

Genome-wide identification and *in silico* gene expression analysis of the related to ABI3/VP1 (RAV) transcription factor family in barley (*Hordeum vulgare* L.)

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Abstract: RAV (Related to ABI3/VP1) transcription factors are unique members of the AP2-ERF superfamily with AP2 and B3 domains and play important roles in the regulation of seed germination, plant growth, and stress response. In the study, 7 RAV genes, named *HvRAVs*, were identified in barley based on the available genome sequences. While five of the seven *HvRAVs* were located on chromosome 3, *HvRAV5* and *HvRAV7* were located on chromosome 1 and 4, respectively. Six of the predicted *HvRAVs* were intron-less, except *HvRAV2*, which had one intron. *HvRAV* proteins have shown basic, instable, and hydrophilic properties. The AP2 domain specific RAYD and WLK motifs were detected in all *HvRAV* proteins. Besides, B3 repression domain, R/KLFGV, is also found in the C-terminal of *HvRAVs*. *HvRAVs* were found to have stress-related cis-acting elements, including MYB, MYC, and W-BOX. *HvRAV2* was predicted to have no GARE motifs, TATCCCA or TAACAA(G/A), and LTREs. Under drought conditions, the expression level of *HvRAVs* did not significantly change in a drought-sensitive barley genotype, whereas *HvRAV5* and *HvRAV7* were dramatically down-regulated in a drought-tolerant genotype. Expression of *HvRAV5* was also inhibited by salinity. *HvRAV7* was strongly induced by plant pathogen attack. Only *HvRAV6* was induced by exogenous gibberellin application and during the germination process. Interestingly, *HvRAV6* transcript was detected higher than other *HvRAVs* in all stress and control conditions as well as during germination. *In silico* analyses have shown that *HvRAVs* play a role in response to different abiotic and biotic stresses as well as in plant development. However, the extensive biological roles of *HvRAV* genes in plant development and in response to abiotic and biotic stresses need further investigation.

Introduction

Transcription factors (TFs), sequence-specific DNA binding proteins, play central roles in plant growth, development, hormone response, and in response to biotic and abiotic stresses by regulating target genes (Liu *et al.*, 2013). The transcription factor RAVs (Related to ABI3/VP1) are plant-specific and belong to the APETALA2/Ethylene Responsive Factor (AP2/ERF) family, which plays a crucial role in the regulation of biotic and abiotic stress-responsive gene expression (Du *et al.*, 2016). The AP2/ERF superfamily is characterized by the AP2/ERF DNA binding domain, which consists of about 60 to 70 highly conserved amino acids (aa) (Sakuma *et al.*, 2002). The AP2/ERF superfamily is divided into 5 subfamilies, including APETALA2 (AP2), Related to ABI3/VP1 (RAV), Dehydration-Responsive Element Binding

(DREB), Ethylene-Responsive Factors (ERF), and soloist (Guo *et al.*, 2016). RAV TFs were first identified in the ABA insensitive-3 (*abi3*) mutant from *Arabidopsis* and the viviparous1 (*vp1*) mutant from *Zea mays* (Giraudat *et al.*, 1992; Suzuki *et al.*, 1997). RAV proteins contain two different DNA-binding domains, the AP2 and B3 domains, differently from other AP2/ERF TFs, which have only AP2 domain (Feng *et al.*, 2005). The AP2 and B3 DNA-binding domains recognize consensus CAACA and CACCTG sequences, respectively, in *Arabidopsis*, so plant RAVs with both AP2 and B3 domains show much higher DNA binding affinity (Kagaya *et al.*, 1999; Wei *et al.*, 2018; Xie *et al.*, 2019).

RAV proteins serve as negative regulators in plant development by repressing the transcriptional activity and forming complexes with other co-repressors. Besides, RAVs have important functions in abiotic and biotic stress responses in plants (Matías-Hernández *et al.*, 2014). It has been found that the 15-aa peptide sequence (GNSKTLRLFGVNMEC), named B3 repression domain (BRD), had a repressive activity among the B3 super-family,

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including auxin response factor (ARF), abscisic acid-insensitive3 (ABI3), high-level expression of sugar inducible (HSI), related to ABI3/VP1 (RAV) and reproductive meristem (REM). Deletions in BRD of some B3 proteins revealed that only a short peptide composed of five amino acids, R/KLFGV, is essential to maintain the repressive activity of the BRD domain (Ikeda and Ohme-Takagi, 2009). A quite similar sequence, MLFGV, has been found in Arabidopsis RAV3 and RAV3-like TFs (Causier et al., 2012).

The number of TFs in the RAV family is relatively less in comparison to other subfamilies of the AP2/ERF superfamily, i.e., in cauliflower, 146 of 171 AP2/ERF TFs belong to the ERF family, whereas 15 and 9 TFs were classified into the AP2 and RAV subfamily, respectively (Li et al., 2017). RAV family members play important roles in many different physiological and developmental pathways, including hormone signaling (ethylene and brassinosteroids), flowering, leaf senescence, bud outgrowth, and response to stress in several plant species (Matías-Hernández et al., 2014). There is little information in the literature about the characterization, expression patterns, and functional roles of barley RAV genes.

Barley (*Hordeum vulgare* L.) ranks fourth after rice, wheat, and maize in terms of cultivated area and production among cereals (FAO, 2018). Barley is majorly consumed as animal feed (seeds or as forage crop) and used as malt in the production of alcohol and non-alcoholic beverages, and a minor portion of production is used in the human diet (Uçarlı and Gürel, 2020). Barley is grown in different environmental conditions with a higher tolerance against biotic and abiotic stresses than other crops (Dawson et al., 2015). Besides its agronomic importance, barley is a well-studied plant in terms of genetics, functional genomics, and breeding for many researchers in the field of cereal crops.

In the present study, genome-wide bioinformatics analyses were conducted to predict cDNA and amino acid sequences of RAV TF family members in barley. Gene structure, cis-acting elements related to abiotic and biotic stress, and highly conserved motifs were determined in HvRAVs. Physicochemical properties of HvRAV proteins were also predicted as well as secondary structures. The phylogenetic trees were generated, and the evolutionary distances were computed between HvRAV proteins and also compared with other monocots. The expression pattern of HvRAV genes at the germination stage and their expression profiles under different stresses, including drought, salinity, and plant-pathogen attacks, were determined using data obtained from distinct microarray experiments. The results will be useful in further study of the RAV family in cereals, especially in the Triticeae tribe.

Materials and Methods

Identification of HvRAV TFs

In addition to the barley genome database, Transcription Factor Databases, including plantTFDB (<http://planttfdb.cbi.pku.edu.cn/>) and iTAK (http://itak.feilab.net/cgi-bin/itak/db_browse.cgi), were used to identify the sequences of barley RAV TFs as well as some monocot RAVs including *Oryza sativa*, *Triticum aestivum*, *Zea mays* and *Brachypodium distachyon* (Jin et al., 2014; Zheng et al., 2016). BLAST programs

(BLASTP, TBLASTN, and BLASTN) which are available on the IPK barley genome database sourced from the assembly Morex V2.0 2019 (<http://webblast.ipk-gatersleben.de/barley/>), Gramene (<http://ensembl.gramene.org/Hordeumvulgare/Info/Index>) and Phytozome (<https://phytozome.jgi.doe.gov/pz/portal.html>) were used to increase the extent of the transcription factor database search results and to determine the location of the RAV genes on barley chromosomes.

