Theor Appl Genet (2010) 121:1117–1131
DOI 10.1007/s00122-010-1376-8

ORIGINAL PAPER

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

Bianca Büttner · Salah F. Abou-Elwafa · Wenying Zhang · Christian Jung · Andreas E. Müller

Received: 28 January 2010 / Accepted: 21 May 2010 / Published online: 20 June 2010

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.

Communicated by T. Luebberstedt.

B. Büttner and S. F. Abou-Elwafa contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s00122-010-1376-8) contains supplementary material, which is available to authorized users.

B. Büttner · S. F. Abou-Elwafa · W. Zhang · C. Jung · A. E. Müller (✉)
Plant Breeding Institute, Christian-Albrechts-University of Kiel, Olshausenstr. 40, 24098 Kiel, Germany
e-mail: a.mueller@plantbreeding.uni-kiel.de

Present Address:
W. Zhang
Agricultural College, Yangtze University, Jingzhou 434025, Hubei Province, People's Republic of China

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; Hautekeete 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 day-length 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 *AP1* (*APETALA1*). *AP1* in *A. thaliana* also affects flowering time but in contrast to *VRN1* in cereals has not been associated directly with vernalization requirement or response (Yan et al. 2003, 2004; Alonso-Blanco et al. 2009) and differs from *VRN1* in regard to temporal and spatial expression profiles (Li and Dubcovsky 2008; Greenup et al. 2009).

In *B. vulgaris*, reverse genetic approaches have identified an *FLC*-like gene (*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. (1) *A. thaliana* has only a facultative requirement for long photoperiods and also flowers under short-day conditions (Koornneef et al. 1998a). *B. vulgaris*, by contrast, is an obligate long-day plant (Curth 1960; Lexander 1980). (2) 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. (3) 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; Hautekeete et al. 2002).

(4) *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. (1) One-locus model: a 3:1 phenotypic segregation ratio for bolting behavior (bolting vs. non-bolting without vernalization), and complete co-segregation of *B* locus marker genotypes and bolting phenotypes (Fig. 1a). (2) Epistatic locus model: assuming that the epistatic locus is not genetically linked to the *B* locus, the phenotypic segregation for bolting behavior would also be expected to occur at a ratio of 3:1. In contrast to the one-locus model, however, we would expect independent segregation of bolting phenotypes and *B* locus marker genotypes (Fig. 1b). 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*.

Materials and methods

Plant material

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

M3 or M4 plants of biennial mutant lines were crossed with individuals of accession 991971 (Table 1). In order to synchronize flowering times the annual crossing partners were vernalized for 12 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 was identified phenotypically by hypocotyl color. In *B. vulgaris* hypocotyl color is encoded by the *R* locus, with the *R* allele encoding red hypocotyl color being dominant over the *r* allele for green hypocotyl color (Butterfass 1968; Barzen et al. 1992). Three mutant families have green hypocotyls (000855, 011763, 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

