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ORIGINAL ARTICLE

A cysteine in the repetitive domain of a high-molecular-weight glutenin subunit interferes with the mixing properties of wheat dough

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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.

Keywords Cysteine · Dough properties · Glutenin polymerisation · High-molecular-weight glutenin · Site-directed mutagenesis · Wheat

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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. HMW-GS have been classified into x-type and y-type subunits. 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) 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). 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). In contrast, H45, an Australian wheat cultivar with the *Glu-B1br* allele, has relatively weak dough. Gao et al. (2012) demonstrated that the *Glu-B1br* allele includes two slightly different $Bx7^{OE}$-like genes (*Bx7.1* and *Bx7.2*), the sequences of which 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 (Fig. 1). Gao et al. (2012) 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) 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. In the research reported here, the objectives were to isolate individual HMW-GS, investigate their effects on the mixing properties of wheat dough, and determine whether the properties of Bx7.1 could be attributed directly to its extra cysteine.

Previous research (Békés et al. 1994b; Xu et al. 2006; Yan et al. 2009; Chen et al. 2011) has demonstrated that glutenin genes can be heterologously expressed in

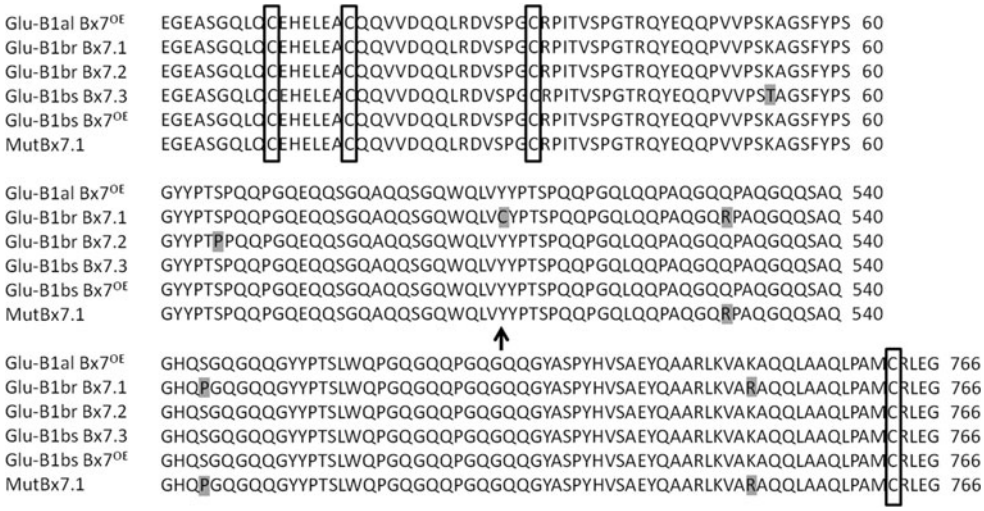

Fig. 1 Alignment of amino acid sequences of high-molecular weight glutenin subunits encoded by the $Bx7^{OE}$ genes of the *Glu-B1al* allele of Glenlea, the *Bx7.1* and *Bx7.2* genes of the *Glu-B1br* allele of H45, and the *Bx7.3* and $Bx7^{OE}$ genes of the *Glu-B1bs* allele of VQ0437 wheat and by a mutant gene (*MutBx7.1*) derived from *Bx7.1* by site-directed mutagenesis

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.

Materials and methods

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) was used as the template. The guanine at 1,598 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 °C for 1 min, followed by 18 cycles of 95 °C for 50 s, 70 °C for 50 s and 68 °C for 6 min, and a final extension step at 68 °C for 7 min. Mutagenesis was confirmed by commercial sequencing of the mutated gene (Australian Genome Research Facility).

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.

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 small-scale dough mixer (4-g micro-doughLAB z-arm mixer, Perten Ltd., NSW, Australia) (Bason et al. 2007). Results were analysed 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.

