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Research Report

Identification of Three *FLOWERING LOCUS C* Genes Responsible for Vernalization Response in Radish (*Raphanus sativus* L.)

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Abstract. *Raphanus sativus* L. is grown worldwide and used as fresh vegetables. In the Brassicaceae family, the *FLOWERING LOCUS C* (*FLC*) gene is a key regulator of flowering time and explains a large part of natural flowering time variation and the vernalization response. Here we report three *FLC* orthologous genes *RsFLC1*, *RsFLC2*, and *RsFLC3* in *R. sativus* identified from the de novo assembled transcriptome. The sequences of three *RsFLC* genes have a high similarity to *Arabidopsis FLC*. Overexpression of each *RsFLC* gene in *Arabidopsis* induced late flowering, suggesting that every *RsFLC* gene functions as a floral repressor. All *RsFLC* genes were highly expressed in non-vernalized plants, whereas their expression levels significantly decreased by the vernalization treatment. Furthermore, the rate of decrease in their expression was proportional to the length of cold exposure. A significant level of sequence variation exists among *RsFLC* alleles derived from a variety of *Raphanus* cultivars, suggesting that *RsFLC* genes have diverged considerably but still retain essential functions.

***Additional key words*:** floral repressor, flowering time, orthologous gene, *RsFLC*, transcriptome

Introduction

Radish (*Raphanus sativus* L., 2n = 18), a member of the family Brassicaceae, is a root vegetable grown and consumed worldwide. In East Asian countries, long-season big root radish varieties are widely grown, where flowering time is an important agronomic trait because bolting results in the failure of harvest while the root is being converted from a nutrient sink to a source. On the other hand, flowering on time is also important for economical seed production. Like most other species in the Brassicaceae family, flowering of radish is accelerated by the cold treatment, a process known as vernalization. During the period of radish cultivation, unexpected exposure to the cold temperature often results in precocious bolting, which causes a significant loss of yield. Thus, better understanding of flowering time genes and the vernalization response are important for radish breeding.

Raphanus belongs to the tribe Brassiceae along with *Arabidopsis* and many economically important *Brassica* species. The *FLOWERING LOCUS C* (*FLC*) gene in *Arabidopsis* is a key regulator of flowering that encodes a MADS box transcription factor (Michaels and Amasino, 1999). Natural variation of flowering time in *Arabidopsis* is often associated with allelic variations of *FLC* (Michaels et al., 2003; Shindo et al., 2005). In *Arabidopsis*, FLC functions as a floral repressor during vegetative growth whereas its reduced expression upon vernalization activates the floral integrators such as *FT, FD,* and *SOC1*. In turn, they activate the expression of floral meristem identity genes *LFY* and *AP1*, initiating a transition from a vegetative to reproductive phase (Helliwell et al., 2006; Kim et al., 2009; Searle et al., 2006; Sheldon et al., 2000). *FLC* expression is primarily controlled by epigenetic modifications at the chromatin level (Kim et al., 2009).

Functions of *FLC* and other flowering genes are well conserved in the family Brassicaceae (Osborn et al., 1997). For example, *B. rapa* contains four copies of *FLC* genes (*BrFLC1-3*

and *5*) (Schranz et al., 2002), whereas *B. oleracea* appears to have five copies (*BoFLC1-5*) (Lin et al., 2005; Okazaki et al., 2007; Schranz et al., 2002). Interestingly, *B. napus*, an allotetraploid between *B. rapa* and *B. oleracea*, is reported to have five copies of *FLC* genes (*BnFLC1*-5) (Tadege et al., 2001; Zou et al., 2012). The whole genome studies revealed that *FLC* gene multiplication is common in the genus *Brassica*, due probably to whole genome duplication events during evolution (Chalhoub et al., 2014; Liu et al., 2014; Wang et al., 2011). In *B. rapa*, three *FLC* paralogs *BrFLC1/2* and *BrFLC5* were found to cosegregate with flowering-time loci in a completely additive manner (Schranz et al., 2002), and among them, *BrFLC2* appears to have a major effect (Okazaki et al., 2007; Xiao et al., 2013; Zhao et al., 2010). This indicates that *BrFLC* genes are somewhat redundant with overlapping functions to control the flowering time in response to vernalization.

In this study, we isolated three *FLC* genes from the de novo assembled transcriptome of *Raphanus* and examined their expression patterns relative to flowering time upon vernalization treatment. We also demonstrated their function as a floral repressor in transgenic *Arabidopsis*, and investigated allelic sequence variations among a variety of radish cultivars.

Materials and Methods

Plant Materials

Big root radish cultivars 'Jinju Daepyung' (JD, Dongwon Nongsan Seed Co., Korea), 'Chung Woon' (CW, Monsanto, Korea), and 'Chung Han' (CH, Danong Co., Korea) were chosen for their commercial importance. A total of 234 accessions obtained from RDA-GENEBANK (www.genebank.go.kr) were examined for flowering time, and five of them that displayed early flowering were selected (Table 1). As a reference accession for radish genome sequencing in Korea, 'WK10039' (WK) was chosen for transcriptome analysis.

