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Genetic localization of four genes for nematode (*Heterodera schachtii* Schm.) resistance in sugar beet (*Beta vulgaris* L.)

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Abstract Sugar beet (*Beta vulgaris* L.) is highly susceptible to the beet cyst nematode (*Heterodera schachtii* Schm.). Three resistance genes originating from the wild beets *B. procumbens* ($Hs1^{pro-1}$) and *B. webbiana* ($Hs1^{web-1}$, $Hs2^{web-7}$) have been transferred to sugar beet via species hybridization. We describe the genetic localization of the nematode resistance genes in four different sugar beet lines using segregating F_2 populations and RFLP markers from our current sugar beet linkage map. The mapping studies yielded a surprising result. Although the four parental lines carrying the wild beet translocations were not related to each other, the four genes mapped to the same locus in sugar beet independent of the original translocation event. Close linkage (0–4.6 cM) was found with marker loci at one end of linkage group IV. In two populations, RFLP loci showed segregation distortion due to gametic selection. For the first time, the non-randomness of the translocation process promoting gene transfer from the wild beet to the sugar beet is demonstrated. The data suggest that the resistance genes were incorporated into the sugar beet chromosomes by non-allelic homologous recombination. The finding that the different resistance genes are allelic will have major implications on future attempts to breed sugar beet combining the different resistance genes.

Key words Sugar beet · *Beta vulgaris* · Nematode resistance · RFLP · Genetic maps · Bulk segregant analysis

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Introduction

The beet cyst nematode (*Heterodera schachtii* Schm.) is one of the major pests in sugar beet (*Beta vulgaris* L.) cultivation. Sugar beet and closely related cultivated beet species (fodder beet, red table beet, swiss chard) are susceptible to this pathogen, and within *B. vulgaris* there is no genetic variability for resistance. Only a partial resistance has ever been found in one accession of the wild species *B. vulgaris* ssp. *maritima* (Lange et al. 1993), and due to its polygenic and recessive mode of inheritance this resistance is difficult to handle in backcross breeding programs.

Attempts have therefore been made worldwide to introduce monogenic resistance genes from three wild species of the *Beta* section IV (*B. procumbens*, *B. patellaris*, *B. webbiana*). These resistances are inherited in a dominant way, thus facilitating the breeding process. Using monosomic analysis Löptien (1984a) located three resistance genes in each of the species *B. procumbens* and *B. webbiana*. These genes are located on chromosome 1 ($Hs1^{pro-1}$, $Hs1^{web-1}$), chromosome 7 ($Hs2^{pro-7}$, $Hs2^{web-7}$, Löptien 1984a; Lange et al. 1993) and chromosome 8 $Hs3^{pro-8}$, $Hs3^{web-8}$ (Löptien 1984a; Reamon-Ramos and Wricke 1992). Only one chromosome in *B. pattellaris* ($Hs1^{pro-1}$, Löptien 1984a) was found to carry a resistance gene. Results from isozyme and molecular marker studies indicate that the resistance-carrying chromosomes from *B. webbiana* and *B. procumbens* are homoeologous (Wagner et al. 1989; Jung et al. 1993).

Gene transfer from wild to cultivated beet has been achieved in the following way. Species' hybrids were backcrossed to sugar beet giving rise to a series of alien monosomic addition lines ($2n = 19$) that contain the original wild beet chromosome (Savitsky 1975; Löptien 1984a; Speckmann et al. 1985). An almost complete lack of homology between sugar beet and wild beet chromosomes was found when metaphase I pollen mother cells were analyzed. The added chromosome almost exclusively formed a univalent. Trivalent formation ranged

between 2% (de Jong et al. 1985) and 5% (Löptien 1984b). Therefore, the transfer of resistance to sugar beet can only be accomplished by rare crossover or translocation events. Among the offspring of monosomic addition lines diploids (2n = 18) have been selected for with a more or less stable inheritance of the resistance trait (Yu 1981; Jung and Wricks 1987; Heijbroek et al. 1988). These lines are intensively used as introduction lines for sugar beet breeding. However, transmission rates have been considerably low, ranging from 70.6% to 100% (Jung and Wricke 1987). With only one exception has resistance been proven to be durable. Lange et al. (1993) presented data on a pathotype which is able to complete its life cycle on beets carrying the resistance gene $Hs1^{pro-1}$ from chromosome 1. However, beets carrying the gene from chromosome 7 were still found to be resistant against this pathotype.

