Theor Appl Genet (2001) 103:1201–1210 © Springer-Verlag 2001

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Establishment of a local map of AFLP markers around the powdery mildew resistance gene *Run*1 in grapevine and assessment of their usefulness for marker assisted selection

Received: 18 October 2000 / Accepted: 4 April 2001

Abstract The monogenic dominant genetic determinism of total resistance to powdery mildew, introduced from *Muscadinia rotundifolia* into *Vitis vinifera*, was further assessed in BC_4 and BC_5 full-sib families. A BC_5 population of 157 individuals was used to select AFLP markers linked to the resistance gene, *Run*1. Thirteen AFLP markers were selected and a local map was constructed around the *Run*1 gene. Ten markers among the 13 were found to co-segregate with the resistance gene. The usefulness of these 13 AFLP markers for the selection of *Run*1-carrying genotypes was further assessed through their analysis in a set of 22 *Run*1-carrying resistant genotypes and 16 susceptible genotypes. Three markers out of the 13 analysed were found to be absent in all susceptible genotypes and present in all resistant individuals, and may thus represent good tools for the marker-assisted selection of grapevine varieties resistant to powdery mildew. A recombination event among the markers that were originally found to co-segregate was observed in one of the resistant individuals, showing that recombination is possible in this region and may therefore be observed in larger populations.

Keywords *Vitis vinifera* · *Uncinula necator* · Resistance · AFLP · Introgression

Introduction

The most-widely cultivated grapevine is the *Vitis vinifera* species which originated in middle Asia and subsequently spread over all of occidental temperate Asia, southern Europe and northern Africa (Olmo 1976). *V. vinifera* is valued for the quality of its berries for wine or table consumption; however, it is susceptible to a number of potentially damaging pathogens. Among the most threatening are the powdery and downy mildews which were introduced during the 19th century together with accessions of the American wild *Vitis* species in which they were endemic (Galet 1977). Powdery mildew is caused by the fungus *Uncinula necator* (Schw. Burr.) which is able to attack species in all the *Vitaceae* family (Boubals 1961). It was first described in 1845 in a greenhouse in Great-Britain and it rapidly spread into Europe, causing severe yield losses (Galet 1977). It is now a world-wide problem in viticulture. Despite the fungicidal effect of sulphur discovered in 1850 and considerable progress made in the development of new organic fungicides, the parasite is still difficult to control for a number of reasons including the appearance of strains resistant to systemic fungicides (Steva et al. 1988; Debieu et al. 1995), the existence of two sources of primary inoculum (hyphae in dormant buds or cleistothecia in bark; Boubals 1961; Diehl and Heintz 1987; Pearson and Gadoury 1987; Gadoury and Pearson 1988; Delye and Corio-Costet 1998), and difficulties in establishing epidemiological models efficient in any climate (Thomas et al. 1994). From a more general point of view, the limitation of systematic chemical sprayings would be of significant benefit to environment and health.

At the turn of the 20th century, the first attempts to select resistant varieties to downy and powdery mildew were conducted by private breeders (Olmo 1976; Galet 1988). Many accessions of American *Vitis* species (*Vitis labrusca*, *Vitis rupestris*, *Vitis riparia*, *Vitis aestivalis*) are partially or totally resistant to powdery mildew. In contrast, *V. vinifera* is classified as susceptible, although different cultivars do show varying levels of susceptibilty (Boubals 1961) and an ontogenetic partial resistance has been reported in developing grape berries (Gadoury et al. 1997; Ficke et al. 1999). The resistant varieties that were bred were thus interspecific complex hybrids between several resistant *Vitis* accessions and *V. vinifera* (Galet 1988). However, good fruit quality was not fully recovered, mainly because of the quantitative inheritance of resistance and quality traits (Boubals

Communicated by P. Langridge

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1961; Eibach et al. 1989). The use of these hybrids was therefore limited until their cultivation was finally banned in Europe (Galet 1988).

The muscadine grape [*M. rotundifolia* (Michx.) Small], which originated from South-East USA, is highly resistant to most of the *V. vinifera* pathogens (Olmo 1971; Bouquet 1986) including *Uncinula necator*. The species was first classified by Planchon (1887) in the genus *Vitis*, section *Muscadinia*, but, due to its anatomical and morphological characteristics, Small (1903) proposed that the section *Muscadinia* be classified as a distinct genus. The discovery that the chromosome numbers were different in *Vitis* (2=38) and *Muscadinia* (2n=40) gave new support to this proposal (Branas 1932). Programs aimed at the introgression of resistance genes from *M. rotundifolia* into *V. vinifera* were developed (Wylie 1871; Detjen 1919a, b) but have been limited by the high sterility of the hybrids (Patel and Olmo 1955; Nesbitt 1966; Olmo 1971; Bouquet 1986). A total resistance to powdery mildew derived from *M. rotundifolia* was demonstrated to be controlled by a single dominant locus by Bouquet (1986). This locus, called *Run*1 (for **R**esistance to ***U**ncinula* ***n**ecator* 1), was introduced into the *V. vinifera* genome using a pseudo-backcross strategy (different *V. vinifera* genotypes are used at each backcross step to avoid inbreeding) aimed at the creation of new good quality grape varieties which are resistant to powdery mildew (Bouquet 1986).

The objectives of the present work were to further assess the monogenic dominant determinism of the resistance, to develop markers tightly linked to the *Run*1 locus and to address the usefulness of these markers for breeding purposes. Such markers would be useful for a number of reasons including: (1) in order to select individuals with the smallest introgressed *Muscadinia*-originated genomic fragment, (2) to be able to monitor its association with partial resistance in breeding programs, (3) to screen other *M. rotundifolia* accessions, and (4) to facilitate isolation of the gene by map-based cloning.

