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## Genome-wide identification and expression analysis of sucrose nonfermenting-1-related protein kinase (*SnRK*) genes in *Triticum aestivum* in response to abiotic stress

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The SnRK gene family is a key regulator that plays an important role in plant stress response by phosphorylating the target protein to regulate subsequent signaling pathways. This study was aimed to perform a genome-wide analysis of the *SnRK* gene family in wheat and the expression profiling of SnRKs in response to abiotic stresses. An in silico analysis identified 174 *SnRK* genes, which were then categorized into three subgroups (SnRK1/2/3) on the basis of phylogenetic analyses and domain types. The gene intron–exon structure and protein-motif composition of *SnRKs* were similar within each subgroup but different amongst the groups. Gene duplication and synteny between the wheat and *Arabidopsis* genomes was also investigated in order to get insight into the evolutionary aspects of the TaSnRK family genes. The result of *cis*-acting element analysis showed that there were abundant stress- and hormone-related *cis*-elements in the promoter regions of 129 *SnRK* genes. Furthermore, quantitative real-time PCR data revealed that heat, salt and drought treatments enhanced TaSnRK2.11 expression, suggesting that it might be a candidate gene for abiotic stress tolerance. We also identified eight microRNAs targeting 16 TaSnRK genes which are playing important role across abiotic stresses and regulation in different pathways. These findings will aid in the functional characterization of TaSnRK genes for further research.

Bread wheat (*T. aestivum* L.) is a major cereal crop and an important source of carbohydrates and protein in the human diet, accounting for 20% of daily calorie consumption. The most significant economic feature is grain yield, which is influenced by a variety of biotic and abiotic stressors. By 2050, it is expected that the demand for wheat will increase by 60%<sup>1</sup>. Plants use a variety of molecular defence mechanisms to deal with abiotic stresses such as salt, drought, and heat. Plants respond to environmental stresses in two ways: gene expression regulation and protein modification<sup>2</sup>. Protein kinase-mediated phosphorylation and dephosphorylation are important in protein modification<sup>3</sup>. SnRKs (Sucrose nonfermenting 1 (SNF1)-related protein kinases) are a group of protein kinase genes that have a role in a variety of physiological activities<sup>4</sup>. Based on their sequence similarities and gene architectures, plant SnRKs may be split into three subfamilies: SnRK1, SnRK2, and SnRK3<sup>4,5</sup>. The SnRK1 subfamily has a highly conserved N-terminal protein kinase (Pkinase) domain, which is similar to SNF1 in yeast and AMPKs in mammals<sup>6</sup>. The SnRK2 and SnRK3 subfamilies are unique to plants, and both show more variability than the SnRK1 subfamily members in terms of plant diversity. A conserved P kinase domain and a C-terminal variable adjusting domain are found in members of the SnRK2 family<sup>7</sup>. SnRK3, known as CIPK (CBL-interacting protein kinases), also has conserved N-terminal protein kinase domains and NAF domains, as well as PPI domains at the C-terminus<sup>8,9</sup>.

Plant cells respond to starvation and metabolic stress through the SnRK1 family of genes. Catalytic components of heterotrimeric complexes, SnRK1 kinases interact with two additional subunits<sup>10</sup>. SnRK1 was shown to be involved in the stimulation of sucrose synthase expression and had a key function in carbohydrate metabolism control in *S. tuberosum*<sup>11</sup>. Low energy stress (e.g., darkness and hypoxia) may also cause SnRK1 nuclear

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translocation and subsequent induction of SnRK1 target genes, allowing for the replenishment of cellular energy for plant development<sup>12</sup>. SnRK1 genes were also shown to be hubs in a network of signalling pathways that included cell cycle control, pathogen responses, and meristem formation, as reported earlier<sup>13</sup>.

The SnRK2 genes, on the other hand, play a significant role in plants' responses to abiotic stressors, particularly osmotic and salt stress. In *A. thaliana*, for example, SnRK2.10 phosphorylates multiple target genes, including the dehydrins ERD10 and ERD14, to deal with osmotic stress<sup>14</sup>. In *N. tabacum*, SnRK2.1 regulates salt tolerance positively<sup>15,16</sup>. SnRK2 subfamily genes in *A. thaliana* may be divided into three groups: ABA-independent kinases, genes responsive to drought stress, and kinases that are substantially activated by ABA<sup>7,17</sup>. The most comprehensive study on ABA-dependent group 3 kinases is now underway. For example, one of the SnRK2 family genes, *AtSnRK2.6* (OST1), is involved in the ABA signalling pathway in stomatal guard cells, and OST1 protein stability may be regulated by the E3-ubiquitin ligase HOS15 in Arabidopsis to reduce ABA signal sensitivity<sup>18,19</sup>.

CBL (calcium sensor calcineurin B-like proteins)-interacting protein kinases (CIPKs), also known as SnRK3 kinases, play an important role in plant stress tolerance<sup>4,20</sup>. The SOS (salt excessively sensitive) system, for example, was the first CBL-CIPK route found in *A. thaliana*. SOS3 (*AtCBL4*) on the cell membrane recognised the calcium signal created by salt stress, and then SOS3 joined with SOS2 (*AtCIPK24*) to phosphorylate SOS1 ( $\text{Na}^+/\text{H}^+$  antiporter) to remove excess  $\text{Na}^+$  from root cells<sup>21,22</sup>. *MdCIPK13* and *MdCIPK22* also improved salt and drought tolerance in apple by phosphorylating the sucrose transporter *MdsUT2.2*<sup>23,24</sup>. In *B. napus*, overexpression of *BnCBL1-BnCIPK6* may improve high salinity tolerance and low potassium tolerance<sup>25</sup>. Finally, growing data has highlighted the relevance of SnRKs function in nutrient consumption and stress response, and researchers may be able to increase plant stress resistance by genetic modification of these genes.

The importance of *SnRK* genes function in nutrient consumption and stress response is becoming clearer, and researchers may be able to improve plant stress resistance by genetically modifying these genes. However, there is no information available on *SnRK* genes in wheat, one of the most important cereals. In the current study, we identified 174 *SnRK* gene family members in the genome of *Triticum aestivum*. We also evaluated their evolutionary relationships, gene architecture, protein motifs, chromosomal position, and *cis*-elements in promoter regions in a systematic way. The differential expression of the *SnRK* genes under abiotic stress was also investigated using qRT-PCR. The results obtained in this study provide important insights into the molecular pathways that underpin stress tolerance and molecular breeding.

## Results

**Identification of SnRK genes of wheat.** A total of 174 *SnRK* genes were identified and further subdivided into three categories: SnRK 1, SnRK 2 and SnRK3 which have 14, 65 and 95 genes, respectively (Supplementary Table S1). The number of SnRK gene in wheat is higher than other plant species e.g. *A. thaliana* and rice. It may be due to hexaploid nature of wheat genome. Each protein's longest amino acid sequence was chosen for further investigation. The parameters of the gene characteristics including Gene IDs, amino acid length, molecular weights (MW), isoelectric points (pI), grand average of hydropathicity (GRAVY) and sub-cellular localization were analysed (Table 1). The molecular weight of all these TaSnRK proteins spans from 26.8 to 146.1 kDa, while the amino acid length spans from 239 to 1335. The isoelectric point of SnRKs family ranged from 4.3 to 9.4 indicating basic nature of proteins. The hydrophobicity and hydrophilicity are revealed by the GRAVY scores. SnRK proteins from *T. aestivum*, on the other hand, show a negative GRAVY score, implying that they are hydrophilic. The degree of hydrophilicity, on the other hand, is proportionate to the increased variability. According to the expected subcellular localization data, most of the *TaSnRK* genes are expressed in the nucleus and cytoplasm, followed by chloroplast and mitochondria (Table 1).

**Phylogenetic analysis of SnRK genes family.** To study the evolutionary relationships among SnRK proteins and their classification, we performed unrooted phylogenetic tree analysis using the full-length amino acid sequences of 174 *SnRKs* genes of *T. aestivum*, 38 of *A. thaliana* and 50 of *O. sativa* (Fig. 1). The clustering of 38 AtSnRKs into three groups was reported earlier<sup>3</sup>. Based on the phylogenetic analysis and domains presence, 174 TaSnRKs were also divided into three groups in this study. Of these, 14 proteins in the TaSnRK1 subfamily have Pkinase (PF00069 of Pfam), UBA (PF00627), and KA1 (PF02149) domains, whereas 65 proteins in the TaSnRK2 subfamily have Pkinase domains with strong resemblance to AtSnRK2 subfamily, and 95 proteins in the SnRK3 subfamily have Pkinase and NAF (PF03822) domain (Fig. 1).

**Motif composition and gene structural analysis of the SnRK gene family in *T. aestivum*.** MEME analysis showed that 10 conserved motifs were identified in TaSnRK proteins (Fig. 2A). The conserved motif's sequence and length details have been listed in Supplementary Table S1. The conserved Pkinase domain including the pattern 1, 2, 3, 5, 6 was discovered in all *TaSnRK* genes in this investigation (Supplementary Fig. 1). Furthermore, *TaSnRK* genes from the same subfamily have comparable pattern compositions, but *TaSnRK* genes from different subfamilies have varied motif compositions. TaSnRK1 subfamily genes have 9 motif (motifs 1, 2, 3, 4, 5, 6, 7, 8 and 10) while TaSnRK2 subfamily genes had either motifs 1, 2, 4, 6, 8 or motifs 1, 2, 3, 4, 5 and 10. TaSnRK3 subfamily genes had 10 motifs while a few of them do not have any motif (Fig. 2A). In conclusion, the comparable gene architectures and conserved motif compositions of SnRK genes within the same subfamily substantially support the phylogenetic analysis based subfamily classifications.

The exon-intron structure of *TaSnRK* genes showed that the TaSnRK1 subfamily genes have more than 10 exons, while the TaSnRK2 subfamily genes have 2–34 exons followed by TaSnRK3 subfamily which have 1–33 exons (Fig. 2B). Notably, 14 genes are intron-less. In addition, the TaSnRK3 family is divided into two subgroups. The genes in SnRK3 subgroup 1 had more than 10 exons, while the genes in subgroup 2 had less than four exons.

