Mol Breeding (2017) 37: 149
https://doi.org/10.1007/s11032-017-0749-y

Molecular cloning, expression analysis, and subcellular localization of *FLOWERING LOCUS T* (*FT*) in carrot (*Daucus carota* L.)

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Received: 24 May 2017 / Accepted: 31 October 2017 / Published online: 14 November 2017
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Abstract Flowering, an important agronomic trait for seed plants, represents the end of vegetative growth and begins to reproduce. In regulating the floral transition, FLOWERING LOCUS T (FT) encoding a mobile floral signal protein that belongs to the phosphatidylethanolamine-binding protein (PEBP) family acts as critical role. Here, an FT-like gene, *DcFT*, was isolated and cloned from European *Daucus carota* L. cultivar "Nantes-H06" (GenBank accession number KY768910), and an alignment of the *DcFT* protein and other FT-homolog proteins showed that it shared 88.00% similarity with CsFT from *Camellia sinensis*. Phylogenetic tree analysis indicated that *DcFT* had the closest relationship with GpFT (*Gypsophila paniculata*). Quantitative RT-PCR was analyzed to show that the expression pattern of *DcFT* in inflorescences sharply increased after 10DAA and then slowly increased reaching the maximum at 30DAA. Subcellular localization made clear that the *DcFT* protein was located in the nucleus and cytoplasm. Broadly speaking, *DcFT* is an FT-like homologous gene in carrot regulating the floral transition and could be a candidate gene for accelerating the process in carrot breeding.

Keywords Carrot · Floral transition · Quantitative RT-PCR · Subcellular localization

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11032-017-0749-y) contains supplementary material, which is available to authorized users.

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Introduction

In angiosperms, flowering, the significant agronomic trait in seed plant, is a critical and refined step for plants and is precisely regulated by various endogenous and exogenous (such as environment) factors (Guo et al. 2015; Tan and Swain 2006; Wilkie et al. 2008; Niu et al. 2016). In model plant *Arabidopsis thaliana*, five primary flowering pathways have been confirmed, incorporating the vernalization, gibberellin, photoperiodic, autonomous, and age pathways (Komeda 2004; Boss et al. 2004; Srikanth and Schimid 2011). *FLOWERING LOCUS T* (*FT*) is a vital integrator gene of these flowering pathways and the mobile protein of it encoding induces flowering (Wigge et al. 2005), and the product of the *FT* gene is widely considered as florigen or the major component of an intricate signal (Zeevaart 2008; Turck et al. 2008; Tamaki et al. 2007; Ahn et al. 2012).

The *FT* gene was first deciphered in *A. thaliana* (Kobayashi et al. 1999). Subsequent research revealed that the FT protein was translocated from leaf tissues to

the apical meristem and was a direct target of the nuclear protein CONSTANS (CO) in *A. thaliana* (Samach et al. 2000; Wigge et al. 2005). In long-day (LD) conditions, CO transcription is adjusted by circadian clock, reaching a peak after dawn and activating the downstream *TWIN SISTER OF FT* (*TSF*) and *FT* genes, promoting their expression (Bohlenius et al. 2006). In the apical meristem, the FT protein interacts with FLOWERING LOCUS D (FD), a bZIP transcription factor, forming a complex part that activates the genes *LEAFY* (*LFY*), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*), and *APETALA1* (*AP1*) in the floral meristem to promote flowering (Abe et al. 2005; Ho and Weigel 2014). At present, homologs of *FT* have been authenticated in many species, including dicotyledonous plants such as sugar beet (Pin et al. 2010), tomato (Lifschitz et al. 2006), soybean (Sun et al. 2011), tobacco (Harig et al. 2012), cucurbits (Lin et al. 2007), onion (Lee et al. 2013), and lettuce (Fukuda et al. 2011), as well as monocots for instance sorghum (Wolabu et al. 2016). This has shown that the FT proteins in various plant species have a conserved functional domain. However, the regulatory mechanism of the vegetative to flowering transition and flower initiation in carrot (*Daucus carota* L.) has not been reported.

