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ORIGINAL PAPER

Distribution of *Hordoindoline* genes in the genus *Hordeum*

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Abstract *Hordoindoline* (*Hin*) genes, which are known to comprise *Hina*, *Hinb-1,* and *Hinb-2*, are associated with grain hardness in barley. However, the interspecific variation in the *Hin* genes in the genus *Hordeum* has not been studied in detail. We examined the variation in *Hin* genes and used it to infer the phylogenetic relationships between the genes found in two *H. vulgare* subspecies (cultivated barley and *H. vulgare* subsp. *spontaneum*) and 10 wild relatives (*H. bogdanii*, *H. brachyantherum*, *H. bulbosum*, *H. chilense*, *H. comosum*, *H. marinum*, *H. murinum*, *H. patagonicum*, *H. pusillum*, and *H. roshevitzii*). The *Hina* and *Hinb* genes of these species were amplified by PCR. We found two *Hinb* genes in three wild species (*H. bogdanii*, *H. brachyantherum*, and *H. roshevitzii*) and preliminarily named them *Hinb-A* and *Hinb-B*. Cluster analysis showed that the 17 *Hinb* genes present in *Hordeum* formed two distinct clusters (named A and B). Seven *Hinb* genes were included in Cluster-A, and 10 *Hinb* genes were included in Cluster-B. All *Hinb-A* genes were included in Cluster-A, while all of the *Hinb-B* genes were included in Cluster-B. In contrast, the *Hinb-1* and *Hinb-2* genes in *H. vulgare* were included in Cluster-B. These results suggest that the *Hinb* genes duplicated during the early stages of diversification in the genus *Hordeum*. On the other hand, the *Hinb-1* and *Hinb-2* genes in *H. vulgare* seem to have been generated by a duplication of the *Hinb* gene after the split of the lineages leading to *H. vulgare* and *H. bulbosum*.

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Introduction

In common wheat (*T. aestivum*), the *puroindoline-a* (*Pina*) and *puroindoline-b* (*Pinb*) genes are located at the *hardness* (*Ha*) locus on the short arm of chromosome **5D** (Mattern et al. 1973; Law et al. 1978). Grain hardness is mainly controlled by these genes in common wheat (Giroux and Morris 1998; Krishnamurthy and Giroux 2001; Beecher et al. 2002; Ikeda et al. 2005). The puroindoline proteins (PINs) encoded by the *puroindoline* (*Pin*) genes belong to a group of cysteine-rich basic proteins, but PINs are unique among plant proteins because they contain a Trp-rich hydrophobic domain (Blochet et al. 1993). This domain is proposed to play two important roles, to control grain hardness, which affects flour particle size (Giroux and Morris 1997 and 1998; Morris 2002), and to produce antimicrobial activity (Dubreil et al. 1998; Jing et al. 2003; Phillips et al. 2011). Recently, Massa and Morris (2006) studied the phylogenetic relationships of the *Pina*, *Pinb*, and *grain softness protein-1* genes in *Triticum* and *Aegilops*.

The presence of *Pin* orthologues in *H. vulgare* subsp. *vulgare* (cultivated barley) has been demonstrated by molecular techniques. An orthologous *Ha* locus has been found on the short arm of the barley **5H** chromosome by Southern hybridization using wheat *puroindoline* cDNA (Rouvés et al. 1996). A quantitative trait locus for genes related to endosperm hardness has been found at the same chromosomal location (Thomas et al. 1996; Beecher et al. 2002). PCR analysis of cultivated barley using primer pairs designed based on *Pin* genes identified the *hordoindoline-a* (*Hina*) and *hordoindoline-b* (*Hinb*) genes, which appear to be orthologues of *Pina* and *Pinb*, respectively, because the hordoindoline proteins (HINs) encoded by *Hina* and *Hinb* also belong to a group of cysteine-rich basic proteins

and contain a Trp-rich hydrophobic domain (Gautier et al. 2000). Furthermore, the *Hinb* gene in cultivated barley consists of two genes (*Hinb-1* and *Hinb-2*), which was demonstrated by Southern blot and PCR analyses (Darlington et al. 2001). A large number of *Hina* and *Hinb* allelic variation were reported to exist among cultivated barley and wild barley (*H. vulgare* subsp. *spontaneum*) (Caldwell et al. 2006; Turuspekov et al. 2008; Li et al. 2010). In addition, direct evidence of the effects of *Hin* genes on grain hardness has been reported by Takahashi et al. (2010), who confirmed that the grain of the *Hinb-2* null mutant was significantly harder than that of the wild type. Grain hardness in barley is also one of the most important quality characters affecting the end-use properties. There was a highly significant correlation of the grain hardness with the malting quality (Allison et al. 1976; Nagamine et al. 2009) and rumen dry-matter digestibility, which is one of the most important traits in feeding of beef cattle (Turuspekov et al. 2008).

