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Molecular Characterization of the *Waxy* Gene in Einkorn Wheat

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Abstract: This study characterizes 15 *waxy* genes from 15 accessions of the einkorn wheats *Triticum urartu*, *T. boeoticum* and *T. monococcum*. The mature protein coding sequences of *waxy* genes were analyzed. Nucleotide sequence variations in these regions resulted from base substitution and/or indel mutations. This work identified 8 distinct haplotypes from the diploid wheat *waxy* gene sequences. A main haplotype was found in 7 gene samples from the A^u genome and A^m genome. The *waxy* gene sequences from the A^u and A^m genomes could be obviously clustered into two clades, but the sequences from the A^m genome of *T. boeoticum* and *T. monococcum* could not be clearly distinguished. The phylogenetic analysis revealed that the *waxy* gene sequences from the A^m genome had accumulated fewer variations and evolved at a slower rate than the sequences from the A^u genome. These results would contribute to the understanding of functional aspects and efficient utilization of *waxy* genes.

Key words: Diploid wheat, phylogenetic analysis, haplotype, SNP, waxy

INTRODUCTION

Diploid wheats, including *Triticum urartu* (2n = 2x = 14, A^uA^u), *T. boeoticum* (2n = 2x = 14, A^mA^m) and *T. monococcum* (2n = 2x = 14, A^mA^m), were the A genome donors of polyploid wheats and they provided many characters or genes of agronomic interest (D'Egidio and Nardi, 1993). Subsequent molecular studies identified *T. urartu* as the A^u genome ancestor of emmer wheat, durum wheat and common wheat, whereas *T. monococcum* is the donor of the A^m genome of *Triticum zhukovskyi* (Dvorak *et al*., 1993; Jiang and Gill, 1994; Feldman, 2001; Baum and Bailey, 2004), although, *T. urartu* and *T. boeoticum* are wild and *T. monococcum* is domesticated.

Granule-bound starch synthase (GBSSI, EC2.4.1.21), also called *waxy* protein (Echt and Schwartz, 1981) is a nuclear-encoded enzyme of about 60 kDa, which plays a crucial role in the amylose synthesis in the plastids of plants (Vos-Scheperkeuter *et al*., 1986). The gene encoding the granule-bound starch synthase (called *waxy* gene) has been cloned from maize (Shure *et al*., 1983; Klosgen *et al*., 1986), potato (Visser *et al*., 1989; Van-Der-Leij *et al*., 1991), barley (Rohde *et al*., 1988), rice (Hirano and Sano, 1991; Okagaki, 1992), pea (Dry *et al*., 1992),

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common wheat (Mason-Gamer *et al*., 1998; Murai *et al*., 1999) and three diploid wheats only (Yan *et al*., 2000). In view of the importance of the *waxy* genes to wheat quality attributes (Zhao *et al*., 1998), it was of interest to analyze more genes from respective diploid progenitors of wheat.

In this study, fifteen partial *waxy* genes from *T. urartu* (designated the *wx-TuA* gene), *T. boeoticum* (designated the *wx-TbA* gene) and *T. monococcum* (designated the *wx-TmA* gene) were cloned. The objectives of this study were to isolate and characterize the partial *waxy* genes from diploid wheats and to investigate the polymorphisms of these genes in the A genome, as well as to discuss the nucleotide variations.

MATERIALS AND METHODS

Plant Materials

In total, 15 accessions of einkorn wheats, including 5 *T. boeoticum*, 5 *T. monococcum* and 5 *T. urartu* accessions, were used to characterize *waxy* genes. The einkorn wheat accessions were kindly provided by Dr. Harold Bockelman of American Germplasm Resources Information Network (GRIN) of USDA. These diploid wheat accessions were collected from various countries, including Turkey, Armenia, Iraq, Asia Minor, Iran and others (Table 1).