In this study, we cloned the coding region of the "florigen" *FT* gene from carrot, *DcFT*, and performed a bioinformatic analysis and expression profile *of DcFT* in different carrot tissues and organs. To inquire into the subcellular localization of the FT protein in carrot, we fused it to a vector containing green fluorescence protein (GFP) report group and bombarded it into the epidermal cell of onion (*Allium cepa* L.) by biolistic transformation. Our studies provide a theoretical basis for *FT* gene in regulating flowering time in carrot (*Daucus carota* L.) breeding process.

Materials and methods

Plant material and growth condition

The European *D. carota* cultivared variety "Nantes-H06" was studied in this experiment. Material was cultivated in the experimental field at the Vegetable Research Center of Beijing Agriculture and Forestry Science Academy (Beijing, China). Roots, stems, and leaves were collected at three different periods: before anthesis (no flower primordium), when a distinct meristem (namely, the flower primordium) had formed and when floral organ formation was complete (that is the flower unfolding). Rachises, petioles, and inflorescences (according to the number of days after anthesis (DAA); classified as 10, 20, and 30 DAA) were got from the complete floral organ stage. All materials were harvested and placed in the fridge with −80 °C for analyzing gene expression levels.

Cloning of *DcFT* from *D. carota*

Total RNA was extracted from the leaves of carrot using Trizol reagent (Xinjingke, Beijing, China) according to the slightly modified instruction and then reversed transcription cDNA with a TIANScript RT Kit (Tiangen, Beijing) following the instructions. For cloning the *DcFT* gene, utilizing the DNAMAN software to contrast with known sequences of the *FT* homolog from different plants, for instance, *Chrysanthemum* × *morifolium* and *A. thaliana*, designed degenerate primers (FT-M-F and FT-M-R) according to the conservative domains. PCR amplification was done with the product of RT-PCR from the leaves as a template. SMART 5′- and 3′-RACE kits (Clontech, Palo Alto, CA, USA) were used to amplify the *DcFT* sequence by nested amplification. For 3′-RACE, the antisense primer was from the 3′-RACE kit, and the gene-specific primers were FT-3-out-64 and FT-3-inner-110, respectively. For 5′-RACE, the sense primers were in the 5′-RACE kit and the FT-5-out-269 and FT-5-inner-119 primers were used as inverse primers. The PCR amplification reaction volume and conditions followed the instructions of the SMART 5′- and 3′-RACE kits. FT-GSP-F and FT-GSP-R primers containing restriction enzyme cutting sites were amplified for getting the open reading frame (ORF) that was used for plant expression vector construction. All primers sequences are listed in Supplement Table S1. The products of PCR amplification were checked on 1% denaturing agarose gels and recycled by using a MiniBEST agarose gel DNA extraction kit (Takara, Dalian, China). The PCR product was ligated into the PMD19-T vector (Takara, Dalian, China) and then transformed into *Escherichia coli* DH5α competent cells (Takara, Dalian, China). After, picking positive clones by using blue-white selection and 5–10 single colonies (white) was sequenced (AuGCT, Beijing, China). Finally, the complete sequences were assembled using DNAMAN to obtain

the full-length cDNA of *DcFT* gene, and then submitted to GenBank.

Bioinformatic and homology comparison of *DcFT*

The deduced amino acid sequences of carrot *DcFT* were analyzed using the NCBI blast program (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). By the online Expasy Protparam tool (http://web.expasy.org/protparam/), the *DcFT* protein physical properties were predicted. The deduced protein of *DcFT* and FT proteins from other plant species were aligned using Clustal W (Thompson et al. 1994) with the default parameters. Phylogenetic tree and molecular evolutionary analyses were performed with the MEGA 6.0 software (Neighbor-Joining (NJ) method) (Tamura et al. 2013; Saitou and Nei 1987). Bootstrap evaluation was analyzed with 1000 bootstrap replicates. The numbers represent the bootstrap support (percentage) at each node.