Vernalization Treatment

Seeds were sterilized in a 50% bleach solution with 0.1% Triton X-100, sown on the MS medium containing 1% sucrose and 0.8% agar, and germinated at 24°C. Nine days after germination, when the primary leaf was about 5 mm in length and two true leaves were visible, the seedlings were moved to a 4°C glass door refrigerator for the vernalization treatment, and then transplanted to soil. Plants were grown in a 10 cm diameter and 6 cm height pot in the growth room at 24°C under the 16-hour day condition. The flowering time of vernalized plants were determined by measuring the days after transplanting (DAT) or by counting the leaf number when the inflorescence was visible.

De Novo Transcriptome Assembly

Total RNA was isolated from the 9-day-old WK seedlings which had been vernalized for 34 days (34V) or non-vernalized (NV). The RNA-seq library was constructed as previously reported (Zhong et al., 2011). A total of 104,522,294 paired-end reads were obtained from the Illumina HiSeq 2000 platform. The de novo assembly was performed on a set of quality-controlled sequence reads using the Velvet (ver. 1.2.08) (Zerbino and Birney, 2008) and Oases (ver. 0.2.08) programs (Schulz et al., 2012) with default parameters. The BLAST analysis and further assembly using Velvet produced a set of unigene contigs that served as templates for gene expression profiling. The Bowtie2 program (ver. 2.1.0) (Langmead and Salzberg, 2012) with default parameters was adopted to map the sequence reads. The fragments per kilobase of exon model per million mapped reads (FPKM) values were calculated for every gene using eXpress (ver. 1.3.1) (Roberts and Pachter, 2013). The RNA-seq data sets were deposited in the NCBI/EBI/DDBJ Sequence Read Archive (DRA001193).

Phylogenetic Analysis

The *FLC* gene sequences in the Brassicaceae family were aligned using ClustalW and the phylogenetic tree was drawn

Table 1. Information for cultivars and accessions of *R. sativus* used in this study.

Name	RDA-genebank number	Flowering time without vernalization (DAT)	Producer or germplasm ID	Origin
WK10039 (WK)	-	~ 259	NIHHS, RDA	Korea
Jinju Daepyung (JD)	-	~ 150	Dongwon Nongsan Seed Co., Ltd.	Korea
Chung Woon (CW)	-	~ 311	Monsanto	Korea
Chung Han (CH)	-	~ 193	Danong Co., Ltd.	Korea
G46	IT102539	< 35	PI311753	Poland
G162	IT218906	< 35	CR 501	Poland
G189	IT247262	< 35	sung 04	Myanmar
G190	IT247265	< 35	sung 126	Myanmar
G219	IT261985	< 35	THA-LSY-2000-71	Thailand

by the neighbor-joining method with a 1,000 bootstrap test by MEGA6 with default parameters (Tamura et al., 2013). Radish genomic scaffolds Rsa1.0_01295.1, Rsa1.0_00493.1, and Rsa1.0_03137.1 (Kitashiba et al., 2014) were used for gene structure analysis.

Generating Transgenic *Arabidopsis*

Coding sequences of *RsFLC* genes were amplified from WK cDNA by PCR using the primer pairs, RsFLC1-1 (5'-gaGGTACCATGGGGAGGAAGAAACTTGAAATCAAGC)/ RsFLC1-2 (5'-tGAGCTCCTAATTAAGCAATGGGAGAGTTACCGG), RsFLC2-1 (5'-gaGGTACCATGGGAAGAAAAAAACTAGAGATCAAGCG)/ RsFLC2-2 (5'-ctGAGCTCCTAATAAAGCAGTGGGAGAGTTACCGGA), and RsFLC3-1 (5'-gaGGTACCATGGGAAGAAAAAAACTAGAAATCAAACG)/ RsFLC3-2 (5'-ctGAGCTCCTAATTAAGCAGCGGGAGAGTTACCG) containing the KpnI and SacI restriction sites (underlined) for subsequent cloning. PCR was performed with 30 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 60 s. The GUS fragment of the 35S:GUS:NOS terminator cassette in the pBI101 vector was replaced with the KpnI/ SacI digested fragments of *RsFLC1*, *2*, and *3* CDS, respectively. The plasmids were introduced into *Agrobacterium tumefaciens* GV3101 strain. Transgenic plants were produced by the 'floral-dip' method in *A. thaliana* ecotype Columbia-0 (Col-0) (Clough and Bent, 1998). Transgenic T1 plants were selected on the MS medium containing 50 $\mu g \cdot mL^{-1}$ kanamycin and transplanted to soil.

