Contribution of High Molecular Weight Glutenin Subunits to Genetic Variance for Gluten Strength

BY

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A Thesis

Submitted to the Faculty of Graduate Studies in Partial Fulfillment of the Requirements for the Degree of

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ABSTRACT

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Gluten strength is very important for bread making quality of wheat. Gluten is a continuous protein network which contains two protein groups: gliadins and glutenins. Glutenins consist of two distinct classes known as high molecular weight (HMW) glutenins and low molecular weight (LMW) glutenins. These two classes interlink by disulphide bonds between cysteine amino acid residues to form large polymers conferring elasticity of wheat dough. Gliadins are mostly monomeric proteins which interact with glutenin polymers by non-covalent bonds.

The contribution of HMW glutenins to gluten strength has long been recognized. The type and amount of these subunits present in wheat cultivars, are very important for gluten strength and bread making quality.

HMW glutenin subunits are encoded at three loci on group 1 chromosomes of hexaploid wheat. These loci have two genes encoding two types of HMW glutenin subunits. As recombination between the genes encoding x- and y- types of subunits on the same chromosome rarely occurs they are designated as loci Glu-A1, Glu-B1 and Glu-D1.

In this study, the proportion of variability for gluten strength contributed by each of these loci was estimated. A doubled haploid (DH) population was produced from a cross between the wheat variety AC Karma, a weaker gluten type and a breeding line 87E03-S2B1 which has extra strong gluten characteristics. The population was grown in the field for two years, at four locations in total. The DH population segregated for HMW glutenins, but the parental lines were very similar for LMW glutenins and gliadins, hence, only a few minor subunits segregated for these two prolamin classes.

HMW glutenin composition of DH lines was determined using SDS-PAGE. DH lines were tested for gluten strength by the SDS-sedimentation and mixograph tests for both years and all sites. The effect of Glu-D1 encoded subunit pair 5+10 was found to have the greatest contribution to gluten strength, followed by the effect of overexpressed Bx7 subunit encoded at Glu-B1 locus.

Estimated variability for gluten strength, contributed by Glu-D1 encoded subunits was 43% for mixing development time. Variability estimate for Glu-B1 locus was 12%. Additive variability for energy to peak contributed by the same loci was 51%. Incorporation of these subunits into a breeding population will utilize a considerable portion of the variability for gluten strength.

In order to assist breeders in early generation selection for gluten strength, PCR primers for HMW glutenin genes at all three loci have been designed and tested .

PCR tests for the HMW glutenins can be used in early plant development i.e., prior to crossing, using DNA from leaf tissue. These markers could also be used during the production of DH lines because the lines can be tested for their HMW genotype on haploid leaf tissue prior to the labor intensive step of chromosome doubling.

FOREWORD

Material presented in this thesis is organized in two manuscripts. Manuscript #1 *"SDS-PAGE and PCR-based marker system applied to breeding for gluten strength"* is written in the format required by the journal, *Molecular Breeding*, Kluwer Academic Publishers. Manuscript #2 entitled: "*Genetic variance for gluten strength contributed by high molecular weight glutenins,*" will be submitted to, *Cereal Chemistry*, American Association of Cereal Chemists.

1.0 INTRODUCTION

Wheat is a widely adapted cereal crop, grown around the world. Globally, wheat makes up almost one-third of annual cereal production in terms of area under cultivation and total yield (Shewry and Tatham 1997). Canada contributes 20% of world market share and produced 26 million tonnes of wheat (Canadian Wheat Board, Report on global wheat market, May 1, 2001). About 95% of world wheat production is bread wheat, while the rest of the production is durum wheat used for pasta and other products (Bushuk 1994). Therefore, suitability for bread making is an important quality criterion that contributes to the commercial value of wheat.

Wheat flour mixed with water forms a dough which has unique characteristics compared to other cereal crops, and may be processed into various products including leavened bread. Wheat dough has elasticity (strength) and extensibility (viscous flow).The balance between these two physical properties makes dough suitable for different end-uses (Shewry and Tatham 1997). Visco-elastic properties are mainly influenced by protein components, but interaction with other components, such as lipids, which have the role of plasticisers, have been reported (Shewry and Tatham 1997). Based on its unique physical characteristics, dough has the ability to retain gases produced as a result of yeast fermentation.

Abundant evidence exists to show that wheat proteins influence rheological dough

properties (Wrigley 1993). Gluten proteins designated as prolamins compose the majority of wheat storage proteins. There are two groups of proteins: gliadins and glutenins. The latter group can be further subdivided into high and low molecular weight glutenins. Quality differences between wheat varieties are influenced by differences in their protein content and composition. Glutenin subunit composition is determined by the genetic make-up of the cultivar. In spite of the tremendous work done in this area, knowledge is not complete due to the large number of prolamin subunits, their complexity and interactions, as well as, contribution of various of other factors. An understanding of glutenin proteins effect, on functional quality, their genetics, biochemistry and properties of subunits, is valuable for plant breeders devoted to the development of new varieties with improved bread making quality.

This thesis project investigated the relationship between high molecular weight glutenin subunits (HMW) and gluten strength. A doubled haploid population derived from the cross between wheat cultivar AC Karma and line 87E03-S2B1 was used in the study. The parental cultivars have different HMW glutenin subunits while the other two classes of gluten proteins i.e., the gliadins and the low molecular weight glutenin subunits (LMW) are similar.

This research had the following objectives:

1. To determine the contribution of individual HMW glutenins and their interactions

to genetic variability for gluten strength using a population derived from a cross between Canadian Prairie Spring Wheat cultivar AC Karma and Canadian Western extra strong line 87E03-S2B1, derived from cultivar Glenlea.

2. To develop molecular markers associated with three high molecular weight glutenin genes and assess their use in the marker assisted selection for gluten strength.

2.0 LITERATURE REVIEW

2.1 Wheat quality

Wheat quality is a very complex trait. In order to define quality and examine factors contributing to this complex and quantitative trait, it is useful to define the basic quality parameters which determine wheat type. These are hardiness, gluten strength and protein content (Bushuk 1994). Some of the quality aspects are controlled exclusively by genetics and some are strongly influenced by the environment (Bekes and Gras 1999). Bread making potential is directly proportional to the protein content (Bekes and Gras 1999). Protein content is strongly influenced by growing conditions, particularly availability of nitrogen (Pechanek et al. 1997). Another quality parameter that is important in bread making is hardness. Both hard and soft wheat exist. Hard wheat has a hard kernel, high gluten strength and protein content and it is suitable for bread production. Soft wheat has a soft kernel, low protein content and is used principally for cookie and biscuit production.

Gluten proteins confer the viscoelastic properties of dough, which are considered critical for bread making properties of wheat varieties. Gluten proteins designated prolamins compose the majority of wheat storage proteins and consist of two distinct groups: gliadins and glutenins. Pomeranz (1965) published one of the first reports on gluten proteins as a contributing factor to bread making quality.

Differences between cultivars in the proportion of the 3 M urea soluble proteins were associated with bread making performance. Good bread making cultivars contained a higher proportion of insoluble glutenins.

2.2 Wheat proteins

Osborn (1907) proposed the first classification of wheat proteins based on their solubility. According to this classification wheat endosperm proteins are divided into four fractions: water-soluble albumins, salt-soluble globulins, alcohol-soluble gliadins and glutenins and proteins insoluble in 70% ethanol, but soluble in dilute aqueous acid or alkali. Albumins and globulins are enzymes and their substrates are endosperm storage components, starch and storage proteins as well as structural components of cell wall, components of lipoproteins and glycoproteins (Wrigley 1993). Gliadins and glutenins are deposited in protein bodies in the endosperm and during kernel development they accumulate disproportionately compared to other proteins (Shewry and Tatham 1997). They serve as a nitrogen source for developing embryo.

2.2.1 Wheat storage proteins- Glutenins and Gliadins

Gliadins and glutenins are the major wheat storage proteins accounting for 80% of total flour protein. They contribute to the viscoelastic properties of dough produced from wheat flour (Gras et al. 1999). Glutenins form very large polymers with a

molecular weighst above one million (Payne and Corfield 1979). Based on electrophoretic mobility in SDS-PAGE glutenin, subunits are classified into two main types: low molecular weight glutenin subunits (LMW-GS) which are electrophoretically faster subunits with molecular weights between 25,000-45,000 daltons, and electrophoretically slower moving high molecular weight glutenin subunits (HMW-GS) with molecular weights of 60,000-90,000 daltons.

Gliadins are monomeric proteins and can be divided into four groups: α, β, γ and ω gliadins. Omega gliadins are a distinct class of gliadins due to a deficiency of sulfur and a different secondary structure (Tatham et al. 1990).

Another classification has been proposed by Shewry et al. (1992), based on the sulfur content. Prolamins are classified into three groups: sulfur-rich prolamins which include α-, β-, γ-gliadins and LMW glutenins; sulfur-poor prolamins such as ω-gliadins and third group containing HMW glutenins. While α- and γ-gliadins contain a considerable level of sulfur containing amino acids (2-3 mol%), ω-gliadins have little or no sulfur containing amino acids. Omega gliadins are rich in glutamine, proline and phenylalanine while the proportion of these amino acids is low in α- and γ-gliadins. Presence or absence of sulfur in their primary structure affects their ability to form disulfide bonds which are crucial in the polymer formation. Classes of α- and γ-gliadins do form intramolecular disulfide bonds. Gluten polypeptides have amino acids with hydrophobic side chains which permit interaction between polypeptides via hydrogen bonds.

In spite of structural differences between prolamins, a general model can be established describing their basic molecular structure. Each has a central repetitive domain flanked by N-terminal and C-terminal domains. Major differences in structural organization are in the length of all three domains and their primary structure. A class of S-rich prolamins (α- and γ- gliadins and LMW-glutenins), along with S-poor prolamins (ω-gliadins) have a tetrapeptide motif PQQP*[1]. In addition, a hexapeptide motif QQQQPVL exists in the repetitive domain of LMW glutenin subunits. Similarly, PQQP tetrapeptide is interspersed with different hexapeptide motif, PQPQPFP in α gliadins. In ω gliadins the PQQPFPQQ motif is the core repeat sequence (Tatham et al. 1990).

Another similarity between α-, γ-gliadins and LMW-glutenins is the presence of a very large C- terminal domain taking up over one-half to two-third of the polypeptide length. Cysteine residues are located in the C-terminal domain of S-rich prolamins while the LMW glutenin subunits contain cysteine residues in the N-terminal domain, as well. Usually, seven cysteine residues are present in the C- terminal domain of LMW glutenin subunits and one cysteine residue in the N-terminal domain or in the repeated domain (Tatham et al. 1990). LMW glutenins have a very short repetitive domain and six of the seven cysteine residues of the C-terminal domain are involved in formation of three intramolecular disulphide bonds. The remaining cysteine residue, along with the cysteine from the N-terminal domain are

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[1]*G-Gly; Q-Gln; P-Pro; Y-Tyr; S-Ser; V-Val ; L-Leu ;F-Phe

available for intermolecular cross linking.

There is another criterion for grouping α-, γ-gliadins and LMW glutenins in the same group, based on similarity in their secondary structure. The C-terminal domain contains α-helix and β-sheet structure and some β-turns. The β- turns are the predominant conformation in the repetitive domain (Shewry et al. 1998). The secondary structure of ω-gliadins has few α-helix and β-sheet structures, but β-turns are abundant.

2.2.2 High molecular weight glutenin subunits

High molecular weight glutenin subunits make up 10-20% of prolamin content and only 5-15% of total protein content (Shewry and Tatham 1997), but they are the single most important factor affecting bread making quality.

HMW glutenin subunits have a primary structure organized in three domains: N-terminal domain consisting of 81-104 amino acid residues, a large central repetitive domain consisting of 480-700 amino acid residues, and a C-terminal domain with only 42 amino acid residues (Shewry et al. 1992). HMW glutenin subunits are divided into x- and y-type according to their electrophoretic mobility. The x-type subunits have a central repetitive domain of 650-700 amino acid residues, while the repetitive domain of y-type subunits ranges from 435 to 540 amino acids (Tatham et al. 1992). The x-type subunits have tripeptide, hexapeptide and nonapeptide

motifs, GQQ, PGQGQQ and GYYPTSPQQ, respectively. The y- type subunits do not have the tripeptide motif, and the hexapeptide PGQGQQ and the nonapeptide consensus motif GYYPTSLQQ are present in their primary structure (Shewry et al. 1992).

The cysteine residues in the HMW glutenin subunits are positioned in the N-terminal and C-terminal domains with an exception of few y-subunits with cysteine residues in the central repetitive domain toward the C-terminal end (Bushuk 1994). The number of cysteine residues is considerably higher in the N-terminal domain. Usually three cysteine residues are found in the x-type subunits and five in the y-type subunits but only one cysteine residue in the C-terminal domain in both subunit types of HMW glutenins.

HMW glutenins have the α-helix in both terminal domains, but β-turns are predominant in the repetitive domain, similarly to the ω-gliadins. The central repetitive domain of HMW glutenins has a form of loose spiral based on repetitive β turns, flanked by N-terminal and C-terminal domains rich in α-helix and cysteine residues (Field et al. 1987). The spiral structure of the central domain of HMW glutenins is considered as a very important contributor to the elastic properties of gluten. Also, the size of the repetitive domain may contribute to elastic properties (Kasarda 1999). The repetitive domain of HMW glutenins contains many glutamine amino acid residues (30-35 mol%) and their amide groups are involved in hydrogen bond formation between the different subunits of HMW glutenins and between

HMW glutenins and other proteins (Shewry et al. 1992).

2.3 Genetics of wheat proteins

Genes encoding wheat storage proteins, are positioned at nine major loci in the wheat genome. All ω-gliadins, most γ-gliadins and a few β-gliadins are encoded by alleles on the group 1 homeologous chromosomes at the Gli-1 locus (Shewry et al. 1992). The group 6 chromosome locus Gli-2, encode all α-, most β- and some γ-gliadins. Gliadin genes are tightly linked and those on the chromosomes 1 are closely linked with genes coding for LMW glutenin subunits.

LMW glutenin subunits are encoded by genes located on the short arms of homeologous group 1 chromosome at the Glu-3 loci. The Glu-3 loci and Gli-1 loci are tightly linked and no recombination has been reported. However, in a doubled haploid population produced in the cross RL 4452 x AC Domain (Agriculture and Agri-food, Canada), recombination of 3% between Gli-1, Glu-3 loci has been observed (O. Lukow and K. Adams, personal communication).

HMW glutenin subunits are encoded by genes located on the long arms of the homoeologous chromosome 1 in wheat. Genes encoding x- and y-type subunits are closely linked and designated as a single locus, Glu-1. Therefore, loci Glu-A1, Glu-B1 and Glu-D1 on the long arms of chromosomes 1A, 1B and 1D, respectively, encode HMW glutenin subunits. In bread wheat cultivars one or both genes on the

locus Glu-A1 are inactive (Payne et al. 1981) thus three to five subunits are usually expressed. The x-type subunits are slower moving in the electrophoretic field compared to faster moving y-type subunits. It was observed that HMW glutenin subunits display considerable allelic variations, which has been used to study the relationship between subunit composition and bread making quality.

2.4 Glutenin polymer

The glutenin subunits polymerize into native glutenin by disulfide bond formation between cysteine residues forming the largest naturally occurring polymers. There is considerable evidence suggesting that both HMW glutenin subunits and LMW glutenin subunits combine to form native glutenin polymer, but some studies where null lines have been used show that polymers might be formed from LMW-GS or HMW-GS, exclusively (Gupta et al. 1995).

Glutenin subunits linked together by disulphide bonds form a glutenin polymer with unique visco-elastic properties. A polymeric fraction of gluten is made up of a diversity of glutenin polymers. Their composition ranges from dimers (Mr 40,000-150,000) up to sizes estimated at several million daltons (Lafiandra et al. 1999). Glutenin polymers are established during the grain development when the synthesis of different proteins is intensified. Glutenin structure is determined primarily at the grain filling stage and maybe further modified by alterations of disulphide bonds resulting in changes in the polymer size distribution (Wrigley and

Bekes 1999). Further modifications of disulphide bonds can take place at the grain ripening stage. Rainy weather during ripening may lead to the changes in the glutenin structure (Wrigley and Bakes 1999). Furthermore, some hydrolases produced during sprouting are proteases that can partially hydrolyse peptide bonds in the glutenin proteins (Wrigley et al. 1999). In addition, grain in the field may be the target of insects from the genera Lygaidae, Eurygaster and Aelia. Proteases originating from insect saliva can cause changes in glutenin polymers and in the extreme cases the result may be a complete loss of bread making potential (Wrigley et al. 1999).

A number of models for glutenin polymer formation have been proposed. Ewart (1979) had proposed a model known as the "linear glutenin hypothesis." According to this model glutenin subunits are linked together in a head-to-tail manner by disulphide bonds formed in random order. In other words one disulphide bond is formed between two polypeptide chains composing linear polymers. Graveland et al.(1985) proposed a model where y-type subunits of HMW glutenins are linked at both ends to different x-type HMW glutenin subunits by disulphide bonds in a head-to-tail fashion. Four clusters, each containing three low molecular weight subunits are attached to the y-type subunits representing the core unit. Units are linked together by disulphide bonds between N-and C- termini of the x- type subunits, forming the backbone of the glutenin polymer. This model was based mainly on the results of partial reduction experiments showing dimers of HMW glutenin subunits. Kasarda (1989) proposed a model assuming that LMW glutenin

subunits are linked together in head-to-head manner by intermolecular disulphide bonds formed between cysteine residues in the C-terminal domains of these subunits. The HMW glutenin subunits are linked to the glutenin polymer by means of different LMW subunits at their N- and C-terminal domains, resulting in antiparallel packing of the central repetitive domains of LMW glutenin subunits. In this model, the polymer ends with the addition of a subunit containing only one cysteine residue available for intermolecular bond formation. Such subunits are designated "chain terminators" as opposed to "chain extenders," which can form two or more disulphide bonds. Some authors have recently confirmed the existence of those types of subunits (Kasarda, 1999). Glutenin polymers mainly consist of HMW and LMW glutenin subunits, but besides those fundamental constituents, gliadin like proteins have been detected in the polymers (Lafiandra et al. 1999). Gliadin-like subunits, containing an odd number of cysteine residues, play the role of "chain terminators" in the polymerization process, due to the presence of only one cysteine residue involved in intermolecular bond formation. Elongation of the glutenin polymer will be terminated with the incorporation of these gliadin-like subunits. These polypeptides are similar to gliadins, but only have one cysteine residue which confers termination of the glutenin polymer (Lafiandra et al. 1999).

Payne and Corfield (1979), extracted flour proteins with SDS buffer containing low levels of the reducing agent β-mercaptoethanol and characterized disulphide linked oligomers using electrophoretic techniques. Results showed that HMW glutenin subunits were involved in oligomer formation. Those results are incompatible with

Kasarda's model, which assumes linkage of HMW glutenin subunits to LMW subunits only. Partial reduction studies (Werner et al. 1992) revealed that x-y type dimers are predominant in the glutenin polymer. The presence of x-y dimers supports the hypothesis proposed by Graveland et al. (1985) who hypothesized that the backbone of the glutenin polymers is formed from x-y dimers of HMW glutenin subunits from which low molecular subunits branch in clusters consisting of LMW subunits, exclusively.

