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Identification and mapping of random amplified polymorphic DNA (RAPD) markers linked to resistance against beet necrotic yellow vein virus (BNYVV) in *Beta* accessions

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Abstract Molecular markers linked to resistance genes are useful to facilitate the introgression of one or more of these genes in breeding materials. Following the approach of bulked segregant analysis, RAPD markers linked to resistance genes against beet necrotic yellow vein virus were identified in the four *Beta* accessions Holly-1-4, R104, R128 and WB42. Two primers were found which generate RAPD markers tightly linked to resistance in segregating families of Holly-1-4, R104 and R128, indicating that the resistance genes in these accessions might be situated at the same locus. Other, specific, primers were identified which generate RAPD markers linked to resistance in each of these accessions. Short-range maps were established around the resistance locus in these accessions. For WB42, RAPD markers were only identified at a relatively large distance from the resistance gene. Conversion of three RAPD primers of Holly-1-4, R104 and R128 into STS primers resulted in STS markers which can be readily used for marker-assisted selection in breeding programmes.

Key words Beet necrotic yellow vein virus · *Beta vulgaris* · Bulked segregant analysis · RAPD markers · Resistance genes · STS markers

Introduction

Rhizomania is a major disease problem in many sugar beet-growing countries in the world (Richard-Molard 1985). The disease is caused by the beet necrotic yellow vein virus (BNYVV) (Tamada 1975), which is transmitted by the soil borne fungus *Polymyxa betae* Keskin. Severe infections with rhizomania can reduce the sugar yield to more than 50% (Johansson 1985; Richard-Molard 1985). Breeding for resistance to rhizomania is the only possible means to counteract the disease (Schlösser 1988; Asher 1993). Resistance to BNYVV was described for accessions of *Beta vulgaris* L. subsp. *vulgaris* and subsp. *maritima* (L.) Arcang. (Lewellen et al. 1987; Whitney 1989), *B. corolliflora* Zos., *B. intermedia* Bunge and *B. lomatogona* Fisch. & Mey (Paul et al. 1993). Monogenic resistance from the subsp. *vulgaris* or *maritima* is the most attractive, because of the relative ease of handling monogenic traits in breeding programmes. However, if molecular markers are available to detect the individual resistance genes, genotypes with two or more resistance genes can be identified. Such genotypes are valuable in breeding programmes, especially as combined resistances may provide higher levels of resistance and enhance the durability of the resistance (Lewellen and Biancardi 1990). The *B. vulgaris* subsp. *vulgaris* accession Holly-1-4 is thought to contain a single dominant major gene for resistance to BNYVV (Lewellen et al. 1987; Scholten et al. 1996). Resistance from the accession *B. vulgaris* subsp. *maritima* WB42 is dominant and perhaps simply inherited (Whitney 1989). This resistance is probably either based on one or two dominant major genes showing distorted segregation, or on two complementary major genes which are both required for resistance (Scholten et al. 1996). Two major accessions with probably a single resistance gene are the *B. vulgaris* subsp. *vulgaris* R104, with resistance originating from *maritima*, and R128, with resistance from Swiss chard (Lewellen, personal communication). Since these four accessions are genetically derived from two subspecies and originate from four different countries (Table 1) it might be that different resistance genes are involved.

To identify rhizomania-resistant *Beta* genotypes, greenhouse or field tests have to be carried out using infested soils. In greenhouse tests the resistance levels of

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Table 1 Origins of the resistant plant material

Resistant accession	Origin of resistance from *Beta vulgaris* subsp.	Country of origin
Holly-1-4[a]	*vulgaris*	USA
R104	*maritima*	Italy
R128[b]	*vulgaris*	Turkey
WB42	*maritima*	Denmark

[a] Holly-1-4 is a selection of the sugar beet accession Holly (Lewellen et al. 1987)
[b] Resistance to BNYVV in R128 originated from Swiss chard

individual plants are determined by estimating the virus concentration in the rootlets after infection using ELISA (Bürcky and Büttner 1985; Giunchedi et al. 1985; Whitney 1986; Paul et al. 1992), whereas in field tests components such as total yield, sugar content, sugar yield and juice purity are evaluated and occasionally ELISA tests are also carried out. The necessary testing of hundreds of lines in commercial breeding programmes is hampered by the laborious nature of both tests. Moreover, in greenhouse tests resistant plants with intermediate levels of virus concentrations appear, which cannot be discriminated as resistant or susceptible. To facilitate breeding programmes, molecular markers linked to resistance genes against BNYVV will be very useful for the introgression of such genes in susceptible cultivars or breeding materials. In the present study, bulked segregant analysis (BSA) (Michelmore et al. 1991) was used to identify RAPD markers (Welsh and McClelland 1990; Williams et al. 1990) linked to resistance against BNYVV in four *Beta* accessions: Holly-1-4, R104, R128 and WB42. As the amplification of RAPD markers using decamer oligonucleotide primers is sometimes difficult to reproduce in other laboratories, some tightly linked markers were converted to sequence-tagged-site (STS) markers, which are more stable in PCR (Olson et al. 1989). The possible existence of different resistance loci in these accessions will be discussed by comparing the RAPD resistance markers found in them.

