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Isolation and expression analysis of the *HvnAnt2* gene in qingke barley (*Hordeum vulgare* L. var. *nudum* Hook. f.) varieties with different grain colours

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Abstract: To investigate the role of the *HvnAnt2* gene in the formation of different qingke barley grain colours, *HvnAnt2* was isolated from the leaves of the White 91-97-3 (white), Blue qingke (blue), Kunlun 17 (black), and Purple qingke (purple). The *HvnAnt2* gene encodes a hydrophilic unstable protein consisting of 561 amino acids, without a transmembrane structure and without a signal peptide, with one bHLH-MYC_N and one HLH domain at amino acids 22–204 and 387–436, respectively. The HvnAnt2 of qingke barley was most closely related to *Hordeum vulgare* and most distantly related to *Triticum monococcum*; it was mainly related to light responsiveness and methyl jasmonate responsiveness. The *Ant2* gene was highly expressed in Kunlun 17 and Purple qingke barley grains during the later stage of development. The protein interaction prediction showed that flavonoid 3'-monooxygenase, anthocyanin biosynthesis gene regulators, and key enzymes in folate metabolism interacted with the Ant2 protein. This study provides a reference for further analysis of the *Ant2* gene in the anthocyanin synthesis pathways of qingke barley with different grain colours.

Keywords: anthocyanin; bHLH; expression pattern; Tibetan hulless barley

Abbreviations: ABA – abscisic acid; ANS – anthocyanidin aynthase; Arg – arginine; Asp – aspartic acid; bHLH – basic helix-loop-helix; CHI – chalcone isomerase; CHS – chalcone synthase; DFR – dihydroflavonol-4-reductase; F3H – flavanone 3-hydroxylase; F3'H – flavonoid 3'-hydroxylase; Glu – glutamic acid; Lys – lysine; MeJA – methyl jasmonate; MYB – MYB transcription factors; MYC – MYC transcription factors; NCBI – National Center for Biotechnology Information; P450 – cytochromeP450; R/B – *B* and *R* are functionally duplicate genes that encode proteins with the basic-helix-loop-helix (b-HLH) motif found in Myc proteins; TT8 – transparent testa 8

Qingke barley (*Hordeum vulgare* L. var. *nudum* Hook. f.), a significant grain crop distributed in the Tibetan Plateau region, is a variety of cultivated barley (*Hordeum vulgare* L.) that is also known as naked barley due to the separation of the inner lemma and glumes of the seed grain at maturity (Yang et al. 2018;

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Guo et al. 2020). Coloured qingke barley accounts for nearly two-thirds of the local varieties of qingke barley, and its colour is related to the formation of rich natural anthocyanin compounds (Abdel-Aal et al. 2018; Xu et al. 2023). The Chinese and international classification of barley seed colour is mainly divided into four colours: yellow, blue, purple, and black (Zeven 1991). When the pericarp (seed outermost layer) and the dextrins (the outermost tissue of the endosperm of cereal seeds) contain no pigment, the seeds are yellow; when the dextrins contain anthocyanin and the pericarp contains no pigment the seeds are blue; when the dextrins and the pericarp both contain anthocyanin the seeds are purple; and when the pericarp contains melanin the seeds are black (Shoeva et al. 2016; Suriano et al. 2018). The blue dextrination trait in barley evolved only recently, with most wild barley varieties displaying a blue dextrination layer, while local varieties and cultivars have dextrination layers of various colours, such as blue, purple, and white (Jia et al. 2020). Due to the thin atmosphere in the Tibetan Plateau region, residents live under high-intensity ultraviolet (UV) radiation for a long period, which can cause problems such as cardiovascular disease as well as nerve and skin damage (Manisalidis et al. 2020). Anthocyanins are flavonoids – a family of compounds based on the nucleus of flavonoids that can appear red, anthocyanins containing phenolic hydroxyl groups in coloured barley have antioxidant properties, α-glucoside inhibitory activity, and free radical scavenging properties, which are remarkably effective in mitigating the risks of UV exposure and treating the aforementioned diseases (Alam et al. 2021; Jin et al. 2022).

The basic helix-loop-helix (bHLH), one of the largest families of transcription factors in plants, regulates numerous cellular processes in eukaryotes and is also involved in some of the vital responses of plants to drought stress (Jin et al. 2014). Members of this family have two conserved motifs: a basic DNA binding region and a helix-loop-helix region (Hao et al. 2021). These proteins contain the bHLH domain and usually function as homodimer or heterodimer to regulate the expression of their target genes, participate in numerous physiological processes, and have a wide range of functions in the biosynthesis, metabolism, and transduction of plant hormones (Hao et al. 2021). Previous studies have shown that the *Ant2* gene encodes a transcription factor with a basic helix loop-repressor structural domain (bHLH), which is considered to be a regulatory gene for the anthocyanin glycoside biosynthesis pathway (Lahaye et al. 1998; Cockram et al. 2010). *Ant2* encodes *HvbHLH1* and shares 35% homology with *TT8* (Himi & Taketa 2015). The *HvAnt2* gene was located on the 2H chromosome in barley, which regulates the biosynthesis of anthocyanosides in the barley seed pericarp and up-regulates the expression of the structural genes *CHS*, *CHI*, *F3H*, *F3'H*, *DFR*, and *ANS* in the barley seed pericarp anthocyanin pathway (Shoeva et al. 2016).

