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Functional Characterization of *DcFT1*, an Ortholog for the *FLOWERING LOCUS T* Gene in Carnation (*Dianthus caryophyllus* L.)

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We analyzed *FLOWERING LOCUS T* (*FT*) orthologs to elucidate the regulatory mechanisms of flowering in carnations. There are six *FT* candidate genes in the carnation genome. Phylogenetic analysis and amino acid alignment suggested that four genes were *FT*-like genes involved in promoting flowering. Of these, Dca19666.1 had the all conserved amino acids necessary for florigen activity. Therefore, this gene was designated as *DcFT1* and used for further analysis. *DcFT1* transcript levels increased as the plants developed from the vegetative to the reproductive growth stages. *DcFT1* was unevenly expressed in the leaves, and was more abundant in young leaves. A splice variant was identified; however, its relationship with flowering could not be determined. The early blooming cultivar showed an early elevation of *DcFT1* compared to the late-blooming cultivar. Treatment to accelerate flowering by cooling at the end of the day also accelerated the increase in *DcFT1* expression. The cloned *DcFT1* was overexpressed in *Arabidopsis* for functional characterization. The resulting transgenic plants began to bolt earlier than the control plants transformed with the empty vector. qRT-PCR analysis of the *DcFT1*-overexpressor showed that several genes related to flowering were upregulated. These results suggest that *DcFT1* is an important regulator of carnation flowering.

Key Words: carnation, *FLOWERING LOCUS T* like genes, flowering time, gene expression, splicing variant, transformants.

Introduction

Carnation (*Dianthus caryophyllus* L.) is one of the most important cut flowers worldwide. As carnations are harvested repeatedly from a single cutting, it is necessary to promote the flowering of each individual shoot and extend harvesting times to increase flower yield. Therefore, early flowering is an important breeding target. Carnations are considered relatively long-day plants, and long-day treatments are effective in promoting flowering; trials have been conducted using

incandescent lights or fluorescent lamps (Harris, 1966; Holley and Baker, 1963, 1991) and light-emitting diodes (LEDs) in more recent years (Kato et al., 2023). However, these have not been widely used in the field due to their negative effects such as stem softening and reduced flower numbers in spray carnations (Kato et al., 2023). Temperature is also important to promote flowering; however, carnations are highly susceptible to high temperatures, negatively affecting cut flower yield and quality (Wan et al., 2015; Yamaguchi, 1991). In Japan's warm area cropping type, with planting in June or July, flowering promotion faces a hot season problem. Unusually high temperatures have serious effects, such as delayed flowering and stem softening (Higashiura et al., 2020). Therefore, we developed techniques to improve stem characteristics under high temperatures using night and short-term chilling techniques (Higashiura et al., 2020, 2021). To develop techniques to promote flowering without loss of quality and to breed cultivars that are less sensitive to day length and

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temperature, it is necessary to understand flowering at the molecular level in carnations.

Molecular mechanisms for flowering and associated genes have been identified in many important agronomic crops (Blümel et al., 2015), but have not yet been reported for carnation. *FLOWERING LOCUS T (FT)* is a strong factor that controls flowering. *FT* encodes the phosphatidylethanolamine binding protein (PEBP)/Raf kinase inhibitor protein (RKIP) (Kardailsky et al., 1999; Kobayashi et al., 1999). In *Arabidopsis thaliana*, *FT* is expressed in the vascular phloem of leaves under long-day conditions (Takada and Goto, 2003). The translated product of *FT* is transported from the leaf to the shoot apex through the phloem (Jaeger and Wigge, 2007) and interacts with the transcription factor FD, which promotes flowering (Lee et al., 2019). *TERMINAL FLOWER 1 (TFL1)* is an *FT* homolog that antagonizes *FT* and interacts with FD (Hanano and Goto, 2011). Several studies have estimated amino acid sequences that are important for flowering regulation by comparing the FT and TFL1 proteins (Ahn et al., 2006; Hanzawa et al., 2005; Ho and Weigel, 2014; Pin et al., 2010). The amino acid sequence of the loop region identified by Ahn et al. (2006) is widely conserved among functional *FT* homologs in various plants (Pin et al., 2010; Wickland and Hanzawa, 2015). The AtFT-FD complex directly promotes the transcription of *SUPPRESSOR OF OVEREXPRESSION OF CO 1 (SOC1)*, which integrates multiple floral signals, and *APETALA1 (AP1)*, a determinant of the floral meristem (FM). The AtFT-FD complex also promotes *LEAFY (LFY)* transcription in floral meristems and *FRUITFULL (FUL)* transcription in the inflorescence meristem (IM) via SOC1 (Blázquez et al., 2006; Srikanth and Schmid, 2011). Gibberellin has been suggested to activate *AtFT* and *SOC1* transcription, and *ASYMMETRIC LEAVES1 (AS1)* forms a complex with CO to promote *AtFT* expression (Song et al., 2012). It regulates flowering by forming different networks (Mutasa-Göttgen and Hedden, 2009).

