Tracing the origin, evolutionary history, and biological functions of CKI genes: a focus on *Gossypium* spp

Yaoyao Li

National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University

Yanlong Li

National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University

Yuanyuan Chen

National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University

Maojun Wang

National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University

Xianlong Zhang

National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University

Longfu Zhu

National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University

Ling Min (✉ lingmin@mail.hzau.edu.cn)

Huazhong Agriculture University <https://orcid.org/0000-0003-4278-0626>

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Abstract

Background: Casein kinase I (CKI) is a kind of serine/threonine protein kinase highly conserved in plants and animals. Although molecular function of individual member of CKI family has been investigated in *Arabidopsis*, little is known about their origin and evolutionary history in the plant kingdom.

Results: In this study, seven representative plant species (with a focus on cotton) are applied to study gene family evolution and characterize the origin of *CKI* genes. Three important insights were gained: (i) the ancestral *CKI* genes were traced back to 250 million years ago; the family expansion occurred in different plant species through independent genome duplication events; (ii) the *CKI* genes were classified into two types, on the basis of their structural characteristics; (iii) expression profile analysis revealed that cotton *CKI* genes had various expression patterns in different tissues and exhibited inducible expression in response to photoperiod (circadian clock), light signal and heat stress during cotton anther development.

Conclusion: This study provides genome-wide insights into the evolutionary history of cotton *CKI* genes and lays a foundation for further investigation of their roles in specific developmental processes and/or environmental stress conditions.

Background

Casein kinase I (CKI) is a serine/threonine specific protein kinase, highly conserved in plant and animal species [1–3]. In mammals, structural analysis of the CKI proteins had showed that the kinase domains were highly conserved, but the length and primary structure of their N-terminal and C-terminal non-catalytic domains were highly diverse [3]. Compared to other protein kinases, CKI proteins contain four short conserved peptides: HIPXR, LPWQGLKA, EXSRDD, and LLGPSLEDLF [4].

Due to the broad distribution of the *CKI* gene family members and their substrates, *CKI* genes have been found to participate in many biological activities. In yeast, CKI plays important roles in gene expression regulation, vesicle trafficking, cell morphogenesis, cell cycle, cell colonization, and DNA repair [5–8]. In mammals, CKI is involved in cell proliferation, cytokine production, and the transduction and regulation of a variety of signaling pathways related to apoptosis and tumor production and development, such as Wnt and Hedgehog [9–11]. In plants, it has been reported that several *CKI* genes have important functions in the regulation of a variety of biological processes related to growth and development, as well as various responses to environmental stimuli [12]. In rice, abscisic acid (ABA) and brassinolide (BR) caused up-regulation of *OsCKI1*, while *OsCKI1* deficiency caused shorter primary roots and fewer lateral and adventitious roots [2]. *Early flowering 1 (EL1)/Heading date 16 (Hd16)*, a CKI, regulated the rice flowering pathway by enhancing the photoperiod response caused by the phosphorylation of DELLA protein SLR1 and Ghd7 [13–14]. In *Arabidopsis*, the casein kinase I-like (AtCKL) *AtCKL2* and *AtCKL3*, are necessary for ABA to regulate seed germination, root growth and gene expression [15–17], and overexpression of either *AtCK1.3* or *AtCK1.4* delayed flowering under long-day conditions in *Arabidopsis* [18]. In addition, MLK3, a

Plant-specific Casein Kinase 1, is critical for maintaining proper flowering time [19]. In cotton, *GhCKI* was speculated to regulate not only tapetal programmed cell death (PCD) and anther dehiscence [20], but also somatic embryogenesis by modulating auxin homeostasis [21]. Since the functions of most *CKI* genes in higher plants are largely unknown, their identification and characterization are particularly relevant for understanding their role and to potentially employ them as genetic resources for improving crop plant defense against biotic and abiotic stresses. In cotton, no genome-wide characterization of the *CKI* gene family has been reported so far. However, the recently published cotton species genomic information is a solid foundation for characterizing *CKI* genes at a genome-wide level. Here we have investigated several fundamental questions regarding the *CKI* gene family evolution: (i) the evolutionary expansion of the *CKI* gene family; (ii) the diversity of gene structure and domain architecture; and (iii) expression profiles of the *CKI* genes under different conditions. In summary, we retraced the evolution of the *CKI* genes to better understand their essential elements and thus be able to exploit this knowledge for plant growth and development.

