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ORIGINAL PAPER

A barley Hordoindoline mutation resulted in an increase in grain hardness

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Abstract Barley seed proteins, Hordoindolines, are homologues of wheat Puroindolines, which are associated with grain hardness. Barley *Hordoindoline* genes are known to comprise *Hina* and *Hinb*, and *Hinb* consists of two *Hinb* genes, *Hinb-1* and *Hinb-2*. Two types of allele were found for *Hina*, *Hinb-1* and *Hinb-2* genes, respectively, among Japanese two- and six-rowed barley lines. One of the alleles of *Hinb-2* (*Hinb-2b*) had a frame-shift mutation resulting in an in-frame stop codon. For two-rowed barley lines, grain hardness was significantly higher among lines with the *Hinb-2b* than those with the wild type *Hinb-2* gene (*Hinb-2a*). Protein spots corresponding to HINa, HINb-1, and HINb-2 were identified by 2D-gel electrophoresis among barley lines with *Hinb-2a.* Among the lines with *Hinb-2b*, HINa and HINb-1 were expressed at similar levels as those in the wild type, but HINb-2 was not detected. A DNA (cleaved amplified polymorphic sequence) marker was developed to distinguish between the *Hinb-2a* and *Hinb-2b* gene sequences. Analysis of grain hardness among F_2 lines derived from a cross between a line with *Hinb-2a* (Shikoku hadaka 115) and a line with the *Hinb-2b* (Shikoku hadaka 84) showed significantly higher grain hardness in the mutant lines. From these results, the *Hinb-2b* frame-shift (null) mutation might play a critical role in barley grain hardness. The DNA marker will be useful in barley breeding to select lines having harder grain texture.

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Introduction

Grain hardness in barley is one of the most important quality characters related to barley grain processing. The amounts of cell wall polysaccharides (β-glucan and arabinoxylan), grain proteins, and grain lipid are associated with the grain hardness. Grain protein and β-glucan contents were positively correlated with hardness (Henry and Cowe 1990). While grain protein and β-glucan contents were negatively correlated with malt extract rate (Swanston and Taylor 1988), grain hardness showed a positive correlation with pearling yield (Iwami et al. 2003). Pearled barley grains are used in miso, distilled spirits, and boiled barley to cook with rice. The efficiency of pearling is one of the most important characteristics to reduce the processing cost.

In common wheat, grain hardness is mainly controlled by a single *Hardness* (*Ha*) locus on the short arm of chromosome 5D (Mattern et al. 1973; Law et al. 1978), where *Puroindoline-a* (*Pina*), *Puroindoline-b* (*Pinb*), and *Grain Softness Protein-1* (*Gsp-1*) genes are tightly linked (Tranquilli et al. 1999). It was reported that mutations in *Pina* or *Pinb* resulted in hard grain texture (Giroux and Morris 1998; Bhave and Morris 2008). Transgenic work

has provided more direct insight into the in vivo roles and interrelationships of PINA and PINB on grain hardness (Krishnamurthy and Giroux 2001; Beecher et al. 2002a). The amount of PIN proteins was related to the degree of grain hardness (Ikeda et al. 2005).

Gautier et al. (2000) reported the significant homology of *Hordoindoline-a* (*Hina*) and *Hordoindoline-b* (*Hinb*) sequences to the wheat counterparts, *Pina* and *Pinb*, respectively. Darlington et al. (2001) reported that two tandemly linked *Hinb* genes (*Hinb-1* and *Hinb-2*), encode the putative proteins HINb-1 and HINb-2, which share 90–91% amino acid sequence identity with PINb-D1.

Beecher et al. (2002b) showed that the largest QTL associated with grain hardness mapped to the *Hina/Hinb/Gsp* region on the short arm of chromosome 5H. On the other hand, Fox et al. (2007) identified loci on chromosomes 2H, 3H, 5H, 6H, and 7H with high LOD scores related to grain hardness, but a significant LOD score was not found for the location of the *Hordoindoline* genes. Therefore, despite the similarities with *Pins*, the relationship of *Hins* to grain hardness and associated phenotypes has been uncertain.

