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Characterization of a Novel *1Ay* Gene and Its Expression Protein in *Triticum urartu*

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Abstract

High molecular weight glutenin subunit (HMW-GS) plays an important role in determining dough property and bread-making quality, and the exploration of novel genes for HMW-GS will be crucial for quality improvement program. A gene coding the y type HMW-GS at *Glu-A1* locus in *Triticum urartu* (AA, 2n=2×=14) with an electrophoretic mobility similar to that of 1Dy12, was cloned, sequenced, and heterologously expressed. This novel active *1Ay* gene FJ404595 was confirmed by structure analyses of nucleotide and deduced amino acid sequences combining with phylogenetic analysis. The open reading frame (ORF) of this gene was 1 830 bp, encoded a protein of 608 amino acid residues containing 46 hexapeptides and 14 nonapeptides, which was mostly similar to the *1Ay* gene AM183223 at a high identity of 99.62% with the two substitutions of both leucine/proline and valine/glutamate, obviously different from the *1Ay* gene EU984504 with 587 residues containing 44 hexapeptides and 13 nonapeptides in *T. urartu*. The amino acid (leucine) at 446 differed from that (proline) of all the eight compared active 1Ay subunits. The predicted secondary protein structure implied that this 1Ay subunit might also have positive impact on flour processing quality.

Key words: *Triticum urartu*, 1Ay subunit, expression, electrophoretic mobility, secondary structure

INTRODUCTION

High molecular weight glutenin subunits (HMW-GS) encoded by the *Glu-1* loci on the long arms of chromosomes 1A, 1B, and 1D are the important wheat seed storage proteins in determining the unique viscoelastic properties of dough and bread-making quality (Lawrence and Shepherd 1980; Payne 1987; Shewry *et al*. 2003a). The combination of 1Dx5+1Dy10 encoded by *Glu-D1* is associated with stronger dough and better bread-making quality, compared with 1Dx2+1Dy12 (Payne *et al*. 1981). In the *Glu-B1* locus, the subunit pair 1Bx17+1By18 is better than 1Bx6+1By8 and 1Bx20 (Lukow *et al*. 1989; Shewry *et al*. 2003a, b). In *Glu-A1* locus, 1Ax1 and 1Ax2* subunits are better than null allele on bread-making quality (Branlard *et al*. 1992, 2001; Cornish *et al*. 2001).

In theory, hexaploid or common wheat (*Triticum aestivum*, AABBDD, 2n=6×=42) could have six HMW-GSs, because each locus on *Glu-A1*, *Glu-B1*, and *Glu-D1* consists of two tightly linked genes encoding one larger x-type and one smaller y-type subunits, respectively. However, HMW-GS genes of hexaploid wheat are not all expressed due to gene silencing. The genes coding for subunits 1Bx, 1Dx, and 1Dy are usu-

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ally expressed, those for subunits 1Ax and 1By sometimes are silent, and that for subunit 1Ay have been deemed to be silent in hexaploid wheat (Forde *et al*. 1985; D'Ovidio *et al*. 1996).

However, the 1Ay subunit are widely existed in wild diploid and tetraploid wheat genotypes (Waines and Payne 1987; Ma *et al*. 2007; Hu *et al*. 2008; Xu *et al*. 2009), which provides the opportunity to isolate and analyze the nucleotide sequence of active *1Ay* genes, and to explore its effect on bread-making quality. Some *T. durum* (AABB, 2n=4×=28) lines containing 1Ay subunit from *T. dicoccoides* (AABB, 2n=4×=28) showed very promising gluten properties (Ciaffi *et al*. 1991). Likewise, the introduction of 1Ay subunit from diploid relative *T. thaoudar* (AA, 2n=2×=14) into hexaploid wheats increased the gluten strength (Rogers *et al*. 1997). Recently, a 1Ay subunit pattern, faster than 1Dy12 in electrophoretic mobility, was observed in the T_0 seed from *1Ay* transgenic common wheat through pollen-tube pathway (Bai *et al*. 2004; Ma *et al*. 2008). These results are undoubtedly encouraging to use 1Ay for enhancing the flour-making quality.

