Two CONSTANS-LIKE genes jointly control flowering time in beet

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Breeding vegetative crops (e.g. beets, cabbage, forage grasses) is challenged by two conflicting aims. For field production, flowering must be avoided while flowering and seed set is necessary for breeding and seed production. The biennial species sugar beet makes shoot elongation ('bolting') followed by flowering after a long period of cold temperatures. Field production in northern geographical regions starts in spring. A thickened storage root is formed only during vegetative growth. It is expected that winter beets, which are sown before winter would have a much higher yield potential. However, field production was not possible so far due to bolting after winter. We propose a strategy to breed winter beets exploiting haplotype variation at two major bolting time loci, B and B2. Both genes encode transcription factors controlling the expression of two orthologs of the Arabidopsis gene FLOWERING LOCUS T (FT). We detected an epistatic interaction between both genes because F₁ plants homozygous for two B/B2 mutant alleles did not bolt even after vernalization. Fluorescence complementation studies revealed that both proteins form a heterodimer in vivo. In non-bolting plants, the bolting activator BvFT2 was completely downregulated whereas the repressor BvFT1 was upregulated which suggests that both genes acquire a CONSTANS (CO) like function in beet. Like CO, B and B2 proteins house CCT and BBX domains which, in contrast to CO are split between the two beet genes. We propose an alternative regulation of FT orthologs in beet that can be exploited to breed winter beets.

The transition from the vegetative to the generative phase is of major interest to crop breeders due to its high relevance for yield and quality. Crop plants show great variation regarding their phenological development. If vegetative parts of the plant are harvested (leaves, roots) they must not enter the reproductive phase, a major step in plant development commonly referred to as floral transition. Sugar beet (Beta vulgaris L.) is a typical vegetative crop with a biennial life cycle. After sowing in spring, it produces huge leaf and root mass until harvest in autumn. As a result of secondary thickening, a storage root is produced with sucrose contents between 17–20%¹. As a biennial plant it enters the reproductive phase only after exposure to a long period of cold temperatures (< 4 °C). Then, the shoot is elongated ('bolting') and flowers are produced. Early bolting under field conditions must be strictly avoided because it gives rise to flowering plants with small roots and low sucrose content. For seed production, plants must bolt and flower early after winter. This follows, that conventional sugar beet cannot be cultivated as a vegetative crop over winter, commonly referred to as 'winter beet'¹.

Quantitative trait loci (QTL) and major genes controlling bolting time have been mapped to the nine beet chromosomes². The bolting time QTL SEASONAL BOLTING-4 and -9 (SBT-4, SBT-9) accounts for up to 52% of the phenotypic variation². The phenotypic effect of SBT-4 is likely caused by the major flowering time regulator BvFT2 because they were mapped to the same position on chromosome 4. SBT-9 was precisely mapped to the position of BR1. This QTL was recently fine mapped by a sequencing approach and a gene similar to CLEAVAGE AND POLYADENYLATION SPECIFICITY FACTOR 73-I (CPSF73-I) from Arabidopsis was suggested as a candidate gene for this QTL². Sugar beet has two sequences which share high homology to FLOWERING LOCUS T (FT) a major integrator of signals from different regulatory pathways triggering floral transition in Arabidopsis⁵. BvFT1 is a floral repressor which is transcriptionally active before winter and prevents bolting. In contrast, BvFT2 is a floral inducer which is activated during vernalization. A high BvFT2 activity is indicative for generative (bolting) beet plants⁶.

Two upstream regulators of the two BvFT orthologs have been cloned. BOLTING TIME CONTROL 1 (BTC1) belongs to the PRR3/7 clade of PSEUDO RESPONSE REGULATOR (PRR) genes that are components of the

