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Genome-wide identification and expression profiling of snakin/GASA genes under drought stress in barley (*Hordeum vulgare* L.)

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Abstract

Gibberellic Acid-Stimulated Arabidopsis (GASA) proteins are present in various plants and have a role in plant growth, stress responses, and hormone crosstalk. GASA coding sequences in barley were discovered in this study. We then investigated gene and protein structure, physicochemical characteristics, evolutionary and phylogenetic relationships, promoter region, post-translational modification, and in silico gene expression. Finally, real-time quantitative PCR (RT-qPCR) was used to examine the expression of GASA genes in root and shoot tissues under drought stress. We found 11 GASA genes spread across six of seven chromosomes in the barley genome. A conserved GASA domain and 12-cysteine residues at the C-terminus were included in the proteins. All GASA genes contained secretory signal peptides. The GASA genes in *Hordeum vulgare* (HvGASA) have been classified into three subfamilies based on evolutionary analysis. According to synteny analyses, segmental duplications are significant in forming the GASA gene family. According to the cis-elements analyses, GASA genes may be induced by a variety of phytohormones and stresses. Tissue-specific expression analysis indicated that GASA genes had varied expression patterns in different tissues. Contrary to common perception, the expression study of GASA genes under biotic and abiotic stresses revealed that GASA genes are more induced by abiotic stresses than biotic stresses. The qPCR confirmed the response of GASA genes to abiotic stresses and showed different expression patterns of these genes under drought stress. Overall, these results can improve our knowledge about the function of GASA genes and provide data for future researches.

Keywords Abiotic stress · Drought treatment · Genome-wide analysis · *Hordeum vulgare* · Snakin/GASA

Introduction

Plants are constantly threatened by biotic and abiotic forces, and to maintain homeostasis, they begin cascade responses (Olga et al. 2020). Antimicrobial peptides (AMPs) are one of the most important plant defense molecules against these challenges. Many antimicrobial peptides have been implicated as part of the innate immune system in many organisms, including microorganisms, arthropods, animals, and plants (Elsbach 2003; Holaskova et al. 2015). AMPs have a distinct structural-activity connection and are divided into several families (snakins, thionins, defensins, hevein like peptides, hairpinins, cyclotides, and lipid transfer proteins) based on their sequence similarities, inclusion or lack of conserved cysteine residues, and protein structures (Olga et al. 2020). The Gibberellic acid-stimulated in Arabidopsis peptide family (also known as Snakin protein) is widely distributed in plants and performs various activities in signal transduction, cell division, fruit ripening, blooming period, seed and plant developmental phases and also in response to environmental stresses (Hou et al. 2018). GASA proteins have three domains, an 18–29 amino acid N-terminal signal peptide, a hydrophilic and variable region of up to 31 amino acids in the middle, and a conserved domain of up to 60 amino acids at the C-terminal, which generally contains 12 cysteine residues (Rezaee et al. 2020; Silverstein et al. 2007; Wang et al. 2009). All biological activities of the GASA proteins are related to the carboxyl terminus (Ahmad et al. 2020; Fan et al. 2017). GASA genes may also have tissue-specific

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expression patterns, according to earlier research. AtGASA1 and AtGASA2 seem to be more expressed in flower buds and siliques of Arabidopsis, whereas AtGASA4 is more expressed in meristematic areas. The OrGASA9 gene in rice (*Oryza sativa*) panicles is highly expressed (Li et al. 2020). Furthermore, the expression of GASA genes is higher in young tissues than in older tissues. GASA genes are also implicated in the reactions to abiotic and biotic stresses and to hormones. By elevating salicylic acid (SA) biosynthesis, ectopic expression of FsGASA4 (GASA gene in *Fagus sylvatica*) improves salinity, ROS, and heat stress resistance during seed germination in transgenic *Arabidopsis thaliana* (Alonso-Ramírez et al. 2009). Different abiotic stimuli, such as salt, drought, cold, heat, and metal stresses, modulated the gene expression levels of OsGASA in rice (Muhammad et al. 2019). Some PtGASA family members (GASA genes in *Populus trichocapa*) are involved in hormone responses, growth and development of vegetative organ, and drought stress responses (Han et al. 2021). By modifying the expression of defensive response genes, members of the soybean (*Glycine max*) GASA gene family boost soybean mosaic virus resistance (He et al. 2017). Citrus tristeza virus infection activates CcGASA4 in citrus (*Citrus clementina*) (Wu et al. 2020). In transgenic Arabidopsis, overexpression of the AtGASA4 gene from European beech improves salinity, ROS, and heat stress tolerance (Alonso-Ramrez et al. 2009). The tomato GASA genes Snakin-1 and Snakin-2 are active against bacteria (*Clavibacter michiganensis*) and fungi (*Fusarium solani* and *Botrytis cinerea*) (Almasia et al. 2008; Balaji and Smart 2012). After being infected with *Colletotrichum gloeosporioides*, HbGASA genes are engaged in innate immunity and control ROS generation in rubber (*Hevea brasiliensis*) (An et al. 2018). As a result, the GASA gene family has several physiological and biological activities. GASA proteins are also stimulated by gibberellin (GA) and other plant hormones such as auxins (IAA), abscisic acid (ABA), brassinosteroids (BRs), and salicylic acid (SA), according to prior research (Li et al. 2011; Qu et al. 2016; Wu et al. 2020). Signal transduction and cross-talk between GA and other phytohormones are crucial functions of GASA genes (Wang et al. 2009). AtGASA4 and AtGASA6 in Arabidopsis are also stimulated by growth hormones including GA, auxin, cytokinin, and BR but suppressed by stress hormones like ABA, SA, and jasmonic acid (JA) (Qu et al. 2016). Furthermore, GASA genes play a crucial role in GA production and signaling, and in various interactions between GASA genes and DELLAs (Li et al. 2020; Sun et al. 2013; Zhang et al. 2009). So far, the expression pattern and evolutionary relations of GASA genes were investigated in common wheat (Cheng et al. 2019), apple (Fan et al. 2017), rice (Muhammad et al. 2019), Arabidopsis (Zhang and Wang 2008), grapevine (Ahmad et al. 2020), soybean (Ahmad et al. 2019), cotton (Qiao et al. 2021),

maize (Zimmermann et al. 2009), cacao (Faraji et al. 2021), tobacco (Li et al. 2022), potato (Nahirñak et al. 2016) and populus (Han et al. 2021). According to the findings, the GASA gene family plays a crucial function in regulating cell signaling in response to environmental stress and is also linked to cell growth regulation.

Drought (water stress) is a severe global issue that substantially limits crop yield, and recent global climate change has worsened this condition (Yang et al. 2021). Water stress is a critical negative factor that affects the area under cultivation and crop productivity in arid and semi-arid locations across the globe. Water stress may happen early or late in the growing season, but its impact on yield decrease is most significant when it happens after anthesis (Samarah 2005). Water stress has a negative impact on morphology, physiology, and metabolism of plants. The type of cultivar, development stage, stress duration, and stress level affect the changes caused by drought (Qiu et al. 2020).

Barley (*Hordeum vulgare* L.), with $2n = 2x = 14$, is the fourth most valuable grain in the world after wheat, rice, and corn (Baum et al. 2007). Since the growth season for barley is quick and its biological, morphological, and genomic studies have been done, and its natural genomic diversity is high; thus, this plant has been selected as a suitable model plant for analyzing biotic and abiotic stresses (Hackenberg et al. 2015).

This study aimed to identify members of GASA gene family in *Hordeum vulgare* and to discover their expression pattern under drought stress. In this study, we first identified the GASA genes throughout the barley genome, and then we performed a detailed bioinformatics investigation (including evaluating several physicochemical characteristics, cis-regulatory elements, gene ontology, subcellular localization, signal peptide, transmembrane helices, disulfide bonds, putative antimicrobial activity, evolutionary analysis, post translational modification prediction, in silico gene expression analysis) on identified genes. In addition, the expression patterns of the GASA genes were studied in response to drought stress.

Materials and methods

Identification of GASA gene family members in the barley genome

All available GASA protein sequences were obtained from the NCBI database protein bank to identify GASA gene family members in the barley genome. The downloaded sequences were aligned using CLC Genomics Workbench 9.0 and Vector NTI 10.3 softwares, and a consensus sequence was obtained from different sequences. The consensus sequence was inserted into the tBLASTn server

and aligned against the barley genome. The results of this alignment were entered into the following stages of analysis as hypothetical GASA genes. The primers were designed to amplify complete gene fragments using two Vector NTI 10.3 and Allele ID 7.0 softwares (Apte and Singh 2007), based on complete open reading frames (ORFs) of identified hypothetical GASA genes (Table 1).

To extract genomic DNA from barley tissues, the CTAB technique with RNase was utilized (Doyle and Doyle 1987). The PCR was performed using Ampliqon *Taq* DNA polymerase master mix with a temperature program, denaturation at 94 °C for 30 s, annealing for 30 s, and extension at 72 °C for 30 s, over 35 cycles. After the reaction, PCR products were loaded on 1.5% agarose gel and confirmed by size. According to the manufacturer's instructions, the amplified fragments were purified using a Thermo Fisher Scientific gel purification kit and sequenced. The sequencing results of each gene were separately analyzed using CLC Genomics Workbench 9.0 and Vector NTI 10.3 softwares. After editing, they were evaluated for complete ORFs and then submitted to NCBI genbank. These sequences were confirmed using NCBI CDD, Pfam, FGENESH, Phytozome, morexGenes, and Ensembl Plants tools and databases to further confirm the GASA proteins in barley (Table 2).

