

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

**Novel Genetic Factors Affecting Bolting and Floral Transition
Control in *Beta vulgaris***

Dissertation
zur Erlangung des Doktorgrades
der Agrar- und Ernährungswissenschaftlichen Fakultät
der Christian-Albrechts-Universität zu Kiel

vorgelegt von

M.Sc. Salah Fatouh Abou-Elwafa

aus Sohag, Ägypten

Kiel, 2010

Dekan: Professor Dr. Karin Schwarz

1. Berichterstatter: Professor Dr. Christian Jung

2. Berichterstatter: Professor Dr. Daguang Cai

Tag der mündlichen Prüfung: 10.02.2010

Tables of Contents

Tables of Contents	I
Abbreviations	V
List of Tables	VII
List of Figures	VIII
1. General introduction	1
1.1 History of sugar beet cultivation	1
1.2 Biology, taxonomy and breeding of sugar beet	1
1.3 Sugar beet cultivation in non-European countries	6
1.4 Genetic analysis of sugar beet	7
1.5 The impact of early bolting on sugar beet production	8
1.6 Environmental and internal factors regulating bolting and flowering ..	9
1.7 Flowering time regulation in model species	10
1.8 Flowering time genes in <i>B. vulgaris</i>	13
1.9 Objectives and scientific hypotheses	13
2. Conservation and divergence of autonomous pathway genes in the flowering regulatory network of <i>Beta vulgaris</i>:	15
2.1 Abstract	15
2.2 Introduction	15
2.3 Materials and Methods	20
2.3.1 Bioinformatic analyses	20
2.3.2 Plant material and growth conditions	21
2.3.3 BAC library screening	22

2.3.4	RT-PCR and RACE	22
2.3.5	Vector construction and transformation of <i>A. thaliana</i>	23
2.3.6	Genetic mapping and statistical analysis	24
2.4	Results	25
2.4.1	<i>Beta vulgaris</i> homologs of autonomous pathway genes	25
2.4.2	Genetic map positions	28
2.4.3	<i>BvFLK</i> accelerates the time to bolting in Arabidopsis and complements the <i>flk1</i> mutation	29
2.4.4	<i>BvFLK</i> represses <i>FLC</i> expression in Arabidopsis	32
2.4.5	<i>BvFVE1</i> does not complement an <i>fve</i> mutation in Arabidopsis ...	32
2.4.6	Transcript accumulation of <i>BvFVE1</i> , but not <i>BvFLK</i> , is under circadian clock control	33
2.5	Discussion	35
2.6	Acknowledgements	41
2.7	References	41
3.	A survey of EMS-induced biennial <i>Beta vulgaris</i> mutants reveals a novel bolting locus which is unlinked to the bolting gene <i>B</i>.....	52
3.1	Abstract	52
3.2	Introduction	52
3.3	Materials and Methods	57
3.3.1	Plant material	57
3.3.2	Phenotypic analysis	58
3.3.3	DNA extraction and genotypic analysis	59

3.3.4 Map construction and statistical analysis	60
3.4 Results	60
3.4.1 Phenotypic segregation for annual bolting	60
3.4.2 Co-segregation analysis of bolting phenotypes and B locus marker genotypes: Evidence for additional bolting loci	62
3.4.2.1 Co-segregation analysis in populations EW1, EW2 and EW3	62
3.4.2.2 Co-segregation analysis in populations EW4a and EW4b .	63
3.4.3 Variation in bolting time among annuals in F ₂ populations	65
3.4.4 Genetic mapping of the bolting locus in population EW2	66
3.4.5 Co-segregation analysis of bolting behavior and the chromosome IX marker MP_R0018 in populations EW1, EW3 and EW4a	67
3.5 Discussion	68
3.6 Acknowledgements	75
3.7 References	75
4. Genetic mapping of a novel bolting locus (<i>B4</i>) linked to the <i>B</i> gene on chromosome II of <i>Beta vulgaris</i>	81
4.1 Abstract	81
4.2 Introduction	81
4.3 Materials and Methods	84
4.3.1 Plant material and phenotypic analysis	84
4.3.2 Marker development and genetic mapping	85
4.3.3 Statistical analysis	86
4.4 Results	86

4.4.1 Phenotypic analysis of bolting behavior in F ₂ populations EW5a and EW5b	86
4.4.2 Co-segregation analysis of bolting phenotypes and <i>B</i> locus marker genotypes	88
4.4.3 Genetic mapping of <i>B4</i> locus on chromosome II	89
4.4.4 Neither <i>B</i> nor <i>B4</i> locus affects bolting time in populations EW5a and EW5b	91
4.5 Discussion	92
4.6 Acknowledgements	95
4.7 References	95
5. Closing Discussion	98
6. Summary	107
7. Zusammenfassung	109
8. References	111
9. Supplementary data	124
10. Acknowledgments	125
11. Curriculum vitae	126

Abbreviations

A	Adenine
ABA	Abscic acid
AFLP	Amplified Fragment Length Polymorphism
ANOVA	Analysis of Variance
BAC	Bacterial artificial chromosome
bp	Basepair(s)
BvCOL1	<i>Beta vulgaris</i> CONSTANS Like1
BvFL1	<i>Beta vulgaris</i> Flowering Locus1
C	Cytosine
°C	Degree celsius
CaMV 35S	Cauliflower Mosaic Virus 35S Promoter
CAPS	Cleaved amplified polymorphic sequence
cM	Centimorgan
cm	Centimeter
CMS	Cytoplasmic male sterility
CO	CONSTANS
Col-0	Arabidopsis Columbia background
DNA	Deoxyribonucleic acid
dNTP	2` - deoxyribonucleotide- 5` - triphosphate
EMS	Ethyl methanesulfonate
EST	Expressed Sequence Tag
F ₁	First generation
F ₂	Second generation
F ₃	Third generation
FCA	FLOWERING LOCUS CA
FLC	Flowering Locus C
FLD	FLOWERING LOCUS D
FLK	Flowering Locus KH domains
FPA	FLOWERING LOCUS PA
FT	Flowering Locus T
FVE	FLOWERING LOCUS VE
FY	FLOWERING LOCUS Y
G	Guanine
GA	Gibberellins
h	Hour
Ha	Hectare
JA	Jasmonic acid
kb	Kilo base pairs = 1,000 bp
LD	LUMINIDEPENDENS

LOD	Logarithm of odds
LSD	Least significant difference
Mb	Mega base pairs = 1,000,000 bp
Mg	Milligram
min	Minute
ml	Milliliter
mM	Millimolar
NASC	Nottingham Arabidopsis Stock Centre
NCBI	National Center for Biotechnology Information
ng	Nanogram
nm	Nanometer
PCR	Polymerase Chain Reaction
qPCR	Quantitative real-time polymerase chain reaction
QTL	Quantitative Trait Loci
RACE	Rapid amplification of complementary DNA ends
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphisms
RNA	Ribonucleic acid
RNAi	RNA interference
RRM	RNA Recognition Motifs
RT-PCR	Reverse transcription polymerase chain reaction
sec	Second
SNP	Single Nucleotide Polymorphism
SOC1	SUPPRESSOR of OVEREXPRESSION of CONSTANS 1
ssp.	subspecies
SSR	Simple Sequence Repeats
SVP	SHORT VEGETATIVE PHASE
SWP1	SWIRM DOMAIN PAO PROTEIN 1
T	Thymine
T-DNA	Transfer DNA
UV	Ultraviolet radiation
µg	Microgram
µl	Microliter
χ ²	Chi square

List of Tables

Table 1: <i>B. vulgaris</i> ESTs with homology to <i>A. thaliana</i> autonomous pathway genes	26
Table 2: 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 <i>BvFLK</i> and <i>FLK</i> into <i>A. thaliana</i> Col-0 and the <i>flk</i> mutant SALK_112850 (<i>flk1</i>).....	30
Table 3: Biennial EMS mutants and generation of F ₂ populations	58
Table 4: Phenotypic segregation for bolting behavior in F ₂ populations	62
Table 5: Co-segregation analysis of bolting behavior and B locus marker genotypes	64
Table 6: Analysis of variance among B locus marker genotypes in annual subpopulations.....	66
Table 7: Co-segregation analysis of bolting behavior and chromosome IX marker genotypes	68
Table 8: Analysis of variance among chromosome IX marker genotypes in annual subpopulations	69
Table 9: Phenotypic segregation for bolting behavior in F ₂ populations	87
Table 10: Co-segregation analysis of bolting behavior and the B locus marker (GJ1001c16) genotypes in populations EW5a and EW5b	88
Table 11: Analysis of variance and T-test among B and B4 locus marker genotypes in annual subpopulations	91

List of Figures

Figure 1: Sequence and structure of the autonomous pathway gene homologs <i>BvFLK</i> and <i>BvFVE1</i>	27
Figure 2: Genetic map positions	28
Figure 3: complementation analysis of <i>BvFLK</i> in the <i>A. thaliana flk1</i> mutant..	31
Figure 4: Expression of <i>BvFLK</i> and <i>BvFVE1</i> in <i>B. vulgaris</i>	34
Figure 5: Test for allelism between EMS mutations and the <i>B</i> locus	56
Figure 6: Phenotypic segregation for bolting behavior in F ₂ populations.....	62
Figure 7: Genetic map position of a major locus for annual bolting on chromosome IX	67
Figure 8: Phenotypic segregation for bolting behavior in F ₂ populations	87
Figure 9: Allelism test between EMS-induced mutation and the <i>B</i> locus	89
Figure 10: Genetic map position of <i>B4</i> locus for annual bolting on chromosome II	90
Figure 11: Genetic map positions of bolting loci and floral transition genes in sugar beet in mapping populations	101

1. General introduction

1.1. History of sugar beet cultivation

Sugar beet (*Beta vulgaris* ssp. *vulgaris* L.) is a herbaceous dicotyledon. The cultivated form is biennial. It grows vegetatively in its first year as a near-rosette plant and develops a large fleshy taproot that contains the food reserve for the second year of growth. In the second year, sugar beet becomes reproductive (Biancardi 2005).

Beets grown as vegetables are shown in 4000-year old Egyptian temple artwork; however, their use as a sugar crop is relatively recent. Beet had been known as a vegetable crop in different civilizations in the Mediterranean region, including Egyptian, Greek and Roman. It had been grown mainly for leaves, and it might be similar to what is known today as Swiss chard. Many names for beet in different ancient languages (e.g. *selg* in Arabic and *silg* in Nabataean) are apparently derived from the Greek word *sicula* used by Theophrastus ca. 300 B.C. (Biancardi 2005; McGrath *et al.*, 2007). More detailed accounts of the history of sugar beet are given by Biancardi 2005.

Sugar beet was cultivated regularly in the field in the seventeenth century, therefore it is much newer than other traditional crops. As early as in the sixteenth century, the French botanist Olivier de Serres extracted a sweet syrup from beet roots. The Prussian chemist Andreas Sigismund Marggraf used alcohol to extract sugar from beets in 1747. Because the sugar content of the roots of *Beta* which he investigated was very low (1.6% of the root fresh weight), this method did not lend itself to industrial scale production (Poggi 1980).

Breeding of sugar beet started in the beginning of the nineteenth century by Franz Carl Achard, a former student and successor of Marggraf, who obtained by mass selection the variety *White Silesian* which had 5-7% sugar content (Bosemark 1993). By the middle of the nineteenth century the first sugar beet variety with a relatively high-sugar content (~14%), "*Imperial Rübe*", had been released. By the beginning of the twentieth century and with the discoveries of Mendelian genetics and Fischer's statistical analysis, breeding of sugar beet was proceeding much faster (Bosemark 1993).

1.2. Biology, taxonomy and breeding of sugar beet

Sugar beet is a biennial species germinating epigeally and forming a rosette of glossy dark green leaves with outstanding petioles and conical storage roots in the first year. The reproductive development by which the plant morphology becomes

Distinctly different from the vegetative plant morphology takes place in the second year, after exposure to reproductive stimuli (vernalization and long days). During reproductive development stems are elongated, leaves become smaller, petioles are shortened and roots become woody and have a low sugar content (Biancardi 2005; McGrath *et al.*, 2007).

Sugar beet is the most important crop species of the genus *Beta* (family: *Amaranthaceae*, subfamily: *Chenopodiaceae*; Angiosperm Phylogeny Group 2009). Species from the genus *Beta*, which are annual, biennial or perennial, are widespread all over the Mediterranean coasts, the Atlantic coast of Europe, the Near and Middle East and parts of Asia including India (Hjerdin *et al.*, 1991; Letschert *et al.*, 1994; Ford-Lloyd 2005). A debate has been going on for years among scientists regarding the classification of the genus *Beta* (Jung *et al.*, 1993; Bruun *et al.*, 1995; Lange *et al.*, 1999; Kadereit *et al.*, 2006). According to the APG III system (Angiosperm Phylogeny Group 2009), the genus *Beta* was classified into three sections; i) *B. vulgaris* which includes three subspecies; *B. vulgaris* ssp. *vulgaris*, *B. vulgaris* subsp. *maritima* (Arcang.) and *B. vulgaris* subsp. *adanensis* (Ford-Lloyd & J. T. Williams), ii) *B. macrocarpa* (Guss.), and iii) *B. patula* (Aiton). All the cultivated forms of the genus *Beta* belong to the section *B. vulgaris* and can be separated on the basis of the morphological features into four groups (Letschert *et al.*, 1994);

- i. Leaf beet (or foliage beet); this group comprises two separate types, spinach beet and Swiss chard.
- ii. Garden beet, beets of this group have succulent storage roots and are grown as root vegetables for human consumption.
- iii. Fodder beet, these beets are characterized by their enlarged hypocotyls and crowns and are used exclusively as stock feed.
- iv. Sugar beet, which is the most recently domesticated and most widely grown crop of the genus *Beta*.

The main objective of sugar beet breeding programs is to produce reliable and stable varieties which produce the highest possible yield of extractable white sugar per area taking into consideration the production costs, and meet the various requirements of both growers and sugar factories. These objectives can only be achieved through selection for different agronomic and technological traits, some of which are complex and some are more simple (Bosemark 1993; Biancardi 2005). Since there is a negative correlation between root yield and sugar content, maximum expression of the two component characters is difficult to be obtained.

Consequently, sugar beet varieties are allocated to three distinct classes; i) E-type (with emphasis on root yield or “*Ertrag*” in German), ii) Z-type (with emphasis on content of sugar or “*Zucker*”) or iii) N-type (Normal) which is an intermediate in both characters (Biancardi 2005). Quality traits in sugar beet which include contents of sugar and impurities (sodium, potassium and α -amino-nitrogen), and negatively affect the gross sugar yield by reducing the extractable white sugar are potential targets for sugar beet breeding programs (Biancardi 2005). Although these traits are influenced to a great extent by environmental factors (El-Geddawy *et al.*, 2008; Petkeviciene 2009; Hoffmann 2010), a considerable genetic variability in quality traits in sugar beet populations was observed. However the genetic variation in sugar content is relatively small, suggesting that increasing root yield is the most promising way to achieve a high sugar yield (Schneider *et al.*, 2002; Biancardi 2005; McGrath and Trebbi 2007).

One of the most critical traits in cultivated sugar beet for sugar production in temperate climates is the breeding against premature bolting (bolting in the first year without prior exposure to prolonged periods of cold over winter (vernalization)) which drastically reduces root yield. The annual habit in *B. vulgaris* was shown to be controlled by a single dominant gene, termed the bolting gene *B*, which promotes the initiation of bolting in long days without vernalization (Munerati, 1931; Abegg, 1936). Marked variations in bolting behavior among populations have been reported, and much of this variability is related to latitude and mainly due to differences in vernalization requirement (Sadeghian and Johansson 1993; VanDijk *et al.*, 1997; Boudry *et al.*, 2002). Bolting frequency was found to be influenced by both environmental and genetic factors (Abegg 1936; Owen and McFarlane J.S. 1958; Sadeghian *et al.*, 1993a; Abou Elwafa *et al.*, 2006). In the last two decades great efforts have been made for understanding the environmental and genetic factors underlying annuality of sugar beet. The *B* locus was mapped to chromosome (linkage group) II of sugar beet using isozymes and RFLP markers (Abe *et al.*, 1993; Boudry *et al.*, 1994). A high-density AFLP-based genetic map of the *B* locus has been published (El-Mezawy *et al.*, 2002), several BACs from the vicinity of the *B* locus were identified (Hohmann *et al.*, 2003), and BAC-derived markers were developed (Hohmann *et al.*, 2003; Gaafar 2005). A candidate for the *B* gene was recently identified by map-based cloning (A. Müller, personal communication).

Sugar beet is characterized to have a high degree of self-incompatibility with low self-fertility. Before the discovery of CMS (cytoplasmic male sterility) in sugar beet, self-sterility was used to maintain heterosis in sugar beet varieties (Biancardi 2005; Brunn *et al.*, 1995). Male sterility in sugar beet as a cross-pollinated species is a pivotal character in sugar beet breeding programs. There are two types of male

sterility in sugar beet; i) genetic CMS which depends on a combination of genetic and cytoplasmic factors, and is the most important for hybrid seed production, ii) genetic male sterility which is used to facilitate cross pollination in only relatively few breeding schemes. Exploiting the CMS character of sugar beet has led to a dramatic increase in sugar beet productivity via the production of commercial hybrid sugar beet (Havey 2004; Biancardi and Skaracis 2005; Fenart *et al.*, 2008; Saxena *et al.*, 2010).

Seed monogermity and quality have a substantial impact on sugar beet crop productivity by influencing the germination and emergence of seedlings and hence affecting the distribution and uniformity of the beet plants in the field which plays a major role in exploiting, capture and perception of light, water and nutrients (Biancardi 2005; Jaggard *et al.*, 2009). The monogerm seed character in sugar beet is controlled by a single recessive gene termed “*m*”, and the action of the *m* gene is modified by other genes with relatively weak effects (Savitisky 1952). Seed germination of sugar beet is not only affected by cultivation practices, environmental and pathological factors, but also by endogenous factors such as dormancy, which is a heritable trait (Jamil *et al.*, 2006; Wagmann *et al.*, 2010).

Sugar beet is vulnerable for several pathogens including viruses, bacteria, fungi, nematodes and insects which attack different developmental stages of beet plants resulting in a significant reduction or a complete destruction of root yield (Biancardi 2005; Chirumamilla *et al.*, 2008; McGrann *et al.*, 2009). Storage of beet roots and high beet processing quality are complex physiological characters and were found to be largely influenced by climatic conditions during storage and resistance to pathogens (Bosemark 1993; Strausbaugh *et al.*, 2008; Strausbaugh *et al.*, 2009). Hence, breeding for resistance to such pathogens is a fundamental aim of sugar beet breeders. Abiotic stresses which are caused by physical factors (heat and UV) or physiochemical factors (drought and nutrient deficiency) are responsible for significant losses in yield and quality of sugar beet (Ober and Luterbacher 2002; Biancardi 2005; Monreal *et al.*, 2007; Shrestha *et al.*, 2010). Therefore, in the light of climate change and its consequences (water shortage, raising temperature and changing the light composition; Bates *et al.*, 2008) breeding of sugar beet varieties which are tolerant or adapted to abiotic stresses has acquired considerable interest.

Additional important characters which must be taken into account in sugar beet breeding programs are morphological and anatomical characters such as root, color, texture and shape, fiber content and cell size which affect harvesting operations, storage properties and the efficiency of sugar processing (Abd Elrahim *et al.*, 2005; Biancardi 2005). Recently, with the increasing demands for renewable energy

resources, breeding sugar beet genotypes with higher ethanol productivity, which possess a high capacity of sugar synthesis and accumulation regardless of other traits that may inhibit sugar extraction, has acquired great interest (Biancardi 2005; Streibig *et al.*, 2009).

Conventional breeding have led to dramatic improvements in sugar beet production. In achieving these improvements, mass selection was superior in sugar beet breeding, followed by more complicated schemes based on progeny evaluation and combining ability assessment (De Biaggi and Skaracis 2005). Further advances in breeding methods in the last fifty years have facilitated the application of various breeding approaches which have been originally developed for other crops. However, breeding of sugar beet is time consuming and requires great efforts, care and efficient practices for plant isolation because of two main reasons; i) sugar beet is a cross-pollinated species, with pollen dispersed by wind (up to 1,200 m) and occasionally by insects, and ii) cultivated sugar beet is a biennial crop and this habit complicates breeding programs and seed multiplication (Darmency *et al.*, 2009).

In the last two decades the advantages offered by cell and tissue culture in selection and breeding work have been widely recognized by sugar beet breeders. Thus, *in vitro* vegetative propagation was used both as a method of preserving valuable genotypes and in the development of improved populations. Since sugar beet is a very recalcitrant species when it comes to plant regeneration from cell or undifferentiated tissue, thus far few results from this kind of work are available (Ivic and Smigocki 2005).

More recently, with the aid of the substantial knowledge of biotechnology and molecular biology and its impressive practical exploitation in plant breeding programs, sugar beet breeders have started to consider and understand the genetic mechanisms underlying the traits of interest (Jung 2004; McGrath *et al.*, 2007; Jung and Müller, 2009). As a result, genes for quality traits, disease, pest and herbicide resistances and stress tolerance were cloned and transformed into sugar beet (Weyens *et al.*, 2004; Tertivanidis *et al.*, 2004; Kishchenko *et al.*, 2005; Liu *et al.*, 2008; Yamada *et al.*, 2009; Thiel and Varrelmann 2009). However, at least partly due to the recalcitrance of sugar beet to the *A. tumefaciens*-mediated gene transformation compared to other dicot species, the modified or acquired traits through genetic modification so far are very limited and related to resistance to herbicide, abiotic and biotic stresses, and carbohydrate metabolism (Jung 2004; May *et al.*, 2005; Norouzi *et al.*, 2005). An alternative protocol for efficient and rapid genetic modification of sugar beet using polyethylene glycol-mediated DNA transformation into protoplast populations enriched specifically for a single totipotent cell type derived from stomatal guard cells was developed by Hall *et al.*

(1996), however this technique is of limited use because it requires a specific genotype with a high rate of regeneration in addition to large amounts of purified guard cells (Jung 2004). In addition, a technique for *A. rhizogenes*-mediated hairy roots transformation of sugar beet was successfully established (Menzel *et al.*, 2003).

1.3. Sugar beet cultivation in non-European countries

Sugar beet is the only sucrose storing crop species that can be grown commercially in a wide variety of temperate climates. In Germany, which is ranked as the second sugar beet producer in Europe, after Russia, sugar beet is one of the most fundamental crops which occupies an area of 385,000 ha in 2010/11 with an average yield of 74.96 t/ha (Polet and Wagner 2010). In the Americas, USA is the biggest sugar beet producer with a cultivated area of 406,552 ha, yielding an average of 66.01 t/ha (www.fao.org). In Asia, sugar beet is cultivated in 14 countries, with Turkey ranked as the first Asian producer of sugar beet, which cultivated about 340,000 ha with an average yield of 48.29 t/ha (Cakiroglu and Gifford 2010). In Africa, sugar beet is only cultivated in three countries, two of which are Mediterranean countries, Egypt and Morocco. The cultivated area of sugar beet in Egypt in 2009/2010 is 98,000 ha and the average root yield is 52.58 t/ha (Güven *et al.*, 2010).

Cultivation of sugar beet in the tropical and subtropical regions, which are mostly developed countries, is a substantial goal (Abou Elwafa *et al.*, 2006; Balakrishnan and Selvakumar 2009). In the second half of the last century, great efforts have been made to introduce and adapt sugar beet cultivation to tropical and subtropical countries in order to replace or supplement the sugar production from sugar cane which is prevalent in such countries because of the following substantial reasons; i) the water requirement of sugar beet, which is a decisive determinant of sustainable cultivation in water-strapped regions, is much lower than that of sugar cane, ii) sugar beet has a shorter growing season (5-6 months) compared to sugar cane which may be more than 12 months, and iii) sugar beet could be a possible solution as tolerant crop of soil alkalinity or for newly reclaimed soils which are common in tropical and subtropical areas, and are not suitable for cane or other crops (Mawusi 2004; Abou Elwafa *et al.*, 2006; Nasr and Abd El-Razek 2008).

Furthermore, the cultivation of sugar beet in developed countries is a profitable business for both farmers, by diversification of their incomes by enabling them to grow an additional cash crop, and sugar factories by efficient usage of the resources in the processing of sugar beet, where the factories could be supplied for up to 10 months of the year with high quality beet (Balakrishnan and Selvakumar 2009).

Recently, tropical sugar beet varieties, which are more adapted for cultivation in the tropical and subtropical regions, have been launched by sugar beet breeding companies, and could be successfully grown under these conditions in India, Kenya and Sudan with a reasonable sugar yield. (Balakrishnan and Selvakumar 2009; Mandere *et al.*, 2009).

Sugar beet has several unique features compared to other common crop species which prompt its use for biofuel production. These features are: i) the net energy of ethanol produced from sugar beet surpasses that from other common crops such as corn, sugar cane, rape seed and wheat (Venturi and Venturi 2003; Koga 2008), ii) both of the aerial and underground parts of sugar beet could be fermented and used for biogas production, and the by-products of this process could be also valuable for live stock feeding (Renouf *et al.*, 2008), iii) with the expansion of genetically modified sugar beet cultivation which leads to a significant increase of the biomass production, it became one of the major crops which could be used for biofuel production (Sexton *et al.*, 2009), and iv) its capability to grow and yield well under a wide variety of environmental conditions including the tropical and subtropical areas (Seebaluck *et al.*, 2008).

1.4. Genetic analysis of sugar beet

Sugar beet is a true diploid species with a basic chromosome number of $x = 9$ ($2n = 2x = 18$) and a haploid genome size of 758 Mb with an estimated number of genes of 25,000 (Herwig *et al.*, 2002; Lange *et al.*, 2008). Sugar beet is a cross-pollinated species with a high degree of self incompatibility which is controlled by a single gene “S”, which has a series of four different alleles (De Biaggi 2005). The degree of self-incompatibility is influenced by the external factors, i.e., under low temperatures or at the end of the flowering season self-sterility become partial or incomplete (pseudocompatibility; De Biaggi, 2005). Although selfing of sugar beet which is the first step towards inbred line production is difficult, it is often possible to obtain a small amount of seeds from a selfed plant. However, this problem could be overridden by introducing the obligate self-fertility allele, S^F .

Genetic analysis of sugar beet has started using isozymes and morphological markers (Jung 2004). The progress achieved in the last two decades in the development of molecular markers, i.e. RAPD, RFLP, AFLP, SSR, CAPS and SNP, played a fundamental role in the development of more precise genetic maps by assigning the linkage groups to the nine chromosomes of sugar beet (Schondelmaier *et al.* 1996; Weiland and Yu 2003; Jung 2004; Gaafar *et al.*, 2005; Lennefors 2006; Schneider *et al.*, 2007; McGrath *et al.*, 2007; Grimmer *et al.*, 2007b; Lange *et al.*, 2010; Smulders *et al.*, 2010). The availability of several genetic

linkage maps of the sugar beet genome enhanced the efficiency of molecular genetic analysis of sugar beet and improved its breeding in the past years for several agronomic traits (Schneider *et al.*, 2002; Hunger *et al.*, 2003; Gaafar *et al.*, 2005; Hagihara *et al.*, 2005; Lein *et al.*, 2007; Grimmer *et al.*, 2007a; Taguchi *et al.*, 2009). In an advanced step of exploiting the molecular markers and genetic maps of sugar beet, physical mapping and map-based cloning approach, were applied to clone genes of sugar beet (Cai *et al.* 1997; Desel *et al.*, 2001; Samuelian *et al.*, 2004; Schulte *et al.*, 2006; Capistrano 2010). Phylogenetic analysis, which is a model of relationships between genes or proteins and organisms based on common ancestry, have been carried out in beet for different purposes such as taxonomy classification of the genus *Beta*, ontology and morphology of inflorescence and floral formation (Jung *et al.* 1993; Biancardi 2005; Mglinets 2008; Olvera *et al.*, 2008).

Marker assisted selection which is the most promising application of molecular markers in accelerating the development of crop varieties and the improvement of the quantitative traits such as yield, quality and tolerance to abiotic and biotic stresses (Xu and Crouch 2008; Collard and Mackill 2008; Choudhary *et al.*, 2008; Jannink *et al.*, 2010), was described as an efficient approach in breeding sugar beet for some economically important traits such as bolting resistance, drought tolerance and resistance to root-knot nematode (Abe *et al.* 1997b; Yu 2003; Frisch and Melchinger 2005; Hajheidari *et al.*, 2005; Mandolino 2007).

1.5. The impact of early bolting on sugar beet production

Cultivated sugar beet grows vegetatively in the first season. The reproductive growth takes place in the second season after the exposure to the environmental stimuli to initiate bolting (stem elongation) and flowering. These environmental stimuli are low temperature (vernalization), which is obligatory in cultivated beets, followed by long-day conditions. By contrast, annual beets do not exhibit a vernalization requirement in order to bolt and flower (Lexander 1980). The term photothermal induction of bolting in sugar beet which outlines the effect of both vernalization and daylength on bolting induction of sugar beet was first used by Owen *et al.* (1940). Under conditions such as short days or rapid exposure to warm ambient temperatures after vernalization, the inductive effect of vernalization will be abolished and the plants revert to vegetative growth (devernalization; Lexander 1980). However, Fife and Price (1953) reported that bolting and flowering might be induced under variable environmental cues such as continuous darkness or much prolonged cold treatment.

Early bolting (premature bolting) is an undesirable agronomic character in cultivated sugar beet for sugar production because of the following reasons: i) it leads to a significant decrease in root yield to up to 50%, ii) it drastically reduces sugar yield by reducing sugar content, iii) bolters cause some difficulties during mechanical harvesting, iv) seeds produced from those plants are a likely major source for weed beet contamination, and v) roots resulting from early bolting plants have woody fibrous roots which negatively affect beet processing in the sugar factories and decrease sugar production (Bartsch *et al.* 1999; Rinaldi and Vonella 2006; Jung *et al.*, 2007; Lewellen 2007; Jaggard *et al.*, 2009; Streibig *et al.*, 2009). Since the sugar accumulation period is not strictly limited and root yield continues to increase as long as the plant did not bolt, extending the growing season of sugar beet via autumn sowing is a fundamental target to improve sugar beet productivity in temperate regions (Jaggard and Werker 1999; Jung *et al.*, 2007; Jaggard *et al.*, 2009). However, autumn sowing of sugar beet requires the development of winter beet varieties, which are able to grow over winter and the summer of the following season without bolting (Jung *et al.*, 2007; Jung and Muller 2009).

