Plant Mol Biol Rep (2014) 32:209–218
DOI 10.1007/s11105-013-0637-5

ORIGINAL PAPER

Identification and Expression Analysis of D-type Cyclin Genes in Early Developing Fruit of Cucumber (*Cucumis sativus* L.)

Li Cui · Ji Li · Tinglin Zhang · Qinwei Guo · Jian Xu · Qunfeng Lou · Jinfeng Chen

Published online: 30 August 2013
© Springer Science+Business Media New York 2013

Abstract Plant D-type cyclin genes (*CYCDs*) are important regulators of cell division. However, little is known on their participation during the early developmental stage of cucumber fruit. In this study, cucumber *CYCD* genes were identified and characterized. The expression levels of these genes during early fruit development were assessed from 0 to 8 days after anthesis (DAA). The results revealed the presence of 13 different *CYCD* genes, which were named according to identity percentages of the corresponding orthologs in *Arabidopsis thaliana* and poplar. The genomic organization of each subgroup *CYCD* was similar to their orthologs in *A. thaliana* and poplar. The expression levels of *CsCYCD* genes were analyzed in cucumber fruits under different treatments including natural parthenocarpic fruit, pollinated fruit, and *N*-(2-chloro-4-pyidyl)-*N′*-phenyurea (CPPU)-induced parthenocarpic fruit. The highest expression levels of most *CsCYCDs* genes were at four DAA in natural parthenocarpic and pollinated fruits. Interestingly, the expression patterns of 8 of 13 *CsCYCD* genes in natural parthenocarpic fruit were similar to those in pollinated fruit, but different from those in CPPU-induced parthenocarpic fruit. Collectively, the results of this study provide insights on the *CYCDs* involved in cucumber parthenocarpic fruit development.

Keywords Cucumber (*Cucumis sativus* L.) · CPPU · D-type cyclin · Fruit development · Parthenocarpy

Electronic supplementary material The online version of this article (doi:10.1007/s11105-013-0637-5) contains supplementary material, which is available to authorized users.

L. Cui · J. Li · T. Zhang · Q. Guo · J. Xu · Q. Lou · J. Chen (✉)
State Key Laboratory of Crop Genetics and Germplasm Enhancement, Nanjing Agricultural University, Nanjing 210095, China
e-mail: jfchen@njau.edu.cn

Introduction

The plant cell cycle plays a crucial role in growth and development (Dewitte et al. 2003). The plant cell cycle is regulated at two points, the G1/S and G2/M phases, and is controlled by cyclin-dependent kinases (CDKs), whose activities are determined by different types of cyclins (A-, B-, and D-types) (Morgan 1997). The D-type cyclin (CYCD) controls both the commitment of cells to cell division and the cellular responses to extracellular signals during the G1 phase (Soni et al. 1995; Riou-Khamlichi et al. 1999). Mitogenic signals stimulate the activities of CDK/CYCD complexes involved in cyclin/retinoblastoma (RB; animals) and RB-related genes (RBR; plants) pathways, which are believed to be involved in regulating the commitment of cells to the mitotic cell cycle (Meijer and Murray 2001; Boonstra 2003). Phosphorylation of RBR by CDK/CYCD complexes results in the release of RBR from promoter-bound E2F/DP complexes, triggering the expression of target genes and the progression of cells into the S phase (Uemukai et al. 2005). This phosphorylation is dependent on a specific RBR-binding motif near the cyclin N-domain, which consists of the LxCxE amino acid sequences. Almost all plants CYCDs have a conserved LxCxE RBR-interaction motif (Soni et al. 1995; Huntley et al. 1998). Plant CYCD contains both a conserved cyclin N-domain and a conserved cyclin region involved in CDK binding (Nugent et al. 1991). However, some D-type cyclins often contain less conserved cyclin-C domains (Buendía-Monreal et al. 2011).

Cell division is necessary for plant development. Plant D-type cyclin genes (*CYCDs*) are important in regulating the commitment of cells to cell division during plant growth and development (Soni et al. 1995; Riou-Khamlichi et al. 1999). A significant advance in our understanding of the role of *CYCDs* in

cell division came from transgenic manipulation. In *Arabidopsis*, the overexpression of *AtCYCDs* genes enhances cell division and accelerates plant development (Koroleva et al. 2004; Cockcroft et al. 2000; Dewitte et al. 2003, 2007; Kono et al. 2007; Collins et al. 2012). Therefore, plant *CYCDs* play important roles in cell division and plant development.

Fruit development depends on the successful completion of pollination and fertilization. However, several varieties of cucumber (*Cucumis sativus* L.) can naturally produce parthenocarpic fruit in the absence of fertilization. Fruit growth and development is determined by cell division and cell expansion (Gillaspy et al. 1993). Cell division plays an essential role during early fruit organogenesis by determining the number of cells and the final size of fruits (Bohner and Bangerth 1988). In cucumber, cell division occurs most rapidly in the period before anthesis to 0–4 days after anthesis (DAA) (Boonkorkaew et al. 2008; Fu et al. 2008; Ando et al. 2012; Fu et al. 2010). Plant *CYCD* genes, which regulate cell division and fruit development, have been reported. Kvarnheden et al. (2000) reported that *LeCYCD3;1*, *LeCYCD3;2*, and *LeCYCD3;3* in tomato (*Lycopersicon esculentum* Mill.) are involved in the transduction of signals that lead to fruit development. Similar studies have been reported in white-flower gourd (*Lagenaria leucantha*) and cucumber; the expression of *LlCYCD3;1*, *LlCYCD3;2*, *CsCYCD3;1*, and *CsCYCD3;2* are abundant in pollinated ovaries and parthenocarpic ovaries (Fu et al. 2010; Li et al. 2003). These results suggest that *CYCDs* play important roles in fruit development by promoting cell division. However, there are few studies on the expression levels of other *CYCDs* during early fruit development.

In cucumber, plant growth regulators including α-naphthalene acetic acid (NAA), *N*-(2-chloro-4-pyidyl)-*N′*-phenyurea (CPPU), and brassinosteroids (BRs) induce parthenocarpic fruits by stimulating *CYCD3* expression (Fu et al. 2008, 2010). In addition, pollination/fertilization, which increase the accumulation of auxins, gibberellins (GAs), and cytokinins, enhance *CYCD3* expression and activate cell division in cucumber ovaries (Boonkorkaew et al. 2008; Fu et al. 2008, 2010). With the exception of *CsCYCD3;1* and *CsCYCD3;2*, D-type cyclin genes are not involved in regulating cucumber fruit development (Fu et al. 2008, 2010). The relationship between transcriptional changes in *CYCD* genes during cell division and early fruit development needs to be further studied in cucumber fruits.