T. urartu (AA, 2n=2x=14) is believed as the donor species of A genome for tetraploid and hexaploid wheat (Dvorak *et al*. 1988). It has a number of valuable traits like high protein content, excellent resistance to biotic and abiotic stresses, which are important to improve the agronomic traits of cultivated wheats (Sharma *et al*. 1981; D'Egidio *et al*. 1993). Polymorphism of HMW-GS compositions in different *T. urartu* genotypes has been reported (Bai *et al*. 2004; Ma *et al*. 2007; Jiang *et al*. 2009; Xu *et al*. 2009). The expressed 1Ay subunits displayed either faster or similar electrophoretic mobilities, compared with that of 1Dy12 (Bai *et al*. 2004; Hu *et al*. 2008; Jiang *et al*. 2009). To date, some expressed *1Ay* genes have been cloned (Wan *et al*. 2002; Bai *et al*. 2004; Jiang *et al*. 2009), but information on heterologous expression of *1Ay* genes needs further investigation. Bai *et al*. (2004) provided the unique bacterial expression information of *1Ay* gene in *T. urartu*, that 1Ay subunit is faster than 1Dy12 in electrophoretic mobility. In the present study, the *T. urartu* accession PI428281 expressing a 1Ay subunit with similar electrophoretic mobility to 1Dy12 (Hu *et al*. 2008) was used to investigate nucleotide and deduced protein structures, heterologous expression, phylogenetic relationships for this gene, and to understand the potential value in improving the processing quality in common wheat.

MATERIALS AND METHODS

Plant materials

T. urartu accession PI428281 was obtained from NPGS (http://www.ars-grin.gov). For identifying HMW-GS compositions, five genotypes of common wheat as references including Chinese Spring (CS), Chuanyu 12 (CY12), Xiaoyan 6 (XY6), and two accessions of Xinjiang rice wheat (Table 1) were provided by Triticeae Research Institute, Sichuan Agricultural University, China.

Table 1 Wheat cultivars and their HMW-GS compositions as references

Cultivar	HMW-GS			Reference
	Glu-A1	Glu-B1	Glu-D1	
Chinese Spring (CS)	Null	1Bx7+1By8	1Dx2+1Dy12	Wei *et al*. (2002)
Chuanyu 12 (CY12)	1Ax1	1Bx7+1By8	1Dx5+1Dy10	Wei *et al*. (2002)
Xiaoyan 6 (XY6)	1Ax1	1Bx14+1By15	1Dx2v+1Dy11	Zhang *et al*. (2001)
Xinjiang rice wheat (AS360)	Null	1Bx7+1By9	1Dx5+1Dy10	Wei *et al*. (2002)
Xinjiang rice wheat (AS363)	Null	1Bx17+1By18	1Dx2+1Dy12	Wei *et al*. (2002)

SDS-PAGE analysis

HMW-GSs were separated by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) according to the procedure of Wan *et al*. (2000).

Cloning of *1Ay* gene

Genomic DNA was extracted from the *T. urartu* accession PI428281 as the method described by Yan *et al*. (2002). The complete coding sequence of *1Ay* gene was amplified using a pair of degenerate primers,

including forward primer P1 with 5′-ATGGCTAAGC GGC/TTA/GGTCCTCTTTG-3′ and reverse primer P2 with 5′-CTATCACTGGCTA/GGCCGACAATGCG-3′. PCR reaction mixture (50 μL) consisted of 300 ng of genomic DNA, 0.2 mmol L^{-1} of dNTPs, 1 μmol L^{-1} of each primer, 5 μL of 10×*Ex*PCR buffer, and 2.5 U of *ExTaq* DNA polymerase with high fidelity (TaKaRa, Dalian, China) and ddH_2O to 50 μL volume. PCR was carried out using a PTC-200 thermocycler (MJ Research, USA) with the condition of 95°C for 4 min, followed by 25 cycles of 94°C, 40 s and 68°C, 8 min, and a final incubation at 72°C for 15 min. The amplified fragment recovered from 1.0% agarose gel was cloned into pMD18-T vector (TaKaRa, Dalian, China) for forming *p1Ay* which was transformed into competent cells of *E. coli* DH10B strain. The sub-clones of the fragment were obtained using the nested deletion method of Yan *et al*. (2002), which were submitted to Sunbiotech Company in Beijing for sequencing. Both cloning and sequencing were repeated for three times to exclude sequencing errors.

Analysis of 1Ay protein

HMW glutenin protein was analyzed using computational biology (McGuffin *et al*. 2000). Amino acid sequence was deduced from the cloned nucleotide sequence and imported to PSIPRED server (http://bioinf.cs.ucl.ac.uk/psipred/) for secondary structure prediction.

