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Comparative and evolutionary analyses of cyclophilins in *Cucumis sativus*, *Phaseolus vulgaris*, and *Vitis vinifera*

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ABSTRACT

Cyclophilins, ubiquitous proteins present in the majority of organisms including bacteria, fungi, higher plants, humans, and so on, are known to play diverse cellular functions. In this study, we have performed genome-wide analyses of cyclophilins from three plant species, *Cucumis sativus*, *Phaseolus vulgaris*, and *Vitis vinifera*. This analysis revealed 21, 26, and 22 cyclophilins in *C. sativus*, *P. vulgaris*, and *V. vinifera*, respectively. The majority of cyclophilins are present in the cytosol and nucleus, while few were observed in mitochondria and vacuole. A total of 15, 19, and 16 single-domain cyclophilins while 6, 7, and 6 multi domain cyclophilins are present in *C. sativus*, *P. vulgaris*, and *V. vinifera*. Furthermore, phylogenetic analysis showed the grouping of cyclophilins in five major clades with relevance to domains and sub cellular localization. Our study also suggests *P. vulgaris* and *V. vinifera* have similar intron exon structures. Eight motifs have been conserved in most cyclophilins out of which six amino acids long motif GSQFFI is prominent. *In-silico* expression studies revealed *CucCYP13* is highly expressed in roots (10 folds higher) and is orthologous to previously reported *GmCYP1* of *G. max*. *CucCYP13* from *C. sativus* has been identified as ortholog of *GmCYP1* which plays important role in disease resistance in soybean. Additionally, *CucCYP2*, *PhvCYP22*, and *GsvCYP5* which have high sequence similarity with *Arabidopsis CYP 71* (AT3g44600) could play a key role in gene repression, organogenesis, and meristem development. Overall, the present study offers key insight into this important class of immunophilin, and the newly identified cyclophilins in *P. vulgaris*, *C. sativus*, and *V. vinifera* may play important roles in abiotic stresses and key physiological traits.

1. INTRODUCTION

Cyclophilins are ubiquitous proteins found among almost all genera of bacteria, higher plants, humans, and fungi. Cyclophilins are a class of immunophilins that possess binding ability towards immunosuppressant drugs cyclosporin A, FK506, and rapamycin impeding translocation of nuclear factor of activated T-cells [1–3]. The complex blocks elicitation of mRNA of interleukin-2, interleukin-4, and interferon, thereby, stopping T-cell activation in animals [4]. Cyclophilins particularly bind to cyclosporin A and show peptidyl-propryl activity that is *cis* to *trans* inter-conversion of proline peptide bond [5]. This *cis* to *trans* inter-conversion plays an important role in protein folding suggesting the role of cyclophilin as a chaperon [6,7]. In plants, cyclophilins have different roles such as pre-mRNA splicing [8], transcription regulation [9], cell division [10], signaling [11,12], and stress tolerance [13–15]. Single-domain cyclophilins have only one CYP domain. However, multi-domain has additional domains like *U*-box for ubiquitination, tetratrico peptide repeats (TPR for protein-protein interactions, and assembly of multi-protein complexes, WD-40 for assembly of multi-protein complexes), RNA recognition motif (RRM) for regulation of transcription, helical bundle for signal transduction, PsbQ-like for plant-specific oxygen-evolving enhancer protein 3 and last but not least RRM + Zf for RNA splicing [16]. WD40, TPR, and F-box were reported to have protein-protein interactions [17,18].

Over the past few years, genome-wide studies in plants have proven to be a great tool aiding in the achievement of cyclophilins. The highest numbers of cyclophilins have been reported in *Brassica napus* (94) with 79 single-domain and 12 were multi-domain [19]. Most cyclophilins were found to be localized in cytoplasm while the least in

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mitochondria. Similarly, a total of 85 cyclophilins were identified from *Triticum aestivum* with the majority of them residing in cytoplasm. Out of which, 27 had single domains and 58 had additional domains [20]. Apart from these, 31 cyclophilins in *Arabidopsis thaliana* [21], 75 in *Gossypium barbadense*, 78 in *Gossypium hirsutum*, 40 in *Gossypium arboreum*, 38 in *Gossypium raimondii* [22], 35 in *Solanum lycopersicum* [23], 33 in *Medicago truncatula* [24], 30 in *Medicago domestica* [25], 29 in *Oryza sativa* [21], and 62 in *Glycine max* [26] have been reported till now.

In this study, we have focused on three important plant species *Cucumis sativus, Phaseolus vulgaris,* and *Vitis vinifera. Phaseolus vulgaris* (common bean), a member of the Fabaceae family is an economic source of protein for a vegetarian staple diet in America, Africa, India, Brazil, and China holding 17%–30% of dry weight protein. *Phaseolus vulgaris* faces approximately 60% yield loss due to drought stress solely. Apart from this high saline concentration results in imbalanced ions and photograph inhibition further contributing to major yield loss [27–29]. According to a 2020 report to the New York Agricultural Experiment Station, common bean crops suffer significant losses from bacterial diseases like common bacterial blight and halo blight, caused by *Xanthomonas axonopodis* and *Pseudomonas syringae pv. Phaseolicola,* respectively. Additionally, common rust, caused by *Uromyces appendiculatus*, and viruses such as bean common mosaic virus and bean yellow mosaic virus also contribute substantially to yield reductions in common beans [30–32].

Cucumis sativus is a creeper plant from the Cucurbitaceae family known for its edible fruit and is native to India. Apart from being consumed as fruit is also possesses ayurvedic and cosmetic properties [33,34]. Cucumber faces oxygen depletion and reduced yield in water logging conditions. High salt stress causes ionic imbalance leading to chlorosis, hindering growth, and affecting fruit quality whereas high temperature degrades protein and seedlings in early stages. Biotic stresses from powdery mildew, downy mildew, anthracnose, fusarium wilt, and bacterial wilt also add to significant production loss in cucumber [35–41].

Problems arising due to various biotic and abiotic stresses are also significant in *V. vinifera*. Grapevine is widely used for the production of wine, and juices and consumed in fresh and dried forms [42]. Drought and saline stress causing leaf shedding and retarded photosynthetic rates leads to poor berry quality with compromised taste, size, and color [43,44]. Apart from this grey molds, powdery mildew, and downy mildew also add to huge yield and quality loss [45–47].

Previous studies on cyclophilins from *A. thaliana* have revealed the roles of *AtCYP21-2, AtCyP18-1,* and *AtCYP20-2* in drought stress, temperature stress, and light stress tolerance. *OsCYP20-2*, *OsCyp2* (Os02g0121300), and *OsCYP18-2* from *O. sativa* showed upregulated levels on light, saline, and drought stress [16,48]. The role of *Arabidopsis* cyclophilin *ROC1/AtCYP18-3* and *GmCYP1* cyclophilin of soybean also provides defense against *P. syringae* and *Phytophthora sojae* infection, proving its multi diverse role in both abiotic and biotic stress tolerance [49,50]. However, only a handful of genome-wide analysis have been conducted so far on various plants in an attempt to identify this important class of proteins.

In view of the above, it is important to study the evolution, expression, and functional role of cyclophilins in *P. vulgaris*, *C. sativus,* and *V. vinifera.* Hence, we have identified cyclophilins from *P. vulgaris*, *C. sativus,* and *V. vinifera* through genome-wide analysis. We hypothesize that our identification and characterization of cyclophilins from these three important plant species could provide crucial information regarding their distribution, expression, and evolution along with their putative role in biotic/abiotic stresses and other physiological traits.

2. MATERIALS AND METHODS

2.1. Protein Sequence Retrieval

Protein sequences of *C. sativus, P. vulgaris,* and *V. vinifera* were obtained from the NCBI database by using At1g01940 as a query sequence against default parameters [51].

2.2. Location, Molecular Weight (MW), Isoelectric Point (PI), and Sub Cellular Localization

Chromosome location, exon, and intron of *PhvCYPs* and *GsvCYPs* were obtained from the phytozome database [52], where parameters like proteome as target type, BLASTP as program type, −1 is selected as threshold, and BLOSUM62 as comparison matrix is used. In the case of *CucCYPs* Ensembl Plants using parameters like TBLASTN, 1e-1 E-value threshold, and BLOSUM62 search matrix were used [53]. The pictorial presentation was carried out using Mapchart software for individual chromosomes [54]. For the prediction of sub-cellular localization of protein sequences, the Wolfsport online tool was used [55]. MW and PI analyses were estimated through Prot pi [56].

2.3. Synonymous and Non-Synonymous Substitution Rates (Ka-Ks)

For synonymous and non-synonymous substitution rate analyses, KaKs_Calculator 2.0 was used utilizing genomic sequences of individual cyclophilin [57]. Genomic sequences were retrieved from the phytozome database [52].

2.4. Multiple Sequence Alignment and Phylogenetic Analysis

All the cyclophilin protein sequences (Supplementary Fig. S1) were aligned using BioEdit 7.2 [58]. The aligned sequences were used to generate a phylogenetic tree with neighbor-joining clustering and iTOL: Interactive tree of life to study the ancestral history of all the identified cyclophilins [59]. A cladogram was also constructed to study sequence similarity between homologous and previously reported cyclophilins with known tolerance against abiotic stresses. Chord diagrams were constructed among the cyclophilins from *C. sativus, P. vulgaris,* and *V. vinifera* with their previously reported counterparts from *A. thaliana*, *G. max*, and *M. truncatula*. This was performed in two steps. Initially, the data was structured using python (3.10.11) to generate a worksheet where all protein sequences were compared against each other and the matched sequences between the proteins were counted. Post formatting, Chord diagrams were created using the Python package d3blocks (https://d3blocks.github.io/d3blocks, Ver. 1.1.5).

2.5. Domain Search

For domain search, a batch-CD tool of NCBI was used to obtain the presence of single and multiple domains in each cyclophilin by searching amino acid sequence in CDD v3.1-62456 PSSMs version utilizing default parameters [60]. A pictorial presentation was made using GSDS 2.0 [61].

2.6. Co-Expression Analysis

Gene expression data of *P. vulgaris* were obtained from phytozome [52], while in case of *C. sativus*, cucurbit genome database (CuGenDB) from bioproject PRJNA80169 was used [62]. Expression data of *V. vinifera* was retrieved from the EMBL-EBI expression atlas [63]. Representation of expression data in heatmap was performed using TB tools software [64].

3. RESULTS

3.1. Genome-Wide Distribution of Cyclophilin Proteins

Genome-wide search of cyclophilins from *C. sativus, P. vulgaris, and V. vinifera* were conducted for the identification of putative cyclophilin proteins. Genome-wide analysis revealed the presence of a total of 21, 26, and 22 cyclophilins in *C. sativus, P. vulgaris,* and *V. vinifera,* respectively (Tables 1–3).