The genus *Hordeum* consists of 31 species. Cytological analyses have defined four different genomes in the genus *Hordeum* (Bothmer et al. 1995; Wang et al. 1996): **H** (27 species including *H. bogdanii*, *H. brachyantherum*, *H. chilense*, *H. comosum*, *H. patagonicum*, *H. pusillum*, and *H. roshevitzii*), **I** (*H. bulbosum* and *H. vulgare*), **Xa** (*H. marinum*), and **Xu** (*H. murinum*). Cultivated barley is the only cultivated species in the genus *Hordeum.* Only two wild species, *H. vulgare* subsp. *spontaneum* and *H. bulbosum*, can intercross with cultivated barley (Kuckuck 1934). Phylogenetic studies of *Hordeum* species have also been carried out using various genes (Blattner 2004; Kakeda et al. 2009). Blattner (2009) proposed a new genome denomination recently. The genome name of *H. vulgare* and *H. bulbosum* is **H** instead of **I**, and that of the 27 species including *H. bogdanii* is **I** instead of **H** in accordance with barley chromosome nomenclature. In this paper, following this new genome denomination, we studied the number of *Hinb* genes among wild species in the genus *Hordeum* and carried out phylogenetic analyses of *Hin* genes.

Materials and methods

Plant materials

Twelve diploid species of the genus *Hordeum* were used in this study (Table 1). All samples except for *H. vulgare* subsp. *spontaneum* were obtained from Dr. Shin Taketa of Okayama University. *H. vulgare* subsp. *spontaneum* was supplied by NIAS (National Institute Agrobiological Science) Genebank. In this study, the seven species (*H. bogdanii*, *H. brachyantherum*, *H. chilense*, *H. comosum*, *H. patagonicum*, *H. pusillum* and *H. roshevitzii*) were used

Table 1 *Hordeum* species used it this study and Genbank accession numbers of the *Hin* genes sequences

Species	Genome	Plant accession no.	Sequence accession no.		
			Hina	*Hinb*	
H. bogdanii	**I**	H4014[a]	AB605713	AB605724 (*Hinb-A*)	AB605721 (*Hinb-B*)
H. brachyantherum subsp. *californicum*	**I**	H3317[a]	AB605712	AB605725 (*Hinb-A*)	AB605722 (*Hinb-B*)
H. roshevitzii	**I**	H9152[a]	AB605714	AB605726 (*Hinb-A*)	AB605723 (*Hinb-B*)
H. chilense	**I**	4010001[a]	AB446468	AB446468	
H. comosum	**I**	H10608[a]	AB605710	AB605727	
H. patagonicum subsp. *patagonicum*	**I**	H6052[a]	AB605711	AB605728	
H. pusillum	**I**	H2038[a]	AB605709	AB605729	
H. marinum subsp. *marinum*	**Xa**	H109[a]	AB605715	AB605719	
H. murinum subsp. *glaucum*	**Xu**	line 71[a]	AB605716	AB605720	
H. bulbosum	**H**	J-1[a]	AB605717	AB605718	
H. vulgare subsp. *spontaneum*	**H**	JP77872[b]	AB611025 (GU591284)[c]	AB611028 (*Hinb-1*) (AY644022)[c]	AB611031 (*Hinb-2*) (AY644058)[c]
H. vulgare subsp. *vulgare*, cv. Betzes	**H**		AB611024 (DQ269851)[c]	AB611027 (*Hinb-1*) (AY644090)[c]	AB611030 (*Hinb-2*) (DQ862334)[c]

[a] Accession number of Okayama University

[b] Accession number of NIAS Genebank

[c] Accession numbers of identical sequences found in Genbank are shown in parentheses

as representative of **I** genome species (the section *Stenostachys*), because these species covers both Asian and American species.

PCR amplification and DNA sequencing

Hinb-1 specific primers (1bhoF and 1bhoR) and *Hinb-2* specific primers (2bhoF and 2bhoR) were designed based on the DNA sequences of the *Hinb-1* and *Hinb-2* genes of cultivated barley (Darlington et al. 2001; Caldwell et al. 2006) using the Primer 3 software (Rozen and Skaletsky 2000). Other primers (3bhoF, 4bhoF, 5bhoF, 3bhoR and 4bhoR) were designed based on the nucleotide sequences identified in this study. *Hina* specific primers (ahoF and ahoR) were designed based on the DNA sequences of the *Hina* genes of two *H. vulgare* subspecies (cultivated barley and *H. vulgare* subsp. *spontaneum*) (Gautier et al. 2000; Caldwell et al. 2006; and Li et al. 2010) and the *Pina* gene sequences of wheat and *Aegilops* species (Massa and Morris 2006). The position and direction of the *Hinb* primers were indicated in Fig. 1 and the *Hina* and *Hinb* primer sequences were listed in Table 2.