PCR Amplification

Genomic DNAs were extracted from the young leaves of single plant. From September 2007 to October 2008, two anchored primers (F: 5'-TTGCTGCAGGTAGCCACAC-3'; R: 5'-CTCAAGTGCTGCCTGGCAGAGAA-3') were selected for amplification the target *waxy* gene (Yan *et al*., 2000), which include the extron 1 to extron 3 regions. Reactions were carried out in 50 μL containing 200 ng of genomic DNA, 1.5 mM $MgCl_2$, 5 pmol of each primer, 200 μM of each dNTP and 1.2 units of Taq polymerase (*TaKaRa*) with high fidelity and 5 μL of 10×PCR buffer. The PCR amplification was run in Peltier Thermal Cycler PTC-220 (MJ Research, USA) with the following program: an initial step of 95°C for 2 min, followed by 39 cycles of 95°C for 1 min, 60°C for 1 min and 72°C for 2 min, then 72°C for 10 min. The expected fragments were recovered and cloned into pMD18-T vector (*TaKaRa*), then transformed into the competent *E. coli* cells (DH5α) *waxy* gene is a single copy gene in the diploid wheats (Gautier *et al*., 2000). Therefore, one positive clone of each accession was sequenced by commercial company (*TaKaRa*) in two directions.

Table 1: Plant materials used in this study

Accessions	Origins	Species
PI 191094	Spain	*Triticum monococcum*
PI 191383	Ethiopia	*Triticum monococcum*
PI 401415	Iran	*Triticum boeoticum*
PI 427461	England	*Triticum boeoticum*
PI 428002	Lebanon	*Triticum boeoticum*
PI 428152	Bulgaria	*Triticum monococcum*
PI 428168	Turkey	*Triticum monococcum*
PI 428176	Turkey	*Triticum monococcum*
PI 428180	Armenia	*Triticum urartu*
PI 428220	Turkey	*Triticum urartu*
PI 428262	Lebanon	*Triticum urartu*
PI 428317	Iran	*Triticum urartu*
PI 503309	Iran	*Triticum boeoticum*
PI 538606	Iraq	*Triticum boeoticum*
PI 538736	Lebanon	*Triticum urartu*

Data Analysis

Multiple sequence alignment were conducted by DNAman (version 5.2.2; Lynnon Biosoft), Clustal W (Higgins *et al*., 1994) and MEGA (version 3.1 Kumar *et al*., 2004). Phylogenetic analysis was carried out by MEGA using neighbor-joining method (Saitou and Nei, 1987).

RESULTS

DNA Sequence Variation

Fifteen sequences were obtained and analyzed using BLASTn in NCBI. It was found that these sequences had the closest homology with *waxy* genes, confirming the new sequences as *waxy*. The 15 gene sequences share a high sequence identity (97.06%), among which the identity values of the coding and the noncoding sequences are 99.31 and 89.57%, respectively. Among the 691 nucleotides of 5 gene sequences from the A^u genome, there were 663 conserved positions, 28 variable positions including 7 polymorphic sites (single nucleotide polymorphisms, SNPs) and 21 singleton positions (mutation in only one sequence). The frequency of SNPs in the *waxy* genes from the A^u genome was 1 in 98 bp. In the 10 A^m genome-coding genes, which from the A^m genome were more conserved than those genes of the A^u genome. Among the 691 nucleotides, there were 682 conserved positions, 9 variable positions, 4 polymorphism sites (SNPs) and 5 singleton positions. The frequency of SNPs in the α-amylase inhibitor genes from the A^m genome was 0.57%.

The SNPs were classified into transitions (C-T and A-G) and transversions (A-C, A-T, C-G and G-T), according to their substitution types; the transitions were more common than the transversions (Cooper and Krawczak, 1990). The distribution and proportion of transitions and transversions in the SNPs of *waxy* genes from diploid wheats were investigated. As expected, most of the SNPs (81.25%, 8/9 in *T. monococcum* and 5/7 in *T. urartu*) were transitions and the rest (18.75%) were transversions.

Haplotype Diversity

A total of 8 haplotypes were defined on the basis of SNP and indel analysis, among which 6 haplotypes only had one gene sample (Table 2). Haplotype 4 was found in two out of 15 gene sequences. Haplotype 1 appeared most frequently, which was involved in

Table 2: Distribution patterns of SNPs and indels of 8 haplotypes in the 15 *waxy* genes from einkorn wheat

	43	52	98	143	215	216-221	236	258	268	299	365	366	378	380	387	396	398	404
Haplotype	----------(bp)----------																	
1	T	A	G	G	C	-	G	A	T	G	C	T	C	G	C	-	A	C
2	T	A	G	G	A	-	G	A	T	A	C	T	C	G	C	-	A	T
3	T	A	G	G	A	-	G	A	T	A	C	T	C	G	C	-	A	T
4	T	A	G	G	A	-	G	A	T	G	T	T	C	G	C	-	A	C
5	T	A	C	G	A	I	G	G	T	G	A	A	T	C	T	T	T	C
6	C	A	C	A	A	-	T	G	A	G	A	A	T	C	T	T	T	C
7	C	A	C	A	A	-	T	G	T	G	A	A	T	C	T	T	T	C
8	C	G	C	A	A	-	T	G	T	G	A	A	T	C	T	T	T	C

