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High-molecular-weight glutenin subunits in the *Cylindropyrum* and *Vertebrata* section of the *Aegilops* genus and identification of subunits related to those encoded by the Dx alleles of common wheat

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Abstract The high-molecular-weight (HMW) glutenin subunit composition of seven species from the *Cylindropyrum* and *Vertebrata* sections of the *Aegilops* genus was studied using SDS-PAGE and Western blot analysis. Two subunits were detected in *Ae. caudata* and three in *Ae. cylindrica*. In both species, subunits showing electrophoretic mobility similar to that of 1Dx2 were present. Western blot analysis using a monoclonal antibody (IFRN 1602) specific for the 1Ax and 1Dx subunits of bread wheat showed that the 1Dx-like subunit of *Ae. caudata* gave only a weak reaction. This indicates that *Ae. caudata* expresses subunits which are more distantly related to the 1Dx subunits. Two subunits were detected in each of the 60 accessions of *Ae. tauschii*, including several $1D^tx$ subunits showing different electrophoretic mobilities from those of the 1Dx subunits commonly found in bread wheat. All of the $1D^tx$ subunits reacted strongly with IFRN 1602, confirming their close relationship to the 1Dx subunits of bread wheat. Three subunits were found in *Ae. crassa* (6 x), four in *Ae. ventricosa* and *Ae. juvenalis* and five in *Ae. vavilovii*. In these four species, the subunits that showed electrophoretic mobility similar, or close, to that of 1Dx2 all reacted with IFRN 1602. In addition, *Ae. ventricosa* contained a subunit showing electrophoretic mobility slower than that of 1Dx2.2, which also reacted with IFRN 1602. These results suggest that the D-genome component in the multiploid *Aegilops* species express at least one HMW glutenin subunit that is structurally related to the 1Dx subunits of bread wheat.

Key words HMW glutenins · *Aegilops tauschii* · *Cylindropyrum* · *Vertebrata*

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Introduction

Wheat gluten is a complex mixture of proteins that are classically divided into two groups: the monomeric gliadins and the polymeric glutenins. After reduction of inter-chain disulphide bonds the component subunits of the glutenin polymers can be separated into two groups, the high-molecular-weight (HMW) and low-molecular-weight (LMW) subunits.

The genes encoding the HMW subunits are present at three complex loci (*Glu-A1*, *Glu-B1*, *Glu-D1*) on the long arms of the group-1 chromosomes of bread wheat (Lawrence and Shepherd 1981; Payne 1987). Each locus consists of two tightly linked genes, encoding a lower molecular mass y-type subunit and a higher molecular mass x-type subunit. Silencing of specific genes results in variation in the number of expressed subunits from three to five, while allelic variation in the subunits encoded by the expressed genes results in multiple forms of x-type and y-type subunits, which can be separated by SDS-PAGE (Payne and Lawrence 1983).

Payne et al. (1981) showed that allelic variation in the subunits encoded by chromosome 1D was associated with differences in the breadmaking quality of European wheats, with the 1Dx5+1Dy10 pair of subunits having superior quality to lines containing the allelic subunits 1Dx2+1Dy10, 1Dx3+1Dy12 or 1Dx4+1Dy12. Further studies have allowed all the major subunits encoded by the A, B and D genomes to be ranked in order of quality and have confirmed the importance of the D-genome-encoded subunits (Payne 1987). Furthermore, analysis of near-isogenic lines has shown that the presence of subunits 1Dx5+1Dy10 is associated with a higher level of gluten viscoelasticity and a greater proportion of

unextractable large glutenin polymers than subunits 1Dx2+1Dy12 (Popineau et al. 1994).

The demonstration of a relationship between HMW subunit composition and grain processing quality has stimulated research aimed at understanding this relationship at the biochemical and molecular levels and at manipulating HMW subunit composition in order to improve quality (Shewry et al. 1995). This may be achieved by conventional plant breeding or genetic engineering (Blechl and Anderson 1996; Altpeter et al. 1996; Barro et al. 1997), but maximum success in both cases is likely to depend on the availability of more genes encoding subunits conferring better processing properties. Because the D genome of bread wheat is derived from *Aegilops tauschii* (*Ae. squarrosa*), researchers have focussed on the identification of novel subunits in this species and their evaluation in synthetic hexaploid wheats (Peña et al. 1991; William et al. 1993; Mackie et al. 1996a, b).

