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Original Paper

SDR6 is involved in regulation of flowering time in *Arabidopsis thaliana*

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Abstract Identification of flowering-time related genes in *Arabidopsis thaliana* has very important and meaningful to understand the regulation mechanism of the flowering-time. One late flowering mutant plant which displays a delayed transition to flowering was obtained from the *Arabidopsis* T-DNA insertion mutant library. *SDR6*, which encodes a short-chain dehydrogenase/reductase containing a NAD(P)-binding domain in *Arabidopsis*, has been identified as a novel flowering-time gene in *Arabidopsis* by further analyzing of *SDR6* complementing plants. The *sdr6* plants displayed later flowering than wild-type plants both in long and short days, but flowered later in short day than in long day. The flowering-time of *SDR6* complementing plants is similar to that of wild-type plants. The late-flowering phenotype of *sdr6* plants was reversed by gibberellin and vernalization treatments, which is similar to those of several mutants in the autonomous pathway. Compared with the wild-type, expression levels of *FLC*, *LD*, *FVE*, and *SOC1* genes, key components of the autonomous pathway, were significantly altered in *sdr6* mutants. However, expression levels of the key genes in the photoperiod, gibberellin, and vernalization pathways were not obviously different. Therefore, this gene may be involved in the autonomous flowering pathway to regulate the *Arabidopsis* flowering.

Key words: *Arabidopsis thaliana*, delay of flowering, *SDR6*, autonomous pathway.

The transition of flowering plants from the vegetative phase to the reproductive phase is an important event. Flowering determination, also known as the induction of flower bud formation, is the first stage of the plant reproductive growth and determines the flowering-time of flowering plants. The flowering-time of some plants is mainly affected by environmental factors such as light, temperature, moisture, and nutrient condition, and they can blossom under optimum conditions. Other plants are not sensitive to environmental factors, and they can blossom under appropriate internal conditions that often determine vegetative growth (Flachowsky et al. 2012).

According to early reports, at least four pathways control the transition of *Arabidopsis* flowering, including the photoperiod pathway, autonomous pathway, gibberellin (GA) pathway, and vernalization pathway (Kemi et al. 2013; Mulekar and Huq 2012; Osnato et al. 2012). In *Arabidopsis*, several pathways converge to regulate the expression of at least three genes that promote flowering, namely, the pathway integrators *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) (Lee J and Lee I 2010), *FLOWERING LOCUS T* (*FT*), and *LEAFY* (*LFY*) (Moyroud et al. 2010). For example, in the photoperiod pathway, the transcription factor CONSTANS (CO) activates the expression of *SOC1* through *FT*. The activation of *SOC1* expression has to overcome the repressive action of one of the most potent inhibitors of flowering in *Arabidopsis*, i.e., the MADS-domain protein encoded by *FLOWERING LOCUS C* (*FLC*) (Deng et al. 2011). The autonomous and vernalization pathways ultimately promote flowering by releasing *SOC1* from repression by *FLC* (Moon et al. 2003; Stinchcombe et al. 2005; Willmann and Poethig 2011). The mutants of genes in the autonomous pathway, such as *LUMINIDEPENDENS* (*LD*) and *FVE*, are late flowering because they fail to reduce the expression of *FLC* (Helliwell et al. 2006).

In our previous research, a susceptible mutant to *Botrytis cinerea* has been achieved by screening from the *Arabidopsis* T-DNA insertion mutant library.

Abbreviations: *CO*, *CONSTANS*; *FLC*, *FLOWERING LOCUS C*; *FT*, *FLOWERING LOCUS T*; GA, gibberellin; *LD*, *LUMINIDEPENDENS*; LD, long days; *LFY*, *LEAFY*; MS, Murashige and Skoog; SDR, short-chain dehydrogenase/reductase; SD, short days; *SOC1*, *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1*; *sdr6*/*SDR6*, *SDR6* complementing plant.

