Journal of Experimental Botany, Vol. 59, No. 10, pp. 2735–2748, 2008
doi:10.1093/jxb/ern129 Advance Access publication 20 May, 2008
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Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Sugar beet contains a large *CONSTANS-LIKE* gene family including a *CO* homologue that is independent of the early-bolting (*B*) gene locus

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Received 15 February 2008; Revised 8 April 2008; Accepted 10 April 2008

Abstract

Floral transition in the obligate long-day (LD) plant sugar beet (*Beta vulgaris* ssp. *vulgaris*) is tightly linked to the *B* gene, a dominant early-bolting quantitative trait locus, the expression of which is positively regulated by LD photoperiod. Thus, photoperiod regulators like *CONSTANS* (*CO*) and *CONSTANS-LIKE* (*COL*) genes identified in many LD and short-day (SD)-responsive plants have long been considered constituents and/or candidates for the *B* gene. Until now, the photoperiod response pathway of sugar beet (a *Caryophyllid*), diverged from the *Rosids* and *Asterids* has not been identified. Here, evidence supporting the existence of a *COL* gene family is provided and the presence of Group I, II, and III *COL* genes in sugar beet, as characterized by different zinc-finger (B-box) and CCT (*CO*, *CO*-like, *TOC*) domains is demonstrated. *BvCOL1* is identified as a close-homologue of Group 1a (*AtCO, AtCOL1, AtCOL2*) *COL* genes, hence a good candidate for flowering time control and it is shown that it maps to chromosome II but distant from the *B* gene locus. The late-flowering phenotype of *A. thaliana co-2* mutants was rescued by over-expression of *BvCOL1* thereby suggesting functional equivalence with *AtCO*, and it is shown that *BvCOL1* interacts appropriately with the endogenous downstream genes, *AtFT* and *AtSOC1* in the transgenic plants. Curiously, *BvCOL1* has a dawn-phased diurnal pattern of transcription, mimicking that of *AtCOL1* and *AtCOL2* while contrasting with *AtCO*. Taken together, these data suggest that *BvCOL1* plays an important role in the photoperiod response of sugar beet.

Key words: *Arabidopsis*, *Beta vulgaris*, *B* gene, *Caryophyllids*, *CONSTANS-LIKE* gene, flowering time.

Introduction

Improved knowledge of how floral induction pathways operate in crop plants is vitally important for both agronomy and quality. In sugar beet, *Beta vulgaris* ssp *vulgaris*, photothermal induction (long day photoperiods and exposure to cold temperatures) is required for reproductive growth (Owen *et al*., 1940). The transition is signified by rapid elongation of the stem (bolting) and is known to be tightly linked to the dominant early-bolting (*B*) gene locus (Munerati, 1931), although the underlying mechanisms are complex and poorly understood. Breeders still largely depend on phenotypic rather than genotypic selection and the development of high yielding cultivars has largely been achieved through the selection of late bolting genotypes (biennials) that are considered 'recessive' at the *B* gene locus (*bb*) and have an obligate requirement for vernalization in order to bolt. That these plants are still able to bolt indicates that the *bb* genotype might not represent a loss-of-function mutation. Expression of the bolting habit in both annual (*BB*, *Bb*) and biennial (*bb*) genotypes is positively regulated by long days (LD) (Bell and Bauer, 1942; Owen, 1954; Abe *et al*., 1997), thus photoperiod genes are considered to interact directly or indirectly with the *B* gene and may include candidates for the bolting gene itself. Furthermore, Abe *et al*. (1997) postulated that bolting is regulated by a gene complex that is formed by the *B* gene and a closely linked gene for LD requirement. Although there is a physiological and temporal distinction between bolting and flowering in

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sugar beet, such that plants can bolt without flowering, for example, under SDs (Fife and Price, 1953), bolting time is frequently used as a measure of flowering time because of the high correlation between induction of bolting and flowering. Hence, the important need for a molecular genetic dissection of potential *B* gene candidates in order to understand flowering time control in sugar beet.

The ability to perceive changes in daylength is dependent on a range of factors including light perception and photoperiodic regulation through the circadian clock. Variation in regulatory gene activation and transcript accumulation in different plant species ultimately results in the release of output signals that trigger flowering under different photoperiods. Regulatory genes in the photoperiod pathway were initially identified by screening for reversion to a late-flowering phenotype under long days (LD) in the rapid-cycling accessions of *A. thaliana* (Koornneef *et al*., 1991). *GIGANTEA* (*GI*) and *CONSTANS* (*CO*) were attributed to the molecular hierarchy of the long day (LD) response with *GI* activating transcription of *CO* which, in turn, positively affects transcription of two floral integrators, *FLOWERING LOCUS T* (*FT*) and the MADS-box gene *SUPPRESSION OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) (Putterill *et al*., 1995; Park *et al*., 1999; Suarez-Lopez *et al*., 2001). The external coincidence model demonstrates the promotive effects of *CO* on *FT* expression and is typified by a distinctive pattern of oscillation for *CO* transcripts (Yanovsky and Kay, 2003; Hayama and Coupland, 2004; Valverde *et al*., 2004). *CO* homologues discovered thus far also exhibit a *CO*-like pattern of transcript accumulation which suggests that a similar mechanism of transcriptional and post-transcriptional regulation also exists in the photoperiod pathway of other plant species (Liu *et al*., 2001; Izawa *et al*., 2002; Nemoto *et al*., 2003; Turner *et al*., 2005). Functional equivalents of *AtCO* homologues have been demonstrated (Robert *et al*., 1998; Yano *et al*., 2000; Griffiths *et al*., 2003; Nemoto *et al*., 2003) and *A. thaliana*-like photoperiodic response pathways have been identified in other plant species through complementation studies (Hayama *et al*., 2002; Izawa *et al*., 2002; Kojima *et al*., 2002).

The downstream effects of CO activation are determined by two highly conserved domains on the protein. Two adjacent basic domains known as B-boxes at the amino-terminus bear similarities to the DNA-binding zinc-finger motifs of GATA-type transcription factors (Ramain *et al*., 1993; Tsuzuki *et al*., 2000) and may be involved in binding to the promoters of *FT* and *SOC1* (Samach *et al*., 2000; Hepworth *et al*., 2002). In *A. thaliana*, mutations within these zinc-finger domains have resulted in a late-flowering phenotype (Redei, 1962; Putterill *et al*., 1995; Robson *et al*., 2001). The second conserved domain that is characteristic of CO proteins is located at the carboxyl terminus of CO. This CCT (*CO*, *CO*-like, *TOC*) domain contains about 45 amino acids with a nuclear localization signal present within the second half of the motif (Strayer *et al*., 2000; Robson *et al*., 2001). CCT domains are also common to other proteins including the circadian oscillator, TOC1 (TIMING OF CHLOROPHYLL A/B BINDING PROTEIN1) (Strayer *et al*., 2000) and may be important for mediating protein–protein interactions (Kurup *et al*., 2000; Yamashino *et al*., 2003). Based on domain structure and sequence information, genes encoding these characteristic motifs have been identified in almost all angiosperms. Extensive database searches and phylogenetic analyses have revealed up to 17 *CO*-like (*COL*) gene members in *A. thaliana*, 16 in rice, nine in barley, and three in wheat (Griffiths *et al*., 2003; Nemoto *et al*., 2003). Members from each of the *COL* multi-gene families can be loosely classified into four different groups (Group I to IV) based on conserved residues within domain structures (Robson *et al*., 2001). Functional studies on individual *COL* genes in *A. thaliana* have not been completed, although limited evidence exists for the function of *AtCOL1*, *AtCOL2, AtCOL3*, and *AtCOL9*. Cheng and Wang (2005) discovered that *AtCOL9* (a Group III gene) represses *AtCO* transcription and hence delays flowering through indirect effects on the abundance of *AtFT* mRNA. It has also been reported that, despite about 67% identity to the AtCO protein, manipulation of transcriptional activity of the Group I genes *AtCOL1* and *AtCOL2* had no effect on flowering time (Ledger *et al*., 2001) whilst mutation in *AtCOL3* (a Group I gene) had a significant effect on light signalling and root growth (Datta *et al*., 2006).

