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ORIGINAL ARTICLE

Molecular characterisation of the low-molecular weight glutenin subunit genes of tall wheatgrass and functional properties of one clone Ee34

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Abstract Wild tall wheatgrass (*Lophopyrum elongatum* L., $2x = 14$) is an important resource for improving bread wheat (*Titicum aestivum* L.), including HMW-GS and LMW-GS relevant to end-use quality of the wheat flour. A set of 14 distinct sequences were amplified from the genomic DNA of the tall wheatgrass, using degenerate primers targeted at *Glu-3*, the locus containing the genes encoding the low-molecular weight glutenin subunits (LMW-GS). Three sequences contained an internal stop codon and were classified as pseudogenes. The other 11 all consisted of a single intron-less intact open-reading frame. An alignment of deduced protein sequences showed that the primary structure of all 11 sequences was similar to that of wheat and other wheat-related grass *Glu-3* genes. All 11 sequences carried the 14 amino acid residue *N*-terminal motif MESNIIISFLK/RPWL, and were classified as LMW-m genes, based on the identity of the first amino acid of the mature protein. All but one of the sequences contained seven cysteine residues (the exception had 6). Their repetitive domain differs significantly from that present in *Glu-3* genes isolated from the close relative intermediate wheatgrass (*Thinopyrum Intermedium*, 6*x*). A phylogenetic analysis showed that the tall wheatgrass sequences were closely related to those of the intermediate wheatgrass, but only distantly so to those from decaploid tall wheatgrass. One of the 11 LMW-GS peptides with a free-cysteine residue was heterologously expressed in *E. coli* and purified in sufficient scale to perform a flour supplementation test. This showed that the dough strength of bread wheat flour was significantly increased by the presence of the tall wheatgrass LMW-GS.

Keywords *Lophopyrum elongatum* (Host) A. Löve · LMW-GS gene · Flour quality · Dough micro-mixing test

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Introduction

Seed storage proteins are important determinants of end-use quality of bread wheat (*Triticum aestivum* L.) (Payne 1987; Shewry et al. 2003). The gluten fraction is composed of a mixture of monomeric gliadins and polymeric glutenins, the latter comprising a set of high-molecular weight (HMW-GS) and low-molecular weight (LMW-GS) subunits linked by disulphide bonds (Shewry and Tatham 1997). The HMW-GS, which account for about 10% of the gluten present, are well characterised at both the nucleotide and peptide levels, and their contribution to end-use quality has been widely explored (Shewry et al. 2003). The LMW-GS account for about 40% of the gluten, but neither their global role nor the contribution to quality of specific subunits have been fully researched, mainly because they are more difficult to be isolated than the HMW-GS, which are both larger in size and fewer in number. They are also less easy to distinguish from some of the gliadin components (Shewry et al. 2003). The LMW-GS are encoded by a set of multigene homoeoloci (*Glu-3*) located on the short arms of chromosomes 1A, 1B and 1D (Gupta and Shepherd 1990). The individual genes present at these loci have been classified as either LMW-s, LMW-m or LMW-i types, based on the first amino acid of the mature protein (Lew et al. 1992; Cloutier et al. 2001; Ikeda et al. 2002). Hybridisation experiments suggest that the model bread

wheat cultivar "Chinese Spring" carries 30–40 individual LMW-GS genes (Cassidy et al. 1998). The majority of LMW-GS contain eight Cys residues, of which six are located within the C-terminal conserved domain, and the other two lie close to the *N*- and *C*-terminal domains. The former group is responsible for three intramolecular disulphide bonds, and the latter for interchain bonds (Shewry et al. 2003).

