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## **The Evolution of Flowering Time Regulation in *Beta* Species**

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## List of Abbreviations

°C	Degree Celsius
μE	Microeinstein
μl	Microliter
μmol m <sup>-2</sup> s <sup>-1</sup>	Micromoles of photons per square meter and second
μM	Micromolar
A	Adenine
<i>A. tumefaciens</i>	<i>Agrobacterium tumefaciens</i>
ANCOVA	Analysis of covariances
<i>At</i>	<i>Arabidopsis thaliana</i>
<i>B</i>	<i>Bolting locus from Beta vulgaris</i>
<i>B2</i>	Second bolting locus from <i>Beta vulgaris</i>
<i>B3</i>	Third bolting locus from <i>Beta vulgaris</i>
<i>B4</i>	Fourth bolting locus from <i>Beta vulgaris</i>
<i>B5</i>	Fifth bolting locus from <i>Beta vulgaris</i>
BBCH	Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie
<i>BBX19</i>	<i>DOUBLE B-BOX TYPE ZINC FINGER 19</i>
BLAST	Basic Local Alignment Search Tool
blastp	Standard protein BLAST
bp	Base pairs
<i>BTC1</i>	<i>BOLTING TIME CONTROL 1</i>
<i>Bv</i>	<i>Beta vulgaris</i>
<i>BvFLI</i>	<i>Beta vulgaris FLC</i> homolog
C	Cytosine
CaMV	Cauliflower Mosaic Virus
CAPS	Cleaved amplified polymorphic sequence
CCT	CONSTANS, CONSTANS-LIKE, TOC1
cDNA	Complementary DNA
cM	Centimorgan
<i>CO</i>	<i>CONSTANS</i>
<i>COL</i>	<i>CONSTANS-LIKE</i>
Col-0	<i>A. thaliana</i> ecotype Columbia
<i>COPI</i>	<i>CONSTITUTIVE PHOTOMORPHOGENIC 1</i>
CT	Cycling threshold
CTAB	Cetyl trimethylammonium bromide
DNA	Deoxyribonucleic acid
DTB	Days to bolting
DTF	Days to flower after sowing
<i>E. coli</i>	<i>Escherichia coli</i>
e.g.	<i>Exempli gratia</i>
EMS	Ethyl methanesulfonate
et al.	<i>Et alii</i>
F <sub>1</sub>	First generation after cross
F <sub>2</sub>	Second generation after cross
F <sub>3</sub>	Third generation after cross
<i>FLC</i>	<i>FLOWERING LOCUS C</i>
<i>FT</i>	<i>FLOWERING LOCUS T</i>
G	Guanine
g	Gram
<i>GAPDH</i>	<i>GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE</i>

gDNA	Genomic DNA
<i>GI</i>	<i>GIGANTEA</i>
h	Hour
<i>Hdl</i>	<i>HEADING DATE 1</i>
<i>Hv</i>	<i>Hordeum vulgare</i>
<i>HY5</i>	<i>LONG HYPOCOTYL 5</i>
i.e.	<i>Id est</i>
InDel	Insertion or deletion marker
l	liter
LD	Long day
<i>Ler</i>	<i>A. thaliana</i> ecotype Landsberg <i>erecta</i>
M1	Marker allele 1
M2	Marker allele 2
M <sub>1</sub>	First generation after mutagenesis
M <sub>2</sub>	Second generation after mutagenesis
mg	Milligram
mRNA	Messenger ribonucleic acid
ng	Nanogram
<i>nptII</i>	<i>Neomycin phosphotransferase II</i>
ORF	Open reading frame
<i>pat</i>	Phosphinothricin acetyltransferase
PCR	Polymerase chain reaction
<i>PRR</i>	<i>PSEUDO RESPONSE REGULATOR</i>
QTL	Quantitative trait locus
R	Recombination frequency
REC	Response regulator receiver
RNA	Ribonucleic acid
RNAi	RNA interference
RT-PCR	Reverse transcription polymerase chain reaction
RT-qPCR	Quantitative real-time polymerase chain reaction
SD	Short day
SNP	Single nucleotide polymorphism
SORLIP	Sequences over-represented in light-induced promoters
subsp.	Subspecies
T	Thymine
T <sub>1</sub>	First generation after transformation
T <sub>2</sub>	Second generation after transformation
T <sub>3</sub>	Third generation after transformation
T35S	Terminator derived from CaMV
T-DNA	Transfer DNA
TNCL	Total number of cauline leaves
TNRL	Total number of rosette leaves
TOC1	TIMING OF CAB EXPRESSION 1
<i>UBI</i>	<i>Ubiquitin</i>
UTR	Untranslated region
Zn	Zinc
ZT	Zeitgeber time
$\chi^2$	Chi square

# 1 General Introduction

## 1.1 The sugar beet crop and its cultivated and wild relatives

Species of the genus *Beta* are mainly dicotyledonous and belong to the order of Caryophyllales (family *Amaranthaceae*, subfamily *Chenopodiaceae*) (Märländer et al. 2011; The Angiosperm Phylogeny 2009). These species are divided into two sections (Kadereit 2006): Section I includes all cultivated forms (*Beta vulgaris* ssp. *vulgaris*) and their wild progenitors (*Beta vulgaris* ssp. *maritima*). Species of this section mainly grow within the coastal region of northwest Europe, and around the Mediterranean. Section II (Corollinae) consists of only wild species growing in mountainous and hilly regions in Turkey and adjacent countries. Besides diploids, this section includes tetraploid, pentaploid and hexaploid species, too (Melzer et al. 2014). The usage of leaves as animal feed and pot herbs was probably the beginning of domestication of *Beta* species (Hancock 2012). Cultivated beets exhibit huge morphological variation in leaves, roots and color and are divided into four agronomically important groups: leaf beet (*B. vulgaris* ssp. *vulgaris* Leaf Beet Group), fodder beet (*B. vulgaris* ssp. *vulgaris* Fodder Beet Group), garden beet (*B. vulgaris* ssp. *vulgaris* Garden Beet Group) and sugar beet (*B. vulgaris* ssp. *vulgaris* Sugar Beet Group) (Kadereit 2006). While leaf beets are mainly harvested for their nutritious, healthy leaves, the other forms are harvested for their roots. Red table beets form a thickened root and are well known for their intense dark red hypocotyl. Their main utilization is for production of natural colors for food additives, as well as their usage as vegetable. Fodder beets are mainly produced for animal nutrition.

The history of sugar beet cultivation in Germany is quite short and was initiated in 1747 by Andreas Sigismund Marggraf's discovery that sugar crystals obtained from beet juice were identical to those of sugar cane. Resting upon these findings, Marggraf's student Franz Carl Archard developed the "White Silesian" as the ancestor of all sugar beet cultivars (Biancardi et al. 2005). Soon, the first beet root processing factory was built in Cunern, Silesia in 1801 (Hancock 2012; Melzer et al. 2014). Sugarcane (*Saccharum officinarum*) has always been the main source of sugar in Europe but during the early 19<sup>th</sup> century, sugar beet became a meaningful alternative. During the Napoleonic war in 1811 the import of cane sugar was substituted and beet sugar became of great importance (Cooke and Scott 1993).

During the years of cultivation, the sugar beet crop itself was steadily improved and the sugar beet industry became insistently established world-wide (Draycott 2006). In 2015/2016 sugar beets were cultivated on 29,508 agricultural holdings with 254,843 ha area under cultivation. The sugar amount produced by sugar beet cultivation was 2,942 million tons in the financial year 2015/2016. However, sugar beet is the only sucrose storing crop plant used for sugar production in Europe. Besides sugarcane which is mainly produced in southern climates, sugar beet accounts for about 20 % of the worldwide sugar production (<http://www.zuckerverbaende.de/zuckermarkt/zahlen-und-fakten/weltzuckermarkt/erzeugung-verbrauch.html>). Nowadays, the sucrose content of sugar beet ranges between 15 and 18% (total dry weight), while at the beginning of cultivation it was estimated to be around 4% (Märländer et al. 2011). Intriguingly, 90% of the sugar derives from the root, while about 10 % derive from hypocotyl (Hancock 2012). Besides sugar production, the production of bioethanol and biogas became of greater importance during the last years.

A major breeding aim of sugar beet breeding for the past 200 years has always been the enhancement of extractable sugar content and its quality. Besides sucrose content exaltation, many differential objectives became important to focus on, such as prevention of premature bolting and disease resistance.

Bolting is an undesirable trait which drastically reduces root yield (Jung and Müller 2009) and interferes with harvest operations (Smyth et al. 2014). Sugar beet plants are usually sown in early spring, when premature bolting can be caused by a higher risk of exposure of young plants to cold temperatures. In addition, resistance to different environmental factors which influence beet growth, such as nematodes, viruses or fungal pathogens became another important breeding aim (Biancardi et al. 2005; Dohm et al. 2013). However, one of the main limiting factors of beet growth and therewith sugar yield still is an insufficient canopy of young sugar beet plants at their beginning of their vegetative period (Hoffmann and Kluge-Severin 2010; 2011). A strategy to overcome this problem would be the production of winter beets. These overwintering sugar beets would be sown in autumn and harvested in the following year. The expectation is that winter beets develop a higher leaf area index during prolonged cultivation (Jaggard and Werker 1999) which enables increased light absorption. The anticipated result would be a yield increase of up to 26% (Hoffmann and Kluge-Severin 2010). Anyway, winter hardiness and bolting control are necessary for an implementation.

## 1.2 The life cycle of *Beta* species

Species of the genus *Beta* have evolved different life cycles to ensure their reproduction. Wild beets (ssp. *maritima*) from Mediterranean environments mainly exhibit an annual life cycle. They germinate, bolt and flower within one season under long day conditions without any requirement for vernalization. In comparison, cultivated beets (ssp. *vulgaris*) as well as wild beets from northern latitudes, show a biennial growth habit and require prolonged exposure to cold temperature over winter to acquire floral competence. In the first year, biennial beets grow vegetative and form taproots and leaf rosettes. In the following year after winter, the plants turn into the generative growth phase and develop reproductive organs. Apart from that, long-lived iteroparous, perennial beets were found in Northern Europe to flower in successive cycles (Hautekèete et al. 2002). The formation of different life cycles in beet which mainly stems to vernalization requirement was suggested as an adaptive response to season length and spring temperatures in particular latitudes (Boudry et al. 2002; Van Dijk et al. 1997). In their studies they examined 93 wild beet populations, sampled along a latitudinal cline around the coasts of France and south-east England and found a total absence of vernalization requirement in southern hemispheres when compared to northern. These studies suggest, that both, life cycle and flowering date may correlate with geographical origin of *Beta* accessions. In the context of the rapidly changing climate as a response to global warming, early flowering is a trait which will be selected for in long day plants in the future (Van Dijk and Hautekèete 2007).

## 1.3 Flowering time regulation in crop species

The transition from vegetative to generative growth in the lifetime of a flowering plant is triggered by a number of genes together with endogenous stimuli as well as environmental cues, such as temperature or day length changes. To ensure optimal reproductive success, flowering plants have developed different life cycles. While annual species complete their life cycle in one year, biennial species need to overwinter and thus complete their life cycle in two years (Andrés and Coupland 2012). Sugar beet is a biennial plant and represents the only crop harvested for sugar production in Europe. During the first year of its life cycle it forms a storage root and a leaf rosette, while shoot elongation (bolting) and subsequent flowering starts after the plant is exposed to a period of low temperatures (a process termed vernalization) in the second year. For the cultivation of sugar beet, bolting and flowering time control are major aims and a substantial knowledge of the involved genes and pathways as well as their interactions is of crucial importance.

However, life cycle adaptation to seasonal and latitudinal variation in photoperiod and temperature is a major determinant of reproductive and evolutionary success in flowering plants. In the model species *Arabidopsis thaliana*, an intricate network of genes controls the transition to flowering in response to endogenous and environmental signals. The response to cold is controlled by two major genes, the *FLOWERING LOCUS C (FLC)* and *FRIGIDA (FRI)* (Johanson et al. 2000; Michaels and Amasino 1999; Sheldon et al. 1999), as well as by an unrelated number of other loci (e.g. *VERNALIZATION 1-3*). A different pathway responds to day length which controls the major gene *CONSTANS (CO)*. Both pathways converge to the central integrators *FLOWERING LOCUS T (FT)* and *SUPPRESSOR-OF-OVEREXPRESSION-OF-CONSTANS 1 (SOC1)* (Searle et al. 2006).

Compared to *A. thaliana*, the understanding of flowering time regulation in the core eudicot species *B. vulgaris*, whose lineage diverged from *A. thaliana* shortly after the monocot-dicot split 140 million years ago (Davies et al., 2004; Chaw et al., 2004) is still in its infancy. In sugar beet, bolting is controlled by a dominant gene at the bolting locus *B* (Abegg et al., 1936; Boudry et al., 1994). Recently remarkable progress has been made by the identification of the gene *BTC1* controlling the annual habit of beet. *BTC1* shares sequence homology with the *PSEUDO RESPONSE REGULATOR 7 (PRR7)* gene from *A. thaliana* which is part of the circadian clock system. *BTC1* was identified by a map-based cloning approach and represents the long sought bolting gene *B* (Pin et al. 2012). It is known that *BTC1* is absolutely necessary for flowering and mediates the response to both vernalization and photoperiod, i.e. long days. Beyond that, *BTC1* was shown to control annuality through regulating the two beet *FT* homologs *BvFT1* and *BvFT2* (Pin et al. 2010; Pin et al. 2012). Moreover it was suggested that the domestication of beets involved the selection of a rare partial loss-of-function *btc1* allele that imparts sensitivity to photoperiod, which is restored by vernalization (Pin et al., 2012). These results illustrate how evolutionary plasticity can enable new life cycle strategies.

Beyond that, evidence of a large family of *CONSTANS-LIKE (COL)* genes in sugar beet was given by Chia et al. (2008). Here, three different *COL* genes differing by their zinc-finger (B-Box) and CCT (*CONSTANS*, *CONSTANS-LIKE* and *TOC1*) domains, which are characteristic features of *CONSTANS* (Andrés and Coupland 2012; Khanna et al. 2009; Suarez-Lopez et al. 2001; Valverde 2011) were detected in beet. Together with *FT*, *CONSTANS (CO)* is a major flowering regulator gene, conserved in many plants. Although *BvCOL1* was identified as an important component of the photoperiod pathway in beet, it could not be shown to represent the true orthologue of *CO* (Chia et al., 2008). Besides *BTC1*, several promising bolting time loci have been mapped or cloned in the beet genome in previous studies, such as *B2 (BvBBX19)* and *B5*, while *B2* has already been cloned and characterized (Dally et al., 2014). Dominant alleles at these loci were shown to promote annual bolting (Büttner et al. 2010; Dally et al. 2014). These loci might house important candidate genes for flowering time regulation in beet while their function is not yet entirely known.

To date, no orthologue of *CO* has been identified in beet. Thus far it is known that *BTC1* contains an N-terminal response regulator receiver (REC) domain and a C-terminal CCT (*CONSTANS*, *CONSTANS-LIKE*, and *TOC1*) motif (Pin et al. 2012). Additionally, a recent study led to the identification of *BvBBX19* at the bolting locus *B2*, which encodes a DOUBLE B-BOX TYPE ZINC FINGER protein, and was shown to have impact on flowering time control in beet, too. Expression analysis of *BvBBX19* revealed that it is diurnally regulated and acts like *BTC1* upstream of *BvFT1* and *BvFT2*. Furthermore, this study pointed that *BTC1* expression was reduced in *BvBBX19* mutants (Dally et al., 2014).

An interaction of a B-Box containing protein with a transcription factor was recently demonstrated in *Arabidopsis* (Gangappa et al., 2013). Withal, another study revealed that

*BBX19* interacts with *CONSTANS* to repress *FT* transcription in *Arabidopsis* (Wang et al., 2014). All together, these results suggest a possible interaction of the B-Box domains of the BvBBX19 protein with the CCT-domain of the BTC1 protein leading to a CONSTANS function in beet to promote bolting and flowering.

#### 1.4 The genome the crop plant sugar beet

Species of the genus *Beta* are diploid ( $2n=18$ ) and have a relatively small genome estimated to be 758 Mbp (Arumuganathan and Earle 1991). In 2013, the sequence of the sugar beet genome, comprising of 567 Mbp, was published by Dohm et al. (2013). Previously, Paesold et al. (2012) published a *B. vulgaris* reference FISH karyotype, which provided a resource for anchoring scaffolds and contigs of the beet genome sequence and enabled the localization of markers. Flavell et al. (1974) estimated that the genome contains at least 63% highly repetitive sequences, mainly consisting of numerous retrotransposons und satellite DNA families (Zakrzewski et al. 2013). The sequence of the sugar beet genome as the first non-asterid and non-rosid eudicot genome from the doubled haploid sugar beet line KWS2320 with all annotation is available at <http://bvseq.molgen.mpg.de/Genome/start.genome.shtml>. They sequenced plant material from leaves and roots on Illumina HiSeq2000, Roche/454 FLX and ABI3730 XL sequencing platforms and the final generated 'RefBeet' sequence assembly comprises of 566.6 Mbp in 2,171 scaffolds and 38,337 unscaffolded contigs ( $N50 = 2.01$  Mbp) (Dohm et al. 2013). In total, 85% of the generated sequence could successfully be assigned to all nine chromosomes. Further analysis revealed the prediction of 27,421 protein-coding genes supported by mRNA evidence, while 252 Mbp of the RefBeet genome was identified as repetitive sequence. Phylogenetic analyses led the authors to suggest a separation of the Caryophyllales before the split of asterids and rosids, as well as lineage-specific gene family expansions.

#### 1.5 Aims and objectives

This project was tended to contribute to the knowledge about the evolution of life cycle variation in *Beta* species. For sugar beet breeding with the long-term goal of breeding winter beets, it is of great importance to extend the knowledge about genetic factors which control bolting behavior and therewith different life cycle regimes in beet. Regarding this, the major objectives of this study were:

- (1) Mapping of the *B5* bolting locus and identifying putative candidate genes
- (2) Detecting natural haplotype diversity of the flowering time genes *BTC1*, *BvBBX19*, *BvFT1* and *BvFT2* among different *Beta* species and unveil possible relationships between life cycle adaptation and haplotype variation
- (3) Investigating *BvBBX19* and *BTC1* by overexpression in *Arabidopsis thaliana* for a possible interaction which alters the flowering phenotype

For mapping of the *B5* bolting locus, I awaited to find a novel bolting time gene which extends the genetic model for bolting time regulation in beet. To further contribute to the understanding of life cycle adaptation, I assumed to uncover sequence variations of the floral regulators *BTC1*, *BvBBX19*, *BvFT1* and *BvFT2* which can be related to the life cycle of different *Beta* species. However, I expected a low conservation of *BvBBX19*, *BvFT1* and *BvFT2* haplotypes, because it is assumed that *BTC1* was the gene which breeders selected for during domestication. Moreover, I assumed to find a non-random distribution of haplotypes across accessions from different geographical origin, associated with certain life cycles, which would underpin the relation of both.

Apart from haplotype variation, I supposed that during evolution of *Beta* (and related) species the *CONSTANS* gene, which is conserved among several plant species, split into two genes (*BvBBX19* and *BTC1*) which both act together to establish a *CO* function in beet. I expected that overexpression of both genes (*BvBBX19* and *BTC1*) in *Arabidopsis* accelerates the plant's phenotype towards earlier flowering, compared to the untransformed wild type, such as it was demonstrated for overexpression of the *AtCO*.

## 2 Sequence variation in the bolting time regulator *BTC1* changes the life cycle of sugar beet

### 2.1 Abstract

Flowering plants have developed different life cycles to ensure optimal reproductive and therewith evolutionary success. The wild beet *Beta vulgaris* ssp. *maritima* commonly exhibits an annual life cycle whereas its close relative sugar beet needs prolonged exposure to cold temperatures over winter to acquire floral competence. In beet breeding, flowering is an undesirable trait which drastically reduces yield. Both species need long days to enter the generative phase. The genetic control of photoperiodic flowering has been elucidated in the model plant *Arabidopsis thaliana* and many genes are structurally conserved among angiosperms. *BOLTING TIME CONTROL 1* (*BTC1*) and *BvBBX19* are major flowering time regulators in *Beta* species. It has been suggested that their proteins interact to acquire a *CONSTANS* function to jointly regulate their downstream targets *BvFT1* and *BvFT2*. Annual and biennial *BTC1* haplotypes differ by six non-synonymous SNPs and a large insertion in the promoter region of the ‘biennial’ *btc1* allele. I describe a new *BTC1* allele (haplotype ‘1’), which displays a particular sequence variant with an 8 bp insertion in the promoter and three single nucleotide polymorphisms (SNPs) in the coding sequence of *BTC1* compared to the annual *BTC1<sub>a</sub>* haplotype. The protein lacks a Low Complexity Region (LCR), which likely alters its ability to bind the *BvBBX19* protein. My findings demonstrate that little sequence variation in *BTC1* is sufficient to turn an annual into a biennial phenotype. My results shed new light on the evolution of cultivated beets and the identified haplotype *btc1<sub>1</sub>* offers new possibilities to breed winter beets.

### 2.2 Introduction

The transition from vegetative to generative growth in the life time of a flowering plant is triggered by a number of genes. They are part of a complex gene interaction network and respond to environmental stimuli such as day length and temperature. Flowering plants have evolved different life cycles for adaptation to different climates. Commonly, wild beets of the genus *Beta* (*Beta vulgaris* L. ssp. *maritima*) exhibit an annual life cycle. They germinate, bolt and flower within one season. Contrary, sugar beets (ssp. *vulgaris*) and wild beets from northern latitudes are biennials which need a prolonged period of cold to bolt and flower. Sugar beet has become of great importance for breeders since it represents the only sucrose-storing crop cultivated in temperate regions which accounts for almost 30% of the world’s annual sugar production (<http://faostat3.fao.org>, 2015). Interestingly, it has a very short history of cultivation which started ~ 200 years ago. In sugar beet cultivation, early bolting (followed by flowering and seed set) drastically reduces yield. Consequently, breeders have been selecting against this trait since the beginning.

In the model species *Arabidopsis thaliana*, an intricate network of genes was identified to control floral transition in response to endogenous and environmental signals. The response to cold is controlled by two major genes, the *FLOWERING LOCUS C* (*FLC*) and *FRIGIDA* (*FRI*) (Johanson et al. 2000; Michaels and Amasino 1999) and an unrelated number of other loci (e.g. *VERNALIZATION 1-3*). A different pathway controlled by the major gene *CONSTANS* (*CO*) responds to day length. Both pathways converge to the central integrators *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR-OF-OVEREXPRESSION-OF-CONSTANS1* (*SOCI*) (Searle et al. 2006). *CO* was identified to directly bind to the *FT* promoter for its activation (Tiwari et al. 2010).

Compared to *A. thaliana*, the understanding of flowering time regulation in the core eudicot species *B. vulgaris*, whose lineage diverged from *A. thaliana* shortly after the monocot-dicot



split ~140 million years ago, is still in its infancy. Nevertheless, during the past years huge progress has been achieved identifying several key regulators. The identification of the two Arabidopsis *FT* paralogs, *BvFT1* and *BvFT2*, having evolved different functions was a breakthrough in examining the life cycle of beets (Pin et al. 2010). Intriguingly, the study demonstrated functional differences between the two *FT* genes turning a floral activator to a floral repressor owing to mutations in critical domains. Upstream of *BvFT1* and *BvFT2*, the pseudo-response-regulator (PRR) gene *BOLTING TIME CONTROL 1 (BTC1)* controls the annual habit through the transcriptional regulation of both *FT* genes in beet (Pin et al. 2012). *BTC1* shares sequence homology with the circadian clock-associated *PSEUDO RESPONSE REGULATOR 7 (PRR7)* gene from *A. thaliana* and the major determinant of LD response in barley, *PHOTOPERIOD 1 (PPD-H1)* (Turner et al. 2005). The authors suggested that domestication of beets involved the selection of a rare partial loss-of-function *BTC1* allele which imparts sensitivity to photoperiod and distinguishes two functional haplotype classes.

Biennial (*btc1<sub>a-c</sub>*) and annual haplotypes (*BTC1<sub>d-k</sub>*) differ by six non-synonymous SNPs and a large insertion (~28kb) within the promoter of biennial *btc1* alleles disrupting light-regulatory elements (Pin et al. 2012). Apart from *BTC1*, other bolting time loci (*B2*, *B3*, *B5*) have been detected among the offspring of an ethyl methanesulfonate (EMS) mutagenized annual beet with the dominant *BTC1<sub>d</sub>* allele (Hohmann et al. 2005). Single mutants exhibiting a biennial life cycle were crossed with annual wild beets (*ssp. maritima*) to generate segregating F<sub>2</sub> populations. Phenotypic and marker studies revealed that dominant alleles at these loci promote annual bolting (Abou-Elwafa et al. 2012; Büttner et al. 2010).

Later, the *BvBBX19* gene encoding a DOUBLE B-BOX TYPE ZINC FINGER protein was cloned from the *B2* locus and added to the floral network (Dally et al. 2014). Expression analysis of *BvBBX19* revealed that it is diurnally regulated and acts upstream of *BvFT1* and *BvFT2* like *BTC1*. Moreover, *BTC1* transcription was partly increased compared to annuals and biennials in *BvBBX19* mutants. Altogether, the authors suggested a physical interaction between both proteins, *BvBBX19* and *BTC1* (Dally et al. 2014). Withal, another study revealed that *BBX19* interacts with *CONSTANS* to repress *FT* transcription in Arabidopsis (Wang et al. 2014b). In sugar beet numerous *CONSTANS-LIKE (COL)* genes differing by their zinc-finger (B-Box) and CCT (*CONSTANS*, *CONSTANS-LIKE* and *TOC1*) domains have been detected (Chia et al. 2008; Dally et al. 2014), while only *BvCOL1* has been functionally characterized by overexpression in Arabidopsis (Chia et al. 2008). However, it was excluded as a functional orthologue of *CO* due to a non-typical expression profile.

In this study, I describe a new *BTC1* allele which displays a particular sequence variant of the annual *BTC1<sub>d</sub>* and *BTC1<sub>f</sub>* haplotypes. In contrast to previous knowledge, minor sequence variations within the coding region of the ‘annual’ *BTC1* allele are sufficient to turn an annual into a biennial beet. The mutations have clear effects on the transcriptional activities of the downstream targets. Following our recently proposed model, which suggests that *BTC1* and *BvBBX19* proteins complement each other to acquire a *CONSTANS* function, I assume that the mutations in *BTC1* change the hypothesized binding capacity between both proteins.

## 2.3 Material and methods

### 2.3.1 Plant material, growth conditions and phenotypic analysis

I used two F<sub>2</sub> populations (070049, 070290), derived from a cross of an annual wild beet (991971) with a biennial sugar beet (020415) (Büttner et al. 2010), expected to segregate for the *B5* bolting locus. 020415 is an M<sub>3</sub> line obtained after selfing a biennial M<sub>2</sub> plant (000855) which had been identified in the offspring of an ethylmethanesulfonate treated annual line (Hohmann et al. 2005). F<sub>1</sub> progeny (061365), selected by hypocotyl color, was selfed by bag

isolation and F<sub>2</sub> seeds were harvested. The two F<sub>2</sub> populations 070290 and 070049 consisted of 60 and 118 plants, respectively. F<sub>2</sub> plants were grown in the greenhouse (9 cm pots) between October 2014 and May 2015 and kept under long day (LD) conditions (16 hours of light/8 hours dark, 22°C, 900 $\mu$ E). As controls, two annual (001684, 991971) and two biennial sugar beet lines (063858, 93161) were used. 063858 derived from self-pollinating a single 020415 plant. All F<sub>2</sub> plants were phenotyped twice a week for bolting (BBCH 51) and flowering (BBCH 60) (Meier 1993). F<sub>2</sub> plants which bolted before vernalization were classified as annual, whereas F<sub>2</sub> plants that bolted after vernalization were classified as biennial. Annual plants were further classified as early bolting (bolting within 13 weeks) and late bolting plants (bolting from week 14 to 22).

To distinguish between homozygous and heterozygous annual F<sub>2</sub> plants, F<sub>3</sub> seeds were produced from annual F<sub>2</sub> plants, by bag isolation. F<sub>3</sub> plants (12-16 plants per family) were grown in 96-well trays in the greenhouse between June and October 2015. They were phenotyped for bolting (BBCH 51) (Meier 1993) under LD conditions (16 hours of light/8 hours dark, 22°C, 900 $\mu$ E). F<sub>2</sub> plants were classified as either homozygous annual (100% bolting frequency without vernalization) heterozygous annual (annual/biennial without vernalization). To verify the effect of the candidate gene, a biennial sugar beet homozygous for the *btc1<sub>l</sub>* allele (063858) was crossed with a biennial sugar beet genotype (930184) carrying the recessive *btc1<sub>a</sub>* allele. F<sub>1</sub> plants were identified by molecular marker analysis. Then, they were grown in the greenhouse between October 2015 and July 2016 for the advent of bolting (BBCH 51) was notified. For expression analysis, the biennial sugar beet genotype (063858), the annual sugar beet genotype (001684), the annual crossing parent (991971), and the biennial sugar beet genotype (93161) were used. Plants were grown in a climate chamber under LD conditions (16 hours of light/8 hours dark, 20°C, 320 $\mu$ E). Leaf samples were taken 27 days after sowing (before vernalization) and 23 days after vernalization.

### 2.3.2 Molecular marker analysis

Leaves from three weeks old plants were harvested and freeze-dried. DNA was isolated using the CTAB method (Rogers and Bendich 1985). For marker development, sequence information of the doubled haploid accession KWS2320 (Refseq1.1) (Dohm et al. 2013) was used. PCR products were analyzed by Sanger sequencing at the Institute of Clinical Molecular Biology (IKMB, CAU Kiel). Sequence analysis was performed using the CLC Bio Workbench 6.9 (CLC bio, Aarhus, Denmark) and DNASTAR Lasergene SeqMan Pro (DNASTAR Inc., Madison, USA). For genetic mapping, 39 markers were developed, spanning all nine chromosomes. In total, I developed 23 insertion or deletion (InDel) markers and 16 cleaved amplified polymorphic sequence (CAPS) markers which were segregating in the F<sub>2</sub> populations (Supplementary Table 1 and Supplementary Table 2). To construct a genetic map, F<sub>2</sub> plants were genotyped with 39 molecular markers and genetic distances were calculated using the Kosambi mapping function (Kosambi 1944) from the JoinMap 4.0 program package (Stam 1993) with a LOD threshold value of 3.89. QTL mapping for days to bolting (DTB) in the F<sub>2</sub> population 070049 was performed with PlabQTL 1.2 (Utz H.F. 2006) using simple interval mapping and a co-dominance effect model.

### 2.3.3 Gene expression analysis

The diurnal gene expression of *BvGAPDH*, *BTC1*, *BvFT1* and *BvFT2* was measured by quantitative Real Time-PCR (RT-qPCR) using leaf material from the biennial sugar beet accession 063858, from the annual sugar beet accession 001684 and from the biennial sugar beet 93161. Leaf samples were taken from 4 weeks old plants every four hours in a time course of 24 hours. RNA was isolated using the Total RNA Kit, peqGOLD (Peqlab,

Germany) and DNase treated using DNaseI (Invitrogen, Karlsruhe, Germany). RNA concentrations were measured with a NanoDrop spectrometer (Thermo Scientific, Germany) and 500ng of total RNA was reverse transcribed using a First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany). The cDNA was diluted 1:3 and 2µl were used as a template for RT-qPCR with three biological replicates and three technical replicates, respectively. RT-qPCR was performed with SYBR qPCR Super mix w/ROX (Invitrogen Corporation, Carlsbad, USA) using a CFX96 Real-Time System (Bio-Rad Laboratories GmbH, Munich, Germany).

