Identification of an Active *1Ay* Gene from *Triticum turgidum* ssp. *dicoccoides*

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Abstract

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The high molecular weight glutenin subunit (HMW-GS), encoded by the *1Ay* gene, unexpressed in common wheat, exists in diploid and tetraploid wheats. An active *1Ay* gene was first cloned from wild emmer wheat (*T. turgidum* ssp. *dicoccoides*, 2*n* = 4*x* = 28, AABB), the oldest species in emmer wheat. Here, a novel subunit encoded by the *1Ay* gene (JF519636) present in *T. turgidum* ssp. *dicoccoides* line D141 was characterized. The protein had 608 amino acids with six cysteine residues and showed faster electrophoretic mobility than 1Dy12 from common wheat. Compared with previously reported 1Ay subunits, it contained 16 single point mutations (SPMs). Comparative and phylogenetic sequence analyses suggested that this gene was more similar to the *1Ay* gene from the diploid species (2*n* = 2*x* = 14, AA) *T. urartu* than from *T. monococcum* ssp. *aegilopoides*. Its predicted secondary protein structure possessed a different content of motifs relative to the *1Ay* gene (AY245578) from *T. urartu*, which had similar electrophoretic mobility. In the central repetitive domain, JF519636 had more β-turns and β-bends than the 1Ay subunit AY245578. These structural characteristics in JF519636 could possibly be associated with specific gluten properties.

Keywords: *1Ay* gene; high molecular weight glutenin subunit (HMW-GS); phylogenetic analysis; wild emmer wheat

HMW glutenin subunits (HMW-GSs) including x- and y-type proteins encoded by the loci *Glu-A1*, *Glu-B1* and *Glu-D1* on the long arms of chromosomes 1A, 1B, and 1D, respectively, in common wheat (*Triticum aestivum*, AABBDD, 2*n* = 6*x* = 42) (Lawrence & Shepherd 1980; Payne *et al*. 1980, 1982) are the major seed storage proteins that determine dough viscoelastic properties and bread-making quality (Payne 1987; Shewry *et al*. 1992, 1995). In common wheat, the gene coding the subunit 1Ay is unexpressed because of a premature stop codon (Forde *et al*. 1985) or the insertion of a transposon-like element within its coding region (Harberd *et al*. 1987). However, this subunit is active in many lines of diploid wheat (AA) and the tetraploid wheats *T. turgidum* (AABB) and *T. timopheevii* (AAGG) (Ciaffi *et al*. 1991, 1998; Ma *et al*. 2007; Jiang *et al*. 2009; Xu *et al*. 2009; Hu *et al*. 2012). Active *1Ay* genes have been cloned from the wild diploid wheats *T. urartu* and *T. monococcum* ssp. *aegilopoides*, and the tetraploid wheats *T. turgidum* ssp. *dicoccon* and *T. timopheevii*.

The tetraploid wild emmer wheat *T. turgidum* ssp. *dicoccoides* is the oldest ancestor in emmer with AABB genomes. Some durum lines with the genome AABB containing the 1Ay subunit transferred from *T. turgidum* ssp. *dicoccoides* showed very promising gluten properties (Ciaffi *et al*. 1991). However, the molecular structural characteristics of the gene *1Ay* in *T. turgidum* ssp. *dicoccoides* are still unclear. The objective of the present study was to characterize an active *1Ay* gene from this subspecies and compare it with previously reported alleles from other species. These results should help to better understand the evolution of the *1Ay* gene and facilitate its use in wheat quality improvement.

MATERIAL AND METHODS

Plant materials. *T. turgidum* ssp. *dicoccoides* line D141 from Israel was used in this study. For identifying HMW-GS compositions, five common wheat lines were used as references, including Chinese Spring (CS) with 1Bx7+1By8 and 1Dx2+1Dy12, Chuanyu 12 (CY12) with 1Ax1, 1Bx7+1By8 and 1Dx5+1Dy10, Xiaoyan 6 (XY6) with 1Ax1, 1Bx14+1By15 and 1Dx2+1Dy11, and two lines of Xinjiang rice wheat (*T. petropavlovskyi*): AS360 with 1Bx7+1By9 and 1Dx5+1Dy10 and AS363 with 1Bx17+1By18 and 1Dx2+1Dy12. They were kept at the Triticeae Research Institute of Sichuan Agricultural University, China.