Currently, studies on qingke barley grain colour have primarily focused on aspects such as gene localization (Yao et al. 2018) and quality analysis (Yang et al. 2018), while fewer studies have been reported on the regulatory mechanism of anthocyanin synthesis. Therefore, in this study, qingke barley varieties with diverse grain colours were used as experimental materials, from which the *HvnAnt2* gene was cloned, and its sequence was analysed using relevant bioinformatics. In addition, the expression of the *HvnAnt2* gene in the grains of qingke barley with diverse grain colours and the expression patterns of this gene in each tissue of barley were analysed. The aim of this study was to explore the gene expression patterns of the *HvnAnt2* gene in the grains and tissues of barley with different grain colours and to provide a reference for the molecular breeding of coloured qingke barley in the Tibetan Plateau area.

MATERIAL AND METHODS

Plant material. The varieties of qingke barley tested were White 91-97-3 (white grain), Blue qingke (blue grain), Kunlun 17 (black grain), and Purple qingke (purple grain). The grain colour is shown in Figure 1.

Isolation of the *HvnAnt2* gene in qingke barley. The *Ant2* gene was obtained from transcriptome expression analysis in our previous study. The total RNA from four qingke barley varieties leaves was extracted using the Plant Extract Total RNA Kit (TaKaRa, Beijing, China); the concentration and purity of RNA were determined using an ultra-micro nucleic acid and protein measurement instrument (Implen, NanoPhotometer, Germany); the quality of RNA was detected using 1.0% agarose gel electrophoresis. The RNA was reverse-transcribed into cDNA with reference to the cDNA Synthesis Kit (TaKaRa, Beijing, China) and stored at –20 °C. Polymerase chain reaction (PCR) amplification was performed using cDNA from qingke barley leaves as a template, and Primer 5.0 was used to design

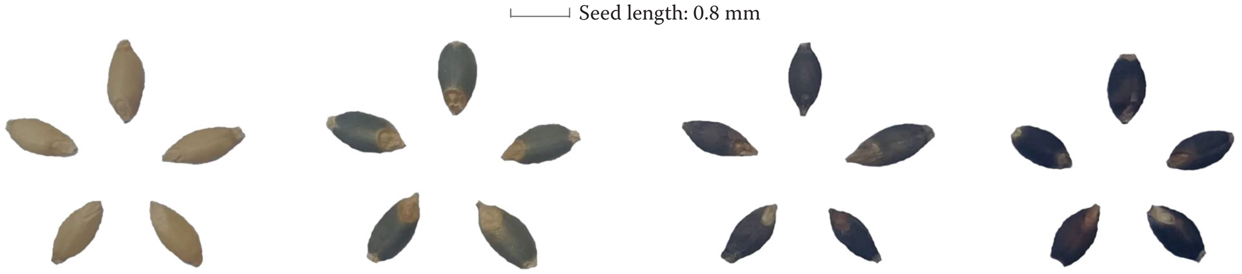

Figure 1. From left to right are white, blue, black, and purple qingke barley grains

the amplification primers for this gene (Table 1). The PCR amplification and agarose gel electrophoresis methods were used as in a previous study (Yao et al. 2021). The target bands were recovered using a column DNA Ge1 Extraction Kit (Sangon Biotech, Shanghai, China), and the target fragments were inserted into the vector of pEasy Blunt (TransGen, Beijing, China) and transformed into *E. coli* Trans-T1 receptive cells. Three positive clones were selected and sent to Sangon Biotech (Sangon Biotech, Shanghai, China) for sequencing.

Bioinformatics analysis of the qingke barley *HvnAnt2* gene. The physicochemical properties of the HvnAnt2 protein were predicted using https://web.expasy.org/. The structural domains were predicted using http://smart.embl-heidelberg.de/. The transmembrane region and signalling peptide were predicted using https://services.healthtech.dtu.dk/ and http://www.cbs.dtu.dk/services/SignalP. The secondary and tertiary structures were predicted using https://npsa-prabi.ibcp.fr/ and http://www.expasy.ch/swissmod/. The subcellular localization was predicted using https://wolfpsort.hgc.jp/. *Cis*-acting elements were predicted using https://bioinformatics.psb.ugent.be/webtools/plantcare/html/. The protein interactions were predicted using https://cn.string-db.org/cgi/.

Homology comparison and phylogenetic analysis of HvnAnt2 proteins. The Blastp function of NCBI was used to query the Ant2 protein sequence of the plant homologs with the HvnAnt2 protein, multi-sequence comparison was performed using DNAMAN 6.0 software, and the maximum likelihood method in MEGA7 software was used to construct the phylogenetic tree.

Expression pattern analysis of the qingke barley *HvnAnt2* gene. Based on the *HvnAnt2* gene sequence obtained via amplification, Primer 5.0 software was employed to design primers for quantitative real-time PCR (qRT-PCR) for the *HvnAnt2* gene (Table 1). cDNA obtained from grains of white, blue, black, and purple qingke were used as a template, 18S rRNA was used as an internal reference gene (Table 1), and TB Green *Premix Ex Taq* II, (TaKaRa) was used as a fluorescent dye. The Light Cycler 480 II System (Roche Diagnostics GmbH, Germany) was utilized to perform qRT-PCR, and the reaction system reference (Su et al. 2019) was used to calculate the relative expression of the *HvnAnt2* gene using the $2^{-\Delta\Delta Ct}$ method. There were three biological replicates and three technical replicates for each sample, and univariate analysis of variance was applied to test the significance of differences. The expression of the *HvnAnt2* gene in barley different tissues was retrieved using https://apex.ipk-gatersleben.de/apex/, and significance analysis was performed used SPSS 19.0.

