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ORIGINAL PAPER

The *Vaccinium corymbosum FLOWERING LOCUS T*-like gene (*VcFT*): a flowering activator reverses photoperiodic and chilling requirements in blueberry

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Abstract

Key message **The blueberry *FLOWERING LOCUS T* (*FT*)-like gene (*VcFT*) cloned from the cDNA of a tetraploid, northern highbush blueberry (*Vaccinium corymbosum* L.) is able to reverse the photoperiodic and chilling requirements and drive early and continuous flowering.**

Abstract Blueberry is a woody perennial bush with a longer juvenile period than annual crops, requiring vernalization to flower normally. Few studies have been reported on the molecular mechanism of flowering in blueberry or other woody plants. Because *FLOWERING LOCUS T* (*FT*) from *Arabidopsis thaliana* plays a multifaceted role in generating mobile molecular signals to regulate plant flowering time, isolation and functional analysis of the blueberry (*Vaccinium corymbosum* L.) *FT*-like gene (*VcFT*) will facilitate the elucidation of molecular mechanisms of flowering in woody plants. Based on EST sequences, a 525-bp*VcFT* was identified and cloned from the cDNA of a tetraploid, northern highbush blueberry cultivar, Bluecrop. Ectopic expression of *35S:VcFT* in tobacco induced flowering an average of 28 days earlier than wild-type plants. Expression of the *35S:VcFT* in the blueberry cultivar Aurora resulted in an extremely early flowering phenotype, which flowered not only during in vitro culture, a growth stage when nontransgenic shoots had not yet flowered, but also in 6–10-week old, soil-grown transgenic plants, in contrast to the fact that at least 1 year and 800 chilling hours are required for the appearance of the first flower of both nontransgenic 'Aurora' and transgenic controls with the *gus*A. These results demonstrate that the *VcFT* is a functional floral activator and overexpression of the *VcFT* is able to reverse the photoperiodic and chilling requirements and drive early and continuous flowering.

Keywords Floral activator · Floral initiation · *FLOWERING LOCUS T* · Flowering time · *Vaccinium corymbosum* L. · Woody plant

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Introduction

Plant flowering is controlled by intrinsic developmental status and environmental signals. Timing of plant flowering is closely related to agricultural productivity. Numerous studies have been undertaken in the past decades to elucidate the molecular basis of flowering in *Arabidopsis thaliana* and other herbaceous plants by investigation into the networks of floral genes (see recent reviews by Trevaskis et al. 2007; Wilkie et al. 2008; Zeevaart 2008; Greenup et al. 2009; Michaels 2009; Amasino 2010; Lee and Lee 2010; Wellmer and Riechmann 2010; Pin 2012). In the proposed networks of floral genes, floral activators

FLOWERING LOCUS T (*FT*) or *FT*-likes, *SUPPRESSOR of OVEREXPRESSION OF CONSTANS 1* (*SOC1*) or *SOC1*-likes, and *LEAFY* (*LFY*) or *LFY*-likes, are three major integrators controlling plant flowering, which integrate flowering signals from multiple pathways (Kardailsky et al. 1999; Kobayashi et al. 1999; Wilkie et al. 2008; Greenup et al. 2009; Michaels 2009; Amasino 2010; Lee and Lee 2010; Pin 2012). Flowering activators such as *FT* or *FT*-likes, *FD* or *FD*-likes, and *SOC1* or *SOC1*-likes, stimulate flowering as their expression levels increase (Samach et al. 2000; Abe et al. 2005; Wigge et al. 2005; Zhang et al. 2010; Pin 2012). Overexpression of the *FT*-likes or *SOC1*-likes is one of the options for shortening juvenility and reducing dependence on vernalization or photoperiod for seasonal flowering; thus, the biotechnological utilization of these genes can improve germplasm and crop production (Borner et al. 2000; Ferrario et al. 2004; Ma et al. 2011; Pin 2012).

Temperate fruit trees are generally poor candidates for studying flowering mechanisms due to their complex genomes (heterozygosity and polyploidy), length of juvenility, and a lack of sufficient genetic and genomic resources. Few model plants have been developed to study the molecular pathways of flowering in woody fruit plants. However, investigations of molecular regulatory pathways controlling seasonal flowering of temperate fruit trees are important for sustainable fruit production. The orthologs to *Arabidopsis* floral genes, such as *FT*, *LFY*, *TERMINAL FLOWER 1* (*TFL1*), and *SOC1*, have been identified from several woody plants such as poplar (*Populus* spp.) (Böhlenius et al. 2006; Mohamed et al. 2010; Rinne et al. 2011), Satsuma mandarin (*Citrus unshiu* Marc.) (Nishikawa et al. 2007), trifoliate orange (*Poncirus trifoliata* L.) (Zhang et al. 2011), sweet orange (*Citrus sinensis* L.) (Pillitteri et al. 2004; Tan and Swain 2007), grapevine (*Vitis vinifera* L.) (Boss et al. 2006), pear (*Pyrus communis* L.) (Freiman et al. 2012), and apple (*Malus* × *domestica* Borkh.) (Hattasch et al. 2008; Flachowsky et al. 2010; Kotoda et al. 2010; Mimida et al. 2011). *FLOWERING LOCUS C* (*FLC*)-like sequences have also been reported in coffee (*Coffea arabica* L.) (Barreto et al. 2012) and trifoliate orange (Zhang et al. 2009). A BLAST search in NCBI showed that orthologs of two vernalization-related flowering repressors *FLC* and *FRIGIDA* (*FRI*) (Michaels and Amasino 1999; Johanson et al. 2000), as well as other flowering activators (*SOC1*, *FT*, *LFY*, *TFL1*, etc.) are all present in *Vitis* spp. This suggests that woody plants may share a conserved flowering-regulation system with *Arabidopsis*. Thus far, few studies on woody perennials have been conducted to unravel the molecular pathways of seasonal flowering, mainly due to insufficient genetic and genomic information, as well as the lack of efficient molecular tools. As a result, little is known about the molecular basis of flowering in woody species. The investigation and characterization of the woody plant-derived orthologs of these genes will facilitate our understanding of seasonal regulation of flowering in woody plants (Michaels 2009; Wang et al. 2009, 2011; Pin et al. 2010; Imamura et al. 2011; Mayfield et al. 2011; Nakano et al. 2011).

