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Isolation and characterization of five novel high molecular weight subunit of glutenin genes from *Triticum timopheevi* and *Aegilops cylindrica*

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Abstract Analysis by SDS-PAGE of total protein fractions from single seeds of *Aegilops cylindrica* (genomes C and D) and *Triticum timopheevi* (genomes A and G) showed the presence of three bands corresponding to high molecular weight subunits of glutenin (HMW subunits) in the former and two major bands and a minor band corresponding to HMW subunits in the latter. Three *Ae. cylindrica* and two *T. timopheevi* HMW subunit gene sequences, each comprising the entire coding region, were amplified by polymerase chain reaction (PCR) and their complete nucleotide sequences determined. A combination of *N*-terminal amino acid sequencing of the proteins identified by SDS-PAGE and alignments of the derived amino acid sequences of the proteins encoded by the PCR products identified the *Ae. cylindrica* HMW subunits as 1Cx, 1Cy and 1Dy, and the *T. timopheevi* HMW subunits as 1Gx, 1Ax and 1Ay. It was not clear whether or not a 1Gy HMW subunit was present in *T. timopheevi*. The PCR products from *Ae. cyclindrica* were derived from 1Cy and 1Dy genes and a silent 1Dx gene containing an in-frame internal stop codon, while those from *T. timopheevi* were derived from 1Ax and 1Ay genes. The 1Cx, 1Gx and 1Gy sequences were not amplified successfully. The proteins encoded by the five novel genes had similar structures to previously characterized HMW subunits of bread wheat (*Triticum aestivum*). Differences and similarities in sequence and structure, and in the distribution of cysteine residues (relevant to the ability of HMW subunits to form high M_r polymers) distinguished the HMW subunits of x- and y-type and of each genome rather than those of the different species. There was no evidence of a change in HMW subunit expression or structure resulting from selective breeding of bread wheat. The novel 1Ax, 1Ay, 1Cy and 1Dy HMW subunits were expressed in *Escherichia coli*, and the expressed proteins were shown to have very similar mobilities to the endogenous HMW subunits on SDS-PAGE. The truncated 1Dx gene from *Ae. cylindrica* failed to express in *E. coli*, and no HMW subunit-related protein of the size predicted for the truncated 1Dx subunit could be identified by immunodetection in seed extracts.

Keywords Wheat · HMW subunit · Breadmaking quality · Storage protein · Repeated sequences

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Introduction

The high-molecular-weight subunits of glutenin (HMW subunits) are particularly important for determining wheat gluten and dough elasticity (Payne 1987; Shewry et al. 1992, 1995). They are encoded by the *Glu-1* loci on chromosomes 1A, 1B and 1D, each locus consisting of two genes encoding an x-type and a y-type subunit (Payne 1987). However, silencing of genes at the *Glu-A1* and *Glu-B1* loci has resulted in variation in the number of HMW subunits in bread wheat cultivars from three to five, with 1Dx, 1Dy and 1Bx subunits always being present and 1By and 1Ax subunits being present in only some cultivars (Payne 1987). 1Ay subunits are not present in any cultivated lines of bread wheat but do occur occasionally in hexaploid wheats (Margiotta et al. 1993; Johansson and Svensson 1995) and more widely in A-genome diploids (Waines and Payne 1987).

The variation in HMW subunit composition has been reported to account for up to 70% of the variation in breadmaking quality between European wheats (Branlard and Dardevet 1985; Payne et al. 1987, 1988), even though they account for only a relatively small proportion of the

total grain protien content (up to about 12%) (Halford et al. 1992; Seilmeier et al. 1991). This impact on quality appears to result from two separate effects: (1) quantitative differences in the total amount of HMW subunit protein associated with the presence of three, four or five subunits; (2) qualitative effects associated with differences in the structure and properties of allelic subunits. In particular, the subunit pair 1Dx5 + 1Dy10 is consistently associated with good breadmaking quality when compared with the allelic pairs 1Dx2 + 1Dy12, 1Dx3 + 1Dy12 and 1Dx4 + 1Dy12 (Payne 1987).

The identification of "quality-associated" HMW subunits in bread wheat has led to wider studies of HMW subunits in gene banks of wheat and related wild species in order to identify novel allelic variants that can be incorporated into bread wheat using genetic or genetic engineering approaches. These include x-type and y-type subunits showing unusually high or low mobilities by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Tahir et al. 1996; Shewry et al. 2001). However, few of these proteins or genes have been characterized in detail (D'Ovidio et al. 1996.

Aegilops cylindrica and *Triticum timopheevi* are both tetraploid species that share one genome with cultivated bread wheat. *T. timopheevi* is an ancient, cultivated wheat that is still grown to a limited extent in the Caucasus and has the A and G genomes, while the wild *Ae. cylindrica* has the C and D genomes. Both species are of potential importance as sources of novel genes for transfer to bread wheat, but only preliminary SDS-PAGE studies of their HMW subunits have been reported (Margiotta et al. 1998; Wan et al. 2000). We have, therefore, used polymerase chain reaction (PCR) technology to isolate genes encoding HMW subunits from both species in order to identify novel variation in the structure of the encoded proteins that could be exploited in bread wheat.

Materials and methods

Seeds

Six accessions of *Aegilops cylindrica* were provided by Dr. Lihui Li, Institute of Crop Germplasm Resources of the Chinese Academy of Agricultural Sciences, Beijing, China. Four accessions of *Triticum timopheevi* were kindly provided by the John Innes Centre, Norwich, UK. *Triticum aestivum* line L88-6 was used as a standard to compare HMW subunit composition.

Protein sequencing

Methods for seed protein extraction and SDS-PAGE were based on those described previously (Wan et al. 2000). Half a grain was crushed in sample buffer containing 0.0625 M Tris-HC1 (pH 6.8), 2% (w/v) SDS, 1.5% (w/v) DTT, 0.0002% bromophenol blue. The extracts were heated for 2 min at 95 °C and centrifuged for 5 min at 12,000 *g*. The supernatant was loaded onto a 10% (w/v) polyacrylamide gel using a Tris-borate buffer system.

After electrophoresis, the proteins were transferred from the gel onto a ProBlott membrane by western blotting. The membrane was saturated with methanol and stained with 0.1% (w/v) Coomassie BBR250 (Sigma, St. Louis, Mo.). The HMW subunit bands were then excised for protein sequencing.

N-terminal amino acid sequences of the HMW subunit proteins were determined by Dr. M. Naldrett (John Innes Centre) using an Applied Biosystems Model 477A pulsed-liquid amino acid sequencer equipped with on-line PTH amino acid analyser Model 120A.

Immunodetection

After SDS-PAGE, proteins were transferred by western blotting onto a ProBlott membrane. The membrane was washed with TBS buffer (20 mM Tris (pH 7.5), 500 mM NaCl), then incubated in blocking solution (5% (w/v) non-fat milk powder in TBS) for 30 min. After being washed twice with TTBS [0.05% (v/v) Tween in TBS], the membrane was transferred into the first antibody solution. The antibody solution used to detect x-type HMW subunits was a 1:50 dilution of monoclonal antibody IFRN 1602 (Mills et al. 2000) in TBS containing 1% (w/v) BSA. The antibody solution for detection of y-type HMW subunits was a 1:5000 dilution of the anti-HMWR2 polyclonal antibody (Denery-Papini et al. 1996) in the same buffer. The membrane was left in the first antibody solution for a period from 1 h to overnight, then washed twice in TTBS and reacted for 1 h with a 1:5000 dilution of either anti-mouse IgG or goat anti-rabbit IgG conjugated with alkaline phosphatase (Sigma). The membrane was washed twice again in TTBS and once in TBS, then immersed in colour development solution (BCIP and NBT in carbonate buffer) for 2–5 min. The reaction was stopped by immersing the membrane in distilled water for 10 min.

