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Genome-wide analysis of the two-component system (TCS) genes in apple and functional identification of the MdHK17 gene in promoting bud regeneration

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Research Article

Keywords: Genome-wide analysis, TCS genes, Functional identification, Gene expression, Apple

Posted Date: June 24th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-9872332/v1

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Additional Declarations: No competing interests reported.

Abstract

Background

The two-component system (TCS) is composed of histidine kinases (HKs), histidine phosphotransfer proteins (HPs), and response regulator proteins (RRs), plays vital roles in cytokinin signal transduction. However, no comprehensive investigation of the TCS genes in apple has been conducted.

Results

A total of 82 *MdTCS* genes were identified, including 29 HK(L)s, 9 HPs, and 44 RRs. Genes within the same phylogenetic tree branch exhibited similar conserved motifs and gene structures. The results of subcellular localization showed that all of the type-B RRs were located in the Nuclear. The relationship of TCS genes between apple and other 9 species showed that apple had a rather closer evolutionary relationship with soybean. Notably, 24 pairs of segmental duplicates were found in all TCS genes from apple, indicating that segmental duplication event was one of the reasons that contributed to the expansion of the *MdTCS*s family. Furthermore, 66 cytokinin-responsive *cis*-elements were found in the promoter regions of the putative apple TCS genes. Microarray data revealed that *MdHKL4*, *MdHKL5, MdHKL10*, *MdRRA9*, and *MdRRB5* were differentially expressed in all tissues. Meanwhile, quantitative real-time PCR (qRT-PCR) indicated that *MdHK17*, *MdHP5*, and *MdRRC4* were up-regulated by exogenous cytokinin from 2 to 8 hours. Ectopic expression of *MdHK17* in tomato indicated that *MdHK17* plays important role in mediating regeneration bud.

Conclusion

This will provide a theoretical basis for further research on the role of the apple TCS gene in regulating the development of regenerative bud.

Introduction

Cytokinin is an important plant hormone, which plays important role in various plant development processes, including seed germination, leaf and root growth, and plant senescence [1–3]. The signal transduction of the cytokinin in plants mainly occurs via the two-component system (TCS). The TCS is initially identified in the bacteria and composed of a histidine kinase (HK) localized in the membrane and a cytoplasmic response regulator (RR) [4–5]. Upon detection the environmental signals, the histidine residue (His) in the HK domain can be phosphorylated, then the phosphate group is transferred to a conserved aspartate (Asp) residue within the RR protein receiver (Rec) domain. Later, another complex plant TCS signaling element with an additional phosphorylation step [6] was identified. Among them, histidine phosphotransfer (HP) protein functions as a mediator to transfer the phosphoryl group from the HK to the RR [7, 8].

TCS signaling has been extensively studied in plants, including *Arabidopsis* [7], rice [9], maize [10], soybean [11], Chinese cabbage [12], tomato [13], melon [14], cucumber [15]. In *Arabidopsis*, three transmembrane HKs, such as *AHK2-4*, play the role of cytokinin receptors in response to different stresses. These receptors can sense the stimulus signals and get autophosphorylated. Then, a phosphate group is transmitted to the Rec domain of type-B RRs through *cis*-elements in the promoter of the putative type-A ARRs repressors, regulating the expression of downstream gene related stress [7, 8].

The TCS pathway also performs crucial functions in response to stress signal transduction, particularly in drought, high salinity, and inappropriate temperature conditions. In heat-stress conditions, the expression of *AHP1-3* is significantly repressed [16]. In addition, each type-A ARRs interact with abscisic acid (ABA) to participate in response to different stress conditions, such as salt, low temperature, and drought conditions [17–25]. A previous study on rice reported that the seedlings with silenced *OsAHP1/2* expression exhibited different patterns of negative response to drought and salt stresses [26]. Another study found that there was a significant association between *OsHK3* and the antioxidant defense mechanism induced by ABA [27]. Most of the TCS genes in soybean exhibit sensitivity to drought [28]. A member of the HK family, *LE-ETR3(Nr)*, isolated from tomato pollen mutant, could respond to heat stress by influencing the carbohydrate metabolism in pollen [29]. In tea plants, *CsRR5* positively regulated the cold stress response through the *CBF* pathway [30].

Apple (*Malus domestica*) is the most widely cultivated temperate fruit trees in the world, possessing extremely high economic and nutritional value. Apple seedlings with branches structures have the advantages of rapid growth, early fruiting, and low labor and effort requirements. The good branching structure of apple is an important indicator for evaluating high-quality apple seedlings. Cytokinin can promote the development of lateral buds and the formation of lateral branches. However, the TCS genes related to cytokinin have not been systematically studied in apple. In the present study, with the assistance of the reference genome of apple, the putative 82 *MdTCSs* were identified. The fundamental analysis was performed using various tools of bioinformatics and molecular biology, such as chromosomal positions, genomic structures, *cis*-elements of promoters, motif components, and the inter-genomic linear relationship. Moreover, the already-known TCS genes from certain important horticultural crops and apple species were also subjected to the phylogenetic analysis. Subsequently, the TCS gene expression patterns in diverse apple tissues were identified, and qRT-PCR was performed to compare the responses of specific genes to cytokinin. Specifically, the *MdHK17* gene, which responds to cytokinin, has been verified to have the effect of promoting bud regeneration. Our comprehensive analysis will provide a foundation for further exploration of the function of the TCS gene and for revealing the potential molecular mechanism of apple response to exogenous hormones.

Materials and Methods

Sequence retrieval and characterization

The TCS protein sequences of rice and *Arabidopsis* were retrieved from Phytozome (http://phytozome.jgj.doe.gov/pz/portal.hhtm) and the National Center for Biotechnology Information (NCBI). Subsequently, these retrieved sequences were utilized as queries for BLASTP searches against the GDR database (https://www.rosaceae.org/species/malus/all) with threshold E-value of 1×10^{-5} [11, 31]. The HMMER3.0 (http://hmmer.janelia.org/) software was used with the expected values of less than 0.1 to select the predicted proteins from the apple genome, which comprised the typical TCS domains such as HATPase (PF02518), HisKA (PF00512), Rec (PF00072), HPt (PF01627) and CHASE (PF03924) [32, 33].

Subsequently, all the 94 putative members were further inspected based on the afore-stated TCS conserved domains using the CDD [34] and SMART (http://smart.embl-heidelberg.de/) tools. Finally, the unique location of *MdTCS* genes on the chromosome were determined, and their amino acid sequences were submitted to the online tool Prot-Param [35] for computing the physicochemical parameters. Meanwhile, the CELLO v2.5 server was used to predict the subcellular localization of MdTCS proteins [36].

Gene structure construction, multiple-sequence alignment, motif analysis, and phylogenetic analysis

The Gene Structure Display Server (GSDS, http://gsds.cbi.pku.edu.cn/) was employed to analyze the intron-exon patterns. The MEME suite (http://meme.nbcr.net/meme/intro.html) was used to found the conserved motifs of apple TCS proteins. Furthermore, multiple-sequence alignment was performed for the predicted TCS proteins using the DNAMAN software to compare the peptide of the conserved domains. The sequences of the TCS proteins in cucumber (45), maize (45), watermelon (48), rice (50), melon (51), *Arabidopsis* (56), tomato (65), Chinese cabbage (85), and soybean (98) were obtained against the NCBI, Cucurbit Genomics (http://cucurbitgenomics.org/), and Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html) databases, and the similarity of these sequences was analyzed using ClustalW. The MEGA 7.0 software was employed to determine the phylogenetic relationships by comparing the evolutionary history between the apple and other different species using the neighbor-joining approach [37].

Candidate promoter regions in the apple TCS genes

In order to identify the *c*is-elements within the apple TCS gene promoter sequences, the upstream sequences (2.0 kb) at the transcription start site were retrieved against the Phytozome database (http://phytozome.jgi.doe.gov/pz/portal.html). Thereafter, the *cis*-elements in the promoter were identified by PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

Plant materials and different treatments

In this study, the leaf samples were obtained from the 'Gala' apple plantlets and were cultured in the MS medium containing 0.05 $mg{\cdot}L^{-1}$ NAA and 0.5 $mg{\cdot}L^{-1}$ 6-BA. The culture conditions were as follows: a temperature of 25 ℃, and a 16-h light/8-h dark photoperiod.

In order to study the response of apple TCS genes under varying degrees of the exogenous hormone, four-week-old apple plantlets were transferred to a nutrient solution supplemented with 100 μM of trans-zeatin (ZT) using a filter paper bridge. Subsequently, the samples of treated seedlings were retrieved at different time points following the treatment: 0 h (control), 2 h, 4 h, and 8 h. The collected plantlet samples were immediately immersed into liquid nitrogen and preserved at − 80 ℃ until the extraction of the total RNA. All samples were analyzed in triplicate.

Gene ontology (GO) analysis and microarray of MdTCSs

The GO enrichment information of the TCS gene family in the apple was obtained from the GDR database (https://www.rosaceae.org/species/malus/all). The apple microarray data published by Celton and colleagues (2014) [38] was downloaded from the Gene Expression Omnibus (GEO) database of NCBI. The downloaded microarray data was further analyzed using TBtools.

RNA extraction, and quantitative real-time PCR (qRT-PCR)

The total RNA was isolated by the Extraction kit (Sangon, Shanghai, China). Subsequently, the reverse-transcribed RNA was removed using the PrimeScript®RT Reagent kit (Takara, Japan), and the first cDNA strand was generated. The specific primers (Supplementary Table S1) were synthesized from Sangon. Moreover, *MdGAPDH* and *SlActin* were used as internal controls for apple and tomato, respectively. The qRT-PCR assay was performed in the LightCycler® 96 Real-Time PCR system (Roche, CH) following a specific protocol. All the qRT-PCR reactions were performed in triplicate in a reaction volume of 20 μL. The relative gene expression was measured using the $2^{-\triangle\triangle CT}$ method [39].

Chromosomal locations, microsynteny and evaluation of duplication time

The data for the *MdTCS* gene positions were obtained by searching the genome database of the apple (https://iris.angers.inra.fr/gddh13/). Accordingly, the distribution of the *MdTCS* gene family on the corresponding apple chromosomes was plotted using the MapGene2Chromosome v2.0 (http://mg2c.iask.in/mg2c_v2.0/) software.

The GDR (https://www.rosaceae.org/) and Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html) databases were used to obtain the genome annotation files and coding sequences (CDS) in apple and *Arabidopsis thaliana*, respectively. Bio-Linux-8.0.7 and Oracle VM VirtualBox were employed to perform synteny comparison and to draw karyotype plots and dot plots between *Arabidopsis thaliana* and apple. Full-length amino acid sequences were aligned in the BLASTP tool of NCBI to detect the gene duplication events. The gene pairs belonging to the tandem or duplication were speculated based on the following

two conditions: the alignment region covered over 80% of the longer gene and up to 80% identity in the aligned regions [40]. The Ka/Ks method is a feasible method to illuminate the evolutionary timelines of different species. The gene pair alignment and the calculation of Ka/Ks were performed using the online tools PAL2NAL (http://www.bork.embl.de/pal2nal/) and ClustalW2 (http://www.genome.jp/tools/clustalw/), respectively. The divergence time of the gene pair was predicted using the equation T = Ks/2r (r = 6.56 × 10^{-9}) [41].

Hormone measurement

A total of 0.5g of regeneration bud from 42-day-old T3-generation 'Micro Tom' transgenic plants were collected for measuring the levels of ZT using an ELISA kit (China Agricultural University, Beijing, China) following the manufacturer's instructions with three biological replicates.