All primer combinations and PCR conditions are listed in Supplementary Table 1, primer sequences are listed in Supplementary Table 2. Resulting data were analyzed using the CFX Manager Software v2.1 (Bio-Rad Laboratories GmbH, Munich, Germany). *BvGAPDH* was used as a reference gene and its geometric mean was used for normalization and calculation of the relative *BTC1*, *BvFT1* and *BvFT2* expression by applying the comparative CT ( $\Delta$ CT) method.

## 2.4 Results

### 2.4.1 Phenotypic analysis of bolting time in F<sub>2</sub> populations

Two sibling F<sub>2</sub> populations (070049 and 070290) were produced from crossing a biennial sugar beet genotype (020415) with an annual wild beet genotype (991971). Both populations segregated for bolting behavior. F<sub>2</sub> plants were classified as annual (bolting within 21 weeks after sowing) or biennial (non-bolting without vernalization). Bolting plants were further classified as ‘early’ bolting (bolting within 13 weeks) and ‘late’ bolting (bolting within 14-21 weeks) (Table 1).

**Table 1:** Phenotypic segregation for bolting behavior in two F<sub>2</sub> populations.

F <sub>2</sub> population	Total number of plants	Number of bolting plants		Number of non-bolting plants	$\chi^2$ -test for H <sub>0</sub> =3:1 (bolting:non-bolting) <sup>a</sup>
		Early bolting	Late bolting		
070049	118	63	31	24	1.37 ns
070290	60	34	10	16	0.09 ns

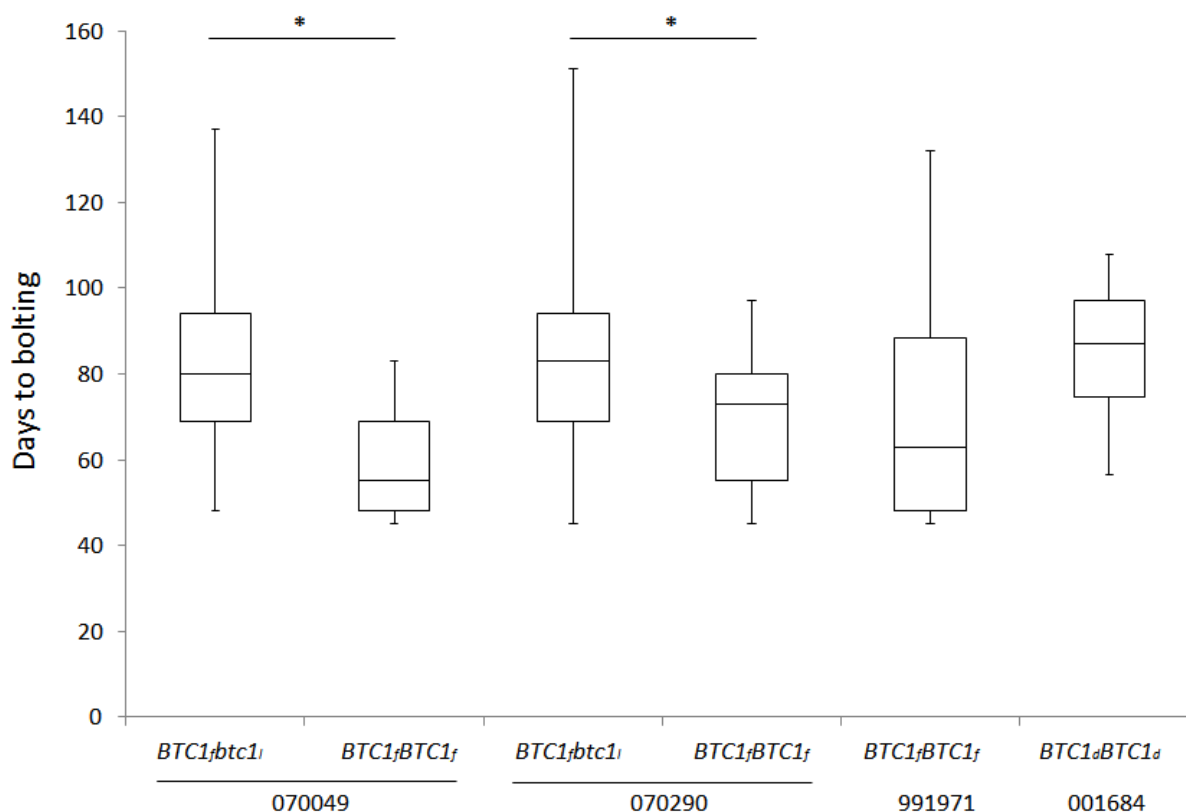
ns= not significant ( $\alpha = 0.01$ )

a= Null hypothesis for monogenic, dominant–recessive inheritance

Plants were grown and phenotyped in the greenhouse and kept under LD conditions (16 hours of light/8 hours dark, 22°C, 900µE). The approximate value for early and late bolting was set according to the mean of days to bolting of the annual controls 001684 and 991971 which were included within the phenotyping experiment. As expected, the null hypothesis for a dominant-recessive inheritance of a monogenic trait was not rejected for both F<sub>2</sub> families, indicating a 3:1 segregation ratio (bolting:non-bolting). For verification of F<sub>2</sub> genotypes of annual plants (homozygous or heterozygous at the bolting locus), F<sub>3</sub> offspring was grown under LD conditions in the greenhouse. I observed 89 segregating (annual/biennial) and 49 non-segregating (100% bolting frequency) F<sub>3</sub> families, respectively. Non-segregating F<sub>3</sub> families were indicative of homozygous F<sub>2</sub> parents. Hence, I revised the selection of F<sub>2</sub> plants for genotypic classification based on phenotypic data from early bolting F<sub>2</sub> plants and F<sub>3</sub> families. Accordingly, I classified 44 F<sub>2</sub> plants as homozygous and 53 F<sub>2</sub> plants as

heterozygous at the bolting locus. In both F<sub>2</sub> populations, plants homozygous for the bolting allele bolted earlier ( $\alpha=0.05$ ) than heterozygous plants (Figure 1).

**Figure 1:** Bolting time measurements of annual F<sub>2</sub> plants in relation to their *BTC1* genotypes. Bolting time was determined as days to bolting (DTB) after sowing (October 24, 2014) without vernalization. F<sub>2</sub> plants were derived from a cross between the biennial sugar beet genotype 020415 (*btc1<sub>i</sub> btc1<sub>i</sub>*) and the annual wild beet 991971 (*BTC1<sub>f</sub> BTC1<sub>f</sub>*). Plants were grown in pots in the greenhouse and kept under LD conditions (16 hours of light/8 hours of darkness, 22°C, 900 $\mu$ E). F<sub>2</sub> plants which did not bolt before cold treatment are not included. The parental genotype 991971 and the annual genotype 001684 were used as controls. The *BTC1* genotypes were determined by their F<sub>3</sub> offspring. Error bars represent the standard error of the mean. Asterisks highlight statistically significant differences in days to bolt (student's *t*-test,  $\alpha = 0.05$ ).



## 2.4.2 Genetic mapping of the bolting locus

I performed a step by step procedure to map the position of the bolting locus. First, I constructed a skeleton map of the beet genome with 39 markers (23 InDel and 16 CAPS markers) evenly spaced across all nine chromosomes with a total length of 464.8 cM (Supplementary Figure 1). The bolting gene causing the early bolting phenotype (resulting from F<sub>2</sub>:F<sub>3</sub> data) was mapped on chromosome II. For fine-mapping with 137 F<sub>2</sub> plants (97 annuals and 40 biennials), I selected eight markers spanning the whole chromosome (Figure 2A). I found that the bolting locus was flanked by the markers CAU3959 and CAU3953 spanning 6.4 cM and including the *BTC1* gene. I reasoned that *BTC1* is responsible for phenotypic variation.

In a next step, I sequenced the whole coding region from the biennial sugar beet genotype 063858 (Figure 2B-C), from the annual crossing parent 991971 and from the annual genotype 001684 which was used as a control for phenotyping. Sequencing revealed that the size of the coding sequence (2.367 bp) was identically between all genotypes and uncovered that the annual crossing parent 991971 exhibits the *BTC1<sub>f</sub>* haplotype, while the annual sugar beet

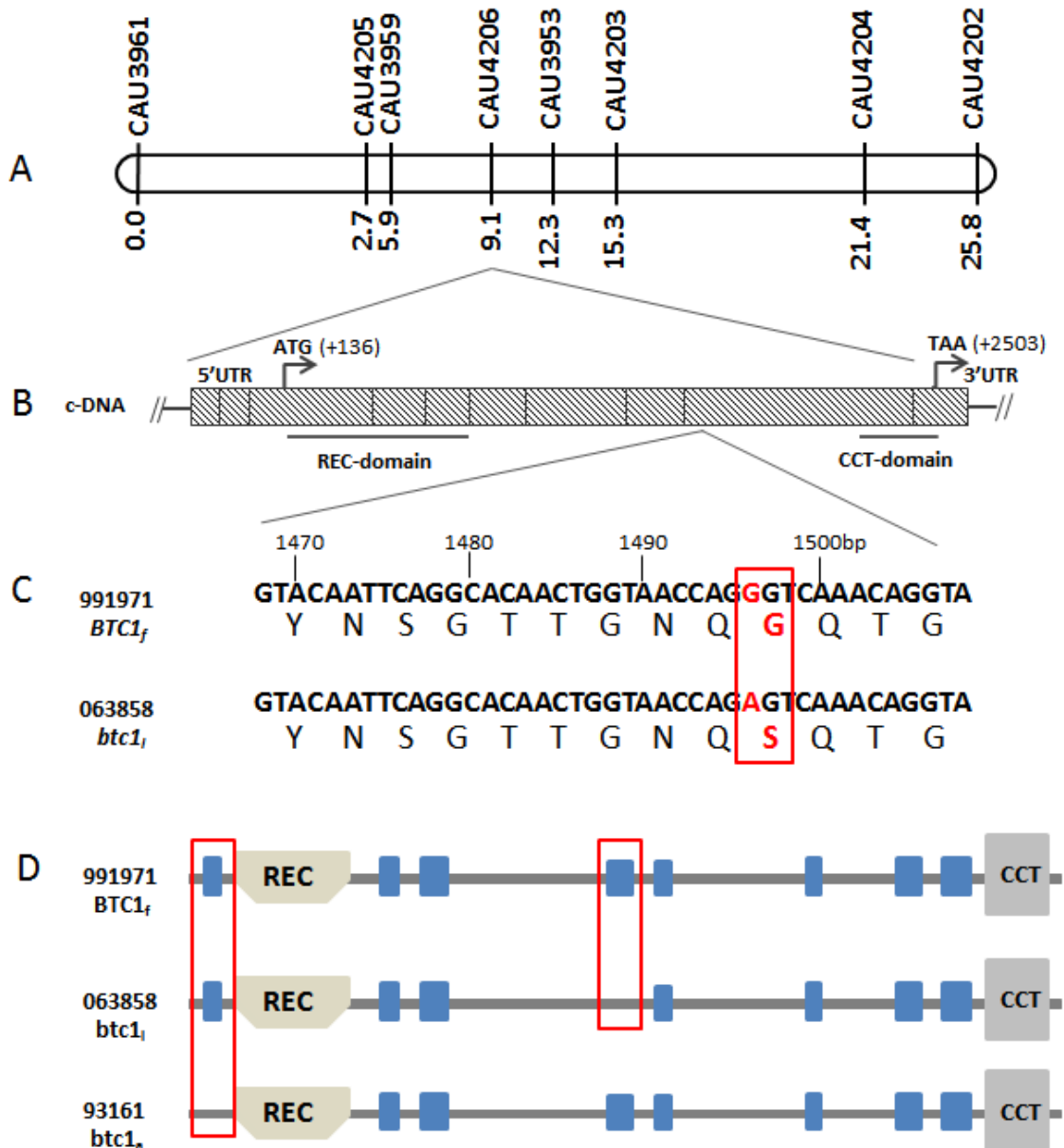
genotype 001684 carries the *BTC1<sub>d</sub>* haplotype (according to Pin et al.(2012)). However, the biennial sugar beet genotype 063858 differed from the annual mapping parent by three missense mutations and one silent mutation (Table 2).

**Table 2:** *BTC1* haplotypes of the mapping parents 063858 and 991971 and of the annual sugar beet genotype 001684. The coding region was sequenced. The position of SNPs is given relative to the translation start site according to the regarding exons.

Exon	8		9				Haplotype
	158	381	29	37	435	686	
991971	A	G	G	G	G	G	<i>BTC1<sub>f</sub></i>
001684	T	A	G	A	A	A	<i>BTC1<sub>d</sub></i>
063858	T	A	A	A	G	G	<i>btc1<sub>l</sub></i>
non-syn. SNP	*	*	*		*	*	

When comparing the *BTC1* coding region of the biennial genotype 063858 with the annual genotype 001684 I identified three missense mutations (Table 2). In total, the biennial genotype 063858 differed from both annual genotypes by six polymorphisms. Five of the six SNP positions in 063858 (position 158 and 381 in exon 8 and position 37 and 435 and 686 in exon 9; Table 2) are common among both annual and biennial haplotypes. By contrast, the third SNP (position 29 in exon 9) was only found in 063858, suggesting that it may be causally involved in the biennial phenotype. This missense mutation comprises a nucleotide exchange from guanine to adenine and results in substitution of a glycine by a serine at position 490 (Figure 2C). Thus, I assigned a novel haplotype (*btc1<sub>l</sub>*) for the *BTC1* allele of 063858 carrying a new polymorphism which was not yet discovered (Table 2). The prediction of the *BTC1* protein structure using SMART (Schultz et al. 1998) disclosed a lack of the 4<sup>th</sup> Low Complexity Region (LCR) within the protein of the biennial sugar beet genotype 063858 (Figure 2D).

**Figure 2:** Genetic map of the bolting locus and a detailed map of the *BTC1* gene with the mutation therein. (A) Genetic map of the bolting locus on chromosome II including markers and their distances in centimorgan (cM). (B) In silico prediction of the *BTC1* gene structure depicting the exonic region and the 3'UTR and 5'UTR. Exons are represented by shaded boxes and the genomic regions coding for the REC-domain and the CCT-domain are highlighted by grey bars. (C) Part of the genomic sequence of exon 9 from the coding region of *BTC1*, including SNP position 29. The amino acid sequences from the annual sugar beet genotype 991971 and the biennial sugar beet genotype 063858 are given. The numbers above the sequence indicate the position in base pairs beginning from the start codon (ATG). The red box highlights the position of the missense mutation from Guanine to Adenine (+1496) and the amino acid change from Glycine to Serine. (D) Predicted structure of three *BTC1* proteins with their REC and CCT domains. 93161 is a reference beet genotype (biennial). The blue boxes indicate the presence of Low Complexity Regions (LCRs). The *btc1<sub>1</sub>* protein lacks the 4<sup>th</sup> LCR as compared to the annual beet genotype 001684 and the biennial *btc1<sub>a</sub>* protein lacks the first LCR, which is highlighted by the red boxes.



To validate the co-segregation between *BTC1* and the bolting gene in the F<sub>2</sub> populations, I designed a CAPS marker (CAU4206) which directly cleaves at position 29 in exon 9 (Figure 2C). Marker genotypes were grouped into three classes each among bolting as well as non-bolting individuals. The expected segregation ratio of the marker genotypes for a complete co-segregation is 0:2:1:1:0:0, while a ratio of 3:6:3:1:2:1 would indicate a random segregation. The observed segregation ratio was tested by  $\chi^2$ -analysis for independent segregation and in both F<sub>2</sub> populations the null hypothesis was rejected, connoting a complete linkage (R=0) between *BTC1* and the bolting gene (Table 3).

**Table 3:** The distribution of genotypic and phenotypic classes in two F<sub>2</sub> populations. The *BTC1* marker CAU4206 was used for genotyping.

F <sub>2</sub> population	Total number of plants genotyped	Bolting						$\chi^2$ -test for H <sub>0</sub> = 3:6:3:1:2:1 <sup>b</sup>
		<i>M1M1</i> <sup>a</sup>	<i>M1M2</i>	<i>M2M2</i>	<i>M1M1</i>	<i>M1M2</i>	<i>M2M2</i>	
070049	117	0	63	30	24	0	0	93.25*
070290	60	0	25	19	16	0	0	68.13*

\*= statistically significant differences for an independent segregation of phenotype and genotype ( $\alpha = 0.01$ )

<sup>a</sup> Marker allele *M1*, which corresponds to the *btc1<sub>l</sub>* allele derived from the biennial sugar beet parent (063858). Marker allele *M2*, corresponding to the *BTC1<sub>f</sub>* allele derived from the annual wild beet parent 991971.

<sup>b</sup>  $\chi^2$ -test for the expected ratio of a monogenic, dominant-recessive trait with an independent segregation of phenotype and *BTC1*

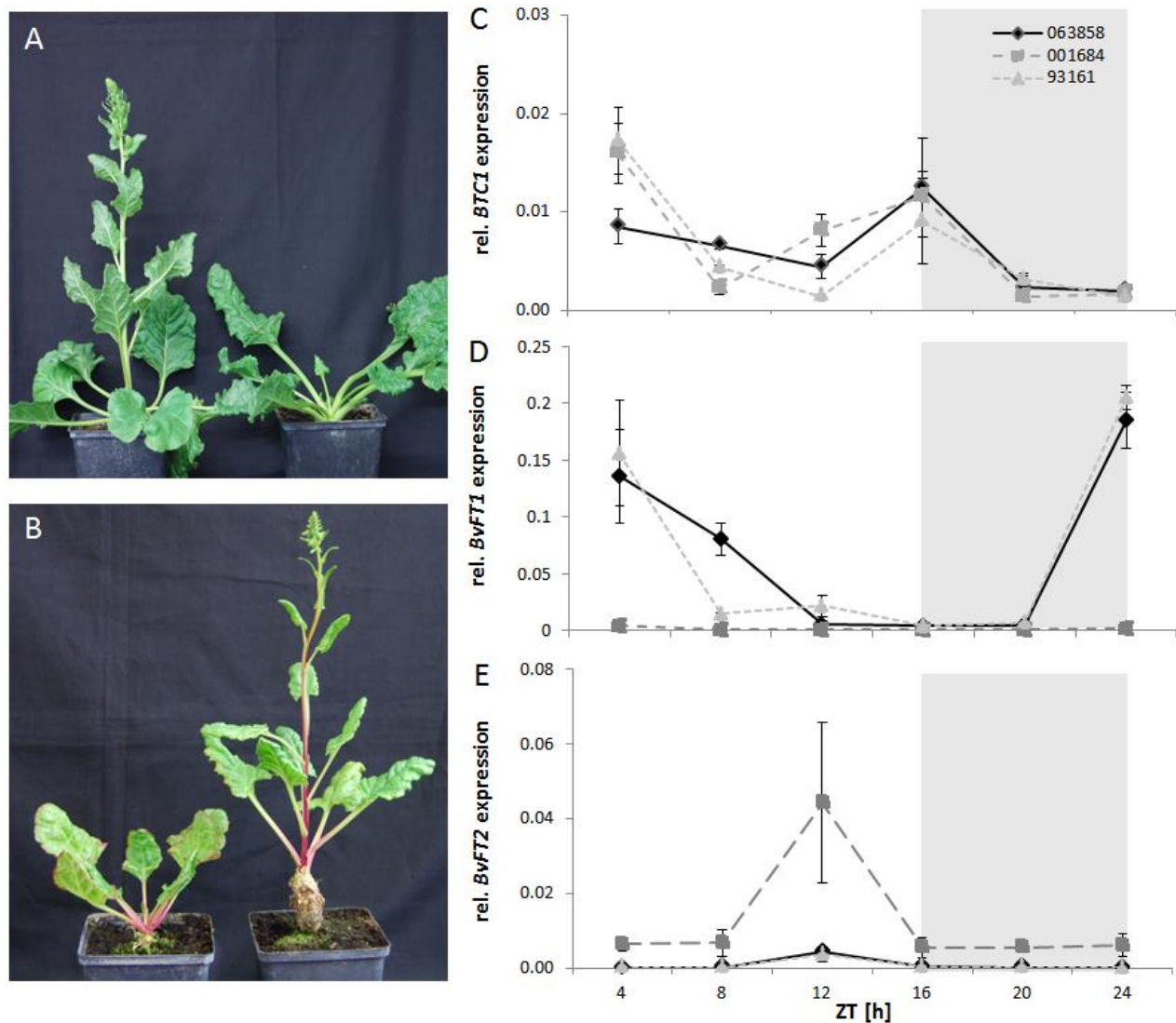
For both F<sub>2</sub> populations, all homozygous annual plants (pertaining to F<sub>3</sub> data) carried the dominant *BTC1<sub>f</sub>* allele (Pin et al. 2012), while all biennial plants carried the mutated *btc1<sub>l</sub>* allele in a homozygous state. To search for other genes causing early bolting in the populations, I performed a whole genome QTL mapping using F<sub>2</sub> and F<sub>3</sub> phenotypic data from population 070049. For F<sub>2</sub> plants that did not bolt without vernalization the value of days to bolting was set to 300 (representing the number of days to bolting in biennial beets after cold treatment in this experiment). With a LOD threshold of 3.89 a single QTL for days to bolting was detected on chromosome II, flanked by the markers CAU3953 and CAU4203 and partially covering the *B* locus, too. This QTL comprises a LOD score of 23.9 at position 9.1 cM (confidence interval 8-12 cM), explaining 71.8% of the observed phenotypic variation.

Taken together, my data confirm that the novel *btc1<sub>l</sub>* haplotype causes the biennial phenotype of the sugar beet genotype 063858. As a verification experiment, I crossed the genotype 063858, which is homozygous for the *btc1<sub>l</sub>* allele with a biennial beet (930184) carrying the homozygous, recessive *btc1<sub>a</sub>* allele, which was denoted to be associated with a non-bolting phenotype (Pin et al. 2012). The resulting F<sub>1</sub> progeny did not bolt without vernalization, even after 30 weeks. This result underpinned my expectation that F<sub>1</sub> progeny carrying both alleles (*btc1<sub>l</sub>* and *btc1<sub>a</sub>*) do not bolt without vernalization, thus confirming the effect of the new haplotype on the non-bolting phenotype. After three months of vernalization at 4°C all F<sub>1</sub> plants as well as the controls (063858 and 930184) bolted.

### 2.4.3 Expression analysis of *BTC1* and its downstream targets *BvFT1* and *BvFT2*

To examine a possible effect of the novel *btc1<sub>l</sub>* allele on the expression profiles of *BTC1* and its downstream targets *BvFT1* and *BvFT2*, I compared the transcriptional activities in leaves of the biennial sugar beet genotype 063858 (*btc1<sub>l</sub>*), the annual sugar beet genotype 001684 (*BTC1<sub>a</sub>*), and the biennial sugar beet genotype 93161 which is homozygous for the recessive *btc1<sub>a</sub>* allele (Pin et al. 2012) (Figure 3).

**Figure 3:** Phenotypes of annual and biennial sugar beets without vernalization and expression analysis of *BTC1*, *BvFT1* and *BvFT2*. (A) The annual sugar beet genotype 001684 (left) bolted without vernalization. The biennial sugar beet genotype 063858 (right) did not bolt without vernalization. Both plants were 12 weeks old. (B) A biennial  $F_2$  plant (left) compared with an annual  $F_2$  plant (right) which bolted without vernalization (population 070049). Both plants are 8 weeks old. Plants were grown in pods in the greenhouse and kept under LD conditions (16 hours of light/8 hours of darkness, 22°C, 900 $\mu$ E). Expression analyses of (C) *BTC1*, (D) *BvFT1* and (E) *BvFT2* in leaves of three different sugar beet genotypes. Plants were grown in a climate chamber and kept under LD conditions (16 hours of light/8 hours of darkness, 20°C, 320 $\mu$ E). The different shadings (white and light grey) of the background indicate light supply (white) and darkness (grey). The x-axis indicates the sampling time points at zeitgeber time (ZT), while the y-axis shows the relative expression of the respective gene normalized against *BvGAPDH*. Each value represents the mean of three biological and three technical replicates. Error bars represent the standard error of the mean (SEM) of the three biological replicates.



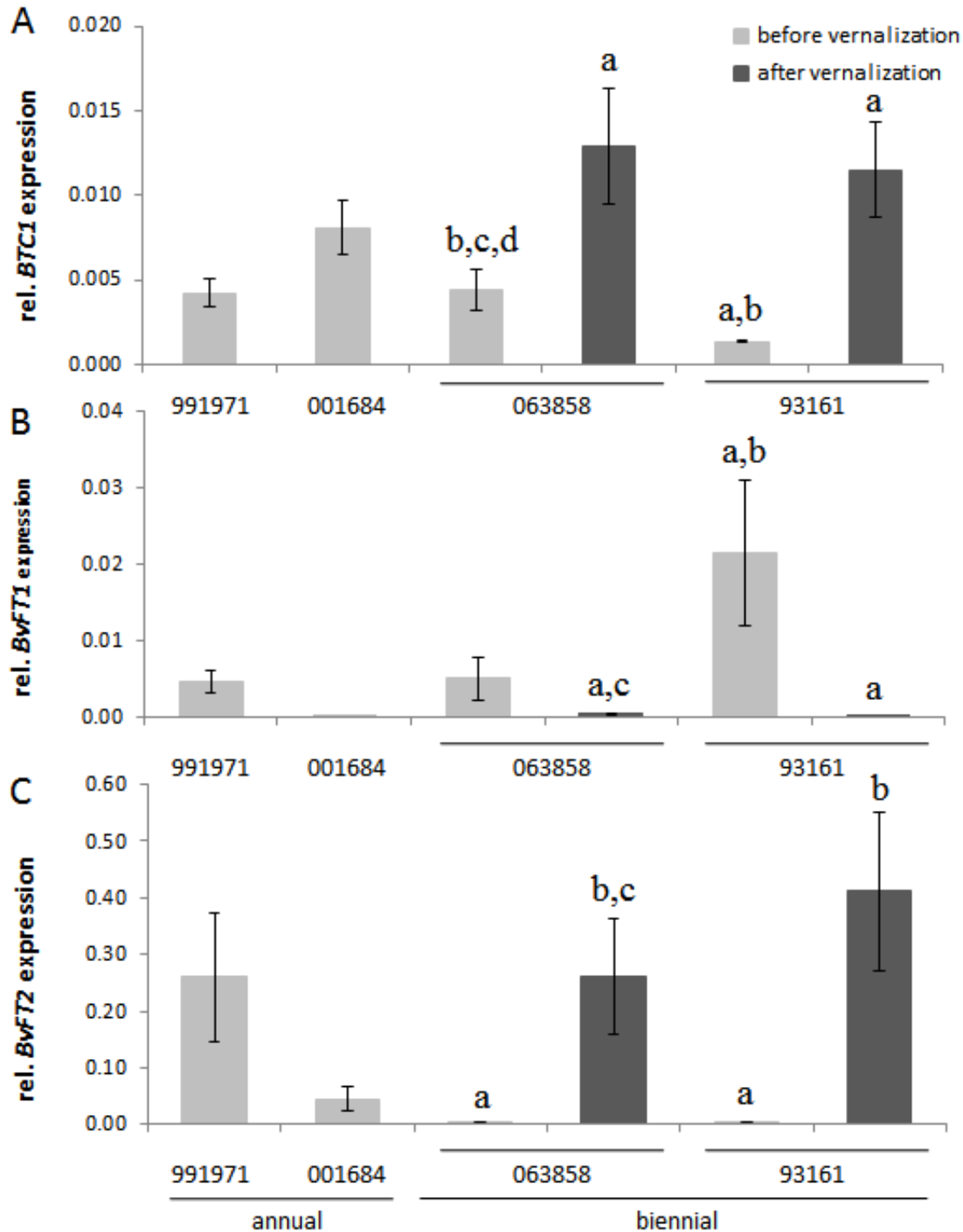
As expected, in both genotypes (001684, 93161) *BTC1* was found to be diurnally regulated. Furthermore it peaked around zeitgeber time (ZT) 4 shortly after dawn and around ZT16 under long day conditions. In the biennial sugar beet genotype 063858, the expression of *BTC1* was similar to the controls. In all genotypes, the expression of *BTC1* decreased rapidly during night (Figure 3C). *BvFT1* was diurnally regulated in the biennial control 93161. As expected, its transcriptional activity was completely downregulated in the annual sugar beet genotype 001684 (Figure 3D). Interestingly, the *BvFT1* expression pattern of the biennial sugar beet genotype 063858 resembles the biennial genotype 93161 (Figure 3D). For both, 063858 and the biennial control 93161, *BvFT1* expression was highly increased during dark with a peak at ZT24 at the end of the night, while the expression decreased during day time.



The diurnal expression of *BvFT2* in both controls 001684 and 93161 corresponded to previous observations (Figure 3E). While *BvFT2* is upregulated in 001684 and peaked at ZT12, it is downregulated in the biennial control. Again, the expression pattern for the *BvFT2* transcriptional activity of the biennial genotype 063858 was like the biennial control.

Subsequently, the transcriptional activities of *BTC1*, *BvFT1* and *BvFT2* after cold treatment (vernalization) were analyzed for both biennial lines, 063858 and 93161. Leaf samples were collected two weeks after vernalization at ZT12. In both lines, the *BTC1* expression was highly increased after vernalization (Figure 4A), while the expression of *BvFT1* was strongly downregulated after vernalization (Figure 4B). Intriguingly, in the biennial genotype 063858 the transcriptional activity of *BTC1* was higher than before vernalization. Concurrently, *BvFT2* was highly expressed after vernalization in the biennial control 93161 and in 063858 (Figure 4C).

**Figure 4:** Expression analyses of (A) *BTC1*, (B) *BvFT1* and (C) *BvFT2* in leaves at ZT12 of four different sugar beet genotypes, two annual (991971, 001684) and two biennial (063858, 93161). Leaf samples were taken 23 days after vernalization. Only the biennial genotypes were kept at 4°C for 12 weeks for vernalization. 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, 991971 and biennial accessions; b, 001684 and biennial accessions; c, nonvernalized and vernalized 063858; d, nonvernalized biennial accessions and e, vernalized biennial accessions.



## 2.5 Discussion

I examined early bolting in two F<sub>2</sub> beet populations which were segregating for the bolting locus *B*. I discovered a novel haplotype of the *BTC1* gene which I termed *btc1<sub>l</sub>*. There are several lines of evidence which demonstrate that this new haplotype causes a biennial life cycle in beet. (i) The bolting locus was mapped to the same position as the *BTC1* gene and no other bolting time QTL was found, (ii) both parents of the mapping population differ by four transitions and therewith their *BTC1* haplotype, (iii) hybrid plants carrying both alleles (*btc1<sub>l</sub>* and *btc1<sub>a</sub>*) do not bolt without vernalization, and (iv) in the biennial sugar beet genotype 063858 the expression profiles of major flowering time regulators are typical for biennial beets. My data provide clear evidence that the *btc1<sub>l</sub>* haplotype causes the biennial life cycle of the 063858 genotype.

