https://doi.org/10.17221/180/2020-PPS

Use of gene family analysis to discover *argonaut* (*AGO*) genes for increasing the resistance of Tibetan hull-less barley to leaf stripe disease

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Citation: Yao X., Wang Y., Yao Y., An L., Bai X., Li X., Wu K., Qiao Y. (2021): Use of gene family analysis to discover *argonaut* (*AGO*) genes for increasing the resistance of Tibetan hull-less barley to leaf stripe disease. Plant Protect. Sci., 57: 226–239.

Abstract: Leaf stripe is a common, but major infectious disease of barley, severely affecting the yield and quality. However, only a few genes have been identified by conventional gene mapping. Gene family analysis has become a fast and efficient strategy for gene discovery. Studies demonstrated that Argonaute (AGO) proteins play an important role in plant disease resistance. Thus, we obtained nine *HvAGO* genes via mRNA sequencing before and after a *Pyrenophora graminea* infection of a disease-resistant variety "Kunlun 14" and a susceptible variety "Z1141". We analysed the physicochemical characteristics, gene structures, and motifs of the *HvAGO* gene sequences and found that these proteins were divided into four clusters by evolutionary distance. There was high consistency in the number of exons, size, and the number and type of motifs in the different clusters. Based on protein phylogenetics, they could be divided into three branches. A collinearity analysis of Tibetan hull-less barley and *Arabidopsis thaliana*, rice, and maize showed that four genes were collinear with respect to the other three species. The qRT-PCR showed the expression levels of *HvAGO1*, *HvAGO2* and *HvAGO4* were significantly increased after infection with *Pyrenophora graminea*. These three members of the *AGO* gene family are, thus, speculated to play an important role in barley leaf stripe resistance. The results provide reference for the application of *HvAGO* genes in the leaf stripe control and the exploration of disease resistance genes in other crops.

Keywords: gene expression; *HvAGO1*; *HvAGO2*; *HvAGO4*; *Pyrenophora graminea*

Tibetan hull-less barley (*Hordeum vulgare* Linnaeus var. *nudum* Hook. F.) belongs to the *Hordeum* genus and Gramineae family and is a variety of cultivated barley. As the lemma and palea of the grains are separated from the caryopsis when it is mature, it is also known as naked barley or qingke on the Tibetan plateau in China (Liang et al. 2012). Since the 5th century, Tibetan hull-less barley has been the staple food for Tibetans and is widely cultivated on the Tibetan plateau, providing food safety for the region (Yao et al. 2018; Zeng et al. 2018). Tibetan hull-less barley is not only rich in nutrients and physiologically active substances, but it is also used as a food colouring in food processing. This barley has a high

Supported by the Qinghai Science and Technology Department, Project No. 2019-ZJ-7075, the Natural Science Foundation of China, Project No. 31660388; 31960427, and by the Agriculture Research System of China, Project No. CARS-05.

https://doi.org/10.17221/180/2020-PPS

nutritional value and excellent medicinal and health effects (Li et al. 2011; Wu et al. 2017).

For many years, leaf stripe has been a major infectious disease that is common in barley and is caused by the fungus *Pyrenophora graminea* (Ito et Kurib.) (Pecchioni et al. 1996; Si et al. 2020). Leaf stripe can lead to a drastic decrease in the barley yield, particularly in the susceptible variety "Arabi Abiad", of which, the yield decreased by 92% in 2004 (Arabi et al. 2004). In 2012, Tibetan hull-less barley leaf stripe affected 52 000 hm of farmland in Qinghai Province, accounting for 60% of the cultivation area (Liu 2012). Leaf stripe, thus, severely affects the yield and quality of barley not only in barley growing regions around the world, but also in hull-less barley growing regions on the Tibetan plateau (Si et al. 2019). At present, the main control method is seed coating (Skorda 1974). However, the ecological environment of the Tibetan plateau is fragile, and there are strict controls on pesticides and chemical fertilisers, which makes it difficult to implement seed coating. Hence, the selective breeding of resistant varieties is the most effective and environmentally friendly method to control leaf stripe. Barley leaf stripe resistance is a complex trait that involves many disease resistance genes (Yang et al. 2016). The quantitative trait loci (QTL) analysis is the most effective method for analysing crucial traits for the selective breeding of disease-resistant barley (Tacconi et al. 2001; Richardson et al. 2006). Inheritance of resistance to barley leaf stripe was studied earlier (Skou et al. 1987). However, only a few leaf stripe-resistant genes were identified by gene mapping. For example, Pecchioni et al. (1996) found two quantitative trait loci (QTLs) on barley chromosome 1 and chromosome 2 using a barley doubled haploid (DH) population derived from the cross 'Proctor' × 'Nudinka', but no leaf stripe-resistant gene was isolated. Gene mapping was also used in the progeny of hybridised leaf stripe-resistant and susceptible varieties to identify two disease resistance genes, *Rdg1a* and *Rdg2a*, which are located on the 2HL and 7HS chromosomes, respectively (Arru et al. 2002, 2003; Richardson et al. 2006; Biselli et al. 2013a, b). Overexpression of these two genes significantly increased the resistance of barley against leaf stripe (Haegi et al. 2008, Biselli et al. 2010; Bulgarelli et al. 2010). Since then, there have been few reports on leaf stripe-resistant genes identified through gene mapping. These findings suggest that we may have reached a bottleneck in barley leaf stripe studies using conventional gene mapping analysis, and, thus, new methods and perspectives are needed to identify new genes that are directly or indirectly related to leaf stripe resistance and the associated mechanisms. Gene family analysis has become a fast and efficient strategy for gene discovery (Yan et al. 2019; Liu et al. 2020). In the discovery of disease-resistant genes, Srideepthi et al. (2020) found some *non-RD* (non-arginine-aspartate) receptors like kinase genes in response to the *Colletotrichum truncatum* stress in hot peppers; Chandra et al. (2020) found certain *NB-ARC* (the nucleotide-binding adaptor shared by APAF-1, R proteins, and CED-4) genes in response to leaf rust infection in wheat. AGO is an important regulatory protein for plant growth, development, and stress resistance (Wu et al. 2015; Kumar et al. 2017). However, the discovery and expression of leaf-related *AGO* genes in the barley genome have not been reported.