For many temperate fruit-bearing trees and shrubs like blueberry (*Vaccinium* spp.), floral buds are usually initiated during the growing season, enter dormancy during late summer or fall, and then flower the following spring after satisfying their chilling requirements and return to favorable growth conditions. Sufficient vernalization is a prerequisite for normal plant growth and synchronized flowering during their life cycle. There is no question about the significant economic importance of normal floral bud initiation, obtaining adequate chilling, and subsequent flowering in tree and shrub fruit production (Wilkie et al. 2008). Global warming is changing the climate, highlighting the importance of the study of the flowering mechanism for sustainable production of temperate fruits.

Blueberry is one of the three major domesticated *Vaccinium* fruit crops (blueberry, cranberry, and lingonberry). It is an economically important fruit crop, due in part to its exceptional nutritional value with high amounts of antioxidants for human health. From 1995 to 2007, worldwide blueberry acreage grew from 23,116 to 58,601 ha (http://fruitgrowersnews.com/index.php/magazine/article/Blueberry-Production-Is-Skyrocketing-Worldwide). Highbush blueberries, including northern and southern ecotypes, are the major cultivated blueberries. Most of the commercial highbush cultivars require vernalization to ensure normal flowering. The northern highbush cultivars require more than 800 chilling units to break dormancy in the spring and generally have better winter hardiness. In contrast, the southern highbush blueberry requires between 150 and 600 chill units to initiate flowering, depending on cultivar, and has better heat tolerance in summer. New cultivars with both low chilling requirement (for the southern US) and good cold tolerance (for the northern and midwestern US) are among the high priorities for blueberry breeding. So far, little research has been conducted to investigate the molecular genetic basis of flowering in blueberry.

To date, we have developed reliable transformation tools for blueberry cultivars (Song and Sink 2004, 2006; Walworth et al. 2012) and the genome of blueberry has been sequenced and is expected to be publically available soon (Dr. Allan Brown, personal communication). With these advantages and as a step toward unraveling gene networks controlling flowering in blueberry, we isolated a *FT*-like gene (*VcFT*) from a northern highbush cultivar Bluecrop, and subsequently conducted functional analysis. We demonstrate that overexpression of *VcFT* significantly accelerates flowering in tobacco and the blueberry cultivar

Aurora. This is the first report on functional analysis of a floral gene from *Vaccinium* plants.

Materials and methods

Plant materials

Vernalized flower buds were collected in May from a northern highbush blueberry (*Vaccinium corymbosum* L.) cultivar Bluecrop maintained in an orchard at the Horticulture Teaching and Research Center of the Department of Horticulture, Michigan State University, East Lansing, MI, USA.

Cloning and phylogenetic analysis of *VcFT*

Total RNA was isolated from vernalized flower buds as they began to swell using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) with the modifications from Dhanaraj et al. (2004). Following isolation, the sample was treated with RQ1 DNase (Promega, Madison, WI, USA) according to the manufacturer's instructions, followed by chloroform:isoamyl alcohol (24:1) extraction, ethanol precipitated, washed with 70 % ethanol, and finally resuspended in DEPC-treated water. Total RNA (0.6 μg) was reverse transcribed into complementary DNA (cDNA) using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). One-tenth of the cDNA was used in one PCR amplification reaction using GoTaq Green Master Mix (Promega).

Blueberry EST sequence "*Vaccinium_corymbosum*_v1_Contig341", which is available in the blueberry EST database (http://www.vaccinium.org/), was used as a reference sequence for designing primers for *VcFT*. All primer sequences used in this study are shown in Table 1. Primers FTF1 and FTR1 were used for the first round PCR amplification; the products were reamplified using primers FTF1 and FTK to add 5′-*Xba*I and *Kpn*I-3′ at the ends of the entire coding region of *VcFT*. The nested PCR products of the second amplification reactions were cloned into a pCR 2.1-TOPO vector (Invitrogen) and then sequenced. The added *Kpn*I site was not used due to a change of our cloning strategy.

To make constructs for plant transformation, 5′-*Xba*I-*VcFT* -*Sac*I-3′ fragments were released from TA cloning vectors by a double digestion with *Xba*I and *Sac*I. They were inserted into the opened *Xba*I and *Sac*I sites in the T-DNA region between the cauliflower mosaic virus (CaMV) 35S promoter and the *Nos* terminator in pBI121, where the GUS (β-glucuronidase) coding region was removed. The resulting *35S:VcFT* was sequenced and subsequently introduced into *Agrobacterium tumefaciens* strain EHA105 (Hood et al. 1993) using the freeze–thaw method.

Amino acid sequences of *VcFT* orthologs were retrieved using the NCBI server (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Selected orthologs were aligned using ClustalW2 multiple sequence alignment program (Thompson et al. 1994) at EBI with default parameters (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Phylogenetic trees were generated using the MEGA 5 software (Tamura et al. 2011).

Plant transformation

35S:VcFT was used for *Agrobacterium tumefaciens* (EHA105)-mediated transformation of leaf explants of tobacco (*Nicotiana tabacum* L. cv Samsun) essentially according to Horsch et al. (1985). Kanamycin resistant shoots from different leaf explants were rooted separately on Murashige and Skoog (1962) medium (MS) containing 100 mgL^{-1} kanamycin monosulfate (Km). Transgenic plants from separate leaf explants were considered as independent transgenic events. Nontransgenic plants were used as controls.

