 2.9	151.3 ± 0.264	0.045 ± 0.01	0 ± 0
C_1 GBLUP	Rep. 1	46	1.605	95.8	150.9	0.208	0.145
	Rep. 2	43	1.491	85.9	151.8	0.225	0.061
	Mean	45	1.548 ± 0.08	90.8 ± 7	151.3 ± 0.598	0.217 ± 0.012	0.103 ± 0.059
C_1 Const.	Rep. 1	43	1.586	93.0	150.7	0.080	0.086
	Rep. 2	46	1.533	85.6	151.7	0.104	0.046
	Mean	44	1.559 ± 0.037	89.3 ± 5.2	151.2 ± 0.733	0.092 ± 0.017	0.066 ± 0.028
C_{1S} GBLUP	Rep. 1	47	1.679	96.1	151.7	0.208	0.272
	Rep. 2	48	1.400	81.6	152.3	0.225	0.140
	Mean	48	1.539 ± 0.198	88.8 ± 10.3	152 ± 0.426	0.217 ± 0.012	0.206 ± 0.094
C_{1S} Const.	Rep. 1	46	1.574	92.3	151.2	0.080	0.125
	Rep. 2	45	1.684	82.9	151.7	0.104	0.123
	Mean	46	1.629 ± 0.078	87.6 ± 6.7	151.5 ± 0.335	0.092 ± 0.017	0.124 ± 0.002
C_2 GBLUP	Rep. 1	12*	1.774	88.6	151.8	-	0.305
	Rep. 2	13*	1.541	78.1	153.0	-	0.160
	Mean	13	1.657 ± 0.165	83.4 ± 7.4	152.4 ± 0.862	-	0.2 ± 0.102
C_2 Const.	Rep. 1	11*	1.829	92.4	151.2	-	0.202
	Rep. 2	9*	1.709	86.3	151.8	-	0.099
	Mean	10	1.769 ± 0.085	89.4 ± 4.3	151.5 ± 0.367	-	0.2 ± 0.073
C_{2S} GBLUP	Rep. 1	47	1.836	95.5	152.1	0.219	0.297
	Rep. 2	35	1.450	79.5	153.2	0.376	0.236
	Mean	41	1.643 ± 0.273	87.5 ± 11.3	152.7 ± 0.809	0.297 ± 0.111	0.266 ± 0.043
C_{2S} Const.	Rep. 1	43	1.632	85.7	151.7	0.115	0.177
	Rep. 2	46	1.751	81.6	152.6	0.145	0.170
	Mean	45	1.691 ± 0.084	83.7 ± 2.9	152.1 ± 0.663	0.13 ± 0.022	0.173 ± 0.005

Gain from Selection for Wheat Grain Fructan Content

Realized gains for wheat grain fructan content between GS cycles ranged between -0.008 - 0.236g/100g (Table 4.3) with total gains of 0.319-0.445g/100g (Figure 4.4). Percent total gains from two generations of GS with, and without, one cycle of subset selection for GBLUP selections were 24.4 ± 20.6 and 25.5 ± 12.5, respectively. For constrained selections, percent total gains from two generations of GS with, and without, one cycle of subset selection were 28.1 ± 6.4 and 34 ± 6.5, respectively (Table 4.3). Total gains were significant for both selection methods ($p < 0.001$). The differences between total expected and realized gains were significant for C_2 ($p = 0.005$) and C_{2S} ($p = 0.022$) (Figure 4.5). No significant differences in gain per cycle were detected between the GS methods ($p = 0.30$-0.88).

Table 4.3: Realized and expected gains from C_0 and previous cycles

Population		Realized Gain from G_0	% Gain from G_0	Expected Gain From G_0	Realized Gains From Previous Cycle	% Gain From Previous Cycle	Expected Gain From Previous Cycle
C_1 GBLUP	Rep. 1	0.284	21.5	0.178	0.284	21.5	0.178
	Rep. 2	0.164	12.4	0.058	0.164	12.4	0.058
	Mean	0.224 ± 0.085	16.9 ± 6.5	0.118 ± 0.085	0.224 ± 0.085	16.9 ± 6.5	0.118 ± 0.085
C_1 Const.	Rep. 1	0.265	20.1	0.167	0.265	20.1	0.167
	Rep. 2	0.207	15.6	0.055	0.207	15.6	0.055
	Mean	0.236 ± 0.041	17.8 ± 3.2	0.111 ± 0.079	0.236 ± 0.041	17.8 ± 3.2	0.111 ± 0.079
C_{1S} GBLUP	Rep. 1	0.359	27.2	0.251	0.075	5.7	0.073
	Rep. 2	0.073	6.0	0.173	-0.091	-6.4	0.116
	Mean	0.216 ± 0.202	16.6 ± 15	0.212 ± 0.055	-0.008 ± 0.117	-0.4 ± 8.5	0.094 ± 0.03
C_{1S} Const.	Rep. 1	0.253	19.2	0.098	-0.012	-0.9	-0.069
	Rep. 2	0.357	27.5	0.082	0.151	12.0	0.026
	Mean	0.305 ± 0.074	23.4 ± 5.9	0.09 ± 0.011	0.07 ± 0.115	5.5 ± 9.1	-0.021 ± 0.067
C_2 GBLUP	Rep. 1	0.453	34.3	0.293	0.169	12.8	0.116
	Rep. 2	0.214	16.7	-0.030	0.050	4.3	-0.087
	Mean	0.334 ± 0.169	25.5 ± 12.5	0.132 ± 0.228	0.11 ± 0.084	8.6 ± 6	0.014 ± 0.144
C_2 Const.	Rep. 1	0.508	38.5	0.181	0.243	18.4	0.014
	Rep. 2	0.382	29.4	0.144	0.176	13.9	0.089
	Mean	0.445 ± 0.089	34 ± 6.4	0.162 ± 0.026	0.21 ± 0.048	16.1 ± 3.2	0.051 ± 0.053
C_{2S} GBLUP	Rep. 1	0.515	39.0	0.306	0.156	11.8	0.055
	Rep. 2	0.123	9.8	0.153	0.051	3.8	-0.021
	Mean	0.319 ± 0.277	24.4 ± 20.6	0.229 ± 0.108	0.103 ± 0.075	7.8 ± 5.7	0.017 ± 0.053
C_{2S} Const.	Rep. 1	0.312	23.6	0.136	0.058	4.42	0.038
	Rep. 2	0.424	32.6	0.070	0.067	5.06	-0.011
	Mean	0.368 ± 0.08	28.1 ± 6.4	0.103 ± 0.046	0.063 ± 0.006	4.7 ± 0.5	0.013 ± 0.035