3.2. *Cucumis sativus*

In *C. sativus,* out of 21 cyclophilins, five cyclophilins are present on chromosomes 2 and chromosome 7 followed by three cyclophilins each on chromosomes 1 and chromosome 6, respectively (Fig. 1a). Two cyclophilins are present on chromosomes 3 and chromosome 5, whereas only one cyclophilin is present on chromosome 4 (Fig. 1a). In *C. sativus*, cyclophilin *CucCYP21 is* the largest protein with 799 amino acids while *CucCYP7* with 111 amino acids is the smallest (Table 1). Out of 21 cyclophilins, 15 cyclophilins are single-domain while six are multi-domain cyclophilins (Supplementary Fig. S2). Cyclophilins viz. *CucCYP1*, *CucCYP4*, *CucCYP6*, *CucCYP7*, *CucCYP8*, *CucCYP9*, *CucCYP10*, *CucCYP11*, *CucCYP12*, *CucCYP13*, *CucCYP14*, *CucCYP15*, *CucCYP18*, *CucCYP19*, and *CucCYP20* has Cyclophilin-like domain (CLD), which is responsible for *cis* to *trans* PPIase activity. The WD40 domain was found in *CucCYP2* while TPRs and TPR-1 domains were observed in *CucCYP3* and *CucCYP16. CucCYP3* has a mitochondrial precursor protein import receptor domain (3a0801s09). Cyclophilin *CucCYP5* is multi-domain protein and has RRM, polyadenylate binding protein (PABP_1234), glycine-rich RNA-binding protein (PLAN03134), transcription termination factor Rho (PRK12678), U2 small nuclear RNA auxiliary factor (U2AF_Ig), and zinc finger domain (Zf_CCHC). Ring_Ubox domain was observed in *CucCYP17* while splicing factor CC1-like family (SF_CC1) domain was observed in *CucCYP21* along with transcription regulator ICP4 (PHA03307) (Fig. 2a). Seven *CucCYPs* are localized in cytosol followed by six in chloroplast and only one was observed in vacuole (Table1).

3.3. *Phaseolus vulgaris*

A total of 26 cyclophilins were identified on nine *P. vulgaris* chromosomes (Fig. 1b). Seven cyclophilins were found on chromosome 1, followed by four and three cyclophilins on chromosome 3 and chromosome 4, respectively. Chromosome 7 had two cyclophilins whereas chromosomes 5 and 6 have only one cyclophilin. In *P. vulgaris*, cyclophilin *PhvCYP5* is the longest with 933 amino acids whereas *PhvCYP14* is the shortest with 136 amino acids (Table 2). Out of 26 cyclophilins, 19 cyclophilins have a single CLD domain while seven cyclophilins are multiple domain proteins (Supplementary Fig. S2). Cyclophilins viz. *PhvCYP1*, *PhvCYP2*, *PhvCYP3*, *PhvCYP4*, *PhvCYP6*, *PhvCYP7*, PhvCYP9, *PhvCYP10*, *PhvCYP11*, *PhvCYP13*, *PhvCYP14*, *PhvCYP16*, *PhvCYP17*, *PhvCYP19*, *PhvCYP20*, *PhvCYP21*, *PhvCYP23*, *PhvCYP25*, and *PhvCYP26* are single-domain cyclophilins. *PhvCYP5* had PHA3307 as an additional domain, while *PhvCYP8* hasRAM signaling pathway protein (SOG2) and104 kDa microneme (PTZ00449) along with PHA3307 domain. Cyclophilin *PhvCYP12* has two pairs of ring_Ubox as extra domains. Cyclophilins *PhvCYP15* and

Table 1. There are 21 cyclophilins identified in *C. sativus*, and for each of them, comprehensive information is available. This includes their ID, amino acid size, PI, MW, sub-cellular localization within the cell, chromosome location, exon-intron count, and specific positions on the chromosome. The gathered data offers a detailed overview of these cyclophilins and their characteristics in the context of *C. sativus*.

Name	ID	Size (AA)	PI	MW (KDa)	Subcellular localization	Chr.	Exon:Intron	Location
CucCYP1	Csa_2G100030	226	6.38	25.7	Vacuole	Chr2	03:02	7,459,112–7,459,855
CucCYP2	Csa_1G153530	624	6.73	70.22	Cytoskeleton	Chr1	13:12	9,959,884–9,970,839
CucCYP3	Csa_7G074830	361	5.86	40.14	Cytosol	Chr7	08:07	5,099,534–5,105,454
CucCYP4	Csa_2G270140	223	6.25	24.89	Cytosol	Chr2	07:06	12,860,308–12,863,860
CucCYP5	Csa_7G237870	601	5.82	70.35	Cytosol	Chr7	14:13	8,565,691–8,571,152
CucCYP6	Csa_3G777640	167	7.95	18.12	Cytoskeleton	Chr3	06:05	29,928,561–9,931,640
CucCYP7	Csa_3G125010	111	9.27	11.99	Cytosol	Chr3	05:04	7,604,494–7,607,432
CucCYP8	Csa_1G690270	175	7.43	18.88	Cytosol	Chr1	01:00	27,710,669–7,712,587
CucCYP9	Csa_4G646250	502	8.39	56.7	Nucleus	Chr4	10:09	22,040,011–2,045,496
CucCYP10	Csa_6G093090	253	8.6	26.73	Chloroplast	Chr6	07:06	6,387,276–6,392,127
CucCYP11	Csa_5G202380	220	6.52	23.94	Vacuole	Chr5	08:07	9,012,193–9,014,711
CucCYP12	Csa_5G128260	252	7.56	27.39	Chloroplast	Chr5	07:06	3,150,462–3,153,911
CucCYP13	Csa_7G009740	272	8.17	18.12	Cytosol	Chr7	01:00	621,837–622,670
CucCYP14	Csa_7G378580	668	10.4 9	74.88	Nucleus	Chr7	13:12	14,024,497–4,030,974
CucCYP15	Csa_7G407760	234	8.61	26.35	Chloroplast	Chr7	07:06	15,769,237–5,771,468
CucCYP16	Csa_2G234600	361	6.82	40.18	Cytosol	Chr2	08:07	11,450,949–11,456,685
CucCYP17	Csa_6G495630	598	7.72	65.02	Nucleus	Chr6	11:10	24,012,186–4,016,268
CucCYP18	Csa_1G042130	320	8.4	35.29	Chloroplast	Chr1	02:01	4,082,353–4,083,693
CucCYP19	Csa_2G009340	309	6.97	33.42	Chloroplast	Chr2	02:01	1,592,194–1,594,166
CucCYP20	Csa_6G185300	204	8.43	21.9	Chloroplast	Chr6	07:06	11,994,395–11,997,135
CucCYP21	Csa_2G380020	799	12.1 3	91.57	Nucleus	Chr2	13:12	19,445,622–19,454,531

Table 2. In total, there are 26 cyclophilins from *P. vulgaris*, each providing details such as ID, size (in amino acids), PI, MW, sub-cellular localization, chromosome location, exon-intron count, and their respective positions on the chromosome.

Name	ID	Size (AA)	PI	MW (KDa)	Subcellular localization	Chr.	Exon:Intron	Location
PhvCYP1	Phvul.007G100400	232	7.72	26.02	Mitochondria	Chr7	07:06	11,083,814–11,088,892
PhvCYP2	Phvul.007G001100	265	9.02	28.81	Chloroplast	Chr7	05:04	52,491–55,392
PhvCYP3	Phvul.001G045200	492	8.15	55.48	Nucleus	Chr1	10:09	3,705,172–3,714,386
PhvCYP4	Phvul.001G155500	663	10.68	74.36	Nucleus	Chr1	13:12	40,661,365–40,668,064
PhvCYP5	Phvul.001G052800	933	12.3	105.6	Nucleus	Chr1	13:12	5,803,310–5,810,235
PhvCYP6	Phvul.001G192400	177	7.83	19.44	Cytosol	Chr1	06:05	45,135,106–45,138,002
PhvCYP7	Phvul.001G219400	259	8.22	28.23	Chloroplast	Chr1	07:06	47,490,701–47,493,183
PhvCYP8	Phvul.001G053000	895	12.23	101.33	Nucleus	Chr1	13:12	5,830,433–5,837,607
PhvCYP9	Phvul.001G243000	234	7.94	25.54	Vacuole	Chr1	07:06	49,537,486–49,540,675
PhvCYP10	Phvul.006G068200.2	204	8.65	21.94	Extracellular	Chr6	07:06	17,960,337–17,963,207
PhvCYP11	Phvul.002G284200	175	8.41	18.95	Cytosol	Chr2	01:00	45,315,832–45,317,843
PhvCYP12	Phvul.002G010800	599	7.93	65.22	Nucleus	Chr2	11:10	1,182,379–1,191,577
PhvCYP13	Phvul.002G088300	226	7.42	25.42	Cytosol	Chr2	08:07	14,508,020–14,513,063
PhvCYP14	Phvul.010G085700.2	136	9.19	14.86	Cytosol	Chr10	05:04	23,736,333–23,739,990
PhvCYP15	Phvul.003G 294200	361	5.83	40.23	Cytoskeleton	Chr3	08:07	53,123,048–53,127,120
PhvCYP16	Phvul.003G007900	228	6.77	25.84	Chloroplast	Chr3	07:06	879,646–884,512
PhvCYP17	Phvul.003G178500	389	5.49	42.21	Chloroplast	Chr3	02:01	40,176,443–40,178,389
PhvCYP18	Phvul.003G246400	360	5.9	40.15	Nucleus	Chr3	08:07	48,320,387–48,324,050
PhvCYP19	Phvul.009G211800.2	289	6.79	31.6	Chloroplast	Chr9	02:01	31,995,362–31,997,191
PhvCYP20	Phvul.009G025000	172	6.86	18.39	Cytosol	Chr9	01:00	6,085,767–6,086,609
PhvCYP21	Phvul.009G120200	232	7.07	25.67	Chloroplast	Chr9	07:06	18,371,554–18,378,981
PhvCYP22	Phvul.005G126100	615	7.01	69.34	Cytoskeleton	Chr5	13:12	36,325,790–36,332,343
PhvCYP23	Phvul.011G203200	196	7.82	21.16	Cytosol	Chr11	07:06	51,898,837–51,902,923
PhvCYP24	Phvul.011G177175	326	4.65	36.7	Cytosol	Chr11	14:13	48,836,652–48,848,744
PhvCYP25	Phvul.011G026900	172	7.83	18.17	Cytosol	Chr11	01:00	2,368,490–2,370,033
PhvCYP26	Phvul.011G034400	234	6.28	25.76	Chloroplast	Chr11	07:06	3,163,429–3,167,392

PhvCYP18 both have TPR_1 and TPR_19 domains. In addition, two more domains namely cytochrome c-type biogenesis protein (NrfG) domain and type IV pilus biogenesis/stability protein (TypeIV_pilW) domain were identified in *PhvCYP18*. WD40 domain was identified only in *PhvCYP22*. Two domains from super-family RRM and RRM_SF, polyadenylate binding protein (PABP_1234), and glycine-rich RNA-binding protein (PLAN03134) were observed in *PhvCY24* (Fig. 2b). Localization of cyclophilins indicated that eight cyclophilins are localized in cytosol followed by seven in chloroplast and only one was localized in vacuole and mitochondria each (Table 2).

