Identification and Characterisation of a Novel Glutenin Subunit in Bread Wheat (*Triticum aestivum* L.)

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List of Abbreviations

AACC	American Association of Cereal Chemists
AGT	Australian Grain Technologies
A-PAGE	Acid polyacrylamide gel electrophoresis
AWCC	Australian Winter Cereals Collection
CSIRO	Australian Commonwealth Scientific and Research Organisation
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EBI	European Bioinformatics Institute
EPP	SDS-extractable polymeric protein
GRIS	Genetic Resources Information System
HMW-GS	High molecular weight glutenin subunit
HPLC	High performance liquid chromatography
HRM	High resolution melting
IPTG	Isopropyl-β-*D*-thiogalactopyranoside
ISBP	Insertion site-based polymorphism
LB	Lysogeny broth
LC-MS	Liquid chromatography-mass spectrometry
LMW-GS	Low molecular weight glutenin subunit
LTR	Long terminal repeat
MAR	Matrix attachment region
MAS	Molecular assisted selection
M_r	Molecular weight

NCBI	National Center for Biotechnology Information
NIRS	Near- infrared spectrophotometry
PCR	Polymerase chain reaction
PEB	Phosphate extraction buffer
PPK	Putative protein kinase
PVDF	Polyvinylidine difluoride
R_{max}	Extensograph maximum resistance
RP-HPLC	Reverse-phase high performance liquid chromatography
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SE-HPLC	Size-exclusion high performance liquid chromatography
SNP	Single nucleotide polymorphism
TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid
TPP	Total polymeric protein
UPP	SDS-unextractable polymeric protein in total polymeric protein
UV	Ultraviolet
4-VP	4-vinylpyridine

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Abstract

Bread is one of the major constituents of the human diet and wheat (*Triticum aestivum* L.) is the most important cereal for bread making. The gluten proteins (glutenins and gliadins) are recognised as important components affecting the processing quality of wheat flour. *Glu-B1al* is an allele that includes a duplication of a gene encoding an x-type high-molecular-weight glutenin subunit, and is thought to increase dough strength through overexpression of that subunit. In this research, a particular glutenin subunit in an Australian cultivar, H45, was investigated. H45 seemed to carry *Glu-B1al*, but it has relatively low unextractable polymeric protein (UPP, an indicator of weak dough). Two Bx genes from H45 were cloned and sequenced. Their sequences differ from each other, and each differ by four single nucleotide polymorphisms (SNPs) from the sequence of the Bx genes of *Glu-B1al* in the Canadian wheat cultivar Glenlea. One of the SNPs leads to an extra cysteine residue in one of the subunits. The *Glu-B1* allele of H45 was designated *Glu-B1br*.

With a restriction digest assay designed to distinguish the *Glu-B1br* allele from other overexpression alleles, it was demonstrated that *Glu-B1br* is co-inherited with low UPP. Among accessions present in the pedigree of H45 and accessions carrying overexpression alleles, *Glu-B1br* was detected only in H45.

Efforts were made to develop alternative markers for *Glu-B1br*. Potential polymorphic regions within or close to *Glu-B1* locus were investigated, but no closely linked polymorphisms were found that could be targeted for marker design.

Individual glutenin subunits encoded by overexpression alleles and a mutant gene (*MutBx7.1*)

derived from the first gene (*Bx7.1*) of *Glu-B1br* were obtained by heterologous expression. Flour incorporation tests showed that the glutenin subunit with the extra cysteine residue (Bx7.1) affects flour and dough mixing properties differently from MutBx7.1 and from the Bx subunits encoded by other overexpression alleles. Given that Bx7.1 and MutBx7.1 differ only with respect to the additional cysteine in Bx7.1, the effects of Bx7.1 on the dough properties of H45 can be attributed directly to that cysteine, which may act by impeding polymerisation.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Chapter 1

General Introduction

Globally, wheat (*Triticum* spp.) is one of the most-produced, consumed and stored cereal grains. The important position of wheat in global agriculture is partly due to work done by wheat breeders to develop improved varieties. Both grain yield and grain quality are important features of wheat varieties (Simmonds 1989). 'Grain quality' includes particular characters required for different end-uses. Hexaploid wheat (*Triticum aestivum* L.), also known as bread wheat, can be processed into different breads, noodles, cakes and biscuits for human consumption. Different end uses require different combinations of quality characters, such as protein content, protein quality, grain hardness, starch quality, lipids and enzymes. For bread making quality, dough strength is considered as an important factor. Dough strength is mainly determined by protein content and composition of gluten proteins (Kramer 1979; Simmonds 1989). Gluten has a critical role in the viscoelasticity of dough. Gluten proteins consist of gliadins and glutenins, which are encoded by alleles located at different loci. It has been demonstrated that these alleles determine glutenin composition and the variation in the composition among wheat varieties is responsible for a large proportion of the variation of loaf volume (Payne 1987; Simmonds 1989).

Thanks to the development of molecular technology, more and more glutenin alleles have been discovered from different wheat varieties (Mclntosh et al. 2008, 2009). It is of interest to discover novel glutenin alleles and study the effect of different glutenin subunits on rheological properties of wheat dough. Thorough understanding of functional properties of

glutenin subunits can be potentially applied in wheat breeding.

Among multiple alleles at the *Glu-B1* high-molecular-weight glutenin locus, the *Glu-B1al* allele has been demonstrated to be associated with the strongest and most extensible dough and longest dough development (Eagles et al. 2004). This allele includes two identical x-type glutenin genes (Ragupathy et al. 2008) that encode the $Bx7^{OE}$ subunit, which is overexpressed relative to high-molecular-weight glutenin subunits.

One Australian wheat cultivar, H45, which seems to carry the *Glu-B1al* allele, differs from other such cultivars in that its dough is not as strong as would be expected if it carries *Glu-B1al*. As a way to exploit novel glutenin alleles in H45 and to study the glutenin subunit effects on dough properties, the study reported in this thesis investigates:

a. the DNA sequences of x-type HMW-GS genes at the *Glu-B1* locus in H45 and the control line VQ0437, and the amino acid sequences of the HMW-GS encoded by those genes;

b. the flour and dough properties of H45 wheat in comparison to wheat lines carrying *Glu-B1al* or similar alleles;

c. development of molecular screening methods to distinguish the *Glu-B1* allele of H45 from other alleles at the *Glu-B1* locus;

d. whether parental differences in flour properties would be co-inherited with contrasting *Glu-B1* alleles in progeny of a cross between H45 and VQ0437;

e. whether the *Glu-B1* allele of H45 can be detected in any of its known ancestors and/or in other Australian wheat varieties;

f. DNA polymorphisms in or close to the *Glu-B1* locus that could be assayed to distinguish H45 from other wheat lines;

g. the effects of individual x-type glutenin subunits encoded by the *Glu-B1* allele of H45 on flour and dough properties; and

h. the effects of a specific amino acid residue in one of these subunits.

Chapter 2

Literature Review

2.1 Introduction

Globally, wheat (*Triticum aestivum* L.) is an important crop for human consumption. Bread is one of the major constituents of human diet and wheat is the most important cereal for bread making. The gluten proteins of wheat are recognised as the most important components governing bread making quality of wheat. Based on their solubility in aqueous alcohol, gluten proteins are classified as gliadins or glutenins. Gliadins are monomeric, while glutenins are polymeric, with both low- and high-molecular-weight subunits (LMW-GS and HMW-GS). These proteins interact to make dough elastic, allowing it to trap the gas bubbles produced by yeast and allowing bread to rise. Variation in the amount and properties of HMW-GS is particularly important, accounting for much of the variation in bread making quality of wheat, even though HMW-GS represent only 5% to 10% of grain protein (Branlard and Dardevet 1985; Payne 1987; Payne et al. 1988; Halford et al. 1992).

The research that is described in this thesis focused on a novel glutenin subunit in the Australian cultivar H45 (sometimes known as Galaxy, or Galaxy H45), which seems to interfere with dough strength. In this chapter, a review of published literature provides an overview of the information of grain quality, endosperm storage proteins, and the functional effect of glutenin alleles.

2.2 Endosperm Storage Proteins in Wheat Grain

Wheat is consumed in many different forms, such as bread, noodles, cakes and pizzas. The ability of wheat flour to be processed into different end uses is largely determined by the gluten proteins, which confer properties of elasticity and extensibility that are essential for functionality of wheat flours (Shewry et al. 1992; Weegels et al. 1996). Wheat flour contains 8% to 20% proteins. Gluten proteins constitute up to 80% to 85% of total flour protein (Shewry et al. 1995). Seed storage proteins were classified by Osborne (1924) on the basis of their solubility: albumins (water), globulins (dilute saline), prolamins (alcohol–water mixtures), and glutelins (dilute acid or alkali). He also showed that prolamins were restricted to the grain of cereals and other grasses. In wheat, prolamins are known as gliadins and glutelins are known as glutenins. Wheat gluten proteins are classically divided into alcohol-soluble (gliadin) and insoluble (glutenin) fractions, each of which constitutes around half of gluten protein (Shewry et al. 1995). Each of these fractions can be further separated by electrophoresis.

2.2.1 Gliadins

The gliadins consist of monomeric proteins which can be separated into α, β, γ, and ω groups according to decreasing mobility in acid polyacrylamide gel electrophoresis (A-PAGE) (Payne 1987). The α-, β- and γ-gliadins all have similar amino-acid compositions and molecular weights in between 30,000 and 45,000 Da, as determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2.1) (Galili and Feldman 1983; Masci et al. 2002). The secondary structure of these gliadins contains α-helices and β-turns. In contrast, the ω-gliadins have higher molecular weights (44,000 to 74,000 Da) and

a distinctive amino acid composition. Their structure has many β-turns, but contains low levels of α-helix or β-sheet (Galili and Feldman 1983; Tatham and Shewry 1985; Tatham et al. 1985; Masci et al. 2002).

2.2.2 Glutenins

Glutenins are polymeric proteins stabilized by interchain disulfide bonds. After reduction of these bonds, the component subunits can be separated into two groups (Fig. 2.1), high molecular weight glutenin subunits (HMW-GS) and low molecular weight glutenin subunits (LMW-GS) (Payne 1987).

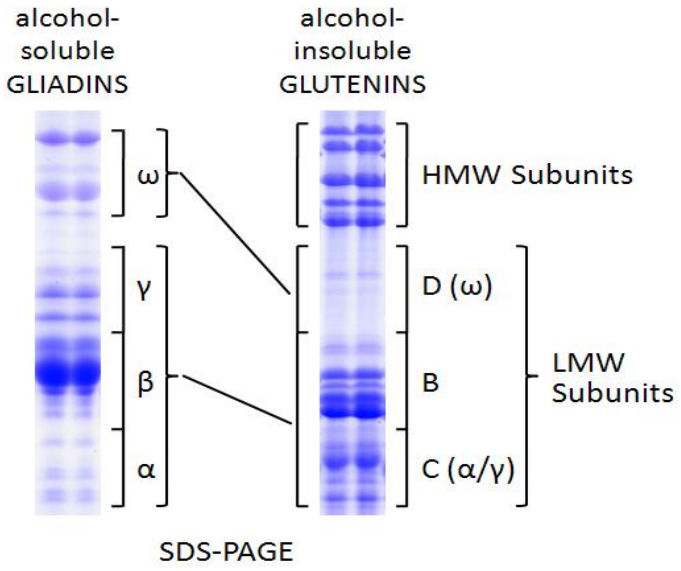

Fig. 2.1 The classification and nomenclature of wheat gluten proteins separated by SDS-PAGE. The D-type LMW subunits are only minor components and are not clearly resolved in the separation shown.

2.2.2.1 Low molecular weight glutenin subunits (LMW-GS)

Low molecular weight glutenin subunits can be further divided into B-, C-, and D-type subunits according to size, isoelectric point, and composition (Payne, 1987). The molecular weight of LMW-GS ranges from 30,000 to about 58,000 Da (30,000 to 40,000 Da for C subunits, 42,000 to 51,000 Da for B subunits and about 58,000 Da for D subunits) (Gianibelli et al. 2001).

2.2.2.2 High molecular weight glutenin subunits (HMW-GS)

High molecular weight glutenin subunits account for only 5% to 10% of grain protein, but they are thought to be the most important components in the process of bread making because they determine gluten elasticity (Tatham et al. 1985; Shewry and Halford 2002). The molecular weight of HMW-GS ranges from 65,000 to 90,000 Da (Shewry and Halford 2002).

High molecular weight glutenin subunits have been classified into x-type and y-type subunits. The x-type subunits generally have a slower electrophoretic mobility in SDS-PAGE and a higher molecular weight than y-type subunits. Both x-type and y-type HMW-GS have a typical three-domain structure consisting of highly conserved N- and C-terminal non-repetitive domains flanking a long central repetitive domain. The repetitive domain consists of tripeptides, hexapeptides and nonapeptides, with the tripeptide motif restricted to the x-type subunits. The C-terminal domain has a constant size (42 amino acid residues) and the N-terminal domain varies only slightly in length (about 80 to 90 amino acid residues for x-type HMW-GS and about 104 amino acid residues for y-type HMW-GS). The length of the repetitive domain is much more variable: between 750 and 850 amino acid residues for x-type HMW-GS and between 600 and 700 amino acid for y-type HMW-GS. The secondary

structure of the N- and C-terminal domains of glutenin subunits is predominantly α-helix. According to Parchment et al. (2001), the repetitive domains, which are rich in proline, glutamine and glycine, are believed to form a series of overlapping β-reverse-turns. Thus, the super secondary structure of the central repetitive domain may form a 'β-spiral'. It is generally accepted that HMW-GS form inflexible rod-like structures due to the specific structure of the central repetitive domain when it is involved in gluten polymers (Tatham et al. 1985). However, Shimoni et al. (1997) reported some flexibility of the central domain of a recombinant HMW-GS that formed an intrachain disulfide bond between its N- and C-terminal domains.

2.3 Chromosomal Locations of Gluten Protein Genes

2.3.1 Gliadins

Bread wheat is hexaploid, containing three different but related genomes (A, B and D), each consisting of 7 chromosome pairs. Gliadin proteins are encoded by genes on the short arms of the homoeologous group-1 and group-6 chromosomes (Boyd and Lee 1967; Payne 1987). Due to poor resolution of one-dimensional separations and overlapping of many gliadin components, only a few gliadin subunits have been assigned to specific chromosomes. It is generally accepted that the ω-and γ-gliadins are encoded by clusters of tightly linked genes at the *Gli-1* loci (*Gli-A1*, *Gli-B1*, and *Gli-D1*) on the short arms of the group-1 chromosomes, whereas the α- and β-gliadins are encoded by the *Gli-2* loci (*Gli-A2*, *Gli-B2*, and *Gli-D2*) on the short arms of the group-6 chromosomes (Payne 1987). A single hexaploid wheat variety can have approximately 15 to 20 α- and β-gliadins (Payne 1987; Metakovsky 1991; Shewry et al. 2003b).

2.3.2 Low molecular weight glutenin subunits (LMW-GS)

Low molecular weight glutenin subunits are encoded by genes at the *Glu-A3*, *Glu-B3* and *Glu-D3* loci on the short arms of the group-1 chromosomes (Gupta and Shepherd 1990; Branlard et al. 2001). Large allelic variation has been reported at the three *Glu-3* loci in bread wheat, with different alleles being designated by the name of the locus followed by letters (Singh et al. 1991). Based on N-terminal sequencing of protein subunits and Southern blot analysis, a multigene family consisting of 30 to 40 LMW-GS genes has been identified (Lew et al. 1992; Cassidy et al. 1998; Cloutier et al. 2001). The LMW-GS are difficult to separate because of their complexity, heterogeneity, and similarity to each other and to some gliadin components.

2.3.3 High molecular weight glutenin subunits (HMW-GS)

The HMW-GS genes are encoded at the *Glu-1* loci on the long arm of group-1 chromosomes (1A, 1B and 1D) (Bietz et al. 1975; Payne et al. 1984; Payne 1987). Each locus contains genes linked together encoding two types of HMW-GS: x- and y-type subunits (Shewry et al. 1992; Shewry and Halford 2002). Therefore, it might be expected that hexaploid bread wheats could contain up to six different HMW glutenin subunits. In fact, only three, four or five subunits are present in most of cultivars of bread wheat, due to the silencing of some genes. All cultivars contain Bx, Dx and Dy subunits, while some cultivars also contain By and/or Ax subunits (Lawrence and Shepherd 1981; Payne et al. 1981). With few exceptions (Janssen et al. 1996; Margiotta et al. 1996), the genes encoding *Glu-A1* y-type subunits are silent in hexaploid wheat (Shewry et al. 2003b; Hu et al. 2012).

Payne and Lawrence (1983) developed a numbering system to identify HMW-GS according

to their mobility on SDS-PAGE. In this system, each subunit has a unique designation (Fig. 2.2). For example, *Glu-B1* (7+8) refers to the presence of subunits Bx7 and By8, both of which are located at the *Glu-B1* locus on chromosome 1B.

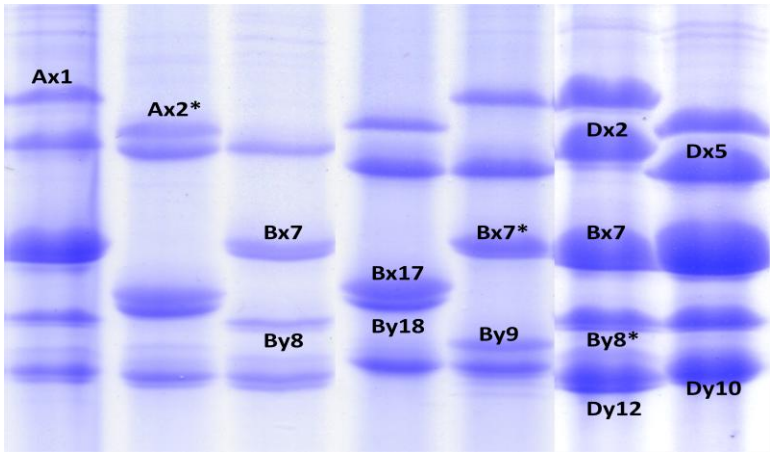

Fig. 2.2 SDS-PAGE of HMW glutenin subunits encoded at the *Glu-1* loci.

2.4 Interactions of Gluten Subunits

Gluten proteins consist of a large number of gliadin and glutenin subunits. The gliadins are single-chain polypeptides, which represent as monomeric proteins. While the glutenins, present in polymers, are multiple-chain polymeric proteins in which individual polypeptides are stabilised into a network by interchain disulphide and hydrogen bonds.

Cysteine residues play an important role in the structure of both gliadins and glutenin subunits. These cysteine residues may be involved in disulfide bonds within the same polypeptide (intra-chain disulfide bonds) or in disulfide bonds between different polypeptides (inter-chain disulfide bonds).

The α-type and γ-type gliadins have six and eight cysteine residues, respectively, all located at highly conserved positions and involved in conserved intra-chain disulfide bonds (Shewry and Tatham 1997). In contrast, ω-type gliadins lack cysteine residues. They also have a very

low level of methionine and are classified as sulfur-poor prolamins (Shewry et al. 1986).

LMW-GS differ from α-, β-, and γ-gliadins in one very important characteristic: their cysteine residues participate in inter-chain and intra-chain disulfide bonds. In each LMW-GS, one cysteine residue in the N-terminal domain and one in the C-terminal domain are available to form inter-chain disulfide bonds. Through covalent linkages with each other and/or with HMW-GS, LMW-GS become incorporated in glutenin polymers (Shewry et al. 1986).

Both gliadins and glutenins contribute to the rheological properties of gluten and dough, but HMW glutenins are thought to be of greater importance (Naeem and MacRitchie 2005). The structures and interactions of the HMW subunits are of great interest for understanding gluten functionality. It was also suggested that the number and locations of cysteine residues in HMW-GS affect the composition of polymeric proteins and bread making quality, due to differences in disulphide bonding capabilities (Köhler et al. 1993; Keck et al. 1995; Lindsay et al. 2000; Pirozi et al. 2008). In HMW-GS, most of the cysteine residues are normally located in the terminal domains. The number and distribution of cysteine residues differs between x-type and y-type HMW-GS. Each of the y-type HMW-GS that has been sequenced to date has seven cysteine residues: five at highly conserved positions in the N-terminal domain, one at a highly conserved position in the C-terminal domain, and one near the C-terminal end of the central repetitive domain (Shewry et al. 2003b; Wieser 2007). The x-type HMW-GS Ax1, Ax2*, Bx7, Bx13, Bx17 and Dx2 are known to have only four cysteine residues: three in the N-terminal domain and one in the C-terminal domain.

Cysteine residues within the same gliadin or glutenin subunits can be linked by intrachain disulphide bonds (Köhler et al. 1993) (Fig. 2.3). Unpaired cysteines in glutenin subunits

remain available to form disulphide bonds with other glutenin subunits to form macropolymer, which makes the molecules act as chain extenders or branchers (Keck et al. 1995; Köhler et al. 1997). In contrast, a subunit with only a single cysteine residue available to form interchain disulphide bonds, can act as a chain terminator, preventing other glutenin subunits from linking with the glutenin macropolymer (Tamás et al. 2002).

For the Bx7 HMW-GS, direct disulphide bond mapping has demonstrated that the first two cysteine residues of the N-terminal domain are linked by an intrachain disulphide bond (Köhler et al. 1993) (Fig. 2.3), and it has been suggested the third N-terminal cysteine residue forms interchain disulfide bonds (Köhler et al. 1997). It has also been reported that the C-terminal domain cysteine links Bx7 with other glutenin subunits by interchain disulfide bonds (Tao et al. 1992; Keck et al. 1995; Lutz et al. 2012).

The HMW-GS Dx5 is unusual in that it has five cysteine residues. Its additional cysteine residue is at the N-terminal end of the repetitive domain (Anderson et al. 1989). This extra cysteine residue was recently found to be linked with cysteine residues in the C-terminal domain of other HMW glutenin subunits by 'head-to-tail' interchain disulfide bonds (Lutz et al. 2012). As suggested by Köhler et al. (1997), the Dx5 HMW-GS forms one or three interchain disulphide in its N-terminal domain, making it act as a chain brancher, promoting a highly cross-linked glutenin network, increasing dough strength and improving baking properties (Tatham et al. 1991; Lafiandra et al. 1993; Darlington et al. 2003).

The Bx14 subunit from *Triticum aestivum* and the Bx20 subunit from *Triticum turgidum* spp. *durum*, differ from other x-type subunits in that they have only two cysteines, with one at each end of the subunit (Shewry et al. 2003a; Li et al. 2004). This affects the pattern of

disulphide cross-links in polymeric glutenin. For Bx20, this has been shown to reduce mixograph dough development time and reduce loaf size (Tatham et al. 1991; Shewry et al. 2003a; Pirozi et al. 2008).

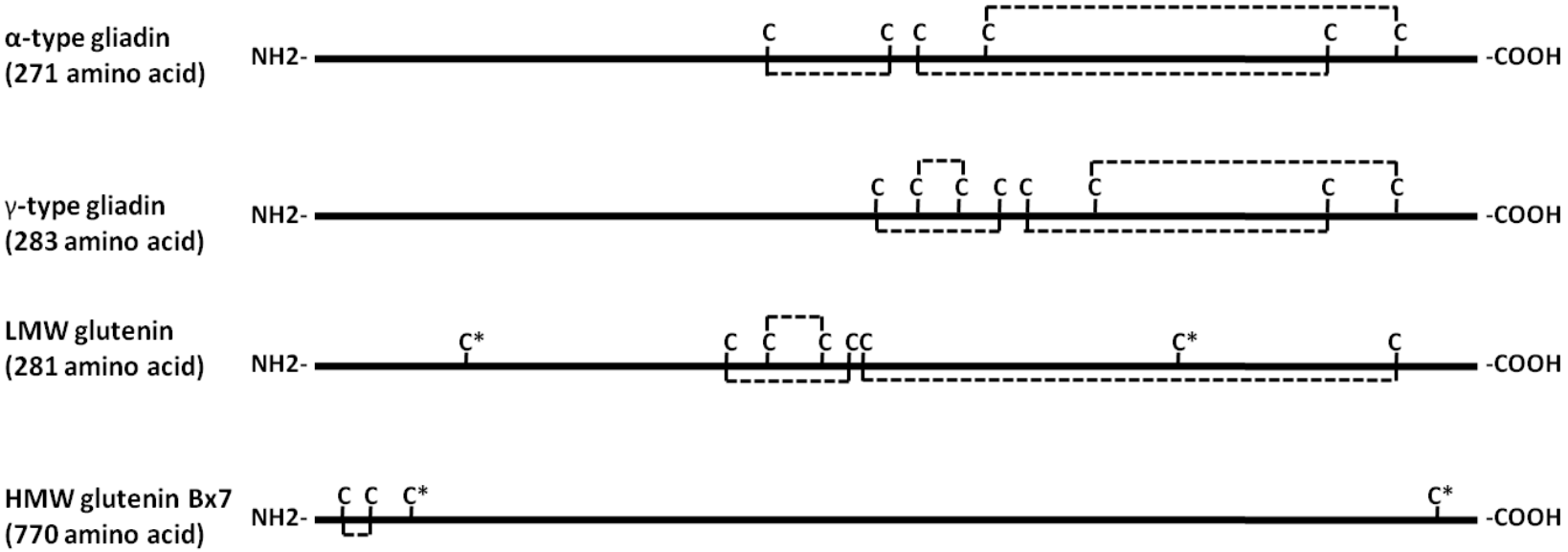

Fig. 2.3 Primary structure of gliadin and glutenin subunit types according to Wieser (2007). Cysteine residues are marked as **'C'**. Putative cysteines involved in inter-molecular disulphide bonds are marked with **'*'**. Dashed lines represent intra-molecular disulphide bonds.

2.5 Determination of Bread Making Quality Characteristics

2.5.1 Physical dough testing

Dough rheological behaviour can be investigated using various instruments, which commonly include the farinograph, mixograph, extensograph and alveograph. The operational principle of these instruments is mostly to trace the torque raised during dough mixing and development (Sahin and Sumnu 2006).

A farinograph provides information about mixing properties of flour by measuring the resistance of dough to mixing blades during prolonged mixing. The output is a consistency curve over time, which indicates dough development time, tolerance to overmixing, stability and optimum water absorption. Dough development time, also called peak time, is the time required to reach the maximum consistency for dough. Tolerance of the dough to overmixing can be indicated by stability (how long the dough maintains maximum consistency), mixing tolerance index (the difference between the height of the curve at its peak and that at a specified time after the peak) and departure time (time required for the top curve to fall below the peak resistance). It has been accepted that higher stability values indicate stronger dough (Rosell et al. 2001).

Mixograph tests quickly analyse small quantities of flour for dough gluten strength. A mixograph records changes in the force required to mix dough as it develops under standardised conditions. The mixograph curve indicates gluten strength, optimum dough development time, mixing tolerance (the resistance of the dough to breakdown during continued mixing), and other dough characteristics. Flour with weak gluten is expected to have a shorter peak time and less mixing tolerance than flour with strong gluten (Martinant et al. 1998).

An extensograph measures the rheological properties of the dough after mixing, providing information about elasticity. Extensograph parameters include extensibility (an indicator of

the viscous component, related to the elongation at the rupture in tensile strength), maximum resistance ($R_{max,}$ corresponding to the force required to stretch the dough) and dough strength (the area under the curve, corresponding to the absolute levels of elastic and viscous components of the dough) (Rosell et al. 2001).

An alveograph, also known as a Chopin Extensograph, measures the functional properties of a piece of dough during a dough being stretched *via* parameters: deformation energy or gluten strength, tenacity or maximum resistance, swelling and extensibility. The gluten strength is related to the area under the curve and the swelling is calculated from the extensibility. The maximum resistance to swelling ratio or tenacity to extensibility ratio provides a better prediction of dough properties than those individual parameters alone (Peña et al. 1995).

2.5.2 Influence of dough rheology on bread making quality

Loaf volume has been reported to be linearly related with flour protein content (Weegels et al. 1996). As gluten proteins generally represent up to 80 to 85% of total wheat protein (Veraverbeke and Delcour 2002), it has long been realized that gluten proteins are important in determining the bread making performance of wheat flour. When mixed with water, gluten proteins form a cohesive visco-elastic mass which has the ability to contain the gas produced during fermentation and oven-rise. Fractionation and reconstitution experiments by Finney (1943) showed clearly that variation in gluten proteins affects bread making performance. It is now generally believed that differences in bread making quality are largely due to differences in gluten composition (Veraverbeke and Delcour 2002). These differences can include differences in the ratio of monomeric gliadin to polymeric glutenin, which helps determine the balance between dough viscosity and elasticity (Khatkar et al. 1995; Janssen et al. 1996), and differences in the composition of glutenin subunits.

2.5.3 Importance of glutenin size

It has been broadly accepted that the degree of aggregation of glutenin subunits determines

the glutenin quality. Relationships of glutenin extractability with dough strength and bread making performance have been confirmed using different solvents (Orth and Bushuk 1972; Gupta et al. 1993; Gras et al. 2001; Ohm et al. 2008). Wheat with strong dough properties contains less acetic-acid-soluble or SDS-soluble glutenin and more insoluble glutenin in its flour (Singh et al. 1990b). Thus, the amount of insoluble glutenin, which is termed residue or gel protein, correlates positively with dough strength, and is the basis of residue protein and SDS sedimentation tests for bread making quality (Orth and Bushuk 1972; Moonen et al. 1982; Branlard and Dardevet 1985; Singh et al. 1990b).

Research using gel permeation chromatography and physical dough testing has indicated that the percentage of total polymeric protein is significantly correlated with mixograph dough development time values and R_{max} (Huebner and Bietz 1985; Gupta et al. 1993). Polymer size is related to the number of unpaired cysteine residues and the ratio between chain brancher/extender and chain terminator subunits (Gupta and MacRitchie 1994; Pirozi et al. 2008). Size-exclusion high-performance liquid chromatography (SE-HPLC) was first applied to study wheat proteins by Bietz (1984). However, the variable extractability of unreduced proteins was a problem associated with SE-HPLC. Singh et al. (1990a; 1990b) developed a simple way to completely extract unreduced flour proteins using mechanical shear with an ultrasonic probe. With this ultrasonication treatment, the large glutenin polymers break down into small polymers, which are still large enough to be separated from the monomeric gliadin and albumin/globulin fractions. By ultrasonication followed by SE-HPLC, the total polymeric protein (TPP) can be separated into SDS-extractable polymeric protein (EPP) and SDS-unextractable polymeric protein (UPP). The percentage of total polymeric protein in total protein has been termed total polymeric protein (%TPP) (Singh et al. 1990b; MacRitchie 1999). Gupta et al. (1993) demonstrated that %UPP (UPP expressed as a percentage of TPP) was positively and significantly correlated with extensograph R_{max} and mixograph dough development time across a set of 74 recombinant inbred wheat lines and a set of 26 wheat

cultivars.

2.5.4 Glutenin subunit composition

Consistent with the idea that the total amount of HMW-GS affects dough properties (Lawrence et al. 1987; Halford et al. 1992), the deletion of HMW-GS encoding genes from wheat has been shown to significantly decrease dough strength (Lawrence et al. 1987; Rogers et al. 1991; Popineau et al. 1994), and the insertion of additional HMW-GS encoding genes has been reported to dramatically increase dough strength (Barro et al. 1997; Rooke et al. 1999).

Based on the results of numerous studies aimed at finding statistical relationships between glutenin composition and quality-related parameters (Payne 1987; Payne et al. 1987; Branlard et al. 2001; Luo et al. 2001; Eagles et al. 2002; Eagles et al. 2004; He et al. 2005; Liu et al. 2005; Eagles et al. 2006; Ohm et al. 2008), it is clear that certain HMW-GS (such as Dx5+Dy10, $Bx7^{OE}$) are generally associated with strong dough properties, while other HMW-GS (such as Dx2+Dy12, Bx20) are associated with weak dough properties (Butow et al. 2003; Ohm et al. 2008). Glutenin subunits or alleles can be used as indicators or predictors of quality for breeding purposes.

2.5.5 Genetic and environmental control of glutenin quality

As reviewed above, properties of glutenin that affect the quality of wheat flour for bread making include structure, size distribution and composition. Variation in these properties is largely determined by genetic variation, but environment can also play a role. Genetic variability is responsible for variation in the sets of HMW-GS and LMW-GS present in different wheat varieties, but the relative amounts of different classes of GS present in wheat appears to depend on a combination of genetic and environmental factors (Kolster et al. 1991; Altenbach et al. 2002; Naeem et al. 2012). Variation in environmental factors such as water

and nutrient availability and temperature stress usually affects bread making quality by changing the protein content and protein composition of the mature wheat grain (Williams et al. 2008; Naeem et al. 2012). Naeem et al. (2012) demonstrated that sulphur deficiency led to an increase in the ratio of HMW- to LMW-GS, which in turn led to greater resistance to extension and less extensibility in dough. Heat stress has been shown to reduce polymerisation of glutenin, reducing dough strength (Stone et al. 1996; Altenbach et al. 2002).

2.6 Genetic Variation of HMW Glutenins

Many different HMW-GS have been detected in wheat and novel HMW-GS are discovered frequently (Shewry et al. 2003b; Wang and Zhang 2006; D'Ovidio et al. 1996; Singh et al. 2007). Allelic variation has been studied in great detail for HMW-GS (Payne 1987; Shewry and Halford 2002; Yan et al. 2003; Liu et al. 2006).

Allelic variation causes differences in molecular weight (Harberd et al. 1986), and plays a crucial role in determining dough viscoelastic properties. Allelic variation at the *Glu-1* loci has been reported to account for up to 70% of the genetic variation for dough quality in wheat: 45 to 70% in Europe (Branlard and Dardevet 1985; Payne et al. 1987; Payne et al. 1988), 35 to 60% in China (He et al. 2005; Liu et al. 2005) and 30 to 50% in southern Australia (Eagles et al. 2002).

At the *Glu-B1* locus, many allelic variants have been detected (Mclntosh et al. 2008, 2009). Butow et al. (2004) classified Bx7-type *Glu-B1* alleles according to the composition of glutenin subunits encoded at *Glu-B1*. Some of the alleles differ only slightly from each other and cannot easily be differentiated from each other by SDS-PAGE. These alleles, which each encode a Bx7 or Bx7* subunit and a By8 or By8* subunit, are designated *Glu-B1b* (7+8), *Glu-B1u* (7*+8), *Glu-B1ak* (7*+8*) and *Glu-B1al* (7^{OE}+8*). When *Glu-B1al* is present, the Bx subunit is overexpressed (Butow et al. 2003; Vawser and Cornish 2004). Butow et al. (2003) showed that reverse-phase HPLC could be used to assess the expression level of

Bx7^{OE} subunit as a percentage of the total amount of HMW-GS present. They also indicated that the high level of expression of Bx7^{OE} increases dough strength. Vawser and Cornish (2004) demonstrated that lines with the *Glu-B1al* allele contain a high proportion of HMW-GS encoded by the B genome. They suggested that the proportion of *Glu-B1* subunits, relative to the total amount of HMW-GS, has a major effect on dough strength. It has also been demonstrated that *Glu-B1al* is associated with significantly stronger and more extensible dough and longer dough development times than any other allele at the *Glu-B1* locus (Eagles et al. 2004). According to Ragupathy et al. (2008), the cultivar Glenlea, which carries the *Glu-B1al* allele, has a duplication of a 10.3-kb region that includes the *Bx7^{OE}* gene. The two copies of the region flank a long terminal repeat (LTR) retroelement.

2.7 DNA Markers for HMW Glutenin Alleles

PCR-based molecular marker assays have been developed to distinguish different HMW-GS genotypes, and some of these have been used in marker-assisted selection (MAS) (McLauchlan et al. 2001; Liu et al. 2008; Gupta et al. 2010).

Allele-specific PCR markers that have been designed for *Glu-A1* genes differentiate among alleles encoding *Ax1*, *Ax-Null* and *Ax2** (Ma et al. 2003; Liu et al. 2008). For the *Glu-D1* locus, markers have been designed to distinguish the *Dx2*, *Dx5*, *Dy10*, *Dy12*, *Dx5+Dy10* genes from each other (D'Ovidio and Anderson 1994; Smith et al. 1994; de Bustos et al. 2001; Ma et al. 2003; Ishikawa and Nakamura 2007; Liu et al. 2008).

Considerable research attention has been paid to the development of molecular markers to distinguish among many allelic variants at the *Glu-B1* locus. Schwarz et al. (2004) reported a PCR-based marker which can distinguish *Bx6* from *Bx7* and *Bx17* based on a 15 bp insertion. A PCR-based marker designed to amplify a 2373 bp fragment that is specific to the *Bx7* gene (Anderson and Greene, 1989) has been applied to distinguish between *Bx7* and *Bx17* (Ahmad, (2000). Ma et al. (2003) later developed a marker that distinguishes between *Bx7* and *Bx17*

based on a 108 bp deletion within the *Bx7* coding sequence. That marker amplifies two products (630 bp and 766 bp) from Bx7 genotypes but only one (669 bp) from Bx17 genotypes. Xu et al. (2008) designed a pair of co-dominant markers to distinguish *Bx14* (one primer pair providing an amplicon of 753 bp and another providing an amplicon of 642 bp) from *Bx17* (337 bp and 534 bp amplicons). A molecular marker based on an 18 bp indel in the coding region can be used to distinguish *Bx*7 and $Bx7^{OE}$ from *Bx*7* (Butow et al. 2004). Markers developed based on a 43 bp indel in the promoter region make it possible to distinguish between *Bx7* and $Bx7^{OE}$. Using the complete DNA sequence of TaBAC1215C06 (EU157184), Ragupathy et al. (2008) designed primer pairs to amplify the right and left junctions of the LTR retroelement, providing markers that were diagnostic of the $Bx7^{OE}$ phenotype across a panel of diploid, tetraploid and hexaploid accessions of *Triticum* spp. These markers generated a 447 bp amplicon from the left junction of the retroelement and an 884 bp amplicon from the right junction of the retroelement in all $Bx7^{OE}$ accessions. A set of the *Glu-B1y* locus-specific primers has been published by Lei et al. (2006). With these markers, *By9* can be detected based on a 45 bp deletion in the coding region, *By8* can be detected using a dominant marker designed based on a single nucleotide polymorphism (SNP), and *By16* can be distinguished from *By8*, *By8**, *By9*, *By18* and *By15*.

