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Isolation, sequencing of the *HvnHID* gene and its role in the purple-grain colour development in Tibetan hulless barley

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Abstract: 2-hydroxyisoflavanone dehydratase (HID) plays an important role in isoflavone biosynthesis. In this study, *HID* was isolated from the seeds of the purple-grained Tibetan hulless barley variety Nerumuzha and the white-grained variety Kunlun 10. The *HvnHID* gene includes the 981 bp open reading frame and encodes a protein of 327 amino acids. It has a typical Abhydrolase_3 domain (78–306) and belongs to the carboxylesterase (CXE) family of the Abhydrolase_3 (α/β hydrolase) superfamily. There are eight nucleotide differences in the *HvnHID* coding sequence and two amino acid differences (one in the Abhydrolase_3 domain) between Nerumuzha and Kunlun 10. The HvnHID of hulless barley has the closest relationship with the HID in *Hordeum vulgare*, and the most distant relationship in *Panicum hallii*. At the early--mid stage of the seed colour development, the *HvnHID* expression levels in the purple and black seeds were significantly higher than in the white and blue ones ($P < 0.01$). During the seed colour development of purple-grained hulless barley, the expression of the key genes (*HvnF3'H*, *HvnDRF*, *HvnANT1,* and *HvnGT*) in the anthocyanidin biosynthetic pathway increased significantly, while the *HvnHID* expression decreased significantly ($P < 0.01$). Thus, it is likely that *HvnHID* negatively regulates the anthocyanidin biosynthesis. This result provides an important basis for further study of the biological functions of *HvnHID* in the anthocyanidin biosynthetic pathway.

Keywords: 2-hydroxyisoflavanone dehydratase; anthocyanidin; isoflavones; purple grain

Hulless barley (*Hordeum vulgare* L. var. *nudum* Hook. f.) is a crop of the genus barley in the Gramineae family. It is also known as naked barley because its seeds are not covered by glumes at ripening (Liang et al. 2012). Naked barley growing on the Qinghai-Tibet Plateau is called Tibetan hulless barley (Qingke). It is the only crop that can mature normally on the plateau and cold areas over 4 000 m a.s.l. The genetic background of hulless barley is highly similar to that of barley (Zeng et al. 2018). Hulless barley is nutritious and has a prominent value for medicine and health, due to its high protein, fibre, and vitamin contents and low fat and sugar contents. Therefore, hulless barley has become increasingly popular in recent years (Li et al. 2011; Wu et al. 2017a).

Coloured barley refers to germplasms that have coloured seeds at ripening; these mainly include black, purple, and blue barley (Shoeva et al. 2016).

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Studies have shown that the appearance of barley with a blue grain is associated with anthocyanidin accumulation in the seed aleurone layer (Akashi et al. 2005; Jia et al. 2020). Meanwhile, the appearance of purple, black, and red grain in barley is associated with anthocyanidin accumulation in the pericarp and glume (Lenfant et al. 2012; Ferreira et al. 2019). Anthocyanidin is the final product of flavonoid metabolism and is a water-soluble pigment that can change colour according to the pH value of the cellular fluid. For example, anthocyanidin exhibits a reddish or bluish colour in acidic or alkaline cellular fluid, respectively (Tanaka et al. 2008). The synthesis of anthocyanins is regulated by many structural and transcription factor genes (Petroni & Tonelli 2011; Zheng et al. 2013). Among plants, early biosynthetic genes including *chalcone synthase* (*CHS*), *chalcone isomerase* (*CHI*), and *flavanone-3-hydroxylase* (F3H) are shared by all of the flavonol biosynthesis processes, whereas late biosynthetic genes including *flavonoid 3'-hydroxylase* (*F3'H*), *flavonoid 3'5'-hydroxylase* (*F3'5'H*), *dihydroflavonol-4-reductase* (*DFR*), *anthocyanidin synthase* (*ANS*) and *UDP-glycose flavonoid glycosyl transferase* (*UFGT*) are specific to different anthocyanin biosynthesis subclass pathways according to the different number of hydroxyl groups in the phenyl group (Zhu & Bao 2021). The structural gene expression is regulated by regulatory genes (Rodas et al. 2014; Hu et al. 2016; Wu et al. 2017b; Kaur et al. 2020), including MYB, basic helix-loop-helix (b HLH), and WD40 classes (MBW complex) (Wang et al. 2015). In barley, the dominant alleles of both the *Ant1* and *Ant2* genes were required for the anthocyanin accumulation in the pericarp (Gordeeva et al. 2019; Zhou et al. 2021). However, the role of 2-hydroxyisoflavanone dehydratase (*HID*) in anthocyanin synthesis has not been reported.

