The *B2* flowering time locus of beet encodes a zinc finger transcription factor

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Sugar beet (*Beta vulgaris*) is a biennial root crop that grows vegetatively in the first year and starts shoot elongation (bolting) and flowering after exposure to cold temperatures over winter. Early bolting before winter is controlled by the dominant allele of the *B* locus. Recently, the *BOLTING TIME CONTROL 1* (*BTC1*) gene has been cloned from this locus. *BTC1* promotes early bolting through repression of the downstream bolting repressor *B. vulgaris FLOWERING LOCUS T1* (*BvFT1*) and activation of the downstream floral activator *BvFT2*. We have identified a new bolting locus *B2* acting epistatically to *B*. *B2* houses a transcription factor which is diurnally regulated and acts like *BTC1* upstream of *BvFT1* and *BvFT2*. It was termed *BvBBX19* according to its closest homolog from *Arabidopsis thaliana*. The encoded protein has two conserved domains with homology to zinc finger B-boxes. Ethyl methanesulfonate-induced mutations within the second B-box caused up-regulation of *BvFT1* and complete down-regulation of *BvFT2*. In *Arabidopsis*, the expression of *FT* is promoted by the B-box containing protein CONSTANS (CO). We performed a phylogenetic analysis with B-box genes from beet and *A. thaliana* but only *BvCOL1* clustered with *CO*. However, *BvCOL1* had been excluded as a *CO* ortholog by previous studies. Therefore, a new model for flowering induction in beet is proposed in which BTC1 and BvBBX19 complement each other and thus acquire a CO function to regulate their downstream targets *BvFT1* and *BvFT2*.

winter beet | sucrose | map-based cloning

In biennial root and leaf crops, avoidance of flowering is of fundamental importance for high yields and good quality. Among these crops are a number of important vegetables such as carrots, red table beet, swedes, or cabbage, where only vegetative parts of the plant are harvested. Other crops are grown for their storage components such as starch, inulin or sucrose. Sugar beet (*Beta vulgaris* L.) is the only sucrose storing crop of northern climates with a relatively short history of cultivation starting in the early 19th century. Shoot elongation, also termed bolting marks the visible onset of floral transition which is followed by flower formation and seed set. Since the early years of beet cultivation ~200 y ago, breeders have been strictly selecting against early bolting because root yield and root quality of flowering plants is low. Typically, beets as other root crops grown in temperate climates need a longer period of cold to flower. In the field, they start bolting right after winter, which is important for seed production. Thus, apart from exposure to cold temperatures to vernalize the plant, long-day (LD) conditions are a second requirement for floral induction.

In the model species *Arabidopsis thaliana*, a network of signaling pathways controls the onset of flowering. Key regulators of flowering time have been found with a similar function also in distantly related species such as rice and tomato (1). Sugar beet belongs to the Amaranthaceae family, which has separated from *A. thaliana* shortly after the monocot–dicot split 140 million years ago. Due to its strict requirement for cold to flower, its life cycle differs much from *A. thaliana*. However, annual accessions are abundant among the closely related wild species of the genus *Beta* (*B. vulgaris* subsp. *maritima*), which start bolting (termed "early bolting") just a few weeks after germination under LD conditions without any requirement for cold temperatures. Recently, the long-sought gene for early bolting from the *B* locus has been cloned from its position on chromosome 2 (2). The *B* locus encodes a pseudo response regulator (PRR) gene *BOLTING TIME CONTROL 1* (*BTC1*). So far, it was assumed that only beets carrying a recessive *b* allele are biennials because biennials with a dominant *B* allele have never been reported. Recently, five more bolting time loci have been detected among the offspring of an ethyl methanesulfonate (EMS) mutagenized annual accession carrying the dominant $BTC1_d$ allele (3). Two loci termed *B2* and *B4* were mapped to chromosome 9 and 2, respectively (4, 5).

In *A. thaliana*, inductive long days are perceived by the photoperiod pathway, which accelerates flowering by activating *GIGANTEA* (*GI*) and *CONSTANS* (*CO*). The CO protein is a transcriptional regulator consisting of two B-box type zinc finger motifs (B-box) and a CCT (CONSTANS, CONSTANS-LIKE, and TOC1) domain and plays a major regulatory role in the photoperiodic pathway (6). Recent studies revealed that CO directly binds to the *FLOWERING LOCUS T* (*FT*) promoter and therefore activates *FT* transcription, required to initiate flowering (7) for which the CCT motif was shown to be required. The B-box domains of the CO protein were thought to be not essential for DNA binding or transcriptional activation. However, *FT* mRNA accumulation is reduced in the late flowering B-box defective *co-2* mutant, indicating that the B-box domain is important for the proper function of CO (7, 8).

CONSTANS-orthologs have been found in all plants investigated so far. Evidence of a large family of *CONSTANS-LIKE* (*COL*) genes in sugar beet was given by Chia et al. (9), who detected three different *COL* genes in sugar beet differing by their B-box and CCT domains, which are characteristic features of *CONSTANS* (1, 10, 11). *BvCOL1* was identified as an important component of the photoperiod pathway in beet (9). However, it is not an ortholog of *CO* because the expression pattern of both genes differed substantially (9). An ortholog of

Significance

Flowering must be strictly avoided in crop plants whose vegetative parts (such as leaves or roots) are harvested. A new flowering time regulator has been cloned from a root crop (sugar beet) which acts epistatically to a recently identified bolting gene. Mutations in each of the two genes resulted in a loss of competence to flower without vernalization. This offers a perspective to breed "never bolting" hybrids by combining two mutations in each of both genes. Those cultivars would have a higher yield potential because they could be grown over winter or early in spring.

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the *FLOWERING LOCUS T* (*FT*) gene from *A. thaliana* has been recently identified (12) as a downstream target of a putative *CO* ortholog. *BvFT2* is a floral activator highly expressed in annual beets and in biennial beets after vernalization (12). Interestingly, the beet genome houses two *FT* paralogs with antagonistic functions. The second one, *BvFT1*, is a floral repressor highly expressed before vernalization and completely down-regulated in annual beets (12). A model has been proposed in which *BvFT1* is repressed in annuals by *BTC1*, whereas *BvFT2* expression is activated by *BTC1* (2, 12).

