Theor Appl Genet (2002) 105:100–105
DOI 10.1007/s00122-001-0859-z

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High-resolution mapping of the bolting gene *B* of sugar beet

Received: 16 August 2001 / Accepted: 16 October 2001 / Published online: 17 May 2002
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Abstract Sugar beet (*Beta vulgaris* L.) is a biennial species. Shoot elongation (bolting) starts after a period of low temperature. The dominant allele of locus *B* causes early bolting without cold treatment. This allele is abundant in wild beets whereas cultivated beets carry the recessive allele. Fifteen AFLP markers, tightly linked to the bolting locus, have been identified using bulked segregant analysis. The F_2-population consisted of 2,134 individuals derived after selfing a single F_1-plant (*Bb*). In a first step, a linkage map was established with 249 markers based on 775 F_2-individuals with a coverage of 822.3 cM. The loci are dispersed over nine linkage groups corresponding to the haploid chromosome number of *Beta* species. Seventeen marker loci were placed at a distance less than 3.2 cM around the bolting gene. In a second step, four of those markers most closely linked to *B* were mapped with the entire F_2-population. Two of the markers were mapped flanking the *B* gene at distances of 0.14 and 0.23 cM. The other two markers were mapped at a distance of 0.5 cM from the gene. The tight linkage could be verified by testing 88 unrelated plants from a breeding program. The closely linked markers will enable breeders to select for the non-bolting character without laborious test crossings. Moreover, these markers are being used for map-based cloning of the bolting gene.

Keywords *Beta vulgaris* · Early bolting · Vernalisation · Bulked segregant analysis · AFLP

Communicated by G. Wenzel

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Introduction

Sugar beet (*Beta vulgaris* ssp. *vulgaris* L.) belongs to the family *Chenopodiaceae*. It is a diploid species with 18 chromosomes (x=9) and a nuclear DNA content of 758 Mbp per haploid genome (Arumuganathan and Earle 1991). Sugar beet is a biennial root crop that grows vegetatively in the first season. It initiates stem elongation (bolting) after exposure to a period of low temperature followed by cultivation under long-day conditions. The *B* locus controlling early bolting (annuality) was first described in a commercial sugar beet cultivar by Munerati (1931). Later it was found that the annual habit in related wild beets (*B. vulgaris* ssp. *maritima* L.) is controlled by the *B* allele, which induces bolting under long-day conditions without requirement for vernalization (Abe et al. 1997). The penetration of gene *B* has been found to be influenced by both environmental and genetic factors (Abegg 1936; Owen and Mc Farlane 1958; Sadeghian 1993). Long days are essential for homozygous *B* plants to initiate bolting; they do not display the annual habit under short-day conditions. A more-complicated behavior was recognized in heterozygous *B* plants. Their bolting behaviour is strongly influenced by environmental factors (Shimamoto et al. 1990; Boudry et al. 1994). Outcrossing of wild beets on seed multiplication plots may introgress the *B* allele into cultivated biennial beets, resulting in varieties contaminated with early bolting plants. As a consequence, root yield and sugar content are reduced. Moreover, bolters cause severe problems during crop harvesting. Although breeders are aware of this problem and take strong action to minimize pollen introgression from wild annual beets, a test system to detect the *B* allele within commercial seed lots would be beneficial. For this, molecular markers tightly linked to the bolting gene are a prerequisite.

The construction of a high-density genetic map for sugar beet (*B. vulgaris* L.) allows the identification of markers tightly linked to the bolting locus. Several genetic maps for sugar beet have been published. The first linkage map was based on RFLP and morphological

markers (Pillen et al. 1992) and the map was extended using additional RFLP and RAPD markers (Barzen et al. 1995). Later, 120 AFLP markers were integrated into this map (Schondelmaier et al. 1996; Schumacher et al. 1997). Mapping and identification of agronomically important genes was first established by a chromosomal linkage map of sugar beet using RFLP markers (Barzen et al. 1992). Gene *B* was located between the markers *pKP591* and *pKP826* with respective distances of 3.8 and 5.2 recombination units; four additional markers located in the same region displayed larger distances (Boudry et al. 1994). The linear order of the RFLP loci was identical in this study to the map of linkage group I (Pillen et al. 1992) which has been assigned later to chromosome II of sugar beet (Schondelmaier and Jung 1997).