Table 2 The genomic information of GASA gene family members in *H. vulgare*

Gene Name	Chromosome	Ensemble ID	Gene length
HvSn2	Chr1H(+)	HORVU1Hr1G074530	515
HvSn3	Chr2H(+)	HORVU2Hr1G001540	393
HvSn4	Chr4H(−)	HORVU4Hr1G013210	397
HvSn5	Chr5H(−)	HORVU5Hr1G097030	455
HvSn7	Chr2H(−)	HORVU2Hr1G081190	539
HvSn8	Chr2H(−)	HORVU2Hr1G034860	437
HvSn9	Chr6H(+)	HORVU6Hr1G094130	1033
HvSn10	Chr7H(+)	HORVU7Hr1G120380	577
HvSn11	Chr5H(+)	HORVU5Hr1G065670	1041
HvSn12	Chr1H(−)	HORVU1Hr1G057570	656
HvSn13	Chr7H(−)	HORVU7Hr1G042390	499

Characterization of gene and protein sequences of barley GASA gene family members

The chromosomal data of all barley GASA gene family members, including their chromosomal number and their position on the chromosomes was obtained from Phytozome, morexGenes, and Ensembl Plants databases. The gene

Table 1 Name, accession number, Ta, amplicon length and sequence of primers used for isolation of complete gene fragments

Gene name	Primer sequence (5′→3′)	Ta (°C)	Amplicon length (bp)	Accession number
HvSn2	F: AATCACCAGAAAGCGAAGCAAACC	59	554	OL677460
	R: GGGATGAGATCACGGGCACTTG			
HvSn3	F: ATATAGGGTCACACATCAGTAGC	60	571	OL677461
	R: GAGATTATTGAACATTGGGACAGG			
HvSn4	F: ACTCACTCGTCGCTCACTCATC	57	568	OL631779
	R: CCGTAGGGTGGAGGTTGAAGAC			
HvSn5	F: CCAAGCAACTCTCAAGAACAC	60	513	OL677462
	R: ATGGGCTGTGGAGGATGG			
HvSn7	F: GCATCGCCACCCAAGCCTAAG	63	719	OL677463
	R: GTCAACACCGTTCCTTCCACACC			
HvSn8	F: GAAGAAGAGAAGACGATGAAGCC	58	590	OL631773
	R: GAAACACTCCTCACTACTCAAACC			
HvSn9	F: ACCGACACTATCTACTACTCCTAC	60	1151	OL631774
	R: GATGATTATTGATGGGTGCTTGG			
HvSn10	F: GCGACCACCAGCCAGAAG	63	611	OL631775
	R: TGAAGGAGAGGAAGAAGAAGATGG			
HvSn11	F: CACTACACTAGCCAGCGTCTTATC	63	1122	OL631776
	R: CAACGGCGAGGCAGAATCC			
HvSn12	F: TACACCTACCTACGGACCTACC	60	798	OL631777
	R: CCTGGCAATGAATCTACATCTACG			
HvSn13	F: TCCTCGTCCTCGCAGTCG	60	526	OL631778
	R: AGTAATAACCATCAACCGCAAGC			

structure display service was used to identify and define the exon–intron boundaries inside all GASA genes (Guo et al. 2007). The cis-regulatory elements inside the 1500 bp promoter region were investigated using PlantCare (Lescot et al. 2002). The CELLO2GO server (Yu et al. 2014) was used to create gene ontology (GO) annotations for barley GASA gene family members. The Protparam server (Wilkins et al. 1999) was used to evaluate several physicochemical characteristics including aliphatic index, isoelectric pH (pI), instability index, molecular weight, and grand average of hydropathy (GRAVY) of the barley GASA proteins (Table 3). The CELLO2GO (Yu et al. 2014), SignalP (Almagro Armenteros et al. 2019), TargetP (Emanuelsson et al. 2007), TMHMM (Krogh et al. 2001), DiANNA (Ferrè and Clote 2005), and CAMP web servers (Thomas et al. 2010) were also used to determine the subcellular localization, signal peptide, signal peptide cleavage site, transmembrane helices, disulfide bonds, and putative antimicrobial activity of barley GASA proteins respectively (Table 3).

The phosphorylation sites of the GASA proteins were predicted using the NetPhos 3.1 server (Blom et al. 2004) with a potential value greater than 0.5. Using default parameters, the N-glycosylation sites were predicted by the NetNGlyc 1.0 server (Gupta and Brunak 2002). After selecting the best models using the 3D-refine software, iterative template-based fragment assembly simulations in I-TASSER (Yang et al. 2015) were performed to construct three-dimensional protein structures of barley GASAs (Bhattacharya et al. 2016).

Evolutionary analyses

The phylogenetic relationships between the barley GASA genes and several main model plant species (Arabidopsis, rice, maize, sorghum and brachypodium) were evaluated and MEGA X software was used to create the maximum likelihood tree. Resulted phylogenetic tree was evaluated by bootstrap method with 1000 replications (Kumar et al. 2018). Multiple sequence alignment of the barley GASA proteins was performed using Clustal Omega (Sievers et al. 2011). The distribution of conserved motif sequences among GASA proteins was determined using the MEME 5.3.0 server.

More than 85% of identity in the nucleotide sequence of genes is evidence of duplication (Zheng et al. 2010). Thus, we used Clustal Omega (Sievers et al. 2011) and Geneious R8.1 softwares to align DNA sequences and to assessing the identity degree (Kearse et al. 2012). Gene duplication events compared to other species were identified through the Plant Genome Duplication Database using the MCScan 0.8 software (Wang et al. 2012). The selection pressure on GASA genes was calculated using the DnaSP 6.0 program by calculating non-synonymous (Ka) and synonymous (Ks)

Table 3 The physico-chemical characteristics of GASA proteins; Length, Molecular weight (MW), Isoelectric point (pI), Instability index (II), Signal peptide length, Subcellular localization, Major amino acids content

Gene Name	Length (aa)	MW (kDa)	pI	II	GRAVY	Aliphatic Index	Signal Peptide Length	Subcellular Localization	Major Amino acids Content
HvSn2	92	9.67	9	41.83	0.04	61.5	29	Extracellular	C (14%), A (11%), G (9.8%)
HvSn3	92	9.94	8.88	43.4	−0.14	73.2	24	Extracellular	C (13%), A (9.8%), K (9.8%)
HvSn4	98	12.63	9.1	26.42	−0.17	74.1	33	Extracellular	C (12.2%), K (11.22%) L (11.22%)
HvSn5	99	24.57	9.18	62.82	−0.32	73.9	33	Extracellular	L (24%), S (21.21%), T (21.21%)
HvSn7	107	11.09	9.25	44.65	0.06	75.8	23	Extracellular	A (15%), C (11.2%), L (10.28%)
HvSn8	111	11.25	8.68	45.38	−0.013	62.5	25	Extracellular	A (12.61%), C (11.7%), G (11.7%)
HvSn9	120	11.09	9.25	44.65	0.069	75.8	28	Extracellular	A (13.3%), L (9.1%), C (10%)
HvSn10	126	17.83	9.21	49.37	−0.33	72.8	36	Extracellular	L (12.7%), Q (10.9%) A (9.7%)
HvSn11	113	12.63	9.1	26.42	−0.177	74.1	29	Extracellular	C (10.6%), K (9.7%), L (8.85%)
HvSn12	120	13.24	9.01	69.87	−0.3	48.2	19	Extracellular	P (15%), A (12.7%), C (11.11%)
HvSn13	101	10.27	9.05	43.23	0.02	59.9	24	Extracellular	A (11%), Q (9.5%), k (9.5%), L (9.5%)

substitutions, as well as the ratio of non-synonymous to synonymous substitutions (Ka/Ks) (Rozas et al. 2017). A synonymous mutation rate of substitutions per synonymous site was used to quantify temporal divergence and duplication. Time divergence and duplication were quantified using a synonymous mutation rate of λ substitutions per synonymous site per year equal to T = (Ks/2λ) (λ = 6.5 × 10^{-9}) × 10^{-6} (Yang et al., 2008). Circos software was used to show the synteny relations of GASA genes between orthologous pairings of barley-rice, barley-Arabidopsis, barley-maize, barley-Brachypodium, barley-sorghum (Krzywinski et al. 2009).

In silico gene expression analysis

In silico gene expression analysis of GASA gene family members under stress conditions was performed using gene expression omnibus datasets from several Affymetrix Barley1 GeneChip microarray (Drought stress: GSE15970, GSE3170, GSE6990 and GSE17669; salt stress: GSE5605; cold stress: GSE6993, GSE10332 and GSE27821; heat stress: GSE23896; fungal stress: GSE14930, GSE20416, GSE33392 and GSE33398; bacteria: GSE17238 and GSE43906; gibberellin (GA) and abscisic acid (ABA): GSE18785 and GSE10328; boron toxicity: GSE14521and mercury toxicity: GSE15295). The expression of GASA gene family members under abiotic stresses was analyzed using expression intensities in drought, salinity, cold, and heat conditions. In addition, the expression profiles of GASA genes under biotic stresses were determined using expression intensities obtained from the treatment of barley plants with pathogens such as Powdery mildew, *Fusarium graminearum*, *Cochliobolus sativus*, and *Puccinia hordei*, *Polymixa gramminis*, *pseudomonas syringae*, and aphid. The heat maps were created using Heatmapper (Babicki et al. 2016).

Plant material and water stress treatment

The seeds of Zahak cultivar of barley &. Seeds were planted in sterile soil-filled pots weighing 2.5 kg (20 cm in height and 15 cm in diameter). To ensure seedlings could expand in soil with enough water, all plants were irrigated daily until they attained the three-leaf stage (i.e., 31 days from germination to the three-leaf stage). The experiment was performed in a completely randomized design (CRD) with three replications. Two irrigation regimes were utilized on pots (control and 20% field capacity (FC)). The control pots were watered every day to maintain the soil moist. The soil moisture content was constant at 75% FC for control treatment plants and 20% FC for drought treatment plants. Three-time periods were chosen for sampling (zero, four, and ten days after applying drought stress). Three controls were collected at each time point to exclude the influence of growth and development processes. After being frozen

in liquid nitrogen, root and shoot samples were kept in a − 80 °C freezer. To evaluate field capacity, the soil was baked at 150 °C for 48 h (FC). Then, it was weighted (A) and saturated with water. Pots were covered with aluminum foil and maintained at room temperature for 24 h to avoid water evaporation (B). The following formula was used to compute the FC: FC = 100*B-A/B**.**

Isolation of total RNA, cDNA synthesis and real-time PCR analysis

The expression of GASA gene family members under water stress condition was investigated using real-time PCR. The SinaPure RNA extraction Kit was used to isolate total RNA from frozen shoot and root samples. Total RNA was quantified using the NanoDrop 2000c Spectrophotometer (Thermo Scientific NanoDrop 2000, USA) and were qualified by 1% agarose gel electrophoresis. The Easy cDNA Synthesis Kit (Parstous, Iran) was used to make the cDNAs. The reactions were performed using iQSYBR Green Supermix kit (Parstous, Iran) on Step One Plus Real-time PCR system (Applied Biosystems, USA) according to the manufacturer's instructions. The real-time PCR reactions were performed in three technical replications. After normalizing Ct values with Actin as endogenous control, relative mRNA abundance (in foldchange) was assessed using the 2$^{-\Delta\Delta Ct}$ approach (Livak and Schmittgen 2001).

