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Genome-wide identification of the calcium-dependent protein kinase gene family in *Fragaria vesca* and expression analysis under different biotic stresses

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Abstract

Calcium-dependent protein kinases (CDPKs) can decode upstream Ca^{2+} flux changes and then phosphorylate downstream signaling molecules while transducing Ca^{2+} signaling, thereby engaging in plant growth, development, and biotic and abiotic stress responses. Although the CDPK gene family has been identified and analyzed in various plants, knowledge concerning CDPKs in woodland strawberry (*Fragaria vesca*) and their expression profiles in response to biotic stresses remain very limited. In this study, we identified 19 CDPK genes in *F. vesca*. Phylogenetic analysis separated the *FvCDPK*s into 4 subgroups. Gene structure and protein motif analysis for these *FvCDPK*s illustrated the different evolutionary relationships within subgroups. Collinearity analysis revealed 18 syntenic pairs between *AtCDPK*s and *FvCDPK*s and 5 syntenic pairs between *OsCDPK*s and *FvCDPK*s. Biotic stress-related cis-acting elements were detected in the promoter regions of *FvCDPK*s. Expression profiling of *FvCDPK*s was performed in different tissues under different biotic stresses, including *Phytophthora cactorum* (PC), *Botrytis cinerea* (BC) and strawberry vein banding virus (SVBV) exposure. The expression pattern of *FvCDPK*s during pathogen infection demonstrated their distinctive functional responses to biotic stresses. In summary, this study provides new insight into *FvCDPK*s and their response under biotic stresses.

Keywords *Fragaria vesca* · CDPK · Gene family · Genome-wide analysis · Expression profiling · Biotic stresses

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Introduction

Strawberry is a popular fruit crop worldwide and has been proposed as a model for functional genomics due to its rapid cycling and fast growth within *Rosaceae* (Amil-Ruiz et al., 2013). However, strawberry cultivars are susceptible to a large number of phytopathogenic organisms and exhibit wide phenotypic diversity in disease resistance, which limits both strawberry fruit quality and plant yield production (Amil-Ruiz et al., 2011). Woodland strawberry (*F. vesca*) is an important experimental model plant for studying the mechanisms of the response to biotic stresses in strawberry (Darwish et al., 2013; Shulaev et al., 2011). The genome of *F. vesca* was published in 2011 and updated in 2019 with more detailed annotations (Folta & Barbey, 2019; Li et al., 2019, b). The new version of the *F. vesca*

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genome was become the foundation for functional genome analysis.

Calcium is known as a universal secondary messenger within the cellular response to environmental stimuli, including phytopathogenic infection. Changes in cytosolic free calcium are needed to transduce a very wide variety of abiotic and biotic signals (Sanders et al., 2002). Various environmental stimuli can cause changes in the concentration of cellular calcium. Then, calcium signals are captured by different calcium sensors that bind Ca^{2+} and become activated (such as CDPKs) or downstream related kinases to transduce intracellular signals. In plants, four classic calcium sensors have been identified: calmodulins (CaMs) and CaM-like proteins (Snedden & Fromm, 1998), calcineurin B-like proteins (CBLs) (Luan et al., 2002), and calcium-dependent protein kinases (CDPKs) (Harper et al., 1994). CDPKs are a special calcium sensor that has both an EF-hand domain and a protein kinase domain. A typical CDPK harbors four domains, including an N-terminal variable domain, a serine/threonine protein kinase domain, an inhibitory-junction domain and a calmodulin-like domain constructed by four EF-hands (Shi et al., 2018). These special functional domains are the key to CDPK identification. Therefore, CDPKs have both Ca^{2+}-sensing ability and protein kinase activities that can convert Ca^{2+} signals into downstream protein phosphorylation (Boudsocq et al., 2010; Ludwig, 2003). CDPKs are thought to function in signal transduction pathways that utilize changes in cellular Ca^{2+} concentration to couple cellular responses to extracellular stimuli (Harmon et al., 2001).

The CDPK gene family was first reported in *Arabidopsis thaliana*, and 34 CDPK genes were found (Hrabak et al., 2003). After that, the CDPK gene family was identified in rice (*Oryza sativa*) (Ray et al., 2007), wheat (*Triticum aestivum*) (Li et al., 2008), pepper (*Capsicum annuum*) (Cai et al., 2015), barley (*Hordeum vulgare*) (Yang et al., 2017), maize (*Zea mays*) (Weckwerth et al., 2015), soybean (*Glycine max*) (Wang et al., 2015) and others. In addition to those in many important commercial crops, the CDPK gene family has also been reported in many horticultural plants, such as grape (*Vitis vinifera*) (Chen et al., 2013), pineapple (*Ananas comosus*) (Zhang et al., 2020), melon (*Cucumis melo L.*) (Zhang et al., 2017) and garden strawberry (*Fragaria x ananassa*) (Crizel et al., 2020). With the identification of the CDPK gene family in various plants, increasing evidence has shown that CDPK genes are differentially expressed in response to diverse biotic stresses and abiotic stresses and in different developmental stages (Li et al., 2019, b). The various subcellular localizations of CDPKs in plants suggest that they might be involved in multiple signal transduction pathways (Martín & Busconi, 2000).

Parallel work in *Arabidopsis* showed that CDPKs played important roles in immune and stress signaling, growth and development, and hormone responses (Zhang et al., 2015). *AtCDPK1* expression was induced by infection with both virulent and avirulent *Pseudomonas syringae*, which indicates that *AtCDPK1* may be involved in the plant defense response (Nie et al., 2015). Melon CDPKs were differentially expressed in response to biotic stress, such as powdery mildew caused by *Podosphaera xanthii* infection, among which *CmCDPK7* upregulation reached 3.8-fold at 24 hpi (Zhang et al., 2017). Pineapple has seventeen CDPKs, most of which are induced by mealybugs, except for *AcoCPK4* and *AcoCPK13* (Zhang et al., 2020). In grape, after *Erysiphe necator* infection, five *VpCDPK*s were upregulated up to 3.0-fold, with different response times and degrees (Zhang et al., 2015). Powdery mildew is a major disease of cultivated strawberry (Peries, 1962), and studies have found that induced loss-of-function in specific MLO genes can confer durable and broad resistance against PM pathogens (Acevedo-Garcia et al., 2014). In a barley mlo mutant genotype, *HvCDPK3* and *HvCDPK4* were found to be antagonistic controls of host cell entry in barley–powdery mildew interactions (Freymark et al., 2007). The strawberry MLO gene family has been identified (Tapia et al., 2021), which will facilitate further studies on PM resistance. Indeed, CDPKs were found to have evolved in plant biotic stress responses among various species, including *Solanum tuberosum* (Fantino et al., 2017), *Solanum habrochaites* (Li et al., 2022), and *Hevea brasiliensis* (Zhang et al., 2022).

In summary, CDPK acts as a calcium sensor that can respond to calcium signal changes caused by different developmental conditions and stresses and induce downstream physiological responses in plants. To date, the identification and verification of CDPK gene family members have been performed genome-wide with evolutionary analysis and molecular structure analysis in many plants. However, to date, no prior work has focused on the identification and analysis of CDPK genes on a genome-wide scale in *F. vesca*. The gene expression pattern of *FvCDPK*s in response to biotic stresses

remains poorly understood. Therefore, in this study, we employed bioinformatics to identify *FvCDPK*s, analyzed their structures and evolutionary relationships, and investigated the expression profiles of *FvCDPKs* under different biotic stresses. This work provides new insight into the functions of *FvCDPKs* in response to biotic stress.

