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**Genetic mapping of flowering time genes and functional
characterisation of an *FVE* homologue from sugar beet**

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List of Abbreviations

°C	degree Celsius
χ^2	chi square
-10PEHVPSBD	-10 promoter element found in the <i>Hordeum vulgare psbD</i> gene promoter
<i>ACGI</i>	<i>ALTERED COLD-RESPONSIVE GENE EXPRESSION 1</i>
AFLP	amplified fragment length polymorphism
ANOVA	analysis of variance
<i>API</i>	<i>APETALA 1</i>
<i>At</i>	<i>Arabidopsis thaliana</i>
<i>ATH1</i>	<i>ARABIDOPSIS THALIANA HOMEODOMAIN 1</i>
<i>ATX1</i>	<i>ARABIDOPSIS TRITHORAX 1</i>
<i>B</i>	bolting locus from <i>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>
BAC	bacterial artificial chromosome
BBCH	Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie
BBH	bidirectional best hit
BLAST	basic local alignment search tool
bp	base pair
BSA	bulked segregant analysis
<i>Bv</i>	<i>Beta vulgaris</i>
BvGI	<i>Beta vulgaris</i> Gene Index
CAF1C	subunit C of chromatin assembly factor 1
<i>CAL</i>	<i>CAULIFLOWER</i>
CAPS	cleaved amplified polymorphic sequence
CaMV	Cauliflower mosaic virus
CBF	CRT/DRE-binding factor
<i>CCA1</i>	<i>CIRCADIAN CLOCK ASSOCIATED 1</i>
CCT	<i>CO</i> , <i>CO</i> -like, <i>TOC1</i>
cDNA	complementary DNA
CFIA	Canadian Food Inspection Agency
<i>CiMFL</i>	<i>Cichorium intybus FLOWERING LOCUS C-like</i>
cM	centiMorgan
<i>CO</i>	<i>CONSTANS</i>
Col-0	Columbia-0 (<i>Arabidopsis thaliana</i> ecotype)
<i>CONZ1</i>	<i>CONSTANS OF ZEA MAYS 1</i>
COR	cold responsive
CRT	C-repeat
<i>CZS</i>	<i>C2H2 ZINC FINGER-SET DOMAIN</i>
DNA	deoxyribonucleic acid
DRE	dehydration responsive element
DREB	DRE-binding protein
<i>drm1</i>	<i>developmentally retarded mutant 1</i>
dt	one tenth of a ton
DTB	days till bolting
E	EMS mutant
<i>E. coli</i>	<i>Escherichia coli</i>
<i>EF2</i>	<i>ELONGATION FACTOR 2</i>
<i>EFS</i>	<i>EARLY FLOWERING IN SHORT DAYS</i>
e.g.	exempli gratia

List of Abbreviations

<i>ELF</i>	<i>EARLY FLOWERING</i>
EMS	ethyl methanesulfonate
EST	expressed sequence tag
et al.	et alii
F ₁	first generation after cross
F ₂	second generation after cross
<i>FCA</i>	<i>FLOWERING LOCUS CA</i>
Fig.	figure
<i>FLC</i>	<i>FLOWERING LOCUS C</i>
<i>FLD</i>	<i>FLOWERING LOCUS D</i>
<i>FLK</i>	<i>FLOWERING LOCUS KH DOMAIN</i>
<i>FPA</i>	<i>FLOWERING LOCUS PA</i>
<i>FRI</i>	<i>FRIGIDA</i>
<i>FT</i>	<i>FLOWERING LOCUS T</i>
<i>FUL</i>	<i>FRUITFUL</i>
<i>FVE</i>	<i>FLOWERING LOCUS VE</i>
<i>fve</i>	<i>FLOWERING LOCUS VE mutant</i>
<i>FY</i>	<i>FLOWERING LOCUS Y</i>
GA	gibberellic acid
<i>Ga3ox1</i>	<i>GIBBERELLIN3-OXIDASE 1</i>
<i>GAPDH</i>	<i>GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE</i>
<i>GRP7</i>	<i>GLYCINE-RICH RNA-BINDING PROTEIN 7</i>
H ₀	null hypothesis
H3	histone H3
H3K4me3	histone H3 trimethylated at lysine 4
H3K9	histone H3 lysine 9
H3K27	histone H3 lysine 27
H4	histone H4
H4-bd	histone H4 binding protein
h	hour
ha	hectare
<i>HAC1</i>	<i>HISTONE ACETYLTRANSFERASE 1</i>
<i>Hd1</i>	<i>HEADING DATE 1</i>
<i>Hd3a</i>	<i>HEADING DATE 3A</i>
HDAC	histone deacetylase complex
<i>HEN4</i>	<i>HUA ENHANCER 4</i>
<i>Hv</i>	<i>Hordeum vulgare</i>
i.e.	id est
InDel	insertion deletion
INRNTPSADB	initiator element found in <i>Nicotiana tabacum psaDb</i> gene
kbp	kilo base pair
KH	K-homology
L.	Linné
LD	linkage disequilibrium
<i>LD</i>	<i>LUMINIDEPENDENS</i>
<i>LDL</i>	<i>LSD1-LIKE</i>
Ler	Landsberg erecta (<i>Arabidopsis thaliana</i> ecotype)
<i>LFY</i>	<i>LEAFY</i>
<i>LHP1</i>	<i>LIKE HETEROCROMATION PROTEIN 1</i>
<i>LHY</i>	<i>LATE ELONGATED HYPOCOTYL</i>
LOD	logarithm of odds
<i>Lp</i>	<i>Lolium perenne</i>

<i>LSD1</i>	<i>LYSINE-SPECIFIC DEMETHYLASE 1</i>
<i>Lt</i>	<i>Lolium temulentum</i>
<i>M</i>	marker allele
<i>M₁</i>	marker alleles present at the <i>B</i> locus in the EMS mutant
<i>M₂</i>	marker alleles present at the <i>B</i> locus in the annual parent
M2	second generation after mutagenesis
M3	third generation after mutagenesis
M4	fourth generation after mutagenesis
μl	micro-litre
MADS	<i>MCMI</i> , <i>AGAMOUS</i> , <i>DEFICIENS</i> and <i>SRF</i>
Mbp	mega base pair
ME	morning element
mRNA	messenger RNA
<i>MSI</i>	<i>MULTICOPY SUPPRESSOR OF IRA</i>
MYA	million years ago
n.a.	not applicable
nb	non bolting
NCBI	National Centre for Biotechnology Information
NF	normalization factor
ng	nanogram
NLS	nuclear localisation signal
ORF	open reading frame
<i>Os</i>	<i>Oryza sativa</i>
P	parental generation
PAF1	RNA polymerase II associated factor 1
PCR	polymerase chain reaction
<i>PEP1</i>	<i>PERPETUAL FLOWERING 1</i>
<i>PEP</i>	<i>PEPPER</i>
PHD	plant homeodomain
QTL	quantitative trait locus
R	recombination rate
<i>R, r</i>	<i>R</i> locus in <i>Beta vulgaris</i> ; <i>R</i> and <i>r</i> allele encoding red and green hypocotyls
RACE	rapid amplification of cDNA ends
RAPD	randomly amplified polymorphic DNA
<i>RbAp</i>	<i>RETINOBLASTOMA ASSOCIATED PROTEIN</i>
<i>REF6</i>	<i>RELATIVE OF EARLY FLOWERING 6</i>
RFLP	restricted fragment length polymorphism
<i>RIK</i>	<i>RS2-INTERACTING KH PROTEIN</i>
RNA	ribonucleic acid
RNAi	RNA interference
ROMA	representational oligonucleotide microarray analysis
RRM	RNA recognition motive
RT-PCR	reverse transcriptase polymerase chain reaction
RT-qPCR	real time quantitative polymerase chain reaction
<i>SHB1</i>	<i>SHORT HYPOCOTYL UNDER BLUE 1</i>
<i>SIY1</i>	<i>SILENE LATIFOLIA Y-GENE 1</i>
<i>SIX1</i>	<i>SILENE LATIFOLIA X-GENE 1</i>
SNP	single nucleotide polymorphism
<i>SOCI</i>	<i>SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1</i>
SORLIP	sequences over-represented in light-induced promoters
SORLREP	sequences over-represented in light-repressed promoters

List of Abbreviations

ssp.	subspecies
SSR	simple sequence repeat
<i>SUF4</i>	<i>SUPPRESSOR OF FRIGIDA 4</i>
suppl.	supplementary
<i>SVP</i>	<i>SHORT VEGETATIVE PHASE</i>
SWIRM	SWI3, RSC8 and MOIRA
<i>SWP1</i>	<i>SWIRM DOMAIN PAO PROTEIN 1</i>
T1	first generation after transformation
T2	second generation after transformation
T3	third generation after transformation
<i>Ta</i>	<i>Triticum aestivum</i>
Tab.	table
TAIR	The Arabidopsis Information Resource
TC	tentative consensus
<i>TFL1</i>	<i>TERMINAL FLOWER 1</i>
TNL	total leaf number
<i>TOC1</i>	<i>TIMING OF CAB1 EXPRESSION</i>
TSSP	transcription start site prediction
<i>TUB</i>	<i>TUBULIN</i>
UTR	un-translated region
<i>VIN3</i>	<i>VERNALIZATION INSENSITIVE 3</i>
<i>VIP</i>	<i>VERNALIZATION INDEPENDENCE</i>
<i>VRN</i>	<i>VERNALIZATION</i>
W	wild type
WD	tryptophan aspartame
WW	tryptophan tryptophan
YAC	yeast artificial chromosome
<i>ZCN8</i>	<i>Zea mays CENTRORADIALIS 8</i>
<i>Zm</i>	<i>Zea mays</i>

1 General introduction

1.1 The systematics and agricultural use of *Beta vulgaris*

Beta vulgaris belongs to the Amaranthaceae family (former Chenopodiaceae). The genus *Beta* comprises the two sections *Beta* and *Corollinae*. The section *Beta* is divided in *Beta vulgaris*, *B. macrocarpa*, and *B. patula*. *B. vulgaris* is further subdivided in wild sea beets (*B. vulgaris* ssp. *maritima*), wild beets (*B. vulgaris* ssp. *adanensis*) and cultivated forms (*B. vulgaris* ssp. *vulgaris*) (Kadereit et al. 2006). The wild sea beet *B. vulgaris* ssp. *maritima* is a perennial coastal subspecies with only a few inland populations, and can be found at the Mediterranean and European Atlantic coasts (Van Dijk et al. 1997). The wild beet *B. vulgaris* ssp. *adanensis* includes annuals and perennials in coastal regions ranging from Greece to Syria (Letschert et al. 1994). Within *B. vulgaris* ssp. *vulgaris*, one distinguishes four cultivated groups: leaf beet, garden beet, fodder beet, and sugar beet (Lange et al. 1999). The wild beet and the cultivated forms within the section *Beta* are cross compatible (Van Dijk et al. 1997; Desplanque et al. 1999).

Sugar beet is the only crop plant used for sugar production in Europe. The roots are used to produce white sugar, pulp, and molasses. In modern sugar beet cultivars, the root contains approximately 75.9% water, 2.6% non-sugars, 18.0% sugar, and 5.5% pulp (CFIA 2001). In 2009 sugar beet in Germany was cultivated on 384,000 ha yielding 675.5 dt/ha (Statistisches Bundesamt 2010). Sugar beet is sown in spring and harvested before winter.



Figure 1: Bolting and non-bolting individual of *Beta vulgaris*. Pictures were taken eight weeks after sowing.

Sugar beet is a biennial plant that forms a storage root and a leaf rosette in the first year and bolts and flowers after vernalization under long-day conditions in the second year (Hohmann et al. 2005; Fig. 1). Vernalization in sugar beet is achieved by exposure to cold temperatures for ten to 14 weeks over winter (Sadeghian and Johansson 1992; Boudry et al. 2002). For details about bolting time regulation in *B. vulgaris* see chapter 1.3. While beet production areas in Europe include central and northern regions, many seed production fields are located in southern France or northern Italy, where the relatively mild winters ensure maximum survival rates.

1.2 Flowering time regulation in *Arabidopsis thaliana*

The timing of the floral transition is of major importance for the reproductive success of the plant, therefore it is controlled by endogenous and exogenous signals to ensure flowering

under favourable conditions (Jung et al. 2007). In *Arabidopsis thaliana* flowering is regulated by four main pathways: the autonomous pathway, the photoperiodic pathway, the vernalization pathway, and gibberellic acid (GA). These four pathways merge in the regulation of the floral integrators *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*), *FLOWERING LOCUS T* (*FT*), and *FD*. The floral integrators activate the floral meristem identity genes *LEAFY* (*LFY*), *APETALA 1* (*AP1*), *CAULIFLOWER* (*CAL*), and *FRUITFUL* (*FUL*) (Fig. 2; Komeda 2004; Puterill et al. 2004; Massiah 2007; Michaels 2008).

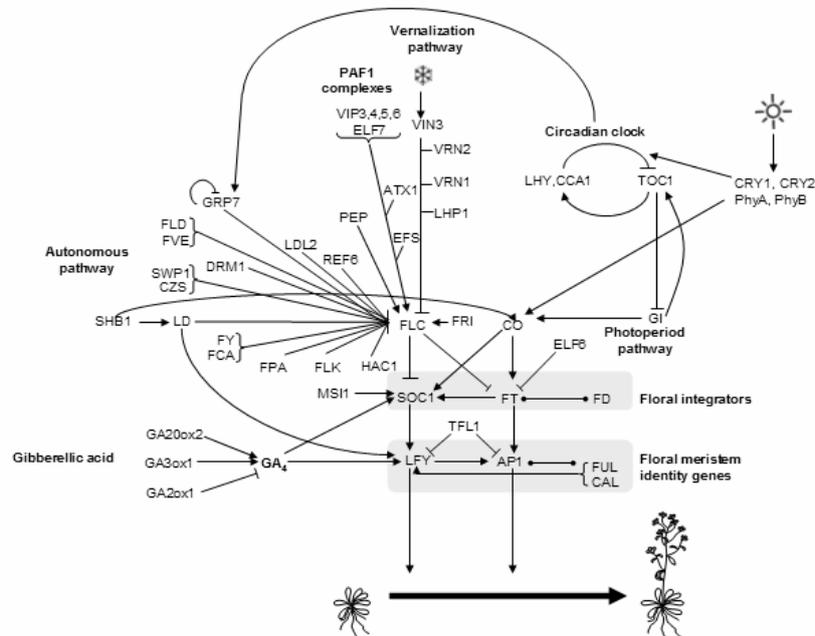


Figure 2: Floral transition in *Arabidopsis thaliana*. Arrows indicate a positive regulatory effect, while lines with bars at the end represent negative effects and lines with filled circles at the end indicate genetic and/or physical interaction. Exogenous signals (cold temperatures and photoperiod) are indicated by symbols.

The vernalization pathway regulates the plant's response to prolonged cold temperatures over winter. Vernalization can accelerate flowering in *Arabidopsis*, but plants can flower without vernalization (Martinez-Zapater and Somerville 1990). Vernalization accelerates flowering by down-regulation of the main repressor of flowering *FLOWERING LOCUS C* (*FLC*) through *VERNALIZATION* (*VRN*) genes (Gendall et al. 2001; Levy et al. 2002). *VRN1* is a plant specific DNA binding protein (Levy et al. 2002) and *VRN2* is a zinc finger protein homologous to polycomb group proteins (Gendall et al. 2001). *VERNALIZATION INSENSITIVE 3* (*VIN3*), a homeodomain (PHD) finger protein whose expression increases proportionally with the length of the cold treatment is necessary for the establishment of *FLC* repression (Sung and Amasino 2004). During vernalization *FLC* is silenced by histone deacetylation and methylation, and these modifications are mitotically stable (Bastow et al. 2004; Sung and Amasino 2004). *LIKE HETEROCHROMATIN PROTEIN 1* (*LHP1*) is needed for the maintenance of the repressed state of *FLC* after the end of the cold period (Sung et al. 2006).

The autonomous pathway comprises at least seven genes: *FLOWERING LOCUS CA* (*FCA*), *FLOWERING LOCUS D* (*FLD*), *FLOWERING LOCUS KH DOMAIN* (*FLK*), *FLOWERING LOCUS PA* (*FPA*), *FLOWERING LOCUS VE* (*FVE*), *FLOWERING LOCUS Y* (*FY*), and *LUMINIDEPENDENS* (*LD*) (Simpson 2004), which regulate *FLC* either at the RNA level by eliminating the accumulation of *FLC* mRNA, or epigenetically by histone modifications (Simpson 2004; Bäurle et al. 2007; Bäurle and Dean 2008). All mutants of this pathway are late flowering under long- and short-day conditions and the phenotypic effect of the mutation

can be overcome by vernalization (Simpson et al. 1999; Velvey and Michaels 2008). *LD* encodes for a homeodomain containing protein associated with DNA/RNA binding (Lee et al. 1994; Simpson 2005). At least one function of *LD* is to participate in the regulation of the meristem identity gene *LFY* (Aukerman et al. 1999). The *FCA* protein contains two RNA binding domains and one WW protein interaction domain, and is assumed to be involved in posttranscriptional regulation (Macknight et al. 1997). The *FCA* transcript is alternatively spliced at two positions leading to four different splice forms (α , β , γ , δ). The alternative splicing regulates the spatial and temporal expression of *FCA* and the amount of functional protein (Macknight et al. 2002). *FY* is an RNA 3' end processing factor and interacts with *FCA* (Simpson et al. 2003). *FPA* is a protein with three RNA recognition motifs (Schomburg et al. 2001) and *FLK* contains K homologous motifs for RNA binding (Lim et al. 2004). These proteins are therefore thought to regulate *FLC* at the RNA level. By contrast to *FLK*, the *FLK* paralogue *PEPPER* (*PEP*) is a floral repressor gene and positive regulator of *FLC* (Ripoll et al. 2009). *FLD* and the WD40 protein *FVE* form a complex which regulates *FLC* by chromatin modifications (Amasino 2004). *FLD* encodes a plant homologue of a component of histone deacetylase complexes in mammals (He et al. 2003), and shows similarities to polyamine oxidases and contains a SWIRM domain (Simpson 2005). For a detailed review of *FVE* function see chapter 1.2.1. In addition to these genes several new genes were assigned to the autonomous pathway more recently on the basis of their mutant phenotypes. Two of these new genes, *SWP1* (*SWIRM DOMAIN PAO PROTEIN 1*) and *CZS* (*C2H2 ZINC FINGER-SET DOMAIN*), interact with each other to form a co-repressor complex that recruits histone deacetylases to *FLC* (Krichevsky et al. 2007). *SWP1* is a homologue of *FLD* and is also referred to as *LSD1-LIKE 1* (*LDL1*). The Arabidopsis genome contains three homologues of *FLD*, *LSD1/SWP1*, *LDL2*, and *LDL3*, and *LDL1* and *LDL2* contribute to the repression of *FLC* (Jiang et al. 2007). Another gene is *REF6* (*RELATIVE OF EARLY FLOWERING 6*), a close relative to *ELF6* (*EARLY FLOWERING 6*). Despite their similar structure, the genes have different roles in flowering time control: *ELF6* acts in the photoperiodic pathway (Noh et al. 2004), while *REF6* represses *FLC* by modification of histone H4 at the *FLC* locus and therefore participates in chromatin regulation of *FLC* (Noh et al. 2004). The gene *SHB1* (*SHORT HYPOCOTYL UNDER BLUE 1*) plays a dual role in the autonomous and photoperiodic pathway. Under long-day conditions *SHB1* activates *CONSTANS*, while under short days it promotes *LD* expression to repress *FLC* (Zhou and Ni 2009). The *developmentally retarded mutant* *drm1* shows up-regulation of *FLC* expression and a pleiotropic phenotype with slower growth and abnormal floral organs, but the mutated gene has not yet been identified (Zhu et al. 2005). *GRP7* (*GLYCINE-RICH RNA-BINDING PROTEIN 7*), an RNA binding protein, negatively auto-regulates its own RNA. This negative regulation is mediated by alternative splicing, similar to the auto-regulation of *FCA* (Streitner et al. 2008). Finally, a mutation in *HAC1* (*HISTONE ACETYLTRANSFERASE 1*) leads to pleiotropic developmental effects in addition to late flowering. The effect of *HAC1* on flowering is mediated by epigenetic modification of factors acting upstream of *FLC* (Deng et al. 2007).

The key gene of the photoperiodic pathway, which regulates the effect of daylength on flowering time, is *CONSTANS* (*CO*). Arabidopsis is a facultative long-day plant that flowers more rapidly under long-day conditions but will flower in short days, too (Koornneef et al. 1998). *CO* shows common characteristics with zinc finger transcription factors and is critical to promote flowering under long-days (Puterill et al. 1995). *CO* belongs to a family with 17 members divided into three groups. All genes share a CCT (*CO*, *CO*-like, *TOCI*) domain. The genes are assigned to groups largely according to the occurrence of B-box zinc finger domains, with group I (including *CO*) having two B-boxes, group II one B-box, and group III one B-box and a second zinc finger domain (Griffiths et al. 2003). In Arabidopsis, light regulation of flowering is mediated by the stabilization of *CO* protein by light. Flowering is

only triggered when high levels of *CO* expression coincide with the external light signal, resulting in induction of the floral integrator gene *FLOWERING LOCUS T (FT)* by CO protein (Imaizumi and Kay 2006). The FT protein is a likely component of the long-sought florigen that moves from the leaves to the shoot apex (Corbesier et al. 2007). There it forms a heterodimer with FD and activates *SOC1* and *API* to induce flowering (Zeevaart 2008). The *FT* homologue *TERMINAL FLOWER 1 (TFL1)* acts independently of *FT* in flowering time regulation. *TFL1* is in contrast to *FT* a repressor of flowering (Hanzawa et al. 2005). The floral integrator gene *SOC1* is needed to prevent premature differentiation of the floral meristem, and mainly regulates *LFY*. *SOC1* is regulated by *CO* and *FLC* via different promoter regions (Hepworth et al. 2002). In addition, *SOC1* can bind to promoters of CBF (C-repeat/dehydration responsive element (DRE)-binding factor) genes *in vivo* and therefore might also negatively regulate the cold response pathway (Lee and Lee 2010). *SOC1* together with *FUL* is also involved in determining the life span of *Arabidopsis* (Melzer et al. 2008).

The fourth major pathway in flowering time regulation is mediated by gibberellins (GA). This phytohormone promotes flowering under long- and short-day conditions but appears to be more important under short days (Parcy 2005). The regulation of flowering is mediated by the active form GA₄ through the activation of *LFY* and *SOC1* (Parcy 2005; Eriksson et al. 2006; Massiah 2007). GA₃ and GA₂₀ oxidases are involved in the synthesis of bioactive GA, while GA₂ oxidase inactivates GA (Schwechheimer 2008).

Besides regulation by these major pathways, floral transition is repressed by the PAF1 (RNA polymerase II associated factor 1) complex. The PAF1 complex recruits the histone methyltransferases *EARLY FLOWERING IN SHORT DAYS (EFS)* and *ARABIDOPSIS TRITHORAX 1 (ATX1)* to *FLC*. This leads to an enrichment of H3K4me₃ of the *FLC* chromatin which is associated with actively transcribed genes. The PAF1 complex consists of *EARLY FLOWERING 7 (ELF7)*, *ELF8/VERNALIZATION INDEPENDENCE 6 (VIP6)*, *VIP3*, *VIP4*, and *VIP5* (Ausin et al. 2005; Adrian et al. 2009; He 2009).

Another repressor of flowering is *FRIGIDA (FRI)* (Johanson et al. 2000). *FRI* delays flowering by activating *FLC* expression and therefore has an antagonistic effect to vernalization (Shindo et al. 2005). Non-functional alleles of *FRI* lead to early flowering, whereas functional alleles of *FRI* and *FLC* confer a winter annual growth habit (Shindo et al. 2005; Scarcelli and Kover 2009).

Flowering time is also regulated by the circadian clock. Circadian clock control involves input pathways which synchronize the clock with the environment, the core oscillator which generates the ~ 24 hour rhythm, and output pathways which regulates numerous biological processes. The input pathway is mediated by light perception of the photoreceptors and temperature. The core oscillator is composed at least of the three genes *CIRCADIAN CLOCK ASSOCIATED 1 (CCA1)*, *LATE ELONGATED HYPOCOTYL (LHY)*, and *TIMING OF CAB1 EXPRESSION (TOC1)* forming a feed back loop (Ausin et al. 2005; Bäurle and Dean 2006). Mutation in *TOC1*, *CCA1*, and *LHY* lead to early flowering (Boss et al. 2004). One of the output pathways is involved in flowering time regulation through *GIGANTEA*, which activates *CO* (Ausin et al. 2005).

1.2.1 The autonomous pathway gene *FVE*

FVE (FLOWERING LOCUS VE) also named *ACG1 (ALTERED COLD-RESPONSIVE GENE EXPRESSION 1)*, Kim et al. 2004) and *MSI4 (MULTICOPY SUPPRESSOR OF IRA 4)*, Ausin et al. 2004) was cloned by a map-based cloning approach and encodes a protein with homology to retinoblastoma associated proteins (Ausin et al. 2004). The protein had previously been described as WD40 repeat protein able to bind metal ions (Kenziar and Folk 1998). *A.*

thaliana FVE consists of 507 amino acids, contains a nuclear localisation signal, six WD domains (Ausin et al. 2004), and upstream of the first WD domain a CAF1C/H4-bd domain (subunit C of chromatin assembly factor 1/histone H4 binding protein; Murzina et al. 2008). WD domains are involved in the formation of protein complexes by serving as binding sites for protein-protein interactions (Nocker and Ludwig 2003). Similar to its human homologue RbAp48, FVE is able to bind at least three atoms of zinc or ions with similar coordination chemistry, although the amino acid sequences do not suggest any known metal binding motifs (Kenzior and Folk 1998). The ability to bind metal ions has been implied in protein complex formation (Kenzior and Folk 1998). FVE was shown to be expressed in Arabidopsis in all tissues analysed i.e. roots, leaves, stem, and flowers (Ausin et al. 2004; Morel et al. 2009) especially in dividing cells (Morel et al. 2009) with the exception of ovules and pollen (Choi et al. 2009). FVE expression is not affected by photoperiod, low temperature or mutations in other autonomous pathway genes (*ld*, *fpa*, *fca*, *fy*) (Ausin et al. 2004). Grafting experiments show that the mRNA of FVE is phloem mobile and that it therefore may act as a systemic floral regulator (Yang and Yu 2010), as was shown for the florigen component *FT* (Turck et al. 2008; Zeevaart 2008).

FVE controls flowering as part of an histone deacetylase complex (HDAC) together with *FLD* (s. 1.2), although FVE itself seems not to bind DNA (Amasino 2004). In addition to controlling *FLC*, FVE and *FLD* play a widespread role in chromatin silencing and act in a target specific manner (Bäurle and Dean 2008). FVE also plays a role in DNA methylation as shown by a loss of DNA methylation at *AtMu1* in the *fve* mutant (Bäurle and Dean 2008). FVE seems to play a role in silencing of some loci (e.g. *AtSN1*, *AtMu1*) associated with high levels of DNA methylation, but it is unlikely that the autonomous pathway has a major role in gene regulation by DNA methylation (Velvey and Michaels 2008). Most autonomous pathway genes are required for the suppression of the transposons *AtSN1* and *AtMu1* (Velvey and Michaels 2008). Although FVE regulates *FLC* independently of the RNA binding protein FCA, FCA seems to act through FVE to repress the transposon *AtMu1* (Bäurle and Dean 2008).

Mutations at the FVE locus were initially isolated by Hussein (1968). Mutation in FVE leads to a late-flowering phenotype in all photoperiods, with a stronger delay under short-day than under long-day conditions (Martinez-Zapater et al. 1995). Short-day conditions, like *fve* mutations, lead to a delay in flowering, but the effect of short days and *fve* mutation are additive (Martinez-Zapater et al. 1995), suggesting that genetic regulation of flowering time by FVE is not epistatic to the photoperiod pathway. The phenotypic effect of FVE mutations can be overcome by vernalization (Ausin et al. 2004). In addition the phenotype can be partially overcome by exposure to far red light (Martinez-Zapater et al. 1995). Vernalization and photoperiod have a similar effect on flowering time and can substitute each other. In vernalization-sensitive ecotypes and late-flowering mutants like *fve* low temperature treatment eliminates light quality and day-length responsiveness (Bagnall 1993). Mutation of FVE also leads to a lengthening of all morphological stages of development (Martinez-Zapater et al. 1995) and therefore an increase in rosette leaf number (Morel et al. 2009). In addition the mutant plants produce more co-inflorescences, larger flowers, and more seeds, but no obvious changes in overall meristem organization were observed (Morel et al. 2009). Although mutations in FVE lead to a delay in flowering the overexpression of FVE is not sufficient to significantly alter flowering time (Ausin et al. 2004). Up to now six mutant alleles of *fve* have been described: *fve-1* and *fve-2* are EMS induced amino acid exchanges in the Ler background (Ausin et al. 2004), *fve-3* and *fve-4* are fast-neutron induced premature stop codons in the Col background (Ausin et al. 2004), *acg1/fve-5* too has a premature stop codon (Kim et al. 2004), and *fve-6* was generated by T-DNA insertion (Morel et al. 2009). The late-flowering mutant phenotype was removed by the introduction of a null allele of *flc* under long- and short-day conditions, showing that the altered expression of *FLC* is the reason for

the delayed flowering (Michaels and Amasino 2001). In all *fve* mutants, *FLC* expression is increased (Ausin et al. 2004; Kim et al. 2004; Morel et al. 2009) and in *fve-3*, *fve-5*, and *fve-6* it was shown that the expression of *SOCI* and *FT* decreased (Kim et al. 2004; Morel et al. 2009), which leads to the delayed flowering. Furthermore, the gene is expressed in a similar pattern like *FLC* in reproductive tissues and the repression of *FLC* in the endosperm is at least partly mediated by *FVE* (Choi et al. 2009). The *FLC* allele present in the Columbia background (*FLC*-Col) in a homozygous state leads to an enhancement of the late flowering phenotype in *fve* heterozygous plants, indicating a synergistic interaction of *FLC* and *FVE* (Sanda and Amasino 1996).

The histone methyltransferase *EFS* (*EARLY FLOWERING IN SHORT DAYS*), inhibits flowering by activation of *FLC* through di- and trimethylation of H3K36 (Xu et al. 2008). *EFS* acts epistatically to *FVE* (and *FCA*) and *FVE* antagonizes the negative effect of *EFS* on flowering (Soppe et al. 1999). *TFL1* delays flowering by repressing the repression of *EFS* by *FVE* (Fig. 3; Ruiz-Garcia et al. 1997; Soppe et al. 1999). Furthermore, autonomous pathway mutant phenotypes are affected by *ATH1* (*ARABIDOPSIS THALIANA HOMEBOX 1*), an activator of *FLC*. The combination of ectopic expression of *ATH1* and a loss-of-function *fve* mutation results in lethality (Proveniers et al. 2007).

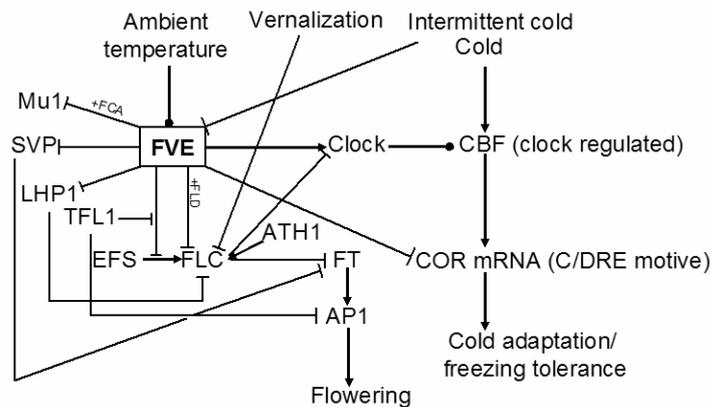


Figure 3: *FVE* regulatory network. Arrows indicate a positive regulatory effect/shortening of the clock period, while lines with bars at the end represent negative effects/extension of the clock period. Lines with filled circles at the end indicate unknown interactions (activation or repression).

Analysis of double mutants show that the autonomous pathway consists of at least two sub-pathways, with *FPA* and *FVE* acting in one epistatic group, and *FCA* and *FY* in another (Koornneef et al. 1998; Rouse et al. 2002). Although *FPA* and *FVE* act in the same sub-pathway, *fve* in contrast to *fpa* has no large effect in combination with *ft* or *fe* on flowering time, but has a stronger effect on *FLC* expression than *fpa* alone (Koornneef et al. 1998; Rouse et al. 2002). The *fpa fve* double mutant shows different pleiotropic phenotypes that are not observed in the single mutants, e.g. reduced chlorophyll content, growth rate or fertility, and appears to be *FLC* independent (Velvey and Michaels 2008). Interestingly, this double mutant did not flower in the absence of vernalization, but the vernalization response appears to be normal. The phenotype of the double mutant indicates that the autonomous pathway has roles independently of flowering time regulation in growth and development (Velvey and Michaels 2008).

Beside its role in flowering time regulation, *FVE* has further functions in the response to intermittent cold and ambient temperature and as meristem regulator. It is a negative regulator of the CBF/DREB (C/DRE-binding factor/DRE-binding protein) pathway, which mediates cold response and cold acclimation (Fig. 3). The C/DRE-binding factor (C-repeat/dehydration-responsive element) is an essential component of this pathway (Kim et al. 2004). Cold acclimation is mainly regulated at the level of gene expression, and histone

acetylation plays a role in cold adaptation (Catala and Salinas 2008). *FVE* is a negative regulator of the CBF regulon. The enhanced cold acclimation capability in the *acg1* mutant correlates with a stronger induction by cold of CBF and target genes (Catala and Salinas 2008). The cold response of *FVE* is independent of *FLC* function (Kim et al. 2004). The dual role of *FVE* in cold and flowering time regulation may be an adaptation to the fluctuating temperatures in spring (Amasino 2004). *FVE* can sense intermitted cold and delay flowering by regulating *FLC* (Kim et al. 2004). *FLD* seems not to have a function in cold regulation, this indicates that *FVE* could function in two different HDAC complexes: in one with *FLD* to regulate flowering and in one without *FLD* to regulate cold response (Amasino 2004; Franklin and Whitelam 2007). The mutant *fve-1* flowers at the same time independently of ambient temperature, while in the wild type flowering is accelerated by elevated temperatures, indicating that ambient temperature is perceived with the help of *FVE*. In addition *FCA* is required for temperature sensing (Blazquez et al. 2003; Balasubramanian et al. 2006; Franklin and Whitelam 2007). In the thermosensory pathway the MADS domain transcription factor *SVP* (*SHORT VEGETATIVE PHASE*), which interacts with *FLC* (Li et al. 2008), is regulated by *FVE* and mediates temperature signalling (Lee et al. 2007). Although *FCA* and *FVE* have partly redundant role in the regulation of flowering time they are not redundant in temperature sensing (Blazquez et al. 2003). *FVE* and *FCA* seem to control flowering in response to temperature changes independently of *FLC*, indicating different roles of the two genes in temperature dependent and independent flowering time control (Blazquez et al. 2003). Independent of *FLC* too is the regulation of the circadian clock, in the *fve* mutant the period of the circadian clock period is increased by more than one hour (Salathia et al. 2006). Moreover *FVE* is a meristem regulator. It is expressed in growing organs and controls the timing and speed of differentiation depending on the organ type. This function is mediated by the negative regulation of *LHP1* and *FLC* during development (Morel et al. 2009). Mutations in *fve* lead to an increase in mature organ size and in biomass, the biomass increase is up to eightfold, therefore *FVE* could be a tool for engineering yield/biomass (Morel et al. 2009).

FVE/MSI4 is part of a small gene family in Arabidopsis. The *MSI* family has five members, divided in two subgroups (*MSI1-3* and *MSI4-5*) (Ausin et al. 2004). *MSI* proteins are part of complexes involved in chromatin assembly and histone modifications (Ausin et al. 2004). *MSI5* shares 83% nucleotide identity with *MSI4* (Hennig et al. 2003). The *MSI* genes are homologous to yeast *MSI* (MULTICOPY SUPPRESSOR OF IRA 1) and mammalian retinoblastoma-associated proteins RbAp64 and RbAp48 (Ausin et al. 2004). The protein family is highly conserved in eukaryotes (Ach et al. 1997). One Arabidopsis homologue of *FVE*, *MSI1*, also regulates flowering. *MSI1* mutants are late flowering, but in contrast to *FVE*, *MSI1* promotes flowering independently of *FLC* (Bouveret et al. 2006). *FVE* and *MSI1* have non-redundant roles in the regulation of flowering and act in different genetic pathways: *MSI1* activates *SOC1* expression independently of *FLC*, and *FVE* acts through *FLC* to activate *SOC1*. Both genes regulate their targets by chromatin modifications (Bouveret et al. 2006). *MSI1* establishes di-methylation and H3K9 acetylation marks that lead to the transcription of *SOC1* (Bouveret et al. 2006). *MSI1* is also involved in gametophyte and seed development (Köhler et al. 2003; Bouveret et al. 2006).

Homologues to *FVE* have been described in other plant species like rice, white campion and maize (Baek et al. 2008; Delichere et al. 1999, Rossi et al. 2001). Like *AtFVE* *OsFVE* is ubiquitously expressed. The function of *OsFVE* is partly conserved: overexpression under the 35S promoter can rescue the late-flowering phenotype of *fve-3* by down-regulating *FLC*, but not the cold response (Baek et al. 2008). Another homologue to *FVE* was identified in *Silene latifolia*, a dioecious plant with XY male and XX female flowers. *FVE* is homologous to *SIY1* (*SILENE LATIFOLIA Y-GENE 1*), one of the first genes cloned from a plant Y chromosome. *SIY1* and the X-chromosome-localized *SIX1* encode almost identical proteins with WD

domains. Both genes are like *AtFVE* expressed in actively dividing cells (Delichere et al. 1999). An orthologue of *FVE* was isolated from *Zea mays* and named *ZmRbAp1*, the protein product of which is able to bind acetylated histones H3 and H4. Like the Arabidopsis gene, *ZmRbAp1* is expressed in all tissues analysed (Rossi et al. 2001). Hecht et al. (2005) identified homologues of autonomous pathway genes in *Pisum sativum*, *Medicago trunculata*, *Lotus japonicus* and *Glycine max* by sequence homology, among others they identified an EST with homology to *FVE* in soybean, lotus and medicago, and isolated a full-length cDNA of pea.

1.3 Bolting time regulation in species of the genus *Beta*

Annuality (bolting without a requirement for vernalization) in beet is controlled by a dominant gene termed *B* (Abegg 1936), currently known as 'bolting gene'. The *B* gene was first described to be present in beet by Munerati (1931). In addition to the *B* locus Owen et al. (1940) described *B'* as a gene for early bolting tendency. Linkage analysis between the two genes and the gene *R* for hypocotyl colour suggested that *B'* is allelic to *B*. Another locus for bolting control was described by Savitsky (1952), who showed that the *lb* allele is responsible for late-bolting tendency after vernalization and linked to the gene *M* for monogermity located on chromosome IV (McGrath et al. 2007). Bolting behaviour in annual beets is controlled by *B* under long days, but under short days it is altered by genes for long-day requirement and one of them was suggested to be closely linked to the *B* gene and to form a complex for annuality (Abe et al. 1997). At the Plant Breeding Institute of the Christian-Albrechts-University of Kiel, a positional cloning project was initiated around 15 years ago to clone and characterize the bolting gene *B* of *B. vulgaris*. The *B* gene was mapped by RFLP and AFLP mapping to chromosome II between markers P04_B193 and P05_b162 (Boudry et al. 1994; El-Mezawy et al. 2002) and a physical map was constructed (Hohmann et al. 2003; Gaafar et al. 2005). In the absence of *B*, induction and timing of flowering depends on vernalization in addition to photoperiod and physiological development. All beets need long-day conditions to initiate flowering (Owen et al. 1940; Fife and Price 1953; Abe et al. 1997). The term photo-thermal induction of bolting was coined to describe the importance of both daylength and temperature on bolting (Owen et al. 1940). It is worth noting that beets are susceptible to de-vernalization, i.e. the promotive effect of vernalization can be neutralized by exposure to temperatures above 20°C directly after vernalization (Margara 1968; Smith 1982; Van Dijk 2009).

Seeds of an annual accession were mutagenised with EMS and 0.5% of the offspring showed altered bolting behaviour. Five lines bolted only after vernalization and therefore behave like biennials. In four additional lines, bolting was delayed by one to four weeks in comparison to the early bolting genotype (Hohmann et al. 2005). Therefore a single mutation may be sufficient to convert an annual beet into a biennial. All mutant lines are useful tools for the study of the annual habit in beets.

Heritability of bolting was estimated using three sets of full-sibs derived from diploid sugar beets that varied in bolting behaviour developed by a factorial cross design. The bolting character is highly heritable with a narrow sense heritability estimated between 0.93 and 0.96. The largest proportion of the genetic variance is due to additive effect, but non-additive effects are involved, too (Sadeghian and Johansson 1992). Environmental and genetic factors have an impact on the penetrance of the *B* gene. Plants heterozygous for *B* (*Bb*) normally bolt without vernalization, but may behave like biennials under unfavourable conditions, e.g. short days and low light intensity (Abegg 1936; Owen 1954; Owen and McFarlane 1958; Shimamoto et al. 1990; Sadeghian et al. 1993; Boudry et al. 1994; Abe et al. 1997). The complicated bolting behaviour of heterozygous plants can be caused by some interacting genes responsible for photo-induction of bolting (Abe et al. 1997).

The early bolting character is widely present among wild beets, but was successfully removed from cultivated forms by breeding. For the cultivation of sugar beet bolting in the first year is an undesirable character, because it decreases sugar yield and disturbs the mechanical harvest. On the other side early bolting and flowering are relevant to speed-up breeding and seed multiplication. In wild beets there is a north south cline for vernalization requirement and quantitative variation in vernalization requirement, with beets derived from more southern parts of the distribution area needing less vernalization than beets from the northern part. The *B* gene has a high occurrence in the Mediterranean region and is absent from the north (Van Dijk et al. 1997; Boudry et al. 2002). The differences in vernalization requirement are probably an adaptive response to the particular latitude (Boudry et al. 2002). In addition to the variation in vernalization requirement there is variation for day length sensitivity in sea beet (Van Dijk and Hautekèete 2007). Vernalization and day-length can at least partially compensate each other (Van Dijk et al. 1997; Van Dijk 2009).

In sugar beet gibberellins (GA) promote bolting, but cannot induce flowering (Mutasa-Göttgens and Hedden 2009). The induction of bolting by GA is independent of the *B* allele and the photoperiod. In beets dominant for the *B* allele bolting needs long-day conditions and the GA pathway is not necessary. However, in beets recessive for *B* GA promotes stem growth of vernalised plants (Mutasa-Göttgens et al. 2010).

1.4 Genetic and genomic resources of sugar beet

Sugar beet is a diploid species ($2n = 18$) with a nuclear DNA content (C-value) of 714-758 Mbp per haploid genome (Bennet and Smith 1976; Arumuganathan and Earle 1991). Presently the genome of the doubled haploid beet genotype KWS2330 is sequenced by the GABI project BeetSeq (<http://www.gabi.de/projekte-alle-projekte-neue-seite-144.php>). The full sequence is expected to be available by 2011.

Highly repetitive DNA sequences comprise ~ 60% of the beet genome (Flavell et al. 1974). The genome of *B. vulgaris* contains all major groups of repetitive elements dispersed over the nine chromosomes, and different families of satellites have been isolated from Beta species, some of them can be used as species-specific probes (Kubis et al. 1997; Dechyeva and Schmidt 2006; Menzel et al. 2006; Menzel et al. 2008; Zakrzewski et al. 2010).

A number of *B. vulgaris* genetic maps have been constructed with molecular markers. The marker types used include RFLP, RAPD, AFLP, SSR, SNP, and ROMA (representational oligonucleotide microarray analysis; Barzen et al. 1992; Pillen et al. 1992; Pillen et al. 1993; Boudry et al. 1994; Barzen et al. 1995; Uphoff and Wricke 1995; Hallden et al. 1996; Schondelmaier et al. 1996; Nilsson et al. 1997; Schumacher et al. 1997; Hansen et al. 1999; Weber et al. 1999; Rae et al. 2000; Möhring et al. 2004; Grimmer et al. 2007; Laurent et al. 2007; McGrath et al. 2007; Schneider et al. 2007; Lange et al. 2010). The map sizes range from 457 cM (Rae et al. 2000) to 1668.6 cM (Lange et al. 2010), with an average of 689 cM. In the most recent studies, Laurent et al. (2007) isolated simple sequence repeat (SSR) markers from two different genomic beet libraries and by data mining of public ESTs at GenBank and constructed a linkage map with the isolated SSR markers. They provided a set of markers for the assignment of linkage groups to the nine chromosomes of beet. McGrath et al. (2007) constructed a publically available framework map including 23 newly mapped SSR markers. A functional map was presented by Schneider et al. (2007). This map contained 524 loci and covers 664.3 cM. EST derived SNP markers were used to construct the map. Lange et al. (2010) showed the feasibility of the novel ROMA approach to generate genetic markers. They developed 511 new dominant markers based on BAC end sequences and ESTs and incorporated them in the map published by Schneider et al. (2007). As part of the beet genome sequencing project, two BAC sequences (in total 254 kbp, with an overlap of 92 kbp

between the two BACs) representing two different sugar beet haplotypes were compared. The sequences differ in the exons at the nucleotide level by only 1% and in the non-coding regions by 9% (Dohm et al. 2009).

Several beet BAC libraries have been constructed. Gindullis et al. (2001) constructed a BAC library of a *Patellifolia procumbens* introgression line consisting of 50,304 clones (8x genome coverage) which was used for centromer analysis of the wild beet (Gindullis et al. 2001). Hohmann et al. (2003) constructed a library with 57,600 clones representing an 8x genome coverage used for map-based cloning of the bolting gene *B* (Hohmann et al. 2003; Gaafar et al. 2005; s. above). A BAC library of a monosomic addition line of *B. corolliflora* in *B. vulgaris*, harbouring 49,920 clones equivalent to 7.5x genome coverage was used for the identification of the alien chromosome (Fang et al. 2004). A further public library of the hybrid line USH20 consists of 36,863 clones, representing a genome coverage of 6.1x (McGrath et al. 2004). The library published by Hagihara et al. (2005), was constructed with the aim to clone the restorer-of-fertility gene *Rf1* by map based cloning and covers the beet genome 3.4x, arranged in 32,180 clones. The library published by Schulte et al. (2006) consists of 61,056 clones, represents a 7.5x genome coverage and was used for the construction of a physical map of a *P. procumbens* translocation in *B. vulgaris*. A further BAC library was constructed by Jacobs et al. (2009), which consists of 36,096 clones (5.7x genome coverage) and was used for the characterization of Beta centromeres.

Sugar beet ESTs are public available through GenBank

(<http://www.ncbi.nlm.nih.gov/genbank/>) at NCBI, including ~ 30,000 ESTs, and the beet gene index DFCI (<http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=beet>), release 3.0 (June 16, 2010) including 17,184 unique sequences. Genomic sequences of sugar beet are publically available via GenBank at NCBI (~ 66,000 sequences).