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photoperiod pathway in Arabidopsis\(^8\). A dominant allele which is highly abundant in wild beet (\textit{B. vulgaris ssp. maritima}) populations from the Mediterranean causes early bolting (without vernalization) resulting in an annual life cycle. Another \textit{PRR7} homolog, \textit{BvPRR7}, is a cold responsive gene with a clock function in beets but not involved in bolting time regulation\(^7\). The second bolting time gene, \textit{BvBBX19}, encodes a putative transcription factor with two B-Box zinc finger motifs but lacking a CCT domain\(^9\). Recently, haplotype variation of the four major bolting time genes from beet have been studied in wild and cultivated beet accessions\(^9\). For \textit{BCT1} and \textit{BvBBX19}, 14 and 7 haplotypes were found, respectively\(^9,10\). They were classified as annual or biennial bolting time regulators. \textit{BCT1} and \textit{BvBBX19} share homology with the transcription factor \textit{CONSTANS} (CO), which regulates floral transition in Arabidopsis in a long day (LD) dependent manner\(^11\). It has two consecutive zinc finger domains which are called B-Boxes\(^12\). Mutants with amino acid alterations in conserved residues of the B-Boxes are late flowering. At the C-terminus, the CO protein has a CCT (CO, CONSTANS-LIKE, and TIMING OF CAB EXPRESSION1) domain which includes a nuclear import signal. By its CCT domain, CO binds to the ubiquitin ligase COP1 and to the FT promoter by forming complexes with other transcription factors\(^13\). This sequence is strictly conserved in proteins which are constituents of the circadian clock\(^15\). CDF (CYCLING DOF FACTORS) transcription factors bind to the CO promoter and inhibit its expression during the morning. Later, they are degraded by the proteasome when GIGANTEA (GI) interacts with FLAVIN BINDING, KELCH REPEAT, F-BOX PROTEIN 1 (FKF1) and ZEITLUPE (ZTL) resulting in strong transcriptional upregulation of CO\(^15\). The CO protein is stabilized by light and degraded in darkness after ubiquitination and proteolysis by the 26S proteasome\(^16\).

In Arabidopsis, apart from CO there are at least 31 genes encoding proteins with B-Box and CCT domains, 16 are CO-Like (COL) proteins with one or two B-Boxes and one CCT domain, the remaining ones are either lacking the CCT domain, or one B-Box and the CCT domain\(^9,10\). \textit{BBX19} and CO are forming dimers which jointly regulate FT in an antagonistic way\(^17\). \textit{BBX32} physically interacts with \textit{COL3} to form a dimer which targets the FT promoter\(^18\). Interestingly, beet has a large \textit{CONSTANS-LIKE} gene family but is lacking a functional CO ortholog with both domains\(^18\). \textit{BCT1} is lacking a B-Box and \textit{BvBBX19} is lacking a CCT domain.

The purpose of this work was to understand the genetic and physical interaction between \textit{BCT1} and \textit{BvBBX19} and to lay the foundations to breed winter beets. We assumed that both proteins work together to acquire a CO-like function. To test our hypothesis, we studied an \textit{F}\textsubscript{2} population segregating for both genes. We found an epistatic interaction between both loci which resulted in three different life cycle regimes. Combining two mutant alleles resulted in plants which completely lost their competence to bolt after vernalization. The genetic data were confirmed by yeast-two-hybrid interaction and \textit{in planta} bimolecular fluorescence complementation studies. Double mutant plants are proposed as prototypes for winter beet breeding which requires complete bolting control after winter.

Results

The \textit{B2} locus is epistatic to \textit{B}. We produced an \textit{F}\textsubscript{2} population from a cross between two biennial beet genotypes, seed code 093187 (\textit{B}\textsubscript{a} \textit{B2}\textsubscript{B2}) and 056822 (\textit{B}\textsubscript{a} \textit{B2}\textsubscript{B2}) which differed by their \textit{B} and \textit{B2} alleles. 145 plants were grown under long day conditions together with their parents and the annual and biennial controls. We determined the genotypes of the \textit{B} and \textit{B2} loci for all \textit{F}\textsubscript{2} plants using the markers CAU4234 and CAU4235 (Supplementary Tables 1 and 2). In accordance with their position on different chromosomes, the observed genotypic segregation fitted a random segregation ratio ($\chi^2 = 15.77$; $\alpha = 0.05$) (Supplementary Tables 3 and 4).

