Key *KdSOC1* gene expression profiles during plantlet morphogenesis under hormone, photoperiod, and drought treatments

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Genet. Mol. Res. 15 (1): gmr.15017579
Received September 2, 2015
Accepted November 4, 2015
Published February 5, 2016
DOI http://dx.doi.org/10.4238/gmr.15017579

ABSTRACT. *Kalanchoe daigremontiana* utilizes plantlet formation between its zigzag leaf margins as its method of asexual reproduction. In this study, *K. daigremontiana* SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (*KdSOC1*), a key intermediate in the transition from vegetative to asexual growth, was cloned. Furthermore, its expression profiles during plantlet formation under different environmental and hormone induction conditions were analyzed. The full-*KdSOC1* cDNA sequence length was 1410 bp with 70% shared homology with *Carya cathayensis SOC1*. The conserved domain search of *KdSOC1* showed the absence of I and C domains, which might indicate novel biological functions in *K. daigremontiana*. The full-*KdSOC1* promoter sequence was 1401 bp long and contained multiple-hormone-responsive cis-acting elements. Hormone induction assays showed that gibberellins and salicylic acid mainly regulated *KdSOC1* expression. The swift change from low to high

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KdSOC1 expression levels during long-day induction was accompanied by the rapid emergence of plantlets. Drought stress stimulated *KdSOC1* expression in leaves both with and without plantlet formation. Together, the results suggested that *KdSOC1* was closely involved in environmental stimulation signal perception and the transduction of *K. daigremontiana* plantlet formation. Therefore, future identification of *KdSOC1* functions might reveal key information that will help elucidate the transition network between embryogenesis and organogenesis during plantlet formation.

Key words: Plantlet formation; Photoperiod; Drought; MADS-box; *KdSOC1*

INTRODUCTION

Kalanchoe daigremontiana is a perennial herbal plant that has zigzag leaf margins in which plantlets form, and this characteristic makes it an ideal model for plantlet morphogenesis research (Garcês et al., 2007). The asexual reproduction of the plant provides it with a quick and complete way to regenerate during a single generation (Garcês et al., 2007; Kulka, 2006, 2008). During the past decades, many studies have described the plantlet formation process at morphological and anatomical levels. However, little was known about the details of this process prior to the functional identification of LEAFY COTYLEDON I (*KdLEC1*), which shed light onto the aspects of this process at the molecular level (Garcês et al., 2007, 2014).

LEC1 stimulates the differentiation of the vegetative cell to a somatic cell, preventing pre-maturation of the somatic cell. However, the loss-of-function mutant of this gene in *Arabidopsis thaliana* exhibited repressed development and desiccation-intolerant somatic cells at the embryonic stage (Lotan et al., 1998; Vicient et al., 2000; Braybrook and Harada, 2008). Interestingly, the silenced *KdLEC1* phenotype of *K. daigremontiana* showed no differences compared to the wild-type plant. However, when the *AtLEC1* promoter was used to drive *KdLEC1* expression in *K. daigremontiana*, disturbance of plantlet formation along the leaf margin was observed, and plantlets were reformed following the addition of exogenous gibberellin (GA) to the leaf. Therefore, self-dysfunction *KdLEC1* suggested that *K. daigremontiana* leaf margin cells might directly differentiate into somatic cells to enhance the asexual reproduction ability of this plant (Garcês et al., 2014). GA was used to activate the dominant stage of the somatic cell in order to initiate plantlet formation (Hays et al., 2001; Ogawa et al., 2003; Mutasa-Göttgens and Hedden, 2009), which indicated that the hormone acts as an un-neglected factor in this process. Recently, the *K. daigremontiana* plantlet formation process was gradually elucidated using the spatial expression analysis of WUSCHEL (*WUS*) (Guo et al., 2015). Although *WUS* was detected in the *K. daigremontiana* shoot apical meristem (SAM) of the leaf margin during plantlet formation, no evidence suggested that this gene regulated the emergence of plantlet formation. Thus, finding the key regulator in this process will signify a breakthrough in future *K. daigremontiana* plantlet research.

Based on these findings and our previous discoveries, we speculated that *K. daigremontiana* plantlet morphogenesis was also sensitive to environmental changes, such as those associated with flowering in *Arabidopsis*. We previously found that sustained plantlets emerged on *K. daigremontiana* leaf margins under drought stress. Thus, a suppression subtractive hybridization technique was applied to screen the differential expression of genes during this process and a *contig1019* sequence was found to be highly homologous to *AtSOC1*. *SOC1* is a member of the MINICHROMOSOME MAINTENANCE 1 (MCM1), AGAMOUS (AG), DEFICIENS (DEF), SERUM

RESPONSE FACTOR (SRF) (MADS) box transcription factor family (Masiero et al., 2011), and it cooperates with AGAMOUS-LIKE 24 (*AGL24*) to promote *LFY* expression, which stimulates the expression of APETALA1 (*AP1*) to initiate flowering organs (Torti et al., 2012). Thus, the unknown mechanisms of somatic cell differentiation that lead to plantlet formation might be elucidated using SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (*KdSOC1*) expression patterns.

In this study, genome walking and rapid amplification of cDNA ends (RACE) techniques were applied to clone promoter and full-length *KdSOC1* cDNA sequences. We utilized bioinformatic databases to analyze the properties of possible conserved domain sequences that encode proteins, including physical and chemical characteristics and putative cis-acting elements in the promoter. Based on the prediction of an essential hormone-responding element in the gene promoter, we checked its expression profile under the induction of different hormones [GA, salicylic acid (SA), abscisic acid (ABA), and methyl jasmonate (MeJa)]. *KdSOC1* expression profiles were checked in 3-month-old *K. daigremontiana* plants cultivated under continuous drought stress conditions. *KdSOC1* expression profiles were also discovered in plants subjected to either long-day (LD) or short-day (SD) conditions.