SDS-PAGE analysis. HMW glutenin subunits were separated by SDS-PAGE as described by Hu *et al*. (2012). The HMW glutenin protein was extracted using two methods. One general method of extraction was used as described by Wan *et al*. (2000). The other, for selective precipitation of HMW glutenin protein, was used as reported by Verbruggen *et al*. (1998) with some modifications by Hu *et al*. (2010).

Cloning and sequencing of the *1Ay* gene. The CTAB method was used to extract genomic DNA from the leaves of 15-day-old plants (Murray & Thompson 1980). The coding regions of HMW glutenin subunits were amplified using the oligonucleotide primers P1 5'-AGCTGCAGAGAGTTCTATCA-3' and P2 5'-ATCACCCACAACACCGAGC-A-3'. A 50 µl reaction mix with *ExTaq* polymerase (Takara Biotechnology Co., Dalian, China) was used, and the reaction was performed at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min and 68°C for 5 min, and then extension at 68°C for 10 min. The PCR products were cloned into the pMD19-T vector (Takara). Then, the recombinant plasmid was transformed into *Escherichia coli* DH10B competent cells. The recombinant plasmids were digested with the restriction endonucleases *Kpn*I and *Xba*I (Takara), and then with exonuclease III (Exo III; Takara) for 1, 2 and 3 min, respectively. The digestion with Exo III resulted in progressive deletion of the 3' end of the insert, leaving a single-stranded 5' overhang that could be removed by treatment with mung bean S1 nuclease (Takara). The blunt ends thus formed were ligated with T4 DNA ligase (Takara), a suitable host was transformed, and colonies were picked at random and screened for the insert size, then a suitable range of inserts was sequenced. The full-length sequence was verified through a series of overlapping subclones using the nested deletion method (Sambrook *et al.* 1989). Nucleotide sequencing was performed by the BGI Company (Shanghai, China). The final nucleotide sequences for each ORF were identified from the sequencing results of three independent clones.

Expression of the *1Ay* gene in *E. coli*. The cloned DNA sequence of the HMW glutenin 1Ay subunit was re-amplified by PCR using the primers BD-AyP1 (5'-ACCCATATGGAAGGTGAGACCTCTAAGC-3') and BD-AyR1 (5'-TTCCTCGAGATATCACTGGTGGCCGAC-3') to remove the signal peptide and add restriction enzyme sites (*Nde*I and *Eco*RI). The resulting fragment was cloned into the bacterial expression vector pET-30a (Invitrogen Corporation, Carlsbad, USA) and transformed into *E. coli* BL21 (DE3) *plysS* cells. The recombinant *E. coli* cells were grown on 2× YT medium (Sambrook *et al.* 1989) with 25 µg/ml kanamycin and 34 µg/ml chloromycetin at 37°C until the OD_{600} reached 0.6. The 1Ay was expressed in *E. coli* by induction with 1 mmol IPTG (isopropylthio-β-D-galactoside) for 4–6 h. The expressed products were extracted as reported by Wan *et al.* (2000). The electrophoretic mobility of the protein expressed in the recombinant cells was compared with that of the native 1Ay subunit extracted from the seeds of wild emmer line D141 using SDS-PAGE.

Sequence comparison and phylogenetic analysis. The amino acid sequence was deduced from the cloned nucleotide sequence. Secondary protein structures were predicted using the protein 8-class secondary structure prediction program SSpro8 (http://scratch.proteomics.ics.uci.edu/). Multiple alignments of amino acid sequences were carried out by DNAMAN (ver. 6.0.3.48) with manual adjustment. A phylogenetic tree was constructed using the N-terminal amino acid sequences according to Wang *et al.* (2007). The software MEGA 4.02 was used to create phylogenetic trees by the neighbour-joining (NJ) method (Tamura *et al.* 2007). Bootstrap values were estimated based on 1000 replications.

RESULTS

SDS-PAGE analysis of HMW-GSs from wild emmer line D141. Four HMW-GS bands were detected by both the general and selective extraction methods (Figure 1). All of the bands had faster electrophoretic mobility than the subunit 1Bx7. The 1Ay subunit migrated faster than 1Dy12 in common wheat.

