RESEARCH PAPER

Three FT and multiple CEN and BFT genes regulate maturity, flowering, and vegetative phenology in kiwifruit

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Abstract

Kiwifruit is a woody perennial horticultural crop, characterized by excessive vegetative vigor, prolonged juvenility, and low productivity. To understand the molecular factors controlling flowering and winter dormancy, here we identify and characterize the kiwifruit PEBP (phosphatidylethanolamine-binding protein) gene family. Five CEN-like and three BFT-like genes are differentially expressed and act as functionally conserved floral repressors, while two MFT-like genes have no impact on flowering time. FT-like genes are differentially expressed, with AcFT1 confined to shoot tip and AcFT2 to mature leaves. Both act as potent activators of flowering, but expression of AcFT2 in Arabidopsis resulted in a greater impact on plant morphology than that of AcFT1. Constitutive expression of either construct in kiwifruit promoted in vitro flowering, but AcFT2 displayed a greater flowering activation efficiency than AcFT1, leading to immediate floral transition and restriction of leaf development. Both leaf and flower differentiation were observed in AcFT1 kiwifruit lines. Sequential activation of specific PEBP genes in axillary shoot buds during growth and dormancy cycles indicated specific roles in regulation of kiwifruit vegetative and reproductive phenologies. AcCEN and AcCEN4 marked active growth, AcBFT2 was associated with suppression of latent bud growth during winter, and only AcFT was activated after cold accumulation and dormancy release.

Key words: Actinidia chinensis, BFT, CEN, development, dormancy, flowering, FT, kiwifruit.

Introduction

The phosphatidylethanolamine-binding proteins (PEBPs), initially identified in animals as Raf-1 kinase inhibitors (Yeung et al., 1999), provide a potential to fine-tune developmental regulatory mechanisms and present opportunities for improvement of agriculturally important traits (Jung and Müller, 2009; Zhang et al., 2010; Yeoh et al., 2011; Iwata et al., 2012; Putterill et al., 2013; Koskela et al., 2016). In angiosperms, this family consists of three phylogenetically distinct groups: the FLOWERING LOCUST (FT)-like proteins (Kardailsky et al., 1999; Kobayashi et al., 1999), the TERMINAL FLOWER1 (TFL1)-like and CENTRORADIALIS (CEN)-like proteins (Bradley et al., 1996, 1997; Ohshima et al., 1997), and the MOTHER OF FT AND TFL1 (MFT)-like proteins (Mimida et al., 2001; Yoo et al., 2004). In addition, the BROTHER OF FT AND TFL1 (BFT)-like proteins (Yoo et al., 2010) represent a separate subclade within the CEN group.
FT- and TFL1/CEN-like genes have conserved functions in regulation of flowering, and the ancestral MFT-like genes (Hedman et al., 2009; Karlgren et al., 2011) have been associated with seed germination (Xi et al., 2010; Footitt et al., 2011; Nakamura et al., 2011). FT-like proteins can act as floralizers (Chailakhyan, 1968; Zeevaart, 2008), moving in the phloem from leaves to the shoot apex in a regulated manner (reviewed in Putterill and Varkonyi-Gasic, 2016) to promote flowering. In contrast, TFL1/CEN-like proteins repress flowering and maintain the inflorescence meristem (Shannon and Meeks-Wagner, 1991; Ratcliffe et al., 1998, 1999; Conti and Bradley, 2007). This antagonistic activity (Shalit et al., 2009; Lfischitz et al., 2014) involves interaction with a common partner FD (Pnueli et al., 2001; Abe et al., 2005; Wigge et al., 2005; Danilevskaya et al., 2010; Hanano and Goto, 2011; Randoux et al., 2014) and is conferred by a divergent external loop and specific amino acids in positions critical for FT function (Hanzawa et al., 2005; Ahn et al., 2006). Other changes resulting in altered surface charge can affect function (Ho and Weigel, 2014), and some FT-like proteins act as repressors of flowering (Pin et al., 2010; Harig et al., 2012; Cao et al., 2016). In many plant species, numerous FT-like genes arose from gene duplication events (Pin and Nilsson, 2012), but only one or two act in flowering control, and FT-like roles besides flowering, such as vegetative growth and storage organ differentiation, have been reported (Hsu et al., 2011; Navarro et al., 2011; Lee et al., 2013), confirming a key role for PEBPs in co-ordination of general growth and development.

