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Report

# The Role of a Pseudo-Response Regulator Gene in Life Cycle Adaptation and Domestication of Beet

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## Summary

**Life cycle adaptation to latitudinal and seasonal variation in photoperiod and temperature is a major determinant of evolutionary success in flowering plants. Whereas the life cycle of the dicotyledonous model species *Arabidopsis thaliana* is controlled by two epistatic genes, *FLOWERING LOCUS C* and *FRIGIDA* [1–3], three unrelated loci (*VERNALIZATION 1–3*) determine the spring and winter habits of monocotyledonous plants such as temperate cereals [4–6]. In the core eudicot species *Beta vulgaris*, whose lineage diverged from that leading to *Arabidopsis* shortly after the monocot-dicot split 140 million years ago [7, 8], the bolting locus *B* [9] is a master switch distinguishing annuals from biennials. Here, we isolated *B* and show that the pseudo-response regulator gene *BOLTING TIME CONTROL 1 (BvBTC1)*, through regulation of the *FLOWERING LOCUS T* genes [10], is absolutely necessary for flowering and mediates the response to both long days and vernalization. Our results suggest that domestication of beets involved the selection of a rare partial loss-of-function *BvBTC1* allele that imparts reduced sensitivity to photoperiod that is restored by vernalization, thus conferring bienniality, and illustrate how evolutionary plasticity at a key regulatory point can enable new life cycle strategies.**

## Results and Discussion

The annual habit in *Beta vulgaris* is characterized by flowering in long days (LDs) without a requirement for vernalization and is commonly found in sea beet (ssp. *maritima*), a wild subspecies from which the domestication of *B. vulgaris* is thought to have originated [11]. By contrast, the cultivated subspecies sugar beet (ssp. *vulgaris*) as well as sea beets from northern latitudes [12] are biennials and require vernalization followed by LDs to initiate flowering. The annual habit is controlled by a dominant gene at the bolting locus *B* [9, 13]. The vernalization response in biennial beets involves the gradual downregulation of an *FT*-like gene (*BvFT1*) that functions as a floral repressor [10]. By contrast, the expression of *BvFT1* in annuals is low throughout plant development, which enables induction of the floral inducer gene *BvFT2* in response to LDs. Here, we have identified *B*, investigated its role in life cycle control and induction of flowering, and discuss its unprecedented regulatory function among flowering plants.

### Map-Based Cloning of the Bolting Locus *B*

To determine the molecular basis of the distinction between annual and biennial life cycles in beets, we isolated *B* by map-based cloning (Figure 1). Genotyping of $F_2$ populations segregating for annuality using several bacterial artificial chromosome (BAC)-derived markers flanking a 0.6 cM genetic window around *B* (Figure 1B; see also Figure S1A and Table S1 available online) identified 107 recombinants for 16,566 gametes analyzed. Bulked segregant analysis (Figures S1B–S1D) identified a codominant marker (A195-A196) that cosegregated with annuality (Figure 1B; Table S2). BAC library screening and chromosome walking allowed the construction of physical maps (Figure 1C). Two marker loci were found to have undergone recombination in a single recombinant each and delimit *B* to a genomic region of approximately 0.2 Mb. Sequencing of the whole interval revealed multiple rearrangements between the annual and the biennial alleles and the presence of five genes in the annual genotype (Figure 1C; Table S3), one of which encodes a pseudo-response regulator (PRR) protein (Figure 1D) with response regulator receiver (REC) and CONSTANS, CONSTANS-LIKE, TIMING OF CAB EXPRESSION 1 (CCT) domains (Figure 1D; Figure S2), and homology to circadian clock-associated genes in *Arabidopsis* and the major determinant of LD response in barley, *PPD-H1* [14]. The gene was considered a strong candidate and named *BOLTING TIME CONTROL 1* (*BvBTC1*). Phylogenetic analysis revealed that *BvBTC1* belongs to the *PRR3*/*PRR7* clade [15], but does not cluster with *PRR3* genes and is less closely related to *PRR7* than another beet gene, *BvPRR7* (Figure 1E; Figure S2), and thus may derive from a third copy of an ancestral PRR gene in the proposed palaeohexaploid ancestor of