Attempts have been made to isolate the resistance gene following a positional cloning strategy. A set of probes has been identified of which all the probes are closely linked to the resistance genes $Hs1^{pro-1}$ and $Hs1^{pat-1}$ (Jung et al. 1992; Salentijn et al. 1992). Also, two RFLP linkage maps of sugar beet have been published (Pillen et al. 1993; Barzen et al. 1995). Some genes of agronomical value have been included like annual behavior (Boudry et al. 1994), restorer (Pillen et al. 1993), rizomania resistance and monogermy (Barzen et al. 1995).

The purpose of the work present here was to localize the wild beet translocations in sugar beet conferring nematode resistance using existing restriction fragment length polymorphism (RFLP) libraries and to select markers differentiating between individuals that are homozygous or heteorzygous at the resistance locus. The resistance genes $Hs1^{pro-1}$, $Hs1^{web-1}$, and $Hs2^{web-7}$ were mapped in four breeding lines of different origin. The mapping data are discussed together with the subsequent implications for future breeding of nematode resistant sugar beet.

Materials and methods

Plant material, resistance test and DNA preparation

Four different F_2 sugar beet populations segregating for nematode resistance were established by selfing individual F_1 plants hemizygous (R•) for nematode resistance. The selection of the donor lines has been described previously (Table 1). Each line originated from an individual translocation event in the offspring of monosomic addition lines. Except for PRO4, translocations were not induced by irradiation. The lines investigated here are not related by descent, they carry genes for nematode resistance from the wild species *B. procumbens* and *B. webbiana* as described in Table 1. A number of plants from each F_2 population were tested for nematode resistance (Table 1) as described by Toxopeus and Lubberts (1979). Genomic DNA was isolated from 5 g of developing green leaves following the protocol of Saghai-Maroof et al. (1984).

RFLP analysis

DNA restriction, Southern blotting and radioactive hybridization were performed as described by Pillen et al. (1993). For bulk segregant analysis (BSA) and most of the mapping experiments, a non-radioactive hybridization system based on enhanced chemiluminescence was used (ECL-Direct, Amersham). Blotting, labelling and hybridization were carried out according to the manufacturer's protocol (Amersham). Non-radioactive autoradiograms were scored after exposure for periods of several hours up to 1 day.

Bulk segregant analyses, linkage analyses

The probe PCR1012 was isolated from *B. procumbens* chromosome 1 and hybridizes with DNA from *Procumbentes* species only (Jung et al. 1992). In this study it was used for distinguishing between different translocation lines carrying the resistance gene $Hs1^{pro-1}$. For BSA (Michelmore et al. 1991) previously mapped RFLP probes were used (Pillen et al. 1993). DNA from resistant and susceptible plants was restricted with the enzymes *Eco*RI, *Eco*RV, *Hin*dIII and *Xba*I, subsequently, bulked in two independent pools and separated on 0.75% agarose gels. The DNA was transferred to a Biodyne B membrane (PALL, Dreiech) using 20 × SSC as transfer buffer. Each bulk consisted of 12 different plants and the total DNA concentration in each lane was about 5 µg.

To shorten the BSA procedure, up to four single-copy RFLP probes were hybridized in parallel to the filters. In this way a representative set of marker loci from each linkage group was analyzed. Probes linked to the resistance genes showed RFLPs between the pools of resistant and susceptible plants. In the next step each of the probes was tested on a survey filter with 24 individuals, and linkage to the resistance gene was estimated by calculating the distance in recombination units. If the RFLP locus was more closely linked to the nematode resistance gene than 25 recombination units, more RFLP loci in the vicinity were mapped.

Linkage was calculated with the computer program MAPMAKER/EXP Ver. 3.0 (Lander et al. 1987) processed on a SUN Sparc 10 workstation (Unix). Linkage groups were formed using the Haldane transformation (Haldane 1919) with thresholds of LOD = 3.0 and 0.3 recombination units.

Statistical analysis of distored segregation of linked marker loci

Segregation ratios for codominant RFLP markers and for the dominant nematode resistance gene were expected to be 1:2:1 and 3:1, respectively. Segregation ratios were tested by χ^2 analysis for the RFLP loci in proximity to the nematode resistance genes and for the nematode resistance locus itself. Also, a test for gametic and zygotic selection (unequal frequencies of the three observed classes) was conducted for the closest linked codominant RFLP locus using the formulas from Wagner et al. (1992) with an $\alpha = 0.01$.