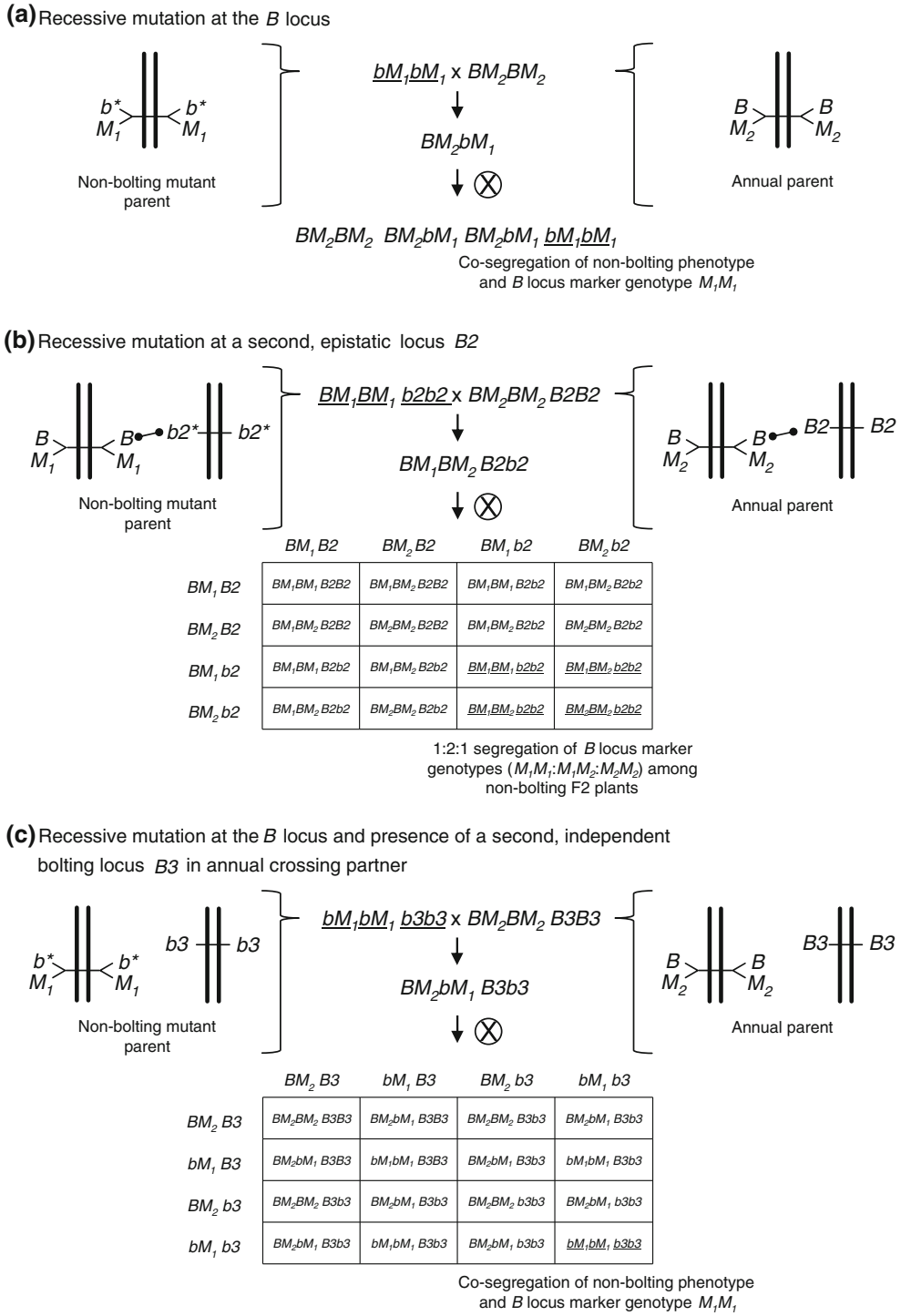

Fig. 1 Test for allelism between EMS mutations and the *B* locus. The expected segregation of bolting phenotypes and *B* locus marker genotypes is shown for crosses between 'non-bolting' (biennial) EMS mutants and annual crossing partners. Three models are shown: **a** The mutation occurred at the *B* 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*

Table 1 Biennial EMS mutants and generation of F2 populations

Mutant family (M2)[a]	Mutant parent of cross	Annual parent of cross	Seed parent of F1 cross progeny	F1 plant selfed	F2 population[c]
000855	020415/15 (M3)[b]	991971/2	020415/15	061365/1	EW1
011763	056822/4 (M4)	991971/9	056822/4	061392/1	EW2
011373	031823/14 (M3)	991971/11	031823/14	061398/1	EW3
000192	020416/14 (M3)	991971/3	991971/3	061373/3	EW4a
000192	020417/16 (M3)	930190/13	020417/16	061460/1	EW4b

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

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

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

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 in winter in the greenhouse without vernalization, and selfed to produce F2 seeds (Table 1).

Phenotypic analysis

Ninety-six plants per F2 population were sown on May 18, 2007, grown in the greenhouse for 1 month under long-day conditions with supplementary lighting (Son-T Agro 400W, Koninklijke Philips Electronics N.V., Eindhoven, The Netherlands) for 16 h, and transplanted to the field on June 20, 2007. Plants were phenotyped every two to 3 days for onset of bolting (BBCH scale code 51; Meier 2001). Phenotypes were scored until November 19, 2007. In populations EW2, EW3, and EW4b, four, six or three plants, respectively, died after transplantation to the field. Population EW1 had a low-germination rate and several plants died at the early seedling stage so that only 78 plants were grown and analyzed. Because the F2 population derived from the cross between 020416/14 and 991971/3 had a germination rate of less than 20%, a new F2 population (EW4a) derived from the same cross, but another F1 plant (061373/3), was sown in the greenhouse on May 16, 2008. Out of 200 seeds sown, 140 germinated. Plants were transplanted to the field on June 20, 2008 and phenotyped in the field until the end of October 2008. As controls, 12 plants each of the annual and mutant parent accessions were grown and phenotyped in parallel to the F2 populations.

DNA extraction and genotypic analysis

For molecular marker analysis, leaf samples were harvested and freeze-dried. Genomic DNA was extracted using the NucleoSpin 96 Plant DNA isolation kit (Macherey and Nagel, Düren, Germany) and a TECAN-Freedom EVO 150® robot (Männedorf, Switzerland). DNA concentration was adjusted to 5 ng/µl. Five markers linked to the *B* locus on chromosome II at $R = 0$ (GJ1001c16, GJ1013c690a, GJ1013c690b), $R = 0.005$ (GJ18T7b) or $R = 0.007$ (Y67L), respectively (Müller et al. unpublished data), were used to differentiate between mutant- and annual parent-derived alleles (Suppl. Tab. 1). SNP markers were genotyped by PCR amplification and sequencing (GJ1013c690a), or converted into CAPS markers (GJ1013c690b, Y67L). GJ1013c690b and Y67L were genotyped by PCR amplification, *Bsa*JI 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 *Hin*fI (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 *EcoR*I 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.

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 an 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).

Results

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 1). As expected for dominant-recessive inheritance of annual bolting, all F1 plants originating from the crosses bolted and flowered without vernalization. F2 populations segregating for the mutant phenotype were tested for co-segregation of bolting behavior with molecular markers located at the *B* locus.

For each of the four crosses, 78–140 F2 plants (populations EW1, EW2, EW3 and EW4a; for population EW4b see below) were phenotyped for bolting behavior under non-vernalizing conditions (bolting or non-bolting; Suppl. Tab. 3). All populations were segregated for bolting behavior and contained both bolting and non-bolting individuals (Fig. 2; Table 2). In two populations (EW1 and 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 2). For the other two populations (EW3 and 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 2), as would be expected for digenic, dominant-recessive inheritance of the trait when only the double-recessive genotype is non-bolting (see 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.