RT-PCR and real-time quantitative PCR

Total RNA (root, stem, leaf, rachis, petiole, and inflorescence (10, 20, and 30 DAA) at the complete floral organ stage and root, stem, and leaf from the other two stages) was extracted by RNAprep pure Plant Kit (Tiangen, Beijing) according to the manufacturer's protocol. *DcFT* expression profiles were analyzed by qRT-PCR. The cDNAs were synthesized with the PrimeScript™ RT reagent kit (TaKaRa, Dalian, China) with gDNA Eraser (Perfect Real Time) according to the instructions. The qRT-PCR primers were designed with the Primer Premier 5 software based on the full-length cDNA sequence of the *DcFT* gene. The β-actin gene (as an internal control) was amplified with the primers to normalize the result (Tian et al. 2015). Before performing the qRT-PCR analysis, the efficiency and specificity of the primers were examined by 1% agarose gel electrophoresis and a preliminary qRT-PCR experiment. The reaction for each sample was repeated in triplicate. The reaction volume was 20 μL, including 2.0 μL cDNA template, 0.4 μL of each primer (10 μM), 7.2 μL PCR-grade water, and 10 μL of SYBR premix Ex Taq II (Perfect Real Time; TaKaRa, Dalian, China). qRT-PCR was performed on a Roche Light Cycler 480 system (Bio-Rad, USA). The program for qRT-PCR was 95 °C for 15 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 20 s. To confirm the credibility of the qRT-PCR results, a melting curve analysis was performed at the end of each PCR reaction at 95 °C for 5 s, 65 °C for 60 s, and 97 °C continuously, prior to termination at 40 °C for 30 s. The dates were analyzed according to the $2^{-\Delta\Delta Ct}$ methods (Schmittgen and Livak 2008).

Construction of the expression vector

Using a transient expression vector pCambia1305-35S-GFP supplied by Jian Ma (Vegetable Research Center, Beijing) analyzed the transient expression of *DcFT* gene. To construct the expression vector *35S::DcFT-GFP*, the primers containing *Xba*I and *Bam*HI sites were designed to amplify the *DcFT* ORF domain. The PCR production was separated on 1% agarose gel and then purified. The empty vector pCambia1305-35S-GFP was digested with restriction enzyme *Xba*I and *Bam*HI at 37 °C for 3 h. Subsequently, the purified PCR product and cleaved *35S::GFP* vector were fused using an In-Fusion HD Cloning kit (TaKaRa, Dalian, China) and then transformed into *E. coli* DH5α competent cells. Positive recombinants were selected and confirmed by sequencing. Ultimately, the *35S::DcFT-GFP* vector was obtained.

Subcellular localization analysis

The obtained *35S::DcFT-GFP* fusion proteins were surveyed by biolistic bombardment of onion epidermal cell as the previous method (Scott et al. 1999). The constructed *35S::DcFT-GFP* DNA was extracted with an EndoFree Maxi Plasmid Kit (Tiangen, Beijing). Gold particles (1-μm diameter) were coated with 5 μg of the DNA as the instruction (Bio-Rad, Hercules, CA, USA), and then it was bombarded into onion epidermal cell at 1200 psi helium pressure utilizing a PDS-1000 system (Bio-Rad). After incubating 24 h in darkness, the onion cells were surveyed by confocal laser scanning microscope (Olympus, Japan). The *35S::GFP* vector was as a reference.

Results

Cloning and characterization of the *DcFT* gene

The full-length cDNA of *DcFT* was obtained from carrot (*D. carota*) by producing the sequence using degenerate primers amplification and the RACE

technology. The full-length cDNA of *DcFT* was 853 bp (GenBank accession number KY768910), comprising a 45-bp 5′-untranslated region (UTR), a 283-bp 3′-UTR, and an ORF 525-bp (Supplemental Fig. S1) encoding 175 amino acid residues with an estimated molecular weight of 20.0 kDa and a theoretical isoelectric point (PI) of 7.75.