Transformation of a northern highbush blueberry cultivar Aurora was performed as previously reported (Song and Sink 2004, 2006). Briefly, leaf explants were co-cultivated with EHA105 for 6 days on co-cultivation medium in the presence of 100 μM acetosyringone at 25 °C in the dark. Selection and regeneration were carried out on regeneration medium (modified McCown's Woody Plant Medium (WPM) containing 4.54 μM thidiazuron, 2.69 μM α-naphthaleneacetic acid, 10 mg L^{-1} Km, and 250 mg L^{-1} cefotaxime) for 12 weeks. Subcultures to fresh selection medium were conducted at 4-week intervals. Transgenic shoots from separate explants were defined as independent transgenic events. Proliferation of individual Km-resistant shoots was conducted on WPM containing 4.56 μM zeatin, 50 mg L^{-1} Km, and 250 mg L^{-1} cefotaxime. Wild-type controls were obtained by regeneration from non-infected leaf explants and transgenic controls were obtained by transformation with a *gus*A gene instead of the *35S:VcFT* construct.

DNA was extracted from leaf tissues using a CTAB method (Doyle and Doyle 1987). Two pairs of primers (Table 1), *npt*II-F and *npt*II-R for the *npt*II gene, and 35S-F (3′ portion of the *CaMV 35S* promoter) and FTK for the *VcFT* gene, were used to verify the presence of transgenes.

Tobacco plant phenotyping

T_0 putative transformants along with nontransgenic control plants were grown under a 16-h photoperiod in 10 × 10 cm pots, containing Suremix potting media (Michigan Grower Products Inc, Galesburg, MI, USA), for 3 weeks before being repotted into 1-gallon pots. The T_0 plants were self-pollinated to obtain T_1 seed used for phenotypic analysis.

In our previous studies, transgenic tobacco plants with pBI121 (the backbone vector) showed no significant

Table 1 Primer sequences used for conventional PCR and real-time RT-PCR

Primer name	Sequence	Target
Conventional PCR primer		
FTF1	5′-tctagaATGCCACGGGATAGGGAT-3′	Blueberry *FLOWERING LOCUS T*-like gene (*VcFT*)
FTR1	5′-CGCGCATGTGTGTAAGTATC-3′	
FTK	5′-ggtaccTCAGCGTCGTCGTCCT-3′	
NPTII-F	5′-GAGGCTATTCGGCTATGACTG-3′	Neomycin phosphotransferase II gene (*npt*II)
NPTII-R	5′-ATCGGGAGCGGCGATACCGTA-3′	
35S-F	5′-TGACGCACAATCCCACTATC-3′	3′ portion of the *CaMV 35S* promoter
Real-time RT PCR primer		
Ntubc2-F	5′-CTGGACAGCAGACTGACATC-3′	Ubiquitin-conjugating enzyme E2 (Ntubc2) (accession AB026056)—from Schmidt and Delaney (2010)
Ntubc2-R	5′-CAGGATAATTTGCTGTAACAGATTA-3′	
BBFT1-F	5′-TCGCATTCGTGTTGTTCCA-3′	*VcFT*
BBFT1-R	5′-CACCCGGGAGCATAGACTGT-3′	

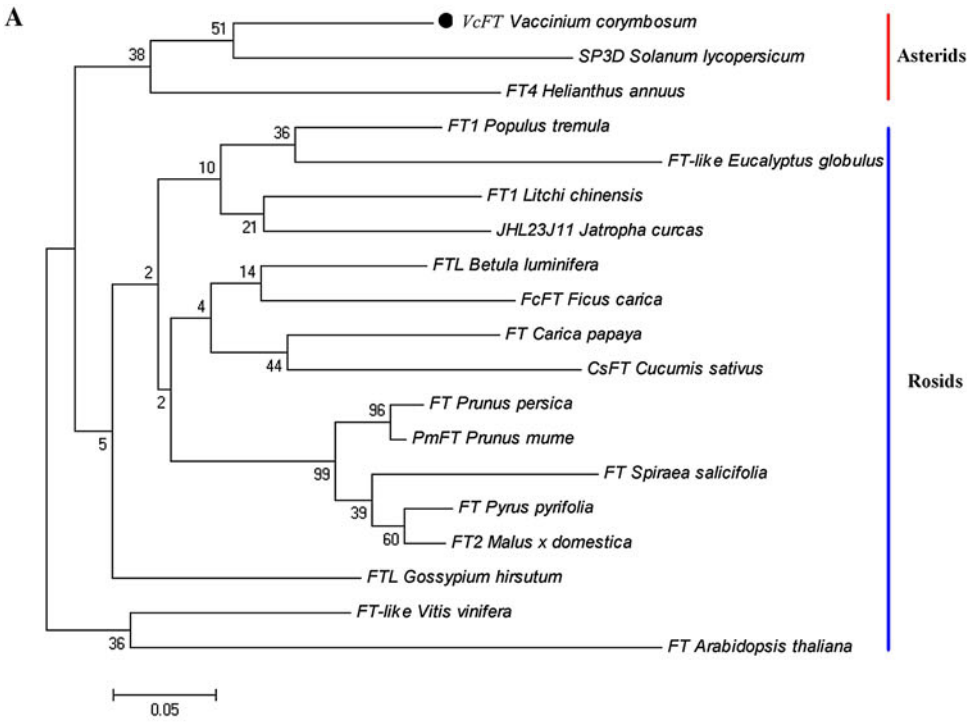
difference in phenotype (i.e. flowering time, plant height, and seed yield) compared to wild-type plants (our unpublished data). Therefore, in this study, transgenic tobacco plants with the pBI121 vector backbone were omitted. T_1 seeds from five transgenic lines were sown along with nontransgenic controls in 48-cell trays with Suremix potting media (Michigan Grower Products Inc.). Plants were maintained under a 16-h photoperiod (long days) with 30 μmol m^{-2} s^{-1} light from cool white fluorescent tubes for 9 weeks. Individual plants were screened by PCR using 35S-F and FTK primers, and selected seedlings of similar size, 10 for each transgenic event or nontransgenic wild type, were transplanted into 10 × 10 cm pots and grown in the greenhouse under natural photoperiod, with an average of 14.9 h of daylight per day. Plants were arranged in a completely randomized design. All plants were grown under a regular schedule of irrigation and fertilization.