1.5 Aims and scientific hypotheses

The scientific hypothesis underlying part of this work was that at least some flowering time genes are conserved between model species, like *Arabidopsis thaliana*, and *Beta vulgaris*. Although the lineage leading to *B. vulgaris* diverged from that leading to *Arabidopsis* around 120 million years ago (Davies et al. 2004), this hypothesis is reasonable because evolutionary conservation of flowering time regulators of *A. thaliana* in other species has been shown (Albert et al. 2005; Hecht et al. 2005; Böhlenius et al. 2006; Jung and Müller 2009). Furthermore *FLC* and *CO* homologues were recently identified in *B. vulgaris*, providing first evidence for the conservation of flowering time regulation (Reeves et al. 2007; Chia et al. 2008). One objective of this work was the isolation and characterisation of previously unidentified homologues of floral transition genes in *B. vulgaris*.

Furthermore I assume that the EMS induced altered bolting behaviour of an annual beet (Hohmann et al. 2005; s. 1.3) is due to a mutation at the bolting locus *B* or at a second locus acting epistatically to *B*. Seeds of an annual beet genotype were treated with EMS and in the offspring beets were identified that bolt only after vernalization and therefore behave like biennials (Hohmann et al. 2005). To define if the *B* gene or an epistatic locus is mutated a co-segregation analysis was conducted. Furthermore, if a mutation is identified which does not co-localise with *B*, this mutation will be mapped.

The aims of the work were

- to generate a new physical map around the *B* gene by BAC analysis, BAC-derived marker development and BAC mapping,

- to clone an *FVE* homologue from sugar beet as a candidate gene for flowering time regulation in beet and characterise it by mapping, expression analysis and transformation into *Arabidopsis*,
- to map EMS mutations in biennial beets and
- to integrate the data from the candidate gene approach and the EMS mutation analysis towards a better understanding of bolting and flowering time control in sugar beet.

2 A survey of EMS-induced biennial *Beta vulgaris* mutants reveals a novel bolting locus which is unlinked to the bolting gene *B*

2.1 Abstract

Beta vulgaris is a facultative perennial species which exhibits large intraspecific variation in vernalization requirement and includes cultivated biennial forms such as the sugar beet. Vernalization requirement is under the genetic control of the bolting locus *B* on chromosome II. Previously, ethyl methanesulfonate (EMS) mutagenesis of an annual accession had yielded several mutants which require vernalization to bolt and behave as biennials. Here, five F₂ populations derived from crosses between biennial mutants and annual beets were tested for co-segregation of bolting phenotypes with genotypic markers located at the *B* locus. One mutant appears to be mutated at the *B* locus, suggesting that an EMS-induced mutation of *B* can be sufficient to abolish annual bolting. Co-segregation analysis in four populations indicates that the genetic control of bolting also involves previously unknown major loci not linked to *B*, one of which also affects bolting time and was genetically mapped to chromosome IX

2.2 Introduction

The species *Beta vulgaris* which comprises several cultivated forms including the sugar beet (*B. vulgaris* L. ssp. *vulgaris*) exhibits large intraspecific variation in vernalization requirement and life span, and includes annual accessions as well as long-lived, iteroparous perennials (Letschert 1993; Hautekèete et al. 2002). Vernalization requirement in wild beets (*B. vulgaris* L. ssp. *maritima*) follows a latitudinal cline, with beets from the southern part of the species' distribution area (the Mediterranean) generally behaving as annuals which do not require vernalization and bolt and flower in the first year. By contrast, wild beets from northern latitudes require vernalization but differ quantitatively in their degree of vernalization requirement (Van Dijk and Boudry 1991; Van Dijk et al. 1997; Boudry et al. 2002). Because bolting drastically reduces root yield, the occurrence of annual bolting in beet crops such as sugar beet has been strongly selected against during the breeding process. Sugar beet cultivars require vernalization to bolt and can thus be sown in spring and grown vegetatively until the beets are harvested in the fall. For seed production, plants are grown over winter (as biennials) and seeds harvested the following summer.

The annual habit in *B. vulgaris* was shown to be under the genetic control of a dominant Mendelian factor termed *B* (Munerati 1931; Abegg 1936), now commonly referred to as the 'bolting gene'. The manifestation of this trait, however, also depends on appropriate environmental conditions and may be influenced by additional, modifying genes (Abegg 1936; Owen et al. 1940; Owen 1954; Boudry et al. 1994; Abe et al. 1997). Owen et al. (1940) coined the term 'photothermal induction' to describe the inductive effects of low temperatures and long photoperiods on bolting in *B. vulgaris* and showed that these environmental cues also promote and accelerate bolting in annual accessions. Plants which are derived from crosses between annual and biennial beets and are heterozygous at the *B* locus (*Bb*) behave as annuals under favorable conditions but may bolt later (Munerati 1931; Abegg 1936). Heterozygotes may also fail to bolt in the first year under suboptimal photothermal conditions as they are present e.g. in late spring, summer or autumn sowings (Owen 1954; Boudry et al. 1994; Abe et al. 1997). Abe et al. (1997) suggested that a second gene closely linked to the *B* locus and regulating long daylength requirement may contribute to the complex control of bolting in heterozygotes. Furthermore, Owen et al. (1940) defined a locus for easy-bolting tendency (*B'*) in a biennial beet accession which does not bolt without prior vernalization under field conditions, but bolts easily and early without vernalization under relatively low

temperatures and long photoperiods in the greenhouse. On the basis of linkage data between the *B* locus and the *R* locus for hypocotyl color, and between *B'* and *R*, the authors concluded that *B'* is allelic to *B*. The *B* locus was mapped by RFLP- and high resolution AFLP-mapping to chromosome II (Boudry et al. 1994; El-Mezawy et al. 2002), and candidates for the bolting gene were recently identified by map-based cloning (Müller et al. unpublished data).

The genetic control of bolting and flowering is best understood in the dicot model species *Arabidopsis thaliana*. Four main regulatory pathways (the vernalization, photoperiod, autonomous, and gibberellic acid pathways) have been described which converge to regulate floral transition through a set of floral integrator genes (for review see Putterill et al. 2004; He and Amasino 2005; Bäurle and Dean 2006; Zeevaart 2008; Jung and Müller 2009; Michaels 2009). Several of the key regulatory genes, including, in particular, the floral integrator *FT* (*FLOWERING LOCUS T*) and the photoperiod pathway gene *CO* (*CONSTANS*) have been shown to be functionally conserved across taxa (Turck et al. 2008; Zeevaart 2008; Jung and Müller 2009). However, for angiosperm species which are only distantly related to *Arabidopsis*, such as the monocots, the general picture which emerged in recent years is that flowering time control often involves related genes and protein domains, but that the regulatory interactions between these genes and their precise function can vary. A prime example is the control of vernalization requirement and response in *A. thaliana* and in temperate cereals. The central regulator of vernalization requirement and response in *A. thaliana* is *FLC* (*FLOWERING LOCUS C*), a MADS box gene which acts as a repressor of flowering and is down-regulated during vernalization by a cascade of regulatory processes (He and Amasino 2005; Bäurle and Dean 2006; Michaels 2009). By contrast, wheat and other cereals do not appear to carry an *FLC*-like gene, and vernalization requirement and response is instead controlled by a regulatory feed-back loop which involves a promoter of flowering that is up-regulated during vernalization (*VRN1*) and a floral repressor gene (*VRN2*), in addition to the *FT*-ortholog *VRN3* (Trevaskis et al. 2007; Colasanti and Coneva 2009; Distelfeld et al. 2009; Greenup et al. 2009; Distelfeld and Dubcovsky 2010). While *VRN2* does not have a close homolog in *A. thaliana* but encodes a domain which is also found in *CO* and other floral regulators, *VRN1* is a MADS box gene with similarity to the *Arabidopsis* floral meristem identity gene *API* (*APETALAI*). *API* in *A. thaliana* also affects flowering time but in contrast to *VRN1* in cereals has not been associated directly with vernalization requirement or response (Yan et al. 2003, 2004; Alonso-Blanco et al. 2009) and differs from *VRN1* in regard to temporal and spatial expression profiles (Li and Dubcovsky 2008; Greenup et al. 2009).

In *B. vulgaris*, reverse genetic approaches have identified an *FLC*-like gene (*BvFLI*; Reeves et al. 2007) and a *CO*-like gene (*BvCOLI*; Chia et al. 2008). Consistent with a conserved role in repression of flowering, expression of *BvFLI* in sugar beet was shown to be down-regulated during vernalization, and overexpression of *BvFLI* in an early-flowering *flc* mutant delayed flowering in *Arabidopsis* (Reeves et al. 2007). Overexpression of *BvCOLI* in a late-flowering *Arabidopsis co* mutant resulted in up-regulation of *FT* and earlier flowering phenotypes similar to those of wild-type plants, which is consistent with a role as a floral inducer gene (Chia et al. 2008). However, despite these similarities, expression analyses of both *BvFLI* and *BvCOLI* also revealed marked differences to *FLC* and *CO*, respectively. In particular, and in contrast to the respective genes in *Arabidopsis*, repression of *BvFLI* expression is not maintained after vernalization but reverts to pre-vernalization levels, and the diurnal expression profile of *BvCOLI* differs from the dusk-phased rhythm of expression which is typical for *CO*.

The use of *A. thaliana* as a model species to understand bolting and flowering time control in *B. vulgaris* has several limitations. i) *A. thaliana* has only a facultative requirement for long

photoperiods and also flowers under short-day conditions (Koornneef et al. 1998a). *B. vulgaris*, by contrast, is an obligate long-day plant (Curth 1960; Lexander 1980). ii) Similarly, with the exception of annual accessions carrying a functional *B* allele, *B. vulgaris* has an obligate requirement for vernalization (Stout 1945), whereas vernalization requirement in *A. thaliana* is facultative. iii) In contrast to *A. thaliana*, *B. vulgaris* is prone to devernalization, i.e. the floral inductive effect of vernalization can be annihilated (under moderately high temperatures and short-day conditions) (Lexander 1980), and comprises iteroparous perennials with a repeated requirement for vernalization (Letschert 1993; Hautekèete et al. 2002). iv) *B. vulgaris* is a species in the Caryophyllales order of angiosperms which belongs to the core eudicots (Angiosperm Phylogeny Group 2009). The phylogenetic lineage leading to the Caryophyllales diverged from that leading to the core eudicot clades, rosids (which includes *A. thaliana*) and asterids, approximately 120 million years ago, i.e., in evolutionary terms, relatively shortly after the divergence of the dicot and monocot lineages approximately 140 million years ago (Davies et al. 2004). In conclusion, the phylogenetic distance between *B. vulgaris* and *A. thaliana* as well as differences in environmental requirements and life history traits of these two species may suggest that distinct regulatory genes and mechanisms may act in *B. vulgaris* which are not present or do not have corresponding functions in *A. thaliana*.

Here we used a forward genetic approach to further elucidate the genetic basis of bolting control in *B. vulgaris*. In a previous study, an annual genotype homozygous for the dominant bolting allele at the *B* locus was mutagenized by ethyl methanesulfonate (EMS) treatment and screened for phenotypic changes in bolting time. This screen and further propagation of mutagenized plants identified several non-segregating M3 families that behave like biennial accessions and require vernalization for induction of bolting (Hohmann et al. 2005). We hypothesized that either the *B* gene is mutated ('one-locus model'), or a second locus is mutated that acts epistatically to *B* and prevents annual bolting even in the presence of *B* ('epistatic locus model'). To distinguish between both models, we analyzed bolting phenotypes and *B* locus markers for co-segregation in five F₂ mapping populations derived from crosses between four biennial mutants and annual crossing partners. Assuming the mutation is recessive and the *B* locus determines annual bolting in the annual crossing partner, the following possible outcomes were expected. i) One-locus model: A 3:1 phenotypic segregation ratio for bolting behavior (bolting vs. non-bolting without vernalization), and complete co-segregation of *B* locus marker genotypes and bolting phenotypes (Fig. 4a). ii) Epistatic locus model: Assuming that the epistatic locus is not genetically linked to the *B* locus, the phenotypic segregation for bolting behavior would also be expected to occur at a ratio of 3:1. In contrast to the one-locus model, however, we would expect independent segregation of bolting phenotypes and *B* locus marker genotypes (Fig. 4b). We present evidence that, in one of the four mutants analyzed, the *B* locus region is mutated. Furthermore, in one population segregating for bolting behavior, we identified and genetically mapped a novel major bolting locus which is unlinked to *B* and appears to act epistatically to *B*. Co-segregation analysis of the remaining populations suggests the presence of at least one additional bolting locus which acts independently of *B*.

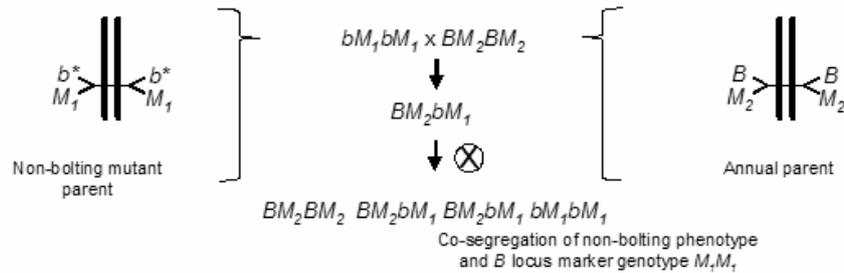
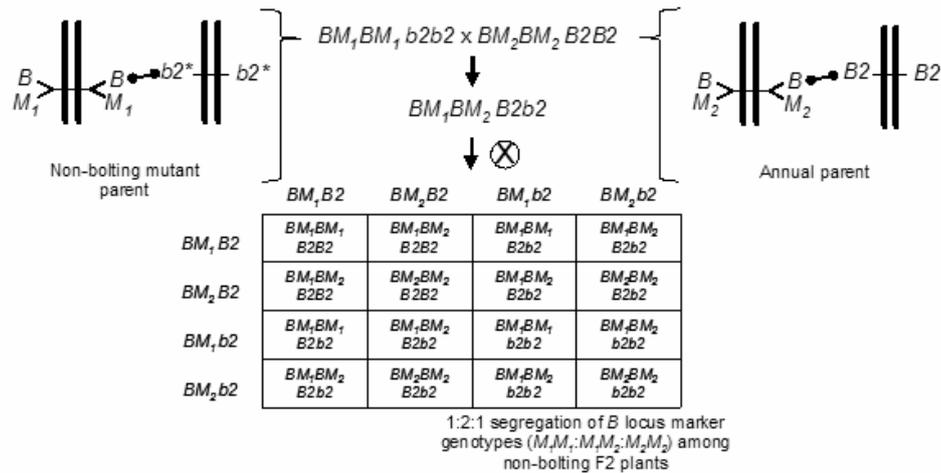
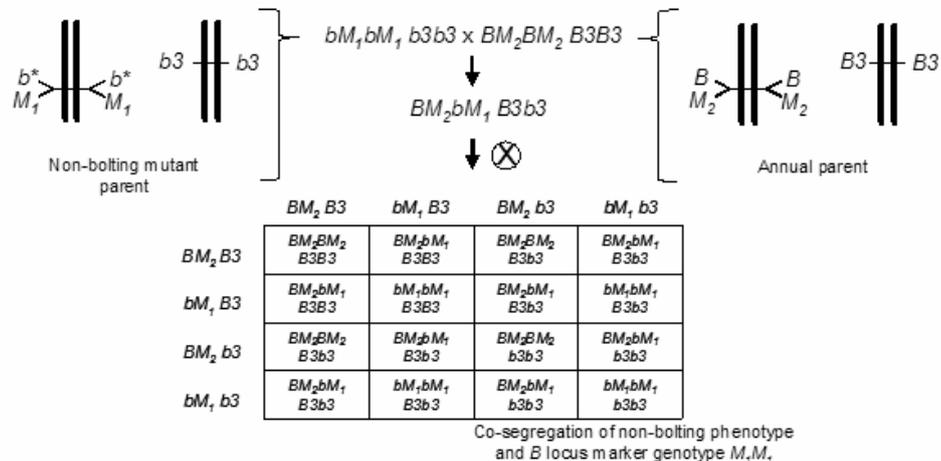
a) Recessive mutation at the *B* locusb) Recessive mutation at a second, epistatic locus *B2*c) Recessive mutation at the *B* locus and presence of a second, independent bolting locus *B3* in annual crossing partner

Figure 4: Test for allelism between EMS mutations and the *B* locus. The expected segregation of bolting phenotypes and *B* locus marker genotypes is shown for crosses between 'non-bolting' (biennial) EMS mutants and annual crossing partners. Three models are shown: a) The mutation occurred at the *B* locus. b) The mutation occurred at a second locus *B2* which acts epistatically to the *B* locus. According to this model, both the *B* locus and the *B2* locus need to carry functional (dominant) alleles for bolting to occur. c) The mutation occurred at the *B* locus, but the annual crossing partner carries an additional bolting locus *B3* which acts independently of the *B* locus. The models assume that the mutations are recessive. For each of the models the allelic constitution of the parents at the various loci is depicted graphically. Black vertical bars represent chromosomes. Recessive alleles which were generated by mutagenesis are marked by asterisks. The dumbbell symbol in b) indicates epistatic interactions. The crossing schemes show plant genotypes in the parent, F₁ and F₂ generations according to the models. Markers are abbreviated as *M*, with *M*₁ and *M*₂ being the marker alleles present at the *B* locus in the EMS mutants or the annual parents, respectively. Biennial genotypes are underlined.

2.3 Material and Methods

2.3.1 Plant material

'Non-bolting', biennial mutants (Tab. 1) had been generated by Hohmann et al. (2005) by EMS mutagenesis of an annual *B. vulgaris* accession (seed code 930190, corresponding to 93167P (El-Mezawy et al. 2002)) which is homozygous for the dominant allele at the *B* locus (*BB*; El-Mezawy et al. 2002). The annual *B. vulgaris* ssp. *maritima* accession 991971 (Gaafar et al. 2005) was originally collected on the Greek island of Khios (USDA-ARS National Plant Germplasm System PI 546521; Hanson and Panella 2003, <http://www.ars-grin.gov/cgi-bin/npgs/acc/display.pl?1441457>). Because the genetic control of annual bolting had been attributed to a single dominant locus, the *B* locus (Munerati 1931; Abegg 1936; Boudry et al. 1994; Hansen et al. 2001; El-Mezawy et al. 2002), it was assumed that the annual accession 991971 carries a functional *B* allele.

M3 or M4 plants of biennial mutant lines were crossed with individuals of accession 991971 (Tab. 1). In order to synchronize flowering times the annual crossing partners were vernalized for twelve weeks at 4°C in a cold chamber in parallel with the mutant plants. All crosses were done by bag isolation in the field in the summer of 2006. Cross progeny were identified phenotypically by hypocotyl color. In *B. vulgaris* hypocotyl color is encoded by the *R* locus, with the *R* allele encoding red hypocotyl color being dominant over the *r* allele for green hypocotyl color (Butterfass 1968; Barzen et al. 1992). Three mutant families have green hypocotyls (000855, 011763, 011373). Individuals from these families were pollinated with 991971 individuals with red hypocotyls. One mutant family has red hypocotyls (000192). The occurrence of a mutant with red hypocotyls is likely to be due to residual heterogeneity at the *R* locus in accession 930190 which was used for mutagenesis and generally has green hypocotyls, but also comprises a small number of individuals with red hypocotyls. Similarly, 991971 plants generally have red hypocotyls but a small subset of plants was found to possess green hypocotyls, indicating heterogeneity at the *R* locus also within this accession. Plant 020416/14 of the mutant line with red hypocotyls was used as pollinator in a cross with a 991971 individual with green hypocotyl color. Two to six progeny plants per cross (corresponding to 3-11% of plants phenotyped for hypocotyl color) had a hypocotyl color indicative of cross progeny.

Table 1: Biennial EMS mutants and generation of F₂ populations.

Mutant family (M2) ^a	Mutant parent of cross	Annual parent of cross	Seed parent of F ₁ cross progeny	F ₁ plant selfed	F ₂ population ^c
000855	020415/15 (M3) ^b	991971/2	020415/15	061365/1	EW1
011763	056822/4 (M4)	991971/9	056822/4	061392/1	EW2
011373	031823/14 (M3)	991971/11	031823/14	061398/1	EW3
000192	020416/14 (M3)	991971/3	991971/3	061373/3	EW4a
000192	020417/16 (M3)	930190/13	020417/16	061460/1	EW4b

^a M2 family numbers correspond to the mutant nomenclature used by Hohmann et al. 2005

^b Six-digit numbers are seed codes, numbers separated by slashes indicate individual plants within a population. The numbers in parentheses indicate mutant generation numbers

^c Seed code numbers for populations EW1 to EW4b are 070047, 070056, 070058, 070292, and 070081, respectively

We hypothesized that the mutant family with red hypocotyls may carry alleles which are rarely present in accession 930190 also at other loci, and that polymorphisms between the mutant and common alleles in 930190 can be used for segregation analysis. We therefore also crossed another individual (020417/16) of this mutant line with accession 930190 by manual

pollination in parallel to the crosses described above. All F₁ plants were propagated over winter in the greenhouse without vernalization, and selfed to produce F₂ seed (Tab. 1).

2.3.2 Phenotypic analysis

96 plants per F₂ population were sown on May 18, 2007, grown in the greenhouse for one month under long day conditions with supplementary lighting (Son-T Agro 400W (Koninklijke Philips Electronics N.V., Eindhoven, The Netherlands) for 16h), and transplanted to the field on June 20, 2007. Plants were phenotyped every two to three days for onset of bolting (BBCH scale code 51; Meier 2001). Phenotypes were scored until November 19, 2007. In populations EW2, EW3 and EW4b, four, six or three plants, respectively, died after transplantation to the field. Population EW1 had a low germination rate and several plants died at the early seedling stage so that only 78 plants were grown and analyzed. Because the F₂ population derived from the cross between 020416/14 and 991971/3 had a germination rate of less than 20%, a new F₂ population (EW4a) derived from the same cross, but another F₁ plant (061373/3), was sown in the greenhouse on May 16, 2008. Out of 200 seeds sown, 140 germinated. Plants were transplanted to the field on June 20, 2008 and phenotyped in the field until end of October 2008. As controls, twelve plants each of the annual and mutant parent accessions were grown and phenotyped in parallel to the F₂ populations.

2.3.3 DNA extraction and genotypic analysis

For molecular marker analysis leaf samples were harvested and freeze dried. Genomic DNA was extracted using the NucleoSpin 96 Plant DNA isolation kit (Macherey and Nagel, Düren, Germany) and a TECAN-Freedom EVO 150® robot (Männedorf, Switzerland). DNA concentration was adjusted to 5 ng/μl. Five markers linked to the *B* locus on chromosome II at R=0 (GJ1001c16, GJ1013c690a, GJ1013c690b), R=0.005 (GJ18T7b), or R=0.007 (Y67L), respectively (Müller et al. unpublished data), were used to differentiate between mutant- and annual parent-derived alleles (Suppl. Tab. 1). SNP markers were genotyped by PCR amplification and sequencing (GJ1013c690a), or converted into CAPS markers (GJ1013c690b, Y67L). GJ1013c690b and Y67L were genotyped by PCR amplification, *Bsa*II or *Hae*III (Fermentas, St. Leon-Rot, Germany) restriction enzyme digestion, respectively, and standard agarose gel electrophoresis. The InDel markers GJ1001c16 and GJ18T7b were genotyped by PCR amplification and electrophoresis on a 3% MetaPhor high resolution agarose gel (Biozym Scientific GmbH, Hessisch Oldendorf, Germany). The chromosome IX marker MP_R0018 (Schneider et al. 2007) was genotyped by PCR amplification and sequencing, or PCR amplification followed by *Hinf*I (Fermentas, St. Leon-Rot, Germany) restriction enzyme digestion and standard agarose gel electrophoresis (Suppl. Tab. 1). The parental origin of marker alleles and segregation in F₂ populations was determined by genotyping the parental accessions and eight randomly chosen F₂ individuals per population. At least one polymorphic marker per population was genotyped in the whole population.

Amplified fragment length polymorphisms were analyzed essentially as described by El-Mezawy et al. (2002), except that for restriction *Pst*I (Fermentas, St. Leon-Rot, Germany) instead of *Eco*RI was used. Pre-amplification was done with primers P01 and M01, and amplification with primers M31-M38 in combination with primers P31-P46 (Vos et al. 1995; <http://wheat.pw.usda.gov/ggpages/keygeneAFLPs.html>). Oligomers were obtained from MWG Biotech AG (Ebersberg, Germany). Genomic DNA from the EMS mutant and the annual accession 991971 was used for pre-selection of suitable primer combinations. Polymorphic fragments were named according to the primer combination used for amplification, followed by an abbreviation of the parent which carried the fragment (E = EMS mutant, W = wild type) and the size of the fragment, e.g. M38xP46_W180. AFLP fragment

sizes were determined by comparison either with the DNA size marker SequaMark® (Invitrogen, Karlsruhe, Germany) or the 50-700 bp sizing standard (LI-COR, LI-COR Biosciences, Lincoln, USA). 38 primer combinations which allowed detection of one to ten AFLPs each were chosen for genotyping.

2.3.4 Map construction and statistical analysis

The genetic map was constructed using the Kosambi mapping function (Kosambi 1944) in JoinMap® 3.0 (Van Ooijen and Voorrips 2001) at a LOD threshold value of 3.0 (rec-value 0.4). Linkage groups were anchored by mapping previously described SSR markers (McGrath et al. 2007; Laurent et al. 2007), EST-based SNP markers (Schneider et al. 2007), and additional sequences with known map positions (Suppl. Tab. 2). Polymorphisms in non-SSR markers were identified by PCR amplification and sequencing, using genomic DNA from the mutant parent and eight randomly selected F₂ plants as template. For mapping, the F₂ population was genotyped at polymorphic sites using SSR marker assays, newly developed CAPS marker assays, or sequencing (Suppl. Tab. 2). Quantitative trait loci (QTL) for bolting time were mapped by composite interval mapping at LOD \geq 3.0, using PLABQTL v 1.2 (Utz and Melchinger 1996).

χ^2 analysis and analysis of variance was performed using SAS 9.1 TS level 1M3 (SAS Institute, Cary, NC, USA). Means of significantly different sample groups were compared using Fisher's least significant difference (LSD) analysis at 5% probability level (SAS 9.1 TS level 1M3).

2.4 Results

2.4.1 Phenotypic segregation for annual bolting

Four 'non-bolting' (biennial) EMS mutants identified by Hohmann et al. (2005) were crossed with the annual *B. vulgaris* ssp. *maritima* accession 991971 (Tab. 1). As expected for dominant-recessive inheritance of annual bolting, all F₁ plants originating from the crosses bolted and flowered without vernalization. F₂ populations segregating for the mutant phenotype were tested for co-segregation of bolting behaviour with molecular markers located at the *B* locus.

Table 2: Phenotypic segregation for bolting behaviour in F₂ populations.

F ₂ population	Total number of plants	Bolting	Non-bolting	χ^2 test for H ₀ = 3:1 (bolting vs. non-bolting) ^a	χ^2 test for H ₀ = 15:1 (bolting vs. non-bolting) ^b
EW1	78	52	26	2.89	97.64**
EW2	92	73	19	0.93	32.57**
EW3	90	76	14	4.28*	13.30*
EW4a	140	135	5	34.29**	0.19
EW4b	93	67	26	0.43	74.80**

^a H₀, null hypothesis for monogenic, dominant-recessive trait

^b H₀, null hypothesis for digenic, dominant-recessive trait

* $\alpha=0.05$; ** $\alpha=0.01$

For each of the four crosses, 78 to 140 F₂ plants (populations EW1, EW2, EW3 and EW4a; for population EW4b s. below) were phenotyped for bolting behavior under non-vernalizing conditions (bolting or non-bolting; Suppl. Tab. 3). All populations segregated for bolting behavior and contained both bolting and non-bolting individuals (Fig. 5, Tab. 2). In two populations (EW1, EW2) the phenotypic segregation ratios did not deviate significantly from

the 3:1 segregation ratio of bolting and non-bolting plants expected for dominant-recessive inheritance of a monogenic trait, as tested by χ^2 analysis (Tab. 2). For the other two populations (EW3, EW4a), the null hypothesis of a 3:1 ratio was rejected at $\alpha=0.05$ or $\alpha=0.01$, respectively. Segregation of bolting and non-bolting plants in population EW4a did not deviate significantly from a ratio of 15:1 (Tab. 2), as would be expected for digenic, dominant-recessive inheritance of the trait when only the double recessive genotype is non-bolting (s. below). Population EW3 also contained an excess of bolting plants, but to a much lesser extent which was not consistent with a 15:1 segregation ratio.

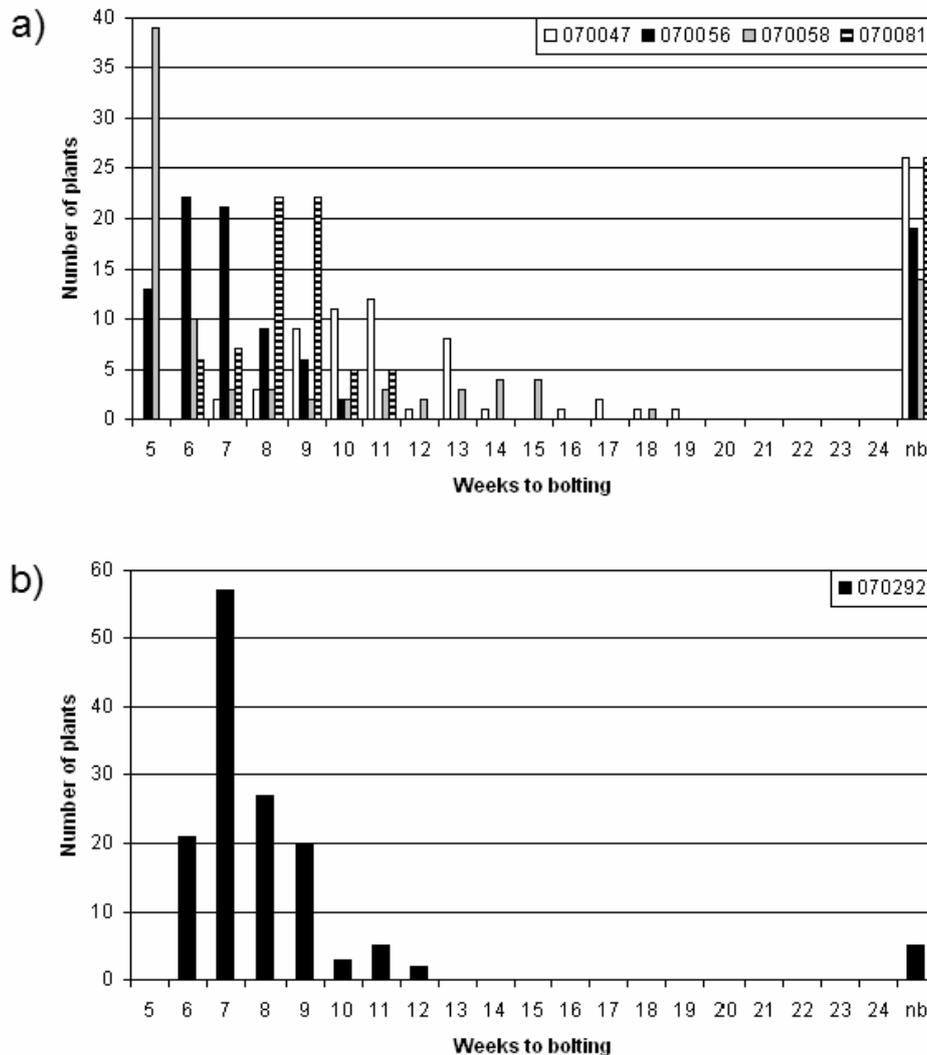


Figure 5: Phenotypic segregation for bolting behavior in F_2 populations. 'Weeks to bolting' indicates the week, counted from the date of sowing, in which stem elongation began (i.e. week 5 corresponds to 29 to 35 days to bolting, etc.; s. Suppl. Tab. 3). 'nb' indicates plants which had not bolted by the end of the experiment in fall. Populations in which bolting and non-bolting plants segregate at a ratio not deviating from 3:1 at $\alpha=0.01$ (EW1, EW2, EW3, EW4b) are shown in a), population EW4a is shown in b).

2.4.2 Co-segregation analysis of bolting phenotypes and *B* locus marker genotypes: Evidence for additional bolting loci

For the genotypic analysis, five co-dominant molecular markers linked to the *B* locus (GJ1001c16, GJ1013c690a, GJ1013c690b, GJ18T7b, Y67L; s. Materials and Methods) were tested for segregation in the F_2 populations. In all populations at least one of the markers segregated and was used for co-segregation analysis (Suppl. Tab. 1). Marker alleles derived from the mutant or annual parents are referred to as M_1 or M_2 , respectively.

2.4.2.1 Co-segregation analysis in population EW1, EW2 and EW3

To distinguish between co-segregation or independent segregation of bolting phenotypes and *B* locus markers in F₂ populations, marker genotypes were grouped into six classes, i.e. *M*₁*M*₁, *M*₁*M*₂ and *M*₂*M*₂ marker genotypes among the bolting individuals of an F₂ population, and *M*₁*M*₁, *M*₁*M*₂ and *M*₂*M*₂ marker genotypes among the non-bolting individuals (Tab. 3). In case of complete co-segregation between bolting phenotypes and *B* locus markers, the segregation ratio of these classes would be expected to be 0:2:1:1:0:0, whereas independent segregation is expected to yield a 3:6:3:1:2:1 segregation ratio. The two populations whose phenotypic segregation ratios did not deviate significantly from 3:1 (EW1, EW2), and population EW3, contained F₂ individuals within each of the six classes, indicating the absence of complete co-segregation between *B* locus marker genotypes and bolting phenotypes. The alternative possibility of independent segregation was tested by χ^2 analysis. In none of the three populations, the null hypothesis (a 3:6:3:1:2:1 segregation ratio, s. above) was rejected, suggesting the presence of at least one additional bolting locus ('*B*2', s. below) which is not genetically linked to the *B* locus.

Table 3: Co-segregation analysis of bolting behaviour and *B* locus marker genotypes.

F ₂ population	<i>B</i> locus marker	Total number of plants genotyped	Bolting			Non-bolting			χ^2 test for H ₀ = 3:6:3:1:2:1 ^b	χ^2 test for H ₀ = 3:8:4:1 ^c
			<i>M</i> ₁ <i>M</i> ₁ ^a	<i>M</i> ₁ <i>M</i> ₂	<i>M</i> ₂ <i>M</i> ₂	<i>M</i> ₁ <i>M</i> ₁	<i>M</i> ₁ <i>M</i> ₂	<i>M</i> ₂ <i>M</i> ₂		
EW1	GJ1013c690a	78	9	28	16	6	15	4	5.59	n.a.
EW2	GJ1001c16	90	8	45	20	3	9	7	10.73	n.a.
EW3	GJ1001c16	90	21	40	15	5	4	5	7.19	n.a.
EW4a	GJ18T7b	140	29	70	36	4	0	1	36.70**	n.a.
	GJ1013c690b	140	30	77	28	4	1	0	39.00**	n.a.
	Y67L	140	33	70	32	5	0	0	34.50**	3.60
EW4b	GJ1013c690b	93	0	39	28	22	3	1	79.80*	n.a.

^a Marker alleles *M*₁ and *M*₂ are derived from the mutant parent or the annual parent, respectively

^b Expected ratio for a monogenic, dominant-recessive trait and independent segregation of phenotype and *B* locus marker

^c Expected ratio for digenic, dominant-recessive trait, co-segregation of phenotype and *B* locus marker

n.a., not applicable

* $\alpha=0.05$; ** $\alpha=0.01$

2.4.2.2 Co-segregation analysis in population EW4a and EW4b

The unexpected observation of a segregation ratio close to 15:1 in population EW4a led us to investigate a third model for the genetic basis of bolting behavior in this population (Fig. 4c). This model assumes the existence of two independent 'bolting genes' at two unlinked loci, the *B* locus and a yet unknown locus which we will refer to as *B*3. For this model to be consistent with a 15:1 segregation ratio, it is further assumed that both loci segregate for dominant and recessive alleles, and that either of the dominant alleles (at the *B* locus or the *B*3 locus) is sufficient to induce bolting. Because the genetic basis for annual bolting in accession 930190 had been mapped to the *B* locus (El-Mezawy et al. 2002) and the biennial mutants had been obtained by EMS mutagenesis of this accession, the model further assumes that the mutant parent of population EW4a carries a mutated, recessive allele (in the homozygous condition) at the *B* locus. The model predicts that all non-bolting plants of the F₂ population carry the *B* locus marker allele derived from the mutant parent in the homozygous condition (*M*₁*M*₁), and that none of the other *B* locus marker genotypes (*M*₁*M*₂, *M*₂*M*₂) occur among non-bolting individuals. The result of genotyping F₂ population EW4a with the *B* locus marker

GJ1013c690b came close to these predictions (Tab. 3). Four of five non-bolting plants in this population carried the *B* locus marker M_1 in the homozygous condition, one plant was heterozygous for the *B* locus marker, and none of the plants carried the M_2 allele in the homozygous condition.

The possibility that one of the two bolting genes in this population predicted by the model is located at or close to the *B* locus was investigated further. To this end, two additional markers flanking the *B* locus on opposite sides (GJ18T7b and Y67L; Suppl. Tab. 1) were tested for co-segregation with the bolting phenotype (Tab. 3). The genotype of marker GJ18T7b deviated from the expectation in the same plant as the genotype of the previously tested marker. For marker Y67L, however, the genotypic data are consistent with the model, i.e. all non-bolting plants carried the mutant-derived allele in the homozygous condition. Furthermore, the null hypothesis for segregation according to the model (a segregation ratio of 3:8:4:1 for the phenotype/marker constellations bolting/ M_1M_1 , bolting/ M_1M_2 , bolting/ M_2M_2 and non-bolting/ M_1M_1) was not rejected by χ^2 analysis (Tab. 3).

Because the small number of non-bolting plants in population EW4a, as a consequence of the high segregation ratio, is somewhat unsatisfactory for statistical analyses, we further tested the possibility of a mutation at the *B* locus by co-segregation analysis in an additional F_2 population (EW4b) derived from a cross between the same mutant line and the annual accession 930190, i.e. the same accession in which *B* was identified as the only (independent) bolting locus by genetic mapping (El-Mezawy et al. 2002, s. Introduction). We took advantage of the apparent genetic divergence of the mutant and the annual accession (s. Materials and Methods) which allowed us to identify a polymorphism between both crossing partners in one of our *B* locus markers (GJ1013c690b, i.e. the same marker which was also used to differentiate between the parental alleles in population EW4a; Suppl. Tab. 1). Out of 93 F_2 plants in population EW4b, 67 bolted and 26 did not bolt without vernalization, and the segregation ratio in this population did not deviate significantly from the 3:1 expectation ($\chi^2 = 0.433$). Co-segregation analysis using the *B* locus marker strongly suggests that marker genotypes and bolting phenotypes do not segregate independently (Tab. 3). Among the 26 non-bolting plants in this population, 22 carry the *B* locus marker genotype (M_1M_1) expected for complete co-segregation between phenotype and the mutant marker allele. Among the remaining four plants, three plants are heterozygous at the marker locus (M_1M_2) and one single plant carries the marker allele derived from the annual parent in the homozygous state (M_2M_2). Among the 67 bolting plants, all plants carry either the M_1M_2 or the M_2M_2 constellation, and none is homozygous for the M_1 allele.

2.4.3 Variation in bolting time among annuals in F_2 populations

Besides annuality, all populations were phenotyped for bolting time of annual individuals (Fig. 5, Suppl. Tab. 3). In populations EW1, EW2 and EW4b, annual plants were approximately normally distributed but the position of the maxima differed between populations (Fig. 5a). In population EW3, the majority of plants bolted early (at five to six weeks from sowing), but a considerable number of plants started to bolt much later (at eleven to 18 weeks; Fig. 5a). The frequency distribution in population EW4a is similar to normal but positively skewed, with bolting in some plants being somewhat delayed (Fig. 5b). To test whether allele composition at the *B* locus affected bolting time in annuals, analysis of variance (ANOVA) was performed for number of days to bolting between the three groups of *B* locus marker genotypes (M_1M_1 , M_1M_2 and M_2M_2). Marker genotypes in populations EW1, EW2 and EW3 did not exhibit significant effects on bolting time (at $\alpha=0.01$). However, ANOVA in population EW4a showed highly significant differences in annual bolting time among the three marker genotypes (Tab. 4). Fisher's Least Significant Difference analysis showed that the mean of days to bolting for the M_1M_1 genotype (M_1 being inherited from the

mutant parent) differed significantly from the other two marker genotypes (M_1M_2 and M_2M_2). The presence of the M_1M_1 genotype correlated with a delay in bolting.

Table 4: Analysis of variance among *B* locus marker genotypes in annual subpopulations.

Marker genotype ^a	Mean (\pm standard deviation) of days to bolting				
	EW1	EW2	EW3	EW4a	EW4b
M_1M_1	88.67 (\pm 24.96)	45.25 (\pm 10.30)	49.05 (\pm 24.23)	58.58 (\pm 9.71) A	n.a. ^b
M_1M_2	74.82 (\pm 12.80)	43.02 (\pm 8.67)	52.80 (\pm 26.18)	48.36 (\pm 7.22) B	56.44 (\pm 8.69)
M_2M_2	71.88 (\pm 20.50)	47.55 (\pm 6.50)	46.20 (\pm 21.03)	46.28 (\pm 6.67) B	56.43 (\pm 8.14)
F (p-value)	2.87 (0.07)	2.08 (0.13)	0.43 (0.65)	25.00 (0.00)	n.a.
LSD _{0.05} ^c	n.a.	n.a.	n.a.	3.53	n.a.

^a *B* locus markers are as indicated in Tab. 3. The *B* locus marker analyzed in population EW4a is GJ1013c690b.

^b Population EW4b did not comprise any M_1M_1 individuals and ANOVA was not performed

^c Fisher's Least Significant Difference at $\alpha=0.05$. Mean values in table cells including the letter 'B' are significantly different from the mean value in the table cell including the letter 'A'
n.a., not applicable

2.4.4 Genetic mapping of the bolting locus in population EW2

Among the populations postulated to carry a bolting locus not genetically linked to *B*, EW2 was selected for AFLP mapping of the locus. A genetic map was constructed which incorporates 141 AFLPs as well as five SSR markers (Laurent et al. 2007, McGrath et al. 2007) and ten SNP-based markers (Schneider et al. 2007; Suppl. Tab. 2) which were used as anchor markers. All linkage groups were anchored to the nine chromosomes of the beet genome (Suppl. Fig. 1). The map covers 571 cM with an average marker interval of 3.65 cM. The sizes of the linkage groups range from 33 cM to 89 cM. The phenotypic marker, i.e. the locus responsible for annual bolting in this population, was mapped to position 52.61 cM on chromosome IX and is flanked by the AFLP marker M34xP46_W160 and the co-dominant SNP-based anchor marker MP_R0018 (Schneider et al. 2007) (Fig. 6). This newly identified bolting locus will be referred to as *B2*.

The presence of a locus responsible for bolting behavior at this location is supported by composite interval QTL analysis of bolting time (as determined by days to bolting) in this population using PLABQTL v 1.2 (Utz and Melchinger 1996). To allow inclusion of individuals which did not bolt without vernalization, the number of days to bolting for these plants was artificially set to 300 (a number which approximates the number of days to bolting in biennial beets subjected to cold treatment over winter). Using this setting and a LOD threshold of 3.0 a single QTL was detected. The QTL co-localizes with the phenotypic marker locus *B2* on chromosome IX at position 52 cM (confidence interval 50-54 cM; Fig. 6), has a LOD score of 46.81 and explains 87.0% of the observed phenotypic variation.

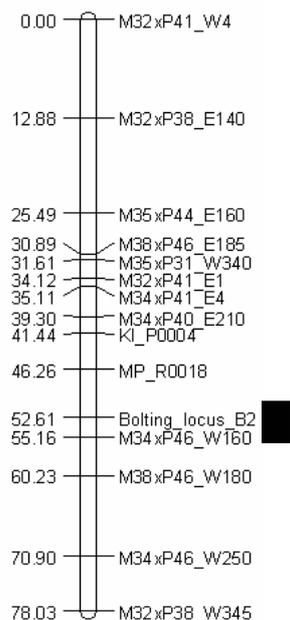


Figure 6: Genetic map position of a major locus for annual bolting on chromosome IX. The map comprises the bolting locus *B2*, twelve AFLP markers, and two co-dominant SNP-based anchor markers (KI_P0004 and MP_R0018). Genetic distances in centiMorgan are given on the left, marker names on the right. The vertical black bar indicates the confidence interval of a quantitative trait locus co-localizing with *B2*.

2.4.5 Co-segregation analysis of bolting behaviour and the chromosome IX marker MP_R0018 in populations EW1, EW3 and EW4a

To investigate the possibility that locus *B2* on chromosome IX also determines bolting behavior in populations EW1 and EW3, and/or co-localizes with the unknown independent bolting locus *B3* in population EW4a, we aimed to test the flanking co-dominant marker MP_R0018 for co-segregation with the phenotypic marker locus in these populations. The marker sequence was found to be polymorphic in all three populations. In populations EW1 and EW4a bolting phenotype and the chromosome IX marker MP_R0018 segregated independently of each other (Tab. 5). However, similar to population EW2, independent segregation was not observed in population EW3. Among 14 non-bolting plants in this population, all but one carried the mutant-derived marker allele in the homozygous condition (M_1M_1), with the remaining plant being heterozygous at the marker locus. Notably, however, among the 76 bolting plants 17 were also of the M_1M_1 genotype at the marker locus. A closer examination of bolting plants which are homozygous for the M_1 marker allele revealed that most of these plants are very late bolting (Suppl. Tab. 3). Analysis of variance of bolting time between the three genotypic classes (M_1M_1 , M_1M_2 and M_2M_2) among the bolting plants of this population revealed that the mean of days to bolting in M_1M_1 individuals (83.12) was significantly higher than the respective means in M_1M_2 (40.66) and M_2M_2 (43.11) individuals (Tab. 6). By contrast, bolting time did not differ significantly between the three genotypic classes among bolting plants in populations EW1 and EW4a, nor was this the case in population EW2.

Table 5: Co-segregation analysis of bolting behavior and chromosome IX marker genotypes.

F ₂ population	Chromosome IX marker	Total number of plants genotyped	Bolting			Non-bolting			χ^2 test for H ₀ = 3:6:3:1:2:1 ^b
			<i>M</i> ₁ <i>M</i> ₁ ^a	<i>M</i> ₁ <i>M</i> ₂	<i>M</i> ₂ <i>M</i> ₂	<i>M</i> ₁ <i>M</i> ₁	<i>M</i> ₁ <i>M</i> ₂	<i>M</i> ₂ <i>M</i> ₂	
EW1	MP_R0018b	77	12	23	17	5	16	4	6.43
EW2	MP_R0018a	92	2	58	13	14	3	2	51.10**
EW3	MP_R0018a	90	17	41	18	13	1	0	26.27**
EW4a	MP_R0018b	138	19	75	29	1	3	1	4.01

^a Marker alleles *M*₁ and *M*₂ are derived from the mutant parent or the annual parent, respectively

^b Expected ratio for monogenic, dominant-recessive trait, independent segregation of phenotype and MP_R0018

* $\alpha=0.05$; ** $\alpha=0.01$

Table 6: Analysis of variance among chromosome IX marker genotypes in annual subpopulations.