Heterologous expression of *1Ay* gene

For bacterial expression of the ORF of *1Ay* gene, the expression vector *pET1Ay* was constructed as described by Wan *et al*. (2002). A pair of primers, B-AyF (5′-ACCCATATGGAAGGTGAGGCCTCTAGGC-3′) and B-AyR (5′-TTCCTCGAGCTATCACTGGCTGGCCG AC-3′) were designed for amplifying the modified *1Ay* ORF in which the nucleotide acid sequence encoding signal peptide was removed. Two restriction enzymes sites (underlined nucleotide) of *Nde* I and *Xho* I (for ligating into the expression vector) were introduced. PCR amplification of the modified ORF was conducted as described above except that the template was plasmid DNA purified from the clone *p1Ay*. The modified ORF was digested with *Nde* I and *Xho* I, and ligated into the expression vector pET-30α (Invitrogen, USA) to construct *pET1Ay*. *E. coli* BL21(DE3) *plysS* cells were transformed with the expression construct *pET1Ay* and grown on 2 × YT medium at 37°C until OD_{600} = 0.6. The expression of 1Ay protein was then induced by 0.1 mmol L^{-1} IPTG (isopropylthio-β-D-galactoside) and the cells were incubated for 4 to 6 h using the cells without IPTG as reference. After the cells were collected by centrifugation at 1 000 r/min for 5 min, the expressed 1Ay protein was extracted by the reported method (Wan *et al*. 2000) and partially modified selective method according to Verbruggen *et al*. (1998), respectively. In order to precipitate the selective HMW glutenin protein, the collected bacterial cells and seed flour were extracted with 70% (v/v) enthanol at room temperature for 30 min, and the suspensions were centrifuged at 15 000 r/min for 5 min at 4°C. The remained residue extracted with 50% (v/v) isopropanol was incubated at 60°C for 1 h, and the suspensions were centrifuged at 15 000 r/m for 5 min at 4°C. Glutenin subunits were extracted from the final residues with 0.08 mol L^{-1} Tris-HCl (pH 8.0) in 50% (v/v) isopropanol containing 3% (v/v) β-Mercaptoethanol for 30 min at 60°C. After centrifugation at 15 000 r/min for 5 min at 4°C, acetone was added to a final concentration of 40%. The residues (the purified glutenin protein) were harvested by centrifugation at 15 000 r/min for 10 min at 4°C, and were dissolved in sample buffer for SDS-PAGE analysis.

Sequence comparisons and phylogenetic analysis

Multiple alignments among amino acid sequences were performed by DNAMAN (ver. 6.0.3.48). The alignments were further improved by visual examination and manual adjustment. A phylogenetic tree was constructed using the amino acid sequences from reported active *1Ay* (Table 2) and other HMW-GS genes from different genomes including 1Ax1 (X61009), 1Ax2* (M22208), 1Bx7 (X13927), 1Dx5 (X12928), 1By8 (AY245797), 1By9 (X61026), 1By15 (EU137874), 1Dy10 (X12929), and 1Dy12 (X03041). The phylogenetic tree was constructed with the aid of MEGA ver. 4.0 software (Tamura *et al*. 2007) using the neighbor joining method (NJ) and the boot-

Table 2 Identities between the 1Ay FJ404595 and eight HMW glutenin 1Ay subunits from different species in full coding region

GenBank accession	Species	Genome	Reference	Identity (%)
FJ404595	*T. urartu*	AA	This study	-
AM183223	*T. urartu*	AA	Unpublished data	99.62
AY245578	*T. urartu*	AA	Bai *et al*. (2004)	99.29
EU984503	*T. urartu*	AA	Jiang *et al*. (2009)	99.40
EU984504	*T. urartu*	AA	Jiang *et al*. (2009)	95.41
EU984506	*T. monococcum* ssp. *aegilopoides*	AA	Jiang *et al*. (2009)	87.39
EU984507	*T. monococcum* ssp. *aegilopoides*	AA	Jiang *et al*. (2009)	95.25
EU984511	*T. turgidum* ssp. *dicoccon*	AABB	Jiang *et al*. (2009)	95.25
AJ306977	*T. timopheevi*	AAGG	Wan *et al*. (2002)	94.32

strap values were estimated based on 1 000 replications.

