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Genetic Diversity of Null Alleles of *Waxy* Gene in *Triticum* L.

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ABSTRACT

In order to exploit new genetic resources for the improving of starch quality of common wheat, the genetic diversity of null alleles of Granule-bound starch synthase I (*waxy* gene) was investigated by special PCR molecular markers in *Triticum* L. The results indicated that there was relative abundant genetic diversity of *waxy* alleles in all accessions. Accession AS2347, AS2356, AS2317 and AS2308 with null allele at *Waxy-B1* locus and AS2310 and AS2335 with null alleles at *Waxy-A1* and *Waxy-B1*, were observed in 81 landraces of *Triticum turdigum* L. from China. In 53 landraces of *Triticum aestivum* L. from Sichuan, China, eight accessions at *Waxy-A1*, *Waxy-B1* and *Waxy-D1* loci and accession AS1668 at *Waxy-D1*, were observed null alleles. In 29 *Triticum macha*, Accession PI361862 and PI572911 at three *Waxy* loci, PI572913 at *Waxy-B1* and *Waxy-D1*, PI572910 at *Waxy-A1* and *Waxy-D1*, PI 290507 at *Waxy-B1* and PI572906 at *Waxy-D1*, respectively, were observed null alleles. Seven accessions with null alleles at *Waxy-B1* locus was observed in 28 *Triticum sphaerococcum*. Specially, the accessions of two regions, Anyue in Sichuan, China and Georgia, had the high frequency of the mutations with null alleles of *waxy* gene. Landraces of *Triticum aestivum* L. with the high frequency of *waxy* wheat, could be considered as a unique genetic resource for improving of waxy wheat. These result suggested that the special molecular marker could be used reliably in evaluation of genetic resources and these mutations also could be directly used in the improving of common wheat.

Key words: *Waxy* gene, null allele, landraces, *Triticum* L., wheat.

INTRODUCTION

Starch, accounts for 80% of wheat grain endosperm, is one of the most important components of cereal production (Hurkman *et al*., 2003). Starch is composed of amylose (20-30%) and amylopectin (70-80%) in cereal. The processing of starch synthesis includes three stages: the produce of ADPG, synthesis of amylose, synthesis of amylopectin (Martin and Smith, 1995). Granule-bound starch synthase I (GBSSI or WAXY protein) play a key role in synthesis of amylose in endosperm tissues of cereals (Shure *et al*., 1983). In wheat, GBSSI proteins were encoded by three genes: *Wx-A1*, *Wx-B1* and *Wx-D1*, located on chromosome 7AS, 4AL and 7DS, respectively (Nakarnura, 1993). Either lack of GBSSI activity or absence of GBSSI protein at three loci will affect the quantitative and quality of amylose and further affect the quality of wheat product.

Especially, the null allele of *Wx-B1* has the most effect for amylase form of wheat, following *Wx-D1* and *Wx-A1* (Miura and Sugawara, 1996). There are eight types for null alleles of three *Wx* loci in wheat: the first type with all three alleles has 20-25% amylose content and the second to the seventh type with any one or two null alleles decreased the 1.7-5.0% amylose and the eighth type with three null alleles at three *Wx* loci lead to very less amylose content in wheat endosperm (Nakamura *et al*., 2002). Common wheat with partial null alleles at *Wx* loci, which lacks one or two WAXY proteins, have been identified widely, but the mutation with three null alleles at *Wx* loci in natural genetic resources was very less mentioned (Yamamori *et al*., 1998; Wang *et al*., 1999). Lacking all three WAXY proteins from three Wx loci could produce the waxy wheat (Nakamura *et al*., 1995). Through crossing the materials of Kanto 107 (null allele at *Wx-A1* and *Wx-B1* loci) and Baihuomiao (null allele at *Wx-D1*), the mutation has been generated with the entire null allele at three *Waxy* loci. Amylose content of this waxy wheat is 0.6-0.7% (Nakamura *et al*., 1993a, b, 1995). Otherwise, the starch characters and potential end use of waxy wheat have been reported (Lee *et al*., 2001; Bhattacharya *et al*., 2002).

The types of waxy proteins could be identified by 1D-SDS-PAGE (Zhao and Sharp, 1996) but the manipulation is complex and the endosperm of wheat is also damaged in the process of prepare sample. Molecular markers were also developed following know the sequence information of waxy gene in wheat (Ren *et al*., 2004). Using molecular marker could not only overcome the limitation of protein electrophoresis, but also detect easily in any stages of wheat development. Molecular assisted selection (MAS) of *waxy* gene can accelerate the improving of way wheat and wheat production. Three molecular marker of *waxy* gene had been applied in the breeding of waxy wheat in Austria (McLauchlan *et al*., 2001) and an improved method by one simple Polymerase Chain Reaction (PCR) to identify three *Wx* loci was also reported (Nakamura *et al*., 2002).