2.5 Role of the Dx5 type subunit in glutenin polymer formation

Gluten polymer size distribution is an important contributor to bread making quality of wheat (Kasarda 1999). The size distribution is influenced by the polymerization of polypeptides and lines with Dx5+Dy10 combination of HMW glutenin subunits (encoded at Glu-D1 locus) have considerably larger polymers than lines with Dx2+Dy12 combination of subunits, encoded at same locus (Kasarda 1999). Some evidence indicates that polymers containing 5+10 subunits are formed earlier than 2+12 subunits polymers suggesting that more rapid formation occurs. The difference between Dx5 and Dx2 subunits, which are almost identical in the primary amino acid sequence in first 100 residues, is in the amino acid residue at the position 97 which has mutated from serine in Dx2 to cysteine in Dx5 (Shewry et al. 1997). Therefore, the Dx5 subunit has 4 cysteine residues in the N-terminal and in the beginning of the repetitive domains as compared to three cysteines in the same region for the Dx2 subunit. The C-terminal domains of both subunits have one

cysteine residue involved in forming one intermolecular disulfide bond. The crucial difference between these two subunits in terms of polymerization ability is in the fourth cysteine in the Dx5 subunit. The fourth cysteine is located at some distance from the other three cysteines and it seems that this is the main factor contributing to the greater ability of Dx5 to form intermolecular bonds as compared to Dx2 (Kasarda 1999), this may explain why Dx5 is associated with larger polymers, but does not explain the rapid formation of polymers containing the 5+10 subunit combination.

The polymer formation speed in the lines containing 5+10 subunits may be explained by the "steric hindrance" hypothesis (Kohler et al. 1997). Their basic assumption was that the cysteine residues at the N-terminal domain are considered as a group. Initial cross-linking might occur from any of those three cysteines and probably that is a rapid event regardless of Dx2 or Dx5 subunit. After the first disulfide bond is formed, steric hindrance will affect any other glutenin subunits approaching the remaining cysteines in that cluster. In addition, the α-helix structure of that region seems to facilitate intramolecular disulfide bond formation between two cysteine residues. The possibility that more than one disulfide bond is formed, including cysteine from this cluster can not be ruled out, however steric hindrance is likely to slow down this process. Otherwise, the Dx2 and Dx5 subunits should have a similar chance to form the first intermolecular bond, but in Dx2 subunits formation of the next intermolecular bond is delayed while in the Dx5 subunit, the distant fourth cysteine residue is readily available for rapid bond formation.

According to this hypothesis, it is likely that the Dx5 subunit forms at least two intermolecular disulfide bonds because of lower steric hindrance, which seems to be the main feature contributing to the more rapid and effective polymerization and branching of Dx5 subunit.

Branching of polypeptide chains is a possible explanation for stronger dough obtained from lines containing three or more cysteine residues involved in intermolecular bonds. The current hypothesis about the contribution of polymer type to gluten strength proposes that linear polymers obtained from subunits with only two available cysteine residues for intermolecular cross links contribute less to dough strength than branched polymers (Kasarda 1999).

2.6 The role of Bx7 type subunit in glutenin polymer formation

The Bx7 subunit has three cysteine residues in the N-terminal domain, but the first two residues are closer to each other as compared to those of the Dx2 and Dx5 subunits. It is likely that the closely positioned cysteines (positions 10 and 17) form an intramolecular disulfide bond while the third cysteine in the N-terminal domain and one residue in the C-terminal domain are available to form intermolecular disulfide bonds (Kasarda 1999). Hence, polymers will be elongated in a linear fashion.

The contribution of the Bx7 subunit to quality has been observed in some cultivars

where this subunit is overexpressed and its effect is considered as quantitative. In cultivars with five expressed HMW glutenin subunits, the proportion of this prolamin class in total extractable protein is roughly 10%. Therefore, individual subunit genes account for approximately 2% of flour protein. Duplication observed in Bx7 gene (D'Ovidio et al. 1997) might explain the overexpression of Bx7 subunit, in which case, positive effects of this subunit on gluten strength can be attributed to an increased amount of subunit.

2.7 Comparison of subunits encoded at Glu-A1, Glu-B1 and Glu-D1 genes

An entire set of HMW glutenin gene sequences from the cultivar Cheyenne has been published (Anderson and Greene 1989, Anderson et al. 1998). A comparison of Ax2*, Bx7, Dx5, By9 and Dy10 sequences reveals that these genes have some similarities. HMW glutenin genes, like other cereal storage genes have no introns and their coding region is among the longest known examples of non-interrupted sequences (Anderson and Greene 1989). Furthermore, the promoter regions contain some conserved sequences involved in tissue specific expression and are found in the 5' flanking region of prolamin genes of other cereal species (Halford et al. 1987). In the Ax2* and Bx7 genes, a consensus polyadenilation signal is positioned 51 bp downstream of double stop codons found in HMW glutenin genes.

Deduced protein sequences revealed similarities between the polypetides, as well. A signal peptide of 21 amino acids was followed by the N-teminal domain consisting

of 87 amino acid residues in Ax2* and 82 in Bx7. Repetitive domains of these subunits contained 666 and 647 residues for Ax2* and Bx7, respectively, followed by the C-terminal domain of 42 amino acid residues in both genes.

The Ax2*, Bx7 and Dx5 subunits are similar to each other in terms of position of cysteine amino acid residues. Three cysteine residues are positioned in N-terminal domain, with the exception of Dx5 subunits which have a fourth cysteine at the beginning of repetitive domain and the C-terminal domain contains 1 cysteine amino acid residue.

Major differences between the amino acid sequences of Ax1 and Ax2* subunits have not been observed, which is consistent with their similar effect on quality (Tatham et al. 1992). The effect of Glu-A1 encoded subunits can be attributed to quantitative contribution, compared to null allele observed in some cultivars. The amount of HMW glutenin subunits is increased in cultivars with Glu-A1 encoded subunit and may be responsible for quality improvement.

2.8 Effect of HMW glutenin subunits on the gluten strength as a quality factor

The relationship between structure of glutenin proteins and their role in the quality aspects and dough properties has been the focus of research effort for several decades. The presence of certain HMW glutenin subunits has been positively correlated with good bread making quality. In an early study by Payne et al.(1979),

progenies from crosses between varieties with good bread making quality and varieties with poor bread making quality were selected for good bread making quality during generations of inbreeding. SDS-sedimentation was used to assess bread making quality because the volume of the sediment and bread making quality were positively correlated. Sediment was obtained after mixing flour with a solution of Sodium Dodecyl Sulfate (SDS) and lactic acid. Their finding was that "band1" was positively correlated with bread making quality. It was assumed that subunit 1 designated as "band 1" on the SDS-PAGE improves the properties of gluten for the bread making process, probably because of its property to form large and stable glutenin polymers. In a subsequent study, it was found that band 1 was controlled by chromosome 1A (Payne et al. 1980). Using the same approach, it was found that bands 5 and 10,and bands 2 and 12 were encoded by chromosome 1D. In order to study the closeness of genes encoding subunits 2 and 5 on the chromosome 1D, F2 progenies from the crosses between cultivar "Chinese Spring" with subunit 2 and "Holdfast" with subunit 5 were tested. Using intensity of endosperm banding pattern four phenotypic classes were detected in the ratio of 1:1:1:1. This phenotypic ratio represents two homozygous classes, for subunits 5 and 2, class of heterozygous endosperm with two doses of the subunits 5 and single dose of subunit 2 and phenotypic class containing doubled dose of the subunit 2 and single dose of subunit 5 in triploid endosperm tissue. It was estimated that maximal distance for genes encoding subunits 2 and 5 is 0.01 cM, suggesting that those genes might be allelic. The presence or absence of HMW glutenin subunits encoded by the alleles of the D genome has a significant effect on bread making quality (Payne et al.

1981). Subunits 2 and 12 were always detected together and subunit 5 was always accompanied by subunit 10. It suggested that genes encoding these subunits are closely positioned on the chromosome. Following the results of positive correlation between band 1 and bread making quality and positive effect of 5+10 subunits encoded by chromosome 1D, it was shown that the presence of all three subunits had a highly positive effect on SDS sedimentation volume as a measure of bread making quality. The effect of these subunits was found to be additive. Payne et al. (1987) have developed a scoring system for HMW glutenin subunits based mainly on the effects of these subunits on the SDS-sedimentation test as an indirect measure of bread making quality. The epistatic effects of alleles at the Glu-1 loci on quality have also been reported (Rousset et al. 1999). One-third to one-half of the variation in bread making quality is attributable to allelic variations at Glu-1 loci (Mir-Ali et al. 1999). Good correlation between dough strength parameters and the amount of insoluble protein were reported in many studies (Gupta et al. 1993, Payne et al. 1979). Flours of good quality have a greater proportion of insoluble glutenin. Allelic effects on dough strength were attributed to the amount and types of produced subunits, variation in quantity and the size distribution of polymeric proteins.

In addition to HMW glutenin subunit composition, the ratio of HMW to LMW glutenin subunits is an important factor in bread making quality. Varieties with greater dough strength were reported to have a higher HMW:LMW glutenin subunit ratio (Gupta and MacRitchie 1994). Dough strength increased with a higher HMW: LMW glutenin

subunit ratio (Weegels and Hamer 1992) indicating that bread making quality is governed not only by the quality of HMW glutenin subunit, but also by other factors such as the amount of glutenin proteins. Size distribution of the polymeric protein is influenced by the HMW:LMW glutenin subunit ratio which in turn is influenced by the quantity of individual subunits and their polymerization behavior, all of which are related to their structure and size (Gupta and MacRitchie 1994).

2.9 Breeding for bread making quality

Broad evidence suggests that bread making quality of wheat is mainly determined by gluten protein composition (Kasarda 1999). Therefore, these proteins should be the major targets in breeding for quality. The important step in the breeding process is the identification of the trait on which the selection process might be based. Subsequently, it is important to identify genotypes which have desirable phenotypes. For early generation selection, the method of choice would ideally be reliable, require a small amount of testing material, be highly correlated with end-use characteristics, be cost-efficient and amenable to screening a large number of samples. The SDS-sedimentation test has been used as an indicator for gluten strength. The use of the mixograph test allows breeders to select directly for physical dough properties in early generations (Gras and O'Brien 1992). Besides a mixograph test which utilizes 10 g of flour, a smaller scale test employing 5 g or 2 g of flour is used in breeding programs (Finney 1989, Gras and O'Brien 1992). Selection of cultivars with strong gluten using the mixograph test has given

considerable progress in quality improvement (Cox et al. 1991). However, the mixograph test is labor intensive because it requires the production of flour by milling. The test is time consuming, and only one sample can be analyzed at a time.

When HMW glutenin subunit composition was identified by SDS-PAGE and quality scores for different subunit compositions were assigned, statistical evaluation of the amount of variability for gluten strength attributable to HMW glutenin could be performed (Payne et al. 1987, Lukow et al. 1989). A few studies reported the effect of HMW glutenin alleles on gluten strength as inferred from the SDS sedimentation test for the alleles encoded at Glu-A1 locus. In the work of Payne et al. (1987) subunits designated as Ax1 and Ax2*, encoded at locus Glu-A1 had similar effect on quality, but were superior when compared to the null allele found in some cultivars. Mir-Ali et al. (1999) obtained different results of the effect of Glu-A1 encoded subunits in groups of genotypes differing in geographical origin. There was a significant effect of the Glu-A1 in two of four groups under study, while the other two groups of genotypes showed no significant effect of Glu-A1 alleles. Other studies (Rogers et al. 1989, Graybosch et al. 1994) also reported no relationship between Glu-A1 encoded subunits Ax1 and Ax2* and quality score. Similarly, contrasting effects for the combination of subunits encoded at Glu-B1 locus were found. Mir-Ali et al. (1999) reported that the Bx7+ By8 combination of subunits encoded at Glu-B1 locus demonstrated positive or negative effects depending on the group of cultivars tested. The most consistent results were reported for a comparison of Dx2+Dy12 subunits and Dx5+Dy10 subunits encoded at the Glu-D1

locus which showed a significantly higher positive effect of the latter combination. Correlations between glutenin subunit composition and gluten strength as quality traits are complex due to interactions between HMW glutenin subunits and between loci encoding other prolamins. Sontag-Strohm et al. (1996) showed a significant interaction between alleles encoding HMW glutenin subunits and LMW glutenin subunits. The effect of HMW glutenins was more evident in lines having the Ax2* subunit than in lines with the null allele at Glu-A1 locus. Mir Ali et al. (1999) suggested that different allelic combinations are differently expressed in different genotypes. The epistatic effects between different loci seem to make significant contribution to gluten strength, indicating that gluten quality is a very complex trait The effect of some subunit combinations has to be considered through its interaction with other alleles present in the particular genotype.

2.10 PCR-based DNA markers for glutenin subunits

HMW glutenin subunits are associated with gluten strength and a significant proportion of the variability can be accounted for by these subunits. Locus pairs encoding x- and y-type subunits of HMW glutenins are closely linked and they have been referred as single loci Glu-A1, Glu-B1, and Glu-D1 on three genomes of bread wheat. In hexaploid bread wheat the y-subunit encoded at the Glu-A1 locus is usually silent. In some bread wheat cultivars, the Ax allele is also null. Silent Glu-A1 pseudogene (Ay) which does not produce a polypeptide was cloned and sequenced by Forde et al. (1985). The Glu-D1 alleles, Dx5 and Dy10, were cloned from

Triticum aestivum L. cv. Cheyenne (Anderson and Greene 1989). The Glu-A1 (Ax2*) and Glu-B1 (Bx7) were cloned as well (Anderson and Greene 1989). DNA sequences from these clones have been made publically available, enabling the development of new methods for glutenin subunit composition assessment. SDS-polyacrylamide gel electrophoresis is commonly used to determine subunit composition in wheat cultivars and this method is widely used to screen breeding lines for glutenin subunit composition using single wheat kernels. Alternatively, Polymerase Chain Reaction (PCR) has been employed for screening alleles encoding glutenin subunits which can be tested using various plant tissue sources. DNA markers for glutenin alleles allow amplification of the fragment with the sequence unique for the particular glutenin subunit gene. DNA isolated from the seedlings of ten wheat cultivars was used to test the efficiency of PCR generated markers for HMW glutenin genes (Smith et al. 1994). A few studies reported the development and testing of PCR based markers for HMW glutenin genes in durum and bread wheat (D'Ovidio et al. 1994, D'Ovidio and Anderson 1994, D'Ovidio et al. 1995a, D'Ovidio et al. 1995b, Lafiandra et al. 1996, DeBustos et al. 2000).

3.0 MANUSCRIPT #1

Gene-assisted selection for gluten strength in wheat doubled haploid breeding programs

3.1 ABSTRACT

High molecular weight glutenin subunits contribute greatly to gluten strength. SDS-PAGE analysis is commonly used to assess HMW glutenin subunit composition of wheat seed. This type of protein analysis is not suitable to screen haploid material or any tissues which do not express the HMW glutenins because it is a protein assay based on seed extract. DNA based molecular markers have been developed to bypass the limitations of SDS-PAGE analysis and broaden the application of marker assisted breeding for HMW glutenin alleles. A doubled haploid population was produced from a cross between a white extra strong wheat line 87E03-S2B1 and Canada Prairie Spring cultivar, "AC Karma", which has much weaker gluten. Molecular markers associated with HMW glutenin alleles Dx5, Ax2* and the overexpressed Bx7 allelic variant were developed. PCR analyses were performed on haploid leaf tissue. Doubled haploid seeds were subjected to SDS-PAGE for characterization of HMW glutenin protein patterns and to confirm the accuracy of the PCR markers. PCR markers showed discrepancies of 2-8.5% when compared to SDS-PAGE. Advantages and disadvantages of DNA markers in breeding programs and doubled haploid line production are discussed.

3.2 INTRODUCTION

Prolamins are seed storage proteins comprising the high molecular weight (HMW) glutenins, the low molecular weight (LMW) glutenins and the gliadins. Glutenin subunits interlinked by disulphide bonds form gluten polymers. Other types of bonds such as hydrogen bonds or tyrosine cross linking are also present (Michon et al. 1999). Glutenin subunits (LMW and HMW) together with monomeric gliadins, which are not covalently linked to glutenin polymers, form a gluten complex with unique visco-elastic properties. Quality of gluten is measured as strength and elasticity. Gluten strength is a very important quality attribute for a number of end-use products, including leavened bread products.

In hexaploid wheat, loci on homeologous group 1 chromosomes of the three wheat genomes (A, B and D) encode polypeptide subunits of HMW glutenins. Hexaploid bread wheat cultivars have six HMW genes (Shewry et al. 1997). There is an x- and a y-type subunit located on the long arm of chromosome 1 of each genome. The genes encoding for x- and y- subunits are found in pairs. Therefore, they are often referred to as single loci named Glu-A1, Glu-B1 and Glu-D1 (Payne et al. 1983).

Most bread wheat cultivars have three to five expressed HMW genes. Gene silencing is one of the factors contributing to the variability of the number of HMW glutenin subunits expressed in wheat cultivars. The x-subunit, located on the A genome and y- subunit of the B genome are not expressed in some cultivars while

the y-type subunit of the A genome is silent in the majority of bread wheat cultivars (Payne et al. 1981). An exception to this trend was reported in a Swedish line where the expression of two Glu-A1 encoded genes was observed (Johansson et al. 1993).

Aside from gene silencing as a source of genetic differences among cultivars, broad allelic variations exist at the three HMW glutenin loci. Based on their mobility in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), subunits have been characterized and a nomenclature system has been proposed (Payne et al. 1987).

HMW glutenins represent approximately 10% of the seed storage proteins, but their effect on gluten strength was reported to be very significant (Payne et al. 1987, Lukow et al. 1989, Gupta et al. 1993). HMW glutenin subunits have molecular weights ranging from 65,000 to 90,000 (Shewry et al. 1997).

In terms of gluten strength, allelic combination 5+10 encoded at locus Glu-D1 has the best quality score, compared to subunit combination 2+12 encoded by contrasting alleles at the same genetic locus (Payne et al. 1981). Also, different Bx+By subunit combinations encoded at the Glu-B1 locus have different effects on gluten quality (Payne et al. 1991). In addition, the amount of subunits expressed in the endosperm might have an effect on gluten quality, but it is more likely that

structure of HMW glutenin subunits is the critical factor influencing size distribution of glutenin polymers and elasticity of the gluten complex.

SDS-PAGE of seed protein has been used for screening of glutenin polypeptides. This method is very efficient as allelic variation at multiple loci can be screened simultaneously. However, it requires seed for extraction of protein and it can not be used for screening of haploid leaf tissue in doubled haploid breeding programs.

The aim of this study was to develop PCR based markers for gluten strength to be used in screening haploid plantlets, to validate the markers and to illustrate application and usefulness of gene-assisted selection method in breeding for gluten strength.