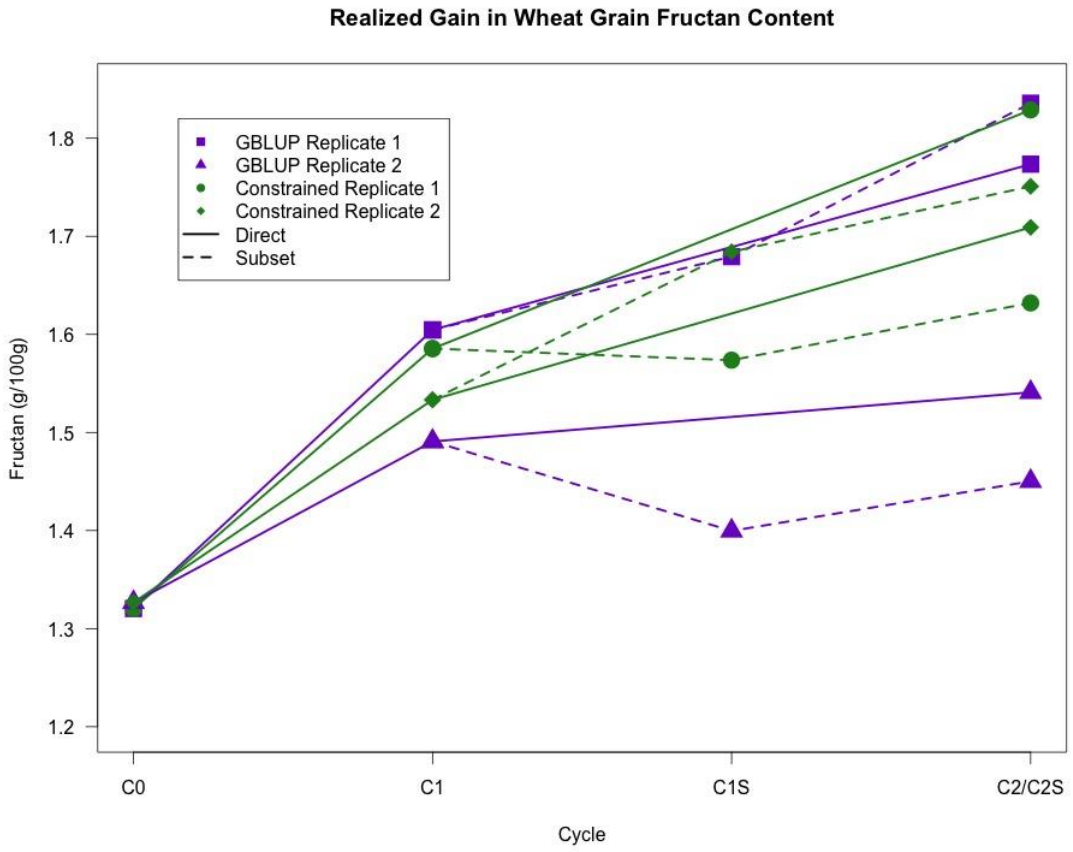

Figure 4.4: Realized gain of wheat grain fructan content over cycles for four populations. The solid lines represent populations selected directly (without random selection). The dashed lines represent populations resulting from random selection at cycle 1S.

