

Genome-wide identification and expression analysis of two-component system genes in switchgrass (*Panicum virgatum* L.)

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Abstract

Composed of Histidine Kinases (HKs), Histidine Phosphotransfer Proteins (HPs), and Response Regulators (RRs), the Two-Component System (TCS) plays an essential role in guiding plant growth, development, and reactions to different environmental factors. Although *TCS* genes have been extensively identified in a variety of plants, the genome-wide recognition and examination of TCS in switchgrass remain unreported. Accordingly, this study identified a total of 87 TCS members in the genome of switchgrass, comprising 20 HK(L)s, 10 HPs, and 57 RRs. Detailed analyses were also conducted on their gene structures, conserved domains, and phylogenetic relationships. Moreover, this study analysed the gene expression profiles across diverse organs and investigated their response patterns to adverse environmental stresses. Results revealed that 87 TCS genes were distributed across 18 chromosomes, with uneven distribution. Amplification of these genes in switchgrass was achieved through both fragment and tandem duplication. *PvTCS* members are relatively conservative in the evolutionary process, but the gene structure varies significantly. Various cis-acting elements, varying in types and amounts, are present in the promoter region of *PvTCS*, all related to plant growth, development, and abiotic stress, due to the TCS gene structure. Protein-protein interaction and microRNA prediction suggest complex interactions and transcriptional regulation among TCS members. Additionally, most TCS members are expressed in roots and stems, with some genes showing organ-specific expression at different stages of leaf and inflorescence development. Under conditions of abiotic stress such as drought, low temperature, high temperature, and salt stress, as well as exogenous abscisic acid (ABA), the expression of most TCS genes is either stimulated or inhibited. Our systematic analysis could offer insight into the characterization of the TCS genes, and further the growth of functional studies in switchgrass.

1. Introduction

The Two-Component System (TCS), which mediates protein phosphorylation, represents a primary signal transduction pathway regulating responses to adverse environmental factors in both bacteria and plants. This system was first identified in *Escherichia coli* [1, 2]; it comprises a membrane-associated histidine protein kinase (HK) and a cytoplasmic response regulator (RR) containing a receiver (REC) domain [3]. In eukaryotic species, including higher-order plants, a complex Two-Component System (TCS) signaling system has been discovered [4]. The three signal elements of plant TCS components are typically Hybrid Histidine Kinases (HKs), Histidine-Containing Phosphotransfers (HPs), and Response Regulators (RRs)[5]. Notably, under stressful conditions, the HK proteins mainly detect these stress signals, thus initiating an autophosphorylation process in the histidine residue (H) of the HK domain. Arabidopsis cytokinin signaling is a prime example of these TCS signal systems. AHK2, AHK3, and AHK4, three embedded histidine kinases, act as cytokinin receptors and show negative reactions to stress in the context of Arabidopsis cytokinin signaling [6]. The transmitter domain's conserved histidine residue triggers autophosphorylation of these kinases, which then transfer the phosphoryl groups to Histidine-containing Phosphotransfer Proteins (HPs) at a conserved aspartate residue. Ultimately, the HPs transmit the signal to the receiver domain in type-B Response Regulators (RRs). The phosphoryl transfer from the histidine of AHPs to the aspartate of the RD of B-ARRs enables the DNA binding capacity of the DBD, thereby allowing a rapid transcriptional response to cytokinin [7]. The promoter of type-A ARR may be influenced by these RRs, which could act as transcription factors that respond to a variety of environmental signals through various cis-elements, thus further influencing the expression of downstream stress-related genes [4–6]. To this point, Arabidopsis has been identified as the TCS system model, and the TCS gene has been found on a

genome-wide basis in various plant species, including rice [8, 9], maize [10], soybean [11], Chinese cabbage [12], tomato [13], cucumber [14], chickpea [15] and sweet potato [16], among others. The classifications and characterizations of Two-Component System (TCS) genes are well-established, with the model plant *Arabidopsis* offering the most extensively studied and understood TCS elements among plants. A genome-wide analysis revealed 55 TCS-related genes in *Arabidopsis*, including 16 AHKs, 6 AHPs, and 33 ARR. The *Arabidopsis* HKs consist of three subfamilies, namely: AHK, ethylene receptor (ETR/EIN), and phytochrome (PHY A-E). The AHK subfamily encompasses three cytokinin receptors (AHK2, AHK3, and AHK4), AHK1, AHK5, and CKI1. Noteworthy, AHPs (AHP1-AHP5) are characterized by a highly conserved XHGXKSSXS motif featuring a Histidine phosphorylation site. However, AHP6, lacking the conserved Histidine residue, is regarded as a pseudo-HP protein. Four subcategories of ARRs, namely type-A, type-B, type-C, and pseudo-RRs [5, 17], can be further divided by both conserved domains and phylogenetic analysis.

The TCS signaling pathway plays an important role in regulating plant growth and development and responding to various environmental stimuli, such as high salinity, drought, and inappropriate temperature [16]. The cytokinin signaling pathway exemplifies the Two-Component System (TCS) signaling system. Studies suggest that three cytokinin receptors act as positive regulators in the transduction of cytokinin signals. Notably, the *ahk2 ahk3 ahk4* triple mutant demonstrates decreased sensitivity to exogenous cytokinin, delayed root and leaf growth, and prompts reproductive developmental defects [8, 18, 19]. In addition, increasing evidence suggests that the TCS signaling pathway is involved in various stress responses. Dehydration induced the transcripts of AHK2, AHK3, and AHK4 quickly, and NaCl and ABA treatment caused an upregulation of AHK2 expression. The expression of AHK3 is also induced under high salinity and cold stress. Further functional studies indicate that these three cytokinin receptors serve as negative regulators in the osmotic stress responses of *Arabidopsis* [20]. Nevertheless, AHK1, a classic osmotic stress protein, is seen to be a positive regulator in both drought and salt stress [21, 22]. Numerous studies have shown that CKI1 is essential for central cell specification in Embryo sacs as well as for vascular patterning [10, 23–25]. AHK5 acts as a negative regulator to control root elongation in an ETR1-dependent ethylene and ABA signaling pathway [26]. Meanwhile, AHK5 positively influences salt sensitivity and enhances biotic pathogen resistance [27]. Similarly, TCS genes in rice, soybean, and tomato have been demonstrated to play a role in responding to drought, high salinity, and extreme temperature stresses [28, 29]. In rice, it was found that *OsAHP1/2* knockdown seedlings exhibited varied response patterns to salt and drought stresses [30]; Furthermore, *OsHK3* was found to participate in ABA-induced antioxidant defense [29]. The roles of certain Tomato Two-Component System (TCS) members in stress responses have been investigated. Notably, the pollens from the tomato 'never-ripe' (Nr) mutant, a histidine kinase mutant, exhibit greater sensitivity to heat stress, a condition that affects pollen carbohydrate metabolism [28]. In soybeans, the expression levels of most TCS genes are induced by water stress [31]. The phytochrome members PHYA, PHYB1, and PHYB2 - of tomatoes act as histidine kinases and are involved in drought stress responses [32]. All type-A ARRs are involved in osmosis-related stress responses through their interaction with abscisic acid (ABA), with either positive or negative effects [14]. Furthermore, numerous RR genes are induced by conditions of drought, high salinity, and low temperature [22, 33]. Among them, B-type response regulatory factor (B-ARR) is widely present in plants and plays a very important role in mediating cytokinin signal transduction [34]. In *Arabidopsis*, B-ARR is also related to sodium accumulation, root elongation, callus formation, salt and cold stress tolerance [35]. In addition, there is a type of pseudogene responsive regulator (PRRs) proteins whose N-terminal signal receiving domain does not have a conserved Asp residue and does not participate in cytokinin signal transduction, but functions in regulating the plant's biological clock [1, 36, 37].

Switchgrass (*Panicum virgatum* L.), a perennial C4 grass known for its high biomass yield, is considered a model bioenergy crop due to its high yield and low input requirements [38, 39]. However, studies on the TCS genes in switchgrass are still limited, and their respective functional roles have not yet been reported. In this research, the switchgrass genome was used to identify potential TCS genes, and their gene structures, conserved domains, phylogenetic connections, collinearity associations, cis-acting components, protein-protein interaction, and microRNA target forecasts were thoroughly scrutinized. Additionally, Quantitative real-time PCR analysis (qRT-PCR) was employed to ascertain the expression profiles of certain identified TCS genes in order to evaluate their reactions to various abiotic stresses and plant hormones. Our thorough examination of the TCS genes in switchgrass could potentially provide a basis for future research to elucidate the role of the TCS family genes in stress tolerance and hormone response in switchgrass.

2. Materials and methods

2.1 Identification of the TCS genes in the switchgrass genome

Obtained from the Phytozome (<https://phytozome.doe.gov>), the sequences of the TCS protein in switchgrass were obtained from the Phytozome (<https://phytozome.doe.gov>) databases. All the TCS members from *Arabidopsis* [5], rice (*Oryza sativa* L.) [8, 9, 40], maize (*Zea mays* L.) [10] and tomato (*Solanum lycopersicum* L.) [13] were obtained from previous studies. Meanwhile, the HMM profiles of the TCS members (HisK :PF00512, HATPase : PF02518, HPt :PF01627, and REC: PF00072) were downloaded from Pfam (<https://pfam.xfam.org/>). The TCS genes were searched with HMMER v. 3.0 (<https://hmmer.org/>) in the local switchgrass database. The threshold E-value was set to less than 0.01. All identified hits were verified using the online databases: SMART (Simple Modular Architecture Research Tool) and NCBI CD-search. The theoretical isoelectric point (PI) and molecular weight (MW) physicochemical properties of all confirmed TCS proteins were determined using the ExPASy ProtParam tool (http://web.expasy.org/compute_pi/). Subcellular localizations were predicted using SubLoc v1.0 website (http://www.bioinfo.tsinghua.edu.cn/SubLoc/eu_predict.htm)

2.2 Gene Structure Construction, Motif Analysis, and Phylogenetic Analysis

Gene Structure Display Server (<http://gsds.cbi.pku.edu.cn>) was utilized to map the exon-intron structures of all switchgrass TCS genes [41]. Each family motif was identified using the MEME program (<http://meme-suite.org/tools/meme>) [42]. These databases are accessed via <https://smart.embl-heidelberg.de/> and <https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>, respectively. The confirmation of the TCS genes was accomplished through multiple sequence alignments executed in ClustalX version 2.1 (<https://www.clustal.org/>). The redundant sequences were removed and a phylogenetic tree was constructed with MEGA11 by the neighbor-joining (NJ) method with 1000 bootstrap replications, a poisson model, and pairwise deletion [42]. Then, the phylogenetic tree was visualized using the EvolView online programmer [43].

2.3 Chromosomal distribution and synteny analysis

The positions of the TCS genes were extracted from JGI database (<https://phytozome-next.jgi.doe.gov/>) [44]. MCscanX was employed to analyse the synteny links using the gene positions as anchors [45]. Next, we visualized the synteny relationships of the TCS family within and between species in the switchgrass using TBtools software [46]. Tandem duplication events were defined as two or more neighboring genes on the same

chromosome. Segmental duplication events were defined as duplicate pairs located on different chromosomes [47]. By utilizing the Codeml process within the PAML program [48], the frequency of duplication events, homologous gene divergence, and the selective pressure on duplicated genes were evaluated by computing the synonymous (Ks) and non-synonymous substitutions (Ka) per site among the duplicated gene pairs. Using Koch et al.'s methodology [49], a neutral substitution rate of 1.5×10^{-8} substitutions per site per year was employed to calculate divergence time for the Chalcone Synthase gene (Chs).

2.4 Promoter region analysis

Selected as the potential promoter regions were the 2,000 base pairs upstream sequences of the initiation codon (ATG) of each TCS gene. The PlantCare database (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) [50] was utilized to probe the promoter region in order to forecast potential cis-acting elements. Microsoft Excel 2010 and TBtools software [51] were utilized to analyze and illustrate the results.

2.5 Protein-protein interaction analysis and microRNA target prediction

Using STRING (<http://string-db.org>) as a reference and the rice protein database as a query, we conducted protein-protein interaction analysis with all identified TCS members' protein sequences. Cytoscape software, with default parameters [52], was used for visualization. MicroRNA targeting prediction for all identified TCS members was carried out using default parameters from the psRNATarget online website [53].

2.6 Plant materials and treatments

The experiment used the typical lowland tetraploid variety "Alamo" of switchgrass, and the seeds were taken from a plant that was grown in our laboratory's field. After seven days of germination on filter paper, plump switchgrass (*P. virgatum* cv. Alamo) seeds were carefully chosen and placed into a hydroponic nutrient solution for further growth. The growth conditions were maintained at 28°C with 14h of light and 23°C with 10h of darkness. Submerging the adventitious roots of the seedlings in a solution of 100 mM NaCl, 20% PEG 6000, and 100µM ABA, salt, drought, and ABA treatments were applied, respectively. For cold and heat treatments, the seedlings were placed under 10/4°C day/night and 40/40°C day/night conditions, respectively. All treatments were repeated three times, with at least 20 seedlings per treatment. The inverted second leaves were then collected at 0, 6, 12, and 24 hours post-treatment. All samples, post-treatment, were instantly frozen in liquid nitrogen and kept in a refrigerator at an -80°C ultralow temperature.