3.3 MATERIALS AND METHODS

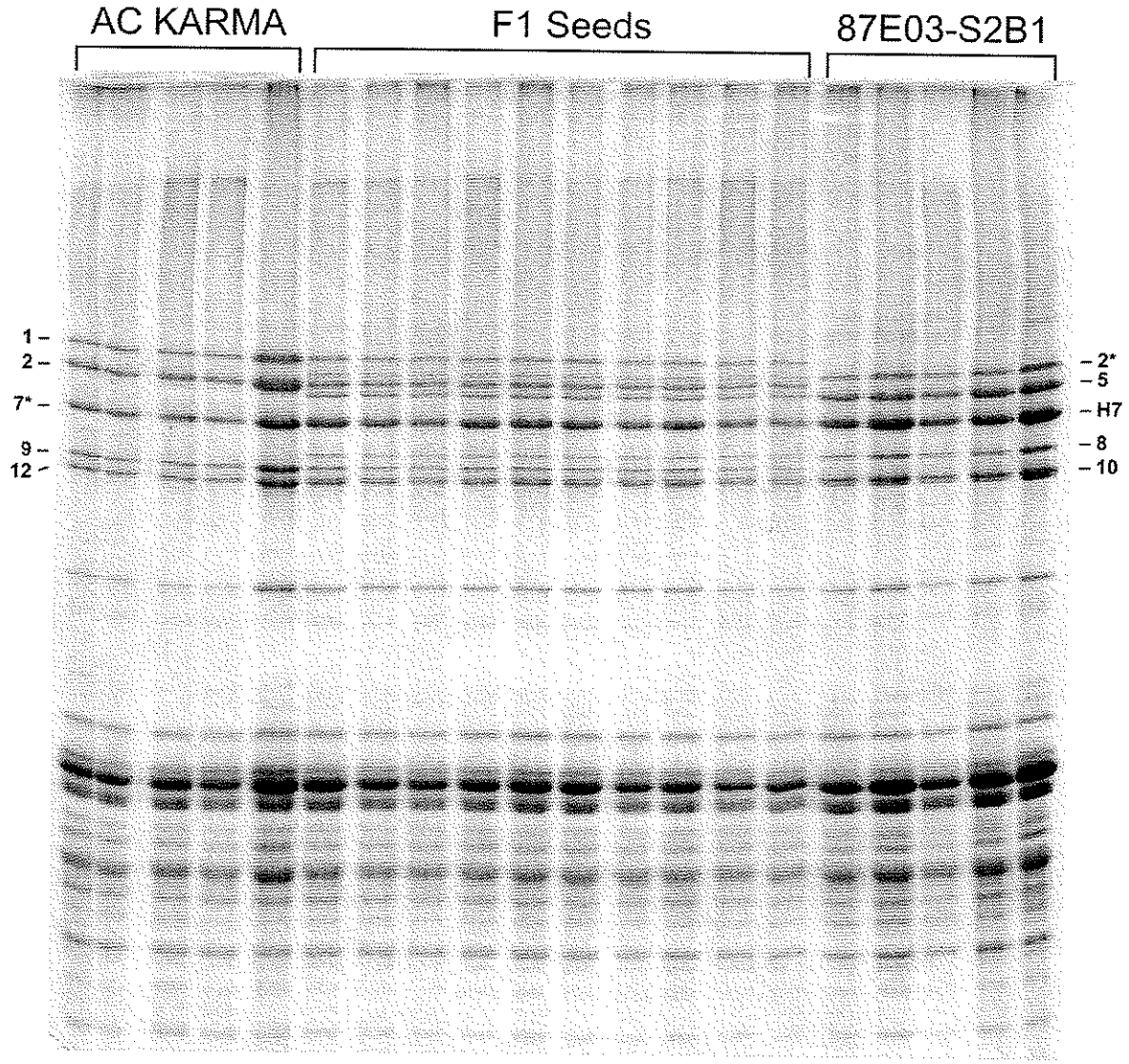
3.3.1 Experimental material

A doubled haploid population was produced from the cross between two genotypes, AC Karma (Canadian Prairie Spring Wheat), and a breeding line 87E03-S2B1. The cultivar AC Karma has a relatively weak gluten and poor bread making quality. Line 87E03-S2B1 was obtained from the following cros: Glenlea*7/C7932. It was derived by seven backcrossing to cultivar Glenlea which is Canadian Western Extra Strong Wheat and was selected for its white seed color. Line 87E03-S2B1 produces an

extra strong gluten. It is mainly used for mixing with weaker flour in order to increase strength. These two genotypes have completely different composition of HMW glutenin subunits, while their LMW glutenins and the gliadins are very similar. Composition of HMW glutenin subunits encoded at three Glu-1 loci is as follows: AC Karma has 1, 7*+9 and 2+12 subunits on the A, B, D genomes, respectively. The line 87E03-S2B1 has contrasting alleles at Glu-1 loci encoding the following subunits: 2*, overexpressed Bx7 subunit (H7+8), and 5+10.

From this cross, ten F_1 hybrid seeds were produced. SDS-PAGE was used to confirm the hybrid nature of these ten seeds (Fig 1-1). A population of 414 haploid lines was produced through maize hybridization technique and embryo rescue. Doubled haploids (DH) were produced through treatment with colchicine (Thomas et al. 1997). The F_1-derived doubled haploid lines from the cross between these two wheat cultivars segregated for HMW glutenin subunits. There was no notable segregation for LMW glutenins and gliadins.

Figure 1-1. SDS-PAGE of five seeds of AC Karma, ten F_1 seeds used for the production of the DH population and five seeds of 87E03-S2B1. A total of ten HMW glutenin subunits were detected in the F_1 endosperms. No segregation for LMW glutenin subunits was observed as shown in the lower portion of the gel. HMW subunits using the numerical code are indicated on both sides of the gel. H7 represents the overexpressed Bx7 subunit from Glenlea.


AC KARMA
F1 Seeds
87E03-S2B1
1
2
7*
9
12
2*
5
H7
8
10

3.3.2 DNA extraction

DNA was extracted from all 414 haploid lines and the parental lines. Haploid plants were sampled at the 2-3 leaf stage, before colchicine treatment. One young leaf per plant was harvested and submerged in liquid nitrogen. The leaf samples were lyophilized and stored dry at -20°C until extraction. DNA was extracted as per Cloutier et al. (2001).The extraction procedure was performed in 96-deep well blocks using hexadecyltrimethylammonium bromide (CTAB). The lyophilized leaf tissue was ground with glass beads and sterile sand in 96 well blocks using a paint shaker. The extraction buffer was pre-warmed to 65°C and added to the ground sample in final concentrations of 100 mM Tris-Cl pH 8.0, 50 mM EDTA, 1.4 M NaCl, 1% CTAB and 0.05 $mgml^{-1}$ of Proteinase K. Following addition of 2% SDS final concentration, samples were incubated for 2 hours at 65°C. DNA was extracted by chlorophorm/ isoamylalcohol (24:1) and precipitated by 0.6 volume isopropanol. After a 70% ethanol wash, DNA was resuspended in distilled water containing RNAase A (0.02 $mgml^{-1}$) and incubated for 1 h at 37°C. The extracted DNA was used for testing PCR-developed markers for HMW glutenin alleles.

PCR reactions were carried out in a PTC-100 thermocycler (MJ Research). Reactions were performed in 96-well microtiter plates in a final volume of 25μL. The reaction mixture contained 1unit Taq DNA polymerase, 1.5 mM $MgCl_2$, 0.8 mM dNTPs, 15 pmol of each primer and 100 ng of the genomic DNA template. Amplification conditions followed by initial denaturation step at 94°C for 5 min, were:

94°C for 30 s, 64°C for 30 s, 72°C for 1 min for 35 cycles and a final extension step of 10 min. PCR products were analyzed using 1% agarose gel in 1xTAE buffer, which was run at 80 V and stained with ethidium bromide.

3.3.3 PCR analysis

The HMW glutenin alleles and as much as 2 kb of the promoter regions for several HMW glutenin alleles from cultivar Glenlea had previously been cloned and sequenced in Dr.S.Cloutier's lab (unpublished) and were available for primer design. Computer software DNAMAN (Lynnon Biosoft) was used for multiple sequence alignment. The primers were synthesized by the primer synthesis unit of Life Technologies. The primer pair Bx7F-428 and Bx7R693 (Bx7-F-428:5'-CAACAACTTGTGGGGGCCTT-3'; Bx7-R693:5'GCGCTTAGCCATCTCAGTGAAC-3') was used in the PCR reaction to amplify a 1116 bp long fragment from the promoter and the proximal end of the N-terminal domain of the gene encoding the overexpressed Bx7 subunit from Glenlea cultivar. Primer Bx7F-428 was designed in a region of the promoter unique to the overexpressed Bx7 allele. This uniqueness permitted the development of a dominant marker specific to the overexpressed allele and which does not amplify the non-overexpressed allele. With this primer, the presence of the Glenlea allele may be distinguished from other alleles encoding HMW glutenin subunits and the allele encoding the non-overexpressed Bx7 subunit from the cultivars other than Glenlea. This dominant marker does not produce an amplification product in lines carrying the non-overexpressed Bx7 subunit.

A co-dominant marker system was also developed to simultaneously test for the presence of the allele associated with higher expression level of Bx7 HMW glutenin subunit and the allele associated with the non-overexpressed Bx7 subunit simultaneously. Two amplified PCR products were always present, but with a difference of 43 bp in size. The forward primer used in the PCR reaction was Bx7F-572: 5'-ACCTCAGCATGCAAACATG-3' and the same reverse primer Bx7-R693 as used in the dominant marker system. PCR conditions were identical. The very small difference in the band size required the use of a 1.7% ethidium bromide containing agarose gel in 1xTAE run at 70 V for 3.5h to separate the amplified products. The bands were visualized by ultra violet and photographed by polaroid.

A GenBank search was performed to obtain Dx5 and Dx2 sequences of *Triticum aestivum*. The sequences were aligned using the DNAMAN software package. The very high similarity of the sequences made primer design very difficult. A dominant marker system was however, successfully developed. To distinguish these two alleles, a forward primer Dx5F384: 5'-CGTCCCTATAAAAGCCTAGCC-3' was chosen because it differed in two nucleotides close to the 3'-end from the corresponding hybridization site of the Dx2 sequence. A 20-mer reverse primer designated Dx5R655 was designed: 5'-GGCTAATGTCTCGGAGCTGT-3', hybridizing to the nucleotide sequence of the Dx5 allele in the N-terminal domain. PCR reaction conditions were the same except that the final $MgCl_2$ concentration was reduced to 1.25 mM to increase stringency and number of cycles was reduced

to 30. PCR products from the Dx5 allele were visualized on a 1% agarose gel stained with ethidium bromide.

To distinguish Ax1 and Ax2* alleles on Glu-A1, two small deletions of 18 bp and 27 bp downstream from the start codon in Ax2* allele were considered for use in a co-dominant marker system. Due to easier visualization of 27 bp differences in amplified PCR fragments that deletion was selected to make PCR products distinguishable. PCR primers were selected 189 bp upstream and 874 bp downstream of the 27 bp deletion in the Ax2* sequence. Thus, PCR products were 1090 and 1063 bp, amplified from Ax1 and Ax2* alleles, respectively. PCR primer sequences were Ax2*F2543: 5'-AAGACAAGGGGAGCAAGGT-3' and Ax2*R3605: 5'-GTGCTCCGCGCTAACATG-3'. PCR conditions were as for Dx5/Dx2 marker. In order to resolve the PCR products, they were separated on 1.6% agarose gel in 1xTAE buffer run at 70 V for 3.5 h, using ethidium bromide staining for visualization. A summary of the PCR primers and conditions as well as marker types is given in appendix 1.

3.3.4 Protein extraction and SDS-PAGE

After chromosome doubling and production of seeds from doubled haploid lines, proteins were extracted from three individual seeds for each DH line. SDS-PAGE was performed to determine HMW glutenin subunit composition. Total protein extraction was used to test the overexpression of Bx7 subunit (HBx7) while glutenin

protein extraction was used to separate individual subunits and score lines for HMW glutenin subunit composition. The two procedures were performed using different halves of the same seed.

The embryo containing half of the kernel was used for total protein extraction. The extraction was performed performed in 96-deep well plates (1 ml). Crushed seed halves were soaked in 0.4 ml of the extraction buffer (3x extraction buffer contained 20% Glycerol, 0.02% Pyronin, 0.125 M Tris-Cl, pH 6.8 and 4% SDS). Extraction was performed using 8% ß-mercaptoethanol. After vortexing at room temperature, samples were incubated at 95°C for 2.5 min. Samples were stored at –20°C until use. A Hoeffer Scientific vertical electrophoresis unit was used to conduct the gel electrophoresis of the total protein extract. Gels were run for 2 h at 40 mA per gel, in a buffer containing 0.1% SDS, 0.192 M Glycine and 0.025 M Tris.

To determine the HMW glutenin subunit profile of the sample and obtain a complete migration pattern of LMW glutenin subunits, the following procedure was applied. The non-embryo containing halves of the seed were crushed and then soaked overnight in 50% 1-propanol. After three steps of sonication at 60°C for 30 min., followed by 50% 1-propanol wash, the majority of gliadins were removed. The remaining pellet was treated with a solution containing 1-propanol, 1M Tris-Cl, pH8.0 and 1% DTT to reduce the proteins, followed by treatment with 0.1 ml of the solution containing 1-propanol, 1M Tris-Cl, pH8.0 and 1.4% 4-vinyl pyridine. After the centrifugation, the supernatant was added to a solution containing 2% SDS,

40% glycerol, 0.02% bromphenol blue, 8% 1M Tris-Cl, pH8.0, and heated for 15 min at 50-65°C. Samples were stored at –20°C until electrophoresis. A 12% SDS-polyacrylamide gel was used to separate LMW and HMW glutenin subunit extracts. Gels were stained with 1% Coomassie blue in ethanol and Blakney stain. After staining, gels were soaked in 5% glycerol and dried at room temperature for several days.

3.3.5 Phenotypic characterization

To validate the usefulness of the markers in breeding for gluten strength, a sub-population of 162 DH lines were grown in the field at one location in 1999 (Glenlea) and three locations in 2000 (Glenlea, Portage La Prairie, Brandon). The entire seeds collected from each plot were harvested, threshed, cleaned and sub-sample of 50 g was tempered to 15 % moisture and milled on a Brabender Quadrimat mill. Mixograph analysis of flour was performed using a 10 g mixograph (Pon et al.1989).

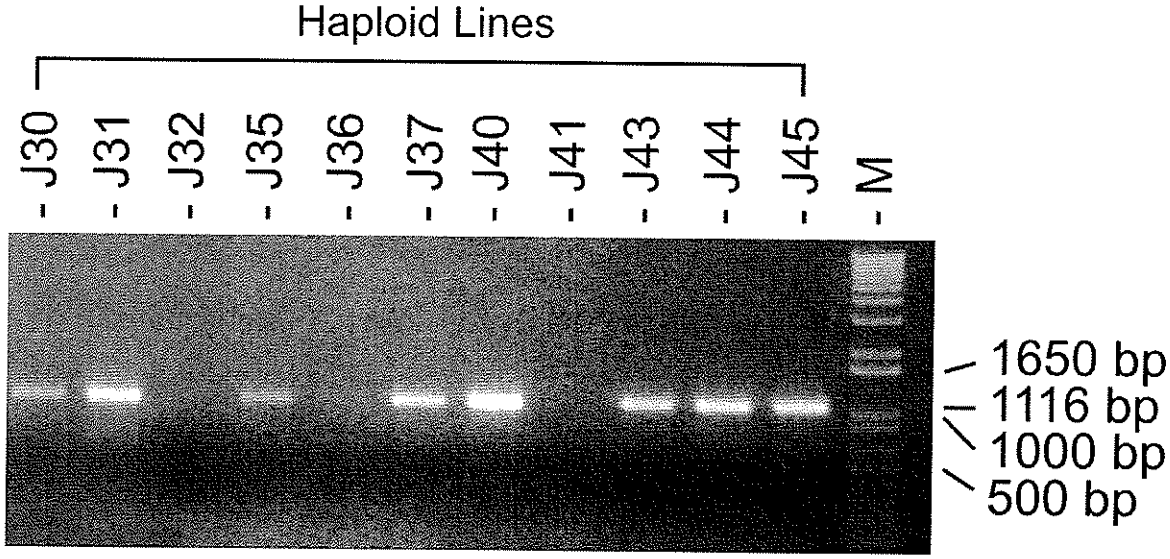
3.4 RESULTS

3.4.1 Marker development

Gel electrophoresis and spectrophotometry of genomic DNA showed that DNA extraction procedure using haploid leaf tissue yielded high quality, high concentration and stable DNA samples. Dominant and co-dominant marker systems

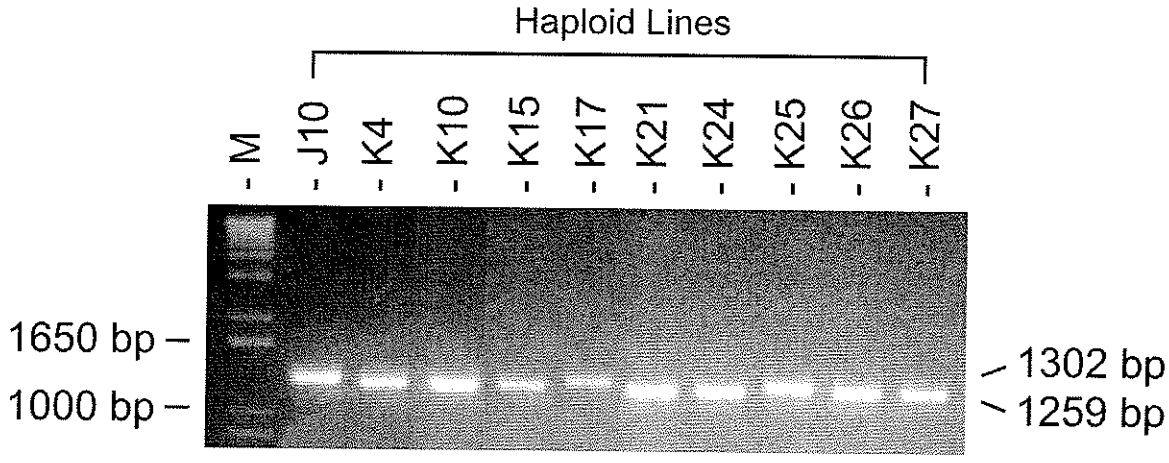
were developed to distinguish the alleles associated with the overexpression of Bx7 from the alleles associated with the non-overexpressed Bx7 subunit. Amplified products obtained using PCR primer pair Bx7F-428 and Bx7-R693 are shown in Fig 1-2A.

Figure 1-2A. Dominant PCR marker system specific to the overexpressed HMW glutenin Bx7 allele. PCR reactions were performed using DNA extracted from haploid leaf tissue. Doubled haploid lines are identified on top. DNA marker is 1 kb plus DNA ladder (Life technologies). DNA size markers are shown in lane M with the size of the bands given in base pairs. Fragment size is 1116 bp.


Haploid Lines
J30
J31
J32
J35
J36
J37
J40
J41
J43
J44
J45
M
1650 bp
1116 bp
1000 bp
500 bp

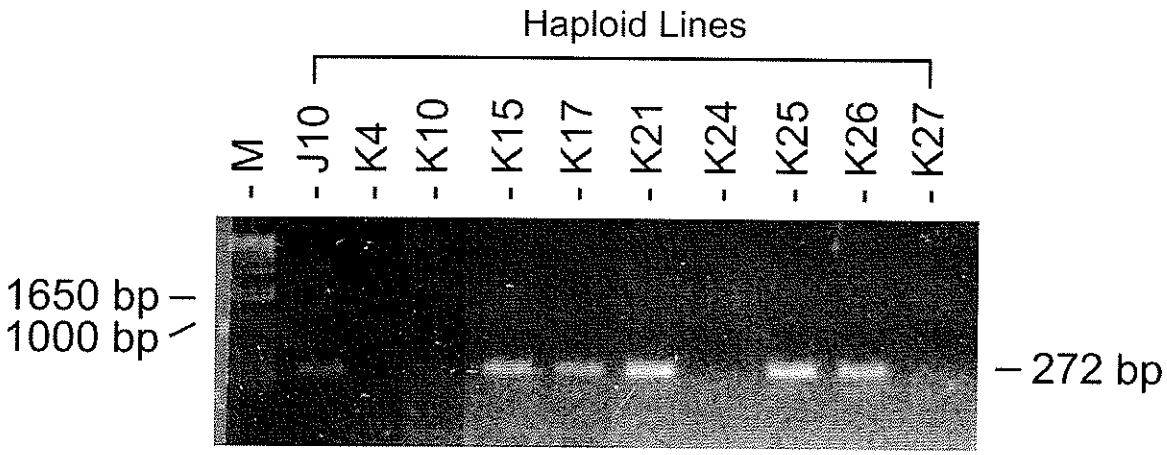
The co-dominant marker system developed using Bx7F-572 and Bx7-R693 primer pair is illustrated in Fig 1-2B. With this primer combination, PCR products were always present, but a difference of 43 bp was observed. The largest of the two amplicons corresponded to HBx7 allele while the smallest represented the Bx7*.

