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ORIGINAL PAPER

Novel *Hina* alleles created by genome editing increase grain hardness and reduce grain width in barley

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Abstract The *hordoindolina* genes (*Hina* and *Hinb*) are believed to play critical roles in barley (*Hordeum vulgare* L.) grain texture. In this study, we created novel alleles of the *Hina* gene using CRISPR/Cas9 (Clustered regularly inter spaced short palindromic repeat-associated protein, CRISPR-Cas) genome editing. Mutagenesis of single bases in these novel alleles led to loss of Hina protein function in edited lines. The grain hardness index of *hina* mutants was 95.5 on average, while that of the wild type was only 53.7, indicating successful conversion of soft barley into hard barley. Observation of cross-sectional grain structure using scanning electron microscopy revealed different adhesion levels between starch granules and protein matrix. Starch granules were loose and separated from the protein matrix in the wild type, but deeply trapped and tightly integrated with the protein matrix in *hina02* mutants. In addition, the grain width and thousand-grain weight of the *hina02* mutant were significantly lower than those of the wild type.

Keywords Barley · Grain hardness · *Hina* · CRISPR/Cas9 · Genome editing

Introduction

Barley (*Hordeum vulgare* L.) is one of the most ancient cereal crops and currently ranks fourth in grain yield and area of cultivation throughout the world. At present, it is mainly used for animal feed and beer brewing, and a small part is used for food and pharmaceutical raw materials (Mantovani et al. 2008). Recent research on the health benefits of β-glucans is rekindling an interest in barley as a major food source for humans (Behall et al. 2004, 2006; Keenan et al. 2007). Moreover, barley is suitable for cultivation under a wide range of environmental conditions. It is highly salt tolerant and can thrive well in regions with very dry, cold climates, where other major crops such as wheat and oat fail to grow (Mahdi et al. 2008).

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Grain hardness, defined as the resistance of the kernel to deformation, is the most important and defining quality of barley and wheat, and is the main standard of international commercial cereal classification (Symes 1969). Cereal kernels are divided into hard kernels and soft kernels according to differences in grain hardness (Turnbull and Rahman 2002). Previous studies have shown that grain hardness significantly affects pearling and milling properties of cereal kernels, flour particle size distribution, starch damage, and flour water absorption (Edney et al. 2002). During dehulling and pearling, loss of endosperm from hard kernels is minimized (Baik and Ullrich 2008). Meanwhile, uniform-sized, bright yellow, plump, thin-hulled, and medium-hard barley kernels are given priority for food consumption. In addition, the malting and feed qualities of barley are strongly associated with grain hardness. (Bowman et al. 2001; Nair et al. 2011; Walker et al. 2011; Mayolle et al. 2012).

Hordoindoline (Hin) and grain softness protein-1 (Gsp-1) are involved in the regulation of grain hardness in barley and are orthologs of wheat Puroindoline (Pin) and Gsp-1 (Gautier et al. 1994, 2000). Mutations in the *Pina* or *Pinb* genes result in hard grain texture (Giroux and Morris 1997; Bhave and Morris 2008); conversely, heterologous expression of *Pina* or *Pinb* in transgenic rice plants confers softer endosperm texture (Krishnamurthy and Giroux 2001). Turuspekov et al. (2008) identified 5 alleles of *Hina*, 6 alleles of *Hinb-1*, 18 alleles of *Hinb-2*, and 18 alleles of *Gsp* in 81 spring barley lines. Grains possessing the most common *Hina*, *Hinb-1*, *Hinb-2*, and *Gsp* genotype combinations had significantly softer texture and increased starch content compared with other genotypes. By contrast, the *Hinb-2* null mutation was associated with increased grain hardness. Although several variations in *Hina* and *Hinb* alleles have been reported in cultivated and wild barley (e.g. *H. vulgare* subsp. *spontaneum*, *Hordeum bogandii*, *Hordeum chilense*, *Hordeum bulbosum*, etc.) (Caldwellet al. 2006; Li et al. 2010; Terasawa et al. 2012; Turuspekov et al. 2008), barley with harder grains is relatively scarce, and the difference in endosperm texture between hard grains and soft grains has received little attention.

