

Article

Genome-Wide Identification, Expression Pattern and Sequence Variation Analysis of *SnRK* Family Genes in Barley

Jiangyan Xiong¹, Danyi Chen¹, Tingting Su¹, Qiufang Shen¹, Dezhi Wu^{2,*} and Guoping Zhang^{1,3,*}

¹ Department of Agronomy, Key Laboratory of Crop Germplasm Resource of Zhejiang Province, Zhejiang University, Hangzhou 310058, China; 11916033@zju.edu.cn (J.X.); 22016182@zju.edu.cn (D.C.); sutingting@zju.edu.cn (T.S.); shengqf@zju.edu.cn (Q.S.)

² College of Agronomy, Hunan Agricultural University, Changsha 410128, China

³ Linyi Institute of Agricultural Sciences, Zhejiang University, Linyi 276000, China

* Correspondence: wudezhi230@163.com (D.W.); zhanggp@zju.edu.cn (G.Z.)

Abstract: Sucrose non-fermenting 1 (SNF1)-related protein kinase (SnRK) is a large family of protein kinases that play a significant role in plant stress responses. Although intensive studies have been conducted on *SnRK* members in some crops, little is known about the *SnRK* in barley. Using phylogenetic and conserved motif analyses, we discovered 46 SnRK members scattered across barley's 7 chromosomes and classified them into 3 sub-families. The gene structures of *HvSnRKs* showed the divergence among three subfamilies. Gene duplication and synteny analyses on the genomes of barley and rice revealed the evolutionary features of *HvSnRKs*. The promoter regions of *HvSnRK* family genes contained many ABRE, MBS and LTR elements responding to abiotic stresses, and their expression patterns varied with different plant tissues and abiotic stresses. *HvSnRKs* could interact with the components of ABA signaling pathway to respond to abiotic stress. Moreover, the haplotypes of *HvSnRK2.5* closely associated with drought tolerance were detected in a barley core collection. The current results could be helpful for further exploration of the *HvSnRK* genes responding to abiotic stress tolerance in barley.

Keywords: *SnRK*; barley; gene structure; expression pattern; SNP distribution



Citation: Xiong, J.; Chen, D.; Su, T.; Shen, Q.; Wu, D.; Zhang, G. Genome-Wide Identification, Expression Pattern and Sequence Variation Analysis of *SnRK* Family Genes in Barley. *Plants* **2022**, *11*, 975. <https://doi.org/10.3390/plants11070975>

Academic Editor: Alexandra S. Dubrovina

Received: 8 March 2022

Accepted: 1 April 2022

Published: 3 April 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Under natural conditions, plants are constantly exposed to a variety of abiotic stresses, such as drought, salinity and extreme temperatures, resulting in the inhibition of growth and development. In responding to these abiotic stresses, plants have evolved a complex genetically regulated system of tolerance or adaptation [1]. For example, barley (*Hordeum vulgare*) could rapidly adapt its enzyme system to scavenge reactive oxygen species (ROS) when exposed to salt stress [2,3]. However, the genetic mechanisms of the tolerance to abiotic stresses in plants are still not fully understood.

Protein kinases and phosphatase-mediated signal transduction are important regulators involved in the genetic networks responding to environmental changes or abiotic stresses [4]. Sucrose non-fermenting 1 (SNF1)-related protein kinase (SnRK) is a protein kinase family involved biochemical response to stresses in plants [5]. All SnRK proteins share a conserved serine/threonine protein kinase domain in the N-terminal, while they are variable in the C-terminal. The SnRK proteins could be grouped into three subfamilies according to the structures of the C-terminal [6]. The SnRK1 subfamily consists of three domains, including a kinase domain in the N-terminal, an ubiquitin-associated (UBA) domain and a kinase-associated 1 (KA1) domain in the C-terminal, which is the homologues of SNF1 in yeasts and AMP-activated protein kinases (AMPK) in animals [7]. The SnRK2 subfamily harbors the regulatory C-terminal domain containing acidic amino acids, either Glu or Asp [8]. The SnRK3 subfamily contains two conserved domains in the C-terminal,

including NAF (named with conserved amino acids N, A and F) and PPI (Protein–protein interacting) domains [9].

It is well documented that the SnRK1 protein kinase is a core regulator in plants to sense energy deficiency [10]. SnRK1 can phosphorylate and inhibit four important enzymes in plant metabolisms, including HMG-CoA reductase (HMGR), sucrose phosphate synthase (SPS), nitrate reductase (NR) and seaweed phosphate synthase 5 (TPS5). SnRK1 regulates the metabolism and development of plants through the above four enzymes [11–13]. SnRK2 plays a key role in responding to ABA and salt stress. In *Arabidopsis* and rice, all member genes of the *SnRK2s* (*AtSnRK2.1-2.10* and *OsSAPK1-10*) could be activated by ABA and salt stresses [14,15]. For instance, *AtSnRK2.6*, also designated as *Open stomata 1*, increased the expression level by ABA in guard cells [16]. *AtSnRK2.4* and *AtSnRK2.10* respond to salt stress by regulating the expression levels of several genes for ROS generation and removal [17]. In rice, *OsSAPK8* is a key positive regulator when exposed to salt, drought and chilling stresses [18]. SnRK3, named as CIPKs (CBL-interacting protein kinases), regulates the transports of Na^+ , K^+ and NO_3^- by combining with Ca^{2+} -dependent CBL to regulate downstream genes and enhance abiotic stress tolerance in plants [19–21]. For example, SOS2 (CIPK24) protein can interact with SOS3 (CBL4) to regulate the activity of plasma membrane H^+/Na^+ antiporter (SOS1) in a Ca^{2+} -dependent way in *Arabidopsis* [22,23]. SnRK3.17/SnRK3.12/SnRK3.23 proteins interact with CBL2/3 at the vacuole membrane, co-chelating Mg^{2+} in the vacuole [24]. In addition, CIPK18 and CBL3 are essential for the functions of NHX5 and NHX6 in maintaining Li^+ homeostasis [25]. Apart from salt stress, a great number of CIPKs were also induced by mild freezing shock treatment [26]. In short, because the *SnRK* gene family is so vast and plays such an important role in responding to abiotic stresses, it is imperative to identify *SnRK* genes and reveal their roles.

Gene families are widespread in plants, and there are the structural and functional similarities among them. In addition, the duplicated family gene pairs among the different genomes can be used to identify the conserved functional genes and determine species relationships, which are beneficial for understanding the mechanisms of gene family evolution and functional differentiation [27]. Recently, many reference genomes of plants are sequenced and assembled, and a variety of *SnRK* family genes have been identified through bioinformatics analysis. However, similar studies are rarely performed on barley. Accordingly, the current study was carried out to identify *HvSnRK* genes and reveal their evolution mechanism, so as to understand the expression patterns of the genes in responding to abiotic stresses in barley.

2. Results

2.1. Identification and Phylogenetic Analysis of SnRKs in Barley

Based on the query protein sequences from *Arabidopsis* and rice (Table S1), totally 46 proteins were identified as the SnRK members in barley (Table S2). The 46 *HvSnRK* proteins consist of 341 to 797 amino acids, with a molecular weight of 38.5 to 89.9 kDa.

To verify the evolutionary relationship of SnRK proteins among diverse plant species, we constructed a phylogenetic tree utilizing 48, 39 and 46 SnRKs from rice, *Arabidopsis* and barley, respectively (Figure 1). The 133 SnRK proteins were clustered into 3 groups, as found previously in *Arabidopsis* [6]. In barley, 4 proteins with Pkinase (PF00069), UBA (PF00627) and KA1 (PF02149) domains are clustered into the *HvSnRK1* subfamily; 11 proteins belonged to the *HvSnRK2* subfamily with high similarity to the *AtSnRK2* and *OsSnRK2* subfamilies; and another 31 proteins with Pkinase and NAF (PF03822) domains were grouped into the *HvSnRK3* subfamily (Figure 2A). Subcellular localization prediction indicated that *HvSnRK1s* were mainly located in cytoplasm, *HvSnRK2s* were mainly located in nuclear and *HvSnRK3s* were mainly located in the plasma membrane (Table S2), suggesting that the three subfamilies of *HvSnRKs* may have the different biological functions.

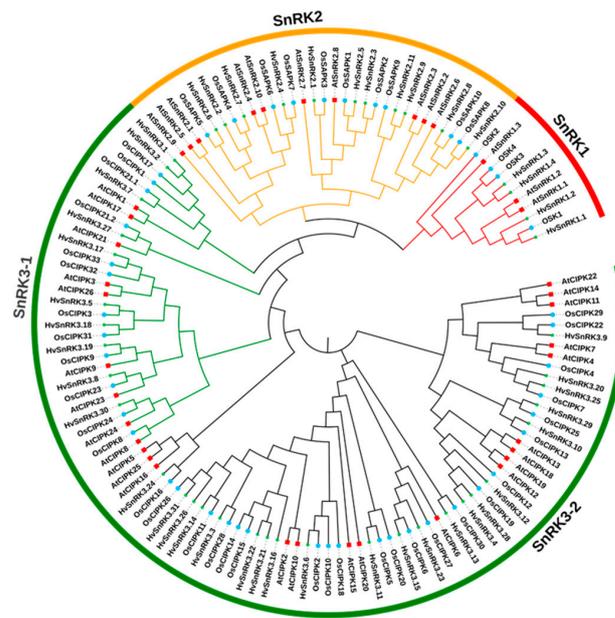


Figure 1. Phylogenetic analysis of SnRK proteins in *O. sativa*, *A. thaliana* and *H. vulgare*. The varied colored arcs represent SnRK protein subfamilies. The 133 SnRK proteins were used to create an unrooted Neighbor-Joining (NJ) phylogenetic tree using MEGA X. The SnRK family proteins from *O. sativa*, *A. thaliana* and *H. vulgare* are represented by the circle, square and star, respectively.

