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Molecular comparison of *waxy* null alleles in common wheat and identification of a unique null allele

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Abstract PCR selection markers for the identification of null *waxy* alleles were used to screen for *waxy* mutations in 168 common wheat cultivars. In all cultivars where the Wx-B1 protein was absent, the *Wx-B1* allele was identical to the previously identified mutation carried by Kanto 107. Although most cultivars missing the Wx-A1 protein also carried the same *Wx-A1* mutation as found in Kanto 107, all of the Turkey *Wx-A1* mutants produced a different PCR fragment, implying the presence of a different mutation. Sequencing of this fragment indicated the mutation, which consisted of a 173-bp insertion in an exon, was in a different location than the previously identified *Wx-A1* mutation. An 8-bp duplication of the *Wx-A1* sequence flanked each end of the insertion, and an element with reverse complementary sequences was present at both ends of the insertion. These structures correspond with the features of class II transposable elements. Hence, the Turkey null *Wx-A1* mutation was likely caused by the movement of a transposon, and this spontaneous mutation appears to be present in a limited geographical area.

Introduction

Waxy protein, or granule-bound starch synthase I, is a key enzyme in amylose synthesis in starch granules. Hexaploid wheat produces three waxy proteins, while waxy wheat is deficient in waxy protein, and partial waxy wheat lacks one or two waxy proteins. Amylose content has a role in noodle quality, and partial waxy wheat is particularly good for Japanese Udon noodle making.

Spontaneous mutations occurring in *waxy* genes have been studied in a number of plant species. In maize, transposable elements or filler DNA between the deletion endpoints have been found in *waxy* mutants (Fedoroff et al. 1983; Wessler et al. 1990; Okagaki et al. 1991; Wessler 1991; Varagona et al. 1992; Purugganan and Wessler 1994; White et al. 1994; Marillonnet and Wessler 1997). A single base mutation leading to an altered splice site and a reduced level of mRNA and protein was found in a rice *waxy* mutant (Cai et al. 1998; Hirano et al. 1998; Isshiki et al. 1998). In barley, the deletion of part of the promoter and 5′ untranslated region (UTR) caused a reduction in *waxy* mRNA levels (Domon et al. 2002; Patron et al. 2002).

In wheat, spontaneous mutations occurring in the *Wx-A1* and *Wx-B1* genes of Kanto 107 (K107) and in the *Wx-D1* gene of Bai Huo have been characterized (Vrinten et al. 1999). A 23-bp deletion and 4-bp insertion of filler DNA occurred in the null *Wx-A1* allele, a 588-bp deletion and 12-bp insertion occurred in the null *Wx-D1* allele, and the entire coding region of the *Wx-B1* gene appeared to be deleted in the null *Wx-B1* allele. Recently, three PCR primer sets capable of distinguishing the mutation occurring in each *waxy* gene were designed (Nakamura et al. 2002). These primers are capable of distinguishing waxy and partial waxy cultivars. Originally, partial waxy wheat was identified by two-dimensional gel electrophoresis (2D-PAGE) (Nakamura et al. 1993a, 1993b; Yamamori et al. 1994). Yamamori et al. (1998) analyzed *waxy* alleles in world common wheat germplasm by 2D-PAGE; however, it has not been determined if those mutations have the same origin as those found in Bai Huo and K107. In this study, we report the results of PCR analysis of these cultivars and discuss the origins of *waxy* mutations.

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Materials and methods

Plant materials

Seed accessions from 168 cultivars of *Triticum aestivum* were obtained from the gene bank of the National Institute of Agrobiological Science (NIAS) (Tsukuba, Japan). Accessions were mainly selected from those analyzed by Yamamori et al. (1998), although several additional cultivars were included to maximize the geographical area covered by the sample.

SDS-PAGE and 2D-PAGE

Preparation of starch granules and separation of waxy proteins by low-bis acrylamide SDS-PAGE and 2D-PAGE were performed as described by Nakamura et al. (1993a, 1993b).