This thesis describes work that investigated a novel glutenin subunit in the Australian cultivar H45 (sometimes known as Galaxy, or Galaxy H45), which has been classified as carrying the *Glu-B1al* allele. It has been observed that H45 does not have the rheological properties that would be expected based on the presence of *Glu-B1al* in combination with the other glutenin alleles of H45. In this research, DNA sequences of x-type HMW-GS genes at the *Glu-B1* locus were investigated from H45 and VQ0437. The flour and dough properties of H45, VQ0437 and progeny of H45 and VQ0437 were tested. A restriction digest assay was designed to distinguish the *Glu-B1* allele of H45 from other alleles at the *Glu-B1* locus and was used to test a panel of wheat cultivars to determine whether some of them carry the same

allele as H45. The same assay was applied to progeny of a cross between H45 and VQ0437 to investigate genetic association of the *Glu-B1* polymorphism with dough properties. Efforts were made to find a DNA polymorphism in or close to *Glu-B1* locus of H45. Individual glutenin subunits were obtained by heterologous expression in *Escherichia coli* and were used to investigate their effects on dough properties.

Chapter 3

A Second 'Overexpression' Allele at the *Glu-B1* High-Molecular-Weight Glutenin Locus of Wheat: Sequence Characterisation and Functional Effects

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The text, figures and tables presented in this chapter are exactly the same as published, except for minor formatting changes such as renumbering of tables and figures for consistency within the thesis. Materials that were included as online resources with the journal article are presented in appendices of this thesis. The statement of acknowledgements and the reference list that were included in the published article are not presented within this chapter; they have been incorporated into the acknowledgements and bibliography of the thesis.

Statement of Authorship

Gao X (Candidate)

Completed the sequencing and sequence assembly, conducted the sequence alignments, designed and applied a restriction digestion assay, extracted protein fractions and separated them on polyacrylamide gels, conducted SE-HLPC analysis, interpreted results and prepared the manuscript.

I hereby certify that the statement of contribution is accurate.

Appelbee MJ

Crossed H45 with VQ0437, developed the F_2 progeny population used in this research, extracted protein fractions and separated them by capillary electrophoresis, trained the candidate in some of the protein analysis methods used in this research, and contributed to revision of the manuscript.

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the article in the thesis.

Mekuria GT

Initiated the cloning and sequencing of *Bx* genes from genomic DNA of Glenlea, H45 and VQ0437 and contributed to revision of the manuscript.

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the article in the thesis.

Chalmers KJ

Provided valuable advice, contributed to research supervision of the candidate and revision of the manuscript.

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Mather DE

Suggested the research topic, oversaw the research, worked closely with the candidate to revise the text and tables of the manuscript prior to publication and acted as corresponding author.

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the article in the thesis.

Abstract

Bread is one of the major constituents of the human diet and wheat (*Triticum aestivum* L.) is the most important cereal for bread making. The gluten proteins (glutenins and gliadins) are recognised as important components affecting the processing quality of wheat flour. In this research, we investigated a particular glutenin subunit allele in an Australian cultivar, H45. Based on protein and DNA assays, the *Glu-B1* allele of H45 seems to be *Glu-B1al,* an allele that includes a functional duplication of a gene encoding an x-type high-molecular-weight glutenin subunit, and is thought to increase dough strength through overexpression of that subunit. Yet H45 does not have the dough properties that would be expected if it carries the *Glu-B1al* allele. After confirming that H45 overexpresses Bx subunits and that it has relatively low unextractable polymeric protein (an indicator of weak dough), we cloned and sequenced two Bx genes from H45. The sequences of the two genes differ from each other, and they each differ by four single-nucleotide polymorphisms (SNPs) from the sequence that has been reported for the *Glu-B1al* x-type glutenin genes of the Canadian wheat cultivar Glenlea. One of the SNPs leads to an extra cysteine residue in one of the subunits. The presence of this additional cysteine may explain the dough properties of H45 through effects on cross-linkage within or between glutenin subunits. We propose that the *Glu-B1* allele of H45 be designated *Glu-B1br,* and we present evidence that *Glu-B1br* is co-inherited with low unextractable polymeric protein.

Key words: Wheat, high-molecular-weight glutenin, overexpression, cysteine

3.1 Introduction

Gluten proteins are important components governing the processing quality of wheat flour for the production of bread and other food products. Based on their solubility in aqueous alcohol, gluten proteins are classified as gliadins or glutenins. Gliadins are monomeric, while glutenins are polymeric, with both high- and low-molecular-weight glutenin subunits (HMW-GS and LMW-GS). These glutenin proteins interact to make dough elastic, allowing it to trap the gas bubbles produced by yeast and enabling the bread to rise. Variation in the amount and properties of HMW-GS accounts for much of the variation in the processing quality of wheat flour (Branlard and Dardevet 1985; Payne et al. 1988; He et al. 2005) even though HMW-GS represent only 8-10% of the protein in wheat grain.

Many different HMW-GS have been detected in wheat (McIntosh et al. 2008). They have highly conserved structures, with each subunit containing a long repetitive region flanked by two highly conserved non-repetitive terminal domains. Within the terminal domains, cysteine residues provide sites for disulphide bonds that connect HMW-GS with each other and with LMW-GS, stabilising glutenin macropolymers and contributing to gluten viscoelasticity (Shewry and Halford 2002). Cross-linkage can also occur between tyrosine residues, but according to Hanft and Koehler (2005), dityrosine residues play only a minor role in the structure of wheat gluten, with less than 0.1% of tyrosine residues being cross-linked. The HMW-GS are known to be encoded at three loci (*Glu-A1*, *Glu-B1* and *Glu-D1* on chromosomes 1A, 1B and 1D, respectively). The alleles at these loci contain tightly linked genes that encode ‘x-type’ and ‘y-type’ subunits (Payne et al. 1981). X-type and y-type HMW-GS differ from each other with respect to their cysteine content and the motifs represented within their repetitive domains (Shewry 2003c).

Allelic variation has been studied in great detail for HMW-GS, particularly for the *Glu-B1* locus, at which many alleles have been discovered (Payne and Lawrence 1983; McIntosh et

al. 2008, 2009). Among the subunits that are encoded at *Glu-B1*, two x-type subunits (designated Bx7 and Bx7*) differ only very slightly in their electrophoretic mobility in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Marchylo et al. 1992). Similarly, two of the y-type subunits (By8 and By8*) are very similar to each other. Accordingly, the *Glu-B1* alleles that encode a Bx7 or Bx7* subunit and a By8 or By8* subunit are not readily distinguished from each other using SDS-PAGE. These alleles are designated *Glu-B1b* (7+8), *Glu-B1u* (7*+8), *Glu-B1ak* (7*+8*) and *Glu-B1al* (7+8*).

When *Glu-B1al* is present, the Bx7 subunit is overexpressed (7^{OE} + 8*) (Marchylo et al. 1992) as a percentage of the total amount of HMW glutenin present, and this overexpression of Bx7 is associated with greater dough strength (Butow et al. 2003; Vawser and Cornish 2004; Eagles et al. 2004). D'Ovidio et al. (1997) presented evidence that the *Glu-B1al* allele includes two copies of its x-type glutenin gene. Sequencing of the BAC clone TaBAC1215C06 (EU157184) later demonstrated that the *Glu-B1al* allele of Glenlea includes two copies of a 10.3-kb region that includes an x-type glutenin gene (Ragupathy et al. 2008). Molecular markers have been developed that detect insertions in the coding region (Butow et al. 2003) and matrix attachment region (MAR) (Radanovic and Cloutier 2003) of the *Glu-B1al* x-type gene that encodes the over-expressed Bx7 subunit. Ragupathy et al. (2008) designed markers that detect the right and left junctions of a long terminal repeat (LTR) retroelement that lines between the two x-type glutenin gene copies in the Glenlea allele and reported that these markers were diagnostic of the $Bx7^{OE}$ phenotype across a panel of diploid, tetraploid and hexaploid accessions of *Triticum* spp.

The Australian cultivar H45 (sometimes known as Galaxy, or Galaxy H45) has been classified as carrying the *Glu-B1al* allele. Nevertheless, it has been observed that H45 does not have the rheological properties that would be expected based on its apparent glutenin alleles. We hypothesised that H45 carries a functionally different allele at the *Glu-B1* locus. We investigated this hypothesis by cloning and sequencing Bx genes from H45, from Glenlea and

from a control line (VQ0437) that carries *Glu-B1al* in combination with the same glutenin, puroindoline, serpin and storage protein activator alleles as H45.

3.2 Materials and Methods

3.2.1 Plant materials

The main plant materials used were the Canadian wheat cultivar Glenlea (pedigree Pembina*2/Bage//CB100), the Australian cultivar H45 (pedigree B1814//WW15/QT7605), the Australian breeding line VQ0437 (pedigree BD200/CD87//Silverstar) and a random sample of 209 F_2 plants derived from a cross between H45 and VQ0437. For some parts of the work, the cultivars Chinese Spring, Gabo, Aroona, Wilgoyne, Janz, Frame, Currawong, Stiletto and/or Glover were used as controls, as they carry known alleles at genes that encode specific grain proteins. Seeds of the cultivars were obtained from the Australian Winter Cereals Collection (Tamworth, NSW, Australia). Seeds of VQ0437 were kindly provided by Dr Russell Eastwood of Australian Grain Technologies Pty. Ltd. (Horsham, VIC, Australia).

3.2.2 Size-exclusion high-performance liquid chromatography (SE-HPLC)

Samples of approximately 30 grains harvested from each of 229 individual glasshouse-grown plants (10 H45 plants, 10 VQ0437 plants and 209 H45/VQ0437 F_2 plants) were milled using a FQC-200 (Metefem, Budapest, Hungary) micromill and sifted for 30 seconds using a 280 μm sieve. After 7 days, the protein was extracted from the sieved flour extracts according to the methods of Singh et al. (1990a; 1990b) and the two-step method of Gupta et al. (1993) with minor modifications. For the first extraction, 1 ml of 0.5% SDS in 0.05 M phosphate extraction buffer (PEB; pH 6.9) was added to 25 mg (± 1 mg) of sample in 1.5-ml microfuge tubes. Samples were vortexed for 30 seconds and subjected to a 10 min centrifugation (10,730 × g) to pellet SDS-insoluble protein and flour residue. The supernatant (SDS-soluble fraction) was decanted into clean 1.5-ml microfuge tubes and set aside.

The remaining pellet was subsequently resuspended in 1 ml of PEB and subjected to a 30 s sonication using a sonifier (Branson model B-12 cell disrupter, Danbury, CT) fitted with a 3 mm diameter stepped microtip probe which generated ultrasonic vibrations with a frequency of 22 kHz. The samples were centrifuged (10,730 × g) for 10 min to pellet the flour residue. The supernatant (SDS-insoluble fraction) was transferred to clean 1.5 ml microfuge tubes. Prior to SE-HPLC, both extracts were filtered into 1-ml glass HPLC vials (Waters Corp., Milford, MA) using a PVDF 0.45 µm Gelman Acrodisk LC13 Minispike (Gelman, Ann Arbor, MI) and placed in an 80 ℃ waterbath for 2 min to inactivate proteases (Larroque and Békés 2000).

SE-HPLC analysis was performed on a Waters (Milford, MA) Protein-Pak 300TM column (C18, 300 Å pore size, 3.5 µm particle size, 150 mm × 4.6 mm id) using a Waters 717plus Auto sampler, a Waters 600 system controller and a Waters 486 detector. Millennium32 (version 3.2) software was used for acquisition and reprocessing of data generated from the detector.

Separation of the SDS-soluble and SDS-insoluble fractions of polymeric protein was achieved in 50 and 40 min, respectively, by loading 10 µl of sample into an eluent of 50% (v/v) acetonitrile and distilled water containing 0.1% trifluoracetic acid (TFA) (Batey et al. 1991) at a flow rate of 0.5 ml/min. Protein was detected by UV absorbance at 214 nm (Stone and Nicolas 1996). The percentage of SDS-unextractable polymeric protein (UPP) (Gupta et al. 1993) was calculated.

3.2.3 SDS sedimentation test

Samples of grain were harvested from glasshouse-grown plants of Glenlea, H45 and VQ0437. A 50 g sample of grain from each line was milled and the flour was sifted as described above for the SE-HLPC separation. SDS sedimentation tests were conducted according to the procedure outlined by Carter et al. (1999) with minor modifications. Samples were placed in

15-ml centrifuge tubes, which were placed in racks with each rack having a measurement scale background. Two stock solutions were prepared as described by Carter et al. (1999). Distilled water (3 ml) was added to each sample. The samples were mixed for 20 s on a high-speed vortex mixer, allowed to hydrate for 5 min, mixed again on the high-speed vortex for 10 s, and then allowed to hydrate for another 5 min. Lactic acid/SDS solution (9 ml) was added to each sample and the tubes were agitated on a Zeleny type rocker (40 cycles/min) for 40 s, rested for 2 min, and agitated again for 40 s. The racks were left in an upright position for 10 min and the height (mm) of the sediment was recorded. Each SDS sedimentation test was performed twice to account for error in procedure.

3.2.4 SDS-PAGE analysis of glutenin and gliadin

SDS-PAGE analysis was conducted to identify individual glutenin and gliadin subunits. Individual grains of each of H45, VQ0437, Glenlea, Gabo, Chinese Spring, Janz, Aroona and Wilgoyne wheat were crushed with a hammer to produce powder samples. Each of these samples was transferred to a 1.5 ml microfuge tube. Extraction of the gliadin fraction was conducted by adding 0.4 ml of 15% (v/v) ethanol to the samples. The samples were incubated at 65 ℃ in an Eppendorf Thermomixer Comfort 1.5 ml (Eppendorf AG, Germany) for 15 min and vortexed for 15 s. The incubation and vortexing steps were then repeated, and the sample residue was pelleted by centrifugation for 10 min (1,968 × g, Eppendorf Centrifuge 5424, Eppendorf AG, Germany). The supernatant was decanted immediately and any excess liquid was removed from the pellet by keeping the tube inverted for 1 minute. The pellet was reserved for subsequent extraction of glutenins. Extraction of gliadins was done by adding 0.4 ml of distilled water to the supernatant and incubating the sample at 4 ℃ for 2 h. This was followed by a 10 min centrifugation (1,968 × g) to pellet the gliadin. This pellet was dissolved in 0.1 ml of 1% SDS at 65 ℃ for 60 min with regular vortexing at approximately 10 min intervals. Once the pellet was completely dissolved, 0.1 ml of sample buffer (0.02% bromophenol blue; 80 mM Tris-HCl (pH 8.0); 69 mM SDS) was added and the sample was

vortexed for 10 s and incubated for 15 min in the Thermomixer at 65℃. These gliadin samples were frozen to be used later in electrophoresis.

The remaining gliadins present in the flour residue were removed by two subsequent wash steps using 1 ml of 50% (v/v) propan-1-ol. The glutenin fraction was extracted from pelleted flour residue using 0.1 ml of 50% (v/v) propan-1-ol: 80 mM Tris-HCl (pH 8.0) containing 1% (w/v) dithiothreitol (DTT) followed by 30 min incubation in the Thermomixer at 65℃. To prevent the reformation of disulfide bonds and improve band resolution, the protein subunits were alkylated for 15 min at 65 ℃ with 0.1 ml of 50% propan-1-ol: 80 mM Tris-HCl (pH 8.0) containing 1.4% (v/v) 4-vinylpyridine (4-VP). This was followed by a 10 min centrifugation (1,968 × g) to pellet the residue. The pellet was discarded and the supernatant was decanted into a clean microfuge tube, then 0.2 ml of sample buffer was added, the sample was vortexed for 10 s and incubated for 15 min in the Thermomixer at 65℃. The resulting glutenin fraction was stored at -20 ℃ until electrophoresis was conducted.

Electrophoresis was conducted using a discontinuous polyacrylamide gel system (Singh et al. 1991) that had been modified (Cornish et al. 2001) to employ a 3% stacking gel and an 8 to 12% acrylamide gradient separating gel with 1.5% cross-linker concentration (bisacrylamide:acrylamide). The electrophoresis apparatus included a Hoeffer SE600 vertical electrophoresis unit (San Francisco, CA), with 16 × 18 cm glass plates separated by a 1 mm spacer. Gels were loaded with 12 µl of sample and electrophoresis was carried out at approximately 10℃ and 40 mA per gel for 3.5 h.

3.2.5 Quantification of Bx7 expression

Quantification of glutenin subunits was performed using the Experion^{TM} automated electrophoresis system using a Pro260 Analysis kit (Bio-Rad Laboratories, USA) following the method described by Wehr et al. (2008). 4 µl of each sample (or molecular-weight ladder) was mixed with 2 µl of Experion^{TM} Pro260 sample buffer containing 0.03%

β-mercaptoethanol, heated at 95 ℃ for 5 min, and then diluted with 84 µl of deionized water and loaded onto a primed chip. In this system, proteins are detected and quantified based on laser-induced fluorescence of a dye that interacts with lithium dodecyl sulphate micelles that coat the proteins.

3.2.6 Molecular marker assays for puroindoline, serpin, storage protein activator and *Glu-B1*

Genomic DNA was isolated from leaf tissues of three-week-old seedlings using a DNA midi-prep method outlined in Rogowsky et al. (1991) with modifications as described by Pallotta et al. (2000). Genomic DNA was quantified with a spectrophotometer (NanoDrop ND-1000, Thermo) and diluted to 100 ng/µl.

Sequence polymorphisms in the *Pina-D1* and *Pinb-D1* puroindoline genes were assayed according to the methods described by Cane et al. (2004) with minor modifications. Reactions were carried out in 12.5 µl volumes containing 12.5 pmol of each primer, 100 µM of each dNTP, 1 × buffer, and 1.5 mM $MgCl_2$ supplied by the manufacturer (Qiagen), 0.1 unit of Taq DNA polymerase (Qiagen), and 50 ng of genomic DNA. The amplification profile involved one cycle at 94 ℃ for 4 min, followed by 35 cycles of 94 ℃ for 1 min, 58 ℃ for *Pina-D1* or 60 ℃ for *Pinb-D1* for 1.5 min and 72 ℃ for 2 min, and a final extension step at 72 ℃ for 10 min. The assay methods and allele classification for the *Srp5B* serpin locus were the same as those used by Cane et al. (2008). A single-nucleotide polymorphism that generates a premature stop codon in the *Spa-B* storage protein activator gene was assayed using the Pmut marker (Guillaumie et al. 2004). PCR reactions were conducted in 10 µl volumes containing 1 pmol of each primer, 100 µM of each dNTP, 1 × buffer, and 1.5 mM $MgCl_2$ supplied by the manufacturer (Qiagen), 0.2 unit of Taq DNA polymerase (Qiagen), and 50 ng of genomic DNA. The amplification profile involved one cycle at 94 ℃ for 5 min, followed by 35 cycles of 94 ℃ for 30 s, 64 ℃ for 40 s and 72 ℃ for 45 s, and a final extension step at 72 ℃ for 5

min. Sequence polymorphisms at the *Glu-B1* locus were assayed using BxFp and MAR markers, both according to methods described by Butow et al. (2004), and using the LTR left-junction and right-junction markers designed by Ragupathy et al. (2008).

All primers were ordered from GeneWorks (Australia) and all PCR products were separated on 1% agarose/TAE gels.

3.2.7 Cloning and sequencing Bx genes from H45, VQ0437 and Glenlea

As the HMW-GS genes have no introns, genomic DNA isolated as described above was used as template for PCR amplification of the entire coding region. In order to amplify both two Bx genes from each of three varieties, degenerate primers were designed based on the DNA sequence of TaBAC1215C06 (EU157184). The primers were

$Bx7^{OE}$copy1-F (5'-CGCGCTCAACTCTTCTAGTCTAA-3') for the first gene,

$Bx7^{OE}$copy2-F (5'-CCACTCCAACTCTCCTTCCA-3') for the second gene, and

$Bx7^{OE}$-R (5'-CACTCTCGTGCCGATCATTA-3') for both genes.

The PCR amplification was carried out using QIAGEN LongRange PCR Kit (QIAGEN). The amplification profile was one cycle at 93 ℃ for 3 min, followed by 35 cycles of 93 ℃ for 15 s, 55 ℃ for 30 s and 68 ℃ for 5 min, and a final extension step at 72 ℃ for 7 min. The amplified products were recovered from 1.0% agarose gels, cloned into the pGEM-T vector, and transformed into *E. coli* JM109 competent cells. Both amplification and cloning were repeated at least three times to minimize the possibility of errors present in amplification and sequencing. For amplification of fragments for sequencing, the following nested primers were designed based on the EU157184 sequence using Vector NTI software (Invitrogen):

P1 (5'-TGAACTCATTTGGGAAGTAAAC-3'),

P2 (5'-CATCCACACTTCTGCAAACAA-3'),

P3 (5'-ATGGCTAAGCGCCTGGTCCTCTTTG-3'),

P4 (5'-CGCAGCAACTCCAACAAATG-3'),

P5 (5'-CGCAGCAGTCGGAACAAG-3') and

P6 (5'-CTATCACTGCCTGGTCGACAATGCG-3').

Sequencing was performed commercially (Australian Genome Research Facility). Sequence analyses were performed using MEGA (Kumar et al. 2004), BLAST and ClustalW2 from the NCBI (http://www.ncbi.nlm.nih.gov/Tools/) and EBI (http://www.ebi.ac.uk/Tools/sequence.html).

3.2.8 Restriction digest assay to detect a DNA polymorphism

Based on the results of the SE-HPLC analysis, the 20 flour samples with the highest UPP and the 20 flour samples with the lowest UPP were selected from the 209 H45/VQ0437 flour samples that had been evaluated for UPP as described above. Each of these samples consisted of flour milled from F_3 grains harvested from an individual F_2 plant. The method described by Pallotta et al. (2003) was used to isolate genomic DNA from subsamples of these flour samples. For each of the resulting 40 DNA samples, primers $Bx7^{OE}$copy1-F, $Bx7^{OE}$copy2-F and $Bx7^{OE}$-R were used to amplify a fragment containing the first Bx gene. Primers P3 and P6 were then used to amplify the entire coding region of the gene. The resulting PCR product was incubated with the restriction enzyme *Rsa* I at 37℃ for 2 h, and the digested products were separated on a 2.5% agarose gel.

3.3 Results

3.3.1 Comparison of the flour properties, protein composition and genotypes of H45 and VQ0437

With SE-HPLC analysis (Appendix 1), the percentage UPP was significantly lower for H45 (30.12 ± 1.78) than for VQ0437 (44.29 ± 1.92) or for a control sample of bakers' flour (45.48 ± 1.39). In the SDS sedimentation test, the SDS sedimentation volume for H45 (19.3 ± 0.14

ml g^{-1}) was significantly lower than for VQ0437 (23.6 ± 0.28 ml g^{-1}) and Glenlea (24.5 ± 0.14 ml g^{-1}), even though all three flour samples had similar protein concentrations.

3.3.2 Comparison of the protein composition of H45 and VQ0437

SDS-PAGE results for H45 and VQ0437 appeared identical to each other in all respects. Both of these lines carry the Ax1, Bx7 (or similar), By8*, Dx2, and Dy12 HMW-GS and the A3c, B3h and D3b LMW-GS (Fig. 3.1). No differences in gliadin profiles were detected (Fig. 3.2). The proportion of Bx subunits relative to total HMW-GS, calculated from the Experion™ automatic electropherogram trace (Fig. 3.3) was 0.543 for H45, 0.563 for VQ0437 and 0.567 for Glenlea, compared to only 0.412 for Chinese Spring, confirming overexpression of the Bx subunits in H45, VQ0437 and Glenlea.

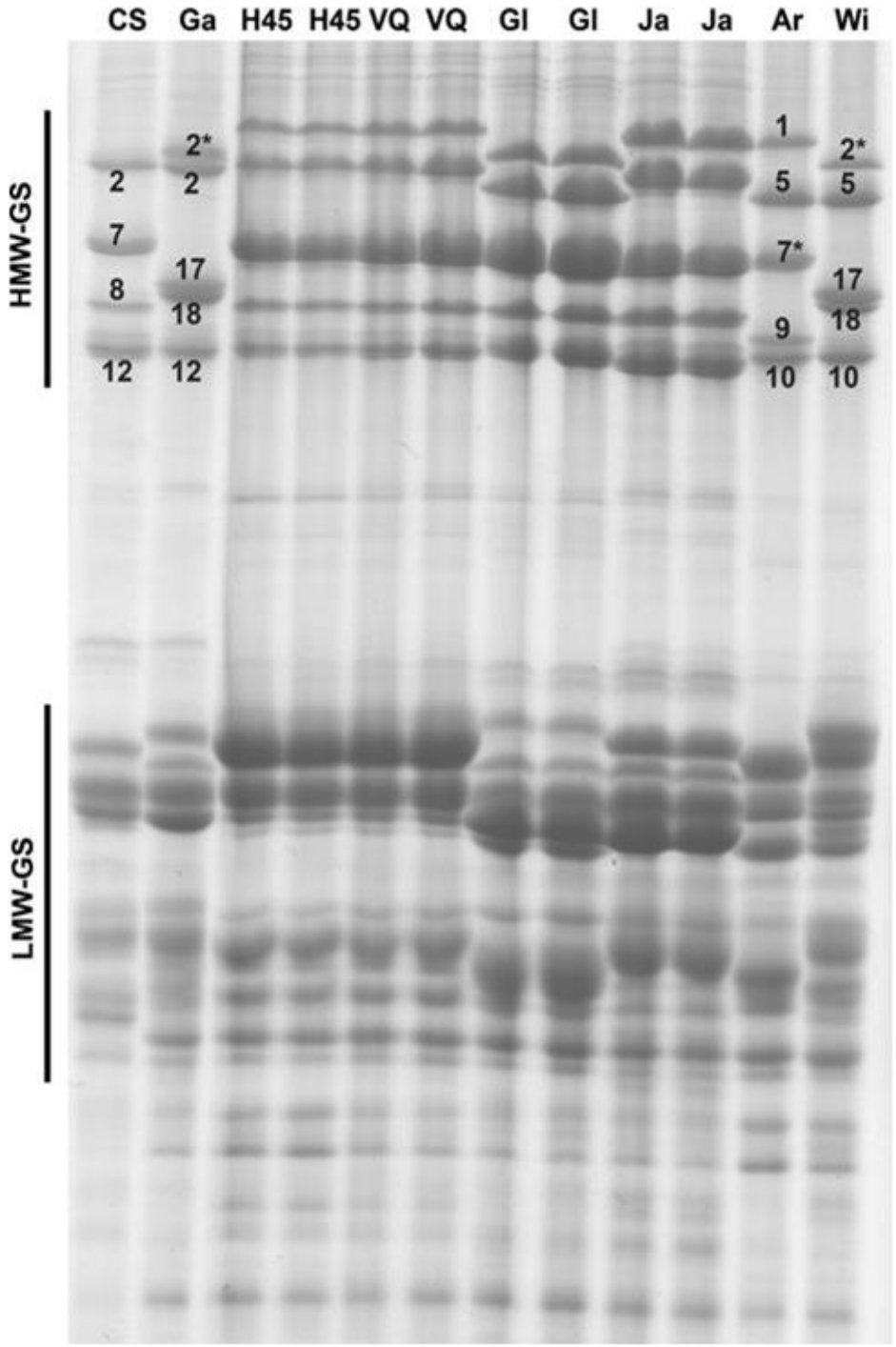

Fig. 3.1 One-dimensional SDS-PAGE of glutenins extracted from flour of Chinese Spring (CS), Gabo (Ga), H45, VQ0437 (VQ), Glenlea (Gl), Janz (Ja), Aroona (Ar) and Wilgoyne (Wi) wheat. HMW-GS and LMW-GS high- and low-molecular-weight glutenin subunits, respectively. 1 and 2* HMW-GS encoded by *Glu-A1a* and *Glu-A1b*, respectively. 7 and 8 HMW-GS encoded by *Glu-B1b*. 17 and 18 HMW-GS encoded by *Glu-B1i*. 7* and 9 HMW-GS encoded by *Glu-B1c*. 2 and 12 HMW-GS encoded by *Glu-D1a*. 5 and 10 HMW-GS encoded by *Glu-D1d*.

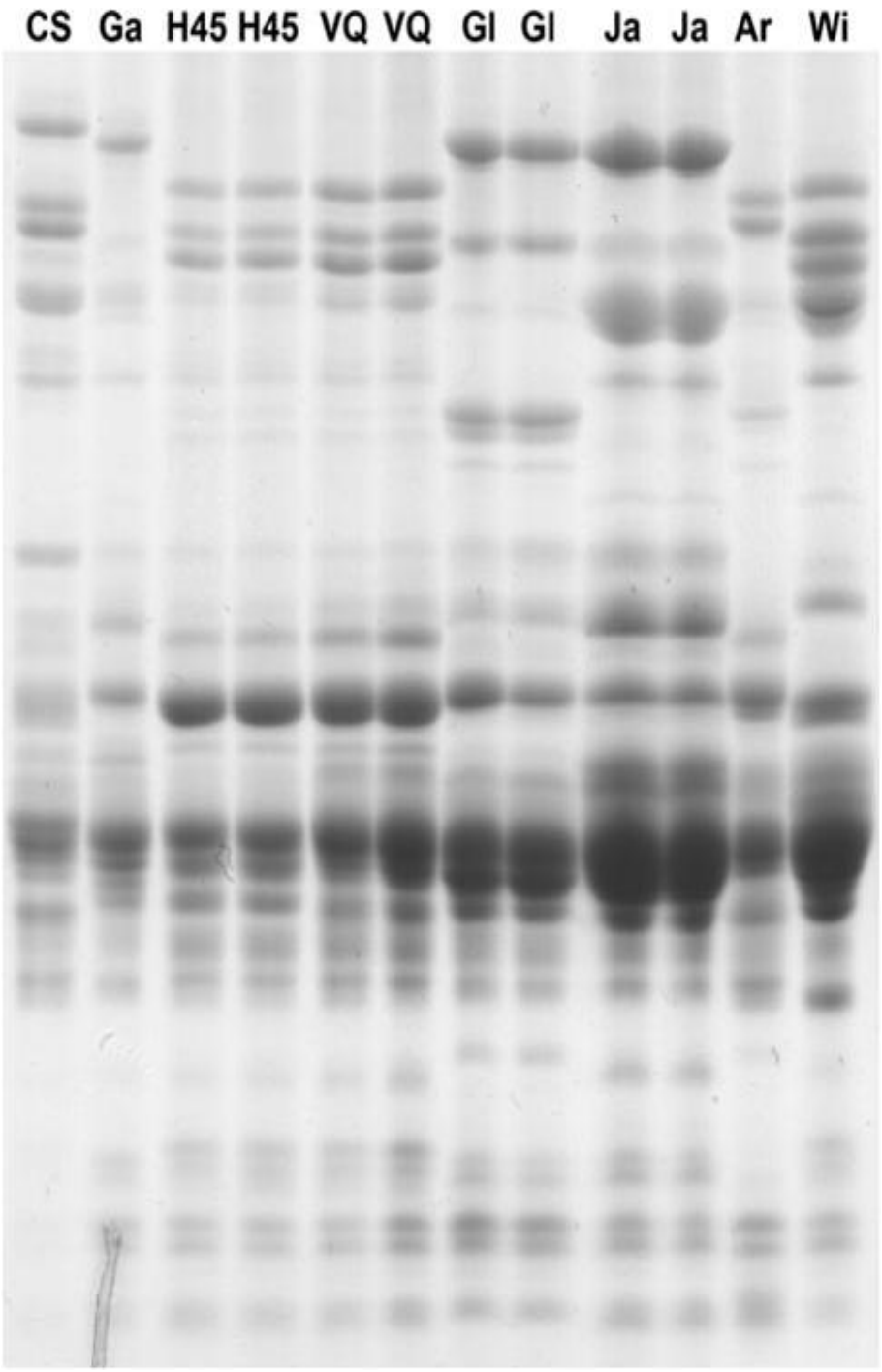

Fig. 3.2 One-dimensional SDS-PAGE of gliadins extracted from flour of Chinese Spring (CS), Gabo (Ga), H45, VQ0437 (VQ), Glenlea (Gl), Janz (Ja), Aroona (Ar) and Wilgoyne (Wi) wheat.

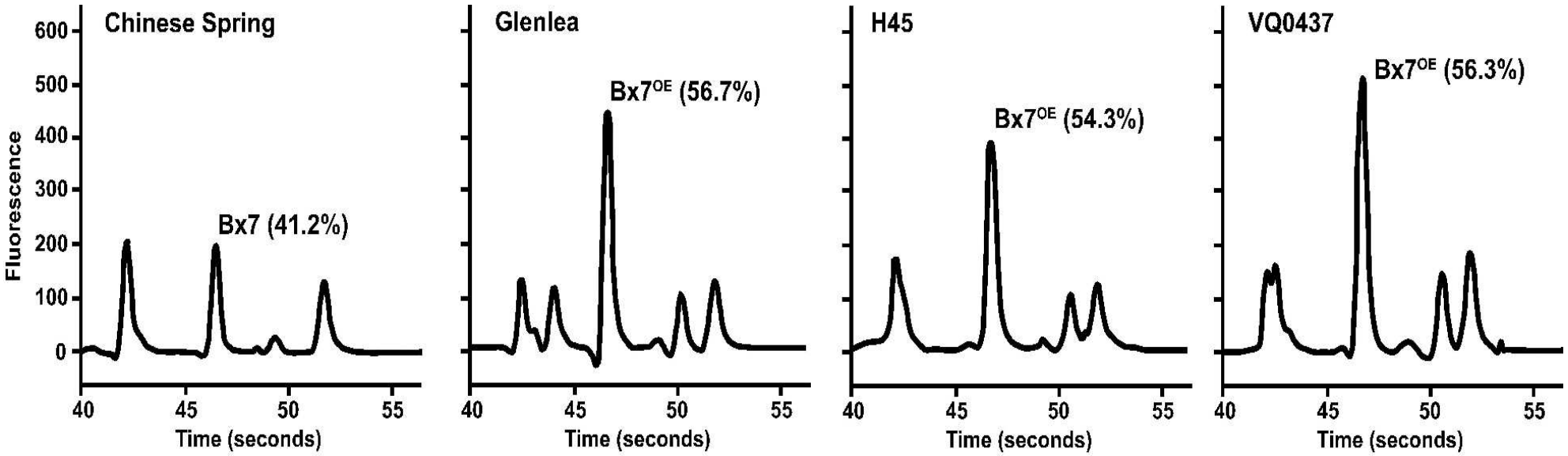

Fig. 3.3 Experion^{TM} automatic electropherograms of high-molecular-weight glutenin subunits extracted from flour of Chinese Spring, Glenlea, H45 and VQ0437 wheat.

3.3.3 Comparison of the genotypes of H45 and VQ0437

None of the molecular marker assays conducted for polymorphisms in puroindoline, serpin and storage protein activator genes detected any differences between H45 and VQ0437. The genes *Pina-D1* and *Pinb-D1* (Giroux and Morris 1998), which are both located at the hardness (*Ha*) locus on chromosome 5D, encode puroindoline a and puroindoline b proteins. Their wild-type alleles have been designated *Pina-D1a* and *Pinb-D1a*. Two alleles with highly conserved mutations have been identified and named *Pina-D1b* and *Pinb-D1b* (Giroux and Morris 1998). With Currawong (*Pina-D1b/Pinb-D1a*) and Stiletto (*Pina-D1a/Pinb-D1b*) as standards, it was shown that the puroindoline genotype of both H45 and VQ0437 is *Pina-D1a/Pinb-D1b* (Fig. 3.4). At the *Srp5B* serpin locus, the genotype of H45 and VQ0437 is the same as the standard Glover: the type named *a* by Cane et al. (2008) (Fig. 3.5). For the *Spa-B* storage protein activator gene, a product of 311 bp (which is indicative of an allele with a premature stop codon) was amplified from Glenlea but no product was amplified from H45, VQ0437 or Chinese Spring (Fig. 3.6).

None of the molecular marker assays conducted for sequence polymorphisms at the *Glu-B1* locus detected any differences among H45, VQ0437 and Glenlea. Using a marker (BxFp) in the Bx coding region, PCR amplicons from both H45 and VQ0437 were the same size as those from Glenlea ($Bx7^{OE}$) and Chinese Spring (Bx7), both of which are known to contain an 18 bp insertion that is not present in Janz (Fig. 3.6). Similarly, PCR amplicons derived using a MAR primer pair showed that H45, VQ0437 and Glenlea all have a 43 bp insertion, which is not present in Chinese Spring or Janz (Fig. 3.6). With primers that Ragupathy et al. (2008) designed to amplify regions flanking a retrotransposon that lies between two copies of the $Bx7^{OE}$ gene in Glenlea, H45, VQ0437 and Glenlea ($Bx7^{OE}$) all exhibited 447 and 884 bp amplicons. These products were not amplified from Chinese Spring (Bx7) or Janz (Bx7*) (Fig. 3.7).