Flowering time is a trait of high agronomic relevance since it strongly affects seed yield and yield potential. To ensure optimal reproductive and therewith evolutionary success, flowering plants have developed different life cycles. Wild species of the genus *Beta* (*B. vulgaris* ssp. *maritima*) which are abundant around the Mediterranean commonly bolt and flower within one season under long day conditions whereas cultivated types require prolonged exposure to cold temperature to acquire floral competence. In the past years, several genes have been identified to control bolting in sugar beet. *BTC1* is told to mainly control the annual habit of beets while during domestication of biennial beets, a partial loss-of-function allele of *BTC1* was selected (Pin et al. 2012). Haplotype analysis of annual and biennial beets revealed the occurrence of two distinct classes of haplotypes. Three ‘biennial’ haplotypes (*btc1<sub>a-c</sub>*) were mainly detected in biennial cultivated beets and at a low frequency in biennial wild beets from northern latitudes. Moreover, there are eight ‘annual’ haplotypes (*BTC1<sub>d-k</sub>*) mainly found in wild beets comprising an annual life cycle (Pin et al. 2012). The biennial *btc1<sub>a</sub>* haplotype, which is only found in cultivated beets, is suggest to result from a spontaneous mutation which happened recently during the early events of beet domestication.

Since the past 200 years, breeders have been strictly selecting for this allele because it guaranteed a non-bolting character during field production. In contrast, biennial wild beets mostly from northern latitudes exhibited the *btc1<sub>b-c</sub>* alleles (Pin et al. 2012). The formerly published *BTC1* haplotype classes for annuals and biennials differ by six non-synonymous SNPs and a large insertion (~28kb) emerging in the promoter region of the biennial haplotypes *btc1<sub>a-c</sub>* (Pin et al. 2012). The polymorphisms we found to constitute the novel haplotype *btc1<sub>l</sub>* causing a biennial phenotype.

In a previous study, Büttner *et al.*, (2010) had crossed an annual wild beet (991971) with a biennial sugar beet (020415) to produce six F<sub>2</sub> populations, expected to segregate for the *B5* locus. In their study, the *B5* locus was not linked to *BTC1*. In my study, we found that segregation for early bolting in two sibling F<sub>2</sub> populations (070049, 070290), which were derived from the same cross but from different F<sub>1</sub> plants was due to the *B* locus housing the *BTC1* gene. This could be explained by heterozygosity of the biennial parent due to unintended pollination by an unknown pollinator because the M<sub>2</sub> plants had been open pollinated to ensure seed set. Thus, we cannot rule out the existence of a *B5* locus and that an unknown *B5* mutant allele is still present in the original M<sub>2</sub> family. The *BTC1* haplotype is definitely not resulting from an EMS mutation because it differs from the mutant parent by three SNPs and an 8bp insertion in the promoter region. However, *btc1<sub>l</sub>* is a new haplotype never found before neither in cultivated nor in wild beet populations. Therefore, it became an interesting candidate for studying the function of *BTC1* and for breeding.

When comparing the novel *btc1<sub>l</sub>* haplotype with the annual *BTC1<sub>d</sub>* of the sugar beet genotype 001684 and the annual *BTC1<sub>f</sub>* of the parental genotype 991971, the residues at five of the six

SNP positions are common among both, annual and biennial *BTC1* haplotypes. Interestingly the SNP at position 29 in exon 9 was only found in the biennial parental sugar beet genotype 063858. This polymorphism results in a substitution of a Glycine to Serine at position 490 and may be causally involved in the biennial phenotype. Serine is a potential phosphorylation target site (confidence score 0.921 according to NetPhos 2.0 (Blom et al. 1999)). In Arabidopsis, the phosphorylation state of all five PRR proteins is diurnally regulated and phosphorylation of two proteins, TOC1/PRR1 and PRR3, has indeed been shown to affect protein-protein interaction and protein stability (Fujiwara et al. 2008). Thus, it seems conceivable that differential phosphorylation of annual and biennial alleles enhances the effects of differential transcript accumulation during the course of the day and thus contributes to differences in protein stability, possibly not dissimilar to regulatory mechanisms involving „coincident“ events as described for CO in *A. thaliana* (Turck et al. 2008)

The pseudo-response regulator gene *BTC1* was identified to host two important evolutionary conserved protein domains as known from the Arabidopsis Pseudo-Response Regulators *PRR3/PRR7* (Takata et al. 2010). The CCT-domain functioning in the photoperiodic pathway (Golembeski and Imaizumi 2015) and the REC-domain. Interestingly, only one of the SNPs (exon 3, nt351) differentiating between the two haplotype classes is located within the sequence encoding the REC-domain. Moreover, the promoter insertion in the biennial *btc1* alleles disrupts a series of sequence motifs which often appear in promoters that are light-regulated, emphasising its importance in life cycle switch. In a previous study, *BTC1* knock down in an annual beet by RNAi resulted in a complete loss of the competence to flower (Pin et al. 2012). My study demonstrates that single polymorphisms within the coding region of an annual *BTC1* allele are sufficient to turn an annual into a biennial phenotype, but not the insertion within the promoter region. I reason that the resulting amino acid change from glycine to serine is likely to alter the structure and function of the BTC1 protein. The predicted BTC1<sub>d</sub> protein displays seven Low Complexity Regions (LCRs) while the *btc1<sub>l</sub>* protein lacks one LCR. Interestingly, the recessive *btc1<sub>a-c</sub>* alleles are also missing one LCR, namely the first one which is located in front of the REC-domain (Figure 2D).

LCRs are highly abundant in eukaryotic proteins and consist of amino acid sequences which contain repeats of either short amino acid motifs or single amino acids (Coletta et al. 2010; Rado-Trilla and Alba 2012). So far, their function is largely unknown, but there is evidence that they play a role in protein subcellular localization (Salichs et al. 2009), protein-nucleic acid interactions (Shen et al. 2004) and protein-protein interactions (Coletta et al. 2010; Xiao and Jeang 1998). For example, it was shown that a low complexity region in the human malaria parasite is responsible for protein-protein interaction of two enzyme-coding genes from the *de novo* pyrimidine biosynthetic pathway (Jmprasittichai et al. 2014). It is of great interest, that LCRs which occur at terminal positions of proteins (t-LCRs) are hypothesized to impact low-specific biological events involving the development of large protein complexes (Coletta et al. 2010). For instance, protein chaperons, known to interact in stress response, were identified to possess low-specificity binding properties as a result of the large variety of binding partners (Tompa and Csermely 2004). Further studies pointed that proteins containing LCRs reveal more protein-protein interactions than those without (Coletta et al. 2010; Jmprasittichai et al. 2014). Pin et al (2012) speculated that the different function of annual and biennial *BTC1* alleles is due to a large promoter insertion in the biennial haplotypes *btc1<sub>a-c</sub>*. This is not supported by my data because the *btc1<sub>l</sub>* allele does not carry the ~28kb insertion in the promoter region.

My data suggest that the lack of one LCR domain alters the function of the BTC1 protein and impacts the transcriptional activities of its downstream targets *BvFT1* and *BvFT2*. I have recently proposed a new model for bolting control in beet, suggesting that BTC1 and BvBBX19 proteins complement each other to acquire a CONSTANS function and jointly

regulate their downstream targets *BvFT1* and *BvFT2* (Dally et al. 2014). In *A. thaliana*, *CO* (*BBX1*) is a key component of the photoperiodic pathway encoding a protein with two major domains: a CCT (CONSTANS, CONSTANS-LIKE and TOC1) domain which was shown to be sufficient for nuclear localization and two zinc-finger B-Box domains towards the amino terminus involved in protein-protein interaction (Gangappa et al. 2013a; Wenkel et al. 2006). Additionally, *CO* was evinced to promote flowering by induction of *FT* through direct binding of the CO protein to the TGTG(N2-3)ATG motif in the *FT* promoter (Cao et al. 2014; Tiwari et al. 2010). Besides *CO*, a huge family of 32 B-Box proteins has been identified in *Arabidopsis* harboring five different subfamilies according to their protein structure (Khanna et al. 2009). All subfamilies house proteins with at least one B-Box. All members of subfamily IV (BBX18-BBX25) carry two B-Boxes such as *AtBBX19* which shares highest homology with *BvBBX19* from beet.

In the past years, several protein-protein interactions of BBX-proteins with others have been proposed in *A. thaliana*. The transcription factor ELONGATED HYOCOTYL 5 (HY5) was discovered to interact with BBX21, 22, 24 and 25 (Datta et al. 2007; Datta et al. 2008; Gangappa et al. 2013a; Gangappa et al. 2013b) and HOMOLOG OF HY5 (HYH) was shown to collude with BBX22, 24 and 25 (Datta et al. 2007; Gangappa et al. 2013b). Moreover, *AtBBX19* was discovered to physically interact with *CONSTANS* in *Arabidopsis* (Wang et al. 2014b). Nevertheless, to date no true orthologue of *CO* in beet has been identified. Recently, I have detected a mutation within the second B-Box of *BvBBX19* turning an annual beet which carries the *BTC1<sub>a</sub>* allele into a biennial. Independent mutations in both genes exhibit a similar effect on the expression of their downstream targets *BvFT1* and *BvFT2*. Interestingly, the expression profiles of the parental genotype carrying the *btc1<sub>i</sub>* allele and a *BTC1 RNAi* plant (Pin et al. 2012) are quite similar. Both displayed a strong increase of *BvFT1* and a decrease of *BvFT2* transcription before vernalization. While the epistatic interaction between *BvBBX19* and *BTC1* has been proven, I hypothesize a joint activity of both proteins. Since the *btc1<sub>i</sub>* protein lacks one LCR, the biennial life cycle in the sugar beet genotype 063858 could be due to an altered binding between both proteins. The novel *btc1<sub>i</sub>* haplotype will be a valuable resource for protein binding studies which I have already initiated.

Early bolting is still a problem in beet production under unfavorable growth conditions. Apparently, all cultivated sugar beets worldwide carry one and the same *BTC1* allele (*btc1<sub>a</sub>*) (Pin et al. 2012). Here I propose a new variant of a *BTC1* allele. It is likely that *BTC1*, as many other flowering time regulators, has pleiotropic effects as for instance on root growth (unpublished data). Therefore, I suggest introducing the new *btc1<sub>i</sub>* allele into beet breeding in combination with the biennial *BvBBX19* allele recently found. Moreover, combining different alleles of both, *BTC1* and *BvBBX19* could be a way to breed winter beets which can be sown before winter (Jung and Müller 2009).

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### 3 Haplotype analysis of flowering time genes of *Beta* species and the impact on life cycle regimes

#### 3.1 Abstract

The species *Beta vulgaris* encompasses wild and cultivated members with a broad range of phenological development. The annual life cycle is commonly found in sea beets (*ssp. maritima*) from Mediterranean environments which germinate, bolt and flower within one season under long day conditions. Biennials such as the cultivated sugar beet (*Beta vulgaris ssp. vulgaris*) as well as sea beets from northern latitudes require prolonged exposure to cold temperature over winter to acquire floral competence. Sugar beet is mainly cultivated for sugar production in Europe and is likely to have originated from sea beet. Flowering time strongly affects seed yield and yield potential and is thus a trait of high agronomic relevance. Besides environmental cues, there are complex genetic networks known to impact life cycle switch in flowering plants. In sugar beet, *BTC1*, *BvBBX19*, *BvFT1* and *BvFT2* are major flowering time regulators. In this study, I phenotyped plants from a diversity *Beta* panel encompassing cultivated and wild species from different geographical origin. Plants were grown under different day length regimes with and without vernalization. Haplotype analysis of *BTC1*, *BvBBX19*, *BvFT1* and *BvFT2* was performed to identify natural diversity of these genes and their impact on flowering. I found that accessions from northern latitudes flowered significantly later than those from southern latitudes. Some plants did not flower at all, indicating a strong impact of latitude of origin on life cycle. Haplotype analysis revealed a high conservation of the CCT-, REC-, BBX- and PEBP-domains with regard to SNP occurrence. I identified sequence variation which may impact life cycle adaptation in beet. My data endorse the importance of *BTC1* in the domestication process of cultivated beets and contribute to the understanding of distribution and adaptation of *Beta* species to different life cycle regimes in response to different environments. Moreover, my data provide a resource for haplotypes identified for the major floral regulators in beet.

#### 3.2 Introduction

To ensure optimal reproductive and therewith evolutionary success, flowering plants have developed different life cycles. Sea beets (*Beta vulgaris* L. *ssp. maritima*) are wild relatives of sugar beet (*B. vulgaris* L. *ssp. vulgaris*). Annual sea beets from Mediterranean environments germinate, bolt and flower within one season under long day conditions whereas most sea beets from northern latitudes are biennial. They need prolonged exposure to cold temperatures (typically during winter) to acquire a floral competent state. There are also perennial wild beets mostly which exhibit an iteroparous life cycle and an increasing life span concomitant with increasing latitude (Hautekèete et al. 2002). The onset of floral transition in beets is indicated by the elongation of the main shoot which is commonly referred to as ‘bolting’. In sugar beet cultivation, early bolting (without vernalization) is a trait of high agronomic relevance because it causes severe yield loss. The genetic control of photoperiodic flowering has been elucidated in the model plant *Arabidopsis thaliana*, while many of the identified genes are structurally conserved in all known plants (Blümel et al. 2015).

In beet, major components of the photoperiodic pathway have been identified. The bolting locus *BOLTING TIME CONTROL 1* (*BTC1*) determines the annual life (Pin et al. 2012). *BTC1* was identified as a pseudo-response regulator (PRR) gene, sharing sequence homology with the *PSEUDO RESPONSE REGULATOR 7* (*PRR7*) gene from *A. thaliana*. It encodes for a protein carrying a response regulator receiver (REC) and a CONSTANS, CONSTANS-Like and TOC1 (CCT) domain. Beets, carrying the dominant *BTC1* allele, mainly reveal an annual

growth habit such as most sea beet genotypes, while beets carrying the recessive *btc1* allele exhibit a biennial life cycle (Pin et al. 2012).

Two *FLOWERING LOCUS T (FT)* genes, *BvFT1* and *BvFT2* which are homologs to the Arabidopsis *FT*, were discovered to act downstream of *BTC1* (Pin et al. 2010). *BvFT1* and *BvFT2*, both belonging to the phosphatidylethanolamine-binding protein (PEBP) gene family, have evolved antagonistic functions. While *BvFT2* promotes flowering and it is required for floral development, *BvFT1* acts as a floral repressor. Pin *et al.* (2012) proposed a model for life cycle control in beet with *BTC1* acting upstream of *BvFT1* and *BvFT2*. In annual beets, the dominant *BTC1* allele represses *BvFT1* while it activates *BvFT2* to induce bolting and flowering. On the contrary, in biennial beets the expression of the recessive *btc1* allele is increasing gradually, leading to a decreasing expression of *BvFT1* during vernalization, what finally enables the promotion of *BvFT2* expression to initiate flowering. The recent discovery of another bolting time regulator *BvBBX19* encoding for a DOUBLE B-BOX TYPE ZINC FINGER protein further extended the model for bolting time regulation in beet (Dally et al. 2014). *BvBBX19* is diurnally regulated and acts epistatically over *BTC1* upstream of *BvFT1* and *BvFT2*. Interestingly, *BTC1* transcription was reduced in *BvBBX19* mutants suggesting a physical interaction of both proteins to jointly regulate *BvFT1* and *BvFT2* (Dally et al. 2014). In addition to those major regulators, several *CONSTANS-LIKE (COL)* genes have been detected, differing by their zinc-finger (B-Box) and CCT domains (Chia et al. 2008; Dally et al. 2014). To date, only *BvCOL1* has been functionally characterized by overexpression in Arabidopsis (Chia et al. 2008) but it was excluded as a functional orthologue of *CO* due to a non-typical expression profile.

The adaptation to different environments is of central importance for the evolutionary success in flowering plants. In *Beta* species, adaptation to different geographical regions is processed through the evolution of different life cycles. It was suggested that the domestication of sugar beet involved the selection of a rare partial loss-of-function allele of *BTC1*, which alters the plant's response to long day conditions (Pin et al. 2012). A *BTC1* haplotype analysis of a large number of *Beta* accessions and cultivars revealed eleven haplotypes divided into two classes, 'annuals' (*BTC1<sub>d-k</sub>*) and 'biennials' (*btc1<sub>a-c</sub>*). These two classes mainly differ by six non-synonymous single-nucleotide polymorphisms (SNPs) as well as a large insertion (~28kb) within the promoter of biennial *btc1* alleles (Pin et al. 2012). Intriguingly, vast majority of cultivated beets carry the recessive *btc1<sub>a</sub>* allele while wild beets mainly exhibited *BTC1* alleles from the 'annual' class.

In contrast, information about *BvBBX19*, *BvFT1* and *BvFT2* haplotypes and their abundance among wild and cultivated species is lacking so far. This study aims to understand the role of four major *Beta* flowering time regulators *BTC1*, *BvFT1*, *BvFT2* and *BvBBX19* on the adaptation to different environments. I assumed that sequence variations within the coding region of these genes have a major impact on the phenological development. Consequently, I expected a non-random distribution of haplotypes across accessions from different geographical origin. Moreover, I reasoned that life cycle changes follow a latitudinal cline. For this purpose, 29 *Beta* accessions from different geographical origin were grown under standardized conditions and the onset of bolting was recorded. I sequenced the coding regions of *BTC1*, *BvFT1*, *BvFT2* and *BvBBX19* from all accessions. I found a high number of polymorphisms in *BTC1*, while sequence variation among the other genes was low. A relationship between haplotype variation and life cycle regime could be established. I found that cultivated beets carry similar combinations of their *BTC1*, *BvBBX19*, *BvFT1* and *BvFT2* haplotypes while wild beets displayed much higher heterogeneity. These results demonstrate that haplotype variations of flowering time regulator genes are main drivers of the adaptive evolution of *Beta* species and the domestication of cultivated beet.

### 3.3 Material and methods

#### 3.3.1 Plant material, growth conditions and phenotypic analysis

*Beta* accessions were selected based on geographical diversity and expected bolting characteristics (annual and biennial) (Table 1). Seeds were sown in 9 cm<sup>2</sup> pots and plants were grown and phenotyped in a climate chamber with 10 plants per accession under different experimental conditions: 22 hours of light, 20°C (experiment 1 (E1)), 16 hours of light, 20°C (experiment 2 (E2)) and 22 hours of light, 20°C interrupted by a cold treatment at 4°C for three months (experiment 3 (E3)). Plants were watered every second day. In experiment 3 plants were fertilized twice, after 119 days directly before vernalization as well as after 210 days directly after vernalization with PERIMOR. The light intensity was held at 315 μmol m<sup>-2</sup>s<sup>-1</sup> and the humidity was about 70%. Bolting (BBCH 51) and flowering (BBCH 60) was recorded according to Meier (1993). Without vernalization, 16 weeks after sowing, plants were classified as annual (bolting) or biennial (non-bolting) (experiment 1 and 2). Plants which did not bolt 16 weeks after vernalization were classified as ‘never bolting’ (experiment 3).

**Table 4:** Plant material used in this study.

Variety	Species name	Seed code	Geographical Origin	Latitude (0°N)
wild beet	<i>B. vulgaris ssp. maritima</i>	080287	Ireland	53.0
		080461	Denmark	56.0
		080468	Egypt	27.0
		080437	Pakistan	31.0
		080418	India	21.0
		100539	Germany	51.0
		991971	Greece	39.0
		080260	Netherlands	52.0
		930034	Spain	40.0
		112787	France	46.0
sugar beet	<i>B. vulgaris ssp. vulgaris</i>	112823	Great Britain	54.0
		080538	Great Britain	54.0
		090023	Germany	51.0
		930176	Germany	51.0
		130333	Germany	51.0
		100043	Germany	51.0
		001684	Germany	51.0
		080394	Iran	32.0
		930181	USA	45.0
		080384	Turkey	39.0
fodder beet	<i>B. vulgaris ssp. vulgaris</i>	091645	Germany	51.0
		080281	Germany	51.0
		080313	Greece	39.0
red table beet	<i>B. vulgaris ssp. vulgaris</i>	080396	Iran	32.0
		092312	Russia	60.0
leaf beet	<i>B. vulgaris ssp. vulgaris</i>	080339	France	45.5
		080238	Iraq	33.0
		081845	China	35.0
		092459	Italy	42.0

### 3.3.2 Molecular analysis

The coding region of the flowering time genes *BvBBX19*, *BTCL1*, *BvFT1* and *BvFT2* was amplified by PCR. Primers and PCR conditions are listed in Supplementary Table 1 and Supplementary Table 2 and 3. In silico prediction of the coding gene structures of *BTCL1*, *BvBBX19*, *BvFT1* and *BvFT2* and primer positions are indicated in Supplementary Figure 1. DNA was isolated from leaves using the CTAB method (Rogers and Bendich 1985) with slight modifications. PCRs were performed for single plants and PCR products of all plants of the same accession were diluted to an equal concentration and pooled. Sanger sequencing of all pools was performed at the Institute of Clinical Molecular Biology (IKMB, CAU Kiel). Sequence analysis was done with the CLC Main Workbench 6.9 (CLC bio, Aarhus, Denmark) and the DNASTAR Lasergene SeqMan Pro (DNASTAR Inc., Madison, USA) program packages. Allelic haplotypes were defined by aligning obtained sequences of the amplified fragments and checking for single nucleotide polymorphisms (SNPs) and insertion/deletion polymorphisms. Pooled sequences were blasted against the beet reference sequence (KWS2320Refseq0.9) (Dohm et al. 2013) using the BLASTN function of the CLC Main Workbench 6.9. All SNP positions were numbered beginning with the translation start site. The evaluation of SNPs and their positions was performed according to the IUPAC code

(Johnson 2010) (Supplementary Table 4). Polymorphisms were categorized as synonymous (no impact on the amino acid sequence) or non-synonymous.

### 3.3.3 Statistical analysis

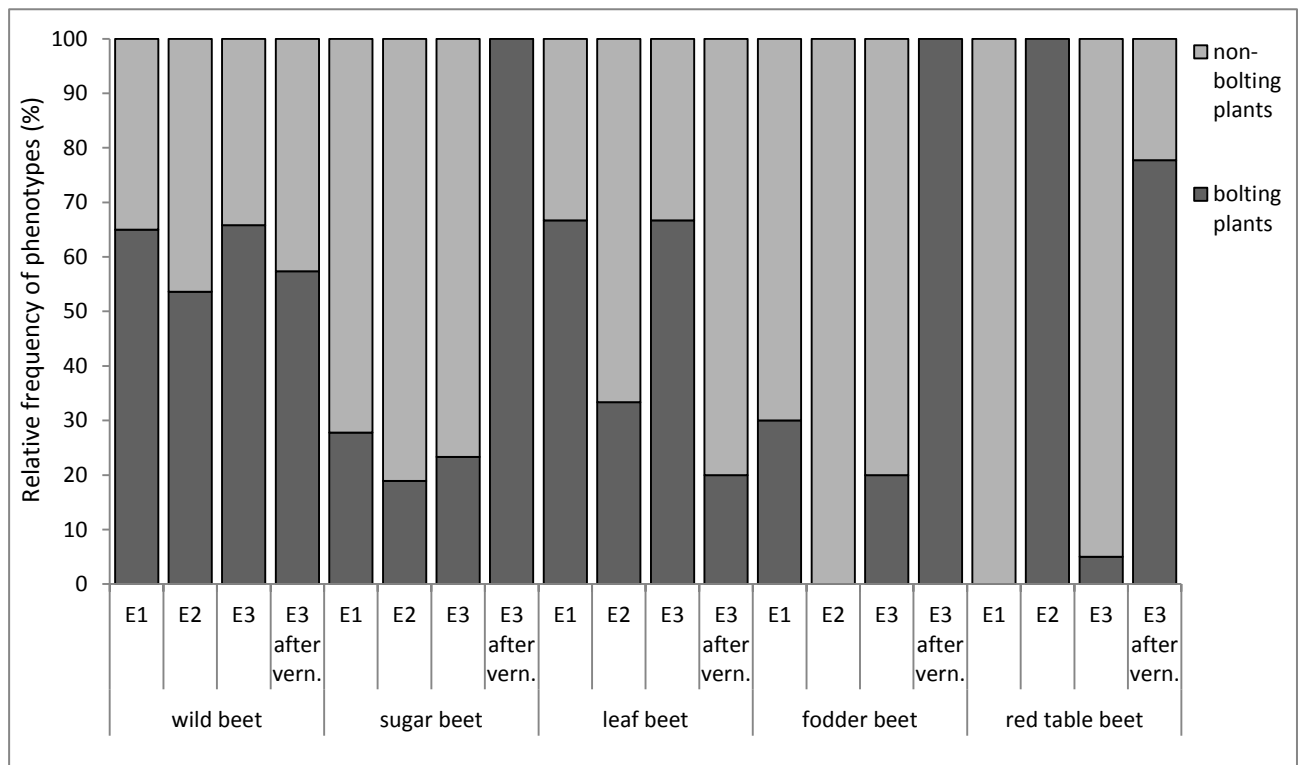
The software R (R Development Core Team 2015) was used for statistical analysis. The data evaluation started with the definition of an appropriate statistical mixed model (Laird and Ware 1982). The data were assumed to be normally distributed and to be heteroscedastic due to the different levels of environments (experiments) and latitude. These assumptions are based on a graphical residual analysis. The statistical model included a pseudo factor (Schaarschmidt and Vaas 2009), consisting of the actual factors experiment (E1, E2, E3), latitude (21°N-60°N) and varieties (wild beet, sugar beet, table beet, fodder beet and leaf beet). This pseudo factor was necessary because the actual factors are not orthogonal. The genotype was regarded as a random factor. Based on this model, multiple contrast tests (Bretz et al. 2011) were conducted in order to compare the several levels of i) variety, ii) latitude, and iii) experiment, respectively. Moreover, a further statistical model was established using latitude and experiment as covariates instead of the pseudo factor. On the basis of this model, an analysis of covariance (ANCOVA) was conducted (Cochran 1957), resulting in (three) different linear regression functions with the same slope.

## 3.4 Results

### 3.4.1 Large phenotypic variation for flowering time in species of the genus *Beta*

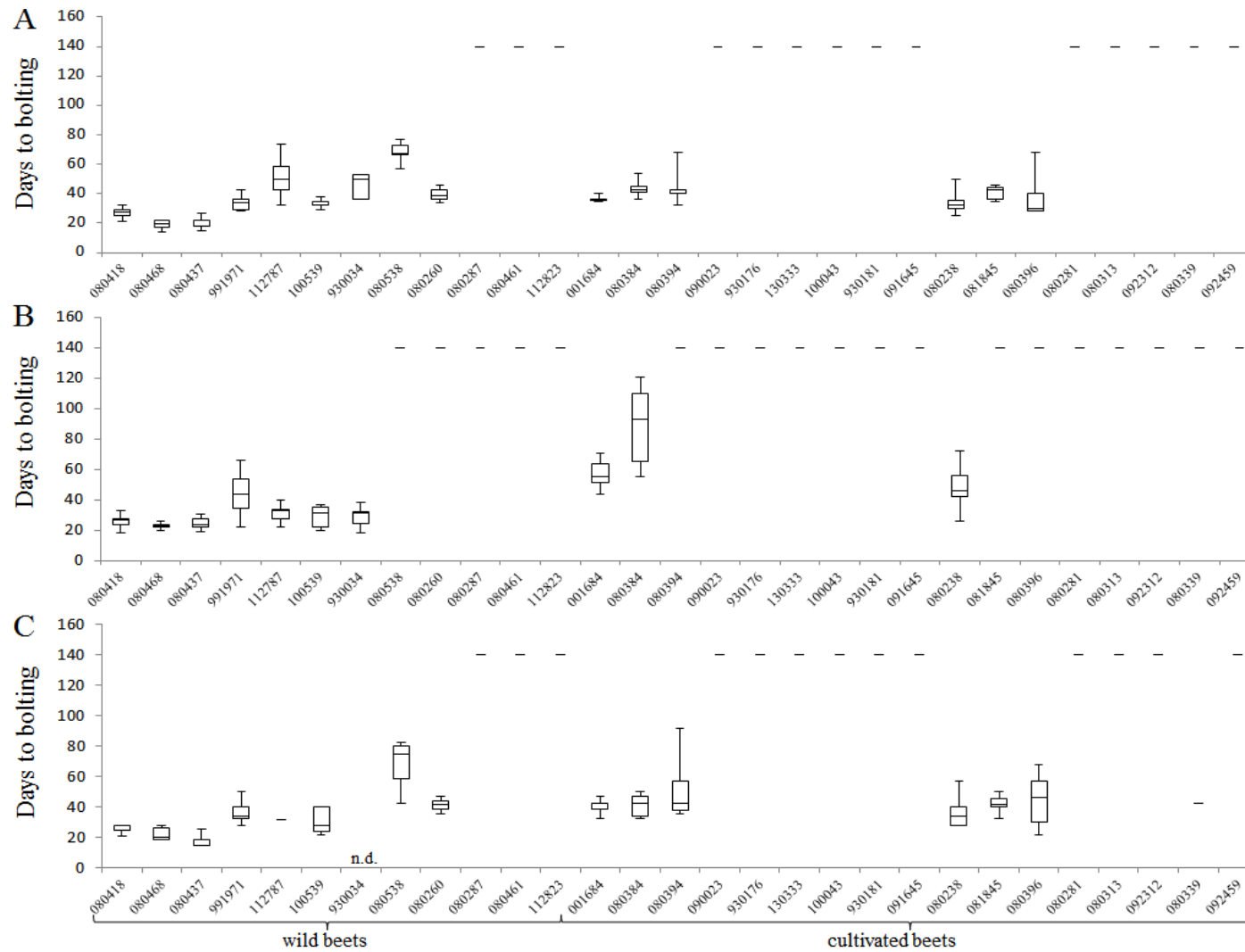
I chose 29 accessions from different geographical origin to represent the genetic diversity of the species *B. vulgaris* (Table 1). Of each accession, 10 plants were grown in a climate chamber under three different environmental conditions. The onset of bolting was assessed as beginning of elongation of the main stem (BBCH51) after Meier (1993). In experiment 1 and 2, plants were held under 22 and 16 hours of light, respectively. In the third experiment, the same day/night regime as in experiment 1 was applied but biennial accessions were subjected to another 12 weeks of cold treatment (4°C). I uncovered annual and biennial bolting behavior in both wild and cultivated accessions (Figure 5, Supplementary Table 5).

**Figure 5:** Phenological development of cultivated beet under three different environments (experiment 1-3). Bolting was determined as the beginning of shoot elongation (BBCH51) (Meier 1993). Plants were grown in pods in a climate chamber and kept under three different LD conditions: 22h light, 20°C (E1), 16h light, 20°C (E2), 22h light, 20°C, interrupted by 12 weeks of 4°C (E3). The number of cultivars used (n) is indicated in light grey. In E1, E2 and E3 ‘non-bolting’ means that plants did not bolt until 16 weeks after sowing. In E3, after vern. ‘non-bolting’ means that plants did not bolt until the end of the experiment (16 weeks after vernalization).