Time to flowering, leaf number, plant height, and number of seed pods were recorded for the T_1 plants. Time to flowering was measured as the number of days between sowing and appearance of the first flower bud. The number of leaves on the central stem and height to the bottom of the central flower cluster was determined at the time when 50 % of flowers in the central cluster were open. Number of seed pods was determined for the central flower cluster when blooming was finished. Statistical analysis was conducted with PROC GLM in SAS (SAS Institute Inc., Cary, NC, USA). Difference from wild type was determined using Dunnett's two-tailed test for difference from a control.

Real-time quantitative reverse-transcription PCR (q-PCR) analyses

For tobacco, two T_0 regenerants and T_2 seedlings derived from two of the earliest flowering transgenic lines were used to confirm transgene expression. T_0 plants were sampled by harvesting newly expanding leaves following transplanting to soil. Non-transformed regenerants were sampled prior to flowering and again after the appearance of flowers. T_2 seeds were sown on selection plates containing Gamborg's B5 medium (Gamborg et al. 1968) without sucrose, with 200 mg l^{-1} kanamycin, and solidified with 0.7 % agar. Seeds from nontransgenic control plants were sown onto identical plates without the addition of kanamycin. Following 4 weeks of selection, seedlings were transferred to soil. At least 10 seedlings were bulked together per biological replicate for RNA isolation 43 days after sowing, with three biological replicates per genotype, and sampling was done by collecting all above-ground tissue.

RNA was isolated using a RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) with on-column RNase-free DNase (Qiagen) treatment according to the manufacturer's instructions. Reverse-transcription of RNA to cDNA was carried out using SuperScript II reverse transcriptase (Invitrogen). Real-time PCR using SYBR Green PCR Core Reagents (Life Technologies, Carlsbad, CA, USA) was carried out on an Applied Biosystems StepOne™ thermocycler (Applied Biosystems, Foster City, CA, USA) with three technical replicates per sample. The amplification conditions were 95 °C for 10 min, followed by 40 cycles of amplification (95 °C for 15 s, 60 °C for 1 min) with plate readings after each cycle and performance of a melting curve analysis. Primers used for the reactions are included in Table 1. Preliminary experiments were conducted to determine the efficiencies of the primers and confirm that the internal control was suitable. Control reactions using the isolated RNA as template without reverse transcriptase were used to verify the absence of detectable genomic DNA in the samples. Data were analyzed by the ΔΔCT method using StepOne Software V2.2 (Applied Biosystems).



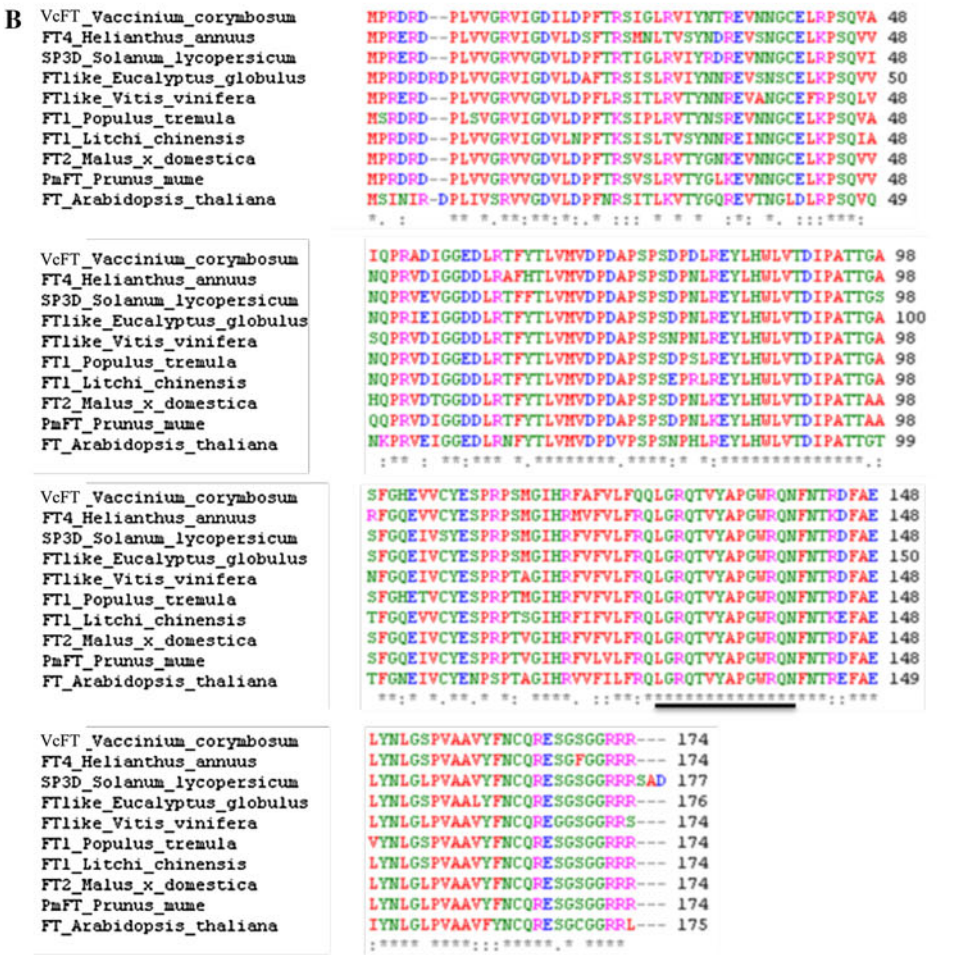


Fig. 1 **a** Phylogenetic analysis of *FT*-like proteins from 19 eudicot plants using the Maximum Likelihood method in MEGA 5. DNA sequences of the *FT*-like proteins were used in multiple sequence alignments. The branch length is drawn to scale, and represents the number of nucleotide substitutions per site. The bootstrap values were obtained from 500 replicates. **b** Multiple sequence alignment of *FT*-like protein sequences from 10 plant species using ClustalW2. *Underlined* sequence identifies the conserved P-loop domain