Figure 1-2B. Co-dominant PCR marker system targeting the HMW glutenin BX7 locus. PCR products are amplified from the promoter and proximal end of the N-terminal domain of Bx7. DNA size markers are shown in lane M with the size of the bands given in base pairs (1 kb plus DNA ladder marker, Life Technologies). The larger fragment (1302 bp) corresponded to the overexpressed Bx7 gene and the smallest fragment (1259 bp) was characteristic of the non-overexpressed Bx7 gene. Doubled haploid lines are identified on top.


Haploid Lines
M
J10
K4
K10
K15
K17
K21
K24
K25
K26
K27
1650 bp
1000 bp
1302 bp
1259 bp

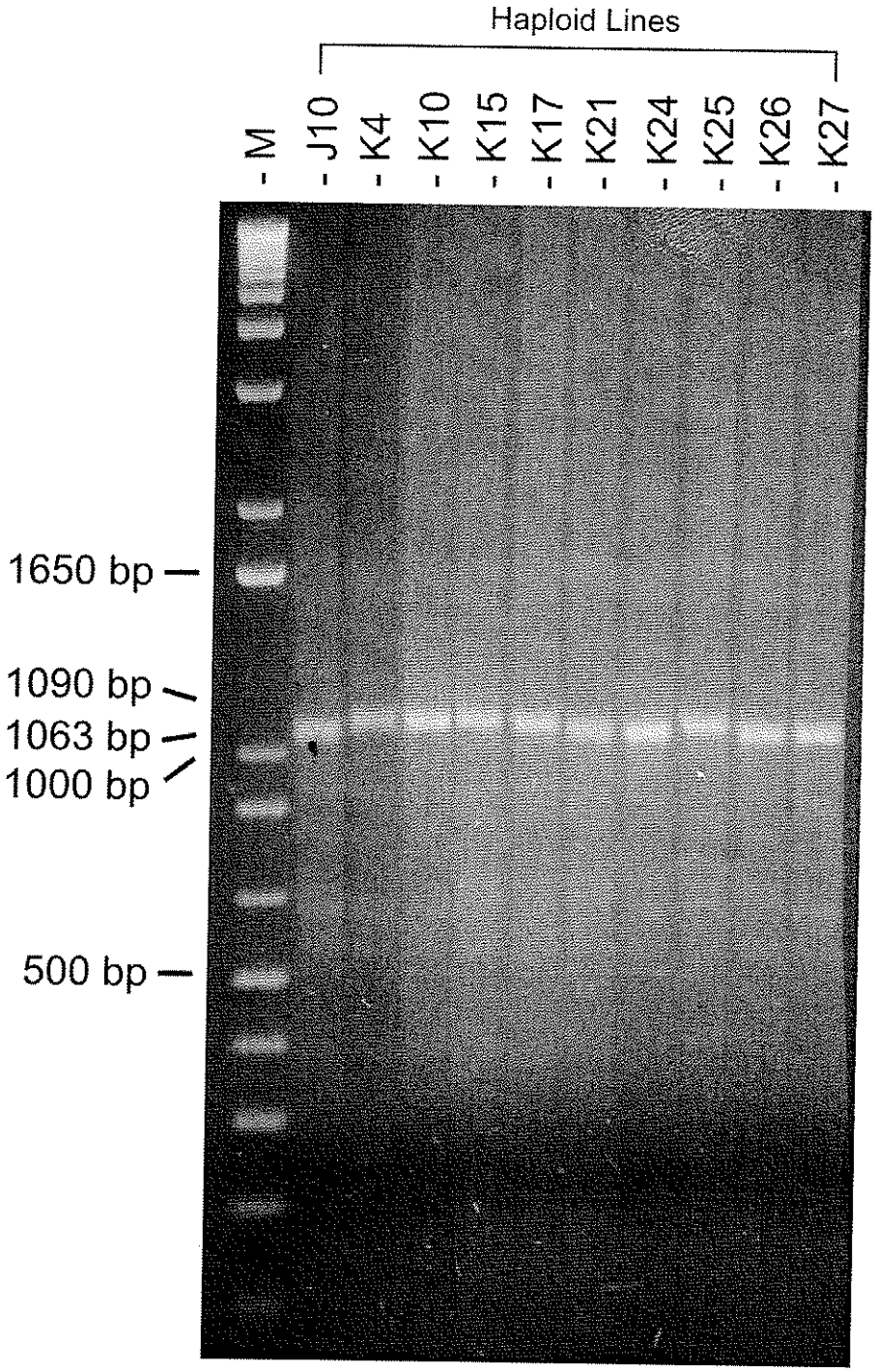
Primers specific to the 1Dx5 allele were used to amplify a 272 bp long fragment in lines carryng this HMW glutenin subunits (Fig 1-3). Electrophoretic analysis of the PCR products performed on genomic DNA of haploid lines showed clearly amplified product which corresponds to the expected product size of 1Dx5 gene while no PCR products were generated with this primer pair in Dx2 lines.

Figure 1-3. Dominant PCR marker associated with the high quality score HMW glutenin allele Dx5. The DH population segregated for alleles Dx5 and Dx2. PCR primer pair Dx5F384 and Dx5R665 did not amplify the Dx2 allele. Agarose gel represents results for the same haploid lines shown in Fig 2B. DNA size markers are shown in lane M with the size of the bands given in base pairs (1 kb plus DNA ladder marker, Life Technologies).


Haploid Lines
M
J10
K4
K10
K15
K17
K21
K24
K25
K26
K27
1650 bp
1000 bp
272 bp

In the doubled haploid population tested, two alleles were detected at the Glu-A1 locus. Amplification products using primer pair Ax2*F2543 and Ax2*R3605 were obtained in all tested lines (Fig 1-4). A small but detectable difference of 27 bp was visualized. The largest amplicons corresponded to HMW glutenin subunit Ax1 which is a slightly larger protein than Ax2*.

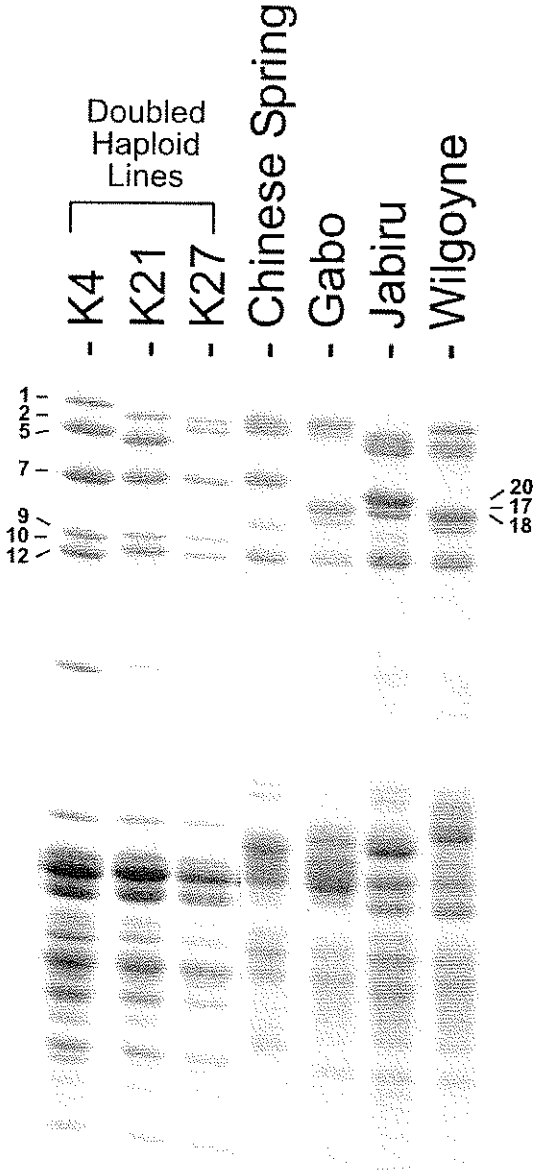
Figure 1-4. Co-dominant PCR marker system designed to differentiate HMW glutenin allele Ax1 and Ax2*. Primer pair Ax2*F2543 and Ax2*R3605 amplified the repetitive domain producing a 1090 bp and 1063 bp fragments for the alleles encoding Ax1 and Ax2*, respectively. Doubled haploid lines are the same as in Fig 1-2B and 1-3. DNA size markers are shown in lane M with the size of the bands given in base pairs (1 kb plus DNA ladder marker, Life Technologies).

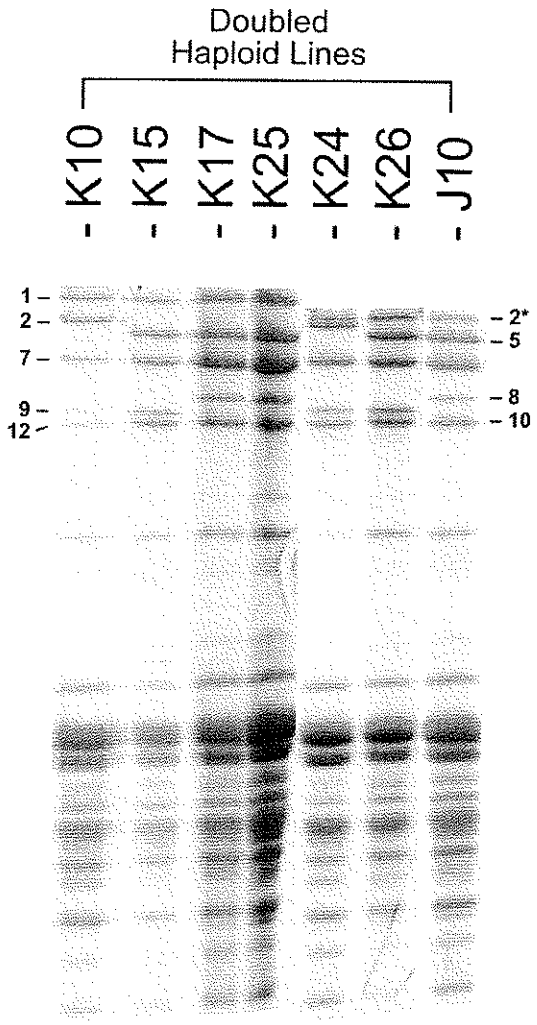

Haploid Lines
M
J10
K4
K10
K15
K17
K21
K24
K25
K26
K27
1650 bp
1090 bp
1063 bp
1000 bp
500 bp

3.4.2 Marker validation by SDS-PAGE

In order to validate the PCR-based method for distinguishing between alleles at the Glu-1 loci, HMW glutenin subunit composition was assessed using SDS-PAGE (Fig 1-5). DH lines segregated into eight different HMW glutenin genotypes i.e., all possible combinations of two alleles for each of the three loci (2^3).

Figure 1-5. SDS-PAGE separation of HMW glutenin subunits of doubled haploid lines. A total of ten different HMW glutenin subunits were detected. Chinese Spring (2, 7, 8, 12), Gabo (2*, 2, 17+18, 12), Jabiru (5, 20, 10) and Wilgoyne (2*, 5, 17+18, 10) were check cultivars. Numbers indicate doubled haploid lines produced from the haploids reported in PCR analyses.


Doubled Haploid Lines
K4
K21
K27
Chinese Spring
Gabo
Jabiru
Wilgoyne
1
2
5
7
9
10
12
20
17
18


Doubled Haploid Lines
K10
K15
K17
K25
K24
K26
J10
1
2
7
9
12
2*
5
8
10

Over production of the Bx7 subunit was visualized by Coomassie staining as a higher intensity band using a total protein extraction procedure (Fig 1-6). The overexpression of Bx7 was always associated with the presence of the By8 subunit because no recombinant was observed in this population.

Figure 1-6. SDS-PAGE analysis of total protein extracts of DH lines. Overexpression of Bx7 subunit is detected as an intensely stained band compared to other HMW glutenin subunits.

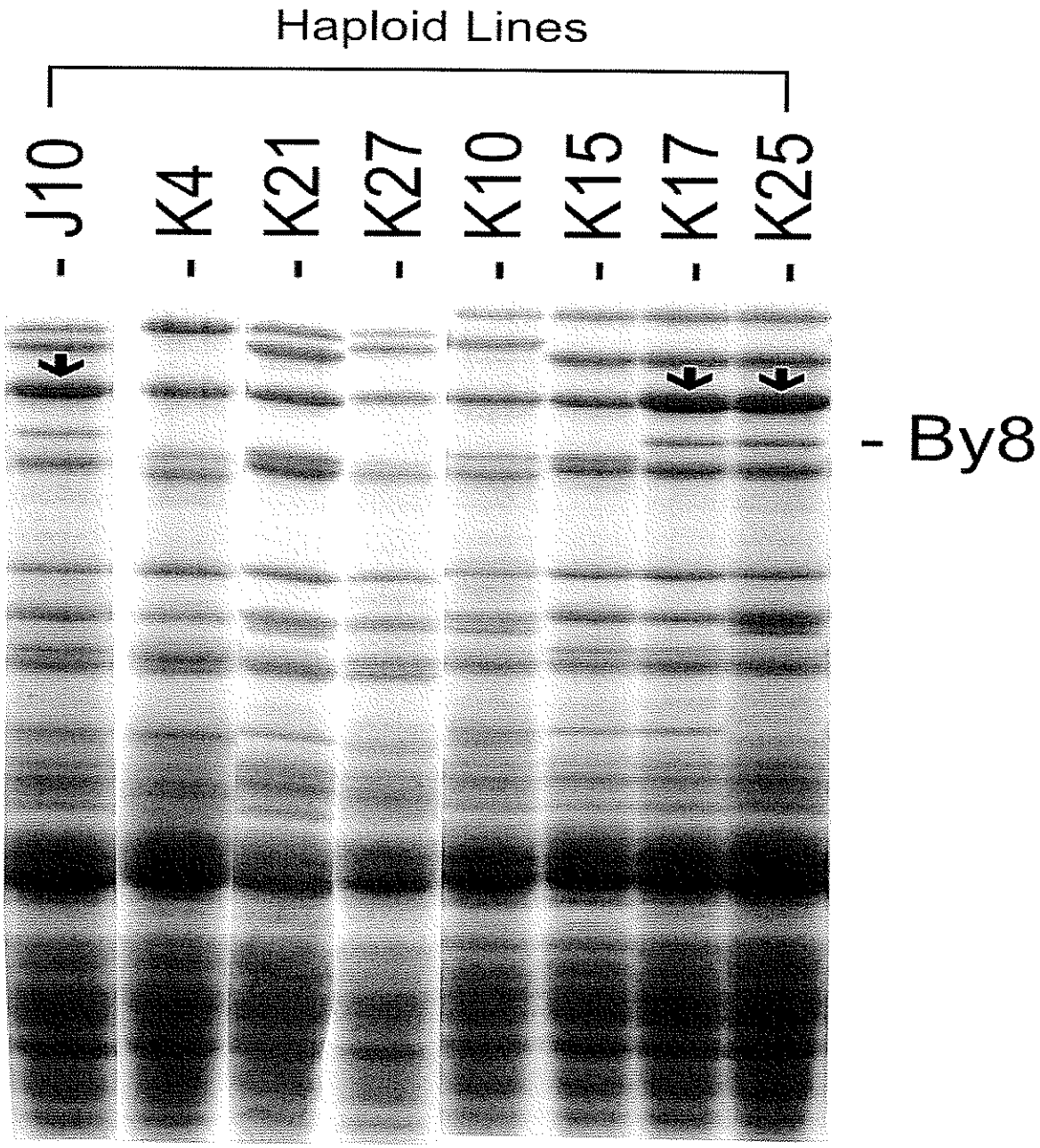

Haploid Lines
J10
K4
K21
K27
K10
K15
K17
K25
By8

Table 1-1. Number of lines for which SDS-PAGE and PCR markers were in agreement (no discrepancies). Discrepancies represent number of lines where PCR gave a contradictory result to SDS-PAGE.

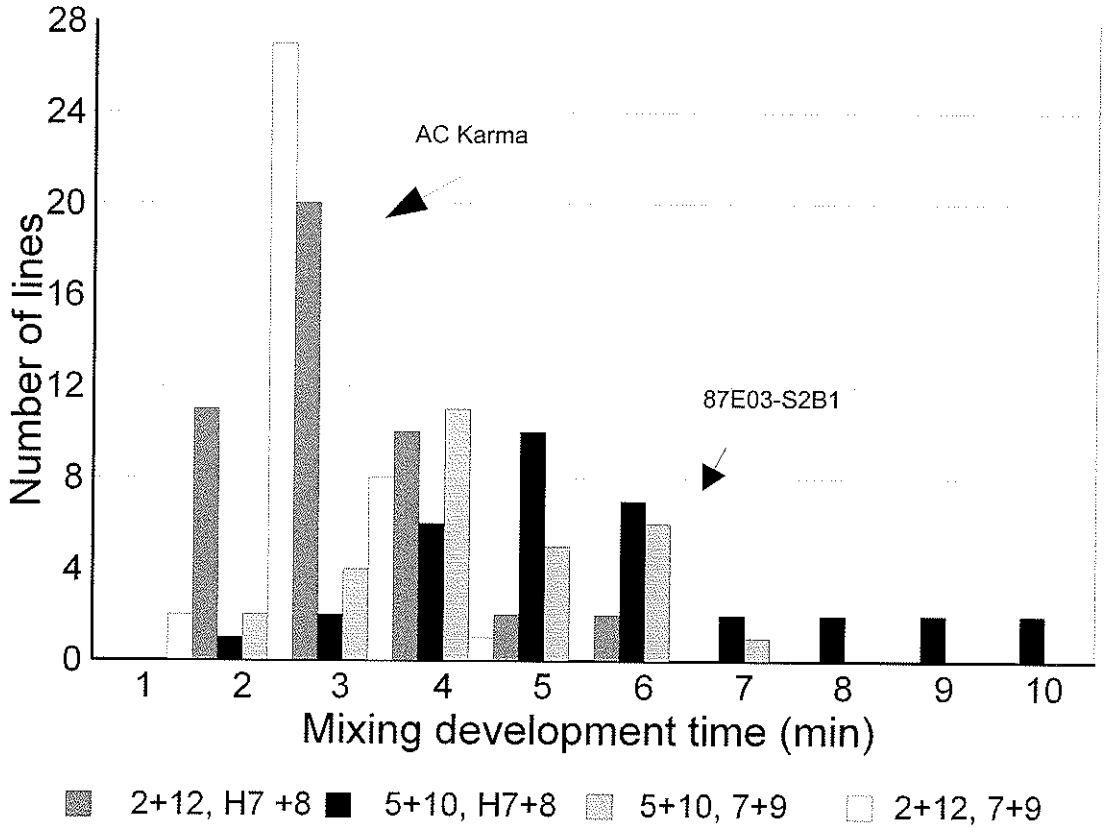
Locus	Allele	No discrepancies	Discrepancies	% Discrepancies
Glu-A1	Ax1	135	2	2
	Ax2*	110	3	
Glu-B1	HBx7	150	7	5.1
	Bx7*	130	8	
Glu-D1	Dx5	104	19	8.5
	Dx2	166	6	

3.4.3 Application of markers to wheat breeding

Complete results of the phenotypic analysis study including analysis of variance component for mixograph, SDS-sedimentation, grain and flour protein are elsewhere (Radovanovic et al. In prep). Mixing development time (MDT) is used here only to illustrate one example of the effect of HMW glutenin composition on quality using a doubled haploid breeding population.