Argonaute proteins are important for the ribonucleic acid-mediated post-transcriptional gene modification complex and were first discovered in *Arabidopsis thaliana* (Bohmert et al. 1998). AGO proteins mainly consist of four domains, namely the variable N-terminal, PAZ, PIWI, and MID domains. Some AGO proteins also contain the DUF1785 and AgoL2 domains. AGO proteins are defined by the presence of functional domains, including the PAZ domain and the PIWI domain at the C-terminal (Hutvagner et al. 2008; Bai et al. 2012). Studies have demonstrated that AGO proteins play an important role in disease resistance in plants by binding different types of small RNAs. An example is in tomato plants, in which miR403 can target the *AGO2* gene to regulate the leaf curl virus disease (Kumar et al. 2017). In rice, virus-induced *OsAGO18* can inhibit miR168, thereby alleviating rice *OsAGO1* inhibition by miR168 and enabling the infected rice to resist the virus (Wu et al. 2015). In tobacco, the differential expression of *AGO1* can alter the resistance of plants towards virus infection, enabling infected tobacco to not only recover, but to also simultaneously increase in tolerance towards cold stress (Banu et al. 2015). Although *HvAGOs* have recently been identified in the barley reference genome (Hamar et al. 2020), it is not clear which ones are associated with leaf stripe. In this study, bioinformatics methods, such as reference genome sequence query, sequence alignment and structure prediction were used to analyse the quantity, chromosome localisation, evolution, and classification of *HvAGO* genes that are associated with barley leaf stripe; to pre-

https://doi.org/10.17221/180/2020-PPS

dict the physiological and biochemical characteristics of the coded proteins; to analyse the response modes of *HvAGO*s to Tibetan hull-less barley leaf stripe; and to screen for Tibetan hull-less barley leaf stripe-related *HvAGO* genes to provide a theoretical foundation for further research on the disease resistance function of *HvAGO*s.

MATERIAL AND METHODS

Plant materials and leaf stripe treatment. Wild Tibetan hull-less barley 'Z1141' and the hybrid variety 'Kunlun14', bred by crossing 'White 91–97–3' × 'Kunlun 12', were used to investigate the leaf stripe resistance. The proportion of the field area (per square meter) of Kunlun14 and Z1141 was calculated from 2015 to 2017. Kunlun14 is a cultivar that can tolerate leaf stripe, while Z1141 is susceptible to leaf stripe. In this study, we used KL14N (Kunlun14 without leaf stripe), KL14S (Kunlun14 with leaf stripe), ZN (Z1141 without leaf stripe) and ZS (Z1141 with leaf stripe) as experimental materials. First, infected leaves of Z1141 were collected to construct the leaf stripe infection system in the laboratory. To complete this process, five pieces (5 × 5 mm) of one typical infected leaf from Z1141 were isolated, rinsed with 75% alcohol for 30 s and 2% sodium hypochlorite for 30 s, and then washed with distilled water three times. Finally, the leaf pieces were cultivated in a sealed Petri dish filled with a potato dextrose agar (PDA) culture medium (200 g of dissolved peeled potato, 17 g of glucose, 16 g of agar added to 1 000 mL of distilled water) after they were dried. The dish was placed in a biochemical incubator at 22–25 °C for 3 days. Non-contaminated mycelia of *Pyrenophora graminea* were transferred to a new PDA culture dish four times until the colony was purified. First, three ensuing fungal cakes were removed with a perforator and cultivated in another new PDA culture medium, which was then placed in a biochemical incubator at 25 °C for 5–7 d until the whole Petri dish was filled with the *Pyrenophora graminea* colony. Second, 300 grains of each Tibetan hull-less barley variety were disinfected with 75% alcohol for 1 min, 150 grains were equally placed into three Petri dishes covered with the *Pyrenophora graminea* colony, and another 150 grains were equally placed in three Petri dishes covered with the PDA culture medium without the *Pyrenophora graminea* colony. The above six dishes were then placed in a low-temperature incubator for two weeks at 6 °C. Finally, 300 grains of each variety were planted in six flowerpots with a vegetative soil and cultured in an artificial climate incubator (daytime: 14 h, at 20 °C; night time: 10 h, at 10 °C) for approximately 10 days. The second infected or normal leaf from the bottom of the seedling was removed, and leaves with at least three biological repeats were refrigerated at –80 °C after freezing in liquid nitrogen.

Identification and analysis of Tibetan hull-less barley leaf stripe-related *HvAGO* genes. We selected all the *HvAGO* genes from the Swiss-Prot gene annotation results after the 'Kunlun 14' and 'Z1141' Tibetan hull-less barley varieties were infected by *Pyrenophora graminea* [Table S1 in electronic supplementary material (ESM)]. SMART software was used for the domain analysis. BLAST was used to align the whole genomes of the rice, *Arabidopsis thaliana*, and maize, and an E-value = $1e^{-10}$ was used for the screening. After removing the sequence repeats and deletions, we obtained the protein sequences of nine *HvAGO* genes. We obtained the CoDing Sequences (CDS), exons and intron structures, and the translated protein sequences of the HvAGO-encoding genes from the Gramene database (http://ensembl.gramene.org). The online tool Expasy Protparma (http://www.expasy.org/tools/protparam.html) was used to analyse the isoelectric point, lipid solubility, hydrophobicity, stability, and other physicochemical properties of the nine *HvAGO* proteins. CELLO v.2.5 (http://cello.life.nctu.edu.tw) was used to predict the subcellular localisation of the HvAGO proteins. A search was conducted in the Gramene database to obtain the chromosome location of the *HvAGO* genes. MapChart software was used to plot the distribution of the *HvAGO* genes on the chromosomes. MEME (http://meme-suite.org/) online software was used to predict the conserved motifs in the protein gene family and determine the conserved amino acid residues in these domains. The maximum motif value was 10 (Table 1), the optimal motif width was 10–100, and the default values were used for the other parameters. The online tools 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 structures and three-dimensional structures of the HvAGO proteins. IQ-TREE software (version 2.0.3) was used for the phylogenetic tree analysis, and iTOL (https://itol.embl.de) was used for the plotting.

Expression level analysis of HvAGO1 under leaf stripe stress. The total RNA per sample was extracted with a TaKaRa MiniBEST Universal

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Table 1. Motif information of the protein gene family

Motif	Width	Best possible match
1	50	FDFYLCSHAGJKGTSRPTHYHVLLDENN-FTADDLQKLVYNLCYVYARCTR
2	36	VTDKPTIIFGADVTHPSPGEDSSPSIAAV-VASMDWP
3	29	RIIFYRDGVSEGQFSQVLNYELDAIRKAC
4	34	RGFYQSJRPTQQGLSLNIDMSATAFVKPG-PVIDF
5	26	GYKPKITFIVVQKRHHTRLFPNDHND
6	29	KMNDQYLANLALKINAKLGGRNTVLADEL
7	29	JVLPEDKNGDLYGDLKRICETELGJVTQC
8	21	PNYLPIELCKIVEGQRYSKKL
9	21	VSLVPPAYYAHLAAYRARFYL
10	24	QWNMNNKKLVNGARVRHWACVNFS

RNA Extraction Kit (Takara, Japan) and this RNA was then reverse-transcribed to cDNA using a PrimeScript 1st Strand cDNA Synthesis Kit (Takara, Japan). Primer 5.0 was used to design the quantitative reverse transcription polymerase chain reaction (qRT-PCR) primers for the *HvAGO* genes, and TC139056 was used as an internal reference for the qRT-PCR analysis (Faccioli et al. 2005). Table 2 shows the primer sequences. The reaction system was 20 μL and contained 10 μL of TB Green premix Ex Taq II (Tli RNaseH Plus) (2×), 2 μL of the cDNA template, 0.8 μL each of PCR forward/reverse primers (10 μmol/L), and 6.4 μL of ddH_2O. The qRT-PCR amplification conditions were as follows: pre-denaturation at 95 °C for 5 min; 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; following by a melting curve at 95 °C for 5 s and 70 °C for 1 minute. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative gene expression levels from the experimental results (Pfaffl et al. 2001).