Materials and methods

Identification of CDPKs in woodland strawberry

The *F. vesca* reference genome (v.4.0) and annotation (v.a2) data were obtained from the GDR database (www.rosaceae.org). The full-length amino acid sequences of *A. thaliana* and *O. sativa* CDPK were downloaded from the UniProt database (https://www.uniprot.org/) and used as query sequences to identify candidate *FvCDPK* by using the NCBI local software BLAST-P (Camacho et al., 2009) with an E-value of e^{-5}. Then, all nonredundant sequences with identity greater than 50% were verified by HMMER 3.0 software (http://hmmer.org/) with the hidden Markov model of the Pkinase domain (PF00069) and EF-hand domain (PF13499) from the Pfam database (http://www.pfam.xfam.org/). The protein sequences with these two domains were considered *FvCDPK* and were further confirmed by the NCBI-CDD database (https://www.ncbi.nlm.nih.gov/cdd) and SMART database (http://www.smart.embl-heidelberg.de/). The 3000 bp upstream and downstream sequences of abnormal genes were reannotated by the FGENESH tool (http://www.softberry.com/). ExPASy online tools (https://www.expasy.org/) were used to predict the molecular weights and isoelectric points of *FvCDPK*s. The positions of the identified *FvCDPK*s were marked on the seven chromosomes of *F. vesca* by using TBtools (Chen et al., 2020). The names of these *FvCDPK*s were retrieved from the position of each gene sorted on the chromosome. Unless otherwise described, default parameters were used for all tools, including those introduced below.

Gene structure and protein motif analysis

Intron–exon structures of 19 *FvCDPK*s were displayed by the online tool GSDS (http://gsds.cbi.pku.edu.cn/) (Hu et al., 2015). The MEME online motif-based sequence analysis tool (http://meme-suite.org/tools/meme) was used to divide protein motifs between 19 *FvCDPK*s with full-length protein sequences in classic mode, and the maximum motif number was set as eight.

Phylogenetic analyses

Phylogenetic analysis was performed on five kinds of plants: *A. thaliana*, *O. sativa*, *V. vinifera*, *M. domestica*, *F. ananassa* and *F. vesca*. We performed CDPK sequence alignment with ClustalW, and neighbor-joining (NJ) tree construction of these five species was performed in MEGA 10.0 software (http://www.megasoftware.net). The ClustalW algorithm with the bootstrap method was used to align sequences and test phylogeny. Except for changing the bootstrap replicates to 1000, the other parameters were set as defaults. Finally, the phylogenetic tree was annotated by iTOL (Letunic & Bork, 2021).

Gene duplication and synteny analysis

For further recognition of the evolutionary relationships within *FvCDPKs* and those from other species, MCscanX software (Wang et al., 2012) was used to detect the collinearity pattern of CDPKs among *A. thaliana*, *O. sativa*, and *F. vesca*. All protein sequences from *F. vesca* were used to make the BLASTP database. The sequences of *A. thaliana* and *O. sativa* were subjected to a search against the protein database of *F. vesca* as queries. The BLASTP parameters were set as E-value < e^{-5}, and the output format was set as table. The BLASTP output file and simplified annotation file of these three species served as the input for MCscanX software to analyze the collinearity relationships and gene duplication patterns with default settings, and the result was visualized by TBtools.

Cis-acting element analysis of the *FvCDPK* promoter sequence

We obtained a 2000 bp 5′ upstream sequence of *FvCDPK*s from woodland strawberry genomic DNA, and then PlantCARE, an online plant cis-acting regulatory element prediction tool (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/), was used to identify the cis-acting elements within the *FvCDPK* promoter

sequences. Stress-related cis-acting elements are displayed.

Expression pattern analysis under biotic stresses

Three RNA-seq datasets of different *F. vesca* organs under different biotic stresses were downloaded from the NCBI SRA database, including *Phytophthora cactorum* (PC)-infected roots (PRJNA327720), strawberry vein banding virus (SVBV)-infected leaves (PRJNA272956) and *Botrytis cinerea* (BC)-infected fruits (PRJNA530684). RNA-seq raw data were converted into fastq format from the SRA format using SRAToolkits (v.2.9.6). Trimmomatic software (v.3.0) was used to filter out low-quality reads and trim adaptors. After quality control, all the clean reads were mapped to the *F. vesca* reference genome (v.4.0) by Hisat2 software (v.2.1.0) (Kim et al., 2015). Then, SAMtools software (v.1.4) (Li et al., 2009) was used to convert the Bam format file into Sam format. We used Cufflinks software (v.2.2.1) (Trapnell et al., 2012) to calculate the FPKM value of each sample and export the total expression matrix. TBtools was used to extract the expression matrix of *FvCDPK*s and plot a heatmap with their FPKM values. The threshold of expression was considered as follows: FPKM<1, no expression; 1 ≤ FPKM<5, low expression; 5 ≤ FPKM <50, moderate expression; and FPKM ≥50, high expression. A 1.5-fold change in expression was considered to indicate a differentially expressed gene.

Results

Identification of CDPK genes in *F. vesca*

After BLAST-P and HMMER searches, a total of nineteen CDPK genes were identified as potential members of the CDPK gene family in *F. vesca*. The molecular weight and isoelectric point of 19 *FvCDPK*s were predicted by ExPASy, and the results are shown in Table 1. The lengths of the *FvCPDK* coding sequences ranged from 1360 bp to 1939 bp, and their predicted numbers of amino acids ranged from 455 to 635. The predicted *FvCDPK* molecular weights (MWs) varied widely from 50.27 to 70.55 kDa. The majority of *FvCDPK* members had pI values below 7.0, except for *FvCDPK*1 and *FvCDPK*2, which had pI values above 7.0.

The names of these 19 *FvCDPK*s were determined based on their locations on seven woodland strawberry chromosomes (Fig. 1). Chromosomes 1, 4, and 7 have only one *FvCDPK*, namely, *FvCDPK1*, *FvCDPK10* and *FvCDPK19*, respectively. Three CDPKs were located on chromosome 5: *FvCDPK11*, *FvCDPK12* and *FvCDPK13*. Both chromosomes 2 and 3 have four CDPKs. *FvCDPK2*, *FvCDPK3*, *FvCDPK4* and *FvCDPK5* were located on chromosome 2, and *FvCDPK6*, *FvCDPK7*, *FvCDPK8* and *FvCDPK9* were located on chromosome 3. Five *FvCDPK*s were identified on chromosome 6: *FvCDPK14* to *FvCDPK18*. According to the distributions of *FvCDPKs* on seven chromosomes of woodland strawberry, no significant *FvCDPK* cluster was found.