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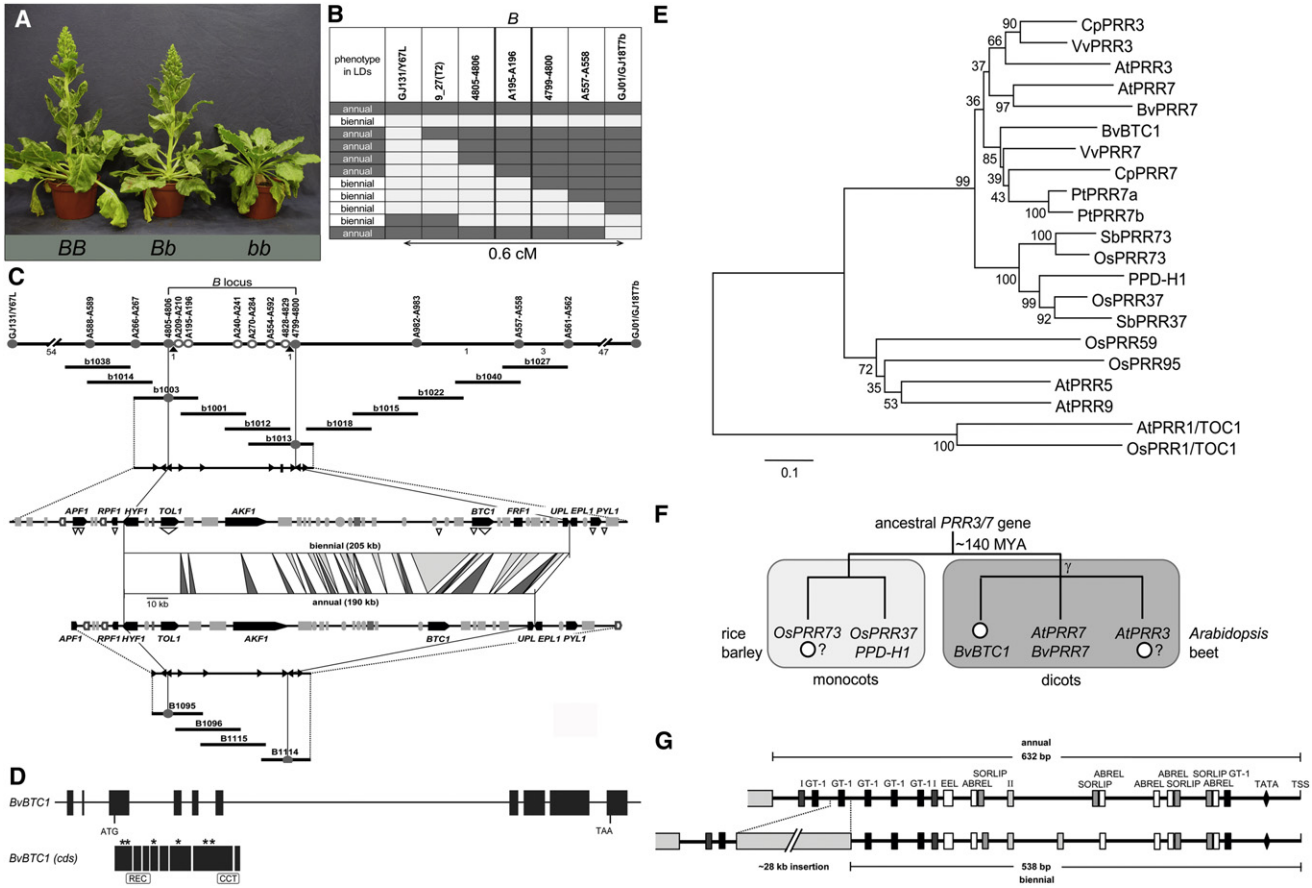


Figure 1. Map-Based Cloning of the *B* Locus in Beet

(A) Phenotypes of nonvernalized annual (*BB*, *Bb*) and biennial (*bb*) plants of the mapping population grown in LDs.

(B) Graphical genotypes of informative fixed recombinants. See also Table S2.

(C) Map of the *B* locus. The number of recombination events between BAC-derived markers (gray circles) is given. Open circles indicate markers that cosegregate with the bolting phenotype. Black triangles, recombination sites on either side of the *B* locus; horizontal bars, BACs; black arrows and rectangles, genes; open arrows, partial or pseudogenes; dark gray rectangles, transposable elements; light gray rectangles, retroelements; ovals, minisatellites and miscellaneous repetitive elements; open triangles, ESTs. Grey triangles indicate rearrangements between biennial and annual genotypes. See also Figure S1, Tables S1–S3.