Heterologous expression of high-molecular weight glutenin subunit genes

The *Bx7.1* and *Bx7.2* genes previously isolated (Gao et al. 2012) 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 °C for 3 min, followed by 35 cycles of 93 °C for 15 s and 68 °C for 3 m, and a final extension step at 72 °C 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 °C, 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 °C 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 mol/m^3 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 °C for 4 h, and harvested by centrifugation (10,000 rpm, 4 °C, 10 min).

Isolation of heterologously expressed proteins

Cell disruption and fractionation were carried out according to Dowd and Békés (2002) with minor modifications.

Cell pellets were resuspended in lysis buffer (50 mol/m^3 Tris-HCl pH 7.3, 100 mol/m^3 NaCl, 1 mol/m^3 EDTA, 1 mol/m^{3}PMSF and 10 mol/m^3 dithiothreitol (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 °C, 15 min), the supernatant was discarded, and the pellet was washed twice with distilled water plus 100 mol/m^3 DTT and 10 mol/m^3 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 mol/m^3 DTT, with the suspension sonicated for 15 s twice to assist solubilisation. The sample was incubated at 65 °C 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.

Analysis of heterologously expressed proteins

The purified protein samples, Bx7^{OE}, Bx7.1 and MutBx7.1 prepared as described above, were completely dissolved in sample buffer (0.02 % bromophenol blue, 80 mol/m^3 Tris-HCl (pH 8.0), 69 mol/m^3 SDS) containing 1 % (w/v) DTT. Electrophoresis was conducted using the methods described by Gao et al. (2012). 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 mol/m^3 ammonium bicarbonate in 30 % acetonitrile. The proteins were reduced with 0.5 µmol DTT in 100 mol/m^3 ammonium bicarbonate and alkylated with 2.75 µmol iodoacetamide in 100 mol/m^3 ammonium bicarbonate. After digestion with 100 ng of sequencing-grade modified trypsin (Promega) in 5 mol/m^3 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.

SDS sedimentation test

For plain flour, SDS sedimentation tests were conducted using the methods described by Gao et al. (2012). For Gabo base flour supplemented with each of the seven heterologously expressed subunits, a reversible reduction–oxidation procedure (Békés et al. 1994a) 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 controls.

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 in triplicate. Each of the seven expressed polypeptides was incorporated into Gabo base flour using a reversible reduction–oxidation procedure (Békés et al. 1994a, b; 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.

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). To determine the protein size

distribution in both extractable and unextractable protein fractions of the dough, SE-HPLC analysis was carried out in duplicate according to Larroque and Békés (2000). The proportion of unextractable polymeric protein (%UPP) was calculated.

Results

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 to 10.5 % and protein contents ranging from 14.8 to 19.2 % (Table 1). Glenlea had the highest SDS sedimentation volume and %UPP value, followed by VQ0437, H45 and Gabo (Table 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 (Online Resource 1). The dough mixing parameters of VQ0437 were intermediate between those of Glenlea and H45 (Table 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 %).

Site-directed mutagenesis and heterologous expression of HMW-GS

DNA sequencing of the mutant gene *MutBx7.1* confirmed that site-directed mutagenesis had successfully replaced the guanine (G) at the 1,598 nt position of the *Bx7.1* gene by an adenine (A), changing the repetitive-domain cysteine codon to a tyrosine codon (Online Resource 2).

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*. Heterologous expression generated 10–20 mg purified protein per litre of LB medium. 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. 2). The LC-eSI-IT mass spectrometry results, which covered between 3 and 10 % of the full sequences of the three subunits, confirmed that peptide sequences were the same as predicted from the nucleotide sequences (Online Resource 2).

Effects of heterologously expressed HMW-GS incorporated into wheat flour

Incorporation of each heterologously expressed protein increased SDS sedimentation volume relative to that of plain Gabo base flour: from 15.3 and 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 2).

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. 3). 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. 3). For these dough properties, the changes observed for the Bx7.1 subunit were substantially less than for the other subunits, including MutBx7.1.