MATERIAL AND METHODS

Plant material

Three-month-old *K. daigremontiana* plants were grown in a greenhouse at 25°C with a 16/8-h photoperiod under conditions that simulated natural drought stress, and soil water content was measured using a Delta T wet sensor (Delta-T Devices Ltd., UK). Newly formed plantlets from the same plant were grown in growth chambers under LD (16-h light and 8-h dark) and SD (8-h light and 16-h dark) conditions.

KdSOC1 cDNA RACE assays

DNA and RNA extractions from *K. daigremontiana* were performed according to the Garcês method (Garcês and Sinha, 2009). Using a partial cDNA sequence, TaKaRa 5'-Full-RACE (TaKaRa, Japan) and TaKaRa 3'-Full-RACE (TaKaRa) kits were used to amplify the 5'- and 3'-ends of the full-*KdSOC1* cDNA sequence. The following primers were used in the two kits: 3'-RACE: GSP1 5'-GTGTCAGACTGTCAGAGAGGGAGCA-3' and GSP2 5'-ATAGAGAACGCCACGAGCAGACAG G-3'; 5'-RACE: GSP1 5'-GAGCAACCTCAGCATCACAGAGAACCG-3' and GSP2 5'-CTCCCTCTCT GACAGTCTGACACCT-3'.

KdSOC1 promoter genome walking assays

Using a partial genomic DNA sequence, a Genome Walking Kit (TaKaRa) was used to amplify the full-*KdSOC1* promoter sequence. The following primers were used: SP1 5'-CTCTTTATCTCTCCACCTTTATTTCCC-3', SP2 5'-TTTTCAGTTTTGCGGCTGTTGTGGTCT-3', and SP3 5'-CTCCCTCTCTGACAGTCTGACACCTAC-3'.

Bioinformatic analyses of *KdSOC1*

The *KdSOC1* open-reading frame (ORF) was predicted using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html), and cis-acting elements in the promoter were predicted using

Plant Cis-Acting Regulatory Element (PlantCARE; http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). Conserved domains were predicted using NCBI Conserved Domain Search (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), and CDS-encoding protein characteristics were predicted using the following programs: ProtParam (http://web.expasy.org/protparam/), TMHMM Server version 2.0 (http://www.cbs.dtu.dk/services/TMHMM/), SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/), iPSORT (http://ipsort.hgc.jp/), PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/), and SWISS-MODEL (http://swissmodel.expasy.org/).

Phylogenetic analysis of *KdSOC1*

The DNAman software using Clustal method was used to perform a multiple-sequence alignment of the KdSOC1 protein-conserved domain sequence and sequences from the following 19 species (Table 1). A phylogenetic tree was constructed using the neighbor-joining method implemented in the MEGA 6 software.

Table 1. Different species of the SOC1 protein from NCBI.

Species	Accession	Species	Accession
Carya cathayensis	AHI85950.1	*Prunus armeniaca*	ACO40488.1
Glycine max	NP 001236377.1	*Mangifera indica*	ADX97324.1
Prunus salicina	AGD88523.1	*Paeonia suffruticosa*	AHJ60268.1
Zea mays	AIR75259.1	*Brassica juncea*	AFH41827.1
Arabidopsis thaliana	NP 182090.1	*Gossypium hirsutum*	AEA29618.1
Brassica napus	AFH41826.1	*Prunus yedoensis*	AEO20233.1
Brassica rapa	NP 001288813.1	*Photinia serratifolia*	AEO20232.1
Malus domestica	NP 001280844.1	*Rosa hybrid cultivar*	AEO20230.1
Spiraea cantoniensis	AEO20234.1	*Prunus mume*	AEO20229.1
Fragaria vesca	NP 001266966.1		

KdSOC1 expression profiles under hormone induction conditions

Three-month-old *K. daigremontiana* plants were divided into five groups that contained three individual plants as biological replicates. All groups were treated using the foliage spray method as follows: Group one was treated with 500 mL H_2O; Group two was treated with 500 mL 100 μM ABA (Sigma); Group three was treated with 500 mL 100 μM SA (Sigma); Group four was treated with 500 mL 100 μM GA (Sigma); and Group five was treated with 500 mL 100 μM MeJa (Sigma). RNA was extracted from the leaves of three individual plants from each group after 2, 4, 8, and 12 h. The cDNA synthesis and real-time PCR analyses were performed using the TransStart® Tip Green qPCR SuperMix (TransGen Biotech), following the manufacturer instructions, and an ABI Step One PCR instrument (Applied Biosystems) was used. The results were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The following primers were used to target *KdSOC1* and *KdActin* (housekeeping gene): *KdSOC1* F: 5'-GAAGACTCAGATGAGGCGTATAGAGA-3' and *KdSOC1* R: 5'-CCCATTTCTGCGCTTGGA-3'; *KdActin* F: 5'-GACTATGAGGCTGAGTTGGAGAC-3' and *KdActin* R: 5'-TCAATGAAGGCTGGAAAAGG-3'.

