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Cloning, identification, and expression analysis of a *Dicer-Like* gene family from *Solanum lycopersicum*

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Abstract

Dicer proteins belong to the RNase III family of proteins, which are key components in small RNA biogenesis. In *Solanum lycopersicum*, seven *Dicer-like* (*DCL*) genes have been identified and have been named *SlDCL*. In this study, we cloned the full-length sequence of the *SlDCL* genes including untranslated regions using RNA ligase-mediated rapid amplification of cDNA ends. Our analysis indicates that 7 *SlDCLs* were located on 5 tomato chromosomes (6, 7, 8, 10, and 11). The gene structure of the *SlDCLs* covered long genomic regions and contained more than 20 exons. Phylogenetic analysis divided the seven *SlDCL* members into four subgroups. In general, all seven *SlDCLs* were expressed in all organs but more in flowers and fruits than in the other parts. Moreover, the expressions of some genes changed slightly after treatment with ethylene or 1-methylcyclopropene suggesting their likely roles in plant responses to ethylene. Our findings provide essential information on *SlDCL* genes in tomato and will aid in the functional classification of *DCL* families in plants.

Additional key words: heterologous expression, phylogenetic analysis, RML-RACE, tomato.

Introduction

Small RNAs are 20 - 30 nucleotides long ribo-nucleotides in eukaryotes and regulate various important biological processes such as response to the environmental stimuli, maintaining genome stability, and defence against viruses (Ramachandran and Chen 2008). Dicer proteins are essential components in small RNA biogenesis pathways and include microRNAs (miRNAs) and small interfering RNAs (siRNAs) (Bouché *et al*. 2006). Dicer or Dicer-like (DCL) proteins generally consist of six conserved domains: dead box, helicase, dicer dimerization, PAZ (Piwi Argonaut and Zwille), RNase III, and dsRNA binding domain (dsRBD). However, lower eukaryotes lack one or two domains (Liu *et al*. 2009). The helicase domain is known to recognize the single strand terminal loop structure of primary miRNA (pre-miRNA) and inhibits rate of substrate cleavage (Zhu *et al.* 2013). The dicer dimerization domain is involved in target selection of small RNA processing (Qin *et al.* 2010); the PAZ and RNase III domains participate in dsRNA substrate cleavage (Liu *et al.* 2009). Interestingly, the distance between PAZ and the cleavage site in the RNase III domain determines the length of mature small RNAs (Hammond 2005).

In general, there are different *Dicer* or *DCL* gene members in eukaryotes. In flowering plants, four DCL proteins have been identified (Parent *et al.* 2015). In *Arabidopsis*, *AtDCL1* mainly contributes to miRNA biogenesis, whereas *AtDCL2*, *AtDCL3*, and *AtDCL4* mediate siRNA processing (Bouché *et al.* 2006). Interestingly, *AtDCL3* and *AtDCL4* have their own preferential substrates. *AtDCL3* prefers short dsRNAs, whereas *AtDCL4* cleaves long dsRNA substrates

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Abbreviations: BK - breaker; *BLAST* - basic local alignment search tool; CDS - coding sequence; DCL - Dicer-like; DPA - days post anthesis; IM - immature green; 1-MCP - 1-methylcyclopropene; miRNA - microRNA; MG - mature green; ORF - open reading frame; PR - pink ripe; RLM-RACE - RNA ligase-mediated rapid amplification of cDNA ends; RR - red ripe; siRNA - small interfering RNA; UTR - untranslated regions.

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(Nagano *et al.* 2014). In addition, different *DCL* genes were found to have redundant roles. *AtDCL1* and *AtDCL3* share a functionally redundant role in *FLOWERING LOCUS C* (*FCL*), a strong floral repressor, repression and thus promote flowering (Schmitz *et al.* 2006). Inactivating both *AtDCL2* and *AtDCL4* is sufficient to restore systemic infection of a suppressor-deficient virus, whereas inhibiting either of them generates an immune response suggesting their redundancy in anti-viral defence (Deleris *et al.* 2006).

Tomato is model plant for studies on fruit development and ripening. Previous studies predicted seven *DCL* genes in tomato by *in silico* analysis (Bai *et al.* 2012). Kravchik *et al.* (2014a,b) have identified *DCL1* and *DCL3* using RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE), and *DCL3* was much longer than the predicted one. In this study, we cloned the full-length cDNA sequences of *DCL2a*, *DCL2b*, *DCL2c*, *DCL2d*, and *DCL4* in *S. lycopersicum*. We then performed a genome-wide analysis of gene structure, conserved domains, phylogeny, and expression analysis of the *DCL* family in tomato. The results can enhance our understanding of *DCL* genes in tomato and constitute a foundation for further investigation.