The genus *Aegilops* is composed of several sections (reviewed by Miller 1987). While species in the *Sitopsis* section contain the S, S^b, S^l, S^{se}, S^{sh} genomes, most species (except *Ae. caudata*) in the *Cylindropyrum* and *Vertebrata* sections possess either the D genome or a D-genome component (Miller 1987; Dvorak et al. 1998a; Linc et al. 1999) derived from *Ae. tauschii* (Miller 1987). Studies on HMW glutenin subunits in *Sitopsis* species have been carried out with the aim of establishing the origin of the B genome (Fernández-Calvín and Orellana 1990; Urbano et al. 1993). On the other hand, analysis of HMW subunits in the *Ae. tauschii*, a member of the *Vertebrata* section, has contributed not only to the elucidation of the phylogenetic relationship between this species and bread wheat but also to the identification of subunits potentially useful in wheat quality breeding (Lagudah and Halloran 1988; Peña et al. 1991; William et al. 1993; Mackie et al. 1996a, b; Dvorak et al. 1998a, b; Allaby et al. 1999). It is now clear that the *Glu-D^{t}1* locus of *Ae. tauschii* most probably encodes two HMW subunits (Mackie et al. 1996b), while molecular cloning and DNA sequencing of the gene encoding the $1Dy^t12$ subunit demonstrated that it was highly related to those of bread wheat (Mackie et al. 1996a). In contrast to these studies of *Ae. tauschii*, little is known about HMW subunit composition in the multiploid species containing the D-genome component, nor is it clear whether the D-genome component in the multiploid species expresses subunits that are structurally related to those encoded by the *Glu-D1* locus. This lack of knowledge can potentially limit our ability to identify and utilise novel *Aegilops* HMW subunits.

We have, therefore, initiated a series of experiments aimed at obtaining a better understanding of HMW subunit composition in the species belonging to the *Cylindropyrum* and *Vertebrata* sections of *Aegilops*, using SDS-PAGE and Western blot analysis with a monoclonal antibody which is specific for 1Ax and 1Dx subunits of bread wheat.

Materials and methods

Eighty-six accessions from seven *Aegilops* species belonging to *Cylindropyrum* and *Vertebrata* sections (Table 1) were supplied by the Institute of Crop Germplasm Resources of the Chinese Academy of Agricultural Sciences (Beijing). The HMW glutenin subunits in five wheat cultivars, Chinese Spring (1Bx7+1By8, 1Dx2+1Dy12), Talon (1Ax1, 1Bx7+1By8, 1Dx5+1Dy10), Abbot (1Ax1, 1Bx6+1By8, 1Dx2+1Dy12), Hereward (1Bx7+1By9, 1Dx3+1Dy12), Shamrock (1Bx13+1By16, 1Dx5+1Dy10), and one wheat line, MG7249 (1Ax2*, 1Bx7+1By8, 1Dx2.2+1Dy12), were used as standards. The monoclonal antibody IFRN1602, which reacts specifically with the 1Ax and 1Dx subunits of bread wheat, has been described previously (Fido et al. 1995; Barro et al. 1997).

Protein extraction

The endosperm portion of a single seed was crushed and suspended in 150 μl of SDS extraction buffer containing 62.5 m*M* Tris, 4% (w/v) SDS, 10% (v/v) glycerol, 2% (v/v) 2-mercaptoethanol and 0.001% (w/v) bromophenol blue (pH 6.8). Samples were allowed to stand at room temperature for at least 2 h with occasional vortexing. They were then placed in a boiling waterbath for 2 min followed by centrifugation (15000 *g*, 15 min). The supernatant was retained for one-dimensional SDS-PAGE.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

HMW glutenin subunits were separated by SDS-PAGE. The separating and stacking gels contained 10% (w/v) and 4.5% (w/v) acrylamide, respectively. The gel was run at a constant current of 20 mA in a mini-Protean II (Bio-Rad). The separating gel was then stained overnight with 0.1% (w/v) Coomassie Brilliant Blue R250, in 25% (v/v) propan-2-ol and 10% (v/v) acetic acid, and destained with 25% (v/v) propan-2-ol and 10% (v/v) acetic acid. Commercial protein markers (Sigma, 36 kDa to approx. 205 kDa) were used as molecular weight standards. At least three seeds of each accession were analysed.