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KdSOC1 expression under different environmental conditions

Total RNA was extracted from the leaves of both 28-day and 37-day LD and SD plants as well as plants under light, moderate, and severe drought stress (three biological replicates for each treatment). cDNA synthesis was performed using the PrimeScript II 1st Strand cDNA Synthesis kit (TaKaRa), following the manufacturer instructions. *KdSOC1* expression profiles were determined using reverse-transcription PCR (RT-PCR), and the conditions were as follows: pre-denaturing at 95°C for 180 s; 30 cycles of denaturing at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 60 s. The following primers were used to target *KdSOC1* and *KdActin*: *KdSOC1* F: 5'-ATGGTGCGAGGGAAGACTCAG-3' *and KdSOC1* R: 5'-GTTGCTTCCATTGTTGGAGCA-3'; *KdActin* F: 5'-GACTATGAGGCTGAGTTGGAGAC-3' and *KdActin* R: 5'-TATTGCCATACAAATCCTTCCTG-3'.

RESULTS

Analyses of *KdSOC1* cDNA and promoter sequences

Sequences amplified using 3'-RACE (Figure 1, lane 1) and 5'-RACE (Figure 1, lane 2) were 426 and 695 bp, respectively. To obtain the full-sequence length, the sequences were assembled using the known cDNA sequence of *contig1019*. The full-cDNA sequence length of *contig1019* was 1410 bp (Figure 1, lane 3), and BLAST results indicated that *contig1019* exhibited 70% shared homology with *C. cathayensis SOC1*. Therefore, *contig1019* was renamed *KdSOC1*. Based on the ORF Finder prediction, the possible ORF sequence length was 684 bp (from 468 to 1151 bp of the *KdSOC1* cDNA sequence 5'-end), which encoded 227 amino acids. The conserved domain search found an MADS-MEF2-like domain (from the third to the 78th amino acid) and a K-box domain (from the 75th to the 170th amino acid) in the KdSOC1 amino acid sequence (Figure 2). Genome walking results indicated that the full promoter sequence of *KdSOC1* was 1401 bp long. The possible multiple-hormone cis-acting element of the *KdSOC1* promoter was predicted by PlantCare, which indicated that there were four essential cis-acting elements, including an ABA-responsive element (ABRE), an MeJa-responsive element (CGTCA-motif), a GA-responsive element (GARE-motif), and an SA-responsive element (TCA-element) (Figure 3).

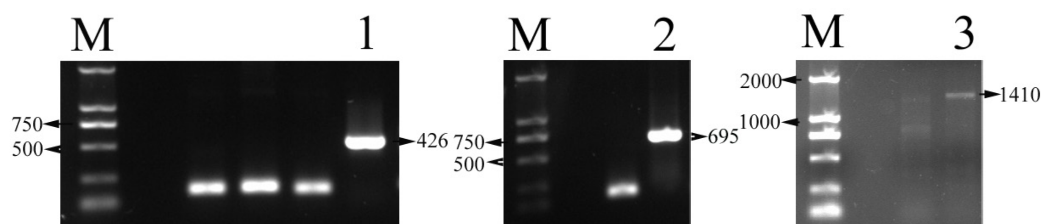

Figure 1. cDNA and gDNA amplification of *KdSOC1*. *Lane 1* = 3'-RACE product; *lane 2* = 5'-RACE product; *lane 3* = genome walking product; *lane M* = DNA marker (units: bp).

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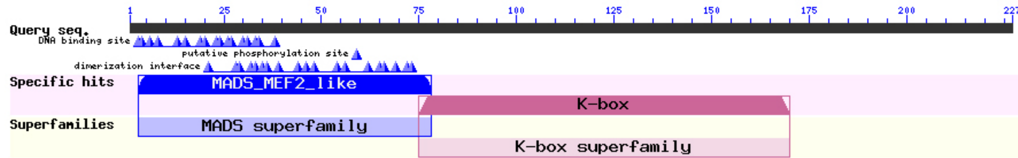

Figure 2. Putative conserved domains of the KdSOC1 protein.

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+ CACAATCTGA TGGACGAAAC GGTAATCAAA TTAAGTAGAC GTATATCTCG CAAATGCGTG CGGACGAGAG
- GTGTTAGACT ACCTGCTTTG CCATTAGTTT AATTCATCTG CATATAGAGC GTTTACGCAC GCCTGCTCTC

+ TAACAGGTTA AACCGATTTA TTTAAAAGTG CAGTTAAAAT CTACTTTAAT ATTATATAGT ACAGTTAGTT
- ATTGTCCAAT TTGGCTAAAT AAATTTTCAC GTCAATTTTA GATGAAATTA TAATATATCA TGTCAATCAA

+ GCCCACTTCA AATTAATTAT TAATGAATGA ATGAAAGAGT TTAAAAAATT TAATTGAAGT TTATGGGGGG
- CGGGTGAAGT TTAATTAATA ATTACTTACT TACTTTCTCA AATTTTTTAA ATTAACTTCA AATACCCCCC

+ AGATGTGTGA CAAACAGGCC CCATTTATAA TTTAGTTTAA TTAAGATGTG ACGTATATAC TTTTTTTCTA
                                                  "CGTCA-motif"
- TCTACACACT GTTTGTCCGG GGTAAATATT AAATCAAATT AATTCTACAC TGCATATATG AAAAAAAGAT

+ GAAAAAGGCC TGGGCAACAC ATAAGGCTAA AAAAGAAAAA AAAAAACCCT AACGAAAGGA CTACATTGAA
- CTTTTTCCGG ACCCGTTGTG TATTCCGATT TTTTCTTTTT TTTTTTGGGA TTGCTTTCCT GATGTAACTT