The recently established gene editing tool, clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9, derived from the adaptive immunity system of *Streptococcus pyogenes*, has provided a breakthrough (Jinek et al. 2012) enabling genomic targeting in many organisms including plants (Cong et al. 2013; Jiang et al. 2013; Shan et al. 2013; Li et al. 2013; Xie and Yang 2013; Mao et al. 2013; Jin et al. 2013; Zhou et al. 2014; Zhang et al. 2014; Fauser et al. 2014; Li et al. 2020a, b; Susana et al. 2018; Zhang et al. 2019a, b; Yang et al. 2020; Zhang et al. 2021; Qiang et al. 2022). In this study, The barley *Hina* gene without introns is located on the chromosome 5H: 1,602,184–1,601,735 of 'Golden Promise' pseudomolecules GPv1, and encodes the 149 amino acids. We created novel *Hina* alleles using the CRISPR/Cas9 system in the commercial barley variety 'Golden Promise' and successfully obtained a barley mutant with increased grain hardness and reduced grain width. In the endosperm of the *hina* mutant, the protein matrix was tightly wrapped around A-type starch and B-type granules, which may give the grain more resistance to deformation.

Materials and methods

Plant materials

The soft spring barley (*Hordeum vulgare* L.) 'Golden Promise' was grown in a greenhouse under controlled conditions of 18 °C during 16 h of light and 15 °C during 8 h of darkness. When immature embryos were 1.5–2 mm in diameter, immature grains were collected from the center of spikes. Immature embryos were used for *Agrobacterium*-mediated transformation.

SgRNA design and plasmid construction

To edit the *Hina* gene in barley, two target sequences (hinT1: CGT GGG TCT GCT TGC TT TGG; hinT2: TGC AAC ATC CCC AGC AC TAC) were designed to recognize the 5′ regions of the conserved coding sequence using the online software CRISPR-GE (http://skl.scau.edu.cn/home). The CRISPR/Cas9 system was provided by Mr. Liu Yaoguang from South China Agricultural University (Guangdong China). The Cas9 endonuclease gene from the Streptococcus pyogenes type II CRISPR/Cas system was optimized according to the codon usage bias of plant. Construction of sgRNA was conducted following

previously published protocols (Ma and Liu 2016; Xie et al. 2017). Two double-stranded target adaptors consisting of target sequence and adaptor were ligated to the *Bsa*I-digested sgRNA vector carrying OsU6 promoter (NEbiolabs, Beijing, China), then overlapping PCR using target sequence-containing chimeric primers and nested PCR using site-specific primers were successively performed to generate the sgRNA expression cassette, respectively. Finally, two sgRNA expression cassettes were assembled into the pYL-CRISPR/Cas9 binary plasmid in one round of cloning using the Golden Gate ligation method (Ma and Liu 2016; Xie et al. 2017). The recombinant vector was transferred into competent cells of *Agrobacterium tumefaciens* EHA105 (Huayueyang, Beijing, China).

Agrobacterium-mediated transformation of barley

Agrobacterium-mediated transformation of 'Golden Promise' was performed as described by Harwood (2019). A single colony of *A. tumefaciens* EHA105 carrying the pYLH-hina vector with Cas9 and two sgRNA cassettes was used to inoculate 10 mL of MG medium. Immature embryos were separated from sterile grains and co-cultivated with *Agrobacterium* EHA105 infection solution for 3 days at 24 °C in the dark. Subsequently, these embryos were transferred to selective medium containing 50 mg/L hygromycin (Coolaber, Beijing, China) and cultured for 6–8 weeks at 24 °C in the dark. Proliferating calli were transferred to transition medium containing 30 mg/L hygromycin and cultured at 24 °C under a 16 h low light/8 h dark photoperiod. Small plantlets were then transferred to regeneration medium containing 30 mg/L hygromycin and cultured for 2 weeks at 24 °C under a 16 h light/8 h dark photoperiod. Putative transgenic plants were transferred to soil and grown to maturity in the greenhouse, under controlled conditions of 18 °C during 16 h light and 15 °C during 8 h darkness.