Table 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	Bx subunits	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	17	19.2	10.0	71.4	3.67	16.2	79.9	15.3	27.9
Glenlea	7^{OE}	17.3	10.0	63.9	9.46	10.2	51.3	24.5	52.7
H45	7.1 and 7.2	14.8	10.5	66.3	3.53	17.2	67.0	19.3	30.1
VQ0437	7.3 and 7^{OE}	16.3	10.4	66.6	5.07	14.0	62.1	23.6	44.3

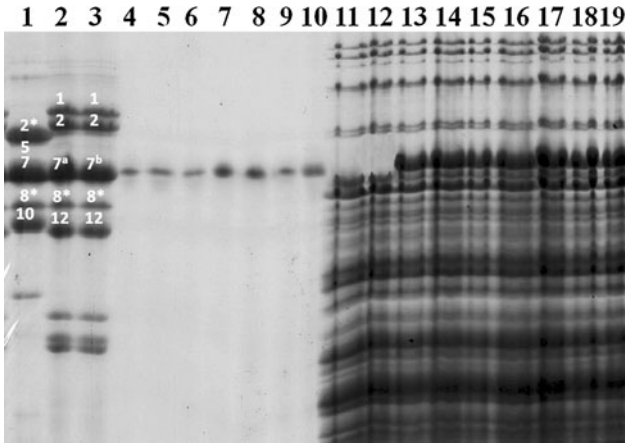

Fig. 2 SDS–PAGE profiles of purified and heterologously expressed HMW-GS. Lanes 1-3, HMW components extracted from Glenlea, H45 and VQ0437 flour, *1* and *2** HMW-GS encoded by *Glu-A1a* and *Glu-A1b*, *7* and *8** HMW-GS encoded by *Glu-B1al*, 7^a and *8** HMW-GS encoded by *Glu-B1br*, 7^b and *8** HMW-GS encoded by *Glu-B1bs*, *5* and *10* HMW-GS encoded by *Glu-D1d*, *2* and *12* HMW-GS encoded by *Glu-B1a*, respectively; 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

Discussion

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. The wild-type genes and the mutant derivative were expressed in *E. coli*, and small amounts of purified HMW glutenin proteins were obtained 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 (Shani et al. 1992; Anderson et al. 1996; Tamás et al. 1998; Dowd and Békés 2002; Xu et al. 2006; Yan et al. 2009; 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. 2009; Liu et al. 2010; Chen et al. 2011; Anderson and Békés 2011; Anderson 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.

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. (1994a). When dough is partially reduced with DTT, it allows the rupture of disulphide 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 disulphide linkages, permitting recovery of dough mixing properties.

Table 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[b] (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[a]	18.1	0.14	118.3