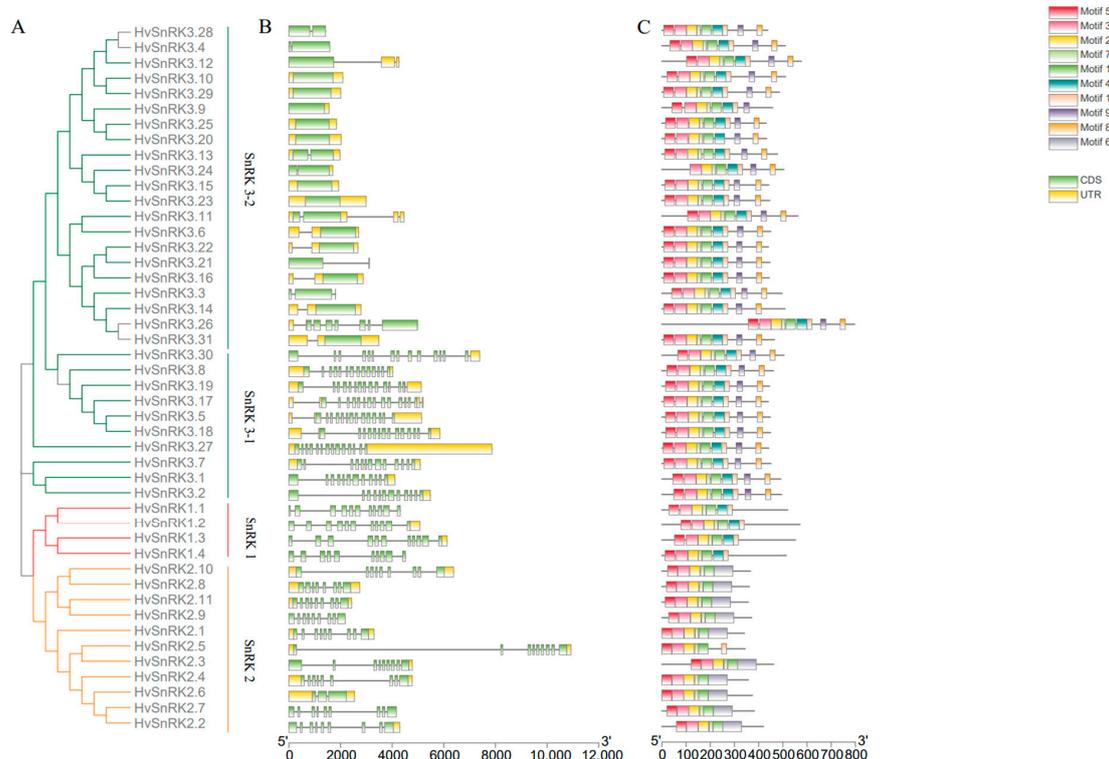


Figure 2. Phylogenetic relationships, gene structures and conserved motifs of the SnRK proteins in *H. vulgare*: (A) Phylogenetic tree of 46 HvSnRK proteins; (B) Gene structures of *HvSnRK* genes. Green boxes: exons. Black lines: introns. Yellow boxes: UTR areas; (C) The motif analysis of *HvSnRK* proteins. The details of the motifs are provided in Table S4.

2.2. Gene Structures, Protein Motifs and 3D Structures of *HvSnRKs*

The gene structures of *HvSnRK* in the same subfamily are similar. Four genes of the *SnRK1* subfamily contain eleven exons. Meanwhile, 10 out of 11 members in the *HvSnRK2* subfamily contain 8 or 9 exons, and only *HvSnRK2.6* contains 3 exons. The *HvSnRK3* subfamily genes have a large variation in exon amount, ranging from 1 to 16 (Figure 2B). In addition, *HvSnRK3s* can be divided to two subgroups, while one subgroup member has at least eight exons and the other group members have no more than four exons, except *HvSnRK3.26*, which contains eight exons (Figure 2B, Table S3).

The protein sequences of 46 *HvSnRKs* were analyzed, and 10 conserved motifs were obtained (Figure 2C). The motifs 1/2/3/5/6 are related to the conserved domains of phosphokinase, while motif 9 is related to the NAF domain (Table S4). The conserved motif analysis reveals that identical conserved motifs are found in the same subfamily. For example, all members in the *HvSnRK1s* contain the motifs 1/2/3/4/5/7/10, and the motifs 1/2/3/4/5/6/7 exist in the *HvSnRK2* subfamily, while the motifs 1/2/3/4/5/7/8/9/10 exist in the *HvSnRK3* subfamily. These results indicate that the same subfamily of *HvSnRK* has high similarity in the gene structure and amino acid sequence. It can be seen from 3D structures of *HvSnRK* proteins that three subfamily proteins have the similar 3D structures in the N terminal but differ in the C terminal (Figure 3).

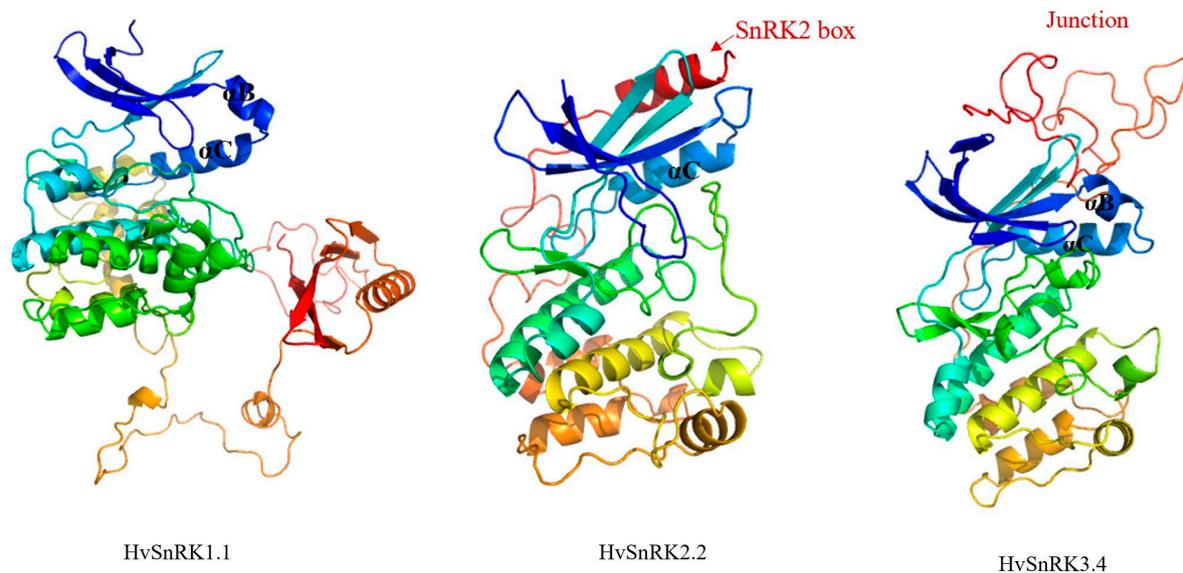


Figure 3. The 3D structure modeling of *HvSnRK* proteins. The pymol software was used to create the structural image.

2.3. Chromosomal Location and Gene Duplication of *HvSnRKs*

In order to reveal the evolution of the barley *SnRK* genes, the chromosome location of the *SnRK* genes was determined (Figure 4). *HvSnRK1* subfamily genes were distributed in chromosomes 1, 3 and 4; *HvSnRK2* subfamily genes are found on chromosomes 1, 2, 3, and 4; and *HvSnRK3* subfamily genes distributed over all chromosomes. Interestingly, *HvSnRK3.12* and *HvSnRK3.13* were clustered within 10 kb in chromosome 3.