Many aspects of PEBP biology remain less well understood in woody perennial plants (Putterill and Varkonyi-Gasic, 2016). Recent studies of the most advanced woody perennial model poplar (Populus spp.) revealed conservation between molecular regulation of flowering in annual plants and seasonal phenology in trees (Ding and Nilsson, 2016). In addition to regulation of flowering, poplar FT- and TFL1/CEN-like genes are implicated in the regulation of vegetative to reproductive transition, growth and dormancy cycles, and the co-existence of vegetative and floral meristems on the same shoot (Böhlenius et al., 2006; Hsu et al., 2006, 2011; Rinne et al., 2011). Similarly, TFL1 is a key regulator of perennial growth and seasonality of flowering in strawberry and rose (Iwata et al., 2012; Koskelä et al., 2012, 2016; Randoux et al., 2014; Rantanen et al., 2015), down-regulation of TFL1-like genes accelerated flowering in apple and pear (Kotoda et al., 2006; Freeman et al., 2012; Yamagishi et al., 2016), ectopic overexpression of a CEN homolog suppressed flowering in kiwifruit (Varkonyi-Gasic et al., 2013), and a connection of TFL1 accumulation with biennial bearing in apple has been established (Haberman et al., 2016).

On the other hand, constitutive or inducible expression of Arabidopsis or poplar FT genes has been attempted and used with some success to accelerate breeding (Srinivasan et al., 2012; Wenzel et al., 2013), although many questions regarding the FT gene, promoter, and feasibility of the approach remain (Zhang et al., 2010). Remarkably, only a handful of endogenous FT genes have been functionally characterized by transgenic approaches in woody perennial species; expression under a strong constitutive promoter resulted in extremely early flowering in trifoliate orange, poplar, apple, and blueberry (Endo et al., 2005; Böhlenius et al., 2006; Hsu et al., 2006; Kotoda et al., 2010; Yamagishi et al., 2011, 2014; Song et al., 2013), and only one example of graft-transmissible early flowering has been reported in a small woody shrub, Barbados nut (Jatropha) (Ye et al., 2014). Even less is known about functional diversification of FT genes in woody perennials. Ectopic expression implied distinctive roles in reproductive and vegetative growth for poplar FT paralogs (Hsu et al., 2011), not yet confirmed by paralog-specific silencing; expression of only one of two FT genes in apple and one of three FT genes in citrus has been associated with transition to flowering (Nishikawa et al., 2007; Kotoda et al., 2010), and expression of a single grape FT gene could be detected irrespective of flowering (Sreekantan and Thomas, 2006; Carmona et al., 2007). Furthermore, some FT genes failed to induce early flowering in woody perennials, despite their ability to promote flowering in model annuals, while affecting dormancy and leaf senescence in apple (Freiman et al., 2015) or shoot vigor in kiwifruit (Varkonyi-Gasic et al., 2013).

Although the ability to regenerate transgenic woody perennials efficiently remains an obstacle, development of new technologies to induce gene-specific biallelic homozygous mutations in trees (Fan et al., 2015) and specifically manipulate members of the PEBP family holds great promise in accelerating the breeding process and improving the architecture and productivity of woody perennials of horticultural importance. Detailed functional characterization of PEBP genes is the necessary first step to improve selection of gene candidates likely to have a key role in maturation and flowering.

Kiwifruit (Actinidia) is one of the most recently domesticated horticultural crops, with a short history of breeding (Datson and Ferguson, 2011), limited genetic resources, and little understanding of molecular regulation or the genetic diversity in flowering responses to internal and environmental signals. Commercial kiwifruit cultivars belong to closely related A. chinesis and A. delicosa, which, together with other members of the Actinidia genus, are woody perennial vines from the order Ericales in the asterid lineage, with features of development specific to temperate woody perennials, including prolonged juvenility, growth spread over two seasons (Brundell, 1975a, b), and a period of winter chilling required to resume growth after winter dormancy (Brundell, 1976). Excessive vegetative growth and a small number of flowers, resulting in low fruit yield and high orchard costs, are key targets in kiwifruit improvement strategies. Flower differentiation occurs during budbreak in spring, from pre-established but undifferentiated overwintering primordia (Walton et al., 1997, 2001; Varkonyi-Gasic et al., 2011). Inflorescences develop in the lower leaf axils of the shoots that emerge after sufficient winter chilling, and photoperiod has no clear role in regulation of flowering in spring (Snelgar et al., 2007).
regulation of kiwifruit maturity, architecture, and phenology. The completion of the draft genome sequence of *A. chinesis* (Huang et al., 2013) provided the opportunity to identify additional sequences belonging to the kiwifruit PEBP family. In this study, we identified 13 Actinidia PEBP genes, five belonging to the *TFL1/CEC* clade, three each belonging to the *BFT* and *FT* clade, and two belonging to the *MFT* clade. All genes with the exception of the previously characterized *AeFT* and *AcCEC* (Varkonyi-Gasic et al., 2013) were characterized here using a combination of expression analysis and functional studies in transgenic Arabidopsis. Two of the previously undescribed *FT*-like genes were further characterized in more detail and their role in activation of flowering was evaluated in transgenic kiwifruit. The information provided help to better understand the diversity and biological function of the PEBP family in woody perennial plants and identifies candidate targets for improvement of kiwifruit architecture, flowering time, and plant determinacy.