Understanding the photoperiodic response in sugar beet presents a new challenge not only because of the physiological complexity of the reproductive transition in beet but also because, unlike *A. thaliana* which is able to flower under both LD and SD (a facultative LD plant), sugar beet has an obligate LD requirement for flowering (Bell and Bauer, 1942). However, sequence information and functional analysis of sugar beet genes involved in flowering time as opposed to bolting time are limited and only just emerging. This includes a concerted effort towards obtaining sequence information for the *B* gene locus (Gaafar *et al*., 2005) and a systematic examination of candidate genes for relationships to *B*. Recently, an *FLC*-like gene, *BvFL1* was isolated by Reeves *et al*. (2007) and was mapped to a different chromosome to the *B* gene. *BvFL1* is alternatively spliced and showed classic responses to vernalization although epigenetic repression was not observed. One variant, *BvFL1-v3* was found to delay flowering time significantly in complementation assays in *A. thaliana* (*Atflc)* mutants, but targets of this repression in sugar beet have not been demonstrated. Here, we have started to dissect the photoperiod pathway and describe the identification of a *COL* gene family in sugar beet. It is shown that one of the genes, *BvCOL1*

resembles Group I *AtCO*/*AtCOL* genes and is a putative homologue of *AtCO* and that it up-regulates transcription of *AtFT* and *AtSOC1* in an *A. thaliana co-2* mutant background. It is also shown that *BvCOL1* does not map to the *B* locus.

Materials and methods

Plant material

Three sugar beet genotypes were used; (i) breeding line C600 (Lewellen, 1989), with an early bolting (annual) habit; (ii) commercial cultivar, Roberta (KWS Saatzucht GmbH, Einbeck, Germany) and (iii) breeding line NF (SESvanderHave, Tiennen, Belgium) which are both late bolting biennials. The annual and biennial habits were attributed to non-obligate and obligate requirements for vernalization, respectively. *Arabidopsis thaliana* wild-type ecotype Landsberg *erecta* (L*er-0*) was obtained from the Nottingham Arabidopsis Stock Centre (NASC ID: NW20, http://arabidopsis.info/). *co-2* mutant plants (Putterill *et al*., 1995) were a kind gift from Professor George Coupland (Max Planck Institute, Cologne, Germany).

Growth conditions and flowering time measurements

Sugar beet was grown in controlled growth chambers at 22 °C with a 16 h LD photoperiod. Photosynthetically active radiation (PAR) with a fluence rate of 220 µmol photons m^{-2} s^{-1} was provided by 125 W white fluorescent lamps (GE Lighting) and 40 W tungsten bulbs (Phillips) for 15 h. Plants were further illuminated for 1 h with tungsten light to enrich far-red light and prevent etiolation. Bolting and flowering were induced by imposing vernalizing conditions (8 weeks in annuals and 18 weeks in biennials) at 6 °C and under continuous low lights (10 µmol m^{-2} s^{-1}). Vernalized plants were thermally buffered at 15 °C for 1 week to prevent de-vernalization before being returned to 22 °C and a 16 h photoperiod as described above. Plants initiated bolting within 2–3 weeks and flowered within 6 weeks of vernalization. *Arabidopsis thaliana* was grown under defined light regimes (LDs of 16 h and SDs of 8 h) in Sanyo Gallenkamp MLR 350 growth chambers at 20 °C. Lighting was supplied by 36 W fluorescent Daystar lamps (CEC Technology) providing 280 µmol m^{-2} s^{-1} of PAR. All leaves (vegetative and cauline leaves excluding young cotyledons) were counted at the first visible sign of budding as a measurement of flowering time.

Phylogenetic analysis

Sequences for *BvCOL1* and *CO*-like family members were identified by BLAST searches of the Genbank database, the *Beta vulgaris* Gene Index, BvGI 1.0 (http://compbio.dfci.harvard.edu/tgi/), and the Sugar Beet EST Database at Michigan State University (http://genomics.msu.edu/sugarbeet/index.html). Sequences were aligned using the Clustal W-based alignment tool in Vector NTI, version 10.0.1 (Invitrogen). Computation of phylogenetic relationships by the Maximum Parsimony (MP) algorithm and bootstrap analysis was carried out using PHYLIP version 3.67 (Felsenstein, 1989).

Southern analysis

Total genomic DNA was extracted according to Smith *et al.* (1990). Aliquots (10 µg) of restriction enzyme digested DNA were analysed by Southern blot hybridization according to standard methods (Sambrook and Russell, 2001). *BvCOL1* gene-specific probes were generated from a 787 bp fragment corresponding to the zinc-finger domains or from 470 bp of the inter-domain (middle) region of the cDNA. *BvCO*-like gene family-specific probes were generated from a 395 bp fragment corresponding to the CCT domain. Transgene-specific probes were generated either from the *CaMV 35S* promoter or *BvCOL1* cDNA sequences.

RNA extraction and quantitative RT-PCR

Total RNA was extracted from at least three individual plants using Plant RNAeasy Kit™ (Qiagen) and DNase treated. Two micrograms of RNA was reverse transcribed using AffinityScript (Stratagene) and diluted 5-fold for QPCR. Primers for QPCR were designed (see Supplementary Table S1 at *JXB* online) and optimized to 98–110% amplification efficiency with products verified by sequencing. Fluorescence of bound SYBR-GREEN (Stratagene) was detected in the MX3000 real-time PCR machine (Stratagene) over 40 cycles at 60 °C annealing temperature. *BvCOL1* transcription levels were measured in triplicate and normalized against *Bv*Tubulin (Karetsou *et al*., 2005), a sugar beet alpha-tubulin gene with low variation in Ct values (18–19 cycles) for all tissues tested. QPCR in *A. thaliana* was normalized against *AtUBC* (At5g25760) (Czechowski *et al*., 2005).

Isolation of BvCOL1 *sequences and generation of transgenic* A. thaliana *plants*

Fragments of 1104 bp (cDNA) and 1771 bp (genomic DNA) were cloned using primers based on TC1427 (see Supplementary Table S2 at *JXB* online) and verified by custom sequencing (John Innes Centre Genome Laboratory, Norwich, UK). *BvCOL1* promoter sequences were obtained by screening sugar beet BAC library filters (McGrath *et al*., 2004) and sequencing positive clones. To create transgenic plants overexpressing *BvCOL1*, the cDNA (1104 bp) was inserted into pEarleyGate 100 (Earley *et al*., 2006), mobilized into *Agrobacterium tumefaciens* strain GV2260 and used to transform the *A. thaliana* late-flowering *co-2* mutant by the floral dip method (Clough and Bent, 1998).

Selection of transgenic A. thaliana *plants*

T_1 progenies of *Agrobacterium*-dipped plants were selected by spraying 2-week-old plants with 0.3 mM glufosinate ammonium (Finale; supplied by Aventis Environmental Science, Essex, UK). Selfed T_2 seeds and progenies were scored for herbicide resistance by painting rosette leaves of 2-week-old plants with a 0.3 mM Finale solution. Leaf necrosis was indicative of herbicide sensitivity and evident after four days whilst resistant plants remained healthy. The presence and expression of *BvCOL1* in resistant lines were verified by PCR and RT-PCR, respectively. Homozygous T_3 seeds were sown together with the wild type (L*er*) and the late-flowering *co-2* mutant under LD and SD conditions. Flowering time was recorded from a minimum of 20 individual plants for each T_3 population and their controls. Statistical analysis of flowering time data was carried out by two-way ANOVA.

Genetic mapping of BvCOL1

To map *BvCOL1* on a genetic map of sugar beet, two parents (K1P1 and K1P2) and an F_1 plant (K1F1) of the F_2 mapping population K1 (kindly provided by Dr B Schulz, KWS Saat AG, Einbeck, Germany) (Mohring *et al*., 2004; Schneider *et al*., 2007) were screened for polymorphisms by PCR amplification of genomic DNA and subsequent sequencing using an ABI PRISM™ 3700 DNA Analyzer. Primers A242 and A245 (see Supplementary Table S2 at *JXB* online) are located approximately 0.6 kb upstream and 0.1 kb downstream of the start codon, respectively. The PCR fragments of 97 F_2 individuals of the mapping population were

sequenced using primer A242 followed by co-dominant scoring of the electropherograms at the sites of four single nucleotide polymorphism (SNP) markers. Marker scores were added to the genotypic data for 305 SNP and RFLP markers in K1 that were generated previously by Schneider *et al*. (2007). The map position of *BvCOL1* was calculated using the Kosambi mapping function (Kosambi, 1944) in JoinMap® 3.0 (Van Ooijen and Voorrips, 2001), as described by Schneider *et al*. (2007).