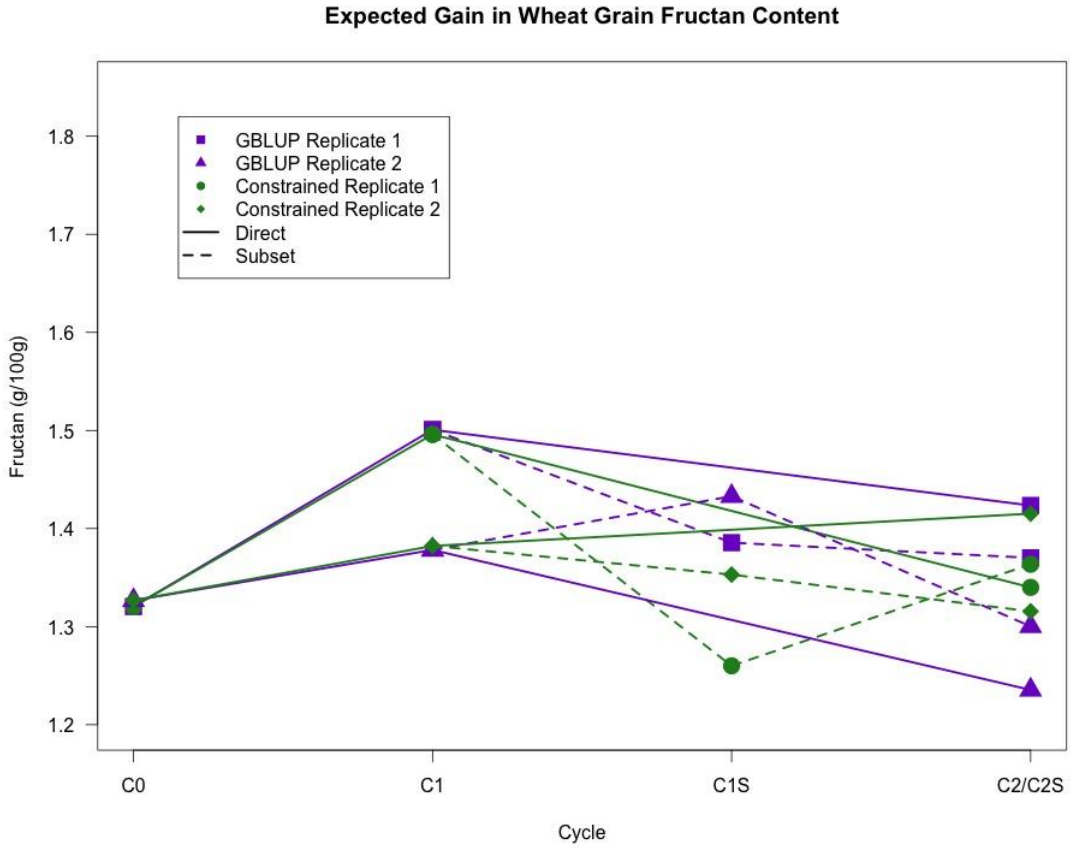

Figure 4.5: Expected gain of wheat grain fructan content over cycles for four populations. The solid lines represent populations selected directly (without random selection). The dashed lines represent populations resulting from random selection at cycle 1S.

Genomic Selection Accuracy

Mean realized GS accuracies among cycles ranged from -0.431 to 0.335 (Table 4.4). The mean correlations between ranks of individual GEBV and TBV values ranged between -0.428 and 0.305. The mean accuracies for the LOOCV with 2016 phenotypes within populations ranged between 0.851 and 0.937.

Table 4.4: Accuracies for GS and correlations of ranks between breeding values.

Population		Realized Accuracy	Rank Correlation Between TBVs, GEBVs	2016 LOOCV Accuracy
C_1 GBLUP	Rep. 1	0.496	0.486	0.945
	Rep. 2	0.173	0.124	0.926
	Mean	0.335 ± 0.229	0.305 ± 0.256	0.936 ± 0.014
C_1 Const.	Rep. 1	0.496	0.486	0.945
	Rep. 2	0.173	0.124	0.926
	Mean	0.335 ± 0.229	0.305 ± 0.256	0.936 ± 0.014
C_{1S} GBLUP	Rep. 1	-0.363	-0.343	0.796
	Rep. 2	-0.500	-0.513	0.905
	Mean	-0.431 ± 0.097	-0.428 ± 0.12	0.851 ± 0.077
C_{1S} Const.	Rep. 1	0.270	0.297	0.931
	Rep. 2	-0.162	-0.160	0.943
	Mean	0.054 ± 0.306	0.068 ± 0.323	0.937 ± 0.008
C_2 GBLUP	Rep. 1	0.738	0.711	0.796
	Rep. 2	-0.445	-0.375	0.905
	Mean	0.146 ± 0.837	0.168 ± 0.768	0.851 ± 0.077
C_2 Const.	Rep. 1	0.071	-0.076	0.931
	Rep. 2	0.596	0.478	0.943
	Mean	0.333 ± 0.371	0.201 ± 0.392	0.937 ± 0.008
C_{2S} GBLUP	Rep. 1	0.267	0.235	0.879
	Rep. 2	-0.091	-0.065	0.961
	Mean	0.088 ± 0.253	0.085 ± 0.213	0.92 ± 0.058
C_{2S} Const.	Rep. 1	0.104	0.196	0.883
	Rep. 2	-0.091	-0.087	0.906
	Mean	0.006 ± 0.138	0.055 ± 0.2	0.895 ± 0.017

Correlated Agronomic Responses

Selection for wheat grain fructan content had no effect on height over multiple cycles of GS ($p = 0.22$-0.64) (Table 4.5). Additionally, no correlation was observed ($r = -0.07$) between grain fructan content and plant height.

