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Molecular Characterization of *Waxy* Gene in *Aegilops tauschii*

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Abstract: In order to exploiting new genetic resources for the wheat breeding, nine *waxy* genes (granule-bound starch synthase, GBSSI) were obtained from wild species, *Aegilops tauschii* (DD) by special primer PCR cloning. These sequences showed the higher similarity to the sequences of *Wx-D*1 locus in common wheat. The analysis of variation and alignment of sequences showed that all the sequences could be distinguished, although from the same species. Out of 35 variable sites of nucleotide in the whole sequences, twelve variable sites located on the region of exon and made six amino acid residues change. Most of variable sites located on transit peptide. Using neighbour-joining method, phylogenetic tree suggested that the sequences of *waxy* gene from *Ae. tauschii* were clustered closely with the sequences of *Wx-D1* locus and far with the sequence of *Wx-A1* and *Waxy-B*1 loci in *Triticum* L.. Moreover, *Ae. tauschii* showed more close relationship with *Triticum* L. and barley and had more close relationship with barley and far distance with rice, potato, pea and *Arabidopsis*. These results would contribute to the deep understanding of functional aspects and evolution of *waxy* gene and the improving of starch quality in common wheat.

Key words: *Aegilops tauschii*, waxy, wheat, phylogenetic tree

INTRODUCTION

Aegilops tauschii (DD, 2n = 14) is considered as not only the ancestor of D genome of hexaploid wheat (McFadden and Sears, 1946; Kihara, 1944), but also the first gene pool for the improving of common wheat (Kimber and Feldman, 1987). This wild diploid species contains some excellent potential traits and easily transfer these characters into tetraploid and hexaploid wheat (Dudnikov, 1998). The original location of this species is the west of Asian. The waxy gene on the D genome had been cloned in common wheat (Yan *et al*., 2000; Murai *et al*., 1999). Granule-bound starch synthase (GBSSI), also called WAXY protein (Echt and Schwartz, 1981), is a key enzyme in the process of amylose synthesis and play an important role in the starch synthesis of cereal (Nakamura *et al*., 1993). The molecular weights of three subunits of GBSSI protein are from 59-60 kDa. They are encoded by three loci of *waxy* gene, *Waxy-A1* (*Wx-A1*, 2,781 bp), *Waxy-B1* (*Wx-B1*, 2,794 bp) and *Waxy-D1* (*Wx-D1*, 2, 862 bp), each consisting of 11 exons and 10 introns, located on chromosome 7AS, 4AL and 7DS, respectively (Nakamura *et al*., 1993; Murai *et al*., 1999). The *waxy* gene had been cloned from barely (Rohde *et al*., 1988), rice (Hirano and Sano, 1991; Wang *et al*., 1990), maize (Shure *et al*., 1983; Klosgen *et al*., 1986), sorghum (Hamblin *et al*., 2007), pea (Dry *et al*., 1992), potato (Van-Der-Leij *et al*., 1991), common wheat (Mason-Gamer *et al*.,1998; Murai *et al*., 1999).

The variation of amylose content is associated with the status of the *waxy* gene expression (Yamamori *et al*., 1994; Urbano *et al*., 2002). The molecular mechanism of variation was also continuously focused for *waxy* gene researches. There are a 19 bp deletion at an exon-intron junction and a deletion including the entire coding region of the *Wx-B1* gene and a 588 bp deletion in the C-terminal region at *Wx-D1* locus in Japanese bread wheat Kanto 107 and Chinese cultivar Baihuo (Vrinten and Nakamura, 2000). A 173 bp insertion of waxy gene also led to a new null *Wx-A1* allele in six Turkish cultivars (Saito *et al*., 2004). Many SNPs (single nucleotide polymorphisms) and/or InDels (insertions or deletions) were detected in the introns of *waxy* gene (Huang and Brule-Babel, 2010). Consequently, although *waxy* is usually considered as conserved gene, the variation of sequences, including exons and introns, could be detected between *Triticum* and *Aegilops* (Mason-Gamer *et al*., 1998).