Sequence characteristics of the 1Ay subunit and its encoding gene. Four DNA bands were produced by PCR from the wild emmer line D141 using the primers P1 + P2 (Figure 2). The smallest DNA fragment (about 1.8 kb) was selected for further cloning

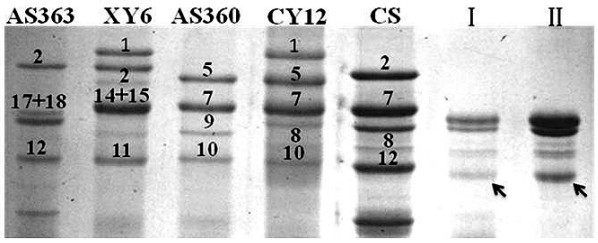

Figure 1. SDS-PAGE analysis of HMW-GSs; samples I and II were derived from the general and selective extraction of HMW glutenins in wild emmer line D141, respectively; the other five lanes are common wheat lines; the HMW-GS encoded by *1Ay* gene is indicated by arrowheads

and sequencing. Its full-length open reading frame (ORF) was 1830 bp. Blast analysis indicated that it belonged to a y-type encoding gene at the *Glu-A1* locus with the highest identity (98%) to the active *1Ay* gene FJ404595 (Table 1). The sequence was deposited in the NCBI database with accession No. JF519636.

JF519636 possessed typical structural characteristics of y-type HMW glutenin subunits. It encoded 608 amino acid residues, including 21 for a signal peptide and 104 in the N-terminal, 438 in the central repetitive, and 45 in the C-terminal domains. It had a similar trend of amino acid variations in the repeat consensus to those of all published active 1Ay subunits, with more variation at positions 1 and 4 in hexapeptides, and 2, 5 and 7 in nonapeptides (Table 2). However, it had the highest variation (1.197) among the repetitive consensus sequences, relative to the criterion hexapeptide PGQGQQ and nonapeptide GYYPTSLQQ (Table 2). It had 16 single point mutations (SPMs) at positions 17 (S/A), 25 (T/A), 27(K/R), 81 (L/V), 102 (H/S) or (H/P), 161 (W/G), 169 (K/Q), 188 (R/G), 297 (V/G), 305 (S/P), 312 (V/G), 320 (L/P), 380 (E/G), 389 (L/P), 531 (P/L)

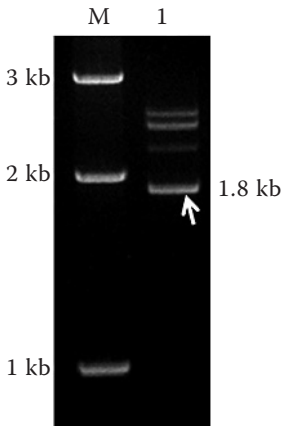

Figure 2. PCR amplification products of HMW-GSs from wild emmer line D141; lane M – DNA ladder; the *1Ay* segment is indicated by the arrowhead in lane 1

Table 1. Sequence comparison between JF519636 and previously published 1Ay proteins

Species	GenBank accession	Genome	Identity (%)	Full length (aa)	Single point mutations (SPMs)				InDels	
					N-terminal domain	repetitive domain	C-terminal domain	total	insertions	deletions
Triticum turgidum ssp. *dicoccoides*	JF519636	AABB	this study	608	/	/	/	/	/	/
T. urartu	FJ404595	AA	98	608	4	19	0	23	0	0
	AM183223	AA	97	608	4	20	1	25	0	0
	AY245578	AA	97	608	4	21	0	25	0	0
	EU984503	AA	97	608	5	19	0	24	0	0
	EU984504	AA	96	587	4	18	0	22	0	2
T. monococcum ssp. *aegilopoides*	EU984506	AA	93	631	5	39	6	50	5	2
	EU984507	AA	96	587	4	21	0	25	0	2
	EU984508	AA	95	732	11	74	12	97	7	1
T. turgidum ssp. *dicoccon*	EU984511	AABB	96	587	4	21	0	25	0	2
T. timopheevii	AJ306977	AAGG	96	587	4	21	3	28	0	2