**Materials and methods**

**Plant materials**

Kiwifruit plant material was sampled from female kiwifruit cultivars, ‘Hort16A’ (*Actinidia chinensis* Planch. var. *chinensis*) and ‘Hayward’ [‘A. delicosa’ (A. Chev.) C.F. Liang et A.R. Ferguson, recently classified as *A. chinensis* var. *delicosa* (A. Chev.) A. Chev.]. Sampling for gene expression analysis was described previously (Wu et al., 2012; Varkonyi-Gasic et al., 2013). Briefly, root, stem, leaf, flower, and fruit tissue were collected from mature plants grown in the Plant & Food Research orchard near Te Puke, New Zealand in the spring and summer season of 2005–2006, basal and distal leaves from mature ‘Hort16A’ plants grown in the Plant & Food Research orchard near Kerikeri, New Zealand in the spring and summer season of 2015 season. Terminal buds were sampled from field-grown ‘Hayward’ plants grown in a commercial orchard near Hamilton, New Zealand during the 1995–1996 season. Amplification of full-length coding sequences was performed on a set of ‘Hort16A’ cDNA described in Ledger et al. (2010). Additional leaf samples (L1–L8) were collected from 1-year-old ‘Hort16A’ plants grown in the Plant & Food Research glasshouse in Auckland, New Zealand in spring 2013. Axillary bud samples used in RNA-seq experiments were collected at monthly intervals from *A. chinensis* plants grown in the Plant & Food Research orchard near Te Puke, New Zealand in the 2014–2015 season. Terminal buds were sampled from the same plants in January and March 2015. Three plants (biological replicates) were used and ~10 buds were sampled at each time point, with the exception of the March terminal bud sample, where only two plants were available. Sampling of tissues was performed at mid-day to avoid variation.

**Isolation of the genes and vector construction**

Gene-specific oligonucleotide primers were designed based on available sequence data (Huang et al., 2013) (see Supplementary Table 1 at JXB online). Full-length coding sequences were amplified from *A. chinensis* ‘Hort16A’ cDNA, cloned into the pUC19 vector, and verified by sequence analysis (GenBank accession nos KX611594–KX611604). After addition of attB sites by PCR, each gene was recombined using Gateway into pDONR221, verified by sequence analysis, then recombined into pHEX2 (Hellens et al., 2005), placing each cDNA between the *CaMV* 35S promoter and the *ocs 3΄* transcriptional terminator. The modified *AcFT1* and *AcFT2* sequences with added attB sites were synthesized by GenScript (http://www.genscript.com) and recombined into pHEX2 as above. For *SUC2 AcFT1* and *SUC2 AcFT2* constructs, the cDNAs were re-amplified to include appropriate restriction sites and cloned as SpeI–XbaI fragments into pSAK778/SUC2 to replace *AcFT* (Varkonyi-Gasic et al., 2013). The 35S:GUS vector control was constructed previously (Drummond et al., 2009). All resulting plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101 by electroporation. 35S:*AcFT1* and 35S:*AcFT2* were also transformed into *A. tumefaciens* strain EHA105 by electroporation.

**Phylogeny**

Predicted protein sequence alignment and phylogenetic analyses were conducted using Vector NTI (Invitrogen) CLUSTAL W, and phylogeny was constructed using a minimum evolution phylogeny test and 1000 bootstrap replicates in MEGA version 5.2 (Tamura et al., 2011).

**Plant transformation and growth**

*Agrobacterium tumefaciens*-mediated Arabidopsis transformation was performed as described (Clough and Bent, 1998; Martinez-Trujillo et al., 2004), using constructs in *A. tumefaciens* strain GV3101. Seeds of transgenic plants were selected on half-strength Murashige and Skoog (1/2 MS) medium supplemented with kanamycin and placed in a growth chamber (LD 21 °C, 16/8 h light/dark) or short-day (SD, 21 °C, 8/16 h light/dark) regime. Plants were grown in soil using a standard potting mix. Transformation of *A. chinensis* ‘Hort16A’ was as previously described (Wang et al., 2007; Voogd et al., 2015), using *A. tumefaciens* strain EHA105.

**RNA extraction and expression studies**

Total RNA from Arabidopsis was isolated using the Trizol reagent (Invitrogen) and from kiwifruit using the Spectrum Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO, USA). Reverse transcription was performed using the QuantiTect Reverse Transcription Kit (Qiagen). Quantifications using real-time PCR were performed with the FastStart DNA Master SYBR Green I mix (Roche Diagnostics, Mannheim, Germany) using the LightCycler 1.5 instrument and the LightCycler Software version 4 (Roche Diagnostics). Oligonucleotide primers (Supplementary Table S1) were designed to produce amplification products of 100–150 nucleotides. The specificity of primer pairs was confirmed by melting curve analysis of PCR products and agarose gel electrophoresis followed by sequence analysis. The expression was normalized to *Actinidia ACTIN* (GenBank accession no. FG403300) or Arabidopsis *ACT2* (At3g18780).