Materials and methods

Tomato (*Solanum lycopersicum* Mill. cv. Ailsa Craig) and tobacco (*Nicotiana benthamiana* Domin) were planted in a commercial soil in a greenhouse at a temperature of 25 °C, a relative humidity of 75 %, a 16-h photoperiod, and an irradiance of 50 - 70 μmol m^{-2} s^{-1}. The tomato fruits were harvested at various ripening stages; IM (immature green, 37 days post anthesis, DPA), MG (mature green, 42 DPA), BK (breaker, 46 DPA), PR (pink ripe, 51 DPA), and RR (red ripe, 56 DPA). The tomato fruits at the BK stage were placed in glass containers and exposed to 60 μM 1-methylcyclopropene (1-MCP) in water or 50 mm^3 dm^{-3} ethylene at 25 °C for 24 h. After the treatment, the fruit samples were stored at -80 °C.

The total RNA was isolated from tomato and *N. benthamiana* samples using a *DeTRNa* reagent (*EarthOx*, San Francisco, CA, USA). The extracted RNA was treated by DNase (*Promega*, Madison, WI, USA) to remove genomic DNA contamination. Concentration and purity of the RNA were measured using a *NAS-99* spectrophotometer (*ATCGene*, Piscataway, NJ, USA) and integrity was checked by gel electrophoresis. The total RNA was used for cDNA synthesis using a cDNA synthesis *SuperMix* kit (*Trans*, Beijing, China).

The full-length cDNA sequences of *SlDCL1* (Genbank accession No. JX962774) and *SlDCL3* (Genbank acc. No. KF840949) were reported by Kravchik *et al.* (2014a,b). To obtain the full-length sequences of the remaining five *SlDCL* genes, we first amplified the *SlDCL* coding sequences (CDSs) from the tomato leaf cDNA under the following cycling conditions: 94 °C for 3 min, 35 cycles of 94 °C for 15 s, 58 °C for 30 s, and 68 °C for 4 min, followed by a final extension at 68 °C for 7 min. The cloned genes perfectly matched the exon regions of genomic sequences in Genbank. To obtain the 5' untranslated regions (*UTRs*) and 3'*UTRs* of the five *SlDCLs*, RLM-RACE was performed, and nested PCR reactions were carried out under the following conditions: 94 °C for 3 min, 35 cycles of 94 °C for 15 s, 60 °C for 30 s, and 68 °C for 1 min, followed by a final extension at 68 °C for 7 min. Oligonucleotide primers and their sequences used in these amplifications are listed in Table 1 Suppl.

For transient gene expression in *N. benthamiana*, the pCAMBIA1300 vector was adopted. A recombinant plasmid was constructed by inserting a 947 bp genomic fragment of *SlDCL2b* (g*SlDCL2b*) into pCAMBIA1300. The DNA fragment covered the hypothetic 5'*UTR* of *SlDCL2b* and the recombinant plasmid was used for heterologous expression. *Agrobacterium tumefaciens* L. strain GV3101 containing pCAMBIA1300-g*SlDCL2b* was cultured at 28 °C in the Lourie-Brot medium (pH 5.6) with 10 mM morpholineethanesulfonic acid and 20 μM acetosyringone for 16 h before harvest and re-suspension in an infiltration buffer (10 mM $MgCl_2$, 200 μM acetosyringone). When a final absorbance at 600 nm reached 1.0, the culture was left at room temperature for 3 h. Then *Agrobacterium* was infiltrated into the leaves of *N. benthamiana* with a 1 cm^3 syringe without a needle.

Full-length cDNA sequences of the seven *SlDCL* genes were searched in the tomato genomic sequences using *basic local alignment search tool* (BLAST) and gene structures were drawn by *FancyGene v. 1.4* (http://bio.ieo.eu/fancygene/) (Zouine *et al.* 2014). The genes were mapped on chromosomes by identifying their chromosomal position and visualized by *MapDraw2* (Liu and Meng 2003, Cao *et al.* 2014). The online *InterPro* software (http://www.ebi.ac.uk/interpro/) was used to analyze the conserved domains in SlDCL proteins, which were subsequently portrayed by *DOG2.0* (Ren *et al.* 2009). The protein sequences were aligned by *ClustalX 1.81* and a phylogenetic tree was constructed using the maximum likelihood method with 1 000 bootstrap replicates and visualized using *MEGA6.06*.