Ectopic-expression of MdHK17 in tomato

Since *MdHK17* responded significantly better to exogenous cytokinin than *MdHP5* and *MdRRC4*, we screened *MdHK17* to verify its ability to regulate bud regeneration. The full-length coding DNA sequence (CDS) of *MdHK17* was cloned into the pCAMBIA1300 plasmid to construct ectopic-expressing *MdHK17* (*MdHK17-OE*) of 'Micro Tom' using the Agrobacterium-mediated leaf disk transformation. The putative transgenic plants resistant to kanamycin were confirmed by qRT-PCR. T3-generation regeneration bud of 'Micro Tom' transgenic plants were used for phenotype observation and gene expression analysis. Primers used for RT-PCR analysis are listed in Supplemental Table S2.

Statistical analyses

Statistical analyses were performed using the one-way ANOVA followed by Duncan's multiple range test. All the data were presented as the mean ± SD from three biological replicates, $P < 0.05$ was considered as statistically significant.

Results

Identification of TCS proteins in apple

BLASTP searches were conducted in the GDR databases to detect the putative *MdTCSs* based on the TCS protein sequences of *Arabidopsis* and rice. In total, 82 putative TCS members were found in apple and divided into 3 subfamilies: I) the HK(L) subfamily, consisting of 19 HKs and 10 HKL members; II) the HP subfamily, consisting of 7 authentic and 2 pseudo members; and III) the RR subfamily, consisting of 14 Type-A, 12 Type-B, 5 Type-C, and 13 pseudo members (Table 1).

Table 1
Characteristics of two-component system (TCS) genes in apple.

Gene			Protein					
Name	Accession	Location	Number of amino acids	Molecular weight	Theoretical pI	Predicted subcellular localization	Family in Arabidopsis	Identities
Histidine Kinase(HK)			(aa)	(KD)				
MdHK1	MDP0000276711	17929435..17934994	1230	137.85	6.54	PlasmaMembrane (2.692)	AHK1	65%
MdHK2	MDP0000258078	8903851..8910652	1203	134.41	6.37	PlasmaMembrane (1.703)	AHK2	62%
MdHK3	MDP0000310800	6459592..6466015	1077	119.78	8.70	PlasmaMembrane (3.763)	AHK3	70%
MdHK4	MDP0000155347	8381572..8389769	1000	111.42	8.80	PlasmaMembrane (4.475)	AHK3	68%
MdHK5	MDP0000151825	928738..933163	1023	114.12	6.66	Cytoplasmic(1.343)	AHK4	69%
MdHK6	MDP0000242242	1540378..1544722	1054	117.75	8.44	PlasmaMembrane (1.268)	AHK4	66%
MdHK7	MDP0000916879	58684..62634	1018	114.61	4.95	Nuclear (3.751)	AHK5	55%
MdHK8	MDP0000285535	5417601..5423757	1285	143.62	5.02	Nuclear(2.189)	AHK5	64%
MdHK9	MDP0000762267	7971284..7974712	503	55.35	8.29	Mitochondrial (1.864)	AHK5	31%
MdHK10	MDP0000226809	35016061..35019940	901	100.03	7.08	Nuclear (2.508)	CKI1	44%
MdHK11	MDP0000242277	19084320..19090084	1266	141.53	7.12	Nuclear(2.605)	CKI1	36%
MdHK12	MDP0000217073	26137185..26140479	530	58.58	7.66	Nuclear(2.392)	CKI1	48.79%
MdHK13	MDP0000267951	7977405..7985599	1072	119.22	8.43	PlasmaMembrane(2.623)	ETR1	78%
MdHK14	MDP0000242413	unanchored	645	72.27	5.83	PlasmaMembrane(3.168)	ETR1	72%
MdHK15	MDP0000288468	34443388..34445756	603	67.78	5.54	PlasmaMembrane(2.767)	ETR1	71%
MdHK16	MDP0000412939	11928018..11929696	497	55.46	6.29	Nuclear(0.975)	ETR1	71%
MdHK17	MDP0000300556	24382604..24387813	618	68.99	8.75	PlasmaMembrane(2.681)	ETR1	82%
MdHK18	MDP0000557234	21574570..21580557	835	93.47	8.45	PlasmaMembrane(2.868)	ERS1	83%
MdHK19	MDP0000257135	32951269..32959899	1153	127.49	7.38	PlasmaMembrane(1.999)	ERS1	65%

Gene			Protein					
MdHKL1	MDP0000195916	21164649..21167699	767	84.77	7.26	PlasmaMembrane(4.088)	ETR2	62%
MdHKL2	MDP0000920189	25334191..25336008	576	64.08	6.74	PlasmaMembrane(4.116)	ETR2	66%
MdHKL3	MDP0000231172	30419688..30422696	756	85.38	6.31	PlasmaMembrane(3.679)	EIN4	64%
MdHKL4	MDP0000170095	26883602..26892411	1896	211.16	6.28	Cytoplasmic(1.696)	PHYA	78%
MdHKL5	MDP0000245413	18271952..18282390	1396	154.76	6.36	Cytoplasmic(1.302)	PHYA	78%
MdHKL6	MDP0000184687	13719624..13725008	1150	127.97	5.86	Cytoplasmic(2.856)	PHYB	77%
MdHKL7	MDP0000285110	8633355..8642606	1195	132.01	5.49	Cytoplasmic(3.028)	PHYB	78%
MdHKL8	MDP0000319396	6243695..6247982	1152	128.40	5.71	Cytoplasmic(1.734)	PHYE	66%
MdHKL9	MDP0000279583	26272589..26276415	1132	126.19	5.71	Cytoplasmic(1.773)	PHYE	65%
MdHKL10	MDP0000786621	11638987..11641767	364	41.28	6.17	Cytoplasmic(2.013)	PDK	83%
Histidine photransfer protein(HP)								
MdHP1	MDP0000061368	16964654..16966057	151	17.35	4.95	Nuclear(1.625)	AHP1	70%
MdHP2	MDP0000170922	13468834..13470232	154	17.70	4.84	Nuclear(1.763)	AHP1	70%
MdHP3	MDP0000216664	29180972..29182318	174	20.06	4.91	Nuclear(2.148)	AHP1	58%
MdHP4	MDP0000123837	33716691..33719728	280	31.29	8.32	Plasma Membrane(2.379)	AHP2	57%
MdHP5	MDP0000231522	31763635..31765566	167	19.59	6.59	Extracellular(1.300)	AHP2	52%
MdHP6	MDP0000186518	20485540..20487038	228	25.5	6.38	Cytoplasmic(2.014)	AHP3	52%
MdHP7	MDP0000538123	13797144..13797752	82	9.48	5.14	Cytoplasmic(1.731)	AHP4	49%
MdPHP1	MDP0000189492	4551543..4552200	182	20.54	5.07	Nuclear(4.121)	AHP4	58%
MdPHP2	MDP0000317420	19154341..19158203	321	37.10	10.14	Nuclear(2.264)	AHP4	71%
Type A response regulator(RR)								
MdRRA1	MDP0000328874	3881112..3882372	314	34.87	9.22	Nuclear(2.993)	ARR3	60%
MdRRA2	MDP0000207048	20240241..20242228	227	25.13	6.45	Nuclear(3.306)	ARR5	70%
MdRRA3	MDP0000705889	14040881..14041540	186	20.53	5.36	Extracellular(2.607)	ARR6	28%
MdRRA4	MDP0000212178	25082409..25084417	223	24.65	6.13	Nuclear(2.849)	ARR6	78%

Gene			Protein					
MdRRA5	MDP0000250737	5469178..5471648	355	39.74	9.98	Nuclear(3.560)	ARR6	73%
MdRRA6	MDP0000625459	2722279..2723701	175	19.61	5.01	Nuclear(2.398)	ARR9	71%
MdRRA7	MDP0000119750	9978510..9980015	223	25.23	5.37	Nuclear(4.047)	ARR9	60%
MdRRA8	MDP0000226231	13641128..13642521	215	24.29	4.88	Nuclear(4.570)	ARR9	61%
MdRRA9	MDP0000509768	20217838..20220762	261	29.05	7.66	Nuclear(3.867)	ARR9	60%
MdRRA10	MDP0000585643	12676869..12683728	305	33.88	6.00	Nuclear(4.215)	ARR9	41%
MdRRA11	MDP0000318452	4602071..4603156	129	14.34	4.55	Cytoplasmic(2.136)	ARR16	56%
MdRRA12	MDP0000922391	3272997..3274023	152	16.80	6.30	Nuclear(2.228)	ARR17	70%
MdRRA13	MDP0000877225	13031381..13032802	167	18.24	5.62	Cytoplasmic(1.545)	ARR17	58%
MdRRA14	MDP0000921402	26232378..26233461	170	18.97	8.58	Nuclear(3.213)	ARR17	64%
Type B response regulator								
MdRRB1	MDP0000290818	11207635..11210754	709	77.74	5.61	Nuclear(3.945)	ARR1	53%
MdRRB2	MDP0000224740	14271909..14275127	670	73.25	5.97	Nuclear(3.998)	ARR1	54.00%
MdRRB3	MDP0000124301	1062119..1064884	527	57.29	6.57	Nuclear(3.504)	ARR2	59%
MdRRB4	MDP0000155328	1090324..1093495	670	72.55	5.92	Nuclear(4.044)	ARR2	59%
MdRRB5	MDP0000311914	1396158..1399893	738	80.26	5.68	Nuclear(4.190)	ARR2	61%
MdRRB6	MDP0000228719	26904623..26909706	641	71.21	5.88	Nuclear(3.795)	ARR2	37%
MdRRB7	MDP0000304202	425559..432660	777	88.31	5.54	Nuclear(3.825)	ARR2	40%
MdRRB8	MDP0000320381	5423460..5427511	637	71.20	5.72	Nuclear(4.194)	ARR11	60%
MdRRB9	MDP0000305167	7367861..7369725	248	28.61	6.31	Nuclear(1.906)	ARR11	73%
MdRRB10	MDP0000307383	7368279..7369681	218	25.22	5.84	Nuclear(2.154)	ARR11	70%
MdRRB11	MDP0000607144	7794452..7797620	699	78.04	6.70	Nuclear(3.914)	ARR12	63%
MdRRB12	MDP0000140568	7839080..7842417	707	79.05	6.96	Nuclear(4.192)	ARR12	63%
Type c response regulator								
MdRRC1	MDP0000235765	unanchored	495	54.93	9.08	Nuclear(4.100)	ARR22	32%
MdRRC2	MDP0000664866	33190830..33193109	537	59.68	8.67	Nuclear(4.319)	ARR22	32%
MdRRC3	MDP0000265206	17041674..17052488	871	93.10	6.37	Cytoplasmic(1.496)	ARR24	65%
MdRRC4	MDP0000664866	9994869..9996058	98	10.76	5.21	Cytoplasmic(1.382)	ARR24	65%
MdRRC5	MDP0000265206	21718245..21718769	174	19.18	5.66	Plasma Membrane(1.947)	ARR24	30%
Pseudo response regulator								
MdPRR1	MDP0000187807	26069292..26070542	207	23.28	5.01	Plasma Membrane(1.676)	APRR1	42%
MdPRR2	MDP0000453272	21309620..21313313	566	62.82	5.58	Nuclear(4.691)	APRR1	49%
MdPRR3	MDP0000286458	26076176..26086064	821	92.58	5.98	Nuclear(3.817)	APRR1	48%
MdPRR4	MDP0000248863	4895438..4901506	665	74.82	5.45	Nuclear(4.171)	APRR2	43%

Gene			Protein					
MdPRR5	MDP0000237396	23515274..23519456	588	65.99	6.18	Nuclear(3.817)	APRR2	47%
MdPRR6	MDP0000248765	25766486..25774676	875	97.13	8.47	Nuclear(3.446)	APRR5	39%
MdPRR7	MDP0000234215	28681467..28683344	260	28.96	6.67	Nuclear(2.222)	APRR5	59%
MdPRR8	MDP0000150823	9676147..9682807	1058	115.8	5.83	Nuclear(1.637)	APRR5	52%
MdPRR9	MDP0000211664	15059630..15063127	765	84.50	5.58	Nuclear(4.231)	APRR5	53%
MdPRR10	MDP0000287839	9687877..9692394	78	85.09	5.75	Nuclear(3.452)	APRR5	65%
MdPRR11	MDP0000918738	33268631..33270362	265	29.43	5.03	Nuclear(4.170)	APRR7	55%
MdPRR12	MDP0000307370	15058624..15067488	1045	115.61	6.08	Nuclear(4.095)	APRR7	37%
MdPRR13	MDP0000149453	17797190..17801039	777	85.01	7.37	Nuclear(4.166)	APRR7	50%

These TCS members were named on the basis of the method, which was widely used in Chinese cabbage (Liu et al., 2014), soybean (Mochida et al., 2010), and melon (Liu et al., 2020). For some important horticultural crops and typical model plant species, TCS genes have been studied and summarized in Table 2.