Estimation of proline content

To determine proline content, shoot and root tissues (100 mg) were homogenized in 3% (w/v) sulphosalicylic acid and centrifuged for 10 min at 4 °C at 10,000 g. In a test tube, ninhydrin (47 mM), phosphoric acid (0.8 M), and glacial acetic acid (0.25 M) were added to the supernatant. In a water bath, the mixture was heated to 98 °C for 60 min and then let to cool at room temperature. The cooled mixture was extracted with toluene. At 520 nm, the absorbance of the reaction mixture was measured (Bates et al. 1973). The quantity of proline was determined using a standard curve made up of known proline concentrations.

Results

Identification of GASA gene family and their chromosomal distributions

We found 11 GASA genes in the *H. vulgare* genome, which spread over 6 of the 7 chromosomes. On chromosomes 1, 5, and 7, two GASA genes were identified. One gene was on each of chromosomes 4 and 6, whereas three genes were found on chromosome 2. This information revealed the

uneven distribution of HvGASA genes on barley genome (Fig S1, Table 2).

Characterization of GASA protein physicochemical properties

The ExPASy server was used to examine protein properties such as molecular weight, isoelectric point, instability index, grand average of hydropathicity (GRAVY), major amino acid content, and aliphatic index. The studied GASA proteins had molecular weight ranging from 9.67 (HvSn2) to 24.57 (HvSn5). Furthermore, most GASA proteins had a molecular weight of less than 13 kDa. In addition, the isoelectric point varied between 8.68 (HvSn8) and 9.25 (HvSn9). Most GASA proteins were unstable, with instability index values above 40, Except for HvSn4 and HvSn11. most GASA proteins were hydrophilic according to GRAVY values except HvSn2, HvSn7, and HvSn9. The aliphatic index values were 75.88 (HvSn9) to 48.25 (HvSn12). Ala, Cys, Lys, and Leu were the most common amino acid residues in GASA proteins, although Ser and Thr were also found (Table 3).

All GASA proteins' transmembrane helices were also examined. HvSn4, HvSn5, HvSn11, and HvSn13 all have at least one transmembrane segment. An N-terminal signal peptide including 19 to 30 amino acids, a variable peptide sequence, and a highly conserved C-terminal region containing approximately 60 amino acids and 12 cysteine residues were found in all GASA proteins (Fig. 1b).

The prediction of antimicrobial characteristics using CAMP server revealed that this trait was present in all the peptides. After removing the signal peptide, the antimicrobial properties of all of the GASA family peptides in the barely were predicted using the Support Vector Machine (SVM) algorithms with a probability of more than 0.98, using the Random Forest (RF) algorithms with a probability of more than 0.8, using the Artificial Neural Network (ANN) algorithms with full confirmation, and using the Discriminant Analysis (DA) algorithms with a probability of more than 0.97 (Table 4).

Checking protein sequences in the CELLO2GO server showed that all proteins accumulate in the extracellular space and are induced in response to abiotic and biotic stressess. Examination of the presence of disulfide bonds in the structure of barley GASAs showed 12 conserved cysteine amino acids that formed six intramolecular disulfide bonds. The binding pattern of cysteine amino acids and the formation of disulfide bonds was the same in HvSn11 and HvSn13, and other GASAs showed specific patterns (Table 5).

The Gene Structure Display Server tool constructed exon–intron structures based on the alignment of cDNA and gene sequences of barley GASAs. Each group of HvGASA genes have same genetic structures that were conserved. The HvSn3, HvSn4, HvSn5, HvSn8, and HvSn9 genes, for example, have one intron and two exons. HvSn11 and HvSn12, on the other hand, were well conserved, with four exons and

Table 4 Predicting antimicrobial properties, using the Support Vector Machine (SVM), Random Forest (RF), Artificial Neural Network (ANN) and Discriminat Analysis (DA) algorithms

Peptide	SVM	RF	ANN	DA
HvSn2	0.987	0.8295	AMP	0.997
HvSn3	0.998	0.8765	AMP	0.94
HvSn4	0.993	0.9585	AMP	1
HvSn5	0.998	0.88	AMP	0.988
HvSn7	0.998	0.9635	AMP	1
HvSn8	1	0.8935	AMP	1
HvSn9	1	0.808	AMP	0.999
HvSn10	0.998	0.938	AMP	0.974
HvSn11	1	1	AMP	1
HvSn12	1	1	AMP	0.996
HvSn13	1	1	AMP	1
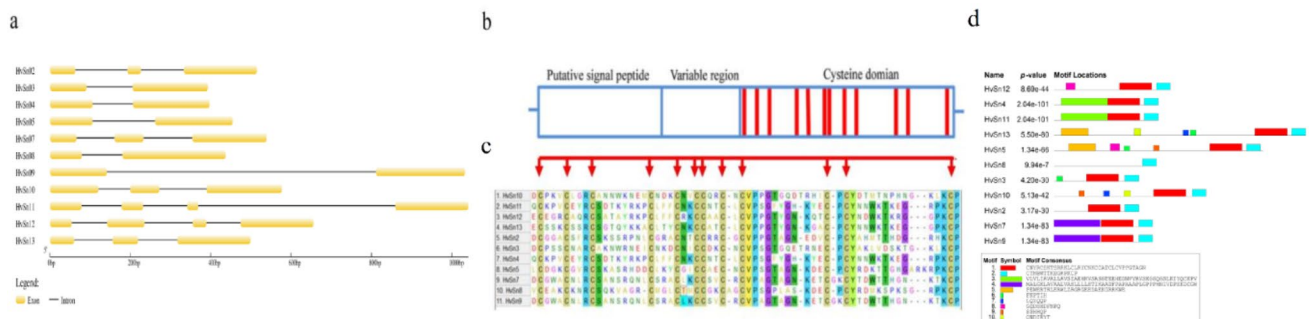
Fig. 1 Analysis of structures of HvGASA genes. **a** Exon–intron composition analysis, yellow boxes and black lines are exon and intron positions, respectively. **b** Analysis of GASA protein structures, red columns represent conserved cysteine residues. **c** Multiple sequence alignments of the HvGASA protein, conserved GASA domains are indicated. **d** Conserved motifs analysis, different conserved motifs are shown in different colors

Table 5 Disulfide bond prediction for GASA proteins

Peptide Name	Bond 1	Bond 2	Bond 3	Bond 4	Bond 5	Bond 6
HvSn2	1–3	2–12	4–7	6–13	8–10	9–11
HvSn3	1–6	2–4	3–5	7–11	8–12	9–10
HvSn4	1–6	2–4	3–10	5–12	7–9	8–11
HvSn5	1–10	2–4	3–10	5–12	7–9	8–11
HvSn7	1–6	2–5	3–4	7–11	8–12	9–10
HvSn8	1–9	2–10	3–5	4–11	6–13	8–12
HvSn9	1–8	2–4	3–6	5–12	7–11	9–10
HvSn10	1–6	2–4	3–5	7–11	8–12	9–10
HvSn11	1–11	2–4	3–6	5–12	7–9	8–10
HvSn12	1–4	2–8	3–13	5–6	7–14	9–10
HvSn13	1–11	2–4	3–6	5–12	7–9	8–10

three introns. The group 3 genes (HvSn2, HvSn7, HvSn10, and HvSn13), which have three exons and two introns, showed similar patterns (Fig. 1a).

We also discovered ten conserved protein motifs in the HvGASA proteins. Except for HvSn8, the majority of members had motifs 1 and 2. However, only motif 2 was found in HvSn8 (Fig. 1d).
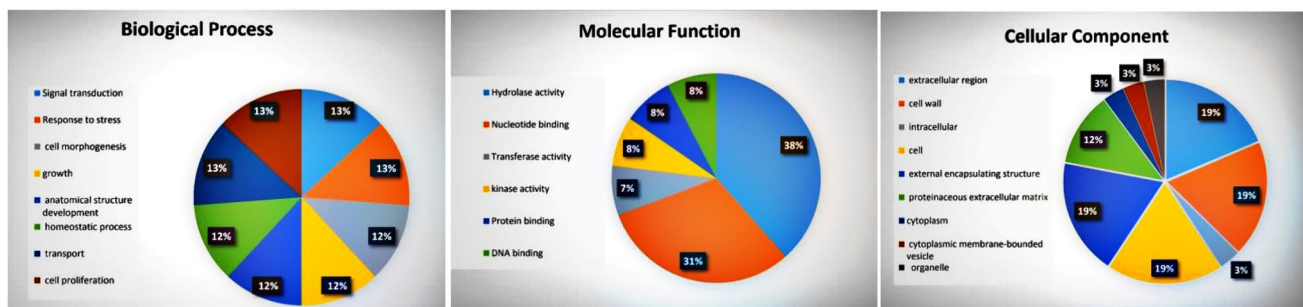
GO analysis showed the involvement of GASA proteins in various biological, cellular, and molecular processes (Fig. 2).