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SDR6 (AT1G01800 or *T1N6_22*), a gene encoding a short-chain dehydrogenase/reductase that contains a NAD(P)-binding domain in *Arabidopsis thaliana*, has been isolated and identified as a resistant-related gene of *Arabidopsis* against *B. cinerea* (Xing et al. 2012). We have also found that *sdr6* plants delay the transition of flowering under long-day conditions, and show no homology with any known flowering-related gene. Thus, we have speculated that *SDR6* is a novel gene involved in the regulation of flowering time. In this study, the function of *SDR6* in regulating the flowering-time of *Arabidopsis* was identified by analyzing the flowering-time of wild-type, *sdr6*, and *SDR6* complementing plants. The responsiveness of *sdr6* to GA and vernalization treatments was analyzed. The expression levels of some critical genes in the different flowering pathways in the wild-type, *sdr6*, and *SDR6* complementing plants and the tissue-specific expression pattern of *SDR6* were analyzed by semi-quantitative RT-PCR. These works will contribute to understand the regulatory mechanisms of *SDR6* in the control of floral transition.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana Columbia (Col-0; wild-type) was provided by Dr. Xia of Hong Kong Baptist University. Based on a bioassay, *sdr6* was screened from the *Arabidopsis* IGDB-XVE mutant library provided by Dr. Jianru Zuo of the Institute of Genetics and Developmental Biology (Chinese Academy of Sciences) and was identified that it is a loss-of-function mutation in which a T-DNA is inserted into the 3′ untranslated region of *SDR6* (AT1G01800 or *T1N6_22*) gene. *SDR6* complementing plant (*sdr6*/*SDR6*) is a gain-of-function mutation in which the *SDR6* cDNA sequence under the control of CaMV 35S promoter is introduced into *sdr6* plants (Xing et al. 2012). The *sdr6* and one independent line of *SDR6* complementing plants were provided by the Mycotoxin and Molecular Plant Pathology Laboratory (Agricultural University of Hebei, China). *Arabidopsis* plants were grown under controlled environmental conditions (22°C and 80% RH) and illumination with cool-white fluorescent lights for a 16 h/8 h light/dark cycle during long days (LD) and 8 h/16 h light/dark cycle during short days (SD).

Domain predictions and phylogenetic analysis

The conserved domain of SDR6 was verified using the Blast-based NCBI conserved domain search engine. Amino acid sequences of SDR6 were obtained from the *Arabidopsis* website. Phylogenetic analysis of the SDR6 and related proteins was performed using DNAMAN software. The three-dimensional mode of SDR6 was constructed by SWISS-MODEL and viewed using 3D Molecule Viewer. For all amino acid analysis, the regions of SDRs used for alignment began at the conserved Rossmann-fold domain.

GA and vernalization treatments

Seeds of wild-type, *sdr6* and *SDR6* complementing plants were surface-sterilized by treating them sequentially in 70% ethanol for 2 min and 30% clorox solution for 10 min and rinsing four times in sterile water. For GA treatments, sterilized seeds were placed on MS (Murashige and Skoog) plates containing 100 μmol/l GA3. After germinating at 4°C for 2 days, the resulting plants were grown under in short- and long-day conditions, and a GA3 solution of 100 μmol/l was sprayed once a week until flowering. To examine vernalization effects, plants were germinated and grown at 4°C for 28 days in the short-day and long-day conditions and transferred to the normal growth temperature (23°C).

Flowering-time measurements

Seeds of wild-type, *sdr6*, and *SDR6* complementing plants were simultaneously sterilized and germinated on MS plates. After 3 weeks, seedlings were transplanted into soil under long- or short-day conditions. Flowering-time was measured as the number of rosette leaves at flowering and the days from sowing to floral initiation. In all, twenty to thirty individual plants were assayed and data were averaged to evaluate statistical significances among treatments.