Gene structure, cis-acting element analysis, and motif recognition

Gene structure was analyzed using the Gene Structure Display Server websites (<http://gsds.cbi.pku.edu.cn/>) (Hu et al., 2015). AP2 and B3 domains were predicted using the domain analysis programs Simple Modular Architecture Research Tool (SMART (<http://smart.embl-heidelberg.de/>)) based on the STRING database (Letunic and Bork, 2018). Conserved motifs of HvRAV TFs were investigated with web-based sequence analysis tool MEME Suite 5.1.1 (<http://meme-suite.org/tools/meme>) with the following parameters: optimum width 6–50 amino acids, any number of repetitions of a motif, the maximum number of motifs set at 10, and all other parameters set at default (Bailey et al., 2009). Cis-acting elements, including abiotic and biotic stress-related, and ABA- and GA-responsive elements were identified using plant cis-acting element databases PlantCARE (Lescot et al., 2002), and PLACE (Higo et al., 1999) in putative promoter regions (2-kb upstream sequences of the transcriptional start site) of HvRAV genes. Putative nuclear localization signal (NLS) sequences were predicted using a cNLS mapper with a cut-off score higher than 3 (Kosugi et al., 2009).

Physicochemical characterization, secondary and three-dimensional structure analysis

The physical and chemical properties of HvRAVs, including molecular weight (MW), theoretical isoelectric point (pI), instability index (II), aromaticity, and grand average of hydropathicity (GRAVY), were determined by ProtParam tools (<http://web.expasy.org/protparam/>). Putative Kinase-specific prediction of protein phosphorylation sites was conducted using NetPhos 3.1 (Blom et al., 2004). Prediction of secondary structure elements and generation of 3-D models of HvRAV proteins were performed with Phyre2 server (Kelley et al., 2015).

Multiple sequence alignment and phylogenetic analysis

The identified protein sequences of HvRAVs and monocot RAVs were aligned using the Clustal T-Coffee program with default parameters (Notredame et al., 2000). The phylogenetic trees were generated by the neighbor-joining (NJ) statistical method with 1000 bootstrap replications using MEGA X software (Kumar et al., 2018). The evolutionary distances were computed using the Poisson correction method with pairwise deletion.

Gene expression profile analysis

Gene Expression profile barley under stress conditions and during germination was downloaded from Gene Expression Omnibus using data obtained from different Affymetrix Barley1 GeneChip microarray experiments (dataset

IDs: Drought stress, GSE15970; salt stress, GSE3097; cold stress, GSE10329; heat stress, GSE23896; fungal stress GSE33401 and GSE33407; germination, GSE9365; gibberellin (GA) and abscisic acid (ABA), GSE18785). The probe sequences of the microarray chip were blasted with cDNA sequences of barley RAV genes. The four RAV genes, namely *HvRAV1*, *HvRAV5*, *HvRAV6*, and *HvRAV7*, hit the four-probe sequences contig7483_at, contig19699_at, contig7483_at, and Hs16k21u_at, respectively. Expression intensities under the control, drought, salinity, cold, and heat conditions were used for expression analysis of *HvRAV* genes under abiotic stresses. In addition, expression intensities obtained from the treatment of barley plants with plant pathogens, including *Fusarium graminearum*, *Cochliobolus sativus*, and *Puccinia hordei*, were used to determine the expression profiles of *HvRAV* genes under biotic stresses. Heat maps were generated using TIGR Multiple Experiment Viewers (Saeed *et al.*, 2003).

Results

Identification of the RAV genes in barley

In the barley genome, seven putative RAV genes were identified using BLAST searches of the plant transcription factor and barley databases (Tab. 1). The coding sequence (CDS) lengths of the predicted barley RAV genes varied, ranging from 927 to 1245 bp. All *HvRAV* genes have both AP2 and B3 domains. While AP2 domains of seven *HvRAV* proteins are 66 aa, the length of B3 domains varied from 99 to 113 residues. It has been found that five *HvRAV* genes, namely *HvRAV1*, *HvRAV2*, *HvRAV3*, *HvRAV4*, and *HvRAV6* located on chromosome 3 and are very close to each other, while *HvRAV5* and *HvRAV7* are located on chromosomes 1 and 4, respectively. All barley RAV genes have one exon except *HvRAV2*, which is disrupted by a 676-bp intron.

Physicochemical characterization, secondary and three-dimensional structure analysis

Detailed information on the physicochemical characteristics of *HvRAV* proteins is shown in Tab. 2. Amino acid lengths of the predicted barley RAV proteins ranged from 308 to

362 aa, and the molecular weight (MW) from 33.53 (*HvRAV7*) to 44.70 (*HvRAV5*) kDa. The theoretical isoelectric point (pI) of the five *HvRAV* proteins was calculated around 9.5, indicating basic character, while the theoretical pI value of *HvRAV4* and *HvRAV7* was calculated as 5.5 considering as acidic protein. Only *HvRAV2* was classified as a stable protein according to the instability index (II) value by 36.95 among the barley RAV proteins (II higher than 40 in other *HvRAVs*). Grand average of hydropathicity (GRAVY) of *HvRAV* proteins was measured between -0.35 and -0.68, indicating hydrophilic proteins. The aliphatic index (AI) values of barley RAV proteins ranged from 59.88 (*HvRAV4*) to 69.53 (*HvRAV6*), thus suggesting protein thermostability. While the net charge of two *HvRAVs*, namely *HvRAV4* and *HvRAV7*, predicted negatively by -4.36 and -3.90, respectively, the rest of *HvRAV* proteins varied from 9.08 and 18.57. The highest Kinase-specific phosphorylation sites were predicted on *HvRAV5* with 57 sites.

Three-dimensional structure of whole *HvRAV* protein, as well as binding domains AP2 and B3, were generated using Phyre2 server (Fig. 1A). Secondary structure analysis showed that all *HvRAV* sequences have 3–4 stranded β -sheet that are antiparallel with one α -helix, relating to the AP2 domain, with a confidence value of 100% (Fig. 1B). It was predicted that the B3 domain contains 5–6 β -sheets that form an open barrel structure with 2 α -helices in barley RAV proteins. The total number of helices and strands ranged 4–9 and 9–12, respectively, in seven *HvRAV* proteins. The percentage of disordered amino acid residues among members of the *HvRAVs* varied from 47% to 52%, meaning that almost half of the *HvRAV* proteins were unfolded (Fig. 1B). The linker region between AP2 and B3 domain was found to have 1 or 2 helices in all barley RAVs except *HvRAV3*. On the other hand, only *HvRAV7* was found to have one-stranded beta sheet on the linker region among the barley RAVs.

Analysis of cis-acting elements in the putative promoter regions of the *HvRAV* genes

Analysis of the promoter regions of *HvRAV* genes showed the presence of different cis-acting elements in different numbers,

TABLE 1

Summary of the *HvRAV* genes identified in the genome of barley (*Hordeum vulgare* L.)

Gene name	Accession No.	CDS length (bp)	Intron	AP2 domain (aa)	B3 domain (aa)	Chromosomal location*
<i>HvRAV1</i>	MLOC_56567.1	1047	0	62	109	Chr3H (17841857–17845827)
<i>HvRAV2</i>	MLOC_75135.1	1089	1	62	109	Chr3H (17784402–17786166)
<i>HvRAV3</i>	MLOC_7940.1	1074	0	62	109	Chr3H (17496009–17498246)
<i>HvRAV4</i>	MLOC_5512.2	963	0	62	111	Chr3H (17050590–17051552)
<i>HvRAV5</i>	MLOC_38822.1	1245	0	62	113	Chr1H (497104728–497105972)
<i>HvRAV6</i>	MLOC_44012.1	1143	0	62	100	Chr3H (449487644–449488786)
<i>HvRAV7</i>	MLOC_63261.1	927	0	62	99	Chr4H (601536592–601537518)

Note: *Chromosomal locations of barley RAV genes were determined according to IPK database.

