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E. Domon · M. Fuijita · N. Ishikawa

The insertion/deletion polymorphisms in the waxy gene of barley genetic resources from East Asia

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Abstract The length polymorphism in the waxy gene, which encodes a granule-bound ADP-glucose-glucosyl transferase [granule-bound starch synthase I (GBSS I), E.C. 2.4.1.11] in barley (*Hordeum vulgare*), was found. The 5′ leader sequence of the waxy gene of barley germplasm from Japan and Korea was analyzed by the polymerase chain reaction (PCR). The waxy gene of these genetic stocks had three types of length polymorphisms, suggesting that there are insertion/deletion mutations at the 5′ leader sequence of the waxy gene. DNA sequence analysis of the polymorphic PCR products showed that: (1) a 403-bp deletion mutation, which included a complete exon I, was found in the *wax* allele and a 193-bp insertion sequence was located in the intron I, and (2) the insertion sequence was also located in intron I of the *Wax* allele. The identity of the insertion sequence was completely conserved between the *wax* allele and the novel *Wax* allele. These finding s implying that the *wax* allele, which was found in indigenous waxy barley, originated in non-waxy barley with the novel *Wax* allele.

Keywords Barley (*Hordeum vulgare L.*) · Waxy gene · Polymorphism · Mutation

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E. Domon · M. Fujita · N. Ishikawa
Shikoku National Agricultural Experiment Station, Sen-yu 1-3-1, Zentsuji, Kagawa 765-8508, Japan

Present addresses:
E. Domon (✉), Kyushu National Agricultural Experiment Station, Suya 2421, Nishigoushi, Kikuchi, Kumamoto 861-1192, Japan
e-mail: domon@affrc.go.jp
Tel.: +81-96-242-1150, Fax: +81-96-249-1002

M. Fujita
National Agricultural Research Centor, Kannondai 3-3-1, Tsukuba, Ibaraki 305-8666, Japan

N. Ishikawa
Chugoku National Agricultural Experiment Station, Nishifukatsu 6-12-1, Fukuyama, Hiroshima 721-8514, Japan

Introduction

The *wax* (formerly called *glx* or *wx*) locus of barley (*Hordeum vulgare*) is responsible for the amylose contents of the endosperm and the pollen grain (Nakao 1950; Ono and Suzuki 1957; Rosichan et al. 1979) and is located on the short arm of chromosome 1 (7H) (Kramer and Blander 1961; Tabata 1961; Kleinhofs 1997). Rohde et al. (1988) reported that the barley waxy gene encodes a granule-bound ADP-glucose-glucosyl transferase [granule-bound starch synthase I (GBSS I), E.C. 2.4.1.11] which, like many other grass species, catalyzes the synthesis of amylose. The starch component in the endosperm that carries the *Wax* allele is composed of about 27% amylose and 73% amylopectin, but the homozygous *wax* endosperm normally contains 2–10% amylose and 98–90% amylopectin (Ishikawa et al. 1995; Washington et al. 2000). The storage starch component in barley endosperm is one of the important grain characteristics affecting malting, food and feed quality (Bhatty 1993; Swanston et al.1995), and the so-called waxy barley is known to have a glutinous endosperm (Ono and Suzuki 1957).

In Japan and Korea, hull-less barley has been traditionally used for food such as miso and rice extender, and ongoing breeding efforts have attempted to produce modern barley cultivars that will best serve this purpose. The indigenous waxy barley cultivars originating in East Asia are mostly of the hull-less type also, and they have been mainly used for luxury foods. Unlike most crop species, the kernels of barley are usually covered with hulls that prevent breeders from visual scoring of the endosperm; the exception is the hull-less genotype. Despite this obstacle, the barley waxy gene has been cloned and characterized (Rohde et al. 1988) like maize (*Zea mays*) and rice (*Oryza sativa*) (Shure et al. 1983; Okagaki and Wessler 1988), and a fine-structure map of the barley *wax* locus has also been reported (Rosichan et al. 1979).