Detection of transgenic plants and mutations

Genomic DNA was isolated from leaves of putative transgenic plants using a TaKaRa MineBEAT Plant Genomic DNA Extraction Kit (TaKaRa, Beijing, China). For detection of T-DNA insertions, PCR was performed with primers ubiF/R (supplementary material Table S1) using 100 ng of DNA in a 20 µL reaction (Sangon Biotech, Shanghai, China). To detect mutations in the *Hina* gene, primers TOMos74T1F/R and TOMos74T2F/R (supplementary material Table S1) were designed against the targeted regions and PCR was performed using a Hi-TOM Gene Editing Detection Kit (Qingxue Biotech, Xi 'an, China). Amplification products from transformed samples were sequenced using an Illumina HiSeq PE150 system (Novogene, China). Sequencing results were analyzed using Hi-TOM (High-throughput Tracking of Mutations, http://www.hi-tom.net/hi-tom/). Nucleotide sequences were analyzed using the AlignX program (Invitrogen, CA, USA). Primers hina02F/R and hinawtF/R (supplementary material Table S1) specific to the mutant sequence of the hina02 line were designed for screening homozygous mutant plants.

Analysis of grain hardness and quality

To analyze phenotypic differences in grain hardness between the wild type and mutant, we used a Single Kernel Characterization System (SKCS) 4100 (Perten Instruments) to measure 300 grains according to American Grain Science Association standard AACC 55-31. The hardness index (HI) of a single grain was obtained. Barley was dehulled before the SKCS test to prevent the SKCS from clogging.

Scanning electron microscopy of endosperm

Grains of the wild type and hina02 line were selected. Transverse sections of dried grain were attached to metallic stubs using carbon stickers and sputter-coated with gold for 30 s. Images were observed and captured using an SU8100 scanning electron microscope (Hitachi, Tokyo, Japan).

Determination of grain quality and agronomic traits

T2 plants generated from hina02 homozygous mutant were grown in a greenhouse under controlled conditions of 18 °C during 16 h light and 15 °C during 8 h darkness. Analysis of the agricultural traits of wild-type and mutant plants was performed using 20 plants. Plant height, number of tillers, spike length, and grains per spike were recorded. Starch content and protein content of grains were determined, respectively, via the acid hydrolysis method and the

Kjeldahl method (Bowman et al. 1988). Every sample was analyzed three times. Three spikes per plant of a minimum of ten mutant and wild type plants were selected for phenotyping. The WSEEN Grain Test System (WSeen, http://www.wseen.com/) was used to measure grain length, grain width, and thousand-grain weight.

Statistical analyses

To analyze the normal distribution of data we performed a Shapiro–Wike (S–W) normality test and then performed an unpaired t-test to test for differences between conditions. Results are expressed as mean ± standard deviation (SD) and considered signifificant at $P < 0.05$. Statistical analyses were conducted using GraphPad Prism 8.0 software (San Diego, CA, USA). Asterisks denote statistical significance (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

Results

Mutation detection

To edit the *Hina* gene in barley, we designed two target sites (hinT1 and hinT2) (Fig. 1a) for the conserved regions of the coding sequence and assembled these into a single vector to generate the construct pYLH-hina using Golden Gate ligation (Fig. 1b). pYLH-hina was transformed into immature barley embryos via *Agrobacterium*-mediated transformation. A total of 12 plantlets were obtained from 355 immature embryos. DNA was isolated from leaves of T0 plants, and 12 independent transgenic plants were identified by PCR amplification (supplementary material Figure S1).