RESULTS

Identification of *HvAGO* genes and analysis of conserved domains in the protein sequences. The gene annotation was carried out on the transcriptome analysis results before and after the *Pyrenophora graminea* infection in the resistant variety 'Kunlun 14' and the susceptible variety 'Z1141'. Sixteen differentially expressed *HvAGO*s were obtained from the screening. After SMART

Table 2. Primer sequences used in the present study

Gene	Description	Primer sequence (5'–3')
HORVU7Hr1G007000	forward-primer	ATGGTCAATGGTGGTAGAG
	reverse-primer	CGGCAGTATAACAATTAGCA
HORVU7Hr1G120600	forward-primer	AGGCAGGAACTAATAGAGGA
	reverse-primer	AATAATACGCTCAGGCTTCT
HORVU4Hr1G004030	forward-primer	GTTCCTCCAGCATACTACG
	reverse-primer	GCCTGACACTCTCCTTAAC
HORVU2Hr1G098650	forward-primer	GTGATGGCGTGAGTGATG
	reverse-primer	CGGTAATCGTTGGAGAGTAG
HORVU2Hr1G098620	forward-primer	TCATTGGCAATATCGTCAGA
	reverse-primer	TCTCGGCATCCTTCTTCA
HORVU3Hr1G038830	forward-primer	GCAGCAGTTGTTGGTTCT
	reverse-primer	CATCATCTTCAGTTCCTTGTG
HORVU3Hr1G021290	forward-primer	ATGAACTCGGTGCTACAAG
	reverse-primer	CAACGGATGGTATATCAGACT
HORVU0Hr1G012490	forward-primer	ATTGCTGCTGTTGTTGGA
	reverse-primer	TGTTGACTGGTTGTGTAGAA
HORVU0Hr1G005350	forward-primer	ACGAGAAGGTGCTATGGA
	reverse-primer	GTGGAATGCTGTGAGTGA
TC139056 (internal reference)	forward-primer	GAAGGATGAGCAAAAGGCCCT
	reverse-primer	GGCAGGCAGACTCATTTCTTCC

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and BLAST were used for further validation, nine protein sequences containing the PIWI, PAZ, or DUF1785 domains were ultimately obtained. Among these sequences, only HORVU2Hr1G098620 contained the PIWI domain, HORVU3Hr1G038830 contained the DUF1875 and PIWI domains, and the remaining seven HvAGOs contained the PIWI, PAZ, and DUF1785.3 domains (Figure 1).

Physiochemical characteristics and subcellular localisation of HvAGO proteins. From Table 3, the CDS lengths of the nine *HvAGO*s ranged from 2 652 to 3 654 bp, and the corresponding protein sequences ranged from 884 to 1 218 amino acids. The predicted molecular weight and isoelectric points ranged from 218.51 to 299.97 kD and from 4.77 to 4.89, respectively. The hydrophilicity of the AGO proteins was 0.731–0.860, indicating that these proteins were hydrophilic. Except for the AGO proteins encoded by HORVU3Hr1G038830 and HORVU0Hr1G012490, which possessed stability indices of less than 40 and were thus stable, the stability indices of the other members of the family were higher than 40, indicating that they were unstable. The lipid solubility index of the AGO proteins was 21.03–29.00.

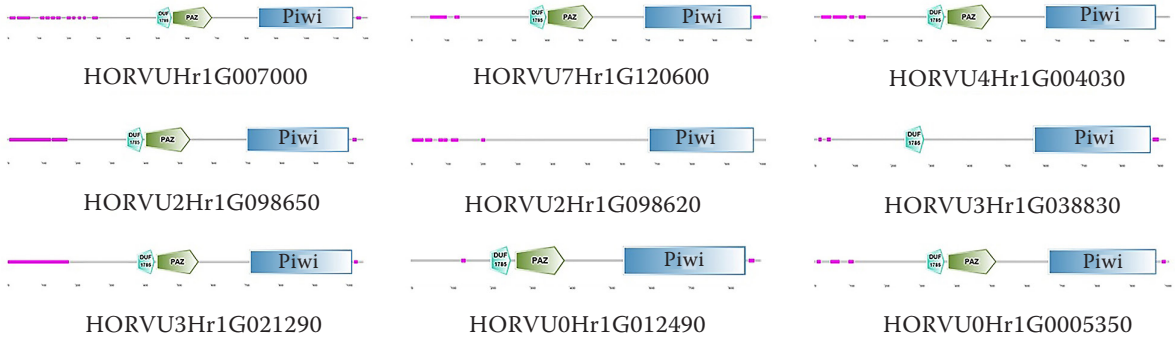

Figure 1. Domain architecture of the predicted barley AGO proteins

The bright blue pentagon represents the DUF1785 domain; the green pentagon represents the PAZ domain; the dark blue quadrilateral represents the DUF1785 PIWI domain; the purple lines represent regions with low complexity

Table 3. The physical and chemical properties of the nine HvAGO protein genes

No.	Gene ID	CDS length (bp)	Intron	Exon	Theoretical pI	Molecular weight (KD)	Subcellular localisation	Instability index	Hydropathicity	Aliphatic index
1	HORVU7Hr1G007000	3 654	22	23	4.78	299.97	peri: 1.488 extr: 1.106	45.23	0.799	25.59
2	HORVU7Hr1G120600	3 195	21	22	4.77	263.25	cyto: 1.592 oute: 1.439 peri: 1.336	46.12	0.860	21.03
3	HORVU4Hr1G004030	3 102	21	22	4.83	252.59	oute: 2.613	41.67	0.744	25.60
4	HORVU2Hr1G098650	3 144	2	3	4.81	255.13	peri: 1.539 oute: 1.444	48.94	0.796	23.73
5	HORVU2Hr1G098620	3 060	2	3	4.77	249.08	peri: 1.822, cyto: 1.382 oute: 1.352	46.78	0.852	25.36
6	HORVU3Hr1G038830	2 769	22	23	4.87	228.29	peri: 2.487	39.49	0.763	27.92
7	HORVU3Hr1G021290	3 156	20	21	4.81	256.32	cyto: 1.929 peri: 1.511	47.03	0.788	24.02
8	HORVU0Hr1G012490	2 652	22	23	4.89	218.51	peri: 2.420	39.76	0.731	29.00
9	HORVU0Hr1G005350	3 039	2	3	4.81	249.88	extr: 1.291	43.70	0.832	25.70

CDS – sequence coding for amino acids in protein; bp – base pair; pI – isoelectric point; KD – kilodalton; peri – periplasmic; extr – extracellular; cyto – cytoplasmatic; oute –outer membrane

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The subcellular localisation of the nine genes was predicted, and the results showed that most of the AGOs located in the periplasm or cytoplasm. Therefore, we hypothesised that these proteins may carry out their functions in the periplasm or cytoplasm.