In the isoflavone biosynthetic pathway of leguminous plants, the shikimic acid pathway produces phenylalanine, which is then converted to ρ-coumaroyl-CoA or naringenin, which are the backbone precursors for the production of flavonoids and isoflavone. Catalysed by a series of enzymes, ρ-coumaroyl-CoA or naringenin are converted to 2-hydroxyisoflavanones, which are the precursors of isoflavones (Sawada et al. 2002; Wang 2011; Vadivel et al. 2016). 2-hydroxyisoflavanones are unstable and can be dehydrated spontaneously, or under the catalytic dehydration activity of HID, to produce isoflavone (Hakamatsuka et al. 1998; Du et al. 2010; Tohge et al. 2017). The *HID* gene is a member of the *Abhydrolase_3* gene family and the enzyme belongs to the α/β hydrolase superfamily (Lenfant et al. 2012). The α/β hydrolases have a spatial structure formed by α/β folds and a highly conserved HGG motif, which is part of an oxyanion hole. The triad residues, tryptophan (Ser), aspartic acid (Asp), and histidine (His), in the conserved sequence, Gly-X-Ser-X-Gly, play a crucial role in the formation and stabilisation of hydrolytic acylase complexes (Akashi et al. 2005; Nomura et al. 2015). Thus far, studies of the *HID* gene have mainly been conducted in legumes, such as the bean (Akashi et al. 2005; Waki et al. 2016; Tohge et al. 2017), *Lotus japonicus* (Shimamura et al. 2007), liquorice (Akashi et al. 1999), alfalfa (Deavours & Dixon 2005) and *Arabidopsis* (Liu et al. 2002), while *HID* has not been reported in barley. With the successful assembly of the barley genome, more and more genes are being identified and cloned (Dai et al. 2018; Jayakodi et al. 2020; Zeng et al. 2020).

In our previous study, a genotyping-by-sequencing (GBS) approach was used to map the gene controlling the purple seed in hulless barley, and an *HID* gene sequence in the candidate chromosome segment was obtained (Yao et al. 2018). In this study, the *HvnHID* gene was isolated from both the purple-grained hulless barley variety Nerumuzha (female parent) and the white-grained hulless barley variety Kunlun 10 (male parent). The sequence characteristics, protein structures, and evolutionary relationships of this gene were analysed using bioinformatics software. The *HvnHID* expression level in the seeds of several coloured hulless barley varieties at the seed colour formation stage, and the expression pattern in the seeds of the purple-grained Nerumuzha variety at different stages of the seed colour development, was analysed using real-time fluorescence-based quantitative polymerase chain reaction (PCR) (RT-qPCR). The results provide a theoretical basis for the further elucidation of the overall role of the isoflavone biosynthesis pathway in the anthocyanidin biosynthesis and of the mechanism of seed colouration in coloured hulless barley.

MATERIAL AND METHODS

Plant material. The plant materials used in this study were the hulless barley varieties Nerumuzha and Dazhangzi (purple grain), Heilaoya and Kunlun 17 (black grain), Kunlun 12 and Kunlun 10 (white grain), and INB0N-7 (blue grain), which were bred by the Hulless Barley Research Laboratory, Institute of Crops, Academy of Agricultural and Forestry

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Sciences, Qinghai University, China. In April 2019, the seeds were planted in the experimental field of the Academy of Agricultural and Forestry Sciences, Qinghai University. In the late milk stage (12 weeks after sowing), the seeds of Nerumuzha and Dazhangzi (purple grain), Heilaoya and Kunlun 17 (black grain), Kunlun 12 and Kunlun 10 (white grain), and INB0N-7 (blue grain), and the seeds of Nerumuzha (purple grain) in the early milk stage (11 weeks after sowing), the late milk stage (12 weeks after sowing), the early dough stage (13 weeks after sowing) and the hard dough stage (14 weeks after sowing) were collected for the gene expression analysis according to Zadoks growth scale (Zadoks, Z73–Z87, early milk to hard dough) (Zadoks et al. 1974). Three biological replicates were taken for each sample. The sampled seeds were immediately frozen in liquid nitrogen and then stored at −80 °C in a freezer.

Extraction of total RNA from hulless barley seeds and cDNA synthesis. A total RNA extraction kit (Tiangen, Beijing, China), designed for plants rich in polysaccharides and polyphenols, was used to extract the total RNA from the hulless barley seeds. The concentration and purity of the total RNA were measured using a NanoPhotometer (Implen GmbH, Germany). The quality of the total RNA was detected by means of 1.5% agarose gel electrophoresis. The first-strand cDNA was synthesised using a PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa, Japan) and the synthesised cDNA was stored at −80 °C.

Isolation of the *HvnHID* gene from hulless barley. The purple seed phenotype of the hulless barley was mapped through GBS. An *HID* gene was isolated from the 77.30–78.50 cM candidate segment on chromosome 7H and was named *HvnHID* (Gene ID: MLOC_71630) (Yao et al. 2018). Primer software Ver. 5.0 was used to design the primers for amplifying the coding sequence (CDS) of this gene (Table S1 in the Electronic Supplementary Material (ESM)). The PCR amplification was carried out using the cDNA template prepared via the reverse transcription of the total RNA from the Nerumuzha and Kunlun 10 seeds. The 25 μL amplification system included 12.5 μL of 2× PCR buffer for KOD Fx, 0.5 μL of each primer (forward and reverse; 10 μmol/L), 5.0 μL of dNTPs (2 mmol/L), 0.5 μL of TOYOBO KOD Fx DNA polymerase (1.0 U/μL), and 1.0 μL of cDNA template; ddH_2O was added to make up the 25 μL. The PCR programme included a pre-denaturation step at 94 °C for 2 min, followed by 35 cycles of denaturation at 98 °C for 10 s, annealing at 56 °C for 30 s, and extension at 68 °C for 1 min 30 s. After a final extension at 72 °C for 10 min, the reaction was terminated at 4 °C for 5 min. The DNA band was recovered by ethanol precipitation, and the concentration and purity of the recovered product were measured using the NanoPhotometer (Implen GmbH, Germany). The product was then ligated to the pEasy-Blunt vector (TransGen Biotech, Beijing, China) and transformed into *Escherichia coli* Trans-T1 Competent cells. Five white colonies were picked for identification, and at least three positive clones were sent to Sangon Biotech (Shanghai, China) for sequencing using the universal primer M13.