Table 1 The *Aegilops* species and number of accessions studied in the investigation

Section	Species	Genome[a]	Number of accessions analysed
Cylindropyrum	*Ae. caudata* L.	C	10
	Ae. cylindrica Host.	C^cD^c	8
Vertebrata	*Ae. tauschii* Coss.	D	60
	Ae. crassa Boiss.(6 x)	DD^2M^{cr}	2
	Ae. ventricosa Tausch.	DM^v	2
	Ae. juvenalis (Thell.) Eig.	DM^j U	2
	Ae. vavilovii (Zhuk.) Chenn.	$DM^{cr}S^p$	2

[a] Genome constitution is based on the work by Miller (1987) and Linc et al. (1999)

Western blotting

The proteins were transferred to Hybond-C membrane by electroblotting for 1 h at a constant voltage of 100 V. The membrane was washed twice with TBST buffer [0.02 M Tris-HCl, 0.5 M NaCl, pH 8.3, 0.05% (v/v) Tween 20] and blocked in TBSTMT (5% milk powder, 0.1% Triton X-100 in TBST) for 30 min. The membrane was incubated in TBSTMT containing a 1:3000 dilution of the monoclonal antibody IFRN1602 for 1 h. After washing three times with TBST, the blot was allowed to react with anti-mouse IgG conjugated with alkaline phosphatase (Sigma, 1:2000 dilution) for 1 h. After a 5-min rinse in substrate buffer (0.1 M Tris-HCl, 0.1 M NaCl, 0.005 M MgCI2, pH 9.5), the membrane was placed in substrate buffer containing 5-bromo-4-chloro-3-indoyl phosphate (BCIP) and nitroblue tetrazolium (NBT) for approximately 30 min. The reaction was stopped by rinsing the membrane with distilled water. Pre-stained protein markers (New England Biolabs, 6.5 kDa to approx. 175 kDa) were used as molecular weight standards.

During the course of this investigation, we also prepared polyclonal antibodies to the 1Dx5 and 1Dy10 subunits. These antibodies recognised multiple HMW glutenin subunits present in both common wheat and *Aegilops* species and were therefore not suitable for identifying *Aegilops* subunits that were related to those encoded by the 1Dx alleles of common wheat.

Results

HMW subunit composition of *Ae. caudata* and *Ae. cylindrica*

The *Cylindropyrum* section of *Aegilops* contains two species: *Ae. caudata* and *Ae. cylindrica* (Table 1). The HMW subunits present in the ten accessions of *Ae. caudata* all showed a similar electrophoretic pattern, consisting of two bands (Fig. 1A). Similarly, there were no differences in HMW glutenin subunit composition among the the eight accessions of *Ae. cylindrica*, all of which had three bands (Fig. 1B). The slow-migrating subunit of *Ae. caudata* had a similar electrophoretic mobility to that of 1Dx2 (Fig. 1A), while the large subunit of *Ae. cylindrica* (Fig. 1B, arrow) was slower than subunits 1Dx2 and 1Dx5. Western blot analysis showed that the large subunit of *Ae. caudata* reacted weakly with IFRN 1602 (Fig. 1C) but that no reaction was observed with the *Ae. cylindrica* subunits (data not shown).