(D) *BvBTC1* gene structure. Filled rectangles, exons; open rectangles, conserved REC and CCT domains. Asterisks indicate amino-acid substitutions between annual and biennial haplotypes. See also Figure S2.

(E) Evolutionary diversification of *PRR3*/*PRR7* genes. Minimum evolution tree (pairwise deletion option) including *BvBTC1*, *BvPRR7*, barley *PPD-H1*, the pseudo-response regulators in the *PRR3*/*PRR7* clade [15], and *Arabidopsis thaliana* and *Oryza sativa PRR1*/*TOC1* and *PRR5*/*PRR9* genes [15]. See also Figure S2.

(F) A model for the evolution of *PRR3*/*PRR7* genes in angiosperms. The partially overlapping roles of *PPD-H1* and *BvBTC1* suggest that the evolution of key functions in the control of photoperiod response predates the monocot-dicot divergence ~140 million years ago [8]. Distinct regulatory roles of *BvBTC1* in beet and *PRR3*/*PRR7* genes in *Arabidopsis* may be the result of sub- or neofunctionalization of different gene copies derived from a common ancestral gene within the respective lineages. Circles represent genes presumed to have been lost during evolution of the respective species. For species in which circles are marked with question marks, the complete genome sequence is not available yet. γ, triplication event [17].

(G) *BvBTC1* promoter. The region from the transcriptional start site (TSS) to an upstream repetitive element (open gray rectangle) is shown. Consensus motifs for light-regulated (box I, box II, GT-1, SORLIP) and cold-regulated (ABREL, EEL) *cis*-regulatory elements are marked by filled or white vertical rectangles, respectively.

rosids and the Caryophyllales clade of eudicots, which includes beet [16, 17] (Figure 1F).

### Haplotype Analysis in Wild and Domesticated Beets

Annual and biennial *BvBTC1* alleles differ by 11 nonsynonymous single-nucleotide polymorphisms (SNPs) (Figure S2) and a large insertion of ~28 kb in the promoter region, which is only present in the biennial allele (Figures 1C and 1G). This indel polymorphism disrupts a series of sequence motifs found in light-regulated promoters [18] and which in the annual allele include five evenly spaced GT-1 elements flanked by two identical I-boxes (Figure 1G). Haplotype analysis of a collection of annual and biennial beets revealed the presence of two classes that were associated with distinct bolting phenotypes (bolting or nonbolting) in the absence of vernalization (Table 1; Table S4). One group comprises eight haplotypes, including the annual parental haplotype, whereas the second group consists of three very similar haplotypes and includes the haplotype in the biennial parents. Remarkably, this same haplotype was present without exception in all cultivated accessions tested, whereas among natural populations, biennial haplotypes occurred at a very low frequency and only in