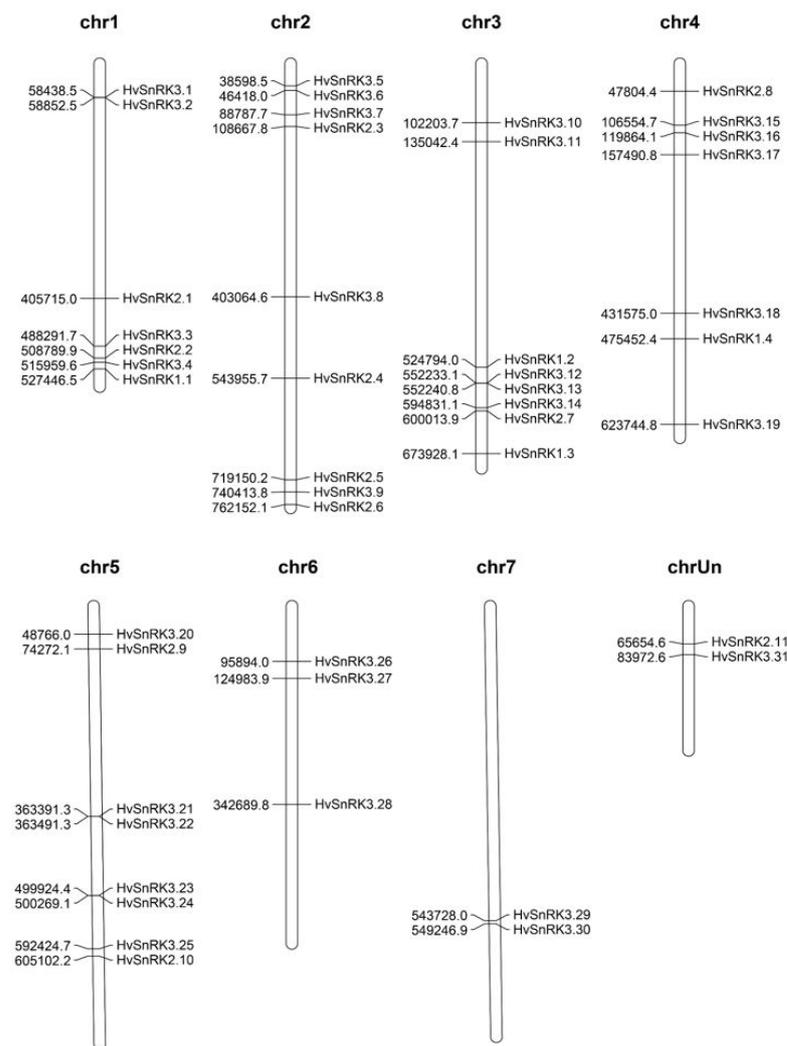


Figure 4. Distribution of *SnRK* genes on the *Hordeum vulgare* genome. The left numbers represent physical location on chromosomes of *HvSnRKs*.

In the colinear segment of the barley genome, six *HvSnRKs* gene pairs were discovered, with the two genes of each pair being located on the distinct chromosomes. Moreover, the collinearity of the *SnRK* family genes between the genomes of barley and rice was also analyzed, with 33 pairs of *SnRK* genes being detected (Figure 5B and Table S6).

2.4. Stress-Related Cis-Elements in the Promoters of *HvSnRK* Genes

For determining the expression pattern of *HvSnRK* genes, 2 kb promoter sequences of all *HvSnRK* genes were extracted from the barley genome database to analyze the *cis*-acting elements. We analyzed the ABA-signal-related components ABRE (ACGTGG/TC), drought-response components MBS (CAACTG) and low-temperature-related components LTR (CCGAAA) (Figure 6, Table S2). As a result, we found 43 of all 46 *HvSnRKs*, except *HvSnRK3.12*, *HvSnRK3.13* and *HvSnRK3.28*, contained the *cis*-elements responding to the 3 abiotic stresses. Among the 43 *HvSnRKs*, 42 genes had ABRE, 19 genes had MBS and 26 genes had LTR. It is indicated that many ABRE, MBS and LTR elements were identified in the promoter of *HvSnRK* family genes, which may respond to abiotic stresses by modulating gene expression.

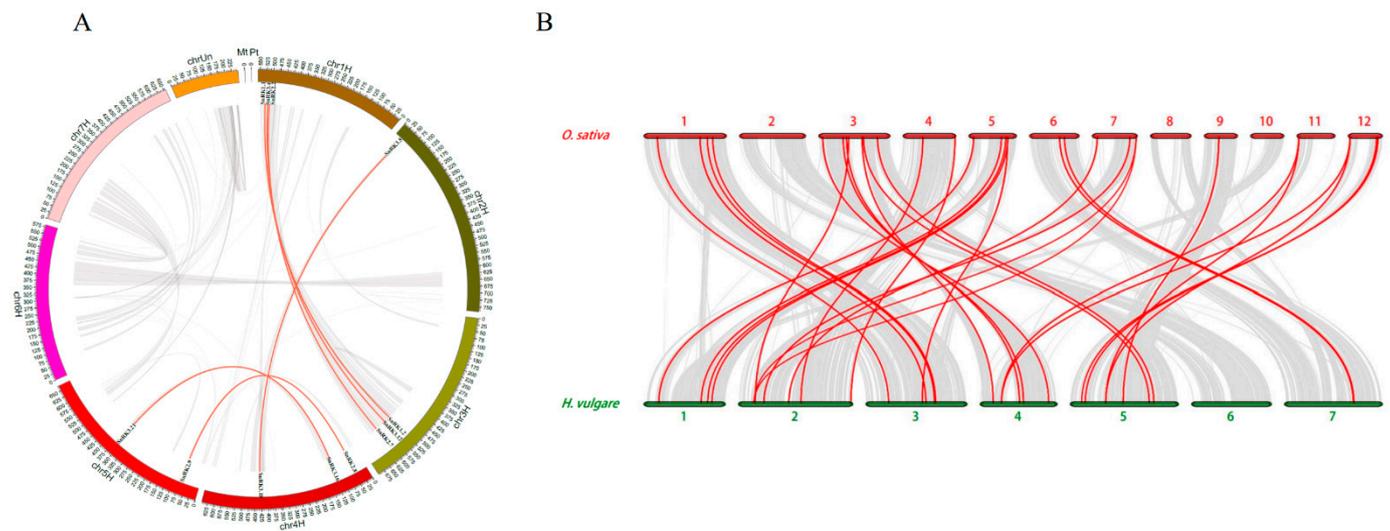


Figure 5. The synteny analysis of *HvSnRK* genes. **(A)** The synteny analysis of *HvSnRK* genes in barley genome. Gray lines: all syntenic blocks in the *H. vulgare* genome. Red lines: duplicated *HvSnRK* gene pairs. **(B)** Synteny analysis of *SnRK* genes between the genomes of *O. sativa* and *H. vulgare*. Gray lines: all collinear blocks within *O. sativa* and *H. vulgare*. Red lines: the synteny of *SnRK* genes between *O. sativa* and *H. vulgare*.

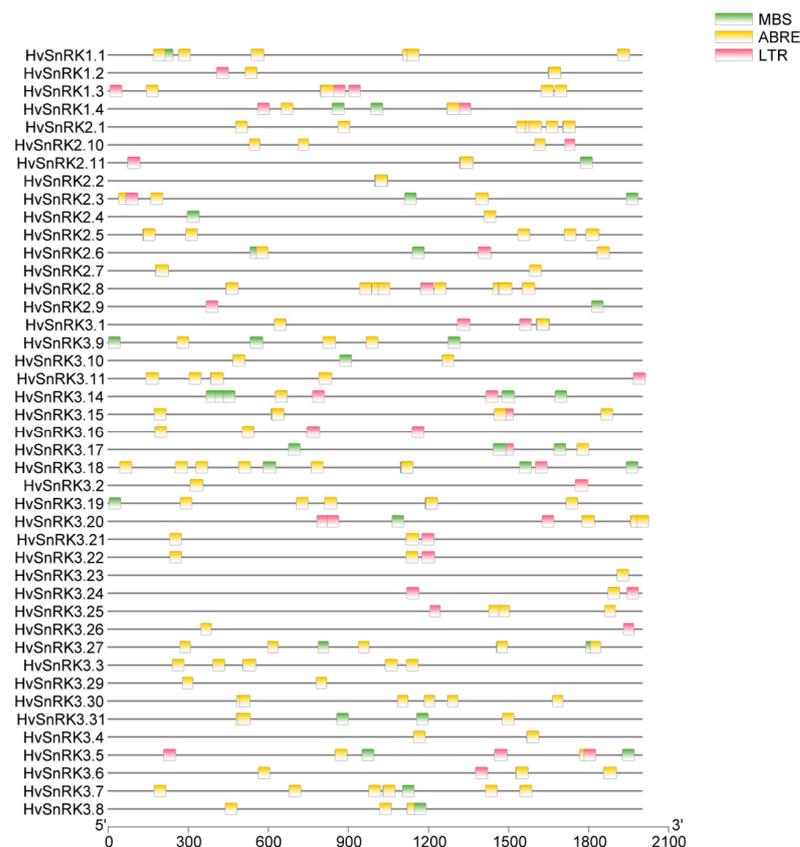


Figure 6. Cis-regulatory elements analysis of *HvSnRK* genes. The green-, yellow- and red-colored boxes indicate MBS, ABRE and LTR *cis*-acting elements, respectively.