| Gene id    | No. of amino acids | Mol. weight | Theoretical pI | Aliphatic index | (GRAVY) | localization | Domains           |
|------------|--------------------|-------------|----------------|-----------------|---------|--------------|-------------------|
| TaSnRK1.1  | 503                | 57,281.36   | 8.77           | 93.62           | - 0.266 | cyto         | PKinase, UBA, KA1 |
| TaSnRK1.2  | 500                | 56,961.98   | 8.77           | 94.18           | - 0.268 | cyto         | PKinase, UBA, KA1 |
| TaSnRK1.3  | 500                | 56,961.98   | 8.77           | 94.18           | - 0.268 | cyto         | PKinase, UBA, KA1 |
| TaSnRK1.4  | 503                | 57,492.46   | 8.58           | 90.89           | - 0.362 | cyto         | PKinase, UBA, KA1 |
| TaSnRK1.5  | 503                | 57,492.46   | 8.58           | 90.89           | - 0.362 | cyto         | PKinase, UBA, KA1 |
| TaSnRK1.6  | 503                | 57,476.47   | 8.58           | 91.29           | - 0.352 | cyto         | PKinase, UBA, KA1 |
| TaSnRK1.7  | 438                | 50,268.93   | 8.7            | 89.5            | - 0.362 | chlo         | PKinase, UBA, KA1 |
| TaSnRK1.8  | 509                | 58,194.2    | 8.65           | 89.27           | - 0.316 | cyto         | PKinase, UBA, KA1 |
| TaSnRK1.9  | 513                | 58,709.82   | 8.55           | 89.71           | - 0.312 | cyto         | PKinase, UBA, KA1 |
| TaSnRK1.10 | 509                | 58,250.26   | 8.55           | 89.84           | - 0.318 | cyto         | PKinase, UBA, KA1 |
| TaSnRK1.11 | 441                | 50,784.85   | 6.33           | 97.01           | - 0.207 | cyto         | PKinase, UBA, KA1 |
| TaSnRK1.12 | 466                | 53,389.7    | 6.86           | 97.04           | - 0.222 | vacu         | PKinase, UBA, KA1 |
| TaSnRK1.13 | 512                | 58,436.59   | 8.65           | 95.18           | - 0.282 | cyto         | PKinase, UBA, KA1 |
| TaSnRK1.14 | 516                | 58,901.13   | 8.53           | 95.58           | - 0.292 | cyto         | PKinase, UBA, KA1 |
| TaSnRK2.1  | 357                | 40,939.6    | 5.55           | 77.84           | - 0.522 | cysk         | PKinase           |
| TaSnRK2.2  | 357                | 40,911.55   | 5.55           | 77.31           | - 0.529 | cyto         | PKinase           |
| TaSnRK2.3  | 357                | 40,911.55   | 5.55           | 77.31           | - 0.529 | cyto         | PKinase           |
| TaSnRK2.4  | 394                | 44,210.97   | 6.15           | 73.3            | - 0.481 | cyto         | PKinase           |
| TaSnRK2.5  | 391                | 43,969.68   | 6.15           | 72.84           | - 0.499 | cyto         | PKinase           |
| TaSnRK2.6  | 391                | 43,927.6    | 6.15           | 72.33           | - 0.511 | cyto         | PKinase           |
| TaSnRK2.7  | 363                | 42,176.97   | 5.86           | 76.5            | - 0.588 | cysk         | PKinase           |
| TaSnRK2.8  | 363                | 42,096.89   | 5.77           | 76.5            | - 0.562 | cysk         | PKinase           |
| TaSnRK2.9  | 363                | 42,177.99   | 5.86           | 77.02           | - 0.574 | cysk         | PKinase           |
| TaSnRK2.10 | 342                | 38,609.93   | 5.43           | 83.6            | - 0.431 | cyto         | PKinase           |
| TaSnRK2.11 | 342                | 38,548.93   | 5.73           | 83.6            | - 0.424 | cyto         | PKinase           |
| TaSnRK2.12 | 342                | 38,663.04   | 5.52           | 83.89           | - 0.435 | cyto         | PKinase           |
| TaSnRK2.13 | 342                | 38,793.3    | 5.59           | 87.13           | - 0.246 | cyto         | PKinase           |
| TaSnRK2.14 | 342                | 38,847.35   | 5.66           | 87.43           | - 0.249 | cyto         | PKinase           |
| TaSnRK2.15 | 343                | 38,998.52   | 5.47           | 84.34           | - 0.278 | cyto         | PKinase           |
| TaSnRK2.16 | 341                | 38,664.29   | 5.45           | 89.97           | - 0.208 | cyto         | PKinase           |
| TaSnRK2.17 | 353                | 40,000.81   | 5.53           | 90.79           | - 0.201 | cyto         | PKinase           |
| TaSnRK2.18 | 353                | 40,014.88   | 5.62           | 90.79           | - 0.197 | cyto         | PKinase           |
| TaSnRK2.19 | 364                | 40,585.43   | 4.89           | 89.73           | - 0.185 | cysk         | PKinase           |
| TaSnRK2.20 | 363                | 40,659.53   | 4.89           | 88.65           | - 0.217 | cyto         | PKinase           |
| TaSnRK2.21 | 360                | 40,327.2    | 4.94           | 90.44           | - 0.191 | cyto         | PKinase           |
| TaSnRK2.22 | 330                | 37,143.13   | 4.68           | 88.64           | - 0.261 | cysk         | PKinase           |
| TaSnRK2.23 | 239                | 26,754.3    | 4.38           | 88.54           | - 0.281 | cyto         | PKinase           |
| TaSnRK2.24 | 361                | 40,631.17   | 4.8            | 88.86           | - 0.271 | cysk         | PKinase           |
| TaSnRK2.25 | 366                | 41,590.39   | 4.87           | 86.83           | - 0.315 | cysk         | PKinase           |
| TaSnRK2.26 | 366                | 41,590.39   | 4.87           | 86.83           | - 0.315 | cysk         | PKinase           |
| TaSnRK2.27 | 366                | 41,590.39   | 4.87           | 86.83           | - 0.315 | cysk         | PKinase           |
| TaSnRK2.28 | 388                | 42,192.41   | 6.76           | 87.96           | - 0.164 | chlo         | PKinase           |
| TaSnRK2.29 | 445                | 48,359.5    | 8.93           | 81.98           | - 0.326 | chlo         | PKinase           |
| TaSnRK2.30 | 448                | 48,647.72   | 8.89           | 80.78           | - 0.343 | nucl         | PKinase           |
| TaSnRK2.31 | 425                | 47,200.17   | 6.43           | 81.22           | - 0.185 | cyto         | PKinase           |
| TaSnRK2.32 | 625                | 70,055.65   | 5.36           | 88.96           | - 0.26  | cyto         | PKinase           |
| TaSnRK2.33 | 626                | 70,584.46   | 5.77           | 92.41           | - 0.267 | cyto         | PKinase           |
| Continued  |                    |             |                |                 |         |              |                   |

| Gene id    | No. of amino acids | Mol. weight | Theoretical pI | Aliphatic index | (GRAVY) | Localization | Domains       |
|------------|--------------------|-------------|----------------|-----------------|---------|--------------|---------------|
| TaSnRK2.34 | 623                | 70,148.93   | 5.73           | 92.23           | - 0.261 | E.R., golg   | PKinase       |
| TaSnRK2.35 | 341                | 38,664.29   | 5.45           | 89.97           | - 0.208 | cyto         | PKinase       |
| TaSnRK2.36 | 481                | 53,582.27   | 6.13           | 84.68           | - 0.299 | cyto         | PKinase       |
| TaSnRK2.37 | 481                | 53,597.33   | 6.13           | 84.89           | - 0.289 | cyto         | PKinase       |
| TaSnRK2.38 | 486                | 53,709.27   | 5.75           | 83.85           | - 0.264 | cyto         | PKinase       |
| TaSnRK2.39 | 486                | 53,751.31   | 5.75           | 84.05           | - 0.265 | cyto         | PKinase       |
| TaSnRK2.40 | 1332               | 145,867.36  | 8.09           | 99.62           | - 0.042 | plas         | PKinase       |
| TaSnRK2.41 | 1335               | 146,114.44  | 8.01           | 99.03           | - 0.049 | plas         | PKinase       |
| TaSnRK2.42 | 1335               | 146,061.45  | 8.01           | 99.03           | - 0.051 | cyto         | PKinase       |
| TaSnRK2.43 | 1027               | 114,149.62  | 5.4            | 82.56           | - 0.418 | cyto         | PKinase       |
| TaSnRK2.44 | 1027               | 114,193.62  | 5.44           | 82.94           | - 0.424 | cyto         | PKinase       |
| TaSnRK2.45 | 1027               | 114,053.56  | 5.42           | 83.6            | - 0.409 | cyto         | PKinase       |
| TaSnRK2.46 | 525                | 60,273.36   | 6.21           | 73.94           | - 0.65  | nucl         | PKinase       |
| TaSnRK2.47 | 527                | 60,480.56   | 6.21           | 73.11           | - 0.654 | nucl         | PKinase       |
| TaSnRK2.48 | 525                | 60,258.4    | 6.12           | 75.05           | - 0.633 | nucl         | PKinase       |
| TaSnRK2.49 | 539                | 61,557.02   | 6.1            | 72.6            | - 0.588 | nucl         | PKinase       |
| TaSnRK2.50 | 542                | 61,867.29   | 6.03           | 71.13           | - 0.62  | nucl         | PKinase       |
| TaSnRK2.51 | 538                | 61,455.88   | 6.1            | 71.65           | - 0.608 | nucl         | PKinase       |
| TaSnRK2.52 | 564                | 65,620.17   | 5.75           | 67.13           | - 0.885 | cyto         | PKinase       |
| TaSnRK2.53 | 564                | 65,294.61   | 5.75           | 67.11           | - 0.878 | cyto         | PKinase       |
| TaSnRK2.54 | 562                | 65,136.4    | 5.78           | 67.54           | - 0.867 | cyto         | PKinase       |
| TaSnRK2.55 | 462                | 53,394.1    | 8.27           | 72.23           | - 0.704 | chlo         | PKinase       |
| TaSnRK2.56 | 462                | 53,394.1    | 8.27           | 72.23           | - 0.704 | chlo         | PKinase       |
| TaSnRK2.57 | 462                | 53,424.13   | 8.27           | 72.23           | - 0.705 | nucl         | PKinase       |
| TaSnRK2.58 | 558                | 63,782.63   | 5.7            | 75.04           | - 0.659 | cyto         | PKinase       |
| TaSnRK2.59 | 558                | 63,919.73   | 5.71           | 74.16           | - 0.67  | cyto         | PKinase       |
| TaSnRK2.60 | 558                | 63,901.75   | 5.71           | 75.56           | - 0.658 | cyto         | PKinase       |
| TaSnRK2.61 | 606                | 68,445.68   | 7.71           | 78.27           | - 0.581 | chlo         | PKinase       |
| TaSnRK2.62 | 948                | 104,031.17  | 6.71           | 71.4            | - 0.579 | nucl         | PKinase       |
| TaSnRK2.63 | 946                | 103,753.05  | 6.85           | 72.38           | - 0.553 | nucl         | PKinase       |
| TaSnRK2.64 | 945                | 103,551.67  | 6.31           | 74.21           | - 0.539 | nucl         | PKinase       |
| TaSnRK2.65 | 311                | 34,927.63   | 5.03           | 87.14           | - 0.317 | chlo         | PKinase       |
| TaSnRK3.1  | 486                | 55,169.05   | 8.99           | 84.67           | - 0.547 | E.R          | PKinase & NAF |
| TaSnRK3.2  | 481                | 54,565.54   | 9.12           | 85.76           | - 0.518 | chlo         | PKinase & NAF |
| TaSnRK3.3  | 491                | 55,735.96   | 9.12           | 87.98           | - 0.485 | chlo         | PKinase & NAF |
| TaSnRK3.4  | 863                | 97,559.7    | 5.95           | 90.71           | - 0.316 | cyto         | PKinase & NAF |
| TaSnRK3.5  | 507                | 57,577.94   | 8.75           | 84.83           | - 0.545 | chlo         | PKinase & NAF |
| TaSnRK3.6  | 507                | 57,470.74   | 8.75           | 84.06           | - 0.541 | cyto         | PKinase & NAF |
| TaSnRK3.7  | 863                | 97,559.7    | 5.95           | 90.71           | - 0.316 | chlo         | PKinase & NAF |
| TaSnRK3.8  | 472                | 53,716.95   | 9.45           | 85.4            | - 0.476 | chlo         | PKinase & NAF |
| TaSnRK3.9  | 472                | 53,612.71   | 9.35           | 84.15           | - 0.488 | chlo         | PKinase & NAF |
| TaSnRK3.10 | 472                | 53,730.79   | 9.39           | 84.98           | - 0.492 | nucl         | PKinase & NAF |
| TaSnRK3.11 | 449                | 51,035.83   | 9.19           | 86.84           | - 0.416 | chlo         | PKinase & NAF |
| TaSnRK3.12 | 362                | 40,942.14   | 8.94           | 87.54           | - 0.436 | chlo         | PKinase & NAF |
| TaSnRK3.13 | 444                | 50,281.78   | 9.03           | 86.28           | - 0.429 | chlo         | PKinase & NAF |
| TaSnRK3.14 | 433                | 48,481.11   | 9.38           | 88.71           | - 0.359 | chlo         | PKinase & NAF |
| TaSnRK3.15 | 438                | 49,256.87   | 9.25           | 86.78           | - 0.387 | chlo         | PKinase & NAF |
| TaSnRK3.16 | 438                | 49,157.79   | 9.29           | 88.33           | - 0.378 | chlo         | PKinase & NAF |
| TaSnRK3.17 | 356                | 40,111.51   | 8.94           | 86.52           | - 0.345 | cyto         | PKinase & NAF |
| TaSnRK3.18 | 452                | 51,009.15   | 9.13           | 83.47           | - 0.391 | nucl         | PKinase & NAF |
| TaSnRK3.19 | 341                | 38,664.29   | 5.45           | 89.97           | - 0.208 | cyto         | PKinase & NAF |
| TaSnRK3.20 | 438                | 49,952.76   | 9.1            | 87.72           | - 0.356 | cyto         | PKinase & NAF |
| TaSnRK3.21 | 466                | 52,443.42   | 9.25           | 82.23           | - 0.445 | chlo         | PKinase & NAF |
| TaSnRK3.22 | 464                | 52,235.09   | 9.34           | 81.96           | - 0.476 | chlo         | PKinase & NAF |
| TaSnRK3.23 | 464                | 52,165.04   | 9.34           | 82.18           | - 0.473 | chlo         | PKinase & NAF |
| TaSnRK3.24 | 447                | 48,011.08   | 9.08           | 88.86           | - 0.143 | plas         | PKinase & NAF |
| Continued  |                    |             |                |                 |         |              |               |