Materials and methods

Plant materials and crosses

The plant materials consisted of the *B. vulgaris* accessions Holly-1-4 and R128, with resistance to BNYVV from the subsp. *vulgaris*, and R104 and WB42, with resistance from the subsp. *maritima*. Resistant F_1 plants, obtained after crosses of Holly-1-4 or WB42 with the susceptible red table beet 'Queen', were crossed with the susceptible male-sterile accession MS-2 to produce segregating backcross (BC) families. For R104 and R128 segregating F_1 families were made by crossing resistant plants of these accessions with MS-2. All accessions are diploid with 2n = 18.

Greenhouse tests

To discriminate between resistant and susceptible plants, individual seedlings were transplanted into a mixture of sand and infested soil and grown in the greenhouse for 1 month. ELISA was used to estimate the virus concentrations, using 100 mg of the rootlets of individual plants (Paul et al. 1992). The remaining parts of the plants were re-planted into soil without BNYVV and vernalized to conduct crosses. Log_{10} virus concentrations were used for statistical analysis. The detection limit for the virus was at a $\log_{10}$ virus concentration of 0.65 ng/ml. Mixture models (Jansen 1993, 1994) were fitted to the data to assist in the classification of the plants as either resistant or suspectible. Simultaneously, the segregation ratios of resistant to suspectible plants were determined to study the number of resistance genes involved. The sugar beet cultivar 'Regina' was used as a susceptible control in all greenhouse tests.

DNA isolation and PCR amplification

Genomic DNA was extracted from fresh or frozen leaves following the procedure of Vanderbeek et al. (1992). DNA concentrations were estimated in the Hoechst Mini Fluorometer (Hoechst Scientific Instruments, San Francisco, USA). PCR was performed in a total volume of 25 µl containing 10 ng genomic DNA, 100 µM each of dATP, dCTP, dGTP and TTP, 25 ng primer (Operon), 2.5 µl 10 × Super*taq* buffer [100 mM Tris-HCl, pH 9.0; 500 mM KCl; 0.1% (w/v) gelatin; 15 mM MgCl; 1% Triton X-100], 0.1 unit Super*taq* Polymerase (SphaeroQ, Leiden, the Netherlands). Each reaction mixture was overlaid with 25µl of mineral oil (Perkin Elmer). DNA amplification was performed in a Hybaid DNA thermal cycler (Biozym, Landgraaf, the Netherlands) in PCR reaction tubes (tube control) or microtiter plates (simulated tube control). The thermal cycles used were: 1 cycle of 5 min 92.5°C, followed by 40 cycles of 5 s at 92.5°C, 45 s at 34.5°C and 45 s at 72°C, then finally 1 cycle of 10 min at 72°C for final extension and 1 s at 28°C. Amplification products were separated by gel electrophoresis using 1.5% agarose gels with TAE to TBE buffers and stained with ethidium bromide.

Bulked segregant analysis (BSA)

BSA (Michelmore et al. 1991) was performed for each segregating family on bulks of DNA of 10–15 of the most-resistant (with virus concentrations up to a $\log_{10}$ of 1.5 ng/ml virus) and of the most-susceptible (with $\log_{10}$ virus concentrations of at least 2.3 ng/ml virus) plants. Primers which amplified a DNA fragment in only one of the bulks were confirmed on the same set of bulks, followed by PCR on six individual resistant and six susceptible plants. RAPD markers with the best linkage to resistance genes were evaluated further on an additional number of individual plants.

Mapping of the RAPD markers

RAPD markers were mapped by analysing the segregating families with the computer programme JoinMap (Stam and Van Ooijen 1995). The segregation of the marker alleles in the families was treated as a cross-pollinating population (aa × ab), which segregates for the dominant b allele, linked to resistance. For each marker at least 34 individual plants were analyzed, distributed over resistant and suseceptible classes. For some markers the entire family was studied. It was assumed that the 30 most-resistant plants, with a maximum $\log_{10}$ virus concentrations of 1.5 ng/ml, all contained the major gene for resistance, whereas the 30 most-susceptible plants, with a minimum $\log_{10}$ virus concentration of 2.3 ng/ml, were assumed to lack the gene. Linkage was considered significant if the logarithm of odds (LOD) score was higher than 3.0. The presence of the markers in individual plants was used to verify the classification of plants as resistant or susceptible based on the mixtures of normal distributions fitted to the virus concentrations.