1.6. Environmental and internal factors regulating bolting and flowering

Timing of floral transition reflects the adaptation of a plant to its environment by adjusting vegetative and reproductive growth phases to local environmental cues (Buckler *et al.*, 2009). Plants adapted their timing of floral transition in response to seasonal changes in the environmental conditions, especially daylength and temperature, which are the most critical environmental cues that affect flowering time synchronization in plants and hence influence the seed set and yield potential. The response of plants to daylength that enables them to be adapted to seasonal changes in their environment, and to latitudinal variation, was observed in 1920 in soybean and tobacco plants by Garner and Allard. Physiological studies on the response of plants to photoperiod revealed that light mediates floral transition through three main aspects; light quality, light quantity and light duration (Carre 2001; Munir *et al.*, 2004; Thomas 2006).

Floral initiation in numerous plant species exhibits a requirement to vernalization. Some species (e.g. *Arabidopsis*) show a facultative requirement; meanwhile other species (e.g. sugar beet and winter cereals) have an obligate requirement to vernalization in order to initiate the reproductive development. The response to vernalization has two characteristic features: i) its quantitative effect, where longer vernalization periods accelerate floral transition, and vice versa; and ii) its saturability, where it reaches a point at which further exposure to cold does not cause additional acceleration of flowering (Dennis and Peacock 2009; Kim *et al.*,

2009). There is a clear temporal separation between exposure of plants to vernalization and the transition to the reproductive development. Floral initiation may occur several weeks after plants return to raised temperatures. Hence, perception of vernalization must be “remembered” by the plant through several mitotic divisions (Sung *et al.*, 2006; Sung and Amasino 2006; Alexandre and Hennig 2008; Michaels 2009; Greenup *et al.*, 2009; Amasino 2010).

Flowering time is also affected by different abiotic stresses, such as nutrient deficiency, heat and drought (Jung and Müller 2009). Drought, nutrient and heat stresses accelerate flowering time, and the acceleration is stronger under SD and is species-dependent (Kolar and Senkova 2008; Zinn *et al.*, 2010; Wada and Takeno 2010; Sangtarash 2010).

Phytohormones play major roles in plant growth and development, and floral transition is affected by exogenous treatment of different types of phytohormones, with GA being the most effective, where it induces bolting and flowering under non-vernalized conditions (Prat *et al.*, 2008; Mutasa-Gottgens and Hedden 2009). Auxin was found to play an important role in regulating the activity of shoot apical meristem (SAM) and floral meristem, and in modulating the response of the plant to unfavorable daylengths (Sundberg and Ostergaard 2009; Halliday *et al.*, 2009; Vernoux *et al.*, 2010). Other plant hormones such as cytokinin, JA and ABA have minor (direct or indirect) roles in regulating floral transition (Bernier and Perilleux 2005; Schroeder and Kuhn 2006; Avanci *et al.*, 2010).

1.7. Flowering time regulation in model species

The transition from the vegetative stage to the flowering development in plants is a complex biological process controlled by many genes. In all seed crops, flowering time is a critical time point in the life cycle of plants which could significantly affect the yield and dry matter. Extensive studies on floral transition in the model species *Arabidopsis thaliana* have shown that floral transition is regulated by the floral integrator genes, i.e. *FLOWERING LOCUS T (FT)*, *TWIN SISTER OF FT (TSF)*, *SUPPRESSOR OF CONSTANS 1 (SOC1)* and *AGAMOUS-LIKE 24 (AGL24)*. These floral integrator genes promote the floral meristem identity genes by integrating the outcomes of genes from the four regulatory pathways, i.e., the photoperiod, vernalization, gibberellin and autonomous pathways, and under favorable conditions promote floral transition (Kim *et al.*, 2008; Adrian *et al.*, 2009; Gregis *et al.*, 2009; Jung and Muller 2009). Two of these four pathways (the vernalization and autonomous pathways) act to regulate the expression of the main floral repressor *FLOWERING LOCUS C (FLC)*, which represses the floral integrator genes (Choi *et al.*, 2009; Seo *et al.*, 2009; Jung and Muller 2009). Other

floral repressors have been reported to act similarly to *FLC* as floral repressors such as *SHORT VEGETATIVE PHASE (SVP)* which is controlled by the autonomous and gibberellin pathways, and directly represses the expression of *FT* and *SOC1*, suggesting that *SVP* is another central regulator of the flowering regulatory network, and that the interaction between *SVP* and *FLC* mediated by various flowering genetic pathways control the convergence of flowering signals (Li *et al.*, 2008).

The main action of vernalization, in winter-annual *Arabidopsis*, is to suppress the expression of *FLC* by changing the chromatin structure of *FLC*, and switch it into a repressed state that is mitotically stable. The promoter region of *FLC* undergoes vernalization-specific histone methylation changes that are associated with the maintenance of transcriptional repression (Bastow *et al.*, 2004). A key component of the vernalization pathway, *VERNALIZATION INSENSITIVE3 (VIN3)*, which is a PHD-domain-containing protein, is involved in initiating the modification of *FLC* chromatin structure (Bond *et al.*, 2009). The stable silencing of *FLC* also requires the DNA-binding protein *VERNALIZATION1 (VRN1)* and the polycomb-group protein *VRN2*. Mutation at both *VRN1* and *VRN2* led to an increase of *FLC* mRNA levels after vernalization, indicating that *FLC* regulation is disturbed in those mutants (Greenup *et al.*, 2009; Distelfeld *et al.*, 2009)

Photoperiod is one of the major environmental cues influencing floral transition. *CONSTANS (CO)* is the central gene in the photoperiod pathway, and is a determined factor in floral transition. *CO* plays a vital role in accelerating floral transition by enhancing the expression of *FT*, *SOC1*, *ACC SYNTHASE (ACS10)* and *AtP5CS2*, and the expression of *FT* is correlated with the expression of *CO* during the day (Samach *et al.*, 2000; Valverde *et al.*, 2004; Turck *et al.*, 2008). *CO* expression is regulated by day length, photoreceptors (*Cry1*, *Cry2*, and *PhyA*) and circadian clock related genes [*LATE ELONGATED HYPOCOTYL (LHY)*, *GIGANTEA (GI)* and *EARLY FLOWERING 3 (ELF3)*, *TIMING OF CAB EXPRESSION 1 (TOC1)*, *PSEUDO RESPONSE REGULATOR 7 (PRR7)/PRR9*] (Hayama and Coupland 2003; Valverde *et al.*, 2004; Kim *et al.*, 2008; Imaizumi 2009).

Studies on mutation either in GA biosynthesis or signaling have shown that GA acts as a floral promoter, with this promotion being more prevalent in SDs where the photoperiodic pathway is inactive (Hytonen *et al.*, 2009). The promotive effect of GA on *Arabidopsis* growth and development was found to be regulated by the GA-signaling components, *GAI* and *RGA* (King *et al.*, 2001; Hou *et al.*, 2008). Each of the *Arabidopsis GA3ox* gene family (*AtGA3ox1–AtGA3ox4*), which regulates the bioactive GA synthesis, has a unique organ-specific expression

pattern, suggesting distinct developmental roles played by individual *AtGA3ox* members in regulating bioactive GA synthesis (Yamaguchi 2008). A coordinative relationship between the GA and the photoperiod pathways was observed. *CO* was suggested to be involved in other GA-related processes and is required for elevated levels of GA biosynthesis in Arabidopsis. GA effect is more drastic in red light than in dark (Putterill 2001;Feng *et al.*, 2008)

The autonomous pathway was originally defined by screening the progeny of EMS or X-ray mutagenized Arabidopsis Landsberg erecta wild type, and consists of at least seven known genes; *LD*, *FCA*, *FY*, *FPA*, *FVE*, *FLD*, and *FLK* (Simpson, 2004). Genes from the autonomous pathway promote floral transition, largely independently of environmental cues, by repressing the expression of *FLC* via RNA-based control mechanisms and/or chromatin modification (Chandler *et al.* 1996;Lim *et al.*, 2004;Simpson 2004;Michaels *et al.*, 2004;Marquardt *et al.*, 2006;Baurle *et al.*, 2007;Baurle and Dean 2008a). Mutations in these genes lead to an increase in the levels of *FLC* mRNA and FLC protein, which in turn lead to a decrease in the expression levels of *SOCI* and *FT*, resulting in late flowering phenotypes, which is photoperiod, GA and vernalization responsive (Chandler *et al.* 1996;Lim *et al.*, 2004;Simpson 2004;Michaels *et al.*, 2004;Marquardt *et al.*, 2006;Baurle *et al.*, 2007;Baurle and Dean 2008a). *FCA*, *FPA*, *FY* and *FLK* regulate *FLC* through RNA regulatory processing. *FCA* and *FPA* are plant-specific RNA binding proteins carrying RNA recognition motifs (RRMs; Macknight *et al.* 1997;Schomburg *et al.*, 2001). In addition to its role in down-regulating the expression of *FLC*, *FCA* was found to be required to regulate its own expression by interacting with the RNA 3' end processing factor *FY* (Simpson *et al.*, 2003;Quesada *et al.*, 2005). *FLD*, which is a plant homolog of a human protein found in histone deacetylase complexes, regulates *FLC* transcriptionally through histone H3K4 demethylation, and is required for the repression of *FLC* by *FCA* and *FPA* (He *et al.*, 2003;Ausin *et al.*, 2004). *LD* encodes a homodomain protein which is known as DNA binding protein but in some rare cases was found to be associated with mRNA and acts as a translational repressor, although its regulatory mechanism of *FLC* is still unknown (Dubnau and Struhl 1996;Rivera-Pomar *et al.* 1996). Both *FCA* and *FPA* were found to interact with *FLD*, suggesting a role of *FCA* and *FPA* in connecting RNA- and chromatin-mediated regulation of *FLC* (Baurle *et al.*, 2007).

FLK encodes a putative RNA binding protein which contains three K-homology (KH) motifs (Lim *et al.*, 2004;Mockler *et al.*, 2004). Although the mechanism of *FLK* is unknown, the basis that KH domains are only present in proteins playing a major role in regulating cellular RNA metabolism, together with biochemical studies, suggested that the KH domains are RNA binding protein which bind to

single stranded RNA in a non-specific manner, are involved in pre-mRNA processing, translation initiation, processive degradation of RNA, and meiosis specific splicing, and are associated with hnRNA (Gibson *et al.* 1993; Adinolfi *et al.* 1999). Furthermore, other KH domains- bearing proteins in Arabidopsis were found to be involved in pre-mRNA processing (e.g. the hnRNA protein which showed high sequence identity to *FLK*, plays an important role in the processing of the AGAMOUS pre-mRNA and floral organ development; (Cheng *et al.*, 2003; Lim *et al.*, 2004)). An analysis of flowering time in different autonomous pathway double mutants showed that *FLK* acts independently from both *FCA* and *FPA*, and the expression of *FLK* was not influenced by any of the other six autonomous pathway mutants analyzed (*fca*, *fpa*, *fy*, *fld*, *fve*, *ld*), and, *vice versa* (Lim *et al.*, 2004; Bäurle and Dean 2008a; Ripoll *et al.*, 2009). Furthermore, Ripoll *et al.* (2009) showed that *PEPPER* (*PEP*), a paralog of *FLK* in Arabidopsis which shares 42.4% amino acid identity with *FLK* and also carries three KH domains, acts as a positive regulator of *FLC*. Mutation at *PEP* could rescue the late flowering phenotype of *flk* mutants, and overexpression of *PEP* resulted in a similar effect on flowering time as mutation of *FLK*. Two lines of evidences suggested that *FLK* may suppress the expression of *FLC* at the transcriptional level via RNA-directed chromatin silencing; i) both correctly spliced *FLC* transcripts and improperly spliced (intron-containing transcripts) were found to accumulate to higher levels in an *flk* mutant compared to wild-type plants (Ripoll *et al.*, 2009), and ii) the repression of the retroelement *AtSNI*, which is subject to RNA-directed chromatin silencing, was partially released in *flk* mutant plants (Bäurle and Dean, 2008; Veley and Michaels, 2008).

1.8. Flowering time genes in *B. vulgaris*

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; Büttner *et al.*, 2010).

A homolog of *FLC* in *B. vulgaris* (*BvFL1*) was cloned and was found to be functionally related to *FLC* (Reeves *et al.*, 2007). However, the genetic map position of *BvFL1* (chromosome VI) in addition to the lack of a clear difference in the expression and regulation of *BvFL1* between the annual and biennial genotypes exclude the possibility that *BvFL1* is the bolting gene. Similarly, evolutionary conservation of *CO* homologs was suggested by overexpression of the *CO*-like gene *BvCOL1* in Arabidopsis. *BvCOL1* complements the late flowering phenotype

of a *co* Arabidopsis mutant and promotes the expression *FT*. The genetic map position of *BvCOL1*, which was mapped to chromosome II, but at a genetic distance of ~25 cM from the *B* locus, excludes the possibility that it corresponds to the bolting gene (Chia *et al.*, 2008).

1.9. Objectives and scientific hypotheses

In the present study we started dissecting the genetic basis of bolting and floral transition of *B. vulgaris* by applying both forward and reverse genetic approaches. The forward genetic approach was used to elucidate the genetic factors underlying bolting behavior in *B. vulgaris*. At the beginning of this work it was known that bolting behavior of sugar beet is controlled by a single dominant gene (*B*). In a previous study, an annual genotype homozygous for the dominant bolting allele at the *B* locus was mutagenized by ethyl methanesulfonate (EMS) treatment. Phenotypic screening for bolting behavior and further propagation of mutagenized plants has led to the identification of several biennial non-segregating M3 families (Hohmann *et al.*, 2005). The aim of this investigation was the identification and genetic mapping of gene(s) affecting bolting time which might be mutated in the biennial EMS-induced genotypes. Assuming that bolting behavior in the annual parent is controlled by the *B* locus, there are at least two conceivable hypothesis; i) the *B* gene is mutated ('one-locus model'), or ii) a second locus is mutated that acts epistatically to *B* and prevents annual bolting even in the presence of *B* ('epistatic locus model'; see Introduction, Chapter 3).

The reverse genetic approach was applied assuming conserved functions between *B. vulgaris* homologs of floral transition genes and their Arabidopsis counterparts in regulating flowering time. The objectives of this investigation were; i) identification of *B. vulgaris* homologs of floral transition genes of the autonomous pathway, ii) genetic mapping of these genes, and iii) functional characterization by overexpression and complementation analysis in Arabidopsis wild type and mutant plants.

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

Published in Journal of Experimental Botany, 2010

2.1. 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* homologs 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, we begin to address this by the identification and genetic mapping of full-length homologs 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*. By contrast, complementation analysis of *BvFVE1* and the presence of a putative paralog in beet suggest evolutionary divergence of *FVE* homologs. It is further shown that *BvFVE1*, unlike *FVE* in *Arabidopsis*, is under circadian clock control. Together, our 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.

2.2. 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 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, i.e. 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. Müller, personal communication), 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 homologs 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 *BvFL1* in beet is regulated by vernalization and delays flowering in transgenic *Arabidopsis* plants, suggesting that *BvFL1* may also be a floral repressor (Reeves *et al.*, 2007). Similarly, evolutionary conservation of *CO* homologs 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*, 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*, *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 *AtSN1*, a retroelement which is subject to RNA-directed chromatin silencing, was at least partially released in mutant plants (Bäurle and Dean, 2008; Veley 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 (Veley and Michaels, 2008; Ripoll *et al.*, 2009). Ripoll *et al.* (2009) further found that *PEPPER* (*PEP*), a paralog 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 homolog of the human histone H3K4 demethylase *LSD1* (*LYSINE-SPECIFIC HISTONE DEMETHYLASE 1*) 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 homologs of *FLD*, *LSD1-LIKE 1* (*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 (Krichevsk *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 IRA4*) and *ACGI* (*ALTERED COLD-RESPONSIVE GENE EXPRESSION 1*)) is homologous to *MSII*

(*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* (Blázquez *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, we start dissecting the components of the autonomous pathway in *B. vulgaris* through a survey of ESTs with homology to autonomous pathway genes and isolation of the corresponding genes. For beet homologs 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 homolog 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. By 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, our 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 homolog.

2.3. Materials and Methods

2.3.1. Bioinformatic analyses

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 homologs of flowering time genes in *Arabidopsis 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* orthologs 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 homologs divided by the total number of residues in the reference gene. For phylogenetic analysis, putative *FLK* and *FVE* orthologs 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).

2.3.2. 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 Suppl. Tab. 1) 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).

2.3.3. 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 PCR₉₆Cleanup 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).

2.3.4. 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 and B043), and 92.9% for *BvFVE1* (primers A066-A042). Fluorescence of Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen Corporation, Carlsbad, 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, California) was detected in an MX3000 real-time PCR machine (Stratagene, La Jolla, Californian) over 40 cycles at 60°C annealing temperature.

The 5' end of *BvFVE1* was identified by 5'-RACE (Frohman *et al.*, 1988) using the GeneRacer™ kit according to the manufacturer's protocol (Invitrogen Corporation, Carlsbad, USA). 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, 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 RNAeasyKit™ (Qiagen, Hilden, Germany) and DNase treated (Fermentas, St. Leon-Rot, Germany). 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 and B337), *FLK* (GenBank accession number AC011437, B281 and B282), and *GAPDH* (GenBank accession number NM_111283; B349 and B350) was 100%, 97.4% and 94.2%, respectively.

2.3.5. 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, Madison USA) 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, 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-CNRGV, Castanet Tolosancedex, 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, Carlsbad, USA) 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 Carlsbad, USA) 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).

2.3.6. 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).

2.4. Results

2.4.1. *Beta vulgaris* homologs of 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 homologs 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 orthologs in beet (*FLK* (BQ586739), *FPA* (BQ489608), *FVE* (BQ592158, EG550040), *LD* (BQ589018, BQ594506); Table 1). Besides *FLK*, one EST (BQ590839) was identified whose closest homolog in *A. thaliana* is *PEP*, an *FLK* paralog (Ripoll *et al.*, 2009; see Introduction). In addition, one EST (CV301493) with homology to *FLD* appears to be orthologous to *LDL1/SWP1*, 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*.

For four putative autonomous pathway genes, named *BvFLK*, *BvFVE1* (corresponding to one of the two homologous ESTs), *BvLD* and *BvLDL1*, 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*, *BvFVE1*, *BvLD* and *BvLDL1* was determined by RT-PCR and sequencing. The number of exons and the sites of introns was found to be conserved between the corresponding *A. thaliana* and *B. vulgaris* genes (Figure 1A; Suppl. Figure 1). 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).

Table 1: *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>LDL1/SWP1</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 *B. 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>).

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

The predicted protein sequences were aligned against the corresponding *A. thaliana* genes (Figure 1B; Suppl. Fig. 1). For *FLK* and *FVE*, homologs 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 homologs 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* homolog) 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). By 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.

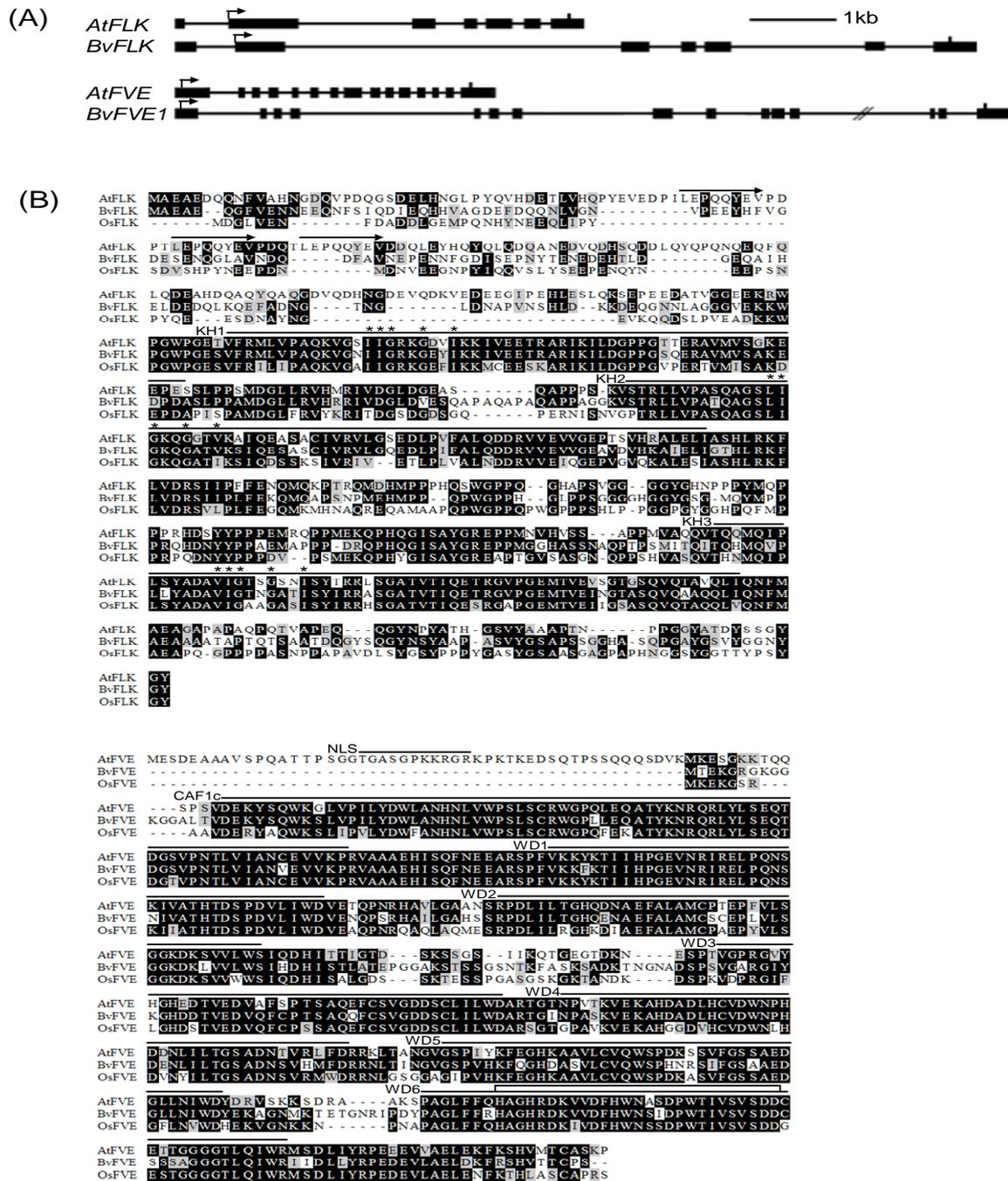


Figure 1: Sequence and structure of the autonomous pathway gene homologs *BvFLK* and *BvFVE1*. (A) Exon-intron structure of *BvFLK*, *BvFVE1* and the respective *A. thaliana* genes (*FLK*, accession number AAX51268; *FVE*, accession number AF498101). Exons are indicated as black rectangles, the position 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.

2.4.2. Genetic map positions

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 (Figure 2). *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.

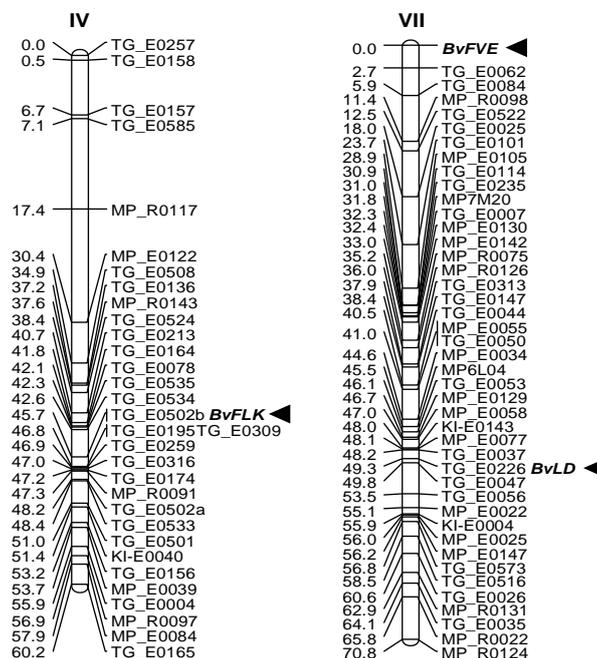


Figure 2: Genetic map positions. *BvFLK* and *BvFVE1* (arrowheads) were mapped to position 45.7 cM on chromosome IV and the top end of chromosome VII, respectively, on a reference map of the sugar beet genome (Schneider *et al.*, 2007). The map position of an EST which had been mapped previously (Schneider *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.

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; Figure 2). *BvLDL1* was mapped to position 10.90 cM on chromosome IX (data not shown).

2.4.3. *BvFLK* accelerates the time to bolting in Arabidopsis and complements the *flk1* mutation

Overexpression and complementation analyses in *A. thaliana* wild-type and mutant plants were employed to assess whether the function of autonomous pathway gene homologs is conserved between beet and Arabidopsis. *BvFLK* and *BvFVE1* were chosen as homologs 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 2).

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 2). 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.

Table 2: 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* mutant SALK_112850 (*flk1*).

Genotype	T1 generation					
	Overexpression in Col-0			Complementation in <i>flk-1</i>		
	Number of plants	DTB (mean ± standard deviation)	TNL (mean ± standard deviation)	Number of plants	DTB (mean ± standard deviation)	TNL (mean ± standard deviation)
35S:: <i>BvFLK</i>	32	29.59 ±2.43 A	10.59 ±1.83 A	25	32.00 ±2.10 A	13.08 ±1.55 A
endo:: <i>BvFLK</i>	15	30.00 ±2.56 A	11.20 ±1.52 A	9	34.89 ±3.79 A	13.33 ±2.29 A
35S:: <i>AtFLK</i>	10	30.90 ±2.08 A	12.00 ±1.94 B	4	31.50 ±4.20 A	10.57 ±1.26 A
Col-0	16	35.69 ±1.70 B	13.31 ±1.14 B	16	35.69 ±1.70 A	13.31 ±1.14 A
<i>flk-1</i> mutant	-	-	-	22	67.71 ±7.15 B	66.35 ±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
Genotype	T2 generation					
	Overexpression in Col-0			Complementation in <i>flk-1</i>		
	Number of plants	DTB (mean ± standard deviation)	TNL (mean ± standard deviation)	Number of plants	DTB (mean ± standard deviation)	TNL (mean ± standard deviation)
35S:: <i>BvFLK</i>	13	27.77 ±2.59 A	15.23 ±1.79 A	14	34.14 ±3.06 A	15.43 ±1.16 A
endo:: <i>BvFLK</i>	13	28.46 ±1.66 A	15.31 ±1.71 A	12	33.92 ±3.62 A	15.51 ±1.29 A
35S:: <i>AtFLK</i>	14	27.23 ±2.05 A	15.00 ±2.00 A	15	34.13 ±5.60 A	15.80 ±1.08 A
Col-0	17	37.88 ±1.50 B	17.53 ±1.01 B	17	37.88 ±1.50 A	17.53 ±1.01 A
<i>flk-1</i> mutant	-	-	-	17	69.82 ±4.63 B	67.82 ±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

^a Fisher'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')

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 five or more days 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; Suppl. Tab. 2).

In the remaining families all plants bolted five or more days 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.

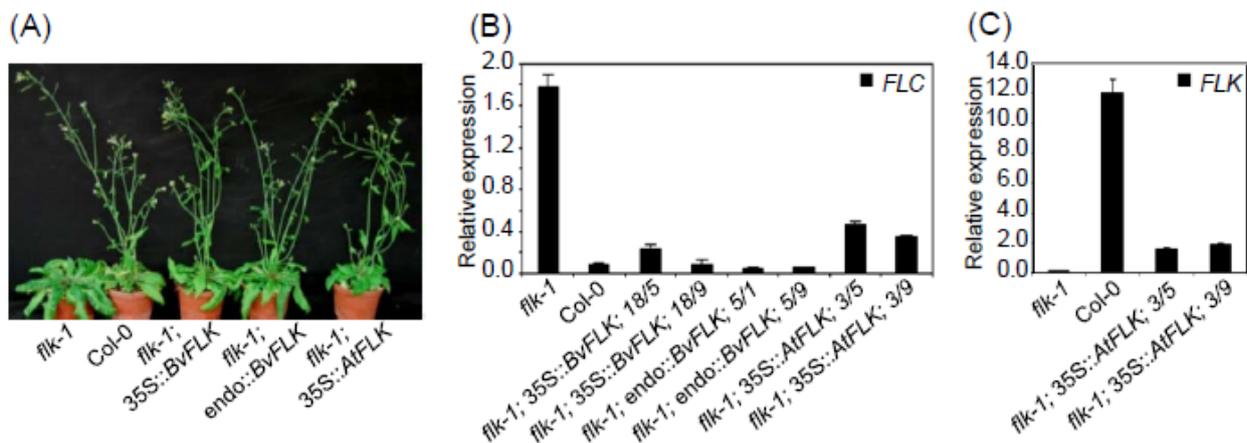


Figure 3: *BvFLK* complements the *A. thaliana flk1* mutant. (A) Phenotypes at 51 days after sowing of the *A. thaliana flk1* mutant, the ecotype Col-0, and the *flk1* mutant transformed with *BvFLK* driven by the CaMV 35S promoter (35S::*BvFLK*), *BvFLK* driven by the endogenous promoter of *BvFLK* 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 *flk1*, Col-0, and transgenic T3 plants carrying the 35S::*BvFLK*, *endo*::*BvFLK*, or 35S::*AtFLK* transgene in the *flk1* 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 *flk1*, Col-0, and transgenic 35S::*AtFLK* T3 plants. Expression levels in (B) and (C) were normalized against *GAPDH* and measured in triplicate.

2.4.4. *BvFLK* represses *FLC* expression in Arabidopsis

To further investigate 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 (Figure 3B). 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 (Figure 3C).

2.4.5. *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 (Suppl. Tab. 4, 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 or higher. 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 (Suppl. Tab. 4). In one exception, a T2 family which carried the 35Spro::*BvFVE1* transgene in the *fve-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; Suppl. Tab. 4). 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).

2.4.6. 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 (Figure 4A). 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 (Figure 4B). 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.