The Cucumber Genome Sequence Project is complete (Huang et al. 2009). The availability of cucumber genomic sequences provides an opportunity to study gene families in a genome-wide manner. To set the foundation for a better understanding of the *CYCD* family in cucumber, we identified the members of the cucumber *CYCD* family and compared their similarity with sequences of the corresponding orthologs in *A. thaliana* and poplar (*Populus trichocarpa*). There is ample information on the chromosomal locations, genomic structures, and expression patterns of cucumber *CYCD* gene family during the early stage of fruit development. This study will help us understand the molecular and biological functions of D-type cyclin genes in cucumber.

Materials and Methods

Identification of CYCD Gene Families in Cucumber

Sequence information for 10 *A. thaliana CYCD* genes and 22 poplar *CYCD* genes were retrieved from the Institute for Genome Research [(TIGR) Annotation Version 5.0 (http://www.tigr.org/tdb/e2k1/ath1/)] and the National Center for Biotechnology Information GenBank (http://www.ncbi.nlm.nih.gov). To assess the corresponding cucumber orthologs, a basic local alignment search tool (BLAST) was performed for each one of the *A. thaliana* and poplar CYCD protein sequences against the Cucumber Genome Database (http://cucumber.genomics.org.cn/page/cucumber/index.jsp). Those with the highest amino acid percentage identity were selected. Cucumber *CYCDs* were named according to the nomenclature of Wang et al. (2004) and Menges et al. (2007). The gene numbers corresponding to every *CYCD* in the Cucumber Genome Database are shown in Supplemental Table S1.

Phylogenetic Analysis

Alignments of the CYCD protein sequences of cucumber, *A. thaliana*, and poplar were performed using ClustalW Multiple Alignment in MEGA 5.0 (Tamura et al. 2011). A Neighbor-Joining phylogenetic tree was constructed by aligning the CYCD protein sequences of *A. thaliana* (10), poplar (22), and cucumber (13). Bootstrap analysis was performed with 5,000 repeats.

Analysis of CYCD Protein Domains and Genomic Organization

To study cyclin-specific domains and motifs, CYCD protein sequences were analyzed using the Functional Site Prediction for Eukaryotic Linear Motif (http://elm.eu.org/) and PFAM (http://pfam.sanger.ac.uk/) databases. The exon–intron organization of cucumber *CYCDs* were analyzed using the Gene Structure Display Server (Guo et al. 2007).

Chromosomal Mapping of Cucumber CYCDs

To determine the location of the *CsCYCD* genes on the cucumber chromosomes, each cDNA sequence was used as a query

sequence for BLAST against cucumber whole genomic scaffolds (http://cmb.bnu.edu.cn/Cucumis_sativus_v20/). According to the information obtained from the whole genomic scaffolds, 13 *CYCDs* were mapped on the corresponding cucumber chromosomes.

Plant Growth and Treatments

Two cucumber cultivars, EC1 (natural parthenocarpy line) and 8419s-1 (non-parthenocarpy line) were used in the experiments. Seedlings were grown in a greenhouse [12-h photoperiod; mean daily air temperatures, 29°C/17°C (day/night); relative humidity, 85%, photosynthetic photo flux density 800 μmol m^{-2} s^{-1}] at the Nanjing Agricultural University in China. The ovaries at the 12–15th nodes of the main stem were isolated one day prior to anthesis to prevent pollen contamination. Ovaries of EC1 were isolated for sampling. Experiments with ovaries of 8419s-1 included two treatments: (1) pollination and (2) parthenocarpic fruit induced with 100 mg L^{-1} of CPPU at anthesis (Fu et al. 2010). Samples were harvested at 0, 2, 4, 6, and 8 DAA; 8419s-1 unpollinated fruits were only harvested at 2 DAA. Samples were frozen in liquid nitrogen and stored at −80°C prior to RNA extraction.

Expression Profile Analysis

In our previous study, expression profiles were obtained from young fruits (2 DAA) consisting of unpollinated EC1, pollinated and unpollinated 8419s-1, and CPPU-inducted 8419s-1. cDNA preparation, Illumine sequencing, and transcript analysis were performed as described by Feng et al. (2012). Differentially expressed genes containing *CYCDs* at 2 DAA in young fruits were retrieved from Cucurbit Genomics Database. The gene expression levels were calculated using the RPKM method (Reads Per kb per Million reads) reported by Mortazavi et al. (2008).

Quantitative Real-Time PCR

Total RNA was extracted from cucumber fruits with Trizol reagent (Invitrogen), according to manufacturer's instructions. for 30 min at 25 °C and purified according to manufacturer's instructions. First-strand cDNA was synthesized from 2 μg of total RNA using a Fermentas Reverse Transcription Kit. The designed primers are shown in Supplemental Table S2. *Cs-Actin* was used as an internal control (GenBank accession number: AB010922). QRT-PCR was performed using SYBR Premix Ex Taq™ Kit (Takara) according to the manufacturer's protocol. The selected genes were analyzed using a Bio-Rad iQ1 real-time PCR. At least three replicates were tested per sample. Relative mRNA (fold) differences were assessed using the $2^{-\Delta\Delta Ct}$ formula (Livak and Schmittgen 2001).

Results

Identification of Cucumber CYCD Genes

Using *A. thaliana* and poplar CYCD protein sequences, we identified 13 candidates D-type cyclins and further analyzed the phylogenetic relationship with *A. thaliana* and poplar CYCDs. The results revealed the presence of cucumber CYCD subgroups: CYCD1, CYCD2/CYCD4, CYCD3, CYCD5, CYCD6, and CYCD7 (Fig. 1). As in *A. thaliana*, CYCD2 and CYCD4 belonged to one subgroup. Five cucumber *CYCD3* genes revealed close homology to *AtCYCD3*; these genes consisted of three pairs of closely related genes arising from genome duplication. In cucumber, CYCD1 and CYCD5 had two members; CYCD6 and CYCD7 had one member. Cucumber CYCDs were named according to phylogenetic results. Of the 13 D-type cyclin genes, two were *CsCYCD3;1* and *CsCYCD3;2* (Fu et al. 2008).

