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ORIGINAL ARTICLE

A novel high molecular weight glutenin subunit from *Australopyrum retrofractum*

Shuwei Liu · Feng Zhao · Xin Gao · Fanguo Chen · Guangmin Xia

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Abstract We describe the sequence of a gene encoding a high molecular weight glutenin subunit (HMW-GS) expressed in the endosperm of the wheat relative *Australopyrum retrofractum*. Although the subunit has a similar primary structure to that HMW-GS genes present in other Triticeae species, its N-terminal domain is shorter, its central repetitive domain includes a unique dodecameric motif, and its C-terminal domain contain an extra cysteine residue. A phylogenetic analysis showed that the *Glu-W1* gene is neither a true x- nor a true y-type subunit, although it is more closely related to the y-type genes present in the K and E genomes than to any other published HMW-GS gene. All these results indicated that this novel subunit may undergo a special evolutionary process different from other Triticeae species. A flour supplementation experiment showed that the *Glu-W1* subunit has a negative effect on dough quality, which might be the result of interaction between the two closely placed cysteine residues in the C-terminal region.

Keywords *Australopyrum retrofractum* · Evolution · *Glu-1* · HMW-GS · W genome

Introduction

The high molecular weight glutenin subunits (HMW-GSs) present in wheat and some of its related grasses are well established as major determinants of dough visco-elasticity (Payne et al. 1987, 1988). The encoding *Glu-1* loci have been mapped to the long arms of chromosomes 1A, 1B and 1D of bread wheat, with each locus comprising two tightly linked genes, designated as the x- and y-type. The gene products of *Glu-1x* and *Glu-1y* are distinguished from one another primarily on the basis of their size (Lawrence and Shepherd 1981; Payne 1987). A number of *Glu-1* genes have been cloned, both from bread wheat itself and some of its relatives (Forde et al. 1985; Sugiyama et al. 1985; Thompson et al. 1985; Halford et al. 1987, 1992; Anderson and Greene 1989; Anderson et al. 1989). Analysis of their coding sequences has shown that the HMW-GS polypeptides are composed of a central repetitive region flanked by two highly conserved terminal non-repetitive domains. The x-type subunit N-terminal domain comprises between 81 and 89 residues, while of the y-type is typically 104 or 105 residues. The central region consists of two duplicated 11–12 residue motifs, followed by an array of tri-, hexa- and nonapeptide repeats, although the tripeptide motif is not found in the y-type subunit. Five conserved cysteine residues are present in the y-type N-terminal domain, two of which have been lost from the x-type subunits due to an 18 residue deletion. The C-terminal domain of all known subunits comprises 42 residues, including one conserved cysteine residue.

Sequence comparisons between various *Glu-1* alleles have been used to make inferences regarding the evolution of species in the grass family. The origin of the linked pair of genes at each locus is thought to reflect the occurrence of a duplication in the ancestral gene (Shewry et al. 2003a), dated to 7.2–10.0 MYA (million years ago), which is earlier than the likely differentiation of the A, B, D and G genomes (5.0–6.9 MYA) (Allaby et al. 1999).

Australopyrum retrofractum (Vickery) Á. Löve ($2n = 14$, WW) is a self-compatible Triticeae species (Connor

S. Liu · F. Zhao · X. Gao · F. Chen · G. Xia (✉)
The Key Laboratory of Plant Cell Engineering and Germplasm Innovation, Ministry of Education, School of Life Sciences, Shandong University, 250100 Jinan, People's Republic of China
e-mail: Xiagm@sdu.edu.cn; fanguo2002@sdu.edu.cn

et al. 1993), endemic to New South Wales (Australia). Its genome (W) is present in a number of Australasian polyploid grasses (Stewart et al. 2005), but the evolutionary relationship of the W to the other Triticeae genomes has not been explored to date. Here, we report the isolation and characterization of *Glu-W1* from *A. retrofractum*. Our purpose for targeting this locus was the possibility that it may prove to be of value for the improvement of the end-use quality of bread wheat, but also that it could extend our current understanding of the evolution of the HMW-GS gene family, and shed some light on speciation within the Triticeae.

Materials and methods

Plant materials

Seeds of *A. retrofractum* (accession number PI531553) collected from Australia were kindly presented by Dr Lu (Fudan University, China). Common wheat Chinese Spring (1Bx7 + 1By8, 1Dx2 + 1Dy12) and Jinan177 (1Bx7 + 1By9, 1Dx2 + 1Dy12) were used for estimating the electrophoresis mobility of the HMW-GS and of *A. retrofractum*.