Targeted mutagenesis in transgenic plants was examined using Hi-TOM Gene Editing Detection, and the resulting sequences were aligned against the wild-type DNA sequence. Four T0 mutant plants (hina02, 06, 07 and 10) and one T1 mutant plant (hina12) were identified by Hi-TOM sequencing, indicating that the CRISPR/Cas9 system continued to function in the offspring, with an editing efficiency of 42%. We detected mutations at the T1 target site; however, no plants carrying a mutation at the T2 site were identified among the transformation events. Line hina02 had a 1-bp (C>A) substitution, a 2-bp (C and A) deletion, and a 12-bp (ACA ACAATTAA) insertion, while line hina06 harbored two types of 2-bp (C>T and C>A) substitutions and a 1-bp (G) insertion and line hina07 had a 1-bp (G) insertion. Line hina12 had a 21-bp (GGGTCTGCT TGCTTTGGT) deletion (Fig. 1c), whereas mutagenesis in lines hina02, 06, and 07 caused a frameshifts

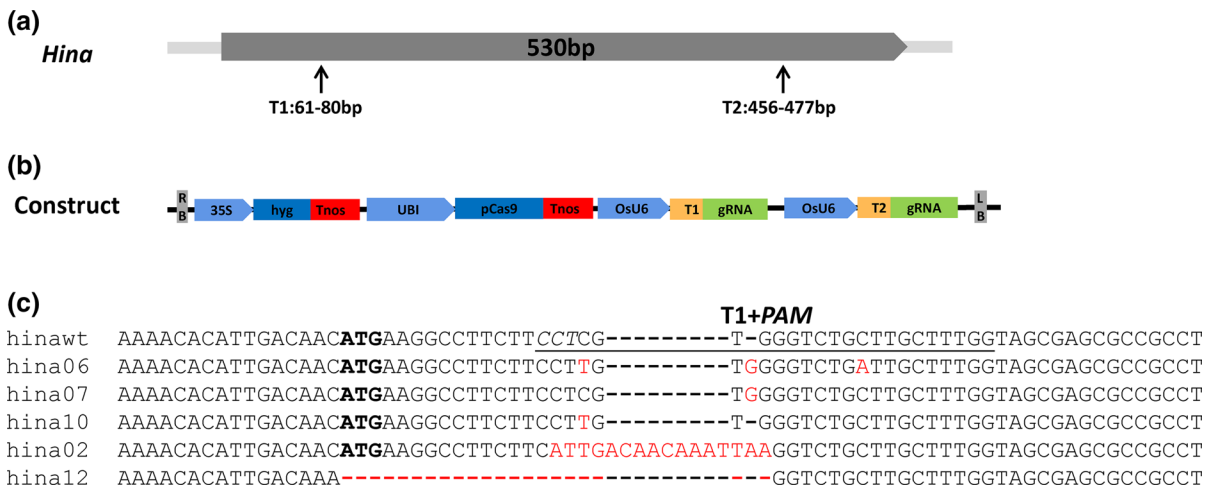

Fig. 1 Schematic diagram of target sites in the *Hina* gene and genome editing vector. **a** Structure of the *Hina* gene and locations of target sites T1 and T2 in the coding domain. **b** Schematic illustration of CRISPR/cas9 construct. PCas9:Cas9 gene modified with plant-optimized codons; OsU6: snRNA gene promoter from rice; gRNA: guide RNA; T: target site. **c** Mutation types in transgenic plants revealed by DNA sequencing; mutant genotypes at theT1 site

and premature termination codon to appear at bp 272 and 232, respectively. The hina12 mutant lacked an initiation codon, while the amino acid sequence of the hina10 mutant was not changed (supplementary material Figure S2).