Conversion of RAPD markers into sequence-tagged-site (STS) markers

RAPD markers were isolated from agarose gels by freeze squeezing and dissolved in 10 µl TE (10 mM Tris-HCL, pH = 8.0; 1 mM

EDTA). An aliquot of 2–3 µl of the solution was re-amplified using the original RAPD primers and the same conditions as for the amplification of genomic DNA. The re-amplified DNA fragment was isolated from an agarose gel by freeze squeezing and purified by phenol/chloroform extraction. The 3'-ends of the RAPD marker were filled in using Klenow polymerase, whereafter the blunt ends were cloned into the de-phosphorylated *Eco*RV site of the plasmid pBluescript Sk^+ (Stratagene) and used to transform *Escherichia coli* DH5α. Plasmid DNA was isolated following the CTAB miniprep method (Del Sal 1988). The ends of the cloned RAPD markers were sequenced in the Applied Biosystem DNA sequencer using Dye terminator (Perkin Elmer). Specific STS primers of about 20-mer oligonucleotides were developed based on these sequences. PCR with the STS primers was performed under similar conditions as for RAPDs; however, the amount of Super*taq* Polymerase was increased to 0.2 units. The thermal cycles used were: 30 cycles of 20 s at 94°C, 30 s at 58–60 °C and 30 s at 72 °C, followed by 1 cycle of 5 min at 72°C.

Results

Resistance tests

To discrimate resistant and susceptible plants of the segregating families of Holly-1-4, R104, R128 and WB42, greenhouse tests were carried out in which the rate of virus multiplication in the rootlets of individual plants was estimated by ELISA. Figure 1 illustrates the distribution of plants in classes of $\log_{10}$ virus concentrations. Although most plants could clearly be classified as resistant or susceptible, plants with intermediate levels of resistance were also found. Mixtures of normal distributions were fitted to the data to estimate the mean virus concentrations (Table 2). The segregation ratios of resistant to susceptible plants approached the expected ratio to support the hypothesis that resistance against BNYVV in Holly-1-4 and R128 is based on a single dominant major gene. For WB42 and R104, however, the segregation is skewed.

Identification of RAPD markers linked to resistance against BNYVV

To identify RAPD markers linked to resistance against BNYVV, bulks of DNA were composed of the most-resistant and most-susceptible plants of the segregating families of Holly-1-4, R104, R128 and WB42. For each set of bulks 580 Operon primers were screened, which resulted in the amplification of more than 3500 DNA fragments per accession. Between 10–30 primers amplified RAPD markers either in a resistant or in a susceptible bulk. These primers were examined further on individual plants. The estimated recombination frequencies of several identified RAPD markers linked to resistance loci in Holly-1-4, R104, R128 and WB42 are presented in Table 3. The primers OP-01 and OP-02 generated DNA fragments that were found to be tightly linked to the resistance locus in Holly-1-4, R104, R128 (Fig. 2, Table 3). Primer OP-01 amplifies the RAPD marker $OP\text{-}01_{1400}$, linked to resistance loci in coupling phase in these three families. This marker was not amplified in the segregat-