Wheat grain fructan content and heading date were negatively correlated ($r = -0.26$) but selection for wheat grain fructan content did not significantly change plant heading date over multiple cycles of GS ($p = 0.12$-0.23; Table 4.4). Realized gains and mean heading date indicate constrained selection populations had significantly earlier heading dates than GBLUP populations in C_{1S} ($p = 0.002$), C_2 ($p = 0.004$), and C_{2S} ($p = 0.039$) but not for C_1 ($p = 0.098$) (Tables: 4.2, 4.5).

Inbreeding

Observed inbreeding levels (f; Table 4.2) in constrained populations were significantly lower ($p < 0.001$) than inbreeding levels in GBLUP populations over all cycles based on marker data (Figure 4.7). Estimates of inbreeding levels inferred from pedigrees were also significantly lower ($p = 0.002$) in constrained populations relative to GBLUP populations for all cycles with the exception of the GBLUP replicate 2 population in C_2.

Observed inbreeding levels were significantly lower than estimated inbreeding values based on marker and pedigree data ($p = 0.01$ and $p < 0.001$, respectively).

Table 4.5: Realized gain for plant height and heading date for each population and each selection method

Population		% Realized Gain from C_0 Height	% Realized Gain from C_0 Heading
C_1 GBLUP	Rep. 1	3.87	-0.125
	Rep. 2	-2.54	0.187
	Mean	0.666 ± 4.53	0.031 ± 0.22
C_1 Const.	Rep. 1	0.82	-0.287
	Rep. 2	-2.84	0.151
	Mean	-1.01 ± 2.59	-0.068 ± 0.31
C_{1S} GBLUP	Rep. 1	4.21	0.388
	Rep. 2	-7.43	0.538
	Mean	-1.613 ± 8.23	0.463 ± 0.11
C_{1S} Const.	Rep. 1	0.11	0.090
	Rep. 2	-5.88	0.156
	Mean	-2.887 ± 4.24	0.123 ± 0.05
C_2 GBLUP	Rep. 1	-3.92	0.477
	Rep. 2	-11.32	1.034
	Mean	-7.62 ± 5.24	0.755 ± 0.39
C_2 Const.	Rep. 1	0.19	0.090
	Rep. 2	-2.02	0.185
	Mean	-0.92 ± 1.56	0.138 ± 0.07
C_{2S} GBLUP	Rep. 1	3.55	0.660
	Rep. 2	-9.77	1.167
	Mean	-3.11 ± 9.42	0.913 ± 0.36
C_{2S} Const.	Rep. 1	-7.06	0.373
	Rep. 2	-7.40	0.744
	Mean	-7.23 ± 0.24	0.559 ± 0.26