The predicted 3D structure of all HvGASA proteins showed that these proteins contain β strands and α helices. The β strands were the most abundant and were more extensive than α helices. There were 1 to 11 β strands and 0 to 6 α helices in the structure of these peptides. The peptides of HvSn2, HvSn4, HvSn11, and HvSn13 only had β strands, and other peptides had β strands and α helices together (Fig. 3).

The post-translational modification of GASAs in terms of phosphorylation and glycosylation was anticipated in this work. Within GASA proteins, we predicted a total of 135 possible phosphorylation events on the aminoacids serine, threonine, and tyrosine. The majority of phosphorylation events were anticipated to be connected to serine (59), next threonine (53), and finally tyrosine (24). Most phosphorylation sites (30 sites) in GASA proteins were anticipated in HvSn5, while phosphorylation events in other proteins varied from 5 to 21 sites. Three GASAs, HvSn7, HvSn9, and HvSn10, also contained a glycosylation site (Fig. 4).

Cis-elements have role in gene expression control. We examined the 1.5 kb upstream of the start codon according to the genome sequence to investigate the regulatory mechanisms behind GASA genes. Cis-elements associated with phytohormones were identified in the promoters of 11 GASA genes, including the TGACG motif, the TATC-box, the P-box, the ABRE, the TGA-element, the AuxRR-core, the ERE, and the TCA element. The majority of these cis-elements were determined to be linked to ABA, GA, and salicylic acid. Cis-elements connected with various kinds of the stress response (LTR and STRE) and disease resistance (TC-rich repeats, W-box, and WUN-motif) were identified in the majority of genes. Cis-elements regulating zein metabolism (O2-site) and seed-specific regulation (RY-element) were also identified in these regions of the promoters. Additionally, promoters of the majority of the GASA genes included cis-elements related to anaerobic respiration (ARE) and light response (GT1-motif, TCT-motif, Box 4, GATA, G-BOX, and AE-box). The findings showed that several plant hormones may control GASA genes to react to various stresses

Fig. 2 Gene ontology showed the involvement of GASA proteins in various biological, cellular, and molecular process
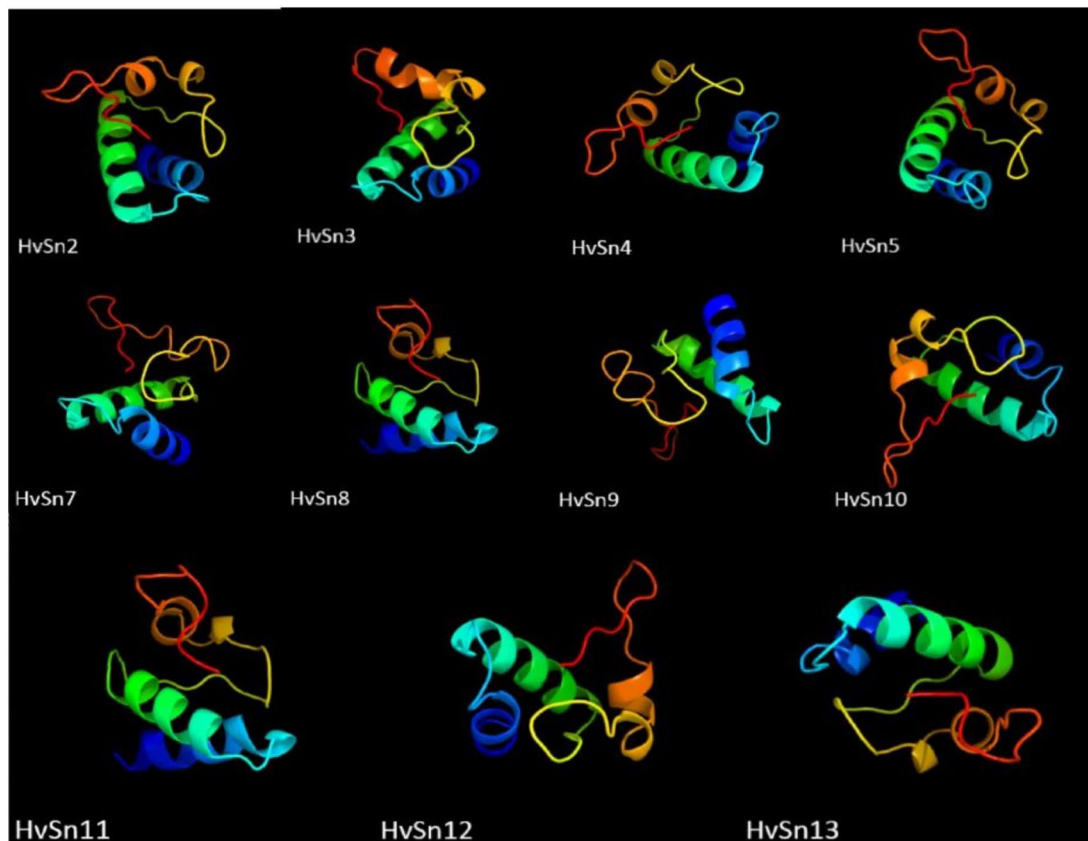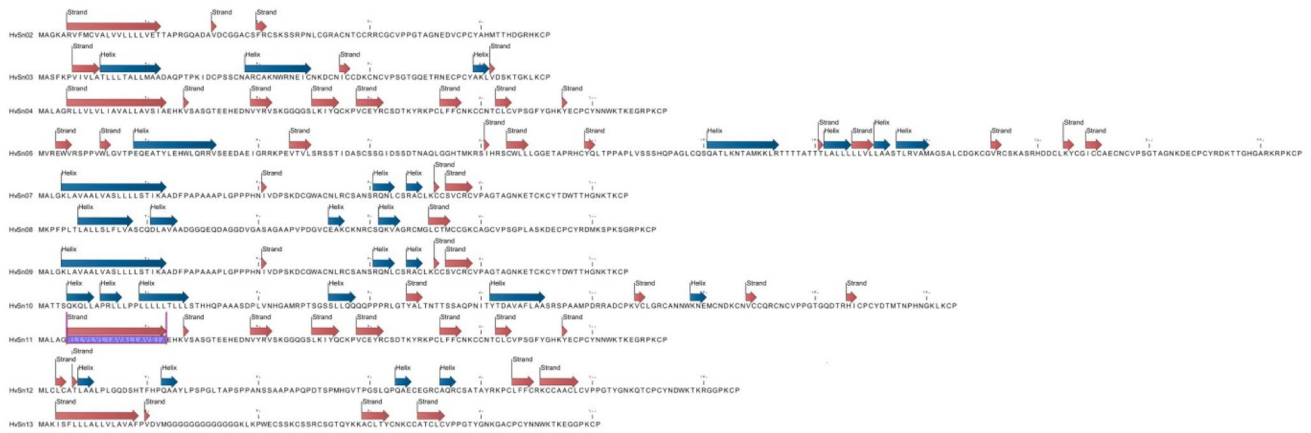
Fig. 3 The protein structure analysis of HvGASA, the blue arrow and the red arrow represent the alpha-helix and the beta-strand, respectively

in barley (Fig. 5). We used Cytoscape 3.8.2 to create a gene-transcription factor interaction network. Strong interactions between Dof, WRKY, ERF, and MYB transcription factors and barley GASA genes were observed in the constructed interaction network (Fig. 6).