Table 1. *BvBTC1* Haplotypes

| Exons | | 3 | | | 5 | 6 | | 7 | | | 8 | | | | | | 9 | | | | | | | | | 10 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Nucleotide position within exon / Haplotype | Promoter upstream region | 92 | 224 | 351 | 89 | 64 | 89 | 23 | 75 | 164 | 9 | 79 | 97 | 154 | 158 | 250 | 37 | 402 | 435 | 476 | 542 | 616 | 670 | 686 | 814 | 72 |
| a | IN | G | A | A | C | G | T | C | A | G | C | C | C | G | T | G | A | A | G | C | G | T | A | G | T | A |
| b | IN | G | A | A | C | G | T | C | A | G | C | C | C | G | T | A | A | A | G | C | G | T | A | G | T | A |
| c | IN | G | A | A | C | G | T | T | A | G | C | C | C | G | T | A | A | A | G | C | G | T | A | G | T | A |
| d | DEL | G | C | T | T | G | G | C | G | T | C | A | C | G | T | A | A | G | A | T | A | T | A | A | T | G |
| e | DEL | G | C | T | T | G | G | C | G | T | C | A | C | G | A | A | A | G | G | T | A | T | A | G | T | G |
| f | DEL | G | C | T | T | G | G | C | G | T | C | A | C | G | A | A | G | G | G | T | A | T | A | G | T | G |
| g | DEL | G | C | T | T | G | G | C | G | T | C | A | C | G | T | A | A | G | G | T | A | T | A | G | T | G |
| h | DEL | G | C | T | T | G | G | C | G | T | T | A | C | G | T | A | A | G | G | T | A | T | A | G | T | G |
| i | DEL | G | C | T | T | G | G | C | G | G | C | A | C | G | T | A | A | G | G | T | G | C | G | G | T | G |
| j | DEL | G | C | T | T | A | G | C | G | T | C | A | C | T | T | A | A | G | G | T | G | T | A | G | C | G |
| k | DEL | T | C | T | T | G | G | C | G | T | C | A | A | G | T | A | A | G | G | T | G | T | A | G | T | G |
| Indels and nonsynonymous SNPs | * | * | * | * | | * | * | * | | * | * | * | * | * | * | * | | * | * | * | * | | * | * | | |
| Indels and nonsynonymous SNPs in mapping populations | * | | * | * | | | * | | | * | | * | | | | * | | * | * | * | * | | | * | | |
| Indels and nonsynonymous SNPs between haplotype group a-c and haplotype group d-k | * | | * | * | | | * | | | | | * | | | | | | * | | * | | | | | | |

The coding sequence of *BvBTC1* was sequenced across a panel of *B. vulgaris* accessions. Eleven haplotypes ("a" to "k") were identified. The position of SNPs in *BvBTC1* is given relative to the translation start site (for exon 3) or the 5′ end of a given exon, respectively. The reference haplotype "a" is present in both biennial mapping parents used in this study as well as all other biennial sugar beet cultivars analyzed. Haplotype "d" is present in both annual mapping parents. Haplotypes "a," "b," and "c" were only found in biennial accessions that require vernalization and LDs for bolting to occur, whereas haplotypes "d"–"k" were all found in accessions that bolt in LDs (22 hr light/2 hr dark). Haplotypes "g" and "j" are exceptional in that these haplotypes were also found in accessions that do not bolt without vernalization, suggesting that other loci in *B. vulgaris* contribute to life cycle control. This is consistent with the recent identification of a second bolting control locus (*B2*), which acts epistatically to *B* and, in the homozygous recessive state, inhibits bolting even in the presence of the dominant allele at the *B* locus [26]. See also Table S4.

accessions from northern latitudes, suggesting that domestication of beet involved the selection of a rare biennial allele. Of the nonsynonymous SNPs present in the mapping populations, six differentiate between the annual and biennial haplotype groups, but none is located at an evolutionarily conserved position among *PRR3/PRR7* genes (Figure S2). The large insertion in the *BvBTC1* promoter was only found in the three biennial haplotypes. By contrast to the distribution of haplotypes at *BvBTC1*, at three (*BvHYF1*, *BvAKF1*, *BvUPL1*) of the remaining four genes at the *B* locus, at least one of the haplotypes found in biennial cultivated accessions also occurred among annual genotypes (Table S5). Furthermore, at two of these genes (*BvHYF1*, *BvAKF1*) and at the fourth gene (*BvTOL1*), two to three different haplotypes were detected among biennial cultivated beets. Thus, only *BvBTC1* has a single, fixed haplotype among all cultivated accessions tested that at the same time exclusively occurs in biennial accessions, which is consistent with a single origin of the biennial allele at the *B* locus in cultivated beets.