3.4. *Vitis vinifera*

In *V. vinifera,* 22 cyclophilins are distributed on 14 different chromosomes (Fig. 1c). Chromosomes 1, 3, 4, 6, 7, and 13 have two cyclophilins each, while chromosomes 5, 8, 11, 14, 15, 17, 18 and 19 have only one cyclophilin. The remaining two cyclophilins are located on two different scaffolds. In *V. vinifera*, cyclophilin *GsvCYP16* is the largest with 700 amino acids while cyclophilin *GsvCYP19* is the smallest with 66 amino acids (Table 3). Out of 22 *V. vinifera* cyclophilins, 16 are single-domain while 6 are multi-domain cyclophilins (Supplementary Fig. S2). The 16 single-domain cyclophilins are *viz. GsvCYP1, GsvCYP2, GsvCYP3, GsvCYP4, GsvCYP7, GsvCYP8, GsvCYP9, GsvCYP10, GsvCYP12, GsvCYP13, GsvCYP15, GsvCYP17, GsvCYP18, GsvCYP19, GsvCYP20,* and *GsvCYP21*. WD40 domain is present in cyclophilin *GsvCYP5* while centriole, cilia, and spindle-associated domain is present in *GsvCYP11*. Domains TPR, TPR_1, TPR_19, 3a0801s09, PEP_TPR_LIPO, and PLN03088 are present in cyclophilin *GsvCYP6*. Two copies of ring_Ubox domain are in cyclophilin *GsvCYP14*. *GsvCYP22* had the highest number of seven domains consisting of RRM, RRM_SF, PABP_1234, PLAN03134, SF_CC1 and two pair of PRK12678 domains (Fig. 2c). Localization of cyclophilins in *V. vinifera* indicated that eight cyclophilins were localized in chloroplast while only one is in mitochondria (Table 3).

3.5. Conserved Domain Analysis

Multiple sequence alignments of *P. vulgaris*, *C. sativus,* and *V. vinifera* cyclophilins showed the presence of conserved regions (Supplementary Fig. S3). The FHR motif is conserved in all cyclophilins of *P. vulgaris*. However, in *V. vinifera*, it is conserved in all cyclophilins except for *GsvCYP5* and *GsvCYP9* (Fig. 3). In *C. sativus*, the FHR motif is conserved in all *CucCYPs* except for *CucCYP5, CucCYP17, CucCYP18*, and *CucCYP19*. The ENF motif is conserved in most cyclophilins, with exceptions including *CucCYP5, CucCYP6, CucCYP9, CucCYP14, CucCYP17, CucCYP18, CucCYP19,* and *CucCYP21* in *C. sativus*, and *PhvCYP3, PhvCYP6, PhvCYP12, and PhvCYP14* in *P. vulgaris*. Additionally, ENF is not

Table 3. In total, there are 22 cyclophilins from *V. vinifera*, each providing details such as ID, size (in amino acids), PI, MW, sub-cellular localization, chromosome location, exon-intron count, and their respective positions on the chromosome.

Name	ID	Size (AA)	PI	MW (KDa)	Subcellular localization	Chr.	Exon:Intron	Location
GsvCYP1	VIT_203s0038g022 00	223	6.32	24.56	Vacuole	Chr3	07:06	1,512,510–1,518,020
GsvCYP2	VIT_206s0004g066 10	200	8.88	21.52	Chloroplast	Chr6	07:06	7,337,828–7,342,997
GsvCYP3	VIT_215s0048g017 80	178	9.04	19.63	Mitochondria	Chr15	05:04	15,962,149–15,964,695
GsvCYP4	VIT_207s0005g024 10	253	7.47	27.4	Chloroplast	Chr7	07:06	4,770,541–4,773,262
GsvCYP5	VIT_206s0061g015 90	649	7.42	73.23	Nucleus	Chr6	11:10	19,509,998–19,533,564
GsvCYP6	VIT_203s0063g010 30	361	6.12	40.3	Cytosol	Chr3	08:07	4,455,952–4,466,907
GsvCYP7	VIT_213s0067g008 70	228	8.64	24.64	Vacuole	Chr13	08:07	497,638–500,735
GsvCYP8	VIT_208s0007g029 00	169	8.12	18.28	Cytoskeleton	Chr8	06:05	16,959,573–16,963,687
GsvCYP9	VIT_205s0077g010 40	234	9.14	26.49	Chloroplast	Chr5	07:06	804,257–807,29 3
GsvCYP10	VIT_204s0008g013 70	498	7.44	56.55	Nucleus	Chr4	10:09	1,119,729–1,127,582
GsvCYP11	VIT_204s0008g05090	438	9.6	49.07	Nucleus	Chr4	13:12	4,566,087–4,576,275
GsvCYP12	VIT_214s0081g007 00	69	9.29	8.1	Cytosol	Chr14	01:00	9,060,994–9,061,519
GsvCYP13	VIT_213s0084g006 20	150	7.18	16.35	Chloroplast	Chr13	06:05	19,663,725–19,668,722
GsvCYP14	VIT_207s0129g000 40	597	7.03	65.3	Cytosol	Chr7	11:10	15,331,993–15,344,208
GsvCYP15	VIT_201s0146g001 10	262	9.4	28.02	Chloroplast	Chr1	07:06	21,897,744–21,913,173
GsvCYP16	VIT_200s0179g002 90	700	10.85	77.49	Nucleus	ChrUn	13:12	7,530,440–7,537,241
GsvCYP17	VIT_200s0513g000 20	331	9.15	35.63	Cytosol	ChrUn	03:02	31,001,212–31,002,813
GsvCYP18	VIT_217s0000g042 70	369	7.89	39.32	Chloroplast	Chr17	02:01	4,509,161–4,510,618
GsvCYP19	VIT_218s0001g144 00	66	7.46	6.69	Chloroplast	Chr18	01:00	12,377,348–12,377,867
GsvCYP20	VIT_211s0118g008 10	194	5.5	20.99	Chloroplast	Chr11	02:01	6,631,087–6,634,009
GsvCYP21	VIT_201s0026g020 70	226	7.38	25.24	Vacuole	Chr1	08:07	11,448,966–11,455,795
GsvCYP22	VIT_219s0027g016 60	633	5.7	73.79	Nucleus	Chr19	14:13	21,921,000–21,933,586

conserved in GsvCYP5, *GsvCYP8, GsvCYP9,* and *GsvCYP10* of *V. vinifera*. The SI and DE motifs are other majorly conserved domains found in all cyclophilins of *P. vulgaris*, but they are missing in *CucCYP17, CucCYP18, CucCYP19*, and *CucCYP21* of *C. sativus*, as well as *GsvCYP9* of *V. vinifera.* The GSQFFI motif is conserved in most cyclophilins of *C. sativus*, except for *CucCYP5, CucCYP17, CucCYP18,* and *CucCYP19*. However, it is not conserved in *PhvCYP5, PhvCY8,* and *PhvCYP23*. *GsvCYP9* and *GscCYP17* also showed no conservation of the GSQFFI motif, whereas it is observed in all other *GsvCYPs*. The QGGD motif is conserved in most cyclophilins, except for *CucCYP5, CucCYP17, CucCYP18*, *CucCYP19,* and *CucCYP21* of *C. sativus*, as well as *PhvCYP3, PhvCYP5, PhvCYP8, PhvCYP22,* and *PhvCYP23* of *P. vulgaris*. Additionally, *GsvCYP5, GsvCYP9,* and *GsvCYP13* do not exhibit conservation of the QGGD motif, while it is conserved in all other *GsvCYPs*. The SMAN motif is also largely conserved, with exceptions including *CucCYP5, CucCYP6, CucCYP9, CucCYP17, CucCYP18, CucCYP19,* and *CucCYP21* of *C. sativus, PhvCYP3, PhvCYP5, PhvCYP8, PhvCYP14,* and *PhvCYP21* of *P. vulgaris,* as well as *GsvCYP1, GsvCYP5, GsvCYP8, GsvCYP9,* and *GsvCYP10* of *V. vinifera.*

Multiple sequence alignment of cyclophilins from *P. vulgaris*, *C. sativus*, *V. vinifera*, *A. thaliana,* and *G. max* showed conservation of WD40 domain in *CucCYP2*, *PhvCYP22*, *GsvCYP5, GmCYP2, GmCYP35,* and *Arabidopsis CYP 71* (AT3g44600) (Supplementary Fig. S4). Conserved domains were also observed between *CucCYP7*, *PhvCYP14,* and *GsvCYP3* cyclophilins and *AT1G01940* (*ATCYP18-1*) (Supplementary Fig. S5A). Similarly, *CucCYP6*, *PhvCYP6* and *GsvCYP8* showed conserved regions with *AT2G36130* (*ATCYP18-2*) (Supplementary Fig. S5B).

3.6. Phylogenetic and Evolutionary Studies of Cyclophilins

Phylogenetic analysis was performed with cyclophilins from *P. vulgaris*, *C. sativus,* and *V. vinifera* which clustered them into 5 different subgroups (Fig. 4a). Seven cyclophilins *viz*. *CucCYP3*, *PhvCYP15*, *GsvCYP6*, *GsvCYP13*, *CucCYP13*, *CucCYP16*, and *PhvCYP18* grouped together on clade 1 and has TPR domains and CLD domain. Clade 2 had 26 cyclophilins while Clade 3 had 17 cyclophilins. Three cyclophilins such as *PhvCYP24*, *CucCYP5*, and *GsvCYP22* grouped together on clade 5 and have glycine-rich RNA-binding protein (PLAN03134), polyadenylate binding protein (PABP_1234), and (RRM) domains. Similarly, three cyclophilins *viz*. *GsvCYP14*, *CucCYP17*, and *PhvCYP12* with ring Ubox domain grouped on clade 4. Three cyclophilins (*GsvCYP10*, *CucCYP9*, and *PhvCYP3*) with nucleus-directed signal domain CYPs clubbed together on clade 5 and chloroplast-directed domains (*CucCYP18, PhvCYP17, GsvCYP18, PhvCYP19, CucCYP19,* and *GsvCYP20*) were on clade 3. A correlation of phylogenetic analysis with the sub-cellular localization of cyclophilins was observed.