Chromosome distribution of the nine *HvAGO* genes. Table 4 and Figure 2 show the chromosome distribution of the nine *HvAGO* genes. Seven *HvAGO* genes were selectively located on chromosomes 2, 3, 4, and 7 in the barley, while the locations of two *HvAGO* genes could not be determined (Figure 2). Among these genes, HORVU7Hr1G007000 and HORVU7Hr1G120600 were located on chromosome 7H, and HORVU4Hr1G004030 was located on chromosome 4H. HORVU3Hr1G038830 and HORVU3Hr1G021290 were located on chromosome 3H, HORVU2Hr1G098650 and HORVU2Hr1G098620 were located on chromosome 2H, and HORVU1Hr1G091720 was located on chromosome 1H. The chromosome locations of HORVU0Hr1G012490 and HORVU0Hr1G005350 were not determined. HORVU2Hr1G098620 and HORVU2Hr1G098650 were in the same gene cluster; therefore, we speculated that they might have similar functions.

Analysis of *HvAGO* gene structures and protein motifs. The phylogenetic analysis of the *HvAGO*s was used to analyse the gene structures of the *HvAGO* family members and we found large differences in the gene structures among the nine *HvAGO* genes (Figure 3). Using the phylogenetic tree of HvAGOs, we analysed the protein motifs of the HvAGO family members and found that although the locations of the ten motifs differed, their arrangements were

Table 4. Chromosome (Chr) distribution of the nine *HvAGO* genes

No.	Gene ID	Chr	Start	End
1	HORVU7Hr1G007000	chr7H	9459266	9467181
2	HORVU7Hr1G120600	chr7H	651899946	651906855
3	HORVU4Hr1G004030	chr4H	8972435	8981437
4	HORVU2Hr1G098650	chr2H	684121508	684127760
5	HORVU2Hr1G098620	chr2H	684080121	684086180
6	HORVU3Hr1G038830	chr3H	226356734	226364526
7	HORVU3Hr1G021290	chr3H	68596766	68603419
8	HORVU0Hr1G012490	chrUn	71291967	71302772
9	HORVU0Hr1G005350	chrUn	29969371	29973587

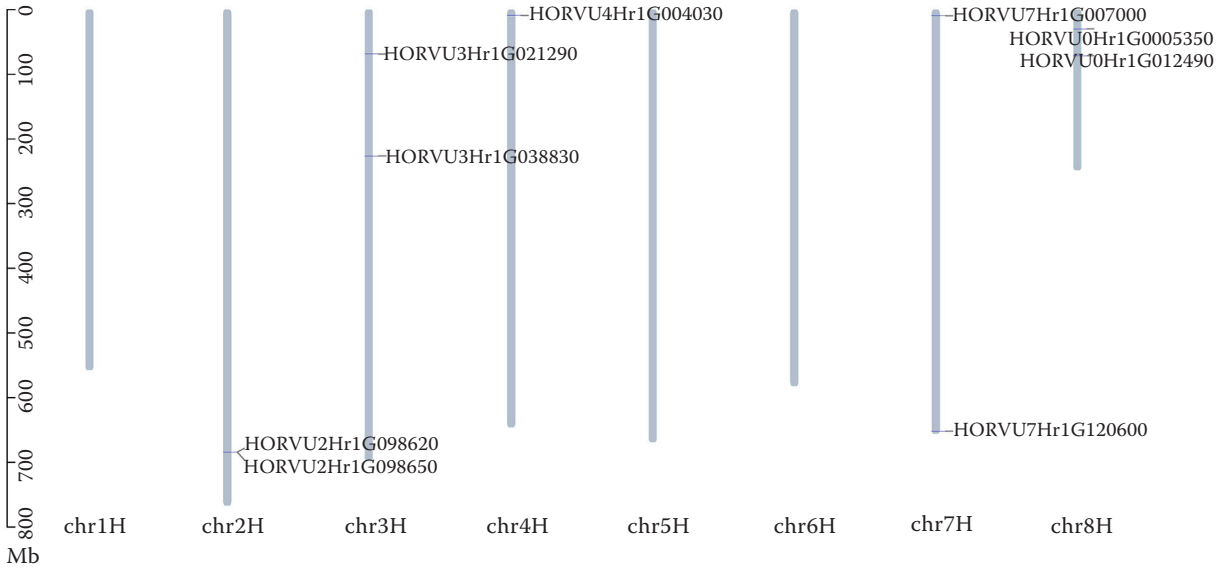

Figure 2. Distribution of 57 *HvAGO* genes on the barley chromosomes

The chromosome numbers are indicated at the bottom of each chromosome; Mb – megabase

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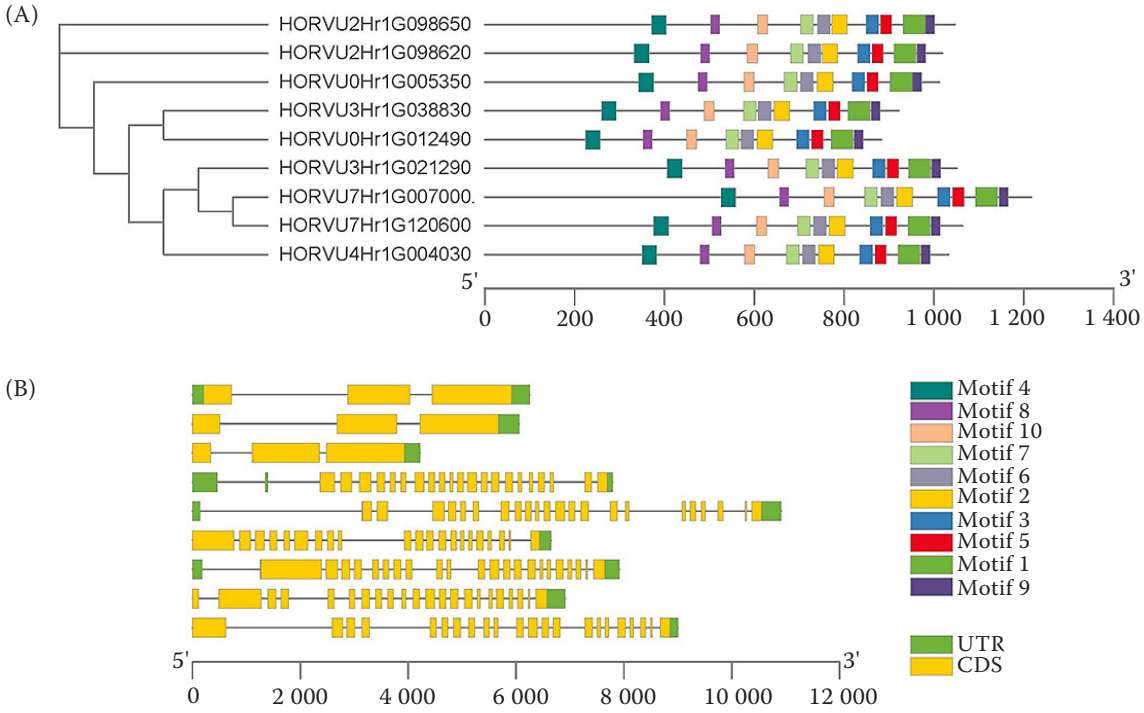

Figure 3. Structure of genes from the *HvAGO* family

(A) Motifs of *HvAGO* genes, (B) UTR (untranslated region) and CDS of *HvAGO* genes

the same among the nine *HvAGO* genes (Figure 3A). Among these genes, HORVU2Hr1G098650, HORVU2Hr1G098620, and HORVU0Hr1G005350 contained two introns, while the remaining HvAGOs were clustered together and contained 20–22 introns (Figure 3B). The structural differences of these *HvAGO* genes may be closely associated with their functions.