Bioinformatic analysis of the *HvnHID* gene. SMART (http://smart.embl-heidelberg.de/) was used to predict the conserved domain in the *HvnHID* CDS. Expasy Protparam (http://www.expasy.org/tools/protparam.html) and Protscale (https://web.expasy.org/protscale/) were used to predict the physicochemical properties and hydrophilicity/hydrophobicity of the deduced proteins. SignalP4.1 (http://www. Detaibio.com/ tools/signal-peptide.html) and TMHMM-2.0 (http://www.cbs.dtu. dk/services/TMHMM-2.0/) were used to predict the signal peptide and transmembrane structure. CELLO Ver. 2.5 (http://cello.life.nctu.edu.tw) was used to predict the subcellular location of the HvnHID protein. SPOMA (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/ npsa_sopma.html) and SWISS-MODEL (https://swissmodel.expasy.org/) were used to predict the secondary and tertiary structure of the HvnHID protein. BlastP in the National Center for Biotechnology Information (NCBI) database was used to search for the amino acid sequences that are homologous to those of the HvnHID protein in other species of the Gramineae family. DNAMAN6.0 software was used to align multiple sequences, and Mega software Ver. 5.1 was used to construct the phylogenetic tree.

Analysis of the *HvnHID* gene expression in the hulless barley. Primer 5.0 was used to design primers for the *HvnHID* (based on the amplified CDS sequence), *HvnF3'H*, *HvnDRF*, *HvnANT1*, *HvnGT*, and *HvnHID* genes (based on the sequence in the barley reference genome, ftp://ftp.ensemblgenomes.org/pub/release-29/plants/fasta/hordeum_vulgare/dna/) for the RT-qPCR analysis (Table S1 in ESM). The templates were the cDNAs (200 ng/μL) prepared from the total RNA of the seeds (collected at 12 weeks after sowing) of the coloured hulless barley varieties Nerumuzha and Dazhangzi (purple grain), Heilaoya

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and Kunlun 17 (black grain), Kunlun 12 and Kunlun 10 (white grain), and INB0N-7 (blue grain). The cDNAs that were prepared from the total RNA of the Nerumuzha (purple grain) seeds sampled at 11, 12, 13, and 14 weeks after sowing, were also used as templates. The *18S rRNA* gene was used as an internal reference (Table S1 in the ESM) (Sun et al. 2010). A 20 µL amplification reaction system included 10 µL of 2× TB Green premix Ex Taq II (Tli RNaseH Plus), 2 µL of the cDNA template, 0.8 µL of each of the forward and reverse primer (10 µmol/L), and 6.4 µL of ddH_2O. The cycling programme included a pre-denaturation step at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 5 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. The temperature protocol of 95 °C for 5 s and 70 °C for 1 min was used to generate the melting curve. The relative expression levels of the genes in the seeds of the coloured hulless barley varieties (at the early-mid stage of grain filling) and in the seeds of Nerumuzha at different stages of the seed colour development, were calculated using the $2^{-\Delta\Delta Ct}$ method (Lenfant et al. 2012). Three biological repeats were set for each sample. SPSS was used for the analysis of variance (ANOVA) and the significance tests (Suantika et al. 2016).

RESULTS

Isolation of the *HvnHID* gene and sequence analysis. The cDNA templates prepared from the total RNA extracted from the seeds of the hulless barley varieties Nerumuzha (purple grain) and Kunlun 10 (white grain), and the primers designed based on the *HvnHID* gene (Table S1 in the ESM), were used for the PCR amplification. An amplicon of approximately 1080 bp was obtained (Figure S1A in the ESM). A 981 bp open reading frame encoding a protein of 327 amino acids was identified using DNAMAN software Ver. 6.0. When comparing Nerumuzha and Kunlun 10, eight nucleotide differences were detected in the gene (99.19% identical) and two amino acids differences were detected in the protein (99.39% identical; Figure S2 in the ESM). This gene was predicted to have a typical Abhydrolase_3 domain (78–306 bp), belonging to the Abhydrolase_3 (α/β hydrolase) superfamily (Figure S1B in the ESM). The Abhydrolase_3 structural domain was 99.56% identical in Nerumuzha and Kunlun 10 with one amino acid difference (Figure S2 in the ESM).