In this DH population, no effect of alleles on Glu-A1 locus was observed (data not shown). A significant main effect was observed for HMW glutenin alleles of the B and D genomes (Appendix 2). No significant interaction between these alleles was observed. As a consequence, the population was divided into four sub-populations based on their allelic variation at the Glu-B1 and Glu-D1 loci. The frequency distribution is shown in Fig 1-7. Lines with 2+12/7*+9 subunit combination had an average MDT of 2.3 min. AC Karma had the same subunit composition and MDT of 2.2 min. In 2+12 background incorporation of H7+8 subunits from 87E03-S2B1 increased the MDT for this sub-population to 3.3 min. Lines with 5+10/7*+9 combination averaged 4.4 min. The longest mean for MDT of 5.5 min was obtained for 5+10/H7+8 sub-population, indicating stronger gluten and dough mixing properties of the lines containing this subunit combination. 87E03-S2B1 parental line had an MDT of 6.4 min.

Figure 1-7. Frequency distribution of DH population derived from the cross between AC Karma x 87E03-S2B1, for mixing development time (MDT).


28
24
20
16
12
8
4
0
Number of lines
AC Karma
87E03-S2B1
1
2
3
4
5
6
7
8
9
10
Mixing development time (min)
2+12, H7 +8
5+10, H7+8
5+10, 7+9
2+12, 7+9

3.5 DISCUSSION

Gluten strength is an important quality parameter for a number of wheat classes. For the extra strong wheat class, high gluten strength is a prerequisite. For the bread wheat class, maintaining a good level of strength is necessary for making high quality products such as leavened bread. For this reason, breeders constantly strive to improve or maintain the quality within wheat classes. Association of the alleles on Glu-1 loci with bread making quality for bread and durum wheat was reported previously (Payne et al. 1981, Lukow et al.1989 Payne et al. 1991, Ammar et al. 2000). Strong association of the 5+10 subunit pair with high dough strength and good quality was reported in many studies (Payne et al. 1979, Payne et al. 1987). Subunit pair 7*+9 had a lower quality score compared to overexpressed H7+8 (Perron et al. 1998). Differences between Ax1 and Ax2* subunits in terms of gluten strength were not consistent and seem to depend on the genetic background (Rogers et al. 1989, Graybosch et al. 1994). Our own research using the described AC Karma x 87E03-S2B1 DH population demonstrated also the positive role of 5+10 and H7+8 on several quality parameters, but did not show significant difference between Ax1 and Ax2* alleles (Radovanovic et al. In prep). An example is illustrated in Fig 1-7. which shows the frequency distribution for the four genotypes for MDT. The sub-population of lines with H7+8/5+10 combination of subunits had the longest mean MDT of 5.5 min.

In the past and still today, breeders have relied heavily on the SDS-sedimentation test for early generation screening because of its ease and low cost. However, the power of this method to distinguish between some gluten strength classes is limited. More recently, SDS-PAGE has been used to select lines with good allelic combinations of HMW and LMW glutenins and gliadins. The main advantage of SDS-PAGE is that the complete HMW glutenin pattern for a given line can be obtained in a single gel lane. Protein extraction from seeds is an established method and can be performed easily in a 96 well format. SDS-PAGE still remains the method of choice for genotype selection when seeds are easily available. Both the above mentioned methods are not universally applicable and can not be used without prior seed production.

HMW glutenins have long repetitive domains making design of a primer pair unique to a given allele difficult (Shewry et al. 1992). This problem is intensified by the fact that allelic variations are small and sometimes multiple close or consecutive point mutations were difficult to find. Promoters can, in some cases, be more divergent than coding sequences and have proved useful in designing primers for both the overexpressed Bx7 and the Dx5 alleles. Long promoter sequences were however not always available. The markers developed and tested herein were based on actual gene and promoter sequences and therefore characterize the allelic variants with no recombination between the marker and the gene they address. In this study, diagnostic PCR markers were developed for Ax1/Ax2*, HBx7/Bx7 and for Dx5. Dominant marker systems were designed for HBx7 and Dx5 (Fig 2A, 3). Co-

dominant marker systems were developed for Ax1/Ax2* and HBx7/Bx7 (Fig 2B, 4). The dominant marker for HBx7 was generated by designing the primer Bx7F-428 over a unique promoter region of the Glenlea overexpressed Bx7 allele. The dominant and co-dominant markers for HBx7 do not detect variation in subunit composition but are diagnostic of the difference of expression of a gene that encode identical subunits. The role of the 43 bp promoter insert in the expression of the Bx7 subunit has not been elucidated.

The advantages of PCR based systems as an alternative to SDS-PAGE for selecting genotypes with particular HMW glutenin genes have been reported (D'Ovidio et al. 1994, D'Ovidio and Anderson. 1994, D'Ovidio et al. 1995a, Lafiandra et al. 1997, DeBustos et al. 2000). PCR is an accurate and a rapid screening method and advanced DNA technology allows efficient screening of hundreds of lines per day necessary for application in breeding programs. Furthermore, PCR analysis may be performed using different plant tissues including haploid tissue as reported in this study.

Doubled haploid populations are extremely advantageous in breeding programs because homozygosity is reached in one generation. In recent years, the maize pollination method has been improved to the point where it is now readily used to produce large breeding populations. The doubled haploid team at the Cereal Research Centre produces more than 10000 DH lines per year for genetic studies and breeding. For this last purpose, it would be advantageous to select lines with

good potential for high quality at the haploid stage. That way, selection would be performed prior to the labor intensive step of colchicine treatment. The need for growth chamber space would also be reduced. SDS-PAGE cannot be used for this purpose because it requires protein to be extracted from seeds which would only be accomplished at the end of the doubled haploid production process.

The major disadvantage of PCR analysis is the requirement for tissue sampling and DNA extraction. However, today's DNA technology market enables higher throughput procedures to be used. Other drawbacks include the need for DNA sequence of multiple alleles for primer design and inherent difficulties associated with multiplex PCR.

In this study, two types of PCR markers were used: dominant and co-dominant. Dominant markers have the advantage of being amenable to simple plus-minus test which can bypass the need for electrophoresis detection. The major drawback of dominant marker is the impossibility to distinguish between a negative response and a failed reaction. Failed reactions can easily be detected with a co-dominant marker system but do require a size separation method such as agarose or acrylamide gel electrophoreses to visualize the different alleles. A capillary system could provide a higher throughput method of separation for co-dominant markers and alleviate the inherent problem of false negative associated with dominant markers.

Accuracy is an important criterion for adoption of any marker system in a breeding program. In order to validate these HMW glutenin PCR markers, the entire population was genotyped by SDS-PAGE. Considering that these markers characterize exactly the gene loci, discrepancies can be mostly attributed to human error or intrinsic problems with the markers themselves rather than genetic recombination. The PCR marker for the Glu-A1 locus and both markers for the Glu-B1 locus were very accurate with a very low percentage of discrepancies when compared with SDS-PAGE (Table 1-1).

In order for markers to be incorporated in breeding programs, they must be selecting a sub-population with desirable characteristics. HMW glutenin subunits are the most important single factor contributing to gluten strength. Selection at only two loci (Glu-B1 and Glu-D1) yielded a sub-population of 34 plants that have an average of MDT of 5.5 min., as compared to only 2.3 min. for the sub-population containing 2+12/7*+8 subunits. Moreover, only lines with 5+10/H7+8 had MDT greater than seven min. In a doubled haploid breeding program, only 25% of the haploid lines would be doubled saving substantial labor cost, green house space and selecting the desirable breeding population.

In conclusion, PCR based markers were developed for alleles at the three HMW glutenin loci which are important in determination of gluten strength. Advantages and disadvantages of marker types were discussed. Their usefulness in breeding

programs was illustrated by their validation, their application in doubled haploid production method and potential for selection of desirable sub-populations.

4.0 MANUSCRIPT #2

Genetic variance for gluten strength contributed by high molecular weight glutenin proteins

4.1 ABSTRACT

A doubled haploid (DH) population from a cross between *Triticum aestivum L.* 'AC Karma' and line 87E03-S2B1, derived from *Triticum aestivum L.* 'Glenlea' was produced to study the genetic contribution of high molecular weight (HMW) glutenin subunits to gluten strength. HMW glutenin subunit composition of each DH line was determined by SDS-PAGE. The population was grown in the field at one location in 1999 and at three locations in 2000. Gluten strength and dough mixing properties were measured by mixograph and SDS-sedimentation tests. Variance components were estimated for each measurement to obtain information about variability contributed by HMW glutenin subunits. Results indicated significant environmental impact on tested mixograph parameters, SDS- sedimentation test, grain and flour protein. A significant main effect of Glu-D1 loci encoded subunit pair 5+10 was obtained for mixograph development time (MDT), energy to peak (ETP), slope after peak (SAP), first minute slope (FMS) as compared to 2+12 subunit combination encoded at the same locus. Low intergenomic interactions were observed for band width energy (BWE), total energy (TEG) and SDS-sedimentation test, involving loci on the B and D genomes only. A proportion of the genetic variability for gluten strength was accounted for by the overexpression of the Bx7 subunit originating from the cv. Glenlea derived line. There was no significant effect of Glu-A1 encoded subunits on mixograph parameters, SDS-sedimentation value, grain protein and flour protein. Estimated genetic variability contributed by Glu-B1 and Glu-D1 encoded HMW glutenins was 55% for MDT and 51% for ETP.

4.2 INTRODUCTION

Breadmaking quality of wheat (*Triticum aestivum* L.) is influenced by protein content and composition. Gluten proteins confer viscoelastic properties of dough and are critical for good breadmaking properties. Wheat storage proteins, designated as prolamins, consist of two protein classes: gliadins and glutenins. Gliadins are small, monomeric proteins comprising the α, β, γ, and ω families. They make up 50% of prolamins (Payne et al. 1984). Glutenins consist of HMW (high molecular weight) or LMW (low molecular weight) glutenin subunits. Even though HMW glutenins constitute only 10% of the total storage proteins as compared to 40% for LMW glutenins (Payne et al. 1984), HMW glutenin subunits have the largest effect on breadmaking quality (Payne et al. 1987).

HMW and LMW glutenin polypeptides interlinked by disulphide bonds between cysteine residues form large polymers with molecular weight that may exceed $1x10^6$ (Werner et al. 1992). Native gluten contains various glutenin polymers differing in the number of subunits joined together, resulting in heterogeneity of glutenin polymers (Kasarda 1989). Besides the covalent disulphide bonds, hydrogen bonds also play a role in protein-protein interactions. They are formed between the long repetitive regions of the HMW glutenins, rich in glutamine amino acid residues (35 mol%) thereby contributing to some of the elastic properties of the gluten (Belton et al. 1999). Furthermore, a less characterized type of crosslinking between side chains, involving two tyrosines, may also play a role in the intrinsic quality of the

polymer (Michon et al. 1999). This type of covalent crosslink is characteristic of other elastic proteins such as elastin and resilin (Shewry et al. 1992).

HMW glutenin subunits are easily distinguished from other prolamins because of their slower mobility in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Their molecular weight is estimated to range from 65,000 to 90,000 (Shewry et al. 1997). SDS-PAGE is commonly used to estimate glutenin subunit composition (Payne et al. 1979).

Genes encoding HMW glutenin subunits are located on the long arms of homoeologous chromosomes 1 in wheat, on loci designated as Glu-1. Therefore, Glu-A1, Glu-B1 and Glu-D1 are loci on chromosomes 1A, 1B and 1D, respectively, encoding HMW glutenin subunits. Each locus contains two tightly linked genes encoding two types of subunits, designated as x- and y-type based on different electrophoretic mobility where x-type subunits are slower, therefore larger subunits (Payne et al. 1981). The two types of subunits also differ in the general composition of their repetitive domain and the number and distribution of their cysteine residues.

Not all subunits are expressed in bread wheat cultivars. Hexaploid wheat usually expresses 3 to 5 HMW glutenin subunits, because one or both genes at locus Glu-A1 are silent. In some cultivars, subunit By is also not expressed (Payne et al. 1981). One exception to this general rule has been reported where six subunits were expressed (Johansson et al. 1993).

Genes encoding HMW glutenin subunits show great allelic variations (Payne et al. 1981). Therefore, HMW glutenin subunits have allelic forms which differ among cultivars. Seventeen different alleles have been described for the x-type subunit at the Glu-B1 locus. The relationship between molecular variation in HMW polypeptides encoded by different alleles and quality parameters has been investigated in many studies (Payne et al. 1987, Lukow et al. 1989, Payne et al. 1991, Cox et al. 1991, Mir-Ali et al. 1999). For example, higher SDS-sedimentation volume was observed when Glu-A1 locus encoded subunits Ax1 or Ax2* was present as compared to the null allele (Payne et al. 1987). However, Mir-Ali et al. (1999) reported a positive effect in only two of the four groups of studied cultivars. In two other studies no relationship between Glu-A1 encoded subunits, Ax1 and Ax2* and the quality score was reported (Rogers et al. 1989, Graybosch et al. 1994). These results point to the importance of the genetic background and many possible interactions between the different subunits and other gluten components as well as environmental effects.

The effect of Bx7+By8 subunits combination encoded at the Glu-B1 locus also depended on the grouping of tested cultivars (Mir-Ali et al. 1999). Presence of the Bx7+By8 subunit was associated with higher dough strength based on several mixograph parameters compared to lines having Bx7+By9 subunit combination (Perron et al 1998). In studies where the effect of Dx2+Dy12 subunits was compared to the Dx5+Dy10 subunits combination encoded at Glu-D1 locus a

positive effect of Dx5+Dy10 combination was reported (Payne et al. 1981, Gupta et al. 1993).

The relative contribution of the various alleles has been characterized qualitatively. The contribution to the genetic variance for a trait such as gluten strength by the common alleles at the Glu-1 loci has not been reported. To estimate such genetic contribution, the use of a large segregating population is necessary.

In this study the effect of HMW glutenin subunits on gluten strength as an important factor of breadmaking quality was studied using a doubled haploid population produced from the cross between two wheat cultivars, AC Karma (Canadian Prairie Spring Wheat) and a line 87E03-S2B. The parents were chosen because they display very different strength characteristics, have different HMW glutenin composition but similar LMW glutenin and gliadin profiles.

4.3 MATERIALS AND METHODS

4.3.1 Plant material

A total of 162 F_1-derived doubled haploid lines produced from the cross between wheat cultivar AC Karma and a breeding line 87E03-S2B1 were produced at Cereal Research Center, Agriculture and Agri-food Canada, using maize pollination system (Thomas et al. 1997).

In 1999, the entire DH population was grown in the field at the experimental station in Glenlea, Manitoba, Canada. Lines were grown in 1 meter long single row plots. Three rows of each parental line was grown. The plots were sprayed with TILT at anthesis to reduce potential infection by fusarium head blight (FHB). During the winter 1999/2000, seed of all DH lines was increased in Palmerston North and Leeston in New Zealand. In the summer 2000 all DH lines and the two parents were grown at three locations, Glenlea, Brandon and Portage La Prairie, Manitoba, Canada. A completely random design with one replicate per location was used. All lines, including parental lines were seeded in 5 row, 4 m long plots. Two rows of winter wheat were seeded between plots to reduce plot edge effects. Control of FHB was done using TILT, as in 1999. Grain was harvested at maturity, threshed and cleaned before quality assessment.

4.3.2 Protein extraction

Two extraction procedures were used to assess HMW glutenin subunit composition of DH lines. Proteins were extracted separately from three seeds of each DH line. SDS-PAGE was carried out for all seeds independently.

Total protein extract was used to detect the overexpression of the Bx7 subunit. The kernel was cut in two latitudinally. The embryo containing half of the kernel was used for total protein extraction. Procedures were performed in 96-deep well plates, using 0.4 ml of 3X extraction buffer containing 20% Glycerol, 0.02% Pyronin, 0.125 M Tris- HCl, pH 6.8. Extraction was performed using 8% ß-mercaptoethanol. Samples were briefly vortexed at room temperature and then incubated at 95°C for 2.5 min. After the centrifugation at 3000 rpm for 10 min., samples were loaded on the gel.

Total endosperm proteins were analyzed on 10% SDS-Polyacrylamide gel electrophoresis, with 3% stacking gel, using a Hoffer Scientific SE 600, standard vertical electrophoretic unit with two gels in each run. Gels were run for 2 hours at constant current of 40 mA per gel, in the buffer containing 0.1% SDS, 0.192 M Glycine and 0.025 M Tris.

To identify HMW glutenin subunits and obtain a complete migration pattern of LMW glutenin subunits, the following protein extraction procedure was applied. The

endosperm halves of the seed were crushed and then soaked in 50% 1-propanol overnight. The gliadin containing supernatant was discarded. Two additional 50% 1-propanol washing steps followed by sonication at 60°C were performed to remove the majority of remaining gliadins. The pellet was then treated with a solution containing 50% 1-propanol, 0.08M Tris-Cl, pH 8.0 and 1% dithiothreitol to reduce the glutenin proteins, sonicated 30 min at 60°C and treated with a solution containing 50% 1-propanol, 0.08M Tris-Cl, pH 8.0 and 1.4% 4-vinyl pyridine. After 15 min incubation at 60°C and centrifugation at 3000 rpm for 5 min, the supernatant was transferred to a solution including 2% SDS, 40 % glycerol, 0.02% bromphenol blue, 0.08M Tris-Cl, pH8.0, followed by heating at 50-65°C, for 15 min. Samples were stored at –20°C until electrophoresis.

To separate LMW and HMW glutenin subunits 12% SDS-PAGE was used. Gels were stained with 1% Coomassie Brilliant blue R-250 in ethanol and 10% trichloroacetic acid, for at least 24 hours. Following the destaining step, gels were stained with Blakney's and then soaked in 5% glycerol prior to being dried at room temperature for several days.

4.3.3 Sample preparation for quality analyses

Fifty grams of clean grain of each of the parental and DH lines from each year and location were used for quality analyses.

Grain protein content was determined by near-infrared reflectance (NIR) (Method 39-25, AACC 2000). Simultaneously, moisture content, required for grain conditioning was determined. Grain was tempered to 15% moisture in two steps. On the first day, moisture was adjusted to 12% and on the second day to 15% according to the AACC standard method (Method 26-95, AACC 2000).

Conditioned grain samples were milled with a Brabender Quadrimat Laboratory Mill. An additional ten grams were milled using the Udy Cyclone Mill. Whole grain flour was used for SDS-sedimentation test.

4.3.4 SDS-sedimentation test

For each sample, 2.5 g of ground whole meal was weighed onto weigh paper and placed into standard 25 x 200 mm cylinder tubes. Two stock solutions were used: I) lactic acid stock solution (85.0 %, v/v) and II) sodium dodecyl sulfate stock solution (12% w/v). Stock solutions were stored at room temperature. Fresh 'working solution' containing 50% SDS stock solution (v/v) and 10% lactic acid stock (v/v) were prepared daily using distilled water to complete volume. Distilled water (25 mL) was added to each 2.5 g sample. After vortexing, samples were allowed to stand for 15 min before adding 25 mL working SDS solution. Tubes were inverted 10 times and the suspension was allowed to settle for 15 min. The sediment height was recorded in millimeters and converted in milliliters using conversion tables. The cultivar "Navigator" was used as a control during the test.