2.5. Expression Profiles of *HvSnRKs* in Different Tissues and under Different Abiotic Stresses

All *HvSnRKs* were expressed at various levels in 15 tissues of the barley cultivar Morex, but there was no expression in the developing young inflorescences (Figure 7A and

Table S7). According to the expression levels in different tissues, *HvSnRKs* can be divided into three groups. Group-1 consists of 18 genes with high expression level, including 3 *HvSnRK1s*, 6 *HvSnRK2s* and 8 *HvSnRK3s*. Group-2 consists of 17 genes with moderate expression level, including 2 *HvSnRK1s*, 3 *HvSnRK2s* and 12 *HvSnRK3s*. Group-3 had 13 genes with the low expression level, including 1 *HvSnRK1s*, 2 *HvSnRK2s* and 10 *HvSnRK3s*. These results indicate the dramatic difference in the expression level among *HvSnRK* genes.

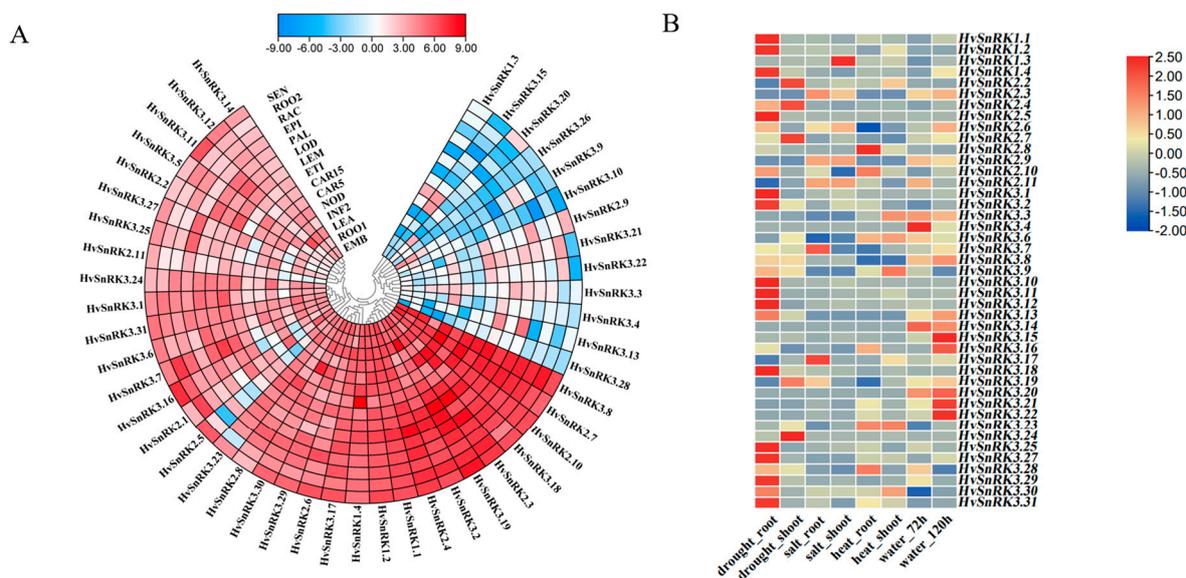


Figure 7. Expression profiles of *HvSnRK* genes. (A) Transcriptional profiles of the *HvSnRK* genes in barley different tissues. (B) Transcriptional profiles of the *HvSnRK* genes under abiotic stresses. The color scale represents expression data with row scale. Blue: Low expression; Red: High expression.

In order to understand the expression profiles of the barley *SnRK* family genes in response to abiotic stresses, we integrated the transcriptome data of barley *SnRK* genes responding to abiotic stresses including drought [28], high salt [29], high temperature [30] and waterlogging [31]. On the whole, most *HvSnRK* genes showed a significant change in expression levels under various abiotic stresses, and a few of them showed little change (Figure 7B, Table S8). For example, *HvSnRK2.5* showed increased expression under drought and salt stresses, while *HvSnRK2.10* had little change under the two stresses. From the expression profiles of *HvSnRK* genes under abiotic stresses, it can be seen that the different *HvSnRK* subfamilies are not consistent in their functions.

2.6. Functions and Regulatory Networks of *HvSnRKs*

The protein–protein interaction (PPI) was predicted to further understand the biological roles and regulatory networks of *HvSnRKs*. A total of 20 functional proteins that interact with *HvSnRK* proteins were discovered (Figure 8). Most of the proteins that interacted with *HvSnRKs* were functionally confirmed ABA signaling components, such as PP2C and PYR/PYL (Figure 8). It was also discovered that the majority of *SnRK3* proteins interacted with Calcineurin B-like proteins (CBLs). These *SnRKs* interaction proteins can be grouped into four categories according to their function annotation, including phosphatase 2C family proteins (ABI5, PP2C6, PP2C9, PP2C30 and PP2C50), ion channel proteins (CBL1, CBL4, CBL6 CBL8 and AKT1), PYR/PYL proteins (PYR1, PYL1, PYL2, PYL4, PYL5, PYL8 and PYL9) and stress- or development-related transcription factors (bZIP27, VP1 and TRAB1).

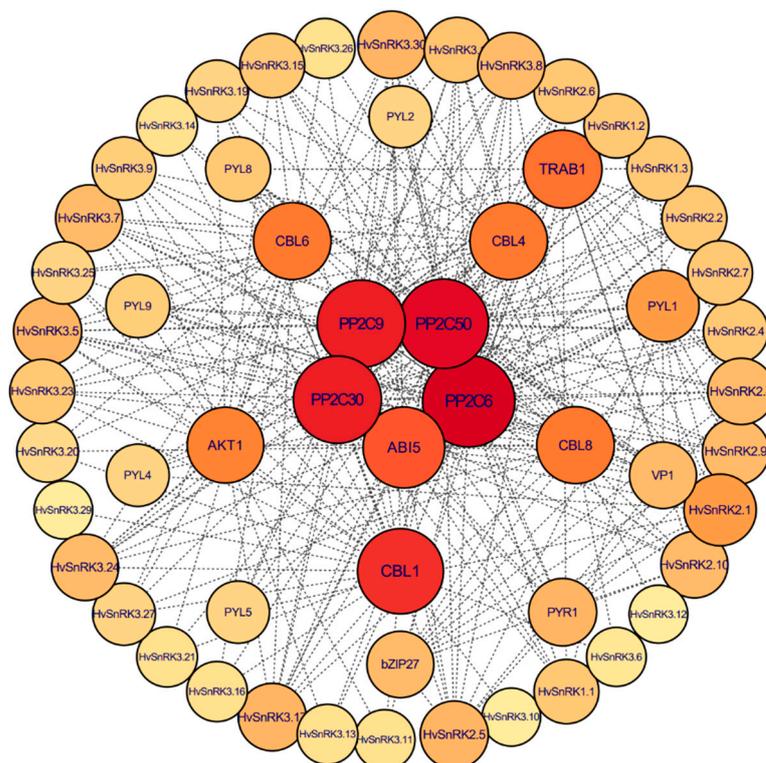


Figure 8. Predicted protein–protein interaction networks of HvSnRK proteins. The external circle represents barley SnRK proteins and the inside circles represent proteins that interact with HvSnRKs. Different layers represent phosphatase 2C family proteins, ion channel protein and PYR/PYL proteins or other transcription factor, respectively. The interaction between these proteins was represented by the gray lines which connected the four circles.

2.7. Sequence Variation of HvSnRKs in a Barley Core Collection

The SNPs of *HvSnRKs* from 100 barley core accessions were detected. On average, a *SnRK* gene contained 10 SNPs (Table S9). However, the SNP density of various subfamilies differed greatly, with the averages of the 3 subfamilies being 7.0, 14.5 and 9.2 SNPs/gene, respectively. Moreover, the SNP density of each *HvSnRK* gene within the same subfamily also showed the obvious difference. Thus, no SNP was detected for *HvSnRK1.4*, while there were 15 SNPs for both *HvSnRK2.5* and *HvSnRK3.24*. Here, the detailed SNP distribution of *HvSnRK2.5* and *HvSnRK3.24* was shown in Figure 9. For *HvSnRK2.5*, 2, 10 and 3 SNPs were detected in the promoter, exon/intron region and 3'UTR regions, respectively (Figure 9A). For *HvSnRK3.24*, 3, 10 and 2 SNPs were detected in the promoter, exon/intron and 3'UTR regions, respectively (Figure 9B).

We analyzed the haplotypes of *HvSnRK2.5* in 100 barley core accessions and found that the expression of *HvSnRK2.5* was highly induced in the leaves under drought treatment (Figure 8). Five haplotypes were identified on the basis of the SNPs in the promoter and exon regions (Table S10). It was found that the barley accessions with *HvSnRK2.5*^{Hap3} kept a higher leaf water content, while the accessions with *HvSnRK2.5*^{Hap4} are quite sensitive to drought stress (Figure 9c). Therefore, it may be suggested that the sequence variation of *SnRKs* affects their expression and response to abiotic stresses.