The axillary bud sequencing libraries were constructed according to the TruSeq RNA sample preparation guide (Illumina, San Diego, CA, USA) and subsequently sequenced by a HiSeq 2500 Sequencing System (Illumina) at Australia Genomics Resource Facility (Brisbane, Australia), obtaining paired-reads of 125 bp. Raw sequence data in FASTQ format were filtered to remove the adapters, low-quality reads, and reads containing ambiguous nucleotides, resulting in an average of 13.8 million reads per library. As the PEBP genes are mostly misannotated in the draft kiwifruit genome and the genome is generally poorly annotated, the mapping was performed on the 13 sequences representing the kiwifruit PEBP gene family, using STAR version 2.5.2a (Dobin et al., 2013), and only uniquely mapped reads with no mismatches per paired alignment were used for gene expression studies. The gene expression unit was calculated as fragments per kilobase of transcript per million reads (FPKM).

**Yeast two-hybrid assay**

*AcFT1* and *AcFT2* coding regions were recombined from entry clones into the GATEWAY destination vectors pDEST32 (pBDGAL4, bait) and pDEST22 (pADGAL4, prey) (Invitrogen). The remaining clones were as described before (Varkonyi-Gasic et al., 2013). These vectors were individually introduced into *Saccharomyces cerevisiae*
strains PJ69-4a (bait) and PJ69-4a (prey) (James et al., 1996) for selection on minimal medium plates (WO) lacking leucine (bait) or tryptophan (prey), followed by mating on YPAD plates and double sequential selection on WO medium lacking both leucine and tryptophan. Final screening was performed on medium lacking tryptophan, leucine, and histidine and supplemented with 0, 1, 2, 5, or 25 mM 3-amino-1,2,4-triazole. Plates were incubated for 4 d at 20 °C, photographed, and scored for growth. Reciprocal tests were performed in duplicate for all combinations.

Results

The Actinidia PEBP gene family

Searches for putative FT-, TFL1/CEN-, and MFT-like genes in the A. chinensis draft genome (Huang et al., 2013), combined with analyses of the Actinidia EST database (Crowhurst et al., 2008), revealed a total of 13 sequences representing the kiwifruit PEBP gene family (Fig. 1A). Five sequences with homology to TFL1/CEN, three with homology to related BFT, three with homology to FT, and two MFT-like sequences were identified, including previously characterized Actinidia FT (AcFT) and CEN (AcCEN) sequences (Varkonyi-Gasic et al., 2013). Amplification and sequencing were further deployed to confirm full-length coding sequences. Predicted protein sequences were used for phylogenetic analysis (Fig. 1B) and for analysis of amino acid positions critical for function. Like the previously described AcFT, newly identified kiwifruit FT-like proteins, annotated AcFT1 and AcFT2, have the conserved glutamine within the external loop (14 amino acids in segment B) and the adjacent tyrosine residue, critical for FT function (Hanzawa et al., 2005; Ahn et al., 2006), but divergence in the external loop of AcFT1 (G137R) was identified (Fig. 1C). In addition, a change of isoleucine at the position highly conserved across the PEBP proteins (II17F) and cysteine at a position mostly conserved across FT-like proteins (CI64G) was identified in AcFT1 (Supplementary Fig. S1). Additional divergent amino acids between AcFT1 and AcFT2 were at positions not highly conserved across FT proteins. With the exception of AcCEN3 and AcBFT1, all kiwifruit CEN- and BFT-like proteins have the conserved residues equivalent to Arabidopsis His88 and Asp144, while in the equivalent
positions both MFT-like sequences have tryptophan and alanine residues (Fig. 1C).

**Differential expression of the Actinidia TFL1/CEN- and BFT-like genes**

Amplification of the full-length coding sequence of *Actinidia* PEBP genes often required cDNA from specific tissues (Supplementary Table S2), suggesting differential expression and mostly low expression levels. Real-time PCR amplification using gene-specific oligonucleotide primers was deployed to study organ-specific expression profiles further. In general, low expression was detected for all transcripts (Fig. 2). *AcCEN3* and *AcCEN4* transcripts were mostly confined to shoot tips, exhibiting a pattern similar to previously described *AcCEN* (Varkonyi-Gasic et al., 2013). *AcCEN2* was detected in all samples, while *AcCEN1*, phylogenetically most similar to Arabidopsis *TFL1* (Fig. 1B), was absent from the shoot tip. All three *AcBFT* transcripts were amplified from the leaf sample, but *AcBFT2* was more abundant in the stem (Fig. 2).