4.3.5 Mixograph

The multichannel, computer-based, 10 g Mixograph was used to test dough physical properties (Pon et al. 1989). Ten grams of Brabender milled flour was mixed with 6.2 mL (62% absorption) distilled water in the mixing bowl. Torque was recorded and data were calculated electronically. Eight parameters from mixograph curve were obtained. These were: PHG, peak height of mean curve; MDT, mixing development time; ETP, energy to peak; FMS, first minute slope; PBW, peak band width; SAP, slope after peak; TEG, total energy; BWE, band width energy.

4.3.6 Flour protein content

Combustion Nitrogen Analysis (Model FP- 428, LECO Corporation) was used to estimate total protein content of Brabender milled flour. About 0.2 g of flour was wrapped in aluminum foil cones which were burnt at high temperature converting nitrogen to elemental nitrogen. Pure EDTA was used as the calibration sample (Nitrogen% 9.57±0.04). Samples of soy flour (N% 8.55±0.04), tryptophan (13.71% N) and lysine (15.32% N) were used as controls. Flour protein content was obtained using a conversion factor of 5.7 of the percent nitrogen expressed on 14% moisture basis.

4.3.7 Statistical analysis

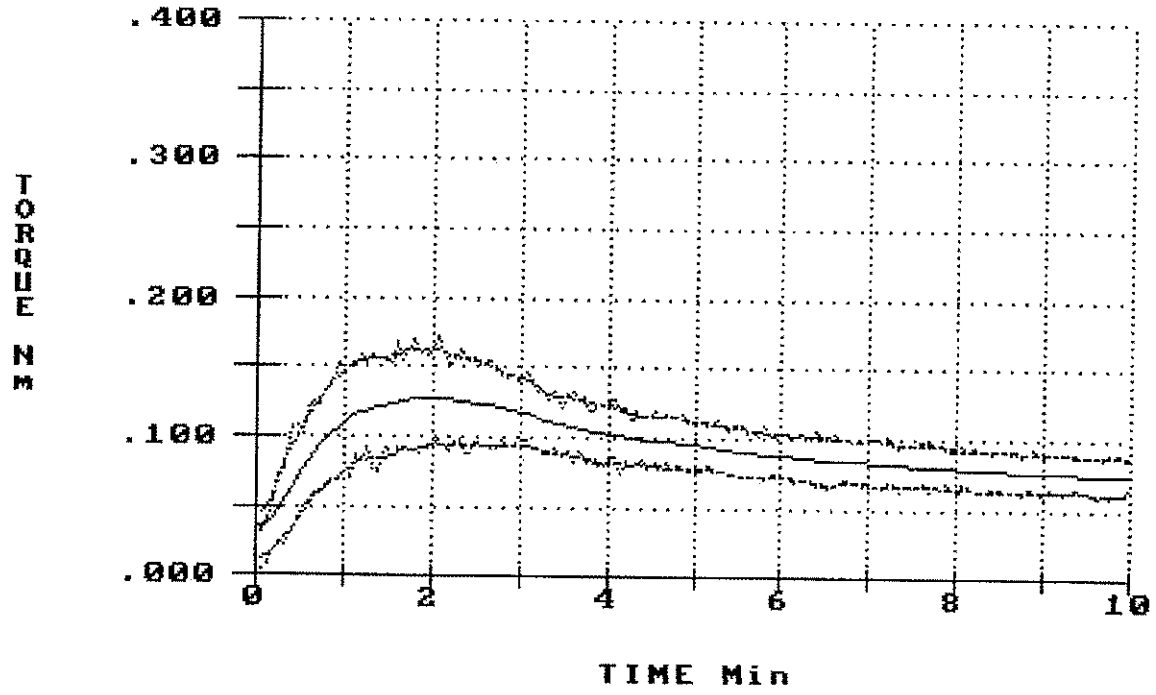
Statistical analysis was carried out using Statistical Analysis System (Version 8, SAS 2000) program. A general linear model was fitted to experimental data. Analysis of variance using Proc GLM was used to test the main effects of subunits encoded at the three Glu-1 loci as well as the two- and three-way interactions. The mean values of the genotypes carrying particular HMW glutenin subunits and specific combinations of subunits were compared to the genotypes carrying contrasting subunits on the same loci. Analysis of variance for 1999 seed increase was performed testing main effects of the subunits encoded at the Glu-A1, Glu-B1, Glu-D1 loci (two level factors), their two-way and three-way interactions. Variance components were calculated using restricted maximum likelihood method in VARCOMP procedure to compare magnitude of variation sources. Data for MDT did not follow a normal distribution and a logarithmic transformation was used.

4.4 RESULTS AND DISCUSSION

4.4.1 Segregation of prolamins in the doubled haploid population

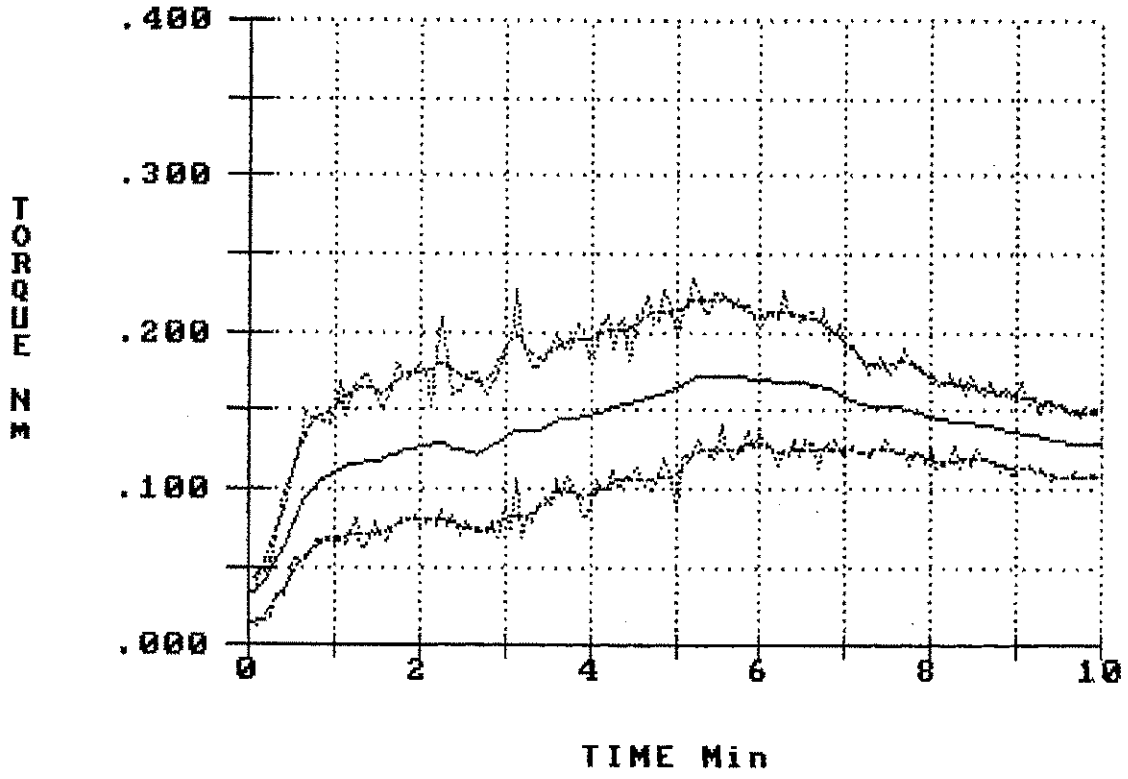
AC Karma is a Canadian Prairie Spring wheat with weaker type strength while 87E03-S2B1 displays strong mixing properties characteristic of the extra strong bread wheat class. Mixograph curves show the mixing characteristics of two parental lines (Fig 2-1A and 2-1B).

Figure 2-1A. Mixograph curve of AC Karma indicates weaker type of gluten, measured by short dough mixing development time (MDT) and big slope after peak (SAP).



PHG: .1276	Nm	MDT: 2.0000	min	ETP: 11.4378	Nm
FMS: .0824	Nm/min	PBW: .0691	Nm	SAP: .0117	Nm/min
TEG: 56.0106	Nm	BWE: 24.8689	Nm	DATE: 12/02/99	

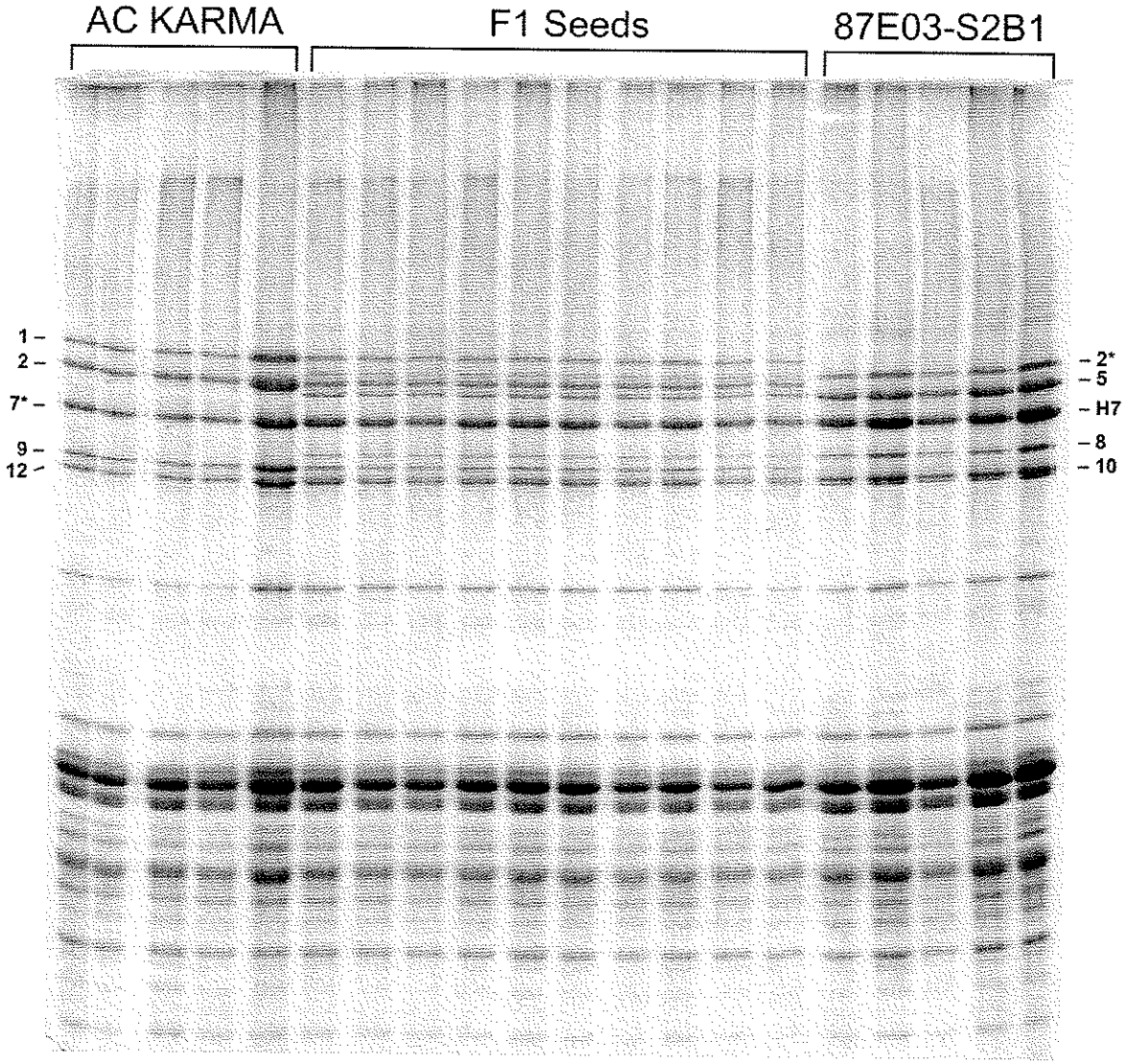
Figure 2-1B. Mixograph curve of 87E03-S2B1 shows strong gluten type. Dough mixing development time (MDT) is long and dough shows resistance to overmixing as seen by stable torque after peak.



PHG:	.1729	Nm	MDT:	5.4667	min	ETP:	41.4686	Nm
FMS:	.0873	Nm/min	PBW:	.0939	Nm	SAP:	.0065	Nm/min
TEG:	82.4482	Nm	BWE:	46.3522	Nm	DATE:	12/02/99	

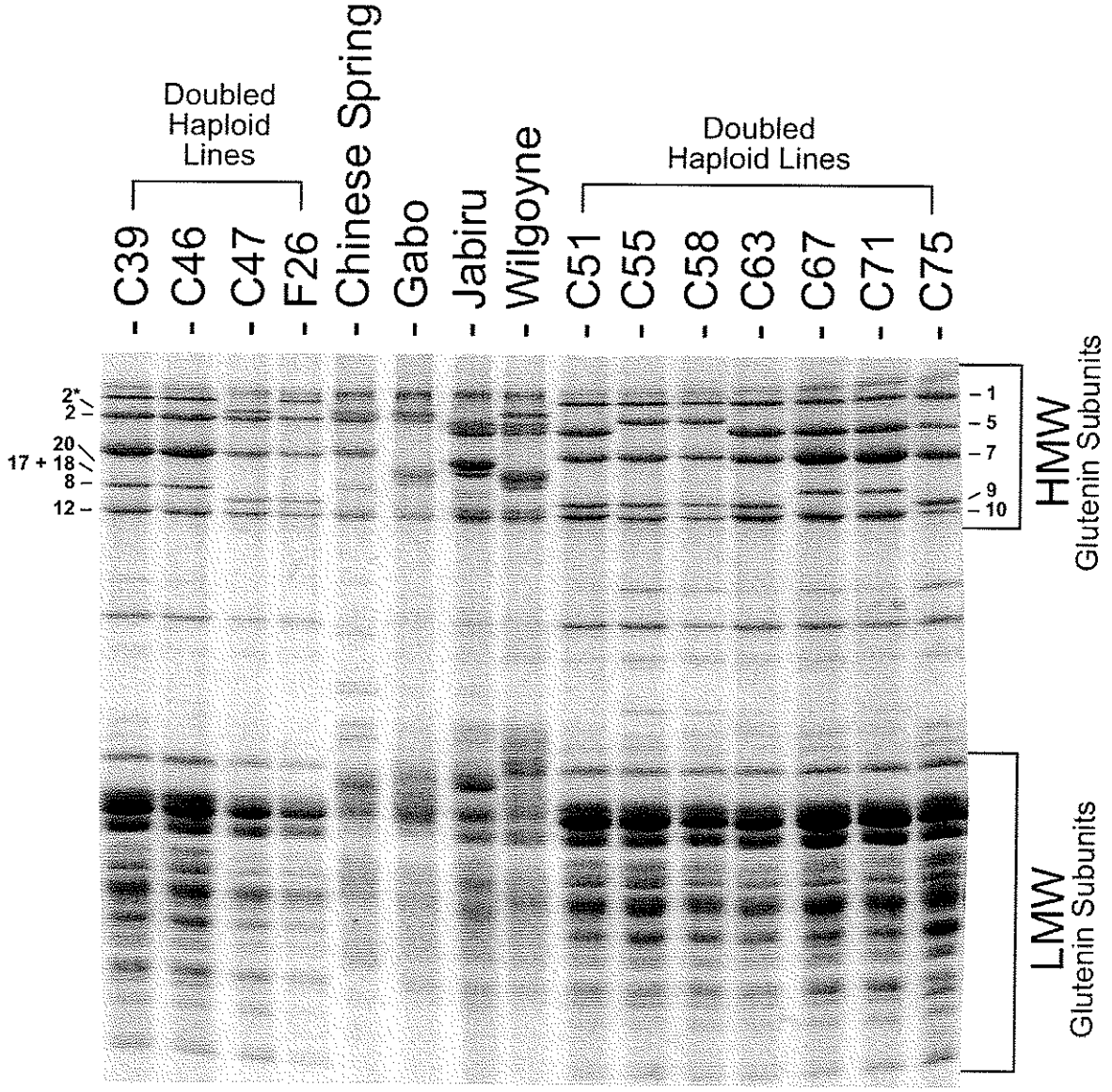
Composition of HMW glutenin subunits encoded by Glu-1 loci of parental lines is as follows: AC Karma has subunits 1, 7*+9, 2+12 (Fig 2-2). The line 87E03-S2B1 has contrasting alleles on Glu-1 loci encoding following subunits: 2*, H7+8, 5+10. The two parental lines have otherwise highly similar LMW glutenin subunits and gliadin profiles as can be visualized on Figure 2-2. This DH population therefore segregated for all HMW glutenin subunits, while being very similar for LMW glutenin subunits and gliadins. SDS-PAGE analysis was used to confirm hybridity of the 10 F_1 seeds used to produce the DH population which was generated using the maize pollination technique (Fig 2-2).

Figure 2-2. 12% SDS-PAGE of ten F1 seeds and five seeds from each parental line. A total of ten HMW bands are detected in hybrid seeds. Subunits H7 and 7* co-migrated. Similar low molecular weight and gliadin composition of the two parental lines can be visualized in the lower portion of the gel.


