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RESEARCH PAPER

Conservation and divergence of autonomous pathway genes in the flowering regulatory network of *Beta vulgaris*

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Abstract

The transition from vegetative growth to reproductive development is a complex process that requires an integrated response to multiple environmental cues and endogenous signals. In *Arabidopsis thaliana*, which has a facultative requirement for vernalization and long days, the genes of the autonomous pathway function as floral promoters by repressing the central repressor and vernalization-regulatory gene *FLC*. Environmental regulation by seasonal changes in daylength is under control of the photoperiod pathway and its key gene *CO*. The root and leaf crop species *Beta vulgaris* in the caryophyllid clade of core eudicots, which is only very distantly related to *Arabidopsis*, is an obligate long-day plant and includes forms with or without vernalization requirement. *FLC* and *CO* homologues with related functions in beet have been identified, but the presence of autonomous pathway genes which function in parallel to the vernalization and photoperiod pathways has not yet been reported. Here, this begins to be addressed by the identification and genetic mapping of full-length homologues of the RNA-regulatory gene *FLK* and the chromatin-regulatory genes *FVE*, *LD*, and *LDL1*. When overexpressed in *A. thaliana*, *BvFLK* accelerates bolting in the Col-0 background and fully complements the late-bolting phenotype of an *flk* mutant through repression of *FLC*. In contrast, complementation analysis of *BvFVE1* and the presence of a putative paralogue in beet suggest evolutionary divergence of *FVE* homologues. It is further shown that *BvFVE1*, unlike *FVE* in *Arabidopsis*, is under circadian clock control. Together, the data provide first evidence for evolutionary conservation of components of the autonomous pathway in *B. vulgaris*, while also suggesting divergence or subfunctionalization of one gene. The results are likely to be of broader relevance because *B. vulgaris* expands the spectrum of evolutionarily diverse species which are subject to differential developmental and/or environmental regulation of floral transition.

Key words: Autonomous pathway, circadian clock, *FLK*, floral transition, *FVE*, sugar beet.

Introduction

Floral transition is a major developmental switch which is tightly controlled by a network of proteins that perceive and integrate environmental and developmental signals to promote or inhibit the transition to reproductive growth. In the model species *Arabidopsis thaliana*, several regulatory pathways which differ in their response to distinct cues have been defined, including the vernalization, photoperiod, and autonomous pathway (for reviews, see He and Amasino, 2005; Bäurle and Dean, 2006; Jung and Müller, 2009; Michaels, 2009). The central regulator of the vernalization response is *FLOWERING LOCUS C* (*FLC*), which acts as a repressor of flowering and is down-regulated in response to prolonged exposure to cold over winter. The promotion of floral transition by long days is mediated by CONSTANS (CO), a key protein of the photoperiod pathway which activates the floral integrator gene *FLOWERING*

LOCUS T (*FT*). Plant genome and expressed sequence tag (EST) sequencing projects in species other than *Arabidopsis*, together with functional studies have begun to unveil the presence and evolutionary conservation of floral regulatory genes across taxa (e.g. Hecht *et al.*, 2005; Albert *et al.*, 2005; Remay *et al.*, 2009; Mouhu *et al.*, 2009). While components of the photoperiod pathway are widely conserved, the regulation of the vernalization requirement and response appears to have diverged considerably during evolution, as exemplified by distinct mechanisms in *Arabidopsis* and temperate cereals (Turck *et al.*, 2008; Colasanti and Coneva, 2009; Distelfeld *et al.*, 2009; Greenup *et al.*, 2009; Jung and Müller, 2009). The phylogenetic lineage leading to *Beta vulgaris*, which includes the biennial crop subspecies sugar beet (*Beta vulgaris* L. ssp. *vulgaris*) as well as annual and perennial wild beets, diverged from that leading to *Arabidopsis* ~120 million years ago—that is, relatively soon after the monocot–dicot divergence (Chaw *et al.*, 2004; Davies *et al.*, 2004). In beet, the vernalization requirement is under the control of the bolting gene *B* (Munerati, 1931; Abegg, 1936; Boudry *et al.*, 1994; El-Mezawy *et al.*, 2002), which is not related to *FLC* (Reeves *et al.*, 2007; A. E. Müller *et al.*, unpublished), and a second, unlinked locus *B2* which may act epistatically to *B* (Büttner *et al.*, 2010). In the absence of the dominant early bolting allele at the *B* locus, beets possess an obligate requirement for both vernalization and long photoperiods, and under high temperature and short-day conditions are prone to reversion to a vegetative state by devernalization. Despite apparent differences in the regulation of floral transition between *A. thaliana* and *B. vulgaris*, the recent identification of beet homologues of *FLC* and *CO* suggest at least partial conservation of the genetic basis of the plants' responses to the environment (Reeves *et al.*, 2007; Chia *et al.*, 2008). The *FLC*-like gene *BvFL1* in beet is regulated by vernalization, and delays flowering in transgenic *Arabidopsis* plants, suggesting that *BvFL1* may also be a floral repressor (Reeves *et al.*, 2007). Similarly, evolutionary conservation of *CO* homologues was suggested by overexpression of the *CO*-like gene *BvCOL1* in *Arabidopsis*, which complements the late-flowering phenotype of a loss-of-function *co* mutation and activates *FT* expression (Chia *et al.*, 2008).

Floral transition in *Arabidopsis* is also regulated by the autonomous pathway of flowering time control whose genes are thought to function largely in parallel to the vernalization pathway upstream of *FLC* and the photoperiod pathway (for reviews, see Boss *et al.*, 2004; Simpson, 2004; Quesada *et al.*, 2005). Autonomous pathway genes repress *FLC* and thus act as promoters of floral transition, and include *FLOWERING LOCUS CA* (*FCA*), *FLOWERING LOCUS D* (*FLD*), *FLOWERING LOCUS KH DOMAIN* (*FLK*), *FLOWERING LOCUS PA* (*FPA*), *FLOWERING LOCUS VE* (*FVE*), *FLOWERING LOCUS Y* (*FY*), and *LUMINIDEPENDENS* (*LD*) (Simpson, 2004). They have in common that mutations in these genes are generally recessive and delay flowering under both long-day and short-day conditions, while the inhibitory effect of the mutations can be overcome by vernalization. Mutations at *FLC* eliminate the late-flowering phenotype caused by mutations in autonomous pathway genes (Koornneef *et al.*, 1994; Lee *et al.*, 1994; Sanda and Amasino, 1996; Michaels and Amasino, 2001).

Although some genes of the autonomous pathway interact genetically and all share a common target, they do not form a single linear pathway with a hierarchical order of activities, but rather constitute different regulatory subgroups (or 'subpathways'; Marquardt *et al.*, 2006). Autonomous pathway genes regulate *FLC* expression through RNA-based control mechanisms and/or by chromatin modification (Boss *et al.*, 2004; Simpson, 2004; Quesada *et al.*, 2005; Bäurle *et al.*, 2007; Bäurle and Dean, 2008). Four genes mediate RNA regulatory processes, *FCA*, *FPA*, *FY*, and *FLK*. *FCA* and *FPA* encode plant-specific RNA-binding proteins which both carry multiple RNA recognition motifs (RRMs; Macknight *et al.*, 1997; Schomburg *et al.*, 2001). *FCA* physically and genetically interacts with the RNA 3' end processing factor *FY*, and this interaction is required both for correct processing of transcripts derived from *FCA* itself and (directly or indirectly) for down-regulation of *FLC* expression (Quesada *et al.*, 2003; Simpson *et al.*, 2003). *FCA* and *FPA* interact genetically with *FLD*, which encodes a chromatin regulatory protein of the autonomous pathway (see below), and at least part of the effect of *FCA* and *FPA* on *FLC* expression and flowering time depends on *FLD* (Liu *et al.*, 2007; Bäurle and Dean, 2008). Thus, *FCA* and *FPA* appear to link RNA and chromatin level control of gene expression.

An analysis of flowering time in various autonomous pathway double mutants indicated that the fourth protein predicted to function in RNA regulation, *FLK*, acts independently of both *FCA* and *FPA* (Bäurle and Dean, 2008; Ripoll *et al.*, 2009). *FLK* expression was not detectably affected in any of the other six autonomous pathway mutants analysed (*fca*, *fpa*, *fy*, *fld*, *fve*, and *ld*), and, vice versa, the expression of all autonomous pathway genes tested (*FCA*, *FPA*, *FVE*, and *LD*) was unaltered in an *flk* mutant (Lim *et al.*, 2004). *FLK* encodes a plant-specific putative RNA-binding protein which contains three K-homology (KH)-type RNA-binding domains (Lim *et al.*, 2004; Mockler *et al.*, 2004). The mode of action of the *FLK* product is not known, but other KH domain proteins in *Arabidopsis*, including HUA ENHANCER 4 (HEN4) (harbouring five KH domains) and RS2-INTERACTING KH PROTEIN (RIK), were shown to be part of protein complexes which mediate pre-mRNA processing or have been implicated in RNA-directed chromatin regulation of gene expression, respectively (Cheng *et al.*, 2003; Phelps-Durr *et al.*, 2005). Also, both correctly spliced *FLC* transcripts and intron-retaining variants accumulated to higher levels in an *flk* mutant than in wild-type plants (Ripoll *et al.*, 2009), and repression of *AtSN1*, a retroelement which is subject to RNA-directed chromatin silencing, was at least partially released in mutant plants (Bäurle and Dean, 2008; Veley and Michaels, 2008). Together, these findings have been interpreted to indicate that *FLK* may suppress *FLC* at least partially at the transcriptional level,

perhaps through RNA-directed chromatin silencing (Veley and Michaels, 2008; Ripoll *et al.*, 2009). Ripoll *et al.* (2009) further found that *PEPPER* (*PEP*), a paralogue of *FLK* in *Arabidopsis*, acts as a positive regulator of *FLC*. The authors showed that *pep* mutations can at least partially rescue the flowering time phenotype of *flk* mutants, and that overexpression of *PEP* resulted in a similar effect to mutation of *FLK* on flowering time. Overexpression of *PEP* in an *flk* mutant background neither further delayed flowering nor led to an increase of *FLC* expression when compared with *flk* mutant plants not carrying the *PEP* transgene, suggesting that *FLK* and *PEP* may interact in the same genetic pathway (Ripoll *et al.*, 2009).