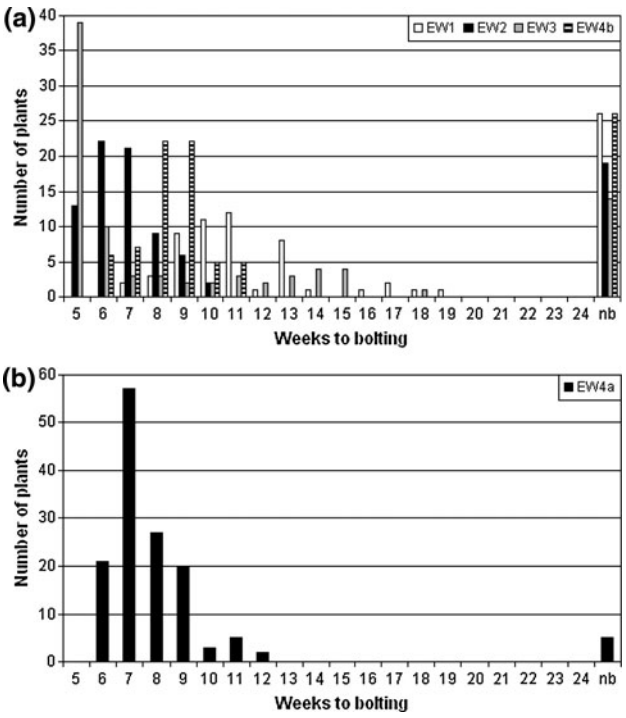

Fig. 2 Phenotypic segregation for bolting behavior in F2 populations. 'Weeks to bolting' indicates the week, counted from the date of sowing, in which stem elongation began (i.e. week 5 corresponds to 29–35 days to bolting, etc.; s. Suppl. Tab. 3). *'nb'* indicates plants which had not bolted by the end of the experiment in fall. Populations in which bolting and non-bolting plants segregate at a ratio not deviating from 3:1 at $\alpha = 0.01$ (EW1, EW2, EW3, EW4b) are shown in **a**, population EW4a is shown in **b**

Table 2 Phenotypic segregation for bolting behavior in F2 populations

F2 population	Total number of plants	Bolting	Non-bolting	χ^2 test for $H_0 = 3:1$ (bolting vs. non-bolting)[a]	χ^2 test for $H_0 = 15:1$ (bolting vs. non-bolting)[b]
EW1	78	52	26	2.89	97.64**
EW2	92	73	19	0.93	32.57**
EW3	90	76	14	4.28*	13.30*
EW4a	140	135	5	34.29**	0.19
EW4b	93	67	26	0.43	74.80**

[a] H_0, null hypothesis for monogenic, dominant-recessive trait

[b] H_0, null hypothesis for digenic, dominant-recessive trait

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

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, and Y67L; see "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.

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, namely, 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 3). In case of complete co-segregation between bolting phenotypes and *B* locus markers, the segregation ratio of these classes would be expected to be 0:2:1:1:0:0, whereas independent segregation is expected to yield a 3:6:3:1:2:1 segregation ratio. The two populations whose phenotypic segregation ratios did not deviate significantly from 3:1 (EW1 and 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, see above) was rejected, suggesting the presence of at least one additional bolting locus ('*B2*', see below), which is not genetically linked to the *B* locus.

Table 3 Co-segregation analysis of bolting behavior and *B* locus marker genotypes

F2 population	*B* locus marker	Total number of plants genotyped	Bolting			Non-bolting			χ^2 test for $H_0 = 3:6:3:1:2:1$[b]	χ^2 test for $H_0 = 3:8:4:1$[c]
			M_1M_1[a]	M_1M_2	M_2M_2	M_1M_1	M_1M_2	M_2M_2		
EW1	GJ1013c690a	78	9	28	16	6	15	4	5.59	n.a.
EW2	GJ1001c16	90	8	45	20	3	9	7	10.73	n.a.
EW3	GJ1001c16	90	21	40	15	5	4	5	7.19	n.a.
EW4a	GJ18T7b	140	29	70	36	4	0	1	36.70**	n.a.
	GJ1013c690b	140	30	77	28	4	1	0	39.00**	n.a.
	Y67L	140	33	70	32	5	0	0	34.50**	3.60
EW4b	GJ1013c690b	93	0	39	28	22	3	1	79.80*	n.a.

[a] Marker alleles M_1 and M_2 are derived from the mutant parent or the annual parent, respectively

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

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

n.a. not applicable

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

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 (Fig. 1c). 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 and 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 3). Four of five non-bolting plants in this population carried the *B* locus marker M_1 in the homozygous condition; one plant was heterozygous for the *B* locus marker, and none of the plants carried the M_2 allele in the homozygous condition.