HMW subunit composition of *Ae. tauschii*

Analysis of 60 accessions of *Ae. tauschii* showed wide variation in HMW glutenin subunit composition. Although all the accessions expressed 2 subunits, 23 different subunit combinations were observed (Table 2). Three of the combinations, present in a total of 30 accessions, were identical to subunit pairs which occur widely in bread wheat (1Dx2+1Dy12, 1Dx5+1Dy10, 1Dx3+1Dy12) while 2 combinations (in 12 accessions) apparently contained subunits present in bread wheat but in different combinations (1Dx5+1Dy12, 1Dx2+ 1Dy10). The remaining 18 accessions contained novel combinations of subunits with different mobilities to those commonly observed in bread wheat (Table 2). This resulted from the presence of either variant x-type subunits (Table 3, Tx1, Tx2 and Tx3 in Fig. 2A) or variant y-type subunits (Table 3, Fig. 2A). Subunits Tx1 and Tx2 had slower mobilities than subunit 1Dx2, while subunit Tx3 showed a faster mobility than subunit 1Dx5 (Fig. 2A). Subunits Ty1 and Ty3 had slower mobilities than subunit 1Dy10, while subunits Ty2 and Ty4 showed faster mobilities than subunit 1Dy12 (Fig. 2A). All of the x-type subunits from the *Ae. tauschii* accessions gave strong reactions with the monoclonal antibody IFRN 1602 (Fig. 2B).

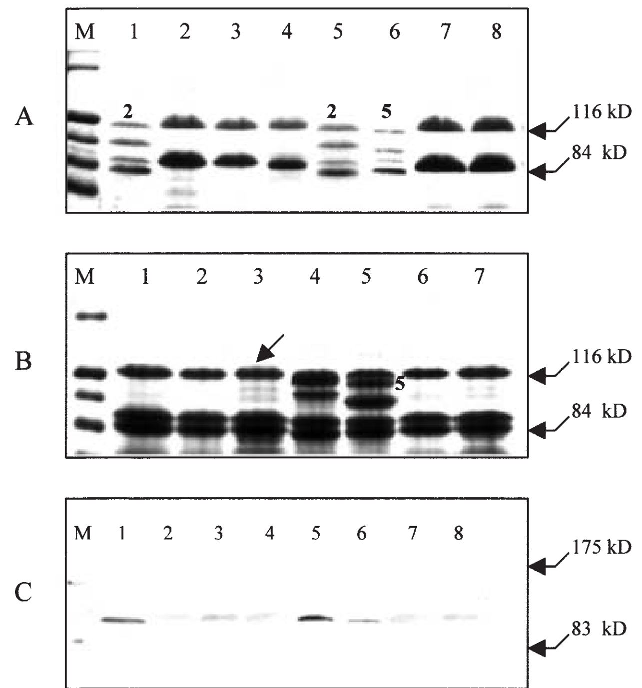

Fig. 1A–C The HMW glutenin subunit composition of *Ae. caudata* (**A**) and *Ae. cylindrica* (**B**) determined by SDS-PAGE (A, **B**) and Western blotting (**C**). The protein bands labelled with *Arabic numerals* (*2*, *5*) represent 1Dx subunits in bread wheat. In **A**, typical results from five accessions of *Ae. caudata* are shown (lanes *2*, *3*, *4*, *7*, *8*). HMW subunits from the bread wheat cvs. Chinese Spring (*lanes 1*, *5*) and Shamrock (*lane 6*) are shown as controls. In **B**, typical results from five accessions of *Ae. cylindrica* are shown (lanes *1*, *2*, *3*, *6*, *7*). HMW subunits from bread wheat cvs. Abbot (*lane 4*) and Talon (*lane 5*) are shown as standards. The large subunit of *Ae. cylindrica* (*arrow*) shows an electrophoretic mobility slower than that of subunits 1Dx2 and 1Dx5 of bread wheat. In **C**, the large subunits from five *Ae. caudata* accessions react weakly with the antibody IFRN 1602 (*lanes 2*, *3*, *4*, *7*, *8*). In contrast, subunits 1Dx2 (*lanes 1*, *5*) and 1Dx5 (*lane 6*) of bread wheat give stronger reactions. In this and subsequent figures, *M* indicates protein markers, while the values on the *right-hand side* of the figures indicate the molecular weights of the protein markers

Table 2 HMW glutenin subunit combinations observed in *Ae. tauschii* accessions

Combination of subunits	2+12	5+10	2+10	5+12	3+12	New
Number of accessions	12	16	5	7	2	18

