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A New Locus Suppresses Bolting under Shortening Daylength in Sugar Beet

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Abstract Bolting tendency in sugar beet is a complex trait has been shown to be controlled by various environmental cues, including prolonged periods of cold temperatures over winter (vernalization) and photoperiod, and multiple genetic factors. Three loci (*B*, *B2* and *B4*) which trigger bolting in the absence of vernalization were identified and genetically mapped in beet. *B4* is linked to the *B* locus and promotes annual bolting independently of *B*. Here, genetic analysis of a large segregated F2 population derived from a cross between a biennial sugar beet and an annual beet accession phenotyped for bolting tendency under three environmental conditions, i.e., long day after vernalization, long day without vernalization and shortening daylength revealed the presence of a major gene which is linked to the gene *B* and suppresses bolting under unfavorable daylength (shortening daylength) and negatively affects bolting time.

Keywords: *Sugar beet, Beta vulgaris, Bolting, Floral transition, vernalization, photoperiod*

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1. Introduction

Timing of the transition from vegetative to reproductive development is determined by various environmental and endogenous factors. Sugar beet (*Beta vulgaris*, L.) is a biennial species requires a combination of environmental stimuli to initiate bolting (stem elongation) and flowering. These environmental stimuli are the exposure to low temperature (vernalization) between 2 and 10º C, followed by long-day conditions [21]. Annual sugar beets do not exhibit a vernalization requirement for bolting, whereas biennial beets have an obligate requirement for vernalization. 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 brought into knowledge [25]. Bolting time is accelerated and number of bolters (inflorescence stalks) is increased as results of vernalization, depending on the genotype. Long days promote reproductive transition of vernalized plants and accelerate the initiation of stem elongation in the apical shoot meristem and bolting [6,14,29,30].

Annual habit in *B. vulgais* was shown to be under the control of a single dominant gene, termed the bolting gene *B*, which promotes the initiation of bolting in long days without prior vernalization [2,22]. Although heterozygous beets at the B locus (Bb) under favorable conditions behave similar to the annual parent in terms of bolting time, the annual beets developed bolters (seedstalk) more rapidly than heterozygotes derived from crosses of annual and biennial beets [2,23]. A complicated behavior of bolting in heterozygote beets is caused by a number of interacting genes responsible for photo-induction of bolting and a gene for long day requirement is closely linked to *B* and they construct a gene complex for annuality has been suggested [1]. [25] hypothesized the presence of a locus responsible for easy-bolting tendency in the biennial beet, termed *B´*. [8] reported a partial penetrance of the annual habit in *B. vulgaris*, where the *B* genotype is present in plants exhibited non-bolting phenotype, and the degree of the penetrance of the *B* gene is depending on the environmental conditions, i.e. daylength and light intensity and other genetic factors.

The *B* locus has been mapped to chromosome II of sugar beet [16]. Recently, a candidate for the bolting locus designated *BOLTING TIME CONTROL 1* (*BvBTC1*) which encodes a pseudo-response regulator (PRR) protein, and homology to circadian clock-associated genes in Arabidopsis and the major determinant of LD response in barley, *PPD-H1* was cloned [27]. Besides, [11] reported the presence of at least two bolting loci (*B2* and *B3*), manipulating bolting behavior in sugar beet; the *B2* locus was shown to regulate bolting via epistatic interaction to the *B* locus, and was mapped by AFLP mapping to chromosome IX, and *B3* locus, which is unlinked either to the *B* or *B2* locus, and was found to regulate bolting behavior independently from the *B* gene. Furthermore, a locus termed *B4* which is genetically linked to the *B* gene on chromosome II and acting independently from the *B* gene in bolting regulation was recently identified [4].