The fine-structure map of the barley *wax* locus showed that the *glx1a* allele of Japanese indigenous

waxy barley had some mutation at the terminal region of the locus (Rosichan et al. 1979). The fine-structure map of the maize *wax* locus has provided accurate positions of mutations in the GBSS I structural gene and was quite useful for isolating transposable elements (Nelson 1968; Wessler and Varagona 1985). Ishikawa et al. (1995) carried out sodium dodecyl sulfate-polyacrylamide gel-electrophoresis of the starch-binding protein and revealed via the silver-staining method that the 60-kDa Waxy protein was reduced but clearly remained in the endosperm of indigenous waxy barley. On the basis of these findings, we presumed that the expression of the *wax* allele from indigenous waxy barley might partially be suppressed by some structural alterations or point mutations in the regulatory region of the waxy gene.

In recent years, intensive breeding efforts in several countries have been made to develop modern waxy barley cultivars (Doi et al. 1998; Rossnagel 2000; Washington et al. 2000); however, molecular information on the structure of the *wax* allele, an important source of the low amylose phenotype, is still limited. Therefore, the objective of the present study was to provide a molecular basis for the *wax* locus of barley genetic stocks from Japan and Korea. In this report we present length polymorphisms in the 5′ leader sequence of the waxy gene from the non-waxy and the waxy barley genetic stocks by means of the polymerase chain reaction (PCR). We cloned the PCR products from the waxy gene and characterized the structural alterations in the *Wax* and *wax* alleles that underlie the length polymorphisms.

Material and methods

Plant material

Two sets of *Wax/wax* barley (*H. vulgare*) near-isogenic lines were subjected to confirm length polymorphism in the *wax* locus, i.e. Shonupana (*Wax*) and Washonupana (*wax*) (Newman et al. 1983), and Oderbrucker (*Wax*) and Waxy Oderbrucker (*wax*) (CI 5899/10*CI 4666, Oderbrucker). Sixty two accessions of cultivated barley genetic stocks (Table 1) were analyzed for length polymorphism in the *wax* locus. One of the waxy hull-less cultivars, CDC Candle, was a kind gift from Dr. B.G. Rossnagel (University of Saskatchewan, Canada). These materials have been maintained in the Shikoku National Agricultural Experiment Station, Zentsuji, Kagawa, Japan.

PCR amplification and sequence analysis

Oligo deoxynucleotide primers were designed using the OLIGO 4.0 program (National Bioscience, Inc.) and purchased from the Funakoshi Company, Ltd. The primers designated p-197 (5′-CAA ACA GAC GAC AAG CGG AGA A-3′) and p+606 (5′-TAG AAA AAG AAA ACA TCA AGC A-3′) were chosen from the genomic DNA sequence of a barley waxy gene (Rohde et al. 1988). The template DNA was extracted from 20-day old seedlings by the CATB procedure (Murray and Thompson 1980) with some modification. The PCR was carried out in a 20-µl volume containing 50 ng of the genomic DNA template; 0.1 µM of each primer; 200 µM of each dATP, dCTP, dGTP and dTTP; 50 mM KCl; 10 mM Tris-HCl; 1.5 mM $MgCl_2$; 0.01% gelatin and 0.5 units of *Taq* polymerase (Takara Shuzo Company, Ltd). PCR steps were processed at 94°C for 4 min, at 52°C for 2 min and at 72°C for 2 min for one cycle, followed by 34 cycles at 94°C for 1 min, at 52° C for 2 min and at 72°C for 2 min, with a final extension step of 72° C for 2 min. The resulting DNA fragments were electrophoresed on a 1.5% agarose gel containing 0.5 µg/ml of ethidium bromide and photographed on an UV transilluminator.

Proof-reading of *pfu* DNA polymerase (Stratagene) was used for the cloning of PCR products. The amplified DNA fragments were electrophoretically separated on an agarose gel as described above and recovered with a QIAquick gel-extraction kit (Qiagen). The DNA fragments were polished and cloned into the *Sma*I site of pBluescript II SK+ phagemid vector (Stratagene) by a Blunting kination ligation kit (Takara Shuzo Company, Ltd), and sequenced with the *Taq* DyeDeoxy terminator cycle sequencing kit on an ABI Prism 377, or an ABI Prism 373A, DNA sequencing system (Perkin Elmer).