*Actinidia* TFL1/CEN- and BFT-like genes are functionally conserved floral repressors

Typically, members of the PEBP family from various flowering plants have conserved roles in the regulation of flowering, so we elected to characterize the kiwifruit PEBP genes by constitutive expression in Arabidopsis. Coding sequences of

![Fig. 2. Relative expression ±SE of *Actinidia* CEN- and BFT-like genes in mature field-grown *A. chinensis* ‘Hort16A’ (black rectangles) and *A. deliciosa* ‘Hayward’ (gray rectangles) tissues, normalized against ACTIN.](https://academic.oup.com/jxb/article-abstract/68/7/1539/3091562)
AcCEN1, AcCEN2, AcCEN3, AcCEN4, AcBFT1, AcBFT2, and AcBFT3 driven by the CaMV 35S promoter were introduced into Arabidopsis Col-0, and a minimum of five kanamycin-resistant primary transgenic plants for each construct were monitored for flowering time in inductive LD conditions. Delayed flowering was observed with all constructs (Fig. 3A), which was correlated with high transgene expression levels (Fig. 3B). A severe delay in bolting combined with change in inflorescence architecture was demonstrated in at least one line for all constructs, with the exception of AcBFT3 which resulted in moderately delayed bolting only. Transgenic plant phenotypes included large rosette leaves, highly branched leafy shoots resulting from proliferation of coflorescences in place of flowers on primary inflorescence stems, leafy axillary shoots developing from axillary meristems of rosette leaves, and third and fourth order branching from the first formed axillary shoots (Fig. 3C, D). The most severe impact was observed in AcCEN4 and AcBFT2 lines, which demonstrated prolonged indeterminacy of the inflorescence meristem (Fig. 3D), and some lines failed to produce flowers and siliques over the duration of the experiment. Development of inflorescences with whorled phyllotaxis of leaf-like organs and flowers with abnormalities such as reduced size, lack of petals, and leaf-like sepals were frequently found (Fig. 3E).

![Fig. 3. Constitutive expression of Actinidia CEN- and BFT-like genes affects flowering in Arabidopsis. (A) The range of flowering time recorded in T1 lines grown in inductive LD conditions. Five to eight lines per construct were monitored for flowering time, recorded as the number of rosette leaves when the primary inflorescence stems were 0.2 cm long. Each dot represents the flowering time of at least one primary transgenic line. (B) Transgene expression in representative late flowering lines, normalized to ACT2. (C) Representative late flowering plants, of the same age and grown under the same conditions as the control Col-0 plant. (D) Examples of altered inflorescence morphology: proliferation of coflorescences on a primary inflorescence stem of a 35S:AcCEN2 plant, leafy axillary shoot produced on a 35S:AcBFT1 plant with abnormal flowers on the primary inflorescence stem, and indeterminate inflorescence meristems of 35S:AcCEN4 and 35S:AcBFT2 plants that failed to produce flowers. Arrows indicate flowers with leaf-like sepals. (E) Examples of inflorescences with whorled phyllotaxis of leaf-like organs (arrowheads) and small abnormal flowers (arrows).](https://academic.oup.com/jxb/article-abstract/68/7/1539/3091562)
Kiwifruit PEBP genes regulate growth, dormancy and flowering

Actinidia MFT-like genes have no impact on flowering time

Arabidopsis MFT is highly expressed in developing seeds, but is also detected in root and leaf tissue (Xi et al., 2010), and its constitutive expression led to slightly earlier flowering under LDs (Yoo et al., 2004). Actinidia MFT-like transcripts were abundant in mature fruit and amplified from seed and flower tissue (Supplementary Table S2). Analysis using real-time PCR also identified expression in vegetative tissues for AcMFT2 and particularly prominent root expression for AcMFT1 (Fig. 4A), but very low expression was detected in axillary buds (Supplementary Table S3). To establish if kiwifruit MFT-like genes affect flowering time, coding sequences of AcMFT1 and AcMFT2, each under the control of the 35S promoter, were introduced into Arabidopsis Col-0. Twelve AcMFT1 and four AcMFT2 independent lines were generated and monitored for flowering time in non-inductive SD conditions. None of the plants bolted by the time they had the minimum of 30 rosette leaves. Wild-type flowering time was also observed in their progeny grown in LD conditions (Fig. 4B); therefore, we conclude that kiwifruit MFT-like genes do not promote flowering in transgenic Arabidopsis.