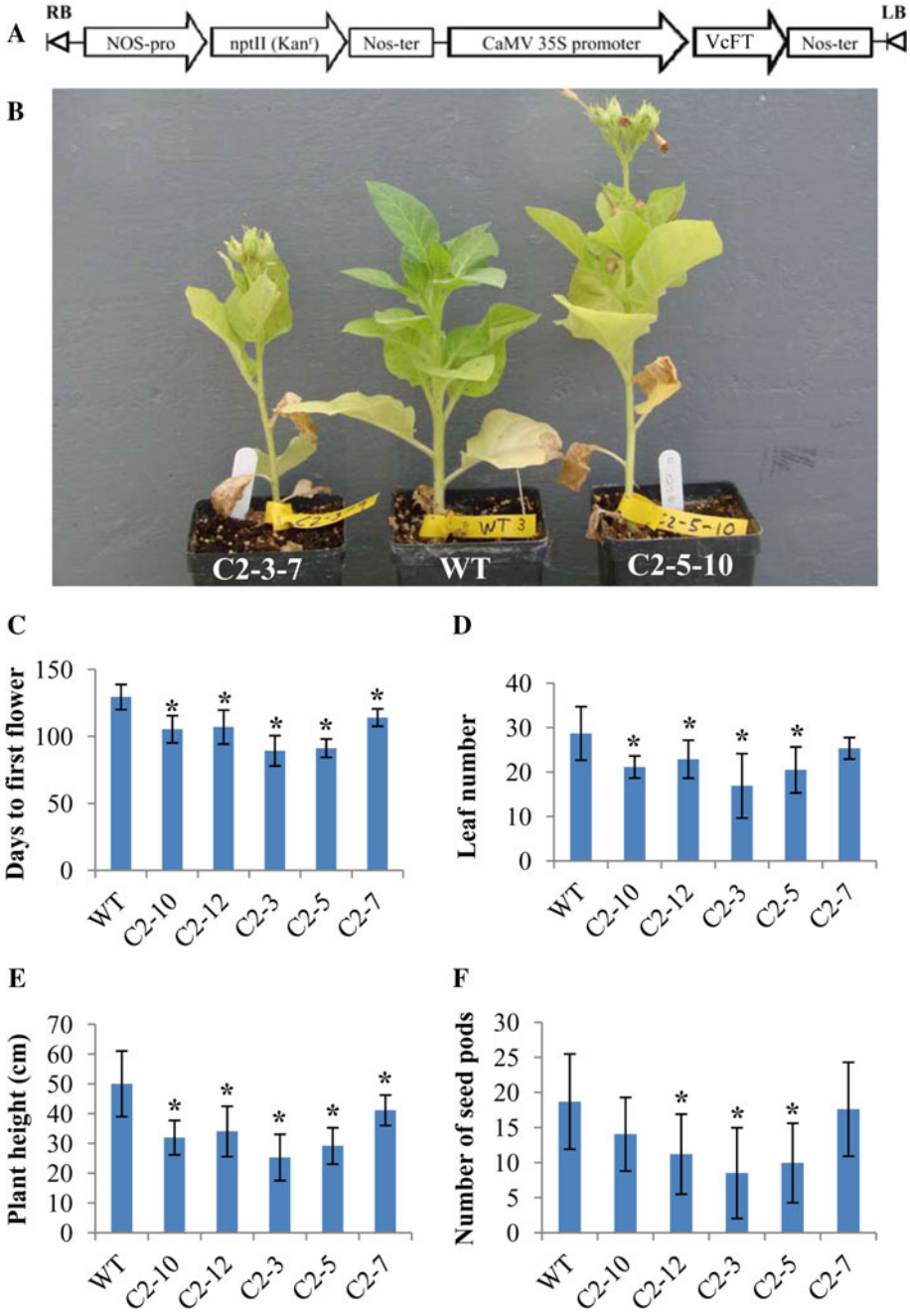


Fig. 2 Effect of ectopic expression of *35S:VcFT* on phenotypic changes in T_1 transgenic plants. **a** Schematic representation of the T-DNA region carrying the *35S:VcFT*. *RB* right border, *LB* left border, *NOS-pro* nos promoter, *nptII* neomycin phosphotransferase gene, *Nos-ter* nos terminator, *CaMV 35S promoter* the cauliflower mosaic virus (CaMV) 35S promoter, *VcFT* a blueberry *FT*-like gene. **b** Early flowering of transgenic (C2-3-7 and C2-5-10) compared with nontransgenic wild-type (WT) plants 93 days after seed germination. **c** Days to emergence of first flowers after the seeds were sown. **d** Leaf numbers when 50 % of flowers in central cluster were open. **e** Plant height (cm) when 50 % of flowers in central cluster were open. **f** Number of seed pods in central flower cluster. Means represent 8–10 plants for each independent transgenic event C2-10, C2-12, C2-3, C2-5, and C2-7, and wild-type control. *Error bars* indicate SD. *Stars* (*) on *error bars* denote significant differences from WT at 95 % confidence level by Dunnett's two-tailed test

Blueberry plant phenotyping

Putative transgenic shoots containing *35S:VcFT*, along with nontransgenic (regenerants) and transgenic (with *gus*A) controls, 3–5 cm in length and 12 shoots per event, were directly rooted in 48-cell trays containing sphagnum peat moss. The trays were covered with transparent plastic covers. The shoots were grown for 6 weeks at 25 °C, 30 μE m^{-2} s^{-1} light for 16 h/day and watered as needed. The plastic covers were then progressively opened in 1 week. The rooted plants were grown under the same environmental conditions for another 4–6 weeks before being repotted into 10 × 10 cm pots. Time to flowering, measured as the number of days between shoot planting and appearance of the first flower, was recorded. Plant height at the appearance of the first flower was measured. The plant height for all plants was measured after all transgenic plants with 35S-*VcFT* flowered.