Table 3 Variant x- and y-type HMW glutenin subunits in *Ae. tauschii*[a]

Subunits	Tx>Dx2	Tx<Dx5	Ty<Dy12	Ty>10	Dy12<Ty<Dy10
Number of accessions	2	1	12	2	1

[a] Tx, x-type subunits; Ty, y-type subunits; Tx>Dx2, the variant x-type subunit in *Ae. tauschii* whose electrophoretic mobility is slower than that of 1Dx2 of bread wheat; Ty<Dy12, the variant y-type subunit of *Ae. tauschii* whose electrophoretic mobility is faster than that of 1Dy12 of bread wheat

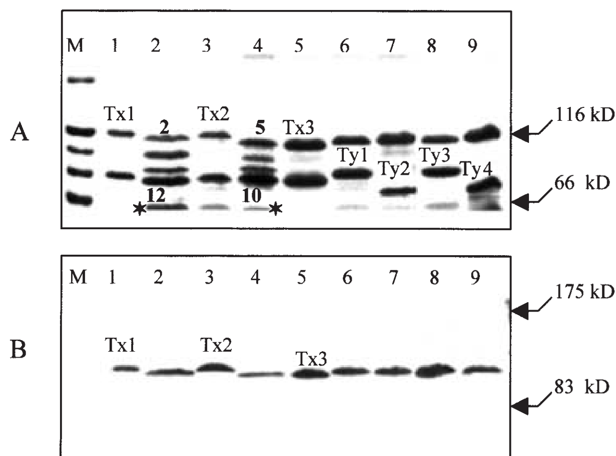

Fig. 2A, B Analysis of variant x- and y-type subunits in *Ae. tauschii* by SDS-PAGE (**A**) and Western blotting (**B**). The protein bands labelled with *Arabic numerals* represent 1Dx subunits (*2*, *5*, *numerals* were positioned on *top* of the bands) or 1Dy subunits (*10*, *12*, *numerals* were placed *under* the bands) in bread wheat. The two bands marked by an *asterisk* (*) are caused by other seed proteins rather than HMW glutenin subunits. In **A**, the electrophoretic mobilities of three variant x-type subunits (*Tx1*, *Tx2*, *Tx3*, *lanes 1*, *3*, *5*) and four variant y-type subunits (*Ty1*, *Ty2*, *Ty3*, *Ty4*, *lanes 6*, *7*, *8*, *9*) are shown. HMW subunits from the bread wheat cvs. Chinese Spring (*lane 2*) and Shamrock (*lane 4*) are shown as controls. In **B**, the different x-type subunits of *Ae. tauschii* (*lanes 1*, *3*, *5*, *6*, *7*, *8*, *9*), together with subunits 1Dx2 (*lane 2*) and 1Dx5 (lane 4) of bread wheat, all react strongly with IFRN 1602