According to the NCBI web server (https://www.ncbi.nlm.nih.gov/Structure/cdd/docs/cdd_search.html), the domain structure of the predicted amino acid sequence showed the *DcFT* was a member of the *CENTRORADIALIS/TERMINAL FLOWER 1/SELF-PRUNING* (*CETS*) gene family, which shares homology to and is alternatively called the PEBP gene family (Supplemental Fig. S2). An alignment of the *DcFT* protein and other FT protein is exhibited in Fig. 1. The amino acid Tyr88 (Y) and Gln144 (Q) belonging to conservative amino acid residues in FT-like proteins are important and are characteristic amino acids for distinguishing FT-like floral promoters (Hanzawa et al. 2005; Ahn et al. 2006). The amino acid sequences all contained the "Segment A domain (14AA)" and LYN, corresponding to a potential ligand-binding pocket in FT/TFL1 family proteins (Ahn et al. 2006). Capitalized letters on the alignment denote functionally vital conservative residues of FT proteins (Manoharan et al. 2016). A BLASTp comparison of the deduced amino acid sequence of *DcFT* with those of other FT proteins revealed that the protein of *DcFT* had the highest identify (88.00%) to that of CsFT and the lowest (62.22%) to that of AfFT (Supplement Table S2).

An alignment of the *DcFT* protein and 18 other plant FT proteins retrieved from NCBI was performed using ClustalW, and then an unrooted phylogenetic tree was constructed by the neighbor-joining method in the MEGA 6.0 software (Fig. 2). The phylogenetic tree indicated that *DcFT* protein had the closest relationship with GpFT and was only distantly related to FT from *Vitis vinifera*. The intimate relationship between *DcFT* and *GpFT* explains that they probably have similar functions in promoting flowering.

Gene expression analysis of *DcFT*

Florigen, which produced in leaves and transmitted to the shoot apex, is primarily seduced by a systemic signal (Chailakhyan 1968; Mathieu et al. 2007). The expression pattern of numerous FT-like homologs has been analyzed, such as *CsatFT1–CsatFT4* in perennial geophyte saffron crocus (*Crocus sativus*) (Tsaftaris et al.