Evolutionary analyses

We created a phylogenetic tree using GASA protein sequences from *A. thaliana*, *H. vulgare*, *Zea mays*, *sorghum* *bicolor*, *Brachypodium distachyon* and *Oryza sativa* to better understand the evolutionary connections between GASA genes. Based on the phylogenetic tree, the GASA genes of *A. thaliana, H. vulgare, Z. mays, S. bicolor, B. distachyon and O. sativa* were classified into three groups comprising 25, 24, and 22 GASA genes, respectively. Group 1 had five barley genes (HvSn2, HvSn3, HvSn7, HvSn9 and HvSn10), Group 2 had four genes (HvSn4, HvSn11, HvSn12, and HvSn13), and Group 3 had two genes (HvSn5 and HvSn8) (Fig. 7).
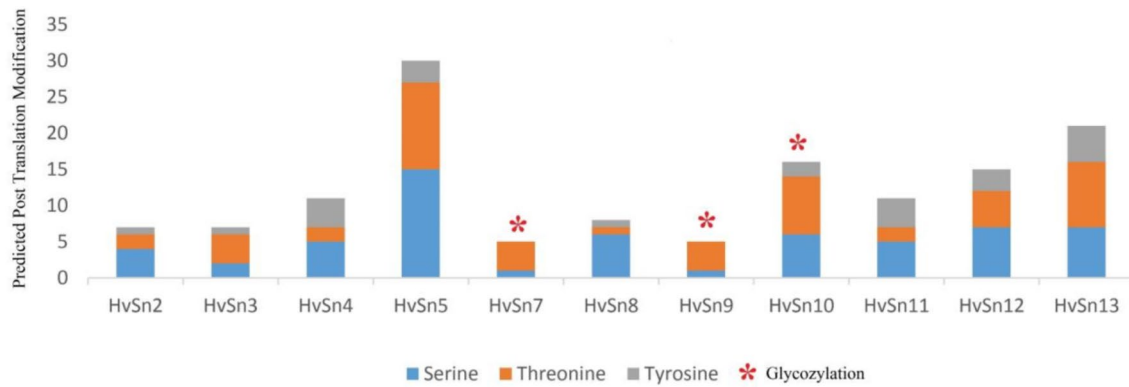
Fig. 4 Post-translational modification of HvGASA. The serine, threonine and tyrosine are phosphorylation sites and the asterisks indicate the occurrence of glycosylation
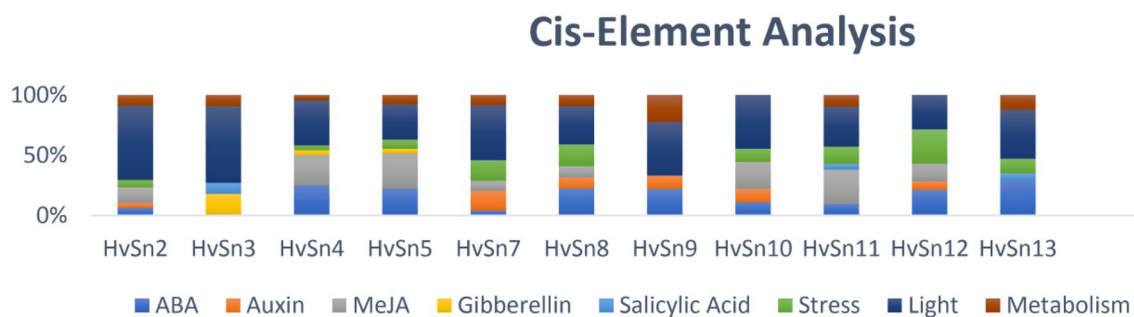
Fig. 5 Predicted cis-elements in the HvGASA promoters. The 1.5 kb upstream of the HvGASA genes were analyzed with the PlantCARE tool.
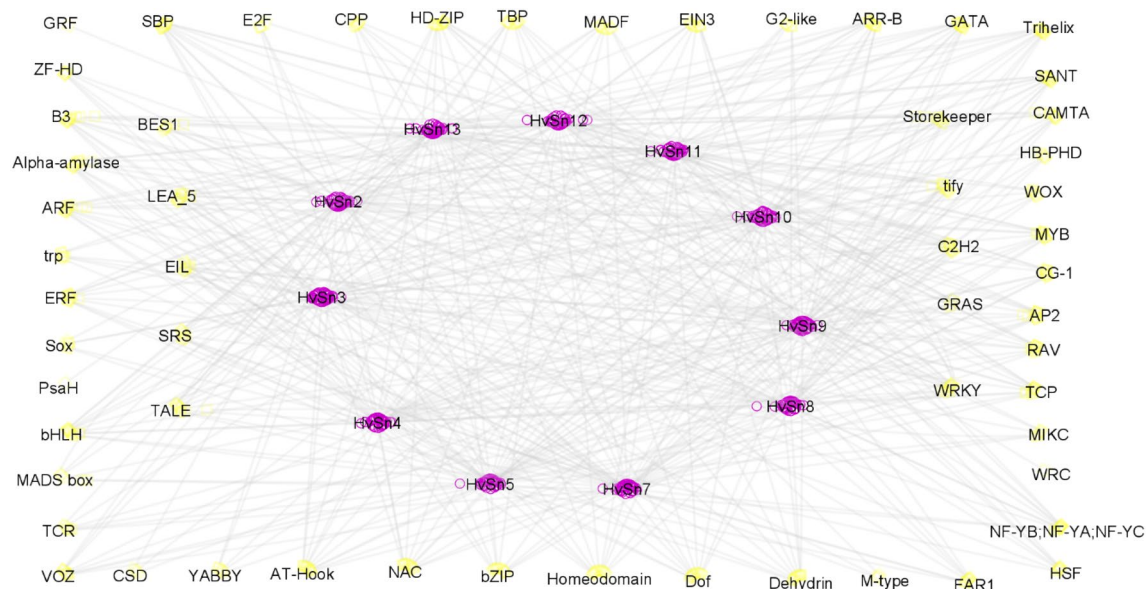
Fig. 6 Interaction network between GASA genes and transcription factors. The HvGASA genes and transcription factors are indicated by purple and yellow colors, respectively
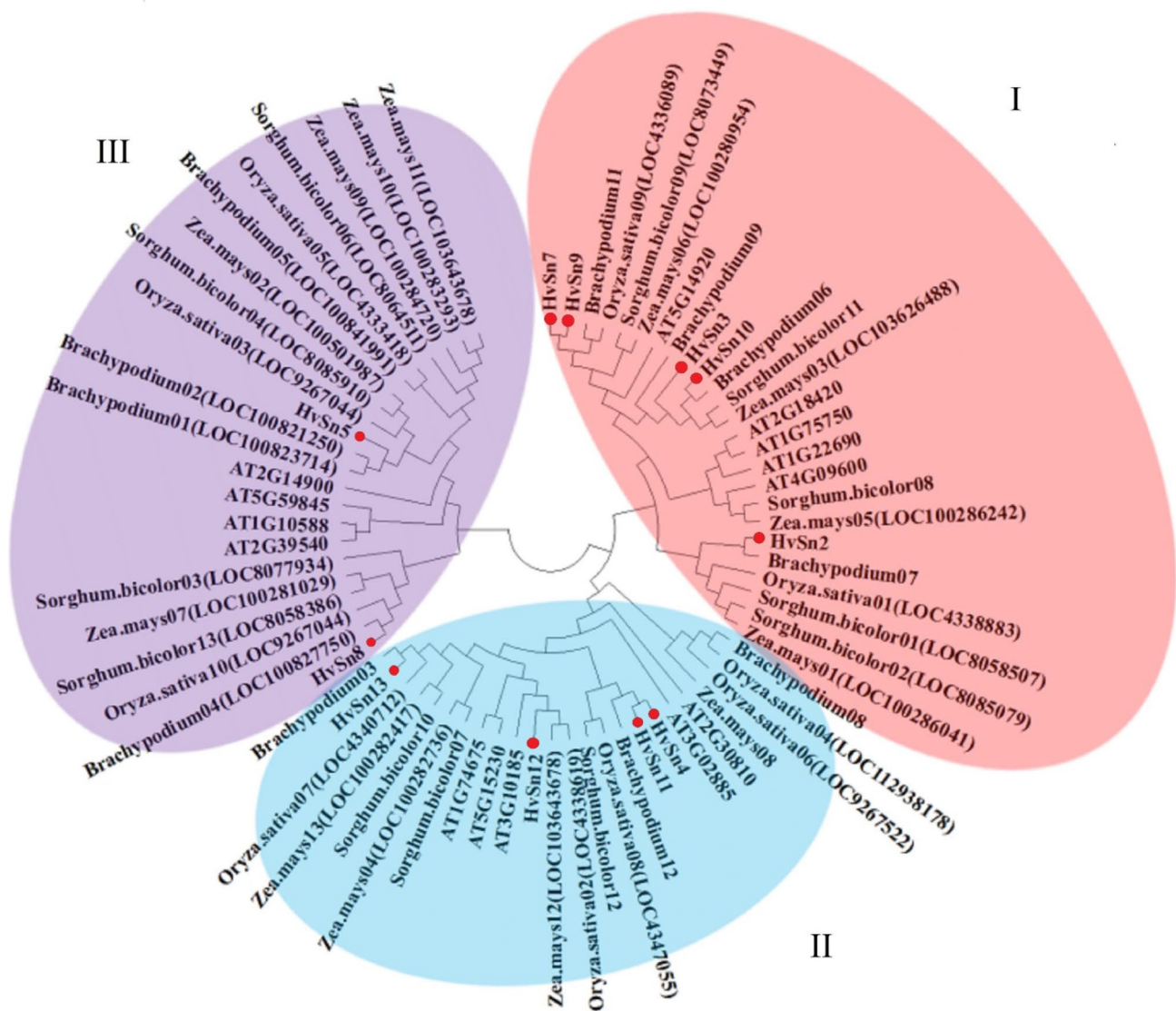
Fig. 7 Phylogenetic analysis of GASA proteins. The phylogenetic tree was generated by Maximum-Likelihood method derived from Clustal Omega alignment of 11, 10, 14, 13, 12 and 11GASA amino acid sequences from *H. vulgare*, *O. sativa*, *A. thaliana*, *Z. mays*, *S. bicolor* and *B. distachyon*, respectively. Different groups are highlighted using different colors

We examined the paralogous relations between GASA genes within barely and the orthologous relations between GASA genes and the other five species (Arabidopsis, brachypodium, rice, Sorghum and maize). Segmental duplications were found in nine GASA genes, divided into three groups: (HvSn3-HvSn9-HvSn10), (HvSn8-HvSn5-HvSn4), and (HvSn11-HvSn12-HvSn13). Except for one (HvSn5HvSn8), these genes have been exposed to high purifying selection pressure after duplication, based on evaluation of synonymous and non-synonymous substitutions (Fig S2), (Table 6).

According to divergence time calculation, the event of duplication of these pairs occurred between 5 and 14 million years ago (MYA). GASA synteny search in other species

Table 6 Synonymous and non-synonymous substitution rates of the duplicated gene pairs in the barley genome

| Gene 1	Gene 2	Ka	Ks	Ka/Ks	Divergence time (MYA)
HvSn3	*HvSn9*	0/073	0/191	0/381543346	14/72981822
HvSn9	*HvSn10*	0/065	0/156	0/417575481	12/00079972
HvSn4	*HvSn5*	0/084	0/095	0/884984274	7/308328245
HvSn5	*HvSn8*	0/093	0/074	1/258155003	5/665870942
HvSn11	*HvSn13*	0/056	0/113	0/496180676	8/673191539
HvSn12	*HvSn13*	0/061	0/079	0/773261433	6/081429161
HvSn11	*HvSn12*	0/081	0/111	0/735954172	8/505171481

indicated high similarity, and orthologous genes were discovered in *H. vulgare*, allowing comparison of species (Fig S3).

