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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.)

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Abstract The annual habit in beet is due to complete or partial absence of the vernalization requirement and can cause severe problems in the beet crop. The absolute vernalization requirement in beet is controlled by a major gene *B* (bolting), known to be linked to the gene *R* (red hypocotyl color), in linkage group I. Segregation for the *B* and *R* genes was studied in several beet progenies. Penetrance of the annual habit in *Bb* genotypes was affected by both environmental and genetic factors. The precise location in linkage group I of the major gene *B* was found by restriction fragment length polymorphism (RFLP) analysis in a back-cross progeny exhibiting partial penetrance of the annual habit. The linkage value between *B* and *R* was in good accordance with previous estimations. Use of the closest RFLP marker (pKP591: 3.8 recombination units) allowed us to estimate the penetrance of the annual habit in this back-cross as 0.62. Evidence of pseudo-compatibility was found in the wild coastal beet (*Beta vulgaris* ssp *maritima*) used as the mother plant of the back-cross: the selfing rate was estimated as 7%.

Key words Bolting gene · Vernalization requirement *Beta vulgaris* L. · RFLP markers · Penetrance

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Introduction

Cultivated beets are either root or leaf crops. Farmers harvest vegetative parts of the plants at the end of the first growing season. Development of reproductive structures, i.e., stem elongation of a former rosette plant, occurs normally during the second growing season after a long period of cold temperature called vernalization. For this reason, beet seed production requires overwintering of the parental lines. Nevertheless, bolting in the field during the first growing season can occur and leads to a yield reduction, a decrease in the sugar content of the roots, and mechanical problems during harvest and sugar extraction processing (Nelson and Deming 1952; Wood and Scoot 1975; Longden and Goddard 1987). Therefore, genetic factors responsible for bolting sensitivity are intensively selected against by sugar beet breeders. On the other hand, seed production of strongly bolting-resistant genotypes can be problematic due to the limited numbers of plants flowering after overwintering in seed production areas (Desprez 1980).

Earliness of flowering is due to the interaction of environmental and genetic components. The main environmental factors are temperature, involved in both vernalization and growth rate, and photoperiod. Knowledge of the flowering-regulating effects of light and temperature and internal physiological mechanisms in sugar beet is reviewed by Lexander (1980). Cultivated beet is characterized as a long-day plant with a vernalization requirement (Vince-Prue 1975). Wild beet populations [*Beta vulgaris* ssp *maritima* (L.) Arcangeli] exhibit a wide range of genetic variability for vernalization requirement (Van Dijk and Boudry 1992).

The absence of any vernalization requirement has been shown to be under the control of a single dominant gene: the bolting gene *B*. As early as 1876, Rimpau provided evidence that the annual habit is dominant. Crosses made by Munerati (1931) showed that the mode of inheritance of the bolting character is controlled by a simple dominant Mendelian factor. These results were confirmed by Abegg

Table 1 Description of the plant material studied (see also Fig. 1)

Name	Phenotypes			Genotypes of the plants used as parents	No. of plants	
	Origin	Life cycle	Hypocotyl color		Autumn sowing	Spring sowing
GP_2	Table beet	Biennial	Red	*Rb/Rb*		1
GP_1	Adventitious beet	Annual	Green	*rB/rB* or *rB/rb*		40
GP_1'	$GP_1 \times GP_1$	Mainly annual	Green		19	72
F_1	$GP_1 \times GP_2$	Annual and biennial	Red		92	132
P_1	$GP_1 \times GP_2$	Annual	Red	*Rb/rB*		1
P_2	Wild coastal beet	Biennial	Green	*rb/rb*		1
$F_{2.1}$	$F_1 \times F_1$	Annual+biennial	Green+red			118
$F_{2.2}$	$F_1 \times F_1$	Annual+biennial	Green+red			135
BC	$P_1 \times P_2$	Annual+biennial	Green+red		165	135