Domain homology comparison of HvAGOs. SMART was used for the online analysis of the domains in the HvAGO. We found that the seven HvAGOs (HORVU0Hr1G005350, HORVU0Hr1G012490,HORVU2Hr1G098650, HORVU3Hr1G021290, HORVU4Hr1G004030, HORVU7Hr1G007000, and HORVU7Hr1G120600) all contained conserved DUF1785, PAZ, and PIWI domains; HORVU3Hr1G038830 contained the DUF1785 and PIWI domains; and HORVU2Hr1G098620 contained the PIWI domain. Through the domain analysis, we confirmed that the above genes belong to the AGO family (Figure 4). The protein homology of the nine identified HvAGOs was 40.91%, of which the domain homology of DUF1875, PAZ, and PIWI was 55.36, 44.04, and 55.86%, respectively. The sequence homology analysis results showed that there were large differences in the HvAGO protein and domain sequences, suggesting that members of the *HvAGO* gene family may have their own unique function and role.

Analysis of the higher-order structures of the HvAGO proteins. The higher-order structures of the HvAGO proteins were predicted (Figure 5). The SPOMA online tool was used to predict the secondary structures of the barley AGOs. Figure 5A shows that the secondary structures of these proteins included α-helices, β-turns, extended chains, and random coils. Random coils were the most important secondary structure components in these proteins, accounting for 44.83–55.38% in the HvAGOs, followed by α-helices (26.21–35.06%), extended chains (13.80–19.93%), and lastly β-turns (3.95–7.23%). The above results showed that the secondary structures of these nine HvAGO proteins consisted mostly of random coils and α-helices. These two secondary structures may play an important role in the function of these proteins. The SWISS-MODEL was used to predict the three-dimensional structures of the HvAGO proteins. According to Figure 5B, the nine HvAGO proteins possessed similar three-dimensional structures, as the functional domain was folded into a hammer and mainly consisted of random coils and α-helices. The body of the hammer contained a PIWI characteristic domain, and the handle of the hammer mainly contained a PAZ characteristic domain.

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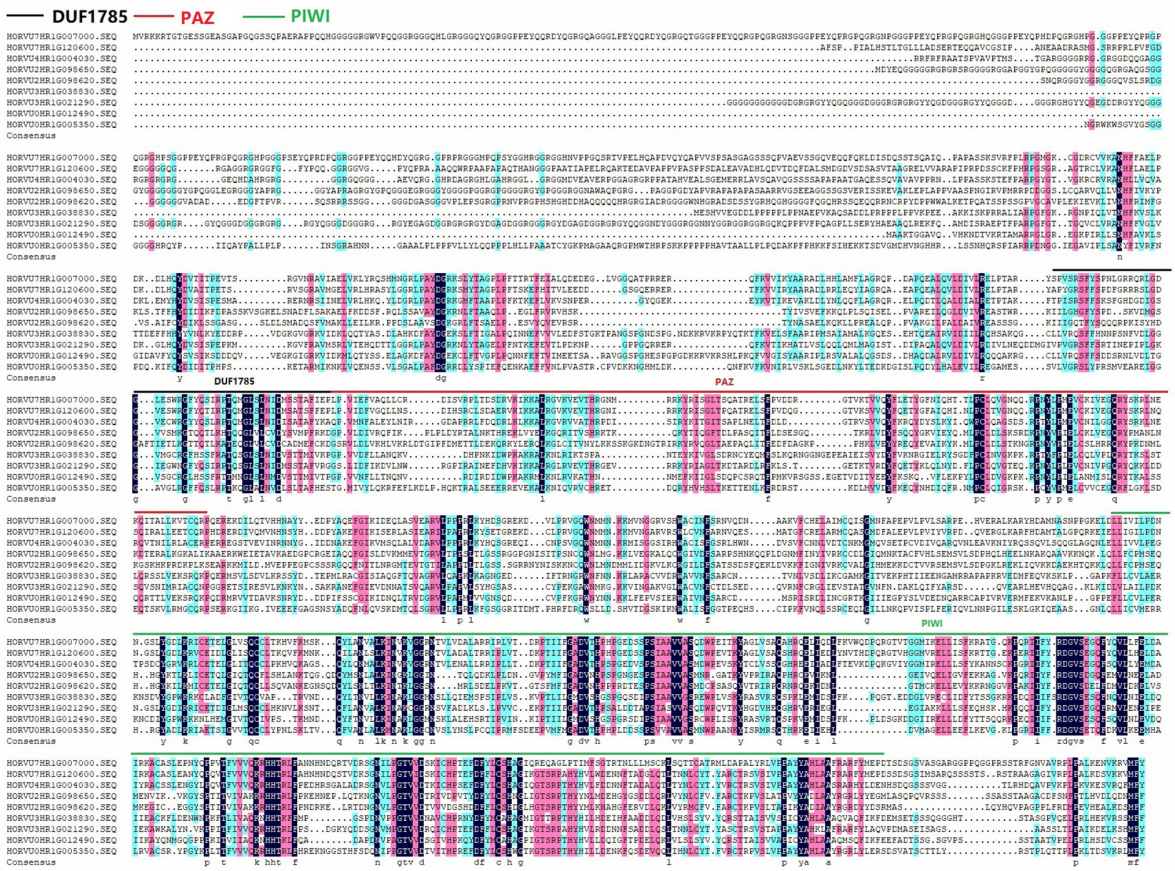

Figure 4. Protein domain and homology comparison of the HvAGOs

Black line – DUF1785 domain; red line – PAZ domain; green line – PIWI domain

Phylogenetic analysis of the HvAGO proteins. The phylogenetic trees were constructed based on the amino acid sequences encoded by nine HvAGO, fifteen rice OsAGO, ten A. thaliana AtAGO, and nineteen maize ZmAGO proteins. As indicated in Figure 6, these fifty-three AGO family members could be divided into three groups. There were twenty-nine members in Group A, of which four, ten, three, and twelve members were from barley, rice, *A. thaliana*, and maize, respectively. There were twelve members in Group B, of which three each were from barley, rice, *A. thaliana*, and maize. There were twelve members in Group C, of which two, two, four, and four were from barley, rice, *A. thaliana*, and maize, respectively. As each group contained an AGO from barley, we hypothesised that the ancestor of barley contained at least three AGOs and that significant gene loss events did not occur after the differentiation of the Gramineae plants. This also indicated that AGOs are evolutionarily conserved, as they are a gene family with important functions in plant growth and development.

