

Aus dem Institut für Pflanzenbau und Pflanzenzüchtung
der Christian-Albrechts-Universität zu Kiel

**Map based cloning and functional analysis of two bolting time loci *BTC1*
and *BvBBX19* in sugar beet (*Beta vulgaris* L.)**

Dissertation
zur Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultät
der Christian-Albrechts-Universität zu Kiel

vorgelegt von

Dipl.-Bioinf. (FH) Nadine Dally

aus Hannover

Kiel, 2013

1. Berichterstatter: Prof. Dr. Christian Jung

2. Berichterstatter: Prof. Dr. Frank Kempken

Tag der mündlichen Prüfung: 04.November 2013

Zum Druck genehmigt: Kiel, den

gez. Prof. Dr. Wolfgang J. Duschl (Dekan)

Table of contents

Table of contents	I
List of Tables.....	IV
List of Figures	VI
List of Abbreviations.....	XI
1 General introduction.....	1
1.1 The sugar beet crop.....	1
1.2 The beet genome.....	1
1.3 General introduction into flowering time regulation.....	2
1.4 Aims and scientific hypotheses	5
2 Map based cloning of <i>BvBBX19</i> , a DOUBLE B-BOX TYPE ZINC FINGER gene from the <i>B2</i> locus on chromosome 9	6
2.1 Introduction	6
2.2 Material and methods	7
2.2.1 Plant material, growth conditions and phenotypic analysis	7
2.2.2 DNA techniques	9
2.2.3 RNA techniques	9
2.2.4 Bioinformatic analysis.....	10
2.2.5 Marker development and genetic mapping	10
2.2.6 Cloning of <i>BvBBX19</i> sequences	11
2.3 Results	11
2.3.1 Phenotypic analysis of bolting time and genetic mapping of the <i>B2</i> locus.....	11
2.3.2 Genetic mapping of the <i>B2</i> locus	14
2.3.3 Candidate gene identification.....	16
2.3.4 Gene structure analysis.....	16
2.3.5 <i>BvBBX19</i> sequence variations	17
2.3.6 Expression analysis of <i>BvBBX19</i> and its putative downstream targets.....	19
2.3.7 Expression of <i>BvBBX19</i> in <i>BTC1</i> -transgenic sugar beets	21

2.4	Discussion.....	22
2.5	References	26
3	Functional analysis of the <i>BOLTING TIME CONTROL 1 (BTC1)</i> gene I: transformation into sugar beet	31
3.1	Introduction	31
3.2	Material and methods	32
3.2.1	Plant material.....	32
3.2.2	Vector construction	35
3.2.3	<i>Agrobacterium</i> mediated transformation	36
3.2.4	Determination of transgene copy numbers.....	37
3.2.5	Growth conditions for primary transformants and phenotypic analysis	37
3.2.6	DNA techniques	40
3.2.7	RNA techniques	41
3.3	Results	42
3.3.1	Transformation and propagation of sugar beet transgenic T ₁ plants.....	42
3.3.2	Copy number determination.....	43
3.3.3	Phenotypic analysis of transgenic T ₁ plants.....	44
3.3.4	Expression analysis of <i>BTC1</i> and downstream targets.....	46
3.4	Discussion.....	49
3.5	References	50
4	Functional analysis of the <i>BOLTING TIME CONTROL 1 (BTC1)</i> gene II: transformation into <i>Arabidopsis thaliana</i>	52
4.1	Introduction	52
4.2	Material and methods	53
4.2.1	Plant material, growth conditions and phenotyping.....	53
4.2.2	PCR, RT-qPCR and DNA sequencing.....	54
4.2.3	Vector construction and <i>A. thaliana</i> transformation	55
4.2.4	Genotyping experiments	57
4.2.5	Expression analysis in transgenic T ₂ plants	57
4.3	Results	58

4.3.1	Molecular and phenotypic identification of T ₁ plants transformed with the <i>BTC1_d/btc1_a</i> genes from sugar beet and the <i>PRR7</i> gene from <i>A. thaliana</i>	58
4.3.2	Expression analysis of <i>BTC1_d</i> , <i>btc1_a</i> and <i>PRR7</i> transgenes in 44 T ₂ families ...	60
4.3.3	Flowering time measurements with transgenic plants	61
4.3.4	Production of homozygous T ₃ lines	63
4.4	Discussion.....	64
4.5	References	65
5	Closing discussion.....	68
6	Summary	71
7	Zusammenfassung.....	73
8	Appendix	75
8.1	Supplementary tables.....	75
8.2	Supplementary figures	129
9	References	135
10	Curriculum vitae and publications	139
10.1	Curriculum Vitae	139
10.2	Publications.....	140
10.2.1	Articles	140
10.2.2	Oral presentations.....	140
10.2.3	Posters	140
11	Declaration of own contribution	142
12	Acknowledgements	143
13	Erklärung.....	145

List of Tables

Table 1: <i>BTCL</i> haplotypes in annual and biennial <i>B. vulgaris</i> accessions. Plants of 106 beet accessions (84 sea beet and 22 cultivated beet accessions) were grown in the greenhouse at 18°C under LD conditions (22h light/ 2h dark) and the onset of bolting was recorded. The coding region of <i>BTCL</i> was sequenced and SNP positions are given relative to the translation start site (for exon 3) or the 5' end of a given exon, respectively. Eleven haplotypes (“a” to “k”) were identified and haplotype “a” and “d” were referred as reference haplotypes for the biennial or annual allele, respectively. Non-synonymous SNPs that differ between these alleles are indicated with asterisks (from Pin et al. 2012, modified).	4
Table 2: Experimental plan for growing and phenotyping conditions of plants and populations conducted during the course of this study.	8
Table 3: Seed materials used in this study.....	9
Table 4: Primer and amplification conditions for PCR, RT-PCR and RT-qPCR performed in this study.	10
Table 5: Non-anonymous and developed sequence based (RefBeet-0.9) molecular markers on chromosome 9 for analysis of co-segregation with bolting phenotypes in the F ₂ -population ₀₅₆₈₂₂	11
Table 6: Phenotypic segregation for annual (early bolting, late bolting with and without flowering) and biennial individuals in the F ₂ -population ₀₅₆₈₂₂ . Plants were phenotyped for bolting (BBCH stage 51) and flowering (BBCH stage 60) under field conditions from May 2010 until October 2010 (Chapter 2.2.1).	12
Table 7: Phenotyping of F ₃ families, derived from annual F ₂ plants. Eight plants/ F ₃ family were sown in soil and grown under natural light conditions in pots outside the greenhouse at Kiel. Annual: bolting until October 19, 2011; Biennial: non-bolting until October 19, 2011. 13	13
Table 8: Co-segregation analysis of annual and biennial individuals and <i>B2</i> locus markers. Analysis was performed with 1301 F ₂ plants, which were determined as being homozygous early bolting annual or biennial according to F ₃ phenotyping (Chapter 2.3.1). Genotyping was performed with molecular markers (Table 5, Supplementary Table 2, Supplementary Figure 3). M ₁ and M ₂ , marker alleles derived from the annual parent or the biennial mutant parent, respectively.....	14
Table 9: Plant material produced and analyzed during this study.	33
Table 10: Experimental plan for cold treatment and phenotyping procedures of transgenic T ₁ plants. All clones/ transformant were first grown for 7 weeks in a greenhouse (18h light, 22°C). Then 3 experiments were performed with different periods of cold treatment (CTP) (Figure 14, Figure 15).	40
Table 11: Primer and amplification conditions for all PCRs performed in this study.	41
Table 12: Generated shoots (transformants) on selectable medium supplemented with kanamycin after sugar beet transformation with two <i>A. tumefaciens</i> strains (AGL1 and GV3101 PMP90RK) carrying the <i>35S:BTCL_d</i> or <i>35S:btcl_a</i> transgenic cassettes and regeneration of clones/ transformant. Each generated shoot represents an independent transformant (T ₁ generation, seed code 120248 - 120308). Shoots were regenerated by micropropagation to yield 20 clones/ transformant. After induction of root yield all obtained	

T₁ plants were transferred to the greenhouse (KWS Saatzucht GmbH, Einbeck, Germany) and grown for 7 weeks under 18h light at 22°C and phenotyped for bolting behavior. 43

Table 13: Transgene copy number of T₁ plants carrying the *35S:BTC1_d* and *35S:btc1_a* transgene cassette measured by qPCR. Two clones/ transformant were analyzed as biological replicates. A single copy hemizygous transgenic plant and a non-transgenic plant were used as positive and negative control, respectively. The mean of the C_T values was compared to those of the controls and converted into a CNI value (Chapter 3.2.4). 43

Table 14: Summary bolting phenotypes and transgene copy number (Chapter 3.3.3) in transgenic beets. 46

Table 15: Genes and primer sequences used for PCR and RT-qPCR experiments 54

Table 16: Vector constructs for Arabidopsis floral dip transformation experiments into the ecotype Col-0 and the mutant *prp7-11*. *BTC1_d*, is the allele from the annual sugar beet line 930190, whereas *btc1_a* stems from the biennial line 940043. 56

Table 17: Results from the *A. tumefaciens* transformation experiments. T₁ seeds were harvested from the ecotype Col-0 and the mutant *prp7-11* after transformation. Transgenic T₁ plants had been transformed with three different constructs. Plants were grown in the greenhouse (16h light/ 8h dark, 22°C, 900μE) and BASTA® treated. Positive T₁ plants carrying the *35S:BTC1_d* or *35S:btc1_a* transgene cassette displayed an 1842 bp fragment whereas positive T₁ plants carrying the *35S:PRR7* transgene cassette showed a 321 bp fragment after PCR. The transformation efficiency was calculated as the number of transgenic plants divided by the total number of plants. T₁ plants were propagated and segregating T₂ families were analyzed. 59

Table 18: Transgene copy number determination in 73 T₂ families over all transformation experiments. A χ^2 -test ($\alpha_{0.05} = 3.84$) was performed to proof whether the observed segregation ratio fits to a 3:1 ratio, expected for inheritance of a monogenic trait. 59

Table 19: Genotyping of T₂ families that were phenotyped. 35 plants/ T₂ family were grown in soil in the climate chamber with 16h light at 22°C. Each plant was genotyped by PCR with DNA extracted from leaves. For genotyping T₂ families, derived from transformation of *35S:PRR7* into Col-0 or *prp7-11*, I used the primers C0294/ C083, whereas primers C017/ A747 were used for genotyping T₂ families, derived from transformation of *35S:BTC1_d* or *35S:btc1_a* into Col-0 or *prp7-11*. The observed segregation ratios (transgenic: non-transgenic) were tested by χ^2 analysis ($\alpha_{0.05} = 3.84$) for a 3:1 ratio, expected for inheritance of single transgene locus. 61

Table 20: Homozygous T₃ lines fixed for the transgenes *BTC1_d/btc1_a* or *PRR7*. T₃ seeds were obtained from single transgenic T₂ plants. From each T3 family, ~60 seedlings were BASTA® sprayed. 64

List of Figures

Figure 1: Proposed model for *BTC1* as a central regulator of life cycle adaptation and induction of flowering in beets. Lines between genes do not imply direct interactions. Weak regulatory effects are indicated by gray lines (from Pin et al. 2012). 4

Figure 2: Phenotypes of F₂ plants from F₂-population₀₅₆₈₂₂. The F₂-population₀₅₆₈₂₂ was phenotyped under non-vernalizing conditions in the field for bolting behavior from May until October 2010. Early bolting: bolting until June 24 followed by flowering; Late bolting: bolting between June 26, and October 25, 2010 and flowering; Late bolting without flowering individuals: bolting between June 26, and October 25, 2010; Biennial: non-bolting until October 25, 2010. 12

Figure 3: Genotypes of the marker locus CAU3784 (A) in homozygous biennial and annual F₂ plants (B). Annual and biennial F₂ plants were determined as being homozygous according to F₃ phenotyping. Genotypes were determined by PCR using the molecular marker CAU3784. The CAU3784 marker allele M₁ derived from the annual parent, whereas the CAU3784 marker allele M₂ derived from the biennial mutant parent. Black arrows indicate the respective PCR fragment size..... 14

Figure 4: Map-based cloning of the *B2* locus in beet. (A) Genetic map position of the *B2* locus on chromosome 9. The map was calculated using 379 annual and 922 biennial F₂ plants of the F₂-population₀₅₆₈₂₂ (JoinMap4.0, LOD threshold value: 5.0). Sequence information for the molecular markers (right) were derived from publically available ESTs (indicated in brackets) or were derived from the sugar beet reference genome sequence (RefBeet-0.9, RefBeet-0.3, **Chapter 2.3.3**). The numbers on the left site display genetic distances in centiMorgan (cM). (B) Physical map of the *B2* locus and the location of the two scaffolds sc00497 and sc00048, covering the *B2* locus. Marker locations are given in grey circles. The number of recombination events between two markers flanking *B2* is given in black triangles (see also **Supplementary Figure 3**). Black boxes with the tip indicate identified gene models (RefBeet-0.3_GeneModels) and their orientated location within the critical region of the scaffold sc00048 (**Chapter 2.3.3**). Locations of molecular markers within are indicated with colored boxes. The two markers CAU3782 and CAU3783 flank the *B2* locus in a genetic distance of 0.276 Mbp. (C) Molecular markers CAU3782, CAU3783 and CAU3784 used for mapping the *B2* locus. The fragment size (bp) of the PCR products is given for each marker genotype with M1, allele derived from the annual parent and M2, allele derived from the biennial mutant parent. Red arrows: primers used for PCR amplification; black vertical arrows: polymorphism site and marker type; InDel: Insertion/ Deletion marker and respective PCR fragment sizes; SNP (single nucleotide polymorphism) with the respective nucleotides for each genotype (G = guanine, C = cytosine, T = thymine). 15

Figure 5: In silico prediction of the *BvBBX19* gene structure based on cDNA and gDNA analysis. Prediction was performed with the gene model g25128.t1 (RefBeet3.0_GeneModels) and the genomic sequence of *BvBBX19* located on scaffold sc00048 (RefBeet0.9). Grey bar: partial cDNA sequence from the gene model g25128.t1 and partial gDNA sequence of scaffold00048, interrupted sequence regions are indicated with a double slash; Dashed grey boxes represent exons; Arrows: transcription and translation starts and ends and respective positions according to the cDNA sequence; Blue bars: locations of sequences with homology to B-box domains (BB1, BB2); Red bars: 3'-UTR and 5'-UTR; Black bars: exonic region belonging to the translated region, respective sizes of the region are given below; Green lines: intronic region, respective size is given above. 17

Figure 6: Sequence variations within *BvBBX19* alleles from the two EMS mutants (seed code 056822 and 031823) and the non-mutated donor line (seed code 930190). A part of the

sequence encompassing the polymorphic region between exon 2 and exon 3 is shown. Grey arrows/ boxes: exons; Green arrows/ lines: intronic region; Black lines: coding region flanked by translation start (ATG) and end (TGA); Red lines: non-coding regions, 3'-UTR and 5'-UTR; SNP positions are indicated as blue boxes and triangles, black triangle: wild type nucleotide derived from the non-mutated donor line (seed code 930190). The respective nucleotide is given above the triangles (A=adenine, G=guanine, C=cytosine, T=thymine); Black asterisk: premature STOP-codon. 18

Figure 7: Multiple alignment of BvBBX19 protein sequences from the EMS mutants (seed code 056822 and 031823), from the non-mutated donor line (seed code 930190) and from *A. thaliana* (BBX19). BvBBX19 cDNA sequences were translated into amino-acid (aa) sequences. The AtBBX19 sequence (TAIR database, At4g38960.1) shares 55% sequence similarity with BvBBX19₉₃₀₁₉₀. Grey bars: B-Box domains (BB1, BB2); blue symbols: predicted highly conserved aa positions within BB2 (Conserved Domains Database, NCBI); aa present in all sequences are highlighted in black; aa present in most aligned sequences are highlighted in grey; aa changes due to EMS mutations are highlighted in red; asterisks: translation termination (stop) codon. 19

Figure 8: Expression analysis of *BvBBX19* in the biennial sugar beet line (seed code 120309) at growth stage BBCH: 61. Plants were grown in a greenhouse after 12 weeks of vernalization. The yellow background indicates day and the grey background indicates night. Each value is the mean of two biological replicates and three technical replicates. Relative gene expression was calculated applying the ΔC_T method and normalized to the geometric mean of *BvGAPDH*. Error bars represent the standard deviation of the mean (\pm SDM) of the two biological replicates. 20

Figure 9: Expression analysis of *BvBBX19*, *BTC1*, *BvFT1*, and *BvFT2* in the annual parent accession 991971 (grey boxes) and the EMS mutant parent (seed code 056822) before and after vernalization. Plants were grown in the greenhouse under LD conditions. Each column is the mean of three biological and three technical replicates. The vertical axis displays the relative expression of the target gene. Relative gene expression was calculated applying the comparative CT (ΔC_T) method and normalized to the geometric mean of the housekeeping gene *BvGAPDH*. Error bars represent the standard deviation of the mean (\pm SDM) of the three biological replicates. 'a', statistically significant different gene expression between the annual parent (991971) and the non-vernalized mutant parent (056822); 'b' statistically significant different gene expression between the vernalized and non-vernalized mutant parent (056822). 21

Figure 10: *BvBBX19* expression in transgenic beets overexpressing the *BTC1_d* (black lines) or *btc1_a* (red lines) allele under the control of the 35S promoter. All plants were cold treated for 12 weeks at 4°C and then grown under LD in the greenhouse. *BvBBX19* expressions of bolting and never bolting beets after vernalization are shown as solid or dashed lines, respectively. Each value is the mean of two biological and three technical replicates. The ΔC_T method was applied to calculate the relative *BvBBX19* expression. For normalization the housekeeping gene *BvGAPDH* was used. Error bars: standard deviation of the mean (\pm SDM) of two biological replicates. 22

Figure 11: A proposed epistatic model for bolting time control in beet with *BvBBX19* and *BTC1_d* acting upstream of *BvFT1* and *BvFT2*. The domain structure of BvBBX19 and BTC1_d proteins is given. Functional or mutated domains are indicated with filled shaded or open boxes, respectively. An interaction between BvBBX19 and BTC1_d proteins to acquire a CO function was speculated. In annuals, the proteins BvBBX19 and BTC1_d interact to repress *BvFT1* and activate *BvFT2* to promote bolting and flowering under LD conditions. In non-

vernalized *B2*-mutants (seed code 056822) an interaction between *BTC1_d* and *BvBBX19* containing an altered BB2 domain, is impaired. Repression of *BvFT1* is not achieved and consequently *BvFT2* is repressed by *BvFT1* and plants grow vegetatively before vernalization. After vernalization and under LD *BvFT1* is not repressed in *B2*-mutants but the floral integrator *BvFT2* is highly activated to initiate bolting and flowering after vernalization. Direct interactions between genes are indicated with red lines; red dotted lines: inhibited direct interaction. Black lines between genes do not imply a direct interaction; dotted black lines: weak regulatory effect. 26

Figure 12: T-DNA region of the transformation vectors A) CAU3713 carrying the *BTC1_d* CDS and B) CAU3714 carrying the *btc1_a* CDS. Arrows in light green represent promoter regions with pNOS: promoter derived from the *nopaline synthase* gene and p35S: cauliflower mosaic virus (CaMV) 35S promoter. The blue arrow represents the selectable marker gene *neomycin phosphotransferase II* gene (*nptII*) for selection of transgenic shoots (see Chapter 3.2.3). In yellow, terminator regions with T35S: 35S terminator derived from CaMV and T-OCS: terminator derived from the *octopine synthase* gene. Red arrow: coding sequence of *BTC1_d* derived from the annual sugar beet line 930190 (A); dark green arrow: coding sequence of *btc1_a* derived from the biennial line 940043 (B). *XmaI* and *SalI*, restriction sites used for cloning the respective *BTC1* CDS. 36

Figure 13: Example for micropropagation of transformant 120301/1 to yield the 20 clones 120301/1-120301/20. After *Agrobacterium* mediated transformation, the transformant 120301/1 was grown on selectable medium supplemented with kanamycin and micropropagated through adventitious shoot regeneration to yield the 20 clones 120301/1 – 120301/20. For induction of root growth plants were grown on selectable medium supplemented with vitamins and then rooted plants were transferred in soil. 37

Figure 14: Workflow for cold treatment experiments of sugar beet transformants. 61 transformants with 20 clones/ transformant were obtained after *A. tumefaciens* mediated transformation and micro-propagation. 20 clones/ transformant were splitted and 3 experiments were performed for different periods of cold treatment (CTP = cold treatment period) (Table 10). 38

Figure 15: Phenotyping and seed production of transformants after different cold treatment periods. Plants of experiment 1 (green box), 2 (yellow box) and 3 (light blue box) were grown on soil and cold treated for 4, 8 and 12 weeks und SD conditions (8h light/ 16h dark, 4°C), respectively (Figure 14, Table 10), and then transferred to the greenhouse for phenotyping (Table 10). The respective conditions are given in grey boxes on the right site. The number of clones/ transformant in experiment 1 and 2 are given in green and yellow boxes, respectively. Phenotyping and seed production for plants of experiment 3 are displayed in the blue box... 39

Figure 16: *In vitro* micropropagation and induction of root growth of a sugar beet transformant. Pictures were taken by Dr. Joseph Kraus, KWS Saatzucht GmbH, Einbeck. Picture A shows the transformant 120301/1 growing on selectable medium after transformation. B shows the clones 120301/2-120301/10 obtained after micropropagation through adventitious shoot regeneration. Therefore shoots were grown on selectable medium supplemented with kanamycin. In picture C root formations of the clones 120301/1 and 120301/2 are shown, therefore shoots were grown on selectable medium supplemented with vitamins. In this stage the clones were transferred on soil. 42

Figure 17 : Bolting and never bolting transgenic T₁ plants after 12 weeks grown in the cold chamber with 8h light at 4°C. The seed codes are shown for each plant. A) 120301/1, bolting after vernalization. B) 120305/1, never bolting after cold treatment. C) 120255/1, never bolting after cold treatment. D) 120268/1, bolting after vernalization. 120301/1 and 120305/1

are clones transformed with *BTC1_d* whereas 120268/1 and 120255/1 are clones transformed with *btc1_a*..... 45

Figure 18: Phenotypic variation of transformants for bolting and never bolting after 2nd cold treatment for 34 weeks at 4°C. Observed phenotypes of A) *BTC1_d* transformed clone 120305/1 and B) *btc1_a* transformed clones 120255/1, 120255/2 and 120255/6. 45

Figure 19: RT-qPCR expression analysis of all *BTC1* genes, transgenic (*BTC1_{transgene}*) and native ones (*BTC1_{native}*) from sugar beet in 4 independent transformants. Two clones/ transformant were analyzed with primers B580/ B581 to specifically amplify a 305 bp fragment of the coding sequence (exon 6 – exon7) of the *BTC1* genes. Each value is the mean of two biological (clones) and three technical replicates. Analyzed clones of transformants 120301 (*35S:BTC1_d*) and 120268 (*35S:btc1_a*) carry 1 transgene copy and bolted after vernalization, whereas clones of transformant 120305 (*35S:BTC1_d*) and 120255 (*35S:btc1_a*) carry >1 transgene copy and were never bolting. As a control the biennial donor line was used (120309). The ΔC_T method was applied to calculate the relative *BTC1* expression. For normalization the housekeeping gene *BvGAPDH* was used. Expression of *BTC1* before vernalization (grey boxes) and after vernalization (black boxes). Error bars: standard deviation of the mean (\pm SDM) of two biological replicates. ZT: zeitgeber time; n.d. not determined. 47

Figure 20: Diurnal Expression analysis of (A) *BTC1_{transgene+native}* expression and (B) *BvFTI* expression in T₁ transformants with bolting and never bolting phenotypes after twelve weeks of cold treatment. Two clones/ transformant were analyzed. The biennial donor line 120309 was used as a control. Plants were grown in a greenhouse at 22°C. Error bars represent the standard deviation of the mean (\pm SDM) of the two biological replicates. The grey background indicates the dark phase. 48

Figure 21: T-DNA region of the transformation vectors CAU3715, CAU3716 and CAU3718 carrying the *BTC1_d*, *btc1_a* and *PRR7* CDS, respectively. Arrows in light green represent promoter regions with p35S: cauliflower mosaic virus (CaMV) 35S promoter. The grey arrow represents the selectable marker *phosphinothricin acetyltransferase (pat)* for selection of transgenic plants (see Chapter 4.2.1). Terminator regions with T35S: 35S terminator derived from CaMV and T-OCS: terminator derived from the *octopine synthase* gene are marked in yellow. Red arrow: coding sequence of *BTC1_d* of the annual sugar beet line 930190; dark green arrow: coding sequence of *btc1_a* of the biennial line 940043; blue arrow: coding sequence of *PRR7* from *A. thaliana*. *Sfi*I restriction sites were used for cloning the respective transgene cassette. 55

Figure 22: Workflow of *A. thaliana* transformations, T₁ and T₂ selection and phenotyping T₂ families. Generation of vectors and the plant transformations are described under Chapter 4.2.1. Selection of T₁ plants and T₂ families as well as the growth conditions of plants and phenotyping procedures are described under Chapter 4.2.1. 56

Figure 23: Transgene genotyping of T₁ plants after transformation of *35S:BTC1_d*, *35S:btc1_a* and *35S:PRR7* into Col-0. DNA from BASTA® resistant T₁ plants transformed with *35S:BTC1_d* (1-4) or *35S:btc1_a* (5-8) was amplified by PCR using the primers C017/ A747. DNA from T₁ plants which were transformed with *35S:PRR7* (9-16) was amplified by PCR using the primers C294/ C083. PCR fragments were separated in a 2% agarose gel. Arrows indicate respective fragment lengths of the PCR products. Non transgenic T₁ plants are marked by asterisks. 58

Figure 24: Gene expression analysis in T₂ plants with single copy insertions of *BTC1_d*, *btc1_a* and *PRR7* transgenes in a Col-0 (A) and *prp7-11* background (B) compared to the non-transformed controls Col-0 and *prp7-11*. The relative expression of *BTC1_d* (red boxes), *btc1_a* (green boxes) and *PRR7* (blue boxes) was measured by RT-qPCR as described in Chapter 4.2.5 and normalized against the housekeeping gene *AtGAPDH* applying the ΔC_T method. From each family, 5 plants were pooled and analyzed. Error bars represent the standard error of the mean of three technical replicates from each analyzed sample. 60

Figure 25: Growth experiments with T₂ families with Col-0 or *prp7-11* background. Plants were grown under 16h of light in a climate chamber. T₂ families with Col-0 background were grown at 22°C, whereas T₂ families with *prp7-11* background were grown at 18°C. Boxes with dashed lines represent 7-13 non-transgenic plants whereas boxes without dashed lines represent 20-27 transgenic T₂ plants from each T₂ family (Supplementary Table 9, Supplementary Table 10). Red: transgenic plants of T₂ families transformed with *35S:BTC1_d*; green: transgenic plants of T₂ families transformed with *35S:btc1_a*; blue: transgenic plants of T₂ families transformed with *35S:PRR7*. Black: non-transformed control Col-0; grey: non-transformed *prp7-11* mutant. Error bars represent the standard error of the mean of phenotyped transgenic or non-transgenic plants/ T₂ family. DTB: days to bolt after sowing (growth stage 5.10). TNRL: total number of rosette leaves at growth stage 5.10. DTF: days to flower after sowing (growth stage 6.10). TNCL: total number of cauline leaves at growth stage 6.10. Numbers above the boxes: mean of phenotyped character. A, significant differences confirmed by Student's *t*-Test at $\alpha=0.05$ between transgenic and non-transgenic T₂ plants of the same family. B, significant differences confirmed by Student's *t*-Test at $\alpha=0.05$ between the control Col-0 and transgenic T₂ plants with Col-0 genetic background or the control *prp7-11* and transgenic T₂ plants with *prp7-11* genetic background. 62

List of Abbreviations

°C	Degree Celsius
μE	Microeinstein
μl	Microliter
μM	Micromolar
A	Adenine
<i>A. tumefaciens</i>	<i>Agrobacterium tumefaciens</i>
aa	Amino acids
AP1	<i>APETALA 1</i>
<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>
BAC	Bacterial artificial chromosome
BB1	B-box domain 1
BB2	B-box domain 2
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
blastn	Standard nucleotide BLAST
blastp	Standard protein BLAST
blastx	Translated BLAST
bp	Base pairs
<i>BTC1</i>	<i>BOLTING TIME CONTROL 1</i>
<i>Bv</i>	<i>Beta vulgaris</i>
bZIP	Basic leucine zipper
C	Cytosine
CaMV	Cauliflower Mosaic Virus
CAPS	Cleaved amplified polymorphic sequence
<i>CCA1</i>	<i>CICADIAN CLOCK ASSOCIATED 1</i>
CCT	CONTANS, CONSTANS-LIKE, TOC1
cDNA	Complementary DNA
CDS	Coding sequence
cM	CentiMorgan
CNI	Copy number indication
CNV	Copy number variation
<i>CO</i>	<i>CONSTANS</i>
<i>COL</i>	<i>CONSTANS-LIKE</i>
Col-0	<i>A. thaliana</i> ecotype Columbia
<i>COP1</i>	<i>CONSTITUTIVE PHOTOMORPHOGENIC 1</i>
CT	Cycling threshold
CTAB	Cetyltrimethylammoniumbromid
CTP	Cold treatment period
DNA	Deoxyribonucleic acid
DTB	Days to bolting

DTF	Days to flower after sowing
<i>E. coli</i>	<i>Escherichia coli</i>
<i>ELF3</i>	<i>EARLY FLOWERING 3</i>
<i>ELF4</i>	<i>EARLY FLOWERING 4</i>
EMS	Ethyl methanesulfonate
EST	Expressed sequence tag
et al.	<i>et alii</i>
F ₁	First generation after cross
F ₂	Second generation after cross
F ₃	Third generation after cross
FISH	Fluorescence <i>in situ</i> hybridization
<i>FL1</i>	<i>FLC-LIKE 1</i>
<i>FLC</i>	<i>FLOWERING LOCUS C</i>
<i>FLD</i>	<i>FLOWERING LOCUS D</i>
<i>FLK</i>	<i>FLOWERING LOCUS K</i>
<i>FT</i>	<i>FLOWERING LOCUS T</i>
<i>FVE</i>	<i>FLOWERING LOCUS VE</i>
G	Guanine
g	Gram
<i>Ga2ox</i>	<i>GIBBERELLIN 2-OXIDASE</i>
<i>GAPDH</i>	<i>GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE</i>
gDNA	Genomic DNA
<i>GI</i>	<i>GIGANTEA</i>
GI	Gene Index
h	Hour
ha	Hectare
<i>Hd1</i>	<i>HEADING DATE 1</i>
<i>Hv</i>	<i>Hordeum vulgare</i>
<i>HY5</i>	<i>LONG HYPOCOTYL 5</i>
i.a.	Inter alia
kbp	Kilo base pairs = 1000 bp
l	liter
LD	Long day
<i>LD</i>	<i>LUMINIDEPENDENS</i>
<i>LDL1</i>	<i>LYSINE-SPECIFIC HISTONE DEMETHYLASE1 LIKE 1</i>
<i>LHY</i>	<i>LATE ELONGATED HYPOCOTYL</i>
M ₁	First generation after mutagenesis
M ₂	Second generation after mutagenesis
M ₃	Third generation after mutagenesis
Mbp	Mega base pairs = 1,000,000 bp
mg	Milligram
mRNA	Messenger ribonucleic acid
NCBI	National Center for Biotechnology Information
ng	Nanogram
<i>nptII</i>	<i>Neomycin phosphotransferase II</i>
ORF	Open reading frame
<i>Os</i>	<i>Oryza sativa</i>
<i>pat</i>	<i>Phosphinothricin acetyltransferase</i>
PCR	Polymerase chain reaction

pM	Picomolar
<i>PPD</i>	<i>PHOTOPERIOD INSENSITIVE</i>
<i>PRR</i>	<i>PSEUDO RESPONSE REGULATOR</i>
REC	Response regulator receiver
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
RT-PCR	Reverse transcription polymerase chain reaction
RT-qPCR	Quantitative real-time polymerase chain reaction
<i>Sb</i>	<i>Sorghum bicolor</i>
SD	Short day
SDM	Standard deviation of the mean
siRNA	Small interfering RNA
SNP	Single nucleotide polymorphism
SORLIP	Sequences over-represented in light-induced promoters
subsp.	Subspecies
T	Thymine
t	Ton
T ₁	First generation after transformation
T ₂	Second generation after transformation
T ₃	Third generation after transformation
T35S	Terminator derived from CaMV
TAIR	The Arabidopsis Information Ressource
T-DNA	Transfer DNA
TF	Transcription factors
TNCL	Total number of cauline leaves
TNRL	Total number of rosette leaves
<i>TOC1</i>	<i>TIMING OF CAB EXPRESSION 1</i>
T-OCS	Terminator derived from the octopin synthase gene
TSS	Transcription start site
UTR	Untranslated region
YAC	Yeast artificial chromosome
Zn	Zinc
ZT	Zeitgeber time
χ^2	Chi square

1 General introduction

1.1 The sugar beet crop

Cultivated beets (*Beta vulgaris* subsp. *vulgaris*) are dicotyledonous plants which belong to the order Caryophyllales (family: Amaranthaceae, subfamily: Chenopodiaceae, genus: *Beta*). There are four agronomically important groups: fodder beet (*B. vulgaris* subsp. *vulgaris* Fodder Beet Group), leaf beet (*B. vulgaris* subsp. *vulgaris* Leaf Beet Group), garden beet (*B. vulgaris* subsp. *vulgaris* Garden Beet Group) and sugar beet (*B. vulgaris* subsp. *vulgaris* Sugar Beet Group) (Lothar Freese, pers. communication; USDA/ARS 2013). Sugar beets show a biennial life cycle. In the first year, plants grow vegetatively forming a leaf rosette and a large storage root. Its sucrose content ranges between 15 and 18% (total dry weight) (Märländer et al., 2011). After winter, plants start stem elongation (bolting) and flowering under long day (LD) conditions. Under artificial conditions, beets need a minimum of 10-14 weeks of cold temperatures (4°C) to start bolting (Boudry et al., 2002). Thus, these have an obligate requirement for vernalization to enter the reproductive phase. Because bolting drastically reduces root yield, sugar beets are cultivated as a spring crop which is sown after winter and harvested next autumn.

The history of European sugar began in 1747 with a remarkable discovery by the German chemist A. S. Marggraf. He demonstrated that the sweet-tasting crystals obtained from beet juice were the same as those from sugarcane. His scholar F. C. Achard reconsidered these findings and finally built up the first sugar factory at Cunern in Lower Silesia in 1801 (Biancardi et al., 2005). In the early 19th century, sugarcane was still the main source of sugar, but sugar beet as an alternative for sugar production in Europe became more important. In 1811, during the Napoleonic war, it was decided to substitute imported cane sugar with beet sugar (Cooke, 1993). During the past 200 years, the sugar beet industry became firmly established world-wide (Draycott, 2006). About 40% of the world's sugar production is from beet and 60% from cane (Asadi, 2007). In Europe, sugar beet is the only crop harvested for sugar production. In Germany, beets were cultivated on 398,000 ha yielding on average 74t/ha in the year 2011 (Statistisches Bundesamt, 2013). Besides sugar production, beets are cultivated for the production of bioethanol and biogas (~30,000 ha, 2011) (Kleffmann, 2011).

The major breeding aim is to increase the amount of extractable sugar, for which a strong selection against premature bolting is crucial. Beet growth is also affected by environmental factors such as fungal pathogens, nematodes, viruses and insects. Therefore, resistance breeding is of great importance (Biancardi et al., 2010; Dohm et al., 2012).

1.2 The beet genome

Sugar beet is a diploid species ($2n = 18$) with a relatively small genome which was estimated to be 758 Mbp in size (Arumuganathan and Earle, 1991). The genome contains at least 63% repetitive sequences (Flavell et al., 1974) consisting of numerous different satellite DNA families and retrotransposons (Falk Zakrzewsky et al., 2013). There is evidence for about 28,000 genes in the sugar beet genome (Weißhaar et al., 2011). The annotation of the *B. vulgaris* genome from the doubled haploid line KWS2320 is in progress and a draft assembly (RefBeet 0.9) is available for download at http://bvseq.molgen.mpg.de/Genome/start_genome.shtml. This sequence has a size of 590 Mbp ($N50 = 759$ kbp). After assembly, 82,305 sequences were obtained. Among these 1964 sequences were scaffolds with a size that ranged from ~3 kbp to ~5 Mbp. The remaining sequences which were not assembled to scaffolds are contigs ($> = 500$ bp) (The *Beta vulgaris* Ressource, 2013). Recently, a reference FISH karyotype was published that enables the

localization of markers and provides a resource for anchoring scaffolds and contigs of the beet genome sequence (Paesold et al., 2012). There are several large insert libraries (bacterial artificial chromosome vectors, BAC) for sugar beet with a genome coverage varying from 5.6 to 8.8 (Gindullis et al., 2001; Hohmann et al., 2003; Jacobs et al., 2009; McGrath et al., 2004). Moreover, 56582 BAC end sequence data are available from the US H20 clones (McGrath et al., 2004; Zakrzewski et al., 2010). One fosmid library of the doubled haploid line KWS2320 with a genome coverage of 5.6, was established by Lange et al. (2008). In total 30,313 *B. vulgaris* expressed sequence tags (ESTs) (June 2013) are publically available (NCBI dbEST, <http://www.ncbi.nlm.nih.gov>). A collection of putative genes and their functional roles had been established during the Gene Index (GI) Project (<http://compbio.dfci.harvard.edu/tgi/>) and the *B. vulgaris* GI (BvGI) includes 17,186 unique entries (June 2013).

Several genetic maps have been constructed for beet. One dense map was published by Schneider et al. (2007). This map comprises 524 loci covering 664.3 cM and integrates three different maps from three mapping populations (K1, K2 and D1). Recently, Dohm et al. (2012) presented the latest high resolution and most dense map based on the K1 mapping population. The map has a total length of 866 cM and comprises 983 SNP markers derived from 283 EST- and 700 BAC-end sequences. In parallel, the authors hybridized gene-derived oligomer probes against BAC libraries to construct a genome-wide and gene-based physical map. In total, 8361 probes and 22,815 BAC clones were integrated and the final map encompasses 535 chromosomally anchored contigs which are publically available (<http://bvseq.molgen.mpg.de/PhysMap/start.physmap.shtml>).

The availability of high density genetic and physical maps and large collections of beet sequences provides a resource for map-based cloning and sequence based identification of genes. In the recent years, next generation sequencing techniques were applied for sequencing the beet transcriptome by ribonucleic acid-sequencing (RNA-Seq), which enables a rapid gene discovery for traits of interest (Mutasa-Göttgens et al., 2012). As an example, RNA-Seq was successfully applied for the identification of the *R* locus gene *CYP76AD1* which encodes a novel cytochrome P450 required for red betalain production (Hatlestad et al., 2012).

1.3 General introduction into flowering time regulation

In plants, the switch from the vegetative to the reproductive phase is of high importance for their reproductive success. This junction is controlled by endogenous and exogenous signals that act together like a network to promote or inhibit the transition to flowering. To ensure seed production, plants developed different strategies during the evolution as adaptation to diverse habitats on Earth. The flowering time regulation in the model species *Arabidopsis thaliana* is well studied and provides an appropriated basis for research of flowering time regulation in other species.

In *A. thaliana*, flowering is regulated by four main pathways: the vernalization, photoperiodic, autonomous and gibberellic acid (GA) pathway (Andrés and Coupland, 2012). Floral induction signals from these pathways are transmitted to two central flowering regulator genes *CONSTANS* (*CO*) and *FLOWERING LOCUS C* (*FLC*). Their proteins antagonistically regulate flowering through regulation of floral integrator genes including *FLOWERING LOCUS T* (*FT*) (Lee and Lee, 2010). *FT* encodes a protein that is transported from the leaves to the shoot apex where it interacts with a basic leucine zipper (bZIP) transcription factor encoded by *FLOWERING LOCUS D* (*FLD*) to trigger flowering through direct activation of the floral meristem identity gene *APETALA 1* (*API*). *FT* expression is negatively regulated by the MADS-box transcription factor encoded by *FLC*, a key gene in the vernalization pathway that is downregulated during vernalization. In contrast, *CO* positively regulates *FT* expression in response to LD (Lee and Lee, 2010). *CO* encodes a zinc finger transcription factor containing two B-box motifs and a CCT (CONSTANS, CONSTANS-LIKE, TOC1) domain and directly binds to the *FT* promoter (Tiwari et al., 2010) (for more details see **Chapter 2.1**).

For induction of *FT*, *CO* needs to be expressed at a similar time of the day. The precise timing of its daily transcription is realized through its regulation by light and the circadian clock. The peak of *CO* expression occurs 12-16 hours after dawn (Andrés and Coupland, 2012). Under short day conditions (for example in winter), the peak occurs at night and the CO protein is degraded. By contrast under long day conditions (for example in summer), the peak coincides with daylight and the CO protein is stabilized and initiates transcription of *FT* (Kinmonth-Schultz et al., 2013). This CO/FT module is a key mechanism for the regulation of flowering in response to changes in day length to ensure plant reproduction during favorable times of the year. Under non-inductive short day (SD) conditions, gibberellins and microRNAs had been identified as playing a crucial role in flowering. Gibberellins are involved in the floral transition through activation of *FT* transcription and other floral integrators (Mutasa-Göttgens and Hedden, 2009). The microRNAs miR156 and miRNA172 were identified as floral integrators or repressors, respectively, that act downstream of the *FT* gene (Eldem et al., 2013).

Species of the genus *Beta* evolved different life cycles to ensure their reproduction. Annual beets, like sea beets (*B. vulgaris* L. subsp. *maritima*) from Mediterranean areas, bolt and flower rapidly under LD conditions within the first year. By contrast, sea beets growing in northern latitudes are biennials and need to be vernalized over winter to start bolting and flowering in the second year under inductive photoperiods to finish their life cycle. Moreover, perennial beets typically flower for several years (Hautekeete et al., 2002). The tendency for bolting without vernalization is under the control of a major dominant Mendelian factor *B*, recently identified as the pseudo-response regulator (PRR) gene *BTC1*, formerly referred to as *BvBTC1* (Pin et al., 2012). Plants carrying the dominant *BTC1* allele exhibit an annual growth habit, whereas biennial plants harbor the recessive *btc1* allele. *BTC1* shares homology with the *A. thaliana* circadian clock gene *PRR7* which encodes for a protein that contains a response regulator receiver (REC) and a CCT domain. Sequencing the coding region of *BTC1* in 84 sea beet and 22 cultivated beet accessions revealed the presence of 11 haplotypes, indicated with letters “a” through “k” (**Table 1**). Among these, three different haplotypes had been identified for biennials (“a-c”) and 8 for annuals (“d-k”). The haplotypes “a” and “d” were derived from biennial and annual reference accessions. All cultivated (biennial) beet accessions tested were found to carry the haplotype “a”. By contrast, *BTC1* alleles similar to the haplotype “d” of the annual reference accession were found in most sea beet accession. The alleles of the reference accessions differ by 11 non-synonymous single nucleotide polymorphisms (SNPs) and a ~28 kbp insertion in the 5' UTR region that is only present in biennials. Pin et al. (2012) demonstrated that *BTC1* is a key regulator controlling life cycle in beet. Downregulation of *BTC1/btc1* expression by RNAi in annual and biennial beets resulted in a continuous vegetative growth habit. Some transgenic plants started to bolt after vernalization but bolting was severely delayed and plants showed a stunted phenotype and did not form flowers.

Table 1: *BTC1* haplotypes in annual and biennial *B. vulgaris* accessions. Plants of 106 beet accessions (84 sea beet and 22 cultivated beet accessions) were grown in the greenhouse at 18°C under LD conditions (22h light/ 2h dark) and the onset of bolting was recorded. The coding region of *BTC1* was sequenced and SNP positions are given relative to the translation start site (for exon 3) or the 5' end of a given exon, respectively. Eleven haplotypes (“a” to “k”) were identified and haplotype “a” and “d” were referred as reference haplotypes for the biennial or annual allele, respectively. Non-synonymous SNPs that differ between these alleles are indicated with asterisks (from Pin et al. 2012, modified).

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

* * * * * * * * * *

In another paper, Pin et al. (2010) had described two paralogous copies of *FT*, *BvFT1* and *BvFT2* as playing a major role in floral transition in beet. The authors demonstrated that *BvFT1* is a floral repressor, because transgenic plants remain vegetative after cold treatment, when *BvFT1* is overexpressed. In contrast, the gene *BvFT2* retained the *FT* function and promotes flowering and transgenic plants overexpressing *BvFT2* flowered early. Complete downregulation of *BvFT2* in annual beets led to a vernalization requirement. Moreover, *BvFT2* is needed for flower development, since plants started stem elongation after vernalization but flowering was completely abolished.

Based on these findings, Pin et al. (2012) proposed a model for life cycle control in annual and biennial beets with *BTC1* acting upstream of *BvFT1* and *BvFT2* (**Figure 1**). Under inductive photoperiods the dominant *BTC1* allele represses *BvFT1* and activates *BvFT2* to promote bolting followed by flowering. By contrast, beets carrying the recessive *btc1* allele do not respond to LD. These plants remain vegetative before winter, because *BvFT1* is active and represses *BvFT2*. During vernalization *btc1* expression increases and *BvFT1* expression gradually decreases. This finally enables the activation of *BvFT2* to initiate the floral transition after vernalization under LD conditions and ensure their reproduction.

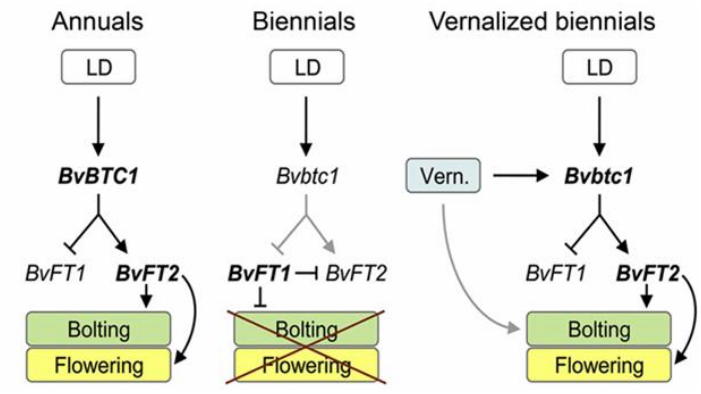


Figure 1: Proposed model for *BTC1* as a central regulator of life cycle adaptation and induction of flowering in beets. Lines between genes do not imply direct interactions. Weak regulatory effects are indicated by gray lines (from Pin et al. 2012).

In the recent years three additional bolting loci termed *B2*, *B3* and *B4* have been identified in populations derived from crosses between an annual sea beet accession and biennial

genotypes which were isolated after EMS mutagenesis (Hohmann et al., 2005). It was shown that dominant alleles at these loci promote annual bolting. While *B3* has not been mapped so far, the locus *B2* was mapped on chromosome 9 like *BvFT1* (Büttner et al., 2010; Pin et al., 2012) (see **Chapter 2.1**). Moreover *B4* was mapped on chromosome 2 and is linked to the *B* locus with a genetic distance of 11 cM and promotes bolting independently of *BTC1* (Abou-Elwafa et al., 2012). Additionally, several *CONSTANS-LIKE (COL)* genes had been identified in beet by Chia et al. (2008). Two genes *BvCOL1* and *BvCOL2* clustered with subgroup I of *COL* genes including *CO* (see **Chapter 2.4**), whereas *BvCOL1* is the closest homolog to the *CO* gene. Overexpression of *BvCOL1* promotes flowering and complements the late flowering phenotype of the *A. thaliana co-2* mutant through activation of *FT*. For *BvCOL2* no complementation experiments were performed. Expression analysis revealed that *BvCOL1* and *BvCOL2* are diurnally regulated but highly expressed at the end of the dark period under both, LD and SD conditions. This expression pattern is more similar to that observed for the genes *COL1* and *COL2*, indicating that *BvCOL1* is no *CO* ortholog.

In *Arabidopsis* the floral repressor gene *FLC* is regulated by a number of genes belonging to the autonomous pathway. In beet, the *FLC-LIKE1* gene *BvFL1* as well as homologs of the autonomous pathway genes *FLOWERING LOCUS K (FLK)*, *FLOWERING LOCUS VE (FVE)*, *LUMINIDEPENDENS (LD)*, and *LYSINE-SPECIFIC HISTONE DEMETHYLASE1 LIKE 1 (LDL1)* had been identified which were termed *BvFLK*, *BvFVE1*, *BvFVE2*, *BvLD*, and *BvLDL1*, respectively (Abou-Elwafa et al., 2010; Reeves et al., 2007). Functional characterization of *BvFLK* and *BvFVE1* in *A. thaliana* revealed an evolutionary conservation as well as evolutionary divergence of their gene functions between *Arabidopsis* and beets. Overexpression of *BvFLK* accelerates flowering in a Col-0 background and complements a late bolting *flk* mutant through repression of the endogenous *FLC* gene (Abou-Elwafa et al., 2010). In contrast, the late bolting phenotype of a *fve* mutant was not rescued by overexpression of *BvFVE1* (Abou-Elwafa et al., 2010).

1.4 Aims and scientific hypotheses

The aims of my work were to

- Fine-map the second bolting locus *B2* on chromosome 9 and to identify and clone a candidate gene
- Functionally characterize the dominant and recessive *BTC1* gene by its ectopic expression in a biennial sugar beet line background
- Functionally characterize the dominant and recessive *BTC1* gene by its ectopic expression in a *A. thaliana* Col-0 and *prr7* mutant background

I assumed that the polypeptides encoded by the dominant and recessive *BTC1* genes are both functional and that the different growth habit is due to different *BTC1* expression levels in annuals and biennials before winter. Moreover, I assumed that the beet gene *BTC1* and the *A. thaliana* gene *PRR7* are orthologs and their proteins share the same function in the regulation of flowering.

2 Map based cloning of *BvBBX19*, a DOUBLE B-BOX TYPE ZINC FINGER gene from the *B2* locus on chromosome 9

2.1 Introduction

Beta vulgaris is a dicotyledonous plant and belongs to the Amaranthaceae family. Species from the genus *Beta* are annual, biennial or perennial plants (see **Chapter 1.1**). The cultivated subspecies sugar beet (*Beta vulgaris* subsp. *vulgaris*) is the only crop harvested for sugar production in Europe and exhibits a biennial life cycle. In the first season, plants grow vegetatively and sucrose is stored in a strong, thickened taproot. In the second year, after winter or artificial cold exposure (vernalization) and long day (LD) conditions plants start shoot elongation (bolting) and flowering to ensure reproduction. Bolting and flowering can also occur without vernalization (annual beets) but is agronomically undesirable because the sugar stored in the root during the first growing season is used as energy resource (see **Chapter 1.1**). Consequently, bolting and flowering time control are major breeding aims for breeding sugar beet and substantial knowledge of the involved genes and pathways as well as their interactions is of crucial importance.