The possibility that one of the two bolting genes in this population predicted by the model is located at or close to the *B* locus was investigated further. To this end, two additional markers flanking the *B* locus on opposite sides (GJ18T7b and Y67L; Suppl. Tab. 1) were tested for co-segregation with the bolting phenotype (Table 3). The genotype of marker GJ18T7b deviated from the expectation in the same plant as the genotype of the previously tested marker. For marker Y67L, however, the genotypic data are consistent with the model, i.e., all non-bolting plants carried the mutant-derived allele in the homozygous condition. Furthermore, the null hypothesis for segregation according to the model (a segregation ratio of 3:8:4:1 for the phenotype/marker constellations bolting/M_1M_1, bolting/M_1M_2, bolting/M_2M_2 and non-bolting/M_1M_1) was not rejected by χ^2 analysis (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, see "Introduction"). We took advantage of the apparent genetic divergence of the mutant and the annual accession (see "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 3). Among the 26 non-bolting plants in this population, 22 carry the *B* locus marker genotype (M_1M_1) expected for complete co-segregation between phenotype and the mutant marker allele. Among the remaining four plants, three plants are heterozygous at the marker locus (M_1M_2) and one single plant carries the marker allele derived from the annual parent in the homozygous state (M_2M_2). Among the 67 bolting plants, all plants carry either the M_1M_2 or the M_2M_2 constellation, and none is homozygous for the M_1 allele.

Variation in bolting time among annuals in F2 populations

Besides annuality, all populations were phenotyped for bolting time of annual individuals (Fig. 2, Suppl. Tab. 3). In populations EW1, EW2, and EW4b, annual plants were approximately normally distributed but the position of the maxima differed between populations (Fig. 2a). In population EW3, the majority of plants bolted early (at 5–6 weeks from sowing), but a considerable number of plants started to bolt much later (at 11–18 weeks; Fig. 2a). The frequency distribution in population EW4a is similar to normal but positively skewed, with bolting in some plants being somewhat delayed (Fig. 2b). 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

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

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

[a] *B* locus markers are as indicated in Table 3. The *B* locus marker analyzed in population EW4a is GJ1013c690b

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

[c] Fisher's Least Significant Difference at $\alpha = 0.05$. Mean values in table cells including the letter 'B' are significantly different from the mean value in the table cell including the letter 'A'

n.a. not applicable

genotypes (M_1M_2 and M_2M_2). The presence of the M_1M_1 genotype correlated with a delay in bolting.

Genetic mapping of the bolting locus in population EW2

Among the populations postulated to carry a bolting locus not genetically linked to *B*, EW2 was selected for AFLP mapping of the locus. A genetic map was constructed which incorporates 141 AFLPs as well as five SSR markers (Laurent et al. 2007; McGrath et al. 2007) and ten SNP-based markers (Schneider et al. 2007; Suppl. Tab. 2), which were used as anchor markers. All linkage groups were anchored to the nine chromosomes of the beet genome (Suppl. Fig. 1). The map covers 571 cM with an average marker interval of 3.65 cM. The sizes of the linkage groups range from 33 to 89 cM. The phenotypic marker, i.e., the locus responsible for annual bolting in this population, was mapped to position 52.61 cM on chromosome IX and is flanked by the AFLP marker M34xP46_W160 and the co-dominant SNP-based anchor marker MP_R0018 (Schneider et al. 2007) (Fig. 3). 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 in winter). Using this setting and an LOD threshold of 3.0 a single QTL was detected. The QTL co-localizes with the phenotypic

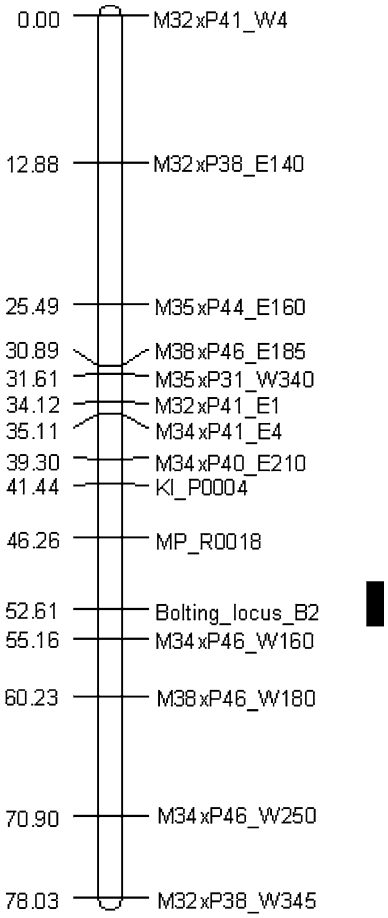

Fig. 3 Genetic map position of a major locus for annual bolting on chromosome IX. The map comprises the bolting locus *B2*, twelve AFLP markers, and two co-dominant SNP-based anchor markers (KI_P0004 and MP_R0018). Genetic distances in centiMorgan are given on the *left*, marker names on the *right*. The *vertical black bar* indicates the confidence interval of a quantitative trait locus co-localizing with *B2*

marker locus *B2* on chromosome IX at position 52 cM (confidence interval 50–54 cM; Fig. 3), has an LOD score of 46.81 and explains 87.0% of the observed phenotypic variation.