Table 2. Comparison of amino acid variations in the repeat consensuses of JF519636 and previously published 1Ay proteins relative to the criterion hexa- and nonapeptides

GenBank accession	Hexapeptide								Nonapeptide											M[b]
	1	2	3	4	5	6	total units	M[a]	1	2	3	4	5	6	7	8	9	total units	M[a]	
	P	G	Q	G	Q	Q			G	Y	Y	P	T	S	L	Q	Q			
JF519636	17	9	1	16	3	2	47	1.021	1	5	2	4	5	1	4	1	2	14	1.786	1.197
AJ306977	18	6	2	14	3	3	45	1.022	0	5	2	0	5	1	2	1	1	13	1.308	1.086
AM183223	13	8	2	14	3	4	47	0.936	1	5	2	2	4	1	5	1	1	14	1.571	1.082
AY245578	15	7	2	14	4	4	47	0.979	1	5	2	2	5	1	5	1	1	14	1.643	1.131
EU984503	15	7	2	14	3	5	47	0.979	1	5	2	2	5	1	5	1	1	14	1.643	1.131
EU984506	16	6	2	16	4	4	52	0.923	1	6	3	0	5	0	6	1	2	14	1.714	1.091
EU984504	16	6	2	14	3	3	45	0.978	1	5	2	2	5	1	3	1	1	13	1.615	1.121
FJ404595	15	8	2	14	3	4	47	0.979	1	5	2	2	4	1	5	1	1	14	1.571	1.115
EU984507	16	6	3	14	3	6	45	1.067	1	5	2	2	5	1	3	1	1	13	1.615	1.190
EU984508	21	6	5	18	2	5	59	0.966	2	8	2	1	7	2	11	0	3	20	1.800	1.177
EU984511	16	6	2	14	3	4	45	1.000	1	5	2	2	5	1	4	2	1	13	1.769	1.172

P – proline; G – glycine; Q – glutamine; Y – tyrosine; T – threonine; S – serine; L – leucine; M – methionine; [a]the average number of amino acid variations per repetitive unit; [b]the average number of amino acid variations per unit including hexapeptides and nonapeptides

and 541 (R/G), compared with previously reported active 1Ay subunits (Figure 3).

The repetitive domain of JF519636 contained 47 hexapeptides and 14 nonapeptides, much like the four sequences AM183223, AY245578, EU984503, and FJ404595 from *T. urartu* (Table 2). However, 23–25 amino acid variations were observed between JF519636 and each of the four *T. urartu* sequences

Table 3. Predicted secondary protein structures of the deduced amino acid sequences of the *1Ay* genes JF51963 (this study) and AY245578 from *T. urartu*

HMW-GS	Structure motifs	Content (%)	Total No.	Dispersal in every region					
				content (%)			No.		
				NT	CR	CT	NT	CR	CT
JF519636	α-helix	21.81	25	55.77	12.47	35.71	5	16	4
	β-strand	11.24	30	15.38	9.75	16.67	4	23	3
	β-turn	18.40	59	6.73	22.00	9.52	4	52	3
	β-bend	0.51	3	0.00	0.68	0.00	0	3	0
	The rest	48.04	80	22.12	55.10	38.10	9	64	8
	3_{10}-helix	0.00	0	0.00	0.00	0.00	0	0	0
	π-helix	0.00	0	0.00	0.00	0.00	0	0	0
	β-bridge	0.00	0	0.00	0.00	0.00	0	0	0
AY245578	α-helix	17.38	21	51.92	6.80	42.86	4	13	4
	β-strand	12.10	36	15.38	10.43	21.43	4	29	3
	β-turn	18.40	57	11.54	21.09	7.14	5	50	2
	β-bend	0.34	2	0.00	0.23	2.38	0	1	1
	The rest	51.79	82	21.15	61.45	26.19	9	68	5
	3_{10}-helix	0.00	0	0.00	0.00	0.00	0	0	0
	π-helix	0.00	0	0.00	0.00	0.00	0	0	0
	β-bridge	0.00	0	0.00	0.00	0.00	0	0	0