Recent studies reported a high level of genetic variation in the barley *Hina*, *Hinb*, and *Gsp* genes (Caldwell et al. 2006; Turuspekov et al. 2008). Turuspekov et al. (2008) identified five alleles of *Hina*, six alleles of *Hinb-1*, 18 alleles of *Hinb-2*, and 18 alleles of *Gsp*, using 81 spring barley lines. They showed that the most common *Hina*, *Hinb-1*, *Hinb-2*, *Gsp* genotype combination was significantly softer grain texture with increasing starch content and dry matter digestibility. Caldwell et al. (2006) found the one of the *Hinb-2* mutants (*Hinb-2b*) had three base substitutions and one base deletion resulting in a null mutant due to generating an in-frame stop codon.

In this study examined the grain hardness among commercial barley varieties, experimental lines, and F_2 lines derived from a cross between lines with *Hinb-2a* and *Hinb-2b* (null mutant) using a *Hinb-2* allele-specific DNA cleaved amplified polymorphic sequence (CAPS) marker. It was also found that lines with *Hinb-2b* lacked HINB-2 protein in the seeds. This is the first report that HINB-2 null mutant increased barley grain hardness.

Materials and methods

Plant material and hardness measurement

Ten barley cultivars/lines were examined. Seven of them were two-rowed barley cultivars/lines, and others were six-rowed barley lines. F_2 lines were derived from a cross between Shikoku hadaka 84 and Shikoku hadaka 115. Grain hardness index (HI), grain weight, grain diameter, and grain moisture content of 100 whole grains were measured by the Single-Kernel Characterization System 4100 (SKCS, Perten Instruments). These were measured three times, and averaged, in ten barley cultivars/lines. For characterization of F_2 lines, we used F_3 seeds. Measurements of each F_2 lines were averaged. All lines were grown under field condition in 2006 at Zentsuji in Kagawa prefecture, Japan. Harvested grains appeared to be sound without sprouting damage.

PCR amplification, restriction enzyme digestion, and DNA sequencing of *Hin* genes

DNA was extracted from seeds and leaves (F_2 lines) using a DNeasy Plant Mini kit (QIAGEN). PCR was performed using total DNA with TaKaRa PCR Thermal Cycler Dice (Takara TP600). A specific primer set was designed based on barley genome sequence on GenBank (accession no. AY644090) to amplify a partial *Hinb-2 gene* as follows: pHinb-2 F1: 5′-AGAGCGGCCAAACCTAGGT-3′; pHinb R: 5′-TCACCAGTAATAGC-3′. The size of amplified products by these primers was 322 bp. Specific primer sets designed based on the barley genome sequence on GenBank (accession no. AY644213, AY644090, AY644090) for full length *Hina*, *Hinb-1*, and *Hinb-2* were as follows: pgHina F1: 5′-GTGTACACAACTGCAGAC AGAAAGC-3′, pgHina R2: 5′-ATTATTCCAAGACCAC TTTTATTTGTC-3′, pgHinb-1 F1: 5′-CAACACCAAAAC AACG-3′, pgHinb-1 R2: 5′-GACCTCATTGATTTGTC-3′, pgHinb-2 F2: 5′-ACCAACACCAAATAAACA-3′, and pgHinb-2 R5: 5′-CCAATATACAAGCGGAATTTTAT TC-3′. The size of amplified products were 631, 535 and 576 bp by *Hina*, *Hinb-1*, and *Hinb-2* primers, respectively. PCR was performed in a volume of 25 µl [1 µl of 10 pmol of each primer, 12.5 µl of AmpliTaq Gold PCR Master Mix (Applied Biosystems)], with cycling parameters of 94°C for 9 min, 35 temperature cycles (94°C, 30 s; 58°C, 30 s; 72°C, 1 min), followed by 7 min final extension at 72°C. To identify *Hinb-2* variations, we performed CAPS analysis; 10 µl of PCR products of *Hinb-2* were digested with a restriction enzyme *Sal*I (8 units) by adding 2 µl of the 10× H buffer (Takara) and 7.6 µl of distilled water, and incubated at 37°C for 3 h. Samples were separated by 3.0% agarose gel (ReadyAgarose Gel, Bio-Rad Lab) electrophoresis containing 10 µg ethidium bromide in 0.5× TBE for 1.5 h, and then visualized by UV irradiation. Digestion of the *Hinb-2* 322 bp product by *Sal*I resulted in 291 and 31 bp restriction fragments. PCR products were purified by QIAquick PCR purification kit (QIAGEN) and sequenced directly using a BigDye Terminator Cycle Sequencing Kit ver. 3.1 (Applied Biosystems) with a 3130 Genetic Analyzer (Applied Biosystems).