Synchronization of flowering time is a complex process that is best studied and understood in the model system Arabidopsis thaliana. In this species, flowering is

determined by four major promoting pathways: long-day photoperiod, gibberellin, the autonomous pathway, and vernalization (Jack, 2004). Although, these pathways can act independently, the balance of their signals is integrated by a common set of genes that determine the appropriate time of flowering [26]. The vernalization requirement of Arabidopsis is mainly controlled by two loci: *FRIGIDA* (*FRI*) and the central repressor of flowering *FLOWERING LOCUS C* (*FLC*) which are down-regulated by vernalization. The natural allelic variation at the *FRI* and *FLC* loci, among different accessions of Arabidopsis, account for most of the difference in flowering time between early and late flowering ecotypes [9,12,19,20,24]. In *A. thaliana*, flowering was shown to be regulated antagonistically by *FLC* and the key photoperiod pathway gene *CONSTANS* (*CO*), both of which regulate the same downstream targets, *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) [17,18,31]. *Beta vulgaris* homologs of *FLC* (*BvFL1*) and its upstream regulator *FLK* (*BvFLK*) were cloned and shown to be functionally related to the respective genes in *A. thaliana* [5,28]. Similarly, functional conservation of a *B. vulgaris* homolog of *CO* (*BvCOL1*) was suggested by [13]. The circadian system is crucial for photoperiodic regulation and daylength perception. Variation in activating regulatory genes in different plant species results in the liberation of output signals that promotes floral transition under different photoperiods [15].

In the current study we aim to; i) further explore the genetic basis of floral transition in sugar beet, and ii) As a long-term goal, providing a tool kit for targeted modification of bolting and flowering time for applications in plant breeding including marker-assisted selection for synchronization of flowering time for hybrid seed production and suppression of early bolting which undesirable for sugar production.

2. Materials and Methods

2.1. Plant Materials

An annual *Beta vulgaris* ssp. *maritima* accessions which were collected on the Mediterranean coasts (Bm11-01; [3] and a self-fertile biennial diploid sugar beet cultivar US H11 provided by Crystal Sugar Company, North Dakota, USA were cross pollinated. Crosses were conducted by bag isolation in the greenhouse. Cross progenies were identified phenotypically by hypocotyl color, so that crossing was performed between plants that have different hypocotyl color (with the red hypocotyl color being dominant over the green one [7,10]. Plants with red hypocotyl served as "father" and that with a green hypocotyl as "mother". F1 seeds from "mother plants" were sown, and F1 plants with red hypocotyl color were considered as hybrids. Hybrid F1 plants were propagated in the greenhouse, and selfed to produce F2 seeds. A large segregated F2 population designated Bvm2012 consists of 558 plants was investigated in the current study.

2.2. Test for the Segregation of Annuality

The segregation of *B* was examined under three environmental conditions in terms of daylength and vernalization exposure. Experiments were carried out in a private farm in Kiel, Germany. The Bvm2012 population was divided into three subpopulations. The first subpopulation (Bvm2012.1) consists of 175 plants were sown on May 2nd, 2012 in the greenhouse. Vernalization was done at 8-leaves stage at 4º C for 12 weeks in a small fridge, and then grown in the greenhouse (ca. 20°C) under SD for one week to avoid devernalization, and finally transplanted into the field in June 2nd, 2012 under long day condition (≥16h light). The second subpopulation (Bvm2012.2) consists of 190 plants was sown on April 1st, 2012 in the greenhouse and transplanted into the field in May 1st, 2012 under long day condition (≥16h light). The third subpopulation (Bvm2012.3) consists of 193 plants was sown on July 15th, 2012 in the greenhouse and transplanted into the field in August 15th, 2012 under shortening daylength condition (where day length is getting short (≤14h light)). Date of bolting initiation (a detectable elongation in the flowering stem) was recorded two weeks after transplanting into the field and continued for 20 weeks after transplanting.

2.3. Statistical Analysis

Analysis of variance (ANOVA) and Chi square analysis were carried out with Proc Mixed of SAS package version 9.2 (SAS 2008). Sample groups with significantly different means were further analyzed using Fisher's least significant difference (LSD) test at a 5% probability level (SAS 9.2).