The analysis of the physicochemical properties and hydrophilicity/hydrophobicity showed that the Nerumuzha HvnHID protein had a molecular formula of $C_{1566}H_{2383}N_{425}O_{453}S_7$, a molecular weight of 34 636.14 Da, a fat solubility index of 81.56, and an average hydrophobicity (GRAVY) of –0.030. The Kunlun 10 HvnHID protein had a molecular formula of $C_{1567}H_{2385}N_{427}O_{453}S_7$, a molecular weight of 34 694.18 Da, a fat solubility index of 81.25, and an average hydrophobicity (GRAVY) of –0.040. The two proteins had an instability index of 28.37, a theoretical isoelectric point of 5.61, 38 negatively charged residues (Asp + Glu), and 30 positively charged residues (Arg + Lys). It is, thus, clear that the HvnHID protein is a stable hydrophilic acidic protein. The protein was predicted to have no transmembrane structure or signal peptide. The CELLO Ver. 2.5 subcellular localisation analysis showed that the two HvnHID proteins may be located in the cytoplasm, with scores of 2.032 and 2.194 for the Nerumuzha and Kunlun 10 proteins, respectively. The order of the possibility of the proteins' location within cellular structures is as follows: cytoplasm > periplasm > inner membrane > extracellular matrix > outer membrane.

The secondary and tertiary structures of the HvnHID proteins were predicted. The proteins had a secondary structure that was mainly composed of random coils, α-helices, extended strands, and β-turns. The order of the proportions of these structures was: random coils > α-helices > extended chain > β-turn (Figure S3A in the ESM). This indicated that the two proteins had mainly random coils and α-helices that may be important to their function. The three-dimensional structures of the HvnHID proteins were predicted using the SWISS-MODEL (Figure S3B in the ESM). The result showed that the HvnHID protein was an oligomer that was mainly composed of random coils and α-helices, and had a conserved propyl carboxylate ligand and α/β folds.

Homology comparison and system evolution. The Nerumuzha HvnHID protein was 100% identical to a predicted protein in hulless barley (GenBank accession number KAE8801614.1) and 99.39% identical to an unknown protein in barley (GenBank accession number AK360742.1). The Nerumuzha HvnHID and the HID proteins in *Aegilops tauschii*, *Brachypodium distachyon*, *Zea mays*, *Oryza sativa*, *Sorghum bicolor*, *Setaria viridis*, *Setaria italica*, and *Panicum hallii* had 91.16%, 83.33%, 75.76%, 75.08%, 74.63%, 65.05%, 60.66%, and 59.11% amino acid identity, respectively. The Kunlun 10 HvnHID protein was 100% identical to an unknown protein in barley (GenBank accession number AK360742.1) and 99.39% identical to a pre-

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dicted protein in hulless barley (GenBank accession number KAE8801614.1). The Kunlun 10 HvnHID and the HID proteins in *Aegilops tauschii*, *Brachypodium distachyon*, *Zea mays*, *Oryza sativa*, *Sorghum bicolor*, *Setaria viridis*, *Setaria italica*, and *Panicum hallii* had 91.16%, 83.33%, 76.06%, 75.68%, 74.93%, 64.78%, 60.96%, and 59.36% amino acid identity, respectively. All these proteins had a relatively conserved Abhydrolase_3 domain, oxyanion pore HGG motif, and Gly-X-Ser-X-Gly sequence (Figure S4 in the ESM). The phylogenetic tree analysis showed that among ten species in the Gramineae family, the HvnHID protein had the closest relationship with the HID proteins in *Hordeum vulgare a*nd *Aegilops tauschii*, and the most distant relationship with those in *Setaria viridis* and *Setaria italica* (Figure S5 in the ESM).

Expression of *HvnHID* in the coloured hulless barley varieties at the early-mid stage of seed ripening. The expression of the *HvnHID* gene was examined in the seeds sampled at 12 weeks after sowing, which corresponds to the early-mid stage of the seed colour development. The seeds were collected from the coloured hulless barley varieties Nerumuzha and Dazhangzi (purple grain), Heilaoya and Kunlun 17 (black grain), Kunlun 12 and Kunlun 10 (white grain), and INB0N-7 (blue grain). The expression level of *HvnHID* was the highest in Nerumuzha (purple grain), followed by Kunlun 17 (black grain), Dazhangzi (purple grain), Heilaoya (black grain), Kunlun 10 (white grain), Kunlun 12 (white grain) and the lowest in INB0N-7 (blue grain). The expression level of *HvnHID* was significantly higher in the purple and black seeds than in the blue and white seeds. Thus, it is speculated that *HvnHID* may be involved in the accumulation of anthocyanidins in the seeds of the purple and black hulless barley varieties (Figure S6 in the ESM).

Expression of *HvnHID* during seed development of the purple-grained Nerumuzha variety. To clarify the role of *HvnHID* during the seed colour development of purple-grained hulless barley, the expression of *HvnHID* and the key genes *HvnF3'H*, *HvnDRF*, *HvnANT1*, and *HvnGT* of the anthocyanidin biosynthetic pathway was analysed. The expression was examined in the seeds of the purple-grained hulless barley variety Nerumuzha that had been collected at 11, 12, 13, and 14 weeks after sowing. To perform this analysis, a RT-qPCR was used. The results showed that the expression levels of *HvnF3'H*, *HvnDRF*, *HvnANT1*, and *HvnGT* significantly increased with the development of the seed colour ($P < 0.01$), and the expression level of *HvnHID* gradually decreased, reaching its lowest value at 14 weeks after sowing ($P < 0.01$; Figure S7 in the ESM). It is speculated that the *HvnHID* gene negatively regulates the accumulation of anthocyanidins during the purple seed formation (Figure S8 in the ESM).