Flowering is controlled by a network of signaling pathways together with environmental cues. Although the knowledge of flowering time regulation in sugar beet is still in its infancy compared to the model species *Arabidopsis thaliana*, various studies in recent years using either map-based cloning or sequence-based cloning approaches have shed some light on beet flowering. The annual habit is controlled by the dominant pseudo response regulator (PRR) gene *BOLTING TIME CONTROL 1 (BTC1)* and represents the long sought bolting gene *B* (Pin et al. 2012) (see **Chapter 1.3**). Furthermore, bolting time loci have been detected by Hohmann et al. (2005), Büttner et al. (2010) and Abou-Elwafa et al. (2012). Hohmann et al. (2005) mutagenized an annual genotype (accession 930190), carrying the dominant *BTC1_d* allele (see **Chapter 1.3**), by ethyl methanesulfonate (EMS). Phenotyping and propagation of these plants resulted in the identification of 5 non-segregating M₃ families, which behave as biennials (Hohmann et al., 2005). In further experiments single plants from four of the five M₃ mutant families were used for crossing experiments with an annual sea beet accession (*B. vulgaris* subsp. *maritima*) to generate F₂ populations, segregating for annual and biennial bolting behavior. Co-segregation analyses of these four F₂ populations revealed 3 loci termed *B2*, *B3* and *B4* which promote annual bolting (Abou-Elwafa et al., 2012; Büttner et al., 2010). Locus *B4* was mapped on chromosome 2 and is linked to *BTC1* but promotes annual bolting independently of *BTC1* (Abou-Elwafa et al., 2012; Büttner et al., 2010). Büttner et al. (2010) further mapped *B2* to chromosome 9 using 2 F₂ populations generated by crossing mutant plants with annual sea beets carrying the dominant *BTC1_d* allele. In the first population the phenotypic segregation ratio of bolting and non-bolting plants did not significantly deviate from a 3:1 segregation ratio, as it was expected for a dominant-recessive inheritance of a monogenic trait. However, an excess of bolting plants was observed in the second population due to the presence of late bolting plants, which started to bolt up to 8 weeks later as plants of the first population. The authors assumed that the respective mutant parent (seed code 031823) carries a mutation in *B2* which impairs but not abolishes gene function in contrast to the other mutant parent (seed code 056822) (Büttner et al., 2010). *B3* has not been mapped so far.

Besides the map-based cloning approaches, sequence-based studies have also revealed detailed information on flowering time genes in sugar beet. The sugar beet *FLOWERING LOCUS C (FLC)* homolog termed *BvFL1 (Beta vulgaris FLC-LIKE 1)*, was identified and functionally characterized by Reeves et al. (2007). The authors assumed that *BvFL1* has the same function as *FLC*, because flowering was repressed in *Arabidopsis flc* mutants overexpressing *BvFL1*. Furthermore, evidence of a large family of *CONSTANS-LIKE (COL)*

genes in sugar beet was given by Chia et al. (2008). In this study, the authors detected three different *COL* genes in sugar beet 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). *CONSTANS* (*CO*) is – together with *FT* – a major flowering regulator gene and conserved in all known plants. *BvCOL1* was identified as an important component of the photoperiod pathway in beet (Chia et al. 2008). Based on their obtained diurnal expression data from *BvCOL1*, which substantially differed from *CO*, the authors presumed *BvCOL1* not to be the true homologue of *CO* (Chia et al. 2008) and to date in beet no homologue of *CO* has been identified.

Recently, remarkable progress has been made in identification of key components of the *CO-FT* regulation in sugar beet. It was shown that the regulation of flowering time in beet is controlled by two paralogous genes of *FT* having antagonistic functions with *BvFT1* acting as a floral repressor and *BvFT2* as a floral activator (Pin et al., 2010). A model was proposed in which *BvFT1* is repressed in annuals by *BTC1*, whereas *BvFT2* expression is activated by *BTC1* (Pin et al., 2010; Pin et al., 2012) (see **Chapter 1.3**).

In *Arabidopsis* the expression of the floral inducer gene *FT* is regulated by *CO*, which functions as a transcriptional activator. In a study by Tiwari et al. (2010) it was reported that the transcriptional activation function derives from the glutamine-rich region between the B-Box and CCT motifs and moreover *CO* was found to directly bind to the *FT* promoter for which the CCT motif was shown to be required. The B-box domains of the *CO* protein were thought to be not essential for DNA binding or transcriptional activation. However *FT* mRNA accumulation is reduced in the late flowering B-box defective *co-2* mutant, indicating that the B-Box domain is important for proper function of *CO* (Kardailsky et al., 1999; Tiwari et al., 2010).

Here, I present the fine-mapping of the locus *B2* and map-based cloning of a candidate gene *BvBBX19*, which encodes for a *DOUBLE B-BOX TYPE ZINC FINGER* protein acting epistatically over *B*. I observed drastic phenotypical changes due to mutation within the second B-box domain which is assumed to be important for proper function of *BvBBX19*. Expression analysis revealed that *BvBBX19* is diurnally regulated and acts upstream of *BvFT1* and *BvFT2*.

2.2 Material and methods

2.2.1 Plant material, growth conditions and phenotypic analysis

All plant materials that were generated and analyzed during the course of this study and all experimental procedures are listed in **Table 2** and **Table 3**.

The F₂ mapping population, segregating for the locus *B2* was developed in the following way: F₁ seeds (seed code 061394) were derived from a cross of the annual *B. vulgaris* subsp. *maritima* accession 991971 (plant No. 10) with red hypocotyl as a pollinator and the biennial *B. vulgaris* mutant accession 056822 (plant No. 5) with green hypocotyl, which carries the mutated *B2* allele (*B2*[']) in the homozygous state, as seed parent (B. Büttner, pers. communication). The M₃ line 056822 was obtained after EMS mutagenesis of the annual accession 930190 (Hohmann et al., 2005). F₁ seeds 056822/5 x 991971/10 were selected by their hypocotyl color and confirmed by PCR using the primers C229/ C230 (**Table 5**). 150 F₁ seeds were grown in the greenhouse under LD conditions (16h light/ 8h dark, 22°C, 900μE). After 4 weeks 21 F₁ plants with red hypocotyl were selected. The F₂ seeds were harvested after 41 weeks and in total 5078 F₂ seeds were obtained.

All F₂ seeds were sown in the greenhouse on April 4, 2010 and finally 6108 F₂ plants were grown under LD conditions (see above). They were transferred into the field on May 17-18, 2010 (**Table 2**). F₂ plants were phenotyped twice a week for bolting (BBCH scale code: 51) and flowering time (BBCH scale code: 60) from May 17, 2010 until October 25, 2010 (Meier, 1993). Plants were classified as annual (bolting without vernalization) or biennial (bolting after vernalization). Annual plants were further classified as early bolting, late bolting and bolting without flowering. Early bolting plants bolted until the end of June, whereas late bolting plants started bolting in July or later. Early bolting and late bolting plants started to flower after bolting. Also late bolting plants without flowering were observed. Annual F₂ plants, which bolted until October 25, 2010, were propagated and F₃ seeds were harvested. Biennial F₂ plants, which did not start bolting up to October 25, 2010, stayed in the field over winter for F₃ seed production in the following summer 2011.

Homozygous *B2B2* or heterozygous *B2B2'* annual F₂ plants were identified by F₃ progeny testing. Eight F₃ plants/ family, derived from annual F₂ plants, were sown in 96er multiport-plates (May 16, 2011) and grown under natural light conditions outside the greenhouse in Kiel (**Supplementary Figure 2**). F₃ families were phenotyped for bolting (BBCH scale code: 51) (Meier, 1993) at three different time points on August 8, September 1 and October 12, 2011.

Table 2: Experimental plan for growing and phenotyping conditions of plants and populations conducted during the course of this study.

Seed Code/ Generation	Sowing Date/ Location	Planting Date/ Location	Time Period Early Bolting	Time Period Late Bolting	Objective	End of Growth Experiment
056822/ M ₃	01.03.12/ greenhouse	05.05.12- 28.08.12/ cold chamber	-	-	expression analyses	31.10.12
991971	01.03.12/ greenhouse	-	-	-	expression analyses	31.10.12
056822/ M ₃	08.04.11/ greenhouse	10.05.11/ field	-	-	phenotyping	12.10.11
991971	08.04.11/ greenhouse	10.05.11/ field	20.05.11- 09.06.11	-	phenotyping	12.10.11
061394/ F ₁	20.10.09/ greenhouse	20.10.09/ greenhouse	11.12.09 - 20.01.09	-	propagation/ genotyping	03.04.10
100155- 100175/ F ₂	09.04.10/ greenhouse	17.05.10- 18.05.10/ field	09.04.10- 24.06.10	25.06.10 - 25.10.10	phenotyping/ propagation/ genotyping	19.08.11 ³
104473- 108214/ F ₃	16.05.11/ field ²	16.05.11/ field ²	16.05.11- 08.08.11	09.08.11- 19.10.11	phenotyping	19.10.11
061394/ F ₁ ⁴	19.04.10/ greenhouse	25.05.10/ field	-	-	propagation/ genotyping	18.10.10
103790- 103792/ F ₂ ⁴	08.04.11/ greenhouse	10.05.11/ field	23.05.11- 23.06.11	24.06.11- 12.10.11	phenotyping/ propagation	12.10.12 ³
111654- 111656/ F ₃ ¹	07.09.11	04.11.11	18.05.12- 23.07.12	-	phenotyping frost tolerance	12.10.12
056822 x 031823 ¹	04.12.12/ greenhouse, cold chamber	14.05.13/ field	-	-	crossing	
056822 x 940043 ¹	03.05.12/ greenhouse, cold chamber		-	-	crossing	

¹ generation of plant material for further projects

² in pots outside the greenhouse under natural light conditions

³ seed production of biennial F₂ plants

⁴ generation and phenotyping of F₂-backup population for fine-mapping *B2*

Table 3: Seed materials used in this study

Seed Code	Population Type	Genotype	Phenotype	Number of Plants Analyzed
056822	M ₃	<i>BBB2'B2'</i>	biennial	10
991971	accession	<i>BBB2B2</i>	annual	10
061394	F ₁	<i>BBB2B2'</i>	annual	21
100155-100175	F ₂	<i>BBB2B2, BBB2B2'</i> <i>BBB2'B2'</i>	segregating: annual/ biennial	6,108
104473-108214	F ₃	<i>BBB2B2, BB2B2'</i> , <i>BB2'B2'</i>	segregating: annual/ biennial	20,392
111654	F ₃	<i>BBB2'B2'</i>	biennial, frost tolerant	9
111655	F ₃	<i>BBB2'B2'</i>	biennial, frost tolerant	19
111656	F ₃	<i>BBB2'B2'</i>	biennial, frost tolerant	20

A second independent biennial mutant family (seed code 031823, M₃ generation), which derived from the same mutagenesis experiment, was previously described by Büttner et al. (2010). For confirmation of mutations within the candidate gene induced through the EMS treatment I used DNA from single plants of this family which was already available.

2.2.2 DNA techniques

Two leaf samples of each F₂ were taken from four weeks old plants and freeze dried for three days. Extraction of genomic DNA was performed using the standard CTAB method (Saghai-Maroo et al., 1984) with slight modifications. DNA was used for PCR in a ten-fold dilution. Standard PCR was performed using *Taq* DNA Polymerase (Invitrogen, Karlsruhe, Germany). All yielded fragments were separated in a 1, 2 or 4% agarose gel. An overview about the used primer combinations as well as the PCR and gel electrophoresis conditions is given in **Table 4** and **Table 5**. Sequencing of PCR products was done by the Institute of Clinical Molecular Biology (IKMB, CAU Kiel, Germany) with a total volume of 25µl of the PCR product.

2.2.3 RNA techniques

To determine the expression of *BvBBX19*, *BTC1*, *BvFT1* and *BvFT2* in the accessions 056822 and 991971, I performed RT-PCR using four weeks old leaf material, taken at zeitgeber time 8 under LD. For diurnal expression analysis of *BvBBX19*, I accomplished RT-qPCR. Leaf material used for this analysis was obtained from the biennial sugar beet line 3DC4156 (seed code 120309) which was obtained from the KWS Saatzucht GmbH (Einbeck, Germany) and transgenic sugar beet plants, which carry either the annual or biennial *BTC1* allele under the control of the constitutive 35S promoter. Samples were collected in 2 h intervals postvernalization (see **Chapter 3.2.7**). Total RNA was extracted using the peqGOLD Plant RNA Kit and DNase treated on column with the peqGOLD DNase I Digest Kit (PEQLAB, Erlangen, Germany). Synthesis of cDNA was achieved with 500ng of total RNA using the First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany). Yielded cDNA was diluted twenty fold, finally 2µl were used for RT-PCR and RT-qPCR as template. For both experiments three independent biological and three technical replicates of each sample were analyzed. RT-PCR was performed with a standard PCR protocol using the *Taq* DNA

Polymerase (Invitrogen, Karlsruhe, Germany). RT-qPCR was realized with the *Power SYBR® Green PCR Master Mix* (Applied Biosystems, Inc, USA) on a CFX96 Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA) with a final reaction volume of 20µl including a final primer concentration of 20pM. An overview about the PCR conditions for the housekeeping gene *BvGAPDH* and *BvBBX19*, *BTC1*, *BvFT1* and *BvFT2* is given in **Table 4**. Resulting data were analyzed with the CFX Manager™ Software v2.1 (Bio-Rad, Hercules, CA, USA). The comparative CT (ΔC_T) method was applied. Relative expression levels were calculated and normalized to the geometric mean of *BvGAPDH*.

Table 4: Primer and amplification conditions for PCR, RT-PCR and RT-qPCR performed in this study.

Gene	Forward Primer	Reverse Primer	PCR Conditions
<i>BvGAPDH</i>	B582 ^a	B583 ^a	95°C, 3' + 40x (95°C, 10"; 61°C, 30"; 72°C, 30") ²
<i>BTC1</i>	B580 ^a	B581 ^a	95°C, 3' + 40x (95°C, 10"; 61°C, 30"; 72°C, 30") ²
<i>BvFT1</i>	B563 ^a	B564 ^a	95°C, 3' + 40x (95°C, 10"; 64.5°C, 30"; 72°C, 30") ²
<i>BvFT2</i>	B584 ^a	B585 ^a	95°C, 3' + 40x (95°C, 10"; 64.5°C, 30"; 72°C, 30") ²
<i>BvBBX19</i>	C565	C566	95°C, 3' + 40x (95°C, 10"; 61°C, 30"; 72°C, 30") ^{1,2}
<i>BvBBX19</i>	BBf	BBr	95°C, 3' + 36x (95°C, 3'; 57°C, 30"; 72°C, 40") + 72°C, 5" ²

¹ RT-qPCR performed with *Power SYBR® Green PCR Master Mix* (Applied Biosystems, Inc, USA)

² PCR and/ or RT-PCR performed with *Taq-DNA-Polymerase* (Invitrogen, Karlsruhe, Germany)

^a (Pin et al., 2010; Pin et al., 2012)

2.2.4 Bioinformatic analysis

For candidate sequence analysis the sugar beet draft genome from the doubled haploid accession KWS2320 was used. A current version (RefBeet-0.9) is available for download under <http://bvseq.molgen.mpg.de>. Moreover, a previous version (RefBeet-0.4) and a collection of predicted gene models (RefBeet-0.3geneModels) were kindly provided by Dr. H. Himmelbauer and his group (Centre for Genomic Regulation, Barcelona, Spain). Published sequences like expressed sequence tags (ESTs) (Schneider et al., 2007) and candidate genes like *BvFT1* (Pin et al., 2010) were used to find scaffolds which are located on chromosome 9. BLAST analyses were performed to map known sequences to the reference sequence using the blastn function in CLC Main Workbench 5.5 (CLC bio, Aarhus, Denmark). Screening the region of interest for sugar beet transcripts was performed with the CLC Genomics Workbench 4.0 (CLC bio, Aarhus, Denmark) using RefBeet-0.3geneModels. Finally, these transcripts were used as queries for a blastx search of the TAIR9 and nt/ nr protein databases at TAIR (<http://www.arabidopsis.org>) and NCBI (<http://www.ncbi.nlm.nih.gov>), respectively.

2.2.5 Marker development and genetic mapping

For fine mapping of the *B2* locus a genetic map on chromosome 9 was generated. Scaffolds, located on chromosome 9 were used to develop sequence based molecular markers, segregating in the F₂ population. Markers, with no detectable insertions or deletions (InDel) but SNPs were converted into cleaved amplified polymorphic sequence (CAPS) markers. The molecular marker GJ1001c16 which is completely linked to the *B* locus (Pin et al., 2012) was used for genotyping. All applied markers in this study are listed in **Table 5** and **Supplementary Figure 3**. The genetic distance in centiMorgan (cM) was calculated using the

Kosambi mapping function (Kosambi, 1943) in JoinMap® 4.0 (Van Oijen and Voorrips, 2001), with a LOD threshold value of 5.0.

Table 5: Non-anonymous and developed sequence based (RefBeet-0.9) molecular markers on chromosome 9 for analysis of co-segregation with bolting phenotypes in the F₂-population₀₅₆₈₂₂.

Marker Name	Marker Type	Marker Assay			Marker Allele in Annual Parent (bp)	Marker Allele in Mutant Parent (bp)
		Primers	PCR Conditions for Marker Assay	Detection		
CAU3782	SNP/CAPS	C375 + C376	95°C, 3' + [(95°C, 30" + 52°C, 30" + 72°C, 20") x 36] + 72°C, 10'	<i>Tsp509I</i> digest + GE ¹ 2%	175	135 and 40
CAU3783	SNP/CAPS	C505 + C470	95°C, 3' + [(95°C, 30" + 55°C, 45" + 72°C, 60") x 36] + 72°C, 10'	<i>BseGI</i> digest + GE 1 %	272 and 252	524
CAU3784	InDel	C507 + C508	95°C, 3' + [(95°C, 30" + 54°C, 30" + 72°C, 20") x 36] + 72°C, 10'	GE 4 %	174	152
CAU3785	SNP/CAPS	C450 + C451	95°C, 3' + [(95°C, 30" + 57°C, 30" + 72°C, 30") x 36] + 72°C, 10'	<i>PstI</i> digest + GE 2 %	553	346 and 207
CAU3786	SNP/CAPS	C442 + C443	95°C, 3' + [(95°C, 30" + 55°C, 30" + 72°C, 30") x 36] + 72°C, 10'	<i>TaqI</i> digest + GE 1 %	465	353 and 112
CAU3787 (MP_E0043 ³)	InDel	C261 + C237	95°C, 3' + [(95°C, 30" + 55°C, 30" + 72°C, 22") x 36] + 72°C, 10'	GE 2 %	405	599
CAU3788 (TG_E0140 ³)	InDel	C229 + C230	95°C, 3' + [(95°C, 30" + 57°C, 30" + 72°C, 30") x 36] + 72°C, 10'	GE 2 %	295	387

¹GE, gel electrophoresis

²Nt pos., nucleotide position within PCR fragment

³EST sequence derived from Schneider et al. (2007)

2.2.6 Cloning of *BvBBX19* sequences

For cloning *BvBBX19* cDNA derived from the accessions 991971 and 056822 was used as template and PCR amplified with the primers BBf and BBr (**Table 4**). These primers have compatible sequence ends that are recognized by the restriction enzyme *BamHI*. The resulting DNA fragments were restricted and ligated into the corresponding restriction sites of the cloning vector pUC18 and then transformed into the *Escherichia coli* strain DH5 alpha (S. Melzer, pers. communication).

2.3 Results

2.3.1 Phenotypic analysis of bolting time and genetic mapping of the *B2* locus

In order to fine-map the locus *B2*, I established a large F₂ mapping population segregating for the locus *B2* on chromosome 9 (**Table 2, Table 3**). In total 5078 seeds were obtained from selfing of 21 F₁ plants (seed code 061394, plant No 1 -21). Subsequently, a total of 6108 F₂ plants divided in 21 F₂-subpopulations (seed code 100155-100175) (**Supplementary Table 1**) was obtained. These plants represent the F₂-population₀₅₆₈₂₂. Plants were grown for six weeks in the greenhouse under LD (16h light, 22°C, 900μE) and then transferred to the field (**Table 2, Supplementary Figure 1**).

The bolting date was determined between May and October 2010. 4415 plants bolted until October 25, 2010 and were thus classified as annual F₂ plants whereas 1042 plants did not

start bolting and were classified as biennial F₂ plants. The remaining 651 plants died and were discarded from further analysis. Among the annuals, I observed 3388 early bolting plants which bolted until June 24, 2010 and 816 late bolting individuals which bolted between June 26, and October 25, 2010. Moreover, I observed 211 late bolting plants, which bolted between June 26, and October 25, 2010 but did not set flowers (**Figure 2, Table 6**). For a dominant-recessive inheritance of a monogenic trait it was expected that the F₂-population₀₅₆₈₂₂ segregates for bolting and non-bolting in a 3:1 ratio. Unexpectedly, the null hypothesis for a 3:1 ratio, tested by χ^2 analysis, was rejected at $\alpha=0.05$, due to an excess of annual plants.

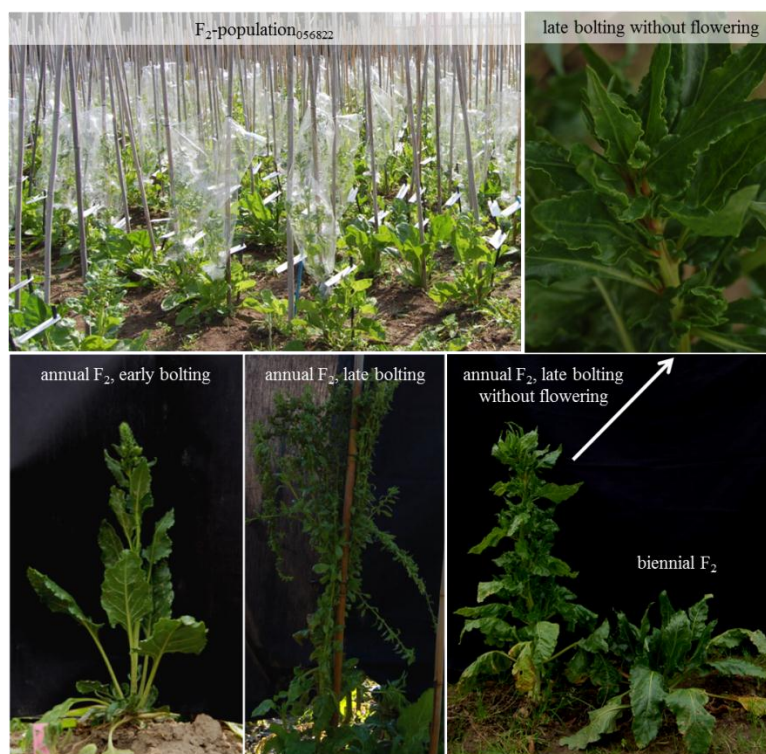


Figure 2: Phenotypes of F₂ plants from F₂-population₀₅₆₈₂₂. The F₂-population₀₅₆₈₂₂ was phenotyped under non-vernalizing conditions in the field for bolting behavior from May until October 2010. Early bolting: bolting until June 24 followed by flowering; Late bolting: bolting between June 26, and October 25, 2010 and flowering; Late bolting without flowering individuals: bolting between June 26, and October 25, 2010; Biennial: non-bolting until October 25, 2010.

Table 6: Phenotypic segregation for annual (early bolting, late bolting with and without flowering) and biennial individuals in the F₂-population₀₅₆₈₂₂. Plants were phenotyped for bolting (BBCH stage 51) and flowering (BBCH stage 60) under field conditions from May 2010 until October 2010 (**Chapter 2.2.1**).

Number Plants Grown	Annual Bolting Phenotypes			Biennial Bolting Phenotypes	χ^2 test for H ₀ = 3:1 (annual vs. biennial)
	Early Bolting	Late Bolting	Late Bolting without Flowering		
5,457	3,388	816	211	1,042	101.49*

* $\alpha=0.05$

I further improved the accuracy of phenotyping by screening F₃ families, derived from annual F₂ plants of the F₂-population₀₅₆₈₂₂. In this way heterozygous annual F₂ plants (*B2B2*') could be clearly distinguished from homozygous (*B2B2*) individuals. We harvested seeds from 3629 annual F₂ plants. In total 2549 F₃ families (8 plants/ family) were grown outside the greenhouse (2011) under natural conditions between May 16 and October 19 without prior cold treatment (**Supplementary Figure 2**). Bolting was determined at three different time points: August 8, September 1 and October 12. F₃ were classified into annuals (bolting until October 19) and biennials (non-bolting until October 19) (**Chapter 2.2.1**). I observed 638

non-segregating annual F₃ families and 1911 F₃ families segregating for annual and biennial individuals (**Table 7**).

Table 7: Phenotyping of F₃ families, derived from annual F₂ plants. Eight plants/ F₃ family were sown in soil and grown under natural light conditions in pots outside the greenhouse at Kiel. Annual: bolting until October 19, 2011; Biennial: non-bolting until October 19, 2011.

	Phenotype		
	Non-Segregating (Annual)	Segregating (Annual/ Biennial)	Total Number of F ₃ Plants Analyzed
Expected Genotype of Annual F ₂ Parent	<i>B2B2</i>	<i>B2B2'</i>	
Number F ₃ Families	638	1,911	2,549

Taking phenotypic data from F₂ and F₃ generations, an annual F₂ plant was classified as homozygous when the F₂ plant bolted early until June 24, 2010 and all plants of the F₃ family bolted until August 8, 2011. In this way, out of 638 annual F₂ plants with non-segregating F₃ offspring, 466 F₂ plants were identified as being homozygous and were expected to have the genotype (*B2B2*). In total 1508 F₂ plants were genotyped, thereof 466 annual F₂ plants (*B2B2*) and 1042 biennial F₂ plants (*B2'B2'*), with 7 *B2* locus markers (**Table 5**). Out of 1042 biennial plants, 120 were excluded for co-segregation analysis because they gave ambiguous PCR results in all markers tested. Moreover and with regard to the low sample size of analyzed F₃ plants/ family, 87 out of 466 annual individuals were heterozygous in all markers. I phenotyped random sampled F₃ families with 35plants/ family and confirmed that these families segregated for annual and biennial individuals.

Accordingly, co-segregation analysis was performed with 1301 homozygous F₂ plants using 7 molecular markers (**Table 5, Table 8, Supplementary Table 2, Supplementary Table 3**). For complete co-segregation between marker genotypes and F₂ phenotypes, I expected that biennial plants carry the marker genotype M₂M₂ derived from the biennial mutant parent whereas annuals carry the marker genotype derived from the annual parent (M₁M₁). The analysis revealed that all marker genotypes co-segregated with F₂ phenotypes. In 6 out of 7 markers analyzed, I observed plants that are heterozygous at the marker loci (M₁M₂) or even homozygous for the other marker allele. The genotypes of one marker CAU3784 (**Figure 3**) showed 100% co-segregation to the phenotypes, because 922 biennial plants carried the marker genotype (M₂M₂) and 373 annuals carried the marker genotype M₁M₁. For the remaining 6 individuals no PCR products were obtained (**Table 8**).

Table 8: Co-segregation analysis of annual and biennial individuals and *B2* locus markers. Analysis was performed with 1301 F₂ plants, which were determined as being homozygous early bolting annual or biennial according to F₃ phenotyping (**Chapter 2.3.1**). Genotyping was performed with molecular markers (**Table 5**, **Supplementary Table 2**, **Supplementary Figure 3**). M₁ and M₂, marker alleles derived from the annual parent or the biennial mutant parent, respectively.

<i>B2</i> Locus Marker (EST)	Annual				Biennial			
	M ₁ M ₁	M ₁ M ₂	M ₂ M ₂	No PCR Product	M ₁ M ₁	M ₁ M ₂	M ₂ M ₂	No PCR Product
CAU3785	338	5	0	36	2	11	645	264
CAU3786	307	3	0	69	1	12	868	41
CAU3782	342	2	0	35	2	8	810	102
CAU3784	373	0	0	6	0	0	922	0
CAU3783	341	9	0	29	0	24	856	42
CAU3787 (MP_E0043)	333	25	1	20	2	53	829	38
CAU3788 (TG_E0140)	335	24	0	20	0	59	791	72

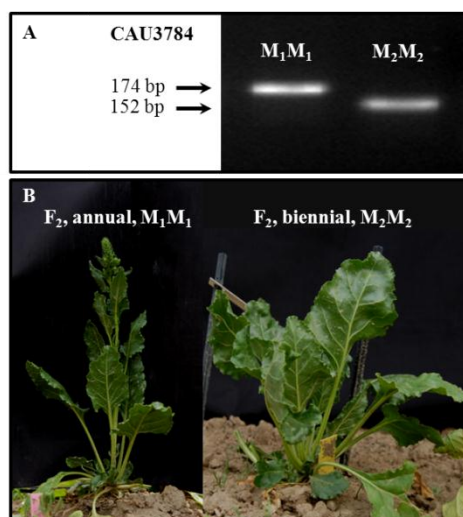


Figure 3: Genotypes of the marker locus CAU3784 (A) in homozygous biennial and annual F₂ plants (B). Annual and biennial F₂ plants were determined as being homozygous according to F₃ phenotyping. Genotypes were determined by PCR using the molecular marker CAU3784. The CAU3784 marker allele M₁ derived from the annual parent, whereas the CAU3784 marker allele M₂ derived from the biennial mutant parent. Black arrows indicate the respective PCR fragment size.

2.3.2 Genetic mapping of the *B2* locus

For development of sequence based molecular markers, sequences were derived from scaffolds, which were likely to be candidate sequences comprising the *B2* locus. Candidate scaffolds were identified in the following way: I mapped the EST markers MP_R0018, linked to the *B2* locus (Büttner et al., 2010) and TG_E0140, MP_E0043 as well as the SNP marker KI_2783 (Schneider et al., 2007), which are located closely to that region, to the RefBeet-0.9 using the blastn function of the CLC Main Workbench (CLC bio, Aarhus, Denmark). In this way, the scaffold sc00048 (RefBeet-0.9) was identified. I further identified the scaffold sc00497 (RefBeet-0.3) that overlaps with scaffold00048 by ~190kb. To determine their order and orientation, molecular markers were developed and genetically mapped to chromosome 9.

Mapping was performed with DNA isolated from 1301 F₂ plants using the Kosambi mapping function (Kosambi, 1943) in JoinMap® 4.0 (Van Oijen and Voorrips, 2001) with a LOD score of 5.0 (**Figure 4**). The high density map around the *B2* region comprises seven markers spanning 4.8 cM on chromosome 9 and does not represent the whole chromosome since we focused only on the *B2* region. Two of these markers are publically available ESTs (TG_E0140 and MP_E0043) (Schneider et al., 2007). The remaining five molecular markers were developed from scaffolds sc00048 and sc00497.

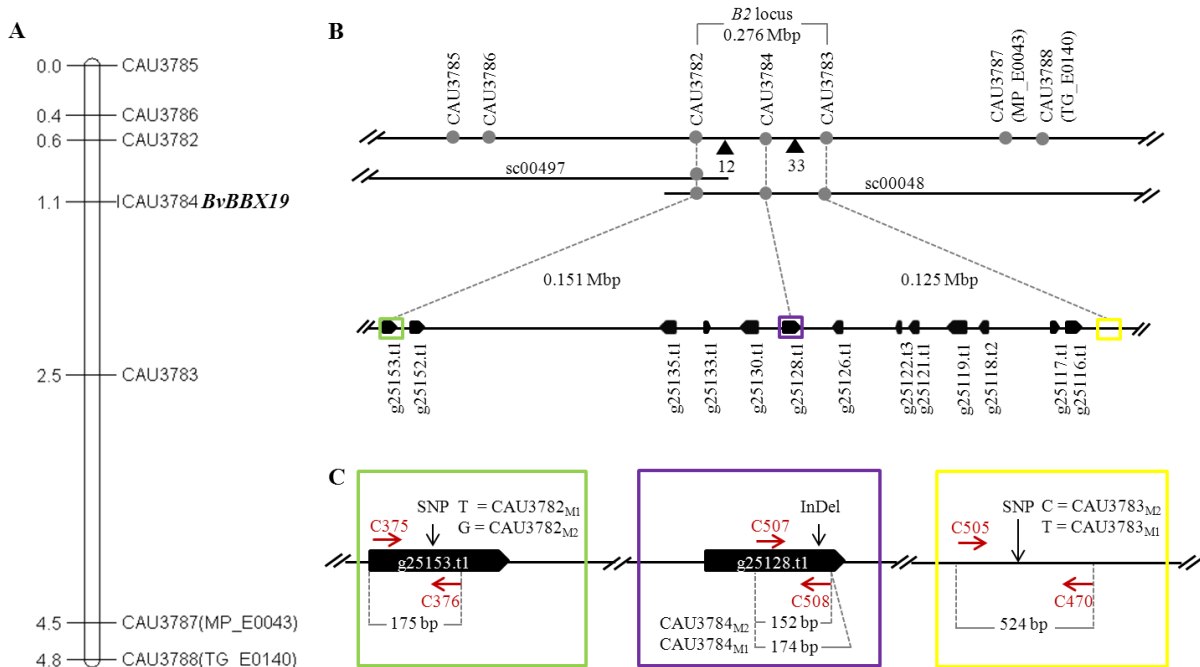


Figure 4: Map-based cloning of the *B2* locus in beet.

(A) Genetic map position of the *B2* locus on chromosome 9. The map was calculated using 379 annual and 922 biennial F₂ plants of the F₂-population₀₅₆₈₂₂ (JoinMap4.0, LOD threshold value: 5.0). Sequence information for the molecular markers (right) were derived from publically available ESTs (indicated in brackets) or were derived from the sugar beet reference genome sequence (RefBeet-0.9, RefBeet-0.3, **Chapter 2.3.3**). The numbers on the left site display genetic distances in centiMorgan (cM).

(B) Physical map of the *B2* locus and the location of the two scaffolds sc00497 and sc00048, covering the *B2* locus. Marker locations are given in grey circles. The number of recombination events between two markers flanking *B2* is given in black triangles (see also **Supplementary Figure 3**). Black boxes with the tip indicate identified gene models (RefBeet-0.3_GeneModels) and their orientated location within the critical region of the scaffold sc00048 (**Chapter 2.3.3**). Locations of molecular markers within are indicated with colored boxes. The two markers CAU3782 and CAU3783 flank the *B2* locus in a genetic distance of 0.276 Mbp.

(C) Molecular markers CAU3782, CAU3783 and CAU3784 used for mapping the *B2* locus. The fragment size (bp) of the PCR products is given for each marker genotype with M1, allele derived from the annual parent and M2, allele derived from the biennial mutant parent. Red arrows: primers used for PCR amplification; black vertical arrows: polymorphism site and marker type; InDel: Insertion/ Deletion marker and respective PCR fragment sizes; SNP (single nucleotide polymorphism) with the respective nucleotides for each genotype (G = guanine, C = cytosine, T = thymine).

The three markers CAU3782, CAU3783, and CAU3784 are located close to the telomeric region of chromosome 9 on scaffold sc00048, which is ~1.6 Mbp in size. The markers CAU3782 and CAU3783 flank the *B2* locus and the marker CAU3784 (**Figure 4**). They are in a distance of ~276 kbp on sc00048. The molecular marker CAU3784 is an InDel marker. The 174 bp fragment (CAU3784_{M1M1}) derived from the annual parent (seed code 991971), whereas the 152 bp fragment (CAU3784_{M2M2}) is from the mutant parent (seed code 056822) (**Figure 4**). Thus CAU3784_{M1} and CAU3784_{M2} are linked in coupling (R=0) with the *B2* and *B2'* alleles. Therefore, the scaffold sc00048 is likely to comprise the sought gene at the locus *B2*.

2.3.3 Candidate gene identification

To identify *B2* candidate sequences, I performed a blastn analysis using the sequence of the critical region of scaffold sc00048 against 33,628 predicted cDNA sequences (including splice variants) termed gene models from the doubled haploid accession KWS2320 (RefBeet3.0_GeneModels), which were kindly provided by H. Himmelbauer (Centre for Genomic Regulation, Barcelona, Spain). In this way 32 gene models were identified (thresholds: e-value 0.0, identity 100%). Then the identified gene models were used as queries for a blastx analysis against the TAIR and NCBI protein databases (threshold: e-value <0.05). In total 13 gene models were obtained which were homologous to genes from other species (Supplementary **Table 4**). I selected the gene model g25128.t1, which is 1458 bp in size, as a sound candidate for *B2*, because the InDel marker CAU3784 is located in the predicted 3'-untranslated region (UTR) of this candidate gene (**Chapter 2.3.4**) and based on its homology to a *B-BOX TYPE ZINC FINGER* gene (*BBX*). *BBX* proteins have been classified to function as transcription factors (Khanna et al., 2009). A blastp analysis against the nr/nt protein database at TAIR revealed 55% identity to the *A. thaliana* protein BBX19 (TAIR, At4g38960). Searching for sugar beet ESTs resulted in the identification two ESTs, BQ589556 and BQ591888, which are homologous to the hypothesized gene model g25128.t1. The gene model g25128.t1 was used to predict open reading frames using the ORF analysis tool in the CLC Bio Main Workbench (CLC bio, Aarhus, Denmark). The predicted ORF encompasses 588 bp and was termed ORF_{B2-KWS2320}. The predicted protein consists of 196 amino acids.

2.3.4 Gene structure analysis

I analyzed the structure of the predicted ORF_{B2-KWS2320} in silico. The transcription start site (TSS) was determined by the software tool FGENESH using the sequence of the putative gene model g25128.t1 as a query. Transcription starts at position 262 and was referred to +1 and ends at position 1438 (+1177, PolyA signal). Thus the transcript was predicted to be 1177 bp in size (**Figure 5**). The TATA-box was predicted as being located at position -28 to -22 relative to the TSS. To determine the exon/intron structure, the sequence of g25128.t1 was aligned against the scaffold sc00048 sequence. The predicted transcript consists of five exons. Translation start and end points were determined according to the predicted ORF_{B2-KWS2320}. The START codon (ATG) is at position +242 (exon 1) and the translation ends (TGA) at position +829 (exon 4). Thus, parts of exon 4 and exon 5 belong to the 3'-UTR, which is supposed to be 348 bp in size (2136 bp in size relative to the genomic DNA, including the intronic region between exon 4 and 5). The 5'-UTR is supposed to be 241 bp in size. The size of the genomic sequence (ATG-STOP) is 4700 bp according to the sequence on scaffold sc00048.

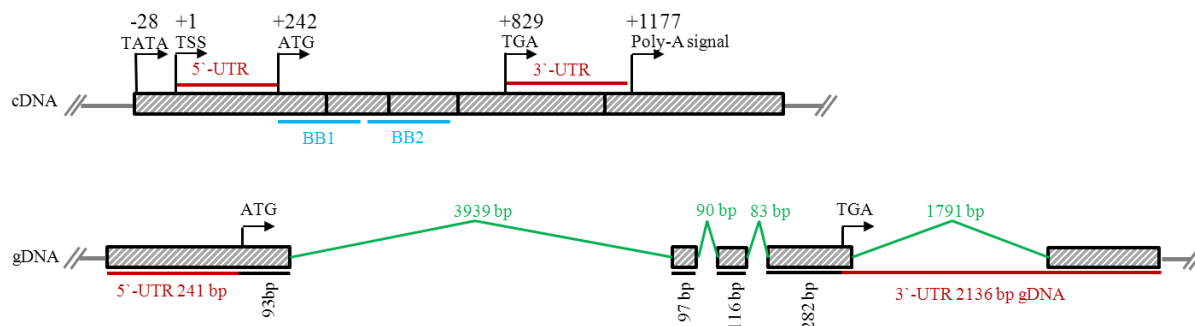


Figure 5: In silico prediction of the *BvBBX19* gene structure based on cDNA and gDNA analysis. Prediction was performed with the gene model g25128.t1 (RefBeet3.0_GeneModels) and the genomic sequence of *BvBBX19* located on scaffold sc00048 (RefBeet0.9). Grey bar: partial cDNA sequence from the gene model g25128.t1 and partial gDNA sequence of scaffold00048, interrupted sequence regions are indicated with a double slash; Dashed grey boxes represent exons; Arrows: transcription and translation starts and ends and respective positions according to the cDNA sequence; Blue bars: locations of sequences with homology to B-box domains (BB1, BB2); Red bars: 3'-UTR and 5'-UTR; Black bars: exonic region belonging to the translated region, respective sizes of the region are given below; Green lines: intronic region, respective size is given above.

The predicted polypeptide sequence of the ORF_{B2-KWS2320} was analyzed with the SMART program (Letunic et al., 2012; Schultz et al., 1998). The sequence shows strong similarity to two B-box-type zinc finger domains. Those domains were shown to be involved in protein-protein interactions (Gangappa et al., 2013). The domains differ in their consensus sequence and length. The B-box domains termed BB1 and BB2 are 47 and 46 amino acids (aa) in size, respectively. The structure of the two B-box domains in the *A. thaliana* protein BBX19 is C-X₂-C-X₈-C-X₇-C-X₂-C-X₄-H-X-C-X₆-H-X₅ (BB1) and C₂-X₂-C-X₈-C-X₇-C-X₂-C-X₄-H-X₆-H-X₅ (BB2). I compared them with the structure of the predicted protein of the ORF_{B2-KWS2320}. Both BB1 domains are 100% identical whereas the BB2 domains differ by one C-residue at the beginning (ORF_{B2-KWS2320}: C-X₂-C-X₈-C-X₇-C-X₂-C-X₄-H-X₆-H-X₅). The protein BBX19 is classified as type IV BBX proteins (Khanna et al., 2009). Following this nomenclature, I designate ORF_{B2-KWS2320} as *BvBBX19*.

2.3.5 *BvBBX19* sequence variations

I hypothesized that DNA sequence variations between both parents of the mapping population are responsible for their phenotypic variation. Therefore, coding and genomic sequences of *BvBBX19* were sequenced. First, coding sequences of *BvBBX19* derived from single plants of the annual parent (seed code 991971) and the mutant parent (seed code 056822) were PCR amplified using the primers BBf/ BBr (**Table 4**). The PCR fragments were cloned into the binary vector pUC18 (S. Melzer, pers. communication). Then, the clones were sequenced from both ends using the primers M13f and M13r (**Supplementary Table 5**). Sequencing revealed that the coding sequence of *BvBBX19* derived from the annual parent (seed code 991971) is 588 bp in size and corresponds to 100% to the predicted ORF_{B2-KWS2320}. In contrast, the sequence derived from the mutant parent (seed code 056822) is 90 bp longer.

Then, I compared the *BvBBX19* alleles (ATG-STOP) of the mutant parent and the line 930190 which is the non-mutagenized progenitor of the EMS mutant line 056822. I found that both sequences were identical with one exception. A polymorphism was found at position 4131 right after the second exon where the donor line (seed code 930190) has a guanine instead of an adenine (seed code 056822) (**Figure 6**). I speculate that this transition was induced through the EMS mutagenesis. The 90 bp insertion in *BvBBX19*₀₅₆₈₂₂ matches perfectly to the second

intron. I assume that the single nucleotide mutation at position 4131 alters the function of the donor splice site in a way that the second intron cannot be removed which results in a longer transcript. This again would result in a shift of the reading frame with a STOP codon within the second intron. Consequently, the truncated *BvBBX19*₀₅₆₈₂₂ would be substantially shorter (72 aa) as the *BvBBX19*₉₃₀₁₉₀ protein (196 aa) (**Figure 7**).

I further analyzed the second mutant 031823, which had been the mutant parent of the population EW3 (Büttner et al., 2010). I compared the *BvBBX19*₀₃₁₈₂₃ sequence with the other *BvBBX19*₀₅₆₈₂₂ and *BvBBX19*₉₃₀₁₉₀ sequences. *BvBBX19*₀₃₁₈₂₃ is identical to *BvBBX19*₉₃₀₁₉₀ with one exception. The *BvBBX19*₀₃₁₈₂₃ allele carries another point mutation within the 3rd exon at position 4253 (**Figure 6**). It is likely that the transition from cytosine to thymine resulted from the EMS treatment. In silico translation of *BvBBX19*₀₃₁₈₂₃ resulted in a 196 aa polypeptide. The only difference in comparison to *BvBBX19*₉₃₀₁₉₀ is an amino acid change at position 75 from leucine to phenylalanine (**Figure 7**).

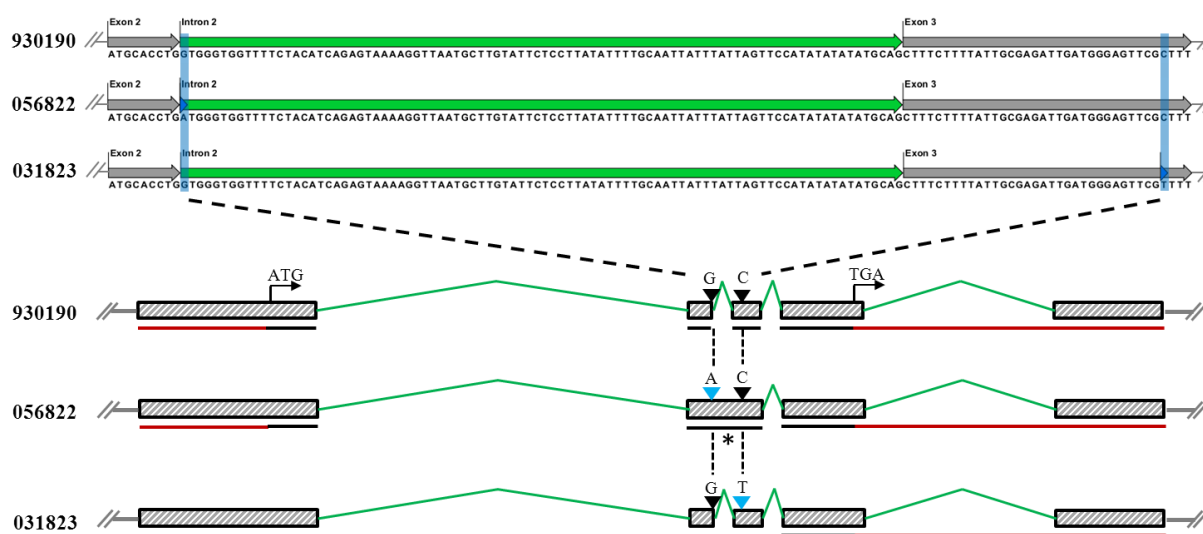


Figure 6: Sequence variations within *BvBBX19* alleles from the two EMS mutants (seed code 056822 and 031823) and the non-mutated donor line (seed code 930190). A part of the sequence encompassing the polymorphic region between exon 2 and exon 3 is shown. Grey arrows/ boxes: exons; Green arrows/ lines: intronic region; Black lines: coding region flanked by translation start (ATG) and end (TGA); Red lines: non-coding regions, 3'-UTR and 5'-UTR; SNP positions are indicated as blue boxes and triangles, black triangle: wild type nucleotide derived from the non-mutated donor line (seed code 930190). The respective nucleotide is given above the triangles (A=adenine, G=guanine, C=cytosine, T=thymine); Black asterisk: premature STOP-codon.

BvBBX19 protein sequences from both mutants (seed code 056822 and 031823) and the non-mutated donor line (seed code 930190) were searched for conserved sequence regions against the Conserved Domains Database at NCBI (<http://www.ncbi.nlm.nih.gov>). This analysis demonstrated that both transitions occurred at highly conserved positions within the BB2 region (**Figure 7**). The zinc finger type structure of the BB2 domain is not altered in the *BvBBX19*₀₃₁₈₂₃ protein. In contrast, the 5'-splice site mutation in the second intron of *BvBBX19*₀₅₆₈₂₂ drastically alters the structure of the BB2 domain to C-X₂-C₁₄.

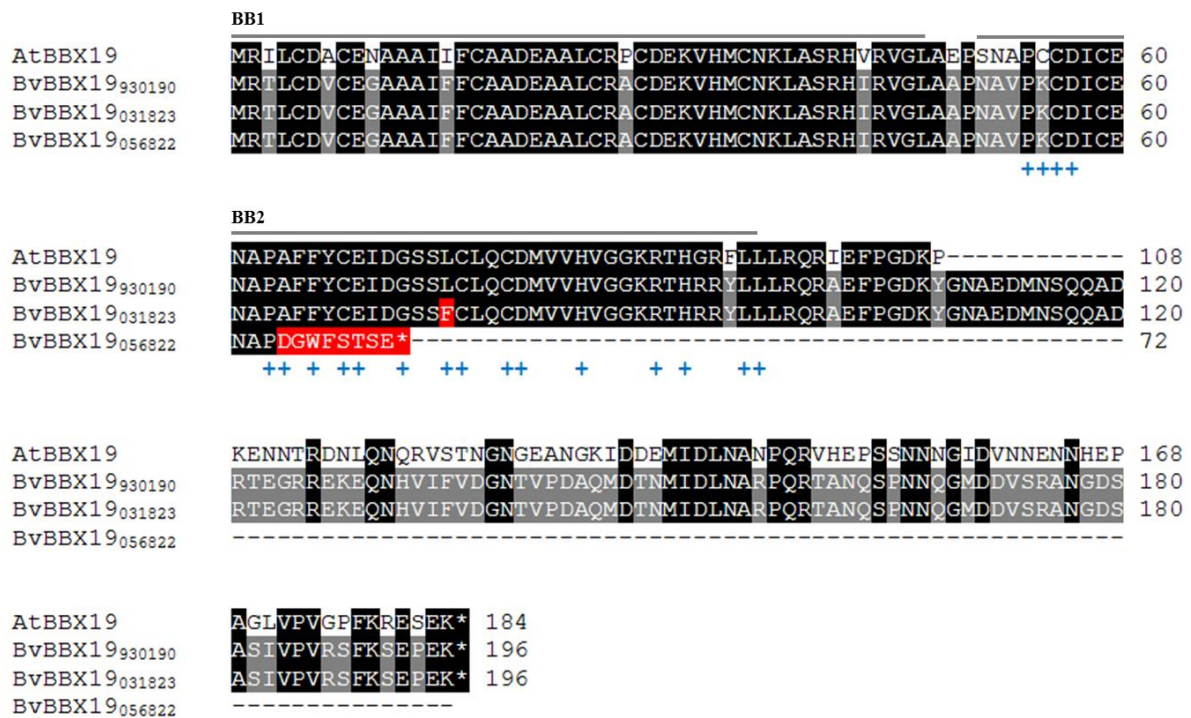


Figure 7: Multiple alignment of BvBBX19 protein sequences from the EMS mutants (seed code 056822 and 031823), from the non-mutated donor line (seed code 930190) and from *A. thaliana* (BBX19). BvBBX19 cDNA sequences were translated into amino-acid (aa) sequences. The AtBBX19 sequence (TAIR database, At4g38960.1) shares 55% sequence similarity with BvBBX19₉₃₀₁₉₀. Grey bars: B-Box domains (BB1, BB2); blue symbols: predicted highly conserved aa positions within BB2 (Conserved Domains Database, NCBI); aa present in all sequences are highlighted in black; aa present in most aligned sequences are highlighted in grey; aa changes due to EMS mutations are highlighted in red; asterisks: translation termination (stop) codon.

2.3.6 Expression analysis of *BvBBX19* and its putative downstream targets

First I measured by RT-qPCR the transcriptional activity of *BvBBX19* in the biennial sugar beet line 120309 (**Chapter 2.2.3**). Plants were vernalized for twelve weeks and then grown in the greenhouse under LD (16h light, 22°C, 900μE). Leaves were taken in a 2 h interval from two single plants which had started flowering (BBCH scale code: 65). The expression analysis revealed that *BvBBX19* is diurnally regulated (**Figure 8**). The expression is highest in the morning and rapidly decreases at zeitgeber time (ZT) 11 until it gradually rises again in the late afternoon and night. Likewise, a rapid down regulation of transcriptional activities had been observed for *BTC1* (see **Chapter 3, Figure 20**).

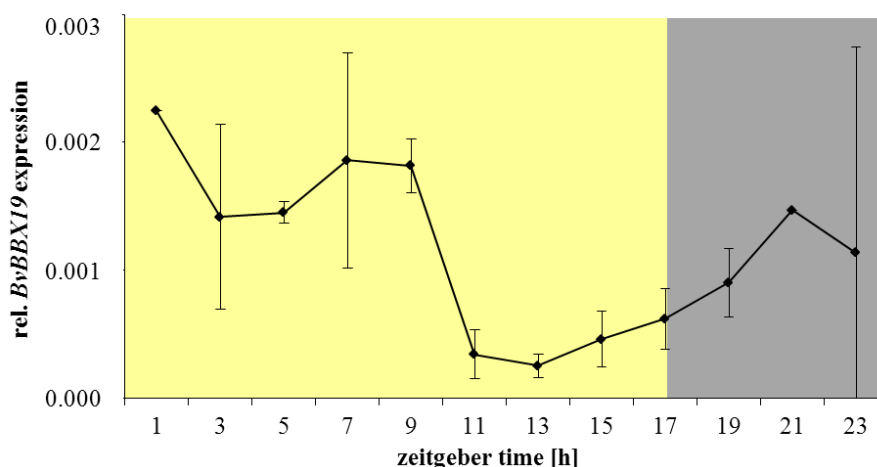


Figure 8: Expression analysis of *BvBBX19* in the biennial sugar beet line (seed code 120309) at growth stage BBCH: 61. Plants were grown in a greenhouse after 12 weeks of vernalization. The yellow background indicates day and the grey background indicates night. Each value is the mean of two biological replicates and three technical replicates. Relative gene expression was calculated applying the ΔC_T method and normalized to the geometric mean of *BvGAPDH*. Error bars represent the standard deviation of the mean (\pm SDM) of the two biological replicates.