Marker genotype ^a	Mean (\pm standard deviation) of days to bolting			
	EW1	EW2	EW3	EW4a
<i>M</i> ₁ <i>M</i> ₁	69.00 (± 9.18)	48.50 (± 4.90)	83.12 (± 25.09) A	46.73 (± 6.44)
<i>M</i> ₁ <i>M</i> ₂	80.04 (± 21.20)	44.53 (± 8.56)	40.66 (± 11.87) B	51.26 (± 9.69)
<i>M</i> ₂ <i>M</i> ₂	74.94 (± 17.84)	43.77 (± 8.68)	43.11 (± 19.35) B	50.14 (± 7.77)
F (p-value)	1.51 (0.23)	0.27 (0.77)	31.10 (0.00)	2.14 (0.12)
LSD _{0.05} ^b	n.a.	n.a.	10.75	n.a.

^a Chromosome IX markers are as indicated in Tab. 4

^b Fisher's Least Significant Difference at $\alpha=0.05$. Mean values in table cells including the letter 'B' are significantly different from the mean value in the table cell including the letter 'A'
n.a., not applicable

2.5 Discussion

The bolting loci in five F₂ populations derived from crosses between four biennial EMS mutants and annual crossing partners and segregating for bolting behavior were tested for allelism to the *B* locus. The main findings of this study are: i) The *B* locus is not the only locus controlling annual bolting in *B. vulgaris*. Bolting control involves at least two other loci not linked to *B* (*B*₂, *B*₃), one of which (*B*₂) was genetically mapped. ii) In one mutant family, the *B* locus (or a locus closely linked to *B*) appears to be mutated, suggesting that an EMS-induced mutation at this locus can be sufficient to convert an annual genotype into a biennial genotype. iii) The annual *B. vulgaris* ssp. *maritima* accession 991971 carries an additional bolting locus (*B*₃) which acts independently of the *B* locus. These findings will be further discussed in the following paragraphs.

i) Two lines of evidence indicate that the bolting locus *B* on chromosome II is not the only locus which controls annual bolting in beets. Firstly, *B* locus markers segregate independently of the phenotypic marker 'annual bolting' in three segregating F₂ populations (EW1, EW2, EW3). Secondly, in another population (EW4a), bolting plants occurred in large excess of what would be expected for monogenic inheritance of this trait, and the observed segregation ratio matched more closely the expectation for digenic inheritance.

One of the novel bolting loci, *B*₂, was mapped to chromosome IX in population EW2. Both the phenotypic segregation data, which do not deviate significantly from the 3:1 ratio (bolting vs. non-bolting) expected for dominant-recessive inheritance of a monogenic trait, and the QTL analysis of bolting behavior in this population suggest that *B*₂ constitutes a major

genetic locus controlling annual bolting in *B. vulgaris*. *A priori*, the existence of a second bolting locus is consistent with our 'epistatic locus' model, according to which the EMS-induced mutation in the mutant parent of this population occurred at a locus which is unlinked to the *B* locus, acts epistatically to *B* and prevents bolting even in the presence of a functional bolting allele at the *B* locus (Fig. 4b). This hypothesis is also in accordance with the phase relationships between bolting phenotypes and mutant-derived or annual parent-derived marker alleles, respectively, i.e., the mutant-derived allele at the MP_R0018 marker locus in the vicinity of *B2* and the non-bolting phenotype are linked in coupling phase and, for example, the homozygous state of the mutant-derived allele ($M_I M_I$) at this marker locus occurs preferentially among non-bolting plants (Tab. 5). *B* and *B2* would have to interact epistatically because the annual accession used for EMS mutagenesis is homozygous for the annual bolting allele at the *B* locus (*BB*), and this allele must still be present in all individuals of the F_2 population if the EMS-induced mutation occurred at *B2*. *A posteriori*, however, we cannot exclude the possibility that annual bolting in the annual parent of this population is not encoded by the *B* locus, but by a different locus which acts independently of *B*. In this scenario, to account for the lack of co-segregation of phenotype and *B* locus marker genotypes, it would have to be assumed that the mutation in the mutant parent occurred at the *B* locus. As discussed below (s. iii), the phenotypic segregation data for population EW4a suggested the presence of an additional independent bolting locus (*B3*) at least in a subset of individuals of the annual parent accession 991971. However, our data are also consistent with one of the two bolting alleles postulated for this population being located at or in very close proximity of the *B* locus and originating from the annual parent (s. ii below), which is in support of our original assumption that a functional *B* allele is indeed present in accession 991971. Our data further suggest that *B3* does not co-localize with *B2* and thus cannot be responsible for annual bolting in population EW2. In conclusion, we regard it as likely that the mutation in the mutant parent of EW2 occurred at *B2* and that, consequently, this locus acts epistatically to *B*.

Two F_2 populations, EW1 and EW3, behaved similarly to population EW2 insofar as bolting phenotypes and *B* locus marker genotypes segregated independently (Tab. 3). The phenotypic marker in population EW1 also segregated independently of the *B2*-linked marker MP_R0018a and may constitute yet another locus. In population EW3, however, the phenotypic marker did not segregate independently of the *B2*-linked marker, and all except one of the non-bolting plants carried the mutant-derived marker allele in the homozygous condition ($M_I M_I$) (Tab. 5), suggesting that *B2* may also affect bolting behavior in this population. However, in contrast to population EW2, the homozygous state of the mutant-derived marker allele not only occurred preferentially among non-bolting plants, but was also frequently found among very late bolting individuals of the population, and individuals of the $M_I M_I$ genotype among bolting plants on average bolted substantially and highly significantly later than individuals of the other two genotypic classes at this locus (Tab. 6). Noteworthy, the bolting plants in population EW2 bolted within 34 to 68 days after sowing (with a mean of 45.51 days), whereas the bolting plants in population EW3 bolted within 34 to 122 days after sowing (with a mean of 50.46 days) (Suppl. Tab. 3). 17 of these plants bolted >68 days after sowing (73 to 122 days, with a mean of 92.12 days; corresponding to weeks eleven to 18 in Fig. 5a). Out of 17 bolting individuals of the $M_I M_I$ genotype in population EW3, 13 belonged to set of 17 late-bolting plants (bolting 73 to 122 days after sowing) and only four bolted earlier (Suppl. Tab. 3).

According to our mapping data the marker locus MP_R0018a is located at a genetic distance of 6.35 cM from the *B2* locus (Fig. 6). This value is in approximate accordance with the number of recombination events in population EW2 (seven among 92 plants analyzed) that are detectable by comparing the genotypes of the dominant phenotypic marker and the co-

dominant molecular marker MP_R0018a (two bolting plants carrying the mutant-derived allele in the homozygous condition (M_1M_1), three non-bolting plants being heterozygous at the marker locus (M_1M_2), and two non-bolting plants carrying the annual parent-derived allele in the homozygous condition (M_2M_2); Tab. 5). In population EW3, the corresponding number of recombination events is 18 (among 90 plants analyzed; Tab. 5), i.e. considerably higher. However, if the frequent occurrence of M_1M_1 genotypes among the late-bolting plants in this population is considered, and if it is thus postulated that the recessive allele at the $B2$ locus in the homozygous state ($b2b2$) is causally involved with the occurrence of either non-bolting or late-bolting phenotypes, the number of recombination events between the marker locus and $B2$ is only nine (four early-bolting plants carrying the mutant-derived allele in the homozygous condition (M_1M_1), one non-bolting plant and two late-bolting plants being heterozygous at the marker locus (M_1M_2), and two late-bolting plants carrying the annual parent-derived allele in the homozygous condition (M_2M_2); Suppl. Tab. 3, Tab. 5), i.e. very similar to the number in population EW2. In conclusion, the segregation data for population EW3 suggest that $B2$ is also the main locus responsible for bolting behavior in this population and provide independent support for the presence of a bolting locus on chromosome IX.

In contrast to population EW2, the homozygous state of the recessive allele at the $B2$ locus in population EW3 appears not to preclude annual bolting, but in $b2b2$ individuals which bolt, bolting is delayed. The occurrence of annual plants among $b2b2$ individuals may also account for the excess of annual plants beyond what would be expected for simple monogenic inheritance of the trait (Tab. 2). The field data for populations EW2 and EW3 (Tab. 2) were obtained with populations sown on the same day and grown side-by-side under identical environmental conditions throughout the entire experiment, suggesting that the differences in bolting behavior between these two populations are largely determined genetically. One possibility is that the mutant parent of population EW3 carries a mutation at $B2$ which impairs gene function, but does not abolish it (under the conditions tested). However, none of the nearly 50 plants of the mutant family phenotyped under field or greenhouse conditions ever bolted without vernalization (Hohmann et al. 2005, and unpublished data), which argues against this possibility. An alternative possibility is that population EW3 contains additional modifying genes which affect bolting behavior, and that certain allele compositions at the corresponding loci and/or certain compositions of alleles at various modifier loci enable (late) bolting even in $b2b2$ individuals. A likely source of allelic variation at such loci between populations EW2 and EW3 is the annual parent accession 991971, given the heterogeneity of this accession. In consideration of the data for both populations, $B2$ appears to function both in the control of annuality *per se*, and in the control of bolting time in annual plants. Analysis of variance of bolting time among the annual plants of population EW3 further suggests that the negative effect of the mutant-derived allele at the $B2$ locus requires this allele to be present in the homozygous condition (Tab. 6).

ii) Evidence that the B locus affects bolting behavior and is mutated in one of the four mutant families analyzed (000192, s. Tab. 1) was obtained from analysis of populations EW4a and EW4b. Firstly, the statistical analysis of the B locus marker-phenotype co-segregation data for population EW4a is consistent with the hypothesis that the B locus is one of the two bolting loci postulated for this population (Tab. 3). Secondly, all non-bolting plants in population EW4a carried the mutant-derived allele at the B -linked marker locus Y67L in the homozygous condition. Thirdly, analysis of variance of bolting time among the annual plants of population EW4a indicated a significant effect of allele composition at the B locus on bolting time (Tab. 4). The presence of the mutant-derived allele at the B locus in the homozygous condition correlated with a delay in bolting, suggesting that, similar to $B2$, the B locus (or a locus linked to B) also affects bolting time in annual plants. Lastly, when a mutant from the same mutant family was crossed with accession 930190 as annual crossing partner, the resulting F_2

population EW4b segregated in accordance with a 3:1 ratio of bolting vs. non-bolting plants, suggesting simple monogenic inheritance of annual bolting in this population and thus facilitating the co-segregation analysis. The marker segregation data for this population (Tab. 3) are largely consistent with the *B* locus being responsible for annual bolting in this population, and the annual parent-derived allele being dominant over the mutant-derived allele (in accordance with the model in Fig. 4a). The genotypes of four non-bolting plants, however, including one which is homozygous for the annual parent-derived *B* locus marker allele (M_2M_2) and three heterozygotes (M_1M_2), deviate from the expectation for a dominant-recessive trait that non-bolting plants are homozygous for the mutant-derived allele. Although we therefore cannot formally exclude the possibility that a locus closely linked to *B* affects bolting behavior in this population, it is conceivable that these individuals did not bolt because of modifying genetic or environmental effects.

iii) Finally, the segregation data for population EW4a indicate that bolting control involves (at least) one additional locus (*B3*). The fact that the segregation ratio does not deviate significantly from 15:1 suggests that this locus is not linked to the *B* locus and acts independently of *B*, but like *B* is also inherited in a dominant-recessive manner (in accordance with the model in Fig. 4c). The following line of evidence suggests that the annual allele at this locus is derived from the annual parent of the population: Genetic mapping of bolting control in the annual accession 930190 identified *B* as the only (independent) bolting locus. The mutant parent of population EW4a was derived from accession 930190 by EMS mutagenesis, is likely to be mutated at the *B* locus (s. ii above) and is biennial, and thus cannot carry a second bolting allele which acts independently of *B*. Because none of the other populations analyzed in the current study provided evidence for an independent bolting locus unlinked to *B*, a functional allele at locus *B3* may be rare in the annual parent accession 991971 and (at least in the homozygous condition) only present in a subset of 991971 individuals. As a consequence of the high ratio of bolting to non-bolting plants in population EW4a, and the resulting small number of non-bolting plants, the phenotypic information content is too small for genetic mapping of *B3*. We also cannot exclude the possibility that the unexpectedly high phenotypic segregation ratio in this population is due to several, quantitative loci. However, because four of five non-bolting plants in this population are not homozygous for the mutant-derived allele at the *B2*-linked chromosome IX marker locus MP_R0018, it seems unlikely that an independent major locus co-localizes with *B2*. The notion that *B2* and *B3* constitute separate loci is also consistent with the genetic modes of action postulated for these loci, with *B2* acting epistatically to *B*, and *B3* acting independently of *B*.

In summary, our data provide evidence for three loci controlling bolting in *B. vulgaris*: The *B* locus on chromosome II, which appears to be mutated in one of four mutant families analyzed, locus *B2* on chromosome IX, which is segregating in two populations and appears to act epistatically to *B*, and locus *B3* which acts independently of *B* and appears to comprise a functional, dominant allele in at least some individuals of the annual accession 991971. The results have implications for our understanding of bolting control in *B. vulgaris*. First, our finding that one biennial mutant family appears to carry a mutation at the *B* locus suggests that a single EMS-induced point mutation of the bolting gene at this locus is sufficient to abolish its function. This mutant is a valuable tool to screen candidates for the bolting gene located at the *B* locus (Müller et al. unpublished data) for sequence variation, and may help to correlate the functional role of the *B* locus in bolting control with a specific gene and/or sequence feature. Second, the genetic control of annual bolting in *B. vulgaris* is more complex than has been described in the past, and involves previously unidentified loci in addition to the well-known *B* locus. Possibly, these additional loci have gone unnoticed as a result of selection by breeders against annual bolting early on in sugar beet breeding, and only limited

genetic research on annual (non-cultivated) beets. In particular, much of the original work on the genetics of bolting behavior was based on related annual beet accessions that were established by Munerati (Munerati 1931; Abegg 1936; Owen et al. 1940). Moreover, the annual accession used in the present study originated from a natural population on a Greek island, i.e. a geographic location within the southern part of the species' distribution area where annuality has its highest frequency and probably a high selective advantage (Van Dijk and Boudry 1991). Lastly, epistatic genes which act in the same genetic pathway as *B* and do not suffice to induce annual bolting in the absence of a functional bolting allele at the *B* locus are impossible to detect in biennial *bb* genotypes as they may be prevalent in cultivated beet breeding material (Gaafar et al. 2005).

The possibility of a second gene controlling annual bolting was considered by Abe et al. (1997). These authors, however, suggested that this second gene is genetically linked to the *B* locus. An additional locus unlinked to *B* is not unlikely given the complexity of floral transition control as it is known for other species. Furthermore, several flowering time genes have been shown to interact epistatically, e.g. in *A. thaliana* (e.g. Koornneef et al. 1991, 1998b; Nilsson et al. 1998; Caicedo et al. 2004). Because the non-bolting mutant phenotype can be overcome by vernalization, the new bolting loci seem unlikely to carry regulatory genes of the vernalization pathway, as it is known for *Arabidopsis*, or other signal transduction cascades that mediate bolting in response to prolonged cold. Furthermore, *BvFL1*, a *B. vulgaris* homolog of the floral repressor gene *FLC* which acts downstream of the vernalization pathway in *Arabidopsis*, can be excluded as a candidate gene for *B2* (but not for *B3* whose map position is not known) because it was mapped to chromosome VI of the beet genome (Reeves et al. 2007). Besides, the annual alleles at the bolting loci in our study are dominant, whereas the recessive, 'non-bolting' alleles are mutant-derived, suggesting that the wild-type alleles do not repress, but promote bolting. The effect of mutagenesis also distinguishes the mutated genes in *B. vulgaris* from *PEP1*, an *FLC* ortholog in the perennial *Brassicaceae* species *Arabis alpina* which determines vernalization requirement in wild-type plants but in its mutated form (following EMS mutagenesis) causes early bolting without a requirement for vernalization (Wang et al. 2009). Conceivably, *B*, *B2* and/or *B3* mediate photoperiod or gibberellic acid control of floral transition, and mutants with an impaired response to the respective exogenous or endogenous cues require the additional stimulus of vernalization for bolting to occur. *B2* cannot correspond to the *CO*-like gene *BvCOL1* because this gene was mapped to chromosome II at a genetic distance of ~25-30 cM from the *B* locus (Chia et al. 2008; Müller et al. unpublished data). The colocalization of *B* and *BvCOL1* on the same linkage group further suggests that *B3* is also unlikely to correspond to *BvCOL1* because our segregation data indicate that *B3* segregates independently of *B*. Candidates for *B2* may include homologs of genes acting upstream or downstream of *CO* in the same genetic pathway, including *FT*-like genes. Although *ft* mutants in *A. thaliana* are only moderately responsive to vernalization (Martinez-Zapater and Somerville 1990; Koornneef et al. 1991; Moon et al. 2005), allelic variation of *FT* orthologs (the *VRN3* genes) (co-)regulates vernalization requirement in cereals (Yan et al. 2006; Trevaskis et al. 2007; Distelfeld et al. 2009). An effort to clone the *B2* locus by a map-based approach using F_2/F_3 populations derived from the original cross has been initiated. Map-based cloning is expected to shed further light on bolting control and regulatory interactions between bolting control genes, and will be greatly facilitated by the sugar beet genome sequence which is expected to be available soon. Finally, an important current breeding goal for sugar beet is the development of novel, high-yielding winter varieties which do not bolt in response to prolonged exposure to cold but which can be artificially induced to bolt and flower for seed production, e.g. through genetic modification (Jung and Müller 2009). The identification of key regulatory genes and a thorough understanding of bolting control is a prerequisite for any approach towards this objective.

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3 Conservation and divergence of autonomous pathway genes in the flowering regulatory network of *Beta vulgaris*

3.1 Abstract

The transition from vegetative growth to reproductive development is a complex process that requires an integrated response to multiple environmental cues and endogenous signals. In *Arabidopsis thaliana*, which has a facultative requirement for vernalization and long days, the genes of the autonomous pathway function as floral promoters by repressing the central repressor and vernalization-regulatory gene *FLC*. Environmental regulation by seasonal changes in day-length is under control of the photoperiod pathway and its key gene *CO*. The root and leaf crop species *Beta vulgaris* in the caryophyllid clade of core eudicots, which is only very distantly related to *Arabidopsis*, is an obligate long-day plant and includes forms with or without vernalization requirement. *FLC* and *CO* homologues with related functions in beet have been identified, but the presence of autonomous pathway genes which function in parallel to the vernalization and photoperiod pathways has not yet been reported. Here, this begins to be addressed by the identification and genetic mapping of full-length homologues of the RNA-regulatory gene *FLK* and the chromatin-regulatory genes *FVE*, *LD* and *LDL1*. When overexpressed in *A. thaliana*, *BvFLK* accelerates bolting in the Col-0 background and fully complements the late-bolting phenotype of an *flk* mutant through repression of *FLC*. In contrast, complementation analysis of *BvFVE1* and the presence of a putative paralogue in beet suggest evolutionary divergence of *FVE* homologues. It is further shown that *BvFVE1*, unlike *FVE* in *Arabidopsis*, is under circadian clock control. Together, the data provide first evidence for evolutionary conservation of components of the autonomous pathway in *B. vulgaris*, while also suggesting divergence or subfunctionalization of one gene. The results are likely to be of broader relevance because *B. vulgaris* expands the spectrum of evolutionarily diverse species which are subject to differential developmental and/or environmental regulation of floral transition.

3.2 Introduction

Floral transition is a major developmental switch which is tightly controlled by a network of proteins that perceive and integrate environmental and developmental signals to promote or inhibit the transition to reproductive growth. In the model species *Arabidopsis thaliana*, several regulatory pathways which differ in their response to distinct cues have been defined, including the vernalization, photoperiod, and autonomous pathway (for review see He and Amasino 2005; Bäurle and Dean 2006; Jung and Müller 2009; Michaels 2009). The central regulator of vernalization response is *FLOWERING LOCUS C (FLC)*, which acts as a repressor of flowering and is down-regulated in response to prolonged exposure to cold over winter. The promotion of floral transition by long days is mediated by *CONSTANS (CO)*, a key protein of the photoperiod pathway which activates the floral integrator gene *FLOWERING LOCUS T (FT)*. Plant genome and expressed sequence tag (EST) sequencing projects in species other than *Arabidopsis*, together with functional studies have begun to unveil the presence and evolutionary conservation of floral regulatory genes across taxa (e.g. Hecht et al. 2005; Albert et al. 2005; Mouhu et al. 2009; Remay et al. 2009). While components of the photoperiod pathway are widely conserved, the regulation of vernalization requirement and response appears to have diverged considerably during evolution, as exemplified by distinct mechanisms in *Arabidopsis* and temperate cereals (Turck et al. 2008; Colasanti and Coneva 2009; Distelfeld et al. 2009; Greenup et al. 2009; Jung and Müller 2009). The phylogenetic lineage leading to *Beta vulgaris*, which includes the biennial crop subspecies sugar beet (*Beta vulgaris* L. ssp. *vulgaris*) as well as annual and perennial wild

beets, diverged from that leading to *Arabidopsis* around 120 million years ago – that is, relatively soon after the monocot-dicot divergence (Chaw et al. 2004; Davies et al. 2004). In beet, vernalization requirement is under control of the bolting gene *B* (Munerati, 1931; Abegg 1936; Boudry et al. 1994; El-Mezawy et al. 2002), which is not related to *FLC* (Reeves et al. 2007; A. E. Müller et al. unpublished), and a second, unlinked locus *B2* which may act epistatically to *B* (Büttner et al. 2010). In the absence of the dominant early bolting allele at the *B* locus, beets possess an obligate requirement for both vernalization and long photoperiods, and under high-temperature and short-day conditions are prone to reversion to a vegetative state by devernalization. Despite apparent differences in the regulation of floral transition between *A. thaliana* and *B. vulgaris*, the recent identification of beet homologues of *FLC* and *CO* suggest at least partial conservation of the genetic basis of the plants' responses to the environment (Reeves et al. 2007; Chia et al. 2008). The *FLC*-like gene *BvFLI* in beet is regulated by vernalization and delays flowering in transgenic *Arabidopsis* plants, suggesting that *BvFLI*, may also be a floral repressor (Reeves et al. 2007). Similarly, evolutionary conservation of *CO* homologues was suggested by overexpression of the *CO*-like gene *BvCOL1* in *Arabidopsis*, which complements the late-flowering phenotype of a loss-of-function *co* mutation and activates *FT* expression (Chia et al. 2008).

Floral transition in *Arabidopsis* is also regulated by the autonomous pathway of flowering time control whose genes are thought to function largely in parallel to the vernalization pathway upstream of *FLC* and the photoperiod pathway (for review see Boss et al. 2004; Simpson 2004; Quesada et al. 2005). Autonomous pathway genes repress *FLC* and thus act as promoters of floral transition, and include *FLOWERING LOCUS CA (FCA)*, *FLOWERING LOCUS D (FLD)*, *FLOWERING LOCUS KH DOMAIN (FLK)*, *FLOWERING LOCUS PA (FPA)*, *FLOWERING LOCUS VE (FVE)*, *FLOWERING LOCUS Y (FY)* and *LUMINIDEPENDENS (LD)* (Simpson 2004). They have in common that mutations in these genes are generally recessive and delay flowering under both long-day and short-day conditions, while the inhibitory effect of the mutations can be overcome by vernalization. Mutations at *FLC* eliminate the late-flowering phenotype caused by mutations in autonomous pathway genes (Koornneef et al. 1994; Lee et al. 1994; Sanda and Amasino 1996; Michaels and Amasino 2001).

Although some genes of the autonomous pathway interact genetically and all share a common target, they do not form a single linear pathway with a hierarchical order of activities, but rather constitute different regulatory sub-groups (or 'sub-pathways'; Marquardt et al. 2006). Autonomous pathway genes regulate *FLC* expression through RNA-based control mechanisms and/or chromatin modification (Boss et al. 2004; Simpson 2004; Quesada et al. 2005; Bäurle et al. 2007; Bäurle and Dean 2008). Four genes mediate RNA regulatory processes, *FCA*, *FPA*, *FY* and *FLK*. *FCA* and *FPA* are plant-specific RNA binding proteins which both carry multiple RNA recognition motifs (RRMs; Macknight et al. 1997; Schomburg et al. 2001). *FCA* physically and genetically interacts with the RNA 3' end processing factor *FY*, and this interaction is required both for correct processing of transcripts derived from *FCA* itself and (directly or indirectly) for down-regulation of *FLC* expression (Quesada et al. 2003; Simpson et al. 2003). *FCA* and *FPA* interact genetically with *FLD*, which encodes a chromatin regulatory protein of the autonomous pathway (see below), and at least part of the effect of *FCA* and *FPA* on *FLC* expression and flowering time depends on *FLD* (Liu et al. 2007; Bäurle and Dean 2008). Thus, *FCA* and *FPA* appear to link RNA and chromatin level control of gene expression.

An analysis of flowering time in various autonomous pathway double mutants indicated that the fourth protein predicted to function in RNA regulation, *FLK*, acts independently of both *FCA* and *FPA* (Bäurle and Dean 2008; Ripoll et al. 2009). *FLK* expression was not detectably

affected in any of the other six autonomous pathway mutants analyzed (*fca*, *fpa*, *fy*, *fld*, *fve*, and *ld*), and, vice versa, the expression of all autonomous pathway genes tested (*FCA*, *FPA*, *FVE* and *LD*) was unaltered in an *flk* mutant (Lim et al. 2004). *FLK* encodes a plant-specific putative RNA binding protein which contains three K-homology (KH)-type RNA binding domains (Lim et al. 2004; Mockler et al. 2004). The mode of action of *FLK* is not known, but other KH domain proteins in Arabidopsis, including HUA ENHANCER 4 (HEN4) (harboring five KH domains) and RS2-INTERACTING KH PROTEIN (RIK), were shown to be part of protein complexes which mediate pre-mRNA processing or have been implicated in RNA-directed chromatin regulation of gene expression, respectively (Cheng et al. 2003; Phelps-Durr et al. 2005). Also, both correctly spliced *FLC* transcripts and intron-retaining variants accumulated to higher levels in an *flk* mutant than in wild-type plants (Ripoll et al. 2009), and repression of *AtSNI*, a retroelement which is subject to RNA-directed chromatin silencing, was at least partially released in mutant plants (Bäurle and Dean 2008; Velely and Michaels 2008). Together, these findings have been interpreted to indicate that *FLK* may suppress *FLC* at least partially at the transcriptional level, perhaps through RNA-directed chromatin silencing (Velely and Michaels 2008; Ripoll et al. 2009). Ripoll et al. (2009) further found that *PEPPER* (*PEP*), a paralogue of *FLK* in Arabidopsis, acts as a positive regulator of *FLC*. The authors showed that *pep* mutations can at least partially rescue the flowering time phenotype of *flk* mutants, and that overexpression of *PEP* resulted in a similar effect on flowering time as mutation of *FLK*. Overexpression of *PEP* in an *flk* mutant background neither further delayed flowering nor led to an increase of *FLC* expression when compared to *flk* mutant plants not carrying the *PEP* transgene, suggesting that *FLK* and *PEP* may interact in the same genetic pathway (Ripoll et al. 2009).

Chromatin level control of *FLC* expression is mediated by *FLD*, *FVE* and *LD*. *FLD* is a homologue of the human histone H3K4 demethylase *LSD1* (*LYSINE-SPECIFIC HISTONE DEMETHYLASE1*) and, in *A. thaliana*, represses *FLC* by H3K4 demethylation and H4 deacetylation of *FLC* chromatin, possibly as part of a co-repressor complex (He et al. 2003; Jiang et al. 2007) and is dependent on the sumoylation state of *FLD* (Jin et al. 2008). The Arabidopsis genome contains three additional homologues of *FLD*, *LSD1-LIKE1* (*LDL1*), *LDL2* and *LDL3*, two of which (*LDL1* and *LDL2*) have been shown to act in partial redundancy with *FLD* to repress *FLC* (Jiang et al. 2007). Furthermore, *LDL1* (also termed *SWP1*, *SWIRM DOMAIN PAO PROTEIN 1*), interacts with the histone methyltransferase *CZS* (*C2H2 ZINC FINGER-SET DOMAIN PROTEIN*) and is part of a co-repressor complex which represses *FLC* by H4 deacetylation and H3K9 and H3K27 methylation at the *FLC* locus (Krichevsky et al. 2007). *LD*, a unique nuclear localized protein in Arabidopsis which contains a homeodomain-like domain (Lee et al. 1994; Aukerman et al. 1999), also appears to regulate *FLC* expression by histone modification, including H3K4 demethylation and H3 deacetylation (Domagalska et al. 2007), but may also repress *FLC* by a negative regulatory interaction with a transcriptional activator of *FLC*, *SUF4* (*SUPPRESSOR OF FRIGIDA 4*; Kim et al. 2006). *FVE* (also termed *MSI4* (*MULTICOPY SUPPRESSOR OF IRA1 4*) and *ACGI* (*ALTERED COLD-RESPONSIVE GENE EXPRESSION 1*)) is homologous to *MSI1* (*MULTICOPY SUPPRESSOR OF IRA1*) in yeast and retinoblastoma-associated proteins in animals, which are components of chromatin assembly complexes. *FVE* is part of a small family of *MSI1*-like WD40 repeat proteins in Arabidopsis (Kenzior and Folk 1998; Ausin et al. 2004; Kim et al. 2004; Hennig et al. 2005). In *fve* mutants, histones H3 and H4 are hyperacetylated in *FLC* chromatin, suggesting that *FVE*, perhaps together with *FLD*, is part of a complex which represses *FLC* expression by chromatin modification (He et al. 2003; Ausin et al. 2004; Amasino 2004). *FVE* and other *MSI1*-like proteins are generally thought of as structural proteins without catalytic function and may provide a scaffold for assembly of larger complexes. *FVE* has also been implicated in temperature-dependent regulation of flowering time, and appears to promote flowering in response to elevated ambient

temperatures through an *FLC*-independent, thermosensory pathway which also includes *FCA* (Blazquez et al. 2003). In addition, *FVE* mediates the plant's response to intermittent cold stress and may provide a link between cold stress response and flowering time control (Kim et al. 2004; Franklin and Whitelam 2007). *FVE* is expressed in all major plant organs but appears to be preferentially expressed in actively dividing cells, and has been assigned a more general role in the regulation of cellular differentiation and developmental transitions (Morel et al. 2009). Recent grafting experiments in *A. thaliana* suggest that *FVE* mRNA is phloem mobile and may contribute to long-distance signalling in plant development (Yang and Yu 2010).

There is increasing evidence that several, if not all, autonomous pathway genes also regulate developmental processes other than floral transition. In particular, double mutant analyses showed that *fpa fld*, *fpa fve* and *fpa ld* mutants have pleiotropic, *FLC*-independent effects on growth rate, chlorophyll content, leaf morphology, flower development and fertility, and that the corresponding genes have partially redundant functions (Veley and Michaels 2008). Furthermore, mutations in *FCA*, *FVE* and *LD* result in an increase in the period length of the circadian clock, thus implicating autonomous pathway genes in the regulation of the clock (Salathia et al. 2006). Finally, transposon and transgene silencing assays in mutants indicated that *FCA*, *FPA*, *FLK* and *FVE* have a more widespread role in RNA-directed chromatin silencing of a range of target genes (Bäurle et al. 2007; Bäurle and Dean 2008; Veley and Michaels 2008). For *FCA*, *FVE*, *FY* and *LD* at least partial conservation of floral regulatory functions was shown in monocots (van Nocker et al. 2000; Baek et al. 2008; Lee et al. 2005; Lu et al. 2006; Jang et al. 2009).

Here, the components of the autonomous pathway in *B. vulgaris* have begun to be dissected through a survey of ESTs with homology to autonomous pathway genes and isolation of the corresponding genes. For beet homologues of four autonomous pathway genes, termed *BvFLK*, *BvFVE1*, *BvLD* and *BvLDL1*, the full-length genomic and coding sequences were identified and the genes were mapped on a reference map of the sugar beet genome. Exon-intron structure and domain organization was found to be conserved between beet and *Arabidopsis* in all four genes. One homologue each of autonomous pathway genes implicated in RNA- or chromatin regulatory mechanisms, *BvFLK* and *BvFVE1*, respectively, was further characterized by overexpression and complementation analysis in *A. thaliana* wild type and mutants. *BvFLK* was able to accelerate bolting time in *A. thaliana* wild type and complement the late-bolting phenotype of an *flk* mutant. In contrast, *BvFVE1* was unable to complement an *fve* mutant, and was found to be under circadian clock regulation in beet, which has not been reported for *FVE* in *Arabidopsis*. Together, data suggest conservation of autonomous pathway components in *B. vulgaris*, while also providing first evidence for divergence or subfunctionalization at least of one autonomous pathway gene homologue.

3.3 Material and Methods

3.3.1 Bioinformatic analysis

The *Beta vulgaris* EST database BvGI (*Beta vulgaris* Gene Index, versions 2.0 and 3.0; <http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=beet>) and the *B. vulgaris* subsets of the NCBI EST and nt/nr databases (<http://www.ncbi.nlm.nih.gov>) were used to identify beet homologues of flowering time genes in *A. thaliana*. Database searches were performed using the tblastn algorithm (Altschul et al. 1990) and *A. thaliana* protein sequences from the Arabidopsis Genome Initiative (AGI; <http://www.arabidopsis.org/tools/bulk/sequences/index.jsp>) as queries. To help infer orthology by bidirectional best hit (BBH) analysis (Overbeek et al. 1999), the beet sequences retrieved through this analysis were used as queries for blastx searches against *A. thaliana*

protein sequences at NCBI (<http://www.ncbi.nlm.nih.gov>) and TAIR (<http://www.arabidopsis.org>). *B. vulgaris* sequences were annotated using pairwise sequence alignments (BLAST2; <http://www.ncbi.nlm.nih.gov>) against putative *A. thaliana* orthologues and the FGENESH+ and FGENESH_C gene prediction programs (<http://linux1.softberry.com/berry.phtml>) for annotation of exon-intron structures, TSSP (<http://linux1.softberry.com/berry.phtml>) and PLACE for annotation of promoter regions (<http://www.dna.affrc.go.jp/PLACE>; Higo et al. 1999), and PFAM (<http://pfam.sanger.ac.uk>) for identification of conserved protein domains. Multiple sequence alignments were made using CLUSTAL W (<http://www.ebi.ac.uk/Tools/clustalw/index.html>). Amino acid identity was calculated as the percentage of identical residues in two homologues divided by the total number of residues in the reference gene. For phylogenetic analysis, putative *FLK* and *FVE* orthologues in other plant species were identified by blastp searches of the NCBI Reference Sequence (RefSeq) protein database (<http://www.ncbi.nlm.nih.gov/refseq>) and BBH analysis essentially as described above, except that the blastp algorithm was used. The sequences were aligned using CLUSTAL W, and unrooted phylogenetic trees were constructed using the neighbor-joining algorithm and the Dayhoff PAM matrix as implemented in the MEGA4 software (Tamura et al. 2007).

3.3.2 Plant material and growth conditions

For expression analysis in different tissues, the biennial *B. vulgaris* accession A906001 (El-Mezawy et al. 2002) was grown in the greenhouse under long-day conditions supplemented with artificial light (Son-T Agro 400W (Koninklijke Philips Electronics N.V., Eindhoven, The Netherlands) for 16h). Six week old plants were vernalized under short day (8h light) conditions at 5°C for three months. Plants were returned to the greenhouse and continued to be grown under the same conditions as before vernalization until the first flowers opened (BBCH scale 60; Meier 2001). For diurnal and circadian expression the commercial biennial cultivar Roberta (KWS Saatzucht GmbH, Einbeck, Germany) was grown under defined light regimes (long days of 16h and short days of 8h) in Sanyo Gallenkamp MLR 350 growth chambers at 22°C. Lighting was supplied by 36 Watt fluorescent Daystar lamps (CEC Technology) providing 300 $\mu\text{mol m}^{-2} \text{sec}^{-1}$ of photosynthetically active radiation.

A. thaliana flowering time gene mutants SALK_112850 (*flk-1*; Alonso et al. 2003; Lim et al. 2004) and SALK_013789 (Alonso et al. 2003), which will be referred to as *fve-7* (following on from the *fve* mutant number in Morel et al. 2009), were received from the Nottingham Arabidopsis Stock Centre (NASC; <http://arabidopsis.info/>). The *fve-7* mutant carries a T-DNA insertion in intron 1 at nucleotide position 2835 of the genomic sequence entry for *FVE* in GenBank (accession number AF498101). Mutants homozygous for the T-DNA inserts in *FLK* or *FVE*, respectively, were identified by PCR using a T-DNA-specific primer (A479 for *flk-1*, B478 for *fve-7*; for primer sequences see Supplementary Table S4 available at JXB online) in combination with a gene-specific primer (A477 for *flk-1*, B476 for *fve-7*). The absence of the wild-type alleles was confirmed by PCR with gene-specific primers which flank the insert on either side (A477 and A478 for *flk-1*, and B476 and B477 for *fve-7*). *A. thaliana* Col-0 (wild-type) plants, mutants, and the T1 and T2 generations of transgenic plants were phenotyped for bolting time under long-day conditions (16h light, Osram L58 W77 Fluora and Osram L58 W840 Lumilux Cool White Hg) at 22°C in a growth chamber (BBC Brown Boveri York, Mannheim, Germany).

3.3.3 BAC library screening

EST sequence information was used to generate genomic fragments for use as probes to screen the *B. vulgaris* BAC library described by Schulte et al. (2006). Probe fragments were generated by PCR amplification using primer combinations A039-A064 for *BvFLK* (717 bp),

A066-A042 for *BvFVE1* (1089 bp), A043-A067 for *BvLD* (589 bp) and A033-A065 for *BvLDL1* (678 bp), and genomic DNA of A906001 as template, and purified using the Montage PCR₉₆ Cleanup Kit (Millipore Corporation, Bedford, USA). The probes were labeled and hybridized to high-density BAC filters essentially as described by Hohmann et al. (2003). Positive clones were verified by PCR analysis using the same primer combinations as for PCR amplification of probe fragments. BAC DNA was isolated using the NucleoBond BAC 100 kit according to the manufacturer's protocol (Macherey-Nagel, Dürren, Germany). The genes-of-interest were sequenced by primer walking (*BvFLK*) or whole BAC sequencing (*BvFVE1*, *BvLD*, *BvLDL1*) on a GS20 sequencing machine (MWG, Ebersberg, Germany).

3.3.4 RT-PCR and RACE

Total RNA was extracted from roots, stems, leaves and flowers of adult plants of accession A906001 using the Plant RNAeasyKitTM (Qiagen, Hilden, Germany) and DNase treated (Ambion, Austin, USA). 1.5 µg of RNA was reverse transcribed using the First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany), and the cDNA was diluted ten times for RT-PCR. The complete coding sequences of *BvFLK*, *BvFVE1* and *BvLDL1* were amplified using leaf cDNA as template and primer combinations A396-A397, A836-A837, and A823-A825, respectively. The coding sequence of *BvLD* was amplified by RT-PCR of several overlapping fragments, using primer combinations A067-A108, B391-A046, A043-B392, A068-A069, and A045-B396. Primers for RT-qPCR were designed and optimized to 94.6% amplification efficiency for *BvFLK* (primers B042-B043), and 92.9% for *BvFVE1* (primers A066-A042). Fluorescence of Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen Corporation, Carlsbad, CA, USA) was measured in a CFX96 real-time PCR machine (Bio-Rad, Munich, Germany) over 40 cycles at annealing temperatures of 65°C for *BvFLK* and *BvFVE1*, and 60°C for glyceraldehyde 3-phosphate dehydrogenase (*BvGAPDH*; BvGI 2.0 accession number TC1351; RT-qPCR amplification efficiency 96.3%). Expression levels were measured in triplicate and normalized against the reference gene *BvGAPDH*.

For diurnal and circadian expression analysis first-strand cDNA was prepared and analyzed by RT-qPCR as described (Chia et al. 2008) except that an in-solution DNase treatment (Ambion, Austin, USA) was used and that expression data were normalized against three *B. vulgaris* housekeeping genes, *BvGAPDH* (see above), elongation factor 1-alpha (*BvEF1α*; BvGI 2.0 accession number TC5), and elongation factor 2 (*BvEF2*; BvGI 2.0 accession number TC64). A normalization factor (NF) was generated for each sample using the geNorm Software v3.5 (<http://medgen.ugent.be/~jvdesomp/genorm/>; Vandesompele et al. 2002). The NF factor was used to normalize and calculate the relative expression values for the genes-of-interest. All primers for normalizer genes and *BvFVE1* were optimized to 98-110% amplification efficiency using serial dilutions of sugar beet leaf cDNA. Amplified fragments were sequenced to confirm specificity. Fluorescence of the bound SYBR-GREEN (Stratagene, La Jolla, CA, USA) was detected in an MX3000 real-time PCR machine (Stratagene) over 40 cycles at 60°C annealing temperature.

The 5' end of *BvFVE1* was identified by 5'-RACE (Frohman et al. 1988) using the GeneRacerTM kit according to the manufacturer's protocol (Invitrogen Corporation). 5'-RACE was performed on double-stranded adaptor-ligated cDNA synthesized from 5 µg total RNA from leaves of four week old sugar beet plants using exon-specific primers (5' RACE primer A830 and 5' RACE nested primer A831). 5' RACE fragments were cloned into the pGEM-T vector (Promega Corporation, Madison, WI, USA) and sequenced using standard Sp6 and T7 primers.

For expression analysis of *FLC* and *FLK* in *A. thaliana*, total RNA was isolated from rosette leaves of 30 day old plants using the Plant RNAeasyKitTM (Qiagen) and DNase treated

(Fermentas). 2 µg of RNA was reverse transcribed using the First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany), and the cDNA was diluted ten times for RT-qPCR. Amplification efficiency of *FLC* (GenBank accession number NM_121052, primers B336-B337), *FLK* (GenBank accession number AC011437, B281 and B282), and *GAPDH* (GenBank accession number NM_111283; B349-B350) was 100%, 97.4% and 94.2%, respectively.

3.3.5 Vector construction and transformation of *A. thaliana*

The coding sequences of *BvFLK* and *BvFVE1* were amplified as described above. The *BvFLK* coding sequence was cloned into pGEM-T (Promega Corporation) to yield plasmid pFT002, and re-amplified from pFT002 with primers A680-*XhoI* and A652-*SpeI*. The PCR product was restricted with *XhoI* and *SpeI* (Fermentas, St. Leon-Rot, Germany) and cloned into the corresponding restriction enzyme sites of the binary vector pSR752Ω (kindly provided by Chonglie Ma and Richard Jorgensen, University of Arizona, Tucson, AZ, USA) which carries the Cauliflower Mosaic Virus (CaMV) 35S promoter. The resulting construct was designated pFT013. A 1813 bp fragment carrying the endogenous promoter region of *BvFLK* and 363 bp of the 5' region of the coding sequence was amplified from BAC DS 794 using primers A896-*EcoRI* and A821-*HpaI*. The fragment was restricted with *EcoRI* and *HpaI* and inserted into the corresponding restriction sites of pFT013, thus effectively replacing the CaMV 35S promoter by the 1435 bp endogenous beet sequence upstream of the *BvFLK* start codon in the resultant plasmid (pFT033). A plasmid carrying the coding region of *FLK* (GenBank accession number BX823281) was kindly provided by the French Genomic Resource Center (INRA-CNRCV, Castanet Tolosan cedex, France). The coding sequence of *AtFLK* was amplified using primers A901-*XhoI* and A938-*SmaI*, and inserted into the corresponding restriction sites of pSR752Ω. The resulting vector carries the *FLK* coding sequence under the control of the CaMV 35S promoter and was designated pFT016. The coding sequence of *BvFVE1* was cloned into pDONOR221 using the Gateway Cloning System (Invitrogen Corporation) to yield plasmid pBS355. The coding sequence was subsequently transferred into the pEarleyGate 100 vector (Earley et al. 2006) to yield the binary vector pBS356, in which the *BvFVE1* coding sequence is under the control of the CaMV 35S promoter.

The intactness of the binary vectors and the sequence of all inserts was confirmed by restriction enzyme digests, PCR amplification and sequencing (Institute of Clinical Molecular Biology, Kiel, Germany). The constructs were transferred by electroporation into *Agrobacterium tumefaciens* LBA 4404 using Electromax (Invitrogen Corporation) competent cells (pFT013, pFT016 and pFT033) or *A. tumefaciens* GV2260 competent cells prepared according to the protocol by Mersereau et al. (1990) (pBS356), and transformed into *A. thaliana* by the floral dip method (Clough and Bent 1998). Primary transformants (T1 plants) were selected by spraying BASTA (Bayer CropScience, Wolfenbüttel, Germany) at a concentration of 1.7 g/l at the two-leaf stage. The presence of the transgene in BASTA resistant plants was confirmed by PCR analysis using primer combinations B042-B043 for pFT013 and pFT033, B281-B282 for pFT016 and A875-A876 for pBS356. The transformants were propagated by selfing to produce T2 seed. Genomic DNA was extracted using the NucleoSpin 96 Plant DNA isolation kit (Macherey and Nagel, Düren, Germany).

3.3.6 Genetic mapping and statistical analysis

Flowering time genes were mapped genetically in the D2 (100 F₂ individuals) and K1 (97 F₂ individuals) reference populations described by Schneider et al. (2007). Polymorphisms were identified by PCR amplification and sequencing of genomic fragments using DNA from the parent and F₁ plants as template. Map positions were calculated using Join Map 3.0 (Van

Ooijen and Voorrips, 2001) and the Kosambi mapping function (Kosambi 1944) at a LOD score of 4.0.

Analysis of variance and t-tests were performed using SAS 9.1 TS level 1M3 (SAS Institute, Cary, NC, USA). Sample groups with significantly different means were further analyzed using Fisher's Least Significant Difference (LSD) test at 5% probability level (SAS 9.1 TS level 1M3).

3.4 Results

3.4.1 *Beta vulgaris* homologues of the autonomous pathway genes

To identify autonomous pathway gene candidates in beet, the *B. vulgaris* EST database BvGI (versions 2.0 and 3.0) and the *B. vulgaris* subsets of the NCBI EST and nt/nr databases were searched for homologues to autonomous pathway genes in *A. thaliana* (see Materials and Methods). Among the seven classical genes assigned to the autonomous pathway in Arabidopsis (*FCA*, *FLD*, *FLK*, *FPA*, *FVE*, *FY*, *LD*) four were found to have putative orthologues in beet [*FLK* (BQ586739), *FPA* (BQ489608), *FVE* (BQ592158, EG550040), *LD* (BQ589018, BQ594506); Table 7]. Besides *FLK*, one EST (BQ590839) was identified whose closest homologue in *A. thaliana* is *PEP*, an *FLK* paralogue (Ripoll et al. 2009; see Introduction). In addition, one EST (CV301493) with homology to *FLD* appears to be orthologous to *LDLI/SWPI*, an *FLD*-like gene in Arabidopsis which recently was also assigned to the autonomous pathway (Jiang et al. 2007; Krichesky et al. 2007; see Introduction). Orthologous ESTs were not identified for *FLD*, *FCA* and *FY*.

Table 7: *B. vulgaris* ESTs with homology to *A. thaliana* autonomous pathway genes.