Both the length of the gluten sequence and the number and location of Cys residues present are probably important for the determination of dough functionality (Shewry and Tatham 1997; Masci et al. 1998, 2002). However, the contribution of few individual LMW-GS has yet been demonstrated experimentally. One example of a beneficial effect has been provided by Xu et al. (2006), working with the cultivar Xiaoyan 6, which contains an LMW-GS gene with nine cysteine residues. An increasing number of LMW-GS genes have been isolated and characterised over recent years. A few of these have been obtained from wheat relatives, most of which do carry *Glu-3* orthologues (D'Ovidio et al. 1999; Johal et al. 2004; Luo et al. 2005; An et al. 2006; Pei et al. 2007; Li et al. 2007; Jiang et al. 2008). *Lophopyrum elongatum* (Host) A. Löve (2*x*, common name: tall wheatgrass) is a wild relative of bread wheat. The presence in this species of a number of favourable traits has made it attractive as a source of genes for wheat improvement, including end-use quality improvement (Dong and Zheng 2000). In this paper, we report the cloning and characterisation of members of the *L. elongatum Glu-3* locus. Our purpose was both to examine the potential of *Glu-E3* genes for the improvement of bread wheat end-use quality, and to investigate the processes underlying the evolution of these loci.

Materials and methods

Plant material

Seeds of *L. elongatum* (Host) A. Löve (E^eE^e, $2n = 14$) were originally collected from France; the accession number is PI531719.

Cloning and sequencing of tall wheatgrass LMW-GS genes

Genomic DNA was extracted from dark grown 14-day-old tall wheatgrass seedlings grown at 23°C, using Murray and Thompson's (1980) CTAB method. This DNA was used as a template for PCR amplification via a pair of degenerate primers, as designed by Luo et al. (2005), in combination with high-fidelity LA *Taq* polymerase in GC buffer (TaKaRa). The PCR programme consisted of an incubation at 95°C for 5 min, followed by ten cycles of 94°C/30 s, 68°C/60 s decreasing by 1°C per cycle, 72°C/90 s, and 35 cycles of 94°C/30 s, 58°C/30 s, 72°C/90 s, with a final extension step of 72°C/7 min. After purification, the PCR products were cloned into pMD 18-T (TaKaRa) and introduced into *E. coli* DH10B competent cells by standard methods (Sambrook et al. 1989). Positive clones were identified by colony PCR, and at least two independent sequences per clone were obtained from a commercial sequencing service (Yingjun, Shanghai, China). The resulting sequences were analysed with the help of MEGA v3.1 software (Kumar et al. 2004) and programs deposited in GenBank and EBI.

Heterologous expression in *E. coli*

The bacterial expression vector pET-24a(+) (Novagen) was used for the heterologous expression of *Ee34*, one of the tall wheatgrass *Glu-E3* sequences in *E. coli*. The coding region of the mature protein was PCR amplified from the recombinant plasmid with primer pairs 5′-GCCCA TATGGAGAGTAACATCATCA and 5′-CTAGAATTCT-TATCAGTAGGCACCA. The former primer introduces an *Nde*I, and the latter an *Eco*RI restriction site, which were used to allow sticky-end ligation of the amplicon to the pET-24a(+) plasmid. After an overnight ligation at 4°C, the vector was transformed into competent *E. coli* strain Rosetta (DE3)-plysS cells using the $CaCl_2$ methods (Sambrook et al. 1989). A single recombinant colony was inoculated into 20 ml LB liquid medium, incubated overnight at 37°C and then transferred to 2 l of the same medium. Heterologous expression was induced by the addition of IPTG to a final concentration of 1 mM once the OD_{600} of the culture had reached ~0.6. The cultured cells were held at 37°C with shaking for 5 h, harvested by centrifugation (10 krpm, 4°C, 10 min) and stored at −20°C.

Separation of heterologously expressed proteins

Cell pellets were resuspended in lysis buffer A (50 mM Tris–HCl pH 7.3, 100 mM NaCl, 1 mM EDTA, 1 mM PMSF, 10 mM DTT) in a ratio of 1 g cells to 5 ml buffer, and disrupted by sonication (2 s sonication, 2 s pause over 20 min on ice at 400 W output). After centrifugation (12 krpm, 4°C, 15 min), the supernatant was discarded and the pellet washed two times in TE and once in 4 M urea, and finally dissolved in 5 ml 8 M urea. The solution was mixed with loading buffer ratio of supernatant to loading buffer 8/2 v/v and was loaded onto an SDS-polyacrylamide gel. After electrophoresis, the gel was stained in cold 0.5 M KCl, the gel segment containing the heterologously expressed protein excised, crushed, resuspended in 50%

(v/v) 1-propanol containing 1% w/v DTT, and incubated at 65°C for 2 h. After centrifugation (12 krpm, 10 min), 5 ml 1-propanol containing 1% DTT was added, the sample was centrifuged (12 krpm, 10 min), and the supernatant discarded. The part of the precipitated material was used to assess product purity and part was resuspended in 50% (v/v) 1-propanol containing 1% DTT and dialysed against 1% (v/v) acetic acid for 72 h. Finally, the sample was freeze-dried.