Extraction of TritonX-114 soluble fraction and 2D-gel electrophoresis

TritonX-114 soluble proteins were extracted from crushed whole barley (about 100 mg) using the method according to Giroux and Morris (1998) and Ikeda et al. (2005). The protein samples were dissolved in 250 µl of IEF sample buffer for very basic proteins (Gorg et al. 1997) containing 16% isopropanol, 8.5 M urea. 2 M thiourea, 4% CHAPS, 25 mM DTT, and 0.5% IEF buffer pH 6–11 (GE Healthcare). After vortexing for 30 min at room temperature, free thiol groups were alkylated by adding 5 µl of 4-vinylpyridine (Branlard et al. 2003). Samples were applied to Immobiline DryStrip pH 6–11, 13 cm (GE Healthcare). After in-gel rehydration for 15 h, IEF was performed for a total of 20 kVh using an Ettan IPGphor IEF System (Amersham Biosciences). The gels were equilibrated with equilibration buffer containing 6 M urea, 2% SDS, 20% glycerol, 10 mM DTT and 50 mM Tris–Cl pH 6.8 and stored at −25°C. For the second dimension, SDS-PAGE was performed using a 14.5% gel 1 mm thickness. Electrophoresis was conducted at 30 mA for 4.5 h. The gels were stained using Coomassie Brilliant Blue G-250 by the method of Neuhoff et al. (1988). For N-terminal amino acid sequence analysis, the gels were blotted onto a Sequi-Blot PVDF Membrane using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad Lab). The N-terminal amino acid sequences of individual spots were determined using a protein sequencer PPSQ-21A (Shimadzu Corporation).

Measurement of protein and β-glucan content

The contents of protein in grains were determined by dry combustion method in a Vario Max CN Elementar analyzer (Elementar Instrument). The sample weight was 150 mg. L-Glutamic acid was used for calibration. Grain nitrogen concentrations were converted to grain protein contents by a conversion factor of 5.83. Total β-glucan content in grains was analyzed according to McCleary and Glennie-Holmes (1985) using a commercial kit (Megazyme Ltd), and presented on the basis of dry weight with 12% of moisture content.

Results

Identification of *Hin* genotypes

The full length sequences of *Hina*, *Hinb-1*, and *Hinb-2* from ten barley cultivars/lines were amplified and sequenced directly. Two type alleles were found for each of the *Hin* genes (Table 1). For *Hina* gene, five and five cultivars/lines had the same nucleotide sequences as Monte Cristo (AY644174) and Morex (AY644179), respectively. Comparing with *Hina* of Monte Cristo, Morex had two base substitutions and one 3 bp deletion resulting in two amino acid substitutions at positions 89 (serine to glycine) and 111 (phenylalanine to valine) and an amino acid deletion at position 145 (Fig. 1). Because the *Hina* amino acid sequence of Monte Cristo had higher similarity to those of the barley wild ancestral species *H. spontaneum* (Caldwell et al. 2006) than those of Morex, *Hina* sequences corresponding to Monte Cristo and Morex were temporarily classified as *Hina-a* and *Hina-b*, respectively (Fig. 1). For *Hinb*, seven and three cultivars/lines had the same combinations of *Hinb-1*/*Hinb-2* as those of Morex (AY644056) and Monte Cristo (AY644051), respectively. Comparing with the Morex sequences, *Hinb-1* of Monte Cristo had two base substitution resulting in an amino acid substitution (isoleucine to leucine) within the signal peptide at position 9 and an amino acid substitution (aspartic acid to glutamic acid) at position 138 (Fig. 2). *Hinb-2* of