Chromatin level control of *FLC* expression is mediated by *FLD*, *FVE*, and *LD*. *FLD* is a homologue of the human histone H3K4 demethylase *LSD1* (*LYSINE-SPECIFIC HISTONE DEMETHYLASE1*) and, in *A. thaliana*, represses *FLC* by H3K4 demethylation and H4 deacetylation of *FLC* chromatin, possibly as part of a co-repressor complex (He *et al.*, 2003; Jiang *et al.*, 2007), and is dependent on the sumoylation state of *FLD* (Jin *et al.*, 2008). The *Arabidopsis* genome contains three additional homologues of *FLD*, *LSD1-LIKE1* (*LDL1*), *LDL2*, and *LDL3*, two of which (*LDL1* and *LDL2*) have been shown to act in partial redundancy with *FLD* to repress *FLC* (Jiang *et al.*, 2007). Furthermore, *LDL1* (also termed *SWP1*, *SWIRM DOMAIN PAO PROTEIN 1*) interacts with the histone methyltransferase *CZS* (*C2H2 ZINC FINGER-SET DOMAIN PROTEIN*) and is part of a co-repressor complex which represses *FLC* by H4 deacetylation and H3K9 and H3K27 methylation at the *FLC* locus (Krichevsky *et al.*, 2007). *LD*, a unique nuclear-localized protein in *Arabidopsis* which contains a homeodomain-like domain (Lee *et al.*, 1994; Aukerman *et al.*, 1999), also appears to regulate *FLC* expression by histone modification, including H3K4 demethylation and H3 deacetylation (Domagalska *et al.*, 2007), but may also repress *FLC* by a negative regulatory interaction with a transcriptional activator of *FLC*, *SUF4* (*SUPPRESSOR OF FRIGIDA 4*; Kim *et al.*, 2006). *FVE* [also termed *MSI4* (*MULTICOPY SUPPRESSOR OF IRA1 4*) and *ACG1* (*ALTERED COLD-RESPONSIVE GENE EXPRESSION 1*)] is homologous to *MSI1* (*MULTICOPY SUPPRESSOR OF IRA1*) in yeast and retinoblastoma-associated proteins in animals, which are components of chromatin assembly complexes. FVE is part of a small family of MSI1-like WD40 repeat proteins in *Arabidopsis* (Kenzior and Folk, 1998; Ausin *et al.*, 2004; Kim *et al.*, 2004; Hennig *et al.*, 2005). In *fve* mutants, histones H3 and H4 are hyperacetylated in *FLC* chromatin, suggesting that FVE, perhaps together with FLD, is part of a complex which represses *FLC* expression by chromatin modification (He *et al.*, 2003; Amasino, 2004; Ausin *et al.*, 2004). FVE and other MSI1-like proteins are generally thought of as structural proteins without catalytic function and may provide a scaffold for assembly of larger complexes. *FVE* has also been implicated in temperature-dependent regulation of flowering time, and appears to promote flowering in response to elevated ambient temperatures through an *FLC*-independent, thermosensory pathway which also includes *FCA* (Blázquez *et al.*, 2003). In addition, *FVE* mediates the plant's response to intermittent cold stress and may provide a link between cold stress response and flowering time control (Kim *et al.*, 2004; Franklin and Whitelam, 2007). *FVE* is expressed in all major plant organs, but appears to be preferentially expressed in actively dividing cells, and has been assigned a more general role in the regulation of cellular differentiation and developmental transitions (Morel *et al.*, 2009). Recent grafting experiments in *A. thaliana* suggest that *FVE* mRNA is phloem mobile and may contribute to long-distance signalling in plant development (Yang and Yu, 2010).

There is increasing evidence that several, if not all, autonomous pathway genes also regulate developmental processes other than floral transition. In particular, double mutant analyses showed that *fpa fld*, *fpa fve*, and *fpa ld* mutants have pleiotropic, *FLC*-independent effects on growth rate, chlorophyll content, leaf morphology, flower development, and fertility, and that the corresponding genes have partially redundant functions (Veley and Michaels, 2008). Furthermore, mutations in *FCA*, *FVE*, and *LD* result in an increase in the period length of the circadian clock, thus implicating autonomous pathway genes in the regulation of the clock (Salathia *et al.*, 2006). Finally, transposon and transgene silencing assays in mutants indicated that *FCA*, *FPA*, *FLK*, and *FVE* have a more widespread role in RNA-directed chromatin silencing of a range of target genes (Bäurle *et al.*, 2007; Bäurle and Dean, 2008; Veley and Michaels, 2008). For *FCA*, *FVE*, *FY*, and *LD* at least partial conservation of floral regulatory functions was shown in monocots (van Nocker *et al.*, 2000; Lee *et al.*, 2005; Lu *et al.*, 2006; Baek *et al.*, 2008; Jang *et al.*, 2009).

Here, the components of the autonomous pathway in *B. vulgaris* have begun to be dissected through a survey of ESTs with homology to autonomous pathway genes and isolation of the corresponding genes. For beet homologues of four autonomous pathway genes, termed *BvFLK*, *BvFVE1*, *BvLD*, and *BvLDL1*, the full-length genomic and coding sequences were identified and the genes were mapped on a reference map of the sugar beet genome. Exon–intron structure and domain organization were found to be conserved between beet and *Arabidopsis* in all four genes. One homologue each of autonomous pathway genes implicated in RNA or chromatin regulatory mechanisms, *BvFLK* and *BvFVE1*, respectively, was further characterized by overexpression and complementation analysis in *A. thaliana* wild type and mutants. *BvFLK* was able to accelerate bolting time in *A. thaliana* wild type and complement the late-bolting phenotype of an *flk* mutant. In contrast, *BvFVE1* was unable to complement an *fve* mutant, and was found to be under circadian clock regulation in beet, which has not been reported for *FVE* in *Arabidopsis*. Together, the data suggest conservation of autonomous pathway components in *B. vulgaris*, while also providing first evidence for divergence or subfunctionalization of at least one autonomous pathway gene homologue.

Materials and methods

Bioinformatic analyses

The *B. vulgaris* EST database BvGI (*Beta vulgaris* Gene Index, versions 2.0 and 3.0; http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=beet) and the *B. vulgaris* subsets of the NCBI EST and nt/nr databases (http://www.ncbi.nlm.nih.gov) were used to identify beet homologues of flowering time genes in *A. thaliana*. Database searches were performed using the tblastn algorithm (Altschul *et al.*, 1990) and *A. thaliana* protein sequences from the Arabidopsis Genome Initiative (AGI; http://www.arabidopsis.org/tools/bulk/sequences/index.jsp) as queries. To help infer orthology by bidirectional best hit (BBH) analysis (Overbeek *et al.*, 1999), the beet sequences retrieved through this analysis were used as queries for blastx searches against *A. thaliana* protein sequences at NCBI (http://www.ncbi.nlm.nih.gov) and TAIR (http://www.arabidopsis.org). *Beta vulgaris* sequences were annotated using pairwise sequence alignments (BLAST2; http://www.ncbi.nlm.nih.gov) against putative *A. thaliana* orthologues and the FGENESH+ and FGENESH_C gene prediction programs (http://linux1.softberry.com/berry.phtml) for annotation of exon–intron structures, TSSP (http://linux1.softberry.com/berry.phtml) and PLACE for annotation of promoter regions (http://www.dna.affrc.go.jp/PLACE; Higo *et al.*, 1999), and PFAM (http://pfam.sanger.ac.uk) for identification of conserved protein domains. Multiple sequence alignments were made using CLUSTAL W (http://www.ebi.ac.uk/Tools/clustalw/index.html). Amino acid identity was calculated as the percentage of identical residues in two homologues divided by the total number of residues in the reference gene. For phylogenetic analysis, putative *FLK* and *FVE* orthologues in other plant species were identified by blastp searches of the NCBI Reference Sequence (RefSeq) protein database (http://www.ncbi.nlm.nih.gov/refseq) and BBH analysis essentially as described above, except that the blastp algorithm was used. The sequences were aligned using CLUSTAL W, and unrooted phylogenetic trees were constructed using the Neighbor–Joining algorithm and the Dayhoff PAM matrix as implemented in the MEGA4 software (Tamura *et al.*, 2007).

Plant material and growth conditions

For expression analysis in different tissues, the biennial *B. vulgaris* accession A906001 (El-Mezawy *et al.*, 2002) was grown in the greenhouse under long-day conditions supplemented with artificial light [Son-T Agro 400W (Koninklijke Philips Electronics NV, Eindhoven, The Netherlands) for 16 h]. Six-week-old plants were vernalized under short-day (8 h light) conditions at 5 °C for 3 months. Plants were returned to the greenhouse and continued to be grown under the same conditions as before vernalization until the first flowers opened (BBCH scale 60; Meier, 2001). For diurnal and circadian expression, the commercial biennial cultivar Roberta (KWS Saatzucht GmbH, Einbeck, Germany) was grown under defined light regimes (long days of 16 h and short days of 8 h) in Sanyo Gallenkamp MLR 350 growth chambers at 22 °C. Lighting was supplied by 36 W fluorescent Daystar lamps (CEC Technology) providing 300 µmol m^{-2} s^{-1} of photosynthetically active radiation.

