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Genome-Wide Identification and Characterization of Cysteine-Rich Receptor-Like Protein Kinase Genes in Tomato and Their Expression Profile in Response to Heat Stress

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Abstract: During plant growth, development and stress adaption, receptor-like protein kinases (RLKs) are essential components in perceiving and integrating extracellular stimuli and transmitting the signals to activate the downstream signaling pathways. Cysteine-rich receptor-like protein kinases (CRKs) are a large subfamily of RLKs and their roles in modulating plant disease resistance are well elucidated. However, the roles of CRKs in plant abiotic stress responses, especially heat stress, are largely unknown. In this study, 35 SlCRK genes were identified in tomato (Solanum lycopersicum) based on the multiple sequence alignment and phylogenetic relationships. SlCRK genes are tandemly distributed on seven chromosomes and have similar exon–intron organization and common conserved motifs. Various phytohormone responsive, stress responsive cis-regulatory elements and heat shock elements are predicted in the promoter regions of SlCRK genes. Transcriptome analysis of tomato fruits under heat stress revealed that most SlCRK genes were downregulated upon heat treatment. GO enrichment analyses of genes that were co-expressed with SlCRK members have identified various stress responses related and proteasomal protein catabolic process related genes, which may be involved in heat stress signaling. Overall, our results provide valuable information for further research on the roles of SICRKs in response to abiotic stress, especially heat stress.

Keywords: CRK; heat stress resistance; Solanum lycopersicum; genome-wide profiling; expression regulation

1. Introduction

Receptor-like protein kinases (RLKs) percept a variety of external and internal stimuli and transmit the input signal to induce the activated expression of appropriate target genes [1]. RLKs generally contain an amino-terminal signal sequence, an extracellular domain, a single transmembrane domain and a cytoplasmic domain with serine/threonine protein kinase activity [2]. RLKs can be divided into several sub-families, including leucine-rich repeat RLKs (LRR-RLKs), cysteine-rich repeat (CRR) RLKs (CRKs), domain of unknown function 26 (DUF26) RLKs, S-domain RLKs, and others [3]. CRKs contain two copies of the conserved C-X8-C-X2-C motif in their extracellular regions [4]. The role of DUF26 domain still remains elusive, while the cysteines have been proposed to form disulfide bridges as potential targets for thiol redox regulation and serve as sensors for reactive oxygen species (ROS), the common signaling molecules produced under various stresses [5]. The extracellular domain of CRKs perceives the extracellular ligands and transduces the signal to intracellular kinase domains. In Arabidopsis thaliana, 46 CRK members have been identified [5]. Many CRKs have been proven to play vital roles in biotic stress response. Over-expression of CRK5 or CRK13 enhances plant resistance...
to Pseudomonas syringae [6,7]. The signaling pathways mediated by several CRKs, such as BR-insensitive 1 (BRI1) [8] and FLAGELLIN-SENSITIVE2 (FLS2) [9], have been well characterized in hormone perception and pathogen response. In addition, many CRKs are transcriptionally induced in response to abiotic stress conditions such as salicylic acid, ozone, UV light, drought and salt treatments [5,6,10–12]. Induced expression of the four structurally closely related CRKs, CRK4, CRK5, CRK19 and CRK20, leads to hypersensitive response-associated cell death in transgenic Arabidopsis [6,11]. CRK6 and CRK7 have been reported to mediate the responses to extracellular ROS production [13]. Two abiotic stress inducible CRK members, CRK36 and CRK45, interact with each other and negatively regulate ABA and osmotic stress signal transduction [14,15]. Besides, CRKs play vital roles in plant immunity. For example, TaCRK2 contributes to leaf rust resistance in wheat (Triticum aestivum) [16]. In cucumber (Cucumis sativus), a dominantly inherited powdery mildew resistance QTL, Pm1.1, contains two tandemly arrayed cysteine-rich receptor-like protein kinase genes [17].

Tomato (Solanum lycopersicum) is a widely cultivated and the most important vegetable worldwide. Tomato is a thermophilic horticultural crop, and sensitive to high temperature. Heat stress poses a serious threat to tomato fruit yield and quality [18–20]. Transcriptional profiling of maturing tomato microspores reveals the involvement of heat shock proteins, ROS scavengers, hormones and sugars in heat stress response [21]. SIMPK1, a mitogen-activated protein kinase, negatively regulates heat stress response by directly interacting with SISPRH1, an antioxidant defense protein [22]. Overexpression of receptor-like kinase ERECTA improves thermotolerance in rice and tomato [23]. Transgenic tomato plants over-expressing betaine aldehyde dehydrogenase (BADH) and choline oxidase A (codA), key enzymes in betaine synthesis, exhibited heat tolerance, along with the elevated expression of heat stress responsive genes and the accumulation of HSP70 protein [24]. However, the regulatory network in response to heat stress in tomato is largely obscure, and the putative roles of CRK genes in tomato abiotic stress acclimation, especially heat stress, have not been investigated.