TABLE 2

Physiochemical characteristics of HvRAV proteins

Protein name	Amino acid length (aa)	MW (kDa)	pI	GRAVY	AI	II	Charge	KSP
HvRAV1	348	38.25	9.67	-0.37	69.54	43.27	17.06	33
HvRAV2	362	39.28	9.44	-0.35	70.11	36.95	16.04	40
HvRAV3	357	38.81	9.46	-0.51	62.66	42.69	15.07	47
HvRAV4	320	34.83	5.50	-0.68	59.88	43.06	-4.36	35
HvRAV5	414	44.70	9.51	-0.45	64.98	46.99	18.57	57
HvRAV6	380	40.07	9.13	-0.38	69.53	42.29	9.08	34
HvRAV7	308	33.53	5.55	-0.56	61.23	43.87	-3.90	33

Note: Molecular Weight (MW), Kinase specific phosphorylation (KSP) sites, Isoelectric Point (pI), Grand Average of Hydropathy (GRAVY), Aliphatic Index (AI), Instability Index (II), and charge of proteins were predicted based on amino acid composition.

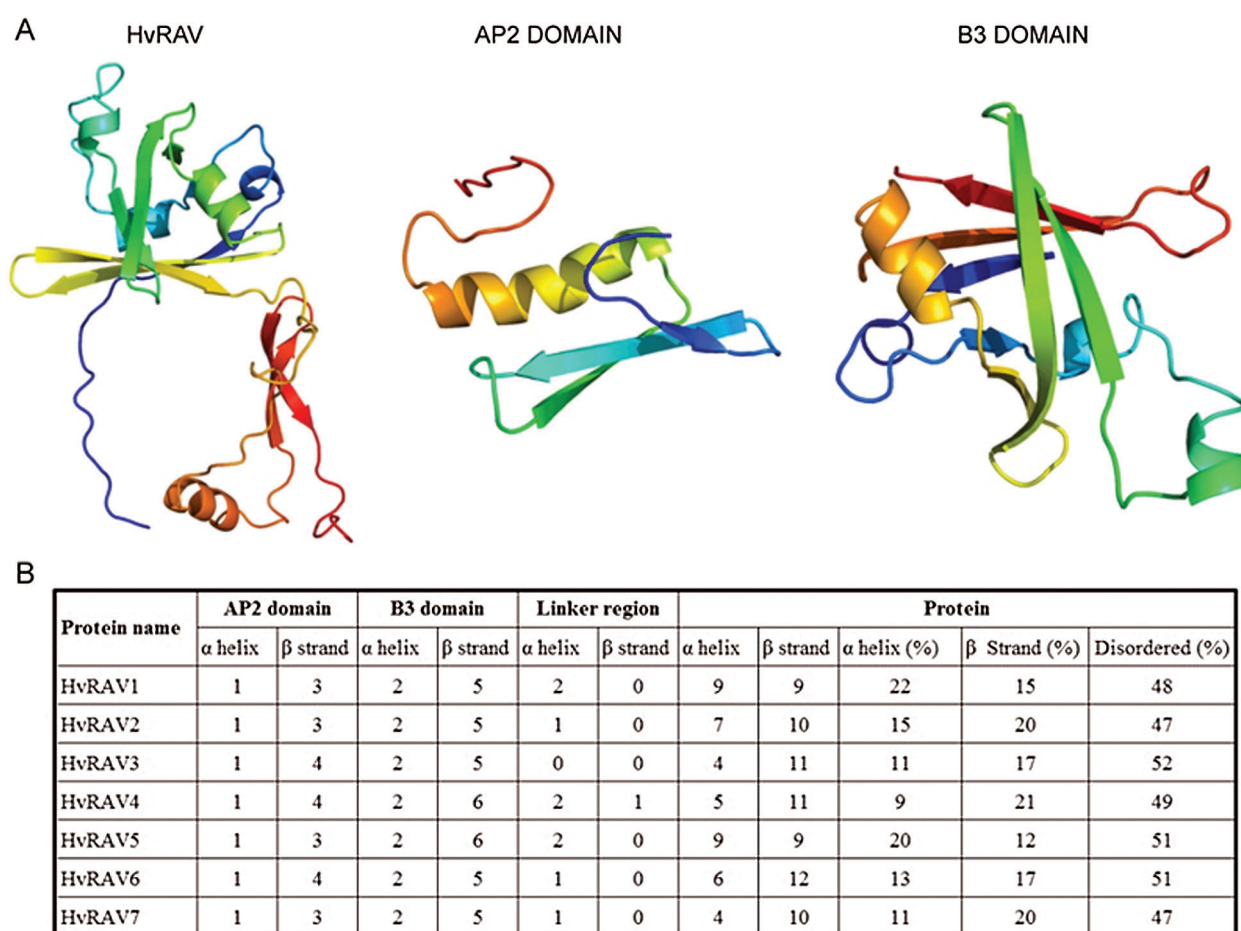


FIGURE 1. Three-dimensional and secondary structures of HvRAVs.

(A) Predicted 3D structures of HvRAV1 protein and highly conserved AP2 and B3 domains modeled at 100% confidence level by using Phyre2 server. (B) Secondary structures of HvRAV proteins and AP2 and B3 domains according to the Phyre 2 server.

which may play a role in abiotic and biotic stress, as well as hormone responsiveness, including ABA and GA (Tab. 3). All *HvRAV* genes contained one or more ABA-responsive elements (ABREs) except *HvRAV5*. Seven *HvRAV* genes contain at least one Dehydration-response element (DRE) site, which has a role in response to drought, salinity, and cold stress. Ethylene-responsive-element-binding factors (ERFs) participate in transcriptional regulation of abiotic and biotic stresses and specifically bind to the GCC-BOX

with the core sequence of AGCCGCC. The GCC-BOX motif was only predicted on the promoter region of *HvRAV5* and *HvRAV6* among seven *HvRAVs*. Cis-acting elements MYB, MYC, and W-BOX, involved in the abiotic stress responsiveness, with high numbers have been found on the promoter of *HvRAVs*. It has been predicted that promoter regions of *HvRAVs* contained two or five low-temperature response elements (LTREs) except *HvRAV2*, which had no LTRE. TC-rich repeats with the core ATTTTC sequence

TABLE 3

Distribution of abiotic and biotic stress-related cis-acting elements in promoter region of barley RAV genes

GENE	ABRE	DRE	ERD1	MYB	MYC	LTRE	W-BOX	GT1	GCC-BOX	TC-RICH REPEATS	GARE
HvRAV1	3	1	7	15	11	5	13	2	0	3	2
HvRAV2	4	1	5	20	5	0	9	4	0	5	3
HvRAV3	1	1	2	10	8	2	12	7	0	6	1
HvRAV4	2	1	5	12	11	2	14	11	0	3	2
HvRAV5	0	4	3	8	8	5	9	2	3	2	1
HvRAV6	4	2	5	17	8	2	14	5	2	3	1
HvRAV7	3	4	3	11	8	5	8	3	0	3	0

have a crucial role in defense and biotic stress response, were determined on the promoter of *HvRAVs*. TATCCCA motif, one of the Gibberellin-responsive elements (GARE), has been found only on the promoter regions of *HvRAV2* with 2 copies and *HvRAV6* with 1 copy, while another GARE motif TAACAA(G/A) found on promoter regions of *HvRAV1*, *HvRAV3*, *HvRAV4*, and *HvRAV5*. No GAREs were detected on the promoter region of *HvRAV7*.