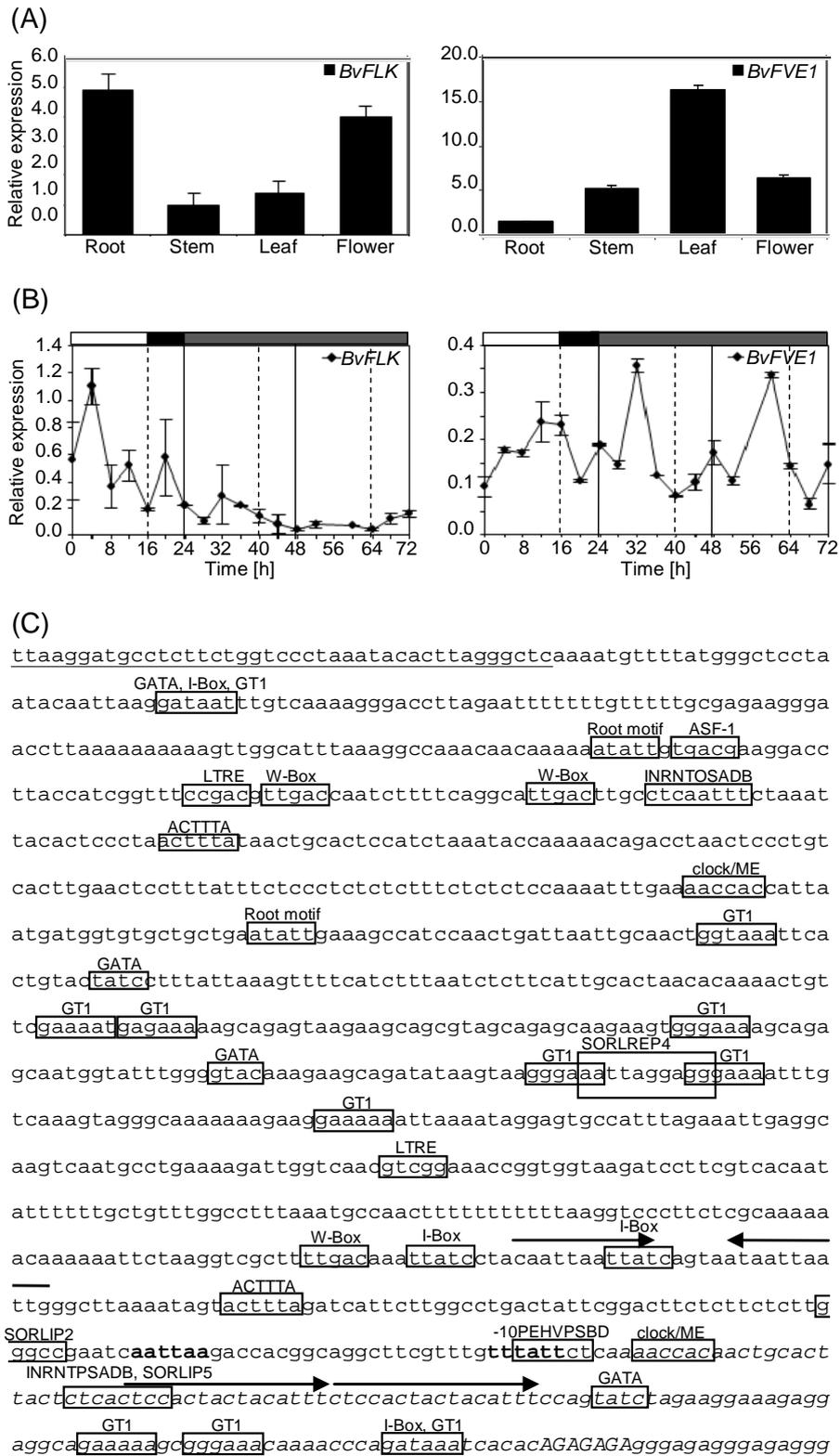


Figure 4: Expression of *BvFLK* and *BvFVE1* in *B. vulgaris*. (A) Expression across major plant organs in the biennial genotype A906001. Plants were vernalized and grown under long-day

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*. (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.

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 (Suppl. Fig. 2).

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 (-10PEHVPSPD) from a circadian clock regulated gene in barley (Thum *et al.*, 2001) (Figure 4C).

2.5. Discussion

The key regulatory genes of the vernalization and photoperiod pathways in Arabidopsis, *FLC* and *CO*, had been reported previously to have functionally related homologs 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* orthologs 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 1). 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 orthologs (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.dfc.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). Orthologs 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 homologs 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 homologs 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 homologs 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 Figure 1B). 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* homolog 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 *BvFL1* in *B. vulgaris* have yet to be experimentally verified. Nevertheless, the complementation data for both *BvFLK* in this study and *BvFL1* 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; Velej 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* ortholog 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 orthologs of *FLK* are present in non-angiosperm species including the gymnosperm *Piceasitchensis* and the lycophyte *Selaginella moellendorffii* (Suppl. Fig. 3A). For the rice ortholog 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 homolog, *OsFVE* (Figure 1B), 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 (Kenzior and Folk, 1998; Murzina *et al.*, 2008) make up most of the protein. The regions outside the

conserved domains vary considerably between *FVE* homologs. 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* homolog 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 1), which may be a paralog 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 Suppl. Fig. 4). 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* (Suppl. Fig. 4). 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 homolog of *FVE* in GenBank was identified. However, several other species were found to carry putative paralogous pairs (or groups) of *FVE* homologs, notably including two species in the *Malpighiales* order of dicotyledonous angiosperms (*Populus trichocarpa*, *PtFVE1 - 3*, and *Ricinus communis*, *RcFVE1 - 2*; Suppl. Fig. 3B, C), 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 paralogs underwent subfunctionalization.

Our 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 (or any of the other classical autonomous pathway genes) has not been reported to be itself 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 homologs 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).

Our survey of autonomous pathway genes provides the first evidence for evolutionary conservation of homologs 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.

2.6. Acknowledgements

The project is funded by the Deutsche Forschungsgemeinschaft under grant no. DFG JU 205/14-1. S. F. Abou-Elwafa is supported by a scholarship from the Ministry of Higher Education, Egypt. Tansy Chia and Effie Mutasa-Göttgens are supported by grants from the UK Biotechnology and Biological Sciences Research Council (BBSRC) and the British Beet Research Organisation (BBRO). We are grateful to Dr. Britta Schulz (KWS Saat AG), Dr. Georg Koch (formerly at Saatzucht Dieckmann GmbH & Co. KG) and Dr. Axel Schechert (StrubeResearch GmbH & Co. KG) for providing the K1 and D2 mapping populations, and we thank Cay Kruse, Kevin Sawford and Andrea Jennings for technical assistance.

2.7. References

1. Abegg, F. A. A genetic factor for the annual habit in beets and linkage relationship. 53, 493-511. 1936. J Agric Res.
2. Albert, V. A., Soltis, D. E., Carlson, J. E., Farmerie, W. G., Wall, P. K., Ilut, D. C., Solow, T. M., Mueller, L. A., Landherr, L. L., Hu, Y., Buzgo, M., Kim, S., Yoo, M. J., Frohlich, M. W., Perl-Treves, R., Schlarbaum, S. E., Bliss, B. J., Zhang, X., Tanksley, S. D., Oppenheimer, D. G., Soltis, P. S., Ma, H., dePamphilis, C. W., and Leebens-Mack, J. H., 2005: Floral gene resources from basal angiosperms for comparative genomics research. BMC.Plant Biol. **5**, 5.
3. Alonso, J. M., Stepanova, A. N., Leisse, T. J., Kim, C. J., Chen, H., Shinn, P., Stevenson, D. K., Zimmerman, J., Barajas, P., Cheuk, R., Gadrinab, C., Heller, C., Jeske, A., Koesema, E., Meyers, C. C., Parker, H., Prednis, L., Ansari, Y., Choy, N., Deen, H., Geralt, M., Hazari, N., Hom, E., Karnes, M., Mulholland, C., Ndubaku, R., Schmidt, I., Guzman, P., guilar-Henonin, L., Schmid, M., Weigel, D., Carter, D. E., Marchand, T., Risseeuw, E., Brogden, D., Zeko, A., Crosby, W. L., Berry, C. C., and Ecker, J. R., 2003: Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. Science **301**, 653-657.

4. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J., 1990: Basic local alignment search tool. *Journal of Molecular Biology* **215**, 403-410.
5. Amasino, R., 2004: Take a cold flower. *Nat Genet* **36**, 111-112.
6. Aukerman, M. J., Lee, I., Weigel, D., and Amasino, R. M., 1999: The *Arabidopsis* flowering-time gene *LUMINIDEPENDENS* is expressed primarily in regions of cell proliferation and encodes a nuclear protein that regulates *LEAFY* expression. *Plant J.* **18**, 195-203.
7. Ausin, I.,onso-Blanco, C., Jarillo, J. A., Ruiz-Garcia, L., and Martinez-Zapater, J. M., 2004: Regulation of flowering time by *FVE*, a retinoblastoma-associated protein. *Nat.Genet.* **36**, 162-166.
8. Baek, I. S., Park, H. Y., You, M. K., Lee, J. H., and Kim, J. K., 2008: Functional conservation and divergence of *FVE* genes that control flowering time and cold response in rice and *Arabidopsis*. *Mol.Cells* **26**, 368-372.
9. Baumann, K., De Paolis, A., Costantino, P., and Gualberti, G., 1999: The DNA binding site of the Dof protein NtBBF1 is essential for tissue-specific and auxin-regulated expression of the rolB oncogene in plants. *Plant Cell* **11**, 323-334.
10. Bäurle, I., Becker, H. C., and Dean, C., 2006: The timing of developmental transitions in plants. *Cell* **125**, 655-664.
11. Bäurle, I. and Dean, C., 2008: Differential interactions of the autonomous pathway RRM proteins and chromatin regulators in the silencing of *Arabidopsis* targets. *PLoS.ONE.* **3**, e2733.
12. Bäurle, I., Smith, L., Baulcombe, D. C., and Dean, C., 2007: Widespread role for the flowering-time regulators *FCA* and *FPA* in RNA-mediated chromatin silencing. *Science* **318**, 109-112.
13. Blazquez, M. A., Ahn, J. H., and Weigel, D., 2003: A thermosensory pathway controlling flowering time in *Arabidopsis thaliana*. *Nat.Genet.* **33**, 168-171.
14. Boss, P. K., Bastow, R. M., Mylne, J. S., and Dean, C., 2004: Multiple pathways in the decision to flower: enabling, promoting, and resetting. *Plant Cell* **16 Suppl**, S18-S31.
15. Boudry, P., Wieber, R., Saumitou-Laprade, P., Pillen, K., Van Dijk, H., and Jung, C., 1994: Identification of RFLP markers closely linked to the bolting gene *B* and their significance for the study of the annual habit in beets (*Beta vulgaris* L.). *Theoretical and Applied Genetics* **88**, 852-858.

16. Büttner, B., Abou-Elwafa, S. F., Zhang, W., Jung, C., and Muller, A. E., 2010: A survey of EMS-induced biennial *Beta vulgaris* mutants reveals a novel bolting locus which is unlinked to the bolting gene *B*. *Theor. Appl. Genet.*
17. Carpenter, C. D., Kreps, J. A., and Simon, A. E., 1994: Genes encoding glycine-rich *Arabidopsis thaliana* proteins with RNA binding motifs are influenced by cold treatment and an endogenous circadian-rhythm. *Plant Physiol.* **104**, 1015-1025.
18. Chan, A. P. and Etkin, L. D., 2001: Patterning and lineage specification in the amphibian embryo. *Current Topics in Developmental Biology*, 1-67. Academic Press.
19. Chaw, S. M., Chang, C. C., Chen, H. L., and Li, W. H., 2004: Dating the monocot–dicot divergence and the origin of core eudicots using whole chloroplast genomes. *Journal of Molecular Evolution* **58**, 424-441.
20. Cheng, Y., Kato, N., Wang, W., Li, J., and Chen, X., 2003: Two RNA binding proteins, *HEN4* and *HUAL*, act in the processing of *AGAMOUS* Pre-mRNA in *Arabidopsis thaliana*. *Developmental Cell* **4**, 53-66.
21. Chia, T. Y. P., Muller, A., Jung, C., and Mutasa-Gottgens, E. S., 2008: Sugar beet contains a large CONSTANS-LIKE gene family including a CO homologue that is independent of the early-bolting (*B*) gene locus. *J. Exp. Bot.* **59**, 2735-2748.
22. Clough, S. J. and Bent, A. F., 1998: Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *The Plant Journal* **16**, 735-743.
23. Colasanti, J. and Coneva, V., 2009: Mechanisms of floral induction in grasses: something borrowed, something new. *Plant Physiol.* **149**, 56-62.
24. Davies TJ, Barraclough TG, Chase MG, Soltis PS, Soltis DE, and Savolainen V. Darwin's abominable mystery: Insights from a supertree of the angiosperms
1020. *PNAS* **101**, 1904-1909. 2004.
25. Dechyeva, D. and Schmidt, T., 2006: Molecular organization of terminal repetitive DNA in Beta species. *Chromosome Research* **14**, 881-897.
26. Distelfeld, A., Li, C., and Dubcovsky, J., 2009: Regulation of flowering in temperate cereals. *Current Opinion in Plant Biology* **12**, 178-184.

27. Domagalska, M. A., Schomburg, F. M., Amasino, R. M., Vierstra, R. D., Nagy, F., and Davis, S. J., 2007: Attenuation of brassinosteroid signaling enhances FLC expression and delays flowering. *Development dev.*
28. Earley, K. W., Haag, J. R., Pontes, O., Opper, K., Juehne, T., Song, K., and Pikaard, C. S., 2006: Gateway-compatible vectors for plant functional genomics and proteomics. *Plant J.* **45**, 616-629.
29. Edwards, K. D., Anderson, P. E., Hall, A., Salathia, N. S., Locke, J. C., Lynn, J. R., Straume, M., Smith, J. Q., and Millar, A. J., 2006: *FLOWERING LOCUS C* mediates natural variation in the high-temperature response of the Arabidopsis circadian clock. *Plant Cell* **18**, 639-650.
30. El-Mezawy, A., Dreyer, F., Jacobs, G., and Jung, C., 2002: High-resolution mapping of the bolting gene B of sugar beet. *Theoretical and Applied Genetics* **105**, 100-105.
31. Elmayan, T. and Tepfer, M., 1995: Evaluation in tobacco of the organ specificity and strength of the rold promoter, domain-A of the 35S promoter and the 35S(2) promoter. *Transgenic Research* **4**, 388-396.
32. Fowler, S. G., Cook, D., and Thomashow, M. E., 2005: Low temperature induction of Arabidopsis CBF1, 2, and 3 is gated by the circadian clock. *Plant Physiol.* **137**, 961-968.
33. Franklin, K. A. and Whitelam, G. C., 2007: Light-quality regulation of freezing tolerance in Arabidopsis thaliana. *Nat Genet* **39**, 1410-1413.
34. Frohman MA, Dush MK, and Martin GR. Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer
932. *PNAS* 85(23), 8998-9002. 1988.
35. Fusada, N., Masuda, T., Kuroda, H., Shimada, H., Ohta, H., and Takamiya, K., 2005: Identification of a novel Cis-element exhibiting cytokinin-dependent protein binding in vitro in the 5'-region of NADPH-protochlorophyllide oxidoreductase gene in cucumber. *Plant Molecular Biology* **59**, 631-645.
36. Gilmartin, P. M., Sarokin, L., Memelink, J., and Chua, N. H., 1990: Molecular Light Switches for Plant Genes. *Plant Cell* **2**, 369-378.
37. Greenup, A., Peacock, W. J., Dennis, E. S., and Trevaskis, B., 2009: The molecular biology of seasonal flowering-responses in Arabidopsis and the cereals. *Annals of Botany* mcp063.

38. Harmer, S. L., Hogenesch, J. B., Straume, M., Chang, H. S., Han, B., Zhu, T., Wang, X., Kreps, J. A., and Kay, S. A., 2000: Orchestrated transcription of key pathways in Arabidopsis by the circadian clock. *Science* **290**, 2110-2113.
39. Harmer, S. L. and Kay, S. A., 2005: Positive and negative factors confer phase-specific circadian regulation of transcription in Arabidopsis. *Plant Cell* **17**, 1926-1940.
40. He, Y., Michaels, S. D., and Amasino, R. M., 2003: Regulation of flowering time by histone acetylation in Arabidopsis. *Science* **302**, 1751-1754.
41. He, Y. H. and Amasino, R. M., 2005: Role of chromatin modification in flowering-time control. *Trends in Plant Science* **10**, 30-35.
42. Hecht, V., Foucher, F., Ferrandiz, C., Macknight, R., Navarro, C., Morin, J., Vardy, M. E., Ellis, N., Beltran, J. P., Rameau, C., and Weller, J. L., 2005: Conservation of Arabidopsis flowering genes in model legumes. *Plant Physiol* **137**, 1420-1434.
43. Heintzen, C., Melzer, S., Fischer, R., Kappeler, S., Apel, K., and Staiger, D., 1994: A light-entrained and temperature-entrained circadian clock controls expression of transcripts encoding nuclear proteins with homology to RNA-binding proteins in meristematic tissue. *Plant Journal* **5**, 799-813.
44. Hennig, L., Taranto, P., Walser, M., Schonrock, N., and Grissem, W., 2003: Arabidopsis MS11 is required for epigenetic maintenance of reproductive development. *Development* **130**, 2555-2565.
45. Herwig, R., Schulz, B., Weisshaar, B., Hennig, S., Steinfath, M., Drungowski, M., Stahl, D., Wruck, W., Menze, A., O'Brien, J., Lehrach, H., and Radelof, U., 2002: Construction of a 'unigene' cDNA clone set by oligonucleotide fingerprinting allows access to 25 000 potential sugar beet genes. *Plant J.* **32**, 845-857.
46. Higo, K., Ugawa, Y., Iwamoto, M., and Korenaga, T., 1999: Plant cis-acting regulatory DNA elements (PLACE) database: *Nucleic Acids Research* **27**, 297-300.
47. Hohmann, U., Jacobs, G., Telgmann, A., Gaafar, R. M., Alam, S., and Jung, C., 2003: A bacterial artificial chromosome (BAC) library of sugar beet and a physical map of the region encompassing the bolting gene B. *Molecular Genetics and Genomics* **269**, 126-136.
48. Hudson, M. E. and Quail, P. H., 2003: Identification of Promoter Motifs Involved in the Network of Phytochrome A-Regulated Gene Expression by Combined Analysis of Genomic Sequence and Microarray Data. *Plant Physiol.* **133**, 1605-1616.

49. Jang, Y. H., Park, H. Y., Kim, S. K., Lee, J. H., Suh, M. C., Chung, Y. S., Paek, K. H., and Kim, J. K., 2009: Survey of Rice Proteins Interacting With OsFCA and OsFY Proteins Which Are Homologous to the Arabidopsis Flowering Time Proteins, FCA and FY. *Plant Cell Physiol.* **50**, 1479-1492.
50. Jiang, D., Yang, W., He, Y., and Amasino, R. M., 2007: Arabidopsis relatives of the human lysine specific demethylase1 repress the expression of *FWA* and *FLOWERING LOCUS C* and thus promote the floral transition. *Plant Cell tpc.*
51. Jin, J. B., Jin, Y. H., Lee, J., Miura, K., Yoo, C. Y., Kim, W. Y., Van Oosten, M., Hyun, Y., Somers, D. E., Lee, I., Yun, D. J., Bressan, R. A., and Hasegawa, P. M., 2008: The SUMO E3 ligase, AtSIZ1, regulates flowering by controlling a salicylic acid-mediated floral promotion pathway and through affects on FLC chromatin structure. *Plant Journal* **53**, 530-540.
52. Jung, C. and Müller, A. E., 2009: Flowering time control and applications in plant breeding
1048. *Trends in Plant Science* **14**, 563-573.
53. Kenzior, A. L. and Folk, W. R., 1998: AtMSI4 and RbAp48 WD-40 repeat proteins bind metal ions. *FEBS Lett.* **440**, 425-429.
54. Kim, H. J., Hyun, Y., Park, J. Y., Park, M. J., Park, M. K., Kim, M. D., Kim, H. J., Lee, M. H., Moon, J., Lee, I., and Kim, J., 2004: A genetic link between cold responses and flowering time through FVE in Arabidopsis thaliana. *Nature Genetics* **36**, 167-171.
55. Kim, S. Y., Yu, X., and Michaels, S. D., 2008: Regulation of Constans and Flowering Locus T Expression in Response to Changing Light Quality. *Plant Physiol.* **148**, 269-279.
56. Koornneef, M., Blankestijndevries, H., Hanhart, C., Soppe, W., and Peeters, T., 1994: The phenotype of some late-flowering mutants is enhanced by a locus on chromosome-5 that is not effective in the Landsberg Erecta wild-type. *Plant Journal* **6**, 911-919.
57. Kosambi, D. D. The estimation of map distances from recombination values. *Ann Eug* **12**, 172-175. 1944.
58. Krichevsky, A., Gutgarts, H., Kozlovsky, S. V., Tzfira, T., Sutton, A., Sternglanz, R., Mandel, G., and Citovsky, V., 2007: C2H2 zinc finger-SET histone methyltransferase is a plant-specific chromatin modifier. *Dev.Biol.* **303**, 259-269.
59. Lee, I., Aukerman, M. J., Gore, S. L., Lohman, K. N., Michaels, S. D., Weaver, L. M., John, M. C., Feldmann, K. A., and Amasino, R. M., 1994:

- Isolation of LUMINIDEPENDENS: A Gene Involved in the Control of Flowering Time in Arabidopsis. *Plant Cell* **6**, 75-83.
60. Lee, J. H., Cho, Y. S., Yoon, H. S., Suh, M. C., Moon, J., Lee, I., Weigel, D., Yun, C. H., and Kim, J. K., 2005: Conservation and divergence of FCA function between Arabidopsis and rice. *Plant Mol. Biol.* **58**, 823-838.
 61. Lim, M. H., Kim, J., Kim, Y. S., Chung, K. S., Seo, Y. H., Lee, I., Kim, J., Hong, C. B., Kim, H. J., and Park, C. M., 2004: A New Arabidopsis Gene, FLK, Encodes an RNA Binding Protein with K Homology Motifs and Regulates Flowering Time via FLOWERING LOCUS C. *Plant Cell* **16**, 731-740.
 62. Liu, F., Marquardt, S., Lister, C., Swiezewski, S., and Dean, C., 2010: Targeted 3' processing of antisense transcripts triggers Arabidopsis FLC chromatin silencing. *Science* **327**, 94-97.
 63. Lu, Q., Xu, Z. K., and Song, R. T., 2006: OsFY, a homolog of AtFY, encodes a protein that can interact with OsFCA-gamma in rice (*Oryza sativa* L.). *Acta Biochimica et Biophysica Sinica* **38**, 492-499.
 64. Macknight, R., Bancroft, I., Page, T., Lister, C., Schmidt, R., Love, K., Westphal, L., Murphy, G., Sherson, S., Cobbett, C., and Dean, C., 1997: FCA, a gene controlling flowering time in Arabidopsis, encodes a protein containing RNA-binding domains. *Cell* **89**, 737-745.
 65. Meier, U. (2001) Growth stages of mono- and dicotyledonous plants. Phenological growth stages and BBCH-identification keys of beet. Federal Biological Research Centre for Agriculture and Forestry.
 66. Mena, M., Cejudo, F. J., Isabel-Lamonedaa, I., and Carbonero, P., 2002: A role for the DOF transcription factor BPBF in the regulation of gibberellin-responsive genes in barley aleurone. *Plant Physiol.* **130**, 111-119.
 67. Mersereau, M., Pazour, G. J., and Das, A., 1990: Efficient transformation of *Agrobacterium tumefaciens* by electroporation. *Gene* **90**, 149-151.
 68. Michaels, S. D. and Amasino, R. M., 2001: Loss of *FLOWERING LOCUS C* activity eliminates the late-flowering phenotype of *FRIGIDA* and autonomous pathway mutations but not responsiveness to vernalization. *Plant Cell* **13**, 935-941.
 69. Michaels, S. D., 2009: Flowering time regulation produces much fruit. *Current Opinion in Plant Biology* **12**, 75-80.
 70. Mockler, T. C., Yu, X., Shalitin, D., Parikh, D., Michael, T. P., Liou, J., Huang, J., Smith, Z., Alonso, J. M., Ecker, J. R., Chory, J., and Lin, C., 2004:

- Regulation of flowering time in Arabidopsis by K homology domain proteins. *Proc.Natl.Acad.Sci.U.S.A* **101**, 12759-12764.
71. Morel, P., Trehin, C., Breuil-Broyer, S., and Negrutiu, I., 2009: Altering *FVE/MSI4* results in a substantial increase of biomass in Arabidopsis – the functional analysis of an ontogenesis accelerator. *Molecular Breeding* **23**, 239-257.
 72. Mouhu, K., Hytonen, T., Folta, K., Rantanen, M., Paulin, L., Auvinen, P., and Elomaa, P., 2009: Identification of flowering genes in strawberry, a perennial SD plant. *BMC Plant Biology* **9**.
 73. Munerati, O. L'eredit`a della tendenza alla annualit`a nellacomune barbabietola coltivata. *Ztschr Z`uchtung, Reihe A,Pflanzenz`uchtung* **17**, 84-89. 1931
 74. Murzina, N. V., Pei, X. Y., Zhang, W., Sparkes, M., Vicente-Garcia, J., Pratap, J. V., McLaughlin, S. H., Ben-Shahar, T. R., Verreault, A., Luisi, B. F., and Laue, E. D., 2008: Structural Basis for the Recognition of Histone H4 by the Histone-Chaperone RbAp46. *Structure* **16**, 1077-1085.
 75. Nag, R., Maity, M. K., and Dasgupta, M., 2005: Dual DNA binding property of ABA insensitive 3 like factors targeted to promoters responsive to ABA and auxin. *Plant Molecular Biology* **59**, 821-838.
 76. Nakamura, M., Tsunoda, T., and Obokata, J., 2002: Photosynthesis nuclear genes generally lack TATA-boxes: a tobacco photosystem I gene responds to light through an initiator. *Plant Journal* **29**, 1-10.
 77. Overbeek, R., Fonstein, M., D'Souza, M., Pusch, G. D., and Maltsev, N., 1999: The use of gene clusters to infer functional coupling. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 2896-2901.
 78. Pater, S., Hensgens, L. A. M., and Schilperoort, R. A., 1990: Structure and expression of a light-inducible shoot-specific rice gene. *Plant Molecular Biology* **15**, 399-406.
 79. Phelps-Durr, T. L., Thomas, J., Vahab, P., and Timmermans, M. C. P., 2005: Maize rough sheath2 and its Arabidopsis orthologue ASYMMETRIC LEAVES1 interact with HIRA, a predicted histone chaperone, to maintain knox gene silencing and determinacy during organogenesis. *Plant Cell* **17**, 2886-2898.
 80. Pierleoni, A., Martelli, P. L., Fariselli, P., and Casadio, R., 2006: BaCellO: a balanced subcellular localization predictor. *Bioinformatics* **22**, e408-e416.

81. Pruneda-Paz, J. L. and Kay, S. A., 2010: An expanding universe of circadian networks in higher plants. *Trends in Plant Science* **15**, 259-265.
82. Quesada, V., Dean, C., and Simpson, G. G., 2005: Regulated RNA processing in the control of Arabidopsis flowering. *Int.J.Dev.Biol.* **49**, 773-780.
83. Quesada, V., Macknight, R., Dean, C., and Simpson, G. G., 2003: Autoregulation of FCA pre-mRNA processing controls Arabidopsis flowering time. *EMBO J.* **22**, 3142-3152.
84. Reeves, P. A., He, Y., Schmitz, R. J., Amasino, R. M., Panella, L. W., and Richards, C., 2006: Evolutionary conservation of the FLC mediated vernalization response: evidence from the sugar beet (*Beta vulgaris*). *Genetics*.
85. Remay, A., Lalanne, D., Thouroude, T., Le Couviour, F., Hibrand-Saint Oyant, L., and Foucher, F., 2009: A survey of flowering genes reveals the role of gibberellins in floral control in rose. *Theoretical and Applied Genetics* **119**, 767-781.
86. Ripoll, J. J., Rodriguez-Cazorla, E., Gonzalez-Reig, S., Andujar, A.,onso-Cantabrana, H., Perez-Amador, M. A., Carbonell, J., Martinez-Laborda, A., and Vera, A., 2009: Antagonistic interactions between Arabidopsis K-homology domain genes uncover PEPPER as a positive regulator of the central floral repressor FLOWERING LOCUS C. *Dev.Biol.*
87. Salathia, N., Davis, S. J., Lynn, J. R., Michaels, S. D., Amasino, R. M., and Millar, A. J., 2006: FLOWERING LOCUS C-dependent and -independent regulation of the circadian clock by the autonomous and vernalization pathways. *BMC.Plant Biol.* **6**, 10.
88. Sanda, S. L. and Amasino, R. M., 1996: Interaction of FLC and late-flowering mutations in Arabidopsis thaliana. *Mol.Gen.Genet.* **251**, 69-74.
89. Santi, L., Wang, Y. M., Stile, M. R., Berendzen, K., Wanke, D., Roig, C., Pozzi, C., Muller, K., Muller, J., Rohde, W., and Salamini, F., 2003: The GA octodinucleotide repeat binding factor BBR participates in the transcriptional regulation of the homeobox gene Bkn3. *Plant Journal* **34**, 813-826.
90. Schneider, K., Kulosa, D., Soerensen, T., M+Âhring, S., Heine, M., Durstewitz, G., Polley, A., Weber, E., Jamsari, Jamsari, Lein, J., Hohmann, U., Tahiro, E., Weisshaar, B., Schulz, B., Koch, G., Jung, C., and Ganal, M., 2007: Analysis of DNA polymorphisms in sugar beet (*Beta vulgaris* L.) and development of an SNP-based map of expressed genes. *Theoretical and Applied Genetics* **115**, 601-615.