FvCDPK gene structure and protein motif identification

The gene structures and protein motifs of the 19 *FvCDPK*s are shown in Fig. 2. Many *FvCDPK*s have 6 or 7 introns, and several genes are distinctly different. The *FvCDPK*s could be divided into 4 subgroups. In subgroup I, *FvCDPK*s have 6 introns, except for *FvCDPK18*. In subgroup II, *FvCDPK*s have 7 introns, except for *FvCDPK6*. Compared with subgroup I members, subgroup II members generally have one more intron. Five members are included in subgroup III, three of which have 7 introns, namely, *FvCDPK10*, *FvCDPK12*, and *FvCDPK17*. *FvCDPK9* has 8 introns, and *FvCDPK14* has 6 introns. Subgroup IV is the most distinguishable subgroup of the *FvCDPK* gene family. Compared with other *FvCDPK*s, *FvCDPK1* and *FvCDPK2* have quite different numbers of introns. *FvCDPK1* has 11 introns, and *FvCDPK2* has 10 introns. *FvCDPK13* has 6 introns, and it is phylogenetically close to *FvCDPK1* and *FvCDPK2*. In addition, 8 explicit motifs were found in many *FvCDPK* family members, except for *FvCDPK1*, *FvCDPK2*, *FvCDPK3*, and *FvCDPK13*. *FvCDPK3* and *FvCDPK13* lacked motif 7, and *FvCDPK1* and *FvCDPK2* lacked motif 6. The results suggested that the gene structures and protein motifs of *FvCDPK* family genes were conserved with a small amount of difference, which indicated the functional redundancy of *FvCDPK* family members.

Phylogenetic analysis of *FvCDPKs*

To detect the evolutionary relationship of *FvCDPK*s, a neighbor-joining (NJ) tree was built using amino acid

Table 1 Characteristics of CDPK genes in woodland strawberry

Gene name	Gene ID	Chr	Group	CDS (bp)	Protein Length (aa)	MW (KDa)	pI
FvCDPK1	FvH4_1g11780	1	IV	1680	550	62.20	8.9
FvCDPK2	FvH4_2g06730	2	IV	1833	600	67.13	8.78
FvCDPK3	FvH4_2g11790	2	I	1360	445	50.27	5.11
FvCDPK4	FvH4_2g16880	2	I	1735	568	63.59	5.77
FvCDPK5	FvH4_2g38010	2	II	1628	533	60.12	5.74
FvCDPK6	FvH4_3g02130	3	II	1695	555	62.42	6.35
FvCDPK7	FvH4_3g22730	3	II	1677	549	61.96	6.53
FvCDPK8	FvH4_3g27800	3	II	1622	531	59.30	5.59
FvCDPK9	FvH4_3g28740	3	III	1662	544	60.89	5.91
FvCDPK10	FvH4_4g11140	4	III	1613	528	60.05	5.39
FvCDPK11	FvH4_5g03610	5	II	1585	519	58.16	5.86
FvCDPK12	FvH4_5g15720	5	III	1671	547	62.21	6.47
FvCDPK13	FvH4_5g27182	5	IV	1598	523	58.21	5.54
FvCDPK14	FvH4_6g11340	6	III	1610	527	59.23	6.07
FvCDPK15	FvH4_6g20840	6	I	1939	635	70.55	5.49
FvCDPK16	FvH4_6g21020	6	I	1817	595	65.93	5.11
FvCDPK17	FvH4_6g23540	6	III	1616	529	59.60	6.15
FvCDPK18	FvH4_6g35360	6	I	1521	498	55.94	4.94
FvCDPK19	FvH4_7g05490	7	I	1717	562	62.55	5.44

sequences of CDPK genes within 6 species. As shown in Fig. 3, *FvCDPK* family members can be divided into four groups, which is consistent with a previous study in *A. thaliana* and other model plants. The four groups included CDPK I, CDPK II, CDPK III and CDPK IV. CDPK I contains 6 *FvCDPK*s, 10 *AtCDPK*s, 11 *OsCDPK*s, 6 *VvCDPK*s, 2 *FaCDPK*s and 16 *MdCDPK* and is the largest subgroup. CDPK II includes 5 *FvCDPK*s, 12 *AtCDPK*s, 8 *OsCDPK*s, 5 *VvCDPK*s, 4 *FaCDPK*s and 8 *MdCDPKs*. CDPK III contains 5 *FvCDPK*s, 8 *AtCDPK*s, 8 *OsCDPK*s, 5 *VvCDPK*s, 2 *FaCDPK*s and 10 *MdCDPKs*. CDPK IV, which is the smallest group, contains 3 *FvCDPK*s, 3 *AtCDPK*s, 4 *OsCDPK*s, 3 *VvCDPK*s, 3 *FaCDPK*s and 1 *MdCDPK*.

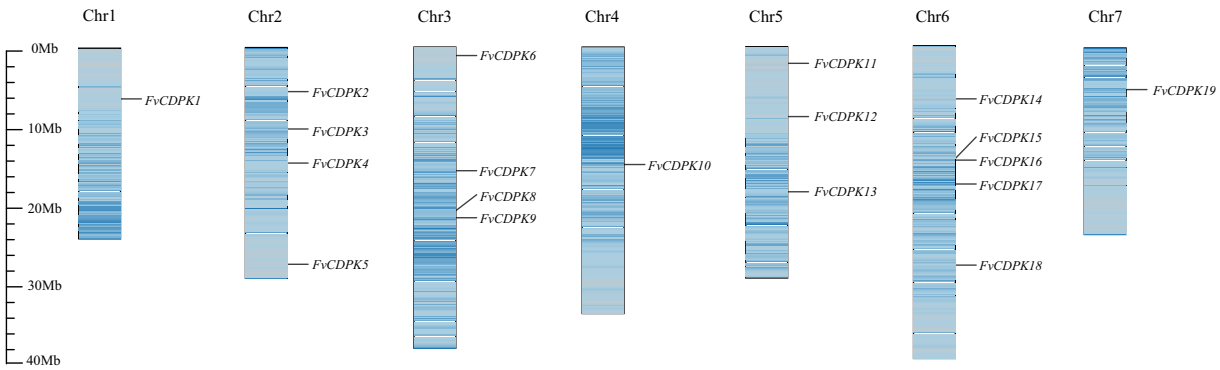

Fig. 1 Localization of *FvCDPK*s on chromosomes. The number of each chromosome is shown at the top of the bar, and the position of each *FvCDPK* is marked by a black line with the gene ID. The length of each chromosome on the graph is proportional to the length of the sequence. The legend on the left shows the length of the chromosome in million bases. The heatmap in each bar shows the gene abundance in every chromosome