SDS-PAGE

The HMW-GSs of *A. retrofractum* and bread wheat (cvs. Chinese Spring and Jinan177) were extracted from mature seeds, as described by Mackie et al. (1996). Each seed was crushed and extracted in 625 µl 50% (v/v) 1-propanol, 1% (w/v) DTT at 60°C for 30 min. After centrifugation (15,000*g*, 10 min), a 400 µl aliquot was removed to a fresh tube and the HMW-GSs precipitated by adding 100 µl 1-propanol containing 1% (w/v) DTT, and leaving at room temperature for 30 min. The precipitate was collected by centrifugation (15,000*g*, 10 min), washed in 60% (v/v) 1-propanol, 1% (w/v) DTT, and resuspended in 100 µl SDS extraction buffer. A 20 µl extract was loaded onto an SDS-PAGE gel, formed from a 4% stacking gel (pH 6.8) overlying a 7.5–10% gradient separating gel (pH 8.5). Separation was carried out at 6 mA for 14–16 h, after which the gel was stained in 0.1% w/v Coomassie Brilliant Blue R250, 10% v/v carbinol, 50% v/v acetic acid for 20 min, and destained by boiling for 1 h in distilled water.

Cloning and sequencing of the *Glu-W1* open reading frame

Genomic DNA was extracted from a single *A. retrofractum* seedling using the CTAB method (Murray and Thompson 1980). A pair of degenerate primers was used to amplify *Glu-W1*, namely P1 (5′-ATGGCTAAGCGGc/tTa/gGTCC TCTTTG) and P2 (5′-CTATCACTGGCTa/gGCCGACA ATGCG). The subsequent PCR employed high fidelity LA Taq polymerase (TaKaRa, Dalian, China) with GC buffer, and the amplicons were separated through 1.0% agarose gels, purified, cloned into pMD18-T (TaKaRa, Dalian, China), and transformed into competent *E. coli* DH10B cells. Both the PCR amplification and the cloning were repeated at least three times. Sub-clones of three randomly selected clones were prepared using the nested deletion method of Sambrook et al. (1989), and submitted for sequencing. Sequences were analysed using MEGA software (Kumar et al. 2004), along with other standard programs mounted on the NCBI (http://www.ncbi.nlm.nih.gov/Tools/) and EBI websites (http://www.ebi.ac.uk/Tools/sequence.html).

Heterologous expression of HMW-GS genes in *E. coli*

To enable the heterologous expression of mature *A. retrofractum* HMW-GS genes in *E. coli*, the primer pair P1 (5′-ACCCATATGGAAGGTGAGGCCTCT) and P2 (5′-CTAGAATTCCTATCACTGGCTGGCCGA) was used to amplify the open reading frame (ORF), and not the signal peptide. The P1 primer includes an *Nde*I and P2 an *Eco*RI restriction site to facilitate subsequent cloning. The amplicon was cloned into pET-24a (Novagen) and transformed into *E. coli* BL21 (DE3) pLysS (Promega). Heterologous gene expression was induced by the addition of 1 mM IPTG, and proteins were extracted from the bacteria by lysis in SDS-PAGE sample buffer (Wan et al. 2002). For the purification of large amounts of heterologously expressed protein, a single transformed colony was inoculated into 20 ml LB liquid medium containing 30 mg/l kanamycin, which was incubated overnight and then combined with 2,000 ml LB liquid medium and held at 37°C. When the OD_{600} of this culture reached 0.6, heterologous protein expression was induced by the addition of IPTG to a final concentration of 1 mM. Bacterial cells were harvested by centrifugation (10,000 rpm, 4°C, 3 min). The heterologous HMW-GS proteins were extracted from these cells following Chen et al. (2009).

N-terminal amino acid sequencing

The purified HMW-GS proteins were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane in preparation for amino acid sequencing. The sequencing was performed using an automatic amino acid sequencer (Applied Biosystems) at the National Key Laboratory of Protein and Plant Genetic Engineering, Beijing University, China.