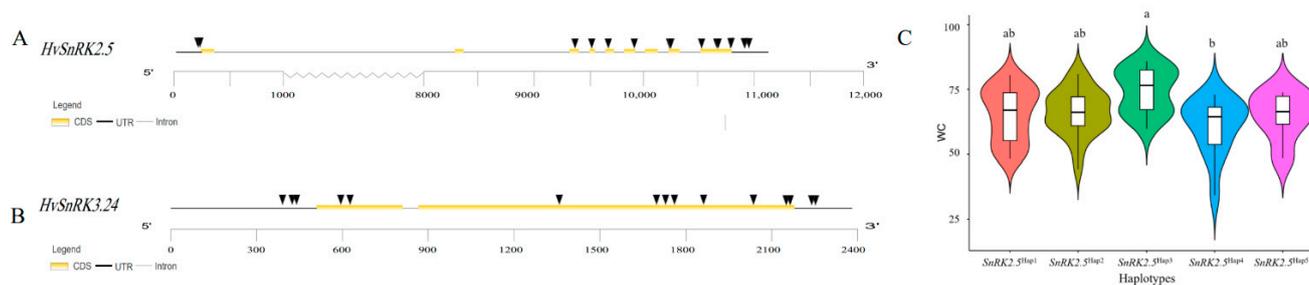


Figure 9. SNPs analysis of *HvSnRK2.5* and *HvSnRK3.24* in 100 accessions of barley core collections. (A) Gene structures and SNPs location of *HvSnRK2.5*. (B) Gene structures and SNPs location of *HvSnRK3.24*. Black triangles indicate the SNP distribution. (C) Water content of different haplotypes of *HvSnRK2.5* in 100 barley accessions under drought treatment. Capital letters represent significant differences according to Tukey's honest significant difference (HSD) test ($p < 0.05$).

3. Discussion

In this research, 46 *HvSnRK* genes classified into three subfamilies were discovered in the barley genome. The comprehensive studies were performed on the *HvSnRK* family genes, including phylogenetic analysis, gene structures, protein 3D structures, gene distribution on genome, gene duplication and *cis*-elements identification in the promoters. Moreover, utilizing publicly available data, the analysis of *HvSnRKs* expression patterns and SNPs distribution was performed. In our understanding, the current study should be helpful for better understanding the functions of *HvSnRK* genes responding to abiotic stresses.

Previously, 39 *AtSnRK* genes were identified in *Arabidopsis*, including 3 *AtSnRK1s*, 10 *AtSnRK2s* and 26 *AtSnRK3s* [6,32–34]; 48 *OsSnRK* genes in *Oryza sativa*, including 4 *OsSnRK1s*, 10 *OsSnRK2s* and 33 *OsSnRK3s* [15]; 34 *EgSnRK* genes in *Eucalyptus grandis*, including 2 *EgSnRK1s*, 8 *EgSnRK2s* and 24 *EgSnRK3s* [35]; 44 *BdSnRK* genes in *Brachypodium distachyon*, including 3 *BdSnRK1s*, 10 *BdSnRK2s* and 31 *BdSnRK3s* [36]; and 114 *BnSnRK* genes in *Brassica napus*, including 10 *BnSnRK1s*, 31 *BnSnRK2s* and 73 *BnSnRK3s* [37], respectively. In the barley genome, 46 *HvSnRK* genes classified into 3 subfamilies were discovered, including 4 *HvSnRK1s*, 11 *HvSnRK2s* and 31 *HvSnRK3s*. Furthermore, the phylogenetic tree of the *AtSnRK*, *OsSnRK* and *HvSnRK* proteins showed that the *HvSnRKs* were closer to the *OsSnRKs* than *AtSnRKs*, as expected.

In *AtSnRK*, *OsSnRK* and *HvSnRK* gene families, various subfamily genes showed the large difference in length and exon-intron structure. In barley, all genes of *HvSnRK1* subfamily contain 11 exons, while 3 of 4 *OsSnRK1* genes have 11 exons. All *HvSnRK2* subfamily genes contain eight or nine exons except for *HvSnRK2.6*, which contains three exons due to intron deletion during the evolution process, being consistent with *SnRK2s* in *Arabidopsis*, rice, maize and sorghum [38,39]. The *SnRK3* subfamily can be divided into 2 subgroups, *SnRK3-1*, with more than 10 exons, and *SnRK3-2*, with fewer than 4 exons. The similarity of exon number in the same subfamily may be attributed to their close evolutionary relationship.

The *cis*-elements in the promoter can regulate gene expression during plant development and the process in response to environmental changes [40,41]. In this study, the promoter sequence analysis showed that various types of *cis*-elements were contained in *HvSnRKs*, such as MBS, ABRE and LTR. The mutations in the MBS element of *ZmSOP2* gene cause the attenuated expression under ABA and dehydration condition in *Zea Mays* [42]. The ABRE element of the *HAHB4* gene was identified to be responsible for ABA, NaCl and drought regulation when the site-directed mutagenesis on the promoter of *HAHB4* was performed on *Arabidopsis* and sunflower [43]. Low temperature response element LTR played a key role in the regulation of *BLT4.9* gene under low temperature stress in barley [44]. We found that most *HvSnRK* genes have at least one of the above *cis*-elements in the promoters.

In addition, 3D structure modeling was constructed to characterize the potential functions of different HvSnRK subfamilies. A noteworthy characteristic of HvSnRK2 is the well-ordered SnRK2 box, which forms a single α -helix and is packed parallel against the α C-helix in the N-terminal lobe. The SnRK2 box– α C interaction has been reported to be crucial for kinase activity [45]. SnRK3 has a junction region in the C terminal, which packs against α B and α C helices, and the central β -sheet was reported to connect the catalytic domain and the regulatory domain [46]. These modeled 3D structures of HvSnRK proteins lay the foundation for us to understand their biological functions.

Gene expression profiles may provide valuable clues for characterizing gene functions. Here, we analyzed the expression levels of *HvSnRKs* in different barley tissues using published transcriptome data. The findings revealed that the expression patterns of 46 genes could be classified into three groups. (Table S7). Interestingly, we found that the *HvSnRKs* in group-3 contained fewer *cis*-elements in their promoters than the other two groups. On average, groups-1, -2 and -3 contain 3.94, 4.29 and 2.69 ABRE, respectively. This indicates that *HvSnRKs* activities may be correlated with the disparities of *cis*-elements in the promoter regions.

In this study, expression patterns of *HvSnRKs* in response to drought, salinity and temperature stresses were also investigated. *HvSnRK3.12* is an orthologous gene of *OsCIPK12* (Figure 5B, Table S6), which regulates the synthesis of proline and soluble sugar in rice exposed to drought stress [47], indicating the identical functions in response to drought stress. However, *HvSnRK2.5*, which was also significantly induced under drought stress, does not have the orthologous gene in rice. According to the predicted PPI networks (Figure 8), *HvSnRK2.5* may respond to drought stress through regulating ABA signaling. Moreover, we determined the SNPs in each *HvSnRK* gene using the re-sequenced data of a barley core collection. As a result, both potential drought-tolerant and sensitive haplotypes were identified in *HvSnRK2.5*. For example, barley accessions with *HvSnRK2.5*^{Hap4} are tolerant to drought stress, while those accessions with *HvSnRK2.5*^{Hap4} are sensitive (Fig9C). Obviously, these drought-tolerant haplotypes could be used as the elite alleles in drought-tolerant breeding.

In conclusion, the current study provides a comprehensive understanding of the *SnRK* family genes in barley. Although phylogenetic relation, structure, expression pattern and sequence variation in *HvSnRK* genes were analyzed, further studies are required to reveal the roles of *HvSnRK* family genes in responding to various stresses.

4. Materials and Methods

4.1. Identification of SnRK Family Proteins in Barley

The amino acid sequences of SnRK proteins from *Arabidopsis thaliana* and rice obtained from the databases TAIR (<https://www.arabidopsis.org/> (accessed on 17 August 2020)) and Ensemble-Plants (<https://plants.ensembl.org/> (accessed on 17 August 2021)) were used as query probes to search for barley SnRKs using BLASTP against the the IBSC_v2 database from the EnsemblePlants (<https://plants.ensembl.org/> (accessed on 17 August 2021)). Meanwhile, using the Hidden Markov Model (HMM) files of SnRKs acquired from the Pfam database (<http://pfam.xfam.org/> (accessed on 27 August 2021)), HMMER 3.0 was used to search the SnRKs from the barley protein sequence dataset. All candidate sequences of the SnRKs were confirmed through the NCBI conserved domain database (<https://www.ncbi.nlm.nih.gov/cdd> (accessed on 27 August 2021)) [48], the SMART database (<http://smart.embl-heidelberg.de/> (accessed on 27 August 2021)) [49] and the Pfam database (<http://pfam.xfam.org/> (accessed on 27 August 2021)) [50]. Then, the candidate sequences were submitted to the ExPASy (http://www.expasy.ch/tools/pi_tool.html (accessed on 28 August 2021)) to analyze physical and chemical properties and isoelectric points of these proteins. Their subcellular locations were predicted by ProtComp v.9.0 (<http://linux1.softberry.com/berry.phtml> (accessed on 28 August 2021)).