3. Results

3.1. Segregation of Annuality

A biennial diploid sugar beet cultivar US H11 provided by Crystal Sugar Company, North Dakota, USA was crossed with the annual *B. vulgaris* ssp. *maritima* accession Bm11-01. As expected for dominant-recessive inheritance of annual bolting, all F_1 plants originating from the crosses as well as the annual parent all bolted and flowered under all environmental conditions (Table 1).

Table 1. Phenotypic segregation for bolting tendency in subpopulations Bvm2012.1, Bvm2012.2 and Bvm2012.3, their parents under variable environmental conditions

Population	Long-day with vernalization			Long-day without vernalization			Shortening-daylength			
	Bolting plants	Non-bolting plants	χ^2 for 3:1	Bolting plants	Non-bolting plants	χ^2 for 3:1	Bolting plants	Non-bolting plants	χ^2 for 3:1	χ^2 for 1:3
Annual parent	41	0	13.67**	31	0	10.33**	35	0	10.79**	113.48**
Biennial parent	36	0	12.00**	0	29	90.00**	0	36	108.00**	12.00**
F1 population	7	0	21.00**	5	0	1.67	7	0	21.00**	2.93**
Bvm2012.1	175	0	58.33**	-	-	-	-	-	-	-
Bvm2012.2	-	-	-	146	44	0.34	-	-	-	-
Bvm2012.3	-	-	-	-	-	-	49	144	280.49**	0.49

** and -; not applicable and highly significant differences, respectively.

From the same F1 cross, 175, 190 and 193 F2 plants (populations Bvm2012.1, Bvm2012.2 and Bvm2012.3) were phenotyped for bolting tendency under long daylength after vernalization, long daylength without vernalization and shortening daylength, respectively. In Bvm2012.1 which was grown under long day after vernalization all plants bolted (Table 1). In the subpopulation Bvm2012.2 which was phenotyped under long day without vernalization exposure, plants segregated for annuality in accordance with a 3:1 ratio of bolting *vs.* non-bolting plants (Table 1), suggesting simple monogenic inheritance of annual bolting in this population and confirming the complete dominance of the *"B"* allele over the *"b"* allele. In contrary to subpopulation Bvm2012.2 which phenotyped under long day, subpopulation Bvm2012.3 which was phenotyped for annuality under shortening daylength exhibited a segregation ratio of 1:3 bolting *vs.* non-bolting plants as tested by Chi square analysis (Table 1).

3.2. Shortening Daylength Delays Bolting Time

Besides bolting tendency, all subpopulations were phenotyped for bolting time of annual individuals (Table 2; Figure 2). In all three subpopulations annual plants were approximately normally distributed but the position of the maxima differed between populations (Figure 1). In Bvm2012.1 which was phenotyped under long day after vernalization all plants bolted in a narrow time of 15 days (45-59 days after sowing (DAS) with a mean of 49.64 days) (Table 2: Figure 2). In Bvm2012.2 subpopulation which was phenotyped under long day without vernalization annual plants bolted within a relatively wide range of 26 days (39-64 DAS with a mean of 51.10 days) (Table 2: Figure 2)). Meanwhile, in Bvm2012.3 subpopulation which was phenotyped under shortening daylength, annual plants showed a significant delay in bolting time and bolted within a time range of 43-87 DAS (with a mean of 60 days) (Table 2; Figure 2).

Table 2. Number of days to bolting in subpopulations Bvm2012.1, Bvm2012.2 and Bvm2012.3, their parents Bm11-01 (the annual parent), US H11 (the biennial parent) and hybrid F1s

	Long-day with vernalization	Long-day without vernalization	shortening-daylength
Annual parent	40.0± 1.32 ^{A}	40.61± 1.82 ^{A}	43.02± 3.75 ^{B}
Biennial parent	44.55± 3.85 ^{B}	n.a.	n.a.
F1 population	44.00± 1.90 ^{B}	44.40± 3.51 ^{B}	47.20± 2.05 ^{C}
Bvm2012.1	49.64± 3.24 ^{D}	n.a.	
Bvm2012.2	n.a.	51.10± 5.16 ^{D}	n.a.
Bvm2012.3	n.a.	n.a.	60.00± 12.90 ^{E}

Fisher's Least Significant Difference at α=0.05 (LSD=1.96). 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.