| Gene id    | No. of amino acids | Mol. weight | Theoretical pI | Aliphatic index | (GRAVY) | localization | Domains       |
|------------|--------------------|-------------|----------------|-----------------|---------|--------------|---------------|
| TaSnRK3.25 | 447                | 48,011.08   | 9.08           | 88.86           | -0.143  | plas         | PKinase & NAF |
| TaSnRK3.26 | 390                | 41,928.22   | 8.73           | 90.62           | -0.084  | plas         | PKinase & NAF |
| TaSnRK3.27 | 439                | 47,836.11   | 8.94           | 92.64           | -0.157  | cyto         | PKinase & NAF |
| TaSnRK3.28 | 439                | 47,867.17   | 9.12           | 92.44           | -0.156  | cyto         | PKinase & NAF |
| TaSnRK3.29 | 439                | 47,879.24   | 9.08           | 92.21           | -0.151  | plas         | PKinase & NAF |
| TaSnRK3.30 | 446                | 47,919.07   | 9.18           | 89.51           | -0.142  | chlo         | PKinase & NAF |
| TaSnRK3.31 | 447                | 49,504.86   | 9.09           | 85.55           | -0.193  | chlo         | PKinase & NAF |
| TaSnRK3.32 | 447                | 49,471.79   | 9              | 85.32           | -0.195  | chlo         | PKinase & NAF |
| TaSnRK3.33 | 432                | 47,928.07   | 6.41           | 92.11           | -0.167  | cyto         | PKinase & NAF |
| TaSnRK3.34 | 432                | 48,069.22   | 7.68           | 88.03           | -0.216  | cyto         | PKinase & NAF |
| TaSnRK3.35 | 433                | 47,848      | 8.46           | 93.23           | -0.173  | cyto         | PKinase & NAF |
| TaSnRK3.36 | 317                | 35,509.72   | 5.34           | 89.81           | -0.162  | cyto         | PKinase & NAF |
| TaSnRK3.37 | 433                | 47,892.11   | 8.46           | 92.1            | -0.156  | cyto         | PKinase & NAF |
| TaSnRK3.38 | 433                | 47,020.58   | 6.72           | 87.69           | -0.133  | chlo         | PKinase & NAF |
| TaSnRK3.39 | 436                | 47,362.85   | 6.48           | 87.32           | -0.171  | chlo         | PKinase & NAF |
| TaSnRK3.40 | 519                | 56,926.68   | 8.06           | 76.18           | -0.31   | mito         | PKinase & NAF |
| TaSnRK3.41 | 520                | 56,965.71   | 8.38           | 76.6            | -0.303  | chlo         | PKinase & NAF |
| TaSnRK3.42 | 522                | 57,208.9    | 8.11           | 76.69           | -0.316  | chlo         | PKinase & NAF |
| TaSnRK3.43 | 456                | 50,799.38   | 8.61           | 85.13           | -0.311  | cyto         | PKinase & NAF |
| TaSnRK3.44 | 555                | 61,507.58   | 8.96           | 82.43           | -0.323  | chlo         | PKinase & NAF |
| TaSnRK3.45 | 521                | 57,993.2    | 8.39           | 76.97           | -0.429  | chlo         | PKinase & NAF |
| TaSnRK3.46 | 519                | 57,926.21   | 8.35           | 77.63           | -0.417  | chlo         | PKinase & NAF |
| TaSnRK3.47 | 522                | 58,085.74   | 8.65           | 82.36           | -0.288  | chlo         | PKinase & NAF |
| TaSnRK3.48 | 525                | 58,523.38   | 8.77           | 82.8            | -0.299  | chlo         | PKinase & NAF |
| TaSnRK3.49 | 353                | 39,277.96   | 5.27           | 77.62           | -0.319  | chlo         | PKinase & NAF |
| TaSnRK3.50 | 462                | 53,394.1    | 8.27           | 72.23           | -0.704  | chlo         | PKinase & NAF |
| TaSnRK3.51 | 477                | 53,260.86   | 9.43           | 80.57           | -0.388  | chlo         | PKinase & NAF |
| TaSnRK3.52 | 477                | 53,276.92   | 9.41           | 82.2            | -0.371  | chlo         | PKinase & NAF |
| TaSnRK3.53 | 431                | 47,128.62   | 8.82           | 96.68           | -0.08   | chlo         | PKinase & NAF |
| TaSnRK3.54 | 431                | 47,227.75   | 8.81           | 96.45           | -0.099  | chlo         | PKinase & NAF |
| TaSnRK3.55 | 431                | 47,146.69   | 8.66           | 96.91           | -0.071  | chlo         | PKinase & NAF |
| TaSnRK3.56 | 432                | 47,695.96   | 9.13           | 92.31           | -0.204  | mito         | PKinase & NAF |
| TaSnRK3.57 | 432                | 47,647.9    | 8.68           | 92.31           | -0.177  | mito         | PKinase & NAF |
| TaSnRK3.58 | 432                | 47,653.86   | 9.19           | 92.52           | -0.196  | mito         | PKinase & NAF |
| TaSnRK3.59 | 433                | 47,621.03   | 9.19           | 91.66           | -0.187  | mito         | PKinase & NAF |
| TaSnRK3.60 | 433                | 47,516.97   | 9.28           | 92.33           | -0.166  | cyto         | PKinase & NAF |
| TaSnRK3.61 | 432                | 47,421.87   | 9.51           | 90.51           | -0.185  | mito         | PKinase & NAF |
| TaSnRK3.62 | 449                | 50,551.69   | 6.59           | 89.02           | -0.337  | cyto         | PKinase & NAF |
| TaSnRK3.63 | 449                | 50,513.77   | 7.67           | 90.33           | -0.33   | cyto         | PKinase & NAF |
| TaSnRK3.64 | 370                | 41,272.64   | 9.38           | 91.16           | -0.209  | nucl         | PKinase & NAF |
| TaSnRK3.65 | 402                | 45,089.86   | 8.9            | 88.78           | -0.264  | nucl         | PKinase & NAF |
| TaSnRK3.66 | 446                | 50,054.68   | 8.87           | 90.07           | -0.214  | cyto         | PKinase & NAF |
| TaSnRK3.67 | 446                | 49,921.74   | 8.78           | 92.04           | -0.18   | cyto         | PKinase & NAF |
| TaSnRK3.68 | 462                | 51,797.34   | 9.15           | 82.53           | -0.429  | chlo         | PKinase & NAF |
| TaSnRK3.69 | 461                | 51,740.29   | 9.15           | 82.71           | -0.429  | chlo         | PKinase & NAF |
| TaSnRK3.70 | 453                | 50,950.38   | 9.16           | 83.31           | -0.434  | chlo         | PKinase & NAF |
| TaSnRK3.71 | 445                | 50,038.32   | 7.67           | 79.33           | -0.407  | chlo         | PKinase & NAF |
| TaSnRK3.72 | 443                | 49,798.06   | 7.23           | 80.14           | -0.388  | chlo         | PKinase & NAF |
| TaSnRK3.73 | 439                | 50,259.7    | 7.66           | 86.58           | -0.475  | chlo         | PKinase & NAF |
| TaSnRK3.74 | 439                | 50,259.7    | 7.66           | 86.58           | -0.475  | chlo         | PKinase & NAF |
| TaSnRK3.75 | 439                | 50,248.75   | 7.19           | 85.69           | -0.466  | chlo         | PKinase & NAF |
| TaSnRK3.76 | 447                | 50,443.98   | 8.69           | 86.4            | -0.371  | chlo         | PKinase & NAF |
| TaSnRK3.77 | 447                | 50,460.98   | 8.7            | 85.97           | -0.392  | chlo         | PKinase & NAF |
| TaSnRK3.78 | 447                | 50,532.98   | 8.6            | 84.88           | -0.414  | chlo         | PKinase & NAF |
| TaSnRK3.79 | 382                | 43,428.98   | 8.06           | 88.38           | -0.363  | chlo         | PKinase & NAF |
| TaSnRK3.80 | 443                | 50,260.79   | 8              | 85.21           | -0.381  | chlo         | PKinase & NAF |

Continued

| Gene id    | No. of amino acids | Mol. weight | Theoretical pI | Aliphatic index | (GRAVY) | localization | Domains       |
|------------|--------------------|-------------|----------------|-----------------|---------|--------------|---------------|
| TaSnRK3.81 | 449                | 50,983.68   | 8.27           | 86.24           | -0.373  | chlo         | PKinase & NAF |
| TaSnRK3.82 | 439                | 48,837.15   | 8.17           | 98.15           | -0.181  | pero         | PKinase & NAF |
| TaSnRK3.83 | 446                | 49,653.72   | 6.19           | 96.61           | -0.224  | nucl         | PKinase & NAF |
| TaSnRK3.84 | 529                | 59,022.41   | 8.38           | 87.88           | -0.233  | cyto         | PKinase & NAF |
| TaSnRK3.85 | 417                | 47,498.45   | 7.57           | 86.02           | -0.432  | cyto         | PKinase & NAF |
| TaSnRK3.86 | 417                | 47,486.49   | 7.58           | 85.78           | -0.43   | cyto         | PKinase & NAF |
| TaSnRK3.87 | 451                | 51,041.42   | 6.84           | 85.81           | -0.416  | cyto         | PKinase & NAF |
| TaSnRK3.88 | 471                | 52,048.56   | 7.68           | 90.91           | -0.263  | cyto         | PKinase & NAF |
| TaSnRK3.89 | 466                | 51,625.04   | 6.95           | 91.89           | -0.261  | chlo         | PKinase & NAF |
| TaSnRK3.90 | 448                | 49,648.95   | 6.95           | 94.26           | -0.229  | cyto         | PKinase & NAF |
| TaSnRK3.91 | 465                | 51,705.23   | 6.74           | 91.25           | -0.277  | cyto         | PKinase & NAF |
| TaSnRK3.92 | 466                | 51,766.51   | 7.65           | 93.11           | -0.243  | cyto         | PKinase & NAF |
| TaSnRK3.93 | 466                | 51,548.11   | 7.22           | 90.82           | -0.238  | cyto         | PKinase & NAF |
| TaSnRK3.94 | 466                | 51,838.48   | 7.64           | 91.24           | -0.267  | cyto         | PKinase & NAF |
| TaSnRK3.95 | 480                | 53,041.06   | 6.9            | 94.9            | -0.159  | cyto         | PKinase & NAF |

**Table 1.** List of the identified TaSnRK family members in *T. aestivum*.

**Analysis of chromosomal location and orthologous genes in *T. aestivum*.** The chromosomal distribution of all *TaSnRK* genes across the genome was investigated which provides useful information on the genomic regions (Fig. 3). The A sub-genome had 56 *TaSnRK* genes, comprising 2 genes from TaSnRK1 subfamily, 21 genes from TaSnRK2 subfamily, and 33 genes from TaSnRK3 subfamily, whereas the B sub-genome had 61 genes, comprising 8 genes from TaSnRK1 subfamily, 21 of TaSnRK2 subfamily, and 32 of TaSnRK3 subfamily. However, 4 genes of TaSnRK1 subfamily, 23 genes of TaSnRK2 subfamily, and 30 genes of TaSnRK3 subfamily were found on the D sub-genome (Supplementary Fig. S2A). These findings suggested that TaSnRK genes were distributed randomly throughout the A, B, and D chromosomes (Supplementary Fig. S2B).