PCRs were performed in a total volume of 25 µl containing, 2.0 mM $MgCl_2$, 2.0 mM dNTP, 0.4 µM of each primer, 0.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems), 1 × PCR Gold buffer (Applied Biosystems), and 100 ng of total DNA. The reactions were performed according to the following protocol using the GeneAmp 9700 PCR System (Applied Biosystems, USA): denaturation at 94°C for 5 min; 35 cycles of 94°C for 30 s, 58°C for 60 s, and 72°C for 60 s; and a final extension step at 72°C for 5 min. The PCR products were electrophoresed in 1.5% Agarose 21 (Nippon Gene) gel in TAE buffer, purified using a QIAquick PCR purification kit (QIAGEN), and sequenced directly using the BigDye Terminator Cycle Sequencing Kit ver.3.1 (Applied Biosystems) using a 3130 Genetic Analyzer (Applied Biosystems).

Phylogenetic analyses

Multiple sequence alignments of *Hina* and *Hinb* nucleotide sequences and the deduced amino acid sequences of these genes were performed using ClustalW (Tompson et al. 1994). Phylogenetic trees were constructed from the DNA sequences using DNASIS Pro Ver. 3.0 (Hitachi Software Engineering) with the neighbor-joining method (Saitou and Nei 1987). The bootstrap analyses were performed with 1,000 samplings.

Results

Analyses of *hordoindoline-b*

PCRs using *Hinb-1*-specific primers (1bhoF and 1bhoR) to amplify *Hinb* were not successful in any sample except for the two *H. vulgare* subspecies. Therefore, PCRs were carried out in all samples using *Hinb-2*-specific primers (2bhoF and 2bhoR). Using these primers, DNA fragments (about 510 bp) were amplified from six species

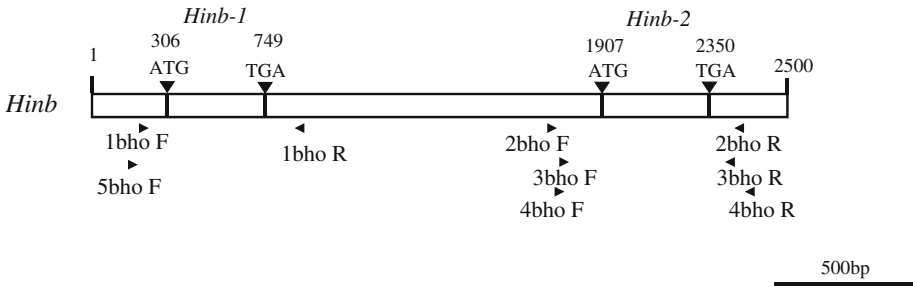

Fig. 1 Schematic representation of the *Hinb* genes and their flanking regions of cultivated barley referred from Darlington et al. (2001). The *arrows* indicate the positions and the directions of the primers. *Scale bar* nucleotide length

Table 2 Genome-specific PCR primers used to amplify the *Hina* and *Hinb* gene sequences

Forward primer		Reverse primer	
1bhoF	5′-TTGCACCATTTCTGTTTGCTTA-3′	1bhoR	5′-AATATGCACACAACCCACCAT-3′
2bhoF	5′-TAGGAAGTCTCAACCCATCTA-3′	2bhoR	5′-GTGGTATGTGACAGTTTATTGG-3′
3bhoF	5′-TCTCAACCCATCTATTCATCTT-3′		
4bhoF	5′-TCATCTTCACCAACACCAAATA-3′		
5bhoF	5′-CTCAACCCATCTATTCATCTCC-3′	3bhoR	5′-GGCTATATCATCACCAGTAATAGCC-3′
		4bhoR	5′-TCACATGTTGTGGTATGTGAC-3′
ahoF	5′-TGTGGCCTCATCTCATCTATT-3′	ahoR	5′-GACCACTTTTATTTGTCACATGCAG-3′

Table 3 PCR primer sets used to amplify the *Hinb* gene sequences

Species	*Hinb, Hinb-1* and *Hinb-A*		*Hinb*, *Hinb-2* and *Hinb-B*	
	Forward primer	Reverse primer	Forward primer	Reverse primer
H. bogdanii	5bhoF	3bhoR	3bhoF	2bhoR
H. brachyantherum	5bhoF	4bhoR	4bhoF	2bhoR
H. bulbosum	n/a		2bhoF	2bhoR
H. chilence	5bhoF	3bhoR	n/a	
H. comosum	5bhoF	3bhoR	n/a	
H. marinum	n/a		2bhoF	2bhoR
H. murinum	n/a		2bhoF	2bhoR
H. patagonicum	5bhoF	4bhoR	n/a	
H. pusillum	5bhoF	3bhoR	n/a	
H. roshevitzii	5bhoF	3bhoR	2bhoF	2bhoR
H. vulgare subsp. *spontaneum*	1bhoF	1bhoR	2bhoF	2bhoR
H. vulgare subsp. *vulgare*	1bhoF	1bhoR	2bhoF	2bhoR