Materials and methods

Plant material

Resistant plants and segregating populations were derived from different steps of the breeding program aimed at the development of resistant grapevine cultivars through the introgression of the *Run*1 gene into *V. vinifera*. The name and origin of populations is summarised in Table 1 and the names and origin of resistant individuals are described in Fig. 1. Throughout this paper, a backcross (BC) is used to define a pseudo-backcross as described in the introduction. It should be noted that the BC_2 individual VRH12–4-80 was named VRH12–4-89 in Bouquet (1986). The search for *Run*1-linked molecular markers, was carried out through the analysis of the population Mtp3294 (VRH3082–1-42×Cabernet-Sauvignon N) derived from the *M. rotundifolia* accession G52 after five pseudo-backcrosses (Bouquet 1986). The susceptible *V. vinifera* cultivars that were used for molecular analysis were: Aranel B, Arriloba B, Aubun N, Baroque B, Cabernet franc N, Cabernet-Sauvignon N, Chasan B, Cot N, Fer N, Grenache N, Marselan N, Merlot N, Pinot N, Riesling B, Semillon B and Syrah N. A partially resistant complex hybrid, Villard blanc, of different *Vitis* species including *V. vinifera* was also analysed (Galet 1988). All plants were grown at the experimental stations of Chapitre and Vassal near Montpellier (France).

Pathogenicity tests

In vivo greenhouse pathogenicity tests were performed in July, by spraying the seedlings with a suspension of conidia made from natural field isolates. The sprays were repeated two or three times at 1-week intervals until the infestation was homogeneous. Resistant and susceptible plants were scored 1 month later as described by Bouquet (1986). In the case of the tests made in 1987, the results obtained in the greenhouse were checked the next year in a nursery field with high disease pressure. In vitro pathogenicity tests were performed using a *U. necator* monoconidial isolate (Mtp1) obtained from an infested grapevine in a greenhouse at Montpellier (France). Cabernet-Sauvignon young leaves were detached from 3-month-old cuttings and surface-sterilised by soaking for 1 min in a 1% v/v sodium hypochloride solution. The leaves were then rinsed two times with sterilised water, dried with sterilised absorbent paper and placed onto medium containing Agar (2% w/v) and Benzimidazol (0.003% w/v) in a Petri dish. The upper side of the leaves was then dry inoculated with spores from the Mtp1 isolate as described by Cartolaro and Steva (1990). The leaves were used as sources of inoculum 10 to 15 days after inoculation. Plants were screened for resistance to powdery mildew, as described by Cartolaro and Steva (1990), by infection of leaf disks (three per genotype) using Cabernet-Sauvignon as a susceptible control. About 8–10 days after inoculation, sporulations on leave disks were observed and scored in two classes: resistant (no conidia or conidiophores) and susceptible (production of conidia).

Preparation of genomic DNA

Total DNA was extracted using the protocol of Lodhi et al. (1994) modified as follows. Grapevine leaves (1.5 g) were harvested and ground in the presence of liquid nitrogen. Ten microliters of extraction buffer (Tris HCl 0.1 M pH8, EDTA 0.02 M, NaCl 1.4 M, CTAB 2%, β-mercaptoethanol 1%) were added. The homogenate was incubated at 65°C for 30 min. Most of the proteins and polysaccharides were removed by centrifugation following the addition of 10 ml of chloroform/isoamyl alcohol (24:1). Thirty microliters of cold 95% ethanol was added to the supernatant in the presence of 1.5 M NaCl. After 5 min, the DNA pellet was extracted with a Pasteur pipette, dried on absorbent paper, dissolved in TE buffer pH 8.0 (Maniatis et al. 1989) and stored at 4°C. DNA concentration was estimated by comparison with known quantities of phage λ DNA (GIBCO-BRL, Gaithersburg, Md., USA).

AFLP analysis

AFLP (amplified fragment length polymorphism) is a molecular marker technique based on selective PCR amplification of restriction fragments first described by Vos et al. (1995). Two hundred and fifty nanograms of genomic DNA were digested during 3 hours at 37°C with 2.5 units of *Eco*RI (Boerhinger Mannheim, Germany) and *Mse*I (GIBCO-BRL). The ligation of double-stranded adapters to the ends of the restriction fragments was performed at 20°C for 2 h according the instructions of the AFLP Analysis System I kit (GIBCO-BRL). Pre-amplification and amplification steps were performed using Promega *Taq* polymerase (Madison, Wisconsin, USA) according to the instructions of the AFLP Analysis System I kit (GIBCO-BRL). The pre-amplification step was performed with primers specific for the *Eco*RI and *Mse*I adapters, including the selective nucleotides A and C respectively (*Eco*RI+A: 5′-GAC TGC GTA CCA ATT C**A**-3′; *Mse*I+C: 5′-GAT GAG TCC TGA GTA A**C**) and the selective amplification step using the same primers with two additional selective nucleo-