Table 2
Summary of two-component system (TCS) gene numbers identified in plants.

Species	HK(L)	HP (Pseudo-HP)	Type-A RR	Type-B RR	Type-C RR	Pseudo RR	Total	Reference
Arabidopsis thaliana	17 (9)	6 (1)	10	12	2	9	56	Schaller et al., 2008
Oryza sativa	11 (3)	5 (3)	20	7	-	5	51	Pareek et al., 2006
Zea mays	11 (3)	9 (2)	21	7	-	-	48	Chu et al., 2011
Glycine max	21(15)	10(3)	18	15	3	13	98	Mochida et al., 2010
Solanum lycopersicum	9(11)	4(2)	7	23	1	8	65	He et al., 2016
Cucumis melo L.	9(8)	6(3)	8	11	-	6	51	Liu et al., 2020
Lotus japonicus	14	7	7	11	1	5	40	Ishida et al., 2009
Brassica rapa	20(9)	8(1)	21	17	4	15	85	Liu et al., 2014
Cucumis sativus L	18(8)	7 (2)	8	8	-	5	46	He et al., 2016
Citrullus lanatus	19(9)	6 (2)	8	10	1	5	49	He et al., 2016

We analyzed physicochemical properties of all the predicted apple TCS genes. For apple HK(L) subfamily, the molecular weights were 41.28–211.16 kDa, with *MdHKL4* encoding the highest molecular weight and longest protein (1896 aa), while *MdHKL10* encoding the lowest molecular weight and shortest protein (364 aa). In addition, the theoretical isoelectric points ranged from 4.95 (*MdHK7*) to 8.80 (*MdHK*4). The protein subcellular localization prediction showed that 14 *MdHK(L)s* were located in the Plasma Membrane. The remaining genes were predicted to be located in the Cytoplasmic (8), Nuclear (6), and Mitochondrial (1) (Table 1). Regarding the apple HP subfamily, *MdPHP2* encoding the longest proteins (321 aa) has the largest molecular weight (37.10 kDa), while *MdHP7* has the lowest molecular weight (9.48 kDa) because of the shortest proteins (82 aa). Meanwhile, more than half of the *MdHPs* were located in the Nuclear. For the apple RR subfamily, *MdPRR8* has the largest molecular weight (115.83 kDa) to encode the longest proteins (1058 aa), whereas *MdRRC4* encode the shortest proteins with 98 aa. Among the apple RR subfamily, members belonging to the type-A apple RRs were basically located in the Nuclear except for *MdRRA3*, *MdRRA11*, and *MdRRA13.* Interestingly, all of the type-B RRs were positioned in the Nuclear. In the apple pseudo RR family, only *MdPRR1* was located in the Plasma Membrane, the others genes were located in the Nuclear.

HK Proteins in apple

All MdHK(L)s had a conserved HK(L) domain, which comprised five typical characteristic motifs, namely, H, N, G1, F, and G2 (Hwang et al., 2002), except for *MdHK17* and *MdHKL10* (Fig. S1). Five cytokinin receptor-like *MdHKs*, namely, *MdHK2*, *MdHK3*, *MdHK4*, *MdHK5*, and *MdHK6*, existed high identity (62%–70%) with their *Arabidopsis* counterparts (Table 1). We observed that these cytokinin receptor-like *MdHKs* fell on one branch and had similar gene structure (Fig. 1A). These 5 MdHKs which included HK, Rec, CHASE, and transmembrane (TM) domains were forecasted to contain 6 motifs, such as motif 1, 3, 4, 5, 6, and 9. (Fig. 1A; Fig. S1).

tree, gene structures of MdHPs. Different motifs of MdHP proteins are indicated by different colored boxes and numbered 1–5 in subfigure of Motif Location. The sequence information for each motif is provided in Fig. S5. (C) Phylogenetic tree, gene structures of MdRRs. Different motifs of MdRR proteins are indicated by different colored boxes and numbered 1–10. The sequence information for each motif is provided in Fig. S6. The phylogenetic trees were constructed using the neighbor-joining method with 1000 bootstrap replicates by MEGA 7.0; Gene structures were analyzed using the Gene Structure Display Server (GSDS); The motif locations were analyzed using MEME software.

Apple ethylene receptors included 10 members (*MdHK13-MdHK19*) and *MdHKL1-MdHKL3* (Table 1). *MdHKL1* showed the lowest sequence identity (62%) with *ETR2*, however, *MdHK18* was homologous with *Arabidopsis ERS1*, with highest identity of 83% (Table 1). Apple ethylene receptors were clustered one branch, suggesting that they had relatively close relationship with each other (Fig. 1A). It is worth noting that *MdHK16*, *MdHKL1*, and *MdHKL3* have similar exon-intron structure, consisting of 1 intron. Additionally, *MdHK14* and *MdHK15* contain 4 introns (Fig. 1A). All predicted apple ethylene receptors possessed 1 characteristic domain (GAF domain) and 2–4 TM helices in the upstream region of GAF domain (Table 1, Fig. S3).

Apple PHY genes containing *MdHKL4–MdHKL9* shared 65%–78% similarity to their corresponding *Arabidopsis* counterparts (Table 1). In our study, these genes fell on one branch based on their similar protein sequences, and *MdHKL6–MdHKL9* possessed 3–5 introns according to the gene structure analysis (Fig. 1A). In addition, 6 apple PHY members included motif 1, 3, and 7, and all contained 3PAS, 1PAC, and 1 GAF domains (Fig. S3).

HP proteins in apple

A total of 9 HPs in apple with 7 authentic HPs and 2 pseudo-HPs were identified (Table 1). All 5 authentic HP members and 2 pseudo HP members (*MdPHP1* and *MdPHP*2), fell on one branch, and another branch was occupied by *MdHP*4 and *MdH5* (Fig. 1B). We found that all the 7 *MdHPs* and *MdPHP2* contained 2–7 introns except *MdPHP1*, the number of motifs varied between 1 and 5 among *Md(P)HPs* (Fig. 1B; Fig.S4).'XHQXKGSSSS' consensus sequence were observed in the His of HPt domain, however, the H site of the Hpt domain was replaced by the Q residue for *MdPHP1* and *MdPHP2* (Fig. S2). *MdPHP2* exhibited high identity (71%) with its *Arabidopsis* homologs *AHP4* in spite of lost the conserved Hpt domain (Table 1).

RR proteins in apple

We found 44 *Md(P)RRs* including 14 type-A RRs, 12 type-B RRs, 5 type-C RRs, and 13 pseudo RRs (Table 1). 44 members were divided into 2 branches, one was composed of *MdPRR1-13* and *MdRRBs*, except for *MdRRB6* and *MdRRB7*, the other included all of the *MdRRAs* and *MdRRCs* (Fig. 1C). Among these *Md(P)RRs*, multiple sequence alignment showed that most of *Md(P)RRs* had a conserved Rec domain, with the exception of *MdRRA11* and *MdRRC4* (Fig. S2). Type-A RRs of apple are characterized with a short C terminal extension following the Rec domain related to motif 1, 2, 4, 5, and 7, whereas *MdRRA4* and *MdRRA11* lost motif 4 (Fig. 1C). The similarities between the 14 type-A *MdRRs* and their homologs in *Arabidopsis* ranged from 28%–78%. For the 12 type-B MdRR proteins, as transcription factors (TFs), had a long C-terminal extension with a *Myb*-like DNA binding domain and 4 conserved motifs, namely motif 1, 2, 3, and 4, except for *MdRRB10* (Fig. 1C; Fig. S5). The *Myb*-like DNA binding domain functions as a signature domain to identify type-B MdRRs and type-A MdRRs. They shared 37%–73% sequence identity to their counterparts of *Arabidopsis*, including *ARR1*, *ARR2*, *ARR11*, and *ARR12* (Table 1). 5 type-C MdRR proteins also were finally screened by a serious of comparisons. Interestingly, *MdRRC4* had an unstable Rec domain instead of a conserved Rec domain although it exhibited high identity (65%) with *ARR24* (Fig. S5).

Phylogenetic analysis of plant TCS proteins

To further clarify the relationship of TCS genes between apple and other plant species including *Arabidopsis*, rice, maize, soybean, Chinese cabbage, melon, cucumber, tomato, and watermelon, we performed multiple sequence alignments and constructed phylogenetic trees. The 198 HK(L) proteins from these selected species were divided into 7 distinct subfamilies, the cytokinin receptor, ethylene receptor, phytochrome-like receptor, AHK1-like, CKI1-like, CKI2/AHK5-like, and PDK-like subfamilies (Fig. 2). By analyzing the evolutionary relationship between apple and other plant species, we observed that apple HK(L)s have the closest relationship with soybean, which was clustered in same subclade with apple. Additionally, all of the maize HK(L) proteins fell on cytokinin receptor subfamily and were distributed in the same branch with rice, so we speculated a close phylogenetic relationship between them (Fig. 2).

The 78 (P)HP proteins from 10 species were mainly divided into 4 different clades (Fig. 3). Clade II was the smallest, no members of *Md(P)HPs* were detected on it. Most members of MdHPs, namely, *MdHP1*, *MdHP2*, *MdHP3*, and *MdHP6* mainly distributed in Clade I. Clade I and IV were composed of only HPs from dicots, however, Clade II and III consisting of the HP members both contained monocots and dicots.