In the present study, the allele component of *Waxy* gene was investigated in landraces of *Triticum aestivum* L. (AABBDD, 2n = 42) from Sichuan, China, landraces of *Triticum turgidum* L. (AABB, 2n = 28) from China, *Triticum macha* Dekapr. et Menabde(AABBDD, 2n = 42) and *Triticum sphaerococcum* Perc. (AABBDD, 2n = 42) by special molecular marker. The results could help us to understanding the diversity of waxy gene in these genetic resources and exploiting new genetic materials to use in breeding of common wheat.

MATERIALS AND METHODS

Materials: A total of 81 accessions of *T. turgidum* L. landraces from China, 53 accession of *T. aestivum* L. landraces from Sichuan, China, 29 accessions of *T. macha* and 28 accessions of *T. sphaerococcum*, were used in this study (Table 1). The materials with AS were provided by the Germplasm Laboratory of Triticeae Research Institute, Sichuan Agriculture University and the other materials were kindly provided by Dr. Harold Bockelaman, USDA-ARS, National Small Grains Collection.

Methods: All seeds were germinated under the dark at 23 for 1 week, young leaves were harvested and crushed into powder with the aid of liquid nitrogen and the genomic DNA extracted by the CTAB method (Wang *et al*., 2008). A pair of primers, derived from the exon 4/6 region of the GBSSI gene (McLauchlan *et al*., 2001), was used as the special molecular markers. The forward primer: 5'-AAG AGC AAC TAC CAG T-3' is located in exon 5 (position 1464-1481) and the reverse primer:

Table 1: Materials used in this study

Species	Chromosome	Accession
T. turgidum L.	AABB	AS2235, AS2236, AS2239, AS2240, AS2325, AS2249, AS2250, AS2251, AS2253, AS2254, AS2258, AS2259, AS2277, AS2282, AS2284, AS2285, AS2291, AS2293, AS2299, AS2300, AS2301, AS2302, AS2304, AS2305, AS2308, AS2309, AS2310, AS2311, AS2312, AS2313, AS2314, AS2315, AS2317, AS2321, AS2322, AS2328, AS2333, AS2335, AS2336, AS2342, AS2343, AS2347, AS2351, AS2352, AS2353, AS2354, AS2355, AS2356, AS2357, AS2370, AS2375, AS2378, AS2379, AS2380, AS2381, AS2241, AS2243, AS2273, AS2283, AS2296, AS2292, AS2290, AS2294, AS2295, AS2297, AS2298, AS2303, AS2306, AS2307, AS2318, AS2320, AS2324, AS2329, AS2327, AS2341, AS2368, AS2372, AS2373, AS2376, AS2374, AS2382.
T. aestivum L.	AABBDD	AS1589, AS1647, AS1657, AS1623, AS1588, AS1587, AS1648, AS1649, AS1676, AS1672, AS1652, AS1577, AS1569, AS1659, AS1579, AS1582, AS1572, AS1625, AS1563, AS1699, AS1591, AS1670, AS1592, AS1598, AS1655, AS1590, AS1556, AS1573, AS1574, AS1593, AS1698, AS1646, AS1641, AS1635, AS1600, AS1702, AS1701, AS1700, AS1596, AS1660, AS1661, AS1663, AS1664, AS1667, AS1668, AS1669, AS1697, AS1597, AS1673, AS1671, AS1627, AS1558, AS1561.
T. macha	AABBDD	PI272554, PI272555, PI278660, PI290507, PI352466, PI355508, PI355509, I355510,PI355511, PI355512, PI355513, PI355514, PI361862, PI428146, PI428148, PI428177, PI428178, PI428179, PI542466, PI572905, PI572906, PI572907, PI572909, PI572910, PI572911, PI572912, PI572913, PI611470, PI140191.
T. sphaerococcum	AABBDD	PI324492, PI282451, AS348, PI277142, PI42013, PI42014, PI330556, AS347, PI168685, PI352498, PI277165, PI40944, PI337997, PI272581, PI277141, PI323439, PI70711, CItr17737, PI40941, PI40943, PI182118, PI115818, PI282452, PI191301, PI277164, CItr8610, PI272580, PI352499.