In our previous studies, we performed QTL analysis of early-late flowering and found a major common QTL on LG 10 for flowering time in carnations, despite using two mapping populations in different years (Yagi et al., 2020). We also identified DNA markers and scaffolds for a neighboring QTL. In the QTL regions, Dca19666.1 is highly homologous to FT. The present study aimed to investigate the role of the Dca19666.1 gene in the flowering process of carnation. We isolated the Dca19666.1 gene from a carnation cultivar and named it *DcFT1*. The relationship between flowering time and *DcFT1* expression levels was analyzed using early- and late-flowering cultivars. Functional analysis was also performed using ectopic expression of *DcFT1* in *Arabidopsis*. Our data suggested a potential role for *DcFT1* in promoting flowering in carnation.

Materials and Methods

Plant material and growth conditions

To examine varietal differences, the early-flowering cultivar ‘Kaneainou 1 go (Kane)’ and the late-flowering cultivar ‘Light Pink Barbara (LPB)’ were grown in a glass greenhouse at Kobe University in 2021. Rooted cuttings from each cultivar were planted on a flowerbed on June 9, 2021. An end-of-day (EOD)-cooling treatment was conducted in a glass greenhouse at the Awaji Agricultural Technology Center (34.31 NE, 134.80 E) in 2020. A standard type carnation, ‘Excerea’, was grown as described by Higashiura et al. (2021). Rooted cuttings were planted on July 1, 2020. EOD-cooling was performed from July 21 at a setting temperature of 21°C for 4 h after sunset. This step was skipped for the non-cooled control. The start time of the cooling system was adjusted weekly based on sunset in Kobe City, Japan. The treatment began on July 21 and was completed on September 24.

The samples were collected from the upper, middle, and lower leaves until flowering. All leaf samples were immediately frozen in a dry ice-ethanol bath or liquid N₂ and stored at –80°C until use.

FT ortholog analysis in silico

A search was performed for *FT* ortholog candidates in the carnation genome database (<http://carnation.kazusa.or.jp/>; Yagi et al., 2014) using sequences of *Arabidopsis FT*, *Gypsophila paniculata FT*, and *Beta vulgaris* ssp. *vulgaris* as queries. Phylogenetic analysis was conducted using the Neighbor-Joining method in Geneious Prime software (Dotmatics) based on the PEBP family proteins from the plant species, Al: *Arabidopsis lyrata*, Am: *Antirrhinum majus*, At: *A. thaliana*, Bv: *Beta vulgaris*, Ci: *Citrus unshiu*, Ej: *Eriobotrya japonica*, Fv: *Fragaria vesca*, Gm: *Glycine max*, Gp: *Gypsophila paniculata*, In: *Ipomoea nil*, Lp: *Lolium perenne*, Md: *Malus domestica*, Nt: *Nicotiana tabacum*, Os: *Oryza sativa*, Pc: *Pyrus communis*, Pn: *Populus nigra*, Pp: *Physcomitrium patens*, Ps: *Pisum sativum*, Ro: *Rosa chinensis*, Sl: *Solanum lycopersicum*, Ta: *Triticum aestivum*, and Vv: *Vitis vinifera*, listed in Table S1.

RNA extraction and cDNA synthesis

Total RNA was extracted from the leaves of carnations (*D. caryophyllus*) and *A. thaliana* using a Maxwell 16 automated purification system (Promega, Madison, WI, USA) according to the manufacturer’s instructions. For sequence analysis with carnation leaves and gene expression analysis with *Arabidopsis* leaves, a ReverTra Ace α - First Strand cDNA Synthesis kit (TOYOBOKO Co., Ltd., Osaka, Japan) and a ReverTra Ace qPCR RT kit (TOYOBOKO Co., Ltd.) were used, respectively, for reverse transcription according to each instruction manual.