A. thaliana flowering time gene mutants SALK_112850 (*flk-1*; Alonso *et al.*, 2003; Lim *et al.*, 2004) and SALK_013789 (Alonso *et al.*, 2003), which will be referred to as *fve-7* (following on from the *fve* mutant number in Morel *et al.*, 2009), were received from the Nottingham Arabidopsis Stock Centre (NASC; http://arabidopsis.info/). The *fve-7* mutant carries a T-DNA insertion in intron 1 at nucleotide position 2835 of the genomic sequence entry for *FVE* in GenBank (accession number AF498101). Mutants homozygous for the T-DNA inserts in *FLK* or *FVE*, respectively, were identified by PCR using a T-DNA-specific primer (A479 for *flk-1*, B478 for *fve-7*; for primer sequences see Supplementary Table S1 available at *JXB* online) in combination with a gene-specific primer (A477 for *flk-1*, B476 for *fve-7*). The absence of the wild-type alleles was confirmed by PCR with gene-specific primers which flank the insert on either side (A477 and A478 for *flk-1*, and B476 and B477 for *fve-7*). *A. thaliana* Col-0 (wild-type) plants, mutants, and the T_1 and T_2 generations of transgenic plants were phenotyped for bolting time under long-day conditions (16 h light, Osram L58 W77 Fluora and Osram L58 W840 Lumilux Cool White Hg) at 22 °C in a growth chamber (BBC Brown Boveri York, Mannheim, Germany).

Bacterial artificial chromosome (BAC) library screening

EST sequence information was used to generate genomic fragments for use as probes to screen the *B. vulgaris* BAC library described by Schulte *et al.* (2006). Probe fragments were generated by PCR amplification using primer combinations A039–A064 for *BvFLK* (717 bp), A066–A042 for *BvFVE1* (1089 bp), A043–A067 for *BvLD* (589 bp), and A033–A065 for *BvLDL1* (678 bp), and genomic DNA of A906001 as template, and purified using the Montage PCR_{96} Cleanup Kit (Millipore Corporation, Bedford, CA, USA). The probes were labelled and hybridized to high-density BAC filters essentially as described by Hohmann *et al.* (2003). Positive clones were verified by PCR analysis using the same primer combinations as for PCR amplification of probe fragments. BAC DNA was isolated using the NucleoBond BAC 100 kit according to the manufacturer's protocol (Macherey-Nagel, Dürren, Germany). The genes of interest were sequenced by primer walking (*BvFLK*) or whole BAC sequencing (*BvFVE1*, *BvLD*, and *BvLDL1*) on a GS20 sequencing machine (MWG, Ebersberg, Germany).

RT-PCR and RACE

Total RNA was extracted from roots, stems, leaves, and flowers of adult plants of accession A906001 using the Plant RNAeasyKit™ (Qiagen, Hilden, Germany) and DNase treated (Ambion, Austin, TX, USA). A 1.5 µg aliquot of RNA was reverse transcribed using the First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany), and the cDNA was diluted 10 times for RT-PCR. The complete coding sequences of *BvFLK*, *BvFVE1*, and *BvLDL1* were amplified using leaf cDNA as template and primer combinations A396–A397, A836–A837, and A823–A825, respectively. The coding sequence of *BvLD* was amplified by RT-PCR of several overlapping fragments, using primer combinations A067–A108, B391–A046, A043–B392, A068–A069, and A045–B396. Primers for RT-qPCR were designed and optimized to 94.6% amplification efficiency for *BvFLK* (primers B042-B043), and 92.9% for *BvFVE1* (primers A066–A042). Fluorescence of the Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen Corporation, Carlsbad, CA, USA) was measured in an CFX96 real-time PCR machine (Bio-Rad, Munich, Germany) over 40 cycles at annealing temperatures of 65 °C for *BvFLK* and *BvFVE1*, and 60 °C for glyceraldehyde 3-phosphate dehydrogenase (*BvGAPDH*; BvGI 2.0 accession number TC1351; RT-qPCR amplification efficiency 96.3%). Expression levels were measured in triplicate and normalized against the reference gene *BvGAPDH*.

For diurnal and circadian expression analysis, first-strand cDNA was prepared and analysed by RT-qPCR as described (Chia *et al.*, 2008) except that an in-solution DNase treatment (Ambion) was used and that expression data were normalized against three *B. vulgaris* housekeeping genes, *BvGAPDH* (see above), elongation factor 1-α (*BvEF1α*; BvGI 2.0 accession number TC5), and elongation factor 2 (*BvEF2*; BvGI 2.0 accession number TC64). A normalization factor (NF) was generated for each sample using the geNorm Software v3.5 (http://medgen.ugent.be/~jvdesomp/genorm/; Vandesompele *et al.*, 2002). The NF was used to normalize and calculate the relative expression values for the genes of interest. All primers for normalizer genes and *BvFVE1* were optimized to 98–110% amplification efficiency using serial

dilutions of sugar beet leaf cDNA. Amplified fragments were sequenced to confirm specificity. Fluorescence of the bound SYBR-GREEN (Stratagene, La Jolla, CA, USA) was detected in an MX3000 real-time PCR machine (Stratagene) over 40 cycles at 60 °C annealing temperature.

The 5' end of *BvFVE1* was identified by 5′-rapid amplification of cDNA ends (RACE) (Frohman *et al.*, 1988) using the GeneRacer™ kit according to the manufacturer's protocol (Invitrogen Corporation). 5′-RACE was performed on double-stranded adaptor-ligated cDNA synthesized from 5 µg of total RNA from leaves of 4-week-old sugar beet plants using exon-specific primers (5′-RACE primer A830 and 5′-RACE nested primer A831). 5′-RACE fragments were cloned into the pGEM-T vector (Promega Corporation, Madison, WI, USA) and sequenced using standard Sp6 and T7 primers.

For expression analysis of *FLC* and *FLK* in *A. thaliana*, total RNA was isolated from rosette leaves of 30-day-old plants using the Plant RNAeasyKit™ (Qiagen) and DNase treated (Fermentas). A 2 µg aliquot of RNA was reverse transcribed using the First Strand cDNA Synthesis Kit (Fermentas), and the cDNA was diluted 10 times for RT-qPCR. Amplification efficiency of *FLC* (GenBank accession number NM_121052, primers B336-B337), *FLK* (GenBank accession number AC011437, B281-B282), and *GAPDH* (GenBank accession number NM_111283; B349-B350) was 100, 97.4, and 94.2%, respectively.

Vector construction and transformation of A. thaliana

The coding sequences of *BvFLK* and *BvFVE1* were amplified as described above. The *BvFLK* coding sequence was cloned into pGEM-T (Promega Corporation) to yield plasmid pFT002, and re-amplified from pFT002 with primers A680-*Xho*I and A652-*Spe*I. The PCR product was restricted with *Xho*I and *Spe*I (Fermentas) and cloned into the corresponding restriction enzyme sites of the binary vector pSR752Ω (kindly provided by Chonglie Ma and Richard Jorgensen, University of Arizona, Tucson, AZ, USA) which carries the cauliflower mosaic virus (CaMV) 35S promoter. The resulting construct was designated pFT013. A 1813 bp fragment carrying the endogenous promoter region of *BvFLK* and 363 bp of the 5′ region of the coding sequence was amplified from BAC DS 794 using primers A896-*Eco*RI and A821-*Hpa*I. The fragment was restricted with *Eco*RI and *Hpa*I and inserted into the corresponding restriction sites of pFT013, thus effectively replacing the CaMV 35S promoter by the 1435 bp endogenous beet sequence upstream of the *BvFLK* start codon in the resultant plasmid (pFT033). A plasmid carrying the coding region of *FLK* (GenBank accession number BX823281) was kindly provided by the French Genomic Resource Center (INRA-CNRGV, Castanet Tolosan cedex, France). The coding sequence of *AtFLK* was amplified using primers A901-*Xho*I and A938-*Sma*I, and inserted into the corresponding restriction sites of pSR752Ω. The resulting vector carries the *FLK* coding sequence under the control of the CaMV 35S promoter and was designated pFT016. The coding sequence of *BvFVE1* was cloned into pDONOR221 using the Gateway Cloning System (Invitrogen Corporation) to yield plasmid pBS355 The coding sequence was subsequently transferred into the pEarleyGate 100 vector (Earley *et al.*, 2006) to yield the binary vector pBS356, in which the *BvFVE1* coding sequence is under the control of the CaMV 35S promoter.

The intactness of the binary vectors and the sequence of all inserts were confirmed by restriction enzyme digests, PCR amplification, and sequencing (Institute of Clinical Molecular Biology, Kiel, Germany). The constructs were transferred by electroporation into *Agrobacterium tumefaciens* LBA 4404 using Electromax (Invitrogen Corporation) competent cells (pFT013, pFT016, and pFT033) or *A. tumefaciens* GV2260 competent cells prepared according to the protocol of Mersereau *et al.* (1990) (pBS356), and transformed into *A. thaliana* by the floral dip method (Clough and Bent, 1998). Primary transformants (T_1 plants) were selected by spraying BASTA (Bayer CropScience, Wolfenbüttel, Germany) at a concentration of 1.7 g l^{-1} at the two-leaf stage. The presence of the transgene in BASTA-resistant plants was confirmed by PCR analysis using primer combinations B042–B043 for pFT013 and pFT033, B281–B282 for pFT016, and A875–A876 for pBS356. The transformants were propagated by selfing to produce T_2 seed. Genomic DNA was extracted using the NucleoSpin 96 Plant DNA isolation kit (Macherey and Nagel, Düren, Germany).

Genetic mapping and statistical analysis

Flowering time genes were mapped genetically in the D2 (100 F_2 individuals) and K1 (97 F_2 individuals) reference populations described by Schneider *et al.* (2007). Polymorphisms were identified by PCR amplification and sequencing of genomic fragments using DNA from the parent and F_1 plants as template. Map positions were calculated using Join Map 3.0 (Van Ooijen and Voorrips, 2001) and the Kosambi mapping function (Kosambi, 1944) at a LOD score of 4.0.