Table 1 *Hin* genotypes and grain characteristics of ten barley cultivars/lines

Barley cultivars/lines	Row	Genotype			Hardness index (HI)[a]	Grain weight (mg)[a]	Diameter (mm)[a]	β-Glucan content (%)[a]
		Hina	*Hinb-1*	*Hinb-2*				
Toyonokaze	Six row	*Hina-a*	*Hinb-1a*	*Hinb-2a*	61.6e	36.1de	2.7d	4.5d
Ichibanboshi	Six row	*Hina-a*	*Hinb-1a*	*Hinb-2a*	68.8c	37.8d	2.8cd	4.4d
Mannenboshi	Six row	*Hina-b*	*Hinb-1a*	*Hinb-2a*	75.2b	35.6e	2.7cd	5.6a
Sukai golden	Two row	*Hina-b*	*Hinb-1a*	*Hinb-2a*	60.4d	46.9b	2.9a	3.8f
Nishinohoshi	Two row	*Hina-b*	*Hinb-1a*	*Hinb-2a*	63.2d	47.5b	2.9a	3.9e
Shikoku hadaka 109	Two row	*Hina-b*	*Hinb-1a*	*Hinb-2a*	53.7f	47.7b	2.9a	4.8c
Shikoku hadaka 115	Two row	*Hina-b*	*Hinb-1a*	*Hinb-2a*	45.2g	48.3b	2.9ab	3.8f
Shikoku hadaka 84	Two row	*Hina-a*	*Hinb-1b*	*Hinb-2b*	79.2a	43.2c	2.8bc	5.0b
Miharu gold	Two row	*Hina-a*	*Hinb-1b*	*Hinb-2b*	69.9c	51.4a	2.9ab	3.7g
Shunrei	Two row	*Hina-a*	*Hinb-1b*	*Hinb-2b*	78.1ab	51.4a	2.9ab	3.8f

[a] The same letters in the same column are not significantly different ($P < 0.05$) by Tukey's test

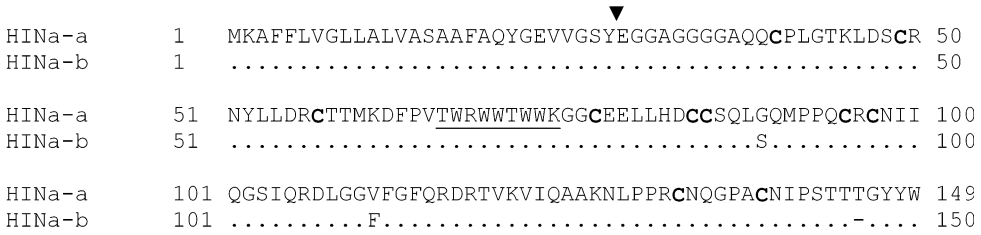

Fig. 1 Alignment of the HINa-a and HINa-b amino acid sequences. The HINa-a sequence is derived from Monte Cristo. The HINa-b sequence is derived from Morex. GenBank accession numbers of HINa are as follows: Monte Cristo (AY644174), Morex (AY644179). The sequences of cysteine (*C*) are marked in *boldface* type, and the sequences of TRD (tryptophan rich domain) are *underlined*. An *arrowhead* indicates the signal peptide cleavage site

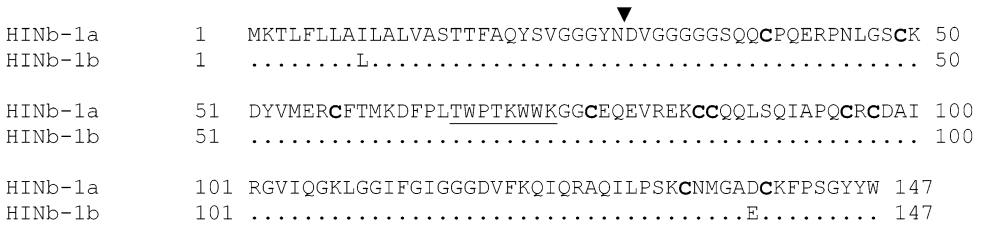

Fig. 2 Alignment of the HINb-1a and HINb-1b amino acid sequences. The HINb-1a sequence is derived from Morex. The HINb-1b sequence is derived from Monte Cristo. GenBank accession numbers of HINb-1 are as follows: Morex (AY644056), Monte Cristo (AY644051). The sequences of cysteine (*C*) are marked in *boldface* type, and the sequences of TRD (tryptophan rich domain) are *underlined*. An *arrowhead* indicates the signal peptide cleavage site

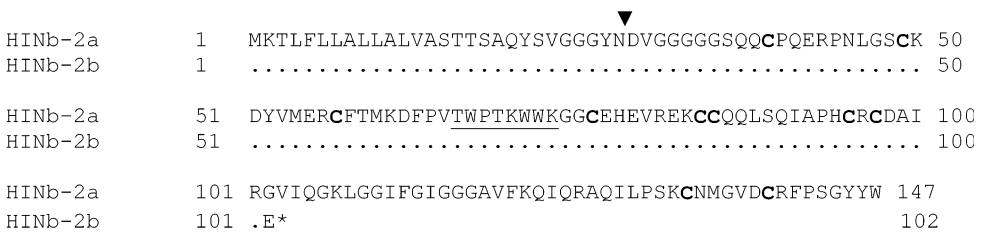

Fig. 3 Alignment of the HINb-2a and HINb-2b amino acid sequences. The HINb-2a sequence is derived from Morex. The HINb-2b sequence is derived from Monte Cristo. GenBank accession numbers of HINb-2 are as follows: Morex (AY644056), Monte Cristo (AY644051). The sequences of cysteine (*C*) are marked in *boldface* type, and the sequences of TRD (tryptophan rich domain) are *underlined*. An *arrowhead* indicates the signal peptide cleavage site