Results

Length polymorphisms

The sequences and the locations of the primers relevant to the 5′ leader sequence of the barley waxy gene were chosen from a barley waxy gene known as the European Molecular Biology Laboratory (EMBL) nucleotide sequence database accession number X07931 (Rohde et al. 1988), as shown in Fig. 1. When these two primers were applied to the genomic DNA extracted from *Wax/wax* near-isogenic lines, DNA fragments of about 800 bp for *Wax* lines and 600 bp for *wax* lines were successfully amplified (Fig. 2). A DNA fragment of expected size from the database (i.e. 800 bp) was exclusively present in the *Wax* lines, but a 600-bp fragment was amplified from both *wax* lines. This result indicates that these polymorphic DNA fragments were specific for the waxy gene and amplified from the *Wax* and *wax* allele s, respectively.

The sixty two accessions of barley genetic stocks shown in Table 1, which mainly consisted of Japanese cultivars, were analyzed for the length polymorphism of the waxy gene. Figure 3 shows three types of alleles which were observed after agarose-gel electrophoresis. The polymorphisms observed in these genetic stocks are also shown in Table 1. All 13 waxy accessions showed the 600- bp fragment, and neither the 800-bp nor the 1,000-bp fragments were observed in the waxy barley analyzed. The 1,000-bp fragment seems to be derived from a *Wax* allele not reported previously, which was exclusively amplified from 33 accessions out of 46 (72%) non-waxy Japanese and Korean barley cultivars analyzed, instead of the 800-bp fragment. Of all these accessions with the 1,000-bp fragment, 32 of them were six-rowed barley. On

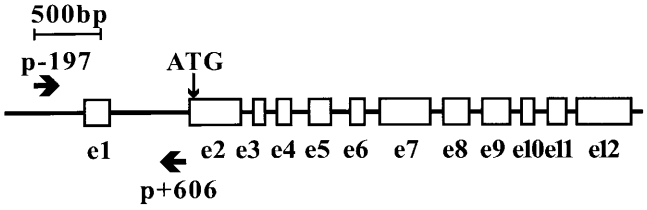

Fig. 1 Schematic representation of the barley GBSS I structural gene. Exons are represented in *open boxes* (e1–e12). The primers p-197 and p+606, used for PCR, are represented by *arrows*. The position of the start codon (ATG) is shown in the exon 2

Table 1 Barley accessions used for investigating length polymorphisms in the *wax* locus by PCR. Length polymorphism in PCR products derived from the wax locus is shown in base pairs