At locus number	Gene	Best hit(s) in <i>B. vulgaris</i> (GenBank accession number) ^a	E-value	Best hit in <i>A. thaliana</i> (At locus number or gene name) ^b
At4g16280	<i>FCA</i>	TC13484	6.1e-17	At1g44910
At3g10390	<i>FLD</i>	CV301493	6.6e-71	<i>LDLI/SWPI</i>
At3g04610	<i>FLK</i>	BQ586739	8.4e-83	<i>FLK</i>
		BQ590839	8.8e-44	<i>PEP</i>
At2g43410	<i>FPA</i>	BQ489608	7.4e-17	<i>FPA</i>
At2g19520	<i>FVE</i>	EG550040	4.4e-85	<i>FVE</i>
		BQ592158	2.4e-62	<i>FVE</i>
At5g13480	<i>FY</i>	BQ588779	1.3e-15	At4g02730
At4g02560	<i>LD</i>	BQ589018	4.0e-27	<i>LD</i>
		BQ594506	1.6e-18	<i>LD</i>

^a*Beta vulgaris* ESTs or TCs (tentative consensus sequences) were identified by tblastn sequence similarity searches in BvGI 3.0 (<http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=beet>)

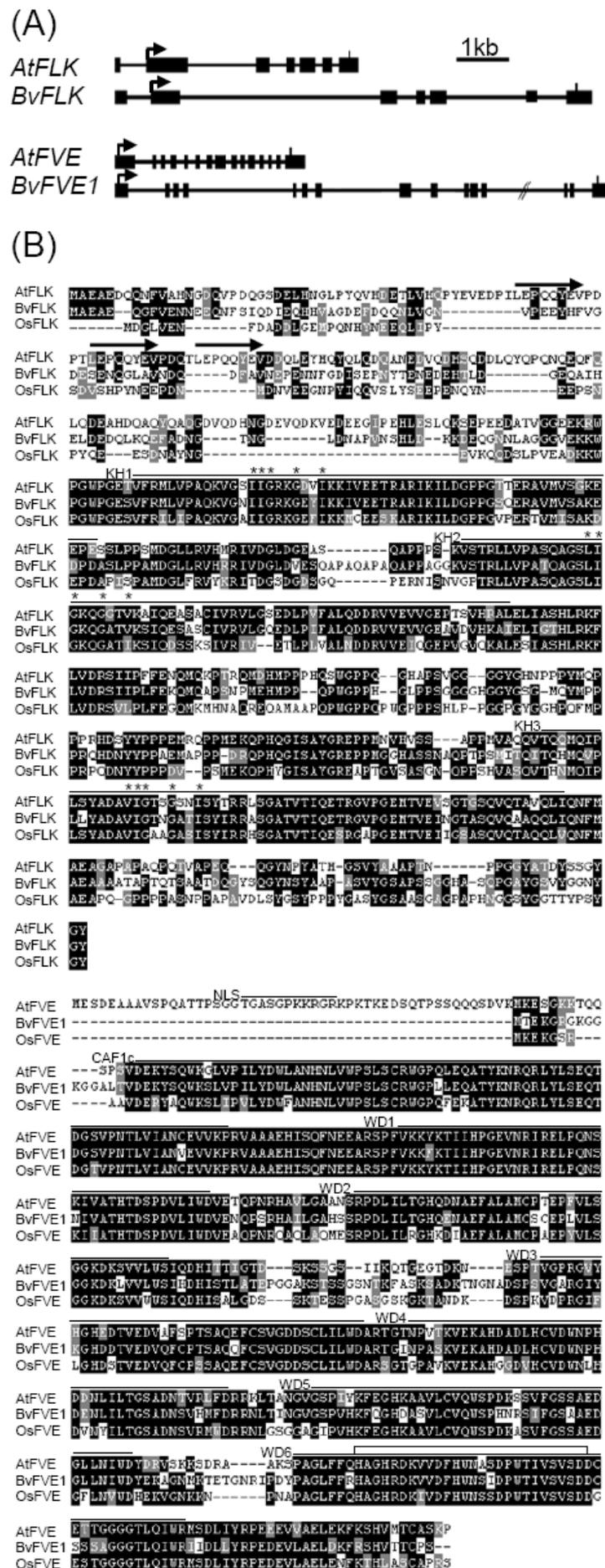
^bBest hits in *A. thaliana* were identified by blastx in the TAIR9 protein database (<http://www.arabidopsis.org>)

For four putative autonomous pathway genes, named *BvFLK*, *BvFVEI* (corresponding to one of the two homologous ESTs), *BvLD* and *BvLDLI*, the complete genomic sequence was identified by BAC library screening and BAC sequencing or primer walking. The two ESTs with homology to *LD* were both found to derive from the same gene. The exon-intron structure of *BvFLK*, *BvFVEI*, *BvLD* and *BvLDLI* was determined by RT-PCR and sequencing. The number of exons and the sites of introns were found to be conserved between the corresponding *A. thaliana* and *B. vulgaris* genes (Fig. 7A; Supplementary Fig. S2 available at JXB online). However, several of the *B. vulgaris* introns are substantially larger than the respective introns in *A. thaliana* and contain repetitive elements such as minisatellites

and various short low complexity and/or simple repeat regions [e.g. (AT)_n], but longer transposons or retroelements were not identified. *BvLDL1*, like *LDL1* in *A. thaliana* (but unlike *FLD*), contains only a single exon. The coding sequences of the four *B. vulgaris* genes are somewhat shorter than those of their Arabidopsis counterparts (*BvFLK* 1674 bp vs. *FLK* 1731 bp, *BvFVE1* 1413 bp vs. *FVE* 1524 bp; *BvLD* 2829 bp vs. *LD* 2859 bp; *BvLDL1* 2487 bp vs. *LDL1* 2532 bp).

The predicted protein sequences were aligned against the corresponding *A. thaliana* genes (Fig. 7B; Supplementary Fig. S2 available at JXB online). For *FLK* and *FVE*, homologues had also been identified in rice (Lim et al. 2004; Baek et al. 2008) and were included in the alignment. The overall amino acid sequence identity between the proteins in *A. thaliana* and *B. vulgaris* was highest for *FVE* (72%), intermediate for *FLK* (57%) and *LDL1* (58%), and relatively low for *LD* (43%). The domain organization of all four proteins was largely conserved, and the degree of sequence conservation between homologues was highest within domains. In particular, all domains in *BvFLK* (three KH-type RNA binding domains, with 77-88% amino acid identity to the corresponding domains in the *A. thaliana* homologue) and in *BvFVE1* were highly conserved [a chromatin assembly factor 1 subunit C (CAF1c) domain with 96% amino acid identity, and six WD40 repeat domains with 83-95% amino acid identity]. In contrast, in *BvFLK*, *BvFVE1* and *BvLDL1*, the N-terminal protein regions are only lowly conserved. Furthermore, 50 amino acids at the N terminus of *FVE* including a putative nuclear localization signal (amino acids 20-30; Ausin et al. 2004) are absent in *BvFVE1*.

Figure 7: (s. next page) Sequence and structure of the autonomous pathway gene homologues *BvFLK* and *BvFVE1*. (A) Exon-intron structure of *BvFLK*, *BvFVE1* and the respective *A. thaliana* genes (*FLK*, accession number AAX51268; *FVE*, accession number AF498101). Exons are indicated as black rectangles, the position of start and stop codons is indicated by arrows and vertical bars, respectively. (B) Pairwise sequence alignments and domain organization. The alignments were generated using ClustalW2 (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>). Identical and similar residues are highlighted by black or grey boxes, respectively. The position of protein domains according to Pfam 22.0 (<http://pfam.sanger.ac.uk/>) is marked by horizontal lines above the alignment. In *FLK*, the position of three perfect 8-residue repeats and the core residues of K-homology RNA binding (KH) domains (Mockler et al. 2004) are indicated by arrows and asterisks, respectively. The first and sixth WD40 repeat domains (WD1 and WD6) were not identified by Pfam and were annotated according to Ausin et al. (2004). A putative nuclear localization signal (NLS; black line) in *FVE* according to Ausin et al. (2004) and a potential zinc binding site (unfilled box) in WD6 (Kenzior and Folk 1998) are also indicated. WD, WD40 repeat domain; CAF1c, CAF1 subunit C /histone binding protein RBBP4 domain.



3.4.2 Genetic map position

The genes were mapped on a single nucleotide polymorphism (SNP)-based genetic reference map of expressed genes in sugar beet (Schneider et al. 2007). *BvFLK* carries a SNP in the 3' UTR and was mapped to chromosome IV, where it colocalizes with marker TG_0502b at a cumulative genetic distance of 45.7 cM (Fig. 8). *BvFVE1* carries three SNPs within a 1457 bp fragment of intron four and was mapped to a position at the very distal end of chromosome VII. This map position is consistent with the presence of several sequences on the BAC clone that carries *BvFVE1*, which show homology to *ApaI* and *RsaI* satellite sequences known to be located in subtelomeric regions in sugar beet (Dechyeva and Schmidt 2006), including several subtelomeric repeats located just upstream of *BvFVE1*. The two ESTs corresponding to *BvLD* had been mapped previously to chromosome VII by Schneider et al. (2007) and are represented by markers TG_E0240 (BQ589018) and TG_E0226 (BQ594506; Fig. 8). *BvLDL1* was mapped to position 10.90 cM on chromosome IX (data not shown).

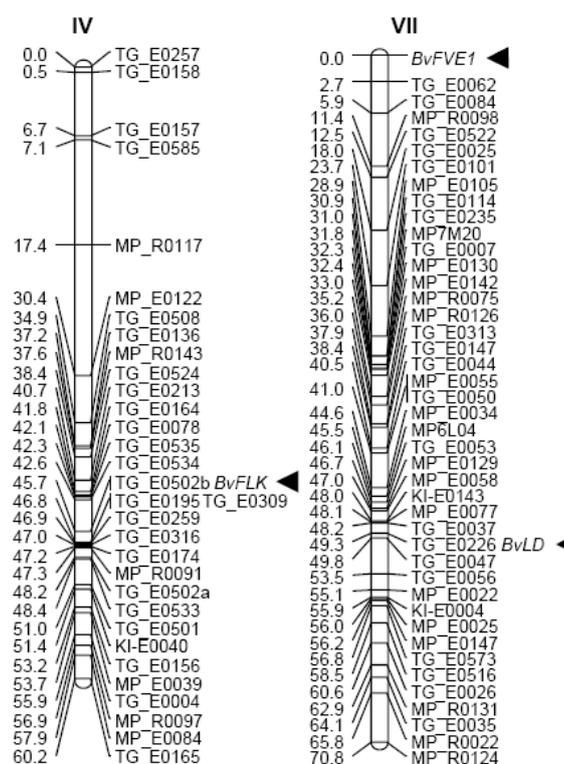


Figure 8: Genetic map positions. *BvFLK* and *BvFVE1* (arrowheads) were mapped to position 45.7 cM on chromosome IV and the top end of chromosome VII, respectively, on a reference map of the sugar beet genome (Schneider et al. 2007). The map position of an EST which had been mapped previously (Schneider et al. 2007) and corresponds to *BvLD* is also indicated. Genetic distances in centiMorgan (cM) are given on the left, marker names on the right.

3.4.3 *BvFLK* accelerates the time to flowering in *Arabidopsis* and complements the *flk1* mutant

Overexpression and complementation analyses in *A. thaliana* wild-type and mutant plants were employed to assess whether the function of autonomous pathway gene homologues is conserved between beet and *Arabidopsis*. *BvFLK* and *BvFVE1* were chosen as homologues of autonomous pathway genes which, respectively, are putative RNA-regulatory genes or exert their function at the level of chromatin.

The coding sequence of *BvFLK* driven by the CaMV 35S promoter ('35S::*BvFLK*'), the *BvFLK* coding sequence driven by the 1435bp genomic region upstream of the *BvFLK* coding sequence in beet, which will be referred to as the endogenous *BvFLK* promoter ('endo::*BvFLK*'), and the coding sequence of *FLK* under the control of the CaMV 35S promoter ('35S::*AtFLK*') were transformed into *A. thaliana* Col-0 and the *flk* mutant SALK_112850 [Alonso et al. 2003; corresponding to *flk-1* in Lim et al. 2004, and *flk-4* in Mockler et al. 2004]. Under long-day conditions, bolting in the *flk-1* mutant was found to be delayed by 31-33 days when compared to Col-0, the genetic background of the mutant (Table 8).

Selection with BASTA and PCR analysis of transgene integration identified nine to 32 primary (T1) transformants derived from transformation of the *BvFLK* transgene cassettes into Col-0 or the *flk-1* mutant, and ten and four transformants, respectively, derived from transformation of the 35S::*AtFLK* transgene cassette. Bolting time in Col-0 plants transformed with either of the transgene cassettes was approximately five to six days earlier than in Col-0 control plants, and this effect did not differ significantly between the three sets of transformants (Table 8). The total number of leaves at bolting was only slightly reduced in the transformants, but differed significantly from the Col-0 control plants in the 35S::*BvFLK* and endo::*BvFLK* transgenic plants. Expression of the transgene cassettes in the *flk-1* mutant background fully rescued the phenotype both in regard to bolting time and numbers of leaves at bolting. All three sets of transformants bolted approximately 33-36 days earlier than the *flk-1* mutant.

Nine to ten T1 plants of each of the sets of transformants carrying the 35S::*BvFLK* or endo::*BvFLK* transgene cassettes in either the Col-0 or *flk-1* mutant background, and all 35S::*AtFLK* transformants were selfed to produce T2 seed. In the T2 families derived from transformation into Col-0, plants which bolted >5 d earlier than the mean of the Col-0 control plants (37.88±1.50 days to bolting) were initially considered as candidates for transgenic segregants. According to this criterium, three to nine of the T2 families from each of the three sets of transformants exhibited segregation of the number of early-bolting plants (24-33 days to bolting) to the number of plants which bolted within the time range observed for the control plants (35-40 days to bolting) of 13:4 to 16:1, which did not deviate significantly from the 3:1 or 15:1 ratios expected for one to two transgene loci (as tested by χ^2 analysis; Supplementary Table S5 at JXB online). In the remaining families all plants bolted >5 d earlier than the control plants. The T2 families derived from transformation into the *flk-1* mutant consisted of plants which bolted either as late as the *flk-1* mutant controls (63-77 days to bolting, with a mean and standard deviation of 69.82±4.63), or much earlier (25-44 days to bolting). In one to three of the T2 families from each of the three sets of transformants, early and late-bolting plants segregated at ratios as above, while the remaining families only contained early-bolting plants.

Table 8: Number of days to bolting (DTB) and total number of leaves at bolting (TNL) of primary transformants (T1 generation) and transgenic plants in segregating T2 populations derived from transformation of *BvFLK* and *FLK* into *A. thaliana* Col-0 and the *flk-1* mutant SALK_112850 (*flk-1*).

T1 generation						
Genotype	Overexpression in Col-0			Complementation in <i>flk-1</i>		
	Number of plants	DTB (mean \pm standard deviation)	TNL (mean \pm standard deviation)	Number of plants	DTB (mean \pm standard deviation)	TNL (mean \pm standard deviation)
35S:: <i>BvFLK</i>	32	29.59 \pm 2.43 A	10.59 \pm 1.83 A	25	32.00 \pm 2.10 A	13.08 \pm 1.55 A
endo:: <i>BvFLK</i>	15	30.00 \pm 2.56 A	11.20 \pm 1.52 A	9	34.89 \pm 3.79 A	13.33 \pm 2.29 A
35S:: <i>AtFLK</i>	10	30.90 \pm 2.08 A	12.00 \pm 1.94 B	4	31.50 \pm 4.20 A	10.57 \pm 1.26 A
Col-0	16	35.69 \pm 1.70 B	13.31 \pm 1.14 B	16	35.69 \pm 1.70 A	13.31 \pm 1.14 A
<i>flk-1</i> mutant	-	-	-	22	67.71 \pm 7.15 B	66.35 \pm 1.32 B
F value (prob.)		27.45 (0.00)	10.03 (0.00)		383.00 (0.00)	3397.00 (0.00)
LSD _{0.05} ^a		2.14	1.46		4.42	2.05

T2 generation						
Genotype	Overexpression in Col-0			Complementation in <i>flk-1</i>		
	Number of plants	DTB (mean \pm standard deviation)	TNL (mean \pm standard deviation)	Number of plants	DTB (mean \pm standard deviation)	TNL (mean \pm standard deviation)
35S:: <i>BvFLK</i>	13	27.77 \pm 2.59 A	15.23 \pm 1.79 A	14	34.14 \pm 3.06 A	15.43 \pm 1.16 A
endo:: <i>BvFLK</i>	13	28.46 \pm 1.66 A	15.31 \pm 1.71 A	12	33.92 \pm 3.62 A	15.51 \pm 1.29 A
35S:: <i>AtFLK</i>	14	27.23 \pm 2.05 A	15.00 \pm 2.00 A	15	34.13 \pm 5.60 A	15.80 \pm 1.08 A
Col-0	17	37.88 \pm 1.50 B	17.53 \pm 1.01 B	17	37.88 \pm 1.50 A	17.53 \pm 1.01 A
<i>flk-1</i> mutant	-	-	-	17	69.82 \pm 4.63 B	67.82 \pm 2.01 B
F value (prob.)		95.38 (0.00)	8.34 (0.00)		222.44 (0.00)	2307.00 (0.00)
LSD _{0.05} ^a		1.85	1.41		4.06	1.86

^aFisher's Least Significant Difference at $\alpha=0.05$. The letters A and B indicate significant differences between the mean values given in a table column (i.e. mean values in table cells including the letter 'B' are significantly different from the mean values in table cells including the letter 'A')

From all six sets of transformants, one T2 family each which segregated for early and late bolting (at a ratio which did not deviate significantly from 3:1) was selected for co-segregation analysis of the early-bolting phenotype and the transgene. As expected, all plants which bolted early were found to be transgenic, whereas none of the remaining plants tested positive for the transgene (Supplementary Table S5 at JXB online). ANOVA of days to bolting and the number of leaves at bolting revealed highly significant differences for both traits between the transgenic subfamilies in T2 and the respective untransformed control plants (Table 8). Similarly, t-tests between the transgenic and non-transgenic individuals within a T2 family showed highly significant differences for both traits in all six families (Supplementary Table S6 at JXB online). For transgenic plants in the *flk-1* mutant background, the values for neither of the traits differed significantly from those for Col-0, thus confirming that the transgenes fully rescue the phenotype (Table 8; Fig. 9A).

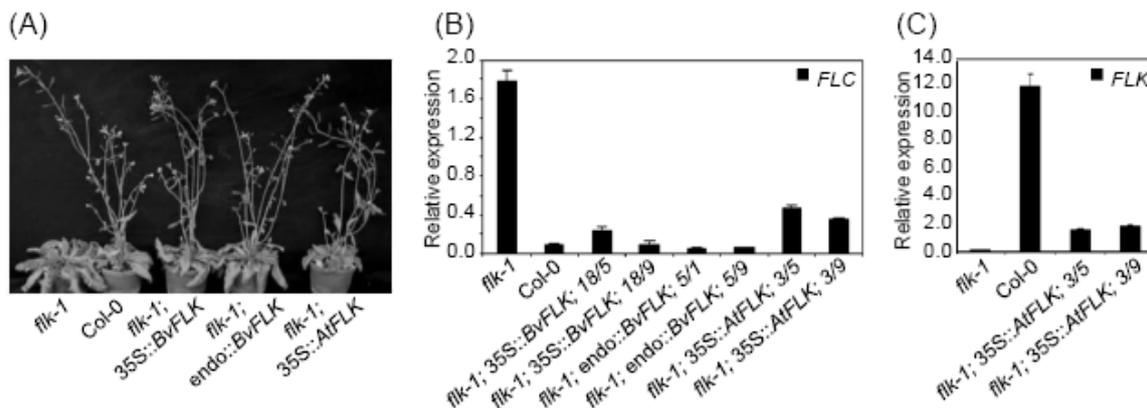


Figure 9: *BvFLK* complements the *A. thaliana flk-1* mutant. (A) Phenotypes at 51 days after sowing of the *A. thaliana flk-1* mutant, the ecotype Col-0, and the *flk-1* mutant transformed with *BvFLK* driven by the CaMV 35S promoter (35S::*BvFLK*), *BvFLK* driven by the endogenous promoter of *BvFLK* in sugar beet (endo::*BvFLK*), or *A. thaliana FLK* driven by the CaMV 35S promoter (35S::*AtFLK*) (T1 plants). Plants were grown under long-day conditions. (B) RT-qPCR expression analysis of *FLC* in *flk-1*, Col-0, and transgenic T3 plants carrying the 35S::*BvFLK*, endo::*BvFLK*, or 35S::*AtFLK* transgene in the *flk-1* mutant background. For each of the transgenic lines, two T3 plants were tested that were derived from different transgenic individuals of a T2 family. (C) RT-qPCR expression analysis of *FLK* in *flk-1*, Col-0, and transgenic 35S::*AtFLK* T3 plants. Expression levels in (B) and (C) were normalized against *GAPDH* and measured in triplicate. Error bars indicate standard deviations of the mean.

3.4.4 *BvFLK* represses *FLC* expression in Arabidopsis

To investigate further the functional conservation of *BvFLK* and *FLK*, it was tested whether the effect of *BvFLK* on bolting time in transgenic *A. thaliana* plants is mediated through *FLC*. Consistent with previous results obtained with different *flk* mutants (Lim et al. 2004; Mockler et al. 2004) *FLC* expression was significantly higher in the *flk-1* mutant than in Col-0 (Fig. 9B). By contrast, *FLC* expression levels in *flk-1* plants carrying the *BvFLK* transgene driven by either the 35S promoter or its endogenous promoter were strongly reduced compared to the untransformed mutant, and resembled the low expression of *FLC* in Col-0. *FLC* expression was also downregulated in *flk-1* plants expressing the 35S::*AtFLK* transgene, although expression was somewhat less reduced than in *BvFLK* transgenic plants or the Col-0 wild type. RT-qPCR of *FLK* confirmed the previous finding that *FLK* is not detectably expressed in *flk-1* (Lim et al. 2004), and showed further that expression of *FLK* in the 35S::*AtFLK* transgenic plants which were assayed for *FLC* expression was lower than in Col-0 wild type (Fig. 9C).

3.4.5 *BvFVE1* does not complement an *fve* mutation in Arabidopsis

To assess the possible role of *BvFVE1* in regulating floral transition, the coding sequence of *BvFVE1* driven by the CaMV 35S promoter (35S::*BvFVE1*) was transformed into *A. thaliana* Col-0 and the *fve* mutant SALK_013789 (Alonso et al. 2003; *fve-7*). Bolting under long-day conditions in *fve-7* was found to be delayed by 29-34 days when compared to Col-0 (Supplementary Table S7 at JXB online, and data not shown). Selection with BASTA and PCR analysis identified 16 and 27 transgenic events in the Col-0 and *fve-7* mutant background, respectively. All primary transformants were phenotyped for initiation of bolting, but showed a similar phenotype as the respective untransformed control plants (data not shown). Ten T1 plants from each set of transformants were selfed for further analysis in the T2 generation. Because the phenotypic data for the primary transformants suggested that the transgene may not or may only weakly affect bolting time, BASTA selection was used to identify T2 families in which the transgene is segregating. For each of the two sets of

transformants, one family was identified in which presence and absence of the transgene segregated at a ratio which did not deviate significantly from 3:1, and one family with a segregation ratio of > 15:1. For each of these four families, an additional 25 plants were grown without selection, phenotyped for bolting time and tested for the presence of the transgene by PCR. However, the time to bolting was generally very similar in the transgenic and the non-transgenic plants, and neither the means of numbers of days to bolting nor the means of numbers of leaves at bolting differed significantly between the two groups within a given family (Supplementary Table S7 at JXB online). In one exception, a T2 family which carried the 35Spro::*BvFVE1* transgene in the *five-7* mutant background and only contained transgenic plants among 21 plants tested (T2 family #32) bolted approximately six days earlier (59.95 ± 7.26 days to bolting) than control plants (65.75 ± 4.33 days to bolting; Supplementary Table S7 at JXB online). This difference was statistically significant by conventional criteria, but only marginally so at a p-value of 0.04. There was no significant difference between the means of total numbers of leaves at bolting between the two groups. The intactness of the 35S::*BvFVE1* transgene cassette and transgene expression was confirmed by PCR amplification and sequencing of the complete promoter and coding sequence of the transgene, and by RT-PCR, respectively (data not shown).

3.4.6 Transcript accumulation of *BvFVE1*, but not *BvFLK*, is under circadian clock control

Expression of *BvFLK* and *BvFVE1* in sugar beet was analyzed in four major plant organs, root, stem, leaf and flower, of adult plants. Both genes were found to be expressed in all samples analyzed. *BvFVE1* is relatively abundant in leaves and only lowly expressed in roots, whereas *BvFLK* is relatively highly expressed in roots and flowers (Fig. 10A). Because various autonomous pathway genes in *Arabidopsis* have been implicated in the regulation of the circadian clock (Salathia et al. 2006; see Introduction), and many regulators are subject to feedback regulation by the clock (Pruneda-Paz and Kay 2010), diurnal and circadian regulation of *BvFLK* and *BvFVE1* expression was investigated by RT-qPCR of transcripts in leaves from eight to ten week old plants (Fig. 10B). Under long-day (16 h light) conditions, *BvFLK* transcript levels fluctuated during the course of the day and appeared to be highest at 4 h of the light phase. To investigate circadian clock regulation, long day-entrained plants were moved to continuous light and transcript levels were monitored over the subsequent 48 hours. However, *BvFLK* expression did not oscillate and remained low throughout this period. *BvFVE1* expression, under long-day conditions, rises to a peak at 12 h, decreases during the first four hours of darkness, and rises again at the end of the night. In sharp contrast to *BvFLK*, *BvFVE1* expression continued to oscillate robustly throughout each 24 h period under continuous light, although peak amplitude was higher than during the entrainment phase. A promoter sequence analysis for both genes identified a region ~0.5-1 kb upstream of the transcription start site of *BvFLK* in which multiple root motifs (Elmayan and Tepfer 1995) and phytohormone-response elements, including four cytokinin-response elements (Fusada et al. 2005), are clustered, whereas consensus sequences for light-regulated elements appear to be relatively scarce (Supplementary Fig. S3 at JXB online). Sequence elements involved in circadian regulation were not identified. By contrast, the *BvFVE1* promoter contains a number of elements which have been implicated in light or circadian regulation, including two SORLIPs (sequences over-represented in light-induced promoters), one SORLREP (sequence over-represented in light-repressed promoters), eleven GT1 consensus sequence motifs (Gilmartin et al. 1990; Zhou 1999; Hudson and Quail 2003), and a -10 promoter element (-10PEHVSPD) from a circadian clock regulated gene in barley (Thum et al. 2001) (Fig. 10C).

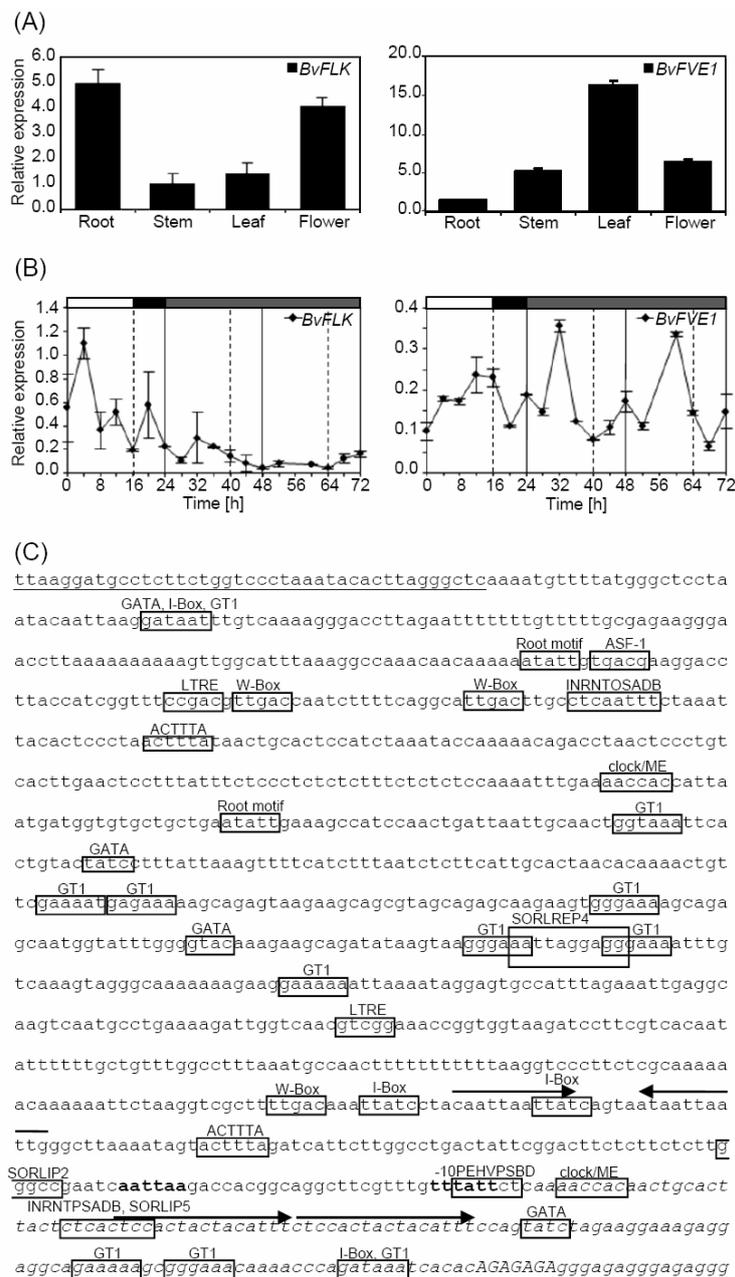


Figure 10: Expression of *BvFLK* and *BvFVE1* in *B. vulgaris*. (A) Expression across major plant organs in the biennial genotype A906001. Plants were vernalized and grown under long-day conditions. RT-qPCR expression levels were normalized against *BvGAPDH* and measured in triplicate. (B) Diurnal and circadian RT-qPCR expression profiles. Relative expression levels in leaves are shown for a 24h period under long-day conditions, followed by 48h under continuous low light at a constant temperature of 22°C. Expression was measured every four hours. Expression was normalized using *BvGAPDH*, *BvEF2* and *BvTUB*. Error bars indicate standard deviations of the mean. (C) *BvFVE1* promoter and 5' UTR. 1116 bp of the genomic sequence upstream of the start codon are shown. The transcription start site was determined by RACE. The 5' UTR sequence is printed in italics. Bold letters at positions -9 and -36 (relative to the transcription start site) indicate a TATA box-like sequence (de Pater et al. 1990) and a TATA-box according to the transcription start site prediction program TSSP (<http://www.softberry.ru/berry.phtml>), respectively. A GA repeat motif (Santi et al. 2003) in the 5' UTR shortly upstream of the ATG start codon is shown in capital letters. Putative light and circadian clock-regulated promoter elements are boxed (SORLIP and SORLREP (Hudson and Quail 2003), GT1 consensus sequence (Terzaghi and Cashmore 1995), IBOX core motif (Terzaghi and Cashmore 1995), GATA box (Gilmartin et al. 1990), INRNTPSADB (Nakamura et al. 2002), -10PEHVPSBD (Thum et al. 2001), and a 6 nt motif (clock/ME) which is common to a promoter element overrepresented in circadian clock regulated genes and a morning element; Harmer and Kay 2005). Arrows above the sequence denote inverted and tandem repeat units. The 3' end of a sequence tract with homology to the subtelomeric satellite AM076746 of *B. vulgaris* (clone pAv34-32; Dechyeva and Schmidt 2006) is underlined.

3.5 Discussion

The key regulatory genes of the vernalization and photoperiod pathways in Arabidopsis, *FLC* and *CO*, had been reported previously to have functionally related homologues in beet (Reeves et al. 2007; Chia et al. 2008). In the present study the question was addressed whether the genes of the third major regulatory pathway of flowering time control in Arabidopsis are conserved between the two species. Putative *B. vulgaris* orthologues of four autonomous pathway genes in Arabidopsis, *FLK*, *FVE*, *LD* and *LDL1*, were identified and genetically mapped. Three of these genes (*BvFVE1*, *BvLD* and *BvLDL1*) are homologous to autonomous pathway genes which are thought to regulate *FLC* expression by chromatin modification, whereas the fourth gene (*BvFLK*) encodes a putative RNA binding protein. The beet genes are highly similar to their Arabidopsis counterparts in terms of exon-intron structure and domain organization. With the exception of *BvLD*, the degree of overall sequence conservation with Arabidopsis is similar to (*BvFLK*, *BvLDL1*) or higher (*BvFVE1*) than that of the only two previously characterized flowering time genes in beet, *BvFL1* and *BvCOL1* (Reeves et al. 2007; Chia et al. 2008). For another gene of the autonomous pathway, *FPA*, a sugar beet EST with only moderate homology was identified (Table 7). However, ~3 kb of the genomic sequence of the corresponding gene (*BvFPA*) was sequenced and found to include the coding region for three RRM-type RNA binding domains also present in *FPA*, which substantiated that *BvFPA* and *FPA* are likely orthologues (data not shown). For the remaining three classical autonomous pathway genes, *FCA*, *FY* and *FLD*, orthologous ESTs were not identified. The currently available EST and transcript sequence collection for *B. vulgaris*, however, only represents 17184 genes (BvGI 3.0, release date June 16, 2010; <http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=beet>), which is approximately one half to two thirds of the total number of genes in beet (Herwig et al. 2002). Orthologues of *FCA* and *FY* with partially conserved functions (Lee et al. 2005; Lu et al. 2006; Jang et al. 2009) and a gene with high homology to *FLD* (Lu et al. 2006) have been identified in rice, suggesting that homologues may also be present in beet. Together, these sequence data and observations indicate that at least some autonomous pathway genes are conserved in beet.

Two genes were chosen, *BvFLK* and *BvFVE1*, whose homologues in Arabidopsis are thought to regulate *FLC* expression either through an RNA-based control mechanism (*FLK*) or by chromatin modification (*FVE*), to test the hypothesis that at least some of the autonomous pathway gene homologues are also functionally conserved. Among the genes identified here, these two genes also showed the highest degree of sequence conservation within conserved domains. Transgenic expression of *BvFLK* from both the constitutive CaMV 35S promoter and the endogenous promoter of *BvFLK* was found to accelerate bolting in *A. thaliana* and, in an *flk* mutant background, to fully rescue the phenotype to the bolting time of wild-type plants. *FLC* expression in transgenic plants carrying the *BvFLK* transgene was strongly reduced compared to untransformed controls, suggesting that the effect of *BvFLK* on bolting time is mediated through repression of *FLC*. In both 35S::*BvFLK* and endo::*BvFLK* transgenic plants, *FLC* expression is similarly low as in Col-0 wild-type plants, which indicates that all regulatory protein domains required for regulation of *FLC* expression are functionally conserved between *FLK* and *BvFLK*. At the sequence level, this is consistent with the high degree of homology within the KH-type RNA binding domains and the strict conservation between Arabidopsis and beet of the core consensus sequence of KH domains (Mockler et al. 2004; see asterisks in Fig. 7B). The low sequence conservation outside the KH domains, in particular in the N-terminal region of the proteins [which in *FLK* contains three perfect 8-residue repeats of unknown function (Mockler et al. 2004) which are not conserved in *BvFLK*], further suggests that this region is less critical for *FLK* function. The *B. vulgaris* homologue of *FLC*, *BvFL1*, complements *FLC* function in *A. thaliana flc* mutants (Reeves et al. 2007), suggesting that *BvFLK* may also regulate *BvFL1*. However, the regulation of

vernalization requirement and response appears to have diverged considerably during evolution (Colasanti and Coneva 2009; Distelfeld et al. 2009; Greenup et al. 2009; Jung and Müller 2009), and possible interactions between *BvFLK* and *BvFLI* in *B. vulgaris* have yet to be experimentally verified. Nevertheless, the complementation data for both *BvFLK* in this study and *BvFLI* by Reeves et al. (2007) suggest that regulatory interactions between these genes contribute to flowering time control in beet.

Our data also show for the first time, to our knowledge, that transgenic expression of the endogenous *A. thaliana* gene complements an *flk* mutant. The fact that full phenotypic complementation was achieved by expression from the CaMV 35S promoter suggests that developmental or environmental regulation of *FLK* transcription is not a precondition for the gene's function in flowering time control. Interestingly, *FLC* expression in 35S::*AtFLK* transgenic plants was strongly reduced but was not as low as in *BvFLK* transformants or wild-type controls. Incomplete repression of *FLC* correlated to some extent with the relatively low expression of *FLK* in 35S::*AtFLK* plants compared to wild type. Because the early bolting phenotype of the wild-type was fully restored in the transformants, the plant appears to tolerate moderately elevated expression levels of *FLC* without a significant delay in bolting.

Expression of *BvFLK* across major plant organs of sugar beet was strongest in roots and flowers but was also clearly detectable in leaves and stems, and resembled the relative expression levels of *FLK* in *A. thaliana* (Lim et al. 2004). While expression in aerial parts of the plant may well be associated with a functional role in flowering time control, the high expression level in roots, which was also observed for *FLK* in Arabidopsis, may reflect an additional, unknown function of *BvFLK* (as has also been suggested for *FLK*; Lim et al. 2004). *FLK* was implicated in RNA-directed chromatin silencing of retroelements (Bäurle and Dean 2008; Veley and Michaels 2008). Although speculative, it is conceivable that *BvFLK* is involved in repression of other targets such as e.g. developmental genes which may not be required at certain developmental stages, e.g. after floral transition, and/or in certain organs such as roots. The clustering of putative phytohormone response elements in the promoter of *BvFLK* may further suggest hormonal regulation of *BvFLK* activity. Finally, *BvFLK* does not appear to be under circadian clock control. Microarray data for *A. thaliana* indicate that expression of *FLK* is not circadian-regulated either (Edwards et al. 2006; <http://affymetrix.arabidopsis.info/narrays/experimentbrowse.pl>, accession number NASCARRAYS-108). Together, our data suggest that *FLK* and *BvFLK* are functionally related regulators of floral transition, and provide the first evidence for evolutionary conservation of *FLK* function outside the model species *A. thaliana*. In the apparent absence of an *FLC* orthologue in rice, sequence conservation in rice of the same regions which are conserved between *A. thaliana* and *B. vulgaris* may support the notion that *FLK* also has additional functions. Consistent with this possibility, putative orthologues of *FLK* are present in non-angiosperm species including the gymnosperm *Piceasitchensis* and the lycophyte *Selaginella moellendorffii* (Supplementary Fig. S4A at JXB online). For the rice orthologue of another RNA regulatory autonomous pathway gene, *OsFCA*, the possibility of other functions has been raised by detection of various protein-protein interactions (Lee et al. 2005; Lu et al. 2006; Jang et al. 2009).

In contrast to *BvFLK*, transgenic expression of *BvFVE1* did not complement the bolting time phenotype of an *A. thaliana* *fve* mutant. This result differs from data reported by Baek et al. (2008) for CaMV 35S promoter-driven expression of a rice homologue, *OsFVE* (Fig. 7B), which was shown to at least partially rescue the flowering time phenotype of an *fve* mutant in Arabidopsis in ~29% of independent primary transformants. So what are the reasons for the apparent absence of functional conservation between Arabidopsis and beet? The degree of sequence conservation between *FVE* and *BvFVE1* (72% amino acid identity) is very similar to

that between *FVE* and *OsFVE* (Baek et al. 2008), and *BvFVE1* and *OsFVE* are also highly similar to each other (71% amino acid identity). The degree of sequence conservation to Arabidopsis is also higher than that for *BvFLK*, *BvFL1* and *BvCOL1*, and much higher than for other autonomous pathway genes in rice (*OsFCA* and *OsFY*) with at least partially conserved functions (Lee et al. 2005; Lu et al. 2006). Thus, the degree of overall sequence conservation alone does not appear to be a good indicator of functional conservation, and may simply be a consequence of the large proportion of amino acid residues within functional or structural domains, as the CAF1c domain and the WD40 repeat domains thought to be required for the formation of a β -propeller structure (Kenziar and Folk 1998; Murzina et al. 2008) make up most of the protein. The regions outside the conserved domains vary considerably between *FVE* homologues. In particular, an N-terminal region which is present in *FVE* is missing in both *BvFVE1* and *OsFVE*. The finding that *OsFVE* at least partially complements *FVE* function suggests that this region is not absolutely required and, by extrapolation, its absence in *BvFVE1* cannot be the reason for the lack of functional complementation. Other regions with low sequence conservation are located between the WD40 repeat domains two and three, and between the WD40 repeat domains five and six. In the human *FVE* homologue RbAp46, the latter region carries a negatively charged loop which contributes to recognition of histone H4 and is not present in other WD40 β -propeller structures (Murzina et al. 2008). It seems possible that this region determines binding specificity and/or the type of interaction with other proteins. For structural proteins such as *FVE* which are vitally involved in protein complex formation, or formation of various protein complexes with different functions, as suggested by Amasino (2004), selection of binding partners is likely to be a crucial determinant of protein function. In addition, specific protein-protein interactions depend on co-evolution of binding sites. It is thus conceivable that the binding specificity determinants of *FVE* and *BvFVE1* have diverged sufficiently to prevent wild type-like interactions between *BvFVE1* and interacting proteins in *A. thaliana*. If the absence of functional complementation of *FVE* by *BvFVE1* is indeed a result of divergent evolution of protein binding sites, as suggested above, the possibility cannot formally be excluded that *BvFVE1* still exerts a similar function in beet as *FVE* in Arabidopsis, e.g. as part of a co-evolved protein complex.

The *B. vulgaris* genome appears to carry a second gene, represented by EST EG550040 (Table 7), which may be a paralogue of *BvFVE1* and will be referred to as *BvFVE2*. In the region represented by the EST, *BvFVE2* shares a similar degree of homology to *FVE* as *BvFVE1* (with the exception of the C-terminal region of the putative partial translation product; see Supplementary Fig. S5 at JXB online). Interestingly, the valine and lysine residues located in the variable region between WD40 repeat domains five and six and conserved between *FVE* and *OsFVE* are also conserved in *BvFVE2* (Supplementary Fig. S5 at JXB online). Although Baek et al. (2008) alluded to the presence of a second rice sequence with homology to *FVE* (OsJ_003110), this sequence was removed from GenBank, and no other close rice homologue of *FVE* in GenBank was identified. However, several other species were found to carry putative paralogous pairs (or groups) of *FVE* homologues, notably including two species in the *Malpighiales* order of dicotyledonous angiosperms (*Populus trichocarpa*, *PtFVE1 - 3*, and *Ricinus communis*, *RcFVE1 - 2*; Supplementary Fig. S4B, C at JXB online), which (like *B. vulgaris*) are also only distantly related to the *Brassicales*. Thus, it is conceivable that a gene duplication event occurred relatively early in the course of dicot evolution, possibly followed by gene loss in some lineages (including the lineage to Arabidopsis), and that the two paralogues underwent subfunctionalization.

The present expression data may provide further indications for subfunctionalization of *BvFVE1*. First, *BvFVE1* appears to be most strongly expressed in leaves of adult plants, whereas the data for *FVE* indicate highest expression in flowers (Ausin et al. 2004). Second,

BvFVE1 is diurnally regulated and under circadian control, a finding which appears consistent with the presence of multiple putative light-regulatable promoter elements. It is worth noting that *BvFVE1* also has an unusual map position close to the telomere and immediately adjacent to subtelomeric repeats, which may have contributed to the evolution of cis-regulation of *BvFVE1* expression. Although *FVE* was shown to affect circadian period length (Salathia et al. 2006), the gene itself (or any of the other classical autonomous pathway genes) has not been reported to be under control of the circadian clock. Furthermore, the microarray data by Edwards et al. (2006) indicate that *FVE* is not circadian-regulated, as determined after entrainment under intermediate day-length conditions (12h light/dark cycles). Interestingly, the small glycine-rich RNA-binding protein GRP7 in Arabidopsis which has long been known to be under circadian-clock control was recently assigned to the autonomous pathway (Streitner et al. 2008). Like *FVE*, this protein has also been implicated in various other biological processes including cold stress response (Carpenter et al. 1994; Heintzen et al. 1994). Circadian regulation of autonomous pathway genes or homologues may indicate a certain level of cross-talk between different floral regulatory pathways upstream of the floral integrator *FT*, as has been observed for *FLC* and the circadian clock (Edwards et al. 2006; Salathia et al. 2006; Spensley et al. 2009). A comparison with the circadian-regulated, putative photoperiod pathway gene *BvCOL1*, which was analyzed for circadian oscillations in the same plant samples as *BvFVE1* (Chia et al. 2008), shows that *BvFVE1* and *BvCOL1* have roughly complementary expression profiles, possibly suggesting regulation by different circadian clock output pathways and/or opposing regulatory roles. Alternatively, circadian clock regulation of *BvFVE1* and *GRP7* may reflect the broader involvement of these genes in other biological processes (e.g. cold stress response; Harmer et al. 2000; Fowler et al. 2005; Franklin and Whitelam 2007).

The present survey of autonomous pathway genes provides the first evidence for evolutionary conservation of homologues in beet as well as divergence and differential regulation of one gene. The results have further implications because i) functional conservation of autonomous pathway genes outside Arabidopsis, with the exception of a few reports for monocots, has not yet been studied in detail, ii) *B. vulgaris*, among dicot plants, is only a very distant relative of the model species *A. thaliana*, and belongs to a eudicot clade which is little understood at the molecular level, and iii) the environmental requirements for floral transition differ markedly between beet and model plants, in particular Arabidopsis and rice. Thus, the findings for *B. vulgaris* expand the spectrum of evolutionary diverse species for which molecular data are available and which are subject to differential environmental regulation of bolting and flowering. Finally, for sugar beet and other root and leaf crops, bolting and flowering is undesirable because it drastically reduces yield. The identification and genetic mapping of floral promoters provides tangible targets for marker-assisted or transgenic approaches towards crop improvement.

3.6 Acknowledgements

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4 Physical mapping of the *B* locus in *Beta vulgaris*

4.1 Abstract

In sugar beet (*Beta vulgaris* L. ssp. *vulgaris*), annuality (bolting and flowering without a requirement for vernalization) is controlled by the bolting locus *B* which had been mapped previously to chromosome II. Using a map-based cloning approach we developed a set of markers that co-segregate with *B*, and a physical map that spans 0.6 Mb of the *B* locus. Lack of recombination within this region in a large mapping population indicates a high physical to genetic distance ratio and recombination suppression.

4.2 Introduction

Sugar beet (*Beta vulgaris* L. ssp. *vulgaris*) is a dicotyledonous crop species whose lineage diverged from that leading to *Arabidopsis* ~120 million years ago (Davies et al. 2004). In *B. vulgaris*, annuality (bolting and flowering without a requirement for vernalization) is controlled by a major dominant genetic factor termed *B* (Munerati 1931; Abegg 1936; Boudry et al. 1994; El-Mezawy et al. 2002) which is now commonly referred to as the bolting gene. In addition bolting is controlled by *B2*, a locus unlinked to *B* which may act epistatically to *B* (Büttner et al. 2010). *B* is allelic to *B'*, a dominant factor for 'easy bolting' that was identified in another early study by Owen et al. (1940). In the absence of the dominant annual or easy bolting alleles at the *B* locus (i.e., in *bb* genotypes), induction of bolting and flowering in sugar beet requires vernalization. This vernalization requirement is obligate but there is also quantitative variation between *bb* genotypes in regard to the temperature and/or length of cold exposure required to induce floral transition, both in cultivated varieties (Sadeghian et al. 1993) and in the wild relative *B. vulgaris* ssp. *maritima* (Boudry et al. 2002). Both the occurrence of annual alleles and quantitative variation in vernalization requirement follow a latitudinal cline, with annuality or a low requirement for vernalization, respectively, being more widespread in wild *B. vulgaris* ssp. *maritima* populations from southern regions within their natural geographic range (Van Dijk et al. 1997; Boudry et al. 2002).