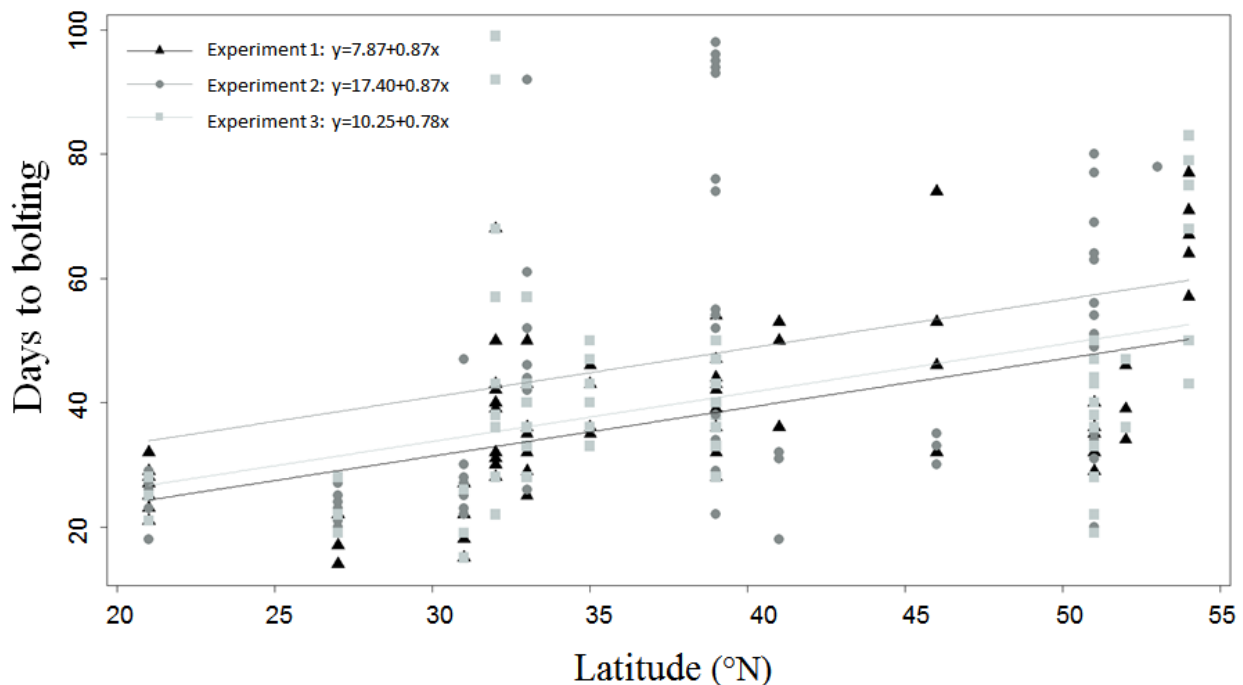
There was a clear tendency for earlier bolting without vernalization under 22h of light (experiment 1 and 3). On average, annual plants bolted 10 days earlier under 22h of light as when grown under 16h. Five accessions were classified as annual under 22h light while they behaved as biennials under 16h light (Figure 6A-C; Supplementary Table 5). The earliest accession under 22h light, 080437 from Pakistan (31°N), bolted 19 days after sowing. Under 16h light it bolted after 27 days. The earliest accession under 16h light was 080468 from Egypt (27°N), which bolted 23 days after sowing. I found that accessions which bolted without cold treatment had a complete absence of vernalization requirement. Contrary, 080538 from Great Britain (54°N) was the latest accession under 22h light which bolted 69 days after sowing. Interestingly, in experiment 3 only seven out of ten plants bolted before vernalization, but the remaining three bolted after vernalization. When grown under 16h light, accession 080538 performed a biennial life cycle. There was considerable phenotypic variation within accessions under the same experimental conditions. The wild beet accessions 080260 (52°N), 080538 (54°N), 100539 (51°N), and 112787 (46°N) from northern latitudes segregated into annual and biennial plants (22h light). Similarly, the cultivated beet accessions 080384 (39°N), 080394 (32°N) and 080396 (39°N) from southern latitudes segregated for bolting under 22h light (Supplementary Table 5).

**Figure 6:** Bolting time measurements for all accessions under different environmental conditions. A) Days to bolting (DTB) of plants of accessions that bolted before vernalization when held in experiment 1 (22h light/2h dark). B) DTB of plants of accessions when held in experiment 2 (16h light/8h dark). C) DTB of plants of accessions when held in experiment 3 (22h light/2h dark). Bolting was determined as DTB after sowing without vernalization. Plants were grown in pods in a climate chamber and kept under LD conditions at 20°C, 315  $\mu\text{mol m}^{-2}\text{s}^{-1}$  and 70% humidity. Plants are separated by wild beet accessions and cultivated beet accessions. For plants of accessions that did not bolt without vernalization the value of days to bolting was set to 140 days. Error bars represent the standard error of the mean (SEM)



I reasoned that the phenological development of *Beta* genotypes depends on latitude of origin. To test this hypothesis, I conducted an analysis of covariances (ANCOVA) (Cochran 1957) with data from experiment 1-3. This analysis revealed three different linear regression functions with the same slope (Figure 7) suggesting that accessions from southern latitudes of origin flowered earlier than those from northern latitudes. As all regression functions revealed the same slope, I concluded that all environments exert a similar effect of latitude on days to bolting. Additionally, I found, that accessions from northern latitudes exhibited a tendency towards biennial bolting (Supplementary Table 5). Surprisingly, not all cultivated beets displayed a biennial behavior. The leaf beet accession 080238 from Iraq (33°N) revealed an annual life cycle under all experimental conditions (without vernalization). Similar as some wild beet accessions, it bolted earlier under 22h light (35 days), than under 16h light (50 days). Moreover, the sugar beet accession 080384 from Turkey (39°N) segregated for bolting and non-bolting before vernalization, while bolting plants had a strong tendency towards early flowering in all experiments. After vernalization, sugar and fodder beets bolted within 26-61 days. However, one leaf beet accession from Italy (42°N) and one table beet accession from Russia (60°N) segregated into bolting and ‘never bolting’ after vernalization. Also, the wild beet accessions 080287, 080461, 112823 and 112787 from Ireland (53°N), Denmark (56°N), Great Britain (54°N), and France (46°N) exhibited a tendency towards ‘never bolting’ (Supplementary Table 10).

**Figure 7:** Days to bolting on a latitudinal cline (°N). Bolting plants from experiment 1 are depicted as black triangles. Bolting plants from experiment 2 are depicted as dark grey, filled circles and bolting plants from experiment 3 are depicted as light grey, filled boxes. The statistical model included experiment and latitude as covariates and an analysis of covariances (ANCOVA) was conducted, resulting in different linear regression functions for all experiments with the same slope. Plants were grown in pods in a climate chamber and kept under LD conditions (either 16 hours of light/8 hours dark, 20°C (experiment 2) or 22 hours of light/2 hours dark (experiment 1 and 3), 20°C, 315  $\mu\text{mol m}^{-2}\text{s}^{-1}$  and 70% humidity). Non-bolting plants are not included in the analysis.





### 3.4.2 Haplotype variation of four major flowering time regulators

Next, I aimed to link sequence variations and phenological development. For haplotype analyses, I sequenced the coding regions of *BTC1*, *BvBBX19*, *BvFT1* and *BvFT2* because they had been identified as major constituents of the bolting time regulatory pathway in beet (Dally et al. 2014; Pin et al. 2010; Pin et al. 2012). I sequenced pooled PCR products from single plants of an accession grown in experiment 1. If a pooled DNA sample turned out to be a mixture of different sequences or if I detected segregation into bolting and non-bolting plants, single plants were sequenced.

First, I sequenced the *BTC1* coding region (2367 bp) for each accession and compared it to the reference sequence (Pin et al. 2012). Twenty-five out of 27 SNPs had already been described by Pin et al. (2012) while two additional polymorphisms (exon 8 nt2 and exon 9 nt29) turned out to be new (Table 5). Four polymorphic nucleotides are located within the sequence encoding the CCT- and the REC-domain (two in each domain). One of these (exon 3, nt351) represents a non-synonymous mutation from Asparagine to Lysine (Pin et al. 2012). In total, I identified 11 different *BTC1* haplotypes among all 29 accessions. Of these, three haplotypes have been unknown so far (*btc1<sub>l</sub>*, *BTC1<sub>m</sub>*, *BTC1<sub>n</sub>*), the remaining eight haplotypes have already been identified by Pin et al (2012) (Table 5).

**Table 5:** Haplotype studies with the *BTC1* gene. 27 SNPs were assembled to eleven haplotypes. The coding sequence of *BTC1* was sequenced from all plants of the *B. vulgaris* panel. According to Pin et al., (2012) the position of the SNPs is given relative to its translation start site according to the regarding exons. The polymorphism exon9nt616 (published by Pin et al., (2012)) could be identified after own database research as erroneously swapped (see HQ709094.1). The different nucleotides are indicated by different colors.

Exon	3			5	6		7			8							9								10		
	92	224	351	89	64	89	23	75	164	2	9	79	97	154	158	250	29	37	402	435	476	542	616	670	686	814	72
<i>BTC1<sub>a</sub></i>	G	A	A	C	G	T	C	A	G	C	C	C	C	G	T	G	G	A	A	G	C	G	A	A	G	T	A
<i>BTC1<sub>d</sub></i>	G	C	T	T	G	G	C	G	T	C	C	A	C	G	T	A	G	A	G	A	T	A	A	A	A	T	G
<i>BTC1<sub>e</sub></i>	G	C	T	T	G	G	C	G	T	C	C	A	C	G	A	A	G	A	G	G	T	A	A	A	G	T	G
<i>BTC1<sub>g</sub></i>	G	C	T	T	G	G	C	G	T	C	C	A	C	G	T	A	G	A	G	G	T	A	A	A	G	T	G
<i>BTC1<sub>h</sub></i>	G	C	T	T	G	G	C	G	T	C	T	A	C	G	T	A	G	A	G	G	T	A	A	A	G	T	G
<i>BTC1<sub>i</sub></i>	G	C	T	T	G	G	C	G	G	C	C	A	C	G	T	A	G	A	G	G	T	G	G	G	G	T	G
<i>BTC1<sub>j</sub></i>	G	C	T	T	A	G	C	G	T	C	C	A	C	G	T	A	G	A	G	G	T	G	A	A	G	C	G
<i>BTC1<sub>k</sub></i>	T	C	T	T	G	G	C	G	T	C	C	A	A	G	T	A	G	A	G	G	T	G	A	A	G	T	G
<i>btcl<sub>l</sub></i>	G	C	T	T	G	G	C	G	T	C	C	A	C	G	T	A	A	A	G	G	T	A	A	A	G	T	G
<i>BTC1<sub>m</sub></i>	G	C	T	T	G	G	C	G	G	T	C	A	C	G	T	A	G	A	G	G	T	G	G	A	G	T	G
<i>BTC1<sub>n</sub></i>	G	C	T	T	G	G	C	G	T	C	C	A	C	G	T	A	G	A	G	G	C	G	A	A	G	T	G
non-syn. SNP	*	*	*		*	*			*	*	*	*	*	*	*	*	*		*	*	*	*		*	*		

As expected, most of the cultivated (biennial) beet accessions carried the *btc1<sub>a</sub>* haplotype (Supplementary Table 6), which has already been attributed as ‘biennial’ *btc1* haplotype (*BTC1<sub>a-c</sub>*) (Pin et al. 2012). Second, I sequenced the coding region of the *BvBBX19* gene (588 bp). Sequence variation was much lower as observed for *BTC1*. I identified one non-synonymous and three synonymous polymorphisms. As expected, none of the accessions carried the EMS mutations which had been published by Dally et al (2014). Interestingly, only one synonymous SNP was located within the region coding for the B-Box-domains. Taken together, all polymorphisms gave rise to seven haplotypes (*BvBBX19<sub>a-g</sub>*) across all accessions analyzed in this study (Table 6).

**Table 6:** Haplotype variation within the *BvBBX19* gene. Four SNPs were assembled to seven haplotypes. The coding sequence of *BvBBX19* was sequenced from all plants of the *B. vulgaris* panel. The position of the SNPs is given relative to its translation start site according to the regarding exons. The different nucleotides are indicated by different colors.

Exon	2		4		
	69	45	59	231	
<i>BvBBX19<sub>a</sub></i>	A	G	C	T	
<i>BvBBX19<sub>b</sub></i>	A	G	C	C	
<i>BvBBX19<sub>c</sub></i>	G	G	C	T	
<i>BvBBX19<sub>d</sub></i>	G	A	C	C	
<i>BvBBX19<sub>e</sub></i>	G	A	C	T	
<i>BvBBX19<sub>f</sub></i>	G	A	T	C	
<i>BvBBX19<sub>g</sub></i>	G	A	T	T	
non-syn. SNP			*		

The non-synonymous SNP was present only in two haplotypes (*BvBBX19<sub>f, g</sub>*). These haplotypes occurred in all leaf beet- and wild beet accessions, but only in one fodder beet- and one sugar beet accession (Supplementary Table 7). Intriguingly, all accessions with the *BvBBX19<sub>f</sub>* or *BvBBX19<sub>g</sub>* haplotypes originate from southern latitudes (21-42°N).

Third, I analyzed the coding region of the floral repressor *BvFTI* (540 bp). In total, I found one synonymous as well as four non-synonymous SNPs. Additionally, I identified an insertion of 3 bp in exon 1 which occurred only in two accessions. These polymorphisms could be assembled to eight haplotypes (*BvFTI<sub>a-h</sub>*) (Table 7). Four of the five polymorphisms are located outside the PEBP -domain region. Only two accessions house a polymorphism within the PEBP coding region (exon 4 nt57, haplotype *BvFTI<sub>c</sub>*) (Supplementary Table 8), indicating a high conservation of this domain.

**Table 7:** Haplotype variation within the *BvFT1* gene. 5 SNPs were assembled to eight haplotypes. The coding sequence of *BvFT1* was sequenced from all plants of the *B. vulgaris* panel. The position of the SNPs is given relative to its translation start site according to the regarding exons. The different nucleotides are indicated by different colors.

Exon	1				4	Insertion
	11	20	70	72	57	
<i>BvFT1<sub>a</sub></i>	<b>G</b>	C	C	C	C	
<i>BvFT1<sub>b</sub></i>	<b>G</b>	C	<b>G</b>	C	C	
<i>BvFT1<sub>c</sub></i>	<b>G</b>	C	<b>G</b>	C	<b>T</b>	
<i>BvFT1<sub>d</sub></i>	<b>G</b>	<b>T</b>	<b>G</b>	C	C	
<i>BvFT1<sub>e</sub></i>	<b>G</b>	<b>T</b>	C	C	C	
<i>BvFT1<sub>f</sub></i>	<b>T</b>	<b>T</b>	C	<b>T</b>	C	X
<i>BvFT1<sub>g</sub></i>	<b>G</b>	<b>T</b>	C	<b>T</b>	C	
<i>BvFT1<sub>h</sub></i>	<b>G</b>	C	C	<b>T</b>	C	
non-syn. SNP	*	*	*		*	

Fourth, I studied the *BvFT2* gene and identified two non-synonymous polymorphisms giving rise to four haplotypes (*BvFT2<sub>a-d</sub>*) (Table 8). One SNP is located within the PEBP-domain region (exon 4 nt39). Remarkably, those haplotypes with the PEBP domain mutation (*BvFT2<sub>b</sub>* and *BvFT2<sub>c</sub>*) were only present in wild beet accessions from northern latitudes (39-56°N). In contrast, *BvFT2<sub>a</sub>* and *BvFT2<sub>d</sub>* are highly abundant in cultivated *Beta* accessions (Supplementary Table 9).

**Table 8:** Haplotype variation within the *BvFT2* gene. Two SNPs were assembled to four haplotypes. The coding sequence of *BvFT2* was sequenced from all plants of the *B. vulgaris* panel. The position of the SNPs is given relative to its translation start site according to the regarding exons. The different nucleotides are indicated by different colors.

Exon	1	4
SNP position/ Haplotype	82	39
<i>BvFT2<sub>a</sub></i>	A	<b>G</b>
<i>BvFT2<sub>b</sub></i>	A	A
<i>BvFT2<sub>c</sub></i>	C	A
<i>BvFT2<sub>d</sub></i>	C	<b>G</b>
non-syn. SNP	*	*

### 3.4.3 Relation between haplotype variation and life cycle regime

I anticipated a link between haplotype variation and life cycle regime which in turn depends on the geographical origin of an accession. First, I looked for a reciprocal relation between haplotypes and phenological development under long day conditions (experiment 1) (Supplementary Table 10). As a general rule, cultivated beets which mainly exhibited a biennial life cycle displayed low genetic variation. In the majority, they carry similar

combinations of their *BTC1*, *BvBBX19*, *BvFT1* and *BvFT2* haplotypes. Most cultivated sugar beet accessions which exhibited a biennial life cycle (seed codes: 090023, 130333, 091645, 100043 and 930176) revealed the ‘biennial’ *BTC1<sub>a</sub>* and *BvBBX19<sub>a</sub>* haplotypes, respectively. Moreover, seven out of nine sugar beet accessions, as well as both red table beet accessions displayed either the *BvFT1<sub>a</sub>* and *BvFT2<sub>a</sub>* or the *BvFT1<sub>a</sub>* and *BvFT2<sub>d</sub>* haplotype combination. Wild beets displayed a much higher heterogeneity (between and within accessions) regarding their *BvBBX19*, *BvFT1* and *BvFT2* haplotypes whereas most accessions were fixed for only one *BTC1* haplotype (exceptions: 991971, 080538, 081845), despite a high sequence variation within this gene across all accessions. Noteworthy, the new *BTC1<sub>m</sub>* haplotype only occurred in wild beet accessions from higher latitudes (21.0-39.0°N).

### 3.5 Discussion

I examined 29 *Beta* accessions from different geographical origins including wild and cultivated beets for phenotypic plasticity under different photoperiodic conditions. Further, I analyzed the haplotypes of *BTC1*, *BvBBX19*, *BvFT1* and *BvFT2* to uncover the relationship between haplotype variation and life cycle adaptation. I found a general southward shift towards earlier flowering. Plants from northern latitudes flowered considerably later or did not flower at all, pointing at a strong coherence of life cycle and geographical origin. Besides, I detected a high conservation of the important protein domains (CCT-, REC-, BBX- and PEBP) for all genes emphasizing their evolutionary relevance for life cycle adaptation in *Beta* species. Withal, haplotype analysis of *BvBBX19*, *BvFT1* and *BvFT2* displayed only a few polymorphisms when compared with the high SNP frequency in *BTC1*, highlighting a strong selection pressure. My data indicate that domestication of cultivated beets mainly stems to breeders selection for the *BTC1* gene, but not inevitably to selection for *BvBBX19*, *BvFT1* or *BvFT2*. The domestication process was reflected by haplotype combinations, as well. While most cultivated beets carried similar haplotype combinations of *BTC1*, *BvBBX19*, *BvFT1* and *BvFT2*, wild beets displayed much higher heterogeneity.

To warrant evolutionary success of a flowering plant, the adaptation to different climates concomitant with life cycle control is of utmost importance. There are several environmental factors which impact phenotypic plasticity of a flowering plant, such as temperature (Andrés and Coupland 2012; McClung et al. 2016; Song et al. 2014) and photoperiod (Andrés and Coupland 2012; Searle and Coupland 2004). Species of the genus *Beta* have evolved different life cycles over the years allowing adaptation to a broad spectrum of latitudes. Besides annuality, beets have evolved biennial and perennial life cycles (Hautekète et al. 2002), which makes this species an ideal study species for life cycle adaptation. However, prior to this study there was only scant knowledge about the relationship between plant phenology and geographical origin in correlation with genetic diversity of known flowering time genes.

Van Dijk et al. (1997) and Boudry et al. (2002) have demonstrated that differences in life cycle due to vernalization requirement seem to be an adaptive response to season length and spring temperatures in particular latitudes. They studied 93 wild beet populations situated along a latitudinal cline around the French coast northwards to south-east England and found, that in southern hemispheres a total absence of vernalization requirement is more common than in northern hemispheres (Van Dijk et al. 1997). Hence, northern plants flowered less in northern sites, than southern plants in southern sites. Similar observations were obtained by Boudry et al. (2002), who sampled seed-balls from seven wild beet populations from different habitats in France with a smaller number of populations but a higher number of plants than Van Dijk et al. (1997).

Even though both studies covered a higher number of populations and plants compared to my study, their observations only focused on wild beets from the Mediterranean northwards to

south-east England. Our *Beta* panel, by contrast, displays a broader range of genotypes from different geographical origin and it includes several cultivated beets. However, my findings of a southward shift towards earlier flowering and a complete absence of vernalization requirement of *Beta* accessions from southern latitudes confirm the findings of Boudry et al. (2002) and Van Dijk et al. (1997). I found that *Beta* accessions from northern hemispheres flowered later or did not flower at all, suggesting a major effect of vernalization requirement whereas accessions from southern latitudes flowered early (e.g. 080437 which bolted 27 days after sowing in experiment 2). Nowadays, the climate changes rapidly due to global warming. Early flowering is a trait which will be selected for in long day plants (Van Dijk and Hautekèete 2007).

Major flowering time regulators associated with life cycle adaptation in beet are *BTC1*, *BvBBX19*, *BvFT1* and *BvFT2* (Dally et al. 2014; Pin et al. 2010; Pin et al. 2012). The impact of natural allelic variation of *BTC1* on the life cycle of beet has already been demonstrated (Pin et al. 2012). The authors suggested that life cycle adaptation results from haplotype diversity of the *BTC1* bolting gene which alters the plants response to long day conditions. They discovered two different classes of *BTC1* alleles which they refer to be associated with beets life cycle: ‘biennial’ haplotypes (*btc1<sub>a-c</sub>*), which are primarily found in cultivated beets, as well as ‘annual’ haplotypes (*BTC1<sub>d-k</sub>*) mainly occurring in wild beets. Both classes differ by a large insertion (~28kb) emerging in the promoter region of the biennial haplotypes *btc1<sub>a-c</sub>* and by six non-synonymous SNPs and several synonymous SNPs (Pin et al. 2012). Interestingly, only one of the non-synonymous SNPs (exon 3, nt351), differentiating between these classes, is located within the sequence encoding for the CCT-domain. Moreover, this polymorphism is only present in cultivated beets carrying the *btc1<sub>a</sub>* haplotype, indicating a potential target during domestication. The dominant *B* allele occurs mainly in species from southern parts, which I could confirm with my findings, but it is expected to increase in frequency as well as in northward movements (Hautekèete et al. 2009). For *BvBBX19* it was shown that polymorphisms derived from EMS mutagenesis turned an annual to a biennial beet (Dally et al. 2014). However, is there natural variation of *BvBBX19*, *BvFT1* and *BvFT2* as it was demonstrated for *BTC1*?

In my study, I show natural variation in all genes investigated, however, the degree of variation was quite different. From 25 polymorphisms identified in *BTC1*, 19 were described as non-synonymous SNPs (Pin et al. 2012), accounting for eleven haplotypes. Among all accessions, I detected eleven *BTC1* haplotypes from which three have not been described before (*btc1<sub>i</sub>*, *BTC1<sub>m</sub>*, *BTC1<sub>n</sub>*). These haplotypes display the highest genetic diversity of the chosen material. Most cultivated beets carried the *btc1<sub>a</sub>* haplotype reflecting the effect of the domestication bottleneck on genetic diversity (Doebley et al. 2006). This is also underpinned by a low genetic variation and similar haplotype combinations among cultivated beets.

The *BvBBX19* haplotypes displayed only four polymorphisms. One of these is a non-synonymous (exon 4, nt59) and one a synonymous SNP within the sequence coding for the B-Box-domain. The remaining two polymorphisms are synonymous and they are located outside of the B-Box-domain coding sequence. For *BvFT1* I identified five polymorphisms as well as two for *BvFT2*. Interestingly, for each gene I found one non-synonymous SNP within the sequence coding for the PEBP-domains. Nevertheless, haplotypes with polymorphisms in the PEBP-domains were observed very rarely for both *BvFT1* and *BvFT2*. Hence, my data argue for a high conservation of the PEBP-and B-Box-domains in *Beta* species. The *BvFT1<sub>f</sub>* which displays a 3bp insertion within the coding region of the PEBP domains was only found in two wild beet accessions (080437 and 080418) from southern latitudes which displayed very early bolting phenotypes. I hypothesize that this insertion may impair the repressing function of *BvFT1* in these plants thus enabling an early flowering phenology.

The gene *BvFT2* displayed the highest sequence conservation underpinning its importance as a floral integrator. This is in line with several findings suggesting, that *FT* homologs that induce flowering are highly conserved in diverse species, such as the rice *FT* orthologue *Heading date3 (Hd3a)* (Tamaki et al. 2007), the tomato *SINGLE FLOWER TRUSS (SFT)* (Lifschitz et al. 2006) or *CENTRORADIALIS8 (ZCN8)* from *Zea mays* (Lazakis et al. 2011; Meng et al. 2011).

I observed a general tendency for increasing sequence heterogeneity in wild beets in comparison to cultivated beets. A reason for this observation might be that all cultivated sugar beets stem from the “White Silesian” as a single-source population (Biancardi et al. 2005). They have been strictly selected against early bolting since breeding started in the early 18<sup>th</sup> century resulting in a selective sweep in this region of the genome. The new haplotypes detected in this study may serve as new resources for beet breeding to broaden the variation for bolting resistance even after winter which is necessary to breed winter beets (Jung and Müller 2009).

Overall, my results clearly demonstrate a strong correlation of phenotypic plasticity with geographical origin. Moreover, I show that flowering time genes of *Beta* species show a high conservation, especially of protein-domain-coding regions in floral regulator genes, emphasizing their evolutionary relevance for life cycle adaptation. My results clearly indicate, that breeders mainly selected for the *BTC1* gene during domestication of cultivated beet accessions. The increased heterogeneity of *BvBBX19*, *BvFT1* and *BvFT2* haplotypes in wild beets reflects the evolutionary pressure they are exposed to in their consistent run with climate change such as global warming adaptation to guarantee their future fitness and existence.

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## 4 Functional analysis of *BvBBX19* after transformation into *Arabidopsis thaliana*

### 4.1 Introduction

In the model species *Arabidopsis thaliana*, the onset of flowering is controlled by a network of genes and many of them are arranged in four main pathways: the autonomous, vernalization, gibberellic acid and the photoperiodic pathway (Blümel et al. 2015; Srikanth and Schmid 2011). Within the photoperiodic pathway, *CONSTANS* (*CO*) is a major key component and was shown to promote flowering under long day (LD) conditions by activating the floral inducer *FLOWERING LOCUS T* (*FT*) through direct binding of the CO protein to the TGTG(N2-3)ATG motif in the *FT* promoter (Cao et al. 2014; Tiwari et al. 2010). Recent analysis revealed that *CO* encodes a protein with two major domains: a CCT (CONSTANS, CONSTANS-LIKE and TOC1) domain which was shown to be sufficient for nuclear localization and two zinc-finger B-Box domains towards the amino terminus involved in protein-protein interaction (Gangappa et al. 2013a; Wenkel et al. 2006). *Arabidopsis thaliana* has a large B-Box family consisting of 32 members (Khanna et al. 2009; Robson et al. 2001). All members are clustered into five subfamilies according to protein sequence analyses. Members of subfamily I-III are termed as *CO* (*BBX1*) and *CO*-like proteins due to their structure with one CCT-domain and at least one B-Box (*BBX1-BBX17*). Members of subfamily IV-V (*BBX18-BBX32*) contain one or two B-Boxes but lack the CCT-domain. The CCT domain is implicated in transcriptional regulation as well as in nuclear protein import (Gendron et al. 2012; Jang et al. 2008; Laubinger et al. 2006; Robson et al. 2001). In contrast, the B-Box motifs were shown to be of importance for transcriptional regulation as well as for heterodimeric formation with other BBX family members and other proteins (Gangappa and Botto 2014; Gangappa et al. 2013a; Qi et al. 2012).

A number of B-Box mutations have been identified within the *CONSTANS* gene. Their mutants showed reduced expression levels of *FT* leading to a late flowering phenotype such as in the B-Box defective *co-2* mutant, pointing the importance of the B-Box domain for the function of CO (Ben-Naim et al. 2006; Kardailsky et al. 1999; Robson et al. 2001; Tiwari et al. 2010). In contrast, constitutive overexpression of *CO* induced an early flowering phenotype under LD conditions (Onouchi et al. 2000). Equally, other BBX family members were also shown to be involved in flowering processes, such as *BBX32*, *BBX24*, *BBX7*, *BBX6* and *BBX5* (Cheng and Wang 2005; Datta et al. 2006; Li et al. 2014b; Park et al. 2011). *BBX19*, another transcription factor with two B-Box motifs, was recently shown to delay flowering under inductive photoperiods by constitutive overexpression, while diminished expression by RNA interference advances flowering. Interestingly, the authors exhibited that *BBX19* is expressed in vasculature like *CO* and its constitutive expression negatively impacts the transcription of *FT*, *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*), *LEAFY* (*LFY*), and *FRUITFUL* (*FUL*), but not the expression levels of *CO*.

As a negative regulator of flowering time, *BBX19* was shown to interact with the CO protein to deplete their active pool which is necessary for *FT* activation and therewith for induction of flowering (Wang et al. 2014a). Hence, their results demonstrate that *BBX19* functions as a checkpoint that prevents *FT* expression shortly after the advent of *CO* expression. The family of *CONSTANS-LIKE* (*COL*) genes is widely distributed among the plant kingdom. For sugar beet, evidence of *COL* genes was given including three different *COL* genes which differ in their B-Box and CCT domains (Chia et al. 2008). Although *BvCOL1* was identified as a potential member of the photoperiod pathway, its function in beet is still unknown and it was not declared as an orthologue of *CO* due to different expression patterns of both genes (Chia et al. 2008). Hence, to date no true *CO* orthologue has been identified in beet.

Lately, remarkable progress has been made in identifying key components of the *FT* regulation in beet: two *FT* paralogs were identified in beet and shown to have antagonistic functions with *BvFT1* acting as a floral repressor and *BvFT2* as a floral integrator (Pin et al. 2010), which indicated neofunctionalization of flowering time genes during the evolution of *Beta* species. Furthermore, the bolting gene *BOLTING TIME CONTROL 1 (BTC1)* was cloned from sugar beet and displayed high sequence homology to a *PSEUDO RESPONSE REGULATOR (PRR)* gene from *Arabidopsis*. *BTC1* belongs to the *PRR3/PRR7* clade of PRRs from both, monocots such as rice (*Oryza sativa*) or barley (*Hordeum vulgare*) and dicots such as poplar (*Populus trichocarpa*) and grapevine (*Vitis vinifera*). Additionally it was discovered to contain a CCT domain and promotes flowering under LD conditions by repression of *BvFT1* and induction of *BvFT2* (Pin et al. 2010; Pin et al. 2012).