Vector construction

Amplified *DcFT1* cDNA was inserted into the *Sfi*I site of the pRAFLentr vector (Ogawa et al., 2008) using an In-Fusion HD Cloning Kit (Takara Bio Inc., Shiga, Japan). The cDNA was sequenced, and no genetic mutations were discovered in the carnation genome database. The coding sequence of *DcFT1* was recombined with the downstream region of the 35S promoter in the pFAST-G02 destination vector (Shimada et al., 2010) using the LR reaction of the Gateway system (Thermo Fisher Scientific Inc., Tokyo, Japan). The vector construct was transformed into the *Agrobacterium tumefaciens* strain GV3101.

Arabidopsis transformation

The transformed *Agrobacterium tumefaciens* GV3101 was used to infect *A. thaliana* using the floral dip method (Clough and Bent, 1998). The infected plants were incubated for 16 h in the dark and grown for approximately three weeks at 23°C with a photoperiod of 16 h. T₂ lines were used to confirm the transformation by selecting biarafof tolerance with a biarafof-targeting antibiotic. gDNA was extracted from T₂ leaves, as described by Li et al. (2012). PCR assays and thermal cycling were performed according to the KOD Plus Neo (TOYOBO) instruction manual. Specific primers were designed that harbored biarafof-resistant genes to select transformants. The primer sequences used in this study are listed in Table S2.

Measurement of flowering time and expression analysis

The T₃ seeds were sterilized and planted in 1/2 Murashige and Skoog (MS) medium. Days to bolting after sowing, the number of rosette leaves at bolting, and days to flowering after sowing were measured. Above ground tissues of T₃ plants were used for RNA extraction and real-time PCR. Real-time PCR was performed using a LightCycler 480 System II (Roche Diagnostics, Basel, Switzerland) or a LightCycler Nano (Roche Diagnostics) using THUNDERBIRD SYBR quantitative PCR (qPCR) Mix (TOYOBO) based on the SYBR Green I dye intercalation method. The primers were designed for the analysis of *DcFT1* cloning and expression (Table S2). The *AtFT* and *AtCO* primers were designed by Deng and Chua (2015), and *AtAPI*, *AtLFY*, *AtFUL*, and *AtSOC1* primers were drafted by Lei et al. (2017). As morphogenesis genes, the *AtKNAT1*, *AtAS1*, *AtAS2*, and *AtGA20ox1* primers were designed as described by Li et al. (2012), Song et al. (2012), Jun et al. (2010), and Ikezaki et al. (2010), respectively. *DcActin* and *AtUBQ10* were used as controls, referring to previous studies by Totsuka et al. (2018) and Krzymuski et al. (2015), respectively. The thermal cycling conditions were as follows: 95°C for 30 s, three-step amplification with 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s, pre-melt hold at 95°C for 10 s, and melting at 60 to 97°C at 0.1°C·s⁻¹.

All data were normalized to each *DcActin* or *AtUBQ10* transcript level.

Statistical analyses

Statistical analyses were performed using JMP 13 software (SAS Institute, Cary, NC, USA). Statistical outliers were detected using the Smirnov-Grubbs test. Significant differences were determined by the Wilcoxon signed-rank test. For the multiple range test, significant differences were determined using the Tukey-Kramer's HSD test or the Steel-Dwass test, depending on the normal distribution of the data. Data shown in figures and tables are the mean ± standard error calculated from the indicated biological and technical replicates.