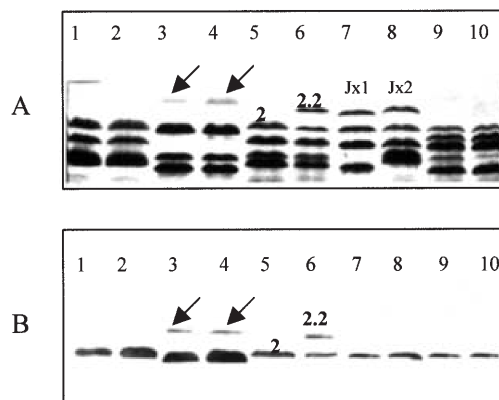

Fig. 3 The HMW glutenin subunit compositions of *Ae. crassa, Ae. ventricosa, Ae. juvenalis* and *Ae. vavilovii* determined by SDS-PAGE (**A**) and Western blotting (**B**). In **A** and **B**, *lanes 1* and *2* are accessions of *Ae. crassa*, *lanes 3* and *4* are accessions of *Ae. ventricosa*, *lanes 7* and *8* are accessions of *Ae. juvenalis*, and *lanes 9* and *10* are accessions of *Ae. vavilovii*. The protein bands labelled with *Arabic numerals* (*2*, *2.2*) represent 1Dx subunits in bread wheat cultivars. HMW subunits from the bread wheat cvs. Chinese Spring (*lane 5*) and MG7249 (*lane 6*) are shown as controls. In *Ae. ventricosa*, a subunit with an electrophoretic mobility slower than that of 1Dx2.2 is indicated by the *arrow* (*lanes 3*, *4*). In *Ae. juvenalis*, two subunits, one with a faster mobility (*Jx1*) than 1Dx2.2 and one with a similar mobility (*Jx2*) to 1Dx2.2 are present. In **B**, the presence of the subunits reactive with IFRN 1602 is detected by Western blot analysis. In *Ae. juvenalis*, the subunit with a slower mobility than subunit 1Dx2.2 also reacted with IFRN 1602 (*arrow*). The positive reactions of subunits 1Dx2 from Chinese Spring (*lane 5*), 1Dx2.2 and 1Ax2* from MG7249 (*lane 6*) confirmed the specificity of IFRN 1602

HMW subunit compositions of multiploid species possessing the D-genome component

The electrophoretic patterns of HMW glutenin subunits in the four multiploid species possessing the D-genome component are shown in Fig. 3A. *Ae. crassa* had 3 subunits, the largest of which was identical in mobility to subunit 1Dx2 (Fig. 3A, lanes 1, 2). *Ae. ventricosa* exhibited 4 subunits (Fig. 3A, lanes 3, 4), including 1 which had a slightly faster mobility than subunit 1Dx2 and 1 that migrated slower than subunit 1Dx2.2 (Fig. 3A, arrow). Four subunits were also detected in the two accessions of *Ae. juvenalis* (Fig. 3A, lanes 7, 8), but the electrophoretic patterns of the subunits differed between the two accessions. The largest subunit in accession RM0281 (Jx1 in Fig. 3A) had a faster mobility than subunit 1Dx2.2, whereas that in accession RM0284 (Jx2 in Fig. 3A) had an identical mobility to subunit 1Dx2.2. Of the 2 subunits common to both *Ae. juvenalis* accessions, 1 had a similar mobility to subunit 1Dx2 (Fig. 3A, lanes 7, 8). *Ae. vavilovii* expressed 5 subunits, the largest of which had mobility close to that of subunit 1Dx2 (Fig. 3A, lanes 9, 10). In Western blot experiments, IFRN 1602 reacted with the 1Dx2-like subunits in *Ae. crassa* and *Ae. juvenalis* (Fig. 3B, lanes 1, 2, 7, 8) and with the subunits which migrated close to subunit 1Dx2 in *Ae. ventricosa* and *Ae. vavilovii* (Fig. 3B, lanes 3, 4, 9, 10). In addition, IFRN 1602 also recognised the subunit that migrated slower than subunit 1Dx2.2 in *Ae. ventricosa* (Fig. 3B, arrow). In contrast, the Jx1 and Jx2 subunits in *Ae. juvenalis* did not show any positive reaction in Western blot analysis (Fig. 3B).

Discussion

Previous studies of HMW glutenin subunits in *Aegilops* species have focussed largely on *Ae. tauschii*. Being diploid and ancestral to the D genome of hexaploid wheat, *Ae. tauschii* encodes fewer HMW subunits, and these are directly comparable to the subunits encoded by the *Glu-D1* locus of bread wheat (Lagudah and Hal-

loran 1988; Mackie et al. 1996a). Compared with *Ae. tauschii,* the multiploid species containing the D-genome component are likely to encode more HMW subunits, and the identification of these subunits is likely to be more difficult due to the fact that some will be encoded by genomes other than the D genome. Nevertheless, analysis of the HMW subunit composition in these species is essential if we wish to identify the full range of HMW subunits encoded by the D genome in different species.