To uncover the potential roles of SlCRK genes in heat stress responses, we performed a genome-wide analysis of the tomato SlCRK gene family. Thirty-five putative SlCRK genes in tomato were identified and named according to chromosomal distributions. All SlCRK genes possessed various stress-responsive cis-elements, suggesting that SlCRK can respond to various environmental stimuli. Phylogenetic tree, gene structure and conserved motif analyses showed that SlCRK genes have conserved structure and motifs, and tandemly clustered SlCRKs are diverse. Transcriptome analyses of tomato fruits treated by heat stress revealed that SlCRK genes were mainly downregulated upon heat. GO enrichment analysis of co-expressed genes suggested that some stress responsive genes were significantly regulated. Our study has identified the tomato CPK gene family and provides important cues for further investigations on CRK-mediated network in plant abiotic stress responses.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

The tomato cultivar “Micro-Tom” is a model cultivar featured by small size, short life circle and capacity to grow under fluorescent lights at a high density. The seedlings were grown in pots inside a greenhouse maintained at 28 °C and 14/10 h (light/dark) photoperiod and 60–70% humidity. For heat stress treatment, four-week seedlings were treated at 42 °C for 0 h, 24 h, 48 h and 96 h, continuously. Leaves were collected at each point and frozen in liquid nitrogen immediately for total RNA extraction.

2.2. RNA Extraction and Expression Analysis

Total RNA was extracted from collected tomato fruit samples using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The RNA concentration and purity of total RNA samples were checked using a Nanodrop2000 instrument (Thermo Fisher Scientific, Waltham, MA, USA) and electrophoresis in agarose gels. For mRNA-seq, the cDNA library was
constructed by Illumina TruseqTM RNA sample prep kit. After analysis of RIN values using an Agilent 2100 Bioanalyzer Lab-on-Chip system (Agilent, Santa Clara, CA, USA), the pair-end sequencing was performed on Illumina HiSeq X Ten platform.

2.3. Expression Analysis of SlCRK Genes and GO Enrichment of Co-Expressed Genes of SlCRK Genes

The mRNA-seq were conducted and analyzed by Oebiotech Corporation (Oebiotech, Shanghai, China). Gene expression levels were estimated from mean FPKM (fragments per kilobase of exon model per million reads mapped) values for each sample and heatmap were constructed with Z-score transforming from FPKM values using the R package (https://www.r-project.org/, accessed on 30 March 2021). Times-series gene expression analyses were conducted by STEM (Short Time-series Expression Miner) software [25]. Pearson correlation coefficient between SlCRK genes and other genes were used to perform co-expressed analysis. The top 30 GO terms were collected to construct GO enrichment analysis of co-expressed SlCRK genes. These bioinformatic analyses were performed using the OECloud tools at https://cloud.oebiotech.cn, accessed on 4 April 2021.

2.4. Identification of SlCRK Family Members in the Tomato Genome

The latest released version of tomato genome SL4.0 and version ITAG4.0 of the annotation were used to search for SlCRK genes and sequence alignment (ftp://ftp.solgenomics.net/tomato_genome/assembly/build_4.00/, accessed on 2 March 2021). According to the annotation “cysteine-rich receptor-like protein kinase”, the gene ID and protein sequence were collected. These sequences and homologous CRK genes in Arabidopsis were used as queries to perform BLASTP searches against the tomato protein sequence database with a maximum E-value of $1 \times 10^{-3}$ to find all remaining putative SlCRK genes. By eliminating genes that are not preserved in the latest genome version or, more likely were considered as belonging to another protein family, the SlCRK gene family was constructed. Genomic sequences, transcript sequences and CDS sequences were all obtained. All SlCRK genes were submitted to EXPASy (https://web.expasy.org/protparam/, accessed on 4 March 2021) to calculate the number of amino acids, molecular weight and theoretical isoelectric points (pI). Subcellular localization predictions were conducted on WoLF PSORT (https://www.genscript.com/wolf-psort.html, accessed on 5 March 2021). The numbers roughly indicate the number of nearest neighbors for the query which localizes each site. The higher the scores, the more likely the localization is.

2.5. Phylogenetic Analysis

The full-length protein sequences of SlCRK genes were used for phylogenetic analysis. All the protein sequences were first aligned by ClustalW with default parameters [26] and then phylogenetic result was generated with MEGA X software with the maximum likelihood method, JTT substitution model with 1000 bootstrap replicates [27]. Genes were mapped on chromosomes by identifying their chromosomal position in the tomato genome database and visualized by mapgene2chromosome v2 (http://mg2c.iask.in/mg2c_v2.0/).