n/a The *Hinb* primers are not applicable

(*H. bulbosum*, *H. marinum*, *H. murinum*, *H. roshevitzii*, and two *H. vulgare* subspecies). We confirmed that these fragments contained *Hinb* genes by sequencing them directly. On the other hand, PCRs were unsuccessful for *Hinb* genes from the rest of six species (*H. bogdanii*, *H. brachyantherum*, *H. chilense*, *H. comosum*, *H. patagonicum*, and *H. pusillum*). Therefore, two new *Hinb-2*-specific primers (3bhoF and 4bhoF) were designed based on the *Hinb-2* flanking sequences of the two *H. vulgare* subspecies (Darlington et al. 2001; Caldwell et al. 2006) and those identified in this study. PCRs were successful in *H. bogdanii* and *H. brachyantherum* using these primers. However, PCRs were not successful for the remaining four species (*H. chilense*, *H. comosum*, *H. patagonicum*, and *H. pusillum*).

Thus, three new primers (3bhoR, 4bhoR, and 5bhoF) were designed based on the previously reported sequences of *Hinb*-1 and *Hinb-2* (Darlington et al. 2001; Caldwell et al. 2006), *Pinb* (*T. aestivum*, Chang et al. 2006 and *Aegilops speltoides*, Lillemo et al. 2002) and the *Hinb* sequences found in this study. PCRs were performed with these primers in all samples. Consequently, DNA fragments (about 520 bp) containing *Hinb* genes were amplified from seven species (*H. bogdanii*, *H. brachyantherum*, *H. chilense*, *H. comosum H. patagonicum, H. pusillum*, and *H. roshevitzii*).

We performed a BLAST search (NCBI) on the all *Hinb* genes identified in this study and confirmed that 13 were novel genes, and these sequences were registered in DDBJ (Table 1). Four *Hinb-1* and *Hinb-2* sequences of two *H. vulgare* subspecies were identical to already published sequences (Table 1). Comparison of the *Hinb* sequences (444 bp) revealed the presence of two *Hinb* genes in three species (*H. bogdanii*, *H. brachyantherum*, and *H. roshevitzii*). Since the sequence similarity between the two *Hinb* genes in the three species was lower than that between *Hinb-1* and *Hinb-2* genes in the two *H. vulgare* subspecies, we temporarily named one of the three *Hinb* genes, which were amplified by three new primes (3bhoR, 4bhoR, and 5bhoF), as *Hinb-A* and the other *Hinb* genes, which were amplified by four *Hinb-2*-specific primers (2bhoF, 2bhoR, 3bhoF, and 4bhoF), as *Hinb-B*. We summarized the identified *Hinb* genes of 12 species and the primer pairs used to amplify them as shown in Table 3.

In a comparison of the *Hinb* sequences with the *Pinb* sequence (AB180737), the ORF of all *Hinb* genes were 3 bp shorter than that of *Pinb*. Among the *Hinb* sequences, 85 sites (19%) displayed variation. The predicted amino acid sequences of *Hinb* are shown in Fig. 2. All of the HINB proteins contained 148 amino acid (a.a.) residues, 10 cysteines at the same positions and a Trp-rich hydrophobic domain spanning from position 68 to 74 (Fig. 2). The Trp-rich hydrophobic domain of eight HINB showed one amino acid substitution at position 71 compared with that of PINB. Seven HINB had a Lys-to-Arg substitution and one HINB had a Lys-to-Ser substitution (Fig. 2).

A phylogenic analysis with the 17 *Hinb* sequences and one *Pinb* DNA sequence showed that the 17 *Hinb* genes of the genus *Hordeum* also formed two distinct clusters (Fig. 3). The *Hinb-A* and *Hinb-B* genes were divided in two clusters. One cluster included all the *Hinb-A* genes, and the other cluster included all the *Hinb-B* genes, which were defined as Cluster-A and Cluster-B, respectively (Fig. 3). Seven *Hinb* genes (*H. bogdanii Hinb-A*, *H. brachyantherum Hinb-A*, *H. chilense Hinb*, *H. comosum Hinb*, *H. patagonicum Hinb*, *H. pusillum Hinb*, and *H. roshevitzii Hinb-A*) that belonged to **I** genome species were included in Cluster-A. The remaining 10 *Hinb* genes, which belonged to the **H**, **I**, **Xa** and **Xu** genome species, were included in Cluster-B. Cluster-B included the *Hinb-1* and *Hinb-2*