Code	Cultivar	*Wax/wax*[a]	Type of allele[b]	Row type	Origin/source
1	Waxy betzes	*wax*	600-bp	Two	USA
2	CDC Candle	*wax*	600-bp	Two	Canada
3	Chalssalbori	*wax*	600-bp	Six	Korea
4	Masan naked	*wax*	600-bp	Six	Korea
5	Dango mugi (000028)	*wax*	600-bp	Six	Japan
6	Dango mugi (OUJ483)	*wax*	600-bp	Six	Japan
7	Hizen	*wax*	600-bp	Six	Japan
8	Izumo mochi	*wax*	600-bp	Six	Japan
9	Mochi mugi D	*wax*	600-bp	Six	Japan
10	Tokushima mochi mugi	*wax*	600-bp	Six	Japan
11	Yatomi mochi	*wax*	600-bp	Six	Japan
12	Yonezawa mochi	*wax*	600-bp	Six	Japan
13	Yonezawa mochi 2	*wax*	600-bp	Six	Japan
14	Betzes	*Wax*	800-bp	Two	Europe
15	Igri	*Wax*	800-bp	Two	Europe
16	Asahi 5	*Wax*	800-bp	Two	Japan
17	Daisen gold	*Wax*	800-bp	Two	Japan
18	Golden melon	*Wax*	800-bp	Two	Japan
19	Hoshi masari	*Wax*	800-bp	Two	Japan
20	Ishuku shirazu	*Wax*	800-bp	Two	Japan
21	Kawa saigoku	*Wax*	800-bp	Two	Japan
22	Ko C	*Wax*	800-bp	Two	Japan
23	Nishino chikara	*Wax*	800-bp	Two	Japan
24	Satuki nijou	*Wax*	800-bp	Two	Japan
25	Shikoku hadaka 84	*Wax*	1,000-bp	Two	Japan
26	Akashinriki	*Wax*	1,000-bp	Six	Japan
27	Asama mugi	*Wax*	1,000-bp	Six	Japan
28	Bandai hadaka	*Wax*	1,000-bp	Six	Japan
29	Beni hadaka	*Wax*	1,000-bp	Six	Japan
30	Benkei mugi	*Wax*	1,000-bp	Six	Japan
31	Biwairo hadaka	*Wax*	1,000-bp	Six	Japan
32	Bouzu	*Wax*	1,000-bp	Six	Japan
33	Chikurin (060119)	*Wax*	1,000-bp	Six	Japan
34	Doriru mugi	*Wax*	1,000-bp	Six	Japan
35	Hagane mugi	*Wax*	1,000-bp	Six	Japan
36	Hinode hadaka	*Wax*	1,000-bp	Six	Japan
37	Boseong covered 8	*Wax*	1,000-bp	Six	Japan
38	Ichibanboshi	*Wax*	1,000-bp	Six	Japan
39	Iwata santoku	*Wax*	1,000-bp	Six	Japan
40	Kagawa hadaka 1	*Wax*	1,000-bp	Six	Japan
41	Kashima mugi	*Wax*	1,000-bp	Six	Japan
42	Kikai hadaka	*Wax*	800-bp	Six	Japan
43	Kobinkatagi (040293)	*Wax*	800-bp	Six	Japan
44	Gwangju sanjaku 36-2	*Wax*	1,000-bp	Six	Korea
45	Kuroshio hadaka	*Wax*	1,000-bp	Six	Japan
46	Minori mugi	*Wax*	1,000-bp	Six	Japan
47	Mochi hadaka (090112)	*Wax*	1,000-bp	Six	Japan
48	Nakate hadaka	*Wax*	800-bp	Six	Japan
49	Nanpu hadaka	*Wax*	1,000-bp	Six	Japan
50	Saga hadaka 1	*Wax*	1,000-bp	Six	Japan
51	Sanuki hadaka	*Wax*	1,000-bp	Six	Japan
52	Sekitori 1	*Wax*	1,000-bp	Six	Japan
53	Senbon hadaka	*Wax*	1,000-bp	Six	Japan
54	Shiro chinko	*Wax*	800-bp	Six	Japan
55	Shishikui zairai	*Wax*	1,000-bp	Six	Japan
56	Taisho mugi	*Wax*	1,000-bp	Six	Japan
57	Takeshita	*Wax*	1,000-bp	Six	Japan
58	Tokushima mochi mugi 2	*Wax*	1,000-bp	Six	Japan
59	Tsumamachi mochi mugi	*Wax*	1,000-bp	Six	Japan
60	Yokozuna	*Wax*	1,000-bp	Six	Japan
61	Yon M kei 48	*Wax*	800-bp	Six	Japan
62	Yuunagi hadaka	*Wax*	1,000-bp	Six	Japan

[a] *Wax/wax:* endosperm phenotype for *wax* locus
[b] Type of allele: approximate fragment size of PCR products were shown in base pairs

Table 2 Length polymorphism in PCR products derived from *wax* locus. Each accession was displayed in code number

Type of allele	Two-rowed	Six-rowed
600-bp	1, 2	3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13
800-bp	14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24	42, 43, 48, 54, 61
1,000-bp	25	26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 44, 45, 46, 47, 49, 50, 51, 52, 53, 55, 56, 57, 58, 59, 60, 62

Type of allele: approximate fragment size of PCR products were shown in base pairs