In this study, 57 orthologous pairings were identified between *T. aestivum* and *H. vulgare*, while 166 between *T. aestivum* and *A. thaliana*. However, 53 orthologous genes were found within wheat species for instance between *T. aestivum* and *T. urartu*, while 102 orthologous genes between *T. aestivum* and *Ae. dicoccoides* and 63 between *T. aestivum* and *Ae. tauschii* (Supplementary Table S3).

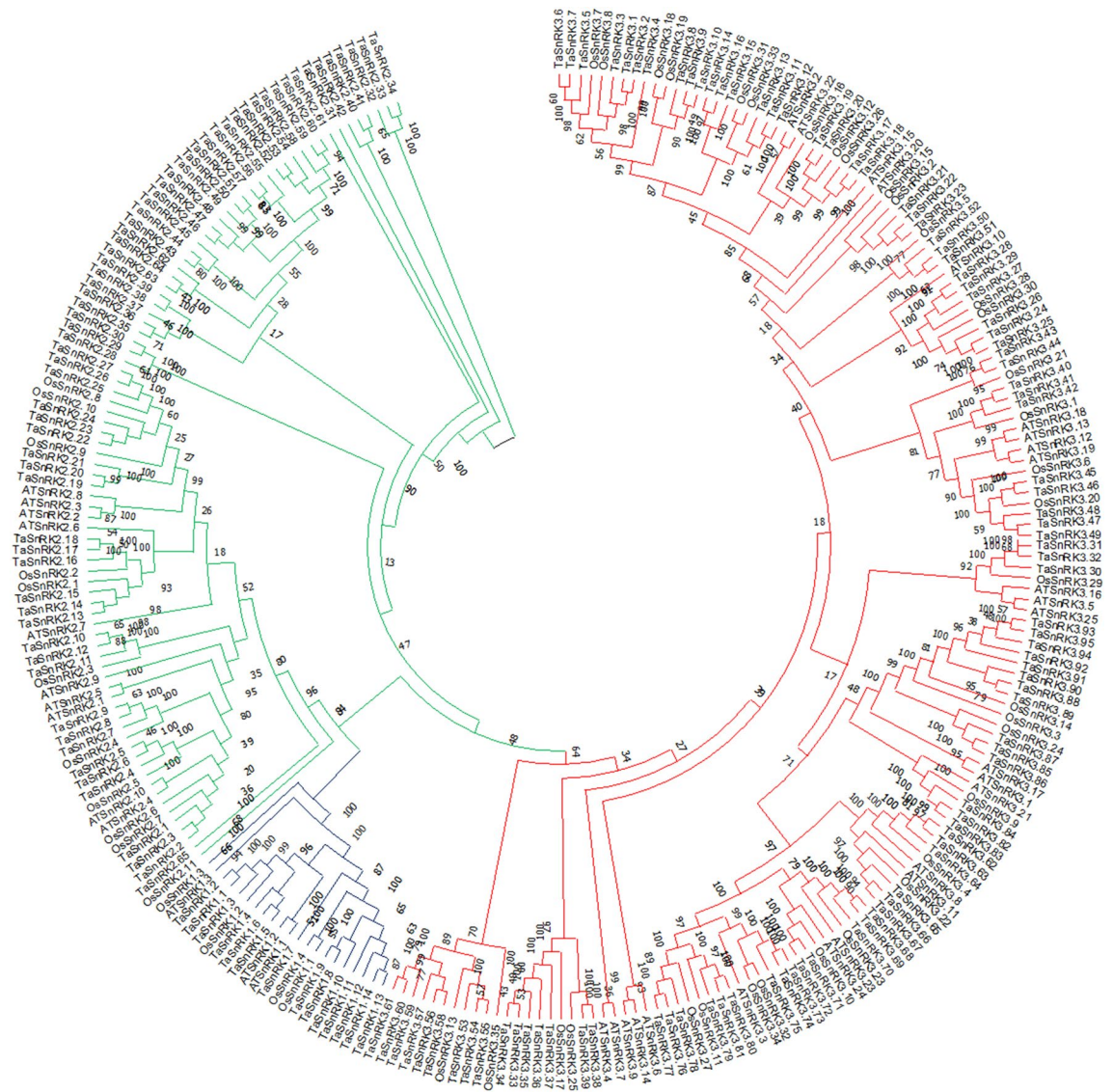
We identified 11 segmental events across different chromosomes and 2 tandem duplication occurrences in the same chromosome using the BLAST and MCScanX techniques. The findings revealed that gene duplication may have produced some *TaSnRK* genes, and that segmental duplication events were important in the growth of *TaSnRK* genes in the wheat genome. We also looked into the frequency of tandem duplication occurrences. There were 45 *TaSnRK* gene pairs found in this area all of which were strongly related. (Supplementary Table S4). However, the identities of these were > 80%, indicating that they were included into tandem duplication occurrences.

We looked at the duplication events of the *TaSnRK* gene in the wheat genome since gene duplication has a big impact on the emergence of new functionalities and gene families. In addition, 55 gene pairs were found to be duplicated as shown in Fig. 4.

Furthermore, the synteny of *SnRK* gene pairs between *T. aestivum* genome and *A. thaliana* genome was performed. The result showed that 166 *SnRK* genes of *T. aestivum* exhibiting syntenic relationship with *AtSnRK* genes (Fig. 5 and Supplementary Table S3), suggesting that these genes might have contributed to the evolution of *TaSnRK* gene family.  $K_s$  values,  $K_a$  values,  $K_a/K_s$  ratios and divergence time of paralogous and orthologous on *SnRK* family genes were estimated to assess the evolutionary constraints undertaken (Supplementary Table S4). The  $K_a/K_s$  ratios of the majority of segmentally duplicated *TaSnRK* gene pairs were < 1, the mean values of TaSnRK3 gene pairs ( $K_a/K_s = 0.30$ ) and TaSnRK2 ( $K_a/K_s = 0.35$ ) were lower than TaSnRK1 ( $K_a/K_s = 0.41$ ). Furthermore, segmental gene divergence time spans from 18.76 to 34.97 Mya. These findings showed that the *TaSnRK* gene family may have been subjected to significant purifying selection during evolution.

**Promoter analysis.** PlantCARE was used to look at *cis*-elements (1.5 kb upstream from ATG) in order to better understand the function and regulatory processes of *TaSnRK* genes. We found 129 out of 174 *TaSnRK* genes had *cis*-elements (Supplementary Fig. S3, Supplementary Table S5). MyB, ABRE, and LTR *cis*-elements were found to be involved in drought, ABA, and low-temperature responses. Auxin (9.77%), MeJA (51.72%), and Gibberallin (11.49%) *cis*-elements were found in phytohormones. It was also shown that most genes have many *cis*-element types. In addition, the TaSnRK1 (30.50) family had more *cis*-elements than the TaSnRK2 (19.29) or TaSnRK3 (23.81) families (Supplementary Table S5). Finally, the *cis*-elements study revealed that most *TaSnRK* genes can respond to a variety of environmental challenges, and that distinct subfamily genes can be regulated in various ways.

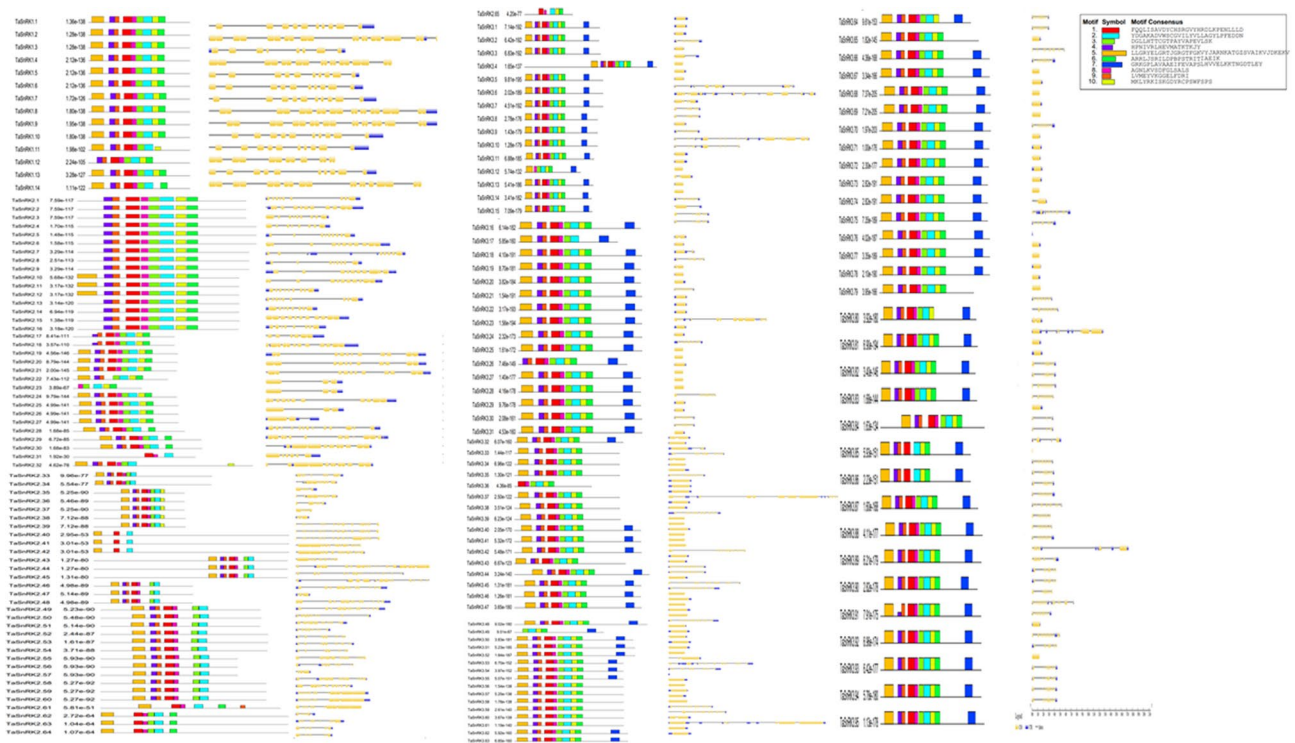
**MicroRNAs targeting TaSnRK.** The role of miRNAs in controlling the expression of *TaSnRK* genes have been investigated using the psRNA Target server. We predicted 16 *TaSnRK* genes as possible targets for eight different miRNAs (Table 2). Tae-miR319 implicated in the regulation of seven *TaSnRK* genes (TaSnRK3.33,