DISCUSSION

Recently, studies of *HID* genes have mainly focused on legumes (Deavours & Dixon 2005; Livingstone et al. 2011). A few authors have reported on *HID* genes in non-legumes, such as *Arabidopsis* (Ayabe et al. 2007; Liu et al. 2002) and the lotus (Shimamura et al. 2007). There have been no reports on *HID* genes in gramineous plants. In this study, the *HvnHID* genes were isolated from the hulless barley varieties Nerumuzha (purple grain) and Kunlun 10 (white grain). The HvnHID protein had a highly conserved oxyanion hole HGG motif and had the catalytic triad residues tryptophan (Ser), aspartic acid (Asp), and histidine (His) in the conserved sequence, Gly-X-Ser-X-Gly, which is a typical structure of α-esterase family members. The HGG motif and Gly-X-Ser-X-Gly sequence were highly conserved between ten gramineous plants, with a homology of 100% and 83.16%, which might be play an important role in the stabilisation of the intermediates in the hydrolysis process (Ollis et al. 1992). In plants, the *Abhydrolase_3* gene family generally included the carboxylesterase (CXE) gene family and the hormone-sensitive lipase-like (HSL) gene family (Lenfant et al. 2012). In this study, the 11–326 amino acids of the HvnHID protein were predicted to give rise to a tertiary structure with typical properties of carboxylic acid propyl ester ligands and with α/β folds. These structural features were in line with those of the Abhydrolase_3 (α/β hydrolase) superfamily, which suggested that *HvnHID* participates in regulating plant growth and dealing with biotic stresses, as are the *CXE* gene family genes (Gershater et al. 2007). The Abhydrolase_3 domain was highly conserved between ten gramineous plants, with a homology of 83.71%. The homology comparison of the *HvnHID* genes from the purple-grained and white-grained hulless barley showed that there are two amino acid differences, one of which was in the Abhydrolase_3 domain. Studies have shown that changes in the sequence of protein coding regions, especially key structural domains, may lead to changes in their function (Eulgem et al. 2000). A T-DNA-inserted

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ugt78d2 mutant accumulated very little anthocyanin and lacked 3-O-glucosylated quercetin in *Arabidopsis* (Lee et al. 2005). Johnson et al. (2001) identified a region that determines the substrate specificity of DFR by analysing chimeric DFRs of the Petunia and Gerbera. Furthermore, by changing a single amino acid in this presumed substrate-binding region, they developed a DFR enzyme that preferentially reduces to dihydrokaempferol, resulting in the production of orange flowers. In this study, it remains to be verified whether the alteration of two amino acids directly or indirectly affects the synthesis of anthocyanins. The synthesis of anthocyanidins in plant cells mainly occurs on the endoplasmic reticulum membrane and is catalysed by a series of enzymes. Anthocyanidin was the final product of the flavonoid metabolism (Tanaka et al. 2008). Isoflavones comprise a class of secondary metabolites that mainly exist in legumes (Wang 2011). These secondary metabolites play roles in the ecological defence, plant resistance to pathogenic microorganisms, and the interaction between plants and biotic environmental factors (Akashi et al. 2005). HID was an important rate-limiting enzyme in the isoflavone biosynthetic pathway, and it regulated the isoflavone synthesis together with the isoflavone synthase (IFS) in plants. Plant flavanone metabolism required a variety of enzymes. Some enzymes competed for a substrate and other enzymes coordinate and promote each other's activity (Jung et al. 2000). In the present study, it was found that the expression of *HvnHID* was significantly higher in the purple and black grains of the hulless barley than in the white and blue grains ($P < 0.01$) at the early-mid stage of the seed colour development (12 weeks after sowing). This suggests that *HvnHID* may play an important role in the anthocyanidin biosynthesis.

Studies have shown that the phenylalanine pathway produces ρ-coumaroyl-CoA and naringenin, which are the backbone precursors for the anthocyanidin and isoflavone biosynthesis. In the isoflavone synthesis pathway, under the catalysis of chalcone synthase (CHS), chalcone isomerase (CHI), and IFS, naringenin was converted to 2-hydroxyisoflavanones. 2-hydroxyisoflavanone was then converted to form isoflavones via spontaneous dehydration or under the catalysis of HID (Sawada et al. 2002; Wang 2011; Vadivel et al. 2016). In the anthocyanidin biosynthesis pathway, ρ-coumaroyl-CoA was catalysed by CHS, CHI, flavonoid 3'-hydroxylase (F3'H), flavonoid 3'5'-hydroxylase (F3'5'H), dihydroflavonol 4-reductase (DRF), anthocyanidin synthase (ANS), glycosyltransferase (GT), and other enzymes to form anthocyanidin or flavonoids (Saito et al. 2013). Therefore, the anthocyanidin synthesis pathway competed with the isoflavone synthesis pathway for ρ-coumaroyl-CoA or naringenin. In this study, the expression of *HvnHID* was analysed in the Nerumuzha seeds (purple grain) sampled at 11, 12, 13, and 14 weeks after sowing. The results indicated that the expression level of *HvnHID* significantly decreased with the development of the seed colour. In contrast, the expression of the anthocyanidin synthesis-related genes, *HvnF3'H*, *HvnDRF*, *HvnANT1,* and *HvnGT,* significantly increased. Both positive and negative regulations have been found for the biosynthesis of anthocyanidins (Clark & Verwoerd 2011). The inhibition of anthocyanidin biosynthesis was mainly caused by transcription factors. In the lignin and anthocyanidin biosynthesis pathway, regulation by a binary complex formed by an R3-MYB repressor and bHLH caused the lignin content to increase and the anthocyanidin content to decrease (Ma & Constabel 2019). Through histone H3 deacetylation, the MYB75 and HAT1-TPL proteins formed a transcription repressor complex and inhibited the anthocyanidin biosynthesis (Zheng et al. 2019). Lou et al. (2014) found that flavonol synthase (FLS) competed with DRF for precursor substances, which blocked the expression of genes downstream of the anthocyanidin biosynthesis pathway, and resulted in the production of blue and white grape hyacinths. Yonekura-Sakakibara et al. (2019) hypothesised that F3'H and CHS might compete with IFS, HID, and flavanone 2-hydroxylase (F2H) for the substrate, ρ-coumaroyl-CoA, during anthocyanidin biosynthesis. Similarly, here, it can be speculated that there may be a competitive relationship between the *HvnHID* gene and the key genes (*HvnF3'H*, *HvnDRF*, *HvnANT1*, and *HvnGT*) in the anthocyanidin biosynthesis pathway during the seed colour development of the purple-grained hulless barley variety Nerumuzha (Figure S8 in the ESM). It is also possible that the *HvnHID* gene negatively regulates the anthocyanidin biosynthesis in purple-grained hulless barley. However, the specific regulating mechanism still needs to be clarified.