Relative expressions of *SlDCLs* in leaves, flowers, and fruits at different stages were analyzed using the total RNA as template in a real-time PCR system *CFX96* (*Bio-Rad*, Hercules, CA, USA) with a *SYBR Green* PCR master mix (*Bio-Rad*). *Actin* was used as internal control. Real time qPCR conditions were as follows: 95 °C for 3 min followed by 40 cycles of 95 °C for 10 s and 60 °C

for 30 s. The relative expression of each gene was determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). All real time qPCR data present three biological replicates. The SPSS statistics 19.0 was used for statistical analysis. Duncan's multiple range test was applied to compare statistical significances among differences in gene expression between the hormone treated and the control groups. A significant level was set to $\alpha = 0.05$. All data are presented as means ± standard errors (SEs).

Results

To identify *DCL* genes in the tomato genome, four *Arabidopsis* DCL protein sequences were used as queries to search against the tomato genome database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the *BLASTP* program. In total, seven genes in the tomato genome were identified as possible members of the *DCL* family. The predicted sequences of *SlDCLs* were used to design primers and amplify the respective genes. Based

Table 1. Identification of SlDCL genes (a - Kravchik *et al*. 2014a, b - Kravchik *et al*. 2014b).

Gene name	Chromosome location	Full length [bp]	Number. of exons	5'UTR [bp]	3'UTR [bp]	Protein length [aa]
SlDCL1[a]	ch10 111707-125292	5745	21	347	253	1914
SlDCL2a	ch06 32061350-32073832	4209	23	57	165	1402
SlDCL2b	ch11 2690171- 2697539	4209	23	62	130	1402
SlDCL2c	ch11 2713473-2724875	4218	23	75	367	1405
SlDCL2d	ch11 2699623-2707130	4113	23	95	467	1370
SlDCL3[b]	ch08 56205205-56253969	4977	25	69	301	1658
SlDCL4	ch07 52944-86564	4863	25	166	326	1620

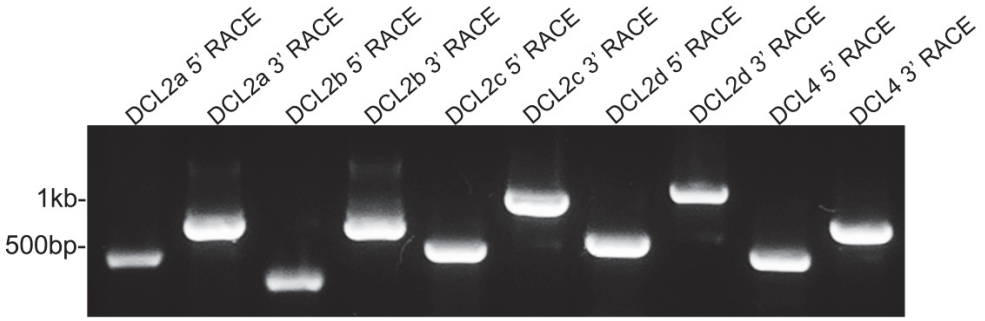

Fig. 1. The 5' and 3' end products of *SlDCLs*. Gel electrophoresis of five *SlDCLs* amplified by nested PCR.

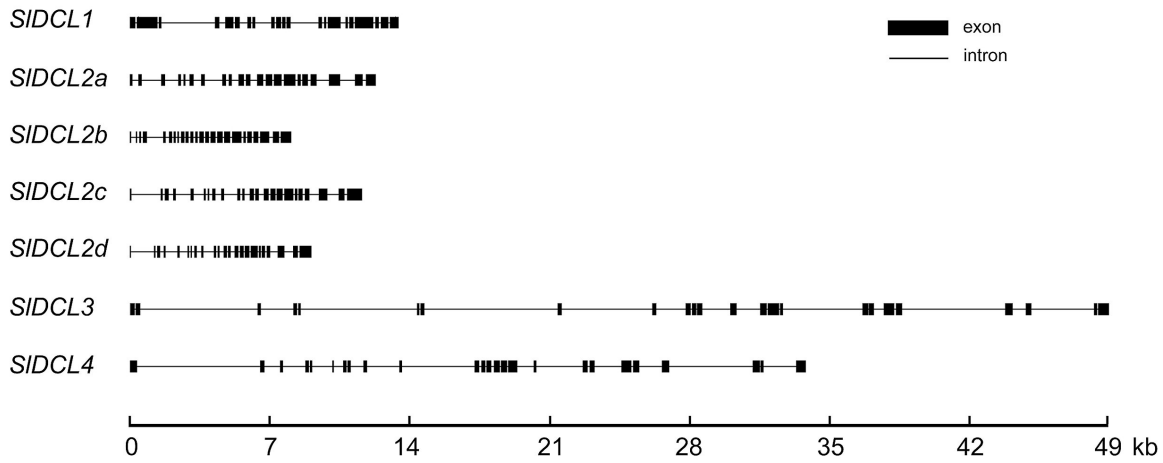

Fig. 2. The exon-intron structure of *SlDCLs*. Exons are represented by *black boxes* and introns by *black lines*. The map scale shows the length of the *SlDCL* genomic DNA.