+ TAATTCAAGA AAAATTATTA CCTGGAGAGG GACCTACTTG CAAAAACCGT AGACAAGTCG GGTCGGGTTT
- ATTAAGTTCT TTTTAATAAT GGACCTCTCC CTGGATGAAC GTTTTTGGCA TCTGTTCAGC CCAGCCCAAA

+ ATGTCTGTCT CCTGACAAAG CGACGATAAT TGTTTAGTAA TCATATTATT TCTAGGTCGC GTTGTCGCCT
- TACAGACAGA GGACTGTTTC GCTGCTATTA ACAAATCATT AGTATAATAA AGATCCAGCG CAACAGCGGA

+ TCTCATTGGC TGCTCCCCCT TACCTCATCG TACGGACGAT GTTGACGTGT CAAAACCTAA CTTTCCTATT
                                           "CGTCA-motif"
- AGAGTAACCG ACGAGGGGGA ATGGAGTAGC ATGCCTGCTA CAACTGCACA GTTTTGGATT GAAAGGATAA

+ TTATTGCTGT TTTTTCTAAT TTTCCACTCC ACTTCACTCC ACTCCACTCC ACCACTACAT TTTTTCCCTT
- AATAACGACA AAAAAGATTA AAAGGTGAGG TGAAGTGAGG TGAGGTGAGG TGGTGATGTA AAAAAGGGAA

+ CTCGCTTTCT TTTCATCAAC AGTAAAATTT TAGCAATAAA ACAATGAAAA AAAATCAAAA GTGGAAATTT
- GAGCGAAAGA AAAGTAGTTG TCATTTTAAA ATCGTTATTT TGTTACTTTT TTTTAGTTTT CACCTTTAAA

+ TAAATATGCA AATTGAGAAT CGAGTAAAGT TTGAATTCTC GGCTCAGGGA TTTGCTTTTG CCATTTATGG
- ATTTATACGT TTAACTCTTA GCTCATTTCA AACTTAAGAG CCGAGTCCCT AAACGAAAAC GGTAAATACC

+ TGCTCTGCGT TTGATTGGTT TTGCTAAAAC AAGACCCGGC TGAGGTTTAG CTTCTTTCAT CTCATTTCAT
- ACGAGACGCA AACTAACCAA AACGATTTTG TTCTGGGCCG ACTCCAAATC GAAGAAAGTA GAGTAAAGTA

+ TCTTAGTTTG GGTTATTTGT GTGTGTGAAA TTACGGTTTT GCCATTGATC GGGTAGATGG ATATTTCTTT
- AGAATCAAAC CCAATAAACA CACACACTTT AATGCCAAAA CGGTAACTAG CCCATCTACC TATAAAGAAA

+ GCGGGGAACT GTGACCACAC AAATAAGAAG GCATCACATA ATAATTTGAT CAATTATTGA TACATTAAAA
- CGCCCCTTGA CACTGGTGTG TTTATTCTTC CGTAGTGTAT TATTAAACTA GTTAATAACT ATGTAATTTT

+ AATTTAAAAA AAATAAATGC ATTAGTTAAT TGATACGTAT AATTTAAATT TTTTCGAAAT GAGATTTGAC
- TTAAATTTTT TTTATTTACG TAATCAATTA ACTATGCATA TTAAATTTAA AAAAGCTTTA CTCTAAACTG
                             "ABRE"
+ AATTTTTCAT TGATGATGTT CACTCACACG TGGTATATTA TGTCGATCCA AAGTCACTTT TATTTTCTTT
- TTAAAAAGTA ACTACTACAA GTGAGTGTGC ACCATATAAT ACAGCTAGGT TTCAGTGAAA ATAAAAGAAA
   "TATA-box"
+ CTATTTTAAT GCTTGTCTTT TCTGGATGTG GAAAGAATTG GGGGTGGACA TGGGGGAAAT AAAGGTGGAG
- GATAAAATTA CGAACAGAAA AGACCTACAC CTTTCTTAAC CCCCACCTGT ACCCCCTTTA TTTCCACCTC
                                                 "CAAT-box"
+ AGATAAAGAG AAAGATGAGA TGGAAAGCTG TGGCGAAGAA ATTATGCAAT GTTGAAGTGA TCAACTAGCA
- TCTATTTCTC TTTCTACTCT ACCTTTCGAC ACCGCTTCTT TAATACGTTA CAACTTCACT AGTTGATCGT

+ GCAGAAGCAC AAGAAGAAGA CCACAACAGC CGCAAAACTG AAAACACATC ATCTTCATCT TCATCTTCCA
- CGTCTTCGTG TTCTTCTTCT GGTGTTGTCG GCGTTTTGAC TTTTGTGTAG TAGAAGTAGA AGTAGAAGGT

+ AACCCACAGC CCCAGAAATT AAGCTGATCA GCCAGAGTTT GAATCTTCAC CTGATTCTGA GCCACCCCAT
- TTGGGTGTCG GGGTCTTTAA TTCGACTAGT CGGTCTCAAA CTTAGAAGTG GACTAAGACT CGGTGGGGTA
             GARE –motif
+ TTCCTCTCAA TCTTAAACAG ACACCAATTT CTAATCTGGG TCTTCTCCTC TACTTCCCTA TTCTGACCTT
                                                         "TCA-element"
- AAGGAGAGTT AGAATTTGTC TGTGGTTAAA GATTAGACCC AGAAGAGGAG ATGAAGGGAT AAGACTGGAA

+ TCTCTTCTTA AATCAGTTGA CCAGGGCCT
- AGAGAAGAAT TTAGTCAACT GGTCCCGGA
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Figure 3. Prediction of cis-acting elements in the *KdSOC1* promoter. Yellow shading: “ABRE”; CACGTG: cis-acting element involved in abscisic acid responsiveness; pink shading: CGTCA-motif; CGTCA: cis-acting regulatory element involved in MeJa responsiveness; brown shading: GARE-motif; AAACAGA: gibberellin-responsive element; red shading: TCA-element; TCAGAAGAGG: cis-acting element involved in salicylic acid responsiveness; blue shading: TATA-box; TTTTA: core promoter element around -30 of the transcription start; green shading: CAAT-box; CAAT: common cis-acting element in promoter and enhancer regions.