Collinearity analysis of *HvAGO* genes with *A. thaliana*, rice, and maize *AGO* genes. We plotted comparative collinear graphs of the barley, *A. thaliana*, rice, and maize genomes to examine the evolutionary history of the barley *AGO* genes. The results showed that there was no collinearity between the barley *AGO* genes and the *A. thaliana AGO* genes, which may be due to the genetic distance between barley and *A. thaliana* is far and the collinearity of the reference genomes is low. In addition, there were nine *HvAGO* and ten *AtAGO* genes, which is a low number. The collinearity analysis results showed that HORVU4Hr1G004030–LOC_Os03g47820, HORVU3Hr1G038830–LOC_Os01g16850, HORVU2Hr1G098620–LOC_Os04g52540 and HORVU2Hr1G098620_LOC_Os04g52540 homologous gene pairs were collinear genes in barley and rice; HORVU7Hr1G120600–Zm00001d014875_T001, HORVU4Hr1G004030 –Zm00001d033518_T001, HORVU4Hr1G004030–Zm00001d013542_T003, HORVU2Hr1G098620–Zm00001d026300_T001, HORVU2Hr1G098620 –Zm00001d002316_T001,

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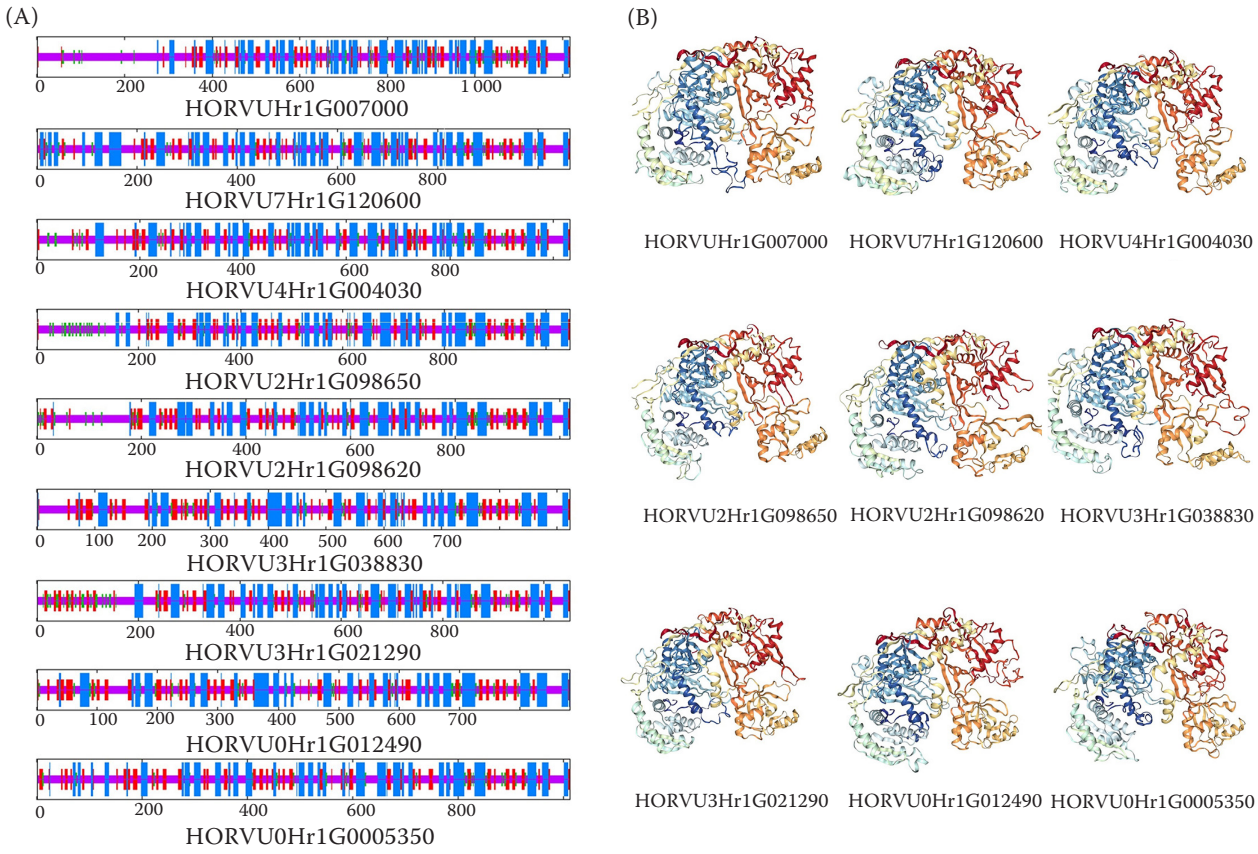

Figure 5. (A) Secondary and (B) tertiary structures of the HvAGO proteins

HORVU3Hr1G038830–Zm00001d040429_T003, and HORVU3Hr1G038830–Zm00001d008249_T012 homologous gene pairs were collinear genes in barley and maize (Figure 7). This demonstrates that these four *HvAGO* genes are homologs of rice and maize *AGO* genes, and we hypothesise that they originated from a common ancestor.

Analysis of the expression level of HvAGO genes in Tibetan hull-less barley leaf stripe. The expression levels of the nine *HvAGO* genes changed differently after the *Pyrenophora graminea* infection (Figure 8). The expression levels of two *HvAGO* genes (HORVU0Hr1G005350 and HORVU7Hr1G120600) decreased after infection, but the expression was low even before infection, whereas the expression levels of the other seven *HvAGOs* increased (Figure 8A). Among these seven genes, the expression levels of HORVU2Hr1G098650, HORVU3Hr1G038830, and HORVU7Hr1G007000 increased significantly after *Pyrenophora graminea* infection ($P < 0.01$). This was particularly true for HORVU2Hr1G098650 in 'Kunlun 14' and 'Z1141' following *Pyrenophora graminea* infection, as the respective expression levels were 47.87 and 15.07 times those of the leaves without the barley leaf stripe disease ($P < 0.01$). Therefore, it is speculated that these three *HvAGO* genes play important roles in the resistance against Tibetan hull-less barley leaf stripe (Figure 8B).