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 5). However, similar to population EW2, independent segregation was not observed in population EW3. Among 14 non-bolting plants in this population, all but one carried the mutant-derived marker allele in the homozygous condition (M_1M_1), with the remaining plant being heterozygous at the marker locus. Notably, however, among the 76 bolting plants, 17 were also of the M_1M_1 genotype at the marker locus. A closer examination of bolting plants which are homozygous for the M_1 marker allele, revealed that most of these plants are very late in bolting (Suppl. Tab. 3). Analysis of variance of bolting time between the three genotypic classes (M_1M_1, M_1M_2, and M_2M_2) among the bolting plants of this population revealed that the mean of days to bolting in M_1M_1 individuals (83.12) was significantly higher than the respective means in M_1M_2 (40.66) and M_2M_2 (43.11) individuals (Table 6). By contrast, bolting time did not differ significantly between the three genotypic classes among bolting plants in populations EW1 and EW4a, nor was this the case in population EW2.

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: (1) The *B* locus is not the only locus controlling annual bolting in *B. vulgaris*. Bolting control involves at least two other loci not linked to *B* (*B2* and *B3*), one of which (*B2*) was genetically mapped. (2) 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. (3) 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:

1) 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 *B. vulgaris*. A priori, the existence of a second bolting locus is consistent with our 'epistatic locus' model, according to which the EMS-induced mutation in the mutant parent of this population occurred at a locus which

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

F2 population	Chromosome IX marker	Total number of plants genotyped	Bolting			Non-bolting			χ^2 test for $H_0 = 3:6:3:1:2:1$[b]
			M_1M_1[a]	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

[a] Marker alleles M_1 and M_2 are derived from the mutant parent or the annual parent, respectively

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

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

is unlinked to the *B* locus, acts epistatically to *B* and prevents bolting even in the presence of a functional bolting allele at the *B* locus (Fig. 1b). 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 5). *B* and *B2* would have to interact epistatically because the annual accession used for EMS mutagenesis is homozygous for the annual bolting allele at the *B* locus (*BB*), and this allele must still be present in all individuals of the 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. 3), 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. 2 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 3). The phenotypic marker in population EW1 also segregated independently of the *B2*-linked marker MP_R0018a and may constitute yet another locus. In population EW3, however, the phenotypic marker did not segregate independently of the *B2*-linked marker, and all except one of the non-bolting plants carried the mutant-derived marker allele in the homozygous condition (M_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 6). Noteworthily, the bolting plants in population EW2 bolted within 34–68 days after sowing (with a mean of 45.51 days), whereas the bolting plants in population EW3 bolted within 34–122 days after sowing (with a mean of 50.46 days) (Suppl. Tab. 3). 17 of these plants bolted >68 days after sowing (73–122 days, with a mean of 92.12 days; corresponding to weeks 11–18 in Fig. 2a). Out of 17 bolting individuals of the M_1M_1 genotype in population EW3, 13 belonged to set of 17 late-bolting plants (bolting 73–122 days after sowing) and only four bolted earlier (Suppl. Tab. 3).

According to our mapping data the marker locus MP_R0018a is located at a genetic distance of 6.35 cM from the *B2* locus (Fig. 3). This value is in approximate accordance with the number of recombination events in population EW2 (7 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-

Table 6 Analysis of variance among chromsome IX marker genotypes in annual subpopulations

Marker genotype[a]	Mean (±SD) of days to bolting			
	EW1	EW2	EW3	EW4a
M_1M_1	69.00 (±9.18)	48.50 (±4.90)	83.12 (±25.09) A	46.73 (±6.44)
M_1M_2	80.04 (±21.20)	44.53 (±8.56)	40.66 (±11.87) B	51.26 (±9.69)
M_2M_2	74.94 (±17.84)	43.77 (±8.68)	43.11 (±19.35) B	50.14 (±7.77)
F (*p* value)	1.51 (0.23)	0.27 (0.77)	31.10 (0.00)	2.14 (0.12)
$LSD^{b}_{0.05}$	n.a.	n.a.	10.75	n.a.

[a] Chromosome IX markers are as indicated in Table 4

[b] Fisher's Least Significant Difference at $\alpha = 0.05$. Mean values in table cells including the letter 'B' are significantly different from the mean value in the table cell including the letter 'A'

n.a. not applicable

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

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

2) Evidence that the *B* locus affects bolting behavior and is mutated in one of the four mutant families analyzed (000192, see Table 1) was obtained from analysis of populations EW4a and EW4b. Firstly, the statistical analysis of the *B* locus marker-phenotype co-segregation data for population EW4a is consistent with the hypothesis that the *B* locus is one of the two bolting loci postulated for this population (Table 3). Secondly, all non-bolting plants in population EW4a carried the mutant-derived allele at the *B*-linked marker locus Y67L in the homozygous condition. Thirdly, analysis of variance of bolting time among the annual plants of population EW4a indicated a significant effect of allele composition at the *B* locus on bolting time (Table 4). The presence of the mutant-derived allele at the *B* locus in the homozygous condition correlated with a delay in bolting, suggesting that, similar to *B2*, the *B* locus (or a locus linked to *B*) also affects bolting time in annual plants. Lastly, when a mutant from the same mutant family was crossed with accession 930190 as annual crossing partner, the resulting F2 population EW4b segregated in accordance with a 3:1 ratio of bolting versus 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 3) are largely consistent with the *B* locus being responsible for annual bolting in this population, and the annual parent-derived allele being dominant over the mutant-derived allele (in accordance with the model in Fig. 1a). 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.