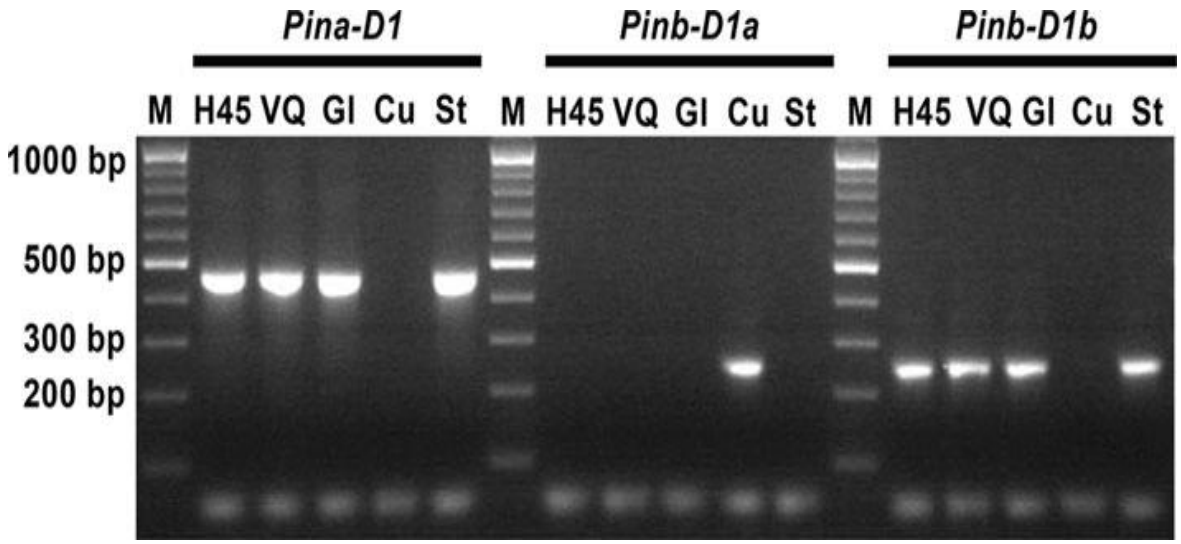

Fig. 3.4 PCR products amplified with primers for the puroindoline genes *Pina-D1* and *Pinb-D1* from genomic DNA of H45, VQ0437 (VQ), Glenlea (Gl), Currawong (Cu; *Pina-D1b / Pinb-D1a*) and Stiletto (St; *Pina-D1a / Pinb-D1b*) wheat. M: 100 bp ladder.

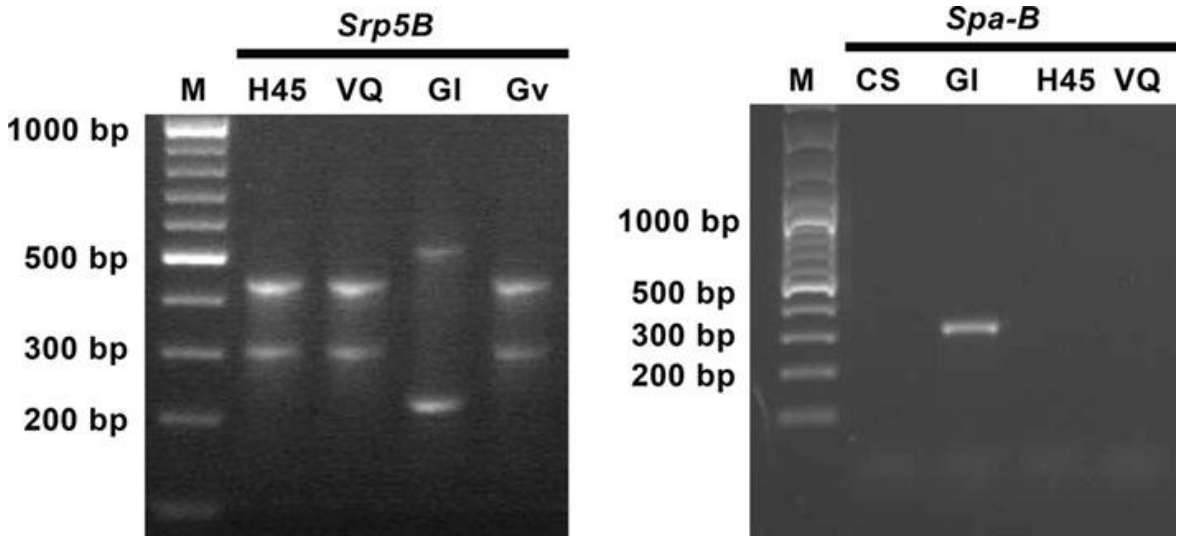

Fig. 3.5 PCR products amplified with primers for the serpin gene *Srp5B* from genomic DNA of H45, VQ0437 (VQ), Glenlea (Gl; *Srp5Bb*) and Glover (St; *Srp5Ba*) wheat and products amplified with primers for the storage protein activator gene *Spa-B* from Chinese Spring (CS), Glenlea (Gl), H45 and VQ0437 (VQ) wheat. M: 100 bp ladder.

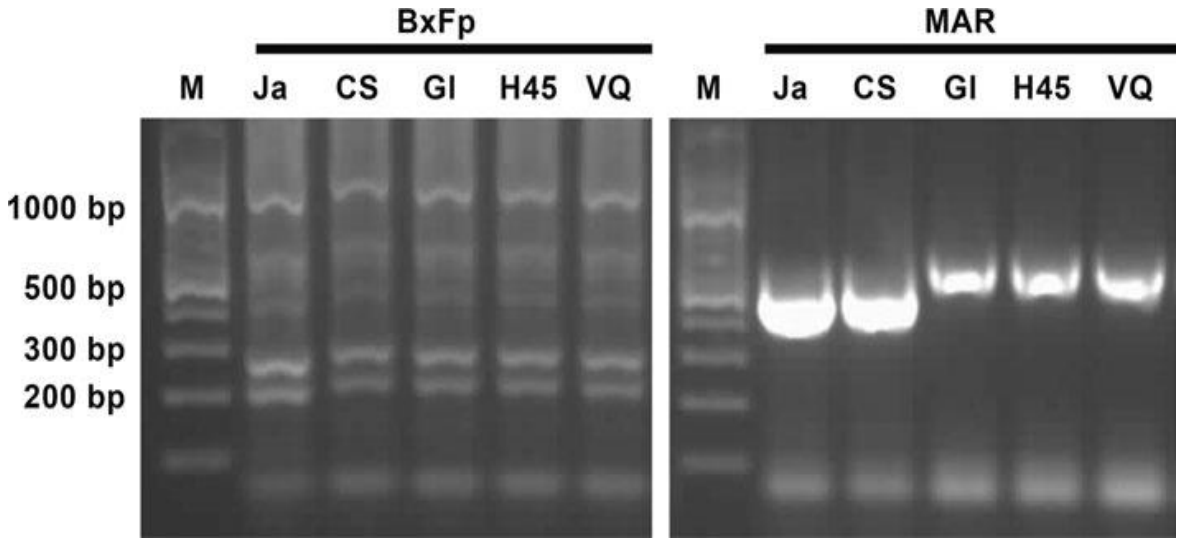

Fig. 3.6 PCR products amplified with BxFp and MAR primers for the Bx glutenin genes from genomic DNA of Janz (Ja, Bx7*), Chinese Spring (CS, Bx7), Glenlea (Gl, $Bx7^{OE}$), H45 and VQ0437 (VQ) wheat. M: 100 bp ladder.

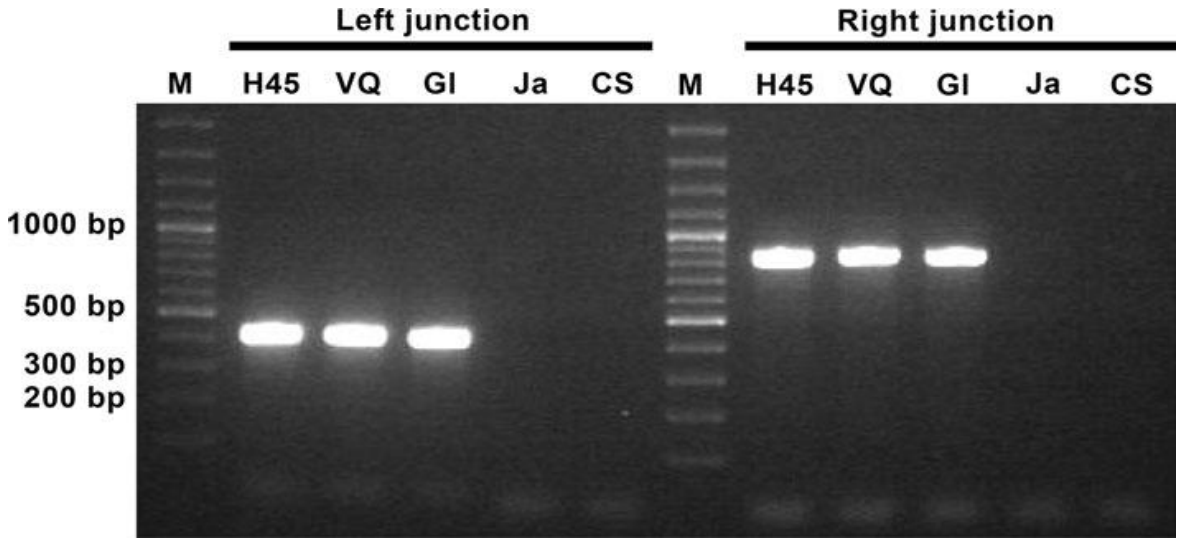

Fig. 3.7 PCR products amplified from genomic DNA of H45, VQ0437 (VQ), Glenlea (Gl; $Bx7^{OE}$), Janz (Ja; Bx7*) and Chinese Spring (CS; Bx7) wheat using primers designed at the left and right junctions of a retroelement between duplicate copies of the Bx gene in the *Glu-B1al* allele of Glenlea. M: 100 bp ladder.

3.3.4 Sequences of Bx7 alleles

Two regions (4.3 kb and 3.9 kb in length), each including a Bx gene, were cloned from each of H45, VQ0437 and Glenlea and were fully sequenced. For Glenlea, the sequences obtained for the two gene copies were the same as each other and the same as those in GenBank (EU157184). Alignment of the H45 sequences (JF938070, JF938071) with those of Glenlea revealed four single nucleotide polymorphisms (SNPs) in the first gene and four SNPs in the second gene (Appendix 2). All four SNPs in the first gene are non-synonymous (Fig. 3.8), with one altering a lysine into an arginine in the C-terminal region of the protein and the others altering amino acids in the repetitive domain: a tyrosine into a cysteine, a glutamine into an arginine, and a serine into a proline. Only one of the four SNPs in the second gene is non-synonymous; it alters a proline into a serine in the repetitive domain. Alignment of the VQ0437 sequences (JF938072, JF938073) with those of Glenlea revealed two SNPs in the first gene and one SNP in the second gene (Appendix 2). Of these, one of the SNPs in the first gene is non-synonymous, altering a lysine into a threonine in the N-terminal region (Fig. 3.8).

According to the sequences obtained for the first gene, the SNP that alters a tyrosine (in Glenlea and VQ0437) into a cysteine (in H45) eliminates an *Rsa* I restriction site. This restriction site polymorphism provided the basis for the design of a molecular marker assay to distinguish the H45 allele from each of the other two alleles. After amplification of a 2,391 bp product representing the coding region products of the first gene, digestion with *Rsa* I should result in 22 products for Glenlea and VQ0437, but only 21 for H45 (Table 3.1). The H45 digestion products should include a 216 bp fragment that is not present in the Glenlea or VQ0437, and should lack a 135 bp fragment and one of three 81 bp fragments that are present in Glenlea and VQ0437.

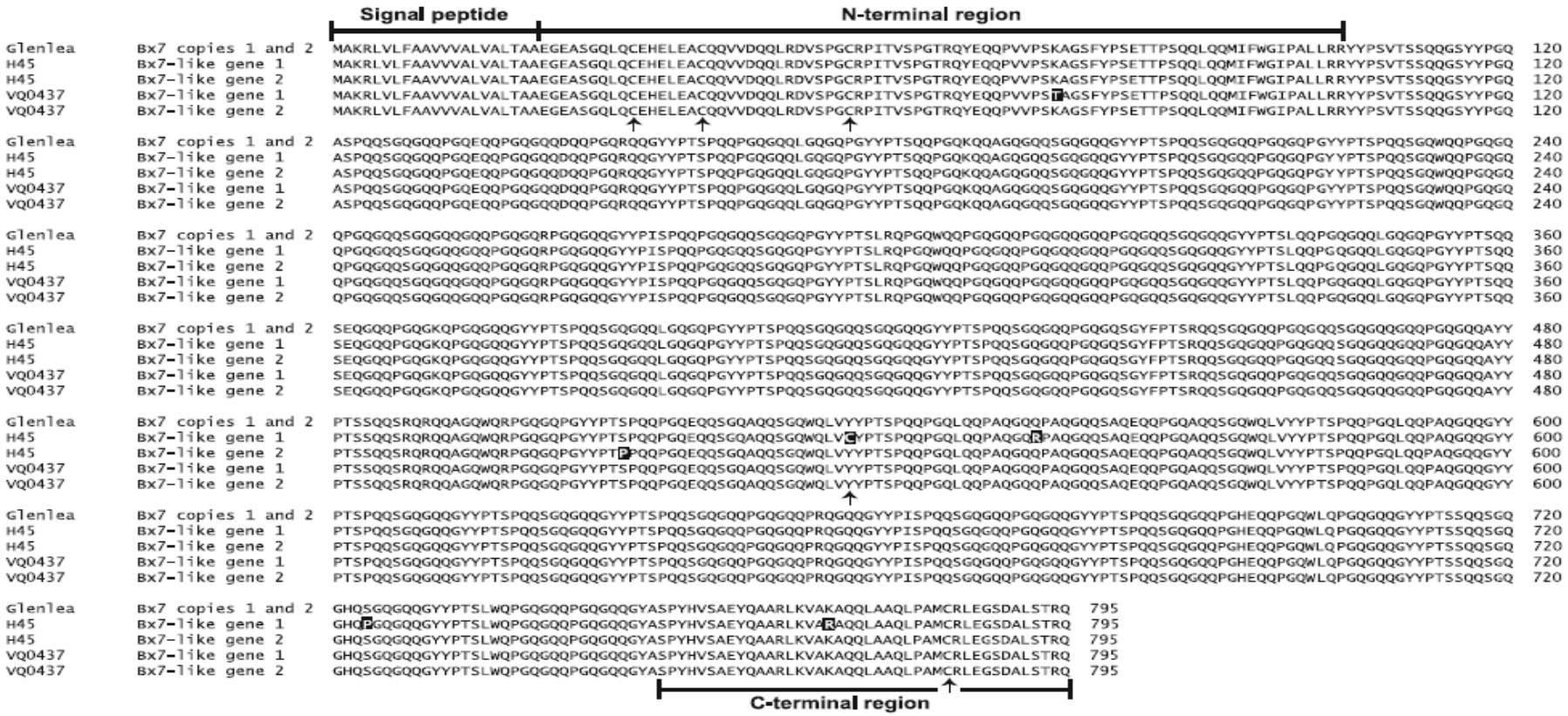

Fig. 3.8 Amino acid sequences translated from the nucleotide sequence of a duplicated gene at the *Glu-B1* locus that encodes the high-molecular-weight glutenin subunit $Bx7^{OE}$ in Glenlea wheat (EU157184), aligned with the amino acid sequences translated from each copy of the corresponding gene from the wheat variety H45 (JF938070, JF938071) and the wheat breeding line VQ0437 (JF938072, JF938073). The positions of cysteine residues are shown by arrows.

Table 3.1 Restriction fragments expected for an assay in which the restriction enzyme *Rsa* I is used to digest a 2,391 bp fragment amplified from the first of two x-type high-molecular-weight glutenin genes at the *Glu-B1* locus of Glenlea, H45 and VQ0437 wheat.

Fragment length (bp)	Wheat lines and numbers of restriction fragments	
	Glenlea and VQ0437	H45
523	1	1
216		1
189	1	1
162	1	1
144	1	1
135	1	
108	1	1
107	1	1
93	1	1
81	3	2
78	2	2
63	7	7
45	2	2

3.3.5 Variation among H45/VQ0437 F_2 progeny

The UPP values of the flour samples milled from the F_3 grains harvested from individual H45/VQ0437 plants exhibited a continuous range of variation from slightly below the value for H45 to slightly above the value for VQ0437 (Fig. 3.9). With the *Rsa* I digestion assay, the H45 allele could be distinguished from that of VQ0437, principally based on the presence of the additional 216 bp fragment (Fig. 3.10). All 20 of the $F_{2:3}$ families plants with the lowest UPP gave the same result as H45, while all 20 of the $F_{2:3}$ families plants with the highest UPP gave the same result as VQ0437 (Fig. 3.10).

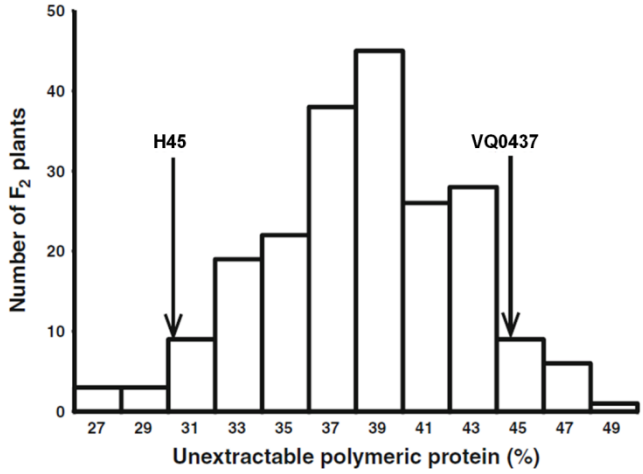

Fig. 3.9 Frequency distribution of percentage SDS-unextractable polymeric protein in flour samples of F_3 grains harvested from F_2 plants from a cross between H45 and VQ0437.

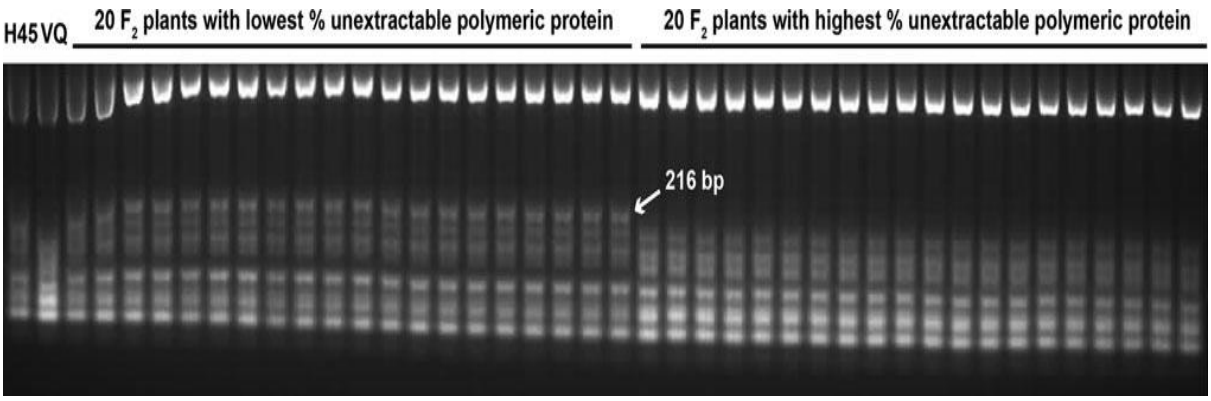

Fig. 3.10[1] Restriction fragments from *Rsa* I digestion of PCR products of first of two x-type high-molecular weight glutenin genes at the *Glu-B1* locus of H45, VQ0437 (VQ) and sets of 20 H45/VQ0437 F_2 plants with low and high SDS-unextractable polymeric protein.

[1] There is a better image in Appendix 3.

3.4 Discussion

The results reported here demonstrate that the Australian wheat cultivar H45 has significantly lower SDS-unextractable polymeric protein and SDS sedimentation volume than Glenlea, which carries the Bx overexpression allele *Glu-B1al*. Further, H45 was found to differ from the breeding line VQ0437 for these traits, even though H45 and VQ0437 seemed to carry exactly the same combination of glutenin alleles. These two lines also carry the same alleles for a storage protein activator gene that is closely linked with *Glu-B1* and has previously been proposed (Guillaumie et al. 2004) as a candidate gene for Bx overexpression, and for puroindoline and serpin genes that affect milling quality and that are routinely assayed in wheat breeding. They also had similar gliadin profiles as assessed by SDS-PAGE. The similarity of these two lines across all of these genes helped ensure that VQ0437 was an appropriate control line to be compared with H45 and to be used as a parent to generate a population in which the effects of *Glu-B1* would not be confounded by effects of other known grain quality genes. With quantification of Bx subunits as a proportion of HMW-GS, we confirmed that both H45 and VQ0437 overexpress Bx subunits. Further, we found that they both carry the tandem segmental duplication that is thought to be diagnostic for the $Bx7^{OE}$ phenotype (Ragupathy et al. 2008). This led us to investigate the sequence of each of the Bx genes from H45, VQ0437 and Glenlea.

By isolating and sequencing both Bx genes from H45, VQ0437 and Glenlea, we discovered several non-synonymous SNPs. Of these, the one most likely to explain the properties of H45 causes a tyrosine-to-cysteine change in the HMW-GS encoded by the first Bx gene. The results from assaying this SNP on DNA extracted from flour milled from F_3 grains harvested from high-UPP and low-UPP F_2 plants demonstrated that the H45 *Glu-B1* allele is strongly

associated with low UPP. Given the continuous range of variation observed for UPP (Fig. 3.9), it seems that *Glu-B1* is not the sole factor affecting this trait. Although there may be genetic factors involved, much of the variation is probably due to error variation among measurements taken on flour samples from individual F_2 plants.

It seems likely that the tyrosine-to-cysteine mutation is the causal polymorphism underlying the association of *Glu-B1* with UPP. Given the minor role that dityrosine residues play in cross-linking within or between glutenin subunits (Hanft and Koehler 2005), the loss of a tyrosine would be unlikely to have a major effect on glutenin polymerisation. The gain of a cysteine would be more significant. Most x-type HMW-GS contain four cysteines: three in the N-terminal domain and one in the C-terminal domain. Normally, two of the cysteines in the N-terminal domain are connected by an intrachain disulphide bond, while the other two cysteines contribute to glutenin polymerisation *via* interchain disulphide bonds with cysteines in other HMW- or LMW-GS (Keck et al. 1995; Wieser 2007). In the subunit encoded by the first Bx gene of H45, there is an extra cysteine, located in the repetitive domain of the protein. Differences in the number and location of cysteine residues in HMW-GS are known to affect the pattern of disulphide bond formation, and hence the composition and functionality of glutenin polymers (Tamás et al. 2002; Pirozi et al. 2008). Depending on the number and positions of cysteine residues available to form disulphide bonds, glutenin subunits may act as chain extenders or chain terminators (Tamás et al. 2002), affecting polymer size and thus dough strength and extensibility .

If intrachain links form more rapidly than interchain links (as has been proposed by Kasarda (1999)), the extra cysteine in the Bx subunit encoded by the first Bx gene of H45 might form intrachain disulphide bonds, leading to a conformation that could impede interchain links. In

that case, the subunit with the extra cysteine might act as a chain terminator, preventing other HMW-GS from bonding to the gluten matrix and counteracting the favourable effects of Bx overexpression. This would be similar to effects observed by Tamás et al. (2002), who found that chain-terminator proteins could inhibit the effects of incorporating supplemental Bx7 protein into dough. This provides a plausible explanation for the low values of UPP and SDS-sedimentation volume that we observed for H45. Another possible explanation is that the 'extra' cysteine participates in interchain bonds, and this affects polymerisation in some other way.

We propose to designate the *Glu-B1* alleles of H45 and VQ0437 as *Glu-B1br* and *Glu-B1bs*, respectively. The discovery of these alleles demonstrates that *Glu-B1al* is not the only *Glu-B1* allele with a duplication of an x-type glutenin gene. *Glu-B1al*, *Glu-B1br* and *Glu-B1bs* all confer overexpression of subunits with the same size and electrophoretic mobility as Bx7, but *Glu-B1br* seems to differ from the others in its effect on glutenin polymerisation. Given the sequence similarity between the coding regions of the three alleles, the fact that the same duplication junctions are detected in all three alleles and that those duplication junctions have been detected in diploid, tetraploid and hexaploid species of *Triticum* (Ragupathy et al. 2008), it seems likely that these alleles originated from a common ancestor. The origin of *Glu-B1al* has previously been traced back to the Argentinean wheat cultivar Klein Universal II (Butow et al. 2004; Vawser and Cornish 2004). VQ0437 probably inherited *Glu-B1bs* from the cultivar CD87 (which has previously been classified as carrying *Glu-B1al*). The origin of *Glu-B1br* is not known. We have not been able to detect the gene duplication in any of the known ancestors of H45.

With the *Rsa* I digestion assay, *Glu-B1br* can be distinguished from *Glu-B1al* and *Glu-B1br* based on the presence or absence of a restriction site caused by the SNP that generates the tyrosine-cysteine amino acid substitution. As this assay requires long-range PCR and a restriction enzyme, it is not very convenient for large-scale screening of germplasm or for routine use in breeding. Nevertheless, it could be useful in situations where it is important to distinguish *Glu-B1br* from other similar alleles.

The discovery of *Glu-B1br* is of practical significance for wheat breeding, especially in Australia, where H45 has been used as a parent and has been particularly important in hybrid wheat breeding. The presence of a cysteine in the repetitive region of first Bx gene of *Glu-B1br* could explain the weaker dough of H45 and some of its progeny relative to other lines that carry two Bx genes at the *Glu-B1* locus and that overexpress Bx subunits. Knowledge of which parents carry *Glu-B1br* and which carry *Glu-B1al* or *Glu-B1bs* could improve the predictability of cross outcomes in wheat breeding.

Chapter 4

Screening of Wheat Germplasm for Bx Overexpression Alleles at the *Glu-B1* Locus

4.1 Introduction

As reported in Chapter 3 of this thesis, two new Bx overexpression alleles, designated *Glu-B1br* (Bx7.1+Bx7.2+By8*) and *Glu-B1bs* (Bx7.3+Bx7^{OE}+By8*), have been discovered and characterised. The effects of the *Glu-B1br* allele on flour and dough properties differ from those of the *Glu-B1al* and *Glu-B1bs* alleles.

Polymerase chain reaction (PCR) based molecular marker assays have been developed to distinguish different HMW-GS genotypes (D'Ovidio and Anderson 1994; Smith et al. 1994; de Bustos et al. 2001; Ma et al. 2003; Ishikawa and Nakamura 2007; Liu et al. 2008). To detect the *Glu-B1al* allele, Ragupathy et al. (2008) designed primer pairs to amplify the right and left junctions of a long terminal repeat (LTR) retroelement that lies between two Bx gene copies in TaBAC1215C06 (EU157184). This provided markers that were diagnostic of the *Bx7^{OE}* phenotype across a panel of diploid, tetraploid and hexaploid accessions of *Triticum* spp. One of these LTR markers has been used by Jin et al. (2011) to screen wheat cultivars from 20 countries.

The LTR markers designed by Ragupathy et al. (2008) do not distinguish *Glu-B1br* or *Glu-B1bs* allele from *Glu-B1al* allele (see Chapter 3 of this thesis). However, a restriction digest assay that was reported in Chapter 3 of this thesis can distinguish the *Glu-B1br* allele from the other Bx overexpression alleles. As this assay requires two long-range PCR steps

followed by a restriction digest, it is not very convenient for large-scale screening of germplasm. To detect the *Glu-B1br* allele wheat germplasm, it is more practical to first use the molecular markers designed by Ragupathy et al. (2008) to detect the Bx gene duplication and then to apply the restriction digest assay only to those lines that carry the gene duplication.

This chapter describes the screening of a panel of wheat cultivars for the presence of the *Glu-B1br* allele and the assessment of unextractable polymeric protein in wheat lines that were confirmed to carry Bx overexpression alleles.

4.2 Materials and Methods

4.2.1 Plant materials

In this research, Glenlea, H45, VQ0437, Chinese Spring and Janz were used as controls because they are known to carry the genes encoding $Bx7^{OE}$ (Glenlea and VQ0437), Bx7.1 (H45), Bx7.2 (H45), Bx7.3 (VQ0437), Bx7 (Chinese Spring) and Bx7* (Janz). The wheat lines Ciano 67, Kalyansona, WW15, WW80, Olympic, and Bluebird (represented by Bluebird #3, Bluebird #4 and a progeny line of Kalyansona/Bluebird), were used because they are ancestors of H45. The wheat cultivars Kukri, Chara, AGT Katana, LongReach Lincoln, Mace, Magenta and Wyalkatchem were included because they have been classified as carrying the $Bx7^{OE}$ subunit (Eagles et al. 2004; Vawser and Cornish 2004 and personal communication with Dr. Howard Eagles and Dr. Marie J. Appelbee). The wheat cultivars Datatine, EGA Stampede, Gladius, Corrigin, IAS 20 Iassul, Veranopolis, Cook and Batavia were also included based on suggestions that they may carry Bx overexpression alleles (personal communication with Dr. Howard Eagles). Seeds of these lines were obtained from the

Australian Winter Cereals Collection (Tamworth, NSW, Australia) (Table 4.1).

4.2.2 Molecular marker assay and restriction digestion assay on a panel of wheat cultivars

Genomic DNA was prepared according to the method reported in section 3.2.6 in this thesis. Molecular marker assays to distinguish the retrotransposon junctions lying between the two *Bx* gene copies on *Glu-B1* locus described by Ragupathy et al. (2008) were conducted on a panel of wheat cultivars. The cultivars confirmed to carry an overexpression allele at the *Glu-B1* locus were then assayed for the *Bx7.1* gene using the restriction digestion method described in section 3.2.8 of this thesis.

4.2.3 SDS-PAGE analysis of HMW glutenins

SDS-PAGE analysis was conducted to identify individual HMW glutenin subunits. Individual grains of Chinese Spring, Janz and each wheat line for which a Bx overexpression allele had been detected were crushed with a hammer to produce powder samples. Each of these samples was transferred to an individual 1.5 ml microfuge tube. Extraction of the HMW glutenin subunits was conducted according to the method described by Mackie et al. (1996). Electrophoresis was conducted using a discontinuous polyacrylamide gel system (Singh et al. 1991) that had been modified (Cornish et al. 2001) to employ a 3% stacking gel and an 8% acrylamide separating gel. The electrophoresis apparatus was described in section 3.2.4 of this thesis.

4.2.4 Reversed phase high-performance liquid chromatography (RP-HPLC)

RP-HPLC analysis of HMW-GS of wheat lines was conducted in duplicate based on the

method described by Vawser et al. (2004). Identification of subunit peaks was based on previously reported examples (Lafiandra et al. 1994; Vawser and Cornish 2004) and SDS-PAGE profiles.

4.2.5 Size-exclusion high-performance liquid chromatography (SE-HPLC)

Samples of 8 to 10 grains of Chinese Spring, Janz and each wheat line for which a Bx overexpression allele had been detected were prepared following the method described in section 3.2.2 of this thesis. SE-HPLC analysis was conducted and UPP value was calculated as described in section of 3.2.2 of this thesis.

Table 4.1 Pedigrees of wheat lines.

Wheat line	Australian Winter Cereals Collection Accession number	Pedigree
Glenlea	AUS15566	Pembina*2/Bage//CB100
H45	AUS29488	B1814//WW15/QT7605[2]
		Kalyansona/Bluebird//Anza*3/WW80/3/Olympic*2/Ciano 67[3]
VQ0437		BD200/CD87//Silverstar[4]
Chinese Spring	NS1806	Selection from Chinese landrace
Janz	AUS24794	3Ag3/4*Condor//Cook
Ciano 67	AUS125	Pitic 62/(SIB)Chris//Sonora 64
Kalyansona	AUS1214	Penjamo 62(SIB)/Gabo 55
WW15	AUS6919	Lerma Rojo 64//(Selection 14)Norin 10/Brevor/3/3*Andes Enano
WW80	AUS6921	Penjamo 62/4*Gabo 56//Tezanos Pintos Precoz/Nainari 60
Olympic	AUS3117	Baldmin/Quadrat
Kalyansona/Bluebird	AUS18584	Kalyansona/Bluebird
Bluebird#3	AUS12055	Ciano 67(SIB)/3/Sonora 64/Klein Rendidor//Siete Cerros 66
Bluebird#4	AUS90595	Ciano 67(SIB)/3/Sonora 64/Klein Rendidor//Siete Cerros 66
Kukri	AUS29472	DRP/5/Frontana/Kenya 58//N10B/G55/3/Nainari 60/4/Tobari 66/Ciano 67//Tobari 66/8156/3/Calidad//Bluebird/Ciano 67/6/Madden/6*RAC177

[2] Pedigree provided by Genetic Resources Information System for Wheat and Triticale (http://wheatpedigree.net/) and database of Australian Plant Breeder's Rights (http://pericles.ipaustralia.gov.au/pbr_db/)

[3] Pedigree provided by Genetic Resources Information System for Wheat and Triticale

[4] Pedigree provided by Dr Russell Eastwood, Australian Grain Technologies

Table 4.1(Cont.) Pedigrees of wheat lines.

Wheat line	Australian Winter Cereals Collection Accession number	Pedigree
Chara	AUS30031	BD225/CD87
AGT Katana	AUS38783	Kukri/Tammin
LongReach Lincoln	AUS99353	96WFHB5568/Otane//Rubric
Mace	AUS99360	Wyalkatchem/Stylet//Wyalkatchem
Magenta	AUS36469	Carnamah/Tammin 18
Wyalkatchem	AUS99280	Machete/3/Gutha//Jacup*2/11th Isepton 135
Datatine	AUS26161	3Ag3/3*Halberd//4*Tincurrin
EGA Stampede	AUS36681	Selection from progeny of complex crosses involving some of the following parents: 11 IBWSN 50, Seri M82, Sunpict, Genaro T81, Batavia, Hartog, Sun290B, Janz, Sunvale and QT4646
Gladius	AUS99336	RAC875/Krichauff//Excalibur/Kukri/3/RAC875/Krichauff/4/RAC875//Excalibur/Kukri
Corrigin	AUS24850	Tincurrin*2//Gamenya/Iassul
IAS 20 Iassul	AUS19525	Colonias//Frontana/Kenya 58
Veranopolis	AUS19533	Trintecinco//Fronteira/Mentana
Cook	AUS20275	Timgalen/(SIB)Condor//Condor
Batavia	AUS25271	Brochis(SIB)/Banks

4.3 Results

4.3.1 Molecular marker assay on a panel of wheat cultivars

With primers that Ragupathy et al. (2008) designed to amplify regions flanking a retrotransposon that lies between two copies of the *Bx7*^{OE} gene in Glenlea, 447 bp and 884 bp products were both amplified from the positive control Glenlea (Fig. 3.7 and Fig. 4.1), from H45 and VQ0437 (Fig. 3.7), and from Kukri, Chara, AGT Katana, Magenta, Mace, LongReach Lincoln and Wyalkatchem (Fig. 4.1). Neither of these products were amplified from the negative controls Chinese Spring and Janz, or from Ciano 67, WW15, WW80, Kalyansona, Olympic, Corrigin, IAS 20 Iassul, Veranopolis, EGA Stampede, Gladius, Kalyansona/Bluebird, Bluebird #3, Bluebird #4, Cook, Batavia or Datatine (Fig. 4.1).

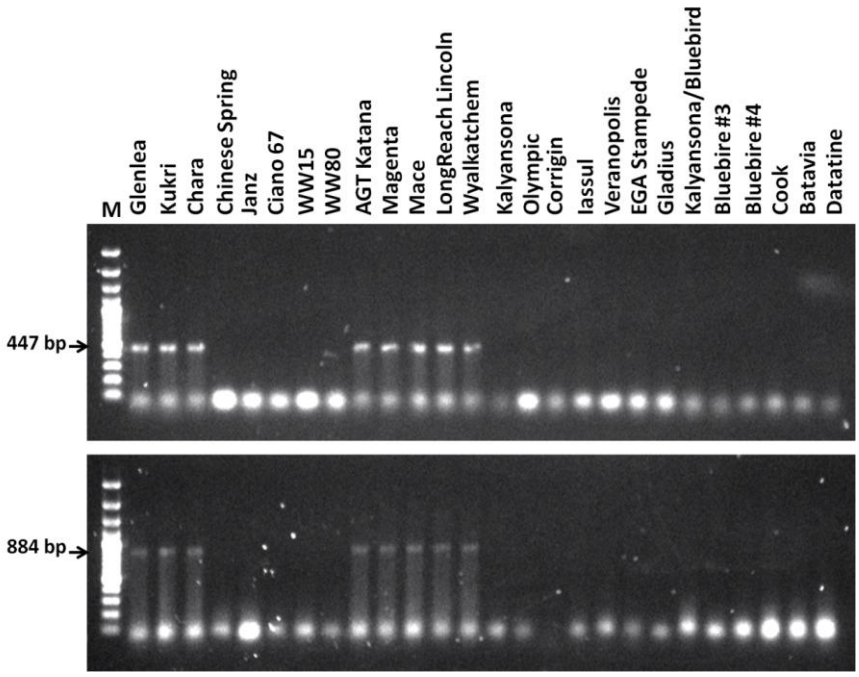

Fig. 4.1 PCR products amplified from genomic DNA of a panel of wheat cultivars using primers designed at the left (447 bp) and right (884 bp) junctions of a retroelement between duplicate copies of the Bx gene in the *Glu-B1al* allele of Glenlea. Positive control Glenlea (*Bx7*^{OE}) and negative controls Chinese Spring (*Bx7*), Janz (*Bx7**) were included. M: 100 bp ladder.

4.3.2 HMW-GS composition analysis and SE-HPLC analysis on a panel of wheat lines

In SDS-PAGE, each of the wheat lines in which the *Glu-B1* retrotransposon junctions were detected (H45, VQ0437, Kukri, Chara, AGT Katana, Magenta, Mace, LongReach Lincoln, and Wyalkatchem) had a band that is similar in mobility to the Bx7, Bx7* and $Bx7^{OE}$ bands of the controls Chinese Spring, Janz and Glenlea (Fig. 4.2). With RP-HPLC elution profiles, the subunits of each line were identified (Fig. 4.3) and the proportions of various alkylated HMW-GS subunits relative to the total HMW-GS area of each line were calculated (Table 4.2). Among the wheat lines in which the *Glu-B1* retrotransposon was detected, the proportion of Bx subunits ranged from 40.5% to 46.5%, with a mean value of 43.9 ± 1.9%, which is significantly higher than the proportions of Bx subunits in Chinese Spring (Bx7, 37.4%) and Janz (Bx7*, 31.0%). With SE-HPLC analysis, it was shown that with one exception (H45), the %UPP of the lines that overexpress Bx subunits ranged between 42.18% and 55.12%, with a mean value of 49.03 ± 4.44%, which is significantly higher than the %UPP of H45 (30.12%), Chinese Spring (19.62%) and Janz (38.65%) (Table 4.2).