Differential and largely opposing expression of AcFT1 and AcFT2

FT-like genes are of particular interest as key activators of flowering and so were studied in detail. AcFT1 was preferentially expressed in terminal buds (Fig. 5A) and in the very small distal leaves during shoot outgrowth in early spring (Fig. 5B). Expression in the shoot tip and very small distal leaves was also observed in rapidly growing large shoots of young glasshouse-grown plants (Fig. 5C; Supplementary Fig. S2). In contrast, AcFT2 was preferentially expressed in the leaf tissue (Fig. 5D). This expression was confined to large basal leaves of larger shoots, declined in mature leaves in late spring, and was detected on branches bearing flower buds, but also on vegetative shoots emerging after the first (floral) flush (Fig. 5E). AcFT2 was also expressed in basal leaves of rapidly growing shoots of young glasshouse-grown plants, with a gradient of expression opposite to that of AcFT1 (Fig. 5C; Supplementary Fig. S2).

AcFT1 and AcFT2 are functionally conserved activators of flowering with differential impact on plant morphology

To assess the potential of AcFT1 and AcFT2 to promote flowering, their coding sequences, driven by the 35S promoter, were introduced into the Arabidopsis wild-type Col-0, and eight independent lines for each construct were monitored for flowering time. Early flowering was detected with all AcFT1 and AcFT2 plants in inductive LD conditions and with kanamycin-resistant progeny of two independent lines of each construct in LD and non-inductive SD conditions (Fig. 6A, B). Both constructs were evaluated for functional conservation in the Ler ft-1 late flowering mutant background. Both 35S:AcFT1 and 35S:AcFT2 complemented the ft-1 mutation, and transgenic plants flowered much earlier than wild-type Ler plants (Fig. 6C, D). To test the potential of AcFT2 to perform the role of the leaf-derived phloem-mobile florigen, its coding sequence was placed under control of the vascular-specific SUCROSE TRANSPORTER 2 (SUC2) promoter and introduced into transgenic Arabidopsis. The kanamycin-resistant lines demonstrated precocity in both day-length conditions (Fig. 6A, E). Similarly, SUC2 promoter-driven AcFT1 promoted flowering in transgenic Arabidopsis (Supplementary Fig. S3).

Fig. 4. Characterization of Actinidia MFT-like genes. (A) Relative expression ±SE in A. chinensis ‘Hort16A’ (black rectangles) and A. delicosa ‘Hayward’ (gray rectangles) tissues, normalized against ACTIN. (B) Wild-type flowering time in 35S:AcMFT lines. Flowering time was recorded as the number of rosette leaves when the primary inflorescence stems were 0.2 cm long. Error bars represent the SD for 10 T2 transgenic lines grown in long-day (LD) conditions.
Voogd et al. 35S:AcFT2 Arabidopsis lines typically flowered slightly earlier and often developed terminal flowers, very small leaves, and thin inflorescence stems, in contrast to mostly wild-type size leaves in early-flowering Col-0 35S:AcFT1 lines (Fig. 6B). We hypothesized that AcFT1 divergence in one of the three previously identified positions (G137R in the external loop and I117F or C164G at positions mostly conserved across FT-like proteins; Supplementary Fig. S1) and altered binding to FD contribute to differential FT activity. However, similar phenotypes were observed when we introduced individual mutations at these positions in AcFT2 and reversed them in AcFT1, and lines with simultaneous mutations at all three positions in AcFT2 also produced relatively small rosette leaves (Supplementary Fig. S4). Similarly, both AcFT1 and AcFT2 were capable of interaction with Arabidopsis and kiwifruit FD proteins in a yeast-two hybrid assay, with similar interaction intensities (Supplementary Fig. S5).
Constitutive expression of AcFT1 and AcFT2 leads to extreme precocity in transgenic kiwifruit

To test if AcFT1 and AcFT2 promote flowering and demonstrate differential activity in kiwifruit, their coding sequences driven by the 35S promoter were introduced into *A. chinensis* using standard regeneration and transformation protocols. Major differences were observed 2 months after inoculation; flower instead of leaf development was observed in the presence of ectopic AcFT2, but both leaf and flower development were recorded in the presence of ectopic AcFT1 and only leaf development was recorded in the control (Fig. 7A). Both constructs gave rise to *in vitro* flowers (Fig. 7B, C), which aborted development soon after initiation of rudimentary reproductive organs (Fig. 7D), while some AcFT1 lines developed leaves and flowers with differentiating floral organs (Fig. 7E).

Seasonal expression patterns in axillary buds

To study the potential roles in dormancy, winter chilling, growth resumption, and initiation of flowering, expression of PEBP genes was further analyzed in kiwifruit axillary buds collected at regular intervals over the season. Interrogation of axillary bud transcriptomes identified differential expression of some *Actinidia* CEN- and BFT-like genes at different stages of bud development. AcCEN and AcCEN4 were abundant in summer, during active growth, but gradually declined at the beginning of autumn, at which stage AcBFT2 transcript abundance started to increase and remained high during the dormancy period. AcCEN4 and to a lesser extent AcCEN and AcCEN3 were also detected in actively growing terminal buds collected in summer and early autumn (Fig. 8A). Very low abundance or total absence of transcripts was observed for the remaining kiwifruit CEN- and BFT-like genes (Supplementary Table S3). Unlike AcFT, which was detected in buds during accumulation of winter chilling and growth resumption in spring, AcFT1 and AcFT2 transcripts were mainly absent from axillary buds and AcFT1 was detected in samples of actively growing terminal buds collected in summer and early autumn (Fig. 8A). Accumulation of AcBFT2 during establishment of dormancy was also detected in ‘Hayward’ axillary buds (Fig. 8B). Transcriptional activity of expressed PEBP genes is summarized in a schematic diagram (Fig. 9).