Table 1. Primers designed for the *HvnAnt2* gene

Primer name	Primer sequence (5'→3')	Purpose
HvnAnt2-F	TGGACTCCGGGCTTCTTG	gene clone
HvnAnt2-R	ACCAGTGTCTCAGCAGCAT	gene clone
HvnAnt2-F1	CGAGATAAAGACGAGGAAGGT	qRT-PCR
HvnAnt2-R1	GGACAGGAGGGACTGGTAGA	qRT-PCR
18S rRNA-F	CTACGTCCCTGCCCTTTGTACA	internal reference primer
18S rRNA-R	ACACTTCACCGGACCATTCAA	internal reference primer

RESULTS

Cloning and sequence analysis of the qingke barley *HvnAnt2* gene. Leaf cDNA of white, blue, black, and purple qingke barley varieties was amplified (Figure 2A) using HvnAnt2-F/R (Table 1) as a primer. The HvnAnt2 protein had one bHLH-MYC_N and one HLH domain (Figure 2B). The molecular formula of the HvnAnt2 protein was $C_{2661}H_{4262}N_{750}O_{830}S_{22}$, the molecular weight was 60.746 kDa, the instability index was 52.01, and the aliphatic index was 85.63, with 70 negatively charged residues (Asp + Glu), 61 positively charged residues (Arg + Lys), and a grand average of hydrophobicity of –0.231. HvnAnt2 protein was hydrophilic and unstable with no transmembrane structure and no signal peptide. The secondary structure of HvnAnt2 protein was composed of α-helices, β-angles, irregular curls, and extended chains, and the functional domain of the tertiary structure was Y-shaped (Figure 2C and D). The nucleotide sequences of white, blue, and purple qingke barley were consistent, while there were three amino acid mutation sites in Kunlun 17, namely, leucine mutation to valine (at amino acid 78), valine mutation to alanine (at amino acid 363), and threonine mutation to methionine (at amino acid 488) (Figure 2E).

Homology comparison and evolutionary tree analysis of the HvnAnt2 protein. The protein sequence similarity of nine homologous monococcum plants, namely, *Hordeum vulgare*, *Aegilops tauschii*, *Triticum aestivum*, *Triticum dicoccoides*, *Lolium perenne*, *Setaria viridis*, *Panicum hallii*, *Panicum miliaceum*, and *Triticum monococcum*, compared with qingke barley HvnAnt2 protein was 100, 99.46, 83.42, 83.07, 68.99, 63.28, 61.21, 60.49, and 60.82%, respectively (Figure 3). Among the nine species, qingke was most closely related to *Hordeum vulgare* and was distantly related to *Triticum monococcum* (Figure 4).

Analysis of the *cis*-regulatory elements of the major promoter of the *HvnAnt2* gene. PlantCARE was used to analyse the *cis*-acting elements of the *HvnAnt2* gene, and the results showed that it contained abundant *cis*-acting elements, such as light response, ABA response, MeJA response, and MYB binding sites, core promoter element and other expression elements. The presence of these elements