2.7 RNA extraction and qRT-PCR analysis

RNAiso Plus (TaKaRa, Dalian, China) was employed to extract total RNA, in accordance with the manufacturer's instructions; the PrimeScript™ RT Reagent Kit with gDNA Eraser (Perfect Real Time, TaKaRa, Dalian, China) was then utilized to synthesize cDNA. Using Primer (version 5.0) software, the primers listed in Supplementary Table S1 were designed. To confirm the specificity of the reactions, melting curve analysis was conducted, and the products were further confirmed by agarose gel electrophoresis. To perform qRT-PCR, a Roche LightCycler®480 II system was employed, with the following conditions: 95°C for 15s, followed by 40 cycles of 95°C for 15 s, 55°C for 15 s, and 72°C for 15s. Using the SYBR Premix ExTaq kit (TOYOBO, Osaka, Japan), two biological replicates and three technical replicates were conducted for each sample, as well as a negative control.

Elongation factor 1α (EF1α) was employed as the reference gene for the calculation of the expression level [54].

The comparative $2^{-\Delta\Delta Ct}$ method was then employed to calculate the relative expression levels of the different genes, and TBtools generated a heat map based on the relative expression data of each gene.

2.8 Analysis of protein subcellular localization

Cloning the full-length coding sequence of TCSs without a terminator codon (TAG) into the EGFP vector to generate a PvTCSs-EGFP fusion protein for investigating subcellular localization. The primers used for plasmid construction are listed in Table S6. To introduce these constructs into *Nicotiana tabacum* plants, both 35S::PvTCSs-EGFP and the nuclear marker gene *OsSK2* [55] were co-transformed using *Agrobacterium tumefaciens* strain GV3101.

3. Results

3.1 Identification and basic information of the TCS Genes in switchgrass

Using the Arabidopsis TCS proteins as query sequences, BLAST P searches were conducted against the local BLAST + program. After eliminating redundant sequences and conserved domains, 87 TCS members, comprising 20 HK(L)s, 10 HPs, and 57 RRs, were identified in the switchgrass genome (Supplementary Table S2). The length of the Histidine Kinases (HKs) protein ranges from 188 to 1202 amino acid (Table 1). Among them, the AHK subfamily comprises 8 members (PvHK1-8), with amino acid lengths ranging from 757aa to 1202aa and isoelectric points ranging from 5.93 to 8.59. There are significant variations in MWs; PvHK2 had an MW of 83.93 kDa and PvHK6 was 133.14 kDa, and the subcellular localization predictions show that they are mainly localized in the plasma membrane and cytoplasm. The ETR subfamily comprises 6 members (PvETR1-6), with amino acid lengths ranging from 602aa to 769aa and isoelectric points ranging from 6.52 to 8.05. There are significant variations in MWs; *PvETR3* had an MW of 67.00 kDa and *PvETR2* was 85.14 kDa, and the subcellular localization predictions show that they are mainly localized in the plasma membrane and chloroplast. The PHY subfamily comprises 6 members (PvPHYA-F), and the amino acid lengths of this subfamily are distributed between 1038aa and 1165aa, except for the PvPHYE amino acid length of 188aa. The isoelectric points of the other members are between 4.75 and 5.88; Similarly, except for PvPHYE, which has an MW of 20.80 kDa, the MW distribution of other members ranged from 115.01 kDa to 127.65 kDa. Predictions of subcellular localization demonstrate that all members of this subfamily are situated in chloroplasts, thus demonstrating that they are all part of the plant's photosensitive pigment signaling system. In addition, the 20 HKs genes were unevenly distributed on 9 chromosomes, including Chr01K, Chr01N, Chr03K, Chr03N, Chr05K, Chr05N, Chr08N, Chr09K, and Chr09N, among which 9 chromosomes had no HKs genes. Analysis of the physicochemical properties of proteins from 10 HPs members revealed that the amino acid lengths of this family of proteins were mainly distributed between 143aa and 196aa, with isoelectric points ranging from 4.55 to 9.13 and the MW distribution of this members ranging from 16.10 kDa to 23.10 kDa. Prediction of subcellular localization revealed that this family of proteins were mainly situated in chloroplasts and nuclei, yet PvHP9 and PvHP10 were situated in extracellular. The 10 *PvAHP* genes were uniformly distributed on 8 chromosomes, including Chr02K, Chr02N, Chr03K, Chr03N, Chr05K, Chr05N, Chr06K and Chr06N, among which 10 chromosomes had no *PvHP* genes (Table 2).

Table 1
Basic information on PvHKs members

Gene name	ID	Chromosome	AA	PI	MW(kDa)	Location	Subcellular localization
PvHK1	Pavir.1KG503600.1	Chr01K	974	5.94	108.48	50664607–50671095	Plasma membrane
PvHK2	Pavir.1NG411200.1	Chr01N	757	5.96	83.93	60372825–60379286	Cytoplasmic
PvHK3	Pavir.5NG264417.1	Chr05N	1045	8.38	117.29	69006473–69014462	Cytoplasmic
PvHK4	Pavir.5KG741600.2	Chr05K	1003	8.59	112.74	59582697–59588917	Plasma membrane
PvHK5	Pavir.9KG032338.1	Chr09K	998	6.15	109.55	9520177–9526697	Chloroplast
PvHK6	Pavir.9KG357000.1	Chr09K	1202	6.51	133.14	31317233–31328475	Plasma membrane
PvHK7	Pavir.9NG129673.1	Chr09N	994	5.93	109.02	10433732–10440687	Plasma membrane
PvHK8	Pavir.9NG401900.1	Chr09N	1201	6.09	132.61	39786999–39798389	Plasma membrane
PvETR1	Pavir.1KG547200.1	Chr01K	769	6.52	85.11	56003616–56008119	Chloroplast
PvETR2	Pavir.1NG554100.1	Chr01N	768	6.67	85.14	66091514–66095116	Chloroplast
PvETR3	Pavir.3KG172400.1	Chr03K	602	8.05	67.00	10608391–10618346	Endoplasmic reticulum.
PvETR4	Pavir.3NG214791.1	Chr03N	632	7.03	70.08	10277643–10282647	Plasma membrane
PvETR5	Pavir.9KG036200.1	Chr09K	634	6.88	70.40	10837255–10841895	Plasma membrane
PvETR6	Pavir.9NG148800.1	Chr09N	641	6.88	71.26	11609919–11614364	Plasma membrane
PvPHYA	Pavir.8NG150600.2	Chr08N	1038	5.43	115.01	13262068–13272029	Chloroplast
PvPHYB	Pavir.9KG029983.1	Chr09K	1130	5.75	125.18	9387722–9393185	Chloroplast
PvPHYC	Pavir.9NG128200.1	Chr09N	1131	5.81	125.33	10238172–10245639	Chloroplast
PvPHYD	Pavir.9NG674300.1	Chr09N	1165	5.88	127.65	66732027–66739482	Chloroplast
PvPHYE	Pavir.9KG432932.1	Chr09K	188	4.75	20.80	57020777–57021852	Chloroplast

Gene name	ID	Chromosome	AA	PI	MW(kDa)	Location	Subcellular localization
PvPHYF	Pavir.9NG097800.1	Chr09N	1135	5.85	126.23	7708806–7715067	Chloroplast

We scrutinized the physicochemical characteristics of the amino acid sequences of 57 Response Regulators (RRs) proteins, noting disparities among the three subfamilies (Table 3). In the Type A RRs subfamily, the amino acid sequence length primarily ranged from 121 to 248 amino acids (aa), implying a relatively small molecular weight (13.58–26.77 kDa). Predictions were that the majority of these proteins would be situated in the cytoplasm, with only a scant amount in the nucleus. For the Type B RRs subfamily, the amino acid sequence length predominantly fluctuated between 330 to 718 aa, and these proteins displayed a molecular weight mainly varying between 37.96 kDa and 78.60 kDa. The Isoelectric Point (PI) primarily ranged from 5.16 to 6.26. Except for PvRR38 and PvRR42, the rest were predicted to localize in the nucleus. In the PRRs subfamily, the amino acid sequence length predominantly oscillated between 134 to 789 aa. The molecular weights exhibited significant variations, with PvPRR7 and PvPRR8 showing weights of 14.53 kDa and 85.51 kDa consecutively. The principal distribution of PI was between 5.89 and 8.91. Visualizing the chromosomal localization of all members of the TCS of switchgrass, Table 3) predicted PvPRR6 and PvPRR7 to be located in the cytoplasm, while the rest were expected to be in the nucleus. Our findings revealed that the majority of the TCS genes were situated in high-density regions close to telomeres (Fig. S1), thus providing a more intuitive representation of the chromosomal localization of all genes. The results revealed that 87 members were spread out unevenly among 18 chromosomes, with the most on Chr01K, Chr01N, Chr09K, and Chr09N, and the least on Chr04N, where only PvRR40 was present.

Table 2
Basic information on PvHPs members

Gene name	ID	Chromosome	AA	PI	MW(kDa)	Location	Subcellular localization
PvHP1	Pavir.2KG217732.1	Chr02K	145	4.55	16.10	20762489–20764700	Chloroplast
PvHP2	Pavir.2NG243700.1	Chr02N	145	4.55	16.17	20504633–20508784	Chloroplast
PvHP3	Pavir.3KG164227.1	Chr03K	196	9.13	23.10	11996850–12001496	Chloroplast
PvHP4	Pavir.3KG340100.1	Chr03K	152	6.16	17.63	18606164–18608602	Nuclear
PvHP5	Pavir.3NG177203.1	Chr03N	151	8.99	17.81	12160085–12162597	Nuclear
PvHP6	Pavir.3NG258154.1	Chr03N	152	5.02	17.39	19177857–19180611	Chloroplast
PvHP7	Pavir.5KG546300.1	Chr05K	151	5.87	17.14	48066554–48069147	Chloroplast
PvHP8	Pavir.5NG491700.1	Chr05N	151	6.59	17.26	56413903–56416272	Chloroplast
PvHP9	Pavir.6KG407400.1	Chr06K	143	6.72	16.23	47708419–47712493	Extracellular
PvHP10	Pavir.6NG369800.1	Chr06N	143	5.65	16.25	52302989–52306023	Extracellular

Table 3
Basic information on PvRRs members

Gene name	ID	Chromosome	AA	PI	MW(kDa)	Location	Aliphatic Index
<i>A-type response regulator</i>							
PvRR1	Pavir.1KG338200.1	Chr01K	241	8.95	26.15	35984744–35987607	Endoplasmic reticulum
PvRR2	Pavir.1KG392600.1	Chr01K	144	8.82	15.35	42736046–42737235	Cytoplasmic
PvRR3	Pavir.1KG554300.2	Chr01K	127	6.73	14.38	56477176–56477792	Nuclear
PvRR4	Pavir.1NG291000.1	Chr01N	248	8.5	26.77	45506257–45509060	Chloroplast
PvRR5	Pavir.1NG358300.1	Chr01N	142	8.82	15.27	51868134–51869783	Cytoplasmic
PvRR6	Pavir.1NG546300.1	Chr01N	132	6.3	14.91	66559760–66560374	Cytoplasmic
PvRR7	Pavir.2NG278900.2	Chr02N	179	5.17	19.37	18467277–18468982	Chloroplast
PvRR8	Pavir.3KG016200.1	Chr03K	200	5.71	22.33	1743605–1745736	Nuclear
PvRR9	Pavir.3NG010100.1	Chr03N	196	6.39	22.14	1332042–1334707	Nuclear
PvRR10	Pavir.5NG632500.1	Chr05N	232	5.37	25.31	70764613–70768207	Nuclear
PvRR11	Pavir.6KG234700.1	Chr06K	123	5.48	13.87	31928649–31929933	Cytoplasmic
PvRR12	Pavir.6KG234800.1	Chr06K	123	6.27	14.00	31882269–31883864	Cytoplasmic
PvRR13	Pavir.6KG235000.1	Chr06K	123	5.55	13.95	31857300–31858901	Cytoplasmic
PvRR14	Pavir.6KG299100.1	Chr06K	122	5.54	13.77	39653675–39654994	Cytoplasmic
PvRR15	Pavir.6KG299200.1	Chr06K	122	5.88	13.77	39660020–39661391	Cytoplasmic
PvRR16	Pavir.6KG305100.2	Chr06K	122	5.54	13.75	39857811–39859179	Cytoplasmic
PvRR17	Pavir.6NG213221.1	Chr06N	123	5.76	13.88	33354744–33356231	Cytoplasmic
PvRR18	Pavir.6NG243100.1	Chr06N	121	4.9	13.63	43326692–43328034	Cytoplasmic