The 289 RRs proteins from 10 species were grouped into 4 subfamilies including type-A, type-B, type-C, and PRRs (Fig. 4). The members of apple type-A RRs showed a fairly close evolutionary relationship with other selected species, particularly with soybean. In detail, monocots and eudicots were distributed alternatively in the type-A RRs subclade, suggesting that type-A RR genes already existed before the divergence of monocot-eudicot (Fig. 4). Type-B RRs, the biggest subgroup, covered 3 subgroups. Type B-1 RR was the largest of the three, and contained the RRs from the all ten species. Type B-2 RRs included one member of MdRRBs, namely, *MdRRB6*. Type B-3 RRs was the smallest, no members of MdRRBs were found in it, but contained 2 *Arabidopsis* members, namely, *ARR19* and *ARR20*. Interestingly, type-C RRs from the seven species had close relationship in phylogenetic tree, melon and cucumber genomes did not contain any members of type-C RRs. All the PRRs were further divided into 3 subclades. According to the evolutionary relationships, type-B PRRs and type-B RRs were clustered on one big branch, declaring that type-B PRRs have close relationship with type-B RRs compared to other subfamilies. Clock PRRs, which was the the largest PRR subgroup, contained PRR members from all selected plant species. APRR8-like subfamily was named on the basis of APRR8 from *Arabidopsis*.

Genomic distribution and gene duplication of TCS members in apple

All identified apple TCS genes were mapped on the 16 chromosomes, except *MdHK14* and *MdRRC1* located on the unanchored chromosomes (Fig. S9). In particular, apple *HK(L)s*, *HP*, and *RR* members were mapped on 12 chromosomes, 8 chromosomes, and 14 chromosomes, respectively. Eight pairs of tandem genes, including *MdHK9/MdHK13*, *MdHK9/MdHK18*, *MdHK13/MdHK18*, *MdRRB3/MdRRB4*, *MdRRB9/MdRRB10*, *MdRRB11/MdRRB12*, *MdPRR8/MdPRR10*, and *MdPRR9/MdPRR12*, were found in duplication events, accounting for 25% of all apple TCS genes (Table 3). Notably, 24 pairs of segmental duplicates were found in all TCS genes from apple. The synonymous rate (Ks) and nonsynonymous rate (Ka) were calculated to evaluate the possible divergence mechanisms by using the 32 paralogous gene pairs of apple TCS (Table 3). For MdHK(L)s, the Ks of 14 duplicated gene pairs ranged from 0.0111 to 0.4862, the corresponding divergent time was 16.206 Mya to 0.370 Mya. For Md(P)HPs, the Ks of 3 duplicated gene pairs varied from 0.0843 to 0.9842, which speculated the divergent time was 32.860 Mya to 2.810 Mya. For MdRRs, the Ks of 15 duplicated gene pairs were 0.0029 to 0.4280, suggesting that these genes were diverged from 14.266 Mya to 0.096 Mya.

Table 3
Inference of duplication time of duplicated TCS paralogous pairs in apple.

Duplicated Gene Pairs	Ka	Ks	Ka/Ks	Evolutionary Selection	Duplication Type	Divergence time (Mya)
MdHK3/MdHK4	0.0800	0.2275	0.3516	purifying	Segmental	7.583
MdHK5/MdHK6	0.0230	0.1197	0.1920	purifying	Segmental	3.990
MdHK7/MdHK8	0.0653	0.2513	0.2599	purifying	Segmental	8.376
MdHK9/MdHK13	0.0232	0.0111	2.0968	diversifying	Tandem	0.370
MdHK9/MdHK18	0.0591	0.4862	0.1215	purifying	Tandem	16.206
MdHK10/MdHK12	0.0592	0.2224	0.2664	purifying	Segmental	7.413
MdHK13/MdHK18	0.0868	0.2217	0.3912	purifying	Tandem	7.390
MdHK14/MdHK15	0.0510	0.1528	0.3340	purifying	Segmental	5.093
MdHK14/MdHK16	0.0795	0.2040	0.3895	purifying	Segmental	6.800
MdHK15/MdHK16	0.0266	0.0535	0.4977	purifying	Segmental	1.783
MdHKL1/MdHKL2	0.0324	0.1727	0.1877	purifying	Segmental	5.756
MdHKL4/MdHKL5	0.0285	0.1662	0.1717	purifying	Segmental	5.540
MdHKL6/MdHKL7	0.0542	0.2343	0.2313	purifying	Segmental	7.810
MdHKL8/MdHKL9	0.0549	0.2270	0.2421	purifying	Segmental	7.566
MdHP1/ MdHP2	0.0030	0.0843	0.0352	purifying	Segmental	2.810
MdHP4/ MdHP5	0.1414	0.2575	0.5492	purifying	Segmental	8.583
MdPHP1/ MdPHP2	0.7162	0.9842	0.7276	purifying	Segmental	32.806
MdRRA2/MdRRA4	0.0296	0.1250	0.2367	purifying	Segmental	4.166
MdRRA7/MdRRA8	0.0447	0.1593	0.2802	purifying	Segmental	5.310
MdRRA12/MdRRA14	0.0348	0.1401	0.2488	purifying	Segmental	4.670
MdRRB1/MdRRB2	0.0411	0.1608	0.2556	purifying	Segmental	5.360
MdRRB3/MdRRB4	0.0042	0.0088	0.4709	purifying	Tandem	0.293
MdRRB4/MdRRB5	0.0597	0.1556	0.3835	purifying	Segmental	5.186
MdRRB8/MdRRB9	0.0255	0.1301	0.1957	purifying	Segmental	4.336
MdRRB8/MdRRB10	0.0647	0.2010	0.3217	purifying	Segmental	6.700
MdRRB9/MdRRB10	0.0364	0.0564	0.6450	purifying	Tandem	1.880
MdRRB11/MdRRB12	0.0054	0.0029	1.8910	diversifying	Tandem	0.096
MdRRC3/MdRRC4	0.2815	0.4280	0.6576	purifying	Segmental	14.266
MdPRR2/MdPRR3	0.0907	0.2463	0.3684	purifying	Segmental	8.210
MdPRR8/MdPRR10	0.0433	0.0796	0.5434	purifying	Tandem	2.653
MdPRR9/MdPRR12	0.0157	0.0138	1.1422	diversifying	Tandem	0.460
MdPRR11/MdPRR12	0.0840	0.2366	0.3550	purifying	Segmental	7.886

Note: Ks: number of synonymous substitutions per synonymous site; Ka: number of nonsynonymous substitutions per nonsynonymous site. When Ka/Ks = 1, neutral evolution; Ka/Ks < 1, purifying selection; Ka/Ks > 1, diversifying selection.

Analysis of cis-elements in putative promoter regions of TCS genes in apple

To better understand the features of apple TCS genes, we analyzed *cis*-elements of upstream 2 kb DNA sequence of promoter regions (Fig. S10). A total of 9 different *cis*-regulatory elements, which were classified into 2 types including hormone-responsive and stress-responsive elements, were detected. Among the total 82 promoter regions of TCS genes, 66 *cis*-acting elements related to cytokinin responses were identified. A large number of *cis*-elements related MeJA-responsiveness were identified in the 60 promoters of apple TCS genes. Meanwhile, we also detected 50 and *cis*-acting elements connected with Drought- and Abscisic acid-responsiveness in *MdTCS* genes, respectively. The rest contained Auxin-, Gibberellin-, Wound-, Low-temperature and Salicylic acid responsive elements, the number of them varied from 18 to 45 predicting apple TCS genes.

Synteny analysis

To compare the colinearity relationship between *Arabidopsis* and apple of TCS gene family, we performed a synteny analysis. For HK(L)s, a total of 13 gene pairs were found. Among them, chromosome 1 and 2 both existed 4 genes with the higher number compared with other 3 chromosomes. In apple, we found that gene pairs were mainly distributed 9 chromosomes, chromosome 3 were presented 3 genes having colinearity relationship with *Arabidopsis* (Fig. 5A). For HPs, only two gene pairs showed collinearity locating to chromosome 9 and 11 of apple (Fig. 5B). Regarding to RRs, 32 gene pairs possess microsynteny distributing almost all of chromosomes of apple and *Arabidopsis* (Fig. 5C). Chromosome 1 of *Arabidopsis* and chromosome 13, 14, and 16 of apple contain the most gene pairs having colinearity relationship.

Expression profiles of apple TCS genes in various tissues

The microarray data related to tissue-specific expression patterns were downloaded from the NCBI. Among them, gene expression levels of seven major tissues originated from two different cultivars were presented by Heatmap. For apple HK(L)s, we grouped them into 3 clades according to their different expression levels (Fig. 6A). Compared with clade Ⅱ, the genes of clade Ⅰ were lowly expressed in leaf, flower, fruit 100daa, and harvested fruit. In clade Ⅱ, MdHK(L)s were observed to express in all tissues, particularly *MdHKL4*, *MdHKL5*, and *MdHKL10* with highest expression levels. The genes from clade Ⅰ of Md(P)HPs were predominantly expressed in all tissues, whereas all of genes from clade Ⅱ were not expressed in all tissues (Fig. 6B). In addition, *MdHP3* and *MdHP5* of clade Ⅰ displayed increased expression in fruit 100daa. Apple MdRRs were further clusterd into 4 groups (Fig. 6C). In clade Ⅳ, the gene expression levels gradually increased with fruit development. There were 17 genes from clade Ⅱ were not expressed in all tissues. *MdRRA9* and *MdRRB5* of clade Ⅰ were displayed highest expression level in all tissues. In clade Ⅲ, the transcripts of *MdRRA1*, *MdRRA10*, and *MdRRA8* were only detected in root and stem, the rest members had the higher expression levels in the late development than the early development.

Gene ontology

GO functional enrichment analysis was carried out using all TCS genes of apple. The results of GO functional analysis were divided into three main categories: molecular function, cellular component, and biological process (Fig. 7). The molecular function was enriched a total of 16 terms, 5 of which were significantly enriched to two-component sensor activity (GO:0000155), two-component response regulator activity (GO:0000156), protein histidine kinase activity (GO:0004673), signal transducer activity (GO:0004871), and protein binding (GO:0005515). In the biological process, GO was enriched in 14 terms, among these terms, two-component signal transduction system (phosphorelay) (GO:0000160), regulation of transcription, DNA-dependent (GO:0006355), signal transduction (GO:0000160), phosphorylation (GO:0016310), and peptidyl-histidine phosphorylation (GO:0018106) had highest propotion. In the cellular component, 7 terms were observed, namely, membrane (GO:0016020), endoplasmic reticulum membrane (GO:0005789), tricarboxylic acid cycle enzyme complex (GO:0045239), endoplasmic reticulum membrane (GO:0005789), intracellular (GO:0005622), integral to membrane (GO:0016021), and cytoplasm (GO:0005737).

TCS genes of apple response to exogenous cytokinin

Verification of the expression changes of 82 TCS members uncovered that majority of the TCS genes could respond to exogenous ZT in apple seedlings (Fig. 8). In the MdHK(L)s subfamilies, except *MdHK14–16*, *MdHKL1/2*, and *MdHKL6–10*, the rest of the detected TCS genes were upregulated by exogenous ZT treatment. In detail, *MdHK5* and *MdHK6* were notably upregulated by cytokinin at 8 h; *MdHK17* were increased 36-fold and 58-fold at 4 h and 8 h compared with control, respectively. *MdHK14–16*, *MdHKL1/2*, and *MdHKL6–10* were downregulated from 2 h to 8 h following ZT treatment (Fig. 8A). Md(P)HPs were differentially expressed at different treatment times, *MdHP1* and *MdHP2* were slightly upregulated from 2 h to 8 h, *MdHP5* increased to high levels at 2 h, which were 4-fold higher than control. *MdHP3/4*, *MdHP6/7*, and *MdPHP1/2* were downregulated by exogenous ZT application (Fig. 8B). Almost all of the type-A RRs could be induced by ZT treatment, except *MdRRA3*. By contrast, type-B RRs were downregulated by ZT treatment. For the MdRRC subfamily, we observed that *MdRRC4* were increased 11-fold and 6-fold at 2 h and 8 h as high as reference group, respectively. *MdRRC5* increased modestly compared with the control. Most of MdPRRs decrease to a low level from 2 h to 8 h, whereas *MdPRR1*, *MdPRR2*, *MdPRR3*, and *MdPRR12* were upregulated at 2 h and downregulated from 4 h to 8 h (Fig. 8C).