Results

Identification and Classification of the Casein Kinase I(CKI) in Gossypium

To extract CKI sequences, BLASTP searches of the complete genomes of three sequenced cotton species (*G. raimondii*, *G. arboreum*, and *G. hirsutum* acc. TM-1) [22–24] were implemented using the homology-based method with 13 *Arabidopsis* CASEIN KINASE 1-LIKE (CKL) and *HEADING DATE 16* (*Hd16*, a CKI protein) encodes as queries. As a result, 31, 30, and 61 CKI members were identified in *G. raimondii* (D genome), *G. arboreum* (A genome) and *G. hirsutum* (AD genome), respectively (Table 1). We named the 31 *G. raimondii* CKI genes *GrCKI1* to *GrCKI31*. Considering *G. hirsutum* is an allotetraploid cotton species which contains A and D genomes, we named the 61 putative *G. hirsutum* CKI genes as *GhCKI1A/D* to *GhCKI31A/D*, following the same nomenclature system applied to *G. raimondii* CKI genes. To get a better understanding of the phylogenetic relationships between CKIs, an unrooted phylogenetic tree was generated using the CKI protein sequences from *G. raimondii*, *G. arboreum* and *G. hirsutum* acc. TM-1 (Figure 1). The CKIs were classified into type I and II. A homolog of most *CKI* genes can be found once in the diploid *G. raimondii*, once in *G. arboreum*, and in two copies in the tetraploid *G. hirsutum* acc. TM-1 (Table 1). The inconsistent presence of single homologous genes among these cotton species might result from gene gains or losses during their individual evolution process or from assembly errors in partial chromosomal regions, which need to be further confirmed.

Tetraploid cotton species such as *G. hirsutum* L. are thought to have formed by a polyploidization event that occurred approximately 1–2 million years ago, which involved D and A genome species (Wendel, 1989) [25]. The sequenced D-progenitor genome (*G. raimondii*) has been well assembled and annotated and its co-linearity with *G. hirsutum* acc. TM-1 genome resulted obvious [22, 24]. Consequently, *G. raimondii* genome information was used to characterize the *CKI* family genes. The phylogenetic tree was independently constructed using the MEGA 6 software (Figure S1). All *G. raimondii* CKI proteins fell into two distinct groups, which was consistent with the results of Figure 1. The type I CKI proteins were further

divided into three subclasses: Group A, B, and C; the type II CKI proteins were classified into two subclasses: Group D and Group E (Figure S1 and Table S1).

Gene Structure, Conserved Motifs and Domains of CKI Genes in G. raimondii

The gene structures were related to their roles. Hence, the gene structure of CKI proteins in *G. raimondii* were investigated. The putative conserved domains of GrCKI proteins were found using the online program Conserved Domain Search Service (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). The type I GrCKI proteins were highly conserved within their kinase domains, but differed significantly in the length and primary structure of their N-terminal (8–53 aa) and C-terminal domains (176 aa up to more than 206 aa) (Figure 2a, Figure S2a). The gene structure of type I GrCKI proteins was consistent with previous reports [3–4, 26]. The type II GrCKI proteins also presented conserved kinase domains. However, contrary to the type I GrCKI proteins, type II possessed a variable N-terminal (1–159 aa) and conserved C-terminal (278–355 aa) (Figure 2b and Figure S2b). In addition, several short sequences are absolutely conserved among the CKIs and not found in other kinases. To further confirm whether the conserved fragments are present in type I and II GrCKI proteins, sequence alignment was performed. The result showed type I GrCKI proteins possess the four short sequences LLGPSLEDLF, HIPXR, EXSRDD, and LPWQGLKA (Figure 2c). Type II GrCKI proteins contained three of the four conserved sequences (LGPSL, SRRDD, and LPWQG). However, compared with the type I CKI proteins, two specific fragments LGKGGFGQV and HGDVKPEN were present in the type II GrCKI proteins only. Comprehensively, LGPSL, SRRDD, and LPWQG appeared in both type I and type II GrCKI proteins.

The 3D structure of GrCKI proteins was predicted by the software SWISS-MODEL (<https://swissmodel.expasy.org>) [27]. The 3D structure of type I GrCKIs was conservative, the same for all 16 of them (Figure S3). However, 3D structures of type II GrCKIs were variable: only some proteins showed similar structures (GrCKI17, GrCKI18, and GrCKI21; GrCKI24, GrCKI25, and GrCKI29). To obtain more insights into the diversity of motif compositions among GrCKI proteins, the conserved motifs in the C-terminal were further analyzed with the program MEME. As shown in Figure 2d, the conserved motifs 1–8 were identified. Based on the motif composition, GrCKI proteins were divided into five groups (A-E); type I GrCKI proteins were divided into groups A to C; type II GrCKI proteins were classified into groups D and E. Most of the type I GrCKI proteins within the same group shared similar motif compositions while high divergence was observed among different groups, implying that the type I GrCKI members within the same group may perform similar functions and that some motifs may play an important role in the specific functions of the group. For example, all the type I GrCKI except GrCKI5 in Group A possessed motifs 1, 2, 3, 4, and 5, while all members in Group C contained motifs 1 and 4 (Figure 2d). GrCKI22 and GrCKI23, which were type II GrCKI proteins, had short sequences and thus showed no motif. Except GrCKI20 and GrCKI29 which contained seven motifs, the vast majority of type II GrCKI proteins presented similar eight motifs composition. Generally, the consistency of motif compositions of GrCKI proteins with the phylogenetic groups further supported the close evolutionary relationships among GrCKIs, as well as the reliability of our phylogenetic analysis. To get a better understanding of the diversification of type I and type II *GrCKI* genes in *G. raimondii*, the exon/intron organization was analyzed. As expected, most