Functionality of the cloned *A. retrofractum* HMW-GS subunit

The purified *A. retrofractum* HMW-GS was tested for functionality by mixing with flour milled from cv. Jinan177. A 10 g sample of normal flour (14.5% moisture and 12.6% protein content) was supplemented with 25 mg heterologously expressed *A. retrofractum* HMW-GS protein, and this was mixed with 4.9 ml water and 0.6 ml 1 mg/ml DTT for 30 s. After a resting period of 4 min, the dough was treated with 0.72 ml 10 mg/ml KIO_3, mixed for 30 s, rested for 5 min, and finally mixed for 10 min. Mixing curves and other dough quality parameters were obtained with a Mixograph device. Four parameters were selected to evaluate the effect of heterologously expressed protein on dough property of bread wheat, out of them, mixing time (MT), TxW and PW are measurement of dough strength while RPS is an inverse measurement of dough stability (Walker and Walker 1992, Lee et al. 1999, Chen et al. 2009).

Results

HMW-GS of *A. retrofractum*

The electrophoretic mobility of the single *A. retrofractum*-specific HMW-GS observed is similar that of the 1Dx2 subunit present in both cvs. Jinan177 and Chinese Spring (Fig. 1). The PCR amplicon consisted of a single fragment of length ~2.5 kb (Fig. 2), but restriction digestion of a series of clones of this amplicon showed that the amplicon was heterogeneous. One of these sequences (W2.5) was selected to represent the sequence of *Glu-W1*. It was transformed into *E. coli* to achieve heterologous expression. The electrophoretic mobility of the heterologous protein was indistinguishable from that of the *Glu-W1* product present in the endosperm of *A. retrofractum* (Fig. 3a); The SDS-PAGE profile of the purified heterologous HMW-GS product consisted of only a single band (Fig. 3b), confirming that the purification procedure had successfully removed all contaminating *E. coli* proteins. Furthermore, the N-terminal amino acid sequencing of the protein used for flour supplementation showed that its N-terminal peptide sequence was EGEASGQLKCER, as expected from the nucleotide sequence of *Glu-W1*.

The deduced peptide sequence of the *Glu-W1* product

The deduced peptide sequence of the *Glu-W1* product comprised four regions: a signal peptide, a central repetitive region and the conserved terminal (N and C) regions. Five cysteine residues were present in the N-terminal region, and their positions were identical to those in y-type

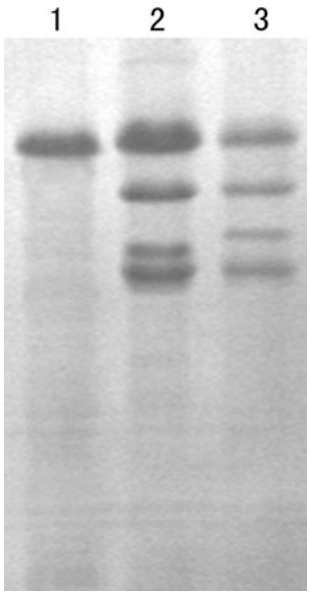

Fig. 1 SDS-PAGE profiles of endosperm proteins. *Lane 1 A. retrofractum*, *lane 2* bread wheat cv. Jinan177, *lane 3* bread wheat cv. Chinese Spring

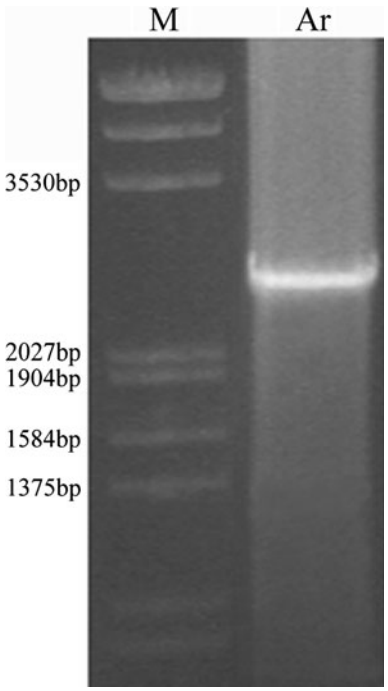

Fig. 2 The *A. retrofractum* HMW-GS amplicon (*lane Ar*). *Lane M* *Eco*RI + *Hin*dIII digested λ DNA

subunits (Fig. 4a). However, the *Glu-W1* product differed from the standard sequence in four ways: (1) Its N-terminal region contained 75 residues, which is rather shorter than most known HMW-GSs (Fig. 4a); (2) an N-terminal conserved glutamine residue (present in all known x-type, but absent from most y-type subunit), was present (Fig. 4a).