RESULTS

SDS-PAGE analysis of *T. urartu* accession PI428281

SDS-PAGE analysis showed that *T. urartu* accession PI428281 had both 1Ax and 1Ay subunits. Compared with the five references which contain seven x-type subunits (Null, 1Ax1, 1Dx2, 1Dx5, 1Bx7, 1Bx14, and 1Bx17) and seven y-type subunits (1By8, 1By9, 1Dy10, 1Dy11, 1Dy12, 1By15, and 1By18), respectively, the HMW glutenin subunit 1Ay in PI428281 showed similar electrophoretic mobility to 1Dy12 in CS (Fig.1 and Table 1).

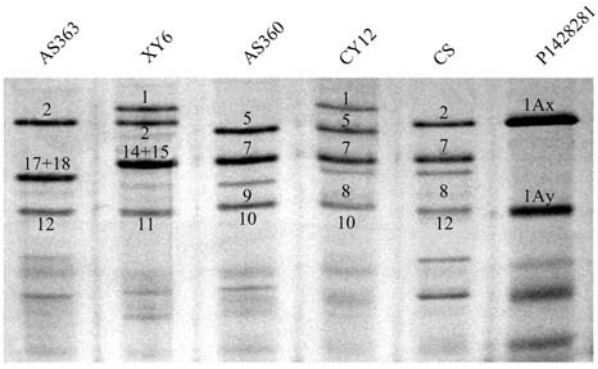

Fig. 1 SDS-PAGE analysis of HMW-GSs in *T. urartu* accession PI428281. CS, Chinese Spring; CY12, Chuanyun 12; XY6, Xiaoyan 6; AS360 and AS363, Xinjiang rice wheat.

Cloning and sequence analysis of *1Ay* gene

During PCR reaction using primers P1 and P2, single amplified fragment about 1.8 kb was obtained in PI428281 (Fig. 2), which was close to the size of complete coding region sequences of y-type HMW-GS genes (Shewry *et al*. 1995; Shewry and Halford 2002). The length of nucleotide sequence was 1 830 bp, encoding 608 amino acids. The nucleotide sequence has been deposited in GenBank under the accession number FJ404595.

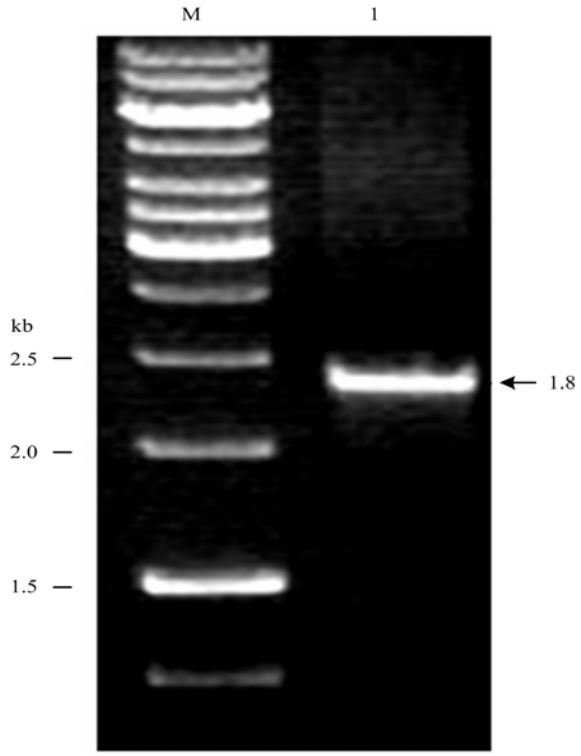

Fig. 2 PCR amplification of y-type HMW-GS coding region from *T. uratu* accession PI428281. Lane M, DNA ladder; lane 1, PCR amplification product indicated by an arrowhead.

The amino acid sequence deduced from the *1Ay* nucleotide sequence included the first 21 residues (signal peptide). The primary structure of deduced 1Ay mature protein included N-terminal, repetitive, and C-terminal domains with 104, 441, and 42 amino acids,

respectively. The deduced amino acid sequence consisted of six cysteine residues, five in N-terminal and the other in the C-terminal (Fig. 3).