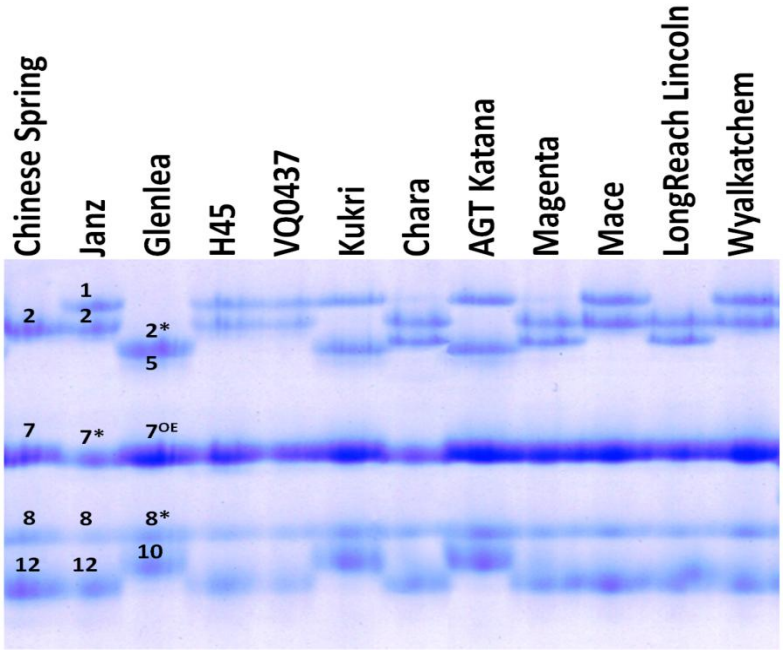

Fig. 4.2 One-dimensional SDS-PAGE of HMW glutenins extracted from flour of Chinese Spring, Janz, Glenlea, H45, VQ0437, Kukri, Chara, AGT Katana, Magenta, Mace, LongReach Lincoln, and Wyalkatchem wheat. 1 and 2* HMW-GS encoded by *Glu-A1a* and *Glu-A1b*, respectively. 7 and 8 HMW-GS encoded by *Glu-B1b*. 7^{OE} and 8* HMW-GS encoded by *Glu-B1al*. 7* and 8 HMW-GS encoded by *Glu-B1u.* 2 and 12 HMW-GS encoded by *Glu-D1a*. 5 and 10 HMW-GS encoded by *Glu-D1d*.

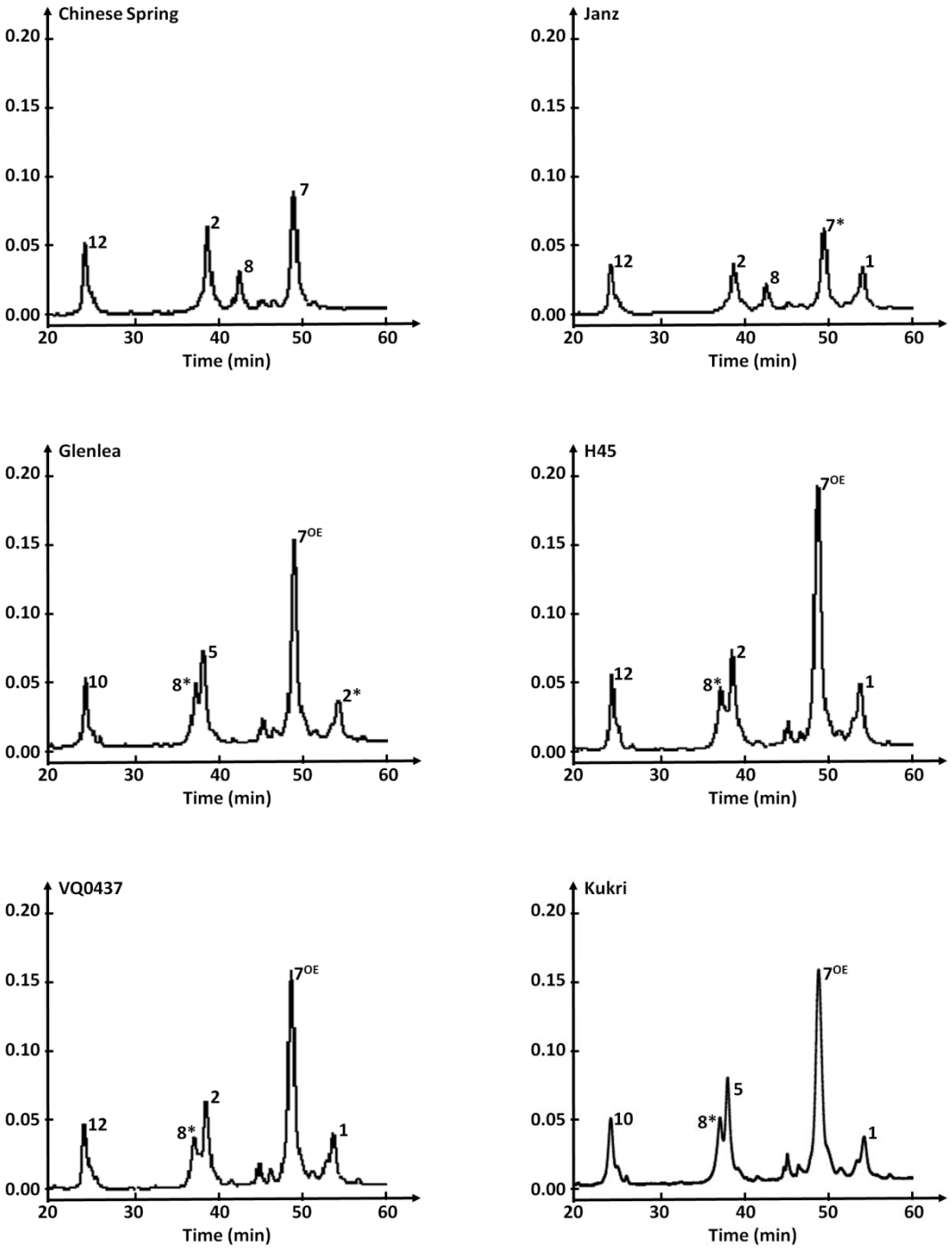

Fig. 4.3 RP-HPLC chromatograms of various wheat lines.

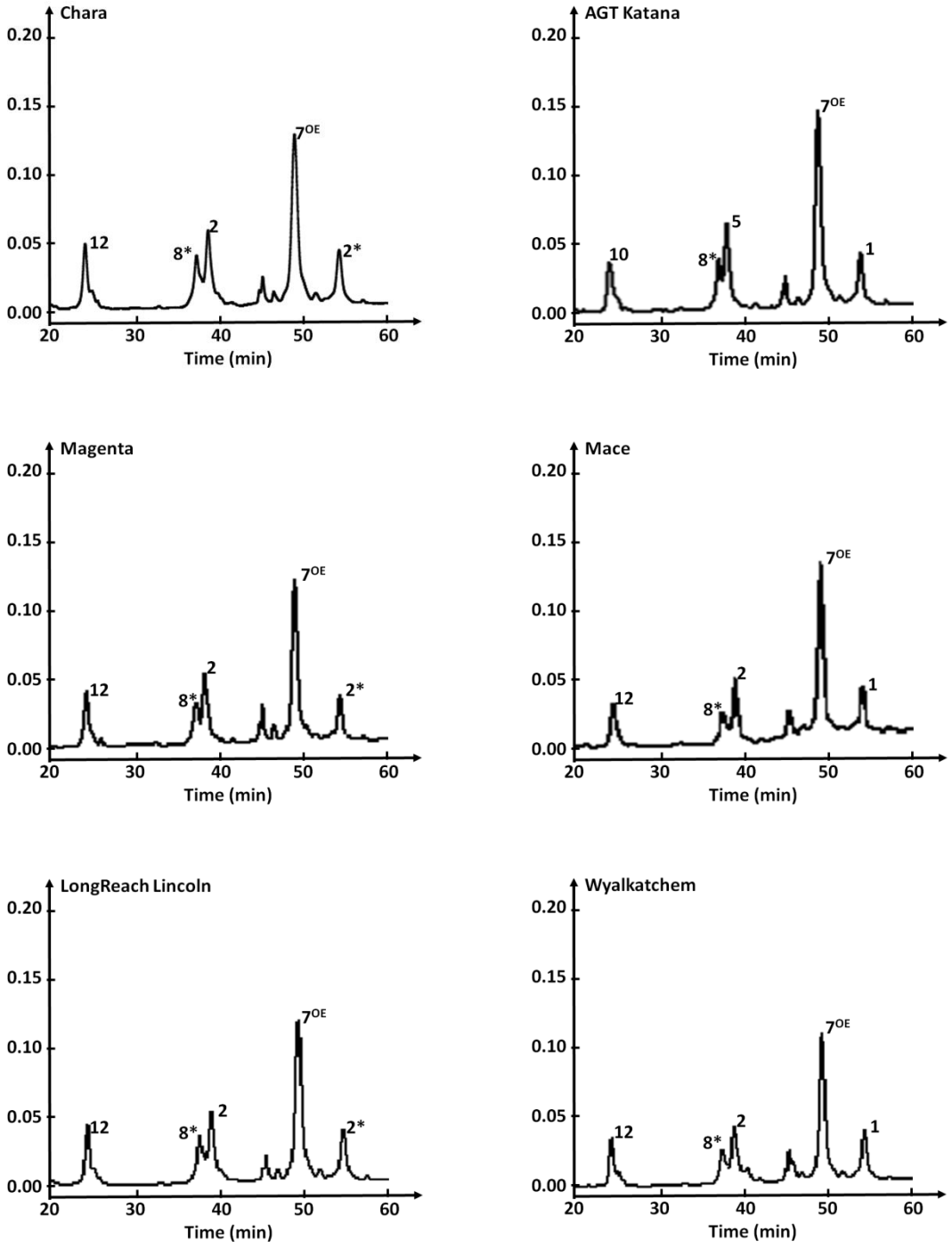

Fig. 4.3 (Cont.) RP-HPLC chromatograms of various wheat lines.

Table 4.2 Quantities of individual alkylated high molecular weight glutenin subunits encoded at the *Glu-A1*, *Glu-B1* and *Glu-D1* loci, expressed as percentages of the total amount of high molecular weight glutenin as assessed by reverse-phase high-performance liquid chromatography, and the percentage of unextractable polymeric protein (UPP) as assessed by size-exclusion high-performance liquid chromatography, for 12 lines of wheat.

Lines	***Glu-A1***		***Glu-B1***				***Glu-D1***				**UPP**
	1x (%)	**2*x (%)**	**7x (%)**	**8*y (%)**	**7*x (%)**	**8y (%)**	**2x (%)**	**12y (%)**	**5x (%)**	**10y (%)**	**(%)**
Chinese Spring			37.4			17.1	25.8	19.7			19.62
Janz	21.8				31.0	10.8	20.8	15.6			38.65
Glenlea		14.6	45.1	11.9					15.3	13.1	55.12
H45	16.7		45.2	12.1			13.8	12.1			30.12
VQ0437	15.6		45.3	11.3			15.3	12.5			44.29
Kukri	12.6		46.5	10.7					18.8	11.5	54.56
Chara		13.7	42.6	12.2			21.7	10.2			48.16
AGT Katana	18.4		44.0	9.9					15.3	12.4	47.76
Magenta		15.8	43.7	9.8			17.5	13.2			49.67
Mace	18.0		44.7	10.6			15.2	11.6			52.70
LongReach Lincoln		16.5	41.6	12.7			15.6	13.7			42.18
Wyalkatchem	21.2		40.5	11.7			14.1	12.4			46.88

4.3.3 Restriction digestion assay on a panel of wheat lines that overexpress Bx subunits

The digested products showed that Kukri, Chara, AGT Katana, Magenta, Mace, LongReach Lincoln and Wyalkatchem all exhibited the same pattern as that of Glenlea and VQ0437. Only H45 has an additional 216 bp fragment and is missing a 135 bp fragment (Fig. 4.4).

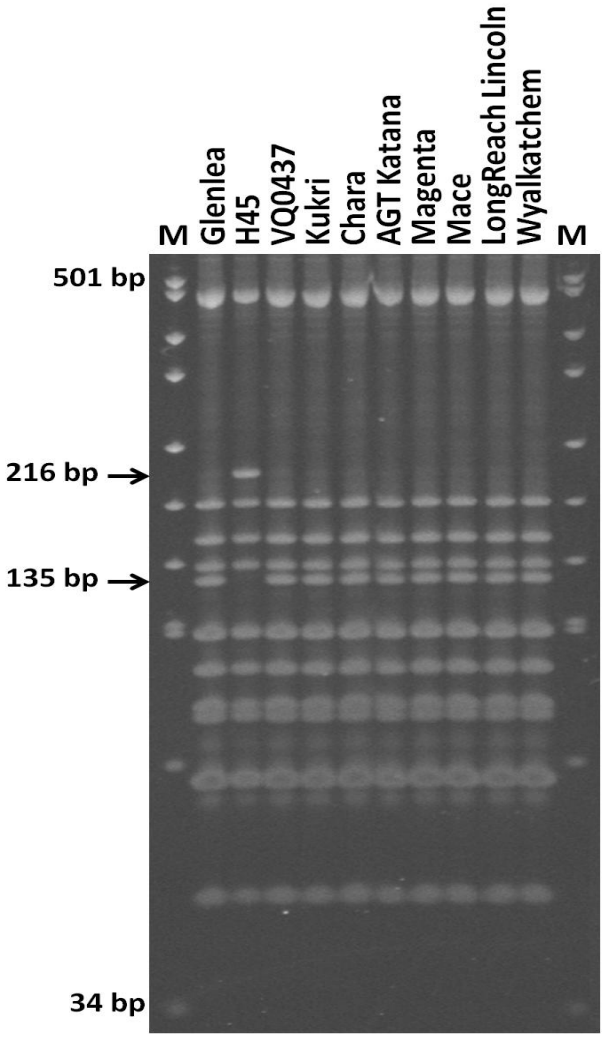

Fig. 4.4 Restriction fragments from *Rsa* I digestion of PCR products of first Bx high-molecular weight glutenin gene of a panel of wheat cultivars, with Glenlea, H45 and wheat line VQ0437 as controls. M: pUC19 DNA/MspI (HpaII) ladder.

4.4 Discussion

4.4.1 Germplasm screening for *Glu-B1br* allele

The *Glu-B1br* allele is of particular interest because its effect on flour and dough properties differs from that of other Bx overexpression alleles. Ancestors of H45 were therefore investigated to attempt to trace the origin of that allele. None of the immediate ancestors of H45 that were assayed here exhibited the amplicons that indicate the presence of retrotransposon junctions (and thus duplication of the Bx gene) within the *Glu-B1* locus (Ragupathy et al. 2008). It was therefore not possible to definitively trace the origin of the *Glu-B1br* allele in this study.

Some other wheat varieties that are thought to carry Bx overexpression alleles were investigated to determine whether any of them also carry *Glu-B1br* allele. With molecular marker assays, SDS-PAGE and RP-HPLC analysis, Glenlea, H45, VQ0437, Kukri, Chara, AGT Katana, Magenta, Mace, LongReach Lincoln and Wyalkatchem were all confirmed to carry a duplication of *Bx* gene and to overexpress Bx subunits. Of these varieties, Glenlea, Kukri, Chara and LongReach Lincoln had been tested with LTR markers by Ragupathy et al. (2008) and/or by Jin et al. (2011) and the results obtained here were consistent with the previously reported results. Contrary to what was observed here, Jin et al. (2011) reported that Wyalkatchem does not carry the gene duplication. This difference can be attributed to heterogeneity within this variety. With SDS-PAGE and RP-HPLC, some plants of Wyalkatchem were found to exhibit patterns expected for the HMW-GS $Bx7^{OE}$ and By8*, while others exhibited patterns expected for Bx13 and By16 (Fig. 4.5 and 4.6). Jin et al. (2011) may have tested a biotype carrying *Glu-B1f* allele, which encodes Bx13 and By16 subunits.

Among the lines for which the Bx gene duplication was detected here, only H45 exhibited the *Glu-B1br* allele pattern when tested with the restriction digest assay.

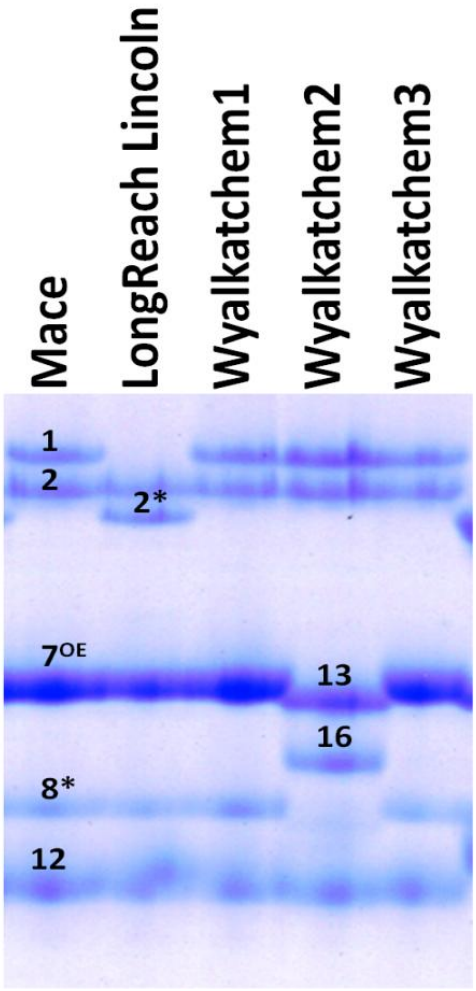

Fig. 4.5 One-dimensional SDS-PAGE of HMW glutenins extracted from flour of single seed of Mace, LongReach Lincoln, three lines of Wyalkatchem from a mixed stock from AWCC. 1 and 2* HMW-GS encoded by *Glu-A1a* and *Glu-A1b*, respectively. 7^{OE} and 8* HMW-GS encoded by *Glu-B1al*.13 and 16 HMW-GS encoded by *Glu-B1f*. 2 and 12 HMW-GS encoded by *Glu-D1a*.

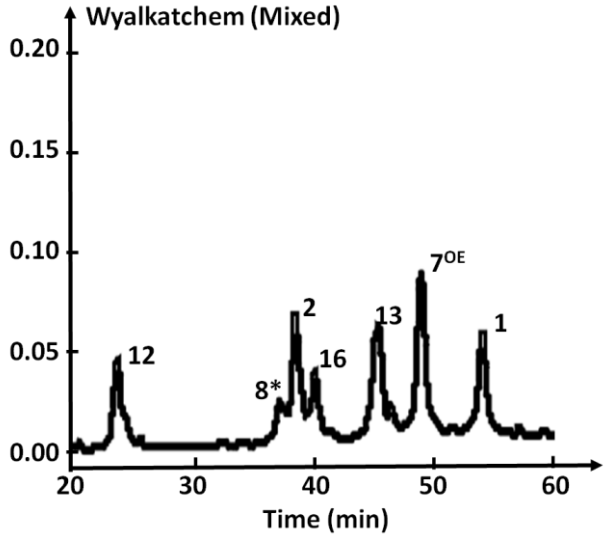

Fig. 4.6 RP-HPLC chromatogram of flour milled from 10 Wyalkatchem grains from AWCC. 1 HMW-GS encoded by *Glu-A1a*. 7^{OE} and 8* HMW-GS encoded by *Glu-B1al*. 13 and 16 HMW-GS encoded by *Glu-B1f.* 2 and 12 HMW-GS encoded by *Glu-D1a*.

4.4.2 Ancestral sources of Bx overexpression alleles

Eagles et al. (2004), Vawser et al. (2004) and Butow et al. (2004) examined the pedigrees of Glenlea and other Bx overexpression varieties and noted that the Argentinean variety Tezanos Pintos Precoz is a common ancestor of most known Bx overexpression lines. They all suggested the variety Klein Universal II as the source of the *Glu-B1al* allele. Vawser et al. (2004) suggested that Americano 44D, also carried that allele, and Ragupathy et al. (2008) later confirmed that Americano 44D has the Bx gene duplication. According to Genetic Resources Information System (GRIS) for Wheat and Triticale, Americano was a landrace from Uruguay, Klein Universal II was a selection from that landrace and Americano 44D and Americano 44 are synonyms for Klein Universal II.

Here, pedigrees of the lines studied in this research were obtained from GRIS and/or the Australian Plant Breeder's Rights database. Glenlea probably inherited its *Glu-B1al* allele

from the Americano landrace *via* Americano 44D, Sin Rival, Sinvalocho, Tezanos Pintos Precos and CB100 (Fig. 4.7). Wyalkatchem could have inherited the overexpression allele from Tezanos Pintos Precoz, which is an ancestor of Machete. Mace and AGT Katana probably obtained their Bx overexpression alleles from Wyalkatchem and Kukri, while Wyalkatchem and Kukri could have inherited their overexpression alleles from Tezanos Pintos Precoz *via* Machete and Kukri, respectively. LongReach Lincoln could have inherited its Bx overexpression allele from Tezanos Pintos Precoz *via* Tobari 66 and Rubric or Otane. In contrast, neither H45 nor Magenta is known to have Tezano Pintos Precoz in its ancestry. H45 has Americano 44D in its ancestry. Magenta does not have Americano 44D in its ancestry but Americano 25E is in the ancestry of both Carmanah and Tammin, the parents of Magenta.

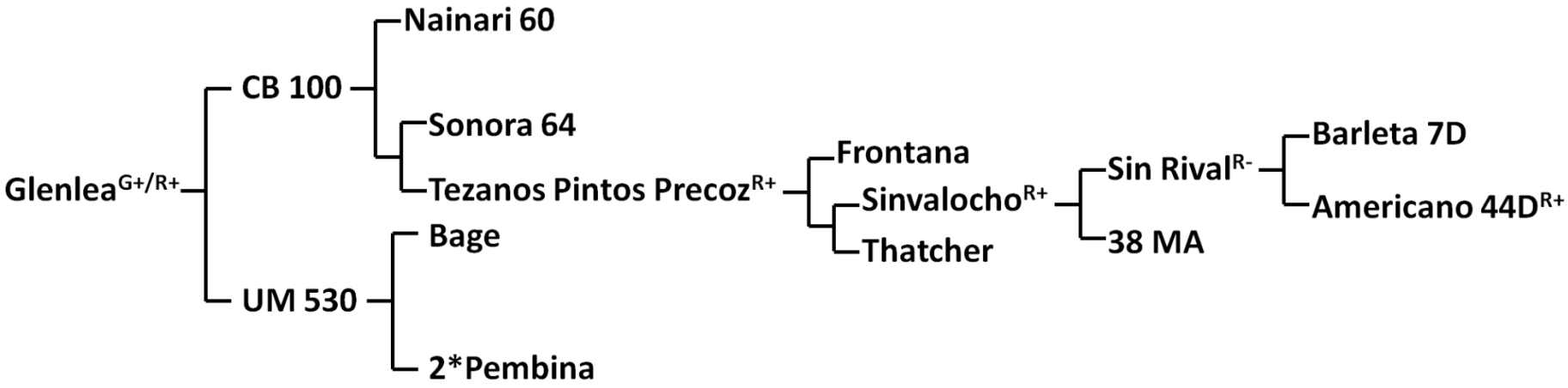

Fig. 4.7 Pedigree of Glenlea. The accessions with detailed pedigrees could be tracked back to the possible accessions carrying the original Bx overexpression allele. Detailed pedigrees not shown for other accessions that unlikely inherited the Bx overexpression allele based on the information provided by GRIS. $^{G+}$ Positive result tested with LTR markers (Ragupathy et al. 2008) in this research, $^{R+/R-}$ Positive/negative result tested with LTR markers (Ragupathy et al. 2008).

According to its variety description (http://pericles.ipaustralia.gov.au/pbr_db/), the pedigree of H45 is B1814//WW15/QT7605. This pedigree is also listed in GRIS, but so is an alternative pedigree, Kalyansona/Bluebird//Anza*3/WW80/3/Olympic*2/Ciano 67. Given that the pedigree of B1814 is Ciano 67/2*Olympic and that of QT7605 is Kalyansona/Bluebird (both according to GRIS), the two versions of the pedigree are very similar, differing only with respect to the directions of crosses and with respect to whether WW15 or an Anza*3/WW80 backcross derivative is a grandparent of H45 (Fig. 4.8 and 4.9). According to GRIS, both WW15 and Anza have the pedigree Lerma Rojo 64//Norin 10/Brevor/3/3*Andes Enano, indicating a very close relationship between WW15 and the Anza backcross derivative. However there is an important difference, in that WW80, the donor parent in the backcrossing, has Tezanos Pintos Precoz in its pedigree (Penjamo 62/4*Gabo 56//Tezanos Pintos Precoz/Nainari 60). If WW80 is an ancestor of H45, then H45 could have inherited its Bx overexpression allele from Tezanos Pintos Precoz *via* WW80. Alternatively, according to either version of the pedigree, H45 might have inherited its Bx overexpression allele from Americano 44D *via* Klein Rendidor, Bluebird and QT6705. In this research, QT6705 could not be tested because it is no longer available. WW80, two Bluebird accessions (Bluebird #3 and Bluebird #4) and a Bluebird/Kalyansona accessions were all tested, and none of them exhibited the Bx gene duplication. As 'Bluebird' is the name of a CIMMYT cross, and not a particular pure line from that cross, it is possible that Bluebird itself was heterogeneous at *Glu-B1*. It therefore seems possible that the *Glu-B1br* allele could have been inherited from Americano 44D *via* Klein Rendidor, Bluebird and QT7605. The breeding line VQ0437, which carries the overexpression allele *Glu-B1bs* allele, probably inherited that allele from its parent CD87, which is known to carry a Bx overexpression allele

(Butow et al. 2003). According to the pedigree of CD87 (Yecora F 70/Tobari F 66//Ciano F 67/Huacamayo/3/Condor, based on personal communication with Dr Howard Eagles), CD87 could have inherited its Bx overexpression allele from Americano 44D *via* Tezanos Pintos Precozor with several other possible intermediaries, or from Klein Universal, or from Americano 25E (Fig. 4.10).

4.4.3 Possible sources that could be tested for the *Glu-B1br* allele

To detect the origin of *Glu-B1br* allele, additional germplasm could be screened with the combination of the two DNA assays used here. More accessions in the two possible pedigrees of H45 (Fig. 4.8 and 4.9) could be tested, such as Klein Rendidor and additional accessions of Bluebird. Among 33 hexaploid accessions that Butow et al. (2004) had identified as carrying a 43 bp indel, 24 were tested with LTR markers by Ragupathy (2008) and 20 of these were confirmed to carry the Bx gene duplication. Across previous studies (Ragupathy et al. 2008; Jin et al. 2011), a total of 57 hexaploid accessions have been found to carry the Bx gene duplication. Without better markers, it would be costly to assay all of these accessions, but further work could start with just few accessions selected from each country (Butow et al. 2004; Ragupathy et al. 2008; Jin et al. 2011), based on pedigrees or the results of phylogenetic studies. Additionally, Americano 25E might be tested to confirm whether it carries the Bx overexpression allele. If the Bx overexpression allele is present in Americano 25E, there will be more possible sources for the *Glu-B1br* allele.

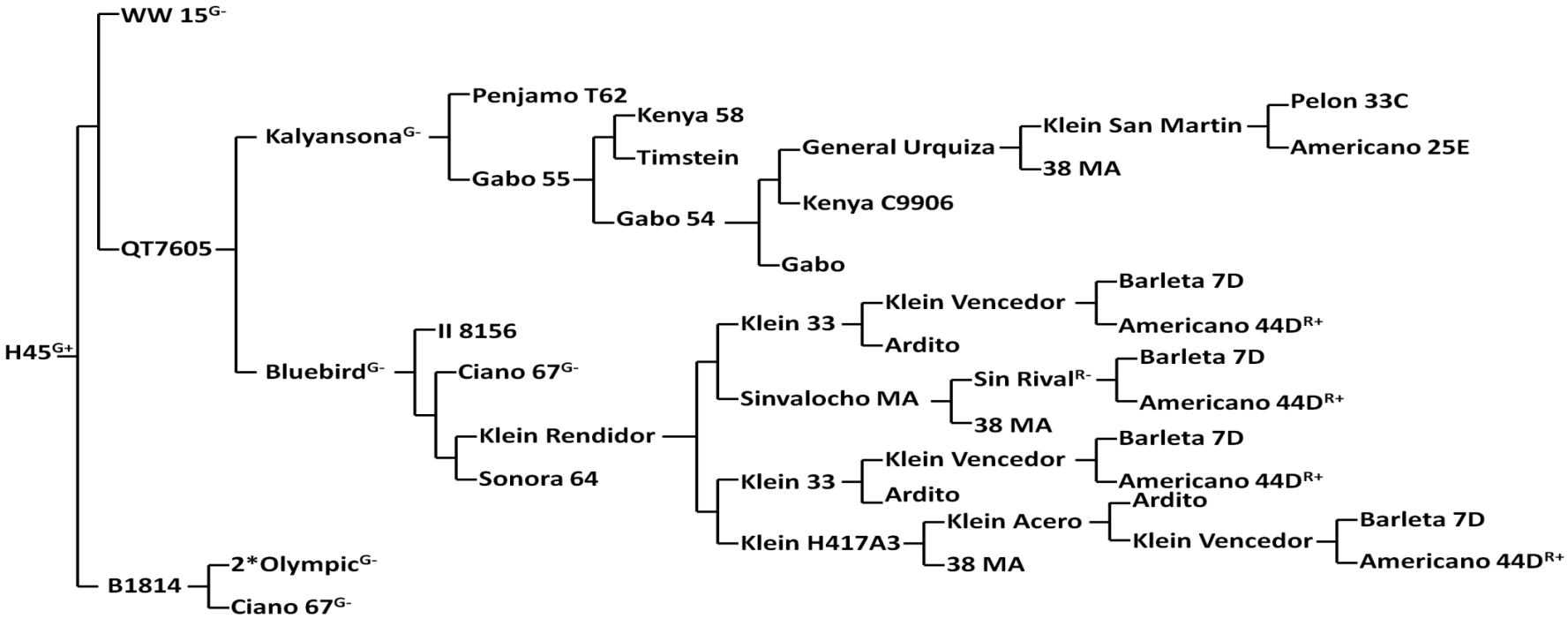

Fig. 4.8 Pedigree of H45: B1814//WW15/QT7605. The accessions with detailed pedigree information could be tracked back to the possible accessions carrying the original Bx overexpression allele. Detailed pedigrees not shown for other accessions that unlikely inherited the Bx overexpression allele based on the information provided by GRIS. $^{G+/G-}$ Positive/negative result tested with LTR markers (Ragupathy et al. 2008) in this research, $^{R+/R-}$ Positive/negative result tested with LTR markers (Ragupathy et al. 2008).

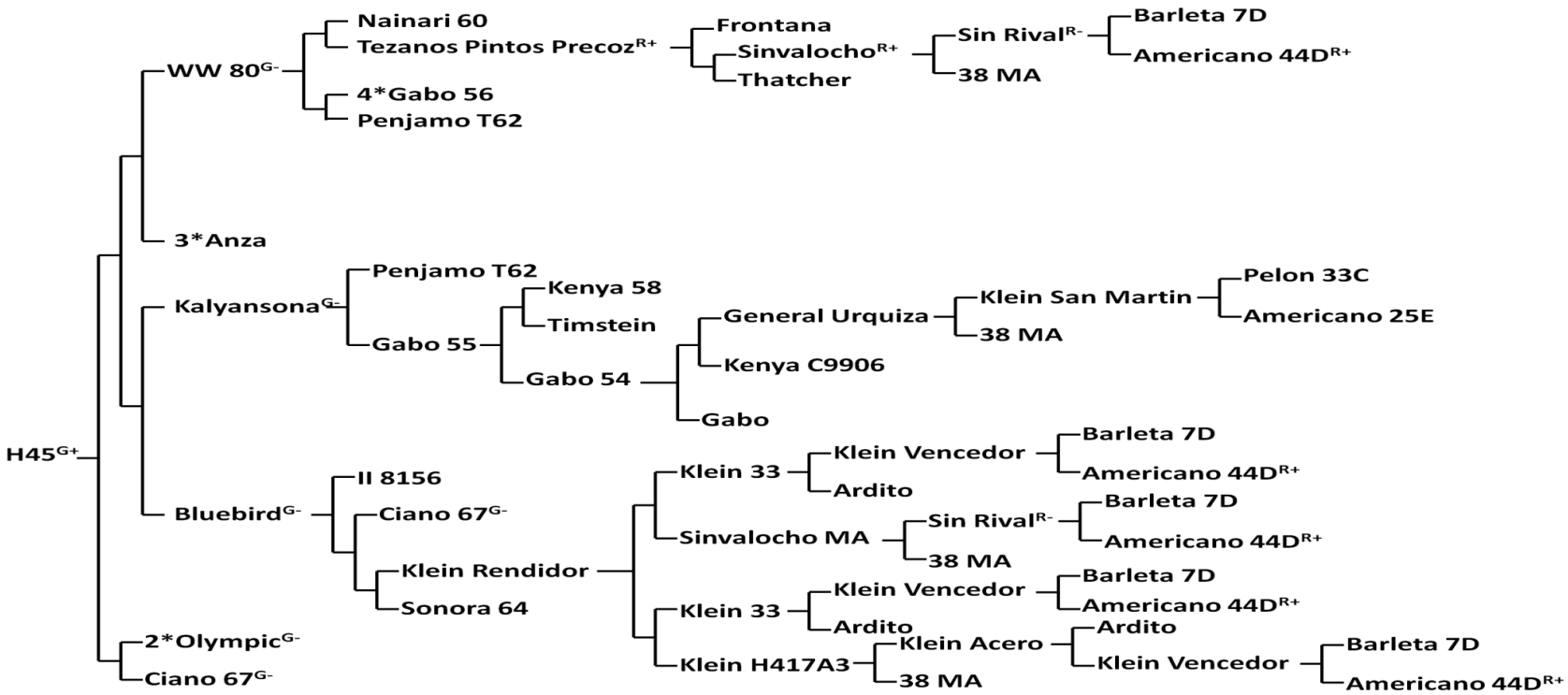

Fig. 4.9 Pedigree of H45: Kalyansona/Bluebird//Anza*3/WW80/3/Olympic*2/Ciano 67. The accessions with detailed pedigree information could be tracked back to the possible accessions carrying the original Bx overexpression allele. Detailed pedigrees not shown for other accessions that unlikely inherited the Bx overexpression allele based on the information provided by GRIS. $^{G+/G-}$ Positive/negative result tested with LTR markers (Ragupathy et al. 2008) in this research, $^{R+/R-}$ Positive/negative result tested with LTR markers (Ragupathy et al. 2008).

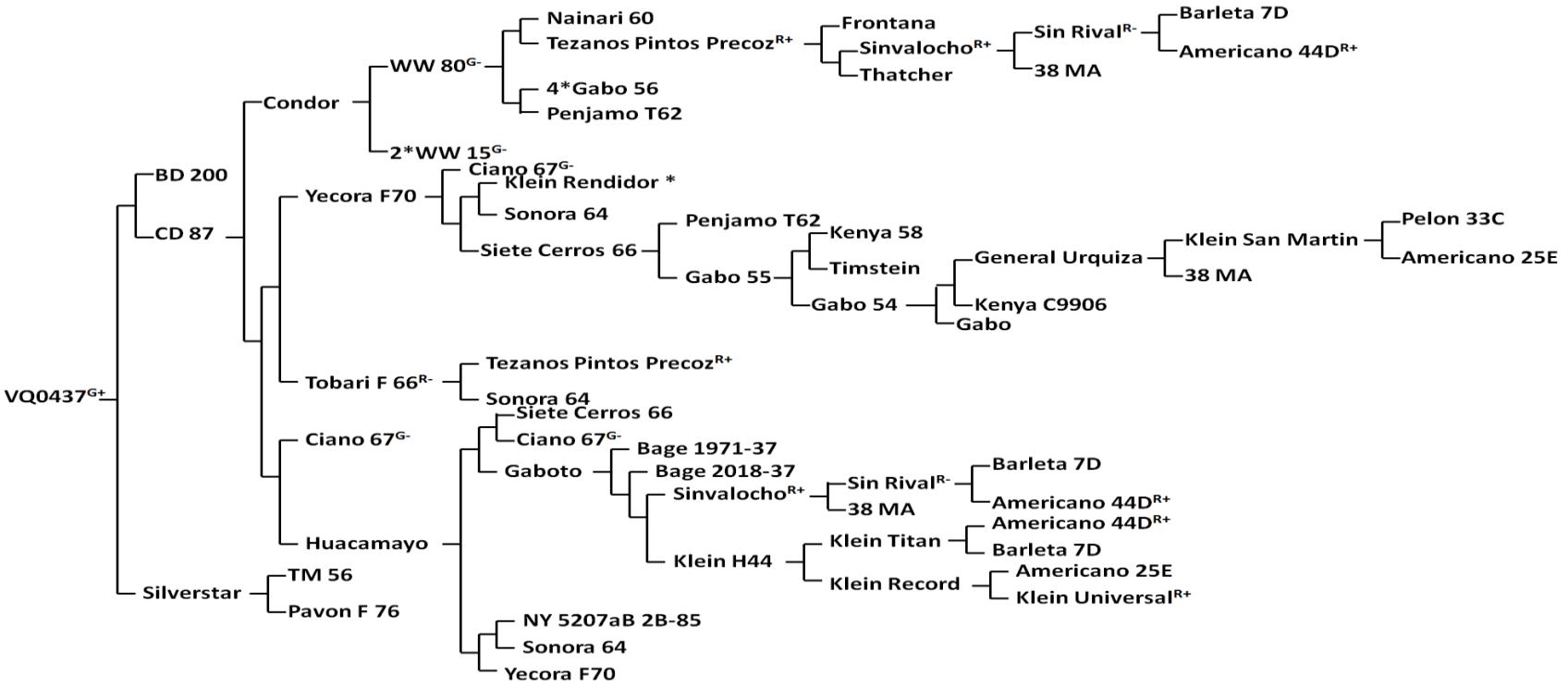

Fig. 4.10 Pedigree of VQ0437. The accessions with detailed pedigrees could be tracked back to the possible accessions carrying the original Bx overexpression allele. *, detailed pedigree of Klein Rendidor was shown in Fig. 4.9. Detailed pedigrees not shown for other accessions that unlikely inherited the Bx overexpression allele based on the information provided by GRIS. $^{G+/G-}$ Positive/negative result tested with LTR markers (Ragupathy et al. 2008) in this research, $^{R+/R-}$ Positive/negative result tested with LTR markers (Ragupathy et al. 2008).