The biennial controls bolted within 3–4 weeks after vernalization whereas the annual controls bolted early (4–6 weeks after sowing) without vernalization required (Fig. 1A). Most \textit{F}\textsubscript{2} plants carrying at least one \textit{Ba} and one \textit{B2} allele were lacking a vernalization requirement because they bolted within 114 days after sowing like plants from the annual controls (Fig. 1A). Six \textit{F}\textsubscript{2} plants with the \textit{BaBa} \textit{B2fB2f} genotype did not bolt prior to cold treatment. However, these plants were the earliest biennials as they bolted already 9–16 days after vernalization. Neither the \textit{F}\textsubscript{2} genotypes carrying the homozygous \textit{Ba} allele in combination with \textit{B2} or \textit{B2} alleles (\textit{B}\textsubscript{a} \textit{B2f} \textit{B2f}, \textit{B}\textsubscript{a} \textit{B2h} \textit{B2h}, \textit{B}\textsubscript{a} \textit{B2h} \textit{B2f}) nor those plants carrying the \textit{B2} allele (heterozygous or homozygous) in combination with the homozygous \textit{B2} allele (\textit{B2} \textit{B2} \textit{B2}, \textit{B}\textsubscript{a} \textit{B2f} \textit{B2f}) were able to bolt without cold treatment. After vernalization, all \textit{F}\textsubscript{2} plants homozygous or heterozygous for the \textit{B2} allele (\textit{B}\textsubscript{a} \textit{B2} \textit{B2}, \textit{B}\textsubscript{a} \textit{B2} \textit{B2}) started shoot elongation within three weeks which is typical for biennial beets.

Consistent with our initial hypothesis, the \textit{F}\textsubscript{2} population displayed a third phenotypic class for bolting time because 27 out of 30 \textit{F}\textsubscript{2} plants that carry the homozygous \textit{B2a} allele in combination with the homozygous and heterozygous \textit{Ba} allele and all 17 \textit{B}\textsubscript{a} \textit{B2} \textit{B2}, \textit{F}\textsubscript{2} plants failed to bolt until the end of the experiment (325 days after sowing). The fact that almost all \textit{F}\textsubscript{2} plants carrying the homozygous \textit{B2} allele were non-bolting after vernalization irrespective of the \textit{B} allele indicates that the \textit{B2} allele is able to ‘mask’ the phenotypic effect of the \textit{Ba} or \textit{B2} alleles.

How can transgressive variation in the \textit{F}\textsubscript{2} population be explained? We tested two genetic hypotheses to explain the phenotypic segregation observed in this experiment (Supplementary Table 4). Our initial hypothesis follows the assumption that all plants carrying at least one \textit{Ba} and \textit{B2} allele are annual, plants which are homozygous for either the \textit{B} or \textit{B2} allele are biennial, and only the double homozygous \textit{F}\textsubscript{2} plants (\textit{B}\textsubscript{a} \textit{B2} \textit{B2}) do not bolt after vernalization giving rise to a phenotypic segregation of 9:6:1 (annual: biennial: non-bolting after vernalization). This hypothesis was rejected after a $\chi^2$ test for goodness of fit to a 9:6:1 ratio ($\chi^2 = 150.03$; $\alpha = 0.01$). The second hypothesis is based on the assumption that the \textit{B2} allele acts epistatically over the \textit{B} locus. In this case, a 9:4:3 phenotypic segregation was to be expected (Supplementary Table 4). As this segregation rate was not rejected ($\chi^2 = 2.24$; $\alpha = 0.01$), we assume that the \textit{B2} allele which was derived from an EMS mutagenesis acts epistatically to \textit{B} resulting in a non-bolting (after vernalization) phenotype (Supplementary Table 4). However, this interaction does not fully explain phenotypic variation because biennial plants were found in the \textit{B}\textsubscript{a}\textit{B2} parent 056822 and among the corresponding \textit{F}\textsubscript{2} genotypes (Fig. 1A). In conclusion, genetic analyses are clearly pointing at a joint activity of both loci to control the onset of bolting.
The floral promoter BvFT2 is completely downregulated in beets which do not bolt after vernalization. We questioned whether the transcript levels of BTC1 and BvBBX19 differ between F2 plants bolting and non-bolting after vernalization. Therefore, the effect of different B/a/Bd and B2/B2, allele combinations on their transcriptional activity was investigated using the same F2 plants as for the genetic experiments. Leaves were taken 23 days after cold treatment every 4 hours over 24 hours. We observed no significant differences in the diurnal expression pattern of BTC1 between double homozygous F2 plants (B/aB/aB/a) and F2 plants that are homozygous for the B2h allele and the biennial controls (Fig. 2A) despite of strikingly different life cycle regimes. BTC1 was upregulated during the day and the transcript levels decreased during the night in controls as well as in F2 individuals bolting after vernalization (B/aB/aB/aB/a) and F2 genotypes non-bolting after vernalization (B/aB/aB/aB/a). For BvBBX19 we detected generally low expression levels during the day with continuously increasing transcript levels during the night in all plants which bolted after vernalization except for the parental genotype 056822 (B/aB/aB/aB/a). In general, the BvBBX19 transcript levels were increased during the day (ZT8) in all plants which is in accordance with previous data. Interestingly, upregulation was also observed in F2 genotypes that failed to bolt after the cold treatment (B/aB/aB/aB/a and B/aB/aB/aB/a) however at a much lower level when compared to the parental genotype 056822 (Fig. 2C).

