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Development of RAPD and SCAR markers linked to the *Pvr4* locus for resistance to PVY in pepper (*Capsicum annuum* L.)

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Abstract Potato Virus Y (PVY) is the only potyvirus infecting pepper (*Capsicum annuum* L.) in Europe. Currently, the development of pepper varieties resistant to PVY seems to be the most-efficient method to control PVY damage. Among the sources of resistance, a monogenic dominant gene *Pvr4* confers resistance against all known PVY pathotypes. In this work, bulked segregant analysis (BSA) was used to search for randomly amplified polymorphic DNA (RAPD) markers linked to the *Pvr4* gene, using segregating progenies obtained by crossing a homozygous resistant ('Serrano Criollo de Morelos-334') with a homozygous susceptible ('Yolo Wonder') cultivar. Eight hundred decamer primers were screened to identify one RAPD marker ($UBC19_{1432}$) linked in repulsion phase to *Pvr4*. This marker was converted into a dominant sequence characterised amplified region (SCAR) marker ($SCUBC19_{1423}$). This marker was mapped into a dense *Capsicum* genetic map in a region where several genes for resistance to different diseases are located. This marker can be useful to identify PVY-resistant genotypes in segregating progenies of pepper in marker-assisted selection (MAS) breeding programs.

Keywords *Capsicum annuum* · PVY · RAPD · SCAR · BSA · Marker assisted selection

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Introduction

Potato Virus Y (PVY) was first described by Smith in 1931 (cited by De Bokx and Huttinga 1981) as infecting potato, and is considered as the type member of *Potyvirus*, included in the *Potiviridae* family, the largest group of virus-affecting plants (Murphy et al. 1995). PVY is able to infect a large number of plant species, including some of economical importance such as potato, tomato and pepper (De Bokx and Huttinga 1981). PVY is distributed worldwide and is the only potyvirus-infecting pepper in Europe, especially in the Mediterranean area (Marchoux et al. 1976; Luis-Arteaga and Gil-Ortega 1986), and is transmitted by at least 25 aphid species (De Bokx and Huttinga 1981). PVY isolates from Mediterranean countries were initially classified within pathotypes 0, 1 and 1-2, based on their differential interactions with pepper varieties carrying the resistance genes $pvr2^+$, $pvr2^1$ and $pvr2^2$ (Gebre-Selassie et al. 1985). More recently, a new PVY pathotype has been proposed and named as PVY-1-3 (Luis-Arteaga et al. 1997). Although several methods, including vector control, have been described to prevent PVY infections, they are insufficient to control the disease. Consequently, the use of pepper varieties with some level of resistance against PVY has been suggested as the most-efficient method to control PVY damage.

Monogenic recessive sources of resistance against PVY in pepper were first described and listed in an allelic series by Cook (1961, 1962 and 1963). Subsequently, some oligogenic resistances were also reported (Nagai and Costa 1972). Later, a monogenic dominant gene named *Pvr4*, that confers resistance against all known PVY pathotypes and pepper mottle virus (PepMoV), was found in the *Capsicum annuum* L. line 'Serrano Criollo de Morelos-334' (SCM-334) (Pasko et al. 1992; Dogimont et al. 1996). Recently, a new dominant source of resistance, *Pvr7*, has been described in *Capsicum chinense* Jacq. 'PI159236'. This gene appears to be tightly linked to *Pvr4* and seems to be active against PepMoV, although it is still not known if it also confers resistance against PVY (Grube et al. 2000).

Standard procedures followed in breeding programmes to introduce resistance genes into genotypes of interest, include successive crosses, together with selection steps based on inoculations with the appropriate isolates, to finally fix and obtain the desired improved material. However, in the case of PVY isolates, it is often difficult to ensure if the inoculated isolate is the appropriate one, due to the high observed rates of changes from one pathotype to another (Arnedo-Andrés 2001). Moreover, currently available serological methods are not sensitive enough to distinguish between PVY pathotypes (Soto et al. 1994). Similarly, the DNA sequences coding for the coat protein show very high similarity among the different PVY pathotypes (Llave et al. 1999). Thus, the availability of molecular markers linked to *Pvr4* would be extremely useful to avoid those drawbacks in pepper breeding programmes aimed to develop PVY resistant lines.