DNA extraction and PCR amplification of HMW subunit gene sequences

Seeds were germinated and grown for 1 week in darkness. Etiolated leaves were harvested, and genomic DNA was prepared from them using a DNeasy plant mini kit (QIAGEN, Valencia, Calif.). HMW subunit sequences were amplified by PCR using the following oligonucleotides as primers: (1) 5′ -ATGGCTAAGCGGTTAGTCCT; (2) 5′ -GCTGCAGAGAGTTCTATC; (3) 5′ -AGATGACTAAGCGGTTGGTTC; (4) 5′ -AGCTGCAGAGAGTTCTATC; (5) 5′ -ATGGCTAAGCGGTTGGTCCT; (6) 5′ -CGACGCATTGTCGGCTAGCC.

A combination of primers 1 and 2 was used to amplify 1Dx HMW subunit sequences, a combination of 3 and 4 to amplify 1Ax HMW subunit sequences and a combination of 5 and 6 to amplify y-type HMW subunit sequences. PCR reactions were performed with a GeneAmp 9700 cycler (Perkin Elmer) in a reaction volume of 50 µl containing 2.5 U'Pfu Turbo' DNA polymerase (Stratagene), 50 pmol each primer and 20 m*M* each dNTP. The reaction mix was heated to 94 °C for 2 min to denature the DNA, cycled 35 times at 94 °C for 1 min, 60 °C for 2 min and 72 °C for 2.5 min, then incubated at 72 °C for 10 min. PCR products were separated by agarose gel electrophoresis, and products of the expected size were purified from the gel using a QIAquick gel extraction kit (Qiagen) and ligated into plasmid pCR-Script AMP SK(+) (Stratagene). The resulting plasmids were transformed into Ultracomp *Escherichia coli* cells (InVitrogen, carlsbad, Calif.).

Nucleotide sequencing of HMW subunit genes

The nucleotide sequences of the HMW subunit genes were obtained by primer walking. Chain-terminator sequencing reactions were performed with an ABI Prism dRhodamine Terminator Cycle Sequencing Reaction Kit using a GeneAmp 9700 cycler (Perkin Elmer), with 25 cycles of 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min. The PCR products were precipitated with ethanol, dissolved in 3 µl formamide loading buffer, denatured by heating at 95 °C for 2 min and loaded onto a 4.45% (w/v) polyacrylamide

gel in an ABI Prism 377XL (PE) sequencing machine. Electrophoresis was carried out for 7 h.

Sequence analyses

Multiple *N*- and *C*-terminal sequence alignments were produced using the PILEUP programme (Wisconsin Package version 10, Genetics Computer Group, Madison, Wis.) with a gap creation penalty of 3.0 and a gap extension penalty of 0.1 and displayed using the PRETTYBOX programme; alignments of the repetitive domain were done by eye. Evolutionary distances were calculated using the DISTANCES programme, correcting for multiple substitutions at a single site, and displayed by the unweighted pair group method using arithmetic averages with the GROWTREE programme.

Expression of HMW subunit genes in *E. coli*

The cloned HMW subunit DNA sequences were amplified by PCR using the following oligonucleotides as primers: (7) 5′ -CTCTCATATGGAAGGTGAGGCCTCTGAGCA; (8) 5′ -CTCTCTCGAGGTTGGGTAGTACCCTGGTTA; (9) 5′ -CTCTCATATGGAAGGTGAGGCCTCTAGGCA; (10) 5′ -CTCTCTCGAGTTACTGGCTAGCCGACAATGCGT; (11) 5′ -CTCTCATATGGAAGGTGAGGCCTCTGGGCA; (12) 5′ -CTCTCTCGAGTTACTGGCTGGCCAACAATGCGT.

A combination of primers 7 and 8 was used to amplify the *Ae. cylindrica* 1Dx gene, a combination of 9 and 10 to amplify the *Ae. cylindrica* 1Cy and 1Dy genes and the *T. timopheevi* 1Ay gene and a combination of 11 and 12 to amplify the *T. timopheevi* 1Ax gene. In each case a *Nde*I restriction site (CATATG) was incorporated at the 5′ end of the PCR product and a *Xho*I restriction site (CTCGAG) at the 3′ end. The PCR reactions were heated to 94 °C for 2 min to denature the DNA, cycled 35 times at 94 °C for 1 min, 68 °C for 4 min, then incubated at 68 °C for 10 min. The PCR products were cloned into pCR-Script Amp SK(+), and their nucleotide sequences were confirmed. The HMW subunit DNA sequences were then excised by digestion with *Nde*I and *Xho*I and ligated into the expression vector pET-24a (Novagen) (Studier 1991). *E. coli* BLR (DE3) plysS cells were transformed with the pET plasmids containing the HMW subunit genes and grown on 2YT medium (Sambrook et al. 1989) containing 35 μg ml^{-1} kanamycin in "baffled" flasks in a shaking incubator at 37 °C until the OD_{600} reached 0.6. HMW subunit protein expression was then induced by the addition of 1 m*M* IPTG and the cells incubated overnight. The cells were collected by centrifugation at 5,000 *g* and 4 °C for 10 min and frozen at –80 °C or used immediately.

HMW subunit protein was extracted from the cells by suspending the latter in 50% (v/v) propanol and 2% (w/v) DTT and then sonicating them using an Ultrasound Liquid Processor XL (Heat Systems). The resulting suspension was incubated at 65 °C for 3 h, and then the cell debris was removed by centrifugation at 10,000 *g* and 4 °C for 10 min. The supernatant was dialysed overnight in 10% acetic acid at 4 °C and lyophilized. The purified protein was dissolved in sample buffer for SDS-PAGE analysis and western blotting.

Small-scale expression of HMW subunit genes in *E. coli*

A single bacterial colony was inoculated into 50 ml 2YT medium containing 35 μg ml^{-1} kanamycin and incubated until the OD_{600} was approximately 0.6. HMW subunit protein expression was induced by the addition of IPTG, and the incubation was continued for another 7 h. The cells from 1 ml of the culture were collected by centrifugation at 10,000 *g* for 1 min and re-dissolved in lysis buffer (0.1 *M* $MgCl_2$, 4% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 0.01% (w/v) bromophenol blue, 100 m*M* Tris-HCl, pH 6.8) (Chen and Christen 1997), and the proteins were analysed by SDS-PAGE and western blotting (Fido et al. 1995)