Results

Isolation and sequence analysis of *VcFT*

We found a 779-bp *FT*-like EST (*Vaccinium_corymbosum*_v1_Contig341) in the blueberry EST database (http://

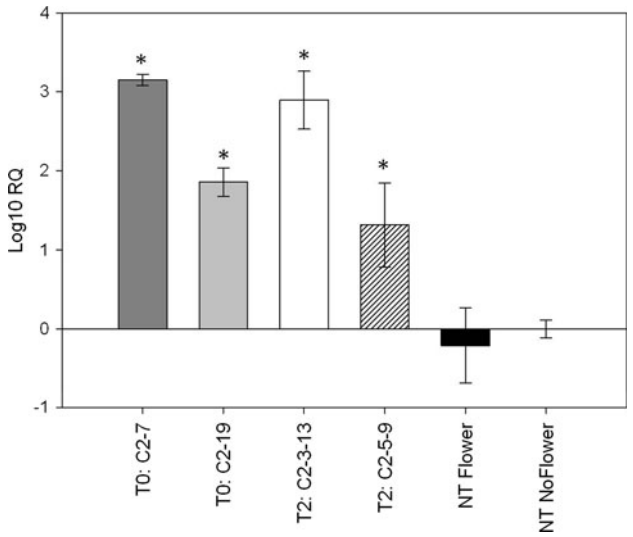


Fig. 3 Real-time RT-PCR analysis of *35S:VcFT* expression. *NT NoFlower* nontransgenic regenerants prior to flowering. *NT Flower* nontransgenic regenerants at flowering stage. *C2-7* and *C2-19* T_0 transgenic regenerants randomly chosen for sampling. *C2-3-13* and *C2-5-9* T_2 plants derived from two independent transgenic events. Transcript levels were normalized to ubiquitin-conjugating enzyme E2 (Ntubc2) (accession AB026056) and are depicted relative to NT NoFlower. *Error bars* represent 95 % confidence intervals based on three technical replicates (T_0 plants) or three biological replicates with three technical replicates (T_2 plants). *Stars* (*) denote significant difference from NT NoFlower

www.vaccinium.org/tools/blast), which is the only EST contig with high similarity, based on E value, to a *Populus tremula* FT-like protein mRNA (GenBank accession QD 387859.1). Sequence analysis of the *FT*-like EST indicated that the first 525-bp of the Contig341, with the first two nucleotides "GN" (as published in the Contig341) replaced by "AT", was an ortholog of *Arabidopsis FT*. Combining RT-PCR with nested PCR, we cloned and sequenced the 525-bp *FT* ortholog (*VcFT*). Only four nucleotides did not match the EST sequence in Contig341. This *VcFT* sequence has a full-length open reading frame (ORF) with an ATG start codon and a TGA stop codon. It encodes 175 amino acids and phylogenetic analysis of cDNA sequences of the selected top nine BLAST hits of FT-like proteins indicates that the *VcFT* is grouped in the clade of *FT*-like genes and shows high similarity (76–90 %) to many other *FT*-likes (Fig. 1).

Ectopic expression of *VcFT* promoted early flowering in tobacco

Early flowering of transformants containing *35S:VcFT* (Fig. 2a) indicate that *VcFT* is a flowering activator. T_1 plants of all five transgenic events tested flowered an average of 28 days (ranging from 16 to 41 days, depending on event) earlier than wild-type plants. Wild-type plants flowered at 130 days after sowing and production of with 29 leaves. In contrast, the flowering time of these *35S:VcFT* transformants occurred about 102 days after sowing and the production of only 21 leaves (averaged across all lines) (Fig. 2b–f).

Quantitative PCR (q-PCR) analysis confirmed expression of *VcFT* in T_0 and T_2 generation transgenic tobacco plants. Selected lines, two T_0 and two T_2, showed significant accumulation of *VcFT* transcripts (Fig. 3). No significant increase in amplification occurred in reactions with nontransgenic regenerants at flowering stage, compared to those at non-flowering stage, using the primers designed for *VcFT*; providing evidence that these primers do not co-amplify an endogenous *FT*-like gene in tobacco (Fig. 3). Melt curve analysis of the PCR products suggests production of a single amplicon for the primer pair.

Overexpression of *VcFT* promoted early flowering in blueberry

Neither control blueberry plants (i.e. nontransgenic, nor six transgenic 'Aurora' events containing a *gus*A gene) flowered in vitro regardless of culture time; in contrast, all five independent transgenic events with *35S:VcFT* flowered after culturing in vitro for 6 weeks (Fig. 4a). The flowers showed normal morphology but did not develop into fruits under in vitro culture conditions (Fig. 4b, c).

Following transfer to soil, all three independent transgenic events, 12 plants/event, flowered after an average of 6, 8, and 10 weeks, respectively, without being subjected to any chilling treatment (Fig. 4d–g). Unlike normally flowering nontransgenic 'Aurora' in which each flower bud often develops into a cluster with 8–10 flowers, we did not observe multiple flowers from a single bud. A single flower was observed from either an apical or an auxiliary bud (Fig. 4d–f). It appeared that flower bud formation and flowering occurred simultaneously (Fig. 4f). Pollinated flowers set fruits (Fig. 4g). In contrast, neither nontransgenic nor transgenic controls flowered after 12 weeks (Fig. 4h, i). In fact, even under the optimal conditions it usually takes at least 1 year plus a chilling period of 800 chilling hours for the in vitro shoots of these 'Aurora' controls to develop into a flowering stage. Without any chilling, 'Aurora' does not flower. These results indicate that overexpression of *35S-VcFT* is highly effective at eliminating chilling requirement and promoting early flowering in blueberry.