A cladogram was constructed using cyclophilins from *P. vulgaris*, *C. sativus,* and *V. vinifera* cyclophilins and their homologs from other species and four major clades were observed (Fig. 4b). Clade 1 has *OsCYP25* (rice) showed 87% protein sequence similarity with *CucCYP8* and 93% similarity with *PhvCYP11*. *PhvCYP11* has 91% similarity with AtCYP*18-1* (Roc2) of *Arabidopsis*. Clade 2 with *PhvCYP25*, *PhvCYP20,* and *CucCYP13* has 96.5%, 92.5% and

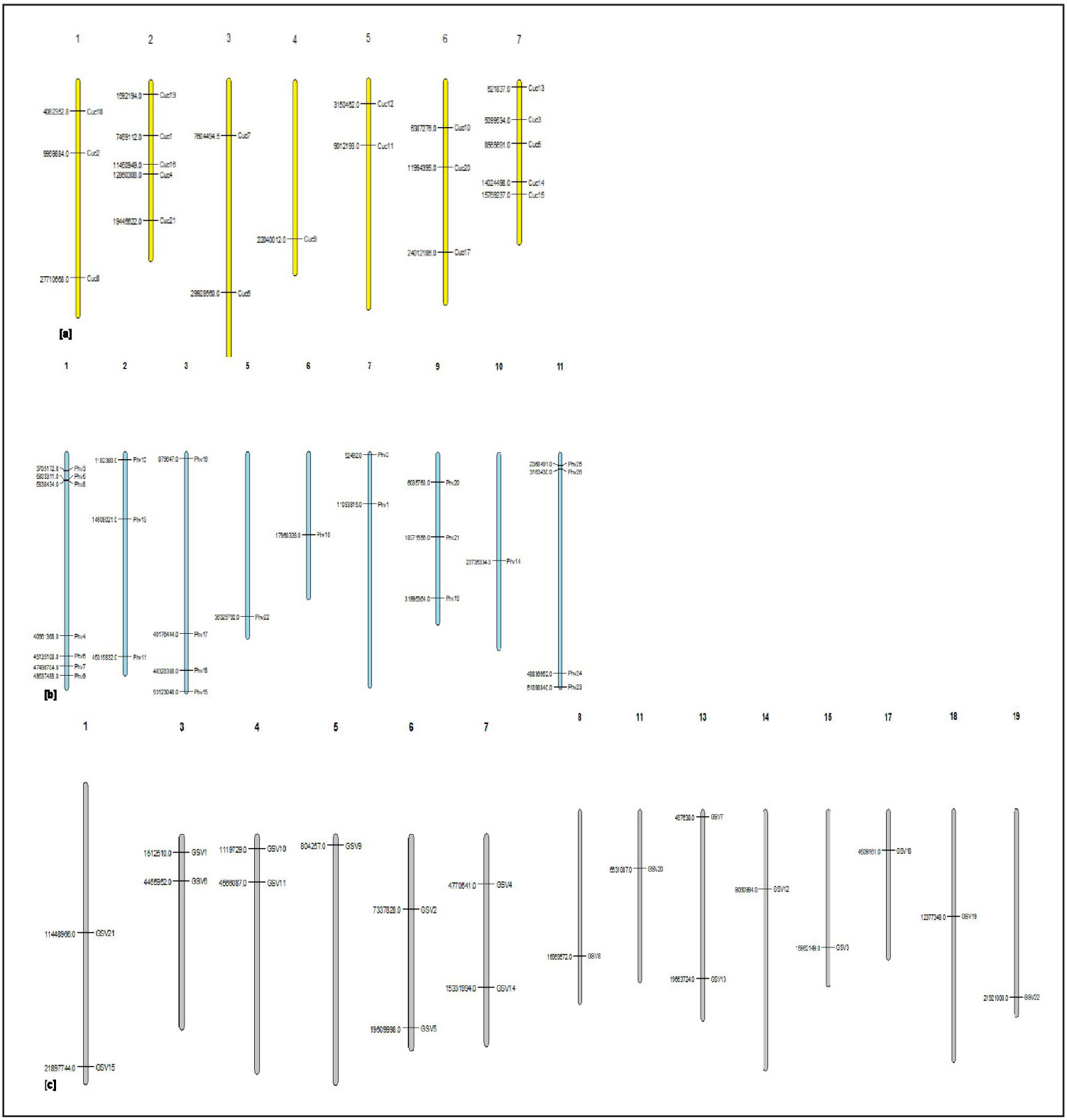

Figure 1. Distribution of the identified cyclophilins on different chromosomes (no. marked on top) with an uneven distribution. (a) Total 21 *C. sativus* cyclophilins (CucCYPs) were on 7 chromosomes, (b) Twenty-six *P. vulgaris* cyclophilins (PhvCYPs) distributed on 9 chromosomes while no cyclophilins detected on chromosomes 4 and 8, (c) Twenty-two *V. vinifera* cyclophilins (GsvCYPs) were on 14 chromosomes while 2 were present on scaffold.

97.7%, similarity to *GmCYP1*. *CucCYP13* has 90.8% similarity with *AtCYP18-3* and 86.1% similarity with *OsCYP2*. Clade3 with *CucCYP10* has 79.5% similarity to *AtCYP20-2* while *GsvCYP6* has 76.6% similar with *OsCYp20-2*. Phylogenetic analysis revealed that *PhvCYP25* and *GmCYP1 are* present in the same clade and have 96.5% amino acid sequence similarity. Similarly, *PhvCYP20*

Table 4. The information is organized in a table format, displaying the CucCYPs, GsvCYPs, and PhvCYPs, alongside their corresponding *A. thaliana* orthologs. Additionally, the table provides a detailed summary of the roles these cyclophilins play in various abiotic and biotic stresses.

Arabidopsis orthologs	Stress factor	CucCYP	PhvCYP	GsvCYP
AT3G62030 (ATCYP20-3)	Light oxidative	Cuc12	Phv7	Gsv4
AT5G13120 (ATCYP20-2)	Light temperature	Cuc10	Phv2	Gsv15
AT3G55920 (AtCYP21-2)	Drought stress	Cuc11	-	-
AT2G29960 (ATCYP19-4)	-	Cuc20	-	-
AT4G34960 (ATCYP21-1)	-	Cuc4	Phv26	Gsv1
AT3G22920	-	-	-	Gsv12
AT4G32420(ATCYP95)	-	-	-	Gsv11
AT3G63400 (ATCYP63)	-	Cuc14	Phv4	Gsv16
AT4G34870	-	-	-	Gsv19
AT5G67530 (ATCYP65)	-	Cuc17	Phv12	-
AT1G01940 (ATCYP18-1)	Heat	Cuc7	Phv14	Gsv3
AT4G33060 (ATCYP57)	*Psuedomonas syringae* infection	Cuc9	Phv3	Gsv10
AT2G36130 (ATCYP18-2)	Drought	Cuc6	Phv6	Gsv8
AT1G26940 (ATCYP23-1)	-	Cuc1	Phv13	Gsv21
AT3G44600 (ATCYP71)	-	Cuc2	Phv22	Gsv5
AT1G53720 (ATCYP59)	-	Cuc5	Phv24	Gsv22
AT3G66654 (ATCYP21-4)	-	Cuc15	-	Gsv9
AT2G38730 (ATCYP22)	-	-	Phv23	Gsv13
AT2G15790 (ATCYP40)	-	-	Phv15	Gsv6
AT1G74070 (ATCYP26-2)	-	Cuc18	Phv17	Gsv18
AT5G35100 (ATCYP28)	-	Cuc19	Phv19	Gsv20
AT3G56070 (ATCYP19-3)	-	Cuc8	Phv11	-
AT2G16600 (ATCYP19-1)	Drought	Cuc13	-	-

and *CucCYP13* have 92.5% and 97.7%, protein sequence similarity with *GmCYP1,* respectively. Cyclophilin *GsvCYP6* has 76.6% protein similarity with thylakoid luminal cyclophilin of rice *OsCYp20-2*.

3.7. Cyclophilin Grouping: Conserved Domains and Common Sub-Cellular Localization

A similar pattern of correlation between sub-cellular localization and domains was observed by constructing phylogenetic tree with all novel cyclophilins and previously reported ones from other plants (such as *A. thaliana*, *O. sativa, M. truncatula*, and *G. max*) (Fig. 5). Eight cyclophilins (*GsvCYP5*, *GmCYPs20*, *GmCYPs35*, *AT3G44600*, *PhvCYP22*, *CucCYP2*, *LOC_ Os08 g44330* and *Medtr2g085075-MtCYP71*) with WD40 domain grouped together on clade 5. ii. Cyclophilins with common ring_UBox domain and nucleus location (*CucCYP17*, *PhvCYP12*, *GmCYP18*, *GmCYP19*, *AT5G67530,* and *Medtr5g015500*) grouped together on clade5.vi, while cyclophilins with ring_UBox domain and cytosol localization (*GsvCYP17*, *GsvCYP14,* and *LOC_Os03g10400*) grouped on another branch of the same clade. All cyclophilins with TPR domain (*Medtr8g079690*, *AT2G15790*, *PhvCYP18*, *GmCYP9*, *GmCYP8*, *CucCYP3*, *CucCYP16*, *GsvCYP6*, *Medtr4g086760*, *PhvCYP15*, *GmCYP17,* and *GmCYP16*) grouped together on clade 1. Similarly, RRM domain containing cyclophilins (*Medtr8g442420*, *Medtr6g084140*, *GsvCYP22*, *CucCYP5*, *PhvCYP24*, *GmCYP59*, *GmCYP56*, *AT1G44478*, *LOC_ Os06 g45900*, *LOC_Os06 g45910*, *Medtr8g442420,* and *AT1G53720*) formed separate subgroup on clade 5.i.