(1936) who defined the Y-R-B linkage group. The cross-over frequency between the *B* and *R* genes (red color of the hypocotyl) was estimated as 15.5%. This linkage group is now known as linkage group one (Theurer 1968; Smed et al. 1989; Pillen et al. 1992) and has been cytologically assigned to chromosome one (Van Geyt et al. 1988). Fundamentally, the *B* gene seems to remove all necessity for thermal induction, but is affected by the presence of other genes and by the environment. Penetrance of the annual habit (the proportion of *Bb* individuals exhibiting an annual habit) was discussed by Owen (1954): "heterozygous *Bb* beets are much more complicated to deal with than homozygous *BB* beets. Much of this complexity is probably due to the influence of modifying genes introduced with the *b* gene". He concluded that genetic investigations need to be carried out under controlled greenhouse conditions (day length and temperature), as the effect of a single gene may range from Mendelian to quantitative depending on the environment. Most of the more recent genetical investigations, carried out by sugar beet breeders, deal with the quantitative vernalization requirement in *bb* genotypes (Marcus 1948; Bolelova et al. 1984; Le Cochec and Soreau 1989) and have been interpreted in terms of a multigenic quantitative trait with dominance effects. The *B* gene, however, is hardly discussed in these studies. The distribution of the *B* allele in wild beet populations shows a north-south cline along the coastlines of Europe (Van Dijk and Boudry 1992).

The presence of undesired bolting plants in the leaf or root crop can be due either to (1) the presence of individuals without any vernalization requirement, a trait under the control of the major gene *B*, or (2) the vernalization of individuals with a low quantitative vernalization requirement permitting floral induction following cold days after early spring sowing (Desprez 1980; Evans and Weir 1981). Individuals without any vernalization requirement have been found in high frequencies in French weed beet populations. These originate from hybridization between cultivated and wild inland beets in seed production areas (Boudry et al. 1993). Control, under greenhouse conditions, of seed lots produced in areas where these inland wild beet populations are present is often required in order to prevent unacceptable bolting rates in the field. This illustrates the strong agronomical importance of the *B* gene.

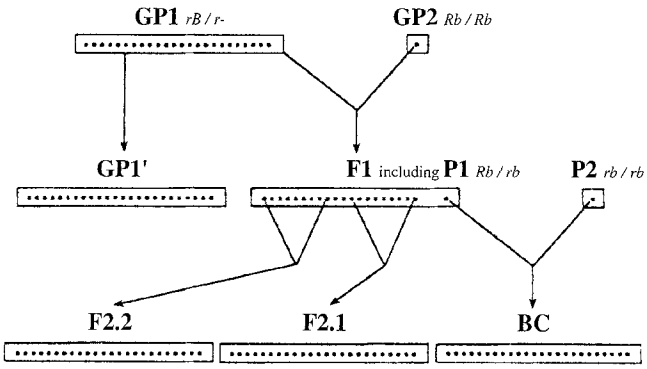


Fig. 1 Crossing scheme used to generate the progenies studied

Dense linkage maps are a prerequisite for the precise localization of a given gene. Recently, RFLP maps for sugar beet have been published by Pillen et al. (1992) and Barzen et al. (1992). Since the *R* gene was already included in the RFLP map published by Pillen et al. (1992), our objective was to precisely localize the *B* gene on this map. DNA markers linked to *R* were used in a linkage analysis on a back-cross segregating for the *R* and the *B* genes. Markers closely linked to the *B* gene were then used to estimate the penetrance of the annual habit in this back-cross. Other probes were tested to evaluate the selfing rate of the mother plant in the back-cross studied.

Materials and methods

Plant material

Different types of beets have been crossed in order to obtain a back-cross progeny (BC) segregating for both *B* and *R* genes, which was then used for the RFLP analysis (Fig. 1, Table 1). The table beet, adventitious beets, and the wild coastal beet which we crossed are taxonomically considered as members of the same species: *Beta vulgaris* L. [see Letschert (1993) for a taxonomical review of the section *Beta*]. The F_1 (*rB/Rb*) was obtained by pollination of a set of wild annual inland beets (*B. vulgaris* L.) from south-western France

(GP_1: *rB/rB* and *rB/rb*) by a red table beet (GP_2: *Rb/Rb*). GP_1' are individuals resulting from crosses between GP_1 annual inland beets. One of the F_1 individuals (P_1: *Rb/rB*) was used as a pollen donor for a coastal wild beet (*B. vulgaris* ssp *maritima*) (P_2: *rb/rb*) to produce the BC. Other F_1 individuals were crossed pairwise to generate $F_{2.1}$ and $F_{2.2}$.