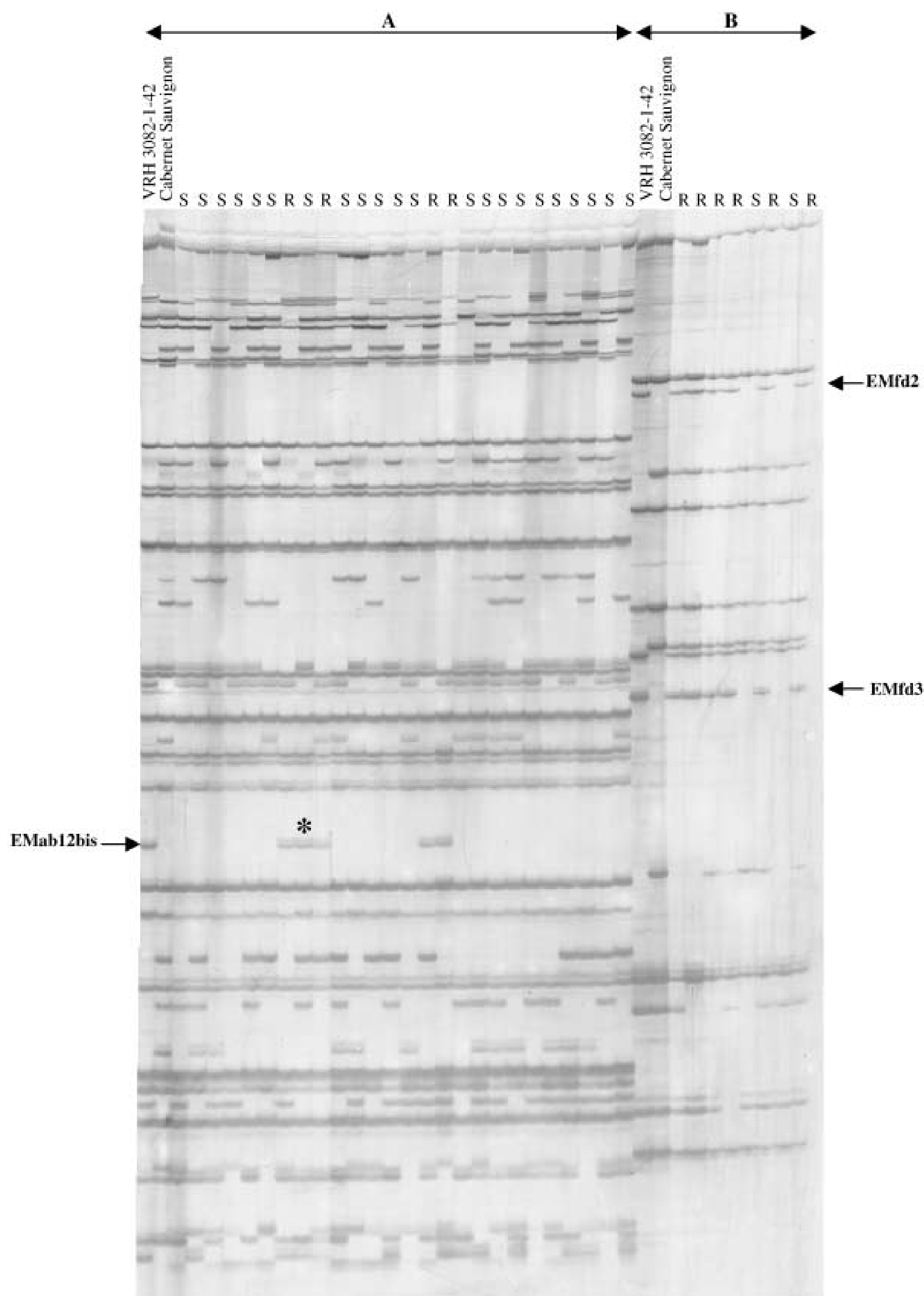

Fig. 1 Example of the AFLP pattern observed using two primer combinations, *Eco*+AAC/*Mse*+CTC (**A**) and *Eco*+AGC/Mse+CAC (**B**), giving respectively one and two markers linked to the *Run*1 gene. For each primer combination, the two parents (VRH3082–1-42 and Cabernet Sauvignon) and a set of individuals of the Mtp3294 population were analysed. "*R*" is for a resistant individual, "*S*" is for a susceptible one. Markers are indicated by an *arrow* followed by their names. The *star* indicates a recombinant individual

tides. Amplification fragments were separated on a 6% denaturing polyacrylamide gel at 60 W for 2.5 h. DNA was observed by silver staining with the Silver Sequence DNA Sequencing System (Promega). The size of the markers was evaluated by comparison to a 30–330-bp ladder (GIBCO-BRL).

Cloning and sequencing of the AFLP markers

A drop of pure water was overlaid on the band corresponding to the marker. The band was excised with a yellow tip and put into a sterile tube with the addition of 200 µl of pure water. The acrylamide was then slashed into pieces with a yellow tip and the tube was incubated at 95°C for 30 min. The acrylamide matrix was pelleted by centrifugation at 13000 rpm for 1 min, 50 µl of the supernatant was removed into a new tube and four different dilutions made (0, 1/10, 1/100, 1/1000). Each dilution was tested for a re-amplification of the fragment using the AFLP pre-amplification protocol except that DTT (10 mM) was also added to the reaction mixture. The best compromise between the absence of other bands than the marker and the quantity of amplification product was used for the further cloning steps. The fragments were ligated into a PGEM-T Easy vector (Promega) according to the manufacturer's instructions. The ligation product was transformed into competent DH5α *Escherichia coli* bacteria prepared according to Hanahan (1983). Transformants were analysed by amplification using bacteria as a template in the AFLP pre-amplification protocol. The size of the products of amplification was compared to the size of the marker after electrophoresis in a 6% denaturing polyacrylamide gel for 2.5 h at 60 W. Candidates were sequenced in forward orientation using the T7 universal primer

and the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit with AmpliTaq DNA polymerase FS (PE Applied Biosystems, Perkin Elmer, Foster City, Calif., USA). The sequences were analysed in an ABI 373 DNA Sequencing System (Perkin Elmer).

Statistical analysis

The goodness-of-fit between observed and expected segregation ratios at both loci were tested by a chi-square analysis. Map distances were estimated using the software JoinMap version 2.0 (Stam 1993). The minimum LOD score of 4.0 and a maximum fraction of recombination of 0.499 were used to form linkage groups. Recombination fractions were converted into centiMorgans (cM) by applying the Kosambi function (Kosambi 1944).

Results

Genetic determinism of resistance to powdery mildew derived from the *M. rotundifolia* accession G52

The monogenic dominant genetic determinism of resistance was previously determined in BC_3 and BC_2F_2 populations (Bouquet 1986). The results of further genetic analysis conducted using the BC_4 and BC_5 generations are presented in Table 1. The results are still in favour of the genetic control of the resistance involving a major dominant locus. In all the resistant parents, a dominant gene was transmitted together with an introgressed fragment, in a heterozygous state. In all populations, it should segregate as if it was a classical backcross population, in a 1:1 ratio. This was indeed the case in most of the analysed populations (Table 1). However, the segregations were biased in two BC_5 populations towards the susceptible class.