4.4.4 Evolution of Bx overexpression alleles

Given that all three of the known Bx overexpression alleles include two very similar Bx genes and that the same two retrotransposon junctions can be amplified from all three alleles, it seems highly unlikely that these three alleles arose independently from each other. Moreover, the two Bx7^{OE} genes of *Glu-B1al* are identical to each other, and the *Bx7*^{OE} gene of *Glu-B1bs* differs from them by just one synonymous SNP. The *Bx7.1* and *Bx7.2* genes of *Glu-B1br* and the *Bx7.3* genes of *Glu-B1bs* differ from each other and from the *Bx7*^{OE} genes by several SNPs. It seems that *Glu-B1al* is the ancestral form and that each of the other two alleles arose from it *via* the accumulation of point mutations.

Based on the available pedigree information, and the limited number of ancestors that have been genotyped for the presence of a Bx gene duplication, it is not possible to conclude when *Glu-B1br* and *Glu-B1bs* diverged from *Glu-B1al*. Glenlea, VQ0437 and possibly H45 have Tezanos Pintos Precoz as a common ancestor, but it seems unlikely that both *Glu-B1br* and *Glu-B1bs* diverged from *Glu-B1al* within just six or seven cycles of crossing since Tezanos Pintos Precoz. Another possibility is that Americano 44D is the common ancestor and that the alleles diverged as they were inherited through three independent lineages. This explanation seems more likely, because it allows up to 12 cycles of crossing for the *Glu-B1br* allele and 16 cycles of crossing for the *Glu-B1bs* allele for mutations to accumulate. Another alternative is that all three alleles may have been present in the landrace Americano, with each one captured in a different selection from the landrace (possibly Americano 44D, Americano 25E and Klein Universal) and passed down unchanged to the modern varieties and lines.

4.4.5 Possible approaches to detect specific alleles for quality selection in wheat breeding

Although H45 is the only cultivar in which *Glu-B1br* has been detected so far, it is possible that it is present in cultivars that have not been assayed yet, and/or in breeding germplasm in which H45 has been used as a parent. As the *Glu-B1br* allele has unique effects on flour and dough properties, it would be useful for breeding programs to be able to distinguish *Glu-B1br* from other *Glu-B1* alleles. Previously developed markers such as the LTR markers of Ragupathy et al. (2008) should be suitable for distinguishing *Glu-B1br* from alleles with no Bx gene duplication, but cannot distinguish *Glu-B1br* from *Glu-B1al* or *Glu-B1bs*. The restriction digest assay developed here could be applied to specifically detect the *Glu-B1br* allele, but it requires two-step long-range PCR and a restriction digest, making it not particularly convenient for large-scale screening of germplasm or for routine use in wheat breeding. Alternatively, if small samples of flour are available, the SDS-sedimentation test and/or SE-HPLC analysis could be used for selection, as their results are consistent with those of the restriction digest assay.

Chapter 5

Efforts Made to Develop Alternative Markers for the *Glu-B1br* Allele

5.1 Introduction

As reported in Chapter 3 of this thesis, a new Bx overexpression allele, designated *Glu-B1br* (*Bx7.1+Bx7.2+By8**), was discovered in the wheat cultivar H45. Due to the unique effects of this allele on dough properties, compared to *Glu-B1al* and *Glu-B1bs* alleles, it would be useful to have a molecular marker to distinguish this particular allele from other alleles in germplasm screening and marker-assisted selection. A restriction digest assay was designed and reported to distinguish *Glu-B1br* allele from other Bx overexpression alleles, but this assay is not suitable for large scale screening. An alternative marker for *Glu-B1br* allele is needed.

Polymerase chain reaction (PCR)-based molecular markers have been developed to distinguish different HMW-GS alleles (D'Ovidio and Anderson 1994; Smith et al. 1994; de Bustos et al. 2001; Ma et al. 2003; Ishikawa and Nakamura 2007; Liu et al. 2008). These markers were designed based on single nuclear polymorphisms (SNPs), insertions or deletions within or close to the coding regions of HMW-GS genes. Relative to the Bx genes of the *Glu-B1al* allele, the Bx genes of the *Glu-B1br* allele each have 4 SNPs. Unfortunately, these SNPs are not good targets for primer design because they are within repetitive regions of the genes and because of sequence similarity among *Glu-B1* alleles and genes within alleles, and sequence similarity of *Glu-B1 Bx* genes with other HMW-GS genes.

This chapter describes efforts that have been made to detect polymorphisms that are

associated with the *Glu-B1br* allele and that could be used as a basis for marker assays.

5.2 Materials and Methods

5.2.1 Plant materials

The plant materials used were the Australian cultivar H45 (pedigree B1814//WW15/QT7605), the Australian breeding line VQ0437 (pedigree BD200/CD87//Silverstar) and a random sample of 209 $F_{2:3}$ families derived from a cross between H45 and VQ0437. The cultivars Chinese Spring, Janz, and Glenlea were used as controls. Seeds of the cultivars were obtained from the Australian Winter Cereals Collection (Tamworth, NSW, Australia). Seeds of VQ0437 were kindly provided by Dr Russell Eastwood of Australian Grain Technologies Pty. Ltd. (Horsham, Vic, Australia). The DNA of Chinese Spring nullisomic-tetrasomic lines N1AT1B, N1AT1D, N1BT1A, N1BT1D, N1DT1A, and N1DT1B was used to identify the chromosomal location of the molecular markers.

5.2.2 Cloning and sequencing left and right junctions of a long terminal repeat (LTR) retroelement at *Glu-B1* locus

Given that the left- and right-junctions of a long terminal repeat (LTR) retroelement (Ragupathy et al. 2008) had already been demonstrated to be specific to alleles that carry a Bx gene duplication, these regions were amplified from genomic DNA of H45, VQ0437 and Glenlea, according to method described by Ragupathy et al. (2008). The amplified products were recovered from 1.0% agarose gels, cloned into the pGEM-T vector, transformed into *E. coli* JM109 competent cells and sequenced commercially (Australian Genome Research Facility). Both amplification and cloning were carried out in duplicate to minimize the possibility of errors present in amplification and sequencing. Sequence analyses were

performed using MEGA (Kumar et al. 2004), BLAST and ClustalW2 from the NCBI (http://www.ncbi.nlm.nih.gov/Tools/) and EBI (http://www.ebi.ac.uk/Tools/sequence.html).

5.2.3 High resolution melting analysis for detection of DNA polymorphism in introns of putative protein kinase gene at *Glu-B1* locus and with ISBP markers in TaBAC1215C06

The TaBAC1215C06 sequence (EU157184) containing the *Glu-B1al* allele was obtained from GenBank (accession number: EU157184). This sequence contains a putative protein kinase gene, in addition to a gene encoding the By8* HMW-GS subunit and duplicate copies of the $Bx7^{OE}$ gene. Four pairs of primers (Table 5.1) were designed to amplify small intron regions within the putative protein kinase gene. PCR products were amplified from genomic DNA of Glenlea, H45, VQ0437 and F_2-3, a progeny of H45 and VQ0437 that had been confirmed to carry *Glu-B1br* allele by testing with the restriction digest assay, with Chinese Spring and its nullisomic-tetrasomic lines N1AT1B, N1AT1D, N1BT1A, N1BT1D, N1DT1A, and N1DT1B and water as controls. The PCR reaction mixture was carried out using 1× Roche LightCycler® 480 Master Mix, 2 mM $MgCl_2$, 200 nM of each primer, 50 ng genomic DNA and PCR grade water in a volume of 12.5 μl. All PCR reactions were conducted in triplicate. The PCR amplification was conducted by a touchdown PCR protocol (Table 5.1) and high resolution melting analysis was performed on the LightCycler® 480 Real-Time PCR System (Roche Applied Science, Australia). The PCR products were denatured at 95 ℃ for 30 s, followed by pre-melting at 52 ℃ (for PPK1, PPK2 and PPK3; 45 ℃ for PPK4) for 30 s, and melt at a ramp of 52 ℃ (for PPK1, PPK2 and PPK3; 45 ℃ for PPK4) to 95 ℃ with 25 acquisitions per ℃, and cool to 37 ℃ for 1 s.

Insertion site-based polymorphism (ISBP) markers were designed using *IsbpFinder* program

provided by Dr Etienne Paux (Genetics, Diversity & Ecophysiology of Cereals, INRA) (Paux et al. 2010) and the DNA sequence of TaBAC1215C06 (EU157184). Fifteen ISBP primer pairs with high confidence scores (Table 5.2) were used to amplify insertion-site-containing regions of TaBAC1215C06. PCR products were amplified as described above from the same samples. The PCR amplification was conducted by a touchdown PCR protocol, starting with one cycle at 95 ℃ for 10 min, followed by 10 cycles of 94 ℃ for 30 s, 64 ℃ for 30 s, with 1 ℃ decrease per cycle, and 72 ℃ for 30s, then 40 cycles of 95 ℃ for 30 s, 54 ℃ for 30 s, and 72 ℃ for 30s, and a final extension step at 72 ℃ for 10 min. High resolution melting (HRM) analysis was performed on the LightCycle® 480 Real-Time PCR System (Roche Applied Science, Australia). The PCR products were denatured at 95 ℃ for 30 s, followed by pre-melting at 51 ℃ for 30 s, and melt at a ramp of 51 ℃ to 95 ℃ with 25 acquisitions per ℃, and cool to 37 ℃ for 1 s.

The fluorescence data were HRM curves analysed with the Gene Scanning analysis mode of the LightCycler® 480 software (Roche Applied Science, Australia) and visually scored, then compared with the PCR products separated on 2% agarose TAE gel. The PCR products for which the HRM curves for H45 looked different from those for Glenlea and/or VQ0437 were commercially sequenced (Australian Genome Research Facility).

Table 5.1 Primers designed to amplify small intron regions of a putative protein kinase gene based on the DNA sequence of TaBAC1215C06 (EU157184).

Primers	Sequence (5'-3')	Amplicon size (bp)	PCR protocol
PPK-1	F: GGTGCTGCTGCTCATGGATT R: GATGTCTCTTGCCCAGCCTC	196	one cycle at 95 ℃ for 10 min, followed by 10 cycles of 95 ℃ for 30 s, 65 ℃ for 30 s, with 1 ℃ decrease per cycle, and 72 ℃ for 30s, then 40 cycles of 95 ℃ for 30 s, 55 ℃ for 30 s, and 72 ℃ for 30s, and a final extension step at 72 ℃ for 10 min
PPK-2	F: TTAGCCTCCTTCATCACCCA R: GCCTCTGGCAGCATCAACT	300	
PPK-3	F: CGACACCACGAAACCAACTC R: GGACGACTGCTTGCTTCTTCT	403	
PPK-4	F: CTACATACAGCAGCAACAC R: GCAGTAACCGAGCAAG	169	one cycle at 95 ℃ for 10 min, followed by 10 cycles of 95 ℃ for 30 s, 58 ℃ for 30 s, with 1 ℃ decrease per cycle, and 72 ℃ for 30s, then 40 cycles of 95 ℃ for 30 s, 48 ℃ for 30 s, and 72 ℃ for 30s, and a final extension step at 72 ℃ for 10 min

Table 5.2 Insertion site-based polymorphism (ISBP) markers within TaBAC1215C06 designed by *IsbpFinder* program.

Primers	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Amplicon size (bp)
ISBP- 2339	GGTCAAACGGTCAAAACCAC	TCCGTTCCATAATATAAGAGCGT	174
ISBP- 2445	CCGTTCCATAATATAAGAGCAGC	CTCGTTTTGAATCGGTCCAT	118
ISBP- 3565	CCGGTTTTGAAGTTGAAGGA	ATCTGTGCTAGAAGCGCCAT	286
ISBP- 5194	TAGCCACCGACATGGAAAAC	CATTGGATTAAGCATGAAACGA	227
ISBP- 11765	ACTCGCGAGCAAATTCTTGT	ACTCCTTCCGTCCCAAAATA	166
ISBP- 11868	CCCTCTGTCCCAAAATAAGTGA	TATCCTTGAAGAGTGGCGCT	166
ISBP- 14667	ATTGAAAGCAGGGAGGGAGT	CAAGTTTGAATTTCCCGCAT	291
ISBP- 18254	CCCACACACCTAGTGCCATA	TCGCTTATCCCTCTCGTGTT	282
ISBP- 24694	CCCACACACCTAGTGCCATA	TGAAACCTTTGGCCAGTCAT	281
ISBP- 35718	CAAGAAACGACGGGAAAAGA	TGCTTGGGTTTTGAATTTCC	288
ISBP- 38013	TATTCGAGAGGGAGTGGTGG	CTAGCGTCAGTGGGCTTGTT	284
ISBP- 41606	GTAGCGCCTTACTGGTAGCG	CCAAACACTGCAACCACAAC	201
ISBP- 42211	TGAAGACAATCTCCAAGCCC	CCGGTCGACTTAACTCCAGA	179
ISBP- 43016	GGTAGCGAAATAGCACCCTG	TGCATCTCCATGGATAGATCATT	165
ISBP- 44295	TTCTTCATCGCACATTGAGG	TCACGACTAGCCATGTCAGC	251

5.2.4 SSR genotyping analysis associated with UPP distribution

Eight-seven SSR markers that had previously been specifically assigned to wheat chromosome 1B (Somers et al. 2004; Sourdille et al. 2004; Sourdille et al. 2005; Hayden et al. 2006) were assayed for polymorphism between H45 and VQ0437. Those that were found to be polymorphic were assessed on the $F_{2:3}$ population of H45/VQ0437. The SSR assays were conducted using the Multiplex-Ready PCR Technology as described by Hayden et al. (2008). Semi-automated SSR allele scoring was carried out with GeneMapper v4.0 software (Applied Biosystems). Marker regression analysis between SSR genotypes and UPP phenotypic values was conducted with MapManager QTXb20 for Windows (Manly et al. 2001).

5.3 Results

5.3.1 Cloning and sequencing left and right junctions of a long terminal repeat (LTR) retroelement at *Glu-B1* locus

Regions including left and right junctions of a long terminal repeat (LTR) retroelement in the Bx overexpression alleles were cloned from H45 and VQ0437, with the same regions from Glenlea used as controls (Fig. 5.1). The sequences of these regions in H45 and VQ0437 were found to be identical to those of the corresponding regions in Glenlea (TaBAC1215C06).

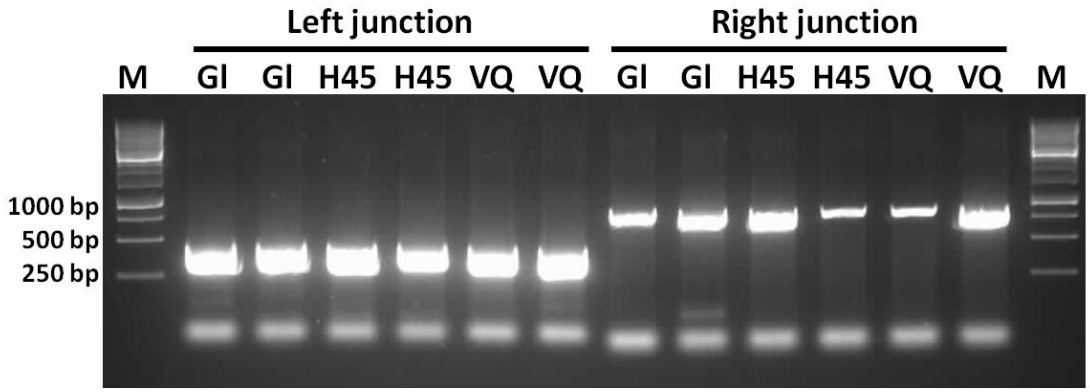

Fig. 5.1 PCR products amplified from genomic DNA of H45, VQ0437 (VQ), Glenlea (Gl; $Bx7^{OE}$) wheat using primers designed at the left and right junctions of a retroelement between duplicate copies of the Bx gene in the *Glu-B1al* allele of Glenlea. M: GeneRuler^{TM} 1 kb DNA ladder.

5.3.2 High resolution melting analysis for detection of DNA polymorphism in introns of putative protein kinase gene at *Glu-B1* locus

Each of the four primer pairs that were designed to flank introns in a putative protein kinase gene within the *Glu-B1al* allele amplified a product of approximately the expected size (Fig. 5.2). According to the results obtained for the nulli-tetrasomic lines, none of these products seem to be specific to chromosome 1B. A second smaller product that was amplified with the PPK-3 primers seems to be specific to chromosome 1A. The HRM curves of the PCR products amplified from genomic DNA of wheat lines carrying *Glu-B1br* allele were very similar to those from PCR products amplified from genomic DNA of wheat lines carrying other Bx overexpression alleles (Fig. 5.2), indicating that HRM analysis could not be used to detect DNA polymorphism among Glenlea, H45 and VQ0437 in the introns of putative kinase gene.

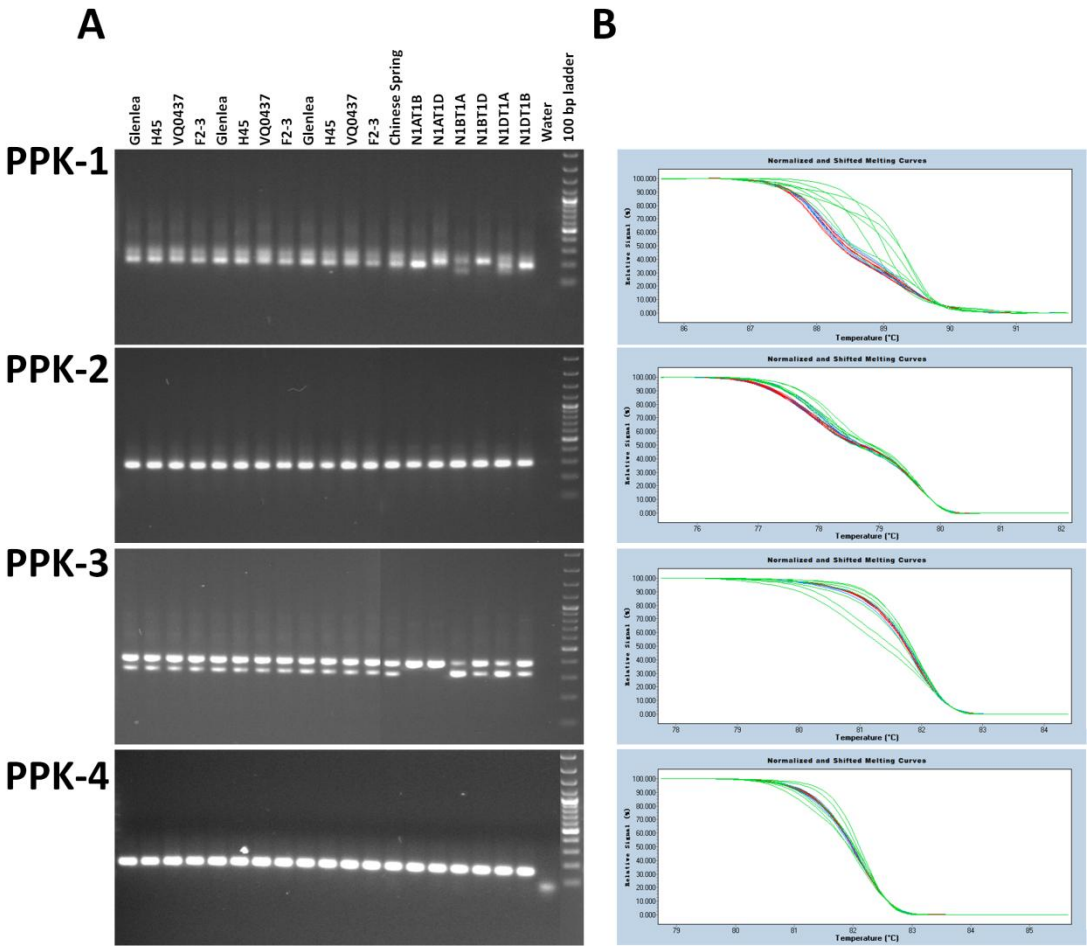

Fig. 5.2 DNA polymorphism analysis in intron regions of putative protein kinase gene at *Glu-B1* locus. A. PCR products amplified with PPK primers from genomic DNA of Glenlea, H45, VQ0437 and a progeny of H45 and VQ0437 F_2-3, with Chinese Spring and its nullisomic-tetrasomic lines N1AT1B, N1AT1D, N1BT1A, N1BT1D, N1DT1A, and N1DT1B and water as controls. B. High-resolution melting curves for these PCR products, with Glenlea and VQ0437 marked in blue, H45 and F_2-3 marked in red and Chinese Spring and its nullisomic-tetrasomic lines marked in green.

5.3.3 High resolution melting analysis for detection of DNA polymorphism with ISBP markers in TaBAC1215C06

With five of the ISBP primer pairs, there was no evidence of specificity to chromosomes 1B (Fig. 5.3A) and all of the samples had very similar melting curves (Fig. 5.3B). With one of these primer pairs (Fig. 5.3A3), PCR failed to amplify any distinct products from three of the DNA samples.

With seven other ISBP primer pairs, there was evidence of specificity to chromosome 1B and of polymorphism between Chinese Spring and the Bx overexpression lines (Fig. 5.4A), but no clear polymorphism among the Bx overexpression lines (Fig. 5.4B). With two of these primer pairs (Fig. 5.4A1 and A2), PCR failed to amplify any distinct products from three of the DNA samples. With another two of these primer pairs (Fig. 5.4A4 and A6) the amplicons of the nullisomic-tetrasomic lines N1BT1A and N1BT1D of Chinese Spring were fainter than those other PCR products, and gave different melting curves from others (Fig 5.4B4 and B6). With another two of these primer pairs (Fig. 5.4A5 and A7) amplicons of the nullisomic-tetrasomic lines N1BT1A and N1BT1D of Chinese Spring were longer than those other PCR products, and had slightly different HRM curves (indicated by arrows in Fig. 5.4B5 and B7).

With the remaining three ISBP primer pairs, there was evidence of specificity to chromosome 1B and there were slight differences in the melting curves between H45 and Glenlea (Fig. 5.5).With one of these primer pairs, the melting curves of H45 and F_2-3 differ slightly from those from the other lines (Fig 5.5B1). With another primer pair, the longer of two PCR products was specific to chromosome 1B (Fig. 5.5A2) and the nulli-tetrasomic lines that lack chromosome 1B had distinct curve shapes. With the other primer pair, melting curves of PCR

products amplified from Glenlea seemed different from the other curves (Fig. 5.5B3). Given the apparent specificity and/or distinct melting curves of amplicons from these three ISBP primer pairs, the amplicons obtained from genomic DNA of Glenlea and H45 were commercially sequenced. None of the amplicons from H45 were found differ in sequence from those from Glenlea (TaBAC1215C06).

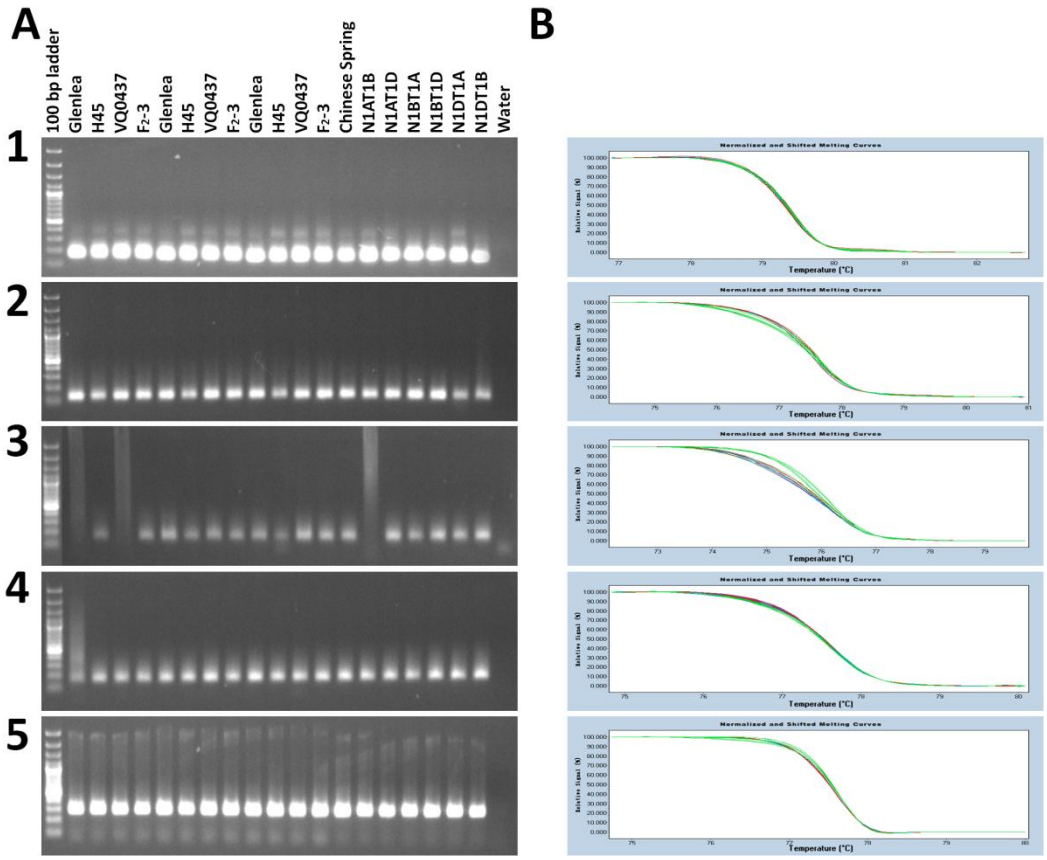

Fig. 5.3 DNA polymorphism analysis with insertion site-based polymorphism markers designed based on sequence of TaBAC1215C06 (EU157184). A. PCR products amplified from genomic DNA of Glenlea, H45, VQ0437 and a progeny of H45 and VQ0437 F_2-3, with Chinese Spring and its nullisomic-tetrasomic lines N1AT1B, N1AT1D, N1BT1A, N1BT1D, N1DT1A, and N1DT1B and water as controls. 1. PCR products amplified with ISBP-2339. 2. PCR products amplified with ISBP-2445. 3. PCR products amplified with ISBP-11765. 4. PCR products amplified with ISBP-11868. 5. PCR products amplified with ISBP-18254. B. High-resolution melting curves for these PCR products, with Glenlea and VQ0437 marked in blue, H45 and F_2-3 marked in red and Chinese Spring and its nullisomic-tetrasomic lines marked in green.

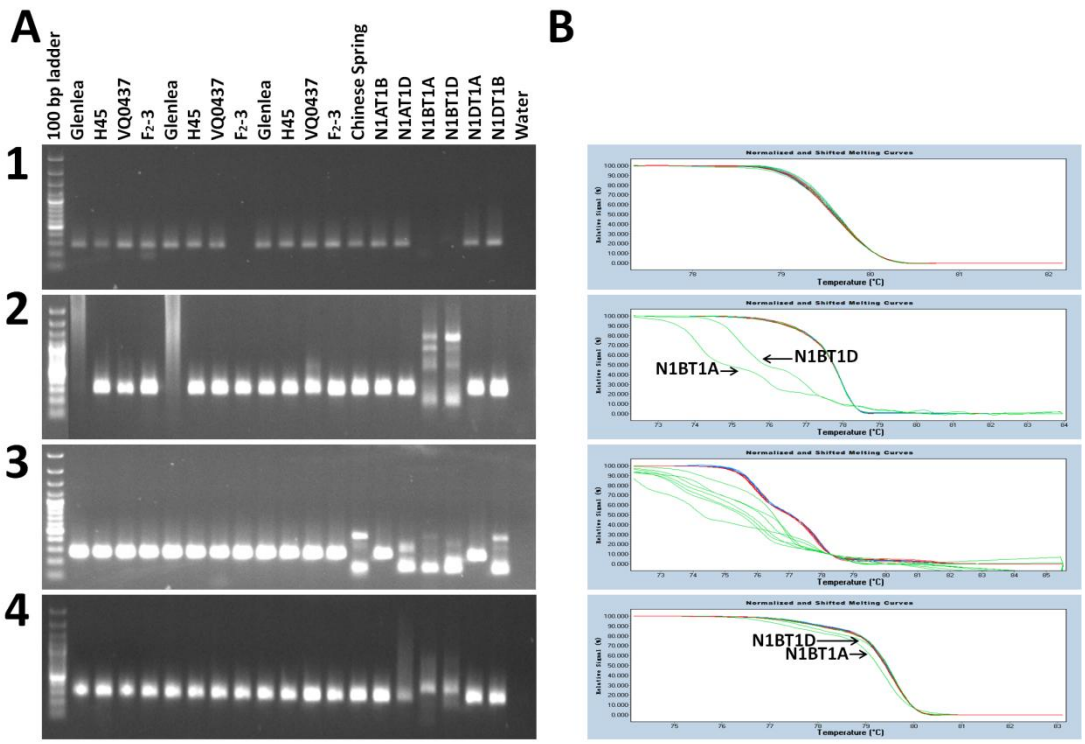

Fig. 5.4 DNA polymorphism analysis with insertion site-based polymorphism markers designed based on sequence of TaBAC1215C06 (EU157184). A. PCR products amplified from genomic DNA of Glenlea, H45, VQ0437 and a progeny of H45 and VQ0437 F_2-3, with Chinese Spring and its nullisomic-tetrasomic lines N1AT1B, N1AT1D, N1BT1A, N1BT1D, N1DT1A, and N1DT1B and water as controls. 1. PCR products amplified with ISBP-3565. 2. PCR products amplified with ISBP-14667. 3. PCR products amplified with ISBP-24694. 4. PCR products amplified with ISBP-35718. B. High-resolution melting curves for these PCR products, with Glenlea and VQ0437 marked in blue, H45 and F_2-3 marked in red and Chinese Spring and its nullisomic-tetrasomic lines marked in green.

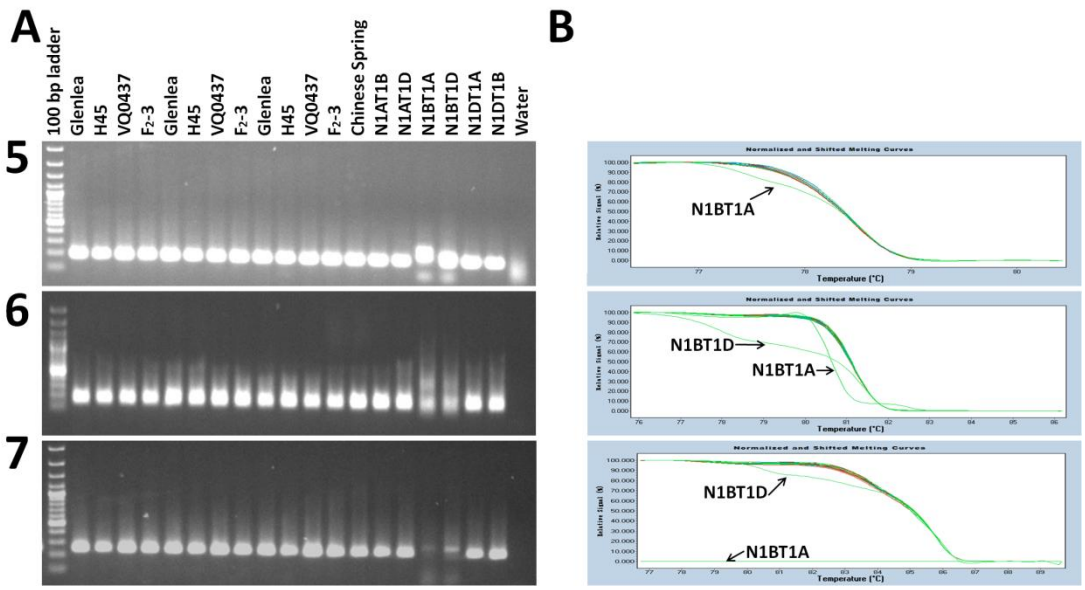

Fig. 5.4 (Cont.) DNA polymorphism analysis with insertion site-based polymorphism markers designed based on sequence of TaBAC1215C06 (EU157184). A. PCR products amplified from genomic DNA of Glenlea, H45, VQ0437 and a progeny of H45 and VQ0437 F_2-3, with Chinese Spring and its nullisomic-tetrasomic lines N1AT1B, N1AT1D, N1BT1A, N1BT1D, N1DT1A, and N1DT1B and water as controls. 5. PCR products amplified with ISBP-41606. 6. PCR products amplified with ISBP-43016. 7. PCR products amplified with ISBP-44295. B. High-resolution melting curves for these PCR products, with Glenlea and VQ0437 marked in blue, H45 and F_2-3 marked in red and Chinese Spring and its nullisomic-tetrasomic lines marked in green.

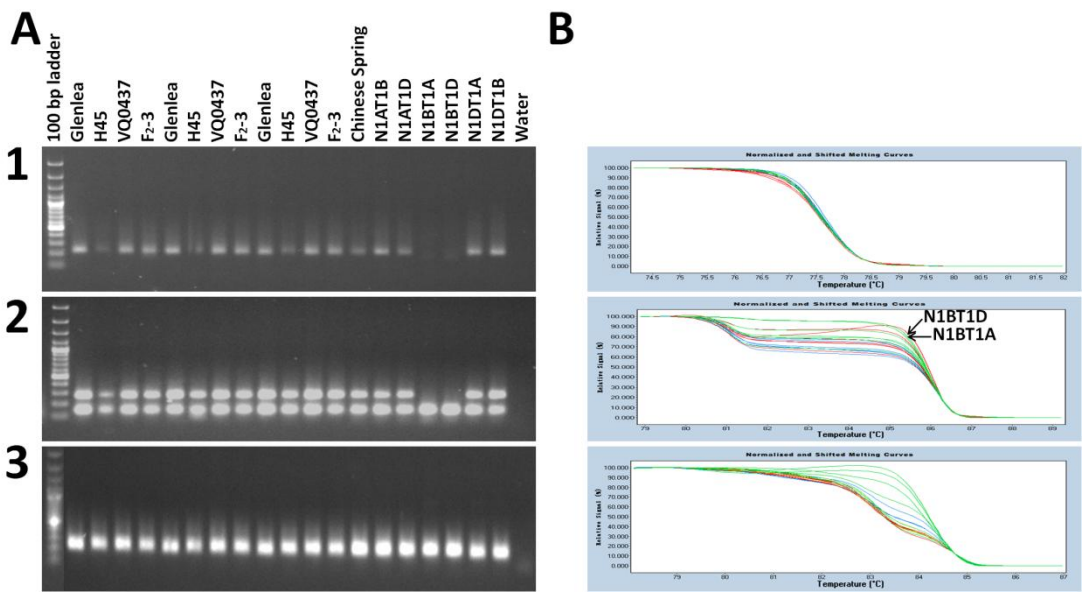

Fig. 5.5 DNA polymorphism analysis with insertion site-based polymorphism markers designed based on sequence of TaBAC1215C06 (EU157184). A. PCR products amplified from genomic DNA of Glenlea, H45, VQ0437 and a progeny of H45 and VQ0437 F_2-3, with Chinese Spring and its nullisomic-tetrasomic lines N1AT1B, N1AT1D, N1BT1A, N1BT1D, N1DT1A, and N1DT1B and water as controls. 1. PCR products amplified with ISBP-5194. 2. PCR products amplified with ISBP-38013. 3. PCR products amplified with ISBP-42211. B. High-resolution melting curves for these PCR products, with Glenlea and VQ0437 marked in blue, H45 and F_2-3 marked in red and Chinese Spring and its nullisomic-tetrasomic lines marked in green.

5.3.4 SSR genotyping analysis and marker regression analysis

Of 87 SSR markers tested, 13 markers detected length polymorphisms between H45 and VQ0437 (Table 5.3). Eight of the markers were grouped into three linkage groups (Fig. 5.6) and the other five markers (barc0174, cfd0059, gdm0028, gdm0126 and wmc0113) were not found to be linked with any other SSR markers. The SSR marker with the strongest association with UPP was gdm0028, which accounted for 14% variation of the UPP values among F_2 progeny of H45 and VQ0437 (P=0.05). Among 20 H45/VQ0437 progeny that were fixed for *Glu-B1br* (as detected by the restriction digest assay reported in Chapter 3 of this thesis), only 15 were fixed for the H45 genotype at gdm0028. The other five exhibited both the H45 and VQ0437 alleles. Similarly, among 20 H45/VQ0437 progeny that were fixed for the VQ0437 allele at *Glu-B1bs* only eight were fixed for the VQ0437 allele at gdm0028, while one had the same genotype as H45, nine exhibited both alleles, and two could not be scored.

Table 5.3 Regression analysis between SSR markers and unextractable polymeric protein among F_2 progeny of H45 and VQ0437.