Test for hypocotyl color and the annual habit

Plants were sown and grown under greenhouse conditions. Annual habit was tested by growing plants under long days (16 h) at a temperature between 20 °C and 25 °C. Under such photo-thermal conditions, only individuals carrying the *B* allele are able to bolt and flower; *bb* genotypes stay vegetative as they require vernalization for flowering induction. Plants were classified as flowering or non-flowering 130 days after germination. The test was performed in two seasons, autumn and spring; light intensity and temperature being slightly higher in spring than in autumn. The tested progenies were grown simultaneously in autumn or spring under identical conditions. Hypocotyl color was recorded as green or red a few days after germination. The proportions of plants were compared using a χ^2 test.

RFLP analysis

Total DNA extraction, restriction digests and blotting procedures were as described in Saumitou-Laprade et al. (1993) with the following specifications: 5 µg of DNA was restricted with *Eco*RI (2u/µg DNA), *Eco*RV (4u/µg DNA), *Hin*dIII (8u/µg DNA), and *Xba*I (8u/µg DNA). For each combination (individual/enzyme) 5 µg of restricted DNA was separated on 0.75% agarose-slab gels overnight. Southern filters were hybridized with radiolabelled probes (Feinberg and Vogelstein 1983) as described in Pillen et al. (1992). Autoradiograms were scored after 5-days exposure. Probes were chosen according to their position relative to the *R* gene on linkage group I as published by Pillen et al. (1992).

Linkage analysis

Linkage analysis was performed with the computer program MAPMAKER (Lander et al. 1987) using linkage criteria of LOD=0.4 and 0.23 recombination units. Multi-point linkage map distances were calculated using the Kosambi transformation (Kosambi 1944). Partial penetrance of the *B* gene led to an unbalanced back-cross. For this reason, only the flowering BC plants were used to estimate probe ordering and linkage values between RFLP probes and the *B* gene. The mapping procedure was not fundamentally modified due to this unbalanced back-cross progeny.

Results

Segregation for hypocotyl color and annual habit: choice of individuals used for the mapping of the *B* gene

Results for the segregation of hypocotyl color and annual habit are presented in Table 2. Growing season (autumn or spring) significantly affected the ratio of annual versus biennial plants in the F_1 progenies (χ^2=110.7; P< 0.001) but not in GP_1' and BC (χ^2=4.9; P=0.27 and χ^2=1.9; P=0.49 respectively). GP_1' showed 84.2% of plants flowering in autumn and 97.2% in spring. This suggests that GP_1 was not totally fixed for the *B* allele and segregated a few *bb* genotypes in the GP_1' progeny. These data are confirmed by the results obtained with the F_1: instead of the expected 100% flowering plants with a total penetrance and dominance of

Table 2 Segregation results for hypocotyl color and annual habit in the different crosses used in this study (see Fig. 1), compared to the expected values for a total penetrance of red hypocotyl and annual habit assuming 15.5% recombination between the genes *B* and *R* (ns, not significant; ***, P< 0.001)

Parents genotypes	Progenies and sowing season	Green hypocotyl, flowering				Red hypocotyl, non-flowering				Green hypocotyl, non-flowering				Red hypocotyl, flowering				Chi-square test	Total number of plants
		Observed		Expected		Observed		Expected		Observed		Expected		Observed		Expected			
		n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%		
rB/r–×*rB/r*–	GP_1' autumn	16	84.2	–		0		–		3	15.8	–		0		–			19
rB/r–×*rB/r*–	GP_1' spring	70	97.2	–		0		–		2	2.8	–		0		–			72
rB/r–×*Rb/Rb*	F_1' autumn	0		–		71	77.2	–		0		–		21	22.8	–			92
rB/r–×*Rb/Rb*	F_1' spring	0		–		11	8.3	–		0		–		121	91.7	–			132
Rb/rB×*Rb/rB*	$F_{2.1}$ spring	36	30.5	28.8	24.4	31	26.3	28.8	24.4	2	1.7	0.7	0.6	49	41.5	59.7	50.6	ns	118
Rb/rB×*Rb/rB*	$F_{2.2}$ spring	33	24.4	32.9	24.4	29	21.5	32.9	24.4	1	0.7	0.8	0.6	68	50.3	68.3	50.6	ns	135
Rb/rB×*rb/rb*	BC autumn	33	20.0	69.7	42.25	82	49.7	69.7	42.25	43	26.1	12.8	7.75	7	4.2	12.8	7.75	***	165
Rb/rB×*rb/rB*	BC spring	32	23.7	57.0	42.25	55	40.7	57.0	42.25	41	30.4	10.5	7.75	7	5.2	10.5	7.75	***	135

the annual habit in *Bb* genotypes, only 22.8% of the F_1 plants flowered for the autumn sowing and 91.7% for the spring sowing. Both $F_{2.1}$ and $F_{2.2}$ show a segregation consistent with expectation taking into account a 15.5% recombination between *R* and *B* (for the two families grouped together: χ^2=3.3; *P*=0.34). These results confirmed that the total penetrance of the *B* gene is only achieved under our spring growing conditions.