BvCOL *sequence accession numbers*

Three full-length *BvCOL* genomic sequences cloned from biennial sugar beet, cultivar Roberta were deposited into GenBank. Accession numbers are EU437782 (*BvCOL1*), EU437783 (*BvCOL2*), and EU437784 (*BvCOL3*).

Results

A CO*-like (*COL*) multi-gene family in sugar beet*

Currently, about 26 887 ESTs for *Beta vulgaris* are available through the dbEST division of Genbank. Stringent cluster analyses have further aligned these ESTs into longer sequences thereby allowing 'Tentative Consensuses' (TC) to be annotated and assigned putative functions (Quackenbush *et al*., 2000). From the collection of ESTs, 13 618 unigenes or TCs may be identified and are presently available through the *Beta vulgaris* Gene Index, BvGI 1.0 (http://compbio.dfci.harvard.edu/tgi/). *BvCOL* genes were identified by first searching this database and confirming the results by querying the sugar beet EST database (http://genomics.msu.edu/sugarbeet/index.html). A TBLASTN search with AtCO and CO-like protein sequences resulted in 13 hits with Expect (E) values <0.5 (Table 1). The nucleotide sequences were first translated to identify conserved domain residues that typify *COL* genes (B-box and CCT) and then compared by alignment against AtCO and AtCOL proteins and homologues from other plant species (Fig. 1A, B).

Three transcripts (TC1427, TC879, TC2903) were found to encode putative full-length coding sequences with predicted B-box and CCT domains (Table 1; Fig. 1A, B). These have been designated *BvCOL1*, *BvCOL2*, and *BvCOL3*, respectively. Seven other EST/TCs appear to be truncated at the 3′ end but encode distinguishable B-boxes. Of these putative transcripts, four (Cn3561, TC1812, BQ593762, and BQ584310) contained B-box 1 and 2. However, incomplete 3′ (BQ593762) and 5′ (BQ584310) sequences meant that only one complete and one partial B-box could be identified reliably for two of these proteins. The three other ESTs (TC3738, BQ591888, BQ594583) contained only a single B-box. Three remaining EST/TCs (BQ582975, TC163, CV301775) were truncated at the 5′ end and encoded only a characteristic CCT domain. To verify expression of all 13 sequences in sugar beet vegetative leaf tissues, RT-PCR (see Supplementary Table S2 at *JXB* online) was carried out and the resultant cDNA fragments were cloned and confirmed by sequencing (data not shown).

The encoded B-boxes have a high level of identity (up to 82%) with the classic zinc-finger motif commonly found in COL proteins (Omichinski *et al*., 1993; Putterill *et al*., 1995) and hence it is predicted that these truncated ESTs are likely to derive from *CO*-like genes in sugar beet. However, zinc binding motifs also exist in other plant proteins (Torok and Etkin, 2001) such as salt tolerance (STO) and salt tolerance homologue (STH)

Table 1. *Beta vulgaris CONSTANS-like (*BvCOL*) expressed sequence tag/tentative consensus (EST/TC) sequences*

Gene sequences were identified by TBLASTN of BvGI 1.0 (http://compbio.dfci.harvard.edu/tgi/) and the sugar beet EST database (http://genomics.msu.edu/sugarbeet/index.html) using AtCO, AtCOL1 to AtCOL16 protein sequences. The presence of CO-like domains is denoted by B1 (B-box 1), B2 (B-box 2) and CCT. Accession numbers with a 'TC' (Tentative Consensus), 'BQ' and 'CV' prefix were obtained from BvGI, and those with a 'Cn' prefix were obtained from the sugar beet EST database at MSU. The E-values given in the table refer to similarity to AtCO.

	Name	ESTs in BvGI 1.0	TCs in BvGI 1.0	ESTs in Bv database at MSU	Domains	E-value
1	**BvCOL1**	BI543628, BI543722 BI543724, BQ488270 BQ588630, BQ589119	**TC1427**	Cn2349	B1, B2, CCT	1.1e-41
2	**BvCOL2**	BQ583937, BQ588069	**TC879**	Cn2600	B1, B2, CCT	4.4e-45
3	**BvCOL3**	BQ487825, BQ487842, BQ583909, BQ583972	**TC2903**	Cn3652	B1, CCT	2.2e-10
4		BQ584191, BQ584479		**Cn3561**	B1, B2	9.5e-22
5		BQ489825, BQ589815 BQ591614	**TC1812**	Cn1811	B1, B2	1.5e-16
6		**BQ593762**			B1, B2 partial	1.9e-13
7		**BQ584310**			B1 partial, B2	7.8e-05
8		BQ489587, BQ489817	**TC3738**	Cn3977	B1	5.0e-11
9		**BQ591888**		Cn1730	B1	5.1e-09
10		**BQ594583**			B1	5.7e-08
11		**BQ582975**			CCT	0.035
12		BQ588535, BQ589202 BQ589272, BQ589370	**TC163**	Cn2315	CCT	0.46
13				**CV301775**	CCT	0.49