Here, we describe the map-based cloning of a candidate gene from the *B2* locus, *BvBBX19*, which is acting epistatically over *B*. The gene *BvBBX19* encodes a *DOUBLE B-BOX TYPE ZINC FINGER* protein. Two *B2* EMS mutants were further analyzed. Both carry point mutations at the *BvBBX19* gene typical for EMS mutagenesis. The mutations cause amino acid changes within the second B-box domain, which is assumed to be important for the proper function of *BvBBX19*. Those mutations have a strong impact on the function of the BTC1 protein turning an annual plant homozygous for the annual *BTC1*-allele into a biennial one which can only flower after cold treatment. Expression analysis revealed that *BvBBX19* is diurnally regulated and acts upstream of *BvFT1* and *BvFT2*. These findings shed new light on the flowering time regulation in a biennial crop species.

Results

Genetic and Physical Mapping of the *B2* Locus. We produced a large F_2 mapping population after crossing a biennial *B. vulgaris B2* mutant (*B2′*) with an annual wild beet (*B2*) (*B. vulgaris* subsp. *maritima*) (Fig. S1). In total, 5,457 F_2 plants were grown in a field in Kiel (Germany) from spring 2010 until autumn 2011. Within 30 wk after sowing, 1,042 plants did not bolt and were classified as biennials (Fig. S1). 4415 F_2 plants started bolting (early bolting) during this time period, but we suspected that some of them had been misclassified as annuals. To verify their F_2 genotypes, we grew the F_3 offspring of annual F_2 plants in 96mer multipot-plates outside the greenhouse between May and October. We observed 638 nonsegregating annual (*B2B2*) and 1,911 segregating F_3 families (Fig. S1). We reassessed the genotypic classification of all F_2 plants with phenotypic data from very early bolting F_2 plants and F_3 families, which were bolting within 12 wk after sowing. As a result, 466 F_2 plants were classified as homozygous (*B2B2*) and 2,083 F_2 plants were classified as heterozygous at the *B2* locus.

Then, we selected seven markers (Tables S1 and S2) from scaffolds sc00048 and sc00497 for fine mapping the *B2* locus with 1,301 F_2 plants (922 biennial *B2′B2′*, 379 annual *B2B2*). We identified the most tightly linked markers as being homozygous in either one or the other phenotypic class of F_2 plants. As a result, only marker CAU3784 was completely linked to *B2* because no recombinants were detected among the homozygous plants analyzed. All biennial plants investigated carried the biennial allele. Likewise, all annuals carried the annual allele suggesting that this marker is closest to the *B2* gene and that all biennials are homozygous for the mutant allele (*B2′*) (Table S1).

The resulting map spans 4.8 cM across the *B2* locus (Fig. 1*A*). The three markers CAU3782, CAU3783, and CAU3784 are located close to the telomeric region of chromosome 9 on scaffold sc00048, which is ∼1.6 Mbp in size. This region of the beet genome has a high recombination frequency typical for telomere-near regions (1 cM/0.145 Mbp). The markers CAU3782 and CAU3783 are located in a distance of ∼276 kbp to each other flanking the *B2* locus and the marker locus CAU3784 (Fig. 1*B*). The molecular marker CAU3784 was genotyped as an InDel marker (Fig. 1*C*). The mutant parent allele ($CAU3784_{M2}$) is linked in coupling phase ($R = 0$) with the *B2′* allele. In conclusion, the scaffold sc00048 is likely to carry the *B2* gene.

Candidate Gene Identification. We identified 32 gene models (thresholds: e-value 0.0, identity 100%) which were used as queries for a BLASTX analysis against the TAIR and NCBI protein databases

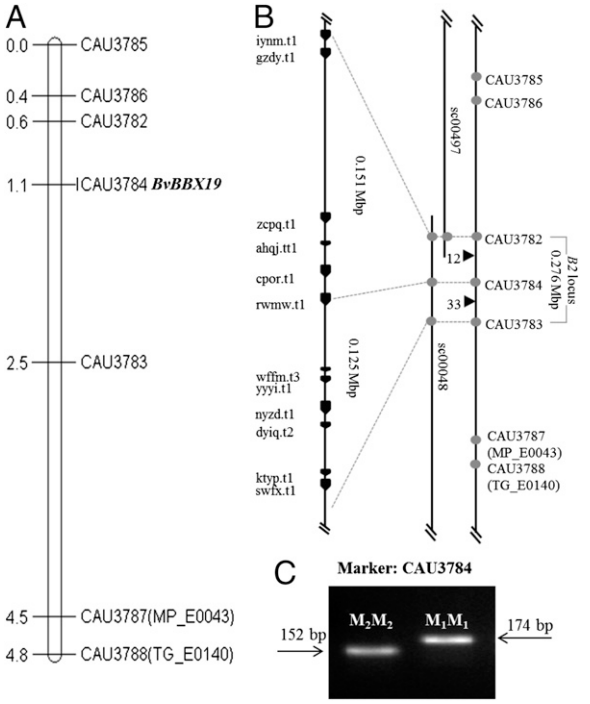

Fig. 1. Map-based cloning of the *B2* gene in beet. (*A*) Genetic map position of the *B2* locus on chromosome 9. (*B*) Physical map of the *B2* locus and the location of the two sequence scaffolds sc00497 and sc00048, covering the *B2* locus. Crossover events are given as black triangles. Black boxes indicate gene models. (*C*) Molecular marker CAU3784 used for mapping the *B2* locus; M_1 and M_2 are the alleles from annual and biennial parents, respectively.