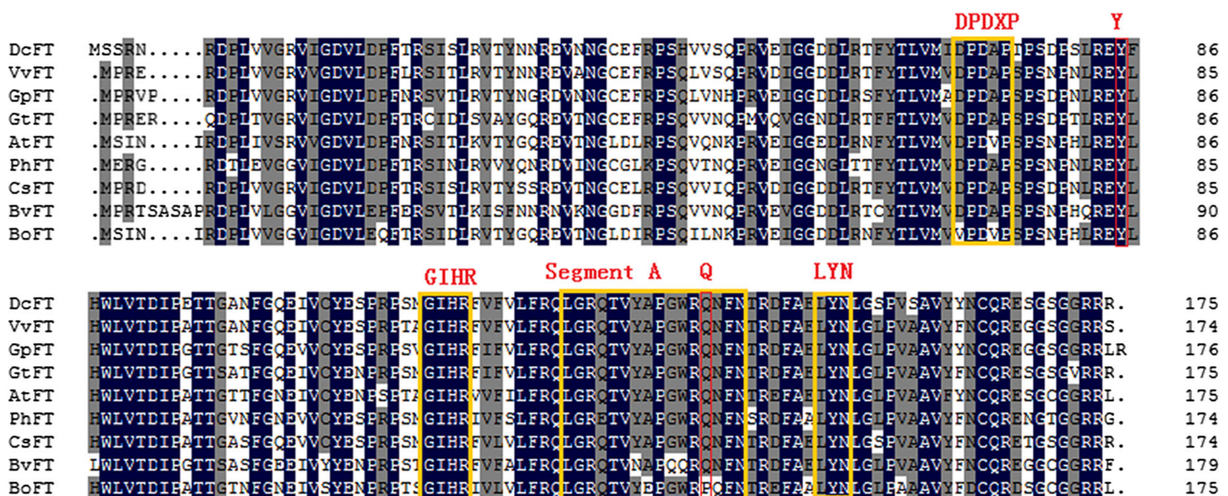

Fig. 1 Alignment of the deduced amino acid sequence of *DcFT* and other homologous proteins in plants. A dark blue background represents amino acid 100% identity, and a light gray background represents amino acid identity > 75%. GenBank accession numbers of sequences in the figure are as follows: *Vitis vinifera* (V*vFT*, ABL98120.1), *Gypsophila paniculata* (*GpFT*, BAK23998.1), *Gentiana triflora* (*GtFT*, BAK40194.1), *Arabidopsis thaliana* (*AtFT*, AAF03936.1), *Petunia* × *hybrida* (*PhFT*, BAV21621.1), *Camellia sinensis* (Cs*FT*, BAM83573.1), *Beta vulgaris subsp. vulgaris* (B*vFT*, AEI55782.1), *Brassica oleracea* (Bo*FT*, ACH86033.1), and *Daucus carota* (*DcFY*, KY768910). The three rectangles in yellow represent conserved regions and amino acids shown in red indicate the Tyr85 (Y) and Gln140 (Q) residues that distinguish all FT-like members

2013). In order to elucidate the *DcFT* expression pattern in carrot, a qRT-PCR experiment was carried out to detect the transcript levels *DcFT* in various tissues/organs of carrot. In the complete floral organ stage, the expression level in rachises and petiole was the highest and lowest, respectively (Fig. 3a). Among the three different growth stages in carrot, the expression level of *DcFT* in leaves was the lowest in the third stage, stem and leaf was the highest in the obvious-meristem stage, and in the pre-anthesis (no flower primordium) stage, the expression level of *DcFT* in root and stem was approximately equality (Fig. 3b). After 10DAA, the expression level of *DcFT* in inflorescence sharply increased and then slowly increased reaching the maximum at 30DAA (Fig. 3c). In conclusion, these results illuminated that the expression of *DcFT* existed among tissues during developmental periods and was different, manifesting that *DcFT* was likely to play a central role in regulating flowering in carrot.

Subcellular localization of *DcFT*

To determine the cellular location of the *DcFT* protein, the fusion protein expression vector *35S::DcFT-GFP* was constructed by fusing the C-terminus of the *DcFT* protein to the green fluorescence protein (*GFP*) with the 35S promoter of the Cauliflower Mosaic Virus (CaMV). Then, the DNA plasmid of *35S::DcFT-GFP* was guided into onion epidermal cells employing a gene gun. An expression vector *35S::GFP* was as a reference. After cultivating for 24 h, *35S::DcFT-GFP* and the empty *35S::GFP* protein signal were detected by fluorescence microscopy, respectively (Fig. 4a–c, d–f). The fusion protein *35S::DcFT-GFP* was clearly distributed in the nucleus and cytoplasm (Fig. 4d–f). This consequence was in line with the localization of NtFT protein from tobacco (*Nicotiana tabacum* L.) (Harig et al. 2012).