Semi-quantitative RT-PCR analysis

Transcript levels of flowering-time genes, e.g., *CO*, *FT*, *GI*, *SOC1*, *FLC*, *FVE*, *LD*, *SPY*, *VRN1*, and *VRN2* in wild-type, *sdr6* and *SDR6* complementing plants were measured using semi-quantitative RT-PCR and gene-specific primers (Table 1). The seedlings were sampled at Zeitgeber time (ZT) 8 h in SD when *CO*, *FT* and *GI* were expressed at their peaks (Wu et al. 2008). To examine tissue-specific expression pattern of *SDR6* in the wild-type, rosette leaves, cauline leaves, flowers, siliques, stems, and roots were collected. Total RNA of the wild-type, *sdr6*, *SDR6* complementing plants and different tissues were prepared with Trizol reagent (Invitrogen, USA) respectively. Reverse-transcription of the total RNA was done with RevertAid M-MuLV Reverse Transcriptase (Sangon, China). Synthesized cDNA served as the PCR template, while 18S rRNA was used for equal loading. The RT-PCR runs were 24 to 31 cycles, depending on the linear range of PCR production for each gene, with each cycle at 94°C for 30 s, annealing temperature for 30 s, and 72°C for 1 min, with a final cycle at 72°C for 10 min. All RT-PCR experiments were performed in three biological replicates and each biological sample was analyzed in triplicate. All date were statistic by SPSS.

Results

sdr6 is late flowering both in long and short days

Under long-day conditions (16 h light), the flowering-time of *sdr6* was later than those of wild-type and *SDR6* complementing plants (*sdr6*/*SDR6*). Consistent with the flowering-time, the number of rosette leaves of *sdr6* was significantly higher than that of wild-type and

Table 1. PCR primers used in this study.

Gene name	TAIR gene No.	Primer sequence	Annealing temperature	Number of PCR cycles
CO	AT5G15840	5′-CTCCTCGGCTTCGATTTCTC-3′ 5′-CATTAACCATAACGCATACATTTC-3′	55°C	27
FT	AT1G65480	5′-ACTATATAGGCATCATCACCGTTCGTTACTCG-3′ 5′-ACAACTGGAACAACCTTTGGCAATG-3′	60°C	30
GI	AT1G22770	5′-TTTTTCCCGAATCATTTGATG-3′ 5′-ATCCAGCCTTAGGCTCTGAAG-3′	54°C	28
SOC1	AT2G45560	5′-ATGCAACAAGCAGACAAG-3′ 5′-CAAGGTAACCCAATGAAC-3′	48°C	30
FLC	AT5G10140	5′-GGTGATATGGTGCTGTGTACAGTTCC-3′ 5′-AGAATGGCACAGGAATGAGC-3′	60°C	31
FVE	AT2G19520	5′-GACGAGAAGTACTCTCAGTGG-3′ 5′-ACAACTGACTTGTCCTTGC-3′	55°C	27
LD	AT4G02560	5′-CGGAAAATAACAATGCC-3′ 5′-TCCCACCTTACTAGAAATGC-3′	47°C	28
SPY	AT3G11540	5′-GCTTCACAAGATTACACCCTC-3′ 5′-CGTAGTATTTGGATAGCCGA-3′	53°C	28
VRN1	AT3G18890	5′-TGTTTGGCGTGTAGGAC-3′ 5′-TGGACTTTGATGAACCC-3′	48°C	30
VRN2	AT4G16845	5′-TTTGCTCTATGCTATGTGG-3′ 5′-AGTGACTGAGGTATTACGG-3′	49°C	30
SDR6	AT1G01800	5′-CGGCAAACTCGATATTCTGGT-3′ 5′-CAAAAGTTGGAGACATTGGCG-3′	55°C	27
18S rRNA	AT3G41768	5′-GTTGCAGTTAAAAAGCTCGT-3′ 5′-TTGATTTCTCATAAGGTGCC-3′	55°C	24