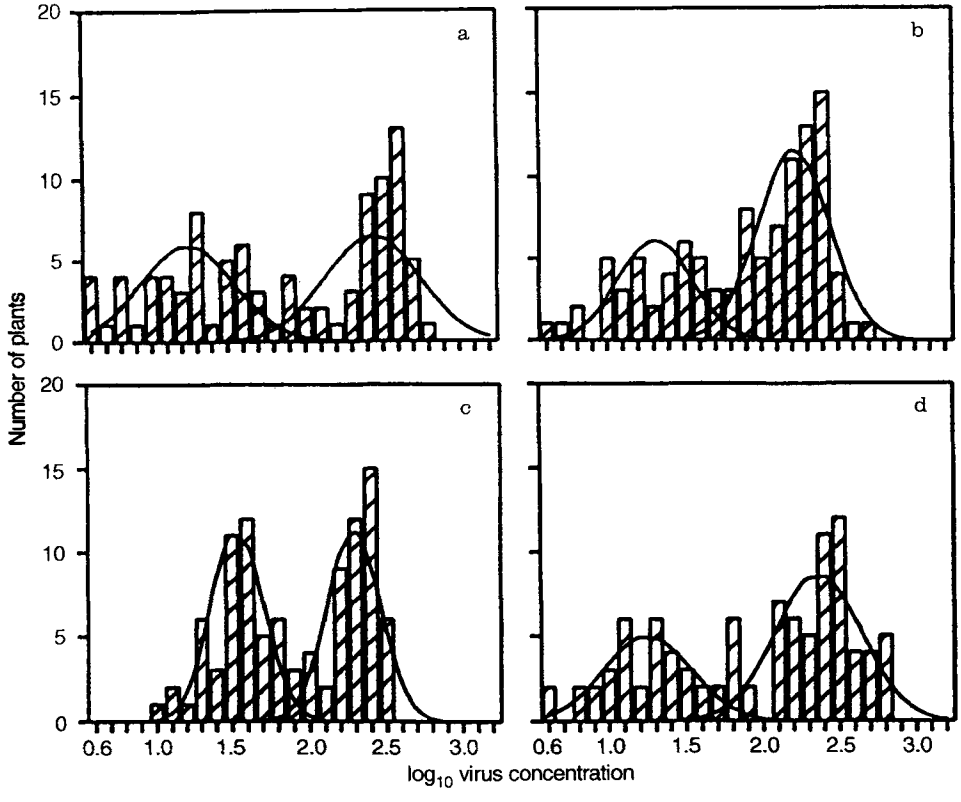

Fig. 1a–d Curves fitted to histograms of $\log_{10}$ BNYVV concentrations of individual plants from segregating families of **a** Holly-1-4, **b** R104, **c** R128, and **d** WB42, showing the segregation between resistant and suspectible classes

Table 2 Estimated mixture model parameters based on $\log_{10}$ of BNYVV concentrations (in ng/ml) in rootlets of individual plants of the segregating families of Holly-1-4, R104, R128 and WB42, and the observed ratios of resistant (R) to susceptible (S) plants with the 95% confidence intervals, demonstrating the possible fit of the expected ratios to the hypothesis that resistance is controlled by a single dominant major gene

Resistant accession	Segregating family	Number of plants	Mean low virus concentration	Mean high virus concentration	SD	Observed ratio R:S	95% confidence interval[a]	Hypothesis: monogenic resistance accepted
Holly-1-4	BC	95	1.33	2.46	0.28	0.48:0.52	±0.16	Yes
R104	F_1	105	1.31	2.21	0.24	0.35:0.65	±0.10	No
R128	F_1	98	1.53	2.29	0.17	0.50:0.50	±0.14	Yes
WB42	BC	112	1.24	2.36	0.29	0.37:0.63	±0.12	No
'Regina'	Control	88		2.57	0.19	0:1		

[a] The 95% confidence interval is based on the mean observed ratios ± 1.96 *standard error. If 0.5 fits within the interval for resistance, it means that the hypothesis is accepted that 50% of the plants can be resistant

Table 3 Recombination frequencies between the BNYVV resistance gene and the RAPD markers, identified by bulked segregant analysis, in segregating families of the accessions Holly-1-4, R104, R128 and WB42

Primer[a]	F_1(Holly-1-4 × 'Queen')			R104			R128			F_1(WB42 × 'Queen')		
	C/R[b]	r[c]	n[d]	C/R	r	n	C/R	r	n	C/R	r	n
OP-01	C	0.03	60	C	0	60	C	0	60	-		
OP-02	R	0.02	59	C	0	60	C	0.02	60	-		
OP-02*	-			R	0	60	-			-		
OP-03	R	0.16	38	-			R	0.16	55	R	0.17	35
OP-04	-			-			-			C	0.18	50
OP-05	-			-			-			C	0.17	52
OP-06	C	0.03	59	-			-			-		
OP-07	-			C	0.05	60	-			-		
OP-08	-			-			C	0.02	60	-		
OP-09	R	0.17	12	C	0.04	54	C	0.02	60	-		
OP-10	R	0.13	31	-			-			-		
OP-11	C	0.15	34	-			-			-		
OP-12	-			C	0.05	60	C	0.05	60	-		
OP-13	-			-			-			R	0.18	34
OP-14	R	0.05	43	-			C	0	58	-		
OP-15	-			C	0	60	-			-		
OP-16	-			C	0.05	60	-			-		
OP-17	-			R	0.02	46	-			-		
OP-18	-			-			-			R	0.17	35
OP-19	C	0.08	12	-			C	0	36	-		
OP-20	-			-			-			R	0.17	48
OP-21	-			-			-			R	0.16	49
OP-22	-			C	0.03	58	-			-		
OP-23	R	0.14	37	-			-			-		
OP-24	-			C	0.04	52	-			-		
OP-25	C	0.09	34	-			-			-		