Multiple sequence alignment and phylogenetic analysis

Multiple sequence alignment (MSA) of seven barley RAV proteins was displayed in Fig. 2. Multiple Sequence alignment showed that all barley RAV transcription factors contained highly conserved AP2 and B3 domains. In the AP2 domain of barley RAV proteins, highly conserved RAYD and YRG elements were predicted. A highly conserved 18 amino acid core region of RAYD was predicted to form an α -helix in the C terminal region of the AP2 domains. A highly conserved YRG element, containing mostly hydrophilic and basic 19–20 amino acids, was predicted to form 2 β sheets in the N terminal region of the AP2 domain. It was also detected that the YRG motif has been replaced with the R(K)F(Y)K motif in AP2 domains of barley RAVs. MSA analysis demonstrated that seven barley RAV proteins contain a WLK motif in the AP2 domain. B3 repression domain (BRD) containing R/KLFGV residues was detected in the C-terminal of all *HvRAVs*. All barley RAV proteins have been found to have at least one bipartite nuclear localization signal (NLS) sequence with scores ranging from 3.0 to 6.1; thus, *HvRAVs* are predicted to be localized in both the nucleus and the cytoplasm. Besides, *HvRAV1*, *HvRAV5*, and *HvRAV6* have monopartite NLS sequences GWFKRQCMA, HAVWKKRCVDF, and VAFKKQCIE, respectively, in the C-terminal region of the proteins (Fig. 2).

The MEME motif search tool was conducted to find motifs in barley RAV proteins. In total, 6 conserved motifs were identified, lengths ranging from 20–50 amino acids in all RAV proteins (Fig. 3). While 50-aa-length motif 1 including F/YKG and WLK sequences was observed on the AP2 domain, the motifs 3, 4, and 5 were predicted on the B3 domain of barleys RAVs. Interestingly, a F/YRG sequence was found at the terminal of motif 1. The highly conserved RAYD (motif 1) element in AP2/ERF superfamily

was converted to CAYD in *HvRAV7*. Motif 2 specifying a linker region between AP2 and B3 domains was highly conserved. Motif 6 was located in the C terminal region of RAV protein and containing the BRD element R/KLFGV.

A phylogenetic tree of seven barley RAV transcription factors was generated based on the full-length amino acid sequences with the neighbor-joining method. According to the phylogenetic analysis, barley RAV proteins were distributed into three groups: Group I, including *HvRAV1*, *HvRAV2*, and *HvRAV3*; Group II, including *HvRAV4* and *HvRAV7*; and Group III, with *HvRAV5* and *HvRAV6* (Fig. 4A). To evaluate evolutionary relationships between RAV proteins in monocots, a phylogenetic analysis of the 7 barley RAVs, 4 Rice RAVs, 7 wheat RAVs, 3 maize RAVs, and 6 *Brachypodium* RAVs were undertaken based on a phylogenetic tree generated with a statistical method using full-length amino acid sequences of RAV proteins. Based on the phylogenetic analysis of RAV proteins, some barley RAVs show closer evolutionary status with RAVs of different species. Barley RAV3 (*HvRAV3*) was more closely related to wheat Ta390BE2090B.1 than other barley RAVs (Fig. 4B). Amino acid composition of AP2 and B3 domains in monocots was aligned in five monocots. It is predicted that AP2 domains were highly conserved with YRG, WLK, and RAYD elements. AP2 domains contain 2–4 beta-sheets with one helix. B3 domains of monocots also demonstrated highly conserved regions. In general, monocots have 2 helices in the B3 domain, but rice RAV (Os01g04750.1) has an extra alpha-helix in the N-terminal region of the domain.

Gene expression profile analysis of barley RAVs

The data obtained from distinct microarray experiments (GEO numbers are mentioned in Materials and Methods section) were used to monitor the expression profiles of the *HvRAV* transcription factors, namely *HvRAV1*, *HvRAV5*, *HvRAV6*, and *HvRAV7*, in response to abiotic and biotic stresses as well as gibberellic acid (GA) and abscisic acid (ABA) treatments (Fig. 5).

Drought-sensitive and tolerant barley genotypes were treated with drought stress in 10% AWC (available water capacity) soil for 0, 1, 3, and 5 days. While the expression of the four *HvRAV* genes was not significantly changed in response to drought stress in drought-sensitive genotype, the drought stress significantly down-regulated the expression

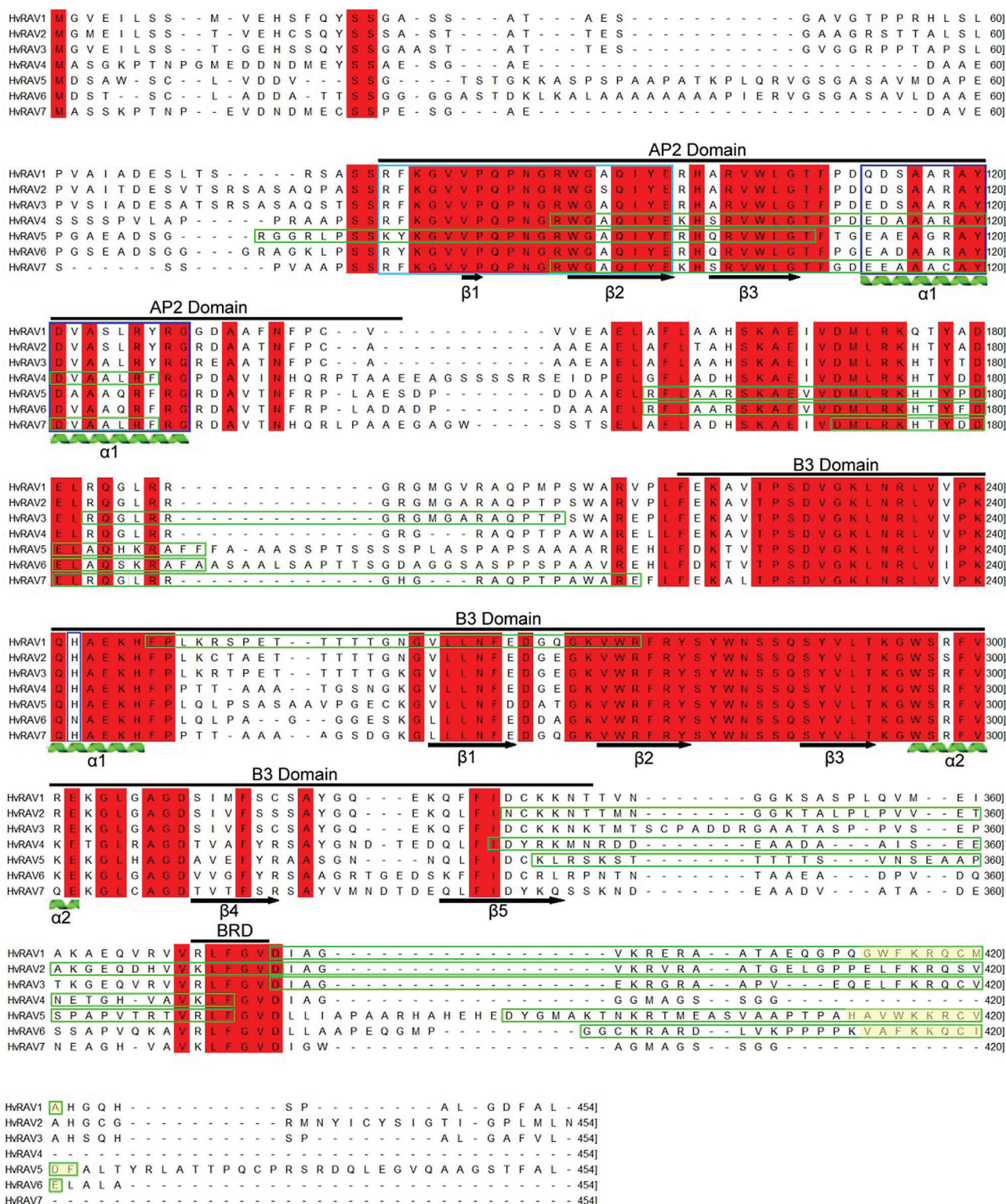


FIGURE 2. Multiple sequence alignment of seven barley RAV proteins according to the Clustal T-Coffee program.