As a result of strong selection by breeders against annuality, commercial sugar beet cultivars do not contain the dominant *B* allele and are generally referred to as biennials. Wild beets, however, are iteroparous perennials that can flower repeatedly over several years (Hautekèete et al. 2002). The perennial life cycle of vernalization-requiring (*bb*) wild beets is thought to be associated with 'devernalization', i.e., reversal of the vernalised (epigenetic) state at the end of the summer (after flowering) when temperatures are still relatively high but days are getting shorter (Van Dijk 2007). Devernalization ensures that flowering in subsequent years again is timed to occur under favorable conditions and only after renewed cold exposure over winter. Experiments under artificial environmental conditions suggest that devernalization can also occur before the onset of flowering when plants that had been vernalised are transferred relatively quickly from the low temperatures required for vernalization to high temperatures (~20°C or higher) under short day conditions.

In addition to the obligate vernalization requirement of biennials, both biennials and wild annual beets are obligate long-day plants. As noted by van Dijk (2007) wild beets exhibit a certain compensatory relationship between vernalization and photoperiod such that lengthening the periods of cold exposure reduces the minimal day length required for flowering to occur.

The recent identification and functional analysis of *FLC*- and *CO*-like genes in sugar beet (which are not located at the *B* locus) provide first evidence for conservation of the genetic basis of the plant's response to the environment (Reeves et al. 2006; Chia et al. 2008).

In previous work, a high resolution AFLP map was constructed that covers a 5 cM window around the *B* locus on chromosome II (El-Mezawy et al. 2002), and a first physical map of BACs in the vicinity of the *B* locus and several BAC-derived markers were developed (Hohmann et al. 2003; Gaafar et al. 2005). Here, I revised the composition of BAC contigs, remapped the contigs onto a map of the *B* locus region, and generated a new physical map of the genomic regions flanking the *B* locus on either side.

4.3 Material and Methods

4.3.1 Plant material

The mapping population that was used in the present study was described previously by El-Mezawy et al. (2002), Gaafar et al. (2005), and Schulze-Buxloh (2006), and was derived from a cross between a biennial (*bb*) sugar beet line (A906001) and an annual (*BB*) line (93167P) as pollinator. 1028 F₂ plants were used in this study.

4.3.2 Marker development and genotyping

BAC and YAC end-derived sequences from opposite ends of individual BAC contigs were amplified from genomic DNA of the parents and the F₁ of the mapping population and sequenced, and polymorphisms were detected by multiple sequence alignments using the AlignX and Assemble modules of the Vector NTI Advance 10 software package (Invitrogen Corporation, Carlsbad, USA). Six new markers were developed, incl. three SNP markers, two InDel markers, and one CAPS marker (Tab. 9). SNP markers and the 1 bp InDel marker GJ70T7b were genotyped by PCR amplification and sequencing, the InDel marker GJ18T7b was genotyped by PCR amplification and electrophoresis on a 3% MetaPhor high resolution agarose gel (Biozym Scientific GmbH, Germany), and the CAPS marker by PCR amplification, *Hae*III (Fermentas, St. Leon-Rot, Germany) restriction enzyme digestion and standard agarose gel electrophoresis.

Table 9: Co-dominant BAC- and YAC-derived markers from the vicinity of the *B* locus.

Marker	Primer combination	PCR conditions	Marker fragment size [bp]	Marker type
Y67L ^{1,2}	A019+A020	94°C, 2' + [(94°, 30" + 63°C, 30" + 72°C, 25") x 32] + 72°C, 7'	240	CAPS (<i>Hae</i> III)
GJ191Sp6	A162-A163	94°C, 2' + [(94°, 30" + 54°C, 30" + 72°C, 45") x 32] + 72°C, 7'	370	SNP
GJ70T7b	016J09tP1+P2	94°C, 2' + [(94°, 30" + 54°C, 30" + 72°C, 30") x 32] + 72°C, 7'	233(bb)	InDel
GJ29Sp6b	A051+A052	94°C, 2' + [(94°, 30" + 58°C, 30" + 72°C, 30") x 32] + 72°C, 7'	382	SNP
GJ18T7b ¹	A015+A016	94°C, 2' + [(94°, 30" + 54°C, 30" + 72°C, 30") x 32] + 72°C, 7'	142(BB)/152(bb)	InDel
GJ243Sp6	B523-B524	94°C, 2' + [(94°, 30" + 51°C, 30" + 72°C, 40") x 32] + 72°C, 7'	218	SNP

¹ GJ18T7b and Y67L were also used by Büttner et al. 2010

² derived from a YAC end (Y67L; Hohmann et al. 2003)

In addition to the newly developed markers, six markers were used that had been developed by Gaafar et al. (2005) (GJ18T7, GJ70co3F4, GJ131co15F6, GJ29T7, GJ18Sp6, GJ01co36F4). In cases where marker assays did not give unambiguous results the polymorphic fragments were genotyped by sequencing. Pairwise sequence comparisons of marker fragments revealed that the b131-derived marker (GJ131co15F6) corresponds to the AFLP markers P05_b162, which is linked in coupling with the *b* allele, and P05_B159, which is linked in coupling with the *B* allele (El-Mezawy et al. 2002). The BAC b1-derived sequence that was used for the development of marker GJ01co36F4 corresponds to the 'R' end of YAC Y102 which had been identified originally by screening with AFLP marker fragment P14_b118, and the b70-derived sequence that was used for the development of marker GJ70co3F4 corresponds to the 'L' end of Y89 which had been identified with AFLP fragment P05_b162 (Hohmann et al. 2003). The recombination rates between individual markers and *B* were calculated by dividing the number of recombination events between these markers and the *B* locus marker GJ1001c16 (Büttner et al. 2010) by the total number of gametes, and multiplication by 100.

4.3.3 BAC contig analysis

Overlaps between BACs and the orientation of BACs within contigs were determined by PCR and sequence analysis. It was assumed that PCR products of the same size with the same primer combination from two different BACs indicate over-lapping of the BACs. Sequence identity between PCR fragments was confirmed by sequencing.

Table 10: PCR and sequencing assays for BAC contig analysis.

BAC end	Primer combination	PCR conditions	PCR fragment size [bp]	Sequencing primer
GJ191Sp6	A162-A163	94°C, 2' + [(94°, 30" + 54°C, 30" + 72°C, 45") x 32] + 72°C, 7'	370	A162
GJ191T7	A164-A165	94°C, 2' + [(94°, 30" + 54°C, 30" + 72°C, 45") x 32] + 72°C, 7'	468	A164
GJ392Sp6	A029-A030	94°C, 2' + [(94°, 40" + 60°C, 40" + 72°C, 40") x 32] + 72°C, 7'	234	A029
GJ392T7	A027-A028	94°C, 2' + [(94°, 40" + 60°C, 40" + 72°C, 40") x 32] + 72°C, 7'	184	A027
GJ18Sp6	072J03s P1+P2	94°C, 2' + [(94°, 40" + 54°C, 40" + 72°C, 40") x 32] + 72°C, 7'	415	072J03s P1
GJ243Sp6	B523-B524	94°C, 2' + [(94°, 30" + 51°C, 30" + 72°C, 40") x 32] + 72°C, 7'	218	B524
GJ243T7	044K24t P1+P2	94°C, 2' + [(94°, 30" + 51°C, 30" + 72°C, 40") x 32] + 72°C, 7'	422	044K24t P2
GJ703T7	A023-A024	94°C, 2' + [(94°, 40" + 60°C, 40" + 72°C, 40") x 32] + 72°C, 7'	227	A023
GJ703Sp6	A025-A026	94°C, 2' + [(94°, 40" + 60°C, 40" + 72°C, 40") x 32] + 72°C, 7'	173	A025

4.4 Results

4.4.1 BAC contig assembly and revision of the physical map around *B*

The relative order and orientation of BACs identified by Hohmann et al. (2003) was further analysed by BAC end sequencing, and PCR and sequence analysis of BAC overlaps. The analysis of BAC overlaps indicates that the Sp6 end of BAC b243 is the outstanding end

because the GJ243Sp6 marker gave a band only with the BAC of which it was derived (Tab. 11). Likewise, the orientation of b703 and b392 was determined (Tab. 11, Fig. 12). B392 and b18 overlap as shown by sequence identity from PCR fragments amplified from the two BACs (Tab. 11).

Table 11: BAC contig analysis.

BAC end	Contig	Fragment on BAC ¹	No fragment on BAC
GJ18Sp6	3	GJ18, GJ392	-
GJ392T7	3	GJ392	GJ018
GJ392Sp6	3	GJ392, GJ018	-
GJ703T7	3	GJ703	GJ243
GJ703Sp6	3	GJ703	GJ243
GJ243T7	3	GJ243, GJ001	-
GJ243Sp6	3	GJ243	-
GJ191Sp6	2	GJ191	-
GJ191T7	2	GJ191	GJ131

¹For all BACs sequence identity to the BAC end given in the first column was verified by sequencing

Moreover, sequence comparison of the BAC sequences of b70 and b131 (Gaafar et al. 2005) revealed sequence identity over about half of the respective sequences and allowed unequivocal assignment of b70 and b131 to the same contig. Contrary to an earlier RFLP fingerprint analysis (Hohmann et al. 2003) the sequence-based analysis indicated that BACs b1 and b22 fall into two distinct contigs (contig 3 and contig 2, respectively) and do not overlap. BAC b29 which previously also had been assigned to the genomic region around *B*, but outside the region for which a minimal tiling path was proposed, and two overlapping BACs constitute a third contig (contig 1).

To establish the order of contigs relative to *B* we used six BAC-derived molecular markers (GJ01co36F4, GJ18Sp6, GJ18T7, GJ29T7, GJ70co3F4, GJ131co15F6) described by Gaafar et al. (2005) and six newly developed markers (GJ18T7b, GJ29Sp6b, GJ70T7b, GJ191Sp6, GJ243Sp6, Y67L) that all fall within the three distinct contigs. By using these markers a total of 16 recombinant plants among 1028 individuals of the F₂ mapping population were verified (960701/s6/10, 960701/s6/67, 960701/s6/244, 960701/s6/198, 950619/116, 950619/319, 950619/526, 950619/902, 960701/s5/112, 960701/s6/53, 960701/s6/300, 950619/298; Gaafar 2005; Schulze-Buxloh 2006) or newly identified (960701/s4/1272, 960701/s4/635, 960701/s4/712, 960701/s4/265). The graphical genotypes of these recombinants at the twelve marker loci and the *B* locus (as derived from the bolting phenotype) are given in Fig. 11 and show that contig 1 and contig 2 flank the *B* locus on either side. Contig 3 is located on the same side as contig 1 but further distal from *B*, as indicated by recombination between the contig 3-derived marker GJ243Sp6 and the contig 1-derived markers. The genetic distances of contig 3 (and the *B*-proximal end of contig 1) to *B* and contig 2 to *B* were calculated as 0.3 cM and 0.3 cM, respectively.

rec. #	F2 plant #	Y67L	GJ191 Sp6	GJ131 co15F6	GJ70 co3F4	GJ70 T7b	B	GJ29 Sp6b	GJ29 T7	GJ18 Sp6	GJ18 T7b	GJ18 T7	GJ01 co36F4	GJ243 Sp6
1	960701/s6/10	A	A	A	A	A	A							
2	960701/s6/67	A	A	A	A	A	A							
3	960701/s6/244	A	A	A	A	A	A							
4	960701/s6/198						A	A	A	A	A	A	A	A
5	950619/116						A	A	A	A	A	A	A	A
6	950619/319						A	A	A	A	A	A	A	A
7	950619/526						A	A	A	A	A	A	A	A
8	950619/902						A	A	A	A	A	A	A	A
9	960701/s4/1272						A	A	A	A	A	A	A	A
10	960701/s4/635													
11	960701/s4/712													
12	960701/s4/265													
13	960701/s5/112													
14	960701/s6/53													
15	960701/s6/300													
16	950619/298													A

Figure 11: Graphical genotypes of recombinants. Shown are the marker genotypes of 16 F₂ individuals (out of 1028 plants from a mapping population) that had undergone recombination in the vicinity of *B*. Also shown are the genotypes at the *B* locus as predicted from bolting phenotypes in F₂ individuals and F₃ progeny plants (El-Mezawy et al. 2002; Gaafar et al. 2005; Schulze-Buxloh 2006), and at one YAC-derived marker locus (Y67L) that co-segregates with contig 2-derived markers. The filled boxes and patterns depict *BB* genotypes and marker alleles that are linked in coupling phase to *B* and are homozygous (black boxes), *bb* genotypes and marker alleles in the homozygous state that are linked in repulsion phase to *B* (white boxes), and *Bb* genotypes and heterozygous marker loci (cross-hatched boxes).

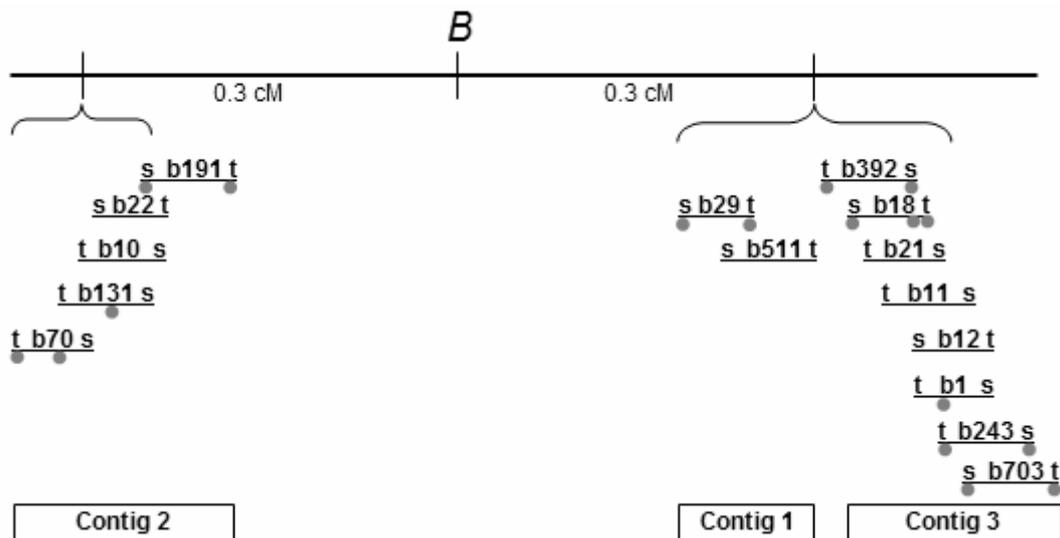


Figure 12: BAC contigs and markers in the vicinity of the *B* locus on chromosome II. Individual BACs are indicated by short horizontal bars, with BAC numbers given above bars. Sp6 and T7 ends are abbreviated as ‘s’ and ‘t’, respectively. Grey circles indicate BAC-derived molecular markers. Included in the brackets are regions of the physical map in which all corresponding markers share the same genetic position, as tested in 1028 F₂ plants of a previously established mapping population (El-Mezawy et al. 2002). The map includes flanking markers described by Gaafar et al. (2005) (GJ18T7, GJ70co3F4, GJ131co15F6, GJ29T7, GJ18Sp6, GJ01co36F4), Büttner et al. (2010) (GJ18T7b, Y67L), given in table 9 (GJ70T7b, GJ29Sp6b, GJ191Sp6, GJ243Sp6), or given in table 10 (GJ191T7, GJ243T7, GJ392Sp6, GJ392T7, GJ703Sp6, GJ703T7). The genetic map distances between contigs 1 and 2 and the *B* are indicated. Due to lack of recombination within contigs 1 and 2, the orientation of these contigs is not known, contig 3 is located at the same site as contig 1 but further away from *B*, as indicated by a recombination event between the b243s-derived marker (GJ243Sp6) in contig 3 and the contig 1 derived markers.

4.5 Discussion

In the present study, a PCR- and sequence-based analysis of BAC overlaps and six newly developed BAC end-derived markers were used to generate a new physical map of the genomic region encompassing the *B* locus in sugar beet (Fig. 12). In contrast to an earlier analysis which was largely based on PCR and RFLP fingerprinting (Hohmann et al. 2003), the high information content of sequence data in combination with recombination analysis allowed us to assign several BACs which previously had been thought to overlap into distinct contigs. In the new map contig 1 is now located on the same side of *B* as contig 3, and contig 2 is on the opposite side of the *B* locus. Contig 3 is on the same side of *B* as contig 1 but further away as indicated by a recombination event between GJ243Sp6 in contig 3 and the contig 1-derived markers. The *B* locus is located between contigs 1 and 2, and thus falls into a genetic window of ~0.6 cM between the markers derived from these flanking contigs. The orientation of contigs 1 and 2 is not known due to the lack of recombination within these contigs (Fig. 12).

The recombination analysis and the new map were the prerequisite for a bulked segregant analysis using informative recombinants shown in Fig. 11 to identify markers co-segregating with the *B* locus, and isolation of the bolting gene (manuscript in preparation).

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5 Closing discussion

In summary, the following main results were obtained:

1. A revised physical map of the genomic region around the *B* locus was established by BAC analysis, BAC marker development, and remapping of opposite ends of individual BAC contigs. The new physical map was the prerequisite for the identification of markers completely co-segregating with the bolting locus *B* and the isolation of the *B* locus by map-based cloning.
2. Two novel loci controlling bolting behaviour in sugar beet were identified in EMS mutagenised plants by co-segregation analysis: *B2* which acts epistatically to *B* and was mapped to chromosome IX, and a putative locus *B3* which acts independently of *B* and for which the map position is unknown.
3. *BvFVE1*, a homologue of the flowering time control gene *FVE* from *Arabidopsis*, was isolated and characterised. *BvFVE1* shows the same exon-intron and domain structure as the *Arabidopsis* homologue, was not able to complement the *Arabidopsis fve* mutant, and in contrast to *AtFVE* is diurnally regulated.

To sum up, new bolting and flowering time genes in *Beta vulgaris* were identified. Bolting time control involves at least two further genes in addition to the known bolting locus *B*. In addition, the first autonomous pathway gene homologue in sugar beet, *BvFVE1* was identified and characterised.

Genetic analysis of flowering time mutants

To analyse the genes underlying bolting behaviour in *B. vulgaris* seeds of an annual beet were mutagenised with EMS and in the offspring plants with altered bolting behaviour were identified (Hohmann et al. 2005). It was assumed that in the mutants bolting only after vernalization either the *B* gene or a locus acting epistatically to *B* is mutated. Because the biennial mutants were only identified on the basis of their phenotype, the proof is missing if the observed biennial phenotype is due to EMS mutagenesis or a result of a natural occurring rare allele (e.g., as a result of heterogeneity of the accession used for mutagenesis). Despite evidence that 931667P is not completely homogeneous, as shown by segregation for hypocotyl colour (s. 2.3.1) there is no hint that bolting behaviour is segregating in this accession. All plants grown under different environmental conditions bolted without vernalization and behave clearly as annuals (Monika Bruisch, personal communication; Hohmann et al. 2005; Büttner et al. 2010). Irrespective of the origin the non-bolting allele is interesting for breeding. Co-segregation analysis indicates that among the mutants analysed both situations are represented – a mutation in the *B* gene, and a mutation in a gene acting epistatically to *B*. Furthermore a gene acting independently of *B* in bolting control was identified. One locus acting epistatically to *B* was named *B2* and mapped by AFLP mapping to chromosome IX. Although AFLP analysis has the disadvantage to be a dominant marker system, it was chosen for mapping *B2* because it has the advantages that it can be applied to any DNA sample without prior sequence information, readily identifies polymorphisms, and allows a quick scan of the whole genome for polymorphism to create a map (Meudt and Clark 2007).

As discussed in chapter 2.5 *B2* may be a gene mediating the response to photoperiod including *FLOWERING LOCUS T (FT)*-like genes or gibberellins (GA). A homologue to *LDL1 (LSD1-LIKE 1)*, a gene of the autonomous pathway was mapped to chromosome IX (s.

3.4.2). In addition, *Ga3ox1* (*GIBBERELLIN3-OXIDASE 1*) and *FT* were mapped to chromosome IX (Schulze-Buxloh et al. personal communication). *Ga3ox1* is a candidate gene for *B2*, because it catalyses the conversion of the GA precursor to the bioactive form (Mitchum et al. 2006). Because *LDL1*, *Ga3ox1*, and *FT* were mapped in another population than *B2* and the map of *B2* is only a rough map, one can not exclude the possibility that *Ga3ox1*, *LDL1* or *FT* co-localise with *B2* and are therefore candidate genes for the *B2* locus.

Besides map based cloning different approaches are applied in other species to analyse the genetic basis of bolting. Cultivation of the biennial species *Allium cepa* faces similar problems like beet cultivation. By analysing protein profiles of two cultivars with different bolting behaviour a protein was isolated with significant homology to the chromodomain of heterochromatin protein 1 (Hyun et al. 2009). In addition to onion and beet *Cichorium intybus* (cultivated forms chicory and radicchio) is a biennial species needing vernalization to flower that is cultivated as an annual plant. A *FLOWERING LOCUS C-like* (*CiMFL*) sequence was isolated. However, the biological function of *CiMFL* was not conserved between Arabidopsis and chicory as tested by complementation analysis of the Arabidopsis mutant (Locascio et al. 2009).

Cloning and characterisation of an *FVE* homologue

In addition to the new bolting time loci sugar beet genes with homology to known flowering time genes were identified. *FVE* (*FLOWERING LOCUS VE*), as all genes of the autonomous pathway, was originally a candidate gene for the *B* locus. The mutation is recessive and leads to late flowering, but the delay can be overcome by vernalization, thus the phenotype is similar to that of a biennial beet. *BvFVE* was chosen among autonomous pathway genes for further analysis, because it shows the highest homology between sugar beet and Arabidopsis of all autonomous pathway gene homologues identified (s. 3.4.1). In addition, *FVE* has in Arabidopsis further functions besides flowering time regulation as regulator of biomass (potential yield increase) (Morel et al. 2009) and as a mediator of intermittent cold stress response (Kim et al. 2004). The full-length cDNA of *BvFVE1* was cloned and characterised by map position, expression analysis and transformation of the late-flowering Arabidopsis *fve-7* mutant. Mapping of *BvFVE1* to chromosome VII in the present study showed that the gene is neither the *B* gene, which is located on chromosome II, nor the *B2* gene, which was mapped to chromosome IX. The genomic sequence of *BvFVE1* was obtained by primer walking followed by whole BAC sequencing. The latter technique was employed because primer walking proved to be inefficient due to large introns and repetitive sequence elements within the gene. BAC sequencing with the 454 technology (Margulies et al. 2005) achieved only 46% sequence coverage. The low sequence coverage is probably due to the repetitive nature of the genomic sequence context of *BvFVE1*, as the 454 technology, due to the short read length, has problems with resolving repeats (Wicker et al. 2006; Quinn et al. 2009; Steuernagel et al. 2009). The presence of repetitive sequence elements in the vicinity of *BvFVE1* was indeed proven. Several BAC sub-contigs show homology to Beta specific *Apal* and *RasI* satellites (Dechyeva and Schmidt 2006), and a non-viral retrotransposon (BNR1; Schmidt et al. 1995) was identified. Assembly of BAC sub-contigs with the help of the sequence data from primer walking yielded two contigs within the genomic region that includes *BvFVE1*, but there is a gap of sequence information between the putative exons 12 and 13. Most likely because of repeats (AT stretches) in that region it was not possible to fill the gap by further walking steps from both sides. There were no further attempts to close the gap because cDNA analysis indicated that the missing genomic sequence is intronic. The sub-telomeric position of *BvFVE1* as shown by mapping and supported by the presence of sub-telomeric repeats (Dechyeva and Schmidt 2006) is worth nothing. This position could influence the gene expression via the telomere position effect (TPE), a repression of genes

adjacent to telomeres (Doheny et al. 2008; Ottaviani et al. 2008). However, our expression data did not support the idea of silencing of *BvFVE1*, as *BvFVE1* was shown to be expressed in all tissues and all conditions analysed. A further interesting observation in relation to the map position is that often genes related to stress response are clustered at sub-telomeric regions. This strategy seems to be evolutionary conserved allowing the reversible silencing and a fast response to changes in the environment (Borst and Ulbert 2001; Robyr et al. 2002; Barry et al. 2003; Dreesen et al. 2007; Ottaviani et al. 2008). *AtFVE* plays a role in the response to intermittent cold stress, and delays flowering in response to cold (Kim et al. 2004), therefore it would be interesting to see if *BvFVE1* has a similar function in cold response.

By comparing *BvFVE1* and *AtFVE* an interesting detail was found in the 5' region of the gene. Here the two genes differ in sequence, but carry similar structural elements with an inverted repeat being followed by a tandem repeat, but up to now no function of these repeats was reported for *AtFVE*. The sequence of the inverted repeats in beet has similarity to a motif which serves as a binding site for homeobox domains (Ohigishi et al. 2001). 5' RACE indicates that part of the sugar beet repeats are expressed, which may suggest a possible function in regulation of mRNA stability and/or translational efficiency. The repeats may form a secondary structure which may have a regulatory function. A mechanism for thermo-regulated expression was described in the bacterium *Listeria monocytogenes*. Here the 5' UTR forms a temperature-dependent secondary structure that regulates the access to the ribosome binding site (Shine-Dalgarno sequence) and therefore translation (Johansson et al. 2002). A similar mechanism is thinkable for the thermoregulation of *FVE*.

BvFVE1 is expressed in all tissues analysed like the homologue from Arabidopsis, but in contrast to Arabidopsis *BvFVE1* has a diurnal and circadian expression profile. This is consistent with the identification of different light regulated elements in the promoter of *BvFVE1* (s. 3.4.6). Yet the SORLIP (sequences over-represented in light-induced promoters) and GT motives are also found in non light induced promoters, so the motif itself is no proof for a promoter to be light inducible (Zhou 1999; Hudson and Quail 2003). Binding to GT and GATA motifs is co-regulated, and GT-1 is necessary but not sufficient to confer light responsiveness (Zhou 1999). Not the single motive is sufficient to make the promoter light inducible, but the combination of different motives (Puente et al. 1996; Chattopadhyay et al. 1998). Interestingly the following light regulated elements were found by PLACE (Higo et al. 1999) in the promoter of *AtFVE* too: GATA-box, GT1 consensus sequence, and the I-box. Although *AtFVE* affects circadian period length (Salathia et al. 2006), diurnal regulation of *AtFVE* or *FVE* homologues in other species has yet to be reported. Additionally microarray data indicate that *AtFVE* is not circadian-regulated (Edwards et al. 2006).

BvFVE1 shows no functional complementation of an *fve* mutant in flowering time control. In contrast to *BvFLK*, *BvCO* (Chia et al. 2008) and *BvFLI* (Reeves et al. 2007) no complementation of the mutant phenotype was achieved by transformation with *BvFVE1*. Possible reason for the lack of complementation of the *fve-7* mutant with *BvFVE1* include – as discussed in more detail in the chapter 3.5 – the missing N-terminal region of the protein including a potential nuclear localisation signal (NLS), co-evolution of binding partner and binding sites, and sub-functionalisation. The lack of complementation does not exclude the possibility that *BvFVE1* has a function in flowering time control in sugar beet. One way to test this possibility would be the transformation of *B. vulgaris* with *BvFVE1* or a *BvFVE1* RNAi construct. Another possibility is that *BvFVE2* plays a role in flowering time regulation instead of *BvFVE1*, and this could be tested by complementation analysis of the Arabidopsis *fve-7* mutant with *BvFVE2*. Furthermore, it would be interesting to see if *BvFVE2* like *BvFVE1* shows a circadian expression profile.

AtFVE has in addition to its role in flowering time control a function in cold response, these two different roles are probably carried out by the formation of two different histone deacetylase complexes (HDAC) (Amasino 2004; Franklin and Whitelam 2007). So it would be informative to see if *BvFVE1* can take over the cold response function in the *Atfve* mutant. This could be tested for instance by measuring expression of cold responsive (COR) genes by quantitative real time PCR. Worth noting is that *OsFVE* only complements the flowering phenotype, but not the cold phenotype of the Arabidopsis *fve* mutant (Baek et al. 2008).

Much is known about flowering time regulation in Arabidopsis, rice and cereals. The line leading to Beta diverged from the line leading to Arabidopsis around 120 million years ago (MYA), while the line leading to *Oryza sativa* (rice), *Triticum aestivum* (wheat), and *Hordeum vulgare* (barley) (all Poaceae) separated from the line leading to Arabidopsis around 140 MYA (Davies et al. 2004) the time of the divergence of monocots and dicots (Chaw et al. 2004). These species need different environmental conditions to flower. *Arabidopsis thaliana* flowers earlier under long-day (16h light) conditions and after vernalization, but will flower even under short-day (8h light) conditions (Koornneef et al. 1998) and without vernalization (Martinez-Zapater and Somerville 1990). *O. sativa* is a short-day plant without vernalization requirement. Cereals are long-day plants; there are varieties with and without vernalization requirement (Greenup et al. 2009). *B. vulgaris* has a vernalization requirement and is like Arabidopsis and cereals a long-day plant. There are similarities and differences in flowering time regulation in Arabidopsis, cereals, and rice. The main regulator of the long-day response in *A. thaliana* is *FLOWERING LOCUS T* (*FT*; Imaizumi and Kay 2006; Jaeger et al. 2006; Zeevaert 2006; Turck et al. 2008). *FT-like* genes were identified in wheat (*TaFT*; Yan et al. 2006), barley (*HvFT*; Yan et al. 2006; Faure et al. 2007), rice (*Hd3a*; Kojima et al. 2002), maize (*ZCN8*; Danilevskaya et al. 2008), and *Lolium temulentum* (*LtFT*; King et al. 2006). The *FT* orthologue of rice is *Hd3a* which has a similar role as *FT* in Arabidopsis, but it is expressed in short-days while *AtFT* is expressed in long-days (Kojima et al. 2002). The role of *FT* is conserved in cereals (Yan et al. 2006). Therefore it appears likely that different genes regulate *FT* homologues across species to account for the seasonal flowering response.

FT is regulated by *CONSTANTS* (*CO*) in Arabidopsis (Samach et al. 2000). *CO-like* genes have been identified in rice (*Hd1*; Yano et al. 2000), barley (*HvCO1* and *HvCO2*; Griffiths et al. 2003), wheat (*TaHD1* and *TaHD2*; Nemoto et al. 2003), maize (*CONZI*; Miller et al. 2008), perennial ryegrass (*LpCO3*; Martin et al. 2004), and sugar beet (*BvCOL*; Chia et al. 2008). *Hd1* from rice is diurnally expressed like *AtCO* (Izawa et al. 2002; Hayama et al. 2003; Shin et al. 2004). In Arabidopsis *CO* activates *FT*, but in rice high *Hd1* expression is not associated with high *Hd3a* (the rice *FT* homologue) expression. The function of *CO* seems to be conserved between Arabidopsis and grasses (Greenup et al. 2009). Sugar beet contains a *CONSTANS-LIKE* (*COL*) gene family with at least ten genes that show homology to the *CO* family in Arabidopsis. *BvCOL1* was analysed further: it maps to chromosome II, but it is not the *B* locus. It can complement the late flowering phenotype of the Arabidopsis *co-2* mutant. The expression pattern of *BvCOL1* is more similar to *AtCOL1* and *AtCOL2* than to *AtCO* (Chia et al. 2008).

Another key regulator of flowering in Arabidopsis is *FLOWERING LOCUS C* (*FLC*), however there are no *FLC-like* genes identified in cereals or rice up to now (Greenup et al. 2009; Higgins et al. 2010). Accordingly the vernalization response must be mediated by different genes in Arabidopsis and crops. Three major loci control the vernalization response in wheat and barley: *VERNALIZATION 1* (*VRN1*), *VRN2*, and *VRN3* (Dubcovsky et al. 1998; Karsai et al. 2005; Yan et al. 2005; Yan et al. 2006). *VRN1* is induced by vernalization (Danyluk et al. 2003; Trevaskis et al. 2003; Yan et al. 2003; von Zitzewitz et al. 2005) and up-regulates *FT* under long day conditions (Hemming et al. 2008). Sequence variations in

VRN1 lead to differences in vernalization requirement (Yan et al. 2003; Fu et al. 2005; von Zitzewitz et al. 2005; Cockram et al. 2007; Szücs et al. 2007). *VRN2* delays flowering by inhibition of *FTI* (Hemming et al. 2008), and its expression decreases after vernalization (Yan et al. 2004; Karsai et al. 2005; Dubcovsky et al. 2006; Trevaskis et al. 2006; Hemming et al. 2008). Down-regulation is mediated by *VRN1* (Trevaskis et al. 2006; Hemming et al. 2008). The *VRN2* gene from cereals is related to *Ghd7* of rice (Yan et al. 2004; Dubcovsky et al. 2006; Trevaskis et al. 2006). *VRN3* is an integrator of the vernalization and the photoperiod response (Yan et al. 2006; Trevaskis et al. 2006). *VRN3* encodes for an orthologue of Arabidopsis *FT* and is collinear with *Hd3a* in rice (Yan et al. 2006). In autumn *VRN3* is repressed by *VRN2*, therefore *VRN1* can not be induced. *VRN1* is up-regulated during winter and represses *VRN2*. Low levels of *VRN2* allow up-regulation of *VRN3* by long-days, *VRN3* then can further promote *VRN1* expression and induce flowering. A homologue to *FLC* was identified in sugar beet. *BvFLI* is a repressor of flowering in transgenic Arabidopsis. *BvFLI* is like *AtFLC* down-regulated in response to cold in sugar beet, but the repression is not maintained after vernalization (Reeves et al. 2007). By contrast, expression data from our group indicate up-regulation of *BvFLI* during vernalization and a return to pre-vernalization levels after vernalization (Schulze-Buxloh and Müller, personal communication). *BvFLI* is alternatively spliced giving rise to four different variants. It was mapped to chromosome VI and is therefore not the *B* locus or the *B2* locus (Reeves et al. 2007).

Homologues of autonomous pathway genes are found in rice and *Brachypodium distachyon*, a model species for temperate grasses, such as barley and wheat (Higgins et al. 2010). Database analysis provides EST data and therefore expression data for all autonomous pathway genes in rice (Lu et al. 2006). Genes with homology to *FCA* (*FLOWERING LOCUS CA*), *FY* (*FLOWERING LOCUS Y*) and *FVE* were isolated from rice (Lee et al. 2005; Lu et al. 2006; Baek et al. 2008). *OsFCA* shows a similar gene structure, expression pattern, and alternative splicing pattern as *AtFCA*, but overexpression of *OsFCA* could only partly complement the late flowering *fca* phenotype in Arabidopsis and was not able to down-regulate *FLC*, but did up-regulate *SOC1* in transgenic Arabidopsis. This implies conservation and divergence of the *FCA* function between rice and Arabidopsis (Lee et al. 2005). *OsFY* and *OsFCA-γ* protein-protein interactions, a key factor in their function in Arabidopsis, were shown by yeast two-hybrid system (Lu et al. 2006). Like *FCA*, *FVE* function seems only to be partially conserved between rice and Arabidopsis. Although the two genes show conserved exon-intron structure, expression pattern and complementation of the flowering phenotype, *OsFVE* could not rescue the cold response of the *Atfve* mutant (Baek et al. 2008).

In summary, flowering time regulation seems to be in part conserved between Arabidopsis and sugar beet: *FLC* one of the central regulators of flowering in Arabidopsis, is present and appears to be functionally conserved (Reeves et al. 2007), in addition to the involvement of *CO* or *CO*-like genes in photoperiodic response (Chia et al. 2008). Genes of the autonomous pathway are present, but only *FLK* (s. 3.4.4), and not *FVE1* (s. 3.4.5) shows functional conservation in flowering time regulation.

Prospects for beet breeding

The long-time aim is a beet genotype which is suitable for winter beet cultivation. Sowing of beets in autumn and harvesting the next summer or autumn, assuming adequate pest control and sufficient water supply, is estimated to have an up to 26% higher yield than spring sown beets (Jaggard and Werker 1999). Winter beet cultivation is at the moment not possible, because beets will get vernalised over winter and will bolt in the next summer leading to yield loss. For winter beet cultivation the beet should not bolt after winter, but for seed multiplication and breeding bolting is necessary. These two conflicting aims may be achieved by genetic engineering (Jung and Müller 2009).

Are the genes presented in this work possible candidate genes for the development of winter beet? Firstly, *B2* could be a potential marker for the selection for or against the annual habit in beets. The absence of annuality is a pre-requisite for the breeding of both traditional spring beets and winter beets. To test if *B2* derived markers are useful for marker assisted selection, the markers could be tested in a panel of wild and cultivated Beta species with known bolting behaviour to look for association of marker and trait. Secondly, for *BvFVE1* no influence in flowering time regulation was shown in Arabidopsis, so it is not a prime candidate for the development of winter beets. If *BvFVE1* had a similar function as in Arabidopsis, then overexpression would not lead to an acceleration of bolting and flowering (Ausin et al. 2004), and knock-out of *FVE* would delay bolting but not abolish it. An interesting aspect of *FVE* is its effect on biomass production – in the Arabidopsis *fve* mutant the biomass was increased up to eightfold depending on light conditions (Morel et al. 2009) – because an increase in leaf biomass would lead to an increase in solar radiation capture and therefore in sugar yield (Jaggard et al. 2009). Thirdly, the analysis of the EMS mutants identified a mutant in which the *B* locus appeared to be mutated, and is a valuable tool for studying the function of *B* and its role in bolting, and how it could be modified for the winter beet cultivation. The newly identified bolting genes *B2* and *B3* are further potential candidate genes for the genetic engineering of winter beets. The *B2* locus is acting epistatically to *B*; therefore only one of the two genes has to be modified for an effect on bolting behaviour. The knock-out of *B2* leads – as shown in the EMS mutant – to a biennial beet if the functional *B* allele is present. The overexpression of *B2* in an annual beet may further accelerate bolting; overexpression in a beet without a functional *B* allele may have an effect on bolting with and/or without vernalization. A map based cloning project was started to clone the *B2* locus. For this project a new mapping population is grown, because of the limited number of individuals in the F₂ populations under study here. The identification of the causal DNA polymorphism for the non-bolting phenotype is expected to greatly advance our understanding of bolting behaviour in sugar beet.

Prior to the current study, the pathways controlling bolting behaviour in *B. vulgaris* were largely unknown. To analyse the bolting behaviour in sugar beet two different approaches were applied: the (co-segregation) analysis of mutations leading to a biennial phenotype in an annual genotype treated with EMS (Hohmann et al. 2005) and the identification and characterisation of candidate genes for the floral transition on the basis of homology. While the candidate gene approach highlighted evolutionary diversification in flowering time control between the two distantly related dicot species *A. thaliana* and *B. vulgaris*, the co-segregation analysis was successful in identifying novel bolting factors in *B. vulgaris*. Future analysis will show whether the underlying genes have functionally equivalent homologues in Arabidopsis, or have evolved a function specific to Beta. In addition to these two approaches the existing physical map around the bolting gene *B* of sugar beet was significantly revised, which laid the groundwork for the subsequent successful cloning of the bolting gene (Müller et al. personal communication).

6 Summary

The timing of floral transition is of major importance for the reproductive success of the plant, therefore it is controlled by endogenous and exogenous signals to ensure flowering under favourable conditions. In the model species *Arabidopsis thaliana*, many floral transition genes have been identified and functionally characterised, and plant genome and EST sequencing projects have started to reveal the presence and evolutionary conservation of these genes across taxa (Albert et al. 2005; Hecht et al. 2005). In the crop species *Beta vulgaris* in the Caryophyllid clade of core eudicots, which is only very distantly related to *Arabidopsis*, however, the corresponding genes are largely unknown (Müller et al. 2006). Sugar beet (*Beta vulgaris* L. ssp. *vulgaris*) is a biennial crop, which forms a leave rosette and a storage root in the first year and bolts after a period of prolonged cold temperature (vernalization) in the second year (Hohmann et al. 2005). For cultivation of sugar beet bolting in the first year is an undesirable character, because it decreases sugar yield and obstructs the mechanical harvest. On the other side early bolting and flowering are relevant to speed-up breeding and seed multiplication. Annual bolting is believed to be controlled mainly by the bolting locus *B* (Abegg 1936), which was mapped by RFLP- and high-resolution AFLP-mapping to chromosome II (Boudry et al. 1994; El-Mezawy et al. 2002).

The objectives of this project were to transfer, and expand, our knowledge of flowering time control to sugar beet. Bolting and flowering time genes in sugar beet were identified by candidate gene and co-segregation analysis.

A revised physical map around the *B* locus was established by BAC analysis and remapping using five newly developed markers from opposite ends of individual BAC contigs. The new physical map contains two large BAC contigs flanking the *B* locus on either side. The genetic distance of these contigs to *B* was estimated as 0.3 cM. The new physical map was the prerequisite for identification of markers completely co-segregating with the bolting locus *B* and map-based cloning of *B*. EMS mutagenesis of an annual genotype and phenotypic mutant screening had previously identified several mutants that require vernalization to bolt and thus behave as biennials (Hohmann et al. 2005). There are at least two hypotheses conceivable to explain the mutant phenotypes. 1.) The *B* gene is mutated ('one-locus model'), and 2.) a second locus is mutated that acts epistatically to *B* and prevents annual bolting even in the presence of *B* ('epistatic locus model'). To test the hypotheses F₂ populations segregating for the mutant phenotype and molecular markers located at the *B* locus were generated. In three populations marker genotypes and bolting phenotypes do not co-segregate, suggesting that in these populations annual bolting is effected by a second locus not genetically linked to *B*. One population was selected for genetic mapping of the second locus, and a genetic map was constructed by AFLP mapping. The novel bolting locus was mapped to chromosome IX and was named *B2*. Co-segregation analysis with a marker flanking the *B2* locus in the remaining F₂ populations suggested that *B2* is responsible for bolting also in one other population, providing further evidence for the presence of *B2* in the beet genome and its functional role in bolting control. In addition a locus controlling bolting independently of *B* was identified and named *B3*.

In a complementary approach, *BvFVE1*, a putative orthologue of a flowering time gene of the autonomous pathway, was identified by homology-based methods. In *A. thaliana* the genes of the autonomous pathway function as floral promoters by repressing the central repressor and vernalization-regulatory gene *FLC*. Exon-intron and domain structure was found to be conserved between orthologues. *BvFVE1* was further functionally characterised by expression profiling in sugar beet and complementation analysis in the *Arabidopsis* mutant. *BvFVE1* was not able to complement the *Arabidopsis fve* mutant. Complementation analysis of *BvFVE1*

and the presence of a putative paralogue in beet suggest evolutionary divergence of *FVE* homologues. It is further shown that *BvFVE1*, unlike *FVE* in Arabidopsis, is under circadian clock control.

The current study increases our knowledge about bolting and flowering time control in *B. vulgaris*. The identification and characterisation of *BvFVE1* suggests evolutionary diversification in flowering time control between the two distantly related dicot species *A. thaliana* and *B. vulgaris*, and the co-segregation analysis was successful in identifying novel bolting factors in *B. vulgaris*. Future work will show whether the underlying genes have functionally equivalent homologues in Arabidopsis, or have evolved a function specific to Beta. Further, the physical map around the bolting gene *B* of sugar beet was significantly revised, which was a prerequisite for the subsequent successful cloning of the bolting gene (Müller et al. manuscript in preparation).

7 Zusammenfassung

Der Zeitpunkt der Blühinduktion ist von überragender Bedeutung für den Vermehrungserfolg von Pflanzen und daher kontrolliert von endogenen und exogenen Signalen, die Blüten unter optimalen Bedingungen sicherstellen (Jung et al. 2007). In der Modellpflanze *Arabidopsis thaliana* wurden viele der wichtigen Blühgene identifiziert und funktionell charakterisiert, darüber hinaus zeigen Genom- und EST-Sequenzierungsprojekte das Vorhandensein und die evolutionäre Konservierung dieser Gene in verschiedenen Taxa (Albert et al. 2005; Hecht et al. 2005). In der Art *Beta vulgaris*, die nur sehr entfernt mit *Arabidopsis* verwandt ist, sind die entsprechenden Gene weitgehend unbekannt (Müller et al. 2006). Zuckerrübe (*Beta vulgaris* L. ssp. *vulgaris*) ist eine zweijährige Art, die im ersten Jahr eine Blattrosette und eine Speicherwurzel bildet und im zweiten Jahr nach anhaltender Kälteeinwirkung (Vernalization) schosst (Hohmann et al. 2005). Für die Kultivierung ist Schossen und Blühen unerwünscht, da es den Ertrag dramatisch reduziert und Probleme bei der Ernte bereitet. Frühes Schossen und Blühen jedoch ist wichtig, um Züchtung und Saatguterzeugung zu beschleunigen. Es wird angenommen, dass Schossen im ersten Jahr hauptsächlich durch das Schossgen *B* kontrolliert wird (Abegg 1936), das mittels RFLP- und hochauflösender AFLP-Kartierung auf Chromosom II kartiert wurde (Boudry et al. 1994; El-Mezawy et al. 2002).

Die Ziele der Arbeit sind das Wissen über Blühzeitkontrolle auf Zuckerrübe zu übertragen und zu erweitern. Blüh- und Schossgene der Zuckerrübe werden mittels Kandidatengen- und Kosegregationsanalyse identifiziert.

Eine überarbeitete physikalische Karte um den *B*-Lokus wurde erstellt, indem einige BACs analysiert und rückkartiert wurden mit der Hilfe von fünf neu entwickelten Markern von den entgegengesetzten Enden der einzelnen BAC-Contigs. Die neue physikalische Karte enthält zwei große BAC-Contigs die den *B*-Lokus auf beiden Seiten flankieren. Die genetischen Abstände dieser Contigs zu *B* wurden auf 0,3 cM geschätzt. Die neue physikalische Karte war die Voraussetzung für die Identifizierung eines Markers, der vollständig mit dem Schosslokus *B* segregiert und die kartengestützte Klonierung von *B*.

EMS-Mutagenese eines einjährigen Genotyps und die anschließende phänotypische Mutantenauswahl identifizierte mehrere Mutanten, die Vernalization brauchen um zu schossen und sich daher wie zweijährige Rüben verhalten (Hohmann et al. 2005). Es gibt mindestens zwei Hypothesen um den Mutantenphänotyp zu erklären: 1.) das Schossgen ist mutiert, und 2.) ein zweites Gen ist mutiert, das epistatisch zu *B* agiert und schossen im ersten Jahr auch in Anwesenheit von *B* verhindert. Um die Hypothesen zu überprüfen wurden F₂-Populationen erzeugt, die für den Mutantenphänotyp und molekulare Marker am *B*-Lokus spalten. In drei Populationen spalten Marker und Phänotyp unabhängig voneinander. Diese ist ein Hinweis, dass in diesen Populationen Einjährigkeit von einem zweiten Locus beeinflusst wird, der nicht mit *B* gekoppelt ist. Eine Population wurde ausgewählt um den zweiten Locus genetisch zu kartieren und eine Karte mittels AFLP-Markern erstellt. Der neue Schosslokus wurde auf Chromosome IX kartiert und *B2* genannt. Eine Spaltungsanalyse mit *B2* flankierenden Markern in einer der verbleibenden Populationen lässt vermuten, dass *B2* für das Schossverhalten in einer weiteren Population verantwortlich ist. Dies ist ein weiterer Beweis für das Vorhandensein von *B2* im Rübengenom und seine Rolle in der Schossregulation. Darüber hinaus wurde ein Locus identifiziert, der Schossen unabhängig von *B* kontrolliert und *B3* genannt.