[a] The sequences of the primers, amplifying RAPD markers linked to resistance, can be given by the authors on request
[b] Linkage to resistance in coupling (C) or repulsion phase (R); - = no difference between the bulks of resistant and susceptible plants
[c] r = recombination frequencies calculated for the most-resistant plants, with a maximum $\log_{10}$ virus concentration of 1.5 ng/ml, and the most-susceptible plants with a minimum $\log_{10}$ virus concentration of 2.3 ng/ml
[d] n = number of plants

ing family of WB42. The second primer, OP-02, generates the RAPD marker OP-02_{750}, linked to resistance loci in coupling phase in R104 and R128, but in repulsion phase in Holly-1-4. In R104 this primer also amplifies a further RAPD marker, OP-02_{1000}, which is linked in repulsion phase to the resistance locus. Together, OP-02_{750} and OP-02_{1000} behave as a single co-dominant marker, segregating for resistance and susceptibility. The primer OP-02 did not produce markers linked to resistance in WB42. The primer OP-03 generated a marker linked to resistance loci in repulsion phase in Holly-1-4, R128 and WB42. However, as was the case for all RAPD markers linked to resistance in WB42, the linkage between RAPD marker OP-03 and the resistance locus was low. The primers OP-04 and OP-05 amplify the most tightly linked RAPD markers in coupling phase for resistance in WB42. Some RAPD markers were linked to resistance in only one of the accessions, like the OP-06 primer in Holly-1-4, the OP-07 primer in R104 and OP-08 primer in R128.

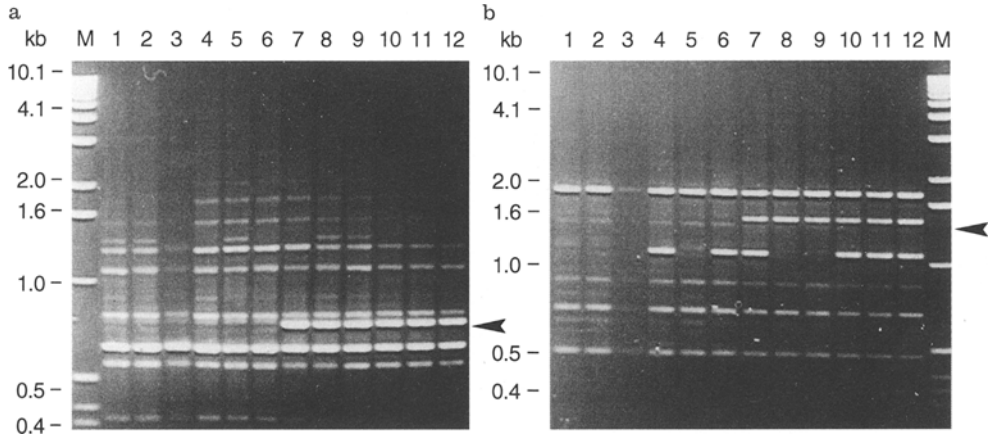

Fig. 2a, b RAPD markers present in individual susceptible (*lanes 1–6*) and resistant (*lanes 7–12*) plants of the segregating family of R128. The *arrows* point to the segregating markers of **a** OP-02_{750} and **b** OP-01_{1400}; *M* = 1-kb DNA ladder

Mapping of the RAPD markers

The presence of the RAPD markers OP-01_{1400} and OP-02_{750} was analyzed in the entire populations of Holly-1-4, R104, and R128, including those plants with intermediate levels of virus concentrations. Both RAPD markers were present in most of the plants in the resistant parts of the frequency distribution (Fig. 3), except for the segregating family of Holly-1-4 in which OP-02_{750} is present in the susceptible plants. These results confirm the analysis of the resistance scores with the mixture models. The distribution of the RAPD markers also supports the hypothesis that resistance in Holly-1-4 and R128 is based on a single major gene. For R104 the distribution of the markers was as skewed as determined by the mixture models (Fig. 3).

RAPD markers linked to resistance were employed for the construction of short-range maps around the resistance locus in the four accessions using the computer program DrawMap mes Joinmap (Stan and Van Ooijen 1995) and (Van Ooijen 1994). In Fig. 4 the maps are shown with the RAPD markers located on both sides of the resistance locus within a distance of 25 cM. As resistance was measured as a quantitative character by analysing the BNYVV concentrations in rootlets of individual plants, the contribution of the RAPD markers to resistance was also determined by a QTL analysis with the computer program MapQTL (Van Ooijen and Maliepaard 1995). For the family of Holly-1-4 the most tightly linked RAPD markers, OP-01_{1400} and the OP-06_{1500}, explain 66% and 61% respectively of the total phenotypic variation, confirming linkage between the markers and a major resistance gene in Holly-1-4. The OP-01 and OP-02 RAPD markers explain 70% and 68% respectively of the variation in R104 and 81% and 74% in R128, indicating linkage between these markers and a major resistance gene in R104 and R128.