Determination of grain hardness and endosperm microstructure

Due to a limited quantity of grains, we first analyzed the distribution of single grain HI in a heterozygous group generated from hina02, hina06, hina07, and hina12 edited plants; these plants produced wild-type grains, heterozygous mutated grains, and homozygous mutated grains. The grain HI of the heterozygous group was from 32.2 to 95.56, with an average of 65, while the HI of wild type was 32.47–71.94, with an average of 53.81 (Fig. 2). The HI of hina10 edited plants was similar to that of the wild type. This result shows that grains of *hina* mutants, except hina10, possessed a higher HI. Homozygous mutations of line hina02 were identified by PCR (supplementary material Figure S3), and the grain HI of the homozygous hina02 mutant was 93.08 (from 55.89 to 105.91) on average (Figs. 2, 4e), representing an increase of 72.97% compared with the wild type.

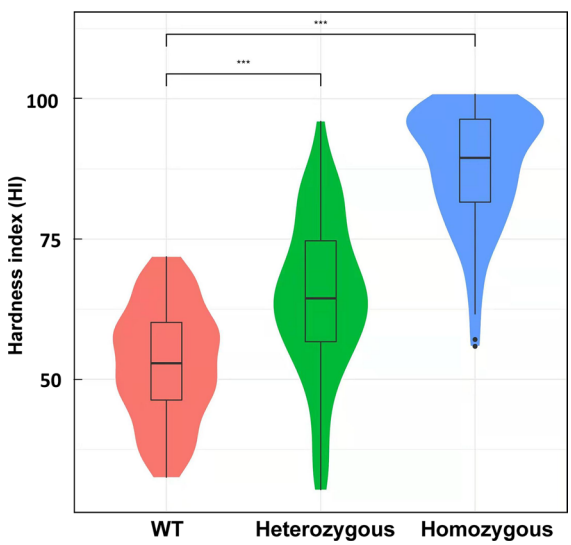

Fig. 2 Single grain hardness index (HI) distribution of wild type and editing plants. WT: wild type; Heterozygous: the group generated from hina02, hina06, hina07, and hina12 edited plants and consisted of wild-type grains, heterozygous mutated grains, and homozygous mutated grains; Homozygous: the grains of homozygous plants screened from hina02 mutant; Error bars indicate standard deviation. ***$P < 0.001$

Scanning electron microscope images of fractured endosperms illustrated differences between the wild type and hina02 mutant. In the wild type, the endosperm possessed large (A-type) starch granules and numerous small (B-type) starch granules. A-type starch granules were smooth and devoid of the associated protein matrix. The starch granule surface in the wild type was relatively free of associated protein material, and the spaces between adjacent starch granules appeared to be largely devoid of matrix protein (Fig. 3a–c). In the hina02 mutant, the spaces between adjacent starch granules were filled with protein matrix, which appeared to coat A-type starch granules and to embed B-type starch granules completely (Fig. 3d–f). These images indicate that starch granules were loose and separated from the protein matrix in the wild type, while they were deeply trapped and tightly integrated with the protein matrix in the mutant.

Agronomic traits and grain quality

Plants generated from the homozygous hina02 mutant shared the same appearance as the wild type. Phenotypically, there were no differences in plant height, number of tillers, spike length, grains per spike, or grain length (Fig. 4a and supplementary material Table S2). However, the grain width and thousand-grain weight of the hina02 mutant were 14.67% and 22.92% lower than those of the wild type, respectively (Fig. 4b–d). The starch and protein content of hina02 were 68.13% and 14.03%, while those of wild type were 63.71% and 11.83% respectively (Fig. 4f, g). These results suggested that knocking-out of *Hina* gene may affect grain development.