Our aim was to identify molecular markers tightly linked to the bolting locus to distinguish bolting plants from non-bolting plants as early as the seedling stage. Secondly, those markers will be the basis for positional cloning of the gene. Here, we present the mapping of 15 AFLP markers and two RFLP markers in close vicinity to the *B* gene. The position of these markers has been verified with sugar beet plants differing with respect to early bolting behaviour.

Materials and methods

Plant material and DNA extraction

A non-bolting (*bb*) sugar beet line (A906001) was crossed with a bolting line (93167P) as a pollinator (*BB*). Both lines were provided by the seed company A. Dieckmann Heimburg (Sülbeck, Germany). The F_2-population (960701) was produced by selfing a single F_1-plant (940081) to obtain 2,134 individuals. The plants were cultivated in the greenhouse under long-day conditions (16-h light) at a temperature of 20 °C. Due to the limited greenhouse capacity the entire population was grown in four fractions at four different dates of sowing. Bolting was determined as stem elongation which began 6 weeks after sowing and extending to 15 weeks after sowing. Plants that did not show stem elongation during this period were scored as non-bolting individuals. The expected segregation ratio of 3:1 (bolting:non-bolting) was examined using a χ^2-test. For verification of the F_2-phenotypic data, 1,359 F_3-families (990001) derived from the selfing of individual F_2-plants were planted in a field near Kiel early in May. Of each F_3-family, 20 plants were investigated for their bolting behaviour. These plants were not investigated with AFLP markers.

A number of 88 single plants from a sugar beet breeding program previously characterized for early bolting were used in a verification experiment. The given phenotypic data were 53 bolting and 35 non-bolting plants. Those plants were kindly supplied by KWS Saat AG (Einbeck, Germany).

From each single plant two to three young leaves were collected and stored in 2-ml microcentrifuge tubes at –80 °C. Leaf materials were freeze-dried for 3 days using a Lyophylizer (VaCo I, Zirbus GmbH, Osterode, Germany). Sugar beet genomic DNA was extracted following a CTAB-procedure (Saghai-Maroof et al. 1984).

Marker analysis

Flanking RFLP markers *pKoeln60* and *pKP826* were used for identifying F_2-plants either homozygous for the dominant or for the recessive bolting alleles. Correspondingly, bulked segregant analysis (Michelmore et al. 1991) was carried out using pooled DNA of eight bulks from the F_2-population 960701, three bulks consisting of *BB* plants and five bulks including only *bb* plants. For the bulks, DNA from either eight or four individuals of the F_2-population was pooled. Bulked groups contained equal amounts of DNA.

AFLP analysis was performed according to a standard protocol (Vos et al. 1995). DNA was digested with *Eco*RI (Pharmacia), and *Mse*I (New England BioLabs), and ligated to the *Eco*RI adapter and *Mse*I adapter. For pre-amplification, ligated DNA was amplified using the *Eco*RI primer + A and the *Mse*I primer + C. The PCR reaction was carried out in a Perkin Elmer 9600 thermocycler. An amplification involving primers with three selective nucleotides was conducted. AFLP products were mixed with an equal volume of tracking dye (98% formamide, 10 mM of EDTA, pH 8.0, xylene cyanol and 0.05% bromophenole blue). Amplicons were denatured at 94 °C for 3 min and chilled on ice prior to gel-electrophoresis. Electrophoresis was done on a Li-Cor sequencer (MWG-Ebersberg). Aliquots (1 µl) of sample solutions were loaded onto a denaturing 8% polyacrylamide gel (Long Ranger, Biozym). 1 × TBE buffer was used for electrophoresis. Gels were run at a constant 35 W and 1,500 V. The resulting ‘tiff’ image of the gel was scored visually for polymorphisms using the program One-Dscan (Scanalytics).

Mapping procedure and statistical analysis

AFLPs from F_2-plants were scored codominantly according to their band intensities. A strong band and a missing band was indicative for homozygosity ‘A’ (inherited by the non-bolting parent, A906001) or ‘B’ (inherited by the *BB*-parent, 93167P), whereas a band of normal intensity was indicative for heterozygosity ‘H’. In the case of ambiguity, the individual was scored as a ‘C’ or ‘D’. ‘C’ was used for a non-distinct band derived from the bolting parent, while ‘D’ was used for a non-distinct band derived from the non-bolting parent. The data sets were assembled using the mapping program JoinMap 2.0 (Stam and Van Ooijen 1995).