CONCLUSIONS

In this study *HID* gene sequences were isolated from the seeds of both the purple-grained Tibetan hulless barley variety Nerumuzha (female parent)

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and the white-grained variety Kunlun 10 (male parent). The expression of *HvnHID* at the early-mid stage of the seed colour development and the key genes (*HvnF3'H*, *HvnDRF*, *HvnANT1*, and *HvnGT*) in the anthocyanidin biosynthetic pathway at three stages of the seed colour development were tested. It is likely that *HvnHID* negatively regulates the anthocyanidin biosynthesis. The result provides an important basis for the further identification of the biological functions of *HvnHID* and lays a foundation for clarifying the mechanism underlying seed colouration in coloured barley.

REFERENCES

Akashi T., Aoki T., Ayabe S.I. (1999): Cloning and functional expression of a cytochrome P450 cDNA encoding 2-hydroxyisoflavanone synthase involved in biosynthesis of the isoflavonoid skeleton in licorice. Plant Physiology, 121: 821–828.

Akashi T., Aoki T., Ayabe S.I. (2005): Molecular and biochemical characterization of 2-hydroxyisoflavanone dehydratase. involvement of carboxylesterase-like proteins in leguminous isoflavone 9biosynthesis. Plant Physiology, 137: 882–891.

Ayabe S. (2007): Polynucleotide encoding 2-hydorxyisoflavanone dehydratase and application of the same. US20070050865.

Clark S.T., Verwoerd W.S. (2011): A systems approach to identifying correlated gene targets for the loss of colour pigmentation in plants. BMC Bioinformatics, 12: 343.

Dai F., Wang X., Zhang X., Chen Z., Zhang G. (2018): Assembly and analysis of a qingke reference genome demonstrates its close genetic relation to modern cultivated barley. Plant Biotechnology Journal, 16: 760–770.

Deavours B.E., Dixon R.A. (2005): Metabolic engineering of isoflavonoid biosynthesis in Alfalfa. Plant Physiology, 138: 2245–2259.

Du H., Huang Y.B., Tang Y.X. (2010): Genetic and metabolic engineering of isoflavonoid biosynthesis. Applied Microbiology and Biotechnology, 86: 1293–1312.

Eulgem T., Rushton P.J., Robatzek S., Somssich I.E. (2000): The WRKY superfamily of plant transcription factors. Trends in Plant Science, 5: 199–206.

Ferreira L.L., Silva T.R., Maturana M.A., Spritzer P.M. (2019): Dietary intake of isoflavones is associated with a lower prevalence of subclinical cardiovascular disease in postmenopausal women: cross-sectional study. Journal of Human Nutrition and Dietetics, 32: 810–818.

Gershater M.C., Cummins I., Edwards R. (2007): Role of a carboxylesterase in herbicide bioactivation in *Arabidopsis thaliana*. Journal of Biological Chemistry, 282: 21460–21466.

Gordeeva E.I., Glagoleva A.Y., Kukoeva T.V., Khlestkina E.K., Shoeva O.Y. (2019): Purple-grained barley (*Hordeum vulgare* L.): marker-assisted development of NILs for investigating peculiarities of the anthocyanin biosynthesis regulatory network. BMC Plant Biology, 19 (S1): 52.

Hakamatsuka T., Mori K., Ishida S., Ebizuka Y., Sankawa U. (1998): Purification of 2-hydroxyisoflavanone dehydratase from the cell cultures of *Pueraria lobata* in honour of Professor G.H. Neil Towers 75th birthday. Phytochemistry, 49: 497–505.

Hu M., Lu Z., Guo J., Luo Y., Li H., Li L., Gao F. (2016): Cloning and characterization of the cDNA and promoter of UDP-glucose: flavonoid 3-O-glucosyltransferase gene from a purple-fleshed sweet potato. South African Journal of Botany, 106: 211–220.