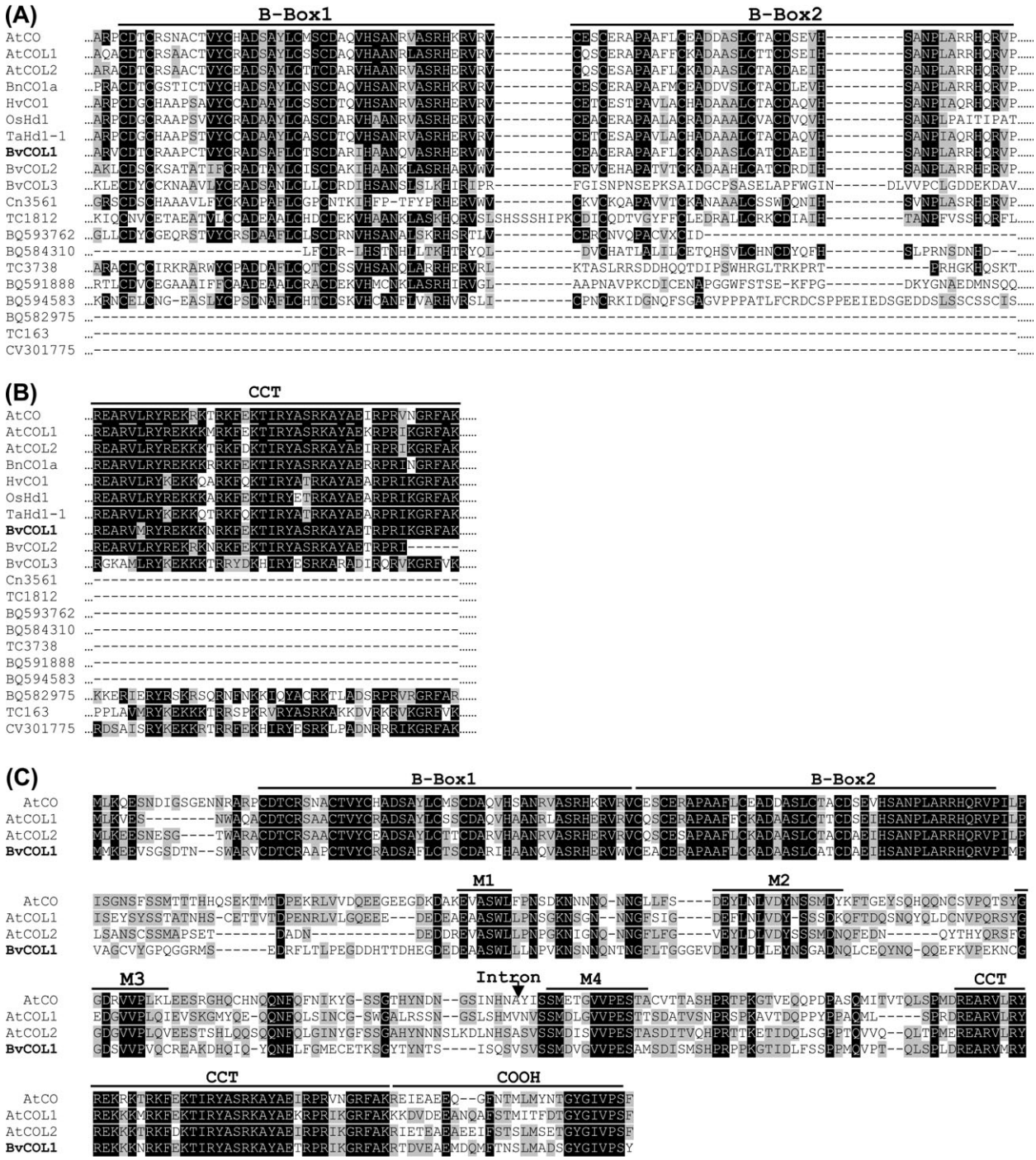

Fig. 1. Multiple sequence alignment of putative CO-like EST/TC protein sequences in sugar beet. Comparison of putative sugar beet CO-like EST/TCs against Group Ia (AtCO/AtCOL1/AtCOL2) proteins of *A. thaliana* and CO/Hd1 homologues for (A) only the B-box domains of 10 EST/TCs and (B) only the CCT domain of six EST/TCs. (C) Full-length protein sequence of BvCOL1 aligned against Group Ia proteins of *A. thaliana*. The position of the intron within the BvCOL1 sequence is indicated by an arrow. Characteristic domains of CO-like proteins are marked by lines over sequences. Identical and similar residues are highlighted in black and grey, respectively. Accessions with a 'TC' or 'BQ' prefix are obtained from BvGI 1.0. Other accession numbers are *AtCO* (At5g15840; NM_121589) (Putterill *et al*., 1995), *AtCOL1* (At5g15850; AY065001) (Putterill *et al*., 1995), *AtCOL2* (At3g02380; NM_111105) (Ledger *et al*., 1996), *BnCOa1* (AY280868) (Robert *et al*., 1998), *OsHd1* (AB041838) (Yano *et al*., 2000), *HvCO1* (AF490469) (Griffiths *et al*., 2003), *TaHd1-1* (AB094490) (Nemoto *et al*., 2003).

(Lippuner *et al*., 1996; Song *et al*., 1998; Holm *et al*., 2001). Similarly, ESTs with predicted CCT domains are equally likely also to belong to genes that are not part of the *COL* family, such as *TOC1* (Strayer *et al*., 2000), *CHLOROPLAST IMPORT APPARATUS* (*CIA2*) (Sun *et al*., 2001), and other 'unknown/hypothetical' proteins.

To identify true *COL* gene members and to understand the relationship of the 13 sugar beet *CO*-like genes, estimation of genetic distance by maximum parsimony (MP) was carried out on B-box and CCT domain nucleotides only. *CO*/*Hd1* homologues (Group I: B-box 1, B-box 2), *AtCOL6* (Group II: B-box 1), *AtCOL9* (Group III: B-box 1, diverged B-box 2), and genes encoding B-box and CCT domains that are outside the *COL* gene family were included in the phylogenetic analysis of sugar beet genes (Fig. 2).

The 10 sugar beet EST/TCs encoding B-boxes fell into four clades/groups that are consistent with the predicted number of zinc-finger domains. *BvCOL1*, *BvCOL2*, and Cn3561 clustered with Group I *CO*-like/*Hd1* genes with two B-boxes. The remaining EST/TCs with two B-boxes (BQ584310 and BQ593762) clustered with *AtCOL9*, a Group III gene with a diverged B-Box 2. Three EST/TCs (*BvCOL3,* BQ594583, TC3738) encoding only a single B-box were allocated as Group II genes together with *AtCOL6*. Interestingly, the phylogenetic tree placed two ESTs (BQ591888 and TC1812) in a cluster with the salt tolerance genes, *AtSTO and AtSTH*. TC1812 is 60% and BQ591888 is 45% identical to *AtSTO* at the nucleotide level and hence could be related to this class of genes. Analysis of the six sugar beet EST/TCs encoding CCT domains placed *BvCOL1* and *BvCOL2* in the same cluster as Group I *CO*/*Hd1* genes. Three ESTs/TCs (CV301775, *BvCOL3*, and TC163) were clustered with *AtCOL9*, a Group III gene. However, one EST (BQ582975) showed 57% identity with *AtTOC1* and is hence likely not to belong to the *CONSTANS* gene family. MP analysis of corresponding protein sequences also consistently allocated each EST/TCs within the same major clusters (data not shown). The presence of a sugar beet multi-gene family was supported further by Southern analysis using a fragment corresponding to the CCT domain of *BvCOL1*. At low stringency, three hybridized bands were present in both biennial and annual sugar beet genotypes suggesting that there are at least three Group I *COL* genes (Fig. 3A). This concurs with the above phylogentic cluster analysis of existing sugar beet ESTs.

BvCOL1 shares common motifs with A. thaliana *Group I CO and CO-like proteins*

A single locus of *BvCOL1* is present in both annual and biennial genotypes (Fig. 3B) and has highest homology to Group I *CO*/*Hd1* genes compared to the other *CO*-like ESTs in sugar beet. An alignment of BvCOL1 protein sequence (Fig. 1C) against Group I CO-like proteins of *A. thaliana* revealed that the closest similarities were to Group I, sub-group Ia, proteins (Griffiths *et al*., 2003). AtCO, AtCOL1, and AtCOL2 are characteristic sub-group Ia proteins due to the presence of four conserved motifs (M1 to M4) in the more diverse inter-domain (middle) region of the peptides. BvCOL1 is highly conserved at these motifs with the exception of M2 where it diverges from the M2a motif (L-V-D-Y) of sub-group Ia proteins. Another variant of the M2 motif (M2c) has been reported

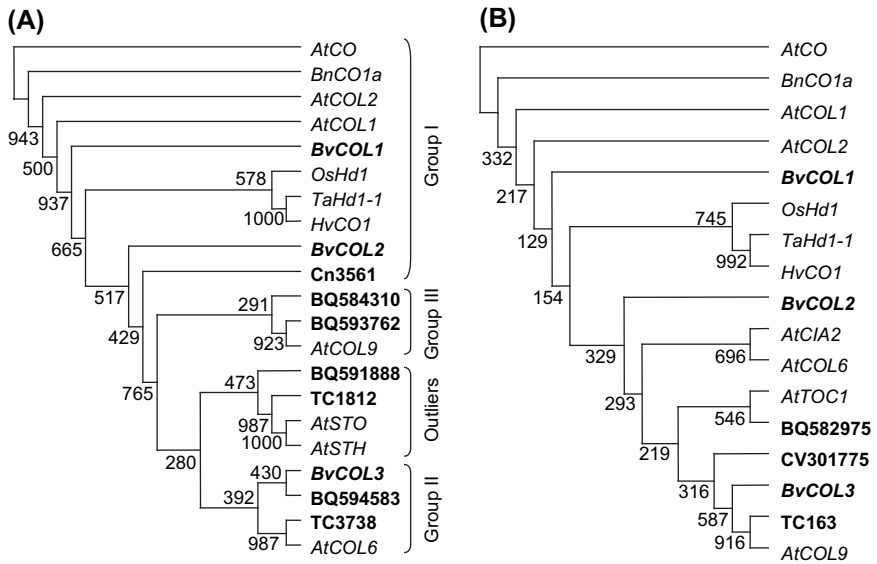

Fig. 2. Phylogenetic analysis of sugar beet *CO*-like genes by maximum parsimony (MP). (A) MP tree of *CO*-like DNA sequences encoding only the B-box domains and two other zinc-binding genes that are not part of the CONSTANS family from *A. thaliana* (*AtSTO*, At1g06040 and *AtSTH*, At2g31380). (B) MP tree of *CO*-like DNA sequences encoding only the CCT domain and two other CCT-containing genes that are not part of the CONSTANS family from *A. thaliana* (*AtTOC1*, At5g61380 and *AtCIA2,* At4g25990). Bootstrap values from 1000 replicates were used to assess robustness of the tree. Group I genes are represented by *CO*/*Hd1* homologues from different species (see Fig. 1 legend for accession numbers.). *AtCOL6* (At1g68520; NM_105523) and *AtCOL9* (At3g07650; NM_111644) (Cheng and Wang, 2005) were included as representatives of Group II and Group III genes, respectively.

in *Physcomitrella patens* ssp. *patens PpCOL* genes (Zobell *et al*., 2005). Comparison of sequence identity at both nucleotide and protein level further revealed that *BvCOL1* is more similar to *AtCOL1* (66% and 59%) and *AtCOL2* (67% and 62%) than *AtCO* (64% and 55%) (data not shown).