Analysis of Protein Sequences in CYCD: Domains and Motifs

Plant cyclins contain a conserved region called the cyclin core, which consists of cyclin N-domain and cyclin C-domain

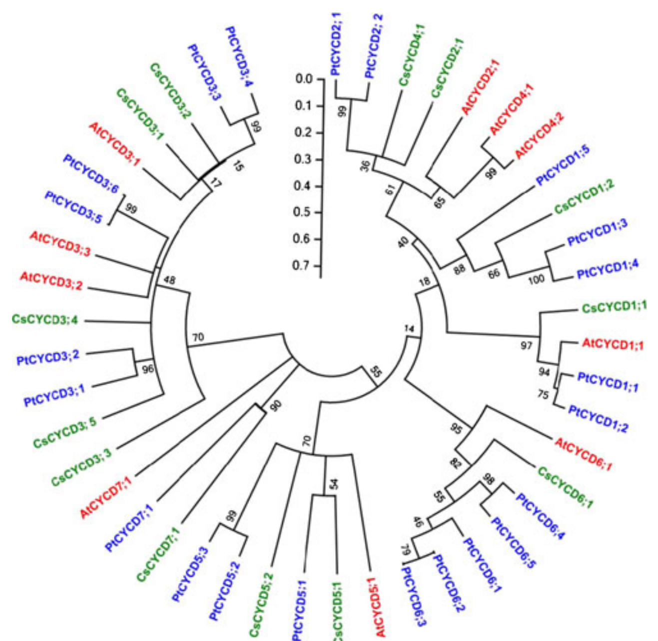

Fig. 1 Phylogenetic tree of 45 CYCD protein sequences from *A. thaliana* AtCYCDs (*red*), poplar PtCYCDs (*blue*), and cucumber CsCYCDs (*dark green*). Gene IDs are listed in Supplemental Table S1. The values above the branches represent bootstrap percentages (5,000 replicates). The *scale bar* represents 0.1 amino acid substitutions per site

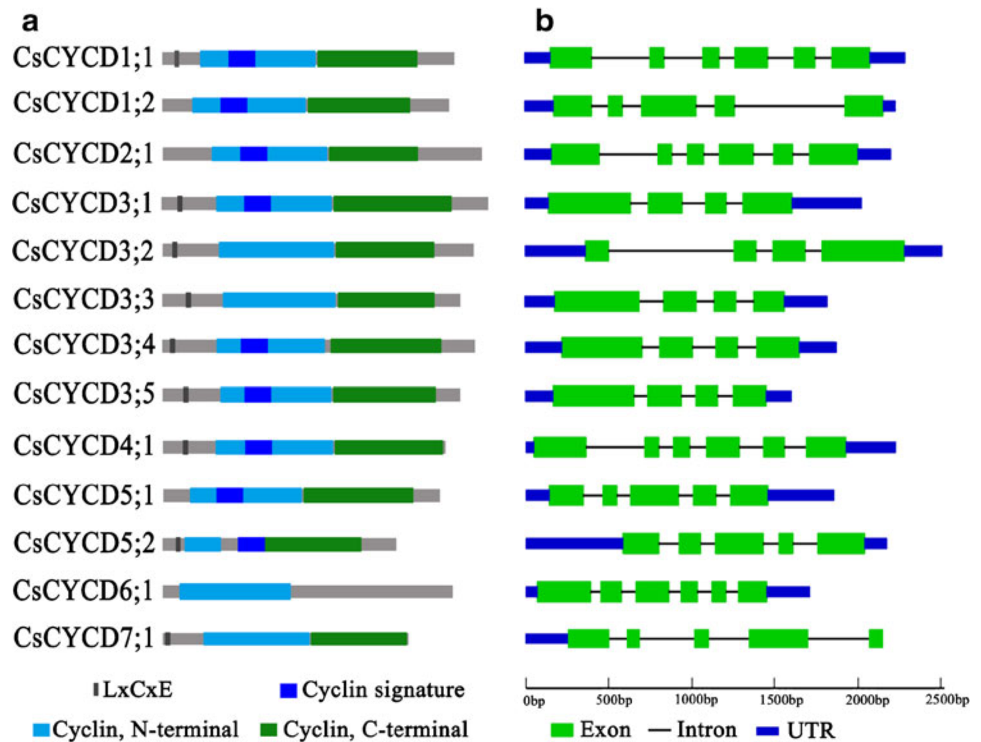

Fig. 2 Genomic organization and protein domains of cucumber D-type cyclins. **a** Characteristic cyclin domains in cucumber CYCDs. **b** Genomic organization with exons (*light green bars*), introns (*gray lines*), and untranscribed regions (UTRs) (*blue bars*)

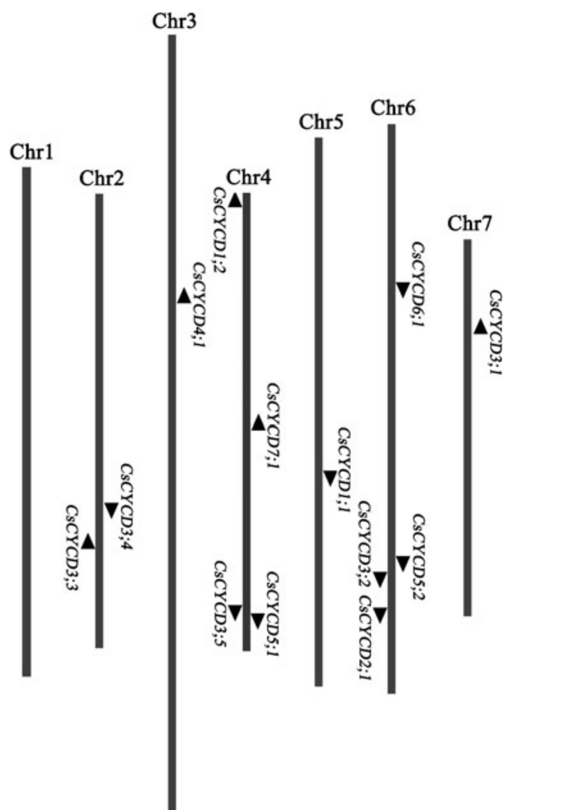

Fig. 3 Genomic localization of cucumber *CYCD* genes. The *arrows* next to the gene names show the direction of the scaffold, which was sequenced in cucumber genome database

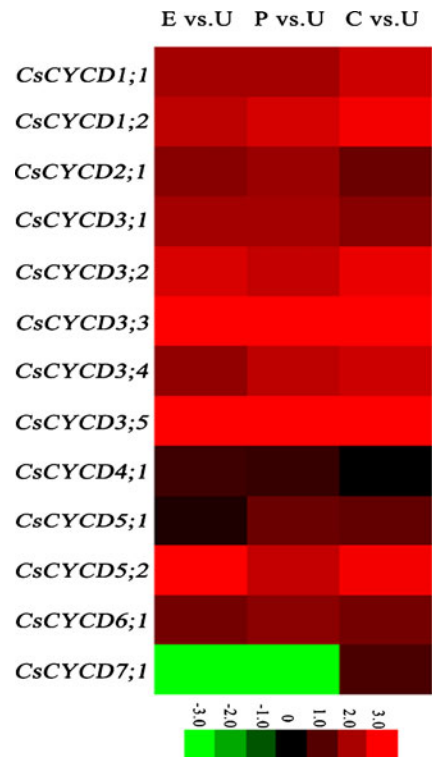

Fig. 4 Expression levels of D-type cyclin genes analyzed by RNA-Seq analysis of ovaries at 2 DDA. The *colors* represent RPKM-normalized $\log_2$ transformed counts. *Red* indicates upregulation, *dark* indicates on differences, and *green* indicates downregulation expression levels in different ovaries. The control consisted of 8419s-1 unpollinated ovary (*U*). Expression levels of *CYCDs* genes in EC1 non-pollinated ovary (*E*), 8419s-1 pollinated ovary (*P*), and CPPU-induced 8419s-1 ovary (*C*)