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

[b] The mean SDS sedimentation volumes were calculated based on two replications

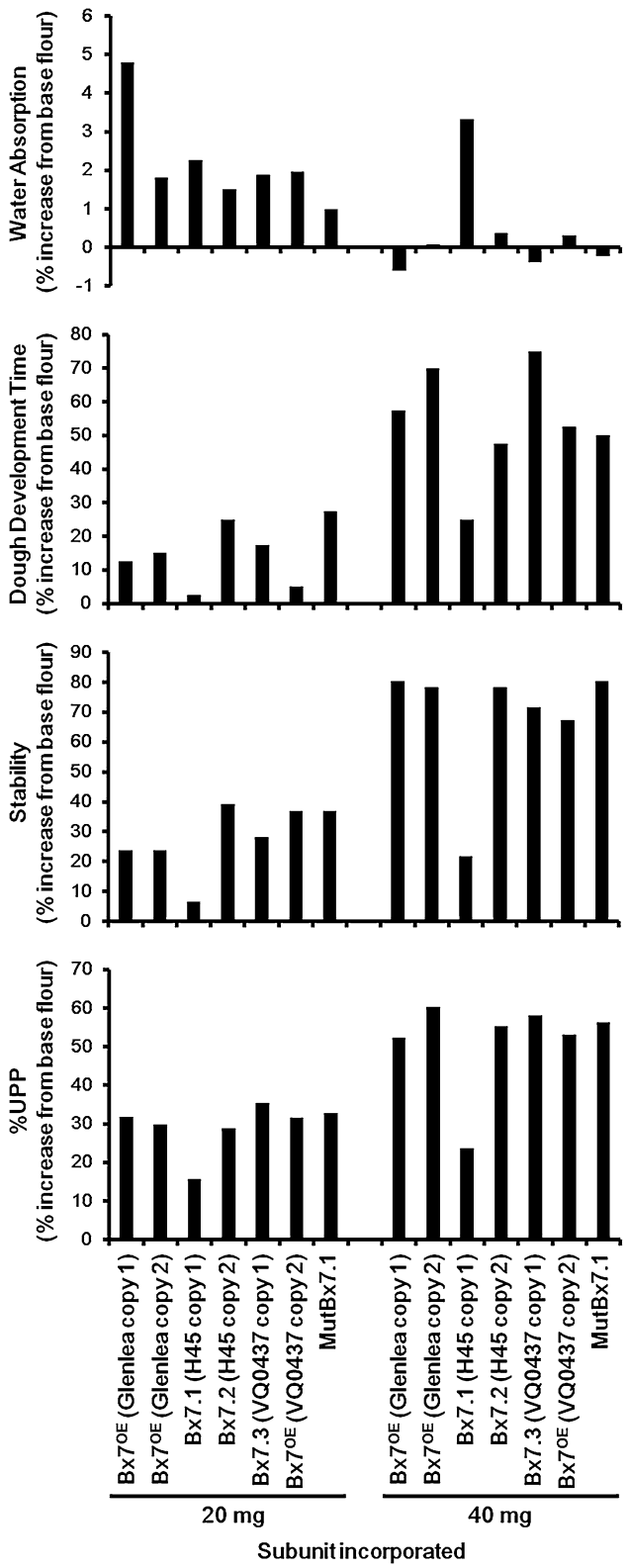

Fig. 3 Percentage changes in dough mixing properties and unextractable polymeric protein with heterologously expressed glutenin subunits incorporated at two levels (20 and 40 mg) into 4 g of Gabo base flour, relative to those of the base flour

This reduction–oxidation procedure allows added glutenin subunits to be incorporated into the polymeric protein of the base flour. This 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. 2003; Xu et al. 2006; Maforimbo et al. 2008; Yan et al. 2009; Liu et al. 2010; Chen et al. 2011).

The flour samples of the wheat lines used here all came from glasshouse-grown plants and were quite high in protein (ranging from 14.8 to 19.2 %) relative to what might be expected for flour milled from wheat grain produced in the field. Nevertheless, the wheat lines ranked as expected with respect to their SDS sedimentation volume, %UPP and dough mixing parameters, with the Gabo base 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. (1994a). 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. (1994a), the concentration of reducing-oxidising 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. (1994a), mixing for 30 s was used to ensure effective dispersion of reducing and oxidisation reagents.

Effects of heterologously expressed HMW-GS 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 (Fig. 1). Consistent with the results of previous SDS sedimentation tests conducted on flour from H45, VQ0437 and Glenlea wheat (Gao et al. 2012), 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.

Effects of the incorporation of the heterologously expressed HMW-GS on dough mixing properties

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), 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) 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.

Effect of heterologously expressed HMW-GS 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 intermolecular cross links in polymeric protein.

Possible mechanisms for the effect of an extra cysteine residue on dough properties

The combined use of site-directed mutagenesis, heterologous expression and reduction–oxidation incorporation of polypeptides into flour has provided sufficient evidence to attribute the unusual effect of the *Glu-B1br* allele to the