Gene name	ID	Chromosome	AA	PI	MW(kDa)	Location	Aliphatic Index
<i>A-type response regulator</i>							
PvRR19	Pavir.6NG243200.1	Chr06N	121	4.9	13.63	43361982–43363319	Cytoplasmic
PvRR20	Pavir.6NG243400.2	Chr06N	121	4.9	13.63	43373513–43374838	Cytoplasmic
PvRR21	Pavir.6NG246800.1	Chr06N	121	5.28	13.58	43182915–43183924	Cytoplasmic
PvRR22	Pavir.7KG168600.1	Chr07K	239	8.58	25.46	33485405–33489551	Endoplasmic reticulum
PvRR23	Pavir.7KG253900.1	Chr07K	139	8.52	15.08	40071483–40073013	Cytoplasmic
PvRR24	Pavir.7KG378200.1	Chr07K	166	6.74	17.87	51421063–51422332	Chloroplast
PvRR25	Pavir.7NG088270.1	Chr07N	123	5.55	13.92	879792–881191	Cytoplasmic
PvRR26	Pavir.7NG096700.1	Chr07N	123	5.55	13.95	286850–291495	Cytoplasmic
PvRR27	Pavir.7NG190900.1	Chr07N	230	8.84	24.47	31254128–31256852	Chloroplast
PvRR28	Pavir.7NG193417.1	Chr07N	238	8.74	25.25	31224958–31227926	Cytoplasmic
PvRR29	Pavir.7NG340100.1	Chr07N	139	7.71	15.00	38049481–38051159	Cytoplasmic
PvRR30	Pavir.7NG435700.1	Chr07N	168	6.15	18.18	49462085–49462709	Cytoplasmic
Gene name	ID	Chromosome	AA	PI	MW(kDa)	Location	Aliphatic Index
PvRR31	Pavir.8KG033800.2	Chr08K	203	5.25	22.71	2776315–2778225	Nuclear
PvRR32	Pavir.8NG029800.1	Chr08N	201	5.76	22.57	1621900–1623780	Nuclear
<i>B-type response regulator</i>							
PvRR33	Pavir.1KG088000.1	Chr01K	636	6.26	69.20	6612450–6617580	Nuclear
PvRR34	Pavir.1KG089500.1	Chr01K	636	6.2	69.16	6528098–6532853	Nuclear
PvRR35	Pavir.1KG518100.1	Chr01K	677	5.97	73.66	4551136–54557045	Nuclear

Gene name	ID	Chromosome	AA	PI	MW(kDa)	Location	Aliphatic Index
<i>A-type response regulator</i>							
PvRR36	Pavir.1NG079300.2	Chr01N	635	6.16	68.90	6549521–6554631	Nuclear
PvRR37	Pavir.4KG074200.2	Chr04K	679	6.2	74.55	6522275–6526885	Nuclear
PvRR38	Pavir.4KG310700.1	Chr04K	718	5.91	78.60	38944024–38949697	Cytoplasmic
PvRR39	Pavir.4KG346800.1	Chr04K	691	6.06	74.97	41103674–41115149	Nuclear
PvRR40	Pavir.4NG179900.1	Chr04N	682	5.57	73.76	41246435–41252355	Nuclear
PvRR41	Pavir.5KG703400.1	Chr05K	575	5.24	64.28	58192270–58206483	Nuclear
PvRR42	Pavir.7KG006300.1	Chr07K	368	5.8	41.73	24921421–24923835	Cytoplasmic
PvRR43	Pavir.7KG006400.1	Chr07K	380	5.16	42.84	24908380–24910902	Nuclear
PvRR44	Pavir.9KG213000.1	Chr09K	330	5.35	37.96	23382295–23412337	Nuclear
PvRR45	Pavir.9KG540900.5	Chr09K	687	6.26	73.87	62548307–62554216	Nuclear
PvRR46	Pavir.9NG196400.7	Chr09N	331	5.29	38.05	25130131–25134127	Nuclear
PvRR47	Pavir.9NG840500.7	Chr09N	685	6.1	73.77	73610285–73615723	Nuclear
Pseudogene responsive regulator (PRRs)							
PvPRR1	Pavir.1KG385300.1	Chr01K	521	6.01	57.98	41744859–41747964	Nuclear
PvPRR2	Pavir.1NG350900.1	Chr01N	522	5.89	58.02	50799219–50802447	Nuclear
PvPRR3	Pavir.2KG379300.1	Chr02K	625	6.63	69.71	53418020–53423227	Nuclear
PvPRR4	Pavir.2NG448600.1	Chr02N	623	6.42	69.53	54763663–54768456	Nuclear
PvPRR5	Pavir.2NG610075.1	Chr02N	746	6.41	80.40	70094034–70106996	Nuclear
PvPRR6	Pavir.5KG544714.1	Chr05K	168	7.59	18.69	48851150–48852705	Cytoplasmic

Gene name	ID	Chromosome	AA	PI	MW(kDa)	Location	Aliphatic Index
<i>A-type response regulator</i>							
PvPRR7	Pavir.5NG470586.1	Chr05N	134	8.91	14.53	57338487–57339126	Cytoplasmic
PvPRR8	Pavir.8KG072800.4	Chr08K	789	8.87	85.51	4588507–4593979	Nuclear
PvPRR9	Pavir.9KG482200.1	Chr09K	757	6.21	82.89	58530952–58541644	Nuclear
PvPRR10	Pavir.9NG690300.1	Chr09N	757	7.01	82.66	68849641–68857961	Nuclear

3.2 Phylogenetic analysis of TCS proteins

To analyse the phylogenetic relationships of PvHKs genes, an unrooted NJ tree was constructed based on monocotyledonous and dicotyledonous plants of 20 HKs proteins from switchgrass, 16 from Arabidopsis, 14 from rice, 17 from maize and 18 from tomato (Fig. 1, Supplementary Table 2). The results showed that the 85 HK(L) proteins from the five land plant species were perfectly divided into six distinct subfamilies, namely, cytokinin receptors, CKI1, AHK1, AHK5, ethylene receptors, and phytochromes. It is interesting that the HKs members of switchgrass and maize are only included in cytokinin receivers, ethylene receivers, and phytochromes, which is similar to previous research results.

Two subfamilies of the authentic and pseudo-HP proteins from these five species were mainly identified, which were marked with red and blue lines in Fig. 2. It can be clearly seen that the HPs phylogenetic relationship between Arabidopsis and tomato, which belong to the same dicotyledonous plant genus, is closer, while the evolutionary relationships of HPs members in rice, corn, and switchgrass, which belong to the same monocotyledonous plant genus, are more similar. The evolutionary relationship of AHP protein between dicotyledonous and monocotyledonous plants is varied, while the evolutionary relationships within each plant are comparatively stable. In the dicotyledonous group, SIHP4 and SIPHP1 are grouped with AHP4, while the other members of tomato are grouped separately with AHP1, AHP2, AHP3, and AHP5, which may also be effective in cytokin signaling [56]. It is speculated that PvHPs are also related to abiotic stresses such as salinity and drought, as evidenced by the aggregation of switchgrass HPs and OsHP1/2 on tree branches [30]. Considering their lack of homologous counterparts with Arabidopsis, further research is needed on their evolutionary mechanisms and functional roles.

The RR proteins of the five species were divided into 5 subfamilies: type-A RR, type-B RR, type-C RR, type-B PRR, and Clock PRR (Fig. 3). The A-type RR protein occupies two separate branches, one of which contains only five members of Arabidopsis and tomato; The main branch is another large one, which includes 5 species, and the A-type RR of three monocotyledonous plants, namely switchgrass, corn, and rice, is only included in this subfamily. All B-type RR proteins occupy a large branch alone, and there are several sub-branches within it. It is evident that monocotyledonous and dicotyledonous species tend to cluster together, indicating that these different sub-branches may occur after the diversification of monocotyledonous and dicotyledonous plants. C-type RR and A-type RR have similar structures, but their genetic distance is not closely related. Studies have indicated that C-type RR is likely the oldest RR, while A-type RR could have been derived from C-type RR through mutations in its

promoter [57]. We divided the PRRs into two subfamilies: type B PRR and Clock PRR, both of which comprise members from five species. Approximately twice the amount of members in the Clock PRR subfamily is that of B-type PRR, and there is no obvious difference between monocotyledonous and dicotyledonous plants, implying that the PRR protein is fairly consistent in the development of monocotyledonous and dicotyledonous plants.

3.3 Conserved motifs and gene structure analysis

The differences in UTR-CDS structure often reflect evolutionary information within gene families, while protein function and evolutionary relationships are typically determined by the type and composition of conserved motifs. Using Evolview, the evolutionary relationships and gene structures of the three gene families (HKs, AHP, and RRs) in TCS were visualized from the genome of switchgrass. The MEME online program was then employed to analyze the conserved motifs (HKs10, AHP10, and RRs20) among the members of these gene families.

Firstly, members of the HK, ETR, and PHY subfamilies in the HKs family cluster in three different branches (Fig. 4A), and the evolutionary relationship between the HK subfamily and the ETR subfamily is closer. Secondly, members within the same subgroup share conserved gene structures, while gene members in different subgroups exhibit some differences (Fig. 4B). Interestingly, although PvPHYE belongs to the PHY subfamily, it has the shortest gene length and the simplest gene structure. The above results indicate that there is rich diversity among members of the HKs family and relatively conservative evolutionary relationships within subfamilies, which enables them to play different functions during plant growth and development. Conservative motif analysis of HKs indicates that PvHKs proteins typically contain 4–6 motifs, with motif 1, motif 2, motif 3, and motif 4 being highly conserved domains in all PvHKs protein sequences except for PvPHY. The HK subfamily contains all 10 motifs, while the ETR subfamily contains the same motif except for motif 6 and motif 9 found in PvETR1 and PvETR2. There is no significant difference in the motif content among the other members of the PHY subfamily except for PvPHY. In addition, specific motifs unique to certain subgroups were identified, such as motif10, which only appeared in the HK subgroup (Fig. 4C, Fig.S2). Visualizing the Protein Domain, a member of the HKs family, revealed significant differences between the PHY subfamily and the other two subfamily members. PAS domain is a unique domain of this family; Although most members of the ETR subfamily and HK subfamily have PPK1107 Domain, which explains the close evolutionary relationship between the two, CHASE Domain only appears in the HK subfamily, and GAF only appears in the ETR subfamily; In addition, some minority domains appear in specific subfamilies, such as REC Domain and ETR-ERS-EIN4 Domain appearing in PvETR1 and PvETR2 (Fig.S3).

On the basis of the phylogenetic tree within the AHP gene family of switchgrass, a comparative analysis was conducted on the UTR-CDS, Motif, and Domain of this family. The gene structures of 10 HPs members were strikingly alike, with the exception of PvHP3, which had an extra exon; the other members only had 6 exons, suggesting that the family's member structure is highly conserved in evolutionary relationships (Fig. 5A, B). AHPs conserved motif analysis shows that PvHPs proteins typically contain 3–4 motifs, with motif1, motif2, and motif3 being highly conserved domains among all PvHPs protein sequences; Meanwhile, motif4 and motif5 also appear relatively conservatively in PvHP3-8 (Fig. 5C, Fig. S4). In addition, a conserved phosphate transfer domain (HPt) containing His was identified in the AHP family, corresponding to motif 1 (Fig. S5).

Evolutionary analysis was conducted on all members of the RRs family in switchgrass, and it was found that similar to the previous evolutionary analysis (Fig. 3), A-type RR, B-type RR, and PRRs were clustered together,

respectively. Simultaneously, the gene structures of members in the same subfamily are comparable, as evidenced by the gene structure (Fig. 6A, B). Considering that there are multiple subfamily types within the RRs family, we selected 20 motifs to analyze their protein structures. RRs family proteins typically contain 4–5 conserved motifs, among which motif 1, motif 2, motif 4, and motif 5 are highly conserved motifs among almost all RRs member sequences. In addition, motif 3 and motif 11 are only present in the B-type RR subfamily, motif 6, motif 12 and motif 17 are only present in the PRRs subfamily, and motif 9 is only present in the A-type RR subfamily (Fig. 6C, Fig. S6). Meanwhile, we analyzed the structural domains of protein sequences of RRs family members. All other members of the 32 type A RRs, apart from PvRR8, PvRR9, PvRR10, PvRR31, and PvRR32, had a REC domain; the type-B RRs, however, were distinguished by one REC domain in the N terminal end and one Myb domain in the C terminal end. All 15 type-B RRs meet this characteristic. In addition, two subgroups of pseudo RRs (PRRs) can be further distinguished: clock type and B-type. Compared to real RR, PRR has a pseudo REC domain. The CCT domain of Clock PRR (Fig. S7) is of great significance in controlling cyclic rhythms and flowering time [58].