Comparison of amino acid sequences

Multiple sequence alignments were performed among the amino acid sequences of the present 1Ay FJ404595 and other eight reported active 1Ay (Fig. 3 and Table 2). They shared similar typical primary structure with a signal peptide, both a conserved N- and C-terminal, and a central repetitive domain with varied residues (Fig. 3). The present 1Ay FJ404595 had the identical polypeptide length to those of the three active 1Ay subunits of AM183223, AY245578, and EU984503 in *T. urartu* with 608 residues containing 46 hexapeptides and 14 nonapeptides, but longer than the four polypeptides with identical residues of 587 containing 44

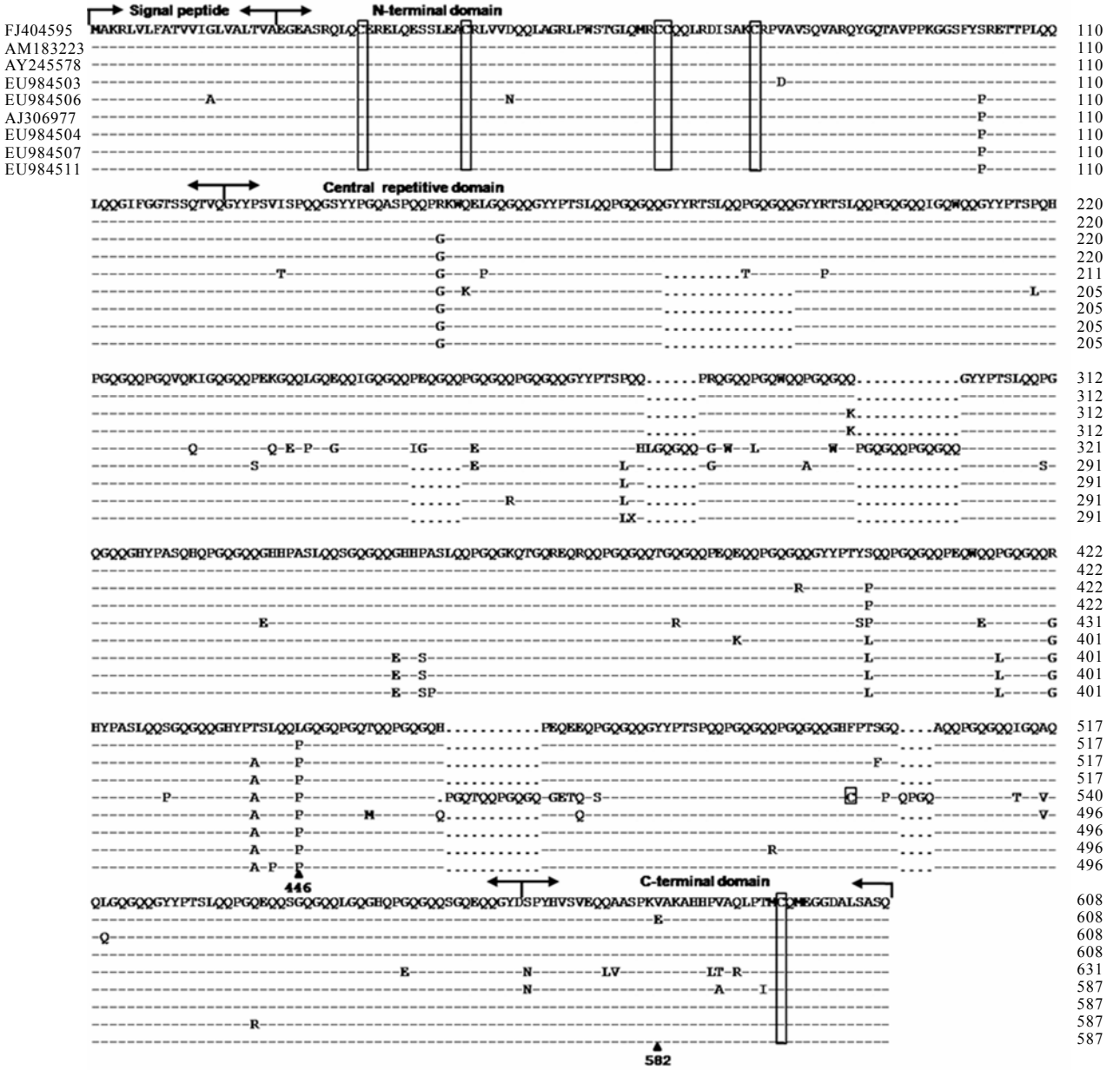

Fig. 3 Comparison of the amino acid sequences between the 1Ay subunit FJ404595 and other reported active 1Ay subunits. The signal peptide, N- and C-terminal domains, and central repetitive domain are indicated. The cysteines are marked by blackframes. Identical and deleted residues are indicated by "-" and ".", respectively. The amino acid substitutions of it with the most similar 1Ay subunits are indicated by no-tailed arrowheads.

hexapeptides and 13 nonapeptides from *T. urartu* EU984504, *T. monococcum aegilopoides* EU984507, *T. turgidum dicoccon* EU984511, and *T. timopheevi* AJ306977, shorter than that from EU984506 of *T. monococcum aegilopoides* with 631 residues containing 52 hexapeptides and 14 nonapeptides. Its amino acid composition was most similar to that of the 1Ay AM183223 from *T. urartu* with a high identity of 99.62%, substituted by leucine/proline at 446 and valine/glutamate at 582. The amino acid (leucine) at 446 differed from that (proline) of all the eight compared active 1Ay subunits (Fig. 3).