N-terminal amino acid sequencing

Protein samples were passively transferred on to a polyvinylidene difluoride membrane after electrophoresis, using 10 mM CAPS pH 4.5 as the transfer buffer. Amino acid sequencing was performed with an automatic amino acid sequencer (Applied Biosystems Procise®), at the National Key Laboratory of Protein and Plant Genetic Engineering, Beijing University, China.

Ten-gram mixograph tests

Mixing tests were conducted with a 10 g Mixograph (National Mfg. Co., Lincoln, NE) according to Walker and Walker (1992). The parameters determined included the time to peak dough resistance (mixing time, MT), 8-min curve width (TxW), peak width (PW) and right of peak slope (RPS). MT, TxW and PW are measurement of dough strength whilst RPS is an inverse measurement of dough stability (Walker and Walker 1992; Lee et al. 1999). HMW-GS 1Dx2 and HMW-GS 1Bx14 were purified from the flour after SDS-polyacrylamide gel electrophoresis according to the above method of the bread wheat cultivar SR3 and Xiaoyan 6 to represent two positive controls since they were assumed to be related to poor and good quality, respectively (Xu et al. 2006). Flour of the cultivar Jinan177 (1Bx7 + 1By9, 1Dx2 + 1Dy12) was chosen as the base flour, which have a poor flour quality (Liu et al. 2006). A reversible reduction/oxidation procedure for incorporating the added polypeptides into glutenin was employed (Bekes et al. 1994). A 10 g sample of base flour (14.5% moisture and 12.6% protein) was supplemented with 50 or 100 mg of test proteins, thoroughly mixed with 4.90 ml water plus 0.6 ml 1 mg/ml DTT for 30 s, and rested for 4 min. At this point, 0.72 ml 10 mg/ml KIO_3 was added and the dough mixed for 30 s, rested for 5 min and mixed for an additional 10 min. Mixing curves and parameters were recorded by a Mixograph device.

Accession numbers

The complete nucleotide sequences and deduced peptide sequences of the following 14 *Glu-E3* genes have been deposited in GenBank under the following accession numbers: *Ee2*, EU822813; *Ee3*, EU822814; *Ee4*, EU822815; *Ee6*, EU822816; *Ee10*, EU822817; *Ee13*, EU822819; *Ee14*, EU822820; *Ee17*, EU822826; *Ee18*, EU822825; *Ee27*, EU822821; *Ee31*, EU822828; *Ee33*, EU822827; *Ee34*, EU82282; and *Ee35*, EU822824.

Results

PCR amplification and cloning of LMW-GS alleles from Glu-E3

A number of amplified fragments containing the complete coding sequence were obtained from tall wheatgrass template. Their length was between 900 and 1,000 bp. Fourteen distinct inserts were identified and designated *Ee2*, *Ee3*, *Ee4*, *Ee6*, *Ee10*, *Ee13*, *Ee14*, *Ee17*, *Ee18*, *Ee27*, *Ee31*, *Ee33*, *Ee34* and *Ee35*. The nucleotide sequence of all of these was highly similar to that of known LMW-GS genes (Table 1), but none was present in any of the public DNA sequence databases. *Ee2*, *Ee6* and *Ee31* were probably pseudo-genes, because each contained an internal stop codon. The remaining 11 all represented a single intact open-reading frame. All were intron-less, with a coding region of length 957–1,071 bp. The sequences were distinct from one another based on the both single-nucleotide polymorphisms and indels, and their pairwise similarity varied from 99 (*Ee3* and *Ee18*) to 96% (*Ee3* and *Ee17*).