We reasoned that the BTC1/BvBBX19 genotype impacts the expression of the two FT paralogs BvFT1 and BvFT2. It had been demonstrated that floral transition in beet is promoted through downregulation of the floral repressor BvFT1 and therewith upregulation of the floral inducer BvFT2, which both are downstream targets of BTC1 and BvBBX19. We observed that the transcriptional activity of BvFT1 and BvFT2 follows the anticipated expression pattern (Fig. 2B,D). As expected, BvFT2 was highly upregulated and BvFT1 completely downregulated after vernalization in the biennial controls and in biennial F2 plants. Interestingly, a contrasting expression pattern was observed in F2 plants which did not bolt after vernalization. The transcriptional activity of BvFT1 was two times higher in non-bolting F2 plants homozygous for B/aB/a as compared to F2 plants homozygous for B/aB/a.

BvBBX19 and BTC1 physically interact with each other. The absence of a gene in sugar beet encoding a canonical CO protein suggested that one or several other proteins fulfill the function of CO in this plant. The most likely candidates are BvBBX19 and BTC1 since they contain two B-Box domains and the CCT domain, respectively resembling the CO domain structure. One likely scenario how BvBBX19 together with BTC1 can replace CO is direct physical interaction between the two proteins resulting in a functional CO ortholog. To test this hypothesis, we performed yeast-two-hybrid studies. Constructs were made containing the full-length coding regions of BvBBX19 and BTC1 fused to either the GAL4 DNA-binding domain (BD) or the GAL4 activation domain (AD) at the N-terminus of the respective proteins. In addition, we included constructs of the previously identified BvBBX19 mutant (BvBBX19m), which contains a premature stop codon resulting in a BvBBX19 variant with only one B-Box. Again, AD or BD-domains were fused to the N-terminus of BvBBX19m. Wild type BvBBX19 and BTC1 constructs showed no autoactivation and were thus useful to study interaction between the two proteins. For both combinations of wild type BvBBX19 with BTC1, we observed growth of
yeast cells on selective plates (-Leu, -Trp, -His) as well as induction of the \( \alpha \)-galactosidase reporter in the quantitative assays (Fig. 3). Thus, BvBBX19 a and BTC1 d interact. In case of BvBBX19 h we observed autoactivation for the BD-BvBBX19 h construct. Thus, this construct was not useful for further interaction studies. However, AD-BvBBX19 h did not result in autoactivation. In combination with BD-BTC1 d, colonies were formed on selective medium and \( \alpha \)-galactosidase activity induced. This result implies that the second C-terminally located B-box in BvBBX19 a, which is missing in BvBBX19 h, is not essential but supportive for the interaction with BTC1 d.

Y2H data strongly suggested direct physical interaction between BvBBX19 and BTC1. For further confirmation, we applied ratiometric bimolecular fluorescence complementation assays (rBiFC). We used constructs where either the 5' or the 3' region of BvBBX19 was fused with the 5'-terminal half of YFP (nYFP). Accordingly, BTC1 d was fused with the C-terminal part of YFP (cYFP) at its N- or C-term. These constructs were co-transfected into Nicotiana benthamiana leaves using Agrobacterium-mediated infiltration. All four combinations resulted in YFP signals (Fig. 4A) in contrast to co-expression of the non-fused nYFP controls with BTC1 d fused to cYFP at its N- or C-term (Fig. 4B). The truncated BBX19 version nYFP-BvBBX19 h co-transfected with BTC1 d carrying cYFP at the N- or C- term also gave a clear YFP signal in contrast to BvBBX19 h constructs carrying nYFP at the C-term (Fig. 4C). The latter result is expected since BvBBX19 h contains a stop codon upstream of the second B-Box and thus does not allow expression of the C-terminal YFP half. Interestingly, in all cases complemented YFP signals were observed in nuclear bodies. Quantification of the YFP against the RFP fluorescence signals from at least 20 images (Fig. 4D) are consistent with the representative pictures presented in Fig. 4A–C.