Observed segregation ratios from each marker were tested by χ^2 analysis for a deviation from the expected ratios (1:2:1, $\alpha = 0.01$). Linkage analysis for all markers (249 marker loci) was performed using JoinMap 2.0. Marker loci were assigned to linkage groups with a two-point analysis based on a LOD score > 20. Map distances were calculated using Kosambi’s mapping function (Kosambi 1944).

Results

Bulked segregant analysis

The flanking RFLP marker loci *pKoeln60* and *pKP826* were used to test 232 F_2-plants which had been previously characterized for early bolting. Crossover events within the interval between both markers were detected in seven non-bolting plants. Bulked segregant analysis was carried out using pooled DNA of eight bulks from the F_2-population 960701: three bulks of *BB* plants and five bulks of *bb* plants. The genotypes were determined according to RFLP markers *pKP826* and *pKoeln60*. In total, 316 AFLP primer combinations were performed in bulked segregant analysis to identify markers linked to the bolting locus. Figure 1 shows AFLP fingerprints of the pooled DNA of bolting and non-bolting bulks derived from the F_2-population 960701 with two AFLP primer combinations (P10 and P11). In total, 123 AFLP markers were able to differentiate between the pooled

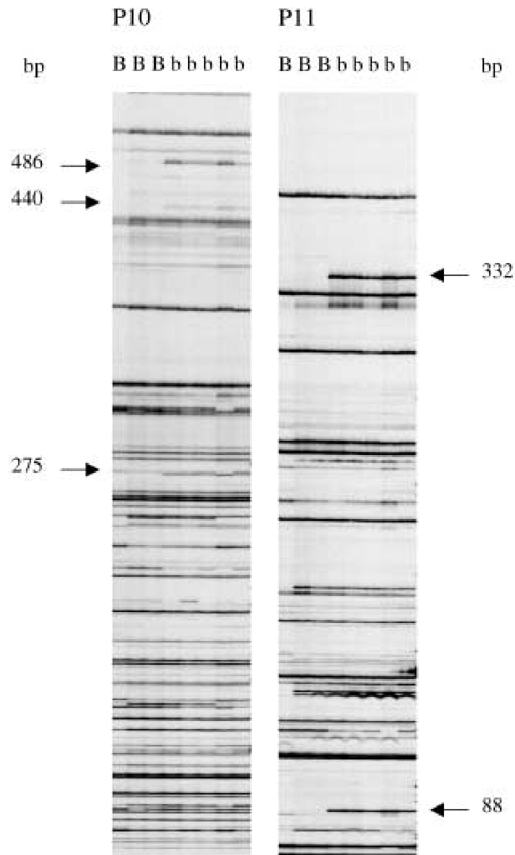

Fig. 1 Bulked segregant analysis with DNA from bolting and non-bolting plants from the F_2-population 960701. AFLPs between three bolting (*B*) and five non-bolting (*b*) bulks were obtained for two AFLP primer combinations (*P10* and *P11*). For P10, *arrows* depict three AFLP markers specific for the b-pool with fragment sizes of 275 bp, 440 bp and 486 bp, respectively. For P11, two markers specific for the b-pool were obtained with fragment sizes of 88 bp and 332 bp, respectively. One microliter of the PCR product was loaded on a 8% polyacrylamide gel. Polymorphisms were detected on a Li-Cor sequencer

DNA of bolting and non-bolting bulks, 18 of them detected a fragment derived from the annual parent, the others detected fragments from the biennial parent.

Linkage analysis

The polymorphic AFLPs were mapped using 775 F_2-plants. On the average, 60–80 bands were visible per gel image, their sizes ranging from approximately 50 to 500 bp. AFLP analysis of the F_2-population with the primer combination P05 shows differences in banding intensities among the bolting plants (Fig. 2), which correlate with the results of the *B*-specific marker P05_B159. We interpreted those plants with strong banding intensities as homozygous at the bolting locus (*BB*) and those with lower intensities as heterozygous ones (*Bb*). In AFLP analysis of the *B*-specific marker P05_b162, as shown in Fig. 2, individuals with strong intensities were scored as homozygous non-bolting plants (*bb*) and those with lower intensities as heterozygous bolting plants (*Bb*). A total of 247 AFLP markers and two RFLP markers was used in this study for linkage analysis.