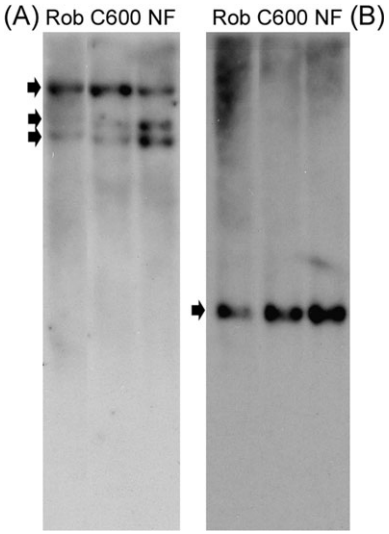

Fig. 3. DNA gel blot analysis of *CO*-like (*COL*) gene copies in sugar beet. Genomic DNA from two biennial (Roberta and NF) and one annual variety of sugar beet (C600) was digested with *Hin*dIII and probed with fragments derived from *BvCOL1* (TC1427). (A) 395 bp fragment corresponding to CCT domain under low stringency, and (B) 787 bp fragment corresponding to B-box 1 and 2 domains under high stringency.

The genomic region spanning the *BvCOL1* coding sequence was amplified and found to contain two exons of similar sizes to Group Ia genes in *A. thaliana* (746 bp and 358 bp) and an intron located between the M3 and M4 motifs as in the Group I *COL* genes (Griffiths *et al*., 2003; Zobell *et al*., 2005). To investigate *cis*-regulatory elements of *BvCOL1*, upstream promoter sequences were isolated by using a short fragment comprising the B-box 1 domain to screen a sugar beet BAC library (McGrath *et al*., 2004) under high stringency. Six positive BACs were identified (consistent with the expected library size), and a 600 bp fragment upstream of the initiation start codon was isolated (Fig. 4A). Multiple sequence alignment with promoters of *A. thaliana* Group Ia *CO*-like genes revealed 44% nucleotide conservation to *AtCO*, 45% to *AtCOL1* and 50% to *AtCOL2* (data not shown). *Cis*-regulatory DNA sequence motifs that are commonly found in circadian- and/or light-regulated genes in *A. thaliana* were also identified: G-box (Martinez-Garcia *et al*., 2000; Michael and McClung, 2003), I-box (Terzaghi and Cashmore, 1995), and SORLIP (‘sequences over-represented in light-induced promoters’) 1 and 2 (Hudson and Quail, 2003). The region containing the three SORLIP 1 motifs and the I-box is largely conserved in *AtCOL1*, *AtCOL2* and, to a lesser extent, *AtCO* (data not shown). CCAAT boxes recently implicated in regulation of flowering (Wenkel *et al*., 2006) and a 57 bp

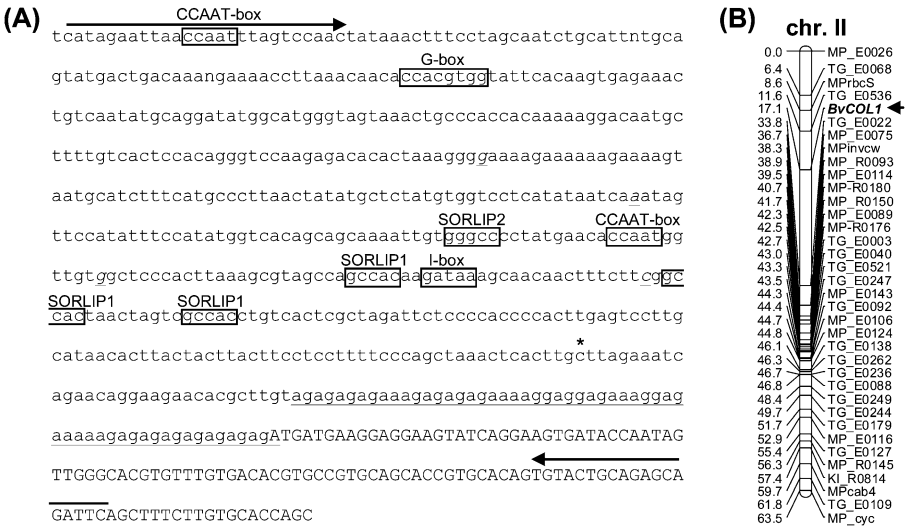

Fig. 4. *BvCOL1* promoter/5′ UTR sequence and genetic map position. (A) *BvCOL1* promoter/5′ UTR sequence. The 600 bp sequence upstream of the ATG start codon is shown in lower case letters. The positions of four single nucleotide polymorphisms in the K1 mapping population that were used for genetic mapping are underlined and in italics. The positions of primers used for SNP detection are indicated by arrows above the sequence. The asterisk indicates the 5′ end of the sequence corresponding to EST TC1427. *Cis*-regulatory DNA sequence motifs that are commonly found in circadian- and/or light-regulated genes are indicated as G-box (Martinez-Garcia *et al*., 2000; Michael and McClung, 2003), I-box (Terzaghi and Cashmore, 1995), SORLIP1 and 2 (Hudson and Quail, 2003) and CCAAT boxes (Wenkel *et al*., 2006). A 57 bp GA-rich tract that includes several GAGA sites and a GA octodinucleotide repeat motif $(GA)_8$, (Santi *et al*., 2003) just upstream of the translation start codon is underlined. (B) Using the four polymorphic sites indicated in (A), the position of *BvCOL1* (arrow) was mapped on a SNP-based genetic map of expressed genes (Schneider *et al*., 2007). *BvCOL1* is located on chromosome II between markers TG_E0536 and TG_E0022.

GA-rich tract that includes several GAGA or GAGAG sites and a GA octodinucleotide repeat motif [$(GA)_8$; (Santi *et al*., 2003)] were also identified upstream of the translation start codon (Fig. 4A).

BvCOL1 *maps to sugar beet chromosome II and is not the bolting gene* B

BvCOL1 was mapped to chromosome II of *B. vulgaris* on a SNP-based genetic map of expressed genes (Schneider *et al*., 2007). PCR amplification yielded a single PCR product and sequencing gave rise to unambiguous sequence electropherograms that revealed four single nucleotide polymorphisms between K1P1 and K1P2 in the genomic region upstream of the start codon (Fig. 4A). At two other positions, the *BvCOL1* BAC sequence differed from K1P1 and K1P2 (Fig. 4A). These two sites are located within the GA-rich simple sequence repeat region upstream of the start codon and are likely to reflect the sites of polymorphisms between the parent plants of the mapping population and the genotype USH20 used for BAC library construction. The *BvCOL1* gene is located between markers TG_E0536 and TG_E0022, at a cumulative genetic distance of 17.1 cM (Fig. 4B). To examine the possibility that *BvCOL1* corresponds to the bolting gene, which was previously also mapped to chromosome II (Boudry *et al*., 1994; El-Mezawy *et al*., 2002), a small subset of the F_2 mapping population that was used by El-Mezawy *et al*. (2002) for genetic linkage between *BvCOL1* and *B* was analysed. Among 20 F_2 plants that are phenotypically and genotypically well characterized for the early bolting character (including phenotypic data for >20 F_3 plants each and extensive molecular marker data), six plants showed recombination between polymorphic sites in *BvCOL1* and the *B* locus (data not shown). This high frequency of recombination indicates that *BvCOL1* is not a constituent of the bolting gene locus.