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Putative KdSOC1 protein structure analysis

The results of the ProtParam tool, which checked the chemical and physical properties of KdSOC1, indicated that its possible chemical formula was $C_{1105}H_{1844}N_{336}O_{360}S_{9}$, and its molecular mass was predicted to be 25885.3 D. Moreover, the protein consisted of 19 amino acids, including 13.2% glutamic acid and 0.4% histidine, and it contained 38 acidic and 41 alkaline amino acids. The theoretical isoelectric point of this protein was 8.61, and the aliphatic index, grand average of hydropathicity, and instability indices were 75.68, -0.864, and 60.65, respectively. There was no transmembrane domain, and a signal peptide was found in the protein (TMHMM Server 2.0 prediction; SignalP4.0 Server).

The PSIPRED prediction of the KdSOC1 secondary structure showed that it contained 40.67% helices and 50.22% coils. The tertiary structures of AtSOC1, PsSOC1, CcSOC1, and KdSOC1 were synthesized using SWISS-MODEL (Figure 4). KdSOC1, PsSOC1, and CcSOC1 proteins shared high similarity between their secondary structures, and no ligands were found in these proteins. AtSOC1 exhibited a coiled secondary structure, which might affect its bioactivity. Therefore, KdSOC1 might manipulate other genes that are located downstream.

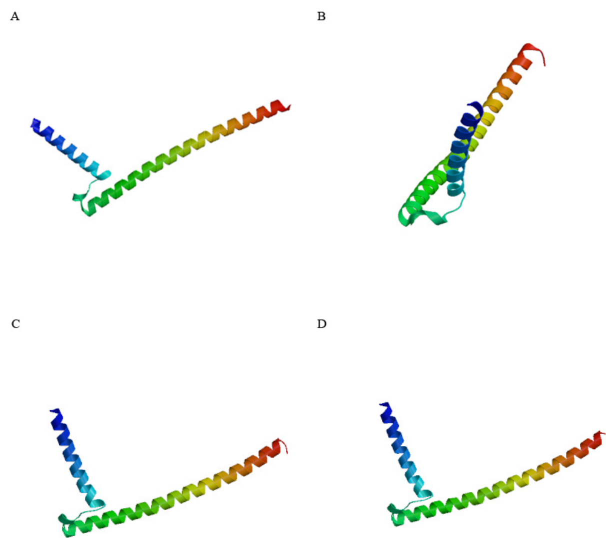

Figure 4. Putative tertiary structure of KdSOC1 and SOC1 proteins from other species. **A.** *Carya cathayensis* SOC1 protein, **B.** *Arabidopsis thaliana* SOC1 protein, **C.** *Kalanchoe daigremontiana* SOC1 protein, and **D.** *Paeonia suffruticosa* SOC1 protein.

Phylogenetic analysis of KdSOC1

The homology analysis (BLAST) of *KdSOC1* and *SOC1* from other species indicated that it shared 70% homology with *C. cathayensis* and *P. suffruticosa*, 69% homology with *P. yedoensis*, and 68% homology with *G. hirsutum*, *P. salicina*, *P. mume*, and *G. max*. The multiple-sequence alignment of KdSOC1 sequences with sequences from 19 different species showed that the most conserved KdSOC1 region was the MADS domain, and divergence in its K-box domain suggested that *KdSOC1* possessed a novel function (Figure 5).