Table 1 Plant material and origin of the resistance genes

Nematode resistant donor line	Resistance gene	Origin of resistance gene	Reference	Number of F_2 individuals investigated
A906001	$Hs1^{pro-1}$	*B. procumbens* chromosome 1	Jung et al. 1992	48
PRO4	$Hs1^{pro-1}$	*B. procumbens* chromosome 1	Jung and Wricke 1987	101
WEB6	$Hs1^{web-1}$	*B. webbiana* chromosome 1	Jung and Wricke 1987	96
WEB11	$Hs2^{web-7}$	*B. webbiana* chromosome 7	Jung and Wricke 1987	61

Results

Resistance tests, BSA

The results of the resistance tests are presented in Table 2. Due to the dominant character of the resistance gene a 3:1 segregation was expected. However, segregation in populations WEB6 and WEB11 deviated significantly from the expected ratio.

Bulks of resistant and susceptible plants were tested with a representative set of probes (Table 3) from each of the nine linkage groups in sugar beet previously identified by Pillen et al. (1993). For each population only markers from linkage group IV yielded an RFLP between both pools of DNA. Consequently, only markers from this linkage group were selected for further mapping studies.

Fine mapping of the nematode resistance loci

A906001

Populations A906001 and PRO4 both carry the resistance gene *Hs1*$^{pro-1}$ from *B. procumbens*. To verify that the translocations are different we tested 4 resistant individuals from each of these two populations with the wild beet-specific probe PCR1012. Only A906001 individuals gave signals, proving that these lines carry translocations different in sizes (Fig. 1).

Seven markers from linkage group IV were tested within this population, only two of them displayed RFLPs (pKP942, pKP1162). The resistance gene was localized at a distance of 4.6 cM from probe pKP942. While linkage order is the same as in that reference map S227, the recombination frequencies were significantly different between both populations (Fig. 2). In population A906001 neither segregation at the nematode resistance locus nor segregation at the closest linked marker locus were significantly distorted. However, a striking reduction in one homozygous class was evident.

PRO4

Out of 15 probes tested from linkage group IV 4 revealed polymorphisms in this populations and could be integrated in the linkage map (Fig. 2). The marker order deviated from the reference population in a similar way as in WEB11. Close linkage to the nematode resistance locus was found for markers pKP557, pKP815 and pKP1107. The probes pKP1107 and pKP815 segregated in a dominant way with the consequence that

Table 2 Segregation ratios for nematode resistance and for the closest linked codominant RFLP loci. Statistical tests for segregation ratios and for gametic and zygotic selection are according to Wagner et al. (1992)

Population	Segregation at the resistance locus			RFLP loci with codominant segregation						
	Resistant	Susceptible	χ^2 3:1	Locus	A	H	B	χ^2 1:2:1	χ^2 Gamete	χ^2 Zygote
A906001	32	16	n.s.	pKP942	7	25	15	n.s.	n.s.	n.s.
PRO4	68	33	n.s.	pKP557	7	47	22	10.2**	n.s.	n.s.
WEB6	53	43	20.1**	pKP942	2	53	37	28.8**	26.6**	n.s.
WEB11	21	40	53.6**	pKP557	2	21	21	16.5**	16.4**	n.s.

** Significant at $P = 0.01$; n.s. = not significant
A, Homozygous (AA); H, heterozygous (Aa); B, homozygous (aa)

Table 3 RFLP probes used in the bulk segregant analyses

Linkage Group	RFLP probe no.
I	pKP1159[a], pKP967[a], pKP826[a], pKP814[a], pKP374[a], pKP490[a], pKP730[a], pKP843[b], pKP959[b]
II	pKP810[a], pKP1009[a], pKP876[a], pKP1172[a], pKP753[a], pKP890[a]
III	pKP1004[a], pKP558[a], pKP613[a], pKP909[a], pKP972[a], pKP603[a], pKP994[a], pKP1238[a], pKP1112[b], pKP995[b], pKP370[b], pKP766[b], pKP918[b], pKP1225[b]
IV	pKP942[a], pKP1107[a], pKP878[a], pKP741[a], pKP501[a], pKP511[a], pKP1180[a], pKP561[d], pKP602[d], pKP569[d], pKP1162[d], pKP557[d], pKP720[d], pKP815[d]
V	pKP975[a], pKP899[a], pKP510[a], pKP575[a], pKP749[a], pKP1151[a], pKP951[b], pKP859[b], pKP953[b], pKP543[b]
VI	pKP1011[a], pKP1227[a], pKP495[a], pKP830[a], pKP937[a], pKp559[b], pKP581[b], pKP917[b]
VII	pKP1184[a], pKP850[a], pKP818[a], pKP1219[a], pKP743[a], pKP911[a], pKP1204[a], pKP722[b], pKP829[b], pKP970[b], pKP562[b], pKP727[b], pKP851[c]
VIII	pKP849[a], pKP782[a], pKP1224[a], pKP954[a], pKP492[a], pKP498[a], pKP519[a], pKP1125[a]
IX	pKP968[a], pKP574[a], pKP922[a], pKP759[a], pKP847[d], pKP1158[d]