The predicted secondary structure

Using PSIPRED protein structure prediction server (McGuffin *et al*. 2000), the secondary structure of 1Ay protein was predicted (Fig. 4). The result revealed helixes existed in the signal peptide, N-terminal, and C-terminal domains, strands in the signal peptide and N-terminal domain, and the majority of coils in the repetitive domain. Of the six cysteine residues, three distributed in helixes, one in strand, and two in coils.

Expression of *1Ay* gene in *E. coli*

To confirm the cloned DNA fragment was the accurate representative of *1Ay* ORF of *T. urartu* PI428281, heterologous expression of the cloned gene was performed in *E. coli*. The bacterial cells induced by IPTG produced the 1Ay protein (Fig. 5, lanes 3, 4) that had identical electrophoretic mobility to that of PI428281 seed

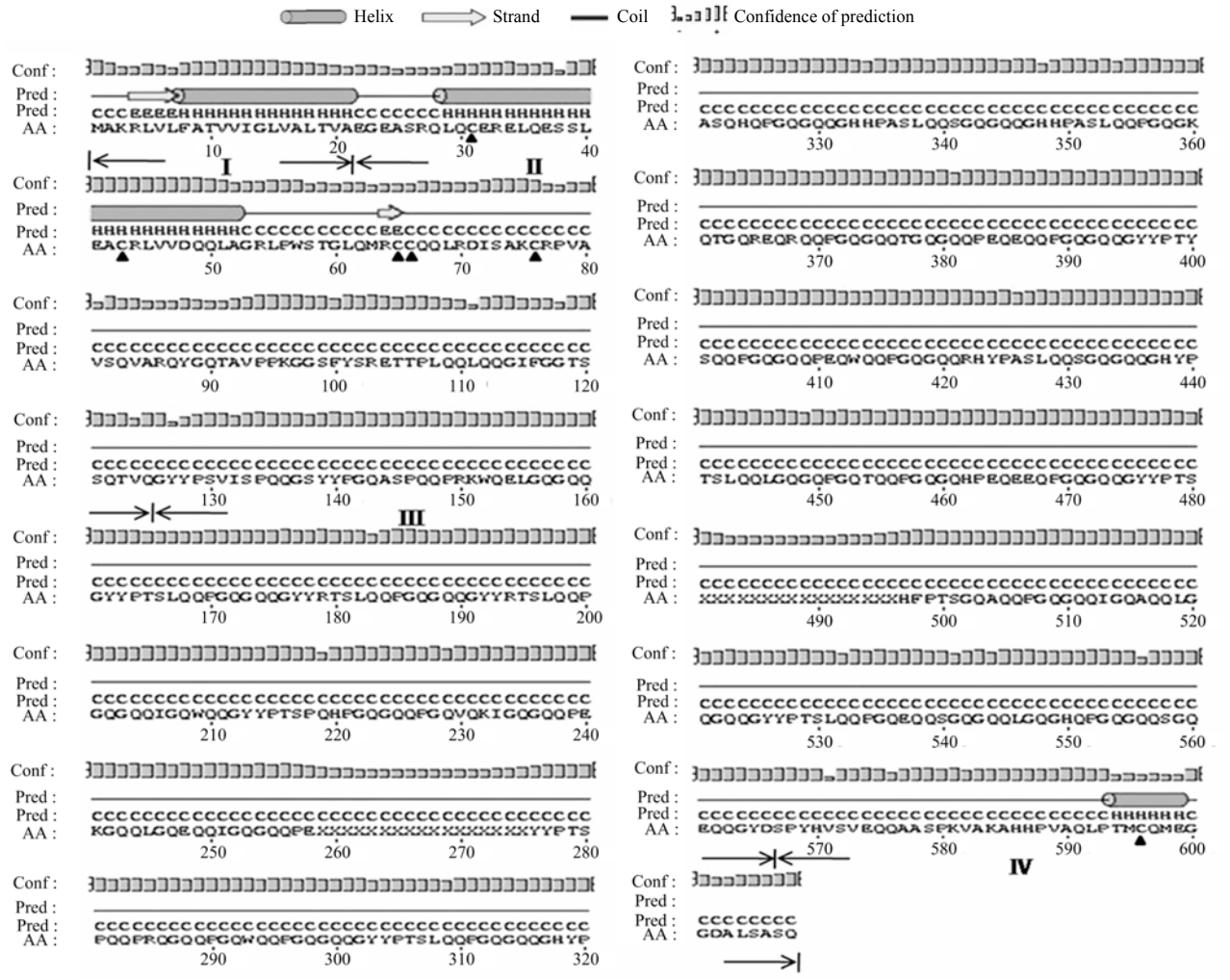

Fig. 4 Predicted secondary structure of HMW-GS 1Ay from *T. urartu* accession PI428281. Pred, predicted secondary structure; AA, target sequence; I, signal peptide; II, N-terminal domain; III, repetitive domain; IV, C-terminal domain. No-tailed arrowheads indicate cysteine residues.

(Fig. 5, lanes 1, 2), while the bacterial cells induced without IPTG did not express the 1Ay protein (Fig. 5, lane 5). All the 1Ay proteins migrated similarly to the 1Dy12 subunit in Chinese Spring (Fig. 5, lane 6).