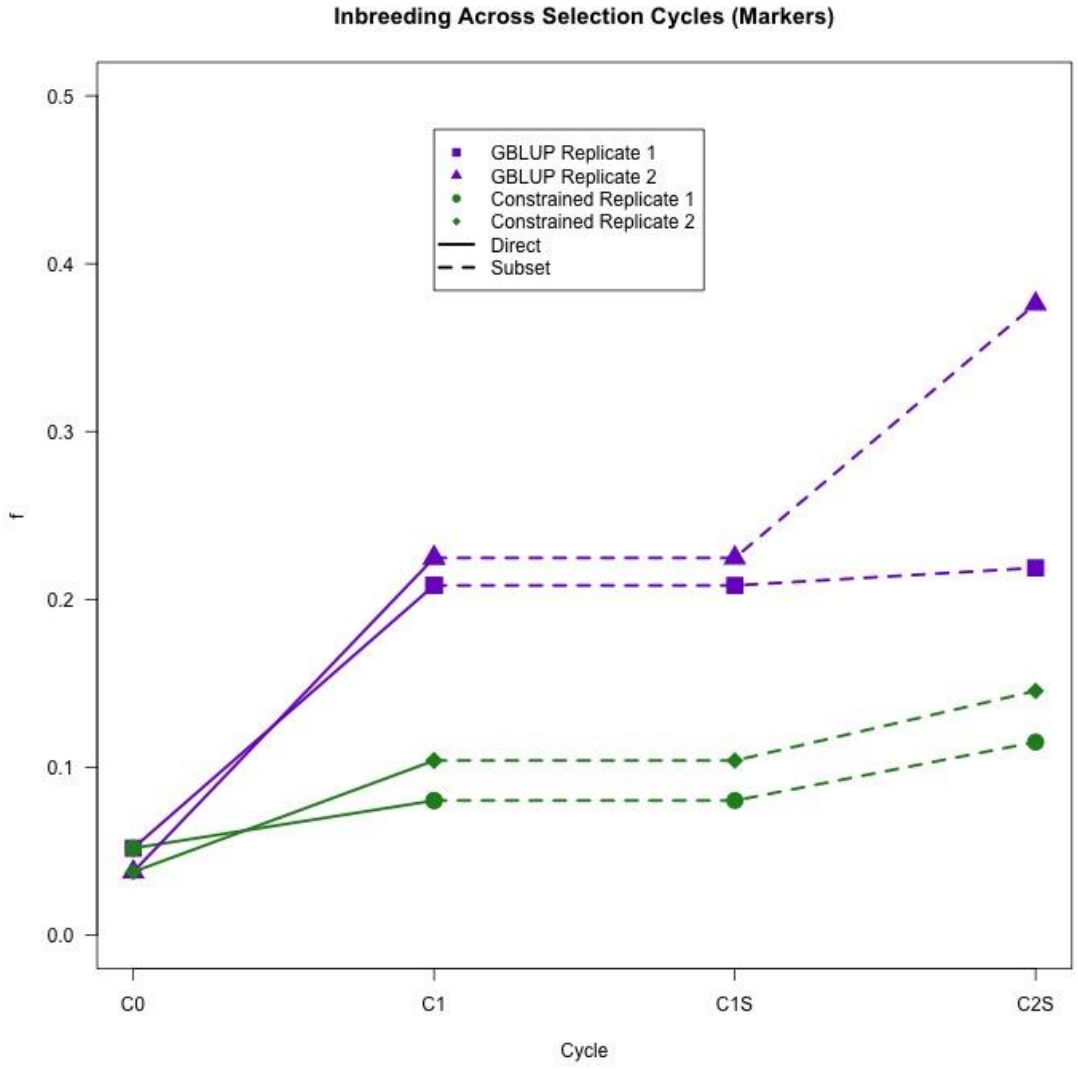

Figure 4.6: Inbreeding levels (f) inferred from GBS markers over cycles for four populations. The solid lines represent populations selected directly (without random selection). The dashed lines represent populations resulting from random selection at cycle 1S.

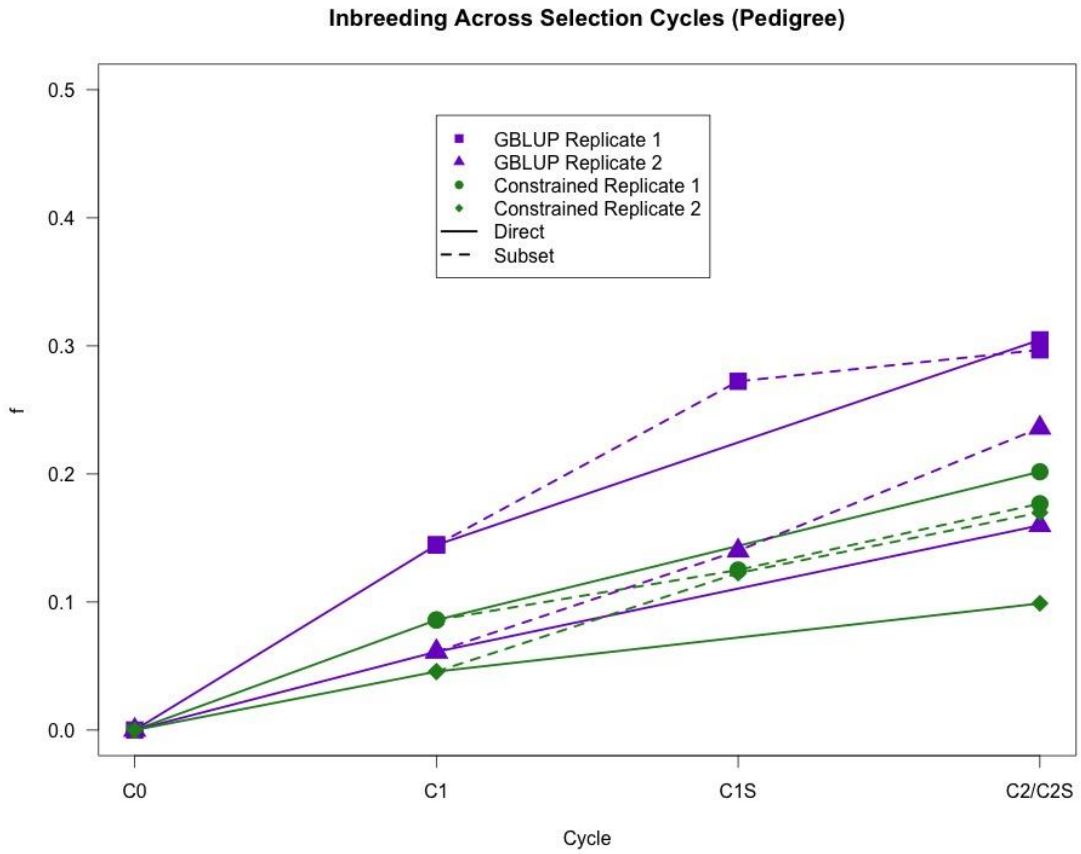

Figure 4.7: Inbreeding levels (f) inferred from pedigrees over cycles for four populations. The solid lines represent populations selected directly (without random selection). The dashed lines represent populations resulting from random selection at cycle 1S

Table 4.6: Expected and observed inbreeding for GBLUP and constrained selection populations.