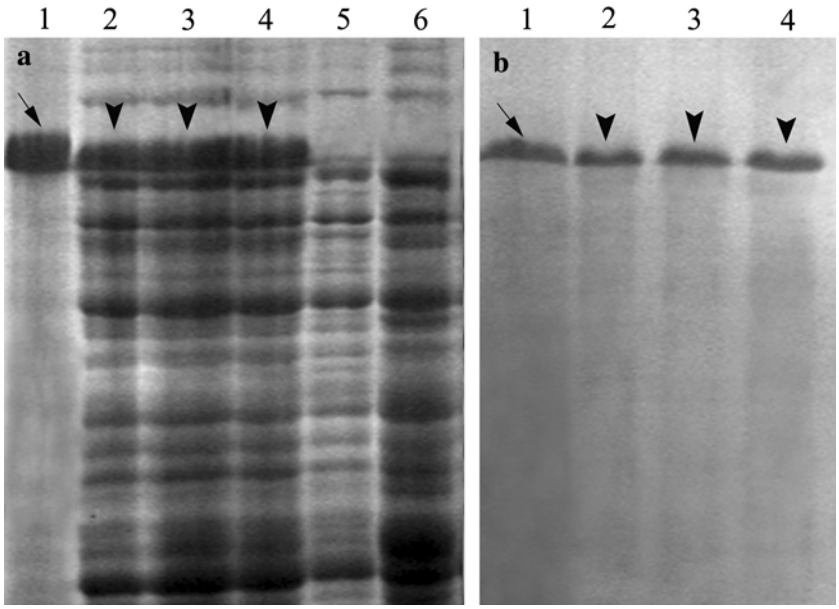

Fig. 3 SDS-PAGE analysis of heterologously expressed *Glu-W1*. The *arrowhead* indicates gene products of M_r similar to that of the *Glu-W1* product expressed in the endosperm of *A. retrofractum* (*arrowed*). **a** Total proteins prepared by dissolving transformed *E. coli* cells directly in SDS-PAGE buffer. *Lane 1 A. retrofractum* endosperm proteins, *lanes 2–4* proteins expressed from IPTG-induced *E. coli* transformed with *Glu-W1*, *lane 5* proteins expressed from non-IPTG-induced *E. coli*, *lane 6* proteins expressed from IPTG-induced *E. coli* carrying an empty vector. **b** Preparative purification of heterologous *Glu-W1* product. *Lane 1 A. retrofractum* endosperm proteins, *lanes 2–4* heterologous proteins purified from IPTG-induced *E. coli* transformed with *Glu-W1*

(3) The length of its C-terminal region (45 residues) is three residues longer than those of published HMW-GSs, since it has a duplication of a cysteine–arginine–leucine tripeptide; this introduces an additional cysteine within the C-terminal domain (Fig. 4b). (4) The two long peptides (11mer, followed by a 12mer) present at the upstream end of the central repetitive region of typical HMW-GS sequences were absent; in addition, a unique hexa- or nonapeptide motif (P/SGQGQPQGQGQQ) was present in this region, as well as an 18 residue (PGQGQPQGQGQPQGQGQQ) motif produced by the duplication of GQPQGQ (Fig. 5).

Evolutionary relationship of *W2.5* with published *Glu-1* genes

A phylogenetic analysis was based on an alignment of both the N-terminal region and the full length sequence. Both generated the expected separation of x- from y-type sequences, and showed that the *Glu-W1* product sequence was closely related to neither group, although it appears to be closer to a y- than to an x-type (Fig. 6). The closest y-type genes to *Glu-W1* are those in the E and K genomes.

Functional properties of heterologously expressed protein of W2.5

The various Mixograph values obtained from the supplemented flour dough are given in Table 1. When the *Glu-W1* product was incorporated into cv. Jinan177 flour, there was a decrease in TxW (curve width), PW (peak width) and RPS (right of peak slope) and a slight increase in MT, compared to dough made from cv. Jinan177. These results suggest that the *Glu-W1* subunit is likely to exert a negative effect on dough quality.