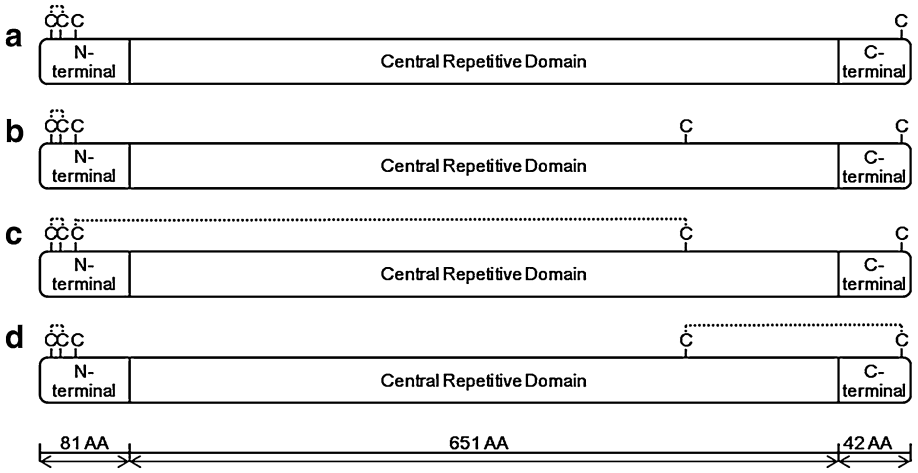

Fig. 4 Schematic representations of **a** a Bx7^{OE} (or MutBx7.1 or Bx7.2 or Bx7.3) glutenin polypeptide showing the positions of its four cysteine residues and an expected intramolecular disulphide bond (*dashed line*), with two terminal-domain cysteines remaining available to participate in intermolecular linkages; **b** a 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 intramolecular disulphide bonds, each leaving one terminal-domain cysteine available to participate in intermolecular linkages

'extra' cysteine of the Bx7.1 subunit. Given the importance of disulphide linkages between cysteine residues in the formation and stabilisation of polymeric glutenin, it seems likely that the mechanism of the *Glu-B1br* effect involves participation of the extra cysteine in disulphide bonds. Intuitively, it might be expected that additional opportunities for cross-linkage would enhance polymerisation, yet the effects observed here all seem more consistent with reduced polymerisation.

Like most x-type HMW glutenin subunits, Bx7^{OE}, MutBx7.1, Bx7.2, Bx7.3 and MutBx7.1 each contain four cysteine residues, three in the N-terminal domain and one in the C-terminal domain. Normally, as shown in Fig. 4a, two of the cysteines in the N-terminal domain would be connected by an intramolecular bond, while the other two cysteines would be available to form end-to-end bonds with cysteines in other glutenin subunits (Keck et al. 1995; Wieser 2007). If the extra cysteine of the Bx7.1 subunits (Fig. 4b) forms an intermolecular (middle-to-end or middle-to-middle) bond, this might interfere with end-to-end linkage among subunits and/or cause branching of the polymer. Alternatively, in accordance with the suggestion of Kasarda (1999) that intramolecular links form more rapidly than intermolecular links, the repetitive-domain cysteine may link with one of the terminal-domain cysteines (Fig. 4c, d) of the same subunit. This would leave only one terminal-domain cysteine available to form an intermolecular bond. This would cause the Bx7.1 subunit to act as a chain terminator, preventing other subunits from linking to the glutenin polymer. Either of these types of interference with glutenin polymerisation could explain the observed functional properties of H45 flour and of Gabo flour supplemented with the Bx7.1 glutenin subunit.

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References

American Association of Cereal Chemists (1995) American Association of Cereal Chemists, Approved Methods of AACC, Methods 54–40A, approved November 1995, 9th edn. The American Association of Cereal Chemists, St Paul

Anderson OD, Békés F (2011) Incorporation of high-molecular-weight glutenin subunits into doughs using 2 gram mixograph and extensigraphs. J Cereal Sci 54:288–295

Anderson OD, Greene FC, Yip RE, Halford NG, Shewry PR, Malpica-Romero JM (1989) Nucleotide sequences of the two high-molecular-weight glutenin genes from the D-genome of a hexaploid bread wheat, *Triticum aestivum* L. cv Cheyenne. Nucleic Acids Res 17:461–462