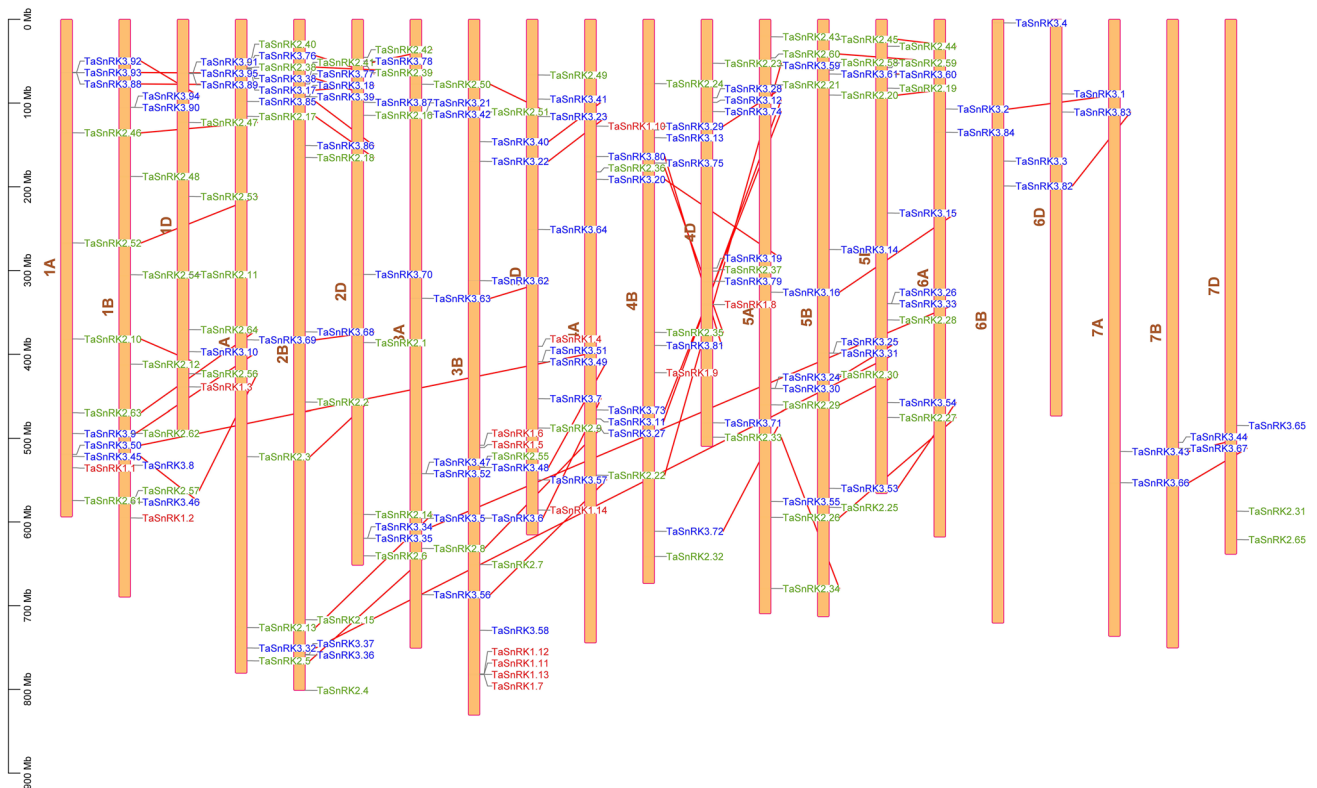
**Figure 1.** Phylogenetic tree of *SnRK* genes from *T. aestivum*, *A. thaliana*, and *O. sativa*. 174 *SnRK* genes from wheat, 38 from *Arabidopsis* and 50 from rice were clustered into three subgroups (SnRK1, SnRK2 and SnRK3). Wheat SnRK1, 2 and 3 subgroups are denoted as blue, green and red, respectively. The tree was generated using MEGA X software using the neighbor-joining method.

TaSnRK3.36, TaSnRK2.37, TaSnRK2.35, TaSnRK2.36, TaSnRK3.38, TaSnRK2.39). Tae-miR408 accounted for regulating expression of three *TaSnRK* genes (TaSnRK3.63, TaSnRK3.64, TaSnRK3.62). The expression of *TaSnRK* genes (TaSnRK1.3, TaSnRK1.2, and TaSnRK2.39, TaSnRK2.38) may be influenced by the Tae-miR164 and Tae-miR167b (Table 2). Tae-miR1119 and Tae-miR160 were predicted to regulate the expression of TaSnRK3.38 and TaSnRK3.74, respectively.

**Functional annotation of Hub genes and interacting network analysis.** Based on their involvement in a biological and cellular process, we studied the function of potential hub genes. String database contains a collection of predicted and experimentally confirmed protein–protein interactions in wheat and other species. However, wheat SnRK found in string database is linked to them as well as numerous metabolic and regulatory processes. SnRK were defined based on the interaction observed. *TaSnRK2.48* is included in the first, which serves as the network's (Fig. 6). Here, the cluster is directly linked to TaSnRK3 subfamily (*TaSnRK3.7*, *TaSnRK 3.14*, *TaSnRK 3.24*, *TaSnRK3.32*, *TaSnRK3.33*, *TaSnRK3.38*, *TaSnRK3.41*, *TaSnRK3.44*, *TaSnRK3.55*, *TaSnRK3.62*, *TaSnRK3.71*, *TaSnRK3.74*, *TaSnRK3.81*, *TaSnRK3.82*, *TaSnRK3.84* and *TaSnRK3.93*). These genes involved in Ca-dependent processes like as autophagy and maintenance. The second interaction with SnRK 2 subfamily (*TaSnRK2.12*, *TaSnRK2.27* and *TaSnRK2.36*) was primarily linked to OST. Whereas the primary role of OST is to act as an activator of the abscisic acid (ABA) signalling pathway, which governs several ABA responses including stomata closure in response to dehydration, plant diseases, or changes in atmospheric relative humidity (RH). Pathogen-associated molecular patterns (PAMPs) (e.g. flg22 and LPS) of pathogenic bacteria such as *P.syringae* pv. tomato (Pst) and *E. coli* O157:H7 are required for stomata closer. *TaSnRK 2.28* and

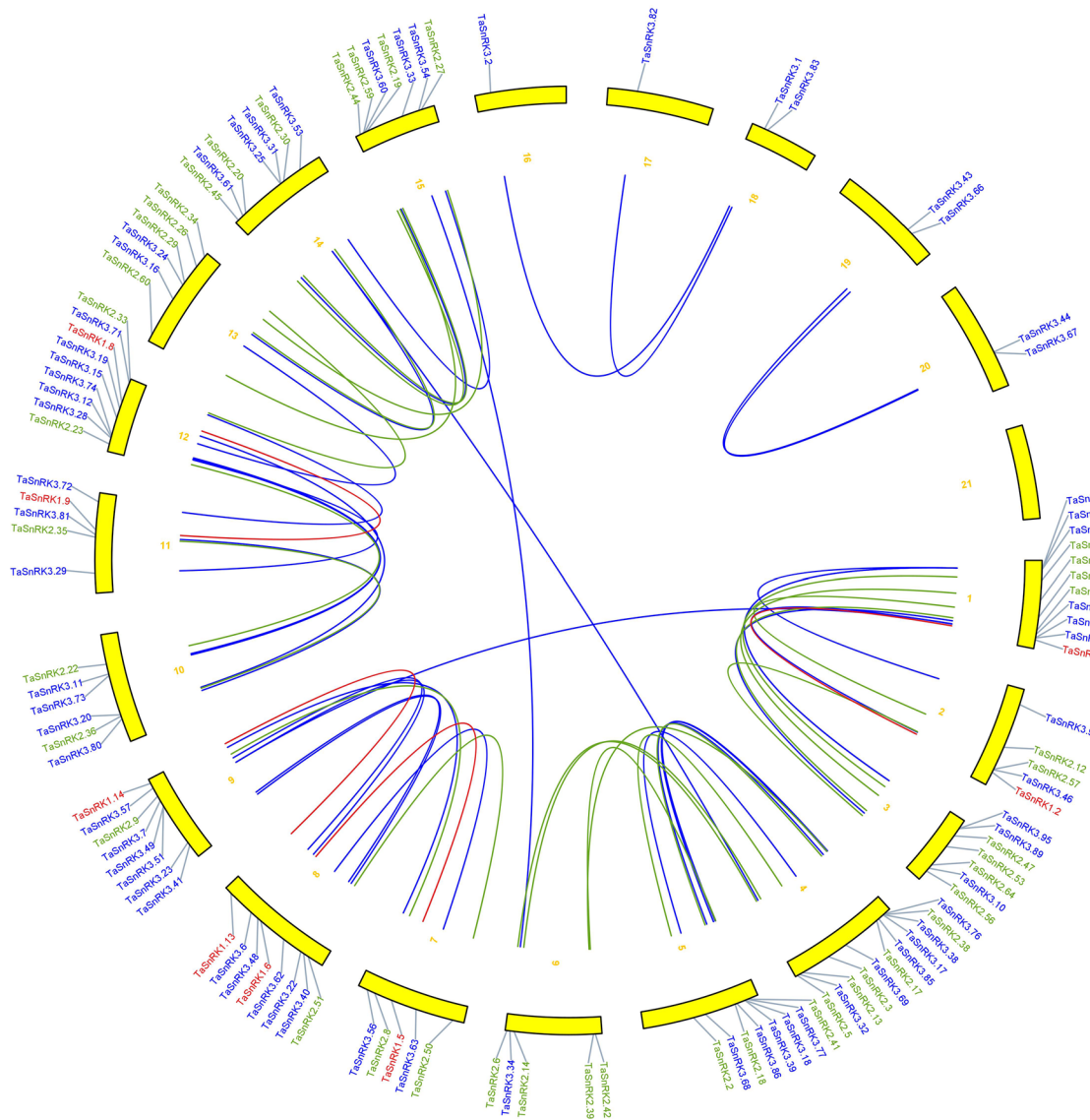


**Figure 2.** (A) Arrangement of ten conserved motifs in the *TaSnRK* genes following MEME analysis. Different colored boxes represent different motifs and their position in each sequence of *TaSnRK* genes. (B) Gene structure of wheat SnRK. Exons are indicated in yellow rectangles and grey line connecting two exons represent introns.

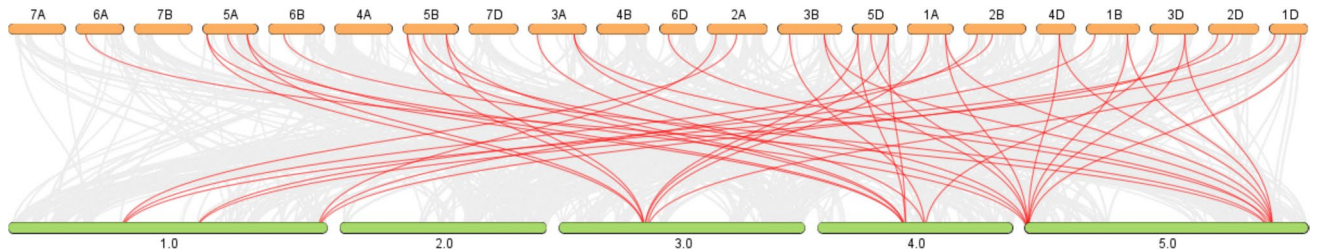


**Figure 3.** Distribution of *TaSnRK* genes on the 21 chromosomes of wheat and within the three sub-genomes. Black lines representing the gene pairs. Physical map showing the chromosomal distribution, with position on the left side scale bar and the name of the gene on right side.