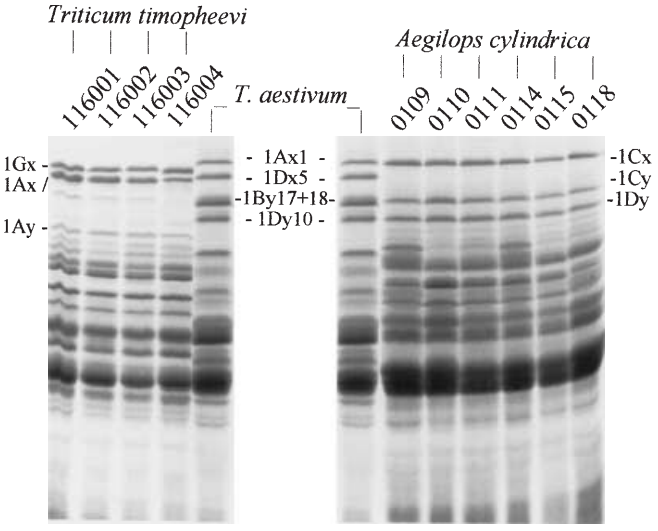

Fig. 1 SDS-PAGE analysis of total protein fractions from single seeds of four accessions of *Triticum timopheevi*, six accessions of *Aegilops cylindrica* and *Triticum aestivum* line L88-6. HMW subunits 1Ax1, 1Dx5, 1By17 + 18 (which did not separate) and 1Dy10 in *T. aestivum* line L88-6 are indicated. The designations shown for the HMW subunits of *T. timopheevi* (1Gx, 1Ax and 1Ay) and *Ae. cylindrica* (1Cx, 1Cy and 1Dy) are based on their size and on subsequent analyses

Northern analysis of E. coli cells expressing a truncated HMW subunit gene

Total bacterial RNA was extracted using an RNeasy kit (Qiagen) following the manufacturer's instructions. The RNA (5 μg) was separated under denaturing conditions in the presence of formlaldehyde on l.4% agarose and transferred to Hybond-C (Amersham) according to Sambrook et al. (1989). A α-[^{32} P]dATP-labelled probe was prepared from the cloned *Ae. cylindrica* 1Dx gene by the random-primed extension method (Feinberg and Vogelstein 1984) using the Prime-It II Random Primer Labelling Kit (Stratagene). The final wash stringency was 0.5 × SSC at 65 °C.

Results and discussion

HMW subunit composition of *Aegilops cylindrica* and *Triticum timopheevi*

SDS-PAGE of total protein fractions from single seeds of six accessions of *Ae. cylindrica* showed similar patterns of HMW subunits (Fig. 1), each line containing three bands with similar mobilities to subunits 1Ax1, 1By17+18 and 1Dy10 from the control *T. aestivum* line L88-6. *N*-terminal amino sequencing of the three bands (Table 1) suggested that the top band was an x-type subunit (with Gly at position 6), while the two lower bands were y-type (with Arg at position 6). Based on this and further studies, the three subunit bands were identified as 1Cx, 1Cy and 1Dy, as indicated in Fig. 1.

Similar analyses of four accessions of *T. timopheevi* also showed identical patterns, with two major bands with similar mobilities to subunit 1Dx5 of bread wheat together with a minor band of faster mobility than subunit 1Dy10 (Fig. 1). *N*-terminal amino acid sequencing

Table 1 Comparison of the *N*-terminal amino acid sequences of the HMW subunits of *Triticum timopheevi* and *Aegilops cyclindrica* with the consensus sequences of x-type and y-type HMW subunits of bread wheat. The *T. timopheevi* and *Ae. cyclindrica* sequences were determined by direct amino acid sequencing of protein bands blotted onto ProBlott membrane and deduced from the DNA sequences of the corresponding genes

Species	Subunit	Residue									
		1	2	3	4	5	6	7	8	9	10
Triticum aestivum	x-type consensus	E	G	E	A	S	G_E	Q	L	Q	(C)
Aegilops cylindrica	1Cx determined	E	G	E	A	S	G	Q	L	Q	(C)
	Silent 1Dx deduced	E	G	E	A	S	E	Q	L	Q	(C)
Triticum timopheevi	1Ax determined	E	G	E	A	S	G	Q	L	Q	(C)
	1Ax deduced	E	G	E	A	S	G	Q	L	Q	(C)
	1Gx determined	E	G	E	A	S	G	Q	L	Q	(C)
Triticum aestivum	1Dy consensus	E	G	E	A	S	R_K	Q	L	Q	(C)
Aegilops cylindrica	1Dy determined	E	G	E	A	S	R	Q	L	Q	(C)
	1Dy deduced	E	G	E	A	S	R	Q	L	Q	(C)
	1Cy determined	E	G	E	A	S	R	Q	L	Q	(C)
	1Cy deduced	E	G	E	A	S	R	Q	L	Q	(C)
Triticum timopheevi	1Ay deduced	E	G	E	A	S	R	Q	L	Q	
	1Gy	Not determined									

suggested that the two high M_r bands were x-type subunits (Table 1), and these were subsequently shown to be 1Gx and 1Ax subunits (as indicated in Fig. 1). Amino acid sequencing of the low M_r band failed to give conclusive results, but subsequent work showed that it corresponded to the 1Ay subunit (Fig. 1).

The designation of each subunit as x- or y-type based on the amino acid residue at position 6 proved accurate. Table 1 shows clearly that the two types differ consistently at this position; x-type subunits have either a glutamic acid residue or neutral glycine residue, whereas y-type subunits have a basic arginine or lysine residue.

While no attempt was made to quantify accurately the total amount of HMW subunit protein in *Ae. cylindrica* and *T. timopheevi*, the intensities of the HMW subunit bands on the gel were comparable with those of *T. aestivum*, with the exception of the y-type subunits of *T. timopheevi*, of which the 1Ay band was relatively faint and the 1Gy band could not be identified clearly.

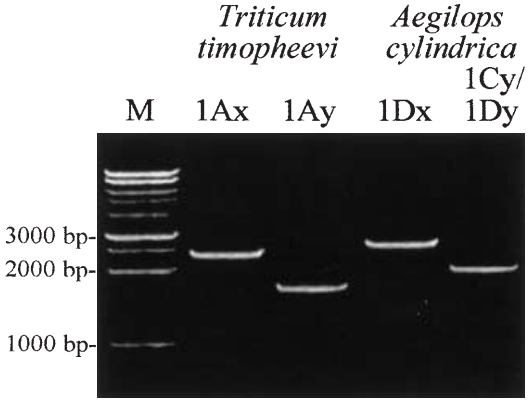

Fig. 2 PCR products derived from the amplification of genomic DNA from accession 116001 of *Triticum timopheevi* and accession 0109 of *Aegilops cylindrica* using primers based on known HMW subunit sequences (refer to Materials and Methods for primer sequences). *M* DNA size markers, *lane 2* a product amplified with 1Ax-specific primers, *lanes 3* and *5* products amplified with y-type-specific primers (the band in *lane 5* was subsequently shown to be a doublet), *lane 4* a product amplified with 1Dx-specific primers

PCR amplification of HMW subunit genes

The close similarity between the *N*-terminal amino acid sequences determined for the HMW subunit bands from *Ae. cylindrica* and *T. timopheevi* and the consensus sequences of x-type and y-type subunits from bread wheat (Table 1) suggested that the genes encoding the proteins would be very similar to their bread wheat homologues. It was, therefore, decided to use non-degenerate oligonucleotides with identical sequences to those at the 5′ and 3′ ends of the coding regions of known HMW subunit genes to prime PCR reactions in order to amplify HMW subunit DNA sequences from genomic DNA of single accessions of each of the two species.