on the CDS sequences, the 5' and 3'RACE primers were designed and RLM-RACE was carried out to obtain the 5'and 3'ends of the cDNA. The 5'*UTR* of *SlDCL2b* was not obtained due to its low expression and a high similarity with *SlDCL2c*. Then, we applied *SlDCL2b* in *N. benthamiana* to eliminate interferences from *SlDCL2c* in tomato. The 5' and 3' *UTR* of five *SlDCLs* were obtained successfully (Fig. 1), and mRNAs of *SlDCL2a*, *2b*, *2c*, *2d*, and *SlDCL4* were submitted to Genbank under accession numbers from KR153939 to KR153943.

Bioinformatics information for each *SlDCL* included the chromosome location, full-length of the genes, exon numbers, lengths of 5' and 3' *UTRs* and number of amino acids (Table 1). The lengths of *SlDCLs* varied from 4 113 (*SlDCL2d*) to 5 745 bp (*SlDCL1*). *SlDCL3* and *SlDCL4* contained 25 exons, which was the most among

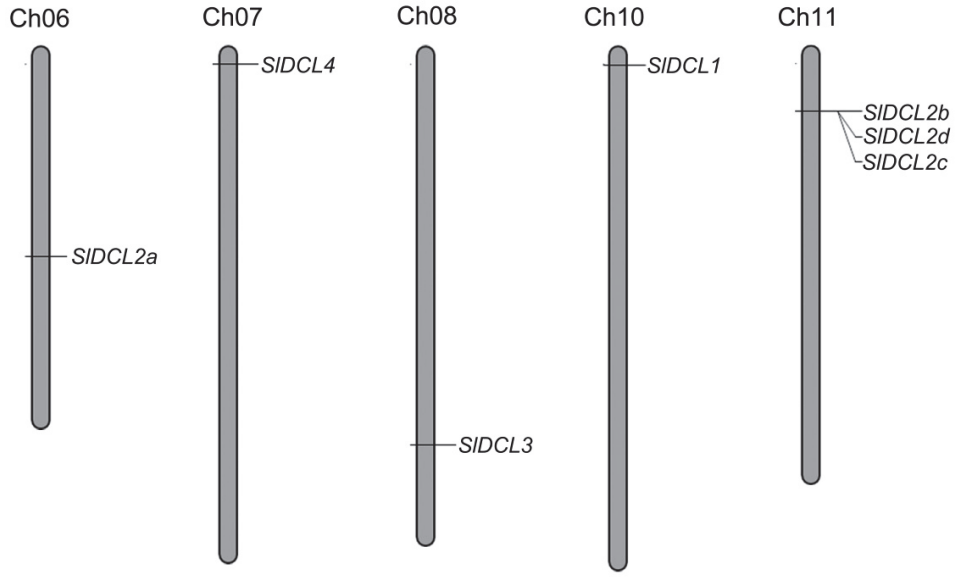

Fig. 3. Chromosomal locations of *SlDCLs.* Seven *SlDCL* genes were mapped on five tomato chromosomes. The chromosome number is specified at the top of each chromosome.

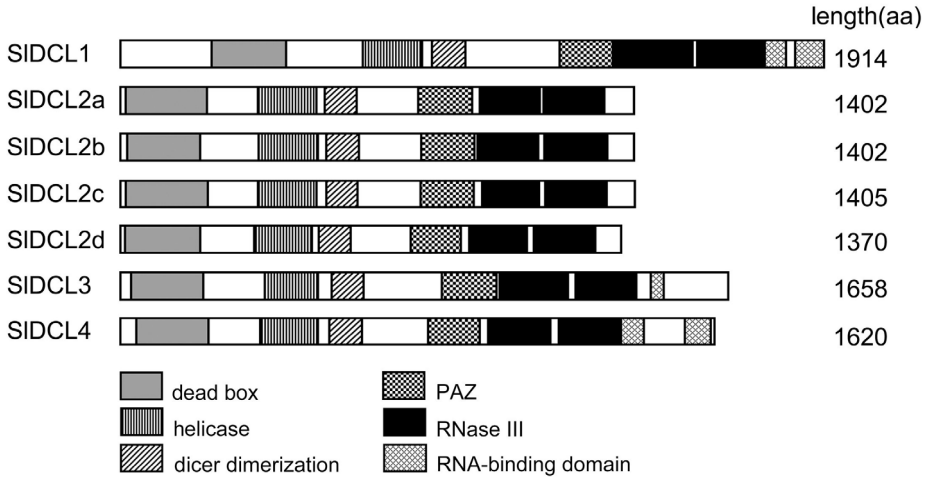

Fig. 4. Conserved domains of SlDCL proteins. The conserved domains are represented by *boxes with different patterns*. Numbers on the right represent the size of SlDCL proteins.

all *SlDCLs*. The lengths of 5'*UTRs* from the seven *SlDCLs* varied between 57 and 347 bp, and 3'*UTRs* varied from 130 to 467 bp. The 7 *SlDCLs* coded 1370 to 1914 amino acids and the largest among them was *SlDCL1*.