The variations of *waxy* gene in wild diploid species, *T. monococcum* L. ssp. *monococcum* and *T.urartu* Thum. Ex Gandil. which was considered as the A genome origin of the all polyploidy wheats, have been well characterized

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at the molecular level (Murai *et al*., 1999; Yan *et al*., 2000; Liu *et al*., 2009). But the less information was reported about the performance of *waxy* gene in *Ae. tauschii* which was considered as the ancestor of D genome of hexaploid wheat. It was blocking to understanding deeply the function and evolution of *waxy* gene in *Triticum* L. and exploiting the new genetic resources.

In present study, the variation of sequence of *waxy* gene was investigated in *Ae. tauschii* which potentially could be an important source for improving starch quality and also provide valuable information on the phylogeny of *waxy* gene in different species.

MATERIALS AND METHODS

A total of nine accessions of *Ae. tauschii*, were used in this study (Table 1). The materials were kindly provided by Dr. Harold Bockelaman, USDA-ARS, National Small Grains Collection.

All seeds were germinated under the dark at 23°C for 1 week, young leaves were harvested and crushed into powder with the liquid nitrogen and the genomic DNA extracted by the CTAB method (Wang *et al*., 2008). A pair of primers which were designed by the conserve sequence region of *waxy* genes (Yan *et al*., 2000), was used as the cloning primers: the forward primer: 5'-TTGCTGCAGGTAGCCACAC-3' and the reverse primer: 5'-CTC AAGTGCTGCCTGGCAGAGAA-3'. The amplified region includes the first, second and the third exons and introns and also includes the partial fourth exon of *waxy* gene. PCR was performed in a 50 μL volume, containing 1.5 UTaq plus DNA polymerase, 100 ng of each template DNA, 5 μL PCR buffer (supplied with Taq plus DNApolymerase), 1.5 mM $MgCl_2$, 100 mM of each dNTP and 150 ng each primer. The reactions were conducted in a PTC-220 (MJ Research, USA) using the following program: 95°C for 3 min, followed by 35 cycles at 94°C for 1 min, at 60°C for 1 min and at 72°C for 2 min and a final extension step of 72°C for 10 min. The PCR products were separated on 1.5% agarose gels. The expected fragments were recovered and cloned into pMD18-T vector (TaKaRa), then transformed into the competent *E. coli* cells (DH5α). Positive clone of each accession was sequenced by commercial company (Invitrogen) in two directions.

Table 1: The information of the cloning *waxy* genes in this study

Name of sequence	Accessions[a]	Species	Origin
D22	PI603222	*Ae. taushii* (DD)	Former soviet union
D23	PI574469	*Ae. taushii* (DD)	India
D28	PI511365	*Ae. taushii* (DD)	Baluchistan,Pakistan
D36	PI603223	*Ae. taushii* (DD)	Mazandaran, Iran
D37	PI508262	*Ae. taushii* (DD)	Xinjiang, China
D38	PI486272	*Ae. taushii* (DD)	Van, Turkey
D41	Clae20	*Ae. taushii* (DD)	Mazandaran, Iran
D42	Clae13	*Ae. taushii* (DD)	Mazandaran, Iran
D43	PI486268	*Ae. taushii* (DD)	Hakkari, Turkey

a: No. of accessions came from USDA-ARS, National Small Grains Collection (http://www.ars-grin.gov/npgs/index.html)

Data analysis: The obtained sequences were compared with known *waxy* sequences using BLAST (http://www.ncbi.nlm.nih.gov/BLAST). Sequences were manually inspected by BioEdit ver 7.0.9 (Hall, 1999). Sequence alignment was completed by ClustalX (Larkin *et al*., 2007) and DNAMAN 5.2.2 (http: //www. lynnon.com). Neighbour-joining trees was constructed based on the alignment of all known sequences, including six sequences from barley (Rohde *et al*., 1988), rice (Wang *et al*., 1990), Pea (Dry *et al*., 1992), potato (Van-Der-Leij *et al*., 1991) and *Arabidopsis* (NM103023), respectively, using Kimura 2-parameter model in MEGA4.0 (Saitou and Nei, 1987; Kimura 1980; Tamura *et al*., 2007).