(Nugent et al. 1991). The cyclin N-domain is approximately 120 amino acids long and comprises the CDK-binding region with a conserved cyclin signature of eight amino acids. The cyclin-C domain is less conserved and is present in most plant cyclins (Wang et al. 2004). In cucumber, it is present in all cyclins with the exception of CYCD6;1 (Fig. 2a). The CYCD structure is characterized by a conserved cyclin signature, which is essential for cyclin binding to CDK. Cyclins are nonfunctional in the absence of an intact cyclin signature. However, with the exception of CsCYCD3;2, CsCYCD3;3, CsCYCD6;1, and CsCYCD7;1, all CsCYCDs have the conserved cyclin signature (Fig. 2a).

Almost all plant CYCDs contained an RBR protein-binding site and an amino acid motif (LxCxE) near the N terminus (Ewen et al. 1993; Soni et al. 1995). Of these 13 D-type cyclins, four had no conserved LxCxE motif: CsCYCD1;2, CsCYCD2;1, CsCYCD5;1, and CsCYCD6;1 (Fig. 2a). With the exception CsCYCD1;1 and CsCYCD7;1, which have the same LLCDE sequence, there were differences in the LxCxE motif among the subgroups. These results suggested that there are subgroup-specific differences in RBR binding.

Genomic Organization of CsCYCD Family Genes

We assessed the exon–intron organization in the 13 *CYCD* cucumber genes (Fig. 2b). The exon–intron organization of ancestral *CYCD* genes contains six exons (Menges et al. 2007; Buendía-Monreal et al. 2011). Our results revealed that *CsCYCD1;1*, *CsCYCD2;1*, *CsCYCD4;1*, and *CsCYCD6;1* contained six exons, which is consistent with the ancestral structure of *CYCD* genes in vascular plants. As in *A. thaliana* and poplar, five cucumber *CYCD3* genes had four exons. Other *CsCYCD* genes contained five exons. This result revealed that some exons of the ancestral *CYCD* genes fused into one.

Chromosomal Distribution of D-type Cyclin Genes in Cucumber

All *CYCD* genes from cucumber were anchored to cucumber chromosomes. The chromosomal locations and scaffold sequence directions of the 13 *CsCYCD* genes were analyzed using BLASTN. Further analysis of the cucumber *CYCD* genes revealed that they were distributed on 6 of the 7 cucumber

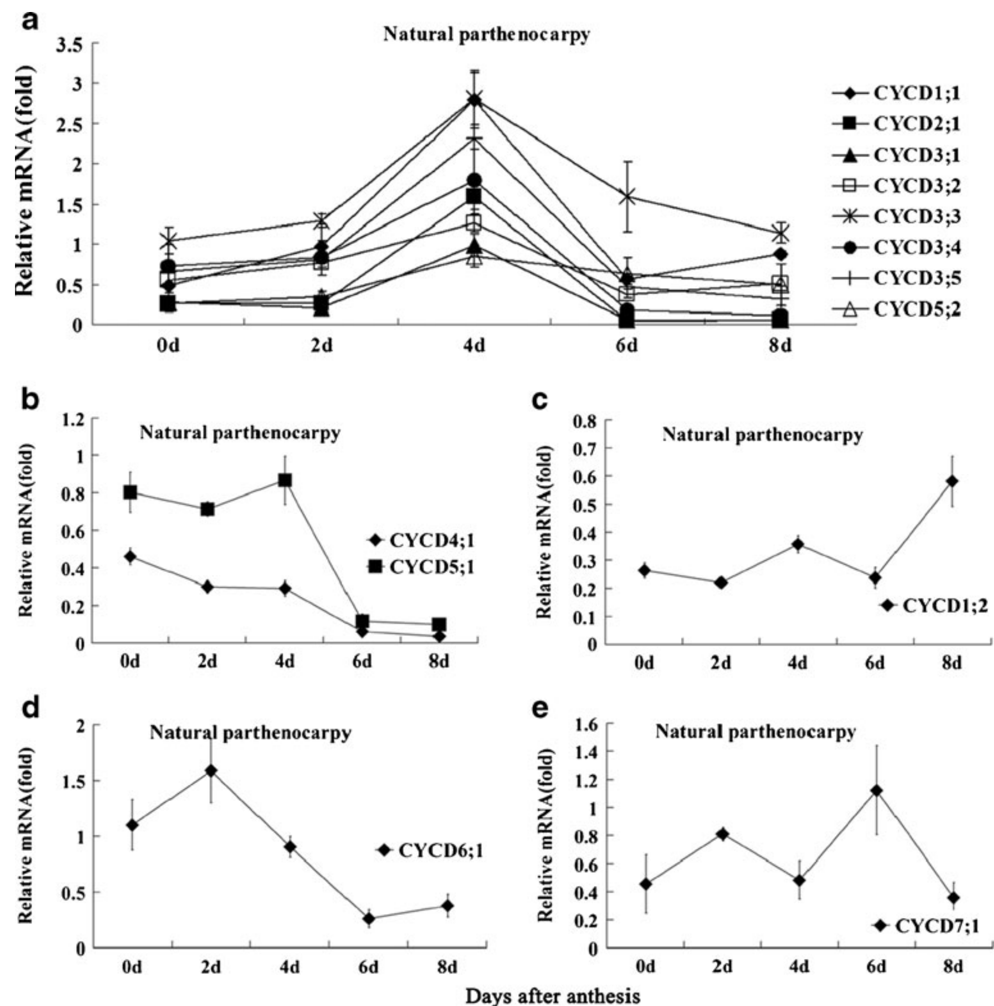

Fig. 5 Expression levels of the 13 *CsCYCD* genes during the early stage of natural parthenocarpic fruit development. Most of the *CsCYCD* expression levels were the highest at 4 DAA (**a**). Other *CsCYCD* genes had different expression levels (**b**, **c**, **d**, and **e**). QRT-PCR analyses were performed using RNA generated from EC1 ovaries at different developmental fruit stages (0, 2, 4, 6, and 8 DAA). The results were expressed relative to mRNA levels of 8419s-1 unpollinated ovary at 0 DAA. Values represent a single experiment consisting of three independent biological replicates. *Bars* indicate SE of the mean of three experimental replicates

chromosomes (Fig. 3). The number of *CsCYCD* genes per chromosome ranged from one to four. Four *CsCYCD* genes were located on chromosome 4, four on chromosome 6, and two on chromosome 2. Three other *CsCYCD* genes were located on chromosomes 3, 5, and 7. However, none of the *CsCYCD* gene was found on chromosome 1. We observed homologous *CsCYCD* genes located on different chromosomes in cucumber, suggesting that duplicated events were potentially involved in the evolution of cucumber *CsCYCDs* (Fig. 3).