### Evaluation of *BvBTC1* Function in Annuals

*BvBTC1* expression is highest in leaves (Figure S3A) and diurnally regulated with a peak of expression around zeitgeber time (ZT) 10 in both short days (SDs) and LDs (Figures 2A and 2B). However, whereas expression levels in both annuals and biennials decline rapidly after this peak in SDs (ZT 12–18, i.e., after nightfall) (Figure 2A), the decline in *BvBTC1* expression is decelerated during the additional daylight hours in LDs, and transcript accumulation at the end of the light phase is higher in annuals than in biennials (Figure 2B). To investigate the regulation of growth habit by *BvBTC1*, we downregulated *BvBTC1* expression by RNAi (Figure 2B). Whereas annuals rapidly initiated bolting when grown in LDs (Figure 2C), *BvBTC1 RNAi* plants derived from independent transformation events (827 and 832) did not bolt for up to 20 months (Figures 2C and 2D), indicating that lack of *BvBTC1* expression results in the loss of the annual habit. As expected [10], in annual control plants, *BvFT1* was repressed, whereas *BvFT2* transcript accumulation peaked in the afternoon in LDs (Figure 2B). Remarkably, the *BvBTC1 RNAi* plants showed a strong increase in *BvFT1* expression and a decrease in *BvFT2* expression, resulting in an apparent switch of the diurnal profile observed for these genes in annuals to that in nonvernalized biennials (Figure 2B). By contrast, no major changes in expression profiles were observed for clock-associated genes (Figure S3B), suggesting that altered regulation of *BvFT1/BvFT2* in the *BvBTC1 RNAi* lines is effected downstream of the circadian clock. The data demonstrate that *BvBTC1* is involved, directly or indirectly, in the transcriptional regulation of *BvFT1* and *BvFT2*.