Quantitative RT-PCR

The RNA was extracted with RNeasy Plant Mini Kit (QIAGEN) according to manufacturer's protocol. Three μg of RNA was treated with 1 U of DNase (Cat. No. M6101, Promega, Madison, WI, USA) and cDNA was synthesized using Superscript III Reverse Transcriptase (Cat. No. 18080, Invitrogen, Carlsbad, CA, USA). Quantitative RT-PCR (qRT-PCR) was performed using the Rotor-Gene Q 2plex HRM platform and QuantiFast SYBR Green PCR kit (Cat. No. 9001560 and 204054, QIAGEN, Hilden, Germany). After 10 min of denaturation at 95°C, 40 cycles of 10 s at 95°C, 15 s at 60°C, and 35 s at 72°C were employed for PCR amplification. Primer pairs used for qRT-PCR are as follows: RsFLC1-q1 (5'-GAGAAATTGCTGGAAGAGGAGAACC)/ RsFLC1-q2 (5'-CCTCCATATTATCAGCTTCAGCTCTCA), RsFLC2-q1 (5'-GAGAAATTGCTGAAAGAAGAAAACCAGGG)/ RsFLC2-q2 (5'-GCAGTGGGAGAGTTACCGGACGAA), RsFLC3-q1 (5'-CTAGAGCTAGGA AGACAGAACTAATGTTA)/RsFLC3-q2 (5'-GCTTCGGCTCCCGCAAGATTG), RsACT-1 (5'-TACCGCAA AGAGCAGTTCGTCAGTG)/ RsACT-2 (5'-GAGCGATGGCTGGAACAGTACTTCAG), and AtUBQ10_561f (5'-CGTTGACTGGGAAAACTATCACT)/ AtUBQ10_637r (5'-GTCCTGGATCTTGGCTTTCA).

Results

Vernalization Response of Korean Radish Cultivars

Vernalization responses of three radish cultivars JD, CW, and CH (Table 1) were examined by measuring the time to flower after 10, 20, 30, 40, and 50 days of cold treatment (Fig. 1). For WK, flowering time of only NV and 30V plants was investigated. We observed that flowering time of the three radish cultivars under NV spanned from 150 to over 300 days. JD, a commercialized landrace cultivar, began flowering relatively early (150 ± 59 DAT, n = 11) even without vernalization, whereas flowering of CW was significantly delayed in the absence of vernalization (311 ± 38 DAT, n = 12). CH went through a moderate length of vegetative growth period before flowering (193 ± 20 DAT, n = 10) without cold treatment. By contrast, flowering of all three cultivars was significantly accelerated in proportion to the length of the vernalization treatment (Fig. 1). Although CW flowered extremely late compared to JD and CH under NV condition, its flowering time was considerably shortened by the cold treatment. That is, even 10 days of cold exposure were enough for CW to flower within 180 DAT. By contrast, CH went through an intermediate length of vegetative phase before flowering under NV condition, but the longer period of cold treatment was required to induce flowering than others (Fig. 1). This indicates that CH has a poor vernalization response compared to JD and CW. WK did not flower until 260 DAT under NV but its flowering was considerably accelerated under 30V condition. Even though there were variations in responsiveness

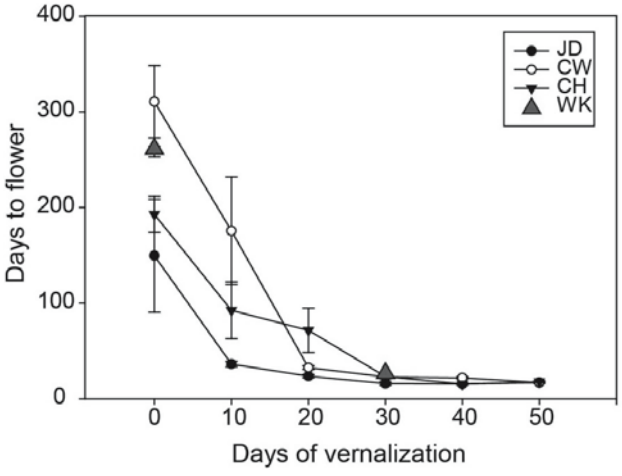

Fig. 1. Accelerated flowering of Korean radish cultivars upon vernalization treatment. Nine-day- old seedlings (n > 10) were cold treated for 10 to 50 days and transplanted into soil. Flowering time was measured by days after transplanting. Values are the means ± SD.

Table 2. Statistics of de novo assembled radish transcriptome.

Reads	Amount
No. of total reads	133,035,984
No. of qualified reads	126,227,670
No. of qualified base pairs (bp)	13,436,634,384
No. of unigenes	34,186
Total unigene length (bp)	38,978,183
Maximum unigene length (bp)	12,266
Minimum unigene length (bp)	200
Average unigene length (bp)	1,140
N50 of unigene (bp)	1,517

to vernalization, all the radish cultivars began flowering within approximately three weeks under 30V condition. These observations imply that all Korean radish cultivars examined in this study are vernalization responsive, and the effect of cold treatment plateaus at 30 days.

De Novo Transcriptome Assembly and the Isolation of Three Radish *FLC* Genes

Because radish genome information was unavailable at the time of this study, we performed a de novo transcriptome assembly in order to obtain sequence information of several radish genes important for flowering and vernalization response. Total RNA was extracted from the aerial parts of WK plants grown under 34V condition, and the RNA-seq library was constructed. A total of 126,227,670 short sequence reads were generated on the Illumina HiSeq2000 platform and finally assembled into 34,186 unigene contigs (Table 2). We tried first to identify putative *FLC* sequences from the de novo assembled contigs because it is one of the most important genes in the vernalization pathway as a floral repressor. We performed a BLAST analysis and identified three putative *FLC* sequences from the radish transcriptome.