2.6. Structural Characterization and Promoter Analysis

The exon–intron organization of 35 SlCRK genes of tomato was analyzed using Gene Structure Display Server (GSDS) (http://gsds.cbi.pku.edu.cn/index.php, accessed on 10 March 2021) [28]. These conserved motifs of SlCRK family members were detected by Multiple Em for Morif Elicitation (MEME) suite 5.3.3 (https://meme-suite.org/meme/tools/meme, accessed on 10 March 2021) [29]. Conserved motifs with an e-value of $<1 \times 10^{-20}$ were subjected to further analysis. These motifs were analyzed on Pfam 34.0 database [30]. The promoter region, containing 2000 bp upstream sequences of SlCRK coding sequences in tomato, were retrieved from the genome sequence and then performed using PlantCARE [31] to identify the cis-regulatory elements. The cis-regulatory elements were represented by TBtools [32].
3. Results

3.1. Identification, Phylogenetic Analysis and Chromosomal Localization of SlCRK Genes in Tomato

As the annotation information of tomato genome is relatively complete, we used “cysteine-rich receptor-like protein kinase” as key words to search for target genes, and these target genes and homologous CRK genes in Arabidopsis were used as queries for BLASTP to search for other putative SlCRK genes. A total of 35 genes were identified as SlCRK family members. The SlCRK family members were named according to their gene IDs, starting from chromosome one to twelve, and their gene coordinates. The length and molecular weight of SlCRK proteins vary from 93 to 856 amino acids and 10.59 kDa to 96.25 kDa, respectively. The theoretical isoelectric point (pI) of 18 SlCRKs was acidic (4.86–6.81), and 17 CRK proteins were alkaline (7.02–9.57). According to the predicted scores, the higher of which implies a greater possibility for localization, the subcellular location of 13 SlCRKs were in the plasma membrane. Chloroplast, cytoplasm, plasma membrane and nucleus were also highly predicted location organelles. These results above were listed in Table 1. These diverse localizations of SlCRKs may indicate their various functions.

An unrooted phylogenic tree was constructed using the maximum likelihood method (Figure 1). The distribution and density of SlCRK genes were not uniform on chromosomes. Chromosomal localization of SlCRK genes are displayed in Figure 2, which shows that most SlCRK genes were tandemly distributed. These genes were mapped to 7 of 12 chromosomes. There are 4 members on ch01, 14 members on ch02, 6 members on ch03, 1 member on ch04, 6 members on ch05, 2 members on ch11 and ch12, respectively. Combined with phylogenic analysis and chromosomal localization, tandemly distributed SlCRK genes did not show high sequence similarity, suggesting they could have different functions, although they are physically related.
Table 1. SICRK gene family members in Solanum lycopersicum.