91. Schomburg, F. M., Patton, D. A., Meinke, D. W., and Amasino, R. M., 2001: FPA, a gene involved in floral induction in Arabidopsis, encodes a protein containing RNA-recognition motifs. *Plant Cell* **13**, 1427-1436.
92. Schulte, D., Cai, D., Kleine, M., Fan, L., Wang, S., and Jung, C., 2006: A complete physical map of a wild beet (*Beta procumbens*) translocation in sugar beet. *Mol.Genet.Genomics* **275**, 504-511.
93. Simpson, G. G., 2004: The autonomous pathway: epigenetic and post-transcriptional gene regulation in the control of Arabidopsis flowering time. *Curr.Opin.Plant Biol.* **7**, 570-574.
94. Simpson, G. G., Quesada, V., Henderson, I. R., Dijkwel, P. P., Macknight, R., and Dean, C., 2004: RNA processing and Arabidopsis flowering time control. *Biochem.Soc.Trans.* **32**, 565-566.
95. Spensley, M., Kim, J. Y., Picot, E., Reid, J., Ott, S., Helliwell, C., and Carre, I. A., 2009: Evolutionarily Conserved Regulatory Motifs in the Promoter of the Arabidopsis Clock Gene LATE ELONGATED HYPOCOTYL. *Plant Cell* **21**, 2606-2623.
96. Streitner, C., Danisman, S., Wehrle, F., Schoning, J. C., Alfano, J. R., and Staiger, D., 2000: The small glycine-rich RNA-binding protein AtGRP7 promotes floral transition in Arabidopsis thaliana. *The Plant Journal*.
97. Sutoh, K. and Yamauchi, D., 2003: Two cis-acting elements necessary and sufficient for gibberellin-upregulated proteinase expression in rice seeds. *Plant Journal* **34**, 635-645.
98. Terzaghi, W. and Cashmore, A., 1995: Light-regulated transcription. *Annu Rev Plant Physiol Plant Mol Biol* **46**, 445-474.
99. Thum, K. E., Kim, M., Morishige, D. T., Eibl, C., Koop, H. U., and Mullet, J. E., 2001: Analysis of barley chloroplast psbD light-responsive promoter elements in transplastomic tobacco. *Plant Molecular Biology* **47**, 353-366.
100. Turck, F., Fornara, F., and Coupland, G., 2008: Regulation and identity of florigen: *FLOWERING LOCUS T* moves center stage. *Annu Rev Plant Biol* **59**, 573-594.
101. Van Ooijen and Voorrips. *JoinMap*® 3.0: Software for the calculation of genetic linkage maps. Plant Research International, Wageningen, the Netherlands . 2001.
102. van, N. S., Muszynski, M., Briggs, K., and Amasino, R. M., 2000: Characterization of a gene from *Zea mays* related to the Arabidopsis flowering-time gene LUMINIDEPENDENS. *Plant Mol.Biol.* **44**, 107-122.

103. Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., and Speleman, F., 2002: Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology* **3**, research0034.
104. Veley, K. M. and Michaels, S. D., 2008: Functional redundancy and new roles for genes of the autonomous floral-promotion pathway. *Plant Physiol.* **147**, 682-695.
105. Yang, H. W. and Yu, T. S., 2010: Arabidopsis floral regulators *FVE* and *AGL24* are phloem-mobile RNAs. *Botanical Studies* **51**, 17-26.
106. Zhou, D. X., 1999: Regulatory mechanism of plant gene transcription by GT-elements and GT-factors. *Trends in Plant Science* **4**, 210-214.

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

Published in Theoretical and Applied Genetics, 2010

3.1. 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.

3.2. 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* (*APETALA1*). *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 *Beta vulgaris*, reverse genetic approaches have identified an *FLC*-like gene (*BvFL1*; Reeves *et al.*, 2007) and a *CO*-like gene (*BvCOL1*; Chia *et al.*, 2008). Consistent with a conserved role in repression of flowering, expression of *BvFL1* in sugar beet was shown to be down-regulated during vernalization, and overexpression of *BvFL1* in an early-flowering *flc* mutant delayed flowering in Arabidopsis (Reeves *et al.*, 2007). Overexpression of *BvCOL1* 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 *BvFL1* and *BvCOL1* also revealed marked differences to *FLC* and *CO*, respectively. In particular, and in contrast to the respective genes in Arabidopsis, repression of *BvFL1* expression is not maintained after vernalization but reverts to pre-vernalization levels, and the diurnal expression profile of *BvCOL1* 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 (Koorneef *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 (Figure 5a). 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 (Figure 5b). 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*.

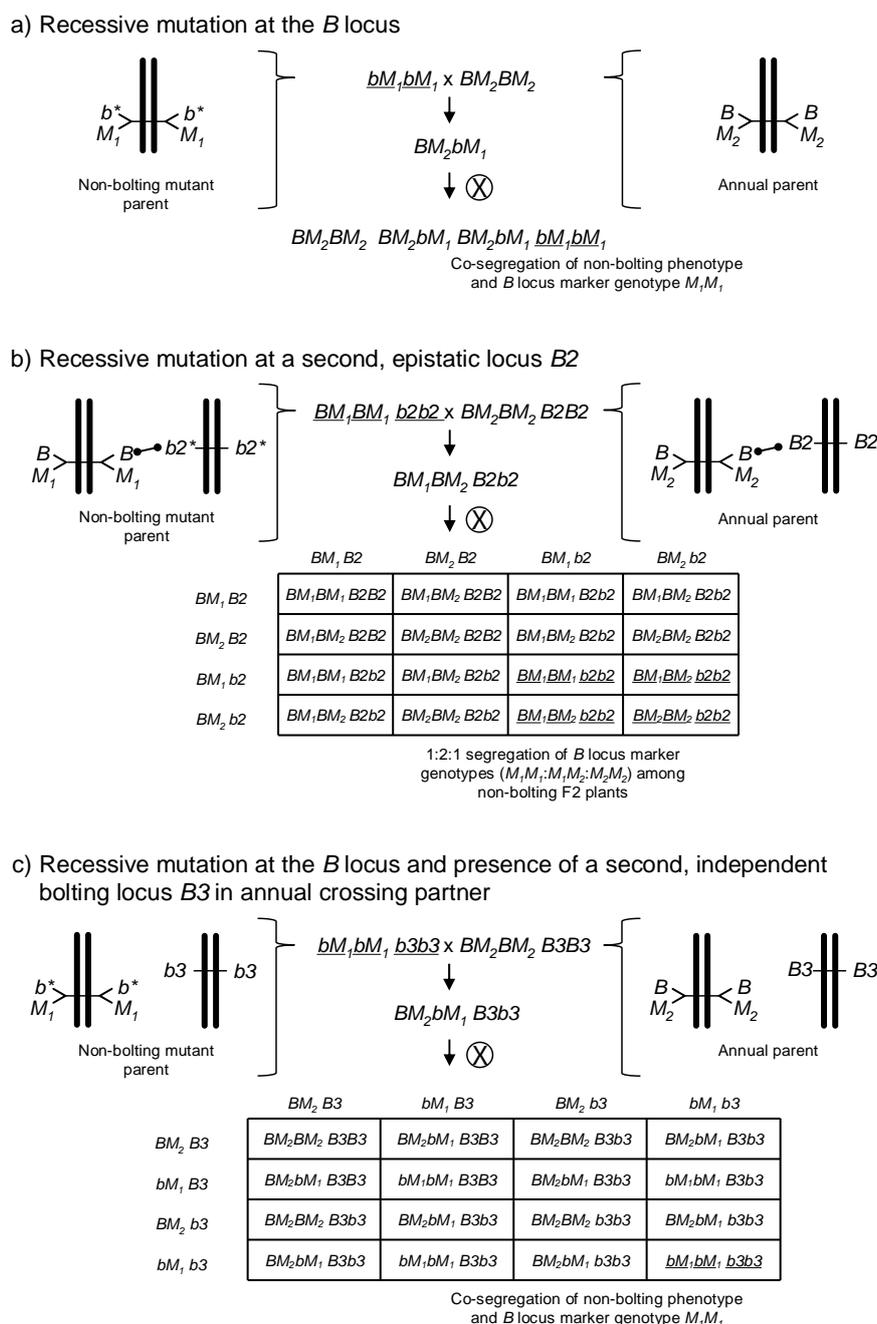


Figure 5: Test for allelism between EMS mutations and the *B* locus. The expected segregation of bolting phenotypes and *B* locus marker genotypes is shown for crosses between 'non-bolting' (biennial) EMS mutants and annual crossing partners. Three models are shown: a) The mutation

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, F_1 and F_2 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.

3.3. Materials and Methods

3.3.1. Plant material

'Non-bolting', biennial mutants (Table 3) 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 (Table 3). 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, Mechelke *et al.* 1992). Three mutant families have green hypocotyls (000855, 011763 and 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.

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 overwinter in the greenhouse without vernalization, and selfed to produce F₂ seed (Table 3).

3.3.2. 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;(Uwe Meier, H.Bleiholder *et al.*, 2001;Hohmann, Jacobs *et al.*, 2005)). Phenotypes were scored until November 19, 2007.

Table 3: Biennial EMS mutants and generation of F₂ populations.

Mutant family (M ₂) ¹	Mutant parent of cross	Annual parent of cross	Seed parent of F ₁ cross progeny	F ₁ plant selfed	F ₂ population
000855	020415/15 (M3) ²	991971/2	020415/15	061365/1	070047
011763	056822/4 (M4)	991971/9	056822/4	061392/1	070056
011373	031823/14 (M3)	991971/11	031823/14	061398/1	070058
000192	020416/14 (M3)	991971/3	991971/3	061373/3	070292
000192	020417/16 (M3)	930190/13	020417/16	061460/1	070081

¹ M₂ family numbers correspond to the mutant nomenclature used by Hohmann *et al.*, 2005

² Six-digit numbers are seed codes, numbers separated by slashes indicate individual plants within a population. The numbers in parentheses indicate mutant generation numbers.

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.

3.3.3. 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 5ng/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 Biosences, Lincoln, USA). 38 primer combinations which allowed detection of one to ten AFLPs each were chosen for genotyping.

3.3.4. 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).

3.4. Results

3.4.1. 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 (Table 3). 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 behavior with molecular markers located at the *B* locus.

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 (Figure 6; Table 4).

Table 4: Phenotypic segregation for bolting behavior 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) ¹	χ^2 test for H ₀ = 15:1 (bolting vs. non-bolting) ²
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**

¹ H₀, null hypothesis for monogenic, dominant-recessive trait

² H₀, null hypothesis for digenic, dominant-recessive trait

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

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 (Table 4). 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 (Table 4), 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.

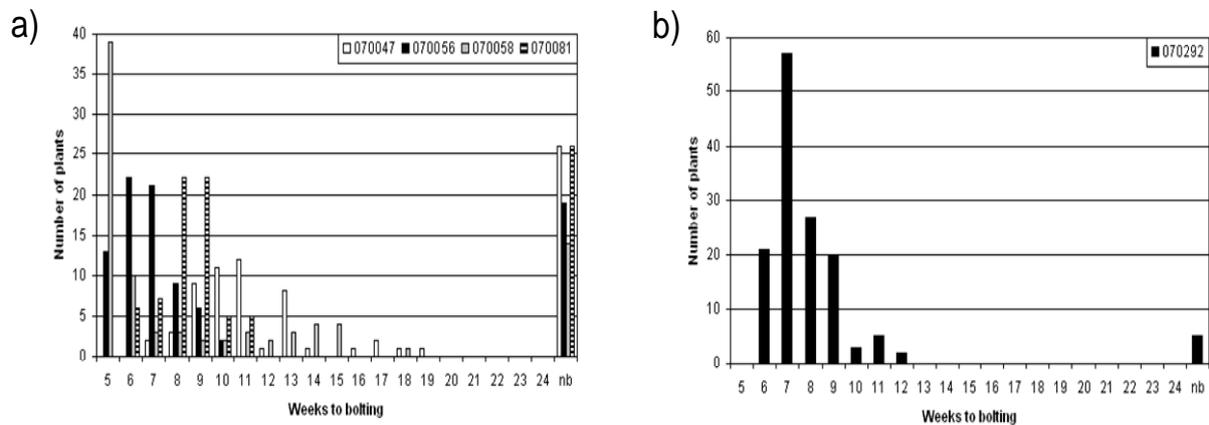


Figure 6: Phenotypic segregation for bolting behavior in F₂ populations. 'Weeks to bolting' indicates the week, counted from the date of sowing, in which stem elongation began (i.e. week 5 corresponds to 29 to 35 days to bolting, etc.; s. Suppl. Table 3). 'nb' indicates plants which had not bolted by 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).

3.4.2. 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 F₂ 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.

3.4.2.1. Co-segregation analysis in populations 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_1M_1 , M_1M_2 and M_2M_2 marker genotypes among the bolting individuals of an F₂ population, and M_1M_1 , M_1M_2 and M_2M_2 marker genotypes among the non-bolting individuals (Table 5). 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 ('*B2*', s. below) which is not genetically linked to the *B* locus.

3.4.2.2. Co-segregation analysis in populations 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 (Figure 5c). 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 *B3*. 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 *B3* 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_1M_1), and that none of the other *B* locus marker genotypes (M_1M_2 , M_2M_2) occur among non-bolting individuals. The result of genotyping F₂ population EW4a with the *B* locus marker GJ1013c690b came close to these predictions (Table 5). 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 (Table 5).

Table 5: Co-segregation analysis of bolting behavior and B locus marker genotypes.

F ₂ population	B locus marker	Total number of plants genotyped	Bolting			Non-bolting			χ^2 test for H ₀ ; 3:6:3:1 :2:1 ²	χ^2 test for H ₀ ; 3:8:4:1 ³
			<i>M</i> ₁ <i>M</i> ₁ ¹	<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. ⁴
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.

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

² Expected ratio for a monogenic, dominant-recessive trait and independent segregation of phenotype and B locus marker

³ Expected ratio for digenic, dominant-recessive trait, co-segregation of phenotype and B locus marker

⁴ n.a., not applicable

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

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*₁*M*₁, bolting/*M*₁*M*₂, bolting/*M*₂*M*₂ and non-bolting/*M*₁*M*₁) was not rejected by χ^2 analysis (Table 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 F₂ 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 F₂ 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 (Table 5). 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.

3.4.3. Variation in bolting time among annuals in F₂ populations

Besides annuality, all populations were phenotyped for bolting time of annual individuals (Figure 6, Suppl. Tab. 3). In populations EW1, EW2 and EW4b, annual plants were approximately normally distributed but the position of the maxima differed between populations (Figure 6a). 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; Figure 6a). The frequency distribution in population EW4a is similar to normal but positively skewed, with bolting in some plants being somewhat delayed (Figure 6b). 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 (Table 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 6: Analysis of variance among B locus marker genotypes in annual subpopulations.

Marker genotype ¹	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. ^{2,3}
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} ⁴	n.a.	n.a.	n.a.	3.53	n.a.

¹ B locus markers are as indicated in Tab. 3. The B locus marker analyzed in population EW4a is GJ1013c690b.

² n.a., not applicable

³ Population EW4b did not comprise any *MIMI* individuals and ANOVA was not performed.

⁴ 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'.

3.4.4. 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. Figure 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) (Figure 7). 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; Figure 7), has a LOD score of 46.81 and explains 87.0% of the observed phenotypic variation.

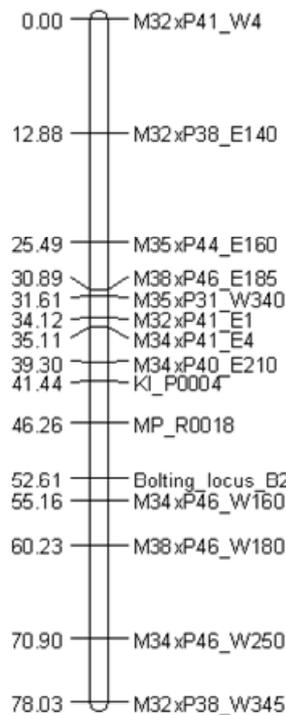


Figure 7: Genetic map position of a major locus for annual bolting on chromosome IX. The map comprises the bolting locus *B2*, twelve AFLP markers, and two co-dominant SNP-based anchor markers (KI_P0004 and MP_R0018). Genetic distances in centiMorgan are given on the left, marker names on the right. The vertical black bar indicates the confidence interval of a quantitative trait locus co-localizing with *B2*.

3.4.5. Co-segregation analysis of bolting behavior 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 (Table 7). 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).

Table 7: 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 $H_0 = 3:6:3:1:2:1^2$
			M_1M_1 ¹	M_1M_2	M_2M_2	M_1M_1	M_1M_2	M_2M_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

¹ Marker alleles M_1 and M_2 are derived from the mutant parent or the annual parent, respectively.

² Expected ratio for monogenic, dominant-recessive trait, independent segregation of phenotype and MP_R0018

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

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 (Table 8). 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.

3.5. 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 *Beta vulgaris*. Bolting control involves at least two other loci not linked to *B* (*B2*, *B3*), one of which (*B2*)

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 (*B3*) which acts independently of the *B* locus. These findings will be further discussed in the following paragraphs.

Table 8: Analysis of variance among chromosome IX marker genotypes in annual subpopulations.

Marker genotype ¹	Mean ± standard deviation of days to bolting			
	EW1	EW2	EW3	EW4a
<i>M₁M₁</i>	69.00 ±9.18	48.50 ±4.90	83.12 ±25.09 A	46.73 ±6.44
<i>M₁M₂</i>	80.04 ±21.20	44.53 ±8.56	40.66 ±11.87 B	51.26 ±9.69
<i>M₂M₂</i>	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} ²	n.a. ³	n.a.	10.75	n.a.

¹ Chromosome IX markers are as indicated in Table 6.

² 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

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, *B2*, 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 *B2* constitutes a major genetic locus controlling annual bolting in *Beta 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 (Figure 5b). 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_1M_1) at this marker locus occurs preferentially among non-bolting plants (Table 7). *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 F₂ 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 F₂ populations, EW1 and EW3, behaved similarly to population EW2 insofar as bolting phenotypes and *B* locus marker genotypes segregated independently (Table 5). 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_1M_1) (Table 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_1M_1 genotype among bolting plants on average bolted substantially and highly significantly later than individuals of the other two

genotypic classes at this locus (Table 5). 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 Figure 6a). Out of 17 bolting individuals of the M_1M_1 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 (Figure 7). 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); Table 7). In population EW3, the corresponding number of recombination events is 18 (among 90 plants analyzed; Table 7), 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. Table 3, Table 7), 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 (Table 4). The field data for populations EW2 and EW3 (Table 4) 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 (Table 8).

ii) Evidence that the *B* locus affects bolting behavior and is mutated in one of the four mutant families analyzed (000192, s. Table 3) 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 (Table 5). 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 (Table 6). 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 F₂ 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 (Table 5) 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 (Figure 5a). 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 (Figure 5c). 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; Koorneef *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 F₂/F₃ 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.

3.6. Acknowledgements

The project was funded by the Deutsche Forschungsgemeinschaft under grant no. DFG JU 205/14-1. S. F. Abou-Elwafa is supported by a scholarship from the Ministry of Higher Education, Egypt. We thank Uwe Hohmann for propagation of mutants, Gretel Schulze-Buxloh for marker sequence information, Monika Bruisch and Erwin Danklefsen for technical assistance in the field and greenhouse, and Martina Bach and Monika Dietrich for technical assistance in the laboratory.

3.7. References

1. Abe, J., Guan, G. P., and Shimamoto, Y., 1997: A gene complex for annual habit in sugar beet (*Beta vulgaris* L.). *Euphytica* **94**, 129-135.

2. Abegg, F. A. A genetic factor for the annual habit in beets and linkage relationship. *53*, 493-511. 1936. *J Agric Res*.
3. Alonso-Blanco, C., Aarts, M. G., Bentsink, L., Keurentjes, J. J., Reymond, M., Vreugdenhil, D., and Koornneef, M., 2009: What has natural variation taught us about plant development, physiology, and adaptation?. *Plant Cell* **21**, 1877-1896.
4. Angiosperm Phylogeny Group. An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG III. *161*, 105-121. 2009. *Botanical Journal of the Linnean Society*.
5. Barzen, E., Mechelke, W., Ritter, E., Seitzer, J. F., and Salamini, F., 1992: RFLP markers for sugar beet breeding: chromosomal linkage maps and location of major genes for rhizomania resistance, monogermity and hypocotyl colour. *The Plant Journal* **2**, 601-611.
6. Baurle, I. and Dean, C., 2006: The timing of developmental transitions in plants. *Cell* **125**, 655-664.
7. Boudry, P., McCombie, H., and Van Dijk, H., 2002: Vernalization requirement of wild beet *Beta vulgaris* ssp *maritima*: among population variation and its adaptive significance. *Journal of Ecology* **90**, 693-703.
8. Boudry, P., Wieber, R., Saumitou-Laprade, P., Pillen, K., Van Dijk, H., and Jung, C., 1994: Identification of RFLP markers closely linked to the bolting gene B and their significance for the study of the annual habit in beets (*Beta vulgaris* L.). *Theoretical and Applied Genetics* **88**, 852-858.
9. Butterfass, T., 1968: Die Zuordnung des Locus R der Zuckerrübe (*Hypokotylfarbe*) zum Chromosom II. *Theoretical and Applied Genetics* **38**, 348-350.
10. Caicedo, A. L., Stinchcombe, J. R., Olsen, K. M., Schmitt, J., and Purugganan, M. D., 2004: Epistatic interaction between *Arabidopsis* FRI and FLC flowering time genes generates a latitudinal cline in a life history trait. *Proceedings of the National Academy of Sciences* **101**, 15670-15675.
11. Chia, T. Y. P., Muller, A., Jung, C., and Mutasa-Gottgens, E. S., 2008: Sugar beet contains a large CONSTANS-LIKE gene family including a CO homologue that is independent of the early-bolting (B) gene locus. *Journal of Experimental Botany* **ern129**.
12. Colasanti, J. and Coneva, V., 2009: Mechanisms of floral induction in grasses: something borrowed, something new. *Plant Physiol* **149**, 56-62.

13. Curth, P. Der Übergang in die reproduktive Phase der Zuckerrübe in Abhängigkeit von verschiedenen Umweltfaktoren. 4, 7-80. 1960. Deutsch Akademie Landwirtschaftswissenschaften zu Berlin.
14. Davies T.J., Barraclough T. G. Chase M. W. Soltis P. S. Soltis D. Savolainen V. Darwin's abominable mystery: Insights from a supertree of the angiosperms. PNAS 101, 1904-1909. 2004.
15. Distelfeld, A. and Dubcovsky, J., 2010: Characterization of the maintained vegetative phase deletions from diploid wheat and their effect on VRN2 and FT transcript levels. Mol.Genet.Genomics **283**, 223-232.
16. Distelfeld, A., Li, C., and Dubcovsky, J., 2009: Regulation of flowering in temperate cereals. Curr.Opin.Plant Biol. **12**, 178-184.
17. El, M., El-Mezawy, A., Dreyer, Dreyer, F., Jacobs, Jacobs, G., Jung, and Jung, C., 2002: High-resolution mapping of the bolting gene B of sugar beet. Theoretical and Applied Genetics **105**, 100-105.
18. Gaafar, R. M., Hohmann, U., and Jung, C., 2005: Bacterial artificial chromosome-derived molecular markers for early bolting in sugar beet. Theor.Appl.Genet. **110**, 1027-1037.
19. Greenup, A., Peacock, W. J., Dennis, E. S., and Trevaskis, B., 2009: The molecular biology of seasonal flowering-responses in Arabidopsis and the cereals. Ann.Bot.(Lond).
20. Hansen, M., Kraft, T., Christiansson, M., and Nilsson, O., 1999: Evaluation of AFLP in Beta. Theoretical and Applied Genetics **V98**, 845-852.
21. Hanson, L. E. and L. Panella. Rhizoctonia root rot resistance of Beta PIs from USDA-ARS NPGS, 2003. Biol Cult Tests 19:FC012 . 2003.
22. Hautekeete, N. C., Piquot, Y., and Van Dijk, H., 2002: Life span in Beta vulgaris ssp maritima: the effects of age at first reproduction and disturbance. Journal of Ecology **90**, 508-516.
23. He, Y. H. and Amasino, R. M., 2005: Role of chromatin modification in flowering-time control. Trends in Plant Science **10**, 30-35.
24. Hohmann, U., Jacobs, G., and Jung, C., 2005: An EMS mutagenesis protocol for sugar beet and isolation of non-bolting mutants. Plant Breeding **124**, 317-321.
25. Jung, C. and Müller, A. E., 2009: Flowering time control and applications in plant breeding. Trends in Plant Science **14**, 563-573.

26. Koornneef, M., Hanhart, C. J., and Veen, J. H., 1991: A genetic and physiological analysis of late flowering mutants in *Arabidopsis thaliana*. *Molecular and General Genetics MGG* **229**, 57-66.
27. Koornneef, M.,onso-Blanco, C., Blankestijn-de Vries, H., Hanhart, C. J., and Peeters, A. J. M., 1998: Genetic Interactions Among Late-Flowering Mutants of *Arabidopsis*. *Genetics* **148**, 885-892.
28. Koornneef, M.,onso-Blanco, C., Peeters, A. J. M., and Soppe, W., 2003: GENETIC CONTROL OF FLOWERING TIME IN ARABIDOPSIS. *Annual Review of Plant Physiology and Plant Molecular Biology* **49**, 345-370.
29. Kosambi, D. D. The estimation of map distances from recombination values. *Ann Eug* **12**, 172-175. 1944.
30. Laurent, V., Devaux, P., Thiel, T., Viard, F., Mielordt, S., Touzet, P., and Quillet, M., Comparative effectiveness of sugar beet microsatellite markers isolated from genomic libraries and GenBank ESTs to map the sugar beet genome. *Theoretical and Applied Genetics*.
31. Letschert, J. P. W., 1993: *Beta* section *Beta*: biogeographical patterns of variation and taxonomy. *Wageningen Agricultural University Papers* **93**, 1-154.
32. Lexander, K. Present knowledge on sugar beet bolting mechanisms. 245-258. 1980. *Proc. Int. Inst. Sugar Beet Res. 43rd Winter Congress*.
33. Li, C. and Dubcovsky, J., 2008: Wheat FT protein regulates VRN1 transcription through interactions with FDL2. *Plant J.* **55**, 543-554.
34. Martinez-Zapater, J. M. and Somerville, C. R., 1990: Effect of Light Quality and Vernalization on Late-Flowering Mutants of *Arabidopsis thaliana*. *Plant Physiol* **92**, 770-776.
35. McGrath, J. M., Trebbi, D., Fenwick, A., Panella, L., Schulz, B., Laurent, V., Barnes, S., and Murray, S. C., 2007: An Open-Source First-Generation Molecular Genetic Map from a Sugarbeet x Table Beet Cross and Its Extension to Physical Mapping. *Crop Science* **47**, S-27.
36. Meier, U., 2001: Phenological growth stages and BBCH-identification keys of beet. Growth stages of mono-and dicotyledonous plants. BBCH Monograph, Federal Biological Research Centre for Agriculture and Forestry, Germany.
37. Michaels, S. D., 2009: Flowering time regulation produces much fruit. *Current Opinion in Plant Biology* **12**, 75-80.
38. Moon, J., Lee, H., Kim, M., and Lee, I., 2005: Analysis of flowering pathway integrators in *Arabidopsis*. *Plant Cell Physiol* **46**, 292-299.

39. Munerati, O. L'eredità della tendenza alla annualità nella commune barbabetola, coltivata. 17, 84-89. 1931. Ztschr Züchtung, Reihe A, Pflanzenzüchtung.
40. Nilsson, O., Lee, I., Blazquez, M. A., and Weigel, D., 1998: Flowering-time genes modulate the response to LEAFY activity. *Genetics* **150**, 403-410.
41. Owen, F. W. The significance of single gene reactions in sugar beets. 8, 392-398. 1954. Proc Amer Soc Sugar Beet Technol.
42. Owen, F. W., Carsner, E., and Stout, M., 1940: Photothermal induction of flowering in sugar beets. *J.Agric.Res.* **61**, 101-124.
43. Putterill, J., Laurie, R., and Macknight, R., 2004: It's time to flower: the genetic control of flowering time. *Bioessays* **26**, 363-373.
44. Reeves, P. A., He, Y., Schmitz, R. J., Amasino, R. M., Panella, L. W., and Richards, C. M., 2007: Evolutionary Conservation of the FLOWERING LOCUS C-Mediated Vernalization Response: Evidence From the Sugar Beet (*Beta vulgaris*). *Genetics* **176**, 295-307.
45. Schneider, K., Kulosa, D., Soerensen, T., M+Åhring, S., Heine, M., Durstewitz, G., Polley, A., Weber, E., Jamsari, Jamsari, Lein, J., Hohmann, U., Tahiro, E., Weisshaar, B., Schulz, B., Koch, G., Jung, C., and Ganal, M., 2007: Analysis of DNA polymorphisms in sugar beet (*Beta vulgaris* L.) and development of an SNP-based map of expressed genes. *Theoretical and Applied Genetics* **115**, 601-615.
46. Stout, M., 1945: Translocation of the reproductive stimulus in sugar beet. *Botanical Gazette* **107**, 86-95.
47. Trevaskis, B., Hemming, M. N., Dennis, E. S., and Peacock, W. J., 2007: The molecular basis of vernalization-induced flowering in cereals. *Trends Plant Sci.* **12**, 352-357.
48. Turck, F., Fornara, F., and Coupland, G., 2008: Regulation and Identity of Florigen: FLOWERING LOCUS T Moves Center Stage. *Annu Rev Plant Biol* **59**, 573-594.
49. Utz, Melchinger. PLABQTL: a program for composite interval mapping of QTL. *J Quant Trait Loci* 2. 1996.
50. Van Dijk H and Boudry P, 1991: Genetic variation for life histories in *Beta maritima*. *Int.Board Plant Genet.Resources* **7**, 9-16.
51. Van Ooijen, JW and Voorrips, RE. *JoinMap*® 3.0: Software for the calculation of genetic linkage *maps*. Plant Research International, Wageningen, the Netherlands . 2001.