(threshold: e-value <0.05). In total, 12 gene models were obtained which were homologous to genes from *A. thaliana* (Table S3). We selected the gene model rwmw.t1 (according to RefBeet-1.1gene-Models; ref. 13), which is 1,458 bp in size, as a candidate sequence for *B2* due to the following reasons. First, the InDel marker CAU3784 is located within the predicted 3′-untranslated region (UTR) of this candidate gene and second, it shares homology with *B-BOX TYPE ZINC FINGER* (*BBX*) genes whose proteins have been proposed to act as transcription factors (10). A BLASTP analysis against the nr/nt protein database revealed 55% identity to the *A. thaliana* protein BBX19 (TAIR, At4g38960) and two sugar beet ESTs were found (BQ589556 and BQ591888) sharing high homology to the hypothetical gene model rwmw.t1. An ORF for rwmw.t1 whose sequence comes from the DH line KWS2320 (later termed $BvBBX19_{2320}$) was predicted encompassing 588 bp (196 amino acids).

Gene Structure Analysis. We analyzed the structure of the predicted ORF with the ab initio gene prediction program FGENESH (14) using $BvBBX19_{2320}$ as a query. The transcription start site (TSS) was localized at position 262 (+1) and the end at position 1438 (+1177, PolyA signal) resulting in a 1,177-bp transcript (Fig. 2*A*). A TATA-box was predicted at position −28 to −22 relative to the TSS. The predicted transcript has five exons; parts of exon 4 and exon 5 belong to the 3′-UTR, which is supposed to be 348 bp in size. The size of the 5′-UTR is 241 bp. In summary, the size of the genomic sequence (ATG-STOP) located within scaffold sc00048 is 4,700 bp.

Then, we analyzed the predicted polypeptide sequence of $BvBBX19_{2320}$ with the web-based protein domains identification tool SMART (15, 16). The sequence shows strong similarity to two B-box-type zinc finger domains. The B-box domains termed BB1 and BB2 (Fig. 2*C*) are 47 and 46 aa in size, respectively. The structure of the two B-box domains in the *A. thaliana* protein BBX19 is $C\text{-}X_2\text{-}C\text{-}X_8\text{-}C\text{-}X_7\text{-}C\text{-}X_2\text{-}C\text{-}X_4\text{-}H\text{-}X\text{-}C\text{-}X_6\text{-}H\text{-}X_6$ (BB1) and $C_2\text{-}X_2\text{-}C\text{-}X_8\text{-}C\text{-}X_7\text{-}C\text{-}X_2\text{-}C\text{-}X_4\text{-}H\text{-}X_6\text{-}H\text{-}X_5$ (BB2). We compared

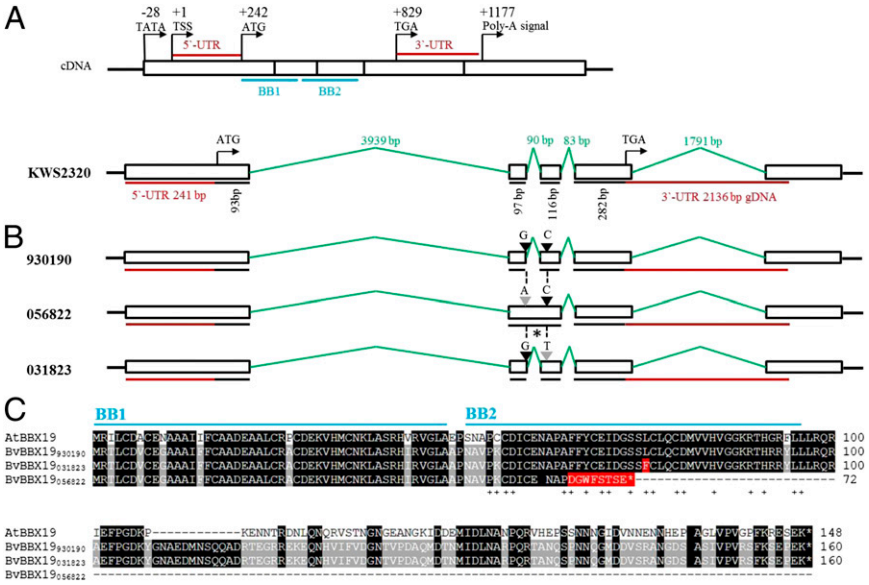

Fig. 2. (*A*) In silico prediction of the *BvBBX19* gene structure. Exons are drawn as open boxes; B-box regions (BB1, BB2) are drawn in blue; red bars: 3′-UTR and 5′-UTR; black bars: translated region; Green bars: introns. (*B*) Sequence variations within *BvBBX19* alleles from the two EMS mutants (seed codes 056822 and 031823) and the nonmutated donor line (930190); gray triangles: SNP positions; black triangles: nucleotide derived from the nonmutated donor line; black asterisk: premature stop codon. (*C*) Multiple alignment of BvBBX19 protein sequences from beet and from *A. thaliana* (AtBBX19); +: highly conserved amino acid positions within BB2 (Conserved Domains Database, NCBI). Amino acid changes due to EMS mutations are highlighted in red; asterisks: stop codon.

the BBX19 sequence with the predicted $BvBBX19_{2320}$ protein. Both zinc finger structures of the BB1 domains are 100% identical, whereas the zinc finger structure of the BB2 domains differs by one C residue at the beginning ($BvBBX19_{2320}$: C-X_2-C-X_8-C-X_7-C-X_2-C-X_4-H-X_6-H-X_5). We named the predicted beet protein *BvBBX19* because it showed highest homology to the BBX19 protein (10).

***BvBBX19* Sequence Variations.** If *BvBBX19* was the sought *B2* gene its sequences were expected to differ between the parents of the mapping population as well as between mutant and nonmutated donor line. We found that the coding sequence from the annual parent was identical in size (588 bp) and structure (100% identity) to the predicted $BvBBX19_{2320}$ ORF whereas the sequence from the mutant parent ($BvBBX19_{056822}$) has a 90-bp insertion perfectly matching to the second intron (Fig. 2*B*).