Linkage Group	Marker	LRS	UPP explained (%)	P value
Unlinked	barc0174	2.9	1	0.09
Unlinked	cfd0059	6.7	3	0.01
Unlinked	gdm0028	30.7	14	0.00
Unlinked	gdm0126	0.1	0	0.82
Unlinked	wmc0113	3.0	1	0.08
Group 1	wmc0419	4.8	3	0.03
Group 1	stm0511tgag	1.9	1	0.16
Group 1	cfa2129	2.1	1	0.14
Group 1	cfd0048	1.0	1	0.32
Group 2	stm0737tcac	7.4	4	0.01
Group 2	gwm0818	0.2	0	0.68
Group 3	barc0131	2.0	1	0.16
Group 3	cfd0002	0.0	0	0.85

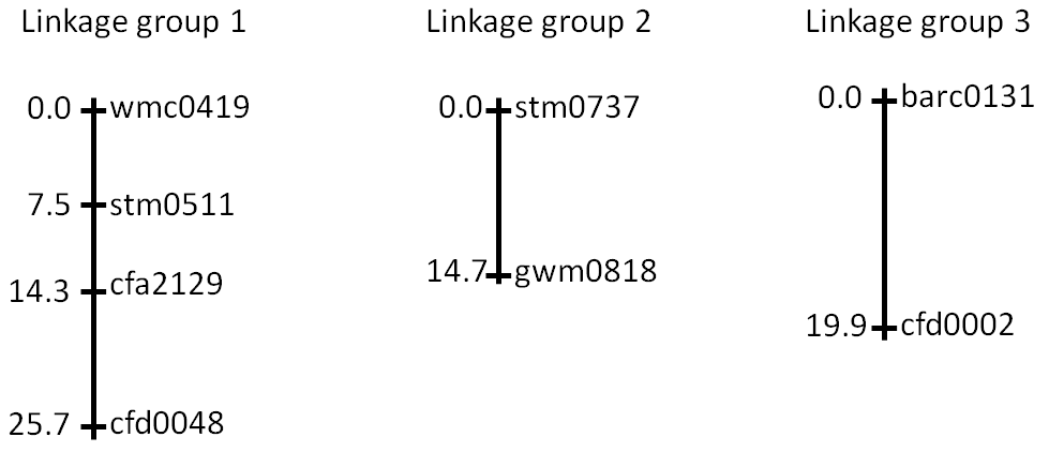

Fig. 5.6 Linkage groups of H45/VQ0437 population with eight SSR markers on Chromosome 1B, with genetic distances shown in cM.

5.4 Discussion

5.4.1 DNA polymorphism targeted for marker design to distinguish the *Glu-B1br* allele

As high molecular weight glutenin genes contain no introns and have highly repetitive sequences, the DNA polymorphism within the Bx genes of H45 cannot easily be targeted to design markers to distinguish the *Glu-B1br* allele from other similar alleles. Therefore, efforts were made to detect DNA polymorphism within other parts of TaBAC1215C06. Considering the common ancestry of the alleles, few polymorphisms were expected. Nevertheless, given that the Bx genes themselves have accumulated several SNPs, it was hoped that other polymorphism might be discovered within the BAC. The first targets were the left and right junctions of a long terminal repeat (LTR) retroelement at *Glu-B1* locus. Although there was no particular reason to expect DNA polymorphism within these regions, they were tested because they were known to be specific to alleles containing the Bx gene duplication, and could be amplified with primers designed by Ragupathy et al. (2008). Unfortunately, sequencing of these regions did not reveal any polymorphisms.

The next targets were intron regions of a putative protein kinase gene in the BAC. The putative protein kinase gene is separated from the Bx gene by only 301 bp, and is within the region that is duplicated in Bx overexpression alleles. The putative protein kinase gene contains five introns. These were targeted for marker design because they might be more polymorphic than exons. However, none of the PCR products amplified by the *PPK* intron-based primers exhibited any polymorphism that was detectable by HRM analysis.

A third strategy involved the detection of possible insertion sites within TaBAC1215C06 and designing primer pairs to flank these sites. The sequence of TaBAC1215C06 contains 53.2 kb,

with 10.3 kb of fragment duplication. Based on a previous report (Paux et al. 2010) that ISBP markers can be detected at a high density (an average of 3.8 kb per marker), one might expect to design 11 high-confidence ISBP markers. In this research, 15 high-confidence ISBP markers were designed within TaBAC1215C06, but none of these detected polymorphism between H45 and VQ0437. In this case, it is believed that the alleles of H45, Glenlea and VQ0437 have common ancestry, so the expected rate of polymorphism would be much lower than 70% reported by Paux et al. (2010) for a panel of 92 European and Australian wheat lines.

5.4.2 SSR genotyping analysis of association between SSR markers and unextractable polymeric protein

Among 13 SSR markers that were assayed on an F_2 population of H45/VQ0437, eight SSR markers were mapped on three individual linkage groups and no linkage was detected for the other five markers. It is surprising that so many of the markers were not found to be linked with any of the others. Even though the markers used had previously been mapped on chromosome 1B, it is possible that some of the polymorphisms detected here may be on other chromosomes, possibly homeoloci on chromosome 1A or 1D. Among the SSR markers, gdm0028 was the most strongly associated with UPP, but its association was not as strong as was found for *Glu-B1* itself (Chapter 3 of this thesis), and the number of recombinants observed between *Glu-B1* and gdm00028 was much higher than expected based on the reported distance of 5.9 cM between these loci (Sourdille et al. 2005).

5.4.3 Possible approaches to detect DNA polymorphism associated with *Glu-B1br* allele

Although no readily assayed DNA polymorphism was detected within or near the *Glu-B1br*

allele in this research, there are other possible approaches that could be pursued to discover DNA polymorphisms linked with *Glu-B1br*. Many SNPs have been detected and mapped in wheat, and SNP markers have been developed and applied in wheat breeding (Khlestkina and Salina 2006; Ganal and Röder 2007; Barker and Edwards 2009; Allen et al. 2011; Trick et al. 2012). One possible approach would be to make use of a recently developed 90k iSelect Infinium HD platform assays (www.illumina.com). The entire H45/VQ0437 population could be assayed on this platform to generate a genome-wide genetic map, or bulks of selected *Glu-B1br* and *Glu-B1bs* progeny could be assayed and compared to detect SNPs that are linked with *Glu-B1*. Another source of wheat SNP assays is the wheat KASPar (Chen et al. 2010) SNP database (http://www.cerealsdb.uk.net/CerealsDB/SNPs/Documents/DOC_snps.php). Based on the Chromosome 1B map of Avalon/Cadenza (Allen et al. 2011), *Glu-B1* was mapped at near the KASPar marker XBS00010536. According to the wheat KASPar SNP database (accessed on 12th, Sep, 2012), there were 25 KASPar markers available within 2.5 cM of XBS00010536. These markers could be assayed individually on H45 and VQ0437 and polymorphic SNPs could then be assayed on the H45/VQ0437 population to test their association with *Glu-B1* and UPP.

Chapter 6

A Cysteine in the Repetitive Domain of a High-Molecular-Weight Glutenin Subunit Interferes with the Mixing Properties of Wheat Dough

Amino Acids; submitted 2012

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The text, figures and tables presented in this chapter are exactly the same as the manuscript, except for minor formatting changes such as renumbering of tables and figures for consistency within the thesis. Materials that were included as online resources with the manuscript are presented in appendices of this thesis. The statement of acknowledgements and the reference list that were included in the manuscript are not presented within this chapter; they have been incorporated into the acknowledgements and bibliography of the thesis.

Statement of Authorship

Gao X (Candidate)

Proposed the use of heterologous expression and site-directed mutagenesis to investigate the Bx7.1 glutenins subunit, grew plants and prepared the base flour, conducted site-directed mutagenesis, heterologous expression and purification of individual glutenin subunits, conducted of SDS sedimentation test, dough mixing test and SE-HPLC analysis on flour incorporated with individual glutenin subunits, interpreted results and prepared the manuscript.

I hereby certify that the statement of contribution is accurate.

Zhang Q

Trained and supervised the candidate in the heterologous expression and protein purification methods used in this research, helped in the interpretation of the results and contributed to revision of the manuscript.

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the manuscript in the thesis.

Newberry MP

Trained and supervised the candidate in assessment of dough mixing properties, contributed to analysis and interpretation of dough mixing data and contributed to revision of the manuscript.

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Chalmers KJ

Contributed to project planning and research supervision of the candidate and to revision of the manuscript.

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Mather DE

Proposed the overall research topic, supervised development and planning of the research, contributed to revision of the manuscript, and acted as corresponding author.

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the manuscript in the thesis.

Abstract

The quality of wheat (*Triticum aestivum* L.) for making bread is largely due to the strength and extensibility of wheat dough, which in turn is due to the properties of polymeric glutenin. Polymeric glutenin consists of high- and low-molecular-weight glutenin protein subunits linked by disulphide bonds between cysteine residues. Glutenin subunits differ in their effects on dough mixing properties. The research presented here investigated the effect of a specific, recently discovered, glutenin subunit on dough mixing properties. This subunit, Bx7.1, is unusual in that it has a cysteine in its repetitive domain. With site-directed mutagenesis of the gene encoding Bx7.1, a guanine in the repetitive domain was replaced by an adenine, to provide a mutant gene encoding a subunit (MutBx7.1) in which the repetitive-domain cysteine was replaced by a tyrosine residue. Bx7.1, MutBx7.1 and other Bx-type glutenin subunits were heterologously expressed in *Escherichia coli* and purified. This made it possible to incorporate each individual subunit into wheat flour and evaluate the effect of the cysteine residue on dough properties. The Bx7.1 subunit affected dough mixing properties differently from the other subunits. These differences are due to the extra cysteine residue, which may interfere with glutenin polymerisation through cross-linkage within the Bx7.1 subunit, causing this subunit to act as a chain terminator.

Key words: cysteine, dough properties, glutenin polymerisation, high-molecular-weight glutenin, site-directed mutagenesis, wheat.

6.1 Introduction

The glutenin polymers of wheat (*Triticum aestivum* L.) grain, flour and dough consist of diverse high- and low-molecular-weight glutenin subunits (HMW-GS and LMW-GS) held together by disulphide bonds between cysteine residues (Kasarda 1999). Together with monomeric gliadin proteins, polymeric glutenin forms gluten, which gives dough the capacity to trap gas bubbles produced during fermentation, enabling bread to rise. The rheological properties of wheat gluten are known to be affected by the quantity of glutenin relative to gliadin and the subunit composition of the glutenin (Janssen et al. 1996; Shewry et al. 2000). Previous research has demonstrated that variation in HMW-GS accounts for much of the variation in the strength and elasticity of wheat gluten and dough, and thus the quality of wheat flour for processing into bread and other food products (Branlard and Dardevet 1985; Payne et al. 1988; He et al. 2005). Functional differences among HMW-GS are thought to be affected by the numbers and positions of cysteine residues, which can determine whether HMW-GS act as chain extenders or chain terminators during polymerisation (Köhler et al. 1993; Kasarda 1999; Lindsay et al. 2000; Pirozi et al. 2008).

The structure of HMW-GS typically consists of relatively small N- and C-terminal domains flanking a long highly repetitive central domain. The N-terminal domain contains one or more (generally three to five) cysteine residues (Tatham et al. 1991; Buonocore et al. 1996; Margiotta et al. 2000; Li et al. 2004; Shewry and Tatham 1997), while the C-terminal domain has only one. All y-type HMW-GS have a cysteine residue present in the repetitive domain close to C-terminal domain (Anjum et al. 2007). In contrast, the repetitive domains of most x-type HMW-GS have no cysteine residues. The only reported exceptions are the Dx5 subunit,

which carries an additional cysteine residue in the first repeat block adjacent to the N-terminal domain (Anderson et al. 1989) and the recently reported Bx7.1 subunit (Gao et al. 2012) [Chapter 3 of this thesis] which has a cysteine residue at the 512th position of its full 774-amino-acid sequence.

The variation among HMW-GS is under genetic control. The genes encoding HMW-GS are located at the *Glu-A1, Glu-B1* and *Glu-D1* loci on chromosomes 1A, 1B and 1D, respectively. At the *Glu-B1* locus, many alleles have been discovered (Payne and Lawrence 1983; Mclntosh et al. 2008, 2009), most of which include one gene encoding an 'x-type' (Bx) HMW-GS and one gene encoding a 'y-type' (By) HMW-GS. However, three *Glu-B1* alleles (*Glu-B1al, Glu-B1br and Glu-B1bs*) have been reported to include two Bx genes and one By gene (Ragupathy et al. 2008; Gao et al. 2012 [Chapter 3 of this thesis]). All three of these alleles are associated with overexpression of Bx subunits relative to other HMW-GS. In the *Glu-B1al* allele, the two Bx genes are identical in sequence (Ragupathy et al. 2008). Each of them encodes a polypeptide (Bx7^{OE}) with three cysteine residues in its N-terminal domain and one in its C-terminal domain. Wheat cultivars with the *Glu-B1al* allele have strong dough (Butow et al. 2003; Eagles et al. 2004; Vawser and Cornish 2004). The *Glu-B1bs* allele, which includes a gene encoding another very similar subunit (Bx7.3) and a gene encoding a Bx7^{OE} subunit, seems to confer similar effects (Gao et al. 2012) [Chapter 3 of this thesis]. In contrast, H45, an Australian wheat cultivar with the *Glu-B1br* allele, has relatively weak dough. Gao et al. (2012) [Chapter 3 of this thesis] demonstrated that the *Glu-B1br* allele includes two slightly different *Bx7^{OE}*-like genes (*Bx7.1* and *Bx7.2*) whose sequences each differ by four single-nucleotide polymorphisms (SNPs) from that of the *Bx7^{OE}* gene. All four

of the *Bx7.1* SNPs and one of the four *Bx7.2* SNPs are non-synonymous. Interestingly, one of the *Bx7.1* SNPs leads to a cysteine residue in the repetitive domain of the protein. Gao et al. (2012) [Chapter 3 of this thesis] suggested that the repetitive-domain cysteine of Bx7.1 subunits could interfere with glutenin polymerisation by forming intra- or intermolecular disulphide linkages.

Through genetic analysis, Gao et al. (2012) [Chapter 3 of this thesis] were able to attribute protein properties of H45 to the *Glu-B1br* allele, but not specifically to the Bx7.1 subunit and its extra cysteine. Due to the very close linkage of the *Bx7.1* and *Bx7.2* genes, the subunits they encode are always both present or both absent in progeny of H45. With gel electrophoresis, $Bx7^{OE}$-like subunits can be readily separated from most other HMW-GS, but not from each other due to their very similar electrophoretic mobilities.

Previous research (Békés et al. 1994b; Xu et al. 2006; Yan et al. 2009a; Chen et al. 2011) has demonstrated that glutenin genes can be heterologously expressed in *Escherichia coli*. This provides a means of obtaining samples of individual subunits. Here, to permit direct investigation of the effects of the extra cysteine of the Bx7.1 subunit, site-directed mutagenesis was used to replace the repetitive-domain cysteine codon of the *Bx7.1* gene by a tyrosine codon. The resulting mutant gene (*MutBx7.1*), the *Bx7.1* gene, and other $Bx7^{OE}$-like genes were then expressed in *E. coli*, to provide sufficient amounts of protein to be incorporated into flour for SDS sedimentation and small-scale dough mixing tests.

6.2 Materials and Methods

6.2.1 Site-directed mutagenesis

A mutant gene (*MutBx7.1*) was prepared using the polymerase chain reaction (PCR) with the Quick Change Site-directed Mutagenesis Kit (Stratagene). A previously constructed plasmid (pGEM-T) containing the gene encoding the Bx7.1 subunit of H45 (Gao et al. 2012) [Chapter 3 of this thesis] was used as the template. The guanine at 1598 nt was substituted with adenine using the following synthetic oligonucleotides as mutagenic primers:

SDM-Forward: 5'- ACAATGGCAACTAGTGTACTACCCAACTTCTCCGC-3' and SDM-Reverse: 5'- GCGGAGAAGTTGGGTAGTACACTAGTTGCCATTGT-3' (mutation site underlined). The amplification profile was one cycle at 95 ℃ for 1 min, followed by 18 cycles of 95 ℃ for 50 s, 70 ℃ for 50 s and 68 ℃ for 6 min, and a final extension step at 68 ℃ for 7 min. Mutagenesis was confirmed by commercial sequencing of the mutated gene (Australian Genome Research Facility).

6.2.2 Preparation of flour samples

Grains harvested from glasshouse-grown plants of each of the wheat cultivars Glenlea, H45, Gabo and the wheat breeding line VQ0437 were conditioned to 15% (w/w) moisture level overnight and milled using a Brabender Quadrumat Senior mill. Gabo, a weak-gluten cultivar that carries the alleles *Glu-A1b*, *Glu-B1i* and *Glu-D1a* (Cinco-Moroyoqui and MacRitchie 2008) and has no Bx7-like HMW-GS was included to provide a suitable base (control) flour for the incorporation of heterologously expressed HMW-GS. The protein and moisture contents of each flour sample were estimated by near infrared reflectance.

6.2.3 Analysis of mixing properties of plain flour samples

Analysis of plain flour of Glenlea, H45, VQ0437 and Gabo was conducted using a 10-g Mixograph pin mixer (National Manufacturing Company, Lincoln, NE, USA) using AACC Approved Method 54-40A (American Association of Cereal Chemists 1995), with dough prepared according to water absorption levels determined with micro-farinograph dough mixer (4-g micro-doughLAB z-arm mixer, Perten Ltd., NSW, Australia) (Bason et al. 2007). Results were analyzed using the MixSmart software (AEW Consulting, Lincoln, NE, USA). The measured torque data were used to determine three parameters: dough development time, resistance at breakdown 5 min after the dough development time, and bandwidth at breakdown 5 min after the dough development time. Stronger mixing flours will have longer dough development times, and be more resistant to over-mixing, as indicated by lower values of resistance and bandwidth at breakdown.

6.2.4 Heterologous expression of high molecular weight glutenin subunit genes

The *Bx7.1* and *Bx7.2* genes previously isolated (Gao et al., 2012) [Chapter 3 of this thesis] from the wheat cultivar H45 (GenBank accessions JF938070 and JF938071), the *Bx7.3* and *Bx7*^{OE} genes isolated from the wheat breeding line VQ0437 (JF938072 and JF938073), the two *Bx7*^{OE} gene copies isolated from the wheat cultivar Glenlea (EU157184) and the mutant gene *MutBx7.1* were all maintained in the plasmid pGEM-T vector (Promega). The coding region of each gene, excluding its signal peptides, was amplified using a QIAGEN LongRange PCR Kit (QIAGEN). The amplification profile was one cycle at 93 ℃ for 3 min, followed by 35 cycles of 93 ℃ for 15 s and 68 ℃ for 3 m, and a final extension step at 72 ℃ for 7 min. The expression primers were 5’-ACCCATATGGAAGGTGAGGCCTCT-3’ and

5’-CTAGAATTCCTATCACTGCCTGGTCGA-3’. These primers introduce *Nde* I and *EcoR* I recognition sites (underlined). These recognition sites were used to perform sticky-end ligation of the amplicons into the bacterial expression vector pET-24a (Novagen). After an overnight ligation at 4 ℃, the constructs were transformed separately into *E. coli* strain BL21 (DE3)-plysS cells by electroporation (Dower et al. 1988). A single recombinant colony was inoculated into 5 ml of lysogeny broth (LB) medium, incubated overnight at 37 ℃ and then transferred to 1000 ml of LB medium. Heterologous expression was induced by the addition of isopropyl-β-*D*-thiogalactopyranoside (IPTG) to a final concentration of 1 mM once the OD_{600} (the optical density of the sample measured at a wavelength of 600 nm) of the culture had reached approximately 0.6. To provide a negative control, bacteria were transformed with an empty pET-24a plasmid. The cultured cells were induced at 37 ℃ for 4 h, and harvested by centrifugation (10,000 rpm, 4 ℃, 10 min).

6.2.5 Isolation of heterologously expressed proteins

Cell disruption and fractionation were carried out according to Dowd and Bękés (2002) with minor modifications. Cell pellets were resuspended in lysis buffer (50 mM Tris-HCl pH 7.3, 100 mM NaCl, 1 mM EDTA, 1 mM PMSF, 10 mM DTT), at a ratio of 1 g cells to 5 ml buffer, placed on ice and disrupted by sonication over a period for 5 min (with cycles of 2 s sonication at 400 W output separated by pauses of 2 s). After centrifugation (12,000 rpm, 4 ℃, 15 min), the supernatant was discarded and the pellet was washed twice with distilled water plus 100 mM DTT and 10 mM PMSF to remove water-soluble cell proteins. The suspension was centrifuged as above and the supernatant was discarded. Then the pellet was resuspended in 5 ml of 50% (v/v) propan-1-ol containing 10 mM DTT, with the suspension

sonicated for 15 s twice to assist solubilisation. The sample was incubated at 65 ℃ for 2 h, with vortexing every 5 min. After centrifugation (12,000 rpm, 10 min), the extracted protein in the supernatant was pooled, dialysed for 48 h against 150 ml of 0.1% acetic acid and freeze-dried.

6.2.6 Analysis of heterologously expressed proteins

Both the cell pellets and purified protein samples prepared as described above were completely dissolved in sample buffer (0.02% bromophenol blue, 80 mM Tris–HCl (pH 8.0), 69 mM SDS) containing 1% (w/v) dithiothreitol (DTT). Electrophoresis was conducted using the methods described by Gao et al. (2012) [Chapter 3 of this thesis]. The gel was stained with staining solution (0.1% Coomassie Brilliant Blue R-250, 50% methanol and 10% glacial acetic acid) and destained with destaining solution (40% methanol and 10% glacial acetic acid). Gel segments containing the heterologously expressed proteins were excised, crushed and destained with 50 mM ammonium bicarbonate in 30% acetonitrile. The proteins were reduced with 0.5 μmol DTT in 100 mM ammonium bicarbonate and alkylated with 2.75 μmol iodoacetamide in 100 mM ammonium bicarbonate. After digestion with 100 ng of sequencing-grade modified trypsin (Promega) in 5 mM ammonium bicarbonate containing 10% acetonitrile, the subsamples were extracted with 1% formic acid in water, 1% formic acid in acetonitrile and 100% acetonitrile for LC-eSI-IT mass spectrometry at the Adelaide Proteomics Centre.

6.2.7 SDS sedimentation test

For plain flour, SDS sedimentation tests were conducted using the methods described by Gao

et al. (2012) [Chapter 3 of this thesis]. For Gabo base flour supplemented with heterologously expressed subunits, a reversible reduction-oxidation procedure (Békés et al. 1994) was employed to incorporate the expressed polypeptides into the base flour, and SDS sedimentation tests were conducted using the methods described by Carter et al. (1999) with minor modifications. A 0.5 g sample of base flour (10.0% moisture and 19.2% protein) was supplemented with 5 mg of heterologous protein, then thoroughly mixed with 2.9 ml distilled water plus 0.1 ml of 0.1 mg ml^{-1} DTT for 20 s on a high-speed vortex mixer, allowed to hydrate for 5 min, mixed again on the high-speed vortex for 10 s, and then allowed to hydrate for another 5 min. At this point, 0.05 ml of 0.5 mg ml^{-1} KIO_3 was added and mixed for 30 s, then the sample was rested for 5 min. Lactic acid/SDS solution (9 ml) was added to each sample and the tubes were agitated on a Zeleny-type rocker (40 cycles min-1e) for 40 s, rested for 2 min, and agitated again for 40 s. The racks were left in an upright position for 10 min and the height of the sediment was recorded. Each SDS sedimentation test was performed twice, with samples of the cultivar Gabo included in each rack as control.

6.2.8 Dough mixing test

A 4 g micro-doughLAB z-arm mixer (Perten Ltd., NSW, Australia) (Bason et al. 2007) was used to carry out reduction-oxidation mixing and to assess optimal water absorption levels. Expressed polypeptides were incorporated into Gabo base flour using a reversible reduction-oxidation procedure (Békés et al. 1994; Oszvald et al. 2009) , with minor modifications. The 4 g base flour (at 10.04% moisture) and 20 mg (0.5% of base flour) or 40 mg (1.0% of base flour) of heterologous protein were mixed with 2.69 ml of water and 0.1 ml water containing 0.5 mg ml^{-1} DTT for 30 s, and rested for 3 min. At this point, 0.05 ml of 2.5

mg ml^{-1} KIO_3 was added and the dough mixed for 30 s, then rested for 4 min, and finally mixed for an additional 10 min. Mixing curves and other dough quality parameters were registered. The mixing parameters selected for evaluation of the effect of heterologously expressed protein on dough properties were: water absorption, which is the water content of the dough as a percentage of the flour weight necessary to achieve a dough of desired consistency; dough development time, which is the time to reach the peak resistance of the dough; and stability, which is the time between the arrival and departure times, and is a measurement of dough strength (Oszvald et al. 2009). Dough samples were collected at peak time from the micro z-arm mixer and freeze-dried for protein analysis.

6.2.9 Size-exclusion high-performance liquid chromatography (SE-HPLC)

Freeze-dried dough samples were ground and the proteins were extracted in two steps following the method described by Gao et al. (2012) [Chapter 3 of this thesis]. To determine the protein size distribution in both extractable and unextractable protein fractions of the dough, SE-HPLC analysis was carried out according to Larroque et al. (2000). The proportion of unextractable polymeric protein (%UPP) was calculated.

6.3 Results

6.3.1 Site-directed mutagenesis of *Bx7.1*

Sequencing of the mutant gene *MutBx7.1* confirmed that site-directed mutagenesis had successfully replaced the guanine (G) at the 1598 nt position of the *Bx7.1* gene by an adenine (A), changing the repetitive-domain cysteine codon to a tyrosine codon (Appendix 4).

6.3.2 Mixing and physiochemical properties of plain flour samples

The plain flour samples milled from Glenlea, H45, VQ0437 and Gabo had moisture contents ranging from 10.0% and 10.5% and protein contents ranging from 14.8% to 19.2% (Table 6.1). Glenlea had the highest SDS sedimentation volume and %UPP value, followed by VQ0437, H45 and Gabo (Table 6.1). Consistent with this, the mixograph analyses confirmed that the sample of Glenlea flour had stronger dough properties than the samples of Gabo and H45 flour, with relatively long dough development times, and good resistance to over-mixing as demonstrated by lower resistance and bandwidth breakdown values at 5 min beyond dough development time) (Appendix 5). The dough mixing parameters of VQ0437 were intermediate between those of Glenlea and H45 (Table 6.1). Gabo flour had a very high water absorption level (71.4%) which would, in part, be due to the very high protein content of this flour (19.2%). H45 and VQ0437 had moderately high water absorption values (around 66%), while Glenlea had the lowest water adsorption value (63.9%).

Table 6.1 Protein and moisture contents, dough mixing properties assessed with a 10-g mixograph, SDS sedimentation volume and SDS-unextractable polymeric protein (UPP) of flour samples milled from four lines of wheat

Wheat line	Protein content (%)	Moisture content (%)	Water absorption (%)	Dough mixing properties			SDS sedimentation volume (ml g^{-1})	UPP (%)
				Dough development time (min)	Resistance breakdown at 5 min (%)	Bandwidth breakdown at 5 min (%)		
Gabo	19.2	10.0	71.4	3.67	16.2	79.9	15.3	27.9
Glenlea	17.3	10.0	63.9	9.46	10.2	51.3	24.5	52.7
H45	14.8	10.5	66.3	3.53	17.2	67.0	19.3	30.1
VQ0437	16.3	10.4	66.6	5.07	14.0	62.1	23.6	44.3

6.3.3 Heterologous expression of HMW-GS

The *MutBx7.1* glutenin gene generated by site-directed mutagenesis and each of the *Bx7*^{OE}-like glutenin genes from Glenlea, H45 and VQ0437 was successfully expressed by in *E. coli*. As expected, the SDS-PAGE mobility of each of the purified heterologously expressed proteins was the same as that of the Bx7^{OE}-like subunits extracted from flour of Glenlea, H45 and VQ0437 wheat (Fig. 6.1). The LC-eSI-IT mass spectrometry results, which covered between 3% and 10% of the full sequences, confirmed that peptide sequences were the same as predicted from the nucleotide sequences.

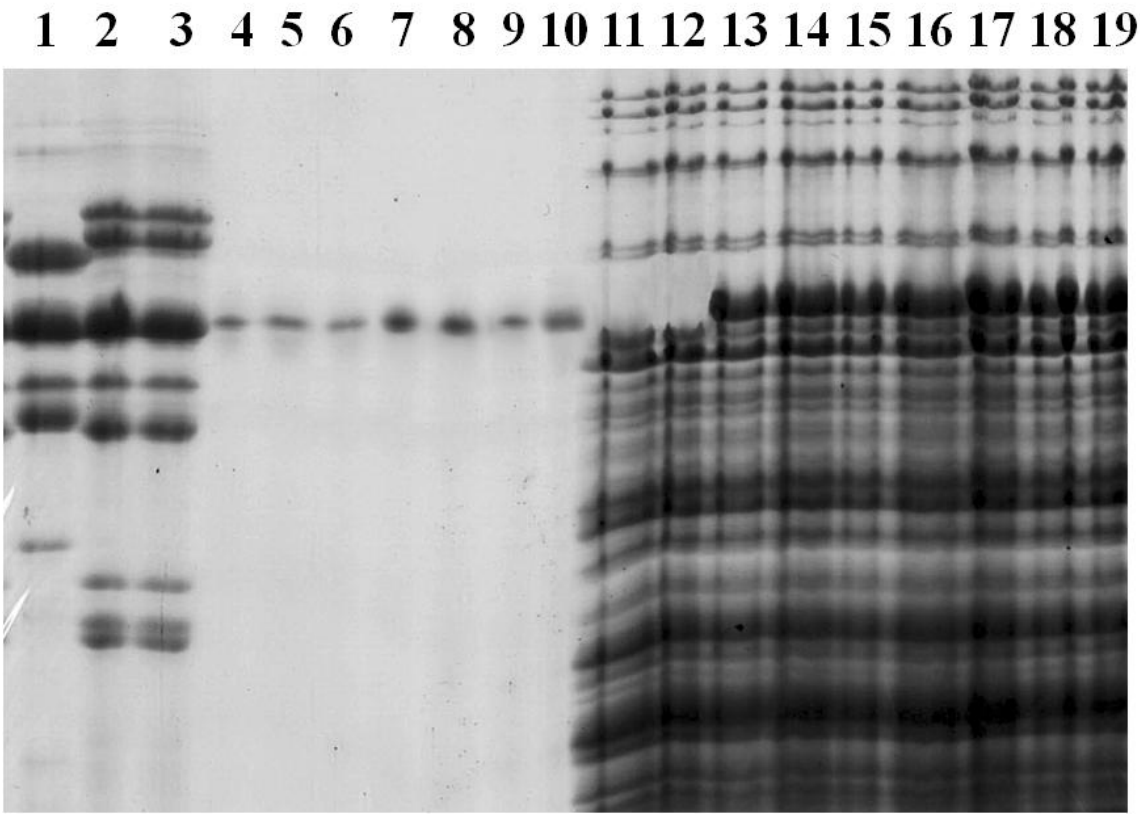


Fig. 6.1 SDS–PAGE profiles of purified and heterologously expressed HMW-GS. Lanes 1-3, HMW components extracted from Glenlea, H45 and VQ0437 flour; lanes 4-10, purified proteins of $Bx7^{OE}$ copy1, $Bx7^{OE}$ copy2, Bx7.1, Bx7.2, Bx7.3, $Bx7^{OE}$ from VQ0437 and MutBx7.1; lane 11, pET-24a in BL21 (DE3)-plysS cells induced by isopropyl-β-*D*-thiogalactopyranoside ; lane 12, pET-24a-Bx7 in BL21 (DE3)-plysS cells not induced by isopropyl-β-*D*-thiogalactopyranoside; lanes 13-19, pET-24a-$Bx7^{OE}$ copy1, pET-24a-$Bx7^{OE}$ copy2, pET-24a-Bx7.1, pET-24a-Bx7.2, pET-24a-Bx7.3, pET-24a-$Bx7^{OE}$-VQ0437 and pET-24a-MutBx7.1 in BL21 (DE3)-plysS cells induced by isopropyl-β-*D*-thiogalactopyranoside

6.3.4 Effects of incorporated proteins on SDS sedimentation volume

Incorporation of each heterologously expressed protein increased SDS sedimentation volume relative to that of plain Gabo base flour: from 15.3 ml g^{-1} to 16.5 mg g^{-1} (7.8% increase) for Bx7.1 and to between 18.1 and 18.5 g ml^{-1} (18.3% to 20.9% increase) for the other proteins, including MutBx7.1 (Table 6.2).

Table 6.2 SDS sedimentation volumes of samples of base flour milled from Gabo wheat with and without incorporation of heterologously expressed high-molecular weight glutenin subunits

Glutenin subunit	Source of glutenin gene	SDS sedimentation volume		
		Mean (ml g^{-1})	Standard deviation	% of Gabo
None		15.3	0.14	100.0
$Bx7^{OE}$ copy1	Glenlea	18.2	0.28	119.0
$Bx7^{OE}$ copy2	Glenlea	18.3	0.42	119.6
Bx7.1	H45	16.5	0.14	107.8
Bx7.2	H45	18.3	0.14	119.6
Bx7.3	VQ0437	18.5	0.42	120.9
$Bx7^{OE}$	VQ0437	18.4	0.28	120.3
MutBx7.1	H45[5]	18.1	0.14	118.3

[5] The *MutBx7.1* gene was obtained by site-directed mutagenesis of the H45 *Bx7.1* gene.

6.3.5 Effects of incorporated proteins on mixing properties and unextractable polymeric protein

Flour incorporated with the lower amount (20 mg) of any of the heterologously expressed glutenin subunits increased water absorption relative to the Gabo base flour (Fig. 6.2). Similarly, flour incorporated with the higher amount (40 mg) of the Bx7.1 subunit increased water absorption. In contrast, incorporation of 40 mg of any of the other subunits (including MutBx7.1) caused little or no change to water absorption (-0.4% to 0.4%).

Addition of expressed glutenin subunits increased dough development time, mixing stability, and % UPP, particularly when the larger amount (40 mg) of protein was added (Fig. 6.2). For these dough properties, the changes observed for the Bx7.1 subunit were substantially less than for the other subunits, including MutBx7.1.

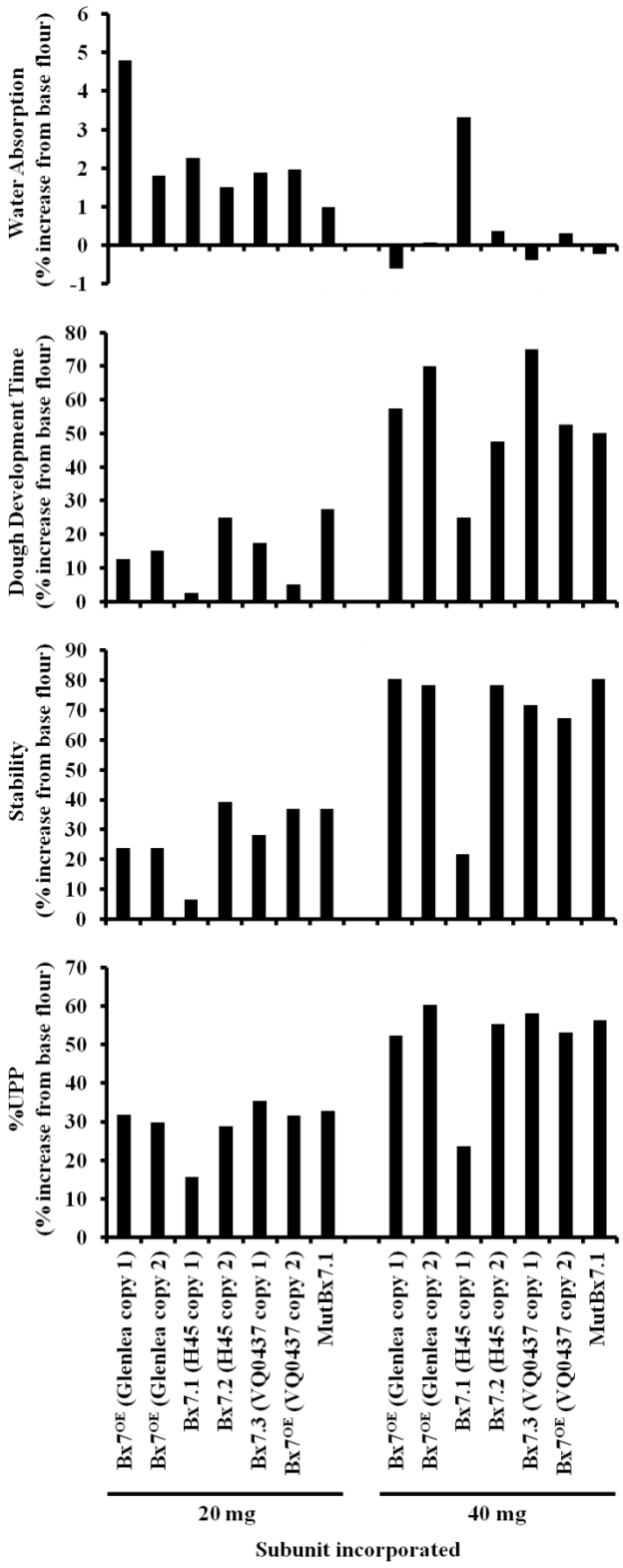


Fig. 6.2 Percentage changes in dough mixing properties and unextractable polymeric protein with heterologously expressed glutenin subunits incorporated at two levels (20 mg and 40 mg) into 4 g of Gabo base flour, relative to those of the base flour

6.4 Discussion

6.4.1 Site-directed mutagenesis and heterologous expression of HMW-GS

In this research, a mutant gene was obtained by site-directed mutagenesis of a native glutenin gene originally isolated from an Australian wheat cultivar, expressed the wild-type gene and a mutant derivative in *E. coli*, and obtained small amounts of purified HMW glutenin proteins for flour and dough property tests. Site-directed mutagenesis has been widely applied in genetic engineering of seed storage proteins of wheat, rice and maize (Washida et al. 1999; Holding and Larkins 2008). This has included some research on glutenin genes of wheat (Shani et al. 1994; Orsi et al. 2001; Lombardi et al. 2009; Saumonneau et al. 2011). Heterologous expression in *E. coli* has been widely used to obtain individual glutenin subunits (Tamás et al. 1998; Dowd and Békés 2002; Xu et al. 2006; Yan et al. 2009a; Liu et al. 2010) and proteins produced in this way have previously been incorporated into flour to study their functional effects on dough mixing properties (Tamás et al. 1998; Xu et al. 2006; Yan et al. 2009a; Liu et al. 2010; Chen et al. 2011). Here, heterologous expression permitted the isolation of subunits encoded by very tightly linked genes and the combination of site-directed mutagenesis with heterologous expression made it possible to investigate the effect of one particular polymorphism within a gene. This approach was effective here and could be useful for studying the effects of other differences among glutenin subunits.