GrCKI genes within the same group showed very similar exon/intron distribution patterns in terms of exon length and intron number (Figure S4). For example, most type I *GrCKI* genes in groups A, B, and C had thirteen or fourteen exons of similar length, whereas members of type II *GrCKI* genes within Group D and E contained sixteen or seventeen exons, except for *GrCKI22* (*Gorai.013G175600.1*), *GrCKI23* (*Gorai.006G042700.1*) and *GrCKI29* (*Gorai.007G343500.1*), which possessed one, one, and fourteen exons, respectively. Thus, similarly to the exon/intron organization, members belonging to the same group also showed similar motif composition, indicating their functional similarities. And the diverse evolutionary patterns in exon numbers of the *CKI* genes may hint at their functional diversifications in gene expression. These results further supported the classification between type I and type II *GrCKI* genes.

The CKI Gene Family mainly Expanded through Genome Duplication

To examine the evolutionary history of the *CKI* genes, we firstly carried out phylogenetic analysis between the eudicots (*G. raimondii* and *Arabidopsis*) and monocot (rice) (Figure S5). Clearly, the *CKI* genes from eudicots (*G. raimondii* and *Arabidopsis*) and monocot (rice) were present in all subgroups (Table S1), indicating that the appearance of most *CKI* genes in plants precedes the divergence between monocots/eudicots. The phylogenetic analysis also showed that the *CKI* members from different plant species were not evenly distributed in the subgroups. Some *CKI* genes in *Arabidopsis* have two or more counterparts in *G. raimondii*. For example, Group D contained five *G. raimondii* *CKIs* but only one *Arabidopsis* member; Group E contained ten *G. raimondii* *CKIs* but there were only three *Arabidopsis* *CKI* genes. The findings may indicate that the *CKIs* from different plant species underwent differential expansion, though the mechanism is still not understood. Plant evolution is characterized by genome duplication events, which in turn resulted in the expansion of many gene families. Four duplication events have been detected in cotton from the ancient angiosperms development period to the stage of tetraploid cotton species: an ancient whole genome duplication (WGD) very early in angiosperm evolution, one triplication event, the recent WGD event specific for cotton, and the tetraploid event [22, 25, 28]. Previous reports calculated the absolute dates for the large-scale gene duplications using the assumed clock-like rates of synonymous substitution of 2.6×10^{-9} substitutions/synonymous site/year [29]. Hence, based on *Ks* values, we calculated the divergence time of homologous *CKI* genes in *G. raimondii*. The result was compared with the estimated time of WGD, which suggests that there were three *CKI* genes for type I and two for type II. During the genome triplication event shared by flowering plants, one of type II *CKIs* was duplicated into three copies (Figure 3a). After the subsequent ancient triplication event, the three type I *CKI* genes and one of the type II genes in Group D tripled. Interesting, only group A of the type I *CKI* gene showed the triploidization: the rest of the *CKI* only duplicated. It is also possible that they all underwent triploidization but then lost some copies, though it is difficult to determine. However, in all these events type II *CKI* genes in Group E did not even undergo duplication (Figure 3a). *G. raimondii* and *Theobroma cacao* originated from a common ancestor 18–58 million years ago [23]. Because of this, the gene numbers of type I or II *CKI* should match those of a common ancestor of *G. raimondii* and *T. cacao*. The *CKI* gene numbers in *T. cacao* were consistent with the speculation (Figure 3b and 3c; Table S1). Also, each *CKI* gene in *T. cacao* had one or more counterparts in *G. raimondii*. Type I and II *CKI* genes

conformed to the rule. After the species divergence, the two types both underwent the duplication events. Furthermore, because of the tetraploid event, one type II *CKI* gene in group E duplicated and resulted in two different genes, *Gorai.006G042700.1* and *Gorai.013G175600.1*. In short, the *CKI* subgroups showed various degrees and patterns of duplications, probably associated with many known WGD events which greatly expanded the number of genes in seed plants.