In einem ergänzenden Ansatz wurde *BvFVE1*, ein Ortholog eines Blühgenes des autonomen Weges der Blühregulation anhand von Homologien identifiziert. In *A. thaliana* sind die Gene des autonomen Weges an der Repression des zentralen Repressors *FLC* beteiligt und

demzufolge induzieren sie das Blühen. Die Exon-Intron und Domain-Struktur ist konserviert zwischen den Orthologen. *BvFVE1* wurde weiter funktionell charakterisiert anhand von Expressionsprofilen in Zuckerrübe und Komplementationsanalyse in der Arabidopsis Mutante. *BvFVE1* war nicht in der Lage die Mutante zu komplementieren. Diese Analyse und das Vorhandensein eines potentiellen Paralogs in Rübe deuten evolutionäre Divergenz zwischen den *FVE* Homologen an. Zusätzlich wurde gezeigt, dass *BvFVE1* im Gegensatz zu *FVE* in Arabidopsis circadian reguliert ist.

Diese Studie erweitert unser Wissen über Schoss- und Blühkontrolle in *B. vulgaris*. Die Identifizierung and Charakterisierung von *BvFVE1* zeigt evolutionäre Diversifikation in der Blühzeitkontrolle zwischen den beiden entfernt verwandten Arten *A. thaliana* und *B. vulgaris* und die Kosegregationsanalyse war erfolgreich in der Identifikation neuer Schossloki in *B. vulgaris*. Weitere Arbeiten werden zeigen ob die zugrunde liegenden Gene funktionell äquivalente Homologe in Arabidopsis haben oder ob sie eine Funktion spezifisch für Beta entwickelte haben. Desweiteren wurde die existierende physikalische Karte des *B*-Lokus grundlegend überarbeitet und dies war die Voraussetzung für die anschließende erfolgreiche kartengestützte Klonierung des Schossgens (Müller et al. Manuskript in Vorbereitung).

8 Supplementary material

8.1 Supplementary material for chapter 2

Supplementary Table 1: Non-anonymous molecular markers for analysis of co-segregation with bolting phenotypes.

Marker name (chromosome)	Marker type	Marker assay			Marker allele in mutant parent (M_1)	Marker allele in annual parent (M_2)	Genotyped in population
		Primers	PCR conditions for marker assay	Detection			
GJ1001c16 (II)	InDel	A195-A196	94°C, 2' + [(94°, 30" + 59°C, 30" + 72°C, 25") x 32] + 72°C, 5'	GE ¹	174 bp fragment	202 bp fragment	EW2, EW3
GJ1013c690a (II)	SNP	A749-A750	94°C, 2' + [(94°, 30" + 61.8°C, 30" + 72°C, 80") x 32] + 72°C, 5'	Sequencing	Nt pos. ² 251 = A	Nt pos. 251 = G	EW1
GJ1013c690b (II)	SNP/CAPS	A749-A750	94°C, 2' + [(94°, 30" + 61.8°C, 30" + 72°C, 80") x 32] + 72°C, 5'	<i>Bsa</i> II digest + GE	1729 bp fragment	405 bp and 1324 bp fragments	EW4a, EW4b
GJ18T7b (II)	InDel	A015-A016	94°C, 2' + [(94°, 30" + 54°C, 30" + 72°C, 30") x 32] + 72°C, 5'	GE	142 bp fragment	152 bp fragment	EW4a
Y67L (II)	SNP/CAPS	A019-A020	94°C, 2' + [(94°, 30" + 63°C, 30" + 72°C, 25") x 32] + 72°C, 5'	<i>Hae</i> III digest + GE	240 bp fragment	110 bp and 130 bp fragments	EW4a
MP_R0018a (IX)	SNP/CAPS	B193-B194	94°C, 2' + [(94°, 30" + 57°C, 30" + 72°C, 90") x 32] + 72°C, 5'	<i>Hin</i> fI digest + GE	250 bp and 320 bp fragments	570 bp fragment	EW2, EW3
MP_R0018b (IX)	SNP	B227-B228	94°C, 2' + [(94°, 30" + 58°C, 30" + 72°C, 35") x 32] + 72°C, 5'	Sequencing	Nt pos. 544 = G	Nt pos. 544 = T	EW1, EW4a

¹ GE, gel electrophoresis

² Nt pos., nucleotide position within PCR fragment

Supplementary Table 2: Anchor markers.

Chromosome	Marker name	Marker type	Marker assay				Marker allele in mutant parent (M_1)	Marker allele in annual parent (M_2)
			PCR primers	PCR conditions for marker assay	PCR fragment size in mutant parent [bp]	Detection		
I	KI_P0003 ¹	SNP /CAPS	A784-A785	94°C, 2' + [(94°, 30" + 58°C, 30" + 72°C, 60") x 32] + 72°C, 5'	575	<i>Hinf</i> I digest + GE ²	575 bp fragment	124 bp and 451 bp fragments
II	TG_E0040 ³	SNP /CAPS	B123-B124	94°C, 2' + [(94°, 30" + 59°C, 30" + 72°C, 40") x 32] + 72°C, 5'	510	<i>Hpa</i> II digest + GE	510 bp fragment	205 bp and 305 bp fragments
II	MP_R0145 ³	SNP /CAPS	B161-B162	94°C, 2' + [(94°, 30" + 55°C, 30" + 72°C, 30") x 32] + 72°C, 5'	509	<i>Pst</i> I digest + GE	509 bp fragment	200 bp and 309 bp fragments
II	BQ584037 ⁴	InDel (SSR)	A974-A975	94°C 1,5' + [(94°C 30" + 58°C 30" + 72°C 1') 13x -0,8°C] + [(94°C 30" + 47°C 30" + 72°C 1') 31x] + 72°C 10'	~135	GE (3% MetaPhor)	~135 bp fragment	~140 bp fragment
III	KI_P0002 ¹	SNP	A626-A622	94°C, 2' + [(94°, 30" + 58°C, 30" + 72°C, 75") x 32] + 72°C, 5'	~1000	Sequencing	Nt pos. ⁵ ~320 = A	Nt pos. ~320 = G
IV	FDSB1023 ⁶	InDel (SSR)	A991-A992	94°C 1,5' + [(94°C 30" + 58°C 30" + 72°C 1') 13x -0,8°C] + [(94°C 30" + 47°C 30" + 72°C 1') 31x] + 72°C 10'	~250	GE (3% MetaPhor)	~250 bp fragment	~245 bp fragment
V	SB156 ⁶	InDel (SSR)	A978-A979	94°C 1,5' + [(94°C 30" + 58°C 30" + 72°C 1') 13x -0,8°C] + [(94°C 30" + 47°C 30" + 72°C 1') 31x] + 72°C 10'	~150	GE (3% MetaPhor)	~150 bp fragment	~155 bp fragment
VI	BvGTT1 ⁶	InDel (SSR)	B011-B012	94°C 1,5' + [(94°C 30" + 58°C 30" + 72°C 1') 13x -0,8°C] + [(94°C 30" + 47°C 30" + 72°C 1') 31x] + 72°C 10'	~125	GE (3% MetaPhor)	~125 bp fragment	~120 bp fragment
VII	KI_P0001 ¹	SNP	A185-A137	94°C, 2' + [(94°, 30" + 53°C, 30" + 72°C, 100") x 32] + 72°C, 5'	1457	Sequencing	Nt pos. 209 = C	Nt pos. 209 = T
VII	TG_E0240 ³	SNP	A108-A109	94°C, 2' + [(94°, 30" + 58°C, 30" + 72°C, 60") x 32] + 72°C, 5'	536	Sequencing	Nt pos. 323 = A	Nt pos. 323 = T
VIII	FDSB1007 ⁶	InDel (SSR)	B015-B016	94°C 1,5' + [(94°C 30" + 58°C 30" + 72°C 1') 13x -0,8°C] + [(94°C 30" + 47°C 30" + 72°C 1') 31x] + 72°C 10'	~270	GE (3% MetaPhor)	~270 bp fragment	~260 bp fragment
IX	MP_R0018a ³	SNP /CAPS	B193-B194	94°C, 2' + [(94°, 30" + 57°C, 30" + 72°C, 90") x 32] + 72°C, 5'	570	<i>Hinf</i> I digest + GE	250 bp and 320 bp fragments	570 bp fragment
IX	KI_P0004 ¹	SNP	B212-B213	94°C, 2' + [(94°, 30" + 55°C, 30" + 72°C, 40") x 32] + 72°C, 5'	542	Sequencing	Nt pos. 342 = C	Nt pos. 342 = T

¹ Unpublished markers (Schulze-Buxloh et al. personal communication), named according to the nomenclature by Schneider et al. (2007): KI, Kiel; P, polymorphism² GE, gel electrophoresis³ Schneider et al. 2007⁴ McGrath et al. 2007⁵ Nt pos., nucleotide position within PCR fragment⁶ Laurent et al. 2007

Supplementary Table 3: F₂ Phenotyping and genotyping data.

F ₂ Population/ Plant number	Days to bolting ¹	GJ1013 c690a ^{2,3}	MP_R0018
EW1/1	nb	B	B
EW1/2	nb	H	H
EW1/3	63	H	H
EW1/4	70	H	H
EW1/6	59	A	A
EW1/7	77	H	H
EW1/8	nb	B	B
EW1/10	73	H	B
EW1/11	61	A	H
EW1/12	66	H	B
EW1/13	nb	H	nd
EW1/15	nb	H	A
EW1/16	89	H	A
EW1/17	47	A	A
EW1/18	89	A	A
EW1/19	47	A	A
EW1/20	61	A	H
EW1/21	59	H	A
EW1/22	73	A	H
EW1/23	nb	B	H
EW1/25	75	H	H
EW1/26	75	B	H
EW1/28	nb	H	H
EW1/29	129	B	H
EW1/30	nb	B	H
EW1/31	nb	B	H
EW1/32	75	H	H
EW1/33	nb	H	H
EW1/35	nb	H	A
EW1/36	nb	A	A
EW1/37	nb	H	H
EW1/39	nb	H	B
EW1/40	87	A	B
EW1/41	63	B	H
EW1/42	nb	B	H
EW1/43	nb	A	H
EW1/44	68	A	A
EW1/45	77	H	B
EW1/46	nb	H	B

F ₂ Population/ Plant number	Days to bolting ¹	GJ1013 c690a ^{2,3}	MP_R0018
EW1/48	122	A	H
EW1/49	70	H	B
EW1/50	nb	A	H
EW1/52	nb	H	H
EW1/54	56	H	H
EW1/55	nb	H	B
EW1/56	nb	H	H
EW1/57	70	H	A
EW1/59	61	A	B
EW1/60	77	H	B
EW1/62	73	H	H
EW1/63	66	H	A
EW1/64	73	A	A
EW1/65	117	H	A
EW1/66	63	H	B
EW1/67	110	B	H
EW1/68	nb	H	H
EW1/69	63	A	B
EW1/71	nb	H	A
EW1/72	89	H	A
EW1/73	73	H	B
EW1/74	100	A	H
EW1/75	84	B	A
EW1/76	nb	A	H
EW1/77	70	H	H
EW1/78	66	H	H
EW1/80	66	H	A
EW1/81	119	B	H
EW1/83	96	H	H
EW1/84	52	A	B
EW1/85	61	B	H
EW1/86	nb	H	H
EW1/87	87	A	A
EW1/88	70	B	H
EW1/91	89	H	A
EW1/92	87	B	H
EW1/94	66	H	B
EW1/95	89	H	H
EW1/96	75	H	A

F ₂ Population/ Plant number	Days to bolting	GJ1001c 16	MP_R0018
EW2/1	42	A	H
EW2/2	40	A	H
EW2/3	40	H	H
EW2/4	45	H	H
EW2/5	nb	H	B
EW2/6	45	A	H

F ₂ Population/ Plant number	Days to bolting	GJ1001 c16	MP_R0018
EW2/50	nb	A	B
EW2/51	34	H	A
EW2/52	34	H	H
EW2/53	59	A	H
EW2/54	nb	H	B
EW2/55	34	B	H

EW2/7	59	A	A
EW2/8	nb	A	B
EW2/9	56	H	H
EW2/10	68	H	H
EW2/11	40	A	H
EW2/12	52	H	H
EW2/13	59	H	A
EW2/14	47	A	H
EW2/15	52	A	A
EW2/16	47	H	H
EW2/17	45	H	H
EW2/18	47	H	A
EW2/19	45	H	H
EW2/20	nb	H	H
EW2/21	47	H	H
EW2/22	45	B	H
EW2/23	34	H	H
EW2/24	40	H	H
EW2/25	40	H	H
EW2/26	52	H	B
EW2/27	nb	B	H
EW2/28	49	A	H
EW2/29	nb	H	B
EW2/30	45	B	B
EW2/32	49	H	H
EW2/33	nb	A	B
EW2/34	47	H	H
EW2/35	34	H	H
EW2/36	40	H	H
EW2/39	42	A	A
EW2/40	34	H	A
EW2/41	34	H	A
EW2/42	34	H	H
EW2/43	34	H	H
EW2/44	52	H	H
EW2/45	38	B	H
EW2/46	38	H	H
EW2/47	38	H	H
EW2/48	nb	H	B
EW2/49	38	B	A

EW2/56	45	A	H
EW2/57	45	H	H
EW2/58	nb	H	A
EW2/59	38	H	H
EW2/60	42	B	H
EW2/61	54	B	H
EW2/62	66	B	H
EW2/63	54	H	H
EW2/64	nb	A	B
EW2/65	40	H	H
EW2/66	45	A	H
EW2/68	42	H	H
EW2/69	38	H	H
EW2/70	40	A	H
EW2/71	45	A	H
EW2/72	45	H	H
EW2/73	45	H	A
EW2/74	nb	A	B
EW2/75	38	H	A
EW2/76	38	H	H
EW2/77	59	H	H
EW2/78	34	H	H
EW2/79	34	H	H
EW2/80	34	H	H
EW2/81	42	A	A
EW2/82	47	A	H
EW2/83	52	A	H
EW2/84	nb	B	H
EW2/85	61	H	H
EW2/86	59	A	H
EW2/87	45	A	A
EW2/88	nb	B	B
EW2/89	nb	H	A
EW2/90	nb	H	B
EW2/91	56	A	H
EW2/92	nb	A	B
EW2/93	nb	H	B
EW2/94	34	H	H
EW2/95	38	H	H
EW2/96	nb	A	B

F ₂ Population/ Plant number	Days to bolting	GJ1001c 16	MP R0018
EW3/1	56	H	H
EW3/2	66	A	H
EW3/3	nb	B	B
EW3/4	nb	B	B
EW3/5	40	H	H
EW3/6	34	H	H
EW3/7	34	H	H
EW3/8	34	H	A
EW3/10	nb	A	B
EW3/11	nb	B	B

F ₂ Population/ Plant number	Days to bolting	GJ1001 c16	MP R0018
EW3/49	34	B	A
EW3/50	34	B	A
EW3/51	nb	H	B
EW3/52	34	H	H
EW3/53	34	B	H
EW3/54	73	H	B
EW3/55	34	B	H
EW3/56	34	B	H
EW3/57	34	B	H
EW3/59	101	B	B

EW3/14	105	H	B	EW3/60	34	A	A
EW3/15	34	H	A	EW3/61	38	H	A
EW3/16	nb	H	B	EW3/62	34	A	A
EW3/17	66	H	A	EW3/63	34	H	H
EW3/18	nb	A	H	EW3/64	nb	H	B
EW3/19	38	A	H	EW3/65	34	A	H
EW3/20	89	H	B	EW3/66	nb	A	B
EW3/21	38	A	H	EW3/67	94	B	B
EW3/22	nb	A	B	EW3/68	34	A	H
EW3/23	98	H	B	EW3/69	34	B	H
EW3/24	38	H	H	EW3/70	34	H	H
EW3/25	101	B	B	EW3/71	34	H	H
EW3/26	56	A	H	EW3/72	34	H	H
EW3/27	80	B	B	EW3/73	34	B	H
EW3/28	47	H	H	EW3/74	77	H	H
EW3/29	34	H	A	EW3/75	34	A	H
EW3/30	34	H	A	EW3/77	34	A	H
EW3/31	63	B	H	EW3/78	nb	B	B
EW3/32	34	H	A	EW3/80	38	A	B
EW3/33	34	B	H	EW3/81	87	H	A
EW3/34	105	H	B	EW3/82	38	H	B
EW3/35	34	H	H	EW3/83	59	B	H
EW3/36	34	B	A	EW3/84	38	H	H
EW3/37	34	A	H	EW3/85	nb	A	B
EW3/38	34	B	A	EW3/86	34	H	H
EW3/39	34	B	H	EW3/87	73	H	H
EW3/40	38	H	H	EW3/88	49	H	B
EW3/41	nb	B	B	EW3/89	34	H	H
EW3/42	47	H	A	EW3/90	52	B	H
EW3/43	34	B	A	EW3/91	34	H	B
EW3/44	89	A	B	EW3/92	122	H	B
EW3/45	34	A	A	EW3/93	38	B	H
EW3/46	34	H	H	EW3/94	96	A	A
EW3/47	78	H	B	EW3/95	98	H	B
EW3/48	nb	H	B	EW3/96	34	H	H

F ₂ Population/ Plant number	Days to bolting	Y67L	GJ1013 c690b	GJ18T7b	MP_R00 18
EW4a/1	48	H	H	H	A
EW4a/2	48	A	H	H	B
EW4a/3	53	H	B	B	A
EW4a/4	41	H	H	H	B
EW4a/5	46	H	H	H	H
EW4a/6	48	B	H	A	A
EW4a/7	46	H	H	H	A
EW4a/8	82	B	H	B	B
EW4a/9	46	A	A	A	A
EW4a/10	58	B	B	B	H
EW4a/11	41	H	H	H	B
EW4a/12	46	H	H	H	H
EW4a/13	51	B	H	H	H
EW4a/14	53	B	B	B	A
EW4a/15	44	A	A	A	A

EW4a/16	76	H	B	B	H
EW4a/17	44	A	H	A	H
EW4a/18	76	B	B	B	H
EW4a/19	53	H	H	H	A
EW4a/20	44	A	A	A	B
EW4a/21	44	H	H	H	H
EW4a/22	58	B	B	B	A
EW4a/23	48	H	H	H	H
EW4a/24	76	H	H	H	H
EW4a/25	44	A	A	A	A
EW4a/26	62	B	B	B	B
EW4a/27	62	H	B	B	H
EW4a/28	46	H	H	H	H
EW4a/29	44	H	H	H	H
EW4a/30	46	H	H	H	H
EW4a/31	55	H	H	A	H
EW4a/32	55	A	A	A	B
EW4a/33	41	A	A	A	B
EW4a/34	39	A	A	A	H
EW4a/35	55	H	B	B	A
EW4a/36	51	H	H	H	H
EW4a/37	74	B	B	B	A
EW4a/38	44	H	H	H	H
EW4a/39	58	B	B	B	H
EW4a/40	41	H	H	H	A
EW4a/41	55	A	A	A	H
EW4a/42	53	B	B	B	H
EW4a/43	55	H	H	H	H
EW4a/44	46	A	A	A	A
EW4a/45	44	H	H	H	H
EW4a/46	48	H	A	A	B
EW4a/47	44	H	H	H	B
EW4a/48	62	B	B	B	H
EW4a/49	41	A	H	H	B
EW4a/50	51	H	H	H	B
EW4a/51	44	H	H	H	H
EW4a/52	58	B	B	B	H
EW4a/53	62	B	B	B	H
EW4a/54	58	H	H	H	H
EW4a/55	55	H	H	H	H
EW4a/56	44	A	A	A	B
EW4a/57	58	B	B	H	A
EW4a/58	nb	B	B	B	H
EW4a/59	48	H	H	H	A
EW4a/60	44	A	A	A	H
EW4a/61	46	H	H	H	B
EW4a/62	62	B	B	B	H
EW4a/63	55	H	H	H	H
EW4a/64	46	H	H	H	H
EW4a/65	58	B	B	B	B
EW4a/66	41	H	H	H	B
EW4a/67	41	H	H	H	H
EW4a/68	51	H	H	H	B
EW4a/69	41	H	H	H	B

EW4a/70	41	A	A	A	H
EW4a/71	48	H	H	H	H
EW4a/72	41	H	H	H	A
EW4a/73	44	B	H	A	A
EW4a/74	58	B	B	B	A
EW4a/75	53	B	H	H	H
EW4a/76	44	H	H	H	H
EW4a/77	83	B	B	A	H
EW4a/78	41	A	H	A	B
EW4a/79	44	H	H	H	H
EW4a/80	44	A	A	A	B
EW4a/81	58	A	A	A	H
EW4a/82	nb	B	B	B	H
EW4a/83	44	H	H	H	H
EW4a/84	65	B	B	B	H
EW4a/85	44	H	H	H	A
EW4a/86	53	H	H	H	A
EW4a/87	51	H	H	H	H
EW4a/88	44	A	A	A	H
EW4a/89	46	H	H	H	A
EW4a/90	44	A	H	A	H
EW4a/91	46	B	B	B	H
EW4a/92	nb	B	H	A	A
EW4a/93	41	A	A	A	B
EW4a/94	44	H	H	H	B
EW4a/95	39	A	A	A	H
EW4a/96	48	A	A	A	nd
EW4a/97	44	A	A	A	B
EW4a/98	44	H	H	H	H
EW4a/99	58	H	H	H	H
EW4a/100	41	H	H	H	B
EW4a/101	51	H	H	H	H
EW4a/102	58	B	B	B	B
EW4a/103	58	B	B	B	A
EW4a/104	44	H	H	H	H
EW4a/105	48	H	H	H	H
EW4a/106	53	B	B	B	A
EW4a/107	41	H	H	H	H
EW4a/108	nb	B	B	B	B
EW4a/109	46	H	H	H	H
EW4a/110	46	H	H	H	H
EW4a/111	62	H	H	H	H
EW4a/112	44	H	H	H	B
EW4a/113	48	H	H	H	H
EW4a/114	41	H	H	H	H
EW4a/115	48	H	H	H	H
EW4a/116	51	H	H	H	H
EW4a/117	62	H	H	H	H
EW4a/118	41	H	H	H	H
EW4a/119	48	H	H	H	A
EW4a/120	44	A	H	A	H
EW4a/121	62	B	B	B	H
EW4a/122	nb	B	B	B	H
EW4a/123	44	A	A	A	H

EW4a/124	62	B	B	B	H
EW4a/125	53	B	B	H	A
EW4a/126	48	H	H	H	H
EW4a/127	55	A	A	A	B
EW4a/128	51	A	A	A	H
EW4a/129	48	H	H	H	H
EW4a/130	41	B	A	A	nd
EW4a/131	51	B	B	B	A
EW4a/132	51	A	A	A	H
EW4a/133	41	H	H	H	H
EW4a/134	72	A	A	A	H
EW4a/135	51	H	H	H	A
EW4a/136	41	A	A	A	B
EW4a/137	65	B	B	B	B
EW4a/138	48	B	H	B	H
EW4a/139	44	H	H	H	A
EW4a/140	44	A	A	A	H

F ₂ Population/ Plant number	Days to bolting	GJ1013 c690b
EW4b/1	52	H
EW4b/2	42	H
EW4b/3	nb	B
EW4b/4	59	H
EW4b/5	nb	B
EW4b/6	61	A
EW4b/7	61	A
EW4b/8	61	H
EW4b/9	49	A
EW4b/10	nb	B
EW4b/11	nb	B
EW4b/12	56	A
EW4b/13	69	A
EW4b/14	52	A
EW4b/15	61	A
EW4b/16	nb	B
EW4b/17	nb	B
EW4b/18	52	A
EW4b/19	61	H
EW4b/20	61	H
EW4b/21	52	H
EW4b/22	nb	B
EW4b/23	61	H
EW4b/24	52	A
EW4b/25	54	H
EW4b/26	52	A
EW4b/27	59	H
EW4b/28	nb	B
EW4b/29	56	A
EW4b/30	56	A
EW4b/31	nb	B
EW4b/32	59	H

F ₂ Population/ Plant number	Days to bolting	GJ1013 c690b
EW4b/48	66	H
EW4b/49	nb	B
EW4b/50	49	A
EW4b/51	77	H
EW4b/52	61	H
EW4b/53	59	H
EW4b/54	54	H
EW4b/55	52	A
EW4b/56	66	H
EW4b/57	42	H
EW4b/58	nb	B
EW4b/59	59	H
EW4b/60	59	H
EW4b/61	nb	B
EW4b/62	nb	B
EW4b/63	56	H
EW4b/64	42	H
EW4b/65	56	A
EW4b/67	59	H
EW4b/68	61	A
EW4b/70	74	A
EW4b/71	56	H
EW4b/72	nb	B
EW4b/73	nb	A
EW4b/74	nb	B
EW4b/75	45	H
EW4b/76	54	H
EW4b/77	61	H
EW4b/78	61	H
EW4b/79	nb	B
EW4b/80	52	A
EW4b/81	52	A

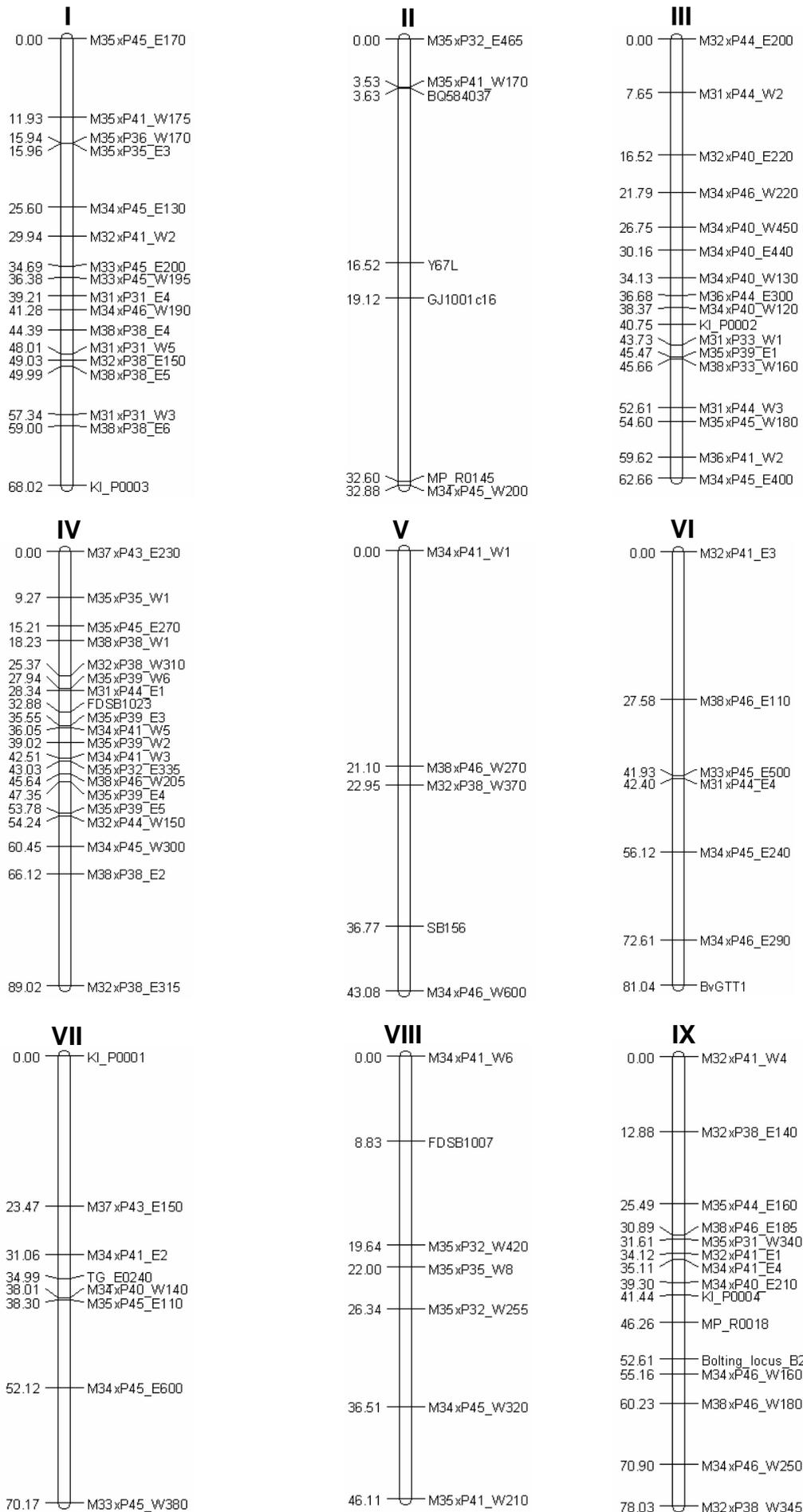
EW4b/33	59	H
EW4b/34	59	A
EW4b/35	72	A
EW4b/36	nb	B
EW4b/37	nb	B
EW4b/38	73	H
EW4b/39	45	H
EW4b/40	nb	B
EW4b/41	nb	H
EW4b/42	nb	B
EW4b/43	52	A
EW4b/44	73	H
EW4b/45	42	A
EW4b/46	59	H
EW4b/47	42	H

EW4b/82	52	A
EW4b/83	nb	H
EW4b/84	42	A
EW4b/85	68	A
EW4b/86	45	H
EW4b/87	nb	H
EW4b/88	68	A
EW4b/89	nb	B
EW4b/90	52	A
EW4b/91	nb	B
EW4b/92	56	H
EW4b/93	47	H
EW4b/94	59	H
EW4b/95	45	H

¹ nb, non-bolting

² 'B' and 'A' indicate *B* locus marker genotypes homozygous for the marker allele (M_1) derived from the mutants, or homozygous for the marker allele (M_2) derived from the annual parent accession, respectively. 'H' indicates heterozygous marker genotypes.

³ nd, not determined



Supplementary Figure 1: AFLP map of the *B. vulgaris* genome. The map comprises 141 AFLP markers, ten SNP-based markers and five SSR markers. All linkage groups were anchored to the nine chromosomes (I-IX). The *B* locus markers GJ1001c16 and Y67L are located on chromosome II, in accordance with the known chromosomal location of this locus. The novel bolting locus *B2* is located on chromosome IX. Map positions are given in centiMorgan.

8.2 Supplementary material for chapter 3

Supplementary Table 4: Primer sequences.

Primer name	Sequence (5'→3')
A479	ATTTTGCCGATTTTCGGAAC
A477	TGCAGAGCTTCATGGAAGT
B476	GGAGCTGCAAATCCCGTCC
A478	ATACCAGTTGCAGGACCAGG
B478	GCGTGGACCGCTTGCTGCAACT
B477	GACTTGTGCCTCCAAGCCTTAA
A039	AACCCTTGAAGAGCAGTTTTGGC
A064	AGAGGTATTATACTATCGATCTACC
A066	GGTAGACGAGAAGTACAGCCAG
A042	CAGCAGCAACTCTAGGTTTAAACAACC
A043	ACTATCCTTCAGAAATCGACATCGG
A067	GCTTCGGGATTCAACAGATCTC
A033	GTTTGGGCTGTAAATAAGGTTGC
A065	CCAAACTCAACCCTCAACAGTG
A396	ATGGCAGAGGCAGACAAGG
A397	ATATCCATAATTTCCACCGTAAACTG
A836	ATGACGGAGAAAGGTAGAGGA
A837	TTAAGAGGGGCATGTCGTTAC
A823	GTTACTTCTTACCATCTGATCC
A825	TTGAACATGAACCCCACCACC
A067	GCTTCGGGATTCAACAGATCTC
A108	CGTGTACATTCAACCTGTTGAAG
B391	CGAAGCTGATTAATCATGGAGG
A046	GCAAGGAGCTCGAGATCAGGC
A043	ACTATCCTTCAGAAATCGACATCGG
B392	GAGCAGTTGACGTCCTAAG
A068	TACCAGAAGTTGGCGCTGGAG
A069	TAGGGGTAGGTGAAGGAAGAG
A045	CCATGGGATATTGAGATAGACTACG
B396	TTACCGCCTCCGATCACCAC
B042	GTCTGTGAACCCTCAAAAGTC
B043	ATGTTGGTACCTGCCAAAAG
A066	GGTAGACGAGAAGTACAGCCAG
A042	CAGCAGCAACTCTAGGTTTAAACAACC
A830	TATGCTCCGCAGCAGCAACTCTAG
A831	TTCAGAGAGGTAAAGGCGCTGTGAT
B336	CGATAACCTGGTCAAGTCC
B337	CAGTCTCAAGGTGTTCTCC
B281	GAGAGACGGTATTCCGTATG
B282	GCATGTGGACTCTAAGAAGG
B349	CTAGCTGCACCACTAAGTGC
B350	CGTTAAGAGCTGGAAGCACC
A680	GCCGCCTCGAGCAATATATGGCAGAGGCAGAG
A652	CACCTTTAATACCTATAATTCAAACCTAGTTAGTAC
A896	CAACGGAATTCCTACCGTACTACCCCTC
A821	TCTAAATGCGAGTTAACTGGAGC
A901	CGACTCGAGCTAGTCATGGCTGAAGC
A938	GACCCGGGTCATCAGTAACCGTAGCCTGAGC
A875	CAAGATCTCTCTGCCGACAGTGG
A876	AATATCATGCGATCATAGGCGTC
B474	GCTTTGACCGACCACTTCGC
B475	ACGCCGAGAGCAACTTGAA

Supplementary Table 5: Days to bolting and total number of leaves at bolting in 53 T2 families derived from transformation with *BvFLK* or *FLK* and non-transformed controls (Col-0 and *flk-1*). For T2 families in the Col-0 background, plants which bolted five or more days earlier than the mean of the Col-0 control plants (37.88±1.50 days to bolting) were classified as early bolting, all other plants as late bolting. For T2 families in the *flk-1* background, plants which bolted as late as the *flk-1* mutant controls (63-77 days to bolting) were considered as late bolting, whereas plants which bolted much earlier (25-44 days to bolting) and thus within a similar time range as Col-0 control plants (35-40 days to bolting) were classified as early bolting. The observed segregation ratios were tested by X² analysis for deviation from the null hypotheses for dominant-recessive inheritance of one or two transgene loci (3:1 or 15:1, respectively). Six putative single transgene-locus families (indicated in bold letters) were tested by PCR for segregation of the transgene.

T2 plants (genetic background; transgene cassette; family number / plant number)	Number of days to bolting	Total number of leaves at bolting	T2 plants (genetic background; transgene cassette; family number / plant number)	Number of days to bolting	Total number of leaves at bolting
Col-0; 35S:: <i>BvFLK</i> ; 3/1	28	16	Col-0; 35S:: <i>BvFLK</i> ; 4/1	25	14
Col-0; 35S:: <i>BvFLK</i> ; 3/2	31	16	Col-0; 35S:: <i>BvFLK</i> ; 4/2	28	11
Col-0; 35S:: <i>BvFLK</i> ; 3/3	28	13	Col-0; 35S:: <i>BvFLK</i> ; 4/3	28	15
Col-0; 35S:: <i>BvFLK</i> ; 3/4	28	13	Col-0; 35S:: <i>BvFLK</i> ; 4/4	28	15
Col-0; 35S:: <i>BvFLK</i> ; 3/5	28	15	Col-0; 35S:: <i>BvFLK</i> ; 4/5	28	16
Col-0; 35S:: <i>BvFLK</i> ; 3/6	31	18	Col-0; 35S:: <i>BvFLK</i> ; 4/6	31	15
Col-0; 35S:: <i>BvFLK</i> ; 3/7	31	15	Col-0; 35S:: <i>BvFLK</i> ; 4/7	40	22
Col-0; 35S:: <i>BvFLK</i> ; 3/8	31	21	Col-0; 35S:: <i>BvFLK</i> ; 4/8	28	17
Col-0; 35S:: <i>BvFLK</i> ; 3/9	28	18	Col-0; 35S:: <i>BvFLK</i> ; 4/9	31	15
Col-0; 35S:: <i>BvFLK</i> ; 3/10	31	16	Col-0; 35S:: <i>BvFLK</i> ; 4/10	31	19
Col-0; 35S:: <i>BvFLK</i> ; 3/11	35	15	Col-0; 35S:: <i>BvFLK</i> ; 4/11	31	17
Col-0; 35S:: <i>BvFLK</i> ; 3/12	28	13	Col-0; 35S:: <i>BvFLK</i> ; 4/12	31	12
Col-0; 35S:: <i>BvFLK</i> ; 3/13	28	13	Col-0; 35S:: <i>BvFLK</i> ; 4/13	31	17
Col-0; 35S:: <i>BvFLK</i> ; 3/14	28	11	Col-0; 35S:: <i>BvFLK</i> ; 4/14	38	16
Col-0; 35S:: <i>BvFLK</i> ; 3/15	31	16	Col-0; 35S:: <i>BvFLK</i> ; 4/15	28	14
Col-0; 35S:: <i>BvFLK</i> ; 3/16	28	14	Col-0; 35S:: <i>BvFLK</i> ; 4/16	31	14
Col-0; 35S:: <i>BvFLK</i> ; 3/17	28	14	Col-0; 35S:: <i>BvFLK</i> ; 4/17	24	14
Segregation ratio (early bolting: late bolting)	16:1		Segregation ratio (early bolting: late bolting)	15:2	
X ² for 3:1	3.31 (ns) ^a		X ² for 3:1	1.59 (ns)	
X ² for 15:1	0.01 (ns)		X ² for 15:1	0.86 (ns)	
Col-0; 35S:: <i>BvFLK</i> ; 5/1	31	20	Col-0; 35S:: <i>BvFLK</i> ; 6/1	31	13
Col-0; 35S:: <i>BvFLK</i> ; 5/2	- ^c	-	Col-0; 35S:: <i>BvFLK</i> ; 6/2	28	16
Col-0; 35S:: <i>BvFLK</i> ; 5/3	31	21	Col-0; 35S:: <i>BvFLK</i> ; 6/3	35	13
Col-0; 35S:: <i>BvFLK</i> ; 5/4	31	19	Col-0; 35S:: <i>BvFLK</i> ; 6/4	-	-
Col-0; 35S:: <i>BvFLK</i> ; 5/5	31	18	Col-0; 35S:: <i>BvFLK</i> ; 6/5	24	9
Col-0; 35S:: <i>BvFLK</i> ; 5/6	31	22	Col-0; 35S:: <i>BvFLK</i> ; 6/6	31	14
Col-0; 35S:: <i>BvFLK</i> ; 5/7	28	17	Col-0; 35S:: <i>BvFLK</i> ; 6/7	28	15
Col-0; 35S:: <i>BvFLK</i> ; 5/8	28	14	Col-0; 35S:: <i>BvFLK</i> ; 6/8	28	13
Col-0; 35S:: <i>BvFLK</i> ; 5/9	31	17	Col-0; 35S:: <i>BvFLK</i> ; 6/9	28	15
Col-0; 35S:: <i>BvFLK</i> ; 5/10	31	18	Col-0; 35S:: <i>BvFLK</i> ; 6/10	28	13
Col-0; 35S:: <i>BvFLK</i> ; 5/11	31	21	Col-0; 35S:: <i>BvFLK</i> ; 6/11	39	17
Col-0; 35S:: <i>BvFLK</i> ; 5/12	31	17	Col-0; 35S:: <i>BvFLK</i> ; 6/12	28	13
Col-0; 35S:: <i>BvFLK</i> ; 5/13	31	12	Col-0; 35S:: <i>BvFLK</i> ; 6/13	31	12
Col-0; 35S:: <i>BvFLK</i> ; 5/14	31	18	Col-0; 35S:: <i>BvFLK</i> ; 6/14	31	14
Col-0; 35S:: <i>BvFLK</i> ; 5/15	39	15	Col-0; 35S:: <i>BvFLK</i> ; 6/15	31	15

Col-0; 35S:: <i>BvFLK</i> ; 5/16	31	15
Col-0; 35S:: <i>BvFLK</i> ; 5/17	31	18
Segregation ratio (early bolting: late bolting)	15:1	
X ² for 3:1	3.00 (ns)	
X ² for 15:1	0.00 (ns)	
Col-0; 35S::<i>BvFLK</i>; 7/1	28	16
Col-0; 35S::<i>BvFLK</i>; 7/2^b	40	18
Col-0; 35S::<i>BvFLK</i>; 7/3	28	11
Col-0; 35S::<i>BvFLK</i>; 7/4	28	13
Col-0; 35S::<i>BvFLK</i>; 7/5	28	16
Col-0; 35S::<i>BvFLK</i>; 7/6	29	15
Col-0; 35S::<i>BvFLK</i>; 7/7	33	15
Col-0; 35S::<i>BvFLK</i>; 7/8	24	15
Col-0; 35S::<i>BvFLK</i>; 7/9	28	14
Col-0; 35S::<i>BvFLK</i>; 7/10^b	39	18
Col-0; 35S::<i>BvFLK</i>; 7/11	24	15
Col-0; 35S::<i>BvFLK</i>; 7/12^b	38	19
Col-0; 35S::<i>BvFLK</i>; 7/13	25	17
Col-0; 35S::<i>BvFLK</i>; 7/14	24	17
Col-0; 35S::<i>BvFLK</i>; 7/15	31	17
Col-0; 35S::<i>BvFLK</i>; 7/16	31	17
Col-0; 35S::<i>BvFLK</i>; 7/17^b	35	19
Segregation ratio (early bolting: late bolting)	13:4	
X ² for 3:1	0.02 (ns)	
X ² for 15:1	8.56 **	
Col-0; 35S:: <i>BvFLK</i> ; 12/1	25	12
Col-0; 35S:: <i>BvFLK</i> ; 12/2	31	18
Col-0; 35S:: <i>BvFLK</i> ; 12/3	25	13
Col-0; 35S:: <i>BvFLK</i> ; 12/4	25	14
Col-0; 35S:: <i>BvFLK</i> ; 12/5	31	18
Col-0; 35S:: <i>BvFLK</i> ; 12/6	25	16
Col-0; 35S:: <i>BvFLK</i> ; 12/7	28	21
Col-0; 35S:: <i>BvFLK</i> ; 12/8	28	12
Col-0; 35S:: <i>BvFLK</i> ; 12/9	28	14
Col-0; 35S:: <i>BvFLK</i> ; 12/10	24	13
Col-0; 35S:: <i>BvFLK</i> ; 12/11	28	12
Col-0; 35S:: <i>BvFLK</i> ; 12/12	25	17
Col-0; 35S:: <i>BvFLK</i> ; 12/13	28	15
Col-0; 35S:: <i>BvFLK</i> ; 12/14	28	14
Col-0; 35S:: <i>BvFLK</i> ; 12/15	28	14
Col-0; 35S:: <i>BvFLK</i> ; 12/16	-	-
Col-0; 35S:: <i>BvFLK</i> ; 12/17	24	17
Segregation ratio (early bolting: late bolting)	16:0	
X ² for 3:1	5.33 **	

Col-0; 35S:: <i>BvFLK</i> ; 6/16	28	13
Col-0; 35S:: <i>BvFLK</i> ; 6/17	28	14
Segregation ratio (early bolting: late bolting)	14:2	
X ² for 3:1	1.33 (ns)	
X ² for 15:1	1.07 (ns)	
Col-0; 35S:: <i>BvFLK</i> ; 10/1	28	11
Col-0; 35S:: <i>BvFLK</i> ; 10/2	31	12
Col-0; 35S:: <i>BvFLK</i> ; 10/3	28	9
Col-0; 35S:: <i>BvFLK</i> ; 10/4	38	15
Col-0; 35S:: <i>BvFLK</i> ; 10/5	28	12
Col-0; 35S:: <i>BvFLK</i> ; 10/6	28	16
Col-0; 35S:: <i>BvFLK</i> ; 10/7	28	16
Col-0; 35S:: <i>BvFLK</i> ; 10/8	28	14
Col-0; 35S:: <i>BvFLK</i> ; 10/9	28	11
Col-0; 35S:: <i>BvFLK</i> ; 10/10	31	14
Col-0; 35S:: <i>BvFLK</i> ; 10/11	28	13
Col-0; 35S:: <i>BvFLK</i> ; 10/12	31	18
Col-0; 35S:: <i>BvFLK</i> ; 10/13	28	21
Col-0; 35S:: <i>BvFLK</i> ; 10/14	-	-
Col-0; 35S:: <i>BvFLK</i> ; 10/15	24	13
Col-0; 35S:: <i>BvFLK</i> ; 10/16	28	17
Col-0; 35S:: <i>BvFLK</i> ; 10/17	28	17
Segregation ratio (early bolting: late bolting)	15:1	
X ² for 3:1	3.00 (ns)	
X ² for 15:1	0.00 (ns)	
Col-0; 35S:: <i>BvFLK</i> ; 13/1	28	14
Col-0; 35S:: <i>BvFLK</i> ; 13/2	31	16
Col-0; 35S:: <i>BvFLK</i> ; 13/3	39	18
Col-0; 35S:: <i>BvFLK</i> ; 13/4	28	13
Col-0; 35S:: <i>BvFLK</i> ; 13/5	31	14
Col-0; 35S:: <i>BvFLK</i> ; 13/6	31	16
Col-0; 35S:: <i>BvFLK</i> ; 13/7	28	14
Col-0; 35S:: <i>BvFLK</i> ; 13/8	28	13
Col-0; 35S:: <i>BvFLK</i> ; 13/9	31	16
Col-0; 35S:: <i>BvFLK</i> ; 13/10	38	18
Col-0; 35S:: <i>BvFLK</i> ; 13/11	31	18
Col-0; 35S:: <i>BvFLK</i> ; 13/12	31	15
Col-0; 35S:: <i>BvFLK</i> ; 13/13	31	16
Col-0; 35S:: <i>BvFLK</i> ; 13/14	31	17
Col-0; 35S:: <i>BvFLK</i> ; 13/15	25	15
Col-0; 35S:: <i>BvFLK</i> ; 13/16	31	18
Col-0; 35S:: <i>BvFLK</i> ; 13/17	28	14
Segregation ratio (early bolting: late bolting)	15:2	
X ² for 3:1	1.59 (ns)	

X ² for 15:1	1.07 (ns)	
Col-0; 35S:: <i>BvFLK</i> ; 14/1	28	15
Col-0; 35S:: <i>BvFLK</i> ; 14/2	28	14
Col-0; 35S:: <i>BvFLK</i> ; 14/3	38	16
Col-0; 35S:: <i>BvFLK</i> ; 14/4	31	15
Col-0; 35S:: <i>BvFLK</i> ; 14/5	31	17
Col-0; 35S:: <i>BvFLK</i> ; 14/6	28	14
Col-0; 35S:: <i>BvFLK</i> ; 14/7	39	12
Col-0; 35S:: <i>BvFLK</i> ; 14/8	31	18
Col-0; 35S:: <i>BvFLK</i> ; 14/9	31	17
Col-0; 35S:: <i>BvFLK</i> ; 14/10	31	15
Col-0; 35S:: <i>BvFLK</i> ; 14/11	31	15
Col-0; 35S:: <i>BvFLK</i> ; 14/12	28	14
Col-0; 35S:: <i>BvFLK</i> ; 14/13	31	16
Col-0; 35S:: <i>BvFLK</i> ; 14/14	31	18
Col-0; 35S:: <i>BvFLK</i> ; 14/15	28	12
Col-0; 35S:: <i>BvFLK</i> ; 14/16	31	14
Col-0; 35S:: <i>BvFLK</i> ; 14/17	31	18
Segregation ratio (early bolting: late bolting)	15:2	
X ² for 3:1	1.59 (ns)	
X ² for 15:1	0.86 (ns)	