Anderson OD, Kuhl JC, Tam A (1996) Construction and expression of a synthetic wheat storage protein gene. Gene 174:51–58

Anderson OD, Békés F, D'Ovidio R (2011) Effects of specific domains of high-molecular-weight glutenin subunits on dough properties by an in vitro assay. J Cereal Sci 54:280–287

Anjum FM, Khan MR, Din A, Saeed M, Pasha I, Arshad MU (2007) Wheat gluten: high molecular weight glutenin subunits—structure, genetics, and relation to dough elasticity. J Food Sci 72:56–63

Bason ML, Dang JMC, Booth RI (2007) Mixing characteristics of dough as determined by the Newport Scientific micro dough-LAB. Cereal Foods World 52:A14

Békés F, Gras PW, Gupta RB (1994a) Mixing properties as a measure of reversible reduction and oxidation of doughs. Cereal Chem 71:44–50

Békés F, Gras PW, Gupta RB, Hickman DR, Tatham AS (1994b) Effects of lBx20 HMW glutenin on mixing properties. J Cereal Sci 19:3–7

Branlard G, Dardevet M (1985) Diversity of grain protein and bread wheat quality. II. Correlation between high molecular weight subunits of glutenin and flour quality characteristics. J Cereal Sci 3:345–354

Buonocore F, Caporale C, Lafiandra D (1996) Purification and characterizaion of high Mr glutenin subunit 20 and its linked y-type subunit from durum wheat. J Cereal Sci 23:195–201

Butow BJ, Ma W, Gale KR, Cornish GB, Rampling L, Larroque O, Morell MK, Békés F (2003) Molecular discrimination of *Bx7* alleles demonstrates that a highly expressed high-molecular-weight glutenin allele has a major impact on wheat flour dough strength. Theor Appl Genet 107:1524–1532

Carter BP, Morris CF, Anderson JA (1999) Optimizing the SDS sedimentation test for end-use quality selection in a soft white and club wheat breeding program. Cereal Chem 76:907–911

Chen FG, Zhao F, Liu RK, Xia GM (2011) Functional properties of two low-molecular-weight glutenin subunits carrying additional cysteine residues from hybrid introgression line II-12 derived from *Triticum aestivum* and *Agropyron elongatum*. Food Chem 127:1773–1776

Cinco-Moroyoqui FJ, MacRitchie F (2008) Quantitation of LMW-GS to HMW-GS ratio in wheat flours. Cereal Chem 85:824–829

Dowd C, Békés F (2002) Large-scale expression and purification of high-molecular-weight glutenin subunits. Protein Expres Purif 25:97–104

Dower WJ, Miller JF, Ragsdale CW (1988) High efficiency transformation of *E.coli* by high voltage electroporation. Nucleic Acids Res 16:6127–6145

Eagles HA, Eastwood RE, Hollamby GJ, Martin EM, Cornish GB (2004) Revision of the estimates of glutenin gene effects at the *Glu-B1* locus from southern Australian wheat breeding programs. Aust J Agr Res 55:1093–1096

Gao X, Appelbee M, Mekuria G, Chalmers K, Mather DE (2012) A second 'overexpression' allele at the *Glu-B1* high-molecular-weight glutenin locus of wheat: sequence characterisation and functional effects. Theor Appl Genet 124:333–343

Haraszi R, Gras PW, Tömösközi S, Salgó A, Békés F (2004) Application of a micro Z-arm mixer to characterize mixing properties and water absorption of wheat flour. Cereal Chem 51:555–560

He ZH, Liu L, Xia XC, Liu JJ, Pena RJ (2005) Composition of HMW and LMW glutenin subunits and their effects on dough properties, pan bread, and noodle quality of Chinese bread wheats. Cereal Chem 82:345–350

Holding DR, Larkins BA (2008) Genetic engineering of seed storage proteins. Advan Plant Biochem Mol Biol 1:107–133

Janssen AM, Tv Vliet, Vereijken JM (1996) Rheological behaviour of wheat glutens at small and large deformations. Effect of gluten composition. J Cereal Sci 23:33–42