Conserved Gene Structures of Radish *RsFLC* Genes

Primer sets were designed based on the contig sequences, and three *RsFLC* genomic fragments were obtained by PCR amplification from nine radish plants including WK, three commercial F_1 hybrid cultivars (JD, CW, and CH), and five early flowering accessions (G46, G162, G189, G190, and G219) collected from RDA-GENEBANK (Table 1). These three *RsFLC* genes were named as *RsFLC1*, *RsFLC2*, and *RsFLC3* based on the sequence similarity with known *Brassica FLC* genes (Figs. 2A and 2B). As shown in Fig. 2A, all three *RsFLC* genes have a high degree of similarity to the *FLC* genes in *Arabidopsis*, *B. rapa*, and *B. oleraceae* in the family Brassicaceae. Phylogenetic analysis allowed us to assign these three *RsFLC* genes to each of *FLC1*, *2*, and *3* clades, respectively (Fig. 2B), indicating that the genus *Raphanus* has significantly diverged after genome triplication, which is thought to have occurred about 18 MYA (Yang et al., 2006). In addition, each *RsFLC* gene outside the *Brassica FLC* gene groups reflects the genetic distance between *Raphanus* and *Brassica* (Fig. 2B), even though *Raphanus* and *Brassica* are the most closely related genera in the Brassicaceae family (Bailey et al., 2006; Johnston et al., 2005). Overall exon-intron structures are highly conserved among *RsFLC* genes with seven exons and six introns (Fig. 2C). The *RsFLC1* has the longest 1st intron and most resembles the *Arabidopsis FLC* gene.

Five out of 234 accessions from RDA-GENEBANK collections began flowering within 35 DAT under NV condition, suggesting that they have little vernalization requirement, and their *FLC* genes might have lost the floral repressor activity. Thus, we searched for sequence variations in the coding regions of *RsFLCs* that might explain difference in vernalization requirements between early and late flowering cultivars. In every *RsFLC* sequence examined, very few sequence variations were found in the exons. By contrast, the intronic sequences, particularly the 6th introns of *RsFLC2* and *RsFLC3*, have a number of variations among these accessions. We could not find any polymorphic sequences associated with the early or late flowering phenotype, suggesting that different vernalization responses among the radish cultivars are likely not determined by sequence variations in the coding regions of *RsFLCs*. Genomic *FLC* sequences of nine radish plants were deposited in GenBank (KP027017- KP027042).

Overexpression of *RsFLC* Gene Induces Late Flowering in *Arabidopsis*

To verify the function of *RsFLC* genes, we generated transgenic *Arabidopsis*, in which each *RsFLC* gene was driven by the cauliflower mosaic virus *35S* promoter. Many of transgenic plants showed severe late flowering phenotypes. Approximately 46% (11/24) of *35S:RsFLC1* T1 plants displayed late flowering, and 45.6% (5/11) of them only produced many rosette leaves (as many as > 50) but never flowered, which was the strongest late-flowering phenotype among the *RsFLC* T1 transgenic plants. For *RsFLC2* and *RsFLC3*, 11% (3/26) and 28% (7/25) of T1 plants showed late flowering, respectively. Two *35S:RsFLC3* T1 plants failed to flower until 270 days after sowing. However, all *35S:RsFLC2* T1 plants eventually flowered after a prolonged period of vegetative growth compared to wild type (Table 3).

The transcript levels of each *RsFLC* gene were examined by qRT-PCR in wild type and transgenic *35S:RsFLC* T1 plants. None or very low levels of *RsFLC* transcripts were detected in non-transgenic plants, whereas *RsFLC* transcripts