I assumed that *BvBBX19* is an upstream regulator of *BTC1* because *B2*-mutants are biennial even in the presence of the dominant *BTC1_d* allele. I performed RT-qPCR to examine the expression of *BvBBX19*, *BTC1*, *BvFT1* and *BvFT2* in both parents of my mapping F_2 -population₀₅₆₈₂₂ (**Chapter 2.2.3**). Three plants of the biennial mutant parent (seed code 056822) and the non-mutated annual parent (seed code 991971) were grown in the greenhouse under LD conditions. After four weeks (BBCH scale code 16) leaf samples were harvested at zeitgeber time (ZT) 8. Then mutant plants (seed code 056822) were vernalized for three months and leaves were harvested four weeks after vernalization at ZT 8.

The transcriptional activity of *BvBBX19* was slightly higher in non-vernalized plants of the biennial mutant as in the annual parent carrying the functional *BvBBX19* allele, whereas the expression of *BTC1* was slightly lower in non-vernalized mutants. The transcriptional activity of the floral repressor *BvFT1* was slightly increased in the mutant before vernalization. The floral promoter *BvFT2* displayed the greatest contrast between non-mutated and mutated genotypes. I observed a complete down-regulation of the *BvFT2* gene in the mutants (without vernalization), whereas *BvFT2* was highly upregulated in the annual parent (**Figure 9**). The transcriptional activity of *BvBBX19*, *BTC1*, and *BvFT2* in vernalized mutant plants was similar to that observed in annuals.

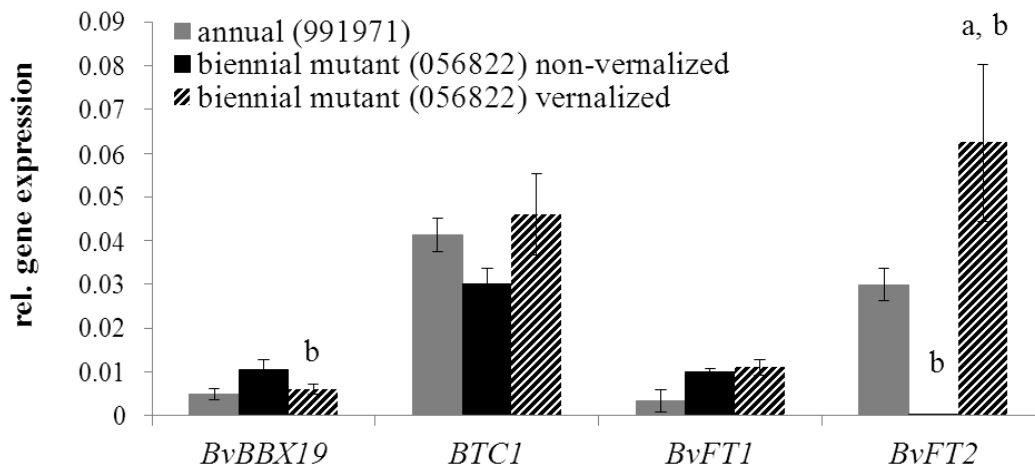


Figure 9: Expression analysis of *BvBBX19*, *BTC1*, *BvFT1*, and *BvFT2* in the annual parent accession 991971 (grey boxes) and the EMS mutant parent (seed code 056822) before and after vernalization. Plants were grown in the greenhouse under LD conditions. Each column is the mean of three biological and three technical replicates. The vertical axis displays the relative expression of the target gene. Relative gene expression was calculated applying the comparative CT (ΔC_T) method and normalized to the geometric mean of the housekeeping gene *BvGAPDH*. Error bars represent the standard deviation of the mean (\pm SDM) of the three biological replicates. ‘a’, statistically significant different gene expression between the annual parent (991971) and the non-vernalized mutant parent (056822); ‘b’ statistically significant different gene expression between the vernalized and non-vernalized mutant parent (056822).

2.3.7 Expression of *BvBBX19* in *BTC1*-transgenic sugar beets

The expression data suggest that *BvBBX19* like *BTC1* acts upstream of *BvFT1* and *BvFT2*. To further investigate the relationship between *BvBBX19* and *BTC1* I measured the *BvBBX19* expression in four *BTC1*-transgenic beets which grew in the greenhouse under LD conditions after twelve weeks of vernalization (see **Chapter 3**). Those beets had been transformed with the coding sequences of the dominant *BTC1_d* gene (seed code 120305, 120301) termed *35S:BTC1_d* and the recessive *btc1_a* gene (seed code 120255, 120268) termed *35S:btc1_a* under the control of the constitutive Cauliflower Mosaic Virus (CaMV) 35S promoter (**Supplementary Table 11**). As a donor plant the biennial sugar beet line 120309 was used. All transgenic beets are primary transformants (T_1). I grew 20 genetically identical plants (clones) from each transformant. The diurnal expression of *BTC1* and its putative downstream targets (*BvFT1* and *BvFT2*) was analyzed in two clones/ transformant by RT-qPCR in the same way as described for the non-transgenic sugar beet line (seed code 120309) (**Chapter 2.2.3**). Clones of two transformants (seed code 120301, 120268) showed high *BTC1* expression and started to bolt after vernalization. In contrast, clones of the other two transformants (seed code 120305, 120255) failed to bolt after cold treatment and were accordingly referred as being never bolting. Moreover, *BTC1* was completely down-regulated in those plants (see **Figure 20**).

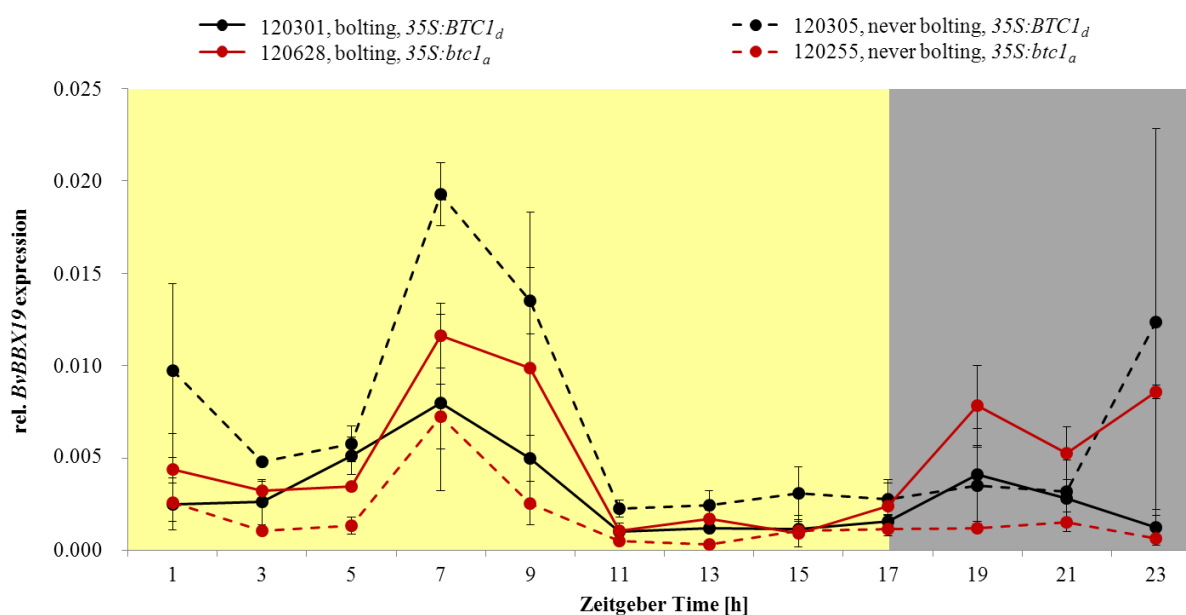


Figure 10: *BvBBX19* expression in transgenic beets overexpressing the *BTC1_d* (black lines) or *btc1_d* (red lines) allele under the control of the 35S promoter. All plants were cold treated for 12 weeks at 4°C and then grown under LD in the greenhouse. *BvBBX19* expressions of bolting and never bolting beets after vernalization are shown as solid or dashed lines, respectively. Each value is the mean of two biological and three technical replicates. The ΔC_T method was applied to calculate the relative *BvBBX19* expression. For normalization the housekeeping gene *BvGAPDH* was used. Error bars: standard deviation of the mean (\pm SDM) of two biological replicates.

I observed transcriptional activity of *BvBBX19* in each transgenic beet. In all plants investigated, the transcriptional activity of *BvBBX19* was quite low as compared to *BTC1*, *BvFT1* and *BvFT2* (Figure 20) but expression was at least three times higher in transgenic plants when compared to the vernalized non transgenic biennial sugar beet (seed code 120309) (Figure 10). Interestingly the expression pattern of *BvBBX19* in transgenic plants differs from that observed in the vernalized sugar beet line 120309 (Figure 10) with a distinct expression peak at ZT 7. That was also observed for *BTC1* in the same transgenic plants (see Chapter 3, Figure 10, Figure 20). As previously in the sugar beet line 120309, *BvBBX19* expression rapidly decreases to a minimum at ZT 11. The transcriptional activities differed between the analyzed transgenic beets. The highest *BvBBX19* expression was observed for plants of the 35S:*BTC1_d* transformant 120305, which failed to bolt after cold treatment, whereas *BvBBX19* was less expressed in plants of the 35S:*BTC1_d* transformant 120301, which bolted after vernalization. In transgenic beets transformed with the recessive *btc1_d* gene, *BvBBX19* was higher expressed in bolting plants (seed code 120268).

2.4 Discussion

Beet has become an interesting model to study the regulation of flowering time genes, because the flowering time genes *BvFT1* and *BTC1* acquire a new function compared to their *A. thaliana* homologs *FT* and *PRR7*. In *A. thaliana*, *FT* promotes flowering (Tiwari et al., 2010). In beet, two paralogs *BvFT1* and *BvFT2* had distinct functions with *BvFT1* acting as a floral repressor whereas *BvFT2* retained the function of the *FT* gene and acts as a floral integrator. Furthermore, *BTC1* controls the life cycle in beet through regulation of *BvFT1* and *BvFT2*. By contrast, in *A. thaliana* the circadian clock gene *PRR7* is not involved in the regulation of *FT*. This provides a totally new regulation of flowering time genes in beet that is different to the regulation known for Arabidopsis (Pin et al., 2010; Pin et al., 2012).

In this study, I present a classical map based cloning approach for cloning the *B2* gene in combination with sequenced based gene identification. I have fine mapped the locus *B2* with

two molecular markers flanking the locus with a genetic distance of 1.9 cM. In contrast to previous studies, I could use the draft assembly of the sugar beet reference sequence and predicted gene models which turned out to be extremely useful for the development of sequenced based molecular markers and gene identification. In this way, I could identify *BvBBX19* as a candidate gene for *B2*. Expression analyses revealed that *BvBBX19* is diurnally regulated and acts like *BTC1* upstream of *BvFT1* and *BvFT2*. Plants carrying the recessive *btc1* or the mutated *BvBBX19* gene showed a biennial growth habit, indicating that both genes act in conjunction to promote annual bolting in beet.

The locus *B2* had been previously mapped on chromosome 9 using a F₂ population termed EW2 consisting of 90 F₂ plants (Büttner et al., 2010). The resulting map of chromosome 9 encompassed 14 markers and has a size of 78.03 cM. The *B2* locus was mapped at position 52.61 cM, what is not near the telomeric region (Büttner et al., 2010). In contrast, I mapped the *B2* locus near the telomeric region. My data are more reliable as the data obtained from population EW2 because the increase of the phenotyped F₂ plants (~6000) lead to the identification of further recombinants. Moreover, phenotyping F₃ plants enabled the discrimination between homozygous and heterozygous annual F₂ plants. This finally improved the resolution of the genetic map on chromosome 9.

Crossover frequencies vary largely among plant chromosomes. They were found to be high in telomeric regions and low in centromeric regions (Dohm et al., 2012; Mayer et al., 2012; Schnable et al., 2009). Consistent with these results, the recombination rate observed for the *B2* locus with 45 recombinants/ 2,606 gametes analyzed is 3 fold higher than that observed for the *B* locus that was mapped near the centromeric region (107 recombinants/ 16,566 gametes) (Pin et al., 2012). Moreover, the observed ratio of the genetic to physical distance for the locus *B2* with 1 cM/ 0.145 Mbp is almost six fold higher as the estimated genome wide average of 1 cM/ 0.855 Mbp (Dohm et al., 2012) and the estimated ratio of 1 cM/ 0.79 Mbp on chromosome 9 (Paesold et al., 2012) whereas the recombination around the *B* locus was thought to be suppressed, because the genetic to physical distance was almost almost 20 fold lower (1cM/ 16.6 Mbp) compared to the genome wide average.

In the present study, the independent biennial mutant lines 056822 and 031823 which derived from an EMS mutagenesis of an annual genotype were molecularly analyzed. I identified a single transition in each of the mutants (seed code 056822, 031823) that occurred at different positions within the candidate gene. These results clearly point to EMS (= ethyl methansulfonate) induced mutation, because EMS causes point mutations from G/C to A/T, termed transitions, randomly in the genome. Accordingly, I assume that sequence variations within the candidate gene are responsible for the biennial character in beet and conclude that the identified candidate gene is the sought gene at the locus *B2*.

In a previous study, Büttner et al. (2010) observed differences in bolting behavior between two F₂ populations derived from crosses of two independent *B2*-mutants as seed parent with an annual sea beet as pollinator (056822/4 x 991971/9 and 031823/14 x 991971/11). These populations had approximately the same size (92 and 90 F₂ plants). In the 1st population (056822/4 x 991971/9), the authors observed only 2 F₂ plants that carried the mutated *B2*₀₅₆₈₂₂ allele in the homozygous state and bolted without vernalization. By contrast, in the 2nd population the amount of annual F₂ plants carrying the mutated *B2*₀₃₁₈₂₃ allele was at least 8 fold higher. It was suggested that phenotypic differences are largely determined genetically, because both F₂ populations were grown and phenotyped under field conditions in parallel. My results indicate that the predicted protein encoded by the candidate gene in the 1st mutant (seed code 056822) is truncated, whereas in the 2nd mutant (seed code 031823) a single codon has been changed. By taking the phenotypic results from Büttner et al. (2010) into account, I

assume that the truncated protein impairs the gene function in a stronger manner and thus results in a stronger phenotypic effect.

The predicted protein BvBBX19, encoded by the identified candidate gene shows high homology to the B-box protein B-BOX TYPE ZINC FINGER 19 (BBX19, AT4G38960.1) of *A. thaliana*. B-box (BBX) proteins are a class of zinc finger transcription factors (TFs) that possess one or two B-box motifs with a certain structure that are stabilized through the binding of Zinc ions (Khanna et al., 2009; Klug and Schwabe, 1995). TFs are proteins activating or repressing the transcription of their target genes and thus play a major role in plant growth and development.

In *Arabidopsis* the BBX protein family consists of 32 members and is divided into five subfamilies according to their protein sequence (Khanna et al., 2009). Subfamily I and II encompass 6 (BBX1-6) and 7 members (BBX7-13), respectively. Members of these subfamilies share the same structure: two B-box zinc finger motifs at the amino terminus and a CCT domain at the carboxy-terminus. Subfamily III is composed of 4 member (BBX14-17) which have one B-box motif and a CCT domain. Eight members (BBX18-25) of another subfamily (subfamily IV) carry two B-box motifs whereas only one B-box motif is present in the 7 members BBX26-32 of subfamily V. Members of subfamily I-III were previously termed as CO and CO-like COL proteins, based on their structure of at least one B-box and the CCT domain.

The first cloned *BBX* gene in *Arabidopsis* was *CO*, which is an important regulator of flowering under LD conditions in the photoperiod pathway and belongs to the subfamily I. The *CO* (BBX1) gene promotes flowering under LD conditions by activating the floral inducer gene *FT* through binding to its promoter (Tiwari et al., 2010). Under short day (SD) conditions, the *BBX6* gene induces flowering by promoting the expression of *FT* (Hassidim et al., 2009). In contrast, *BBX7* represses flowering under LD conditions by reducing the expression of *CO* and *FT* (Cheng and Wang, 2005). Besides the regulation of flowering, *BBX* genes have uncovered roles in the regulation of the circadian rhythm (*BBX2*) (Ledger et al., 2001) and recently, *BBX16* was shown to promote branching and suppress hypocotyl elongation which can be efficiently suppressed by shade (Wang et al., 2013). Moreover, 7 genes play a role in photomorphogenesis (*BBX4*, *BBX20*, *BBX21*, *BBX22*, *BBX24*, *BBX25*, *BBX32*) and contribute to the regulation of the key regulator genes of photomorphogenesis *LONG HYPOCOTYL 5 (HY5)* and its interacting partner *CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1)*, a suppressor of photomorphogenesis (Bowler, 2013; Datta et al., 2006; Fan et al., 2012; Holtan et al., 2011). *BBX18* has been related to flower development and additionally is involved in the regulation of basal and acquired thermo tolerance (Sarmiento, 2013). And finally, *BBX19* was characterized as being a negative regulator of photomorphogenesis, because transgenic plants overexpressing the *BBX19* coding sequence had shorter hypocotyls compared to Col-0 when grown under a wide range of continuous red or far-red light rates (Kumagai et al., 2008).

BBX genes were also identified in several other species only a few of these were functionally characterized so far. In rice (*Oryza sativa*), the BBX family consists of 30 *OsBBX* genes (Huang et al., 2012). The *CO* ortholog *Hd1* was shown to promote flowering under SD conditions and suppresses flowering under LD conditions (Yano et al., 2000). The *COL* gene *OsCOL4* functions as a floral repressor under both SD and LD conditions (Lee et al., 2010). Another *COL* gene, *OsCO3* which is not found in *Arabidopsis*, negatively regulates flowering in rice under SD conditions (Kim et al., 2008). Griffith et al. (2003) identified 9 *COL* genes in barley (*Hordeum vulgare*). Recently, the closest *CO* homolog *HvCO1* was shown as being a floral inducer (Campoli et al., 2012). In *B. vulgaris*, 13 *BBX* genes were identified, among these the closest *CO* homolog *BvCOL1* was functionally characterized. The authors demonstrated that overexpression of *BvCOL1* complements the late flowering *Arabidopsis*

co2 mutant. However, the diurnal expression profile of *BvCOL1* is different of *CO* that peaks in the late evening and more similar to that observed for *COL1* and *COL2* with highest expression before dawn, therefore the authors suggested that *BvCOL1* is not functional ortholog to *CO* (Chia et al., 2008).

Although it has not been reported that *BBX19* has an effect on flowering time in Arabidopsis, I suggest that the identified beet homolog *BvBBX19* is involved in the regulation of flowering time. In Arabidopsis and other species, an effect on flowering time was just reported for *CO* and *COL* genes, so far. This raises the possibility that *BvBBX19* has evolved another function similar to *CO* and *COL* genes. In contrast to *CO* and *COL* proteins, *BvBBX19* harbors two B-box domains but lacks the CCT domain. The single mutations found in each biennial mutant line occurred within the second B-box domain. These results strongly indicate that the second B-box domain is important for proper function of the *BvBBX19* protein to initiate floral transition. In Arabidopsis, the importance of B-box domains of the *CO* protein was demonstrated before by Robson et al. (2001) through analyzing several *CO* mutants that carry mutations in each the B-Box domains, respectively. They figured out that in all mutants flowering was delayed concluding that all B-box domains are important for proper *CO* function to initiate floral transition (Robson et al., 2001). By contrast, in *H. vulgare* the second B-box domain of *HvCO1* seems to be not essential for its function, because complementation of the late flowering Arabidopsis *co2* mutant carrying a T-DNA insertion within the second B-box was not achieved, whereby overexpression of the *HvCO1* gene in spring barley resulted in early flowering (Campoli et al., 2012).

Like other *CO* and *COL* genes, *BvBBX9* is also diurnal regulated. Moreover, I could demonstrate that it acts upstream of *BvFT1* and *BvFT2*. A regulation of *BvFT1* and *BvFT2* was previously demonstrated for *BTC1* by Pin et al. (2012). Expression analysis of *BTC1* and *BvBBX19* in *B2*-mutants and transgenic sugar beet plants demonstrated that misexpression of one gene affects the expression of the other gene. Taking these results into account and with regard to the fact that annual bolting is only promoted in plants carrying the dominant *BTC1_d* allele and the functional *BvBBX19* allele it could be suggested that proteins of these alleles interact to promote bolting through regulation of *BvFT1* and *BvFT2*. Protein-protein interactions involving BBX proteins have been demonstrated before. In a recent study by Gangappa et al. (2013) the B-box containing protein BBX25 from Arabidopsis was found to interact with the basic domain/leucine zipper (bZIP) transcription factor HY5. The authors clearly demonstrated by yeast two-hybrid and pull-down assays that BBX25 interacts with HY5 through its B-Boxes, because single substitutions in the B-boxes resulted in complete abolition of the interaction.

Regarding these results, I speculate that the interaction between *BTC1_d* and the mutated *BvBBX19* protein is abolished, due to the mutated B-box domain. Consequently the regulation of the flowering time genes *BvFT1* and *BvFT2* is not achieved and plants remain vegetative before winter. Although in the *B2*-mutant (seed code 056822) the floral repressor *BvFT1* is not down-regulated after vernalization, *BvFT2* is up-regulated and initiates bolting and flowering. It might be possible that after vernalization other genes contribute to the activation of *BvFT2* in a stronger manner than *BvFT1* represses *BvFT2*.

For biennials, carrying the functional *BvBBX19* but the recessive *btc1_a* allele it could be further speculated that an interaction is not achieved due to the lack of specific binding sites within the recessive *btc1_a* allele which are in turn present in the dominant *BTC1_d* allele. Proteins encoded by the dominant and recessive *BTC1* alleles differ in several non-synonymous polymorphisms. Moreover, the promoters differ by the presence of few *cis*-regulatory elements (SORLIP, box II) and a large insertion that is only present in the recessive allele and interrupts a series of GT-1 elements (Pin et al., 2012). It might be

suggested that at least one of these polymorphisms/ elements or its composition promotes the protein-protein interaction, whereas the absence of polymorphisms/ elements or differences of the composition inhibits this interaction and thus regulation of *BvFT1* and *BvFT2* is abolished before winter.

Regarding the known regulation of *FT* expression by the CO protein in *Arabidopsis* (Andrés and Coupland, 2012) and under the assumption that in beet the protein BvBBX19 interacts with *BTC1_d*, it is conceivable that this protein complex acquires a CO function. This protein complex would consist of two B-box domains, derived from BvBBX19 and a CCT as well as a response regulator receiver domain (REC) domain, derived from *BTC1_d* and thus resembles largely the protein structure of CO. Taking this into account, I proposed a new model for bolting and flowering control in beet (**Figure 11**) which provides an incentive for further studies like yeast two-hybrid to understand the proposed interaction between BvBBX19 and *BTC1_d*.

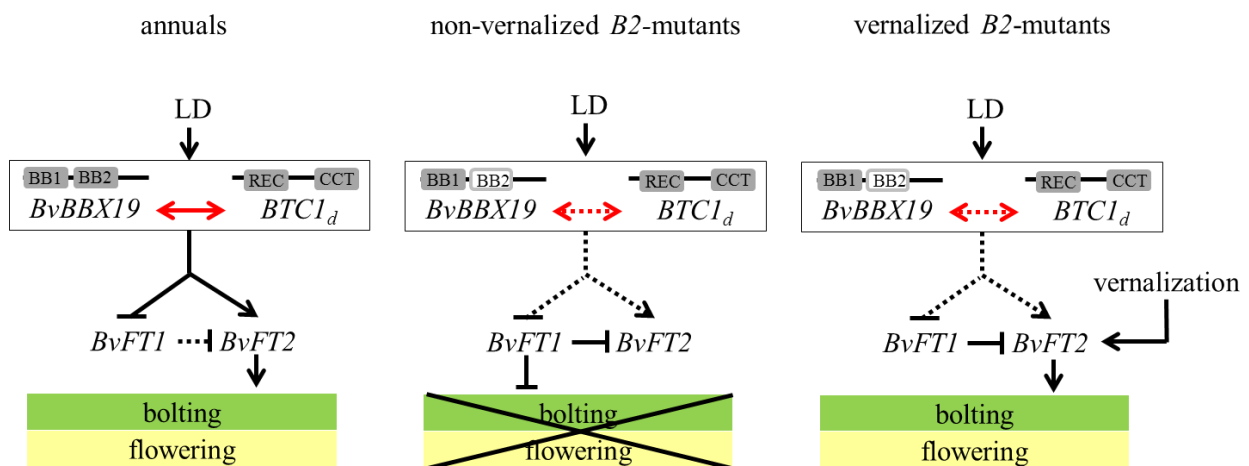


Figure 11: A proposed epistatic model for bolting time control in beet with *BvBBX19* and *BTC1_d* acting upstream of *BvFT1* and *BvFT2*. The domain structure of *BvBBX19* and *BTC1_d* proteins is given. Functional or mutated domains are indicated with filled shaded or open boxes, respectively. An interaction between *BvBBX19* and *BTC1_d* proteins to acquire a CO function was speculated. In annuals, the proteins *BvBBX19* and *BTC1_d* interact to repress *BvFT1* and activate *BvFT2* to promote bolting and flowering under LD conditions. In non-vernalized *B2*-mutants (seed code 056822) an interaction between *BTC1_d* and *BvBBX19* containing an altered BB2 domain, is impaired. Repression of *BvFT1* is not achieved and consequently *BvFT2* is repressed by *BvFT1* and plants grow vegetatively before vernalization. After vernalization and under LD *BvFT1* is not repressed in *B2*-mutants but the floral integrator *BvFT2* is highly activated to initiate bolting and flowering after vernalization. Direct interactions between genes are indicated with red lines; red dotted lines: inhibited direct interaction. Black lines between genes do not imply a direct interaction; dotted black lines: weak regulatory effect.

2.5 References

- Abou-Elwafa, S., B. Büttner, F. Kopsisch-Obuch, C. Jung, and A. Müller, 2012: Genetic identification of a novel bolting locus in *Beta vulgaris* which promotes annuality independently of the bolting gene B. *Molecular Breeding* 29, 989-998.
- Andrés, F., and G. Coupland, 2012: The genetic basis of flowering responses to seasonal cues. *Nature Reviews Genetics* 13, 627-639.
- Bowler, C., 2013: Photomorphogenesis, B-Box Transcription Factors, and the Legacy of Magnus Holm. *The Plant Cell* Vol. 25, 1192-1195
- Büttner, B., S.F. Abou-Elwafa, W. Zhang, C. Jung, and A.E. Müller, 2010: A survey of EMS-induced biennial *Beta vulgaris* mutants reveals a novel bolting locus which is unlinked to the bolting gene B. *Theor Appl Genet* 121, 1117-31.

- Campoli, C., B. Drosse, I. Searle, G. Coupland, and M. von Korff, 2012: Functional characterisation of *HvCO1*, the barley (*Hordeum vulgare*) flowering time ortholog of *CONSTANS*. *The Plant Journal* 69, 868-80.
- Cheng, X.F., and Z.Y. Wang, 2005: Overexpression of *COL9*, a *CONSTANS*-LIKE gene, delays flowering by reducing expression of *CO* and *FT* in *Arabidopsis thaliana*. *The Plant Journal* 43, 758-768.
- Chia, T.Y., A. Müller, C. Jung, and E.S. Mutasa-Göttgens, 2008: Sugar beet contains a large *CONSTANS*-LIKE gene family including a *CO* homologue that is independent of the early-bolting (*B*) gene locus. *J Exp Bot* 59, 2735-48.
- Datta, S., G. Hettiarachchi, X.W. Deng, and M. Holm, 2006: *Arabidopsis* *CONSTANS*-LIKE3 is a positive regulator of red light signaling and root growth. *Plant Cell* 18, 70-84.
- Dohm, J.C., C. Lange, D. Holtgräwe, T. Rosleff Sorensen, D. Borchardt, B. Schulz, H. Lehrach, B. Weisshaar, and H. Himmelbauer, 2011: Palaeohexaploid Ancestry for Caryophyllales Inferred from Extensive Gene-Based Physical and Genetic Mapping of the Sugar Beet Genome (*Beta vulgaris*). *The Plant Journal* 70, 528-540.
- Fan, X.Y., Y. Sun, D.M. Cao, M.Y. Bai, X.M. Luo, H.J. Yang, C.Q. Wei, S.W. Zhu, Y. Sun, K. Chong, and Z.Y. Wang, 2012: BZS1, a B-box Protein, Promotes Photomorphogenesis Downstream of Both Brassinosteroid and Light Signaling Pathways. *Molecular Plant* 5, 591-600.
- Gangappa, S.N., C.D. Crocco, H. Johansson, S. Datta, C. Hettiarachchi, M. Holm, and J.F. Botto, 2013: The *Arabidopsis* B-BOX Protein BBX25 Interacts with HY5, Negatively Regulating *BBX22* Expression to Suppress Seedling Photomorphogenesis. *Plant Cell* 25, 1243-1257.
- Griffiths, S., R.P. Dunford, G. Coupland, and D.A. Laurie, 2003: The evolution of *CONSTANS*-like gene families in barley, rice, and *Arabidopsis*. *Plant Physiology* 131, 1855-67.
- Hassidim, M., Y. Harir, E. Yakir, I. Kron, and R.M. Green, 2009: Over-expression of *CONSTANS*-LIKE 5 can induce flowering in short-day grown *Arabidopsis*. *Planta* 230, 481-491.
- Hohmann, U., G. Jacobs, and C. Jung, 2005: An EMS mutagenesis protocol for sugar beet and isolation of non-bolting mutants. *Plant Breeding* 124, 317-321.
- Holtan, H.E., S. Bandong, C.M. Marion, L. Adam, S. Tiwari, Y. Shen, J.N. Maloof, D.R. Maszle, M.A. Ohto, S. Preuss, R. Meister, M. Petracek, P.P. Repetti, T.L. Reuber, O.J. Ratcliffe, and R. Khanna, 2011: BBX32, an *Arabidopsis* B-Box Protein, Functions in Light Signaling by Suppressing HY5-Regulated Gene Expression and Interacting with STH2/BBX21. *Plant Physiology* 156, 2109-2123.
- Huang, J., X. Zhao, X. Weng, L. Wang, and W. Xie, 2012: The Rice B-Box Zinc Finger Gene Family: Genomic Identification, Characterization, Expression Profiling and Diurnal Analysis. *PLoS One* 7, e48242.
- Kardailsky, I., V.K. Shukla, J.H. Ahn, N. Dagenais, S.K. Christensen, J.T. Nguyen, J. Chory, M.J. Harrison, and D. Weigel, 1999: Activation tagging of the floral inducer *FT*. *Science* 286, 1962-1965.

- Khanna, R., B. Kronmiller, D.R. Maszle, G. Coupland, M. Holm, T. Mizuno, and S.H. Wu, 2009: The *Arabidopsis* B-box zinc finger family. *The Plant cell* 21, 3416-20.
- Kim, S.-K., C.-H. Yun, J.H. Lee, Y.H. Jang, H.-Y. Park, and J.-K. Kim, 2008: *OsCO3*, a *CONSTANS*-LIKE gene, controls flowering by negatively regulating the expression of *FT*-like genes under SD conditions in rice. *Planta* 228, 355-365.
- Klug, A., and J.W. Schwabe, 1995: Protein motifs 5. Zinc fingers. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 9, 597-604.
- Kosambi, D.D., 1943: The estimation of map distances from recombination values. *Annals of Human Genetics* 12, 172-175.
- Kumagai, T., S. Ito, N. Nakamichi, Y. Niwa, M. Murakami, T. Yamashino, and T. Mizuno, 2008: The common function of a novel subfamily of B-box zinc finger proteins with reference to circadian-associated events in *Arabidopsis thaliana*. *Bioscience, Biotechnology, and Biochemistry* 72, 1539-1549.
- Ledger, S., C. Strayer, F. Ashton, S.A. Kay, and J. Putterill, 2001: Analysis of the function of two circadian-regulated *CONSTANS*-LIKE genes. *The Plant Journal* 26, 15-22.
- Lee, Y.-S., D.-H. Jeong, D.-Y. Lee, J. Yi, C.-H. Ryu, S.L. Kim, H.J. Jeong, S.C. Choi, P. Jin, J. Yang, L.-H. Cho, H. Choi, and G. An, 2010: *OsCOL4* is a constitutive flowering repressor upstream of *Ehd1* and downstream of *OsphyB*. *The Plant Journal* 63, 18-30.
- Letunic, I., T. Doerks, and P. Bork, 2012: SMART 7: recent updates to the protein domain annotation resource. *Nucleic acids research* 40, D302-5.
- Mayer, K.F.X., R. Waugh, P. Langridge, T.J. Close, R.P. Wise, A. Graner, T. Matsumoto, K. Sato, A. Schulman, G.J. Muehlbauer, N. Stein, R. Ariyadasa, D. Schulte, N. Poursarebani, R.N. Zhou, B. Steuernagel, M. Mascher, U. Scholz, B.J. Shi, P. Langridge, K. Madishetty, J.T. Svensson, P. Bhat, M. Moscou, J. Resnik, T.J. Close, G.J. Muehlbauer, P. Hedley, H. Liu, J. Morris, R. Waugh, Z. Frenkel, A. Korol, H. Berges, A. Graner, N. Stein, B. Steuernagel, S. Taudien, M. Groth, M. Felder, M. Platzer, J.W.S. Brown, A. Schulman, M. Platzer, G.B. Fincher, G.J. Muehlbauer, K. Sato, S. Taudien, D. Sampath, D. Swarbreck, S. Scalabrin, A. Zuccolo, V. Vendramin, M. Morgante, K.F.X. Mayer, A. Schulman, and I.B.G.S. Conso, 2012: A physical, genetic and functional sequence assembly of the barley genome. *Nature* 491, 711-716.
- Meier, U., 1993: Growth stages of mono- and dicotyledonous plants. Phenological growth stages and BBCH-identification keys of beet. Federal Biological Research Centre for Agriculture and Forestry, Braunschweig, Germany.
- Paesold, S., D. Borchardt, T. Schmidt, and D. Dechyeva, 2012: A sugar beet (*Beta vulgaris* L.) reference FISH karyotype for chromosome and chromosome-arm identification, integration of genetic linkage groups and analysis of major repeat family distribution. *The Plant Journal* 72, 600-11.
- Pin, P.A., R. Benlloch, D. Bonnet, E. Wremerth-Weich, T. Kraft, J.J.L. Gielen, and O. Nilsson, 2010: An Antagonistic Pair of *FT* Homologs Mediates the Control of Flowering Time in Sugar Beet. *Science* 330, 1397-1400.
- Pin, P.A., W. Zhang, S.H. Vogt, N. Dally, B. Büttner, G. Schulze-Buxloh, N.S. Jelly, T.Y. Chia, E.S. Mutasa-Göttgens, J.C. Dohm, H. Himmelbauer, B. Weisshaar, J. Kraus, J.J. Gielen, M. Lommel, G. Weyens, B. Wahl, A. Schechert, O. Nilsson, C. Jung, T. Kraft, and A.E.

Müller, 2012: The role of a pseudo-response regulator gene in life cycle adaptation and domestication of beet. *Curr Biol* 22, 1095-101.

Reeves, P.A., Y. He, R.J. Schmitz, R.M. Amasino, L.W. Panella, and C.M. Richards, 2007: Evolutionary conservation of the *FLOWERING LOCUS C*-mediated vernalization response: evidence from the sugar beet (*Beta vulgaris*). *Genetics* 176, 295-307.

Robson, F., M.M. Costa, S.R. Hepworth, I. Vizir, M. Pineiro, P.H. Reeves, J. Putterill, and G. Coupland, 2001: Functional importance of conserved domains in the flowering-time gene *CONSTANS* demonstrated by analysis of mutant alleles and transgenic plants. *The Plant Journal* 28, 619-31.

Saghai-Marooif, M.A., K.M. Soliman, R.A. Jorgensen, and R.W. Allard, 1984: Ribosomal DNA spacer-length polymorphisms in barley: mendelian inheritance, chromosomal location, and population dynamics. *Proc Natl Acad Sci U S A* 81, 8014-8.

Sarmiento, F., 2013: The BBX subfamily IV: Additional cogs and sprockets to fine-tune light-dependent development. *Plant Signal Behav* 8.

Schnable, P.S., D. Ware, R.S. Fulton, J.C. Stein, F. Wei, S. Pasternak, C. Liang, J. Zhang, L. Fulton, T.A. Graves, P. Minx, A.D. Reily, L. Courtney, S.S. Kruchowski, C. Tomlinson, C. Strong, K. Delehaunty, C. Fronick, B. Courtney, S.M. Rock, E. Belter, F. Du, K. Kim, R.M. Abbott, M. Cotton, A. Levy, P. Marchetto, K. Ochoa, S.M. Jackson, B. Gillam, W. Chen, L. Yan, J. Higginbotham, M. Cardenas, J. Waligorski, E. Applebaum, L. Phelps, J. Falcone, K. Kanchi, T. Thane, A. Scimone, N. Thane, J. Henke, T. Wang, J. Ruppert, N. Shah, K. Rotter, J. Hodges, E. Ingenthron, M. Cordes, S. Kohlberg, J. Sgro, B. Delgado, K. Mead, A. Chinwalla, S. Leonard, K. Crouse, K. Collura, D. Kudrna, J. Currie, R. He, A. Angelova, S. Rajasekar, T. Mueller, R. Lomeli, G. Scara, A. Ko, K. Delaney, M. Wissotski, G. Lopez, D. Campos, M. Braidotti, E. Ashley, W. Golser, H. Kim, S. Lee, J. Lin, Z. Dujmic, W. Kim, J. Talag, A. Zuccolo, C. Fan, A. Sebastian, M. Kramer, L. Spiegel, L. Nascimento, T. Zutavern, B. Miller, C. Ambroise, S. Muller, W. Spooner, A. Narechania, L. Ren, S. Wei, S. Kumari, B. Faga, M.J. Levy, L. McMahan, P. Van Buren, M.W. Vaughn, K. Ying, C.-T. Yeh, S.J. Emrich, Y. Jia, A. Kalyanaraman, A.-P. Hsia, W.B. Barbazuk, R.S. Baucom, T.P. Brutnell, N.C. Carpita, C. Chaparro, J.-M. Chia, J.-M. Deragon, J.C. Estill, Y. Fu, J.A. Jeddloh, Y. Han, H. Lee, P. Li, D.R. Lisch, S. Liu, Z. Liu, D.H. Nagel, M.C. McCann, P. SanMiguel, A.M. Myers, D. Nettleton, J. Nguyen, B.W. Penning, L. Ponnala, K.L. Schneider, D.C. Schwartz, A. Sharma, C. Soderlund, N.M. Springer, Q. Sun, H. Wang, M. Waterman, R. Westerman, T.K. Wolfgruber, L. Yang, Y. Yu, L. Zhang, S. Zhou, Q. Zhu, J.L. Bennetzen, R.K. Dawe, J. Jiang, N. Jiang, G.G. Presting, S.R. Wessler, S. Aluru, R.A. Martienssen, S.W. Clifton, W.R. McCombie, R.A. Wing, and R.K. Wilson, 2009: The B73 Maize Genome: Complexity, Diversity, and Dynamics. *Science* 326, 1112-1115.

Schneider, K., D. Kulosa, T.R. Soerensen, S. Mohring, M. Heine, G. Durstewitz, A. Polley, E. Weber, Jamsari, J. Lein, U. Hohmann, E. Tahiro, B. Weisshaar, B. Schulz, G. Koch, C. Jung, and M. Ganal, 2007: Analysis of DNA polymorphisms in sugar beet (*Beta vulgaris* L.) and development of an SNP-based map of expressed genes. *Theor Appl Genet* 115, 601-15.

Schultz, J., F. Milpetz, P. Bork, and C.P. Ponting, 1998: SMART, a simple modular architecture research tool: identification of signaling domains. *Proceedings of the National Academy of Sciences of the United States of America* 95, 5857-64.

- Suarez-Lopez, P., K. Wheatley, F. Robson, H. Onouchi, F. Valverde, and G. Coupland, 2001: *CONSTANS* mediates between the circadian clock and the control of flowering in *Arabidopsis*. *Nature* 410, 1116-1120.
- Tiwari, S.B., Y. Shen, H.C. Chang, Y. Hou, A. Harris, S.F. Ma, M. McPartland, G.J. Hymus, L. Adam, C. Marion, A. Belachew, P.P. Repetti, T.L. Reuber, and O.J. Ratcliffe, 2010: The flowering time regulator *CONSTANS* is recruited to the *FLOWERING LOCUS T* promoter via a unique cis-element. *New Phytologist* 187, 57-66.
- Valverde, F., 2011: *CONSTANS* and the evolutionary origin of photoperiodic timing of flowering. *Journal of Experimental Botany* 62, 2453-2463.
- Van Oijen, J., and R. Voorrips, 2001: JoinMap® 3.0, software for the calculation of genetic linkage maps. Plant Research International. Wageningen, The Netherlands.
- Wang, H.G., Z.L. Zhang, H.Y. Li, X.Y. Zhao, X.M. Liu, M. Ortiz, C.T. Lin, and B. Liu, 2013: *CONSTANS-LIKE 7* regulates branching and shade avoidance response in *Arabidopsis*. *Journal of Experimental Botany* 64, 1017-1024.
- Yano, M., Y. Katayose, M. Ashikari, U. Yamanouchi, L. Monna, T. Fuse, T. Baba, K. Yamamoto, Y. Umehara, Y. Nagamura, and T. Sasaki, 2000: *Hdl*, a major photoperiod sensitivity quantitative trait locus in rice, is closely related to the *Arabidopsis* flowering time gene *CONSTANS*. *Plant Cell* 12, 2473-2483.

3 Functional analysis of the *BOLTING TIME CONTROL 1 (BTC1)* gene I: transformation into sugar beet

3.1 Introduction

The species *Beta vulgaris* (*B. vulgaris*) comprises annual, biennial and perennial forms which differ in their life cycle. Annuals exhibit an annual growth habit resulting in stem elongation (bolting) and flower formation under inductive photoperiods within one year. In contrast, biennials require a prolonged period of cold temperatures (vernalization) to initiate bolting and flowering in the following year under inductive photoperiods. Perennials can exhibit long juvenile periods and typically flower for several years (Lange et al., 1999).

Sugar beet exhibits a biennial life cycle and is cultivated for sugar production and biomass production (see **Chapter 1.1**). Under central European climates, sugar beets are sown in spring and harvested in autumn. To improve the biomass and sugar yield a prolonged vegetative growth phase is essential. Since a decade, winter beet production under central European climates has been discussed (Eichholz and Röstel, 1962). Winter beets would be sown in August and harvested in the following year. This would require winter-hardiness and never bolting (non-bolting after a prolonged period of cold temperatures). On the other hand, seed production of winter beets must also be ensured and therefore they have to bolt and flower.

The annual growth habit is controlled by the dominant *BOLTING TIME CONTROL 1 (BTC1)* gene. By contrast, biennial beets carry the recessive *btc1* gene. There are three different haplotypes in biennials (*btc1_{a-c}*) and 8 different haplotypes in annuals (*BTC1_{d-k}*) (see **Chapter 1.3**) (Pin et al., 2012). Annual and biennial *BTC1* genes differ in several non-synonymous single nucleotide polymorphisms (SNPs) and differ in their promoter sequence in a 28 kbp large insertion, which is only present in the biennial promoter (see **Chapter 1.3, Table 1**) (Pin et al., 2012).

Both, the dominant and the recessive *BTC1* genes are diurnally regulated with highest expression around zeitgeber time (ZT) 10 under long days (LD). In annuals, *BTC1* is higher expressed at the end of the light phase when compared to non-vernalized biennials. In biennials, *btc1* expression will be enhanced during the vernalization and thus *btc1* expression levels were higher throughout most of the light phase (Pin et al., 2012). *BTC1* is an upstream regulator of two *FLOWERING LOCUS T (FT)* paralogs *BvFT1* and *BvFT2*. In annuals, the dominant *BTC1* gene mediates the downregulation of the floral repressor gene *BvFT1* and upregulation of the floral inducer gene *BvFT2* to induce bolting and flowering under inductive photoperiods. In biennials, the regulation of *BvFT1* and *BvFT2* is not achieved by the recessive *btc1* gene (Pin et al., 2012). The authors suggested that the reduced response to inductive photoperiods is due to a partial loss-of-function mutation of the biennial allele (Pin et al., 2012).

Several transgenic plants were generated either by overexpression of *BvFT1* or downregulation of *BTC1*, *btc1* or *BvFT2* by RNAi. None of these plants bolted before cold treatment and even after cold treatment bolting was suppressed or delayed. These plants could be a suitable resource for development of a winter beet but unfortunately no transgenic plant flowered and thus seed production was not ensured (Pin et al., 2010; Pin et al., 2012).

Besides the three major genes *BTC1*, *BvFT1* and *BvFT2* more flowering time genes have been cloned so far. Their function was analyzed in Arabidopsis. The sugar beet gene *FLOWERING LOCUS C – LIKE (BvFLI)*, a homolog of Arabidopsis *FLOWERING LOCUS C (FLC)*, a key

regulator of the vernalization response, was shown to be negatively regulated by vernalization and delayed flowering in transgenic *Arabidopsis* plants (Reeves et al., 2007). Additionally, overexpression of the *B. vulgaris* *CONSTANS LIKE 1* (*BvCOL1*) gene, a homolog of the photoperiodic pathway gene *CONSTANS* (*CO*), complements the flowering time phenotype of *A. thaliana* loss-of-function *co2* mutant (Chia et al., 2008). Furthermore, three additional loci termed *B2*, *B3* and *B4* have been identified in populations derived from crosses between an annual sea beet accession and biennial genotypes, isolated after EMS mutagenesis (see **Chapter 2.1**). It was shown that dominant alleles at these loci promote annual bolting (Abou-Elwafa et al., 2012; Büttner et al., 2010; Hohmann et al., 2005). Abou-Elwafa et al. (2010) described the identification of the *FLOWERING LOCUS K* (*FLK*) homolog *BvFLK*. Overexpression in *Arabidopsis* accelerates bolting in the Col-0 background and a complete complementation of the late-bolting phenotype of a *flk* mutant through repression of *FLC* could be shown. Moreover, it was possible to identify full-length homologues of the *Arabidopsis* chromatin-regulatory genes *FLOWERING LOCUS VE* (*FVE*), *LUMINIDEPENDENS* (*LD*), and *LYSINE-SPECIFIC HISTONE DEMETHYLASE1 LIKE1* (*LDL1*) in sugar beet (Abou-Elwafa et al., 2010).

In the present study, I used a reverse genetic approach to further investigate putative functional differences between the annual and biennial *BTC1* gene in transgenic sugar beet plants. Therefore, I overexpressed the respective *BTC1* allele under the control of the constitutive CaMV 35S promoter in a biennial background. I hypothesized that constitutive expression of each allele in a biennial background results in an annual growth habit of transgenic plants. Consequently, I further hypothesized that the annual as well as the biennial *BTC1* allele is functional and different growth habits are due to low *btc1* expression before winter in biennials. My data indicate that constitutive high expression of *BTC1* in the generated transgenic plants cannot be achieved. Consequently all transgenic plants had a certain requirement for vernalization. I observed no phenotypic differences between plants carrying the annual or biennial transgene, suggesting that both alleles are functional. Nevertheless my results demonstrate for the first time transgene-mediated co-suppression in transgenic sugar beet plants carrying multiple integrations of the *BTC1* transgene, which results in a never bolting phenotype after cold treatment. The data of my study provide a suitable resource with deep impact on breeding winter beets, to generate never bolting hybrids for which seed production could be ensured.

3.2 Material and methods

3.2.1 Plant material

All plant materials and obtained seeds generated and analyzed during this study are listed in **Table 9**. Reverse transcribed RNA derived from the sugar beet lines 930190 and 940043 was used for amplification of the *BTC1* coding sequence, respectively. The annual line 930190 corresponds to 93167P (El-Mezawy et al., 2002). It carries the homozygous dominant *BTC1_a* allele, while the biennial line 940043 carries the homozygous recessive *btc1_a* allele. For sugar beet transformation (**Chapter 3.2.3**) the biennial sugar beet line 120309 (according to 3DC4156, KWS Saatzucht GmbH, Einbeck, Germany) was used. Transgenic plants (T₁ generation) were phenotyped (**Chapter 3.2.5**) and propagated to yield T₂ seeds.

Table 9: Plant material produced and analyzed during this study.