Domain AP2, domain B3, and B3 repression domain (BRD) are marked above the corresponding sequences with a black line. Fully conserved regions are indicated in red highlight. In AP2 and B3 domains, common beta-sheet (β) and alpha-helix (α) are underlined with arrow and helix, respectively. Bipartite nuclear localization signals (NLS) are shown in green rectangles, and monopartite ones are in yellow highlight. Highly conserved YRG motif (light blue rectangle) and RAYD element (dark blue rectangle) are predicted in the AP2 domain.

of *HvRAV5* and *HvRAV7* with 14.3- and 25-fold, respectively, in drought-tolerant genotype (Fig. 5A). In another study, 17-day-old barley plants were exposed to 100 mM NaCl for 0, 3, 8, and 27 h. The expression of *HvRAV5* was changed with a significant decrease at 27 h of NaCl treatment

(Fig. 5B). The expression of the other *HvRAV* genes did not change or slightly decreased depending on exposure time. The changes in expression of *HvRAV* genes under low temperatures were investigated by subjecting the 7-old-day barley seedlings of cv. Dicktoo to chilling (4°C for 5 days),

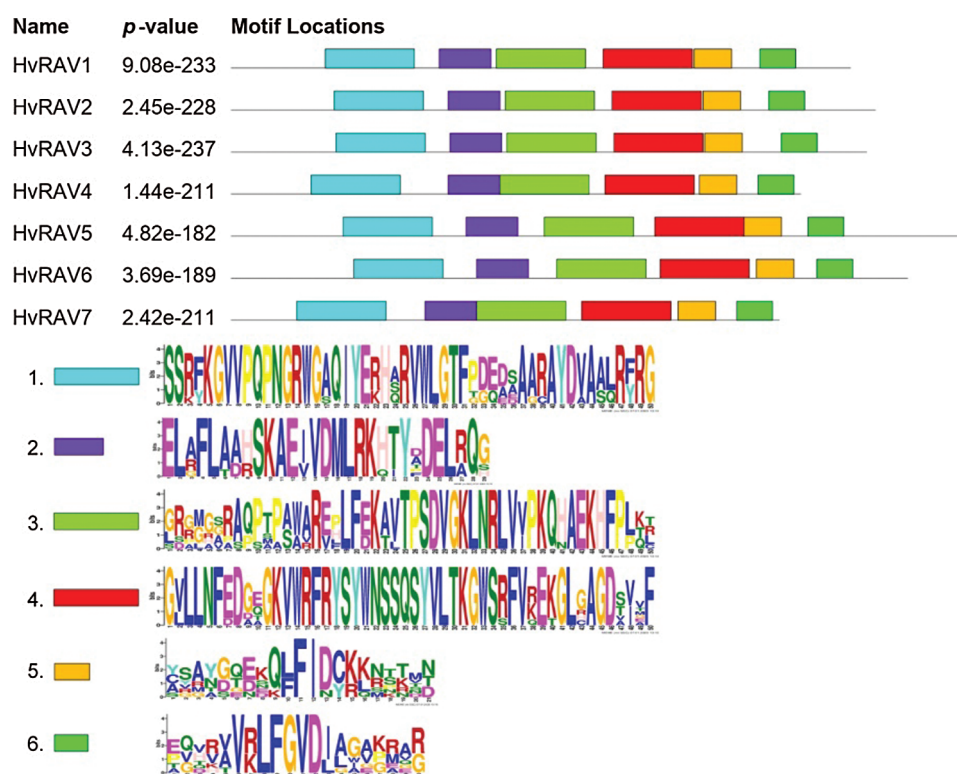


FIGURE 3. Distribution of conserved motifs in barley RAV proteins. Sites and number of motif distribution in genes; E-value and statistical significance of motif.

subzero (-4°C for 8 days), and deacclimation (20°C for 3 days) conditions. The mRNA level of *HvRAV1* did not change in all cold conditions. On the other hand, the chilling and subzero treatments resulted in the upregulation of the *HvRAV7* gene with 2.9- and 6.3-fold, respectively, while the expression of *HvRAV5* was upregulated under subzero and deacclimation conditions compared to the control conditions (Fig. 5C). Developing barley caryopses were harvested at 0, 0.5, 3, and 6 h during heat stress (42°C). Heat stress has been found to decrease the expression of *HvRAV5* and *HvRAV7* at 0.5 h of treatment, but by time expression level was upregulated. On the other hand, the expression of *HvRAV1* and *HvRAV6* almost did not change during heat stress (Fig. 5C). The expression profile of *HvRAVs* in response to biotic stress was also monitored using gene chip arrays. *Hordeum vulgare* ssp. *spontaneum* was inoculated with *Cochliobolus sativus*, causing spot blotch, and *Puccinia hordei*, causing leaf rust, for 0, 12, 24, 36, and 48 h. The expression of *HvRAV7* was up-regulated at 12 h of both pathogen attacks, but by the time mRNA level has been decreased. Besides, it has been found that the pathogen attacks have affected the expression patterns of *HvRAV6* and *HvRAV7* antagonistically. In another study, the spikes of cv. Morex inoculated with *Fusarium graminearum*, causing head blight in cereals for 0, 24, 48, 72, 96, and 144 h. *HvRAV7* has been found to express differentially at different time points after *F. graminearum* inoculation, while the expression of *HvRAV1* and *HvRAV6* were not significantly changed (Fig. 5D).

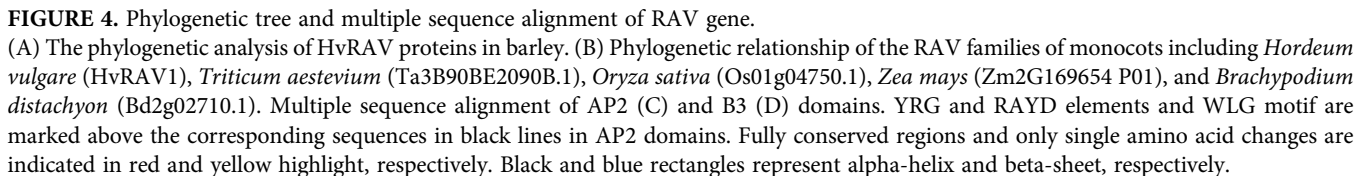
The expression profile of *HvRAVs* was investigated during germination at 0, 24, 48, and 72 h after imbibition (HAI) in endosperm and embryo tissues. Only *HvRAV6* was observed to differentially expressed in endosperm and

embryo tissues during the germination of barley seeds among the *HvRAVs*. *HvRAV6* was dramatically upregulated and reached the highest peak at 24 HAI but then decreased in endosperm tissues. In embryo tissues, the mRNA level of *HvRAV6* rapidly increased at 24 HAI and maintained a high transcript level during the germination process (Fig. 6A). It has been observed that *HvRAV6* was upregulated in response to exogenous GA ($1\text{ }\mu\text{M}$) treatment, whereas its expression did not significantly change after exogenous ABA ($50\text{ }\mu\text{M}$) treatment in barley aleurone cells. SLN1 is a DELLA protein, which is a repressor in the G signaling pathway. In *sln1* mutants, the transcript level of *HvRAV6* was lower than GA-treated samples. In addition, the transcript level of *HvRAV6* has been found very high in all conditions when compared to the other three barley RAVs.