AC KARMA
F1 Seeds
87E03-S2B1
1
2
7*
9
12
2*
5
H7
8
10

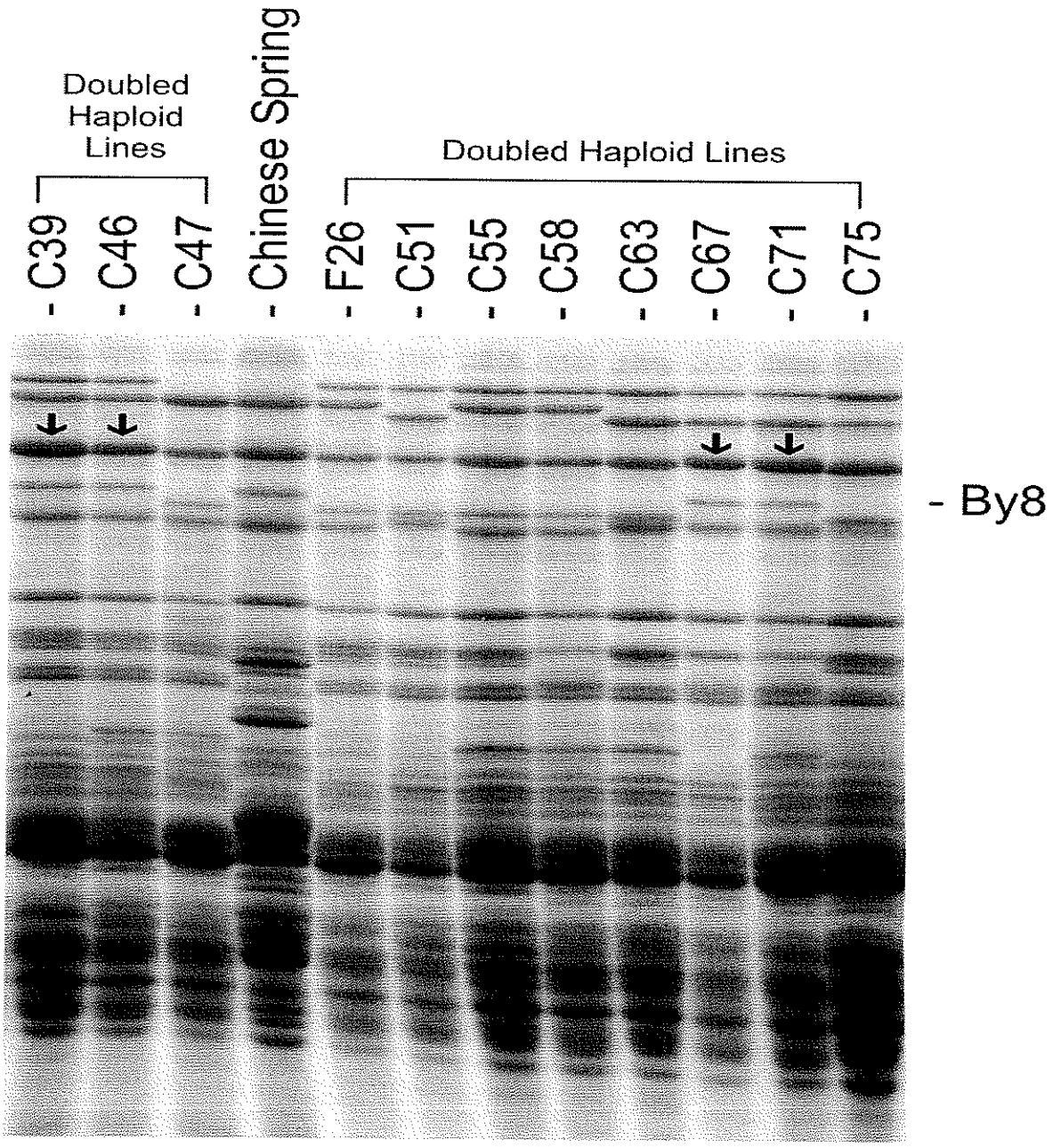
From the original DH population produced from the cross between AC Karma and 87E03-S2B1, a total of 150 DH lines was used for statistical analysis. A total of five HMW glutenin subunits representing three genetic loci were expressed in parental lines and in the F_1-derived DH population. Due to the prolamin composition of the parental lines, the DH population segregated for all HMW glutenin subunits, while only a few minor gliadins and LMW glutenins segregated as seen by SDS-PAGE (Fig 2-3). HMW glutenin subunits were scored based on a previously proposed system (Payne et al. 1983). A total of ten different HMW glutenin subunits were detected and all lines were homozygous for their HMW glutenin alleles confirming that they were true doubled haploid lines.

Figure 2-3. 12% SDS-PAGE of HMW glutenin subunits of DH population. Chinese Spring, Gabo, Jabiru and Wilgoyne are check cultivars. Their HMW glutenins composition is as follows: Chinese Spring 2,7,8,12; Gabo 2*, 2, 17+18, 12; Jabiru 5, 20,10; Wilgoyne 2*, 5, 17+18, 10. HMW glutenin subunits identified in this population are indicated.


Doubled Haploid Lines
C39
C46
C47
F26
Chinese Spring
Gabo
Jabiru
Wilgoyne
Doubled Haploid Lines
C51
C55
C58
C63
C67
C71
C75
2*
2
20
17 + 18
8
12
1
5
7
9
10
HMW Glutenin Subunits
LMW Glutenin Subunits

Subunit Bx7* from AC Karma and 87E03-S2B could not be resolved on the basis of their electrophoretic mobility in 12% SDS-PAGE. They could, however, be distinguished using 10% SDS-PAGE of total protein extract, where the overproduction of the Bx7 subunit from 87E03-S2B (Glenlea*7/C7932) displayed a higher staining intensity as compared to the AC Karma Bx7* subunit (Fig 2-4). This was a qualitative assessment of the overexpression of the Glu-B1 x-type subunit based on its amount relative to other subunits in the same lane.

Figure 2-4. 10% SDS-PAGE of total protein extract was used to identify the overexpressed Bx7 subunit. Chinese Spring was the check cultivar (Bx7 not overexpressed).


Doubled Haploid Lines
- C39
- C46
- C47
- Chinese Spring
Doubled Haploid Lines
- F26
- C51
- C55
- C58
- C63
- C67
- C71
- C75
- By8

Overexpression of the Bx7 subunit also always co-segregated with the By8 subunit from 87E03-S2B while the non-overexpressed Bx7* always co-segregated with By9, confirming the absence of recombination at the Glu-B1 locus. In this cross, a total of eight (2^3) different HMW genotypes can be seen in the DH population. The eight genotypes and their observed frequency in the DH population are given in (Table 2-1).

Table 2-1. HMW glutenin subunits composition of parental lines and number of lines in the DH population segregating for the eight potential genotypes

	HMW glutenin subunits			
Glu-1loci	1A	1B	1D	
Parental lines				
AC Karma	Ax1	Bx7*+By9	Dx2+Dy12	
87E03-S2B1(Glenlea*7/C7932)	Ax2*	HBx7+By8	Dx5+Dy10	
Genotypes				Number of lines
1	Ax1	Bx7*+By9	Dx2+Dy12	21
2	Ax1	Bx7*+By9	Dx5+Dy10	16
3	Ax1	HBx7+By8	Dx2+Dy12	22
4	Ax1	HBx7+By8	Dx5+Dy10	17
5	Ax2*	Bx7*+By9	Dx2+Dy12	18
6	Ax2*	Bx7*+By9	Dx5+Dy10	14
7	Ax2*	HBx7+By8	Dx2+Dy12	24
8	Ax2*	HBx7+By8	Dx5+Dy10	18
				150

4.4.2 Genetic variance for gluten strength

Analysis of variance was performed for the combined sites, one in 1999 and three sites in 2000. Loci were fixed effects. Environment and DH lines within genotypes were random effects. The ANOVA table for four quality parameters is shown in Table 2-2. Genetic effect was subdivided into the main effect of Glu-A1, Glu-B1 and Glu-D1 loci, their two-way and three-way interactions.

Subunits encoded by Glu-D1 showed the greatest relative contribution to six of the eight mixograph parameters measured, with the exception of BWE (Table 2-2).The effect of Glu-D1 encoded subunits on TEG was not significant. The effect of Glu-D1 encoded subunits was highly significant ($P< 0.0001$) for mixograph parameters, MDT, PKH, ETP, FMS and SAP, while PBW and BWE were significant at 0.001, and 0.01 levels of significance, respectively. Glu-B1 encoded subunits had significant effect on most mixograph parameters, with the exception of mixograph peak height. The significance levels were somewhat lower as a whole than for the Glu-D1 locus. Band width energy was the only mixograph derived parameter for which the relative contribution of the subunits encoded at Glu-B1 locus was higher than the effect of Glu-D1 encoded subunits (Table 2-2).

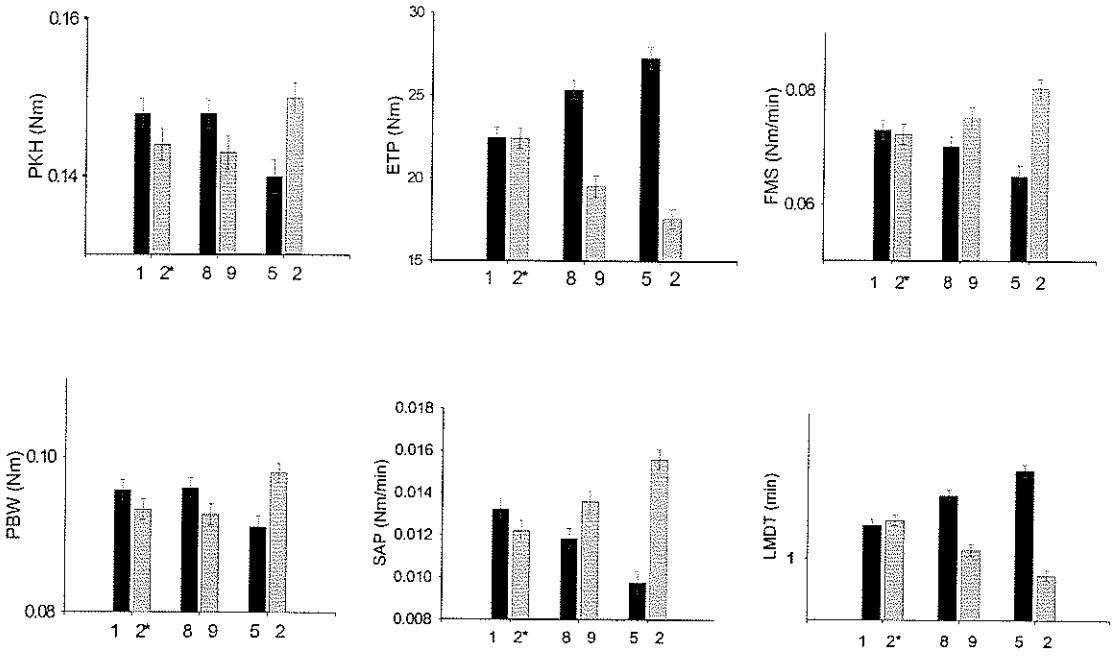
Table 2-2. Analyses of variance for mixing development time (MDT), energy to peak (ETP), SDS-sedimentation test, flour protein, peak height (PKH), first minute slope (FMS), band width energy (BWE) and slope after peak (SAP)

		Mean square							
Source of variation	DF	MDT	ETP	SDS-sedimentation	Flour protein	PKH	FMS	BWE	SAP
Environment	3	7.92**	2509.16**	9000.64**	119.11**	0.01**	0.02**	5126.5**	0.002**
Lines (Genotypes)	142	0.21**	106.32**	102.73**	2.82**	0.16**	0.001**	91.78**	0.0001**
A	1	0.08	0.62	3.68	0.22	0.003	0	24.62	0.0002
B	1	10.11**	4883.14**	2438.71**	17.93*	0.003	0.004**	1663.91**	0.0005**
D	1	37.17**	13538.00**	1715.77**	0.02	0.03**	0.03**	802.33**	0.005**
A* B	1	0	28.71	73	3.75	0	0	3.76	0.0001
A* D	1	0.12	7.85	86.01	0.01	0.002	0.002	14.16	0
B* D	1	0.4	16.77	787.74**	8.01	0.001	0.001	386.35*	0
A * B * D	1	0.05	11.18	0.24	0.17	0	0	53.81	0
Error	440	0.03	23.75	23.23	0.57	0	0.0003	23.23	0.00003

*, ** Significant at $P = 0.05$, $P = 0.01$, respectively.

Comparisons of means of lines having H7+8 and 7*+9 combinations of subunits showed that the overexpressed Bx7 subunit had a positive effect on mixing development time, energy to peak, peak band width contributing to the stronger dough as inferred from these parameters (Fig 2-5). The positive effect of the H7+8 subunit combination may be explained by a quantitative contribution of overexpressed Bx7 subunit in the total amount of HMW glutenin subunits. A higher amount of HMW glutenins has been reported to improve gluten strength (Shewry et al. 1992).

Figure 2- 5. Comparisons of means of all DH lines with given HMW glutenin patterns for peak height (PKH), energy to peak (ETP), first minute slope (FMS), peak band width (PBW), slope after peak (SAP) and logarithmically transformed mixing development time (LMDT) of DH lines having contrasting HMW glutenin subunits at the Glu-1 loci (combined 1999 and 2000 data). Parameters with a significant main effect at a minimum of one Glu-1 locus and no intergenomic interactions are reported.



1= Ax1 subunits at Glu-A1 locus
2*= Ax2*subunits at Glu-A1 locus
8= By8 subunit at Glu-B1 locus. By8 co-segregates with overexpressed Bx7*
9= By9 subunit at Glu-B1 locus. By9 co-segregates with Bx7*
5= Dx5 subunit at Glu-D1 locus, co-segregates with Dy10
2= Dx2 subunit at Glu-D1 locus, co-segregates with Dy12

Grain protein content was not significantly affected by any HMW glutenin composition. Glu- B1 encoded subunits had a small, but significant positive effect on flour protein content (Table 2-2). Mean values for flour protein for lines with H7+8 was 13.17% while lines with 7*+9 averaged 12.82% flour protein. If HMW glutenins comprise 10% of total protein in cultivars with five HMW glutenin subunits, this means that each individual subunit contributes an average to 2% of the total protein. If the overexpression of the Bx7 subunit is the result of duplication, then a small increase of 2% of the protein fraction, i.e., 0.26 % on a 13% protein basis, is expected. Observed difference of 0.35% fits this hypothesis well. Molecular evidence for duplication of the Bx7 gene has been published (D'Ovidio et al. 1997). In this genetic background, comparison of HMW glutenin Ax1 and Ax2* did not contribute significantly to any of the tested quality parameters nor to the grain or flour protein content (Table 2-2). Evidence for a significant role of the Glu-A1 encoded subunits has been shown with different genetic material. Higher SDS-sedimentation volume was observed when Ax1 or Ax2* were present as compared to the null allele (Payne et al. 1987, Gupta et al. 1989). No significant difference in main effects of Glu-A1 alleles were reported by Cox et al. (1991) for two parameters, mixing time and mixing tolerance, but significant interaction between the Glu-A1 and Glu-B1 encoded subunits was observed.

Comparisons of means of genotypes within lines containing contrasting subunits at Glu-1 loci are shown in Fig 2-5. Comparisons are shown only for parameters with

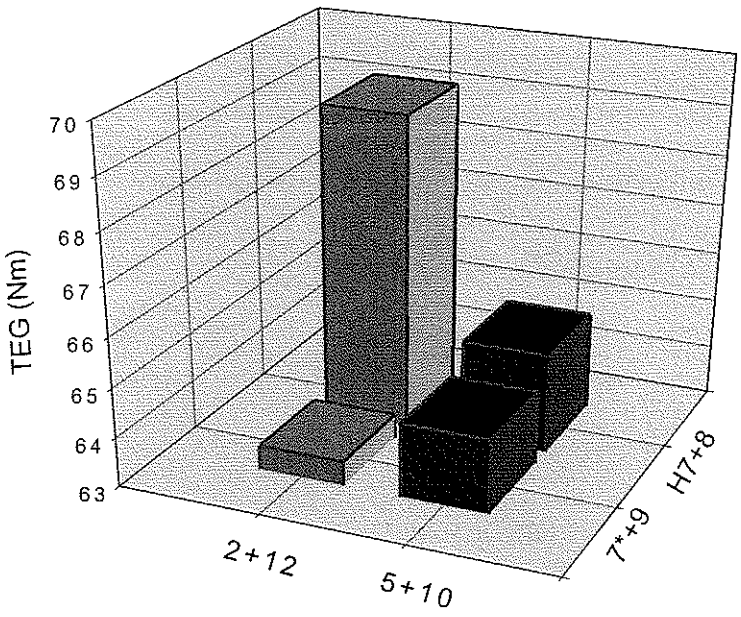
significant main effect of any Glu-1 loci and no significant interactions between loci. Significantly higher ETP, PBW and LMDT were obtained for DH lines carrying H7+8 subunits combination compared to 7*+9 containing lines. FMS and SAP were however higher for lines with the 7*+9 combination of subunits. For ETP and LMDT, higher values were obtained for genotypes with 5+10 subunits, while PKH, PBW, FMS and SAP were significantly higher for genotypes carrying 2+12. Significant interactions between Glu-B1 and Glu-D1 encoded subunits were observed for SDS sedimentation volume, TEG and BWE (Figure 2-6). No other two-way or three-way interactions were observed (Table 2-2).

The SDS-sedimentation test has been used in many studies as a measure of gluten strength (Payne et al. 1981, Mir Ali. et al 1999, Sontag-Strohm et al. 1996). While this test may not be as accurate as the mixograph test in estimating gluten strength parameters, it offers other advantages. The test requires little starting material, is inexpensive, fast, and requires no specialized equipments which makes it well suited for early generation screening in a breeding program. Higher sediment volume correlates with stronger dough and better bread making properties (Lorenzo et al. 1987). In this study high sedimentation volumes (>63 ml) were obtained for all glutenin compositions except 7*+9 with 2+12 which interacted in negative fashion giving a lower sedimentation volume, averaging only 57 ml (Fig 2-6C). Results obtained in SDS-sedimentation test indicating high gluten strength of 5+10 and H7+8

containing lines. This is in agreement with mixograph derived results for MDT, ETP, BWE(Figure2-5 and Figure 2-6B).

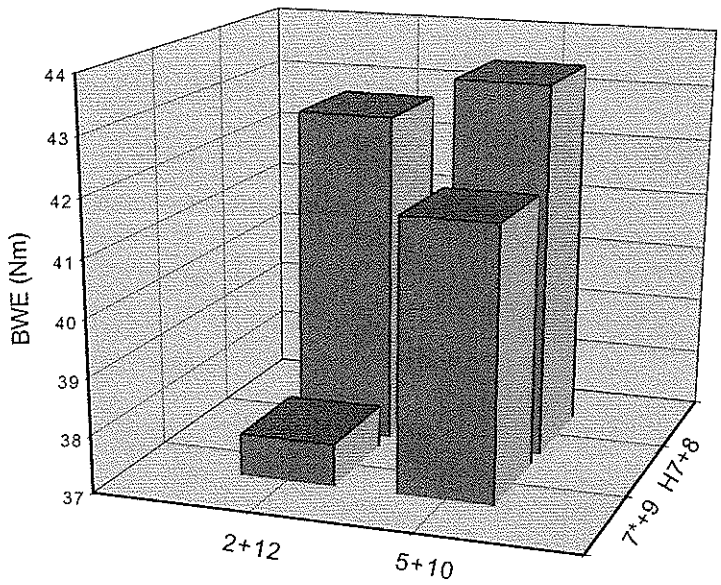
Figure 2-6 A-C. Comparisons of means for A) total energy (TEG), B) band width energy (BWE) and C) SDS-sedimentation and of lines having the combination of Glu-1B and Glu-1D encoded subunits where significant two-way interaction was observed. Results for 150 DH lines, for 1999 and 2000 data are presented.

A



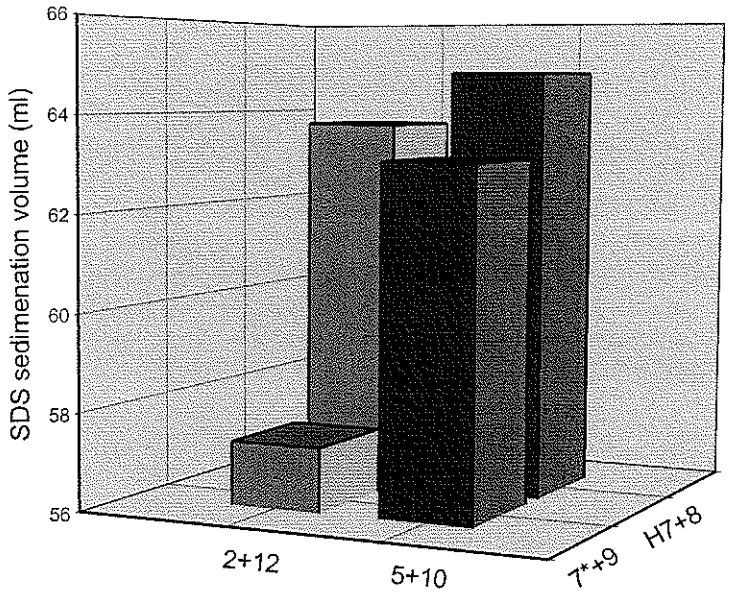
2+12= Dx2+Dy12 at Glu-D1 locus
5+10= Dx5+Dy10 at Glu-D1 locus
H7+8= overexpressed Bx7+By8
7*+9= Bx7*+By9

B



2+12= Dx2+Dy12 at Glu-D1 locus
5+10= Dx5+Dy10 at Glu-D1 locus
H7+8= overexpressed Bx7+By8
7*+9= Bx7*+By9

C



2+12= Dx2+Dy12 at Glu-D1 locus
5+10= Dx5+Dy10 at Glu-D1 locus
H7+8= overexpressed Bx7+By8
7*+9= Bx7*+By9

Intergenomic BxD interaction was also observed for TEG. A H7+8 subunit composition had the highest average value of 69.3 Nm interacting in a positive way with 2+12 and 5+10 subunits (65 Nm).The 7*+9 interaction with 5+10 subunits yielded the 64.5 Nm and AC Karma 7*+9/2+12 combination was shown to be not advantageous based on TEG values. Observed differences in TEG were significant at P=0.05).