Analysis of variance (ANOVA) and *t*-tests were performed using SAS 9.1 TS level 1M3 (SAS Institute, Cary, NC, USA). Sample groups with significantly different means were further analysed using Fisher's least significant difference (LSD) test at a 5% probability level (SAS 9.1 TS level 1M3).

Results

Beta vulgaris *homologues of autonomous pathway genes*

To identify autonomous pathway gene candidates in beet, the *B. vulgaris* EST database BvGI (versions 2.0 and 3.0) and the *B. vulgaris* subsets of the NCBI EST and nt/nr databases were searched for homologues to autonomous pathway genes in *A. thaliana* (see Materials and methods). Among the seven classical genes assigned to the autonomous pathway in *Arabidopsis* (*FCA*, *FLD*, *FLK*, *FPA*, *FVE*, *FY*, and *LD*), four were found to have putative orthologues in beet [*FLK* (BQ586739), *FPA* (BQ489608), *FVE* (BQ592158, EG550040), and *LD* (BQ589018, BQ594506); Table 1]. Besides *FLK*, one EST (BQ590839) was identified whose closest homologue in *A. thaliana* is *PEP*, an *FLK* paralogue (Ripoll *et al.*, 2009; see Introduction). In addition, one EST (CV301493) with homology to *FLD* appears to be orthologous to *LDL1*/*SWP1*, an *FLD*-like gene in *Arabidopsis* which recently was also assigned to the autonomous pathway (Jiang *et al.*, 2007; Krichesky *et al.*, 2007; see Introduction). Orthologous ESTs were not identified for *FLD*, *FCA* and *FY*.

For four putative autonomous pathway genes, named *BvFLK*, *BvFVE1* (corresponding to one of the two homologous ESTs), *BvLD*, and *BvLDL1*, the complete genomic sequence was identified by BAC library screening and BAC sequencing or primer walking. The two ESTs with homology to *LD* were both found to derive from the same gene. The exon–intron structure of *BvFLK*, *BvFVE1*, *BvLD*, and *BvLDL1* was determined by RT-PCR and sequencing. The number of exons and the sites of introns were found to be conserved between the corresponding *A. thaliana* and *B. vulgaris* genes (Fig. 1A; Supplementary Fig. S1 at *JXB*

Table 1. Beta vulgaris *ESTs with homology to* Arabidopsis thaliana *autonomous pathway genes*

At locus number	Gene	Best hit(s) in *B. vulgaris* (GenBank accession no)[a]	E-value	Best hit in *A. thaliana* (At locus no or gene name)[b]
At4g16280	*FCA*	TC13484	6.1e-17	At1g44910
At3g10390	*FLD*	CV301493	6.6e-71	*LDL1/SWP1*
At3g04610	*FLK*	BQ586739	8.4e-83	*FLK*
		BQ590839	8.8e-44	*PEP*
At2g43410	*FPA*	BQ489608	7.4e-17	*FPA*
At2g19520	*FVE*	EG550040	4.4e-85	*FVE*
		BQ592158	2.4e-62	*FVE*
At5g13480	*FY*	BQ588779	1.3e-15	At4g02730
At4g02560	*LD*	BQ589018	4.0e-27	*LD*
		BQ594506	1.6e-18	*LD*

[a] *Beta vulgaris* ESTs or TCs (tentative consensus sequences) were identified by tblastn sequence similarity searches in BvGI 3.0 (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=beet).
[b] Best hits in *A. thaliana* were identified by blastx in the TAIR9 protein database (http://www.arabidopsis.org).

online). However, several of the *B. vulgaris* introns are substantially larger than the respective introns in *A. thaliana* and contain repetitive elements such as minisatellites and various short low complexity and/or simple repeat regions [e.g. $(AT)_n$], but longer transposons or retroelements were not identified. *BvLDL1*, like *LDL1* in *A. thaliana* (but unlike *FLD*), contains only a single exon. The coding sequences of the four *B. vulgaris* genes are somewhat shorter than those of their *Arabidopsis* counterparts (*BvFLK* 1674 bp versus *FLK* 1731 bp; *BvFVE1* 1413 bp versus *FVE* 1524 bp; *BvLD* 2829 bp versus *LD* 2859 bp; *BvLDL1* 2487 bp versus *LDL1* 2532 bp).

The predicted protein sequences were aligned against the corresponding *A. thaliana* genes (Fig. 1B; Supplementary Fig. S1 at *JXB* online). For *FLK* and *FVE*, homologues had also been identified in rice (Lim *et al.*, 2004; Baek *et al.*, 2008) and were included in the alignment. The overall amino acid sequence identity between the proteins in *A. thaliana* and *B. vulgaris* was highest for FVE (72%), intermediate for FLK (57%) and LDL1 (58%), and relatively low for LD (43%). The domain organization of all four proteins was largely conserved, and the degree of sequence conservation between homologues was highest within domains. In particular, all domains in BvFLK (three KH-type RNA-binding domains, with 77–88% amino acid identity to the corresponding domains in the *A. thaliana* homologue) and in BvFVE1 were highly conserved [a chromatin assembly factor 1 subunit C (CAF1c) domain with 96% amino acid identity, and six WD40 repeat domains with 83–95% amino acid identity]. In contrast, in BvFLK, BvFVE1, and BvLDL1, the N-terminal protein regions are only slightly conserved. Furthermore, 50 amino acids at the N-terminus of FVE including a putative nuclear localization signal (amino acids 20–30; Ausin *et al.*, 2004) are absent in BvFVE1.

Genetic map positions

The genes were mapped on a single nucleotide polymorphism (SNP)-based genetic reference map of expressed genes in sugar beet (Schneider *et al.*, 2007). *BvFLK* carries a SNP in the 3′ untranslated region (UTR) and was mapped to chromosome IV, where it colocalizes with marker TG_0502b at a cumulative genetic distance of 45.7 cM (Fig. 2). *BvFVE1* carries three SNPs within a 1457 bp fragment of intron 4 and was mapped to a position at the very distal end of chromosome VII. This map position is consistent with the presence of several sequences on the BAC clone that carries *BvFVE1*, which show homology to *Apa*I and *Rsa*I satellite sequences known to be located in subtelomeric regions in sugar beet (Dechyeva and Schmidt, 2006), including several subtelomeric repeats located just upstream of *BvFVE1*. The two ESTs corresponding to *BvLD* had been mapped previously to chromosome VII by Schneider *et al.* (2007) and are represented by markers TG_E0240 (BQ589018) and TG_E0226 (BQ594506; Fig. 2). *BvLDL1* was mapped to position 10.90 cM on chromosome IX (data not shown).

BvFLK *accelerates the time to bolting in* Arabidopsis *and complements the* flk-1 *mutation*

Overexpression and complementation analyses in *A. thaliana* wild-type and mutant plants were employed to assess whether the function of autonomous pathway gene homologues is conserved between beet and *Arabidopsis*. *BvFLK* and *BvFVE1* were chosen as homologues of autonomous pathway genes which, respectively, are putative RNA regulatory genes or exert their function at the level of chromatin.

The coding sequence of *BvFLK* driven by the CaMV 35S promoter ('35S::*BvFLK*'), the *BvFLK* coding sequence driven by the 1435 bp genomic region upstream of the *BvFLK* coding sequence in beet, which will be referred to as the endogenous *BvFLK* promoter ('endo::*BvFLK*'), and the coding sequence of *FLK* under the control of the CaMV 35S promoter ('35S::*AtFLK*') were transformed into *A. thaliana* Col-0 and the *flk* mutant SALK_112850 [Alonso *et al.*, 2003; corresponding to *flk-1* in Lim *et al.* (2004) and to *flk-4* in Mockler *et al.* (2004)]. Under long-day conditions, bolting in the *flk-1* mutant was found to be delayed by 31–33 d when compared with Col-0, the genetic background of the mutant (Table 2).

Selection with BASTA and PCR analysis of transgene integration identified 9–32 primary (T_1) transformants derived from transformation of the *BvFLK* transgene cassettes into Col-0 or the *flk-1* mutant, and 10 and 4 transformants, respectively, derived from transformation of the 35S::*AtFLK* transgene cassette. Bolting time in Col-0 plants transformed with either of the transgene cassettes was ~5–6 d earlier than in Col-0 control plants, and this effect did not differ significantly between the three sets of transformants (Table 2). The total number of leaves at bolting was only slightly reduced in the transformants, but differed significantly from the Col-0 control plants in the