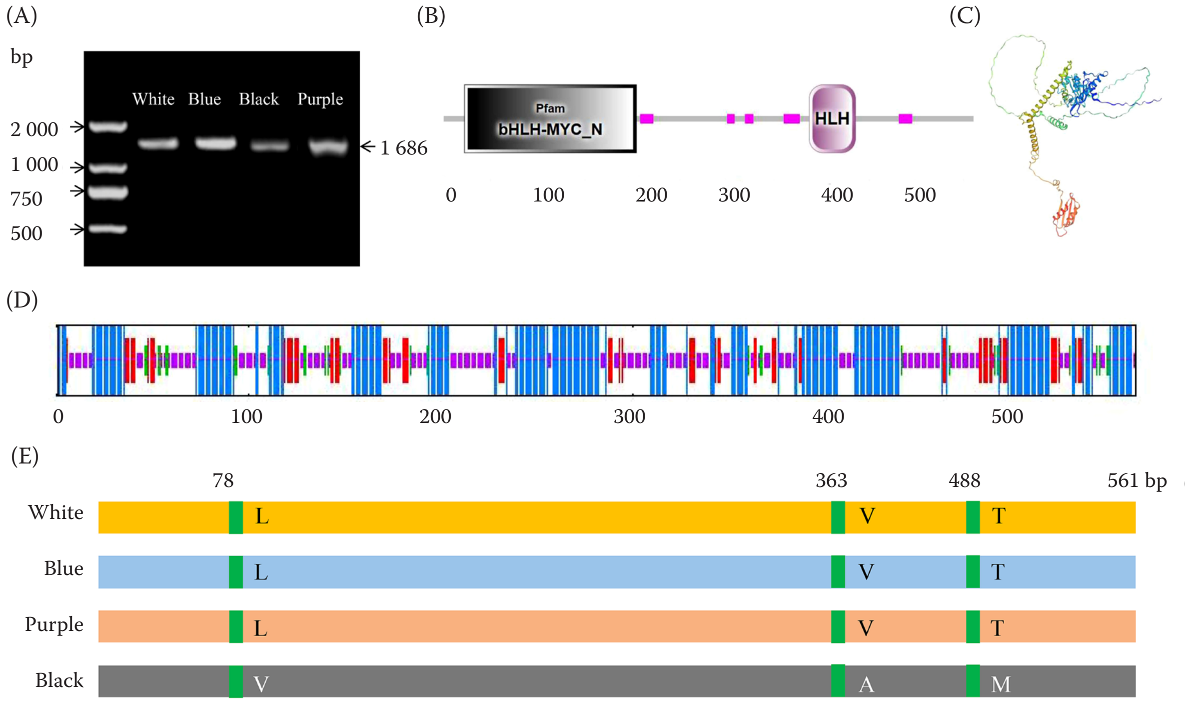

Figure 2. PCR amplification and protein sequence prediction of the *HvnAnt2* gene in qingke barley leaves: amplification of the *HvnAnt2* gene (A); prediction of the domain (B); prediction of the tertiary structure (C); prediction of the secondary structure, where blue colour indicates α-helices, green colour indicates β-turns, orange colour indicates irregular curls, and red colour indicates extended chains (D); multisequence alignment (L – leucine; V – valine; T – threonine; A – alanine; M – methionine) (E)

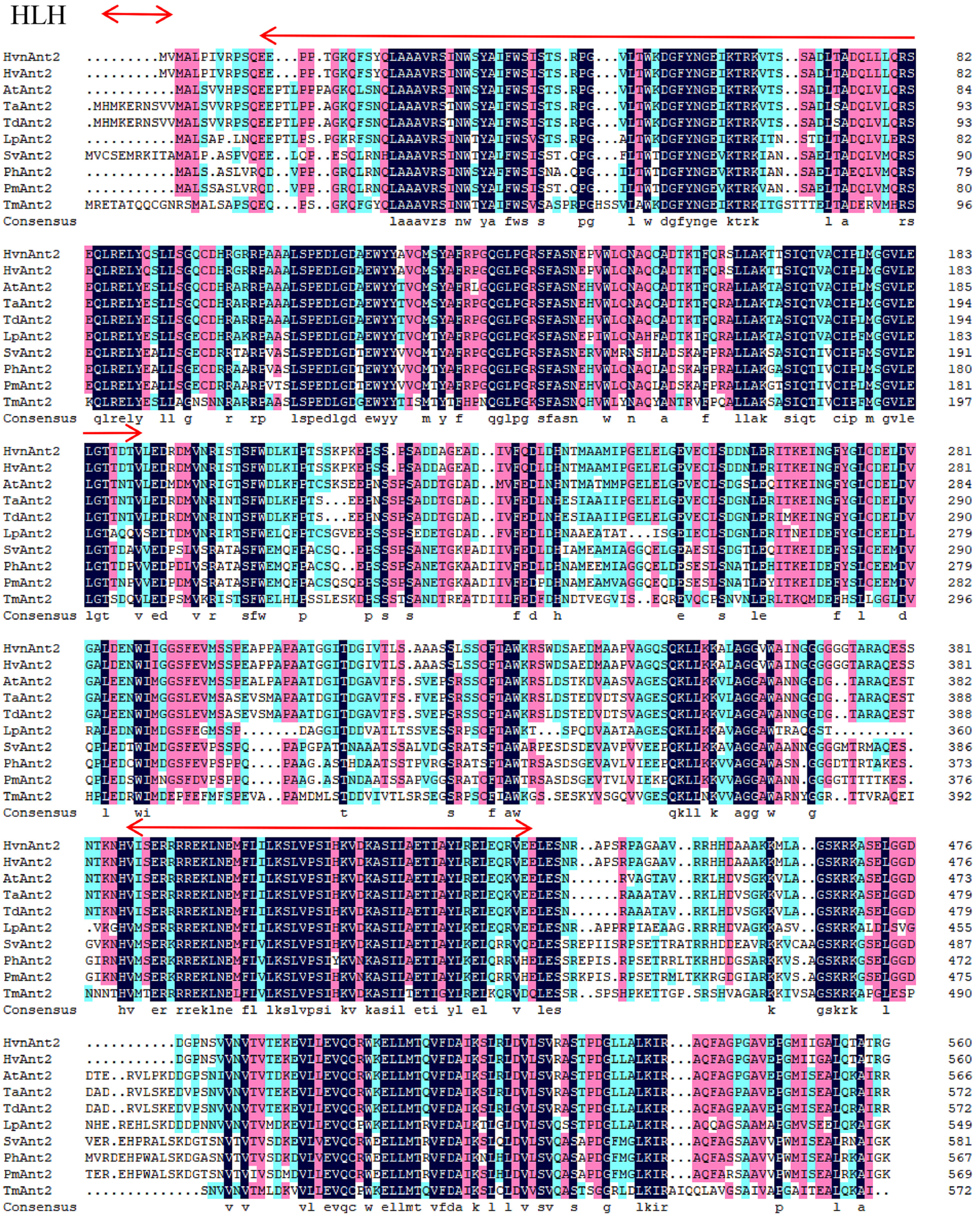

Figure 3. Multiple comparisons of amino acid sequences of HvnAnt2 with other homologs plant proteins