RESULTS

Variation of sequences of waxy gene: A total of nice sequences with 925 bp were obtained after cloning from all the materials. Blast in NCBI confirmed all the sequences belonged to the partial *waxy* gene, suggested that the *waxy* gene was successfully cloned from *Ae. tauschii* (Table 1). The average value of similarity between the sequences was 99.12% and showed 98.85% and 98.76% similar with *Wx-TtD* (AF110375) and *Wx-D1a* (AB019624). The substitution and indels of nuclear bases were detected at thirty-five base sites in the whole sequences. The substitution types included A-G, T-C, A-C, G-C and G-T. The indels types mainly focused on one or two bases deletion.

Based on the variation of bases, all the sequences could be clustered into four groups. There was difference only at one SNP site between sequences from the same group, likes group 1 (D23 and D43), group 2 (D37 and D38) and group 3 (D36 and D41), indicated that these sequences have the higher similarity each other. Furthermore, sequences from group 4 showed the highest variation, compared with other groups. Group 3 consisted of the sequences from accession PI603223 and clase 20 which came from Mazandaran, Iran. Interestingly, the accession clase 13 which also came from the Mazandaran, Iran, was clustered into group 4 (D22, D28 and D42). These results indicated although the accessions came from the same region, the variation of sequence of *waxy* gene could be observed in the same species, *Ae. stauschii.*

Variation of amino acid residue of *waxy* gene: All the sequences included three introns and four exons. The

lengths of the first, second, third and fourth exon were 321, 81, 99 and 102 bp while the first, second and third intron were 90, 96 and 104 bp, respectively. Twelve variable nucleotide sites were observed in the region of exons, but only six sites lead the change of amino acid residue among the sequences *waxy* gene. Two types of amino acid residues was detected at 5 bp (Ala\Val), 25 bp (Leu\Pro), 63 bp (Val\Phe), 154 bp (Lys\Thr), 184 bp (Arg\Gln) and 213 bp (Thr\Ala), respectively (Table 2). Interestingly, most of variable amino acid residues located on the transit peptide of WAXY protein (Fig. 1). The change of nucleotide mainly happened on the first and third base in the codon.

Table 2: The distribution of variable amino acid residues of *waxy* gene in *Ae. tauschii*

Location of sequence (bp)	Variation of nucleotide	Variation of amino acid
5	GCG\GTG	Ala\Val
25	TCG\CCG	Leu\Pro
63	GGG\GGT	Val\Phe
154	AGC\CGC	Lys\Thr
184	GGC\AGC	Arg\Gln
213	CCA\CCG	Thr\Ala

Phylogenetic analysis: In order to estimate the relationship of *waxy* gene between *Ae. tauschii* and other species, the phylogenetic tree was constructed by Neighbor-joining method (Fig. 2). A total of 21 sequences were used in this tree, including sequences from barely, rice, potato, pea and *Arabidopsis*, respectively. All the sequences could be clustered to six groups. All sequences from *Ae. tauschii* and sequence AF110375 from *Ae. tauschii* and sequence AF163319 from *Wx-D1* locus of common wheat, were clustered into one group, suggested that *wax*y gene from D genome showed higher conserved characterization. Interestingly, sequence D28, D39 and D22 which were collected from different location, were clustered closely. Sequence of *waxy* gene from potato and rice were clustered into one group while sequences from *Arabidopsis* and pea were clustered into one group. Sequence from barely was more similar to sequence of waxy gene from *Triticum* L. and *Aegilops* species. Sequence from *Ae. speltoides* (AF110374), *T. monococcum* (AF110373), common wheat (EU719660 and AB019622), suggested that *waxy* gene at *Wx-B1*