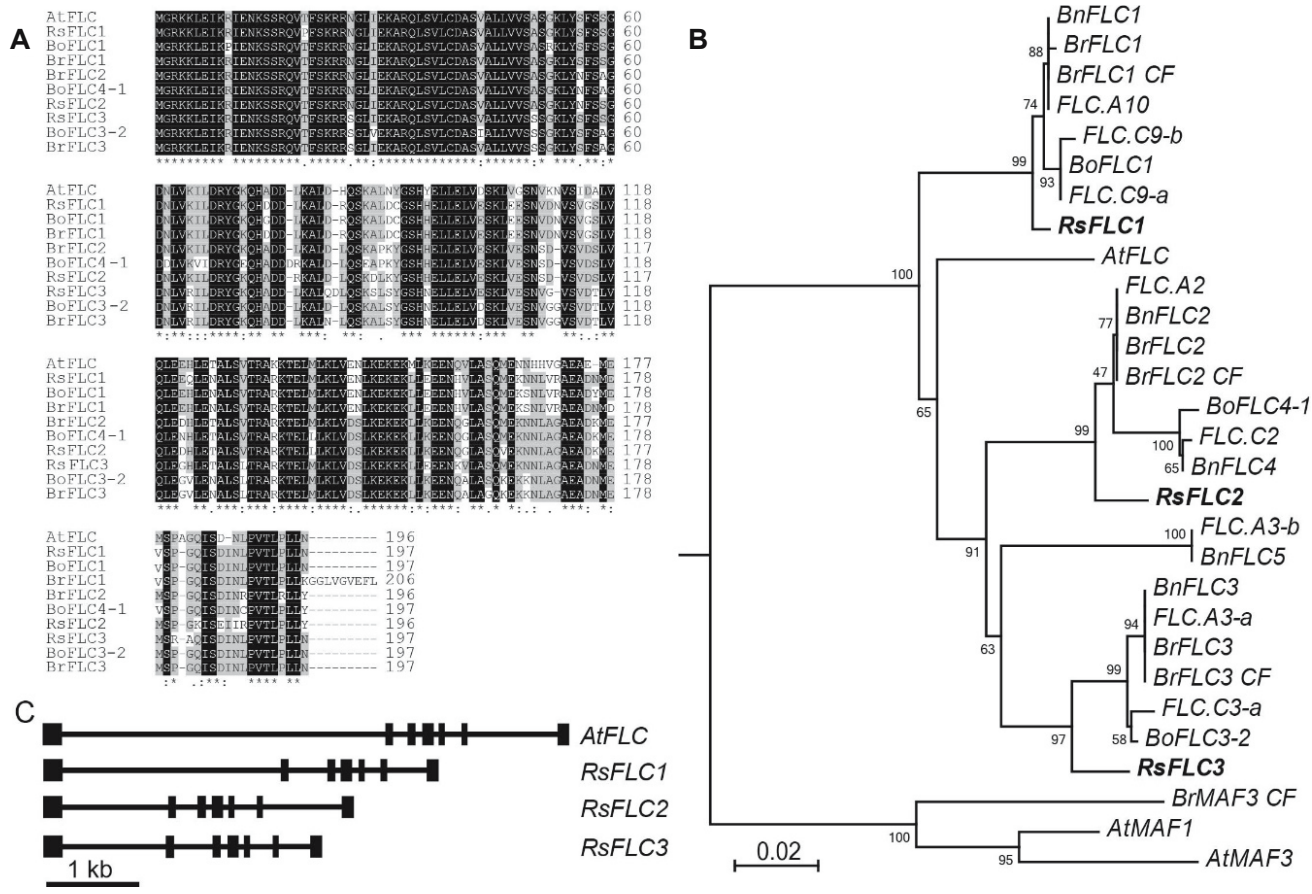

Fig. 2. Structures of *RsFLC* genes and their phylogeny in the Brassiceae family: (A) Sequence alignment of RsFLC proteins with *Brassica* and *Arabidopsis* FLC proteins; (B) Phylogenetic analysis on *FLC* genes in the Brassiceae family using neighbor-joining method numbers on each branch are bootstrap values. *RsFLC* genes are designated with bold characters. Accession numbers of the genes used in the analysis are as follows (in parentheses): *BrFLC1* (DQ866874.1), *BrFLC2* (DQ866875.1), *BrFLC3* (DQ866876.1), *BrFLC1 CF* (Bra009055), *BrFLC2 CF* (Bra028599), *BrFLC3 CF* (Bra006051), *FLC.A10* (JQ255390.1), *FLC.C9-a* (JQ255391.1), *FLC.C9-b* (JQ255392.1), *FLC.A2* (JQ255393.1), *FLC.C2* (JQ255394.1), *FLC.A3-a* (JQ255395.1), *FLC.C3-a* (JQ255396.1), *FLC.A3-b* (JQ255397.1), *BnFLC1* (AY036888.1), *BnFLC2* (AY036889.1), *BnFLC3* (AY036890.1), *BnFLC4* (AY036891.1), *BnFLC5* (AY036892.1), *BoFLC1* (AM231517.1), *BoFLC3-2* (AY306125.1), *BoFLC4-1* (AY306122.1), *BrMAF3 CF* (Bra024350), *AtFLC* (NM_121052.2), *AtMAF1* (NM_125905.2), *AtMAF3* (NM_106358.3); (C) Schematic representations of *RsFLC* gene structures. Closed boxes and lines represent exons and introns, respectively.

Table 3. Flowering time of transgenic *Arabidopsis* transformed with *35S:RsFLCs*.

Genotype	No. of late flowering T1[z]	Leaves		Days to flower	
		Average	Range	Average	Range
Col-0	0/28 (0 %)	9.39 ± 1.83	7-13	26.29 ± 4.99	20-33
35S:RsFLC1	11/24 (45.8 %)	45.60 ± 14.52	27-58	55.60 ± 9.96	42-75
35S:RsFLC2	3/26 (11.5 %)	29.67 ± 2.52	27-32	45 ± 3.46	43-49
35S:RsFLC3	7/25 (28 %)	22.33 ± 1.53	22-24	39 ± 2.35	37-43

[z]Five of *35S:RsFLC1* T1 plants and two of *35S:RsFLC3* T1 plants did not flower during the experiment which was over 270 days after planting.

were highly abundant in every late-flowering transgenic plant (Fig. 3B). These results indicate that late flowering was primarily caused by ectopic expression of each *RsFLC* gene, suggesting that they are all functional orthologs of *Arabidopsis FLC* even though their floral repression activities may differ.