Seed Code/ Generation	Experiment ¹	Total Number of Clones/ T ₁ Generated	Transgene Cassette	Phenotype ²	T ₂ Seed Code of Propagated Clones	Seeds Available
930190	RT-PCR <i>BTC1_d</i> CDS	-	-	annual		
940043	RT-PCR <i>btc1_a</i> CDS	-	-	biennial		
120309	3	6	-	biennial	130333	514
120248/ T ₁	1, 2, 3	20	<i>35S:btc1_a</i>	segregating	-	-
120249/ T ₁	1, 2, 3	20	<i>35S:btc1_a</i>	biennial	122439 122440	189 553
120250/ T ₁	1, 2, 3	20	<i>35S:btc1_a</i>	segregating	-	-
120251/ T ₁	1, 2, 3	20	<i>35S:btc1_a</i>	biennial	122441 122442	324 518
120252/ T ₁	1, 2, 3	18	<i>35S:btc1_a</i>	biennial	122443 122444	260 373
120253/ T ₁	1, 2, 3	20	<i>35S:btc1_a</i>	biennial	122445 122446	698 208
120254/ T ₁	1, 2, 3	20	<i>35S:btc1_a</i>	biennial	122447 122448	278 700
120255/ T ₁	1, 2, 3	20	<i>35S:btc1_a</i>	never bolting	-	-
120256/ T ₁	1, 2, 3	20	<i>35S:btc1_a</i>	biennial	122449 122450	315 488
120257/ T ₁	1, 2, 3	20	<i>35S:btc1_a</i>	biennial	122451	0
120258/ T ₁	1, 2, 3	20	<i>35S:btc1_a</i>	biennial	122452 122453	56 0
120259/ T ₁	1, 2	10	<i>35S:btc1_a</i>	n.a. ³	-	-
120260/ T ₁	1, 2	10	<i>35S:btc1_a</i>	n.a. ³	-	-
120261/ T ₁	1, 2	10	<i>35S:btc1_a</i>	n.a. ³	-	-
120262/ T ₁	1, 2	10	<i>35S:btc1_a</i>	n.a. ³	-	-
120263/ T ₁	1, 2	10	<i>35S:btc1_a</i>	n.a. ³	-	-
120264/ T ₁	1, 2, 3	20	<i>35S:btc1_a</i>	segregating	122456	55
120265/ T ₁	1, 2, 3	20	<i>35S:btc1_a</i>	biennial	122457	0
120266/ T ₁	1, 2, 3	20	<i>35S:btc1_a</i>	segregating	-	-
120267/ T ₁	1, 2, 3	20	<i>35S:btc1_a</i>	biennial	-	-
120268/ T ₁	1, 2, 3	20	<i>35S:btc1_a</i>	biennial	122458 122459	0 0
120269/ T ₁	1, 2, 3	20	<i>35S:btc1_a</i>	segregating	122460 122461	0 76
120270/ T ₁	1, 2, 3	20	<i>35S:btc1_a</i>	biennial	122462 122463	43 428
120271/ T ₁	1, 2, 3	20	<i>35S:btc1_a</i>	segregating	122464	56
120272/ T ₁	1, 2, 3	17	<i>35S:btc1_a</i>	biennial	122465	87

Seed Code/ Generation	Experiment ¹	Total Number of Clones/ T ₁ Generated	Transgene Cassette	Phenotype ²	T ₂ Seed Code of Propagated Clones	Seeds Available
120273/ T ₁	1, 2, 3	20	<i>35S:btc1_a</i>	segregating	-	-
120274/ T ₁	1, 2, 3	17	<i>35S:btc1_a</i>	biennial	122466 122467	500 450
120275/ T ₁	1, 2, 3	20	<i>35S:btc1_a</i>	biennial	122468 122469	858 470
120276/ T ₁	1, 2, 3	20	<i>35S:BTC1_d</i>	biennial	122470 122471	503 153
120277/ T ₁	1, 2, 3	20	<i>35S:BTC1_d</i>	segregating	122472 122473	0 502
120278/ T ₁	1, 2, 3	20	<i>35S:BTC1_d</i>	biennial	122474 122475	407 289
120279/ T ₁	1, 2, 3	20	<i>35S:BTC1_d</i>	biennial	122476	378
120280/ T ₁	1, 2, 3	20	<i>35S:BTC1_d</i>	biennial	122477	379
120281/ T ₁	1, 2, 3	20	<i>35S:BTC1_d</i>	biennial	122478 122479	71 122
120282/ T ₁	1, 2, 3	20	<i>35S:BTC1_d</i>	biennial	122480	240
120283/ T ₁	1, 2, 3	20	<i>35S:BTC1_d</i>	biennial	122481	15
120284/ T ₁	1, 2, 3	20	<i>35S:BTC1_d</i>	biennial	122482 122483	0 87
120285/ T ₁	1, 2, 3	20	<i>35S:BTC1_d</i>	biennial	122484 122485	157 523
120286/ T ₁	1, 2, 3	20	<i>35S:BTC1_d</i>	segregating	-	-
120287/ T ₁	1, 2, 3	20	<i>35S:BTC1_d</i>	biennial	122486 122487	153 83
120288/ T ₁	1, 2, 3	20	<i>35S:BTC1_d</i>	biennial	122488 122489	471 189
120289/ T ₁	1, 2, 3	20	<i>35S:BTC1_d</i>	biennial	122490	313
120290/ T ₁	1, 2, 3	20	<i>35S:BTC1_d</i>	biennial	122491 122492	196 276
120291/ T ₁	1, 2, 3	20	<i>35S:BTC1_d</i>	biennial	122493 122494	545 316
120292/ T ₁	1, 2, 3	20	<i>35S:BTC1_d</i>	segregating	-	-
120293/ T ₁	1, 2, 3	17	<i>35S:BTC1_d</i>	segregating	-	-
120294/ T ₁	1, 2, 3	20	<i>35S:BTC1_d</i>	segregating	122495	74
120295/ T ₁	1, 2, 3	20	<i>35S:BTC1_d</i>	never bolting	-	-
120296/ T ₁	1, 2, 3	20	<i>35S:BTC1_d</i>	biennial	122496 122497	351 579
120297/ T ₁	1, 2, 3	20	<i>35S:BTC1_d</i>	segregating	-	-
120298/ T ₁	1, 2, 3	20	<i>35S:BTC1_d</i>	biennial	122498 122499	34 176
120299/ T ₁	1, 2, 3	20	<i>35S:BTC1_d</i>	biennial	122500 122501	0 532

Seed Code/ Generation	Experiment ¹	Total Number of Clones/ T ₁ Generated	Transgene Cassette	Phenotype ²	T ₂ Seed Code of Propagated Clones	Seeds Available
120300/ T ₁	1, 2, 3	20	<i>35S:BTC1_d</i>	biennial	-	-
120301/ T ₁	1, 2, 3	20	<i>35S:BTC1_d</i>	biennial	122502 122503	340 191
120302/ T ₁	1, 2, 3	20	<i>35S:BTC1_d</i>	biennial	122504 122505	621 755
120303/ T ₁	1, 2, 3	20	<i>35S:BTC1_d</i>	segregating	-	-
120304/ T ₁	1, 2, 3	20	<i>35S:BTC1_d</i>	segregating	-	-
120305/ T ₁	1, 2, 3	20	<i>35S:BTC1_d</i>	never bolting	-	-
120306/ T ₁	1, 2, 3	20	<i>35S:BTC1_d</i>	never bolting	-	-
120307/ T ₁	1, 2, 3	20	<i>35S:BTC1_d</i>	segregating	-	-
120308/ T ₁	1, 2, 3	12	<i>35S:BTC1_d</i>	biennial	122506 122507	362 304

¹ Experiment 1: 5 clones/ T₁ cold treated for 4 weeks; Experiment 2: 5 clones/ T₁ cold treated for 8 weeks; Experiment 3: 10 clones/ T₁ cold treated for 12 weeks; Experimental design see **Figure 14**, **Figure 15**, **Table 10** and **Supplementary Table 6**.

² Phenotypes were set according to phenotypic results after 12 weeks of cold treatment in Kiel (CAU Kiel, Germany) biennial: bolting after 12 weeks vernalization; never bolting: non bolting after 12 weeks of cold treatment; segregating: biennial and never bolting individuals within 10 analyzed clones/ transformant.

³ n.a.: not available; no phenotyping/ seed production performed after 12 weeks of cold treatment (Experiment 1), because 10 clones dropped down during the transport to Kiel

3.2.2 Vector construction

Total RNA was extracted from leaves of adult plants of the sugar beet lines 930190 and 940043 and reverse transcribed (**Chapter 3.2.7**). Complete coding sequences of *BTC1* were amplified by RT-PCR using cDNA as template and the gene specific primers C011/ C012 (C. Tränkner, pers. communication). Primers were derived from the exonic region of the *BTC1* sequence flanking the open reading frame (ORF) (**Table 11**).

The PCR fragments were ligated into the cloning vector pJet1.2 (Fermentas, St. Leon-Rot, Germany) and transformed into a heat-shock competent *E. coli* strain to yield the plasmids CAU3668, carrying the *btc1_a* coding sequence and CAU3669 (*BTC1_d*). Integrated coding sequences *BTC1_d* and *btc1_a* were amplified from the plasmids CAU3668 and CAU3669 using the primers A984/ A985. The resulting PCR product was used as template for a second PCR with the primers Xma_BTC1_F/ Sal_BTC1_R. These primers have overlapping ends with a certain sequence, which will be recognized by the enzymes *XmaI* and *SalI*, respectively. In a next step these PCR products were restricted with *Cfr9I* (an isoschizomer of *XmaI*) and *SalI* (Fermentas, St. Leon-Rot, Germany).

Digested PCR fragments were subcloned into the corresponding restriction enzyme sites of the binary vector p9N-35S (DNA cloning service, Hamburg, Germany) behind the constitutive Cauliflower Mosaic Virus (CaMV) 35S promoter and finally transformed into a heat shock competent *E. coli* strain. As a selectable marker the binary vector carries the *neomycin phosphotransferase II* (*nptII*) gene, which encodes for a protein conferring resistance to the antibiotic agent kanamycin. The recombinant plasmids were named as follows: CAU3713, carrying the *35S:BTC1_d* transgenic cassette and CAU3714 (transgenic cassette: *35S:btc1_a*) (**Figure 12**). The cloned inserts were verified by Sanger sequencing at the Institute for Clinical Molecular Biology (IKMB, Kiel, Germany). Whole transgenic cassettes

including the promoter and terminator of the plasmids CAU3713 and CAU3714 were sequenced using the primers A747, p35S_F, pLH_F, pLH_R, A592 and BvBTC1_SeqAdd_F. Sequencing was performed with 3 μ l (100ng/ μ l) plasmid DNA (CAU3713 and CAU3714) and a final primer concentration of 4.8 μ M. Revealed sequences were assembled and compared to the predicted sequence using the alignment and assembly function in the CLC Main Workbench (version: 5.7.1). Finally, the recombinant plasmids CAU3713 and CAU3714 were transformed into *A. tumefaciens* GV3101 PMP90 RK (Koncz and Schell, 1986) and the hypervirulent *A. tumefaciens* strain AGL1 (Lazo et al., 1991) (KWS Saatzucht GmbH, Einbeck, Germany).

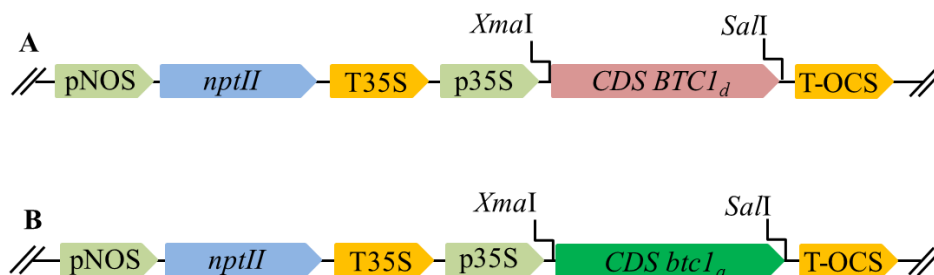


Figure 12: T-DNA region of the transformation vectors A) CAU3713 carrying the *BTC1_d* CDS and B) CAU3714 carrying the *btc1_a* CDS. Arrows in light green represent promoter regions with pNOS: promoter derived from the *nopaline synthase* gene and p35S: cauliflower mosaic virus (CaMV) 35S promoter. The blue arrow represents the selectable marker gene *neomycin phosphotransferase II* gene (*nptII*) for selection of transgenic shoots (see Chapter 3.2.3). In yellow, terminator regions with T35S: 35S terminator derived from CaMV and T-OCS: terminator derived from the *octopine synthase* gene. Red arrow: coding sequence of *BTC1_d* derived from the annual sugar beet line 930190 (A); dark green arrow: coding sequence of *btc1_a* derived from the biennial line 940043 (B). *XmaI* and *SalI*, restriction sites used for cloning the respective *BTC1* CDS.

3.2.3 *Agrobacterium* mediated transformation

Transformation experiments, identification of transgenic shoots, micropropagation, induction of root growth as well as the determination of the copy number (Chapter 3.2.4), have been performed by Dr. Joseph Kraus (KWS Saatzucht GmbH, Einbeck, Germany). Binary vector constructs CAU3713 and CAU3714 were used for *Agrobacterium*-mediated transformation into the biennial sugar beet line 120309. The transformation was performed according to the protocol of Lindsey and Gallois (1990). Kanamycin was used as selectable agent for the identification of transgenic sugar beet shoots. In total eight transformations were performed from September 14 until October 5, 2010 using the two *A. tumefaciens* strains AGL1 and GV3101 PMP90 RK. Generated transgenic shoots growing on selectable medium (supplemented with kanamycin) were termed as transformants (T₁ generation). Each transformant obtained an individual seed code listed in Table 9. In total 61 independent transformants were obtained after transformation, thereof 33 carrying the 35S:*BTC1_d* and 28 carrying the 35S:*btc1_a* transgene cassette.

All independent transformants were micropropagated by adventitious shoot regeneration to yield 20 genetically identical shoots/transformant, which were termed as clones. The clones were named according to the transformant seed code with a serial single plant number e.g. 120301/1 - 120301/20. Clones were transferred to medium supplemented with vitamins to induce root growth (Figure 13). Clones with root formation were transferred to soil on November 7, 2011 and from then on shoots were referred to as plants. As a control, the donor line 120309 was used, which underwent the same regeneration process without transformation and were grown in parallel with the transformants.

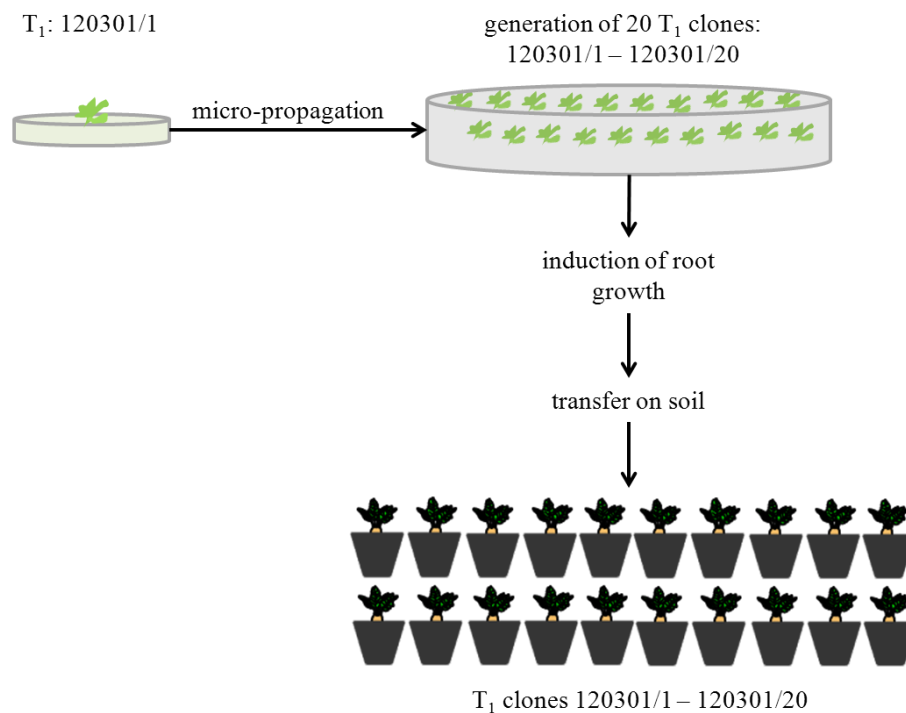


Figure 13: Example for micropropagation of transformant 120301/1 to yield the 20 clones 120301/1-120301/20. After *Agrobacterium* mediated transformation, the transformant 120301/1 was grown on selectable medium supplemented with kanamycin and micropropagated through adventitious shoot regeneration to yield the 20 clones 120301/1 – 120301/20. For induction of root growth plants were grown on selectable medium supplemented with vitamins and then rooted plants were transferred in soil.

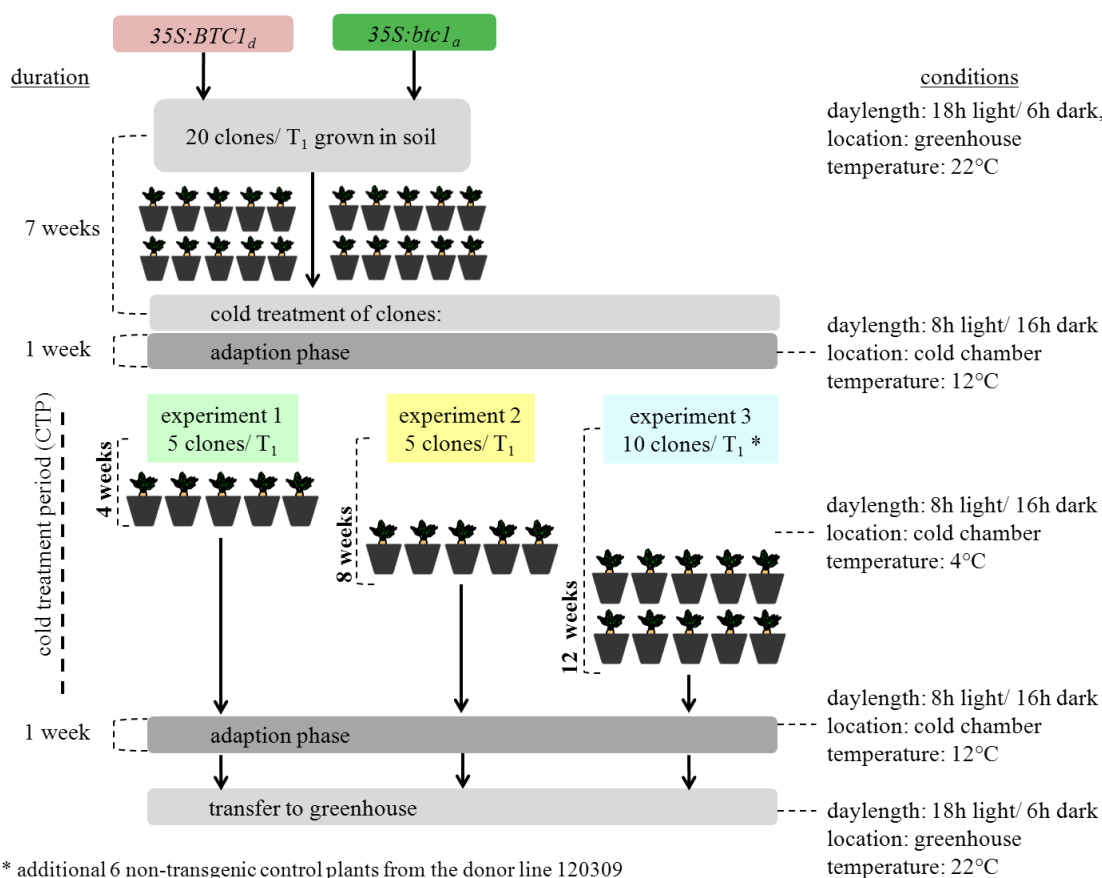
3.2.4 Determination of transgene copy numbers

Genomic DNA was isolated from leaves and qPCR experiments were performed with specific primers amplifying the *nptII* gene (J. Kraus, pers. communication). A single copy transgenic plant hemizygous for the *nptII* gene in a genetic background of the biennial sugar beet line 120309 was used as a positive control and the donor plant 120309 as a negative control. Two biological replicates/ control were analyzed and the mean of the resulting C_T values was calculated. In the same way means of the C_T values derived from two clones/ transformant were calculated. In a next step the means were compared to those of the positive and negative control. C_T means of the two biological replicates derived from the positive (1 transgene copy integrated) or negative (no transgene copy integrated) control were accordingly set to the values 1.0 or 0.0, respectively. In the following, I termed these values copy number indication (CNI) values. In this way copy numbers of the transgene were estimated for each transformant. Transformants were supposed to carry a single copy of the transgene, when the CNI values range from 0.6-1.3 and multiple integrations were expected, when the CNI value was at least 1.5 (J. Kraus, pers. communication).

3.2.5 Growth conditions for primary transformants and phenotypic analysis

Twenty clones of each transformant were grown for seven weeks under long day (LD) conditions (18h light/6h dark, 22°C) in the greenhouse in Einbeck (J. Kraus, pers. communication). None of the clones started to bolt within seven weeks. Thus for all clones a cold treatment was performed as follows: cold chamber under short day (SD) conditions (8h light/16h dark) at 4°C with one week of adaptation at 12°C. The 20 clones/ transformant were split and three different experiments were performed in which the cold period (CTP) was varied (**Figure 14**). Experiment 1 was performed with 5 clones/ transformant with a CTP of 4 weeks, experiment 2 was performed with 5 clones/ transformant with a CTP of 8 weeks and

experiment 3 was performed with 10 clones/ transformant and 6 non-transgenic plants of the donor line 120309 as a control with a CTP of 12 weeks (Table 10). Then, plants were grown in a greenhouse in Einbeck (KWS Saatzucht GmbH, Germany) under LD with 18h light/ 6h dark, 22°C (J. Kraus, pers. communication).



* additional 6 non-transgenic control plants from the donor line 120309

Figure 14: Workflow for cold treatment experiments of sugar beet transformants. 61 transformants with 20 clones/ transformant were obtained after *A. tumefaciens* mediated transformation and micro-propagation. 20 clones/ transformant were splitted and 3 experiments were performed for different periods of cold treatment (CTP = cold treatment period) (Table 10).

The number of plants per experiment as well as the cold treatment conditions and duration is given.

Bolting (BBCH scale code: 51) (Meier, 1993) was recorded as days to bolting (DTB) after cold treatment for all T₁ plants under the following conditions: plants of experiment 1 and experiment 2 were phenotyped in the greenhouse in Einbeck (18h light/ 6h dark, 22°C, KWS Saatzucht GmbH, Germany). Plants of experiment 3 were first grown for 4 weeks in the greenhouse in Einbeck (under the same conditions as in experiment 1 and 2) and then transferred to the greenhouse in Kiel on March 27, 2012 for phenotyping and seed production (16h light/ 8h dark, 22°C, 900μE, CAU Kiel, Germany) (Figure 15, Table 10, Supplementary Figure 4).

In experiment 1, all plants were phenotyped at five time points from February 9, 2012 until March 13, 2012 whereas in experiment 2 all plants were phenotyped at four time points from March 15, 2012 until April 13, 2012 (J. Kraus, pers. communication). In experiment 3, I phenotyped 8 out of 10 clones/ transformant twice a week from March 27, 2012 until May 16, 2012. The remaining two clones were propagated by selfing with bag isolations in July 2012 to yield T₂ seeds for further experiments (Figure 15, Table 10).

Plants which underwent 12 weeks of cold treatment (experiment 3) and did not start to bolt up until May 16, 2012, underwent a second cold treatment for additionally 34 weeks in a cold chamber in Kiel (16h light/ 8h dark, 4°C, 200 μE, CAU Kiel, Germany) (Table 10, Supplementary Figure 4).

Then, plants were transferred to the greenhouse where they grew under 16h light at 22°C. To analyze whether differences in bolting time between the transformants and the non-transgenic control are statistically significant, I performed a T-test with the program Student's *t*-Test ($p = 0.05$) (<http://www.physics.csbsju.edu/stats/t-test.html>). For this analysis I used the DTB data from transformants where all analyzed clones either bolted after vernalization or were never bolting. Those transformants that segregated for bolting after vernalization or never bolting were not tested.

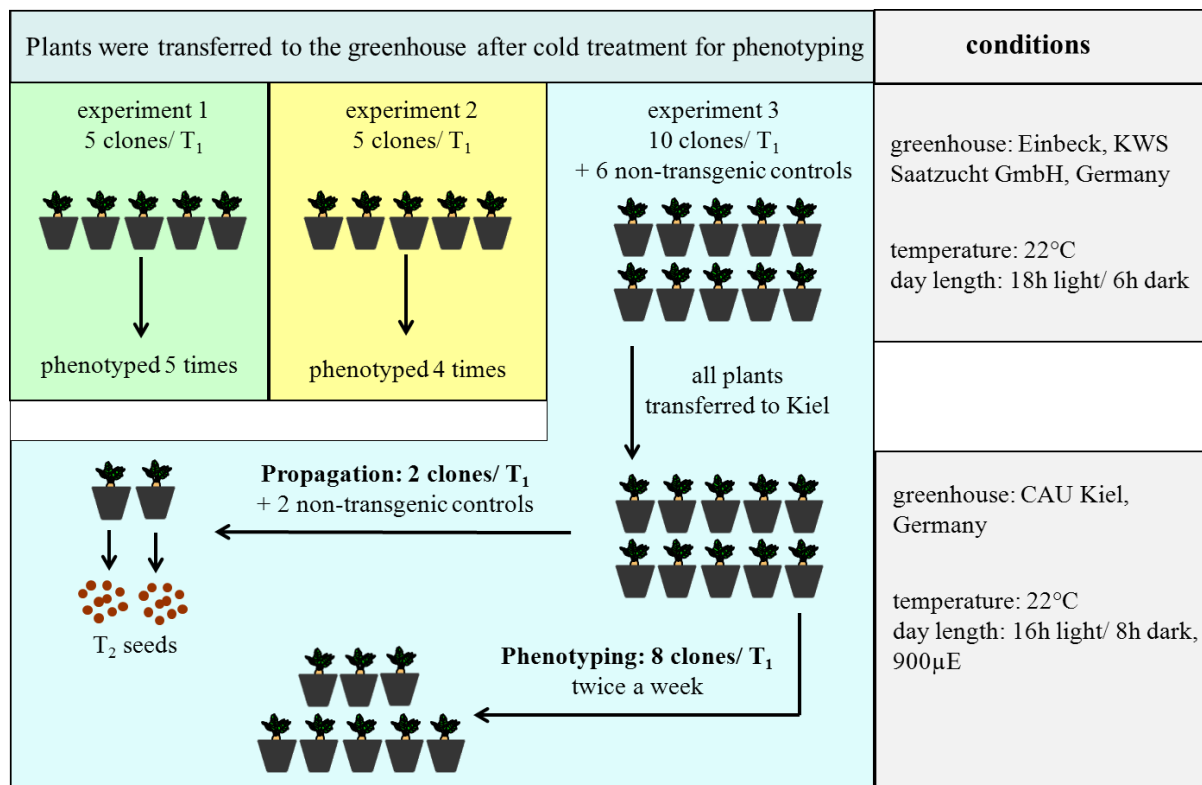


Figure 15: Phenotyping and seed production of transformants after different cold treatment periods. Plants of experiment 1 (green box), 2 (yellow box) and 3 (light blue box) were grown on soil and cold treated for 4, 8 and 12 weeks and SD conditions (8h light/ 16h dark, 4°C), respectively (**Figure 14**, **Table 10**), and then transferred to the greenhouse for phenotyping (**Table 10**). The respective conditions are given in grey boxes on the right site. The number of clones/ transformant in experiment 1 and 2 are given in green and yellow boxes, respectively. Phenotyping and seed production for plants of experiment 3 are displayed in the blue box.

Table 10: Experimental plan for cold treatment and phenotyping procedures of transgenic T₁ plants. All clones/ transformant were first grown for 7 weeks in a greenhouse (18h light, 22°C). Then 3 experiments were performed with different periods of cold treatment (CTP) (Figure 14, Figure 15).

Experiment/ CTP (weeks)	Number of Clones/ Transformant Analyzed	Total Number of T ₁ Plants Analyzed	Cold Treatment	Phenotyping	Transfer to Kiel	Second Cold Treatment	End of Experiment
1/ 4	5/ 61	299 ^{a,c}	05.12.11- 02.01.12	09.02.12 14.02.12 20.02.12 02.03.12 13.03.12	-	-	13.03.12
2/ 8	5/ 61	295 ^{a,b}	05.12.11- 30.01.12	15.03.12 22.03.12 29.03.12 13.04.12	-	-	13.04.12
3/ 12	8/ 56 ¹	451 ^{d,e}	05.12.11- 27.02.12	27.02.12- 16.05.12	27.03.1 2	16.05.12- 09.01.13	26.06.13

¹ Clones of 5 transformants (120259-120263) dropped down during the transfer to Kiel and were discarded

^a only 1 clone for transformant 120308 available

^b only 2 clones for transformants 120272 and 120274 available

^c only 3 clones for transformant 120252 available

^d only 5 clones for transformant 120293 available

^e including 6 clones of the non-transgenic control (seed code 120309)

3.2.6 DNA techniques

I used the NucleoBond® Xtra Midi Kit (Macherey Nagel GmbH & Co. KGI, Düren, Germany) for extraction of plasmid DNA. For leaf DNA extraction, leaf samples were treated with liquid nitrogen and grinded into a fine powder. For extraction of genomic DNA I applied the standard CTAB method (Saghai-Marouf et al., 1984) with slight modifications. DNA was stored in double-distilled water (ddH₂O) at -20°C. For PCR, DNA was diluted 1:10 with ddH₂O. I performed PCR with *Taq*-DNA-Polymerase (Invitrogen, Karlsruhe, Germany) or recombinant *Pfu*-DNA-Polymerase (Fermentas, St.Leon-Rot, Germany). All yielded fragments were separated in 1 or 2% agarose gels. PCR fragments, which were later cloned into vectors, were purified with the NucleoSpin® Gel and PCR Clean-up (Macherey Nagel GmbH & Co. KGI, Düren, Germany). All primer combinations and PCR conditions are given in **Table 11**. PCR products were sequenced applying the Sanger method at the Institute of Clinical Molecular Biology (IKMB Kiel, Germany) with a final sample volume of 25µl and a final primer concentration of 3.2µM.

Table 11: Primer and amplification conditions for all PCRs performed in this study.

Gene/ Construct	Forward Primer	Reverse Primer	PCR Conditions
<i>BTC1_d/btc1_a</i>	BTC_F	BTC_R	95°C, 5' + 35 x (95°C, 1'; 58°C, 30"; 72°C, 5') ²
<i>BTC1_d/btc1_a</i>	A984	A985	95°C, 3' + 10 x (95°C, 30"; 50°C, 30"; 72°C, 5') ²
<i>BTC1_d/btc1_a</i>	Xma_BTC1_F	Sal_BTC1_R	95°C, 3' + 28 x (95°C, 30"; 62°C, 45"; 72°C, 5') ²
35S: <i>BTC1_d/btc1_a</i>	A747	P35S_F	95°C, 3' + 28 x (95°C, 30"; 58°C, 30"; 72°C, 1') ¹
<i>nptII</i>	nptII_F	nptII_R	95°C, 3' + 32 x (95°C, 30"; 66°C, 30"; 72°C, 30") ¹
<i>BvGAPDH</i>	B582	B583	95°C, 3' + 40 x (95°C, 10"; 61°C, 30"; 72°C, 30") ³
<i>BTC1</i>	B580	B581	95°C, 3' + 40 x (95°C, 10"; 61°C, 30"; 72°C, 30") ³
<i>BvFTI</i>	B563	B564	95°C, 3' + 40 x (95°C, 10"; 64.5°C, 30"; 72°C, 30") ³
5'-35S: <i>BTC1_d/btc1_a</i>	B615	A882	95°C, 3' + 36 x (95°C, 3'; 58°C, 30"; 72°C, 35") ¹
3'-35S: <i>BTC1_d/btc1_a</i>	B616	B619	95°C, 3' + 36 x (95°C, 3'; 55°C, 30"; 72°C, 35") ¹

¹ PCR performed with *Taq*-DNA-Polymerase (Invitrogen, Karlsruhe, Germany)

² PCR performed with Recombinant *Pfu*-DNA-Polymerase (Fermentas, St.Leon-Rot, Germany)

³ RT-qPCR performed with *Power SYBR®* Green PCR Master Mix (Applied Biosystems, Inc, USA)

3.2.7 RNA techniques

For expression analysis of *BTC1* and *BvFTI* in transformants, 50 mg of leaf material was taken on June 29, 2011 from transgenic shoots, growing on selectable medium supplemented with kanamycin in a growth chamber (16h light, 24°C) before cold treatment at zeitgeber time (ZT) 4 and stored in *RNAlater®* (Qiagen, Hilden, Germany) (J. Kraus, pers. communication). Zeitgeber time means a standard of time that is based on the light/dark cycle. The time when lights on is defined as ZT 0 and thus ZT 4: 4 hours after light on. For sampling 5 weeks after vernalization, leaf samples were taken on April 3, 2012 at ZT 7 from plants that were grown in the greenhouse under 22°C with 16h light. The biennial donor line 120309 was used as a control. I kept the leaves in liquid nitrogen for 30 min and stored them at -70°C. For diurnal expression analyses plants were grown for 6 weeks in the greenhouse in Kiel under 16h light/ 8h dark, 22°C, 900µE (CAU Kiel, Germany). Leaves were sampled every two hours (May 9 to May 10, 2012) and kept in liquid nitrogen before storing at -70°C. Total RNA was extracted using the peqGOLD Plant RNA Kit and DNase-treated on a column with the peqGOLD DNase I Digest Kit (PEQLAB, Erlangen, Germany). I employed 500ng of total RNA (DNase treated) in cDNA synthesis using the First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany). The resulting cDNA was resolved in 20µl and diluted twenty fold with ddH₂O. 2 µl were used as template for RT-PCR and RT-qPCR experiments. RT-PCR was performed with *Taq*-DNA-Polymerase (Invitrogen, Karlsruhe, Germany) and the primer combinations B615/ A882 and B616/ B619. For RT-qPCR, two independent biological replicates and three technical replicates of each sample were analyzed. RT-qPCR was performed with the *Power SYBR®* Green PCR Master Mix (Applied Biosystems, Inc, USA) on a CFX96 Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA) with a final reaction volume of 20µl including a final primer concentration of 20 pM. The

housekeeping gene *BvGAPDH* was used as a reference (**Table 11**). Data were analyzed with the CFX Manager™ Software v2.1 (Bio-Rad, Hercules, CA, USA) and the comparative CT (ΔC_T) method was applied. Relative expression levels were calculated and normalized to the geometric mean of *BvGAPDH*.

3.3 Results

3.3.1 Transformation and propagation of sugar beet transgenic T₁ plants

The dates of conducted transformation experiments, the number of shoots regenerating on selectable medium and the number of rooted plants are listed in **Table 12**.

Regenerated shoots were regarded as independent transformants. In total, 61 independent transformants were identified, 33 were transformed with construct CAU3713 (transgene cassette *35S:BTC1_d*) and 28 with construct CAU3714 (transgene cassette *35S:btcl_a*), which matches to known transformation efficiencies with sugar beet (J. Kraus, pers. communication) and indicates that no *BTC1* allele was preferentially introduced into the sugar beet genome.

From each of 61 generated shoots, 20 clones were produced by micropropagation (**Figure 16**) with 5 exceptions (transformant 120308 : 12; transformants 120272, 120274 and 120293: 17; transformant 120252: 18). The transformants 120308 and 120293 carry the *35S:BTC1_d* transgenic cassette while the remaining three transformants carry the *35S:btcl_a* transgenic cassette. To ensure that clones are non-chimeric each shoot was grown on selectable medium and the transgene integration was determined by PCR (J. Kraus, pers. communication).

Moreover, I verified the integration of the complete transgene in each of the 61 independent transformants by PCR followed by sequencing. I amplified genomic DNA of one clone/transformant by PCR using the primers pLH_F/ A882, A883/ B616 and B619/ p9-T-OCS. PCR products were sequenced with the same primers and additionally p35S_F, A592, A747 and BvBTC1_SeqAdd_F. Sequence comparison confirmed the transgene integrity in all transformants. Eventually, 1203 plants from 61 independent transformation events were transferred to the greenhouse (KWS Saatzucht GmbH, Einbeck, Germany) (**Table 12**).

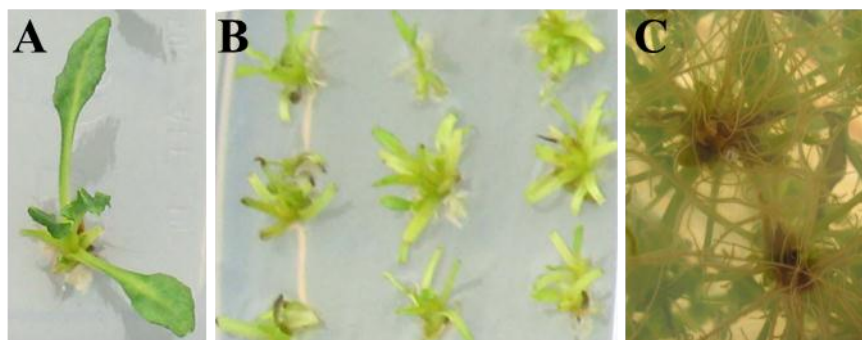


Figure 16: *In vitro* micropropagation and induction of root growth of a sugar beet transformant. Pictures were taken by Dr. Joseph Kraus, KWS Saatzucht GmbH, Einbeck. Picture **A** shows the transformant 120301/1 growing on selectable medium after transformation. **B** shows the clones 120301/2-120301/10 obtained after micropropagation through adventitious shoot regeneration. Therefore shoots were grown on selectable medium supplemented with kanamycin. In picture **C** root formations of the clones 120301/1 and 120301/2 are shown, therefore shoots were grown on selectable medium supplemented with vitamins. In this stage the clones were transferred on soil.

Table 12: Generated shoots (transformants) on selectable medium supplemented with kanamycin after sugar beet transformation with two *A. tumefaciens* strains (AGL1 and GV3101 PMP90RK) carrying the *35S:BTCI_d* or *35S:btcI_a* transgenic cassettes and regeneration of clones/ transformant. Each generated shoot represents an independent transformant (T₁ generation, seed code 120248 - 120308). Shoots were regenerated by micropropagation to yield 20 clones/ transformant. After induction of root yield all obtained T₁ plants were transferred to the greenhouse (KWS Saatzucht GmbH, Einbeck, Germany) and grown for 7 weeks under 18h light at 22°C and phenotyped for bolting behavior.

Construct/ Transgenic Cassette	Transformation Date	Bacterial Strain	Number of Generated Shoots (Transformants)	Number of Clones/ Transformant	Total Number of Obtained T ₁ Plants	Seed Code
CAU3714/ <i>35S:btcI_a</i>	04.09.10 - 05.10.10	AGL1	14	20	278 ²	120248- 120275
		GV3101 PMP90RK	14	20	274 ¹	
CAU3713/ <i>35S:BTCI_d</i>	04.09.10 - 05.10.10	AGL1	13	20	254 ³	120276- 120308
		GV3101 PMP90RK	20	20	397 ¹	
Total			61		1,203	

¹ only 17 clones for transformants 120293, 120274 and 120272 available

² only 18 clones for transformants 120252 available

³ only 12 clones for transformant 120308 available

3.3.2 Copy number determination

The transgene copy number was determined for all independent transformants by qPCR (**Chapter 3.2.4**). The mean of resulting C_T values derived from two clones, representing biological replicates of one transformant, was calculated and compared to the controls. As expected a variation was found. To simplify the interpretation, the C_T means were set to the CNI values 1.0 (positive control) and 0.0 (negative control) (**Chapter 3.2.4**). For single copy transformants it was expected that the values range from 0.6 - 1.3, and values of at least 1.5 were expected in transformants carrying multiple integrations (J. Kraus, pers. communication) (**Chapter 3.2.4**). Accordingly, 20 transformants are supposed to carry a single copy of the transgene whereas 18 transformants carry >1 copy. Because the quantified value was below the threshold of 0.6, 18 transformants gave ambiguous results and copy numbers could not be determined (**Table 13**). A table with data from all measurements is given as **Supplementary Table 7**.

Table 13: Transgene copy number of T₁ plants carrying the *35S:BTCI_d* and *35S:btcI_a* transgene cassette measured by qPCR. Two clones/ transformant were analyzed as biological replicates. A single copy hemizygous transgenic plant and a non-transgenic plant were used as positive and negative control, respectively. The mean of the C_T values was compared to those of the controls and converted into a CNI value (**Chapter 3.2.4**).

Copy Number	Number of Transformants		CNI Value
	<i>35S:BTCI_d</i> Transgenic Cassette	<i>35S:btcI_a</i> Transgenic Cassette	
single copy	12	10	0.6 – 1.3
>1 copy	13	7	> 1.5
not determined	13	6	< 0.6

3.3.3 Phenotypic analysis of transgenic T₁ plants

All 1203 transgenic plants that were obtained after transformation (**Table 12**) were grown in the greenhouse under 18h light at 22°C (**Figure 14**). I expected under the assumption that the polypeptides have a different function that those plants transformed with the CAU3713 construct start shoot elongation without any vernalization requirement and in contrast those plants transformed with the construct CAU3714 start shoot elongation after vernalization. Six plants of the biennial donor line 120309 were used as a control. No transgenic plant started shoot elongation indicating no functional differences between the dominant and the recessive *BTC1* genes. Accordingly, after 7 weeks plants were transferred to the cold chamber (4°C, 8h light) (**Figure 14**) whereby clones of each transformant were split into 3 experiments varying in the period of cold treatment (experiment 1 = CTP 4 weeks, experiment 2 = CTP 8 weeks, experiment 3 = CTP 12 weeks) (**Figure 14, Table 10**), to examine whether the transgenic plants have a reduced vernalization requirement. Plants of the control (120309) were cold treated for 12 weeks (experiment 3) in parallel with the transgenic plants. After vernalization, plants of each experiment were phenotyped under different conditions (**Chapter 3.2.5, Table 10, Figure 15**). In total, 1203 transgenic plants were cold treated. Of these, 1041 plants were phenotyped after the cold treatment. Of the remaining 162 plants, 112 (2 clones/ transformant, experiment 3) were used for seed production and 50 plants, derived from 5 independent transformants (120259-120263) were discarded.

Among 1041 phenotyped plants, 581 started to bolt and 460 did not bolt within a period of 11 weeks. In the following, the phenotypic results of plants that underwent 12 weeks of vernalization (experiment 3) will be summarized, because this is the only experiment that was performed with non-transgenic biennial controls.

In experiment 3, a total of 447 transgenic plants, derived from 56 independent transformants were phenotyped with 6 control plants. I observed 349 plants that bolted 34-53days after vernalization and 98 plants that did not bolt within a period of 11 weeks (**Figure 17, Supplementary Table 6**). No phenotypic differences with regard to days to bolting were observed between transgenic plants carrying the dominant or recessive *BTC1* transgene (**Supplementary Table 6**). I expected that clones of one transformant do not differ in their bolting behavior, based on their identical genetic information. Surprisingly, in 16 out of 56 transformants I observed clonal variation, 9 were transformed with *BTC1_d* and 7 with *btc1_a* (**Figure 18, Supplementary Table 6**). Those transformants were not used for further analyses. No clonal variation was observed in 36 transformants that started bolting after vernalization, 21 of which carry the *35S:BTC1_d* and 15 the *35S:btc1_a* transgene cassette (**Supplementary Table 6**). Among these transformants, one transformant that carries the *BTC1_d* transgene (seed code 120301) and two with the *btc1_a* transgene (seed code 120254 and 120270) bolted 34 days after vernalization and thus 2 days earlier when compared to the control. Although this difference is quite low it is statistically significant (T-test, p = 0.05). Late bolting transformants were also observed, two plants transformed with *BTC1_d* (seed code 120296 and 120300) started to bolt after 41 or 51 days, respectively, which is statistically significant later as the control. Moreover, 4 transformants did not start bolting within a period of 11 weeks, 3 carried the *35S:BTC1_d* transgene cassette (seed code 120295, 120305 and 120306) and one carried the *35S:btc1_a* transgene cassette (seed code 120255) (**Supplementary Table 6**). These plants were again transferred to the cold chamber and grown under 16h light at 4°C for another 34 weeks to ensure seed production (**Table 10**).



Figure 17 : Bolting and never bolting transgenic T₁ plants after 12 weeks grown in the cold chamber with 8h light at 4°C. The seed codes are shown for each plant. A) 120301/1, bolting after vernalization. B) 120305/1, never bolting after cold treatment. C) 120255/1, never bolting after cold treatment. D) 120268/1, bolting after vernalization. 120301/1 and 120305/1 are clones transformed with *BTC1_d* whereas 120268/1 and 120255/1 are clones transformed with *btc1_a*.

After this period, they were transferred to the greenhouse and kept under 16h light at 22°C. These transformants showed phenotypic variation. All clones transformed with *btc1_a* (seed code 120255) failed to bolt within 24 weeks after the 2nd cold treatment. Accordingly, these plants were termed ‘never bolting’. The *BTC1_d* transformed clones (seed code 120305) segregated for never bolting and bolting plants, whereas bolting clones showed a stunted phenotype (**Figure 18**). None of the bolting plants produced flowers until June 26, 2013.



Figure 18: Phenotypic variation of transformants for bolting and never bolting after 2nd cold treatment for 34 weeks at 4°C. Observed phenotypes of A) *BTC1_d* transformed clone 120305/1 and B) *btc1_a* transformed clones 120255/1, 120255/2 and 120255/6.

I compared the phenotypic data obtained from clones of the 3 transformants (seed code 120301, 120254 and 120270) that underwent 12 weeks of cold treatment and bolted significant earlier with those obtained from clones of the same transformants that underwent 4 or 8 weeks of cold treatment (**Figure 14, Figure 15, Supplementary Table 6**). All *BTC1_d* transformed clones (seed code 120301) that were phenotyped after 4 or 8 weeks of cold treatment bolted, whereas only a few clones transformed with the *btc1_a* (seed code 120254 and 120270) bolted after the shortened period of cold treatment (**Supplementary Table 6**). Furthermore, I compared the phenotypic data from never bolting clones (seed code 120295, 120305, 120306 and 120255) of the three cold treatment experiments and figured out that none of the clones started bolting (**Supplementary Table 6**). It has to be noted that phenotypic data, obtained after shortened cold treatment period (CTP 4 and CTP 8) were

generated without controls. However these data give a tendency that some plants bolt after shortened vernalization.

In a next step I compared the CNI-values (**Supplementary Table 7**) with the bolting data of T₁ plants derived from 40 transformants that showed no clonal variation after 12 weeks of cold treatment (**Supplementary Table 6**) to examine the relationship between transgene copy number and phenotype.

I divided the transformants into three classes, biennial early bolting, biennial late bolting and never bolting. Biennial early bolting plants bolted earlier or equally as the non-transgenic control. By contrast, biennial late bolting plants bolted significantly later as the control. Among the first class, 46% carry one copy of the transgene, whereas in the 2nd and 3rd class 100% had >1 transgene copy (**Table 14**). The remaining plants of the first class had either >1 transgene copies or the transgene copy number could not be determined, because the CNI-value was below 0.6 (**Chapter 3.2.4, Supplementary Table 7**).

Table 14: Summary bolting phenotypes and transgene copy number (**Chapter 3.3.3**) in transgenic beets.

Copy Number	Phenotypes ¹		
	Biennial Early Bolting ²	Biennial Late Bolting ³	Never Bolting
single copy	16	-	-
>1 copy	2	1	4
ambiguous results	17	-	-

¹ Phenotypes determined according to phenotyped clones after 12 weeks of cold treatment, excluded: transformants with clonal variation

² biennial early bolting: plants bolting statistical significant earlier as the control or as the control

³ biennial late bolting: plants bolting statistical significant later as the control

3.3.4 Expression analysis of *BTC1* and downstream targets

First, I analyzed the expression of all *BTC1* genes, transgenic (*BTC1*_{transgene}) and native ones (*BTC1*_{native}), in T₁ transformants before and after 12 weeks of cold treatment by RT-qPCR. Growth conditions, sampling time and RT-qPCR procedures are described in **Chapter 3.2.7**. I analyzed 4 transformants, two of these were early bolting after vernalization (seed code 120301 and 120268) and two transformants were never bolting (seed code 120305 and 120255). The biennial donor line 120309 was used as a control.

I observed differences between the *BTC1*_{transgene+native} expression in transgenic plants and the *BTC1*_{native} expression in the control. Before cold treatment, in all transformants, except transformant 120255, the *BTC1*_{transgene+native} expression was 1.4 or 4.0 fold increased when compared to the *BTC1*_{native} expression of the control. Highest *BTC1*_{transgene+native} expression was observed in transformant 120301, which carries one copy of the *BTC1*_d transgene (**Figure 19**). For transformant 120255 the *BTC1*_{transgene+native} expression could not be determined, because the leaves were too small for sampling before vernalization (J. Kraus, pers. communication).

After cold treatment the *BTC1*_{native} expression was increased 3.4 fold in the control. Also in plants of transformant 120268 (1 copy), I observed an upregulation of the *BTC1*_{transgene+native} from 0.007 to 0.013 after vernalization, but the expression was not as high as in the control. Interestingly, in the remaining transgenic plants that were analyzed the *BTC1*_{transgene+native} expression was decreased when compared to the *BTC1*_{transgene+native} expression before vernalization (seed code 120301, with 1 copy and 120305 with >1 copy). However, for transformant 120301 a large standard deviation (SDM) was calculated. Lowest *BTC1*_{transgene+native} expression was observed in both transformants carrying >1 transgene copy (seed code 120305 and 120255) (**Figure 19**).

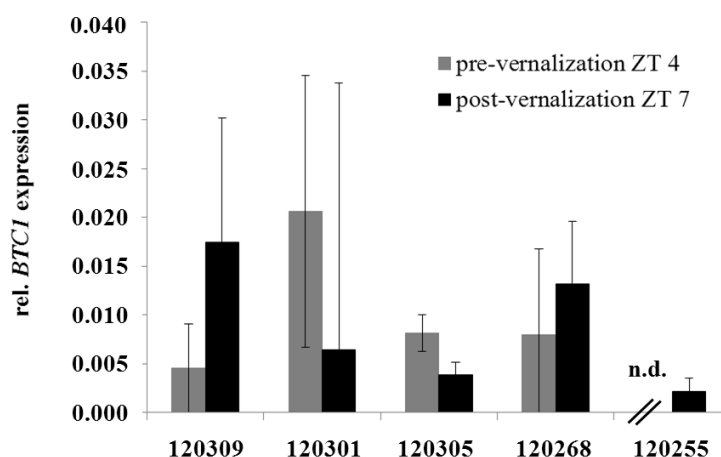


Figure 19: RT-qPCR expression analysis of all *BTC1* genes, transgenic (*BTC1*_{transgene}) and native ones (*BTC1*_{native}) from sugar beet in 4 independent transformants. Two clones/ transformant were analyzed with primers B580/ B581 to specifically amplify a 305 bp fragment of the coding sequence (exon 6 – exon7) of the *BTC1* genes. Each value is the mean of two biological (clones) and three technical replicates. Analyzed clones of transformants 120301 (*35S:BTC1_d*) and 120268 (*35S:btcl_a*) carry 1 transgene copy and bolted after vernalization, whereas clones of transformant 120305 (*35S:BTC1_d*) and 120255 (*35S:btcl_a*) carry >1 transgene copy and were never bolting. As a control the biennial donor line was used (120309). The ΔC_T method was applied to calculate the relative *BTC1* expression. For normalization the housekeeping gene *BvGAPDH* was used. Expression of *BTC1* before vernalization (grey boxes) and after vernalization (black boxes). Error bars: standard deviation of the mean (\pm SDM) of two biological replicates. ZT: zeitgeber time; n.d. not determined.

In a next step, I analyzed the diurnal expression of the *BTC1*_{transgene} and *BTC1*_{native} sequences as well as its downstream target gene *BvFTI* after 12 weeks of cold treatment in the same transgenic plants of the 4 transformants and of the biennial control by RT-qPCR (**Figure 20**). Growth conditions of the plants, time point of leaf sampling and RT-qPCR procedure are described in **Chapter 3.2.7**. According to the model proposed by Pin et al. (2012), *BvFTI* is downregulated by *btcl_a* in biennials after vernalization and plants start bolting. Therefore, I expected high expression rates of *BvFTI* in never bolting plants after cold treatment.

A diurnal expression of *BTC1*_{native} was observed in the control with highest expression at ZT 9, whereas *BvFTI* is complete downregulated (**Figure 20**), which is consistent with previous observations (Pin et al., 2012).

Also in plants of the transformants 120301 and 120268, which carry 1 copy of the transgene and bolted after vernalization, *BTC1*_{transgene+native} was diurnally regulated (**Figure 20**). In both transformants, I observed a shift of the peak which occurs at ZT 7 and the expression rate of *BTC1*_{transgene+native} at ZT 7 was doubled when compared to the *BTC1*_{native} expression of the control at ZT 9. Similar to the control, in both transformants *BTC1*_{transgene+native} expression decreases at ZT 11 and rises again at ZT 13. However, *BTC1*_{transgene+native} was also upregulated during the early dark phase and was at least doubled at ZT 19 in both transformants when compared to the control. Similar to the control, *BvFTI* was complete downregulated in plants of transformant 120301 that carries the *BTC1_d* transgene. Interestingly, no downregulation of *BvFTI* was observed in plants of transformant 120268, carrying the *btcl_a* transgene and the diurnal expression of *BvFTI* in this transformant is similar to that, previously observed in non-vernalized biennials (Pin et al., 2012).

By contrast, a complete downregulation of $BTCI_{\text{transgene+native}}$ was observed in never bolting plants of the transformants 120305 and 120255, which carry >1 copy of the transgene (**Figure 20**). As expected, in those plants $BvFTI$ is highly upregulated and the diurnal expression is similar to that observed for transformant 120268.