52. VanDijk, H., Boudry, P., McCombie, H., and Vernet, P., 1997: Flowering time in wild beet (*Beta vulgaris* ssp. *maritima*) along a latitudinal cline. *Acta Oecologica-International Journal of Ecology* **18**, 47-60.
53. Vos, P., Hogers, R., Bleeker, M., Reijans, M., Lee, T. v. d., Hornes, M., Friters, A., Pot, J., Paleman, J., Kuiper, M., and Zabeau, M., 1995: AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* **23**, 4407-4414.
54. Wang, R., Farrona, S., Vincent, C., Joecker, A., Schoof, H., Turck, F.,onso-Blanco, C., Coupland, G., and Albani, M. C., 2009: PEP1 regulates perennial flowering in *Arabis alpine*. *Nature* **459**, 423-427.
55. Yan, L., Fu, D., Li, C., Blechl, A., Tranquilli, G., Bonafede, M., Sanchez, A., Valarik, M., Yasuda, S., and Dubcovsky, J., 2006: The wheat and barley vernalization gene *VRN3* is an orthologue of *FT*. *Proc.Natl.Acad.Sci.U.S.A* **103**, 19581-19586.
56. Yan, L., Helguera, M., Kato, K., Fukuyama, S., Sherman, J., and Dubcovsky, J., 2004: Allelic variation at the *VRN-1* promoter region in polyploid wheat. *Theor.Appl.Genet.* **109**, 1677-1686.
57. Yan, L., Loukoianov, A., Tranquilli, G., Helguera, M., Fahima, T., and Dubcovsky, J., 2003: Positional cloning of the wheat vernalization gene *VRN1*. *Proc.Natl.Acad.Sci.U.S.A* **100**, 6263-6268.
58. Zeevaart, J. A., 2009: My journey from horticulture to plant biology. *Annu.Rev.Plant Biol.* **60**, 1-19.

Genetic mapping of a novel bolting locus (*B4*) linked to the bolting gene *B* on chromosome II of *Beta vulgaris*

4.1. Abstract

Bolting tendency in the crop species *Beta vulgaris*, which includes the sugar beet, is a complex trait governed by various environmental cues, including prolonged periods of cold temperatures over winter (vernalization) and photoperiod, and multiple genetic factors. Two loci which promote bolting in the absence of vernalization are known in beet, the bolting locus *B* on chromosome II and the *B2* locus on chromosome IX. Here, two sibling F_2 populations derived from a cross between a biennial genotype, which requires vernalization to bolt, and an annual beet were phenotyped for bolting behavior. Co-segregation analysis between bolting phenotypes and the genotypes of a molecular marker located at the *B* locus revealed the presence of a novel bolting locus (*B4*), which is linked to the *B* locus on chromosome II and was genetically mapped in both populations. The genetic distance between the *B* and *B4* loci was found to be ~9 cM. Two molecular markers are flanking the *B4* locus with genetic distances of 0.1 and 0.3 cM, with the closest one exhibiting complete co-segregation with the phenotypic marker.

4.2. Introduction

Sugar beet (*Beta vulgaris* L. ssp. *vulgaris*) is a biennial crop and requires a combination of environmental stimuli to initiate bolting (stem elongation) and flowering. These environmental stimuli are the exposure to low temperatures between 2°C and 10°C (vernalization), followed by long-day conditions (Alexander 1980). Owen *et al.* (1940) introduced the term photothermal induction of bolting in sugar beet which refers to the effect of both vernalization and daylength on bolting induction of sugar beet. In general, bolting time is accelerated and the number of bolters is increased as a result of vernalization (Sadeghian *et al.* 1993; Sadeghian and Johansson 1993; Crosthwaite and Jenkins 1993; Abou Elwafa *et al.*, 2006). Long days promote the transition to reproductive development of vernalized plants and accelerate the initiation of stem elongation in the apical shoot meristem. Under non-inductive conditions such as short days or rapid exposure to warm ambient temperatures after vernalization, the inductive effect of vernalization is abolished and the plants revert to vegetative growth, a phenomenon known as “devernalization” (Alexander 1980). However, Fife and Price (1953) reported that bolting and flowering might be induced under variable environmental cues such as continuous darkness or prolonged cold treatment. They added that light is not required for bolting initiation but is required for rapid development of bolters after

stem elongation was initiated, which can occur after a long period of thermal induction regardless of daylength. For short-day conditions, Mutasa-Göttgens *et al.* (2010) reported that application of gibberellin growth hormones (GA) also promotes stem elongation in plants which had been vernalized prior to GA treatment.

Wild beet populations of the subspecies *Beta vulgaris* ssp. *maritima* exhibit great variation in life cycle and bolting behavior. These variations are latitude-dependent, and vary from perennial populations in northern areas to annual populations in the Mediterranean region (Sadeghian *et al.*, 1993; Boudry *et al.*, 1994; VanDijk *et al.*, 1997). Variation in bolting behavior among populations was found to be mainly due to differences in their requirement for vernalization. In contrast to biennial cultivated beets which have an obligate requirement for vernalization, annual wild beets bolt without prior vernalization. Early bolting is an undesirable trait in cultivated sugar beet varieties because it drastically reduces root yield and interferes with mechanical harvesting, and breeders have successfully selected for the biennial habit (Bartsch *et al.*, 1999; Jaggard and Werker, 1999; Rinaldi and Vonella, 2006). The annual habit in *B. vulgaris* was shown to be controlled by a single dominant gene, termed the bolting gene *B*, which promotes the initiation of bolting in long days without vernalization (Munerati, 1931; Abegg, 1936). Although heterozygous beets resulted from crossing of annual and biennial beets under favorable conditions behaved similar to the annual parent in terms of bolting time, the annual beets developed bolters more rapidly than either F₁ or F₂ plants (Munerati, 1931; Abegg, 1936). The authors attributed this observation to the presence of some genes which modify the action of the gene *B* in inducing bolting initiation. (Abe *et al.* 1997) further suggested that bolting tendency in sugar beet is regulated by two genes, i.e. the *B* gene which is responsible for the thermal induction of bolting, and a modifying gene which is responsible for photo-induction of bolting (termed *Lr*) and is closely linked to the *B* locus. These authors hypothesized that the annual habit in sugar beet is mainly controlled by the *B* gene under favorable conditions for bolting, but that the tendency for annual bolting may be modified by the *Lr* gene under unfavorable daylength conditions. Sadeghian *et al.* (1993) suggested that bolting tendency in sugar beet is controlled by genes affected by vernalization and photoperiod and which may act independently or interact epistatically, and that a large proportion of the genetic effect is due to additive effects. Moreover, they suggested a synergistic effect between low temperature and daylength and a possible substitution for each other under specific environmental conditions. Owen *et al.* (1940) hypothesized the presence of a locus responsible for easy-bolting tendency in the biennial beet, termed *B'*, which the authors suggested to be allelic to *B*. Boudry *et al.* (1994)

reported partial penetrance of the annual habit in *B. vulgaris*, where the dominant *B* allele is present in plants exhibiting a non-bolting phenotype, and suggested that the degree of the penetrance of the *B* gene depends on environmental conditions and other genetic factors.

The *B* locus was mapped to chromosome II of sugar beet (El-Mezawy *et al.*, 2002), and a candidate for the *B* gene was recently identified by map-based cloning (A. Müller, personal communication). Besides, Büttner *et al.* (2010) reported the presence of a second bolting locus (*B2*) which co-regulates bolting behavior in sugar beet. The *B2* locus was suggested to regulate bolting via epistatic interactions with the *B* locus, and was mapped by AFLP mapping to chromosome IX. The data by Büttner *et al.* (2010) also indicated the possibility of a third locus (*B3*), which is unlinked to the *B* locus and the *B2* locus, and was suggested to regulate bolting behavior independently from the *B* gene.

The use of the facultative long-day plant *Arabidopsis thaliana* as a model has led to the identification of four major regulatory pathways, i.e., the vernalization, photoperiod, autonomous, and gibberellic acid pathways, which control bolting and flowering time via regulating floral integrator genes (Putterill *et al.*, 2004; He and Amasino 2005; Baurle and Dean 2006; Michaels 2009; Jung and Müller 2009). In *A. thaliana*, bolting and flowering was shown to be regulated antagonistically by *FLC*, a central repressor of flowering which is down-regulated by vernalization, and the photoperiod pathway, which regulate the same downstream targets, *SOC1* and *FT*. Modulating light quality conditions by far-red enrichment can overcome the *FLC*-mediated floral repression (Hepworth *et al.*, 2002; Searle *et al.*, 2006; Helliwell *et al.*, 2006). Recently, a homolog of *FLC* in *B. vulgaris* (*BvFL1*) was cloned and was shown to be functionally related to *FLC* (Reeves *et al.*, 2007). Similarly, functional conservation of a *B. vulgaris* homolog of *CO* (*BvCOL1*) was suggested by Chia *et al.* (2008) who showed that *BvCOL1* complements the late-flowering phenotype of a loss-of-function *co* mutant of *A. thaliana* and activates *FT* expression. *BvFL1* and *BvCOL1* were mapped to chromosomes XI and II, respectively.

In the current study we further investigated the genetic basis of bolting behavior 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. Phenotypic screening for bolting behavior and further propagation led to the identification of five non-segregating biennial M₃ families (Hohmann *et al.*, 2005). In this study, one of those EMS-mutagenized biennial genotypes was crossed with the annual wild type *B. vulgaris* ssp. *maritima* accession 991971. F₁ hybrids were identified and F₂ populations were established as described in Büttner *et al.* (2010). Assuming that annuality in the annual parent is encoded by the *B*

locus, there are at least three conceivable hypotheses; i) the *B* locus is alone responsible for bolting and is mutated or nonfunctional in the EMS-mutagenized biennial parent (one locus model) which is expected to yield a 3:1 phenotypic segregation ratio (bolting: non-bolting) in the F_2 generation and complete cosegregation between bolting phenotypes and *B* locus marker genotypes ii) the presence of a second, unlinked locus which is mutated or nonfunctional in the EMS-mutagenized parent and acts epistatically with the *B* locus (epistatic locus model, i.e., the second locus prevents annual bolting even in the presence of a functional *B* allele at the *B* locus). The expected phenotypic segregation ratio in F_2 is 3:1 (bolting: non-bolting) but *B* locus marker alleles and bolting phenotypes would be expected to segregate independently, iii) the presence of an epistatic locus which is mutated or non-functional in the biennial parent, but, in contrast to ii), is linked to *B*. The phenotypic segregation ratio expected from this hypothesis is 3: 1 (bolting: non-bolting), and the segregation ratio of the *B* locus marker genotypes among the biennial individuals and among annual plants would depend on the recombination frequency between the two loci.

4.3. Materials and methods

4.3.1. Plant material and phenotypic analysis

F_2 populations were established by crossing a biennial genotype (000992) resulting from EMS mutagenesis of an annual *B. vulgaris* accession (Hohmann *et al.*, 2003), with the annual wild type 991971, which was collected on the Greek island of Khios (Gaafar *et al.*, 2005); USDA-ARS National Plant Germplasm System PI 546521; Hanson and Panella, 2003, <http://www.ars-grin.gov/cgi-bin/npgs/acc/display.pl?1441457>). Crossing was done between plants that have different hypocotyl color (with the red hypocotyl color being dominant over the green one, (Butterfass 1968; Barzen *et al.* 1992). F_1 seeds from the parent that has a green hypocotyl were sown, and plants that have red hypocotyls were considered as hybrids. F_2 seeds were produced by selfing of F_1 plants essentially as described by Büttner *et al.* (2010).

Two sibling F_2 populations were investigated. 96 plants from population EW5a, derived by selfing of F_1 plant 061388/1, 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. Six plants died after transplanting into the field. 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 population EW5b, derived by selfing of F₁ plant 061388/2, 208 plants were sown on July 2, 2009, grown in 96-well trays until August 10, 2009, transplanted into larger pots (13 cm diameter) and grown in the greenhouse under long day conditions with supplementary lighting (16h, Son-T Agro 400W (Koninklijke Philips Electronics N.V., Eindhoven, The Netherlands) for 16h July 2 - October 27, 2009, and for 22h October 28, 2009 – June 14, 2010. Plants were phenotyped for onset of bolting as described above.

121 F₃-families (a total of 2736 F₃ plants, with 20-23 plants from each F₃ family) derived from the selfing of individual annual F₂ plants from population EW5b which bolted before October 27, were sown on February 12, 2010, in 35-well trays (Hermann Meyer KG, Germany) and phenotyped for onset of bolting in the greenhouse until May 6, 2010 under long day conditions with supplementary lighting (16h, Son-T Agro 400W). The F₃ families were distinguished into non-segregating families (100% bolting frequency) and segregating families. The phenotypic segregation ratio of bolting vs. non-bolting plants did not deviate significantly from 3:1, as tested by Chi square analysis.

4.3.2. Marker development and genetic mapping

For molecular marker analysis leaf samples were harvested from 3 weeks old plants and freeze-dried. Genomic DNA was isolated using the NucleoSpin 96 Plant DNA isolation kit (Macherey and Nagel, Düren, Germany) and a TECAN-Freedom EVO 150® robot (Männedorf, Switzerland). Genomic DNA was isolated from 90 and 208 plants from EW5a and EW5b F₂ populations, respectively. In order to differentiate the *B* locus alleles derived from the annual wild type parent and the EMS-mutagenized biennial parent a molecular marker (GJ1001c16) linked to the *B* locus at *R*=0 (A. Müller, personal communication) was used essentially as described by Büttner *et al.* (2010). Recombination frequencies were calculated using the maximum likelihood estimate with the following formulae:

$$r = R / N$$

where *r* denotes recombination frequency, *R* denotes number of recombinants, and *N* denotes total number of individuals tested (Adama and Joly, 1980).

The corresponding LOD score was calculated according to the following general formula:

$$LOD = \log_{10} \frac{\theta^k \times (1 - \theta)^{n-k}}{0.5^n}$$

Where n denotes the total number of offspring, and k denotes the number of recombinant offspring. θ is the recombinant fraction, i.e. k/n (Speed and Zhao 2007).

To generate a genetic map of chromosome II, a *BvCOL1* gene marker (A242-A245; Chia *et al.*, 2008), a publicly available SSR marker (FDSB1300; Laurent *et al.*, 2007), and twelve EST-derived markers located on chromosome II in the K1 mapping population (Schneider *et al.*, 2007) were used to analyze population EW5a. The EST markers were converted to CAPS markers (Suppl. Tab. 3). The EST loci were amplified and the PCR products were restriction digested with the appropriate restriction enzyme, followed by analysis on 1% or 2% agarose gels. 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 and a maximum recombination value (rec-value) of 0.4.

For construction of a genetic map for population EW5b, 208 F₂ plants were analyzed with three markers at or in the vicinity of the *B* locus, GJ18T7b, GJ1001c16 and Y67L, and five EST-derived CAPS markers located on chromosome II (Schneider *et al.*, 2007). The majority of the annual F₂ plants were scored codominantly according to the phenotypic data of their F₃ progenies.

4.3.3. Statistical analysis

Chi square (χ^2) analysis, T-test and analysis of variance (ANOVA) were 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).

4.4. Results

4.4.1. Phenotypic analysis of bolting behavior in F₂ populations EW5a and EW5b

Two sibling F₂ populations, EW5a consisting of 90 plants and EW5b consisting of 208 plants, were derived from a cross between a biennial genotype, identified after EMS mutagenesis of an annual genotype (Hohmann *et al.*, 2005), and an annual wild beet accession. The two F₂ populations were phenotyped for bolting behavior under field or greenhouse conditions (see Materials and Methods), and both populations segregated for bolting behavior (Figure 8, Suppl. Table 1 and 2). The null hypothesis for dominant-recessive inheritance of a single dominant gene as it was described for the bolting locus *B* (Munerati *et al.*, 1931; Abegg, 1936; see Introduction), i.e. a 3:1 phenotypic segregation ratio of bolting and non-bolting individuals, was not rejected by Chi square analysis for the field-grown population

EW5a (Table 9). In population EW5b, which was grown in the greenhouse, the observed phenotypic segregation at the end of growth under 16h light conditions (130 bolting plants vs. 78 non-bolting plants) deviated significantly from the expectation for a 3:1 segregation ratio. However, continued growth under 22h light conditions resulted in bolting of an additional 38 plants (which will be referred to as late-bolting plants) by the end of the experiment. When the late-bolting plants are included in the subpopulation of bolting plants, the segregation ratio of population EW5b (168 bolting plants vs. 40 non-bolting plants) did not deviate significantly from the expected ratio of 3:1 (Table 9).

Table 9: Phenotypic segregation for bolting behavior in two F₂ populations.

F ₂ population	Total number of plants	Number of bolting plants	Number of non-bolting plants	χ^2 test for H ₀ = 3:1 (bolting vs. non-bolting) ¹
EW5a	90	73	17	1.79 ns
EW5b	208	168	40	3.68 ns

¹ H₀, null hypothesis for monogenic, dominant-recessive trait
ns, non-significant ($\alpha=0.01$)

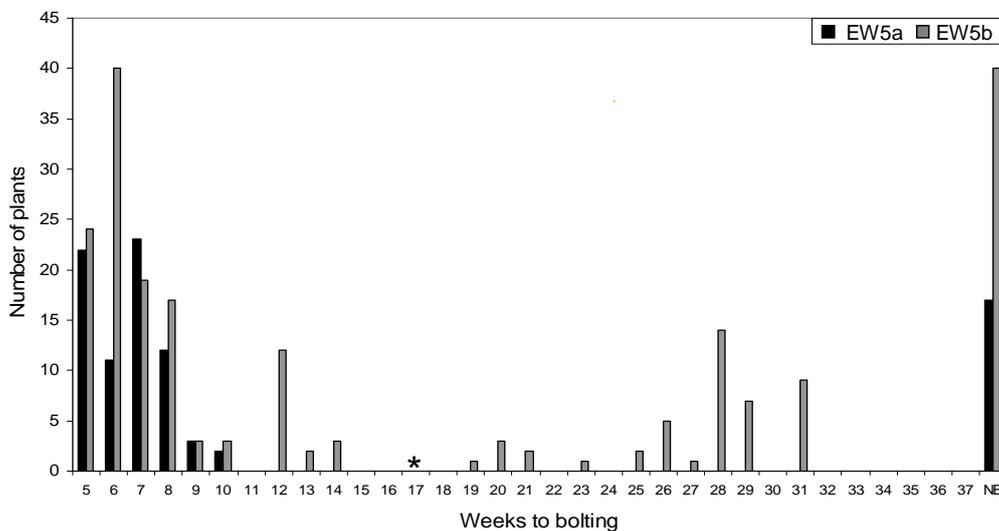


Figure 8: Phenotypic segregation for bolting behavior in F₂ populations EW5a and EW5b. Number of days to bolting is indicated as weeks to bolting (week 5 corresponds to 29–35 days to bolting, etc.; s. Suppl. Tab. 1). ‘nb’ indicates plants which had not bolted by the end of the experiment. Population EW4a was grown in the field, whereas population EW5b was grown in the greenhouse. Supplementary lighting in the greenhouse was increased to 22h starting in week 17 (indicated by the asterisk).

4.4.2. Co-segregation analysis of bolting phenotypes and *B* locus marker genotypes

The two F₂ populations were analyzed for co-segregation between bolting phenotypes and co-dominant markers located at the *B* locus. To distinguish between complete co-segregation and independent segregation between the *B* locus marker genotypes and the bolting phenotypes, marker genotypes were grouped into six classes, i.e., *M*₁*M*₁, *M*₁*M*₂ and *M*₂*M*₂ genotypes of the *B* locus marker present among the bolting individuals of an F₂ population, and *M*₁*M*₁, *M*₁*M*₂ and *M*₂*M*₂ genotypes present among the non-bolting individuals. The occurrence of co-segregation between the bolting behavior and the *B* locus marker genotypes would be expected to result in a 0:2:1:1:0:0 segregation ratio, while independent segregation would yield a 3:6:3:1:2:1 segregation ratio for the six classes. Genotypic analysis of populations EW5a and EW5b with the *B* locus marker GJ1001c16 (Büttner *et al.*, 2010) showed that, in both populations, all six classes were present. According to Chi square analysis both hypotheses were rejected (Table 10), indicating that neither complete co-segregation (hypothesis i), s. Introduction) nor independent segregation between the *B* locus marker genotype and bolting behavior (hypothesis ii) had occurred and that neither the *B* locus nor an epistatic, unlinked locus is responsible for bolting behavior in the F₂ populations.

Table 10: Co-segregation analysis of bolting behavior and the *B* locus marker (GJ1001c16) genotypes in populations EW5a and EW5b.

F ₂ Population	Marker	Total number of plants genotyped	Bolting <i>M</i> ₁ <i>M</i> ₁ ¹	Bolting <i>M</i> ₁ <i>M</i> ₂	Bolting <i>M</i> ₂ <i>M</i> ₂	Non-bolting <i>M</i> ₁ <i>M</i> ₁	Non-bolting <i>M</i> ₁ <i>M</i> ₂	Non-bolting <i>M</i> ₂ <i>M</i> ₂	χ ² test for H ₀ = 3:6:3:1:2:1	χ ² test for H ₀ = 0:2:1:1:0:0
EW5a	GJ1001c16	90	7	51	15	12	3	2	30.40**	69.90**
EW5b	GJ1001c16	208	14	92	62	33	3	4	89.00**	231.0**

0:2:1:1:0:0, the expected ratio for monogenic, dominant recessive trait, co-segregation of phenotype and *B* locus marker genotype

3:6:3:1:2:1, the expected ratio for monogenic, dominant recessive trait, independent segregation of phenotype and *B* locus marker genotype

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

* α=0.05; ** α=0.01

The phenotypic segregation data and the lack of either complete co-segregation or independent segregation between the bolting phenotypes and the genotypes of the *B*

locus marker suggested that bolting behavior in populations EW5a and EW5b is (co-)regulated by a second locus which is genetically linked to *B* and thus located on chromosome II, in accordance with hypothesis iii). This locus will be referred to as *B4*.

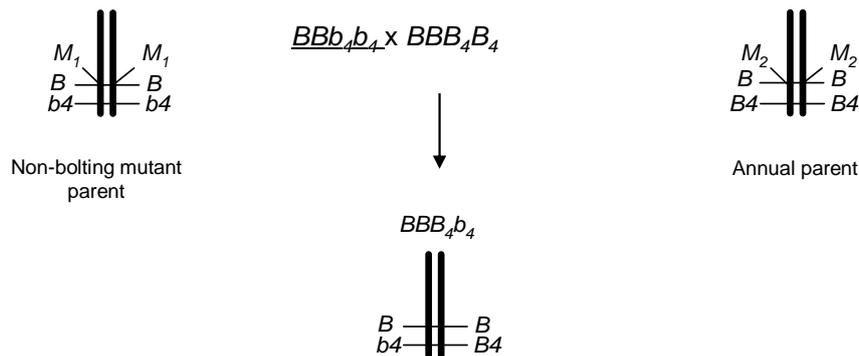


Figure 9: Proposed bolting loci on chromosome II and their allelic composition in the parents and F_1 plant of the segregating F_2 populations analyzed (hypothesis iii). M_1 and M_2 are two different alleles of a marker which is tightly linked to the *B* locus ($R = 0$).

4.4.3. Genetic mapping of the *B4* locus on chromosome II

In order to map the *B4* locus, a genetic map was constructed containing 15 molecular markers, including twelve newly developed CAPS markers (Suppl. Tab. 3) derived from a set of ESTs which had been previously mapped to chromosome II (Schneider *et al.*, 2007), the *B* locus marker GJ1001C16 (Büttner *et al.*, 2010), the *BvCOL1* marker (Chia *et al.*, 2008), and the SSR marker FDSB1300 (Laurent *et al.*, 2007). The *B4* locus was mapped between markers TG_E0092 and MP_R0145 at a cumulative genetic distance of 55.58 cM (Figure 10a). Among the two closest, flanking markers, marker MP_R0145 showed complete co-segregation with the non-bolting phenotype among the non-bolting individuals. The closest marker to *B4* is TG_E0092 at a genetic distance of 1.27 cM. The genetic distance between the *B* locus and the *B4* locus is 10.36 cM. The calculated recombination frequency between the *B* locus and the *B4* locus in population EW5a is 0.13 at a LOD score of 8.12.

To verify the position of *B4*, population EW5b was genotyped with eight markers, including the five EST-derived CAPS markers TG_E0068, TG_E0179, TG_E0562, TG_E0092 and MP_E0023 (Suppl. Tab. 3), two markers flanking the *B* locus

(GJ18T7b and Y67L), and the *B* locus marker GJ1001c16 (Büttner *et al.*, 2010). Similar to population EW5a, marker TG_0092 is the closest marker on one side of the *B4* locus. Moreover, the marker TG_E0562, which is not polymorphic in EW4a, showed complete co-segregation with *B4*, i.e., all the non-bolting individuals exhibited the biennial parent-derived allele in the homozygous state (M_1M_1), while all the bolting individuals exhibited the annual parent-derived allele either in the homozygous or heterozygous state (M_1M_2 or M_2M_2). The phenotypic data obtained from 121 F_3 families (2736 plants) were used here. The occurrence of 100% bolting plants within an F_3 family indicated homozygosity at the locus responsible for bolting in the corresponding F_2 plant, whereas the occurrence of a phenotypic segregation ratio in F_3 which did not significantly deviate from 3:1 (bolting: non-bolting) was indicative of heterozygosity of the F_2 individual. Using the data obtained from F_3 families the *B4* locus could be precisely located to a 0.42 cM genetic interval between the markers TG_E0562 and TG_E0092 (Figure 10b). Furthermore, TG_E0562 marker was the closest to *B4* locus with a genetic distance of 0.09 cM, while the genetic distance between the *B4* locus and marker TG_E0092 was 0.33 cM.

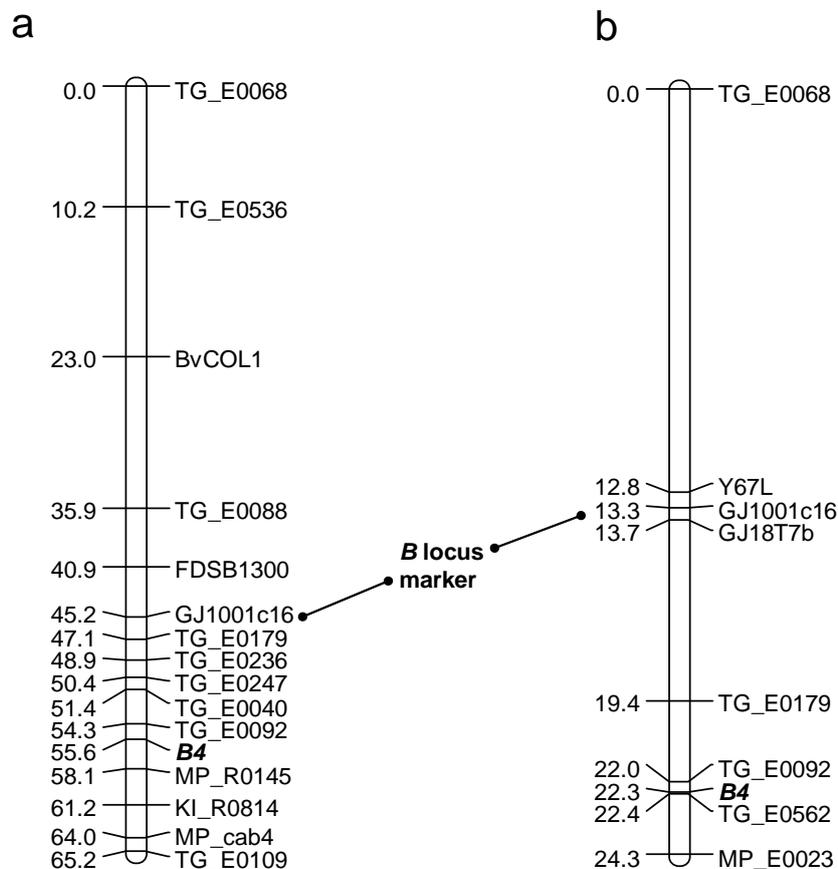


Figure 10: Genetic map position of *B4* locus. a) *B4* locus was mapped to position 55.58 cM in population EW5a. TG_E0092 is the closest marker to *B4* locus with a genetic distance of 1.3 cM, and b) genetic map position of *B4* locus in population EW5b based on F₃ phenotypic data. Marker TG_E0562 is the closest marker to *B4* with a genetic distance of 0.1 cM.

4.4.4. Neither *B* nor *B4* locus affects bolting time in populations EW5a and EW5b

The number of days from sowing to the date of bolting was scored in both populations (Suppl. Table 1 and 2; Figure 8). Analysis of variance (ANOVA) of number of days to bolting was performed for the three genotypes of the *B* locus marker (GJ1001C16; M_1M_1 , M_1M_2 and M_2M_2) in both populations. Similarly, ANOVA was performed to analyze the effect of the genotype of the *B4* locus flanking marker TG_E0092 on the number of days to bolting in populations EW5a and EW5b. Since, there is complete co-segregation between the *B4* locus marker TG_E0562 and the bolting phenotypes in population EW5b, i.e., none of the bolting individuals exhibited the M_1M_1 genotype of the marker, only a t-test is applicable to assess the effect of the two alleles of the *B4* locus marker on bolting time. The statistical tests revealed no significant differences between the genotypes of either the *B* or the *B4* locus markers in terms of bolting time in either of the populations (Table 11).

Table 11: Analysis of variance and t-test among *B* and *B4* locus marker genotypes in annual subpopulations.

Marker genotype	Mean (\pm standard deviation) of days to bolting				
	EW5a		EW5b		
	GJ1001c16	TG_E0092	GJ1001c16	TG_E0092	TG_E0562
M_1M_1	47.87 (± 9.62)	48.40 (± 9.49)	115.00 (± 64.74)	79.51 (± 60.50)	- ¹
M_1M_2	49.90 (± 7.94)	49.73 (± 8.07)	88.32 (± 64.60)	91.74 (± 66.80)	81.62 (± 62.63)
M_2M_2	45.29 (± 9.67)	43.40 (± 8.76)	78.82 (± 60.90)	81.00 (± 00.00)	90.37 (± 65.71)
F/T value (P-value) ¹	1.10 (0.34)	1.33 (0.27)	1.92 (0.15)	1.41 (0.24)	0.85 (0.40)
LSD _{0.05} ²	n.a. ³	n.a.	n.a.	n.a.	n.a.

¹ Marker TG_E0562 did not exhibit M_1M_1 genotype in population EW5b, and unpaired t-test was performed instead of ANOVA.

² Fisher's Least Significant Difference at $\alpha=0.05$.

³ n.a., not applicable

4.5. Discussion

Two sibling F_2 populations derived from a cross between a biennial genotype and an annual genotype were analyzed. The biennial genotype was identified by Hohmann *et al.* (2005) in a screen for biennial phenotypes following EMS mutagenesis of an annual accession, but there is no clear evidence whether the biennial life cycle of the biennial parent of the F_2 populations resulted from EMS mutagenesis or is due to a natural allele which may be present at low frequency within the accession that had been subjected to EMS mutagenesis. However, the origin of bienniality in a given parent should not influence the inheritance of bolting behavior in progenies.