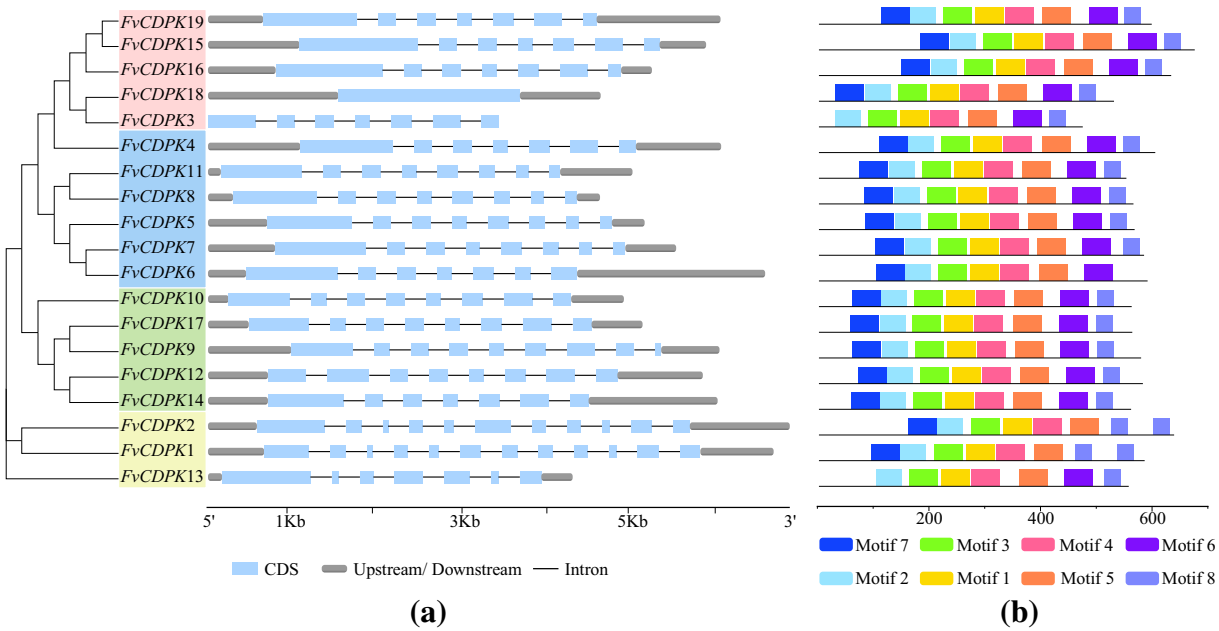

Fig. 2 Phylogenetic relations, gene structures and protein motifs of *FvCDPK*s. **a** The unrooted phylogenetic tree was constructed by the use of full-length amino acid sequences of 19 *FvCDPK*s with the neighbor-joining method. The four subgroups are marked with distinct colors (CDPK I: pink, CDPK II: blue, CDPK III: green, and CDPK IV: yellow). **b** The motifs of the respective CDPK genes are marked with different colors. The consensus sequence of each motif is shown below the motif panel

Intriguingly, *F. vesca* has the same distribution and number of CDPK genes as *V. vinifera*. The other three species have more CDPK genes, except for *F. ananassa,* which has only 11 CDPKs. This may be due to the constriction of the cultivated strawberry genome during domestication. Moreover, in addition to *FaCDPK*s, *FvCDPK*s have a close evolutionary relationship with *MdCDPK*s, which both belong to Rosaceae. In subgroup IV, only *FvCDPK*1 has homologs in apple, which may indicate that subgroup IV was constricted in the long-term evolution of *M. domestica* or expanded in the *F. vesca* genome.

Analysis of gene duplication and synteny in *FvCDPK*

After phylogenetic analysis of *AtCDPKs* and *OsCDPKs*, we investigated the potential *FvCDPKs* duplication events in the *F. vesca* genome and collinearity between the two model plants. The collinearity patterns are shown in Fig. 4. Surprisingly, neither segmental nor tandem duplication events were found among *FvCDPK*s. This indicates that *FvCDPKs* were not amplified in the *F. vesca* genome. Moreover, eighteen pairs of collinearity relationships between *FvCDPKs* and *AtCDPKs* were detected. These syntenic gene pairs between *FvCDPKs* and *AtCDPKs* can be divided into three patterns. Pattern one represents a single *FvCDPK* corresponding to one *AtCDPK*, including *FvCDPK5-AtCDPK29*(AT1G76040), *FvCDPK8-AtCDPK17*(AT5G12180), *FvCDPK9-AtCDPK7*(AT5G12480), *FvCDPK11-AtCDPK3*(AT4G23650) and *FvCDPK14-AtCDPK13*(AT3G51850). For the second pattern, a single *FvCDPK* corresponded to two *AtCDPK*s, including *FvCDPK12-AtCDPK10*(AT1G18890)/*AtCDPK30*(AT1G74740) and *FvCDPK17-AtCDPK14*(AT2G41860)/*AtCDPK32*(AT3G57530). The third pattern represents a single *FvCDPK* corresponding to three *AtCDPK*s, including *FvCDPK1-AtCDPK28*(AT5G66210)/*AtCDPK18*(AT4G36070)/*AtCDPK16*(AT2G17890), *FvCDPK4-AtCDPK6*(AT2G17290)/*AtCDPK26*(AT4G38230)/*AtCDPK5*(AT4G35310) and *FvCDPK6-AtCDPK15*(AT4G21940)/*AtCDPK21*(AT4G04720)/*AtCDPK19*(AT1G61950). Compared to that with *AtCDPK*s, the collinearity between *FvCDPKs* and *OsCDPKs* showed lower complexity. Six pairs of collinearity relationships were found between *FvCDPKs* and *OsCDPKs*. Two *FvCDPK*s correspond to two *OsCDPK*s, including *FvCDPK15-*