Then, we compared the coding sequences from the mutant parent and the nonmutagenized donor line 930190. Both sequences are identical except one polymorphism at position 4131 right after the second exon where the nonmutagenized donor line ($BvBBX19_{930190}$) has a guanine instead of an adenine (Fig. 2*B*). We reason that this transition had been induced by EMS mutagenesis. It is conceivable that this single nucleotide mutation alters the function of the donor splice site in a way that the second intron cannot be removed resulting in a longer transcript which carries a stop codon within the second intron due to a shifted reading frame (Fig. 2*C*).

We assumed that *BvBBX19* is a flowering time regulator from the *B2* locus. As a verification experiment, we analyzed another EMS mutant (seed code 031823) with the same bolting time phenotype whose mutation had been mapped exactly to the *B2* locus (5). This mutant had been found independently of the 056822 mutant (3). We compared the $BvBBX19_{031823}$ sequence with $BvBBX19_{056822}$ and $BvBBX19_{930190}$. $BvBBX19_{031823}$ is identical to $BvBBX19_{930190}$ with one exception. The $BvBBX19_{031823}$ allele carries a point mutation within the third exon at position 4253 (Fig. 2*B*). This transition from cytosine to thymine is likely to result from the EMS treatment. The predicted polypeptide only differs from $BvBBX19_{930190}$ by an amino acid exchange at position 75 from leucine to phenylalanine (Fig. 2*C*).

We searched the Conserved Domains Database at the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov/Structure/cdd/cdd_help.shtml) for conserved sequence regions using the BvBBX19 protein sequences from both mutants (seed code 056822 and 031823) and the nonmutated donor line (seed code 930190) as queries. This analysis demonstrated that both transitions occurred at highly conserved positions within the BB2 region (Fig. 2*C*). The zinc finger type structure of the BB2 domain is not altered in the $BvBBX19_{031823}$ protein. In contrast, the 5′-splice site mutation in the second intron of $BvBBX19_{056822}$ drastically alters the structure of the BB2 domain to C-X_2-C_{14}.

***BBX*-Like Sequences in Beet.** We identified 15 BBX genes (BvBBXs) plus two splice variants in the sugar beet genome (RefBeet-0.9) after a BLASTP search using the first B-box region of the CO protein sequence as query (Table S4). The presence of conserved domains in each of the BBX protein sequences has been confirmed by SMART (15) and CD-search (17). Among the 15 BvBBXs, six contain two B-boxes and one CCT domain, two contain one B-box and one CCT domain, six contain two B-box domains, and one contains only one B-box domain (Table S4).

A maximum likelihood based phylogenetic tree (Fig. 3) of the 15 BvBBXs was constructed using Mega5.2 (18) after model test. To clarify the orthologous relationship of the BBXs from *B. vulgaris* and from *A. thaliana*, another phylogenetic tree was constructed comprising 30 AtBBXs from Khanna et al. (10) and 15 BvBBXs. As a result, none of the BvBBXs clustered with CONSTANS (Fig. S2). Interestingly, many BvBBXs such as BvCOL2, BvBBX19 (B2) are clustered with two *Arabidopsis* proteins demonstrating an evolutionary conservation between ancient copies in beet and duplicated copies in *A. thaliana*.

***BvBBX19* Expression Analysis and Putative Downstream Targets.** We measured the transcriptional activity of *BBX19* in leaves of nonvernalized plants. *BvBBX19* shows diurnal expression and the expression peaks at dawn (zeitgeber time, ZT 0). Then, it gradually decreases over day until ZT 8 followed by a rapid increase during the night at ZT 18 (Fig. 4). During the night, *BvBBX19* is higher expressed in biennial beets as in annual beets. Interestingly, both mutant lines showed a second peak of *BvBBX19* expression at ZT 4 and ZT 6, respectively (Fig. 4).

B2 mutants are biennial even in the presence of the dominant *B* allele (haplotype $BTC1_d$) pointing at an epistatic interaction between both loci. Therefore, we hypothesized that *BvBBX19* mutations impact the transcriptional activity of *BTC1* and their putative downstream targets. We examined the expression of *BvBBX19*, *BTC1*, *BvFT1*, and *BvFT2* in annual beets carrying the dominant *BTC1* and the functional *B2* allele (seed code 991971, 001684). We also investigated biennial beets, which carry either the recessive *btc1* (seed code 93161P) or the mutated *b2* (seed code 056822) allele. We found that *BvBBX19* is higher expressed in the mutant compared with the nonmutated annual beet

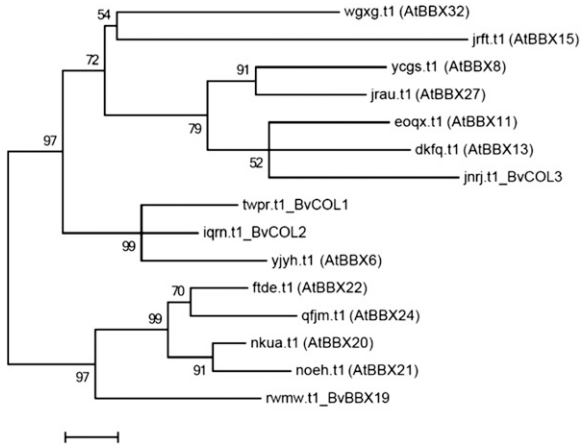

Fig. 3. Maximum likelihood phylogenetic tree of 15 BBX deduced amino acid sequences identified in the sugar beet genome. The respective domain structures of the proteins and their chromosomal locations in the sugar beet genome are given in Table S4. The bootstrap consensus tree inferred from 1,000 replicates was constructed by Mega5.2 after aligning the predicted protein sequences from 15 BvBBXs by MAFFT. The percentage bootstrap values are indicated at the branch points. Scale bar represents 0.2 amino acid substitutions per site. BvCOL1, BvCOL2, and BvCOL3 have been already published by Chia et al. (9). Best *Arabidopsis* homologs of uncharacterized BvBBXs are indicated in brackets.