In addition, sequence similarity amongst cyclophilins was observed in *C. sativus, P. vulgaris, and V. vinifera* sequences (Fig. 6). Multiple shared sequences were observed among cyclophilins of *C. sativus, P. vulgaris, and V. vinifera* sequences when compared with previously reported cyclophilins from *A. thaliana*, *M. truncatula*, and *G. max*. *GsvCYP16* and *PhvCYP20* showed most repeated sequences (Fig. 6a). Family-wise sequence similarity was observed among cyclophilins of leguminosae *viz*. *P. vulgaris, G. max,* and *M. truncatula* (Fig. 6b). High similarities were also observed between cyclophilins from *C. sativus A. thaliana* (Fig. 6c).

3.8. Comparative Analysis of Cyclophilins With Arabidopsis Orthologous

The cyclophilins *CucCYP12, PhvCYP7*, and *GsvCYP4*, which share a common single CLD domain, are orthologous to *AT3G62030 (ATCYP20-3)* in *A. thaliana*. This gene has been associated with a role in light-induced oxidative stress [65] (Table 4). Additionally, *AT5G13120 (ATCYP20-2)*, which plays a similar role in light stress, was found to be orthologous to *CucCYP10, PhvCYP2,* and *GsvCYP15* [66]. *AT4G33060 (ATCYP57)*, known for its role in *P. syringae* infection, is orthologous to *CucCYP9, PhvCYP3*, and *GsvCYP10* [67]. Furthermore, three *A. thaliana* cyclophilins, *AT2G16600 (ATCYP19-1)*, *AT2G36130 (ATCYP18-2),* and *AT3G55920 (ATCYP21-2)* which are up regulated multiple folds, enhance stress tolerance against drought. They were orthologous to CucCYP*13*, *CucCYP6*, *PhvCYP6*, *GsvCYP8,* and *CucCYP11* [68–70]. Another important cyclophilin in *A. thaliana*, *AT1G01940*

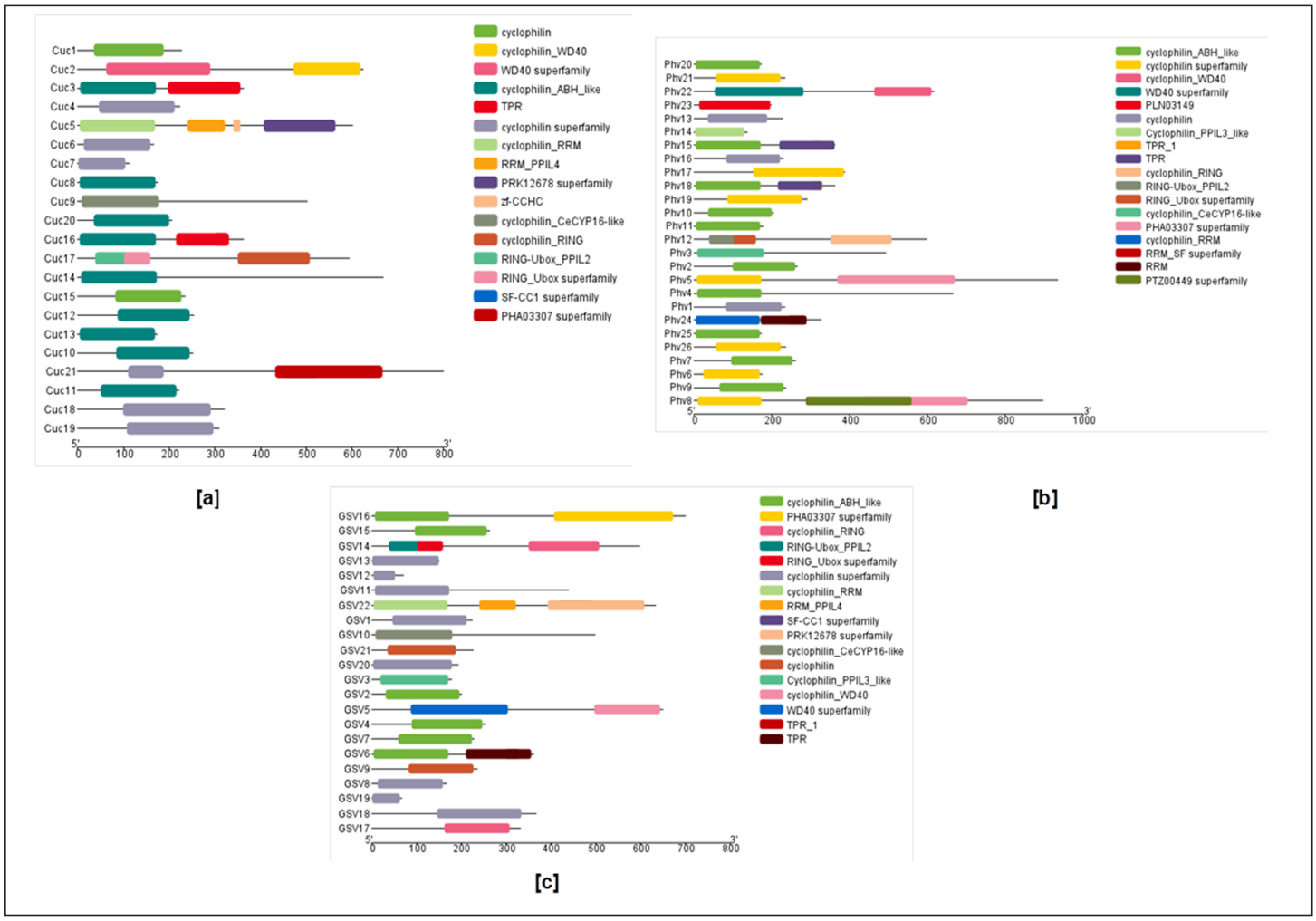

Figure 2. Schematic representation of single and multi-domain in the identified cyclophilins. Domain analysis was done by using NCBI batch-CD tool (https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi) for the protein sequence of cyclophilins derived from (A) *C. sativus*, (B) *P. vulgaris*, and (C) *V. vinifera*. In *C. sativus*, out of the 21 cyclophilins identified, 15 were found to be single domain, and the remaining 6 were categorized as multi-domain cyclophilins. In the case of *P. vulgaris*, 19 cyclophilins were classified as single domain, and 7 were identified as multi-domain cyclophilins. Similarly, *V. vinifera* exhibited 16 single domain cyclophilins, with the remaining 6 being categorized as multi-domain cyclophilins.

(ATCYP18-1), known to confer tolerance against heat stress, is orthologous to *CucCYP7, PhvCYP14*, and *GsvCYP3* [71].

3.9. Intron-Exon Structure in Cyclophilins

The distribution of intron-exon was analyzed among *C. sativus, P. vulgaris, and V. vinifera* in the present study where the number of introns varied in the range of 0–13 in all three plant species (Tables 1–3). In *C. sativus*, *CucCYP5* had the highest number of 14 exons followed by 13 exons in *CucCYP2*, *CucCYP14,* and *CucCYP21* while only one exon was present in *CucCYP8*. However, *PhvCYP24* has 14 exons followed by 13 exons each in *PhvCYP4*, *PhvCYP5*, *PhvCYP8,* and *PhvCYP22*. Interestingly, no introns were found in *PhvCYP20* and *PhvCYP25*. In *V. vinifera*, *GsvCYP22* has 14 exons followed by 13 exons each in *GsvCYP11* and *GsvCYP16*. On the other end, *GsvCYP12* and *GsvCYP19* have no introns.

3.10. Synonymous and Non-Synonymous Substitution Rate (Ka-Ks)

The Ks values of 21 cyclophilins from *C. sativus* ranged from 0.2 (for gene pair *CucCYP3-CucCYP16*) to 0.57 (for pair *CucCYP19-CucCYP7*) (Supplementary Fig. S6). Meanwhile, the Ks values of 26 cyclophilins from *P. vulgaris* ranged from 0.2 (for gene pair *PhvCYP16- PhvCYP1*) to 0.56 (for gene pair *PhvCYP24-PhvCYP19*). In the case of *V. vinifera* Ks values ranged from 0.03 (for gene pair *GsvCYP17-GsvCYP14*) to 0.6 (for gene pair *GsvCYP22- GsvCYP9*). The divergence time (in MYA) of *C. sativus* ranged from 59.6 to 130.9 MYA, with the lowest divergence observed in the *CucCYP3-CucCYP16* gene pair and the highest in the *CucCYP19-CucCYP7* gene pair. In *P. vulgaris*, the divergence time ranged from 12.32 to 33.59 MYA, with the lowest divergence observed in the *PhvCYP21-PhvCYP26* gene pair and the highest in the *PhvCYP24-PhvCYP19* gene pair. In *V. vinifera*, the *GsvCYP22-GsvCYP9* gene pair showed the highest divergence time, while the *GsvCYP17-GsvCYP14* gene pair exhibited the lowest divergence time, approximately around 7.5 MYA, indicating a recent divergence event between *GsvCYP17* and *GsvCYP14*.

3.11. In silico Expression Analysis of Cyclophilins

Gene expression analysis indicated differential expression of key cyclophilins from various plant species. Transcriptomic data of *C. sativus* from different tissues (root, male, female, ovary, fertilized

Figure 3. Schematic representation of multiple sequence alignment for cyclophilins protein family showing conserved motifs. Protein sequences of cyclophilin family derived from (a) *C. sativus*, (b) *P. vulgaris* and (c) *V. vinifera* were aligned by using Clustal Omega tool (https://www.ebi.ac.uk/Tools/msa/clustalo) further visualization was done in Mview (https://www.ebi.ac.uk/Tools/msa/mview) and conserved regions are shown in different colour. The conservation of motifs was evident in the cyclophilins of all three plant species.