Discussion

We have successfully cloned the gene encoding the one HMW-GS in the *A. retrofractum* endosperm detected by SDS-PAGE. The primary structure of this subunit is similar to that of other HMW-GSs cloned from wheat and its various relatives. The *Glu-W1* locus was expected, on the basis of the typical composition of *Glu-1* loci, to encode both an x- and a y-type subunit, but the only HMW-GS gene cloned from *A. retrofractum* could not be classified as either an x- or a y-type. The presence of five conserved cysteine residues in the N-terminal domain and the absence of the conserved tripeptide motifs in the central repetitive region of the *Glu-W1* subunit imply that it is more likely to be a y- than an x-type. On the other hand, x-type subunits have a conserved glutamine residue within their N-terminal domains, and this feature was also present in *Glu-W1*. A small number of y-type subunits (E, K and Ta genomes) also share this feature, however. Overall, the phylogenetic analysis suggested that the

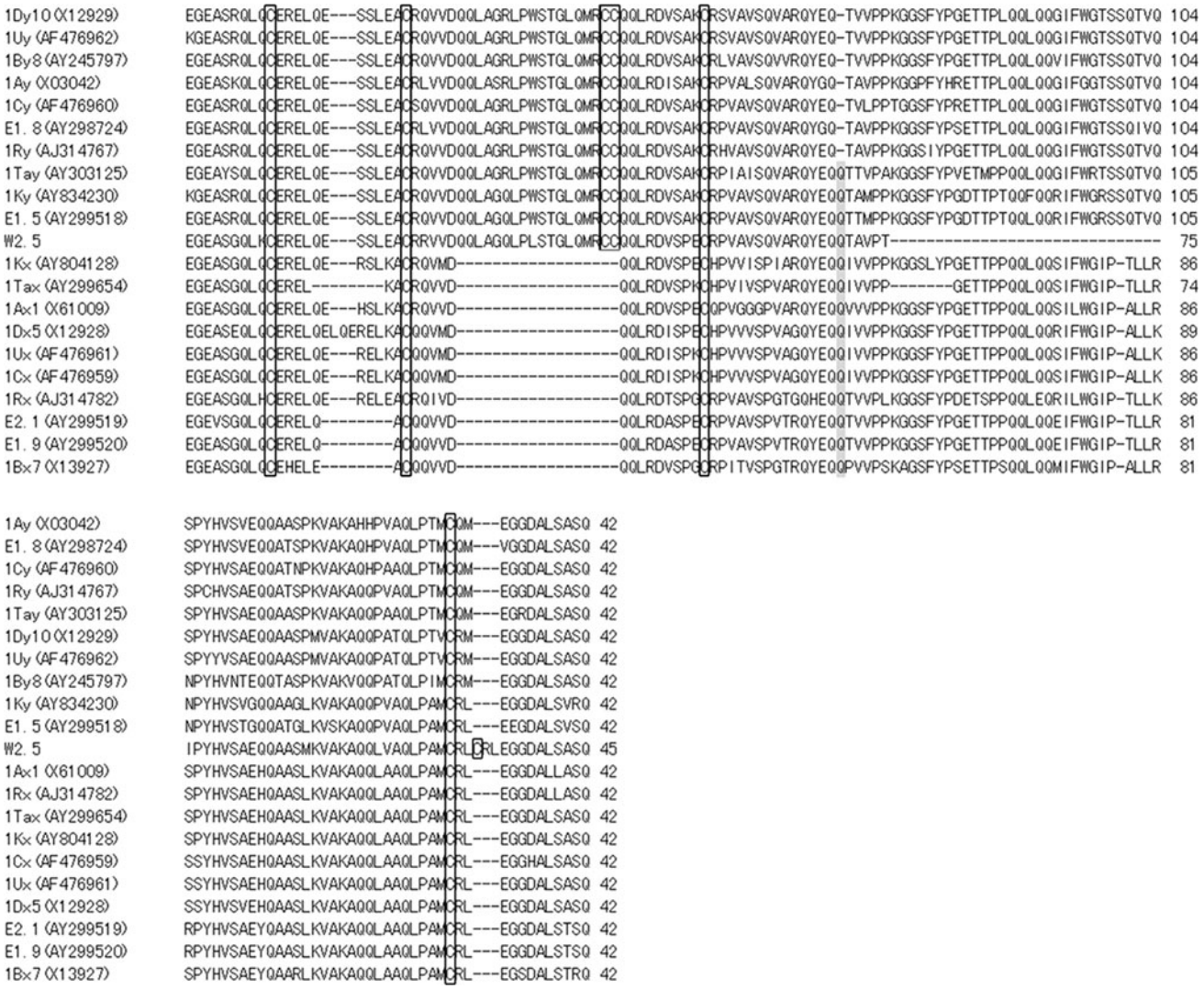

Fig. 4 Alignment of the N- (**a**) and C-terminal (**b**) domains of *Glu-W1* with those of other HMW-GS sequences. Conserved cysteine residues are shown *boxed*, and the glutamine (*Q*) residue conserved in the N-terminal domain of all x-type and a few y-type subunits as *shaded*

Glu-W1 subunit is intermediate between an x- and a y-type gene.