Discussion

Grain hardness has great influence on the end-use properties of cereal kernels (Ma et al. 2017). In wheat, hard kernels (Grain HI > 60) with larger flour particle, more damaged starch and higher water absorption is suitable for making breads and noodles, conversely hard kernels (Grain HI < 40) with smaller flour particle, fewer damaged starch and lower water absorption is suitable for making biscuits, cakes and other sweet

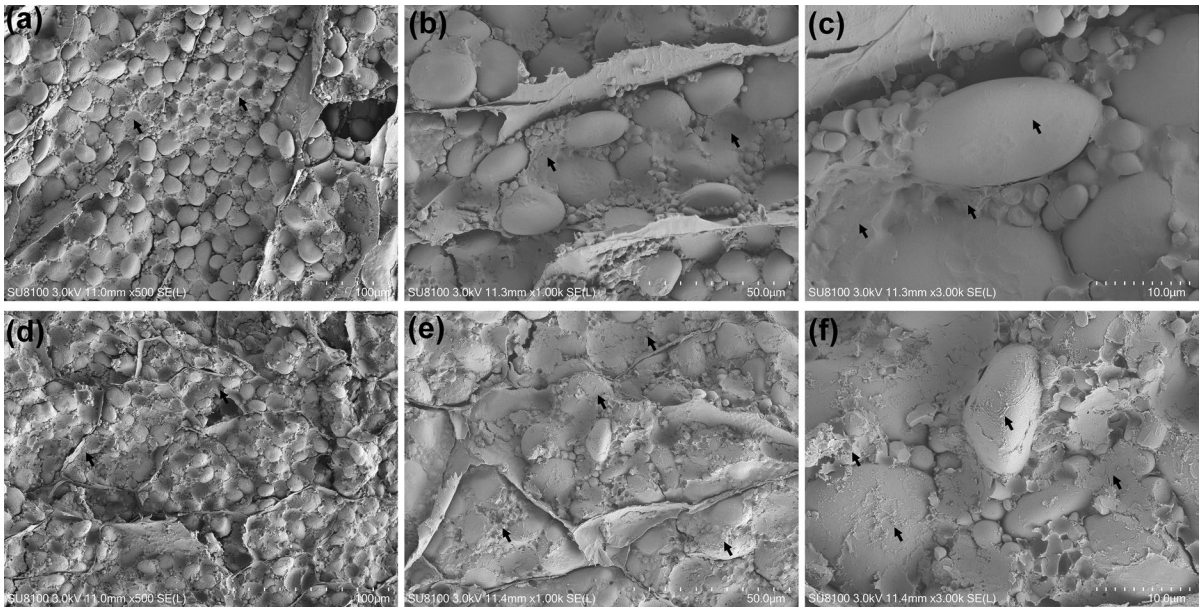

Fig. 3 Scanning electron microscope images of fractured endosperms illustrating differences in starch–protein binding between the wild type and hina02 mutant. **a**–**c** Endosperm of wild type. **d**–**f** Endosperm of hina02 mutant. A: A-type starch granules; B: B-type starch granules; P: protein matrix