HvnAnt2 – qingke barley; HvAnt2 – *Hordeum vulgare*; AtAnt2 – *Aegilops tauschii* – TaAnt2 – *Triticum aestivum*; TdAnt2 – *Triticum dicoccoides*; LpAnt2 – *Lolium perenne*; SvAnt2 – *Setaria viridis*; PhAnt2 – *Panicum hallii*; PmAnt2 – *Panicum miliaceum*; TmAnt2 – *Triticum monococcum*

The red arrow represents the helix-loop-helix (HLH) domain

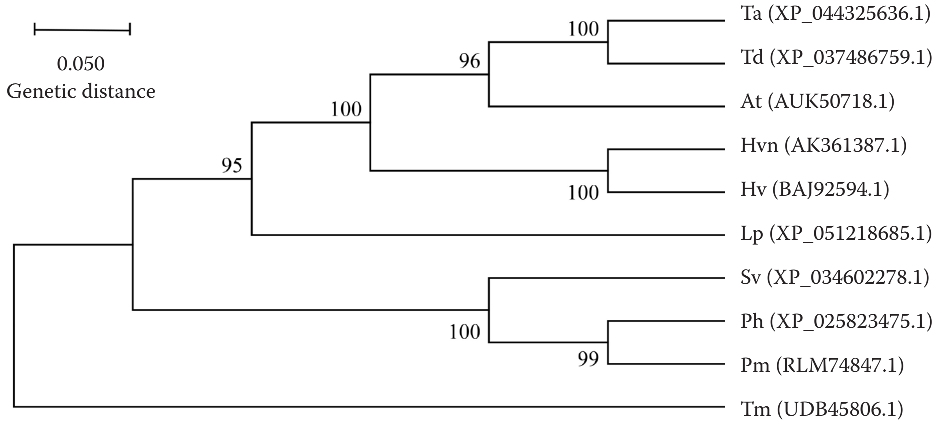

Figure 4. Phylogenetic relationship between qingke barley HvnAnt2 protein and Ant2 proteins in other plants
Ta – *Triticum aestivum*; Td – *Triticum dicoccoides*; At – *Aegilops tauschii*; Hvn – qingke barley; Hv – *Hordeum vulgare*; Lp – *Lolium perenne*; Sv – *Setaria viridis*; Ph – *Panicum hallii*; Pm – *Panicum miliaceum*; Tm – *Triticum monococcum*

in the *HvnAnt2* gene suggests that it is involved in plant physiological and abiotic stress as well as immune responses (Figure 5) (Table 2).

Gene expression analysis of *HvnAnt2*. This study conducted RNA-sequencing (RNA-seq) analysis of four qingke barley varieties grains at the milk, soft dough, and mature stages. The *Ant2* gene expression was the highest in Purple qingke and Kunlun 17 (Figure 6A and B); the expression level of *Ant2* gene gradually increased with the maturity of grain; and there was no significant difference between different varieties ($P < 0.05$). During barley development, developing tillers at the third internode and leaflet-like inflorescences revealed significantly higher expression levels than the other 14 tissues (Figure 6C) ($P < 0.05$).

Ant2 protein interaction analysis. The protein–protein interaction prediction results showed that 10 proteins interacted closely with A0A287J214 in *Hordeum vulgare*. These consisted of proteins containing the Spc97_Spc98 domain (A0A287MLD4, A0A287ISL8, and A0A287EVW5), protein containing Tubulin_C (A0A287EWW5), unidentified protein (A0A287KIR4), protein containing DHFR_1 and DHFR1 Thymidylat_synt (A0A287JIX6), proteins con-

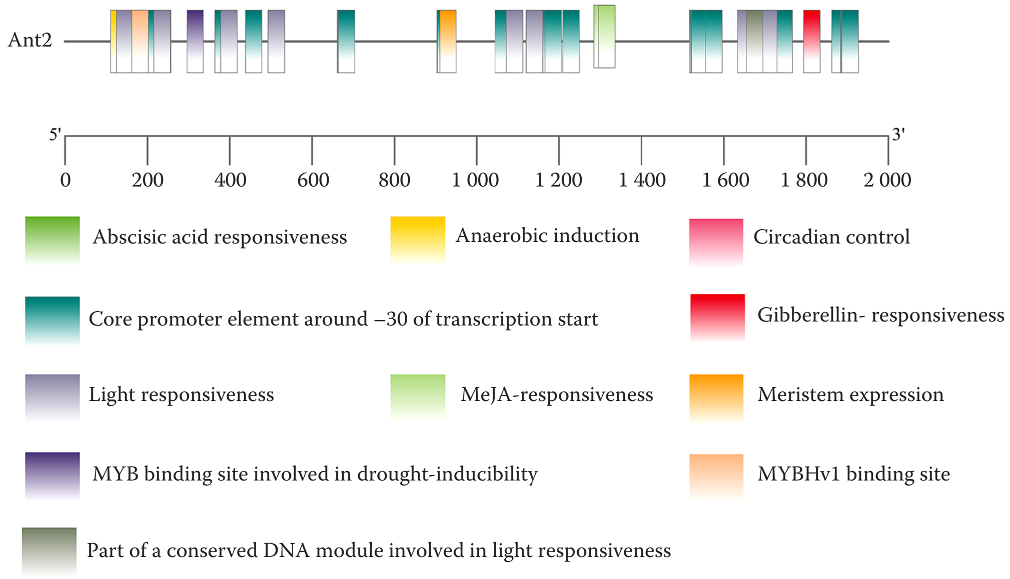

Figure 5. Structure of the *cis*-acting elements in the promoter region of the *HvnAnt2* gene