4.2. Analysis of Protein Motifs and Gene Structures

The amino acid sequences of the SnRKs in barley were uploaded to the MEME online tool (<http://meme.sdsc.edu/meme/itro.html> (accessed on 29 August 2021)) [51] to analyze the conserved motifs. The settings were selected based on numerous analyses' experience value: the motif length was set to 6–100 bp, and the maximum number of motifs was set to 10. InterProScan (<http://www.ebi.ac.uk/Tools/pfa/iprscan/> (accessed on 29 August 2021)) was used to annotate the motifs. The sequences of barley SnRK genes were extracted from EnsemblePlants database (<http://plants.ensembl.org/index.html> (accessed on 17 August 2021)), and the gene structure map was created using the Tbttools software [52]. The upstream (−2 kb) sequences of the promoters of *HvSnRK* genes were submitted to the PlantCARE database (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/> (accessed on 29 August 2021)) [53] to analyze the *cis*-acting elements related to abiotic stress such as drought response components MBS (CAACTG) and low temperature response components LTR (CCGAAA).

4.3. Phylogenetic Analysis of SnRK Family Proteins in Barley

The full-length SnRK protein sequences of *Arabidopsis*, rice and barley were aligned with Clustal X for multiple sequence alignment (MSA) [54], and 1000 bootstrap tests were performed with MEGA-X software to construct the phylogenetic tree by the Neighbor-Joining (NJ) method [55].

4.4. Modeling of 3D Structures of *HvSnRKs*

The 3D structures of *HvSnRK* proteins were predicted by homology modeling method. Firstly, the most similar homology of each *HvSnRK* protein were found in the PDB database (<http://www.rcsb.org/> (accessed on 30 August 2021)) by the position-specific iterated BLAST algorithm (PSI-BLAST) [56], then the 3D structures of *HvSnRK* proteins were predicted by the Swiss-Model interactive tool (<https://swissmodel.expasy.org/interactive/> (accessed on 30 August 2021)) [57]. In addition, the 3D structure quality of the *HvSnRK* proteins was tested by SAVES server (<http://nihserver.mbi.ucla.edu/SAVES/> (accessed on 30 August 2021)). Finally, the 3D structures were displayed by the Pymol software.

4.5. Chromosomal Location and Gene Duplication of *HvSnRKs*

The chromosomal location data of *HvSnRKs* was retrieved from the EnsemblePlants, and their distribution was mapped to 7 barley chromosomes using the MapChart v. 2.3 software [58]. *SnRK* duplication patterns, including segmental and tandem duplications, were classified using the Multiple Collinearity Scan toolkit (MCScanX) software [59]. A tandem duplication was defined as a chromosomal area of less than 200 kb containing two or more genes with more than 70% sequence identity between them [60]. Moreover, the synteny relationships of *SnRK* genes between *H. vulgare* and *O. sativa* were performed with python. Non-synonymous (K_a) and synonymous (K_s) substitutions of each duplicated SnRK gene pair were calculated using the KaKs _Calculator 2.0 [61].

4.6. Expression Patterns and Interaction Networks Analysis of *HvSnRKs*

The transcriptome data of 16 different tissues of barley cultivar Morex were obtained from the barley transcriptome database (<https://apex.ipk-gatersleben.de/apex/?p=284> (accessed on 3 October 2021)), and the FPKM (Fragments Per Kilobase of exon model per Million mapped fragments) values of *HvSnRKs* were normalized by log₂ for further analysis. In addition, barley transcriptome data of drought [28], salt [29], heat [30] and waterlogging [31] treatments were obtained from published references. The heatmaps were performed by the Tbttools software. Moreover, the interaction networks of barley SnRK genes were identified based on the database STRING (<http://string-db.org/cgi> (accessed on 3 October 2021)) [62] and the predicted interaction networks were created with the Cytoscape software [63].

4.7. Sequence Variation and Haplotypes of *SnRKs* in a Barley Core Collection

SNPs in the coding regions of the *HvSnRK* genes were identified in 100 barley accessions from the International Barley Core Selected Collection according to previously re-sequenced data (Table S11) [64]. For further analysis, SNPs with Minor Allele Frequencies (MAF) more than 5% and a missing rate of less than 20% were employed. Moreover, we performed a soil-culture experiment using the core collections to investigate the relationship between the haplotypes of *SnRK* genes and their drought tolerance. Barley seeds were sowed and grown in the mixture of peat soil and vermiculite with the ratio of 2:1, and watering was stopped for 2 weeks when seedlings were at the 3-leaf stage. The drought tolerance of barley accessions was evaluated based on water content of shoots. Then, we compared shoot water content in each haplotype of the *HvSnRK* genes.

5. Conclusions

In this study, 46 *SnRK* family genes were found and classified into 3 subfamilies in barley. Analysis on phylogenetic tree, conserved motifs, gene structure and protein 3D structure of these *SnRK* family genes were performed. The expression profiles showed that the *HvSnRK* genes differed greatly in their expression patterns in 15 barley tissues and in response to various abiotic stresses. The expression of *HvSnRK2.5* and *HvSnRK3.24* were significantly upregulated under drought stress. A protein–protein interaction analysis showed that *HvSnRK* proteins probably responded to abiotic stresses through ABA signaling. Moreover, the SNPs in each *HvSnRK* gene were detected, and the haplotypes showing drought tolerance and sensitivity were identified in some genes. The current results could be helpful for further exploration of the *HvSnRK* genes associated with abiotic stress tolerance in barley.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/plants11070975/s1>, Table S1: The information of *SnRKs* in *A. thaliana* and *O. sativa*. Table S2: Characteristics of *SnRK* family genes in barley. Table S3: List of *HvSnRK* genes identified in this study. Table S4: Conserved amino acid motifs and annotation of *HvSnRKs*. Table S5: Syntenic blocks and Ka/Ks values of *HvSnRKs*. Table S6: Orthologous relationships of *SnRKs* between barley and rice. Table S7: The expression profiles of *HvSnRKs* in different barley tissues. Table S8: The expression profiles of *HvSnRKs* under abiotic stresses. Table S9. List of SNPs in *HvSnRKs* identified in this study. Table S10. List of haplotypes of *HvSnRK2.5* identified in this study. Table S11. The information of 100 barley core collection accessions.

Author Contributions: Conceptualization, J.X. and G.Z.; methodology, J.X., D.C., T.S., and G.Z.; formal analysis, J.X.; investigation, D.W.; resources, J.X., Q.S., D.W., and G.Z.; writing—original draft preparation, J.X. and D.W.; writing—review and editing, J.X. and G.Z.; visualization, D.W. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by National Key R&D Program of China (2018YFD1000704-4), Zhejiang Science and Technology Major Project on Breeding New Agricultural Varieties (2021C02064-3), China Agriculture Research System (CARS-05) and Jiangsu Collaborative Innovation Center for Modern Crop Production (JCIC-MCP).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Bohnert, H.J.; Gong, Q.; Li, P.; Ma, S. Unraveling abiotic stress tolerance mechanisms—getting genomics going. *Curr. Opin. Plant Biol.* **2006**, *9*, 180–188. [CrossRef]
2. Shen, Q.F.; Fu, L.B.; Qiu, L.; Xue, F.; Zhang, G.P.; Wu, D.Z. Time-course of ionic responses and proteomic analysis of a Tibetan wild barley at early stage under salt stress. *Plant Growth Regul.* **2017**, *81*, 11–21. [CrossRef]