Kasarda DD (1999) Glutenin polymers: the in vitro to in vivo transition. Cereal Foods World 44:566–571

Keck B, Köhler P, Wieser H (1995) Disulfide bonds in wheat gluten: cysteine peptides derived from gluten proteins following peptic and thermolytic digestion. Z Lebensm Unters Forsch 200: 432–439

Köhler P, Belitz HD, Wieser H (1993) Disulfide bonds in wheat gluten: further cysteine peptides from high molecular weight (HMW) and low molecular weight (LMW) subunits of glutenin and from γ-gliadins. Z Lebensm Unters Forsch 196:239–247

Larroque OR, Békés F (2000) Rapid size-exclusion chromatography analysis molecular size distribution for wheat endosperm proteins. Cereal Chem 77:451–453

Li W, Wan Y, Liu Z, Liu K, Liu X, Li B, Li Z, Zhang X, Dong Y, Wang D (2004) Molecular characterization of HMW glutenin subunit allele *1Bx14*: further insights into the evolution of *Glu-B1-1*alleles in wheat and related species. Theor Appl Genet 109:1093–1104

Lindsay MP, Tamás L, Appels R, Skerritt JH (2000) Direct evidence that the number and location of cysteine residues affect glutenin polymer structure. J Cereal Sci 31:321–333

Liu SW, Zhao F, Gao X, Chen FG, Xia GM (2010) A novel high molecular weight glutenin subunit from *Australopyrum retrofractum*. Amino Acids 39:385–392

Lombardi A, Barbante A, Cristina PD, Rosiello D, Castellazzi CL, Sbano L, Masci S, Ceriotti A (2009) A relaxed specificity in interchain disulfide bond formation characterizes the assembly of a low-molecular-weight glutenin subunit in the endoplasmic reticulum. Plant Physiol 149:412–423

Maforimbo E, Skurray G, Uthayakumaran S, Wrigley C (2008) Incorporation of soy proteins into the wheat-gluten matrix during dough mixing. J Cereal Sci 47:380–385

Margiotta B, Pfluger L, Roth MR, MacRitchie F, Lafiandra D (2000) Isogenic bread wheat lines differing in number and type of high Mr glutenin subunits. In: Shewry PR, Tatham AS (eds). Wheat Gluten Royal Society of Chemistry, London, pp 29–33

Mclntosh RA, Yamazaki Y, Dubcovsky J, Roger J, Morris C, Somers DJ, Appels R, Devos KM (2008) Catalogue of gene symbols for wheat: 2008 supplement. KoMUGI Integrated Wheat Science Database. Available at http://wwwshigennigacjp/wheat/komugi/genes/downloadjsp Wheat Genetic Resource Database Komugi-Natl. BioResource Res. Project, Japan

Mclntosh RA, Yamazaki Y, Dubcovsky J, Roger J, Morris C, Somers DJ, Appels R, Devos KM (2009) Catalogue of gene symbols for wheat: 2009 supplement. KoMUGI Integrated Wheat Science Database Available at http://www.shigen.nig.ac.jp/wheat/komugi/genes/download.jsp. Wheat Genetic Resource Database Komugi-Natl. BioResource Res. Project, Japan

Ohm JB, Ross AS, Peterson CJ, Ong YL (2008) Relationships of high molecular weight glutenin subunit composition and molecular weight distribution of wheat flour protein with water absorption and color characteristics of noodle dough. Cereal Chem 85: 123–131

Orsi A, Sparvoli F, Ceriotti A (2001) Role of individual disulfide bonds in the structural maturation of a low molecular weight glutenin subunit. J Biol Chem 276:32322–32329

Oszvald M, Tamás C, Rakszegi M, Tömösközi S, Békés F, Tamás L (2009) Effects of incorporated amaranth albumins on the functional properties of wheat dough. J Sci Food Agric 89:882–889

Payne PI, Lawrence GJ (1983) Catalogue of alleles for the complex gene loci, *Glu-A1*, *Glu-B1*, and *Glu-D1* which code for the high molecular weight subunits of glutenin in hexaploid wheat. Cereal Res Commun 11:29–35