NT – N-terminal domain; CR – central repetitive domain; CT – C-terminal domain

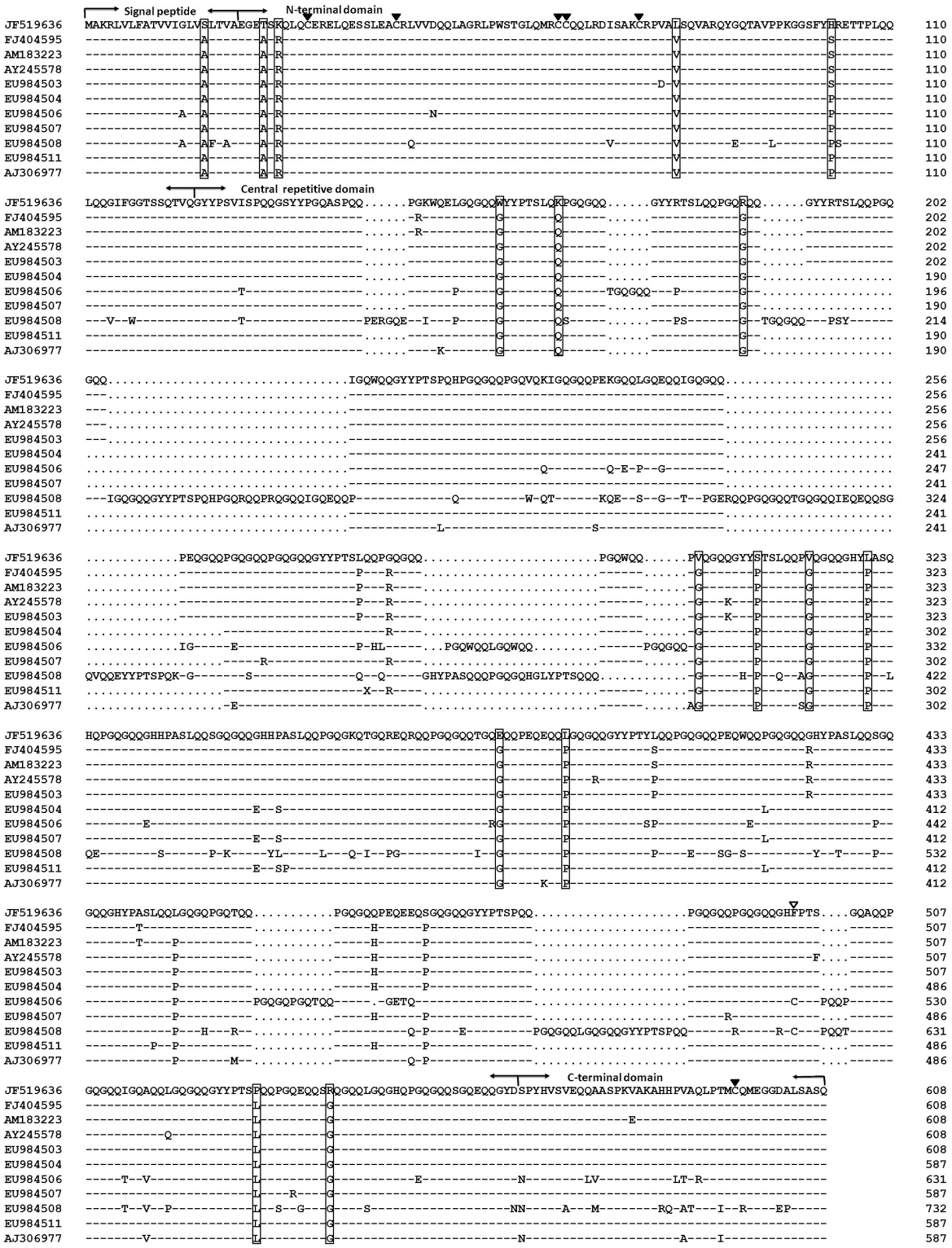

Figure 3. Comparison of amino acid sequences between JF519636 and the 10 previously reported 1Ay subunits; single point mutations (SPMs) are marked by boxes; conserved cysteine residues in the N- and C-terminal domains are marked by solid triangles; the position of the extra cysteine residue in the central repetitive regions of two 1Ay subunits from *T. monococcum* ssp. *aegilopoides*, EU984506 and EU984508, is marked by a hollow triangle; identical and deleted residues are indicated by "*-*" and "*.*", respectively; JF519636 comes from *T. turgidum* ssp. *dicoccoides*; FJ404595, AM183223, AY245578, EU984503 and EU984504 from *T. urartu*; EU984506, EU984507 and EU984508 from *T. monococcum* ssp. *aegilopoides*; EU984511 from *T. turgidum* ssp. *dicoccon*; AJ306977 from *T. timopheevii*