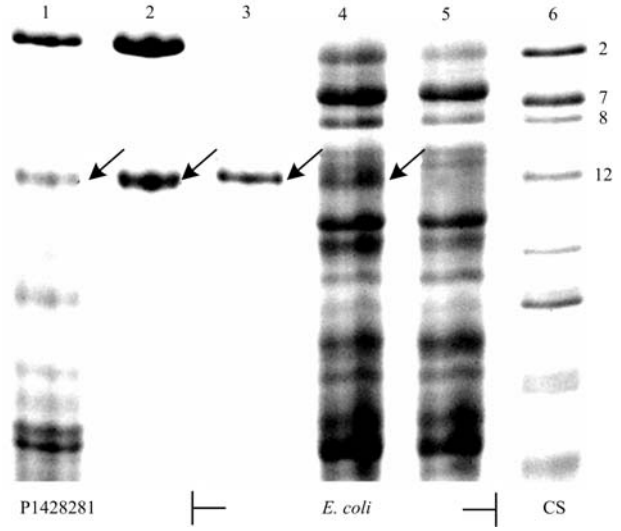

Fig. 5 SDS-PAGE analysis of bacterial expression of the *1Ay* ORF from *T. urartu* accession PI428281. Lanes 1 and 2, the total and HMW glutenins extracted generally and selectively from the seed, respectively; lanes 3 and 4, the selective and general extraction of 1Ay proteins induced by IPTG, respectively; lane 5, the bacterial protein not induced by IPTG; lane 6, CS (Chinese Spring) as reference. The arrowheads indicate 1Ay protein.

Evolutionary relationships of HMW-GS 1Ay

To determine the evolutionary relationships between the present 1Ay subunit (FJ404595) and other HMW-GS, a phylogenetic tree was constructed. As shown in Fig. 6, the phylogentic tree was clearly clustered into two separate groups, one by y-type and the other by x-type of genes. The present 1Ay (FJ404595) is more closely related to other 1Ay rather than to 1By, 1Dy and x-type subunits. The seven 1Ay subunits encoded by *Glu-A1* loci from wild diploid and tetraploid wheats had very close evolutionary relationships with a very high bootstrape value of 99. The detailed analysis showed that it was clustered with three (AM183223, AY245578, and EU984503) of the four compared *1Ay* genes in *T. urartu* in clade I with a very high bootstrape value of 92 , while estranged from those in *T. monococcum aegilopoides*, *T. turgidum dicoccon*, and *T. timopheevi*.