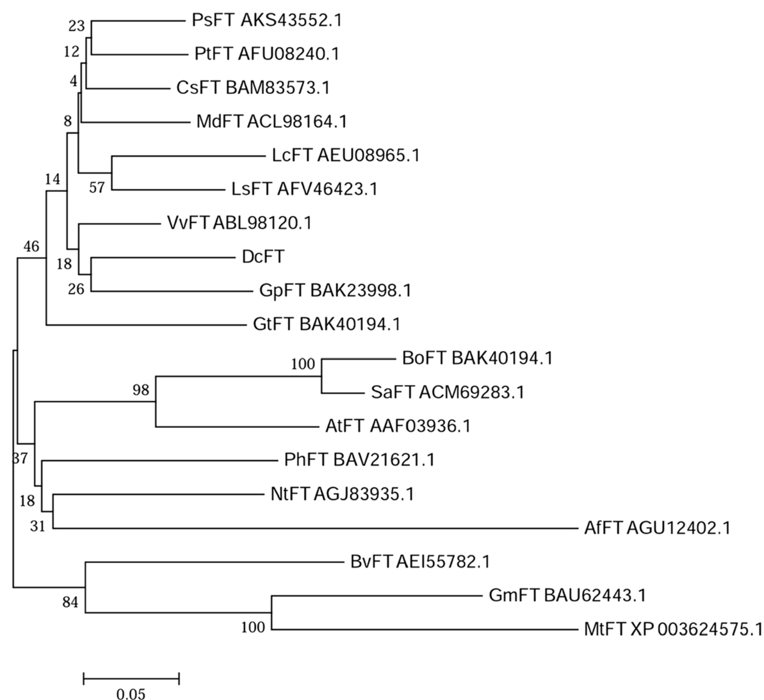

Fig. 2 Phylogenetic tree of *DcFT* and other plant FT proteins. Numbers at branch points show bootstrap support. GenBank accession numbers of sequences in the figure are as follows: *Paeonia suffruticosa* (PsFT, AKS43552.1), *Populus tomentosa* (PtFT, AFU08240.1), *Camellia sinensis* (CsFT, BAM83573.1), *Malus domestica* (MdFT, ACL98164.1), *Litchi chinensis* (LcFT, AEU08965.1), *Lactuca sativa* (LsFT, AFV46423.1), *Vitis vinifera* (Vv*FT*, ABL98120.1), *Gypsophila paniculata* (*GpFT*, BAK23998.1), *Gentiana triflora* (GtFT, BAK40194.1), *Brassica oleracea* (Bo*FT*, ACH86033.1), *Sinapis alba* (SaFT, ACM69283.1), *Arabidopsis thaliana* (AtFT, AAF03936.1), *Petunia × hybrida* (PhFT, BAV21621.1), *Nicotiana tabacum* (NtFT, AGJ83935.1), *Allium fistulosum* (AfFT, AGU12402.1), *Beta vulgaris subsp. vulgaris* (BvFT, AEI55782.1), and *Medicago truncatula* (MtFT, XP_003624575.1)