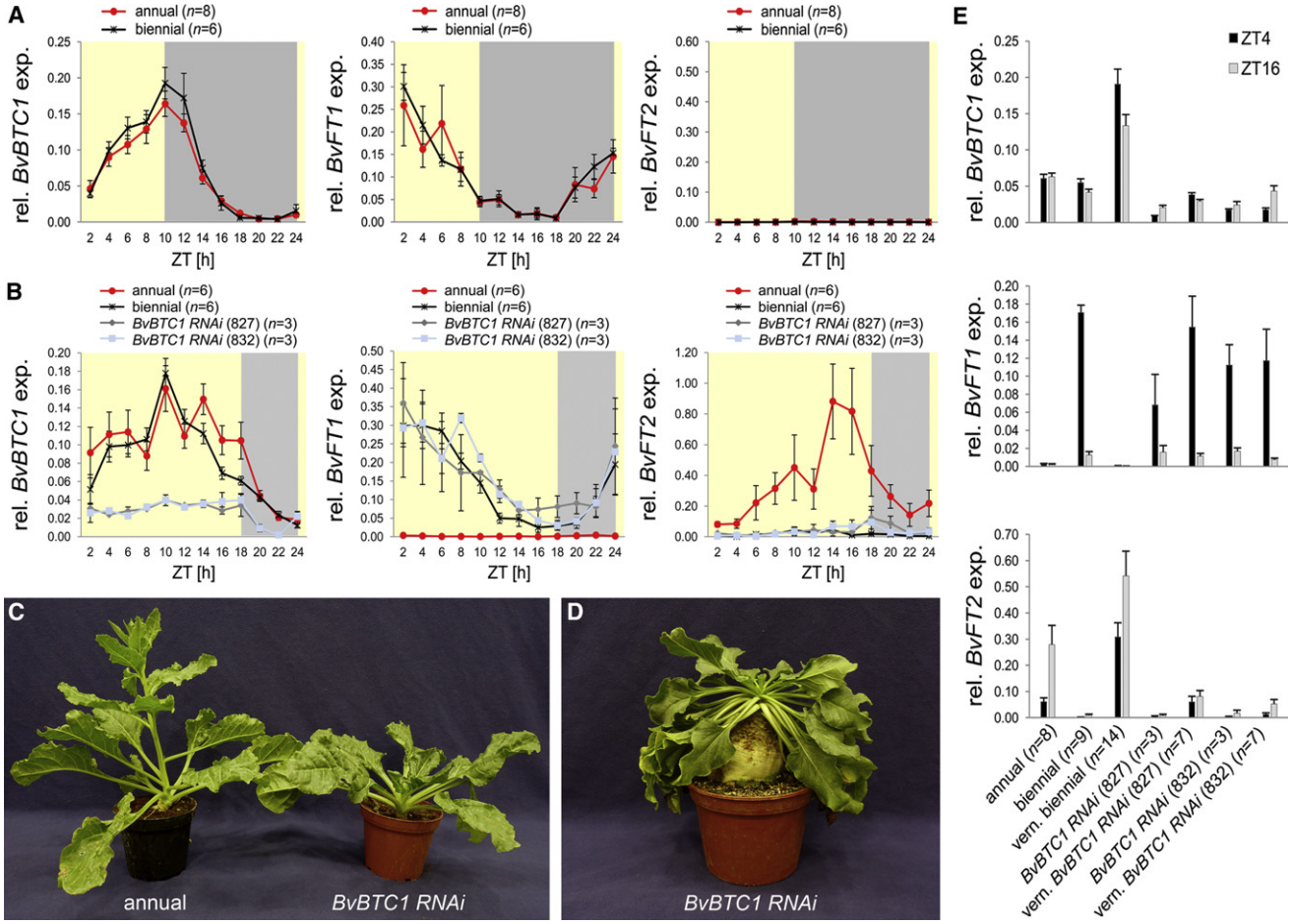


Figure 2. *BvBTC1* Controls Annuality through Regulation of the *BvFT1*/*BvFT2* Module

(A and B) Diurnal RT-qPCR expression profiles in annuals and biennials in SDs (A) and LDs (B), and in *BvBTC1 RNAi* lines in LDs (B). ZT, zeitgeber time. Error bars, mean ± SEM. See also Figure S3B.

(C) Phenotype of annual and *BvBTC1 RNAi* plants grown in LDs for five weeks.

(D) Nonbolting phenotype of *BvBTC1 RNAi* plants grown in LDs for six months.

(E) Transcript accumulation in *BvBTC1 RNAi* plants and controls in LDs at ZT4 and ZT16 before and after vernalization. Error bars represent mean ± SEM.

Vernalization gradually enhanced *BvBTC1* expression (Figure 2E; Figures S3C and S3D) and resulted in higher transcript levels throughout most of the light phase in biennials than in nonvernalized biennials (Figure 3A). Expression of *BvFT1* was downregulated by vernalization in biennials as expected [10], whereas expression levels in the *BvBTC1 RNAi* plants remained high after vernalization and coincided with low *BvFT2* expression (Figure 2E). After vernalization, the *BvBTC1 RNAi* plants bolted, but bolting was delayed and most plants showed a stunted phenotype, i.e., an arrest of stem growth. None of the stunted plants proceeded to flower, whereas those with normal-sized inflorescence stems developed aberrant intermediates between flowers and shoots. This altered flowering phenotype is identical to that observed for *BvFT2 RNAi* plants [10], corroborating our result that *BvBTC1* is an upstream regulator of *BvFT2*. These data indicate that in beet, unlike other plants where PRR function has been characterized [19–24], *BvBTC1* activity is necessary for promotion of flowering, most likely due to the fact that *BvBTC1* is required for *BvFT2* expression.

## Evaluation of *Bvbtc1* Function in Biennials

To evaluate a possible functionality of the recessive allele (*Bvbtc1*), the gene was downregulated by RNAi in a biennial genetic background (*Bvbtc1 RNAi* lines, Figure 3B). *BvFT1* expression was elevated in the *Bvbtc1 RNAi* lines and at the end of vernalization was strongly increased compared to the control, whereas *BvFT2* transcript accumulation was very low after vernalization (Figure 3B). Several independent *Bvbtc1 RNAi* lines failed to bolt for more than twelve weeks after vernalization, whereas the nontransgenic controls bolted 37–46 days after vernalization (Figure 3C). Consistent with the phenotypes, *BvFT1* and *BvFT2* in vernalized *Bvbtc1 RNAi* plants followed the typical diurnal expression profiles of nonvernalized biennials (Figure 3D). A few *Bvbtc1 RNAi* plants bolted, but stem elongation was severely impaired, resulting in a stunted appearance of the stem similar to that observed for vernalized RNAi plants with an annual background (Figures 3E–3G), and none of the plants set flowers. These results demonstrate that *Bvbtc1* retains a role as a promoter of bolting in biennials and that reduced expression of *Bvbtc1* compromises the vernalization response. Importantly, the