Ectopic expression of MdHK17 in tomato

To further investigate the regeneration bud activity of *MdHK17*, we obtained transgenic tomato plants over-expressing *MdHK17*, namely two homozygous overexpression lines OE-5 and OE-6 (Fig. 9A). qRT-PCR assay showed a obvious increase in the expression level of *MdHK17* transgenic lines (Fig. 9B). The number of regeneration bud in transgenic lines was significantly more than that in the WT (Fig. 9C). The content of ZT is higher in OE-5 and OE-6 transgenic lines than in WT (Fig. 9D).

Discussion

Prediction and Phylogenetic Analysis of the TCSs in Apple

In apple, 82 TCS members were screened, which were more than that in the reported plant species except *Glycine max* and *Brassica rapa* (Table 2). The main reason originated from the larger genome size and duplication events of apple genome. Meanwhile, the HK(L)s subfamily of apple including 29 gene members, which is slightly bigger than that in the identified species, except *Glycine max*. The type-A *RR*s possibly originated from the mutations of promoters of type-C RR, which were deemed as the oldest RRs [42]. In particular, 5 type-C RRs were detected in apple, while there are no type-C RRs identified in melon, cucumber, rice, maize, and wheat. The reasons for the occurrence or absence of type-C RRs deserved to be explored in the future. The CHASE domain plays a vital role to recognize and bind cytokinin [7] and was highly conserved among the apple cytokinin receptors (Fig. S1). Simultaneously, we found that, except for *MdHK2*, all of the apple cytokinin receptors possessed 1 TM helix at the downstream region of CHASE domain and had 1–3 TM helix at the upstream region of CHASE domain (Fig. S1, Table 1), which was similar to previous studies [43, 44]. The PHY members in *Arabidopsis*, which act as red or far-red light photoreceptors participate in various photomorphogenic processes [45]. Therefore, we predicted PHY members of apple involved in the process of morphogenesis.

Duplications of the TCS Gene Family in Apple

Gene duplication including segmental and tandem duplication is deemed as the driving force to expand gene family [7, 11, 12]. Tandem and segmental duplication was found in TCS gene of apple, which accounted for 25% and 75% in apple TCS genes, respectively. This result suggested that both segmental and tandem duplication contributed to the expansion of TCS genes in apple. Simultaneously, tandem duplication gene pairs located on the same chromosome have conserved gene structure and domain. Similar phenomenon was identified in previous studies. In detail, four pairs of segment duplicates and a tandem duplicate cluster were found in tomato [13]. A pair of *CsRR13*/*CsRR14* serving as tandem duplication was identified in cucumber, while *ClHKL2*/*ClHKL3* and *ClRR16*/*ClRR17* serving as tandem duplications and one couple segment duplicate including *ClHP1*/*ClHP2* were found in watermelon [15]. In contrast, 20, 61, and 66 TCS genes were observed from *Arabidopsis*, Chinese cabbage, and soybean, which demonstrated segmental duplication played a critical role in the expansion of TCS genes in their genomes [7, 11, 14]. Intriguingly, we also found diversifying selection occurred in tandem duplication of TCS genes from apple, except for *MdHK9/MdHK18* and *MdHK13/MdHK18*, implying that the type of evolutional selection may be connected with the mechanism of duplication events. Compared with three representative members of Cucurbitaceae family including melon, cucumber, and watermelon, there are more duplication events identified in apple, indicating that whole genome duplication may be appeared during evolution. Through the synteny analysis of three subfamilies of apple TCS gene, it is found that there is an obvious collinear relationship of RR subfamily between *Arabidopsis* and apple.

Roles of MdTCSs in Apple Growth and Development

The microarray data analysis found that 26 out of the 82 genes have been observed to be expressed in apple roots (Fig. 6), implying that these genes might be related to the synthesis of cytokinin. Previous studies such as *Arabidopsis*, Chinese cabbage, soybean, tomato, and melon have consistent results [8, 12, 28]. *MdHK10–12* detected in flowers and fruits were homologous with *Arabidopsis CKI1*, which was involved in the development of ovules and female gametophyte. Notably, all ethylene receptors including *MdHK13–19* and *MdHKL1*/*MdHKL2* displayed higher expression level in apple flowers and fruits (Fig. 6A). This result has been verified in the development of flowers and fruits in tomato and cucumber [46, 47]. Additionally, except *MdPHP1*, *MdPHP2*, *MdHP4*, *and MdHP7*, the rest TCS genes exhibit preferential expression in flowers. Meanwhile, TCS members of *Arabidopsis* could be regulated female gametophyte and fruit development [48, 49]. In soybean, *GmHK01*, *GmHP02*, *GmRR17*, and *GmRR34* were observed to express in inflorescence or seed tissues [11]. In tomato, *SlPRR8* possibly plays vital roles in regulating plastid development and fruit ripening [50].

Regulation of MdTCSs in Response to cytokinin

Cis-acting elements associated with cytokinin accounted for 80% of promoter regions of apple TCS genes, implying that TCS genes play important roles in cytokinin signal transduction. The expression profiles of 82 genes were analyzed by qRT-PCR to verify whether these genes could respond to cytokinin. We discovered that more than half of the TCS gens negatively respond to cytokinins (Fig. 8). In detail, *MdHK15*, *MdHKL1*, *MdHKL6*, *MdHKL7*, and *MdHKL9* were downregulated by ZT treatment (Fig. 8). *MdHK5*, *MdHK6*, *MdHK17*, *MdHP5*, and *MdRRA4* are obviously induced by ZT treatment, while *MdHKL5*, *MdRRA9*, *MdRRA13*, *MdRRA14* existed significant fluctuations in gene expression (Fig. 8). This evidence indicated that these predicted apple TCS genes are complex in cytokinin signal transduction. In tea plant, *CsRR5* may regulate cold stress by integrating phytohormone signals through the *CBF-COR* pathway (Dong et al., 2025). In our study, the ectopic expression of *MdHK17* in tomato has been proven to promote bud regeneration. These results indicated that the TCS genes not only participate in the differentiation of buds but also is involved in the response process to environmental stress through its interaction with plant hormones. This will lay a solid foundation for future in-depth research on the apple TCS gene family in terms of plant growth and differentiation.

Conclusion

A total of 82 TCS genes of apple were annotated based on the apple genome. We used bioinformatic methods to analyze physicochemical properties of apple TCS gene. We forecasted some internal connection between apple and 9 identified species through phylogenetic analysis. The enlargement of apple TCS family was caused by tandem and segmental duplications. Many *MdTCSs* showed obvious expression patterns under different varieties and different growth stages. Additionally, qRT-PCR analysis showed that *MdHK17*, *MdHP5*, *MdRRA2*, *MdRRA4*, and *MdRRC4* may be involved in the regulation of ZT transport in apple seedling. It is important to note that *MdHK17* may be involved in regulating the differentiation of regenerative buds. These data reveal the potential role of the TCS gene in apple and provide new insights for promoting the differentiation of regenerative buds in apple production.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Table S2

Primers used for RT-PCR analysis

Funding

This work was supported by the Gansu Province Youth Science and Technology Foundation, grant number 24JRRA966.

Author Contribution

P.W. and S.X.L. conceived and planned the research. W.G.F. and P.W. supervised the research. P.W., S.Z.J., Y.P.H. and S.X.L. performed the experiments. P.W. conducted data analysis. P.W. wrote the manuscript. P.W. and S.X.L. edited the manuscript. All authors have read and agreed to the published version of the manuscript.

Data availability

No datasets were generated or analysed during the current study.

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Figures

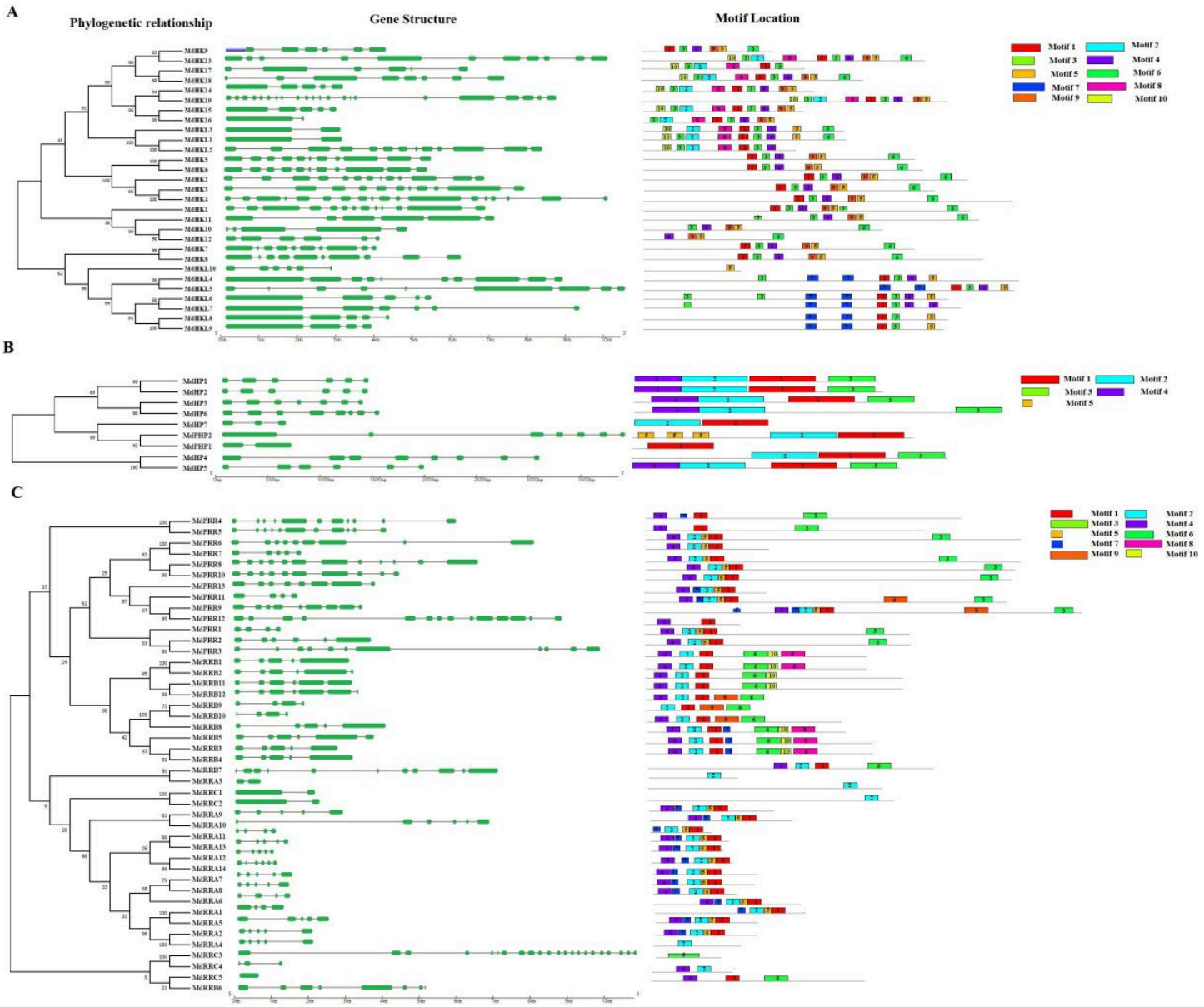


Figure 1

Phylogenetic relationships, gene structures and conserved motifs of TCS genes in apple. (A) Phylogenetic tree, gene structures of MdHK(L)s. The closed green boxes and black lines represent exons and introns in subfigure of gene structures, respectively. Different motifs of MdHK(L) proteins are indicated by different colored boxes and numbered as 1–10 in subfigure of Motif Location. The sequence information for each motif is provided in Fig. S4. (B) Phylogenetic

tree, gene structures of MdHPs. Different motifs of MdHP proteins are indicated by different colored boxes and numbered 1–5 in subfigure of Motif Location. The sequence information for each motif is provided in Fig. S5. (C) Phylogenetic tree, gene structures of MdRRs. Different motifs of MdRR proteins are indicated by different colored boxes and numbered 1–10. The sequence information for each motif is provided in Fig. S6. The phylogenetic trees were constructed using the neighbor-joining method with 1000 bootstrap replicates by MEGA 7.0; Gene structures were analyzed using the Gene Structure Display Server (GSDS); The motif locations were analyzed using MEME software.