DNA extraction

Plant DNA was extracted from young leaf tissue using the Nucleon Phytopure Plant DNA Extraction kit (Amersham Biosciences, Little Chalfont, UK) according to the manufacturer's instructions.

Primers and PCR amplification

The primers and PCR conditions were the same as those used by Nakamura et al. (2002). The primer pairs used to identify each waxy mutation were AFC (5′-TCGTGTTCGTCGGCGCCGA-GATGG-3′) and AR2 (5′-CCGCGCTTGTAGCAGTGGAAGTA-CC-3′) for the *Wx-A1* allele, BDFL (5′-CTGGCCTGCTACCT-CAAGAGCAACT-3′) and BRD (5′-CTGACGTCCATGCCGT-TGACGA-3′) for the *Wx-B1* allele, and BDFL and DRSL (5′-CTGTTTCACCATGATCGCTCCCCTT-3′) for the *Wx-D1* allele. PCR was conducted using a Perkin Elmer 2400 or 9700 thermal cycler (Perkin Elmer, Foster City, Calif.).

Sequence analysis

PCR products were cloned into the vector pCR2.1 (Invitrogen, Carlsbad, Calif.). Inserts were sequenced using an ABI Prism 310 Genetic Analyzer (Perkin Elmer). The T3 and T7 primers, which direct sequencing from within the vector, were used as sequencing primers.

Results

Identification of null alleles by PCR

Common wheat cultivars can be categorized into eight types, including wild and waxy types, based on the presence or absence of the three waxy proteins (Table 1). More than 2,100 cultivars selected from the gene bank of NIAS have been classified in this manner (Yamamori et al. 1998; our unpublished data). To include cultivars originating from the widest possible geographical area, we used 133 classified and 35 unclassified cultivars in this study. The unclassified cultivars were analyzed by 2D-PAGE and six were identified as type 3, while the others were categorized as wild type (Table 2). Therefore, we believed there were 23 cultivars carrying null *Wx-A1* alleles and 48 cultivars carrying null *Wx-B1* alleles within the 168 cultivars.

Table 1 Presence (+) or absence (−) of waxy gene products

	Wx proteins		
	Wx-A1	Wx-B1	Wx-D1
Type 1	+	+	+
Type 2	−	+	+
Type 3	+	−	+
Type 4	+	+	−
Type 5	+	−	−
Type 6	−	+	−
Type 7	−	−	+
Type 8	−	−	−

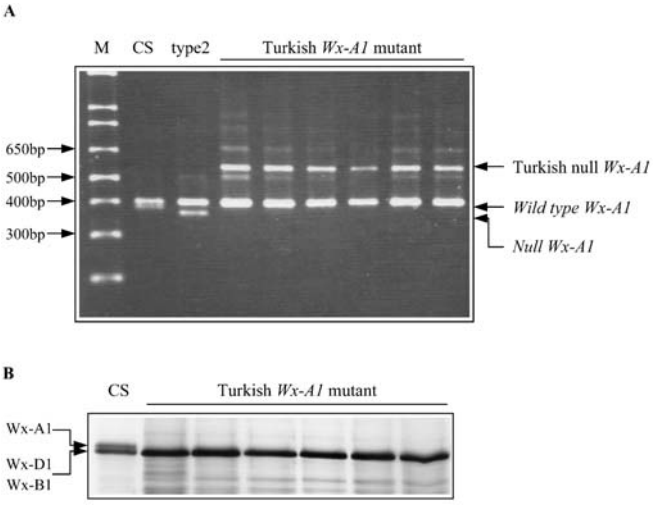


Fig. 1A, B *Wx-A1* mutations in Turkish cultivars. **A** PCR assays for detection of *Wx-A1* null alleles in Turkish cultivars. With the primer set AFC and AR2, a 370-bp fragment was amplified from the null *Wx-A1* allele (type 2) and a 389-bp fragment from the wild-type allele of Chinese Spring (CS). However, Turkish null *Wx-A1* donor plants produced a fragment about 200-bp longer than the fragment from wild type. **B** Wx proteins of CS and Turkish *Wx-A1* mutants analyzed by SDS-PAGE. The Wx-A1 protein was not present in Turkish cultivars that carried an insertion in the *Wx-A1* gene