Table 2. Structure of the *cis*-acting elements in the promoter region of the *HvnAnt2* gene

Site name	Sequence	Starting position	Ending position	Function
ABRE	ACGTG	217 1 120	257 1 160	abscisic acid responsiveness
ARE	AAACCA	111 1 700	151 1 740	anaerobic induction
circadian	CAAAGATATC	150	190	circadian control
TATA-box	ATATAA	178	218	core promoter element around −30 of transcription start
	TATA	179	219	
	TAAAGATT	364	404	
	TATA	438	478	
	TATATAA	661	701	
	TATATA	662	702	
	TATA	664	704	
	TATAA	903	943	
	TATA	904	944	
	TAAAGATT	1 044	1 084	
	TATA	1 155	1 195	
	TATA	1 166	1 206	
	TATA	1 209	1 249	
	taTATAAAtc	1 516	1 556	
	TATATA	1 520	1 560	
	ATATAA	1 521	1 561	
	TATA	1 522	1 562	
	TATA	1 556	1 596	
	TATATA	1 725	1 765	
	TATA	1 727	1 767	
	TATA	1 862	1 902	
	ATATAT	1 884	1 924	
	TATATA	1 885	1 925	
	TATA	1 887	1 927	
GARE-motif	TCTGTTG	1 794	1 834	gibberellin-responsive
G-Box	CACGTT CACGAC TACGTG	216 493 1 120	256 533 1 160	light responsiveness
GT1-motif	GGTTAA	1 633	1 673	
GA-motif	ATAGATAA	125	165	
Gap-box	CAAATGAA(A/G)A	378	418	
TCT-motif	TCTTAC	1 072 1 689	1 112 1 729	
CGTCA-motif	CGTCA	1 285 1 296	1 325 1 336	MeJA-responsiveness
TGACG-motif	TGACG	1 285 1 296	1 325 1 336	
CAT-box	GCCACT	910	950	meristem expression
MBS	CAACTG	296	336	MYB binding site involved in drought-inducibility
CCAAT-box	CAACGG	162	202	MYBHv1 binding site
Box 4	ATTAAT	1 655	1 695	part of a conserved DNA module involved in light responsiveness

taining WD40 domain (A0A287E369, A0A287WVU6, and A0A287R8E9), and one SANT domain-containing protein (F2E3X7_HORVV) (Figure 7A). There were 10 proteins interacted closely with Q7XPS3_ORYSJ in *Oryza sativa*, namely, proteins containing the Spc97_Spc98 domain (Q60F24_ORYSJ, Q0JBZ2_ORYSJ, A0A0P0YC23, and A0A0P0VJP8), protein containing the Tubulin and Tubulin_C domains (TUBG2), proteins containing epimerase domains (Q0J9V0_ORYSJ and A0A0P0V5N7), protein containing the Tmemb_14 domain (B7FAK3_ORYSJ), protein containing the WD40 domain (Q3V826_ORYSJ), and Os01g0619900 protein (A0A0P0V5D3) (Figure 7B). There were 10 proteins interacted closely with A0A287J214 in *Zea mays*, namely, proteins containing the p450 domain (K7TYB1, A0A1D6HC18, B7ZYZ2, CYP92C5, A0A1D6PZR7, and A0A1D6ENZ1), protein containing Sulfotranssfer_3 (A0A1D6Q726), protein containing the NmrA domain (A0A1D6JF41), protein containing the SANT domain (MYBR97), and a protein containing the WD40 domain (pac1) (Figure 7C).

DISCUSSION

Anthocyanidin is commonly found in plants as a water-soluble flavonoid secondary metabolite, it is a colourful glycoside obtained by hydrolysis of anthocyanin (Smeriglio et al. 2016). The structure of anthocyanins in plants primarily includes brick-red pelargonidin, red cyanidin, blue delphinidin, purple peonidin, blue-violet malvidin, and purple petunidin derived from cyanidin and selphinidin (Tanaka & Ohmiya 2008; Wang et al. 2015). Anthocyanins have a wide range of biological functions, such as plant colouring, resisting UV damage, and protecting plants from various biotic and abiotic stresses (Ahmed et al. 2014), and can also be used in food (Li et al. 2016), medicine (Liu et al. 2018), and cosmetics industries (Nizioł-Łukaszewska et al.