| Gene Name | Gene ID      | Gene Length, bp | Transcript Length, bp | CDS Length, bp | Protein Length, aa | pI    | MW, kDa | Subcellular Location          |
|-----------|--------------|-----------------|-----------------------|----------------|--------------------|-------|--------|------------------------------|
| SlCRK1    | Solyc01g007960 | 2678            | 2002                  | 1956           | 651                | 7.49  | 72.17  | plas: 8, E.R.: 3, nucl: 1, extr: 1 |
| SlCRK2    | Solyc01g007970 | 3469            | 2108                  | 1917           | 638                | 8.59  | 71.30  | plas: 9, E.R.: 2, nucl: 1, extr: 1 |
| SlCRK3    | Solyc01g007980 | 2798            | 2235                  | 1917           | 638                | 8.05  | 70.72  | plas: 9, golg_plas: 6, vacu: 3 |
| SlCRK4    | Solyc01g007990 | 3007            | 1478                  | 705            | 137                | 4.88  | 15.40  | extr: 5, vacu: 5, E.R.: 3 |
| SlCRK5    | Solyc02g067770 | 2538            | 1652                  | 1488           | 495                | 6.07  | 55.96  | chlo: 7, nucl: 3, cyto: 2, plas: 1 |
| SlCRK6    | Solyc02g067780 | 2538            | 1652                  | 1488           | 495                | 6.07  | 55.96  | plas: 10.5, golg_plas: 6.5, vacu: 2 |
| SlCRK7    | Solyc02g079990 | 3067            | 2242                  | 2001           | 666                | 6.52  | 74.19  | vacu: 9, cyto: 1, plas: 1, E.R.: 1 |
| SlCRK8    | Solyc02g080010 | 4369            | 2654                  | 2571           | 856                | 7.02  | 96.25  | chlo: 4, E.R.: 4, nucl: 2, mito: 2, cyto: 1 |
| SlCRK9    | Solyc02g080020 | 624             | 504                   | 504            | 167                | 8.56  | 18.41  | vacu: 9, cyto: 1, plas: 1, E.R.: 1 |
| SlCRK10   | Solyc02g080030 | 2272            | 1203                  | 1203           | 400                | 5.29  | 45.05  | chlo: 3, nucl: 3, cyto: 3, plas: 2.5, golg_plas: 2.5, vacu: 1.5 |
| SlCRK11   | Solyc02g080040 | 2630            | 1629                  | 1338           | 445                | 8.16  | 49.77  | chlo: 8, nucl: 2, extr: 2, cyto: 1 |
| SlCRK12   | Solyc02g080050 | 462             | 462                   | 462            | 153                | 4.86  | 17.35  | vacu: 5, extr: 4, golg: 2, nucl: 1, cyto: 1 |
| SlCRK13   | Solyc02g080060 | 1156            | 1104                  | 1104           | 367                | 8.81  | 41.31  | plas: 8.5, golg_plas: 6.5, golg: 3.5 |
| SlCRK14   | Solyc02g080070 | 4178            | 2364                  | 2031           | 676                | 6.16  | 75.45  | plas: 10.5, golg_plas: 6.5, golg: 1.5 |
| SlCRK15   | Solyc02g080080 | 3451            | 2139                  | 2016           | 671                | 6.02  | 74.63  | chlo: 6, extr: 5, cyto: 2 |
| SlCRK16   | Solyc02g080090 | 2985            | 1176                  | 1176           | 391                | 6.27  | 43.28  | nucl: 7, cyto: 5, chlo: 1 |
| SlCRK17   | Solyc02g082310 | 2197            | 1185                  | 1185           | 394                | 4.95  | 42.37  | plas: 3, mito: 2, vacu: 2, E.R.: 2, golg: 2, chlo: 1, cyto: 1 |
| SlCRK18   | Solyc02g086590 | 3939            | 2496                  | 1995           | 664                | 8.56  | 72.79  | cyto: 8, nucl: 3, mito: 2 |
| SlCRK19   | Solyc03g031540 | 2260            | 681                   | 681            | 227                | 6.45  | 25.56  | chlo: 4, extr: 4, vacu: 2.5, E.R., vacu: 2.5, mito: 2 |
| SlCRK20   | Solyc03g031550 | 453             | 453                   | 453            | 150                | 6.11  | 17.16  | plas: 7.5, golg_plas: 6, golg: 3.5, E.R.: 2 |
| SlCRK21   | Solyc03g111530 | 2698            | 981                   | 966            | 321                | 7.42  | 36.75  | plas: 8.5, golg_plas: 6, golg: 2.5 |
| SlCRK22   | Solyc03g111540 | 2863            | 2028                  | 2028           | 675                | 6.17  | 75.90  | E.R.: 3, chlo: 2, mito: 2, plas: 2, extr: 2, cyto: 1, mito: 1 |
| SlCRK23   | Solyc03g112730 | 2717            | 2230                  | 1851           | 616                | 6.00  | 67.94  | nucl: 8, cyto: 3, chlo: 1, mito: 1 |
| SlCRK24   | Solyc03g119340 | 3129            | 1676                  | 1188           | 395                | 6.35  | 43.69  | nucl: 7, cyto: 3, chlo: 1, mito: 1, pero: 1 |
| SlCRK25   | Solyc04g007780 | 4040            | 1468                  | 1200           | 399                | 9.57  | 44.41  | vacu: 4, chlo: 3, plas: 3, extr: 3 |
| SlCRK26   | Solyc05g005050 | 1962            | 967                   | 900            | 299                | 8.80  | 32.85  | mito: 11.5, cyto_mito: 6.5, chlo: 1 |
| SlCRK27   | Solyc05g005060 | 1534            | 1209                  | 990            | 329                | 7.13  | 37.11  | plas: 9, nucl: 2, vacu: 2 |
| SlCRK28   | Solyc05g005070 | 3802            | 2434                  | 2160           | 719                | 6.81  | 80.58  | cyto: 5, golg: 3, nucl: 2, E.R.: 2, chlo: 1 |
| SlCRK29   | Solyc05g018910 | 1543            | 750                   | 750            | 249                | 6.31  | 27.44  | chlo: 9, cyto: 2, vacu: 2 |
| SlCRK30   | Solyc05g018920 | 348             | 282                   | 282            | 93                 | 8.86  | 10.59  | chlo: 4, E.R.: 4, nucl: 2, cyto: 1 |
| Gene Name  | Gene ID     | Gene Length, bp | Transcript Length, bp | CDS Length, bp | Protein Length, aa | pI | MW, kDa | Subcellular Location               |
|------------|-------------|-----------------|-----------------------|----------------|--------------------|----|--------|-----------------------------------|
| SlCRK31    | Solyc05g018930 | 613             | 555                   | 555            | 184                | 5.29 | 20.24 | chloroplast: 11, nucleus: 1, mitochondria: 1 |
| SlCRK32    | Solyc11g011870 | 2769            | 1368                  | 1368           | 455                | 7.97 | 50.43 | cytoplasm: 5.5, cytoplasm-nucleus: 3.5, plastid: 3, mitochondria: 2, chloroplast: 1, endoplasm: 1 |
| SlCRK33    | Solyc11g011880 | 3209            | 1929                  | 1929           | 642                | 8.36 | 71.20 | plastid: 5.5, endoplasm: 4, golgi-plastid: 4, vacuole: 2, golgi: 1.5 |
| SlCRK34    | Solyc12g087910 | 723             | 507                   | 507            | 168                | 8.85 | 19.27 | cytoplasm: 8, extracellular: 3, nucleus: 2 |
| SlCRK35    | Solyc12g087920 | 3352            | 1755                  | 1755           | 584                | 6.29 | 66.27 | chloroplast: 4, extracellular: 4, cytoplasm: 2, vacuole: 2, plastid: 1 |
3.2. Analysis of Cis-Regulatory Elements in SICRK Genes