[a] Probes tested in the populations A906001, PRO4, WEB6, WEB11
[b] Probes tested in the populations A906001, WEB11, PRO4
[c] Probes tested in the population A906001
[d] Probes tested in the populations WEB11 and PRO4

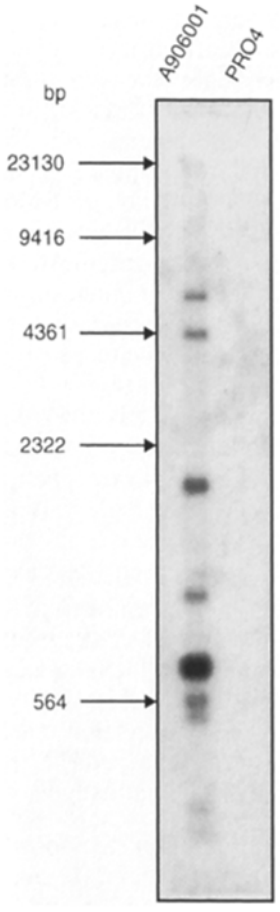

Fig 1 Southern blot analysis with DNA from resistant individuals of populations A906001 and PRO4. The DNA was restricted with *Hin*dIII; plasmid clone PCR1012 was used as a probe; exposure time was 2 days

testing for zygotical or gametical selection was not possible. However, segregation distortion was obvious for the codominant RFLP marker pKP557.

WEB6

The population WEB6 segregated for marker loci pKP942, pKP557, pKP1180, pKP1162, pKP563 and pKP815. For each of the corresponding probes RFLPs were visible between resistant and susceptible plants. An example of a non-radioactive Southern hybridization is given in Fig. 3. Three-point analysis revealed that the order of markers corresponds to that of reference map S227. Five tightly linked markers (pKP942, pKP557, pKP1180, pKP1162 and pKP563) were mapped adjacent to the nematode resistance locus (Fig. 2). Segregation at the marker locus pKP942 was distorted (Table 2), and a striking reduction in one homozygous class is evident.

WEB11

Bulk segregant analysis was not successful within this population since there were no prominent RFLPs between the DNA pools. Therefore, probe candidates from linkages groups II and IX were also included in the linkage analysis. However, clear linkage relationships were only found when using markers from linkage group IV. Among these, loci pKP815 and pKP1107 showed tight linkage with the resistance locus (0 cM). The marker order deviated from that of the reference