In addition, significant BxD interaction was obtained for BWE (Fig 2-6B). In this case, the lowest BWE was observed for the AC Karma type of genotypes carrying 7*+9 and 2+12 subunits (37.7 Nm) showing that this combination of subunits interacts unfavorably as measured by BWE. The combination of subunits 7*+9 with 5+10 resulted in an increase of BWE to 41.7 Nm. Higher BWE values were obtained in the H7+8 background, averaging 42.8 Nm for 2+12 combination and 43.5 Nm for Glenlea (H7+8/5+10) subunits composition (significantly different at P=0.05).

The genotypes expressing 5+10 and H7+8 combinations of subunits had stronger gluten and better mixing properties as was shown from higher values for SDS-sedimentation test, mixing development time, energy to peak, and smaller values for first minute slope and slope after peak. These parameters are commonly used in breeding programs to assess gluten strength of breeding lines.

It has been reported previously that cultivars with 5+10 subunit composition have stronger dough characteristics than cultivars carrying 2+12 subunits (Payne et al. 1991, Branlard et al. 1985, Lorenzo et al. 1987, Lukow et al. 1989, Cox et al. 1991, MirAli et al. 1999) and stronger gluten was observed for lines having an overexpresed Bx7 subunit (Perron et al. 1998).

How Glu-B1 and Glu-D1 encoded subunits influence gluten strength and dough mixing properties is not completely understood at the molecular level. Subunits Dy10 and Dy12 differ from one another by a few amino acids and the presence of two extra hexapeptides in Dy12 (Shewry et al. 1992). All variation resides in the repetitive domain. These minor differences seem unlikely to be significant for glutenin polymer formation and dough properties. A near isogenic line lacking the Dx5 subunit was compared to NIL lacking the Dy10 which showed the largest loss in the bread making potential occurred for the line missing the Dx5 subunit (Payne et al. 1991). Therefore, x- type subunits appear to have an important role in the observed quality differences between 5+10 and 2+12 subunit combinations. Number and position of cysteine residues in glutenin polypeptides are fundamental for polymer formation and subsequently mixing and baking performance. Derived and compared amino acid sequences of Ax2*, Bx7 and Dx5 from cultivar Cheyenne (Anderson et al. 1989) revealed that the Dx5 subunit has an extra Cys residue in 5' non repetitive domain. Therefore, Dx5 and Dx2 subunits are identical in the first 124 amino acid residues (Kohler et al. 1997) with the exception of Ser amino acid residue at position 97 in

Dx2 which has mutated to Cys in Dx5 subunit. This additional cysteine has been proposed to contribute to the better quality associated with the 5+10 subunit pair by allowing branching of the polymer and therefore formation of a larger polymer, influenced by the presence of three or more cysteine residues available for the formation of intermolecular disulphide bonds (Kasarda 1999). In terms of cysteine residues available for formation of intermolecular disulphide bonds, Bx7 subunit is equivalent, or less efficient compared, to Dx2 and less efficient than Dx5 subunit (Kohler et al. 1997). Therefore, it seems that the increased gluten strength associated with HBx7 may be due to the increased amount of the x-type subunits as opposed to structural characteristics of the subunit. Furthermore, the Dx5 subunit has a larger repetitive domain than the Dx2 subunit which is an important factor contributing to the elastic properties of the gluten.

It was shown in this study that during dough mixing and development, time required for dough to reach peak (maximal resistance), which is a measure of dough strength is longer for 5+10 subunits than for the lines containing 2+12 subunits (Figure 2-5). In contrast, accumulation of large polymers associated with peak height on a mixograph curve was significantly higher for 2+12 lines than for 5+10. Both Glu-B1 and Glu-A1 encoded subunits did not have significant effect on peak height. Accumulation of larger polymers for the 2+12 combination of subunits compared to 5+10 has been previously reported (Zhu et al. 1999). However, all 2+ 12 lines with high peak showed very low dough resistance and fast breakdown suggesting that

type of the polymers formed involving these subunits are easy to break during the continuous mixing. Peak height comparison between AC Karma and 87E03-S2B1 (Table 2-3) revealed that 87E03-S2B1 has a higher mixograph peak than AC Karma showing.

Table 2-3. Means for tested quality parameters for DH lines and parental lines for 1999 and 2000 data

	DH lines		AC	87E03-S2B
			Karma	
	Mean	Range	Mean	Mean
Mixing development time (min)	1.15	0-2.95	0.95	2.17
Mixograph peak height (cm)	0.15	0.072-0.271	0.12	0.16
Energy to peak (Nm)	22.01	7-63	12.20	37.26
First minute slope (Nm/min)	0.07	0.015-0.177	0.06	0.07
Peak band width (Nm)	0.10	0.027-0.175	0.09	0.11
Total energy (Nm)	65.95	34-110.9	57.07	73.00
Band width energy (Nm)	41.46	20.1-81.3	36.59	52.82
Slope after peak (Nm/min)	0.01	0-0.048	0.012	0.008
SDS-sedimentation value (mL)	62.10	35-91	50.50	66.20
Grain protein (%)	13.56	11.3-17.1	12.875	14.04
Flour protein (14% mb)	12.97	9.73-16.27	12.00	13.41

The importance of HMW glutenin subunits for gluten strength and breadmaking quality is well documented. Consequently information of the variability contributed by individual loci is valuable in breeding for gluten strength.

Variance components were estimated for each of the eight mixograph measurements as well as SDS-sedimentation value, grain protein and flour protein (Table 2-4). For all tested parameters, variation due to environment was significant (data not shown). The proportion of the variability contributed by the environment was very high for some parameters, i.e., over 50% for SDS-sedimentation test suggesting that breeding lines need to be tested over multiple environments if this test is used for selection. Mixograph parameters like ETP and MDT had the lowest proportion of environmental variance and a relatively low proportion of variability contributed by sources other than HMW glutenins. Subunits encoded at Glu-B1 and Glu-D1 loci account for 12% and 43% of total variability for MDT. Therefore, contribution of the Glu-B1 and Glu-D1 loci which significantly contribute to gluten strength is 55%. Additive contribution of Glu-B1 and Glu-D1 encoded subunits resulted in 51% of the variability for ETP with individual 13.5% and 37.5% for each locus respectively.

Table 2-4. The amount of variation for each source and the percentage of the total variation

Variance components	PKH	ETP	FMS	PBW	SAP	TEG	BWE	LMDT	SDS	GPro	FPro
Environment	8.8	13.4	15.4	21.7	15.7	4.1	32.1	18.0	52.3	52.0	41
A	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0
B	1.1	13.5	1.2	1.3	2.2	1.7	4.2	11.9	4.8	0.3	2.1
D	11.8	37.5	17.9	5.0	24.7	0.0	1.2	43.0	2.6	0.0	0.0
AXB	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0
AXD	0.9	0.0	0.1	0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0
BXD	0.0	0.0	0.1	0.0	0.0	3.2	1.8	0.4	4.2	0.2	0.4
AXBXD	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Lines(Genotype)	25.7	16.4	23.9	11.7	15.8	25.5	7.2	15.2	16.5	25.1	27.8
Residual	51.8	19.2	41.4	59.7	41.4	65.5	53.4	11.6	19.6	22.3	28.7

HMW glutenin subunits are the single most important factors which determine gluten strength. Therefore, they can be used in early generation selection for strong gluten. They are easy to assess using a single seed. In early generations, breeding lines can be screened for desirable HMW glutenins, followed by small scale quality tests in subsequent generations, screening for agronomic traits and yield tests in advanced stages.

4.5 CONCLUSIONS

This study showed that some HMW glutenin subunits and their combinations are more effective than others in conferring gluten strength and dough mixing properties as determined by SDS-sedimentation test and mixograph analysis. For most of the tested parameters, the effects of Glu-B1 and Glu-D1 loci were additive. Also, some intergenomic interactions were observed but only the B and D genomes were involved. Knowledge about those allelic variants which positively contribute to gluten strength, their additive effect, and effects due to intergenomic interactions, are beneficial to breeding programs that aim to produce cultivars with improved gluten strength. Favorable subunits can be brought together in the breeding process and when alleles for these subunits are fixed in the population lines can be screened for agronomic traits.

The effect of Glu-A1 encoded subunits Ax1/Ax2* was nonsignificant for gluten strength. Glu-D1, which encoded the subunit pair 5+10, had a positive effect on MDT, ETP, SAP, FMS, mixograph parameters commonly used for screening lines in breeding for gluten strength. A negative effect of this subunit pair was obtained for PKH. The greatest contribution to gluten strength was obtained for the Glu-D1 locus, accounting 43% of total variation for MDT. Estimated genetic variance of Glu-D1 locus for ETP is 37.5 %.

The effect of overexpression of Bx7 conferred by Glu-B1 locus was significant for most of the parameters, contributing 12% to the variability for MDT and 13.5 % for ETP. Therefore, the additive effect of H7+8 and 5+10 subunits accounted for 55% of the variability for MDT. A total of 51% of the total variability for ETP was determined by the additive effect of Glu-D1 and Glu-B1 loci.

The relatively large variance due to HMW glutenin subunits encoded at Glu-D1 for ETP (37.5%) and Glu-B1 encoded subunits (13.5%) compared to the other sources [lines(genotype) and residual] suggest that a large portion of the variability for gluten strength could be exploited by selecting for specific HMW glutenin subunits in a breeding populations. Similarly, a significantly higher proportion of the variability for MDT was contributed by Glu-B1 and Glu-D1 compared to the contribution of other sources, indicating that the incorporation of H7+8 and 5+10 subunits would be desirable for gluten strength.

5.0 GENERAL DISCUSSION AND CONCLUSIONS

Wheat flour has unique visco-elastic properties influenced mainly by protein component. Proteins comprise only 10-15% of flour dry weight and seed storage proteins called prolamines contribute a significant proportion to total protein. Prolamins are deposited in protein bodies, in the endosperm, during kernel development. They determine functional properties of dough, particularly its gluten component. Other components of dough, such as carbohydrates and lipids may affect dough properties, but predominantly through interactions with gluten.

Prolamin polypeptides by means of covalent and noncovalent interactions form a diversity of polymers which together with monomeric gliadin polypeptides, starch granules and other minor components of dough form a very complex substance known as the gluten matrix. Cysteine amino acid residues are points where covalent disulfide cross links can be formed within or between polypeptides. Also, covalent bonds between tyrosine amino acids are involved in polymer formation. Prolamins are rich in glutamine amino acid which facilitates formation of hydrogen bonds, stabilizing protein-protein interactions.

When flour is mixed with water, flour components become hydrated and a process known as dough development results in the formation of the gluten matrix. The matrix consists of covalently and noncovalently linked polypeptides and starch

granules, which are incorporated in a network where lipid components play a role as plasticizers. Developed dough has elastic and viscous (extensible) properties. Balance between these two physical properties makes dough suitable for different end-use products. For bread making, strong and elastic dough is required. Dough strength is predominantly dependant on gluten strength.

Gluten proteins are diverse in terms of structure and functional characteristics. The class of gluten proteins known as glutenins consists of LMW and HMW glutenins which can form polymers. Gliadins are another class of gluten proteins which form intramolecular bonds only, and interact with glutenin polymers through noncovalent bonds. Gluten quality ultimately depends on these two classes of proteins, their amounts and types.

HMW glutenins are quantitatively a small class of prolamins making up only 10% of flour protein content, but appear to make the largest contribution to gluten strength. Also, the number of HMW glutenin subunits expressed in a wheat cultivar and the amount of each subunit contribute to dough strength. Differences in the amount of HMW glutenins and number of expressed subunits are mainly caused by differences in the promoters of genes The promoters contain elements responsible for tissue specific expression, expression levels and gene silencing. Other factors such as gene duplication can also play a role. Bread wheat varieties usually have 3 -5 HMW glutenin subunits, 15 LMW glutenins and more than 30 gliadins.

HMW glutenins are the largest prolamin polypeptides at 60-90 kDa. Size of the repetitive domains of HMW glutenin subunits with specific secondary structure is an important contributor to elasticity and strength. Evolution of the repetitive domain has been influenced by single base changes which do not necessarily influence amino acid change. The addition or deletion of a single base in repeats would lead to a frame shift which might result in a premature end in gene translation and gene silencing. Furthermore, addition or deletion of whole repeats, or blocks of repeats, are possible events which have led to differences in the sizes of repetitive domains between subunits. Mutations have driven a certain level of diversity of HMW glutenins in terminal domains, giving them distinct properties in terms of the presence or absence of some amino acid residues which are important for their conformation and bond formation.

HMW glutenins are encoded at loci on the long arm of chromosome 1 in the A, B and D genomes of wheat. Each locus has two linked genes. A very low frequency of recombination has been observed within loci and each is designated as a single locus Glu-A1, Glu-B1 and Glu-D1.

While these genes show a high level of homology, they also show some distinct characteristics resulting in different forms known as HMW glutenin alleles and subsequently HMW glutenin subunits. Distinct characteristics of these subunits,

along with their number and amount, affect their behavior in polymerization and dough formation.

In this project, the effect of different HMW glutenin subunits was studied using a doubled haploid population which segregated into eight genotypes minimizing the genetic background for HMW glutenins. This population had a uniform LMW glutenin and gliadin profile. Doubled haploid lines are beneficial for genetic studies of wheat quality because homozygosity is obtained in the generation following the F1. Doubled haploid lines were tested for gluten strength by SDS-sedimentation test and mixograph test and statistical analysis was performed in order to obtain information about the effect of each locus and their interactions.

Mixograph development time (MDT), energy to peak (ETP), band width energy (BWE) and slope after peak (SAP) are commonly used by breeders to assess mixing properties of dough and its strength. Results from this study indicate a significant effect of the D genome encoded subunits on a majority of parameters used to evaluate gluten quality. In this population, the effects of 5+10 vs. 2+12 subunit pairs encoded at the D genome locus were compared and a clear positive effect of 5+10 was obtained for MDT, ETP and SAP along with SDS-sedimentation test.

A significant, if slightly lower effect was obtained for B genome encoded subunits for MDT, ETP, SAP. A comparison was made between H7+8 and 7*+9 pairs. Observed

differences appear to be due to the positive effect of overexpression of Bx7 (H7) subunit on gluten strength. Besides the main effect of B and D genomes, their intergenomic interaction for BWE, TEG and SDS-sedimentation test was obtained.

The objective of this research study with respect to breeding programs was to determine which allelic variants contribute to gluten strength. With an assumption that a great portion of the variability for gluten strength is accounted by HMW glutenins, it was important to estimate variability contributed by each locus separately. For mixing development time and energy to peak, the largest proportion of the variability is contributed by HMW glutenins B and D subunits accounting for 51% of the variability for ETP and 55% for MDT.

While wheat quality is a complex trait, results from this and many other studies suggest that a strong relationship between particular subunits and dough quality exist. Hence, these subunits can be used as markers in early breeding generations, followed by functional tests in later generations while screening for yield and agronomic traits.

Once the positive effect of a particular subunit on gluten strength is established and favorable subunits are introduced to a breeding population, it is important that reliable and fast screening method for HMW glutenins composition exist. Effective

early generation screening for HMW glutenin composition will reduce population size and provide more efficient use of breeding resources.

In this study, genotypes of doubled haploid lines were determined using SDS-PAGE. This method has been widely used in the last decade to assess glutenin subunit composition in breeding lines. SDS-PAGE allows one to determine the complete HMW glutenin profile using endosperm tissue of a single seed. However, this method has the disadvantage that only seed endosperm can be used. As an alternative, PCR technology was employed for early generation screening for HMW glutenins. A prerequisite for PCR primer design was the availability of HMW gene sequences. All HMW glutenin alleles had been cloned and sequenced which allowed the study of sequence homology and possibilities for primers design. In spite of high similarity within this gene family, sets of primers for HMW glutenin genes were developed providing means for gene assisted selection and early generation screening for gluten strength. PCR analysis was performed using haploid leaf tissue which is beneficial in doubled haploid production system because screening of desired lines can be performed before the step of chromosome doubling.

Strong linkage between the genes at HMW glutenin loci allows PCR screening to be done only for one gene at a locus while providing selection of the entire locus. At this time, PCR screening for HMW glutenins must be done in three separate PCR

reactions which is a disadvantage as compared to simultaneous subunit detection in SDS-PAGE.

Conclusions:

Difference between Glu-A1 encoded subunits Ax1 and Ax2 * on gluten quality was neutral. Glu-D1 locus encoded subunits had the greatest contribution to gluten strength. Subunit pair 5+10 showed positive effect on most mixograph parameters related to stronger dough and SDS-sedimentation test.

Comparison of H7+8 and 7*+9 pairs at Glu-B1 loci showed positive effects of the H7+8 pair. Its positive contribution is the result of the overexpressed Bx7 subunit originating from the 87E03-S2B1(Glenlea*7/C7932) line. Also, higher flour protein content was obtained for H7+8 containing lines.

PCR primers for three HMW glutenin loci have been developed and efficiency of their use was tested using DNA extracted from haploid leaf tissue. To distinguish Ax1/ Ax2* alleles, a co-dominant marker system was designed. Differences between HBx7/Bx7* alleles can be tested in both, co-dominant and dominant marker systems. Glu-D1 encoded Dx5/ Dx2 alleles can be distinguished using a dominant PCR-based marker system.

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Appendix1.

Allele	Forward Primer	Reverse Primer	Co-dominant vs. dominant	Fragment size (bp)
HBx7	Bx7-F-428:5'-CAACAACTTGTGGGGGCCTT-3'	Bx7-R693:5'GCGCTTAGCCATCTCAGTGAAC-3'	Dominant	1116
HBx7/Bx7*	Bx7F-572: 5'-ACCTCAGCATGCAAACATG-3'	Bx7-R693:5'GCGCTTAGCCATCTCAGTGAAC-3'	Co-dominant	1302/1259
Dx5	Dx5F384: 5'-CGTCCCTATAAAAGCCTAGCC-3'	Dx5R655: 5'-GGCTAATGTCTCGGAGCTGT-3'	Dominant	272
Ax1/Ax2*	Ax2*F2543: 5'-AAGACAAGGGGAGCAAGGT-3'	Ax2*R3605: 5'-GTGCTCCGCGCTAACATG-3'	Co-dominant	1090/1063

Appendix 2.

ANOVA for MDT (1999 data)

MDT

Source	DF	Sum of Squares	Mean Square	F Value	Pr> F
Model	7	230.98	32.9	20.8	0.0001
Error	138	218.18	1.58		
Corrected Total	145	449.16			

Source	DF	Type III Sum of Squares	Mean Square	F Value	Pr> F
A	1	1.62	1.62	1.02	0.3133
B	1	45.2	45.2	28.6	0.0001
D	1	179	179	113.2	0.0001
A x B	1	0.11	0.11	0.07	0.7904
A x D	1	2	2	1.12	0.2922
B x D	1	0.7	0.7	0.44	0.506
A x B x D	1	0.09	0.09	0.06	0.8132