Jayakodi M., Padmarasu S., Haberer G., Bonthala V.S., Stein N. (2020): The barley pan-genome reveals the hidden legacy of mutation breeding. Nature, 588: 284–289.

Jia Y., Selva C., Zhang Y., Li B., Mcfawn L.A., Li C.D. (2020): Uncovering the evolutionary origin of blue anthocyanins in cereal grains. The Plant Journal, 101: 1057–1074.

Johnson E.T., Ryu S., Yi H., Shin B., Cheong H., Choi G. (2001): Alteration of a single amino acid changes the substrate specificity of dihydroflavonol 4-reductase. Plant Journal, 25: 325–333.

Jung W., Yu O., Lau S.-M.C., O'Keefe D.P., Odell J., Fader G., McGonigle B. (2000): Identification and expression of isoflavone synthase, the key enzyme for biosynthesis of isoflavones in legumes. Nature Biotechnology, 18: 208–220.

Kaur R., Aslam L., Kapoor N., Mahajan R. (2020): Identification and comparative expression analysis of chalcone synthase, flavanone 3-hydroxylase and dihydroflavonol 4-reductase genes in wild pomegranate (*Punica granatum* L.) organs. Brazilian Journal of Botany, 43: 1–14.

Lee Y., Yoon H.R., Yong S.P., Liu J.R., Choi G. (2005): Reciprocal regulation of arabidopsis ugt78d2 and banyuls is critical for regulation of the metabolic flux of anthocyanidins to condensed tannins in developing seed coats. Journal of Plant Biology, 48: 356–370.

Lenfant N., Hotelier T., Velluet E., Bourne Y., Marchot P., Chatonnet A. (2012): ESTHER, the database of the α/β-hydrolase fold superfamily of proteins: tools to explore diversity of functions. Nucleic Acids Research, 41: D423–D429.

Li Y.L., Long C.L., Kato K.J., Yang C.Y., Sato K. (2011): Indigenous knowledge and traditional conservation of hulless barley (*Hordeum vulgare*) germplasm resources in the Tibetan communities of Shangri-la, Yunnan, SW China. Genetic Resources and Crop Evolution, 58: 645–655.

https://doi.org/10.17221/34/2021-CJGPB

Liang J.J., Deng G.D., Long H., Pan Z.F., Wang C.P., Cai P., Xu D.L., Nimaand Z.X., Yu M.Q. (2012): Virus-induced silencing of genes encoding LEA protein in Tibetan hulless barley (*Hordeum vulgare* ssp. *vulgare*) and their relationship to drought tolerance. Molecular Breeding, 30: 441–451.

Liu C.J., Blount J.W., Steele C.L., Dixon R.A. (2002): Bottlenecks for metabolic engineering of isoflavone glycoconjugates in *Arabidopsis*. Proceedings of the National Academy of Sciences, 99: 14578–14583.

Livingstone J.M., Zolotarov Y., StröMvik M.V. (2011): Transcripts of soybean isoflavone 7-O-glucosyltransferase and hydroxyisoflavanone dehydratase gene homologues are at least as abundant as transcripts of their well known counterparts. Plant Physiology and Biochemistry, 49: 1071–1075.

Lou Q., Liu Y., Qi Y., Jiao S., Tian F., Jiang L., Wang Y. (2014): Transcriptome sequencing and metabolite analysis reveals the role of delphinidin metabolism in flower colour in grape hyacinth. Journal of Experimental Botany, 65: 3157–3164.

Ma D., Constabel C.P. (2019): MYB repressors as regulators of phenylpropanoid metabolism in plants. Trends in Plant Science, 24: 275–289.

Nomura T., Murase T., Ogita S., Kato Y. (2015): Molecular identification of tuliposide B-converting enzyme: a lactone-forming carboxylesterase from the pollen of tulip. The Plant Journal, 83: 252–262.

Ollis D.L., Cheah E., Cygler M., Dijkstra B., Frolow F., Franken S.M., Harel M., Remington S.J., Silman I., Schrag J. (1992): The α/β hydrolase fold. Protein Engineering Design & Selection, 5: 197–211.

Petroni K., Tonelli C. (2011): Recent advances on the regulation of anthocyanin synthesis in reproductive organs. Plant Science, 181: 219–229.

Rodas F.R., Rodriguez T.O., Murai Y., Iwashina T., Sugawara S., Suzuki M., Nakabayashi R., Yonekura-Sakakibara K., Saito K., Kitajima J., Toda K., Takahashi R. (2014): Linkage mapping, molecular cloning and functional analysis of soybean gene Fg2 encoding flavonol 3-O-glucoside (1→6) rhamnosyltransferase. Plant Molecular Biology, 84: 287–300.

Sawada Y., Kinoshita K., Akashi T., Aoki T., Ayabe S.I. (2002): Key amino acid residues required for aryl migration catalysed by the cytochrome p450 2-hydroxyisoflavanone synthase. Plant Journal, 31: 555–564.

Shimamura M., Akashi T., Sakurai N., Suzuki H., Saito K., Shibata D., Ayabe S., Aoki T. (2007): 2-Hydroxyisoflavanone dehydratase is a critical determinant of isoflavone productivity in hairy root cultures of *Lotus japonicus*. Plant and Cell Physiology, 48: 1652–1657.