**Discussion**

We have performed a genetic study with all combinations of BTC1 (B) and BvBBX19 (B2) alleles in an F2 population. B2 is a typical annual allele only found in wild beet populations from the Mediterranean. B2h carries six non-synonymous SNPs and a large insertion in the promoter compared to 'annual' alleles6. B2f is found in annual beets9 while B2h is a nonsense EMS mutant allele8.

Beet is a typical long day plant. Bolting even in the presence of the early bolting alleles or after vernalization is strongly delayed in short days19. Therefore, all experiments were performed under long day conditions. An annual life cycle requires an annual B allele and a functional B2 allele (B2/B2). The competence for early flowering is
lost in plants homozygous for the B2 mutant allele $BvBBX19h$ irrespective of the $B$ allele. Likewise, $B$ knockdown (RNAi) plants cannot flower any more even after vernalization.

Double mutants homozygous for the biennial $B$ and the non-functional B2 allele completely lost their competence to bolt which confirmed our initial hypothesis that $B$ and B2 jointly regulate the onset of bolting in sugar beet. By combining two mutant alleles, we could select plants that did not bolt even after cold treatment. We found an incomplete epistatic interaction between bolting resistant plants among all $B2h$ homozygous genotypes, but the presence of the $Bd$ alleles modified the $B2h$ effect because biennial plants were present in the $BdB2h$ parent and F2 plants. We assume that apart from these two major bolting time regulators, additional genes can modify bolting time. Moreover, the rare occurrence of spontaneously bolting plants in production fields points at environmental factors modifying the activities of $B$ and B2. These factors together may explain the presence of biennial plants in the 056822 parent.

The genus *Beta* comprises iteroparous perennials with an annually repeated requirement for vernalization.

Future studies with perennial wild beets will resolve the question whether the BTC1/$BvBBX19$ module and the $BRI$ QTL control the perennial life cycle. However, strictly non-bolting genotypes are likely to be a dead end of

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**Figure 3.** Yeast-2-Hybrid analysis showing interaction of BTC1 with BvBBX19. The proteins were fused at their N-terminus to either the DNA binding domain (BD) or the activation domain (AD) of the GAL4 transcription factor. (A) Yeast cells were transformed with vectors harboring the indicated constructs. Aliquots of overnight cultures were spotted on non-selective (-Leu, -Trp) or selective (-Leu, -Trp, -His) plates and tested for His auxotrophy. Nine clones of each plasmid combination were tested and one representative result is shown. (B) Quantification of BvBBX19/BTC1 interaction using α-galactosidase assay. Means ± SD of three technical replicates are displayed. The BvBBX19 mutant (BvBBX19h) with a premature stop codon after the first B-Box domain was included in these studies. BvBBX19h showed autoactivation (indicated by asterisks).
Figure 4. Interaction between BvBBX19 and BTC1 analyzed by ratiometric bimolecular fluorescence complementation (rBiFC). (A–C) Confocal pictures of *Nicotiana benthamiana* leaves three days after *Agrobacterium tumefaciens* infiltration with the rBiFC constructs. The C-terminal part of YFP (cYFP) or the N-terminal part of YFP (nYFP) was fused to the target proteins (X) at their N-terminus (c/nYFP-X) or at their C-terminus (X-c/nYFP). Fluorescence was detected with a Leica TCS SP5 Confocal Laser Scanning Microscope. YFP was excited with a 488 nm laser and RFP with a 561 nm laser. YFP fluorescence was detected between 535 nm and 560 nm. RFP fluorescence was detected between 600 nm and 625 nm. (A) shows BvBBX19a interaction with BTC1d, (B) negative controls for BTC1d, and (C) interaction of mutant BvBBX19 (BvBBX19h) withBTC1d. Scale bars, 10 µm. (D) Quantification of the mean fluorescence of split-YFP normalized against RFP. Data are means ± SD of at least 20 images selected at random.
evolution because they cannot reproduce sexually in contrast to iteroparous plants which flower and set seeds in subsequent years after winter. Thus, it is no surprise that despite of extensive screenings Btc1/BvbBX19 double mutants have not been found in nature so far.