6.4.2 Effects of heterologously expressed glutenin subunits on SDS sedimentation volume

As the two $Bx7^{OE}$ genes in the *Glu-B1al* allele of Glenlea are identical, there are no differences between the ‘copy1’ and ‘copy2’ $Bx7^{OE}$ constructs. The subunits generated from

these constructs are essentially replicates of each other. Similarly, the subunits generated by the *Bx7*^{OE} gene in the *Glu-B1bs* allele of VQ0437 are identical to those from the Glenlea *Bx7*^{OE} genes, because the DNA polymorphisms are synonymous. In contrast, the Bx7.1, Bx7.2, Bx7.3 and MutBx7.1 subunits all differ in sequence from each other and from Bx7^{OE}. Most notably, the Bx7.1 subunit (one of the subunits from the *Glu-B1br* allele of H45) has a cysteine residue in its repetitive domain. Consistent with the results of previous SDS sedimentation tests conducted on flour from H45, VQ0437 and Glenlea wheat (Gao et al. 2012) [Chapter 3 of this thesis], the Bx7.1 subunit did not increase the SDS sedimentation volume as much as Bx7^{OE}, Bx7.2 or Bx7.3 subunits when incorporated into a base flour. Further, the MutBx7.1 subunit, which differs from the Bx7.1 subunit by only one amino acid residue, was similar in effect to the Bx7^{OE}, Bx7.2 and Bx7.3 subunits. These results are consistent with the hypothesis that it is the extra cysteine in the Bx7.1 subunit that affects the functional properties of glutenin in the wheat cultivar H45. Given the differences obtained with this predictive test, further work was done to investigate the effects of the individual subunits on mixing properties of dough and on the %UPP of glutenins in the dough.

6.4.3 Conditions and base flour for polypeptide incorporation

The reversible reduction-oxidation procedure for incorporation of polypeptides into base flour was first established by Békés et al. (1994). When dough is partially reduced with DTT, it allows the rupture of disulfide bonds within the dough structure, which reduces the average molecular weight of the peptides and interferes with dough mixing properties. When the dough is reoxidised with potassium iodate, free sulfhydryl groups participate in disulfide linkages, permitting recovery of dough mixing properties. This reduction-oxidation procedure

allows added glutenin subunits to be incorporated into the polymeric protein of the base flour. This reduction-oxidation procedure has previously been used to study the functional effect of proteins, especially LMW-GS and HMW-GS from wheat, on dough mixing properties (Tamás et al. 1998; Shewry et al. 2003a; Xu et al. 2006; Maforimbo et al. 2008; Yan et al. 2009a; Liu et al. 2010; Chen et al. 2011).

The base flour used for a polypeptide incorporation test should not have very strong or very weak dough properties, and should not contain the same protein subunit(s) as the supplementation subunit(s). Here, the base flour used was milled from grain of the wheat cultivar Gabo. Gabo was chosen for this purpose because it does not carry Bx7, $Bx7^{OE}$ or $Bx7^{OE}$-like HMW-GS. Gabo is not a current cultivar and its grain and flour are not commercially available. Plants of Gabo wheat therefore had to be grown specifically for the purpose of obtaining base flour. This was done in a glasshouse. The protein content of the Gabo flour sample obtained was high (19.2%), possibly due to the plants having been grown under conditions that differ from typical field conditions. In wheat, grain protein content is affected by environmental conditions, such as the amount of nitrogenous fertilizer, the temperature, light intensity and water availability Kramer (1979). The plain flour samples of the other wheat lines used here also came from glasshouse-grown plants and were quite high in protein (ranging from 14.8% to 17.3%). Nevertheless, the wheat lines ranked as expected with respect to their SDS-sedimentation volume, %UPP and dough mixing parameters, with the Gabo flour having the lowest values for all traits, yet still having a dough development time longer than the 1-min minimum recommended by Békés et al. (1994). Thus the Gabo flour sample was suitable as base flour for incorporation of subunits from the other three

lines.

As indicated by Békés et al. (1994), the concentration of reducing-oxidizing agents required varies according to protein content and glutenin composition of the base flour and the ratio of incorporated glutenin proteins to base flour. In most studies, the amounts of glutenin protein incorporated have ranged from 0.25% to 1.0%, expressed as a percentage of the amount of base flour used. Given the very high protein content of the Gabo base flour, the dough mixing tests were conducted at two supplementation levels (0.5% and 1.0%). As recommended by Békés et al. (1994), mixing for 30 s was used to ensure effective dispersion of reducing and oxidization reagents.

6.4.4 Effects of the incorporation of the heterologously expressed Bx7^{OE} and Bx7^{OE}-like proteins in dough mixing tests

As expected, flour milled from H45 produced much weaker dough than flour milled from Glenlea. This could be due to differences between the Bx7.1 and Bx7.2 subunits of H45 and the Bx7^{OE} subunit of Glenlea (Vawser and Cornish 2004; Gao et al. 2012 [Chapter 3 of this thesis]), but also to other differences in glutenin composition between the two cultivars. In particular, Glenlea expresses the Dx5 and Dy10 subunit combination, which has been reported to give stronger dough properties than the Dx2 and Dy12 combination (He et al. 2005; Ohm et al. 2008), which is present in H45. Thus, plain-flour results are not sufficient to attribute these differences directly to *Glu-B1* polymorphism.

Differences between H45 and VQ0437 are more likely to be due to *Glu-B1* alone, as these two lines have the same genotypes at other loci affecting grain quality. Further, Gao et al.

(2012) [Chapter 3 of this thesis] reported that *Glu-B1br* co-segregated with low %UPP in the progeny of a cross between H45 and VQ0437. Here, SDS-sedimentation, %UPP and dough mixing parameters were all lower for flour milled from H45 than for flour milled from VQ0437, indicating that H45 has weaker dough properties than VQ0437.

Even for comparisons of H45 with VQ0437, the plain flour results are not sufficient to attribute effects to individual subunits. Attribution of effects to individual subunits required the isolation of those subunits and the incorporation individual subunits into base flour.

Consistent with the results of the SDS sedimentation test, incorporation of the Bx7.1 subunit into dough did not improve dough mixing properties as much as incorporation of $Bx7^{OE}$, Bx7.2, Bx7.3 or MutBx7.1. The contrast between the effects of Bx7.1 and MutBx7.1 is particularly significant, as these two subunits differ by only one amino acid residue. The dough properties expected for $Bx7^{OE}$ were apparently recovered by replacing the repetitive-region cysteine of Bx7.1 by a tyrosine. Furthermore the dough with the Bx7.1 subunit incorporated yielded the lowest %UPP value, indicating that a less extensive polymeric polypeptide structure was obtained with Bx7.1 than with any of the other subunits investigated here. The MutBx7.1 subunit generated %UPP levels similar to those obtained with the $Bx7^{OE}$, Bx7.2 and Bx7.3 subunits. This provides strong evidence that the presence of a cysteine in the repetitive region of a $Bx7^{OE}$-like subunit is sufficient to interfere with the functional properties of polymeric glutenin.

6.4.5 Effect of Bx7 subunits on water absorption properties

Incorporation of glutenin subunits into dough mixed using a z-arm configuration affords the

ability to determine the water absorption properties of the incorporated glutenin subunits and base flour. This is in contrast to previous studies that have used only a mixograph pin mixer, with the amount of water added based on the protein and moisture contents of the flour and added protein. This study is the first to investigate effects of glutenin subunit incorporation on dough water absorption behaviour. Incorporation of glutenin subunits at the 0.5% level increased water absorption (for all subunits) but incorporation of glutenin subunits at the 1.0% level only slightly altered water absorption (for all subunits except Bx7.1). In an earlier investigation of the effect of protein supplementation (as opposed to incorporation) on z-arm mixing properties, increasing the amount of protein added elevated water absorption, but increasing glutenin-to-gliadin ratios did not (Haraszi et al. 2004). The behaviour observed here at 0.5% addition is consistent with conclusions from the supplementation studies but the behaviour at 1.0% addition is not. Incorporation of the higher level of glutenin subunits into the polymeric polypeptide structure of the base flour reduced the ability of the polymeric polypeptide to absorb water, probably *via* the formation of additional cross links. This is in contrast to the continual increase in water absorption that is observed as increasing amounts of protein are added without incorporation into the polymeric polypeptide structure. The anomalous behaviour of the Bx7.1 subunit, which showed increases in water absorption at both the 0.5 and 1.0% addition levels, is consistent with the low %UPP values obtained at both levels and with the hypothesis that this subunit has a limited ability to form interchain cross links in polymeric protein.

6.4.6 The extra cysteine residue and its effect on dough properties

While the results presented here do not provide direct evidence for the exact mechanism of the effect of the additional cysteine, it is likely that the mechanism involves the participation of the repetitive-domain cysteine in intramolecular or intermolecular disulphide linkages. Like most x-type HMW glutenin subunits, $Bx7^{OE}$ contains four cysteine residues, three in the N-terminal domain and one in the C-terminal domain. Normally, as shown in Fig. 6.3A, two of the cysteines in the N-terminal domain of $Bx7^{OE}$ would be connected by an intrachain disulphide bond, while the other two cysteines would be available to contribute to glutenin polymerisation by forming interchain disulphide bonds with cysteines in other glutenin subunits (Keck et al. 1995; Wieser 2007). If the repetitive-domain cysteine links with cysteines in other glutenin subunits, this might interfere with end-to-end linkages among subunits or cause branching of the polymer (Fig. 6.3B). Alternatively, in accordance with the suggestion of Kasarda (1999) that intrachain links form more rapidly than interchain links, the repetitive-domain cysteine may link with one of the terminal-domain cysteines (Fig. 6.3C and D) of the same subunit. This would leave only one terminal-domain cysteine available to form an interchain disulphide bond. This would cause the Bx7.1 subunit to act as a chain terminator, preventing other subunits from linking to the glutenin polymer. This interference with glutenin polymerisation could explain the observed functional properties of H45 flour and of Gabo flour supplemented with the Bx7.1 glutenin subunit.

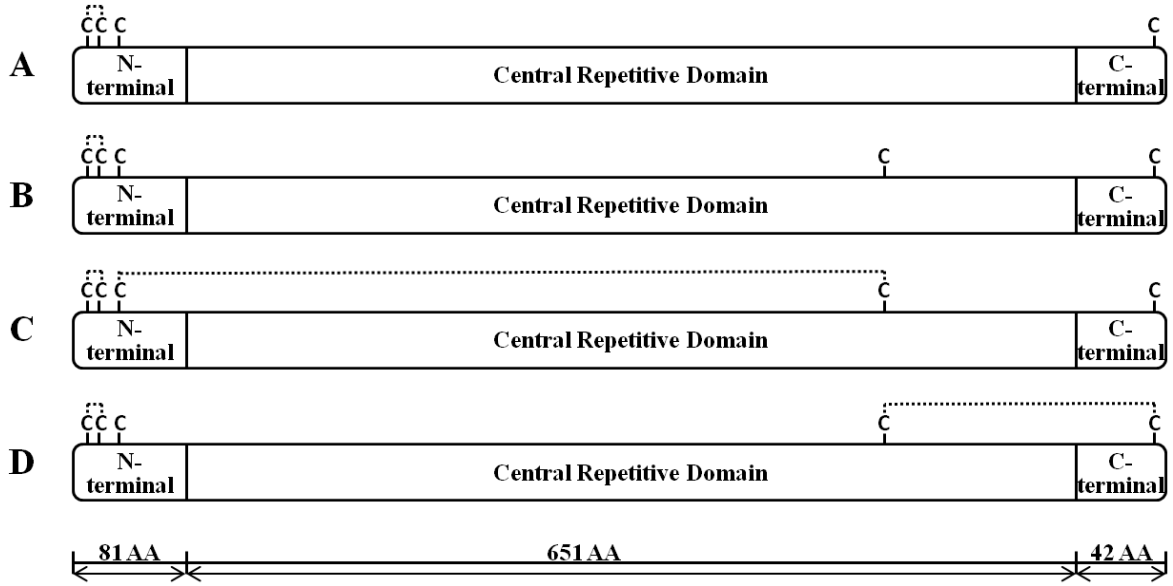


Fig. 6.3 Schematic representations of (A) the $Bx7^{OE}$ glutenin polypeptide showing the positions of its four cysteine residues and an expected intramolecular disulphide bond (dashed line), with two terminal-domain cysteines remaining available to participate in intermolecular linkages; (B) the Bx7.1 glutenin subunit showing the positions of its five cysteine residues and the same intramolecular disulphide bond that is expected in $Bx7^{OE}$, leaving two terminal-domain cysteines and one repetitive-domain cysteine available to participate in intermolecular linkages; (C) and (D) the Bx7.1 glutenin subunit showing two hypothetical combinations of intrachain disulphide bonds, each leaving one terminal-domain cysteine available to participate in intermolecular linkages.

Chapter 7

General Discussion

Even though high molecular weight glutenin subunits take up only small amount of grain protein, they have been considered to be the most important components governing bread making quality as they determine gluten elasticity (Tatham et al. 1985; Shewry and Halford 2002). Prior to my research presented here, many different HMW-GS had been detected in wheat (D'Ovidio et al. 1996; Shewry et al. 2003b; Wang and Zhang 2006; Singh et al. 2007; Yan et al. 2009b; Hu et al. 2012). Variation in the properties of individual HMW-GS (Shewry et al. 2003a; Darlington et al. 2003; Li et al. 2004; Yan et al. 2009b) and glutenin subunit composition (Branlard and Dardevet 1985; Payne 1987; Payne et al. 1988; Halford et al. 1992) had been studied in great detail, and some of these had been found to explain considerable variation in bread making quality among wheat lines. The *Glu-B1al* allele had been reported to have a particularly important effect on dough mixing properties (Eagles et al. 2004), which had been attributed to an overexpression Bx7 subunit (Butow et al. 2003; Vawser and Cornish 2004) encoded by *Bx7* gene duplication at the *Glu-B1* locus (D'Ovidio, 1997; Ragupathy, 2008).

The research reported in Chapter 3 of this thesis demonstrated that *Glu-B1al* is not the only allele that confers overexpression of Bx HMW-GS relative to other HMW-GS. Two new Bx overexpression alleles were discovered and named: *Glu-B1br* in the Australian cultivar H45 and *Glu-B1bs* in a wheat breeding line, VQ0437. Like *Glu-B1al*, each of these alleles includes two genes encoding x-type glutenin subunits, but these genes each differ from the duplicated $Bx7^{OE}$ gene in the *Glu-B1al* allele by a few SNPs.

Prior to this research, the *Glu-B1al* allele of Glenlea wheat had been traced back to an Argentinian cultivar, Klein Universal II (Butow et al. 2004; Eagles et al. 2004; Vawser and Cornish 2004), which is separated from Glenlea by at least seven generations (Figure 4.7 of this thesis). Here, examination of pedigrees information for H45 and VQ0437 revealed that Klein Universal II is also in their ancestry (Figures 4.8, 4.9 and 4.10 of this thesis). Thus the *Glu-B1br* and *Glu-B1bs* alleles may have diverged from the *Glu-B1al* allele by accumulation of mutants as they were inherited from Klein Universal II *via* different lineages, involving up to 12 generations between Klein Universal II and H45 and up to 16 generations between Klein Universal II and VQ0437. Another possibility is that all three of the overexpression alleles were already present in the Americano landrace from which Klein Universal II was derived and that one or both of *Glu-B1br* and *Glu-B1bs* were inherited *via* another derivative of that landrace, possibly Americano 25E. Americano 25E has itself not been included in any previous research to identify whether it carries a Bx overexpression allele.

The restriction digest assay reported in Chapter 3 of this thesis was designed to distinguish the *Glu-B1br* allele from the other two overexpression alleles. It reliably detected the *Glu-B1br* allele in H45 and its progeny, but when it was assayed on a limited number of lines, it did not detect *Glu-B1br* in any of them. Further application of this assay, perhaps on accessions which have been reported to carry *Bx* overexpression allele (Ragupathy et al. 2008; Jin et al. 2011) and/or on additional ancestors of H45 (Figure 4.8 and 4.9 of this thesis) might reveal additional carriers of *Glu-B1br*, and possibly shed light on the origin of this allele. Similarly, it is possible that an assay could be developed to distinguish *Glu-B1bs* from *Glu-B1al* and could be used to provide insights into the origin of that allele.

Amino acid sequences Bx7.1 and Bx7.2 were translated from the two x-type glutenin genes of the *Glu-B1br* allele. Compared to the amino acid sequence of $Bx7^{OE}$, which is encoded by *Glu-B1al*, four and one amino acid polymorphisms were discovered in Bx7.1 and Bx7.2, respectively. The most interesting of these was a tyrosine-to-cysteine change in the repetitive domain of the Bx7.1 subunit which is encoded by the first Bx gene of H45. It seemed likely that the additional cysteine residue could affect the pattern of disulphide bond formation, and hence the composition and functionality of glutenin polymers (Tamás et al. 2002; Pirozi et al. 2008). This was a challenging hypothesis to test, for several reasons. Firstly, the tight linkage of the Bx genes is likely to prevent genetic separation of *Bx7.1* from *Bx7.2*. Secondly, given the similar amino acid sequences of Bx7.1 and Bx7.2, it is unlikely that they could be separated from natural flour. Thirdly, even within Bx7.1, there are four amino acid polymorphisms compared to $Bx7^{OE}$, making it difficult to separate the effects of the additional cysteine from possible effects of the other three polymorphisms. Thus, heterologous expression was conducted to obtain individual glutenin subunits and site-directed mutagenesis was used to generate a mutant gene encoding a subunit different from Bx7.1 by only the tyrosine-to-cysteine change of interest. Heterologous expression, which has been previously used to demonstrate the functional effects of specific glutenins (Xu et al. 2006; Yan et al. 2009a; Liu et al. 2010; Chen et al. 2011) was successful here. Compared with the chromatography isolation methods which have been used to obtain individual glutenin subunits (Tatham et al. 1991), heterologous expression allows for isolation of very similar subunits, such as those encoded by the tightly linked genes within the Bx overexpression alleles. Another merit of heterologous expression in this research is that it allowed the generation of an artificial subunit encoded by the mutant gene *MutBx7.1*, providing clear

evidence that the effects of *Glu-B1br* were due to the presence of a cysteine in the repetitive domain of the Bx7.1 HMW-GS. Genetic transformation of plants has also been used to compare individual subunits (Blechl and Anderson 1996; Barro et al. 1997), but this is more time-consuming than heterologous expression of subunits in *E.coli*.

As heterologous expression generated only 10 to 20 mg purified protein per litre of LB medium, small scale tests were needed to investigate the functional properties of both flour derived from wheat grains and flour incorporated with individual glutenin subunit heterologously expressed from *E.coli*. The flour samples for small scale measurements used here (SE-HPLC analysis, SDS-sedimentation test, dough mixing test and mixograph test) ranged from 25 mg to 10 g. The results derived from different measurements demonstrated consistent variation among different samples. For flour incorporated with individual glutenin subunits, the SDS-sedimentation test modified with a reversible reduction/oxidation procedure provided an option for preliminary study on the properties of individual glutenin subunits, which could reduce the sample scale to 0.5 g. This provided a reliable measurement method to determine the effect of individual subunits in smaller amount, compared to the dough mixing test. Among 12 accessions used in this research, the %UPP values analysed by SE-HPLC were consistent with restriction digest results used to determine the genotype of Bx overexpression alleles. If no better DNA marker for *Glu-B1br* allele could be designed, this may provide an option to distinguish the *Glu-B1br* allele from the other overexpression alleles. Analysis of UPP by SE-HPLC in duplicate samples would require only 25 mg of flour (approximately 3 to 5 grains) and would be less expensive than application of the restriction digest assay reported in Chapter 3 of this thesis.

In this research, the effects on glutenin polymerisation and dough properties of the *Glu-B1br* and *Glu-B1bs* alleles have been studied. It seems that one of the HMW-GS encoded by *Glu-B1br* interferes with the formation of glutenin polymers, leading to poor flour and dough properties in H45 and some of its progeny. The understanding of this effect generated in this thesis research will help wheat breeders understand, predict and manage the outcomes of crosses involving H45.

With the combination methods of site-directed mutagenesis and heterologous expression, subunits Bx7.1 and MutBx7.1 were generated that differed only at the 512th position (cysteine in Bx7.1; tyrosine in MutBx7.1). The direct comparison between Bx7.1 and MutBx7.1 showed that the functional properties of the two subunits are significantly different from each other, which unambiguously demonstrated that the cysteine residue at position 512th of the Bx7.1 affects functional properties. The poor functional properties of the Bx7.1 subunit are probably due to cross-linkages involving the cysteine residue located in the repetitive domain. Based on some previous research (Köhler et al. 1993; Anderson and Békés 2011; Lutz et al. 2012), it might have been expected that availability of larger numbers of cysteine residues to form inter-chain disulfide bonds would have a favourable effect on polymerisation, flour and dough properties. The opposite effect observed here is similar to what was observed by Liu et al. (2010) for an HMW-GS from *Australopyrum retrofractum*, a wheat-related grass. That subunit carries an additional cysteine residue in its C-terminal domain. In dough mixing tests, it had detrimental effects on dough properties. Liu et al. (2010) suggested that the additional cysteine residue may form an intra-molecular cross-linkage with the conserved cysteine in its C-terminal domain, inhibiting the conserved cysteine from forming inter-chain disulfide bonds with other glutenin subunits.

Possible cross-linkages that may be formed by the additional cysteine in the Bx7.1 subunit were presented in Chapter 6 of this thesis, showing several possible explanations of its functional effects. As there is no direct evidence of which type of cross links are actually formed by the additional cysteine residue in Bx7.1 of H45, future research could be conducted with the purified Bx7.1 and MutBx7.1 subunits, to study the disulfide bonds formed by different numbers and locations of cysteine residues. One possible approach would be that used by Lutz et al. (2012). Heterologously expressed proteins could be reoxidized by KIO_3 and digested with thermolysin. Digested fragments could be separated by liquid chromatography-mass spectrometry (LC-MS) to distinguish between some of the possible disulfide cross-linkages.

Other site-directed mutant subunits could also be generated and investigated. The conserved cysteine residues in HMW-GS could be mutated by different numbers of non-cysteine residues (one to three for most of x-type HMW-GS and one to six for y-type HMW-GS) at different locations. This will provide an option to study the cross-linkages between each two cysteine residues, to determine whether they form an inter- or intra-chain disulfide bond. The simplest way would start with just one cysteine mutant at different locations in one subunit. Thus, compared with the wild-type subunit, the normal cross-linkages may not exist in mutant subunits because of the lack of the cysteine residue, which will have different functional properties with dough mixing tests and have different profiles in LC-MS (Lutz et al. 2012) analysis. Furthermore, different types of cross-linkages could be deduced according to the comparison of functional properties and amino acid sequences. However, different cross-linkages may appear according to the different positions of the existing cysteine residues. By this way, the study of the formation of disulfide bonds between different cysteine

residues could be simplified and addressed in detail with high confidence.

Moreover, novel artificial glutenin genes could be derived from natural glutenin genes by altering DNA sequences encoding other amino acids into those encoding cysteine residues at different positions within glutenin subunits. The novel glutenin subunits could be analysed on their functional properties with the methodology reported in this thesis. Novel glutenin genes encoding subunits with favourable properties could be selected to be transformed into wheat, to extend the diversity of glutenin composition available for wheat breeding.

Chapter 8

Conclusions

The research reported in this thesis led to the following conclusions:

1. The Australian wheat cultivar H45 has lower SDS-sedimentation volume, lower UPP value, and weaker dough mixing properties than the Canadian cultivar Glenlea and the Australian breeding line VQ0437.

2. Like the previously characterised *Glu-B1al* allele of Glenlea, the *Glu-B1* alleles in H45 and the breeding line VQ0437 each include two genes encoding x-type HMW-GS.

3. The *Glu-B1* allele of H45 (designated *Glu-B1br*) differs from the *Glu-B1al* allele by four SNPs in each of its two x-type genes.

4. One of the SNPs between the first x-type gene of the *Glu-B1br* and the *Glu-B1al* x-type genes alters a tyrosine into a cysteine in the repetitive domain of the subunit.

5. The *Glu-B1br* allele can be distinguished from other Bx overexpression alleles by a restriction digest assay and was strongly associated with low UPP among 20 plants of F_2 H45/VQ0437 progeny.

6. Of 10 wheat lines found to overexpress Bx HWM-GS, only H45 carries the *Glu-B1br* allele. H45 had lower UPP value than the other nine lines.

7. Reductions of SDS-sedimentation volume, UPP value and dough mixing parameters caused by *Glu-B1br* are due to the tyrosine-to-cysteine substitution in the repetitive domain of the

Bx7.1 subunit.

Chapter 9

Contributions to Knowledge

The research reported in this thesis was targeted to study the flour and dough properties of an Australian wheat H45, to explain the relatively poor dough properties of H45 and to provide information that could be applied for quality selection in wheat breeding. The new contributions to knowledge of this thesis are:

1. Discovery of two glutenin alleles at the *Glu-B1* locus, *Glu-B1br* (in H45) and *Glu-B1bs* (in an Australian breeding line, VQ0437) and demonstration that, like the previously known *Glu-B1al* allele, these alleles contain two x-type genes and confer overexpression of Bx subunits relative to other HMW-GS.

2. Development of a restriction digest assay that detects a SNP controlling a tyrosine-to-cysteine change in the first x-type gene of the *Glu-B1br* allele. This assay can be used to distinguish the *Glu-B1br* allele from *Glu-B1al* and *Glu-B1bs*.

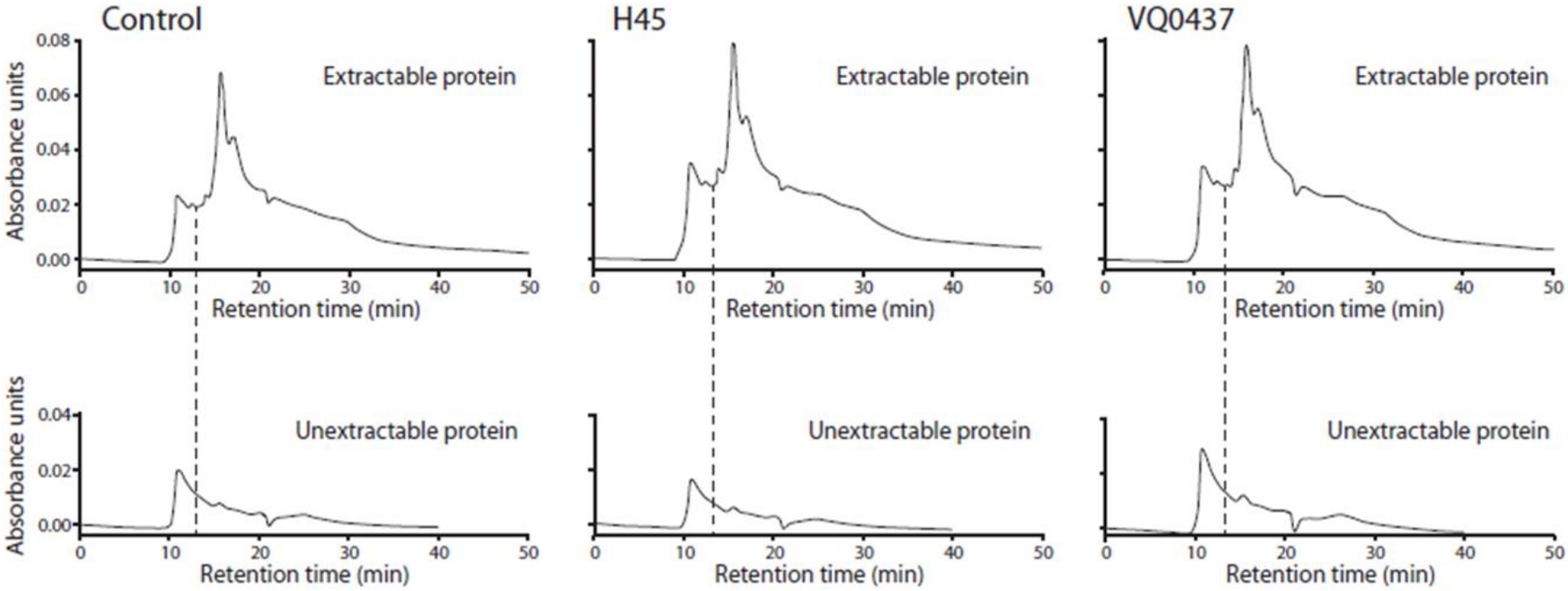
3. Discovery that variation in SDS-unextractable polymeric protein (a predictor of dough strength) was strongly associated with segregation of the *Glu-B1br* and *Glu-B1bs* alleles among progeny of a cross between H45 and VQ0437.

4. Knowledge of the effects on the flour and dough functional properties of individual glutenin subunit encoded by three Bx overexpression glutenin alleles and a mutant gene derived from the first x-type gene of the *Glu-B1br* allele and the property differences caused by the additional cysteine. To my knowledge, this is the first report of the application of the

combined methods of site-directed mutagenesis and heterologous expression to rapidly produce glutenin subunits with only the targeted amino acid differences.

Appendices

Appendix 1. Size-exclusion HPLC separations of extractable and unextractable protein from a control sample of bakers' flour and from flour milled from H45 and VQ0437 wheat. The areas to the left of the vertical dashed lines indicate polymeric protein.