Using AFC and AR2 primers, a 389-bp amplification product was expected from the wild-type *Wx-A1* allele. A 370-bp fragment, containing a 19-bp deletion (Vrinten et al. 1999), was expected from the 23 null *Wx-A1* alleles. However, of the 23 cultivars, 17 produced the expected fragment, but six cultivars instead showed a fragment approximately 200 bp longer than the fragment from wild-type alleles (Fig. 1). These six cultivars all belong to the Turkish group (Table 2).

The presence or absence of a 425-bp amplified fragment using the BDFL and BRD primers was used to identify the mutation in null *Wx-B1* alleles. These primers produce three polymorphic fragments from the three homeologous *waxy* genes, and the disappearance of the shortest 425-bp fragment is associated with null *Wx-B1* alleles in cultivars from several countries (Nakamura et al. 2002). However, in this study, although we selected 48 cultivars classified as type 3 or type 7, which lack the Wx-B1 protein, the 425-bp fragment was missing in only

Table 2 Identification of partial waxy wheat by 2D-PAGE and PCR

Country	Type[a]	PCR fragments			Type[b]	Cultivar
		Wx-A1	*Wx-B1*	*Wx-D1*		
Afghanistan	3	W	M	W	3	AF 9, AF 38, AF 48, AF 51-1, AF 57
Afghanistan	1	W	W	W	1	AF 3, AF 18, AF 77
USA	2	M	W	W	2	California, Sturdy
USA	3	W	M	W	3	Fox, Palo Duro, Yukon
USA	1	W	W	W	1	Fulton, Newturk
UK	1	W	W	W	1	Cama, Essex Conqueror, Hope, Kloka, Maris Nimrod, Tosscambier, Ulka, Vogel 253
Italy	1	W	W	W	1	Combine, Demar 4, Flavio, Jacometti, Marimp 3, Reno, Sangiorgio, Turano
India	2	M	W	W	2	India 3
India	3	W	M	W	3	C 306, Moti, NP 52, NP 852
India	1	W	W	W	1	Cawnpore, Indian, Sharbati Sonora
Egypt	3	W	M	W	3	Giza 156 (15)[c], Mengaui 8516 (R)[c], Sakha 8[c], Tob 8156 (R)
Egypt	1	W	W	W	1	Giza 155[c], Giza 155 Tanta (A Nouka)[C], Giza 157[c], Sakha 69 (20)[C], Up 301[c]
Ethiopia	3	W	M	W	3	Ethiopia 218[c], Ethiopia 220[c], Tob 8156
Ethiopia	1	W	W	W	1	Echiopia Komugi 72–200[c], Echiopia Komugi 72–390[c], Ku 9446 (Micturum Alef)[C]
Australia	2	M	W	W	2	Shino
Australia	3	W	M	W	3	Aroona, Heron, Mengavi
Australia	1	W	W	W	1	Bencubbin, Mintlor, Timgalen
Canada	3	W	M	W	3	Red Man, Reward
Canada	1	W	W	W	1	Ankesu, Ankra, Bindokku, Coronation II, Hastings, Losprout, Max[d], Opal
Colombia	1	W	W	W	1	Bonza 55[c], Crespo 63[c], Napo 63[c], Pal 1, Samaca 68[c], Zipa 68[c], Zipa 68[c]
Sweden	3	W	M	W	3	Algat, Apu[c]
Sweden	1	W	W	W	1	Folke[c], Fylgia[c], Kadett[c], Lulea[c], Norre[c], Sammet Kuff Sweden[c], Svalofs Bore 2[c]
USSR	3	W	M	W	3	Soren (Ussr) Komugi 33586, Soren (Ussr) Komugi 41961, Strela
USSR	1	W	W	W	1	Bezostaja 1, Cikotaba, Ekurishipusu, Skala, Taganrog, Volhynia
Tunisia	3	W	M	W	3	Tob 8156 (R)
Tunisia	1	W	W	W	1	Bt 2281[c], Bt 2288[c], K338 Etoile De Choisy/Koudie[c], Soltane=Bt 2296[c], Tac Penjamo 62[c], Tac Penjamo 62-1[c], Tac Penjamo 62-2[c], Zaafrane[c]
Germany	3	W	M	W	3	Lichtis (Fruher), Sonop
Germany	1	W	W	W	1	Bersee, Disponent, Fasan, Fubav D, Kleintron, Rimpaus Bastard 2, Strubes Dickkopf, Vagued Epis
Turkey	2	M	W	W	2	Turkey-124[e], Turkey-140[e], Turkey-171[e], Turkey-256[e], Turkey-280[e], Turkey-299[e]
Turkey	1	W	W	W	1	Surak 1593/5l, Turkey-225
Pakistan	3	W	M	W	3	Barani 70, B.D 63, KSH S-M. Pak 66, Tanab 107
Pakistan	1	W	W	W	1	C 299, Q 308
France	1	W	W	W	1	Beauchamp, Ble Tourneur 1610, Courtot, Cretan[d], Futsu (France) 40, Koazvi, Talent
China	2	M	W	W	2	Xiang An Xiao Mai1
China	3	W	M	W	3	Jing 1–38 (S)
China	4	W	W	M	4	Bai Huo
China	1	W	W	W	1	Bai Tuzi[d], Beijing 11, Hing Sh[d], Hong Guang Tou[d], Hong Ma Zha, Laochun Mai[d], Manshuu 5
Japan	2	M	W	W	2	Komugi Shin 1, Norin 18, Sekitori 1, Shiro Daruma
Japan	3	W	M	W	3	Chikushi Komugi, Norin 75
Japan	7	M	M	W	7	Chikugoizumi, Hakufu Yuubou
Japan	1	W	W	W	1	Aka Bouzu, Akasabi Shirazu 1, Danchi Komugi
Korea	2	M	W	W	2	Chousen 22, Chousen 23, Namkwang, Susun 6, Suwon 88, Won Gwang
Korea	3	W	M	W	3	Suyuk 44
Korea	1	W	W	W	1	Korea 1, Suwon 121, Suyuk 126, Yungkwang