To obtain insights into the structural diversity of *SlDCLs*, we analyzed the exon-intron organization of individual *DCL* genes in tomato (Table 1). The cDNA length of *SlDCL1* was the longest. For the genomic sequence, however, *SlDCL3* spanned the largest area. It extended approximately 49 kb. In addition, all 7 *SlDCLs* contained more than 20 introns and there were 24 introns

in both *SlDCL3* and *SlDCL4* (Fig. 2).

The chromosome map of *SlDCLs* reveals that seven genes were distributed unevenly on the five chromosomes (Fig. 3). *SlDCL1*, *SlDCL2a*, *SlDCL3*, and *SlDCL4* were distributed on chromosome 10, chromosome 6, chromosome 8, and chromosome 7, respectively. It should be noted that *SlDCL2b*, *2c*, and *2d* were distributed on the same chromosome, and they were located close to each other. Meanwhile, *SlDCL2b*, *2c*, and *2d* displayed high similaritis (from 75.8 to 82.8 %).

To gain further insight into SlDCLs, we employed *InterPro* to detect conserved domains in the SlDCL family (Fig. 4). SlDCL proteins primarily contain six conserved domains: dead box, helicase, dicer

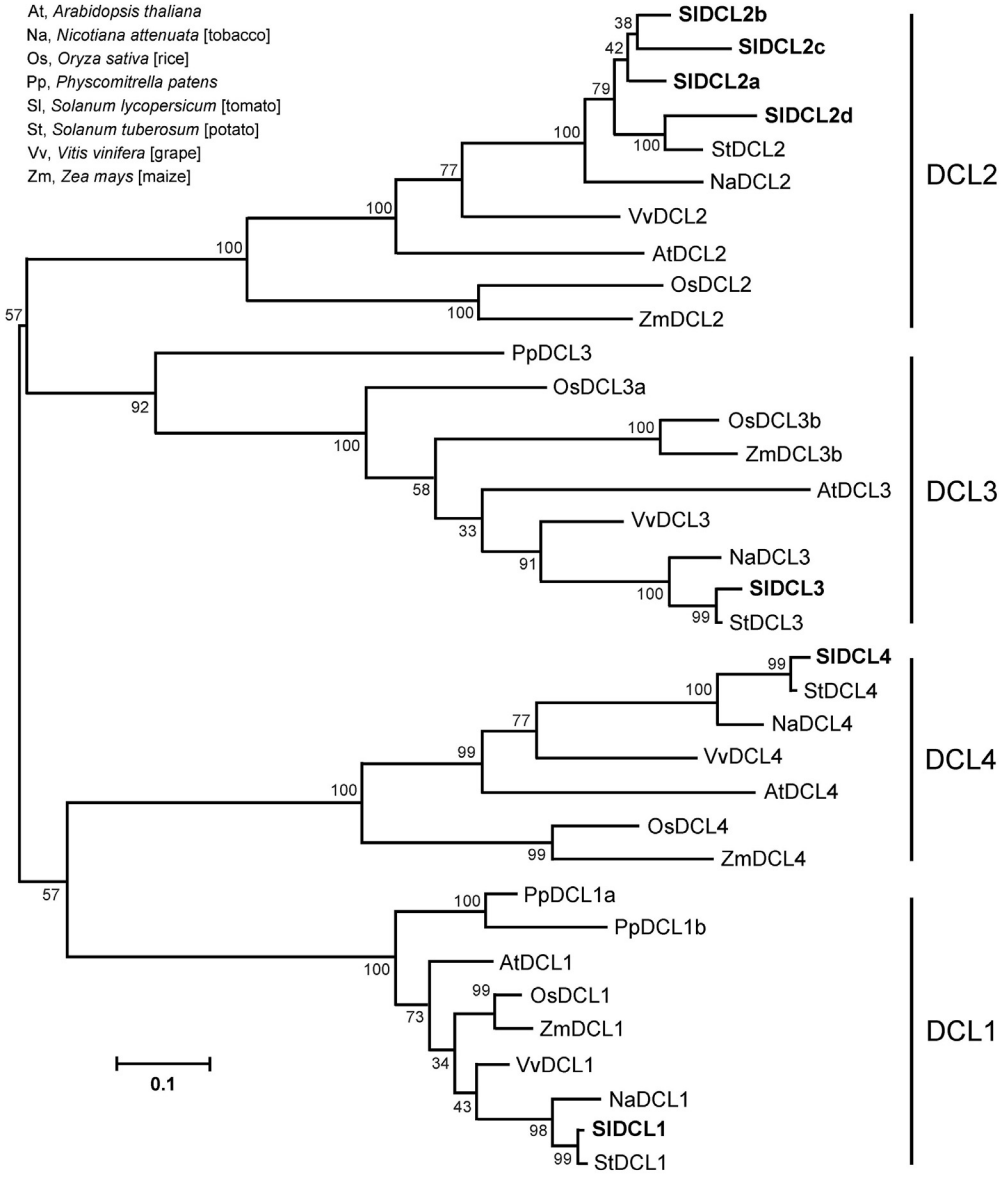

Fig. 5. Phylogenetic analysis included seven DCL proteins from tomato (SlDCL1, JX962774; SlDCL2a, KR153939; SlDCL2b, KR153940; SlDCL2c, KR153941; SlDCL2d, KR153942; SlDCL3, KF840949; SlDCL4, KR153943), four from tobacco (NaDCL1, XM_009608701; NaDCL2, XM_009627603; NaDCL3, XM_009590630; NaDCL4, XM_009599559), four