3) Finally, the segregation data for population EW4a indicate that bolting control involves (at least) one additional locus (*B3*). The fact that the segregation ratio does not deviate significantly from 15:1 suggests that this locus is not linked to the *B* locus and acts independently of *B*, but like *B* is also inherited in a dominant-recessive manner (in accordance with the model in Fig. 1c). 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 (see 2 above) and is biennial, and thus cannot carry a second bolting allele which acts independently of *B*. Because none of the other populations analyzed in the current study provided evidence for an independent bolting locus unlinked to *B*, a functional allele at locus *B3* may be rare in the annual parent accession 991971 and (at least in the homozygous condition) only present in a subset of 991971 individuals. As a consequence of the high ratio of bolting to non-bolting plants in population EW4a, and the resulting small number of non-bolting plants, the phenotypic information content is too small for genetic mapping of *B3*. We also cannot exclude the possibility that the unexpectedly high phenotypic segregation ratio in this population is due to several, quantitative loci. However, because four of five non-bolting plants in this population are not homozygous for the mutant-derived allele at the *B2*-linked chromosome IX marker locus MP_R0018, it seems unlikely that an independent major locus co-localizes with *B2*. The notion that *B2* and *B3* constitute separate loci is also consistent with the genetic modes of action postulated for these loci, with *B2* acting epistatically to *B*, and *B3* acting independently of *B*.

In summary, our data provide evidence for three loci controlling bolting in *B. vulgaris*: The *B* locus on chromosome II, which appears to be mutated in one of four mutant families analyzed, locus *B2* on chromosome IX, which is segregating in two populations and appears to act epistatically to *B*, and locus *B3* which acts independently of *B* and appears to comprise a functional, dominant allele in at least some individuals of the annual accession 991971. The results have implications for our understanding of bolting control in *B. vulgaris*. First, our finding that one biennial mutant family appears to carry a mutation at the *B* locus suggests that a single EMS-induced point mutation of the bolting gene at this locus is sufficient to abolish its function. This mutant is a valuable tool to screen candidates for the bolting gene located at the *B* locus (Müller et al. unpublished data) for sequence variation, and may help to correlate the functional role of the *B* locus in bolting control with a specific gene and/or sequence feature. Second, the genetic control of annual bolting in *B. vulgaris* is more complex than has been described in the past, and involves previously unidentified loci in addition to the well-known *B* locus. Possibly, these additional loci have gone unnoticed as a result of selection by breeders against annual bolting early on in sugar beet breeding, and only limited genetic research on annual (non-cultivated) beets. In particular, much of the original work on the genetics of bolting behavior was based on related annual beet accessions that were established by Munerati (Munerati 1931; Abegg 1936; Owen et al. 1940). Moreover, the annual accession used in the present study originated from a natural population on a Greek island, i.e., a geographic location within the southern part of the species' distribution area where annuality has its highest frequency and probably a high selective advantage (Van Dijk and Boudry 1991). Lastly, epistatic genes which act in the same genetic pathway as *B* and do not suffice to induce annual bolting in the absence of a functional bolting allele at the *B* locus are impossible to detect in biennial *bb* genotypes as they may be prevalent in cultivated beet breeding material (Gaafar et al. 2005).

The possibility of a second gene controlling annual bolting was considered by Abe et al. (1997). These authors, however, suggested that this second gene is genetically linked to the *B* locus. An additional locus unlinked to *B* is not unlikely given the complexity of floral transition control as it is known for other species. Furthermore, several flowering time genes have been shown to interact epistatically, e.g., in *A. thaliana* (e.g. Koornneef et al. 1991, 1998b; Nilsson et al. 1998; Caicedo et al. 2004). Because the non-bolting mutant phenotype can be overcome by vernalization, the new bolting loci seem unlikely to carry regulatory genes of the vernalization pathway, as it is known for Arabidopsis, or other signal transduction cascades that mediate bolting in response to prolonged cold. Furthermore, *BvFL1*, a *B. vulgaris* homolog of the floral repressor gene *FLC*, which acts downstream of the vernalization pathway in Arabidopsis, can be excluded as a candidate gene for *B2* (but not for *B3* whose map position is not known) because it was mapped to chromosome VI of the beet genome (Reeves et al. 2007). Besides, the annual alleles at the bolting loci in our study are dominant, whereas the recessive, 'non-bolting' alleles are mutant-derived, suggesting that the wild-type alleles do not repress, but promote bolting. The effect of mutagenesis also distinguishes the mutated genes in *B. vulgaris* from *PEP1*, an *FLC* ortholog in the perennial *Brassicaceae* species *Arabis alpina* which determines vernalization requirement in wild-type plants but in its mutated form (following EMS mutagenesis) causes early bolting without a requirement for vernalization (Wang et al. 2009). Conceivably, *B*, *B2* and/or *B3* mediate photoperiod or gibberellic acid control of floral transition, and mutants with an impaired response to the respective exogenous or endogenous cues require the additional stimulus of vernalization for bolting to occur. *B2* cannot correspond to the *CO*-like gene *BvCOL1* because this gene was mapped to chromosome II at a genetic distance of ~25–30 cM from the *B* locus (Chia et al. 2008; Müller et al. unpublished data).