RsFLC Genes Are Differentially Regulated in Response to Vernalization

The amounts of *RsFLC* transcripts were determined in six different tissues in early vegetative stage plants (leaf, hypocotyl, and root) and flowering plants (flower, inflorescence stem, cauline leaf) of WK10039. Transcripts of *RsFLC1* and

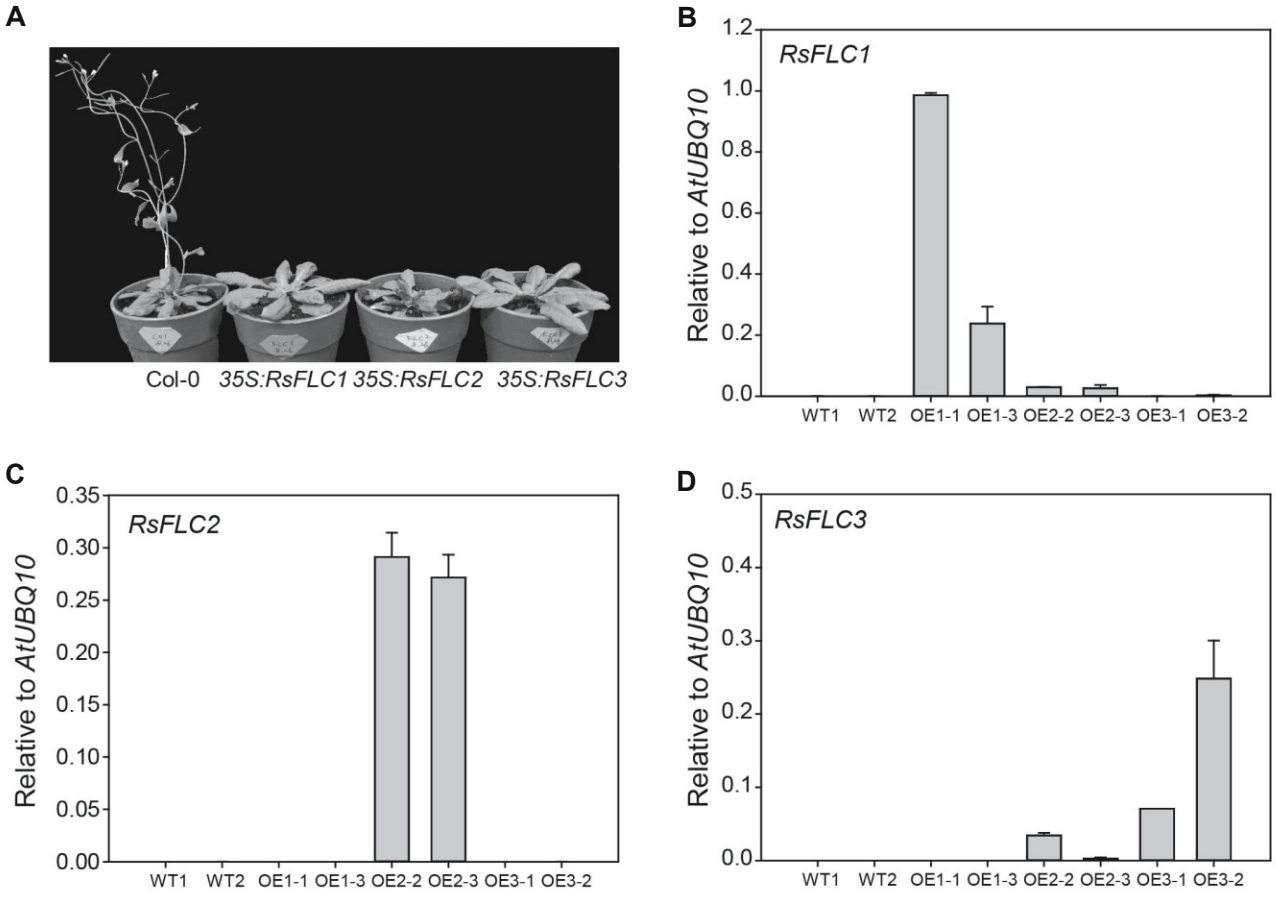

Fig. 3. Ectopic expression of *RsFLC* genes delay flowering in *Arabidopsis*. (A) Late flowering phenotype of T1 transgenic plants overexpressing (OE) *RsFLC* genes; (B - D) Expression levels of each *RsFLC* transgene in representative individuals. OE1, 2, and 3 plants are transformed with *35S:RsFLC1*, *2*, and *3*, respectively. Ectopic transgene expression was determined by qRT-PCR relative to *AtUBQ10*. Columns with error bars indicate means ± SD of two technical replicates.