In silico expression profile analysis

The data obtained from distinct microarray experiments were used to monitor the expression profiles of the GASA genes in response to abiotic and biotic stresses as well as GA and ABA treatments (Fig. 8). In GSE15970 study, drought stress (10% available water capacity (AWC) for 0, 1, 3, and 5 days, induced HvSn2 (foldchange = − 1.4), HvSn4 (foldchange = 3.45), HvSn5 (foldchange = − 1.5), HvSn9 (foldchange = − 1.8), HvSn10 (foldchange = − 1.72), and HvSn13 (foldchange = − 1.6). In all cases, HvSn4 was up-regulated, and HvSn5 was down-regulated. In GSE3170 experiment, HvSn4 gene was up-regulated (foldchange = 1.36) and HvSn7 (foldchange = − 2.46), HvSn8 (foldchange = − 1.16), and HvSn13 (foldchange = − 1.9) genes were down-regulated in response to drought stress. In GSE6990 study drought stress at 7% soil water content (SWC) increased the expression of HvSn5, HvSn10, and HvSn13 genes. Decreased expression of HvSn4 gene was observed at 19% SWC and 38% SWC. HvSn4 (foldchange = − 2.8) and HvSn7 (foldchange = − 1.4) genes were down-regulated at 68% SWC. In GSE17669 experiment, HvSn8 and HvSn13 (foldchange = − 1.1) were down-regulated, and HvSn4 genes (foldchange = 3.53) were up-regulated in awn tissue. In palea tissue, HvSn2 (foldchange = − 1.16), HvSn10 (foldchange = − 1.2), and HvSn13 (foldchange = − 2.4), was down-regulated and the expression of the HvSn13 gene (foldchange = − 1.15) was decreased in seed tissue. Like other abiotic stress, high-temperature stress affects plant physiology and development. In GSE23896 study 3 h after heat treatment, expression of HvSn4 (foldchange = − 1.2), HvSn7 genes (foldchange = − 1.3) were decreased, while expression of HvSn5 (foldchange = 1.17), HvSn8 (foldchange = 1.66), and HvSn13 genes (foldchange = 2.76) were increased. At 6 h after heat treatment, an up-regulation in HvSn13 gene (foldchange = 1.5) and a down-regulation in HvSn7 gene (foldchange = − 1.13) was observed. The GA is a phytohormone that promotes seed germination in plants. In GSE18758 experiment, the HvSn2 (foldchange = − 3.11), HvSn4 (foldchange = − 5.01), and HvSn13 genes (foldchange = − 6.16) were down-regulated in the GA and GA + ABA treated aleurone. In GSE10328 study, long-term ABA treatment increased the expression of HvSn5 gene (foldchange = 1.1), while, the HvSn13 gene (foldchange = − 1.16) showed down-regulation.

In GSE14521 experiment, only HvSn2 gene (foldchange = 3.47) was induced and up-regulated under boron toxicity. In GSE27821 study, HvSn2 (foldchange = 1.44), HvSn4 (foldchange = − 1.7), and HvSn13 (foldchange = − 2) genes were induced under cold stress. In GSE14930 experiment, the powdery mildew infection for 16 h, 20 h, 24 h, and 32 h, increased the expression of the HvSn5 gene (foldchange = 2.25) and decreased the expression of the HvSn4 gene (foldchange = − 1.9).
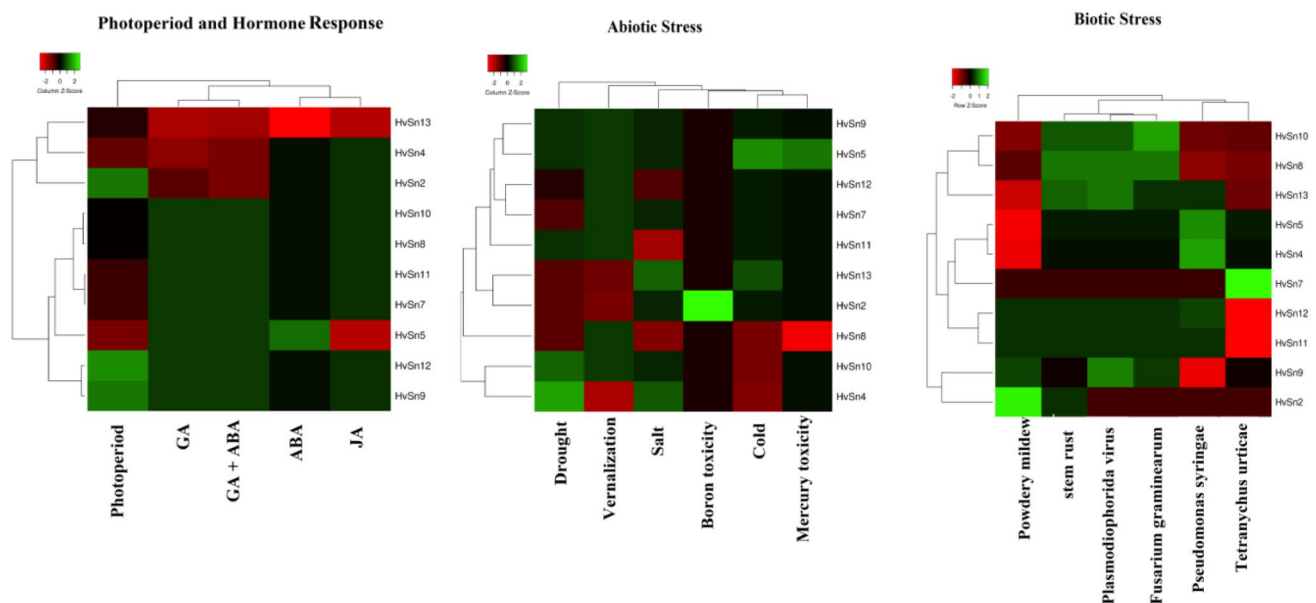
Fig. 8 The expression pattern of the barley GASA genes. **a** In response to photoperiod and hormone treatments. **b** In response to abiotic stresses and **c** in response to biotic conditions. The expression changes in the heatmaps are based on fold change, and green and red colors indicate up-regulation and down-regulation, respectively

In GSE15295 study, the HvSn5 gene (foldchange = 1.5) was up-regulated, whereas the HvSn8 gene (foldchange = 1.68) was down-regulated under mercury treatment (HgCl2). In GSE5605 experiment, HvSn4 (foldchange = 1.1) and HvSn13 genes (foldchange = 1.23) were up-regulated under salinity stress. Also in this experiment, HvSn5 (foldchange = − 1.1) and HvSn13 genes (foldchange = − 1.1) were down-regulated in response to JA treatment. In GSE33398 study, the expression of HvSn4 (foldchange = − 1.7), HvSn5 (foldchange = − 1.4), and HvSn13 (foldchange = − 1.1) genes were decreased under Fusarium infection, whereas HvSn9 (foldchange = 1.15) and HvSn10 (foldchange = 1.14) genes showed up-regulation. In GSE17238 experiment, expression of HvSn9 (foldchange = 2.17) and HvSn13 (foldchange = 1.5) genes was increased in barley roots after host contact with the *Polymyxa graminis*. In GSE43906 study, HvSn4 (foldchange = 1.8) and HvSn5 (foldchange = 1.3) genes were up-regulated in response to *Pseudomonas* syringae infection, while HvSn8 (foldchange = − 1.1), HvSn9 (foldchange = − 1.3), HvSn10 (foldchange = − 1.1), and HvSn13 (foldchange = − 1.3) were down-regulated.

Gene expression analysis with real-time PCR

At the time of reaching FC20%, the HvSn2 gene was up-regulated (foldchange = 2.5) in shoot tissue and down-regulated (foldchange = 0.2) in root tissue. Expression of this gene four days after storage in FC20% significantly increased in both root (foldchange = 2) and shoot (foldchange = 6.5) tissues compared to the control. After ten days of storage in FC20%, a significant decrease in expression of HvSn2 (foldchange = 0.45) gene was observed in both tissues (Fig. 9a). The transcript level of the HvSn3 gene, when reaching FC20%, increased in shoot tissue (foldchange = 2.18) and decreased in root tissue (foldchange = 0.2). Four days after storage in FC20%, this gene down-regulated in shoot tissue (Foldchange = 0.04) and significantly up-regulated in root tissue (foldchange = 5.46) (Fig. 9b). The expression level of the HvSn4 gene in root tissue (Foldchange = 0.15) showed a significant reduction at reaching FC20%. Expression of this gene was significantly increased in root tissue (foldchange = 1.7) four days after storage in FC20% compared to the control. After ten days of storage in FC20%, expression of HvSn4 gene was increased in shoot tissue (foldchange = 4.28) and decreased in root tissue (foldchange = 0.55) (Fig. 9c). At FC20%, the HvSn5 gene was significantly down-regulated in shoot tissue (foldchange = 0.27) and up-regulated in root tissue (foldchange = 1.84) compared to control. Expression of this gene significantly decreased in shoot tissue four (foldchange = 0.12) and ten (foldchange = 0.43) days after storage in FC20% (Fig. 9d). The expression level of the HvSn7 gene at the time of reaching FC20% showed a significant

decrease in root tissue (foldchange = 0.18), while four and ten days after storage in FC20% showed a significant reduction in both shoot and root tissues compared to the control (foldchange = 0.3) (Fig. 9e). At FC20%, the expression level of the HvSn8 gene was elevated in shoot tissue (foldchange = 22.4) and decreased in root tissue (foldchange = 0.51). Expression of this gene was significantly decreased in root tissue four days after storage in FC20% compared to the control (foldchange = 0.5). Ten days after storage in FC20%, HvSn8 was up-regulated in shoot tissue (foldchange = 5.6) and down-regulated in root tissue (foldchange = 0.02) (Fig. 9f). The HvSn9 transcript level after ten days of storage in FC20% showed a significant increase in shoot tissue (Fig. 9g). The expression level of the HvSn10 gene showed a significant increase in root tissue when reaching FC20%. Ten days after storage in FC20%, HvSn10 gene up-regulated in shoot tissue (foldchange = 2) (Fig. 9h). At reaching FC20%, the HvSn11 gene showed a significant down-regulation in root tissue (foldchange = 0.32). After four and ten days of storage in FC20%, this gene significantly down-regulated in both shoot (foldchange = 0.26 and 0.56) and root tissues (foldchange = 0.4) (Fig. 9i). The HvSn12 gene showed a significant up-regulation in both shoot (foldchange = 1.36) and root tissues (foldchange = 112.7) when reaching FC20%. This gene significantly up-regulated in shoot tissue (foldchange = 1.36) after four days of storage in FC20%, while ten days after storage in FC20% showed down-regulation in shoot tissue (foldchange = 0.45) (Fig. 9j). At reaching FC20%, the HvSn13 gene was up-regulated in shoot tissue (foldchange = 3.49) and down-regulated in root tissue (Foldchange = 0.34). After four days of storage in FC20%, the expression level of this gene decreased in shoot tissue (foldchange = 0.2) and significantly increased in root tissue (foldchange = 3.18). Whereas, ten days after storage in FC20% showed decreased expression in both shoot (foldchange = 0.29) and root tissues (foldchange = 0.41) (Fig. 9k).