[a] Identified by 2D-PAGE
[b] Identified by PCR
[c] Not analyzed by Yamamori et al. (1998)
[d] Reexamined proteins by 2D-PAGE
[e] Different size of PCR fragment from other null *Wx-A1* PCR fragment

42 cultivars and was detected in six cultivars collected from Canada, France, and China (Table 2). This implied that there is a different type of mutation in the null *Wx-B1* alleles of these six cultivars.

To verify the absence of the Wx proteins, these cultivars were re-analyzed by SDS-PAGE or 2D-PAGE using starch extracted from seed set on the plants used for the PCR analysis. Wx-A1 protein was not present in the

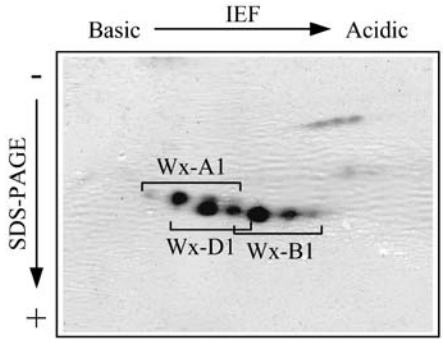


Fig. 2 Analysis of Wx protein by 2D-PAGE to verify the presence of null alleles. Wx-B1 protein was present in the Canadian cultivar Max, which produced a *Wx-B1*-specific fragment in PCR assays. The cultivars Cretan, Bau Tuzi, Hing Sh, Hong Guang Tou, and Laochun Mai also produced Wx-B1 protein (data not shown)