Discussion

The expansion and diversification of *Actinidia* floral repressor genes

Evolution of the PEBP family has had an important role in plant diversification and adaptation. *Actinidia* have undergone two recent whole-genome duplication events, which followed the ancient hexaploidization event shared by core eudicots (Huang et al., 2013). As a result of these events, the *Actinidia* PEBP gene family contains multiple *FT*-, *CEN*- and *BFT*-like genes. The expanded family of floral repressors in kiwifruit might be of significant relevance to plant growth, architecture, and flowering. All kiwifruit CEN- and BFT-like genes drive indeterminacy and delay flowering in Arabidopsis, and at least one of them, AcCEN, inhibits flowering in *Actinidia eriantha* (Varkonyi-Gasic et al., 2013). Together with AcCEN, AcCEN3 and AcCEN4 appear to be preferentially expressed...
in the buds and are thus likely to perform a role in regulation of flowering, architecture, and determinacy as functional homologs of Arabidopsis TFL1. Determinacy is an agronomically important trait associated with domestication, and homologs of TFL1/CEN genes have been implicated in agricultural adaptation in crops such as tomato, barley, grapevine, bean, soybean, and strawberry (Pnueli et al., 1998; Liu et al., 2010; Fernandez et al., 2010; Tian et al., 2010; Comadran et al., 2012; Iwata et al., 2012; Koskela et al., 2012; Repinski et al., 2012). Kiwifruit has been developed into a horticultural crop only recently (Ferguson, 1990). Most cultivars are either direct selections from the wild or are only a few generations removed from the wild, with only a short history of systematic breeding (Datson and Ferguson, 2011). Currently, excessive vegetative vigor is managed through costly manual pruning. For that reason, combined with generally low harvest index of current kiwifruit cultivars, manipulation of the shoot architecture remains a significant breeding target. Therefore, AcCEN, AcCEN4, and AcCEN3 represent prime candidates for kiwifruit improvement.

In contrast to kiwifruit CEN-like transcripts, which were associated with active growth and declined before the establishment of dormancy, expression of a member of the kiwifruit BFT clade is highest during winter dormancy and peaks at the time of leaf drop. The role of BFT proteins remains poorly understood. Arabidopsis BFT has a TFL1-like activity (Yoo et al., 2010) and is associated with stress response (Ryu et al., 2011, 2014). Up-regulation of Arabidopsis BFT following abscisic acid (ABA), drought, or osmotic stress treatment (Chung et al., 2010) resembles AcBFT2 transcript accumulation during winter dormancy, a process which is also potentially mediated by ABA and involves cold-, drought-, and stress-regulated genes (Horvath et al., 2008). In Arabidopsis, high BFT expression causes suppression of axillary inflorescence development (Yoo et al., 2010). In kiwifruit, AcBFT2 transcript accumulation is associated with suppression of latent bud growth during unfavorable winter conditions. Thus, AcBFT2 may repress vegetative growth, but could also maintain the undifferentiated state of meristems in the axils of leaf primordia in the dormant bud (Fig. 9), which develop as inflorescences after sufficient chilling. Interestingly, apple MdBFTa and MdBFTb were expressed at higher levels in dwarfing rootstocks, commonly used to reduce the scion vigor, suggesting a conserved role in suppression of axillary shoot development (Foster et al., 2013). Similarly, expression of a grapevine BFT homolog, VvTFL1C, increased progressively during latent bud development (Carmona et al., 2007), suggesting that aspects of BFT function may be conserved in woody perennials.

AcCEN1, phylogenetically closest to TFL1, and AcCEN2 appear to be expressed in a range of tissues, including the fruit. The relevance of this is unclear at this stage, but is in line with reports of TFL1/CEN-like gene expression in fleshy fruit (Lindqvist et al., 2006; Mimida et al., 2009; Li et al., 2014). The current study provides no evidence that MFT-like genes contribute towards regulation of flowering and architecture in kiwifruit. Their expression in seed may be similar to a proposed role in hormonal signaling regulating seed germination in Arabidopsis (Xi et al., 2010; Footitt et al., 2011) and wheat (Nakamura et al., 2011), but the universality of this MFT-like role is yet to be confirmed in kiwifruit.