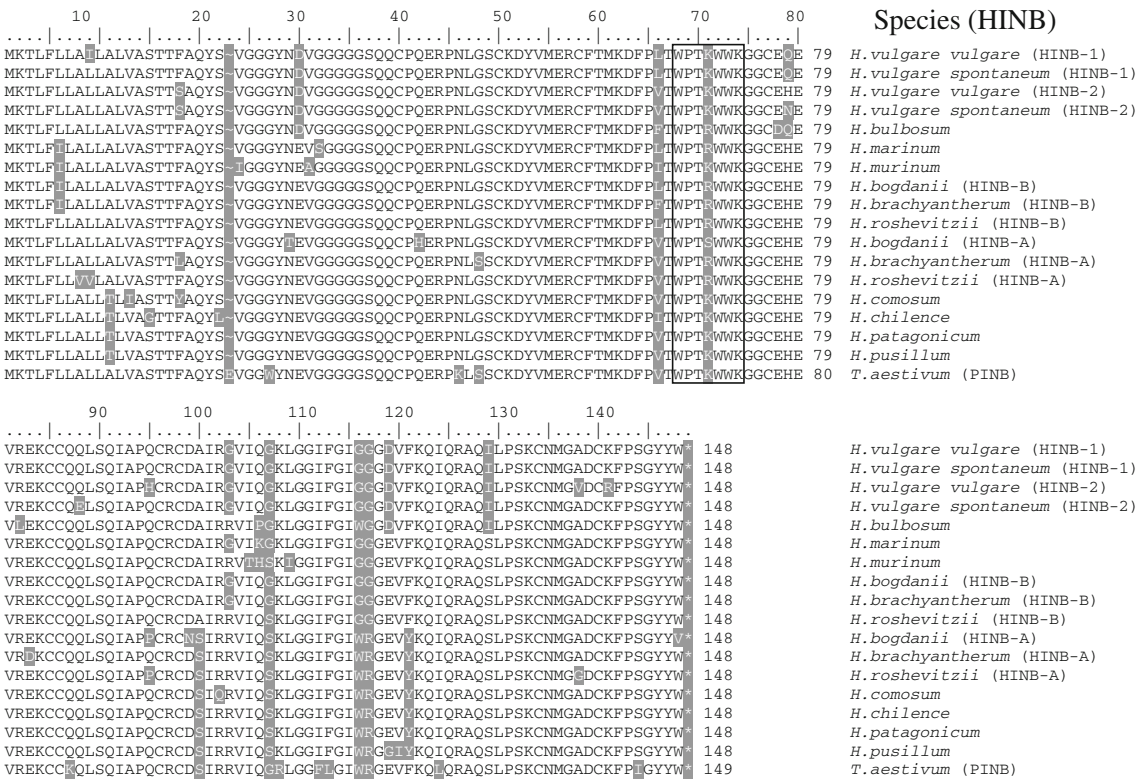

Fig. 2 Alignment of predicted amino acid sequences of the HINB and PINB. The conserved Trp-rich hydrophobic domains at positions from 68 to 74 are indicated by a *rectangle*. Polymorphic sites are *shaded*. Amino acid numbering is based on the methionine (M) start codon. *Dashes* indicate gaps

genes of the *H. vulgare* subspecies, and these genes formed a single clade in the phylogenic tree (Fig. 3). The *T. aestivum Pinb* lineage branched in between Cluster-A and Cluster-B (Fig. 3).

Analyses of *hordoindoline-a*

PCRs were carried out in all samples using *Hina*-specific primers (ahoF and ahoR). DNA fragments (about 560 bp) were amplified using all samples with *Hina*-specific primers. We confirmed that these DNA fragments contained *Hina* genes by sequencing them directly.

We performed a BLAST search (NCBI) on the 12 *Hina* genes identified in this study and confirmed that 10 *Hina* were novel genes, and these sequences were registered in DDBJ (Table 1). The *Hina* sequences of two *H. vulgare* subspecies were identical to already published sequence (Table 1). The eight *Hina* gene sequences (*H. bogdanii*, *H. brachyantherum*, *H. chilense*, *H. comosum*, *H. marinum*, *H. pusillum*, *H. patagonicum* and *H. roshevitzii*) were 447 bp in length, whereas the four *Hina* genes (*H. bulbosum*, *H. murinum*, cultivated barley, and *H. vulgare* subsp. *spontaneum*) were 450 bp long. Among the *Hina* nucleic acid sequences, 77(17%) sites displayed variation.

The predicted amino acid sequences of *Hina* are shown in Fig. 4. Eight HINA (*H. bogdanii*, *H. brachyantherum*, *H. chilense*, *H. comosum*, *H. marinum*, *H. pusillum*, *H. patagonicum*, and *H. roshevitzii*) possessed stop codons at position 149; on the other hand, four HINA (*H. bulbosum*, *H. murinum*, cultivated barley, and *H. vulgare* subsp. *spontaneum*) contained Trp at the same position and a stop codon at position 150 (Fig. 4).