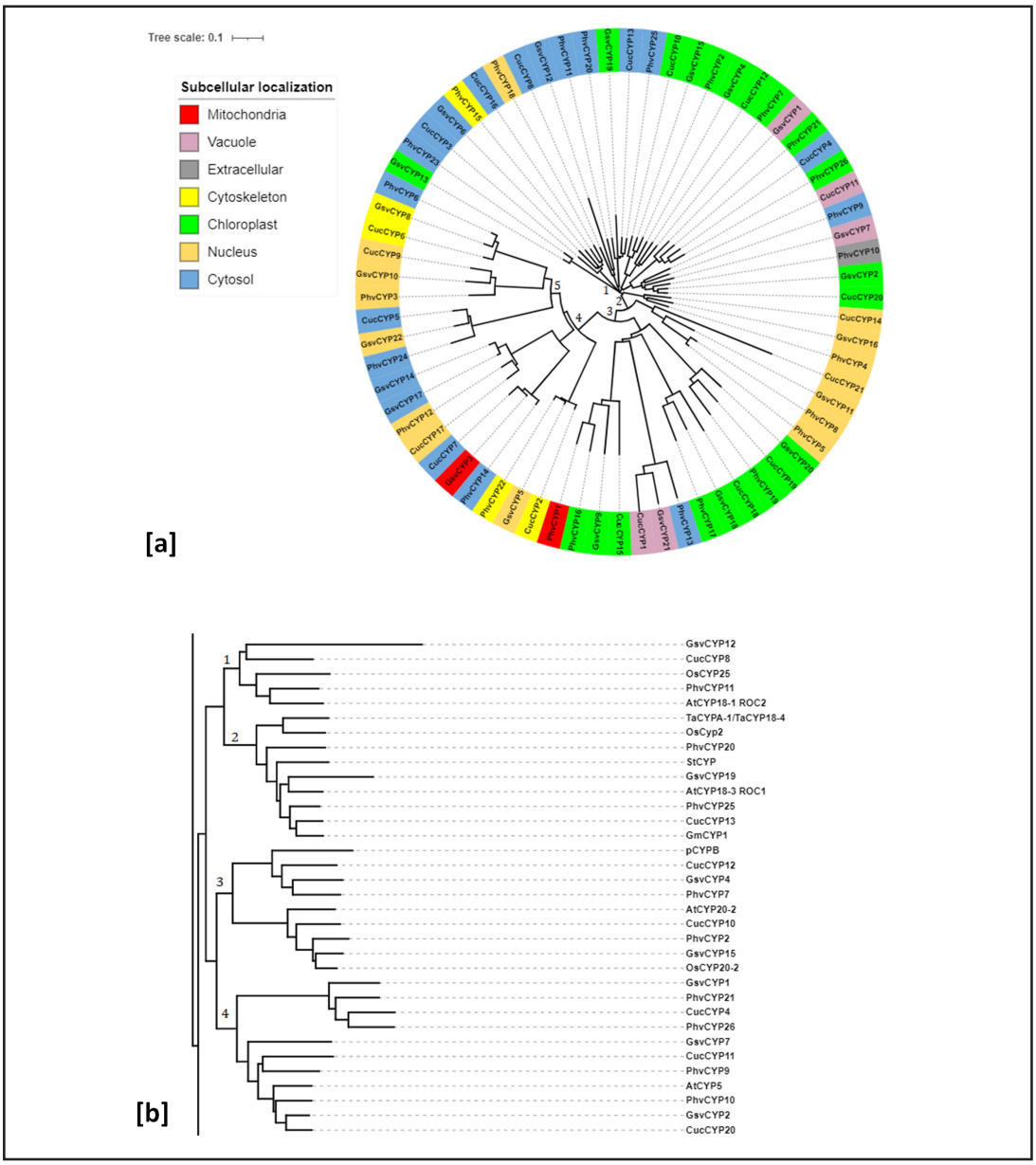

Figure 4. Phylogenetic relationship of newly identified cyclophilin (a) Phylogenetic relationship among the identified cyclophilins of *C. sativus*, *G. max*, *P. vulgaris* and *V. vinifera*. Subcellular localization analysed by wolfpsort online tool where all cyclophilins were found to be localized in mitochondria, vacuole, extracelluar space, cytoskeleton, chloroplast, nucleus and cytosol in uneven manner. Color range represents predicted subcellular localization of cyclophilins. (b) Cladogram showing relationship among cyclophilins from *C. sativus, G. max*, *P. vulgaris* and *V. vinifera* with cyclophilins from various plants having role in abiotic stress.

ovary, unfertilized ovary, stem, tendril base, tendril, and leaf) was obtained from cucurbit genome database (CuGenDB), bio project PRJNA80169 and analyzed to see the differences/similarities in gene expression pattern (Fig. 7a) (Supplementary Fig. S7). Expression analysis revealed that cyclophilins in leaves have higher gene expression as compared to the root. Gene expression of *Csa1G042130*, *Csa2G234600*, *Csa1G153530*, *Csa1G690270*, *Csa2G380020*, and *Csa5G202380* cyclophilins increased in fertilized ovary as compared to the unfertilized one while the level stayed nearly unchanged on fertilization for *Csa7G407760*, *Csa7G237870*, *Csa2G270140,* and *Csa2G009340*. Similarly, *Csa5G128260* showed higher expression of cyclophilins in female plants as compared to the male plants. Cyclophilin *Csa7G009740* (*CucCYP13*) had overall higher expression in roots as compared to any other tissue and was also 97.7% similar with *GmCYP1*.

In silico gene expression analyses were also carried out in *P. vulgaris* and data was retrieved from the phytozome database. The expression level of cyclophilins from *P. vulgaris* was studied in flower bud, flower, green mature pods, leaves, nodules, root on 10th day, root on 19th day, stem on 10th day, stem on 19th day, young pods and young trifoliate. The young pod showed high levels of mRNA transcript, which was the highest amongst all cyclophilin transcript data of *P. vulgaris* obtained from various tissues (Fig. 7b) (Supplementary Fig. S7). Results indicated that cytosol-localized *Phvul.011G026900* (*PhvCYP25*) has higher levels (10–100x) of transcripts than the rest of cyclophilins and shared phylogenetic relationship with *CucCYP13* and *GmCYP1* (Fig. 4b).

RNA seq data of *V. vinifera* was retrieved from the expression atlas database of EMBL-EBI for *GsvCYP* [72], where high-throughput

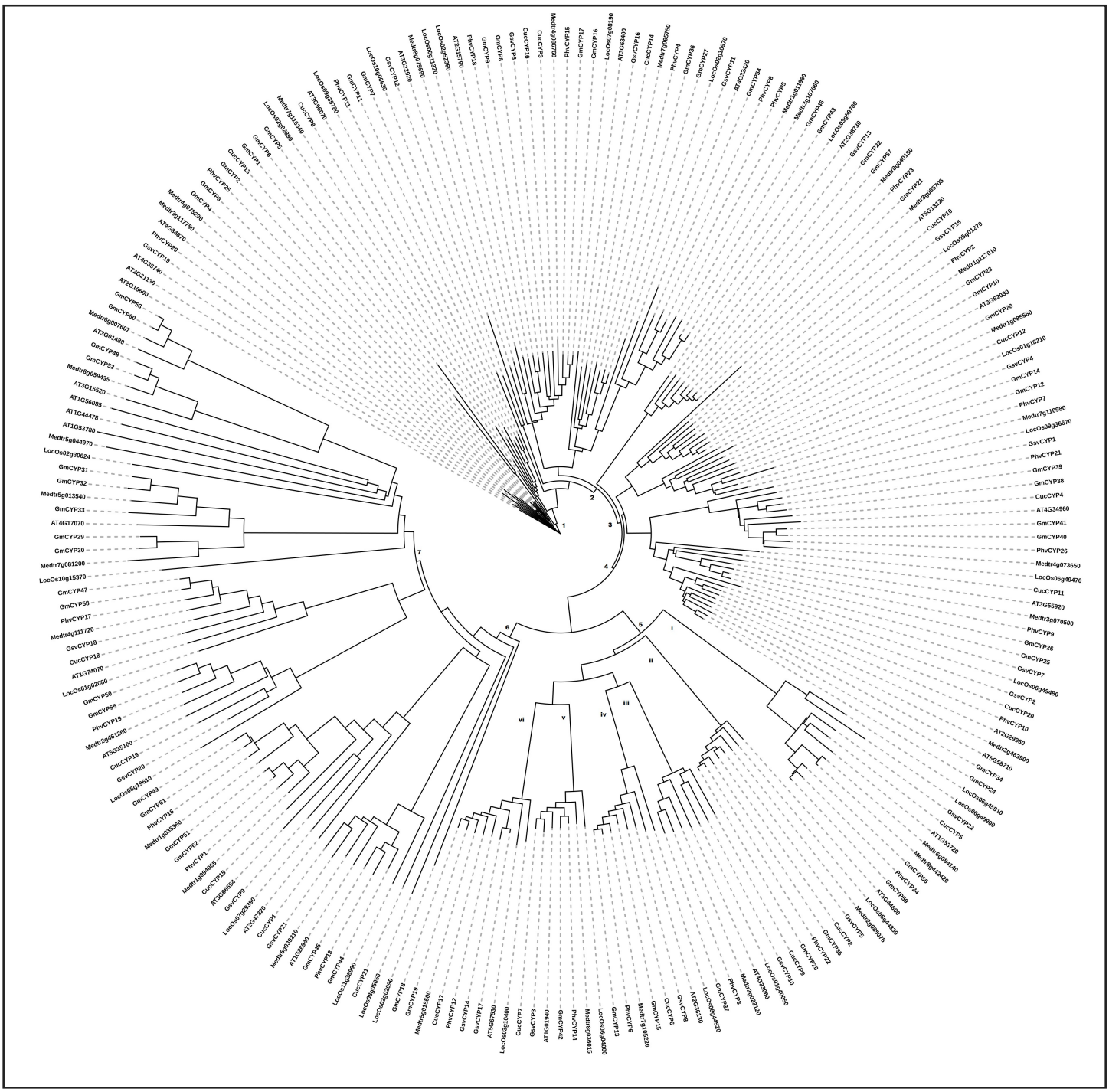

Figure 5. Phylogenetic relationship of cyclophilin protein family for different plants. Phylogenetic correlation of cyclophilins from *C. sativus, P. vulgaris* and *V. vinifera* with previously reported *G. max*, *A. thaliana, M. truncatula* and *O. sativa*. Multiple sequence alignment of all amino acid sequences was carried out in MegaX using clustral W and tree was constructed using iTOL: Interactive tree of life.

sequencing was conducted using samples from three distinct stages of berry development, each infected with *Botrytis cinerea* rot. Stage 1 represents the initial infection phase, characterized by a color change from yellow to pink. In Stage 2, the infection progressed further, resulting in softer berry skin and dark pink coloration. Stage 3 samples were fully rotten but not yet dry. Expression data is presented in fold change against control. Results indicated a down regulation of *GsvCYPs* when infected with *B. cinerea. VIT_211s0118g00810 (GsvCYP20)* and *VIT_201s0146g00110* (*GsvCYP15)* were found to be most down regulated *viz.* 160% on stage 3 and stage 2 of infection, respectively (Fig. 7c) (Supplementary Fig. S7). The least down regulated *GsvCYPs* are *VIT_215s0048g01780 (GsvCYP3), VIT_204s0008g01370 (GsvCYP10), VIT_214s0081g00700 (GsvCYP12)* and *VIT_218s0001g14400 (GsvCYP19).*