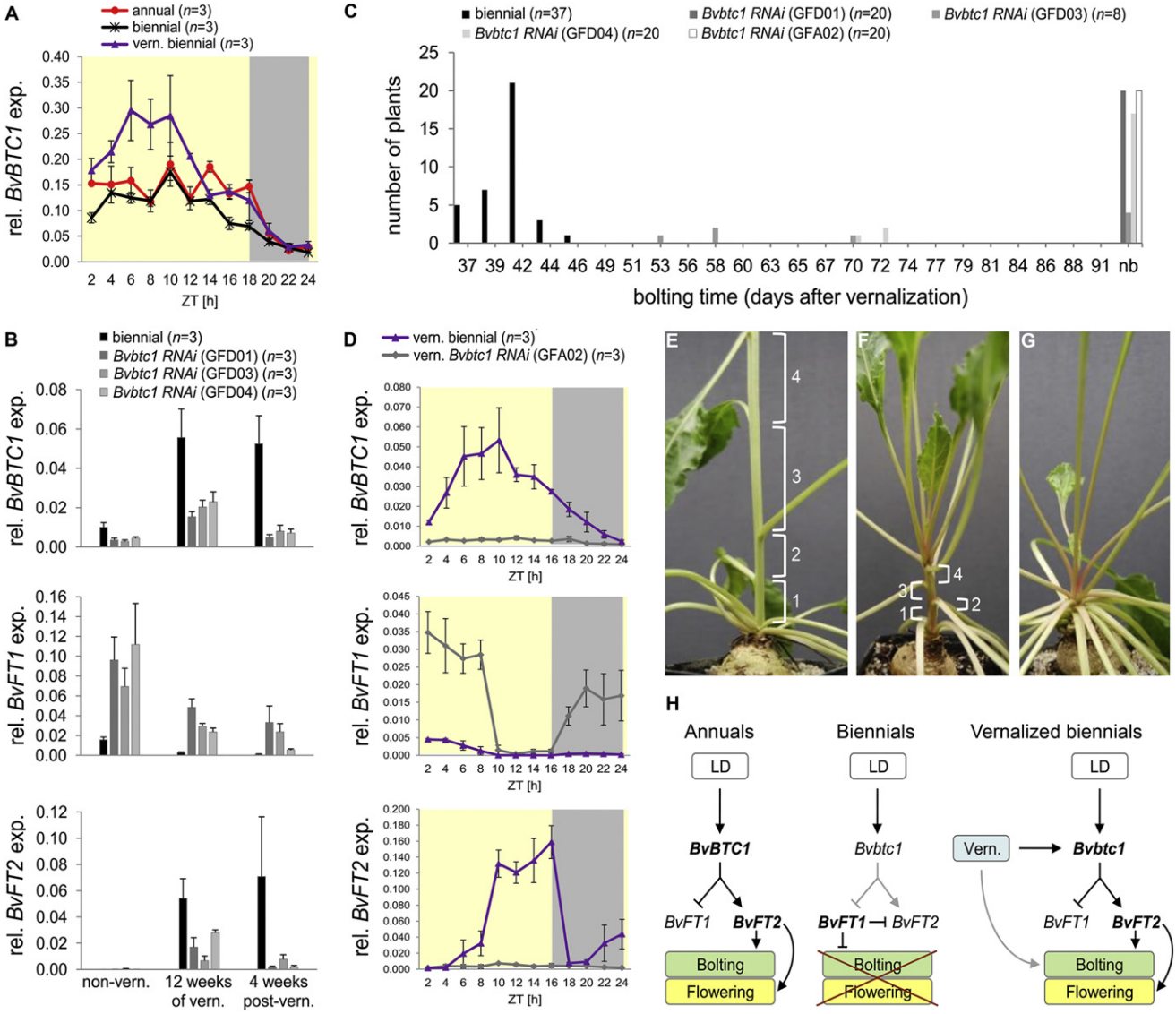


Figure 3. *BvBTC1* Mediates Bolting in Response to Vernalization and Is a Functional Regulator of the *FT* Genes in Biennials. See also Figures S3C and S3D

(A) Diurnal expression profiles of *BvBTC1* in annuals, biennials, and vernalized biennials in LDs. Error bars represent mean ± SEM.

(B) Transcript accumulation in *Bvbtc1 RNAi* plants in LDs at ZT6 before, at the end of, and after vernalization. Error bars represent mean ± SEM.

(C) Bolting time after vernalization. nb, nonbolting.

(D) Diurnal expression profiles in *Bvbtc1 RNAi* and biennial plants in LDs 4 weeks after vernalization. Error bars represent mean ± SEM.

(E–G) Close-up views of a bolting nontransgenic biennial control plant (E) and stunted (F) and nonbolting (G) *Bvbtc1 RNAi* plants in LDs 12 weeks after vernalization. The four lowest internodes are numbered.