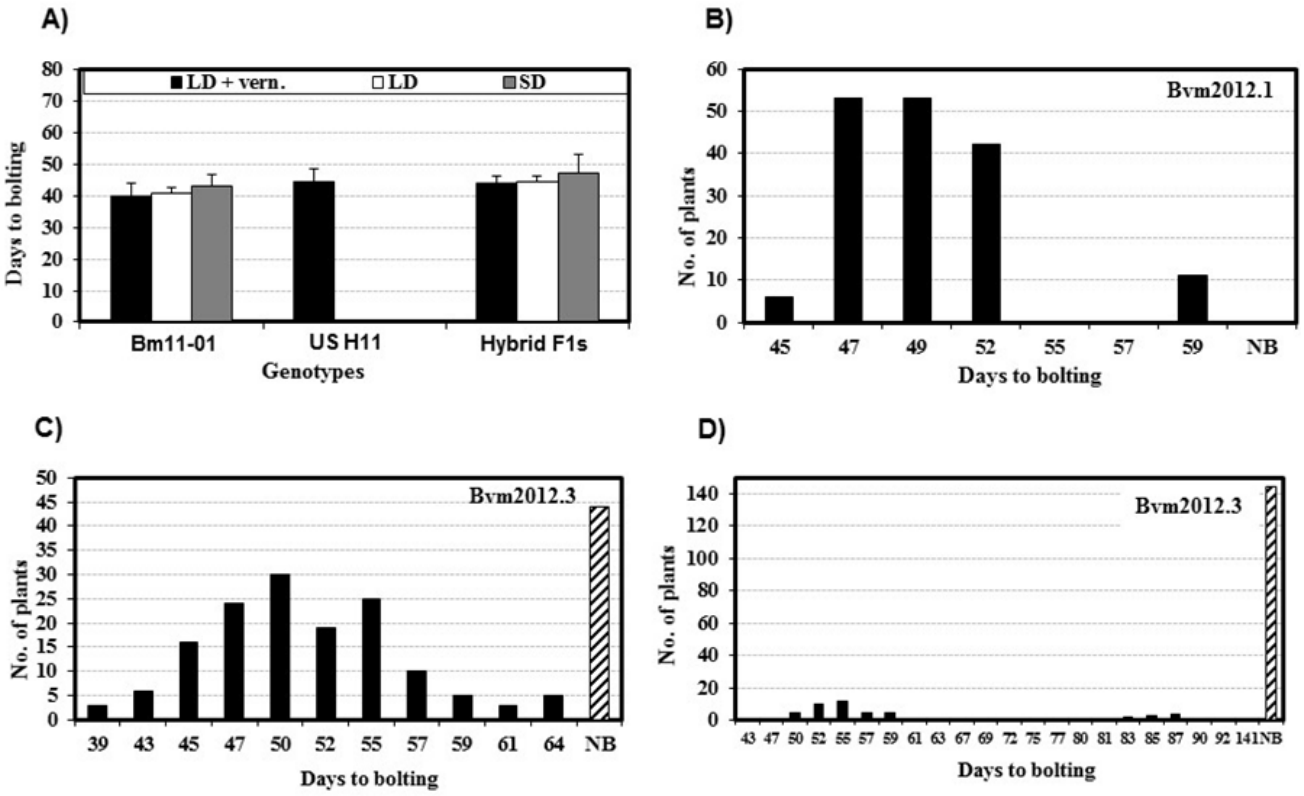

Figure 1. Phenotypic segregation for bolting behavior in F2 subpopulations Bvm2012.1, Bvm2012.2 and Bvm2012.3, their parents Bm11-01 (the annual parent), US H11 (the biennial parent) and hybrid F1s phenotyped under long days after vernalization (LD+ver.), long days without vernalization (LD) and shortening daylength (SD)