To date, eleven haplotypes of *BTC1* have been identified and grouped into two classes: the ‘biennial’ haplotypes (*btc1<sub>a-c</sub>*) mainly found in cultivated beets as well as the ‘haplotypes (*BTC1<sub>d-k</sub>*) which primarily occur in wild beet accessions (Pin et al. 2012). Recently, a novel bolting time regulator in sugar beet was identified by a map-based cloning approach and termed *BvBBX19* according to its closest homolog of *Arabidopsis thaliana* (55% identity to the BBX19 protein from *A. thaliana* according to BLASTP analysis). *BvBBX19* encodes for a DOUBLE B-BOX TYPE ZINC FINGER protein and acts epistatically over *BTC1*. In their study, the authors claim that a single transition within the second B-Box of the *BvBBX19* gene turns an annual into a biennial beet, which flowers only after cold treatment. Furthermore, *BvBBX19* is diurnally regulated such as *BTC1* and acts upstream of *BvFT1* and *BvFT2* (Dally et al. 2014). A functional mutation in *BvBBX19* leads to upregulation of *BvFT1* as well as to complete downregulation of the floral integrator *BvFT2*. Consequently, the authors proposed a new model by which *BvBBX19* and *BTC1* complement each other and acquire a *CO* function, to regulate their downstream targets *BvFT1* and *BvFT2*. Those findings are reminiscent of the results from *Arabidopsis* (Wang et al. 2014a).

Key regulators of flowering pathways are highly conserved and function similar in other distantly related species, such as tomato or rice (Andrés and Coupland 2012). 140 million years ago, shortly after the monocot-dicot split, the family of *Amaranthaceae*, to which sugar beet belongs to, separated from *Arabidopsis*. There have been several complementation studies in which the phenotypes of *Arabidopsis* flowering time mutants could be rescued or altered by integration of a homologous gene from another species. For instance, the *BnCO1* allele from *Brassica napus* was shown to complement the late flowering phenotype of the *co-2* mutant in *Arabidopsis* (Robert et al. 1998). Similarly, it was demonstrated that overexpression of *BvFT2* in *A. thaliana* strongly promotes flowering, while overexpression of *BvFT1* represses flowering (Pin et al. 2010).

I reasoned that the beet floral regulators *BvBBX19* and *BTC1* could accomplish a *CO* function after transformation into *Arabidopsis*. To test this hypothesis, I generated overexpression lines of *A. thaliana* wildtype (Col-0) and the late flowering *CONSTANS* mutant (*co-2*), constitutively overexpressing *BvBBX19* and *BTC1<sub>d</sub>* transgenes. My expectation was, that overexpression of both genes would accelerate the flowering phenotype as it was already demonstrated for overexpression of *CO* in *Arabidopsis* (An et al. 2004; Ayre and Turgeon 2004; Onouchi et al. 2000). I found that overexpression of *BvBBX19* and *BTC1<sub>d</sub>* does not influence the *Arabidopsis* phenotype, neither in the Col-0 wildtype, nor in the *co-2* mutant, when comparing transgenic with non-transgenic plants in segregating T<sub>2</sub> families.

## 4.2 Material and methods

### 4.2.1 Plant material, growth conditions and phenotyping

I used the *A. thaliana* wildtype Col-0 (ecotype Columbia) and the flowering time mutant *co-2* for plant transformation. Besides Col-0, I used the *Ler* (Landsberg *erecta*) ecotype as another control. The *co-2* mutant constitutes an EMS mutant derived from mutagenesis of the *Ler* ecotype. It features a mutation in the first B-Box of the *CO* gene leading to a late flowering phenotype under LD conditions (Robson et al., 2001). The *co-2* mutant was kindly provided by Prof. George Coupland (MPI for Plant Breeding Research, Cologne). For transformation, seeds were sown on soil and cold treated for 24h in a cold chamber (16h light, 4°C) in order to allow simultaneous germination. Then, plants were grown in a climate chamber under LD conditions (16h light/8h dark, 22°C, with a light intensity of 120μE) and transformation was performed using the floral dip method (Clough and Bent 1998).

T<sub>1</sub> plants (grown from seeds after floral dip transformation) were grown in the climate chamber (16h light/8h dark, at 22°C) and T<sub>2</sub> seeds were harvested. T<sub>1</sub> plants transformed with *BTC1<sub>d</sub>* were sprayed with BASTA® (1.7 g/l) (Bayer CropScience, Mohnheim, Germany). Within two weeks, the treatment was performed two times at the four leaf stage (two cotyledons and two true leaves). T<sub>1</sub> plants transformed with *BvBBX19* were tested for kanamycin resistance by growing on agarose plates with a kanamycin concentration of 50 mg/ml. Segregating T<sub>2</sub> families and the controls were grown in multi-pot trays. After cold treatment (4°C) for 24 hours, plants were grown in a randomized design in a climate chamber and phenotyped every day for days to bolting (DTB) for days to flowering (DTF) at first flower bud visible (BBCH growth stage 5.10) and first flower open (BBCH growth stage 6.00) as well as for total number of rosette leaves (TNRL) at growth stage 5.10 (Boyes et al. 2001; Meier 1993).

**Table 9:** Seed codes of all T<sub>2</sub> families used for the analysis.

Seed code	Generation	Background	Other name	Transgene integration
070044	wildtype	-	Col-0	-
132458	EMS mutant	-	<i>co-2</i>	-
143081	T <sub>2</sub> /Col-0	Col-0	L27	<i>35S::BTC1<sub>d</sub> + Ubi::BvBBX19</i>
143082	T <sub>2</sub> /Col-0		L28	<i>35S::BTC1<sub>d</sub> + Ubi::BvBBX19</i>
143083	T <sub>2</sub> /Col-0	Col-0	L29	<i>Ubi::BvBBX19</i>
143084	T <sub>2</sub> /Col-0		L30	<i>Ubi::BvBBX19</i>
142999	T <sub>2</sub> / <i>co-2</i>		L41	<i>35S::BTC1<sub>d</sub></i>
143003	T <sub>2</sub> / <i>co-2</i>		L45	<i>35S::BTC1<sub>d</sub></i>
143005	T <sub>2</sub> / <i>co-2</i>	<i>co-2</i>	L47	<i>35S::BTC1<sub>d</sub></i>
143007	T <sub>2</sub> / <i>co-2</i>		L49	<i>35S::BTC1<sub>d</sub></i>
143012	T <sub>2</sub> / <i>co-2</i>		L54	<i>35S::BTC1<sub>d</sub></i>
143037	T <sub>2</sub> / <i>co-2</i>		L79	<i>Ubi::BvBBX19</i>
143040	T <sub>2</sub> / <i>co-2</i>		L82	<i>Ubi::BvBBX19</i>
143050	T <sub>2</sub> / <i>co-2</i>	<i>co-2</i>	L92	<i>Ubi::BvBBX19</i>
143063	T <sub>2</sub> / <i>co-2</i>		L107	<i>Ubi::BvBBX19</i>
143066	T <sub>2</sub> / <i>co-2</i>		L127	<i>Ubi::BvBBX19</i>

### 4.2.2 Vector construction and *A. thaliana* transformation

For transformation of the complete coding sequences of both, *BTC1<sub>d</sub>* and *BvBBX19*, from the annual sugar beet genotype 930190, into Arabidopsis two transformation plasmids were constructed. For *BTC1<sub>d</sub>*, the complete transgene including promoter and terminator was cut by

*Sfi*I restriction enzyme and cloned into the corresponding restriction sites of the binary vector p7UG-AB (DNA cloning service, Hamburg, Germany). The final construct was named *p7-35S::BTC1<sub>d</sub>* (CAU3715) (Dally 2013). The vector CAU3715 carries the *BTC1<sub>d</sub>* coding sequence driven by a CaMV 35S promoter and the *pat* gene for herbicide-selection. For *BvBBX19*, the coding sequence was cloned into the p9i-UbiATm-vector (DNA cloning service, Hamburg, Germany). As a selectable marker the vector carries the *neomycin phosphotransferase* II (*nptII*) providing kanamycin resistance. The final vector carries the *BvBBX19* coding sequence driven by an *UBIQUITIN* promoter (pUbi) from maize and was kindly provided by Dr. Siegbert Melzer (Plant Breeding Institute, Kiel University, Germany). Both plasmids were transformed into *A. thaliana* Col-0 and into the *co-2* mutant by the floral dip method (Clough and Bent 1998).

### 4.2.3 PCR and sequencing techniques

Leaf samples were taken from four weeks old *A. thaliana* T<sub>1</sub> and T<sub>2</sub> plants and freeze-dried for 24 hours. Genomic DNA was isolated using the CTAB method (Rogers and Bendich 1985) with slight modifications. The DNA was diluted ten-fold and its quality was checked by gel-electrophoresis as well as by PCR using the primer combination B349/B350 amplifying the housekeeping gene *AtGAPDH* (Table 10). The insertion of the transgene as well as the insertion of the *pat* and *nptII* gene in T<sub>1</sub> and T<sub>2</sub> plants was tested by PCR using the primer combinations A883/A747, pUBI\_F/pUBI\_R, C015/C016 and C162/C163. PCR fragments were checked on 1% or 2% agarose gels. The presence of the EMS mutation within the *CONSTANS* gene of the *co-2* mutant was verified by Sanger sequencing at the Institute for Clinical Molecular Biology (IKMB, Kiel, Germany) using the primer combination NH\_CO\_F/NH\_CO\_R (Table 10). Primer sequences are listed in Supplementary Table 2.

**Table 10:** Primer combinations for determination of t-DNA insertion.

Target gene	Forward Primer	Reverse Primer	PCR conditions
<i>CO</i>	NH_Co_F	NH_Co_R	95°C, 3' + 40 x (95°C, 10"; 55°C, 30"; 72°C, 30") +72°C, 5'
<i>BvBBX19</i>	pUBI_F	pUBI_R	95°C, 3' + 36 x (95°C, 30"; 55°C, 30"; 72°C, 30") +72°C, 5'
<i>nptII</i>	C015	C016	95°C, 3' + 36 x (95°C, 30"; 60°C, 30"; 72°C, 30") +72°C, 5'
<i>BTC1</i>	A883	A747	95°C, 3' + 36 x (95°C, 30"; 57°C, 30"; 72°C, 30") +72°C, 5'
<i>AtGAPDH</i>	B349	B350	95°C, 3' + 40 x (95°C, 10"; 61°C, 30"; 72°C, 30") +72°C, 5'
<i>pat</i>	C162	C163	95°C, 3' + 36 x (95°C, 30"; 59°C, 30"; 72°C, 30") +72°C, 5'
<i>BvBBX19</i>	C565	C566	95°C, 3' + 36 x (95°C, 30"; 60°C, 30"; 72°C, 30") +72°C, 5'
<i>BTC1</i>	B580	B581	95°C, 3' + 36 x (95°C, 30"; 60°C, 30"; 72°C, 30") +72°C, 5'

### 4.2.4 Expression analysis by RT-PCR

In order to examine the expression of *BvBBX19* and *BTC1* in transgenic T<sub>2</sub> plants, rosette leaves were harvested from 30 days old plants of five transgenic plants of each family and pooled for each family. Total RNA was extracted using the PeqGOLD plant RNA kit (Peqlab, Germany). Genomic DNA was removed by DNase treatment (Ambion, Austin, USA). The concentration of the extracted RNA was measured using the NanoDrop 2000 spectrophotometer (PeqLab, Germany), and the integrity of the nucleic acid was checked on a 1% agarose gel. For cDNA synthesis, 500ng of total DNase-treated RNA was reverse transcribed using the First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany). The cDNA was diluted twenty-fold and its quality was checked by PCR using the primers B349/B350 for the housekeeping gene *AtGAPDH*. For RT-PCR, 2µl of the diluted cDNA was used as a template. The expression of *BTC1<sub>d</sub>* and *BvBBX19* was measured using the primer combinations B580/B581 and C565/C566, respectively (Table 10). PCR products were tested on 2% agarose gels.

### 4.3 Results

#### 4.3.1 Phenotypic and molecular identification of T<sub>1</sub> plants transformed with the *BTC1<sub>d</sub>* and *BvBBX19* coding sequences

Altogether, 286.890 seeds were obtained after floral dip experiments of Col-0 and *co-2* mutant plants with *BTC1<sub>d</sub>* and *BvBBX19* (Table 11). In order to determine primary transgenic plants (T<sub>1</sub>) with an integration of the *BvBBX19* transgene, 60.520 seeds were sown on agarose plates containing kanamycin (50mg/l). For the identification of transgenic plants with an integration of the *BTC1<sub>d</sub>* transgene 15.000 seeds were sown on soil. All plants were grown in the climate chamber under controlled LD conditions. After BASTA<sup>®</sup> treatment, 16 seedlings (T<sub>1/col-2</sub>) survived and were selected for further propagation. An integration of the *BTC1<sub>d</sub>* transgene was asserted for 14 BASTA<sup>®</sup> resistant T<sub>1</sub> plants by PCR, using the primer combinations A883/A747 and C162/C163 (Table 10). For transformants, carrying the *BvBBX19* transgene, concomitant with the *nptII* gene for kanamycin resistance, 83 T<sub>1</sub> plants with a *co-2* mutant background (T<sub>1/co-2</sub>) and twelve with a Col-0 background (T<sub>1/Col-0</sub>) were positively selected and further tested by PCR using the primer combinations C565/C566 and C565/C566. In total, 85 T<sub>1</sub> plants (77 T<sub>1/co-2</sub> and 8 T<sub>1/Col-0</sub>) exhibited an integration of the *BvBBX19* transgene. Altogether, 99 T<sub>1</sub> transformants have been positively identified for an integration of either *BTC1<sub>d</sub>* or *BvBBX19* (Table 11) and were bag isolated for harvesting T<sub>2</sub> seeds.

**Table 11:** Results of the transformation experiments of *A. thaliana* ecotype Col-0 and the *co-2* mutant with *35S::BTC1<sub>d</sub>* and *UBI::BvBBX19*. After transformation T<sub>1</sub> seeds were harvested and the efficiency for transformation was calculated as the number of transgenic T<sub>1</sub> plants (PCR confirmed) per seeds sown.

Background	Construct	# of T <sub>1</sub> seeds harvested	# of T <sub>1</sub> seeds analyzed	Kan./ BASTA <sup>®</sup> positive	PCR positive T <sub>1</sub> plants	Transform. efficiency [%]	Seed code of T <sub>2</sub> families analyzed	
<i>co-2</i>	<i>Ubi::BvBBX19</i>	104.650	50.000	83	77	0.15*	143030, 143040, 143050, 143063, 143066	
<i>co-2</i>	<i>35S::BTC1<sub>d</sub></i>	71.630	15.000	16	14	0.09**	142999, 143003, 143005, 143007, 143012	
Col-0	<i>Ubi::BvBBX19</i>	32.005	5.375	5	4	0.07*	143081, 143082	
Col-0 +	<i>35S::BTC1<sub>d</sub></i>	<i>Ubi::BvBBX19</i>	78.605	5.145	7	4	0.08*	143083, 143084
Total:		286.890	75.520	111	99			

#### 4.3.2 Genotypic and phenotypic analysis of segregating T<sub>2</sub> families

In total, 14 different T<sub>2</sub> families could be investigated more closely (Table 12). I determined the transgene copy number in all T<sub>2</sub> families. A 3:1 segregation ratio (transgenic:non-transgenic) is expected for a single copy integration, while a 15:1 segregation ratio is expected for the integration of two transgene copies. Seedlings were treated with BASTA<sup>®</sup> and counted for resistant and susceptible plants. For T<sub>2</sub> families with an integration of the

*BvBBX19* transgene, seeds were sown on agarose plates containing kanamycin. Again, resistance or susceptibility was assessed and counted: vially grown plants were expected to be transgenic (kanamycin resistant) and those that died on the plates were assessed as non-transgenic. To identify the segregation of transgenic and non-transgenic plants and therewith deduce the copy number integration,  $\chi^2$ -analysis was performed. At a significant level of 5% ( $\alpha_{0.05} = 3.84$ )  $\chi^2$ -analysis was not significant for twelve out of 14  $T_2$  families, implying that the observed proportion of transgenic and non-transgenic plants did not differ significantly from the expected 3:1 ratio. Contrary, in the remaining two  $T_2$  families ( $T_{2/co-2}$ ) the segregation ratio significantly deviates from the expected ratio of 3:1, due to an excess of transgenic plants, suggesting that more than one copy of the transgene is integrated (Table 12).

**Table 12:** Genotyping segregating  $T_2$  families. Plants with an integration of the *Ubi::BvBBX19* transgene were grown on agarose plates containing 50mg/l kanamycin. *35S::BTC1* transgenic plants were sown in soil and treated with the BASTA® herbicide. To test whether the observed segregation ratio fits to a 3:1 ratio (transgenic : non-transgenic), as expected for inheritance of a single transgene, a  $\chi^2$ -test ( $\alpha_{0.05} = 3.84$ ) was performed.

Background	Construct	Seed code	Kan./ BASTA® positive	Kan./ BASTA® negative	$\chi^2$ -test for $H_0=3:1$ (t:nt) <sup>1</sup>	$\chi^2$ -test for $H_0=15:1$ (t:nt) <sup>1</sup>
Col-0	<i>Ubi::BvBBX19</i>	143081	24	3	2.78	1.09
		143082	16	2	1.85	0.726
	<i>Ubi::BvBBX19</i> + <i>35S::BTC1<sub>d</sub></i>	143083	20	4	0.89	4.44*
		143084	22	5	0.6	6.9**
<i>co-2</i>	<i>35S::BTC1<sub>d</sub></i>	142999	12	5	0.18	15.56**
		143003	29	3	4.17**	0.53
		143005	23	6	0.29	10.32**
		143007	23	6	0.29	10.32**
		143012	26	2	4.76**	0.04
<i>co-2</i>	<i>Ubi::BvBBX19</i>	143037	16	6	0.06	16.59**
		143040	29	5	1.92	4.15*
		143050	15	6	0.14	17.86**
		143063	20	7	0.01	17.84**
		143066	25	5	1.11	5.55

t= transgenic; nt= non-transgenic;  $\chi^2$ -test: \*  $\alpha=0.05$ , \*\*  $\alpha=0.025$

### 4.3.3 Overexpression of the *BTC1<sub>d</sub>* and *BvBBX19* coding sequence does not alter the phenological development of Arabidopsis

It had been shown that the B-Box defective *co-2* mutant has a late flowering phenotype (Robson et al. 2001). Moreover, overexpression of *CO* in a wildtype background lead to an early flowering phenotype (Onouchi et al. 2000). On these grounds, I expected that if *BvBBX19* and *BTC1* complement each other and acquire a *CO* function in a Col-0 wildtype background, plants would flower earlier than the untransformed controls.

Moreover, I awaited that overexpression of only *BvBBX19* would not affect the late flowering phenotype of the *co-2* mutant. To proof this hypothesis, 14 segregating  $T_2$  families (35 plants per family) (Table 12) were grown in the climate chamber under controlled LD conditions together with the controls Col-0, *Ler* and *co-2*. The wildtype Col-0 and the *Ler* ecotype started bolting after 19 days and flowering after 26 days. The total number of rosette leaves was twelve for Col-0 and nine for the *Ler* ecotype. The *co-2* mutant bolted after 26 days, flowered after 37 days and had on average a total number of 21 rosette leaves (Table 13).

**Table 13:** Results of the phenotypic analysis of all investigated T<sub>2</sub> families in a Col-0 or *co-2* mutant background. DTB = days to bolting; DTF = days to flowering; TNRL = total number of rosette leaves.

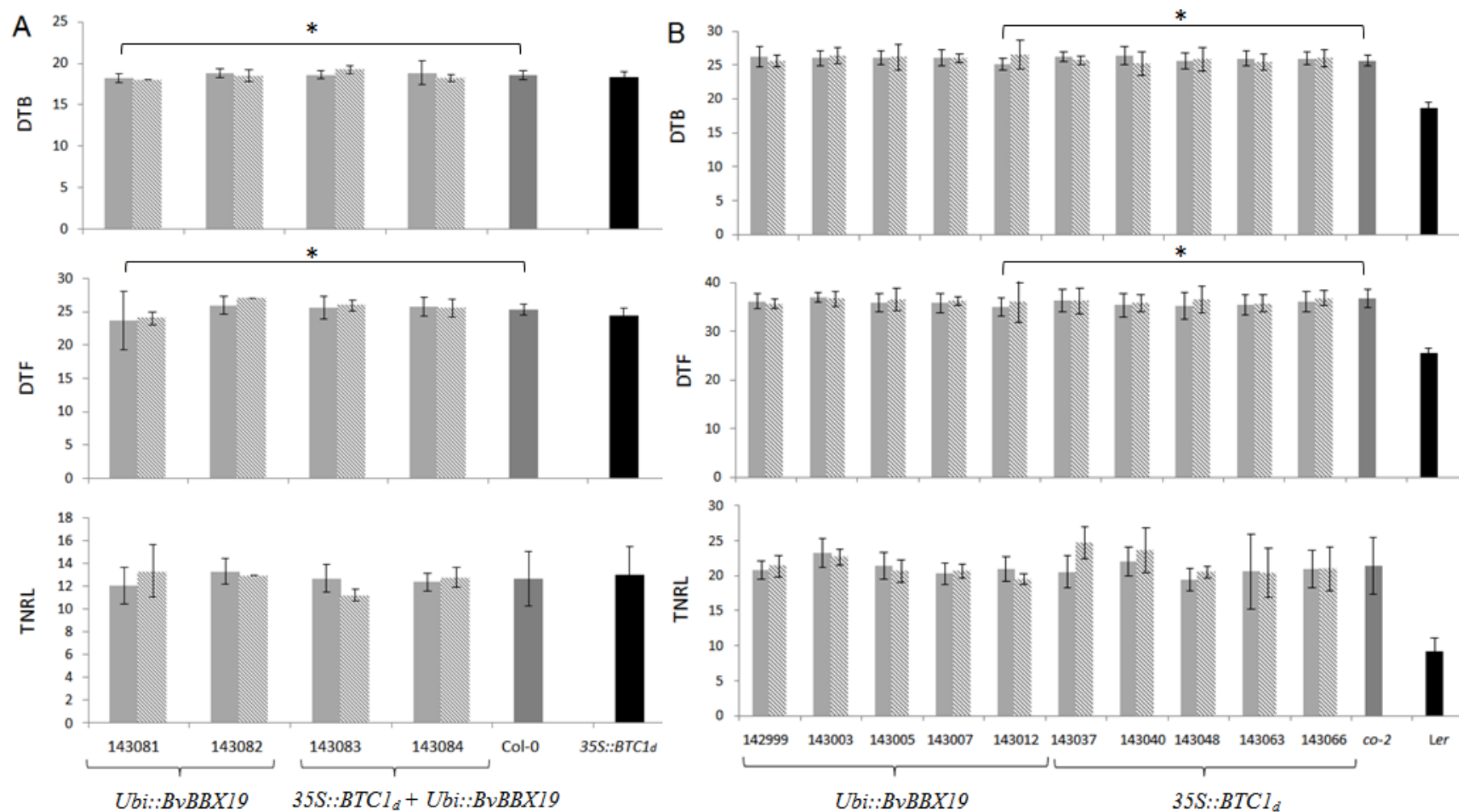
Background	Construct	Seed code			
		T <sub>2</sub> family	DTB	DTF	TNRL
<i>co-2</i>	<i>35S::BTC1<sub>d</sub></i>	142999	26.18	36.09	20.81
		143003	26.04	36.91	22.20
		143005	26.11	35.77	21.38
		143007	26.05	35.75	20.3
		143012	25.11*	34.96***	20.96
<i>co-2</i>	<i>Ubi::BvBBX19</i>	143037	26.28**	36.35	20.57
		143040	26.33**	35.29	22.03
		143049	25.57	35.14	19.38
		143063	25.94	35.36	20.57
		143066	25.96	36.08	20.96
<i>co-2</i>			25.91	36.62	21.43
<i>Ler</i>			18.81	25.63	8.90
Col-0	<i>Ubi::BvBBX19</i>	143081	18.2***	23.7***	12.08
		143082	18.8	26.00	12.31
	<i>Ubi::BvBBX19</i> + <i>35S::BTC1<sub>d</sub></i>	143083	18.6	25.65	12.7
		143084	18.85	25.76	12.38
Col-0			18.75	25.75	12.31

Students *t*-test: \*  $\alpha=0.05$ , \*\*  $\alpha=0.025$ , \*\*\*  $\alpha=0.001$

First, I analyzed days to bolting and flowering as well as the total number of rosette leaves in T<sub>2</sub> families within a Col-0 background (T<sub>2Col-0</sub>). Both, transgenic and non-transgenic T<sub>2</sub> plants bolted on average after 19 days, flowered after 26 days and revealed a total number of 12 rosette leaves, similar to the controls Col-0 and *Ler* (Figure 8A). Nevertheless, transgenic plants of the T<sub>2</sub> family 143081 revealed an average of 23 days to flowering, implying significant differences to the non-transgenic control Col-0. Then, I analyzed DTB, DTF and TNRL in T<sub>2</sub> families within a *co-2* background (T<sub>2co-2</sub>). Both, transgenic (independent of an integration of *BTC1* or *BvBBX19*) and non-transgenic T<sub>2</sub> plants bolted on average after 26 days, flowered after 36 days and revealed a total number of rosette leaves of 20 to 22, similar to the untransformed *co-2* mutant control (Figure 8B; Table 13). Although, some of the T<sub>2</sub> families displayed significant differences in either DTB or DTF (143012, 143037 and 143040), the differences account less than one day (Table 13). According to these results I concluded, that neither an integration of *BTC1<sub>d</sub>* or *BvBBX19* as a single transgene, nor an integration of both coding sequences in *Arabidopsis* alters the plants phenological development and none of the transgenic plants bolted or flowered earlier than non-transgenic plants.



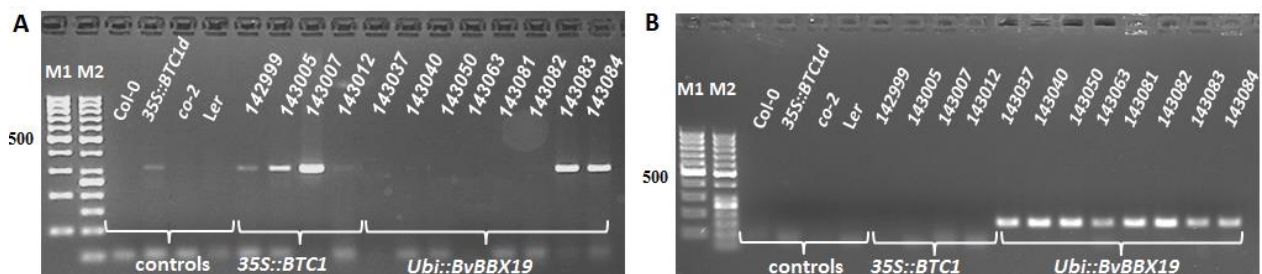
**Figure 8:** Growth experiments of  $T_2$  families with either a Col-0 (A) or *co-2* background (B). Boxes with dashed lines represent non-transgenic plants whereas filled light grey boxes represent transgenic plants for each  $T_2$  family. Black and dark grey bars picture the controls Col-0, *co-2*, *Ler* as well as Col-0 plants homozygous for an integration of *35S::BTC1<sub>d</sub>*, *co-2*. Error bars display the standard error of the mean of phenotyped transgenic or non-transgenic plants/  $T_2$  family. DTB: days to bolt after sowing (BBCH growth stage 5.10 (Meier 1993)) DTF: days to flower after sowing (BBCH growth stage 6.10 (Meier 1993)), total number of rosette leaves (TNRL) at growth stage 5.10 (Meier 1993). Asterisks highlight statistically significant differences in days to bolt (student's *t*-test,  $\alpha = 0.05$ ).



### 4.3.4 Expression analysis of segregating T<sub>2</sub> families by RT-PCR

In a next step, I measured the transcriptional activities of *BTC1<sub>d</sub>* and *BvBBX19* in transgenic T<sub>2</sub> plants by RT-PCR by pooling five transgenic plants per T<sub>2</sub> family. First, all samples were tested for expression of the house-keeping gene *AtGAPDH* using primers B582/B583 to confirm the successful transcription of mRNA to cDNA. Second, the expression of *BTC1<sub>d</sub>* was measured using the primers B580/B581 and *BvBBX19* expression was measured using the primer combination C565/C566 (Table 10). As expected, expression analysis revealed expression levels of *BTC1<sub>d</sub>* for all pools of transgenic plants of T<sub>2</sub> families (142999, 143005, 143007, 143012, 143083 and 143084) (Figure 9A) as well as I could detect a high expression of *BvBBX19* (143083, 143084, 143037, 143040, 143050, 143063 and 143066) for all investigated T<sub>2</sub> families, too (Figure 9B).

**Figure 9:** Gene expression analysis in T<sub>2</sub> plants by RT-PCR. A) Expression analysis of the *BTC1<sub>d</sub>* transgene using the primer combination B580/B581. B) Gene expression analysis of the *BvBBX19* transgene using the primer combination C565/C566. Products were detected by gel-electrophoresis using a 2% Agarose gel, 90V, 40 min.



## 4.4 Discussion

Flowering as the change from vegetative to reproductive growth is a key developmental switch in several plants. In the model organism *A. thaliana*, the onset of flowering is driven by a complex network of signaling pathways (Blümel et al. 2015; Srikanth and Schmid 2011), comprising various flowering time genes such as *CO* or *FT*. Due to high homology there have been a lot of studies investigating flowering time genes from other species by complementation analysis in *Arabidopsis* (Chia et al. 2008; Kloosterman et al. 2013; Lazakis et al. 2011; Li et al. 2014a; Pin et al. 2010). The CO protein consists of two domains, a CCT domain, sufficient for nuclear localization of the protein, as well as two zinc-finger B-Box domains which were shown to be involved in protein-protein interactions (Gangappa et al. 2013a; Wenkel et al. 2006). In the species *Beta vulgaris*, so far no true orthologue of *CO* has been found (Chia et al. 2008). However, the bolting gene *BTC1* encodes for a protein with a CCT domain (Pin et al. 2012) and *BvBBX19* as another bolting time regulator encodes for a DOUBLE B-BOX TYPE ZINC FINGER protein (Dally et al. 2014). Moreover, expression analysis of *BTC1* and *BvBBX19* revealed that both genes act upstream of *BvFT1* and *BvFT2* which led us to hypothesize an interaction of both to acquire a *CO* function in beet. To investigate my hypothesis, I transformed the *Arabidopsis* wildtype Col-0, as well as the late flowering *co-2* mutant with *BTC1* and *BvBBX19*. I expected, that overexpression of both genes in an *Arabidopsis* wildtype background would alter the flowering phenotype to early flowering, as it was shown for overexpression of *CO* (An et al. 2004; Ayre and Turgeon 2004; Onouchi et al. 2000).

My data revealed no phenotypic changes of Col-0 wildtype plants which exhibited an insertion of both, *BTC1<sub>d</sub>* and *BvBBX19*, when compared with non-transgenic plants in segregating T<sub>2</sub> families. Besides, a single integration of neither *BvBBX19* nor *BTC1<sub>d</sub>* in the late-flowering *co-2* mutant accelerated the phenotype. Hence, my results suggest that there is no prominent effect of *BvBBX19*, on flowering time when transformed into *A. thaliana*, because no major differences of DTB and DTF were observed between transgenic and non-transgenic plants, neither in combination with *BTC1<sub>d</sub>*. The only differences I detected consisted of less than one day and could have occurred due to measuring errors. I assume that the difference of 2.05 days earlier flowering of transgenic plants from 143081 could possibly represent an artefact due to unexpected mutations occurred during the transformation process or ascribed to the insertion side of the transgene.