Global Correlation Analysis of CsCYCD Expression Profiling in Young Fruits at 2 DDA

According to previous work in our lab, the 13 D-type cyclin genes can be identified from the expression profiles in young fruits at 2 DAA. Of the 13 D-type cyclin genes, 11 were upregulated in EC1 natural parthenocarpic fruit, 8419s-1 pollinated fruit, and CPPU-induced parthenocarpic fruit at 2 DAA (8419s-1 unpollinated fruit served as the control) (Fig. 4). In addition, the expression level of *CsCYCD4;1* was similar among the different treatments. However, at 2 DAA, *CsCYCD7;1* was slightly upregulated in CPPU-induced fruits, whereas it was downregulated in EC1 parthenocarpic and pollinated young fruits compared to the control. These results indicated that cell division in parthenocarpic and pollinated fruits at 2 DAA was very rapid.

Expression Levels of CsCYCDs During the Early Development of Cucumber Fruits

To assess the role of D-type cyclins during the early development of natural parthenocarpic fruits, we analyzed the

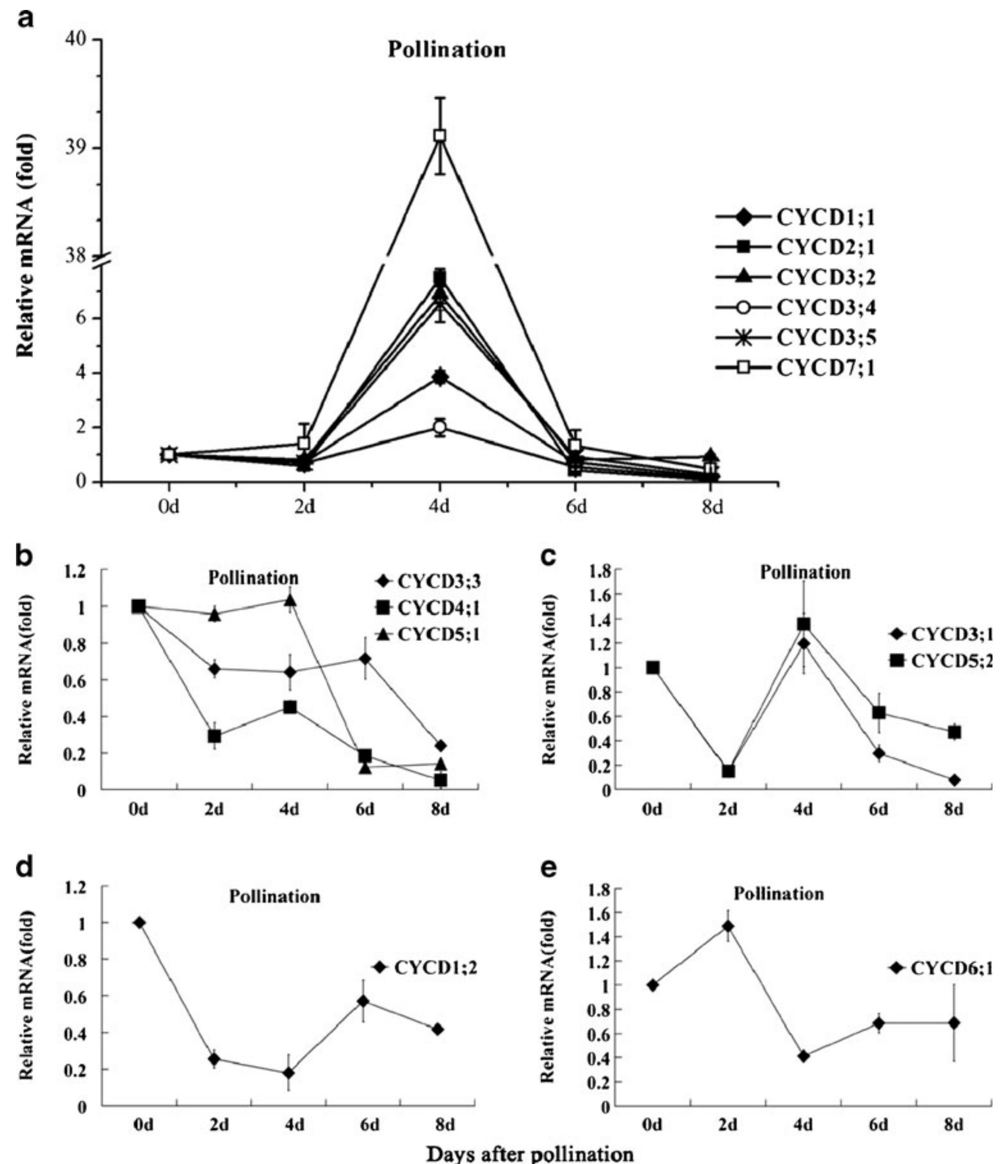

Fig. 6 Expressional levels of 13 *CsCYCD* genes during the early developmental stage of pollinated fruits. QRT-PCR analyses were performed using RNA generated from 8419s-1 pollinated fruits at different developmental stages (0, 2, 4, 6, and 8 DAA). For more details see Fig. 5

expression levels of the 13 cucumber *CYCD* genes. In EC1 natural parthenocarpic fruit, the highest expression levels of *CsCYCD1;1*, *CsCYCD2;1*, *CsCYCD3;1*, *CsCYCD3;2*, *CsCYCD3;3*, *CsCYCD3;4*, *CsCYCD3;5*, and *CsCYCD5;2* were obtained at 4 DDA and then began to decrease with the completion of cucumber fruit set (Fig. 5a), suggesting that the period of cell division may occur from anthesis to 4 DAA in natural parthenocarpic fruits. Furthermore, the expression patterns of these genes were similar from 0 to 8 DAA. *CsCYCD4;1* and *CsCYCD5;1* were gradually downregulated after anthesis (Fig. 5b). On the other hand, *CsCYCD1;2* was upregulated after anthesis (Fig. 5c). The highest expression level of *CsCYCD6;1* was at 2 DAA and then declined (Fig. 5d). In addition, the expression level of *CsCYCD7;1* was upregulated at 2 and 6 DAA during the early developmental stage of natural parthenocarpic fruit (Fig. 5e).