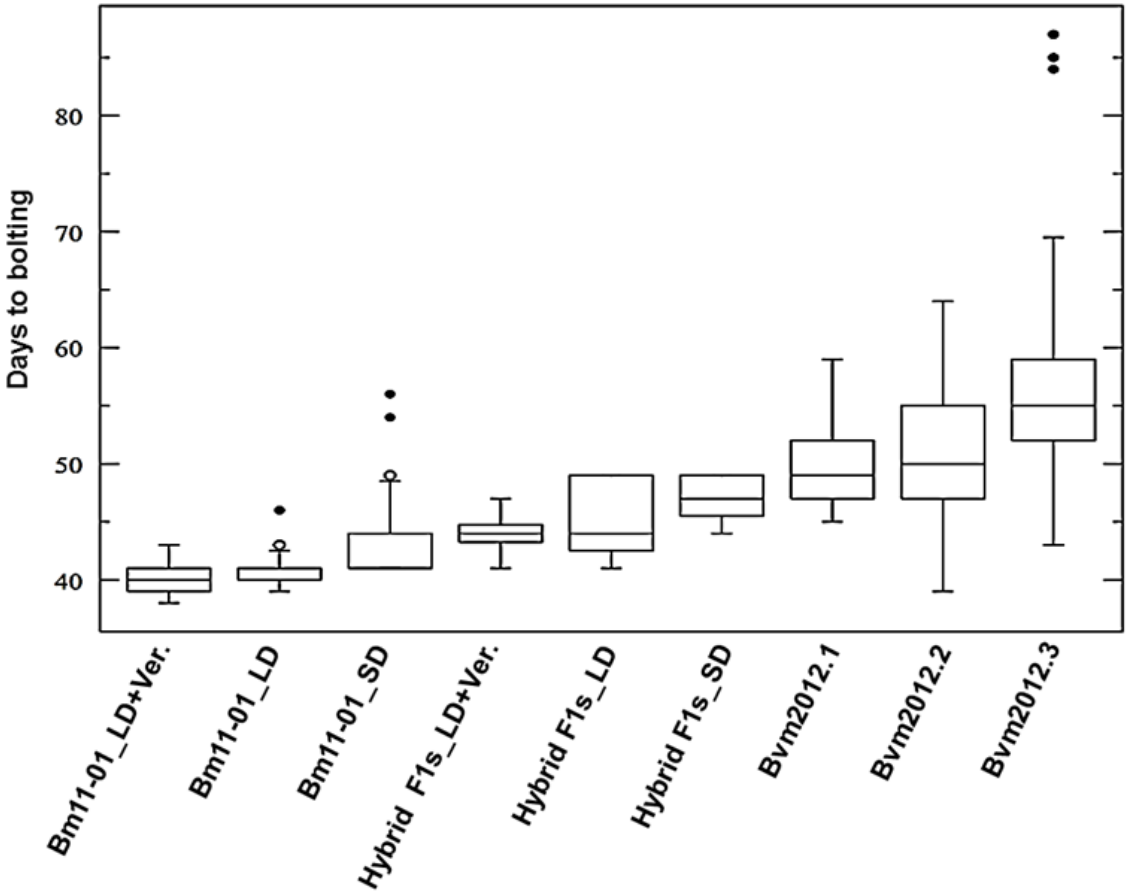

Figure 2. Boxplot for days to bolting of the annual parent Bm11-01, hybrid F1s and annual F2 plants of subpopulations Bvm2012.1, Bvm2012.2 and Bvm2012.3 grown under long days after vernalization (LD+Ver.), long days without vernalization (LD) and shortening daylength (SD)

To test the significance of differences in bolting time between annual plants in all three subpopulations, analysis of variance (ANOVA) was performed for number of days to bolting. Highly significant differences between annual plants in the three subpopulations were observed. The mean of days to bolting for population Bvm2012.3 which was phenotyped under shortening daylength (60 DAS) was significantly higher than that of population Bvm2012.1 (49.64 DAS) and population Bvm2012.2 (51.10 DAS) (Table 2; Figure 2). Fisher's Least Significant Difference (LSD) revealed no significant difference in bolting time between annual plants of population Bvm2012.1 and population Bvm2012.2 (Table 2).