Population		Expected Inbreeding (Markers)	f (Markers)	Expected Inbreeding (Pedigree)	f (Pedigree)
C_1 GBLUP	Rep. 1	0.236	0.208	0.422	0.145
	Rep. 2	0.240	0.225	0.281	0.061
	Mean	0.238 ± 0.002	0.217 ± 0.012	0.351 ± 0.1	0.103 ± 0.059
C_1 Const.	Rep. 1	0.123	0.080	0.231	0.086
	Rep. 2	0.133	0.104	0.178	0.046
	Mean	0.128 ± 0.007	0.092 ± 0.017	0.204 ± 0.038	0.066 ± 0.028
C_{1S} GBLUP	Rep. 1	0.230	0.208	0.604	0.272
	Rep. 2	0.351	0.225	0.367	0.140
	Mean	0.291 ± 0.086	0.217 ± 0.012	0.486 ± 0.168	0.206 ± 0.094
C_{1S} Const.	Rep. 1	0.134	0.080	0.355	0.125
	Rep. 2	0.171	0.104	0.257	0.123
	Mean	0.153 ± 0.026	0.092 ± 0.017	0.306 ± 0.07	0.124 ± 0.002
C_2 GBLUP	Rep. 1	0.527	-	0.667	0.3046875
	Rep. 2	0.448	-	0.427	0.15985577
	Mean	0.488 ± 0.056	-	0.547 ± 0.17	0.2 ± 0.102
C_2 Const.	Rep. 1	0.220	-	0.379	0.202
	Rep. 2	0.220	-	0.253	0.099
	Mean	0.22 ± 0	-	0.316 ± 0.089	0.2 ± 0.073
C_{2S} GBLUP	Rep. 1	0.343	0.219	0.767	0.297
	Rep. 2	0.631	0.376	0.662	0.236
	Mean	0.487 ± 0.204	0.297 ± 0.111	0.714 ± 0.075	0.266 ± 0.043
C_{2S} Const.	Rep. 1	0.174	0.115	0.448	0.177
	Rep. 2	0.256	0.145	0.391	0.170
	Mean	0.215 ± 0.058	0.13 ± 0.022	0.419 ± 0.04	0.173 ± 0.005

Genetic Variance

Estimated genetic variances between C_0 and C_1, as well as C_1 and C_2, within each selection method indicated significant reductions ($p < 0.001$; Table 4.7). There was no significant reduction in genetic variance estimates between C_1 and C_{1S} (p = 0.16-0.97) or C_{1S} and C_{2S} (p = 0.16-0.44) within each selection method. While the average level genetic variance of GBLUP selection populations after multiple cycles of selection was 50% lower than genetic variance in constrained selection populations, genetic variances within cycles were not significantly different between selection methods (p: 0.21 – 0.57; Table 4.7).

Table 4.7: Contrast of estimated genetic variances of genomic selection methods based on likelihood ratio test. G: GBLUP; C: Constrain.

Selection Methods	Difference in Variance	*P*-value†	Difference in Variance	*P*-value	Difference in Variance	*P*-value	Difference in Variance	*P*-value
	-Cycle 0 vs. Cycle 1 -		-Cycle 1 vs. Cycle 1S -		-Cycle 1 vs. Cycle 2 -		-Cycle 1S vs. Cycle 2S -	
GBLUP	-0.1021	<.0001	0.001271	0.9723	-0.06955	0.0805	-0.04366	0.4405
Constrain	-0.1148	<.0001	-0.03115	0.1626	-0.1074	0.0053	-0.04915	0.1646
	----------Cycle 1----------		----------Cycle 1S----------		----------Cycle 2----------		---------Cycle 2S----------	
G vs. C	-0.01272	0.5708	-0.04514	0.2166	-0.05054	0.3132	-0.05062	0.3649

Subset Selection

Mean GEBVs for selected individuals for in C_{1S} populations were not significantly different than the mean of randomly sampled GEBVs with the exception of replicate 1 of GBLUP (Table 4.8). A one-tailed *t*-test of replicate 1 of GBLUP found the selected individuals had a lower mean than the comparable random sample ($p = 0.013$).

Table 4.8: Mean GEBVs for selected individuals and 1,000 random samples in C_{1S}

Population		Selected Individual Mean GEBVs	Random Sample Mean GEBVs	p-value
C_{1S} GBLUP	Rep. 1	0.032	0.076	0.028
	Rep. 2	0.066	0.091	0.075
	Mean	0.049 ± 0.019	0.084 ± 0.004	
C_{1S} Const.	Rep. 1	0.079	0.062	0.409
	Rep. 2	0.048	0.084	0.054
	Mean	0.063 ± 0.011	0.073 ± 0.007	

Discussion

Effectiveness of Selection

For both GS methods, percent total gain was high (23.6-38.5%) with the exception of GBLUP replicate 2 (9.8% in C_{2S}) (Table 4.3). While subset selection did impact gain per unit time, it did not have a negative impact on overall gain as total gains for C_2 and C_{2S} were not significantly different ($p = 0.50$). It is worth noting that training populations were updated based on greenhouse material whereas the effectiveness of selection was evaluated within the 2016 field environment. The significant gains in wheat grain fructan content ($p < 0.001$) for both selection methods suggest that RGS is an effective breeding strategy for increasing wheat grain fructan content.

For GS cycles, percent gain per cycle was higher in C_1 (16.9-17.8%) than C_2 (8.6-16.1%). The large observed deviations in realized and percentage gains in GBLUP populations in C_2 were due to the small gains observed in replicate 2 (Table 4.3).