Deduced and direct LMW-GS peptide sequence

The deduced peptide sequences of the 11 genes consisted of 319 (*Ee4* and *Ee33*) to 357 (*Ee17* and *Ee27*) peptides, giving a range in predicted molecular weight of between 33.6 (*Ee33*) and 38.3 kDa (*Ee17*) (Table 1). Sequence alignment showed that their primary structure was similar to that of wheat and other wheat-related grass *Glu-3* genes. This was made up of a 20 amino acid residues signal peptide, a 14 amino acid residues *N*-terminal motif, a repetitive domain rich in glutamine and proline, and a C-terminal domain consisting of subregion I (cysteine-rich), II (glutamine-rich) and III (conserved) (Fig. 1). The *N*-terminal 14 amino acid residues of all 11 genes were MESNIIISFLK/RPWL (Fig. 1). On the basis of the first amino acid residue of the mature protein, all belonged to the LMW-m type, but none contained the METSHIPG *N*-terminal conserved motif, which is common for this type; all except *Ee13* contained seven cysteine residues (Table 1; Fig. 1). BLASTn searches of these sequences (Table 1) showed that the *Ee17*, *Ee27* and *Ee34* sequences were 97% identical to AY214452 (Xu et al. 2004), whilst the identity of the other eight ranged from 93 to 97%.

Table 1 Basic properties of LMW-GS genes isolated from tall wheatgrass

Genes	Amino acid number	Deduced PI	Deduced molecular weight (kD)	Number of Cys	Similar published number sequence (identity %)
Ee3	327	8.21	36.97	7	AY214454 (95)
Ee4	299	8.21	33.76	7	AY214454 (95)
Ee10	305	8.53	34.42	7	AY214454 (97)
Ee13	314	8.86	35.60	6	AY214454 (94)
Ee14	305	8.21	34.52	7	AY214454 (97)
Ee17	337	8.98	38.28	7	AY214452 (97)
Ee18	327	8.21	37.01	7	AY214454 (95)
Ee27	337	7.60	38.24	7	AY214452 (97)
Ee33	299	6.87	33.66	7	AY214454 (95)
Ee34	336	8.78	38.14	7	AY214452 (97)
Ee35	313	8.21	35.41	7	AY214454 (94)

Phylogenetic analysis of tall wheatgrass LMW-GS genes

A phylogenetic analysis was carried out based on the nucleotide sequence of the 11 tall wheatgrass *Glu-D3* genes along with eight other *Triticeae* species LMW-m type, three LMW-s type, six LMW-i type genes, and four intermediate tall wheatgrass genes. The 11 tall wheatgrass genes all clustered closely with one another, and appear closely related to three of the four intermediate tall wheatgrass sequences, but only distantly so to those from decaploid tall wheatgrass (Fig. 2).

Large-scale preparation of LMW-GS protein

The transformed bacteria, Rosetta (DE3)-plysS cells, carrying the recombinant plasmids were identified and used to express the target protein, whilst the bacteria contained the expression vector pET-24a(+) was used as control. Total 8 l cultures used to prepare batches of proteins were collected after about ~10 h incubation. The results of N-terminal amino acid sequencing and gel analyses confirmed that there were no modifications (Fig. 3); Ee34 had an *N*-terminal peptide sequence MESNIIISFL, as predicted from the DNA sequence.

Functional properties of protein Ee34

A micro-mixing test of supplemented flour was used to examine any contribution to functionality of the heterologously expressed tall wheatgrass LMW-GS protein. The mean values of four mixing parameters (mixing time, MT; 8 min curve width, TxW; peak width, PW, and right of peak slope, RPS) of three replicate analyses are given in Table 2. As indicated from Table 2, when the LMW-GS was incorporated into the base flour, it caused significantly increase in MT, TxW and PW and decrease in RPS values ($P < 0.01$). Poor HMW-GS 1Dx2 and strong HMW-GS 1Bx14 were included as two positive controls, and showed higher mixing time and peak width when 50 mg of alternative protein was incorporated, and the parameter values of 1Bx14 were higher than those of 1Dx2, as expected. The LMW-GS was more effective in those parameters than two positive controls. It is notable that the effects of the LMW-GS on mixing time, peak width, and 8-min curve width were largely strengthened when 100 mg of the protein was incorporated. Figure 4 showed the comparison of these incorporations.