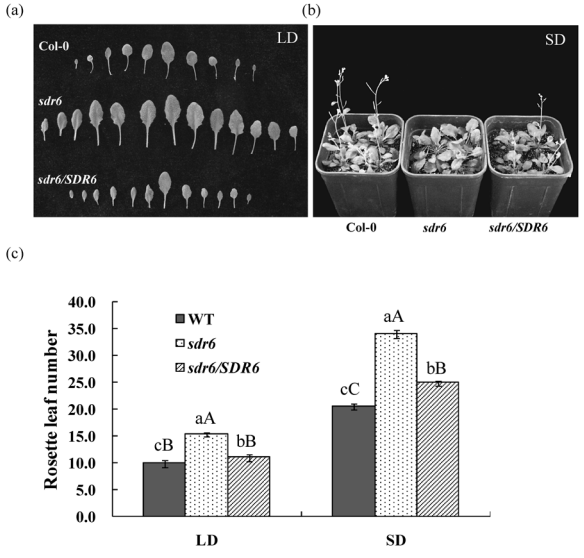

Figure 1. Daylength effects on flowering of *sdr6*. (a) 35-day-old wild-type (Col-0), *sdr6*, and *SDR6* complementing plants grown in long days (LD). (b) Plants grown in short days (SD). (c) Flowering-time measurements of *sdr6*, Col-0 and *SDR6* complementing plants under LD or SD. For each line, twenty to thirty plants were measured and data were averaged to evaluate statistical significances among treatments. The different letters in the figure indicate significant difference at $p<0.05$ and $p<0.01$ (lowercase letter at $p<0.05$, uppercase letter at $p<0.01$).

sdr6/*SDR6* plants (Figures 1a and 1c). Also under short-day conditions (8 h light), the *sdr6* flowered significantly later than wild-type and *sdr6*/*SDR6* plants (Figures 1b and 1c). In addition, we also obtained a loss-of-function mutation in which a T-DNA is inserted into the fifth exon of the same gene (SALK_100214; *sdr6*-1). The *sdr6*-1 was also late flowering to a similar degree as *sdr6* both in long and short days (data not shown). These observations unequivocally confirm that the *SDR6* played an important role in the flowering of *Arabidopsis*.

SDR6 encodes a NAD(P)-binding Rossmann-fold superfamily protein

Sequence searches in databases revealed that SDR6 encoded a NAD(P)-binding Rossmann-fold superfamily protein, which belongs to the short-chain dehydrogenase/reductase (SDR) family of proteins (Figure 2a). SDRs are a functionally diverse family of oxidoreductases that have a single domain with a structurally conserved Rossmann fold (alpha/beta folding pattern with a central beta-sheet), a NAD(P)(H)-binding region, and a structurally diverse C-terminal region. Classical SDRs are typically about 250 residues long, whereas extended SDRs are approximately 350 residues. Sequence identity between different SDR enzymes typically ranges within 15–30%. The 3D structure of SDR6 domains suggested significant structural homology with *Arabidopsis* SDR4 (Figure 2b). Using SDR6 and other SDR domain proteins, a phylogenetic tree was constructed to update new functional clades by DNAMAN (Figure 2c). SDR6 protein (At NP_001077442.1) displayed high homology with other SDR domain proteins from *Arabidopsis* (At retSDR4) and (At SDR NP_563635.1). To date, the possible involvement of SDR6 in regulating flowering in *Arabidopsis* has not been studied.