4. Discussion

Three F_2 subpopulations derived from a cross between a biennial sugar beet genotype and an annual wild beet genotype was phenotyped for bolting tendency under variable environmental cues. Heterozygote F1 plants as well as the annual parent all bolted under all environmental conditions (Table 1; Figure 1), confirming that the *"B"* allele is completely dominant over *"b"* allele. The results presented here provide two findings in determining the genetic basis of the bolting suppression observed in selfed F2 progenies. First, shortening daylength was the determinant factor for bolting suppression in the F2 subpopulation Bvm2012.3 because a normal segregation for annuality (3:1, bolting *vs.* non-bolting) was observed in the subpopulation Bvm2012.2 grown under long-day (Table 1). Second, it seems likely that heterozygous *B* plants were susceptible for bolting suppression under shortening daylength than homozygous *B* plants, suggesting that the gene responsible for bolting suppression is tightly associated with heterozygosity for *B*. A similar complicated behavior of heterozygous *B* plants (*Bb*) to environmental cues was observed [1,8,30].

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, with a possible delay, annual beets initiated bolters more rapidly than either F1 or F2 plants. This observation could be ascribed to the presence of a second gene which is responsible for the photo-induction of bolting and closely linked to the *B* locus, which is responsible for the thermal-induction of bolting, manipulating bolting time. However, we cannot exclude the possibility of the presence of some genes that modify the action of the gene *B* in inducing bolting initiation. Bolting tendency was shown to be affected by genes acts epistatically [30]. They suggested that bolting tendency in sugar beet is controlled by genes affected by vernalization and photoperiod, which act independently or interact epistatically, and a large proportion of the genetic effect is mainly due to additive effects. The authors suggested two different physiological mechanisms for daylength and vernalization in regulating bolting initiation which influenced the effects of genes and resulted in epistatic effects on bolting initiation. Moreover, two genes acting either independently or epistatically to the B gene in bolting tendency control of sugar beet were identified [11].

Two lines of evidences suggest that bolting suppression is controlled by a gene responsible for photo-induction

rather thermal-induction and is closely linked to the gene *B* and could easily modify its effect under shortening daylength; i) As reported that the gene *B* is responsible for thermal-induction of bolting [1,25,27], the annual habit in *B. vulgaris* thus is controlled by bolting gene *B* for cold requirement under long days, and ii) the phenotypic segregation ratio for bolting tendency (non-bolting *vs.* bolting) in subpopulation Bvm2012.3 which did not deviates significantly from 3:1, as tested by Chi square analysis (Table 1). Through genetic linkage and quantitative trait locus analyses in two populations derived from a cross between a biennial genotype and an annual wild beet accession, the presence of a novel major bolting locus *B4* which is linked to the *B* locus but promotes annual bolting independently of *B* has been reported [4]. The genetic distance between *B* and *B4* on chromosome II is 11 cM. Moreover, this hypothesis is in accordance with the results of [1] that the annual habit in sugar beet is controlled by the *B* gene under a favorable condition for bolting (vernalization and long days), but it could be easily modified by a gene responsible for daylength requirement under short days. Besides its role in bolting suppression under shortening daylength, the observed delay in bolting time of annual plants under shortening daylength suggest that the new gene is photoperiod-dependent and is most likely a circadian-clock regulated gene (further investigations are required).

In conclusion, our data reveal the presence of a major locus for bolting tendency in *Beta vulgaris*, which is closely linked to the *B* gene and suppresses bolting under unfavorable photoperiodic conditions (shortening daylength). However, quantitative trait locus analysis and a construction of a genetic linkage map may be particularly useful for: i) synchronization of bolting and flowering which is in outbreeding plant species with a reliable environmental cue such as the photoperiod is essential for out-breeding and genetic recombination and for hybrid seed production as an ultimate goal, ii) efficient marker-assisted selection against weed beet in breeding programs or seed quality control assays, and iii) induction of bolting without a requirement for vernalization to promote breeding programs and seed production for cultivation of sugar beet cultivars adapted to tropical and subtropical areas.

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