Expression Profiles of CKI Genes in Different Tissues from G. hirsutum

Several findings suggested that *CKI* genes are involved in plant development [16, 17, 20]. To further associate the biological functions of *CKI* genes with specific developmental processes in cotton, the expression profiles in different organs/tissues (including roots, stems, leaves, petals, anthers, and 5 DPA [days post anthesis] ovules) were examined by quantitative RT-PCR (qRT-PCR). Because of the high sequence similarity between *GhCKIA* and *GhCKID* cDNAs, we designed one common primer pair for analyzing *CKIA/D* gene expression. After the specificity for each primer pair was verified, suitable qRT-PCR primer pairs for 44 (26 type I *CKI* genes and 18 type II *CKI* genes) of the 61 *CKI* genes (33 type I *CKI* genes and 28 type II *CKI* genes) were obtained (Table S2). As shown in Figure S6, the majority of the type I and type II *CKI* genes exhibited different tissue expression. *GhCKI2A/D*, *GhCKI3A/D*, *GhCKI8A/D*, *GhCKI10A/D*, *GhCKI11A/D*, *GhCKI14A/D*, *GhCKI15A/D*, *GhCKI18A/D*, *GhCKI20A/D*, and *GhCKI27A/D* were constitutively expressed in every tested tissue, implying that these genes may play regulatory roles at multiple developmental stages. In addition, some genes were highly expressed in specific tissues only. For example, the expression levels of *GhCKI1A/D*, *GhCKI5A/D*, *GhCKI9A/D*, *GhCKI12A/D*, *GhCKI13A/D*, *GhCKI19A/D*, *GhCKI26A/D*, *GhCKI28A/D*, *GhCKI29A/D*, *GhCKI30A/D*, and *GhCKI31A/D* were very high in anthers. The transcripts of some other genes, such as *GhCKI4A/D*, *GhCKI14A/D*, *GhCKI20A/D*, and *GhCKI27A/D*, were preferentially expressed in leaves. These results provide additional insight into their roles during different growth and development processes in cotton.

CKI Genes Respond to High Temperature during Cotton Anther Development Process

CKI genes exhibited the highest expression in cotton anthers, except for *GhCKI14A/D* and *GhCKI27A/D* (Figure S6). Our previous studies showed that one member of the *CKI* gene family, *GhCKI* (*Gh_A07G0121/GhCKI11A*) was induced in H05 (the high temperature (HT)-sensitive line) anthers, but not in 84021 (the HT-tolerant line) anthers under HT condition [20]. Genome-wide analyses of *G. hirsutum* *CKI* genes in response to HT during anthers development may lay a foundation for further understanding the mechanisms involved in HT tolerance or HT sensitivity. In the present study, a heat-map representing expression profiles was produced using the transcriptomic data which contained the three different anther development stages (TS, tetrad stage; TDS, tapetal degradation stage; ADS, anther dehiscence stage) of 84021 and H05 under HT and NT [30]. It was observed that 34 of 61 *GhCKI* genes (19 from type I and 15 from type II) were differently expressed in 84021 and H05 under HT and NT, even after filtering with an absolute threshold $\log_2\text{ratio} \geq 1$ (Figure 4a). We found that most genes were more expressed in H05 after HT exposure, such as type I genes (*GhCKI10* and *GhCKI11*) and type II genes (*GhCKI20* and *GhCKI27*) (Figure 4a). To verify the result, the qRT-PCR experiments were also performed (Figure 4b), but no

obvious differences were observed after HT treatment in anthers, consistently with the RNA-seq data. These results indicated that HT influenced the expression of both type I and type II *GhCKI* genes during anther development.

Circadian Rhythm and Light Signal Regulation of GhCKI Gene Expression

To determine whether the expression of cotton *CKI* genes is regulated by photoperiod (circadian clock), the transcription level of *G. hirsutum* *CKI* genes under different diurnal conditions were investigated. Thirty-six *G. hirsutum* *CKI* genes were expressed at a sufficient level to evaluate their circadian regulation (Figure 5). Twelve type I genes (*GhCKI1A/D*, *GhCKI2A/D*, *GhCKI3A/D*, *GhCKI8A/D*, *GhCKI13A/D*, and *GhCKI14A/D*) and ten type II genes (*GhCKI19A/D*, *GhCKI20A/D*, *GhCKI27A/D*, *GhCKI28A/D*, and *GhCKI30A/D*) showed a high expression peak at 12:00 under long-day (LD) conditions. The expression peaks of eight type I genes (*GhCKI4A/D*, *GhCKI11A/D*, *GhCKI12A/D*, and *GhCKI15A/D*) and six type II genes (*GhCKI18A/D*, *GhCKI26A/D*, and *GhCKI31A/D*) were at 16:00 under long-day (LD) conditions. Under short-day (SD) conditions, all the 36 *CKI* genes showed a high expression peak at 8:00, and the expression of *GhCKI* genes increased gradually in the dark and decreased in the light. However, the expression of the 36 *GhCKI* genes showed different results under LD conditions, the expression was higher in the light than in the dark. These results identified a set of clock-regulated *GhCKI* genes showing different phases of expression and provided additional insight to understand the underlying mechanisms of modulation in light signaling.