(Figure 3, Table 1). All five sequences contained six cysteine residues, five in the N-terminus and one in the C-terminus, differing from the two sequences EU984506 and EU984508 from *T. monococcum* ssp. *aegilopoides* in possessing an extra cysteine residue in the repetitive region (Figure 3). JF519636 was also obviously different from the two sequences of *T. monococcum* ssp. *aegilopoides* because of a high number of SPMs (50 and 97), as well as many InDels (Figure 3, Table 1).

Predicted secondary structure of the protein encoded by the *1Ay* gene. Because JF519636 from this study and AY245578 from *T. urartu* line IZ29-1 (Bai *et al.* 2004) showed similar electrophoretic mobility in SDS-PAGE, their protein secondary structures were predicted and compared. As shown in Table 3, similar to the 1Ay subunit AY245578, JF519636 had four kinds of secondary structure motifs, including α-helix (21.81%), β-strand (11.24%), β-turn (18.40%), and β-bend (0.51%), with the rest comprising 48.04%. The former two were mainly distributed in the conserved N-terminal (55.77 and 15.38%) and C-terminal (35.71 and 16.67%) domains, compared with the central repetitive domain (12.47 and 9.75%). However, the latter two motifs and unstructured sequence mainly existed in the central repetitive domain (22.00, 0.68 and 55.10%), compared with the N-terminus (6.73, 0.00 and 22.12%) and the C-terminus (9.52, 0.00 and 38.10%). However, the relative content and number of these motifs differed from AY245578, not only over the entire polypeptide but also in each of the three domains. In the central repetitive domain, JF519636 had 52 β-turn residues (22.00%) and 3 β-bend residues

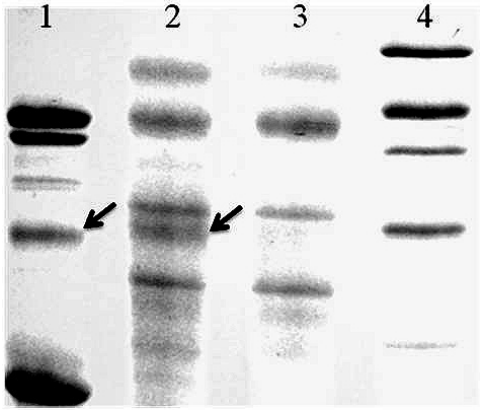

Figure 4. Bacterial expression analysis of the *1Ay* ORF from *T. turgidum* ssp. *dicoccoides* line D141; lane 1 – HMW glutenins from D141 seeds; lane 2 and 3 – the proteins from recombinant cells induced with and without IPTG, respectively; lane 4 – Chinese Spring as a reference; arrowheads indicate the 1Ay protein