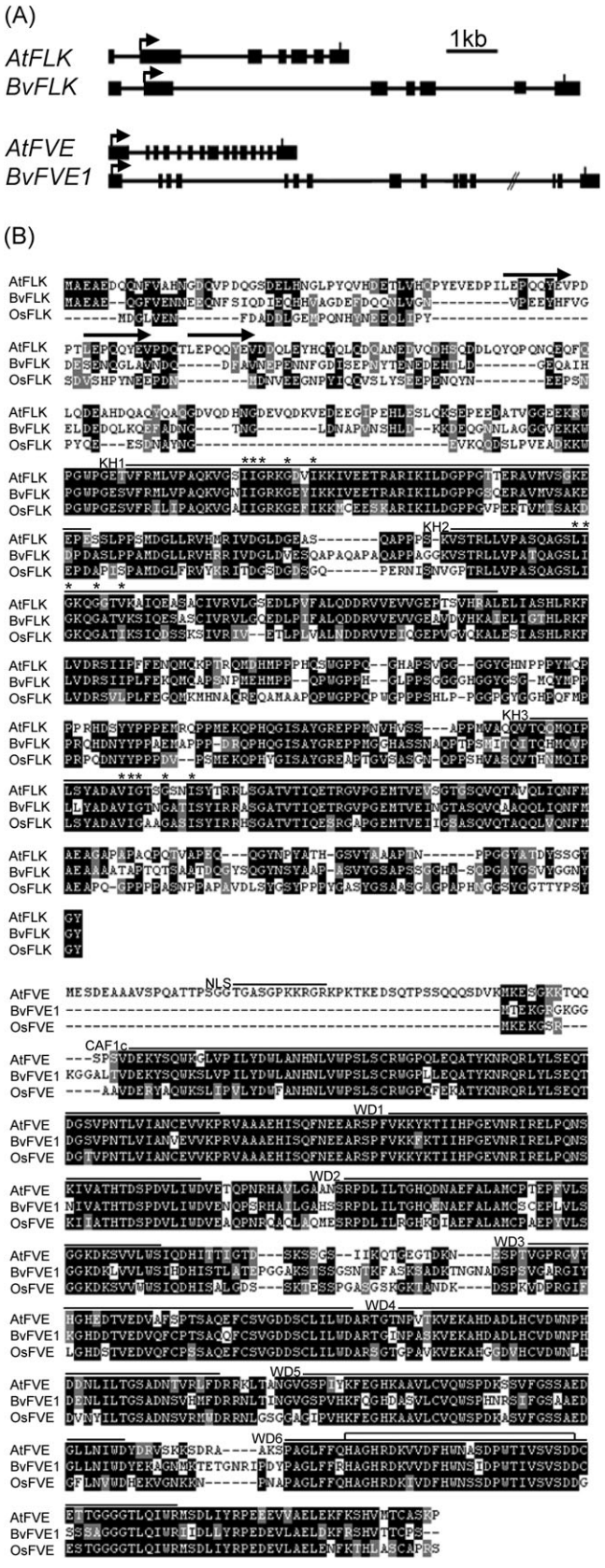

Fig. 1. Sequence and structure of the autonomous pathway gene homologues *BvFLK* and *BvFVE1*. (A) Exon–intron structure of *BvFLK*, *BvFVE1*, and the respective *A. thaliana* genes (*FLK*, accession number AAX51268; *FVE*, accession number AF498101). Exons are indicated as black rectangles, and the position of start and stop codons is indicated by arrows and vertical bars, respectively. (B) Pairwise sequence alignments and domain organization. The alignments were generated using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Identical and similar residues are highlighted by black or grey boxes, respectively. The position of protein domains according to Pfam 22.0 (http://pfam.sanger.ac.uk/) is marked by horizontal lines above the alignment. In *FLK*, the positions of three perfect eight-residue repeats and the core residues of K-homology RNA-binding (KH) domains (Mockler *et al.*, 2004) are indicated by arrows and asterisks, respectively. The first and sixth WD40 repeat domains (WD1 and WD6) were not identified by Pfam and were annotated according to Ausin *et al.* (2004). A putative nuclear localization signal (NLS; black line) in *FVE* according to Ausin *et al.* (2004) and a potential zinc-binding site (unfilled box) in WD6 (Kenzior and Folk, 1998) are also indicated. WD, WD40 repeat domain; CAF1c, CAF1 subunit C/histone-binding protein RBBP4 domain.

35S::*BvFLK* and endo::*BvFLK* transgenic plants. Expression of the transgene cassettes in the *flk-1* mutant background fully rescued the phenotype in regard to both bolting time and numbers of leaves at bolting. All three sets of transformants bolted ~33–36 d earlier than the *flk-1* mutant.

Nine to 10 T_1 plants of each of the sets of transformants carrying the 35S::*BvFLK* or endo::*BvFLK* transgene cassettes in either the Col-0 or *flk-1* mutant background, and all 35S::*AtFLK* transformants were selfed to produce T_2 seed. In the T_2 families derived from transformation into Col-0, plants which bolted ⩾5 d earlier than the mean of the Col-0 control plants (37.88±1.50 d to bolting) were initially considered as candidates for transgenic segregants. According to this criterion, 3–9 of the T_2 families from each of the three sets of transformants exhibited segregation of the number of early-bolting plants (24–33 d to bolting) to the number of plants which bolted within the time range observed for the control plants (35–40 d to bolting) of 13:4 to 16:1, which did not deviate significantly from the 3:1 or 15:1 ratios expected for one to two transgene loci (as tested by χ^2 analysis; Supplementary Table S2 at *JXB* online). In the remaining families, all plants bolted ⩾5 d earlier than the control plants. The T_2 families derived from transformation into the *flk-1* mutant consisted of plants which bolted either as late as the *flk-1* mutant controls (63–77 d to bolting, with a mean and SD of 69.82±4.63), or much earlier (25–44 d to bolting). In 1–3 of the T_2 families from each of the three sets of transformants, early- and late-bolting plants segregated at ratios as above, while the remaining families only contained early-bolting plants.

From all six sets of transformants, one T_2 family each which segregated for early and late bolting (at a ratio which did not deviate significantly from 3:1) was selected for co-segregation analysis of the early-bolting phenotype and the transgene. As expected, all plants which bolted early were found to be transgenic, whereas none of the remaining plants tested positive for the transgene (Supplementary Table S2 at *JXB* online). ANOVA of days to bolting and

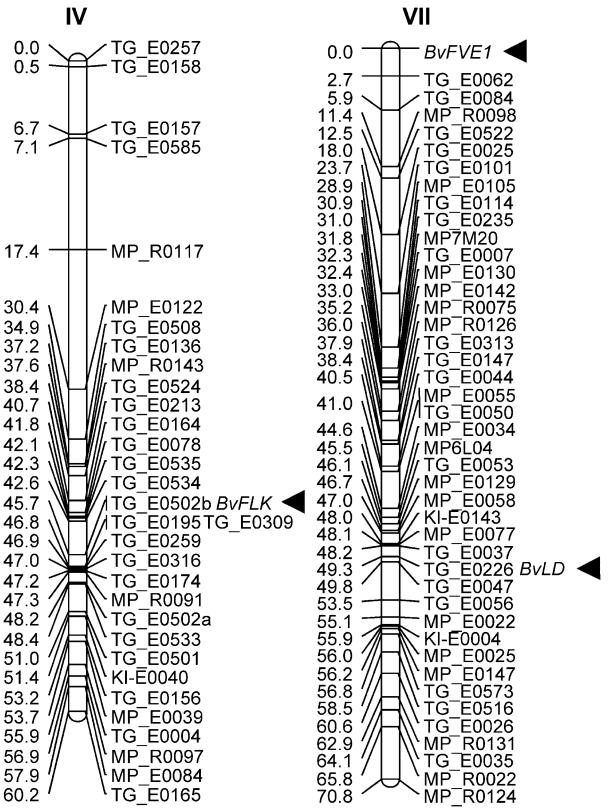

Fig. 2. Genetic map positions. *BvFLK* and *BvFVE1* (arrowheads) were mapped to position 45.7 cM on chromosome IV and the top end of chromosome VII, respectively, on a reference map of the sugar beet genome (Schneider *et al.*, 2007). The map position of an EST which had been mapped previously (Schneider *et al.*, 2007) and corresponds to *BvLD* is also indicated. Genetic distances in centiMorgans (cM) are given on the left, and marker names on the right.

the number of leaves at bolting revealed highly significant differences for both traits between the transgenic subfamilies in T_2 and the respective untransformed control plants (Table 2). Similarly, *t*-tests between the transgenic and non-transgenic individuals within a T_2 family showed highly significant differences for both traits in all six families (Supplementary Table S3 at *JXB* online). For transgenic plants in the *flk-1* mutant background, the values for neither of the traits differed significantly from those for Col-0, thus confirming that the transgenes fully rescue the phenotype (Table 2; Fig. 3A).

BvFLK *represses* FLC *expression in* Arabidopsis

To investigate further the functional conservation of *BvFLK* and *FLK*, it was tested whether the effect of *BvFLK* on bolting time in transgenic *A. thaliana* plants is mediated through *FLC*. Consistent with previous results obtained with different *flk* mutants (Lim *et al.*, 2004; Mockler *et al.*, 2004), *FLC* expression was significantly higher in the *flk-1* mutant than in Col-0 (Fig. 3B). In contrast, *FLC* expression levels in *flk-1* plants carrying the *BvFLK* transgene driven by either the 35S promoter or its endogenous promoter were strongly reduced compared with the untransformed mutant, and resembled the low expression of *FLC* in Col-0. *FLC* expression was also down-regulated in *flk-1* plants expressing the 35S::*AtFLK* transgene, although expression was somewhat less reduced than in *BvFLK* transgenic plants or the Col-0 wild type. RT-qPCR of *FLK* confirmed the previous finding that *FLK* is not detectably expressed in *flk-1* (Lim *et al.*, 2004), and showed further that expression of *FLK* in the 35S::*AtFLK* transgenic plants which were assayed for *FLC* expression was lower than in Col-0 wild type (Fig. 3C).