To further investigate whether CKIs are involved in light signaling, the expression of *G. raimondii* (Figure S7) and *G. hirsutum* (Figure 6) *CKI* genes in cotyledon under light and dark conditions was examined by qRT-PCR. The expression of most *CKI* genes both in *G. raimondii* and *G. hirsutum* was up-regulated under light, except for *GhCKI19A/D* (Figure 6), *GrCKI6*, *GrCKI14*, *GrCKI25*, and *GrCKI26* (Figure S7). However, there was only a slight difference between the two types of *CKI* genes. The results indicated both type I and type II might be involved in plant light signal.

Discussion

Structural Characteristics of G. raimondii CKI genes

In animals, certain characteristics of *CKI* that certainly influence its activity have been identified at the protein level: structure-related regulation, subcellular localization, interaction with other proteins, and post-translational modifications [31]. As a member of the superfamily of serine/threonine-specific kinases, the function of phosphorylation is a priority to focus on. However, the characteristics of phosphorylation and sequences of plant *CKI* genes were not systematically analyzed in this study. Instead, we systematically identified *CKI* genes in the eudicots (*Arabidopsis thaliana*, *Theobroma cacao*, *G. raimondii*, *G. arboreum*, and *G. hirsutum* acc. TM-1) and in one monocot (*Oryza sativa*) (Figure 1, Figure 3, and Figure S5). Based on sequence comparison and phylogenetic analysis, plant *CKI* genes were first divided into two groups, namely type I and type II *CKI* genes (Figure 1, Figure S1, and Figure S5). Motif compositions, 3D protein

structure, and exon/intron distribution patterns in terms of exon number in *G. raimondii* agreed with our hypothesis (Figure 2, Figure S3 and Figure S4).

Regarding the functional characteristics of the N-terminal and conserved C-terminal regions, previous reports in mammals showed that CKI presents a β -sheets N-terminal lobe and mainly a α -helical C-terminal lobe, which are connected by a hinge region forming a catalytic cleft for substrate and ATP binding [32–33]. Within the C-terminal region, a specific phosphate moiety binding motif has been identified allowing the recognition of phosphorylated protein substrates, which is believed to be involved in *CKI* regulatory interactions. These reports showed that the N-terminal and C-terminal lobes play an important role in substrate phosphorylation. In this study, type I CKI proteins resulted highly conserved within their kinase domains, but significantly differing in the length and primary structure of their N-terminal and C-terminal domains (Figure 2a), which is consistent with previous reports [3, 4, 26, 31, 34]. Interestingly, contrary to type I, type II CKI proteins also presented conserved kinase domains and possessed variable N-terminal and conserved C-terminal regions (Figure 2b). Furthermore, the 3D structures of type I CKI proteins were always identical, while type II were inconsistent (Figure S3). Based on the characteristic of plant *CKI* genes, we hypothesis the type I and type II *CKI* genes possess different functions on account of the difference in structures, especially regarding the function of phosphorylation. Functional studies will be needed in the future to further explore this difference.

Evolutionary Expansion of the G. raimondii CKI Gene Family

The evolution of *Gossypium CKI* genes was characterized by a history of multiple gene duplications at different stages as in the four duplication events (an ancient angiosperm WGD event, one triplication event, a specific and recent cotton WGD event, and the tetraploid event) [22, 25, 28]. The phylogeny obtained in this study suggested that *Gossypium CKI* genes may be traced back to 250 million years ago, before the ancestral angiosperm WGD event (Figure 3a). At this node, duplication created five ancestral genes (three type I *CKI* genes and two type II *CKI* genes) (Figure 3a). Thereafter, with the divergence of angiosperms, independent duplications occurred in different lineages (such as eudicots [*G. raimondii* and *Arabidopsis*] and monocots [rice]), generating specific duplication events. The five ancestral genes duplicated in the four events to reach the current *CKI* gene numbers. However, all the 31 members of the *CKI* gene family in *G. raimondii* did not equally derived from the five ancestral genes. Two of the five ancestral genes (Group A and Group E) duplicated vigorously, creating 19 members of the *CKI* gene family in *G. raimondii*. At another key node, the rapid amplification of *CKI* genes was due to a whole-genome duplication (WGD) event in the *Gossypium* genus after its separation from *T. cacao* (Figure 3b) [23]. In addition, the *CKI* members from eudicots (*G. raimondii* and *Arabidopsis*) and monocot (rice) were not equally distributed among the subgroups (Figure S5), which indicated that the *CKI* genes from different plant species went through differential expansion. Therefore, we speculated that the origin of the *CKI* genes was very ancient and the expansion might have occurred in different plant species and arose from independent duplications. Besides, the gene families which possess an ancient evolutionary history have important functions, such as *MADS-box* genes[35], *Auxin response factors* [36], or *ASYMMETRIC LEAVES2-LIKE/LOB-DOMAIN* transcription factors[37]. Thus, we speculate that *CKI* genes

may play important roles in plant development and be involved in very diverse biological roles to adapted to environmental stress.