In 8419s-1 pollinated fruit, the highest expression levels of *CsCYCD1;1*, *CsCYCD2;1*, *CsCYCD3;2*, *CsCYCD3;4*, *CsCYCD3;5*, and *CsCYCD7;1* were obtained at 4 DDA (Fig. 6a). The expression levels of *CsCYCD3;3*, *CsCYCD4;1*, and *CsCYCD5;1* were gradually downregulated after anthesis (Fig. 6b). The expression levels of *CsCYCD3;1* and *CsCYCD5;2* increased at 0 and 4 DDA (Fig. 6c). *CsCYCD1;2* was downregulated at from 0 to 4 DAA, whereas it was upregulated at 6 DAA (Fig. 6d). Figure 6e shows that the expression level of *CsCYCD6;1* was similar to that in natural parthenocarpic fruit. Interestingly, we observed that the expression levels of certain *CsCYCD* genes were consistent during the early development of natural parthenocarpic fruit and pollinated fruit. These genes included *CsCYCD1;1*, *CsCYCD2;1*, *CsCYCD3;2*, *CsCYCD3;4*, *CsCYCD3;5*, *CsCYCD4;1*, *CsCYCD5;1*, and *CsCYCD6;1*. This result suggested that the period of cell division in natural parthenocarpic fruit may coincide with that in pollinated fruits.

To assess whether the above results exist in CPPU-induced parthenocarpic fruits, we analyzed the expression levels of cucumber *CYCD* genes during the early developmental stage of CPPU-induced parthenocarpic fruits. The expression levels of *CsCYCD1;1*, *CsCYCD1;2*, *CsCYCD3;5*, *CsCYCD5;1*, and *CsCYCD5;2* were downregulated from 0 to 6 DAA and upregulated at 8 DAA (Fig. 7a). The expression levels of *CsCYCD2;1* and *CsCYCD3;1* were downregulated after anthesis (Fig. 7b), whereas the expression level of *CsCYCD3;2* was upregulated after anthesis (Fig. 7d). The expression levels of *CsCYCD3;3*, *CsCYCD3;4*, *CsCYCD4;1*, *CsCYCD6;1*, and *CsCYCD7;1* remained constant at 2 DAA and subsequently began to decline (Fig. 7c). Therefore, the results indicated that the expression levels of D-type cyclins genes in CPPU-induced parthenocarpic fruit were different among natural parthenocarpic fruit and pollinated fruit, with the exception of *CsCYCD4;1* (Figs. 5b, 6b, and 7c).

Discussion

In this study, we identified 13 genes that encode D-type cyclins in cucumber. Of the 13 genes, two were *CsCYCD3;1* (EU122163) and *CsCYCD3;2* (EU195880) (Fu et al. 2008). The other genes were named on the basis of identity percentages with *A. thaliana* and poplar genes. A phylogenetic analysis revealed that six subgroups of cucumber *CYCDs* were similar with those in *A. thaliana* and poplar; each subgroup of D-type cyclins contained at least one gene (Fig. 1). The CYCD4 cyclins were considered to be members of the CYCD2 subgroup (Wang et al. 2004). The similarity between the CYCD2 and CYCD4 subgroups was further confirmed in our phylogenetic analyses.

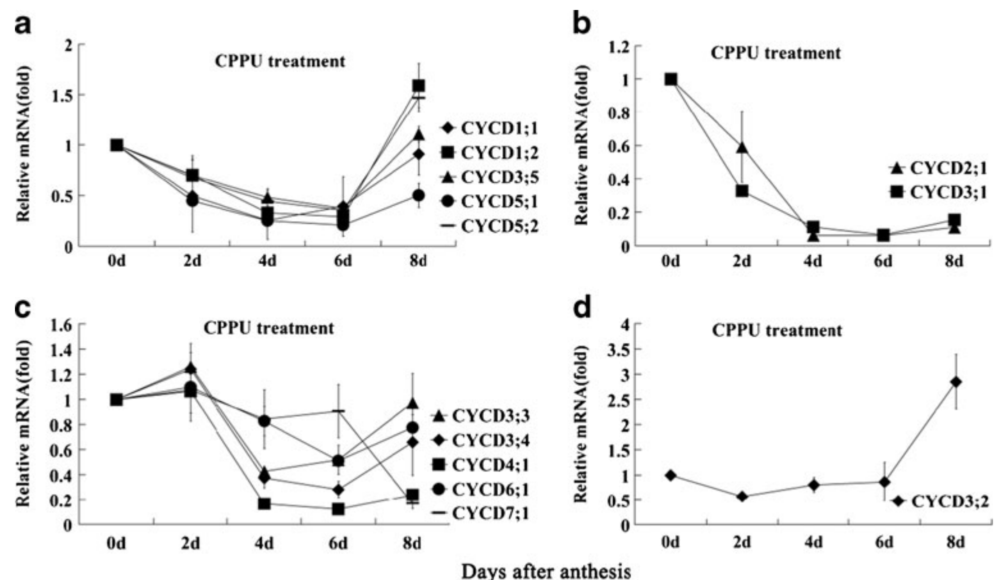

Fig. 7 Expression levels of 13 *CsCYCD* genes obtained from CPPU-treated 8419s-1 ovaries at different developmental fruit stages (0, 2, 4, 6, and 8 DAA). For more details see Fig. 5

Plant RBR was identified by the LxCxE-containing D-type cyclins (Boniotti and Gutierrez 2001; Nakagami et al. 2002). However, *A. thaliana* CYCD5 contains a similar FxCxE motif whereas CYCD4;2 and CYCD6;1 have no apparent RBR-interaction motif (Vandepoele et al. 2002). In poplar, CYCD1;4, CYCD5;3, and CYCD6 have no LxCxE motifs (Menges et al. 2007). All cucumber D-type cyclins had LxCxE motifs with the exception of CYCD1;2, CYCD2;1, CYCD5;1, and CYCD6;1. This result indicated that there are differences among plants.

The genomic organization of *CYCD* genes and *CYCD1*, *CYCD2/4*, and *CYCD6* groups is conserved in angiosperms (Buendía-Monreal et al. 2011). In this study, *CsCYCD1;1*, *CsCYCD2;1*, *CsCYCD4;1*, and *CsCYCD6;1* had six exons (Fig. 2b). Previous studies have reported that all members of the *CYCD3* subgroup in *Arabidopsis* and poplar have four exons with a conserved length in the central exon and exon 1, which represent exons 1–3 of the ancestral structure (Menges et al. 2007). In cucumber, the *CsCYCD3* subgroup had four exons, suggesting that a similar evolution took place in dicotyledons. Other cucumber *CYCD* genes contained five exons. Menges et al. (2007) reported that the ancestral exons 3 and 4 have fused into one exon in all *CYCD5* genes of angiosperms. A similar phenomenon was observed in cucumber *CsCYCD1;2*, *CsCYCD5;1*, *CsCYCD5;2*, and *CsCYCD7;1*.