Realized and Expected Gains

Realized total gains were significant for both selection methods ($p < 0.001$) with total increase in grain fructan content ranging from 0.32 to 0.45g/100g over two cycles of selection.

Estimates of total realized gains exceeded total expected gains for C_2 ($p = 0.005$) and C_{2S} ($p = 0.022$). Expected gains from selection for cycles following C_0 were negatively influenced by low selection accuracies (Table 4.4) and small selection

differentials (Table 4.1). These small values can be attributed to the differences in individual performance between the greenhouse and 2016 field environments (Table S4.1).

Realized Accuracies

Realized accuracies of GS across populations averaged 0.109. While there was a wide range of realized GS accuracies observed (-0.431 to 0.335), the realized accuracies were low given the significant positive realized gains observed for wheat grain fructan content. Further examination of the correlations of ranks between GEBVs of material grown in the greenhouse and TBVs of material grown in the 2016 field season found correlations were similar to the realized GS accuracies (-0.428 to 0.305) with an average of 0.095.

The 2016 growing season from which TBVs for GS accuracies were estimated was an abnormally dry growing season. Observed rainfall between planting (October 2015) and harvest (July 2016) averaged 75% of normal. During the peak of the growing season, May through July, observed rainfall was 45.3% of normal. Given that fructans act as osmoregulators and are involved in physiological functions under drought conditions, it is possible some crossover genotype-by-environment interactions (GxE) occurred in the realized gain trial which resulted in low GS accuracies. The low correlation of genetic values between the greenhouse and 2016 field environments (r: 0.28-0.29) support the hypothesis that crossover GxE was present in this study.

To assess GS accuracies without the presence of confounding crossover GxE interactions, a leave-one-out cross validation was performed for each population with phenotypic data collected in the 2016 realized gain trial. Accuracies of the LOOCV were high with an average accuracy of 0.91. The similarity between the correlation of performance ranks and realized GS accuracies along with high LOOCV accuracies also support the hypothesis that crossover GxE was present between environments in which TP materials were grown and the 2016 realized gain trial.

Impact of Selection on Inbreeding and Genetic Variance

Inbreeding levels for all selected populations increased throughout the study. Though inbreeding increased, inbreeding levels in constrained populations were significantly lower than inbreeding levels in GBLUP populations based on markers ($p < 0.001$) and pedigree records ($p = 0.002$)..

Observed inbreeding levels were significantly lower ($p \leq 0.01$) than estimated inbreeding values for all cycles. Expected inbreeding levels were estimated based on empirical contributions of selected individuals to the subsequent generation under the assumption of random mating. Given that individual plants were selected and intermated, it is entirely possible that mating was not fully randomized within the study thereby leading to lower than expected levels of observed inbreeding. Use of more plants representing selected individuals would have facilitated more random mating and potentially resulted in observed inbreeding levels that were more in line with expected values.

Genetic variance significantly decreased ($p < 0.001$) with the first cycle of GS (C_0 to C_1) for both methods and with the second cycle of GS ($p = 0.005$; C_1 to C_2) for the constrained method. This loss of genetic variance is in accordance with previous studies which found the empirical application of GS results in significant decreases in genetic variance (Asoro et al., 2013; Rutkoski et al., 2015). The populations resulting from the constrained selection approach contained 50% more genetic diversity than GBLUP populations. Though constrained populations contained more genetic diversity than GBLUP populations after two cycles of selection, the difference between populations was not significant for C_2 ($p = 0.31$) or C_{2S} ($p = 0.36$).

Correlated Responses

Selection for wheat grain fructan content was not associated with a change in plant height ($p = 0.22$-0.64) or heading date ($p = 0.13$-0.23). Heading dates for constrained populations were significantly earlier than GBLUP populations for all selection cycles ($p = 0.002$-0.039) with the exception of C_1 ($p = 0.098$); however, the differences in mean heading date between the populations were no more than one day.

Conclusion

This study showed GS for wheat grain fructan content with two selection methods was successful over two cycles of selection. Though subset selection did decrease the gain per unit time, there was no significant difference in total gains between C_2 and C_{2S}. Additionally, no significant difference was observed in total gains between GBLUP and constrained selection methodologies. Populations

developed with the OCS selection methodology exhibited lower levels of inbreeding and greater levels of genetic variance relative to populations developed with GBLUP selection.

Realized gains far exceeded expected gains estimated with field computed selection differentials. The expected gains from selection were negatively influenced by low selection accuracy and small selection differentials. Realized GS accuracies were lower than expected based on observed realized gain. Further investigation of individual performance between greenhouse and 2016 environments found non-correspondence of ranks for selected individuals and low correlations of genetic values between greenhouse and 2016 field environments. The non-correspondence and low correlations in combination with high cross-validation prediction accuracies within 2016 environments support the hypothesis that crossover GxE was present between environments for GEBV and TBV estimation. The presence of crossover GxE may also explain lack of improvement in selection effectiveness in later cycles of selection with updated TPs. The influence of selection for wheat grain fructan content and plant height were negligible, whereas, the influence on heading date were more pronounced with constrained populations exhibiting earlier heading dates relative to GBLUP selection populations with the exception of C_1.