A high-density map was established in two steps. In the first step, a linkage map based on 775 F_2-individuals was constructed. Linkage relationships of the 249 segregating markers were determined using a three-point analysis. Under these conditions nine linkage groups were established. The linkage map derived from 775 F_2-plants covered 822.3 cM. The linkage group corresponding to chromosome II was 90.7 cM in size including 50 marker loci. Gene *B*, as shown in Fig. 3A, was located within an interval of 5 cM, containing 17 marker loci. The most interesting markers were those that showed the lowest recombination frequencies among 775 genotypes of F_2-population 960701. These markers loci were P04_B174, P04_B193, P05_B159 and P05_b162. They showed 4, 0, 1 and 2 recombinants among 775 individuals, respective-

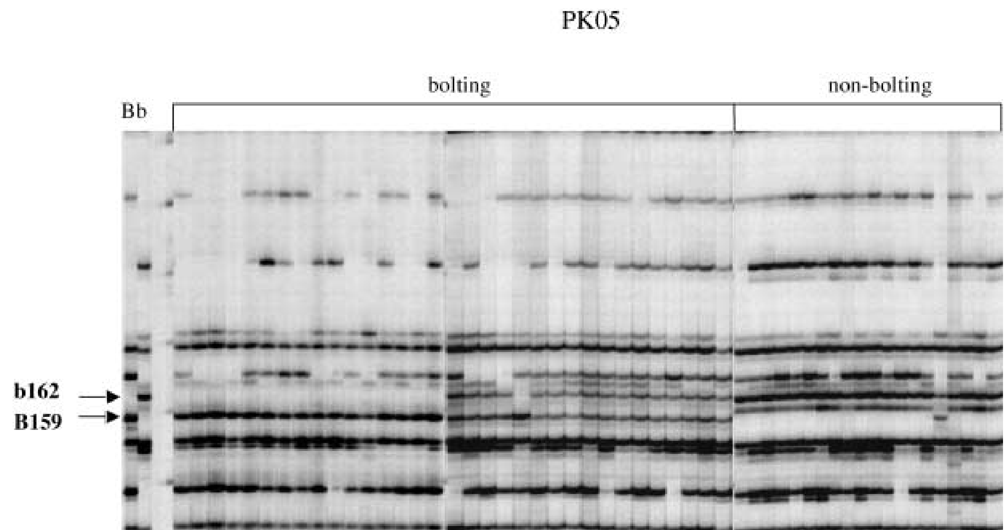

Fig. 2 AFLPs from individual plants of F_2-population 960701 using primer combination P05. The bolting (*B*) and non-bolting (*b*) parents were used as a control. Two AFLPs closely linked to the *B*-gene are indicated by *arrows*: B159 displays a fragment from the bolting parent, b162 from the non-bolting parent. In each case, 1 μl of the PCR amplification product was loaded on an 8% polyacrylamide gel. Polymorphisms were detected on a Li-Cor sequencer

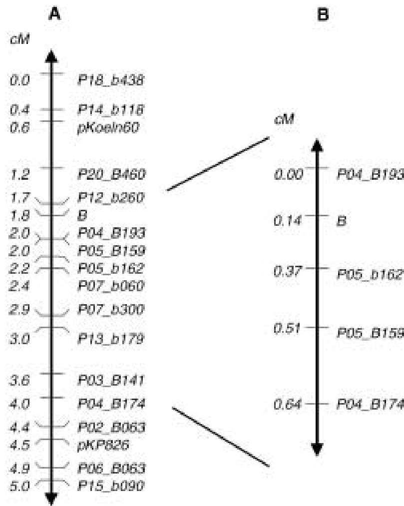

Fig. 3A, B Genetic maps around the *B*-locus on chromosome II of sugar beet. **A** A genetic interval of 5 cM including 17 marker loci and the bolting gene *B*. The map is based on 775 plants of F_2-population 960701. **B** A genetic interval of 0.64 cM around the bolting locus *B* encompassing four closely linked AFLP loci, based on the evaluation of 2,134 plants of the same population

ly. As shown in Fig. 3A, the marker P04_B174 was mapped with a distance of 2.2 cM to gene *B*. The markers P04_B193 and P05_B159 were mapped in the same region with a distance of 0.2 cM from gene *B*. The marker P05_b162 was localized with a distance of 0.4 cM from the bolting locus.