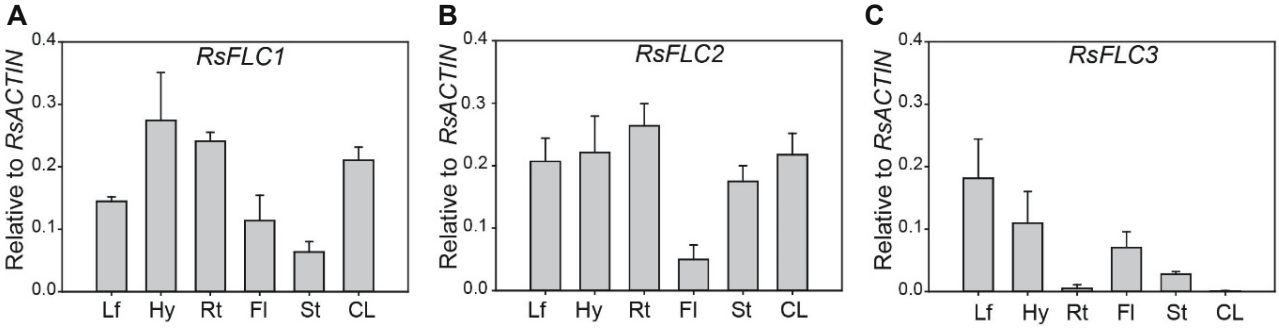

Fig. 4. Tissue specific expression of three *RsFLC* orthologs: (A - C) Transcripts abundance of *RsFLC1* (A), *RsFLC2* (B), and *RsFLC3* (C) was determined by qRT-PCR. Leaf (Lf), hypocotyl (Hy), and root (Rt) were harvested from three plants at the early vegetative stage and flower (Fl), stem (St), and cauline leaf (CL) were obtained from three flowering plants of WK.

RsFLC2 were detected in all tissues examined (Figs. 4A and 4B). In contrast, *RsFLC3* was detected in very low level in roots and cauline leaf (Fig. 4C).

In order to test whether *RsFLC* genes are responsible for vernalization response in radish, we performed a transcriptome analysis to compare the expression levels of three *RsFLC* genes between NV and 34V WK plants. In order to assess *RsFLC* gene expressions, the FPKM values were calculated according to read abundance. All three *RsFLC* genes had a relatively high abundance of transcripts under NV condition, where *RsFLC3* was the most but *RsFLC2* was the least abundant (Fig. 5A). Under 34V condition, however, the expression levels of all *RsFLC* genes dramatically decreased (Fig. 5A). In order to confirm that a decrease in *RsFLC*