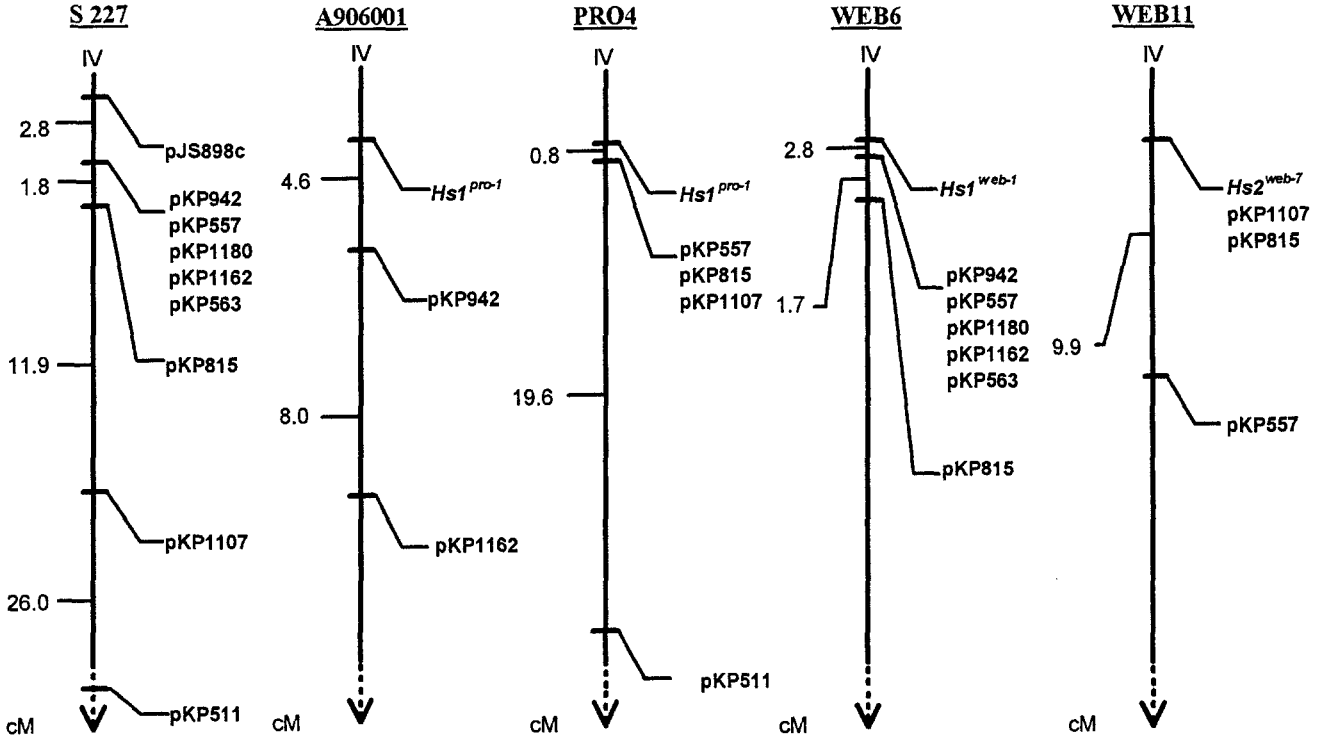

Fig 2 Partial linkage maps from four populations segregating for nematode resistance in comparison to the reference map S227 (Pillen et al. 1993)

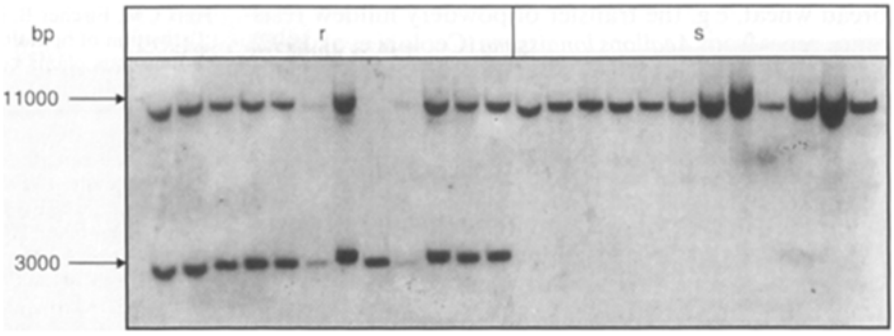

Fig. 3 Non-radioactive Southern blot analysis with DNA from resistant (*r*) and susceptible (*s*) plants from population WEB6. The DNA was digested with *Xba*I; plasmid clone pKP942 was used as a probe; exposure time was 6 h

population, and segregation at the resistance locus was heavily disturbed. Likewise, genotypic classes at the marker locus pKP557 were non-randomly distributed due to gametic selection (Table 2).

Discussion

Linkage analyses were conducted in four sugar beet populations segregating for different genes for nematode resistance from species of the section *Procumbentes*. The lines are not related by descent with respect to the introduced wild beet segment because they originate from different alien addition lines. Only PRO4 and A906001 share the same gene in common, i.e. $Hs1^{pro-1}$ from *B. procumbens* chromosome 1. However, their wild beet translocations do differ in size, as revealed by marker analysis with the wild beet-specific probe PCR1012, indicating that they do not result from the same translocation event. A clear correlation between the number of repetitive loci homologous to probe PCR1012 and the size of the wild beet segments introduced into sugar beet was found by Jung et al. (1992), which suggested that the PRO4 translocation is significantly smaller in size than the translocation of A906001.