The colocalization of *B* and *BvCOL1* on the same linkage group further suggests that *B3* is also unlikely to correspond to *BvCOL1* because our segregation data indicate that *B3* segregates independently of *B*. Candidates for *B2* may include homologs of genes acting upstream or downstream of *CO* in the same genetic pathway, including *FT*-like genes. Although *ft* mutants in *A. thaliana* are only moderately responsive to vernalization (Martinez-Zapater and Somerville 1990; Koornneef et al. 1991; Moon et al. 2005), allelic variation of *FT* orthologs (the *VRN3* genes) (co-)regulates vernalization requirement in cereals (Yan et al. 2006; Trevaskis et al. 2007; Distelfeld et al. 2009). An effort to clone the *B2* locus by a map-based approach using 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.

Acknowledgments 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.

References

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

Abegg FA (1936) A genetic factor for the annual habit in beets and linkage relationship. J Agric Res 53:493–511

Alonso-Blanco C, Aarts MGM, Bentsink L, Keurentjes JJB, Reymond M, Vreugdenhil D, Koornneef M (2009) What has natural variation taught us about plant development, physiology, and adaptation? Plant Cell 21:1877–1896

Angiosperm Phylogeny Group (2009) An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG III. Bot J Linn Soc 161:105–121

Barzen E, Mechelke W, Ritter E, Seitzer JF, Salamini F (1992) RFLP markers for sugar beet breeding: chromosomal linkage maps and location of major genes for rhizomania resistance, monogermy and hypocotyl colour. Plant J 2:601–611

Bäurle I, Dean C (2006) The timing of developmental transitions in plants. Cell 125:655–664

Boudry P, Wieber R, Saumitou-Laprade P, Pillen K, Van Dijk H, 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.). Theor Appl Genet 88:852–858

Boudry P, McCombie H, Van Dijk H (2002) Vernalization requirement of wild beet *Beta vulgaris* ssp. *maritima*: among population variation and its adaptive significance. J Ecol 90:693–703

Butterfass T (1968) Die Zuordnung des Locus *R* der Zuckerrübe (Hypokotylfarbe) zum Chromosom II. Theor Appl Genet 38:348–350

Caicedo AL, Stinchcombe JR, Olsen KM, Schmitt J, Purugganan MD (2004) Epistatic interaction between Arabidopsis *FRI* and *FLC* flowering time genes generates a latitudinal cline in a life history trait. Proc Natl Acad Sci USA 101:15670–15675

Chia TYP, Muller A, Jung C, Mutasa-Gottgens ES (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

Colasanti J, Coneva V (2009) Mechanisms of floral induction in grasses: something borrowed, something new. Plant Physiol 149:56–62

Curth P (1960) Der Übergang in die reproduktive Phase der Zuckerrübe in Abhängigkeit von verschiedenen Umweltfaktoren. Deutsch Akademie Landwirtschaftswissenschaften zu Berlin, pp 7–80

Davies TJ, Barraclough TG, Chase MW, Soltis PS, Soltis DE, Savolainen V (2004) Darwin's abominable mystery: insights from a supertree of the angiosperms. Proc Natl Acad Sci USA 101:1904–1909

Distelfeld A, 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(3):223–232

Distelfeld A, Li C, Dubcovsky J (2009) Regulation of flowering in temperate cereals. Curr Opin Plant Biol 12:178–184

El-Mezawy A, Dreyer F, Jacobs G, Jung C (2002) High-resolution mapping of the bolting gene *B* of sugar beet. Theor Appl Genet 105:100–105

Gaafar RM, Hohmann U, Jung C (2005) Bacterial artificial chromosome-derived molecular markers for early bolting in sugar beet. Theor Appl Genet 110:1027–1037

Greenup A, Peacock WJ, Dennis ES, Trevaskis B (2009) The molecular biology of seasonal flowering-responses in Arabidopsis and the cereals. Ann Bot (Lond) (Epub ahead of print) doi:10.1093/aob/mcp063

Hansen M, Kraft T, Ganestam S, Sall T, Nilsson NO (2001) Linkage disequilibrium mapping of the bolting gene in sea beet using AFLP markers. Genet Res 77:61–66

Hanson LE, Panella L (2003) Rhizoctonia root rot resistance of Beta PIs from USDA-ARS NPGS, 2003. Biol Cult Tests 19:FC012. doi:10.1094/BC19

Hautekeete NC, Piquot Y, Van Dijk H (2002) Life span in *Beta vulgaris* ssp. *maritima*: the effects of age at first reproduction and disturbance. J Ecol 90:508–516

He YH, Amasino RM (2005) Role of chromatin modification in flowering-time control. Trends Plant Sci 10:30–35

Hohmann U, Jacobs G, Jung C (2005) An EMS mutagenesis protocol for sugar beet and isolation of non-bolting mutants. Plant Breed 124:317–321

Jung C, Müller AE (2009) Flowering time control and applications in plant breeding. Trends Plant Sci 14:563–573

Koornneef M, Hanhart CJ, Veen JH (1991) A genetic and physiological analysis of late flowering mutants in *Arabidopsis thaliana*. Mol Gen Genet 229:57–66

Koornneef M, Alonso-Blanco C, Peeters AJM, Soppe W (1998a) Genetic control of flowering time in Arabidopsis. Annu Rev Plant Biol 49:345–370

Koornneef M, Alonso-Blanco C, Blankestijn-de Vries H, Hanhart CJ, Peeters AJM (1998b) Genetic interactions among late-flowering mutants of Arabidopsis. Genetics 148:885–892

Kosambi DD (1944) The estimation of map distances from recombination values. Ann Eugen 12:172–175