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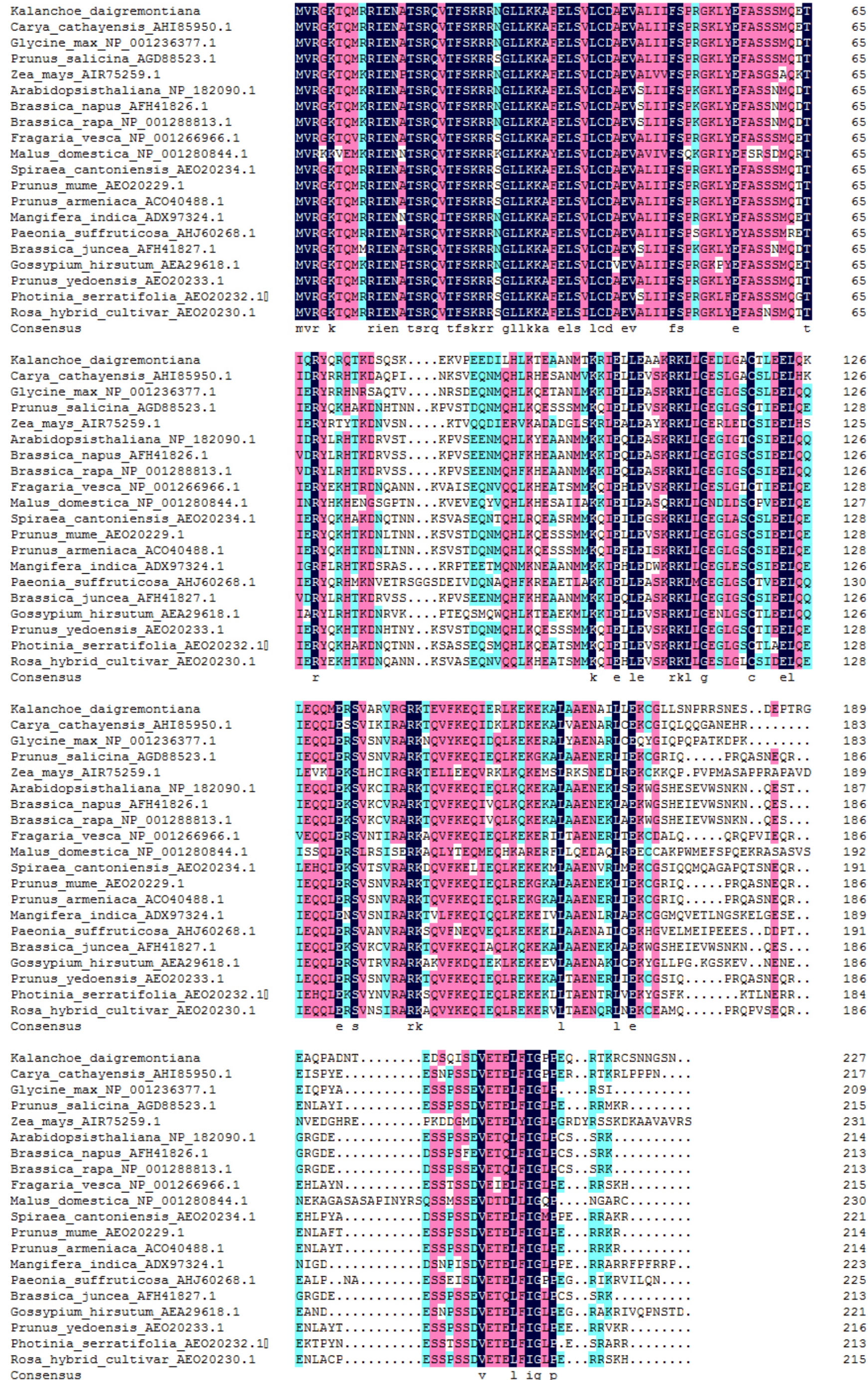

Figure 5. Multiple-sequence alignment of *KdSOC1*. Black shading: 100% homology; pink shading: >75% homology; blue shading: >50% homology.

Based on the results of the KdSOC1 multiple-sequence alignment, a phylogenetic tree was constructed using the neighbor-joining method implemented in MEGA 6 (Figure 6). This result indicated that *KdSOC1* can be classified using standard plant taxonomy, and it suggests that its encoded protein reflects close evolutionary relationships with *SOC1* from *P. suffruticosa* and *G. hirsutum*.

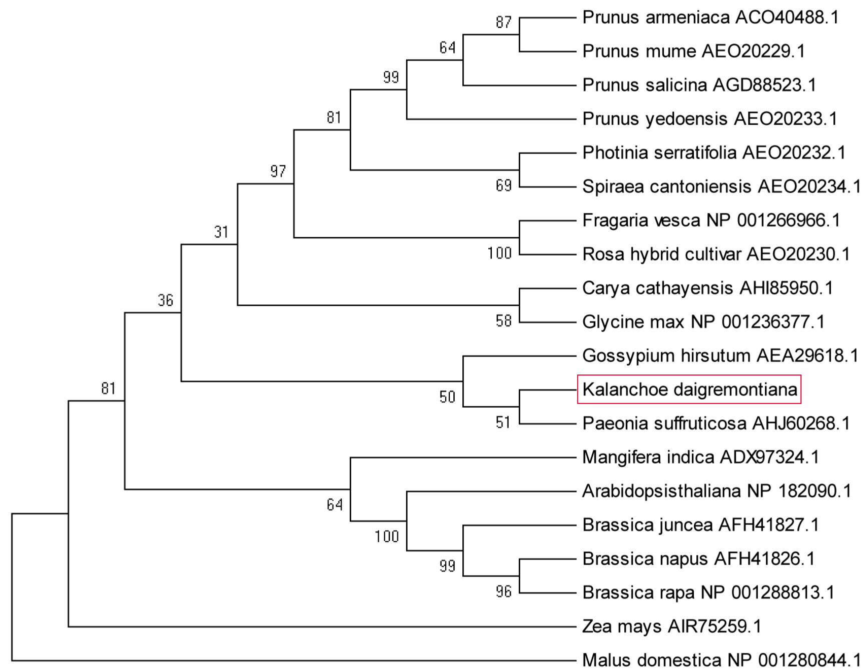

Figure 6. KdSOC1 phylogenetic tree. The tree was constructed using the neighbor-joining method with 1000 bootstrap replicates as implemented in the MEGA6 software.

Hormone induction of *KdSOC1* expression

With the prediction of multiple-cis-acting elements in the *KdSOC1* promoter and its transcription factor identity, hormones should play essential roles in the regulation of its expression. *KdSOC1* was upregulated to the highest level at 4 h after ABA, GA, and MeJa treatments, and it gradually decreased from 8 to 12 h after hormone treatments (Figure 7). The gene expression patterns following SA treatment exhibited the same trend seen in other hormones. However, expression peaked at a relatively higher level than others, and no obvious repression effects were observed 2 h after treatment (Figure 7). In general, *KdSOC1* could be induced 4 h after treatments with the four hormones, but SA and GA had long-lasting effects on gene expression. Moreover, ABA, GA, and MeJa had negative effects on *KdSOC1* expression during the first 2 h after hormone treatment.