Selection of AFLP markers linked to the *Run*1 gene

A subset of 157 (74 resistant and 84 susceptible) individuals of the Mtp3294 population was re-tested using an in vitro test on detached leaves. This subset was used for the search of molecular markers linked to the *Run*1 gene. Two bulks, one of ten resistant plants and one of ten susceptible plants were constituted in order to develop a bulked segregant analysis strategy (Michelmore et al. 1991). Sixty four combinations of selective primers were used to amplify pooled DNA from individuals of the two bulks. Seventeen primer combinations produced 19 polymorphic markers between the two bulks. Those markers were first analysed on the available progenitors of the Mtp3294 population (NC6–15, VRH8628, VRH5–18–79, VRH3082–1-42, Cabernet-Sauvignon, Grenache, Aubun and Merlot). Thirteen of the markers described in Table 2 were consistently present in all the resistant progenitors and absent in the Cabernet-Sauvignon (Table 3). Two of them were also present in Grenache (EMhb11, EMff1) and Aubun (only EMhb11) (Table 3). As AFLP markers are non-specific, two bands of the same size in two unrelated genotypes may not represent the same sequence. The band corresponding to EMhb11 was cloned in both VRH3082–1-42 and in Grenache, and sequenced. Only three nucleotide changes

Table 1 Origin of the different full-sib families tested for their resistance to powdery mildew; occurrence of susceptible and resistant genotypes; χ^2 of goodness of fit to the expected 1:1 segregation

Population	Cross at the origin of the populations	Year of the cross	Backcross step	Number of tested plants	Susceptible	Resistant	χ^2 (1*df*)
Mtp3082[a]	Aubun N×VRH1–28–82	1986	BC_4	312	153	159	0.115
Mtp3083[a]	Aubun N×VRH1–34–82	1986	BC_4	175	86	89	0.051
Mtp3084[a]	Aubun N×VRH1–11–82	1986	BC_4	140	64	76	1.028
Mtp3169	Grenache N×VRH3082–1-24	1989	BC_5	20	9	11	0.2
Mtp3179	Grenache N×VRH3082–1-32	1989	BC_5	280	131	149	1.157
Mtp3180	Grenache N×VRH3082–1-72	1989	BC_5	164	80	84	0.097
Mtp3274	VRH3082–1-42×Grenache N	1994	BC_5	147	76	71	0.170
Mtp3275	VRH3082–1-42×Carignan N	1994	BC_5	146	91	55	8.877[b]
Mtp3276	VRH3082–1-42×Syrah N	1994	BC_5	150	89	61	5.227[c]
Mtp3277	VRH3082–1-42×Cabernet-Sauvignon N	1994	BC_5	141	76	65	0.858
Mtp3294	VRH3082–1-42×Cabernet-Sauvignon N	1995	BC_5	350	165	185	1.142

[a] This test was performed over 2 consecutive years. The compiled results are given
[b] Significant at the 0.001 probability level
[c] Significant at the 0.01 probability level

Table 2 List and size of the markers linked in coupling with the *Run*1 gene. *Eco+* corresponds to the three selective bases of the *Eco*R1 primer. *Mse+* corresponds to the three selective bases of the *Mse*1 primer. The size of the fragments was determined by the sequence of the fragment except for those indicated by a star for which it was determined by comparison with a size marker

	EMab12b	EMhb11	EMaa10	EMba5	EMhb1	EMad8	EMbd4	EMfd2	EMfd3	EMhe4	EMge10	EMff1	EMeb2
Eco+	AAC	AAG	AAC	ACA	AAG	AAC	ACA	AGC	AGC	AAG	AGG	AGC	ACT
Mse+	CTC	CTC	CTA	CTA	CTC	CAC	CAC	CAC	CAC	CAG	CAG	CAT	CTC
Size (pb)	118	143	157	235*	475	110*	221	248	145	205*	141	330*	>330*

Table 3 Genotypes at the 13 AFLP markers in coupling with the *Run*1 gene of the susceptible and resistant ancestors of the Mtp3294 population. + is for the presence of the marker associated band and – for its absence. "BC step" stands for the step of the pseudo-backcross at which the individual was obtained

BC step	Genotypes	EMab12b	EMhb11	EMaa10	EMba5	EMhb1	EMad8	EMbd4	EMfd2	EMfd3	EMhe4	EMge10	EMff1	EMeb2
Resistant ancestors														
F_1	NC6–15	+	+	+	+	+	+	+	+	+	+	+	+	+
BC_1	VRH8628	+	+	+	+	+	+	+	+	+	+	+	+	+
BC_2	VRH5–18–79	+	+	+	+	+	+	+	+	+	+	+	+	+
BC_4	VRH3082–1-42	+	+	+	+	+	+	+	+	+	+	+	+	+
Susceptible ancestors														
	Aubun	–	+	–	–	–	–	–	–	–	–	–	–	–
	Cabernet-Sauvignon	–	–	–	–	–	–	–	–	–	–	–	–	–
	Grenache	–	+	–	–	–	–	–	–	–	–	–	+	+
	Merlot	–	–	–	–	–	–	–	–	–	–	–	–	–