	407	408-409	424	427	429	432-433	434	437	438	445	476	541	590	593	605	615	626
Haplotype	----------(bp)----------																
1	T	II	G	A	A	-	G	C	T	A	T	T	G	G	G	C	G
2	T	II	G	A	A	-	G	C	T	A	C	T	G	G	A	C	G
3	T	II	G	A	A	-	G	C	T	A	T	T	G	G	A	C	G
4	T	II	G	A	A	-	G	C	T	A	T	T	A	A	G	T	G
5	C	-	A	T	G	II	A	G	C	G	T	T	A	A	G	C	A
6	C	-	A	T	G	II	A	G	C	G	T	T	A	A	G	C	A
7	C	-	A	T	G	II	A	G	C	G	T	T	A	A	G	C	A
8	C	-	A	T	G	II	A	G	C	G	T	C	A	A	G	C	A

I: GGTACA, II: TG, -: Deletion

7 gene samples. One and 4 haplotypes were identified in *T. urartu*, *T. boeoticum* and *T. monococcum*, respectively. Meanwhile, 2, 3, 5, 6, 7 and 8 haplotypes were only found in *T. urartu*, *T. boeoticum* and *T. monococcum*, respectively. Haplotypes 2, 3, 5, 6, 7 and 8 were observed in one species or subspecies. Haplotype 1 was shared by the two species or subspecies (5 gene samples form *T. boeoticum*; 2 gene samples from *T. monococcum*). Haplotypes 4 was shared by both *T. monococcum* and *T. urartu*. It can be concluded that *waxy* genes from *T. urartu* were more diverse. Compared the *waxy* alleles of einkorn wheat with that of bread wheat (Murai *et al*., 1999), new alleles were frequently observed in einkorn wheat. Therefore, einkorn wheat could be potentially valuable sources of novel *waxy* alleles to improve the amylose synthesis in bread wheat.

Deduced Amino Acid Polymorphism

Analysis of the deduced amino acid sequences of the *waxy* protein found that the sequences of all the haplotypes from *T. urartu, T. boeoticum and T. monococcum* could encode proteins. Changes in the amino acid residues at 6 positions could be attributed to the polymorphic sites in the nucleotide sequences (Table 3). There were 6 different putative waxy protein amino acid residues in einkorn wheats.

Phylogenetic Analysis

Phylogenetic distances were calculated and used to construct the neighbor-joining trees showing phylogenetic relationships among the *waxy* genes in diploid wheats. In addition to the NJ method, the same data set was analyzed by Minimum Evolution (ME) and Maximum Parsimony (MP) methods. As the results of the 3 methods were similar, only the NJ tree is presented. It was found that the *waxy* genes were obviously divided into two groups. Four genes from the A^u genome of *T. urartu* clustered into one group, the other genes were included in the other group (Fig. 1). Furthermore, the genes, which are from *T. urartu* (Accession number was PI538736) and *T. monococcum* (Accession number was

Table 3: Variation of amino acids caused by the nucleotide changes in genes

Substitution	Amino acid position	Variation	Frenquency	Codon position
GCC/GTC	5	Ala/Val	3/12	2nd
CGG/CAG	8	Arg/Gln	1/14	2nd
GGC/AGC	75	Gly/Ser	4/11	1st
AAG/ATG	78	Lys/Met	1/14	2nd
ACC/ATC	117	Thr/Ile	1/14	2nd
ATC/GTC	138	Ile/Val	4/11	1st