Monte Cristo had four base substitutions and one base deletion resulting a frame-shift mutation (Fig. 3). Because *Hinb-1* and *Hinb-2* sequences of Morex had higher similarity to those of *H. spontaneum* (Caldwell et al. 2006) than those of Monte Cristo, *Hinb-1* and *Hinb-2* sequences corresponding to Morex and Monte Cristo were temporarily classified as *Hinb-1a* and *Hinb-2a*, and *Hinb-1b* and *Hinb-2b*, respectively (Table 1).

SKCS and β-glucan content

Among seven two-rowed barley cultivars/lines, the HI of the cultivars/lines with *Hina-a*/*Hinb-1b*/*Hinb-2b* was significantly higher than those with *Hina-b*/*Hinb-1a*/*Hinb-2a* (Table 1). Among these cultivars/lines, HI had no significant correlations with β-glucan content, grain weight, or grain diameter. Among three six-rowed barley cultivars/lines, Mannenboshi with *Hina-b*/*Hinb-1a*/*Hinb-2a* showed significantly higher HI than Ichibanboshi and Toyonokaze with *Hina-a*/*Hinb-1a*/*Hinb-2a*. However, Mannenboshi also showed significantly higher β-glucan content and smaller grain weight than Ichibanboshi and Toyonokaze.

Expression of HIN protein

The effects of *Hin* gene mutations on HIN proteins, HINa, HINb-1, and HINb-2 were studied by 2D-gel electrophoresis (IPG × SDS-PAGE) using the TritonX-114 soluble fraction, and identified these proteins by N-terminal amino

acid sequencing. Using Ichibanboshi (*Hina-a*/*Hinb-1a*/*Hinb-2a*), a single spot was identified corresponding to HINa (EGGAGGGGAQQ) and two spots corresponding to HINb (DVGGGGGSQQ) as major spots among the basic proteins (spots A, B1 and B2 in Fig. 4). Their apparent molecular weights were around 15 kDa. HINb spots had slightly lower molecular weight than the HINa spot. There were eight amino acid substitutions between HINb-1 and HINb-2. Their deduced p*I*s of the mature proteins were 8.56 and 8.73, respectively. Therefore, the spot with higher p*I* was expected to be HINb-2. Although cultivars/lines with *Hinb-2a* had three spots corresponding to HINa, HINb-1, and HINb-2 products, cultivars/lines with *Hinb-2b* lacked a spot of HINb-2 (Table 1; Fig. 4).

CAPS analysis of F_2 lines with *Hinb-2* gene

To clarify the effects of the *Hinb-2* gene on grain characteristics, a *Hinb-2* allele-specific CAPS marker was developed to distinguish between *Hinb-2a* and *Hinb-2b* by the *Sal*I site at position 410, which was present in *Hinb-2a* but absent in *Hinb-2b* due to the point mutation at position 410. Using genomic DNA of the parental lines and 51 F_2 lines, *Hinb-2* genes were amplified with a primer combination of pHinb-2 F1 and pHinb R. The molecular sizes of the amplified products of *Hinb-2a* and *Hinb-2b* were 322 and 321 bp, respectively. Digestion of the *Hinb-2a* 322 bp fragment by *Sal*I resulted in 291 and 31 bp restriction fragments. On the other hand, the *Hinb-2b* 321 bp product was not digested due to lack of the *Sal*I site. Homozygous and heterozygous plants among these lines were identified by this method (Fig. 5). Of 51 F_2 lines, 15 and 13 F_2 lines had homozygous *Hinb-2a* and *Hinb-2b*, respectively, and the other lines were heterozygous. Because *Hina*, *Hinb-1*, and *Hinb-2* are tightly linked, the lines with *Hinb-2b* should have *Hina-a* and *Hinb-1b*. The *Hinb-2* allele by the CAPS marker agreed with the results of expression of HINB-2 by 2D-electrophoresis.