3.4 Synteny analysis of *PvTCS* genes in switchgrass

Switchgrass is a homologous tetraploid with two subgenomes. We used a database downloaded from JGI (Walnut Creek Joint Genomics Institute, California, USA) to determine the positions and chromosome lengths of three gene family members, PvHKs, PvHPs, and PvRRs, in TCS. Here, all 87 *PvTCS* are assigned to 18 chromosomes, and the distribution of *PvTCS* on chromosomes is uneven, with significant differences in chromosome distribution among different families (Fig. 7). Moreover, in the collinearity analysis of the TCS gene in switchgrass, a total of 36 fragment repeats and one tandem repeat were identified, involving 44 *PvTCS* genes (Fig.S1). Members of the HKs family are mainly distributed on the Chr09K and Chr09N chromosomes, with PvHK1 and PvHK2 located on Chr01K and Chr01N, respectively. They are homologous genes, and PvHK3 and PvHK4, as well as PvETR3 and PvETR4, have the same relationship; A-type RRs are mainly distributed on Chr06K, Chr06N, Chr07K, and Chr07N, and segmental and tandem repeats are the modes of gene replication in this subfamily; The distribution of B-type RR on 8 chromosomes is relatively uniform, with fragment duplication being the main mode. Similarly, PRRs mainly appear in the form of fragment replication on 9 chromosomes of switchgrass, with a relatively uniform distribution of their numbers. The results of Synteny analysis show that certain *PvTCS* pairs are close to each other on the same chromosome, with similar structures and evolutionary relationships.

Calculations of the synonymous rate (Ks), non-synonymous rate (Ka), and Ka/Ks of the duplicates were made, and the divergence time was determined using the Ks values (Supplementary Table S3). These Ks values spanned from 0.04219–1.590162, which is equivalent to the divergence time from 0.402–53.005 MYA (million years ago). The long time span suggests that TCS gene expansion and evolution occurred over millions of years, and not only at specific time points. Furthermore, the Ka/Ks values of the segmental duplications, all of them less than 1, suggest that they all underwent purification selection. To further predict the species evolution mechanism of *PvTCS* family members, TCS genes from switchgrass, Arabidopsis, and rice were selected for synteny analysis (Fig. 8). Among them, switchgrass and Arabidopsis have 7 tandem repeat genes, with fewer collinearity genes and 64 tandem repeat genes with rice, indicating that the TCS gene family proteins are relatively conserved in Poaceae and monocotyledonous plants.

3.5 Analysis of *cis*-elements in the putative promoter regions of TCS genes in switchgrass

The 2000 bp regions upstream of the transcriptional start sites were selected for the purpose of identifying *cis*-elements, which act as TF binding sites and can be used to determine tissue-specific or stress-responsive gene expression patterns [59]. To further investigate the transcriptional regulation and potential functionalities of these TCS genes, these *cis*-regulatory elements were situated upstream of the genes. Predicted *cis*-acting regulatory elements, mainly related to plant abiotic stress and hormones (Figs. 8–12), include light responsive elements such as AE box, G-box, Sp1, as well as *cis*-elements associated with cold stress (LTR), drought response (MBS), heat stress (STRE), anaerobic stress (ARE, GC motif), injury stress (WUN motif), and more. *Cis*-acting elements, such as those involved in abscisic acid (ABRE), auxin (IAA) response (AuxRR core, TGA element), jasmonic acid (JA) response (CGTCA motif, TGACG motif), gibberellin (GA) response (GARE motif, P-box, TATC box), salicylic acid (SA) TCA element, and ethylene responsive element, are typically found in promoters of *PvTCS* genes, and regulate hormone responses. Incorporating seed-specific expression (RY-element), cell cycle regulation (MSA-like), and meristem expression (CAT box) as *cis*-elements, the *TCS* gene of switchgrass may be implicated in the control of various abiotic stresses, plant hormone reactions, and cell growth and development. Moreover, *cis*-elements (circadian) related to circadian rhythms were also identified. To sum up, this gene may be involved in the regulation of growth and development.

3.6 Expression analysis of TCSs indifferent tissues and growth stages

Figure 9 Expression profiles of TCSs in different tissues and growth stages. Note: IN3 was bundle. E4 represents the vascular bundle isolated from the 1/5 fragment of internode 3. These tissues of pool leaf blade. E4, pool leaf sheath. E4, pool nodes. E4, pool whole crown. E4 and whole root sys were collected from the leaf blade, leaf sheath, nodes, and root system in elongation growth stage 4 (4 nodes). DAP0, DAP5, DAP10, DAP20, DAP25 and DAP30 represent the whole forest at 0, 5, 10, 20, 25, and 30 days post fertilization, and V1, V3, and V5 represent vegetative growth stage 1 (1 leaf), vegetative growth stage 3 (3 leaves), and vegetative growth stage 5 (5 leaves)

The expression patterns of TCS genes in 26 tissues, obtained from the JGI database, were visualized with R in order to investigate the possible roles of TCS genes in plant growth and development (Fig. 9, Supplementary Table 4). The majority of the TCS genes in switchgrass, out of the 87 members, have higher expression levels in the roots, followed by stems and leaves. In the early flowering stage, some members also showed higher expression levels, while members with lower expression levels mainly appeared in the tissues between seeds and stems. Further analysis indicates significant tissue expression specificity among the HKs, AHP, and RRs families. At the outset, apart from *PvPHYA* and *PvPHYC*, all HKs exhibited a considerable amount of expression in nutrient organs, such as roots and stems, and during nutrient development phases. However, they showed relatively low expression in reproductive organs and reproductive growth stages. Secondly, there are similarities in the relative expression levels between AHP family members and HKs family members, with a slight difference being that their relative expression levels in leaves are also relatively high. In addition, due to the large number of members and numerous subgroups in the RRs family, there are significant differences in expression levels among members. For example, PRRs members not only have relatively high expression levels in roots and stems, but also in stem tips and inflorescences. Some RRs members show significant high expression levels at various stages of inflorescence and post flowering (*PvRR12-26*, *PvRR39-40*, 42, 43), and show a trend of first

increasing and then decreasing during flower development, indicating that A-type RRs and B-type RRs also play an important role in the development of switchgrass flowers. The above results suggest that TCS members may have different functions at different stages of plant growth and development.

3.7 Functional enrichment analysis of TCS genes

This study identified 87 TCS genes for which Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis were conducted. Analysis of GO enrichment revealed that the TCS gene was primarily regulated by hormone signal transduction (e.g. cytokinin and ethylene), histidine phosphorylation, phosphate transduction, meristem development, embryonic tissue development, root development, light signal response, nitrogen compound metabolism, leaf senescence, and circadian rhythm. Meanwhile, the molecular functions involved in *TCS* genes are mainly divided into four categories, namely protein kinase activity, phosphotransferase activity, molecular transducer activity, and hormone binding receptor related activity. It is worth noting that these genes are mainly located in the nucleus, intracellular, reticular and endometrial systems, as well as organelles associated with the cell membrane (Fig. 10A). In addition, the results of KEGG enrichment analysis showed that the main enriched pathways for all *TCS* genes included plant hormone signaling transduction (ko: 04075), circadian rhythm (ko: 04712), and MAPK signaling pathway (ko: 04016) (Fig. 10B). The evidence points to TCS genes being primarily responsible for plant reactions to environmental stimuli, hormone signal transduction, and circadian rhythms.

3.8 Prediction of protein-protein interaction and miRNAs targeting of TCS members

This study employed the rice protein database as a reference to anticipate protein interactions between TCS members of switchgrass, due to the two-component system's critical part in plant reactions to abiotic stress and hormone signal transduction. The results indicated a variety of protein interactions between HKS and AHPs, particularly among histidine protein kinase members (PvHKSs) and phosphate transfer proteins (PvHPSs), which serve as cytokinin signal receptors (purple line connection). Additionally, there are diverse interactions between pvhps and response regulators (RRs), with the interaction between PvHP1/2/3/5 and pvrrs being notably complex. It is likely that PvHPSs are primarily linked to A-type ARRAs, as they are thought to be the primary response genes of cytokinins [60, 61]. Furthermore, there are various interactions among pvrrs (Fig. 11A). Therefore, it is speculated that there is a complex interaction within the TCS of switchgrass, offering a reference for further research on the molecular mechanisms of TCS in regulating plant growth, development, and responses to abiotic stress.

The regulation of plant growth, development, and stress is greatly impacted by microRNA (miRNA), which has been demonstrated to be involved in the timing of flowering, the formation of flower organs, the formation of apical meristem [62], and lateral root development [63] during growth and development. Additionally, miRNA is involved in the regulation of drought stress, salt stress, and temperature stress during abiotic stress [63–65]. In this study, by using the mRNA sequence of switchgrass TCS members as input, miRNAs targeting switchgrass TCS members were retrieved from the rice miRNA database. The prediction results showed that a total of 133 miRNAs targeted 63 TCS members (Fig. 11B, Supplementary Table 5). Among them, seven *PvHKSs* are mainly targeted by genes such as miR2919, miR2871, and miR2873. *PvETRs* are mainly regulated by miRNAs such as miR444 and miR2100. *PvHPSs* are regulated by 11 miRNAs, such as miR1425-5p, miR5148, and miR5512. A-type ARRAs are regulated by a variety of miRNAs. For example, *PvRR26* is mainly regulated by miR164 and miR5836.

PvRR22 is the target gene of miR169. Some miRNAs can regulate multiple target genes simultaneously, such as miR1858 regulating *PvRR4* and *PvRR27*, and miR2919 regulating the expression of *PvRR10/22/28*. Similarly, B-type ARR genes are also regulated by a variety of miRNAs, such as miR168, miR397, and miR319. PRRs are the target genes of miR1871, miR5075, and miR5501. The prediction of TCS member microRNAs is of great significance for the further study of the post-transcriptional regulation of switchgrass TCS members in the future. A basis for further exploration of its molecular operation is thus established.

3.9 Expression profiles of TCS genes in response to various abiotic stresses

Studies have increasingly revealed that TCS genes are involved in plant responses to abiotic stressors. Consequently, due to the disparities in gene structure and cis-acting elements in the promoter area, we studied the expression of 31 genes from 87 PvTCSs in the leaves of switchgrass when exposed to drought, low temperature, salt, high temperature, and ABA stress. Except for two unexpressed genes *PvRR42* and *PvRR43*, the expression patterns of the remaining 29 genes in the leaves of switchgrass under different stress treatments were focused on the heatmap (Fig. 12). As treatment time elapsed for drought stress, most TCS genes displayed an augmented expression pattern, 13 of which (*PvHK1/2/3*, *PvETR1/3*, *PvHP1*, *PvRR1/9/38/40/45*, and *PvPRR6/7*) attained their peak expression after 12 hours of treatment, and their expression levels began to decline after 24 hours. Similarly, the relative expression levels of nine TCS genes (*PvHK4*, *PvPHYB/E/F*, *PvHP3*, *PvRR7/33/41*, *PvPRR3*) showed a weak upward trend within 0–12 hours but reached their maximum value after 24 hours of drought treatment. However, three clustered genes, *PvPHYA* and *PvHP4/9*, reached their expression peak after 6 hours of drought treatment and then showed a decreasing trend (Fig. 12A). Following 12 hours of low-temperature treatment, the expression of the majority of TCS genes was significantly augmented, and the drought treatment that followed caused a decrease in their expression levels. Eight genes (*PvHK2*, *PvPHYA*, *PvHP1/4*, *PvRR9/37/38/41*) still maintained high expression levels after 24 hours of drought (Fig. 12B). Unlike low-temperature treatment, high-temperature treatment at 40°C resulted in four expression patterns of TCS genes. Among them, seven genes (*PvPHYB/E/F*, *PvRR40*, *PvPRR3/7/9*) showed a gradually decreasing trend in expression levels with the passage of high-temperature stress time. After 6 hours of high-temperature treatment, a marked upregulation of expression levels was observed in *PvETR3* and *PvPRR1/6*, yet this trend began to diminish as the stress period progressed. Five genes (*PvETR1*, *PvHK2/4*, *PvRR38/45*) showed a gradual increase in expression levels within 12 hours of treatment, and their expression levels began to decrease again after 12 hours, but their relative expression levels at 24 hours were still significantly higher than those in the control group (0 h). The expression of the 12 genes (*PvHK1/3*, *PvHP1/3/4/9*, *PvRR1/7/9/33/37/44*) gradually increased within 24 hours, reaching its peak at 24 hours (Fig. 12C). The expression levels of TCS genes under salt stress can be divided into four distinct patterns; *PvRR1* and *PvPHYA/E* were notably higher in the initial stage of stress treatment than after 12 hours. The expression levels of *PvHK4*, *PvHP4*, and *PvRR7/37/40* remained almost unchanged in the first 12 hours of treatment, but were significantly upregulated at 24 hours. *PvRR33/41*, *PvPHYB*, and *PvHP1/3/9* showed a decreasing and then increasing expression trend. However, *PvRR9/39/45*, *PvETR1*, *PvPHYF*, and *PvPRR1/3/9*'s relative expression levels gradually rose over the course of 24 hours, reaching their peak at 24 hours, while the other members showed a decreasing and then increasing expression trend. The expression level gradually increased with time within 12 hours of drought treatment, but showed a decreasing trend after 12 hours. At 24 hours, the expression level was still significantly greater than the control group (0 h) (Fig. 12D). ABA, as an important plant hormone, participates in regulating various environmental stress responses in plants. Studies have shown that there is a close interaction between

cytokinins and ABA, which jointly regulate plant response to environmental signals and growth and development processes [66]. Investigating the influence of exogenous ABA on the expression of *TCS* genes in switchgrass, Fig. 11E revealed that the majority of the detected *TCS* genes were generally increased after ABA treatment, which is in agreement with the widespread presence of ABA responsive elements (ABRE) in the promoter region of *TCS* genes in switchgrass.