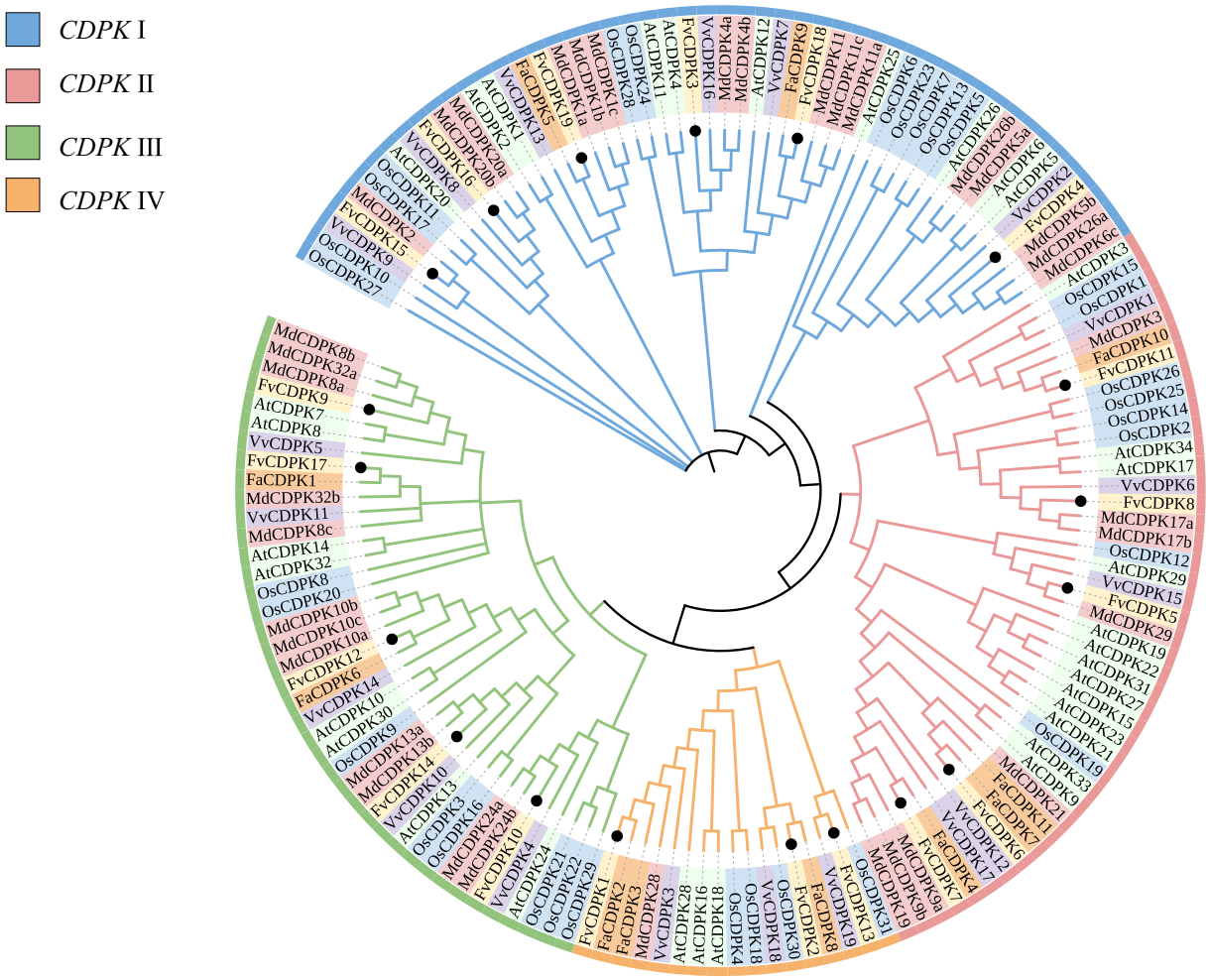

Fig. 3 Phylogenetic analysis of CDPK genes in 6 species (*A. thaliana*, *O. sativa*, *Vitis vinifera*, *M. domestica F. ananassa* and *F. vesca*). CDPKs are shown in different shapes, and the four subgroups are marked with distinct colors and Roman numerals

OsCDPK10(Os03g0788500) and *FvCDPK16*-*OsCDPK17*(Os07g0161600)/*OsCDPK11*(Os03g0789000). Three *FvCDPK*s were found to have collinearity to both *AtCDPKs* and *OsCDPKs*. The first pair is *FvCDPK14*-*OsCDPK3*(Os01g0832300), which showed the first pattern in both *AtCDPKs* and *OsCDPKs*. Then, *FvCDPK*4 had one collinear gene in rice, *FvCDPK4*-*OsCDPK11*(Os03g0128700), and was collinear with three corresponding *AtCDPK* genes. *FvCDPK6* is quite similar to *FvCDPK4*, sharing the same pattern in rice and *Arabidopsis*, and *FvCDPK6*-*OsCDPK2*(Os07g0515100) is a unique pair found in rice. Collinearity patterns across these three species indicate that these orthologous genes may have been derived from the same ancestor. The second and third patterns may illustrate the constriction of *FvCDPKs* that was also found in the phylogenetic analysis.

Analysis of cis-acting elements in *FvCDPKs*

To understand the regulated expression of *FvCDPK*s induced by upstream cis-acting elements, we obtained a 2000 bp upstream sequence of each *FvCDPK*. As shown in Fig. 5, the promoter region sequence analysis showed that there were elements related to various stresses. In particular, we were interested in biotic stress-related elements, such as the WUN motif, TC-rich repeats and the W box. In these three cis-acting elements, the number ranged from 0 to 3, with a differential distribution across *FvCDPK*s. *FvCDPK9*, *FvCDPK13*, *FvCDPK16* and *FvCDPK19* lack these

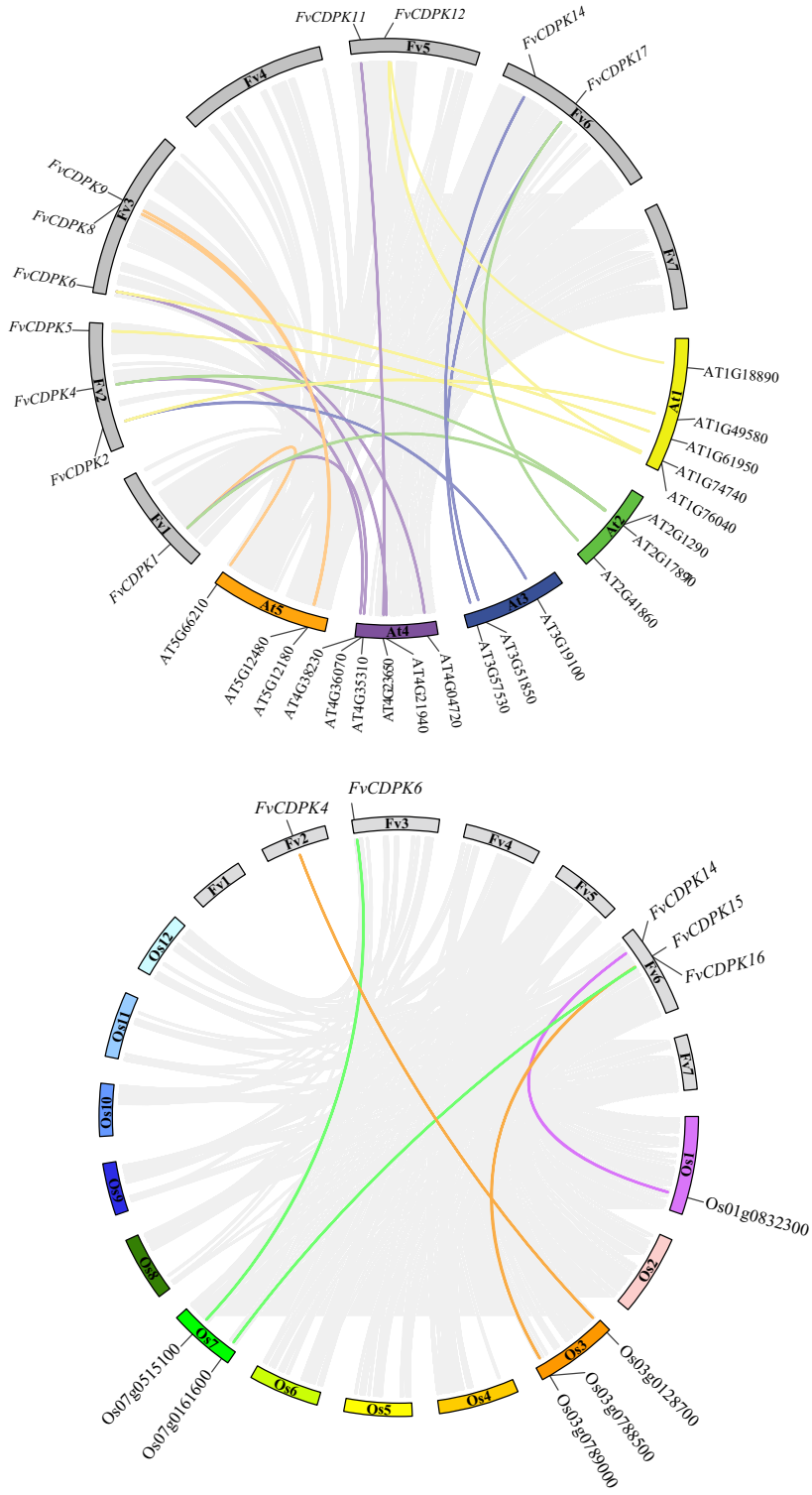

Fig. 4 Localization and collinearity of *AtCDPK*s, *OsCDPK*s, and *FvCDPK*s. **a** Collinearity circle plot between *A. thaliana* and *F. vesca*. **b** Collinearity circle plot between *O. sativa* and *F. vesca*. The circle plot represents the chromosome with the numbers marked. Chromosomes of *F. vesca* are shown in gray, and the chromosomes of other species are shown in different colors. The lines inside the circle connect two genes from different species, illustrating their synteny. The distribution of each *FvCDPK*, *AtCDPK* and *OsCDPK* is marked on the chromosome