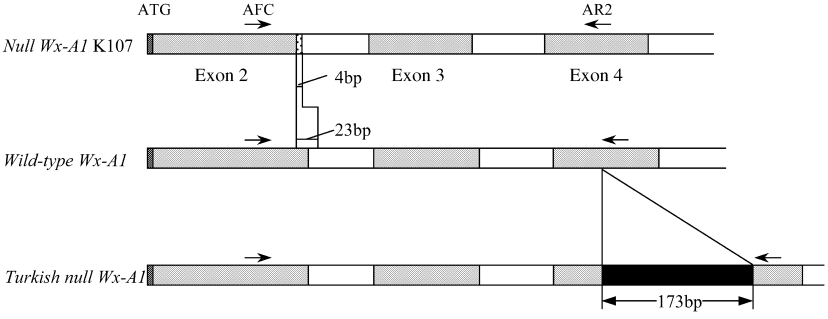
six Turkish cultivars that produced the longer PCR fragment from the *Wx-A1* gene (Fig. 1B), which corresponded to the previous analysis (Yamamori et al. 1998). In contrast, the six cultivars in which a *Wx-B1*-specific fragment was amplified clearly showed the presence of Wx-B1 protein in the 2D-PAGE analysis (Fig. 2). Therefore, we reclassified these six cultivars as type 1, and concluded that all cultivars classified into type 3 or type 7 possess the same mutation in the *Wx-B1* gene.

Analysis of Turkish *Wx-A1* mutant

The longer fragment observed in the null *Wx-A1* alleles of six Turkish cultivars suggested an insertion was present in the *Wx-A1* gene, resulting in the absence of Wx-A1 protein. Sequencing of the fragment showed the presence of an extra 173-bp in the middle of an exon of the *Wx-A1* gene (Fig. 3A). The 19-bp sequence deletion observed in

Fig. 3A, B The insertion in the null *Wx-A1* allele of Turkish cultivars. **A** Diagrammatic representation of the null mutations in the *Wx-A1* gene. The *black box* represents the insertion in the null *Wx-A1* allele of Turkish cultivars, and the *stippled box* represents filler DNA in the null *Wx-A1* allele of K107. **B** Comparison of genomic DNA sequences of *Wx-A1* alleles between the primers AFC and AR2. The sequence of the intron of the *Wx-A1* gene is shown in *lower-case letters*. The filler DNA in K107 and the insertion in Turkish cultivars are shown in *gray boxes*. Terminal inverted repeats are shown in *bold* and target site duplications are *underlined*

A



B

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Null Wx-A1 (K107)    TCGTGTTCGT CGGCGCCGAG ATGGCGCCCT GGAGCAAGAC TGGCGGCCTC GGCGACGTCC TCGGGGGCCT
Wild-type Wx-A1      TCGTGTTCGT CGGCGCCGAG ATGGCGCCCT GGAGCAAGAC TGGCGGCCTC GGCGACGTCC TCGGGGGCCT
Null Wx-A1 (Turkey)  TCGTGTTCGT CGGCGCCGAG ATGGCGCCCT GGAGCAAGAC TGGCGGCCTC GGCGACGTCC TCGGGGGCCT

Null Wx-A1 (K107)    CCCCGCCGCG GAC.....gccttctt ataaatgttt cttcctgcag ccatgcctgc
Wild-type Wx-A1      CCCCGCCGCC ATGGCCgtaa gcttgcgcca ctgccttctt ataaatgttt cttcctgcag ccatgcctgc
Null Wx-A1 (Turkey)  CCCCGCCGCC ATGGCCgtaa gcttgcgcca ctgccttctt ataaatgttt cttcctgcag ccatgcctgc