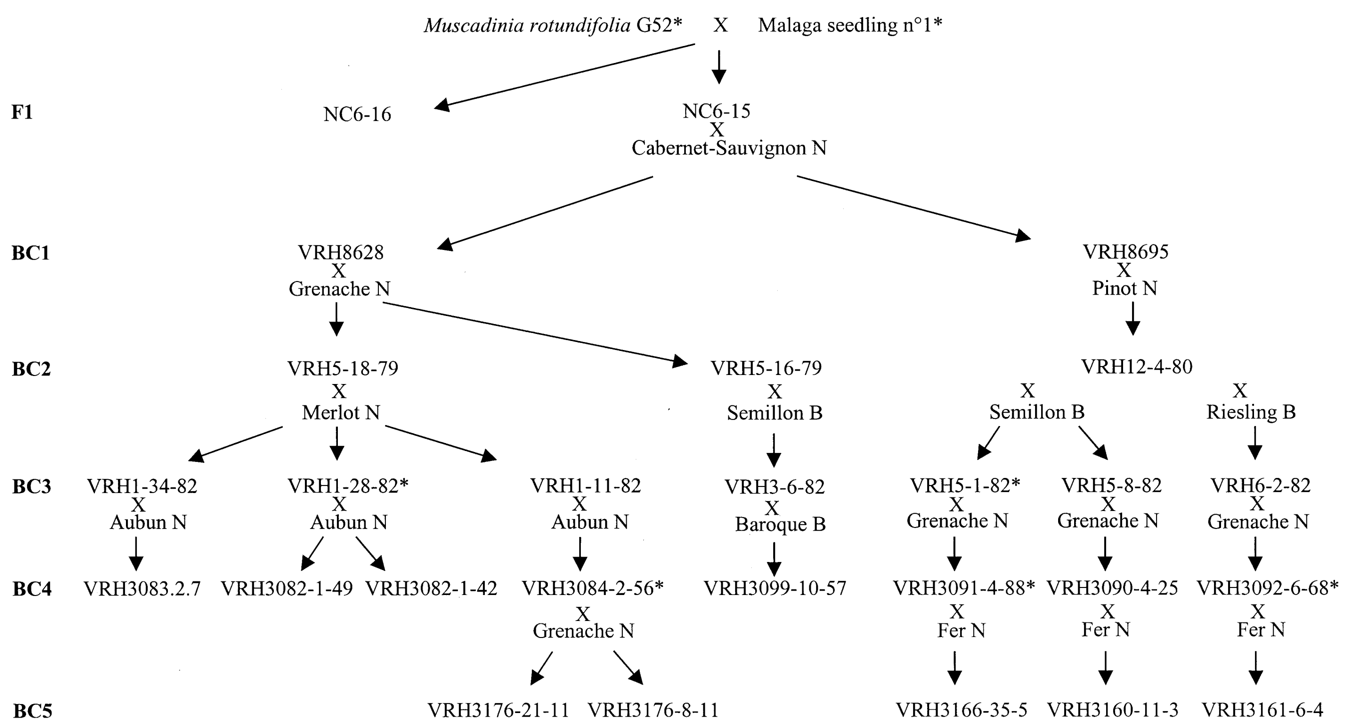

Fig. 2 Parentage relationships of the different individuals studied. G52 is the original resistant *M. rotundifolia* genotype. Stars indicate the genotypes that were not analysed because they are no longer available. NC6–15, NC6–16 and all genotypes whose name begins with the prefix VRH are resistant. The others are susceptible. *BCn* represents the pseudo-backcross step at which the individual was obtained

were observed between the two fragments, indicating that they correspond to the same genomic region.

Establishment of a local genetic map with markers in coupling, around the *Run*1 gene

The 11 AFLP primer combinations that yielded the 13 consistently polymorphic markers were further analysed on a subset of 157 plants of the Mtp3294 population (Fig. 2). Eleven recombinant plants were detected which allowed three of the markers (EMab12bis, EMhb11, EMaa10) to be ordered, with all the other markers co-segregating with the *Run*1 gene (Fig. 3). It should be noted that the segregation of those markers occurred in the resistant parent (VRH3082–1-42) in the same way as detected in the backcross population.

Detection of markers in repulsion in the *Run*1 region

The amplification with the above analysed AFLP primer combinations yielded, on average, 60 bands among which an average of ten polymorphic markers between the two parents of the population could be analysed (106 in total). Six markers were discarded because they were

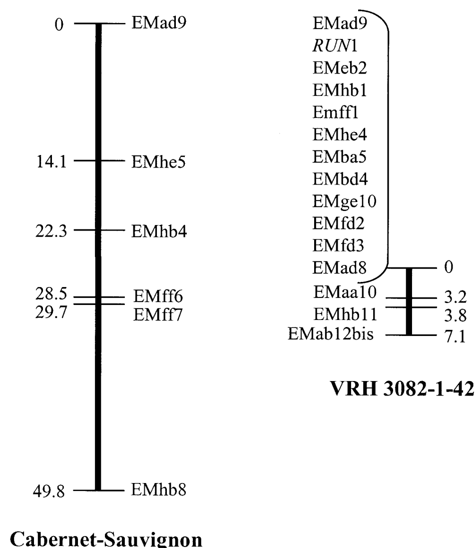

Fig. 3 Local map of the resistant genotype VRH3082–1-42 and in Cabernet-Sauvignon of the *Run*1 region. The 11 loci in *brackets* together with *Run*1 at the top of the VRH3082–1-42 linkage group are all co-segregating: their order is thus arbitrary. Note also that the orientation of the two linkage groups is arbitrary and may not be correct

strongly biased ($P<0.005$). Among the remaining 100 markers, 13 were biased (at the 0.05 significance level). Classically, in F_1 crosses, two maps are to be made, one for each parent (Ritter et al. 1990). Markers heterozygous in both parents are used afterwards to find the homologous linkage groups and to construct a consensus map (Ritter et al. 1990; Stam 1993; Lodhi et al. 1995). In our case, a consensus map was directly constructed using the JoinMap software (Stam 1993). This map consisted of 26 linkage groups (data not shown) and is thus not saturated. This allowed us to detect a new marker (EMad9) linked to the *Run*1 gene and heterozygous in both parents (the band was present both in Cabernet-Sauvignon and in VRH3082–1-42 and segregated in a 3:1 ratio). EMad9 is co-segregating with the *Run*1 gene in repulsion, but it should be noted that the precision for the calculation of the percentage of recombination is far less important for such markers harbouring the same alleles in both parents because it is impossible to know the parental origin of the alleles (Ritter et al. 1990; Lodhi et al. 1995). EMad9 allowed the detection of the linkage group of the Cabernet-Sauvignon's map homologous to the *Run*1 linkage group (Fig. 3). A second marker in common in the two homologous linkage groups would be necessary to know their relative orientation and to confirm that they are indeed homologous.

Analysis of the linked markers in 22 *Run*1 genotypes, 16 *V. vinifera* varieties and an interspecific hybrid

The purpose of this analysis was to make a preliminary study to determine to what extent those AFLP markers would be useful for the selection of: (1) *Run*1-carrying resistant individuals (later on called VRH individuals), and (2) resistant individuals retaining the narrowest *M. rotundifolia* introgressed genomic fragment. Only the 13 markers detected through the bulked segregant analysis in coupling with the *Run*1 gene were used for this purpose (Table 2). The different tested genotypes were derived from our breeding program: VRH individuals thus shared common ancestors at different levels, and the susceptible genotypes were used at different backcross steps (Fig. 1). Villard blanc is an interspecific complex hybrid that may be used as a source for the introgression of polygenic resistance to powdery mildew.