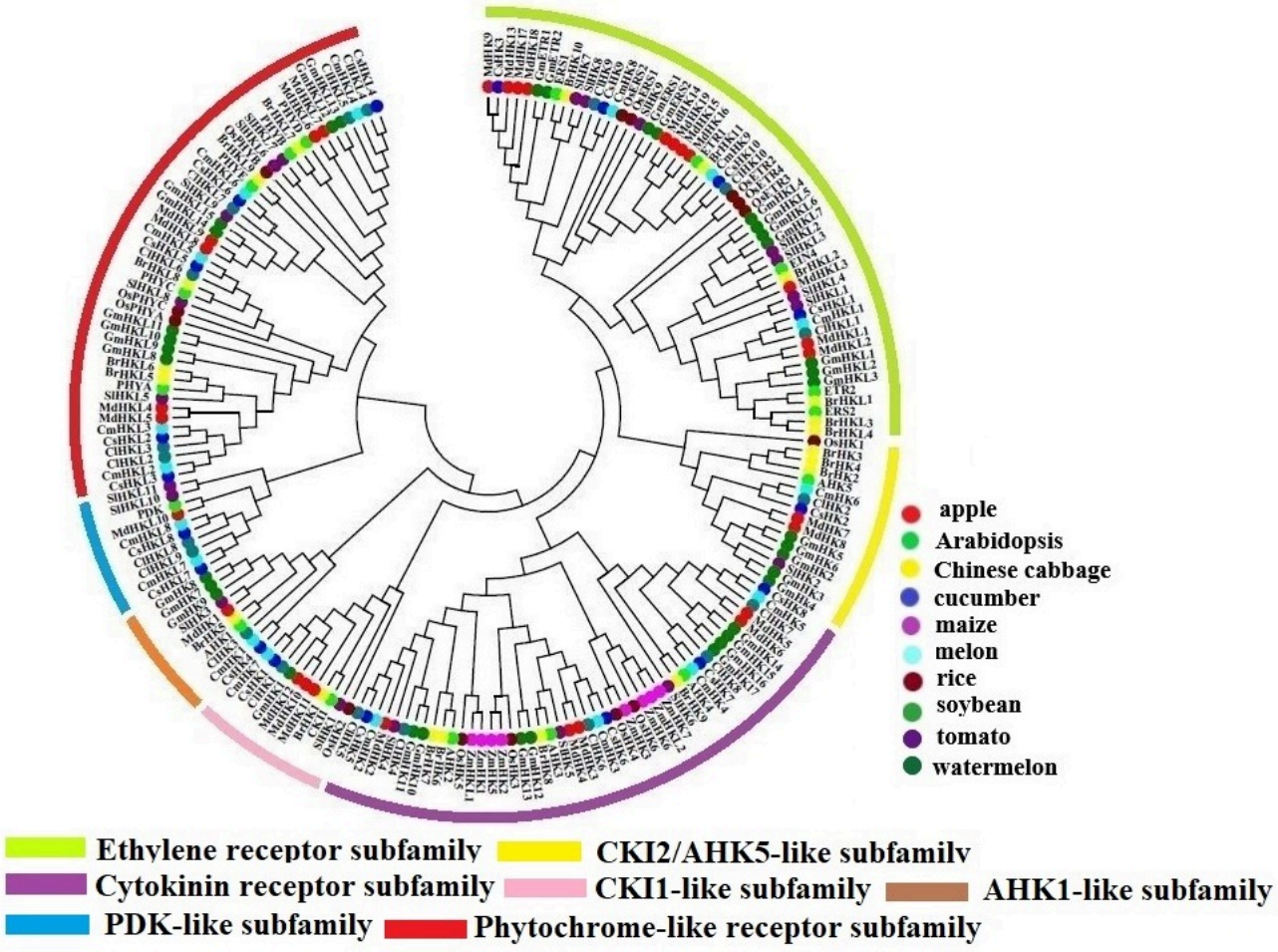


Figure 2

P**hylogenetic relationship of HK(L) proteins in *Arabidopsis*, rice, maize, Chinese cabbage, soybean, tomato, cucumber, watermelon, melon, and apple.** The phylogenetic tree was constructed using the neighbor-joining method with 1000 bootstrap replicates by MEGA 7.0 The diverse subgroups of HK(L) proteins are marked by different colors.

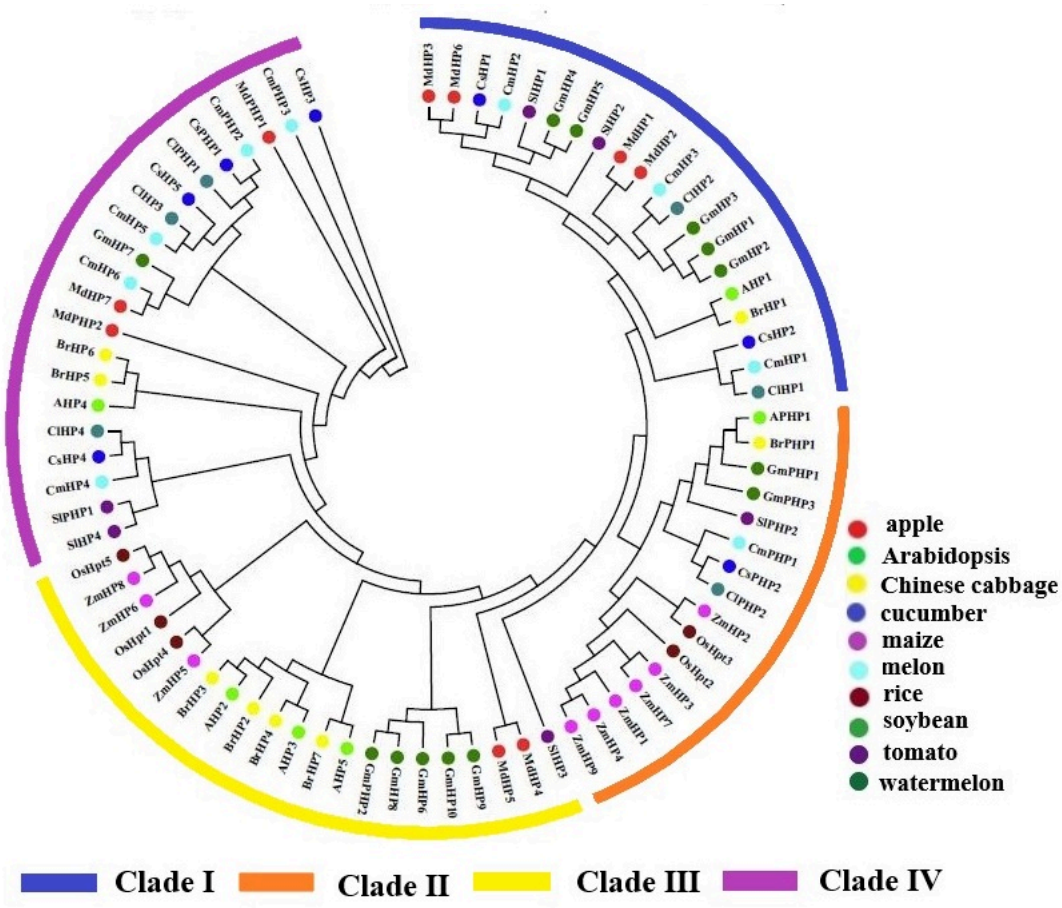


Figure 3

Phylogenetic analysis of HP proteins in *Arabidopsis,* rice, maize, Chinese cabbage, soybean, tomato, cucumber, watermelon, melon, and apple.The phylogenetic tree was constructed using the neighbor-joining method with 1000 bootstrap replicates by MEGA 7.0 The diverse subgroups of HP proteins are marked by different colors.