BvFVE1 *does not complement an* fve *mutation in* Arabidopsis

To assess the possible role of *BvFVE1* in regulating floral transition, the coding sequence of *BvFVE1* driven by the CaMV 35S promoter ('35S::*BvFVE1*') was transformed into *A. thaliana* Col-0 and the *fve* mutant SALK_013789 (Alonso *et al.*, 2003; *fve-7*). Bolting under long-day conditions in *fve-7* was found to be delayed by 29–34 d when compared with Col-0 (Supplementary Table S4 at *JXB* online, and data not shown). Selection with BASTA and PCR analysis identified 16 and 27 transgenic events in the Col-0 and *fve-7* mutant background, respectively. All primary transformants were phenotyped for initiation of bolting, but showed a similar phenotype to the respective untransformed control plants (data not shown). Ten T_1 plants from each set of transformants were selfed for further analysis in the T_2 generation. Because the phenotypic data for the primary transformants suggested that the transgene may not or may only weakly affect bolting time, BASTA selection was used to identify T_2 families in which the transgene is segregating. For each of the two sets of transformants, one family was identified in which the presence and absence of the transgene segregated at a ratio which did not deviate significantly from 3:1, and one family with a segregation ratio of $\geqslant$15:1. For each of these four families, an additional 25 plants were grown without selection, phenotyped for bolting time, and tested for the presence of the transgene by PCR. However, the time to bolting was generally very similar in the transgenic and the non-transgenic plants, and neither the means of numbers of days to bolting nor the means of numbers of leaves at bolting differed significantly between the two groups within a given family (Supplementary Table S4). In one exception, a T_2 family which carried the 35Spro::*BvFVE1* transgene in the *fve-7* mutant background and only contained transgenic plants among 21 plants tested (T_2 family #32) bolted ~6 d earlier (59.95±7.26 d to bolting) than control plants (65.75±4.33 d to bolting; Supplementary Table S4). This difference was statistically significant by conventional criteria, but only marginally so at a *P*-value of 0.04. There was no significant difference between the means of total numbers of leaves at bolting between the two groups. The intactness of the 35S::*BvFVE1* transgene cassette and transgene expression was confirmed by PCR amplification and sequencing of the complete promoter and coding sequence

Table 2. Number of days to bolting (DTB) and total number of leaves at bolting (TNL) of primary transformants (T_1 generation) and transgenic plants in segregating T_2 populations derived from transformation of *BvFLK* and *FLK* into *A. thaliana* Col-0 and the *flk* mutant SALK_112850 (*flk-1*)

	Overexpression in Col-0			Complementation in *flk-1*		
Genotype	**No. of plants**	**DTB (mean± SD)**	**TNL (mean ±SD)**	**No. of plants**	**DTB (mean ±SD)**	**TNL (mean ±SD)**
T_1 generation						
35S::*BvFLK*	32	29.59±2.43 a	10.59±1.83 a	25	32.00±2.10 a	13.08±1.55 a
endo::*BvFLK*	15	30.00±2.56 a	11.20±1.52 a	9	34.89±3.79 a	13.33±2.29 a
35S::*AtFLK*	10	30.90±2.08 a	12.00±1.94 b	4	31.50±4.20 a	10.57±1.26 a
Col-0	16	35.69±1.70 b	13.31±1.14 b	16	35.69±1.70 a	13.31±1.14 a
flk-1 mutant	–	–	–	22	67.71±7.15 b	66.35±1.32 b
F-value (probability)		27.45 (0.00)	10.03 (0.00)		383.00 (0.00)	3397.00 (0.00)
$LSD_{0.05}$[a]		2.14	1.46		4.42	2.05
T_2 generation						
35S::*BvFLK*	13	27.77±2.59 a	15.23±1.79 a	14	34.14±3.06 a	15.43±1.16 a
endo::*BvFLK*	13	28.46±1.66 a	15.31±1.71 a	12	33.92±3.62 a	15.51±1.29 a
35S::*AtFLK*	14	27.23±2.05 a	15.00±2.00 a	15	34.13±5.60 a	15.80±1.08 a
Col-0	17	37.88±1.50 b	17.53±1.01 b	17	37.88±1.50 a	17.53 ±1.01 a
*flk-1*mutant	–	–	–	17	69.82±4.63 b	67.82±2.01 b
F-value (probability)		95.38 (0.00)	8.34 (0.00)		222.44 (0.00)	2307.00 (0.00)
$LSD_{0.05}$[a]		1.85	1.41		4.06	1.86

[a] Fisher's least significant difference at α=0.05. The letters a and b indicate significant differences between the mean values given in a table column (i.e. mean values in table cells including the letter 'b' are significantly different from the mean values in table cells including the letter 'a')

of the transgene, and by RT-PCR, respectively (data not shown).

Transcript accumulation of BvFVE1*, but not* BvFLK*, is under circadian clock control*

Expression of *BvFLK* and *BvFVE1* in sugar beet was analysed in four major plant organs, root, stem, leaf, and flower, of adult plants. Both genes were found to be expressed in all samples analysed. *BvFVE1* is relatively abundant in leaves and only slightly expressed in roots, whereas *BvFLK* is relatively highly expressed in roots and flowers (Fig. 4A). Because various autonomous pathway genes in *Arabidopsis* have been implicated in the regulation of the circadian clock (Salathia *et al.*, 2006; see Introduction), and many regulators are subject to feedback regulation by the clock (Pruneda-Paz and Kay, 2010), diurnal and circadian regulation of *BvFLK* and *BvFVE1* expression was investigated by RT-qPCR of transcripts in leaves from 8- to 10-week-old plants (Fig. 4B). Under long-day (16 h light) conditions, *BvFLK* transcript levels fluctuated during the course of the day and appeared to be highest at 4 h of the

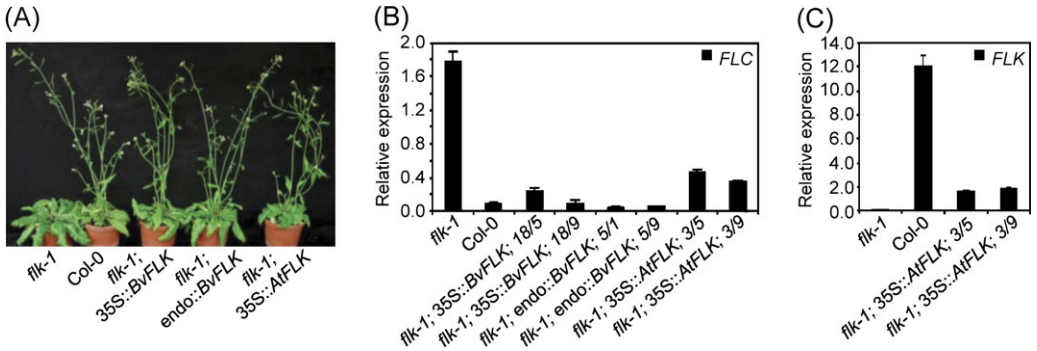

Fig. 3. *BvFLK* complements the *A. thaliana flk-1* mutant. (A) Phenotypes at 51 d after sowing of the *A. thaliana flk-1* mutant, the ecotype Col-0, and the *flk-1* mutant transformed with *BvFLK* driven by the CaMV 35S promoter (35S::*BvFLK*), *BvFLK* driven by the endogenous promoter of *BvFLK* in sugar beet (endo::*BvFLK*), or *A. thaliana FLK* driven by the CaMV 35S promoter (35S::*AtFLK*) (T_1 plants). Plants were grown under long-day conditions. (B) RT-qPCR expression analysis of *FLC* in *flk-1*, Col-0, and transgenic T_3 plants carrying the 35S::*BvFLK*, endo::*BvFLK*, or 35S::*AtFLK* transgene in the *flk-1* mutant background. For each of the transgenic lines, two T_3 plants were tested that were derived from different transgenic individuals of a T_2 family. (C) RT-qPCR expression analysis of *FLK* in *flk-1*, Col-0, and transgenic 35S::*AtFLK T3* plants. Expression levels in B and C were normalized against *GAPDH* and measured in triplicate. Error bars indicate the standard deviations of the mean.