To date, only three distinct motifs have been found in the central repetitive region of the family of HMW-GS proteins (Shewry et al. 2003a). The dodecamer motif present in *Glu-W1* is therefore novel. Its presence implies that the W genome has been able to evolve independently of the other Triticeae ones. No doubt this reflects the fact that the geographical range of *Australopyrum* spp. is limited to Australia, which has been geographically isolated from other landmasses for many hundreds of millions of years.

The HMW-GSs are a major component of the large glutenin polymers, the properties of which are so important for the determination of dough strength (Shewry et al. 2002). It has been suggested that the alternation of x-type and y-type subunits facilitates the formation of the "backbone" structure of the glutenin polymers (Shewry et al. 2003b). The number and distribution of covalent disulphide bonds within this backbone, and the properties of non-covalent interactions between the repetitive domains are probably major factors underlying the influence of the HMW-GS over the size and structure of the glutenin macromolecule (Shewry et al. 2002). The presence of an additional cysteine residue in the repetitive region of subunit 1Dx5 is thought to be responsible for the correlation of this particular HMW-GS with good bread-making quality (Lafiandra et al. 1993; Gupta and MacRitchie 1994). Here we have shown that the addition of the *Glu-W1* subunit to normal flour has a detrimental effect on key dough properties. Galili and co-workers have found