To further investigate the potential regulatory mechanisms of SICRKs during abiotic stress responses, the 2 kb upstream sequences from the translation start sites of SICRKs were used to identify the cis-regulatory elements (Figure 3). In addition to missing sequence information on the promoter regions of some SICRKs, the results showed that they had common upstream promoter elements, including MYB (myeloblastosis related proteins) and MYC (myelocytomatosis related proteins) binding sites, heat shock elements (HSEs)
and various responsive elements, including defense and stress responsive, light responsive, drought-inducibility, low-temperature responsive and phytohormone responsive elements. There are 359 light responsive elements and 116 HSEs in SlCRK promoter regions. MYB and MYC transcription factors are always associated with plant stress tolerance. There are 209 and 127 binding sites of MYB and MYC proteins in SlCRK upstream sequences, respectively. Besides, 61 abscisic acids responsive elements, 82 JA-responsive and 18 SA-responsive elements were predicted in SlCRK upstream sequences. These results indicated that SlCRK genes may be activated by various developmental and environmental stimuli, and SlCRK genes may be involved in many complex regulatory pathways.

3.3. Structure Analysis of SlCRKs

To study the gene structure of SlCRKs, we analyzed exon–intron organization and conserved motifs. The exon–intron organization analysis using a GSDS database indicated that the number and distribution of exon–intron locations were highly conserved among SlCRK homologs in tomato (Figure 4). SlCRKs have one to nine exons per gene. SlCRK2 and SlCRK28 have nine exons; SlCRK5, SlCRK12 and SlCRK20 have only one exon. Nine genes have seven exons which show similar distribution and exon length for each exon. Besides, phylogenetic clustered SlCRKs showed similar exon–intron organization while tandemly distributed SlCRKs did not, which is consistent with the results of the phylogenetic analysis.

![Figure 3. Cis-regulatory elements in SlCRK gene promoter regions, containing the upstream 2 kb region of SlCRK coding sequence.](image-url)
Figure 4. Gene structure analysis of SlCRK genes. Yellow boxes, blue boxes and lines indicate exons; untranslated regions indicate introns.

We then searched for conserved motifs through the MEME suite (Figure 5). SlCRKs have common conserved motifs, including salt stress response/anti-fungal domain (PF01657), protein kinase domains (PF00069) and protein tyrosine kinase (PF07714). Twenty-five of thirty-five SlCRKs have salt stress response/anti-fungal domain (PF01657); among them, 18 SlCRKs have two salt stress response/anti-fungal domains (PF01657). Twenty-four SlCRKs have tyrosine kinase domain (PF07714) and 26 SlCRKs have protein kinase domain (PF00069). SlCRK30 does not contain one of these characteristic conserved motifs from MEME, but its protein sequence is homologous to cysteine-rich receptor-like protein kinase (PTHR27002: SF128) through PANTHER (Protein ANalysis THrough Evolutionary Relationships) classification.
Figure 5. Identification of conserved motifs in SlCRK protein sequences. Significantly overrepresented motifs are graphically depicted by bars corresponding to their predicted positions. The red boxes (Motif 1) represent kinase domains (PF00069). The sapphire and grass green boxes (Motif 2 and 3) represent protein tyrosine kinase domain (PF07714) and the purple boxes (Motif 4) represent salt stress response/anti-fungal domain (PF01657). The remaining 6 motifs showed no match in the Pfam IDs.
3.4. Expression Patterns of SlCRKs in Response to Heat Stress