(H) Proposed model for *BvBTC1* as a central regulator of life cycle adaptation and induction of flowering in beets. In annuals, *BvBTC1* activity mediates bolting and flowering through the regulation of *BvFT1* and *BvFT2*. The recessive allele in biennials (*Bvbtc1*) is not sufficiently sensitive to LDs or encodes a protein that is less active than in annuals and thus cannot transduce the inductive LD signal in the first growing season. The cold-induced upregulation of *Bvbtc1* during winter and/or an enhancing activity of additional vernalization response factors restores the functionality of the gene and enables *Bvbtc1* to stably repress *BvFT1* and activate *BvFT2*, possibly also involving *BvFT1*-independent interactions. Lines between genes do not imply direct interactions. Weak regulatory effects are indicated by gray lines.

data further show that the promotive effect of *Bvbtc1* activity in biennials is also mediated through the regulation of *BvFT1* and *BvFT2* expression and thus strongly suggest that the biennial gene product is functional.

### A Model for Life Cycle Control by *BvBTC1*

The current data suggest a model whereby *BvBTC1* acts upstream of *BvFT1* and *BvFT2* (Figure 3H). We hypothesize that annuals, carrying the dominant *BvBTC1* allele, respond to LDs without prior vernalization owing to elevated *BvBTC1* expression levels at a critical time of the day (possibly at the end of the light phase). Perhaps not dissimilar to the coincidence model proposed for the regulation of CONSTANS in *A. thaliana* [25], *BvBTC1* expression above a certain threshold level may lead to activation and/or stabilization of the gene product, e.g., by posttranslational modification or protein complex formation. The activated and/or stable form of BvBTC1 protein may then be able to stably repress *BvFT1*

and enable induction of *BvFT2* expression. By contrast, the recessive *Bvbtc1* allele in biennial beets may not be sufficiently expressed in LDs and cannot release the repression of *BvFT2*, and therefore, the plants remain vegetative before winter. The gradual upregulation of *Bvbtc1* in winter and increased post-vernalization expression levels during most of the day may again result in accumulation of the functional gene product above a threshold level and could thus compensate for the lack of efficient induction by LDs alone. Alternatively, or further adding to differences in transcriptional regulation of *BvBTC1* in annuals and biennials, the protein product of the biennial allele may be less active than its counterpart in annuals. In this scenario, induction of bolting by vernalization may require additional vernalization-responsive genes that either increase the activity of *Bvbtc1* or its protein product in biennials or act independently of *Bvbtc1* to promote bolting. The possibility that other regulatory genes contribute to the vernalization response in biennials is indicated by our observation that a subset of *Bvbtc1 RNAi* plants initiated bolting after vernalization.

## Conclusions

Our results indicate that a partial loss-of-function mutation of *BvBTC1* resulted in reduced sensitivity to inductive photoperiods before winter in biennials, thus imposing an obligate requirement for vernalization that acts on *BvBTC1* itself and restores the responsiveness to LDs, and that selection of a rare biennial allele carrying a large insertion in the promoter has been a key factor in the domestication of beets. The data also reveal an unexpected parallel between *Beta* and cereals, suggesting that the evolution of a key regulatory function in the control of long-day response by *PRR3*/*PRR7* genes predates the monocot-eudicot divergence. However, unlike *PRR3*/*PRR7* genes in cereals, which control photoperiod response [14, 24] but have not been implicated in life cycle control or vernalization response, *BvBTC1* has adopted a new role as a regulator of growth habit, possibly in coevolution with the downstream *BvFT1*/*BvFT2* module and other coregulatory genes. Importantly, *BvBTC1* responds to vernalization and thus is able to integrate both photoperiod and temperature signals, suggesting that *BvBTC1* plays a central part in mediating the long known compensatory effects of these environmental cues in beets. Our results for a taxon that is phylogenetically distant from both *Arabidopsis* and the monocots reveal a novel mode of life cycle control in flowering plants and illustrate how evolutionary plasticity can shape adaptation to changing climates by acting at different nodes of regulatory networks.

### Accession Numbers

Nucleotide sequences used in this study have been deposited with GenBank under accession numbers HQ709091–HQ709096 and HQ709099. See also Table S1.

### Supplemental Information

Supplemental Information includes three figures, five tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.cub.2012.04.007.

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