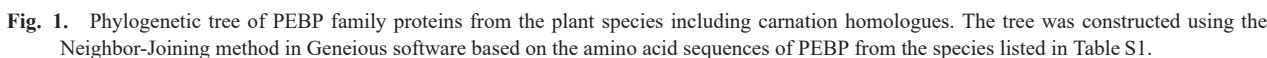
Results

Conserved FT orthologs in carnation

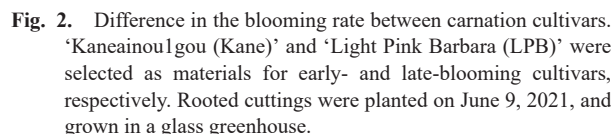
Six FT sequence homologs (Dca10963.1, Dca19666.1, Dca25456.1, Dca48544.1, Dca55347.1, and Dca58192.1) were found in "Francesco" by BLASTP search using the amino acid sequence of FT paralogs in *A. thaliana*, *G. paniculata* or *B. vulgaris* ssp. *vulgaris* as a query in Carnation DB. Among them, Dca19666.1 and Dca58192.1 scored >200 and >70% identities for all query sequences, respectively (Table S3). Phylogenetic analysis using PEBP family proteins from different plant species showed that Dca10963.1, Dca19666.1, Dca25456.1 and Dca58192.1 were grouped in the FT-like gene family clade (Fig. 1). Dca48544.1 and Dca55347.1 were grouped in the BROTHER of FT and TFL1 (BFT1) and Mother of FT and TFL1 (MFT)-like gene family, respectively. In the TERMINAL FLOWER 1 (TFL1)/CENTRORADIALIS (CEN)-like group, none of the genes were found. In Dca19666.1, all major amino acids involved in the FT protein that promotes flowering were conserved (Table S4). In contrast, both Dca10963.1 and Dca25456.1 had long branches in the phylogenetic tree (Fig. 1). This could be caused by their shorter sequences as compared to those of other FT candidates, probably due to an exon/intron misprediction, an unreadable region in the sequence, or a pseudogene. In Dca58192.1, a mutation was found in three of seven amino acids involved in flower promotion. Because Dca19666.1 can affect the early-late nature of the plant (Yagi et al., 2020), it was selected as the first candidate gene for further analysis and named *DcFT1*.

Expression analysis of *DcFT1*

Cloning of *DcFT1* revealed a splice variant. This variant had a longer sequence than the coding genome sequence due to a first intron insertion and was named *DcFT1a* (Fig. S1). The relationship between flowering time and *DcFT1* expression levels was analyzed using the early-flowering cultivar 'Kane' and the late-flowering cultivar 'LPB'. Flowering started at seven



The expression of *DcFTI* was investigated using end-of-day-cooling treatment (EOD). The EOD during the four hours after sunset effectively lowered the average temperature by about 5.5°C (Fig. S2). EOD accelerated the first flowering by approximately three weeks compared to the control (Fig. 4A). There was a signifi-



cant difference in average days to flowering between the control (149 ± 2.4) and EOD (134 ± 3.5) groups. The *DcFTI* transcripts began to accumulate 12 and 11 weeks after planting in the control and EOD groups, respectively. The marked increase in the transcription of *DcFTI* in EOD started one week earlier than that in the control (Fig. 4B). A significant difference in *DcFTI* expression was observed between the control and EOD groups 11 weeks after planting. The gap gradually narrowed toward blooming after the EOD treatment.

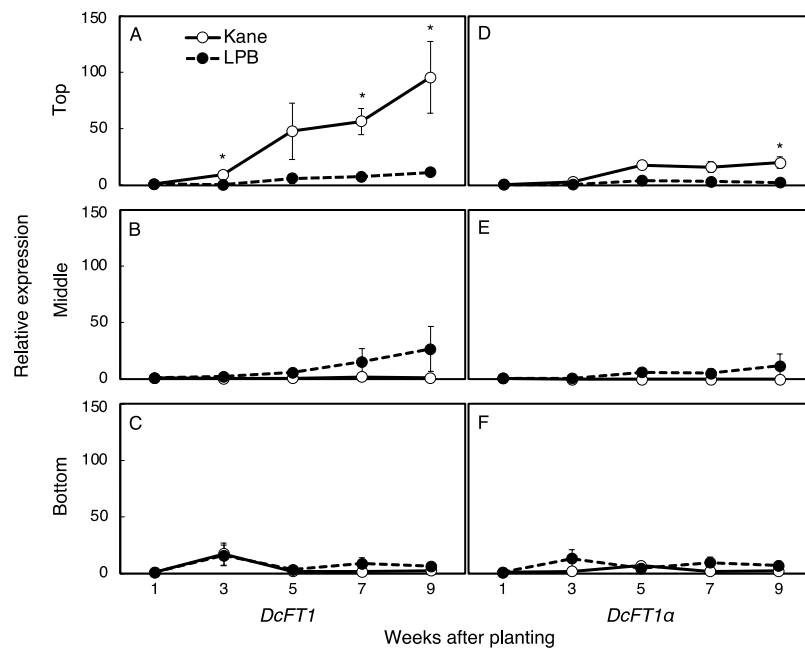


Fig. 3. Periodic changes in the expression of *DcFT1* and its splice variant before flowering. The expression levels of *DcFT1* (A, B, C) and its splice variant (*DcFT1a*; D, E, F) were quantified by qRT-PCR and normalized to those of *ubiquitin*. ‘Kaneainoulgou (Kane)’ and ‘Light Pink Barbara (LPB)’ cultivars were selected as materials for early- and late-blooming, respectively. Leaves were collected every two weeks from the stems at the top (A, D), middle (B, E), and bottom (C, F) until flowering ($n = 4$). Significant differences between cultivars are indicated by asterisks (Wilcoxon rank-sum test, $P < 0.05$).