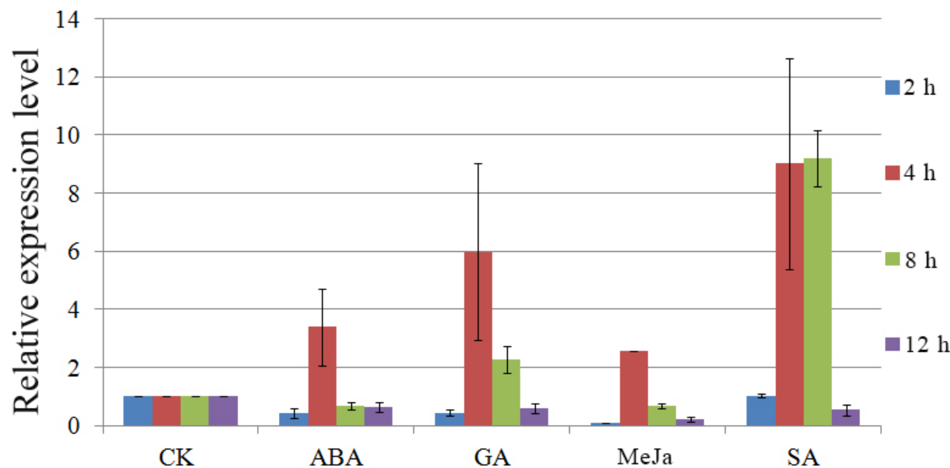

Figure 7. *KdSOC1* expression under different hormone induction conditions. Mean fold-change was calculated relative to CK (treated with H_2O). Error bar represents SE (N = 3). ABA = abscisic acid; GA = gibberellin; MeJa = methyl jasmonate; SA = salicylic acid.

KdSOC1 expression under varied photoperiod conditions

Despite a common understanding of constitutive formation types of *K. daigremontiana* plantlets, other environmental conditions should also be considered because of the presence of *KdSOC1*. The photoperiod is an essential environmental factor associated with the embryogenesis of SAM and organogenesis of flower meristems, and *SOC1* is an integrator for photoperiod signal transduction and activation of SAM differentiation. The leaf curve margin for 28-day LD plants (Figure 8A) was more obvious than 28-day SD plants (Figure 8B), and the leaf color of 28-day LD plants was greener than that observed in SD plants. The *de novo* formation of plantlets first appeared in 37-day LD plant leaf margins (Figure 8C), but there were no signs of plantlets in 37-day SD plants (Figure 8D). In 37-day LD and SD plants, *KdSOC1* was highly expressed in the leaves (Figure 8E). However, its expression was sharply downregulated in 28-day LD plants and silenced in 28-day SD plants. Therefore, LD was a core stimulus for *K. daigremontiana* plantlet morphogenesis, particularly regarding the regulation of *KdSOC1* expression.

KdSOC1 expression under drought stress conditions

The drought-responsive element in *KdSOC1* inspired us to examine *KdSOC1* expression under drought stress conditions. The *K. daigremontiana* plants under light drought stress (20% soil water content) possessed more mature plantlets than well-watered plants (CK 1, Figure 9, first panel). However, plants under moderate (10% soil water content) and severe (3% soil water content) drought stress exhibited significantly fewer plantlets and small, curly leaves compared to well-watered plants (CK 2, Figure 9, second and third panels). The *KdSOC1* RT-PCR results indicated that *KdSOC1* was expressed more in the leaves (with plantlets) of plants cultivated under stress conditions than in well-watered plants (Figure 9, fourth panel). Moreover, increasing levels of drought stress elevated *KdSOC1* expression in leaves both with and without plantlets, but the

expression levels were lower than those of well-watered plants (CK 2 and 3), with the exception of light drought-stressed plants (Figure 9, fourth panel). Taken together, the results suggested that *KdSOC1* was closely involved in the drought stress stimulation of *K. daigremontiana* plantlet formation.

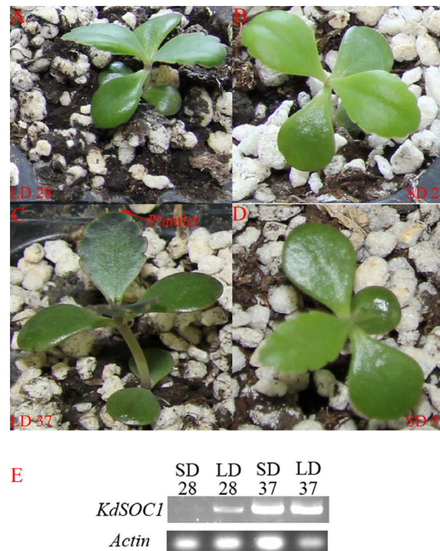

Figure 8. *KdSOC1* expression patterns during plantlet formation under LD and SD induction conditions. **A. B.** 28-day LD and SD plants. **C. D.** 37-day LD and SD plants. **E.** RT-PCR *KdSOC1* expression results in *Kalanchoe daigremontiana* leaves during LD and SD induction. LD = long-day condition; SD = short-day condition.