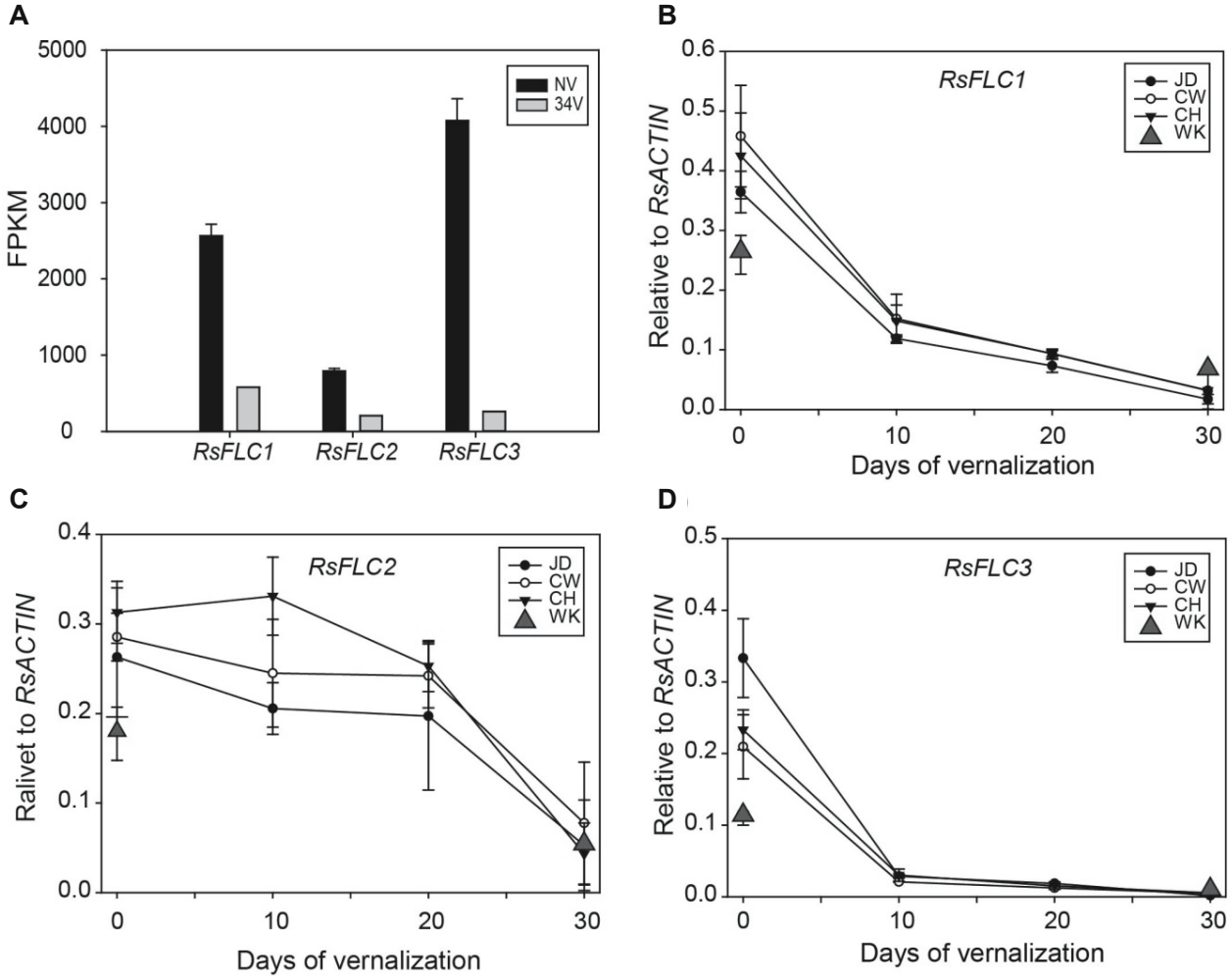

Fig. 5. Responses of *RsFLC* genes to vernalization treatment: (A) FPKM values of three *RsFLC* transcripts in WK at NV and 34V; (B, C, and D) Relative amount of three *RsFLC* transcripts abundance compared to the internal control *RsACTIN* were measured by qRT-PCR.

expression was indeed a response to cold, the expression levels of *RsFLC* genes relative to that of *ACTIN* were determined by qRT-PCR in a 30-day time window with 10-day intervals (Fig. 5). When compared to NV condition, the expression levels of *RsFLC1* and *RsFLC3* were considerably lowered even after 10 days of cold treatment (10V) (Figs. 5B and 5D), and were marginal in 30V WK plants (Figs. 5B and 5D). However, *RsFLC2* did not show a rapid decrease in expression after vernalization (Fig. 5C). Expression of *RsFLC2* started to decrease after 20V, which is quite a slow response compared to *RsFLC1* and *RsFLC2*.

In order to determine whether the *RsFLC* expression levels can explain flowering time, we monitored *RsFLC* expression changes in three commercial cultivars JD, CW, and CH. In CW and CH, expression levels of *RsFLC1* ($R^2 = 0.861$ and 0.951) and *RsFLC3* ($R^2 = 0.904$ and 0.996) showed strong correlations with flowering time. By contrast, *RsFLC2* expression was not likely associated with flowering time. In JD, any of *RsFLC* did not show a significant correlation with flowering time. The expression studies on three *RsFLC* genes upon vernalization showed that expression of all three *RsFLC* genes was reduced in response to vernalization. However, the timing and patterns of reduction in gene expression were different. *RsFLC1* and *RsFLC3* genes, but not *RsFLC2*, showed strong correlations with flowering time in response to vernalization (Fig. 5).

Discussion

In this study, we report three *FLC* orthologous genes *RsFLC1-3* isolated from a de novo assembled transcriptome of radish. A recently published *R. sativus* genome also contains three copies of *FLC* genes (Kitashiba et al., 2014). It is postulated that chromosome rearrangement and segmental duplication after genome triplication resulted in more than three copies of *FLC* genes in *B. rapa* and *B. oleracea* ge-

nomes (Lin et al., 2005; Okazaki et al., 2007; Schranz et al., 2002; Yang et al., 2006). Thus, our study suggests that similar events have also occurred to radish during evolution leading to the duplication of the *FLC* genes and their divergence thereafter. Our phylogenetic analysis also suggests that *FLC* gene multiplication has preceded the speciation and functional diversification events in the Brassicaceae family (Fig. 2B).

We observed a high degree of allelic variations among radish *FLC* genes. At least six alleles for each *RsFLC* gene were identified by genomic sequencing on nine radish accessions. However, sequence comparison of *RsFLC* genes between late and early flowering accessions did not reveal any association of flowering time with allelic variations. This implies that regulation of flowering time in radish is primarily governed by *RsFLC* genes but there must be other factors to control the vernalization effect in response to the cold temperature.

We revealed that *RsFLC* genes were all responsive to cold. Their expression levels significantly decreased upon exposure to the low temperature (Fig. 5), and at the same time, flowering was considerably accelerated (Fig. 1). This vernalization effect also depends on the length of cold treatment. Even though additional studies are required to reveal specialized functions of *RsFLC* genes, it is highly plausible that they have functions very similar to those of *Arabidopsis FLC* considering its comparable expression patterns and response to vernalization. Therefore, it will be of great interest to identify downstream targets of RsFLCs and to study how they are regulated at the chromatin level.

Overexpression of each *RsFLC* gene was able to delay flowering in transgenic *Arabidopsis* plants, which indicates that three *RsFLC* genes all function as a floral repressor. However, it is speculated that not all *RsFLC* genes are equally functional. Our study suggests that *RsFLC1* and *RsFLC3*, but not *RsFLC2* are likely to have a major effect on flowering time regulation in response to vernalization. Further genetic studies are required to determine the major *FLC* gene in *Raphanus* and how they respond to the cold weather. This is also commercially important because flowering time is one of the important traits in radish breeding.

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