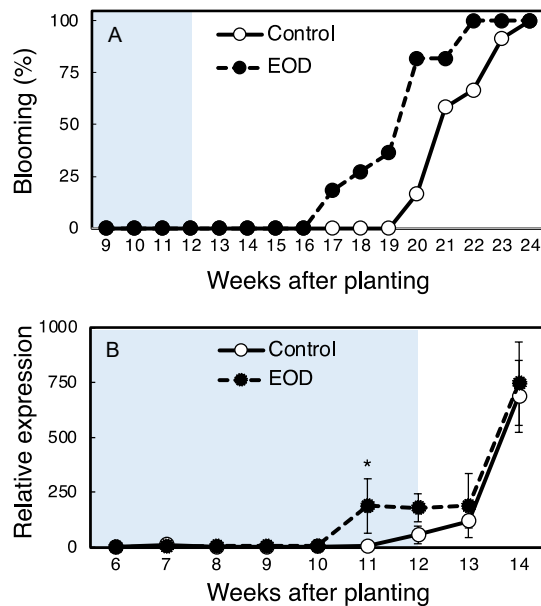


Fig. 4. The effect of end-of-day-cooling treatment on early blooming and *DcFT1* expression. The blooming rate (A) was calculated as a percentage of the total number of plants ($n = 11-12$) in the control (control) and end-of-day-cooling treatments (EOD). EOD-cooling was performed at 21°C for 4 h after sunset from July 21 to September 24, 2020, as shown in pale blue highlights in the graphs. The expression levels of *DcFT1* were quantified by qRT-PCR (B) and normalized to those of *ubiquitin*. The leaves were collected weekly from the top of the stem until blooming ($n = 4$). Significant differences between treatments are indicated by asterisks (Wilcoxon rank-sum test; $P < 0.05$).

Analysis of *DcFT1*-overexpressed *Arabidopsis*

Functional analysis was performed using ectopic expression of *DcFT1* in *Arabidopsis*. The 35S promoter-driven *DcFT1* construct was introduced into *Arabidopsis*, which resulted in three overexpression lines with varying expression levels (Fig. 5A, B). The transcript level of *DcFT1* was markedly high in *DcFT1*-ox3, followed by -ox2 and -ox1 in that order. A comparison of the phenotypes of the three *DcFT1*-ox lines and the vector control line (VC) revealed that all *DcFT1*-ox lines had a significantly lower number of days to bolting (Fig. 5C). *DcFT1*-ox2 and *DcFT1*-ox3 reduced the number of rosette leaves compared to the VC at the time of bolting (Fig. 5D).

For further analysis, *DcFT1*-ox3 was selected as it exhibited the highest expression level. The number of days to flowering was significantly higher in *DcFT1*-ox3 (38 days) than in VC (29 days). In the wild type (WT), the formation of flower buds was visually confirmed during bolting (Fig. 6A, B). However, this was not confirmed in *DcFT1*-ox3, in which flower bud formation was delayed (Fig. 6B). In addition, *DcFT1*-ox3 was dwarfed compared to WT (Fig. 6C, D), and the stem and leaves were asymmetric (Fig. 6E, F). To identify the genes responsible for the promotion of bolting in *DcFT1*-ox plants, we performed expression analysis of flowering-related genes (Fig. S3) in T_3 individuals with empty VC on day 11 after sowing and in *DcFT1*-ox3. In the transformant *DcFT1*-ox3 used in this experiment, the introduced *DcFT1* was expressed at