DISCUSSION

The proportion of the field area (per square meter) of Kunlun14 and Z1141 was calculated by us from 2015 to 2017. The incidence of Kunlun 14 was less than 10%; While 1141 had an incidence of more than 90%. Kunlun14, a hybrid variety that can tolerate leaf stripe, while Z1141, a wild variety that is susceptible to leaf stripe. Thus, it could be seen that although many resistance genes were derived from wild varieties, the disease resistance of the hybrid varieties was significantly higher than that of the wild varieties after long-term natural and artificial selection (Gerechter-Amitai et al. 1971; Li et al. 2018). In recent years, AGO gene family members have been isolated and identified from many higher plants such as *A. thaliana*, apples, maize, and rice through bioinformatics analyses (Kapoor et al. 2008; Kim et al. 2011; Qian et al. 2011; Xu et al. 2016). RNA

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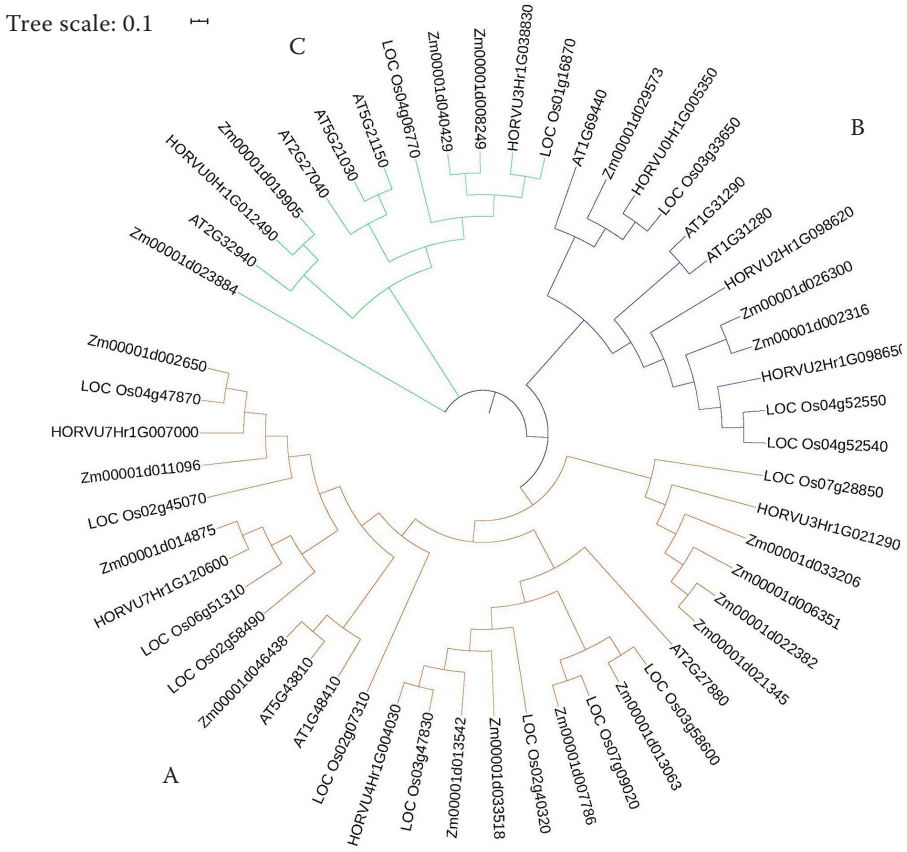

Figure 6. Unrooted phylogenetic tree of the HvAGO proteins in *Hordeum*, *Oryza* and *Arabidopsis*

Group A is indicated by orange lines, group B is indicated by blue lines, and group C is indicated by green lines; the gene IDs of *AGOs* are shown on the outside of the circle; the scale bar represents 0.1 amino acid substitutions per site

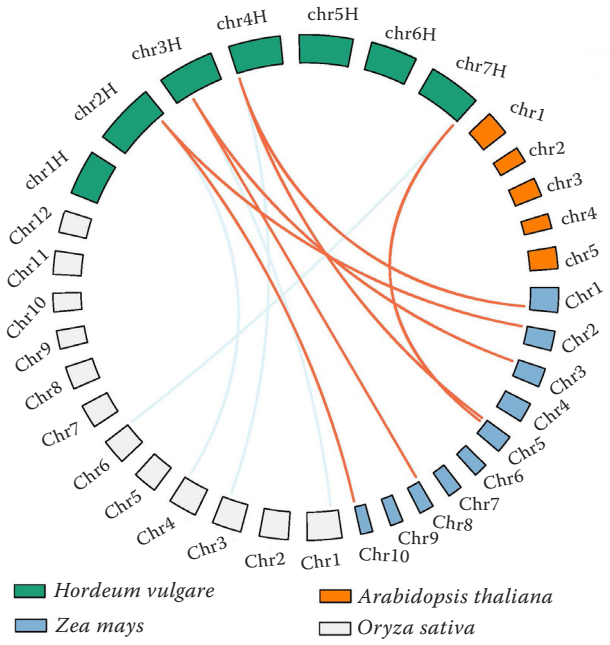

Figure 7. Synteny analysis of the AGO genes between barley and *Arabidopsis thaliana*, barley and rice, and barley and maize

The sister paralogous pairs are indicated by the coloured lines

interference plays an important role in plant growth and development. Studies on rice and maize have confirmed that AGO proteins are integral to known small RNA regulatory pathways and inhibit the target gene expression to regulate developmental processes (Kapoor et al. 2008; Qian et al. 2011). In the barley genome, Hamar et al. (2020) identified eleven *HvAGOs*, among which seven were the same as in our study, and a nSew *HvAGO* (HORVU2Hr1G098620) was found in this study. We are the first to isolate nine leaf stripe-related *HvAGOs* in the barley genome. Compared with *A. thaliana*, we found four Group A members of the leaf stripe-related *HvAGO* genes, which is one more than *A. thaliana*; three Group B members, which is identical to *A. thaliana;* and two Group C members, which is two less than *A. thaliana*. Further analysis is required to determine if there is a functional redundancy in these genes or whether these new members have different functions or regulatory mechanisms during a *Pyrenophora graminea* infection.

Previous studies showed that AGO proteins contain highly conserved domains, such as DUF1785,

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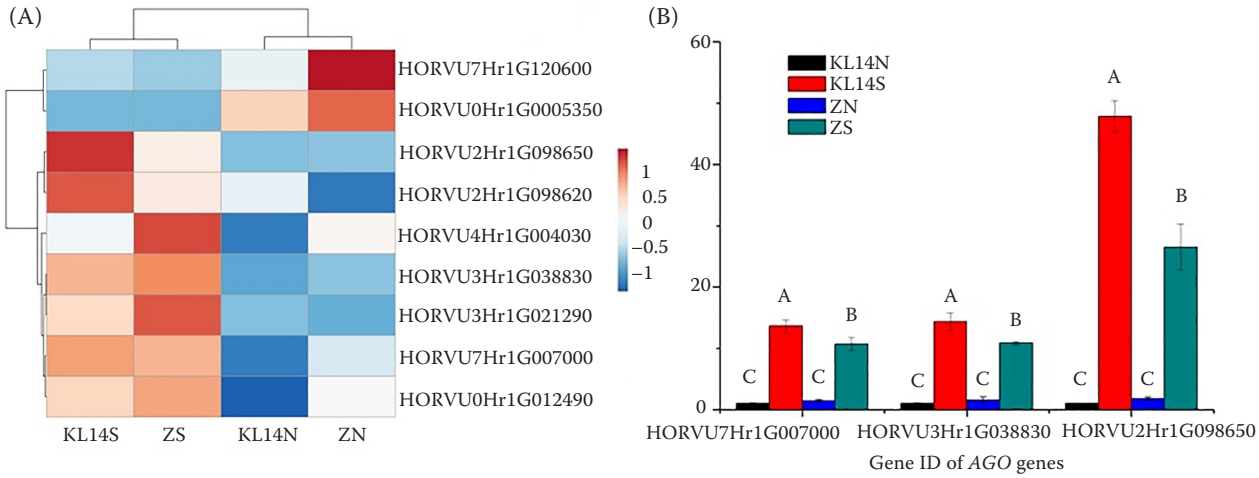