In addition, the expression of *35S:VcFT* resulted in smaller plant sizes at the time of flowering due to a slower growth rate compared to nontransgenic and transgenic (with the *gus*A) controls (Fig. 5). At the time of first flower, three transgenic events had average plant heights of 3.3 cm for BBFT1, 3.2 cm for BBFT2, and 2.7 cm for BBFT3; in contrast, by the time of our data

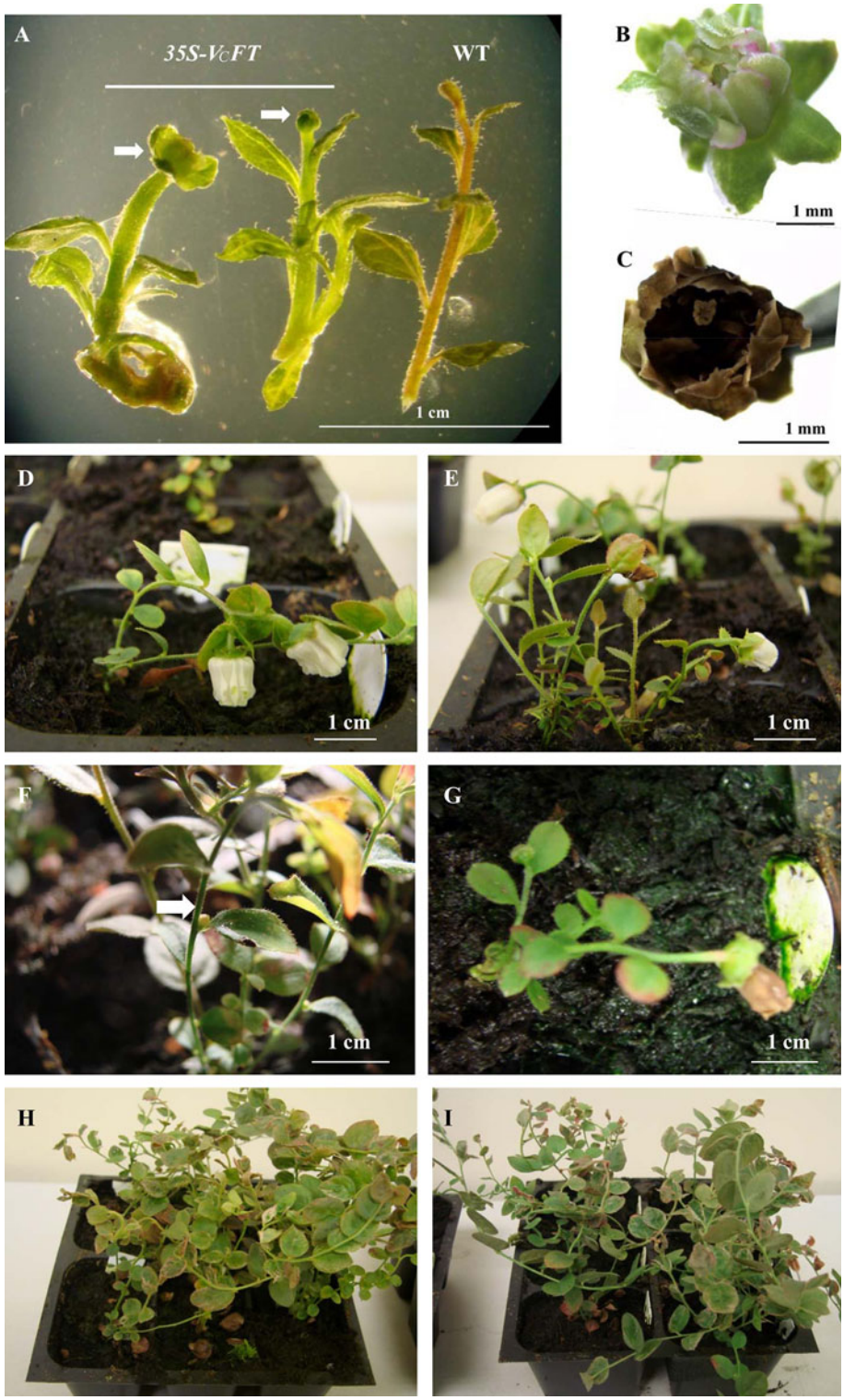


Fig. 4 Phenotype of blueberry cultivar Aurora expressing *35S:VcFT*. **a** In vitro flowering of transgenic shoots with *35S:VcFT* (*arrows* indicate the floral organs). *WT* wild-type 'Aurora'. **b** An in vitro shoot derived flower at the bud break stage. **c** A normal in vitro shoot derived flower at late flowering stage after it senesced. **d** Early flowering of an 8-week old plant (after the in vitro shoot was planted in soil) of transgenic event BBFT1. **e** Early flowering of a 6-week old plant of transgenic event BBFT2. **f** Early flowering of an auxiliary bud (see the *arrow*) in a 10-week old plant of transgenic event BBFT3. **g** A fruit set in an 8-week old plant of transgenic event BBFT2 after pollination using 'Legacy' pollen. **h** Ten-week old nontransgenic 'Aurora' plants. **i** Ten-week old transgenic 'Aurora' plants with the *gus*A

collection 12-week old nontransgenic and transgenic controls (with *gus*A) did not flower and their average plant heights were 13.3 and 12.5 cm, respectively (Fig. 5). In fact, based on our previous observation, few flower buds were observed in 40–60 cm-height 'Aurora' cultured at 25 °C, 30 μE m^{-2} s^{-1} of light for 16 h/day due to the long-day photoperiod and a lack of chilling. For all 12-week old plants, all three transgenic 'Aurora' events with *35S:VcFT* showed a significantly lower height than nontransgenic and transgenic (with *gus*A) controls, indicating slower growth of the plants after the expression of *35S:VcFT*.