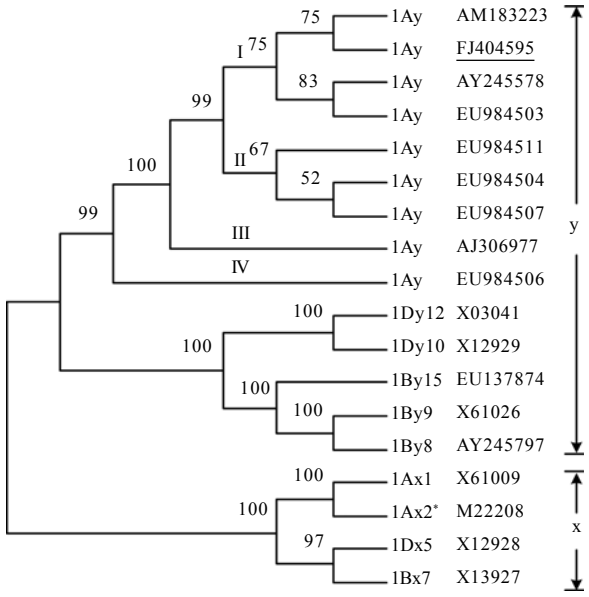

Fig. 6 Phylogenetic tree constructed through the whole amino acid sequences from the 1Ay subunit FJ404595 and other reported HMW-GSs.

DISCUSSION

Not all members in the genes encoding HMW-GS are expressed in cultivated wheat varieties, in particular, *1Ay* gene, which is regarded as always silencing (D'Ovidio *et al*. 1996). However, 1Ay subunits widely existed in wild diploid and tetraploid wheat genotypes and possessed genetic variations (Waines and Payne 1987; Ma *et al*. 2007; Hu *et al*. 2008; Xu *et al*. 2009), which made it possible to analyze the structural features of *1Ay* genes and their corresponding proteins. To date, 17 *1Ay* genes from different species have been successfully isolated and eight of them are inactive (Wan *et al*. 2002; Bai *et al*. 2004; Sun *et al*. 2004; Jiang *et al*. 2009). In the present study, the active *1Ay* gene (FJ404595) in *T. urartu* PI428281 was cloned and analyzed. The deduced protein sequence had the typical feature of y-type subunit (Fig. 3). Phylogenetic analysis showed that it was clustered with 1Ay, 1By, and 1Dy rather than x-type subunits (Fig. 6), confirming it belonging to y-type subunit. It was clustered with three of the four compared 1Ay subunits from *T. urartu* with a very high bootstrap value. Moreover, the present 1Ay subunit (FJ404595) was very similar to other active 1Ay subunits in *T. urartu* in amino acid compositions,

residue numbers, and the peptide length (Fig. 3).

Till date, there have been only two reports on heterologous expression of *1Ay* genes from *T*. *urartu* accession IZ29-1 with a faster electrophoretic mobility than 1Dy12 (Bai *et al*. 2004) and from *T*. *timopheevi* with a faster electrophoretic mobility than 1Dy10 (Wan *et al*. 2002). In this study, the *1Ay* gene from *T*. *urartu* PI428281 was successfully expressed in *E*. *coli*, and yielded a polypeptide showing the identical electrophoretic mobility with the 1Ay subunit from its seed (Fig. 5). This is the first heterologous expression of *T*. *urartu* 1Ay protein with a similar electrophoretic mobility to that of 1Dy12. If this 1Ay subunit could exist in hexaploid wheat, it might mix/cover/confuse with 1Dy12 because of their similar electrophoretic mobilities, and therefore difficult to be distinguished, possibly leading to mistake *1Ay* gene as unexpressing or silencing in *T*. *aestivum* (Forde *et al*. 1985; D'Ovidio *et al*. 1996). Actually, the kind of 1Ay subunit migrating faster than 1Dy12, has been observed in hexaploid wheat background because of the absence of interference (Margiotta *et al*. 1996; Bai *et al*. 2004; Ma *et al*. 2008). However, it is unclear whether or not these characters of 1Ay are associated with its "inactivity" (Forde *et al*. 1985; Harberd *et al*. 1987; Wan *et al*. 2002; Sun *et al*. 2004; Jiang *et al*. 2009).

D'Ovidio and Anderson (1994) reported that y-type subunits had major influence on dough quality than x-type subunits. More numerous coils in the repetitive domain could benefit the formation of gluten polymer and therefore enhance the functional properties of HMW glutenins (Li *et al*. 2007). The secondary structures of HMW-GS 1Dx5 and 1Dy10 in hexaploid wheat are very simliar (Pandey *et al*. 2008). In this study, the secondary structure of 1Ay subunit in *T*. *urartu* PI428281 was firstly predicted (Fig. 4). The result showed that this 1Ay subunit was made up of coils for 91%, similar to the secondary structure of 1Ax1 (Pandey *et al*. 2008) which benefits to flour quality (Branlard *et al*. 1992, 2001; Cornish *et al*. 2001). Thus, the existence or transfer of the active 1Ay subunit into common wheats might also have positive impact on flour processing quality.

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