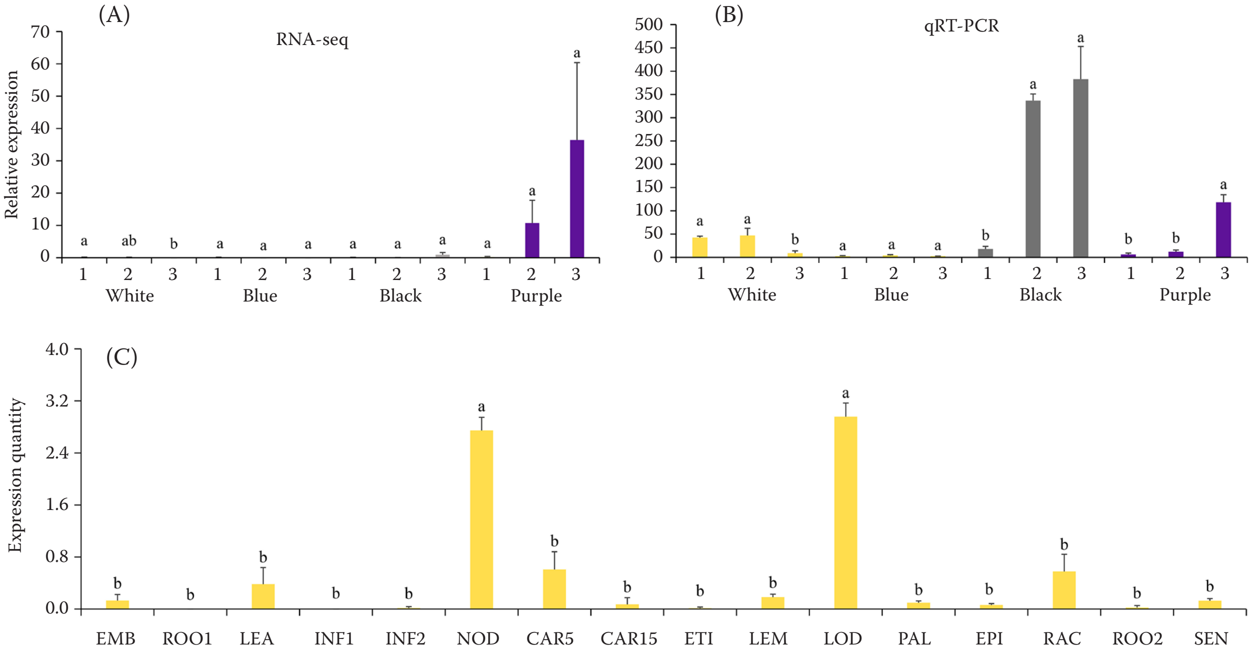

Figure 6. Expression of *Ant2* gene in different qingke barley varieties and barley tissues: the relative expression levels of RNA-seq (A) and qRT-PCR (B) of white, blue, black, and purple qingke barley (one-way analysis of variance was used to test the significance of differences; different lowercase letters represent significant differences in the same variety in the three periods and the amount of expression in different tissues ($P < 0.05$)); expression of Ant2 gene in different tissues of barley (C) EMB – 4-day-old embryos; ROO1 – roots from seedlings (10-cm shoot stage); LEA – shoots from seedlings (10-cm shoot stage); INF1 – Young developing inflorescences (5 mm); LEA – shoots from seedlings (10-cm shoot stage); NOD – developing tillers, third internode (42 days after pollination (DAP)); CAR5 – developing grain (5 DAP); CAR15 – developing grain (15 DAP); ETI – etiolated seedling, dark conditions (10 DAP); LEM – inflorescences, lemma (42 DAP); LOD – inflorescences, lodicule (42 DAP); PAL – dissected inflorescences, palea (42 DAP); EPI – epidermal strips (28 DAP); RAC – inflorescences, rachis (35 DAP); ROO2 – roots (28 DAP); SEN – senescing leaves (56 DAP)

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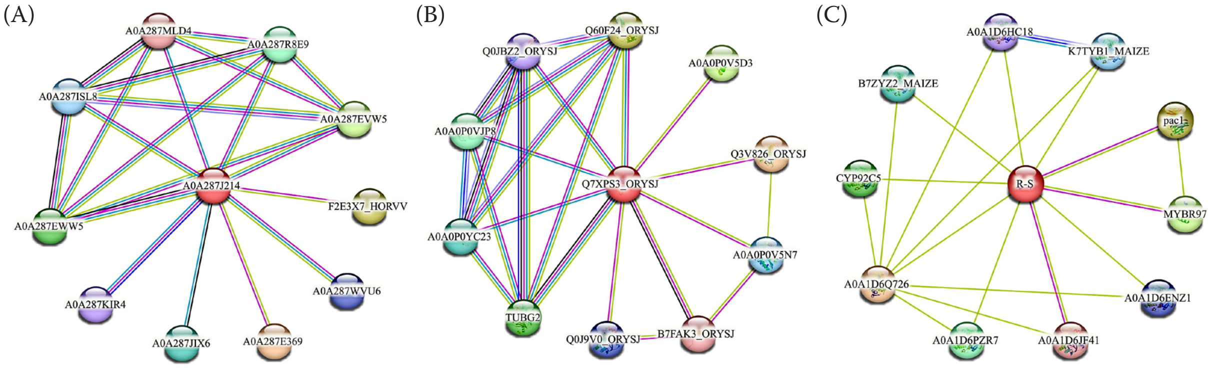

Figure 7. Predictive analysis of Ant2 protein interactions among *Hordeum vulgare* (A), *Oryza. Sativa* (B), and *Zea mays* (C)
Each node represents all the proteins produced by a single, protein-coding gene locus; colored nodes – query proteins and first shell of interactors; white nodes – second shell of interactors; empty nodes – proteins of unknown three-dimensional (3D) structure; filled nodes – a 3D structure is known or predicted; blue line – obtained from curated databases; light red – experimentally determined; green – gene neighborhood; red – gene fusions; purple – gene co-occurrence; light green – text-mining; black – co-expression; light purple – protein homology

2018). The Ant2 in this study belongs to the bHLH family of transcription factors, which are considered to be the regulatory genes of the anthocyanin glycoside biosynthesis pathway (Lahaye et al. 1998; Shoeva et al. 2016; Cockram et al. 2010). Therefore, it is of great significance to explore this gene to elucidate the synthesis of anthocyanins in qingke barley.