X ² for 15:1	0.86 (ns)	
Col-0; 35S:: <i>BvFLK</i> ; 21/1	38	18
Col-0; 35S:: <i>BvFLK</i> ; 21/2	28	19
Col-0; 35S:: <i>BvFLK</i> ; 21/3	28	17
Col-0; 35S:: <i>BvFLK</i> ; 21/4	24	15
Col-0; 35S:: <i>BvFLK</i> ; 21/5	25	14
Col-0; 35S:: <i>BvFLK</i> ; 21/6	28	13
Col-0; 35S:: <i>BvFLK</i> ; 21/7	28	14
Col-0; 35S:: <i>BvFLK</i> ; 21/8	24	12
Col-0; 35S:: <i>BvFLK</i> ; 21/9	28	12
Col-0; 35S:: <i>BvFLK</i> ; 21/10	24	14
Col-0; 35S:: <i>BvFLK</i> ; 21/11	28	16
Col-0; 35S:: <i>BvFLK</i> ; 21/12	25	16
Col-0; 35S:: <i>BvFLK</i> ; 21/13	25	15
Col-0; 35S:: <i>BvFLK</i> ; 21/14	24	13
Col-0; 35S:: <i>BvFLK</i> ; 21/15	25	16
Col-0; 35S:: <i>BvFLK</i> ; 21/16	39	21
Col-0; 35S:: <i>BvFLK</i> ; 21/17	28	17
Segregation ratio (early bolting: late bolting)	15:2	
X ² for 3:1	1.59 (ns)	
X ² for 15:1	0.86 (ns)	

T2 plants (genetic background; transgene cassette; family number / plant number)	Number of days to bolting	Total number of leaves at bolting
Col-0; endo:: <i>BvFLK</i> ; 2/1	25	14
Col-0; endo:: <i>BvFLK</i> ; 2/2	38	17
Col-0; endo:: <i>BvFLK</i> ; 2/3	28	16
Col-0; endo:: <i>BvFLK</i> ; 2/4	28	16
Col-0; endo:: <i>BvFLK</i> ; 2/5	25	14
Col-0; endo:: <i>BvFLK</i> ; 2/6	35	6
Col-0; endo:: <i>BvFLK</i> ; 2/7	24	14
Col-0; endo:: <i>BvFLK</i> ; 2/8	-	-
Col-0; endo:: <i>BvFLK</i> ; 2/9	25	13
Col-0; endo:: <i>BvFLK</i> ; 2/10	24	14
Col-0; endo:: <i>BvFLK</i> ; 2/11	28	15
Col-0; endo:: <i>BvFLK</i> ; 2/12	25	12
Col-0; endo:: <i>BvFLK</i> ; 2/13	31	18
Col-0; endo:: <i>BvFLK</i> ; 2/14	25	14
Col-0; endo:: <i>BvFLK</i> ; 2/15	28	15
Col-0; endo:: <i>BvFLK</i> ; 2/16	24	12
Col-0; endo:: <i>BvFLK</i> ; 2/17	25	14
Segregation ratio (early bolting: late bolting)	16:0	
X ² for 3:1	5.33 **	

T2 plants (genetic background; transgene cassette; family number / plant number)	Number of days to bolting	Total number of leaves at bolting
Col-0; endo:: <i>BvFLK</i> ; 3/1	31	18
Col-0; endo:: <i>BvFLK</i> ; 3/2	31	14
Col-0; endo:: <i>BvFLK</i> ; 3/3	28	15
Col-0; endo:: <i>BvFLK</i> ; 3/4	31	16
Col-0; endo:: <i>BvFLK</i> ; 3/5	31	18
Col-0; endo:: <i>BvFLK</i> ; 3/6	28	16
Col-0; endo:: <i>BvFLK</i> ; 3/7	28	15
Col-0; endo:: <i>BvFLK</i> ; 3/8	28	17
Col-0; endo:: <i>BvFLK</i> ; 3/9	31	18
Col-0; endo:: <i>BvFLK</i> ; 3/10	28	13
Col-0; endo:: <i>BvFLK</i> ; 3/11	28	15
Col-0; endo:: <i>BvFLK</i> ; 3/12	31	18
Col-0; endo:: <i>BvFLK</i> ; 3/13	31	19
Col-0; endo:: <i>BvFLK</i> ; 3/14	31	15
Col-0; endo:: <i>BvFLK</i> ; 3/15	31	17
Col-0; endo:: <i>BvFLK</i> ; 3/16	-	-
Col-0; endo:: <i>BvFLK</i> ; 3/17	31	15
Segregation ratio (early bolting: late bolting)	16:0	
X ² for 3:1	5.33 **	

X ² for 15:1	1.07 (ns)		X ² for 15:1	1.07 (ns)	
Col-0; endo:: <i>BvFLK</i> ; 4/1	28	13	Col-0; endo:: <i>BvFLK</i> ; 5/1	28	16
Col-0; endo:: <i>BvFLK</i> ; 4/2	31	18	Col-0; endo:: <i>BvFLK</i> ; 5/2	35	15
Col-0; endo:: <i>BvFLK</i> ; 4/3	31	17	Col-0; endo:: <i>BvFLK</i> ; 5/3	28	17
Col-0; endo:: <i>BvFLK</i> ; 4/4	28	16	Col-0; endo:: <i>BvFLK</i> ; 5/4	28	13
Col-0; endo:: <i>BvFLK</i> ; 4/5	28	15	Col-0; endo:: <i>BvFLK</i> ; 5/5	31	17
Col-0; endo:: <i>BvFLK</i> ; 4/6	28	18	Col-0; endo:: <i>BvFLK</i> ; 5/6	38	15
Col-0; endo:: <i>BvFLK</i> ; 4/7	25	9	Col-0; endo:: <i>BvFLK</i> ; 5/7	31	19
Col-0; endo:: <i>BvFLK</i> ; 4/8	25	12	Col-0; endo:: <i>BvFLK</i> ; 5/8	28	18
Col-0; endo:: <i>BvFLK</i> ; 4/9	28	16	Col-0; endo:: <i>BvFLK</i> ; 5/9	31	19
Col-0; endo:: <i>BvFLK</i> ; 4/10	31	15	Col-0; endo:: <i>BvFLK</i> ; 5/10	28	16
Col-0; endo:: <i>BvFLK</i> ; 4/11	28	17	Col-0; endo:: <i>BvFLK</i> ; 5/11	28	14
Col-0; endo:: <i>BvFLK</i> ; 4/12	31	19	Col-0; endo:: <i>BvFLK</i> ; 5/12	28	18
Col-0; endo:: <i>BvFLK</i> ; 4/13	28	14	Col-0; endo:: <i>BvFLK</i> ; 5/13	31	16
Col-0; endo:: <i>BvFLK</i> ; 4/14	24	13	Col-0; endo:: <i>BvFLK</i> ; 5/14	28	14
Col-0; endo:: <i>BvFLK</i> ; 4/15	31	18	Col-0; endo:: <i>BvFLK</i> ; 5/15	28	16
Col-0; endo:: <i>BvFLK</i> ; 4/16	28	15	Col-0; endo:: <i>BvFLK</i> ; 5/16	28	13
Col-0; endo:: <i>BvFLK</i> ; 4/17	28	14	Col-0; endo:: <i>BvFLK</i> ; 5/17	28	19
Segregation ratio (early bolting: late bolting)	17:0		Segregation ratio (early bolting: late bolting)	15:2	
X ² for 3:1	6.08 **		X ² for 3:1	1.59 (ns)	
X ² for 15:1	1.14 (ns)		X ² for 15:1	0.86 (ns)	
Col-0; endo::<i>BvFLK</i>; 6/1^b	38	18	Col-0; endo:: <i>BvFLK</i> ; 11/1	31	23
Col-0; endo::<i>BvFLK</i>; 6/2	28	15	Col-0; endo:: <i>BvFLK</i> ; 11/2	31	24
Col-0; endo::<i>BvFLK</i>; 6/3	28	15	Col-0; endo:: <i>BvFLK</i> ; 11/3	31	18
Col-0; endo::<i>BvFLK</i>; 6/4	25	12	Col-0; endo:: <i>BvFLK</i> ; 11/4	28	15
Col-0; endo::<i>BvFLK</i>; 6/5	28	14	Col-0; endo:: <i>BvFLK</i> ; 11/5	28	13
Col-0; endo::<i>BvFLK</i>; 6/6^b	28	16	Col-0; endo:: <i>BvFLK</i> ; 11/6	-	-
Col-0; endo::<i>BvFLK</i>; 6/7	28	16	Col-0; endo:: <i>BvFLK</i> ; 11/7	31	19
Col-0; endo::<i>BvFLK</i>; 6/8	38	16	Col-0; endo:: <i>BvFLK</i> ; 11/8	31	19
Col-0; endo::<i>BvFLK</i>; 6/9	28	17	Col-0; endo:: <i>BvFLK</i> ; 11/9	28	18
Col-0; endo::<i>BvFLK</i>; 6/10	28	18	Col-0; endo:: <i>BvFLK</i> ; 11/10	31	20
Col-0; endo::<i>BvFLK</i>; 6/11	28	13	Col-0; endo:: <i>BvFLK</i> ; 11/11	31	18
Col-0; endo::<i>BvFLK</i>; 6/12	31	14	Col-0; endo:: <i>BvFLK</i> ; 11/12	31	17
Col-0; endo::<i>BvFLK</i>; 6/13^b	37	16	Col-0; endo:: <i>BvFLK</i> ; 11/13	31	21
Col-0; endo::<i>BvFLK</i>; 6/14^b	38	18	Col-0; endo:: <i>BvFLK</i> ; 11/14	28	15
Col-0; endo::<i>BvFLK</i>; 6/15	28	16	Col-0; endo:: <i>BvFLK</i> ; 11/15	28	16
Col-0; endo::<i>BvFLK</i>; 6/16	31	17	Col-0; endo:: <i>BvFLK</i> ; 11/16	31	20
Col-0; endo::<i>BvFLK</i>; 6/17	31	16	Col-0; endo:: <i>BvFLK</i> ; 11/17	31	19
Segregation ratio (early bolting: late bolting)	13:4		Segregation ratio (early bolting: late bolting)	16:0	
X ² for 3:1	0.02 (ns)		X ² for 3:1	5.33 **	
X ² for 15:1	8.56 **		X ² for 15:1	1.07 (ns)	
Col-0; endo:: <i>BvFLK</i> ; 12/1	31	15	Col-0; endo:: <i>BvFLK</i> ; 13/1	28	16
Col-0; endo:: <i>BvFLK</i> ; 12/2	38	15	Col-0; endo:: <i>BvFLK</i> ; 13/2	28	18

Col-0; endo:: <i>BvFLK</i> ; 12/3	31	18
Col-0; endo:: <i>BvFLK</i> ; 12/4	35	15
Col-0; endo:: <i>BvFLK</i> ; 12/5	31	17
Col-0; endo:: <i>BvFLK</i> ; 12/6	28	15
Col-0; endo:: <i>BvFLK</i> ; 12/7	31	17
Col-0; endo:: <i>BvFLK</i> ; 12/8	31	16
Col-0; endo:: <i>BvFLK</i> ; 12/9	31	18
Col-0; endo:: <i>BvFLK</i> ; 12/10	28	11
Col-0; endo:: <i>BvFLK</i> ; 12/11	31	18
Col-0; endo:: <i>BvFLK</i> ; 12/12	31	11
Col-0; endo:: <i>BvFLK</i> ; 12/13	31	17
Col-0; endo:: <i>BvFLK</i> ; 12/14	31	19
Col-0; endo:: <i>BvFLK</i> ; 12/15	31	17
Col-0; endo:: <i>BvFLK</i> ; 12/16	31	18
Col-0; endo:: <i>BvFLK</i> ; 12/17	31	19
Segregation ratio (early bolting: late bolting)	15:2	
X ² for 3:1	1.59 (ns)	
X ² for 15:1	0.86 (ns)	
Col-0; endo:: <i>BvFLK</i> ; 15/1	31	18
Col-0; endo:: <i>BvFLK</i> ; 15/2	28	13
Col-0; endo:: <i>BvFLK</i> ; 15/3	28	16
Col-0; endo:: <i>BvFLK</i> ; 15/4	-	-
Col-0; endo:: <i>BvFLK</i> ; 15/5	25	14
Col-0; endo:: <i>BvFLK</i> ; 15/6	28	16
Col-0; endo:: <i>BvFLK</i> ; 15/7	25	16
Col-0; endo:: <i>BvFLK</i> ; 15/8	28	12
Col-0; endo:: <i>BvFLK</i> ; 15/9	24	18
Col-0; endo:: <i>BvFLK</i> ; 15/10	38	18
Col-0; endo:: <i>BvFLK</i> ; 15/11	31	12
Col-0; endo:: <i>BvFLK</i> ; 15/12	-	-
Col-0; endo:: <i>BvFLK</i> ; 15/13	31	14
Col-0; endo:: <i>BvFLK</i> ; 15/14	31	14
Col-0; endo:: <i>BvFLK</i> ; 15/15	28	15
Col-0; endo:: <i>BvFLK</i> ; 15/16	25	15
Col-0; endo:: <i>BvFLK</i> ; 15/17	31	13
Segregation ratio (early bolting: late bolting)	15:0	
X ² for 3:1	5.00 *	
X ² for 15:1	1.00 (ns)	

T2 plants (genetic background; transgene cassette; family number / plant number)	Number of days to bolting	Total number of leaves at bolting
Col-0; 35S:: <i>AtFLK</i> ; 1/1	31	17
Col-0; 35S:: <i>AtFLK</i> ; 1/2	31	18

Col-0; endo:: <i>BvFLK</i> ; 13/3	28	14
Col-0; endo:: <i>BvFLK</i> ; 13/4	24	14
Col-0; endo:: <i>BvFLK</i> ; 13/5	24	12
Col-0; endo:: <i>BvFLK</i> ; 13/6	28	14
Col-0; endo:: <i>BvFLK</i> ; 13/7	28	12
Col-0; endo:: <i>BvFLK</i> ; 13/8	28	16
Col-0; endo:: <i>BvFLK</i> ; 13/9	28	16
Col-0; endo:: <i>BvFLK</i> ; 13/10	31	23
Col-0; endo:: <i>BvFLK</i> ; 13/11	28	16
Col-0; endo:: <i>BvFLK</i> ; 13/12	25	14
Col-0; endo:: <i>BvFLK</i> ; 13/13	28	18
Col-0; endo:: <i>BvFLK</i> ; 13/14	31	20
Col-0; endo:: <i>BvFLK</i> ; 13/15	31	17
Col-0; endo:: <i>BvFLK</i> ; 13/16	25	13
Col-0; endo:: <i>BvFLK</i> ; 13/17	-	-
Segregation ratio (early bolting: late bolting)	16:0	
X ² for 3:1	5.33 **	
X ² for 15:1	1.07 (ns)	
Col-0; endo:: <i>BvFLK</i> ; 16/1	31	15
Col-0; endo:: <i>BvFLK</i> ; 16/2	38	15
Col-0; endo:: <i>BvFLK</i> ; 16/3	31	18
Col-0; endo:: <i>BvFLK</i> ; 16/4	35	15
Col-0; endo:: <i>BvFLK</i> ; 16/5	31	17
Col-0; endo:: <i>BvFLK</i> ; 16/6	28	15
Col-0; endo:: <i>BvFLK</i> ; 16/7	31	17
Col-0; endo:: <i>BvFLK</i> ; 16/8	31	16
Col-0; endo:: <i>BvFLK</i> ; 16/9	31	18
Col-0; endo:: <i>BvFLK</i> ; 16/10	28	11
Col-0; endo:: <i>BvFLK</i> ; 16/11	35	18
Col-0; endo:: <i>BvFLK</i> ; 16/12	31	11
Col-0; endo:: <i>BvFLK</i> ; 16/13	31	17
Col-0; endo:: <i>BvFLK</i> ; 16/14	31	19
Col-0; endo:: <i>BvFLK</i> ; 16/15	31	17
Col-0; endo:: <i>BvFLK</i> ; 16/16	31	18
Col-0; endo:: <i>BvFLK</i> ; 16/17	31	19
Segregation ratio (early bolting: late bolting)	17:0	
X ² for 3:1	6.08 **	
X ² for 15:1	1.14 (ns)	

T2 plants (genetic background; transgene cassette; family number / plant number)	Number of days to bolting	Total number of leaves at bolting
Col-0; 35S:: <i>AtFLK</i> ; 2/1	25	12
Col-0; 35S:: <i>AtFLK</i> ; 2/2	25	13

Col-0; 35S::AtFLK; 1/3	31	15	Col-0; 35S::AtFLK; 2/3	25	15
Col-0; 35S::AtFLK; 1/4	28	14	Col-0; 35S::AtFLK; 2/4	25	16
Col-0; 35S::AtFLK; 1/5	31	17	Col-0; 35S::AtFLK; 2/5	25	13
Col-0; 35S::AtFLK; 1/6	31	15	Col-0; 35S::AtFLK; 2/6	25	13
Col-0; 35S::AtFLK; 1/7	38	15	Col-0; 35S::AtFLK; 2/7	25	15
Col-0; 35S::AtFLK; 1/8	31	15	Col-0; 35S::AtFLK; 2/8	25	17
Col-0; 35S::AtFLK; 1/9	35	14	Col-0; 35S::AtFLK; 2/9	25	13
Col-0; 35S::AtFLK; 1/10	31	15	Col-0; 35S::AtFLK; 2/10	28	15
Col-0; 35S::AtFLK; 1/11	31	15	Col-0; 35S::AtFLK; 2/11	28	12
Col-0; 35S::AtFLK; 1/12	28	13	Col-0; 35S::AtFLK; 2/12	28	12
Col-0; 35S::AtFLK; 1/13	40	18	Col-0; 35S::AtFLK; 2/13	28	15
Col-0; 35S::AtFLK; 1/14	28	14	Col-0; 35S::AtFLK; 2/14	24	12
Col-0; 35S::AtFLK; 1/15	31	17	Col-0; 35S::AtFLK; 2/15	28	15
Col-0; 35S::AtFLK; 1/16	31	16	Col-0; 35S::AtFLK; 2/16	28	16
Col-0; 35S::AtFLK; 1/17	28	14	Col-0; 35S::AtFLK; 2/17	28	14
Segregation ratio (early bolting: late bolting)	15:2		Segregation ratio (early bolting: late bolting)	17:0	
X ² for 3:1	1.59 (ns)		X ² for 3:1	6.08 **	
X ² for 15:1	0.86 (ns)		X ² for 15:1	1.14 (ns)	
Col-0; 35S::AtFLK; 3/1	25	11	Col-0; 35S::AtFLK; 4/1	25	11
Col-0; 35S::AtFLK; 3/2	28	13	Col-0; 35S::AtFLK; 4/2	28	15
Col-0; 35S::AtFLK; 3/3	31	18	Col-0; 35S::AtFLK; 4/3	31	14
Col-0; 35S::AtFLK; 3/4	28	15	Col-0; 35S::AtFLK; 4/4	28	16
Col-0; 35S::AtFLK; 3/5	25	13	Col-0; 35S::AtFLK; 4/5	31	15
Col-0; 35S::AtFLK; 3/6	28	12	Col-0; 35S::AtFLK; 4/6	28	14
Col-0; 35S::AtFLK; 3/7	25	13	Col-0; 35S::AtFLK; 4/7	28	12
Col-0; 35S::AtFLK; 3/8	25	15	Col-0; 35S::AtFLK; 4/8	35	15
Col-0; 35S::AtFLK; 3/9	28	14	Col-0; 35S::AtFLK; 4/9	28	13
Col-0; 35S::AtFLK; 3/10	25	12	Col-0; 35S::AtFLK; 4/10	31	14
Col-0; 35S::AtFLK; 3/11	24	12	Col-0; 35S::AtFLK; 4/11	28	15
Col-0; 35S::AtFLK; 3/12	28	13	Col-0; 35S::AtFLK; 4/12	31	16
Col-0; 35S::AtFLK; 3/13	28	12	Col-0; 35S::AtFLK; 4/13	31	18
Col-0; 35S::AtFLK; 3/14	28	11	Col-0; 35S::AtFLK; 4/14	38	17
Col-0; 35S::AtFLK; 3/15	25	13	Col-0; 35S::AtFLK; 4/15	28	14
Col-0; 35S::AtFLK; 3/16	24	13	Col-0; 35S::AtFLK; 4/16	28	12
Col-0; 35S::AtFLK; 3/17	25	13	Col-0; 35S::AtFLK; 4/17	28	14
Segregation ratio (early bolting: late bolting)	17:0		Segregation ratio (early bolting: late bolting)	15:2	
X ² for 3:1	6.08 **		X ² for 3:1	1.59 (ns)	
X ² for 15:1	1.14 (ns)		X ² for 15:1	0.86 (ns)	
Col-0; 35S::AtFLK; 5/1	25	15	Col-0; 35S::AtFLK; 6/1	31	13
Col-0; 35S::AtFLK; 5/2	27	14	Col-0; 35S::AtFLK; 6/2	31	15
Col-0; 35S::AtFLK; 5/3	27	15	Col-0; 35S::AtFLK; 6/3	31	15
Col-0; 35S::AtFLK; 5/4	27	18	Col-0; 35S::AtFLK; 6/4	28	15
Col-0; 35S::AtFLK; 5/5	27	13	Col-0; 35S::AtFLK; 6/5	31	15
Col-0; 35S::AtFLK; 5/6	25	13	Col-0; 35S::AtFLK; 6/6	38	15

Col-0; 35S::AtFLK; 5/7	25	15
Col-0; 35S::AtFLK; 5/8	28	14
Col-0; 35S::AtFLK; 5/9	28	14
Col-0; 35S::AtFLK; 5/10 ^b	38	18
Col-0; 35S::AtFLK; 5/11	31	14
Col-0; 35S::AtFLK; 5/12 ^b	39	18
Col-0; 35S::AtFLK; 5/13 ^b	38	19
Col-0; 35S::AtFLK; 5/14 ^b	40	21
Col-0; 35S::AtFLK; 5/15	28	16
Col-0; 35S::AtFLK; 5/16	25	14
Col-0; 35S::AtFLK; 5/17	31	20
Segregation ratio (early bolting: late bolting)	13:4	
X ² for 3:1	0.02 (ns)	
X ² for 15:1	8.56 **	
Col-0; 35S::AtFLK; 7/1	31	18
Col-0; 35S::AtFLK; 7/2	28	14
Col-0; 35S::AtFLK; 7/3	28	16
Col-0; 35S::AtFLK; 7/4	28	14
Col-0; 35S::AtFLK; 7/5	35	13
Col-0; 35S::AtFLK; 7/6	31	14
Col-0; 35S::AtFLK; 7/7	28	13
Col-0; 35S::AtFLK; 7/8	28	11
Col-0; 35S::AtFLK; 7/9	25	11
Col-0; 35S::AtFLK; 7/10	28	13
Col-0; 35S::AtFLK; 7/11	31	10
Col-0; 35S::AtFLK; 7/12	-	-
Col-0; 35S::AtFLK; 7/13	28	15
Col-0; 35S::AtFLK; 7/14	31	18
Col-0; 35S::AtFLK; 7/15	28	15
Col-0; 35S::AtFLK; 7/16	28	13
Col-0; 35S::AtFLK; 7/17	31	17
Segregation ratio (early bolting: late bolting)	15:1	
X ² for 3:1	3.00 (ns)	
X ² for 15:1	0.00 (ns)	
Col-0; 35S::AtFLK; 9/1	28	17
Col-0; 35S::AtFLK; 9/2	28	19
Col-0; 35S::AtFLK; 9/3	31	17
Col-0; 35S::AtFLK; 9/4	25	15
Col-0; 35S::AtFLK; 9/5	28	17
Col-0; 35S::AtFLK; 9/6	25	15
Col-0; 35S::AtFLK; 9/7	28	15
Col-0; 35S::AtFLK; 9/8	25	17
Col-0; 35S::AtFLK; 9/9	31	17
Col-0; 35S::AtFLK; 9/10	28	15

Col-0; 35S::AtFLK; 6/7	28	16
Col-0; 35S::AtFLK; 6/8	25	12
Col-0; 35S::AtFLK; 6/9	28	14
Col-0; 35S::AtFLK; 6/10	39	16
Col-0; 35S::AtFLK; 6/11	31	15
Col-0; 35S::AtFLK; 6/12	28	13
Col-0; 35S::AtFLK; 6/13	28	16
Col-0; 35S::AtFLK; 6/14	31	16
Col-0; 35S::AtFLK; 6/15	28	14
Col-0; 35S::AtFLK; 6/16	31	17
Col-0; 35S::AtFLK; 6/17	28	18
Segregation ratio (early bolting: late bolting)	15:2	
X ² for 3:1	1.59 (ns)	
X ² for 15:1	0.86 (ns)	
Col-0; 35S::AtFLK; 8/1	31	18
Col-0; 35S::AtFLK; 8/2	31	17
Col-0; 35S::AtFLK; 8/3	28	16
Col-0; 35S::AtFLK; 8/4	28	14
Col-0; 35S::AtFLK; 8/5	25	12
Col-0; 35S::AtFLK; 8/6	27	16
Col-0; 35S::AtFLK; 8/7	27	17
Col-0; 35S::AtFLK; 8/8	25	12
Col-0; 35S::AtFLK; 8/9	28	18
Col-0; 35S::AtFLK; 8/10	28	16
Col-0; 35S::AtFLK; 8/11	28	15
Col-0; 35S::AtFLK; 8/12	29	13
Col-0; 35S::AtFLK; 8/13	28	13
Col-0; 35S::AtFLK; 8/14	25	14
Col-0; 35S::AtFLK; 8/15	31	17
Col-0; 35S::AtFLK; 8/16	31	16
Col-0; 35S::AtFLK; 8/17	28	15
Segregation ratio (early bolting: late bolting)	17:0	
X ² for 3:1	6.08 **	
X ² for 15:1	1.14 (ns)	

Col-0; 35S:: <i>AtFLK</i> ; 9/11	35	16
Col-0; 35S:: <i>AtFLK</i> ; 9/12	35	15
Col-0; 35S:: <i>AtFLK</i> ; 9/13	28	20
Col-0; 35S:: <i>AtFLK</i> ; 9/14	25	15
Col-0; 35S:: <i>AtFLK</i> ; 9/15	25	15
Col-0; 35S:: <i>AtFLK</i> ; 9/16	28	16
Col-0; 35S:: <i>AtFLK</i> ; 9/17	28	21
Segregation ratio (early bolting: late bolting)	15:2	
X ² for 3:1	1.59 (ns)	
X ² for 15:1	0.86 (ns)	

T2 plants (genetic background; transgene cassette; family number / plant number)	Number of days to bolting	Total number of leaves at bolting
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 1/1	28	13
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 1/2	28	12
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 1/3	28	16
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 1/4	28	17
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 1/5	28	14
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 1/6	28	15
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 1/7	28	20
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 1/8	25	15
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 1/9	28	15
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 1/10	28	15
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 1/11	38	15
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 1/12	25	14
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 1/13	28	15
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 1/14	25	14
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 1/15	28	17
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 1/16	28	14
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 1/17	28	18
Segregation ratio (early bolting: late bolting)	17:0	
X ² for 3:1	6.08 **	
X ² for 15:1	1.14 (ns)	
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 5/1	28	19
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 5/2	28	13
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 5/3	28	14
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 5/4	28	17
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 5/5	28	17
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 5/6	28	15
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 5/7	28	18
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 5/8	28	17
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 5/9	31	16
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 5/10	28	17
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 5/11	35	13
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 5/12	25	18
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 5/13	28	16
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 5/14	31	15

T2 plants (genetic background; transgene cassette; family number / plant number)	Number of days to bolting	Total number of leaves at bolting
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 2/1	31	18
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 2/2	31	17
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 2/3	28	18
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 2/4	-	-
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 2/5	28	20
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 2/6	25	16
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 2/7	28	17
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 2/8	25	21
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 2/9	25	18
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 2/10	28	15
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 2/11	31	14
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 2/12	31	16
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 2/13	28	14
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 2/14	25	13
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 2/15	31	17
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 2/16	28	15
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 2/17	28	16
Segregation ratio (early bolting: late bolting)	16:0	
X ² for 3:1	5.33 **	
X ² for 15:1	1.07 (ns)	
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 7/1	35	11
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 7/2	35	16
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 7/3	35	14
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 7/4	35	14
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 7/5	31	11
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 7/6	28	17
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 7/7	31	17
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 7/8	31	18
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 7/9	28	15
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 7/10	28	16
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 7/11	38	16
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 7/12	31	19
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 7/13	31	17
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; 7/14	31	20

<i>flk-1; 35S::BvFLK; 5/15</i>	25	20	<i>flk-1; 35S::BvFLK; 7/15</i>	28	14
<i>flk-1; 35S::BvFLK; 5/16</i>	25	18	<i>flk-1; 35S::BvFLK; 7/16</i>	28	17
<i>flk-1; 35S::BvFLK; 5/17</i>	28	19	<i>flk-1; 35S::BvFLK; 7/17</i>	28	18
Segregation ratio (early bolting: late bolting)	17:0		Segregation ratio (early bolting: late bolting)	17:0	
X ² for 3:1	6.08 **		X ² for 3:1	6.08 **	
X ² for 15:1	1.14 (ns)		X ² for 15:1	1.14 (ns)	
<i>flk-1; 35S::BvFLK; 9/1</i>	31	15	<i>flk-1; 35S::BvFLK; 10/1</i>	28	16
<i>flk-1; 35S::BvFLK; 9/2</i>	28	16	<i>flk-1; 35S::BvFLK; 10/2</i>	28	17
<i>flk-1; 35S::BvFLK; 9/3</i>	28	16	<i>flk-1; 35S::BvFLK; 10/3</i>	28	15
<i>flk-1; 35S::BvFLK; 9/4</i>	28	12	<i>flk-1; 35S::BvFLK; 10/4</i>	28	16
<i>flk-1; 35S::BvFLK; 9/5</i>	24	13	<i>flk-1; 35S::BvFLK; 10/5</i>	28	17
<i>flk-1; 35S::BvFLK; 9/6</i>	28	18	<i>flk-1; 35S::BvFLK; 10/6</i>	28	15
<i>flk-1; 35S::BvFLK; 9/7</i>	28	18	<i>flk-1; 35S::BvFLK; 10/7</i>	28	20
<i>flk-1; 35S::BvFLK; 9/8</i>	24	116	<i>flk-1; 35S::BvFLK; 10/8</i>	28	16
<i>flk-1; 35S::BvFLK; 9/9</i>	24	16	<i>flk-1; 35S::BvFLK; 10/9</i>	28	15
<i>flk-1; 35S::BvFLK; 9/10</i>	28	17	<i>flk-1; 35S::BvFLK; 10/10</i>	25	16
<i>flk-1; 35S::BvFLK; 9/11</i>	28	17	<i>flk-1; 35S::BvFLK; 10/11</i>	25	17
<i>flk-1; 35S::BvFLK; 9/12</i>	25	16	<i>flk-1; 35S::BvFLK; 10/12</i>	28	14
<i>flk-1; 35S::BvFLK; 9/13</i>	28	19	<i>flk-1; 35S::BvFLK; 10/13</i>	28	18
<i>flk-1; 35S::BvFLK; 9/14</i>	24	17	<i>flk-1; 35S::BvFLK; 10/14</i>	25	16
<i>flk-1; 35S::BvFLK; 9/15</i>	28	15	<i>flk-1; 35S::BvFLK; 10/15</i>	28	21
<i>flk-1; 35S::BvFLK; 9/16</i>	28	18	<i>flk-1; 35S::BvFLK; 10/16</i>	28	18
<i>flk-1; 35S::BvFLK; 9/17</i>	28	16	<i>flk-1; 35S::BvFLK; 10/17</i>	25	17
Segregation ratio (early bolting: late bolting)	17:0		Segregation ratio (early bolting: late bolting)	17:0	
X ² for 3:1	6.08 **		X ² for 3:1	6.08 **	
X ² for 15:1	1.14 (ns)		X ² for 15:1	1.14 (ns)	
<i>flk-1; 35S::BvFLK; 11/1</i>	25	17	<i>flk-1; 35S::BvFLK; 12/1</i>	28	16
<i>flk-1; 35S::BvFLK; 11/2</i>	25	17	<i>flk-1; 35S::BvFLK; 12/2</i>	28	18
<i>flk-1; 35S::BvFLK; 11/3</i>	28	17	<i>flk-1; 35S::BvFLK; 12/3</i>	31	15
<i>flk-1; 35S::BvFLK; 11/4</i>	28	15	<i>flk-1; 35S::BvFLK; 12/4</i>	28	14
<i>flk-1; 35S::BvFLK; 11/5</i>	28	18	<i>flk-1; 35S::BvFLK; 12/5</i>	28	18
<i>flk-1; 35S::BvFLK; 11/6</i>	25	13	<i>flk-1; 35S::BvFLK; 12/6</i>	28	13
<i>flk-1; 35S::BvFLK; 11/7</i>	28	17	<i>flk-1; 35S::BvFLK; 12/7</i>	35	18
<i>flk-1; 35S::BvFLK; 11/8</i>	28	13	<i>flk-1; 35S::BvFLK; 12/8</i>	28	16
<i>flk-1; 35S::BvFLK; 11/9</i>	28	17	<i>flk-1; 35S::BvFLK; 12/9</i>	28	19
<i>flk-1; 35S::BvFLK; 11/10</i>	35	20	<i>flk-1; 35S::BvFLK; 12/10</i>	28	17
<i>flk-1; 35S::BvFLK; 11/11</i>	28	17	<i>flk-1; 35S::BvFLK; 12/11</i>	28	17
<i>flk-1; 35S::BvFLK; 11/12</i>	25	17	<i>flk-1; 35S::BvFLK; 12/12</i>	28	15
<i>flk-1; 35S::BvFLK; 11/13</i>	28	18	<i>flk-1; 35S::BvFLK; 12/13</i>	28	16
<i>flk-1; 35S::BvFLK; 11/14</i>	28	19	<i>flk-1; 35S::BvFLK; 12/14</i>	31	12
<i>flk-1; 35S::BvFLK; 11/15</i>	225	17	<i>flk-1; 35S::BvFLK; 12/15</i>	28	16
<i>flk-1; 35S::BvFLK; 11/16</i>	35	18	<i>flk-1; 35S::BvFLK; 12/16</i>	38	15
<i>flk-1; 35S::BvFLK; 11/17</i>	28	17	<i>flk-1; 35S::BvFLK; 12/17</i>	28	16
Segregation ratio (early bolting: late bolting)	17:0		Segregation ratio (early bolting: late bolting)	17:0	
X ² for 3:1	6.08 **		X ² for 3:1	6.08 **	
X ² for 15:1	1.14 (ns)		X ² for 15:1	1.14 (ns)	
<i>flk-1; 35S::BvFLK; 14/1</i>	31	19	<i>flk-1; 35S::BvFLK; 18/1</i>	28	18
<i>flk-1; 35S::BvFLK; 14/2</i>	28	17	<i>flk-1; 35S::BvFLK; 18/2</i>	35	17

<i>flk-1; 35S::BvFLK; 14/3</i>	31	17
<i>flk-1; 35S::BvFLK; 14/4</i>	31	19
<i>flk-1; 35S::BvFLK; 14/5</i>	28	16
<i>flk-1; 35S::BvFLK; 14/6</i>	28	17
<i>flk-1; 35S::BvFLK; 14/7</i>	35	18
<i>flk-1; 35S::BvFLK; 14/8</i>	31	17
<i>flk-1; 35S::BvFLK; 14/9</i>	35	16
<i>flk-1; 35S::BvFLK; 14/10</i>	28	18
<i>flk-1; 35S::BvFLK; 14/11</i>	28	17
<i>flk-1; 35S::BvFLK; 14/12</i>	35	17
<i>flk-1; 35S::BvFLK; 14/13</i>	35	17
<i>flk-1; 35S::BvFLK; 14/14</i>	35	15
<i>flk-1; 35S::BvFLK; 14/15</i>	28	17
<i>flk-1; 35S::BvFLK; 14/16</i>	31	15
<i>flk-1; 35S::BvFLK; 14/17</i>	31	14
Segregation ratio (early bolting: late bolting)	17:0	
X ² for 3:1	6.08 **	
X ² for 15:1	1.14 (ns)	

<i>flk-1; 35S::BvFLK; 18/3</i>	35	17
<i>flk-1; 35S::BvFLK; 18/4</i>	35	16
<i>flk-1; 35S::BvFLK; 18/5</i>	35	15
<i>flk-1; 35S::BvFLK; 18/6</i>	35	16
<i>flk-1; 35S::BvFLK; 18/7</i>	38	15
<i>flk-1; 35S::BvFLK; 18/8</i>	38	16
<i>flk-1; 35S::BvFLK; 18/9</i>	28	17
<i>flk-1; 35S::BvFLK; 18/10</i>	35	18
<i>flk-1; 35S::BvFLK; 18/11^b</i>	69	71
<i>flk-1; 35S::BvFLK; 18/12</i>	31	12
<i>flk-1; 35S::BvFLK; 18/13</i>	35	18
<i>flk-1; 35S::BvFLK; 18/14</i>	35	15
<i>flk-1; 35S::BvFLK; 18/15</i>	35	18
<i>flk-1; 35S::BvFLK; 18/16^b</i>	66	65
<i>flk-1; 35S::BvFLK; 18/17</i>	-	-
Segregation ratio (early bolting: late bolting)	14:2	
X ² for 3:1	1.33 (ns)	
X ² for 15:1	1.07 (ns)	

T2 plants (genetic background; transgene cassette; family number / plant number)	Number of days to bolting	Total number of leaves at bolting
<i>flk-1; endo::BvFLK; 1/1</i>	28	23
<i>flk-1; endo::BvFLK; 1/2</i>	28	13
<i>flk-1; endo::BvFLK; 1/3</i>	35	18
<i>flk-1; endo::BvFLK; 1/4</i>	28	18
<i>flk-1; endo::BvFLK; 1/5</i>	31	13
<i>flk-1; endo::BvFLK; 1/6</i>	31	15
<i>flk-1; endo::BvFLK; 1/7</i>	31	13
<i>flk-1; endo::BvFLK; 1/8</i>	28	19
<i>flk-1; endo::BvFLK; 1/9</i>	28	15
<i>flk-1; endo::BvFLK; 1/10</i>	31	14
<i>flk-1; endo::BvFLK; 1/11</i>	31	15
<i>flk-1; endo::BvFLK; 1/12</i>	31	15
<i>flk-1; endo::BvFLK; 1/13</i>	35	17
<i>flk-1; endo::BvFLK; 1/14</i>	28	16
<i>flk-1; endo::BvFLK; 1/15</i>	28	15
<i>flk-1; endo::BvFLK; 1/16</i>	28	17
<i>flk-1; endo::BvFLK; 1/17</i>	35	17
Segregation ratio (early bolting: late bolting)	17:0	
X ² for 3:1	6.08 **	
X ² for 15:1	1.14 (ns)	
<i>flk-1; endo::BvFLK; 3/1</i>	28	19
<i>flk-1; endo::BvFLK; 3/2</i>	35	16
<i>flk-1; endo::BvFLK; 3/3</i>	31	14
<i>flk-1; endo::BvFLK; 3/4</i>	35	14
<i>flk-1; endo::BvFLK; 3/5</i>	35	15
<i>flk-1; endo::BvFLK; 3/6</i>	35	16

T2 plants (genetic background; transgene cassette; family number / plant number)	Number of days to bolting	Total number of leaves at bolting
<i>flk-1; endo::BvFLK; 2/1</i>	31	17
<i>flk-1; endo::BvFLK; 2/2</i>	31	15
<i>flk-1; endo::BvFLK; 2/3</i>	-	-
<i>flk-1; endo::BvFLK; 2/4</i>	31	14
<i>flk-1; endo::BvFLK; 2/5</i>	35	13
<i>flk-1; endo::BvFLK; 2/6</i>	31	17
<i>flk-1; endo::BvFLK; 2/7</i>	35	16
<i>flk-1; endo::BvFLK; 2/8</i>	31	15
<i>flk-1; endo::BvFLK; 2/9</i>	35	28
<i>flk-1; endo::BvFLK; 2/10</i>	31	14
<i>flk-1; endo::BvFLK; 2/11</i>	38	12
<i>flk-1; endo::BvFLK; 2/12</i>	35	16
<i>flk-1; endo::BvFLK; 2/13</i>	31	15
<i>flk-1; endo::BvFLK; 2/14</i>	31	16
<i>flk-1; endo::BvFLK; 2/15</i>	28	15
<i>flk-1; endo::BvFLK; 2/16</i>	31	15
<i>flk-1; endo::BvFLK; 2/17</i>	31	14
Segregation ratio (early bolting: late bolting)	16:0	
X ² for 3:1	5.33 **	
X ² for 15:1	1.07 (ns)	
<i>flk-1; endo::BvFLK; 4/1</i>	28	18
<i>flk-1; endo::BvFLK; 4/2</i>	28	15
<i>flk-1; endo::BvFLK; 4/3</i>	28	13
<i>flk-1; endo::BvFLK; 4/4</i>	28	16
<i>flk-1; endo::BvFLK; 4/5</i>	31	17
<i>flk-1; endo::BvFLK; 4/6</i>	31	19

<i>flk-1; endo::BvFLK; 3/7</i>	31	14	<i>flk-1; endo::BvFLK; 4/7</i>	28	15
<i>flk-1; endo::BvFLK; 3/8</i>	38	16	<i>flk-1; endo::BvFLK; 4/8</i>	28	15
<i>flk-1; endo::BvFLK; 3/9</i>	31	16	<i>flk-1; endo::BvFLK; 4/9</i>	25	14
<i>flk-1; endo::BvFLK; 3/10</i>	28	14	<i>flk-1; endo::BvFLK; 4/10</i>	28	16
<i>flk-1; endo::BvFLK; 3/11</i>	28	12	<i>flk-1; endo::BvFLK; 4/11</i>	25	18
<i>flk-1; endo::BvFLK; 3/12</i>	31	17	<i>flk-1; endo::BvFLK; 4/12</i>	28	17
<i>flk-1; endo::BvFLK; 3/13</i>	-	-	<i>flk-1; endo::BvFLK; 4/13</i>	38	15
<i>flk-1; endo::BvFLK; 3/14</i>	28	18	<i>flk-1; endo::BvFLK; 4/14</i>	28	15
<i>flk-1; endo::BvFLK; 3/15</i>	28	16	<i>flk-1; endo::BvFLK; 4/15</i>	28	16
<i>flk-1; endo::BvFLK; 3/16</i>	28	13	<i>flk-1; endo::BvFLK; 4/16</i>	28	14
<i>flk-1; endo::BvFLK; 3/17</i>	31	13	<i>flk-1; endo::BvFLK; 4/17</i>	28	17
Segregation ratio (early bolting: late bolting)	16:0		Segregation ratio (early bolting: late bolting)	17:0	
X ² for 3:1	5.33 **		X ² for 3:1	6.08 **	
X ² for 15:1	1.07 (ns)		X ² for 15:1	1.14 (ns)	
<i>flk-1; endo::BvFLK; 5/1</i>	35	13	<i>flk-1; endo::BvFLK; 6/1</i>	28	14
<i>flk-1; endo::BvFLK; 5/2</i>	35	15	<i>flk-1; endo::BvFLK; 6/2</i>	39	15
<i>flk-1; endo::BvFLK; 5/3</i>	35	15	<i>flk-1; endo::BvFLK; 6/3</i>	38	15
<i>flk-1; endo::BvFLK; 5/4</i>	31	17	<i>flk-1; endo::BvFLK; 6/4</i>	38	16
<i>flk-1; endo::BvFLK; 5/5</i>	28	15	<i>flk-1; endo::BvFLK; 6/5</i>	28	16
<i>flk-1; endo::BvFLK; 5/6</i>	38	14	<i>flk-1; endo::BvFLK; 6/6</i>	38	11
<i>flk-1; endo::BvFLK; 5/7</i>	35	15	<i>flk-1; endo::BvFLK; 6/7</i>	31	15
<i>flk-1; endo::BvFLK; 5/8^b</i>	71	64	<i>flk-1; endo::BvFLK; 6/8</i>	38	15
<i>flk-1; endo::BvFLK; 5/9</i>	35	21	<i>flk-1; endo::BvFLK; 6/9</i>	39	15
<i>flk-1; endo::BvFLK; 5/10</i>	28	13	<i>flk-1; endo::BvFLK; 6/10</i>	38	14
<i>flk-1; endo::BvFLK; 5/11^b</i>	67	69	<i>flk-1; endo::BvFLK; 6/11</i>	35	16
<i>flk-1; endo::BvFLK; 5/12</i>	38	18	<i>flk-1; endo::BvFLK; 6/12</i>	31	11
<i>flk-1; endo::BvFLK; 5/13^b</i>	69	68	<i>flk-1; endo::BvFLK; 6/13</i>	31	15
<i>flk-1; endo::BvFLK; 5/14</i>	28	14	<i>flk-1; endo::BvFLK; 6/14</i>	35	15
<i>flk-1; endo::BvFLK; 5/15^b</i>	67	66	<i>flk-1; endo::BvFLK; 6/15</i>	38	17
<i>flk-1; endo::BvFLK; 5/16</i>	31	19	<i>flk-1; endo::BvFLK; 6/16</i>	31	15
<i>flk-1; endo::BvFLK; 5/17</i>	31	17	<i>flk-1; endo::BvFLK; 6/17</i>	31	16
Segregation ratio (early bolting: late bolting)	13:4		Segregation ratio (early bolting: late bolting)	17:0	
X ² for 3:1	0.02 (ns)		X ² for 3:1	6.08 **	
X ² for 15:1	8.56 **		X ² for 15:1	1.14 (ns)	
<i>flk-1; endo::BvFLK; 7/1</i>	31	14	<i>flk-1; endo::BvFLK; 8/1</i>	38	18
<i>flk-1; endo::BvFLK; 7/2</i>	28	17	<i>flk-1; endo::BvFLK; 8/2</i>	28	14
<i>flk-1; endo::BvFLK; 7/3</i>	28	20	<i>flk-1; endo::BvFLK; 8/3</i>	31	15
<i>flk-1; endo::BvFLK; 7/4</i>	28	20	<i>flk-1; endo::BvFLK; 8/4</i>	31	17
<i>flk-1; endo::BvFLK; 7/5</i>	28	18	<i>flk-1; endo::BvFLK; 8/5</i>	31	15
<i>flk-1; endo::BvFLK; 7/6</i>	28	17	<i>flk-1; endo::BvFLK; 8/6</i>	31	16
<i>flk-1; endo::BvFLK; 7/7</i>	28	16	<i>flk-1; endo::BvFLK; 8/7</i>	28	15
<i>flk-1; endo::BvFLK; 7/8</i>	28	21	<i>flk-1; endo::BvFLK; 8/8</i>	31	19
<i>flk-1; endo::BvFLK; 7/9</i>	28	16	<i>flk-1; endo::BvFLK; 8/9</i>	31	15
<i>flk-1; endo::BvFLK; 7/10</i>	28	16	<i>flk-1; endo::BvFLK; 8/10</i>	38	18
<i>flk-1; endo::BvFLK; 7/11</i>	28	17	<i>flk-1; endo::BvFLK; 8/11</i>	31	17
<i>flk-1; endo::BvFLK; 7/12</i>	28	15	<i>flk-1; endo::BvFLK; 8/12</i>	28	11
<i>flk-1; endo::BvFLK; 7/13</i>	28	15	<i>flk-1; endo::BvFLK; 8/13</i>	38	14
<i>flk-1; endo::BvFLK; 7/14</i>	28	18	<i>flk-1; endo::BvFLK; 8/14</i>	31	16