3.10 Subcellular localization analysis

Confocal laser microscopy was employed to detect fluorescence signals in *Nicotiana benthamiana* epidermal cells, and transient expression of green fluorescent protein (EGFP) fusion protein was used to analyze the subcellular localization of TCS protein. The results showed that histidine protein kinase (PvHK2) was mainly expressed in the cytoplasm and cell membrane, revealing that PvHK2 may be a membrane-bound receptor protein (Fig. 13A-D). PvETR3 has been identified as an ethylene receptor, and the fluorescence signal of PvETR3-EGFP is mainly detected on the cell membrane, while there is also weak fluorescence in the cytoplasm. This may be due to the functional role of ETR protein in sensing ethylene signals on the cell membrane and endoplasmic reticulum membrane (Fig. 12E-H). The cell membrane and cytoplasm are the primary sites of expression for the fluorescence signal of the light receptor protein PvPHYE EGFP, with chloroplasts also displaying fluorescence signals (Fig. 12I-L). PvHP1 EGFP, the phosphate transfer protein, is mainly localized in the cell membrane and nucleus, though weak fluorescence signals were also detected in the cytoplasm (Fig. 13M-P). Only in the nucleus was the fluorescence signal of PvRR9 EGFP detected (Fig. 13Q-T); however, the B-type regulatory factor PvRR45's fluorescence signal was only seen in the nucleus (Fig. 13U-X), indicating its role as a transcription factor. To confirm this, we fused the reported nuclear marker gene OsSK2 with ERFP (OsSK2-ERFP) and co-expressed it with TCS-EGFP in tobacco epidermal cells. The overlap of green and red fluorescence signals suggested that PvRR9 and PvRR45 are both nuclear localization proteins. In *Arabidopsis*, all RR proteins were localized exclusively to the nucleus, apart from ARR3 and ARR16, which are also located in the cytoplasm [67]. Similar results were obtained for subcellular localization of tomato TCS protein [16]. Consequently, the subcellular localization findings of six TCS proteins in switchgrass in this study are strikingly analogous to prior studies and show distinct subcellular targeting, implying that they serve distinct roles in the two-component system.

4. Discussions

Plant Two-Component System (TCS) is primarily involved in receiving and transmitting cytokinins, ethylene, and light signals, playing a crucial role in plant growth, development, and response to abiotic stress. In this study, 87 *TCS* genes were identified from the switchgrass genome, including 20 HK (L)s, 10 HPs, and 57 RRs (Table 1–3). The number of TCS members in the switchgrass genome was significantly higher than that in *Arabidopsis* (56) [5], rice (52) [68], maize (59) [10], and tomato (65) [14], but lower than that in soybean (98) [11] and sweet potato (90) [16]. The expansion of the switchgrass TCS, as observed in this investigation, aligns with an intermediate evolutionary trajectory when compared with previous findings.

This is consistent with previously reported classification results [5, 16, 69]. Moreover, PvHK (L) members are only included in three subgroups, namely, cytokinin receptors (PvHKs), ethylene receptors (PvETRs), and phytochrome receptors (PvPHYs), which is similar to maize HK (L) members, also a member of the genus Gramineae (*Zea mays*) [10]. For the phylogenetic evolution of phosphate transfer proteins (AHPs), monocotyledons and dicotyledons are obviously classified into two different branches, indicating that the

protein family is highly conservative in monocotyledons and dicotyledons, respectively. The phylogeny of RRs also showed significant species differences. In addition to the three major subgroups of A-type RRs, B-type RRs, and PRRs identified by most species, there were also C-type RRs subgroups, but no switchgrass TCS member was found. PRRs members of five species all appeared in B-type PRRs, but the number of PRRs was less than that of Clock PRRs, which was similar to the evolution results of sweet potato RRs members [16].

Fragment and tandem replication, which contribute to the enlargement of gene, are typically seen in the evolution of plant genomes [70, 71]. Recent studies have emphasized that segmental duplication is the primary mechanism in the evolution of the TCS gene family, while tandem duplication is secondary [11, 12, 14]. In the collinearity analysis of switchgrass, a total of 36 fragment repeats and a tandem duplication involving 44 *PvTCS* genes were identified (Fig. S1, Supplementary Table S3). This indicates that the evolution of TCS members is relatively conservative. To further understand the evolutionary relationships of TCS members, we conducted a collinearity analysis of TCS members in Arabidopsis, rice, and switchgrass. All collinearity events between switchgrass and Arabidopsis or rice showed that a switchgrass gene exhibited collinearity with one or more Arabidopsis or rice genes, but more collinearity was observed with rice (Fig. 8), indicating significant species differences in the evolution of TCS family genes.

Since two-component systems (TCS) function in a coordinated and unified system, there are often protein interactions between TCS members [40, 67, 72]. Through bioinformatics prediction of the protein interactions among switchgrass HK (L)s, AHPs, and RRs, it was found that *PvHPPs* often accepted the phosphate signal as an intermediate and coordinated with RRs. Similarly, *Phyllostachys pubescens PheRRb8* also interacted with most *PheHPPs* [73]. In short, it is speculated that there are complex protein interactions among TCS members. This similar interaction has been verified in sweet potato using yeast two-hybrid technology [16]. Regulating gene expression is a critical function of microRNAs. This study utilized the rice microRNA database to predict miRNAs that potentially regulate the switchgrass TCS gene based on homologous gene sequence similarity. *PvHKs* are primarily targeted by miR2919, miR2871, and miR2873. The *PvETR*s gene is predominantly regulated by miR444, miR2100, and other miRNAs. *PvHPPs* are regulated by 11 miRNAs. A-type RRs, as target genes of miR164, miR5836, and miR169, are also regulated by various miRNAs like miR168, miR397, and miR319. *PRRs* are targeted by miR1871, miR5075, and miR5501. Furthermore, certain miRNAs, such as miR1858 and miR2919, can simultaneously regulate multiple target genes (Fig. 11B). Extensively studied in other crops are numerous miRNAs implicated in plant growth and development. For instance, miR156 promotes tiller and root development [74, 75], miR396 regulates auxin synthesis, miR164b [76, 77], miR397b [78], miR398b [79, 80], and miR529b [63, 81] regulate panicle growth, development, and rice yield. Simultaneously, many miRNAs involved in abiotic stress may also regulate the expression of TCS members. For example, miR168b, miR393, miR319, miR1425, and miR398b are mainly involved in regulating low-temperature stress. MiR408, miR171, miR164e, miR414, and miR528 are associated with plant salt stress [63, 81], while miR169 and miR166 are linked to plant drought resistance and cadmium tolerance [82, 83]. MiR397, miR169d, and miR396c are connected to plant responses to high-temperature stress [78]. Therefore, TCS members are likely to serve as target genes of corresponding microRNAs in regulating switchgrass growth, development, and resistance to abiotic stress. Further research is warranted to explore the molecular mechanisms underlying this effect.

We conducted an analysis of the expression profiles of Two-Component System (*TCS*) genes in switchgrass roots, stems, leaves, and flowers, in order to ascertain their response patterns under drought, low temperature, high temperature, salinity, and ABA stress. This was in response to prior bioinformatics analyses which have

indicated that the TCS signaling pathway is a major factor in controlling plant growth, development, and reactions to environmental stimuli. The expression levels of *TCS* genes in roots were largely high, in agreement with the expression profiles of TCS genes in other plants, such as *Arabidopsis* [69], tomato [14], soybean [11], Chinese cabbage [12], sweet potato [16], and ginseng [84]. Importantly, TCS members displayed different expression patterns under stress treatment. The expression of *pvhks* significantly increased after drought stress treatment (Fig. 12A), which was consistent with the role of cotton *GhHK8* in drought resistance [85]. The expression of *PvRRs* was low at the beginning of salt stress treatment but significantly increased after 24 hours (Fig. 12D), indicating its potential function in plant salt stress. Recently, it was found that rice B-type *RRs* *OsRR26* participated in cytokinin-mediated ROS signal transduction and then regulated rice salt tolerance [86], suggesting that two-Component system (TCS) may play an important role in drought resistance and salt tolerance of switchgrass [87], which is further confirmed by the research on the regulation of drought resistance and salt tolerance of kidney bean by TCS members [88]. At the same time, the expression levels of TCS members also showed significant differences under low temperature, high temperature, and abscisic acid treatment. Similar results have also been widely studied in *Arabidopsis*, rice, tomato, and soybean [11, 20, 28, 29, 89, 90]. However, it should be noted that TCS contains complex member categories, and the expression patterns of histidine kinases (HKs), AHPs, and *RRs* under the same stress treatment are significantly different, which is closely related to the cis-acting element (Figs. 8–12) and subcellular localization of their promoter region (Fig. 13) [13]. Therefore, in the next molecular mechanism study, we should focus on elucidating the interactions and functional variances among members. This will enable a more comprehensive analysis of the role of TCS in plant growth, development, and response to abiotic stress.

5. Conclusion

This study identified 87 TCS family members in the genome of switchgrass, including 20 HK (L)s, 10 HPs, and 57 *RRs*. Comparative analysis of different plant species showed that the evolution of TCS members is highly conserved. A total of 36 pairs of fragment repeats and 1 pair of tandem repeats were identified, indicating that fragment repeats are the main mode of *TCS* gene amplification, although there are also a small number of tandem repeats. Most *TCS* genes exhibit tissue specificity and are preferentially expressed in root tissues, followed by certain expression levels during stem and leaf development as well as flowering processes. Importantly, the promoter region of the *PvTCS* gene contains a large number of hormones and abiotic stress response elements, suggesting that they play crucial roles in plant perception of hormone signals and response to abiotic stress. GO enrichment, KEGG enrichment, and microRNA prediction analysis also support this speculation. Therefore, we validated their response patterns to drought, cold, heat, salinity, and exogenous ABA, further confirming the role of *TCS* genes in regulating plant abiotic stress adaptation. Additionally, we predicted possible protein interactions between TCS members, which provides a reference for future research on their molecular mechanisms of action. These findings can deepen our understanding of the *TCS* gene characteristics in switchgrass and provide a theoretical basis for further exploring the role of these genes in regulating the abiotic stress adaptation ability of switchgrass.

Declarations

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Author contributions

W.B. performed the experiments, calculated the data and drafted the manuscripts; S.M., Z.T. and Z.J. performed the experiments; L.T., Y.Y., C.X. and N.R. assisted in the data collation and analysis; S.F. and Z.C. provided guidance during the experiments and manuscript writing; X.Y. designed the experiment. All authors have read and approved the manuscript for publication.

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Availability of data and materials

The datasets supporting the conclusions of this article are included within the article.