Expression Patterns of the CKI Genes Suggest Functional Diversification

To date, although the functions of only one *CKI* gene have been characterized in tetraploid cotton [20], no systematic functional analysis of expression patterns for different groups of the tetraploid cotton *CKI* gene family was done. In this study, we demonstrated that *CKI* genes displayed expression divergence in roots, leaves, and anthers (Figure S6). For instance, *GhCKI2A/D*, *GhCKI3A/D*, *GhCKI8A/D*, *GhCKI10A/D*, *GhCKI11A/D*, *GhCKI15A/D*, and *GhCKI18A/D* were constitutively expressed in every tested tissue (such as roots), implying that these genes may play regulatory roles at multiple development stages. In *Arabidopsis*, *AtCKL2* and *AtCKL3* were required for ABA regulation of seed germination, root growth, and gene expression [16, 17]. In rice, *OsCKI1* deficiency resulted in shorter primary roots and fewer lateral and adventitious roots [2]. Similar expression patterns suggest that these preferentially or specifically expressed *G. hirsutum* *CKI* genes might play important roles in root formation and development. Among these 61 identified *CKI* genes, *GhCKI* (namely *GhCKI11A*) is the only one that was speculated to regulate tapetal programmed cell death and anther dehiscence in cotton [20]. Under HT condition, *AtCKL2* and *AtCKL7* were expressed in the tapetum, in anther microspores at stages 9–12, and in anther pollen grains at stages 13–14, which imply *AtCKL2* and *AtCKL7* may be key regulators of tapetal development under HT [38]. Apart from *GhCKI11A* (formerly *GhCKI*), the *GhCKI1A/D*, *GhCKI5A/D*, *GhCKI9A/D*, *GhCKI12A/D*, *GhCKI13A/D*, *GhCKI19A/D*, *GhCKI26A/D*, *GhCKI28A/D*, *GhCKI29A/D*, *GhCKI30A/D*, and *GhCKI31A/D* genes were exclusively expressed in anther. This finding suggested that *CKI* genes were components of a complex transcriptional network regulating anther development.

Previous studies had showed that HT stress causes premature programmed cell of the tapetum, resulting in male sterility and catastrophic loss of crop production [30, 39–41]. However, the mechanism underlying successful male reproductive development under HT remains largely unknown. Except for the *GhCKI11A* (formerly *GhCKI*) gene, which was reported to regulate tapetum development under HT [20], no other *CKI* genes have demonstrated to participate in the regulation of anther development. This study, using the transcriptomic data of cotton anther in response to HT, found that 34 *CKI* genes changed the expression at different anther development stages under HT (Figure 4). These are now further *CKI* genes that participate in regulating stamen development under HT and that will be employed in future works.

Circadian clocks are molecular timekeepers. Many plants use photoperiod (circadian clock) information to prepare for daily environmental changes and increase their fitness in changing environments [42]. The circadian clocks share similar network architecture of feedback loops that form by transcriptional and post-translational regulation among the clock components [43, 44]. Phosphorylation is a common post-translational modification and is an integral part of circadian regulation [44]. Another function of *CKI* is to take a series of biological process via phosphorylating various substrates [3]. In rice, Early flowering 1 (EL1)/ Heading date 16 (Hd16), a CKI protein, regulated the rice flowering pathway by enhancing the photoperiod response caused by the phosphorylation of DELLA protein SLR1 and Ghd7 [13, 14]. In

Arabidopsis, both *CK1.3* and *CK1.4* showed a high expression peak before dark under LD conditions, which indicated that the expression of *CK1.3* and *CK1.4* was strictly regulated by a circadian rhythm; overexpression of either *CK1.3* or *CK1.4* delayed flowering under LD conditions [18]. However, in cotton, it is not clear that the systematic function of the tetraploid cotton *CKI* gene family is involved in photoperiod (circadian clock). In our present study, the expression of *CKI* genes is circadian. Under long-day (LD) conditions, the expression in the light is higher than in the dark. Under short-day (SD) conditions, the expression of all the 36 *CKI* genes showed a high expression in dark. (Figure 5). The results indicated that the expression of *CKI* was strictly regulated by a circadian rhythm. According to these evidences, we propose that at least some *CKI* genes are components of a diurnal rhythm complex network. The functions of these *GhCKI* genes in regulating the circadian clock on the diurnal rhythms will be further characterized in future works.