**Figure 4.** The synteny analysis of TaSnRK family in *T. aestivum*. Different colours represent SnRK subfamilies red lines indicate duplicated TaSnRK1 subfamily gene pairs, green lines indicated TaSnRK2 subfamily gene pairs and blue represented TaSnRK3 subfamily. The chromosome number is indicated at the bottom of each chromosome.



**Figure 5.** Synteny analysis of SnRK genes between *Triticum aestivum* (Orange colour) and *Arabidopsis thaliana* (green colour). Gray lines in the background indicate the collinear blocks within *T. aestivum* and *A. thaliana*, while the red lines highlight the syntenic SnRK gene pairs.

| miRNA_Acc   | Target_Acc | Target_start | Target_end | miRNA_aligned_fragment       | Target_aligned_fragment      | Inhibition  |
|-------------|------------|--------------|------------|------------------------------|------------------------------|-------------|
| tae-miR167b | TaSnRK2.39 | 233          | 253        | UGAAGCUGACAGCAU<br>GAUCUA    | AUGAUCCUGCGGUCA<br>UCUUCA    | Translation |
|             | TaSnRK2.38 | 1610         | 1630       | UGAAGCUGACAGCAU<br>GAUCUA    | AUGAUCCUGCGGUCA<br>UCUUCA    | Translation |
| tae-miR408  | TaSnRK2.65 | 876          | 896        | CUGCACUGCCUCUUC<br>CCUGGC    | AGCACCCGAGGAGGCA<br>GAGCAG   | Cleavage    |
| tae-miR1119 | TaSnRK3.38 | 50           | 73         | UGGCACGGCGUGAUG<br>CUGAGUCAG | AGUGAGCAGCAGCGC<br>GUCGUGUUA | Cleavage    |
| tae-miR160  | TaSnRK3.74 | 332          | 352        | UGCCUGGCUCUUCU<br>AUGCCA     | AGGCAGGCAGGCAGG<br>CAGGCA    | Translation |
| tae-miR319  | TaSnRK3.33 | 905          | 925        | UUGGACUGAAGGGAG<br>CUCUCCU   | AUGGAGCACCCUUGG<br>GUACAA    | Cleavage    |
|             | TaSnRK3.36 | 898          | 918        | UUGGACUGAAGGGAG<br>CUCUCCU   | AUGGAGCACCCUUGG<br>GUACAA    | Cleavage    |
|             | TaSnRK2.38 | 2022         | 2042       | UUGGACUGAAGGGAG<br>CUCUCCU   | GAGGUACUCCUUUCA<br>GACCAA    | Cleavage    |
|             | TaSnRK2.39 | 645          | 665        | UUGGACUGAAGGGAG<br>CUCUCCU   | GAGGUACUCCUUUCA<br>GACCAA    | Cleavage    |
|             | TaSnRK2.37 | 1269         | 1289       | UUGGACUGAAGGGAG<br>CUCUCCU   | GAGGUACUCCUUUCA<br>GACUAA    | Cleavage    |
|             | TaSnRK2.35 | 1219         | 1239       | UUGGACUGAAGGGAG<br>CUCUCCU   | GAGGUACUCCUUUCA<br>GACUAA    | Cleavage    |
|             | TaSnRK2.36 | 1031         | 1051       | UUGGACUGAAGGGAG<br>CUCUCCU   | GCGGUACUCCUUUCA<br>GACUAA    | Cleavage    |
| tae-miR164  | TaSnRK1.3  | 1677         | 1697       | UGGAGAAGCAGGGCA<br>CGUGCA    | GACCUGUGUUCUGCC<br>UUUCUA    | Cleavage    |
|             | TaSnRK1.2  | 1579         | 1599       | UGGAGAAGCAGGGCA<br>CGUGCA    | GACCUGUGUUCUGCC<br>UUUCUA    | Cleavage    |
| tae-miR408  | TaSnRK3.63 | 184          | 204        | CUGCACUGCCUCUUC<br>CCUGGC    | GGAGGGGGAGGGGCG<br>CUGCGG    | Cleavage    |
|             | TaSnRK3.64 | 101          | 121        | CUGCACUGCCUCUUC<br>CCUGGC    | GGAGGGGGAGGGGCG<br>CUGCGG    | Cleavage    |
|             | TaSnRK3.62 | 120          | 140        | CUGCACUGCCUCUUC<br>CCUGGC    | GGAGGGGGAGGGGCG<br>CUGCGG    | Cleavage    |

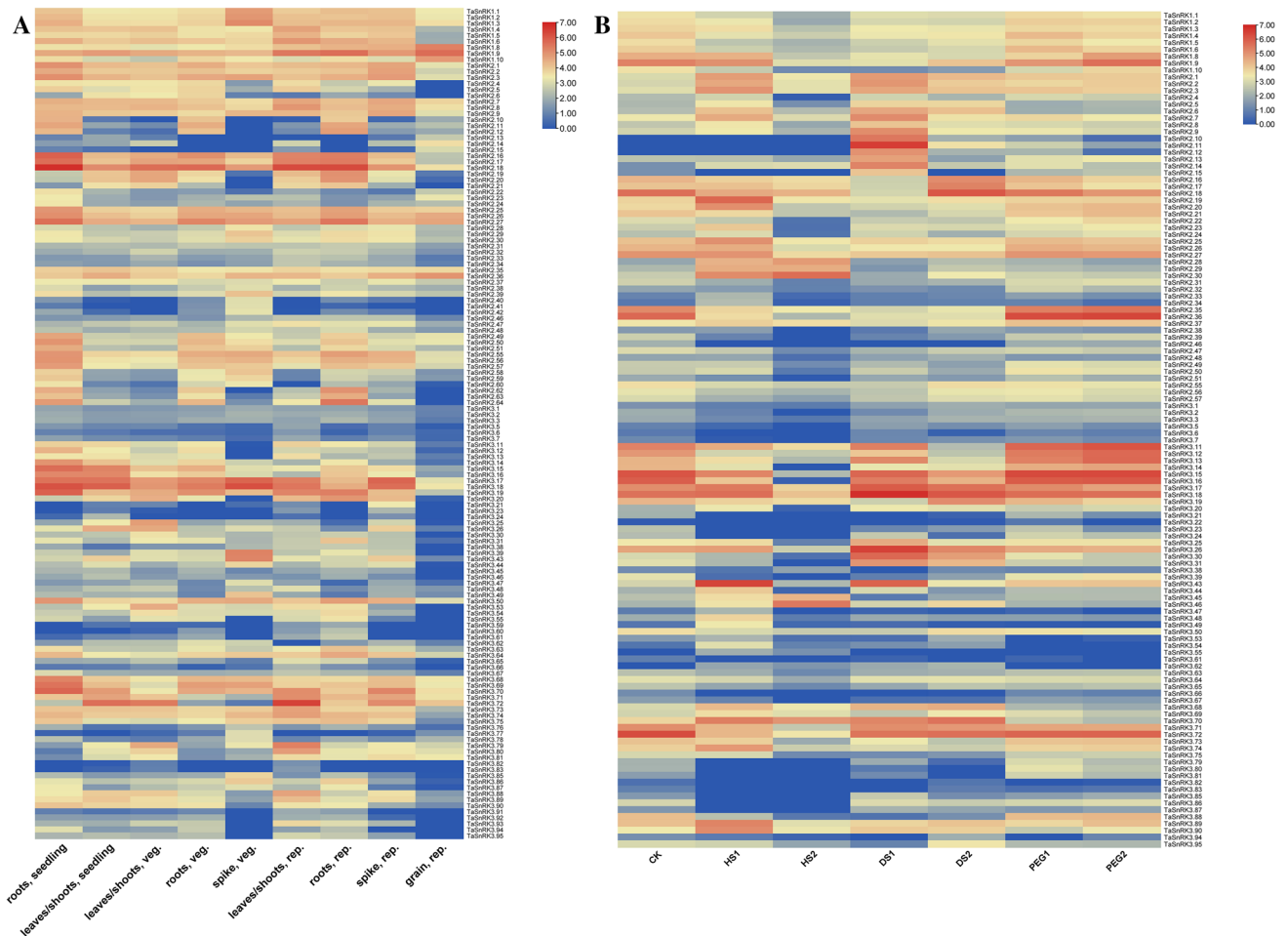
**Table 2.** Prediction Tae-MIR genes and their targets by using the psRNATarget server with default parameters.

*TaSnRK1.9* have a direct connection that plays a crucial function in fatty acid synthesis. Further, there is no direct evidence of a direct interaction between *TaSnRK2.61* and *TaSnRK2.48* (Fig. 6). Apart from that, *TaSnRK2.61* is the network's second hub gene, which mainly interacts with SnRK2 subfamily, such as (*TaSnRK2.16*, *TaSnRK2.28*, *TaSnRK2.31*, *TaSnRK2.38* and *TaSnRK3.71*), which are expressed preferentially in guard cells and appear to be involved ABA signalling mediating by reactive oxygen species.

**Expression profile of SnRK genes in different tissues under abiotic stress.** To understand more about how *TaSnRKs* are involved in development and stress responses, we utilized the expVIP database to retrieve TPM values for all *TaSnRKs* from experiments including abiotic stress and various developmental stages. These TPM data were used to generate Heatmaps. The expression patterns of 174 *TaSnRK* genes in various wheat tissues were evaluated.

Five different tissues at three different developmental stages were taken for this study. The time points are the Zadoks scale (Z-scale). Different *TaSnRK* sub-families show differential induction in different tissues (Fig. 7A). *TaSnRKs* such as *TaSnRK1.1*, *TaSnRK1.2*, *TaSnRK1.3*, *TaSnRK1.4*, *TaSnRK1.5*, *TaSnRK1.6*, *TaSnRK1.8*, *TaSnRK1.9* and *TaSnRK1.10*, *TaSnRK2.1*, *TaSnRK2.2*, *TaSnRK2.3*, *TaSnRK2.4*, *TaSnRK2.5*, *TaSnRK2.6*, *TaSnRK2.7*, *TaSnRK2.8*, *TaSnRK2.9*, *TaSnRK2.10*, *TaSnRK2.11*, *TaSnRK2.12*, *TaSnRK2.16*, *TaSnRK2.17*, *TaSnRK2.18*, *TaSnRK2.25*, *TaSnRK2.26*, *TaSnRK2.27*, *TaSnRK2.50*, *TaSnRK2.51*, *TaSnRK2.55*, *TaSnRK2.56*, *TaSnRK2.57*, *TaSnRK2.62*, *TaSnRK2.64*, *TaSnRK3.16*, *TaSnRK3.17*, *TaSnRK3.18*, *TaSnRK3.19*, *TaSnRK3.30*, *TaSnRK3.31*, *TaSnRK3.43*, *TaSnRK3.44*, *TaSnRK3.50*, *TaSnRK3.53*, *TaSnRK3.54*, *TaSnRK3.55*, *TaSnRK3.68*, *TaSnRK3.69*, *TaSnRK3.70*, *TaSnRK3.71*, *TaSnRK3.72*, *TaSnRK3.86*, *TaSnRK3.87*, *TaSnRK3.89*, *TaSnRK3.90* and *TaSnRK3.95* showed induction in spike tissues at Z39 stage. Whereas, *TaSnRK1.1*, *TaSnRK1.2*, *TaSnRK1.3*, *TaSnRK1.4*, *TaSnRK1.5*, *TaSnRK1.6*, *TaSnRK1.8*, *TaSnRK1.9* and *TaSnRK1.10* etc. were up regulated in leaves tissues at Z75 stage. Multiple *TaSnRKs* were induced at different spike stages especially at Z39 stage. Forty genes of *TaSnRK3* subfamily were induced in root tissues at Z10 stage. This suggests different *TaSnRKs* might be involved in development of different tissues at different stages. Many *TaSnRKs* showed induction at the dough development stage in wheat such as *TaSnRK1.1*, *TaSnRK1.2*, *TaSnRK1.3*, *TaSnRK1.8* to, *TaSnRK1.9* and *TaSnRK1.10*, *TaSnRK2.1*, *TaSnRK2.2*, *TaSnRK2.3*, *TaSnRK2.7*, *TaSnRK2.8*, *TaSnRK2.9*, *TaSnRK2.10*, *TaSnRK2.11*, *TaSnRK2.12*, *TaSnRK2.14*, *TaSnRK2.15*, *TaSnRK2.16*, *TaSnRK2.17*, *TaSnRK2.18*, *TaSnRK2.25*, *TaSnRK2.26*, *TaSnRK2.27*, *TaSnRK2.35*, *TaSnRK2.36*, *TaSnRK2.37*, *TaSnRK2.38*, *TaSnRK2.39*, *TaSnRK2.49*, *TaSnRK2.50*, *TaSnRK2.51*, *TaSnRK2.55*, *TaSnRK2.56*, and *TaSnRK2.57*. However, the expression of *TaSnRK3* subfamily was down regulated in Z85 stage