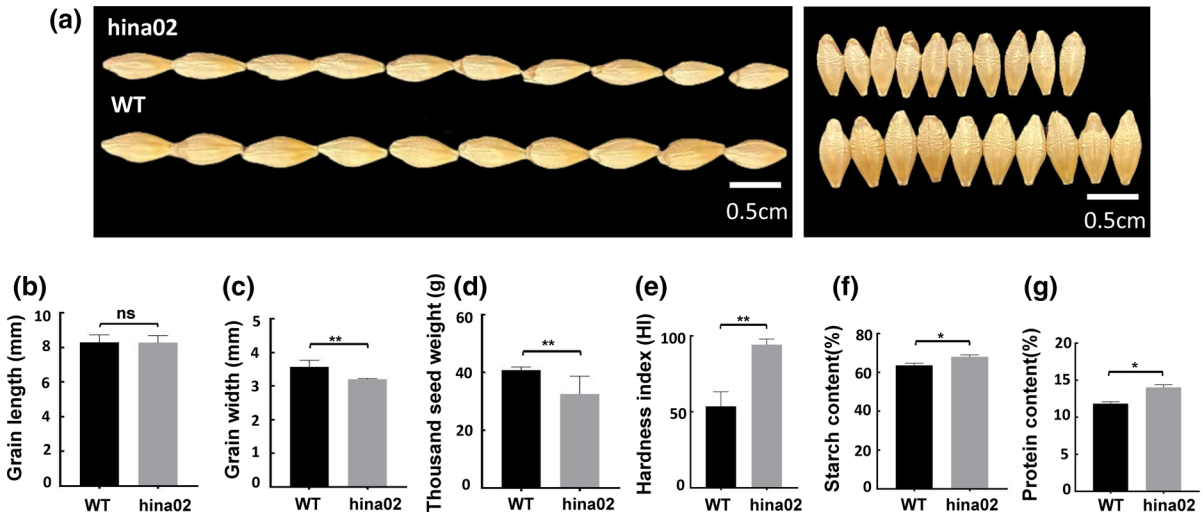

Fig. 4 Phenotypic and quality difference between the wild type and hina02 mutant. **a**, **b** the grains of wild type and hina02 mutant; **c**–**g** Comparison of grain length, grain width, thousand-grain weight, hardness index, starch and protein content of grain between wild type and hina02 mutant. WT: wild type; error bars indicate standard deviation; ns: no significance; $*P < 0.05$; $**P < 0.01$

food (Chen et al. 2005). Grain hardness of common wheat is mainly controlled by *Puroindoline* genes, *Pina* and *Pinb*. Much information is available on the wheat *Pin* alleles and their causal association with texture (Bhave and Morris 2008); however, information about the orthologous *Hin* genes of barley

is limited to a few reports (Walker et al. 2011). The role of *Hin* genes in influencing barley grain texture has only been shown so far by Takahashi et al (2010). Mutation of the *Hinb-2* gene leads to a minor increase in grain hardness. The diversity and potential role of these proteins in barley grain structure therefore needs further analysis.

Here, we investigated *Hina* gene to gain an understanding of the genetic diversity of it and its contribution to textural variations in barley. The *Hina* gene of barley was knocked out using the CRISPR/Cas9 system. Grain hardness and structure were compared between wild type and *hina* mutants sharing the same genetic background. Grain HI based on the SKCS was elevated in all *hina* mutants except hina10, which possessed a synonymous mutation. The HI of the hina02 mutant was 95.5 on average and higher than that of varieties (51.3–80.1) containing *Hinb* alleles across different geographic regions (Beecher et al. 2001; Takahashi et al. 2010; Iwami et al. 2005; Fox et al. 2007). Mutation of *Hina* alleles in the 5′ region of the coding sequence resulted in loss of protein function, which may have important influence in the formation of grain texture. Microscopic observations of endosperms further confirmed our hypothesis. Compared with the wild type, starch granules were deeply trapped and tightly integrated with the protein matrix in the mutant. These structural characteristics may endow the grain with greater hardness.

Furthermore, previous studies have shown that the environment had a significant effect on the SKCS value, this variation could be explained partially by the protein content of the grain, i.e. increasing protein causes the grain to harden (Fox et al. 2007). In our study, all plants were grown in a greenhouse under consistent condition. So the increase of Grain HI in mutant was largely coused by knocking-out of *Hina* gene. Interestingly, the protein content of hina02 were18.59% higher than it of the wild type. However, the relation of *Hina* gene and protein content still needs further research. Finally, this new cultivar with a much higher grain harness provides a good germplasm resource for improving the quality of barley and understanding the formation of endosperm structure.

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Author contributions JL and BL conceived and designed the experiments; YJ and YL performed the experiments and wrote the main manuscript text; YC constructed the expression plasmid; DC and YZ analyzed the data.

Declarations

Competing interests The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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