W2.5	1By8	1Dy12	1Bx7
	GYYPSVSSPQQ	GYYPSVTSPRQ	RYYPSVTSSQQ
	GPYYPGQASPQQ	GSYYPGQASPQQ	GSYYPGQASPQQ
	PGQGQQ	PGQGQQ	SGQGQQ
	PGKWQE	PGKWQE	PGQEQQ
KGQGQQ GYYPTSPQQ			PGQGQQ DQQ
SGQGQQ			PGQRQQ GYYPTSPQQ
SGQKQPQGQGQQ			PGQGQQ
GYYPTFPQQ			LGQGQP GYYPTSQQ
PEQEQQ			PGQKQQ
PGQGQQ GYYPTSPQQ			AGQGQQ
SGQGQPQGQGQQ			SGQGQQ GYYPTSPQQ
PGKGQQ GYYPTSPQQ	LGQGQQ GYYPTSLHQ	PGQGQQ WYYPTSLQQ	SGQGQQ
PGQGQQ GYYSTSPQQ	SGQGQQ GYYPSSLQQ		PGQGQP GYYPTSPQQ
PGQGQQ	PGQGQQ	PGQGQQ	SGQWQQ
PGQGQQ GYYPTSPQQ	IGQGQQ GYYPTSLQQ	IGKGKQ GYYPTSLQQ	PGQGQQ
PGQGQQ	PGQGQQ	PGQGQQ	PGQGQQ
PGQGQQ GHYPTSPQQ	IGQGQQ GYYPTSPQH	IGQGQQ GYYPTSPQH	SGQGQQ GQQ
PGQGQQ	PGQRQQ	TGQRQQ	PGQGQR
PGQGQQ GYYPTSPQQ	PGQGQQ	PVQGQQ	PGQGQQ GYYPISPQQ
PGQGQPQGQGQQ	IGQGQQ	IGQGQQ	PGQGQQ
GYYPTSPQQ	LGQGRQ	PEQGQQ	SGQGQP GYYPTSLRQ
PGLGQQ	IGQGQQ		PGQWQQ
PGKGQQ GYFPTSPQQ	SGQGQQ GYYPTSPQQ	PGQWQQ GYYPTSPQQ	PGQGQQ
PGQGQQ GYYPTSPQQ	LGQGQQ	LGQGQQ	PGQGQQ GQQ
PGQGQPQGQGQQ	PGQWQQ	PGQWQQ	PGQGQQ
PGRGQQ GYYPTSPQQ	SGQGQQ GYYPTSQQQ	SGQGQQ GHYPTSLQQ	SGQGQQ GYYPTSLQQ
PGQGQQ	PGQGQQ GQYPASQQQ	PGQGQQ GHYLASQQQ	PGQGQQ
PGQGQQ GYYPTSSQQ	PGQGQQ GQYPASQQQ	PAQGQQ GHYPASQQQ	LGQGQP GYYPTSQQ
PGQEQQ GYYPTSPQQ	PGQGQQ GQYPASQQQ	PGQGQQ GHYPASQQQ	SEQGQQ
PGQGQQ	PAQGQQ GQYPASQQQ	PGQGQQ GHYPASQQE	PGQGKQ
PGKGQQ GYYPTSPQQ	PGQGQQ GHYLASQQQ	PGQGQQ GQ IPASQQQ	PGQGQQ GYYPTSPQQ
PGKGQQ	PGQGQQ RHYPASLQQ	PGQGQQ GHYPASLQQ	SGQGQQ
PGQGQQ GYYPTSPQQ	PGQGQQ GHYTASLQQ	PGQQ GHYPTSLQQ	LGQGQP GYYPTSPQQ
PGQGQQ	PGQGQQ GHYPASLQQ		SGQGQQ
PGQGQPQGQGQQ	VGQGQQ	LGQGQQ	SGQGQQ GYYPTSPQQ
GYYPTSPQQ	IGQ	IGQ	SGQGQQ
PGQGQPQGQGQQ	LGQRQQ	PGQKQQ	PGQGQS GYFPTSRQQ
PGKGQQ GYYPTSPQQ	PGRGQQ	PGQGQQ	SGQGQQ
PGKGQQ	TRQGQQ	TGQGQQ	PGQGQQ
PGQGQQ GYYPTSPQQ	LEQGQQ	PEQEQQ	SGQGQQ GQQ
PGQGQPQGQGQQ	PGQGQQ		PGQGQQ AYYPTSSQQ
GYYPTSPQQ	TRQGQQ		SRQRQQ
PGKGQQ GYYPTSPQQ	LEQGQQ		AGQWQR
PGKGQQ GYYPTSPQQ	PGQGQQ GYYPTSPQQ	PGQGQQ GYYPTSLQQ	PGQGQP GYYPTSPQQ
PGQGQQ GYYPTSPQQ	SGQGQQ		PGQEQQ
PGQGQQ	PGQSQQ	PGQGQQ	SGQAQQ
PGQGQPQGQGQPQGQGQQ	PGQGQQ GYYSSSLQQ	QGQGQQ GYYPTSLQQ	SGQWQL VYYPTSPQQ
GYYPTSPQQ	PGQGLQ GHYPASLQQ	PGQGQQ GHYPASLQQ	PGQLQQ
PGQGQQ	PGQGHP	PGQGQQGQ	PAQGQQ
SGQGQQ GYYPTSPQQ	GQRQQ-	PGQRQQ	PAQGQQ
PGQGQQ	PGQGQQ	PGQGQH	SAQEQQ
PGQGQPQGQGQQ	PEQGQQ	PEQGQQ	PGQAQQ
GFYPTSPRQ	PGQGQQ GYYPTSPQQ	PGQGQQ GYYPTSPQQ	SGQWQL VYYPTSPQQ
PGQGQQ GHYPTSPQQ	PGQGKQ	PGQGQQ	PGQLQQ
PGQGQQ	LGQGQQ GYYPTSPQQ	LGQGQQ GYYPTSPQQ	PAQGQQ GYYPTSPQQ
PGQEQQ	PGQGQQ	PGQGQQ	SGQGQQ GYYPTSPQQ
PGKGQQ GYYPTSPQQ	PGQGQQ GHCPTSPQQ	PGQGQQ GHCPMSPQQ	SGQGQQ GYYPTSPQQ
PGQGQQ GYYPTSPQQ	TGQAQQ	TGQAQQ	SGQGQQ
PGQGQQ	PGQGQQ	LGQGQQ	PGQGQQ
PGQGQQ GYYPTSPQQ			PRQGQQ GYYPISPQQ
PGQGQQ	IGQVQQ	IGQVQQ	SGQGQQ
SGQGQQ GYYPTSPQQ	PGQGQQ GYYPISLQQ	PGQGQQ GYYPTSLQQ	PGQGQQ GYYPTSPQQ
	SGQGQQ	PGQGQQ	SGQGQQ
	SGQGQQ	SGQGQQ	PGHEQQ
PGQGQQ	SGQGHQ	SGQGHQ	PGQWLQ
TGQGQQ	LGQGQQ	PGQGQQ	PGQGQQ GYYPTSSQQ
SGQEQQ	SGQEQQ	SGQEKQ	SGQGHQ
GYG	GYD	GYD	SGQGQQ GYYPTSLWQ
			PGQGQQ
			GYA