Effects of *Hinb-2* mutation on grain characteristics

Using 24 F_2 lines classified into homozygous *Hinb-2a* (12 lines) and *Hinb-2b* (12 lines), HI, grain weight, grain diameter, protein content, and β-glucan content were measured. HI ranged from 41.1 to 63.0. Kernel weight ranged from 43.7 to 52.3 mg and diameter ranged from 2.8 to 3.1 mm. Protein content ranged from 7.0 to 9.3% and β-glucan content ranged from 2.9 to 4.0%. Significant differences were found between *Hinb-2* allelic classes for grain hardness and β-glucan content (Table 2). The lines with the *Hinb-2b* alleles showed much higher average HI (59.7) than those with the *Hinb-2a* alleles (45.8) (Table 2). Lines with *Hinb-2b* alleles had slightly smaller average β-glucan content (3.4%) than those with *Hinb-2a* alleles (3.6%). Lines with *Hinb-2a*/*Hinb-2b* heterogeneous alleles were characterized as intermediate types between the *Hinb-2b* alleles and the *Hinb-2b* alleles for grain hardness (49.2). The distribution of HI between F_2 lines with *Hinb-2a* and

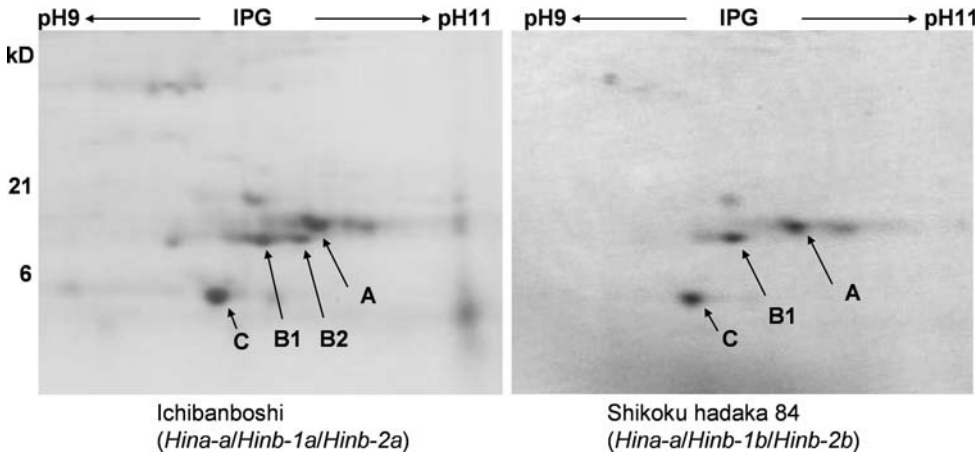

Fig. 4 Identification of HINa, HINb-1, and HINb-2 proteins by 2D-gel electrophoresis using. Ichibanboshi and Shikoku hadaka 84 with different HIN genotypes. HINa (*A*), HINb-1 (*B1*), HINb-2 (*B2*), LTP (lipid transfer protein) (*C*), are indicated with *arrows*

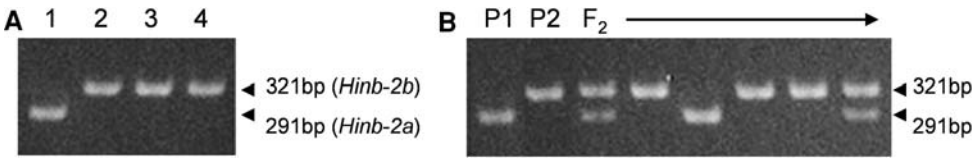

Fig. 5 Electrophoresis of CAPS products by 3% agarose. **a** *Lane 1* Shikoku hadaka 115, *lane 2* Shikoku hadaka 84, *lane 3* Shunrei, *lane 4* Miharu gold. **b** *P1* Shikoku hadaka 115, *P2* Shikoku hadaka 84, *F_2* F_2 lines from the cross Shikoku hadaka 84/Shikoku hadaka 115

those with *Hinb-2b* showed that two *Hinb-2* classes distinctly differed (Fig. 6a). However, the β-glucan content of F_2 lines between the two *Hinb-2* allelic classes showed overlapped distribution (Fig. 6b).

Discussion

The relationship between grain hardness and *Hin* genotypes was studied using commercial varieties, experimental lines, and F_2 lines derived from a cross between lines with *Hinb-2a* and *Hinb-2b*. This is the first report that the HINb-2 null mutation increased barley grain hardness.