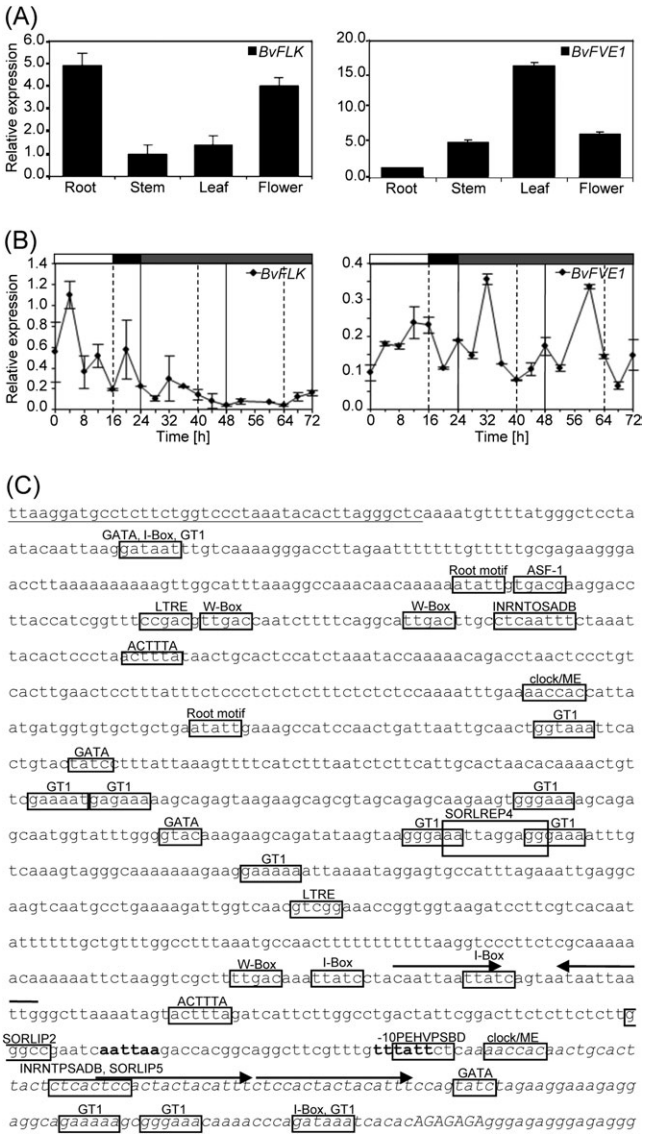

Fig. 4. Expression of *BvFLK* and *BvFVE1* in *B. vulgaris*. (A) Expression across major plant organs in the biennial genotype A906001. Plants were vernalized and grown under long-day conditions. RT-qPCR expression levels were normalized against *BvGAPDH* and measured in triplicate. (B) Diurnal and circadian RT-qPCR expression profiles. Relative expression levels in leaves are shown for a 24 h period under long-day conditions, followed by 48 h under continuous low light at a constant temperature of 22 °C. Expression was measured every 4 h. Expression was normalized using *BvGAPDH*, *BvEF2*, and *BvTUB*. Error bars indicate the standard deviations of the mean. (C) *BvFVE1* promoter and 5′ UTR. A total of 1116 bp of the genomic sequence upstream of the start codon are shown. The transcription start site was determined by RACE. The 5′ UTR sequence is printed in italics. Bold letters at positions –9 and –36 (relative to the transcription start site) indicate a TATA-box-like sequence (de Pater *et al.*, 1990) and a TATA-box according to the transcription start site prediction program TSSP (http://www.softberry.ru/berry.phtml), respectively. A GA repeat motif (Santi *et al.*, 2003) in the 5′ UTR just upstream of the ATG start codon is shown in upper case letters. Putative light- and circadian clock-regulated promoter elements are boxed [SORLIP and SORLREP (Hudson and Quail, 2003), GT1 consensus sequence (Terzaghi and Cashmore, 1995), IBOX core motif (Terzaghi and Cashmore, 1995), GATA box (Gilmartin *et al.*, 1990), INRNTPSADB (Nakamura *et al.*, 2002), –10PEHVPSBD (Thum *et al.*, 2001), and a six nucleotide motif (clock/ME) which is common to a promoter element over-represented in circadian clock-regulated genes and a morning element (Harmer and Kay, 2005)]. Arrows above the sequence denote inverted and tandem repeat units. The 3′ end of a sequence tract with homology to the subtelomeric satellite AM076746 of *B. vulgaris* [clone pAv34-32 (Dechyeva and Schmidt, 2006)] is underlined.

light phase. To investigate circadian clock regulation, long day-entrained plants were moved to continuous light and transcript levels were monitored over the subsequent 48 h. However, *BvFLK* expression did not oscillate and remained low throughout this period. *BvFVE1* expression, under long-day conditions, rises to a peak at 12 h, decreases during the first 4 h of darkness, and rises again at the end of the night. In sharp contrast to *BvFLK*, *BvFVE1* expression continued to oscillate robustly throughout each 24 h period under continuous light, although peak amplitude was higher than during the entrainment phase. A promoter sequence analysis for both genes identified a region ~0.5–1 kb upstream of the transcription start site of *BvFLK* in which multiple root motifs (Elmayan and Tepfer, 1995) and phytohormone-response elements, including four cytokinin-response elements (Fusada *et al.*, 2005), are clustered, whereas consensus sequences for light-regulated elements appear to be relatively scarce (Supplementary Fig. S2 at *JXB* online). Sequence elements involved in circadian regulation were not identified. In contrast, the *BvFVE1* promoter contains a number of elements which have been implicated in light or circadian regulation, including two SORLIPs (sequences over-represented in light-induced promoters), one SORLREP (sequence over-represented in light-repressed promoters), 11 GT1 consensus sequence motifs (Gilmartin *et al.*, 1990; Zhou, 1999; Hudson and Quail, 2003), and a –10 promoter element (–10PEHVPSPD) from a circadian clock-regulated gene in barley (Thum *et al.*, 2001) (Fig. 4C).

Discussion

The key regulatory genes of the vernalization and photoperiod pathways in *Arabidopsis*, *FLC* and *CO*, had been reported previously to have functionally related homologues in beet (Reeves *et al.*, 2007; Chia *et al.*, 2008). In the present study the question was addressed of whether the genes of the third major regulatory pathway of flowering time control in *Arabidopsis* are conserved between the two species. Putative *B. vulgaris* orthologues of four autonomous pathway genes in *Arabidopsis*, *FLK*, *FVE*, *LD*, and *LDL1*, were identified and genetically mapped. Three of these genes (*BvFVE1*, *BvLD*, and *BvLDL1*) are homologous

to autonomous pathway genes which are thought to regulate *FLC* expression by chromatin modification, whereas the fourth gene (*BvFLK*) encodes a putative RNA-binding protein. The beet genes are highly similar to their *Arabidopsis* counterparts in terms of exon–intron structure and domain organization. With the exception of *BvLD*, the degree of overall sequence conservation with *Arabidopsis* is similar to (*BvFLK* and *BvLDL1*) or higher than (*BvFVE1*) that of the only two previously characterized flowering time genes in beet, *BvFL1* and *BvCOL1* (Reeves *et al.*, 2007; Chia *et al.*, 2008). For another gene of the autonomous pathway, *FPA*, a sugar beet EST with only moderate homology was identified (Table 1). However, ~3 kb of the genomic sequence of the corresponding gene (*BvFPA*) was sequenced and found to include the coding region for three RRM-type RNA-binding domains also present in *FPA*, which substantiated that *BvFPA* and *FPA* are likely orthologues (data not shown). For the remaining three classical autonomous pathway genes, *FCA*, *FY*, and *FLD*, orthologous ESTs were not identified. The currently available EST and transcript sequence collection for *B. vulgaris*, however, only represents 17 184 genes (BvGI 3.0, release date June 16, 2010; http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=beet), which is approximately one-half to two-thirds of the total number of genes in beet (Herwig *et al.*, 2002). Orthologues of *FCA* and *FY* with partially conserved functions (Lee *et al.*, 2005; Lu *et al.*, 2006; Jang *et al.*, 2009) and a gene with high homology to *FLD* (Lu *et al.*, 2006) have been identified in rice, suggesting that homologues may also be present in beet. Together, these sequence data and observations indicate that at least some autonomous pathway genes are conserved in beet.

Two genes were chosen, *BvFLK* and *BvFVE1*, whose homologues in *Arabidopsis* are thought to regulate *FLC* expression either through an RNA-based control mechanism (*FLK*) or by chromatin modification (*FVE*), to test the hypothesis that at least some of the autonomous pathway gene homologues are also functionally conserved. Among the genes identified here, these two genes also showed the highest degree of sequence conservation within conserved domains. Transgenic expression of *BvFLK* from both the constitutive CaMV 35S promoter and the endogenous promoter of *BvFLK* was found to accelerate bolting in *A. thaliana* and, in an *flk* mutant background, to fully rescue the phenotype to the bolting time of wild-type plants. *FLC* expression in transgenic plants carrying the *BvFLK* transgene was strongly reduced compared with untransformed controls, suggesting that the effect of *BvFLK* on bolting time is mediated through repression of *FLC*. In both 35S::*BvFLK* and endo::*BvFLK* transgenic plants, *FLC* expression is similarly low as in Col-0 wild-type plants, which indicates that all regulatory protein domains required for regulation of *FLC* expression are functionally conserved between *FLK* and *BvFLK*. At the sequence level, this is consistent with the high degree of homology within the KH-type RNA-binding domains and the strict conservation between *Arabidopsis* and beet of the core consensus sequence of KH domains (Mockler *et al.*, 2004; see asterisks in Fig. 1B). The low sequence conservation outside the KH domains, in particular in the N-terminal region of the proteins [which in FLK contains three perfect eight-residue repeats of unknown function (Mockler *et al.*, 2004) which are not conserved in BvFLK], further suggests that this region is less critical for *FLK* function. The *B. vulgaris* homologue of *FLC*, *BvFL1*, complements *FLC* function in *A. thaliana flc* mutants (Reeves *et al.*, 2007), suggesting that *BvFLK* may also regulate *BvFL1*. However, the regulation of vernalization requirement and response appears to have diverged considerably during evolution (Colasanti and Coneva, 2009; Distelfeld *et al.*, 2009; Greenup *et al.*, 2009; Jung and Müller, 2009), and possible interactions between *BvFLK* and *BvFL1* in *B. vulgaris* have yet to be experimentally verified. Nevertheless, the complementation data for both *BvFLK* in this study and *BvFL1* in the study of Reeves *et al.* (2007) suggest that regulatory interactions between these genes contribute to flowering time control in beet.

The present data also show for the first time, to our knowledge, that transgenic expression of the endogenous *A. thaliana* gene complements an *flk* mutant. The fact that full phenotypic complementation was achieved by expression from the CaMV 35S promoter suggests that developmental or environmental regulation of *FLK* transcription is not a precondition for the gene's function in flowering time control. Interestingly, *FLC* expression in 35S::*AtFLK* transgenic plants was strongly reduced but was not as low as in *BvFLK* transformants or wild-type controls. Incomplete repression of *FLC* correlated to some extent with the relatively low expression of *FLK* in 35S::*AtFLK* plants compared with the wild type. Because the early bolting phenotype of the wild type was fully restored in the transformants, the plant appears to tolerate moderately elevated expression levels of *FLC* without a significant delay in bolting.