from potato (StDCL1, XP_006352611; StDCL2, ; XR_367699.1; StDCL3, XP_006361520; StDCL4, XP_006343691), five from rice (OsDCL1, XP_006650986; OsDCL2, XP_006651573; OsDCL3a, NP_001045148; OsDCL3b, NP_001045148; OsDCL4, BAF80150), four from grape (VvDCL1, XP_010661522; VvDCL2, XP_010649218; VvDCL3, XP_010648401; VvDCL4, XP_002264486), four from maize (ZmDCL1, DAA43005; ZmDCL2, AFW68290; ZmDCL3b, DAA49563; ZmDCL4, AFW58817), four from *Arabidopsis* (AtDCL1, NP_001184881; AtDCL2, AEE73926 ; AtDCL3, NP_001154663; AtDCL4, NP_001190348), and three from *Physcomitrella patens* (PpDCL1a, ABV31244; PpDCL1b, ABG74922; PpDCL3, ABV31245). The *scale bar* represents 0.1 amino acid substitution per site. The *numbers* on the branches represents bootstrap values.

dimerization, PAZ, RNase III, and dsRBD. In general, the closely related members within the same clade had similar conserved domains. SlDCL2a, 2b, 2c, and 2d lacked the dsRBD, whereas SlDCL3 had only one dsRBD.

In order to explore evolutionary relationships among the DCL proteins in plants, 35 DCL family members from tomato, tobacco, potato, *Arabidopsis*, grape, rice, maize, and *Physcomitrella patens* were aligned and a phylogenetic tree was constructed. The phylogenetic tree of DCLs could be clarified into four groups; DCL1, DCL2, DCL3, and DCL4 (Fig. 5). The four classes point out clearly that tomato DCLs are close to their homologs in potato and tobacco from *Solanaceae*.