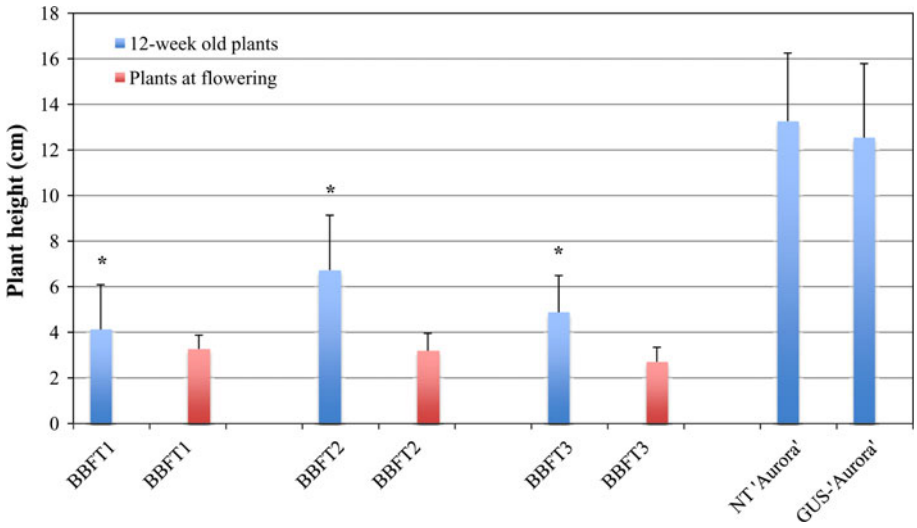


Fig. 5 Effect of overexpression of *35S:VcFT* on plant growth in T_0 transgenic blueberry cultivar Aurora. Means represent 12 plants for each of nontransgenic (NT) 'Aurora', transgenic control (GUS-'Aurora'), and three independent transgenic event: BBFT1, BBFT2, and BBFT3. *Error bars* indicate SD. *Stars* (*) denote significant differences from NT 'Aurora' at $\alpha = 0.05$ by Dunnett's two-tailed test

Discussion

A genetic map of *Vaccinium corymbosum* is currently being developed, but at present is not sufficiently dense to undertake map-based cloning of floral genes from blueberry (Boches et al. 2005). Because of the availability of more and more genomic information, we demonstrate in this study that EST-based cloning is a short-cut approach to isolate candidate genes. Successful cloning and functional analysis of *VcFT* are a necessary step toward unraveling gene networks controlling flowering in blueberry as well as other woody plant species. In addition, because *VcFT* is a flowering activator, it can potentially be used in woody crops for shortening juvenile period and manipulating the time of flowering through genetic engineering.

Considered as a major *florigen* regulating plant development through mobile protein signals, functional analysis of *FT* and *FT*-likes has been reported in numerous studies for 19 plant species of 14 families (for a recent review, see Pin 2012). These include four woody plant species: poplar (Böhlenius et al. 2006; Hsu et al. 2006, 2011), apple (Trankner et al. 2010; Mimida et al. 2011; Trankner et al. 2011), orange (Endo et al. 2005), and grapevine (Sreekantan and Thomas 2006; Carmona et al. 2007). A P-loop domain (LGRQTVYAP…GWRQN) is highly conserved in these woody plants (Pin 2012). Similarly, in tetraploid blueberry, the sequence of this P-loop domain is also conserved in *VcFT*. In addition, ectopic expression and overexpression of the *VcFT* promoted early flowering in tobacco and blueberry, respectively. Similar results were also observed for the *FT*-likes genes derived from the other four woody plants mentioned above. Overexpression of a *FT*-like from poplar (*P. trichocarpa*) in plum (*Prunus domestica*) resulted in continuous flowering plants with altered plant architecture and dormancy requirement (Srinivasan et al. 2012). Similarly, in this study the new flowering pattern (Fig. 4d–i), decreased plant size (Fig. 5), and altered photoperiodic and chilling requirement in transgenic 'Aurora' with *VcFT* suggests that the *VcFT* functions like *FT*, a major flowering pathway integrator and a universal promoter of flowering. Theoretically, overexpressing *FT* breaks the control of the major upstream genes, in which *CONSTANS* (*CO*)-like gene mainly responds to inductive photoperiod and *FLC*-like gene is regulated by autonomous control and vernalization (Hsu et al. 2011; Pin 2012; Posé et al. 2012). This explains the phenotype changes associated with the overexpression of the *VcFT* in blueberry or the poplar *FT*-like in plum. On the other hand, these phenotype changes, such as promotion of flower bud formation, dwarf plant, and altered chilling requirement, are desirable for temperate fruit production. Our transgenic 'Aurora' plants with the *VcFT* are growing in greenhouse. More investigations are still needed to find out the overall effect of the overexpressed *VcFT* on blueberry plant growth and fruit production.

Further investigation of expression of putative downstream blueberry genes such as *AP1*-like(s) and *LFY*-like(s) following overexpression of the *VcFT* in transgenic blueberry plants will help to elucidate the interactions of major floral genes in blueberry. In *P. trichocarpa*, two *FT*-like genes were identified possibly due to ancient genome duplication (Hsu et al. 2006, 2011). Although only one *FT*-like sequence (*Vaccinium_corymbosum*_v1_Contig341) was found in the current blueberry EST database, it is likely that there exist multiple homologs of *VcFT* because 'Bluecrop' is a tetraploid ($2N = 4X = 28$).

In conclusion, as an initial step to investigate flowering mechanism in *Vaccinium*, we isolated a blueberry *FT*-like gene (*VcFT*). Functional analyses of *VcFT* through ectopic expression in tobacco and overexpression in blueberry

revealed that *VcFT* functions as a floral activator. As such, it has potential applications for manipulating flowering time in *Vaccinium* species through genetic engineering.

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