The presence of all markers was strongly dependant on the presence of the *Run*1 resistance gene except for EMff1 that was also amplified in 11 *V. vinifera* varieties and in Villard blanc (Tables 4 and 5). Including EMff1, seven markers were amplified in *V. vinifera* varieties and in Villard blanc (Table 5). Villard blanc showed a presence/absence pattern of markers similar to the pattern of the *V. vinifera* varieties (Table 5). On the whole, in VRH genotypes, 5 to 13 markers were present whereas, in *V. vinifera* varieties and in Villard blanc, only 0 to 5 markers were amplified (Tables 4 and 5). EMhb1, EMbd4 and EMfd3 were present in all VRH individuals tested and absent in Villard blanc and all the *V. vinifera* varieties analysed (Tables 4 and 5). Thus these markers may be specific for the presence of the *Run*1 gene.

Taking into account the order of the markers shown in Fig. 3, those showing no recombination with the *Run*1 gene in the previous analysis were present in all VRH genotypes except NC6–16 and VRH3161–6-4, whereas EMab12b, EMhb11 and EMaa10 were all missing in 11 genotypes (Table 4). All of them are derived from the same BC_1 ancestor, VRH8695, which did not carry the three markers (Table 4, Fig. 1). In those 11 genotypes, the presence of markers EMab12b, EMhb11 or EMaa10 could be explained by transmission from the *V. vinifera* parent: for instance, in VRH3166–35–5, only EMab12b is present, given by Fer N (Tables 4, 5, Fig. 1). This suggested that a recombination event occurred between EMaa10 and the *Run*1 gene at the BC_1 step, and thus that the introgressed fragment may be shorter in all their progenies. Another recombination event may have occurred at the BC_4 step and been transmitted to the BC_5 individual VRH3161–6-4 between a region containing the *Run*1 gene, EMhb1, EMbd4, EMfd3, EMge10, EMff1 and EMeb2 (markers present, Table 4), and a region containing the markers EMba5, EMad8, EMfd2 and EMhe4 (markers absent, Table 4).

Finally, NC6–16 showed an absence of the markers EMab12b, EMhb11, EMaa10, EMad8, EMhe4, EMge10, EMff1 and EMeb2 (Table 4). Taking into account the genotype of NC6–16 and VRH3161–6-4 and the local map of the *Run*1 region (Fig. 2), markers can be sorted into five blocks: (1) EMab12b, EMhb11, EMaa10, already ordered in respect of the *Run*1 gene and absent in NC6–16 and VRH3161–6-4, (2) EMha4, EMad8, absent in NC6–16 and VRH3161–6-4, (3) EMba5, EMfd2,

Table 4 Genotypes at the 13 AFLP markers in coupling with the *Run*1 gene in different introgressed genotypes. + is for the presence of the marker–associated band and – for its absence. "BC step" stands for the step of the pseudo-backcross at which the individual was obtained

BC step	Genotypes	EMab12b	EMhb11	EMaa10	EMba5	EMhb1	EMad8	EMbd4	EMfd2	EMfd3	EMhe4	EMge10	EMff1	EMeb2
F_1	NC6–15	+	+	+	+	+	+	+	+	+	+	+	+	+
F_1	NC6–16	–	–	–	+	+	–	+	+	+	–	–	–	–
BC_1	VRH8628	+	+	+	+	+	+	+	+	+	+	+	+	+
BC_1	VRH8695	–	–	–	+	+	+	+	+	+	+	+	+	+
BC_2	VRH5–16–79	+	+	+	+	+	+	+	+	+	+	+	+	+
BC_2	VRH5–18–79	+	+	+	+	+	+	+	+	+	+	+	+	+
BC_2	VRH12–4-80	–	–	–	+	+	+	+	+	+	+	+	+	+
BC_3	VRH1–11–82	+	+	+	+	+	+	+	+	+	+	+	+	+
BC_3	VRH1–34–82	+	+	+	+	+	+	+	+	+	+	+	+	+
BC_3	VRH3–6-82	–	+	+	+	+	+	+	+	+	+	+	+	+
BC_3	VRH5–8-82	–	–	–	+	+	+	+	+	+	+	+	+	+
BC_3	VRH6–2-82	–	–	+	+	+	+	+	+	+	+	+	+	+
BC_4	VRH3082–1-42	+	+	+	+	+	+	+	+	+	+	+	+	+
BC_4	VRH3082–1-49	+	+	+	+	+	+	+	+	+	+	+	+	+
BC_4	VRH3083–2-7	+	+	+	+	+	+	+	+	+	+	+	+	+
BC_4	VRH3090–4-25	–	–	–	+	+	+	+	+	+	+	+	+	+
BC_4	VRH3099–10–57	–	+	+	+	+	+	+	+	+	+	+	+	+
BC_5	VRH3161–6-4	–	–	–	–	+	–	+	–	+	–	+	+	+
BC_5	VRH3160–11–3	–	–	–	+	+	+	+	+	+	+	+	+	+
BC_5	VRH3166–35–5	+	–	–	+	+	+	+	+	+	+	+	+	+
BC_5	VRH3176–8-11	+	+	+	+	+	+	+	+	+	+	+	+	+
BC_5	VRH3176–21–11	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 5 Genotypes at the 13 AFLP markers in coupling with the *Run*1 gene of different *V. vinifera* susceptible genotypes and a partially resistant complex hybrid (Villard blanc). + is for the presence of the marker-associated band and – for its absence