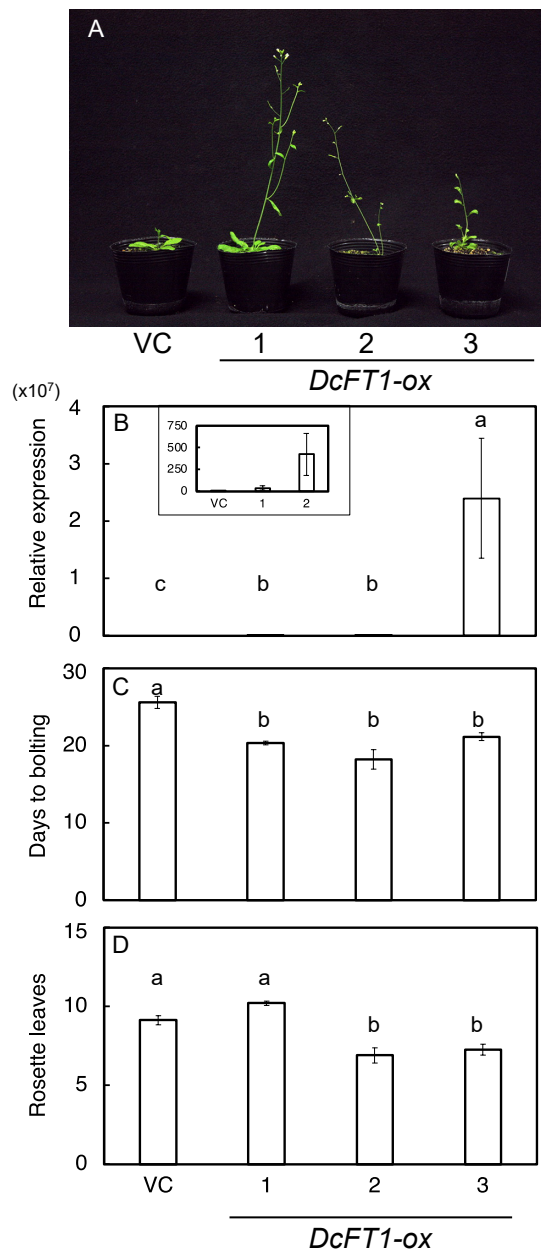


Fig. 5. Analysis of *DcFT1*-overexpressed *Arabidopsis*. Early bolting phenotypes were observed in the three lines of *DcFT1*-overexpressed *Arabidopsis* (A). The differences in *DcFT1* expression levels between empty vector control (VC) and overexpression of *DcFT1* (B) were compared. Expression levels were estimated by qRT-PCR and normalized to the internal control *AtUBQ10*. The relative expression is represented as the calculated value against a VC of 1.0. The y-axes of the outer large graph and the inner small graph have different scales, $\times 10^7$ and $\times 1.0$, respectively. Days to bolting (C) and the number of rosette leaves (D) were evaluated in the four transgenic lines. Different letters indicate significant differences according to Tukey-Kramer's HSD or Steel-Dwass tests ($P < 0.05$).

significantly higher levels than in the empty VC (Fig. 5B). The expression levels of endogenous *AtFT* and *CO* did not differ significantly between the VC and *DcFT1*-ox3 plants (Fig. 7A). In contrast, the expression levels of *API*, *SOC1*, and *FUL*, which are downstream

genes of *AtFT*, were significantly higher in *DcFT1*-ox3 plants. There was no significant difference in the expression of *LFY* between the VC and *DcFT1*-ox3. These results suggest that flower bud differentiation had not started due to environmental conditions, such as long days for seedlings 11 days after sowing.

Next, we investigated the factors that caused *DcFT1*-ox3 to dwarf and form asymmetric cauline leaves. We analyzed the expression of *ASYMMETRIC LEAVES1* (*AS1*), *ASYMMETRIC LEAVES 2* (*AS2*), *KNOTTED-like Arabidopsis thaliana 1* (*KNAT1*), and *GIBBERELLIN 20-OXIDASE 1* (*GA20ox1*) in *DcFT1*-ox3 cells and VC. The expression of *AS1*, *AS2*, and *KNAT1* was not significantly different between the WT and *DcFT1*-ox plants, whereas the expression of *GA20ox1* was significantly lower in *DcFT1*-ox (Fig. 7B).

Discussion

BLAST analysis revealed six orthologs of the *FT* sequence in carnation. Phylogenetic analysis and amino acid alignment revealed that *Dca19666.1* (*DcFT1*) were FT-like genes and all amino acid sequences involved in the promotion of flowering were conserved (Fig. 1; Table S4). Several studies have reported that FT homologs and QTLs for flowering time are co-localized in numerous crops, including domesticated rice (Kojima et al., 2002), wheat (Yan et al., 2006), barley (Wang et al., 2010), rose (Kawamura et al., 2011; Otagaki et al., 2015), sunflower (Blackman et al., 2010), and watermelon (McGregor et al., 2014). *DcFT1* is one gene in the candidate region of the QTL for flowering time and is believed to be involved in the regulation of flowering in carnations (Yagi et al., 2020). In this study, overexpression of *DcFT1* promoted bolting in *A. thaliana* (Fig. 5A, C). However, in *DcFT1* overexpressing plants, *DcFT1*-ox3, flower bud formation was delayed and not visually confirmed at the time of bolting (Fig. 6A, B). Flowering and bolting occur continuously in the dark and are controlled by various factors. In contrast, both occur in parallel under suitable day-length conditions, and bolting is controlled by the same factors under both short- and long-day conditions, making bolting a strong indicator of the transition to the reproductive phase (Pouteau and Albertini, 2009). Furthermore, because the expression of *API* and *FUL*, which determine flower bud tissue, increased significantly in *DcFT1*-ox plants (Fig. 7A), we concluded that *DcFT1* is a flowering-promoting gene.