3. Shen, Q.F.; Yu, J.H.; Fu, L.B.; Wu, L.Y.; Dai, F.; Jiang, L.X.; Wu, D.Z.; Zhang, G.P. Ionic, metabolomic and proteomic analyses reveal molecular mechanisms of root adaption to salt stress in Tibetan wild barley. *Plant Physiol. Biochem.* **2018**, *123*, 319–330. [[CrossRef](#)] [[PubMed](#)]
4. Hunter, T. Protein kinases and phosphatases: The yin and yang of protein phosphorylation and signaling. *Cell* **1995**, *80*, 225–236. [[CrossRef](#)]
5. Zhu, J.K. Abiotic stress signaling and responses in plants. *Cell* **2016**, *167*, 313–324. [[CrossRef](#)] [[PubMed](#)]
6. Estelle, M.H.; Catherine, W.M.C.; Michael, G.; Jeffrey, F.H.; Jung, H.C.; Nigel, H.; Jorg, K.; Sheng, L.; Hugh, G.N.; Michael, R.S.; et al. The *Arabidopsis* CDPK-SnRK superfamily of protein kinases. *Plant Physiol.* **2003**, *132*, 175–183.
7. Halford, N.G.; Hardie, D.G. SNF1-related protein kinases: Global regulators of carbon metabolism in plants? *Plant Mol. Biol.* **1998**, *37*, 735–748. [[CrossRef](#)] [[PubMed](#)]
8. Kulik, A.; Wawer, I.; Krzywińska, E.; Bucholc, M.; Dobrowolska, G.Y. SnRK2 protein kinases-key regulators of plant response to abiotic stresses. *OMICS A J. Integr. Biol.* **2011**, *15*, 859–872. [[CrossRef](#)]
9. Albrecht, V.; Ritz, O.; Linder, S.; Harter, K.; Kudla, J. The NAF domain defines a novel protein-protein interaction module conserved in Ca²⁺-regulated kinases. *EMBO J.* **2001**, *20*, 1051–1063. [[CrossRef](#)]
10. Celenza, J.L.; Carlson, M. A yeast gene that is essential for release from glucose repression encodes a protein kinase. *Science* **1986**, *233*, 1175–1180. [[CrossRef](#)]
11. Dale, S.; Wilson, W.A.; Edelman, A.M.; Hardie, D.G. Similar substrate recognition motifs for mammalian AMP-activated protein kinase, higher plant HMG-CoA reductase kinase-A, yeast SNF1, and mammalian calmodulin-dependent protein kinase I. *FEBS Lett.* **1995**, *361*, 191–195. [[CrossRef](#)]
12. Christopher, S.; Paul, G.D.; Halford, N.G.; Hardie, D.G. Two SNF1-related protein kinases from spinach leaf phosphorylate and inactive at 3-hydroxy-3-methylglutaryl-coenzyme a reductase, nitrate reductase, and sucrose phosphate synthase in vitro. *Plant Physiol.* **1999**, *120*, 257–274.
13. Harthill, J.E.; Meek, S.E.M.; Morrice, N.; Pegg, M.W.; Borch, J.; Wong, B.H.C.; Mackintosh, C. Phosphorylation and 14-3-3 binding of *Arabidopsis* trehalose-phosphate synthase 5 in response to 2-deoxyglucose. *Plant J.* **2006**, *47*, 211–223. [[CrossRef](#)]
14. Boudsocq, M.; Barbier-Brygoo, H.; Laurière, C. Identification of nine sucrose nonfermenting 1-related protein kinases 2 activated by hyperosmotic and saline stresses in *Arabidopsis thaliana*. *J. Biol. Chem.* **2004**, *279*, 41758–41766. [[CrossRef](#)]
15. Kobayashi, Y.; Yamamoto, S.; Minami, H.; Kagaya, Y.; Hattori, T. Differential activation of the rice sucrose nonfermenting1-related protein kinase 2 family by hyperosmotic stress and abscisic acid. *Plant Cell* **2004**, *16*, 1163–1177. [[CrossRef](#)] [[PubMed](#)]
16. Fujii, H.; Chinnusamy, V.; Rodrigues, A.; Rubio, S.; Antoni, R.; Park, S.; Cutler, S.R.; Sheen, J.; Rodriguez, P.L.; Zhu, J.K. In vitro reconstitution of an abscisic acid signaling pathway. *Nature* **2009**, *462*, 660–664. [[CrossRef](#)] [[PubMed](#)]
17. Szymańska, K.P.; Polkowska-Kowalczyk, L.; Lichocka, M.; Maszkowska, J.; Dobrowolska, G. SNF1-related protein kinases SnRK2.4 and SnRK2.10 modulate ROS homeostasis in plant response to salt stress. *Int. J. Mol. Sci.* **2019**, *20*, 143. [[CrossRef](#)]
18. Zhong, R.L.; Wang, Y.X.; Gai, R.N.; Xi, D.D.; Mao, C.J.; Ming, F. Rice SnRK protein kinase *OsSAPK8* acts as a positive regulator in abiotic stress responses. *Plant Sci.* **2020**, *292*, 110373. [[CrossRef](#)] [[PubMed](#)]
19. Kim, K.N.; Cheong, Y.H.; Grant, J.J.; Luan, P.S. CIPK3, a Calcium sensor-associated protein kinase that regulates abscisic acid and cold signal transduction in *Arabidopsis*. *Plant Cell* **2003**, *15*, 411–423. [[CrossRef](#)] [[PubMed](#)]
20. Batistic, O.; Kudla, J. Integration and channeling of calcium signaling through the CBL calcium sensor/CIPK protein kinase network. *Planta* **2004**, *219*, 915–924. [[CrossRef](#)] [[PubMed](#)]
21. Zhu, S.Y.; Yu, X.C.; Wang, X.J.; Zhao, R.; Li, Y.; Fan, R.C.; Shang, Y.; Du, S.Y.; Wu, F.Q.; Xu, Y.H.; et al. Two calcium-dependent protein kinases, CPK4 and CPK11, regulate abscisic acid signal transduction in *Arabidopsis*. *Plant Cell* **2007**, *19*, 3019–3036. [[CrossRef](#)] [[PubMed](#)]
22. Qiu, Q.S.; Guo, Y.; Dietrich, M.A.; Schumaker, K.S.; Zhu, J.K. Regulation of SOS1, a plasma membrane Na⁺/H⁺ exchanger in *Arabidopsis thaliana*, by SOS2 and SOS3. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 8436–8441. [[CrossRef](#)] [[PubMed](#)]
23. Shi, H.; Quintero, F.J.; Pardo, J.M.; Zhu, J.K. The putative plasma membrane Na⁺/H⁺ antiporter SOS1 controls long-distance Na⁺ transport in plants. *Plant Cell* **2002**, *14*, 465–477. [[CrossRef](#)] [[PubMed](#)]
24. Tang, R.J.; Zhao, F.G.; Garcia, V.J.; Kleist, T.J.; Yang, L.; Zhang, H.X.; Luan, S. Tonoplast CBL–CIPK calcium signaling network regulates magnesium homeostasis in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* **2015**, *112*, 3134. [[CrossRef](#)] [[PubMed](#)]
25. Zhang, X.; Li, Z.; Li, X.; Xu, Y.; Xie, H.; Qiu, Q. CBL3 and CIPK18 are required for the function of NHX5 and NHX6 in mediating Li⁺ homeostasis in *Arabidopsis*. *J. Plant Physiol.* **2020**, *255*, 153295. [[CrossRef](#)] [[PubMed](#)]
26. Wang, X.L.; Wu, D.Z.; Yang, Q.; Zeng, J.B.; Jin, G.L.; Chen, Z.H.; Zhang, G.P.; Dai, F. Identification of mild freezing shock response pathways in barley based on transcriptome profiling. *Front. Plant Sci.* **2016**, *7*, 106. [[CrossRef](#)]
27. Tang, H.; Bowers, J.E.; Wang, X.; Ming, R.; Alam, M.; Paterson, A.H. Synteny and collinearity in plant genomes. *Science* **2008**, *320*, 486–488. [[CrossRef](#)] [[PubMed](#)]
28. Koká, F.; Vojta, P.; Galuszka, P. Dataset for transcriptional response of barley (*Hordeum vulgare*) exposed to drought and subsequent re-watering. *Data Brief* **2016**, *8*, 334–341. [[CrossRef](#)] [[PubMed](#)]
29. Fu, L.B.; Shen, Q.F.; Kuang, L.H.; Wu, D.Z.; Zhang, G.P. Transcriptomic and alternative splicing analyses reveal mechanisms of the difference in salt tolerance between barley and rice. *Environ. Exp. Bot.* **2019**, *166*, 103810. [[CrossRef](#)]
30. Andrzej, P.; Maria, B.P.; Aleksandra, S.B.; Katarzyna, K.; Pawel, S.; Kaja, M.; Iver, J.; Artur, J.; Zofia, S.K. Heat stress affects pi-related genes expression and inorganic phosphate deposition/accumulation in barley. *Front. Plant Sci.* **2016**, *7*, 926.