Ethics approval and consent to participate

We declare that all the experimental plants were collected with permission from local authorities of agricultural department and all experimental studies on plants were complied with relevant institutional, national, and international guidelines and legislation.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Figures

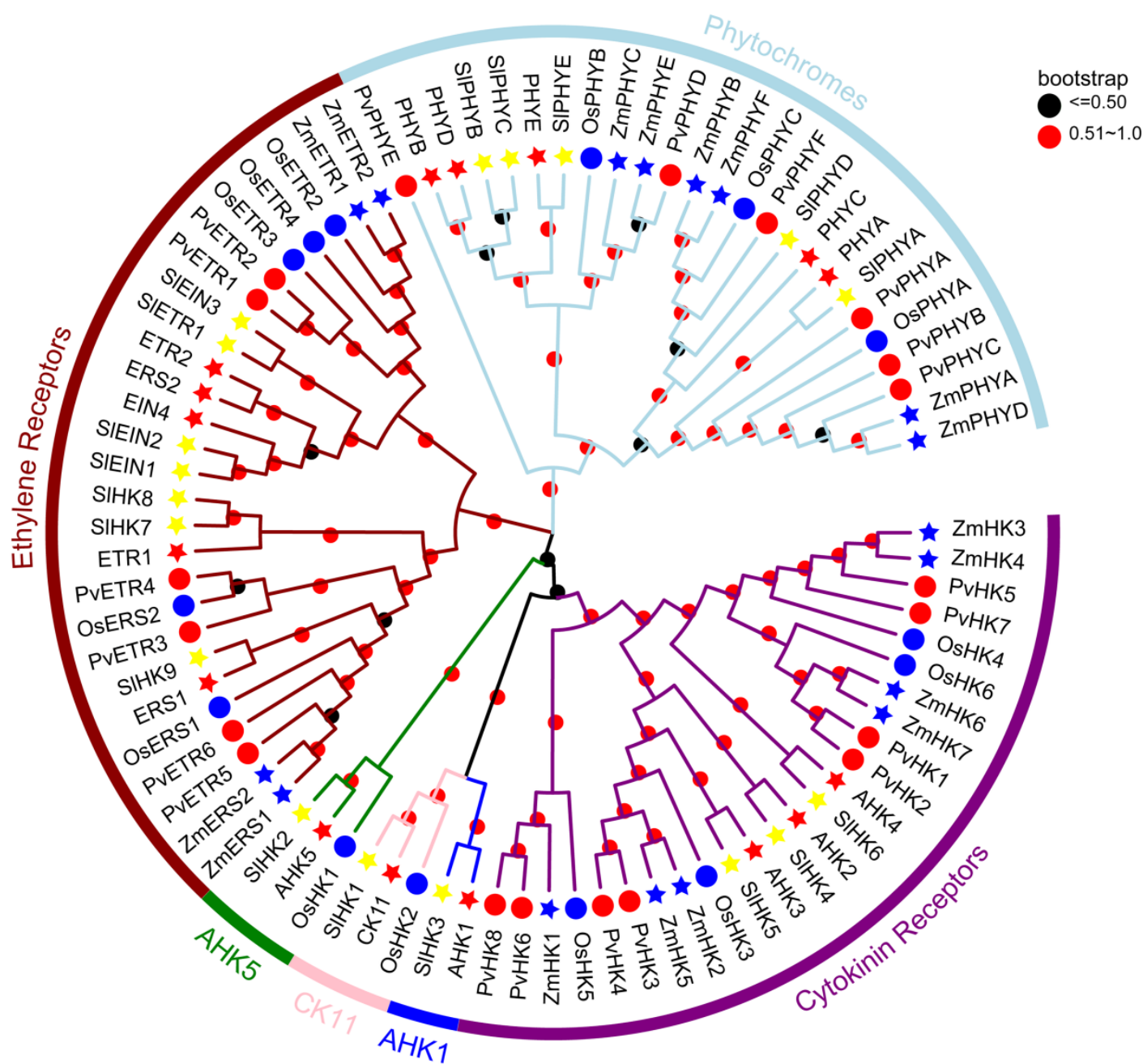


Figure 1

Phylogenetic relationships of HK(L)s in Arabidopsis, rice, maize, tomato and switchgrass. Red stars represent Arabidopsis, blue circles represent rice, blue stars represent maize, yellow stars represent tomato, and red circles represent switchgrass. The outer circle represents six subgroups

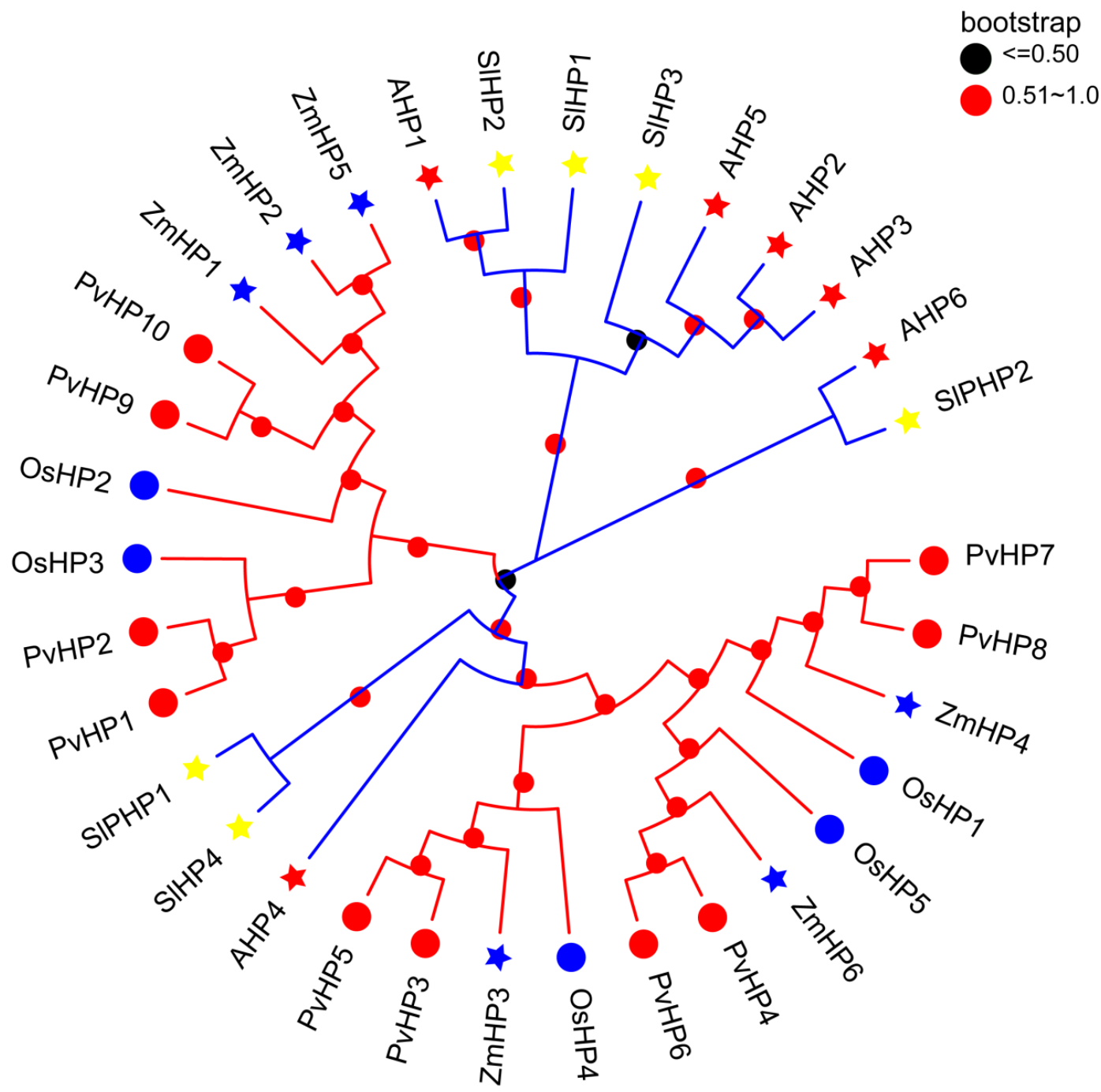


Figure 2

Phylogenetic relationships of HPs in Arabidopsis, rice, maize, tomato and switchgrass. For other details, see Fig. 1

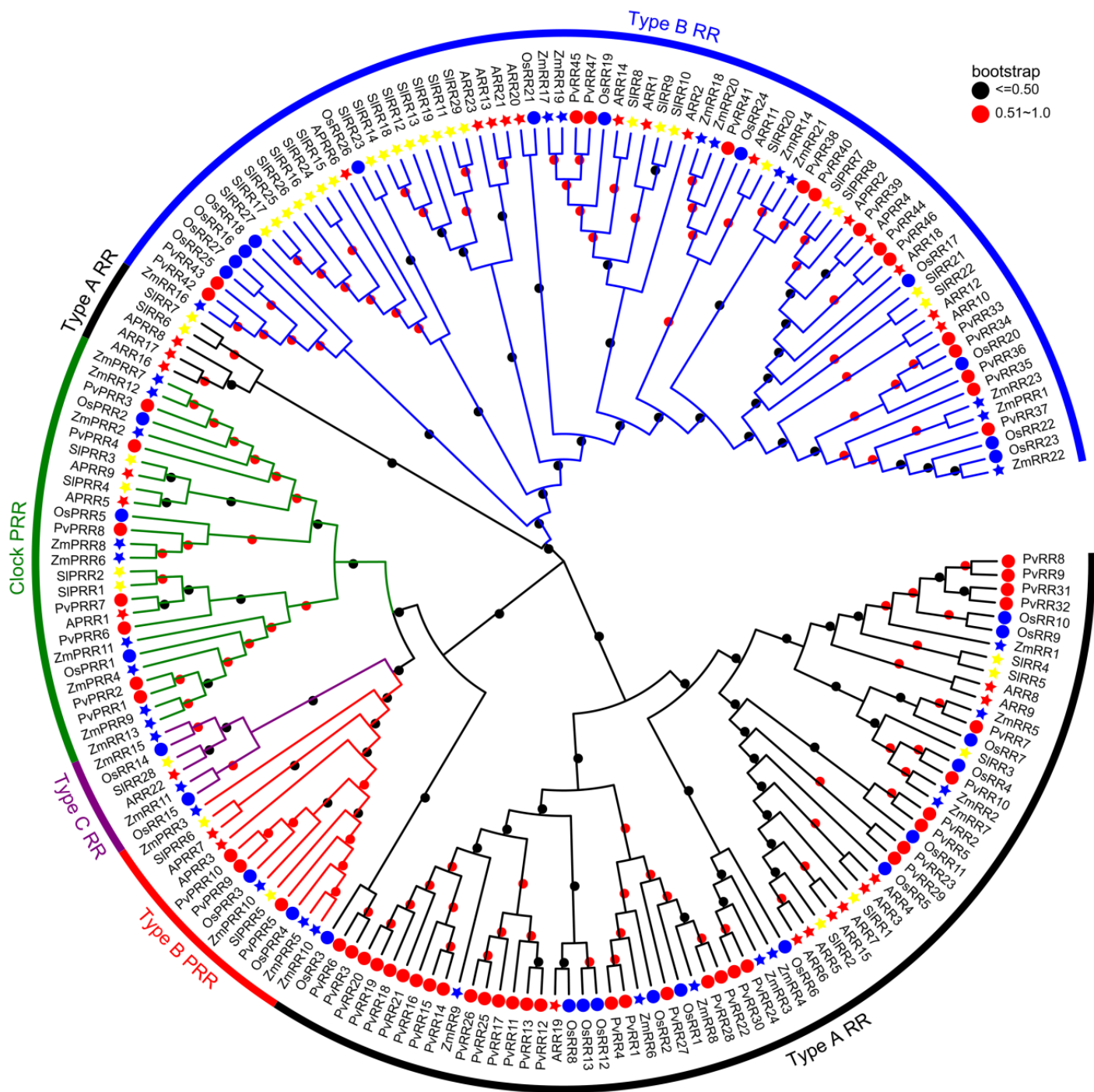


Figure 3

Phylogenetic relationships of RRs in Arabidopsis, rice, maize, tomato and switchgrass. For other details, see Fig. 1

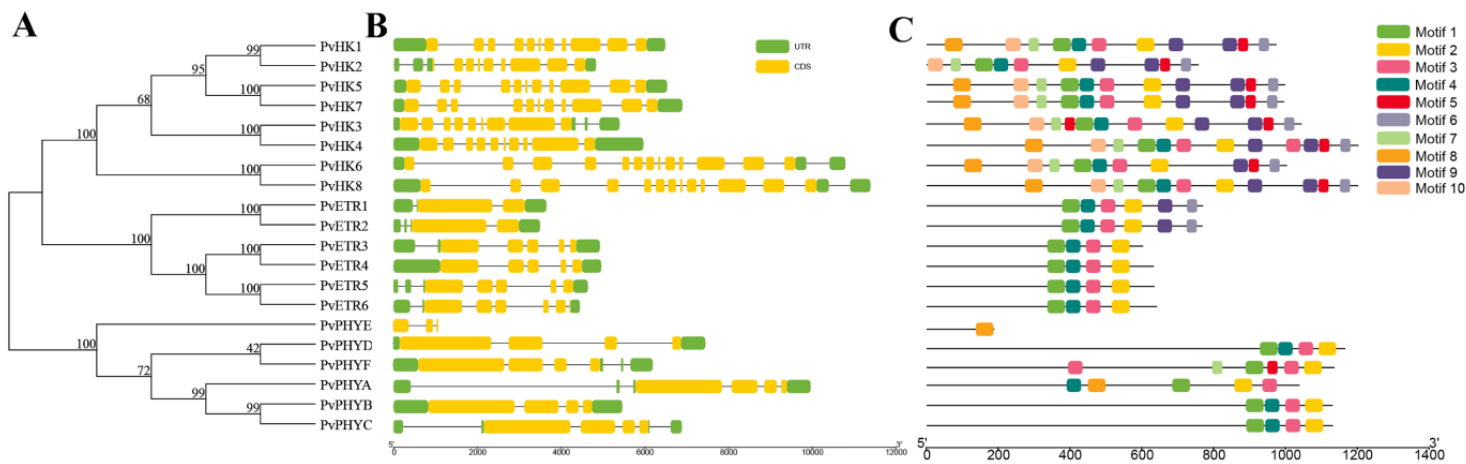


Figure 4

Phylogenetic relationships, conserved motifs, and gene structure analyses of switchgrass HKs members. A, The phylogenetic tree was constructed based on the full-length sequences of 20 PvHKs proteins. B, CDS-UTR structure of PvHKs genes. introns are indicated single lines. The number below refers to the length of the genes. C, Ten conserved motifs from HKs proteins are displayed in different colored boxes. The number below refers to the length of the protein