CYCDs were regulated by multiple hormones at the G1 to S transition phases, thereby affecting the commitment to cell division (De Veylder et al. 1999; Sorrell et al. 1999). *CYCD3* subgroup genes were regulated by pollination and cytokinin (Riou-Khamlichi et al. 1999; Fu et al. 2010). This was consistent with the expression levels of *CsCYCD3* subgroup in pollinated fruits and parthenocapic fruits, which were higher than in aborted fruits at 2 DAA (Fig. 4). In our study, *CsCYCD3;1*, *CsCYCD3;2*, *CsCYCD3;4*, and *CsCYCD3;5* were upregulated in natural parthenocarpic and pollinated fruits at 4 DAA. However, *CsCYCD3;1* was downregulated in young fruits by CPPU treatment at the day of anthesis (Fig. 7b); *CsCYCD3;2* was upregulated from 0 to 8 DAA (Fig. 7d). Therefore, the *CsCYCD3* subgroup may be an important regulator of cell division in cucumber fruit. In addition, some studies have reported that BRs target *CYCD3* expression (Hu et al. 2008; Fu et al. 2008). In our expression profiling experiments, certain *CsCYCD* genes were involved in BRs signal transduction pathways; these genes were not exclusively from the *CsCYCD3* subgroup (Supplemental Figure S1 and Supplemental Table S3). Interestingly, *CsCYCD7;1* was significantly upregulated in pollinated fruits at 4 DAA, resulting in the embryo and endosperm in cucumber fruits (Fig. 6a). This finding is supported by the effects that ectopic *CYCD7;1* expression has on cell divisions and growth of *Arabidopsis* embryos (Collins et al. 2012).

Expression levels of most D-type cyclin genes were the highest at 4 DAA in natural parthenocarpic fruit and pollinated fruit (Figs. 5a and 6a). These genes are among those associated with mitosis and post-mitosis (M and G1) in *Arabidopsis* (Fu et al. 2010; Ando et al. 2012). In cucumber, the number of cells in parthenocarpic fruits did not differ significantly from those in pollinated fruits, partly because of high levels of zeatin and zeatin riboside at 4 DAA (Boonkorkaew et al. 2008). This suggests that expression levels of most *CsCYCDs* were the highest at 4 DAA, partly because of high level of cytokinins in natural parthenocarpic fruits and pollinated fruits, which is consistent with the results obtained by other researchers (Takeno et al. 1992; Boonkorkaew et al. 2008).

Interestingly, the expression levels of cucumber *CYCD* genes revealed that the period of cell division during the early stage of natural parthenocarpic fruit was similar to that of pollinated fruit. However, the expression levels of *CsCYCD* genes in CPPU-induced parthenocarpic fruit were different from those in pollinated fruit and natural parthenocarpic fruit. It is possible that pollination increased the levels of indole-3-acetic acid, zeatin, and gibberellin, which promote cell division and thus to fruit set (Sjut and Bangerth 2006; Kim et al. 1992; Lewis et al. 2006; Ben-Cheikh et al. 1997). Natural parthenocarpic fruit development is controlled by regulation of hormones; a balance of several hormones may partly imitate hormonal actions during pollinated fruit development. This result indicated that cucumber parthenocarpic fruit development may be a process subject to complex hormonal regulation.

In summary, the identification of cucumber *CYCD* genes and the analysis of their protein domain and genomic organization revealed that six CYCD subgroups are conserved across angiosperms. The expression levels of cucumber *CsCYCD* genes were analyzed in cucumber fruits. The expression patterns of Cs*CYCDs* revealed that the time of cell division in natural parthenocarpic fruits is similar to that in pollinated fruits, but different to that in CPPU-induced parthenocarpic fruits. Future studies should focus on assessing the specific functions of D-type cyclins in cucumber parthenocarpic fruit.

Acknowledgments This study was supported by funds from the National Natural Science Foundation of China grant (The 973 Program: 2012CB3904), the Youth Science and Technology Innovation Fund program of Nanjing Agricultural University (No. KJ2012013), the Fundamental Research of Nanjing Agricultural University (Y0201100253) program, and the Ph.D. Program Foundation of Ministry of Education of China (20120097120037).

References

Ando K, Carr K, Grumet R (2012) Transcriptome analyses of early cucumber fruit growth identifies distinct gene modules associated with phases of development. BMC Genomics 13(1):518–534

Ben-Cheikh W, Perez-Botella J, Tadeo FR, Talon M, Primo-Millo E (1997) Pollination increases gibberellin levels in developing ovaries of seeded varieties of citrus. Plant Physiol 114(2):557–564

Bohner J, Bangerth F (1988) Effects of fruit set sequence and defoliation on cell number, cell size and phytomone levels of tomato fruits (*Lycopersicon esculentum* Mill.) with a truss. Plant Growth Regul 7:141–155

Boniotti MB, Gutierrez C (2001) A cell-cycle-regulated kinase activity phosphorylates plant retinoblastoma protein and contains, in *Arabidopsis*, a CDKA/cyclin D complex. Plant J 28(3):341–350

Boonkorkaew P, Hikosaka S, Sugiyama N (2008) Effect of pollination on cell division, cell enlargement, and endogenous hormones in fruit development in a gynoecious cucumber. Sci Hortic 116(1):1–7

Boonstra J (2003) Progression through the G1-phase of the on-going cell cycle. J Cell Biochem 90(2):244–252

Buendía-Monreal M, Rentería-Canett I, Guerrero-Andrade O, Bravo-Alberto CE, Martínez-Castilla LP, García E, Vázquez-Ramos JM (2011) The family of maize D-type cyclins: genomic organization, phylogeny and expression patterns. Physiol Plant 143(3):297–308

Cockcroft CE, den Boer BGW, Healy JMS, Murray JAH (2000) Cyclin D control of growth rate in plants. Nature 405(6786):575–579

Collins C, Dewitte W, Murray JAH (2012) D-type cyclins control cell division and developmental rate during *Arabidopsis* seed development. J Exp Bot 63(10):3571–3586

De Veylder L, de Almeida Engler J, Burssens S, Manevski A, Lescure B, Van Montagu M, Engler G, Inzé D (1999) A new D-type cyclin of *Arabidopsis thaliana* expressed during lateral root primordia formation. Planta 208(4):453–462

Dewitte W, Riou-Khamlichi C, Scofield S, Healy JMS, Jacqmard A, Kilby NJ, Murray JAH (2003) Altered cell cycle distribution, hyperplasia, and inhibited differentiation in *Arabidopsis* caused by the D-type cyclin CYCD3. Plant Cell 15(1):79–92

Dewitte W, Scofield S, Alcasabas AA, Maughan SC, Menges M, Braun N, Collins C, Nieuwland J, Prinsen E, Sundaresan V (2007) *Arabidopsis* CYCD3 D-type cyclins link cell proliferation and endocycles and are rate-limiting for cytokinin responses. Proc Nati Acad Sci USA 104(36):14537–14542