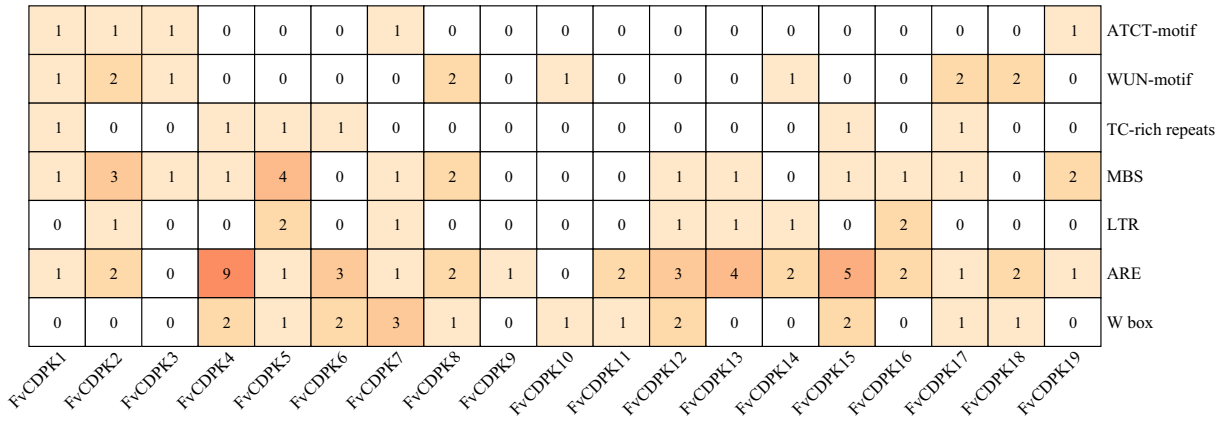

Fig. 5 Stress-related cis-acting element in the promoter region of *FvCDPK*s. The cis-acting element number of each *FvCDPK* is shown as a heatmap. WUN motif, stress response element. TC-rich repeats, involved in defense and stress responses. W box, pathogen-responsive WRKY binding site

three elements, which may be due to functional differentiation. *FvCDPK2*, *FvCDPK3*, *FvCDPK7*, *FvCDPK11*, *FvCDPK12* and *FvCDPK14* only have one of these three elements. Moreover, *FvCDPK1*, *FvCDPK4*, *FvCDPK5*, *FvCDPK6*, *FvCDPK8*, *FvCDPK10*, *FvCDPK15* and *FvCDPK18* have two of these three elements. Only *FvCDPK17* has all three potential pathogen-responsive cis-acting elements.

Expression of *FvCDPK* genes in different tissues under different biotic stresses

RNA-seq data from the NCBI SRA database were downloaded and analyzed to obtain the expression of *FvCDPK*s in response to different biotic stresses, as shown in Fig. 6. Cluster analysis showed that the samples from the same tissues were clustered together, indicating that the expression of *FvCDPKs* has tissue specificity. More specifically, *FvCDPK8*, *FvCDPK10* and *FvCDPK16* showed low expression in all tissues, *FvCDPK13* and *FvCDPK15* were only expressed in roots, and *FvCDPK5* was not expressed in red fruit. Other *FvCDPK*s were expressed in all four tissues, with different expression levels in each tissue. For example, *FvCDPK6* was highly expressed in leaves and moderately expressed in other tissues.

In the expression matrix of *P. cactorum*-infected roots, 7 *FvCDPK* genes were differentially upregulated, including *FvCDPK1*, *FvCDPK4*, *FvCDPK7*, *FvCDPK15*, *FvCDPK17*, *FvCDPK18*, and *FvCDPK19*. In contrast, 3 *FvCDPK*s were downregulated, namely, *FvCDPK2*, *FvCDPK9* and *FvCDPK14*. In the expression data from SVBV-infected leaves, 5 *FvCDPK*s were upregulated, including *FvCDPK1*, *FvCDPK6*, *FvCDPK9*, *FvCDPK12* and *FvCDPK19*, and none showed downregulated expression. The results suggested that *FvCDPK* genes could be expressed in response to *P. cactorum* and SVBV infection. However, no differentially expressed genes could be found in *B. cinerea*-inoculated white and red fruits, which may indicate that *FvCDPK* genes in white and red fruits were not expressed in response to *B. cinerea*. For upregulated *FvCDPK*s, *FvCDPK1*, *FvCDPK4*, *FvCDPK7*, *FvCDPK17*, *FvCDPK18* and *FvCDPK19* were highly expressed and *FvCDPK15* was moderately expressed in *P. cactorum*-infected strawberry roots; *FvCDPK1* and *FvCDPK6* were highly expressed, while *FvCDPK9*, *FvCDPK12* and *FvCDPK19* had moderate expression levels due to SVBV infection in strawberry leaves. These *FvCDPK* genes may play significant roles in *F. vesca* in response to *P. cactorum* and SVBV infection.

Discussion

Strawberry is an economically important crop grown worldwide that faces various phytopathogenic organism threats. Woodland strawberry is a vital experimental material for pathogen-plant interactions and a suitable model for detecting differential gene expression in response to various pathogen infections (Shulaev et al., 2011). Increasing evidence illuminates the functions of CDPKs in biotic and abiotic stresses. Related work in

F. ananassa identified 11 CDPKs and revealed their involvement in abiotic stress, including salt and drought stress (Crizel et al., 2020). However, strawberry CDPKs engaged in biotic stress responses have not yet been determined.

In this work, we used a genome-wide gene family identification method based on conserved domains and sequence similarities. The latest *F. vesca* genome (v.4.0) and annotation (v.a2) were used for the identification of *FvCDPK*s. As a result, we finally found 19 *CDPK* genes in *F. vesca*, which were named *FvCDPK1* to *FvCDPK19* based on their distributions on chromosomes (Fig. 1). The evolutionary relationship of *CDPK*s shows that the numbers of these family members have distinct distributions. For instance, *A. thaliana* has 34 *CDPK* members (Hrabak et al., 2003), while pineapple has only 17 *CDPK*s (Zhang et al., 2020). Thirty-one *OsCDPK*s were identified in *O. sativa*, which is similar to that for *AtCDPK*s. Intriguingly, grape has a more similar number of *CDPK*s to woodland strawberry, and 19 *VvCDPK*s were found within the *V. vinifera* genome, which is similar to the number of *FvCDPK*s. High variability in the number of gene copies present in each genome can lead to various gene family sizes in different species due to successive phases of gene gains and losses (Chauve et al., 2008). However, *M. domestica* and *F. vesca* belong to Rosaceae, and the number of their *CDPK* genes shows obvious differences. *MdCDPK*s (37) are almost two times more abundant than *FvCDPK*s, which may be caused by whole-genome duplications within the apple genome. The kinome identification in wild strawberry shows that 27 FvCDPKs were identified by hidden Markov models of pkinase (Liu et al., 2020). Compared to this recent study, fewer *FvCDPK*s were found in our study, which may be because of the different identification methods. We used both HMMs and BLAST for *FvCDPK* identification, and the lower numbers may be due to the stricter method. Moreover, only 11 *FaCDPK*s were identified in the *F. ananassa* genome, which is less than the number of *FvCDPK*s in our study. This difference may be because *F. vesca*, as a diploid progenitor of *F. ananassa*, maintained more ancestral genes than this octoploid strawberry (Edger et al., 2019). During the hybridization of the *F. ananassa* ancestor, approximately 8 *FvCDPK*s were lost, and only some *CDPK*s remained in its genome. In CDPK I, two homologous gene pairs were detected: *FaCDPK5*/*FvCDPK9* and *FaCDPK9*/*FvCDPK18*. In CDPK II, the homologous genes of *FvCDPK7* and *FvCDPK11* were found to be *FaCDPK4* and *FaCDPK10*. *FvCDPK6* has two homology genes, *FaCDPK7* and *FaCDPK11*. In CDPK III, two homologous pairs were found: *FaCDPK1*/*FvCDPK17* and *FaCDPK6*/*FvCDPK12*. In CDPK IV, *FvCDPK1* has two homologous genes, *FaCDPK2* and *FaCDPK3*. The homologous gene of *FvCDPK2* is *FaCDPK8*. This evolutionary history may lead to the specific functional loss of corresponding *CDPK*s in *F. ananassa*, and the study of *FvCDPK*s is still needed.