5'-TCG TAC CC G TCG ATG AAG TCG A-3' is located in exon 6(1508-1530) (Yan *et al*., 2000). PCR was performed in a 50 μL volume, containing 1.5 U*Taq* plus DNA polymerase, 100 ng of each template DNA, 5μL 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: 94°C for 3 min, followed by 12 cycles at 94°C for 1 min, at 64°C for 1 min and at 72°C for 30 sec and followed by 34 cycles at 94°C 1 min, 58°C 1 min and 72°C 30 sec and a final extension step of 72°C for 5 min. The PCR products were separated on 3% agarose gel.

RESULTS

Allele variation of *Waxy* gene in landraces of *T. turgidum* L.: Two bands could be amplified at *Wx-A1* and *Wx-B1* loci in the landraces of *T. turgidum* L. from China (Fig. 1a). The band with bigger size came from *Wx-A1* locus and the small one from *Wx-B1* locus. The different size of PCR product resulted from the different length between the fifth exon and the sixth exon of *waxy* gene. Both two bands were obtained in 75 accessions of all 81 accessions of tetraploid landraces. No band was obtained in accession AS2310 (Nanmai, Renshou) and AS2335 (Dabaimai, Anxi), indicated that there were null alleles at *Wx-A1* and *Wx-B1* loci in these two accessions. Four accessions (4.9%), AS2347 (Fushoumai, Jianhu), AS2356 (Fushoumai, Qishan), AS2317 (Zaozhuo, Batang) and AS2308 (Wugongnanmai, Suining), were identified as the mutation with null allele at *Wx-B1* locus. These results suggested that the frequency of mutations with null alleles at *Wx* loci was very lower in landraces of *T. turgidum* L. from China.

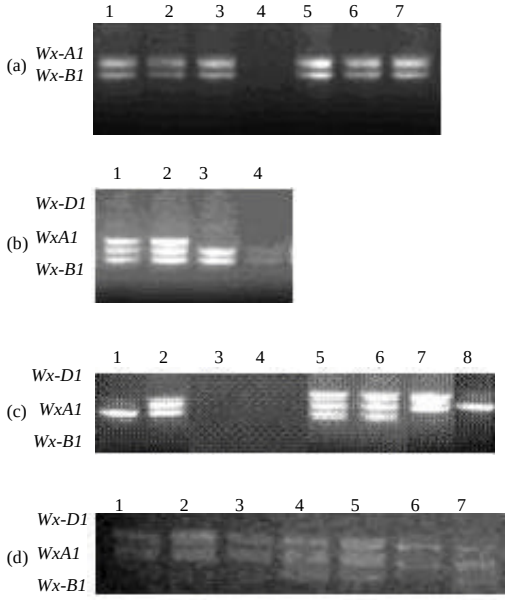

Fig. 1(a-d): PCR assays for detection of null alleles of the waxy genes in *Triticum* L. (a) Landraces of *T. turgidum* L., 1. AS2236, 2. AS2239, 3. AS2240, 4. AS2310, 5.AS2311, 6. AS2312, 7. AS2313. (b) Landraces of *T.* aestivum L., 1. AS1589, 2. AS1647, 3. AS1668, 4. AS1591. (c) *T. macha*, 1. PI572910, 2. PI572906, 3. PI361862, 4. PI572911, 5. PI272554, 6. PI272555, 7. PI290507, 8. PI572913. (d) *T. sphaerococcum*, 1. PI324492, 2. PI282451, 3. AS348, 4. PI277142, 5. PI42013, 6. PI42014, 7. PI330556

Allele variation of *Waxy* gene in landraces of *T. aestivum* L.: Three bands were identified at *Wx-A1*, *Wx-B1* and *Wx-D1* loci in *T. aestivum* L. from Sichuan, China (Fig.1b). The band with the biggest size was amplified from *Wx-D1* locus and the one with the second bigger size from *Wx-A1* locus and the least band from *Wx-A1* locus. No bands were amplified from eight accessions (15%) in 53 landraces of common wheat. These mutations with null alleles at three *Wx* loci included AS1591 (Hongxiaomai, Anyue), AS1670 (Shihonghuamai, Pengxian), AS1592 (Nverhong, Anyue), AS1598 (Dahongpao, Zizhong), AS1655 (Baixiaomai, Dazhu), AS1590 (Dongmai, Anyue), AS1556 (Tuomai, Bazhong) and AS1573 (Liulengmai, Changning). Lacking the band from *Wx-D1* locus was only observed in one accession, AS1668 (Youmangmai, Xinjing). Whole three bands were amplified from 43 accessions of landraces of common wheat. Three accessions from Anyue, Sichuan, China, were the mutations with null alleles at three *Wx* loci. The results suggested that there was abundant allele diversity of W*axy* gene in landraces of common wheat from Sichuan, China.