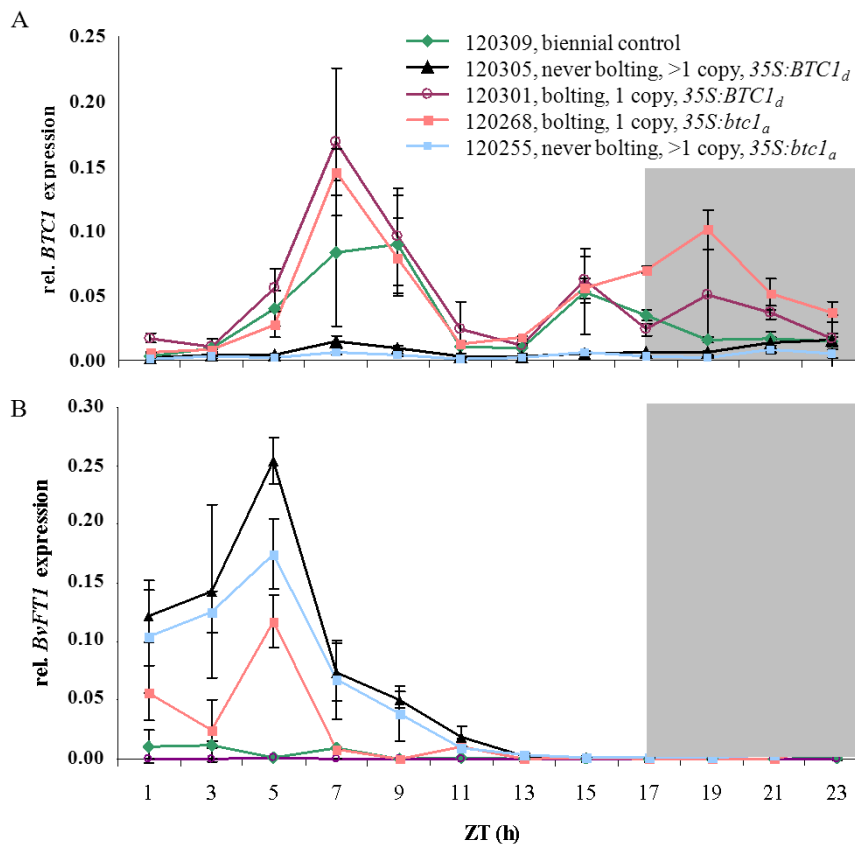


Figure 20: Diurnal Expression analysis of (A) $BTCI_{\text{transgene+native}}$ expression and (B) $BvFTI$ expression in T_1 transformants with bolting and never bolting phenotypes after twelve weeks of cold treatment. Two clones/transformant were analyzed. The biennial donor line 120309 was used as a control. Plants were grown in a greenhouse at 22°C. Error bars represent the standard deviation of the mean (\pm SDM) of the two biological replicates. The grey background indicates the dark phase.

In summary, I could clearly demonstrate that $BTCI_{\text{transgene+native}}$ is complete downregulated whereas $BvFTI$ is highly upregulated in never bolting plants, carrying >1 copy of the transgene. In contrast, $BTCI_{\text{transgene+native}}$ is diurnally expressed and highly upregulated in T_1 plants which carry one copy of the transgene. Moreover, $BvFTI$ is completely downregulated in bolting plants carrying the $BTCI_d$ transgene, whereas in $btc1_a$ transformants $BvFTI$ was not completely downregulated after vernalization.

First investigations of T_2 families (seed code 122502 and 122504) that were derived from selfing one clone of 1-copy-transformants 120301 and 120302, respectively, revealed that the families are segregating for bolting and never bolting T_2 plants after cold treatment. Preliminary data of the $BTCI_{\text{transgene+native}}$ expression in T_2 plants pointed out that all non-transgenic T_2 plants bolted and $BTCI_{\text{native}}$ was upregulated. By contrast transgenic T_2 plants were bolting as well as never bolting. In bolting transgenic plants $BTCI_{\text{transgene+native}}$ was upregulated, whereas in never bolting plants $BTCI_{\text{transgene+native}}$ is completely downregulated. It could be suggested, that never bolting T_2 plants carry the homozygous $BTCI_d$ transgene whereas bolting T_2 plants carry the heterozygous $BTCI_d$ transgene. Certainly, further experiments are essential to investigate the relationship between the phenotype and the allele composition in those transgenic beets.

3.4 Discussion

The bolting behavior of transgenic sugar beets overexpressing *BTC1_d* or *btc1_a* coding sequence has been described together with the copy number integration of the transgene and the expression of *BTC1_{transgene+native}* and *BvFT1*. The findings are surprising in a way that in transgenic plants which carry >1 copy of the *BTC1_d* or *btc1_a* transgene, *BTC1_{transgene+native}* was completely downregulated and *BvFT1* highly upregulated after 12 weeks of cold treatment and consequently those transgenic beets showed a never bolting phenotype.

A similar phenotype and gene expression was previously observed in transgenic plants in which *BTC1* genes were silenced by RNA interference (RNAi) (Pin et al., 2012). In that study *BTC1_d/ btc1* RNAi plants with an annual or biennial genetic background were generated. In *BTC1_d* RNAi plants, the annual growth habit was completely abolished and plants did not start bolting up to 20 months. After 15 weeks of vernalization plants bolted but bolting was delayed. Moreover, most of these bolting plants showed a stunted phenotype and never started flowering. The authors analyzed the *BTC1_d*, *BvFT1* and *BvFT2* expression and demonstrated that *BTC1_d* is completely downregulated whereas *BvFT1* is highly upregulated before and after vernalization. Furthermore, *BvFT2* was completely downregulated before vernalization and a slight increase of the expression level was observed after cold treatment. Similarly, no *btc1* RNAi plant started to bolt after vernalization within 12 weeks of observation. Only a few plants started bolting but none of them started flowering. Diurnal expression analyses of *btc1*, *BvFT1* and *BvFT2* revealed complete downregulation of *btc1* and *BvFT2* before and after the cold treatment. By contrast, *BvFT1* was diurnally and highly upregulated (Pin et al., 2012). In a further study by Pin et al. (2010), *BvFT2* was downregulated in annual plants by RNAi. These plants failed to bolt for more than 57 weeks. After 15 weeks of vernalization plants started to bolt, but compared to the non-transgenic control, *BvFT2* RNAi plants were very late bolting and like the transformants described in the present study as well as *BTC1_d/ btc1* RNAi plants, some *BvFT2* RNAi plants did never start flowering (Pin et al., 2010).

Considering the RNAi mechanism the translation of the mRNA is inhibited by incorporation of double stranded small interfering RNA (siRNA) molecules into the RNA-induced silencing complex (RISC) which targets the respective mRNA and causes its degradation (Hammond et al., 2000). Multiple integrations of a transgene were also found to produce double stranded RNA, which can also be converted into siRNA molecules and thus mediate degradation of the respective mRNA (Mourrain et al., 2007; Müller, 2010).

Taking this into account, there is strong evidence that in the present study degradation of the *btc1_a* native and *BTC1_d/ btc1_a* transgenes is mediated by >1 transgene copy integrations. Consequently *BvFT1* and *BvFT2* cannot be regulated anymore through *BTC1* and plants remain vegetative after vernalization and thus behave as vernalized *btc1* RNAi plants.

Ectopic expression of flowering time genes using the 35S promoter was demonstrated before but was never shown to mediate gene silencing in beet. Overexpression of *BvFT1* in an annual or biennial background resulted in high upregulation of *BvFT1* in transgenic beets. According to the repressor function of *BvFT1*, *BvFT2* was completely downregulated. Transgenic plants with an annual genetic background failed to bolt for more than 6 months and transgenic plants with a biennial genetic background bolted at least 6 months later after vernalization when compared to the non-transgenic biennial control (Pin et al., 2010). Furthermore, overexpression of *BvFT2* in a biennial genetic background revealed that *BvFT2* was highly expressed in transgenic plants and its biennial growth habit was completely abolished. Thus plants bolted and flowered after 18 weeks while still in tissue culture (Pin et al., 2010).

Recently, transgene mediated gene silencing of the endogenous gene and transgene was demonstrated in toad lily (*Tricyrtis* sp.) by overexpressing the *GIBBERELLIN 2-OXIDASE* (*GA2ox*) gene under the control of the 35S promoter (Otani et al., 2013). The authors demonstrated that in plants carrying 1 copy of the transgene, *GA2ox* was overexpressed and plants showed a severe dwarf phenotype. In contrast, plants that have 3 to 4 transgene copies integrated into the genome showed decreased *GA2ox* expression levels and a moderate dwarf phenotype. They assumed that the phenotypic differences are due to co-suppression of the transgene and the endogenous gene, mediated by multiple transgene integrations which results in gene silencing (Otani et al., 2013). These results support my observations that the transgene copy number is negatively associated with the gene expression and bolting phenotypes.

Concluding, my data demonstrate for the first time transgene-mediated gene silencing through co-suppression of the transgene and the native *BTC1_d/ btc1_a* genes in the presence of multiple transgene copies, which results in a never bolting phenotype after vernalization.

In plant breeding research the development of non-bolting/ flowering crops is a crucial component to prevent the loss of energy while bolting and flowering. On the other hand, seed production must be ensured for which flowering is indispensable. Thus a major aim in sugar beet breeding is the development of an autumn sown winter beet with bolting control and winter hardiness. All previously generated transgenic plants (*BTC1_d/ btc1* RNAi, *BvFT1-ox* and *BvFT2* RNAi) (Pin et al., 2010; Pin et al., 2012) remain vegetative after vernalization or bolted without flowering. Accordingly, reproduction of those plants is not feasible and this material is not suitable for developing a winter beet. By contrast, based on the results of my study a new strategy could be developed to generate never bolting hybrids. For this approach two different bolting plants that carry a single copy of the transgene would be crossed to yield a never bolting hybrid that carries two transgene copies. In this way seed production for hybrid generation is ensured, because single copy transformants bolted after vernalization. Moreover, hybrids could be grown for a prolonged vegetative growth period, which increases the root yield and accordingly the sucrose yield. Subsequently, these never bolting hybrids represent a suitable resource for breeding winter beets.

3.5 References

Abou-Elwafa, S., B. Büttner, F. Kopisch-Obuch, C. Jung, and A. Müller, 2012: Genetic identification of a novel bolting locus in *Beta vulgaris* which promotes annuality independently of the bolting gene *B*. *Molecular Breeding* **29**, 989-998.

Abou-Elwafa, S.F., B. Büttner, T. Chia, G. Schulze-Buxloh, U. Hohmann, E. Mutasa-Göttgens, C. Jung, and A.E. Muller, 2010: Conservation and divergence of autonomous pathway genes in the flowering regulatory network of *Beta vulgaris*. *J Exp Bot.* **62**, 3359-3374.

Büttner, B., S.F. Abou-Elwafa, W. Zhang, C. Jung, and A.E. Muller, 2010: A survey of EMS-induced biennial *Beta vulgaris* mutants reveals a novel bolting locus which is unlinked to the bolting gene *B*. *Theor Appl Genet* **121**, 1117-31.

Chia, T.Y., A. Müller, C. Jung, and E.S. Mutasa-Göttgens, 2008: Sugar beet contains a large *CONSTANS*-LIKE gene family including a *CO* homologue that is independent of the early-bolting (*B*) gene locus. *J Exp Bot* **59**, 2735-48.

Eichholz, W., and H.J. Röstel, 1962: Züchtung der Winterzuckerrübe. *Theoretical and Applied Genetics* **32**, 281-290.

El-Mezawy, A., F. Dreyer, G. Jacobs, and C. Jung, 2002: High-resolution mapping of the bolting gene *B* of sugar beet. *Theoretical and Applied Genetics* **105**, 100-105.

- Hammond, S.M., E. Bernstein, D. Beach, and G.J. Hannon, 2000: An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature* **404**, 293-296.
- Hohmann, U., G. Jacobs, and C. Jung, 2005: An EMS mutagenesis protocol for sugar beet and isolation of non-bolting mutants. *Plant Breeding* **124**, 317-321.
- Koncz, C., and J. Schell, 1986: The Promoter of Tl-DNA Gene 5 Controls the Tissue-Specific Expression of Chimeric Genes Carried by a Novel Type of *Agrobacterium* Binary Vector. *Molecular & General Genetics* **204**, 383-396.
- Lange, W., W.A. Brandenburg, and T.S.M. De Bock, 1999: Taxonomy and cultonomy of beet (*Beta vulgaris* L.). *Botanical Journal of the Linnean Society* **130**, 81-96.
- Lazo, G.R., P.A. Stein, and R.A. Ludwig, 1991: A DNA transformation-competent *Arabidopsis* genomic library in *Agrobacterium*. *Biotechnology (N Y)* **9**, 963-7.
- Meier, U., 1993: Growth stages of mono- and dicotyledonous plants. Phenological growth stages and BBCH-identification keys of beet. Federal Biological Research Centre for Agriculture and Forestry, Braunschweig, Germany.
- Mourrain, P., R. van Blokland, J.M. Kooter, and H. Vaucheret, 2007: A single transgene locus triggers both transcriptional and post-transcriptional silencing through double-stranded RNA production. *Planta* **225**, 365-379.
- Müller, A.E., 2010: Gene Silencing in Plants: Transgenes as Targets and Effectors. *Genetic Modification of Plants: Agriculture, Horticulture and Forestry* **64**, 79-101.
- Otani, M., S. Meguro, H. Gondaira, M. Hayashi, M. Saito, D.S. Han, P. Inthima, K. Supaibulwatana, S. Mori, Y. Jikumar, Y. Kamiya, T. Li, T. Niki, T. Nishijima, M. Koshioka, and M. Nakano, 2013: Overexpression of the *gibberellin 2-oxidase* gene from *Torenia fournieri* induces dwarf phenotypes in the liliaceous monocotyledon *Tricyrtis* sp. *J Plant Physiol.*, in press
- Pin, P.A., R. Benlloch, D. Bonnet, E. Wremerth-Weich, T. Kraft, J.J.L. Gielen, and O. Nilsson, 2010: An Antagonistic Pair of *FT* Homologs Mediates the Control of Flowering Time in Sugar Beet. *Science* **330**, 1397-1400.
- Pin, P.A., W. Zhang, S.H. Vogt, N. Dally, B. Büttner, G. Schulze-Buxloh, N.S. Jelly, T.Y. Chia, E.S. Mutasa-Göttgens, J.C. Dohm, H. Himmelbauer, B. Weisshaar, J. Kraus, J.J. Gielen, M. Lommel, G. Weyens, B. Wahl, A. Schechert, O. Nilsson, C. Jung, T. Kraft, and A.E. Müller, 2012: The role of a pseudo-response regulator gene in life cycle adaptation and domestication of beet. *Curr Biol* **22**, 1095-101.
- Reeves, P.A., Y. He, R.J. Schmitz, R.M. Amasino, L.W. Panella, and C.M. Richards, 2007: Evolutionary conservation of the *FLOWERING LOCUS C*-mediated vernalization response: evidence from the sugar beet (*Beta vulgaris*). *Genetics* **176**, 295-307.
- Saghai-Marouf, M.A., K.M. Soliman, R.A. Jorgensen, and R.W. Allard, 1984: Ribosomal DNA spacer-length polymorphisms in barley: mendelian inheritance, chromosomal location, and population dynamics. *Proc Natl Acad Sci U S A* **81**, 8014-8.

4 Functional analysis of the *BOLTING TIME CONTROL 1 (BTC1)* gene II: transformation into *Arabidopsis thaliana*

4.1 Introduction

Recently, the bolting gene *B* has been cloned from sugar beet and was termed *BOLTING TIME CONTROL 1 (BTC1)* with high sequence homology to a *PSEUDO RESPONSE REGULATOR GENE (PRR)* from *Arabidopsis thaliana* (Pin et al., 2010; Pin et al., 2012). *BTC1* was shown to control annuality through regulating two beet *FT* homologs *BvFT1* and *BvFT2* (Pin et al., 2010; Pin et al., 2012) (see **Chapter 1.3**). There are 8 different haplotypes in annual *B. vulgaris* accessions and 3 haplotypes in biennial beets which were named with the letters ‘a’ through ‘k’ (Pin et al., 2012) (see **Chapter 1.3**). Like all PRR proteins, *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). Sequencing the 5′ upstream region of *BTC1*_{a-k} in annual and biennial accessions revealed an insertion within the 5′ untranslated region (5′UTR) of the biennial allele with a size of ~28 kb. Moreover, the predicted polypeptides of the annual and biennial alleles differed by several amino acid substitutions (Pin et al., 2010; Pin et al., 2012) (see **Chapter 1.3**). The authors found, that the *BTC1* expression differs between annual and biennial beets, when grown under long day conditions (18h light/ 6h dark). *BTC1* expression in biennial plants rapidly decreases after zeitgeber time (ZT) 10, whereas the decrease of expression is delayed in annuals. Furthermore, vernalization enhances expression in biennials and thus results in higher transcription levels in vernalized biennials compared to annual and non-vernalized biennials. These results indicate that the annual and biennial alleles may have different functions.

Phylogenetic analysis revealed that *BTC1* belongs to the *PRR3/PRR7* clade of PRRs from monocots as barley (*Hordeum vulgare*) and rice (*Oryza sativa*) and dicots as *Arabidopsis*, grapevine (*Vitis vinifera*), poplar (*Populus trichocarpa*) and papaya (*Carica papaya*). Monocots and dicots diverged ~140 million years ago. Based on similar function of the *PRR7* homolog *PPD-H1* from *H. vulgare* and *BTC1* it was suggested that the evolution of the key functions in the control of photoperiod response was prior the monocot-dicot split (Chaw et al., 2004; Pin et al., 2012). The predicted protein sequence of the *BTC1* gene shares 42% amino-acid identity with the *PRR7* protein. Furthermore, another beet sequence has been identified (*BvPRR7*) whose protein shares even higher sequence similarity with *PRR7* (46%) indicating that its function might be even closer related to the *Arabidopsis* protein (Pin et al., 2012).

In *Arabidopsis thaliana* the regulation of flowering time has been extensively studied. The four main pathways are the vernalization, autonomous, gibberellic acid and photoperiodic flowering pathways (Srikanth and Schmid, 2011). In the photoperiodic flowering pathway, *CONSTANS (CO)* plays a major regulatory role (Kinmonth-Schultz et al., 2013). Recent studies revealed that *CO* directly binds to the *FLOWERING LOCUS T (FT)* promoter and therefore activates *FT* transcription, required to initiate flowering (Tiwari et al., 2010). *CO* is activated by light and the circadian clock (Suarez-Lopez et al., 2001). The circadian clock represents an autonomous mechanism regulating gene activity in a rhythm of about 24h. This mechanism allows plants to adapt to daily environmental changes, e.g. day/night cycle (Dodd et al., 2005). This is achieved by a rhythmic gene regulation involving two interacting feedback-loops, the so called “morning loop” and the “evening loop”. Both loops contain several genetic regulators acting as activators or repressors, which are preferentially expressed at the respective time of the day (Locke et al., 2005; Zeilinger et al., 2006). The morning loop is composed of *LATE ELONGATED HYPOCOTYL (LHY)*, *CIRCADIAN CLOCK ASSOCIATED 1 (CCA1)* together with the *PSEUDO RESPONSE REGULATOR (PRR)* genes *PRR5*, 7 and 9, whereas the evening loop comprises the *TIMING OF CAB EXPRESSION 1*

(*TOC1*), *GIGANTEA* (*GI*), *EARLY FLOWERING 3* (*ELF 3*) *EARLY FLOWERING 4* (*ELF 4*) and *LUX ARRATHMO* (*LUX*) (Anwer and Davis, 2013). Recent studies revealed that morning and evening loops are connected through the key clock regulator gene *TIME OF CAB EXPRESSION 1* (*TOC1*), which inhibits expression of morning and evening oscillator genes (Huang et al., 2012; Wang and Ma, 2012).

In Arabidopsis, *PRR7* has an impact on the circadian clock function and consequently in the regulation of flowering. It has been reported that *PRR7* in conjunction with *PRR9* and *PRR5* represses *CCA1* and *LHY* during the day and thus, indirectly induces *TOC1* expression. In turn, *TOC1* expression inhibits *PRR5*, *PRR7* and *PRR9* expression in the evening (Carre and Veflingstad, 2013). Mutant plants, carrying T-DNA insertions within *PRR5* and/or *PRR7* and/or *PRR9* showed late flowering phenotypes. Single *prr5*, *prr7* or *prr9* mutants all showed a slightly late flowering phenotype and the delay in flowering time increases in the *prr5prr7* and *prr7prr9* double mutants or *prr5prr7prr9* triple mutants (Nakamichi et al., 2005).

Homologs of the *A. thaliana* *PRR7* gene were identified in several other species. In *H. vulgare*, *Ppd-H1* controls the expression of the *CO* orthologs *HvCO1* and *HvCO2* under LD (Campoli et al., 2012; Turner et al., 2005). In the short day (SD) species sorghum (*Sorghum bicolor*), *SbPRR37* was identified to be a repressor of flowering under LD (Murphy et al., 2011). Moreover, the *O. sativa* homolog *OsPRR37* has been shown to display a conserved clock-associated function and complements the late flowering *A. thaliana* *prr7-11* mutant (Murakami et al., 2007). The authors demonstrated that plants overexpressing *OsPRR37* in the *prr7-11* mutant could complement the mutation, and they bolted like the Col-0 ecotype after 29 days, whereas the *prr7-11* mutant bolted after 36 days.

In this study, I aimed to investigate the evolutionary conservation of the *BTC1* and *PRR7* function and moreover to characterize the function of two alleles, *BTC1_d* and *btc1_a* by overexpression in Arabidopsis. My first hypothesis was that amino acid substitutions between *BTC1_d* and *btc1_a* are responsible for the annual or biennial phenotype. Alternatively, the *BTC1_d* and *btc1_a* genes may be differentially regulated by their respective endogenous promoters. For complementation analysis, I overexpressed the *BTC1_d* and *btc1_a* coding sequences in the *A. thaliana* Col-0 ecotype and the *prr7-11* mutant. I measured days to flowering of transgenic T₂ plants in comparison with the non-transgenic T₂ plants of the same family and the non-transformed Col-0 and *prr7-11* mutants. I found differences between T₂ families and the wild type, however the different T₂ genotypes showed the same flowering time behavior. In conclusion, there is no effect of the *BTC1_d* and *btc1_a* gene in *A. thaliana*.

4.2 Material and methods

4.2.1 Plant material, growth conditions and phenotyping

For amplification of *BTC1* coding sequences the annual sugar beet line 930190 carrying the dominant *BTC1_d* allele and the biennial line 940043 carrying the recessive *btc1_a* allele were used. For plant transformation, I used 40 plants of the *A. thaliana* ecotype Col-0 and the *A. thaliana* flowering time gene mutant SALK_030430 referred to as *prr7-11* (Nakamichi et al., 2005), which was kindly provided by Takeshi Mizuno (Nagoya University, Japan). The homozygous *prr7-11* mutant line contains multiple T-DNA insertions within the first exon at position +203/247, which cause a delay in flowering of two days compared to Col-0, when grown in a climate chamber under 16h of light at 22°C (Yamamoto et al., 2003). The presence of the T-DNA insertion was determined by PCR using the primer combinations C015/ C016 and C084/ C015 (**Table 15**).

For plant transformation, seed production from T₁ and herbicide resistance tests with T₂ and T₃ families, plants were sown in soil and cold treated for 24h in a cold chamber (16h light, 4°C) and then grown in the greenhouse under long day conditions (LD, 16h light/ 8h dark, 22°C, 900µE). Herbicide resistance test were performed by BASTA® (Bayer CropScience, Mohnheim, Germany) spraying with a concentration of 1.7 g/l. The treatment was performed twice within two weeks at the four leaf stage (two cotyledons and two true leaves).

T₂ plants (**Supplementary Figure 6**) and the controls Col-0 and *prp7-11* were grown in soil in multi-pot trays, cold treated (see above) and grown in a randomized design in a climate chamber (BBC Brown Boveri York, Mannheim, Germany) under LD with 16h of light (200 µE) at 22°C or 18°C. Phenotyping was performed daily according to Boyes et al. (2001) between growth stage 5.10 (first flower bud visible) and 6.00 (first flower open). At stage 5.10 the number of rosette leaves and at stage 6.00, the number of cauline leaves was counted.

4.2.2 PCR, RT-qPCR and DNA sequencing

All PCR experiments conducted for the vector construction were performed using the (proofreading) recombinant *Pfu*-DNA-Polymerase (Fermentas, St.Leon-Rot, Germany). PCR products exhibiting the expected fragment sizes were sequenced at the Institute for Clinical Molecular Biology (IKMB, Kiel, Germany) with a final sample volume of 25µl and a final primer concentration of 3.2µM. In all PCRs conducted for genotyping *Taq*-DNA-Polymerase (Invitrogen, Karlsruhe, Germany) was used. RT-qPCR experiments were performed with *AtGAPDH*, *PRR7* and *BTC1* genes using the *Power SYBR®* Green PCR Master Mix (Applied Biosystems, Inc, USA) (**Table 15**).

Table 15: Genes and primer sequences used for PCR and RT-qPCR experiments

Gene, Construct	Forward Primer	Reverse Primer	PCR Conditions
<i>AtGAPDH</i>	B349	B350	95°C, 3' + 40 x (95°C, 10"; 61°C, 30"; 72°C, 30")
<i>BTC1</i>	B580	B581	95°C, 3' + 40 x (95°C, 10"; 61°C, 30"; 72°C, 30")
<i>PRR7</i>	C294	C083	95°C, 3' + 40 x (95°C, 10"; 60°C, 30"; 72°C, 30")
<i>PRR7</i>	C156	C157	95°C, 3' + 36 x (95°C, 30"; 54°C, 30"; 72°C, 6') +72°C, 10'
<i>PRR7</i>	C158	C159	95°C, 3' + 36 x (95°C, 30"; 54°C, 30"; 72°C, 6') 72°C, 10'
<i>nptII</i>	C015	C016	95°C, 3' + 36 x (95°C, 30"; 60°C, 30"; 72°C, 30") +72°C, 5'
<i>35S:BTC1_d/btc1_a</i>	C017	A747	95°C, 3' + 36 x (95°C, 30"; 57°C, 30"; 72°C, 2') +72°C, 10'
<i>35S:PRR7</i>	C294	C083	95°C, 3' + 36 x (95°C, 30"; 60°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'
T-DNA <i>prp7-11</i>	C084	C015	95°C, 3' + 36 x (95°C, 30"; 67°C, 45"; 72°C, 4') +72°C, 10'

4.2.3 Vector construction and *A. thaliana* transformation

We used the binary vector p7UG-AB (DNA cloning service, Hamburg, Germany) which carries the *pat* gene. Thus, transgenic plants could be selected by spraying the herbicide BASTA®. Since this vector does not contain a CaMV 35S promoter, we used our own vectors CAU3713 and CAU3714 (see **Chapter 3.2.2, Supplementary Table 11, Supplementary Figure 5**), carrying the *35S:BTC1_d* and *35S:btcl_a* transgene cassette, respectively. The complete transgene cassette including promoter/ terminator was released from the vector by *Sfi*I restriction. Transgene cassettes were cloned into the corresponding restriction sites of the binary vector p7UG-AB. The resulting constructs were named *p7-35S::BTC1_d* (CAU3715) and *p7-35S::btcl_a* (CAU3716), respectively (**Table 16**). The vector *p7-35S::PRR7* (CAU3718) which carries the *PRR7* coding sequence was constructed in the following way: in a first step, the complete coding sequence of *PRR7* was PCR-amplified using the primers C156/ C157 (**Table 15**) and the binary vector *pUNI51:PRR7* (Nottingham Arabidopsis Stock Centre, Loughborough, United Kingdom) as a template. Then, the PCR fragment was again PCR-amplified using the primers C158/ C159 (**Table 15**) with compatible sequence ends that are recognized by the restriction enzymes *Xma*I and *Sal*I. The resulting DNA fragments were restricted and ligated into the corresponding restriction sites of the binary vector p9N-35S (DNA Cloning Service, Hamburg, Germany). The complete transgene cassette including the promoter/terminator was cloned into the p7UG-AB vector (**Figure 21, Supplementary Figure 5**). All vectors designed during this study (**Supplementary Table 11**) were transformed into the heat shock competent *Escherichia coli* strain DH5α and *Agrobacterium tumefaciens* strain GV3101 PMP90 RK, respectively. The intactness of all vectors and sequences was confirmed by PCR amplification (**Chapter 4.2.2**) and sequencing (Institute for Clinical Molecular Biology, Kiel Germany). Subsequently, the vectors CAU3715, CAU3716 and CAU3718 were transformed into 40 Col-0 and 40 *prp7-11 A. thaliana* plants, respectively, by the floral dip method (Clough and Bent, 1998) (**Table 16**). Plants were grown in the greenhouse as described in **Chapter 4.2.1** and T₁ seeds were harvested after bag isolation (**Figure 22**).

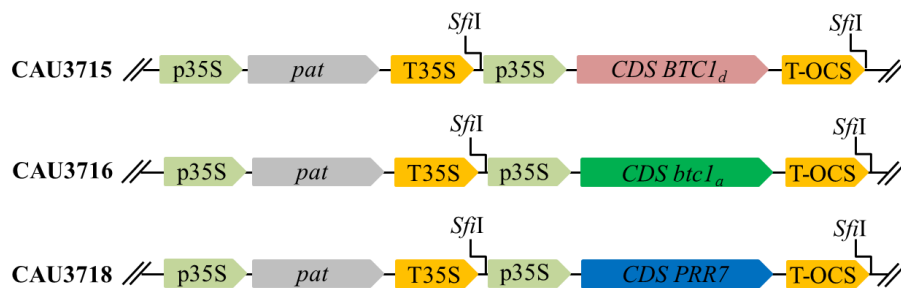


Figure 21: T-DNA region of the transformation vectors CAU3715, CAU3716 and CAU3718 carrying the *BTC1_d*, *btcl_a* and *PRR7* CDS, respectively. Arrows in light green represent promoter regions with p35S: cauliflower mosaic virus (CaMV) 35S promoter. The grey arrow represents the selectable marker *phosphinothricin acetyltransferase* (*pat*) for selection of transgenic plants (see **Chapter 4.2.1**). Terminator regions with T35S: 35S terminator derived from CaMV and T-OCS: terminator derived from the *octopine synthase* gene are marked in yellow. Red arrow: coding sequence of *BTC1_d* of the annual sugar beet line 930190; dark green arrow: coding sequence of *btcl_a* of the biennial line 940043; blue arrow: coding sequence of *PRR7* from *A. thaliana*. *Sfi*I restriction sites were used for cloning the respective transgene cassette.

Table 16: Vector constructs for *Arabidopsis* floral dip transformation experiments into the ecotype Col-0 and the mutant *prp7-11*. *BTC1_d* is the allele from the annual sugar beet line 930190, whereas *btc1_a* stems from the biennial line 940043.

Construct	Donor Plant	Vector Name	Transgene Cassette
<i>p7-35S::BTC1_d</i>	Col-0	CAU3715	<i>35S::BTC1_d</i>
<i>p7-35S::btc1_a</i>	Col-0	CAU3716	<i>35S::btc1_a</i>
<i>p7-35S::PRR7</i>	Col-0	CAU3718	<i>35S::PRR7</i>
<i>p7-35S::BTC1_d</i>	<i>prp7-11</i>	CAU3715	<i>35S::BTC1_d</i>
<i>p7-35S::btc1_a</i>	<i>prp7-11</i>	CAU3716	<i>35S::btc1_a</i>
<i>p7-35S::PRR7</i>	<i>prp7-11</i>	CAU3718	<i>35S::PRR7</i>

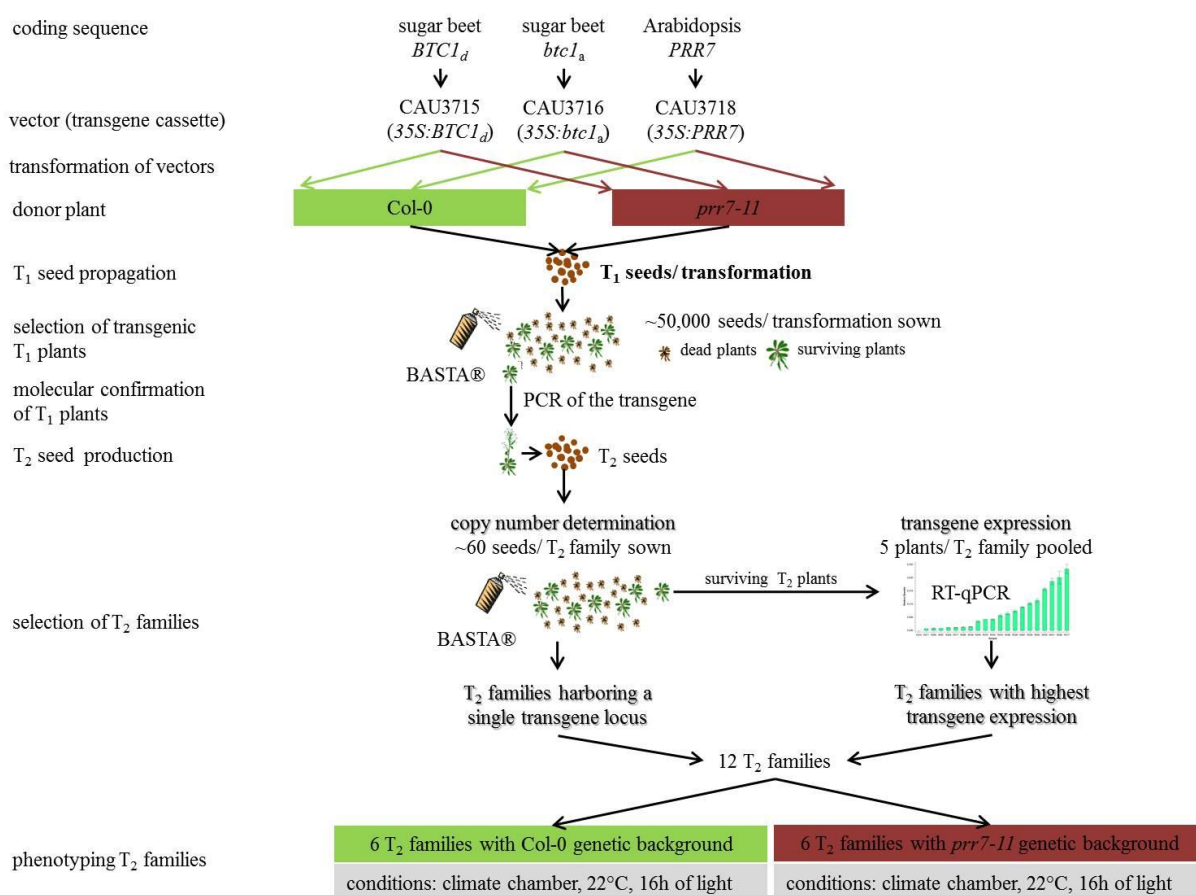


Figure 22: Workflow of *A. thaliana* transformations, T₁ and T₂ selection and phenotyping T₂ families. Generation of vectors and the plant transformations are described under **Chapter 4.2.1**. Selection of T₁ plants and T₂ families as well as the growth conditions of plants and phenotyping procedures are described under **Chapter 4.2.1**.

In a next step T₁ seeds were sown, grown and BASTA® sprayed and transgenic T₁ plants that survived the treatment were bag isolated and T₂ seeds were harvested (**Figure 22**). The T₂ plants should carry single copy insertions, in order to minimize the number of T₂ plants per family for phenotyping. The copy number was determined as follows: ~60 T₂ seedlings/family were grown in soil (conditions see **Chapter 4.2.1**) and plants were BASTA® treated after two weeks as previously described. Then, the number of surviving/dead plants was counted and a χ^2 -test was performed to test for a 3:1 segregation ratio ($\alpha = 0.05$). The expression of the transgene was measured by RT-qPCR in BASTA®-resistant T₂ plants using the primers B580/ B581 and C294/ C083 (**Table 15**).

For identification of homozygous T₃ lines, five transgenic T₂ plants of each family were bag isolated and T₃ seeds harvested. Then, ~60 T₃ plants/ family were grown and BASTA® treated. Those T₃ families in which all plants survived the BASTA® application were defined as homozygous T₃ lines.

4.2.4 Genotyping experiments

Two leaf samples were taken from four weeks old *A. thaliana* T₁ and T₂ plants and freeze-dried for 24h. Extraction of genomic DNA was performed using the CTAB method (Saghai-Maroo et al., 1984) with slight modifications. DNA was diluted ten-fold prior to PCR (**Table 15**). The quality of extracted gDNA was determined by PCR using the primer combination C294/ C083, yielding a 418 bp fragment of the endogenous *PRR7* gene. T₁ and T₂ plants were tested for the presence of the *pat* gene and the transgenes *35S:BTC1_d*, *35S:btc1_a* or *35S:PRR7*, respectively, by PCR amplification with primer combinations C162/ C163, C017/ A747 and C294/ C083 (**Table 15**). In non-transgenic plants, only a 418 bp fragment from the endogenous *PRR7* gene was expected. Transgenic plants were supposed to show two additional fragments: a 491 bp fragment from the *pat* gene and additionally a 321 bp fragment as a consequence of the integration of the *35S:PRR7* transgene or the 1842 bp fragment from the *35S:BTC1_d* or *35S:btc1_a* transgene. PCR fragments were checked on 1% or 2% agarose gels.

4.2.5 Expression analysis in transgenic T₂ plants

For expression analysis of *BTC1* and *PRR7* in transgenic *A. thaliana* T₂ plants, I harvested rosette leaves from five 30 days old transgenic plants of each family and pooled them. Samples were taken three days after BASTA® treatment at zeitgeber time (ZT) 6 and immediately transferred to liquid nitrogen. Total RNA was isolated using the Plant RNeasyKit™ (Qiagen, Hilden, Germany) and treated with DNase using the DNA-free™ Kit (Ambion, Austin, USA) following the supplier's instructions. cDNA synthesis was performed with 500ng of total DNase-treated RNA using the First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's instructions. The resulting cDNA was diluted twenty fold and 2µl were used as template for RT-qPCR. Three technical replicates of each pooled sample were applied to RT-qPCR using the *Power SYBR® Green PCR Master Mix* (Applied Biosystems, Inc, USA) in a final reaction volume of 20µl including a final primer concentration of 20pM on a CFX96-Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA). The housekeeping gene *AtGAPDH* was used as a control and its expression was measured using the primer combination B349/ B350. The expression of *BTC1_d*/ *btc1_a* was measured using the primer combination B580/ B581, whereas the *PRR7* expression was quantified with primer combination C294/ C083 (**Table 15**). Analysis of cycling threshold (CT) values for each reaction was performed using the CFX Manager™ Software v2.1 (Bio-Rad, Hercules, CA, USA). Resulting data were normalized to the geometric mean of *AtGAPDH* expression using the comparative C_T (ΔC_T) method.

4.3 Results

4.3.1 Molecular and phenotypic identification of T₁ plants transformed with the *BTC1_d*/*btc1_a* genes from sugar beet and the *PRR7* gene from *A. thaliana*

In total 2,147,500 seeds were obtained after floral dip experiments of *Col-0* and *prp7-11* plants transformed with *BTC1_d*, *btc1_a* and *PRR7*, respectively. For the identification of primary transgenic plants (T₁), 190,500 seeds were sown in soil and grown in the greenhouse under LD with a 16h light/ 8h dark cycle at 22°C. Seedlings were treated with the herbicide BASTA® and 366 surviving plants were selected. To confirm the transgene integration of each integrated transgene cassette, I analyzed all BASTA® resistant T₁ plants by PCR using the primers C017/ A747 and C294/ C083 (**Table 15, Chapter 4.2.2, Figure 22**). In total 204 T₁ transformants were identified. Of these, 118 T₁ plants showed an 1842 bp fragment demonstrating the integration of the *35S:BTC1_d* or *35S:btc1_a* transgene cassette and 86 T₁ plants showed a 418 bp fragment derived from the endogenous *PRR7* gene and a 321 bp fragment derived from the integrated *35S:PRR7* transgene cassette (**Figure 23, Table 16**). All *BTC1_d*, *btc1_a* and *PRR7*-positive T₁ plants were bag isolated and T₂ seeds were harvested. The remaining plants were discarded.

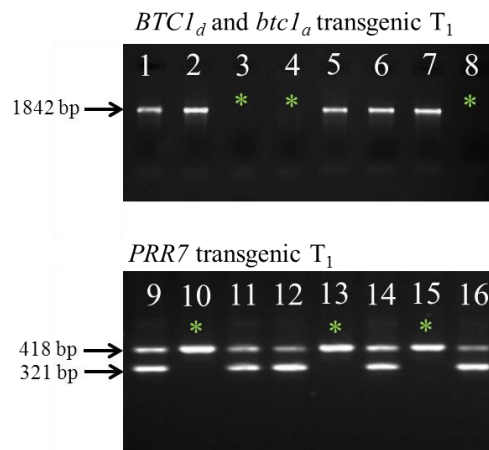


Figure 23: Transgene genotyping of T₁ plants after transformation of *35S:BTC1_d*, *35S:btc1_a* and *35S:PRR7* into *Col-0*. DNA from BASTA® resistant T₁ plants transformed with *35S:BTC1_d* (1-4) or *35S:btc1_a* (5-8) was amplified by PCR using the primers C017/ A747. DNA from T₁ plants which were transformed with *35S:PRR7* (9-16) was amplified by PCR using the primers C294/ C083. PCR fragments were separated in a 2% agarose gel. Arrows indicate respective fragment lengths of the PCR products. Non transgenic T₁ plants are marked by asterisks.

Table 17: Results from the *A. tumefaciens* transformation experiments. T₁ seeds were harvested from the ecotype Col-0 and the mutant *prp7-11* after transformation. Transgenic T₁ plants had been transformed with three different constructs. Plants were grown in the greenhouse (16h light/ 8h dark, 22°C, 900μE) and BASTA® treated. Positive T₁ plants carrying the *35S:BTC1_d* or *35S:btc1_a* transgene cassette displayed an 1842 bp fragment whereas positive T₁ plants carrying the *35S:PRR7* transgene cassette showed a 321 bp fragment after PCR. The transformation efficiency was calculated as the number of transgenic plants divided by the total number of plants. T₁ plants were propagated and segregating T₂ families were analyzed.

Donor Line	Construct	Number of T ₁ Seeds Harvested	#T ₁ Seeds Analyzed	BASTA® Resistant T ₁ Plants	PCR-Positive T ₁ Plants	Transformation Efficiency	Name of Analyzed T ₂ Family
Col-0	<i>35S:BTC1_d</i>	162,500	50,000	14	13	0.026 %	R347, R355
	<i>35S:btc1_a</i>	140,000	50,000	147	86	0.172 %	R241, R338
	<i>35S:PRR7</i>	125,000	50,000	160	78	0.156 %	R099, R117
<i>prp7-11</i>	<i>35S:BTC1_d</i>	545,000	13,500	37 (15 ¹)	7	n.d.	R454, R457
	<i>35S:btc1_a</i>	520,000	13,500	71 (15 ¹)	12	n.d.	R463, R467
	<i>35S:PRR7</i>	655,000	13,500	75 (15 ¹)	8	n.d.	R480, R483
total:		2,147,500	190,500	504 (366 ¹)	204		

¹T₁ plants selected for further analysis
n.d., not determined

The transgene copy number was determined in 73 randomly selected T₂ families from all transformation experiments with ~60 seeds/ T₂ family. Seeds were sown in soil and grown in the greenhouse under 16h light and 22°C. To analyze the segregation of the *pat* gene in T₂ families I treated the seedlings with BASTA® and counted the transgenic (BASTA® resistant) and non-transgenic (BASTA® susceptible) plants (**Figure 22**). In 44 T₂ families the χ^2 -value ranged from $\chi^2 = 0.00$ to $\chi^2 = 3.70$ ($\alpha_{0.05} = 3.84$) (**Supplementary Table 8**) confirming that the segregation ratio of transgenic : non-transgenic plants does not deviate significantly from a 3:1 segregation ratio, which indicates that a single copy of the transgene is integrated in these families. Transgenic plants within the 44 T₂ families were further used for expression analysis of the integrated transgene (**Chapter 4.3.2**). In the remaining 29 T₂ families the segregation ratio deviates significantly from a 3:1 ratio due to an excess of transgenic plants, suggesting the integration of more than one transgene copy and were discarded for further analyses (**Supplementary Table 8**).

Table 18: Transgene copy number determination in 73 T₂ families over all transformation experiments. A χ^2 -test ($\alpha_{0.05} = 3.84$) was performed to proof whether the observed segregation ratio fits to a 3:1 ratio, expected for inheritance of a monogenic trait.

Donor Plant	Construct	Number of T ₂ Families	Copy Number	
			1	>1
Col-0	<i>35S:BTC1_d</i>	13	9	4
	<i>35S:btc1_a</i>	20	12	8
	<i>35S:PRR7</i>	20	13	7
<i>prp7-11</i>	<i>35S:BTC1_d</i>	6	2	4
	<i>35S:btc1_a</i>	8	5	3
	<i>35S:PRR7</i>	6	3	3
Total		73	44	29

4.3.2 Expression analysis of *BTC1_d*, *btc1_a* and *PRR7* transgenes in 44 T₂ families

RT-qPCR was performed to measure the expression of *BTC1_d*, *btc1_a* and *PRR7* in transgenic T₂ plants. The expression was determined in 44 single copy T₂ families and compared to the respective non-transgenic controls Col-0 and *prr7-11*. I pooled 5 plants/ T₂ family, which survived the BASTA® treatment (**Chapter 4.3.1**). To investigate the transgene expression in T₂ plants transformed with *35S:BTC1_d* and *35S:btc1_a*, I used the primers B580/ B851 amplifying a 305 bp fragment of the *BTC1_d* or *btc1_a* transgenes. T₂ plants transformed with *35S:PRR7*, were analyzed using the primers C294/ C083 amplifying 321 bp fragments of the transgenic *PRR7* and the endogenous coding sequence. As expected, I observed *BTC1_d*/*btc1_a* and *PRR7* expression in all respective transgenic plants. For further studies I selected two T₂ families/ construct as independent replicates that show highest transgene expression in a Col-0 and *prr7-11* background (**Figure 24**). The remaining families were discarded.

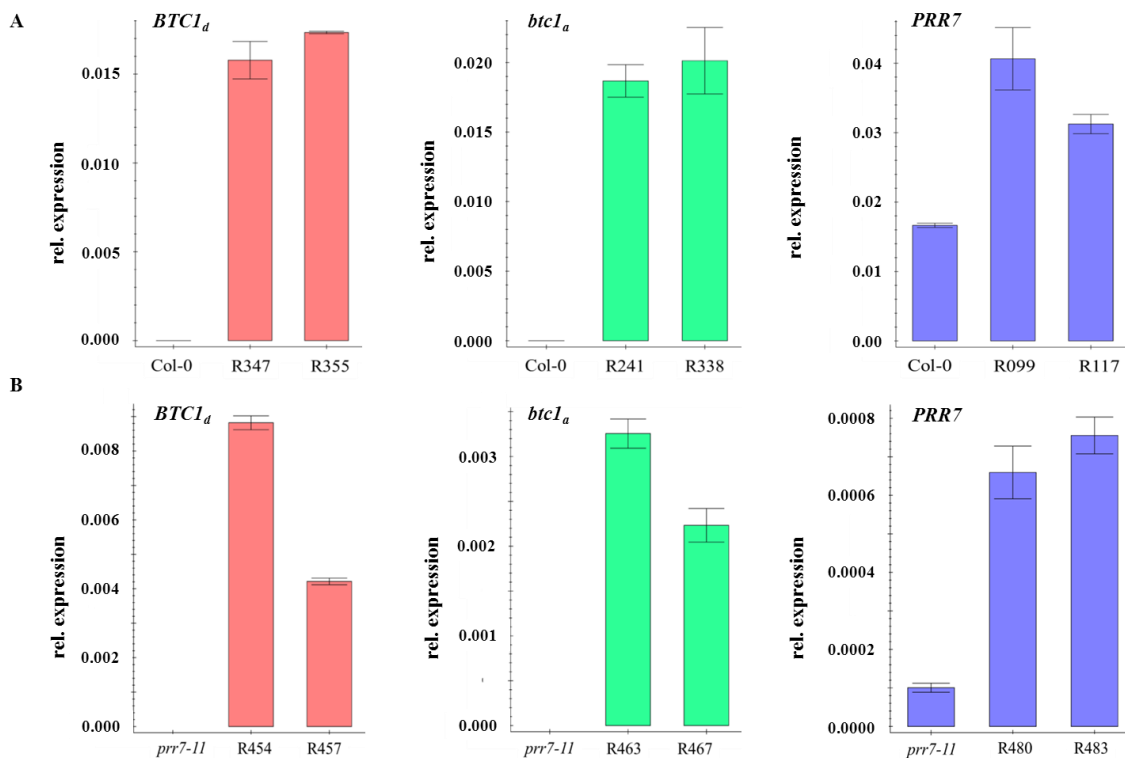


Figure 24: Gene expression analysis in T₂ plants with single copy insertions of *BTC1_d*, *btc1_a* and *PRR7* transgenes in a Col-0 (A) and *prr7-11* background (B) compared to the non-transformed controls Col-0 and *prr7-11*. The relative expression of *BTC1_d* (red boxes), *btc1_a* (green boxes) and *PRR7* (blue boxes) was measured by RT-qPCR as described in **Chapter 4.2.5** and normalized against the housekeeping gene *AtGAPDH* applying the ΔC_T method. From each family, 5 plants were pooled and analyzed. Error bars represent the standard error of the mean of three technical replicates from each analyzed sample.

T₂ families R347, R355, 241 and R338 were derived from transformation of *35S:BTC1_d* or *35S:btc1_a* into Col-0. And the families R454, R457, R463 and R467 were generated by transformation of *35S:BTC1_d* or *35S:btc1_a* into the *prr7-11* mutant. In transgenic plants of these families, *BTC1_d*/*btc1_a* was expressed, whereas no expression was observed in the non-transgenic controls Col-0 and *prr7-11*, demonstrating ectopic expression of the transgenes under the control of the CaMV 35S promoter. This was also observed in T₂ families R099, R117, R480 and R483 which were obtained by transformation of *35S:PRR7* into Col-0 and *prr7-11*, respectively. In transgenic plants of families R099 and R117 the expression of *PRR7* increased at least twofold compared to Col-0. Transgenic plants belonging to family R480 and R483 showed up to eight times higher expression levels of *PRR7* compared to *prr7-11* (**Figure 24**). These 12 T₂ families were selected for phenotyping.

4.3.3 Flowering time measurements with transgenic plants

For phenotyping, 30-35 plants per T₂ family were grown in a climate chamber under 16h of light and phenotyped for bolting and flowering (**Chapter 4.2.1**). As a control, 35 Col-0 and *prp7-11* plants were grown. It had been previously shown that the *prp7-11* mutant, when grown under 22°C, started flowering two days later compared to Col-0 (Yamamoto et al., 2003). I found that the delay in flowering of the *prp7-11* mutant increases up to two weeks when grown at 18°C. Col-0 started flowering after 51 ± 2.3 days after sowing while the *prp7-11* mutant started to flower after 65 ± 5.1 (**Figure 25, Supplementary Table 10**).

Consequently, T₂ families with a Col-0 genetic background were grown at 22°C and T₂ families with a *prp7-11* genetic background were grown at 18°C. For identification of transgenic and non-transgenic plants, each T₂ family was genotyped by PCR as described for the identification of T₁ plants (**Chapter 4.3.1**). Genotyping of 30-35 plants/ T₂ families followed by χ^2 -analysis ($\alpha_{0.05} = 3.84$) revealed that the segregation ratio of transgenic and non-transgenic plants did not deviate significantly from the expected 3:1 ratio in 9 families (**Table 19**). In two families the ratio deviates significantly from a 3:1 ratio and one family R467 could not be genotyped due to poor DNA quality (**Table 19**). These three families were not phenotyped.

Table 19: Genotyping of T₂ families that were phenotyped. 35 plants/ T₂ family were grown in soil in the climate chamber with 16h light at 22°C. Each plant was genotyped by PCR with DNA extracted from leaves. For genotyping T₂ families, derived from transformation of *35S:PRR7* into Col-0 or *prp7-11*, I used the primers C0294/ C083, whereas primers C017/ A747 were used for genotyping T₂ families, derived from transformation of *35S:BTC1_d* or *35S:btc1_a* into Col-0 or *prp7-11*. The observed segregation ratios (transgenic: non-transgenic) were tested by χ^2 analysis ($\alpha_{0.05} = 3.84$) for a 3:1 ratio, expected for inheritance of single transgene locus.