Conversion of RAPD markers into sequence-tagged-site (STS) markers

To obtain a more reliable PCR assay for resistance against BNYVV, pairs of STS primers were synthesized on the basis of the nucleotide sequence of the ends of the RAPD markers OP-01_{1400}, OP-02_{750} and OP-09_{640} linked to resistance loci in Holly-1-4, R104 and R128. Using the STS/OP-01 primers, a single DNA fragment is amplified in resistant plants of the three accessions (Fig. 5 a). The STS/OP-02 primers amplify a marker linked to resistance in coupling phase in R128 (Fig. 5 b). In the R104 family the STS/OP-02 primers generate two fragments, that behave as a single co-dominant marker. As expected from the RAPD results, the STS/OP-02 primers amplify an STS marker in the susceptible plants of the segregating family of Holly-1-4. However, in resistant plants of this family a band of the same size is also sometimes amplified, albeit with a lower intensity than the STS marker in the susceptible plants. The STS/OP-09 marker based on RAPD marker OP-09_{640} is generated in the resistant plants of the segregating families of R104 and R128, and in susceptible plants of the backcross of Holly-1-4 (Fig. 5 c). Another STS marker without linkage to resistance is also produced in these progenies.

Discussion

Resistance to plant viruses has been classified as dominant, incompletely dominant, or recessive (Fraser 1990). Due to the preference of breeders for simple inheritance, the resistance to viruses employed in breeding programmes is often controlled by a single locus. In wild populations, however, inheritance of resistance to viruses may well be more complex (Fraser 1990). The observed segregation ratios of resistant to susceptible plants in the families of Holly-1-4 and R128 suggested that resistance to BNYVV is based on a single dominant major gene. However, resistant plants were found in a broad range of virus concentrations, which might be caused by the involvement of minor genes or by environmental variation. For the families of R104 and WB42, however, the segregation was skewed and did not fit the expected ratio for a single major resistance gene.

Following the strategy of bulked segregant analysis (Michelmore et al. 1991), RAPD markers linked to resistance against BNYVV were identified in four *Beta* accessions. These markers were used to develop short-