Allele variation of *Waxy* gene in *T. macha*: Three bands could be obtained from 23 accessions of *T. macha* (Fig. 1c). No band was amplified in two accessions, PI361862 (from Danmark) and PI572911 (from Georgia), indicated that they belonged to the mutations with null alleles at three *Wx* loci. Only one band was amplified in two accessions, PI572913 (from Georgia), which showed null alleles at *Wx-B1* and *Wx-D1* loci and PI572910 (from Georgia), which showed null alleles at *Wx-A1* and *Wx-D1* loci. Only one accession, PI290507 (from Hungary), showed the null allele at *Wx-B1* locus and one accession, PI572906 from Georgia, had the null allele at *Wx-D1* locus. Four out of six mutations with at least one null allele at three *Wx* loci came from Georgia, suggested that it was very important region for genetic resources of *Waxy* gene.

Allele variation of *Waxy* gene in *T. sphaerococcum*: Three bands were amplified in 21 accessions of *T. sphaerococcum* (Fig. 1d). Null allele at *Wx-B1* locus was identified in five accession from India, which including PI324492, PI282451, PI42014, PI352498 and PI282452 and two from Pakistan, which including PI40941 and PI40943. The result suggested that there was relative simple genetic diversity of allele of *Waxy* gene in *T. sphaerococcum*.

DISCUSSION

Very less information about natural genetic resources of common wheat with null alleles at three *Wx* loci was reported (Yamamori *et al*., 1998; Wang *et al*., 1999). The waxy wheat with three null alleles at *waxy* gene locus was produced by crossing between mutations with partial null alleles at different *Wx* loci (Nakamura *et al*., 1993a; b 1995). In recent years, waxy wheat is becoming more and more interesting for wheat breeders and industry, because of its unique quality characters (Lee *et al*., 2001; Bhattacharya *et al*., 2002). In addition, modification of cereal starch by various methods could provide different production suitable for food and various industrial applications (Kaur *et al*., 2012). *Waxy* gene plays an important role in synthesis of cereal starch and its activity directly affects the quantity and quality of starch. Thus, exploiting the new allele of *waxy* gene in genetic resources of common wheat is very important work for the improving of wheat and modification of starch. In the present study, the allele variation was investigated in the four important genetic resources of common wheat, landraces of *T. turgidum* L., landraces of *T. aestivum* L., *T. macha*, *T. sphaerococcum*. Landraces of *T. turgidum* L., which was grown widely in China before the 1950's, showed the unique performance about the gliadin, HMW-glutenin, protein content and microsatellite (Li *et al*., 2006a, b). Landraces of *T. aestivum* L. from Sichuan, China, also known as "Sichuan White Wheat", were widely planted and conserved by the peasants in the province ago (Liu *et al*., 2005). This population is considered to be a unique resource for genetics and breeding, because of broad hybrid compatibility with rye (Luo *et al*., 1992; Liu *et al*., 2003). *T. macha* and *T. sphaerococcum* were also displayed abundant genetic diversity and contain gene resource for quality and disease resist of wheat (Cao *et al*., 1998; Chen *et al*., 2012).

In present study, abundant genetic diversity of alleles of *Waxy* gene was observed in *Triticum* L. species. In tetraploid, *T. turgidum* L., two accessions with null alleles at both *Wx-A1* and *Wx-B1* loci, four accessions with null allele at *Wx-B1* locus, suggested that there was relative lower diversity of allele of *waxy* gene in this landraces. In hexaploid, *T. sphaerococcum* also showed the similar performance of alleles of *waxy* gene, as only null allele at *Wx-B1* locus was observed in seven accessions. Landraces of *T. aestivum* L. from Sichuan, China, showed the highest percentage (15%) of null alleles at three *Wx* loci. Including the mutations with null alleles at three, two and one *Wx* loci, were detected in *T. macha*, suggested that this hexaploid species had abundant genetic diversity of alleles of *waxy* gene. Actually, this species with high genetic diversity was also observed by RAPD (Cao *et al*., 1998). In addition, the mutations with different null allele at *Wx* loci, which were identified now, could be used in the improving of common wheat. The next steps for these mutations with null alleles of *waxy* gene should be focus on sequence cloning and gene expression to understanding the molecular mechanism of these null alleles.