Genetic Background	Transgene Cassette	T ₂ Family	Plants Grown	Transgenic	Non-Transgenic	No PCR Product	χ^2 -test for H ₀ ¹ = 3:1 (transgenic:non-transgenic)
Col-0	<i>35S:PRR7</i>	R099	35	27	7	1	0.35
	<i>35S:PRR7</i>	R117	35	20	13	2	3.65
	<i>35S:btc1_a</i>	R241	35	29	1	5	7.51*
	<i>35S:btc1_a</i>	R338	35	24	11	0	0.77
	<i>35S:BTC1_d</i>	R347	35	25	9	1	0.04
	<i>35S:BTC1_d</i>	R355	35	26	7	2	0.25
<i>prp7-11</i>	<i>35S:PRR7</i>	R480	35	18	13	4	4.74*
	<i>35S:PRR7</i>	R483	35	21	13	1	3.17
	<i>35S:btc1_a</i>	R463	35	22	7	6	0.01
	<i>35S:btc1_a</i>	R467	35	6	7	22	-
	<i>35S:BTC1_d</i>	R454	35	22	7	6	0.01
	<i>35S:BTC1_d</i>	R457	35	22	9	4	0.27

¹ H₀, null hypothesis for inheritance of a single transgene locus

* significant deviation at $\alpha_{0.05}$ ($\chi^2 = 3.84$) of observed segregation from an expected 3:1 segregation.

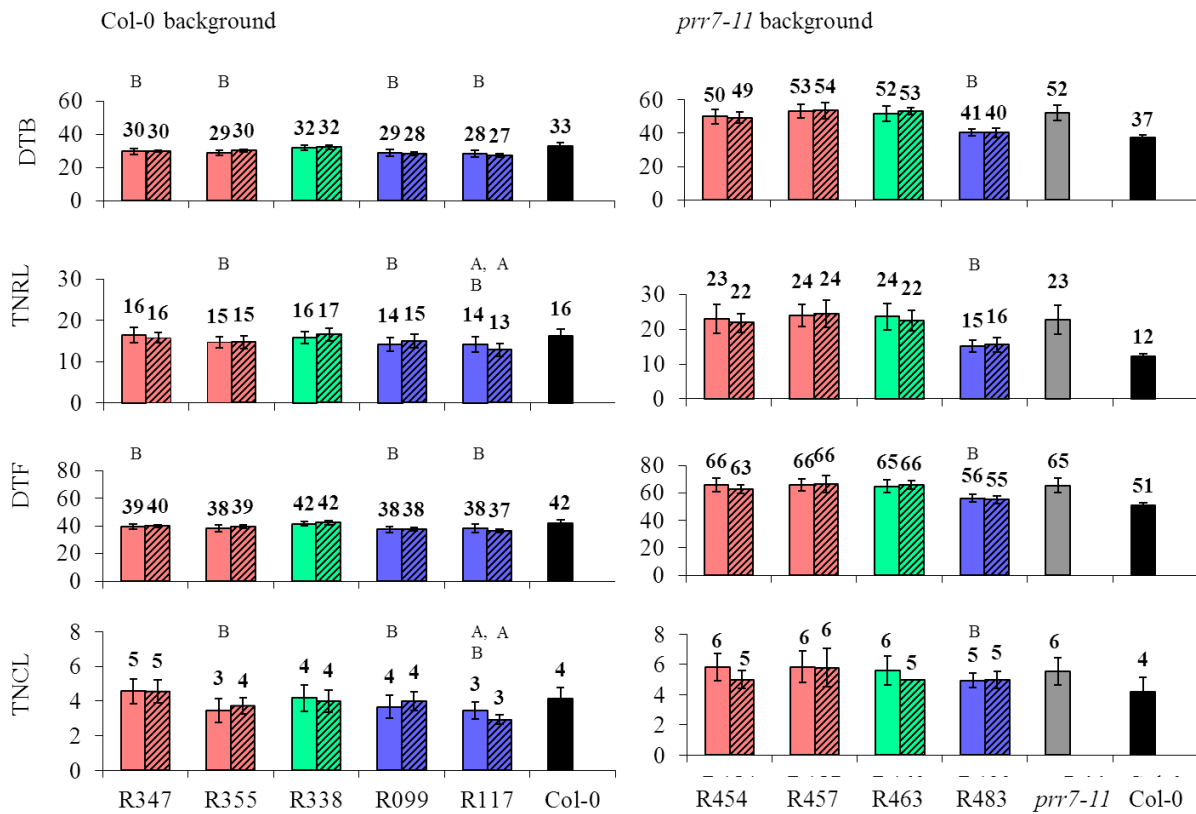


Figure 25: Growth experiments with T₂ families with Col-0 or *prr7-11* background. Plants were grown under 16h of light in a climate chamber. T₂ families with Col-0 background were grown at 22°C, whereas T₂ families with *prr7-11* background were grown at 18°C. Boxes with dashed lines represent 7-13 non-transgenic plants whereas boxes without dashed lines represent 20-27 transgenic T₂ plants from each T₂ family (**Supplementary Table 9, Supplementary Table 10**). Red: transgenic plants of T₂ families transformed with *35S:BTC1_d*; green: transgenic plants of T₂ families transformed with *35S:btc1_a*; blue: transgenic plants of T₂ families transformed with *35S:PRR7*. Black: non-transformed control Col-0; grey: non-transformed *prr7-11* mutant. Error bars represent the standard error of the mean of phenotyped transgenic or non-transgenic plants/T₂ family. DTB: days to bolt after sowing (growth stage 5.10). TNRL: total number of rosette leaves at growth stage 5.10. DTF: days to flower after sowing (growth stage 6.10). TNCL: total number of cauline leaves at growth stage 6.10. Numbers above the boxes: mean of phenotyped character. A, significant differences confirmed by Student's *t*-Test at $\alpha=0.05$ between transgenic and non-transgenic T₂ plants of the same family. B, significant differences confirmed by Student's *t*-Test at $\alpha=0.05$ between the control Col-0 and transgenic T₂ plants with Col-0 genetic background or the control *prr7-11* and transgenic T₂ plants with *prr7-11* genetic background.

Phenotyping T₂ families with a Col-0 genetic background (**Figure 25, Supplementary Table 9, Supplementary Table 10**) revealed no significant differences in bolting and flowering time between transgenic and non-transgenic plants in each segregating T₂ family. Days to bolting (DTB) ranged from 28 to 32 in transgenic plants and from 27 to 32 in non-transgenic T₂ plants in mean. Transgenic plants flowered 38 to 42 days after sowing (mean of DTF) and non-transgenic flowered 37 to 42 days after sowing. Regarding TNRL and TNCL, transgenic and non-transgenic plants of family R117 showed significant differences (Student's *t*-Test; $\alpha=0.05$). Transgenic plants had 14.25 ± 1.84 rosette leaves at growth stage 5.10, whereas non-transgenic plants had 12.92 ± 1.54 . Moreover, the number of cauline leaves at growth stage 6.10 was increased in transgenic plants (3.45 ± 0.50) compared to non-transgenic plants (2.92 ± 0.27). In the remaining T₂ families, TNRL ranged from 14 to 16 in transgenic plants and from 15 to 17 in non-transgenic plants, whereas TNRL ranged from 3 to 5 in transgenic plants and 4 to 5 in non-transgenic plants with no significant differences, respectively.

Interestingly, I observed significant differences, (Student's *t*-Test; $\alpha=0.05$), between transgenic plants of the T₂ families and non-transformed Col-0 control plants, except for

family R338 (transgene integration *35S:btc1_a*). Col-0 plants started to bolt 33 ± 2.09 days after sowing, whereas DTB in transgenic plants of 4 families ranged from 28 to 30 and thus plants of these families bolted 3 to 5 days earlier than the control. Moreover the control plants started flowering 42 days after sowing, while transgenic plants over all T₂ families, except family R338, started flowering after 38 to 39 days. Also the number of rosette and cauline leaves was significantly different in 3 families (**Figure 25, Supplementary Table 9, Supplementary Table 10**) when compared to Col-0. On average, the Col-0 had 16 rosette leaves at growth stage 5.10 and 4 cauline leaves at growth stage 6.10, whereas in transgenic plants of 3 families (**Figure 25, Supplementary Table 9, Supplementary Table 10**) TNRL ranged from 14 to 15 and TNCL ranged from 3 to 4. For family R347 no significant differences were observed for TNRL and TNCL when compared to Col-0.

The *A. thaliana prr7-11* mutant contains multiple T-DNA insertions within the first exon of *PRR7* and flowers 2-7 days later when compared to the ecotype Col-0 (Yamamoto et al., 2003; Murakami, et al., 2007). I expected under the assumption that *BTC1_d* is an ortholog of *PRR7* and the *BTC1* genes have different functions that overexpression of *BTC1_d* as well as *PRR7* but not of *btc1_a* in *prr7-11* mutant plants complements the phenotype of the *prr7-11* mutant and transgenic plants of the selected T₂ families bolt and flower as Col-0, whereas non-transgenic T₂ plants bolt and flower as *prr7-11*. However, I observed no significant differences in bolting time, flowering time and the respective number of cauline or rosette leaves between transgenic and non-transgenic T₂ plants (**Figure 25, Supplementary Table 9, Supplementary Table 10**). On average, transgenic plants over all T₂ families bolted after 41 to 53 days and non-transgenic T₂ plants after 40 to 54 days. At growth stage 5.10 transgenic T₂ plants had 15-24 rosette leaves and non-transgenic T₂ individuals 16-24 leaves. Flowering was initiated in transgenic T₂ after 56-66 days and in non-transgenic plants after 55-66. The number of cauline leaves at growth stage 6.00 ranged from 5-6 in both transgenic and non-transgenic T₂ plants.

When compared to the non-transformed control *prr7-11*, I observed no significant differences (Student's *t*-Test, $\alpha = 0.05$) between transgenic T₂ plants and the control in three out of four T₂ families (**Figure 25, Supplementary Table 9, Supplementary Table 10**). In one family (R483) transgenic T₂ individuals bolted and flowered significantly earlier with a reduced number of leaves than the *prr7-11* mutant. In this family transgenic T₂ plants bolted and flowered 12 and 9 days earlier than the *prr7-11* mutant. Moreover, transgenic T₂ individuals had on average 7 rosette leaves and one cauline leaf less. However, these plants did not bolt and flower as early as Col-0 and the number of leaves was also not as low as in the ecotype (**Figure 25, Supplementary Table 9, Supplementary Table 10**).

4.3.4 Production of homozygous T₃ lines

T₃ seeds were harvested from 5 transgenic plants/ T₂ family after bag isolation. In total 45 T₃ families with ~60 seedlings/ T₃ family were grown in the greenhouse (16h light, 22°C). After 3 weeks seedlings were sprayed with BASTA®. As a control ~60 seedlings of Col-0 were grown and BASTA® treated in parallel. A T₃ family in which 100% seedlings survived the herbicide application was fixed for the transgene and was termed as a homozygous T₃ line. In summary, 9 homozygous T₃ lines were found, where all T₃ plants survived the BASTA® treatment, derived from seeds of single T₂ plants. Seed numbers ranged from 500 seeds to 20,000 (**Table 20**). The remaining 36 T₃ families were segregating for the transgene, thus they were derived from heterozygous T₂ plants.

Table 20: Homozygous T₃ lines fixed for the transgenes *BTC1_d/btc1_a* or *PRR7*. T₃ seeds were obtained from single transgenic T₂ plants. From each T₃ family, ~60 seedlings were BASTA® sprayed.

T ₃ Line Seed Code	Construct	Genetic Background	T ₂ Seed Parent	Number of Seeds Harvested
124287	<i>35S:BTC1_d</i>	Col-0	R355/15	20,000
124285	<i>35S:btc1_a</i>	Col-0	R338/7	10,000
123287	<i>35S:PRR7</i>	Col-0	R117/3	3,000
124284	<i>35S:PRR7</i>	Col-0	R117/8	12,500
123290	<i>35S:PRR7</i>	Col-0	R117/34	4,500
123399	<i>35S:BTC1_d</i>	<i>prp7-11</i>	R457/11	500
123411	<i>35S:btc1_a</i>	<i>prp7-11</i>	R463/10	2,500
123438	<i>35S:PRR7</i>	<i>prp7-11</i>	R483/5	2,000
123439	<i>35S:PRR7</i>	<i>prp7-11</i>	R483/8	500

4.4 Discussion

Arabidopsis has been well studied as a model for flower development and thus offers important advantages for basic research in genetics. Often, genes are functionally characterized by transformation into Arabidopsis, because the transformation procedure is well established and generations can be produced in a short time, due to the short life cycle of Arabidopsis.

I transformed the coding sequence of two *BTC1* alleles and the *PRR7* gene under the control of the constitutive 35S promoter into Arabidopsis Col-0 and *prp7-11* mutant plants. Multiple T-DNA insertions within the first exon of *PRR7* result in a late flowering phenotype of the *prp7-11* mutant (Yamamoto et al., 2003). I expected, if changes in the polypeptide sequence alter the flowering phenotype, that overexpression of *btc1_a* will not restore the flowering phenotype in the *prp7-11* mutant whereas transgenic plants overexpressing *BTC1_d* or *PRR7* in a *prp7-11* genetic background will flower as early as Col-0 and thus will complement the late flowering phenotype of the *prp7-11* mutant. In addition, I assumed that overexpression of *BTC1_d* and *PRR7* in a Col-0 genetic background accelerates flowering under the assumption that the *BTC1_d* protein has the same function in *A. thaliana* as in *B. vulgaris*. In both cases, my assumptions could not be confirmed within segregating T₂ families. None of the two *BTC1* alleles complemented the *prp7-11* mutant and *BTC1_d* did not accelerate flowering in a Col-0 genetic background. These results suggest that *BTC1_d* is not a functional ortholog of *PRR7*.

The current study is based on data from segregating T₂ families. Surprisingly, non-transgenic T₂ plants and non-transformed Col-0 or *prp7-11* plants differed in their bolting and flowering behavior which can only be explained by mutations within the genome in non-transgenic T₂ plants induced by the *A. tumefaciens* mediated transformation (Wilson et al., 2006). In previous studies the effect of *PRR7* or homologs from other species on flowering time was based on data revealed from homozygous T₃ lines. In these studies T₃ lines were phenotyped and data compared to those revealed from non-transformed donor plants (Matsushika et al., 2007; Murakami et al., 2007). It would have been an alternative to phenotype homozygous T₃ lines in comparison to non-transformed controls in this study. In turn and under the assumption that mutations might be induced during the transformation process, non-transformed controls may not be suitable as controls, because they do not carry these mutations.

In *A. thaliana*, *PRR7* is diurnally regulated. The diurnal regulation of genes allows the activation or repression of target genes at a certain time point at the course of 24 h and thus enables to respond to environmental conditions, i.e. day and night (Thomas and Vince-Prue, 1997). In a study by Murakami et al. (2007) the coding sequence of the *PRR7* homolog from rice (*Oryza sativa*), *OsPRR37* was overexpressed in *prp7-11* plants under the control of the endogenous *PRR7* promoter. Homozygous T₃ lines were generated and flowering time was determined in comparison to Col-0 and non-transformed *prp7-11* plants. They could show that *prp7-11* mutants bolted after 36 days with 17 rosette leaves, whereas the independent transgenic lines L#71 and L#72 bolted after 29 days as well as Col-0 with 12 rosette leaves. With regard to these results, it could be suggested that ectopic expression of the *BTC1* genes under the endogenous *PRR7* promoter would have result in a phenotypic effect. However, overexpression of *PRR7* under the control of the 35S promoter was in turn successfully demonstrated by Matsushika et al. (2007). In that study T₃ lines overexpressing the genomic DNA of *PRR7* in a Col-0 genetic background bolted ~7 days earlier as compared to the non-transformed Col-0 plants.

In my experiment, the T₂ families were grown under LD conditions. However, also SD conditions have been used in earlier experiments. Matsushika et al. (2007) observed the early flowering phenotype in transgenic lines overexpressing *PRR7*, in SD (10h light/ 14h dark) but not in LD (16h light/ 8h dark). In contrast, complementation of the late flowering *prp7-11* mutant through overexpression of *OsPRR37* was demonstrated under LD conditions (16h light/ 8h dark) while no growth experiments under SD conditions were mentioned (Murakami et al., 2007).

In conclusion, I assume that *PRR7* and *BTC1_d* are no functional orthologs, because *BTC1_d* could not complement the late flowering phenotype in the *prp7-11* mutant. A putative functional ortholog of the *PRR7* gene in *B. vulgaris* could be the *BvPPR7* gene which shares higher sequence similarity to *PRR7* as *BTC1* and thus is closer related to *PRR7* than *BTC1* to *PRR7* (Pin et al., 2012). It is conceivable that overexpression of *BvPPR7* will complement the late flowering phenotype of the *prp7-11* mutant. Complementation studies for analyzing the effect of *BvPPR7* in *A. thaliana* are in progress (O. Omolade, pers. communication).

4.5 References

- Anwer, M.U., and S.J. Davis, 2013: An overview of natural variation studies in the *Arabidopsis thaliana* circadian clock. *Semin Cell Dev Biol.* 24, 422-429.
- Boyes, D.C., A.M. Zayed, R. Ascenzi, A.J. McCaskill, N.E. Hoffman, K.R. Davis, and J. Grolach, 2001: Growth stage-based phenotypic analysis of *Arabidopsis*: A model for high throughput functional genomics in plants. *Plant Cell* 13, 1499-1510.
- Campoli, C., M. Shtaya, S.J. Davis, and M. von Korff, 2012: Expression conservation within the circadian clock of a monocot: natural variation at barley *Ppd-H1* affects circadian expression of flowering time genes, but not clock orthologs. *BMC Plant Biology* 12, 97.
- Carre, I., and S. Veflingstad, 2013: Emerging design principles in the *Arabidopsis* circadian clock. *Semin Cell Dev Biol.* 24, 393-398.
- Chaw, S.M., C.C. Chang, H.L. Chen, and W.H. Li, 2004: Dating the monocot-dicot divergence and the origin of core eudicots using whole chloroplast genomes. *J Mol Evol* 58, 424-441.
- Clough, S.J., and A.F. Bent, 1998: Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *The Plant Journal* 16, 735-43.

- Dodd, A.N., N. Salathia, A. Hall, E. Kevei, R. Toth, F. Nagy, J.M. Hibberd, A.J. Millar, and A.A. Webb, 2005: Plant circadian clocks increase photosynthesis, growth, survival, and competitive advantage. *Science* 309, 630-633.
- Huang, W., P. Perez-Garcia, A. Pokhilko, A.J. Millar, I. Antoshechkin, J.L. Riechmann, and P. Mas, 2012: Mapping the Core of the *Arabidopsis* Circadian Clock Defines the Network Structure of the Oscillator. *Science* 336, 75-79.
- Kinmonth-Schultz, H.A., G.S. Golembeski, and T. Imaizumi, 2013: Circadian clock-regulated physiological outputs: Dynamic responses in nature. *Semin Cell Dev Biol.* 24, 407-413.
- Locke, J.C., M.M. Southern, L. Kozma-Bognar, V. Hibberd, P.E. Brown, M.S. Turner, and A.J. Millar, 2005: Extension of a genetic network model by iterative experimentation and mathematical analysis. *Mol Syst Biol* 1, 2005.0013.
- Matsushika, A., M. Murakami, S. Ito, N. Nakamichi, T. Yamashino, and T. Mizuno, 2007: Characterization of circadian-associated pseudo-response regulators: I. Comparative studies on a series of transgenic lines misexpressing five distinctive PRR genes in *Arabidopsis thaliana*. *Bioscience, Biotechnology, and Biochemistry* 71, 527-534.
- Murakami, M., Y. Tago, T. Yamashino, and T. Mizuno, 2007: Characterization of the rice circadian clock-associated pseudo-response regulators in *Arabidopsis thaliana*. *Bioscience, Biotechnology and Biochemistry* 71, 1107-10.
- Murphy, R.L., R.R. Klein, D.T. Morishige, J.A. Brady, W.L. Rooney, F.R. Miller, D.V. Dugas, P.E. Klein, and J.E. Mullet, 2011: Coincident light and clock regulation of pseudoresponse regulator protein 37 (PRR37) controls photoperiodic flowering in *Sorghum*. *Proceedings of the National Academy of Sciences of the United States of America*.
- Nakamichi, N., M. Kita, S. Ito, T. Yamashino, and T. Mizuno, 2005: PSEUDO-RESPONSE REGULATORS, PRR9, PRR7 and PRR5, together play essential roles close to the circadian clock of *Arabidopsis thaliana*. *Plant Cell Physiol* 46, 686-98.
- Pin, P.A., R. Benlloch, D. Bonnet, E. Wremmerth-Weich, T. Kraft, J.J.L. Gielen, and O. Nilsson, 2010: An Antagonistic Pair of *FT* Homologs Mediates the Control of Flowering Time in Sugar Beet. *Science* 330, 1397-1400.
- Pin, P.A., W. Zhang, S.H. Vogt, N. Dally, B. Büttner, G. Schulze-Buxloh, N.S. Jelly, T.Y. Chia, E.S. Mutasa-Gottgens, J.C. Dohm, H. Himmelbauer, B. Weisshaar, J. Kraus, J.J. Gielen, M. Lommel, G. Weyens, B. Wahl, A. Schechert, O. Nilsson, C. Jung, T. Kraft, and A.E. Müller, 2012: The role of a pseudo-response regulator gene in life cycle adaptation and domestication of beet. *Curr Biol* 22, 1095-101.
- Saghai-Marouf, M.A., K.M. Soliman, R.A. Jorgensen, and R.W. Allard, 1984: Ribosomal DNA spacer-length polymorphisms in barley: mendelian inheritance, chromosomal location, and population dynamics. *Proc Natl Acad Sci U S A* 81, 8014-8.
- Srikanth, A., and M. Schmid, 2011: Regulation of flowering time: all roads lead to Rome. *Cellular and Molecular Life Sciences* 68, 2013-2037.
- Suarez-Lopez, P., K. Wheatley, F. Robson, H. Onouchi, F. Valverde, and G. Coupland, 2001: *CONSTANS* mediates between the circadian clock and the control of flowering in *Arabidopsis*. *Nature* 410, 1116-1120.
- Thomas, B., and D. Vince-Prue, 1997: *Photoperiodism in plants*. Academic Press, New York.

Tiwari, S.B., Y. Shen, H.C. Chang, Y. Hou, A. Harris, S.F. Ma, M. McPartland, G.J. Hymus, L. Adam, C. Marion, A. Belachew, P.P. Repetti, T.L. Reuber, and O.J. Ratcliffe, 2010: The flowering time regulator *CONSTANS* is recruited to the *FLOWERING LOCUS T* promoter via a unique cis-element. *New Phytologist* 187, 57-66.

Turner, A., J. Beales, S. Faure, R.P. Dunford, and D.A. Laurie, 2005: The pseudo-response regulator *Ppd-H1* provides adaptation to photoperiod in barley. *Science* 310, 1031-1034.

USDA/ARS 2010. National Genetic Resources Program. Germplasm Resources Information Network–(GRIN) [On-line Databank]. National Germplasm Resources Laboratory, Beltsville MD. <http://www.ars-grin.gov/cgi-bin/npgs/html/taxfam.pl?language=de>. Access August 2013

Wang, X., and L. Ma, 2012: Unraveling the circadian clock in *Arabidopsis*. *Plant Signal Behav* 8.

Wilson, A.K., J.R. Latham, and R.A. Steinbrecher, 2006: Transformation-induced mutations in transgenic plants: analysis and biosafety implications. *Biotechnol Genet Eng Rev* 23, 209-37.

Yamamoto, Y., E. Sato, T. Shimizu, N. Nakamich, S. Sato, T. Kato, S. Tabata, A. Nagatani, T. Yamashino, and T. Mizuno, 2003: Comparative genetic studies on the *APRR5* and *APRR7* genes belonging to the *APRR1/TOC1* quintet implicated in circadian rhythm, control of flowering time, and early photomorphogenesis. *Plant Cell Physiol* 44, 1119-1130.

Zeilinger, M.N., E.M. Farre, S.R. Taylor, S.A. Kay, and F.J. Doyle, 3rd, 2006: A novel computational model of the circadian clock in *Arabidopsis* that incorporates *PRR7* and *PRR9*. *Mol Syst Biol* 2, 58.

5 Closing discussion

In *B. vulgaris* an effective control of bolting and flowering is of substantial importance for the development of varieties with high root yield that can be achieved by a prolonged vegetative growth period, and moreover, to ensure their seed production. Thus, profound knowledge of flowering time genes and their regulation has a great impact on sugar beet breeding and cultivation.

The results of my work considerably extend our understanding of flowering time regulation in beet. I could successfully identify the *DOUBLE B-BOX TYPE ZINC FINGER* gene *BvBBX19* as a candidate gene at the bolting locus *B2* (see **Chapter 2**). I could show that *BvBBX19* acts upstream of the flowering regulators *BvFT1* and *BvFT2* and functions epistatically to the bolting gene *BTC1* to promote bolting and flowering. In **Chapters 3** and **4**, I described putative functional differences between the dominant and recessive *BTC1* alleles in transgenic *A. thaliana* and *B. vulgaris* plants. I figured out, that the *PRR7* homolog *BTC1* has a different function in beet as in *A. thaliana* and I could demonstrate identical functions of the polypeptides encoded by the dominant and the recessive alleles of the *BTC1* gene. For the first time, I observed transgene mediated co-suppression in beet by overexpressing the *BTC1* genes of the dominant or recessive alleles which resulted in a never bolting phenotype (see **Chapter 3**).

At the beginning of my work, the pseudo response regulator gene *BTC1* was known to be a homolog of the *A. thaliana* circadian clock gene *PRR7*. Therefore, I designed experiments to investigate a functional conservation of *BTC1* and *PRR7*. **Chapter 4** describes the functional analysis of *BTC1* in the *A. thaliana* wildtype and a *prp7-11* mutant by constitutively overexpressing the annual or biennial *BTC1* coding sequences. No phenotypic differences were observed in T₂ families between transgenic and non-transgenic plants. These data suggest that neither the annual nor the biennial *BTC1* coding sequence under the control of the 35S promoter can accelerate bolting and flowering in the *A. thaliana* wildtype or can complement the late flowering *prp7-11* mutant. In conclusion, there is no orthology between *BTC1* and *PRR7*. My conclusion is further supported by the fact that in *A. thaliana* *PRR7* regulates genes from the photoperiodic pathway but does not play a major role in flowering control (Carre and Veflingstad, 2013), whereas during the course of my study, *BTC1* was demonstrated as being a key gene controlling the beet life cycle and thus plays a major role for the induction of bolting (Pin et al., 2012).

In *B. vulgaris* the annual growth habit is controlled by the dominant *BTC1* allele by regulation of the flowering time genes *BvFT1* and *BvFT2*, i.e. promotion of bolting and flowering in response to LD (Pin et al., 2010; Pin et al., 2012). Biennial beets carry the recessive *btc1* allele for which a partial-loss of-function mutation was suggested to impair the response to inductive photoperiods and thus the regulation of *BvFT1* and *BvFT2* without vernalization (Pin et al., 2012; Pin et al., 2010). Dominant and recessive *BTC1* coding sequences were shown to differ by several non-synonymous SNPs (Pin et al., 2012). To investigate, whether protein sequence differences in the *BTC1* alleles alter the protein functions, coding sequences of the annual and biennial *BTC1* genes were transformed into a biennial *B. vulgaris* line under the control of the constitutive 35S promoter (see **Chapter 3**). I expected an annual phenotype in transgenic plants overexpressing the dominant *BTC1* gene. However, I observed no phenotypic differences between transgenic plants (T₁ generation) carrying either the annual or the biennial *BTC1*- transgene and no plant bolted without cold treatment. Interestingly, after cold treatment I observed bolting and non-bolting (referred as never bolting) individuals and I could clearly demonstrate a correlation between the transgene copy number, the *BTC1/btc1* expression and the bolting phenotypes. In bolting plants carrying single integrations of the transgene, I observed upregulation of *BTC1/btc1* (endogenous and transgene) and

downregulation of *BvFT1* after vernalization. Despite this ectopic expression *BTC1* genes were not highly expressed and it could be speculated, that high expression may be harmful for the plant and consequently constitutive expression is not tolerated.

In never bolting plants which carry multiple integrations of the transgene, *BTC1* or *btc1* were completely downregulated and *BvFT1* was highly upregulated before and after vernalization. The same never bolting phenotype was previously observed by Pin et al. (2012) when *BTC1* was downregulated in annual and biennial beets by RNAi. In these plants the regulation of *BvFT1* and *BvFT2* was completely abolished. Regarding these results, I conclude that multiple copies of the *BTC1* genes cause transgene-mediated silencing (co-suppression) of the endogenous *btc1* gene as well as of the transgenes. Co-suppression was demonstrated before in several plants (Agrawal et al., 2003). These results together demonstrate that the annual as well as the biennial *BTC1* gene have the same function and both are essential for the induction of bolting and flowering through regulation of *BvFT1* and *BvFT2*. In addition, a fine-tuned *BTC1* expression is essential for its proper function.

A differential regulation of the *BTC1* genes by their endogenous promoter could be further suggested. A previous study demonstrated differences between the promoter of annual and biennial *BTC1* alleles and revealed a large insertion of ~28 kb within the promoter region of the biennial allele (Pin et al., 2012). This insertion interrupts a series of *cis*-regulatory sequence motifs (GT-1) which were supposed to stabilize gene transcription while overrepresented (Gilmartin et al., 1990; Pin et al., 2012; Zhou, 1999). By taking these results into account, the reduced number of evenly spaced GT-1 elements in the biennial promoter may not be able to stabilize *btc1* transcription and thus *btc1* is not sufficiently expressed before winter to regulate *BvFT1* and *BvFT2*. This would moreover explain the results shown by Pin et al. (2012) that the dominant *BTC1* is higher expressed at the end of the light phase than the recessive *btc1*.

Furthermore, the contribution of other genes besides *BTC1* to the induction of bolting and flowering in beet could be suggested. Up to date, three additional bolting loci (*B2*, *B3* and *B4*) have been identified after EMS mutagenesis which were supposed to act epistatically or independently of *BTC1* to promote bolting, because mutant plants showed a biennial phenotype although they carry the dominant *BTC1* allele (Abou-Elwafa et al., 2012; Büttner et al., 2010; Hohmann et al., 2005).

Prior to my work two of these loci were mapped to chromosome 2 and 9. I managed to isolate a candidate gene at the bolting locus *B2* on chromosome 9 (see **Chapter 2**). After fine-mapping the locus *B2*, I selected *BvBBX19* as a candidate gene. There are three lines of evidence suggesting that *BvBBX19* encodes the *B2* phenotype, (1) a molecular marker within the 3'-UTR of this gene completely co-segregates with the F₂ phenotype, (2) the mutant and the non-mutated EMS donor line differ by a single non-synonymous transition, potentially induced through the EMS treatment, and (3) the sequence shares high homology to the transcriptional regulator gene *BBX19* from *A. thaliana*. So far *BBX19* has not been shown to play a role in bolting and flowering time. The *BBX19* from *A. thaliana* is involved in the regulation of photomorphogenesis (Kumagai et al., 2008).

It could be suggested that the protein encoded by *BvBBX19* acts as a transcriptional regulator of *BTC1*. Transcription factors bind to *cis*-regulatory elements within the promoter of target genes to transcriptionally regulate their gene expression (Priest et al., 2009). The regulatory function might be abolished while the *BvBBX19* protein is mutated and consequently a fine-tuned *BTC1* expression is not achieved to initiate bolting. Regarding biennial plants (*btc1*) carrying the functional *BvBBX19* allele, it is conceivable that the interruption of *cis*-regulatory elements within the *btc1* promoter abolishes the binding of the *BvBBX19* protein to the

promoter. This suggestion would perfectly complement my previous assumption that different *BTC1* proteins have the same function but the corresponding genes are differentially regulated due to their 5' sequence variations. However, I could not clearly demonstrate a different expression of *BvBBX19* in transgenic bolting and never bolting (after cold treatment) plants (see **Chapter 3**), indicating a regulation of *BvBBX19* expression through the activity of the *BTC1* gene concluding that the *BvBBX19* protein does not act as a transcriptional regulator of the *BTC1* gene.

Furthermore, I could demonstrate antagonistic expression profiles for *BvBBX19*, *BTC1*, *BvFT1* and *BvFT2* by comparing the biennial mutant and non-mutated annual plants, indicating that *BvBBX19* acts upstream of these genes which is another strong indication that *BvBBX19* controls bolting and flowering in beet (see **Chapter 2**). Taking these results into account, I conclude that *BTC1* and *BvBBX19* act together to regulate the flowering time genes *BvFT1* and *BvFT2*. While this is true before cold treatment, after vernalization, more genes are likely to be involved in bolting induction due to the fact that biennial mutants bolt and flower after vernalization.

In conclusion, my results substantially extended the knowledge about bolting and flowering time regulation in beet. The finding that multiple copy integrations of *BTC1* led to a never bolting phenotype in beet provides a suitable resource for breeding winter beets which do not bolt after winter. Several transgenic beets have been generated by overexpressing *BvFT1* or silencing *BTC1* or *BvFT2* by Pin et al. (2010; 2012). All of these beets were never bolting (even after cold treatment) and seed production of these plants was impossible. By contrast, the transgenic beets from this study are able to produce seeds in the case of a single copy integration of *BTC1*, thus they behave like normal sugar beet. Accordingly, these plants can be used as seed parents and pollinators for hybrid seed production. Those hybrids will be 'never bolting' as a consequence of multiple copy integrations. Beside this transgenic approach, non-transgenic plants could be obtained with never bolting or delayed bolting phenotypes (after winter). Since bolting before winter is abolished in plants carrying the recessive *B* or the mutated *B2* allele (*B2'*), it could be speculated that double recessive plants (*bbB2'B2'*) fail to bolt after winter, or are at least severely delayed in bolting. Crossing to produce double mutants have already been initiated.

In the future, for breeding winter beets, further investigations are indispensable to understand the bolting and flowering time regulation in beet. To further analyze the function and the putative interaction of *BvBBX19* and *BTC1*, I suggest the following experiments. For the verification of a putative interaction between the proteins *BvBBX19* and *BTC1* I recommend a yeast two-hybrid analysis. For the functional characterization of *BvBBX19*, I propose its transformation into *A. thaliana* wildtype and suitable mutant plants. The generation of double transformants containing the *BvBBX19* gene and the *BTC1* gene under the control of its endogenous promoter is also conceivable. In addition to this, transformation of the *BTC1* genes under the control of their endogenous promoter followed by phenotypic analysis would extend our knowledge whether the *BTC1* genes are differentially regulated by their promoter. Moreover, the identification of further bolting and flowering time loci as well as the isolation of the known bolting loci *B3* and *B4* would help to extend the knowledge of the bolting and flowering time network in beet. To speed up the identification of candidate genes in beet, the development of a new bulked segregant approach that is based on a next generation sequencing method is in progress at the University of Kiel (K. Xiao, pers. communication).

6 Summary

Sugar beet (*Beta vulgaris* subsp. *vulgaris*), a biennial long day (LD) plant belonging to the family *Chenopodiaceae*, stores sucrose in a thickened taproot and is the only crop cultivated for sugar production in Europe. Full bolting time control is of substantial agronomic importance to prevent stem elongation (bolting) and flowering which result in decreasing root yield.

The regulation of flowering time is well studied in the model species *Arabidopsis thaliana* and the induction of flowering through the zinc finger transcriptional regulator *CONSTANS* (*CO*) and the *FLOWERING LOCUS T* (*FT*) gene appears to be conserved in various plant species. In *B. vulgaris*, a new mechanism of flowering time regulation has been observed. Two *FT* paralogs *BvFT1* and *BvFT2* have evolved antagonistic functions, in which *BvFT1* is a floral repressor and *BvFT2* acts as a floral inducer. Annuals that flower under long days (LD) within one season carry the dominant pseudo response regulator (PRR) gene *BOLTING TIME CONTROL 1* (*BTC1*), which shows homology to the *PRR7* gene in *A. thaliana*. *BTC1* promotes bolting and flowering through repression of *BvFT1* and activation of *BvFT2*. By contrast, in biennials carrying the recessive *btc1* gene, *BvFT1* is not repressed and plants require a period of prolonged cold temperature over winter (vernalization) followed by LD for bolting induction. Protein sequences of the dominant and recessive *BTC1* genes differ by several amino acid substitutions. Besides *BTC1*, three additional bolting loci *B2*, *B3* and *B4* have been previously identified after EMS mutagenesis of an annual genotype. So far, the two loci *B2* and *B4* were mapped to chromosome 9 and 2, respectively. A *B2* mutant displays a biennial phenotype and thus requires vernalization for bolting induction.

The aims of my study were (1) to fine-map the bolting locus *B2* and to identify putative candidate genes, and (2) to investigate the function of *BTC1* in transgenic *A. thaliana* and *B. vulgaris* plants to clarify whether the polypeptides encoded by the dominant and recessive *BTC1* gene have the same function in beet and whether *BTC1* and *PRR7* are orthologs.

In order to fine-map the *B2* locus, 5457 F₂ plants from a cross of a biennial *B2* mutant and an annual wild beet and 2549 F_{2,3} families were phenotyped for bolting behavior. Finally, 1301 F₂ plants were genotyped with molecular markers. I used the draft assembly of the sugar beet reference sequence to develop molecular markers and identified two markers flanking the *B2* locus in a genetic distance of 1.9 cM. I screened this region for candidate genes using a collection of predicted gene models of the sugar beet reference sequence. This enabled the identification of a candidate gene which shows an overall sequence identity of 55% on amino acid level to BBX19, a B-box zinc finger family protein of *A. thaliana*, comprising two B-box domains. Accordingly, I termed the newly detected candidate gene *BvBBX19*. A molecular marker located in the 3'-UTR of *BvBBX19* completely co-segregates with the F₂ phenotypes (1295 F₂ plants). I identified a single transition in each of two independent mutants that occurred at different positions within the candidate gene. Both mutations are likely to be induced through the EMS treatment and alter the amino acid sequence of the *BvBBX19* protein. These results give strong evidence that *BvBBX19* is likely to be the sought gene encoding the *B2* phenotype.

The function of the different *BTC1* alleles was first verified by constitutive expression of both *BTC1* coding sequences in *A. thaliana* wildtype and late flowering *prp7* mutant plants. I observed no significant differences for bolting and flowering in segregating T₂ families. *BTC1* neither accelerated flowering in the wildtype nor complemented the late flowering *prp7* mutant. These results suggest that *BTC1* is not an ortholog of *PRR7* and has a different function in *B. vulgaris* compared to *A. thaliana*. Second, I investigated the *BTC1* function in

B. vulgaris plants (T_1 generation). I observed no phenotypic differences in plants carrying the dominant *BTC1* transgene compared to those carrying the recessive *btc1* transgene, before as well as after cold treatment. However, after cold treatment I observed bolting and never bolting (non bolting after cold treatment) individuals. Expression analysis and copy number determination revealed an upregulation of *BTC1* and a downregulation of *BvFT1* in bolting plants that carry a single copy of the transgene. By contrast, *BTC1* is completely downregulated and *BvFT1* is highly upregulated in never bolting plants carrying multiple integrations of the transgene. These results demonstrate for the first time transgene-mediated co-suppression in transgenic sugar beet plants and thus confirm also that both *BTC1* genes have the same function.

Beyond the aims of my study, I further investigated the expression of *BvBBX19* in bolting and never bolting transgenic beets as well as in the parental accessions of the F_2 population, in comparison with *BTC1*, *BvFT1* and *BvFT2*. The results of this analysis indicate that *BvBBX19* acts in conjunction with *BTC1* upstream of *BvFT1* and *BvFT2* to induce bolting and flowering in beet. Under the assumption that *BvBBX19* and *BTC1* proteins interact with each other, the resulting protein complex would have i.a. two B-box and a CCT domain and thus resemble the domain structure of CO. Accordingly I hypothesize that in beet a CO function is achieved through interaction of *BvBBX19* and *BTC1*.

The results of my work largely extended our knowledge about bolting and flowering time regulation in beet which is of substantial importance for breeding winter beets with controlled bolting behavior.

7 Zusammenfassung

Die einzige Kulturpflanze, die in Europa zur Zuckerproduktion angebaut wird, ist die Zuckerrübe (*Beta vulgaris* subsp. *vulgaris*). Diese zweijährige Langtagpflanze speichert Zucker in ihrer Pfahlwurzel, wobei durch die Streckung der Sprossachse (Schossen) und anschließendes Blühen der Wurzelertrag und somit auch der Zuckerertrag reduziert werden. Für die Kultivierung von Zuckerrüben ist folglich die Blühzeitkontrolle, die bisher in der Modellpflanze *Arabidopsis thaliana* am besten untersucht worden ist, von erheblicher agronomischer Bedeutung.

In *A. thaliana* wird die Blühinduktion durch die Expression des Zinkfinger-Transkriptionsfaktors *CONSTANS* (*CO*) sowie durch das von *FLOWERING LOCUS T* (*FT*) kodierte Protein reguliert, ein Mechanismus, der in vielen anderen Spezies konserviert zu sein scheint. In *B. vulgaris* wurde ein gegenläufiger Prozess der Blühzeitpunkt-Regulation durch zwei *FT* Paraloge *BvFT1* und *BvFT2* nachgewiesen, bei dem *BvFT1* als Blührepressor fungiert, während *BvFT2* die Blühphase induziert. Einjährige *B. vulgaris* Pflanzen, die unter Langtag-Bedingungen im ersten Jahr blühen, tragen das dominante Allel des Pseudo Response Regulator (PRR) Gens *BOLTING TIME CONTROL 1* (*BTC1*). Dieses ist homolog zu *PRR7* in *A. thaliana* und induziert Schossen und Blühen durch Repression von *BvFT1* und Aktivierung von *BvFT2*. Zweijährige Pflanzen hingegen, die das rezessive *btc1* Gen tragen, zeigen keine Repression des *BvFT1* Gens und schossen und blühen somit erst nach einer längeren winterlichen Kälteperiode (Vernalisation), gefolgt von Langtag-Bedingungen. Die Sequenz des vom dominanten *BTC1* Gen abgeleiteten Proteins unterscheidet sich von der des rezessiven in einigen Aminosäuren. Durch chemische Mutagenese eines einjährigen Genotyps wurden bisher drei weitere Genloci (*B2*, *B3* und *B4*) identifiziert, von denen zwei auf Chromosom 9 (*B2*) bzw. Chromosom 2 (*B4*) lokalisiert sind. Es standen *B2* Mutanten zu Verfügung, die einen zweijährigen Phänotyp aufzeigten und entsprechend erst nach Vernalisation schossten.

Ziel meiner Arbeit war es (1) eine Feinkartierung des zweiten Schosslokus (*B2*) durchzuführen und potentielle Kandidatengene in dieser Region zu identifizieren, sowie (2) die Funktion des *BTC1* Gens zu bestimmen mit der Fragestellung, ob die von dem dominanten und rezessiven *BTC1* Gen kodierten Polypeptide sich funktionell unterscheiden und ob *BTC1* und *PRR7* funktionell orthologe Gene sind.

Für die Feinkartierung des Locus *B2* wurde eine 5457 Pflanzen umfassende F₂ Population aus der Kreuzung einer zweijährigen *B2* Mutante mit einer einjährigen Wildrübe genutzt. Daraus wurden durch Selbstung 2549 F_{2:3} Familien erzeugt. Die F₂ Population und F_{2:3} Familien wurden hinsichtlich ihres Schosserverhaltens phänotypisiert. Insgesamt wurden 1301 F₂ Pflanzen mit molekularen Markern genotypisiert. Für die Entwicklung molekularer Marker verwendete ich eine vorläufige Version der Zuckerrüben Referenzsequenz. Auf diese Weise konnten zwei Marker, die den Locus *B2* in einem genetischen Abstand von 1.9 cM flankieren, identifiziert werden. Zur Identifikation potentieller Kandidatengene innerhalb dieser Region verwendete ich eine Kollektion vorhergesagter Genmodelle innerhalb der Referenzsequenz. Dadurch konnte ein Kandidatengen mit zwei B-Box Domänen, dessen Proteinsequenz zu 55% homolog zu dem *A. thaliana* BBX19 Protein aus der Familie der B-Box Zinkfinger-Transkriptionsfaktoren ist, identifiziert werden. Dieses in *B. vulgaris* gefundene Gen bezeichnete ich als *BvBBX19*. Ein Marker, der aus der *BvBBX19* 3'-UTR abgeleitet worden war, war vollständig mit dem *B2* Locus gekoppelt (1295 F₂ Pflanzen). In zwei unabhängigen Mutanten konnte ich die Anwesenheit von zwei unterschiedlich lokalisierten einzelnen Transitionen in *BvBBX19* nachweisen, die auf chemische Mutagenese hinweisen und jeweils zu einer Veränderung der Proteinsequenz führen. Diese Ergebnisse deuten stark

darauf hin, dass es sich bei *BvBBX19* um das Gen handelt, welches für den *B2* Phänotyp kodiert.

Um die Funktion des dominanten und rezessiven *BTC1* Gens zu analysieren, wurden zunächst die kodierenden Sequenzen der *BTC1* Gene im *A. thaliana* Wildtyp und in spät blühenden *prp7* Mutanten konstitutiv exprimiert. *BTC1* förderte in spaltenden T₂ Familien weder das Blühen im Wildtyp- Hintergrund noch komplementierte es die spät blühende *prp7* Mutante. Diese Ergebnisse weisen darauf hin, dass *BTC1* kein funktionelles Ortholog von *PRR7* ist und somit in *B. vulgaris* eine andere Funktion hat als in *A. thaliana*. Auch in einer zweijährigen *B. vulgaris* Linie wurden die kodierenden Sequenzen der *BTC1* Gene konstitutiv exprimiert. *B. vulgaris* Pflanzen (T₁ Generation), die das dominante *BTC1* Transgen trugen, zeigten hinsichtlich ihrer Schossneigung, sowohl vor als auch nach Vernalisation, keinen signifikanten Unterschied zu solchen, die das rezessive Transgen trugen. Allerdings beobachtete ich nach der Kältebehandlung schossende und nicht schossende („nie schossende“) Pflanzen. Mit Hilfe von Expressionsanalysen und Bestimmung der Genkopien-Anzahl konnte ich zeigen, dass schossende Pflanzen nur eine Kopie des Transgens trugen, was mit einer Hochregulation von *BTC1* bei gleichzeitiger Herabregulation von *BvFT1* einherging. Im Gegensatz dazu zeigten nie schossende Pflanzen, die mehrere Kopien des Transgens trugen, eine vollständige Herabregulation von *BTC1* und eine Hochregulation von *BvFT1*. Diese Ergebnisse zeigen erstmals eine durch das *BTC1* Transgen vermittelte Co-Suppression in transgenen Zuckerrüben und belegen die Funktions-Gleichheit der beiden *BTC1* Proteine aus ein- und zweijährigen Pflanzen.

Weiterhin untersuchte ich die Expression von dem identifizierten *BvBBX19* Gen in schossenden und nie schossenden transgenen Pflanzen sowie in den Eltern der F₂ Population und verglich sie mit der Expression der *BTC1*-, *BvFT1*- und *BvFT2*- Gene. Die Ergebnisse dieser Analyse deuten darauf hin, dass es sich bei *BvBBX19* um ein Gen handelt, das gemeinsam mit dem dominanten *BTC1* Allel die Regulation der Gene *BvFT1* und *BvFT2* übernimmt um Schossen in Zuckerrüben zu induzieren. Unter Berücksichtigung einer potentiellen Protein-Protein Interaktion von *BvBBX19* und *BTC1* würde dieser Proteinkomplex unter anderem zwei B-Box und eine CCT-Domäne enthalten und somit der Domänen-Struktur des CO-Proteins ähneln. Somit könnte die Funktion von CO in Zuckerrüben durch eine Interaktion der Proteine *BvBBX19* und *BTC1* übernommen werden.

Durch die Ergebnisse meiner Arbeit konnten grundlegende neue Erkenntnisse zur Regulation des Schoss- und Blühzeitpunktes in der Zuckerrübe gewonnen werden, die für die Züchtung von Winter-Zuckerrüben mit kontrolliertem Schossverhalten von wesentlicher Bedeutung sind.

8 Appendix

8.1 Supplementary tables

Supplementary Table 1: Generation of the *B. vulgaris* F₂-population₀₅₆₈₂₂, consisting of 21 F₂ subpopulations. Subpopulations were derived from single F₁ plants of the accession 061394, which were propagated by bag isolation. F₂ plants were cultivated for six weeks in the greenhouse (16h light, 22°C, 900μE) and then transferred to the field.