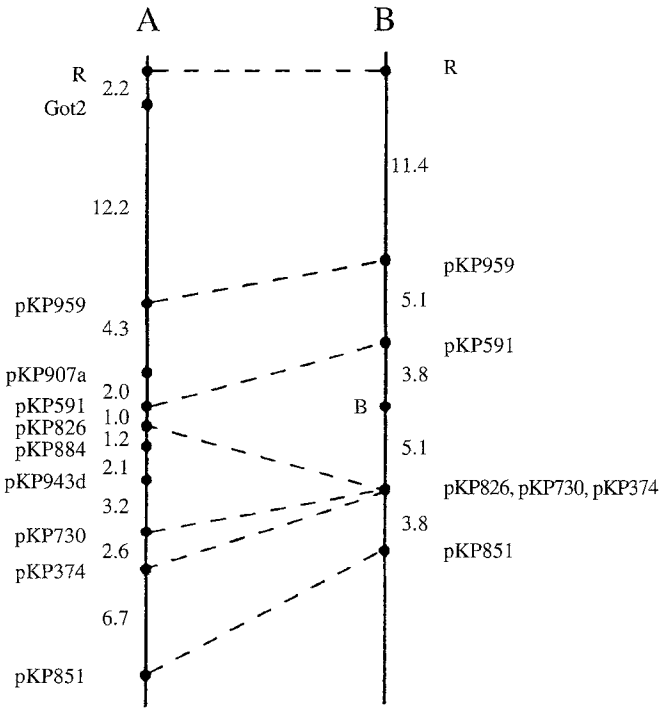
The back-cross, made by a cross between a *Rb/rB* F_1 plant and a *rb/rb* coastal wild beet, showed a significant deviation from the expected 1:1 segregation for the annual habit (for spring and autumn sowing grouped together: χ^2 = 192.7; *P*< 0.0001). Therefore, the search of RFLP markers for the *B* gene and mapping of the *B* gene was performed only on flowering plants, as a fraction of the non-flowering plants were known to carry the *B* allele.

Mapping of the *B* gene

Nine RFLP markers located around the *R* gene on linkage group I were used for the mapping procedure of the *B* gene. All the probes tested showed polymorphic patterns due to the various origins of the plants used in the crossing scheme. However, two high-copy probes (pKP907, pKP943) could not be used due to their complex banding patterns, leading to the impossibility of allelic identification. pKP884, a low-copy probe, showed no linkage with the other probes tested; the fragment linked to the *B* gene was presumably monomorphic in our BC progeny. Additionally, the *Got* izozyme system was tested in the autumn-sowing BC progeny: *Got-3* was found to be polymorphic with no linkage with the R gene while *Got-2* was found to be monomorphic.

Linkage between six RFLP probes (pKP959, pKP591, pKP826, pKP730, pKP374, pKP851) and the *B* gene was estimated as follows. In a first step, among the observed allelic bands, we selected those preferentially associated with flowering plants, as linked to the *B* allele coming from the P_1 parent. These allelic bands were also present in some of the non-flowering plants but were absent in P_2 and GP_2 (*bb* parental plants). Linkage was then estimated within the flowering *Bb* plant fraction (79 individuals). Despite partial penetrance of the annual habit, all the markers used showed significant linkage to the *B* gene. The linkage map based on the segregating back-cross progeny is presented in Fig. 2 together with the corresponding map as obtained by Pillen et al. (1992), slightly corrected according to the most recent available data. We located *B* between the markers pKP591 and pKP826 with respective distances of 3.8 and 5.3 recombination units. The linear order of the RFLP loci was identical in our back-cross with the map of chromosome I developed by Pillen et al. (1992).