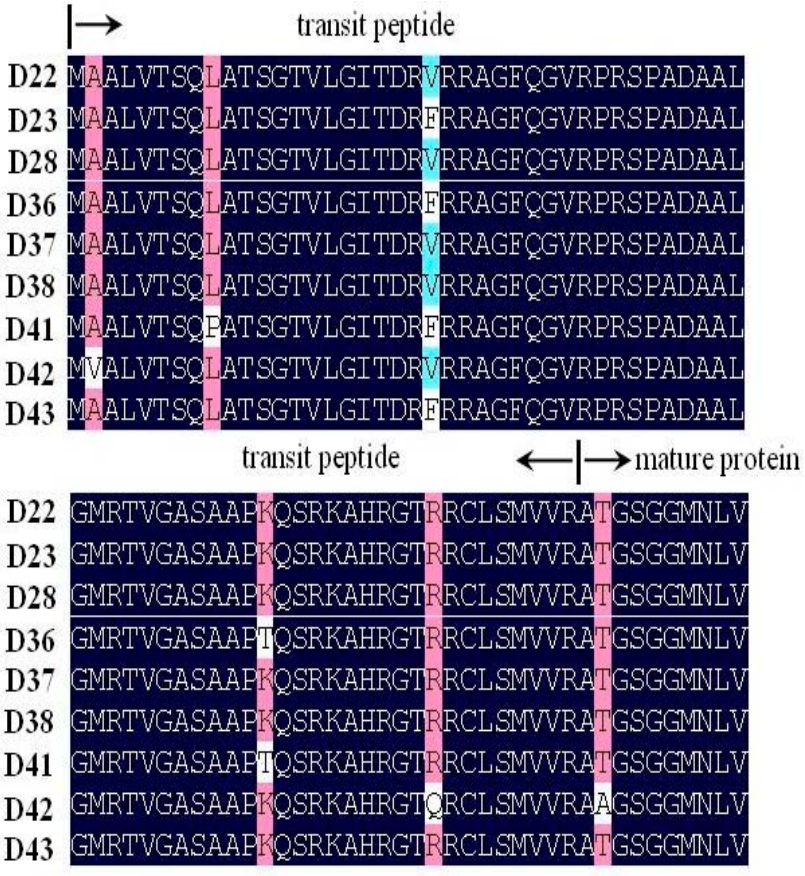

Fig. 1: Partial alignment of amino acids residues of *waxy* gene. Conservative positions are indicated by dark shading. Names of sequences were referenced in Table 1

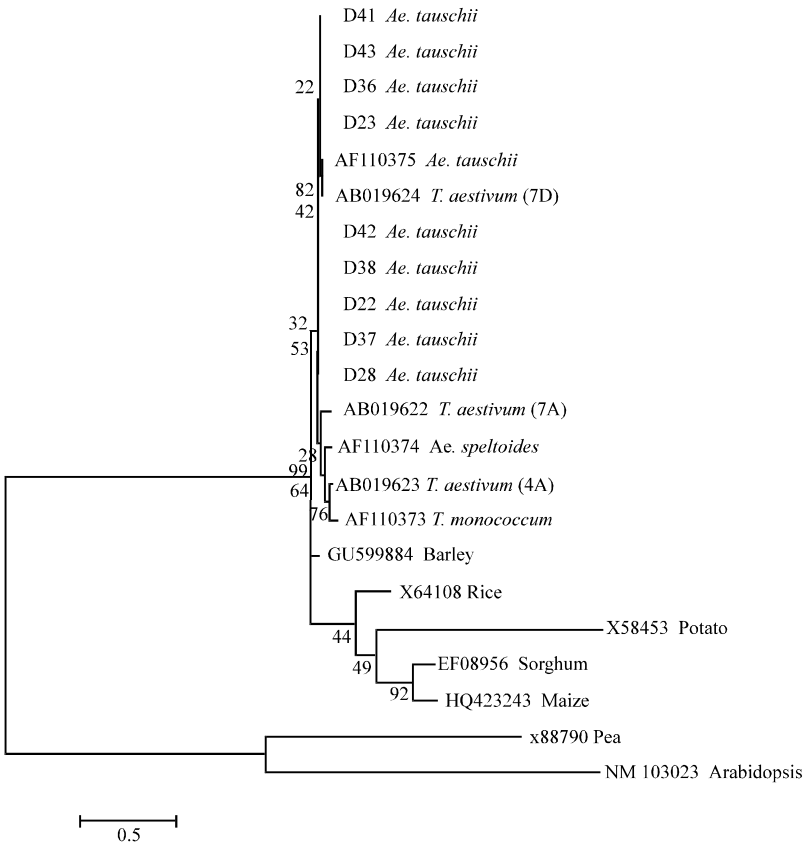

Fig. 2: Neighbour-joining tree based on sequences of waxy gene was constructed by using Kimura 2-parameter model in MEGA 4.0. Bootstrap tree was obtained by 1, 500 repeat calculation