Fig. 5 The sequence of the central repetitive region of the *Glu-W1* product with those of bread wheat subunits 1By8 (AY245797), 1Dy12 (X03041) and 1Bx7 (X13927). The unique repeat motif in *Glu-W1* is *underlined*, and the two long peptides conserved at the start of the repetitive region is *boxed*

that a mutant 1Dx2 subunit lacking the conserved cysteine residue in the C-terminal domain could be assembled into oligomers linked by intermolecular disulphide bonds but not into large polymers in transgenic tobacco (Shani et al. 1994). The basis for this effect may be associated with the close position of an additional cysteine residue to that of a common cysteine residue in the C-terminal region. The interaction between these two cysteines may inhibit the

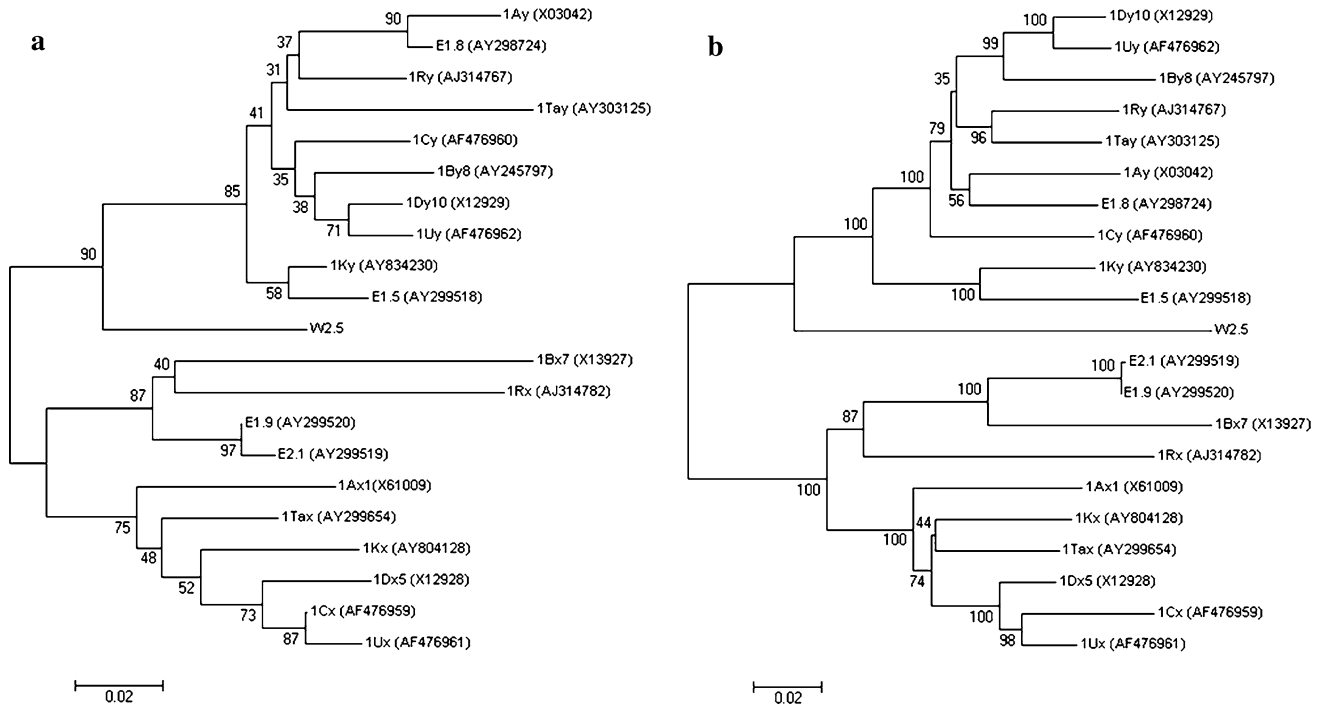

Fig. 6 A phylogenetic analysis of *Glu-W1* and other HMW-GS encoding genes. Trees **a** and **b** were constructed based on, respectively, the N-terminal domain and the full length sequence. Bootstrap values were obtained from 500 replications

Table 1 Rheological properties of dough made from normal flour (control) and flour supplemented with the *Glu-W1* product, as determined by Mixograph analysis

Item	MT	TxW	PW	RPS
Control	1.77	5.614	34.661	−4.076
W2.5	1.83	3.997	30.001	−6.130

MT Mixing time, *TxW* 8 min curve width, *PW* peak width, *RPS* right of peak slope

formation of the critical inter-chain disulphide bonds in this region of the subunit, thereby affecting the glutenin backbone structure and hence the functional properties of the gluten.

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