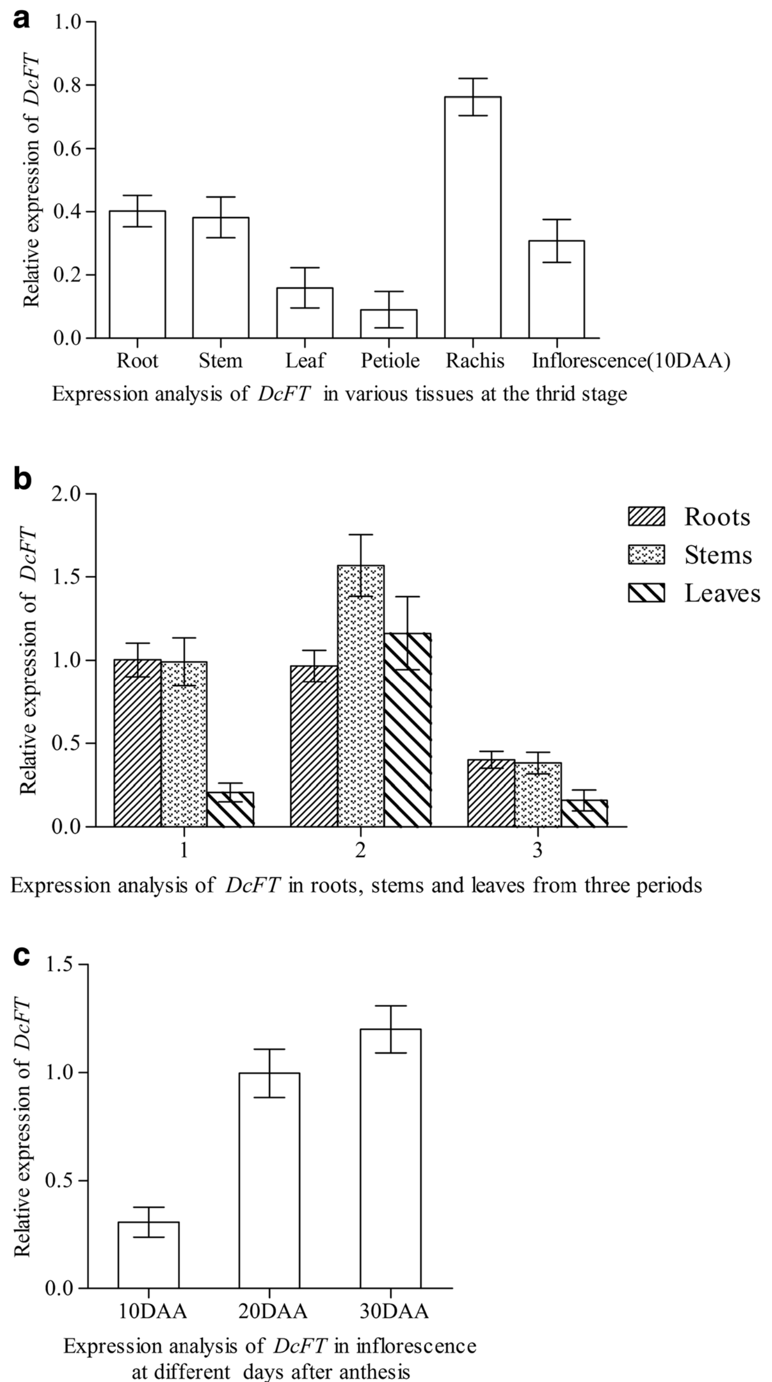

Fig. 3 Expression patterns of *DcFT* in various tissues and organs; roots, stems, and leaves from three periods and inflorescences at different times. qRT-PCR was used to analyze the relative expression level of *DcFT* relative to β-actin protein as a control. Data are mean values and error bars represent the standard deviation from three biological replicates. 1–3 represent the pre-anthesis (no flower primordium), obvious-meristem (namely, the flower primordium), and complete floral organ stages (that is the flower unfolding)

Discussion

Genetic and molecular analyses of various horticultural plants have indicated that FT homologs play a center role in regulating flowering time, for example, in Chinese *Cymbidium*, *Sinapis alba*, and *Carya cathayensis* (Huang et al. 2012; Chen et al. 2009), but the molecular mechanism of *FT* gene has not been studied in carrot. In this present research, isolating and cloning a homologous *FT* gene (*DcFT*) from carrot according to the conserved sequences of FT-like gene that was from various plants. The deduced protein