Previous studies revealed, that *BvBBX19* and *BTC1<sub>d</sub>* are diurnally regulated in beet (Dally et al. 2014; Pin et al. 2012). During my experiments, both transgenes were expressed in a constitutive manner. Even though, for the diurnally regulated beet genes *BvFT1* and *BvFT2*, constitutive overexpression in Arabidopsis accelerated the flowering phenotype (Pin et al. 2010), I cannot exclude, that a diurnal regulation is necessary for an interaction between *BvBBX19* and *BTC1<sub>d</sub>*. However, constitutive overexpression of *CO* in Arabidopsis under the control of the CaMV 35S promoter caused an early flowering phenotype (Hepworth et al. 2002; Onouchi et al. 2000), too. Moreover, the diurnal pattern of *BTC1* from annual beets has highest similarity to that of the ‘biennial’ *btc1*, although their function is absolutely contrary (Pin et al. 2012), suggesting that such pattern may not affect its ability to interact with *BvBBX19*.

In Arabidopsis, *CO* is expressed in the plants phloem (An et al. 2004). In my study, I used two different promoters: *BTC1* was driven by the CaMV 35S promoter, while *BvBBX19* was held under an ubiquitin promoter from maize. Presumably, the CaMV 35S promoter allows expression within the phloem but the ubiquitin promoter from maize does not (George Coupland, pers. communication, 2016). Another explanation for the lack of phenotypic differences in transgenic plants overexpressing *BvBBX19* and *BTC1* is that the protein structures of both are too distinct from the protein structures in Arabidopsis. Nevertheless, other complementation analyses using flowering time genes from beet have been successful (Abou-Elwafa et al. 2010; Chia et al. 2008; Pin et al. 2010; Vogt et al. 2014). Further explanations could be that *BTC1<sub>d</sub>* and *BvBBX19* do not act together to acquire a *CO* function or do not interact at all. Besides it is also conceivable that the two genes may interact with each other in sugar beet but not in an *A. thaliana* background.

Summarizing, the results demonstrate, that *BvBBX19* and *BTC1<sub>d</sub>* do not have a prominent effect on flowering, bolting or number of rosette leaves when expressed constitutively under two different promoters in *A. thaliana* neither when integrated in particular nor when integrated together. Nevertheless, it is not a strong indication that both genes can't act together to acquire a *CO* function for initiating flowering in sugar beet. To proof an interaction, further analyses have to be considered, such as a yeast two-hybrid or a BiFC approach. Additionally I suggest a repetition of the described experiments using either the same promoter for both constructs (CaMV 35S) or the original *CO*-promoter which is diurnally regulated due to its light-regulatory elements.

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## 5 Closing Discussion

One of the most substantial questions faced by all organisms is when is the best time to reproduce for maximizing the evolutionary success of their offspring. Since reproductive success is accompanied by evolutionary success, flowering plants have developed different life cycles. While annual species germinate and flower within one season, biennials need prolonged exposure to cold temperatures over winter to acquire floral competence and fulfill their life cycle. Perennial species are able to reproduce for several times by cycling through vegetative and reproductive phases repeatedly. The fruits and flowers of a flowering plant are part of its life cycle. For millennia, survival of human and animal being have depended upon flowering plants, mainly for nutrition but also for medical compounds. To overcome the widespread concern that we will soon break over the yield barriers of many crop plants, the production of novel varieties which are better adapted to different environments and changing climate conditions, is a major goal in plant breeding. In vegetative crops, such as fodder grasses (e.g. *Lolium perenne*), cabbage (*Brassica olearacea*) and sugar beet (*Beta vulgaris*), flowering is an undesirable trait because it limits yield potential and interferes with harvest operations. Moreover, a major aim in sugar beet breeding is to breed winter beets, which are sown in autumn and harvested in the following year to enable yield increase due to longer cultivation and increased light absorption (Hoffmann and Kluge-Severin 2010). For the aim of breeding winter beets, winter hardiness and bolting control are required. Hence, a profound knowledge of genetic mechanisms triggering life cycle switch is the basis for an effective control of bolting and flowering.

In the model organism *Arabidopsis thaliana*, extensive studies on floral transition unveiled a huge network integrating interactions between proteins, transducing environmental stimuli for impeding or promoting floral transition. By contrast with *Beta* species, the understanding of genetic mechanisms subjecting floral transition and therewith life cycle switch is still at its onset, although several genes are structurally conserved among angiosperms.

The results of my work provide major contributions to our knowledge of flowering time regulation and evolution of major flowering time genes in *Beta* species. I mapped a novel *BTC1* allele (haplotype '1') which is associated with loss of annuality due to minor sequence variation in the coding region (chapter 2). My findings demonstrate for the first time, that mutations within the coding region of an 'annual' *BTC1* allele are sufficient to secure life cycle switch in beet. I show that the novel *btc1<sub>1</sub>* allele alters the expression profiles of its downstream targets *BvFT1* and *BvFT2* to a biennial expression profile before and after vernalization. The *btc1<sub>1</sub>* allele provides an excellent resource for breeding winter beets (chapter 2). Besides *BTC1*, the results of my work demonstrate that natural allelic diversity occurs for *BvBBX19*, *BvFT1* or *BvFT2*, as well. However, my findings endorse that domestication of cultivated beets is mainly attributed to *BTC1* but not to *BvBBX19*, *BvFT1* or *BvFT2* (chapter 3). I discovered a high conservation of the CCT-, REC-, BBX- and PEBP-domains with regard to SNP occurrence, emphasizing their evolutionary relevance for life cycle adaptation. Beyond, the results of my work demonstrate that geographical origin has a strong impact on life cycle adaptation in beet. *Beta* accessions from northern latitudes flower significantly later or did not flower at all when compared with accessions from southern latitudes (chapter 3).

At the beginning of my work, three bolting loci (*B2*, *B3* and *B5*) had been identified by EMS mutagenesis, which were stated to act epistatically or independently to *BTC1* (Büttner et al. 2010; Hohmann et al. 2005). *B2* was identified housing the *BvBBX19* gene which was suggested to act as a transcriptional regulator of *BTC1* (Dally et al. 2014).

In chapter 2, I describe mapping of a bolting locus which was supposed to act independently of *BTC1* (Büttner et al. 2010). In a previous study, a biennial EMS mutant with a mutation at the *B5* locus was crossed with an annual sea beet accession,  $F_1$  plants were propagated by bag isolation and a  $F_2$  population consisting of 78 plants was investigated for phenotypic segregation and co-segregation with the *B* locus. The authors had proposed an independent segregation between bolting phenotypes and the *B* locus (Büttner et al. 2010). Surprisingly, when investigating sibling  $F_2$  populations, from the same cross from Büttner et al. (2010), to the contrary I discovered that the bolting phenotypes co-segregated with the *B* locus. I found that phenotypic variation was directly attributed to a new *BTC1* allele. I could confirm this finding in two sibling  $F_2$  populations consisting of 178 plants. Moreover phenotypic data of bolting  $F_2$  plants, homozygous or heterozygous for the bolting locus, were validated by phenotypic analysis of  $F_3$  families. Additionally, I could rule out the possibility for another bolting locus by QTL analysis and hybrid plants carrying both alleles, *btc1<sub>l</sub>* and *btc1<sub>a</sub>*, did not bolt without vernalization.

Although one mapping parent was originally identified in a phenotypic screen for biennial beet genotypes after an EMS mutagenesis (Hohmann et al. 2005), the number and type of polymorphisms I detected indicate, that the *BTC1* sequence variant represents a naturally occurring *BTC1* allele. An explanation could be foreign pollination of the original mutant, which can occur even during bag isolation (NPZ Innovation GmbH, Holtsee, *pers. communication*). The mutation frequency after EMS treatment depends on the treatment conditions and differs from one crop to another. In Arabidopsis, a frequency of one mutation per 170 kb has been identified (Greene et al. 2003), in barley the frequency is one mutation per Mb (Caldwell et al. 2004) and in polyploid wheat one mutation per 25 kb was found (Slade et al. 2005). In sugar beet the frequency should be in between these frequencies, indicating that three mutations within one gene, as I detected in the biennial sugar beet genotype 063858, are quite inconceivable to occur after EMS treatment. Even though, only one of the three identified mutations which differ between the EMS donor plant 001684 and the ‘expected’ EMS mutant 063858 is a transition from guanine to adenine, which is likely to occur from EMS treatment (Harloff et al. 2012).

Interestingly, one of the polymorphisms I found may be causally involved in the occurrence of the biennial phenotype, because it was only found in the biennial parental mapping genotype. This mutation comprises an amino acid change from glycine to serine which alters the *BTC1* protein structure, because it leads to the lack of a low complexity region (LCR). Usually, *BTC* proteins of the ‘annual’ haplotype group (*BTC1<sub>d-k</sub>*) display seven LCRs, while those of the ‘biennial’ haplotype group (*btc1<sub>a-c</sub>*) hold six LCRs. Both haplotype groups differ by six non-synonymous SNPs as well as a large insertion of ~28 kb within the promoter region of the biennial alleles (Pin et al. 2012). Due to the fact, that the promoter insertion disrupts a series of *cis*-regulatory sequence motifs (GT-1) which are suggested to stabilize gene transcription (Zhou 1999), the different functionality of *BTC1* alleles is supposed to be primarily credited to the promoter function (Pin et al. 2012). My results demonstrate for the first time, that polymorphisms within the coding region cause a biennial life cycle, but not the large insertion within the promoter.

I reason that, because the novel discovered *btc1<sub>l</sub>* allele shows highest similarity of its promoter region to those of the promoter region found in ‘annual’ *BTC1* alleles. The only difference I spotted is an 8 bp insertion within the promoter region of the biennial mapping parent. However, I suppose that the biennial phenotype of the mapping parent occurs due to its altered protein structure. Although primary functions of low complexity regions are unknown, some studies give evidence that LCRs are involved in protein-protein interactions (Jmprasittichai et al. 2014). Moreover, Coletta et al. (2010) claimed that LCRs impact the development of large protein complexes.



*BTC1* was suggested to interact with *BvBBX19* in beet, to acquire a *CO* function and jointly regulate its downstream targets *BvFT1* and *BvFT2* (Dally et al. 2014). Taking into account these findings and the knowledge about LCRs from previous studies, my results support this assumption. I hypothesize, that the lack of a low complexity region in the *btc1<sub>l</sub>* protein alters its function to interact with *BvBBX19* for building a protein complex. I suppose that only proteins of functional, ‘annual’ *BTC1* alleles are suitable to build a protein complex with the *BvBBX19* protein before vernalization. The ‘biennial’ *btc1* protein lacking one low complexity region as well, argue for this hypothesis. Additionally, recessive *btc1* alleles differ from dominant *BTC1* alleles by six non-synonymous polymorphisms and are lacking some transcription factor binding sites (Pin et al. 2012). It can be speculated, that after vernalization other unknown proteins probably impact the regulation of *BvFT1* and *BvFT2* together with *BvBBX19* and *BTC1* to turn the plant to floral competence as it was shown for *GI* which interacts with *CO* in *Arabidopsis* (Song et al. 2014). Although it was shown that *BvFLI*, a vernalization-responsive *FLC* homolog in beet, does not play a major role in the vernalization response of beet (Vogt et al. 2014), other uncharacterized genes cannot be ruled out. In *Arabidopsis* an *FLC*-independent vernalization response pathway was already suggested, because *flc* mutants were shown to be vernalization-responsive (Michaels and Amasino 2001). Subsequently, other MADS-box genes were associated with vernalization response independent of *FLC* (Alexandre and Hennig 2008).

Nevertheless, I detected a substitution from glycine to serine at a potential phosphorylation target site in *btc1<sub>l</sub>*, which may be causally involved in the biennial phenotype. Intriguingly, in barley, a causative polymorphism in *PPD-H1* in a similar region of the protein accounts for natural variation in flowering time, too (Jones et al. 2008). It is of great interest, that in a haplotype screening for *BTC1* (chapter 3), two accessions (930181 and 080313) were detected which exhibited the same *btc1<sub>l</sub>* allele accompanied by a consistent biennial life cycle under different experimental long day conditions. These data additionally underpin the correlation of the *btc1<sub>l</sub>* haplotype with a biennial phenotype.

In contrast to *BTC1* it was shown, that polymorphisms derived from EMS mutagenesis in *BvBBX19* alter the beet’s life cycle, too (Dally et al. 2014). However, information about natural variation of the floral regulator genes *BvBBX19*, *BvFT1* and *BvFT2* from beet was missing so far. For this purpose, the major aim of my experiments described in chapter 3 was to detect natural allelic variation of these genes and concatenated the results with observed life cycles of different *Beta* species. Indeed, I found natural variation for all genes and a high conservation of the protein domains, indicating their evolutionary relevance for life cycle adaptation. My data clearly point, that the process of beet domestication during breeder’s selection mainly stems from variation in *BTC1*, but not from *BvBBX19*, *BvFT1* or *BvFT2*. Although cultivated beets primarily display a similar combination of *BTC1*, *BvBBX19*, *BvFT1* and *BvFT2* haplotypes, for wild beets I found a much higher heterogeneity. Due to a higher SNP occurrence in *BTC1* compared to other genes, my findings propounded, that *BvBBX19*, *BvFT1* and *BvFT2* played an important role during evolution, propounded by their high conservation. These findings are supported by several studies which identified highest conservation of *FT* homologs in other species. In tomato for example, Lifschitz et al. (2006) found, that *SINGLE FLOWER TRUSS* (*SFT*) plays a major role for flowering and is highly conserved. Similar results were obtained by characterization of *FT* homologs from rice (Tamaki et al. 2007) or maize (Lazakis et al. 2011; Meng et al. 2011). Interestingly, compared to *BTC1*, the occurrence of more than one haplotype for *BvBBX19*, *BvFT1* and *BvFT2* within one accession is quite high in wild beets, alluding once more that life cycle adaptation is not profoundly at the bottom of these genes.

Besides haplotype variation, the results from phenotypic analysis of the different *Beta* accessions under different long day conditions revealed a strong correlation of latitude of origin and bolting behavior. I found a general southward shift towards an annual life cycle and earlier flowering. These results suggest a major effect of the vernalization pathway as it was already speculated by Van Dijk et al. (2014) for wild beets along the coast of France. In similar studies by Van Dijk et al. (1997) and Boudry et al. (2002) differences in life cycle due to vernalization requirement was suggested as an adaptive response to season length and spring temperatures in particular latitudes. They detected early flowering concomitant with a total absence of vernalization requirement in southern hemispheres when investigating wild beet populations from the French coast northwards to south-east England.

However, the accessions investigated in my study include several cultivated beets and display a broader range of genotypes from different geographical origin. Moreover, the relation of flowering time variation with molecular variation of certain genes has not been discussed for *B. vulgaris* in detail. Interestingly I found that some haplotypes, e.g. *BvFT1<sub>f</sub>* or *BvBBX19<sub>f</sub>*, could only be detected in accessions from southern latitudes, hinting at different adaptation pressure in different geographic regions. My results suppose a distinct effect of climate adaptation on both, life cycle adaptation and haplotype variation in *Beta* species.

In chapter 4, I investigated whether *BvBBX19* and *BTC1<sub>d</sub>* complement each other and acquire a *CO* function, to accelerate the flowering phenotype of an Arabidopsis plant. In Arabidopsis, *CO* represents the major photoperiod-dependent sensor and is the central regulator of flowering (Putterill et al. 1995; Suarez-Lopez et al. 2001). Previous studies displayed that constitutive expression of *CO* in the companion cells of the phloem results in an early flowering phenotype, disregarding day length, while mutations in *CO* account for late flowering, under LD but not SD (An et al. 2004; Ayre and Turgeon 2004; Valverde et al. 2004). To date, homologs of *CO* have been identified in several species, such as *HvCO1* in barley (*Hordeum vulgare*) (Campoli et al. 2012), *Hd1* (*HEADING DATE 1*) in rice (Yano et al. 2000) or *LpCO* in ryegrass (*Lolium perenne*) (Martin et al. 2004). In sugar beet, a large family of CONSTANS-LIKE genes has been detected. Although *BvCOL1* was identified as a potential member of the photoperiod pathway, its function in beet is still unknown and it was not declared as an orthologue of *CO* due to different expression patterns of both genes (Chia et al. 2008). Hence, to date no true *CO* orthologue has been identified in beet.

My results show, that overexpression of *BvBBX19* and *BTC1<sub>d</sub>* in the *A. thaliana* wildtype Col-0 does not result in an earlier flowering phenotype. No phenotypic differences could be detected between transgenic and non-transgenic plants in segregating T<sub>2</sub> families. These results propose, that neither a single integration of one transgene nor an integration of both genes, *BvBBX19* and *BTC1<sub>d</sub>*, impact flowering in Arabidopsis. An explanation could be, that both transgenes were expressed in a constitutive manner under two different promoters, although previous studies revealed that both *BvBBX19* and *BTC1* are diurnally regulated (Dally et al. 2014; Pin et al. 2012). Is a diurnal regulation through light regulatory promoters necessary for the ability of *BvBBX19* and *BTC1* proteins to interact?

An aspect speaking against is that *BTC1* from both, annual and biennial (*btc1*) beets, show a similar diurnal pattern when compared, although their functionality is said to be absolutely contrary (Pin et al. 2012). Moreover, overexpression of *CO* under the control of the constitutive 35S promoter in Arabidopsis generated an early flowering phenotype (Hepworth et al. 2002). Besides, constitutive overexpression of *BvFT1* and *BvFT2* in Arabidopsis under control of the 35S promoter of the Cauliflower Mosaic Virus (CaMV) accelerated the flowering phenotype, although both genes in beet were shown to be diurnal regulated (Pin et al. 2010). *BTC1* was held under the CaMV 35S promoter, while *BvBBX19* was driven by the maize ubiquitin promoter.

Although both promoters are known to constitutively express genes of interest, there are slight differences which might have impacted the assumed interaction of *BvBBX19* and *BTC1<sub>d</sub>*. Previous studies uncovered, that plant promoters exhibit different functionality in monocots and dicots. For expression of a reporter gene, the ubiquitin promoter from maize conferred higher activity in maize (monocot) protoplasts, than in tobacco (dicot) protoplasts (Christensen et al. 1992).

In angiosperms, several studies revealed that reporter genes driven by the CaMV 35S promoter displayed unequal developmental and tissue patterns (Halfhill et al. 2003; Sunilkumar et al. 2002). Furthermore, in moss (*Physcomitrella patens*) the CaMV 35S promoter displayed much weaker activity as compared to the maize ubiquitin-1 promoter (Horstmann et al. 2004; Saidi et al. 2005). The CaMV 35S promoter is regularly used to drive the expression of recombinant proteins in plants and was shown to successfully engender phenotypic variation in Arabidopsis. Presumably, using this promoter for overexpressing both transgenes might be more promising. In Arabidopsis, *CO* is expressed in the plants phloem (An et al. 2004). Reasonably, the CaMV 35S promoter allows expression within the phloem but the maize ubiquitin promoter doesn't (George Coupland, pers. communication, 2016). Another assumption is that the protein structure of *BTC1<sub>d</sub>* and *BvBBX19* are too different from the protein structures of Arabidopsis homologs, such as *CO*. However, several complementation analyses in Arabidopsis with beet homologs have been successful in the past (Abou-Elwafa et al. 2010; Chia et al. 2008; Pin et al. 2010; Vogt et al. 2014), denoting that flowering time genes from beet can be functional in other species.

Nevertheless, compared to *BBX19* and *BTC1* homologs, it is well known that *FT* homologs are highly conserved in diverse species (Lazakis et al. 2011; Lifschitz et al. 2006; Meng et al. 2011; Tamaki et al. 2007). Conceivably, overexpression of both genes did not accelerate the phenotype, because the potentially formed protein-complex itself was not able to activate the *FT* transcription in Arabidopsis. There are certain studies that demonstrated an interaction of *CO* with several proteins to initially activate *FT*. An interaction of *CO* with *BBX19* through their B-Box domains was shown to inactivate *CO* through seclusion into a protein complex (Wang et al. 2014a). A similar interaction was reported for *TOE* (TARGET OF EAT1), which prevents *CO* from activating *FT* during the morning (Zhang et al. 2015). Recently it was demonstrated, that several *DELLA* proteins interact with *CO* too, for limiting the transcriptional activity of *FT* (Wang et al. 2016; Xu et al. 2016). My findings, that overexpression of *BvBBX19* and *BTC1<sub>d</sub>* does not accelerate the Arabidopsis wildtype phenotype do not generally refuse my hypothesis of an interaction of both.

In summary, the results of my work substantially contribute to our understanding about bolting time regulation in beet. The identification of the novel *btc1<sub>l</sub>* haplotype (chapter 2) leads for the first time to the assumption that sequence variation within the coding region of this gene seem to display life cycle regulation in beet, but not the large insertion within the promoter of biennial *btc1* allele as previously supposed (Pin et al. 2012). The novel allele is an excellent resource for further breeding approaches, since beets carrying this allele do not bolt even under long day conditions such as 20 hours of light, respectively (chapter 2). I showed that genetic variation is highly dependent on geographical origin, indicating the importance of life cycle adaptation due to climate change (chapter 3). However, natural variation occurs in the beet's floral regulator *BvBBX19*, *BvFT1* and *BvFT2*, too, but domestication is clearly attributed to *BTC1*. Even though I could not detect an interaction of both *BTC1* and *BvBBX19* in Arabidopsis by overexpression (chapter 4), the possibility of an interaction of both to acquire a *CO* function cannot be ruled out.

Withal, a lot of questions remain for future experiments. What are other floral regulators in beet? How do those attribute to life cycle adaptation? Might there be a real functional orthologue of CONSTANS in beet except for our hypothesis that during evolution the *CO* gene split to two genes, namely *BTC1* and *BvBBX19* which interact? To answer these questions further analyses are needed. For example, I suggest the investigation of genetic factors from the vernalization pathway, such as *BvFL1*, which displays an *FLC-LIKE1* gene in beet. I suppose that analysis of genes from this pathway will uncover an invariable relationship of haplotype variation and its effect on life cycle adaptation as it was shown for *FRI* and *FLC* in *Arabidopsis* (Caicedo et al. 2004; Stinchcombe et al. 2004). I further propose exploring of the novel *BvFTI<sub>f</sub>* haplotype because it may display a natural *BvFTI* variant which repeals the repressive function of this floral regulator and procures floral transition without vernalization. Diurnal expression analysis of *BvFTI<sub>f</sub>* or complementation analysis in *Arabidopsis* may uncover unknown functions of *BvFTI* and its interplay with *BvFT2* due to allelic variation which occurred during evolution and probably due to adaptation. For finally elucidating an interaction of *BTC1* and *BvBBX19*, I suggest protein studies of both, by a yeast two-hybrid or a BiFC approach which have already been initiated (N. Dally, pers. communication).

## 6 Summary

The *Amaranthaceae* plant family gave rise to several crops such as spinach (*Spinacia oleracea*), quinoa (*Chenopodium quinoa*) or sugar beet (*Beta vulgaris* ssp. *vulgaris*). Species of the genus *Beta* are commonly cultivated today as leaf beet, fodder beet, red table beet or sugar beet. They display large variation with respect to root and shoot morphology and phenological development. *Beta* species represent an interesting resource in order to study life cycle adaptation, because they harbor annual, biennial and perennial species. Biennial species mainly include cultivated beets, such as sugar beet, while annual and perennial life cycles are abundant among the wild progenitors (*B. vulgaris* ssp. *maritima*). Sugar beet is the only sucrose-storing crop grown for sugar production in Europe. It is strictly biennial, starting bolting and flowering only after exposure to a long period of cold temperatures. Sucrose yield and resistance to early bolting have been the major aims in sugar beet breeding. A visionary approach to further increase yield is to extend the cultivation period of beets over winter. Currently, sugar beet is sown in spring and harvested in autumn. For an extended growth period, winter beets are needed which are sown in autumn and stay in the field over winter. However, breeding winter beets requires (i) the improvement of winter hardiness and (ii) a sophisticated system for bolting time control along with an extended knowledge of the genetic factors behind bolting.

From *Arabidopsis*, we learned about the prominent module of *CONSTANS* (*CO*) and the *FLOWERING LOCUS T* (*FT*), which induces flowering and appears conserved among various plant species. In *Beta vulgaris*, a novel regulation of flowering time has been observed. Two *FT* paralogs *BvFT1* and *BvFT2* have been detected, having evolved antagonistic functions. Despite, *BOLTING TIME CONTROL 1* (*BTC1*) was identified to control the switch from an annual to a biennial life cycle in beet. Furthermore, three additional bolting time loci, *B2*, *B3* and *B5* have been described after an EMS mutagenesis of an annual genotype and *B2* was shown to house the *BvBBX19* transcription factor, a DOUBLE B-BOX TYPE ZINC FINGER protein. *BvBBX19* is diurnally regulated and acts upstream of *BvFT1* and *BvFT2*, epistatically over *BTC1*. However, to date a true orthologue of *CO* has not yet been found.

The aims of my study were (1) to map the bolting locus *B5* with the identification of putative candidate genes, and (2) to detect natural haplotype diversity of the bolting time genes *BTC1*, *BvBBX19*, *BvFT1* and *BvFT2* among different *Beta* species and unveil possible relationships between haplotype variation and life cycle adaptation. Moreover, a third aim was to investigate whether *BTC1* and *BvBBX19* act together to alter the flowering phenotype of *Arabidopsis thaliana*.

Towards mapping the *B5* bolting locus, I phenotyped two large F<sub>2</sub> population derived from a cross of an annual wild beet with a biennial sugar beet genotype. Mapping led to the identification of the novel *btc1<sub>l</sub>* allele which caused a life cycle switch in beet, due to minor sequence variation in the coding region. There are 5 lines of evidence demonstrating that sequence variation in the *btc1<sub>l</sub>* haplotype cause a biennial life cycle in beet: (i) The *B5* bolting locus mapped to the same position as the *BTC1* gene, (ii) QTL analysis excluded the possibility for another bolting locus, (iii) both mapping parents differ by their *BTC1* haplotype and (iv), hybrid plants carrying both alleles, *btc1<sub>l</sub>* and the 'biennial' *btc1<sub>a</sub>* allele, do not bolt without vernalization and (v) the expression profiles of the flowering regulators *BvFT1* and *BvFT2* observed for the biennial sugar beet genotype 063858 are typical for biennial beets. Surprisingly, the biennial mapping parent was originally identified in a phenotypic screen for biennial individuals after ethylmethanesulfonate mutagenesis of an annual beet genotype.

However, the number and type of polymorphisms I discovered indicate, that the novel *btc1<sub>l</sub>* allele represents a naturally occurring allele which could provide a new resource for breeding winter beets.

When growing and genotyping a panel of 29 *Beta* accessions from different geographical origin, I uncovered sequence variations of the floral regulators *BTC1*, *BvBBX19*, *BvFT1* and *BvFT2*. My results point at a high conservation of the BBX- and PEBP-domains of these genes with regard to SNP occurrence, indicating their evolutionary relevance for life cycle adaptation. Moreover the results of my work emphasize, that the geographical origin has a strong impact on the life cycle of a *Beta* genotype, probably due to climate and seasonal adaptation. I found that genotypes from northern latitudes flowered significantly later than those from southern latitudes or did not flower at all. With regard to haplotype combinations, cultivated beets mainly displayed a similar combination of *BTC1*, *BvBBX19*, *BvFT1* and *BvFT2* haplotypes, while wild beets exhibited a much higher heterogeneity. Mainly, a large variation was found among the *BTC1* haplotypes while sequence variations within *BvBBX19*, *BvFT1* and *BvFT2* were much lower. This suggests that the latter genes did not play a major role during life cycle adaptation. I reason that after domestication beet breeding fixed only one *BTC1* allele which prevents early bolting in the field.

To further elucidate the interplay of the floral regulators *BvBBX19* and *BTC1* to acquire a *CO* function, I investigated whether over-expression of either one or both genes in *Arabidopsis* impacts the flowering phenotype. After transforming the Col-0 wildtype with both, *BvBBX19* and *BTC1*, and a late flowering *co-2* mutant with *BvBBX19* or *BTC1*, I did not observe any phenotypic differences for bolting in a segregating T<sub>2</sub> family, neither in single nor in double-transformants. My findings suggest that *BvBBX19* and *BTC1* from beet do not accelerate flowering in *Arabidopsis*.

The results of my work notably contribute to our knowledge about the evolution of life cycle variation in *Beta* species under natural conditions and under breeding selection. Moreover, the identified *btc1<sub>l</sub>* allele may provide a future resource for breeding winter beets.

## 7 Zusammenfassung

Die Familie der Amaranthaceae (Fuchsschwanzgewächse) umfasst zahlreiche, bedeutsame Kulturarten, wie Spinat (*Spinacia oleracea*), Quinoa (*Chenopodium quinoa*) und Amarant (*Amaranthus*). Auch Spezies der Gattung *Beta* stammen aus dieser Familie und werden heutzutage häufig als Mangold, Rote Beete, Futter-, und Zuckerrüben kultiviert. Diese Gattung ist von besonderem Interesse für die Untersuchung der Entstehung und Anpassung verschiedener Lebenszyklen, da sie ein-, zwei- und mehrjährige Arten umfasst. Während ein zweijähriger Lebenszyklus meist bei kultivierten Arten wie der Zuckerrübe (*Beta vulgaris* ssp. *vulgaris*) auftritt, wird Ein- und Mehrjährigkeit häufig bei Wildrüben (*B. vulgaris* ssp. *maritima*), den Vorfahren der kultivierten Rübe, beobachtet. Zweijährige Pflanzen bilden im ersten Jahr vegetative Organe aus und beginnen im zweiten Jahr nach Kältereiz über Winter mit der Blühinduktion, um ihren Zyklus mit der Reproduktion zu vollenden. Einjährigkeit bedeutet, dass eine Pflanze ihren Lebenszyklus innerhalb eines Jahres vollendet, ohne dabei auf einen Kältereiz angewiesen zu sein. Die Zuckerrübe ist die einzige Kulturpflanze, die in Europa für die Zuckerproduktion angebaut wird. Ein Hauptziel der Zuckerrübenzüchtung ist, neben der Resistenzzüchtung, vor allem die Steigerung des Zuckerertrags. Um dies zu erreichen wird der Anbau von sogenannten Winterrüben in Betracht gezogen, die im Gegensatz zum derzeitigen Anbausystem schon im Herbst des Erntevorjahres ausgedrillt werden. Durch die Verlängerung der Anbauphase im Feld wird eine effektivere Aufnahme photosynthetischer Strahlung für die Zuckerbildung ermöglicht. Um den Anbau von Winterrüben zu gewährleisten, ist neben der Winterhärte die Kontrolle der Blühinduktion von größter Bedeutung.

Durch Forschung an der Modellpflanze *Arabidopsis thaliana* wurden bereits viele Erkenntnisse über die genetischen Mechanismen der Blühinduktion erlangt. Eines der bekanntesten Module, welches in vielen Spezies konserviert erscheint, ist die Blühinduktion durch die Interaktion der Gene *CONSTANS* (*CO*) und *FLOWERING LOCUS T* (*FT*). In *B. vulgaris* wurde bis heute kein ‚wahres‘ Ortholog von *CO* gefunden. Allerdings konnten zwei *FT* Paraloge, *BvFT1* und *BvFT2* mit gegenläufiger Funktion identifiziert werden. Während *BvFT1* als Blührepressor dient, induziert *BvFT2* das Blühen. Beide Paraloge werden von dem Pseudo Response Regulator (PRR) Gen *BOLTING TIME CONTROL 1* (*BTC1*) reguliert. Einjährige Pflanzen, meist Wildrüben, tragen das dominante *BTC1* Allel, während zweijährige Pflanzen, meist kultivierte Rüben, das rezessive *btc1* Allel tragen. Beide Allele unterscheiden sich hauptsächlich durch sechs nicht-synonyme Polymorphismen in der kodierenden Sequenz, sowie durch eine lange Insertion in der Promotorregion (~28kb) des zweijährigen *btc1* Allels. Neben *BTC1* wurden durch chemische Mutagenese eines einjährigen *Beta* Genotyps noch drei weitere Genloci (*B2*, *B3* und *B5*) identifiziert. Eine Feinkartierung des *B2* Locus führte zu der Entdeckung des *BvBBX19* Transkriptionsfaktors, ein DOUBLE B-BOX TYPE ZINC FINGER Protein. *BvBBX19* ist diurnal reguliert und agiert vorgeschaltet vor *BvFT1* und *BvFT2*.