Tissue-specific expression analysis

The expression pattern of HvGASA genes in various tissues was obtained from publically accessible transcriptome data in BARLEX (Barley Genome Explorer) to provide a preliminary understanding of the functional significance of GASA genes in the barley developmental process. In developing tillers tissue (NOD), the expression of all genes was reported to be extremely low (0—1.07 logFPKM). In embryos tissue (EMB), all genes showed moderate expression (0—2.13 logFPKM), indicating involvement of these genes in germination. In shoots from seedlings tissue (LEA), the expression of HvSn2 (0.11 logFPKM), HvSn3 (0 logFPKM), HvSn11 (0.041 logFPKM), and HvSn12 (1.35 logFPKM) genes was deficient, and the expression of other genes was moderate (1.8—2 logFPKM). In root tissue, the transcription level for
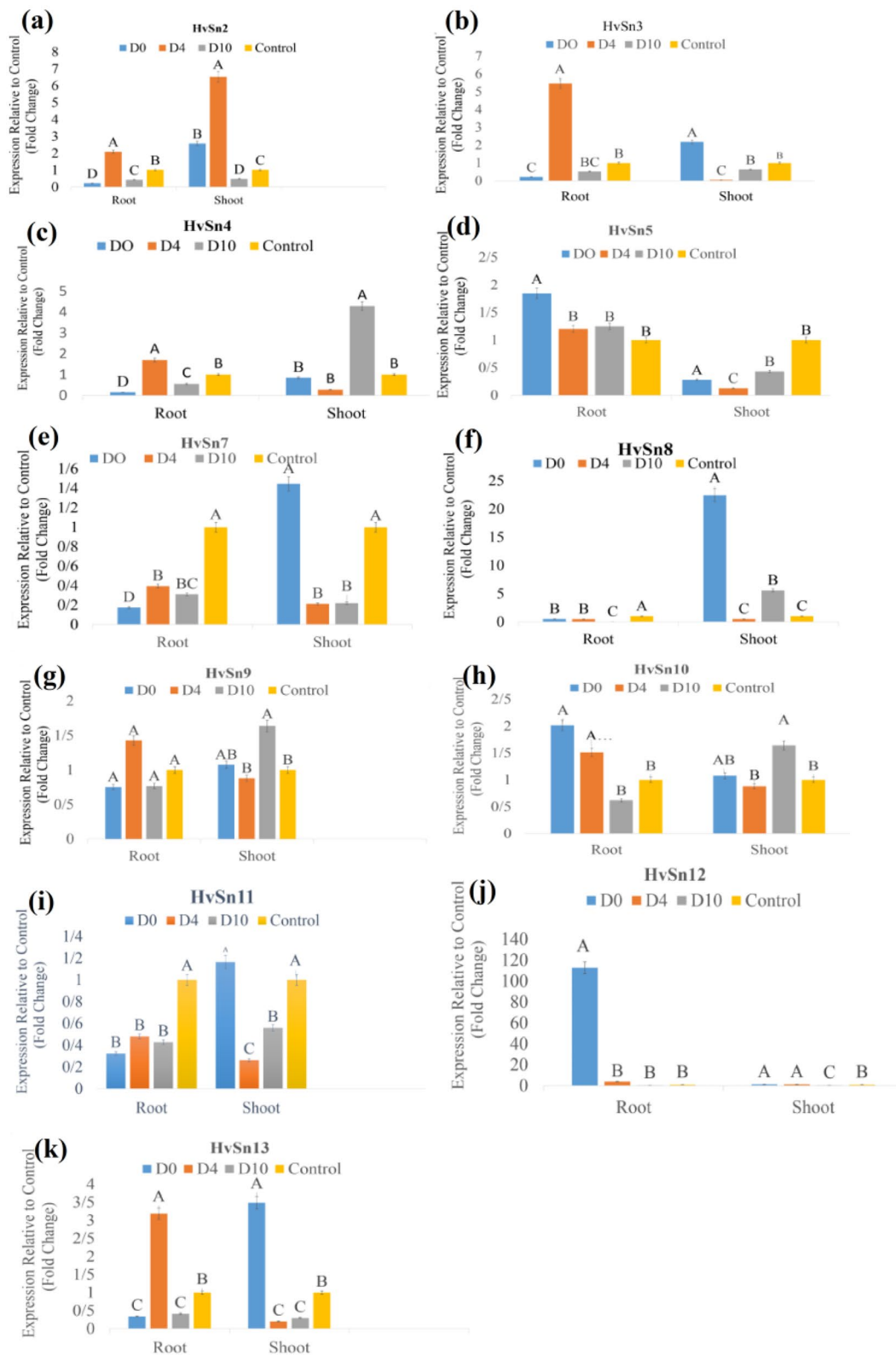
Fig. 9 Real-time PCR analysis of barley GASAs expression under drought stress in the shoot and root tissues and at the time of reaching FC20% (D0), fourth day after reaching FC20% (D4) and tenth day after reaching FC20% (D10). Bars represent standard errors. Letters above the error bars show significant differences. Similar letters show non-significance and non-similar letters indicate the significance ($p < 0.05$)

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HvSn2 (0.75 logFPKM), HvSn3 (0 logFPKM), and HvSn12 (1.33 logFPKM) genes was low, for HvSn11 (2.1 logFPKM) gene was high, and for other genes was intermediate (0.9—1.6 logFPKM). The expression of HvSn2 (0.4 logFPKM), HvSn3 (0 logFPKM), and HvSn12 (1.07 logFPKM) genes was very low, HvSn4 (1.22 logFPKM) and HvSn8 (1.7 logFPKM) was moderate, and other genes had high expression (1.7—2.4 logFPKM) in developing inflorescence tissue (INF) at both sampling times. In developing grain tissue (CAR5), the expression level of HvSn2 (0.14 logFPKM), HvSn3 (0.2 logFPKM), and HvSn7 (1.23 logFPKM) genes was very low, and other genes was very high (1.5—2.27

logFPKM). In CAR15 tissue, HvSn3 (0 logFPKM) and HvSn7 (0.25 logFPKM) genes lacked expression; HvSn5 (1.5 logFPKM) gene expression was increased, and moderate expression of other genes was observed (Fig. 10).

Proline content

Accumulation of proline in plants under drought stress is due to proline synthesis and inactivation of its degradation. Increasing the proline content under stress protects cell membranes, proteins and cytoplasmic enzymes, inhibits reactive oxygen species, and scavenges free radicals.
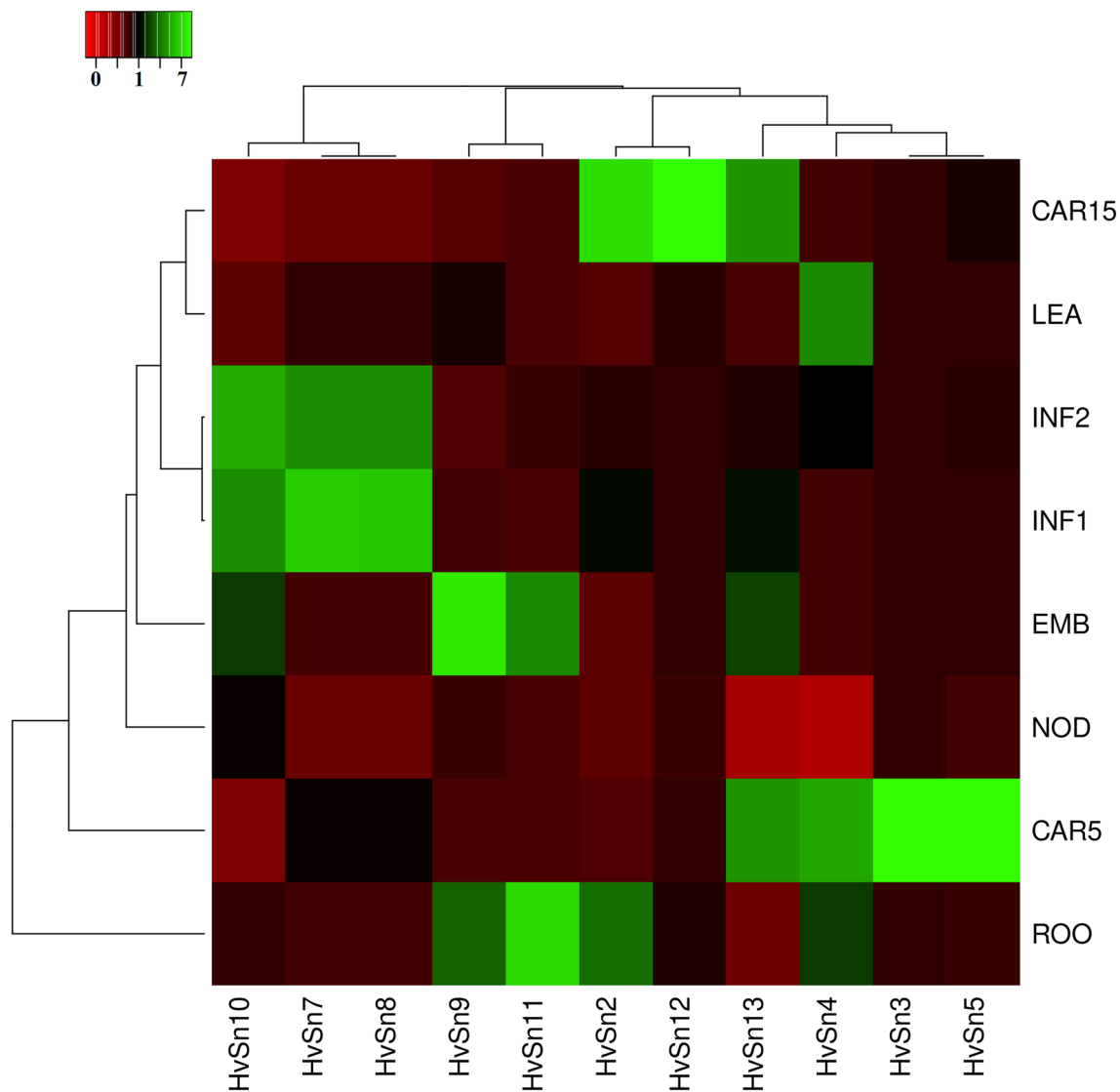
Fig. 10 Tissue-specific gene expression analysis of barley GASAs. EMB, 4-day embryos; ROO, roots from seedlings (10 cm shoot stage); LEA, shoots from seedlings (10 cm shoot stage); INF1: young developing inflorescence (5 mm); INF2, developing inflorescences (1–1.5 cm); NOD, developing tillers, 3rd internode (42 DAP); CAR5, developing grain (5 DAP); CAR15, developing grain (15 DAP). The expression of GASA genes in the heatmap are based on $\log_2$FPKM and are in the range of 0 (red color) to 7 (green color). Color key represents the transcript abundance of the GASA genes in eight barley tissues