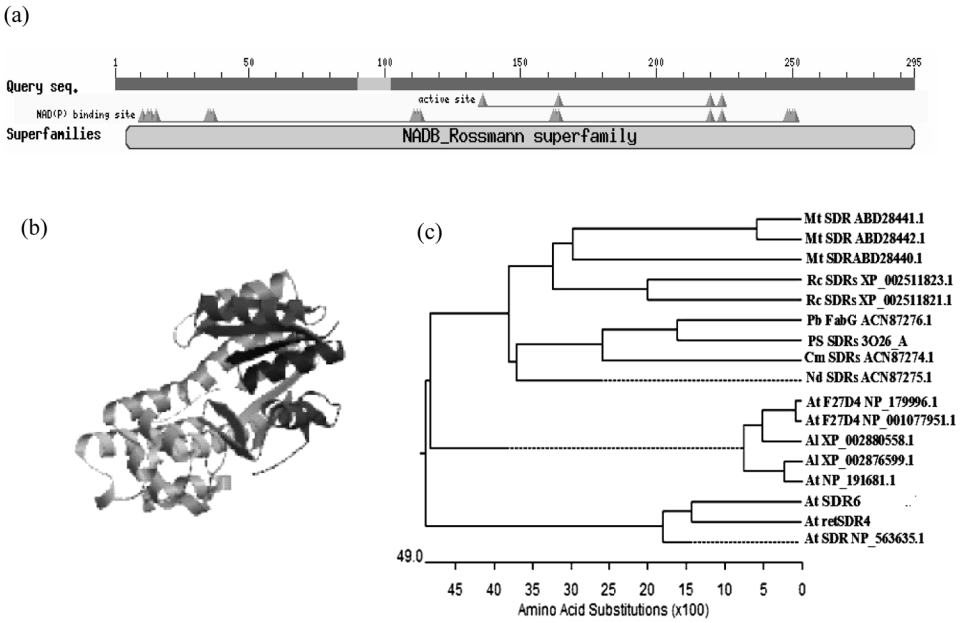

Figure 2. Sequence and phylogenetic analysis of SDR6. (a) Conserved domains of the SDR6 protein of *Arabidopsis*. (b) The 3D structure of SDR6 predicted by SWISS-MODEL. (c) Phylogenetic tree showing the relationship between SDR6 and other SDR domain proteins. At, *Arabidopsis thaliana*; Al, *Arabidopsis lyrata*; Mt, *Medicago truncatula*; Rc, *Ricinus communis*; Pb, *Papaver bracteatum*; Cm, *Chelidonium majus*; Nd, *Nandina domestica*.

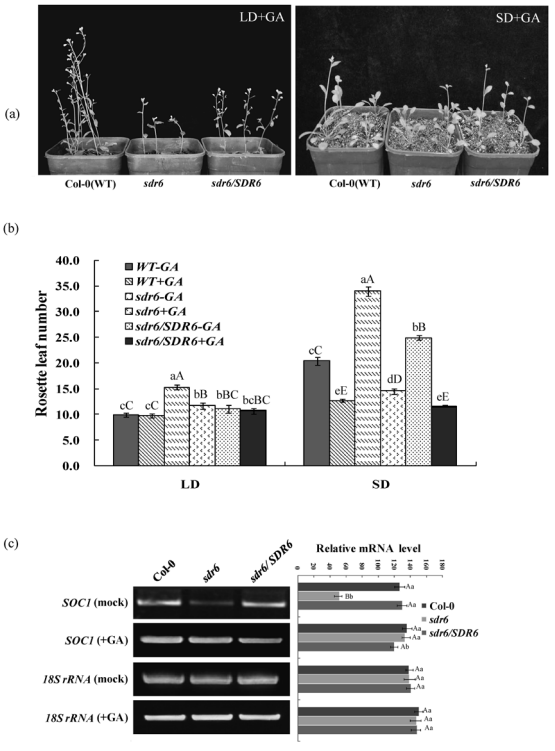

Figure 3. Effects of GA on flowering-time. (a) Flowering phenotype of Col-0, *sdr6* and *SDR6* complementing plants under LD or SD. Plants treated with GA3 once a week and grown under LD and SD. (b) Flowering-time measurements of Col-0, *sdr6* and *SDR6* complementing plants under LD or SD. For each line, twenty to thirty plants were scored. (c) Expression level of *SOC1* in Col-0, *sdr6* and *SDR6* complementing plants treated with GA3. RNA was isolated from 21-day-old grown Col-0, *sdr6* and *SDR6* complementing plants at Zeitgeber time (ZT) 8 h in SD and these plants with GA3 once a week. *18S rRNA* was used as a control.

sdr6 is responsive to GA treatments

A phenomenon consistent with that of autonomous pathway mutants such as *flk* and *fca*, which respond to GA (Lim et al. 2004; Mouradov et al. 2002), was observed. With GA treatment, the flowering-time of *sdr6* plants became earlier than that of untreated plants, at a rosette leaf of 11.8 (LD) and 15 (SD) (Figures 3a and 3b), although it is later than the wild-type. The untreated plants flowered at a rosette leaf number of 15 (LD) and 34 (SD) (Figure 3b). The days to flowering were also shortened by GA. Consistent with this phenotype, expression of *SOC1* was significant up-regulated in GA-treated *sdr6* plants, and was similar to that of the wild-type and *sdr6*/*SDR6* plants treated with GA (Figure 3c).

sdr6 is responsive to vernalization treatments

To examine the responsiveness of *sdr6* to vernalization, the *sdr6* plants were germinated and grown at 4°C for 4 weeks and transferred to normal growth condition (23°C). Vernalization also greatly stimulated the flowering of *sdr6* (Figure 4a). The vernalization-treated *sdr6* initiated flowering at a rosette leaf number of 11.1 (LD) and 14 (SD), which is comparable to that of the wild-type (Figure 4b). These findings suggested that vernalization treatment rescues the *sdr6* phenotype. We also noted that the expression level of *SOC1* in the vernalization-treated *sdr6* is similar to that of the wild-type and *sdr6*/*SDR6* plants treated with vernalization (data not show).