Waxy mutations with null alleles occur spontaneously in cereals (Eriksson, 1970). Null alleles at the *Wx-A1* locus have been found in samples from Japan, Korea and Turkey, while null alleles at the *Wx-B1* locus are very common in the materials from Australia and India (Yamamori *et al*., 1994, Yamamori and Endo, 1996) and Italy (Boggini *et al*., 2001). Null alleles at the *Wx-D1* locus were more rarely and identified only in Baihuo (Yamamori *et al*., 1994) and one Italian landrace

(Boggini *et al*., 2001). Wang *et al*. (1999) also found one accessions with null allele at *Wx-D1*, six accessions with null alleles at *Wx-B1*, in 900 landraces and cultivars. These results suggested that the mutations with null alleles at *Wx* loci had the very lower frequency in genetic resources. In our results, all the accessions still showed the relative lower percentage of null alleles of *waxy* gene. In a total of 191 accessions, only two hexaploid accessions showed null alleles at both two *Wx* loci and 12 accessions showed null alleles at *Wx-B1* locus and two accessions showed null alleles at *Wx-D1* locus. But the mutations with null alleles at three *Wx* loci had higher percentage, especially in landraces of *T. aestivum* L. from Sichuan, China (15%), suggested that this landraces population is unique genetic resource for improving of waxy wheat. The reason that the landraces had high frequency of null alleles could be related with the special dietetic habit of people living in Sichuan.

The usual method to breeding waxy wheat is to crossing these materials with different null alleles of *Waxy* gene (Yamamori *et al*., 1995; Urbano *et al*., 2002). Now 10 accessions with null alleles at three *Wx* loci were identified from *Triticum* L. genetic resources. All the mutations could be directly used in breeding of common wheat and save the time of selection. Furthermore, the variation of *Waxy* gene was only investigated at DNA level. The performance of amylose and starch and the quality in these mutation materials should be further investigated. The influence of the variation of *Waxy* gene loci for the amylose and the relating quality characters also should be focused in the future.

Identifying null allele types of *waxy* gene of the materials is very important for the breeding of *waxy* wheat. The main methods for waxy gene expressing included measuring the amylose content, swelling power, RVA pasting characteristics, iodine staining and waxy protein electrophoresis (Nakamura *et al*., 1993a, b; Crosbie, 1991; McCormick *et al*., 1994; Sulaiman and Morrison, 1990). Zhao and Sharp (1996) improved the 1D-SDS-PAGE method and can easy identify three WAXY protein and suggested that this method could be used in wheat breeding. Wang *et al*. (1999, 2000) further improved SDS-PAGE method and could identify four WAXY subunits (*Wx-A1, Wx-E1, Wx-D1* and *Wx-B1*). Pan *et al*. (2000) also used the 2D-SDS-PAGE to distinguish WAXY protein. But SDS-PAGE method is difficult to apply widely in breeding of wheat, because the process of preparative of samples and manipulation process is very complex and lower effective. The molecular marker method based on PCR was applied widely after the cDNA sequence of Waxy gene reported. Shariflou and Sharp (1999) designed SSR primers to identify the *Wx-D1* locus and Briney *et al*. (1998) designed STS-PCR primers to identify the *Wx-B1* locus. Nakamura *et al*. (2002) confirm that the PCR markers could identify the *waxy* wheat with different null alleles at Wx loci and McLauchlan *et al*. (2001) reported that the special PCR markers, which could identify null alleles of waxy gene, were applied in Australian wheat breeding program. The present study also further confirmed that these molecular markers also could be used reliably in evaluation of genetic resources of *Triticum* L.

CONCLUSION

In this study, the mutations with null alleles at Wx loci were identified in landraces of *T. turgidum* L. from China, landraces of *T. aestivum* L. from Sichuan, China, *T. macha* and *T. sphaerococcum*, by one pair of specific primer. In all 191 accessions, only two hexaploid accessions showed null alleles at both two *Wx* loci and 12 accessions showed null alleles at *Wx-B1* locus and two accessions showed null alleles at *Wx-D1* locus. Interestingly, 10 hexaploid accessions with null alleles at three *Wx* loci and two tetraploid accessions with null alleles at two *Wx* loci, were observed. The mutations with null alleles of *Waxy* gene were easily obtained in the accessions from Anyue,

Sichuan, China and from Georgia. Landraces of *T. aestivum* L. from Sichuan, China, is unique genetic resource for improving of waxy wheat, because of the high frequency of *waxy* wheat. The reason could be related with the special dietetic habit of people living in this region. Further research could help to deeply understand the performance of these mutations about sequence variation, gene expression and quality. These result suggested that the special molecular markers could be used reliably in evaluation of genetic resources and these mutations also could be used directly in the improving of common wheat.

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