BvCOL1 *transcription is under circadian regulation*

Diurnal regulation of *BvCOL1* expression was profiled by QRT-PCR in vegetative leaves of sugar beet plants grown under LD and SD photoperiods. Under both light regimes, *BvCOL1* transcript level was highest at the end of the dark period (time 0 h) which is immediately before the beginning of the light period. At dawn, transcript levels dropped rapidly within the first two hours and remained at low or undetectable levels until night occurred (Fig. 5A). In the dark, *BvCOL1* transcription began immediately in LD-grown plants but SD-grown plants only began transcription after 6 h before they both reached a maximum level at the end of the night. This pattern of diurnal regulation closely mimics that of *AtCOL1* and *AtCOL2* (Ledger *et al*., 2001) and not *AtCO* where transcription peaks in the light under LDs and decreases in the dark (Suarez-Lopez *et al*., 2001). Except for the last 8 h in the dark and the first 2 h in the light, there was little difference between *BvCOL1* transcription levels in plants grown under both photoperiods (Fig. 5A). To further determine if *BvCOL1* is regulated by the endogenous circadian clock, plants were entrained under LD before transfer to continuous light where *BvCOL1* transcription was followed for 48 h (Fig. 5B). Continuous oscillation of transcript levels with a 24 h rhythm was maintained but gradual decrease in peak amplitude was observed. Robust cycling of transcripts under a free-running period suggests that *BvCOL1* is indeed under regulation by the circadian clock.

To confirm diurnal and circadian control of gene expression in the same tissues, we followed transcription of a sugar beet EST (TC3208) with homology to *AtTOC1* (Boxall *et al*., 2005). It was demonstrated that this sequence is 76% identical to the amino-terminus signal-receiver domain of *AtTOC1* at the protein level (Fig. 5F) and 70% identical at the nucleotide level. TC3208 mRNA transcripts oscillated over a 24 h period and peaked 4 h before the end of the day (LD) or at the beginning (after 2 h) of the night (SD), respectively (Fig. 5C) as has been observed for *AtTOC1* (Strayer *et al*., 2000). Robustness of clock regulation was also examined under continuous light and maintenance of the rhythmic cycling of this putative *BvTOC1* transcript was consistent with circadian-clock regulated genes albeit at reduced amplitude (Fig. 5D).

BvCOL1 *transcription is developmentally regulated*

The presence of *BvCOL1* transcripts in vegetative leaf, cauline leaf (on elongating reproductive stems), and in fibrous roots of plants at the 10-leaf stage under LD was determined using QRT-PCR. Figure 5E shows that the amount of *BvCOL1* mRNA increased as the plants matured until the 12-leaf stage when we normally consider the plants to have reached the adult phase. Following vernalization-induced bolting (reproductive transition), *BvCOL1* transcription was also found to be high in cauline leaves but low in the first emerging floral bud and in fibrous roots (Fig. 5E). Expression profiles of the remaining *BvCOL* genes (see Supplementary Table S2 at *JXB* online) were also examined in these tissues but only *BvCOL2* (another Group I gene) matched that of *BvCOL1*. By contrast, the salt-tolerant gene homologues, TC1812 and BQ591888 were preferentially expressed in roots compared to vegetative leaves (data not shown).

BvCOL1 *complements the late-flowering phenotype of the* A. thaliana co-2 *mutant*

To assess a possible role of *BvCOL1* in flowering control, the *BvCOL1* cDNA driven by the strong *CaMV 35S* promoter was transformed into the late-flowering *co-2* mutant. Sixteen T_1 herbicide-resistant and transgene positive transformants exhibited a range of advanced

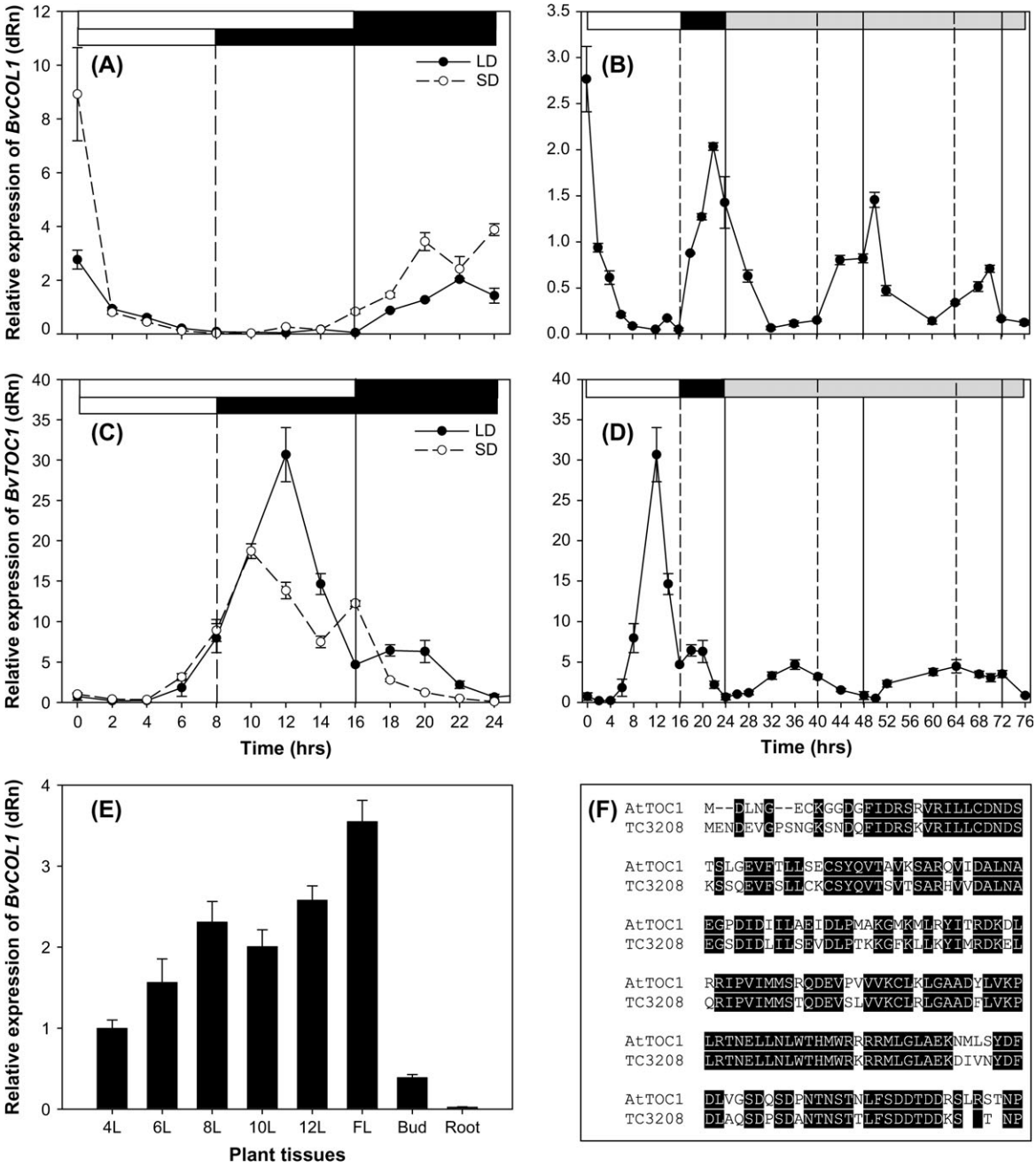

Fig. 5. Expression profiles of *BvCOL1* and a putative *BvTOC1* in biennial sugar beet. Quantified transcription of *BvCOL1* and *BvTOC1* (TC3208) (Boxall *et al*., 2005) by QRT-PCR. *BvCOL1* (A, B) and *BvTOC1* (C, D) transcription in leaf tissues from 1-month-old plants (with eight fully-expanded leaves) over a 24 h period (A, C) and from plants moved to continuous light following entrainment under LDs (B, D). *BvCOL1* transcription in developing leaves (4L to 12L) and in different organs (leaves of elongating stems; FL, floral bud; bud; and roots) sampled in LDs (E). Alignment of protein sequence of *BvTOC1* against *AtTOC1* showing a high degree of homology (76% amino acid identity) in the signal-receiver domain (F). Bars above represent the light (white), dark (black) or subjective light (grey) periods of the day. Solid and dashed lines represent expression levels for plants grown under LD and SD, respectively. Error bars represent standard error of three biological and three technical repetitions.