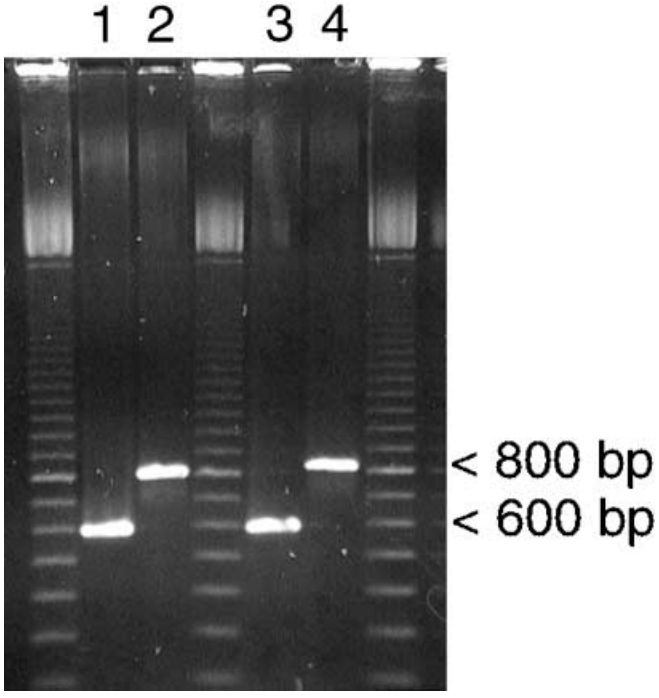

Fig. 2 Detection of the length polymorphism in two sets of *Wax*/*wax* near-isogenic lines. The PCR-amplified DNA fragments derived from the *wax* locus were fractionated in a 2% agarose gel. The *wax* genotypes showed 600-bp fragments (*lanes 1, 3*), whereas the *Wax* genotypes showed 800-bp fragments (*lanes 2, 4*), respectively. *Lanes 1* Washonupana (*wax*), *2* Shonupana (*Wax*), *3* Waxy Oderbrucker (*wax*), *4* Oderbrucker (*Wax*)

the other hand, 11 accessions of two-rowed non-waxy barley originating in Japan and Europe showed the 800-bp fragment, and five accessions of six-rowed barley originating in Japan showed the 800-bp fragment also. The exception for two-rowed non-waxy barley was Shikoku hadaka 84, which showed the 1,000-bp fragment.

Sequence analysis

The PCR products derived from the three alleles were cloned in phagemid vector and sequenced. The DNA sequences from PCR products of Shonupana, Senbon hadaka and Washonupana are aligned in Fig. 4.

The DNA sequence of the 800-bp fragment from Shonupana was identical to that of the barley waxy gene, EMBL nucleotide database accession number X07931, demonstrating that the PCR products were derived from the barley waxy gene. The 600-bp fragment from Washonupana had a 403-bp deletion, spanning from position –129 bp to position +274, relative to the deduced starting point of transcription in the barley waxy gene. This region of deletion contained exon 1 and the deduced transcription starting point for GBSS I of the *Wax* allele. Another structural alteration found in this 600-bp fragment was a 193-bp insertion sequence located within intron I. This sequence was conserved in the 1,000-bp fragment from the novel *Wax* allele of Senbon hadaka. The underlined sequence ga in Fig. 4 was changed in