Null Wx-A1 (K107)    cgttacaacg ggtgccgtgt ccgtgcagGC CAACGGTCAC CGGGTCATGG TCATCTCCCC GCGCTACGAC
Wild-type Wx-A1      cgttacaacg ggtgccgtgt ccgtgcagGC CAACGGTCAC CGGGTCATGG TCATCTCCCC GCGCTACGAC
Null Wx-A1 (Turkey)  cgttacaacg ggtgccgtgt ccgtgcagGC CAACGGTCAC CGGGTCATGG TCATCTCCCC GCGCTACGAC

Null Wx-A1 (K107)    CAGTACAAGG ACGCCTGGGA CACCAGCGTC ATCTCCGAGg tatatatccg ccacatgaat tatcacaatt
Wild-type Wx-A1      CAGTACAAGG ACGCCTGGGA CACCAGCGTC ATCTCCGAGg tatatatccg ccacatgaat tatcacaatt
Null Wx-A1 (Turkey)  CAGTACAAGG ACGCCTGGGA CACCAGCGTC ATCTCCGAGg tatatatccg ccacatgaat tatcacaatt

Null Wx-A1 (K107)    cacatgctcc tgcacatttc tgcaagactt tactgactgg ctggatctcg cagATCAAGG TCGTTGACAG
Wild-type Wx-A1      cacatgctcc tgcacatttc tgcaagactt tactgactgg ctggatctcg cagATCAAGG TCGTTGACAG
Null Wx-A1 (Turkey)  cacatgctcc tgcacatttc tgcaagactt tactgactgg ctggatctcg cagATCAAGG TCGTTGACAG

Null Wx-A1 (K107)    GTACGAGAGG G.....
Wild-type Wx-A1      GTACGAGAGG G.....
Null Wx-A1 (Turkey)  GTACGAGAGG GCAGTGGCGT AGCTAGGGGG TGGCCAGGGT GGTCCATGGA CCACCCTGGA ATTTCCCCAT

Null Wx-A1 (K107)    .....
Wild-type Wx-A1      .....
Null Wx-A1 (Turkey)  AGCTTGTATA TAGTGTAGTA AAAAATATTT CTTTGAAAAT AAATAAACGT ATGTAAATAT TTCTATTGAT

Null Wx-A1 (K107)    .....TGAGGT ACTTCCACTG CTACAAGCGC
Wild-type Wx-A1      .....TGAGGT ACTTCCACTG CTACAAGCGC
Null Wx-A1 (Turkey)  GGACCACCTT GATTTATTGG GCTGGCTACG CCACTGCGAG AGGGTGAGGT ACTTCCACTG CTACAAGCGC

Null Wx-A1 (K107)    GG
Wild-type Wx-A1      GG
Null Wx-A1 (Turkey)  GG
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other null *Wx-A1* alleles was not detected, indicating that the null *Wx-A1* allele of the Turkish cultivars has a different origin.

The insertion has the characteristics of a transposable element (TE), including subterminal sequences resembling terminal inverted repeats (TIRs) and target site duplications (TSDs) at the insertion site (Fig. 3B). This element appears to possess structural features of class II TEs (Feschotte et al. 2002).

In wheat, several types of class II TEs, such as *Stowaway* (Bureau and Wessler 1994) and *CACTA* (Wicker et al. 2003), have been identified. Recently, a new type of miniature inverted TE, *Waffle*, was found in the flanking sequences of high-molecular-weight glutenin genes (Anderson et al. 2002). However, the insertion identified here is not similar to the other class II TEs found in wheat, nor did database searches identify any reported TEs with homology to this insertion.