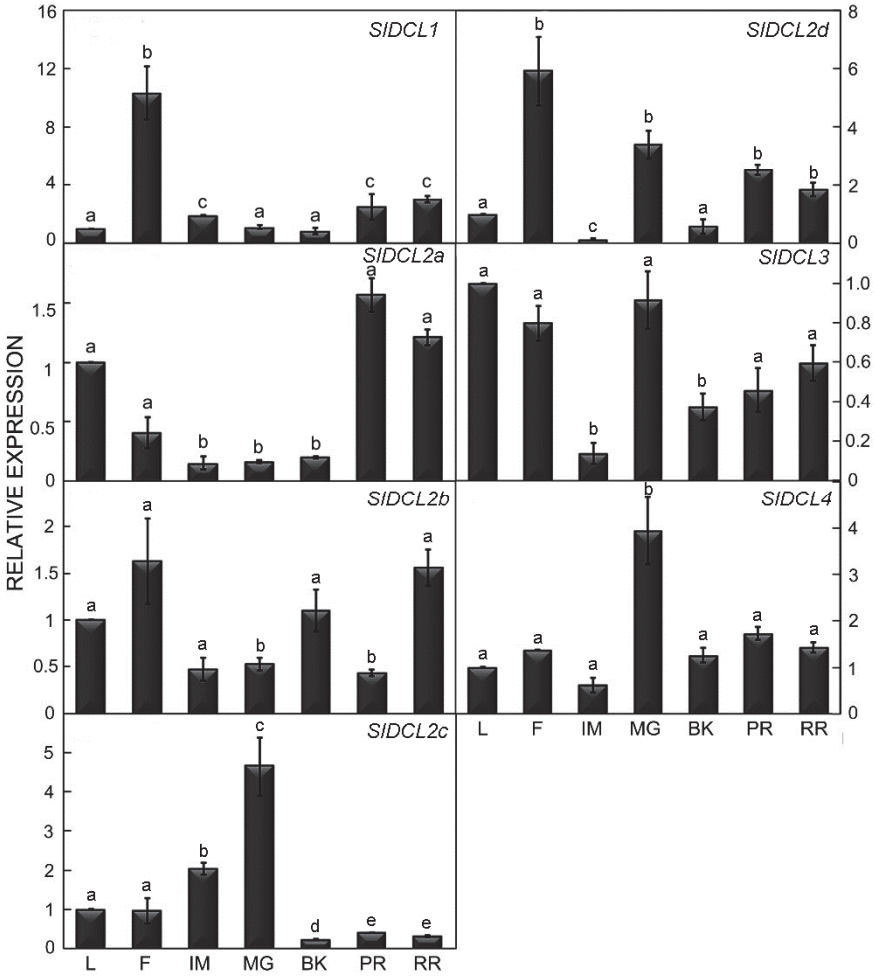

Fig. 6. The relative gene expression of *SlDCLs* in different tomato organs analyzed by real time qPCR. Means ± SEs, $n = 3$. L - leaf; F - flower; IM - immature green fruit; MG - mature green fruit; BK - breaker fruit; PR - pink ripe fruit; RR - red ripe fruit. Lowercase letters on the top of each bar indicate a significance level ($\alpha = 0.05$) of each gene in different tissues.

To analyze transcriptional abundance of *SlDCL* genes in different tomato tissues, we performed real time qPCR to detect gene expression. Expressions of *SlDCLs* were highly variable in leaves, flowers, and fruits in different growth stages (Fig. 6). In general, all seven *SlDCLs* were expressed in all organs, whereas flowers and MG fruits displayed higher expressions than the other parts. The expression of *SlDCL1* in flowers was about 10 times higher than in leaves. The expression of *SlDCL2a* increased gradually as the fruits matured, and reached the highest level in the PR stage, whereas *SlDCL2c* was expressed at low levels in later developmental stages such as the PR and RR. *SlDCL2d* expression fluctuated throughout development, but the levels did not correspond to the developmental periods. Expressions of *SlDCL3* and *SlDCL4* were quite low in the IM stage and their expression pattern varied in fruits.

To detect the response of *SlDCLs* to the plant hormones, transcript accumulation was assessed by real time qPCR in tomato BK fruits treated with ethylene and its receptor inhibitor, 1-MCP. We first identified expression of *ACS2*, a critical positive gene in ethylene

biogenesis, which accumulated in ethylene-treated tomato fruits and was significantly down-regulated in 1-MCP-treated fruits (Fig. 7). These results indicate the differential response of *SlDCLs* to 1-MCP and ethylene. Expressions of *SlDCL2a*, *SlDCL3*, and *SlDCL4* decreased in 1-MCP-treated fruits. However, in ethylene-treated fruits, they did not increase. Moreover, *SlDCL1* and *SlDCL2b* and *2c* were up-regulated in ethylene-treated fruits while they did not respond to the 1-MCP treatment.

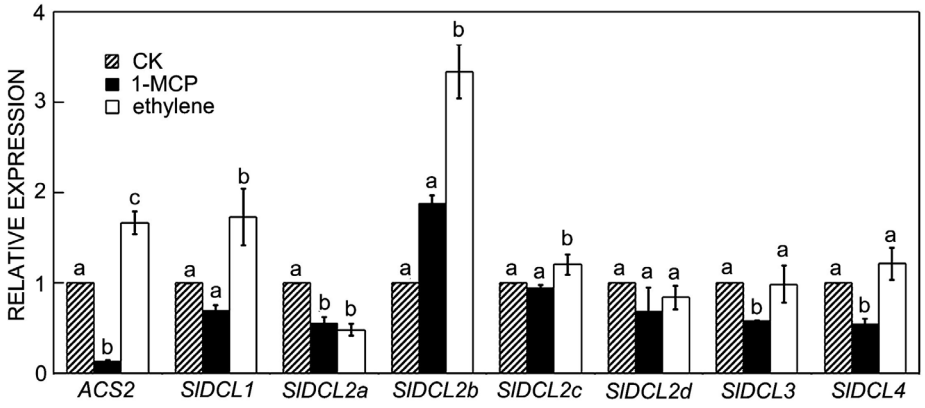

Fig. 7. Expression patterns of *SlDCLs* after treatment with ethylene and 1-methylcyclopropene (1-MCP) analyzed by real time qPCR. Means ± SEs, $n = 3$. CK - control. Different letters indicate significant differences between gene expressions in the same group.