locus showed more diversity in *Triticum* L. and *Aegilops* species. Interestingly, sequence EU719608 (*Wx-1Aa*) was clearly distinguished with other sequences. Otherwise, the sequence of waxy gene from *Ae. tauschii* had the close relationship with the sequence at *Wx-D1* locus and far relationship with the sequence at *Wx-A1* and *Wx-B*1 from *Triticu*m L.

DISCUSSION

Waxy gene encodes from 59-60 kDa protein and is a key regulate gene of the synthesizing process of amylose which is one of main components of seed endosperm (Yamamori *et al*., 1994). Alternatively, *Ae. tauschii*, the donor of D genome in common wheat, is one of the most important gene resources for the improving of common wheat, as it is easy to transform gene to common wheat by directly cross (Kimber and Feldman, 1987).Whereas, exploiting allele of waxy gene in *Ae. tauschii* could provide more useful genetic resources and understand the function and evolution of *waxy* gene. In the previous studies, null mutation at *Wx-D1* locus (*Wx-D1b*) could lead to the distinct decreasing of amylose content in *Ae. tauschii* (Ainsworth *et al*., 1993). Yan *et al*. (2000) obtained the sequences of waxy gene from diploid species containing genome A, B and D (*Wx-TmA*, *Wx-TsB* and *Wx-TtD*), 2803, 2795 and 2862 bp and Murai *et al*. (1999) cloned the sequence of *Wx-D1* locus (2862 bp), containing 11 exons and 10 introns. McLauchlan *et al*. (2001) developed five PCR-molecular markers based on the alignment of sequences from emmer wheat (2n = 14, AA), *Ae. tauschii* (2n = 14, DD), barely, maize, rice, potato. Briney *et al*. (1998) compared the gDNA sequences from rice and barely and sequences of mRNA from wheat, barley and rice and design the STS-PCR primers for *Wx-B1* locus. Fujita *et al*. (1996) thought that there was conserve at the N terminal of *Wx-D1* subunit between common wheat (AABBDD) and *Ae. tauschii*. Although *waxy* gene are considered as high conserved sequences in *Triticum* L., in the present study, the sequences of *waxy* gene from *Ae. tauschii* still showed variation of nucleotide bases, even changed the amino acid residues at six sites in the expressing region. The effect of variation of amino acid residues, especially on transit peptide region, for the

function of *waxy* gene, or amylose content and starch quality, should be further investigated. It could help to exploit unique gene resources from *Ae. tauschii* for the improving of common wheat.

Although, the alignment of *waxy* gene from diploid wheats showed very less variation, the phylogenetic tree can distinguished the sequences of *T. urartu*, *T. boeoticum* and *T. monococcum* (Liu *et al*., 2009). In the present study, the sequences of *waxy* gene from *Ae. tauschii* were clustered closely with sequences at *Wx-D1* locus in common wheat and were far with the sequences at *Wx-A1* and *Wx-B1* loci in *Triticum* L.. Thereby, although amino acid sequences and structural motifs are highly conserved, the sequences of waxy gene can showed the specificity of genomes not only at *Wx-A1*, *Wx-B1* and *Wx-D1* loci of hexaploid wheat, but also in diploid wheats which is usually considered as the ancestor of A genome and *Ae. speltoides* which is usually considered as the ancestor of B genome and *Ae. tauschii* which is usually considered as the donor of D genome. The results showed that the variation of exon was enough to provide extensive resolution within very closely related species. It is also similar to the results reported by Mason-Gamer *et al*. (1998). Many SNPs and/or InDels (insertions or deletions) have been observed in the regions of intron (Huang and Brule-Babel, 2010), also provide an approach to analysis the genetic diversity and exploit the molecular markers of *waxy* gene. The new variant sites of amino acid residuces of *waxy* gene, compared with the previous report (Fujita *et al*., 1996), suggested that exploiting the variation of waxy gene in new genetic resources is necessary to understanding the genetic diversity and function of waxy gene.