(001684) and that *BvBBX19* expression drops markedly after vernalization (Fig. 5). Also, *BTC1* is up-regulated in biennial *B2* mutants compared with the annual line. Its expression pattern before and after vernalization resembles the biennial control 93161P (Fig. 5). The *B2* mutation had a striking effect on the expression of the floral repressor *BvFT1*. In stark contrast to the annual parent, *BvFT1* is highly up-regulated before vernalization. Accordingly, we observed no transcriptional activity of the floral promoter *BvFT2* in *B2* mutants before vernalization. In conclusion, the *BvBBX19* mutation (in the presence of an annual *B* allele) turned the annual expression pattern of all four genes into a biennial expression pattern, which fits well to the transition from an annual to a biennial growth behavior.

Discussion

***BvBBX19* Encodes the Bolting Time Regulator from the *B2* Locus.** In the past years, a number of genes have been identified which add to our understanding of bolting time regulation in beet. *BvFT2* retained the function of the *FT* gene and acts as a floral integrator (12). The finding that another *FT* homolog *BvFT1* is acting as a floral repressor demonstrates neofunctionalization of flowering time regulators during the evolution of *Beta* species (12). Furthermore, *BTC1* in contrast to its *A. thaliana* homolog *PRR7* controls annual life cycle in beet through regulation of *BvFT1* (2). Here, we add *BvBBX19* as a novel key regulator of bolting time whose closest sequence homologs in *A. thaliana* have not been reported to act in this way.

Three lines of evidence demonstrate that *BvBBX19* is the bolting time regulator gene from the *B2* locus. First, it is completely linked ($R = 0$) to the *B2* locus. Second, the two parents of the mapping population differ within the candidate sequence. Third, two mutants with clear phenotypic effects differ from the nonmutated parent line 930190 by point mutations typically for EMS mutagenesis. Our study was based on two EMS mutants that had been selected after seed mutagenesis of an annual beet line carrying the dominant *BTC1* allele (3). We identified a single transition in each of the mutants that occurred at different positions within the candidate gene. These results clearly point to EMS induced mutations because EMS typically causes transitions such as G/C → A/T.

The finding that two genes upstream of an *FT* ortholog jointly regulate the onset of bolting in a biennial root crop might have some practical implications. Resistance to bolting after cold exposure is an important breeding aim, not only for sugar beet but also for other crops where vegetative parts of the plant are harvested such as leaves, tubers or roots. Combining two mutant alleles from each gene could result in a hybrid which lost the competence to bolt even after cold exposure. This hybrid could be sown earlier in spring where cold temperatures can be expected or even before winter ("winter beet") as has already been suggested (19). Detecting allelic variation in each of the two bolting time regulators among the gene pool of the genus *Beta* to produce "never bolting" hybrids will be a task for the future.

Transcription Factors *BvBBX19* and *BTC1* Jointly Control *BvFT2* Expression. What do we know about the function of the *BvBBX19* gene as a bolting time control gene in beet? In the following, we will first analyze the structure and putative function of the predicted protein and then discuss evidence from genetic studies. The predicted protein shows high homology to the transcription factor B-BOX TYPE ZINC FINGER 19 (BBX19, AT4G38960.1) from *A. thaliana*. The BBX protein family from *Arabidopsis* consists of 32 members and is structured into five subfamilies based on protein sequence analyses (10). *CONSTANS* was the first *BBX* gene found in *Arabidopsis* (20). Accordingly, members of subfamily I-III (BBX1-BBX17) were termed CO and CO-like (COL) proteins, based on their structure of at least one B-box and a CCT domain, whereas members of subfamily IV–V (BBX18–BBX32) harbor at most two B-box domains (10). *BBX19* is not known to be involved in flowering time regulation (21). In contrast, *CO* is an important regulator and promotes flowering under LD conditions by activating the floral inducer gene *FT* through binding to its promoter (7).

CO orthologs were identified in several other plant species but only a few have been functionally characterized. In the short day plant rice (*Oryza sativa*), the *CO* ortholog *Hd1* promotes flowering under SD conditions and suppresses flowering under LD conditions (22). Griffiths et al. (23) identified nine *COL* genes in barley (*Hordeum vulgare*), which were termed *HvCO1–HvCO9*. The closest *CO* homolog *HvCO1* was shown to be a floral inducer, because overexpression of *HvCO1* in spring barley accelerated the time to flowering under LD and SD conditions (24).

Does sugar beet have a *CO* ortholog or do *BTC1* and *BvBBX19* jointly act in a way as *CO* does in *A. thaliana*? To answer this question, we made a sequence survey of the sugar beet genome. We identified 15 *BBX* genes. *BvCOL1* has been proposed as the closest *CO* homolog (9), which was confirmed by our phylogenetic analysis. Overexpression of *BvCOL1* complemented a late flowering *Arabidopsis* mutant (*co2*) (9). Unlike *CO, BvCOL1* expression peaks in the late evening similar to

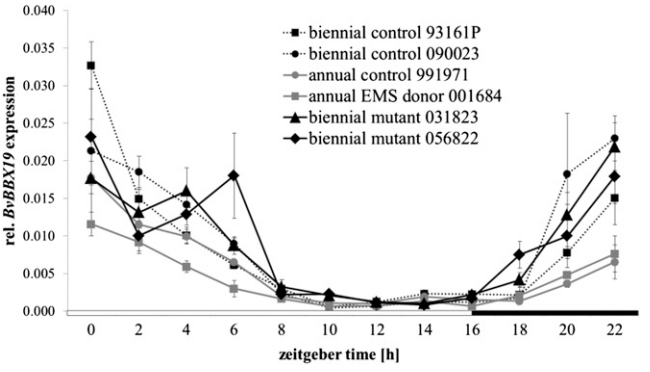

Fig. 4. Diurnal expression analysis of *BvBBX19* in beet. Leaves were harvested 44 d after sowing. Each value is the mean of three biological and three technical replicates. The relative *BvBBX19* expression is given on the left vertical axis. The bar at the bottom indicates light (open bar) and dark (black bar) phases. Error bars represent the SEM of three biological replicates.