4. RESULT SUMMARY

In summary, we identified 21, 26 and 22 cyclophilins from *C. sativus, P. vulgaris* and *V. vinifera*. *C. sativus* has 15 single domain cyclophilins

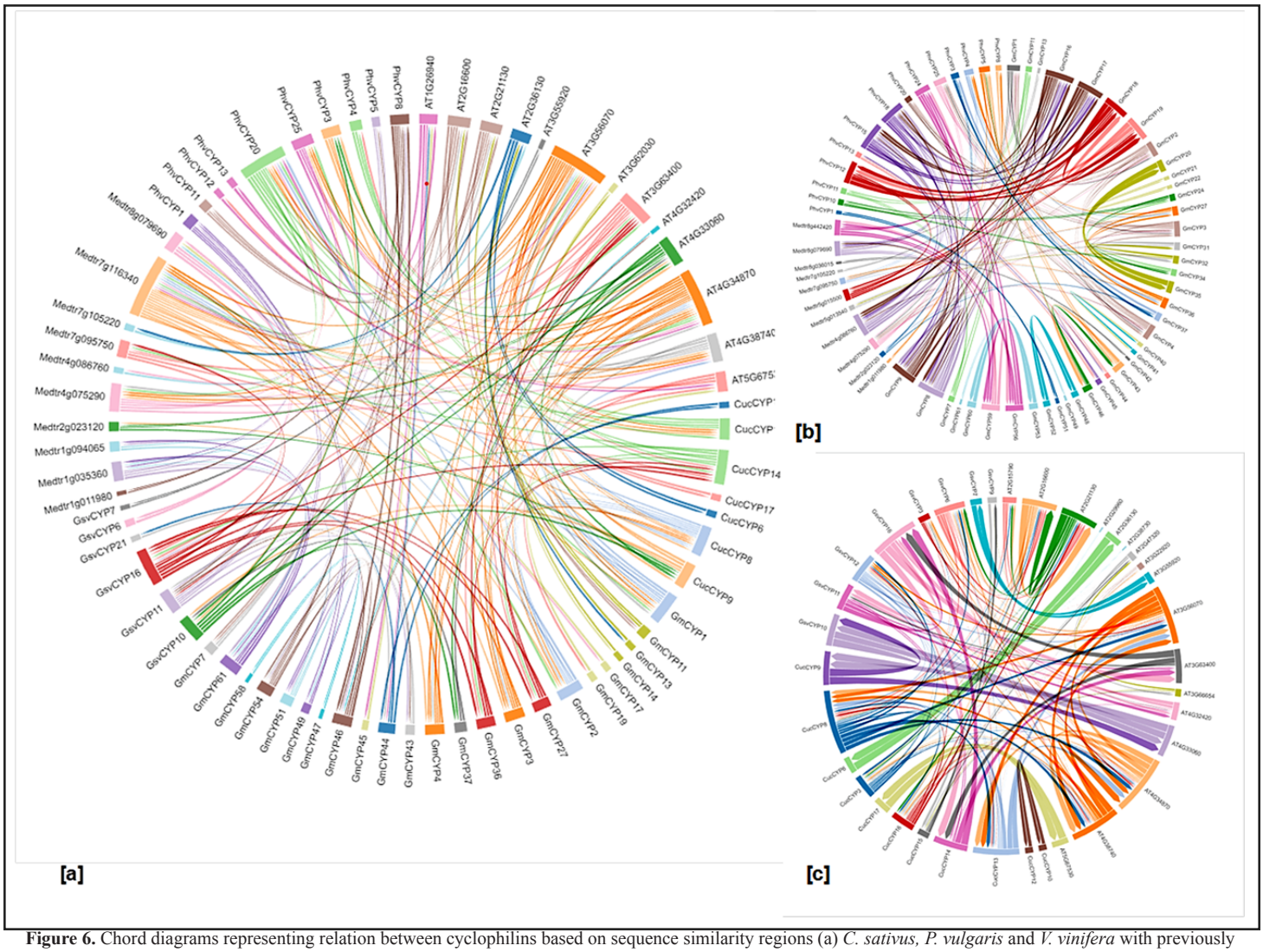

Figure 6. Chord diagrams representing relation between cyclophilins based on sequence similarity regions (a) *C. sativus, P. vulgaris* and *V. vinifera* with previously reported *G. max*, A. thaliana and *M. truncatula* (b) family wise orthology of leguminosae *P. vulgaris*, *G. max* and *M. truncatula* (c) *A. thaliana* and *C. sativus*. The count of colorful segments within each cyclophilin. indicates the presence of shared similarity regions among cyclophilins, which is determined by their amino acid sequences. The highest degrees of similarity were evident in PhvCYP20, GsvCYP16, and CucCYP14 when compared to other cyclophilins, represented by the presence of colored chords.

while 6 cyclophilins are multi-domain proteins. *Phaseolus vulgaris* has 19 single domain cyclophilins while 7 are multi-domain cyclophilins. In case of *V. vinifera* 16 cyclophilins have single CLD domain while rest 6 cyclophilins are multi-domain. Multiple sequence analysis revealed conservation of eight motifs in all reported cyclophilins widespread in different regions. GSQFFI motif has been found to be longest with six amino acids one while SI and DE were smallest with only two amino acids. Phylogenetic analysis revealed grouping of cyclophilins into five major clades where cyclophilins having common domains and sub-cellular localization have close phylogenetic relationships. Evolutionary studies of cyclophilins with previously reported *A. thaliana* cyclophilins were also conducted where *AT3G62030 (ATCYP20-3), AT5G13120 (ATCYP20-2), AT4G33060 (ATCYP57)*, *AT2G16600 (ATCYP19-1)*, *AT2G36130 (ATCYP18-2)* and *AT3G55920 (ATCYP21-2), AT1G01940 (ATCYP18-1)* associated with light stress, *P. syringae* infection, drought and heat stress are orthologous to various cyclophilins in our study. Intron-exon analysis revealed number of introns ranges from 0 to 13 in *C. sativus*, *P. vulgaris* and *V. Vinifera.* All three plant species have maximum 14 exons however minimum number of exons varies in each. Synonymous and non- synonymous substitution analysis revealed the Ks values from cyclophilins from *C. sativus* ranged from 0.2 to 0.57. While the Ks values of *P. vulgaris* cyclophilins ranged from 0.2 to 0.56. In case of *V. vinifera* Ks values ranged from 0.03 to 0.6. Lastly, results from *in-silico* expression analysis in *C. sativus* showed higher expression of (*CucCYP13*) in roots in comparison to other cyclophilins. Cytosol-localized *Phvul.011G026900* (*PhvCYP25*) also expressed multiple times in *P. vulgaris* than other cyclophilins. Whereas expression data of *V. vinifera* infected from *B. cinerea* revealed negative mRNA levels in all cyclophilins.

5. DISCUSSION

Cyclophilins are ubiquitous proteins found in all genera ranging from bacteria, fungi, and higher plants to humans [73]. Previously, a wide range of diversity was reported in cyclophilins from several crops such as 94 cyclophilins in *Brassica napus,* 83 in *Triticum aestivum,* 62 in *Glycine max,* 78 in *G. hirsutum,* 75 in *G. barbadense,* 40 in *G. arboreum,* 38 in *G. raimondii,* 33 in *M. truncatula,* and 29 in *O. sativa*

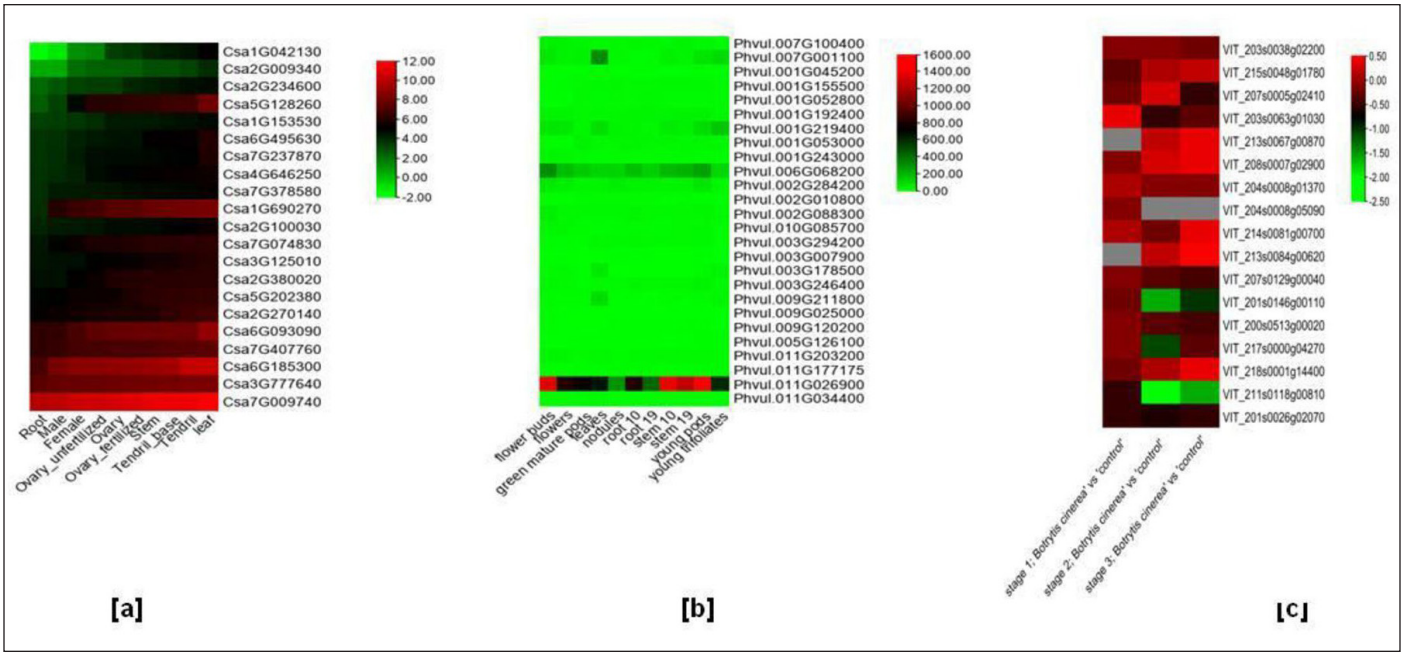

Figure 7. Heatmaps of cyclophilin transcript expression variation in *C. sativus* and *P. vulgaris*. Expression data of *P. vulgaris* was obtained from phytozome (https://phytozome- next.jgi.doe.gov/) and *C. sativus* by using cucurbit genome database (CuGenDB) from bioprojectPRJNA80169 (http://cucurbitgenomics.org/rnaseq/cu/3). Heatmap for (a) *C. sativus* and (b) *P. vulgaris* (c) *V. vinifera* was constructed using TB tools software (https://bio.tools/tbtools).