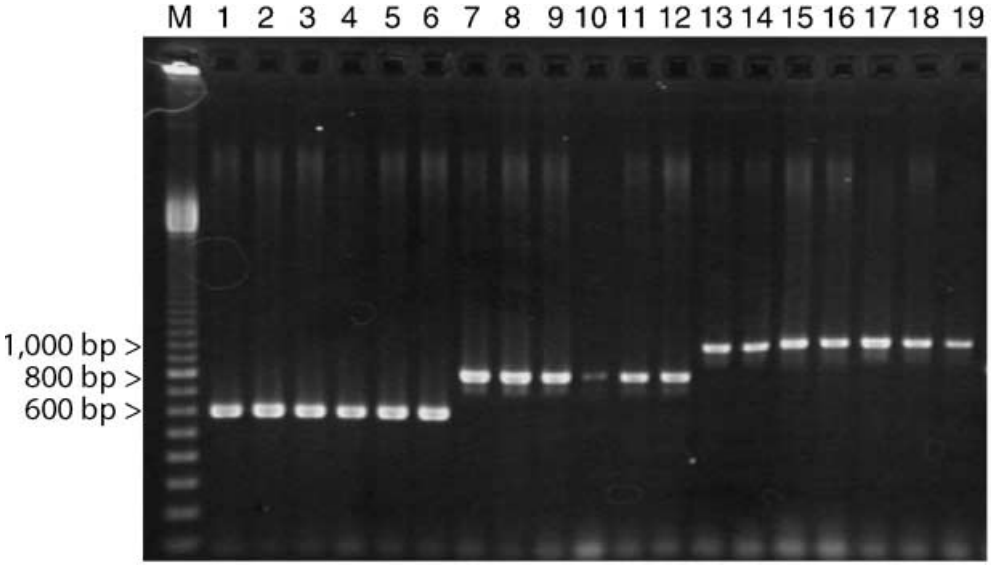

Fig. 3 Example of length polymorphisms in the *wax* locus. Genomic DNA was isolated from each accession and used as a template in PCR for amplifying the DNA fragment derived from the *wax* locus, as shown in Fig. 1. *Lanes M*:100-bp ladder, *1* Hizen, *2* Dango mugi (OUJ483), *3* Izumo mochi, *4* Mochi mugi D, *5* Tokushima mochi mugi, *6* Yatomi mochi, *7* Daisen gold, *8* Asahi 5, *9* Ishuku shirazu, *10* Nishino chikara, *11* Shiro chinko, *12* Kikai hadaka, *13* Yokozuna, *14* Iwata santoku, *15* Saga hadaka 1, *16* Akashinriki, *17* Senbon hadaka, *18* Ichibanboshi, *19* Chikurin (060119). *Lanes 1–6* are waxy barley accessions and lanes 7–19 are non-waxy barley accessions