Divergences in exon-intron structure are widespread across duplicate gene evolution, especially those that can change the function of the gene, which can be caused by amino acid-altering substitutions and/or alterations in exon–intron structure (Xu et al., 2012). In this study, we found that most *FvCDPK*s have 6 or 7 introns, with different evolutionary relationships within the distribution of *CDPK* subgroups. *FvCDPK*s were divided into four subgroups according to previous phylogenetic analysis in *A. thaliana* and *O. sativa*. Compared with subgroup I members, subgroup II members have one more exon that causes intron insertion, contributing to the generation of subgroup II and the expansion of *FvCDPK*s.

Eukaryotic genomes differ in the degree to which genes remain on corresponding chromosomes (synteny) and in corresponding orders (collinearity) over time (Tang et al., 2008). Whole-genome duplications play a critical role in gene expansions, especially in gene families (Cannon et al., 2004). Moreover, in previous works, tandem duplications and segmental duplications were detected in various plants and showed important roles in *CDPK* gene expansion (Zhang & Wang, 2005). However, in the strawberry *CDPK* gene family, we did not find any tandem or segmental duplications, which is a plausible reason for the relatively lower number of *CDPK*s in *F. vesca* than in *M. domestica*, which is also a Rosaceae plant. In this study, 18 pairs of collinear genes were detected between *FvCDPK*s and *AtCDPK*s. However, only 6 collinear gene pairs were found between *FvCDPK*s and *OsCDPK*s. Intriguingly, we found that both phylogenetic analysis and syntenic analysis can indicate correlated functional connections in woodland strawberry and two other model species. *OsCDPK2* stability is subject to light regulation and repressed by light in leaves (Morello et al., 2000). *FvCDPK6* is the collinear gene of *OsCDPK2*, and the expression of *FvCDPK6* was upregulated after SVBV infection in strawberry

leaves, which may indicate that *FvCDPK6* can be transcribed during some pathogenic virus infections, even though it was repressed by light under normal conditions. Synteny analysis among *A. thaliana*, *O. sativa* and *F. vesca* may provide insights into the prediction of gene function for the *CDPK* gene family.

Increasing evidence indicates that CDPKs are engaged in the biotic stress response (Boudsocq & Sheen, 2013). SVBV is one of the major viruses infecting strawberry and has been reported worldwide (Converse, 1992). RNA-seq results suggested that strawberry disease caused by SVBV may affect multiple processes, including pigment metabolism, photosynthesis and plant-pathogen interactions (Chen et al., 2016). Other strawberry diseases, such as crown rot, caused by *P. cactorum* can also give rise to yield reductions in strawberry cultivation. After inoculation of the roots of *F. vesca* with *P. cactorum*, major reprogramming of the root transcriptome occurred, involving 30% of the studied genes (Toljamo et al., 2016). Moreover, the transcriptome of different tissues interacting with biotic stress at diverse developmental stages can demonstrate more about *F. vesca* antipathogen methods. Genes involved in defense response pathways that sense and combat gray mold caused by *Botrytis cinerea* were differentially regulated within 24 hpi in both white and red fruits (Haile et al., 2019). In this study, we retrieved the same raw RNA-seq data from these projects to obtain insight into the characteristics of *FvCDPK*s under viral and fungal stresses.

The expression patterns of *FvCDPK*s under different pathogen infections and in different tissues show distinction patterns in Fig. 6. Compared to SVBV infection of leaves, after root inoculation with *P. cactorum*, the only two *FvCDPK*s that were commonly upregulated were *FvCDPK1* and *FvCDPK19*. Moreover, the other five *FvCDPK*s that were upregulated upon *P. cactorum* inoculation showed expression changes only in *F. vesca* roots. The different expression patterns of *FvCDPK*s under viral and fungal infection implied that *FvCDPK*s can respond to upstream Ca^{2+} signals caused by diverse pathogen attacks. However, after comparing the two fungal pathogen infection treatments, the differential expression of *FvCDPK*s suggested that invasions dominated by tissue-specific phytopathogenic organisms can trigger antipathogen transduction pathways in various tissues and can be captured by different *FvCDPK*s. According to the irregular expression level changes in *FvCDPK*s under these three biotic stresses, microorganisms, including viruses and fungi, can stimulate diverse signal transduction pathways controlled by different *FvCDPK*s in *F. vesca*. Upregulated *FvCDPK*s may participate in different pathogen-related signal transduction pathways as calcium sensors and trigger downstream protein modifications. Only *FvCDPK1* and *FvCDPK19* commonly participate in phytopathogenic viral and fungal responses, which may illustrate that they are engaged in similar pathogen response signal transduction pathways.

Cis-acting element analysis provided some evidence to support the gene expression profiles. In this study, most of the upregulated *FvCDPK*s had biotic stress-related cis-acting elements in their 2000 bp upstream sequences, including WUN motifs, TC-rich repeats and W boxes. TC-rich repeats are involved in the expression regulation of plant disease-resistance genes (Xu et al., 2010). *FvCDPK1*, *FvCDPK4*, *FvCDPK6*, *FvCDPK15* and *FvCDPK17* have TC-rich repeats and are upregulated by pathogen infection. The W box is a WRKY binding element and can mediate the response to pathogen elicitors (Laloi et al., 2004). Seven upregulated FvCDPKs had W boxes, including *FvCDPK4*, *FvCDPK6*, *FvCDPK7*, *FvCDPK12*, *FvCDPK15*, *FvCDPK17* and *FvCDPK18*. Evidence has shown that wound-responsive transcription factors can mediate defense responses to biotic stresses, and the WUN motif can be found in related genes (Chen et al., 2012; Sasaki et al., 2007). *FvCDPK1*, *FvCDPK17* and *FvCDPK18* have WUN-motif elements and are induced by *P. cactorum* infection. However, only *FvCDPK1* was upregulated after SVBV infection. The transcriptional regulatory mechanisms of these pathogen-induced *FvCDPK*s need to be further studied.