Construction of a high-density map around the bolting locus

In a second step, an additional 1,359 plants of F_2-population 960701 were tested with AFLP markers P04_B174, P04_B193, P05_B159 and P05_b162. The phenotypic data obtained from F_3-families were used here. A frequency of 100% bolting plants in the F_3-generation ensured that the original F_2-individual was homozygous for the bolting allele, whereas a ratio of 3 bolting : 1 non-bolting plants was indicative of a heterozygous F_2-individual and a frequency of 100% non-bolting plants indicated that the original F_2-individual was homozygous for the recessive allele (*bb*). In this way, the bolting score of 48 F_2-genotypes that had behaved as non-bolting had to be corrected as bolting (*Bb*) due to 3:1 (bolting:non-bolting) segregation in the F_3-generation. Similarly, three F_2-genotypes that had behaved as non-bolting were rescored as bolting (*BB*) because only bolting plants appeared in the F_3-generation.

The screening of the four closely linked marker loci in a total number of 2,134 genotypes (775 + 1,359) increased the resolution of the genetic map under study from 0.13 cM using 775 genotypes to 0.05 cM. Figure 3B shows the order and the distances among the four marker loci, P04_B174, P04_B193, P05_B159, P05_b162, and gene *B* based on the recombination frequencies in 2,134 genotypes. The marker P04_B193 showed the lowest recombination frequency among all markers with three recombinants among 2,134 individuals, which corresponds to a distance of 0.14 cM to gene *B*, while the marker P05_b162 showed five recombinants which corresponds to a distance of 0.23 cM from gene *B*. The marker P05_B159 showed eight recombinants corresponding to 0.37 cM, whereas the marker P04_B174 showed the highest recombination frequency of 11 recombinants corresponding to a distance of 0.5 cM from the bolting locus.

Verification experiment with breeding material

Four AFLP markers, P04_B174, P04_B193 and P05_B159, which were linked in coupling phase to gene *B*, and P05_b162 linked in repulsion, were evaluated on a set of 88 sugar beet plants differing in bolting behavior. The given phenotypic data were 53 bolting and 35 non-bolting plants. Whether bolters were heterozygous or homozygous at the *B*-locus was unknown before the marker test. In most cases marker genotypes were correlated

Table 1 Evaluation of four closely linked AFLP markers using a panel of 88 sugar beet plants from nine different lines differing in bolting behavior. Line F was represented by ten plants showing no correlation between marker genotype and bolting phenotype. Lines G through I were used as controls where phenotypes and genotypes had been confirmed by test crossing. The AFLP markers were scored codominantly according to the following scheme. B: homozygous individual for the *B* allele, H: heterozygous individual for the *B* and *b* allele, and A: homozygous individual for the *b* allele. Putative recombinants are typed in italics

Population	Phenotype	AFLP Markers				Expected genotype	No. of individuals
		P04_B193	P05_b162	P05_B159	P04_B174		
A	Bolting	B	B	B	B	B/H	14
B	Bolting	H	H	H	H	B/H	30
C	Bolting	H	H	H	*A*	B/H	1
D	Non-bolting	A	A	A	A	A	16
E	Non-bolting	A	A	*H*	A	A	7
F	Non-bolting	H	H	H	H	A	10
G	Bolting	B	B	B	B	B	6
H	Bolting	H	H	H	H	H	2
I	Non-bolting	A	A	A	A	A	2

with bolting-phenotypes, i.e. bolting plants were either heterozygous or homozygous and non-bolting plants were homozygous at the marker loci (Table 1). Except for line F, marker P04_B193 and the *b*-linked marker P05_b162 showed perfect linkage. The other two markers which had been mapped at a higher distance to *B* displayed seven and one recombinants, respectively. No correlation was found with line F. Ten out of 88 plants were identified by all four markers as heterozygous for the bolting locus, although those plants had been scored as non-bolting plants under long-day conditions (16-h light) and a temperature of 20 °C.

Discussion

In this study, a fine-scale map around the bolting gene *B* of sugar beet has been established. We demonstrate that the combined strategy using bulked segregant analysis and AFLP markers is an efficient means to identify tightly linked markers to the gene of interest, as has been previously shown for other crop species (Ballvora et al. 1995; Meksem et al. 1995; Thomas et al. 1995).