Discussion

Contribution of LMW-GS genes to dough quality

The number and position of cysteine residues within the glutenin monomers appears to have a substantial effect on their contribution to end-use quality in wheat (Masci et al. 1998, 2002; Shewry et al. 2003). Most mature *Glu-3* gene products contain eight cysteine residues (Shewry et al. 2003), and of these, the six which are rather well conserved are thought to be involved in the formation of intramolecular disulphide bonds. Such linkages within the monomer have the effect of producing a compactly folded globular protein (Miiller and Wieser 1995; Khatkar et al. 2002). The other two cysteine residues participate in intermolecular disulphide bonds (Masci et al. 1998, 2002; Shewry and Halford 2002; Shewry et al. 2003). Of the 11 tall wheatgrass *Glu-3* mature proteins described here, ten have seven cysteine residues, which include the six conserved ones. It has been suggested that α-gliadins having an odd number of cysteine residues are more likely to

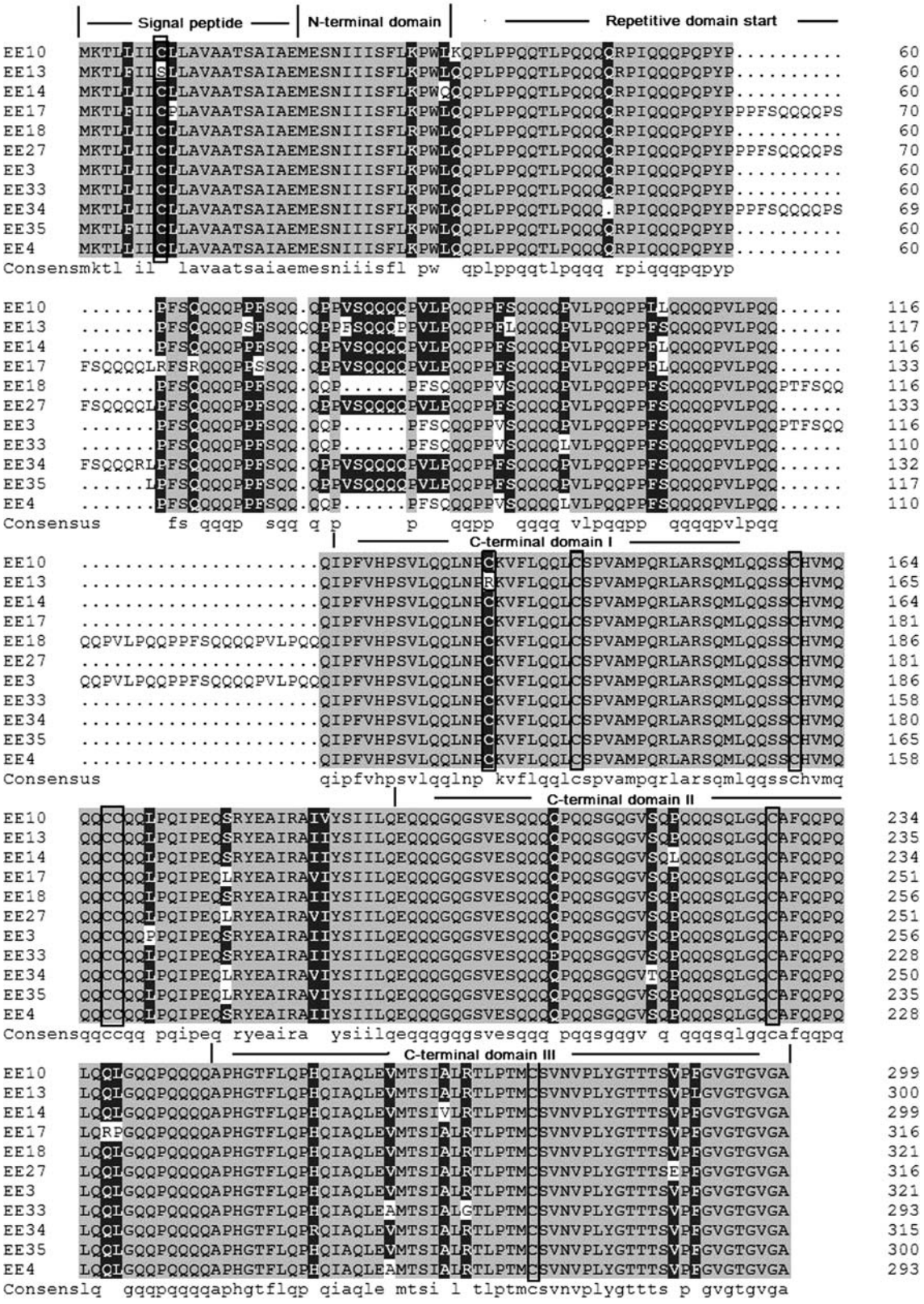

Fig. 1 Deduced peptide sequence alignment of 11 tall wheatgrass *Glu-D3* genes. Cysteines are shown in *boxes*. Deletions are indicated by *points*