All HINA proteins contained 10 cysteines at the same positions and the Trp-rich hydrophobic domain spanning from position 67 to 74 (Fig. 4). The Trp-rich hydrophobic domain of all HINA showed one amino acid substitution compared with that of PINA: Two HINA had a Lys-to-Arg substitutions at position 71, and 10 HINA had a Lys-to-Thr substitution at the same position (Fig. 4).

A phylogenic analysis of the *Hina* and *Pina* genes constructed from 13 nucleic acid sequences showed that 12 *Hina* genes did not form distinct clusters, as was found in the *Hinb* tree (Fig. 5), but rather formed divergent clusters corresponding to the four genome groups (**H**, **I**, **Xa**, and **Xu** genomes). The cluster consisting of the **Xu** genome species was closest to that of the **H** genome species, and the cluster containing the **I** genome species was most distantly related to that containing the **H** genome species.

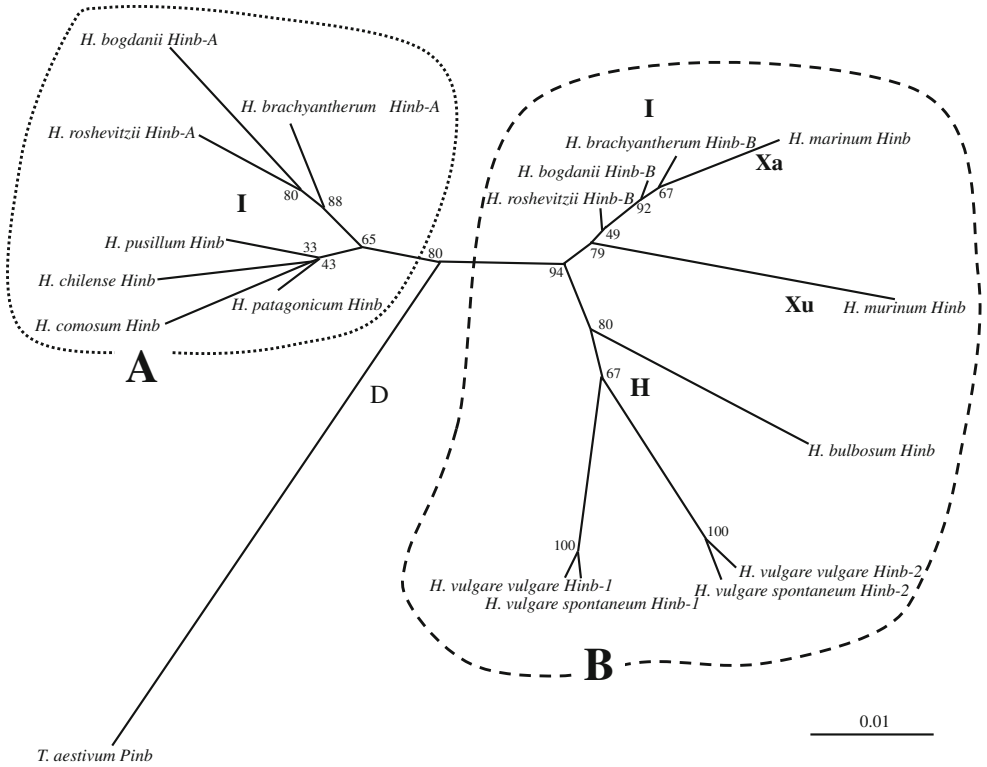

Fig. 3 Phylogenetic tree of the *Hinb* and *Pinb* genes based on 18 sequences from 13 species of the genus *Hordeum* and *T. aestivum*. *Hordeum* genomes (**H**, **I**, **Xa**, and **Xu**) and one *Triticum* genome (**D**) are indicated. The numbers beside the branches indicate bootstrap values (bootstrap reps 10,000). *Scale bar* ratio of nucleotide substitutions. *A* and *B* indicate clusters