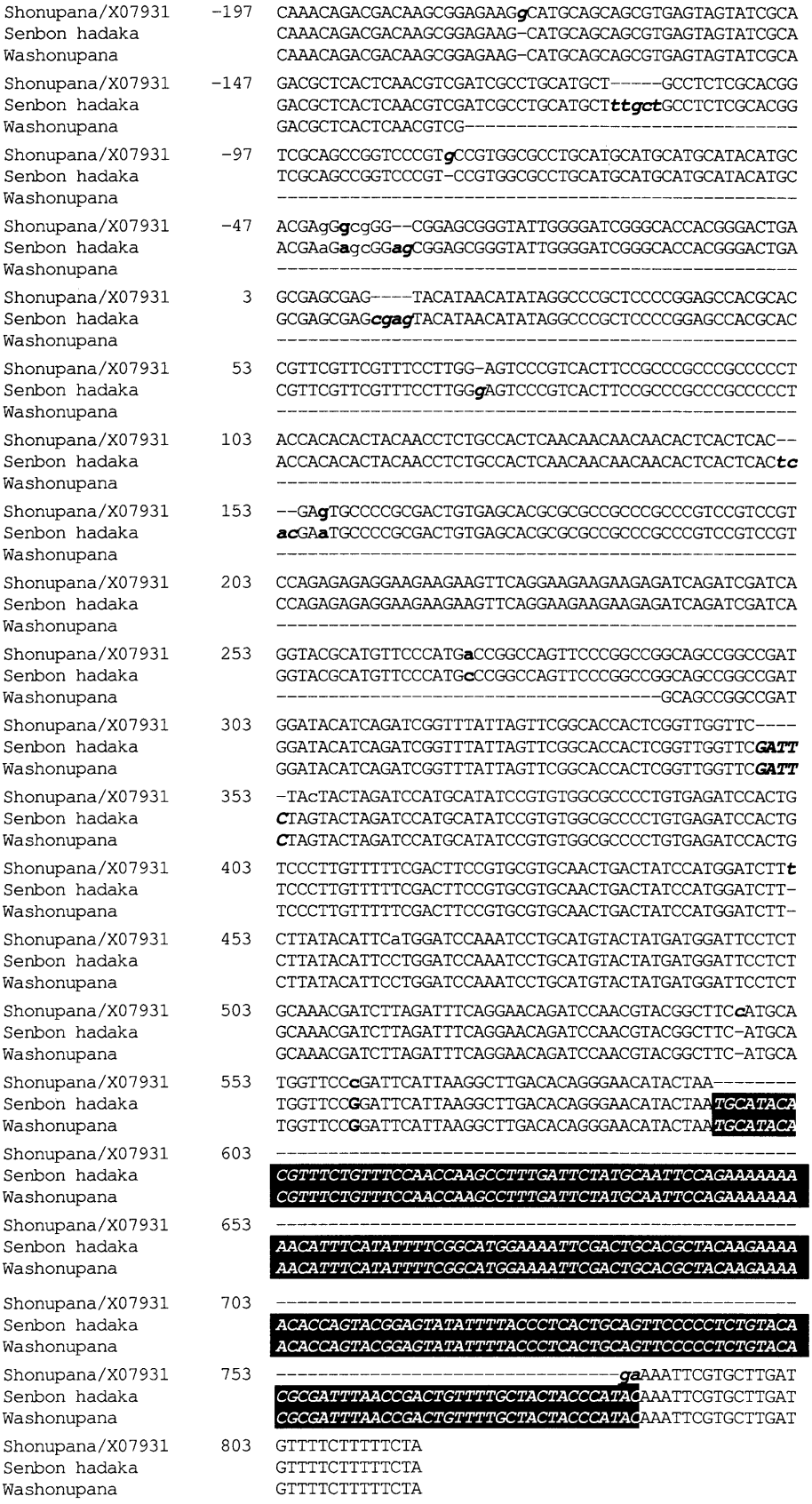

Fig. 4 DNA sequence of the PCR products from the wax locus. Shonupana and Senbon hadaka are non-waxy barley, and Washonupana is a waxy barley. **Boldface letters** indicate the nucleotide sequence altered by base substitution. The nucleotide sequences inserted by mutation are shown in **boldface italics**. The 193-bp insertion sequences are indicated by **solid black boxes**

these alleles; that is, the 193-bp sequence that contained three initiation codons (ATG) was inserted instead of the 2-bp sequence ga (Fig. 4). In addition, the 5-bp sequence GATTC was inserted within intron I of the 600-bp and 1,000-bp fragments. In addition, four single nucleotide polymorphisms (SNPs) and another five insertion/deletion mutations common to both fragments are also shown in Fig. 4.

Discussion

The fine-mapping of the *wax* locus based on intragenic recombination in the GBSS I structural gene has been reported for rice, maize and barley (Li et al. 1965; Nelson 1968; Rosichan et al. 1979). The *wax* locus of maize has provided accurate positions of mutations in the GBSS I structural gene (Wessler and Varagona 1985). In barley, the *glx1a* allele that originated in a Japanese indigenous waxy cultivar was shown to have a mutation in the terminal region of the *wax* locus (Rosichan et al. 1979).

In this study, we showed that the barley *wax* allele has a 403-bp deletion mutation in the 5′ terminal part of the GBSS I structural gene, based on DNA sequence analysis (Fig. 4). The deletion was found to include a TATA box and a deduced transcription starting point of the wild-type *Wax* allele, which suggested the influence of the deletion mutation over the low amylose content in endosperm and pollen starch by reducing transcription from the *wax* allele. We also showed that the *wax* allele and the novel *Wax* allele have a 193-bp insertion in intron I in common (Fig. 4). The novel *Wax* allele was found from among non-waxy barley; therefore, the insertion is probably neutral to the phenotype. Similar spontaneous insertion/deletion events in the GBSS I structural gene were reported by Wessler et al. (1990) in waxy maize. The spontaneous mutants of the waxy maize were shown to have insertion sequences of 1 to 131 bp, called filler DNA, located between deletion endpoints. In the spontaneous waxy barley mutant, such filler DNA was not found at the 403-bp deletion. Instead, the two-base sequence *ga*, which was located in the equivalent position to the 193-bp insertion of the *wax* and novel *Wax* alleles, was found in the 800-bp fragment from the *Wax* allele (Fig. 4). Our experiment was focused on accession s from Japan and Korea; therefore, an ancestral type of *Wax* allele was not determined based on geographical distribution. Further analysis in a wide range of cultivated barley and its wild relatives (*H. vulgare* ssp. *spontaneum*) will allow us to trace the origin of the insertion.