Laurent V, Devaux P, Thiel T, Viard F, Mielordt S, Touzet P, Quillet M (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

Letschert JPW (1993) *Beta* section *Beta*: biogeographical patterns of variation and taxonomy. Wageningen Agric Univ Papers 93:1–154

Lexander K (1980) Present knowledge on sugar beet bolting mechanisms. In: Proc Int Inst Sugar Beet Res 43rd Winter Congress, pp 245–258

Li CX, Dubcovsky J (2008) Wheat *FT* protein regulates *VRN1* transcription through interactions with *FDL2*. Plant J 55:543–554

Martinez-Zapater JM, Somerville CR (1990) Effect of light quality and vernalization on late-flowering mutants of *Arabidopsis thaliana*. Plant Physiol 92:770–776

McGrath JM, Trebbi D, Fenwick A, Panella L, Schulz B, Laurent V, Barnes S, Murray SC (2007) An open-source first-generation molecular genetic map from a Sugarbeet × Table Beet cross and its extension to physical mapping. Crop Sci 47:27–44

Meier U (2001) Growth stages of mono-and dicotyledonous plants. Phenological growth stages and BBCH-identification keys of beet, 2nd edn. Federal Biological Research Centre for Agriculture and Forestry, Germany

Michaels SD (2009) Flowering time regulation produces much fruit. Curr Opin Plant Biol 12:75–80

Moon J, Lee H, Kim M, Lee I (2005) Analysis of flowering pathway integrators in Arabidopsis. Plant Cell Physiol 46:292–299

Munerati O (1931) L'eredità della tendenza alla annualità nella commune barbabietola, coltivata, Ztschr Züchtung, Reihe A. Pflanzenzüchtung 17:84–89

Nilsson O, Lee I, Blazquez MA, Weigel D (1998) Flowering-time genes modulate the response to *LEAFY* activity. Genetics 150:403–410

Owen FW (1954) The significance of single gene reactions in sugar beets. Proc Am Soc Sugar Beet Technol 8:392–398

Owen FW, Carsner E, Stout M (1940) Photothermal induction of flowering in sugar beets. J Agric Res 61:101–124

Putterill J, Laurie R, Macknight R (2004) It's time to flower: the genetic control of flowering time. Bioessays 26:363–373

Reeves PA, He Y, Schmitz RJ, Amasino RM, Panella LW, Richards C (2007) Evolutionary conservation of the *FLC* mediated vernalization response: evidence from the sugar beet (*Beta vulgaris*). Genetics 176:295–307

Schneider K, Kulosa D, Soerensen T, Möhring S, Heine M, Durstewitz G, Polley A, Weber E, Jamsari J, Lein J, Hohmann U, Tahiro E, Weisshaar B, Schulz B, Koch G, Jung C, Ganal M (2007) Analysis of DNA polymorphisms in sugar beet (*Beta vulgaris* L.) and development of an SNP-based map of expressed genes. Theor Appl Genet 115:601–615

Stout M (1945) Translocation of the reproductive stimulus in sugar beet. Bot Gaz 107:86–95

Trevaskis B, Hemming MN, Dennis ES, Peacock WJ (2007) The molecular basis of vernalization-induced flowering in cereals. Trends Plant Sci 12:352–357

Turck F, Fornara F, Coupland G (2008) Regulation and identity of Florigen: *FLOWERING LOCUS T* moves center stage. Annu Rev Plant Biol 59:573–594

Utz HF, Melchinger AE (1996) PLABQTL: a program for composite interval mapping of QTL. J Quant Trait Loci 2:1–5

Van Dijk H, Boudry P (1991) Genetic variation for life histories in *Beta maritima*. Int Board Plant Genet Resour 7:44–55

Van Dijk H, Boudry P, McCombre H, Vernet P (1997) Flowering time in wild beet (*Beta vulgaris* ssp. *maritima*) along a latitudinal cline. Acta Oecol 18:47–60

Van Ooijen JW, Voorrips RE (2001) JoinMap® 3.0. Software for the calculation of genetic linkage maps. Plant Research International, Wageningen, The Netherlands

Vos P, Hogers R, Bleeker M, Reijans M, Lee Tvd, Hornes M, Friters A, Pot J, Paleman J, Kuiper M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res 23:4407–4414

Wang R, Farrona S, Vincent C, Joecker A, Schoof H, Turck F, Alonso-Blanco C, Coupland G, Albani MC (2009) *PEP1* regulates perennial flowering in *Arabis alpina*. Nature 459:423–427

Yan L, Loukoianov A, Tranquilli G, Helguera M, Fahima T, Dubcovsky J (2003) Positional cloning of the wheat vernalization gene *VRN1*. Proc Natl Acad Sci USA 100:6263–6268

Yan L, Helguera M, Kato K, Fukuyama S, Sherman J, Dubcovsky J (2004) Allelic variation at the *VRN-1* promoter region in polyploid wheat. Theor Appl Genet 109:1677–1686

Yan L, Fu D, Li C, Blechl A, Tranquilli G, Bonafede M, Sanchez A, Valarik M, Yasuda S, Dubcovsky J (2006) The wheat and barley vernalization gene *VRN3* is an orthologue of *FT*. Proc Natl Acad Sci USA 103:19581–19586

Zeevaart JA (2008) Leaf-produced floral signals. Curr Opin Plant Biol 11:541–547