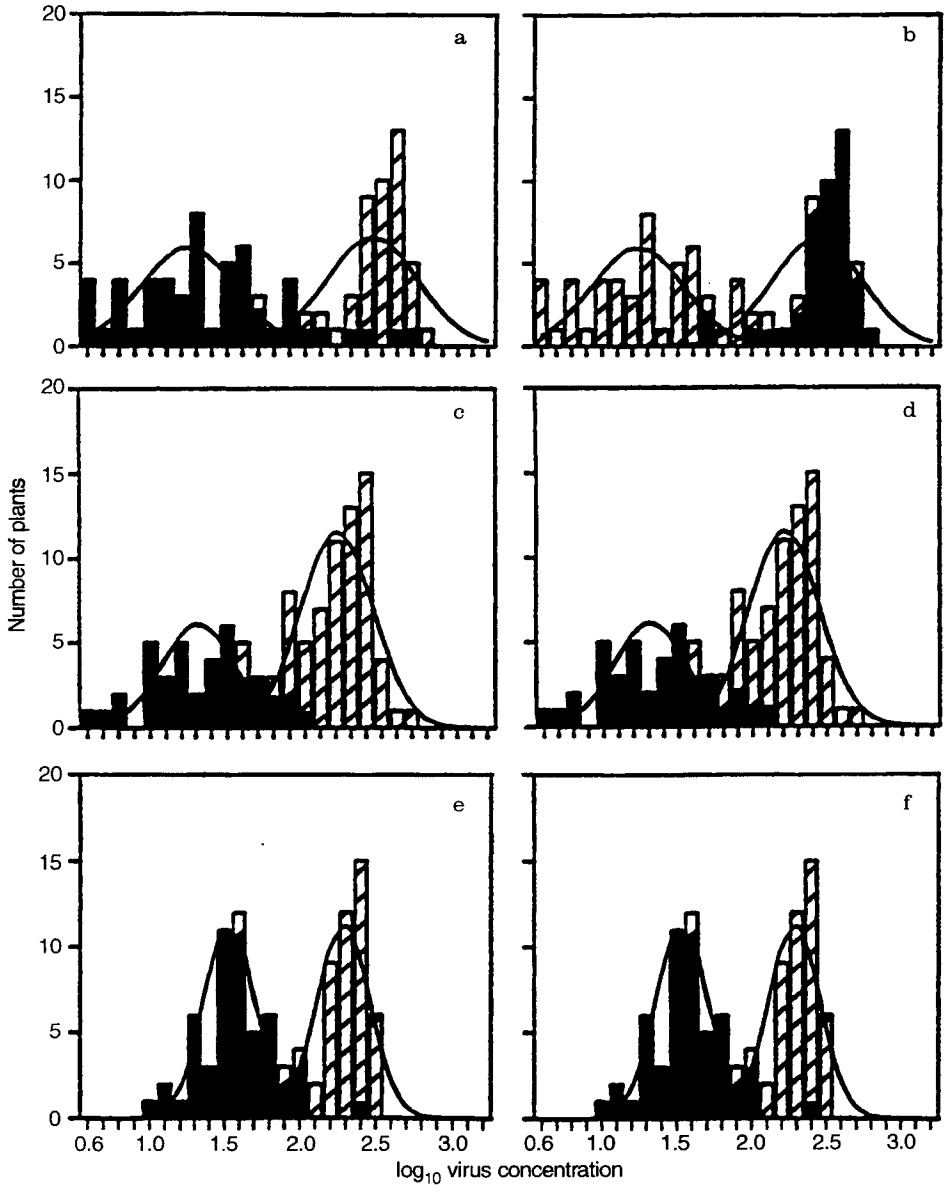

Fig. 3a–f Histograms of $\log_{10}$ BNYVV concentrations of individual plants from segregating families of **a, b** Holly-1-4, **c, d** R104 and **e, f** R128. The *black bars* show the presence of the RAPD markers OP-01_{1400} (a, c, e) and OP-02_{750} (b, d, f) in these individual plants and demonstrate the high correlation between the classification of plants as resistant or susceptible based on these RAPD markers compared to the classification based on the mixtures of normal distributions fitted to the $\log_{10}$ virus concentrations

range maps for the resistance locus of each accession. In Holly-1-4 and R128 linkage was confirmed between the identified RAPD markers and the major resistance gene. Lewellen (1988) suggested the name *Rz* gene for the resistance from Holly-1-4. Although observed segregation ratios did not enable the identification of a major resistance gene in R104, the fact that tightly linked markers co-segregating with the resistance were found, suggested a major resistance gene in this family as well. The skewed segregation ratio in R104 is probably a result of distorted segregation in crosses between the subsp. *maritima* and *vulgaris*. The contribution of the two most-tightly linked markers to the virus concentrations of individual plants of Holly-1-4, R104 and R128 was estimated by QTL mapping. The contribution of the RAPD marker OP-01_{1400} to the explained total phenotypic variation differed only slightly in the three accessions.

The RAPD markers found in the accessions were compared to study whether the resistance loci studied are likely to be different or the same. Linkage between, for example, the RAPD markers OP-01_{1400} and OP-02_{750} and the resistance loci in Holly-1-4, R104 and R128 are an indication for the existence of identical or tightly linked resistance loci in these three accessions. Combining of resistance genes from these three *B. vulgaris* accessions to improve the durability of resistance might then become rather difficult, but still possible if different alleles are involved. On the other hand, the existence of identieal resistant loci in R128, with resistance from Swiss chard, and the other accessions would be surprising since Jung et al. (1993) showed a relatively

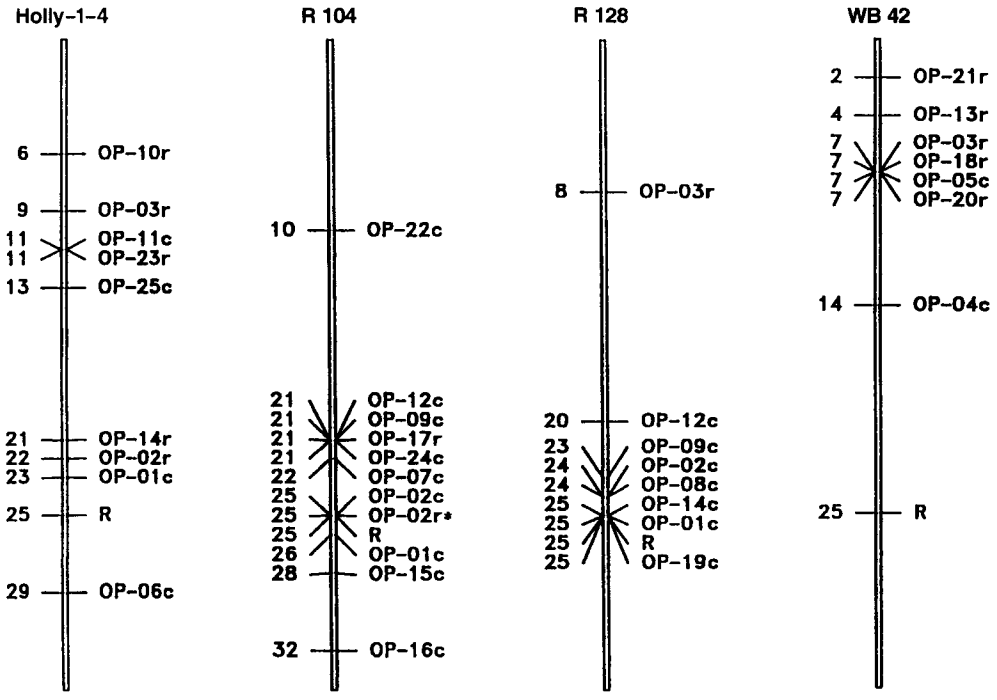

Fig. 4 Short-range maps showing the position of the RAPD makers linked to the resistance loci in the segregating families of Holly-1-4, R104, R128 and WB42. Map distances are in cM. *OP-01r* refers to the primer OP-01, which amplifies a RAPD marker linked to resistance in repulsion phase, whereas *OP-02c* is linked to resistance in coupling phase. In R104 OP-02c and OP-02r* behave as a single co-dominant marker