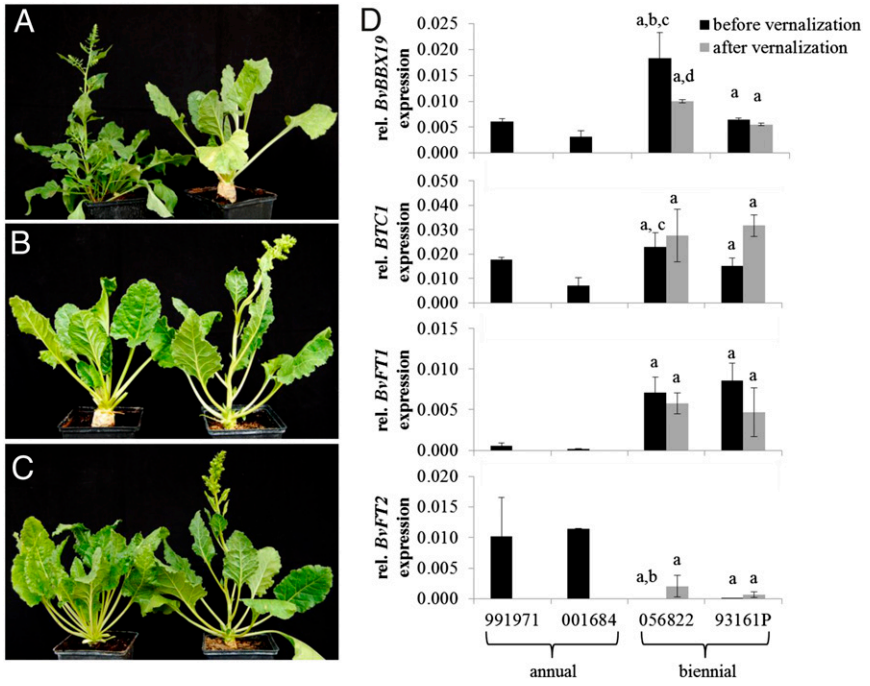

Fig. 5. Phenotypes of annual and biennial beets (nonvernalized and vernalized) and the expression of *BvBBX19* and three putative downstream targets. (*A*) The annual wild beet accession 991971 (*Left*) carries the dominant *BTC1* allele and bolts without vernalization. The biennial sugar beet accession 93161P (*Right*) carries the recessive *btc1* allele and does not bolt without vernalization. (*B*) The biennial sugar beet accession 93161P does not bolt without vernalization (*Left*) but bolts after vernalization (*Right*). (*C*) The *B2* mutant plant from the M_3 line 056822 with the dominant *BTC1* allele and the mutated *B2* allele does not bolt without vernalization (*Left*) but bolts after vernalization (*Right*). (*D*) Expression analysis of *BvBBX19*, *BTC1*, *BvFT1*, and *BvFT2* in annual (seed codes 991971 and 001684) and biennial accessions (seed codes 056822 and 93161P). The biennial *B2* mutant 056822 was obtained after EMS treatment of the annual accession 001684. All plants were grown in a greenhouse under LD conditions for 7 wk. Then, biennials were vernalized for 12 wk and grown in the greenhouse under LD conditions. Black boxes, gene expression 44 d after sowing; gray boxes, gene expression 2 wk after vernalization. Each value is the mean of three biological and three technical replicates. The relative gene expression is given on the left vertical axis. Error bars represent the SEM of three biological replicates. Statistically significant different gene expression was analyzed applying student's *t* test (confidence interval 95%) between: a, the annual EMS donor 001684 and biennial accessions (seed code 056822, 93161P); b, nonvernalized and vernalized mutant 056822; c, nonvernalized biennial accessions; and d, vernalized biennial accessions.

COL1 and *COL2* which are highly expressed before dawn. Thus, it has been suggested that *BvCOL1* is not a functional ortholog of *CO* (9). Moreover, the genetic position of *BvCOL1* does not coincide with a major flowering time QTL on chromosome 2 and none of the flowering time mutations described in beet (3) has been mapped to the *BvCOL1* locus. Hence, there is no evidence to support the role of *BvCOL1* in flowering time regulation in beet.

Evidently, *BvBBX19* is a major flowering time regulator, because mutant plants showed a biennial phenotype despite the presence of the *B* allele. In the following, we will discuss whether *BBX19* could be a functional ortholog of *CO*. Among the BBX class of genes, only *CO* and *COL* genes and their homologs have evolved a function as flowering time regulators with the exception of *BBX24* (21). The BvBBX19 protein is clearly different from CO and COL proteins as it harbors two B-box domains but lacks the CCT domain. To analyze the function of *BvBBX19*, we will compare the two mutants with each other. The single mutations found in each biennial mutant line occurred within the second B-box domain. This strongly indicates that the second B-box domain is important for the proper function of the BvBBX19 protein to initiate floral transition. In *Arabidopsis*, the importance of B-box domains of the CO protein was established by Robson et al. (25) through analyzing several *CO* mutants carrying mutations within the B-box domain. Since all these mutants showed severely delayed flowering they reasoned that all B-box domains are important for the proper function of CO to initiate floral transition.