Appendix 2. The nucleotide sequence of a duplicated gene at the *Glu-B1* locus that encodes the high-molecular-weight glutenin subunit Bx7 in Glenlea wheat (EU157184), aligned with the sequences of corresponding genes from the wheat variety H45 (JF938070, JF938071) and the wheat breeding line VQ0437 (JF938072 JF938073). In the H45 and VQ0437 sequences, nucleotides that differ from the corresponding nucleotide in the Glenlea sequence are shown in white on a black background.

```
Glenlea  Bx7 copies 1 and 2  ATGGCTAAGCGCCTGGTCCTCTTTGCGGCAGTAGTCGTCGCCCTCGTGGCTCTCACCGCC 60
H45      Bx7-like copy 1     ATGGCTAAGCGCCTGGTCCTCTTTGCGGCAGTAGTCGTCGCCCTCGTGGCTCTCACCGCC 60
H45      Bx7-like copy 2     ATGGCTAAGCGCCTGGTCCTCTTTGCGGCAGTAGTCGTCGCCCTCGTGGCTCTCACCGCC 60
VQ0437   Bx7-like copy 1     ATGGCTAAGCGCCTGGTCCTCTTTGCGGCAGTAGTCGTCGCCCTCGTGGCTCTCACCGCC 60
VQ0437   Bx7-like copy 2     ATGGCTAAGCGCCTGGTCCTCTTTGCGGCAGTAGTCGTCGCCCTCGTGGCTCTCACCGCC 60

Glenlea  Bx7 copies 1 and 2  GCTGAAGGTGAGGCCTCTGGACAACTACAATGTGAGCACGAGCTCGAGGCATGCCAACAG 120
H45      Bx7-like copy 1     GCTGAAGGTGAGGCCTCTGGACAACTACAATGTGAGCACGAGCTCGAGGCATGCCAACAG 120
H45      Bx7-like copy 2     GCTGAAGGTGAGGCCTCTGGACAACTACAATGTGAGCACGAGCTCGAGGCATGCCAACAG 120
VQ0437   Bx7-like copy 1     GCTGAAGGTGAGGCCTCTGGACAACTACAATGTGAGCACGAGCTCGAGGCATGCCAACAG 120
VQ0437   Bx7-like copy 2     GCTGAAGGTGAGGCCTCTGGACAACTACAATGTGAGCACGAGCTCGAGGCATGCCAACAG 120

Glenlea  Bx7 copies 1 and 2  GTGGTGGACCAGCAACTCCGAGACGTTAGCCCCGGGTGCCGCCCCATCACCGTCAGCCCG 180
H45      Bx7-like copy 1     GTGGTGGACCAGCAACTCCGAGACGTTAGCCCCGGGTGCCGCCCCATCACCGTCAGCCCG 180
H45      Bx7-like copy 2     GTGGTGGACCAGCAACTCCGAGACGTTAGCCCCGGGTGCCGCCCCATCACCGTCAGCCCG 180
VQ0437   Bx7-like copy 1     GTGGTGGACCAGCAACTCCGAGACGTTAGCCCCGGGTGCCGCCCCATCACCGTCAGCCCG 180
VQ0437   Bx7-like copy 2     GTGGTGGACCAGCAACTCCGAGACGTTAGCCCCGGGTGCCGCCCCATCACCGTCAGCCCG 180

Glenlea  Bx7 copies 1 and 2  GGCACGAGACAATACGAGCAGCAACCTGTGGTGCCGTCCAAGGCCGGATCCTTCTACCCC 240
H45      Bx7-like copy 1     GGCACGAGACAATACGAGCAGCAACCTGTGGTGCCGTCCAAGGCCGGATCCTTCTACCCC 240
H45      Bx7-like copy 2     GGCACGAGGCAATACGAGCAGCAACCTGTGGTGCCGTCCAAGGCCGGATCCTTCTACCCC 240
VQ0437   Bx7-like copy 1     GGCACGAGACAATACGAGCAGCAACCTGTGGTGCCGTCCACGGCCGGATCCTTCTACCCC 240
VQ0437   Bx7-like copy 2     GGCACGAGACAATACGAGCAGCAACCTGTGGTGCCGTCCAAGGCCGGATCCTTCTACCCC 240
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Glenlea  Bx7 copies 1 and 2  AGCGAGACTACGCCTTCGCAGCAACTCCAACAAATGATATTTTGGGGAATACCTGCACTA 300
H45      Bx7-like copy 1     AGCGAGACTACGCCTTCGCAGCAACTCCAACAAATGATATTTTGGGGAATACCTGCACTA 300
H45      Bx7-like copy 2     AGCGAGACTACGCCTTCGCAGCAACTCCAACAAATGATATTTTGGGGAATACCTGCACTA 300
VQ0437   Bx7-like copy 1     AGCGAGACTACGCCTTCGCAGCAACTCCAACAAATGATATTTTGGGGAATACCTGCACTA 300
VQ0437   Bx7-like copy 2     AGCGAGACTACGCCTTCGCAGCAACTCCAACAAATGATATTTTGGGGAATACCTGCACTA 300

Glenlea  Bx7 copies 1 and 2  CTAAGAAGGTATTACCCAAGTGTAACTTCTTCGCAGCAGGGGTCATACTATCCAGGCCAA 360
H45      Bx7-like copy 1     CTAAGAAGGTATTACCCAAGTGTAACTTCTTCGCAGCAGGGGTCATACTATCCAGGCCAA 360
H45      Bx7-like copy 2     CTAAGAAGGTATTACCCAAGTGTAACTTCTTCGCAGCAGGGGTCATACTATCCAGGCCAA 360
VQ0437   Bx7-like copy 1     CTAAGAAGGTATTACCCAAGTGTAACTTCTTCGCAGCAGGGGTCATACTATCCAGGCCAA 360
VQ0437   Bx7-like copy 2     CTAAGAAGGTATTACCCAAGTGTAACTTCTTCGCAGCAGGGGTCATACTATCCAGGCCAA 360

Glenlea  Bx7 copies 1 and 2  GCTTCTCCCCAACAGTCAGGACAAGGACAGCAGCCAGGACAAGAACAGCAACCAGGACAA 420
H45      Bx7-like copy 1     GCTTCTCCCCAACAGTCAGGACAAGGACAGCAGCCAGGACAAGAACAGCAACCAGGACAA 420
H45      Bx7-like copy 2     GCTTCTCCCCAACAGTCAGGACAAGGACAGCAGCCAGGACAAGAACAGCAACCAGGACAA 420
VQ0437   Bx7-like copy 1     GCTTCTCCCCAACAGTCAGGACAAGGACAGCAGCCAGGACAAGAACAGCAACCAGGACAA 420
VQ0437   Bx7-like copy 2     GCTTCTCCCCAACAGTCAGGACAAGGACAGCAGCCAGGACAAGAACAGCAACCAGGACAA 420

Glenlea  Bx7 copies 1 and 2  GGGCAACAAGATCAGCAGCCAGGACAAAGACAACAAGGATACTACCCAACTTCTCCGCAA 480
H45      Bx7-like copy 1     GGGCAACAAGATCAGCAGCCAGGACAAAGACAACAAGGATACTACCCAACTTCTCCGCAA 480
H45      Bx7-like copy 2     GGGCAACAAGATCAGCAGCCAGGACAAAGACAACAAGGATACTACCCAACTTCTCCGCAA 480
VQ0437   Bx7-like copy 1     GGGCAACAAGATCAGCAGCCAGGACAAAGACAACAAGGATACTACCCAACTTCTCCGCAA 480
VQ0437   Bx7-like copy 2     GGGCAACAAGATCAGCAGCCAGGACAAAGACAACAAGGATACTACCCAACTTCTCCGCAA 480

Glenlea  Bx7 copies 1 and 2  CAGCCAGGACAAGGGCAACAACTGGGACAAGGGCAACCAGGGTACTACCCAACTTCACAG 540
H45      Bx7-like copy 1     CAGCCAGGACAAGGGCAACAACTGGGACAAGGGCAACCAGGGTACTACCCAACTTCACAG 540
H45      Bx7-like copy 2     CAGCCAGGACAAGGGCAACAACTGGGACAAGGGCAACCAGGGTACTACCCAACTTCACAG 540
VQ0437   Bx7-like copy 1     CAGCCAGGACAAGGGCAACAACTGGGACAAGGGCAACCAGGGTACTACCCAACTTCACAG 540
VQ0437   Bx7-like copy 2     CAGCCAGGACAAGGGCAACAACTGGGACAAGGGCAACCAGGGTACTACCCAACTTCACAG 540
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Glenlea  Bx7 copies 1 and 2  CAGCCAGGACAAAAGCAGCAGGCAGGACAAGGGCAACAATCAGGACAAGGACAACAAGGG 600
H45      Bx7-like copy 1     CAGCCAGGACAAAAGCAGCAGGCAGGACAAGGGCAACAATCAGGACAAGGACAACAAGGG 600
H45      Bx7-like copy 2     CAGCCAGGACAAAAGCAGCAGGCAGGACAAGGGCAACAATCAGGACAAGGACAACAAGGG 600
VQ0437   Bx7-like copy 1     CAGCCAGGACAAAAGCAGCAGGCAGGACAAGGGCAACAATCAGGACAAGGACAACAAGGG 600
VQ0437   Bx7-like copy 2     CAGCCAGGACAAAAGCAGCAGGCAGGACAAGGGCAACAATCAGGACAAGGACAACAAGGG 600

Glenlea  Bx7 copies 1 and 2  TACTACCCAACTTCCCCGCAACAGTCAGGACAAGGGCAACAACCGGGACAAGGGCAACCA 660
H45      Bx7-like copy 1     TACTACCCAACTTCCCCGCAACAGTCAGGACAAGGGCAACAACCGGGACAAGGGCAACCA 660
H45      Bx7-like copy 2     TACTACCCAACTTCCCCGCAACAGTCAGGACAAGGGCAACAACCGGGACAAGGGCAACCA 660
VQ0437   Bx7-like copy 1     TACTACCCAACTTCCCCGCAACAGTCAGGACAAGGGCAACAACCGGGACAAGGGCAACCA 660
VQ0437   Bx7-like copy 2     TACTACCCAACTTCCCCGCAACAGTCAGGACAAGGGCAACAACCGGGACAAGGGCAACCA 660

Glenlea  Bx7 copies 1 and 2  GGGTACTACCCAACTTCTCCGCAGCAGTCAGGACAATGGCAGCAACCAGGACAAGGGCAA 720
H45      Bx7-like copy 1     GGGTACTACCCAACTTCTCCGCAGCAGTCAGGACAATGGCAGCAACCAGGACAAGGGCAA 720
H45      Bx7-like copy 2     GGGTACTACCCAACTTCTCCGCAGCAGTCAGGACAATGGCAGCAACCAGGACAAGGGCAA 720
VQ0437   Bx7-like copy 1     GGGTACTACCCAACTTCTCCGCAGCAGTCAGGACAATGGCAGCAACCAGGACAAGGGCAA 720
VQ0437   Bx7-like copy 2     GGGTACTACCCAACTTCTCCGCAGCAGTCAGGACAATGGCAGCAACCAGGACAAGGGCAA 720

Glenlea  Bx7 copies 1 and 2  CAACCAGGACAAGGGCAGCAATCAGGACAAGGGCAACAAGGTCAGCAGCCAGGACAAGGG 780
H45      Bx7-like copy 1     CAACCAGGACAAGGGCAGCAATCAGGACAAGGGCAACAAGGTCAGCAGCCAGGACAAGGG 780
H45      Bx7-like copy 2     CAACCAGGACAAGGGCAGCAATCAGGACAAGGGCAACAAGGTCAGCAGCCAGGACAAGGG 780
VQ0437   Bx7-like copy 1     CAACCAGGACAAGGGCAGCAATCAGGACAAGGGCAACAAGGTCAGCAGCCAGGACAAGGG 780
VQ0437   Bx7-like copy 2     CAACCAGGACAAGGGCAGCAATCAGGACAAGGGCAACAAGGTCAGCAGCCAGGACAAGGG 780

Glenlea  Bx7 copies 1 and 2  CAACGACCAGGACAAGGACAACAAGGGTACTACCCAATTTCTCCGCAACAGCCGGGACAA 840
H45      Bx7-like copy 1     CAACGACCAGGACAAGGACAACAAGGGTACTACCCAATTTCTCCGCAACAGCCGGGACAA 840
H45      Bx7-like copy 2     CAACGACCAGGACAAGGACAACAAGGGTACTACCCAATTTCTCCGCAACAGCCGGGACAA 840
VQ0437   Bx7-like copy 1     CAACGACCAGGACAAGGACAACAAGGGTACTACCCAATTTCTCCGCAACAGCCGGGACAA 840
VQ0437   Bx7-like copy 2     CAACGACCAGGACAAGGACAACAAGGGTACTACCCAATTTCTCCGCAACAGCCGGGACAA 840
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Glenlea  Bx7 copies 1 and 2  GGGCAACAATCAGGACAAGGGCAACCAGGGTACTACCCAACTTCTTTGCGGCAGCCAGGA 900
H45      Bx7-like copy 1     GGGCAACAATCAGGACAAGGGCAACCAGGGTACTACCCAACTTCTTTGCGGCAGCCAGGA 900
H45      Bx7-like copy 2     GGGCAACAATCAGGACAAGGGCAACCAGGGTACTACCCAACTTCTTTGCGGCAGCCAGGA 900
VQ0437   Bx7-like copy 1     GGGCAACAATCAGGACAAGGGCAACCAGGGTACTACCCAACTTCTTTGCGGCAGCCAGGA 900
VQ0437   Bx7-like copy 2     GGGCAACAATCAGGACAAGGGCAACCAGGGTACTACCCAACTTCTTTGCGGCAGCCAGGA 900

Glenlea  Bx7 copies 1 and 2  CAATGGCAGCAACCAGGACAAGGGCAGCAACCAGGACAAGGGCAACAAGGTCAGCAGCCA 960
H45      Bx7-like copy 1     CAATGGCAGCAACCAGGACAAGGGCAGCAACCAGGACAAGGGCAACAAGGTCAGCAGCCA 960
H45      Bx7-like copy 2     CAATGGCAGCAACCAGGACAAGGGCAGCAACCAGGACAAGGGCAACAAGGTCAGCAGCCA 960
VQ0437   Bx7-like copy 1     CAATGGCAGCAACCAGGACAAGGGCAGCAACCAGGACAAGGGCAACAAGGTCAGCAGCCA 960
VQ0437   Bx7-like copy 2     CAATGGCAGCAACCAGGACAAGGGCAGCAACCAGGACAAGGGCAACAAGGTCAGCAGCCA 960

Glenlea  Bx7 copies 1 and 2  GGACAAGGACAACAATCAGGACAAGGACAACAAGGATACTACCCAACTTCTCTGCAACAG 1020
H45      Bx7-like copy 1     GGACAAGGACAACAATCAGGACAAGGACAACAAGGATACTACCCAACTTCTCTGCAACAG 1020
H45      Bx7-like copy 2     GGACAAGGACAACAATCAGGACAAGGACAACAAGGATACTACCCAACTTCTCTGCAACAG 1020
VQ0437   Bx7-like copy 1     GGACAAGGACAACAATCAGGACAAGGACAACAAGGATACTACCCAACTTCTCTGCAACAG 1020
VQ0437   Bx7-like copy 2     GGACAAGGACAACAATCAGGACAAGGACAACAAGGATACTACCCAACTTCTCTGCAACAG 1020

Glenlea  Bx7 copies 1 and 2  CCAGGACAAGGGCAACAACTGGGACAAGGGCAACCAGGGTACTACCCAACTTCGCAGCAG 1080
H45      Bx7-like copy 1     CCAGGACAAGGGCAACAACTGGGACAAGGGCAACCAGGGTACTACCCAACTTCGCAGCAG 1080
H45      Bx7-like copy 2     CCAGGACAAGGGCAACAACTGGGACAAGGGCAACCAGGGTACTACCCAACTTCGCAGCAG 1080
VQ0437   Bx7-like copy 1     CCAGGACAAGGGCAACAACTGGGACAAGGGCAACCAGGGTACTACCCAACTTCGCAGCAG 1080
VQ0437   Bx7-like copy 2     CCAGGACAGGGGCAACAACTGGGACAAGGGCAACCAGGGTACTACCCAACTTCGCAGCAG 1080

Glenlea  Bx7 copies 1 and 2  TCGGAACAAGGGCAGCAGCCAGGACAAGGAAAACAACCAGGACAAGGACAACAAGGGTAC 1140
H45      Bx7-like copy 1     TCGGAACAAGGGCAGCAGCCAGGACAAGGAAAACAACCAGGACAAGGACAACAAGGGTAC 1140
H45      Bx7-like copy 2     TCGGAACAAGGGCAGCAGCCAGGACAAGGAAAACAACCAGGACAAGGACAACAAGGGTAC 1140
VQ0437   Bx7-like copy 1     TCGGAACAAGGGCAGCAGCCAGGACAAGGAAAACAACCAGGACAAGGACAACAAGGGTAC 1140
VQ0437   Bx7-like copy 2     TCGGAACAAGGGCAGCAGCCAGGACAAGGAAAACAACCAGGACAAGGACAACAAGGGTAC 1140
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Glenlea  Bx7 copies 1 and 2  TACCCAACTTCTCCGCAACAGTCAGGACAAGGGCAACAACTGGGACAAGGGCAACCAGGG 1200
H45      Bx7-like copy 1     TACCCAACTTCTCCGCAACAGTCAGGACAAGGGCAACAACTGGGACAAGGGCAACCAGGG 1200
H45      Bx7-like copy 2     TACCCAACTTCTCCGCAACAGTCAGGACAAGGGCAACAACTGGGACAAGGGCAACCAGGG 1200
VQ0437   Bx7-like copy 1     TACCCAACTTCTCCGCAACAGTCAGGACAAGGGCAACAACTGGGACAAGGGCAACCAGGG 1200
VQ0437   Bx7-like copy 2     TACCCAACTTCTCCGCAACAGTCAGGACAAGGGCAACAACTGGGACAAGGGCAACCAGGG 1200

Glenlea  Bx7 copies 1 and 2  TACTACCCAACTTCTCCACAGCAGTCAGGACAAGGACAACAATCAGGACAAGGACAACAA 1260
H45      Bx7-like copy 1     TACTACCCAACTTCTCCACAGCAGTCAGGACAAGGACAACAATCAGGACAAGGACAACAA 1260
H45      Bx7-like copy 2     TACTACCCAACTTCTCCACAGCAGTCAGGACAAGGACAACAATCAGGACAAGGACAACAA 1260
VQ0437   Bx7-like copy 1     TACTACCCAACTTCTCCACAGCAGTCAGGACAAGGACAACAATCAGGACAAGGACAACAA 1260
VQ0437   Bx7-like copy 2     TACTACCCAACTTCTCCACAGCAGTCAGGACAAGGACAACAATCAGGACAAGGACAACAA 1260

Glenlea  Bx7 copies 1 and 2  GGGTACTACCCAACTTCTCCGCAACAGTCAGGACAAGGGCAACAACCGGGACAAGGGCAA 1320
H45      Bx7-like copy 1     GGGTACTACCCAACTTCTCCGCAACAGTCAGGACAAGGGCAACAACCGGGACAAGGGCAA 1320
H45      Bx7-like copy 2     GGGTACTACCCAACTTCTCCGCAACAGTCAGGACAAGGGCAACAACCGGGACAAGGGCAA 1320
VQ0437   Bx7-like copy 1     GGGTACTACCCAACTTCTCCGCAACAGTCAGGACAAGGGCAACAACCGGGACAAGGGCAA 1320
VQ0437   Bx7-like copy 2     GGGTACTACCCAACTTCTCCGCAACAGTCAGGACAAGGGCAACAACCGGGACAAGGGCAA 1320

Glenlea  Bx7 copies 1 and 2  TCGGGGTACTTCCCAACTTCTCGGCAGCAGTCAGGACAAGGGCAGCAGCCAGGACAAGGA 1380
H45      Bx7-like copy 1     TCGGGGTACTTCCCAACTTCTCGGCAGCAGTCAGGACAAGGGCAGCAGCCAGGACAAGGA 1380
H45      Bx7-like copy 2     TCGGGGTACTTCCCAACTTCTCGGCAGCAGTCAGGACAAGGGCAGCAGCCAGGACAAGGA 1380
VQ0437   Bx7-like copy 1     TCGGGGTACTTCCCAACTTCTCGGCAGCAGTCAGGACAAGGGCAGCAGCCAGGACAAGGA 1380
VQ0437   Bx7-like copy 2     TCGGGGTACTTCCCAACTTCTCGGCAGCAGTCAGGACAAGGGCAGCAGCCAGGACAAGGA 1380

Glenlea  Bx7 copies 1 and 2  CAACAGTCGGGACAAGGGCAACAAGGTCAGCAACCAGGACAAGGACAACAAGCGTACTAC 1440
H45      Bx7-like copy 1     CAACAGTCGGGACAAGGGCAACAAGGTCAGCAACCAGGACAAGGACAACAAGCGTACTAC 1440
H45      Bx7-like copy 2     CAACAGTCGGGACAAGGGCAACAAGGTCAGCAACCAGGACAAGGACAACAAGCGTACTAC 1440
VQ0437   Bx7-like copy 1     CAACAGTCGGGACAAGGGCAACAAGGTCAGCAACCAGGACAGGGACAACAAGCGTACTAC 1440
VQ0437   Bx7-like copy 2     CAACAGTCGGGACAAGGGCAACAAGGTCAGCAACCAGGACAAGGACAACAAGCGTACTAC 1440
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Glenlea  Bx7 copies 1 and 2  CCAACTTCTTCGCAACAGTCAAGACAAAGGCAACAGGCAGGACAATGGCAACGACCGGGA 1500
H45      Bx7-like copy 1     CCAACTTCTTCGCAACAGTCAAGACAAAGGCAACAGGCAGGACAATGGCAACGACCGGGA 1500
H45      Bx7-like copy 2     CCAACTTCTTCGCAACAGTCAAGACAAAGGCAACAGGCAGGACAATGGCAACGACCGGGA 1500
VQ0437   Bx7-like copy 1     CCAACTTCTTCGCAACAGTCAAGACAAAGGCAACAGGCAGGACAATGGCAACGACCGGGA 1500
VQ0437   Bx7-like copy 2     CCAACTTCTTCGCAACAGTCAAGACAAAGGCAACAGGCAGGACAATGGCAACGACCGGGA 1500

Glenlea  Bx7 copies 1 and 2  CAAGGGCAACCAGGGTACTACCCAACCTCTCCACAGCAGCCAGGACAAGAGCAACAATCA 1560
H45      Bx7-like copy 1     CAAGGGCAACCAGGGTACTACCCAACCTCTCCACAGCAGCCAGGACAAGAGCAACAATCA 1560
H45      Bx7-like copy 2     CAAGGGCAACCAGGGTACTACCCAACCCCTCCACAGCAGCCAGGACAAGAGCAACAATCA 1560
VQ0437   Bx7-like copy 1     CAAGGGCAACCAGGGTACTACCCAACCTCTCCACAGCAGCCAGGACAAGAGCAACAATCA 1560
VQ0437   Bx7-like copy 2     CAAGGGCAACCAGGGTACTACCCAACCTCTCCACAGCAGCCAGGACAAGAGCAACAATCA 1560

Glenlea  Bx7 copies 1 and 2  GGACAAGCGCAACAATCAGGACAATGGCAACTAGTGTACTACCCAACTTCTCCGCAACAG 1620
H45      Bx7-like copy 1     GGACAAGCGCAACAATCAGGACAATGGCAACTAGTGTGCTACCCAACTTCTCCGCAACAG 1620
H45      Bx7-like copy 2     GGACAAGCGCAACAATCAGGACAATGGCAACTAGTGTACTACCCAACTTCTCCGCAACAG 1620
VQ0437   Bx7-like copy 1     GGACAAGCGCAACAATCAGGACAATGGCAACTAGTGTACTACCCAACTTCTCCGCAACAG 1620
VQ0437   Bx7-like copy 2     GGACAAGCGCAACAATCAGGACAATGGCAACTAGTGTACTACCCAACTTCTCCGCAACAG 1620

Glenlea  Bx7 copies 1 and 2  CCAGGCCAATTGCAACAACCAGCACAAGGGCAACAACCAGCACAAGGGCAACAATCAGCA 1680
H45      Bx7-like copy 1     CCAGGCCAATTGCAACAACCAGCACAAGGGCAACGACCAGCACAAGGGCAACAATCAGCA 1680
H45      Bx7-like copy 2     CCAGGCCAATTGCAACAACCAGCACAAGGGCAACAACCAGCACAAGGGCAACAATCAGCA 1680
VQ0437   Bx7-like copy 1     CCAGGCCAATTGCAACAACCAGCACAAGGGCAACAACCAGCACAAGGGCAACAATCAGCA 1680
VQ0437   Bx7-like copy 2     CCAGGCCAATTGCAACAACCAGCACAAGGGCAACAACCAGCACAAGGGCAACAATCAGCA 1680

Glenlea  Bx7 copies 1 and 2  CAAGAGCAACAGCCAGGACAAGCGCAACAATCAGGACAATGGCAACTAGTGTACTACCCA 1740
H45      Bx7-like copy 1     CAAGAGCAACAGCCAGGACAAGCGCAACAATCAGGACAATGGCAACTAGTGTACTACCCA 1740
H45      Bx7-like copy 2     CAAGAGCAACAGCCAGGACAAGCGCAACAATCAGGACAATGGCAACTAGTGTACTACCCA 1740
VQ0437   Bx7-like copy 1     CAAGAGCAACAGCCAGGACAAGCGCAACAATCAGGACAATGGCAACTAGTGTACTACCCA 1740
VQ0437   Bx7-like copy 2     CAAGAGCAACAGCCAGGACAAGCGCAACAATCAGGACAATGGCAACTAGTGTACTACCCA 1740
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Glenlea  Bx7 copies 1 and 2  ACTTCTCCGCAACAGCCAGGACAATTGCAACAACCAGCACAAGGGCAACAAGGGTACTAC 1800
H45      Bx7-like copy 1     ACTTCTCCGCAACAGCCAGGACAATTGCAACAACCAGCACAAGGGCAACAAGGGTACTAC 1800
H45      Bx7-like copy 2     ACTTCTCCGCAACAGCCAGGACAATTGCAACAACCAGCACAAGGGCAACAAGGGTACTAC 1800
VQ0437   Bx7-like copy 1     ACTTCTCCGCAACAGCCAGGACAATTGCAACAACCAGCACAAGGGCAACAAGGGTACTAC 1800
VQ0437   Bx7-like copy 2     ACTTCTCCGCAACAGCCAGGACAATTGCAACAACCAGCACAAGGGCAACAAGGGTACTAC 1800

Glenlea  Bx7 copies 1 and 2  CCAACTTCTCCACAACAGTCAGGACAAGGGCAACAAGGGTACTACCCAACTTCTCCGCAA 1860
H45      Bx7-like copy 1     CCAACTTCTCCACAACAGTCAGGACAAGGGCAACAAGGGTACTACCCAACTTCTCCGCAA 1860
H45      Bx7-like copy 2     CCAACTTCTCCACAACAGTCAGGACAAGGGCAACAAGGGTACTACCCAACTTCTCCGCAA 1860
VQ0437   Bx7-like copy 1     CCAACTTCTCCACAACAGTCAGGACAAGGGCAACAAGGGTACTACCCAACTTCTCCGCAA 1860
VQ0437   Bx7-like copy 2     CCAACTTCTCCACAACAGTCAGGACAAGGGCAACAAGGGTACTACCCAACTTCTCCGCAA 1860

Glenlea  Bx7 copies 1 and 2  CAGTCAGGACAAGGGCAACAAGGGTACTACCCAACTTCTCCGCAACAGTCAGGACAAGGG 1920
H45      Bx7-like copy 1     CAGTCAGGACAAGGGCAACAAGGGTACTACCCAACTTCTCCGCAACAGTCAGGACAAGGG 1920
H45      Bx7-like copy 2     CAGTCAGGACAAGGGCAACAAGGGTACTACCCAACTTCTCCGCAACAGTCAGGACAAGGG 1920
VQ0437   Bx7-like copy 1     CAGTCAGGACAAGGGCAACAAGGGTACTACCCAACTTCTCCGCAACAGTCAGGACAAGGG 1920
VQ0437   Bx7-like copy 2     CAGTCAGGACAAGGGCAACAAGGGTACTACCCAACTTCTCCGCAACAGTCAGGACAAGGG 1920

Glenlea  Bx7 copies 1 and 2  CAGCAGCCAGGACAAGGACAACAGCCAAGACAAGGGCAACAAGGGTACTACCCAATTTCT 1980
H45      Bx7-like copy 1     CAGCAGCCAGGACAAGGACAACAGCCAAGACAAGGGCAACAAGGGTACTACCCAATTTCT 1980
H45      Bx7-like copy 2     CAGCAGCCAGGACAAGGACAACAGCCAAGACAAGGGCAACAAGGGTACTACCCAATTTCT 1980
VQ0437   Bx7-like copy 1     CAGCAGCCAGGACAAGGACAACAGCCAAGACAAGGGCAACAAGGGTACTACCCAATTTCT 1980
VQ0437   Bx7-like copy 2     CAGCAGCCAGGACAAGGACAACAGCCAAGACAAGGGCAACAAGGGTACTACCCAATTTCT 1980

Glenlea  Bx7 copies 1 and 2  CCGCAGCAGTCAGGACAAGGGCAACAACCAGGACAAGGGCAACAAGGATACTACCCAACT 2040
H45      Bx7-like copy 1     CCGCAGCAGTCAGGACAAGGGCAACAACCAGGACAAGGGCAACAAGGATACTACCCAACT 2040
H45      Bx7-like copy 2     CCGCAGCAGTCAGGACAAGGGCAACAACCAGGACAAGGGCAACAAGGATACTACCCAACT 2040
VQ0437   Bx7-like copy 1     CCGCAGCAGTCAGGACAAGGGCAACAACCAGGACAAGGGCAACAAGGATACTACCCAACT 2040
VQ0437   Bx7-like copy 2     CCGCAGCAGTCAGGACAAGGGCAACAACCAGGACAAGGGCAACAAGGATACTACCCAACT 2040
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Glenlea  Bx7 copies 1 and 2  TCTCCGCAGCAGTCAGGACAAGGGCAACAACCAGGACATGAGCAACAGCCAGGACAATGG 2100
H45      Bx7-like copy 1     TCTCCGCAGCAGTCAGGACAAGGGCAACAACCAGGACATGAGCAACAGCCAGGACAATGG 2100
H45      Bx7-like copy 2     TCTCCGCAGCAGTCAGGACAAGGGCAACAACCAGGACATGAGCAACAGCCAGGACAATGG 2100
VQ0437   Bx7-like copy 1     TCTCCGCAGCAGTCAGGACAAGGGCAACAACCAGGACATGAGCAACAGCCAGGACAATGG 2100
VQ0437   Bx7-like copy 2     TCTCCGCAGCAGTCAGGACAAGGGCAACAACCAGGACATGAGCAACAGCCAGGACAATGG 2100

Glenlea  Bx7 copies 1 and 2  CTGCAACCAGGACAAGGGCAACAAGGGTACTATCCAACTTCTTCACAGCAGTCAGGACAA 2160
H45      Bx7-like copy 1     CTGCAACCAGGACAAGGGCAACAAGGGTACTATCCAACTTCTTCACAGCAGTCAGGACAA 2160
H45      Bx7-like copy 2     CTGCAACCAGGACAAGGGCAACAAGGGTACTATCCAACTTCTTCACAGCAGTCAGGACAA 2160
VQ0437   Bx7-like copy 1     CTGCAACCAGGACAAGGGCAACAAGGGTACTATCCAACTTCTTCACAGCAGTCAGGACAA 2160
VQ0437   Bx7-like copy 2     CTGCAACCAGGACAAGGGCAACAAGGGTACTATCCAACTTCTTCACAGCAGTCAGGACAA 2160

Glenlea  Bx7 copies 1 and 2  GGGCATCAATCAGGACAAGGGCAACAAGGGTACTACCCAACTTCTCTGTGGCAACCAGGA 2220
H45      Bx7-like copy 1     GGGCATCAACCAGGACAAGGGCAACAAGGGTACTACCCAACTTCTCTGTGGCAACCAGGA 2220
H45      Bx7-like copy 2     GGGCATCAATCAGGACAAGGGCAACAAGGGTACTACCCAACTTCTCTGTGGCAACCAGGA 2220
VQ0437   Bx7-like copy 1     GGGCATCAATCAGGACAAGGGCAACAAGGGTACTACCCAACTTCTCTGTGGCAACCAGGA 2220
VQ0437   Bx7-like copy 2     GGGCATCAATCAGGACAAGGGCAACAAGGGTACTACCCAACTTCTCTGTGGCAACCAGGA 2220

Glenlea  Bx7 copies 1 and 2  CAAGGGCAACAACCAGGACAAGGGCAACAAGGCTACGCCAGCCCATACCATGTTAGCGCG 2280
H45      Bx7-like copy 1     CAAGGGCAACAACCAGGACAAGGGCAACAAGGCTACGCCAGCCCATACCATGTTAGCGCG 2280
H45      Bx7-like copy 2     CAAGGGCAACAACCGGGACAAGGGCAACAAGGCTACGCCAGCCCATACCATGTAAGCGCG 2280
VQ0437   Bx7-like copy 1     CAAGGGCAACAACCAGGACAAGGGCAACAAGGCTACGCCAGCCCATACCATGTTAGCGCG 2280
VQ0437   Bx7-like copy 2     CAAGGGCAACAACCAGGACAAGGGCAACAAGGCTACGCCAGCCCATACCATGTTAGCGCG 2280

Glenlea  Bx7 copies 1 and 2  GAGTACCAGGCGGCCCGCCTAAAGGTGGCAAAGGCGCAGCAGCTCGCGGCACAGCTGCCG 2340
H45      Bx7-like copy 1     GAGTACCAGGCGGCCCGCCTAAAGGTGGCAAGGCGCAGCAGCTCGCGGCACAGCTGCCG 2340
H45      Bx7-like copy 2     GAGTACCAGGCGGCCCGCCTAAAGGTGGCAAAGGCGCAGCAGCTCGCGGCACAGCTGCCG 2340
VQ0437   Bx7-like copy 1     GAGTACCAGGCGGCCCGCCTAAAGGTGGCAAAGGCGCAGCAGCTCGCGGCACAGCTGCCG 2340
VQ0437   Bx7-like copy 2     GAGTACCAGGCGGCCCGCCTAAAGGTGGCAAAGGCGCAGCAGCTCGCGGCACAGCTGCCG 2340
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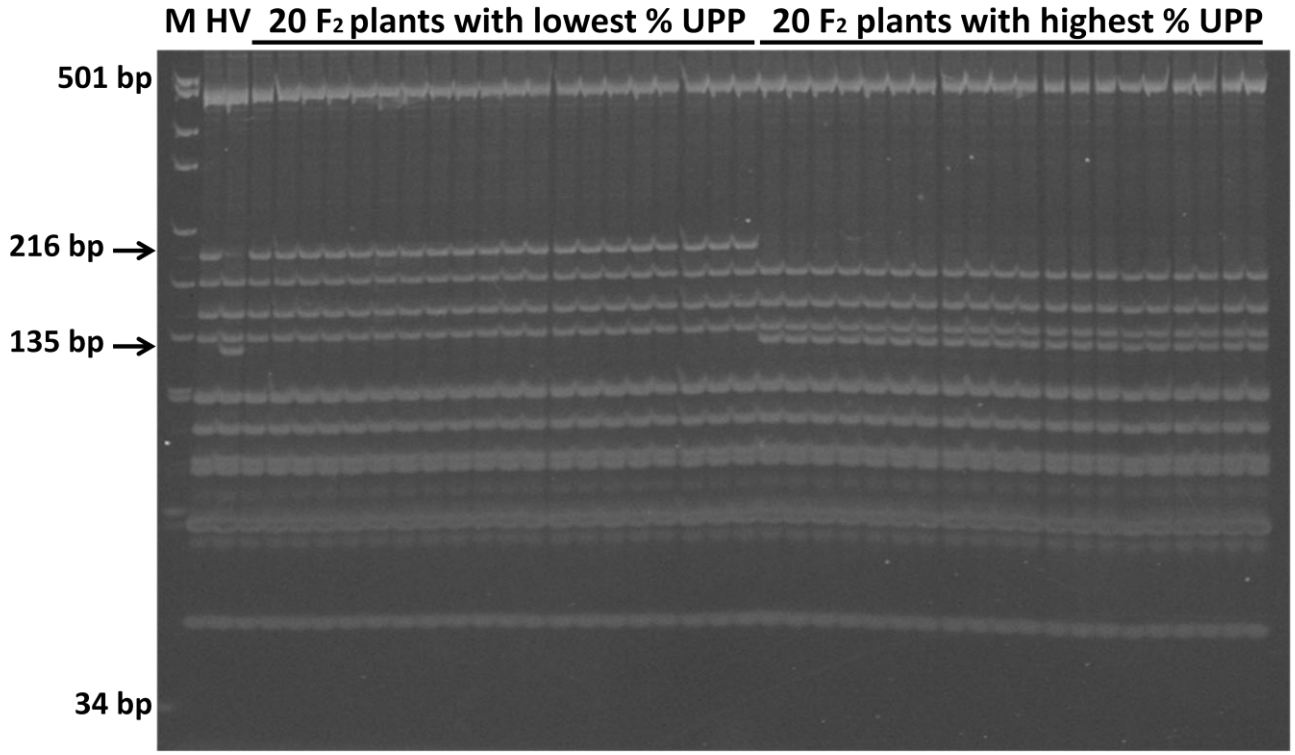
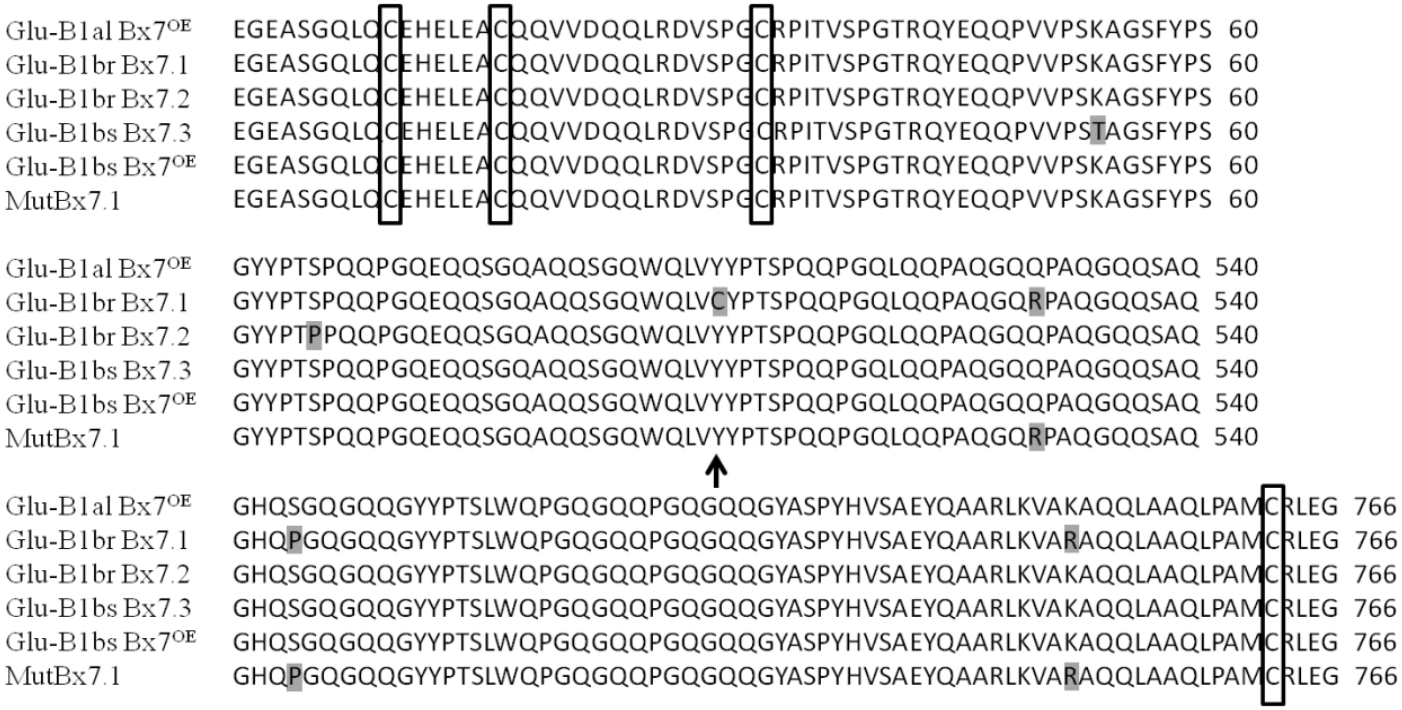
Glenlea	Bx7 copies 1 and 2	GCAATGTGCCGGCTGGAGGGCAGCGACGCATTGTCGACCAGGCAGTGATAG	2391
H45	Bx7-like copy 1	GCAATGTGCCGGCTGGAGGGCAGCGACGCATTGTCGACCAGGCAGTGATAG	2391
H45	Bx7-like copy 2	GCAATGTGCCGGCTGGAGGGCAGCGACGCATTGTCGACCAGGCAGTGATAG	2391
VQ0437	Bx7-like copy 1	GCAATGTGCCGGCTGGAGGGCAGCGACGCATTGTCGACCAGGCAGTGATAG	2391
VQ0437	Bx7-like copy 2	GCAATGTGCCGGCTGGAGGGCAGCGACGCATTGTCGACCAGGCAGTGATAG	2391

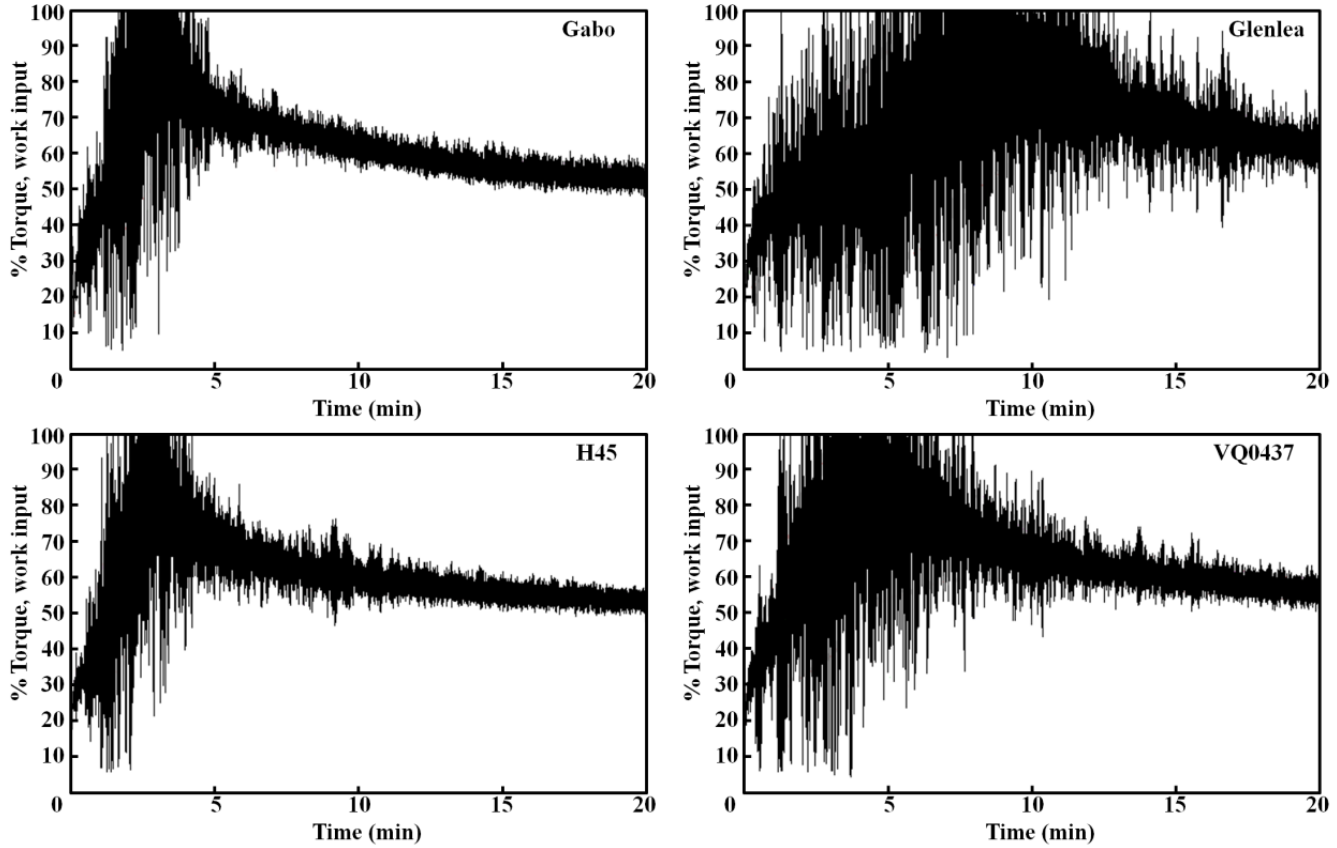
Appendix 3. Restriction fragments from *Rsa* I digestion of PCR products of first copy of the Bx high-molecular weight glutenin gene of H45 (H), VQ0437 (V) and sets of 20 H45/VQ0437 F_2 plants with low and high SDS-unextractable polymeric protein separated on 8% PAGE gel. M: pUC19 DNA/MspI (HpaII) ladder.



Appendix 4. Alignment of amino acid sequences of high-molecular weight glutenin subunits encoded by the *Bx7*^{OE} genes of the *Glu-B1al* allele of Glenlea), the *Bx7.1* and *Bx7.2* genes of the *Glu-B1br* allele of H45, and the *Bx7.3* and *Bx7*^{OE} genes of the *Glu-B1bs* allele of VQ0437 wheat and by a mutant gene (*MutBx7.1*) derived from *Bx7.1* by site-directed mutagenesis. The regions of the protein shown are those that contain cysteine residues and/or in which there are sequence differences among the proteins. Conserved cysteine residues are boxed, and an extra cysteine residue is indicated by an arrow. Amino acid differences are highlighted in grey.



Appendix 5. 10-g mixograph mixing curves of Gabo, Glenlea, H45 and VQ0437.



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