Six oligonucleotides (1–6, see Materials and methods) were prepared. One pair (1 and 2) was designed to amplify 1Dx HMW subunit DNA sequences corresponding to the 1Dx2 (Sugiyama et al. 1985; EMBL accession number X03346) and 1Dx5 (Anderson et al. 1989; X12928) genes of bread wheat from the translation-initiating ATG to position +20 with respect to the last codon (CAG). A second pair (3 and 4) was designed to amplify chromosome 1Ax HMW subunit DNA sequences corresponding to the HMW subunit genes 1Ax1 (Halford et al. 1992; X61009) and 1Ax2* (Anderson and Greene 1989) from position –2 with respect to the translation-initiating ATG to +20 with respect to the last codon (CAG). A third pair (5 and 6)was designed to amplify y-type HMW subunit DNA sequences corresponding to the HMW subunit genes 1Ay (Forde et al. 1985; X03042), 1By9 (Halford et al. 1987; X61026), 1Dy10 (Anderson et al. 1989; X12929) and 1Dy12 (Thompson et al. 1985; X03041) of bread wheat from the translation-initiating ATG to the last codon (CAG).The sequences of these primers were very similar to those designed and used by D'Ovidio and co-workers to amplify HMW subunit genes from bread wheat (D'Ovidio et al. 1995).

HMW subunit genes contain no introns, so genomic DNA can be used as a template for PCR amplification of the entire coding region with no intervening sequences.

Amplification of sequences from *Ae. cylindrica* genomic DNA gave rise to two PCR products (Fig. 2) – one putative x-type HMW subunit sequence produced using oligonucleotides 1 and 2, and one putative y-type HMW subunit sequence produced using oligonucleotides 5 and 6. The PCR products were cloned, and the putative y-type product was found to be a mixture of two products of almost identical size, so three different PCR products out of the four (1Cx, 1Cy, 1Dx and 1Dy) expected to be present in *Ae. cylindrica* had been amplified and cloned successfully.

Amplification of sequences from *T. timopheevi* also produced two products (Fig. 2) – one putative x-type HMW subunit sequence produced with oligonucleotides 3 and 4 and one putative y-type HMW subunit sequence produced with oligonucleotides 5 and 6. Both were cloned and each was found to contain a single product, so two different PCR products out of the four (1Ax, 1Ay, 1Gx, 1Gy) expected to be present in *T.timopheevi* had been amplified and cloned successfully.

A high-fidelity polymerase (*Pfu Turbo*, Stratagene) was used instead of *Taq* polymerase in the amplification procedure in order to reduce the risk of introducing errors into the sequence. The different genes were amplified several times during the course of the study in order to synthesize more DNA for analysis, with genomic DNA being used as a template each time, and no evidence of either single base errors or changes in repeat structure was detected.

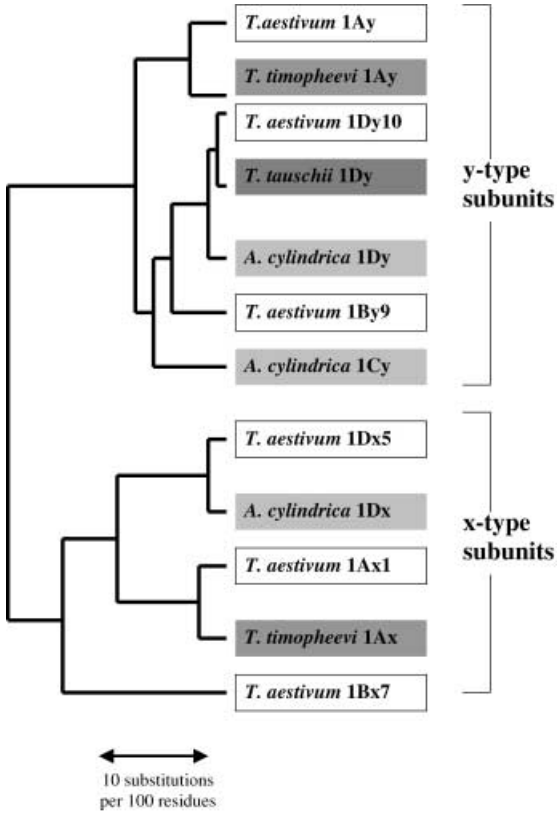

Fig. 3 Dendrogram showing the relationships between the novel HMW subunits from *Aegilops cylindrica* and *Triticum timopheevi* and previously characterized bread wheat (*Triticum aestivum*) HMW subunits 1Ax1 (EMBL accession X61009), 1Bx7 (X13927), 1Dx5 (X12928), 1Ay (not expressed) (X03042), 1By9 (X61026) and 1Dy10 (X12929) and a 1Dy subunit from *Triticum tauschii* (U39229). The tree was derived from multiple *N*- and *C*-terminal sequence alignments produced using the PILEUP programme with a gap creation penalty of 3.0 and a gap extension penalty of 0.1. Evolutionary distances were calculated using the DISTANCES programme, correcting for multiple substitutions at a single site, and displayed by the unweighted pair group method using arithmetic averages with the GROWTREE programme (Wisconsin Package version 10, Genetics Computer Group, Madison, Wis.)

Nucleotide sequencing of the coding regions of five novel HMW subunit genes and characteristics of the encoded HMW subunits

The nucleotide sequences of all five HMW subunit genes were determined by primer walking. The putative x-type subunit gene from *Ae. cylindrica* consisted of 2,535 bp, while the two putative y-type subunit genes consisted of 1,998 bp and 1,962 bp. The putative x-type HMW subunit gene from *T. timopheevi* consisted of 2,425 bp and the putative y-type gene of 1,761 bp. Each comprised a single open reading frame from beginning to end. The nucleotide sequences have been deposited in the EMBL database under accession numbers AJ306973–AJ306977.

The proteins encoded by the five novel genes had essentially similar structures to previously characterized HMW subunits. Each comprised a 21 amino acid signal peptide and short *N*- and *C*-terminal domains flanking a more extensive repetitive domain. The *N*- and *C*-terminal domain sequences were used to produce a dendrogram of the five novel HMW subunit proteins together with bread wheat HMW subunits 1Ax1 (Halford et al. 1992; EMBL accession number X61009), 1Bx7 (Anderson and Greene 1989; X13927), 1Dx5 (Anderson et al. 1989; X12928), 1Ay (silent) (Forde et al. 1985; X03042), 1By9 (Halford et al. 1987; X61026) and 1Dy10 (Anderson et al. 1989; X12929), and a 1Dy subunit from *T. tauschii* (Mackie et al. 1996; U39229) (Fig. 3). This confirmed them as x- and y-type and enabled them to be assigned to genomes, the x-type subunit from *Ae. cylindrica* to the D genome, the y-type subunit encoded by the 1,962-bp gene from *Ae. cylindrica* to the D genome and both the x- and y-type subunits from *T. timopheevi* to the A genome. The y-type subunit encoded by the 1,998-bp gene from *Ae. cylindrica*, while clustering with the y-type subunits, did not cluster with the 1Ay, 1By or 1Dy subunits, and it was therefore assigned to the C genome. This is the first C-genome-encoded HMW subunit to be reported and, to the best of our knowledge, the first gene of any type encoded by this genome. The isolation of an expressed gene from *T.timopheevi* encoding a 1Ay subunit is also of particular interest as 1Ay genes are rarely expressed in bread wheat (Payne 1987; Margiotta et al. 1993; Johansson and Svensson 1995). Neither the 1Gx nor the 1Gy HMW subunit genes from *Triticum timopheevi* were amplified and cloned, suggesting that their DNA