We have demonstrated that *BvBBX19* acts upstream of *BvFT2* which is down-regulated by *BvFT1* (12). A regulation of *BvFT1* and *BvFT2* by *BTC1* has already been reported by Pin et al. (2). This raises the question, whether *BvBBX19 and BTC1* jointly regulate the expression of their downstream targets. The following lines of evidence show that both genes do not act independently. First, mutations in *B2* (*B2′*) compromise the annual *B* allele turning all *B2′* genotypes into biennials irrespective of the *B* genotype. Thus, *BvBBX19* seems to act epistatically over *BTC1*. Second, mutations in both genes have similar effects on the expression of *BvFT1* and *BvFT2*. Third, annual bolting is only promoted in plants carrying both the dominant *BTC1* allele and the functional *BvBBX19* allele. Thus, we suggest that both proteins act together to promote bolting through the regulation of *BvFT1* and *BvFT2*. Protein–protein interactions involving BBX proteins have been demonstrated. In a recent study, Gangappa et al. (26) could prove by yeast two-hybrid and pull-down assays that BBX25 interacts with HY5 through its B-boxes, because

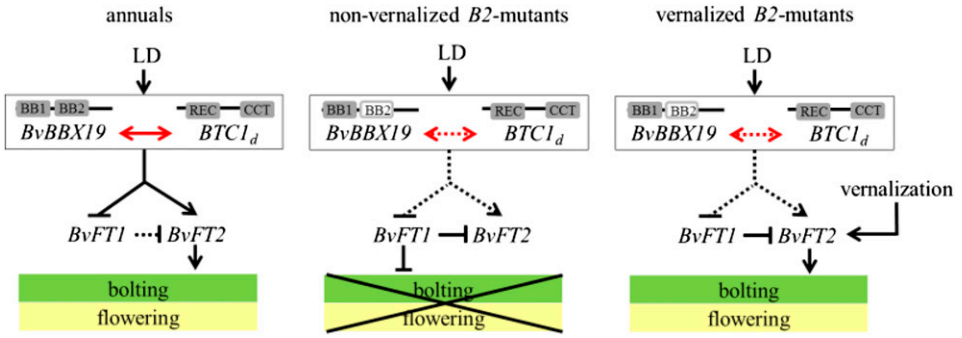

Fig. 6. A proposed epistatic model for bolting time control in beet with *BvBBX19* and $BTC1_d$ acting upstream of *BvFT1* and *BvFT2*. The domain structure of BvBBX19 and $BTC1_d$ proteins is indicated by boxes. The open box represents the mutated domain. An interaction between the two proteins BvBBX19 and $BTC1_d$ or binding of the *BvBBX19* protein to the $BTC1_d$ promoter to acquire a CO function is anticipated. In annuals, the proteins BvBBX19 and $BTC1_d$ act together to repress *BvFT1* and activate *BvFT2* to promote bolting and flowering under LD conditions, whereas a mutation in *B2* mutants prevents floral transition without vernalization, because the floral activator *BvFT2* is repressed by *BvFT1*. After vernalization and under LD conditions *BvFT2* is activated to initiate bolting and flowering.

single substitutions in the B-boxes resulted in complete abolition of the interaction.

It is conceivable that mutations within the B-box domains compromise the interaction between BTC1 and BvBBX19 proteins. Likewise, mutations within the *BTC1* gene could have the same effect resulting in a biennial phenotype. Numerous *BTC1* haplotypes have been found, and the proteins encoded differ by several nonsynonymous polymorphisms. Moreover, their promoters differ by the presence of *cis*-regulatory elements (SORLIP, box II) and a large insertion that is only present in the recessive allele that interrupts a series of GT-1 elements (2). Regarding the known regulation of *FT* expression by the CO protein in *Arabidopsis* (1) and under the assumption that in beet the protein BvBBX19 interacts with BTC1 (encoded by the dominant allele), it is tempting to speculate that this protein complex acquires a CO function. This protein complex would consist of two B-box domains, derived from BvBBX19 as well as a CCT and a response regulator receiver domain (REC) domain, derived from BTC1 and thus resembles largely the protein structure of CO. Hence, we proposed a new model for bolting and flowering control in beet (Fig. 6) which provides an incentive for further studies to understand the proposed interaction between BvBBX19 and BTC1.

Materials and Methods

Plant Material and Genetic Mapping. An annual wild beet homozygous at both bolting loci (*BB*/*B2B2*) was crossed with the biennial *B2* mutant which is homozygous for the mutated *B2* allele (*BB*/*B2′B2′*) (Fig. S1). The F_2 population was sown on April 4, 2010 in a greenhouse and transferred to the field in Kiel on May 17/18, 2010. F_3 families were sown on May 16, 2011 and grown in 96mer multipot-plates under natural light conditions outside the greenhouse from May until October 2011 (*SI Materials and Methods*). Molecular markers were genotyped as InDels or cleaved amplified polymorphic sequences (CAPS) (Table S5).

Gene Expression Analysis. Total RNA from leaves was extracted and cDNA synthesized for quantitative RT-PCR (RT-qPCR). Three independent biological and three technical replicates of each sample were analyzed. RT-qPCR was performed with the *Power* SYBR Green PCR Master Mix (Applied Biosystems) on a CFX96 Real-Time PCR detection system (Bio-Rad) with a final reaction volume of 20 μl including a final primer concentration of 20 pM (Table S6). Resulting data were analyzed with the CFX Manager Software v2.1 (Bio-Rad). The comparative CT (ΔC_T) method was applied. Relative expression levels were calculated and normalized to the geometric mean of *BvGAPDH*.

Bioinformatic Analysis. We used the physical map (27) and different versions of the sugar beet draft genome (http://bvseq.molgen.mpg.de) and a collection of preliminary predicted gene models (RefBeet-0.3geneModels) for which the latest version is now available (13). Published sequences like expressed sequence tags (ESTs) (28) and *BvFT1* (12) were used to find scaffolds which are located on chromosome 9. BLAST analyses (29) were performed to map known sequences to the reference sequence using the BLASTN function of the CLC Main Workbench 5.5 (CLC Bio). Screening the region of interest for sugar beet transcripts was performed with the CLC Genomics Workbench 4.0 (CLC Bio) using RefBeet-0.3geneModels. Finally, these transcripts were used as queries for a BLASTX search against the *Arabidopsis* nt/ nr protein databases of TAIR10 (The *Arabidopsis* Information Resource, www.arabidopsis.org/Blast/index.jsp) and GenBank (NCBI; http://blast.st-va.ncbi.nlm.nih.gov/Blast.cgi), respectively. Multiple alignments of the BvBBXs protein sequences were performed with MAFFT Version 7 (30) using the l-ins-i strategy for a more precise conserved domain align. A maximum likelihood phylogeny tree was constructed with Mega5.2 (18) using the WAG model.