Fig. 2 Partial linkage map of a part of chromosome I obtained from the segregating back-cross (*B*) as compared to the map obtained by Pillen et al. (1992) using an F_2 (*A*). Distances are given in recombination units



Evidence of selfing of the P_2 coastal wild beet (*B. vulgaris* ssp *maritima*)

A few individuals within the non-flowering plants with green hypocotyls exhibited banding patterns which led to the hypothesis that they were selfed progenies of the mother plant P_2. These individuals did not interfere in the mapping of the *B* gene because non-flowering plants were not included in the linkage analysis. Within the non-flowering plants with green hypocotyls, estimation of the selfing rate was needed to determine the penetrance of the *B* gene in the BC progeny in order to distinguish between *rb/rb* genotypes arising through selfing and *rb/rb* genotypes due to crossing-over between *B* and *R*. The probe pKP1195, located close to the *R* gene (Pillen et al. 1992), was used on non-flowering plants with a green hypocotyl to evaluate the selfing rate in this cross. This allowed us to distinguish 20 individuals due to the selfing of the P_2 mother plant out of 84 non-flowering plants with a green hypocotyl (Table 3). The segregation for hypocotyl color (green:red) has, therefore, to be changed from 149:151 to 129:151, which is not significantly different from the expected 1:1 ratio (*P*=0.19). All the other probes, tested on a subset of non-flowering plants with a green hypocotyl, confirmed the hypothesis of selfing of the P_2 mother plant as being the origin of these individuals. The selfing rate of the mother plant P_2 can therefore be estimated as 20/300=7%.

Estimation of the penetrance of the annual habit

As expected, due to the partial penetrance of the annual habit, allelic bands linked to *B* were also present in non-

Table 3 Estimation of the number of selfings and the penetrance of the annual habit in *Bb* individuals in the BC progeny

Item	Autumn sowing		Spring sowing		Total number of plants
	Flowering	Non-flowering	Flowering	Non-flowering	
Plants with green hypocotyl (*rB/rb* and *rb/rb*) divided into:	33	43	32	41	149
Plants due to selfing (*rb/rb*)[a]	0	9	0	11	20
Recombinants between *R* and *B* (*rb/rb*)	0	9	0	15	24
rB/rb genotypes in the "true BC"[b]	33	25	32	15	105
Penetrance estimated: (flowering *rB/rb*)/ (total *rB/rb*)	33/58=0.57		32/47=0.68		65/105=0.62

[a] As revealed by the probe pKP1195
[b] As revealed by the probe pKP591

flowering plants. The probe pKP591 was used to evaluate the number of *B* genotypes in the plants with a green hypocotyl (*rr*). These results are summarized in Table 3. Within the 129 green hypocotyl BC plants, 105 (81%) showed the band linked to the *B* allele. This ratio is consistent with a recombination value of 16.5 between the *R* gene and this probe (P=0.52) and we can conclude that no significant distortion of segregation occurred for this locus. Thus, the reduced number of flowering plants in the back-cross progeny is therefore not due to segregation distortion of the B gene but rather to partial penetrance of the annual habit. This penetrance can be estimated to be 0.62 within the *rrBb* genotypes, *B* being checked by the probe pKP591.

Discussion

Advances in dense RFLP maps for the major crop species have been reviewed by Tanksley et al. (1989). For sugar beet, two maps have been recently published (Barzen et al. 1992; Pillen et al. 1992). A few genes relevant to the expression of agronomically-interesting traits were localized on these maps (hypocotyl color, monogermy, rhizomania resistance). Despite its identification and location on the *Y-R-B* linkage group more than 60 years ago, and its strong agronomical importance, the precise location of the *B* gene is still unknown. Tests for the annual habit in sugar beet commercial seed lots are time and money consuming and not always foolproof since the penetrance of the *B* allele is not always complete and depends on environmental growing conditions. Our determination of the map position of the *B* gene provides genetic markers of the annual habit in beet which could be useful in both sugar beet breeding, seed-lot control, and wild beet population studies. Tightly-linked markers assure the starting point for cloning and sequencing the *B* gene, in order to determine the gene product and its action, both of which are still unknown. Stout (1945), Margara (1960), and Curtis (1964) demonstrated the critical action of a biochemical substance by grafting biennial beets to annual beets; and an extensive review of the possible involvement of gibberellins (GA) in vernalization is provided by Pharis and King (1985). Changes in GA occur soon after vernalization and during flower initiation in bolting-susceptible sugar beets but not in bolting-resistant ones (Pocock and Lenton 1980). GA applications on non-vernalized *bb* genotypes induce bolting but not flowering (Margara 1960). One of the major problems with assigning a role to phytohormones in vernalization is to separate the numerous different developmental and biochemical effects on photo-inductibility, shoot elongation, and flower initiation. Cloning and sequencing of *B* might therefore provide a valuable insight into the physiological and biochemical basis of vernalization requirement in plants.