Genotypes	EMab12b	EMhb11	EMaa10	EMba5	EMhb1	EMad8	EMbd4	EMfd2	EMfd3	EMhe4	EMge10	EMff1	EMeb2
Aranel	+	–	–	–	–	–	–	–	–	–	–	+	+
Arriloba	+	+	–	–	–	–	–	–	–	–	–	+	–
Aubun	–	+	–	–	–	–	–	–	–	–	–	–	–
Baroque	–	+	–	–	–	–	–	+	–	–	–	+	+
Cabernet franc	–	–	–	–	–	–	–	–	–	–	–	+	–
Cabernet-Sauvignon	–	–	–	–	–	–	–	–	–	–	–	–	–
Chasan	+	+	–	–	–	–	–	–	–	–	–	–	+
Cot	–	–	–	–	–	–	–	–	–	–	–	+	–
Fer	+	–	–	–	–	–	–	–	–	–	–	+	–
Grenache	–	+	–	–	–	–	–	–	–	–	–	+	+
Marselan	–	–	–	–	–	–	–	–	–	–	–	+	–
Merlot	–	–	–	–	–	–	–	–	–	–	–	–	–
Pinot	+	–	–	–	–	–	–	+	–	+	–	–	–
Riesling	+	–	+	–	–	–	–	+	–	–	–	+	+
Semillon	+	–	–	–	–	–	–	–	–	–	–	+	–
Syrah	–	–	–	–	–	–	–	–	–	–	–	+	–
Villard blanc	–	–	+	–	–	–	–	+	–	–	–	–+	–

present in NC6–16 and absent in VRH3161–6-4, (4) EMhb1, EMbd4, EMfd3, present in NC6–16 and VRH3161–6-4, and (5) EMge10, EMff1, EMeb2, present in VRH3161–6-4 and absent in NC6–16. If *Run*1 is present in NC6–16, block (4) may contain the closest markers to the resistance gene as all resistant individuals harbour them and all susceptible plants do not. Markers of blocks (1), (2) and (5) may either have been in a heterozygous state in G52 (as they are absent in NC6–16) or part of them may have been brought into NC6–15 by Malaga seedling no. 1. As both the G52 and Malaga seedling n°1 are no longer available these hypotheses could not be checked.

Discussion

Wild species are often valuable sources of resistance to crop pathogens. This is obviously the case for the grapevine where *V. vinifera* is susceptible to most pathogens whereas resistance to the same pathogens can be found in wild grapevine species (Boubals 1959, 1961, 1966; Galet 1977; Eibach et al. 1989). Among these wild species, *M. rotundifolia* offers the highest level of resistance against the widest range of pathogens. As most introgression programs are based on backcrosses of the wild species with the crop species, the more the wild species are phylogenetically distant from the crop species, the more the intro-

gression of the resistance is technically difficult and may even require the use of molecular-biology techniques (see McGrath et al. 1995; Fedak 1999, and references in both). In the case presented here, the resistance to powdery mildew was introduced into *V. vinifera* from the related genus, *M. rotundifolia* (Detjen 1919a, b). The haploid number of chromosomes in the two genera is different and the homology between the genomes may be incomplete (Branas 1932; Patel and Olmo 1955), making it difficult to recover fertile hybrids in the first generations of introgression (Jelenkovic and Olmo 1968; Dunstan 1962; Bouquet 1986). Patel and Olmo (1955) proposed that the two genera may share 13 homoeologous pairs of chromosomes and six (*Vitis*) or seven (*Muscadinia*) specific chromosomes. Based on the observation of the abnormal and irregular pairing of F_1 hybrids, the same authors proposed that those chromosomes may also present structural differences such as inversion or deletions. This may favour the linkage drag of wild genomic fragments in successive generations of backcrosses.

Resistance to a pathogen, introduced from a distant species, may rely on mechanisms associated with non-host resistance, especially when biotrophic pathogens are concerned, and thus may be non-race specific and more durable (Hadwiger and Culley 1993). The resistance conferred by the *Run*1 gene has been effective in the field against the most-frequent genotypes of the fungus present either in Bordeaux or Montpellier since 1974 (data not shown). It is difficult to make inferences from these observations about its durability, as the surfaces cultivated with the resistant genotypes are limited. This may also indicate that the *Run*1 locus is complex and contains several specific resistance genes. Indeed, *Vitis*×*Muscadinia* hybrids resistant to the fungus in France were found to be susceptible in North-Carolina (Bouquet 1986). On the other hand, no clear specialisation of the fungus isolate was observed across species and genera (Gadoury and Pearson 1991). The use of this gene in variety improvement may, therefore, be valuable in Europe together with a few chemical treatments to control the populations of *Uncinula necator* or in combination with other resistance genes. Finally, resistance genes which have been overcome by pathogens have been shown to have a residual effect (Martin and Ellingboe 1976; Durel et al. 2000), and most of the dominant resistance genes cloned so far were shown to be clustered with homologous sequences which may represent a potential source of resistance to the same or to another pathogen (Richter et al. 1995; Michelmore and Meyers 1998; Wang et al. 1998).

The aim of the present work was to develop molecular markers linked to a resistance gene, helpful for the selection of good quality genotypes containing the smallest *M. rotundifolia* genomic fragment around the *Run*1 gene, and thus help to avoid the linkage drag of genes conferring a lower quality from *M. rotundifolia*. In the absence, in Europe, of strains of *U. necator* overcoming the *Run*1 resistance gene that would allow a differential screening of resistance, molecular markers specific for the *Run*1 gene are essential to be able to combine it with other sources of resistance. Such markers should be in strong linkage disequilibrium with the resistance gene.

The segregation of the resistance to powdery mildew derived from NC6–15 was further studied in several populations. Like Olmo (1978), Bouquet (1986) observed the occurrence of susceptible and resistant plants to powdery mildew in F_1 and BC_1 progenies originating from different *M. rotundifolia* accessions. However, segregation ratios were too distorted, due to chromosome disequilibrium and the mortality of the plants, to deduce the genetic determinism of the resistance (Bouquet 1986). The segregation of a single dominant locus (*Run*1) for resistance to powdery mildew was observed in the subsequent BC_2, BC_3 and BC_2F_2 populations (Bouquet 1986). The segregation of the resistance in the further generations of introgression (BC_4 and BC_5) is presented here and confirms the monogenic dominant determinism of the resistance. Only two populations presented a distorted segregation ratio with an excess of susceptible genotypes. The same phenomenon had previously been observed in BC_3 and BC_2F_2 populations where the susceptible parent was the variety Riesling (Bouquet 1986). This may be due to an influence of the genetic background in which the resistance gene was introgressed, as has previously been observed in wheat, tomato and apple (Bai and Knott 1991; Kellerhals et al. 1993; Hammond-Kosack and Jones 1994; Fischer 1995). In the present case, further experiments should be conducted in order to discriminate between genotypic effects and sampling effects in rather small populations.