In the present study we analyzed two F_2 populations and one F_3 population. The principal results from this study are i) the finding that, beside the *B* locus, there is a second locus (*B4*) which is linked to *B* on chromosome II and co-regulates bolting behavior, and ii) the genetic map position of the novel bolting locus and identification of molecular markers closely linked to *B4*.

The non-significant deviation of the observed phenotypic segregation ratio in populations EW5a and EW5b from the 3:1 segregation ratio, suggested that, based on our hypotheses (see Introduction and Büttner *et al.*, 2010), bolting behavior in both populations is either monogenically inherited, with the causative gene being most likely the bolting gene at the *B* locus (hypothesis i), or co-regulated by a second bolting locus which acts epistatically to the *B* locus and is either unlinked (hypothesis ii) or linked to *B* on chromosome II (hypothesis iii). To distinguish between these hypotheses, genotypic analyses of both populations with a genetic marker which is closely linked to the *B* locus ($R=0$) was employed. The non-complete co-segregation between the bolting phenotypes and the *B* locus marker genotypes in both populations indicates that the *B* gene is not, or not alone responsible for bolting behavior in both populations. On the other hand, the absence of an independent segregation between the bolting phenotypes and the *B* locus marker genotypes (Table 10) rejected the second hypothesis, i.e., the presence of a second locus which acts epistatically to the *B* locus and is unlinked to the *B* locus. However, the lack of independent segregation would be consistent with the presence of an epistatic locus which is genetically linked to locus *B* on chromosome II (Figure 9). Consistent with the presence of a second bolting gene on chromosome II outside the *B* locus, a single major QTL was detected in both populations which co-localizes with the phenotypic marker locus *B4* on chromosome II (data not shown). The presence of a second bolting locus which is linked to the *B* locus on chromosome II is consistent with hypothesis iii). According to this hypothesis, both parents carry functional bolting alleles at the *B*

locus, and the *B4* locus is mutated or non-functional (either by EMS mutagenesis or as a result of natural variation) in the biennial parent. Since the annual accession used for EMS mutagenesis is homozygous for the annual bolting allele at the *B* locus (*BB*), the *B4* locus would have to act epistatically with *B* and prevents bolting even in the presence of a functional bolting allele at the *B* locus (Figure 9). This possibility is largely consistent with a previous study (Abe *et al.* 1997) according to which a second locus affecting bolting (termed *Lr*, see Introduction) is genetically linked to the *B* locus. The authors showed that the annual habit of sugar beet is controlled by two genes, *B* and *Lr*, each of which affects different stages of bolting induction, i.e., the *B* gene was implicated in the thermal induction of bolting and *Lr* was suggested to regulate photoperiod responsiveness. Furthermore, and consistent with our hypothesis, the authors reported that the *Lr* gene acts epistatically to *B* and modifies bolting tendency under unfavorable daylength. Taken together, our data and the previous findings of Abe *et al.* (1997) suggest that the *B4* locus may correspond to the *Lr* locus. In contrast to *B4*, the *Lr* locus has not been placed on a genetic map of chromosome II.

Although our data are consistent with epistatic interactions between *B* and *B4*, we cannot exclude the possibility that both parents carry non-functional alleles at the *B* locus, and a functional allele of the *B4* locus is inherited from the annual parent, which would also be consistent with the observed phase relationships between bolting phenotypes and the molecular marker alleles derived from either the annual or the biennial parent. In this scenario, *B4* would act independently of *B* (i.e., *B4* would not require the presence of a functional allele at the *B* locus to promote bolting). Furthermore, although the observed phenotypic segregation ratio in both populations did not deviate significantly from the 3:1 expected ratio, there is a large excess of bolting individuals compared to the expectation in both populations. Interestingly, the observed phenotypic segregation ratio in both populations (with ratios of 4.29:1 and 4.20:1, respectively) is also consistent with an alternative model, i.e., bolting behavior in both populations is controlled by two independent linked loci, *B* and *B4*, and each of the two loci is sufficient to induce bolting independently from the other. According to this model, both loci would be non-functional in the biennial parent, whereas both loci carry functional, dominant alleles in the annual parent. Assuming a recombination value between *B* and *B4* of 0.13 (see Results) as an approximation, the expected phenotypic segregation ratio would be 4.28:1 (bolting: non-bolting) which is very similar and did not exhibit significant deviation from the observed phenotypic segregation ratios in both populations EW5a and EW5b, as tested by Chi square analysis (not shown). In the light of this model, it would appear unlikely that the *B4* locus acts in the same pathway as *B*. A model for two alternative pathways regulating bolting in

Arabidopsis (i.e., vernalization and light quality-dependent pathways) was reported by (Wollenberg *et al.*, 2008) according to which far-red light enrichment can induce floral transition by bypassing the *FLC*-controlled pathway which mediates vernalization requirement, and by promoting the expression of *CONSTANS* (*CO*) and *GIGANTEA* (*GI*). The genetic map position of the *B4* locus precludes the possibility that it carries the *CO* homolog *BvCOL1* (Chia *et al.*, 2008) or the *GI* homolog *BvGI* (G. Schulze-Buxloh, personal communication), but it is conceivable that the *B4* locus carries a gene located upstream *CO* or *GI*, which may be involved in light quality responsiveness.

It was shown that the EST-derived marker, TG_E0092, is the closest marker on one side of the *B4* locus in both populations with genetic distances of 1.27 cM (EW5a) and 0.33 cM (EW5b), respectively. Using the F₃ phenotypic data from population EW5b the *B4* locus could be further delimited to a genetic interval of 0.42 cM (Figure 10). Marker TG_E0562 is the closest to *B4* locus with a genetic distance of 0.09 cM and shows a complete co-segregation with the bolting phenotype, and could be used as a marker for map-based cloning of *B4* gene and for marker-assisted selection against bolting tendency in sugar beet.

In contrast to previous results (Büttner *et al.*, 2010; Mutasa-Gottgens *et al.*, 2010), analysis of variance of number of days to bolting showed that the *B* locus does not have a significant effect on bolting time in either of the populations. This finding is largely consistent with our model for genetic control of bolting behavior in these two populations according to hypothesis iii), according to which both parents have a functional allele at the *B* locus (which would not be expected to have different effects on bolting time. Similarly, analysis of variance of number of days to bolting suggested that the *B4* locus also does not affect bolting time in our populations.

Our results add more complexity to the genetic control of bolting and floral transition in *B. vulgaris*. In a previous study (Büttner *et al.*, 2010), at least two additional loci (*B2*, *B3*) were suggested to regulate bolting behavior in sugar beet. The observed phenotypic segregation ratio and absence of independent segregation between the bolting phenotype and the *B* locus marker genotypes preclude the possibility that either locus *B2* or locus *B3* (which appears not to be linked to *B*; Büttner *et al.*, 2010) determines bolting behavior in the two F₂ populations (EW5a and EW5b).

In summary, our results revealed the presence of a novel locus for bolting control in sugar beet, which is closely linked to the *B* gene on chromosome II with a genetic distance of ~10 cM. A genetic map around the *B4* locus and closely linked molecular markers, may be beneficial for i) marker-assisted selection against

bolting tendency in sugar beet, ii) positional cloning of *B4* gene as a step towards modification of bolting behavior of sugar beet for the development of winter beets, a major objective in sugar beet breeding (Jung and Müller, 2009), and iii) induction of bolting without a requirement for vernalization to facilitate breeding programs and seed production for cultivation of sugar beets adapted to warm climate conditions and tropical and subtropical areas.

4.6. Acknowledgment

The project was funded by the Deutsche Forschungsgemeinschaft under grant no. DFG JU 205/14-1. Salah F. Abou-Elwafa is supported by a scholarship from the Ministry of Higher Education, Egypt. We thank Uwe Hohmann for propagation of mutants and Monika Bruisch and Erwin Danklefsen for excellent technical assistance in the field and greenhouse.

4.7. References

1. Abe, J., Guan, G. P., and Shimamoto, Y., 1997: A gene complex for annual habit in sugar beet (*Beta vulgaris* L). *Euphytica* **94**, 129-135.
2. Abou Elwafa, S. F., H.M.Abdel-Rahim, A.M.Abou-Salama, and E.A.Teama, 2006: Sugar Beet Floral Induction and Fertility: Effect of Vernalization and Day-length Extension. *Sugar Tech* **8**, 281-287.
3. Barzen, E., Mechelke, W., Ritter, E., Seitzer, J. F., and Salamini, F., 1992: RFLP markers for sugar beet breeding: chromosomal linkage maps and location of major genes for rhizomania resistance, monogerm and hypocotyl colour. *The Plant Journal* **2**, 601-611.
4. Baurle, I. and Dean, C., 2006: The timing of developmental transitions in plants. *Cell* **125**, 655-664.
5. Butterfass, T., 1968: Die Zuordnung des Locus R der Zuckerrübe (Hypokotylfarbe) zum Chromosom II. *TAG Theoretical and Applied Genetics* **38**, 348-350.
6. Büttner, B., Abou-Elwafa, S. F., Zhang, W., Jung, C., and Muller, A. E., 2010: A survey of EMS-induced biennial *Beta vulgaris* mutants reveals a novel bolting locus which is unlinked to the bolting gene *B*. *Theor.Appl.Genet.*
7. Chia, T. Y. P., Muller, A., Jung, C., and Mutasa-Gottgens, E. S., 2008: Sugar beet contains a large CONSTANS-LIKE gene family including a CO homologue that is independent of the early-bolting (*B*) gene locus. *J.Exp.Bot.* **59**, 2735-2748.

8. Crosthwaite, S. and Jenkins, I. G., 1993: The Role of Leaves in the Perception of Vernalizing Temperatures in Sugar Beet. *J. Exp. Bot.* **44**, 801-806.
9. El-Mezawy, A., Dreyer, F., Jacobs, G., and Jung, C., 2002: High-resolution mapping of the bolting gene *B* of sugar beet. *Theoretical and Applied Genetics* **105**, 100-105.
10. Fife, J. M. and Price, C., 1953: Bolting and Flowering of Sugar Beets in Continuous Darkness. *Plant Physiol.* **28**, 475-480.
11. Gaafar, R. M., Hohmann, U., and Jung, C., 2005: Bacterial artificial chromosome-derived molecular markers for early bolting in sugar beet. *Theor. Appl. Genet.* **110**, 1027-1037.
12. He, Y. H. and Amasino, R. M., 2005: Role of chromatin modification in flowering-time control. *Trends in Plant Science* **10**, 30-35.
13. Helliwell, C. A., Wood, C. C., Robertson, M., James, P. W., and Dennis, E. S., 2006: The Arabidopsis FLC protein interacts directly in vivo with SOC1 and FT chromatin and is part of a high-molecular-weight protein complex. *Plant J.* **46**, 183-192.
14. Hepworth, S. R., Valverde, F., Ravenscroft, D., Mouradov, A., and Coupland, G., 2002: Antagonistic regulation of flowering-time gene SOC1 by CONSTANS and FLC via separate promoter motifs. *EMBO J.* **21**, 4327-4337.
15. Jung, C. and Müller, A. E., 2009: Flowering time control and applications in plant breeding
1048. *Trends in Plant Science* **14**, 563-573.
16. Kosambi, D. D., 1944: The estimation of map distances from recombination values. *Ann Eug* **12**, 172-175.
17. Laurent, V., Devaux, P., Thiel, T., Viard, F., Mielordt, S., Touzet, P., and Quillet, M. C., 2007: Comparative effectiveness of sugar beet microsatellite markers isolated from genomic libraries and GenBank ESTs to map the sugar beet genome. *Theor. Appl. Genet.* **115**, 793-805.
18. Lexander, K., 1980: Present knowledge on sugar beet bolting mechanisms. *Proc. Int. Inst. Sugar Beet Res. 43rd Winter Congress* 245-258.
19. Meier, U., 2001: Phenological growth stages and BBCH-identification keys of beet. Growth stages of mono- and dicotyledonous plants. BBCH Monograph, Federal Biological Research Centre for Agriculture and Forestry, Germany.
20. Michaels, S. D., 2009: Flowering time regulation produces much fruit. *Current Opinion in Plant Biology* **12**, 75-80.

21. Mutasa-Gottgens, E. S., Qi, A., Wenying, Z., Schulze-Buxloh, G., Jennings, A., Hohmann, U., Muller, A. E., and Hedden, P., 2010: Bolting and flowering control in sugar beet: Relationships and effects of gibberellin, the bolting gene *B* and vernalization. *AoB Plants* lq012.
22. Putterill, J., Laurie, R., and Macknight, R., 2004: It's time to flower: the genetic control of flowering time. *Bioessays* **26**, 363-373.
23. Reeves, P. A., He, Y., Schmitz, R. J., Amasino, R. M., Panella, L. W., and Richards, C. M., 2007: Evolutionary Conservation of the FLOWERING LOCUS C-Mediated Vernalization Response: Evidence From the Sugar Beet (*Beta vulgaris*). *Genetics* **176**, 295-307.
24. Sadeghian, S. Y., Becker, H. C., and Johansson, E., 1993: Inheritance of Bolting in 3 Sugar-Beet Crosses After Different Periods of Vernalization. *Plant Breeding* **110**, 328-333.
25. Sadeghian, S. Y. and Johansson, E., 1993: Genetic-Study of Bolting and Stem Length in Sugar-Beet (*Beta-Vulgaris* L) Using A Factorial Cross Design. *Euphytica* **65**, 177-185.
26. Searle, I., He, Y., Turck, F., Vincent, C., Fornara, F., Krober, S., Amasino, R. A., and Coupland, G., 2006: The transcription factor *FLC* confers a flowering response to vernalization by repressing meristem competence and systemic signaling in *Arabidopsis*. *Genes Dev.* **20**, 898-912.
27. Speed, T. P. and Zhao, H., 2007: Chromosome Maps. In: D. J. Balding, M. Bishop, and C. Cannings (eds.), *Handbook of Statistical Genetics*, 3-39. John Wiley & Sons, Ltd, West Sussex, England.
28. Van Ooijen, JW and Voorrips, RE. *JoinMap*® 3.0: Software for the calculation of genetic linkage *maps*. Plant Research International, Wageningen, the Netherlands . 2001.
29. Wollenberg, A. C., Strasser, B., Cerdan, P. D., and Amasino, R. M., 2008: Acceleration of Flowering during Shade Avoidance in *Arabidopsis* Alters the Balance between FLOWERING LOCUS C-Mediated Repression and Photoperiodic Induction of Flowering. *Plant Physiol.* **148**, 1681-1694.

5. Closing Discussion

Both forward and reverse genetic approaches were implemented for further uncovering the genetic basis of bolting and flowering control in sugar beet. The forward genetic approach (also known as classical genetics) is an approach which is usually used to identify new genes underlying a phenotype of interest by surveying a population segregating for the phenotype to identify the gene and sequence change that underlies a specific mutant (or natural variant) phenotype and selecting individuals which possess this phenotype (Kevei *et al.*, 2006). The implementation of the forward genetic approach includes i) establishing a population with variation for a phenotype of interest by exploiting natural variation, exposing individuals to a mutagen such as a chemical (e.g., EMS), or radiation (e.g., gamma radiation), or insertional (T-DNA or transposon) mutagenesis, ii) phenotypic screening of the population for the given phenotype (this phenotypic screening also could be done under different environmental conditions, where these environmental conditions serve either as a promotive or repressive factor to the phenotype of interest), iii) crossbreeding of the individuals resulting from this screening with genotypes which possess the opposite phenotype of the investigated trait, iv) genetic mapping of the gene(s) underlying this trait, and v), in an advanced step of the study, using the established genetic map as beneficial tool in marker-assisted selection for/against the phenotype of interest or positional cloning of the gene(s) underlying this phenotype (Medina *et al.*, 2006; Peters *et al.*, 2003; Schneider and Leister 2006; Weigel and Glazebrook 2006; Zhong *et al.*, 2010).

Although forward genetics has been applied as a useful approach to uncover new genes underlying phenotypes in model species such as Arabidopsis, Drosophila and mice (e.g. Kevei *et al.*, 2006; Merte *et al.*, 2010), its application is limited as mutations in many genes usually cause only moderate or weak phenotypes (Lee *et al.*, 2010). Furthermore, the identification of the gene underlying a given phenotype depends to a great extent on the mutagen, i.e., the identification of a gene of interest where a mutation is caused by T-DNA or transposon insertion is at least theoretically much faster and possible by determining the sequence tag and analyzing its neighboring sequences, whereas identification of a gene where the mutation has occurred as a result of natural variation or resulted from chemical or radiation treatment requires a more tedious and laborious work in order to delimit the gene via positional cloning. However, the great progress achieved in the development of molecular markers, DNA sequencing and detection methods of DNA polymorphisms facilitate positional cloning of a gene of interest via the implementation of the forward genetic approach (Matsuo *et al.*, 2008; Peters *et al.*, 2003; Wong *et al.*, 2010).

In Chapters 3 and 4 of this study, we applied the forward genetic approach to further dissect the genetic factors underlying bolting behavior in *B. vulgaris*. In a previous

study, an annual *B. vulgaris* line homozygous for the dominant bolting allele *B* was mutagenized by ethyl methanesulfonate (Hohmann *et al.*, 2005). Phenotypic screening for bolting behavior in this mutant population resulted in a number of EMS-mutagenized biennial *B. vulgaris* genotypes. To identify gene(s) underlying bolting behavior in the EMS-mutagenized biennial *B. vulgaris* genotypes, individuals from non-segregating biennial M3 or M4 families were crossed with annual *B. vulgaris* genotypes, and segregating F₂ populations were established by selfing the F₁ hybrids. Seven F₂ populations derived from crosses between five EMS-mutagenized biennial genotypes and annual beets were phenotyped for bolting behavior (Chapters 3 and 4). All seven F₂ populations were genotyped with molecular markers that are closely linked to the bolting locus *B* on chromosome II of *B. vulgaris*. The focus of the current thesis was on four F₂ populations which led to the detection of a putative locus affecting bolting control (*B3*, Chapter 3), and identification and genetic mapping of a new bolting locus, *B4* (Chapter 4)

The major findings described in Chapters 3 and 4 are: i) contrary to what had been thought, the *B* locus is not the only locus controlling annual bolting in *B. vulgaris*. Bolting control involves at least three other loci, i.e., *B2* [Büttner *et al.*, 2010 (Chapter 3)], *B3* and *B4*, ii) the *B* locus (or a locus closely linked to *B*) appears to be mutated or not functional in one mutant family, suggesting that an EMS-induced mutation at this locus can be sufficient to convert an annual genotype into a biennial genotype, iii) locus *B3* is unlinked to the *B* gene and appears to act independently of the *B* locus, and seems to be inherited from the annual *B. vulgaris* ssp. *maritima* accession, and iv) the *B4* locus was genetically mapped to chromosome II of sugar beet (~9 cM from the *B* gene).

Locus *B3* was proposed to act independently from the *B* locus in regulating bolting behavior because the F₂ population where the *B3* locus was identified (EW4a; Chapter 3) exhibited a digenic phenotypic segregation ratio (15:1) with a complete co-segregation between the *B* locus marker allele derived from the biennial parent and the non-bolting phenotype. Similarly to the *B2* locus (Chapter 3), the *B4* locus was suggested to act epistatically to the *B* locus because; i) the two F₂ populations where the *B4* locus was identified and genetically mapped exhibited phenotypic segregation ratios which did not deviate significantly from the 3: 1 expected ratio, ii) the non-complete co-segregation between the bolting phenotypes and the *B* locus marker genotypes in both populations indicates that the *B* gene is not, or not alone responsible for bolting behavior in both populations, iii) the absence of an independent segregation between the bolting phenotypes and the *B* locus marker genotypes would be consistent with the presence of an epistatic locus which is genetically linked to locus *B* on chromosome II. On the other hand, the possibility that the *B4* locus acts independently from the *B* gene cannot be excluded. This possibility was supported by the phenotypic segregation ratio in both populations which exhibited an excess in the proportion of the non-bolting individuals

(4.29:1 and 4.20:1, bolting: non-bolting, in populations EW5a and EW5b, respectively). This phenotypic segregation ratio is consistent with the expected ratio resulted from two linked loci each acting independently from the other.

The genetic control of bolting and flowering is best understood in the dicot model species *A. thaliana* and temperate cereals (Bäurle and Dean 2006; Colasanti and Coneva 2009; Distelfeld *et al.*, 2009; Greenup *et al.*, 2009a; Jung and Müller 2009). In *Arabidopsis*, which is distantly related to *B. vulgaris*, bolting and flowering is controlled by the central regulator of vernalization requirement and response *FLOWERING LOCUS C (FLC)*, which acts as a floral repressor and is down-regulated during vernalization. Furthermore, according to Wollenberg *et al.* (2008) far-red light enrichment can induce floral transition by bypassing the *FLC*-controlled pathway which mediates vernalization requirement, and by promoting the expression of *CONSTANS (CO)* and *GIGANTEA (GI)*. The effect of light quality on floral transition has been reported in several plant species including rice and soybean (Izawa *et al.*, 2002; Liu *et al.*, 2008). In the light of our results, with identifying three new genes involved in bolting control of sugar beet, it would not be unlikely that these genes are involved in manipulating different bolting regulatory pathways including the photoperiod pathway. Loci acting epistatically to *B*, i.e. *B2* and possibly *B4*, would appear to function in the same flowering control pathway as *B*, whereas *B3* (and possibly *B4*), which appears to act independently of *B*, can be assumed to function in a different pathway than *B*.

In *B. vulgaris*, the genetic basis of bolting and floral transition regulation has started to be dissected. Two pathways were proposed to induce bolting independently, i.e., the photoperiod-dependent pathway (involving the *B* gene; A. Müller, personal communication) and the vernalization-dependent pathway which is also influenced by gibberellic acid (“GA”) signalling (Mutasa-Gottgens *et al.*, 2010). Under non-inductive short days, GA treatment promotes bolting initiation in both annual and biennial plants. Furthermore, GA promotes stem elongation in biennial plants irrespective of photoperiod, but did so only after vernalization, suggesting an independent role of vernalization on GA-induced stem elongation (Mutasa-Gottgens *et al.*, 2010). However, neither vernalization nor GA treatment could replace long days required for flowering, suggesting a distinct separation between bolting and flowering. Each of these two developmental stages requires a combination of different regulatory stimuli. A second bolting locus (termed *Lr*) which was speculated to be closely linked to the *B* locus was suggested to be involved in the photoperiodic regulation of bolting and flowering in sugar beet via epistatic interaction to the *B* gene as a modifying gene under unfavorable daylength conditions (Abe *et al.*, 1997). A homolog of *FLC* in *B. vulgaris* (*BvFL1*) was cloned and was found to be functionally related to *FLC* (Reeves *et al.*, 2007). A homolog of the main gene of the photoperiod pathway *CO* (*BvCOL1*) was cloned and

genetically mapped to chromosome II in *B. vulgaris*, and was reported to not correspond to the *B* gene (Chia *et al.*, 2008).

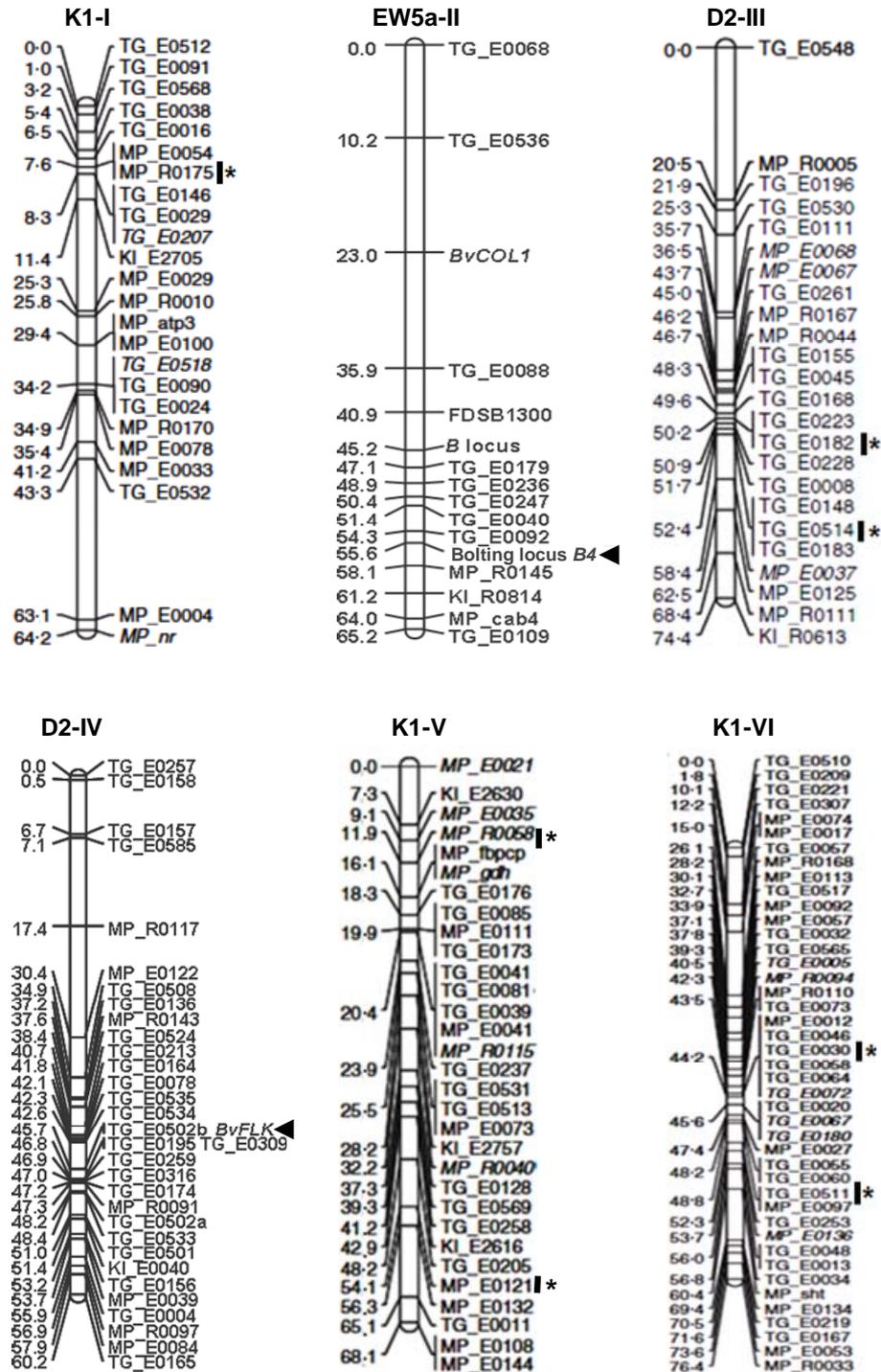
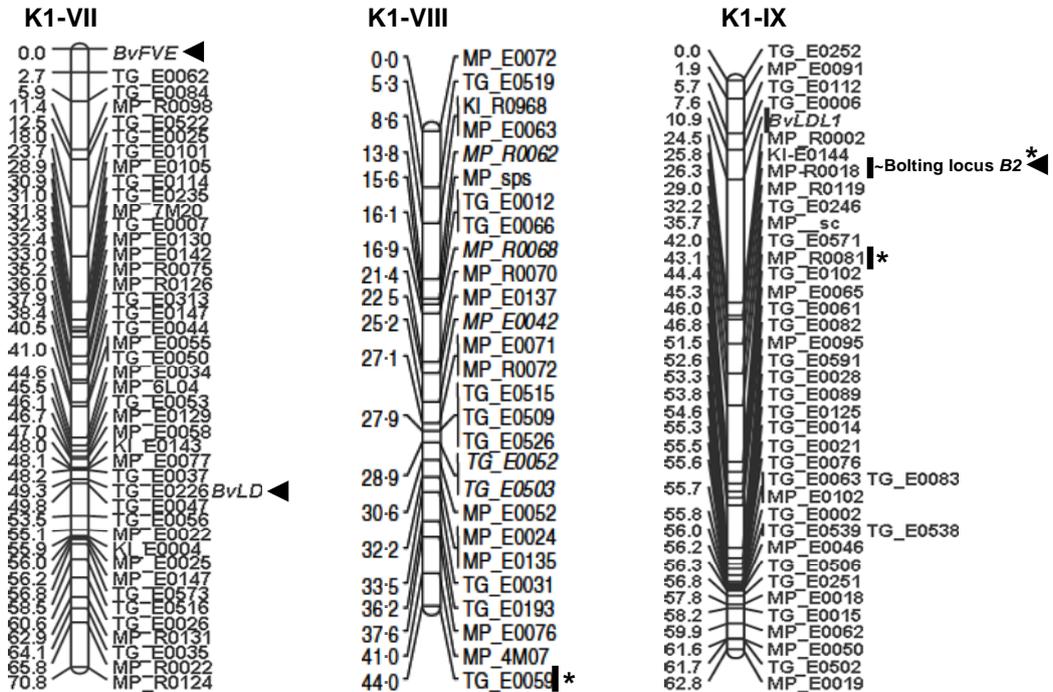


Figure 11: Genetic map positions of bolting loci and floral transition genes in sugar beet in mapping populations K1, D2 (Schneider *et al.*, 2007) and EW5a (Chapter 4). Marker names are indicated at the right side of each linkage group, and cumulative genetic distances are given at the left side. Asterisks

indicate the closest markers to floral transition genes in various mapping populations by G. Schulze-Buxloh (personal communication). Arrows indicate bolting loci and genes described in Chapters 2-4.

Fig. 1 continued



The possibility that *BvFL1* is the *B* gene was excluded, although conserved mechanisms between the two genes in terms of vernalization responsiveness and bolting time regulation in transgenic Arabidopsis was reported, because; i) the genetic map position of *BvFL1* (chromosome VI), and ii) the lack of clear difference in the expression and regulation of *BvFL1* between the annual and biennial genotypes of *B. vulgaris* (Reeves *et al.*, 2007). Because the *B* gene is not *BvFL1*, it is unlikely that the *B2* gene is a homolog of a gene acting epistatically to *FLC* in Arabidopsis. The genetic map position of *B2* locus also rules out the possibility that it is a homolog of either the autonomous pathway genes *BvLD*, *BvLDL1*, *BvFVE1*, or *BvFLK* (Chapter 2). Furthermore, a comparison of the current mapping data for *B2* with unpublished mapping data by Schulze-Buxloh (personal communication) in other populations suggest that *B2* also does not co-localize with the gibberellic acid pathway gene *BvGA3ox1* or the floral integrator gene *BvFT* which were mapped to different regions of chromosome IX (Figure 11; G. Schulze-Buxloh, personal communication). However, the absence of co-localization has yet to be verified in population EW2 and in a newly established larger population segregating for *B2* (Nadine Dally, personal communication). From the fact that the *B* gene is a homolog of a gene regulating photoresponse in Arabidopsis (A. Müller, personal communication) it is not unlikely that the *B2* gene is also homolog of a

gene which epistatically co-regulates photoresponse in Arabidopsis. Candidates for *B2* may thus include homologs of photoreceptor, circadian clock and photoperiod pathway genes such as , *CIRCADIAN CLOCK ASSOCIATED 1 (CCA1)*, *LATE AND ELONGATED HYPOCOTYL (LHY)*, *TIMING OF CAB EXPRESSION (TOC1)*, *ZEITLUPE (ZTL)* and *CYCLING DOF FACTOR 1 (CDF1)*.