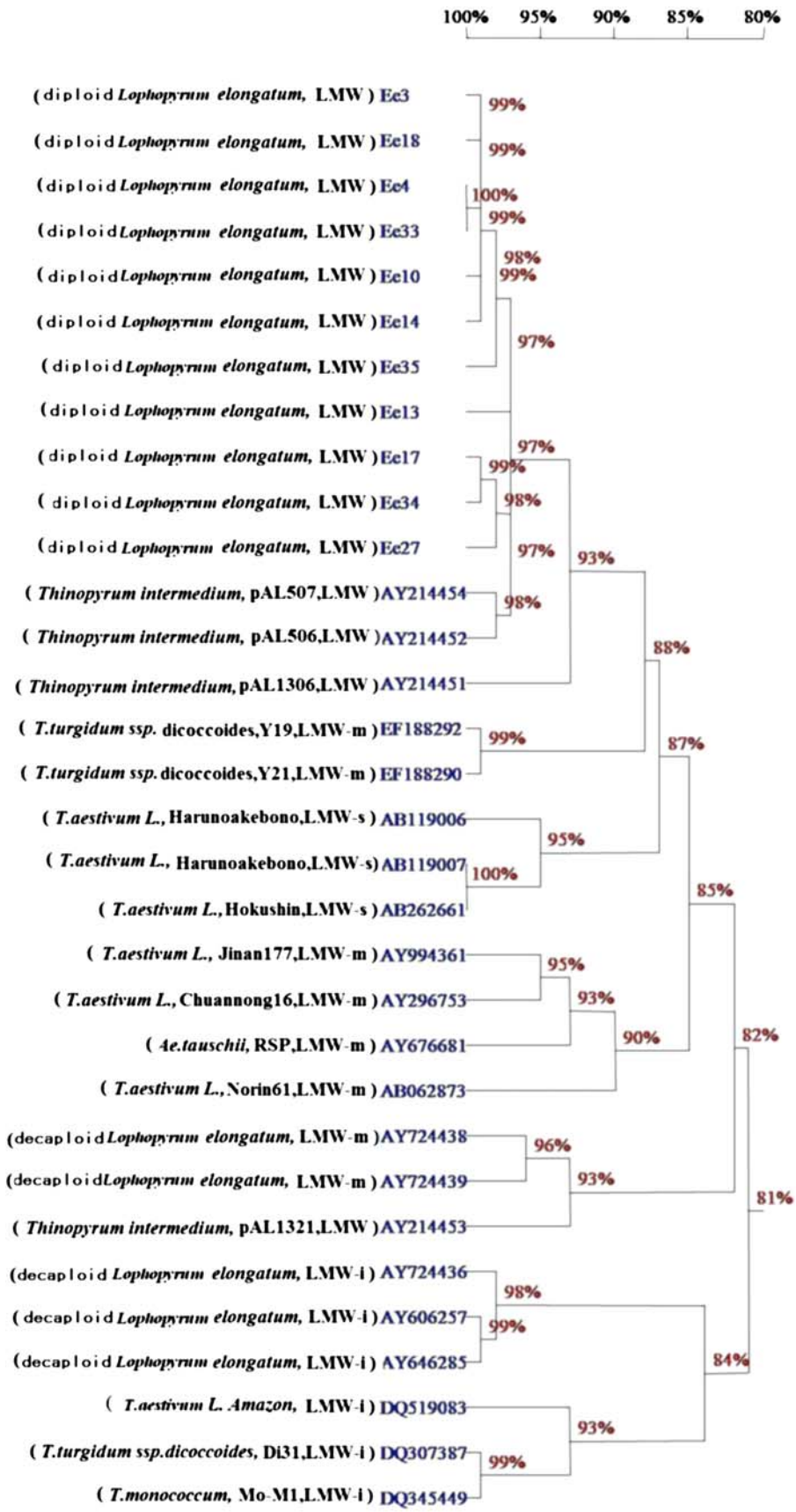

Fig. 2 Phylogenetic analysis of the coding regions of 32 *Glu-D3* genes. The identity of each nucleotide sequence is specified by its GeneBank accession number. The species origin and type of LMW-GS are shown in *brackets*. Sequences AY214451-4, AB119006-7, EF188290 and EF188292 are cDNAs, the rest are genomic DNAs

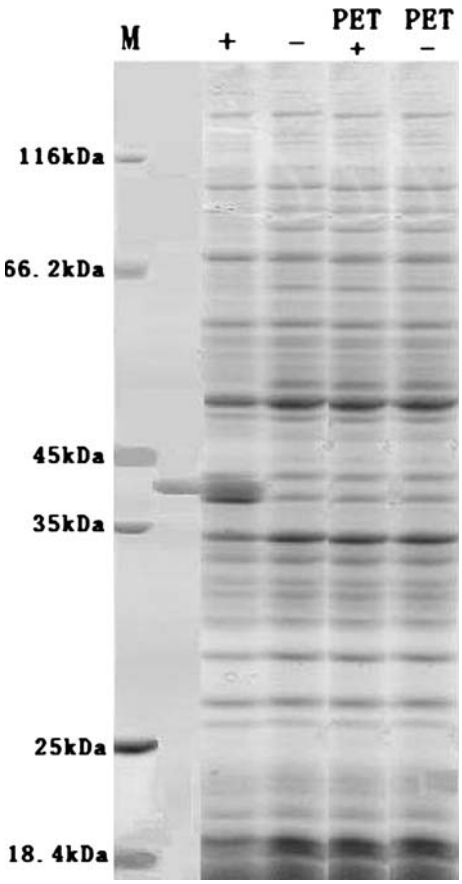