Expression of *BvFLK* across major plant organs of sugar beet was strongest in roots and flowers, but was also clearly detectable in leaves and stems, and resembled the relative expression levels of *FLK* in *A. thaliana* (Lim *et al.*, 2004). While expression in aerial parts of the plant may well be associated with a functional role in flowering time control, the high expression level in roots, which was also observed for *FLK* in *Arabidopsis*, may reflect an additional, unknown function of *BvFLK* (as has also been suggested for *FLK*; Lim *et al.*, 2004). *FLK* was implicated in RNA-directed chromatin silencing of retroelements (Bäurle and Dean, 2008; Veley and Michaels, 2008). Although speculative, it is conceivable that *BvFLK* is involved in repression of other targets such as, for example, developmental genes which may not be required at certain developmental stages (e.g. after floral transition, and/or in certain organs such as roots). The clustering of putative phytohormone response elements in the promoter of *BvFLK* may further suggest hormonal regulation of *BvFLK* activity. Finally, *BvFLK* does not appear to be under circadian clock control. Microarray data for *A. thaliana* indicate that expression of *FLK* is not circadian regulated either (Edwards *et al.*, 2006; http://affymetrix.arabidopsis.info/narrays/experimentbrowse.

pl, accession number NASCARRAYS-108). Together, the data suggest that *FLK* and *BvFLK* are functionally related regulators of floral transition, and provide the first evidence for evolutionary conservation of *FLK* function outside the model species *A. thaliana*. In the apparent absence of an *FLC* orthologue in rice, sequence conservation in rice of the same regions which are conserved between *A. thaliana* and *B. vulgaris* may support the notion that *FLK* also has additional functions. Consistent with this possibility, putative orthologues of *FLK* are present in non-angiosperm species including the gymnosperm *Piceasitchensis* and the lycophyte *Selaginella moellendorffii* (Supplementary Fig. S3A at *JXB* online). For the rice orthologue of another RNA regulatory autonomous pathway gene, *OsFCA*, the possibility of other functions has been raised by detection of various protein–protein interactions (Lee *et al.*, 2005; Lu *et al.*, 2006; Jang *et al.*, 2009).

In contrast to *BvFLK*, transgenic expression of *BvFVE1* did not complement the bolting time phenotype of an *A. thaliana fve* mutant. This result differs from data reported by Baek *et al.* (2008) for CaMV 35S promoter-driven expression of a rice homologue, *OsFVE* (Fig. 1B), which was shown to at least partially rescue the flowering time phenotype of an *fve* mutant in *Arabidopsis* in ~29% of independent primary transformants. So what are the reasons for the apparent absence of functional conservation between *Arabidopsis* and beet? The degree of sequence conservation between *FVE* and *BvFVE1* (72% amino acid identity) is very similar to that between *FVE* and *OsFVE* (Baek *et al.*, 2008), and *BvFVE1* and *OsFVE* are also highly similar to each other (71% amino acid identity). The degree of sequence conservation with *Arabidopsis* is also higher than that for *BvFLK*, *BvFL1*, and *BvCOL1*, and much higher than for other autonomous pathway genes in rice (*OsFCA* and *OsFY*) with at least partially conserved functions (Lee *et al.*, 2005; Lu *et al.*, 2006). Thus, the degree of overall sequence conservation alone does not appear to be a good indicator of functional conservation, and may simply be a consequence of the large proportion of amino acid residues within functional or structural domains, as the CAF1c domain and the WD40 repeat domains thought to be required for the formation of a β-propeller structure (Kenzior and Folk, 1998; Murzina *et al.*, 2008) make up most of the protein. The regions outside the conserved domains vary considerably between *FVE* homologues. In particular, an N-terminal region which is present in *FVE* is missing in both *BvFVE1* and *OsFVE*. The finding that *OsFVE* at least partially complements *FVE* function suggests that this region is not absolutely required and, by extrapolation, its absence in *BvFVE1* cannot be the reason for the lack of functional complementation. Other regions with low sequence conservation are located between the WD40 repeat domains 2 and 3, and between the WD40 repeat domains 5 and 6. In the human *FVE* homologue RbAp46, the latter region carries a negatively charged loop which contributes to recognition of histone H4 and is not present in other WD40 β-propeller structures (Murzina *et al.*, 2008). It seems possible that this region determines binding specificity and/or the type of interaction with other proteins. For structural proteins such as *FVE* which are vitally involved in protein complex formation, or formation of various protein complexes with different functions, as suggested by Amasino (2004), selection of binding partners is likely to be a crucial determinant of protein function. In addition, specific protein–protein interactions depend on co-evolution of binding sites. It is thus conceivable that the binding specificity determinants of *FVE* and *BvFVE1* have diverged sufficiently to prevent wild-type-like interactions between *BvFVE1* and interacting proteins in *A. thaliana*. If the absence of functional complementation of *FVE* by *BvFVE1* is indeed a result of divergent evolution of protein-binding sites, as suggested above, the possibility cannot formally be excluded that BvFVE1 still exerts a similar function in beet to that of FVE in *Arabidopsis*, for example as part of a co-evolved protein complex.

The *B. vulgaris* genome appears to carry a second gene, represented by EST EG550040 (Table 1), which may be a paralogue of *BvFVE1* and will be referred to as *BvFVE2*. In the region represented by the EST, *BvFVE2* shares a degree of homology to *FVE* similar to that of *BvFVE1* (with the exception of the C-terminal region of the putative partial translation product; see Supplementary Fig. S4 at *JXB* online). Interestingly, the valine and lysine residues located in the variable region between WD40 repeat domains 5 and 6 and conserved between *FVE* and *OsFVE* are also conserved in *BvFVE2* (Supplementary Fig. S4 at *JXB* Online). Although Baek *et al.* (2008) alluded to the presence of a second rice sequence with homology to *FVE* (OsJ_003110), this sequence was removed from GenBank, and no other close rice homologue of *FVE* in GenBank was identified. However, several other species were found to carry putative paralogous pairs (or groups) of *FVE* homologues, notably including two species in the Malpighiales order of dicotyledonous angiosperms (*Populus trichocarpa*, *PtFVE1–PtFVE3*, and *Ricinus communis*, *RcFVE1 and RcFVE2*; Supplementary Fig. S3B, C at *JXB* Online), which (like *B. vulgaris*) are also only distantly related to the Brassicales. Thus, it is conceivable that a gene duplication event occurred relatively early in the course of dicot evolution, possibly followed by gene loss in some lineages (including the lineage to *Arabidopsis*), and that the two paralogues underwent subfunctionalization.

The present expression data may provide further indications for subfunctionalization of *BvFVE1*. First, *BvFVE1* appears to be most strongly expressed in leaves of adult plants, whereas the data for *FVE* indicate highest expression in flowers (Ausin *et al.*, 2004). Secondly, *BvFVE1* is diurnally regulated and under circadian control, a finding which appears consistent with the presence of multiple putative light-regulatable promoter elements. It is worth noting that *BvFVE1* also has an unusual map position close to the telomere and immediately adjacent to subtelomeric repeats, which may have contributed to the evolution of *cis* regulation of *BvFVE1* expression. Although *FVE* was shown to affect circadian period length (Salathia *et al.*, 2006), the gene itself (or any of the other classical

autonomous pathway genes) has not been reported to be under control of the circadian clock. Furthermore, the microarray data of Edwards *et al.* (2006) indicate that *FVE* is not circadian regulated, as determined after entrainment under intermediate daylength conditions (12 h light/dark cycles). Interestingly, the small glycine-rich RNA-binding protein GRP7 in *Arabidopsis* which has long been known to be under circadian clock control was recently assigned to the autonomous pathway (Streitner *et al.*, 2008). Like *FVE*, this protein has also been implicated in various other biological processes including cold stress response (Carpenter *et al.*, 1994; Heintzen *et al.*, 1994). Circadian regulation of autonomous pathway genes or homologues may indicate a certain level of cross-talk between different floral regulatory pathways upstream of the floral integrator *FT*, as has been observed for *FLC* and the circadian clock (Edwards *et al.*, 2006; Salathia *et al.*, 2006; Spensley *et al.*, 2009). A comparison with the circadian-regulated, putative photoperiod pathway gene *BvCOL1*, which was analysed for circadian oscillations in the same plant samples as *BvFVE1* (Chia *et al.*, 2008), shows that *BvFVE1* and *BvCOL1* have roughly complementary expression profiles, possibly suggesting regulation by different circadian clock output pathways and/or opposing regulatory roles. Alternatively, circadian clock regulation of *BvFVE1* and *GRP7* may reflect the broader involvement of these genes in other biological processes (e.g. cold stress response; Harmer *et al.*, 2000; Fowler *et al.*, 2005; Franklin and Whitelam, 2007).

The present survey of autonomous pathway genes provides the first evidence for evolutionary conservation of homologues in beet as well as divergence and differential regulation of one gene. The results have further implications because (i) functional conservation of autonomous pathway genes outside *Arabidopsis*, with the exception of a few reports for monocots, has not yet been studied in detail; (ii) *B. vulgaris*, among dicot plants, is only a very distant relative of the model species *A. thaliana*, and belongs to a eudicot clade which is little understood at the molecular level; and (iii) the environmental requirements for floral transition differ markedly between beet and model plants, in particular *Arabidopsis* and rice. Thus, the findings for *B. vulgaris* expand the spectrum of evolutionarily diverse species for which molecular data are available and which are subject to differential environmental regulation of bolting and flowering. Finally, for sugar beet and other root and leaf crops, bolting and flowering are undesirable because they drastically reduce yield. The identification and genetic mapping of floral promoters provides tangible targets for marker-assisted or transgenic approaches towards crop improvement.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Sequence and structure of the autonomous pathway gene homologues *BvLD* and *BvLDL1*.

Figure S2. *BvFLK* promoter and 5' UTR.

Figure S3. Phylogenetic analysis of *FLK* (A) and *FVE* (B, C).

Figure S4. Multiple sequence alignment including *A. thaliana* FVE (AtFVE), BvFVE1, the putative translation product of the largest open reading frame in the *B. vulgaris* EST EG550040 (BvFVE2), and OsFVE.

Table S1. Primer sequences.

Table S2. Days to bolting and total number of leaves at bolting in 53 T_2 families derived from transformation with *BvFLK* or *FLK* and non-transformed controls (Col-0 and *flk-1*).

Table S3. Unpaired *t*-test for number of days to bolting (DTB) and total number of leaves at bolting (TNL) between *BvFLK* or *FLK* transformants and non-transgenic *A. thaliana* plants within six T_2 populations.

Table S4. Unpaired *t*-test for number of days to bolting (DTB) and total number of leaves at bolting (TNL) between *BvFVE1* transformants and non-transgenic *A. thaliana* plants within four T_2 populations.

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