Ziel meiner Arbeit war es (1) eine Feinkartierung des *B5* Locus mit einer Identifizierung putativer Kandidatengene durchzuführen, sowie (2) die Detektion natürlicher Sequenzvariationen (Haplotypen) der Blühgene *BTC1*, *BvBBX19*, *BvFT1* und *BvFT2* in verschiedenen *Beta* Spezies, um diese mit der Ausprägung unterschiedlicher Lebenszyklen in Verbindung zu bringen. Ein weiteres Ziel (3) war es zu untersuchen, ob die Blühgene *BTC1* und *BvBBX19* nach Transformation in *Arabidopsis thaliana* gemeinsam interagieren und, ähnlich wie bei einer Überexpression von *AtCO*, den Blüh-Phänotyp verändern. Für die Feinkartierung des *B5* Locus wurden zwei große F<sub>2</sub> Populationen untersucht, die aus einer Kreuzung einer einjährigen Wildrübe mit einem zweijährigen Zuckerrüben-Genotyp entstanden sind. Dadurch konnte das neue *btc1<sub>1</sub>* Allel identifiziert werden, welches für den zweijährigen Lebenszyklus der Rübe verantwortlich ist. Insgesamt sprechen fünf Argumente

dafür, dass die identifizierten Sequenzvariationen in *btc1<sub>1</sub>* für den zweijährigen Lebenszyklus der untersuchten Zuckerrübe verantwortlich sind: (i) Die Kartierung ergab eine Übereinstimmung des *B5* Locus mit der Position des *BTC1* Gens, (ii) die Möglichkeit eines weiteren Schoss-Locus konnte durch eine QTL-Analyse ausgeschlossen werden, (iii) beide Kreuzungseltern wiesen unterschiedliche *BTC1* Haplotypen auf, (iv) Hybride, die beide Allele tragen, *btc1<sub>1</sub>* und das ‚zweijährige‘ *btc1<sub>a</sub>* schossen und blühen nicht bevor sie einem Kältereiz ausgesetzt wurden und (v) die diurnalen Expressionsprofile der Blühgene *BvFT1* und *BvFT2* des zweijährigen Kreuzungselter der *F<sub>2</sub>* Populationen entsprechen denen einer ‚typischen‘, zweijährigen Zuckerrübe. Interessanterweise wurde genau dieser Kreuzungselter ursprünglich nach einer Ethylmethansulfonat Mutagenese entdeckt. Die Anzahl und Arten der Polymorphismen weisen jedoch darauf hin, dass es sich bei *btc1<sub>1</sub>* um ein natürlich vorkommendes Allel handelt, welches eine neue Ressource für die Züchtung von Winterrüben darstellen könnte.

Die Anzucht und genetische Analyse von 29 *Beta* Akzessionen verschiedener geografischer Herkunft führte zu der Identifizierung natürlicher Sequenzvariationen der Blühgene *BTC1*, *BvBBX19*, *BvFT1* und *BvFT2*. Dabei konnte ich zeigen, dass die kodierende Sequenz für die BBX- und PEBP-Domänen dieser Gene eine besonders niedrige Anzahl an Polymorphismen aufweist, was auf deren evolutionäre Bedeutung bei der Anpassung verschiedener Lebenszyklen von *Beta* Spezies hinweisen könnte. Des Weiteren deuten meine Ergebnisse darauf hin, dass die geografische Herkunft eines *Beta* Genotyps einen starken Einfluss auf dessen Lebenszyklus hat, was möglicherweise mit der Anpassung an die verschiedenen Klimata zusammenhängt. So wiesen Genotypen, die aus nördlichen Breitengraden stammten einen wesentlich späteren Blühzeitpunkt auf oder blühten erst nachdem sie einer längeren Kälteperiode ausgesetzt waren. Solche mit einer Abstammung aus südlichen Breitengraden dagegen blühten besonders früh. Bei der Betrachtung verschiedener Haplotypen-Kombinationen stellte sich heraus, dass besonders unter den Wildrüben eine große Variation bestand, während die kultivierten Rüben meist ähnliche Kombinationen von *BTC1*, *BvBBX19*, *BvFT1* und *BvFT2* Allelen aufwiesen. Darüber hinaus zeigen meine Ergebnisse, dass *BTC1* im Vergleich zu *BvBBX19*, *BvFT1* und *BvFT2* eine besonders hohe Anzahl an Polymorphismen innerhalb der untersuchten Akzessionen aufwies, was darauf hinweist, dass dieses Gen eine tragende Rolle im Prozess der Domestikation der Zuckerrübe spielte.

Zur Untersuchung einer möglichen Interaktion der Blühgene *BvBBX19* und *BTC1* analysierte ich, ob eine Überexpression der beiden Gene in *Arabidopsis* eine Auswirkung auf den Blüh-Phänotyp zeigt. Nach erfolgreicher Transformation des Col-0 Wildtyps mit beiden Genen, sowie nach Transformation der spät blühenden *co-2* Mutante mit entweder *BTC1* oder *BvBBX19*, konnte ich keine phänotypischen Unterschiede im Vergleich zur den nicht-transgenen Kontrollen innerhalb spaltender *T<sub>2</sub>* Familien feststellen. Diese Ergebnisse weisen darauf hin, dass die Rüben-Gene *BvBBX19* und *BTC1* keinen Einfluss auf das Blühverhalten von *Arabidopsis* haben.

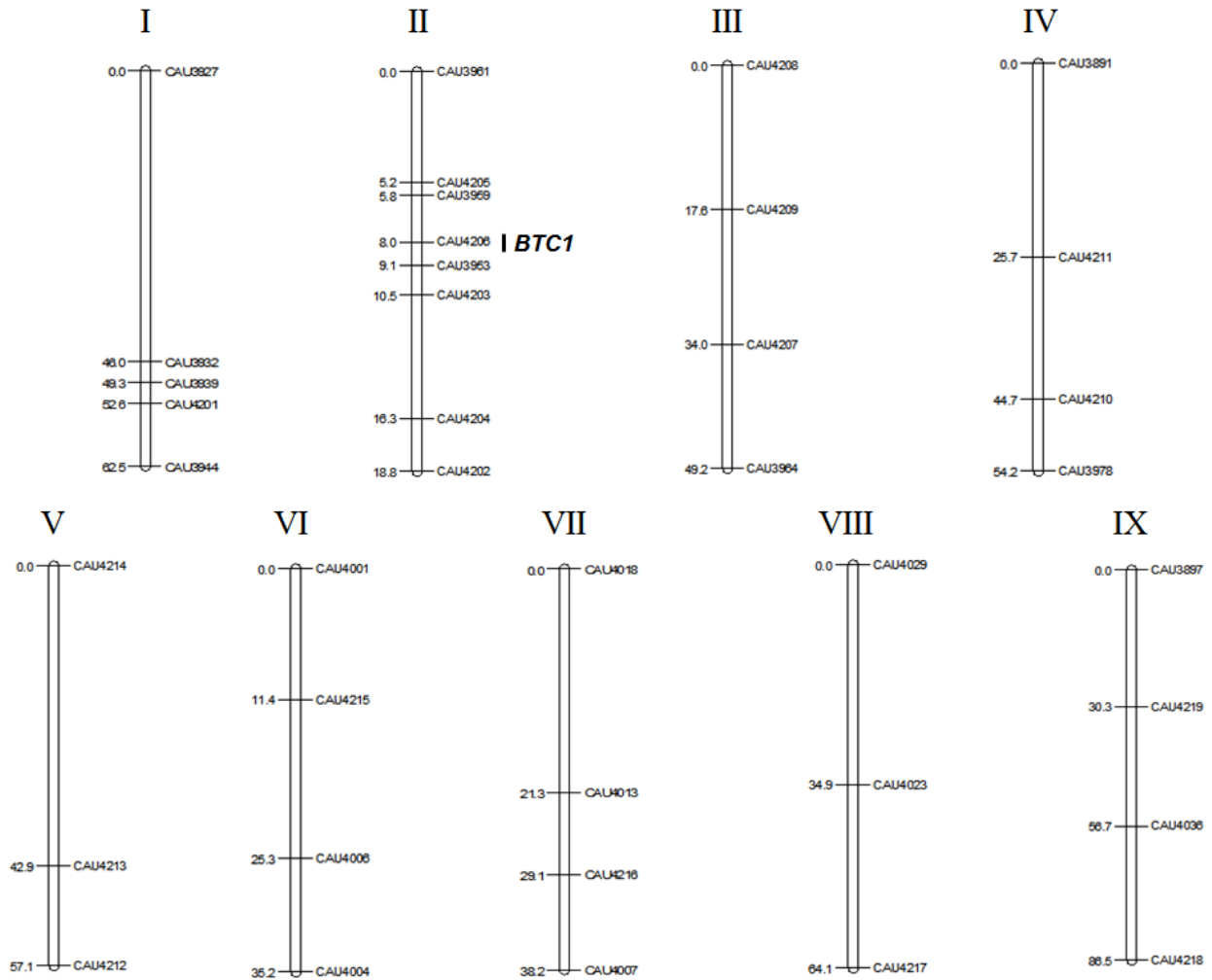
Durch die Ergebnisse meiner Arbeit konnten grundlegende neue Erkenntnisse über die Evolution verschiedener Lebenszyklen von *Beta* Spezies unter natürlichen Bedingungen, sowie unter züchterischer Selektion erlangt werden. Besonders in der Züchtung von Winterzuckerrüben könnte das in dieser Arbeit von mir identifizierte *btc1<sub>1</sub>* Allel zukünftig eine große Rolle spielen und zur Erzeugung von Hybriden eingesetzt werden.



## 8 Appendix

### 8.1 Supplementary Figures

**Supplementary Figure 1:** Genetic map of *Beta vulgaris* constructed with the F<sub>2</sub> family 070049. The program package Joinmap 4.0 was used. All marker names are provided at the right side, while the genetic distances in cM are indicated at the left side of each linkage group. The roman numerals above each linkage group are representing the respective chromosome numbers. The marker CAU4206 within *BTC1* on chromosome II revealed complete linkage to the bolting locus.



## 8.2 Supplementary Tables

**Supplementary Table 1:** Sequence based (RefBeet1.1) molecular markers and marker assays.

Marker	Marker type	Primers	Marker assay		PCR product in annual parent	PCR product in biennial parent
			PCR conditions	Detection		
CAU3927	InDel	X0063 + X0064	PCR: 94°C, 3' + [(94°, 30" + 60°C, 30" + 72°C, 30") x 32] + 72°C, 5'	GE*: 3%, 120V 25 min	360 bp	320 bp
CAU3939	InDel	X0093 + X0094	PCR: 94°C, 3' + [(94°, 30" + 56°C, 30" + 72°C, 30") x 32] + 72°C, 5'	GE: 3%, 120V 25 min	140 bp	120 bp
CAU3932	InDel	X0073 + X0074	PCR: 94°C, 3' + [(94°, 30" + 60°C, 30" + 72°C, 30") x 32] + 72°C, 5'	GE: 3%, 120V 25 min	235 bp	200 bp
CAU4201	CAPS	NH364 + NH365	PCR: 94°C, 3' + [(94°, 30" + 55°C, 30" + 72°C, 30") x 32] + 72°C, 5'	BseGI digest + GE: 3%, 120V 25 min	340 bp	164+176 bp
CAU3944	InDel	X0103 + X104	PCR: 94°C, 3' + [(94°, 30" + 60°C, 30" + 72°C, 30") x 32] + 72°C, 5'	GE: 3%, 120V 25 min	320 bp	291 +29 bp
CAU4206	CAPS	NH619 + NH620	PCR: 94°C, 3' + [(94°, 30" + 55°C, 30" + 72°C, 30") x 32] + 72°C, 5'	Hinfl digest + GE: 3%, 120V 25 min	537 bp	457+80 bp
CAU4202	CAPS	NH520 + NH382	PCR: 94°C, 3' + [(94°, 30" + 55°C, 30" + 72°C, 30") x 32] + 72°C, 5'	RsaI digest + GE: 3%, 120V 25 min	247 bp	132+115 bp
CAU4204	InDel	NH432 + NH392	PCR: 94°C, 3' + [(94°, 30" + 55°C, 30" + 72°C, 30") x 32] + 72°C, 5'	GE: 3%, 120V 25 min	172 bp	141+31 bp
CAU3953	InDel	X0133 + X0134	PCR: 94°C, 3' + [(94°, 30" + 60°C, 30" + 72°C, 30") x 32] + 72°C, 5'	GE: 3%, 120V 25 min	118 bp	145 bp
CAU4203	CAPS	NH402 + NH403	PCR: 94°C, 3' + [(94°, 30" + 55°C, 30" + 72°C, 30") x 32] + 72°C, 5'	PstI digest + GE: 3%, 120V 25 min	559 bp	398+161bp
CAU4205	InDel	NH94 + NH95	PCR: 94°C, 3' + [(94°, 30" + 55°C, 30" + 72°C, 30") x 32] + 72°C, 5'	GE: 3%, 120V 25 min	250 bp	229 bp
CAU3959	InDel	X0155 + X0156	PCR: 94°C, 3' + [(94°, 30" + 62°C, 30" + 72°C, 30") x 32] + 72°C, 5'	GE: 3%, 120V 25 min	246 bp	218 bp
CAU3961	InDel	X0159 + X0160	PCR: 94°C, 3' + [(94°, 30" + 61°C, 30" + 72°C, 30") x 32] + 72°C, 5'	GE: 3%, 120V 25 min	256 bp	236 bp
CAU3964	InDel	X0165 + X0166	PCR: 94°C, 3' + [(94°, 30" + 61°C, 30" + 72°C, 30") x 32] + 72°C, 5'	GE: 3%, 120V 25 min	322 bp	298 bp
CAU4207	CAPS	NH552 + NH553	PCR: 94°C, 3' + [(94°, 30" + 55°C, 30" + 72°C, 30") x 32] + 72°C, 5'	BseJI digest + GE: 3%, 120V 25 min	425 bp	264+161bp
CAU4208	CAPS	NH643 + NH644	PCR: 94°C, 3' + [(94°, 30" + 55°C, 30" + 72°C, 30") x 32] + 72°C, 5'	EarI digest + GE: 3%, 120V 25 min	339+170 bp	519bp
CAU4209	CAPS	NH579 + NH580	PCR: 94°C, 3' + [(94°, 30" + 55°C, 30" + 72°C, 30") x 32] + 72°C, 5'	RsaI digest + GE: 3%, 120V 25 min	514+47 bp	561 bp

**Supplementary Table 2:** Primers used for PCR and RT-qPCR.

Marker name	Primer name	5'-3' sequence forward	Primer name	5'-3' sequence reverse
CAU3927	X0063	GTCACATGGAGCTAGCAATAG	X0064	CTCAAATTTTCGGGCATAGTAATG
CAU3939	X0093	CAGATTCTTCAGTACTCACTC	X0094	CATCTTCCTACCTAATAAATC
CAU3932	X0073	GCACACAATGTCCCTTACTAG	X0074	CTGGAATCTCTCCTTTCTTGTC
CAU4201	NH364	ACGAACAGGGTTTAGGGCCTCTC	NH365	GCTGCTTACAAGAAGTTGATATAGG
CAU3944	X0103	CGCTATCCAGAAGTAACTCAC	X0104	AGGCTGGATCAGTCATTAAG
CAU4206	NH619	GGAGCATCAAAATGGGACTGC	NH620	CCCAATGGGAAAGGATTTGGAG
CAU4202	NH520	GATTGTATAAGGTGGTTATTGTCTG	NH382	CGAGTTTGAGGTTCTGTCGGAATC
CAU4204	NH432	CCTACTGCATAAAGTCCTACTTC	NH391	CATGTGCTTGGGGAGAACGATCG
CAU3953	X0133	GTGACCATTAACCCTACTGAG	X0134	CTACGTCAATGTGATGCATGTC
CAU4203	NH402	CATTCCACCAGGAACATCCACATC	NH403	CGCATGTGATAGTTGATGATACGG
CAU4205	NH94	TTTCTAAGCTCCTCGCTGTTTG	NH95	CTATGATGTTTGGATGGACTCAG
CAU3959	X0155	CTTTAACTTGCTGGGGTGGTC	X0156	GAGATTCGGATCGAGTCCCAG
CAU3961	X0159	GTTTGCACATAAGAGACGGAC	X0160	CATTATTACCCGCTCAGGTG
CAU3964	X0165	GTCAATTCAGACGCAATGAC	X0166	GCTCAAATGCTGCTCCTACTC
CAU4207	NH552	CATAGCATTGACATCCTCGGGAC	NH553	AGGTTTTGGAGAGCGTCTTCAGC
CAU4208	NH643	GAGGGAACGAAGATGCATGACAA	NH644	AGTGATAAGAGCAAACGGGGCAC
CAU4209	NH579	GGAAAGGCTTGAAAGTCATTACC	NH580	CGTTCTAACGTTCCGAGTTCCT
CAU3978	X0239	GTGTAATGTTGCGAAAGTATTC	X0240	CAGTGCACCTTTTGTGTATTG
CAU4210	NH327	CGACGGATTGGTTATTGGGGTTTG	NH328	GGTCGTTGATAGAGTCAATGGGAT
CAU4211	NH480	GGATCAGAGTTACACCTTACCAT	NH481	AACTAGAGTCATCATTACATGCAG
CAU3891	X0021	GGCAGTGGTATAACTTGTATG	X0022	GATTCCTATTGCTCCTTACAC
CAU4212	NH487	GGTTTCTGTGGTTGGGCGGTT	NH522	AGCTCTGAATGTTGAGTGGGTGC
CAU4213	NH492	CAACACCTCCTTCTAAATCCTCC	NH493	CCAACATTTATCTCACGCTCAT
CAU4214	NH594	AGCATGAGTAAGCAACAGGGCAC	NH595	CTGAACAAGTTATGATCTCCAGG
CAU4001	X0383	CACTGCCGCAAAGAATGTAAG	X0384	CTTTGTAATTAGGTTTACATTGC
CAU4215	NH510	GCTTAGCTGAAGGGCAACCAGTA	NH544	GTTAGGCCAATGAACAAAGGGGC
CAU4004	X0409	GTAACAGCCCATAGGTGTAAG	X0410	GTAGTAGAGCTAGTGATACTG
CAU4006	X0417	GAGGAAGCACCTACTTTCAAC	X0418	GTCAAGCTGGCATCCTCAGTC
CAU4007	X0419	GATATGTTAGCTAGAGGAAAC	X0420	GTTAAGCACATATAGGGCAAG
CAU4216	NH514	GAATTTGGGTACCTTCTGAAGAG	NH515	CTTAGCAGGGTTTCTATTTGCG
CAU4013	X0441	CTTTCTCCCCTAATCCTTTCTC	X0442	CTATGGCTGACTGGATAATAC
CAU4018	X0455	GTGGGCATAACAAGCACTTAG	X0456	GTAAGGGAAGGACTTATTGTC
CAU4217	NH585	TGGTGGCATCCTTCATCAAGAGG	NH586	CCTTCAAGACTGAGTGATGACGA
CAU4023	X0475	GATTTGCGGTAGTTTGTCTTC	X0476	CGGACAAACCATACCAGCATG
CAU4029	X0491	CTGACAAATTGCTCCACAATG	X0492	GAAGATGAGCTCTGAAATGG
CAU4218	NH539	GCCAATACCGATAAAGGCTTCAC	NH540	CAGATCCTCATTTCTCAATTGGAC
CAU4036	X0525	CGAATGGCCATTATGTAACAG	X0526	CAAAATAACATCTTTCCTTACC
CAU4219	NH635	GATGGAGTGTGGCGTGTTCATGAG	NH636	GAATGTCACCCGTTGTTTCGCCA
CAU3897	X0053	GACGAGGAAACACCTAGTTTC	X0054	GTGGGCGTACTTTTCTTGTTTC
<i>BvGAPDH</i>	B582*	GCTTTGAACGACCACTTCGC	B583*	ACGCCGAGAGCAACTTGAAC
<i>BTC1</i>	B580*	GTGAAAGCTGTGTAAGGAATGG	B581*	AAGTTCCTGCATGGATCCAG
<i>BTC1</i>	A883	AGAAAGTGATGACTCAATCG	A747	TCCTGGTGAGTTCATAGCCTG
<i>BTC1</i>	A881	CATACCGAAGGCGTATTCTC	A882	GTGACTCATTATCTTGACAG
<i>BTC1</i>	A894	ACAATGAGACCTTAGTATCCG	A895	GAGCATCAAAATGGGACTGC
<i>BTC1</i>	A884	GTGCCAAATGGCATAGAAGC	A886	CCCTAACTATCAATAGCGAGC
<i>BTC1</i>	A749	CAACCAGGAAAATGATGGTCCG	A750	CAGAATCACTGAGGTTTGACAG
<i>BvFT1</i>	B563*	GCATCATTTGGAGAAGAGATTGTTTA	B564*	GGCGTTGTTGTGGAGCATTTA
<i>BvFT1</i>	NH340	CCATCTATACTTGTGATGACCCTT	NH351	ATTGAAATGGAGAGGTGGAATTGG
<i>BvFT1</i>	NH338	GGAACACATCAAAAGGTGAGAAATC	NH339	CTTCCATGCCCCACTACATCTTG
<i>BvFT1</i>	NH334	GTCTCTGGAAGTTTCTTTATCGATTG	NH335	TTAGATAGTGAAGTATTCCTTAGCTT
<i>BvFT2</i>	NH362	CGATCATGCCTAGAGCACCAAGA	NH356	ATCACTAAAATATCTACTACCAG
<i>BvFT2</i>	B584*	GAGCCCAAGTAATCCACACTTG	B585*	GTGTTGAAGTTTGTACGCCAC
<i>BvFT2</i>	FT2h	CTAGTTGTCTCCCATGTTGCTAC	FT2e	AGCAACAATGCTCTTCAATGGCC
<i>BvFT2</i>	NH358	GTGTAGTAAATTTACAACCTTCTTC	NH357	GTGTGTAGGCCAAGAAGTTGTCTG

<i>AtCO</i>	NH_Co_F	GAGAGTAACGACATAGGTAGTG	NH_Co_R	TGTCTGAATTAGGGAACAGCCAC
<i>BvBBX19</i>	C565	TGAGGACTCTTTGTGATGTTTGTGAG	C566	GGTACAGCATTAGGGGCAGCAAG
<i>BvBBX19</i>	NH284	CTTCGCCCATATTGGAGATGTATC	N0169R	GTAAATGGAGATTTAAGAAGGTCC
<i>BvBBX19</i>	N0150F	ATGCATCTTATACTATGGGACCAC	N0150R	GGAACAATGCTTGCCGAATCAC
<i>BvBBX19</i>	N0160F	GATCTCAATGCTAGACCCCAAAG	NH283	CTTCGCCCATATTGGAGATGTATC
<i>BvBBX19</i>	N0162F	CAACGACGACTCAAAGAGACAG	N0162R	CTCAAATTTGCGGCATAGTAATG
<i>BvBBX19</i>	pUBI_F	CGAGTCTAACGGACACCAACC	pUBI_R	GCAATGCTCATTATCTCTAGAG
<i>nptII</i>	C015	CGGCAGGAGCAAGGTGAGATGAC	C016	GGGTTTCGCTCATGTGTTGAGC
<i>PAT</i>	C162	GGTCTGCACCATCGTCAACCAC	C163	AGATCTCGGTGACGGGCAGGAC

\*(Pin et al. 2010; Pin et al. 2012)

**Supplementary Table 3:** Primers and amplification protocol for PCR.

Target gene	Forward Primer	Reverse Primer	PCR conditions
<i>BvBBX19</i>	N0162_F	N0162_R	95°C, 3' + 36 x (95°C, 30"; 55°C, 30"; 72°C, 30") +72°C, 5'
<i>BvBBX19</i>	N0150_F	N0150_R	95°C, 3' + 36 x (95°C, 30"; 55°C, 30"; 72°C, 30") +72°C, 5'
<i>BvBBX19</i>	NH284	N0169R	95°C, 3' + 36 x (95°C, 30"; 55°C, 30"; 72°C, 30") +72°C, 5'
<i>BvBBX19</i>	N0160_F	NH283	95°C, 3' + 36 x (95°C, 30"; 55°C, 30"; 72°C, 30") +72°C, 5'
<i>BTC1</i>	A881	A882	95°C, 3' + 36 x (95°C, 30"; 57°C, 30"; 72°C, 80") +72°C, 5'
<i>BTC1</i>	A894	A895	95°C, 3' + 36 x (95°C, 30"; 57°C, 30"; 72°C, 60") +72°C, 5'
<i>BTC1</i>	A884	A886	95°C, 3' + 36 x (95°C, 30"; 57°C, 30"; 72°C, 60") +72°C, 5'
<i>BTC1</i>	A749	A750	95°C, 3' + 36 x (95°C, 30"; 57°C, 30"; 72°C, 120") +72°C, 5'
<i>BvFT1</i>	NH340	NH351	95°C, 3' + 36 x (95°C, 30"; 62°C, 30"; 72°C, 30") +72°C, 5'
<i>BvFT1</i>	NH338	NH339	95°C, 3' + 36 x (95°C, 30"; 60°C, 30"; 72°C, 30") +72°C, 5'
<i>BvFT1</i>	NH334	NH335	95°C, 3' + 36 x (95°C, 30"; 60°C, 30"; 72°C, 40") +72°C, 5'
<i>BvFT2</i>	NH362	NH356	95°C, 3' + 36 x (95°C, 30"; 60°C, 30"; 72°C, 30") +72°C, 5'
<i>BvFT2</i>	FT2_h	FT2_e	95°C, 3' + 36 x (95°C, 30"; 60°C, 30"; 72°C, 30") +72°C, 5'
<i>BvFT2</i>	NH358	NH357	95°C, 3' + 36 x (95°C, 30"; 60°C, 30"; 72°C, 30") +72°C, 5'

**Supplementary Table 4:** IUPAC code for incomplete nucleic acid specification (Johnson 2010).

Symbol	Mnemonic	Translation
A		A (adenine)
C		C (cytosine)
G		G (guanine)
T		T (thymine)
U		U (uracil)
R	puRine	A or G (purines)
Y	pYrimidine	C or T/U (pyrimidines)
M	aMino group	A or C
K	Keto group	G or T/U
S	Strong interaction	C or G
W	Weak interaction	A or T/U
H	not G	A, C or T/U
B	not A	C, G or T/U
V	not T/U	A, C or G
D	not C	A, G or T/U
N	aNy	A, C, G or T/U

**Supplementary Table 5:** Phenotypic data from three different experiments. Plants were classified as annual (bolting within 16 weeks after sowing) or biennial (bolting only after vernalization). Plants that did not bolt 16 weeks after vernalization were classified as never bolting. Plants were grown and phenotyped in a climate chamber and kept under LD conditions with a light intensity of 900 $\mu$ E.

			Experiment 1 (22 h light)		Experiment 2 (16 h light)		Experiment 3 (22 h light+vernalization)			
Variety	Seed code	Expected bolting behavior	Bolting without vern.	Non-bolting without vern.	Bolting without vern.	Non-bolting without vern.	Bolting without vern.	Non-bolting without vern.	Bolting after vern.	Never bolting
	080287	biennial	-	10	-	10	-	10	4	6
	080461	biennial	-	10	-	10	-	10	3	7
	080260	biennial	3	7	-	10	2	8	8	-
	080538*	biennial	8	-	-	10	7	3	3	-
wild	080468	annual	10	-	10	-	10	-	-	-
beet	080437	annual	10	-	10	-	10	-	-	-
	080418	annual	10	-	10	-	10	-	-	-
	991971	annual	10	-	10	-	10	-	-	-
	930034*	annual	5	-	3	-	-	-	-	-
	100539	annual	10	-	10	-	9	1	1	-
	112787*	perennial	4	4	3	6	-	1	-	1
	112823*	perennial	-	6	1	9	-	6	1	5
	001684	annual	10	-	10	-	10	-	-	-
	090023*	biennial	-	10	-	10	-	5	5	-
sugar	930176*	biennial	-	10	-	9	-	10	10	-
beet	130333	biennial	-	10	-	10	-	7	7	-
	100043*	biennial	-	10	-	10	-	8	8	-
	080394	biennial	7	3	-	10	5	5	5	-
	930181	biennial	-	10	-	10	-	10	10	-
	080384	biennial	8	2	7	3	6	4	4	-
	091645	biennial	-	10	-	10	-	10	10	-
fodder	080313	biennial	-	10	-	10	-	10	10	-
beet	080281	biennial	-	10	-	10	-	10	10	-
	080396	biennial	9	1	-	10	6	4	4	-
table	092312	biennial	-	10	-	10	1	9	5	4
beet	080339	biennial	-	10	-	10	-	10	10	-
leaf	080238	biennial	10	-	10	-	10	-	-	-
beet	081845*	biennial	7	-	-	10	10	-	-	-
	092459*	biennial	-	8	-	7	-	5	1	4
<b>Total</b>			<b>121</b>	<b>151</b>	<b>84</b>	<b>194</b>	<b>106</b>	<b>146</b>	<b>119</b>	<b>27</b>

\*partly less than 10 plants due to low germination rate; vern.= vernalization

**Supplementary Table 6:** *BTC1* haplotypes of all plants from the *Beta* panel. The coding region was sequenced. The position of the SNPs is given relative to the translation start site according to the regarding exons. The polymorphism exon9nt616 (published by Pin et al., (2012)) could be identified after own database research as erroneously swapped (see HQ709094.1). For each accession, PCR products of 10 plants were pooled and sequenced for haplotype analysis. In case of sequence heterogeneity, all single plants were sequenced and haplotypes were assembled which could result in more than one haplotypes per accession. The nomenclature of polymorphisms was given according to the IUPAC code (Johnson 2010).