Fig. 3 SDS-PAGE profile of the purified expressed LMW-GS Ee34. M, standard protein marker, + induced pET-24a-*Ee34* in *Rosetta* (DE3)-plysS by 1 M IPTG, − non-induced pET-24a-*Ee34* in *Rosetta* (DE3)-plysS by IPTG; PET+, induced pET-24a(+) in *Rosetta* (DE3)-plysS by 1 M IPTG; PET−, non-induced pET-24a(+) in *Rosetta* (DE3)-plysS by IPTG

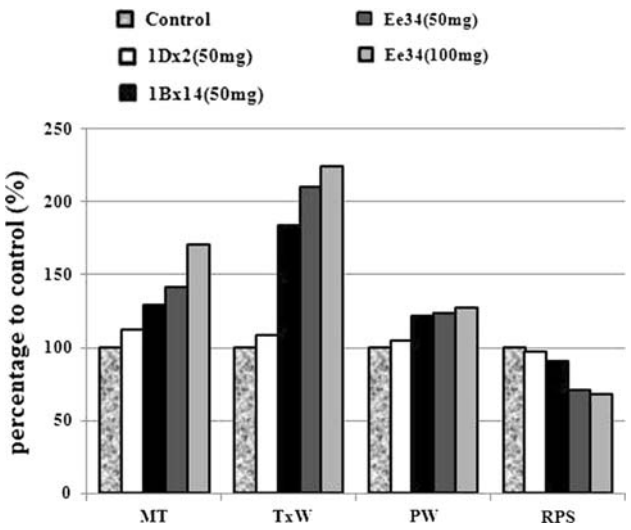

Fig. 4 A comparison of the functional properties of flour supplemented by Ee34 LMW-GS and two standard HMW-GS