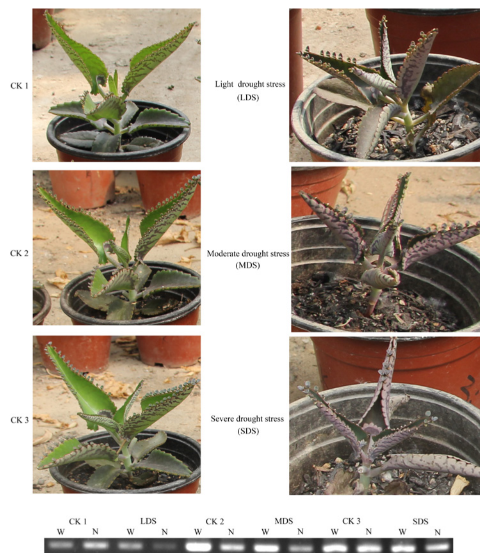

Figure 9. *KdSOC1* expression patterns during plantlet formation under drought stress conditions. CK1, CK2, and CK3 are well-watered treatment counterparts to light, moderate, and severe drought-stressed plants. W: leaves with plantlets. N: leaves without plantlets.

DISCUSSION

Distinctive molecular structure of *KdSOC1*

The typical structure of MADS family genes consists of four domains, including the MADS (M) domain, the intervening (I) domain, the coiled-coil keratin-like (K) domain, and the C-terminal (C) domain (Theissen et al., 1996; Kaufmann et al., 2005). The M domain is a specific DNA-binding unit, which also helps dimerize MADS-box proteins (Immink et al., 2002), and the K domain is important for protein interaction in that it shapes the complexity of protein formation (Egea-Cortines et al., 1999). The I domain is interspaced between the M and K domains, and it determines the specific binding between protein and DNA binding dimers (Masiero et al., 2002). The C-terminal is downstream of the K domain, and it is a key component of protein complex formation (Honma and Goto, 2001). Regarding *KdSOC1*, the I and C domains were absent, and the M domain overlapped the K domain (Figure 2), which might represent an evolutionary consequence in *K. daigremontiana*. Furthermore, these changes might allow *KdSOC1* to encode a protein with a simplified conformation and broad downstream binding ability, thus suggesting a new biological function of *KdSOC1*.

KdSOC1 expression under different hormone induction conditions

According to the results of this study, *KdSOC1* mainly responded to GA and SA induction. GA is an essential endogenous hormone that regulates plant growth, cell division, and elongation, and it acts as a switch between vegetative and sexual growth (Bonhomme et al., 2000; Mutasa-Göttgens and Hedden, 2009). In *Arabidopsis*, GA induces the expression of the GAMYB protein, which activates the expression of *AtSOC1* and *AtLFY*. Thus, the cooperation of GA and *SOC1* might be a key factor for the tight control of flowering in *Arabidopsis* (Mutasa-Göttgens and Hedden, 2009; Mouhu et al., 2013). GA-induced expression of *KdSOC1* might function in the SAM of the leaf margin, which sequentially recruits other genes to complete plantlet formation. SA-induced effects on *KdSOC1* expression provided additional information regarding the role of hormones in plantlet morphogenesis regulation in that SA signaling is a key regulator associated with plant biotic and abiotic resistance mechanisms, including pathogen invasion (Shah, 2003), drought (Chini et al., 2004), and cold stress (Kang and Saltveit, 2002). However, the results of another study indicated that SA was also involved in plant morphogenesis, including root and stem elongation (Stevens et al., 2006).

KdSOC1 expression under different photoperiod and drought stress conditions

The high expression levels of *KdSOC1* under LD conditions were associated with the rapid emergence of plantlets, and similar results were observed in studies of *Arabidopsis* flowering. For instance, *AtSOC1* is an integrator associated with the perception of photoperiod changes, and it acts as an essential player in both embryogenesis and organogenesis (Kimura et al., 2015). Plantlet formation can be seen as a simplified flowering process where somatic cells formed in the SAM of the leaf margin can differentiate into new plantlets. In this study, the obvious leaf curve structure and greener leaf color in 28-day LD plants also indicated the fast maturation of the pre-organogenesis status of leaves. Therefore, based on its swift expression changes in LD plants from 28 to 37 days and its absence in 28-day SD plants, *KdSOC1* might be involved in the embryogenesis and organogenesis of plantlet transition. This phenomenon was also found in *FaSOC1*, which was overexpressed in *Fragaria vesca*, and this result indicated that only certain

FaSOC1 expression patterns could lead to successful growth phase transitions (Mouhu et al., 2013). The elevated *KdSOC1* expression levels in 37-day SD plants also suggested an unknown compensation mechanism associated with *K. daigremontiana* plantlet morphogenesis.

The drought-induced plantlet formation, accompanied by *KdSOC1* upregulation, was a novel finding in this study, and it might represent another answer regarding the functional evolution of the gene in *K. daigremontiana*. The habitat of *K. daigremontiana* in tropical areas might increase the pace of embryo maturation and organ differentiation during plantlet formation, but it may also confer superior adaptability to *K. daigremontiana* under drought stress conditions. Furthermore, *KdSOC1* might stimulate plantlet formation under light drought stress conditions. It might also sustain plantlet maturation and survival during moderate to severe drought stress because of the continuous upregulation of *KdSOC1* in leaves both with and without plantlets. Therefore, the future identification of *KdSOC1* functions might reveal additional key points that will help elucidate the transition network between the embryogenesis and organogenesis of plantlet formation in response to different environmental conditions.

ACKNOWLEDGMENTS

Research supported by the “Graduate Training and Development Program of Beijing Municipal Commission of Education” (#BLCXY201514).

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