A BC_5 progeny was used to select AFLP markers linked to the resistance gene, following the Bulked Segregant Analysis strategy (Michelmore et al. 1991) and 13 AFLP markers were identified in coupling with the gene. On average, each AFLP primer combination identified 60 bands. Thus around 3800 loci have been analysed. Given the size of the grapevine genome (475 Mbp, Lodhi and Reisch 1995), the screening of 3600 loci would theoretically be enough to detect a marker in a 400-kbp interval around the gene with an 0.95 probability (Tanksley et al. 1995). One of the 13 linked markers detected here may therefore be very tightly linked to the *Run*1 gene. Indeed, 10 markers out of the 13 co-segregated with the gene. If the *Run*1 genomic region is too divergent from its *V. vinifera* homologue, recombination may be suppressed and the screening of large progenies would not be informative for the ordering of markers as was the case for the *Hs1^{pro1}* gene for resistance to nematodes in sugar beet (Cai et al. 1997). This may have two consequences. First, any of the ten co-segregating markers may be useful for indirect selection of the *Run*1 genotype. In wheat, for example, markers linked to genes derived from distant species appeared to be more reliable across the genetic backgrounds than markers linked to genes derived from the wheat gene pool (Schachermayr et al. 1997). Second, it may be impossible to reduce the size of the introgressed fragment by conventional breeding and, thus, completely break any linkage with genes unfavourable for the quality of vintage (if any are present on this fragment). The effective tight linkage of the ten markers co-segregating

with the *Run*1 gene remains to be further examined in larger progenies. It is estimated that up to 1000 individuals need to be screened in order to have a 0.95 probability to find markers in a 100-kbp interval (Lodhi et al. 1995; Tanksley et al. 1995). Another way to address the tight linkage between the markers and the resistance gene was to examine them in a set of resistant and susceptible genotypes. Even if resistant genotypes shared parentage relationships, they represent numbers of potential recombination events and different genetic backgrounds. This study confirmed that EMab12b, EMhb11 and EMaa10 recombine frequently with the *Run*1 gene and may thus not be useful for marker-assisted selection (MAS) purposes as seven genotypes did not harbour them (Table 4). A recombination event sharing in two groups the markers co-segregating with the *Run*1 gene in the Mtp3294 population was observed in a BC_5 individual (VRH3161–6-4, Table 4) showing that recombination is possible. In parallel, the screening of a small set of *V. vinifera* cultivars showed that some of the AFLP markers linked to the *Run*1 gene could also be found in some susceptible genotypes (Table 5). The risk of scoring non-homologous sequences as if they were homologous is far less important for AFLP than for RAPD, due to a more-sensitive method of electrophoresis and to more-specific conditions of amplification (Vos et al. 1995). However, experiments aimed at the conversion of AFLP markers into sequence-specific PCR markers showed that the cloning of a single AFLP band may result in heterogeneous recombinant colonies (Shan et al. 1999). In the present study, at least in one case, the fragment amplified in the resistant and susceptible genotypes were shown to be homologous. This result also indicates that homologous sequences may be present in this region in both *V. vinifera* and *M. rotundifolia* genomes, which would favour recombination. Other evidence of an introgression of the *Run*1 gene via homologous recombination events came out of the genotyping of the two F_1 individuals (NC6–15, NC6–16), the two BC_1 individuals deriving from NC6–15 and VRH3161–6-4. It is possible to build models that explain these results involving only simple and homologous events of recombination, with part of the markers being in a heterozygous state in the original *Muscadinia* accession (G52). The chromosome originating from G52 and carrying the *Run*1 gene, and the chromosome originating from Malaga seedling no. 1 that was introgressed, may thus have been structurally not very different. However, this hypothesis would have to be confirmed using more co-dominant markers along the chromosome and BC_1 full-sib families originating from NC6–15 and NC6–16 (McGrath et al. 1995; Fedak 1999). The first requirement may soon be available as microsatellite maps should be available in grapes (Riaz and Meredith 2000) but as F_1 NC6–15 and NC6–16 are nearly sterile it may remain difficult to achieve. When all of these results are considered together, they are in favour of the hypothesis that suspected recombination inhibition in the *Run*1 region may thus not be as strong as in the case of the region carrying the *Hs1*^{pro1} gene. On the whole, three markers, EMhb1, EMbd4 and EMfd3, are always present in resistant genotypes and always absent in *V. vinifera* genotypes, and thus represent good candidates for the MAS of *Run*1 carrying grapevine varieties.

Finally, this study provides interesting observations regarding the marker-assisted management for a program of gene introgression. It is noteworthy that all genotypes that presented a recombination event between EMaa10 and the *Run*1 gene were derived from a single BC_1 individual (VRH8695, Table 4, Fig. 1). The BC_5 individual, VRH3161–6-4, that showed a recombination event between markers that co-segregated in the Mtp3294 full-sib family, is also derived from VRH8695. This illustrates the fact that recombination rates in a particular region of the genome are dependent on the genetic background (Williams et al. 1995) and suggest that markers would be useful to select in early generations individuals with high recombination rates. In the present case, the best generation may have been the BC_2 rather than the BC_1 because of the sterility of F_1 individuals.

Acknowledgements The authors warmly thank M.-F. Corio-Costet, I. Dry, L. Grivet and J.-L. Notteghem for critical reading of the manuscript. They also thank P. François, Y. Danglot and S. Rousseau for technical assistance. The authors declare that the experiments complied with the current laws of France.

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