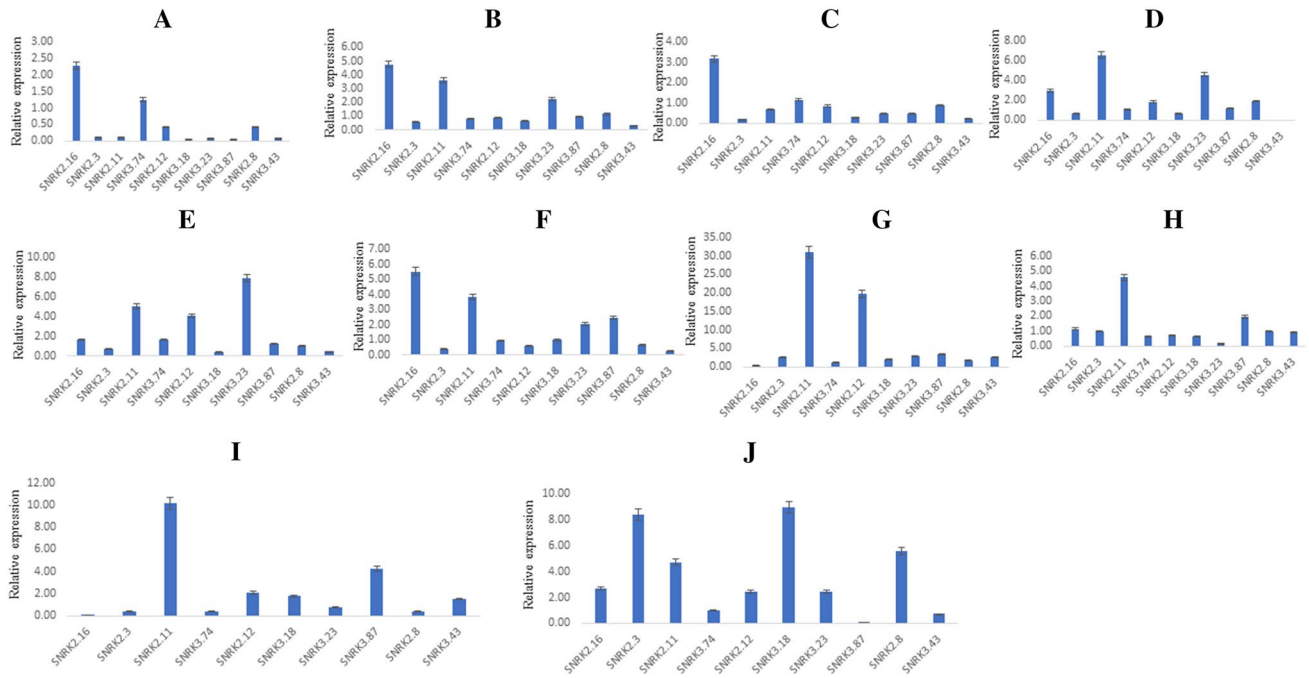
**Figure 7.** Heatmap of *TaSnRK* genes (A) tissue specific expression in leaf, root, shoot and spike at seedling, vegetative and reproductive stages (B) abiotic stress specific expression under drought, heat and osmotic stress at seedling stage. The color scale represents  $\log_2$  expression values. Ck-control; HS1-heat stress at 1hr; HS2-heat stress at 6hr; DS1- drought stress at 1hr; DS2- drought stress at 6hr; PEG1- osmotic stress at 2hr; PEG2- osmotic stress at 12 hr.

expression was tenfold up-regulated, but *TaSnRK3.87* expression was fourfold up-regulated (Fig. 8I), whereas *TaSnRK2.3*, *TaSnRK2.11*, *TaSnRK3.18*, and *TaSnRK2.8* was more than fourfolds (Fig. 8J) in WL711. Abiotic tolerance in wheat might be improved by utilizing a higher expression level of *TaSnRK2.11* which has been shown to increase under salinity, drought, and heat stress conditions. We analysed the RNA-seq and qRT-PCR data of *SnRK* genes to determine the magnitude of RNA transcription level (Fig. 9). A total of ten transcripts were chosen for this. The results revealed that the log fold-change values of these selected transcripts were found comparable in correspondence with RNA-seq results (Fig. 9A,B).

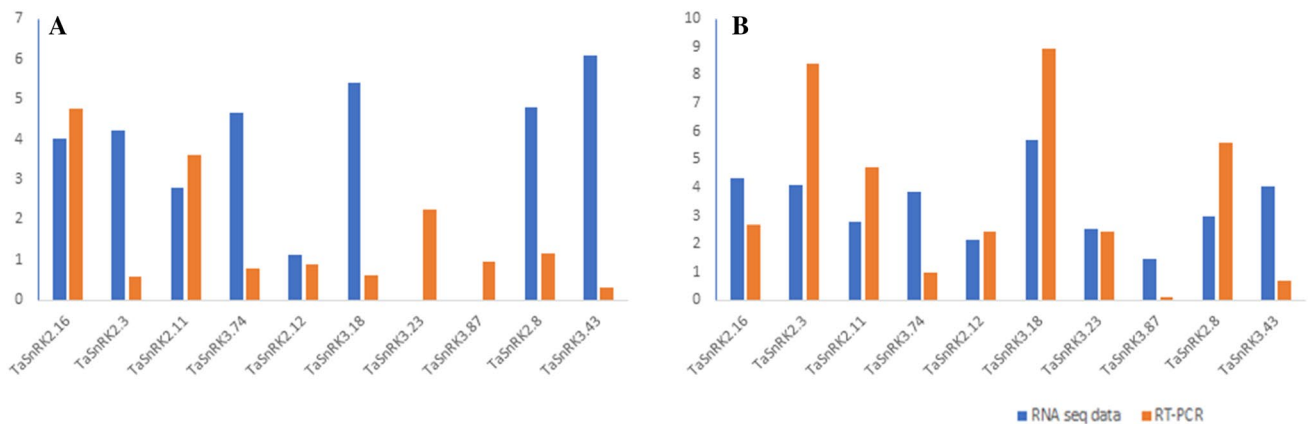
## Discussion

This study identified 174 *TaSnRK* genes in the *T. aestivum* genome, which were classified as *TaSnRK1*, *TaSnRK2*, or *TaSnRK3* based on their subfamily classification. The *TaSnRK* gene family was carefully searched, including evolutionary linkages, protein patterns, gene architectures, gene duplication, distributions of chromosomal, and the promoter *cis*-elements. This research progresses towards the future functionality of *SnRK* genes in order to improve abiotic stress adaption of plant. *SnRK* genes were already reported in *A. thaliana*<sup>4</sup>, *O. sativa*<sup>27</sup>, *B. distachyon*<sup>28</sup>, and *E. grandis*<sup>29</sup> having 39, 48, 44, and 34, respectively. The number of *TaSnRK* genes in the genome of *T. aestivum* is substantially higher than in diploid plants. There were 14 *TaSnRK1* genes, 65 *TaSnRK2* genes, and 95 *TaSnRK3* genes were discovered and classified into three subfamilies. According to more detailed description, *T. aestivum* and other species have similar member proportions for each subfamily. *T. aestivum* is a naturally occurring amphidiploid evolved from *T. urartu* (AA), *A. speltoides* (BB), and *A. tauschii* (DD). There were 56, 61, and 57 *TaSnRK* genes discovered in the A, B, and D sub-genomes, respectively demonstrating that *SnRK* genes had a similar functional role in progenitor species.

Although the conserved domains of the *SnRK* subfamily genes differ, the N-terminal protein kinase domain is retained. It has been discovered that *SnRK3* subfamily genes interact with CBLs in a calcium-dependent manner due to the NAF domain. Furthermore, the NAF domain identifies a set of heterologous kinases that CBL calcium sensor protein targets and participate in a range of signalling cascades<sup>8</sup>. According to this study, distinct



**Figure 8.** qRT-PCR based differential expression analysis of 10 *TaSnRK* genes under abiotic stress. Heat stress treatment at 37 °C (A) HS240, & (B) RAJ3765; Heat stress treatment at 42 °C (C) HS240, & (D) RAJ3765; Salt stress treatment at 150 mM NaCl (E) Kharchia65, (F) HD2687 at 12 h and (G) Kharchia 65, (H) HD 2687 for 24 h; drought stress at 25% (v/v) PEG treatment (I) C306 and (J) W1711 for 24 h. The error bars indicate the standard deviation.



**Figure 9.** Comparison between magnitude of gene expression of RNA-seq and qRT-PCR data of heat stress (A) and drought stress (B).

*TaSnRKs* subfamily genes shared different types of conserved domains. This might imply that the *TaSnRK* genes family is functionally diverse based on the domains contained.

In the *AtSnRK* and *TaSnRK* genes, certain subfamily genes showed substantial structural exon–intron divergences and gene length differences. In genes with fewer introns, increased expression in plants has been previously reported<sup>30,31</sup>. A compact gene structure with few introns has enabled rapid activation and responsiveness to different environmental conditions<sup>31</sup>. However, when the transcriptome data used in this study was combined, we found no evidence that *TaSnRK* gene with fewer introns had shown higher expression levels than the other *TaSnRKs* genes.

According to accumulating data, gene activity was often linked to discrepancies in the promoter region<sup>32</sup>. In gene promoter regions, *cis*-elements played a major role in controlling gene expression during development and environmental changes<sup>33,34</sup>. *TaSnRKs* had several *cis*-elements, including growth hormones, MyB, ABRE, and LTR according to promoter analysis in this investigation. Most gene promoters contained at least one of these components, demonstrating that many *TaSnRKs* were capable of responding to a variety of abiotic stresses while

also promoting growth. When gene expression profiles from TaSnRKs with MyB and ABRE *cis*-elements were merged under drought stress, TaSnRKs with MyB and ABRE *cis*-elements increased by an average of 6.3-fold, but TaSnRKs without *cis*-elements only increased by 3.5-fold. As a result, *cis*-elements analysis can help with gene function studies, particularly gene expression patterns under different stress conditions.