The phylogenetic relationship between *A. thaliana* and *F. vesca* demonstrates that, compared to strawberry, the expansion of *AtCDPK*s produced more *AtCDPK* paralogs than *FvCDPK* paralogs, and the total number of *AtCDPK*s is larger than that of *FvCDPK*s. Moreover, similar expansions occurred in *O. sativa*, resulting in significantly more *OsCDPK*s than *FvCDPK*s. From the perspective of evolutionary genomics, orthologous genes are considered to have similar functions, and paralogous genes may have divergent functions (Koonin, 2005). According to the phylogenetic tree, *FvCDPK1* was divided into the same subbranch as *AtCDPK16*, *AtCDPK18*, *AtCDPK28*, *OsCDPK4* and *OsCDPK18*. Studies on *AtCDPK28* and *OsCDPK4*

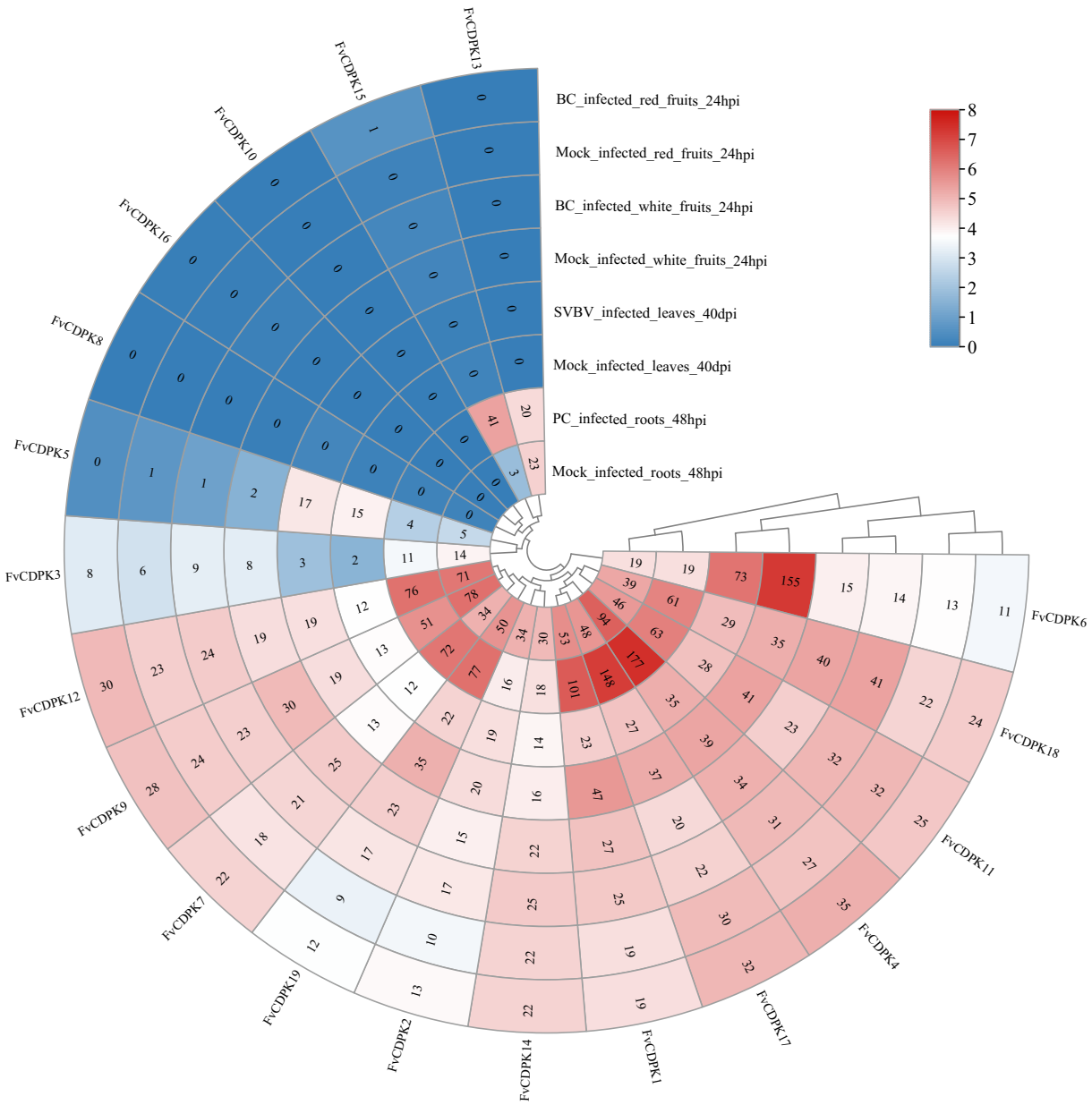

Fig. 6 Expression profiles of *FvCDPK*s in different tissues in response to different biotic stresses. SVBV represents strawberry vein banding virus, BC represents *B. cinerea* and PC represents *P. cactorum*. The value in each single cell is the FPKM value, and the heatmap uses the base 2 logarithm of FPKM as a parameter

revealed their conserved interplay between phosphorylation and ubiquitination in plant immune homeostasis (Monaghan, 2018). *AtCDPK28* is a negative regulator of immune signaling by phosphorylating PUB25/26, which negatively regulates immune responses and disease resistance controlling the pattern recognition receptor (PRR) complex BIK1 (Monaghan et al., 2014). However, a study showed that overexpression of *OsCPK4* in rice enhances resistance to blast disease by preventing fungal penetration (Bundó & Coca, 2016). These differences regulated by biotic stress may indicate that *AtCDPK28* had functional variation during evolution and transformed into a biotic stress-sensitive gene. In addition, we found that *FvCDPK19*, *AtCDPK1* and *AtCDPK2* belong to the same subbranch. Previous studies showed that *AtCDPK1* is engaged in the plant

defense response. The expression of *AtCDPK1* is rapidly induced by fungal infection, and overexpressed *AtCDPK1* leads to an SA-regulated innate immune system to defend against pathogens (Coca & San Segundo, 2010). *AtCDPK1* regulates the *Arabidopsis* response to both pathogen infection and plant hormone signaling, and these two processes are probably mediated by the synergetic action between two important signaling molecules, salicylic acid (SA) and abscisic acid (ABA) (Nie et al., 2015). However, no *OsCDPK* was found in this branch, which may indicate that the ancestral gene was duplicated in Arabidopsis but lost in *O. sativa.* Orthologs can be used to predict the function of proteins in different species, and the upregulation of *FvCDPK1* and *FvCDPK19* in both fungus inoculation and virus infection treatment demonstrates that they may participate in plant defense response signaling and plant immune signaling. *FvCDPK1* obtained resistance to biotic stress similar to *OsCDPK4*, and *FvCDPK19* may have similar functions to *AtCDPK1*. *FvCDPK4* and two *Arabidopsis* orthologs, *AtCDPK5* and *AtCDPK6*, have been divided into the same subbranch, but no orthologous *O. sativa* gene was found. In *Arabidopsis*, simultaneous loss of *AtCDPK5*, *AtCDPK6* and *AtCDPK11* suppressed resistance to *Botrytis cinerea* infection (Gravino et al., 2015). *AtCDPK6* was found to have positive functions induced by yeast elicitor, and it is a convergent hub for signaling pathways related to stomatal closure in response to biotic stress (Ye et al., 2013). Both of the orthologous genes of *FvCDPK4*, *AtCDPK5* and *AtCDPK6*, were reported to positively regulate biotic stress. In addition, the expression of *FvCDPK4* was upregulated in response to *P. cactorum* infection. These results strongly indicated that *FvCDPK4* may have functions in biotic stress. *FvCDPK1*, *FvCDPK4* and *FvCDPK19* can be considered candidates for further functional verification against biotic stresses.

In conclusion, the functions of *FvCDPK*s in biotic stresses need to be determined by more experimental investigation. There are still many unknown biological roles of *FvCDPK*s in various Ca^{2+}-related transduction pathways that need to be deeply understood.

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Declarations

Conflict of interest The authors declare no conflict of interest.

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