CONCLUSION

In this study, nine *waxy* genes were cloned from the donor of D genome of common wheat, *Aegilops tauschii* (DD), by special primer PCR cloning. These sequences were not only higher similar to the gene of *Wx-D1* locus in common wheat, but also showed the different variation each other, although in the same species. Out of 35 variable sites of nucleotide in the whole sequences, twelve variable sites located on the region of exon and made six amino acid residues change. Most of variable sites located on transit peptide. Based on the neighbour-joining method, phylogenetic tree also showed that the sequences of *waxy* gene from *Ae. tauschii* were clustered closely with the sequences of *Wx-D1* locus and far with the sequence of *Wx-A1* and *Waxy-B*1 loci in *Triticum* L. Moreover, *Ae. tauschii* showed more close relationship with *Triticum* L. and barley and had more close relationship with barley and far distance with rice, potato, pea and *Arabidopsis*. The present results would contribute to the deep understanding of functional aspects and evolution of *waxy* gene in *Triticum* L. In the future, the further research for the quality effect of these genes could help to improving starch quality of common wheat.

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REFERENCES

Ainsworth, C.C., J. Clark and J. Balsdon, 1993. Expression, organization and structure of the genes encoding the waxy protein (granule-bound starch synthase) in wheat. Plant Mol. Biol., 22: 67-82.

Briney, A., R. Wilson, R.H. Potter, I. Barclay, G. Crosbie, R. Appels and M.G.K. Jones, 1998. A PCR-based marker for selection of starch and potential noodle quality in wheat. Mol. Breeding, 4: 427-433.

Dry, I., A. Smith, A. Edwards, M. Bhattacharyya, P. Dunn and C. Martin, 1992. Characterization of cDNAs encoding two isoforms of granule-bound starch synthase with differential expression in developing storage organs of pea and potato. Plant J., 2: 193-202.

Dudnikov, A.J., 1998. Allozyme variation in Transcaucasian populations of *Aegilops squarrosa*. Heredity, 80: 248-258.

Echt, C.S. and D. Schwartz, 1981. Evidence for the inclusion of controlling elements within the structural gene at the Waxy locus in maize. Genetics, 99: 275-284.

Fujita, N., A. Wadnao, S. Kozaki, K. Takaoka, S. Okabe and T. Taira, 1996. Comparison of the primary structure of waxy proteins (granule-bound starch synthase) between polyploid wheats and related diploid species. Biochem. Genet., 34: 403-413.

Hall, T.A., 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. Nucleic Acid Symp. Ser., 41: 95-98.

Hamblin, M.T., M.G.S. Fernandez, M.R. Tuinstra, W.L. Rooney and S. Kresovich, 2007. Sequence variation at candidate loci in the starch metabolism pathway in Sorghum bicolor: Prospects for LD mapping. Crop Sci., 47: 125-134.

Hirano, H. and Y. Sano, 1991. Molecular characterization of the waxy locus of rice (*Oryza sativa*). Plant Cell Physiol., 32: 989-997.

Huang, X.Q. and A. Brule-Babel, B.M.C. Res. N. Development of genome specific primers for homoeologous genes in allopolyploid species: The waxy and starch synthase II genes in allo-hexaploid wheat (*Triticum aestivum* L.) as examples. BMC Res. Notes, Vol. 3. 10.1186/1756-0500-3-140

Kihara, H., 1944. Discovery of the DD analyzer, one of the ancestors of *Triticum vulgare* (in Japanese). Agric. Hortic., 19: 13-14.

Kimber, G. and M. Feldman, 1987. Wild Wheat: An Introduction. College of Agriculture, University of Missouri-Columbia, USA., Pages: 146.

Kimura, M., 1980. A simple method for estimating evolutionary rate of base substitution through comparative studies of nucleotide sequences. J. Mol. Evol., 16: 111-120.