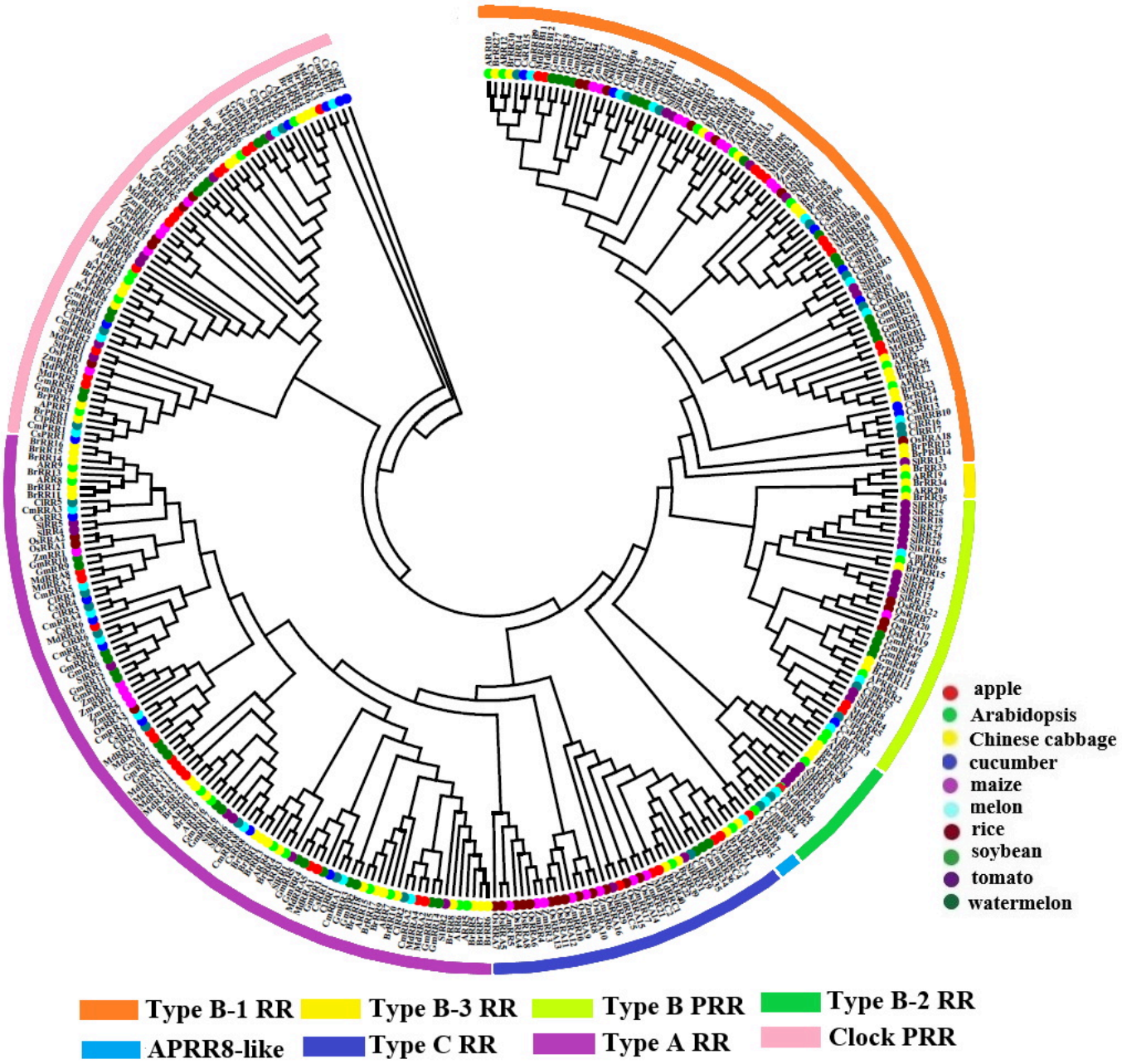


Figure 4

Phylogenetic analysis of RR proteins in *Arabidopsis,* rice, maize, Chinese cabbage, soybean, tomato, cucumber, watermelon, melon, and apple. The phylogenetic tree was constructed using the neighbor-joining method with 1000 bootstrap replicates by MEGA 7.0 The diverse subgroups of RR proteins are marked by different colors.

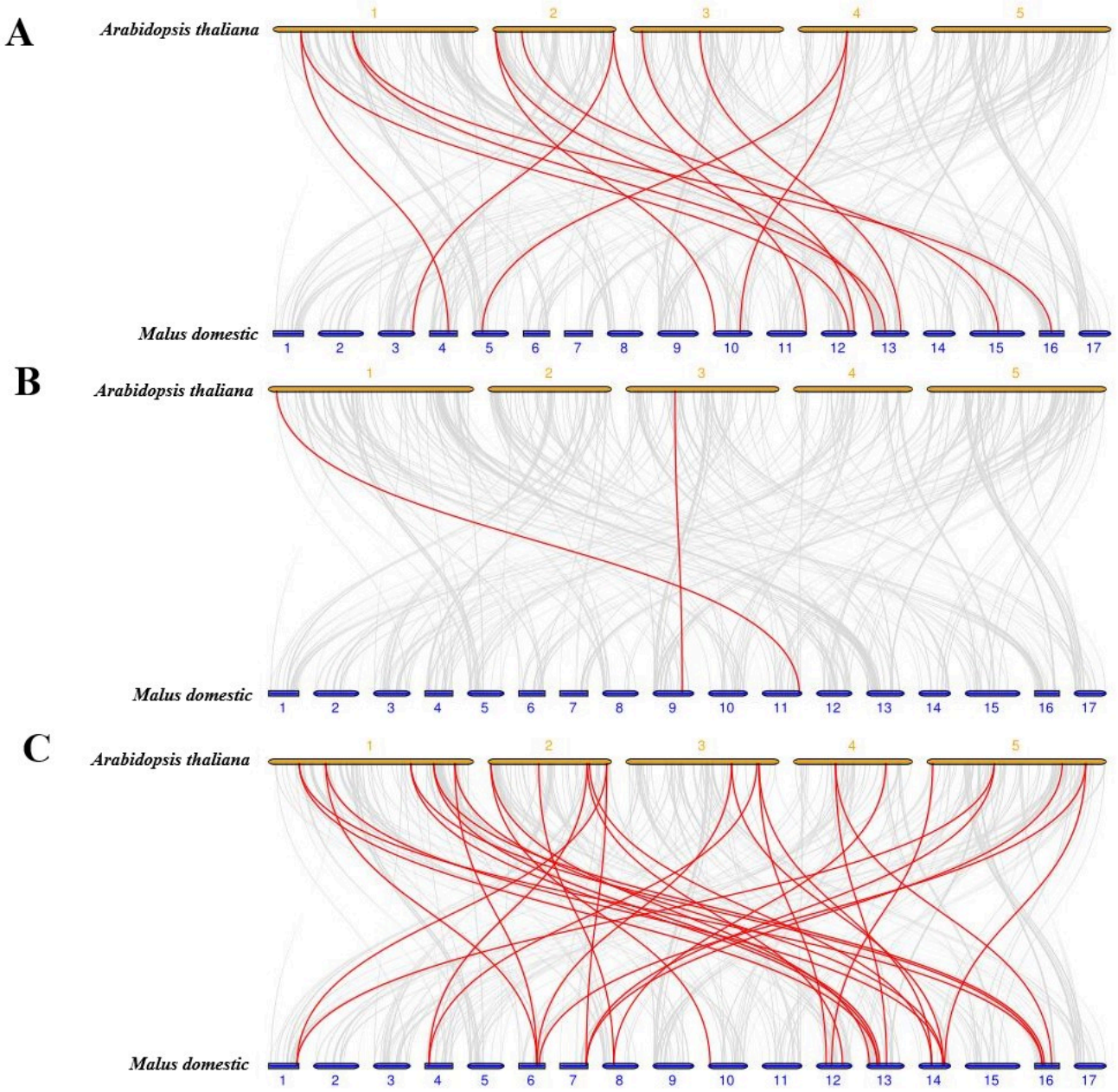


Figure 5

Synteny analyses between apple and *Arabidopsis* genomes. The colour variations represents the extent of similarity and homology among the genes. (A) Represent syntenic genes of HKs in apple and *Arabidopsis*. (B) Represent syntenic genes of HPs in apple and *Arabidopsis*. (C) Represent syntenic genes of RRs in apple and *Arabidopsis.*

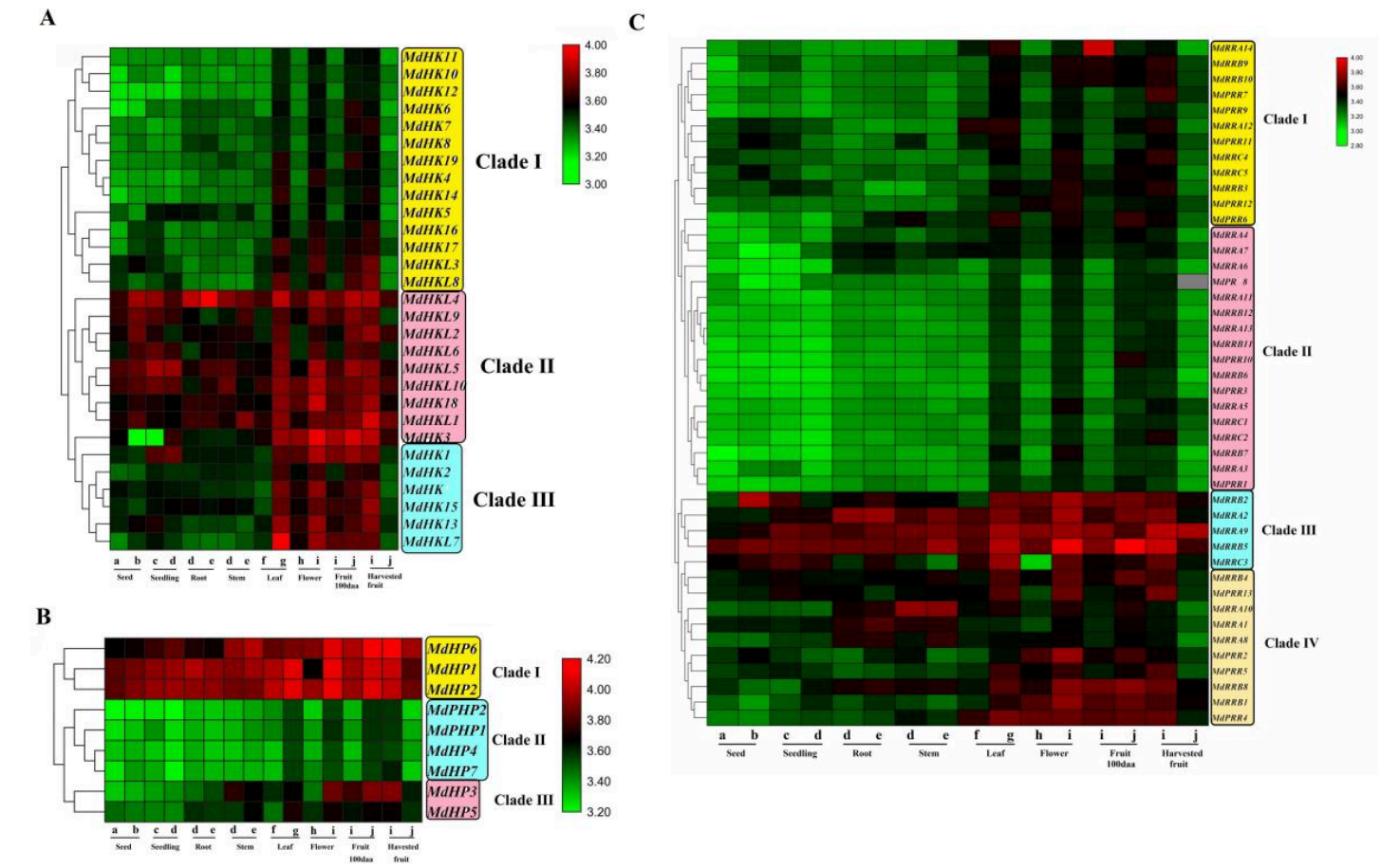


Figure 6

Tissue specific expressions of TCS genes in apple. (A) Tissue specific MdHK(L)s expression profiles in apple. (B) Tissue specific MdHPs expression profiles in apple. (C) Tissue specific MdRRs expression profiles in apple. Data of gene expressions were extracted from NCBI database. a: X4442 × X2596; b: X3069 × X922; c: X4102; d: Gold Delicious; e: X8877; f: M14; g: M49; h: M67; i: M74; j: M20. 100DAA: 100 days after anthesis. Green represents low expression, black represents medium expression, and red represents strong expression.