Discussion

Waxy mutations have been reported in several cereals, such as maize, rice, and barley, as early as the nineteenth century (Eriksson 1970), while in wheat, *waxy* mutations have been identified and studied only within the last 10 years. Common wheat has three homeologous *waxy* loci, and spontaneous *waxy* mutations in each locus were identified after separation of the three Wx proteins, Wx-A1, -B1, and -D1 (Nakamura et al. 1993a). Null *waxy* alleles that do not produce waxy protein were found at the *Wx-A1* and *Wx-B1* loci of Japanese cultivars and at the *Wx-D1* locus of a Chinese cultivar (Nakamura et al. 1993a, 1993b; Yamamori et al. 1994). The eight possible combinations of the three null alleles produce wild-type wheat, waxy wheat, and six types of partial waxy wheat which possess one or two null alleles (Nakamura et al. 1995). The differences in amylose content among partial waxy wheat types affect noodle-making quality, and the selection of desired partial waxy wheat types has become important in wheat breeding programs (Nakamura et al. 2002). The geographical distribution of partial waxy wheat cultivars was examined by analyzing waxy proteins in 1,929 common wheat cultivars (Yamamori et al. 1998). Null *Wx-A1* and *Wx-B1* alleles were found not only in Asian, but also in European and North American cultivars. While null *Wx-A1* alleles were observed frequently in Turkish cultivars, null *Wx-B1* alleles were often found in Australian and Indian cultivars. From an evolutionary standpoint, we wondered whether the mutations within a gene arose independently in different areas, or if a single mutation occurred and spread geographically. Recently, the origin and evolution of the waxy phenotypes in rice (Olsen and Purugganan 2002), barley (Domon et al. 2002; Patron et al. 2002) and foxtail millet (Fukunaga et al. 2002) were analyzed using molecular data. Sequence information for the mutations in waxy wheat (Vrinten et al. 1999) and PCR marker sets to detect the mutations (Nakamura et al. 2002) were used here to provide similar information for the *waxy* mutations in common wheat. Our previous work using these marker sets with a number of cultivars from Japan, Australia, the United States, and Canada indicated the presence of single mutations in all null *Wx-A1* alleles and in all null *Wx-B1* alleles (Nakamura et al. 2002). In this study, we studied cultivars from 20 countries to investigate the origins of waxy mutations. According to our waxy protein analysis data (Yamamori et al. 1998), 23 cultivars from seven countries carried null *Wx-A1* alleles, 48 cultivars from 16 countries carried null *Wx-B1* alleles, and one cultivar from China carried a null *Wx-D1* allele.

Using the primer set AFC and AR2, we expected to detect a 19-bp deletion in the *Wx-A1* allele. However, the deletion was present only in 17 cultivars and was not present in null *Wx-A1* alleles from six Turkish cultivars (Fig. 1). Electrophoresis of PCR products (Fig. 1A) clearly indicated an insertion of close to 200-bp between the AFC and AR2 sequences of the Turkish null *Wx-A1* donor plants that are missing the Wx-A1 protein (Fig. 1B). Sequencing of the fragment revealed that a 173-bp insertion existed in an exon of the *Wx-A1* gene of these cultivars**.** We concluded that the mutation in the *Wx-A1* gene in Turkish germplasm has a separate origin. The higher proportion of Turkish cultivars carrying a null mutation for the *Wx-A1* gene (Yamamori et al. 1998) could be due to the presence of this mutation in Turkish germplasm. It is notable that the insertion was conserved only in Turkish cultivars, while the deletion spread to several areas of the world.

The 173-bp insertion sequence has a 12-bp TIR flanking a 8-bp TSD sequence on either side of the insertion (Fig. 3B). These features are characteristic of class II TEs. Therefore, we concluded the TE identified here likely belongs to the class II category and it was designated "*Hikkoshi*." Although *Hikkoshi* does not resemble any TEs previously identified in Triticeae, further searches of databases allowed us to identify a similar class II TE in rice. Nagano et al. (2002) analyzed repetitive sequences (RSs) in a 200-kbp region around the rice *waxy* locus, identifying 75 RSs, of which 26 were newly discovered TEs likely belonging to the class II category. Seven of the 26 RSs did not resemble any known class II families and were placed in five new families: *Akan*, *Mashu*, *Saroma*, *Shikotsu*, and *Toya*. *Toya*, like *Hikkoshi*, is 176 bp in length with an 8-bp TSD, and the TIR sequence of *Hikkoshi* is very close to that of *Toya* (data not shown).