The B2 genotype exhibited a strong requirement for vernalization even in the presence of the early bolting allele B3. This indicates that there are upstream regulators of the Btc1/BvbBX19 module which respond to cold temperatures and to alterations of the B2 protein. This makes B2 a primary target of a putative vernalization regulatory pathway. However, no further mutants have been detected so far. Searching for orthologous genes from Arabidopsis has not been successful and beet lacks a functional ortholog of FLOWERING LOCUS C which is a major integrator of signals from the vernalization pathway in Arabidopsis. Seemingly, divergent vernalization pathways have evolved in both species. Because vernalization has an epigenetic basis, genes responding to methylation might be interesting candidates. Consequently, two genes, SHORT VEGETATIVE PHASE (BvSVP) and BvVIN3 come into focus as upstream regulators of the Btc1/BvbBX19 module because they are hypomethylated and/or differentially expressed after cold exposure.\(^{31,32}\) How can the bolting-resistant phenotype be explained by protein-interaction and expression studies? BTC1 requires a functional BvbBX19 protein consistent with our data that beets carrying the annual B3 allele do not bolt in the presence of the mutant B2 allele. Loss of competence to bolt is due to downregulation of the floral inducer BvFT2 and upregulation of the floral repressor BvFT1. The non-bolting phenotype of the B2 mutant is not caused by a lack of protein-interaction because binding between BvbBX19s and BTC1 is demonstrated (Figs 3 and 4). Y2H data imply that BBX19 and BTC1 do not require another beet protein for their interaction. However, it cannot be excluded that BTC1 and BvbBX19 interact with other proteins as CO does with PHYTOCHROME INTERACTING FACTOR 4\(^{33}\). We propose a model where Btc1 and BvbBX19 (mutated and wild type) dimerize to bind to the BvFT2 promoter. Likewise, the interaction between BTC1,BvFT1 and/or differentially expressed after cold exposure 21,22.

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Materials and Methods

Plant material and growth conditions. We performed a cross between two single plants of the biennial beet lines seed code 056822 (plant #15) and 093187 (plant #8). The female parent 056822 carries the BTC1 haplotype BTC1d allele only found in annuans beets which confers early bolting without vernalization3 and a mutated BvBBX19 allele4, which we termed BvBBX19f, following the haplotype nomenclature described by Höft et al.5. The pollinator parent 093187/8 carries the bct1a allele and the functional BvBBX19 allele. For ease of understanding we will use the allele nomenclature as B1 (haplotype BTC1d), B2 (haplotype bct1a), B2 (haplotype BvBBX19) and B2 (haplotype BvBBX19f).

For phenotyping, 145 plants of the F1 population 142063 were grown in a climate chamber under long day conditions (16 h light/8 h dark, 320 μmol m−2 s−1) for 325 days. The two parent lines 056822 and 133703 (selfing progeny of 093187), three biennial (seed codes 092492, 930184, 930176) and two annual genotypes (001684, 991971) were grown as controls (five plants per line). Plants were first grown in 9 cm pots for 135 days at 20 °C and then cold treated at 4 °C for 12 weeks, followed by an acclimatization phase at 12 °C for three days. For the rest of the experiment, they grew again in 11 cm pots at 20 °C for another 102 days. Every second day, plants were randomized and the onset of bolting was recorded (BBCH scale code: 51) according to Meier et al.6. Finally, plants were classified as follows: (1) annual plants which bolted within 135 days, (2) biennial plants which bolted only after cold treatment, and (3) plants, which did not bolt until the end of the experiment after 325 days.

DNA techniques. For DNA isolation, leaves were harvested from six-weeks-old F1 plants and freeze dried. Genomic DNA was isolated applying the CTAB method7. A 10-fold dilution of the extracted DNA was later used for PCR using Taq DNA Polymerase (Invitrogen). We used the InDel marker CAU4234 and the CAPS marker CAU4235 for genotyping the BTC1 and BvBBX19 locus, respectively (Supplementary Table 2). PCR products were separated on 1% agarose gels.