31. Borrego-Benjumea, A.; Carter, A.; Tucker, J.R.; Yao, Z.; Xu, W.; Badea, A. Genome-wide analysis of gene expression provides new insights into waterlogging responses in barley (*Hordeum vulgare* L.). *Plants* **2020**, *9*, 240. [[CrossRef](#)] [[PubMed](#)]
32. Kolukisaoglu, U.; Weinl, S.; Blazevic, D.; Batistic, O.; Kudla, J. Calcium sensors and their interacting protein kinases: Genomics of the *Arabidopsis* and rice CBL-CIPK signaling networks. *Plant Physiol.* **2004**, *134*, 43–58. [[CrossRef](#)] [[PubMed](#)]
33. Weinl, S.; Kudla, J. The CBL–CIPK Ca²⁺-decoding signaling network: Function and perspectives. *New Phytol.* **2009**, *184*, 517–528. [[CrossRef](#)]
34. Lyzenga, W.J.; Liu, H.; Schofield, A.; Alexandria, M.; Stone, S.L. *Arabidopsis* CIPK26 interacts with KEG, components of the ABA signaling network and is degraded by the ubiquitin-proteasome system. *J. Exp. Bot.* **2013**, *64*, 2779–2791. [[CrossRef](#)] [[PubMed](#)]
35. Wang, Y.J.; Yan, H.F.; Qiu, Z.F.; Hu, B.; Fan, C.J. Comprehensive analysis of *SnRK* gene family and their responses to salt stress in *Eucalyptus grandis*. *Int. J. Mol. Sci.* **2019**, *20*, 2786. [[CrossRef](#)] [[PubMed](#)]
36. Wang, L.; Hu, W.; Sun, J.; Liang, X.; Yang, X.; Wei, S.; Wang, X.; Zhou, Y.; Xiao, Q.; Yang, G.; et al. Genome-wide analysis of *SnRK* gene family in *Brachypodium distachyon* and functional characterization of *BdSnRK2.9*. *Plant Sci.* **2015**, *237*, 33–45. [[CrossRef](#)]
37. Zhu, W.Z.; Wu, D.Z.; Jiang, L.X.; Ye, L.Z. Genome-wide identification and characterization of *SnRK* family genes in *Brassica napus*. *BMC Plant Biol.* **2020**, *20*, 287. [[CrossRef](#)]
38. Huai, J.L.; Wang, M.; He, J.G.; Zheng, J.; Dong, Z.G.; Lv, H.K.; Zhao, J.F.; Wang, G.Y. Cloning and characterization of the *SnRK2* gene family from *Zea mays*. *Plant Cell Rep.* **2008**, *27*, 1861–1868. [[CrossRef](#)]
39. Li, L.; Zhang, Y.R.; Liu, K.C.; Ni, Z.F.; Fang, Z.J.; Sun, Q.X.; Gao, J.W. Identification and bioinformatics analysis of *SnRK2* and *CIPK* family genes in sorghum. *Agric. Sci. China* **2010**, *9*, 19–30. [[CrossRef](#)]
40. Li, N.; Wei, S.T.; Chen, J.; Yang, F.F.; Kong, L.G.; Chen, C.X.; Ding, X.H.; Chu, Z.H. *OsASR2* regulates the expression of a defence-related gene, *Os2H16*, by targeting the GT-1 *cis*-element. *Plant Biotechnol. J.* **2018**, *16*, 771–783. [[CrossRef](#)]
41. Wei, M.; Xu, X.M.; Li, C.H. Identification and expression of *CAMTA* genes in *Populus trichocarpa* under biotic and abiotic stress. *Sci. Rep.* **2017**, *7*, 17910. [[CrossRef](#)] [[PubMed](#)]
42. Xu, Z.W.; Wang, M.P.; Guo, Z.T.; Zhu, X.F.; Xia, Z.L. Identification of a 119-bp promoter of the maize sulfite oxidase gene (*ZmSO*) that confers high-level gene expression and ABA or drought inducibility in transgenic plants. *Int. J. Mol. Sci.* **2019**, *20*, 3326. [[CrossRef](#)] [[PubMed](#)]
43. Manavella, P.A.; Dezar, C.A.; Ariel, F.D.; Chan, R.L. Two ABREs, two redundant root-specific and one W-box *cis*-acting elements are functional in the sunflower *HAHB4* promoter. *Plant Physiol. Biochem.* **2008**, *46*, 860–867. [[CrossRef](#)] [[PubMed](#)]
44. Dunn, M.A.; White, A.J.; Vural, S.; Hughes, M.A. Identification of promoter elements in a low-temperature-responsive gene (*blt4.9*) from barley (*Hordeum vulgare* L.). *Plant Mol. Biol.* **1998**, *38*, 551–564. [[CrossRef](#)]
45. Ng, L.; Soon, F.; Zhou, X.E.; West, G.M.; Kovach, A.; Suino-Powell, K.M.; Chalmers, M.J.; Li, J.; Yong, E.; Zhu, J.; et al. Structural basis for basal activity and autoactivation of abscisic acid (ABA) signaling SnRK2 kinases. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 21259–21264. [[CrossRef](#)]
46. Chaves-Sanjuan, A.; Sanchez-Barrena, M.J.; Gonzalez-Rubio, J.M.; Moreno, M.; Ragel, P.; Jimenez, M.; Pardo, J.M.; Martinez-Ripoll, M.; Quintero, F.J.; Albert, A. Structural basis of the regulatory mechanism of the plant CIPK family of protein kinases controlling ion homeostasis and abiotic stress. *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 4532–4541. [[CrossRef](#)]
47. Xiang, Y.; Huang, Y.M.; Xiong, L.Z. Characterization of stress-responsive *CIPK* genes in rice for stress tolerance improvement. *Plant Physiol.* **2007**, *144*, 1416–1428. [[CrossRef](#)]
48. Marchler-Bauer, A.; Lu, S.N.; Anderson, J.; Chitsaz, F.; Derbyshire, M.; DeWeese-Scott, C.; Fong, J.; Geer, L.; Geer, R.; Gonzales, N.; et al. CDD: A conserved domain database for the functional annotation of proteins. *Nucleic Acids Res.* **2011**, *39*, 225–229. [[CrossRef](#)]
49. Letunic, I.; Khedkar, S.; Bork, P. SMART: Recent updates, new developments and status in 2020. *Nucleic Acids Res.* **2020**, *49*, 458–460. [[CrossRef](#)] [[PubMed](#)]
50. Finn, R.D.; Coghill, P.; Eberhardt, R.Y.; Eddy, S.R. The Pfam protein families database: Towards a more sustainable future. *Nucleic Acids Res.* **2016**, *44*, D279–D285. [[CrossRef](#)] [[PubMed](#)]
51. Bailey, T.L.; Boden, M.; Buske, F.A.; Frith, M.; Grant, C.E.; Clementi, L.; Ren, J.; Li, W.W.; Noble, W.S. MEME SUITE: Tools for motif discovery and searching. *Nucleic Acids Res.* **2009**, *37*, 202–208. [[CrossRef](#)] [[PubMed](#)]
52. Chen, C.J.; Chen, H.; Zhang, Y.; Thomas, H.R.; Xia, R. TBtools: An integrative toolkit developed for interactive analyses of big biological data. *Mol. Plant* **2020**, *13*, 1194–1202. [[CrossRef](#)]
53. Magali, L.; Patrice, D.; Gert, T.; Kathleen, M.; Yves, M.; Yves, V.D.P.; Pierre, R.; Stephane, R. PlantCARE, a database of plant *cis*-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. *Nucleic Acids Res.* **2002**, *30*, 325–327.
54. Larkin, M.A.; Blackshields, G.; Brown, N.P.; Chenna, R.; Mcgettigan, P.A.; Mcwilliam, H.; Valentin, F.; Wallace, I.M.; Wilm, A.; Lopez, R. Clustal W and Clustal X version 2.0. *Bioinformatics* **2007**, *23*, 2947–29348. [[CrossRef](#)] [[PubMed](#)]
55. Stecher, G.; Tamura, K.; Kumar, S. Molecular evolutionary genetics analysis (MEGA) for macOS. *Mol. Biol. Evol.* **2020**, *37*, 1237–1239. [[CrossRef](#)] [[PubMed](#)]
56. Altschul, S.F.; Madden, T.L.; Schäffer, A.A.; Zhang, J.H.; Zhang, Z.; Miller, W.; Lipman, D.J. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res.* **1997**, *25*, 3389–3402. [[CrossRef](#)] [[PubMed](#)]
57. Arnold, K.; Bordoli, L.; Kopp, J.; Schwede, T. The SWISS-MODEL workspace: A web-based environment for protein structure homology modelling. *Bioinformatics* **2006**, *22*, 195–201. [[CrossRef](#)]

58. Voorrips, R. MapChart: Software for the graphical presentation of linkage maps and QTLs. *J. Hered.* **2002**, *93*, 77–78. [[CrossRef](#)] [[PubMed](#)]
59. Wang, Y.P.; Tang, H.B.; Debarry, J.D.; Tan, X.; Li, J.P.; Wang, X.Y.; Lee, T.H.; Jin, H.Z.; Barry, M.; Guo, H.; et al. MCScanX: A toolkit for detection and evolutionary analysis of gene synteny and collinearity. *Nucleic Acids Res.* **2012**, *40*, 49. [[CrossRef](#)] [[PubMed](#)]
60. Holub, E.B. The arms race is ancient history in *Arabidopsis*, the wildflower. *Nat. Rev. Genet.* **2001**, *2*, 516–527. [[CrossRef](#)] [[PubMed](#)]
61. Wang, D.P.; Zhang, Y.B.; Zhang, Z.; Zhu, J.; Yu, J. KaKs_Calculator 2.0: A toolkit incorporating gamma-series methods and sliding window strategies. *Genom. Proteom. Bioinform.* **2010**, *8*, 77–80. [[CrossRef](#)]
62. Damian, S.; Gable, A.L.; Nastou, K.C.; David, L.; Rebecca, K.; Sampo, P.; Doncheva, N.T.; Marc, L.; Fang, T.; Peer, B. The STRING database in 2021: Customizable protein-protein networks, and functional characterization of user-uploaded gene/measurement sets. *Nucleic Acids Res.* **2020**, *49*, D605–D612.
63. Shannon, P.; Markeil, A.; Ozier, O.; Baliga, N.S.; Wang, J.T.; Ramage, D.; Amin, N.; Schwikowski, B. Cytoscape: A software environment for integrated models of biomolecular interaction networks. *Genome Res.* **2003**, *13*, 2498–2504. [[CrossRef](#)]
64. Wu, D.Z.; Sato, K.; Ma, J.F. Genome-wide association mapping of cadmium accumulation in different organs of barley. *New Phytol.* **2015**, *208*, 817–829. [[CrossRef](#)] [[PubMed](#)]