F ₂ Subpopulation	F ₁ Plant	F ₂ Seeds Harvested	F ₂ Plants Grown in the Field
100155	061394/1	264	267
100156	061394/2	123	151
100157	061394/3	166	222
100158	061394/4	58	55
100159	061394/5	329	283
100160	061394/6	279	476
100161	061394/7	73	120
100162	061394/8	185	332
100163	061394/10	376	512
100164	061394/13	606	514
100165	061394/14	241	266
100166	061394/18	188	148
100167	061394/20	133	131
100168	061394/21	310	378
100169	061394/22	323	445
100170	061394/23	242	275
100171	061394/24	318	169
100172	061394/25	316	171
100173	061394/26	32	25
100174	061394/27	8	2
100175	061394/28	508	1,166
Total: F₂-population₀₅₆₈₂₂		5,078	6,108

Supplementary Table 2: Genotyping data of homozygous *B. vulgaris* F₂ plants according to F₃ phenotyping. ‘b’ and ‘a’ indicate marker genotypes homozygous for the marker allele (M₁) derived from the annual, or homozygous for the marker allele (M₂) derived from the biennial mutant parent accession, respectively. ‘h’ indicates heterozygous marker genotypes. ‘nd’: not determined

F ₂ Single Plant No.	CAU3785	CAU3786	CAU3782	F ₂ Phenotype	CAU3784	CAU3783	CAU3787 (MP_E0043)	CAU3788 (TG_E0140)
6	nd	b	b	b	b	b	b	b
22	b	b	b	b	b	b	b	b
32	b	b	b	b	b	b	a	nd
50	b	nd	b	b	b	b	b	b
190	b	b	b	b	b	b	b	b
233	b	b	b	b	b	b	b	b
319	b	b	nd	b	b	h	h	h
339	nd	nd	b	b	b	b	b	b
342	b	b	b	b	b	b	b	b
406	b	nd	b	b	b	b	b	b
408	b	b	b	b	b	b	b	b
417	b	nd	b	b	b	b	b	b
422	b	b	b	b	b	b	b	b
446	nd	nd	b	b	b	b	b	b
828	b	nd	b	b	b	b	b	b
831	b	nd	b	b	b	b	b	b
852	b	nd	b	b	b	b	b	b
856	b	nd	b	b	b	b	b	b
906	b	b	b	b	b	b	b	b
986	b	b	b	b	b	b	b	b
996	b	b	b	b	b	b	b	b
1000	b	b	nd	b	b	b	b	b
1012	b	nd	b	b	b	b	b	b
1014	b	b	b	b	b	b	b	b
1024	b	nd	b	b	b	b	b	b
1037	b	b	b	b	b	b	b	b
1043	b	nd	b	b	b	b	b	b
1044	b	nd	b	b	b	b	b	b
1101	nd	nd	b	b	b	nd	h	h
1125	b	nd	b	b	b	b	b	b
1127	b	nd	b	b	b	b	b	b
1132	b	nd	b	b	b	b	h	h
1146	b	b	b	b	b	b	b	b
1164	b	b	b	b	b	b	b	b
1169	b	nd	b	b	b	b	b	b

F ₂ Single Plant No.	CAU3785	CAU3786	CAU3782	F ₂ Phenotype	CAU3784	CAU3783	CAU3787 (MP_E0043)	CAU3788 (TG_E0140)
1173	b	b	b	b	b	b	b	b
1179	nd	nd	b	b	b	b	b	b
1194	nd	nd	b	b	b	b	b	b
1218	b	nd	b	b	b	b	b	b
1246	nd	nd	b	b	b	b	b	b
1250	b	nd	b	b	nd	nd	b	b
1258	b	b	b	b	b	b	b	b
1259	b	nd	b	b	nd	nd	nd	b
1268	b	nd	b	b	b	b	nd	b
1275	nd	nd	b	b	b	b	b	b
1276	nd	nd	b	b	b	b	b	b
1296	b	nd	b	b	b	b	b	b
1302	b	nd	b	b	b	b	b	b
1303	b	nd	b	b	b	b	b	b
1310	b	b	b	b	b	b	b	b
1322	b	b	b	b	b	b	b	b
1325	nd	b	b	b	b	b	b	b
1336	b	b	b	b	b	b	nd	b
1357	b	b	b	b	b	b	b	b
1367	b	b	b	b	b	b	b	b
1374	b	b	b	b	b	b	b	b
1379	b	b	b	b	b	b	b	b
1384	b	nd	nd	b	b	b	b	b
1456	b	b	b	b	b	b	b	b
1466	b	b	b	b	b	nd	b	b
1467	b	b	b	b	b	b	b	b
1474	b	b	b	b	b	b	b	b
1484	b	b	b	b	b	b	b	b
1488	b	b	b	b	b	b	b	b
1491	b	b	b	b	b	b	b	b
1493	b	b	b	b	b	b	b	b
1516	b	b	b	b	b	b	b	b
1518	b	nd	b	b	b	b	h	h
1523	b	b	b	b	b	b	b	b
1557	b	b	b	b	b	b	b	b
1569	b	b	b	b	b	b	b	b
1572	b	nd	b	b	b	b	nd	b

F ₂ Single Plant No.	CAU3785	CAU3786	CAU3782	F ₂ Phenotype	CAU3784	CAU3783	CAU3787 (MP_E0043)	CAU3788 (TG_E0140)
3334	b	b	b	b	b	b	b	b
3350	b	b	b	b	b	b	b	b
3371	b	b	b	b	b	b	b	b
3384	b	b	b	b	nd	b	b	b
3388	nd	nd	nd	b	b	nd	nd	nd
3389	b	b	b	b	b	b	b	b
3395	b	b	b	b	b	b	b	b
3416	b	b	b	b	b	b	b	b
3470	b	b	b	b	b	b	b	b
3532	b	b	nd	b	b	b	b	b
3540	b	b	b	b	b	b	b	b
3546	b	b	b	b	b	b	b	b
3553	nd	b	b	b	b	b	b	b
3554	b	b	b	b	b	b	b	b
3569	b	b	b	b	nd	nd	nd	b
3589	b	nd	b	b	b	b	b	b
3655	b	b	b	b	b	b	b	b
3658	b	b	b	b	b	b	b	b
3659	b	b	b	b	b	b	b	b
3661	b	b	b	b	b	b	b	b
3662	b	b	b	b	b	b	b	b
3673	b	b	b	b	b	h	h	h
3674	b	b	b	b	b	b	b	b
3705	b	b	b	b	b	b	b	b
3726	b	b	b	b	b	b	b	b
3734	b	nd	b	b	b	b	b	b
3735	b	b	b	b	b	b	b	b
3739	b	b	b	b	b	b	b	b
3744	b	b	b	b	b	b	b	b
3765	b	b	b	b	b	b	b	b
3768	b	b	b	b	b	b	b	b
3781	b	b	nd	b	b	b	b	b
3805	b	b	b	b	b	b	h	h
3819	b	b	b	b	b	b	b	b
3823	b	b	b	b	b	b	b	b
3830	b	b	b	b	b	b	b	b
3839	b	nd	b	b	b	b	nd	b

F ₂ Single Plant No.	CAU3785	CAU3786	CAU3782	F ₂ Phenotype	CAU3784	CAU3783	CAU3787 (MP_E0043)	CAU3788 (TG_E0140)
810	a	a	a	a	a	a	a	a
817	a	a	a	a	a	a	a	a
818	nd	a	a	a	a	a	a	a
819	a	a	a	a	a	nd	a	a
853	a	a	a	a	a	a	a	a
857	a	a	nd	a	a	a	a	a
860	a	a	a	a	a	a	a	a
862	a	a	a	a	a	nd	nd	a
872	a	a	nd	a	a	a	a	a
873	a	a	a	a	a	a	a	a
882	a	a	a	a	a	h	h	h
896	a	a	nd	a	a	a	a	a
899	a	a	nd	a	a	a	a	a
904	a	a	nd	a	a	a	a	a
905	a	a	nd	a	a	a	a	a
908	a	a	nd	a	a	a	a	a
910	a	a	nd	a	a	a	a	a
917	a	a	nd	a	a	a	a	a
923	a	a	nd	a	a	nd	a	a
927	a	a	nd	a	a	a	a	nd
935	a	a	nd	a	a	nd	a	nd
945	a	a	nd	a	a	a	a	nd
950	a	a	nd	a	a	a	h	h
953	a	a	nd	a	a	a	h	h
954	a	a	nd	a	a	a	a	a
959	a	a	nd	a	a	a	a	a
971	a	a	nd	a	a	a	a	a
979	a	a	a	a	a	a	a	a
984	a	a	nd	a	a	a	a	a
988	a	a	a	a	a	a	a	a
994	a	a	a	a	a	a	a	a
1008	a	a	nd	a	a	a	a	a
1010	a	a	a	a	a	a	a	a
1011	a	a	a	a	a	a	h	h
1015	a	a	nd	a	a	a	a	a
1020	a	a	nd	a	a	a	a	a
1021	a	a	a	a	a	nd	a	a
1022	a	h	nd	a	a	a	a	a

F ₂ Single Plant No.	CAU3785	CAU3786	CAU3782	F ₂ Phenotype	CAU3784	CAU3783	CAU3787 (MP_E0043)	CAU3788 (TG_E0140)
1933	a	a	a	a	a	a	a	a
1935	a	a	a	a	a	a	a	a
1939	a	a	a	a	a	a	a	a
1942	a	a	a	a	a	a	a	a
1943	a	a	a	a	a	a	a	nd
1954	a	a	a	a	a	a	a	a
1955	a	a	a	a	a	a	a	a
1967	a	a	a	a	a	a	a	a
1985	a	nd	a	a	a	nd	nd	a
1990	a	a	a	a	a	a	a	a
1991	a	a	a	a	a	a	a	a
1997	a	nd	a	a	a	a	a	a
2000	a	a	a	a	a	a	a	a
2016	a	a	a	a	a	a	a	a
2028	a	a	a	a	a	a	a	a
2030	nd	a	a	a	a	a	a	a
2032	a	a	a	a	a	a	a	a
2039	a	a	a	a	a	a	a	a
2040	a	a	a	a	a	a	a	a
2058	a	a	a	a	a	a	a	a
2064	a	a	a	a	a	a	a	a
2072	a	nd	nd	a	a	a	nd	nd
2074	nd	a	nd	a	a	a	a	a
2077	a	a	a	a	a	a	a	a
2084	a	a	nd	a	a	a	a	a
2089	a	a	nd	a	a	a	a	a
2092	nd	a	nd	a	a	a	a	a
2099	a	nd	a	a	a	a	a	a
2100	nd	a	a	a	a	a	a	a
2101	nd	a	a	a	a	a	a	a
2104	nd	a	a	a	a	a	a	a
2107	nd	a	a	a	a	a	a	a
2114	nd	a	a	a	a	a	a	a
2115	nd	a	a	a	a	a	a	a
2124	nd	a	a	a	a	a	a	a
2127	nd	a	a	a	a	a	a	a
2128	nd	a	a	a	a	a	a	a

F ₂ Single Plant No.	CAU3785	CAU3786	CAU3782	F ₂ Phenotype	CAU3784	CAU3783	CAU3787 (MP_E0043)	CAU3788 (TG_E0140)
2129	nd	a	a	a	a	h	h	h
2130	nd	a	a	a	a	a	a	a
2132	nd	a	a	a	a	a	a	a
2141	nd	a	a	a	a	a	a	a
2147	nd	a	a	a	a	a	a	a
2154	nd	a	a	a	a	a	a	a
2160	nd	a	nd	a	a	a	a	nd
2167	nd	nd	nd	a	a	a	a	nd
2169	nd	nd	nd	a	a	a	a	nd
2170	nd	nd	nd	a	a	a	a	nd
2173	nd	nd	nd	a	a	a	a	nd
2177	nd	nd	nd	a	a	a	h	nd
2182	nd	nd	nd	a	a	a	a	a
2186	nd	nd	nd	a	a	a	a	a
2187	nd	a	nd	a	a	a	a	nd
2191	nd	nd	nd	a	a	a	nd	a
2197	nd	a	nd	a	a	a	a	a
2202	nd	nd	nd	a	a	a	a	nd
2211	nd	a	a	a	a	a	a	a
2212	nd	a	nd	a	a	a	a	a
2216	nd	a	a	a	a	a	nd	a
2219	nd	a	a	a	a	a	a	nd
2226	nd	a	a	a	a	a	a	a
2233	nd	a	a	a	a	a	a	a
2240	nd	a	a	a	a	a	a	nd
2249	nd	a	a	a	a	a	a	a
2260	nd	a	a	a	a	a	a	a
2261	nd	a	a	a	a	a	a	nd
2264	nd	a	a	a	a	a	a	a
2265	nd	a	a	a	a	a	a	a
2269	nd	a	a	a	a	a	a	a
2271	nd	a	a	a	a	a	a	nd
2274	nd	a	a	a	a	a	a	nd
2275	nd	a	a	a	a	a	a	nd
2278	nd	a	a	a	a	a	a	a
2280	nd	a	a	a	a	a	a	a
2288	nd	a	a	a	a	a	a	a
2290	nd	a	a	a	a	a	a	a

F ₂ Single Plant No.	CAU3785	CAU3786	CAU3782	F ₂ Phenotype	CAU3784	CAU3783	CAU3787 (MP_E0043)	CAU3788 (TG_E0140)
2296	nd	a	a	a	a	a	a	a
2314	nd	a	a	a	a	a	h	nd
2324	nd	a	nd	a	a	h	h	h
2333	nd	a	a	a	a	a	a	a
2334	nd	a	a	a	a	a	a	a
2337	nd	a	a	a	a	a	a	a
2338	nd	a	a	a	a	a	a	a
2342	nd	nd	nd	a	a	a	a	a
2343	nd	a	a	a	a	a	a	a
2346	nd	a	a	a	a	a	a	a
2395	nd	nd	nd	a	a	a	h	nd
2415	nd	a	nd	a	a	h	h	h
2419	nd	a	nd	a	a	a	a	nd
2420	nd	a	nd	a	a	a	a	nd
2444	nd	a	a	a	a	a	h	h
2451	nd	a	a	a	a	a	a	a
2461	nd	a	a	a	a	a	h	h
2490	nd	a	nd	a	a	nd	a	nd
2492	nd	a	a	a	a	a	a	a
2499	nd	a	a	a	a	a	a	a
2502	nd	a	a	a	a	a	a	a
2509	nd	a	a	a	a	a	a	a
2514	nd	a	a	a	a	a	a	a
2516	nd	a	nd	a	a	a	a	a
2523	nd	a	a	a	a	a	a	a
2527	nd	a	a	a	a	a	a	a
2535	nd	a	a	a	a	a	a	a
2544	nd	a	a	a	a	a	a	nd
2550	nd	a	a	a	a	a	a	nd
2555	nd	a	a	a	a	a	a	a
2560	nd	nd	a	a	a	a	nd	a
2576	nd	nd	nd	a	a	a	a	a
2561	nd	nd	nd	a	a	nd	a	a
2578	nd	a	a	a	a	a	nd	h
2585	nd	a	a	a	a	a	a	a
2587	nd	a	a	a	a	a	a	nd
2591	nd	a	a	a	a	a	a	a

F ₂ Single Plant No.	CAU3785	CAU3786	CAU3782	F ₂ Phenotype	CAU3784	CAU3783	CAU3787 (MP_E0043)	CAU3788 (TG_E0140)
2957	nd	nd	a	a	a	h	nd	h
2966	nd	a	a	a	a	a	a	h
2970	nd	a	a	a	a	a	a	h
2979	a	a	a	a	a	h	h	h
2981	a	a	a	a	a	a	a	a
2994	a	a	a	a	a	a	a	a
2996	a	a	a	a	a	a	a	a
3001	a	a	a	a	a	a	a	a
3002	a	a	a	a	a	a	a	a
3011	nd	a	a	a	a	a	a	a
3018	nd	a	a	a	a	a	a	a
3019	nd	a	a	a	a	a	a	a
3022	nd	nd	a	a	a	a	a	a
3037	nd	a	a	a	a	a	a	a
3039	nd	a	a	a	a	a	a	a
3043	nd	a	a	a	a	h	h	h
3049	nd	a	a	a	a	a	a	a
3051	nd	a	a	a	a	h	h	h
3057	nd	a	a	a	a	a	a	a
3064	nd	a	a	a	a	a	a	a
3066	nd	a	a	a	a	a	a	a
3068	nd	a	a	a	a	a	a	a
3070	nd	a	a	a	a	a	a	a
3075	nd	a	a	a	a	a	a	a
3081	nd	a	a	a	a	a	a	a
3086	nd	a	a	a	a	a	a	a
3096	nd	a	a	a	a	a	a	a
3097	nd	a	a	a	a	a	a	a
3099	nd	h	h	a	a	a	a	a
3104	nd	a	a	a	a	nd	a	a
3111	nd	a	a	a	a	a	a	a
3123	nd	a	a	a	a	a	a	h
3127	nd	a	a	a	a	a	a	a
3133	nd	a	a	a	a	a	a	a
3135	nd	a	a	a	a	a	a	a
3139	nd	a	a	a	a	a	a	a
3151	nd	a	a	a	a	a	h	h
3167	nd	a	a	a	a	a	nd	a

F ₂ Single Plant No.	CAU3785	CAU3786	CAU3782	F ₂ Phenotype	CAU3784	CAU3783	CAU3787 (MP_E0043)	CAU3788 (TG_E0140)
3171	nd	a	a	a	a	a	nd	a
3193	nd	a	a	a	a	a	a	a
3203	nd	a	a	a	a	a	a	a
3206	nd	a	a	a	a	a	a	a
3207	nd	a	a	a	a	a	a	a
3208	nd	a	a	a	a	a	a	a
3211	nd	h	h	a	a	a	a	a
3213	nd	a	a	a	a	a	a	a
3224	nd	a	a	a	a	a	a	a
3245	nd	a	a	a	a	h	h	h
3266	nd	a	a	a	a	a	a	a
3273	nd	a	a	a	a	a	a	a
3275	nd	a	a	a	a	a	nd	a
3278	nd	a	a	a	a	a	nd	a
3281	nd	nd	a	a	a	a	nd	a
3285	nd	a	a	a	a	a	a	a
3287	a	a	a	a	a	a	a	a
3292	a	a	nd	a	a	a	a	a
3294	a	a	a	a	a	a	a	a
3300	a	a	a	a	a	a	a	a
3302	a	a	a	a	a	a	a	a
3304	a	a	nd	a	a	a	a	a
3307	nd	a	a	a	a	a	nd	a
3309	a	a	a	a	a	a	a	a
3310	a	a	a	a	a	a	a	a
3311	a	a	a	a	a	a	a	a
3319	a	nd	a	a	a	a	a	a
3323	a	a	a	a	a	a	a	a
3331	a	a	a	a	a	h	h	h
3333	a	a	a	a	a	a	a	a
3336	a	a	a	a	a	a	a	a
3346	a	a	a	a	a	a	a	a
3362	a	a	a	a	a	a	a	a
3369	a	a	a	a	a	a	a	a
3373	a	a	a	a	a	a	a	a
3377	a	a	a	a	a	a	a	a
3379	nd	nd	nd	a	a	a	nd	nd

F ₂ Single Plant No.	CAU3785	CAU3786	CAU3782	F ₂ Phenotype	CAU3784	CAU3783	CAU3787 (MP_E0043)	CAU3788 (TG_E0140)
3865	a	a	a	a	a	a	a	a
3870	h	a	nd	a	a	a	a	a
3871	a	a	a	a	a	a	a	a
3874	a	a	a	a	a	a	a	a
3881	a	a	a	a	a	a	a	a
3886	nd	a	a	a	a	a	h	h
3893	a	a	a	a	a	a	a	a
3895	a	a	a	a	a	a	a	a
3898	a	a	a	a	a	a	a	a
3901	nd	a	a	a	a	a	a	a
3908	a	a	a	a	a	a	a	a
3912	a	a	a	a	a	a	a	a
3926	a	a	a	a	a	a	a	a
3930	nd	a	a	a	a	nd	nd	a
3933	a	a	a	a	a	a	a	a
3942	a	a	a	a	a	a	a	a
3945	a	a	a	a	a	a	a	a
3947	a	a	a	a	a	a	a	a
3949	a	a	a	a	a	a	a	a
3957	a	a	a	a	a	a	a	a
3960	a	a	a	a	a	a	a	a
3961	a	a	a	a	a	a	a	a
3970	a	a	a	a	a	a	a	a
3991	a	a	a	a	a	a	a	a
4001	a	a	a	a	a	a	a	a
4018	a	a	a	a	a	a	a	a
4019	a	a	a	a	a	a	a	a
4024	a	a	a	a	a	nd	a	a
4030	a	a	a	a	a	a	a	a
4048	nd	a	a	a	a	nd	nd	a
4066	a	a	a	a	a	a	a	a
4114	a	a	a	a	a	a	h	h
4117	a	a	a	a	a	a	a	nd
4133	a	a	a	a	a	a	a	a
4136	a	a	a	a	a	a	a	a
4141	nd	a	a	a	a	nd	nd	a
4142	nd	a	a	a	a	a	a	a
4145	a	a	a	a	a	nd	a	a

F ₂ Single Plant No.	CAU3785	CAU3786	CAU3782	F ₂ Phenotype	CAU3784	CAU3783	CAU3787 (MP_E0043)	CAU3788 (TG_E0140)
4147	a	a	nd	a	a	a	a	a
4151	a	a	a	a	a	a	a	a
4153	nd	a	a	a	a	a	a	a
4176	a	a	a	a	a	a	a	a
4177	a	a	a	a	a	a	a	a
4189	b	a	a	a	a	a	a	a
4201	nd	a	nd	a	a	a	a	a
4204	nd	a	a	a	a	nd	nd	a
4205	nd	a	a	a	a	a	a	a
4227	nd	a	a	a	a	a	a	a
4242	nd	a	nd	a	a	a	a	a
4244	nd	nd	a	a	a	a	a	a
4247	nd	a	a	a	a	a	a	a
4253	nd	a	a	a	a	a	a	a
4264	nd	a	a	a	a	a	a	a
4268	nd	a	a	a	a	a	a	a
4275	nd	a	a	a	a	a	a	a
4284	nd	a	a	a	a	a	a	a
4286	nd	a	a	a	a	a	a	a
4292	nd	a	a	a	a	a	a	a
4299	nd	a	a	a	a	a	a	a
4300	nd	a	a	a	a	a	a	a
4304	nd	a	a	a	a	a	a	a
4306	nd	a	a	a	a	a	a	a
4312	nd	a	a	a	a	a	a	a
4316	nd	a	a	a	a	a	a	a
4318	nd	a	a	a	a	a	a	a
4321	nd	a	a	a	a	a	a	a
4329	nd	a	a	a	a	a	a	a
4331	nd	a	a	a	a	a	a	a
4333	nd	a	a	a	a	a	a	a
4337	nd	a	a	a	a	a	a	a
4338	nd	a	nd	a	a	h	h	h
4341	nd	a	a	a	a	a	a	a
4342	nd	a	a	a	a	a	a	a
4349	nd	a	a	a	a	a	a	a
4353	nd	a	a	a	a	a	a	a

F ₂ Single Plant No.	CAU3785	CAU3786	CAU3782	F ₂ Phenotype	CAU3784	CAU3783	CAU3787 (MP_E0043)	CAU3788 (TG_E0140)
5586	a	a	a	a	a	a	a	a
5610	a	a	a	a	a	a	a	a
5616	a	a	a	a	a	nd	a	a
5621	a	a	a	a	a	a	a	a
5623	nd	a	a	a	a	a	a	a
5625	nd	a	a	a	a	a	a	a
5628	a	a	a	a	a	a	a	a
5636	nd	a	a	a	a	a	a	a
5640	a	a	a	a	a	a	a	a
5644	a	a	a	a	a	a	a	a
5653	a	a	a	a	a	a	a	a
5666	a	a	a	a	a	a	a	a
5672	a	a	a	a	a	a	a	a
5673	a	a	a	a	a	a	a	a
5679	a	a	a	a	a	a	a	a
5684	nd	a	a	a	a	a	a	a
5686	a	a	a	a	a	a	a	a
5688	a	a	a	a	a	a	a	a
5698	a	a	a	a	a	a	a	a
5713	a	a	a	a	a	a	a	a
5718	a	a	a	a	a	a	a	a
5745	a	a	a	a	a	a	a	a
5747	a	a	a	a	a	a	a	a
5753	nd	a	a	a	a	a	a	a
5769	a	a	a	a	a	a	a	a
5782	a	a	a	a	a	a	a	a
5785	a	a	a	a	a	a	a	a
5797	a	a	a	a	a	a	a	a
5808	a	a	a	a	a	a	h	h
5820	a	a	a	a	a	a	a	a
5821	nd	a	a	a	a	a	a	a
5822	nd	a	a	a	a	nd	nd	a
5827	a	a	a	a	a	a	a	a
5830	a	a	a	a	a	a	a	a
5836	a	a	a	a	a	a	a	a
5863	a	nd	a	a	a	h	h	nd
5869	a	a	a	a	a	h	h	nd

Supplementary Table 3: Graphical genotypes of recombinants in F₂ plants of F₂-population₀₅₆₈₂₂; Related to **Figure 4** and **Table 8**. Bolting phenotypes and marker genotypes for 116 F₂ recombinants, which were identified by screening 1301 F₂ plants of the F₂-population₀₅₆₈₂₂. Bolting phenotypes were determined by phenotyping F₃. ‘b’ (in white) and ‘a’ (in dark grey) indicate marker genotypes homozygous for the marker allele (M₁) derived from the annual, or homozygous for the marker allele (M₂) derived from the biennial mutant parent accession, respectively. ‘h’ (in light grey) indicates heterozygous marker genotypes. ‘nd’ (dashed lines): not determine. The markers CAU3782 and CAU3783 flank the *B2* locus (flanking region indicated with black bars).

F ₂ Plant Number	CAU3785	CAU3786	B2 Locus				CAU3787 (MP_E0043)	CAU3788 (TG_E0140)
			CAU3782	Phenotype	CAU3784	CAU3783		
4934	b	b	b	annual	b	b	b	h
4427	b	b	b	annual	b	b	b	h
2486	b	b	b	annual	b	h	h	h
2579	b	b	b	annual	b	h	h	h
2758	b	b	b	annual	b	h	h	h
2764	b	b	b	annual	b	h	h	h
5264	b	b	b	annual	b	h	h	h
2297	b	b	b	annual	b	h	h	h
3673	b	b	b	annual	b	h	h	h
319	b	b	nd	annual	b	h	h	h
5031	b	b	nd	annual	b	h	h	h
5191	b	b	b	annual	b	b	h	h
3280	b	b	b	annual	b	b	h	h
3320	b	b	b	annual	b	b	h	h
5685	b	b	b	annual	b	b	h	h
3805	b	b	b	annual	b	b	h	h
3845	b	b	b	annual	b	b	h	h
3888	b	b	b	annual	b	b	h	h
1132	b	nd	b	annual	b	b	h	h
1518	b	nd	b	annual	b	b	h	h
1879	b	nd	b	annual	b	b	h	h
4841	b	b	b	annual	b	b	h	h
4883	b	b	nd	annual	b	b	h	h
1101	nd	nd	b	annual	b	nd	h	h
4770	b	b	b	annual	b	b	h	nd
32	b	b	b	annual	b	b	a	nd
4036	nd	b	b	annual	b	b	h	b
4041	h	b	b	annual	b	b	h	b
5073	h	b	b	annual	b	b	b	b
2309	h	h	h	annual	b	b	b	b
5475	h	h	h	annual	b	b	b	b
2213	h	h	nd	annual	b	b	b	b
3428	a	a	a	biennial	a	a	b	h
4614	a	a	a	biennial	a	nd	b	h
73	a	a	a	biennial	a	h	h	h

F ₂ Plant Number	CAU3785	CAU3786	B2 Locus				CAU3787 (MP_E0043)	CAU3788 (TG_E0140)
			CAU3782	Phenotype	CAU3784	CAU3783		
782	a	a	a	biennial	a	h	h	h
882	a	a	a	biennial	a	h	h	h
1222	a	a	a	biennial	a	h	h	h
1850	a	a	a	biennial	a	h	h	h
2979	a	a	a	biennial	a	h	h	h
2806	a	a	a	biennial	a	h	h	h
3833	a	a	a	biennial	a	h	h	h
5569	a	a	a	biennial	a	h	h	h
6061	a	a	a	biennial	a	h	h	h
3331	a	a	a	biennial	a	h	h	h
3402	a	a	a	biennial	a	h	h	h
2129	nd	a	a	biennial	a	h	h	h
3245	nd	a	a	biennial	a	h	h	h
3043	nd	a	a	biennial	a	h	h	h
3051	nd	a	a	biennial	a	h	h	h
2415	nd	a	nd	biennial	a	h	h	h
2324	nd	a	nd	biennial	a	h	h	h
4338	nd	a	nd	biennial	a	h	h	h
2957	nd	nd	a	biennial	a	h	nd	h
5863	a	nd	a	biennial	a	h	h	nd
5869	a	a	a	biennial	a	h	h	nd
1545	a	a	a	biennial	a	h	h	nd
1563	a	a	a	biennial	a	h	h	nd
2752	nd	a	a	biennial	a	a	h	h
2395	nd	nd	nd	biennial	a	a	h	nd
187	a	a	a	biennial	a	a	h	h
1011	a	a	a	biennial	a	a	h	h
1049	a	a	a	biennial	a	a	h	h
1066	a	a	a	biennial	a	a	h	h
1306	a	a	a	biennial	a	a	h	h
1330	a	a	a	biennial	a	a	h	h
1341	a	a	a	biennial	a	a	h	h
1834	a	a	a	biennial	a	a	h	h
3539	a	a	a	biennial	a	a	h	h
3572	a	a	a	biennial	a	a	h	h
3663	a	a	a	biennial	a	a	h	h
4114	a	a	a	biennial	a	a	h	h
5277	a	a	a	biennial	a	a	h	h
5940	a	a	a	biennial	a	a	h	h
5986	a	a	a	biennial	a	a	h	h
5987	a	a	a	biennial	a	a	h	h
5808	a	a	a	biennial	a	a	h	h

F ₂ Plant Number	CAU3785	CAU3786	B2 Locus				CAU3787 (MP_E0043)	CAU3788 (TG_E0140)
			CAU3782	Phenotype	CAU3784	CAU3783		
5149	a	a	a	biennial	a	a	h	h
3886	nd	a	a	biennial	a	a	h	h
2444	nd	a	a	biennial	a	a	h	h
2461	nd	a	a	biennial	a	a	h	h
3151	nd	a	a	biennial	a	a	h	h
2177	nd	nd	nd	biennial	a	a	h	nd
2314	nd	a	a	biennial	a	a	h	nd
950	a	a	nd	biennial	a	a	h	h
953	a	a	nd	biennial	a	a	h	h
1233	a	a	nd	biennial	a	a	h	h
5998	h	a	a	biennial	a	a	h	h
1345	a	a	a	biennial	a	a	a	h
1410	a	a	a	biennial	a	a	a	h
4906	a	a	a	biennial	a	a	a	h
2911	a	a	a	biennial	a	a	a	h
3123	nd	a	a	biennial	a	a	a	h
2970	nd	a	a	biennial	a	a	a	h
2966	nd	a	a	biennial	a	a	a	h
2578	nd	a	a	biennial	a	a	nd	h
5084	a	a	a	biennial	a	nd	nd	h
5262	nd	a	a	biennial	a	nd	nd	h
4189	b	a	a	biennial	a	a	a	a
3870	h	a	nd	biennial	a	a	a	a
1022	a	h	nd	biennial	a	a	a	a
1389	h	h	a	biennial	a	a	a	a
1565	h	h	h	biennial	a	a	a	a
1625	h	h	h	biennial	a	a	a	a
5561	h	h	h	biennial	a	a	a	a
4537	h	h	h	biennial	a	a	a	a
1067	h	h	nd	biennial	a	a	a	a
5501	h	h	a	biennial	a	a	a	a
2860	h	nd	a	biennial	a	a	a	nd
1440	h	nd	a	biennial	a	nd	a	a
2742	nd	h	h	biennial	a	a	a	a
3099	nd	h	h	biennial	a	a	a	a
3211	nd	h	h	biennial	a	a	a	a
3746	nd	h	h	biennial	a	a	a	a
5162	b	b	b	biennial	a	a	a	a
4413	nd	a	b	biennial	a	a	a	nd

Supplementary Table 4: Predicted gene models (RefBeet3.0_GeneModels) for *B. vulgaris*, located in the critical region, in which *B2* is supposed to be located. Blastn analysis was performed using the critical region of scaffold sc00048 (RefBeet0.9) as query against the RefBeet0.3_GeneModels (CLC Main Workbench, version 5.7.1, blastn thresholds: e-value: 0.0; identity: 100%). Then, the obtained gene models were used for blastx analysis against the TAIR protein database (BLASTX version 2.2.24, threshold e-value < 0.05).

Gene Model	Size (bp)	Sugar Beet EST	<i>Arabidopsis thaliana</i> Homolog (At locus #)	<i>Arabidopsis thaliana</i> Homolog	At Gene: GO Molecular Function	At Gene: GO Biological Process
g25153.t1	3045	BQ588456, BI543524	At4g34710.2	Arginin decarboxylase 2, ADC2	arginine decarboxylase activity, catalytic activity	spermidine biosynthetic process, arginine catabolic process
g25152.t1	1527	n.a.	At2g21270.3	ubiquitin fusion degradation 1, UFD1	unknown	ubiquitin-dependent protein catabolic process
g25135.t1	1571	BQ586826	At2g21280.1	GC1, ATULA, SULA, NAD(P)-binding Rossmann-fold superfamily protein	coenzyme binding, nucleotide binding, catalytic activity	cellular metabolic process
g25133.t1	463	BF011044	At2g21290.1	unknown protein, located in:mitochondrion	unknown	unknown
g25130.t1	3345	BQ592067	At2g21300.2	ATP-binding microtubule motor family protein	microtubule motor activity, ATP binding	microtubule-based movement
g25128.t1	1458	BQ589556, BQ591888	At4g38960.1	B-Box type zinc finger family protein	sequence-specific DNA binding transcription factor activity, zinc ion binding	regulation of transcription
g25126.t1	2591	n.a.	At1g21280.1	Retrotransposon gaga protein	unknown	unknown
g25122.t3	1120	BQ487817, BQ587936, BQ489572	At4g38970.1	Fructose-bisphosphate aldolase 2, FBA2	fructose-bisphosphate aldolase activity, catalytic activity	response to cadmium ion, glycolysis
g25121.t1	1142	BQ490562	At4g38980.1	unknown protein	unknown	unknown
g25119.t1	2232	n.a.	At4g39010.1	glycosyl hydrolase 9B18	hydrolase activity, hydrolyzing O-glycosyl compounds, catalytic activity	carbohydrate metabolic process
g25118.t2	2118	BQ590473, BQ590482	At1g75560.2	zinc knuckle (CCHC-type) family protein	nucleic acid binding, zinc ion binding	unknown
g25117.t1	4793	BQ589661	At2g16485.1	nucleic acid binding; zinc ion binding, DNA binding	siRNA-dependent DNA methylation	unknown
g25116.t1	1259	BQ586688	At2g16460.1	protein of unknown function (DUF1640)	metal ion binding	unknown

Supplementary Table 5: Primers used for PCR and sequencing experiments during the course of this study. Primer sequences are displayed in 5'-3' orientation.

Primer Name	Primer Sequence (5' → 3')
M13_F	GTAAAACGACGGCCAGT
M13_R	CAGGAAACAGCTATGACC
A592	CCAATGGGAAAGGATTTGGAG
A747	TCCTGGTGAGTTCATAGCCTG
A882	GTGACTCATTATCTTGGACAG
A984	TGGATCTGTTGAATGAGGTTG
A985	GGTGTTAGCTATCGGTATTCC
B349	CTAGCTGCACCACTAACTGC
B350	CGTTAAGAGCTGGAAGCACC
B563	GCATCATTTGGAGAAGAGATTGTTTAC
B564	GGCGTTGTTGTGGAGCATTTA
B580	GTGAAAGCTGTGTAAGGAATGG
B581	AAGTTCCTGCATGGATCCAG
B582	GCTTTGAACGACCACTTCGC
B583	ACGCCGAGAGCAACTTGAAC
B615	ACACTAGTTGGATCCCCCG
B616	CTCATTAAAGCAGGGTCGAC
B619	GCTGCTGTGAATGTTGAAGG
C011	GACATGATGGGTTAAAGTTGCAGG
C012	GGCAGCAACTTGTGGAAAGAATTGG
C015	GGTTCTCCGGCCGCTTGGGTG
C016	CGGCAGGAGCAAGGTGAGATGAC
C017	GGGTTTCGCTCATGTGTTGAGC
C018	GGCATGCACATACAAATGGACG
C021	CCCACTATCCTTCGCAAGACCC
C052	GTAGCCCGGATCTGTTGAATGAGGTTGATTC
C053	GTAAGTCGACGGTGTTAGCTATCGGTATTCCTTC
C076	CCTGCTATGGACATTGATAAGACACC
C083	CACTGATGAAGATCCTGCTGAGC
C084	CCAACCGTTAGCTGATTGTGCTAGATCC
C156	ATGAATGCTAATGAGGAGGGGGAG
C157	TTAGCTATCCTCAATGTTTTTTTATGTCTG
C158	AAGCCCGGGATGAATGCTAATGAGGAGGGGGAG
C159	GCGTCGACTTAGCTATCCTCAATGTTTTTTTATGTCTG
C162	GGTCTGCACCATCGTCAACCAC
C163	AGATCTCGGTGACGGGCAGGAC
C229	CCTCATCAGCACACAATCTCC
C230	CGCACCCCTGACACATTTACC
C237	CTCATCAGTCCACCATATTTTCAGAAG
C261	GTGCACACTTTCTTGCCACAGG
C262	GCATCTGGATACTACTATTGTTGCAAC
C263	GAGTAGGGACATCTAACTATCTAAGG

Primer Name	Primer Sequence (5' → 3')
C294	GGGACACTAGTTAGGGATGATCG
C317	TGATTTTAAGCATATGCAGCGAGG
C318	CTACTACTCAAAGGGGCAAATGG
C375	TTCAGCATGCAGATCTGGG
C376	CTCGCCATCTCCTCCATC
C442	AAAGTTTATTGGGGATGGAGGAAG
C443	CGAATAATATCTCTACGTCAGCAGATG
C450	CCACTCCATCTTCGACCTCATATC
C451	CAGCTCAGGGTCAAAACCAACC
C470	GACTTTGAGTGCCCACTATGTG
C505	GTAAATAGCCCCTACCATCTC
C507	CTACTTCCTCTGTTCACTTTTACTTG
C508	CCTTCATTCTTTTTACTTGCCAC
C565	TGAGGACTCTTTGTGATGTTTGTGAGG
C566	GGTACAGCATTAGGGGCAGCAAG
BBf	ACTGTGGATCCATGAGGACTCTTTGTGATGTTTG
BBr	ACTGTGGATCCCTCATTTTTCTGGCTCGCTTTTG

Supplementary Table 6: Phenotyping *B. vulgaris* T₁ plants after cold treatment. Plants were first grown for seven weeks under 18h light, 22°C and then cold treated for 12 (CTP 12), 8 (CTP 8) and 4 weeks (CTP4). Then plants were transferred to the greenhouse and phenotyped for bolting (BBCH scale code: 51) (Meier, 1993). Growth conditions and phenotyping dates/ duration are given in **Table 10**, **Figure 14**, **Figure 15**. The copy number was determined by qPCR (**Chapter 3.2.4**)

Seed Code	Number of Plants	Cold Treatment	Number of Bolting Plants	Days to Bolting ¹	Number of Never Bolting Plants	Phenotype ²	Copy Number
<i>35S:btc1_a</i>							
120248	8	CTP 12	5	41 ± 6.15	3	segregating	>1
	5	CTP 8	1	45 ± 0.00	4		
	5	CTP 4	0		5		
120249	8	CTP 12	8	36 ± 0.00	0	biennial	n.d. ³
	5	CTP 8	5	46 ± 2.80	0		
	5	CTP 4	5	44 ± 2.40	0		
120250	8	CTP 12	6	53 ± 2.88	2	segregating	>1
	5	CTP 8	0		5		
	5	CTP 4	0		5		
120251	8	CTP 12	8	37 ± 1.49	0	biennial	>1
	5	CTP 8	5	45 ± 0.00	0		
	5	CTP 4	0		5		
120252	8	CTP 12	8	36 ± 0.71	0	biennial	1
	5	CTP 8	5	45 ± 0.00	0		
	3	CTP 4	3	38 ± 0.00	0		
120253	8	CTP 12	8	36 ± 0.00	0	biennial	n.d.
	5	CTP 8	5	45 ± 0.00	0		
	5	CTP 4	3	43 ± 0.00	2		
120254*	8	CTP 12	8	34 ± 0.00	0	biennial	n.d.
	5	CTP 8	4	45 ± 0.00	1		
	5	CTP 4	0		5		
120255*	8	CTP 12	0		8	never bolting	>1
	5	CTP 8	0		5		
	5	CTP 4	0		5		
120256	8	CTP 12	8	37 ± 1.85	0	biennial	1
	5	CTP 8	4	45 ± 0.00	1		
	5	CTP 4	1	43 ± 0.00	4		
120257	8	CTP 12	8	38 ± 1.77	0	biennial	1
	5	CTP 8	4	45 ± 0.00	1		
	5	CTP 4	0		5		
120258	8	CTP 12	8	36 ± 0.00	0	biennial	1
	5	CTP 8	5	45 ± 0.00	0		
	5	CTP 4	1	43 ± 0.00	4		
120259	0	CTP 12	n.a.	n.a.	n.a.	n.a.	>1
	5	CTP 8	0		5		
	5	CTP 4	0		5		

Seed Code	Number of Plants	Cold Treatment	Number of Bolting Plants	Days to Bolting ¹	Number of Never Bolting Plants	Phenotype ²	Copy Number
120260	0	CTP 12	n.a.	n.a.	n.a.		
	5	CTP 8	0		5	n.a.	>1
	5	CTP 4	0		5		
120261	0	CTP 12	n.a.	n.a.	n.a.		
	5	CTP 8	5	45 ± 0.00	0	n.a.	n.d.
	5	CTP 4	4	43 ± 0.00	1		
120262	0	CTP 12	n.a.	n.a.	n.a.		
	5	CTP 8	0		5	n.a.	1
	5	CTP 4	0		5		
120263	0	CTP 12	n.a.	n.a.	n.a.		
	5	CTP 8	3	45 ± 0.00	2	n.a.	1
	5	CTP 4	1	60 ± 0.00	4		
120264	8	CTP 12	2	36 ± 2.83	6		
	5	CTP 8	3	59 ± 11.84	2	segregating	>1
	5	CTP 4	2	46 ± 3.00	3		
120265	8	CTP 12	8	37 ± 3.54	0		
	5	CTP 8	3	45 ± 0.00	2	biennial	n.d.
	5	CTP 4	0		5		
120266	8	CTP 12	1	51 ± 0.00	7		
	5	CTP 8	0		5	segregating	>1
	5	CTP 4	0		5		
120267	8	CTP 12	8	36 ± 2.62	0		
	5	CTP 8	4	45 ± 0.00	1	biennial	n.d.
	5	CTP 4	0		5		
120268	8	CTP 12	8	37 ± 1.49	0		
	5	CTP 8	5	52 ± 6.26	0	biennial	1
	5	CTP 4	0		5		
120269	8	CTP 12	7	39 ± 1.07	1		
	5	CTP 8	5	58 ± 9.62	0	segregating	1
	5	CTP 4	0		5		
120270*	8	CTP 12	8	34 ± 0.71	0		
	5	CTP 8	5	45 ± 0.00	0	biennial	n.d.
	5	CTP 4	1	60 ± 0.00	4		
120271	8	CTP 12	1	40 ± 0.00	7		
	5	CTP 8	0		5	segregating	1
	5	CTP 4	0		5		
120272	8	CTP 12	8	38 ± 2.14	0		
	2	CTP 8	1	45 ± 0.00	1	biennial	1
	5	CTP 4	1	43 ± 0.00	4		
120273	8	CTP 12	1	49 ± 0.00	7		
	5	CTP 8	0		5	segregating	>1
	5	CTP 4	0		5		

Seed Code	Number of Plants	Cold Treatment	Number of Bolting Plants	Days to Bolting ¹	Number of Never Bolting Plants	Phenotype ²	Copy Number
120274	8	CTP 12	8	37 ± 1.83	0	biennial	1
	2	CTP 8	2	45 ± 0.00	0		
	5	CTP 4	3	41 ± 2.36	2		
120275	8	CTP 12	8	36 ± 0.00	0	biennial	1
	5	CTP 8	5	45 ± 0.00	0		
	5	CTP 4	2	49 ± 0.00	3		
<i>35S:BTC1_d</i>							
120276	8	CTP 12	8	37 ± 1.41	0	biennial	n.d.
	5	CTP 8	5	45 ± 0.00	0		
	5	CTP 4	1	49 ± 0.00	4		
120277	8	CTP 12	2	41 ± 7.07	6	segregating	>1
	5	CTP 8	0		5		
	5	CTP 4	0		5		
120278	8	CTP 12	8	36 ± 0.00	0	biennial	1
	5	CTP 8	5	45 ± 0.00	0		
	5	CTP 4	0		5		
120279	8	CTP 12	8	38 ± 2.92	0	biennial	1
	5	CTP 8	5	45 ± 0.00	0		
	5	CTP 4	0		5		
120280	8	CTP 12	8	37 ± 1.49	0	biennial	1
	5	CTP 8	5	45 ± 0.00	0		
	5	CTP 4	0		5		
120281	8	CTP 12	8	36 ± 0.00	0	biennial	n.d.
	5	CTP 8	5	45 ± 0.00	0		
	5	CTP 4	2	46 ± 3.00	3		
120282	8	CTP 12	8	36 ± 0.00	0	biennial	n.d.
	5	CTP 8	5	45 ± 0.00	0		
	5	CTP 4	3	45 ± 2.83	2		
120283	8	CTP 12	8	36 ± 0.00	0	biennial	>1
	5	CTP 8	5	45 ± 0.00	0		
	5	CTP 4	1	43 ± 0.00	4		
120284	8	CTP 12	8	36 ± 0.00	0	biennial	n.d.
	5	CTP 8	5	45 ± 0.00	0		
	5	CTP 4	2	43 ± 0.00	3		
120285	8	CTP 12	8	36 ± 0.00	0	biennial	n.d.
	5	CTP 8	5	45 ± 0.00	0		
	5	CTP 4	0		5		
120286	8	CTP 12	2	41 ± 7.07	6	segregating	1
	5	CTP 8	1	45 ± 0.00	4		
	5	CTP 4	0		5		

Seed Code	Number of Plants	Cold Treatment	Number of Bolting Plants	Days to Bolting ¹	Number of Never Bolting Plants	Phenotype ²	Copy Number
120287	8	CTP 12	8	36 ± 0.71	0	biennial	n.d.
	5	CTP 8	5	45 ± 0.00	0		
	5	CTP 4	0		5		
120288	8	CTP 12	8	37 ± 1.07	0	biennial	1
	5	CTP 8	5	46 ± 2.80	0		
	5	CTP 4	0		5		
120289	8	CTP 12	8	37 ± 1.49	0	biennial	n.d.
	5	CTP 8	5	45 ± 0.00	0		
	5	CTP 4	1	49 ± 0.00	4		
120290	8	CTP 12	8	36 ± 0.00	0	biennial	n.d.
	5	CTP 8	5	45 ± 0.00	0		
	5	CTP 4	4	47 ± 7.36	1		
120291	8	CTP 12	8	36 ± 0.00	0	biennial	n.d.
	5	CTP 8	5	45 ± 0.00	0		
	5	CTP 4	5	38 ± 0.00	0		
120292	8	CTP 12	7	49 ± 2.34	1	segregating	>1
	5	CTP 8	0		5		
	5	CTP 4	0		5		
120293	7	CTP 12	5	39 ± 1.79	2	segregating	n.d.
	5	CTP 8	0		5		
	5	CTP 4	0		5		
120294	8	CTP 12	1	36 ± 0.00	7	segregating	1
	5	CTP 8	0		5		
	5	CTP 4	0		5		
120295*	8	CTP 12	0		8	never bolting	>1
	5	CTP 8	0		5		
	5	CTP 4	0		5		
120296*	8	CTP 12	8	41 ± 3.18	0	biennial	1
	5	CTP 8	1	45 ± 0.00	4		
	5	CTP 4	5		0		
120297	8	CTP 12	5	53 ± 3.21	3	segregating	>1
	5	CTP 8	0		5		
	5	CTP 4	0		5		
120298	8	CTP 12	8	39 ± 1.04	0	biennial	1
	5	CTP 8	1	74 ± 0.00	4		
	5	CTP 4	2	43 ± 0.00	3		
120299	8	CTP 12	8	36 ± 0.00	0	biennial	n.d.
	5	CTP 8	4	47 ± 3.03	1		
	5	CTP 4	0		5		
120300*	8	CTP 12	8	51 ± 3.06	0	biennial	>1
	5	CTP 8	0		5		
	5	CTP 4	0		5		

Seed Code	Number of Plants	Cold Treatment	Number of Bolting Plants	Days to Bolting ¹	Number of Never Bolting Plants	Phenotype ²	Copy Number
120301*	8	CTP 12	8	34 ± 0.71	0	biennial	1
	5	CTP 8	5	45 ± 0.00	0		
	5	CTP 4	5	38 ± 0.00	0		
120302	8	CTP 12	8	36 ± 0.00	0	biennial	1
	5	CTP 8	5	45 ± 0.00	0		
	5	CTP 4	5	43 ± 0.00	0		
120303	8	CTP 12	5	47 ± 7.02	3	segregating	>1
	5	CTP 8	0		5		
	5	CTP 4	1	60 ± 0.00	4		
120304	8	CTP 12	7	45 ± 8.25	1	segregating	>1
	5	CTP 8	0		5		
	5	CTP 4	0		5		
120305*	8	CTP 12	0		8	never bolting	>1
	5	CTP 8	0		5		
	5	CTP 4	0		5		
120306*	8	CTP 12	0		8	never bolting	>1
	5	CTP 8	0		5		
	5	CTP 4	0		5		
120307	8	CTP 12	4	46 ± 6.90	4	segregating	>1
	5	CTP 8	0		5		
	5	CTP 4	0		5		
120308	8	CTP 12	8	36 ± 0.00	0	biennial	n.d.
	1	CTP 8	1	45 ± 0.00	0		
	1	CTP 4	1	49 ± 0.00	0		
120309	6	CTP 12	6	36 ± 0.00	0	biennial	-
	0	CTP 8			0		
	0	CTP 4			0		

¹ only bolting plants, days to bolting were shown with standard deviation of the mean

² Phenotype according to phenotyping clones after 12 weeks of cold treatment, biennial: all clones/ transformant bolted after vernalization; never bolting: no clones/ transformant bolted after 1st vernalization,; segregating: bolting and never bolting clones/ transformant

³ n.d. not determined, CNI-value < 0.6 (**Supplementary Table 7, Chapter 3.2.4**)

* statistical significant differences in days to bolting (T-Test, p = 0.05) between non-transgenic control and transgenic plants (CTP 12, n = 8, without clonal variation for bolting and never bolting).

Supplementary Table 7: Copy number determination in 61 independent *B. vulgaris* transformants. Copy number was determined as described in **Chapter 3.2.4**. CNI-value: copy number indication-value; n.d.: not determined, CNI-value > 0.6

Seed Code	CNI-Value	Copy Number
120248	3.00	>1
120249	0.50	n.d.
120250	3.00	>1
120251	2.00	>1
120252	1.00	1
120253	0.50	n.d.
120254	0.50	n.d.
120255	1.70	>1
120256	1.00	1
120257	0.70	1
120258	0.70	1
120259	3.00	>1
120260	3.00	>1
120261	0.50	n.d.
120262	1.20	1
120263	1.30	1
120264	3.00	>1
120265	n.a.	n.d.
120266	3.00	>1
120267	0.00	n.d.
120268	1.30	1
120269	0.80	1
120270	0.50	n.d.
120271	1.00	1
120272	0.80	1
120273	1.80	>1
120274	0.60	1
120275	0.60	1
120276	0.50	n.d.
120277	3.00	>1
120278	0.70	1
120279	1.25	1
120280	0.70	1
120281	0.20	n.d.
120282	0.40	n.d.
120283	1.70	>1
120284	0.40	n.d.
120285	0.30	n.d.
120286	1.30	1
120287	0.50	n.d.
120288	0.60	1
120289	0.50	n.d.
120290	0.00	n.d.
120291	0.50	n.d.
120292	3.00	>1

Seed Code	CNI-Value	Copy Number
120293	n.a.	n.d.
120294	1.00	1
120295	3.00	>1
120296	1.00	1
120297	3.00	>1
120298	0.70	1
120299	0.50	n.d.
120300	3.00	>1
120301	1.10	1
120302	0.60	1
120303	3.00	>1
120304	2.40	>1
120305	3.00	>1
120306	3.00	>1
120307	3.00	>1
120308	0.50	n.d.

Supplementary Table 8: Determination of single transgene integrations in 73 randomly selected *A. thaliana* T₂ families. ~60 seeds/ T₂ family were sown in soil and seedlings were grown under 16h light and 22°C in the greenhouse. After 3 weeks seedlings were treated with BASTA® and the number of herbicide resistant (transgenic) and susceptible (non-transgenic) plants was scored for each T₂ family. The observed segregation ratios were tested by χ^2 -analysis for a 3:1 ($\alpha_{0.05} = 3.84$) ratio, which was expected for inheritance of a single transgene locus. The *BTC1_d/btc1_a* coding sequences were derived from the annual line 930190 or biennial line 940043, respectively. The *PRR7* coding sequence stemmed from *A. thaliana*.