In the light of phenotypic and genotypic analysis of the two F₂ populations where *B4* was identified and genetically mapped, there are two conceivable modes of actions of the *B4* gene as described above. According to the first model whereby *B4* acts epistatically to the *B* gene, candidate genes for *B4* include the same genes as listed above for *B2*. According to the second model whereby the *B4* gene acts independently from the *B* gene, it is tempting to speculate that *B4* belongs to a different regulatory pathway. The genetic map position of *B4* locus excludes the possibility that it corresponds to either *BvCOL1* gene or the *GI* homolog *BvGI* (Figure 11; G. Schulze-Buxloh, personal communication). However, *B4* locus is a possible homolog for a gene which might be involved in other floral transition regulatory pathways, e.g. the gibberellic acid pathway, which acts independently from the *B* gene (Mutasa-Gottgens *et al.*, 2010).

The unknown genetic map position of locus *B3* widens the spectrum of speculation for possible candidates. However, the phenotypic segregation ratio and the co-segregation analysis of the F₂ population where *B3* locus was identified (EW4a) preclude the possibility that it is located on chromosome II. In this regard, locus *B3* could be a homolog of any of the flowering time genes located outside chromosome II, and acting in an independent pathway from the *B* gene, including *FLC*. The colocalization of *B* with *BvCOL1* and *BvGI* on the same chromosome suggests that *B3* is not corresponding to either *BvCOL1* or *BvGI*.

In conclusion, the forward genetic approach was successfully applied in sugar beet and provides the first evidence, in contrast to what had been thought previously, that the *B* locus is not the only locus responsible for bolting behavior in *B. vulgaris*. In addition to locus *B2* (Büttner *et al.*, 2010 (Chapter 3); B. Büttner (Ph.D. thesis in preparation)), two additional bolting loci, *B3* and *B4*, were identified to be involved in bolting behavior regulation; locus *B3* acts independently of *B* and is unlinked to *B*, and locus *B4* acts epistatically to or independently from the *B* locus and was mapped to chromosome II. These bolting loci could be targeted to modify the responsiveness to environmental cues in order to manipulate bolting tendency of sugar beet varieties towards the development of winter sugar beet. Although the effect of these loci on bolting time after vernalization has not yet been investigated, these genes may also prove to be candidate genes for the facilitation of induction and synchronization of bolting and flowering for hybrid seed production.

Contrary to the procedure of the forward genetic approach, the reverse genetic approaches seek to uncover the function of a gene of interest by analyzing the organism for developmental or behavioral effects resulting from sequence variation in a gene of interest identified by DNA sequencing, or from perturbing the function of the gene of interest e.g. by overexpression or silencing. Altering the function of a given gene, which includes physical, chemical and biological methods, is a common technique in both forward and reverse genetic approaches. A typical reverse genetic approach starts with identifying unknown genes based on their sequence similarities to genes of known functions in another organism (Alonso and Ecker, 2006; Gilchrist *et al.*, 2006). Reverse genetics is an important approach to complement the forward genetics approach, and could be efficiently used in different aspects including; i) to investigate the function of all genes in a gene family, which is inapplicable in forward genetics, ii) to analyze the function of a gene of interest which is known to be involved in a given biological process in another organism, for which no forward genetic mutants have yet been identified, and iii) since only a limited number of genes have been mutated in most species, reverse genetics could be a useful approach to study the function of those non-mutated genes (Winkler *et al.*, 1998; Burnett *et al.*, 2003; Fatland *et al.*, 2005; Gilchrist *et al.*, 2006; Gilchrist and Haughn, 2010). Although genome sequence projects of many organisms have revealed a huge number of genes with unidentified function which allows the study of every gene by reverse genetics, identifying a specific phenotype for many plant genes is not feasible (Alonso *et al.*, 2003). In plants, there are many approaches and resources which facilitate the implementation of reverse genetics (May *et al.*, 2002; An *et al.*, 2005). The best reverse genetic tool differs among plant species and depends on the species and the questions to be addressed. In addition to the availability of T-DNA and transposon insertion mutants which make them attractive resources, RNAi and Virus-Induced Gene Silencing (VIGS) are the most attractive approaches of reverse genetics. The huge amount of sequence information that has been generated by genome sequencing of model plant species (e.g., *A. thaliana* and *Oryza sativa*), in addition to the information available from genome-wide expression databases, have underlined the importance of reverse genetics as a powerful approach in dissecting the genetic control of various plant-specific biological processes (Alonso *et al.*, 2006; Gilchrist and Haughn, 2010). Several examples highlight the recent progress that has been achieved via the application of the reverse genetic approaches including different economically important species such as wheat and barley (Slade *et al.*, 2005; Faure *et al.*, 2007). With the development of efficient sequencing technologies (now referred to as second and third generation sequences), the most powerful and cost-effective technique to study the function of a given gene in plants in the future could be achieved via direct sequencing of part or complete genomes (Winkler *et al.*, 1998).

Here, the reverse genetic approach was used to identify *B. vulgaris* homologs of the *A. thaliana* autonomous pathway genes *FLK*, *FVE*, *LD* and *LDL1* (Chapter 2). The reverse

genetic approach was to a great extent a successful approach in identifying several *B. vulgaris* ESTs with high degree of homology to genes of the autonomous pathway in *A. thaliana*. The focus of the current thesis is the identification, genetic mapping and functional characterization of *BvFLK*, in addition to identification and genetic mapping of *BvLDL1*. *BvFLK* was functionally characterized by expression analysis and overexpression and complementation analyses in Arabidopsis wild type and mutant plants, respectively. My results showed that *BvFLK* is able to fully rescue the late bolting phenotype of the Arabidopsis *flk1* mutant and accelerate bolting time of the Arabidopsis wild type Col-0 when it was expressed under either the 35S promoter or its own promoter (endo.). Besides, *BvFLK* was found to mediate flowering time in Arabidopsis through *AtFLC*, where *AtFLC* expression levels in the Arabidopsis *flk1* mutant carrying the *BvFLK* transgene driven by either the 35S promoter or its own promoter were strongly reduced compared to the untransformed mutant, suggesting a conserved mechanism of the autonomous pathway gene homolog *BvFLK* in regulating floral transition in sugar beet, possibly by repressing the expression of the sugar beet *FLC* homolog, *BvFLI*. Contrary to the effect of *BvFLK*, *BvFVE1* was not able to either rescue the late bolting phenotype of the Arabidopsis *fve-7* mutant plants or to accelerate bolting time in Arabidopsis wild type plants when it was expressed under the CaMV 35S promoter [(Abou-Elwafa *et al.*, 2010 (Chapter 2)].

The conserved function between *AtFLK* and its *B. vulgaris* homolog *BvFLK* in floral transition regulation revealed that reverse genetics is a powerful approach in discovering gene function of a particular gene based on sequence similarity to a gene of known function. However, the apparent functional divergence between *AtFVE* and the *B. vulgaris* homolog *BvFVE1*, in terms of flowering time regulation, despite a high sequence similarity compared to the other studied gene homologs, highlights the fact that sequence conservation may not be reliable predictor of functional conservation and that the usefulness of reverse genetic approaches has limitations.

In summary, the implementation of both forward and reverse genetic approaches brought new insights towards understanding the regulation of bolting and floral transition in *B. vulgaris*. Two novel loci, *B3* and *B4*, were implicated in bolting behavior regulation of *B. vulgaris*. Genetic map positions of the autonomous pathway homologs of *B. vulgaris* revealed that there is no co-localization between these genes and the novel bolting loci, suggesting that none of these genes corresponds to any of the newly identified loci. Locus *B3* is unlinked to the *B* gene and acts independently of *B* in bolting behavior control, suggesting that the gene may regulate floral transition not through the photoresponse pathway which includes the *B* gene. Locus *B4* is a potential candidate for a flowering time gene homolog which is linked to the *B* locus (~9 cM), and acts either independently or epistatically to the *B* gene. Finally, functional conservation of a homolog of an autonomous pathway gene in *B. vulgaris* was reported here for the first

time, where *BvFLK* was shown to be evolutionarily and functionally related to the *Arabidopsis* gene *FLK*.

In this study we started to dissect the genetic basis of reproductive transition in *B. vulgaris*, however more experiments are needed for further uncovering the genetic control of bolting and flowering of sugar beet and the genetic interaction among the newly identified loci. Positional cloning experiments of the new bolting genes, *B2* and *B4*, are required as an initial step for targeting modification of bolting behavior of sugar beet towards the development of winter sugar beet which is the fundamental goal of sugar beet breeders, and could only be achieved via modifying the responsiveness to environmental cues by genetic manipulation of bolting and flowering genes (Jung and Müller 2009). In addition, these new bolting and floral transition genes could be used as tools in different breeding aspects such as marker-assisted selection against bolting tendency in sugar beet, synchronization of bolting and flowering time for hybrid seed production purposes, and breeding programs for sugar beet production adapted to tropical and subtropical areas.

6. Summary

Floral transition is a critical developmental switch in the plant life cycle which requires an integrated response to multiple environmental cues and endogenous signals. In *A. thaliana*, several major regulatory pathways, including the vernalization, photoperiod, and autonomous pathways, have been described which converge to regulate floral transition through a set of floral integrator genes. In *Beta vulgaris*, induction and timing of bolting and flowering directly affects both yield potential and seed production. *B. vulgaris* homologs of the central floral repressor gene in *A. thaliana*, *FLOWERING LOCUS C (FLC)*, which functions downstream of the vernalization pathway, and the photoperiod pathway gene *CONSTANS (CO)* were cloned and shown to be functionally related to the respective genes in Arabidopsis. This provides first evidence for the conservation of the genetic basis of floral transition in response to environmental cues. However, the presence of an autonomous pathway which functions in parallel to the vernalization and photoperiod pathways and promotes floral transition by repression of *FLC* has not yet been reported for *B. vulgaris*. The annual habit in *B. vulgaris* was shown to be under the genetic control of a dominant Mendelian factor termed *bolting gene (B)*. The *B* locus was mapped by RFLP- and high resolution AFLP-mapping to chromosome II.

In the present study both forward and reverse genetic approaches were implemented for further discovering the genetic basis of bolting and flowering control in sugar beet. In the forward genetic approach, four F₂ populations segregating for bolting behavior derived from crosses between two EMS-mutagenized biennial and annual parents were analyzed for bolting behavior. Co-segregation analysis of bolting phenotypes with genotypic markers located at the *B* locus in all four F₂ populations revealed at least two previously unidentified bolting control loci, *B3* and *B4*. The data suggest that, locus *B3* acting independently from the *B* locus in bolting behavior control. Locus *B4* was found to be linked to the *B* locus on chromosome II (at a genetic distance of ~9 cM), and may act epistatically to or independently from the *B* gene in bolting control.

In the reverse genetic approach, *BvFLK*, a putative ortholog of the RNA-regulatory autonomous pathway gene *FLOWERING LOCUS KH DOMAIN (FLK)*, was identified, genetically mapped, and analyzed in more detail. Exon-intron structure and domain organization was found to be conserved between *BvFLK* and *FLK*. Similar to *FLK* expression in Arabidopsis, *BvFLK* transcript accumulation was detected in all plant tissues analyzed, with the highest levels of expression in flowers and roots. *BvFLK* accelerates bolting in transgenic *A. thaliana* plants and fully complements the late-bolting phenotype of an Arabidopsis *flk* mutant when expressed either under control of the CaMV 35S promoter or its own promoter. This acceleration in bolting time was shown to be mediated through repression of *FLC* expression. Together, our data suggest evolutionary conservation of the autonomous pathway gene *FLK* between *A. thaliana*

and *B. vulgaris*. The results are likely to be of broader relevance because *B. vulgaris* is only distantly related to model plants and expands the spectrum of evolutionarily diverse species for which genetic information on the regulation of floral transition is increasingly available.

7. Zusammenfassung

Der Übergang zum Blühen ist kritisch in der Entwicklung der Pflanze und benötigt eine integrierte Antwort auf verschiedene ökologische und endogene Signale. In *A. thaliana* wurden einige der wichtigen regulatorischen Wege beschrieben, inklusive des Vernalisations-, des Photoperioden- und des autonomen Regulationswegs, die Blühen gemeinsam durch eine Gruppe von Blühintegrationsgenen regulieren. In *Beta vulgaris* beeinflusst der Zeitpunkt des Schossens und Blühens den Ertrag und die Saatgutproduktion. *B. vulgaris*-Homologe des zentralen Blührepressors in *A. thaliana* *FLOWERING LOCUS C (FLC)*, der oberhalb des Vernalisationswegs arbeitet, und das Photoperioden-Gen *CONSTANS (CO)* wurden kloniert, und es konnte gezeigt werden, dass sie funktionell mit den entsprechenden Genen aus *Arabidopsis* verwandt sind. Dies sind erste Hinweise für die Konservierung der genetischen Grundlage der Blühregulation in Antwort auf Umweltsignale. Jedoch wurde die Anwesenheit eines autonomen Wegs, der parallel zu dem Vernalisations- und dem Photoperiodenweg wirkt und Blühen durch die Repression von *FLC* fördert, bisher nicht in *B. vulgaris* beschrieben. Es wurde gezeigt, dass Einjährigkeit in *B. vulgaris* unter der Kontrolle eines einzelnen Mendelschen Faktors steht, des Schossgens *B*. Das Schossgen wurde mittels RFLP- und hochauflösender AFLP-Kartierung auf Chromosom II kartiert.

In der vorliegenden Studie wurden „forward“ und „reverse“ genetische Ansätze verwendet, um die genetische Grundlage der Schoss- und Blühkontrolle in Zuckerrübe weiter zu untersuchen. Im ersten Ansatz wurden vier F₂-Populationen, die für Schossverhalten spalten und aus Kreuzungen zwischen EMS-mutagenisierten und einjährigen Eltern stammen, auf Schossverhalten untersucht. Die Kosegregationsanalyse des Schossphänotypes mit genetischen Markern am *B*-Locus in allen vier F₂-Populationen identifizierte zumindest zwei vorher unbekannte Loci, *B3* und *B4*. Die Daten deuten an, dass der *B3*-Locus Schossen unabhängig von *B* kontrolliert. Für den *B4*-Locus wurde gefunden, dass er mit dem *B*-Locus auf Chromosom II gekoppelt ist (in einem genetischen Abstand von ~9 cM), und Schossen epistatisch oder unabhängig von *B* regulieren könnte.

Im zweiten Ansatz wurde *BvFLK*, ein putatives Ortholog des RNA-regulierenden Gens *FLOWERING LOCUS KH DOMAIN (FLK)*, ein Gen des autonomen Wegs, identifiziert, genetisch kartiert und im Detail analysiert. Es wurde gezeigt, dass die Exon-Intron-Struktur und Domänenorganisation zwischen *BvFLK* und *FLK* konserviert ist. Ähnlich der *FLK* Expression in *Arabidopsis* wurde eine Anhäufung der *BvFLK* Transkripte in allen untersuchten Geweben nachgewiesen, wobei die höchste Expression in Blüten und Wurzeln auftrat. *BvFLK* beschleunigt Schossen in transgenen *A. thaliana*-Pflanzen und komplementiert den spät-schossenden Phänotyp einer *Arabidopsis flk*-Mutante vollständig, wenn es entweder unter der Kontrolle des CaMV 35S-Promoters oder des eigenen Promoters exprimiert wird. Es wurde gezeigt, dass die Beschleunigung des

Schossens durch die Repression der *FLC*-Expression vermittelt wird.

Zusammengenommen weisen unsere Daten auf die evolutionäre Konservierung von *FLK* zwischen *A. thaliana* und *B. vulgaris* hin. Das Ergebnis ist vermutlich von weiterer Bedeutung, da *B. vulgaris* nur sehr entfernt mit Modellpflanzen verwandt ist und das Spektrum der evolutionär verschiedenen Arten erweitert, für die genetische Information über Blühzeitkontrolle vorhanden ist.

8. References

1. Abd Elrahim, A.M., Abou-Salama, E.A., Teama, and S.F., Abou-Elwafa. Effect of planting and harvesting dates on growth, yield and quality of sugar beet varieties in Middle Egypt. Political, Economic and Technological Challenges for Sugar and its Integrated Industries in the Arab Region, the Middle East, Africa and the European Union. April 3-6, 2005, Alexandria, Egypt, P5 . 3-4-2005.
2. Adinolfi, S., Bagni, C., Castiglione Morelli, M. A., Fraternali, F., Musco, G., and Pastore, A., 1999: Novel RNA-binding motif: the KH module. *Biopolymers* **51**, 153-164.
3. Adrian, J., Torti, S., and Turck, F., 2009: From Decision to Commitment: The Molecular Memory of Flowering. *Mol Plant* **2**, 628-642.
4. Alonso, J. M. and Ecker, J. R., 2006: Moving forward in reverse: genetic technologies to enable genome-wide phenomic screens in Arabidopsis. *Nat.Rev.Genet.* **7**, 524-536.
5. Alonso, J. M., Stepanova, A. N., Leisse, T. J., Kim, C. J., Chen, H., Shinn, P., Stevenson, D. K., Zimmerman, J., Barajas, P., Cheuk, R., Gadrinab, C., Heller, C., Jeske, A., Koesema, E., Meyers, C. C., Parker, H., Prednis, L., Ansari, Y., Choy, N., Deen, H., Geralt, M., Hazari, N., Hom, E., Karnes, M., Mulholland, C., Ndubaku, R., Schmidt, I., Guzman, P., guilar-Henonin, L., Schmid, M., Weigel, D., Carter, D. E., Marchand, T., Risseuw, E., Brogden, D., Zeko, A., Crosby, W. L., Berry, C. C., and Ecker, J. R., 2003: Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science* **301**, 653-657.
6. An, G., Jeong, D. H., Jung, K. H., and Lee, S., 2005: Reverse genetic approaches for functional genomics of rice. *Plant Mol.Biol.* **59**, 111-123.
7. Avanci, N. C., Luche, D. D., Goldman, G. H., and Goldman, M. H., 2010: Jasmonates are phytohormones with multiple functions, including plant defense and reproduction. *Genet.Mol.Res.* **9**, 484-505.
8. Balakrishnan, A. and Selvakumar, T., 2009: Evaluation of Suitable Tropical Sugarbeet Hybrids with Optimum Time of Sowing. *Sugar Tech* **11**, 65-68.
9. Bartsch, D., Lehen, M., Clegg, J., Pohl-Orf, M., Schuphan, I., and Ellstrand, N., 1999: Impact of gene flow from cultivated beet on genetic diversity of wild sea beet populations. *Molecular Ecology* **8**, 1733-1741.
10. Bastow, R., Mylne, J. S., Lister, C., Lippman, Z., Martienssen, R. A., and Dean, C., 2004: Vernalization requires epigenetic silencing of FLC by histone methylation. *Nature* **427**, 164-167.

11. Bates, B., Kundzewicz, S. W., Palutikof, J., and Wu, S. Climate change and water. Technical Paper of the Intergovernmental Panel on Climate Change, IPCC Secretariat, Geneva, 210 pp . 2008.
12. Bernier, G. and Perilleux, C., 2005: A physiological overview of the genetics of flowering time control. *Plant Biotechnol.J.* **3**, 3-16.
13. Biancardi, E., 2005: Objectives of Sugar Beet Breeding. In: E. Biancardi, G. L. Campbell, G. N. Skaracis, and M. De Biaggi (eds.), *Genetics and Breeding of Sugar Beet*, 58-163. Science, Enfield, (NH), USA.
14. Bosemark, N. O., 1993: Genetics and Breeding. In: D. A. Cooke and R. K. Scott (eds.), *The Sugar Beet Crop*, 66-119. CHAPMAN & HALL, London, UK.
15. Bruun, L., Haldrup, A., Petersen, S. G., Frese, L., den Bock, Th. S. M., and Lang, A., 1995: Self-incompatibility reactions in wild species of the genus *Beta* and their relation to taxonomical classification and geographical origin. *Genetic Resources and Crop Evolution* **42**, 293-301.
16. Buckler, E. S., Holland, J. B., Bradbury, P. J., Acharya, C. B., Brown, P. J., Browne, C., Ersoz, E., Flint-Garcia, S., Garcia, A., Glaubitz, J. C., Goodman, M. M., Harjes, C., Guill, K., Kroon, D. E., Larsson, S., Lepak, N. K., Li, H., Mitchell, S. E., Pressoir, G., Peiffer, J. A., Rosas, M. O., Rocheford, T. R., Romay, M. C., Romero, S., Salvo, S., Sanchez, V. H., da Silva, H. S., Sun, Q., Tian, F., Upadhyayula, N., Ware, D., Yates, H., Yu, J., Zhang, Z., Kresovich, S., and McMullen, M. D., 2009: The genetic architecture of maize flowering time. *Science* **325**, 714-718.
17. Burnett, E. and Tattersall, P., 2003: Reverse Genetic System for the Analysis of Parvovirus Telomeres Reveals Interactions between Transcription Factor Binding Sites in the Hairpin Stem. *J.Virol.* **77**, 8650-8660.
18. Cai, D., Kleine, M., Kifle, S., Harloff, H. J., Sandal, N. N., Marcker, K. A., Klein-Lankhorst, R. M., Salentijn, E. M., Lange, W., Stiekema, W. J., Wyss, U., Grundler, F. M., and Jung, C., 1997: Positional cloning of a gene for nematode resistance in sugar beet. *Science* **275**, 832-834.
19. Cakiroglu, O. and Gifford, R. Turkey Sugar Annual 2010. Gloabl Agricultural Information network. 15-4-2010. USDA Foreign Agricultural Service.
20. Capistrano, G. A candidate sequence for the nematode resistance gene Hs1-2 in sugar beet. Ph.D thesis. 2010. Plant Breeding Institute, Christian-Albrechts-Universität zu Kiel.
21. Chandler, J., Wilson, A., and Dean, C., 1996: Arabidopsis mutants showing an altered response to vernalization. *Plant J.* **10**, 637-644.

22. Chirumamilla, A., Yocum, G. D., Boetel, M. A., and Dregseth, R. J., 2008: Multi-year survival of sugarbeet root maggot (*Tetanops myopaeformis*) larvae in cold storage. *J. Insect Physiol* **54**, 691-699.
23. Choi, J., Hyun, Y., Kang, M. J., In, Y. H., Yun, J. Y., Lister, C., Dean, C., Amasino, R. M., Noh, B., Noh, Y. S., and Choi, Y., 2009: Resetting and regulation of Flowering Locus C expression during *Arabidopsis* reproductive development. *Plant J.* **57**, 918-931.
24. Choudhary, K., Choudhary, O. P., and Shekhawat, N. S., 2008: Marker Assisted Selection: A Novel Approach for Crop Improvement. *American-Eurasian Journal of Agronomy* **1**, 26-30.
25. Collard, B. C. and Mackill, D. J., 2008: Marker-assisted selection: an approach for precision plant breeding in the twenty-first century. *Philos. Trans. R. Soc. Lond B Biol. Sci* **363**, 557-572.
26. Darmency, H., Klein, E. K., De Garanbe, T. G., Gouyon, P. H., Richard-Molard, M., and Muchembled, C., 2009: Pollen dispersal in sugar beet production fields. *Theor. Appl. Genet.* **118**, 1083-1092.
27. De Biaggi, M., 2005: Objectives of Sugar Beet Breeding: Self-fertility and Self-incompatibility. In: E. Biancardi, G. L. Campbell, N. G. Skaracis, and M. De Biaggi (eds.), *Genetics and Breeding of Sugar Beet*, 169-212. Science, Enfield, (NH), USA.
28. Dennis, E. S. and Peacock, W. J., 2009: Vernalization in cereals. *J. Biol.* **8**, 57.
29. Desel, C., Jung, C., Cai, D., Kleine, M., and Schmidt, T., 2001: High-resolution mapping of YACs and the single-copy gene *Hs1(pro-1)* on *Beta vulgaris* chromosomes by multi-colour fluorescence in situ hybridization. *Plant Mol. Biol.* **45**, 113-122.
30. Distelfeld, A., Li, C., and Dubcovsky, J., 2009: Regulation of flowering in temperate cereals. *Curr. Opin. Plant Biol.* **12**, 178-184.
31. Dubnau, J. and Struhl, G., 1996: RNA recognition and translational regulation by a homeodomain protein. *Nature* **379**, 694-699.
32. El-Geddawy, I. H., Kheiralla, K. A., Darweish, Y. Y. I., and Sharaf, A. M., 2008: Agricultural practices in relation to yield and quality of sugar beet: Yield and yield components. *Sugar Tech* **10**, 227-233.
33. Fatland, B. L., Nikolau, B. J., and Wurtele, E. S., 2005: Reverse Genetic Characterization of Cytosolic Acetyl-CoA Generation by ATP-Citrate Lyase in *Arabidopsis*. *Plant Cell* **17**, 182-203.
34. Faure, S., Higgins, J., Turner, A., and Laurie, D. A., 2007: The FLOWERING LOCUS T-like gene family in barley (*Hordeum vulgare*). *Genetics* **176**, 599-609.

35. Fenart, S., Arnaud, J. F., De, C., I, and Cuguen, J., 2008: Nuclear and cytoplasmic genetic diversity in weed beet and sugar beet accessions compared to wild relatives: new insights into the genetic relationships within the *Beta vulgaris* complex species. *Theor.Appl.Genet.* **116**, 1063-1077.
36. Feng, S., Martinez, C., Gusmaroli, G., Wang, Y., Zhou, J., Wang, F., Chen, L., Yu, L., Iglesias-Pedraz, J. M., Kircher, S., Schafer, E., Fu, X., Fan, L. M., and Deng, X. W., 2008: Coordinated regulation of *Arabidopsis thaliana* development by light and gibberellins. *Nature* **451**, 475-479.
37. Ford-Lloyd, B., 2005: Sources of genetic variation, genus *Beta*. In: E. Biancardi, G. L. Campbell, G. N. Skaracis, and M. De Biaggi (eds.), *Genetics and breeding of sugar beet*, 25-33. Enfield (NH), USA.
38. Frisch, M. and Melchinger, A. E., 2005: Selection theory for marker-assisted backcrossing. *Genetics* **170**, 909-917.
39. Gaafar, R. M. Fine mapping of the bolting gene of sugar beet (*Beta vulgaris* L.) using BAC-derived sequences. 2005. Christian-Albrechts-Universität zu Kiel.
40. Gibson, T. J., Thompson, J. D., and Heringa, J., 1993: The KH domain occurs in a diverse set of RNA-binding proteins that include the antiterminator NusA and is probably involved in binding to nucleic acid. *FEBS Lett.* **324**, 361-366.
41. Gilchrist, E. J., O'Neil, N. J., Rose, A. M., Zetka, M. C., and Haughn, G. W., 2006: TILLING is an effective reverse genetics technique for *Caenorhabditis elegans*. *BMC.Genomics* **7**, 262.
42. Gilchrist, E. and Haughn, G., 2010: Reverse genetics techniques: engineering loss and gain of gene function in plants. *Briefings in Functional Genomics* **9**, 103-110.
43. Gregis, V., Sessa, A., Dorca-Fornell, C., and Kater, M. M., 2009: The *Arabidopsis* floral meristem identity genes AP1, AGL24 and SVP directly repress class B and C floral homeotic genes. *Plant J.* **60**, 626-637.
44. Grimmer, M. K., Bean, K. M., and Asher, M. J., 2007: Mapping of five resistance genes to sugar-beet powdery mildew using AFLP and anchored SNP markers. *Theor.Appl.Genet.* **115**, 67-75.
45. Grimmer, M. K., Trybush, S., Hanley, S., Francis, S. A., Karp, A., and Asher, M. J., 2007: An anchored linkage map for sugar beet based on AFLP, SNP and RAPD markers and QTL mapping of a new source of resistance to Beet necrotic yellow vein virus. *Theor.Appl.Genet.* **114**, 1151-1160.
46. Guven, C. I., Sherif, S. I., and Gressel, J. P. Egypt Sugar Annual. Gloabl Agricultural Information network. 22-4-2010. USDA Foreign Agricultural Service.

47. Hagihara, E., Itchoda, N., Habu, Y., Iida, S., Mikami, T., and Kubo, T., 2005: Molecular mapping of a fertility restorer gene for Owen cytoplasmic male sterility in sugar beet. *Theor.Appl.Genet.* **111**, 250-255.
48. Hajheidari, M., bdollahian-Noghabi, M., Askari, H., Heidari, M., Sadeghian, S. Y., Ober, E. S., and Salekdeh, G. H., 2005: Proteome analysis of sugar beet leaves under drought stress. *Proteomics.* **5**, 950-960.
49. Halliday, K. J., Martinez-Garcia, J. F., and Josse, E. M., 2009: Integration of light and auxin signaling. *Cold Spring Harb.Perspect.Biol.* **1**, a001586.
50. Havey, M. J., 2004: The use of Cytoplasmic Male Sterility for Hybrid Seed Production. In: H. Daniell and C. D. Chase (eds.), *Molecular Biology and Biotechnology of Plant Organelles*, 623-634. Springer, The Netherlands.
51. Hayama, R. and Coupland, G., 2003: Shedding light on the circadian clock and the photoperiodic control of flowering. *Curr.Opin.Plant Biol.* **6**, 13-19.
52. Hjerdin, A., Säll, T., Nilsson, N. O., Borman, C. H., and Hallden, C., 1991: Genetic Variation Among Wild and Cultivated Beets of the Scetion Beta as Revealed by RFLP Analysis. *Journal of Sugar Beet Research* **312**, 59-67.
53. Hoffmann, C. M., 2010: Sucrose Accumulation in Sugar Beet Under Drought Stress. *J.Agronomy & Crop Science* 1-10.
54. Hunger, S., Di, G. G., Mohring, S., Bellin, D., Schafer-Pregl, R., Borchardt, D. C., Durel, C. E., Werber, M., Weisshaar, B., Salamini, F., and Schneider, K., 2003: Isolation and linkage analysis of expressed disease-resistance gene analogues of sugar beet (*Beta vulgaris* L.). *Genome* **46**, 70-82.
55. Hytonen, T., Elomaa, P., Moritz, T., and Junttila, O., 2009: Gibberellin mediates daylength-controlled differentiation of vegetative meristems in strawberry (*Fragaria x ananassa* Duch). *BMC.Plant Biol.* **9**, 18.
56. Imaizumi, T., 2009: Arabidopsis circadian clock and photoperiodism: time to think about location. *Curr.Opin.Plant Biol.*
57. Ivic, H. D. S. and Smigocki, C. A., 2005: Biolistic transformation of highly regenerative sugar beet (*Beta vulgaris* L.) leaves. *Plant Cell Rep* **23**, 699-704.
58. Jackson, S. D., 2009: Plant responses to photoperiod. *New Phytol.* **181**, 517-531.
59. Jaggard, K. W., Qi, A., and Ober, E. S., 2009: Capture and use of solar radiation, water, and nitrogen by sugar beet (*Beta vulgaris* L.). *J.Exp.Bot.* **60**, 1919-1925.
60. Jaggard, K. W. and Werker, A. R., 1999: An evaluation of the potential benefits and costs of autumn-sown sugarbeet in NW Europe. *Journal of Agricultural Science, Cambridge* **132**, 91-102.