SDR6 deficiency altered the expression of critical genes in autonomous pathway

To investigate further the regulation mechanism underlying the late-flowering phenotype of *sdr6*, transcript levels of critical genes in the different flowering pathways were examined by using semi-quantitative RT-PCR. Significant down-regulation in *sdr6* was noted for the autonomous pathway genes *SOC1*, *FVE* and *LD* (Figure 5). Note that the flowering repressor gene *FLC* is significant up-regulated in *sdr6* (Figure 5). However, expression levels of key components *CO* and *GI* in the photoperiod pathway; *SPY* in the GA pathway (Tseng et al. 2004); and *VRN2* in the vernalization pathway (Gendall et al. 2001; Levy et al. 2002) were not significantly different from the wild-type and *sdr6*/*SDR6* plants. These results suggested that the autonomous pathway is compromised in *sdr6* plants, which led to later flowering when compared with the wild-type.

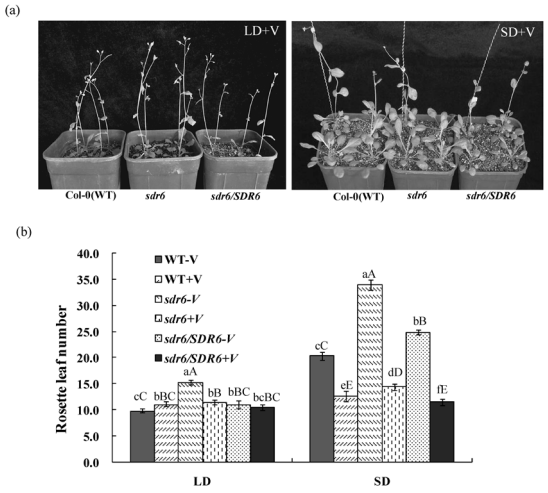

Figure 4. Effects of vernalization on flowering-time. (a) Flowering phenotype of *sdr6*, Col-0 and *SDR6* complementing plants under LD or SD. Plants germinated and grown at 4°C for 4 weeks and transferred to LD or SD. (b) Flowering-time measurements of *sdr6*, Col-0 and *SDR6* complementing plants under LD or SD. For each line, twenty to thirty plants were scored.

SDR6 is expressed constitutively in Arabidopsis

The tissue-specific expression pattern of *SDR6* in the wild-type was examined by semi-quantitative RT-PCR. The results revealed that *SDR6* was highly expressed in rosette leaves, cauline leaves, flowers, stems, and roots, but less expressed in siliques (Figure 6). Similar expression profiles of *SDR6* were obtained from TAIR (http://www.arabidopsis.org/home.html).