Figure 8. Expression patterns of the *HvAGO* genes in the barley leaves infected by *Pyrenophora graminea* (A) expression patterns of the *HvAGO* genes tested by mRNA-seq and (B) expression patterns of the *HvAGO* genes tested by qRT-PCR

(A) the colour scale is shown on the right of the heat map, indicating the expression values, blue indicates the low transcript abundance, red indicates high levels; (B) the analysis values represent the means ± SD of the three replicates; the bars with the same letter are not significantly different ($P \leq 0.01$)

PAZ, and PIWI. These domains play important roles in the binding of sRNA double strands and in the lysis of target RNAs (Song et al. 2006; Wang et al. 2008; Sabbione et al. 2019). We identified nine leaf stripe-related *HvAGO* genes in the reference genome of barley, and their protein homology was 30.91%. The homology of the DUF1785, PAZ, and PIWI domains was 55.36, 44.04, and 55.86%, respectively. It can be seen that the homology of the three domains was not high in the nine HvAGO proteins. This suggests that the three gene types in the *HvAGO* gene family may have their own unique function and effector modes. However, from phylogenetic classification, it is evident that the nine HvAGO proteins and three domains possessed high homology in the same group, and the homology of Group A and Group C exceeded 50%. In Group A, the homology of four HvAGO proteins was 56.90%, of which the homology of the DUF1785, PAZ, and PIWI domains was 74.53, 68.30, and 77.77%, respectively. The homology was higher in Group C; the homology of the four HvAGO proteins was 57.84%, and the homology of the DUF1785, PAZ, and PIWI domains was 73.58, 54.68, and 72.03%, respectively. The homology of the nucleotide sequences of the members in Group B was the lowest, and the protein homology of the four identified HvAGOs was 45.15%, of which the domain homology of DUF1875, PAZ, and PIWI was 46.43, 46.16, and 64.32%, respectively. The high degree of conservation in the amino acid sequences between the members in the same branch also indicated that complementary gene functions may be present when these proteins specifically bind to small RNAs for the cleavage of the target genes (Shao et al. 2013). In addition, the genes encoding the same group of proteins maintain high homology in the number and similarity of introns as well as the length and structure of the sequences. This also shows that the genetic origin of the same group of AGO proteins is somewhat related to their structure and functions.

Homologs of *HvAGO* genes are present in *A. thaliana*, rice, and maize, and the gene annotation results for the same branch were similar, suggesting that the origins and even the functions of these genes have some correlation. For example, in the Group A branch, Zm00001d011096, Zm00001d002650, LOC Os02g45070, LOC Os04g4780, AT1G48410, and AT5G43810, which have the shortest genetic distance with HORVU7HR1G007000, encode the ZmAGO1, ZmAGO1C, OsAGO1, OsAGO1B, AtAGO1, and AtAGO10 proteins, respectively. A previous study showed that there is a partial functional redundancy in AtAGO10 and AtAGO1 (Vaucheret 2008). In addition, in the Group A branch, HORVU7HR1G120600 and HORVU7HR1G007000 had the closest phylogenetic relationship, and the protein homology of these two genes was 57.11%. However, the homology of their PAZ and PIWI domains was higher, being 81.75 and 76.47%, respectively, and were annotated as *AGO1* in Swiss-Prot. Studies have indicated that *AGO1* participates in pathways such

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as the miRNA pathway, posttranscriptional gene silencing (PTGS) pathway, virus-induced gene silencing (VIGS) or antiviral silencing pathway, and the tasiRNA pathway (Niedojadło et al. 2020). AGO1 mainly binds to miRNA in the cytoplasm to cleave the target mRNA or mediate the translation inhibition at the posttranscriptional level to inhibit the target gene expression (Liu et al. 2018). Therefore, it is speculated that proteins encoded by HORVU7HR1G120600 and HORVU7HR1G007000 have similar roles in Tibetan hull-less barley as the AGO1 proteins in other plants.

Following the *Pyrenophora graminea* infection, the expression levels of three *HvAGO*s (HORVU7Hr1G00700, HORVU2Hr1G098650, and HORVU3Hr1G038830) in the diseased leaves increased significantly compared to the non-diseased leaves. The proteins encoded by these three *HvAGO* genes were annotated as *AGO1*, *AGO2*, and *AGO4* in Swiss-Prot, respectively, and were present in Groups A, B, and C, respectively. Studies have demonstrated that *AtAGO1* can bind to the viral siRNA to form an RNA silencing complex, which plays an important role in the disease resistance in plants. *AtAGO2* can catalyse the cleavage of the viral RNA to achieve antiviral responses and can complement *AtAGO1* in the antiviral responses in a non-redundant manner (Jaubert et al. 2011). The susceptibility of '*Atago1* (*ago1-27, ago1-33*) and *Atago2* (*ago2-1*) mutant plants towards *Sclerotinia sclerotiorum* was significantly increased (Banu et al. 2015; Cao et al. 2016), and the *AtAGO4* gene mutation increased the susceptibility of the *A. thaliana* plants (Agorio et al. 2007). The AtAGO4 protein has been proven to possess a cleavage activity and shows a partial redundancy with AtAGO1 (Vaucheret 2008). These three *AGO* genes are clearly different, but are also associated with each other in disease resistance in *A. thaliana.* It is hypothesised that they have similar functions in the leaf stripe resistance in Tibetan hull-less barley. However, further analysis is required to determine their specific functional relationships and regulatory mechanisms.

CONCLUSION

In this study, we obtained nine *HvAGO* genes from the mRNA sequencing analysis before and after the disease-resistant variety 'Kunlun 14' and susceptible variety 'Z1141' were infected with *Pyrenophora graminea*. We analysed the structures and motifs of the *HvAGO* gene sequences and found that these genes contained four gene clusters. There was high consistency in the number and size of the exons and the number and type of the motifs between the different clusters, suggesting that their origins and even functions may have some correlation. Through the phylogenetic analysis of the HvAGO protein sequences, we found that these genes were divided into three branches, with homologous genes in *A. thaliana*, rice, and maize, suggesting that the origins and functions of these genes are somewhat correlated. qRT-PCR was used to measure the expression levels of the nine *HvAGO* genes following the *Pyrenophora graminea* infection of Tibetan hull-less barley, and the expression levels of the *HvAGO1*, *HvAGO2* and *HvAGO4* genes were significantly increased. Therefore, these three genes are speculated to play an important role in the leaf stripe resistance in Tibetan hull-less barley. The results provide a foundation for the application of *HvAGO* genes in barley leaf stripe and the exploration of disease resistance genes in other crops.

Acknowledgement: We would like to thank Accdon (www.accdon.com) for editing this manuscript. We are grateful to the reviewers for their valuable comments.

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Received: December 9, 2020
Accepted: February 20, 2021
Published online: June 9, 2021