Gene expression analysis. We measured the diurnal expression of the four flowering time genes BTC1, BvBBX19, BvFT1 and BvFT2 by qRT-PCR in F1 plants with the BTC1 and BvBBX19 haplotypes B1, B2, B2, B2, B2, B2, B2 and B1, B2, B2, B2, B2, B2, B2, and in the biennial controls 056822 (B1, B2, B2, B2), 133703 (B1, B2, B2, B2) and 930176 (B1, B2, B2, B2). Total RNA was isolated from young leaves that were harvested 23 days after cold treatment in a 4 hours interval over 24 hours (first measurement at ZT 0, the time of lights on). Total RNA was extracted with thepeqGOLD Plant RNA Kit (PeqLab) and subsequently treated with DNase. 500 ng of total RNA was reverse transcribed using a First Strand cDNA Synthesis kit (Fermentas). Resulting cDNA was diluted 10-fold and 2 µl of the diluted solution were used for PCR detection system (Bio-Rad) with a final reaction volume of 20 µl. Three independent biological and three technical replicates were described using a First Strand cDNA Synthesis kit (Fermentas). Resulting cDNA was diluted 10-fold and 2 µl of the diluted solution were used for PCR detection system (Bio-Rad) with a final reaction volume of 20 µl. Three independent biological and three technical replicates were described with the CFX Manager Software (Bio-Rad). Data were analyzed with the CFX Manager Software v2.1 (Bio-Rad). Expression levels were first calculated with the comparative CT (ΔCT) method and then normalized to the geometric mean of BvGAPDH to calculate the relative expression levels.

Yeast-2-Hybrid assays. Yeast-2-Hybrid experiments were performed using the Matchmaker Gold Yeast-Two-Hybrid System (Clontech). The proteins of interest were fused at their N-terminus to either the DNA binding domain (BD) or the activation domain (AD) of the GAL4 transcription factor by insertion of the full-length coding sequences of BvBBX19p, BvBBX19n (BvBBX19 mutant) and BTC1 into the NcoI and XhoI sites of the vector pACT2 or the Ncol and SaI sites of the vector pAS2-1. Full-length coding sequences of BvBBX19p, BvBBX19n, BTC1p, and BTC1n were obtained by PCR with primers listed in Supplementary Table 2. The correctness of the amplified sequences was verified by sequencing. Yeast cells (strain Y2H Gold, Clontech) were transformed according to the supplier’s manual. Screening for histidine auxotrophy was done with nine clones of each trans-construct combinations mentioned above and with p19 to suppress gene silencing37. Three days after infiltration YFP complementation was analyzed using a Leica TCS SP5 Confocal Laser Scanning Microscope (Leica).
YFP was excited with a 488 nm laser and RFP with a 561 nm laser. YFP fluorescence was detected between 535 nm and 560 nm, RFP fluorescence between 600 nm and 625 nm. Quantification of the mean fluorescence of split-YFP was done by normalization against RFP. Data were calculated as means of at least 20 images selected at random. Relative fluorescence was determined using ImageJ estimating the mean grey value of the different pictures within an area of around 5 pixels. The maximum grey value per pixel of YFP fluorescence was set as 225.

Expression of BrBBX19, or BvBBX19, fused to nYFP or unfused nYFP (as negative control for rBiFC) was detected via the HA-tag positioned at the C-terminus of nYFP (Fig. S1). Proteins were extracted from infiltrated leaf tissues by TCA precipitation. 40 μg of proteins per lane were separated by SDS-PAGE. After blotting, the nitrocellulose membrane was blocked with 7% milk powder in TBS and probed with rat anti-HA antibody (Roche, 11867423001, 1:1,500 in TBS-T).

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Author Contributions
N.D. and M.E. designed the experiments and analysed the data. N.D. performed phenotyping, genotyping and expression analysis. M.E. performed yeast-2-hybrid assays, ratiometric Bimolecular Fluorescence Complementation (rBiFC) and immunoblots. C.J., A.B. supervised the project and together with N.H. wrote the manuscript with input from all authors.

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