61. Jamil, M., Lee, D. B., Jung, K. Y., Ashraf, M., Lee, S. C., and Rha, E. S., 2006: Effect of Salt (NaCl) Stress on Germination and early seedling Growth of Four Vegetable Species. *Journal of Central European Agriculture* **7**, 273-281.
62. Jannink, J. L., Lorenz, A. J., and Iwata, H., 2010: Genomic selection in plant breeding: from theory to practice. *Briefings in Functional Genomics* **9**, 166-177.
63. Jeong, S. and Clark, S. E., 2005: Photoperiod Regulates Flower Meristem Development in *Arabidopsis thaliana*. *Genetics* **169**, 907-915.
64. Jung, C., 2004: Genome Analysis: Mapping in Sugar Beet. In: H. Lörz and G. Wenzel (eds.), *Molecular Marker Systems in Plant Breeding and Crop Improvement*, 121-138. Springer, Heidelberg, Germany.
65. Jung, C., Pillen, A., Frese, L., Fahr, L., and Melchinger, A. E., 1993: Phylogenetic relationships between cultivated and wild species of the genus *Beta* revealed by DNA "fingerprinting". *Theoretical and Applied Genetics* **86**, 449-457.
66. Jung, C., Qian, W., Büttner, B., Hohmann, U., Mutasa-Gottgens, 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.
67. Kadereit, G., Hohmann, S., and Kadereit, J. W., 2006: A synopsis of *Chenopodiaceae* subfam. *Betoideae* and notes on the taxonomy of *Beta*. *Willdenowia* **36**, 9-19.
68. Kevei, E., Gyula, P., Hall, A., Kozma-Bognar, L., Kim, W. Y., Eriksson, M. E., Toth, R., Hanano, S., Feher, B., Southern, M. M., Bastow, R. M., Viczian, A., Hibberd, V., Davis, S. J., Somers, D. E., Nagy, F., and Millar, A. J., 2006: Forward genetic analysis of the circadian clock separates the multiple functions of ZEITLUPE. *Plant Physiol* **140**, 933-945.
69. Kim, D. H., Doyle, M. R., Sung, S., and Amasino, R. M., 2009: Vernalization: winter and the timing of flowering in plants. *Annu.Rev.Cell Dev.Biol.* **25**, 277-299.
70. Kim, S. Y., Yu, X., and Michaels, S. D., 2008: Regulation of CONSTANS and Flowering Locus T Expression in Response to Changing Light Quality. *Plant Physiol.* **148**, 269-279.
71. King, K. E., Moritz, T., and Harberd, N. P., 2001: Gibberellins are not required for normal stem growth in *Arabidopsis thaliana* in the absence of GAI and RGA. *Genetics* **159**, 767-776.
72. Kishchenko, E. M., Komarnitskii, I. K., and Kuchuk, N. V., 2005: Production of transgenic sugarbeet (*Beta vulgaris* L.) plants resistant to phosphinothricin. *Cell Biol.Int.* **29**, 15-19.

73. Koga, N., 2008: An energy balance under a conventional crop rotation system in northern Japan: Perspectives on fuel ethanol production from sugar beet. *Agriculture, Ecosystems and Environment* **125**, 101-110.
74. Kolar, J. and Senkova, J., 2008: Reduction of mineral nutrient availability accelerates flowering of *Arabidopsis thaliana*. *J.Plant Physiol* **165**, 1601-1609.
75. Lange, C., Holtgrawe, D., Schulz, B., Weisshaar, B., and Himmelbauer, H., 2008: Construction and characterization of a sugar beet (*Beta vulgaris*) fosmid library. *Genome* **51**, 948-951.
76. Lange, W., Brandenburg, W., and den Bock, Th. S. M., 1999: Taxonomy and cultonomy of beet (*Beta vulgaris* L.). *Botanical Journal of the Linnean Society* **130**, 81-96.
77. Lee, I., Ambaru, B., Thakkar, P., Marcotte, E. M., and Rhee, S. Y., 2010: Rational association of genes with traits using a genome-scale gene network for *Arabidopsis thaliana*. *Nat.Biotechnol.* **28**, 149-156.
78. Lee, J. H., Yoo, S. J., Park, S. H., Hwang, I., Lee, J. S., and Ahn, J. H., 2007: Role of SVP in the control of flowering time by ambient temperature in *Arabidopsis*. *Genes Dev.* **21**, 397-402.
79. Lein, J. C., Asbach, K., Tian, Y., Schulte, D., Li, C., Koch, G., Jung, C., and Cai, D., 2007: Resistance gene analogues are clustered on chromosome 3 of sugar beet and cosegregate with QTL for rhizomania resistance. *Genome* **50**, 61-71.
80. Lennefors, BL. Molecular breeding for resistance to rhizomania in sugar beets. 2006. Swedish University of Agricultural Sciences, Sweden.
81. Letschert, J. P. W., Lange, W., Frese, L., and van den Berg, R. G., 1994: Taxonomy of *Beta* Section *Beta* . *Journal of Sugar Beet Research* **31**, 69-85.
82. Lewellen, R. T., 2007: Registration of CN927-202, CN926-11-3-22, and CN921-306 Sugarbeet Cyst Nematode Resistant Sugarbeet Lines. *Journal of Plant Registrations* **1**, 167-169.
83. Li, D., Liu, C., Shen, L., Wu, Y., Chen, H., Robertson, M., Helliwell, C. A., Ito, T., Meyerowitz, E., and Yu, H., 2008: A repressor complex governs the integration of flowering signals in *Arabidopsis*. *Dev.Cell* **15**, 110-120.
84. Liu, H., Wang, Q., Yu, M., Zhang, Y., Wu, Y., and Zhang, H., 2008: Transgenic salt-tolerant sugar beet (*Beta vulgaris* L.) constitutively expressing an *Arabidopsis thaliana* vacuolar Na/H antiporter gene, AtNHX3, accumulates more soluble sugar but less salt in storage roots. *Plant Cell Environ.* **31**, 1325-1334.
85. Mandere, M. N., Andreas Persson, Stefan Anderberg, and Petter Pilesjo, 2009: Tropical sugar beet land evaluation scheme: development, validation and application under Kenyan condi. *GeoJournal*.

86. Mandolino, G., 2007: Marker assisted selection and genomics of industrial plants. In: P. Ranalli (ed.), *Improvement of Crop Plants for Industrial End Uses*, 59-82. Springer, Dordrecht, The Netherlands.
87. Marquardt, S., Boss, P. K., Hadfield, J., and Dean, C., 2006: Additional targets of the Arabidopsis autonomous pathway members, FCA and FY. *J.Exp.Bot.* **57**, 3379-3386.
88. Matsuo, T., Okamoto, K., Onai, K., Niwa, Y., Shimogawara, K., and Ishiura, M., 2008: A systematic forward genetic analysis identified components of the Chlamydomonas circadian system. *Genes Dev.* **22**, 918-930.
89. Mawusi, S. E. *Farmers' Knowledge and Perception towards a Sustainable Adoption of Sugar Beet in Kenya*. 2004. Lund University, Sweden.
90. May, M. J., Champion, G. T., Dewar, A. M., Qi, A., and Pidgeon, J. D., 2005: Management of genetically modified herbicide-tolerant sugar beet for spring and autumn environmental benefit. *Proc.Biol.Sci.* **272**, 111-119.
91. May, S. T., Clements, D., and Bennett, M. J., 2002: Finding your knockout: reverse genetics techniques for plants. *Mol.Biotechnol.* **20**, 209-221.
92. McGrann, G. R., Grimmer, M. K., Mutasa-Gottgens, E. S., and Stevens, M., 2009: Progress towards the understanding and control of sugar beet rhizomania disease. *Mol.Plant Pathol.* **10**, 129-141.
93. McGrath, J. M., Saccomani, M., Stevanato, P., and Biancardi, E., 2007: Beet. In: C. Kole (ed.), *Genome Mapping and Molecular Breeding in Plants*, 191-207. Springer-Verlag Berlin, Heidelberg, Germany.
94. McGrath, J. M. and Trebbi, D. *Genetics of Water Content in Sugarbeet Roots*. Proceedings of American Society of Sugar Beet Technologists 2007 Biennial Meeting 44(135). 2007.
95. Medina, P. M., Swick, L. L., Andersen, R., Blalock, Z., and Brenman, J. E., 2006: A novel forward genetic screen for identifying mutations affecting larval neuronal dendrite development in *Drosophila melanogaster*. *Genetics* **172**, 2325-2335.
96. Menzel, G., Harloff, H. J., and Jung, C., 2003: Expression of bacterial poly(3-hydroxybutyrate) synthesis genes in hairy roots of sugar beet (*Beta vulgaris* L.). *Appl.Microbiol.Biotechnol.* **60**, 571-576.
97. Merte, J., Wang, Q., Vander Kooi, C. W., Sarsfield, S., Leahy, D. J., Kolodkin, A. L., and Ginty, D. D., 2010: A forward genetic screen in mice identifies Sema3A(K108N), which binds to neuropilin-1 but cannot signal. *J.Neurosci.* **30**, 5767-5775.

98. Mglinets, A. V., 2008: Phylogenetic relationships of genus beta species based on the chloroplast trnK (matK) gene intron sequence information. *Dokl.Biochem.Biophys.* **420**, 135-138.
99. Michaels, S. D., Bezerra, I. C., and Amasino, R. M., 2004: FRIGIDA-related genes are required for the winter-annual habit in Arabidopsis. *Proc.Natl.Acad.Sci.U.S.A* **101**, 3281-3285.
100. Monreal, J. A., Jim'enez, E. T., Remesal, E., Morillo-Velarde, R., Garc'ya-Mauri'no, S., and Echevarr'ya, C., 2007: Proline content of sugar beet storage roots Response to water deficit and nitrogen fertilization at field conditions. *Environmental and Experimental Botany* **60**, 257-267.
101. Mutasa-Gottgens, E. and Hedden, P., 2009: Gibberellin as a factor in floral regulatory networks. *J.Exp.Bot.* **60**, 1979-1989.
102. Nasr, M. I. and Abd El-Razek, A. M., 2008: Sugar beet performance under newly reclaimed soils conditions of Sinai, Egypt. *Sugar Tech* **10**, 210-218.
103. Noh, B., Lee, S. H., Kim, H. J., Yi, G., Shin, E. A., Lee, M., Jung, K. J., Doyle, M. R., Amasino, R. M., and Noh, Y. S., 2004: Divergent roles of a pair of homologous jumonji/zinc-finger-class transcription factor proteins in the regulation of Arabidopsis flowering time. *Plant Cell* **16**, 2601-2613.
104. Norouzi, P., Malboobi, M. A., Zamani, K., and Yazdi-Samadi, B., 2005: Using a Competent Tissue for Efficient Transformation of Sugarbeet (*Beta vulgaris* L.). *In Vitro Cell.Dev.Biol.Plant* **41**, 11-16.
105. Ober, E. S. and Luterbacher, C., 2002: Genotypic variation for drought tolerance in *Beta vulgaris*. *Annals of Botany* **89**, 917-924.
106. Olvera, H. F., Smets, E., and Vrijdaghs, A., 2008: Floral and inflorescence morphology and ontogeny in *Beta vulgaris*, with special emphasis on the ovary position. *Ann.Bot.* **102**, 643-651.
107. Peters, J. L., Cnudde, F., and Gerats, T., 2003: Forward genetics and map-based cloning approaches. *Trends Plant Sci.* **8**, 484-491.
108. Petkeviciene, B., 2009: The effects of climate factors on sugar beet early sowing timing. *Agronomy Research* **7**, 436-443.
109. Poggi, M. E., 1980: The German Sugar Beet Industry. *Economic Geography* **6**, 81-93.
110. Polet, Y. and Wagner, E. EU-27 Sugar Annual 2010. Gloabl Agricultural Information network. E50033. 2010. USDA Foreign Agricultural Service .

111. Prat, L., Botti, C., and Fichet, T., 2008: Effect of plant growth regulators on floral differentiation and seed production in Jojoba (*Simmondsia chinensis*(Link) Schneider). *Industrial Crops and Products* **27**, 44-49.
112. Putterill, J., 2001: Flowering in time: genes controlling photoperiodic flowering in *Arabidopsis*. *Philos.Trans.R.Soc.Lond B Biol.Sci.* **356**, 1761-1767.
113. Renouf, M. A., Wegener, M. K., and Nielsen, L. K., 2008: An environmental life cycle assessment comparing Australian sugarcane with US corn and UK sugar beet as producers of sugars for fermentation. *Biomass and Bioenergy* **32**, 1144-1155.
114. Rinaldi, M. and Vonella, A. V., 2006: The response of autumn and spring sown sugar beet (*Beta vulgaris* L.) to irrigation in Southern Italy: Water and radiation use efficiency. *Field Crops Research* **95**, 103-114.
115. Rivera-Pomar, R., Niessing, D., Schmidt-Ott, U., Gehring, W. J., and Jackle, H., 1996: RNA binding and translational suppression by bicoid. *Nature* **379**, 746-749.
116. Samuelian, S., Kleine, M., Ruyter-Spira, C. P., Klein-Lankhorst, R. M., and Jung, C., 2004: Cloning and functional analyses of a gene from sugar beet up-regulated upon cyst nematode infection. *Plant Mol.Biol.* **54**, 147-156.
117. Sangtarash, M. H., 2010: Responses of different wheat genotypes to drought stress applied at different growth stages. *Pak.J.Biol.Sci.* **13**, 114-119.
118. Savitisky, V. F., 1952: A genetic study of monogerm and multigerm characters in beets. *Proc.Amer.Soc.of Sugar Beet Techn.* 331-338.
119. Saxena, K. B., Sultana, R., Mallikarjuna, N., Saxena, R. K., Kumar, R. V., Sawargaonkar, S. L., and Varshney, R. K., 2010: Male-sterility systems in pigeonpea and their role in enhancing yield. *Plant Breeding* **129**, 125-134.
120. Schneider, A. and Leister, D., 2006: Forward genetic screening of insertional mutants. *Methods Mol.Biol.* **323**, 147-161.
121. Schneider, K., Schafer-Pregl, R., Borchardt, C., and Salamini, F., 2002: Mapping QTLs for sucrose content, yield and quality in a sugar beet population fingerprinted by EST-related markers. *Theor.Appl.Genet.* **104**, 1107-1113.
122. Schroeder, J. I. and Kuhn, J. M., 2006: Plant biology: abscisic acid in bloom. *Nature* **439**, 277-278.
123. Seebaluck, V., Mohee, R., Sobhanbabu, P. R. K., Rosillo-Calle, F., Leal, M. R. L. V., and Johnson, F. X. Bioenergy for Sustainable Development and Global Competitiveness: The case of Sugar Cane in Southern Africa. CARENSA/SEI 2008-02. 2008. Cane Resources Network for Southern Africa (CARENSA).

124. Seo, E., Lee, H., Jeon, J., Park, H., Kim, J., Noh, Y. S., and Lee, I., 2009: Crosstalk between cold response and flowering in *Arabidopsis* is mediated through the flowering-time gene *SOC1* and its upstream negative regulator *FLC*. *Plant Cell* **21**, 3185-3197.
125. Sexton, S., Zilberman, D., Rajagopal, D., and Hochman, G., 2009: The Role of Biotechnology in a Sustainable Biofuel Future. *AgBioForum* **12**, 130-140.
126. Shrestha, N., Geerts, S., Raes, D., Horemans, S., Soentjens, S., Maupas, F., and Clouet, F., 2010: Yield response of sugar beets to water stress under Western European conditions. *Agricultural Water Management* **97**, 346-350.
127. Slade, A. J., Fuerstenberg, S. I., Loeffler, D., Steine, M. N., and Facciotti, D., 2005: A reverse genetic, nontransgenic approach to wheat crop improvement by TILLING. *Nat.Biotechnol.* **23**, 75-81.
128. Smulders, M. J., Esselink, G. D., Everaert, I., De, R. J., and Vosman, B., 2010: Characterisation of sugar beet (*Beta vulgaris* L. ssp. *vulgaris*) varieties using microsatellite markers. *BMC.Genet.* **11**, 41.
129. Strasser, B. +., S+ínchez-Lamas, M., Yanovsky, M. J., Casal, J. J., and Cerd+ín, P. D., 2010: *Arabidopsis thaliana* life without phytochromes. *Proceedings of the National Academy of Sciences* **107**, 4776-4781.
130. Strausbaugh, C. A., Eujayl, I. A., Rearick, E., Foote, P., and Elison, D., 2009: Sugar Beet Cultivar Evaluation for Storability and Rhizomania Resistance. *Plant Disease* **93**, 632-638.
131. Strausbaugh, C. A., Rearick, E., and Camp, S., 2008: Influence of Curly Top and Poncho Beta on Storability of Sugarbeet. *Journal of Sugar Beet Research* **45**, 31-47.
132. Streibig, J. C., Ritz, C., Pipper, C., Yndgaard, F., Fredlund, K., and Thomsen, J. N., 2009: Sugar beet, bioethanol, and climate change. *Earth and Environmental Science* **6**.
133. Sundberg, E. and Ostergaard, L., 2009: Distinct and dynamic auxin activities during reproductive development. *Cold Spring Harb.Perspect.Biol.* **1**, a001628.
134. Sung, S., He, Y., Eshoo, T. W., Tamada, Y., Johnson, L., Nakahigashi, K., Goto, K., Jacobsen, S. E., and Amasino, R. M., 2006: Epigenetic maintenance of the vernalized state in *Arabidopsis thaliana* requires LIKE HETEROCHROMATIN PROTEIN 1. *Nat.Genet.* **38**, 706-710.
135. Sung, S. and Amasino, R. M., 2006: Molecular genetic studies of the memory of winter. *J.Exp.Bot.* **57**, 3369-3377.
136. Taguchi, K., Ogata, N., Kubo, T., Kawasaki, S., and Mikami, T., 2009: Quantitative trait locus responsible for resistance to *Aphanomyces* root rot (black

- root) caused by *Aphanomyces cochlioides* Drechs. in sugar beet. *Theor.Appl.Genet.* **118**, 227-234.
137. Tertivanidis, K., Goudoula, C., Vasilikiotis, C., Hassiotou, E., Perl-Treves, R., and Tsaftaris, A., 2004: Superoxide dismutase transgenes in sugarbeets confer resistance to oxidative agents and the fungus *C. beticola*. *Transgenic Res.* **13**, 225-233.
138. Thiel, H. and Varrelmann, M., 2009: Identification of Beet necrotic yellow vein virus P25 pathogenicity factor-interacting sugar beet proteins that represent putative virus targets or components of plant resistance. *Mol.Plant Microbe Interact.* **22**, 999-1010.
139. Thomas, B., 2006: Light signals and flowering. *J.Exp.Bot.* **57**, 3387-3393.
140. Valverde, F., Mouradov, A., Soppe, W., Ravenscroft, D., Samach, A., and Coupland, G., 2004: Photoreceptor regulation of CONSTANS protein in photoperiodic flowering. *Science* **303**, 1003-1006.
141. VanDijk, H., Boudry, P., McCombie, H., and Vernet, P., 1997: Flowering time in wild beet (*Beta vulgaris* ssp. *maritima*) along a latitudinal cline. *Acta Oecologica-International Journal of Ecology* **18**, 47-60.
142. Venturi, P. and Venturi, G., 2003: Analysis of energy comparison for crops in European agricultural systems. *Biomass and Bioenergy* **25**, 235-255.
143. Vernoux, T., Besnard, F., and Traas, J., 2010: Auxin at the shoot apical meristem. *Cold Spring Harb.Perspect.Biol.* **2**, a001487.
144. Wada, K. C. and Takeno, K., 2010: Stress-induced flowering. *Plant Signal.Behav.* **5**.
145. Wagmann, K., Hautekeete, N. C., Piquot, Y., and Van, D. H., 2010: Potential for evolutionary change in the seasonal timing of germination in sea beet (*Beta vulgaris* ssp. *maritima*) mediated by seed dormancy. *Genetica*.
146. Weigel, D. and Glazebrook, J., 2006: Forward Genetics in Arabidopsis: Finding Mutations that Cause Particular Phenotypes. *Cold Spring Harb Protoc* **2006**, db.
147. Weiland, J. J. and Yu, M. H., 2003: A Cleaved Amplified Polymorphic Sequence (CAPS) Marker Associated with Root-Knot Nematode Resistance in Sugarbeet. *Crop Sci* **43**, 1814-1818.
148. Weyens, G., Ritsema, T., Van, D. K., Meyer, D., Lommel, M., Lathouwers, J., Rosquin, I., Denys, P., Tossens, A., Nijs, M., Turk, S., Gerrits, N., Bink, S., Walraven, B., Lefebvre, M., and Smeekens, S., 2004: Production of tailor-made fructans in sugar beet by expression of onion fructosyltransferase genes. *Plant Biotechnol.J.* **2**, 321-327.

149. Winfield, M., Lu, C., Wilson, I., Coghill, J., and Edwards, K., 2009: Cold- and light-induced changes in the transcriptome of wheat leading to phase transition from vegetative to reproductive growth. *BMC Plant Biology* **9**, 55.
150. Winkler, R. G., Frank, M. R., Galbraith, D. W., Feyereisen, R., and Feldmann, K. A., 1998: Systematic Reverse Genetics of Transfer-DNA-Tagged Lines of *Arabidopsis*. Isolation of Mutations in the Cytochrome P450 Gene Superfamily. *Plant Physiol.* **118**, 743-750.
151. Wong, A. C., Sandesara, R. G., Mulherkar, N., Whelan, S. P., and Chandran, K., 2010: A Forward Genetic Strategy Reveals Destabilizing Mutations in the Ebolavirus Glycoprotein That Alter Its Protease Dependence during Cell Entry. *J.Virol.* **84**, 163-175.
152. Xu, Y. and Crouch, J. H., 2008: Marker-Assisted Selection in Plant Breeding: From Publications to Practice. *Crop Sci* **48**, 391-407.
153. Yamada, N., Promden, W., Yamane, K., Tamagake, H., Hibino, T., Tanaka, Y., and Takabe, T., 2009: Preferential accumulation of betaine uncoupled to choline monooxygenase in young leaves of sugar beet--importance of long-distance translocation of betaine under normal and salt-stressed conditions. *J.Plant Physiol* **166**, 2058-2070.
154. Yamaguchi, S., 2008: Gibberellin metabolism and its regulation. *Annu.Rev.Plant Biol.* **59**, 225-251.
155. Yu, H. M., 2003: Development of Root-Knot Nematode-Resistant Sugarbeet. 1s joint IIRB-ASSBT Congress, 26thFeb.-1 March 2003, San Antonio (USA) 763-765.
156. Zhong, R., Thompson, J., Ottesen, E., and Lamppa, G. K., 2010: A forward genetic screen to explore chloroplast protein import in vivo identifies Moco sulfurase, pivotal for ABA and IAA biosynthesis and purine turnover. *Plant J.* **63**, 44-59.
157. Zinn, K. E., Tunc-Ozdemir, M., and Harper, J. F., 2010: Temperature stress and plant sexual reproduction: uncovering the weakest links. *J.Exp.Bot.* **61**, 1959-1968.

9. Supplementary data

The supplement material is available on CD.

Contents:

File name	Content	Format
<i>BvFLK</i>	The whole <i>BvFLK</i> genomic sequence	.txt
pFT001	A binary vector (pSR752 modified)	.txt
pFT002	The CDS of <i>BvFLK</i> in pGEM-T vector	.txt
pFT003	The CDS of <i>BvFLK</i> in pGEM-T vector (<i>XhoI</i> site has incorporated)	.txt
pFT011	The CDS of <i>BvFLK</i> in pGEM-T vector (containing <i>AscI</i> and <i>SpeI</i> recognition sites)	.txt
pFT013	The CDS of <i>BvFLK</i> in pFT001 vector (driven by the 35S promoter)	.txt
pFT014	The endogenous promoter of <i>BvFLK</i>	.txt
pFT015	The CDS of <i>AtFLK</i> in pGEM-T vector	.txt
pFT016	The CDS of <i>AtFLK</i> in pFT001 vector (driven by the 35S promoter)	.txt
pFT033	The CDS of <i>BvFLK</i> in pFT001 vector (driven by its endogenous promoter)	.txt
pFT066	The CDS of <i>BvLDL1</i> in pGEM-T vector	.txt
pGADT7 + <i>AtFLK_CDS</i>	The CDS of <i>AtFLK</i> in pGADT7 vector	.txt
pCMV- Sport6.1+ <i>AtFLK_cDNA</i>	The CDS of <i>AtFLK</i> in pCMV-Sport6.1 vector	.txt

10. Acknowledgements

It is a great pleasure to thank the people who made this thesis possible.

First, I thank my principal supervisor Prof. Dr. Christian Jung for giving me the great opportunity to study my Ph.D. at the honor institute of Plant Breeding at Christian-Albrechts University of Kiel and for his help, support and patience. I appreciate all his contributions of time, ideas, and funding to make my Ph.D. experience productive and encourage.

I would like to express my special, deep and sincere gratitude to the good advice, support and friendship of my second supervisor Dr. Andreas Müller, has been a precious on both personal and academic levels, for which I am extremely grateful. It is difficult to overstate my gratitude to Dr. A. Müller. With his inspiration, his enthusiasm, and his great efforts to speculate and interpret things clearly and simply, I gained and learned too much from him. Besides my advisors, I would like to thank the committee of my thesis: Prof. Dr. Henning Kage, Prof. Dr. Karl H. Mühling, Prof. Dr. Daguang Cai and Prof. Dr. Christian Jung for giving insightful comments and reviewed my work on a very short notice.

I would like to acknowledge Deutsche Forschungsgemeinschaft (DFG) and the Ministry of Higher Education, Egypt for financial support. Furthermore, I would to thank the Institute of Clinical Molecular Biology (IKMB), Kiel, Germany for sequencing. I am very grateful also to all kind members of the plant breeding institute, University of Kiel for their kind help during the course of this work.

I deeply grateful to my entire extended family: my parents, my sisters and brothers, who were supportive for providing a loving environment for me, despite missing me for a long time. I wish to express my warm, loving and sincere thanks to my wife and my sons

Last, but not least, I would to thank all my professors, colleagues and friends at the Agronomy Dept., Assiut University, Egypt

11. Curriculum Vitae

Name: Salah Fatouh Abou-Elwafa

Date of birth: June, 23, 1975

Place of birth: Sohag, Egypt

Gender: Male

EDUCATION:

2006-2011 Ph.D. in Plant Breeding Institute, Christian-Albrechts University of Kiel, Germany.

Dissertation: Novel Genetic Factors Affecting Bolting and Floral Transition Control in *Beta vulgaris*.

2002 M.Sc. in Agronomy, Faculty of Agriculture, Assiut University, Egypt.

Dissertation: Effect of planting and harvesting dates on growth, yield and quality of sugar beet.

1997 B.S. in Agronomy and Plant Breeding (with honor degree), Faculty of Agriculture, Assiut University, Egypt.

SCIENTIFIC CAREER:

1998-2006 Assistant Professor; Agronomy Department, Assiut University, Egypt.

LIST OF PUBLICATIONS:

1. **Abou-Elwafa, S.F.**, B.Büttner, T.Chia, G.Schulze-Buxloh, U.Hohmann, E.Mutasa-Göttgens, C.Jung and AE.Müller. Conservation and divergence of the autonomous pathway of flowering time regulation in *Beta vulgaris*. ***Journal of Experimental Botany* (2010)**.
2. Büttner, B., **S.F.Abou-Elwafa**, W.Zhang, C.Jung, and AE.Müller. 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* (2010)**.
3. **Abou-Elwafa, S.F.**, H.M.Abdel-Rahim, A.M.Abou-Salama, and E.A.Teama. Sugar Beet Floral Induction and Fertility: Effect of Vernalization and Day-length Extension. ***Sugar Tech*, 8, 281-287 (2006)**.

4. **Abou-Elwafa, S.F.**, H.M. Abdel-Rahim, E.A.Teama and A.M.Abou-Salama. Studies on sugar beet floral induction and fertility II- Effect of root age and day-length extension. *International symposium on the technologies to improve sugar productivity in developing Countries, Guilin, P.R. China, 5-8th December, 2006.*
5. Abd Elrahim H.M., A.M.Abou-Salama, E.A.Teama and **S.F.Abou-Elwafa**. Effect of planting and harvesting dates on growth, yield and quality of sugar beet varieties in Middle Egypt. *International conference on: "Political, Economic and Technological Challenges for Sugar and its Integrated Industries in the Arab Region, the Middle East, Africa and the European Union", Alexandria, Egypt, 3-6 April 2005.*
6. Abd Elrahim H.M., A.M. Abou-Salama, E.A. Teama and **S.F. Abou-Elwafa**. Sugar beet performance and interaction with planting dates, varieties and harvesting dates in Middle Egypt. *International conference on: "Political, Economic and Technological Challenges for Sugar and its Integrated Industries in the Arab Region, the Middle East, Africa and the European Union", Alexandria, Egypt, 3-6 April 2005.*