flowering time phenotypes compared to the *co-2* mutant. Two lines that flowered earliest (*BvCOL1-1*) and latest (*BvCOL1-9*) were selected for further characterization. Following segregation in the T_2 generation, flowering time of homozygous T_3 lines were compared to the *co-2* mutant and wild-type (L*er*) under both LDs (16 h light) and SDs (8 h light). Under our conditions, the vegetative phase of *co-2* mutants was prolonged with an almost 5-fold increase in leaf number before flowering was initiated under both photoperiods compared to L*er* (Table 2). This

vegetative phase is significantly reduced when *BvCOL1* is over-expressed in the mutant background. Under LD, lines *BvCOL1-1* and *BvCOL1-9* flowered significantly earlier than untransformed *co-2* mutants, with flowering time being close to but not completely restored to wild-type numbers ($P < 0.01$) (Table 2; Fig. 6A). However, the acceleration of flowering time by *BvCOL1* was most obvious under non-inductive SD where line *BvCOL1-1* not only flowered earlier than the *co-2* mutant, but was also slightly earlier than wild-type plants grown under the same conditions ($P < 0.05$) (Table 2). By contrast, line *BvCOL1-9* only partially restored flowering time under SD. Southern blot analysis revealed that line *BvCOL1-1* contained two transgene copies but only one copy was found in line *BvCOL1-9* (data not shown). Consistent with this difference in transgene copy number, the level of *BvCOL1* transcript accumulation was found to be two-fold higher in transgenic line *BvCOL1-1* than in line *BvCOL1-9* (Fig. 6B). Thus it is possible that gene dosage and/or positional effects may have contributed to the observed differences in the degree of phenotypic complementation.

Table 2. *Flowering time of transgenic* 35S::BvCOL1 A. thaliana *plants*

Effect of *BvCOL1* driven by the *CaMV 35S* promoter on the flowering time of *A. thaliana co-2* mutants under LD (16/8 h light/dark) and SD (8/16 h light/dark) conditions. Data were collected from at least 20 individual homozygous T_3 plants selected from 16 original transformants and are presented as the mean ±SE.

Long days	Leaf number
L*er-0*	6.6±0.2
co-2	37.3±0.7
BvCOL1-1	8.4±0.3[a]
BvCOL1-9	11.2±0.5[a]
Short days	**Leaf number**
L*er-0*	9.6±0.7
co-2	58.9±1.2
BvCOL1-1	8.5±0.5[a]
BvCOL1-9	23.0±2.8[a]

[a] Student's *t* test showed significant difference ($P < 0.01$).

BvCOL1 *up-regulates* AtFT *and* AtSOC1 *transcription*

The effect of *BvCOL1* transcription in transgenic *A. thaliana* lines was investigated by QRT-PCR using tissues from plants grown under inductive LD. In the untransformed *co-2* mutant, endogenous *AtFT* transcription was undetectable compared with basal levels in the wild type (L*er*). By comparison, high transgene expression observed in line *BvCOL1-1* correlated with a significantly higher up-regulation of *AtFT* expression compared to the lower expresser, *BvCOL1-9*. Furthermore, *AtFT* transcripts detected in line *BvCOL1-1* exceeded levels detected in the wild-type (L*er*) background (Fig 6C). In addition to

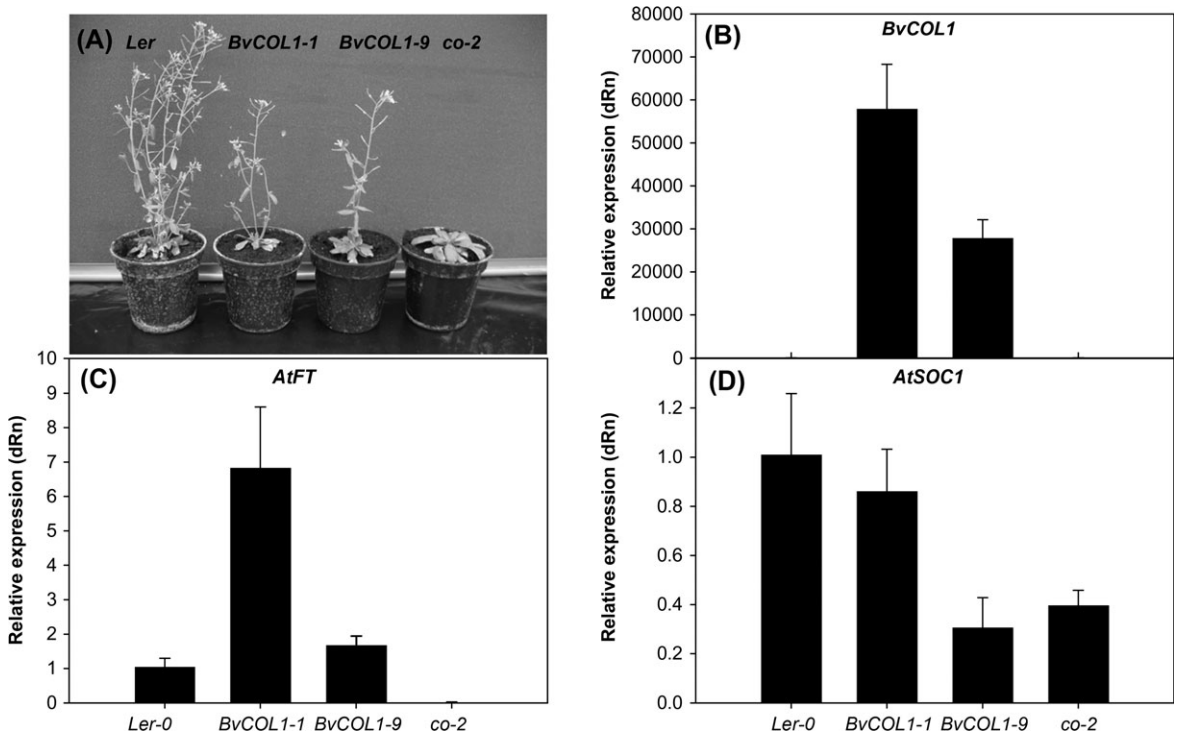

Fig. 6. Flowering time and QRT-PCR analysis of gene expression in transgenic and untransformed *A. thaliana*. (A) Phenotype of 6-week-old wild-type, transgenic, and *co-2* mutant lines. (B–D) Quantification of the *BvCOL1* transgene and endogenous *AtFT* and *AtSOC1* gene transcription in 2-week-old *A. thaliana* plants grown under LD by QRT-PCR. Error bars represent standard error of three biological and three technical repetitions.

direct effects on levels of *AtFT* expression, *AtCO* has also been shown to up-regulate *AtSOC1* expression. In accordance with previous findings (Samach *et al*., 2000), it was found that endogenous *AtSOC1* transcription in the *co-2* mutant was down-regulated compared to the wild type (Fig. 6D). By over-expressing *BvCOL1*, *AtSOC1* transcription was almost fully restored only in line *BvCOL1-1* and not in line *BvCOL1-9* (Fig. 6D).

Discussion

The effects of the environment on floral initiation in sugar beet have been observed for years but the molecular mechanisms that underlie its control remain to be fully elucidated. We have started the dissection of the obligate LD response in biennial beet by identifying a family of *CO*-like genes that play an important role in mediating between external light signals and flowering time. An extensive search of publicly available EST sequences and phylogenetic analysis has identified at least 10 genes (partial and full-length) that bear homology to the *CO* gene family of *Arabidopsis* with characteristic zinc-finger and/or CCT domains. Southern blot analysis and expression in vegetative leaves further verified the presence of a multigene family in sugar beet, as had also been shown to exist in monocots, dicots, and even in the moss, *Physcomitrella* (Robert *et al*., 1998; Yano *et al*., 2000; Liu *et al*., 2001; Griffiths *et al*., 2003; Kim *et al*., 2003; Nemoto *et al*., 2003; Hecht *et al*., 2005; Zobell *et al*., 2005).