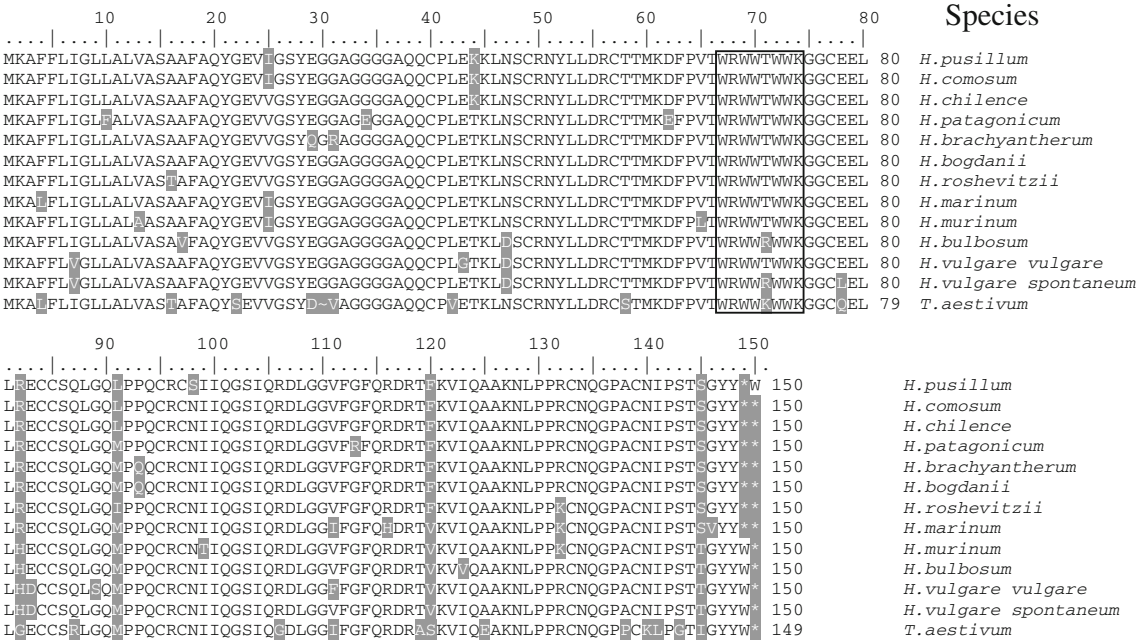

Fig. 4 Alignment of the predicted amino acid sequences of the HINA and PINA. The sequence description and labels are the same as in Fig. 2

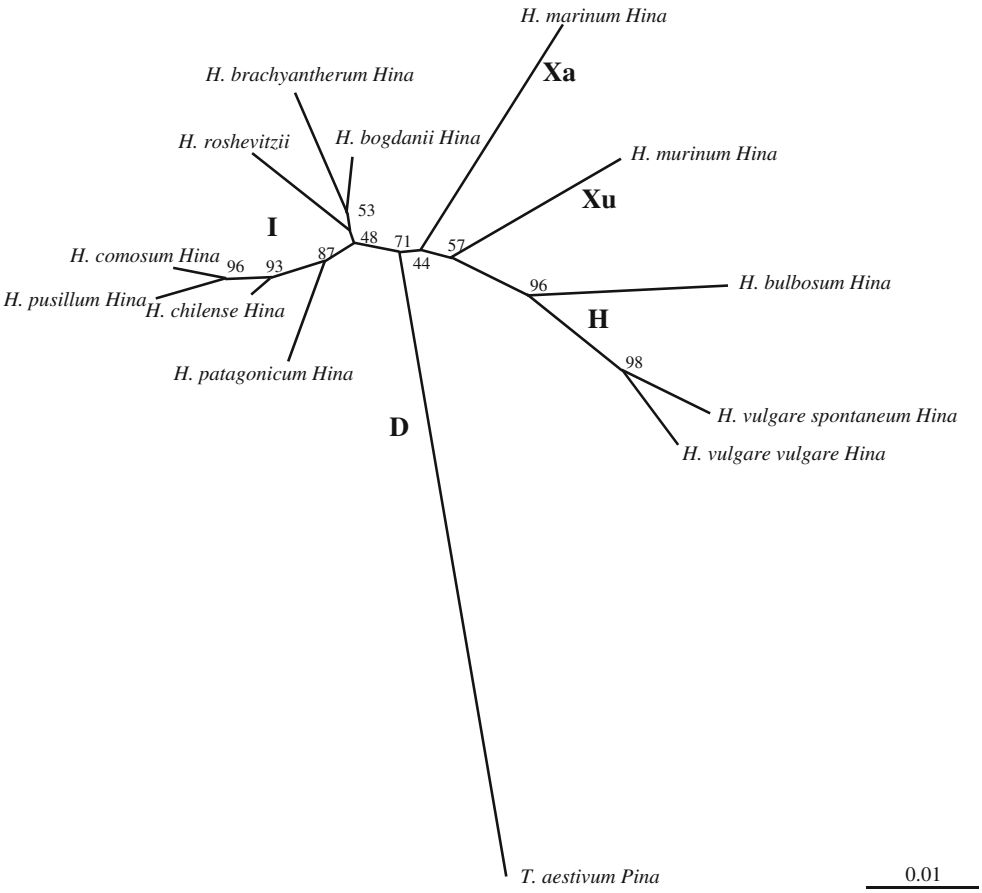

Fig. 5 Phylogenetic tree of the *Hina* and *Pina* genes based on 14 sequences from 13 species of the genus *Hordeum* and *T. aestivum*. Tree description and labels are the same as in Fig. 3

Discussion

Previous studies identified the *Hin* genes and revealed their distribution within *H. vulgare* subspecies (Darlington et al. 2001; Caldwell et al. 2006). However, the interspecific variation in the *Hin* genes among the genus *Hordeum* has not been studied in detail. We studied the *Hin* genes and the distribution of the *Hin* genes in two *H. vulgare* subspecies and 10 wild relatives.