Besides the 193-bp insertion, four SNPs and another five insertion/deletion mutations existed in the *wax* allele and the novel *Wax* allele (Fig. 4). In addition, our survey on length polymorphisms of the *wax* locus showed that the novel *Wax* allele with the 193-bp insert was the present in the majority (72%) of Japanese non-waxy barley (Table 1). The only exception for two-rowed barley that showed the 1,000-bp fragment was Shikoku hadaka 84 (Table 1), which is derived from the single cross of two-row-covered and six-row-hull-less parents. The *wax* locus and the *nud* locus, which dominate naked caryopsis (Fedak et al. 1972), are also located on chromosome 1 (7H); therefore, the 1,000-bp fragment from Shikoku hadaka 84 might be a result of introgression of the novel *Wax* allele from six-rowed parents. It seemes unlikely that the *wax* allele, which conserves common insertion sequences and SNPs with the novel *Wax* allele, had been derived from the *Wax* allele with the 800-bp fragment by a single mutational event. The insertion sequences and the SNPs that were conserved between the *wax* allele and the novel *Wax* allele suggest that the former was derived from the latter by a deletion mutation.

The barley *wax* locus is highly polymorphic in the microsatellite with an AT repeat (Saghai Maroof et al. 1994; Becker and Heun 1995). A similar polymorphic microsatellite, composed of a CT repeat, is found in the rice *wax* locus (Ayres et al. 1997). However, these polymorphisms themselves were thought to be neutral to the phenotype of the endosperm (Saghai Maroof et al. 1994; Ayres et al. 1997). Washington et al. (2000) reported that a microsatellite marker Bmag206 for the *wax* locus showed no polymorphism among elite waxy cultivars. We analyzed the 5′ leader sequence of the waxy gene of indigenous waxy barley from distinct sources via PCR and showed that all of them are monomorphic in length polymorphism (Table 1). Therefore, most likely, these *wax* alleles from the indigenous waxy barley have not been differentiated and that they are virtually an identical allele.

Our results suggested that the *wax* allele derived from the different indigenous waxy barley cultivars might have a common origin, unlike those of rice or maize, and that the amylose content of the endosperm might be controlled by the genetic background, which affects expression of the waxy gene. The variance in the amylose content of the waxy genotype may probably be supported by variegations and sectoring, which stained blue black with I/KI, in the endosperm of waxy barley (Ono and Suzuki 1957; Schriber and Habekuß 1996). If expression of the *wax* allele, which is derived from indigenous waxy barley, is essentially unstable, novel genetic resources that completely lack amylose will offer the stable alternative. Zero-amylose waxy genotypes have been developed by the sodium azide treatment, and one of the mutants had a novel *wax* allele (Ishikawa et al. 1995). Bhatty and Rossnagel (1997) also reported the development of novel zero-amylose waxy breeding lines that derived from a cross between waxy parents. Because both the parents were assumed to have the indigenous *wax* allele, these breeding lines may have a gene, or genes, that affect expression of the waxy gene in their genetic background. These breeding lines will provide new breeding materials for developing stable zero-amylose cultivars.

Our results showed that: (1) the *wax* allele had deletion and insertion mutations in the 5′ leader sequence of the GBSS I gene, and (2) the *Wax* allele with the insertion sequence was frequently found in the barley genetic stocks from Japan and Korea. The deletion in the *wax* al-

lele described above will provide an effective genetic marker, which should be useful for selecting the *wax* allele at the seedling stage in a breeding program for waxy barley cultivars. Finally, these results will provide detailed information concerning the differentiation of the waxy gene for genetic and evolutionary studies on cultivated barley.

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