Plant reception of light signal and its subsequent reaction is important for both growth and development. In *Arabidopsis*, *CK1* genes are involved in the light signaling pathway mainly through phosphorylation; casein kinase 1 proteins *CK1.3* and *CK1.4* phosphorylate CRYPTOCHROME 2 (CRY2) which is the blue-light receptor, to regulate blue light signaling [18]. Our results showed that the expression of most type I and type II *CKI* genes was upregulated under light conditions compared to dark (Figure 6, Figure S7). We thus believe that both type I and type II *CKI* gene are involved in the response to light signals. But we do not know how these genes respond to light signals in cotton, and whether they participate in light signals by phosphorylating photoreceptors CRY2 or light signal components, including HY5, HF5, HFR1, COP1, and PIF1, which in *Arabidopsis* are phosphorylated by casein kinase 2 proteins [45]. We also do not know whether the pathways and capabilities of these two types of genes are the same.

Conclusion

Our study offers a promising landscape to unravel the underlying structural characteristics and evolutionary expansion of *CKI* genes and further elucidate their expression patterns in different tissues and various conditions; this is crucial to better understand their characteristics and to elucidate their precise functions in regulating various facets of plant growth and development.

Methods

Database search and identification of CKI genes

The genomic database of three cotton species *G. raimondii*, *G. arboreum* and *G. hirsutum* acc. TM-1 were downloaded from <https://www.cottongen.org/>. The protein database of *A. thaliana*, *O. sativa* and *T. cacao* were obtained from The *Arabidopsis* Information Resource (TAIR: <http://www.arabidopsis.org/>) and <http://www.phytozome.net/>, respectively. BLAST searches were performed using 13 *CKL* genes within the *Arabidopsis* casein kinase 1-like gene family and *heading date 16 (Hd16)* encoding a casein kinase I protein as queries. The candidate *CKI* proteins were further aligned to remove redundant

sequences. Subsequently, the four specific fragments (LLGPSLEDLF, HIPXR, EXSRDD, and LPWQGLKA) were used to further confirm the presence of the CKI protein sequences.

Gene structure and phylogenetic analysis

Sequence alignments were generated with CLUSTALX [46], and the alignments among CKIs were adjusted before the tree was constructed. The online Gene Structure Display Server 2.0 [47] (<http://gsds.cbi.pku.edu.cn/>) was used to identify the exon/intron organization. *G. raimondii* CKI protein sequences were submitted to online MEME (Multiple EM for Motif Elicitation) program [48] (<http://meme-suite.org/tools/meme>) to identify conserved protein motifs. The 3D structure of GrCKI proteins was predicted by SWISS-MODEL (<https://swissmodel.expasy.org>) [27]. Phylogenetic trees were constructed by the Maximum likelihood method in MEGA 6 [49].

Plant materials, growth conditions and stress treatments

Four cotton accessions used in this experiment are provided by Huazhong Agricultural University. For the expression profiles in different organs/tissues, various samples of *G. hirsutum* cv. YZ1 were extracted from roots, stems, leaves, petal, anther, and ovules excised carefully from bolls five DPA. To analysis the expression patterns of *Gossypium hirsutum* CKI genes at different anther developmental stages under NT and HT conditions, two cotton (*Gossypium hirsutum*) lines with obvious differences in performance under HT were employed in this study: 84021, which is tolerant to HT, and H05, which is sensitive to HT [30]. The plants were grown in a greenhouse at 28 °C to 35 °C/20 °C to 28 °C day/night as a normal condition. During HT treatment, the plants were cultivated at 35 °C to 39 °C/29 °C to 31 °C day/night in a greenhouse. When the plants were treated with HT for 7 d, buds of different lengths (6–7, 9–14, and more than 24 mm) were collected under HT and NT. The anthers were excised and immediately frozen in liquid nitrogen; they were then stored at –80 °C until use. The transcriptome profiles of CKI genes were isolated from the RNA-seq data [30]. In order to analyze the diurnal regulation of *G. hirsutum* CKIs gene expression, *G. hirsutum* cv. YZ1 was grown under short-day conditions (8 h light/16 h dark) and long-day conditions (16 h light/8 h dark), respectively. The leaves of four leaves period cotton plants were harvested. We also collected cotyledons under light and dark conditions, that from *G. raimondii* and *G. hirsutum* cv. YZ1 respectively.

qRT-PCR

Various plant samples were collected and immediately frozen in liquid nitrogen and stored at –80°C. total RNA was isolated from the collected cotton tissues using previously published methods [50]. First-strand cDNA was generated from 3 µg total RNA using the M-MLV reverse transcriptase (Invitrogen). The cDNA was used as a template for qRT-PCR. The qRT-PCR reactions were performed using the 7500 Real-Time PCR System (Applied Biosystems). The primers used in this study were listed in Table S2, Table S3.