Ewen ME, Sluss HK, Sherr CJ, Matsushime H, Kato J, Livingston DM (1993) Functional interactions of the retinoblastoma protein with mammalian D-type cyclins. Cell 73(3):487

Feng C, Chen M, Xu C, Bai L, Yin X, Li X, Allan AC, Ferguson IB, Chen K (2012) Transcriptomic analysis of Chinese bayberry (*Myrica rubra*) fruit development and ripening using RNA-Seq. BMC Genomics 13(1):19–34

Fu FQ, Mao WH, Shi K, Zhou YH, Asami T, Yu JQ (2008) A role of brassinosteroids in early fruit development in cucumber. J Exp Bot 59(9):2299–2308

Fu F, Mao W, Shi K, Zhou Y, Yu J (2010) Spatio-temporal changes in cell division, endoreduplication and expression of cell cycle-related genes in pollinated and plant growth substances-treated ovaries of cucumber. Plant Biol 12(1):98–107

Gillaspy G, Ben-David H, Gruissem W (1993) Fruits: a developmental perspective. Plant Cell 5(10):1439–1451

Guo A-Y, Zhu Q-H, Chen X, Luo J-C (2007) GSDS: a gene structure display server. Yi Chuan 29(8):1023–1026

Hu Y, Bao F, Li J (2008) Promotive effect of brassinosteroids on cell division involves a distinct *CycD3*-induction pathway in *Arabidopsis*. Plant J 24(5):693–701

Huang S, Li R, Zhang Z, Li L, Gu X, Fan W, Lucas WJ, Wang X, Xie B, Ni P (2009) The genome of the cucumber, *Cucumis sativus* L. Nat Genet 41(12):1275–1281

Huntley R, Healy S, Freeman D, Lavender P, de Jager S, Greenwood J, Makker J, Walker E, Jackman M, Xie Q (1998) The maize retinoblastoma protein homologue ZmRb-1 is regulated during leaf development and displays conserved interactions with G1/S regulators and plant cyclin D (CycD) proteins. Plant Mol Biol 37(1):155–169

Kim IS, Okubo H, Fujieda K (1992) Endogenous levels of IAA in relation to parthenocarpy in cucumber (*Cucumis sativus* L.). Sci Hortic 52(1–2):1–8

Kono A, Umeda-Hara C, Adachi S, Nagata N, Konomi M, Nakagawa T, Uchimiya H, Umeda M (2007) The *Arabidopsis* D-type cyclin CYCD4 controls cell division in the stomatal lineage of the hypocotyl epidermis. Plant Cell 19(4):1265–1277

Koroleva OA, Tomlinson M, Parinyapong P, Sakvarelidze L, Leader D, Shaw P, Doonan JH (2004) *CycD1*, a putative G1 cyclin from *Antirrhinum majus*, accelerates the cell cycle in cultured tobacco BY-2 cells by enhancing both G1/S entry and progression through S and G2 phases. Plant Cell 16(9):2364–2379

Kvarnheden A, Yao JL, Zhan X, O'Brien I, Morris BA (2000) Isolation of three distinct *CycD3* genes expressed during fruit development in tomato. J Exp Bot 51(352):1789–1797

Lewis DH, Burge GK, Hopping ME, Jameson PE (2006) Cytokinins and fruit development in the kiwifruit (*Actinidia deliciosa*). II. Effects of reduced pollination and CPPU application. Physiol Plant 98(1):187–195

Li Y, Yu JQ, Ye QJ, Zhu ZJ, Guo ZJ (2003) Expression of *CycD3* is transiently increased by pollination and *N*-(2-chloro-4-pyridyl)-*N′*-phenylurea in ovaries of *Lagenaria leucantha*. J Exp Bot 54(385):1245–1251

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25(4):402–408

Meijer M, Murray JAH (2001) Cell cycle controls and the development of plant form. Curr Opin Plant Biol 4(1):44–49

Menges M, Pavesi G, Morandini P, Bögre L, Murray JAH (2007) Genomic organization and evolutionary conservation of plant D-type cyclins. Plant Physiol 145(4):1558–1576

Morgan DO (1997) Cyclin-dependent kinases: engines, clocks, and microprocessors. Annu Rev Cell Dev Biol 13(1):261–291

Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B (2008) Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nat Methods 5(7):621–628

Nakagami H, Kawamura K, Sugisaka K, Sekine M, Shinmyo A (2002) Phosphorylation of retinoblastoma-related protein by the cyclin D/cyclin-dependent kinase complex is activated at the G1/S-phase transition in tobacco. Plant Cell 14(8):1847–1857

Nugent J, Alfa CE, Young T, Hyams JS (1991) Conserved structural motifs in cyclins identified by sequence analysis. J Cell Sci 99(3):669–674

Riou-Khamlichi C, Huntley R, Jacqmard A, Murray JAH (1999) Cytokinin activation of *Arabidopsis* cell division through a D-type cyclin. Science 283(5407):1541–1544

Sjut V, Bangerth F (2006) Effect of pollination or treatment with growth regulators on levels of extractable hormones in tomato ovaries and young fruits. Physiol Plant 53(1):76–78

Soni R, Carmichael JP, Shah ZH, Murray J (1995) A family of cyclin D homologs from plants differentially controlled by growth regulators and containing the conserved retinoblastoma protein interaction motif. Plant Cell 7(1):85–103

Sorrell DA, Combettes B, Chaubet-Gigot N, Gigot C, Murray JAH (1999) Distinct cyclin D genes show mitotic accumulation or constant levels of transcripts in tobacco bright yellow-2 cells. Plant Physiol 119(1):343–352

Takeno K, Ise H, Minowa H, Dounowaki T (1992) Fruit growth induced by benzyladenine in *Cucumis sativus* L.: influence of benzyladenine on cell division, cell enlargement and indole-3-acetic acid content. J Jap Soc Hort Sci 60:915–920

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: Molecular evolutionary genetics analysis using maximum

likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28(10):2731–2739

Uemukai K, Iwakawa H, Kosugi S, de Uemukai S, Kato K, Kondorosi E, Murray JAH, Ito M, Shinmyo A, Sekine M (2005) Transcriptional activation of tobacco E2F is repressed by co-transfection with the retinoblastoma-related protein: cyclin D expression overcomes this repressor activity. Plant Mol Biol 57(1):83–100

Vandepoele K, Raes J, De Veylder L, Rouzé P, Rombauts S, Inzé D (2002) Genome-wide analysis of core cell cycle genes in *Arabidopsis*. Plant Cell 14(4):903–916

Wang G, Kong H, Sun Y, Zhang X, Zhang W, Altman N, Ma H (2004) Genome-wide analysis of the cyclin family in *Arabidopsis* and comparative phylogenetic analysis of plant cyclin-like proteins. Plant Physiol 135(2):1084–1099