The diversification of Actinidia FT genes

The FT-like genes have emerged with the evolution of the angiosperms, consistent with their key role in regulation of
Kiwifruit PEBP genes regulate growth, dormancy and flowering

Flowering (Shalit et al., 2009; Karlsgren et al., 2011), while subsequent functional divergence contributed to the redundant, similar, or novel, and in some cases antagonistic FT functions (Blackman et al., 2010; Pin et al., 2010; Hsu et al., 2011; Navarro, 2011; Harig et al., 2012; Lee et al., 2013; Cao et al., 2016). The three Actinidia FT genes probably reflect two recent whole-genome duplication events in kiwifruit (Huang et al., 2013), after which these genes have evolved divergent expression patterns. The previously described AcFT gene expression in response to cold correlated with winter chilling requirement and bloom time of kiwifruit cultivars, but it failed to promote flowering in kiwifruit (Varkonyi-Gasic et al., 2013). This expression at later stages of dormancy coincided with reduction in AcBFT2 transcript accumulation, suggesting that antagonism between these two genes may be of importance for bud dormancy and growth cycles.

In contrast, AcFT1 and AcFT2 both act as potent activators of flowering. This function is conserved across diverse
plant species and may be non-cell autonomous, as demonstrated for AcFT2, thus fulfilling the criteria for florigen (Chailakhyan, 1968). The severity of phenotypes in both Arabidopsis and kiwifruit plants constitutively expressing high levels of AcFT2 suggests that it may act in a dosage-dependent manner across long distances, when high expression may provide sufficient amounts to be delivered to the meristems in latent buds, to balance out the repressors of flowering and establish floral fate. Conversely, AcFT1 expression differs from the florigen concept in that its expression appears restricted to kiwifruit sink tissue. Therefore, it might be fulfilling a role other than flowering control, perhaps in regulation of terminal bud expansion rate and abortion, which underlies high developmental plasticity of kiwifruit (Foster et al., 2007). Alternatively, both AcFT1 and AcFT2 may act locally; in particular, AcFT2 may have a role in termination of leaf expansion and development, while AcFT1 may regulate termination of the shoot apical meristem after establishment of leaf fate.

Comparison of amino acid sequences identified divergence of AcFT1 residues in positions highly conserved in other FT-like proteins, which did not abolish its function and had no effect on the interaction with FD proteins, further confirming the robust nature of FT proteins as activators of the flowering process. The substitution for polar-positive arginine in the position of non-polar-neutral glycine in the conserved segment B (G137R) had little effect on AcFT1 activity, as recently demonstrated in Arabidopsis (Ho and Weigel, 2014) and consistent with findings in sugar beet (Klintenäs et al., 2012). A conservative I117F substitution did not affect FT function, despite conservation of isoleucine in this position in FT and most other PEBP proteins (Supplementary Fig. S1) (Ho and Weigel, 2014). Similarly, introduction of a glycine in the position of highly conserved cysteine (C164G) had no impact on AcFT activity. Therefore, the sum of differences in these and other positions and interactions with proteins other than FD, including TEOSINTE-BRANCHED1/CYCLOIDEA/PCF (TCP) transcription factors (Mimida et al., 2011; Niwa et al., 2013; Ho and Weigel, 2014), and binding of unknown ligand(s) might provide the basis for different flowering time and architecture of 35S:AcFT1 and 35S:AcFT2 lines.

In summary, multiple differentially expressed and functionally divergent FT- and CEN-like genes regulate flowering and architecture in kiwifruit, and a BFT-like gene is associated with a lack of growth during dormancy. Specific expression of FT-like activators and CEN/BFT-like repressors (Fig. 9) may ensure adequate timing of growth and dormancy, leaf, flower, and fruit development, shoot tip growth, and establishment of latent buds. Manipulation of their dosage and functionality may provide practical means to reduce juvenility, hasten the breeding process, improve orchard management, and increase kiwifruit productivity.

Supplementary data

Supplementary data are available at JXB online.

Table S2. Expression of PEBP genes in specific kiwifruit tissues.

Table S3. Expression of PEBP genes in buds during the season.

Fig. S1. Alignment of PEBP protein sequences from a range of plant species.

Fig. S2. Relative expression of AcFT1 and AcFT2 in the leaves of fast growing shoots collected from juvenile glasshouse-grown plants.

Fig. S3. A representative early flowering SUC2:AcFT1 Arabidopsis plant, of the same age and grown under the same LD conditions as the control Col-0 plant.

Fig. S4. Mutagenesis of divergent AcFT1 residues had little impact on flowering time and leaf size.

Fig. S5. Yeast two-hybrid assays were used to evaluate interactions between FT and FD proteins.

Fig. S6. Relative expression ±SE in A. deliciosa axillary buds collected at monthly intervals during the growth and dormancy season.

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