To investigate the transcript levels of SlCRKs in response to heat stress, we analyzed mRNA-seq results after high temperature treatment at 0 h, 24 h, 48 h and 96 h. Despite some SlCRK transcripts not being detected, including SlCRK19, SlCRK20 and SlCRK29, most SlCRKs were significantly down-regulated under high temperature treatment. The expression profiles were displayed as a heat map (Figure 6A). Two significant expression models were identified through short time-series expression miner (STEM) software (Figure 6B,C). Compared with expression level at 0 h, eight SlCRKs (SlCRK3, SlCRK8, SlCRK9, SlCRK11, SlCRK12, SlCRK14, SlCRK15 and SlCRK25) were down-regulated by two fold at 24 h and 48 h, and further by three fold at 96 h after high temperature treatment. Four SlCRKs (SlCRK1, SlCRK7, SlCRK13 and SlCRK35) exhibited one fold down-regulated expression change at 24 h and 48 h, then decreased to three fold down-regulated at 96 h. SlCRK2, SlCRK4 and SlCRK7 showed no significant expression change at 24 h and 48 h but 1.68-fold, 1.70-fold and 1.53-fold down-regulated at 96 h. SlCRK28 was steadily two-fold down-regulated. Adversely, SlCRK31 exhibited close expression levels at 24 h and 96 h along with that at 0 h, but decreased to 1.83-fold at 48 h. Only two SlCRKs were up-regulated in response to heat stress. SlCRK17 were up-regulated at 48 h and 96 h, and SlCRK32 were up-regulated at 24 h. Therefore, plants adjust the expression levels of SlCRKs to cope with heat stress. Many SlCRKs were down-regulated in response to high temperature and their expression levels reached their lowest point at 96 h, especially tandemly distributed SlCRKs on chr02.

3.5. Enrichment of Co-Expressed Genes Associated with SlCRKs in Tomato under High Temperature Treatment

To further investigate the regulatory network of SlCRKs, we screened the genes co-expressed with SlCRKs and performed GO enrichment analyses. By calculating the Pearson correlation coefficient between all the mRNAs and SlCRK genes, a total of 6337 genes were identified, of which mRNAs with correlation coefficient greater than 0.9 and p < 0.05 are considered as co-expressed genes with SlCRKs. The top 30 GO enrichment results were shown in Figure 7A. There were several GO terms related to disease resistance, such as response to chitin (GO: 0010200), regulation of salicylic acid biosynthetic process (GO: 0009042), response to wounding (GO: 0009611), defense response (GO: 0006952), defense response to bacterium (GO: 0042742) and response to molecule of bacterial origin (GO: 0002237). Besides, phytohormone-related GO terms, including ethylene-activated signaling pathway (GO:0009873) and regulation of salicylic acid biosynthetic process (GO:00080142), and proteasomal protein catabolic process (GO:0043161, 0010498, 0010499) GO terms, were identified. Besides, calmodulin binding (GO:0005516) related genes were also co-expressed with SlCRKs. In addition, we screened SlCRK genes with significant expression model. GO enrichment of co-expressed SlCRK genes in model 1 was similar to that of all SlCRK genes (Figure 7B). Surprisingly, GO enrichment of co-expressed SlCRK genes in model 2 was centered on chloroplast and photosynthesis (Figure 7C). Therefore, these results showed that there were various signaling related genes co-expressed with SlCRKs.
3.4. Expression Patterns of SlCRKs in Response to Heat Stress

To investigate the transcript levels of SlCRKs in response to heat stress, we analyzed mRNA-seq results after high temperature treatment at 0 h, 24 h, 48 h and 96 h. Despite some SlCRKs transcripts not being detected, including SlCRK19, SlCRK20 and SlCRK29, most SlCRKs were significantly down-regulated under high temperature treatment. The expression profiles were displayed as a heat map (Figure 6A). Two significant expression models were identified through short time-series expression miner (STEM) software (Figure 6B,C). Compared with expression level at 0 h, eight SlCRKs (SlCRK3, SlCRK8, SlCRK9, SlCRK11, SlCRK12, SlCRK14, SlCRK15 and SlCRK25) were down-regulated by two fold at 24 h and 48 h, and further by three fold at 96 h after high temperature treatment. Four SlCRKs (SlCRK1, SlCRK7, SlCRK13 and SlCRK35) exhibited one fold down-regulated expression change at 24 h and 48 h, then decreased to three fold down-regulated at 96 h. SlCRK2, SlCRK4 and SlCRK7 showed no significant expression change at 24 h and 48 h but 1.68-fold, 1.70-fold and 1.53-fold down-regulated at 96 h. SlCRK28 was steadily two-fold down-regulated. Adversely, SlCRK31 exhibited close expression levels at 24 h and 96 h along with that at 0 h, but decreased to 1.83-fold at 48 h. Only two SlCRKs were up-regulated in response to heat stress. SlCRK17 were up-regulated at 48 h and 96 h, and SlCRK32 were up-regulated at 24 h. Therefore, plants adjust the expression levels of SlCRKs to cope with heat stress. Many SlCRKs were down-regulated in response to high temperature and their expression levels reached their lowest point at 96 h, especially tandemly distributed SlCRKs on chr02.