Exon	3			5	6		7			8						9										10	Haplotypes in 10 plants / accession	
	92	224	351	89	64	89	23	75	164	2	9	79	97	154	158	250	29	37	402	435	476	542	616	670	686	814		72
090023	G	A	A	C	G	T	C	A	G	C	C	C	C	G	T	G	G	A	A	G	C	G	A	A	G	T	A	<i>BTC1<sub>a</sub></i>
930176	G	A	A	C	G	T	C	A	G	C	C	C	C	G	T	G	G	A	A	G	C	G	A	A	G	T	A	<i>BTC1<sub>a</sub></i>
130333	G	A	A	C	G	T	C	A	G	C	C	C	C	G	T	G	G	A	A	G	C	G	A	A	G	T	A	<i>BTC1<sub>a</sub></i>
091645	G	A	A	C	G	T	C	A	G	C	C	C	C	G	T	G	G	A	A	G	C	G	A	A	G	T	A	<i>BTC1<sub>a</sub></i>
100043	G	A	A	C	G	T	C	A	G	C	C	C	C	G	T	G	G	A	A	G	C	G	A	A	G	T	A	<i>BTC1<sub>a</sub></i>
092312	G	A	A	C	G	T	C	A	G	C	C	C	C	G	T	G	G	A	A	G	C	G	A	A	G	T	A	<i>BTC1<sub>a</sub></i>
080339	G	A	A	C	G	T	C	A	G	C	C	C	C	G	T	G	G	A	A	G	C	G	A	A	G	T	A	<i>BTC1<sub>a</sub></i>
080281	G	A	A	C	G	T	C	A	G	C	C	C	C	G	T	G	G	A	A	G	C	G	A	A	G	T	A	<i>BTC1<sub>a</sub></i>
001684	G	C	T	T	G	G	C	G	T	C	C	A	C	G	T	A	G	A	G	A	T	A	A	A	A	T	G	<i>BTC1<sub>d</sub></i>
100539	G	C	T	T	G	G	C	G	T	C	C	A	C	G	A	A	G	A	G	G	T	A	A	A	G	T	G	<i>BTC1<sub>e</sub></i>
930034	G	C	T	T	G	G	C	G	T	C	C	A	C	G	A	A	G	A	G	G	T	A	A	A	G	T	G	<i>BTC1<sub>e</sub></i>
080384	G	C	T	T	G	G	C	G	T	C	C	A	C	G	A	A	G	A	G	G	T	A	A	A	G	T	G	<i>BTC1<sub>e</sub></i>
092459	G	C	T	T	G	G	C	G	T	C	C	A	C	G	A	A	G	A	G	G	T	A	A	A	G	T	G	<i>BTC1<sub>e</sub></i>
080238	G	C	T	T	G	G	C	G	T	C	C	A	C	G	T	A	G	A	G	G	T	A	A	A	G	T	G	<i>BTC1<sub>e</sub></i>
080396	G	C	T	T	G	G	C	G	T	C	T	A	C	G	T	A	G	A	G	G	T	A	A	A	G	T	G	<i>BTC1<sub>h</sub></i>
080461	G	C	T	T	A	G	C	G	T	C	C	A	C	T	T	A	G	A	G	G	T	G	A	A	G	C	G	<i>BTC1<sub>i</sub></i>
080287	G	C	T	T	A	G	C	G	T	C	C	A	C	T	T	A	G	A	G	G	T	G	A	A	G	C	G	<i>BTC1<sub>j</sub></i>
112787	T	C	W	T	G	G	C	G	T	C	C	A	A	G	T	A	G	A	G	G	T	G	A	A	G	T	G	<i>BTC1<sub>k</sub></i>
112823	T	C	W	T	G	G	C	G	T	C	C	A	A	G	T	A	G	A	G	G	T	G	A	A	G	T	G	<i>BTC1<sub>k</sub></i>
080260	T	C	W	T	G	G	C	G	T	C	C	A	M	K	T	A	G	A	G	G	T	G	A	A	G	T	G	<i>BTC1<sub>k</sub></i>
930181	G	C	T	T	G	G	C	G	T	C	C	A	C	G	T	A	A	A	G	G	T	A	A	A	G	T	G	<i>BTC1<sub>l</sub></i>
080313	G	C	T	T	G	G	C	G	T	C	C	A	C	G	T	A	A	A	G	G	T	A	A	A	G	T	G	<i>BTC1<sub>l</sub></i>
080468	G	C	T	T	G	G	C	G	G	T	C	A	C	G	T	A	G	A	G	G	T	G	G	A	G	T	G	<i>BTC1<sub>m</sub></i>
080437	G	C	T	T	G	G	C	G	G	T	C	A	C	G	T	A	G	A	G	G	T	G	G	A	G	T	G	<i>BTC1<sub>m</sub></i>
080418	G	C	T	T	G	G	C	G	G	T	C	A	C	G	T	A	G	A	G	G	T	G	G	A	G	T	G	<i>BTC1<sub>m</sub></i>
991971	G	C	T	T	G	G	C	G	G	Y	C	A	C	G	W	A	G	R	G	G	T	G	R	A	G	T	G	<i>BTC1<sub>e</sub>, BTC1<sub>f</sub>, BTC1<sub>m</sub></i>
080394	G	C	T	T	G	G	C	G	K	C	C	A	C	G	T	A	G	A	G	G	C	G	A	A	G	T	G	<i>BTC1<sub>n</sub></i>
080538	G	C	T	T	R	G	C	G	K	C	C	A	C	K	T	A	G	A	G	G	Y	G	A	A	G	M	G	<i>BTC1<sub>j</sub>, BTC1<sub>n</sub></i>
081845	G	C	T	T	G	G	C	G	K	C	C	A	C	G	T	A	G	A	G	G	Y	G	R	A	R	T	G	<i>BTC1<sub>j</sub>, BTC1<sub>m</sub>, BTC1<sub>n</sub></i>
non-syn. SNP	*	*	*		*	*	*		*		*	*	*	*	*	*	*		*	*	*	*		*	*			



**Supplementary Table 7:** *BvBBX19* haplotypes of all plants from the *Beta* panel. The coding region was sequenced. The position of the SNPs is given relative to the translation start site according to the regarding exons. For each accession, PCR products of 10 plants were pooled and sequenced for haplotype analysis. In case of sequence heterogeneity, all single plants were sequenced and haplotypes were assembled which could result in more than one haplotypes per accession.

Exon	2	4			
Seed code	69	45	59	231	Haplotypes in 10 plants / accession
001684	A	G	C	T	<i>BvBBX19<sub>a</sub></i>
090023	A	G	C	T	<i>BvBBX19<sub>a</sub></i>
091645	A	G	C	T	<i>BvBBX19<sub>a</sub></i>
130333	A	G	C	T	<i>BvBBX19<sub>a</sub></i>
100043	A	G	C	T	<i>BvBBX19<sub>a</sub></i>
930176	A	G	C	T	<i>BvBBX19<sub>a</sub></i>
112823	A	G	C	T	<i>BvBBX19<sub>a</sub></i>
080339	A	G	C	T	<i>BvBBX19<sub>a</sub></i>
100539	A	G	C	T	<i>BvBBX19<sub>a</sub></i>
092312	A	G	C	T	<i>BvBBX19<sub>a</sub></i>
112787	A	G	C	Y	<i>BvBBX19<sub>a</sub>, BvBBX19<sub>b</sub></i>
930181	G	A	C	T	<i>BvBBX19<sub>e</sub></i>
930034	G	A	C	T	<i>BvBBX19<sub>e</sub></i>
080384	G	A	C	T	<i>BvBBX19<sub>e</sub></i>
080287	G	A	C	C	<i>BvBBX19<sub>d</sub></i>
080538	G	A	C	C	<i>BvBBX19<sub>d</sub></i>
080461	G	A	C	C	<i>BvBBX19<sub>d</sub></i>
080281	G	A	C	C	<i>BvBBX19<sub>d</sub></i>
092459	G	A	T	C	<i>BvBBX19<sub>f</sub></i>
080468	G	A	T	C	<i>BvBBX19<sub>f</sub></i>
080396	G	A	T	C	<i>BvBBX19<sub>f</sub></i>
080394	G	A	Y	C	<i>BvBBX19<sub>d</sub>, BvBBX19<sub>f</sub></i>
081845	G	A	Y	T	<i>BvBBX19<sub>f</sub>, BvBBX19<sub>g</sub></i>
080437	G	A	Y	C	<i>BvBBX19<sub>d</sub>, BvBBX19<sub>f</sub></i>
080418	G	A	Y	C	<i>BvBBX19<sub>d</sub>, BvBBX19<sub>f</sub></i>
991971	G	A	Y	Y	<i>BvBBX19<sub>d</sub>, BvBBX19<sub>f</sub>, BvBBX19<sub>g</sub></i>
080313	G	R	C	Y	<i>BvBBX19<sub>c</sub>, BvBBX19<sub>d</sub></i>
080238	G	R	Y	Y	<i>BvBBX19<sub>c</sub>, BvBBX19<sub>f</sub>, BvBBX19<sub>g</sub></i>
080260	R	R	C	Y	<i>BvBBX19<sub>a</sub>, BvBBX19<sub>d</sub></i>
non-syn. SNP			*		

**Supplementary Table 8:** *BvFT1* haplotypes of all plants from the *Beta* panel. The coding region was sequenced. The position of the SNPs is given relative to the translation start site according to the regarding exons. For each accession, PCR products of 10 plants were pooled and sequenced for haplotype analysis. In case of sequence heterogeneity, all single plants were sequenced and haplotypes were assembled which could result in more than one haplotypes per accession.

Exon	1				4	Haplotypes in 10 plants/accession
	11	20	70	72	57	
100539	G	C	C	C	C	<i>BvFT1<sub>a</sub></i>
991971	G	C	C	C	C	<i>BvFT1<sub>a</sub></i>
930034	G	C	C	C	C	<i>BvFT1<sub>a</sub></i>
090023	G	C	C	C	C	<i>BvFT1<sub>a</sub></i>
130333	G	C	C	C	C	<i>BvFT1<sub>a</sub></i>
091645	G	C	C	C	C	<i>BvFT1<sub>a</sub></i>
080384	G	C	C	C	C	<i>BvFT1<sub>a</sub></i>
930181	G	C	C	C	C	<i>BvFT1<sub>a</sub></i>
100043	G	C	C	C	C	<i>BvFT1<sub>a</sub></i>
001684	G	C	C	C	C	<i>BvFT1<sub>a</sub></i>
080313	G	C	C	C	C	<i>BvFT1<sub>a</sub></i>
092312	G	C	C	C	C	<i>BvFT1<sub>a</sub></i>
080396	G	C	C	C	C	<i>BvFT1<sub>a</sub></i>
080287	G	Y	C	C	C	<i>BvFT1<sub>a</sub>, BvFT1<sub>e</sub></i>
080461	G	Y	C	C	C	<i>BvFT1<sub>a</sub>, BvFT1<sub>e</sub></i>
080538	G	Y	C	C	C	<i>BvFT1<sub>a</sub>, BvFT1<sub>e</sub></i>
080260	G	Y	G	C	C	<i>BvFT1<sub>b</sub>, BvFT1<sub>d</sub></i>
930176	G	C	G	C	C	<i>BvFT1<sub>b</sub></i>
080468	G	C	G	C	T	<i>BvFT1<sub>c</sub></i>
092459	G	C	G	C	Y	<i>BvFT1<sub>b</sub>, BvFT1<sub>c</sub></i>
080339	G	C	S	C	C	<i>BvFT1<sub>a</sub>, BvFT1<sub>b</sub></i>
081845	G	C	S	C	C	<i>BvFT1<sub>a</sub>, BvFT1<sub>b</sub></i>
080281	G	C	S	C	C	<i>BvFT1<sub>a</sub>, BvFT1<sub>b</sub></i>
112823	G	C	S	C	C	<i>BvFT1<sub>a</sub>, BvFT1<sub>b</sub></i>
080394	G	Y	S	T	C	<i>BvFT1<sub>g</sub>, BvFT1<sub>h</sub></i>
112787	G	Y	S	C	C	<i>BvFT1<sub>a</sub>, BvFT1<sub>b</sub>, BvFT1<sub>d</sub></i>
080238	G	C	C	T	C	<i>BvFT1<sub>h</sub></i>
080437	T	T	C	T	C	<i>BvFT1<sub>f</sub></i>
080418	T	T	C	T	C	<i>BvFT1<sub>f</sub></i>
non-syn. SNP	*	*	*		*	

**Supplementary Table 9:** *BvFT2* haplotypes of all plants from the *Beta* panel. The coding region was sequenced. The position of the SNPs is given relative to the translation start site according to the regarding exons. For each accession, PCR products of 10 plants were pooled and sequenced for haplotype analysis. In case of sequence

heterogeneity, all single plants were sequenced and haplotypes were assembled which could result in more than one haplotypes per accession.

<b>Exon</b>	<b>1</b>	<b>4</b>	
Seed code	82	39	Haplotypes in 10 plants/ accession
090023	C	G	<i>BvFT2<sub>d</sub></i>
930176	C	G	<i>BvFT2<sub>d</sub></i>
130333	C	G	<i>BvFT2<sub>d</sub></i>
091645	C	G	<i>BvFT2<sub>d</sub></i>
080394	C	G	<i>BvFT2<sub>d</sub></i>
080313	C	G	<i>BvFT2<sub>d</sub></i>
080384	C	G	<i>BvFT2<sub>d</sub></i>
080281	C	G	<i>BvFT2<sub>d</sub></i>
092312	C	G	<i>BvFT2<sub>d</sub></i>
080238	C	G	<i>BvFT2<sub>d</sub></i>
080538	C	A	<i>BvFT2<sub>c</sub></i>
080437	A	G	<i>BvFT2<sub>a</sub></i>
080418	A	G	<i>BvFT2<sub>a</sub></i>
001684	A	G	<i>BvFT2<sub>a</sub></i>
100043	A	G	<i>BvFT2<sub>a</sub></i>
080396	A	G	<i>BvFT2<sub>a</sub></i>
092459	A	G	<i>BvFT2<sub>a</sub></i>
080287	C	R	<i>BvFT2<sub>d</sub>, BvFT2<sub>c</sub></i>
080461	C	R	<i>BvFT2<sub>d</sub>, BvFT2<sub>c</sub></i>
112823	C	R	<i>BvFT2<sub>d</sub>, BvFT2<sub>c</sub></i>
100539	A	R	<i>BvFT2<sub>a</sub>, BvFT2<sub>b</sub></i>
930034	A	R	<i>BvFT2<sub>a</sub>, BvFT2<sub>b</sub></i>
930181	M	G	<i>BvFT2<sub>a</sub>, BvFT2<sub>d</sub></i>
080339	M	G	<i>BvFT2<sub>a</sub>, BvFT2<sub>d</sub></i>
080468	M	G	<i>BvFT2<sub>a</sub>, BvFT2<sub>d</sub></i>
081845	M	G	<i>BvFT2<sub>a</sub>, BvFT2<sub>d</sub></i>
080260	M	R	<i>BvFT2<sub>a</sub>, BvFT2<sub>b</sub>, BvFT2<sub>d</sub></i>
991971	M	R	<i>BvFT2<sub>a</sub>, BvFT2<sub>b</sub>, BvFT2<sub>d</sub></i>
112787	M	R	<i>BvFT2<sub>a</sub>, BvFT2<sub>b</sub>, BvFT2<sub>c</sub>, BvFT2<sub>d</sub></i>
non-syn. SNP	*	*	

**Supplementary Table 10:** Complete list of all haplotype combinations and phenotypic data. The table also comprises information on growth type and latitude of origin. Rows shaded in light grey indicate accessions which were non-bolting without vernalization under all environments. Cells shaded in dark grey indicate accessions which revealed a non-bolting phenotype under 16 hours of light (experiment 2) but an annual phenotype or mixed phenotype (annual + biennial) under 22 hours light (experiment 1+3) before vernalization.

Variety	Seed code	Latitude of origin (°N)	<i>BTC1</i> haplotype	<i>BvBBX19</i> haplotype	<i>BvFT1</i> haplotype	<i>BvFT2</i> haplotype	Experiment 1 (22h light)		Experiment 2 (16h light)		Experiment 3 (22h light+vernalization)			
							Bolting without vern.	Non-bolting without vern.	Bolting without vern.	Non-bolting without vern.	Bolting without vern.	Non-bolting without vern.	Bolting after vern.	Never bolting after vern.
sugar beet	090023	51.0	<i>BTC1<sub>a</sub></i>	<i>BvBBX19<sub>a</sub></i>	<i>BvFT1<sub>a</sub></i>	<i>BvFT2<sub>d</sub></i>	-	10	-	10	-	5	5	-
	130333	51.0	<i>BTC1<sub>a</sub></i>	<i>BvBBX19<sub>a</sub></i>	<i>BvFT1<sub>a</sub></i>	<i>BvFT2<sub>d</sub></i>	-	10	-	10	-	7	7	-
	091645	51.0	<i>BTC1<sub>a</sub></i>	<i>BvBBX19<sub>a</sub></i>	<i>BvFT1<sub>a</sub></i>	<i>BvFT2<sub>d</sub></i>	-	10	-	10	-	10	10	-
	100043	51.0	<i>BTC1<sub>a</sub></i>	<i>BvBBX19<sub>a</sub></i>	<i>BvFT1<sub>a</sub></i>	<i>BvFT2<sub>a</sub></i>	-	10	-	10	-	8	8	-
	930176	51.0	<i>BTC1<sub>a</sub></i>	<i>BvBBX19<sub>a</sub></i>	<i>BvFT1<sub>b</sub></i>	<i>BvFT2<sub>d</sub></i>	-	10	-	9	-	10	10	-
	930181	45.0	<i>BTC1<sub>l</sub></i>	<i>BvBBX19<sub>e</sub></i>	<i>BvFT1<sub>a</sub></i>	<i>BvFT2<sub>av</sub></i> <i>BvFT2<sub>d</sub></i>	-	10	-	10	-	10	10	-
	001684	51.0	<i>BTC1<sub>d</sub></i>	<i>BvBBX19<sub>a</sub></i>	<i>BvFT1<sub>a</sub></i>	<i>BvFT2<sub>a</sub></i>	10	-	10	-	10	-	-	-
	080384	39.0	<i>BTC1<sub>e</sub></i>	<i>BvBBX19<sub>e</sub></i>	<i>BvFT1<sub>a</sub></i>	<i>BvFT2<sub>d</sub></i>	8	2	7	3	6	4	4	-
	080394	32.0	<i>BTC1<sub>n</sub></i>	<i>BvBBX19<sub>db</sub></i> <i>BvBBX19<sub>f</sub></i>	<i>BvFT1<sub>g</sub></i> <i>BvFT1<sub>h</sub></i>	<i>BvFT2<sub>d</sub></i>	7	3	-	10	5	5	5	-
red table beet	092312	60.0	<i>BTC1<sub>a</sub></i>	<i>BvBBX19<sub>a</sub></i>	<i>BvFT1<sub>a</sub></i>	<i>BvFT2<sub>d</sub></i>	-	10	-	10	1	9	5	4
	080339	45.5	<i>BTC1<sub>a</sub></i>	<i>BvBBX19<sub>a</sub></i>	<i>BvFT1<sub>av</sub></i> <i>BvFT1<sub>b</sub></i>	<i>BvFT2<sub>av</sub></i> <i>BvFT2<sub>d</sub></i>	-	10	-	10	-	10	10	-
fodder beet	080281	51.0	<i>BTC1<sub>a</sub></i>	<i>BvBBX19<sub>d</sub></i>	<i>BvFT1<sub>av</sub></i> <i>BvFT1<sub>b</sub></i>	<i>BvFT2<sub>d</sub></i>	-	10	-	10	-	10	10	-
	080313	39.0	<i>BTC1<sub>l</sub></i>	<i>BvBBX19<sub>cv</sub></i> <i>BvBBX19<sub>d</sub></i>	<i>BvFT1<sub>a</sub></i>	<i>BvFT2<sub>d</sub></i>	-	10	-	10	-	10	10	-
	080396	32.0	<i>BTC1<sub>h</sub></i>	<i>BvBBX19<sub>f</sub></i>	<i>BvFT1<sub>a</sub></i>	<i>BvFT2<sub>a</sub></i>	9	1	-	10	6	4	4	-
leaf beet	092459	42.0	<i>BTC1<sub>e</sub></i>	<i>BvBBX19<sub>f</sub></i>	<i>BvFT1<sub>b</sub></i> <i>BvFT1<sub>c</sub></i>	<i>BvFT2<sub>a</sub></i>	-	8	-	7	-	5	1	4

leaf beet	080238	33.0	<i>BTC1<sub>g</sub></i>	<i>BvBBX19<sub>c</sub></i> <i>BvBBX19<sub>f</sub></i> <i>BvBBX19<sub>g</sub></i>	<i>BvFT1<sub>h</sub></i>	<i>BvFT2<sub>d</sub></i>	10	-	10	-	10	-	-	-
	081845	35.0	<i>BTC1<sub>i</sub></i> <i>BTC1<sub>m</sub></i> <i>BTC1<sub>n</sub></i>	<i>BvBBX19<sub>f</sub></i> <i>BvBBX19<sub>g</sub></i>	<i>BvFT1<sub>a</sub></i> <i>BvFT1<sub>b</sub></i>	<i>BvFT2<sub>a</sub></i> <i>BvFT2<sub>d</sub></i>	7	-	-	10	10	-	-	-
wild beet	080287	53.0	<i>BTC1<sub>j</sub></i>	<i>BvBBX19<sub>d</sub></i>	<i>BvFT1<sub>a</sub></i> <i>BvFT1<sub>e</sub></i>	<i>BvFT2<sub>b</sub></i> <i>BvFT2<sub>c</sub></i>	-	10	-	10	-	10	4	6
	080461	56.0	<i>BTC1<sub>j</sub></i>	<i>BvBBX19<sub>d</sub></i>	<i>BvFT1<sub>a</sub></i> <i>BvFT1<sub>e</sub></i>	<i>BvFT2<sub>b</sub></i> <i>BvFT2<sub>c</sub></i>	-	10	-	10	-	10	3	7
	112823	54.0	<i>BTC1<sub>k</sub></i>	<i>BvBBX19<sub>a</sub></i>	<i>BvFT1<sub>a</sub></i> <i>BvFT1<sub>b</sub></i>	<i>BvFT2<sub>b</sub></i> <i>BvFT2<sub>c</sub></i>	-	6	1	9	-	6	1	5
	100539	51.0	<i>BTC1<sub>e</sub></i>	<i>BvBBX19<sub>a</sub></i>	<i>BvFT1<sub>a</sub></i>	<i>BvFT2<sub>a</sub></i> <i>BvFT2<sub>b</sub></i>	10	-	10	-	9	1	1	-
	930034	40.0	<i>BTC1<sub>e</sub></i>	<i>BvBBX19<sub>e</sub></i>	<i>BvFT1<sub>a</sub></i>	<i>BvFT2<sub>a</sub></i> <i>BvFT2<sub>b</sub></i>	5	-	3	-	-	-	-	-
	080468	27.0	<i>BTC1<sub>m</sub></i>	<i>BvBBX19<sub>f</sub></i>	<i>BvFT1<sub>c</sub></i>	<i>BvFT2<sub>a</sub></i> <i>BvFT2<sub>d</sub></i>	10	-	10	-	10	-	-	-
	080437	31.0	<i>BTC1<sub>m</sub></i>	<i>BvBBX19<sub>b</sub></i> <i>BvBBX19<sub>f</sub></i>	<i>BvFT1<sub>f</sub></i>	<i>BvFT2<sub>a</sub></i>	10	-	10	-	10	-	-	-
	080418	21.0	<i>BTC1<sub>m</sub></i>	<i>BvBBX19<sub>b</sub></i> <i>BvBBX19<sub>f</sub></i>	<i>BvFT1<sub>f</sub></i>	<i>BvFT2<sub>a</sub></i>	10	-	10	-	10	-	-	-
	991971	39.0	<i>BTC1<sub>m</sub></i>	<i>BvBBX19<sub>b</sub></i> <i>BvBBX19<sub>f</sub></i> <i>BvBBX19<sub>g</sub></i>	<i>BvFT1<sub>a</sub></i>	<i>BvFT2<sub>a</sub></i> <i>BvFT2<sub>b</sub></i> <i>BvFT2<sub>d</sub></i>	10	-	10	-	10	-	-	-
	080260	52.0	<i>BTC1<sub>k</sub></i>	<i>BvBBX19<sub>a</sub></i> <i>BvBBX19<sub>d</sub></i>	<i>BvFT1<sub>b</sub></i> <i>BvFT1<sub>d</sub></i>	<i>BvFT2<sub>a</sub></i> <i>BvFT2<sub>b</sub></i> <i>BvFT2<sub>d</sub></i>	3	7	-	10	2	8	8	-
	112787	46.0	<i>BTC1<sub>k</sub></i>	<i>BvBBX19<sub>a</sub></i> <i>BvBBX19<sub>b</sub></i>	<i>BvFT1<sub>a</sub></i> <i>BvFT1<sub>b</sub></i> <i>BvFT1<sub>d</sub></i>	<i>BvFT2<sub>a</sub></i> <i>BvFT2<sub>b</sub></i> <i>BvFT2<sub>c</sub></i> <i>BvFT2<sub>d</sub></i>	4	4	3	6	-	1	-	1
	080538	54.0	<i>BTC1<sub>j</sub></i> <i>BTC1<sub>n</sub></i>	<i>BvBBX19<sub>d</sub></i>	<i>BvFT1<sub>a</sub></i> <i>BvFT1<sub>e</sub></i>	<i>BvFT2<sub>c</sub></i>	8	-	-	10	7	3	3	-

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## 10 Curriculum vitae and publications

### 10.1 Curriculum vitae

#### Personal data

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Name	Nadine Höft
Date of birth	20.01.1987
Place of birth	Rendsburg, Germany
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#### Professional experience

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Since 2017/02	Administrative Manager “SPP1530 – Flowering Time Control: from Natural Variation to Crop Improvement”, Plant Breeding Institute, Christian-Albrechts University of Kiel, Germany
2013/09-2015/09	PhD representative of the International Max Planck Research School (IMPRS) for Evolutionary Biology

#### Education

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Since 2013	PhD student of the International Max Planck Research School (IMPRS) for Evolutionary Biology, Plant Breeding Institute, Christian-Albrechts University of Kiel, Germany
2012/09-2012/12	PhD program of the International Max Planck Research School for Evolutionary Biology
2012/07	M. Sc. in Biology, Plant Breeding Institute, Christian Albrechts-University of Kiel, Germany Master thesis: „Untersuchung von transgenen Arabidopsis Pflanzen bei denen über das FLP/FRT Rekombinationssystem Repressorgene der Blühinduktion aktiviert werden“
2009-2012	M. Sc. courses in Biology, Christian Albrechts-University of Kiel, Germany
2009/10	B. Sc. in Biology, Christian Albrechts-University of Kiel, Germany Bachelor thesis: „Charakterisierung des octopaminergen Systems des Nematoden <i>C. elegans</i> “
2006-2009	B. Sc. courses in Biology, Christian Albrechts-University of Kiel, Germany
2006-2007	B. Sc. courses in Anglistics, Christian Albrechts-University of Kiel, Germany
2003-2006	„Fachgymnasium Gesundheit und Soziales, Berufliche Schulen Rendsburg“, grammar school, Rendsburg, Germany
1997-2003	„Bertolt-Brecht-Schule, Büdelsdorf“, middle school, Büdelsdorf, Germany
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## 10.2 Publications

### 10.2.1 Oral presentations

\***Höft, N.**, Dally, N., Hasler, M., Jung C. (2016). Sequence variations in flowering time genes from species of the genus *Beta* with different geographical origin. PP1530 Workshop: Circadian rhythms, flowering time regulation and crop plant adaptation 20.-22.01.2016.

\***Höft, N.**, Dally, N., Jung C. (2013). The evolution of flowering time genes in *Beta* species. IMPRS retreat, Gut Ankelohe, Germany, 01.-03.09.2013.

### 10.2.2 Posters

\***Höft, N.**, Dally, N., Hasler M., Jung C. (2017). Haplotype variation of three major flowering time genes and their impact on life cycle regimes of *Beta* species. SPP1530-Workshop “Genome Engineering and Epigenetic Variation of Flowering Time Genes”, IPK Gatersleben, 22-23.06.2017

Dally, N., Eckel M, Pandya A, Batschauer A, **Höft, N.**, Jung C. (2017). Unraveling the molecular mechanism of floral transition on beet for the development of winter beets. PP1530-Workshop “Genome Engineering and Epigenetic Variation of Flowering Time Genes”, IPK Gatersleben, 22-23.06.2017

\***Höft, N.**, Dally, N., Jung C. (2016). The impact of geographical origin of *Beta* species on its different life cycle regimes. PP1530 Workshop: Circadian rhythms, flowering time regulation and crop plant adaptation 20.-22.01.2016.

Dally, N., Eckel M, **Höft, N.**, Batschauer A, Jung C. (2016). Functional analysis of two key regulators for bolting time in *Beta* species to breed winter sugar beets. PP1530 Workshop: Circadian rhythms, flowering time regulation and crop plant adaptation 20.-22.01.2016.

\***Höft, N.**, Dally, N., Jung C. (2015). Towards understanding the evolution of different life cycle regimes in *Beta* species. IPMB 2015: 11th Congress of International Plant Molecular Biology, Bourbon Cataratas Convention & Spa Resort, Iguazú Falls, Brazil, 25-30.10.2015

\***Höft, N.**, Dally, N., Jung C. (2015). Unraveling the geographic origin of sugar beet. IMPRS Retreat, Gut Ankelohe, Germany, 16.-18.09.2015

\***Höft, N.**, Dally, N., Jung C. (2014). Life cycle adaption in beet identification of a new bolting time gene. GPZ 2014: Genetic Variation in Plant Breeding, University Kiel 23.-25.09.2014

\***Höft, N.**, Dally, N., Xiao K., Jung C. (2014). Functional characterization of a new bolting time gene from sugar beet. IMPRS Retreat, Eckernförde, Germany, 01.-03.09.2014

\***Höft, N.**, Dally, N., Jung C. (2014). Functional characterization of the *B4* bolting locus from sugar beet. II. International Symposium: Genetic Variation of Flowering Time Genes and Applications for Crop Improvement, University Bielefeld 24.-26.03.2014

\***Höft, N.**, Tränkner C., Jung C., Melzer S. (2012). Control of flowering in sugar beet and Arabidopsis through activation of flowering time repressors by FLP-FRT recombination. "Breeding crops for sustainable agricultural production" Symposium of GPZ e.V., Gießen 28.2.-1.3.2012

\* Presented by N. Höft

## 11 Declaration of own contribution

Chapter 2, 3 of this thesis will be submitted for publication. The doctoral student Nadine Höft contributed to the chapters as follows:

**Chapter 2: Sequence variation in the bolting time regulator *BTC1* changes the life cycle of sugar beet**

Designing experiments: 80 %

Results: 100 %

Writing the chapter: 80 %

Discussion and revision: 70 %

**Chapter 3: Haplotype analysis of flowering time genes of *Beta* species and the impact on life cycle regimes**

Designing experiments: 80 %

Results: 90 %

Writing the chapter: 80 %

Discussion and revision: 80 %

**Chapter 4: Functional analysis of *BvBBX19* after transformation into *Arabidopsis thaliana***

Designing experiments: 80 %

Results: 100 %

Writing the chapter: 100 %

Discussion and revision: 90 %

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### 13 Erklärung

Hiermit erkläre ich, dass ich die hier vorliegende Dissertation mit dem Titel: ‚The Evolution of Flowering Time Regulation in *Beta Species*‘ in Form und Inhalt selbstständig, mit der Unterstützung meines Betreuers, verfasst habe. Ich habe keine anderen als die angegebenen Hilfsmittel und Quellen verwendet und die Arbeit unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft erstellt. Diese Arbeit wurde an keiner anderen Stelle im Rahmen eines Prüfungsverfahrens vorgelegt, veröffentlicht oder zur Veröffentlichung eingereicht. Dies ist mein bisher erstes und einziges Promotionsverfahren.

Kiel, \_\_\_\_\_

(Datum)

\_\_\_\_\_

(Unterschrift Kandidat)