Discussion

Characterization of the barley RAVs

RAV (Related to ABI3/VP1) proteins are members of the APETALA2/Ethylene Responsive Factor (AP2/ERF) family and play a crucial role in response to biotic and abiotic stresses by regulating gene expression as well as seed germination (Zhao *et al.*, 2017; Wei *et al.*, 2018). In barley, seven RAV genes were identified based on barley genome and transcription factor databases. In plants, the number of RAVs is less than AP2/ERF superfamily members, including AP2, B3, and DREB. Barley has predicted to have 19 HvAP2, 41 DREB, and 54 HvERF. Besides, in Arabidopsis, 146 AP2/ERF genes are distributed into 6 RAV, 18 AP2, 57 DREB, and 65 ERF (Guo *et al.*, 2016). The RAVs are unique transcription factors with two DNA binding domains, including AP2 and B3 in AP2/ERF superfamily. All seven



some chromosomes contain more than two *RAV* genes, i.e., 4 *RAVs* on chromosome 13. Besides, six of eleven *RAV* genes (three pairs) are located as a tandem at the telomere ends of chromosomes. Analysis revealed that these *RAV* genes originated from tandem duplication events (Liu and Zhang, 2017).

Seven HvRAV proteins ranged from 33.53 to 44.70 kDa in molecular weight (MW) with hydrophilic and thermostable properties. Major of the HvRAVs were predicted to have basic characteristics with around 9.5 pI and positive charge, except HvRAV4 and HvRAV7 with 5.5 pI. All the soybean GmRAVs were predicted basic characteristics with higher than 7 pI (Zhao *et al.*, 2017). Only HvRAV2 was predicted as a stable protein according to instability index value (36.95) among HvRAV proteins. The HvRAV proteins were calculated to have a high number of kinase-specific phosphorylation (KSP) sites, i.e., HvRAV5 had 57 KSP sites. Protein phosphorylation is a post-translational modification that regulates the function of a protein and plays a crucial role in cellular control of biological processes (Blom *et al.*, 2004).

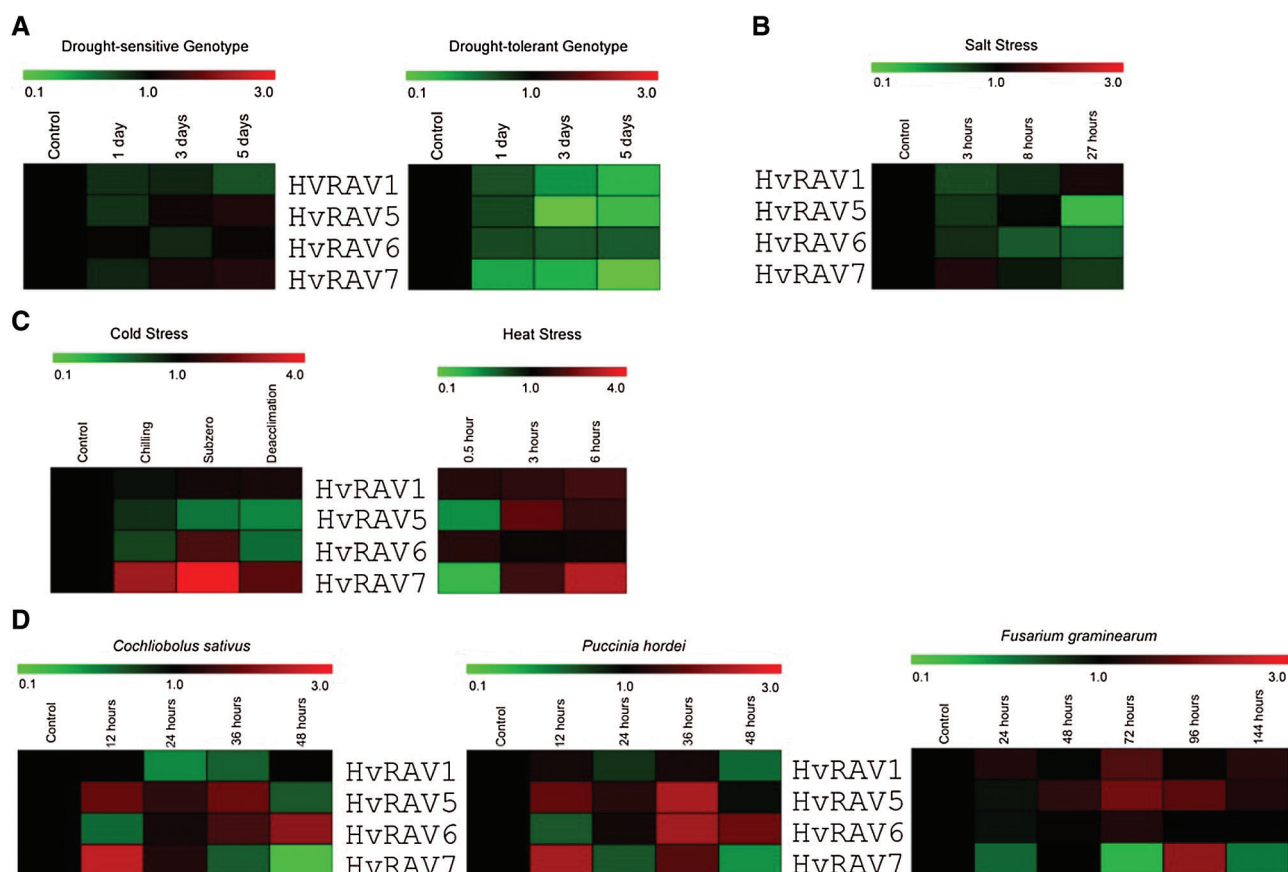


FIGURE 5. Expression profiles of the four *HvRAV* genes, namely *HvRAV1*, *HvRAV5*, *HvRAV6*, and *HvRAV7*, in response to abiotic and biotic stresses.

Heat map represents the differential gene expression of *HvRAV* genes in response to (A) drought stress, (B) salt stress, (C) cold and heat stress, (D) pathogen attacks, including *C. sativus*, *P. hordei*, and *F. graminearum*. While the red color indicates the upregulation of the *HvRAVs*, the green color shows the downregulation of the *HvRAVs*.