<i>flk-1; endo::BvFLK; 7/15</i>	28	16
<i>flk-1; endo::BvFLK; 7/16</i>	31	19
<i>flk-1; endo::BvFLK; 7/17</i>	35	20
Segregation ratio (early bolting: late bolting)	17: 0	
X ² for 3:1	6.08 **	
X ² for 15:1	1.14 (ns)	
<i>flk-1; endo::BvFLK; 9/1</i>	35	17
<i>flk-1; endo::BvFLK; 9/2</i>	28	15
<i>flk-1; endo::BvFLK; 9/3</i>	38	16
<i>flk-1; endo::BvFLK; 9/4</i>	31	16
<i>flk-1; endo::BvFLK; 9/5</i>	38	17
<i>flk-1; endo::BvFLK; 9/6</i>	28	15
<i>flk-1; endo::BvFLK; 9/7</i>	38	13
<i>flk-1; endo::BvFLK; 9/8</i>	31	19
<i>flk-1; endo::BvFLK; 9/9</i>	28	11
<i>flk-1; endo::BvFLK; 9/10</i>	28	10
<i>flk-1; endo::BvFLK; 9/11</i>	28	12
<i>flk-1; endo::BvFLK; 9/12</i>	28	12
<i>flk-1; endo::BvFLK; 9/13</i>	28	16
<i>flk-1; endo::BvFLK; 9/14</i>	28	19
<i>flk-1; endo::BvFLK; 9/15</i>	28	13
<i>flk-1; endo::BvFLK; 9/16</i>	31	14
<i>flk-1; endo::BvFLK; 9/17</i>	28	20
Segregation ratio (early bolting: late bolting)	17: 0	
X ² for 3:1	6.08 **	
X ² for 15:1	1.14 (ns)	

<i>flk-1; endo::BvFLK; 8/15</i>	28	13
<i>flk-1; endo::BvFLK; 8/16</i>	28	14
<i>flk-1; endo::BvFLK; 8/17</i>	28	16
Segregation ratio (early bolting: late bolting)	17: 0	
X ² for 3:1	6.08 **	
X ² for 15:1	1.14 (ns)	

T2 plants (genetic background; transgene cassette; family number / plant number)	Number of days to bolting	Total number of leaves at bolting
<i>flk-1; 35S::AtFLK; 1/1</i>	28	18
<i>flk-1; 35S::AtFLK; 1/2</i>	28	15
<i>flk-1; 35S::AtFLK; 1/3</i>	28	16
<i>flk-1; 35S::AtFLK; 1/4</i>	28	16
<i>flk-1; 35S::AtFLK; 1/5</i>	28	12
<i>flk-1; 35S::AtFLK; 1/6</i>	28	12
<i>flk-1; 35S::AtFLK; 1/7</i>	28	13
<i>flk-1; 35S::AtFLK; 1/8</i>	35	15
<i>flk-1; 35S::AtFLK; 1/9</i>	-	-
<i>flk-1; 35S::AtFLK; 1/10</i>	28	10
<i>flk-1; 35S::AtFLK; 1/11</i>	31	17
<i>flk-1; 35S::AtFLK; 1/12</i>	28	15
<i>flk-1; 35S::AtFLK; 1/13</i>	28	14
<i>flk-1; 35S::AtFLK; 1/14</i>	31	17
<i>flk-1; 35S::AtFLK; 1/15</i>	31	19
<i>flk-1; 35S::AtFLK; 1/16</i>	35	18
<i>flk-1; 35S::AtFLK; 1/17</i>	31	16
Segregation ratio (early bolting: late bolting)	16:0	

T2 plants (genetic background; transgene cassette; family number / plant number)	Number of days to bolting	Total number of leaves at bolting
<i>flk-1; 35S::AtFLK; 2/1</i>	35	15
<i>flk-1; 35S::AtFLK; 2/2</i>	38	13
<i>flk-1; 35S::AtFLK; 2/3</i>	28	14
<i>flk-1; 35S::AtFLK; 2/4</i>	35	18
<i>flk-1; 35S::AtFLK; 2/5</i>	38	17
<i>flk-1; 35S::AtFLK; 2/6</i>	44	18
<i>flk-1; 35S::AtFLK; 2/7</i>	31	15
<i>flk-1; 35S::AtFLK; 2/8</i>	31	13
<i>flk-1; 35S::AtFLK; 2/9</i>	31	19
<i>flk-1; 35S::AtFLK; 2/10</i>	28	13
<i>flk-1; 35S::AtFLK; 2/11</i>	44	19
<i>flk-1; 35S::AtFLK; 2/12</i>	31	14
<i>flk-1; 35S::AtFLK; 2/13</i>	28	14
<i>flk-1; 35S::AtFLK; 2/14</i>	35	18
<i>flk-1; 35S::AtFLK; 2/15</i>	38	17
<i>flk-1; 35S::AtFLK; 2/16</i>	38	16
<i>flk-1; 35S::AtFLK; 2/17</i>		
Segregation ratio (early bolting: late bolting)	16:0	

X ² for 3:1	5.33 **	
X ² for 15:1	1.07 (ns)	
<i>flk-1; 35S::AtFLK; 3/1</i>	28	16
<i>flk-1; 35S::AtFLK; 3/2</i>	38	18
<i>flk-1; 35S::AtFLK; 3/3</i>	35	18
<i>flk-1; 35S::AtFLK; 3/4</i>	38	17
<i>flk-1; 35S::AtFLK; 3/5</i>	35	21
<i>flk-1; 35S::AtFLK; 3/6</i>	25	15
<i>flk-1; 35S::AtFLK; 3/7</i>	44	22
<i>flk-1; 35S::AtFLK; 3/8</i>	44	18
<i>flk-1; 35S::AtFLK; 3/9</i>	31	15
<i>flk-1; 35S::AtFLK; 3/10</i>	31	19
<i>flk-1; 35S::AtFLK; 3/11</i>	28	17
<i>flk-1; 35S::AtFLK; 3/12</i>	35	16
<i>flk-1; 35S::AtFLK; 3/13</i>	31	16
<i>flk-1; 35S::AtFLK; 3/14</i>	31	15
<i>flk-1; 35S::AtFLK; 3/15</i>	38	14
<i>flk-1; 35S::AtFLK; 3/16^b</i>	71	73
<i>flk-1; 35S::AtFLK; 3/17^b</i>	68	64
Segregation ratio (early bolting: late bolting)	15:2	
X ² for 3:1	1.59 (ns)	
X ² for 15:1	0.86 (ns)	

X ² for 3:1	5.33 **	
X ² for 15:1	1.07 (ns)	
<i>flk-1; 35S::AtFLK; 4/1</i>	31	16
<i>flk-1; 35S::AtFLK; 4/2</i>	-	-
<i>flk-1; 35S::AtFLK; 4/3</i>	28	12
<i>flk-1; 35S::AtFLK; 4/4</i>	25	17
<i>flk-1; 35S::AtFLK; 4/5</i>	35	19
<i>flk-1; 35S::AtFLK; 4/6</i>	31	15
<i>flk-1; 35S::AtFLK; 4/7</i>	28	13
<i>flk-1; 35S::AtFLK; 4/8</i>	31	17
<i>flk-1; 35S::AtFLK; 4/9</i>	28	10
<i>flk-1; 35S::AtFLK; 4/10</i>	28	15
<i>flk-1; 35S::AtFLK; 4/11</i>	35	15
<i>flk-1; 35S::AtFLK; 4/12</i>	35	15
<i>flk-1; 35S::AtFLK; 4/13</i>	35	16
<i>flk-1; 35S::AtFLK; 4/14</i>	35	14
<i>flk-1; 35S::AtFLK; 4/15</i>	35	12
<i>flk-1; 35S::AtFLK; 4/16</i>	-	-
<i>flk-1; 35S::AtFLK; 4/17</i>	35	13
Segregation ratio (early bolting: late bolting)	15:0	
X ² for 3:1	5.00 *	
X ² for 15:1	1.00 (ns)	

Genotype / plant number	Number of days to bolting	Total number of leaves at bolting
Col-0/1	37	18
Col-0/2	40	17
Col-0/3	39	17
Col-0/4	38	16
Col-0/5	38	19
Col-0/6	39	18
Col-0/7	39	17
Col-0/8	40	16
Col-0/9	38	16
Col-0/10	35	17
Col-0/11	36	19
Col-0/12	40	19
Col-0/13	36	18
Col-0/14	37	18
Col-0/15	38	18
Col-0/16	37	18
Col-0/17	37	17

Genotype / plant number	Number of days to bolting	Total number of leaves at bolting
<i>flk-1/1</i>	74	67
<i>flk-1/2</i>	74	67
<i>flk-1/3</i>	74	68
<i>flk-1/4</i>	77	67
<i>flk-1/5</i>	67	72
<i>flk-1/6</i>	69	69
<i>flk-1/7</i>	66	71
<i>flk-1/8</i>	77	67
<i>flk-1/9</i>	71	69
<i>flk-1/10</i>	69	71
<i>flk-1/11</i>	72	67
<i>flk-1/12</i>	65	65
<i>flk-1/13</i>	74	65
<i>flk-1/14</i>	65	67
<i>flk-1/15</i>	65	69
<i>flk-1/16</i>	65	67
<i>flk-1/17</i>	63	65

^a Non-significant ($\alpha > 0.05$)

^b Non-transgenic T2 plants as tested by PCR

^c '1' indicates plants which died before bolting

* $\alpha = 0.05$; ** $\alpha = 0.01$

Supplementary Table 6: Unpaired t-test for number of days to bolting (DTB) and total number of leaves at bolting (TNL) between *BvFLK* or *FLK* transformants and non-transgenic *A. thaliana* plants within six T2 populations.

T2 family (genetic background; transgene cassette; family number)	DTB (mean \pm standard deviation)		T-test value for DTB (probability)	TNL (mean \pm standard deviation)		T-test value for TNL (probability)
	Transgenic plants ^a	Non- transgenic plants		Transgenic plants	Non- transgenic plants	
Col-0; 35S:: <i>BvFLK</i> ; #7	27.77 \pm 2.89 (n=13 ^b)	38.00 \pm 2.16 (n=4 ^b)	6.48 (0.00)	15.23 \pm 1.79	18.50 \pm 0.58	3.53 (0.00)
Col-0; endo:: <i>BvFLK</i> ; #6	28.46 \pm 1.66 (n=13 ^b)	38.00 \pm 0.82 (n=4 ^b)	10.89 (0.00)	15.31 \pm 1.70	17.00 \pm 1.15	1.84 (0.09)
Col-0; 35S:: <i>AtFLK</i> ; #5	27.23 \pm 2.05 (n=13 ^b)	38.75 \pm 0.96 (n=4 ^b)	10.71 (0.00)	15.00 \pm 2.00	19.00 \pm 1.41	3.69 (0.00)
<i>flk-1</i> ; 35S:: <i>BvFLK</i> ; #18	34.14 \pm 3.06 (n=14 ^b)	67.50 \pm 2.12 (n=2 ^b)	14.70 (0.00)	16.29 \pm 1.68	68.00 \pm 4.24	34.56 (0.00)
<i>flk-1</i> ; endo:: <i>BvFLK</i> ; #5	32.92 \pm 3.62 (n=13 ^b)	68.50 \pm 1.91 (n=4 ^b)	18.60 (0.00)	15.85 \pm 2.41	66.75 \pm 2.22	37.52 (0.00)
<i>flk-1</i> ; 35S:: <i>AtFLK</i> ; #3	34.13 \pm 5.60 (n=15 ^b)	69.50 \pm 2.12 (n=2 ^b)	8.63 (0.00)	17.13 \pm 2.26	68.50 \pm 6.36	24.94 (0.00)

^a Transgenic plants were identified by PCR analysis as described in Materials and Methods

^b Number of, respectively, transgenic or non-transgenic plants within a given T2 population

Supplementary Table 7: Unpaired t-test for number of days to bolting (DTB) and total number of leaves at bolting (TNL) between *BvFVE1* transformants and non-transgenic *A. thaliana* plants within four T2 populations.

T2 family (genetic background; transgene cassette; family number)	DTB (mean \pm standard deviation)		T-test value for DTB (probability)	TNL (mean \pm standard deviation)		T-test value for TNL (probability)
	Transgenic plants ^a	Non- transgenic plants		Transgenic plants	Non- transgenic plants	
Col-0; 35S:: <i>BvFVE1</i> ; #9	35.22 \pm 3.95 (n=23 ^b)	36.50 \pm 9.19 (n=2 ^b)	0.40 (0.69)	15.65 \pm 3.39	19.00 \pm 8.49	1.57 (0.13)
Col-0; 35S:: <i>BvFVE1</i> ; #16	31.43 \pm 4.24 (n=14 ^b)	31.73 \pm 1.68 (n=11 ^b)	0.22 (0.82)	12.86 \pm 2.57	12.36 \pm 1.12	0.59 (0.56)
<i>fve-7</i> ; 35S:: <i>BvFVE1</i> ; #1	66.62 \pm 7.08 (n=13 ^b)	65.75 \pm 4.33 (n=8 ^b)	0.31 (0.76)	33.31 \pm 9.27	36.88 \pm 6.24	0.38 (0.70)
<i>fve-7</i> ; 35S:: <i>BvFVE1</i> ; #32	59.95 \pm 7.26 (n=21 ^b)	- ^c	2.11 (0.04) ^d	32.76 \pm 7.91	- ^c	1.32 (0.20) ^d

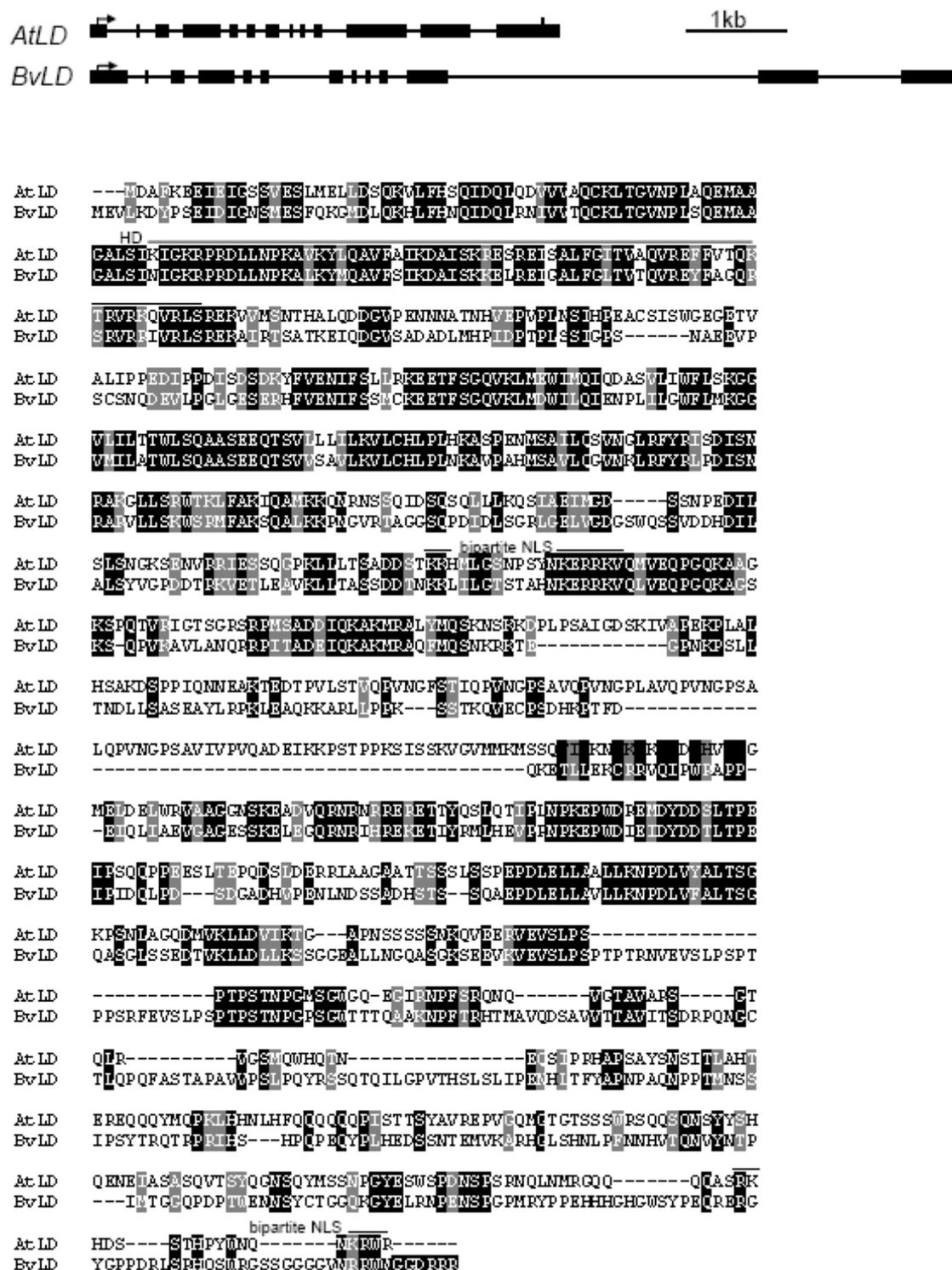
^a Transgenic plants were identified by PCR analysis as described in Materials and Methods

^b Number of, respectively, transgenic or non-transgenic plants within a given T2 population

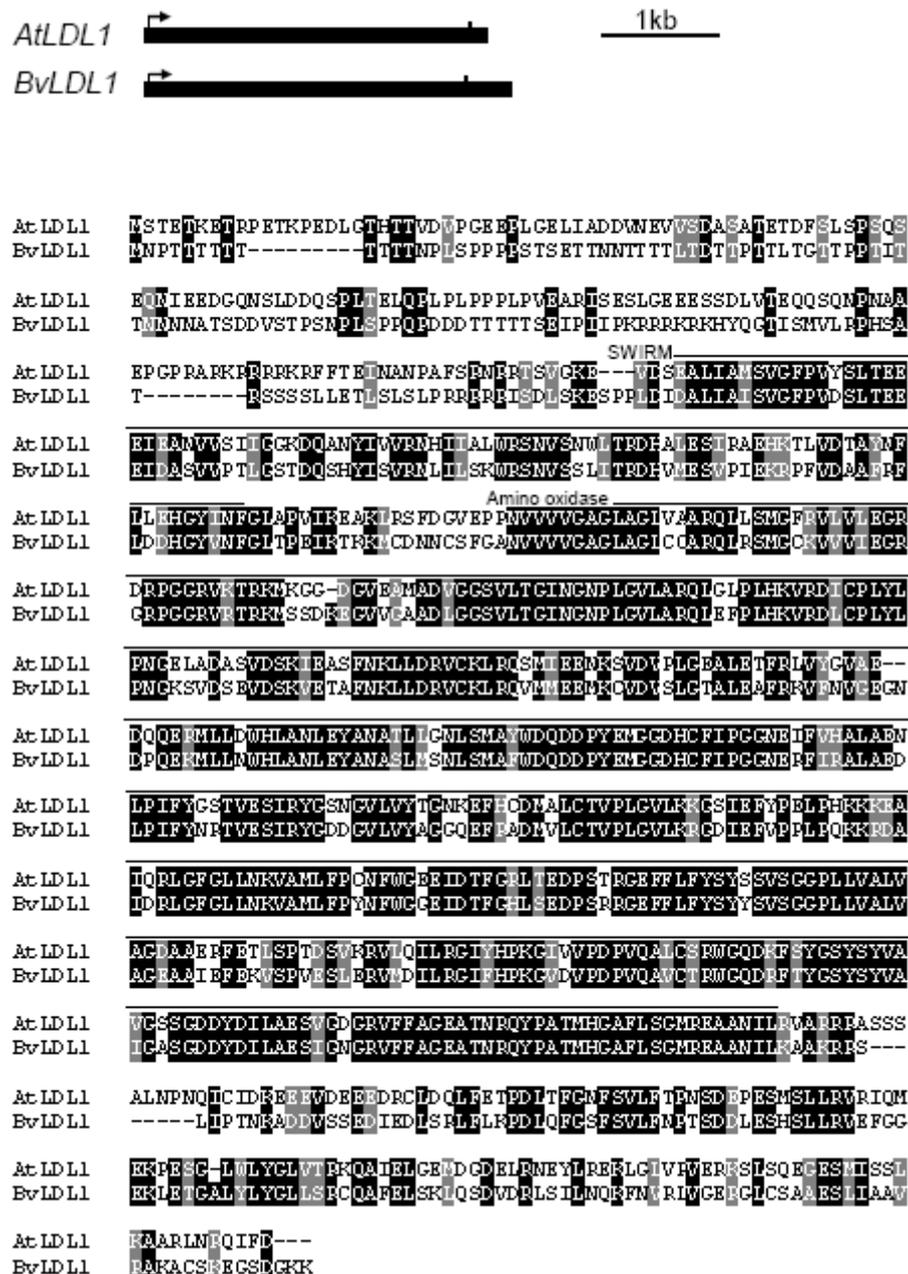
^c T2 family #32 did not include non-transgenic plants

^d The means of DTB and TNL in transgenic plants was compared against the respective means in the non-transgenic plant group of family #1 which was grown in parallel

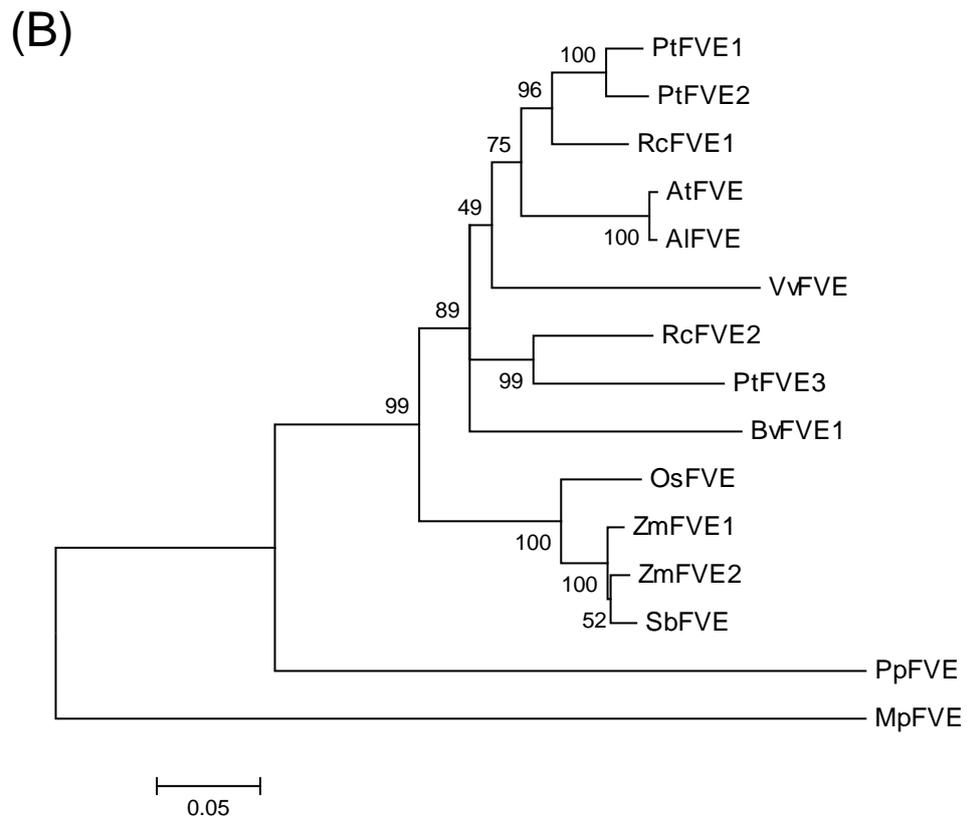
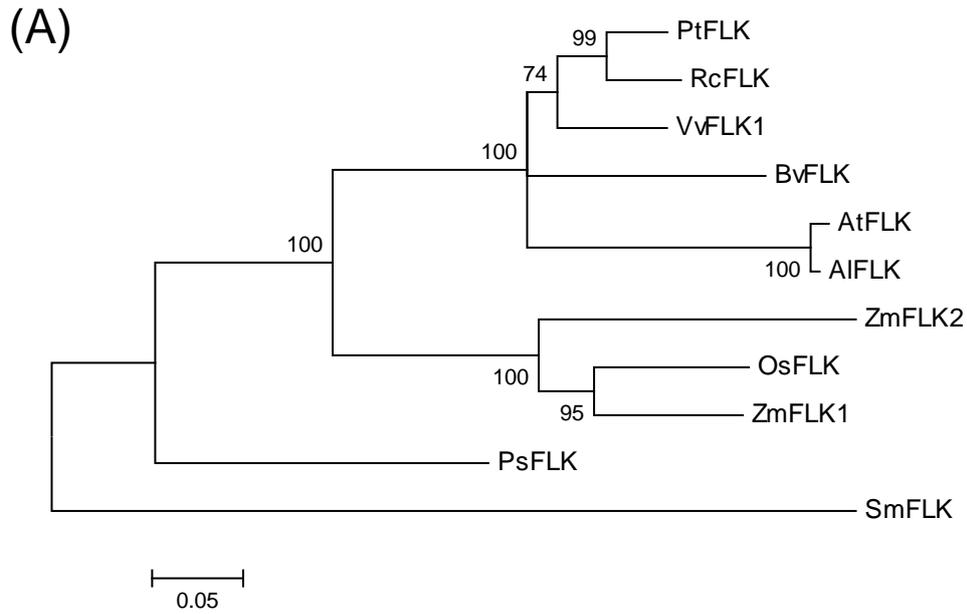
(A)

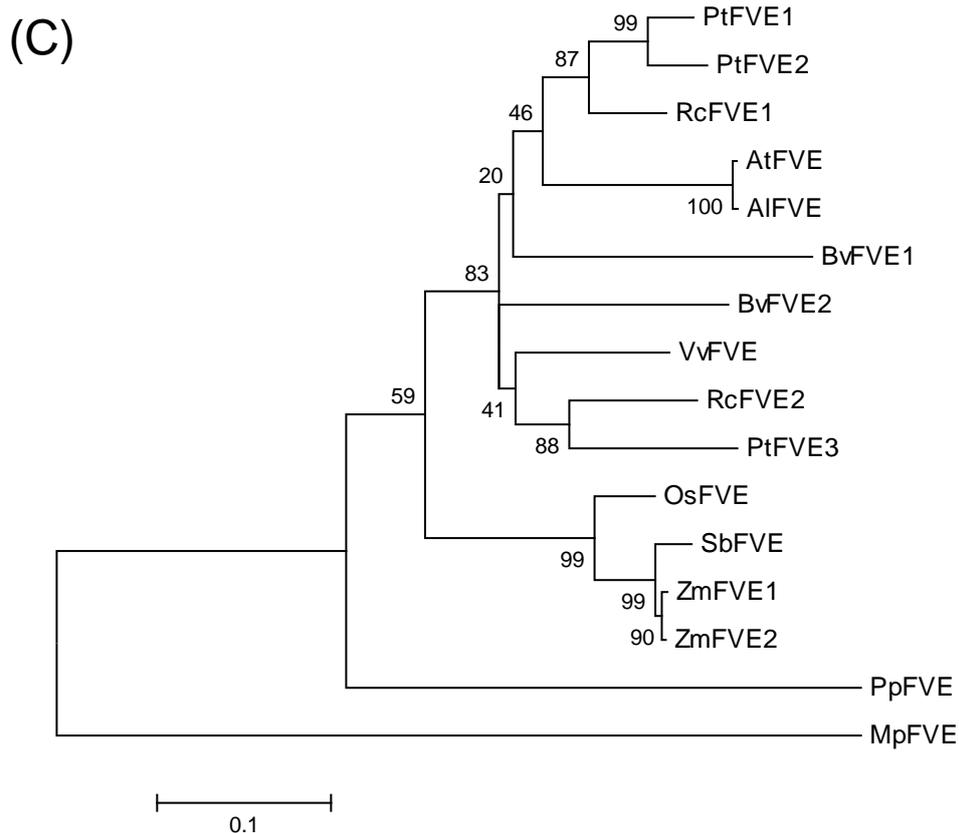


(B)



Supplementary Figure 2: Sequence and structure of the autonomous pathway gene homologs *BvLD* and *BvLDL1*. (A) Exon-intron structure of *BvLD*, *BvLDL1* and the respective *A. thaliana* genes (*LD*, accession number CAJ53849; *LDL1*, accession number NP_176471). Exons are indicated by black rectangles, the position of start and stop codons is indicated by arrows and vertical bars, respectively. (B) Pairwise sequence alignments and domain organization. The alignments were generated using ClustalW2 (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>). Identical and similar residues are highlighted by black or grey boxes, respectively. The position of protein domains according to Pfam 22.0 (<http://pfam.sanger.ac.uk/>) is marked by horizontal lines above the alignment. Two putative nuclear localization signals (NLS) in *LD* according to Lee et al. (1994) are indicated. HD, homeodomain; SWIRM, SWI3, RSC8 and MOIRA domain.





Supplementary Figure 4: Phylogenetic analysis of *FLK* (A) and *FVE* (B, C). Included are all putative plant orthologues retrieved from the NCBI RefSeq protein database (<http://www.ncbi.nlm.nih.gov/refseq>) using blastp and bidirectional best hit analysis as described in Materials and Methods. (A) Unrooted neighbor-joining tree including *A. thaliana* FLK (AtFLK), BvFLK, *Arabidopsis lyrata* FLK (AIFLK, XP_002884454.1), *Oryza sativa* FLK (OsFLK, AAL31692.1), *Picea sitchensis* FLK (PsFLK, ABK24418.1), *Populus trichocarpa* FLK (PtFLK, XP_002319087.1), *Ricinus communis* FLK (RcFLK, XP_002521945.1), *Selaginella moellendorffii* FLK (SmFLK, XP_002968912.1), *Vitis vinifera* FLK (VvFLK, XP_002269249.1), *Zea mays* FLK1 (ZmFLK1, NP_001151605.1), and *Z. mays* FLK2 (ZmFLK2, NP_001148654.1). (B) Unrooted neighbor-joining tree including *A. thaliana* FVE (AtFVE), BvFVE1, *A. lyrata* FVE (AIFVE, XP_002886035.1), *Micromonas pusilla* FVE (MpFVE, XP_003058120.1), *Physcomitrella patens* FVE (PpFVE, XP_001776478.1), *P. trichocarpa* FVE1 (PtFVE1, XP_002332825.1), *P. trichocarpa* FVE2 (PtFVE2, XP_002330494.1), *P. trichocarpa* FVE3 (PtFVE3, XP_002327543.1), *R. communis* FVE1 (RcFVE1, XP_002514113.1), *R. communis* FVE2 (RcFVE2, XP_002527406.1), *Sorghum bicolor* FVE (SbFVE, XP_002456237.1), *V. vinifera* FVE (VvFVE, XP_002282044.1), *Z. mays* FVE1 (ZmFVE1, NP_001105067.1), and *Z. mays* FVE2 (ZmFVE2, NP_001105191.1). (C) Unrooted neighbor-joining tree including a partial predicted protein sequence of BvFVE2 (corresponding to nucleotide positions 3 – 629 of EST EG550040) and all proteins in (B), except that for tree construction only the partial amino acid sequences were used that align to the partial BvFVE2 sequence. Bootstrap values as percentages of 1000 replicates are given at the branching points of the trees.

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                                NLS
AtFVE  MESDEAAAVSPQATTPSGGTGASGPKKRGRKPKTKEDSQTTPSSQQQSDVKMKESSGKKTQQ
BvFVE1  -----MTEKGRGKGG
BvFVE2  -----
OsFVE   -----MKEKGSR-----

CAF1c
AtFVE  ---SPSVDEKYSQWKGLVPILYDWLANHNLVWVPSLSCRWGPQLEQATYKNRQRLYLYSEQT
BvFVE1  KGGALTVDEKYSQWKS LVPILYDWLANHNLVWVPSLSCRWGPLLEQATYKNRQRLYLYSEQT
BvFVE2  -----
OsFVE  ---AAVDERYAQWKS LIPVLYDWFANHNLVWVPSLSCRWGPQFEKATYKNRQRLYLYSEQT

                                WD1
AtFVE  DGSVPNTLVIANCEVVKPRVAAAEHISQFNEEARSPFVKKYKTI IHPGEVNRIRELPQNS
BvFVE1  DGSVPNTLVIANVEVVKPRVAAAEHISQFNEEARSPFVKKFKTI IHPGEVNRIRELPQNS
BvFVE2  -----
OsFVE  DGTVPNTLVIANCEVVKPRVAAAEHISQFNEEARSPFVKKYKTI IHPGEVNRIRELPQNS

                                WD2
AtFVE  KIVATHDSDPDLIWDVETQPNRHAVLGAANSRPDLILTGHQDNAEFALAMCPTPEFVLS
BvFVE1  NIVATHDSDPDLIWDVENQPSRHALLGAHSSRPDLILTGHQENA EFALAMCSCEPLVLS
BvFVE2  -----TREP YVLS
OsFVE  KIIATHDSDPDLIWDVEAQPNRQAQLAQMESRPDLILRGHKDIAEFALAMCPAEPYVLS

                                WD3
AtFVE  GGKDKSVVLSIQDHIITIGTD--SKSSGS--IKQTGEGTDKN---ESPTVGPGRGVY
BvFVE1  GGKDKLVVLSIQDHIISTLATEPGGAKSTSSGSNTKFAKKSADKTNGNADSPSVGARGIY
BvFVE2  GGKDRSVVLSIQDHIITATASDPSNKPQGS GATIVKQNG-----ESPSTIGPRGIF
OsFVE  GGKDKSVVWWSIQDHIISALGDS--SKTESSPGASGSKGKTANDK---DSPKVDPRGIF

                                WD4
AtFVE  HGHEDTVEDVAFSP TSAQEFCSVGDDSDCLILWDARTGTNPVTKVEKAHADLHCVDWNP
BvFVE1  KGHDDTVEDVQFCPTSAQQFCSVGDDSDCLILWDARTGINPASKVEKAHADLHCVDWNP
BvFVE2  QGHEDTVEDVQFCPKSSQEFCSVGDDSDCLILWDARSGSDPVIKVEKAHADLHCVDWNPV
OsFVE  LGHDS TVEDVQFCPSSAQEFCSVGDDSDCLILWDARSGTGPAVKVEKAHGGDVHCVDWNLH

                                WD5
AtFVE  DDNLI L TGS ADNTVRLFDRRKLTA NGVGSPIYKFEFGHKA AVL CVQWSPDKSSVFGS SAED
BvFVE1  DENLI L TGS ADNSVHMFDRRNLTI NGVGS PVHKFQGHDA SVLCVQWSPHNRSIFGSA AED
BvFVE2  DENYI L TGS ADSVSRMFDRRNLTSAGVGS PVHKFEGHKA AVL CVQWSPDNP TVFGS A AED
OsFVE  DVNYI L TGS ADNSVRMWRDRRNLGSGGAGIPVHKFEGHKA AVL CVQWSPDKASVFGS SAED

                                WD6
AtFVE  GLLNIWDYDRVSKKSDRA----AKSPAGLFFQHAGHRDKVDFHWNASDPWTIVSVSDDC
BvFVE1  GLLNIWDYEKAGNMKTETGNRI PDYPAGLFFRHAGHRDKVDFHWN SIDPWTIVSVSDDC
BvFVE2  GVLNIWDYEKVGKKKERG-ARTPNAPPGLFFLMLG-----KWIRG----LISLE---
OsFVE  GFLNVDWHEKVGKKN-----PNAPAGLFFQHAGHRDKIVDFHWNSSDPWTIVSVSDDG

AtFVE  EITGGGGT LQIWRMSDLIYRPEHEVVAELEKFKSHVMT CASKP
BvFVE1  SSSAGGGT LQIWR I IDLLYRPEDEVLAELDKFRSHVTT CPS--
BvFVE2  -----
OsFVE  ESTGGGGT LQIWRMSDLIYRPEDEVLAEL ENFKTHLASCAPRS

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Supplementary Figure 5: Multiple sequence alignment including *Arabidopsis thaliana* FVE (AtFVE), BvFVE1, the putative translation product of the largest open reading frame in the *B. vulgaris* EST EG550040 (BvFVE2), and OsFVE. Asterisks indicate a valine and a lysine residue which are conserved between FVE, BvFVE2 and OsFVE. NLS, putative nuclear localization signal (Ausin et al. 2004); CAF1c, CAF1 subunit C / histone binding protein RBBP4 domain; WD, WD40 repeat domain. A potential zinc binding site (unfilled box) in WD6 (Kenzior and Folk 1998) is also indicated.

9 References

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Characterization of a novel *developmentally retarded mutant (drm1)* associated with the
autonomous flowering pathway in Arabidopsis. Cell Research **15**, 133-140.

10 Curriculum Vitae and Publications

10.1 Curriculum Vitae

Personal data

Name: Bianca Büttner
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Education and work history

Since 07/05 PhD student at the Plant Breeding Institute at Christian-Albrechts-University of Kiel

03/05 – 03/05 Research assistant at the Institute for Pharmaceutical Biology at the Julius-Maximilians-University of Würzburg
Work group Prof. Dr. Thomas Roitsch

11/04 – 01/05 Research assistant at the Institute for Pharmaceutical Biology at the Julius-Maximilians-University of Würzburg
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07/04 – 09/04 Student research assistant at the Institute for Pharmaceutical Biology at the Julius-Maximilians-University of Würzburg
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10/99 – 09/04 Julius-Maximilians-University of Würzburg
Studies in biology
Degree: diploma

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10.2 Publications

Articles

Büttner, B.#, Abou-Elwafa, S.#, Zhang, W., Jung, C., and Müller, A., 2010: A survey of EMS-induced biennial *Beta vulgaris* mutants reveals a novel bolting locus which is unlinked to the bolting gene *B*. *Theoretical and Applied Genetics* **121** (6), 1117-1131.

Abou-Elwafa, S.#, Büttner, B.#, Chia, T., Schulze-Buxloh, G., Hohmann, U., Mutasa-Göttgens, E., Jung, C., and Müller, A., 2010: Conservation and divergence of autonomous pathway genes in the flowering regulatory network of *Beta vulgaris*. *Journal of Experimental Botany* DOI 10.1093/jxb/erq321.

Jung, C., Qian, W., Büttner, B., Hohmann, U., Mutasa-Göttgens, E., Chia, T., and Müller, A., 2007: Using genomic information for altering bolting and flowering behaviour of crop plants. *Molecular Plant Breeding* **5**, 156-158.

Müller, A., Büttner, B., Hohmann, U., and Jung, C., 2006: Functional genomics of floral transition in sugar beet. In 'Bericht über die 57. Tagung der Vereinigung der Pflanzenzüchter und Saatgutkaufleute Österreichs', HBLFA Raumberg-Gumpenstein, Austria, November 21-23, 2006; Tagungsband pp. 41-42.

These authors contributed equally to this work.

Oral presentations

Abou-Elwafa, S., Büttner, B., Hohmann, U., Chia, T., Mutasa-Göttgens, E., Jung, C., and Müller, A., 2010: Evolutionary conservation of gene function in the autonomous pathway of flowering time control in sugar beet. Annual Meeting of the Gesellschaft für Pflanzenzüchtung (GPZ)/AG Genomanalyse 'Genomic-based breeding', October 26-28, 2010, Gießen, Germany.

*Büttner, B., Abou-Elwafa, S., Jung, C., and Müller, A., 2010: A second bolting factor in *Beta vulgaris* that acts epistatically to the bolting gene *B*. 10. Haupttagung der Gesellschaft für Pflanzenzüchtung (GPZ) 'Innovations in Breeding Methodology', March 15-17, 2010, Freising-Weihenstephan, Germany.

Müller, A., Zhang, W., Büttner, B., Schulze-Buxloh, G., Abou-Elwafa, S., Vogt, S., Chia, T., Mutasa-Göttgens, E., and Jung, C., 2010: Identification of key regulators for flowering time regulation in a biennial crop. XVIII Plant & Animal Genome Conference, January 9-13, 2010, San Diego, California, USA.

Zhang, W., Büttner, B., Schulze-Buxloh, G., Abou-Elwafa, S., Vogt, S., Chia, T., Mutasa-Göttgens, E., Winter, P., Jung, C., and Müller, A., 2009: Functional genomics of floral transition in sugar beet. Keystone Symposium on Plant Sensing, Response and Adaptation to the Environment, January 11-16, 2009, Big Sky, Montana, USA.

Schulze-Buxloh, G., Abou-Elwafa, S., Büttner, B., Lejealle, F., Stich, B., Koch, G., Wolf, M., Schechert, A., Jung, C., and Müller, A., 2009: Towards synchronization of flowering time for hybrid seed production: genetic mapping of floral transition genes and QTL in sugar beet (*Beta vulgaris*). XVII Plant & Animal Genome Conference, January 10-13, 2009, San Diego, California, USA.

*Büttner, B., Abou-Elwafa, S., Schulze-Buxloh, G., Hohmann, U., Chia, T., Mutasa-Göttgens, E., Jung, C., and Müller, A., 2008: Flowering time control in sugar beet: identification and functional analysis of floral transition genes for applications in plant breeding. Annual Meeting of the Gesellschaft für Pflanzenzüchtung (GPZ)/AG Genomanalyse 'Plant Breeding: from High Throughput Genotyping to Whole Genome Selection', November 10-11, 2008, Göttingen/Einbeck, Germany.

Zhang, W., Büttner, B., Schulze-Buxloh, G., Abou-Elwafa, S., Hohmann, U., Chia, T., Mutasa-Göttgens, E., Jung, C., and Müller, A., 2008: Functional genomics of floral transition in sugar beet. Control of Flowering Time and Applications for Plant Breeding, September 22-24, 2008, Salzgau, Germany.

*Büttner, B., Schulze-Buxloh, G., Abou-Elwafa, S., Hohmann, U., Jung, C., and Müller, A., 2008: Blühzeitkontrolle in Zuckerrübe: Identifizierung von Kandidatengenen für die Blühinduktion. 21. Tagung Molekularbiologie der Pflanze, February 26-29, 2008, Dabringhausen, Germany.

* Presented by B. Büttner

Posters

Dally, N., Büttner, B., Müller, A., Tränkner, C., and Jung, C., 2010: Towards positional cloning of the *B2* gene controlling annual bolting in beets (*Beta vulgaris*). Annual Meeting of the Gesellschaft für Pflanzenzüchtung (GPZ)/AG Genomanalyse 'Genomic-based breeding', October 26-28, 2010, Gießen, Germany.

Müller, A., Zhang, W., Büttner, B., Schulze-Buxloh, G., Abou-Elwafa, S., Frerichmann, S., Chia, T., Mutasa-Göttgens, E., Dohm, J., Himmelbauer, H., and Jung, C., 2009: Identification of flowering time regulators for breeding sugar beet with altered vernalization requirement. 9th IPMB Congress 'Leading Biology through Plant Science', October 25-30, 2009, St. Louis, Missouri, USA.

Vogt, S., Zhang, W., Schulze-Buxloh, G., Büttner, B., Abou-Elwafa, S., Dietrich, M., Wolf, M., Schechert, A., Winter, P., Jung, C., and Müller, A., 2009: GABI-GENOFLORE: functional genomics of floral transition for targeted genetic modification of flowering time in sugar beet. GABI-Statusseminar, March 3-5, 2009, Potsdam, Germany.

*Büttner, B., Abou-Elwafa, S., Schulze-Buxloh, G., Hohmann, U., Chia, T., Mutasa-Göttgens, E., Jung, C., and Müller, A., 2008: Flowering time control in sugar beet: identification and functional analysis of floral transition genes for applications in plant breeding. Control of Flowering Time and Applications for Plant Breeding, September 22-24, 2008, Salzgau, Germany.

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*Büttner, B., Hohmann, U., Schulze-Buxloh, G., Kruse, C., Jung C., and Müller, A., 2006: Positional cloning of the bolting gene from *Beta vulgaris*. Plant Genetics Conference, September 20-23, 2006, Kiel, Germany.

* Presented by B. Büttner

11 Declaration: own contribution to each manuscript

The chapters of this thesis are published (Chapter 2 and Chapter 3) or in preparation (Chapter 4). The doctoral student Bianca Büttner contributed to the publications as follows:

Chapter 2: A survey of EMS-induced biennial *Beta vulgaris* mutants reveals a novel bolting locus which is unlinked to the bolting gene *B*

Büttner, B.#, Abou-Elwafa, S.#, Zhang, W., Jung, C., and Müller, A., 2010
Theoretical and Applied Genetics **121** (6), 1117-1131

These authors contributed equally to this work.

- Conduction of all crosses, identification of cross progeny (F₁), seed propagation, and generation of three F₂ populations
- All experimental work concerning the F₂ populations EW1, EW2, and EW3 (phenotyping, DNA isolation, genotyping, data analysis)
- AFLP map construction, anchor marker development and anchoring, QTL analysis
- Preparation of the respective figures and tables
- Writing of the draft of the manuscript, revision of the manuscript under supervision of Dr. Andreas Müller

Chapter 3: Conservation and divergence of autonomous pathway genes in the flowering regulatory network of *Beta vulgaris*

Abou-Elwafa, S.#, Büttner, B.#, Chia, T., Schulze-Buxloh, G., Hohmann, U., Mutasa-Göttgens, E., Jung, C., and Müller, A., 2010
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These authors contributed equally to this work.

- Bioinformatic identification of sugar beet ESTs with homology to autonomous pathway genes
- All experimental work concerning *BvFVE1* (mapping, cloning, complementation analysis, expression analysis across tissues, promoter analysis, data analysis) except diurnal and circadian expression analysis, 5'-RACE, and phylogenetic analysis
- Preparation of the respective figures and tables
- Writing of the draft of the manuscript part about *BvFVE1*, revision under supervision of Dr. Andreas Müller

Chapter 4: Physical mapping of the *B* locus in *Beta vulgaris*

Pin, P., Zhang, W., Büttner, B., Schulze-Buxloh, G., Dally, N., Jelly, N., Chia, T., Mutasa-Göttgens, E., Dohm, J., Himmelbauer, H., Weisshaar, B., Gielen, J., Nilsson, O., Jung, C., Kraft, T., and Müller, A.

- Marker development and remapping

- Preparation of the respective figures and tables
- Writing of Chapter 4 for incorporation into a manuscript on map-based cloning of the bolting gene (Pin et al. manuscript in preparation), revision under supervision of Dr. Andreas Müller

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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 oder befunden habe.

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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.

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