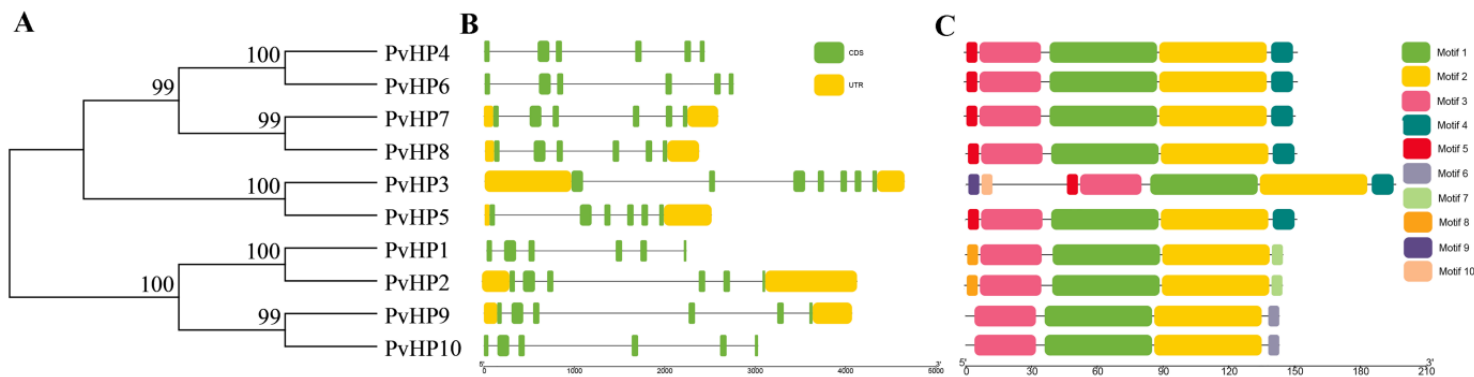


Figure 5

Phylogenetic relationships, conserved motifs, and gene structure analyses of switchgrass AHPs members. A, The phylogenetic tree was constructed based on the full-length sequences of 10 PvHPs proteins. B, CDS-UTR structure of PvHPs genes. introns are indicated single lines. The number below refers to the length of the genes. C, Ten conserved motifs from HPs proteins are displayed in different colored boxes. The number below refers to the length of the protein

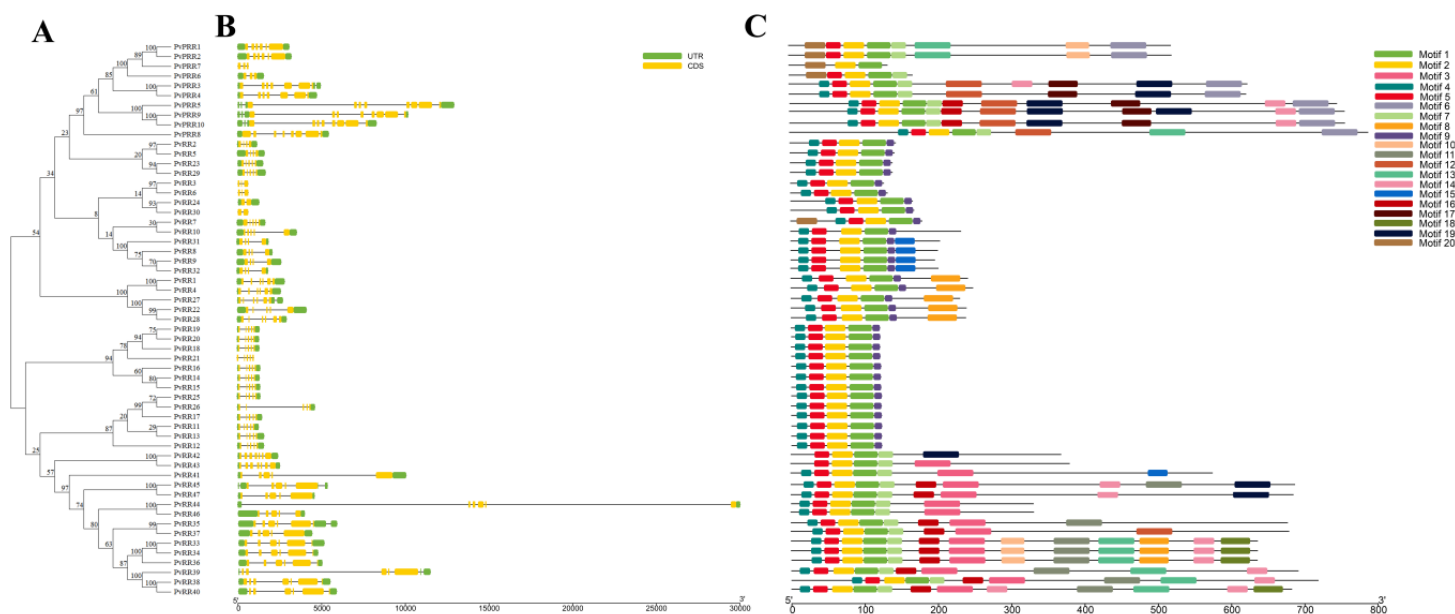


Figure 6

Phylogenetic relationships, conserved motifs, and gene structure analyses of switchgrass RRs members. A, The phylogenetic tree was constructed based on the full-length sequences of RRs proteins. B, CDS-UTR structure of RRs genes. introns are indicated single lines. The number below refers to the length of the genes. C, 20 conserved motifs from RRs proteins are displayed in different colored boxes. The number below refers to the length of the protein

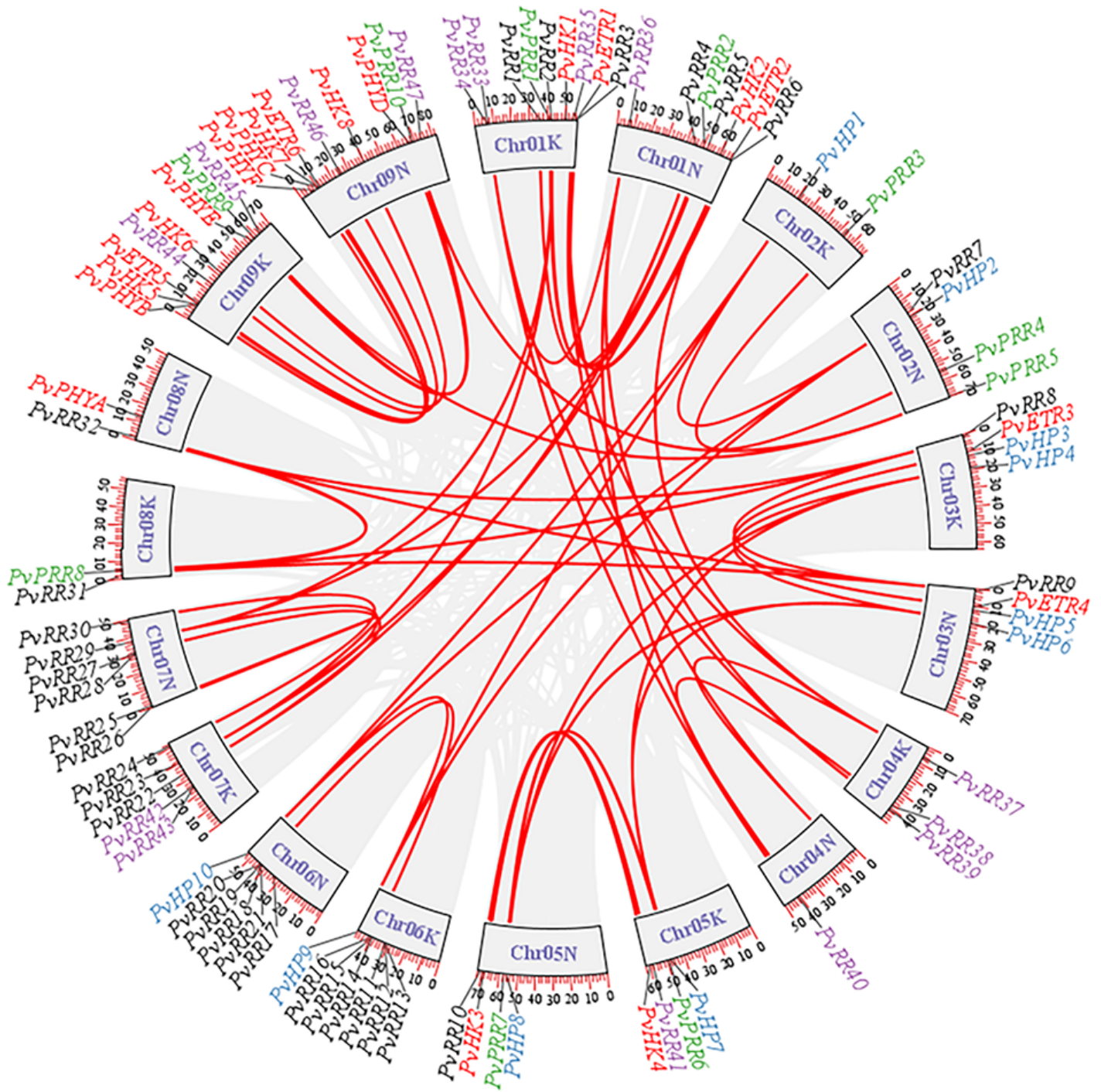


Figure 7

Syntenic relationships of duplicated TCS genes in switchgrass. Lines between the chromosomes indicate duplicated gene pairs. Bars located in the chromosomes represent the chromosome length (cM). HK(L)s, HP and RR gene family members were highlighted with different color fonts

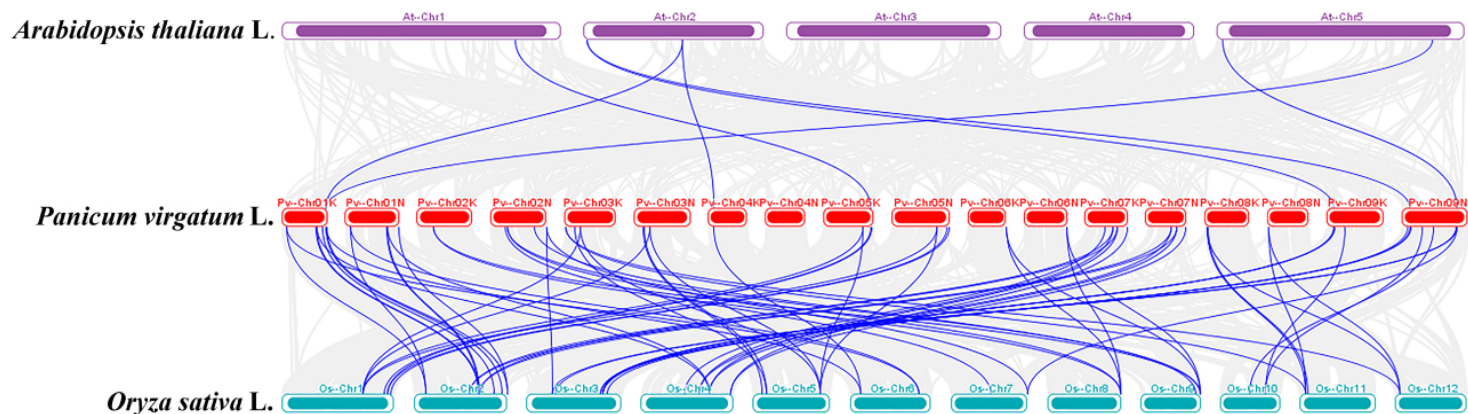


Figure 8

Syntenic relationships of duplicated TCS genes in switchgrass, Arabidopsis, and rice trilobal. Lines between the chromosomes indicate duplicated gene pairs