DcFT1-ox was dwarfed, probably because of the decreased expression of *GA20ox1*, a gibberellin synthesis gene (Fig. 7B). *KNAT1* suppresses the transcription of *GA20ox1*. In the *Arabidopsis* line overexpressing *KNAT1*, the leaves are asymmetric, and the rosette leaves are largely lobed (Lincoln et al., 1994), while in wild type, the cotyledons are small and round, and the true leaves are large and serrated (Bowman et al., 1994). *KNAT1* is a class-I KNOTTED 1-like homeobox

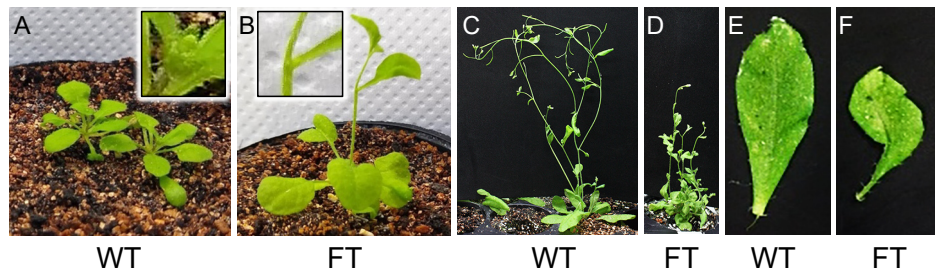


Fig. 6. Phenotypes caused by a strong overexpression of *DcFT1* in *Arabidopsis*. *DcFT1-ox3* (FT) (B) bolted earlier than the wild type (WT) (A) without bud formation (as shown in the inner boxes). Conversely, it was dwarfed at flowering (D) compared to the wild type (C). *DcFT1-ox3* differs from the wild type (E) by forming asymmetric leaves (F).

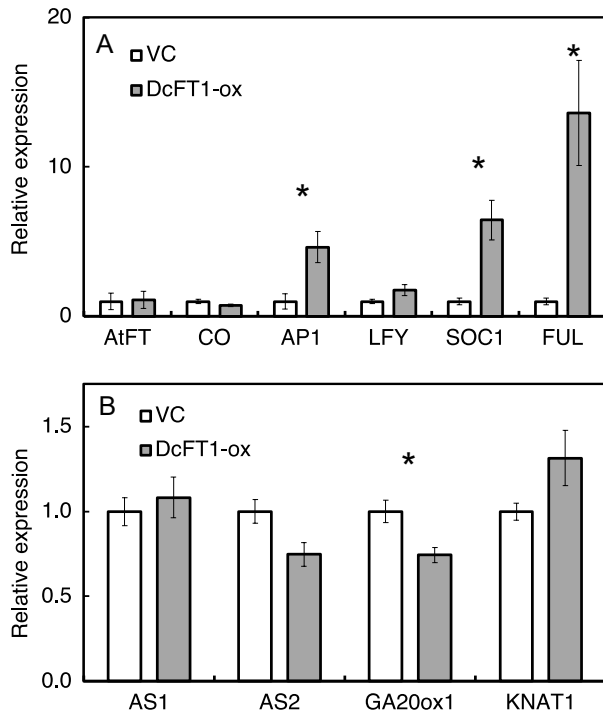


Fig. 7. Transcriptional changes in genes around *FT* in *Arabidopsis thaliana* by overexpressing *DcFT1*. Gene expression levels of flowering-related genes (A) and leaf morphogenesis-related genes (B) around *FT* were compared between an empty vector control (VC) and *DcFT1* on the expressor (*DcFT1-ox*) plants. Expression levels were estimated by qRT-PCR and normalized to the internal control *AtUBQ10*. The relative expression is presented as the calculated value against a VC of 1.0. Significant differences between VC and *DcFT1-ox* are indicated by asterisks (Wilcoxon rank-sum test; $P < 0.05$).