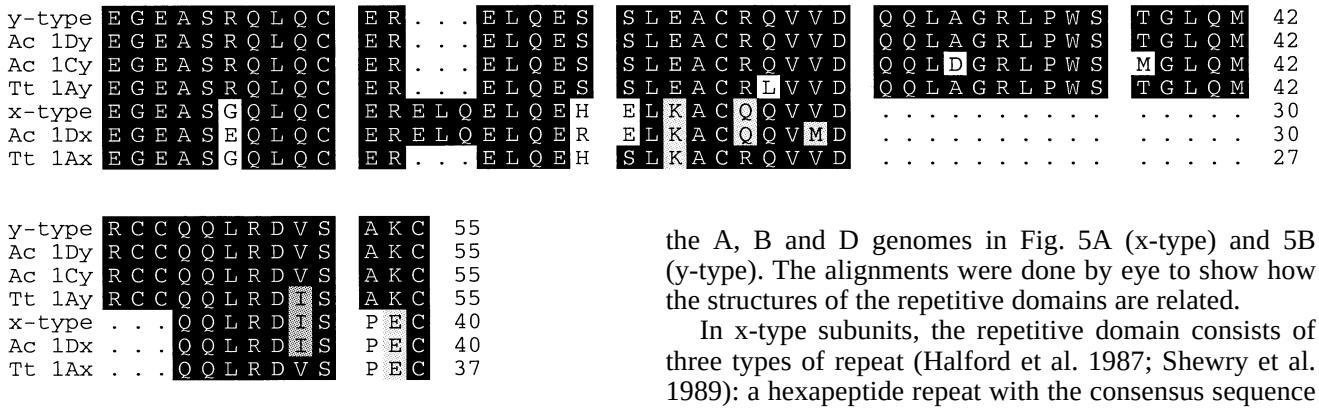

Fig. 4 Alignments of the *N*-terminal amino acid sequences of HMW subunits from *Aegilops cylindrica* and *Triticum timopheevi* with x- and y-type HMW subunit consensus sequences, up to the end of a deletion present in the x-type sequences. The alignment was produced using the PILEUP programme with a gap creation penalty of 3.0 and a gap extension penalty of 0.1 and displayed using the PRETTYBOX programme (Wisconsin Package version 10, Genetics Computer Group, Madison, Wis.)

sequences were sufficiently different from the primer sequences for the PCR reaction not to work.

The sizes and cysteine contents of the whole proteins and their three domains are summarized in Table 2. The x-type HMW subunits of bread wheat have a deletion in the *N*-terminal domain that includes two cysteine residues (Halford et al. 1987). This deletion is also present in the 1Dx subunit of *Ae. cylindrica* and the 1Ax subunit of *T. timopheevi* and is shown clearly in Fig. 4, in which the amino acid sequences of the *N*-terminal domains of the *Ae. cylindrica* and *T. timopheevi* HMW subunits are aligned with the x- and y-type consensus sequences up to the end of this deletion.

The complete amino acid sequences of the *Ae. cylindrica* and *T. timopheevi* HMW subunits are aligned with those of x- and y-type bread wheat HMW subunits from the A, B and D genomes in Fig. 5A (x-type) and 5B (y-type). The alignments were done by eye to show how the structures of the repetitive domains are related.

In x-type subunits, the repetitive domain consists of three types of repeat (Halford et al. 1987; Shewry et al. 1989): a hexapeptide repeat with the consensus sequence Pro Gly Gln Gly Gln Gln, a nine amino acid repeat consisting of the hexapeptide plus a tripeptide with the consensus sequence Gly Gln Gln and a 15 amino acid repeat consisting of the hexapeptide plus a nonpeptide with the consensus sequence Gly Tyr Tyr Pro Thr Ser Pro Gln Gln. In y-type subunits the repetitive domain contains only the hexapeptide and hexapeptide/nonapeptide repeats and in the nonapeptide leucine is more prevalent than proline at position 7 (Shewry et al. 1989). There are 23 amino acid residues at the *N*-terminal end of the repetitive domain in both x- and y-type HMW subunits that show similarity with the other repeats but do not have the same structure, and a truncated hexapeptide/nonapeptide repeat at the *C*-terminal end of the repetitive domain.

The size variation of HMW subunits is due almost entirely to the insertion and duplication of blocks of these repeats. This process appears to be quite rapid in some regions in some subunits; for example the last stretch of hexapeptide/tripeptide repeats in the 1Ax subunits consists of only three repeats, whereas the equivalent region in the 1Dx subunit of *Ae. cylindrica* consists of nine repeats, and in 1Dx5 of *T. aestivum* it consists of ten repeats. This region is missing entirely in subunit 1Bx7, and this subunit also lacks a number of other blocks of

Table 2 Characteristics of proteins encoded by novel HMW subunit genes from *Triticum timopheevi* and *Aegilops cyclindrica*, and 'typical' HMW subunits from bread wheat (*Triticum aestivum*)

	M_r	Number of residues				Number of cysteine residues			
		Total	*N*-terminal domain	Repetitive domain	*C*-terminal domain	Total	*N*-terminal domain	Repetitive domain	*C*-terminal domain
Triticum aestivum 1Ax1	87,680	809	86	681	42	4	3	0	1
Triticum timopheevi 1Ax	86,581	797	86	469	42	4	3	0	1
Triticum aestivum 1Bx7	82,865	770	80	648	42	4	3	0	1
Triticum aestivum 1Dx5	88,137	827	89	696	42	5	3	1[a]	1
Aegilops cylindrica 1Dx (not expressed)	87,317	824	88	694	42	4	3	0	1
Triticum aestivum 1Ay (not expressed)	63,027	581	104	435	42	6	5	0	1
Triticum timopheevi 1Ay	61,184	566	104	420	42	6	5	0	1
Triticum aestivum 1Dy10	67,495	627	104	481	42	7	5	1	1
Aegilops cylindrica 1Dy	68,319	633	104	487	42	8	5	2	1
Aegilops cylindrica 1Cy	69,439	645	104	499	42	7	5	1	1
Triticum aestivum 1By9	73,518	684	104	538	42	7	5	1	1