Abbreviations

CKI, Casein kinase I; ABA, abscisic acid; BR, brassinolide; EL1, Early flowering 1; Hd16, Heading data 16; PCD: programmed cell death; MLK3: Mut9-like kinases 3; CKL, Casein Kinase 1-like; Gr, *G. raimondii*; Gh, *G. hirsutum*; WGD, whole genome duplication; DPA, days post anthesis; HT, high temperature; NT, normal temperature; TS, tetrad stage; TDS, tapetal degradation stage; ADS, anther dehiscence stage; LD, long-day; SD, short-day;

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interest

The authors declare no conflicts of interest.

Acknowledgments

Not applicable

Author contributions

Y.L. carried out the experiment with Y.L. and wrote the main manuscript text, Y.C. and M.W. analysed the data, L.M, X.Z and L.Z. designed and supervised the research and revised the manuscript. All authors reviewed the manuscript.

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Availability of data and materials

The data that support the results are included within the article and its additional files. Other relevant materials are available from the corresponding authors on reasonable request.

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Tables

Due to technical limitations, Table 1 is provided in the Supplementary Files section.

Figures

Figure 1

Phylogenetic tree of the Casein Kinase I (CKI) in *Gossypium* spp. The phylogenetic tree was constructed using 122 cotton CKI protein sequences from *G. hirsutum* (61), *G. arboreum* (30) and *G. raimondii* (31) with the Maximum likelihood method in MEGA 6. The three different symbols represent the three cotton species: green for *G. arboreum*, red for *G. raimondii*, and blue for *G. hirsutum*. The GenIDs of CKI genes from *G. hirsutum*, *G. arboreum*, and *G. raimondii* were listed in Table 1.

Figure 2

Gene structure and protein motif of the Casein Kinase I (CKI) in *G. raimondii*. (a) and (b) Multiple sequence alignments of type I and type II GrCKI proteins, respectively, in *G. raimondii* using ClustalX (c) The specific fragments exist in type I and type II GrCKI proteins. The red font indicates the specific fragments from type II GrCKI proteins. (d) Motif composition of GrCKI proteins. Conserved motifs in the GrCKI proteins are indicated by colored boxes. The GenIDs of type I and type II GrCKI genes were listed in Table S1

Figure 3

Evolutionary expansion of the *G. raimondii* CKI gene family and phylogenetic analysis of CKIs in *G. raimondii* and *T. cacao*. (a) The putative evolutionary history of the GrCKI genes. The four colored boxes represent the four duplication events. (b) Phylogenetic analysis of type I and type II CKI genes in *G. raimondii* and *T. cacao*, respectively. (c) Number of CKI genes in *G. raimondii* and *T. cacao* in different groups. The Gene IDs of type I and type II CKI genes from *G. raimondii* and *T. cacao* were listed in Table S1.

Figure 4

Expression profiles of *G. hirsutum* CKI genes in anthers at different development stages under NT and HT conditions. (a-b) Expression patterns of *G. hirsutum* CKI genes in 84021 and H05 at different anther developmental stages under NT and HT conditions from RNAseq (a) and Quantitative RT-PCR experiment (b). 8N and 8H refer to 84021 (the HT-tolerant line) under NT and HT conditions, respectively; HN and HH refer to H05 (the HT-sensitive line) under NT and HT conditions, respectively; TS: tetrads stage; TDS: tapetal degradation stage; FPKM: fragments per kb of transcript per million fragments mapped. The RNAseq data of the expression profiles was cited from Min et al. [30]. Error bars indicate $\pm$ standard deviations of triplicate experiments. Asterisks indicate statistically significant differences (* $P < 0.05$, ** $P < 0.01$) by Student's t-test. The GhUB7 gene was used as the reference gene to normalize the total amount of cDNA in each reaction.

Figure 5

Expression profiles of CKI genes under different diurnal conditions. (a) and (b) Expression profiles of the type I and type II CKI genes in *G. hirsutum* under different diurnal conditions, respectively. Diurnal changes in transcript levels of 36 *G. hirsutum* CKI genes under short-day (SD) and long-day (LD) conditions. SD, short day (8 h light); LD, long day (16 h light); Black boxes denote dark, while the solid red boxes show light. Values are means $\pm$ standard deviations. Asterisks indicate statistically significant differences (* $P < 0.05$, ** $P < 0.01$) by Student's *t* test. The *Gh18S* gene was used as the reference gene to normalize the total amount of cDNA in each reaction.

(a)

Type I

(b)

Type II

Figure 6

Expression profiles of GhCKI genes under dark and light conditions. (a) and (b) Expression profiles of the type I GhCKI genes (26 genes) and type II GhCKI genes (18 genes) in upland cotton cotyledons under dark and light conditions, respectively. Error bars indicate $\pm$ standard deviations of triplicate experiments. Asterisks indicate statistically significant differences (*P<0.05, **P<0.01) by Student's t-test. The GhUB7 gene was used as the reference gene to normalize the total amount of cDNA in each reaction.

Supplementary Files

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