The coding region of the *waxy* gene appears to be missing in the null *Wx-B1* allele found in K107 (Vrinten et al. 1999), and a 425-bp fragment between the BDFL and BRD primer sequences of the *Wx-B1* gene was employed to identify this mutation. Of 48 cultivars reported to have null *Wx-B1* alleles, 42 did not produce the 425-bp PCR fragment, while the fragment was amplified in six cultivars, suggesting that a different mutation might be present in these cultivars. However, reanalysis of waxy protein patterns showed these six cultivars possessed Wx-B1 protein (Fig. 2). Therefore,

there are no conflicts between the absence of Wx-B1 protein and the PCR results. For both the original analysis and this study, we obtained materials from the gene bank of NIAS. Likely, the samples of these six cultivars contain a mixture of wild-type and null *Wx-B1* seed.

Since only one mutation was identified in *Wx-B1* genes, this spontaneous mutation probably has a single origin. In addition, from the geographical distribution pattern of null *Wx-B1* alleles (Yamamori et al. 1994, 1998), we speculate that the mutation originally arose in western Asia and spread first to other Asian countries, and modern breeding programs accelerated the distribution of the allele to cultivars bred in Australia and Europe and North America. Further analysis of old domestic cultivars from western Asian will be necessary to support this hypothesis.

Mutants lacking Wx-B1 protein were not found among 42 cultivars from Italy or 155 cultivars from Turkey in our studies (Yamamori et al. 1998), whereas Urbano et al. (2002) analyzed only 56 common wheat cultivars and found cultivars form Nepal, Italy, and Turkey carrying null *Wx-B1* alleles. While they identified a cultivar from Nepal that appears to carry an identical mutation to the one detected in this study, PCR analysis indicated that this mutation was not present in *Wx-B1* genes of the Italian and Turkish cultivars. This suggests that at least two different mutations occurred separately at the *Wx-B1* locus.

Using the primers BDFL and DRSL, the expected 2,307-bp fragments were amplified in all cultivars possessing Wx-D1 protein, while only Bai Huo, which carries a null allele, showed a 576-bp shorter fragment (Vrinten et al. 1999). Shariflou et al. (2001) identified a wheat line with a null allele for Wx-D1 protein that does not contain this deletion. Therefore, to-date, two different mutations that occurred independently at the *Wx-D1* locus have been identified. However, there is no evidence that these mutations spread to other areas, and both mutations may have occurred relatively recently. Urbano et al. (2002) also recently identified two mutants, one a cultivar from Iran and one from Italy, which lack the Wx-D1 protein. However, since information on the presence of the 588-bp deletion in these two cultivars is not available, the origins of these mutations are unknown. Further molecular analysis of these cultivars will provide insight into the origins and geographical distribution of mutations in the *Wx-D1* locus.

The waxy phenotypes of foxtail millet appear to have a polyphyletic origin (Fukunaga et al. 2002). Analysis of the *waxy* genes of 79 landraces from east to south Asian areas showed the presence of three types of mutations that resulted in a waxy phenotype, and the geographical distribution of each type was distinct. In rice, the spontaneous waxy mutation is caused by a single base pair mutation in the 5′ splice end of the first intron of the *waxy* gene (Cai et al. 1998; Hirano et al. 1998; Isshiki et al. 1998). Based on this evidence, Olsen and Purugganan (2002) traced the geographical distribution and origin of the waxy phenotype, concluding that a single mutation that arose in Southeast Asia is the only source of *waxy* null alleles that spread to other areas. In barley, a low amylose mutant carries a spontaneous deletion in the promoter and 5′UTR of the *waxy* gene (Domon et al. 2002; Patron et al. 2002). This mutation also likely originated in China, from where it was probably distributed to other Asian countries (Patron et al. 2002). Compared to these diploid plants, the origins and spread of *waxy* mutations in common wheat appear to be more complex. The presence of two or more types of mutations at each of the three *waxy* loci suggests that the hexaploid nature of common wheat may increase the diversity of mutations, although single locus mutations are masked by the other two homeologous loci.

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