[a] Not present in the allelic subunit 1Dx2

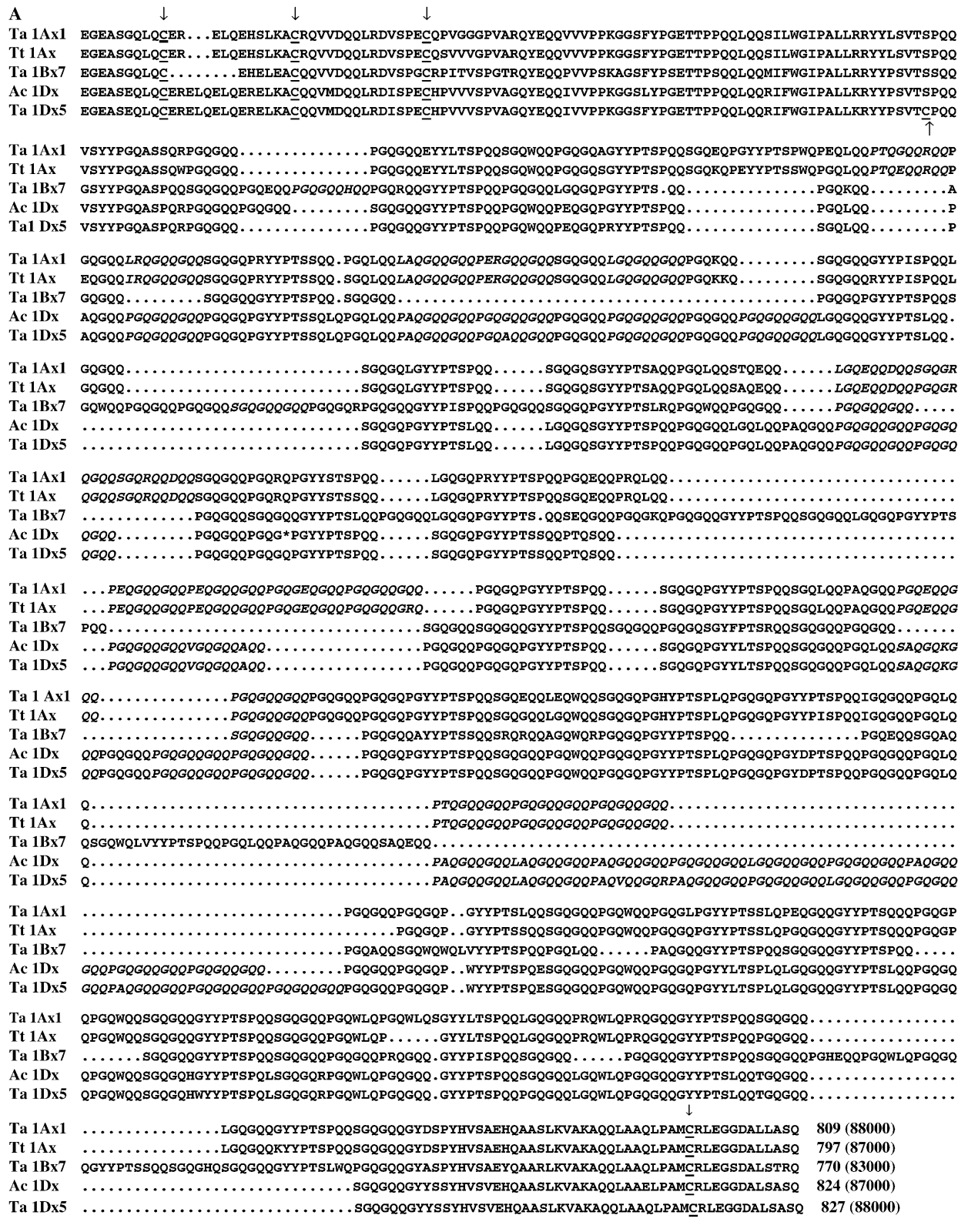

Fig. 5 Multiple sequence alignments of the derived amino acid sequences of x-type (**A**) and y-type (**B**) HMW subunits. The alignments were assembled by eye to demonstrate the repeat structure of the central domains. Cysteine residues are *underlined* and their positions indicated with an *arrow*; *N*- and *C*-terminal domains are shown in *bold*; hexapeptide repeat motifs are shown in *bold*; hexapeptide/nonapeptide 15 amino acid repeat motifs are shown in *upright, normal print*, and hexapeptide/tripeptide 9 amino acid repeat motifs are shown in *italics*. The sequences shown are the *Triticum timopheevi* (Tt) 1Ax and 1Ay subunits and *Aegilops cylindrica* (Ac) 1Dx, 1Cy and 1Dy subunits characterized in this study, and the *Triticum aestivum* (Ta) HMW subunits 1Ax1 (EMBL accession number X61009), 1Bx7 (X13927), 1Dx5 (X12928), 1Ay (X03042), 1By9 (X61026) and 1Dy10 (X12929)

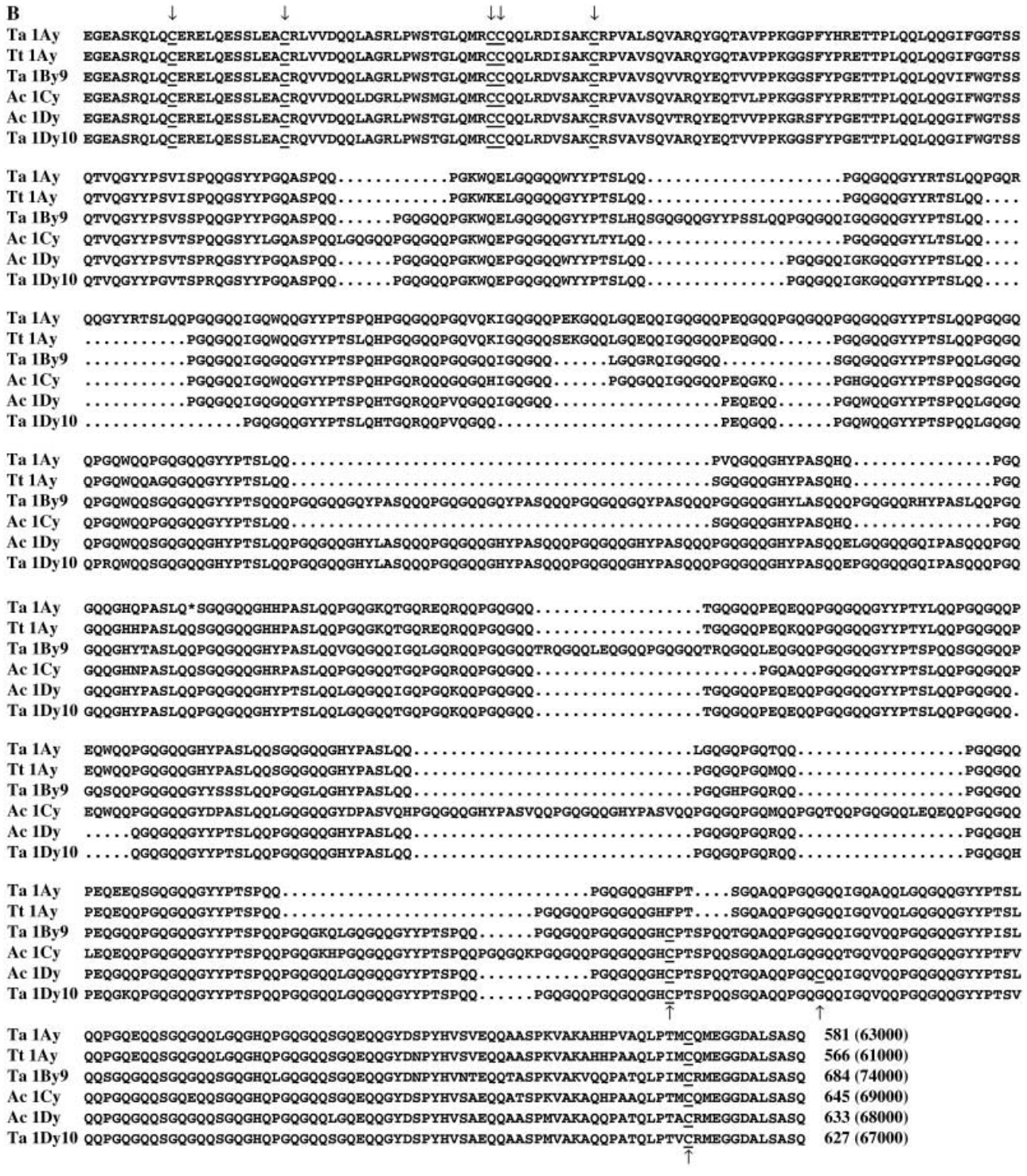

Fig. 5 Continued

repeats present in the other x-type subunits and contains several that are not present in the others. Subunit 1Bx7 also appears to have diverged more rapidly in the *N*- and *C*-terminal domains (Figs. 3, 4 and 5A). Intriguingly, the same is not true of subunit 1By9, so this cannot be attributed simply to the B genome having diverged earlier than A, C and D.