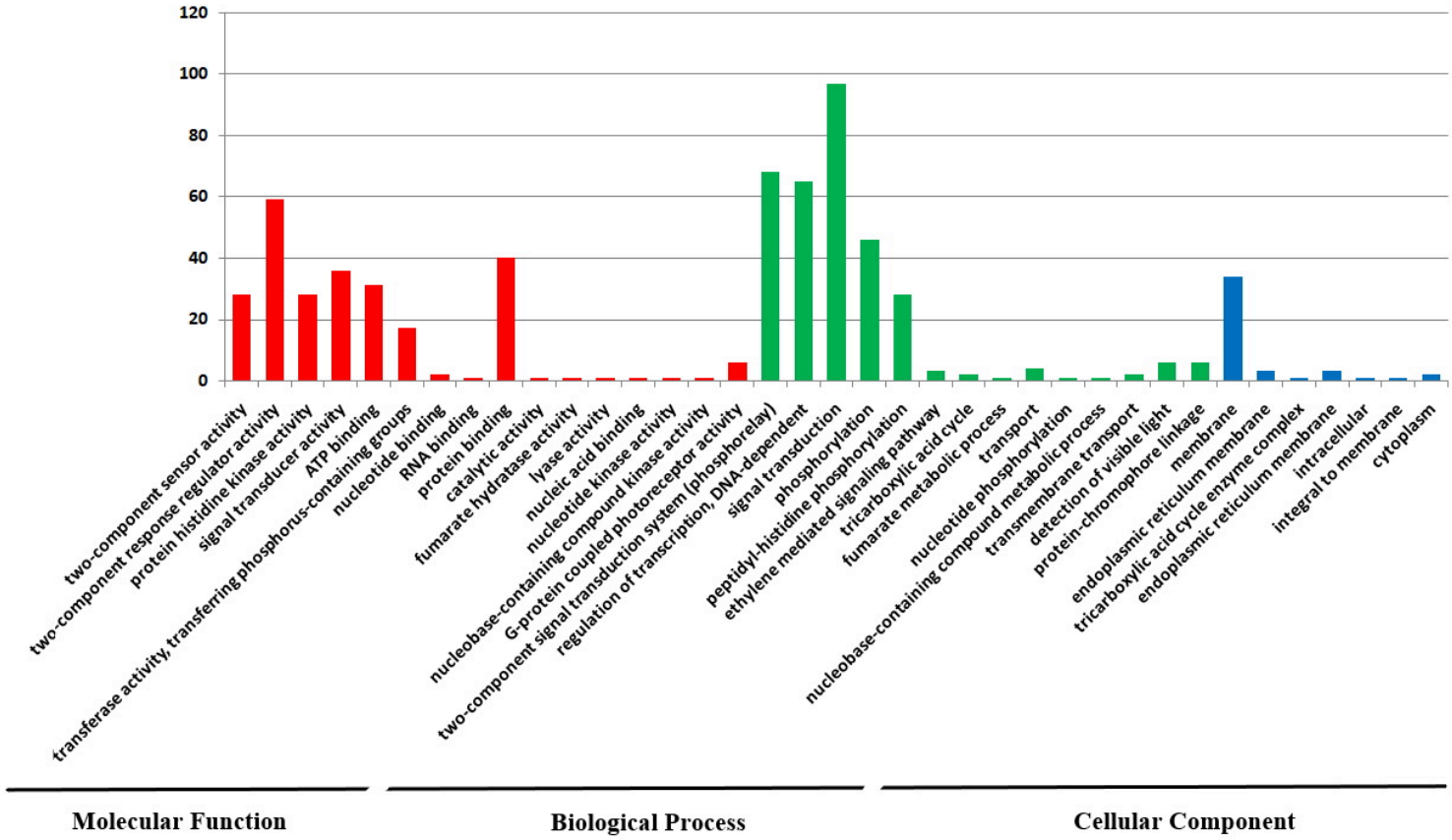


Figure 7

GO (Gene ontology) analysis of *TCS* gene family in apple.

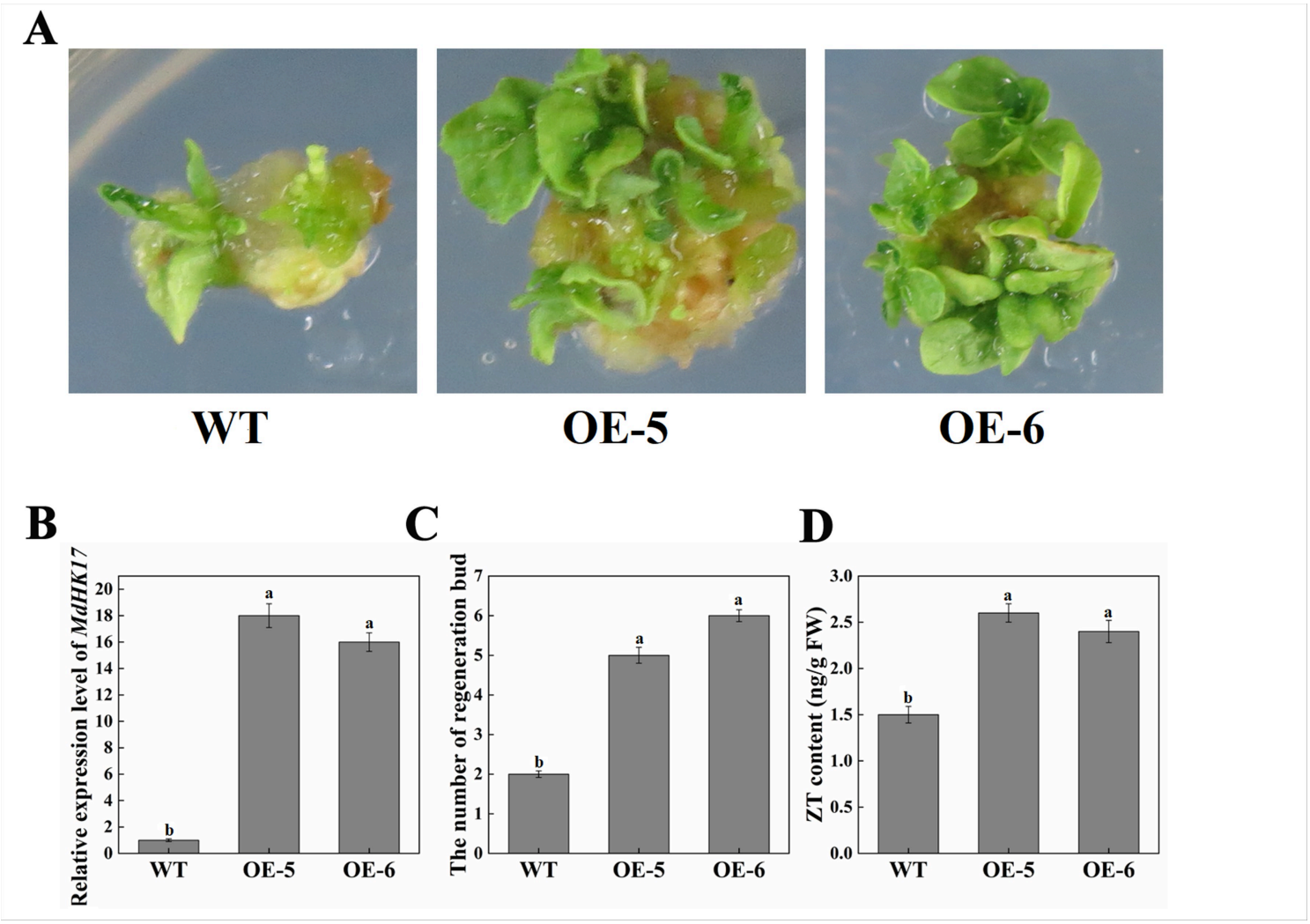


Figure 8

The phenotype of tomato overexpressing *MdHK17*. (A) Phenotype of in WT and transgenic lines tomatoes. (B) Relative expression level of *MdHK17* in WT and transgenic lines tomatoes. (C) The number of regeneration bud in WT and transgenic lines tomatoes (D) ZT content in WT and transgenic lines tomato. Values are means ± SD of three biological replicates. Different lowercase letters indicate significant differences by Duncan's test ($P < 0.05$).

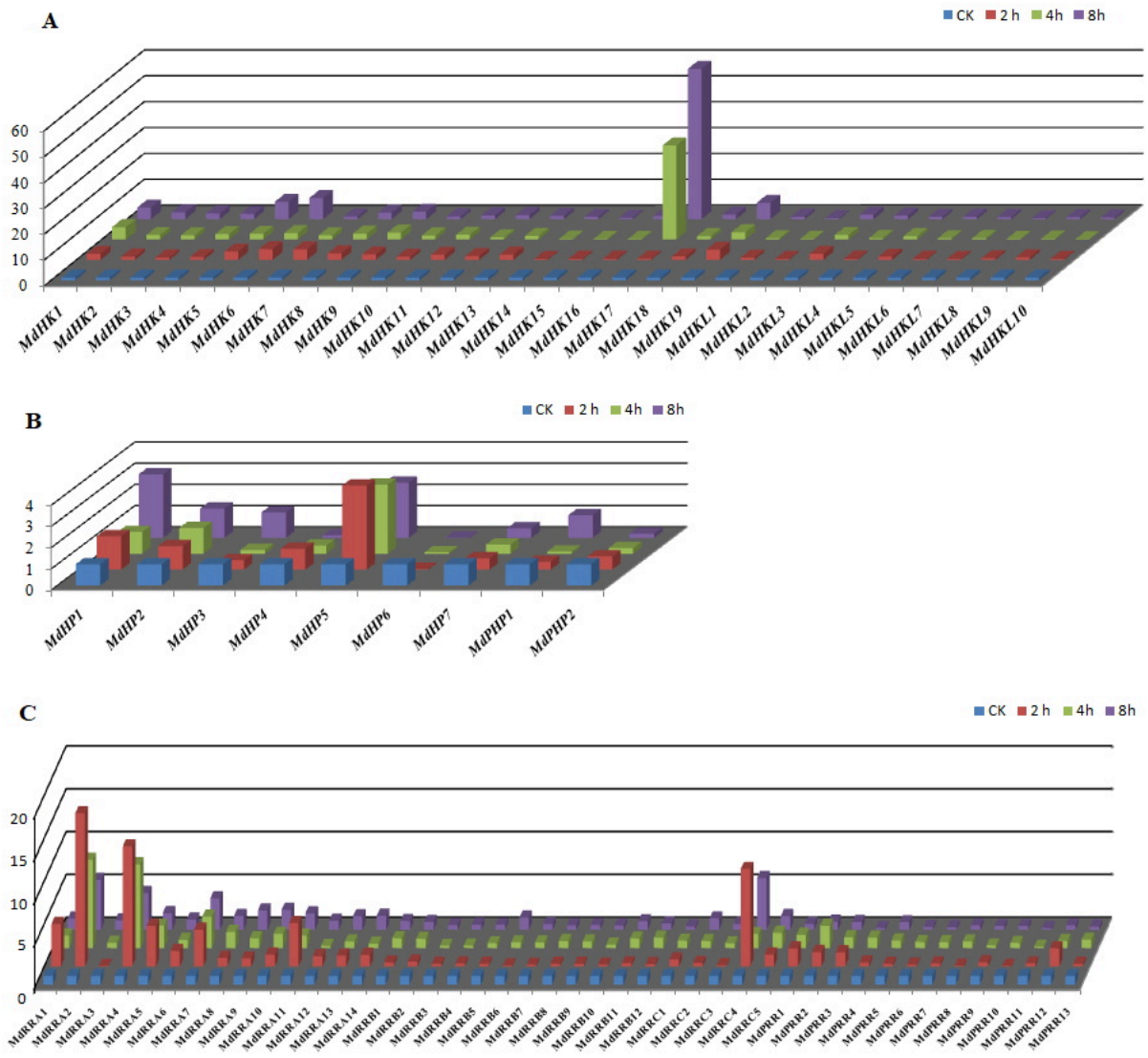


Figure 9

Expression profiles of TCS genes in response to ZT treatment.(A) The expression patterns of MdHK(L)s in response to ZT treatment. (B) The expression patterns of MdHPs in response to ZT treatment. (C) The expression patterns of MdRRs in response to ZT treatment. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analyses were used to assess TCS genes transcript levels in leaves sampled at 2 h, 4 h and 8 h after ZT treatment in four-old-week seedlings. Control (CK) seedlings were grown with normal irrigation. The mean of three experiments stands for their relative expression levels.

Supplementary Files

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