For HINa, comparing deduced amino acid sequence of HINa-a with HINa-b, there were amino acid substitution at positions 89 (glycine to serine) and 111 (valine to phenylalanine) and a deletion at position 144 (Fig. 1). While the HI of Mannenboshi (*Hina-b*/*Hinb-1a*/*Hinb-2a*: six-rowed) was significantly higher than that of Ichibanboshi (*Hina-a*/*Hinb-1a*/*Hinb-2a*: six-rowed), the β-glucan content of Mannenboshi was also significantly higher, and the grain weight of Mannenboshi was significantly smaller that of Ichibanboshi. For HINb-1, comparing the deduced amino acid sequence of HINb-1a with HINb-1b, there were amino acid substitutions at position 9 (isoleucine to leucine) in the signal peptide region and at position 138 (aspartic acid to glutamic acid) (Fig. 2). Scarlett, Harrington, and Morex have *Hina-b* (Beecher et al. 2002b; Caldwell et al. 2006). The HI of these cultivars was not as high as those of other cultivars (Beecher et al. 2002b; Iwami et al. 2005; Fox et al. 2007). The ten important cysteine residues and a tryptophan rich domain (TRD) involved in the starch binding site were conserved among these alleles, and the amount of HINa and HINb-1 protein between these alleles did not appear to be different in 2D-electrophoresis analysis. These results indicated that the amino acid sequence variation of HINa and HINb-1 did not affect grain hardness.

For HINb-2, because a nonsense mutation at position 103 resulted in the deletion of the C terminal region including two cysteine residues, HINb-2b protein was assumed to have lost its function. One of the parent of Shikoku hadaka 84 was Mona, which is two-rowed barley

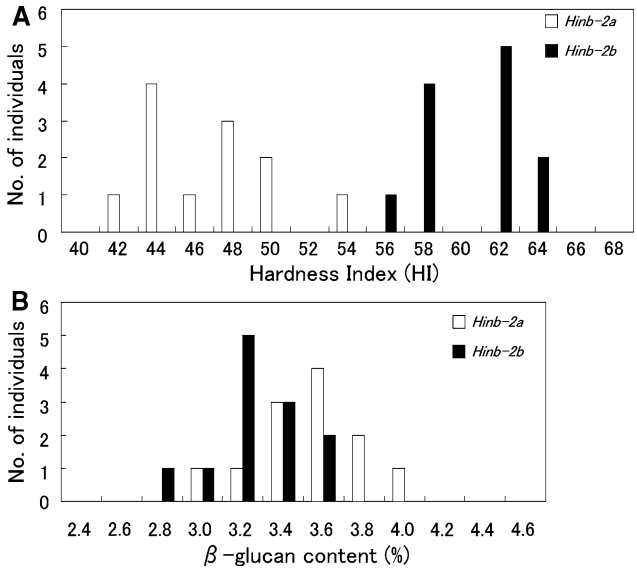

Fig. 6 Frequency distribution of F_2 lines with different *Hinb-2* genotypes. The distributions of hardness index (**a**), β-glucan content (**b**). *Hinb-2a* homozygotes of *Hinb-2a*, *Hinb-2b* homozygotes of *Hinb-2b*

classified into *Hinb-2b*. Monte Cristo (*Hina-a*/*Hinb-1b*/*Hinb-2b*) was Mona's parent. Therefore, the *Hinb-2b* allele of Shikoku hadaka 84 was assumed to have originated from Monte Cristo.

Based on phylogenetic analysis, the *Hina* amino acid sequence of Monte Cristo was evaluated to be closer to those of the barley wild ancestral species *H. spontaneum* than those of Morex: the amino acid sequence identity of *Hina* of Morex and Monte Cristo is 96.6 and 97.3%, respectively, to that in the line AY644094, which belonged to the majority of the *H. spontaneum* sequences. Therefore, the *Hina* sequences corresponding to Monte Cristo and Morex were designated as *Hina-a* and *Hina-b*, respectively. For *Hinb*, since *Hinb-1* and *Hinb-2* sequences of Morex were classified as being closer to those of *H. spontaneum* than those of Monte Cristo by phylogenetic analysis: the amino acid sequence identity of *Hinb-1* in Morex and Monte Cristo, and *Hinb-2* in Morex and Monte Cristo is 99.3, 99.3, 98.6, and 68.7%, respectively, to the lines AY644024 and AY643971. Therefore, the *Hinb-1* and *Hinb-2* sequences corresponding to Morex and Monte