There is considerable degeneracy within the repeat sequences, although the length of the repeats and the residues at certain positions (notably 3, 5 and 6 of the hexapeptide, 1–3 of the tripeptide and 1, 6 and 8 of the nonapeptide) are tightly conserved (Shewry et al. 1989; Feeney et al. 2001). Studies using structure prediction (Tatham et al. 1984), CD spectroscopy (Tatham et al. 1990) and NMR spectroscopy (van Dijk et al. 1997a, 1997b) have indicated that the predominant structures in the repetitive domains of HMW subunits are β-turns.

These are in equilibrium with poly-L-proline-II like structures (Gilbert et al. 2000). Synthetic peptides based on repeat motifs from 'real' HMW subunits have been shown to form type I/III β-turns (Gilbert et al. 2000), but long synthetic peptides consisting of perfect repeats may adopt a structure based on class II β-turns (Feeney et al. 2001). This indicates that degeneracy within the repeat motifs can affect the secondary structure of HMW subunits, perhaps explaining why residues at some positions within the repeats are more highly conserved than others.

Despite the fact that *T. timopheevi* is considered to be a primitive wheat and *Ae. cylindrica* is not cultivated at all, the repeat motifs of the HMW subunits from these species are not significantly more or less degenerate than those of the HMW subunits from *T. aestivum*. Rather, variants in particular repeat motifs of HMW subunits from *T. timopheevi* and *Ae. cylindrica* are usually also present in the corresponding repeat motif of the homologous subunit from *T. aestivum* (Fig. 5). This is true even when comparing expressed and non-expressed subunits, despite the fact that the latter are presumably not under any selective constraint. There is also no pattern in the differences in size of the repetitive domains between the subunits from the cultivated and wild species. This indicates that the sequence, structure and length of the repetitive domains of the HMW subunits have not been altered greatly, if at all, by selective breeding of bread wheat.

Figures 4 and 5 show the close relationship between the *N*- and *C*-terminal domains of the novel subunits and the corresponding x-and y-type subunits of bread wheat. The x-type subunits contain an 18 amino acid deletion in the *N*-terminal domain that includes two cysteine residues. The 1Dx subunits contain a duplication of the Glu Leu Gln peptide at position 13, while 1Bx7 lacks this peptide and single residues immediately before and after it. As with the repetitive domains, there is nothing to distinguish the HMW subunits of the cultivated species from the HMW subunits of its primitive and wild relatives.

The number and distribution of cysteine residues in the HMW subunit proteins are relevant to their ability to form high M_r polymers stabilized by inter-chain disulphide bonds (Shewry and Tatham 1997). In particular, effects on the size and branching pattern of the glutenin polymers could be expected, with consequent effects on their biophysical and functional properties. It is, therefore, of interest that the 1Dy HMW subunit protein from *Ae. cylindrica* contains an additional cysteine residue when compared with all other characterized y-type subunits. This cysteine is at position 535, in the repetitive domain, and substitutes for a glycine residue in the 1Cy subunit of *Ae. cylindrica*, the 1Ay subunit of *T. timopheevi* and the y-type subunits of bread wheat (Fig. 5B).

In contrast, both the *Ae. cylindrica* and *T. timopheevi* 1Dx HMW subunits lack the additional cysteine residue that is present at position 97 in subunit 1Dx5 (at the *N*-terminal end of the repetitive domain) but not in any other subunit characterized to date, including 1Dx2 (Sugiyama et al. 1985). This difference between subunits

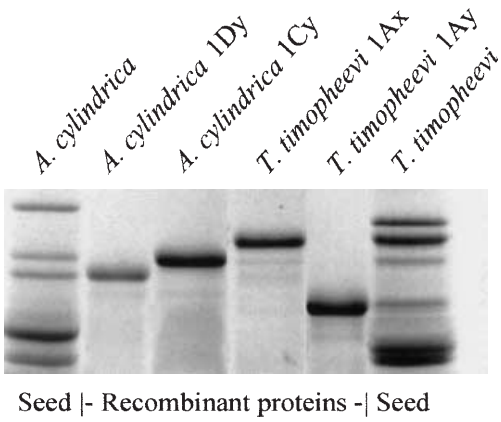

Fig. 6 SDS-PAGE showing co-migration of recombinant 1Ax, 1Ay, 1Cy and 1Dy HMW subunits expressed in *E. coli* and HMW subunits present in seed proteins from *Triticum timopheevi* and *Aegilops cylindrica.*

IDx5 and 1Dx2 has been proposed to contribute to the differences in quality associated with the allelic subunit pairs lDx5 + 1Dy10 (good) and 1Dx2 + 1Dy12 (poor) (Shewry et al. 1992).

Expression of novel 1Ax, 1Ay, 1Cy and 1Dy genes in *E. coli*

In order to confirm that the novel 1Ax, 1Ay, 1Cy and 1Dy genes expressed proteins that corresponded to those in the grain and that the designation of HMW subunit proteins identified by SDS-PAGE (Fig. 1) was correct, the regions of the 1Ax, 1Ay, 1Cy and 1Dy genes encoding the mature proteins (i.e. proteins lacking the signal peptide and beginning with the amino acid sequences determined directly) were expressed in *E. coli*. The DNA fragments were amplified by PCR, checked by nucleotide sequencing, cloned into expression plasmid pET24a and transformed into bacterial strain BLR (DE3) plysS. The expressed proteins were purified by extraction with 50% (v/v) propanol containing 2% (w/v) DTT.

The recombinant HMW subunits were then separated by SDS-PAGE next to total protein extracts from single seeds of *T. timopheevi* and *Ae. cylindrica* (Fig. 6). They showed very similar mobilities to the endogenous subunits, and the designations of the endogenous subunits shown in Fig. 1 were clearly confirmed.