Discussion

The DCL proteins are key components of the miRNA and siRNA biogenesis pathways in plants. They process long dsRNAs or hairpin RNAs into mature small RNAs (Chapman and Carrington 2007). In this study, we identified seven *DCL* genes in tomato (Table 1). Compared to *Arabidopsis* (Vazquez 2006), rice (Song *et al.* 2012), maize (Qian *et al.* 2011), tobacco (Bozorov *et al.* 2012), grape (Zhao *et al.* 2015) and *Physcomitrella patens* (Arif *et al.* 2012), the *DCL* family was by far the biggest in tomato. Protein sequence analysis shows that DCL proteins generally contained six conserved domains, whereas one or two were lacking in several species. For instance, RNase III was not found in grape DCL1 (VvDCL1); RNase III and dsRBD were absent in VvDCL2 (Zhao *et al.* 2015); DCL2 in maize (ZmDCL2) lacked one dsRBD (Qian *et al.* 2011), and in the DCL2 clade in tomato, dsRBD was missing (Fig. 4). In *Arabidopsis*, dsRBD is involved in pre-miRNA recognition and subcellular localization (Burdisso *et al.* 2012) indicating that the SlDCL2 clade might lack the function of pre-miRNAs identification. However, it is not known how the lack of dsRBD influences plants.

To further evaluate the evolutionary association of *SlDCLs* with homologous genes from other plant species, we constructed an unrooted tree by aligning their full-length protein sequences. Evolutionary studies based on sequences from 35 proteins of two monocots (maize and rice), five dicots (tomato, tobacco, potato, grape, and *Arabidopsis*), as well as *P. patens* show that DCL1 was more closely related to DCL4, whereas DCL2 was closer to DCL3. Each protein subfamily in the bryophyte *P. patens* and the six angiosperms clustered distinctly on different branches (Fig. 5) indicating segregation. In addition, a functional diversity of DCLs occurred prior to the divergence of monocots and dicots (Henderson *et al.* 2006). Among the five dicots, DCL families of the *Solanaceae* (tomato, potato, and tobacco) were closely related. Interestingly, some orthologs showed a closer relationship than paralogs between tomato and potato, rice and maize, such as SlDCL2d and StDCL2, and OsDCL3b and ZmDCL3b, indicating that these genes forming the ortholog pairs could have originated from their common ancestor by ancient duplication events that occurred during tomato and potato or rice and maize divergence (Liu *at al.* 2013). Gene families might evolve through tandem duplication (Cannon *et al*. 2004). Gu *et al*. (2002) conducted a detailed analysis of duplicate genes in yeast, *Drosophila*, and *Caenorhabditis elegans*. According to the above analysis, we suppose that SlDCL2b, 2c, and 2d represented tandem duplications and SlDCL2a might derive from them. The DCL1s were reported to be involved in miRNA biogenesis in *Arabidopsis*, rice, and tomato (Liu *at al.* 2005, Bouché *et al.* 2006, Kravchik *et al.* 2014a). In *Arabidopsis*, AtDCL2 was known to contribute to anti-virus defence (Deleris *et al.* 2006). Similarly, *ZmDCL2* mRNAs was up-regulated in virus-infected maize plants indicating its role in anti-viral capability (Xia *at al.* 2014).

All seven *SlDCL* genes were expressed in all organs and tissues (Fig. 6). Previous studies demonstrated that different *DCLs* display diverse expression patterns in various species such as grape (Zhao *et al.* 2015), maize

(Qian *et al.* 2011), rice, and *Arabidopsis* (Kapoor *et al.* 2008). In grape, *VvDCL4* was found to have a robust expression in fruits, whereas the remaining genes are expressed in two or more tissues or organs (Zhao *et al.* 2015). Rice *DCL1* and *DCL3b* depict lower expressions than the others (Kapoor *et al.* 2008). In tomato, *SlDCL2a* showed relatively high expressions in the late fruit developmental stages (PR and RR). Nevertheless, *SlDCL2c* expression was markedly reduced in these two stages, approximately one-tenth of that in the MG stage. In addition, peak expressions of *SlDCL1* and *SlDCL2d* were in flowers indicating that these two genes could be involved in regulation of flower development. *SlDCL2a*, *2b*, *2c* and *2d*, belonged to the *DCL2* clade and were also most closely related to each other within tomato. However, they exhibited various expression patterns in different tissues. These findings suggest that homologous *DCL* genes in different plants could play different roles in regulation of plant growth and development.

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