Table 2 SKCS, protein content, and β-glucan content of F_2 lines with different *Hinb-2* genotypes

F_2 genotype	Hardness index (HI)[a]	Kernel weight (mg)[a]	Diameter (mm)[a]	Protein content (%)[a]	β-Glucan content (%)[a]
Hinb-2a	45.8 ± 3.3c	47.2 ± 2.3a	2.9 ± 0.1a	8.1 ± 0.6a	3.6 ± 0.2a
Hinb-2b	59.7 ± 2.4a	47.9 ± 1.9a	3.0 ± 0.1a	8.2 ± 0.7a	3.4 ± 0.2b
Hinb-2a/Hinb-2b	49.2 ± 3.2b	47.3 ± 2.4a	2.9 ± 0.1a	–	–

F_2 lines from the cross Shikoku hadaka 84/Shikoku hadaka 115

[a] The same letters in the same column are not significantly different ($P < 0.05$) by Student's t test

Cristo were temporarily classified as *Hinb-1a* and *Hinb-2a*, and *Hinb-1b* and *Hinb-2b*, respectively (Table 1).

Protein content and grain diameter are known to relate to grain hardness in barley. Gamlath et al. (2008) and Henry and Cowe (1990) reported that an increase in β-glucan content resulted in higher grain hardness. However, in the present study, the correlation between β-glucan content and grain hardness was not significant among the ten cultivars/lines. In the F_2 lines, the β-glucan content of lines with *Hinb-2b* was slightly lower than those with *Hinb-2a* (Table 2; Fig. 6b), indicating that higher grain hardness of lines with *Hinb-2b* was not due to higher β-glucan content. No significant differences were found for the grain weight or grain diameter between the F_2 lines with *Hinb-2a* and those with *Hinb-2b*. The HI of the heterozygotes (*Hinb-2a/Hinb-2b*) was intermediate between that of homozygotes of *Hinb-2a* and *Hinb-2b* (Table 2). Our preliminary analysis also showed that the lines with the *Hinb-2b* allele showed significantly higher average HI (51.6) than those of the *Hinb-2a* allele (41.8), using F_3 lines derived from the same cross. Because *Hina*, *Hinb-1*, and *Hinb-2* genes are closely linked (*Hina*/*Hinb-1*: 77 kbp; *Hinb-1*/*Hinb-2*: 1.2 kbp), the *Hin* genotypes of F_2 lines with *Hinb-2b* identified by CAPS markers was assumed to be *Hina-a*/*Hinb-1b*/*Hinb-2b*. These results are the first clear evidence that HINb-2 is involved in grain hardness in barley.

Barley HINs are known to be orthologues of wheat PINs. Darlington et al. (2001) reported that ten cysteines and TRD found in PINs were also conserved in HINs. They also reported that the nucleotide sequence identity of *Pinb* with *Hinb-1* and *Hinb-2* was 91 and 90%, respectively, and that the 5′ flanking sequences of *Hinb* is very similar to that of *Pinb*. These results strongly suggested that HINs has the same role as PINs, which bind to endosperm starch and weaken the adhesion between the starch and surrounding protein matrix. Ikeda et al. (2005) reported that common wheat cultivars lacking PINb protein (*PINb-D1c*) showed higher grain hardness that those expressing PINb protein (*Pinb-D1b*). Turuspekov et al. (2008) suggested that the expression of HIN is related to the grain hardness in barley.

Further analysis is necessary to clarify the relationship between the variation of HIN protein amount and *Hin* gene variation.

The *Ha* locus in barley encodes grain softness protein-1 (GSP-1), which is homologous to wheat GSP-1 and is expected to be related to grain hardness. The analysis of the relationship between *Gsp-1* gene variation and barley grain hardness will be required. Because the grain size in six-rowed barley is generally smaller than that in two-rowed barley, the relationship between *Hin* gene variation and grain hardness in six-rowed barley also requires further analysis.

For barley processing, soft grains are easily broken during pearling; broken grains are not appropriate for processing to produce as miso or boiled pearled barley. Iwami et al. (2003) indicated that the HI was negatively correlated with the broken grain ratio. To develop barley cultivars appropriate for various food processing, it is necessary to further clarify the relationship between genetic variation in *Hin* and grain hardness.

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