A deficit of resistant individuals was obvious for all four populations investigated, thereby confirming previous results (Jung and Wricke 1987). There are three mechanisms possible to explain this reduction in size of the resistant fraction. (1) Loss of the translocation as a whole, as has been speculated to occur during meiosis (Jung and Wricke 1987). (2) Gene inactivation or gene silencing (e.g. methylation), which has been observed for transgenes after transformation (Hart et al. 1992). (3) The gene itself or flanking genes residing on the translocation may act as lethal genes reducing the survival rates of gametes. There is evidence, at least for populations WEB6 and WEB11, that our data fit well to the last model because not only the resistance character itself but also the neighboring loci were affected by distorted segregation.

A nematode resistance gene of uncertain origin has previously been mapped using random amplified polymorphic DNA (RAPD) markers to linkage group I at a distance of 21.6 $\pm$ 10.9 recombination units from the *R* gene for red hypocotyl color (Uphoff and Wricke 1992). We did not observe any linkage to red hypocotyl color in our populations (data not shown). Also, no linkage was found with markers from linkage group I.

It is a surprising finding from our studies that the different wild beet genes all map to the same position of the sugar beet genome. Obviously, there is a preferential point of integration for the wild beet segment carrying the resistance gene distal to marker locus pKP942 on chromosome IV in sugar beet. Therefore, the earlier hypothesis that the mechanism of incorporation from the wild beet is purely coincidental (Jung and Wricke 1987), like random translocation following breakage and reunion of non-homologous chromosomes, can be ruled out leaving open the question whether there is a general mechanism controlling gene exchange between *Beta* species. Our data suggest that the distal region on sugar beet linkage group IV shares homology with the wild beet, thereby proliferating gene exchange between chromosomes. Comparative mapping studies with markers from our sugar beet linkage map are under way to map chromosomes I and VII in *B. procumbens* where the nematode resistance genes are located.

Rearrangements within eucaryotic genomes can be induced by homologous exchange between repetitive elements in somatic cells. This mechanism is unlikely in our case because of the low degree of homology between both species with respect to repetitive elements (Schmidt et al. 1991). Our data suggest that the incorporation of the wild beet genes is triggered by non-allelic homologous recombination as has been found in plants (Quin et al. 1994). Only short sequences of homology between donor and recipient DNA are required for homologous recombination events. This homology is also likely to exist between different *Beta* species. Chromosome pairing during meiosis is a prerequisite for crossover events. Pairing between wild and sugar beet chromosomes is a rare event and was observed at low frequencies in monosomic addition lines, ranging from 2% (de Jong et al. 1985) to 5% (Löptien 1984b). Therefore, large populations of offspring of monosomic addition lines had to be screened for possible recombinants. No gene system able to promote homoeologous pairing has been described for *Beta* species so far. In wheat breeding *ph*-mutants favoring chromosome pairing between homoeologous chromosomes have been used successfully for transferring genes from wild species into

bread wheat, e.g. the transfer of powdery mildew resistance genes from *Aegilops longissima* (Ceoloni et al. 1992).

The data presented here will have major implications on future attempts to breed a nematode-resistant sugarbeet. Marker-assisted selection of resistant beets is feasible with wild beet-specific markers cloned from the translocation itself (Jung et al. 1992). The repetitive nature of these sequences lends itself to squash dot selection using leaf pieces or polymerase chain reaction-based selection techniques (Jung and Herrmann 1991). Due to their linkage to the gene, recombination between these marker loci and the resistance gene is rare resulting in a highly reliable marker-assisted selection. However, the squash dot test does not allow any differentiation among the resistant beets with respect to homozygosity. Now, the probes closely linked to the translocation offer the possibility for marker-assisted selection of homozygous beets from segregating populations. This is essential for determining the transmission rate of the resistance gene and for selecting true-breeding resistant plants.

The appearance of virulent pathotypes is expected in the future. A first report has been given of a pathotype able to break the resistance from *B. procumbens* chromosome 1 (Lange et al. 1993) while beets carrying the $Hs2^{web\text{-}7}$ gene were still resistant.Therefore, breeding programs have been initiated that aim to combine the different genes from *B. procumbens* and *B. webbiana* in one hybrid component. In the light of the data presented here these programs cannot ever be crowned with success because of the allelic relationship between both characters. The only way to combine both genes will be to breed a hybrid variety derived from a cross between parents that are homozygous for each of the two genes.

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