Figure 6. Expression pattern of SlCRK genes. (A) Heat map expression profiles of SlCRK genes in high temperature treatment at 0 h, 24 h, 48 h, and 96 h. The scores are Z-score transforming FPKM values. (B,C) Significant expression model (0, −2, −2, −3) and (0, −1, −1, −3).
Figure 7. GO enrichment analysis of co-expressed genes with SlCRK genes. The Y axis corresponds to the GO term. The X axis corresponds to the Enrichment Score. Different shapes correspond to different GO categories (BP, CC, MF). The size of the dot corresponds to the number of different genes in the GO term and the bubble color is changed from purple-blue-green-red. The smaller the enrichment pValue, the greater the significance. GO enrichment analysis of co-expressed genes with all SlCRK genes (A), SlCRK genes of model 1 (B) and model 2 (C).
4. Discussion

RLKs in plants are a large superfamily of proteins that are structurally similar. RLKs are involved in development, growth, hormone perception and the response to pathogens [33]. CRKs are one of the largest families of RLKs, which are pivotal players in plant defense response, immunity, oxidative stress, ultraviolet radiation response, abscisic acid sensitivity and drought tolerance [5–7,10,13,15,34–37]. Their roles have been functionally uncovered in *Arabidopsis*, rice, wheat, cucumber, soybean and apple [16,17,37–40]. In *Arabidopsis*, CRK5 functions as a regulator of growth, development and ultraviolet radiation responses, enhances abscisic acid sensitivity and confers drought tolerance [35,38]. Overexpression of CRK13 results in enhanced resistance to *Pseudomonas syringae* [7]. The semi-dominant mutation in the cysteine-rich receptor-like kinase gene, *ALS1*, confers a constitutive defense response in rice [41]. TaCRK2 contributes to leaf rust resistance in wheat [16]. Genome-wide analysis of the apple CRK family have illustrated their annotation, genomic organization, and expression profiles in response to fungal infection [38]. However, the function of CRKs in response to abiotic stress, especially heat stress, have not been investigated. Moreover, its roles in tomato have not been reported yet. Hence, to understand the putative role of SICRKs under heat stress, we conducted the genome-wide identification and expression profiling of the SICRK gene family in tomato.

In this study, we identified 35 SICRK family members in tomato. As in *Arabidopsis*, common bean and apple [38], SICRK genes were tandemly distributed in the genome of tomato (Figure 2). There are 10 SICRK genes tandemly clustered on ch02. SICRKs on ch01, ch03, ch05, ch11 and ch12 are also clustered, suggesting gene duplications. Only SICRK17, SICRK18, SICRK23, SICRK24 and SICRK25 are separately distributed. Gene duplications, such as tandem, whole-genome, segmental duplications, are believed to have a significant impact on the expansion of gene family in flowering plants [42]. Tandem and whole-genome duplication (WGD)/segmental duplication are very common and function as a main resource of gene family expansion and genome complexity [43]. Gene clusters generally promote the recombination and accelerate evolution of the associated traits. Phylogenetic analysis (Figure 1) showed that tandemly clustered genes were not necessarily evolutionarily conserved in protein sequences, which suggested that clustered genes may not be redundant and have diverse biological functions.

Physical and biochemical property analyses were conducted on SICRK proteins (Table 1). The length and molecular weight of SICRK proteins vary and the theoretical isoelectric points (pI) of 18 SICRKs were acidic and 17 SICRKs were alkaline. The subcellular localization of 13 SICRKs were predicted in the plasma membrane. Some were predicted in the chloroplast, nucleus, etc. The scores were listed in Table 1. These diverse locations of SICRKs may indicate their various functions. Typical RLKs contain an extracellular ligand-binding domain, a transmembrane domain and an intracellular protein kinase domain. These results indicated that SICRKs may have various biological functions thanks to their physically different properties.