Klosgen, R.B., A. Gierl, Z.S. Schwarz-Sommer and H. Saedler, 1986. Molecular analysis of the waxy locus of *Zea mays*. Mol. Gen. Genet., 203: 237-244.

Larkin, M.A., G. Blackshields, N.P. Brown, R. Chenna and P.A. McGettigan *et al*., 2007. Clustal W and clustal X version 2.0. Bioinformatics, 23: 2947-2948.

Liu, Y.X., W. Li, Y.M. Wei, G.Y. Chen and Y.L. Zheng, 2009. Molecular characterization of the *Waxy* gene in einkorn wheat. J. Plant Sciences, 4: 114-121.

Mason-Gamer, R.J., R.J. Weil and E.A. Kellogg, 1998. Granulebound starch synthase: Structure, function and phylogenetic utility. Mol. Biol. Evol., 15: 1658-1673.

McFadden, E.S. and E.R. Sears, 1946. The origin of *Triticum spelta* and its free threshing hexaploid relatives. J. Hered, 37: 81-89.

McLauchlan, A., F.C. Ogbonnaya, B. Hollingsworth, M. Carter and K.R. Gale *et al*., 2001. Development of robust PCR-based DNA markers for each homoeoallele of granule-bound starch synthase and their application in wheat breeding programs. Aust. J. Agric. Res., 52: 1409-1416.

Murai, J., T. Taira and D. Ohta, 1999. Isolation and characterization of the three waxy genes encoding the granule-bound starch synthase in hexaploid wheat. Genet, 234: 71-79.

Nakamura, T., M. Yamamori, H. Hirano and S. Hidaka, 1993. Identification of three wX proteins in wheat (*Triticum aestivum* L.). Biochem. Genet, 31: 75-86.

Rohde, W., D. Becker and F. Salamini, 1988. Structural analysis of the waxy locus from Hordeum vulgare. Nucleic Acids Res., 16: 7185-7186.

Saito, M., M. Konda, P. Vrinten, K. Nakamura and T. Nakamura, 2004. Molecular comparison of waxy null alleles in common wheat and identification of a unique null allele. Theor. Applied Genet., 108: 1205-1211.

Saitou, N. and M. Nei, 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol., 4: 406-425.

Shure, M.S., A. Wessle and N. Federoff, 1983. Molecular identification and isolation of the waxy locus in maize. Cell, 35: 225-233.

Tamura, K., J. Dudley, M. Nei and S. Kumar, 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol., 24: 1596-1599.

Urbano, M., B. Margiotta, G. Colaprico and D. Lafiandra, 2002. Waxy proteins in diploid, tetraploid and hexaploid wheat. Plant Breeding, 121: 465-469.

Van-Der-Leij, F.R., R.G.F. Visser, A.S. Ponstein, E. Jacobsen and W.J. Feenstra, 1991. Sequence of structural gene for granulebound starch synthase of potato (*Solanum tuberosum* L.). Mol. Gen. Genet., 228: 240-248.

Vrinten, P.L. and T. Nakamura, 2000. Wheat granule-bound starch synthase I and II are encoded by separate genes that are expressed in different tissues. Plant Physiol., 122: 255-264.

Wang, J.R., Y.M. Wei, X.Y. Long, Z.H. Yan, E. Nevo, B.R. Baum and Y.L. Zheng, 2008. Molecular evolution of dimeric α-amylase inhibitor genes in wild emmer wheat and its ecological association. BMC Evol. Biol., Vol. 8 10.1186/1471-2148-8-91

Wang, Z., Z. Wu, Y. Xing, F. Zheng, X. Guo, W. Zhang and M. Hong, 1990. Nucleotide sequence of rice waxy gene. Nucleic Acids Res., 18: 5898-5898.

Yamamori, M., T. Nakamura, T.R. Endo and T. Nagamine, 1994. Waxy protein deficiency and chromosomal location of coding genes in common wheat. Theor. Applied Genet., 89: 179-184.

Yan, L.L., M. Bhave, R. Fairclough, C. Konik, S. Rahman and R. Appels, 2000. The genes encoding granule-bound starch synthases at the waxy loci of the A, B and D progenitors of common wheat. Genome, 43: 264-272.