Shoeva O.Y., Mock H.P., Kukoeva T.V., Börner A., Khlestkina E.K. (2016): Regulation of the flavonoid biosynthesis pathway genes in purple and black grains of *Hordeum vulgare*. PLoS ONE, 11: e0163782.

Suantika G., Situmorang M.L., Aditiawati P., Khakim A., Suryanarayan S., Nori S.S., Kumar S. Ferisca P. (2016): Effect of red seaweed on growth, salinity stress tolerance and vibriosis resistance in Shrimp *Litopenaeus vannamei* Hatchery. Journal of Fisheries and Aquatic Science, 12: 127–133.

Sun M., Wang Y.S., Yang D.Q., Wei C.L., Gao L.P., Xia T., Shan Y., Luo Y. (2010): Reference genes for real-time fluorescence quantitative PCR in *Camellia sinensis*. Chinese Bulletin of Botany, 45: 579–587.

Tanaka Y., Sasaki N., Ohmiya A. (2008): Biosynthesis of plant pigments: anthocyanins, betalains and carotenoids. Plant Journal, 54: 733–749.

Tohge T., de Souza L.P., Fernie A.R. (2017): Current understanding of the pathways of flavonoid biosynthesis in model and crop plants. Journal of Experimental Botany, 68: 4013–4028.

Vadivel A.K.A., Sukumaran A., Li X.Y., Dhaubhadel S. (2016): Soybean isoflavonoids: role of GmMYB176 interactome and 14-3-3 proteins. Phytochemistry Reviews, 15: 391–403.

Waki T., Yoo C.Y., Fujino N., Mameda R., Denessiouk K., Yamashita S., Motohashi R., Akashi T., Aoki T., Ayabe S., Takahashi S., Nakayama T. (2016): Identification of protein-protein interactions of isoflavonoid biosynthetic enzymes with 2-hydroxyisoflavanone synthase in soybean (*Glycine max* (L.) Merr.). Biochemical and biophysical research communications, 469: 546–551.

Wang H., Li M.F., Yang Y., Jin W.M. (2015): Recent advances on the molecular mechanisms of anthocyanin synthesis in fruits. Plant Physiology Journal, 51: 29–43.

Wang X.Q. (2011): Structure, function, and engineering of enzymes in isoflavonoid biosynthesis. Functional and Integrative Genomics, 11: 13–22.

Wu K.L., Yao X.H., Yao Y.H., Chi D.Z., Feng Z.Y. (2017a): Analysis of the relationship between *Wx* gene polymorphisms and amylose content in hulless barley. Czech Journal of Genetics and Plant Breeding, 53:144–152.

Wu X.X., Gong Q.H., Ni X.P., Zhou Y., Gao Z.H. (2017b): Ufgt: the key enzyme associated with the petals variegation in japanese apricot. Frontiers in Plant Science, 8: 108.

Yao X.H., Wu K.L., Yao Y.H., Bai Y.X., Ye J.X., Chi D.Z. (2018): Construction of a high-density genetic map: genotyping by sequencing (GBS) to map purple seed coat color (Psc) in hulless barley. Hereditas, 155: 37.

Yonekura-Sakakibara K., Higashi Y., Nakabayashi R. (2019): The origin and evolution of plant flavonoid metabolism. Frontiers in Plant Science, 10: 943.

https://doi.org/10.17221/34/2021-CJGPB

Zadoks J.C., Chang T.T., Konzak C.F. (1974): A decimal code for the growth stages of cereals. Weed Research, 14: 415–421.

Zeng X., Guo Y., Xu Q., Mascher M., Guo G., Li S., Mao L., Liu Q., Xia Z., Zhou J., Yuan H., Tai S., Wang Y., Wei Z., Song L., Zha S., Li S., Tang Y., Bai L., Zhuang Z., He W., Zhao S., Fang X., Gao Q., Yin Y., Wang J., Yang H., Zhang J., Henry R.J., Stein N., Tashi N. (2018): Origin and evolution of qingke barley in Tibet. Nature Communications, 9: 5433.

Zeng X., Xu T., Ling Z., Wang Y., Li X., Xu S., Xu Q., Zha S., Qimei W., Basang Y., Dunzhu J., Yu M., Yuan H., Nyim T. (2020): An improved high-quality genome assembly and annotation of Tibetan hulless barley. Scientific Data, 7: 139.

Zheng T., Tan W., Yang H., Zhang L.E., Li T.T., Liu B.H., Zhang D.W., Lin H.H. (2019): Regulation of anthocyanin accumulation via MYB75/HAT1/TPL-mediated transcriptional repression. PLoS Genetics, 15: e1007993.

Zheng Y., Li J.H., Xin N., Wang L., Guan B.H., Li S.H. (2013): Anthocyanin profile and gene expression in berry skin of two redvitis viniferagrape cultivars that are sunlight dependent versus sunlight independent. Australian Journal of Grape and Wine Research, 19: 238–248.

Zhou C., Zeng Z., Suo J., Li X., Han N. (2021): Manipulating a single transcription factor, ant1, promotes anthocyanin accumulation in barley grains. Journal of Agricultural and Food Chemistry, 69 (18).

Zhu Y., Bao Y. (2021): Genome-wide mining of myb transcription factors in the anthocyanin biosynthesis pathway of *Gossypium Hirsutum*. Biochemical Genetics, 59: 678–696.

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