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1. Andrés F, Coupland G (2012) The genetic basis of flowering responses to seasonal cues. *Nat Rev Genet* 13(9):627–639.
2. Pin PA, et al. (2012) The role of a pseudo-response regulator gene in life cycle adaptation and domestication of beet. *Curr Biol* 22(12):1095–1101.
3. Hohmann U, Jacobs G, Jung C (2005) An EMS mutagenesis protocol for sugar beet and isolation of non-bolting mutants. *Plant Breed* 124(4):317–321.
4. Abou-Elwafa S, Büttner B, Kopisch-Obuch F, Jung C, Müller AE (2012) Genetic identification of a novel bolting locus in *Beta vulgaris* which promotes annuality independently of the bolting gene *B*. *Mol Breed* 29(4):989–998.
5. Büttner B, Abou-Elwafa SF, Zhang W, Jung C, Müller AE (2010) A survey of EMS-induced biennial *Beta vulgaris* mutants reveals a novel bolting locus which is unlinked to the bolting gene *B*. *Theor Appl Genet* 121(6):1117–1131.
6. Kinmonth-Schultz HA, Golembeski GS, Imaizumi T (2013) Circadian clock-regulated physiological outputs: Dynamic responses in nature. *Semin Cell Dev Biol* 24(5):407–413.
7. Tiwari SB, et al. (2010) The flowering time regulator CONSTANS is recruited to the *FLOWERING LOCUS T* promoter via a unique *cis*-element. *New Phytol* 187(1):57–66.
8. Ben-Naim O, et al. (2006) The CCAAT binding factor can mediate interactions between CONSTANS-like proteins and DNA. *Plant J* 46(3):462–476.
9. Chia TY, Müller A, Jung C, Mutasa-Göttgens ES (2008) Sugar beet contains a large *CONSTANS-LIKE* gene family including a *CO* homologue that is independent of the early-bolting (*B*) gene locus. *J Exp Bot* 59(10):2735–2748.
10. Khanna R, et al. (2009) The Arabidopsis B-box zinc finger family. *Plant Cell* 21(11):3416–3420.
11. Valverde F (2011) CONSTANS and the evolutionary origin of photoperiodic timing of flowering. *J Exp Bot* 62(8):2453–2463.
12. Pin PA, et al. (2010) An antagonistic pair of *FT* homologs mediates the control of flowering time in sugar beet. *Science* 330(6009):1397–1400.
13. Dohm JC, et al. (2014) The genome of the recently domesticated crop plant sugar beet (*Beta vulgaris*). *Nature* 505(7484):546–549.
14. Solovyev V, Kosarev P, Seledsov I, Vorobyev D (2006) Automatic annotation of eukaryotic genes, pseudogenes and promoters. *Genome Biol* 7(Suppl1:S10):1–12.
15. Letunic I, Doerks T, Bork P (2012) SMART 7: Recent updates to the protein domain annotation resource. *Nucleic Acids Res* 40(Database issue):D302–D305.
16. Schultz J, Milpetz F, Bork P, Ponting CP (1998) SMART, a simple modular architecture research tool: Identification of signaling domains. *Proc Natl Acad Sci USA* 95(11):5857–5864.
17. Marchler-Bauer A, et al. (2005) CDD: A Conserved Domain Database for protein classification. *Nucleic Acids Res* 33(Database issue):D192–D196.
18. Tamura K, et al. (2011) MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 28(10):2731–2739.
19. Jung C, Müller AE (2009) Flowering time control and applications in plant breeding. *Trends Plant Sci* 14(10):563–573.
20. Putterill J, Robson F, Lee K, Simon R, Coupland G (1995) The *CONSTANS* gene of *Arabidopsis* promotes flowering and encodes a protein showing similarities to zinc finger transcription factors. *Cell* 80(6):847–857.
21. Gangappa SN, Botto JF (2014) The BBX family of plant transcription factors. *Trends Plant Sci*, 10.1016/j.tplants.2014.01.010.
22. Yano M, et al. (2000) *Hd1*, a major photoperiod sensitivity quantitative trait locus in rice, is closely related to the *Arabidopsis* flowering time gene *CONSTANS*. *Plant Cell* 12(12):2473–2484.
23. Griffiths S, Dunford RP, Coupland G, Laurie DA (2003) The evolution of *CONSTANS*-like gene families in barley, rice, and *Arabidopsis*. *Plant Physiol* 131(4):1855–1867.
24. Campoli C, Drosse B, Searle I, Coupland G, von Korff M (2012) Functional characterisation of *HvCO1*, the barley (*Hordeum vulgare*) flowering time ortholog of *CONSTANS*. *Plant J* 69(5):868–880.
25. Robson F, et al. (2001) Functional importance of conserved domains in the flowering-time gene CONSTANS demonstrated by analysis of mutant alleles and transgenic plants. *Plant J* 28(6):619–631.
26. Gangappa SN, et al. (2013) The *Arabidopsis* B-BOX protein BBX25 interacts with HY5, negatively regulating *BBX22* expression to suppress seedling photomorphogenesis. *Plant Cell* 25(4):1243–1257.
27. Dohm JC, et al. (2012) Palaeohexaploid ancestry for Caryophyllales inferred from extensive gene-based physical and genetic mapping of the sugar beet genome (Beta vulgaris). *Plant J* 70(3):528–540.
28. Schneider K, et al. (2007) Analysis of DNA polymorphisms in sugar beet (*Beta vulgaris* L.) and development of an SNP-based map of expressed genes. *Theor Appl Genet* 115(5):601–615.
29. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* 215(3):403–410.
30. Katoh K, Standley DM (2013) MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Mol Biol Evol* 30(4):772–780.