The 1Dx gene from *Ae. cylindrica* is silent

The 1Dx gene from *Ae. cylindrica* contains an in-frame stop codon resulting from a CAA (Gln) → TAA (Stop) mutation replacing residue 343 in the repetitive domain (indicated by an asterisk in line 24 of Fig. 5A). This indicates that it is highly unlikely to be expressed as a full-length protein, consistent with there being only one x-type *Ae. cylindrica* HMW subunit (1Cx) identified by SDS-PAGE (Fig. 1). Stop codons have been reported

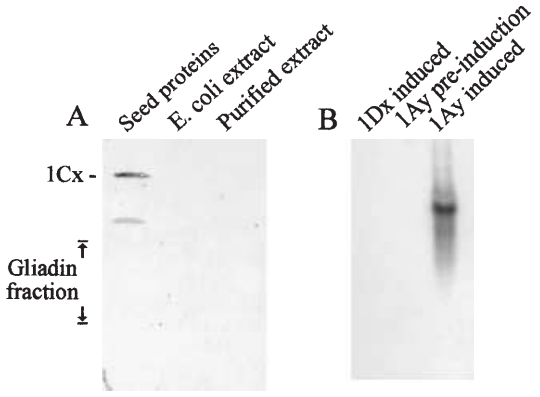

Fig. 7 **A** Western analysis of HMW subunit protein in *Aegilops cylindrica* seed extract and crude and purified extracts of *E. coli* cells harbouring an expression plasmid containing the truncated 1Dx HMW subunit gene from *Ae. cylindrica*. The blot was reacted with antibody IFRN 1602. The endogenous 1Cx subunit is indicated, and the position of the gliadin fraction is shown because the truncated 1Dx HMW subunit (*M*r 36,692) would be expected to co-migrate with this fraction if it were present. **B** Northern analysis of total RNA extracted from *E. coli* cells harbouring an expression plasmid containing the 1Ay HMW subunit gene from *Ae. cylindrica* before and after expression of the HMW subunit gene was induced, and from *E. coli* cells harbouring an expression plasmid containing the truncated 1Dx HMW subunit gene after induction. The blotted RNA (5 μg per track) was hybridized with a α-[^{32} P]dATP-labelled probe prepared from the cloned *Ae. cylindrica* 1Dx gene

previously in silent 1Ay and 1Ax genes from bread wheat (Forde et al. 1985; Bustos et al. 2000), resulting from similar CAG (Gln) → TAG (Stop) and CAA (Gln) → TAA (Stop) mutations, respectively. They are also present in gliadin and LMW subunit pseudogenes (Anderson and Greene 1997; Rafalski 1986).

If a truncated 1Dx subunit ending at residue 342 were present in *Ae. cylindrica* seed extracts it would not necessarily be immediately apparent on SDS-PAGE because of the presence of many other proteins of a similar size (its predicted size would be 36,692). Therefore, it was decided to screen total seed proteins extracted from single seeds of *Ae. cylindrica* accession 0109 for the presence of a HMW subunit-related protein of this size. The proteins were separated by SDS-PAGE, western-blotted and reacted with the monoclonal antibody IFRN 1602. This antibody was raised against a synthetic peptide (Gly Ser Val Thr Cys Pro Gln Gln Val) based on the amino acid sequence of HMW subunit 1Dx5 from residues 93–101 and has been shown to recognize the sequence ^{94} Ser Val Thr Ser Pro Gln Gln^{100} (Mills et al. 2000), which is present in other x-type HMW subunits and would be present in the truncated protein encoded by the 1Dx gene from *Ae. cylindrica.*

In order to determine exactly where the truncated protein would be expected to run on the gel, the truncated 1Dx gene was expressed in *E. coli* using the same system as had been used for the 1Ax, 1Ay, 1Cy and 1Dy genes, and crude and purified extracts from the bacteria were loaded alongside the seed proteins. However, the only proteins on the blot that reacted well with the antibody were in the seed extract and corresponded to the putative 1Cx subunit and a smaller, less reactive protein that was too large to be the truncated 1Dx subunit but may have been one of the y-type subunits (Fig. 7A). There was no evidence at all for the presence of the truncated HMW subunit in the *E. coli* extract. This meant that we could not be certain exactly where the truncated protein would run if it were present in the seed extract. However, given its size, it would be expected to co-migrate with the gliadin fraction (indicated on Fig. 7A), and no protein of that mobility reacted with the antibody. It was, therefore, concluded that the truncated 1Dx HMW subunit was not present in the seed extract or was present at such a low level that it could not be detected.

The nucleotide sequence of the recombinant truncated 1Dx HMW subunit gene in the *E. coli* expression plasmid had been checked in its entirety, and there was no obvious reason why it should not be expressed. In order to investigate this further, RNA was extracted from the bacterial cells containing the 1Dx expression plasmid and from cells expressing the 1Ay gene. Northern analysis of the RNA using a probe synthesized from the cloned 1Dx gene showed clearly that HMW subunit transcripts were present in the cells expressing the 1Ay gene but not in those containing the 1Dx gene (Fig. 7B)

It is possible that the mRNA transcribed from the 1Dx gene was unstable. This could also explain the absence of a truncated 1Dx subunit in the grain. A more obvious explanation for the lack of a 1Dx subunit in *Ae. cylindrica* would be that the promoter of the 1Dx gene were inactive, but this would not explain the failure of the gene to express in *E. coli*. The promoter of the 1Ay gene from *T. aestivum* has been shown to be inactive when linked to a reporter gene and expressed in tobacco. In contrast, the 1Dx5 gene promoter is not only active but extremely powerful both in tobacco (Halford et al. 1989) and wheat (Lamacchia et al. 2001). However, the 1Ay gene promoter contains all of the regulatory elements present in active HMW subunit genes, and its inactivity has never been explained satisfactorily.

Conclusions

We have isolated five novel genes encoding HMW subunits from *Triticum timopheevi* and *Aegilops cylindrica*, including a ‘silent’ 1Dx gene from the latter species. Perhaps the most striking conclusion to be drawn from this study is that several thousand years of bread wheat selection and breeding has not had any obvious effect on HMW subunit expression levels or structure, despite the qualitative and quantitative link between HMW subunit composition and grain quality (the y-type HMW subunits of *T. timopheevi* were present in relatively low amounts compared with y-type HMW subunits in bread wheat, but this was not the case with the 1Cy and 1Dy subunits of *Ae. cylindrica*). This may reflect the relatively low priority given to quality compared with yield in wheat breeding until recent times. Alternatively, it may indicate that changes in the expression levels or structure

of individual subunits did not make enough difference to grain quality to be detected and selected for until the advent of modern genetic techniques and the analysis of large numbers of cultivars.

Of the four expressed genes, three are of potential interest for expression in bread wheat. Firstly, 1Ay genes are only rarely expressed in bread wheat and the gene from *T. timopheevi* is, therefore, of considerable interest. Similarly, the 1Dy gene from *Ae. cylindrica* is of interest as the encoded protein contains an additional cysteine residue which could have an impact on the structure and properties of glutenin polymers. Finally, although the protein encoded by the 1Cy gene from *Ae. cylindrica* does not show any unusual features, it is the first HMW subunit gene encoded by the C genome to be isolated, and it would be interesting to determine how the protein interacts with those encoded by the A, B and D genomes when expressed in bread wheat.

The 1Dx gene of *Ae. cylindrica* contained an in-frame stop codon, and mRNA transcribed from it failed to accumulate in *E. coli*. However, these could be secondary effects with the primary cause of its lack of expression in the grain being inactivity of the promoter as described for a silent 1Ay gene from bread wheat (Halford et al. 1989).

Cluster analysis of the HMW subunit *N*- and *C*-terminal sequences using standard computer software clearly distinguished between the subunits of different genomes, including a 1Dy subunit of the ancestral D genome species, *Triticum tauschii*, and the wild and primitive species used in this study. Such analyses of HMW subunits could be useful in answering questions on the evolution of wheat and its related species.

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