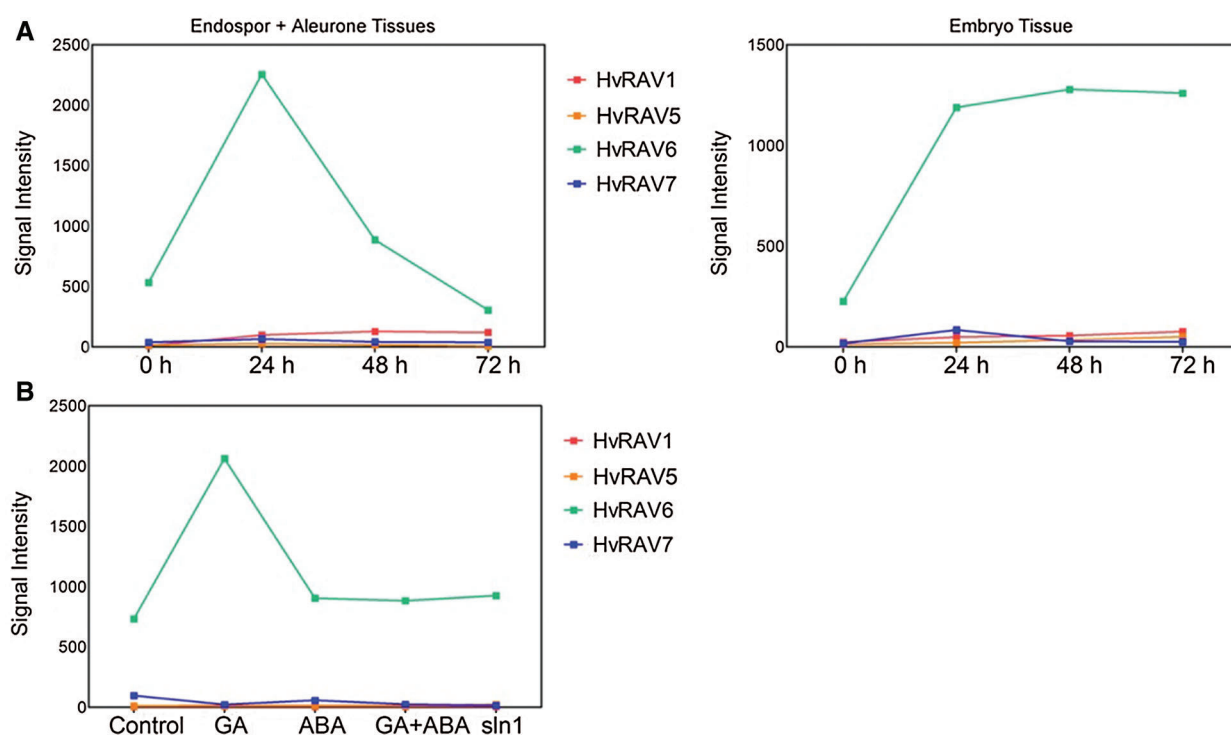


FIGURE 6. Signal intensities of *HvRAV* genes obtained from microarray experiments.

(A) Signal intensity of *HvRAV* genes in endosperm+aleurone and embryo tissues during germination at different hours. (B) Signal intensities of *HvRAV* genes after GA and/or ABA treatment and in *sln1* mutants.

A high number of phosphorylation sites in HvRAVs may promote the interaction with the co-repressor proteins and bind to cis-regulatory elements of stress-responsive genes.

Multiple sequence alignment revealed that all predicted barley RAV transcription factors contained highly conserved AP2 and B3 DNA binding domains. Highly conserved YRG and RAYD elements were predicted on the N-terminal and C-terminal of the AP2 domain of barley RAV proteins, respectively. The YRG element contains basic and hydrophilic 20 amino acids in the N-terminal region of the AP2 domain and plays an important role in DNA binding activity (Okamuro *et al.*, 1997). In the study, the YRG motif has been found to replace with the R(K)F(Y)K motif in AP2 domains of barley RAVs (Fig. 2). Similar results have been also observed in the RAVs of other plants including wheat, rice, maize, *Brachypodium distachyon* (Fig. 3C). It has been reported that the YRG motif was highly conserved in the AP2 domain of other AP2/ERF superfamily members including ERF, DREB, and AP2 in *Triticum aestivum* and *Arabidopsis thaliana* (Zhuang *et al.*, 2011). Another highly conserved region in the AP2 domain is RAYD element which is about 40 aa lengths and plays a crucial role in protein-protein interactions and DNA binding through α -helix (Okamuro *et al.*, 1997). The 18-amino-acid core region of the RAYD element was predicted to form an α -helix in the C-terminal region of the AP2 domain in barley RAVs. But the arginine (R) residue of RAYD motif was replaced with cysteine (C), thus forming CAYD motif in HvRAV7. The RAYD motif was also detected in the AP2 domains of other monocots RAVs including wheat, rice, and maize. In AP2 domains of barley AP2/ERF superfamily, RAYD motif was highly conserved, but also replaced with CAYD, MAYD, LAYD, RAHD, and RAND in DREB subfamily, as well as EAYD, KAYD, GAYD, and RAYI in AP2 subfamily (Guo *et al.*, 2016). WLK is another important motif in AP2 domain and part of the β 3 sheet. All barley RAVs contained highly conserved WLK residues, same as the ERF and DREB subfamilies. However, the W (tryptophan) residue of WLK was replaced with another aromatic and hydrophilic residue tyrosine (Y) in the AP2 subfamily. Similar results have been reported in maize, rice, and Arabidopsis (Nakano *et al.*, 2006).

RAV TFs have an additional B3 DNA binding domain, which consists of around 110 residues, as well as a single AP2 domain (Nakano *et al.*, 2006). B3 domain is widespread in plant genomes and contains seven-stranded β -sheet that constitutes an open β -barrel with two short α -helices at the ends of the barrel (Waltner *et al.*, 2005). Similarly, two short α -helices and 5–6 β -sheets were predicted in the B3 domains of barley. The β 1, β 2, and β 3 are on highly conserved sequences and located between two short α -helices (Fig. 2). AP2/ERF super family TFs can function as transcriptional activator and repressor proteins. Two major repressor motifs are the ERF-associated amphiphilic repression (EAR) motif (DLNxxP or LxLxLx) and the B3 repression domain (BRD) motif (GNSKTLRLFGVNMEC) in the C-terminal (Ohta *et al.*, 2001; Ikeda and Ohme-Takagi, 2009; Matías-Hernández *et al.*, 2014). It has been reported that only a short peptide of five amino acids, R/KLFGV, is important as a transcriptional repression activity in B3 proteins. In this

study, BRD, containing R/KLFGV residues, was found in the C-terminal of all HvRAVs as well as in some monocot RAVs including wheat, rice, maize. Repression domains (RD) function as transcriptional repression activity by interacting with the co-repressor proteins as well as by binding DNA directly. Causier *et al.* (2012) have found that variations of the R/KLFGV including MLFGV, RLFGI, and RLFGF showed interaction with co-repressor TPL (TOPLESS), while mutations in leucine (L) and phenylalanine (F) of the RLFGV sequence, new RSSGV motif, and prevented interaction with TPL in Arabidopsis.

Gene expression in response to abiotic and biotic stresses

To evaluate the potential functions of the HvRAV genes in response to biotic and abiotic stresses, the expression patterns of HvRAV1, HvRAV5, HvRAV6, and HvRAV7 were investigated using available microarray gene expression data. The RAV genes play a crucial role in response to environmental stresses, including salinity, drought, and pathogen attacks, by regulating the expression of target genes (Liu and Zhang, 2017). Chen *et al.* (2016) have reported RAV orthologs *Bradi2g1760* and *Bradi2g47220* were significantly induced by salinity stress, while another ortholog *Bradi2g02720* was repressed by salt stress at 3 time points (1, 2, and 3 h). On the contrary, in barley, HvRAVs were not induced by salinity stress, even expression of HvRAV5 significantly decreased at 27 h after 100 mM NaCl (Fig. 5B). The HvRAV genes exhibited different expression patterns under long-term drought stress in drought-sensitive and drought-tolerant barley genotypes. While the expression level of the four HvRAV genes did not show considerable changes in drought-sensitive genotype, the drought stress significantly down-regulated the expression of HvRAV5 and HvRAV7 with 14.3- and 25-fold, respectively, in drought-tolerant genotype. The RAV's negative regulation in response to salinity and drought has been reported using both *rav* mutants and RAV-overexpressing transgenic plants (Zhao *et al.*, 2017). Under salt conditions, RAV-overexpressing transgenic plants showed more inhibited seed germination than the wild type, while *rav* mutants exhibited higher seed germination as well as better growth after germination compared to wild types in the Arabidopsis. In addition, transpirational water loss was found higher in RAV-overexpressing transgenic plants than in wild types under drought conditions (Fu *et al.*, 2014). On the contrary, positive effects of heterologous expression of some RAV genes in Arabidopsis also have been reported, i.e., overexpression of soybean *GmRAV3* and pepper *CaRAV1* improved salinity and drought tolerance in transgenic Arabidopsis (Sohn *et al.*, 2006; Zhao *et al.*, 2017). The contradictory results in expression patterns also have suggested that homologous RAV genes may exhibit various responses against the specific stresses in different levels. There is no clear explanation about the various responses of RAV genes against salinity and drought. Being involved in different signaling and regulation pathways may result in various responses in similar stress conditions.