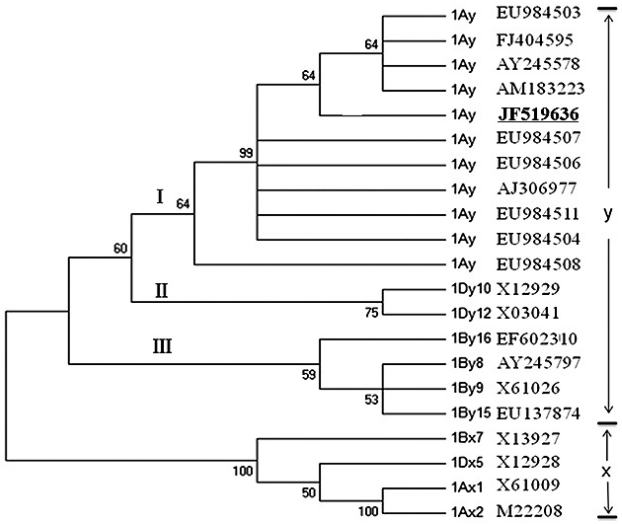

Figure 5. Phylogenetic tree based on N-terminal domains from the present 1Ay subunit JF519636 and previously published sequences; JF519636, EF602310 come from *T. turgidum* ssp. *dicoccoides*; FJ404595, AM183223, AY245578, EU984503, and EU984504 from *T. urartu*; EU984506, EU984507, and EU984508 from *T. monococcum* ssp. *aegilopoides*; EU984511 from *T. turgidum* ssp. *dicoccon*; AJ306977 from *T. timopheevii*; AY245797 from *T. turgidum* ssp. *durum*; X12929, X03041, X61026, EU137874, X13927, X12928, X61009, and M22208 from *T. aestivum*

(0.68%), more than AY245578, which had 50 (21.09%) and 1 (0.23%), respectively.

Expression of the *Ay* gene in *E. coli*. The authenticity of the cloned *1Ay* gene was confirmed by successful expression of the coding regions in *E. coli.* Expression of the mature protein was detected in the IPTG-induced bacterial cells, which showed a band identical to that of 1Ay extracted from seeds of *T. turgidum* ssp. *dicoccoides* line D141. In contrast, the mature protein was not observed in the control bacterial culture that was not induced by IPTG (Figure 4).

Phylogenetic analysis. The phylogenetic tree consisting of 21 HMW glutenin subunits was clearly separated into two clusters, one of x-type and the other of y-type subunits (Figure 5). The present 1Ay JF519636 was more closely clustered with four *1Ay* genes (EU984503, FJ404595, AY245578 and AM183223) from *T. urartu* than with those from *T. monococcum* ssp. *aegilopoides*, *T. turgidum* ssp. *dicoccon* and *T. timopheevii* (Figure 5).

DISCUSSION

The HMW-GS encoded by the *1Ay* gene, unexpressed in common wheat, exists widely in wild

diploid and tetraploid wheats (Forde *et al.* 1985; D' Ovidio *et al.* 1996; Hu *et al.* 2012). Ten previously cloned active *1Ay* genes were derived from the wild diploid wheats *T. urartu* and *T. monococcum* ssp. *aegilopoides*, and from the domesticated tetraploid wheats *T. turgidum* ssp. *dicoccon* and *T. timopheevii* (Table 1). In the present study, an active *1Ay* gene was cloned from the wild tetraploid wheat *T. turgidum* ssp. *dicoccoides*, which is the oldest species of emmer wheat with the genome AABB (Feldman *et al.* 1995; Chantret *et al.* 2005). The 1Ay subunit JF519636 was similar to that of AY245578 from *T. urartu* line IZ29-1, but differed from the other nine *1Ay* genes in electrophoretic mobility in SDS-PAGE (Wan *et al.* 2002; Bai *et al.* 2004; Jiang *et al*. 2009; Hu *et al.* 2010). However, its predicted protein secondary structure was different from that of AY245578 in the relative content and number of motifs because of 25 different amino acids (Figure 3, Tables 1 and 3). Moreover, compared with the 10 previously reported 1Ay proteins, the present subunit had 16 SPMs and had the highest amino acid variation in the repetitive consensus region relative to the criterion hexa- and nonapeptides (Figure 3).

Comparative sequence analysis indicated that the present 1Ay subunit JF519636 from *T. turgidum* ssp. *dicoccoides* was more similar to those from *T. urartu* than to those from *T. monococcum* ssp. *aegilopoides* (Figure 3, Tables 1 and 2). The phylogenetic tree also showed that it was clustered together with four *T. urartu* lines (Figure 5). These results support the idea that *T. urartu* provided the donor A genome in *T. turgidum* ssp. *dicoccoides* (Chapman *et al.* 1976; Dvorak *et al.* 1988).

The present 1Ay subunit JF519636 had more α-helixes in the conserved N- and C-terminal domains, and more β-turns and β-bends in the central repetitive domain, similar to the secondary structures of other HMW-GSs (Tatham *et al.* 1985, 1990). Bekes and Gras (1999) suggested that β-turns might endow HMW-GSs with unique elastic properties. In the central repetitive domain, JF519636 had more β-turns and β-bends than the 1Ay subunit AY245578 (Table 3). These structural characteristics in JF519636 may possibly be associated with special gluten properties.

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