We have determined the HMW subunit composition of seven species from the *Cylindropyrum* and *Vertebrata* section of *Aegilops* (Table 1). The results obtained with *Ae. tauschii* were similar to those reported by previous investigators (Lagudah and Halloran 1988; William et al. 1993; Mackie et al. 1996b). Compared to the *Glu-D1* locus of bread wheat, the *Glu-D^t1* locus of *Ae. tauschii* encoded a wider range of HMW subunits (Tables 2, 3). This is consistent with the proposal that the genes in the *Glu-D^t1* locus are more variable than those in the *Glu-D1* locus (Lagudah and Halloran 1988; William et al. 1993). The relationship between the putative variant x- and y-type subunits observed in *Ae. tauschii* with those described by William et al. (1993) is not clear. Further work is needed to determine the extent to which the D^tx subunits differ from the commonly observed Dx subunits of bread wheat. However, we demonstrated that the different types of D^tx subunits all reacted strongly with IFRN 1602, a monoclonal antibody which recognises short peptide sequences located towards the *N*-terminal end of the repetitive domains of 1Ax and 1Dx subunits. This demonstrates that, despite a greater level of variation associated with the genes in the *Glu-D^t1* locus, there is some sequence conservation between the x-type subunits of *Ae. tauschii* and bread wheat. However, some differences between the $1D^t$-encoded subunits and the 1D-encoded subunits showing similar electrophoretic mobilities can be expected, particularly since *Ae. tauschii* apparently contained subunit combinations which have not been detected in bread wheat (i.e. subunits 1Dx2+1Dy10, 1Dx5+1Dy12). Only 1 such combination (1Dx5+1Dy12) has been reported in bread wheat (in the cv. Fiorello), but detailed analysis showed this comprised subunit 1Dy12 combined with a mutant form of subunit 1Dx2, the latter showing similar mobility on SDS-PAGE to subunit 1Dx5 and initially being misidentified (Lafiandra et al. 1993). Analysis of another diploid species, *Ae. caudata*, showed that all accessions expressed 2 subunits, the larger of which had an electrophoretic mobility similar to that of subunit 1Dx2 of bread wheat. This subunit reacted only weakly with IFRN 1602, indicating a greater degree of sequence divergence from the 1Dx subunits.

As expected, multiple HMW subunits were detected in the multiploid D-genome species. The tetraploid species expressed 3 (*Ae. cylindrica*) or 4 (*Ae. ventricosa*) subunits while the hexaploid species expressed 3 (*Ae. crassa*), 4 (*Ae. juvenalis*) or 5 (*Ae. vavilovii*) subunits. In the case of *Ae. cylindrica*, none of the subunits from the eight accessions reacted with IFRN 1602. Considering that the C^c genome of *Ae. cylindrica* is derived from the C genome of *Ae. caudata* and that the D^c genome of this species is derived from the D genome of *Ae. tauschii* (Linc et al. 1999), the absence of any reaction with IFRN 1602 indicates that sequence divergence may have occurred in the HMW subunits encoded by *Ae. cylindrica*. A further possibility for the absence of any 1Dx-related subunit in *Ae. cylindrica* may be the silencing of the gene encoding the x-type subunit in the D genome of this species. In contrast to what was found for *Ae. cylindrica*, the subunits in *Ae. crassa, Ae. juvenalis, Ae. vavilovii* and *Ae. ventricosa*, which had eletrophoretic mobilities identical, or close, to that of subunit 1Dx2, reacted specifically with IFRN 1602, indicating that these subunits may be closely related to those encoded by the D genome of bread wheat. It is also of interest that a subunit larger than Dx2.2 was observed in *Ae. ventricosa*. Although this band was less intensively stained by Coomassie blue than the other subunits, it gave a clear, specific reaction with IFRN 1602 in Western blot analysis (Fig. 3B, arrow).

In conclusion, we have presented a relatively detailed description of HMW glutenin subunit composition in the seven species from the *Cylindropyrum* and *Vertebrata* section of *Aegilops*. The successful identification of *Aegilops* subunits related to those encoded by the 1Dx genes of bread wheat constitutes a starting point for further analysis of HMW subunits in the multiploid D-genome component-containing species. The future application of more sophisticated approaches, such as gene cloning, will provide further information on the genetic control and relationships of HMW glutenin subunits in *Aegilops* species and identify components suitable for exploitation in wheat quality improvement using genetic transformation.

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