We identified 13 novel *Hinb* genes and revealed that three species (*H. bogdanii*, *H. brachyantherum*, and *H. roshevitzii*) possessed two *Hinb* genes (*Hinb-A* and *Hinb-B*). To confirm whether *Hinb-A* and *Hinb-B* are tandemly located at the *Ha* locus, as was found for *Hinb-1* and *Hinb-2* (Caldwell et al., 2004), PCR analyses in the three species were carried out using primers (5bhoF and 2bhoR in Fig. 1) and new primers (data not shown) spanning *Hinb-1* and *Hinb-2* to amplify longer DNA fragments. This attempt was not successful at amplifying specific PCR products (data not shown). We consider that these genes might not be tandemly located as found in *H. vulgare*.

Cluster analysis showed that the 17 *Hinb* genes found to be present among *Hordeum* species formed two clusters (Fig. 3). Furthermore, the three **I** genome species (*H. bogdanii*, *H. brachyantherum* and *H. roshevitzii*) with two *Hinb* genes (*Hinb-A* and *Hinb-B*) were occured in both clusters (Fig. 3). On the other hand, the *Pinb* branch was not included in these clusters, and its branching point was located in between the two clusters (Fig. 3). Therefore, we consider that the *Hinb-A* and *Hinb-B* genes in the **I** genome, sharing common ancestor with wheat *Pinb* gene, did not duplicate after the speciation of the **I** genome species. Instead, we consider that *Hinb* gene duplication occurred during the early stages of the speciation of the genus *Hordeum* and that the ancestral species of the genus *Hordeum* probably had two *Hinb genes*. Since our phylogenic analysis of the *Hinb* gene showed that the *Hinb-1* and *Hinb-2* genes of *H. vulgare* formed a single clade within Cluster-B (Fig. 3), the *Hinb-1* and *Hinb-2* genes in *H. vulgare* might have been generated by a duplication of the *Hinb* gene after the speciation of *H. vulgare*. It was also suggested that all of the *Hordeum* species except the **I** genome species lost one of their *Hinb* genes during the evolutionary process.

All HINs identified in this study possessed 10 cysteine sites and a Trp-rich hydrophobic domain, as are found in PINs (Figs. 2, 4). Therefore, HINs probably have the same function as PINs, which are involved in grain hardness and antimicrobial activity (Giroux and Morris 1997, 1998;

Morris 2002; Jing et al. 2003; Phillips et al. 2011). It is interesting that the Trp-rich hydrophobic domains of all HINA and eight HINB showed one amino acid substitution compared with those of PINA and PINB, respectively. At position 71, 10 HINA had Thr and two HINA had Arg instead of Lys found at the corresponding position in PINA (Fig. 4). In recent study, Phillips et al. (2011) reported that a Lys-to-Thr substitution in HINA resulted in a general increase in antifungal activity. Moreover, Massa and Morris (2006) reported PINA of *Aegilops* species have Arg instead of Lys and discussed that both Arg and a Lys can interact with Trp through cation-π interactions. HINB analyzed in this study have an Arg in seven species or Ser in one species instead of Lys at position 71 (Fig. 2). None of a Lys-to-Ser substitution at this domain was reported previously in PINB and HINB, therefore this is the first report of this substitution. Further analysis is necessary to clarify the effect of these substitutions on HINs functions.

The phylogenic trees of *Hina* and *Hinb* were compared with those based on rDNA internal transcribed spacer (ITS) sequences (Blattner 2004) and thioredoxin-like (HTL) gene sequences (Kakeda et al., 2009). Both the HTL and ITS trees showed four clearly divergent clusters corresponding to the four genomes (**H**, **I**, **Xa** and **Xu**). The *Hina* tree also showed clusters for the four genome groups. On the other hand, the *Hinb* phylogenic tree showed two distinct clusters due to gene duplication during the early stages of the speciation of the genus *Hordeum* (Fig. 3). Interestingly, all seven *Hinb* genes within Cluster-A were **I** genome species (Fig. 3). We consider that the ancestral species of the **H**, **Xa**, and **Xu** genomes lost one of their *Hinb* genes during the evolutionary process. For Cluster-B, we found distinct clades consisting of species from the **H**, **I**, **Xa**, and **Xu** genomes as found for the HINA tree.

This study revealed the distribution of the *Hin* genes in the genus *Hordeum*. Our results strongly suggest that *Hinb-1* and *Hinb-2* arose by gene duplication after the split of the *H. vulgare*-lineage from the *H. bulbosum*-lineage. Moreover, this study shows that the ancestral species of the genus *Hordeum* most probably had two *Hinb genes*. Further studies are necessary to clarify the evolution and function of the *Hina* and *Hinb* genes at the *Ha* locus.

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