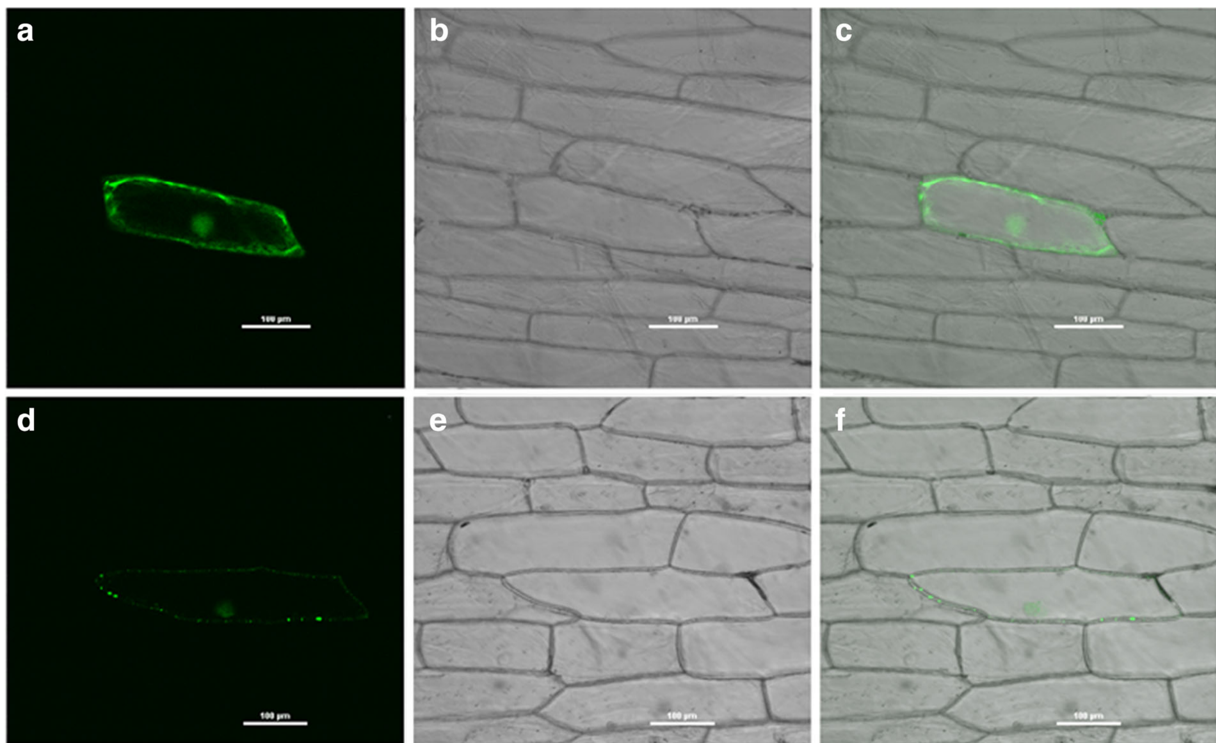

Fig. 4 Subcellular localization of *35S::DcFT-GFP*. **a**–**c** Subcellular localization of *35S::GFP*; the panels represent fluorescence signal (excitation, 488 nm), bright field, and merged images, respectively. **d**–**f** Subcellular localization of *35S::DcFT-GFP*; the panels represent fluorescence signal (excitation, 488 nm), bright field, and merged images, respectively

sequence of *DcFT* was by BLAST search in the NCBI database indicating that *DcFT* proteins belong to the PEBP family and have the conserved domain sequences that promote flowering (Ahn et al. 2006). Furthermore, phylogenetic analysis distinctly revealed an intimate genetic relationship between *DcFT* and *GpFT* or *VvFT*. This conclusion was likely to suggest *DcFT* has similar functions to *GpFT* and *VvFT*, which participated in the control of flowering (Debener and Winkelmann 2010; Vergara et al. 2016).

The expression pattern of *DcFT* was very wide in various tissues but was predominantly expressed in inflorescences (30DAA) and rachises (Fig. 3a, c), which was consistent with the rest of FT homologs (Huang et al. 2012). In numerous plants, the expression patterns of FT-like homologs are evidently different in various tissues. In tobacco, *NtFT1*–*NtFT4* were mostly expressed in leaves (Harig et al. 2012). In onion (*Allium cepa* L.), the expression level of *FT* genes (*AcFT1*-Ac*FT7*) was detected in seed, leaf, bulb, and flowering bulb (Lee et al. 2013; Manoharan et al. 2016). In seedlings and mature period of *A. thaliana*, *AtFT* was expressed in every tissue, and especially, in flowers and immature siliques, the expression levels were higher (Kobayashi et al. 1999). In this study, the relative expression level of *DcFT* in leaf was the highest at the obvious-meristem (namely, flower pirmordium) stage and decreased immediately to basal levels at the complete floral organ stage (Fig. 3b), which may suggest that *DcFT* has a function in photosynthesis for regulating flowering time in carrot (*Daucus carota* L.). With the growth, the expression pattern of *DcFT* in inflorescences sharply increased and then slowly increased reaching the maximum at 30DAA (Fig. 3c), which suggest that the *FT* gene transfers to floral organ and promotes flowering with gradually accumulating after flowering. How the FT gene promotes flowering and the related mechanism need to further studied. Now, an experiment was under way involving transgenic tobacco plants, comparing short-day contition (8-h light/16-h dark) with long-day condition (16-h light/8-h dark) to study the mechanisms of *FT* gene intensively in carrot (*Daucus carota* L.).

Our data indicates that the protein of *DcFT* is localized in cytoplasm and nucleus (Fig. 4) which is in line with that of CsFT and FaFT (Higuchi et al. 2013; Lei

et al. 2015), suggesting *DcFT* may play an important role in flowering regulation.

Funding information This work was funded by grants from the National Key Technology Research and Development Program of China (2012BAD01B00, 2012BAD50G01, 2014BAD01B08, and JNKYT201601). Our research was also supported by the Technological Innovation Capacity Program of the Beijing Academy of Agricultural and Forestry Sciences (KJCX20150111, KJCX20170102).

Compliance with ethical standards The experiments described here comply with the current laws of China.

Conflict of interest The authors declare that they have no conflict of interest.

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