*TaSnRK* gene expression levels were analysed using transcriptome data in different tissues and organs of *T. aestivum*<sup>26</sup>. The research results showed that expression patterns of these genes are divided into three categories (Fig. 7). In this study, it was found that subgroup-2 TaSnRKs contain fewer *cis*-elements than TaSnRKs in subgroups-1 and -3 in their promoters. Every gene in subgroup-1 has an average of 5.08 Auxin, 13.55 MeJA, and 8.47 ABRE, 1.69 MYB, 3.38 LTR compared with each gene in subgroup3 and averages of 2.05 in Auxin, 11.08 of MeJA, 1.43 of Gibberellins and 6.67 ABRE, 1.43 MYB, 0.61 LTR, each gene in subgroup 2 with a total of 0.35 Auxin, 9.12 MeJA, 4.56 Gibberellin, 3.50 ABRE, 1.40 MYB, 0.35 LTR. These data showed that *TaSnRK* activity is linked to promoter region differences.

The roles and functions of several TaSnRKs in response to different abiotic stresses were also determined. Drought stress findings showed that ABA production and *A. thaliana* signals in response to drought were orthologous to *AtSnRK2.3*<sup>39</sup> and imply that *TaSnRK2.11* and *AtSnRK2.3* play the same role in response to drought stress. The extreme expression changes in *T. aestivum* against effects of drought, salt, thermal stress and ABA induction were reported in *TaSnRK2.8*, *TaSnRK2.12*, *TaSnRK3.11*, *TaSnRK2.16*, *TaSnRK3.23* and *TaSnRK3.83* whereas its orthologs, *AtSnRK2.2*, could also respond to osmotic stress and ABA induction in *A. thaliana*. This shows that under different conditions the *TaSnRK2.11* gene may be activated substantially.

Previous research has shown that ABA-independent regulation of SnRKs occurs in terrestrial plants, such as Arabidopsis *SnRK2.1*, *SnRK 2.4*, *SnRK 2.5*, *SnRK 2.9*, and *SnRK 2.10*, which are activated by osmotic stress following an ABA-independent pathway<sup>7</sup>. Arabidopsis ABA-independent SnRKs control stress related gene/transcripts under hyperosmotic conditions, complementing the action of ABA-dependent SnRK2s function<sup>35</sup>. As reported in other plants, ABA-independent SnRK2s in wheat also showed sensitive reaction to osmotic stress. Specific responses to low nitrogen or sulfur deprivation<sup>36</sup>, however, appear to be initiated in an ABA-independent manner, as in other plants. These findings support the hypothesis that the plant-specific SnRK2 subfamily is important in stress response signalling both in Arabidopsis and wheat. These pathways are not solely responsible for energy-saving decisions, but they do resulting complex remodelling of cell metabolism, as evidenced by the interactions with DNA repair and maintenance pathways and TOR in Arabidopsis<sup>37</sup> network as indicated by STRING studies. The fact that a single stress induces the expression of a large number of SnRK2 genes implies that there is a significant compensating impact or pleiotropy within this family in wheat. It is well known that most stresses result in oxidative damage<sup>38,39</sup>. The third SnRK subfamily, the other hand, played an important role in response to osmotic, salt, and heat stresses, because it consists of proteins kinases interacting with calcineurin B-like calcium binding domains<sup>7</sup> which are mostly involved in drought and salt resistance, being the SOS (salt overly sensitive) mechanism being the best-known<sup>40</sup>.

The findings of this study provide a thorough description of the *SnRK* gene family in *T. aestivum*. It helps us to better understand the biological role of specific *TaSnRK* genes in *T. aestivum*. The study presented just a fundamental characterisation of the *TaSnRK* genes and a comprehensive functional validation would be required to hold the importance of the SnRK family.

## Conclusions

*SnRK* genes are involved in a variety of signalling pathways, including responses to biotic and abiotic stresses. In this study, *SnRK* gene family has been intensively investigated in wheat. A total of 174 *TaSnRK* genes were discovered and categorized into three subgroups based on motif composition and gene structural similarity within each subfamily. Phylogenetic analyses of *SnRK* genes in *A. thaliana* and *O. sativa* can also be used to derive the evolutionary characteristics of the *TaSnRK* genes. Furthermore, the TaSnRK family's microRNA targeting, *cis*-acting elements, and gene expressions were investigated in order to better understanding the biological role of *TaSnRK* genes in *T. aestivum*.

## Materials and methods

**Identification and characterization of SnRKs.** Protein sequences of SnRKs identified in related plant species were obtained from the Phytozome database (<http://www.phytozome.net/>) and the Rice Annotation Project (RAP) (<https://rapdb.dna.affrc.go.jp/>). BLASTP searches were performed against the bread wheat protein sequences ([ftp://ftp.ensemblgenomes.org/pub/plants/release-51/fasta/triticum\\_aestivum/pep/](ftp://ftp.ensemblgenomes.org/pub/plants/release-51/fasta/triticum_aestivum/pep/)) using an e-value cut-off of 0.0001 and bit-score > 100. Potential SnRK candidates were discovered using the methods described above. Following the removal of duplicate results, the final sequences were checked for the existence of SnRK related domains using HMMscan (<https://www.ebi.ac.uk/Tools/hmmer/search/hmmscan>), the SMART database (<http://smart.embl-heidelberg.de/>)<sup>41</sup> PFAM<sup>42</sup> NCBI CDD (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>)<sup>43</sup>. Additionally, tools from ExpASY (<http://www.expasy.ch/tools/pitool.html>) were used to compute the number of amino acids, molecular weights (MW), and isoelectric point (pI) of each SnRK protein.

**Phylogenetic analysis of SnRK genes family.** A multiple sequence alignment was performed with ClustalW with default settings<sup>44,45</sup> of 174 non-redundant TaSnRK amino acid. Using MEGA X and the Neighbor-Joining (NJ) technique<sup>45</sup>, a phylogenetic tree was created utilizing the poisson model, pairwise deletion, and 1000 replications of bootstrap. MEGA X has been used to create an unrooted NJ tree that includes all *A. thaliana* and *O. sativa* SnRKs protein sequences.

**Motif composition and gene structural analysis of the SnRK gene family in *T. aestivum*.** The Multiple Expectation Maximization for Motif Elicitation (MEME) online tool<sup>46</sup> (<http://meme.sdsc.edu/meme/itro.html>) was used to find conserved motifs in TaSnRK proteins with the following parameters: The number of repetitions is unlimited, the maximum number of motifs is ten, and the ideal motif length is six to one hundred residues. The exon–intron structures of TaSnRK family genes were analysed using the Gene Structure Display Server online application (GSDS v.2.0: <http://gsds.cbi.pku.edu.cn>) based on the gff3 data file<sup>47</sup>.

**Analysis of chromosomal location and orthologous genes in *T. aestivum*.** Using MapChart version 3.0<sup>48</sup>, the chromosomal coordinates of all TaSnRK genes were mapped to 21 chromosomes of the wheat genome based on physical location from the Plant ensemble database. All *T. aestivum* gene sequences were aligned using BLASTP with an e value of  $1e^{-10}$  to find gene duplication. The pattern of duplicated SnRK were classified as segmental, tandem duplications using MCScanX with default parameters<sup>49</sup>. A tandem duplication is defined as a chromosomal area of less than 200 kb that contains two or more genes<sup>50</sup>. TBtools were used to exhibit synteny relationships of the orthologous SnRK genes between *T. aestivum* and *A. thaliana*, the syntenic analysis maps and synonymous (Ks) and non-synonymous (Ka) substitution of each duplicated TaSnRK gene<sup>51</sup>.

**cis-Elements in promoter regions of TaSnRKs.** The wheat genome database was used to extract upstream sequences (1500 bp) from the start codon of each TaSnRK gene, which were subsequently utilised for cis-element distributions in promoter regions using PlantCARE software (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>)<sup>52</sup>.

**Prediction of MIR genes targeting TaSnRK.** The TaSnRK gene transcript sequences were obtained from the wheat genome database. The psRNATarget service was used to examine the matured miRNA sequences<sup>53,54</sup> and the TaSnRK transcript sequences with default settings<sup>55,56</sup>.

**Analysis of protein–protein interactions.** The STRING v1054 databases were used to identify protein–protein functional interactions.

SnRK protein sequences were uploaded to the STRING<sup>57</sup> application, and the database was searched with *A. thaliana* as the reference organism. All identified interaction partners were gathered and searched against the *A. thaliana* genome using blast software at e-value  $1-e10$ .

Using Cytoscape<sup>58</sup>, the one best-hit for each gene was selected for the creation of a PPI network. Finally, the top hub gene from the interaction network was predicted using Cytoscape's cytoHubba plugin (Cytoscape Consortium 2016).

**RNA-seq derived gene expression profiling.** The value of TPM (transcripts per million) for each TaSnRK was obtained from the expVIP database (<http://www.wheat-expression.com/>). Clustvis (<https://biit.cs.ut.ee/clustvis/>) was used to create heatmaps<sup>59</sup>.

**Plant material and growth conditions.** In this experiment, seeds of bread wheat genotypes (C-306, WL-711, RAJ3765, HS240, Kharchia 65, and HD2687) were procured from Germplasm Unit, ICAR-Indian Institute of Wheat and Barley Research, Karnal, India. Seeds were sterilised with 1% sodium hypochloride for 10 min and then rinsed with distilled water three times and germinated in petri plates at 22 °C under controlled conditions. Seedlings were moved to a culture bottle filled with full-strength Hoagland's solution after 5 days of germination and allowed to grow for seven days. Each genotype was seeded in two sets of three biological replications. For drought stress, two contrasting wheat genotypes, C306-drought tolerant and WL711, drought susceptible were used. After 14 days of growth in Hoagland's solution, one set of seedlings was given osmotic shock using 25% (v/v) polyethylene glycol (PEG) 6000 for 24 h, while untreated used as control<sup>60</sup>. Leaf samples from control and stressed seedlings were harvested at above mentioned time intervals for expression analysis. For heat stress, two contrasting wheat genotypes were chosen: RAJ3765-heat resistant and HS240-heat sensitive. These plants were kept at 42 °C for 3 h (at Basal), followed by at 37 °C for 3 h (at Normal), and finally at 42 °C for 3 h (at Acquired). At the time interval stated above, leaf samples from the basal and acquired stress levels were collected. Kharchia 65-salt resistant and HD2687-salt sensitive wheat genotypes were utilised to study salt stress. Two week old seedlings of both genotypes were subjected to 150 mM NaCl treatment. The leaf samples were collected 12 h and 24 h after the treatment. All obtained samples were wrapped in foil and immediately frozen in liquid nitrogen at – 80 °C for total RNA isolation.

**RNA isolation and real time PCR analysis.** RNA was extracted using TRIzol Reagent according to the manufacturer's instructions. To get DNA-free RNA, the extracted RNA was treated with DNase I (NEB, USA). The first strand cDNA synthesized from 1 g of total RNA by using Superscript-III reverse transcriptase (Invitrogen, USA). The cDNA was diluted to 1:2 for real-time qRT-PCR analysis, and 1.5 µL of the diluted cDNA was utilised as a template in a 20 µL reaction volume according to the manufacturer's instructions. Real time quantitative RT-PCR analysis was carried out by using the BIO-RAD CFX96 using SYBR Green (Bio-Rad). The endogenous control  $\beta$ -actin was used to standardise the expression data<sup>61</sup>. Using the  $2^{-\Delta\Delta Ct}$  method, the expression was measured as a relative fold change<sup>62</sup>. The error bars show the standard deviation of the three biological replicates' expression.

**Research statement.** Experimental research and field studies on plants complies with relevant institutional, national, and international guidelines and legislation.

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## Author contributions

P.S. conceived the study. S.M. and P.S. analysed the data, S.M., R.S., P.S. wrote the first draft. P.S., R.S., R.T., G.P.S. edited the manuscript. All authors have reviewed and approved the final manuscript.

## Competing interests

The authors declare no competing interests.

## Additional information

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