Structure analysis and conserved motifs of SICRKs showed many SICRKs exhibit conserved structure and have common conserved motifs. Phylogenetic clustered SICRKs, instead of tandemly clustered SICRKs, showed similar exon–intron organization (Figure 4). Consistent with previous study in *Arabidopsis* and common bean, SICRKs typically contain protein kinase domain (PF00069), tyrosine kinase domain (PF07714) and salt stress response/anti-fungal domain (PF01657) (Figure 5).

The upstream cis-regulatory elements of SICRKs showed that they could be triggered by various environmental stimuli (Figure 3). The heat shock transcription factor (HSF) binds to HSEs to regulate the transcriptional response of HSF target genes [44]. There were 116 HSEs in SICRK promoter regions. Plant hormone, including auxin, abscisic acid, gibberellin, Methyl jasmonate, play crucial roles in thermotolerance. To adapt to high temperature, seedlings may elevate the photosynthetic and meristematic tissues away from heat-adsorbing soil by elongating hypocotyls and therefore taking advantage of the cooling effect of moving air [45]. By blocking the auxin transport using the inhibitor of
polar auxin transport 1-aphthylphthalamic acid, the high-temperature-induced hypocotyl elongation response is abolished, indicating that auxin transport is essential to a response to increased temperature [46]. Suppressed biosynthesis of GAs interferes high-temperature-induced hypocotyl elongation in Arabidopsis [47]. This implies that rapid modulation of GA pathway is essential for high-temperature-induced hypocotyl elongation along with auxin. SA-dependent signaling improves basal thermotolerance but is not required for acquired thermotolerance in Arabidopsis [48]. Exogenous application of SA increases certain HSPs gene expression and eventually promotes heat tolerance in Arabidopsis [49]. In tomato, ABA-deficient mutant notabilis (not) tomato genotype is sensitive to heat stress (42 °C for 24 h), as evidenced by decreased photochemical efficiency (Fv/Fm), and increased lipid peroxidation compared with wild-type Ailsa Craig [50]. In Arabidopsis, JA has a positive regulatory role in basal thermotolerance, while ethylene shows the reverse effect [49,51]. It is reported that CRK5 in Arabidopsis is the regulator of UV light responses [35]. Light responsive cis-regulatory elements are another common motif encountered in the promoters of most of the SlCRKs. Defense and stress responsive and drought inducibility elements are also found in the promoter regions of SlCRKs, suggesting that the expression of SlCRKs may be triggered by biotic and abiotic stress. Besides, MYB and MYC transcription factors are key regulators in plant stress tolerance [52,53], whose binding sites occurred 209 and 127 times in SlCRK upstream sequences, respectively.

Expression profiles of SlCRK genes were visualized by heat map (Figure 6A) and two significant models in response to heat stress were identified, (0, −1, −1, −3) and (0, −2, −2, −3) (Figure 6B,C). Other SlCRKs’ expression levels have been found to decrease at 24 h, 48 h and/or 96 h. These results revealed that SlCRKs down-regulate their expressions in response to heat stress, with different temporal distribution. Pearson correlation coefficient was calculated, and 6337 genes were identified to be co-expressed genes with SlCRKs. GO enrichment analysis (Figure 7) showed that under heat stress, genes of biotic stress response related GO terms (GO: 0010200, response to chitin; GO: 0009873, ethylene-activated signaling pathway; GO: 0009611, response to wounding; GO: 0006952, defense response; GO: 0042742, defense response to bacterium; GO: 0002237, response to molecule of bacterial origin) have related expression pattern with SlCRKs. These results indicated that SlCRKs may respond to heat stress through similar pathway as biotic stress signaling. There may be a basal regulatory network between biotic and abiotic stress response mechanisms.

5. Conclusions

In summary, we performed a genome-wide analysis of SlCRK family members in tomato and 35 SlCRK genes were identified. Biochemical and physical properties of SlCRK proteins were characterized. SlCRKs are mostly tandemly clustered in tomato genome, with the maximum number on ch02. Gene clustering indicated the possibility of gene duplication as a factor of CRK gene family expansion. The phylogeny of SlCRKs showed that there are multiple clustered SlCRKs and tandemly clustered SlCRKs vary in protein sequence. SlCRKs share common conserved protein domain, such as protein kinase domain (PF00069), tyrosine kinase domain (PF07714) and salt stress response/anti-fungal domain (PF01657). Phylogenetic clustered SlCRKs have similar exon-intron organization and conserved motifs distribution. Promoter analysis identified several responsive cis-regulatory elements, including auxin, gibberellin, light and defense responsive elements. Further, most SlCRKs down-regulate their expression levels in response to heat stress. GO enrichment of co-expressed genes with SlCRKs showed that disease stress responsive genes have similar expression patterns with SlCRKs in response to heat stress. Taken together, our results provide a foundation for the functional characterization of SlCRK proteins in tomato.

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