The GS scheme used in this experiment was well adapted for the generation time for winter wheat; however, the implementation of this GS scheme on a larger scale will require further logistical considerations. The genotyping, selection, and crossing of individual plants can limit the ability to cross plants with varying

maturities. Further research is needed determine if OCS methods provide reduce inbreeding rates relative to GBLUP selection in long term empirical GS studies.

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Supplementary Materials

Table S4.1: Mean ranks of selection candidates by cycle in the greenhouse (GH) and 2016 field trial

Population		Number of Selection Candidates	Number of Selected Candidates	GH Rank Selected Candidates	2016 Rank Selected Candidates
C_1 G-BLUP	Rep. 1	41	12	6.5	12.5
	Rep. 2	37	12	6.5	18.5
	Mean	39 (2)	12 (0)	6.5 (0)	15.5 (3)
C_1 Const.	Rep. 1	41	17	27.7	15.1
	Rep. 2	37	15	13.1	18.3
	Mean	39 (2)	16 (1)	20.4 (7.3)	16.7 (1.6)
C_{1S} G-BLUP	Rep. 1	46	12	35.2	13.0
	Rep. 2	43	12	17.4	32.2
	Mean	44.5 (1.5)	12 (0)	26.3 (8.9)	22.6 (9.6)
C_{1S} Const.	Rep. 1	43	20	26.3	23.8
	Rep. 2	44	19	25.3	22.0
	Mean	43.5 (0.5)	19.5 (0.5)	25.8 (0.5)	22.9 (0.9)
C_2 G-BLUP	Rep. 1	43	18	6.5	32.3
	Rep. 2	43	12	6.5	30.3
	Mean	43 (0)	15 (3)	6.5 (0)	31.3 (1)
C_2 Const.	Rep. 1	46	12	9.5	18.2
	Rep. 2	44	18	13.8	25.8
	Mean	45 (1)	15 (3)	11.6 (2.1)	22 (3.8)
C_{2S} G-BLUP	Rep. 1	45	12	6.5	22.1
	Rep. 2	48	12	6.5	27.3
	Mean	46.5 (1.5)	12 (0)	6.5 (0)	24.7 (2.6)
C_{2S} Const.	Rep. 1	46	20	11.0	21.7
	Rep. 2	40	19	11.2	23.2
	Mean	43 (3)	19.5 (0.5)	11.1 (0.1)	22.4 (0.8)

CHAPTER 5

CONCLUSION

The overall goal of this work was to apply genomic selection for nutritional breeding in wheat. Wheat grain fructans were selected as the breeding target due to their physiological roles in plants and humans, and their potential application in developing climate resilient, nutritionally improved wheat varieties. Before this work, genotypic variation for fructan content in wheat was known; however, the variation had not been widely capitalized on within wheat breeding programs. There were few empirical studies examining the application of genomic selection (GS) in wheat, especially in breeding for nutritional quality. The existence of genotype-by-environment (GxE) interactions for wheat grain fructan content was unclear based on literature. In order to perform GS in a time-efficient manner for grain composition in winter wheat, selection and crossing of materials must be performed in the greenhouse with optimal training population updates relying on phenotypes from greenhouse seed. If strong GxE existed for grain fructan content, the use of GS within the greenhouse environment would potentially limit the translation of gains from selection to field environments. Additionally, the observed loss of genetic variance and rapid rates of inbreeding within simulation and empirical studies of GS caused widespread concern regarding the long term sustainability of selection gains from GS. Several methods of inbreeding constraint through optimal contribution methods had been proposed in animal breeding; however, the constrained selection methodologies had never been applied in empirical plant breeding studies.

Before generalizing how this work has contributed to the greater body of knowledge, it must be emphasized that the results and conclusions presented here were drawn based on a small number of populations which may not be representative of other wheat populations.

This work has helped to fill several gaps in knowledge about wheat grain fructans and application of GS methodologies for nutritional breeding. Wheat grain fructan content is significantly influenced by genotype, environment, and GxE; however, the impact of GxE interactions on wheat grain fructan content is small. The genetic architecture underlying wheat grain fructan content is supportive of GS. Gain from selection with GS in greenhouse environments translated to the field environment. Evaluating new germplasm developed with GS over the span of multiple years in outdoor field trials is crucial to ensuring extreme growing season conditions do not result in crossover GxE which can negatively impact realized selection accuracies and gains per cycle of selection. Additionally, the use optimum contribution selection (OCS) methods in GS controls inbreeding levels and retains more genetic variance relative to GBLUP methods.

Several topics worthy of additional research have arisen as a result of this work. The changes in grain composition (i.e. proteins, lipids, other carbohydrates) as a result of selection for fructan content within the wheat grain were not evaluated in this study. While total wheat grain fructan content was increased in this study, it is unknown what fructan structures are present within the grains of improved germplasm. Further examination of the distribution of fructan structures present in the wheat grains of high fructan varieties will provide valuable information regarding varieties which are most

resistant to yeast and heat mediated fructan degradation in the development of wheat-based food products. Lastly, the development and empirical evaluation of methods to adequately control inbreeding over multiple generations of GS is needed. While OCS methods aided in conservation of genetic variance and control of inbreeding, development of methods to control inbreeding with the comparison of with equal numbers of selected individuals between selection methodologies will be valuable in finding truly effective inbreeding constraints to be imposed within GS framework.