(*KNOX*) gene, that is thought to be central to leaf morphogenesis (Hay et al., 2002). The *KNOX* gene family is negatively regulated by the *AS1/AS2* gene, and the *as1* mutant has a phenotype similar to that of *KNAT1-ox* (Ori et al., 2000). Because the rosette leaves of *DcFT1-ox* plants were asymmetric, we analyzed the expression of *GA20ox1* upstream genes *AS1*, *AS2*, and *KNAT1* in relation to leaf morphogenesis. However, the expression levels of these genes were not different between the WT and *DcFT1-ox* plants, suggesting that *GA20ox1* is regulated by different pathways and that

there are other genes involved in the abnormal morphology of *DcFT1-ox* leaves.

FT homologs in tomato (*Solanum lycopersicum* L.) and lettuce (*Lactuca sativa* L.) are highly expressed in mature leaves and unevenly expressed according to leaf position (Fukuda et al., 2011; Shalit et al., 2009). Therefore, in a preliminary study, the leaves closest to the base of the first lateral branch that developed after pinching the shoots were sequentially sampled and used for analysis. However, the expression level of *DcFT1* was low, and the fluorescence level detected by real-time PCR was close to the detection limit. Therefore, in this study, site-specific expression analysis was performed, which showed that *DcFT1* in carnation is highly expressed in the upper part of the plant (Fig. 3). *FT* homologs exhibit age-dependent leaf expression in several plant species. In tomato, *SFT*, which promotes floral development, is most highly expressed in the second mature leaf, with expression levels decreasing in the fourth, sixth, eighth, tenth, and younger leaves. *SP*, a homolog of *Arabidopsis* *TFL1* that suppresses floral development, showed an opposite trend to *SFT* (Shalit et al., 2009). When lettuce was grown under high temperatures to induce abscission and floral development, *LsFT* expression was higher in mature larger leaves than in immature smaller leaves, and this trend was more pronounced during floral development than during floral transition (Fukuda et al., 2011). Floral developmental signals have been speculated to be generated in mature expanded leaves (Fukuda et al., 2011). The expression of *DcFT1* was higher in the leaves near the shoot apex of carnations in this study. The expression was high in mature leaves of tomato and lettuce, suggesting a different regulatory mechanism for flowering in carnation.

The EOD-cooling treatment accelerated flowering and the expression of *DcFT1* (Fig. 4A, B). In a previous study on chrysanthemum, diurnal variations in heat sensitivity were correlated with the regulation of the *FT* ortholog (*FTL3*) (Nakano et al., 2013, 2015). This temperature-dependent regulation has also been observed in *Arabidopsis*, for which transcript levels increase at the end of cooler nights (Kinmonth-Schultz et al., 2016). This study is the first to show a relation-

ship between EOD-cooling techniques and FT expression, which could be a marker for further optimization of bloom control in carnation.

Splice variants are often found in FT homologs and may be involved in the regulation of flower growth. In chrysanthemum (*Chrysanthemum morifolium*), the expression of splice variants has been suggested to regulate flowering, and multiple CmFTL1 splicing variants have different functions and are involved in the regulation of floral development (Mao et al., 2016). In addition, in *Brachypodium distachyon* L., there are two splicing variants of BdFT2 α and β . The flower growth-promoting BdFT2 β and the repressive BdFT2 α are involved in, and regulate, the transition to the reproductive growth phase (Qin et al., 2017). In carnation, the coding sequences and splice variants of DcFT1 show similar expression patterns, making it unlikely that splice variants are involved in the regulation of flowering.

A comparison of the coding sequence of Dca19666.1 between the early maturing ‘Kane’ and late maturing ‘LPB’ showed no variation (Fig. S4). The splice variant DcFT1 α contained the full length of the first intron, and an SNP was located in the same region (Fig. S4). When the promoter sequence of about 5 kbp and the 3’ flanking region of about 2 kbp of *DcFT1* were compared between the two cultivars, we found an 804 bp indel at 3.7 kbp upstream of the start codon and some variants in the 3’ regions (data not shown). To date, no correlations between the SNP or indel sequences and flowering time have been observed despite using several early-late cultivars. This study and Yagi et al. (2020) suggest that *DcFT1* is a central factor in the regulation of floral development and is likely to influence differences in early-late flowering. However, we could not find the causal sequence regions. In recent years, high-quality genome sequences for carnation have been reported (Jiang et al., 2023; Zhang et al., 2022). Using this information will accelerate gene isolation. Now that neighboring genes and detailed gene sequences have been identified by high-precision genomes, the identification of causal genes will soon be pursued by map-based cloning and expression analysis approaches.

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