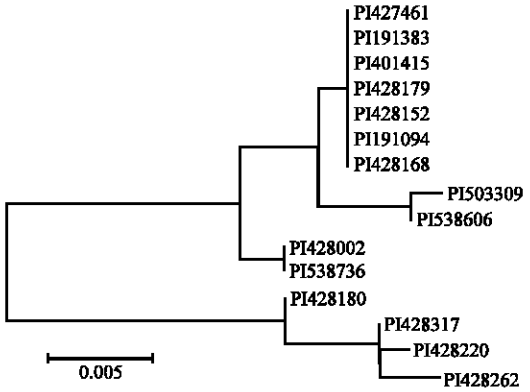

Fig. 1: Neighbor-joining tree of *waxy* genes

PI428002), could be separated with the other genes from *T. boeoticum* and *T. momococcum* in the subgroup by phylogenetic analysis. Neighbor-joining trees were also constructed for haplotypes of *waxy* genes from *T. boeoticum, T. monococcum* and *T. urartu*. Both the phylogenetic trees and haplotypes show that the *waxy* genes from the A^u genome were more divergent than those from the A^m genome. Moreover, the species *T. urartu* contained more haplotypes than the species *T. monococcum* and *T. boeoticum*.

DISCUSSION

The information in this study describes a set of homoeologous genes encoding granule-bound starch synthase (*waxy* gene), from *T. urartu*, *T. boeoticum* and *T. monococcum* (closely related to the A genome donors of hexaploid wheat). The *wx-TuA, -TbA* and *-TmA* genes possessed high homology in exons, compared with other known *waxy* genes (Ainsworth *et al*., 1993). The alignment of the fifteen genes showed only one gap in exon1 and the observed variation among the *wx-A* genes provides a basis for primer design and PCR-based assays for assaying the mutants of the *waxy* genes in common wheat.

It has been found that the A genome of polyploid wheat is complex; obviously, more than one diploid wheat was involved in its formation (Metakovsky and Baboev, 1992). Therefore, a comparative intraspecific study of polymorphism is needed for a better understanding of the phylogenetic relationships between wheat species. An obvious feature of the waxy genes from diploid wheats with A^m and A^u genomes is the single copies gene. Isoelectric focusing, two-dimensional gel electrophoresis and both direct and clone sequencing also revealed single copies of the waxy genes in polyploid wheat (Zhao and Sharp, 1996; Murai *et al*., 1999; Yan *et al*., 2000). In our study, 15 *waxy* gene sequences were obtained from 15 diploid wheat accessions, indicating that there were *waxy* genes in the diploid wheats and all of them were single copy.

The gene sequences of the *waxy* gene from the diploid wheats with A^m and A^u genomes were obviously clustered in two groups with high bootstrap values and the genes from *T. boeoticum* and *T. monococcum* could not be distinguished by phylogenetic analysis. Several studies indicate a high level of similarity between the chromosomes of *T. monococcum* and *T. aestivum* (Dubcovsky *et al*., 1995; Luo *et al*., 2000), suggesting that A^u and A^m genomes, notwithstanding their high sterility when crossed, have not diverged very significantly since their separation from a common progenitor. In previous investigations, it was possible to distinguish *T. urartu* from *T. boeoticum* and *T. monococcum* by microsatellite and RFLP markers (Corre and Bernard, 1995; Hammer *et al*., 2000). In recent studies, the A genome bearing *T. urartu* clearly differs from the A bearing *T. boeoticum* and *T. monococcum* in that it lacks the short A1 5S DNA gene unit class (Baum and Bailey, 2004). Earlier studies have shown that A^m and A^u are related but distinct genomes because the hybrids between *T. urartu* and *T. monococcum* are not fertile (Johnson and Dhaliwal, 1976). The AFLP markers were exploited to assess the differences among A genomes of diploid A^m and A^u wheats and their polyploid relatives (Brandolini *et al*., 2006). This indicated that all A^m genome samples (*T. monococcum*) clustered together and were separated from the A^u diploids (*T. urartu*). Isoenzyme analysis also showed a very low level of genetic variation within populations of *T. monococcum* or *T. boeoticum* (Smith-Huerta *et al*., 1989; Moghaddam *et al*., 2000). The RFLP analysis, however, showed high levels of polymorphism (Castagna *et al*., 1994; Corre and Bernard, 1995). Thus, *T. monococcum* and *T. boeoticum* could be regarded as two subspecies of one species with the same A^m genome.

The haplotype analysis revealed 8 haplotypes in 15 diploid wheat *waxy* gene samples. Haplotype 1 was found to be the main haplotype, occurring in 7 samples from *T. urartu* and *T. monococcum*. Out of the 8 haplotypes, 6 were found in only one single gene sample; haplotype 2 only had 2 samples, indicating that the *waxy* genes in einkorn wheats had less diversity.

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