aggregate with the disulphide cross-linked gluten matrix, and thereby have a positive effect on end-use quality (Kasarda et al. 1984). A contrasting model has been proposed by Porceddu et al. (1998), in which gliadins having an odd number of cysteines can induce chain termination, with a negative effect on pasta quality. Here, we have provided some preliminary evidence that the Ee34, with seven cysteine residues (6 conserved and 1 free), had a positive effect on flour quality. The similar result was obtained using different base flour and LMW-GS containing 8 or 9 Cys residues (Lee et al. 1999; Xu et al. 2006). Out of these Cys residues, only two or three participated in intermolecular disulphide bonds, which had a large bearing on the flour quality. These results supported the hypothesis that Ee34 might act as "chain brancher", enhance the protein to join into the glutenin network and form larger glutenin polymers, which contribute to the dough elasticity and high-quality property of the bread wheat.

Evolutionary relationship of Glu-3 alleles

Hexaploid wheat carries 30–40 LMW-GS genes at its three *Glu-3* loci, equivalent to 10–13 per diploid genome. Given that all *Glu-3* homoeoloci—in both wheat and other *Triticeae* species—share this structure, it is reasonable to propose that the wheat and tall wheatgrass progenitor species also had a complex *Glu-3* locus, composed of ~10 genes. The function of the storage proteins is a passive one—to provide nutrition to the germinating zygote. Thus, their coding sequence is probably under little selection pressure, and furthermore it may be of some selective advantage for a plant to evolve multiple genes encoding storage proteins,

Table 2 The means of mixing time (MT), 8-min curve width (TxW), peak width (PW), right of peak slope (RPS) of the dough of base flour (control) and the dough incorporated with 1Dx2, 1Bx14, LMW-GS, determined from triplicate mixing experiments

Item	Control	1Dx2 (50 mg)	1Bx14 (50 mg)	Ee34 (50 mg)	Ee34 (100 mg)
MT (min)	1.7^{A}	1.9^{B}	2.2^{C}	2.4^{D}	2.9^{E}
TxW (%)	5.6^{A}	6.1^{B}	10.3^{C}	11.8^{D}	12.6^{E}
PW (A.U.)	162^{A}	170^{B}	197^{C}	200^{C}	207^{D}
RPS (%min^{-1})	-4.1^{A}	-4.0^{A}	-3.7^{B}	-2.9^{C}	-2.8^{C}

Means with different capital letters are significantly different at $P < 0.01$ level

as this increases the supply of nutrition to the germinating zygote. Following the speciation from the progenitor species, these ~10 genes most likely will have evolved independently, both in number and in sequence, so that a phylogenetic analysis would be expected to recognise groups of gene copies related by descent, rather than groups of gene copies based on the species in which they currently reside. However, our finding was that the tall wheatgrass sequences clustered with one another, rather than being dispersed throughout the phenogram, as expected. Several PCR amplifications have been done and a large number of clones were isolated and sequenced. All the 11 sequences were homologous in different amplification and sequencing; however, the primers were not designed specifically for the m-class LMW-GS genes, but did according to the published data including m-, s- and i-class LMW-GS ones. We are not sure whether the degenerate primers can amplify all the members of the *Glu-3* family or not under altered PCR parameters. Maybe they were selective in this species—so we are not seeing the full set of tall wheatgrass members. Within the LMW-i clade (Fig. 2), the *T. turgidum* DQ307387 and the *T. monococcum* DQ345449 sequences were 99% homologous, and clustered closely with *T. aestivum* DQ519083. Within the LMW-m/LMW-s group, the 11 tall wheatgrass genes described here were all highly similar to one another (93–97% homologous), and were more closely related to three sequences AY214451, AY214452 and AY214454, respectively, from intermediate tall wheatgrass than to any from decaploid *Th. ponticum*. Thus, the E genome from diploid *L. elongatum* may be not the ancestor of E genome of decaploid *Th. Ponticum*.

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