Barley HvRAV7 was expressed highly after long-term chilling and subzero treatment (Fig. 5C). In another microarray experiment (GSE41513), HvRAV6 and HvRAV7

were immediately induced by cold treatment within hours in both roots and shoot tissues of barley. Similarly, RAV genes have been reported to be induced by cold in *Brassica napus* and Chinese cabbage (Zhuang *et al.*, 2011; Li *et al.*, 2013). On the other hand, Fu *et al.* (2014) have reported that RAV1 and RAV2 genes were downregulated by cold in Arabidopsis. High temperature also changed the expression profile of HvRAV genes irregularly. The expression of HvRAV5 and HvRAV7 was repressed at 0.5 h after heat treatment but then beginning to increase in barley caryopses (Fig. 5C). BraRAV14, a member of the Chinese cabbage RAV subfamily, has been shown to be induced by heat stress (38°C) and reached peak level at 4 h of treatment (Li *et al.*, 2013). In another study, it has been reported that the expression level of tea CsRAV (Cs088-RAV) was increased in response to heat stress in 4 tea cultivars compared to the controls. However, expression profiles of the Cs088-RAV were varied in 4 tea cultivars in low- and high-temperature stress (Wu *et al.*, 2015). The same RAV gene may show different responses to adverse temperature changes in cultivars of the plants.

Plants defend themselves against a wide variety of pathogens including fungi, bacteria, and viruses by regulating multiple signal pathways and inducing defense-related genes (Sohn *et al.*, 2006). Transcription factors have significant roles in controlling the expression of these target genes. Sohn *et al.* (2006) showed that over-expression of pepper CaRAV1 strongly induced some pathogenesis-related genes including PR1, PR2 and PR5. This result conferred resistance to bacterial pathogen *Pseudomonas syringae* in transgenic Arabidopsis. RAV transcription factors play role in disease resistance against various plant pathogens by inducing the genes involved in melatonin, a signal molecule in plant defense mechanism, biosynthesis, thus improved the endogenous melatonin level (Lee *et al.*, 2014). The cassava MeRAV1 and MeRAV2 have been shown to improve the disease resistance against cassava bacterial blight by activating melatonin biosynthesis genes and increasing the melatonin level (Wei *et al.*, 2018). In barley, HvRAV7 was induced by fungal pathogens *C. sativus* and *P. hordei* at early hours after inoculation but then decreased. HvRAV6 showed an antagonistic expression pattern compared to HvRAV7 after both pathogen attacks (Fig. 5D). The transcript level of the HvRAV7 gene demonstrated to oscillate after each 24 h (for 144 h): decreasing mRNA level at 24 h and increasing at 48 h after *F. graminearum* treatment in barley cv. Morex. In long-term conditions, it has been reported that only BnRAV12 was significantly over-expressed among 15 BrRAVs in response to fungal plant pathogen *L. biglobosa* treatment at 7 d after inoculation in *Brassica napus* (Owji *et al.*, 2017). The activation of defense responses in plants is initiated by host recognition of primary pathogen-derived elicitors. Different RAV genes are involved in response to different pathogen attacks. Distinct pathogen elicitor activates different signal transduction pathways and defense genes and transcription factors. Besides, the type and number of pathogen-responsive cis-acting elements in the promoter region may lead to differential expression of some RAV genes. In barley, RAV genes have been predicted to have a different

number of pathogen-responsive cis-acting elements including W-BOX, GT1, TC-rich repeats and GCC-BOX (Tab. 3). HvRAV6 has been predicted to contain 14 W-BOX, 5 GT1 and 2 GCC-BOX binding elements, whereas HvRAV7 has a lower number of W-BOX and GT1, and no GCC-BOX.

Gene expression during germination and in response to ABA and GA treatments

Arabidopsis RAV1 (*AtRAV1*) has been reported to regulate the ABA signaling negatively during seed germination and establishment of the seedling. *AtRAV1* repressed the expression of *ABI3*, *ABI4*, and *ABI5*, which are ABA-responsive genes during seed germination. On the other hand, exogenous application of ABA also suppressed the expression of *AtRAV1* (Feng *et al.*, 2014). In barley aleurone cells, it has been observed that ABA treatment did not significantly change the expression of barley RAVs (Fig. 6B). Opposite results also have been reported in the literature. Exogenous ABA application induced the soybean RAVs and overexpression of *GmRAV3* improved the plant insensitivity in response to exogenous ABA during seed germination and early seedling development in Arabidopsis (Zhao *et al.*, 2017). These different results emphasize the functional diversity of RAVs in plants. On the other hand, exogenous application of GA, which promotes seed germination, significantly induced the expression of HvRAV6 compared to the controls. SLN1 (Slender1) is a DELLA protein, which negatively regulates the GA signaling pathway in barley (Chandler *et al.*, 2002). Chen *et al.* (2010) have reported that the genes induced by GA and highly expressed genes in *sln1* mutant are highly similar genes. GA mostly regulates the expression of TFs via SLN1 in the barley aleurone cells. The mRNA level of HvRAV6 was dramatically increased after GA treatment (Fig. 6B). Interestingly, however, in *sln1* mutant barley, the transcript level of HvRAV6 as high as ABA treated samples and lower than GA treated samples. The results may suggest that HvRAV6 was also regulated by GA but not dependent on SLN1.

In barley, the transcript level of the HvRAV6 gene was dramatically increased and reached peak point at 24 h after imbibition in endosperm tissues, but then decreased until the end of the germination process (Fig. 6A). In embryo tissues, mRNA level of HvRAV6 began to increase by imbibition, reached the highest level at 48 h after imbibition, and remained stable and high level for the next 24 h during post-germination. However, the transcripts of three RAV genes (*HvRAV1*, *HvRAV5*, and *HvRAV7*) were only detectable at a low level and their expression did not change during germination in both tissues. On the other hand, the transcript level of HvRAV6 was detected very high when compared to *HvRAV1*, *HvRAV5*, and *HvRAV7* in both tissues and plant hormone (ABA and GA) treatments.

Conclusion

RAVs are unique members of the AP2/ERF superfamily with two DNA binding domains, AP2 and B3. They have important roles in the regulation of different plant processes

including germination and stress responses. There is little information about barley *RAV* genes. In this study, 7 *RAV* genes were identified and characterized using bioinformatics tools and databases in barley. Conserved motifs and protein characteristics are determined using bioinformatics tools. Cis-acting elements that have a role in stress were predicted in the promoter region of *HvRAV* genes. Based on the microarray analysis, the expression profiles of 4 *HvRAVs* (*HvRAV1*, *HvRAV5*, *HvRAV6*, and *HvRAV7*) were determined under adverse environmental conditions, including drought, salinity, low and high temperature, and pathogen attack. Besides, the transcript level of *HvRAVs* was also determined in germination and in response to GA and ABA treatments. *In silico* analyses have demonstrated that *HvRAVs* play roles in response to different abiotic and biotic stress conditions as well as plant growth. However, the extensive biological roles of *HvRAV* genes in plant development and in response to abiotic and biotic stresses need further investigation.

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Authors' Contribution: The author confirms sole responsibility for the following: study conception and design, data collection, analysis and interpretation of results, and manuscript preparation.

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