Therefore, plant responses to drought stress through increasing proline level and inducing the activity of antioxidant enzymes. Mean comparison results showed a significant increase of proline in shoot tissue after 4 and 10 days of storage in FC20% and in root tissue after 10 days in FC20%. While the amount of proline in both shoot and root tissues did not change significantly when reaching FC20% compared to the control (Fig S4).

Discussion

We discovered 11 GASA genes that were unevenly dispersed across 6 chromosomes. GASA proteins have a low molecular weight and cysteine domains that are conserved. GASA genes are found in lower numbers, have a low molecular weight, alkaline and are unequally distributed on chromosomes within genomes in rice (Muhammad et al. 2019), grapevine (Ahmad et al. 2020), Arabidopsis (Fan et al. 2017), and tomato (Rezaee et al. 2020), according to previous research. However, in other cases, such as in apple (Fan et al. 2017) and soybean (Ahmad et al. 2019), a relatively high number of GASA genes identified. In a study, 545 Snakin/GASA protein-coding genes were identified in 33 plant species (Su et al. 2020). There are differences in the number of genes in GASA family (between 5 and 37) across various plant species. All angiosperm plant species have the multigene family of snakin/GASA peptides, whereas animals, mosses, and green algae do not possess these peptides. On the other hand, various bacteria, including *Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Soehngenia saccharolytica*, *Glycocaulis profundi*, and *Staphylococcus warneri*, possess similar gene sequences. This suggests that the origin of Snakin/GASA may be the result of adaptation of ancient plants to the soil (Silverstein et al. 2007).

The number of introns varied within GASA genes that clustered together in phylogeny. The loss and gain of introns occur in plant protein-coding genes throughout evolution, and it has also been seen in the GASA of other plant species (Ahmad et al. 2019). The peptide length of barley GASAs was determined in the range of 62 to 126 amino acids. Previous studies reported that the length of Snakin/GASA peptides is varied and is in the range of 64 to 120 amino acids (Zhang and Wang 2017). The basic properties, hydrophilicity, and protein stability were predicted for barley GASAs. The stability shows the half-life of proteins in their cellular enzymatic activities (Abdullah et al. 2021). All barley GASAs except HvSn4 and HvSn11 were unstable proteins that show these two proteins may be an important part of various enzymatic functions. There are differences in the physicochemical properties of Snakin/GASA peptides across various plant species.

Subcellular localization prediction also provide information on protein function (Zhang and Wang 2008). The extracellular localization was predicted for all barley GASAs. GASA proteins have previously been seen outside the cells in various plants. The accumulation in the extracellular environment may help GASA proteins to play their role in plants, including antimicrobial activity against pathogens (Ahmad et al. 2019; Rezaee et al. 2020; Zhang et al. 2009).

Post-translational modifications are chemical changes of proteins that result in differences in structure and function, such as subcellular localization and allosteric regulation of enzyme activity (Duan and Walther 2015; Nahirñak et al. 2016). Phosphorylation sites were predicted in all barley GASAs, ranging from 7 to 30. Protein phosphorylation is also crucial for cell signaling, protein regulation and serves as a substrate for different kinases (Silva-Sanchez et al. 2015). Similarly, glycosylation is a common and diverse alteration that is critical for biochemical and physiological processes of an organism (Abdullah et al. 2021). We discovered glycosylation sites on the N-terminal of HvSn7, HvSn9, and HvSn10 genes. These glycosylated GASAs may have an important role in plant processes.

We compared barley GASAs to GASA from five other species (Arabidopsis, rice, maize, sorghum, and bachypodium) in phylogenetic analysis. GASA proteins were divided into three groups by phylogenetic analysis, and barley GASAs were also divided into three groups. The function of GASA was not connected with its phylogenetic grouping, and we found gene with varied function, even within the same phylogenetic group. These data suggest that protein function is linked to numerous mechanisms other than close evolutionary relationships. In *Nicotiana tabacum* L., similar observations have been reported (Nawaz et al., 2019). Some research (Nawaz et al. 2014; Nuruzzaman et al. 2010) have suggested that closely related proteins on a phylogenetic tree had similar functions.

In evolution, domestication, functional regulation, and biotic and abiotic stressors, tandem and segmentally duplicated genes play a crucial role (Ahmad et al. 2020). We discovered segmental duplication in nine genes divided into three categories groups. Segmentally duplicated genes have also reported in previous research. As a result, functional investigations of segmentally duplicated genes may provide accurate information about their functions in various physiological and biochemical processes. Furthermore, our findings, together with recent research (Ahmad et al. 2019), show that genes belonging to the same family are more likely to experience segmental duplication events. As previously observed, the Ka/Ks < 1 suggests that purifying selection pressure exists on the GASA genes following duplication (Ahmad et al. 2020). On barley GASAs, we mainly found purifying selection pressure and replicating genes.cis-acting regulatory elements (CREs) at promoter

region of genes that are recognized by certain transcription factors play an important function in controlling the expression pattern (Ahmadizadeh and Heidari, 2014; Nawaz et al., 2019). In the promoter site of barley GASAs, we discovered numerous critical cis-regulatory elements in response to light, hormones, stresses, growth and development. Drought, anaerobic induction, low-temperature, and plant defense cis-regulatory elements were also found. The presence of several cis-regulatory elements in promoter regions of barley GASAs suggests that these genes play role in various plant processes. The functions of these cis-regulatory components may be better understood with further research.

The expression of GASA genes varies according to the developmental stage. For example, most SlGASA genes are expressed during fruit development in tomatoes (Rezaee et al. 2020), whereas in Arabidopsis, most AtGASA genes are expressed during germination and bolting stages (Rubinovich et al. 2014). Additionally, grapevine (*Vitis vinifera* L.) vegetative parts exhibit higher VvGASA gene expression levels than bloom and fruit tissues (Ahmad et al. 2020). The OsGASA genes are also highly expressed in seedlings, booting, and flower initiation stages of rice (Rezaee et al. 2020). Some studies reported that GASA genes play role in regulation of flower formation in the potato, soybean, and Arabidopsis plants (Ahmad et al. 2019; Berrocal-Lobo et al. 2002). The results of the present study were consistent with previous studies and showed that the identified GASAs have different expression in different tissues and developmental stages of barley.

Although gibberellic acid is the primary inducer of GASA genes, other phytohormones such as auxin, abscisic acid, brassinosteroid, and salicylic acid have also been shown to stimulate the expression of these genes (Berrocal-Lobo et al. 2002; Muhammad et al. 2019; Nahirñak et al. 2016; Qu et al. 2016). In the current study, GASA genes displayed differential expression in response to hormone treatments, suggesting that hormone crosstalk change the expression levels of GASA genes. On the other hand, GASA genes play a critical role in hormone induced responses in plants.

The GASA genes investigated in this study displayed a variety of expression patterns in response to biotic and abiotic stresses. The involvement of GASA genes in response to abiotic and biotic stresses has also been observed in previous research. The GASA gene family, for example, is implicated in the reaction of rubber plant (*Hevea brasiliensis*) to the fungal disease *Colletotrichum gloeosporioides* by inducing plant innate immunity through the generation of reactive oxygen species (An et al. 2018). Over-expression of GASA members may also improve Arabidopsis tolerance to abiotic stimuli such as heat, salt, and oxidative stresses (Alonso-Ramrez et al., 2009). In addition, considerable research have shown that salt (Muhammad et al. 2019) and heat stresses alter the expression of GASA genes (Zhang et al. 2017).

Overall, our findings suggest that GASA genes play various roles in response to biotic, abiotic, and exogenous hormone treatments.

Conclusion

The current study is the first report of the identification and characterization of members of the GASA gene family in barley. In this study for the first time, the GASA gene family was identified and characterized in the Zahak cultivar of barley. The gene structures of 11 HvGASAs and their chromosomal locations and evolutionary relations, as well as their expression pattern were studied. The presence of GASA domain, extracellular signal peptide, six disulfide bridges, and antimicrobial activity were observed for all GASA genes. Different expression pattern in different tissues and induction under both biotic and abiotic stresses were other characteristic of these genes. As previously mentioned, GASA genes have been hypothesized to be sensitive to various stresses, but our findings demonstrate that GASA genes are more induced by abiotic stresses than by biotic stresses. GASA genes have been involved in various plant growth and development processes through the hormone-related pattern, suggesting these genes engage in regulatory processes and hormonal cross-talks. Overall, the information found in this study can expand our information about the potential role of GASA genes in plant biological and physiological processes and can help future researchers to better understand the role of these genes.

Declarations

Ethical standards The authors declare that they have no conflict of interest or personal relationships that could have appeared to influence the work reported in this paper. The present study does not involve human participants and/or animals.

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