Payne PI, Holt LM, Krattiger AF, Carrillo JM (1988) Relationships between seed quality characteristics and HMW glutenin subunit composition determined using wheats grown in Spain. J Cereal Sci 7:229–235

Pirozi MR, Margiotta B, Lafiandra D, MacRitchie F (2008) Composition of polymeric proteins and bread-making quality of wheat lines with allelic HMW-GS differing in number of cysteines. J Cereal Sci 48:117–122

Ragupathy R, Naeem HA, Reimer E, Lukow OM, Sapirstein HD, Cloutier S (2008) Evolutionary origin of the segmental duplication encompassing the wheat *GLU-B1* locus encoding the overexpressed Bx7 ($Bx7^{OE}$) high molecular weight glutenin subunit. Theor Appl Genet 116:283–296

Saumonneau A, Rottier K, Conrad U, Popineau Y, Gueguen J, Francin-Allami M (2011) Expression of a new chimeric protein

with a highly repeated sequence in tobacco cells. Plant Cell Rep 30:1289–1302

Shani N, Steffen-Campbell J, Anderson OD, Greene FC, Galili G (1992) Role of the amino- and carboxy-terminal regions in the folding and oligomerization of wheat high molecular weight glutenin subunits. Plant Physiol 98:433–441

Shani N, Rosenberg N, Kasarda DD, Galili G (1994) Mechanisms of assembly of wheat high molecular weight glutenins inferred from expression of wild-type and mutant subunits in transgenic tabacco. J Biol Chem 269:8924–8930

Shewry PR, Tatham AS (1997) Disulphide bonds in wheat gluten proteins. J Cereal Sci 25:207–227

Shewry PR, Popineau Y, Lafiandra D, Belton P (2000) Wheat glutenin subunits and dough elasticity: findings of the EURO-WHEAT project. Trends Food Sci Tech 11:433–441

Shewry PR, Gilbert SM, Savage AWJ, Tatham AS, Wan Y-F, Belton PS, Wellner N, D'Ovidio R, Békés F, Halford NG (2003) Sequence and properties of HMW subunit 1Bx20 from pasta wheat (*Triticum durum*) which is associated with poor end use properties. Theor Appl Genet 106:744–750

Tamás L, Békés F, Greenfield J, Tatham AS, Gras PW, Shewry PR, Appels R (1998) Heterologous expression and dough mixing studies of wild-type and mutant C hordeins. J Cereal Sci 27: 15–22

Tatham AS, Field JM, Keen JN, Jackson PJ, Shewry PR (1991) Purification and characterization of HMW glutenin subunits encoded by chromosome 1B of durum wheat (*Triticum durum*). J Cereal Sci 14:111–116

Vawser MJ, Cornish GB (2004) Over-expression of HMW glutenin subunit *Glu-B1* 7x in hexaploid wheat varieties (*Triticum aestivum*). Aust J Agr Res 55:577–588

Washida H, Wu C, Suzuki A, Yamanouchi U, Akihama T, Harada K, Takaiwa F (1999) Identification of *cis*-regulatory elements required for endosperm expression of the rice storage protein glutelin gene *Glu-B1*. Plant Mol Biol 40:1–12

Wieser H (2007) Chemistry of gluten proteins. Food Microbiol 24: 115–119

Xu H, Wang RJ, Shen X, Zhao YL, Sun GL, Zhao HX, Guo AG (2006) Functional properties of a new low-molecular weight glutenin subunit gene from a bread wheat cultivar. Theor Appl Genet 113:1295–1303

Yan YM, Jiang Y, An XL, Pei YH, Li XH, Zhang YZ, Wang AL, He Z, Xia X, Békés F, Ma W (2009) Cloning, expression and functional analysis of HMW glutenin subunit *By8* gene from Italy pasta wheat (*Triticum turgidum* L. ssp. durum). J Cereal Sci 50:398–406