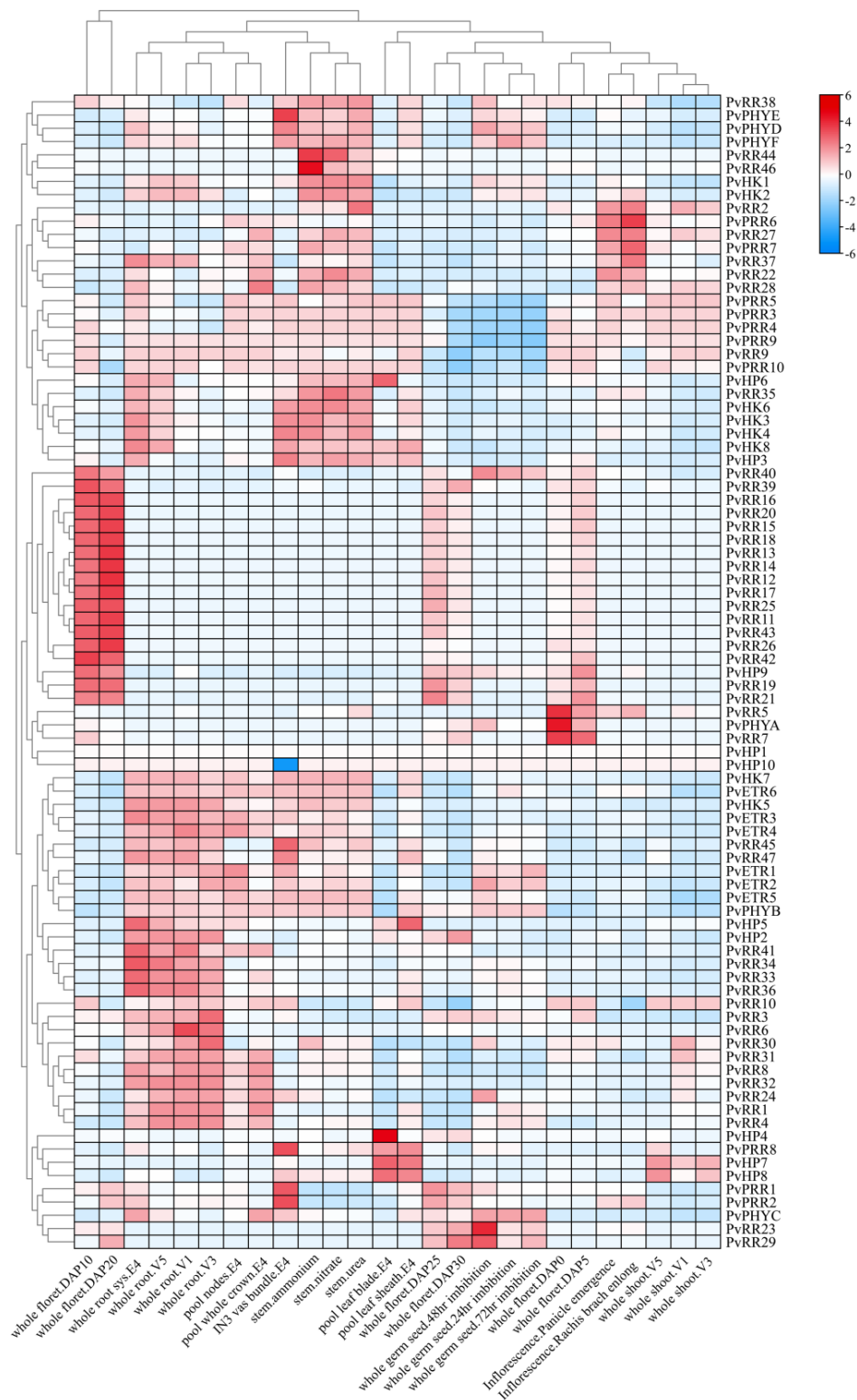


Figure 9

Expression profiles of TCSs in different tissues and growth stages. Note: IN3 vas bundle. E4 represents the vascular bundle isolated from the 1/5 fragment of internode 3. These tissues of pool leaf blade. E4, pool leaf sheath. E4, pool nodes. E4, pool whole crown. E4 and whole root sys were collected from the leaf blade, leaf sheath, nodes, and root system in elongation growth stage 4 (4 nodes). DAP0, DAP5, DAP10, DAP20, DAP25 and DAP30 represent the whole foret at 0, 5, 10, 20, 25, and 30days post fertilization, and V1, V3, and V5 represent

vegetative growth stage 1 (1 leaf), vegetative growth stage 3 (3 leaves), and vegetative growth stage 5 (5 leaves)

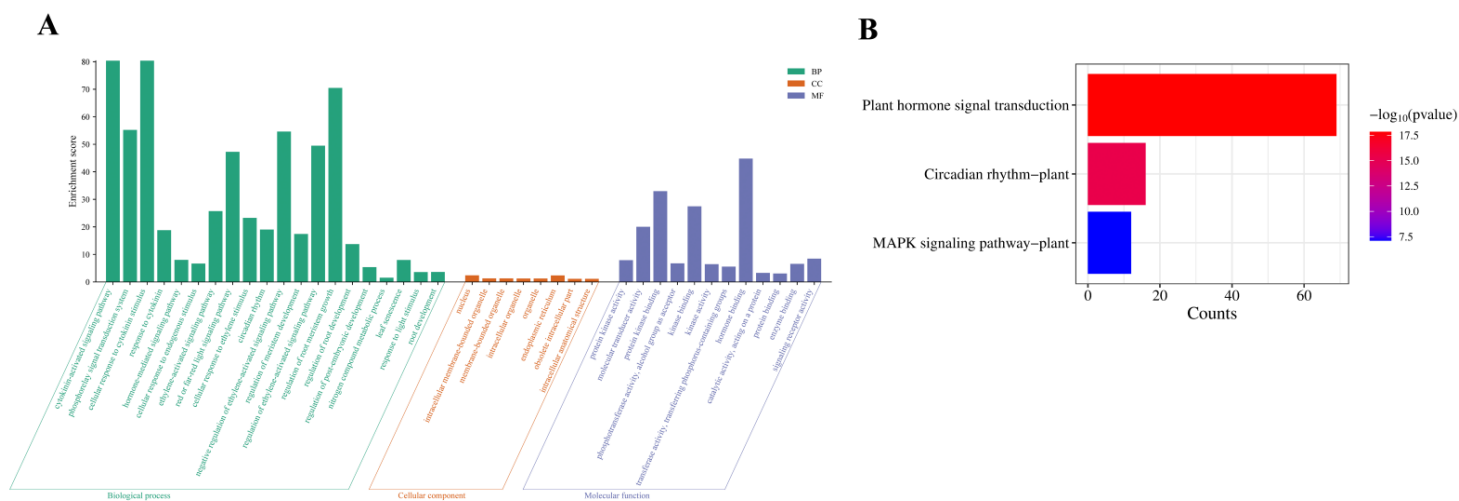


Figure 10

GO and KEGG enrichment analysis of TCS genes in switchgrass. **A**, GO term enrichment analysis, Biological processes (BP), Cellular component(CC), Molecular function (MF) . **B**, KEGG pathway enrichment analysis

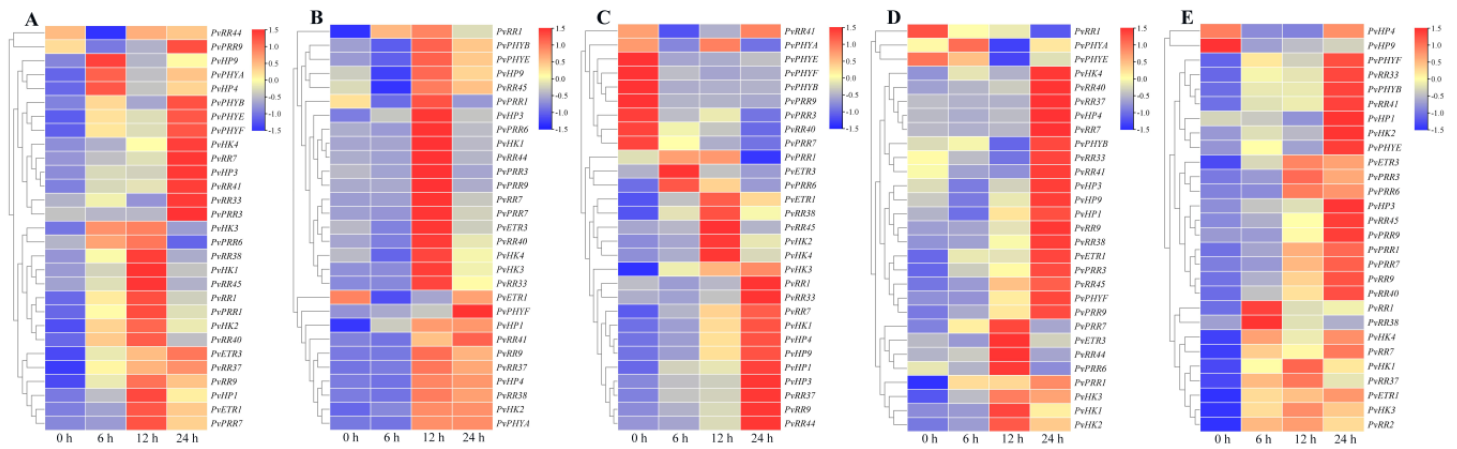


Figure 12

Heat map representation for the response patterns to drought (A), cold stress (B), heat stress (C), salt stress (D) and exogenous ABA (E) treatment TCS genes in switchgrass. The expression levels of genes are presented using fold-change values transformed to Log^2 format compared to control

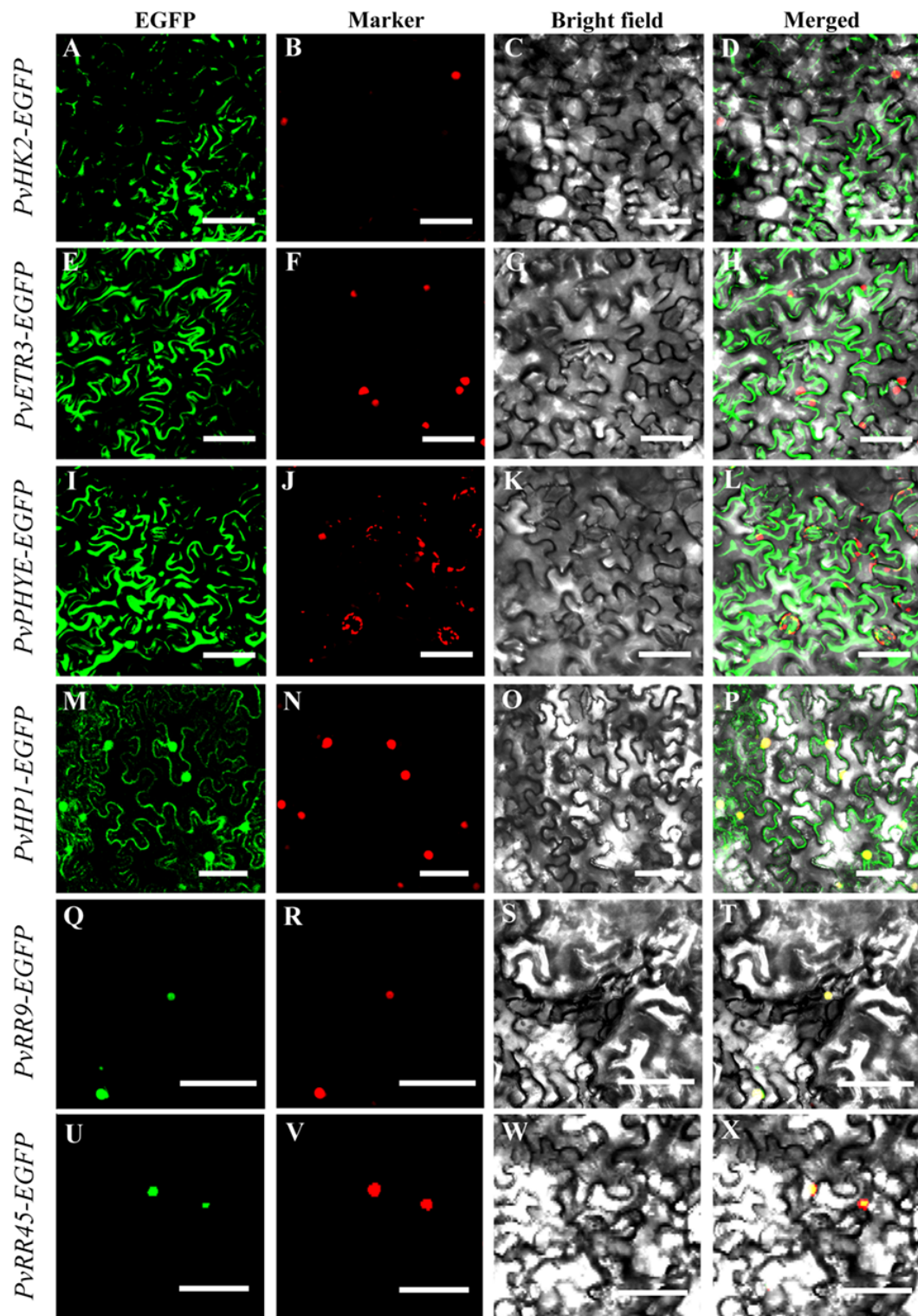


Figure 13

Subcellular localization of *PvTCSs*. *PvHK2-EGFP*, *PvETR3-EGFP*, *PvPHYEE,-GFP*, *PvHP1-EGFP*, *PvRR9-EGFP*, *PvRR45-EGFP* and nucleus-mCherry marker were transiently expressed in tobacco epidermal cells, scale bar = 50µm

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryFigure.pdf](#)
- [SupplementaryTable.xlsx](#)