The cross-over frequency between the *R* and *B* genes was first estimated as 15.5% (Abegg 1936). This value was approximated from several segregating progenies (backcrosses and F_2s) giving different values ranging from 9.6±1.30 to 20.0±1.60%. Owen and Ryser (1942) reported a value of 15.9±0.58% recombination between *R* and *B*. Our result is in good accordance with these estimations, the two-point analysis giving a value of 18% and the multipoint analysis a total of 20.3%. Discrepancies in recombination frequencies between crosses can also be found for the presently-studied RFLP probes. Recombination values, given by the two-point analyses, between the RFLP probes pKP591 and pKP826 flanking the *B* gene are 1% in the F_2 (Pillen et al. 1992) and 9% in our backcross. These values are significantly different (P=0.003) and might be related to the fact that different subspecies were used to estimate recombination frequencies. Genetic variability for recombination frequencies has been documented within and among several plant subspecies (e. g., Beavis and Grant 1991; Ritter et al. 1991; Wang et al. 1991; Fatmi et al. 1993). Recombination frequencies tend to decrease when the individuals involved in the cross are taxonomically distant, due either to reduction of recombination itself or else to the partial elimination of zygotes originating from recombinant gametes (Gebhardt et al. 1991). Distorted mapping might therefore be greater in crosses involving wild and cultivated forms of beet than in crosses within each form. On the other hand, no consistent hybridization barrier was found between cultivated beets and *B. vulgaris* ssp *maritima* (see Abe et al. 1987) and no significant

segregation distortion was found for the *R* gene in our study.

B. vulgaris ssp *maritima* is usually reported as self-incompatible (Dale and Ford-Lloyd 1983). The gametophytic self-incompatibility system is known to be under the control of two loci, *X* and *Z* both with multi-allelic series (Owen 1942). The dominant allele S^f for self-compatibility is largely used in breeding programs. Pseudo-compatibility, defined as low selfing rates in the absence of allopollen competition, has been reported in both cultivated beets (3.2–8.3%, Barocka 1985) and wild beets (see Knapp 1958). The observed 7% selfing of the mother plant P_2 could be explained by relatively-low pollen production of the annual P_1 parent due to its smaller size. RFLP markers, such as probe pKP1195, provide a good tool for the study of selfing rates and auto- and allo-pollen competition in beets.

As emphasized by Owen (1954), the influence of growing conditions is greater in *Bb* genotypes than in *Bb* genotypes. This is clearly illustrated in our study by the comparison of F_1 (*Bb*) and GP_1' (*BB*) progenies (Table 2). Furthermore, the annual habit of the *Bb* genotypes has to be studied in relation not only to environmental conditions but also to genetic factors. In our experiments, the seasonal effect on the penetrance of the annual habit could be related to differences in light intensity between the two growing periods. The availability of RFLP markers of the B gene allows us to distinguish between the action of the B gene, the influence of the environment, and the action of other genes. Knowing the linkage value between *R* and *B*, the normal 1:1 segregation for the *R* gene in the back-cross progeny (129:151 plants), and the inductive spring growing conditions as shown by $F_{2.1}$ and $F_{2.2}$, we can deduce that partial penetrance of the *B* gene in this back-cross progeny (Table 3) is due to genetic factors. Therefore, we can conclude that genes exist which modify the penetrance of the annual habit in beets. These epistatic genes appear to be dominant and present in the wild coastal beet P_2 but not in the red table beet GP_1 as penetrance of the *B* gene was complete in the two F_2s tested. Further crosses and segregation analyses are in progress in order to estimate the number of genes involved and their position on the genetic map. Such dominant genetic factors epistatic to the B gene have consequences for both sugar beet breeding progams and wild beet population studies. These dominant genes can reduce the efficiency of greenhouse tests to detect seed lots where, due to accidental hybridization with annual beets, *Bb* genotypes are present in too-high frequencies, giving unacceptable bolting rates in the field. Similarly, the existence of such modifying genes decreasing the penetrance of the annual habit could lead to an easier protection of this polymorphism at the within-population level, especially in populations where the *B* allele is present at low frequencies, such as the Atlantic coast populations from where the P_2 plant originates.

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