[20–24]. Cyclophilins has been associated with different physiological processes such as transcription regulation, hormonal signaling, organogenesis, and plant-pathogen interactions [74, 75].

In the present work, comparative and evolutionary studies were conducted in order to understand the role of cyclophilins family in three distinct species *C. sativus*, *P. vulgaris* and *V. vinifera.* We performed a comprehensive study that resulted in the identification of 21, 26 and 22 cyclophilins from *C. sativus*, *P. vulgaris*, and *V. vinifera*, respectively. Results from chromosomal distribution, sub-cellular localization and domain analysis revealed that cyclophilins are wide spread in cytosol and nucleus while few were detected in mitochondria and vacuole. In this study, we identified several cyclophilins from *C. sativus*, *P. vulgaris* and *V. vinifera*, which are orthologs to cyclophilins of other crops which play key roles in various biotic and abiotic stress tolerances. For example, orthologs of WD 40 domain containing cyclophilin were identified from all three plant species. The WD40 repeat (WDR) domain in cyclophilins from *C. sativus, P. vulgaris, V. vinifera* is conserved similar to previously reported *G. max* and *A. thaliana* (Supplementary Fig. S6). (WDR) domains are typically known for its β-propeller domains that were responsible for protein interaction scaffolds in multi-protein complexes [76]. Additionally, they could have a role in cell division, meristem organization, development of floral parts, regulation of secondary metabolites, and innate immunity [77,78]. The three cyclophilins *viz*. *CucCYP2*, *PhvCYP22,* and *GsvCYP5* which are grouped with previously reported *Arabidopsis CYP 71* (AT3g44600) which plays a key role in gene repression and organogenesis [79]. Disruption of *CYP71* had resulted in ectopic activation of homeotic genes that regulate meristem development. The *cyp71* mutant plants displayed dramatic defects, including reduced apical meristem activity, delayed and abnormal lateral organ formation, and arrested root growth. *CYP71* was also associated with the chromatin of target gene loci and physically interacted with histone [79]. Similarly, other cyclophilins were also identified, which play a key role in other important traits. Cyclophilins *GsvCYP6* in *V. vinifera* has 76.6% protein sequence similarity with previously reported OsCYP20 protein which regulated spliceosome assembly along with its interacting part OsSYF2 and thereby could contribute to long grain size and aid sugar metabolism in *O. sativa* [80]. Previous results have shown that *OsCYP20–2t1*, a knock out mutant of *OsCYP20–2* resulted in shorter grain and lacked cell elongation suggesting possibility for similar role of *GsvCYP6*.

Additionally, cyclophilins have been reported to play key role in drought, light, salt, heat, chilling stress tolerance [81–85]. In this study, cyclophilins from *C. sativus, P. vulgaris,* and *V. vinifera viz. CucCYP7*, *PhvCYP14* and *GsvCYP3* cyclophilins shared conserved domains with *AT1G01940* (*ATCYP18-1*) (Supplementary Fig. S7A), which was up regulated several folds and conferred heat tolerance [71]. Similarly, *CucCYP6*, *PhvCYP6* and *GsvCYP8,* respectively, grouped together with *AT2G36130* (*ATCYP18-2*) (Supplementary Fig. S7B), which interacted with *AtSKIP* and translocated to the nucleus from cytoplasm providing resistance against drought [69].

Genome duplication is base of evolution in plants and contributes to the expansion of gene families. Polyploidy has been the driving force in this evolution and expansion in plants. Angiosperms are reported to have many polyploidization events along with divergence that has resulted in varying gene families in different plant species. Here, we have identified 21 cyclophilins in *C. sativus*, 26 in *P. vulgaris* and 22 in *V. vinifera*. Species *P. vulgaris* and *V. vinifera* has only experienced core eudicot common hexa-ploidy (γ event) around 100 MYA and no recent whole genome duplication while entire Cucurbitaceae family had a tetra polyploidization event shortly after paleo-hexaploidy 90-102 MYA [86]. Both *A. thaliana* and *G. max* have experienced two additional polyploidization events, apart from the γ event. Specifically, in *A. thaliana*, these events occurred approximately 65–100 million years ago (α event) and 180 million years ago (β event), while in *G. max*, they occurred around 59 million years ago and 13 million years ago. These polyploidization events likely contributed to the expansion of the cyclophilin gene family in both species [87,88].

Some cyclophilins in *C. sativus* could have emerged from gene duplication or inter-chromosome rearrangement. All the three plant species showed Ka/Ks >1 denoting genes have gone through purifying selection. Varying number of cyclophilins in *P. vulgaris* and *V. vinifera* showed chromosomal level duplication or deletion [89,90]. The lowest divergence time between the gene pair *GsvCYP17-GsvCYP14*, compared to other gene pairs, indicates a probable recent divergence in *V. vinifera*.

Cyclophilin are also crucial in plant–microbe interactions. Recent studies have provided insights on how cyclophilins offer resistance against fungus like *Magnaporthe oryzae*, *B. cinerea*, *Cryphonectria parasitica*, and *Puccinia triticina* [91]. Cyclophilins hold high affinity for cyclosporin A, an immunosuppressive drug known for its antifungal properties [92]. Expression profile of cyclophilins from *C. sativus* was analyzed from publicly available data from cucurbit genome database (CuGenDB). *CucCYP13* is highly expressed in roots and has highly similarity to *GmCYP1* (97.7%) from *G .max*. Interestingly, *GmCYP1*conferres resistance against *P. sojae, an oomycete that causes root and stem rot in soybean. GmCYP1 interactes with effector Avr3b* which has PPIase-dependent enzymatic activity. *Avr3b* triggeres hypersensitive response and contributes in virulence against *P. sojae* strain P6497 [50,93]. Studies on *CucCYP13* can be further conducted to study its role in biotic stress against *P. sojae*.

Cyclophilins play an important role in abiotic stress tolerance. The gene up-regulation of cyclophilins provides resistance against stress in *M. domestica* in response to abiotic stress [25]. Eight cyclophilins were responsive to salt stress and ten cyclophilins to drought stress by increasing expression. *MdCYP16* showed upregulated response to both salt and drought indicating resistance to abiotic stresses [25]. Apart from this, cyclophilins from *Cucumis* family are believed to have an influence in biosynthesis of volatile compound resulting in fragrant aroma of melon. *Cucumis melo* shares consensus chloroplast simple sequence repeats in common with *C. sativus* and has recent divergence history [94,95]. Recent study indicated that overexpression of *GmCYP2* from *G. max* confers salt tolerance by regulating photosynthetic and ionic homeostasis [96].

Cyclophilins have also been associated in improving biotic stress tolerance. Expression data of *GsvCYs* suggest a potential role of cyclophilins in the gray mold fungus *B. cinerea* as indicated by the down regulation of *VIT_211s0118g00810 (GsvCYP20)* and *VIT_201s0146g00110 (GsvCYP15)*. Viaud *et al.* [97] demonstrated that mutants deficient in the *CyP1* gene exhibited decreased formation of appressoria and was unable to penetrate the plant cuticle effectively. Restoring the wild-type *CyP1* gene in the mutant strain reinstated virulence, confirming the crucial role of *CyP1* as a virulence determinant in *Magnaporthe grisea* [97]. A similar pattern was also observed by Sun *et al.* [98] where deletion of the *BcCyp2* gene in *B. cinerea* significantly impaired the fungus's ability to infect its host plants, including tomato and *Arabidopsis*. Mutants lacking the *BcCyp2* gene exhibited defects in infection-related development, such as reduced conidiation, decreased appressorium formation, and impaired penetration of plant tissues. Complementation of the mutant strain with the wild-type *BcCyp2* gene restored virulence, conclusively confirming the role of *BcCyp2* as a virulence factor in *B. cinerea* [98].

Similarly, isolation of 475 bp long cyclophilin from *Gossypium arboretum* has also proven to have links with epicuticular wax production in cotton. This wax loads provided tolerance against leaf curl virus in cotton and transmission via whitefly provides a new and important aspect of cyclophilin in plant defense mechanism [99]. Overexpression of cyclophilins from *Panax ginseng* reduces spore formation of *Phytophthora cactorum*, enhances proline synthesis in salt stress conditions and slows chlorophyll level to combat saline stress in *A. thaliana* [100].

Future studies on stress responsive expression, transcriptomics and proteomics can provide more significant role of reported cyclophilins in *C. sativus, P. vulgaris* and *V. vinifera* against various abiotic and biotic stress tolerance. Variations in cyclophilins can also be utilized as molecular marker to produce stress tolerant cultivars.

6. CONCLUSION

Cyclophilins are widespread proteins present in various organisms, spanning a wide range from bacteria and animals to plants. Genome-wide analysis and identification of cyclophilins from few plants revealed their varying numbers in genome but their role remained ambiguous. We have conducted genome-wide search and identification of cyclophilins for the first time in three plant species *C. sativus*, *P. vulgaris* and *V. vinifera* which revealed presence of 21 cyclophilins in *C. sativus*, 26 in *P. vulgaris* and 22 in *V. vinifera*. Subsequently, a detailed analysis on chromosome location, domain, sub-cellular localization, gene structure, genome duplication, phylogeny, orthologous studies and expression analysis provided key insight on the evolution of cyclophilins in these three plant species. Results revealed that majority of cyclophilins in *C. sativus*, *P. vulgaris* and *V. vinifera* are single-domain containing cyclophilins with localization in cytosol. Additionally, evolutionary studies of these cyclophilins with previously reported cyclophilins from *Arabidopsis* suggested potential role of *CucCYP2, PhvCYP22* and *GsvCYP5* cyclophilins in organogenesis, meristem development and conferring immunity against oomycete. Leveraging the knowledge generated from present study combined with *in-silico* expression studies under stress conditions can be harnessed in advance crop breeding techniques and generation of superior varieties to combat global stress challenge and food security. This study provides comprehensive information for further research on cyclophilins from *C. sativus*, *P. vulgaris* and *V. vinifera.*

7. LIST OF ABBREVIATIONS

CucCYP, *Cucumis sativus* cyclophilin; CYP, Cyclophilin; *GsvCYP, Vitis vinifera* cyclophilin; MD, Multiple domain; *PhvCYP*, *Phaseolus vulgaris* cyclophilin; SD, Single domain.

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9. CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

10. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

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12. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

13. DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

14. PUBLISHER'S NOTE

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15. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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SUPPLEMENTARY MATERIAL

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