Among the *BvCOL* genes that were identified, the closest homologue to Group 1a *CO*-like genes is *BvCOL1* with promoter and coding regions most similar to *AtCOL2* at 50% and 62%, respectively. In common with the *COL* genes, we found that *BvCOL1* was under circadian control. It is estimated that the circadian clock controls between 2% to 36% of plant gene expression and plays an important intermediary role between light regulation and plant gene expression (Harmer *et al*., 2000; Schaffer *et al*., 2001; Michael and McClung, 2003). Some common *cis*-regulatory motifs have been attributed to clock response such as the CCA1-binding site (CBS, AAAAATCT) and evening element (EE, AAAATATCT) (Harmer *et al*., 2000). CBS and a morning element (ME, AACCACGA) (Harmer and Kay, 2005) are present in genes exhibiting a dawn-phased rhythmic expression pattern such as *LHY* (Piechulla *et al*., 1998; Michael and McClung, 2002, 2003). In the *BvCOL1* promoter fragment identified in this study, none of these circadian-clock response elements (CCRE) are present, but circadian regulation of transcript oscillation was observed, indicating that other CCREs governing morning-phased rhythmic expression are yet to be identified. However, several light-response motifs are present in our *BvCOL1* promoter fragment such as G-boxes which are known to be targets of the light-transducing factor, PIF3 (Martinez-Garcia *et al*., 2000). PIF3s are known to bind G-boxes located in the promoters of the MYB transcription factors *LHY* and *CCA1*, thereby activating their transcription.

It was observed that, when sugar beet plants are grown under different photoperiods, the diurnal regulation of *BvCOL1* was strikingly different from the characteristic late evening (dusk) peak of *AtCO* and *Hd1* homologues. Instead, *BvCOL1* transcripts peak just before dawn, thereby closely resembling *AtCOL2* (and *AtCOL1*) but *AtCOL2* (and *AtCOL1*) does not control flowering as mis-expression had no effect on flowering time in *A. thaliana* (Ledger *et al*., 2001). This raises the possibility that *BvCOL1* is not the true homologue of *CO* as the diurnal profile of mRNA accumulation in existing *CO* homologues consistently supports the external coincidence model of photoperiod-dependent activation. However, It was observed that overexpressed *BvCOL1* can complement the loss-of-function *co-2* mutant thereby demonstrating a functional equivalence with the *AtCO*/*Hd1* homologues. It may be argued that the disparity in the apparent degree of conservation in gene sequence and regulation between *A. thaliana* and *B. vulgaris* on the one hand and gene function on the other hand is due to duplication of an ancestral gene bearing similarities to *AtCOL1*/*AtCOL2* followed by subsequent functional diversification, and/or gene loss (e.g. in the lineage leading to *B. vulgaris*).

Under both LD and SD photoperiods, *BvCOL1* was also found to be equally transcribed in the 14 h period that immediately followed the first 2 h after dawn. The differences in transcription under different day-lengths were in the last 8 h when *BvCOL1* mRNA is more abundantly expressed under SD than LD. This is surprising as *CO* homologues in the long-day plants *Arabidopsis* (*AtCO)* and wheat (*TaHd1-1*) are more highly expressed under LD (Putterill *et al*., 1995; Nemoto *et al*., 2003) and raises another possibility that *BvCOL1* may also act as a repressor of flowering under SDs. Such a lack of significant differences in mRNA abundance under different photoperiods is not unusual as *OsHd1* in the SD-rice plant is also equally transcribed under both photoperiods (Yano *et al*., 2000). Further, phylogenetic analyses consistently placed *BvCOL1* in a separate group to *AtCOL9*, a known repressor of flowering (Cheng and Wang, 2005) and we observed that *BvCOL1* advances flowering time under SD and LD but with a more pronounced effect under SD in transgenic *Arabidopsis*. This suggests that *BvCOL1* is unlikely to be a repressor of flowering. Certainly, the level of transcription under inductive photoperiods does not directly indicate functional activation nor does it define the period length at which the plant is responsive to reproductive switches. Instead, the activation of *CO*/*Hd1* in response to light

signals is more likely due to the balance of endogenous trans-activating/repressive factors induced by exposure to inductive and non-inductive photoperiods. This notion was supported by complementation analysis of the short-day rice *Hd1* mutant line with a genomic fragment of the long-day wheat gene, *TaHd1-1* (Nemoto *et al*., 2003). In addition, the rice *Hd1* homologue is able to not only promote heading (flowering) under SD but also to inhibit flowering in LD, termed a bifunctional response (Yano *et al*., 2000). To understand the upstream regulatory domains necessary for LD-responsive activation in sugar beet will require further work such as expression under the *BvCOL1* promoter and mis-expression in transgenic plants.

Although complementation of null mutants may be regarded with caution as a tool for identifying gene homologues, it does nevertheless provide useful information about protein function. Thus, functional equivalence between BvCOL1 and AtCO/Hd1 proteins was supported by two observations. One, the differences in flowering time of lines *BvCOL1-1* and *BvCOL1-9* indicate that gene dosage and/or positional effects may have significantly influenced complementation of the *A. thaliana* mutant. Southerns and qPCR showed that the degree of phenotypic complementation and the level of *BvCOL1* expression correlate with copy number of the transgene (one versus two copies) thereby suggesting that CO activity is a limiting factor in flowering induction. This has been observed by Putterill *et al*. (1995) in *A. thaliana* where over-expression of *AtCO* in wild-type plants further advanced flowering time. The same conclusion may be inferred from the intermediate flowering phenotype of heterozygous plants expressing any of the seven classical *co* mutations (Robson *et al*., 2001). It appears therefore that the genetic composition of *BvCOL1* functions similarly to the endogenous *AtCO* in its ability quantitatively to determine flowering time by copy number and expression level. Two, the expressed BvCOL1 protein in *A. thaliana* is able to interact positively with the two downstream genes *AtFT* and *AtSOC1* in the absence of the endogenous AtCO activator. Heterologous interaction between *BvCOL1* with *AtFT* and *AtSOC1* may be due in part to functional domains within BvCOL1. Despite a higher degree of homology to AtCOL2, BvCOL1 is also highly similar to the AtCO protein, with 77% amino acid identity within the B-box domains and 86% within the CCT domain. It may be that BvCOL1 can substitute for the absence of AtCO by being recruited to promoters of downstream genes to affect transcription, but an indirect effect of the transgene cannot be ruled out at this point. It is interesting also to note that the conservation of domain residues between group Ia proteins in *A. thaliana* are between 85–89% (B-box) and 88–93% (CCT) and yet, only AtCO has been demonstrated to control flowering time. It is, however, possible that like *BvCOL1*, *AtCOL1* and *AtCOL2* may play a role in flowering time in the absence of *AtCO* although complementation analysis of the classical *co* mutants has yet to be done.

So far it has been shown that *BvCOL1* is a light-responsive gene that is regulated by the circadian clock and encodes a protein that is able to mediate the LD response in *A. thaliana*. Molecular mapping has demonstrated that *BvCOL1* is located on chromosome II, but is not incorporated in the early bolting *B* gene locus. Preliminary haplotype analysis of the *BvCOL1* gene from different *Beta* accessions (including wild species) has only detected synonymous substitutions resulting in silent mutations (data not shown). Hence, the difference in bolting response of annuals and biennials could not be attributed to *BvCOL1*. However, data from Abe *et al*. (1997) and Owen (1954) suggested that the annual bolting habit conferred by *B* is modified by LD-responsive genes. Therefore, the quantitative effects of *CO*-like or other photoperiod genes on bolting time, rather than the bolting response *per se*, cannot be excluded and this is, therefore, currently still under investigation. The question remains as to whether *BvCOL1* is the main determinant of the LD photoperiod response in sugar beet and we are working to address this by transgenic manipulation of *BvCOL1* in sugar beet and by screening of TILLING populations for mutations in *BvCOL1*. In order to interpret results from this work correctly, the molecular hierarchy of *GI – CO – FT* in governing LD response in sugar beet must be proven. We are therefore also engaged in studies to identify these genes and examine their relationships and physiological significance in sugar beet.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Table 1. Sequences of primers for QPCR.

Supplementary Table 2. Sequences of primers for PCR and RT-PCR of *BvCOL* EST/TCs and *BvCOL1* promoter.

Acknowledgements

This work is funded by Biotechnology and Biological Sciences Research Council Core Strategic Grant 4668. ES Mutasa-Gottgens is supported by The British Beet Research Organization (BBRO grant No. 06/20). We thank Andrea Jennings and Cay Kruse for technical assistance, Professor George Coupland (Max Planck Institute, Cologne, Germany) for providing the *co-2* mutants, Dr Britta Schulz (KWS Saat AG, Einbeck, Germany) for providing the K1 mapping population and Professor Eberhard Weber (Martin-Luther-University Halle-Wittenberg, Germany) for providing the SNP and RFLP marker data set for K1. A Müller is financially supported by the German research foundation (DFG, grant No. Ju 205/14-1).

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