Genetic Background	Transgene Cassette	T ₂ Family	# Plants Grown	# Plants Survived	# Plants Died	χ^2 -test for H ₀ ¹ = 3:1 (transgenic:non-transgenic)
Col-0	<i>35S:PRR7</i>	R039	40	30	10	0.00
	<i>35S:PRR7</i>	R041	46	37	9	0.73
	<i>35S:PRR7</i>	R055	42	40	2	9.17*
	<i>35S:PRR7</i>	R062	42	27	15	2.57
	<i>35S:PRR7</i>	R067	40	29	11	0.13
	<i>35S:PRR7</i>	R072	51	49	2	12.08*
	<i>35S:PRR7</i>	R076	33	29	4	2.91
	<i>35S:PRR7</i>	R077	49	40	9	1.15
	<i>35S:PRR7</i>	R081	45	28	17	3.92*
	<i>35S:PRR7</i>	R091	37	35	2	7.58*
	<i>35S:PRR7</i>	R098	35	34	1	9.15*
	<i>35S:PRR7</i>	R099	32	24	8	0.00
	<i>35S:PRR7</i>	R112	48	37	11	0.11
	<i>35S:PRR7</i>	R117	36	30	6	1.33
	<i>35S:PRR7</i>	R125	36	22	14	3.70
	<i>35S:PRR7</i>	R137	52	42	10	0.92
	<i>35S:PRR7</i>	R139	49	40	9	1.15
	<i>35S:PRR7</i>	R149	32	23	9	0.17
	<i>35S:PRR7</i>	R174	51	50	1	14.44*
<i>35S:PRR7</i>	R175	59	51	8	4.12*	
Col-0	<i>35S:btc1a</i>	R217	66	28	38	37.35*
	<i>35S:btc1a</i>	R226	42	38	4	5.37*
	<i>35S:btc1a</i>	R232	42	42	0	14.00*
	<i>35S:btc1a</i>	R234	32	32	0	10.67*
	<i>35S:btc1a</i>	R241	42	34	8	0.79
	<i>35S:btc1a</i>	R244	43	32	11	0.01
	<i>35S:btc1a</i>	R245	45	45	0	15.00*
	<i>35S:btc1a</i>	R266	41	30	11	0.07
	<i>35S:btc1a</i>	R271	64	47	17	0.08
	<i>35S:btc1a</i>	R287	36	24	12	1.33
	<i>35S:btc1a</i>	R293	63	63	0	21.00*
	<i>35S:btc1a</i>	R301	51	50	1	14.44*
	<i>35S:btc1a</i>	R302	46	34	12	0.03
	<i>35S:btc1a</i>	R308	62	50	12	1.05
	<i>35S:btc1a</i>	R309	31	22	9	0.27
<i>35S:btc1a</i>	R317	47	40	7	2.56	

Genetic Background	Transgene Cassette	T ₂ Family	# Plants Grown	# Plants Survived	# Plants Died	χ^2 -test for H ₀ ¹ = 3:1 (transgenic:non-transgenic)
Col-0	<i>35S:btc1a</i>	R320	42	30	12	0.29
	<i>35S:btc1a</i>	R338	66	55	11	2.44
	<i>35S:btc1a</i>	R339	47	43	4	6.82*
	<i>35S:btc1a</i>	R342	45	28	17	4.36*
Col-0	<i>35S:BTC1_d</i>	R344	65	51	14	0.42
	<i>35S:BTC1_d</i>	R345	39	26	13	1.44
	<i>35S:BTC1_d</i>	R346	50	38	12	0.03
	<i>35S:BTC1_d</i>	R347	56	44	12	0.38
	<i>35S:BTC1_d</i>	R348	35	19	16	8.01*
	<i>35S:BTC1_d</i>	R349	50	48	2	11.76*
	<i>35S:BTC1_d</i>	R350	47	37	10	0.35
	<i>35S:BTC1_d</i>	R351	38	34	4	4.25*
	<i>35S:BTC1_d</i>	R352	55	46	9	2.19
	<i>35S:BTC1_d</i>	R353	36	28	8	0.15
	<i>35S:BTC1_d</i>	R354	43	34	9	0.38
	<i>35S:BTC1_d</i>	R355	51	37	14	0.16
	<i>35S:BTC1_d</i>	R357	41	40	1	11.13*
<i>pr7-11</i>	<i>35S:PRR7</i>	R480	63	48	15	0.05
	<i>35S:PRR7</i>	R482	61	44	17	0.27
	<i>35S:PRR7</i>	R483	58	42	16	0.21
	<i>35S:PRR7</i>	R484	80	77	3	19.27*
	<i>35S:PRR7</i>	R486	50	48	2	11.76*
	<i>35S:PRR7</i>	R488	55	34	21	5.09*
<i>pr7-11</i>	<i>35S:btc1_a</i>	R463	59	46	13	0.28
	<i>35S:btc1_a</i>	R464	75	63	12	3.24
	<i>35S:btc1_a</i>	R466	68	61	7	7.84*
	<i>35S:btc1_a</i>	R467	65	50	15	0.13
	<i>35S:btc1_a</i>	R468	74	0	74	24.67*
	<i>35S:btc1_a</i>	R471	61	56	5	9.19*
	<i>35S:btc1_a</i>	R472	59	47	12	0.68
	<i>35S:btc1_a</i>	R474	59	41	18	0.95
<i>pr7-11</i>	<i>35S:BTC1_d</i>	R450	50	30	20	6.00*
	<i>35S:BTC1_d</i>	R452	94	87	7	15.45*
	<i>35S:BTC1_d</i>	R453	58	31	27	14.37*
	<i>35S:BTC1_d</i>	R454	56	46	10	1.52
	<i>35S:BTC1_d</i>	R457	57	46	11	0.99
	<i>35S:BTC1_d</i>	R458	57	54	3	11.84*

Supplementary Table 9: Phenotyping *A. thaliana* T₂ families for bolting and the number of rosette leaves. 29-35 plants/ T₂ family and as a control 35 plants of Col-0 and *prrr7-11*, respectively, were grown under 16h of light in a climate chamber. T₂ families with a Col-0 genetic background were grown at 22°C and T₂ families with *prrr7-11* genetic background were grown at 18°C. Bolting was recorded as days to bolt (DTB) after sowing, when the first flower bud was visible (growth stage 5.10) and additionally the total number of rosette leaves (TNRL) at this stage was scored. T₂ plants were genotyped by PCR as described in (Chapter 4.2.4).

Genetic Background, Transgene Cassette	T ₂ Family	DTB (mean ± standard deviation)				TNRL (mean ± standard deviation)			
		Transgenic T ₂	Non-Transgenic T ₂	Control Col-0	Control <i>prrr7-11</i>	Transgenic	Non-Transgenic	Control Col-0	Control <i>prrr7-11</i>
Col-0, <i>35S:AtPRR7</i>	R099	28.81 ± 1.84 (n=27) ^B	28.43 ± 0.90 (n=7)	33.00 ± 2.09 (n=35)	-	14.15 ± 1.65 (n=27) ^B	15.00 ± 1.60 (n=7)	16.21 ± 1.64 (n=35)	-
Col-0, <i>35S:AtPRR7</i>	R117	28.40 ± 2.18 (n=20) ^B	27.31 ± 1.07 (n=13)			14.25 ± 1.84 (n=20) ^{A, B}	12.92 ± 1.54 (n=13) ^A		
Col-0, <i>35S:BTC1_d</i>	R347	29.68 ± 1.71 (n=25) ^B	30.00 ± 0.47 (n=9)			16.44 ± 1.88 (n=25)	15.89 ± 1.20 (n=9)		
Col-0, <i>35S:BTC1_d</i>	R355	28.88 ± 1.40 (n=26) ^B	30.14 ± 0.83 (n=7)			14.69 ± 1.29 (n=26) ^B	14.71 ± 1.48 (n=7)		
Col-0, <i>35S:btc1_a</i>	R338	31.88 ± 1.62 (n=24)	32.40 ± 1.28 (n=11)			15.83 ± 1.43 (n=24)	16.60 ± 1.50 (n=11)		
<i>prrr7-11</i> , <i>35S:AtPRR7</i>	R483	40.57 ± 2.13 (n=21) ^B	40.31 ± 2.89 (n=13)	37.46 ± 1.23 (n=35)	51.97 ± 4.54 (n=35)	15.19 ± 1.79 (n=21) ^B	15.54 ± 2.10 (n=13)	12.14 ± 0.87 (n=35)	22.77 ± 4.09 (n=35)
<i>prrr7-11</i> , <i>35S:BTC1_d</i>	R454	50.00 ± 4.29 (n=22)	49.17 ± 3.34 (n=7)			22.95 ± 4.18 (n=22)	21.83 ± 2.67 (n=7)		
<i>prrr7-11</i> , <i>35S:BTC1_d</i>	R457	53.15 ± 4.17 (n=22)	53.50 ± 4.92 (n=9)			24.00 ± 3.18 (n=22)	24.40 ± 3.93 (n=9)		
<i>prrr7-11</i> , <i>35S:btc1_a</i>	R463	51.67 ± 4.48 (n=22)	53.20 ± 2.04 (n=7)			23.62 ± 3.71 (n=22)	22.40 ± 2.94 (n=7)		

^A significant differences confirmed by Student's *t*-Test at $\alpha = 0.05$ between transgenic and non-transgenic T₂ plants of the same family

^B significant differences confirmed by Student's *t*-Test at $\alpha = 0.05$ between the control Col-0 and transgenic T₂ plants with Col-0 genetic background or the control *prrr7-11* and transgenic T₂ plants with *prrr7-11* genetic background

n, number of plants

Supplementary Table 10: Phenotyping *A. thaliana* T₂ families for flowering and the number of cauline leaves. 29-35 plants/ T₂ family and as a control 35 plants of Col-0 and *prrr7-11*, respectively, were grown under 16h of light in a climate chamber. T₂ families with a Col-0 genetic background were grown at 22°C and T₂ families with *prrr7-11* genetic background were grown at 18°C. Flowering was recorded as days to flower (DTB) after sowing, when the first flower bud opened (growth stage 6.10) and additionally the total number of cauline leaves (TNCL) at this stage was scored. T₂ plants were genotyped by PCR as described in (Chapter 4.2.4).

Genetic Background, Transgene Cassette	T ₂ Family	DTF (mean ± standard deviation)				TNCL (mean ± standard deviation)			
		Transgenic T ₂	Non-Transgenic T ₂	Control Col-0	Control <i>prrr7-11</i>	Transgenic	Non-Transgenic	Control Col-0	Control <i>prrr7-11</i>
Col-0, <i>35S:AtPRR7</i>	R099	37.63 ± 2.17 (n=27) ^B	37.57 ± 1.05 (n=7)	42.21 ± 2.53 (n=35)	-	3.67 ± 0.67 (n=27) ^B	4.00 ± 0.53 (n=7)	4.15 ± 0.61 (n=35)	-
Col-0, <i>35S:AtPRR7</i>	R117	38.25 ± 2.84 (n=20) ^B	36.54 ± 1.28 (n=13)			3.45 ± 0.50 (n=20) ^{A, B}	2.92 ± 0.27 (n=13) ^A		
Col-0, <i>35S:BTC1_d</i>	R347	39.48 ± 2.06 (n=25) ^B	40.00 ± 1.05 (n=9)			4.56 ± 0.70 (n=25)	4.56 ± 0.68 (n=9)		
Col-0, <i>35S:BTC1_d</i>	R355	38.12 ± 2.41 (n=26)	39.43 ± 1.05 (n=7)			3.46 ± 0.69 (n=26) ^B	3.71 ± 0.45 (n=7)		
Col-0, <i>35S:btc1_a</i>	R338	41.50 ± 1.53 (n=24)	42.20 ± 1.72 (n=11)			4.17 ± 0.75 (n=24)	4.00 ± 0.63 (n=11)		
<i>prrr7-11</i> , <i>35S:AtPRR7</i>	R483	56.05 ± 2.65 (n=21) ^B	55.15 ± 2.74 (n=13)	50.66 ± 2.33 (n=35)	65.43 ± 5.09 (n=35)	4.95 ± 0.49 (n=21) ^B	5.00 ± 0.55 (n=13)	4.20 ± 0.96 (n=35)	5.54 ± 0.92 (n=35)
<i>prrr7-11</i> , <i>35S:BTC1_d</i>	R454	65.71 ± 4.99 (n=22)	62.67 ± 3.20 (n=7)			5.81 ± 0.91 (n=22)	5.00 ± 0.58 (n=7)		
<i>prrr7-11</i> , <i>35S:BTC1_d</i>	R457	65.75 ± 4.50 (n=22)	66.30 ± 6.20 (n=9)			5.85 ± 1.06 (n=22)	5.80 ± 1.25 (n=9)		
<i>prrr7-11</i> , <i>35S:btc1_a</i>	R463	64.62 ± 4.56 (n=22)	65.60 ± 3.01 (n=7)			5.62 ± 0.95 (n=22)	5.00 ± 0.00 (n=7)		

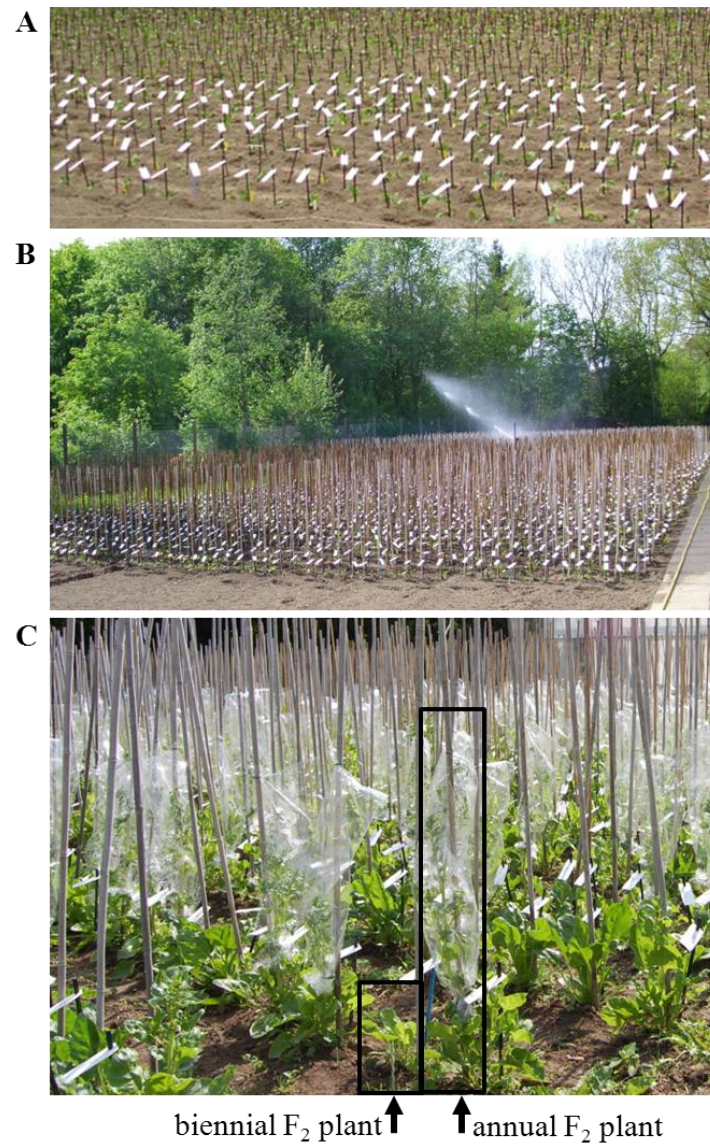
^A significant differences confirmed by Student's *t*-Test at $\alpha = 0.05$ between transgenic and non-transgenic T₂ plants of the same family

^B significant differences confirmed by Student's *t*-Test at $\alpha = 0.05$ between the control Col-0 and transgenic T₂ plants with Col-0 genetic background or the control *prrr7-11* and transgenic T₂ plants with *prrr7-11* genetic background
n, number of plants

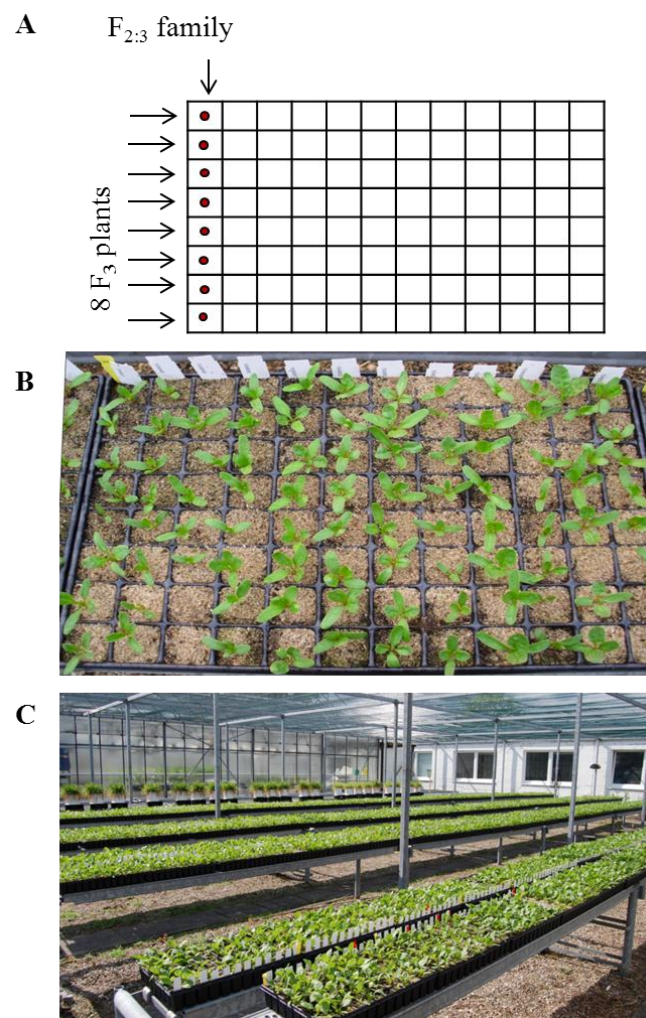
Supplementary Table 11: Binary vectors constructed during the study.

Construct	Integrated Gene	Promoter	Selectable Marker Gene	Vector Backbone	Vector Backbone Reference
CAU3668	<i>btc1_a</i>	-		pJet1.2	Fermentas, St. Leon Rot, Germany
CAU3669	<i>BTC1_d</i>	-		pJet1.2	Fermentas, St. Leon Rot, Germany
CAU3713	<i>BTC1_d</i>	35S	<i>nptII</i>	p9N-35S	DNA cloning Service, Hamburg, Germany
CAU3714	<i>btc1_a</i>	35S	<i>nptII</i>	p9N-35S	DNA cloning Service, Hamburg, Germany
CAU3715	<i>BTC1_d</i>	35S	<i>pat</i>	p7UG-AB	DNA cloning Service, Hamburg, Germany
CAU3716	<i>btc1_a</i>	35S	<i>pat</i>	p7UG-AB	DNA cloning Service, Hamburg, Germany
CAU3718	<i>PRR7</i>	35S	<i>pat</i>	p7UG-AB	DNA cloning Service, Hamburg, Germany

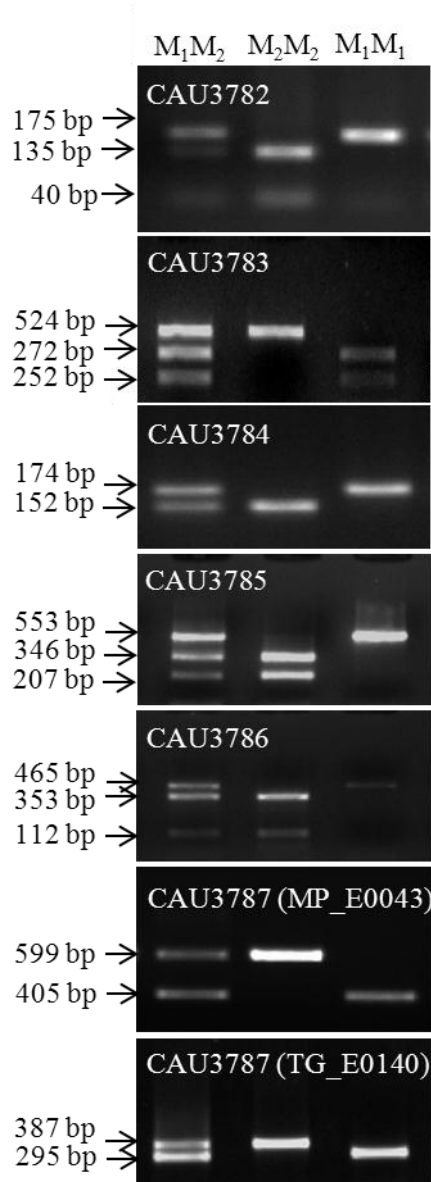
8.2 Supplementary figures



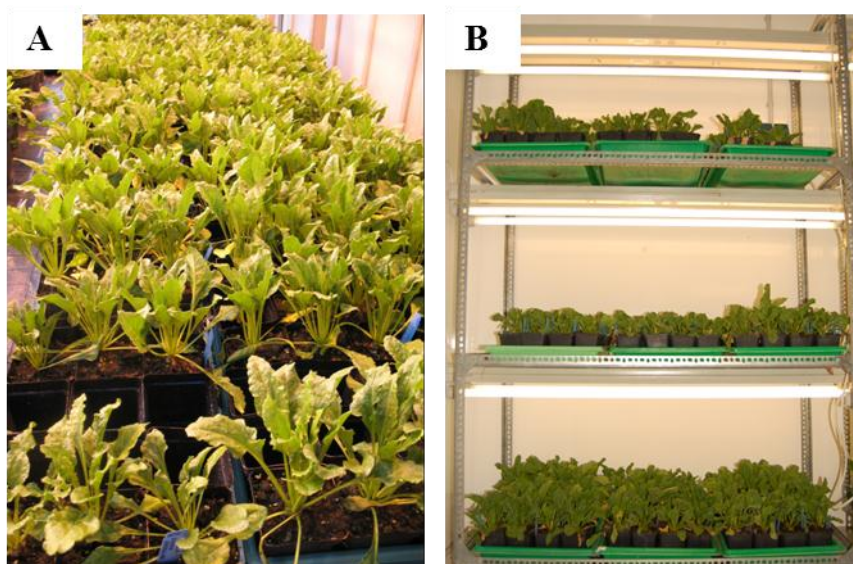
Supplementary Figure 1: F₂-population₀₅₆₈₂₂ growing in the field from May 17, 2010 until 19.08.2011 which were phenotyped for bolting and flowering behavior from May 17, 2010 until October 25, 2010. (A) F₂ plants were transplanted to the field after cultivation for 6 weeks in the greenhouse (16h light, 22°C). (B) Overview about the field. (C) Annual and biennial F₂ plants of F₂-population₀₅₆₈₂₂. Annual plants that started bolting were propagated by bag isolation in the field for seed production. Biennial plants that survived the winter in the field were also propagated by bag isolation.



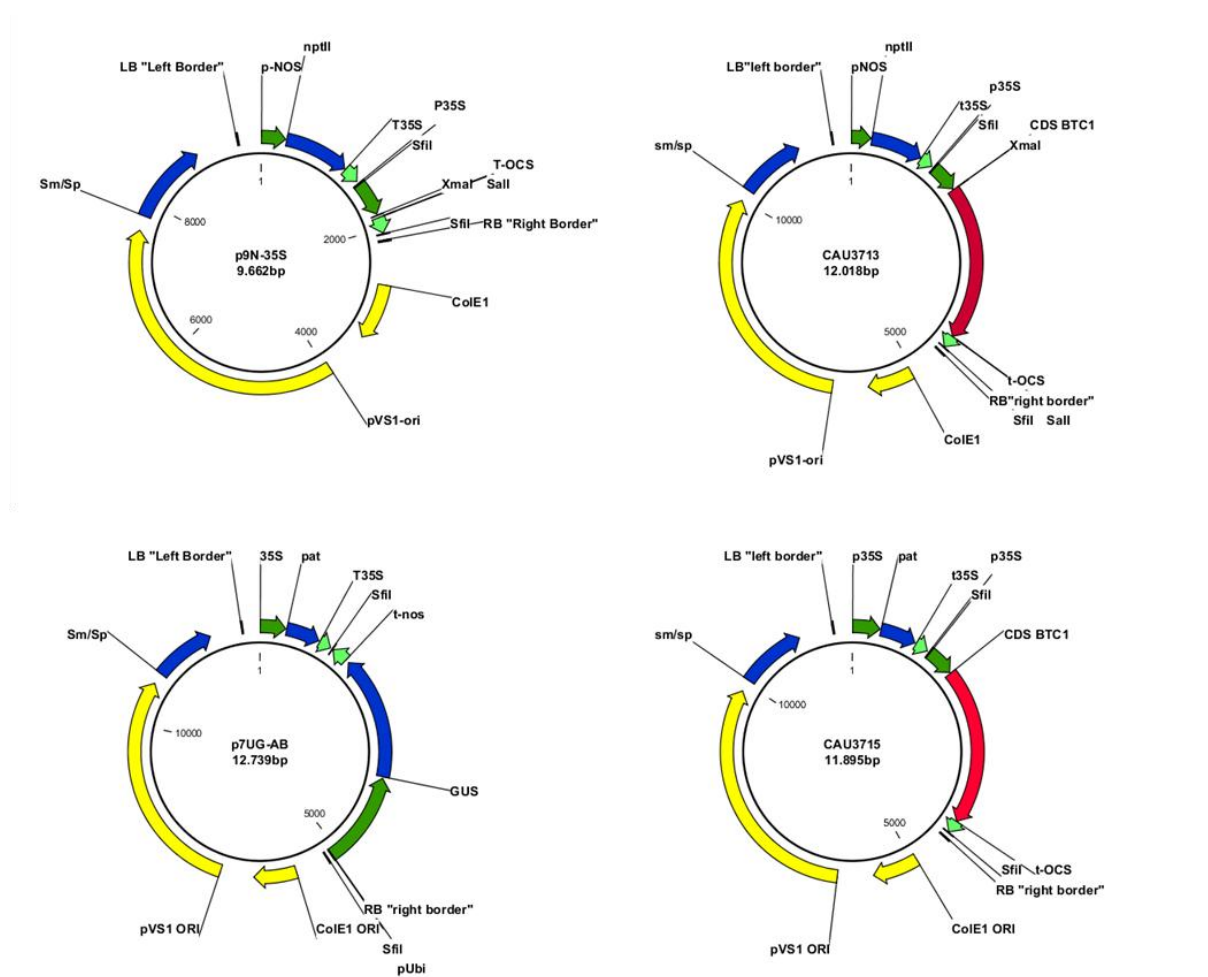
Supplementary Figure 2: Phenotyping $F_{2:3}$ families that were derived from annual F_2 plants. (A) For phenotyping, 8 F_3 plants per $F_{2:3}$ family were grown in a 96er multipot plate. (B) Picture of a 96er multipot plate with 12 $F_{2:3}$ families. (C) F_3 plants were grown under natural conditions outside the greenhouse and phenotyped for bolting behavior from May 16, 2011 until October 12, 2011.



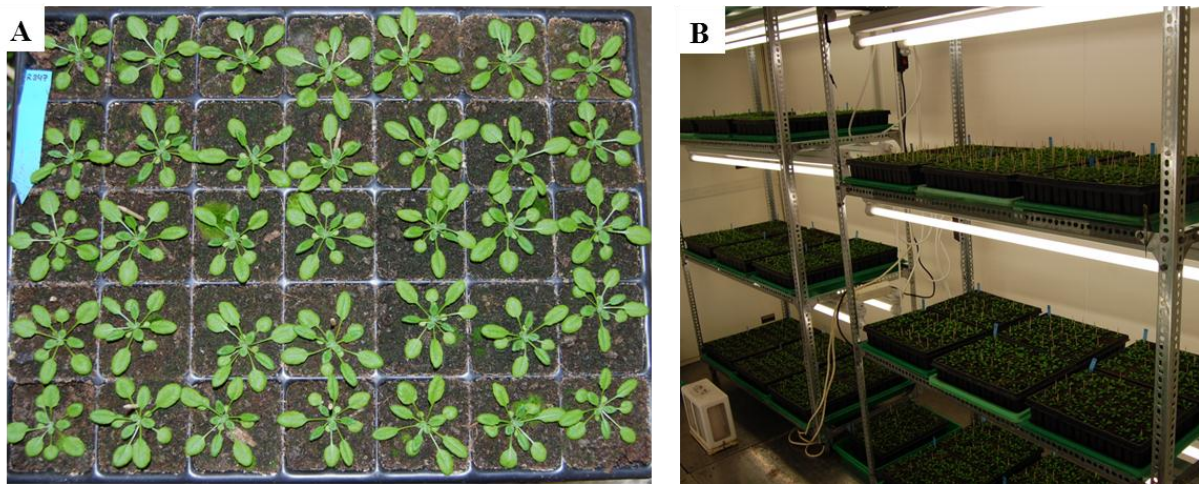
Supplementary Figure 3: Genotypes of marker loci (given on top of each picture) that were used for fine-mapping the *B2* locus in *B. vulgaris* in heterozygous annual F₂ plants, homozygous annual F₂ plants and homozygous biennial F₂ plants of the F₂-population₀₅₆₈₂₂. Genotypes were determined by PCR (InDel) or PCR and a subsequent digest with specific restriction enzymes (CAPS). All yielded fragments were analyzed on agarose gels (see **Table 5**). The marker allele M₁ derived from the annual parent, whereas the marker allele M₂ derived from the biennial mutant parent. Black arrows indicate the respective fragment size in base pairs (bp).



Supplementary Figure 4: Transgenic *B. vulgaris* T₁ plants carrying the *35S:BTC1_d* and *35S:btC1_d* transgene cassette. (A) Plants growing in the greenhouse under 16h light and 22°C in Kiel (Germany). (B) Plants growing in a cold chamber for cold treatment at 4°C and 8h light in Kiel (Germany).



Supplementary Figure 5: Binary vectors p9-35S and p7UG-AB and constructed transformation vectors CAU3713 and CAU3715. Yellow arrows: replication origin; dark green arrows: promoter; light green arrows: terminator; red arrows: integrated coding sequence of *BTC1*; blue arrows: region encoding the reporter gene β -Glucuronidase (GUS) or resistance-conferring genes for streptomycin/ spectinomycin (Sm/Sp) and glufosinate (pat).



Supplementary Figure 6: Phenotyping transgenic *A. thaliana* plants carrying the *35S:BTC1_a*, *35S:btc1_a* and *35S:PRR7* transgene cassette. (A) T₂ plants of each family were grown in 35er multipot plates with 35 T₂ plant/ family (here family R347). (B) Plants were phenotyped under LD conditions (16h light, 22°C) in a climate chamber for bolting, flowering and the number of cauline and rosette leaves (see **Chapter 4**).

9 References

- Abou-Elwafa, S., B. Büttner, F. Kopisch-Obuch, C. Jung, and A. Müller, 2012: Genetic identification of a novel bolting locus in *Beta vulgaris* which promotes annuality independently of the bolting gene *B*. *Molecular Breeding* 29, 989-998.
- Abou-Elwafa, S.F., B. Büttner, T. Chia, G. Schulze-Buxloh, U. Hohmann, E. Mutasa-Göttgens, C. Jung, and A.E. Muller, 2010: Conservation and divergence of autonomous pathway genes in the flowering regulatory network of *Beta vulgaris*. *J Exp Bot.* 62, 3359-3374.
- Agrawal, N., P.V. Dasaradhi, A. Mohammed, P. Malhotra, R.K. Bhatnagar, and S.K. Mukherjee, 2003: RNA interference: biology, mechanism, and applications. *Microbiol Mol Biol Rev* 67, 657-685.
- Andrés, F., and G. Coupland, 2012: The genetic basis of flowering responses to seasonal cues. *Nature Reviews Genetics* 13, 627-639.
- Arumuganathan, K., and E.D. Earle, 1991: Nuclear DNA content of some important plant species. *Plant Molecular Biology Reporter* 9, 208-218.
- Asadi, M., 2007: Beet-Sugar Handbook. John Wiley & Sons, Inc.
- Biancardi, E., L.G. Campbell, G.N. Skaracis, and M. De Biaggi, 2005: Genetics and Breeding of Sugar Beet. Science Publishers Inc., Enfieldt, NH, USA.
- Biancardi, E., J.M. McGrath, M. Saccomani, and P. Stevanato, 2010: Sugar Beet. In J. E. Bradshaw (ed.) Root and Tuber Crops. Handbook of Plant Breeding Springer Science+Business Media, LLC, New York, NY USA 7, p. 173-219.
- Boudry, P., H. McCombie, and H. Van Dijk, 2002: Vernalization requirement of wild beet *Beta vulgaris* ssp *maritima*: among population variation and its adaptive significance. *Journal of Ecology* 90, 693-703.
- Büttner, B., S.F. Abou-Elwafa, W. Zhang, C. Jung, and A.E. Muller, 2010: A survey of EMS-induced biennial *Beta vulgaris* mutants reveals a novel bolting locus which is unlinked to the bolting gene *B*. *Theor Appl Genet* 121, 1117-1131.
- Carre, I., and S. Veflingstad, 2013: Emerging design principles in the *Arabidopsis* circadian clock. *Semin Cell Dev Biol.* 24, 393-398.
- Chia, T.Y., A. Müller, C. Jung, and E.S. Mutasa-Göttgens, 2008: Sugar beet contains a large *CONSTANS*-LIKE gene family including a *CO* homologue that is independent of the early-bolting (*B*) gene locus. *J Exp Bot* 59, 2735-2748.
- Cooke, D.A., 1993: The Sugar Beet Crop. Chapman & Hall.
- Dohm, J.C., C. Lange, D. Holtgräwe, T. Rosleff Sorensen, D. Borchardt, B. Schulz, H. Lehrach, B. Weisshaar, and H. Himmelbauer, 2011: Palaeohexaploid Ancestry for Caryophyllales Inferred from Extensive Gene-Based Physical and Genetic Mapping of the Sugar Beet Genome (*Beta vulgaris*). *The Plant Journal* 70, 528-540.
- Draycott, P., 2006: Sugar Beet. Blackwell Publishing Ltd.

- Eldem, V., S. Okay, and T. Ünver, 2013: Plant microRNAs: new players in functional genomics. *Turkish Journal of Agriculture and Forestry*.
- Eyers, M., K. Edwards, and W. Schuch, 1992: Construction and characterisation of a yeast artificial chromosome library containing two haploid *Beta vulgaris* L. genome equivalents. *Gene* 121, 195-201.
- Falk Zakrzewsky, B.W., Thomas Schmidt, 2013: A Molecular Cytogenetic Analysis of the Structure, Evolution, and Epigenetic Modifications of Major DNA Sequences in Centromeres of Beta Species, In: Jiming Jiang, James A. Birchler, (eds.) *Plant Centromere Biology* John Wiley+Sons, Inc.
- Flavell, R.B., M.D. Bennett, J.B. Smith, and D.B. Smith, 1974: Genome size and the proportion of repeated nucleotide sequence DNA in plants. *Biochem Genet* 12, 257-269.
- Gilmartin, P.M., L. Sarokin, J. Memelink, and N.H. Chua, 1990: Molecular light switches for plant genes. *Plant Cell* 2, 369-378.
- Gindullis, F., D. Dechyeva, and T. Schmidt, 2001: Construction and characterization of a BAC library for the molecular dissection of a single wild beet centromere and sugar beet (*Beta vulgaris*) genome analysis. *Genome* 44, 846-855.
- Hatlestad, G.J., R.M. Sunnadeniya, N.A. Akhavan, A. Gonzalez, I.L. Goldman, J.M. McGrath, and A.M. Lloyd, 2012: The beet *R* locus encodes a new cytochrome P450 required for red betalain production. *Nat Genet* 44, 816-820.
- Hautekeete, N.C., Y. Piquot, and H. Van Dijk, 2002: Life span in *Beta vulgaris* ssp *maritima*: the effects of age at first reproduction and disturbance. *Journal of Ecology* 90, 508-516.
- Hohmann, U., G. Jacobs, and C. Jung, 2005: An EMS mutagenesis protocol for sugar beet and isolation of non-bolting mutants. *Plant Breeding* 124, 317-321.
- Hohmann, U., G. Jacobs, A. Telgmann, R.M. Gaafar, S. Alam, and C. Jung, 2003: A bacterial artificial chromosome (BAC) library of sugar beet and a physical map of the region encompassing the bolting gene *B*. *Molecular Genetics and Genomics* 269, 126-136.
- Jacobs, G., D. Dechyeva, T. Wenke, B. Weber, and T. Schmidt, 2009: A BAC library of *Beta vulgaris* L. for the targeted isolation of centromeric DNA and molecular cytogenetics of *Beta* species. *Genetica* 135, 157-167.
- Kinmonth-Schultz, H.A., G.S. Golembeski, and T. Imaizumi, 2013: Circadian clock-regulated physiological outputs: Dynamic responses in nature. *Semin Cell Dev Biol.* 24, 407-413.
- Kleffmann, 2011: <http://www.kleffmann.com/opencms/opencms/de/mediacenter/Pressemitteilungen/Zuckerruebe>.
- Kleine, M., D. Cai, C. Elbl, R.G. Herrmann, and C. Jung, 1995: Physical Mapping and Cloning of a Translocation in Sugar-Beet (*Beta-Vulgaris* L) Carrying a Gene for Nematode (*Heterodera-Schachtii*) Resistance from *Beta-Procumbens*. *Theoretical and Applied Genetics* 90, 399-406.
- Kleinlankhorst, R.M., E.M.J. Salentijn, W.G. Dirkse, M. Arensdereuver, and W.J. Stiekema, 1994: Construction of a Yac Library from a *Beta vulgaris* Fragment Addition and Isolation of a Major Satellite DNA Cluster Linked to the Beet Cyst-Nematode Resistance Locus *Hs1*(Pat-1). *Theoretical and Applied Genetics* 89, 426-434.

- Kumagai, T., S. Ito, N. Nakamichi, Y. Niwa, M. Murakami, T. Yamashino, and T. Mizuno, 2008: The common function of a novel subfamily of B-box zinc finger proteins with reference to circadian-associated events in *Arabidopsis thaliana*. *Bioscience, Biotechnology, and Biochemistry* 72, 1539-1549.
- Lange, C., D. Holtgräwe, B. Schulz, B. Weisshaar, and H. Himmelbauer, 2008: Construction and characterization of a sugar beet (*Beta vulgaris*) fosmid library. *Genome* 51, 948-951.
- Lange, W., W.A. Brandenburg, and T.S.M. De Bock, 1999: Taxonomy and cultonomy of beet (*Beta vulgaris* L.). *Botanical Journal of the Linnean Society* 130, 81-96.
- Lee, J., and I. Lee, 2010: Regulation and function of *SOCI*, a flowering pathway integrator. *Journal of Experimental Botany* 61, 2247-2254.
- Märländer, B., T. Lange, and B. Wulkow, 2011: Dispersal Principles of Sugar Beet from Seed to Sugar with Particular Relation to Genetically Modified Varieties. *JOURNAL FÜR KULTURPFLANZEN* 63 (11), 349–373.
- McGrath, J.M., R.S. Shaw, B.G. de los Reyes, and J.J. Weiland, 2004: Construction of a sugar beet BAC library from a hybrid with diverse traits. *Plant Molecular Biology Reporter* 22, 23-28.
- Mutasa-Gottgens, E., and P. Hedden, 2009: Gibberellin as a factor in floral regulatory networks. *Journal of Experimental Botany* 60, 1979-1989.
- Mutasa-Göttgens, E.S., A. Joshi, H.F. Holmes, P. Hedden, and B. Gottgens, 2012: A new RNASeq-based reference transcriptome for sugar beet and its application in transcriptome-scale analysis of vernalization and gibberellin responses. *BMC Genomics* 13, 99.
- Paesold, S., D. Borchardt, T. Schmidt, and D. Dechyeva, 2012: A sugar beet (*Beta vulgaris* L.) reference FISH karyotype for chromosome and chromosome-arm identification, integration of genetic linkage groups and analysis of major repeat family distribution. *The Plant Journal* 72, 600-611.
- Pin, P.A., R. Benlloch, D. Bonnet, E. Wremerth-Weich, T. Kraft, J.J.L. Gielen, and O. Nilsson, 2010: An Antagonistic Pair of *FT* Homologs Mediates the Control of Flowering Time in Sugar Beet. *Science* 330, 1397-1400.
- Pin, P.A., W. Zhang, S.H. Vogt, N. Dally, B. Buttner, G. Schulze-Buxloh, N.S. Jelly, T.Y. Chia, E.S. Mutasa-Gottgens, J.C. Dohm, H. Himmelbauer, B. Weisshaar, J. Kraus, J.J. Gielen, M. Lommel, G. Weyens, B. Wahl, A. Schechert, O. Nilsson, C. Jung, T. Kraft, and A.E. Müller, 2012: The role of a pseudo-response regulator gene in life cycle adaptation and domestication of beet. *Curr Biol* 22, 1095-101.
- Priest, H.D., S.A. Filichkin, and T.C. Mockler, 2009: Cis-regulatory elements in plant cell signaling. *Curr Opin Plant Biol* 12, 643-649.
- Reeves, P.A., Y. He, R.J. Schmitz, R.M. Amasino, L.W. Panella, and C.M. Richards, 2007: Evolutionary conservation of the *FLOWERING LOCUS C*-mediated vernalization response: evidence from the sugar beet (*Beta vulgaris*). *Genetics* 176, 295-307.
- Schneider, K., D. Kulosa, T.R. Soerensen, S. Mohring, M. Heine, G. Durstewitz, A. Polley, E. Weber, Jamsari, J. Lein, U. Hohmann, E. Tahiro, B. Weisshaar, B. Schulz, G. Koch, C. Jung,

and M. Ganal, 2007: Analysis of DNA polymorphisms in sugar beet (*Beta vulgaris* L.) and development of an SNP-based map of expressed genes. *Theor Appl Genet* 115, 601-15.

Statistisches Bundesamt, 2013: Land- und Forstwirtschaft, Fischerei.

Wachstum und Ernte -Feldfrüchte. Fachserie 3 Reihe 3.2.1 Artikelnummer: 2030321132014.

The *Beta vulgaris* Ressource, 2013: <http://bvseq.molgen.mpg.de/index.shtml>.

Tiwari, S.B., Y. Shen, H.C. Chang, Y. Hou, A. Harris, S.F. Ma, M. McPartland, G.J. Hymus, L. Adam, C. Marion, A. Belachew, P.P. Repetti, T.L. Reuber, and O.J. Ratcliffe, 2010: The flowering time regulator *CONSTANS* is recruited to the *FLOWERING LOCUS T* promoter via a unique cis-element. *New Phytologist* 187, 57-66.

Weißhaar, B., J. Dohm, A. Minoche, B. Schulz, T. Kraft, M. Wolf, D. Holtgraewe, and H. Himmelbauer, 2011: The draft genome sequence of sugar beet (*Beta vulgaris*). *Plant & Animal Genomes XIX Conference W563: Sugar Beet*.

Zakrzewski, F., T. Wenke, D. Holtgrawe, B. Weisshaar, and T. Schmidt, 2010: Analysis of a c0t-1 library enables the targeted identification of minisatellite and satellite families in *Beta vulgaris*. *BMC Plant Biol* 10, 8.

Zhou, D.X., 1999: Regulatory mechanism of plant gene transcription by GT-elements and GT-factors. *Trends Plant Sci* 4, 210-214.

10 Curriculum vitae and publications

10.1 Curriculum Vitae

Personal data

Name Nadine Dally
 Date of birth 27.08.1984
 Place of birth Hanover, Germany
 Marital status Single
 Nationality German

Education

1991/ 1995 "Grundschule Kreuzriede" elementary school, Hanover, Germany
 1995/ 1997 "Orientierungsstufe Entenfangweg" orientation stage, Hanover, Germany
 1997 "Gymnasium Lutherschule" grammar school, Hanover, Germany
 1997/ 2004 "Gymnasium Goetheschule" grammar school, Hanover, Germany
Degree: Abitur

2004/ 2009 Study of Bioinformatics at the University of applied science OOW Emden, Germany
Degree: Diploma
 Practical and diploma semester at the University of Veterinary Medicine Hannover, Foundation (Supervisor: Prof. Dr. Nina Baltes)
Practical thesis: "Construction and analysis of isogenic *Actinobacillus pleuropneumoniae* serotype 7 (strain AP76) deletion mutants based on autotransporter adhesins"
Diploma thesis: "Differentiation of several *Actinobacillus pleuropneumoniae* serotypes based on autotransporter adhesins using DNA sequencing and RFLP analysis"

Since 2009 PhD student at the Christian-Albrechts University of Kiel, Plant Breeding Institute

10.2 Publications

10.2.1 Articles

Pin, P.A., W. Zhang, S.H. Vogt, N. Dally, B. Buttner, G. Schulze-Buxloh, N.S. Jelly, T.Y. Chia, E.S. Mutasa-Gottgens, J.C. Dohm, H. Himmelbauer, B. Weisshaar, J. Kraus, J.J. Gielen, M. Lommel, G. Weyens, B. Wahl, A. Schechert, O. Nilsson, C. Jung, T. Kraft, and A.E. Müller, 2012: The role of a pseudo-response regulator gene in life cycle adaptation and domestication of beet. *Curr Biol* 22, 1095-101.

10.2.2 Oral presentations

*Dally, N., AE. Minoche, JC. Dohm, H. Himmelbauer, D. Holtgräwe, B. Weisshaar, AE. Müller, C. Jung. "New bolting genes from sugar beet (*B. vulgaris*) which control early bolting independent of *BvBTC1*." Plant and Animal Genome XXI, San Diego CA, 12.-16.01.2013.

*Dally, N., AE. Minoche, JC. Dohm, H. Himmelbauer, D. Holtgräwe, B. Weisshaar, AE. Müller, C. Jung. "New bolting genes from sugar beet (*B. vulgaris*) which control early bolting independent of *BvBTC1*." Flower Crop- SPP1530 Flowering Time Control, Cologne 22.01.2013.

Müller A, S. Vogt, W. Zhang, B. Büttner, A. Abou-Elwafa, G. Schulze-Buxloh, N. Dally, C. Jung. "A new perspective on floral transition and life cycle control: The view from a non-model species." "Plant Stress and Genomics" Conference of GPZ e.V., Halle 18.-20.09.2012

Jung, C., Frerichmann, S., Dally, N., Pfeiffer, N., Vogt, S., Müller, A. E. "Key regulators for flowering time control in a biennial crop species." *11. Botanikertagung*, Berlin September 18-23, 2011.

Tränkner, C., M. Kirchhoff, N. Dally, F. Kopisch-Obuch, C. Jung. "Gene expression of flowering time control genes in autumn sown beet". Genomics-based breeding, Symposium of GPZ e.V. Gießen 26.-28.10.2010.

Tränkner, C., M. Kirchhoff, N. Dally, F. Kopisch-Obuch, C. Jung. „Züchtung von Zuckerrüben für den Winteranbau. Pflanzenbiotechnologie in Deutschland – Wo stehen wir?“ Tagung des Arbeitskreises Deutsche In-vitro-Kulturen (ADIVK) und der Gesellschaft für Pflanzenbiotechnologie e.V., Leibniz Universität Hannover 13.-15.9.2010.

* Presented by N. Dally

10.2.3 Posters

*Dally N., J. Kraus, AE. Müller, C. Jung. „Modification of bolting behavior in biennial beets by constitutive expression of *BvBTC1*." Plant and Animal Genome XXI", San Diego (CA) 12.-16.01.2013.

K. Xiao, Dally N., Jung C. "Identification of bolting genes of sugar beet by NGS of genotypic bulks." Plant 2030 Status Seminar, Potsdam 06.-08.03.2013.

Müller, A., S. Vogt, W. Zhang, N. Dally, G. Schulze-Buxloh, M. Lommel, G. Weyens, M. Lefèbvre, B. Wahl, A. Schechert, C. Jung. „The genetic control of bolting in *Beta vulgaris*." ECPGR Working on Beta and WBN, Capelle-en-Pévèle, France 20.-22.06. 2012.

*Dally N., B. Büttner, AE. Minoche, JC. Dohm, H. Himmelbauer, AE. Müller, C. Jung. "Fine-mapping of the *B2* gene controlling annual bolting in beets (*Beta vulgaris*). "Plant Stress and Genomics Conference of GPZ e.V. Halle 18.-20.09.2012.

*Dally, N., B. Büttner, A. Müller, C. Tränkner, C. Jung. „Towards positional cloning of the *B2* gene controlling annual bolting in beets (*Beta vulgaris*).”Genomics-based breeding Symposium of GPZ e.V. Gießen 26.-28.10.2010.

* Presented by N. Dally

11 Declaration of own contribution

Chapter 2, 3 and 4 of this thesis are not published. The doctoral student Nadine Dally contributed to the chapters as follows:

Chapter 2: Map based cloning of *BvBBX19*, a DOUBLE B-BOX TYPE ZINC FINGER gene from the *B2* locus on chromosome 9

Designing experiments: 100 %

Results: 100 %

Writing the chapter: 100 %

Discussion and revision: 100 %

Chapter 3: Functional analysis of the *BOLTING TIME CONTROL 1 (BTC1)* gene I: transformation into sugar beet

Designing experiments: 100 %

Results: 85 %

Writing the chapter: 100 %

Discussion and revision: 100 %

Chapter 4: Functional analysis of the *BOLTING TIME CONTROL 1 (BTC1)* gene II: transformation into *Arabidopsis thaliana*

Designing experiments: 100 %

Results: 100 %

Writing the chapter: 100 %

Discussion and revision: 100 %

12 Acknowledgements

Now it is time to express my deepest gratitude to all those who provided me the possibility to complete my thesis, and who made the past years to a wonderful time.

There are no proper words to convey my deep gratitude and respect for my PhD supervisor Prof. Dr. Christian Jung for the opportunity to conduct my doctoral thesis at the Plant Breeding Institute, and for the support throughout my thesis with his knowledge whilst allowing me the room to work in my own way. Especially, I thank you for the appreciation of my work.

I am greatly thankful for the supervision of Conny Tränkner and Andreas Müller. Their support, discussions and suggestions allowed me a fast incorporation into the plant research.

Friedrich, I thank you so much for your support, encouragement and foremost I thank you for being the person you are.

Further I would like to offer my special thanks to the company KWS Saatzucht GmbH and especially to Joseph Kraus for giving me the opportunity to present my data on an international conference, and for the comprehensive collaboration.

Another special thank goes to Daniela Holtgräwe from the University Bielefeld for all fruitful discussions, your help and for the nice moments we had during meetings.

The DFG is kindly acknowledged for financial support.

Hilke, Bettina, Antje, I am greatly thankful for your support during my project and especially Antje, for your commitment to my following project.

I like to convey my special thanks to special persons: Sebastian “the Lovely Guy”, Sarah “the Elk Woman”, Nozi “the Purple Lady”, Gina “the... (sorry Gina, I cannot say it here but I am sure that you know what I want to say)”, Sabrina “the Sunny Woman”, Martina “the Flower”, Nadine “the Höfti”, Conny “the Woman Who Grills A Single Sausage”, Steffi “the Hanover-Connection” and Yuan “the Coolest China-Woman”. Thank you, for your support, encouragement, friendship and the nice time with you.

A warm thank goes to my friends for their patience. You have been waiting for calls and visits for a so long time. But... can you hear me singing: “and now, the end is near ... “

Closing 100 tubes filled with leaf samples takes about 5 minutes for one person. Can you imagine how long it will take to close 12,000 tubes? This is just the beginning of the story but at this point I would like to express my sincere appreciation to ALL my colleagues! Without your support a project like this would have been impossible.

A special warm thank goes to the “green area”. In the laboratory, you can work as hard as you would like... if nobody cares about your plants you will never succeed. With this in mind and much much more, I thank Erwin, Bärbel, Cotschi, Kirsten, Thomas, Carlotta, Katharina, Victoria, Cornelius, Christopher, Felix and especially Moni.

Brigitte, Moni and Sigi to all of you I would like to show my deepest appreciation for your outstanding support in the lab, in the field, and in my life! I learned and will further learn a lot from you. Without your support, your patience, your encouragement and your engagement,

we would still be singing: “But we still haven` t found what we` re looking for”. I cannot thank you enough for all these things and much, much more.

I would like to show my GREATEST appreciation to Nina. I am trying, but I am not able to say or even write anything, otherwise I will start crying.... (too late). I will miss you, my “Antagonist”!

Last but not least, I like to express wholehearted thanks to my family. You cannot imagine how glad I am to have such a family. You believed in me and encouraged me at any time. I thank you for all the love and support you gave me.

“Dally, du weißt doch, kein Bier vor Vier”



Monika Bruisch, May 10, 2012 (2:47 AM)

13 Erklärung

Ich versichere an Eides statt, dass ich bis zum heutigen Tage weder an der Christian-Albrechts-Universität zu Kiel noch an einer anderen Hochschule ein Promotionsverfahren endgültig nicht bestanden habe oder mich in einem entsprechenden Verfahren befinde.

Kiel, _____
(Datum)

(Unterschrift Kandidat)

Ich versichere an Eides statt, dass ich die Inanspruchnahme fremder Hilfen aufgeführt habe, sowie, dass ich die wörtlich oder inhaltlich aus anderen Quellen entnommen Stellen als solche gekennzeichnet habe.

Kiel, _____
(Datum)

(Unterschrift Kandidat)