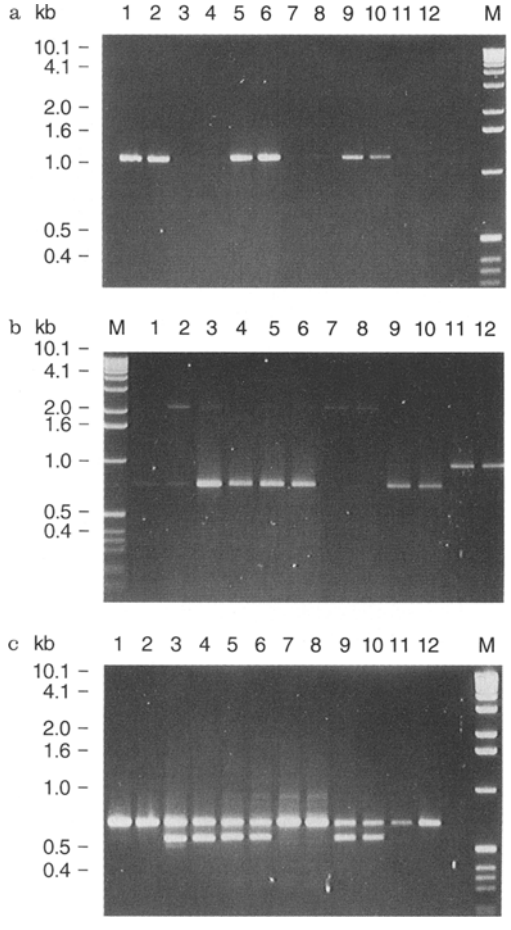

Fig. 5a–c The presence of STS markers in segregating families using bulks of resistant plants of Holly-1-4 (*lanes 1, 2*), R128 (*lanes 5, 6*) and R104 (*lanes 9, 10*) and bulks of susceptible plants of Holly-1-4 (*lanes 3, 4*), R128 (*lanes 7, 8*) and R104 (*lanes 11, 12*) with **a** STS/OP-01, **b** STS/OP-02 and **c** STS/OP-9; *M* = 1-kb DNA ladder

large distance at the DNA level between *B. vulgaris* cultivars and Swiss chard.

In earlier studies it was found that resistance to BNYVV in WB42 may be based either on one (or more) dominant major gene(s), showing distorted segregation in crosses with *B. vulgaris* subsp. *vulgaris*, or on two unlinked complementary major genes, which are both required for resistance (Scholten et al. 1996), as has been proposed for resistance in potato to potato leaf roll virus (Barker et al. 1994). If two complementary genes are responsible for resistance in WB42, markers linked to one of the resistance genes would also be amplified on about one-third of the susceptible plants, which could explain the low number of markers that was found. However, the occurrence of complementary genes conferring resistance to viruses is not very likely. The hypothesis of two unlinked dominant resistance genes in WB42 could also explain the low number of markers linked to resistance in this accession. In that case the plants in the resistant bulk may contain one or both resistance loci. The genetic window around the resistance loci will then become larger, resulting in the identification of markers farther away from the loci of interest. The existence of two unlinked resistance loci in WB42, however, is not in agreement with the markers that have been identified so far, since all these markers were found to be linked. Therefore, it seems most likely that resistance in WB42 is based on one resistance locus, showing distorted segregation in crosses with subsp. *vulgaris*. The result that the bulks of resistant and susceptible plants of WB42 did not generate the $OP\text{-}01_{1400}$ and the $OP\text{-}02_{750}$ RAPD marker might be an indication of the existence of a different resistance locus in WB42 compared to Holly-1-4, R104 and R128. To clarify whether different loci are involved, the alleles have to be

studied using F_2 families of crosses between the various sources of resistance.

As a result of this study, indirect selection for the resistance gene from Holly-1-4, R104 or R128 can be done in breeding programmes, without the need for disease testing, simply by the selection of resistant individuals possessing two flanking RAPD or STS markers. The RAPD markers linked to resistance in repulsion phase might be useful to analyze selfings of resistant plants in order to discriminate between homozygous and heterozygous resistant plants.

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