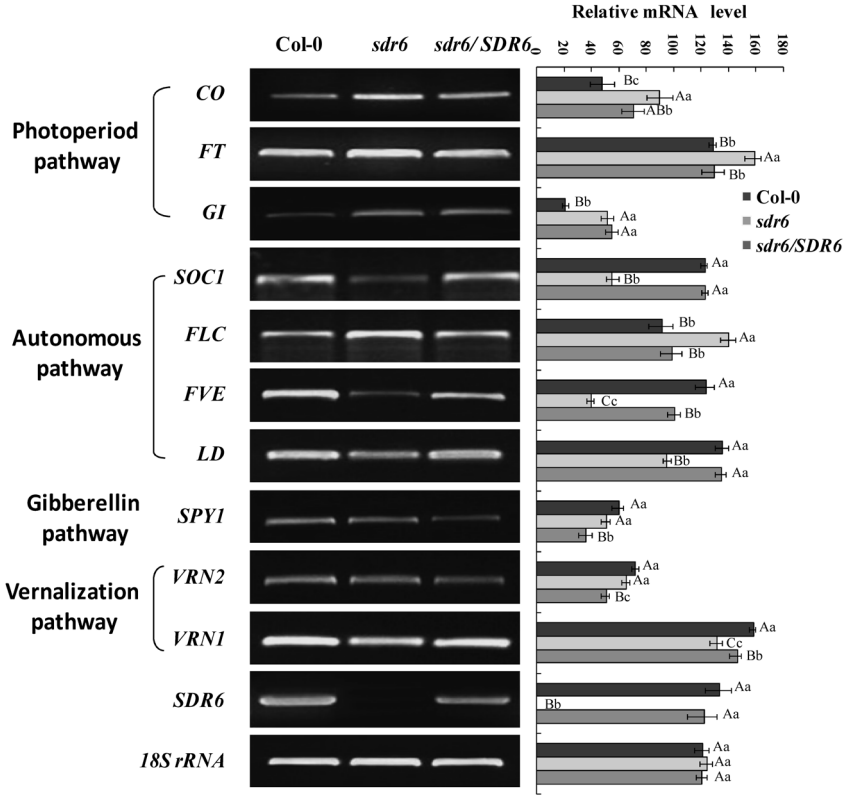

Figure 5. Expression levels of flowering-related genes in wild-type, *sdr6* and *SDR6* complementing plants as determined by semi-quantitative RT-PCR. Semi-quantitative RT-PCR data were normalized to 18S *rRNA*. Error bars represent standard deviations.

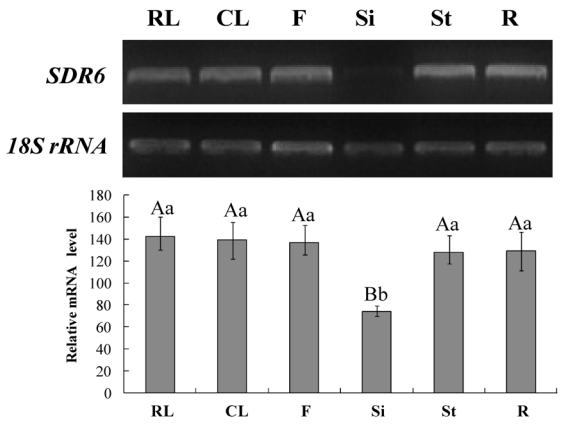

Figure 6. Tissue-specific expression patterns of *SDR6* in the wild-type. Plants were grown until flowering, and plant parts were separately harvested for total RNA isolation. RL, rosette leaves; CL, cauline leaves; F, flowers; Si, siliques; St, stems; R, roots.

Discussion

SDR6, which encodes a protein containing a NAD(P)-binding domain in *Arabidopsis*, shows no homology with any known flowering-related gene. In this study, we found that *sdr6* display a delayed transition to flowering both in long and short days and the flowering-time of *SDR6* complementing plants was similar to that of wild-type plants. We also noted that *SDR6* deficiency alter the expression of flowering-related genes, i.e., *FLC*, *LD*, *FVE*, and *SOC1*. These results fully demonstrated that *SDR6* is an important gene involved in the regulation of flowering-time in *Arabidopsis*.

The photoperiod, vernalization, GA, and autonomous pathways were identified as major factors that regulated the flowering-time in *Arabidopsis* (He and Amasino 2005; Komeda 2004). Accordingly, we sought to identify the *SDR6*-mediated flowering pathway by analyzing the flowering-time and expression of some critical genes in the different flowering pathways. We subjected the wild-type, *sdr6*, and *SDR6* complementing plants to different physiologic conditions. The *sdr6* plants displayed later flowering under either long-day or short-day conditions, especially under short-day conditions (Figure 1). Thus, the *sdr6* plants were sensitive to the photoperiod pathway. GA treatment reversed, in part, the delayed flowering of *sdr6* plants (Figure 3). After exposure to vernalization, the *sdr6* plants flowered earlier than the plants grown in LD and SD (Figure 4). This responsiveness of the *sdr6* plants to GA and vernalization were similar to previous results for several autonomous pathway mutants (Ausin et al. 2004; Henderson et al. 2005; Mockler et al. 2004). In addition, transcript levels of critical genes in the autonomous pathway, i.e., *FLC*, *LD*, *FVE*, and *SOC1* were significantly altered in *sdr6* plants. However, expression levels of the key genes in the photoperiod, gibberellin, and vernalization pathways were not obviously different. Taken together, these observations suggested that *SDR6* is a new component of the autonomous pathway and prevented late flowering.

Acknowledgements

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