In sugar beet, marker analysis is facilitated by the high degree of polymorphisms, typical for an outbreeding species. An evaluation of AFLPs in sugar beet had been carried out screening different accessions of sugar beet and wild beet (Hansen 1998). Among 15 accessions, 96.4% of the AFLP loci turned out to be polymorphic. In our experiment 316 primer combinations were initially used for pre-selecting the most-informative ones. For mapping, 25 primer combinations were used yielding 247 polymorphic AFLP markers.

The correct position of mapped markers strongly depends on the quality of the phenotypic data obtained. On a first view, bolting is an easy to score character. However, environmental factors have to be considered, such as temperature and day length. Also environment × genotype interactions have been observed. Here, we have verified the phenotypic data from individual F_2-plants using F_3-families. Within the F_2-population under study, 51 F_2-genotypes out of 1,359 were regarded as wrongly classified during phenotypic evaluation (3.75%). After evaluating the F_3-offspring of the single F_2-individuals, it was possible to correct the wrong phenotypic data.

In addition, four tightly linked markers were tested with unrelated breeding material. For 8 out of 9 lines tested, the potential of our markers for marker-assisted selection of beets homozygous for the bolting locus was clearly demonstrated, thus avoiding time-consuming test crossings. One line previously scored as non-bolting under field conditions was found to be heterozygous at the four linked marker loci. The reasons for this may be the false scoring of plants due to field conditions unfavorable for early bolting. Recombination events at such a high frequency are unlikely to explain those disagreeing results because of the small distances between markers and the bolting gene. However, if all those individuals have inherited the same chromosome region around the *B* gene, a single recombination event that had occurred earlier might be an explanation.

The final aim of our study is to clone the bolting gene. One way would be a candidate gene-approach using previously cloned vernalization or flowering genes from *Arabidopsis thaliana*. The *FRI* (Johanson et al. 2000) gene causes late flowering whereas *FCA* (Macknight et al. 1997) strongly promotes flowering in that species. It was reasonable to believe that one of these genes might be an ortholog of *B*, thus sharing some degree of homology (Levy and Dean 1998). cDNA clones from both genes kindly supplied by Dr. Caroline Dean (Norwich, UK) were hybridized as RFLP probes to sugar beet bulks from our segregating F_2-population (data not shown). While the FRI probe gave signals only on *A. thaliana* DNA, the FCA probe hybridized also to sugar beet DNA. However, no polymorphism between bolting and non-bolting bulks was observed. The absence of linkage between the putative FCA homolog in beet and the *B*-locus indicates that *B* is not an FCA homolog, which implies that the *B*-gene can only be cloned by a positional cloning approach. This strategy strongly relies on tight relationships between genetic and physical distances in the given genome region. The *B*-gene is obviously located near the centromere as judged from its position on the genetic map. Also, clustering of markers around the gene may indicate a centromere-near position. Clusters of markers at centromeric and possibly telomeric regions were found in other species like tomato (Tanksley et al. 1992), barley and wheat (Chao et al. 1989; Qi et al. 1996). In the present AFLP map of sugar beet, several markers clustering around the putative centromere were identified. The centromeric suppression of recombination may be one of the main reasons for the regional concentration of markers in genetic maps (Frary et al. 1996). This has to be taken into consideration when cloning the gene from its position. Our map relies on an extraordinary high number of recombination events thus offering the possibility for constructing a physical map around the *B*-locus. We have already started sceening YAC- and BAC-libraries and a first, yet incomplete, contig around the gene has been established.

The cloned bolting gene itself could be used as a marker for identifying heterozygous individuals, avoiding problems with recombination between marker loci and the target locus. Even more interesting from a breeder's view is the manipulation of early bolting by means of genetic manipulation. This could be achieved by transformation of beets with the *B*-gene under the transcriptional control of an inducible promotor. Then, the early bolting/early flowering character could be utilized to shorten the breeding process while varieties for commercial beet production will still have the non-bolting phenotype. Alternatively, antisense technology (Arndt and Rank 1997) could be used for completely suppressing the bolting gene in beet varieties. In this way winter beets can be bred which are sown before winter even in moderate climates like those of central Europe. Complete resistance to bolting even after cold treatment is a

prerequisite for the breeding of winter beets which could only be handled by genetic manipulation of the *B*-gene. Due to the longer growing period, winter beets have a higher yielding potential offering an interesting alternative to beet production in the future.

Acknowledgements We thank Monika Bruisch for her excellent technical assistance. This project was funded by the seed companies A. Dieckmann Heimburg (Sülbeck, Germany) and KWS Saat AG (Einbeck, Germany).

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