Variations in pigmentation patterns are mainly based on the allelic variation of regulatory genes rather than structural genes (Gordeeva et al. 2019). Previous research has demonstrated that the *Ant2* gene plays a key role in anthocyanin synthesis (Cockram et al. 2010). In barley, the transcription of the *Ant* gene was almost undetectable in the glume and peel of strains containing the recessive allele of the gene (Gordeeva et al. 2019). It was concluded that *ant2* mutant barley plants had no anthocyanin accumulation in stems, auricles, awns, or lemmas, whereas the *Ant2* gene regulated anthocyanin glycoside colouration in purple and white grain auricles, awns, or lemmas (Himi & Taketa 2015; Cockram et al. 2010). The Ant2 transcript level of coloured barley varieties Kunlun Purple and Kunlun Black were significantly higher than in the colourless barley variety GP (Zhou et al. 2021). In this study, the expression of the *Ant2* gene in black qingke barley was the highest, and the expression was significantly higher in developing tillers at the third internode (42 DAP) and leaflet-like inflorescences (42 DAP) than in the other 14 tissues examined, the expression levels of different grain colour qingke barley and qingke barley tissues were variety-specific and tissue-specific, which was consistent with the study (Zhou et al. 2021; Chen et al. 2023). *Ant2* gene in black and purple qingke barley, there was no study on blue qingke barley (Zhou et al. 2021). The structural differences between the *Ant2* and *ant2* alleles may underlie their different expression levels in the peel of barley grains (Shoeva et al. 2016). Through genome-wide association analysis of barley, and sequence analysis showed that *Ant2* encoded bHLH-class transcription factors, while a 16-bp fragment deletion in the *ant2* exon of the defunct allele led to the premature termination of transcription of the predicted protein (Cockram et al. 2010). In this experiment, the *Ant2* gene was cloned in the leaves of four varieties of qingke barley with different grain colours. Only three amino acids were changed in black qingke barley: leucine was changed to valine, valine was changed to alanine, and threonine was changed to methionine. The function of this gene needs to be further studied.

Studies have shown that the rearrangement of *Ant2* gene promoter region in common barley Bowman reduces the transcription level of this gene, resulting in the loss of barley grain phenotype (Shoeva et al. 2016). In this study, the white phenotype of white qingke barley might have also been caused by the rearrangement of the *Ant2* gene promoter region. *Cis*-acting elements indicated that the *HvnAnt2* gene contains ABA response, MeJA response, and MYB binding sites, and might be related to plant physiological and abiotic stress as well as immune response, suggesting that the *HvnAnt2* gene is an inducible promoter, the presence of *cis*-acting elements in the gene promoter might mean that the *HvnAnt2* gene has a different function. There was no specific transcriptional regulation of flavonoid synthesis in black barley, which indicated that flavonoid pigments are not involved in the formation of black lemma and pericarp characters (Hao et al. 2021). Elevated transcript levels of the *Ant2* gene in barley purple pericarp (Shoeva et al. 2016) and its homologs in other cereals (Khlestkina 2013; Jiang et al. 2018) suggest that *Ant2* also controls anthocyanin biosynthesis in the pericarp. The positive regulatory interaction between *Ant* genes at the transcriptional level predetermines the transcriptional activity of each regulatory gene and the structural gene for late anthocyanin biosynthesis (*F3'H*, *ANS*). When the *Ant1* and *Ant2* gene alleles were in a dominant constitution, the transcription levels of *F3'H* and *ANS* genes were significantly up-regulated (Gordeeva et al. 2019). Flavonoid 3'-monooxygenase belongs to the cytochrome P450 family, and cytochrome P450 plays an important role in the biosynthesis of flavonoids and their coloured compounds, anthocyanins (Han et al. 2010; Tanaka & Brugliera 2013). Furthermore, *ANS* is expressed in most tissues of plants, and its expression varies at diverse times in various plants in a tissue-specific, and variety-specific manner (Zhou et al. 2010; Wang et al. 2011). It is speculated that the regulatory effect of the *Ant* gene on the *ANS* gene may be a crucial cause of barley grain colouration. The barley *HvANT2* candidate gene belonged to a branch containing bHLH proteins encoded by genes at the R/B locus, which controlled anthocyanin pigmentation in maize (Goff et al. 1992). Protein interaction prediction indicated that anthocyanin regulatory protein (R-S), flavonoid 3'-monooxygenase, anthocyanidin reductase, and MYB-related transcription factor and other proteins and enzymes related to anthocyanin synthesis existed in the *Ant2* gene. The results laid a foundation for further understanding of the *Ant2* gene function.

CONCLUSION

In this study, the *HvnAnt2* gene was cloned from the white, blue, black, and purple qingke barley varieties. The *cis*-acting elements of the promoter showed that the *HvnAnt2* gene was primarily related to light responsiveness and MeJA-responsiveness. The *Ant2* gene was highly expressed in black and purple qingke barley grains during the later stage of development, and was significantly higher in developing tillers at the third internode and leaflet-like inflorescences of barley. The presence of flavonoid 3'-monooxygenase, anthocyanin biosynthesis regulatory genes interacted with the Ant2 protein. It is speculated that *HvnAnt2* gene may be related to plant physiological and abiotic stress as well as immune response, and participates in the formation of coloured qingke barley.

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