Several strategies to develop different types of molecular markers useful for tagging resistance genes have been described (Staub 1996). Among these methods, BSA (Michelmore et al. 1991), combined with several types of molecular markers, has been extensively used to find markers linked to genes of interest, especially when near-isogenic lines (NILs) are not available for those genes. The transformation of these markers into more stable and easily applicable markers, such as SCARs (Paran and Michelmore 1993) or cleaved amplified polymorphic sequences (CAPS) (Konieczny and Ausubel 1993), is generally the next step before their routine application in MAS programmes.

Several molecular markers linked to different potyvirus resistance genes have been found in *Capsicum* spp. in the last few years. The loci *pvr1* (Murphy et al. 1998), *pvr2* (Caranta et al. 1997), *pvr6* (Caranta et al. 1996) and *Pvr7* (Grube et al. 2000), conferring different spectra of resistances against several potyviruses, have been tagged with different molecular markers and following different location strategies. In the case of *Pvr4*, two different strategies, using AFLPs and RAPDs, were designed in order to obtain useful markers for MAS. One group (Caranta et al. 1999) used BSA combined with AFLPs, and our group used BSA combined with RAPDs.

In this work, we report the localisation of a RAPD marker linked to the *Pvr4* locus and its transformation into a SCAR marker, which is more useful for practical breeding purposes. Moreover, the markers were also studied on several pepper lines with different responses after PVY inoculations. Finally, the marker linked to *Pvr4* was mapped on a high-density pepper genetic map.

Materials and methods

Plant and viral materials and inoculations

Two pepper varieties, 'Serrano Criollo de Morelos-334' (SCM-334), resistant to PVY and homozygous for the allele *Pvr4*, and 'Yolo Wonder' (YW), susceptible to PVY and homozygous for the allele *Pvr4*+, were crossed in both directions to produce F_1 populations (SCM-334 × YW and YW × SCM-334). F_1 plants from the cross SCM-334 × YW were selfed to obtain 110 F_2 plants. These were also selfed to obtain a total of 96 F_3 lines, with 25 to 30 plants per line. Backcrosses were also made by crossing the two F_1 populations with the parental genotypes.

The PVY isolate P-22-88 belonging to the PVY-1-2 pathotype was used as an inoculum. This strain was isolated in Málaga (Spain) from infected pepper plants. It was maintained and multiplied in tobacco plants before its use in the inoculations.

To ensure that the inoculum only contained PVY particles, the following species were used as differential hosts: *Vigna unguiculata* L., *Cucumis sativus* L., *Cucurbita pepo* L., *Chenopodium quinoa* Willd., *Chenopodium amaranticolor* Coste & Reyn., *Datura stramonium* L., *Nicotiana glutinosa* L., *Nicotiana rustica* L., *Nicotiana sylvestris* Speg. et Comes, *Nicotiana tabacum* L. 'Xanthi nc', *Petunia hybrida* Vilm., *Physalis floridana* Rydb. and *Ocimum basilicum* L. Moreover, to confirm that the viral isolate was a member of PVY pathotype 1-2, the following pepper differential varieties were inoculated: 'Doux des Landes', 'Yolo Wonder', 'Yolo Y', 'Florida VR2', 'Puerto Rico Wonder' and 'Serrano Veracruz'.

Virus inoculum for mechanical transmissions was prepared by grinding 1 g of fresh infected leaves from tobacco plants in 4 ml of 0.03 M phosphate buffer, pH 8.5, containing 0.2% sodium diethyldithiocarbamate (DIECA) and 75 mg/ml of mesh carborundum and activated charcoal (Marrou 1967). The plants were inoculated by manually rubbing the leaves with the sap extract; excess inoculum was removed by rinsing the leaves with water.

All the plant materials derived from the crosses between 'SCM-334' and 'YW' were inoculated twice, first in the stage of two true leaves, on one cotyledon and on the first two true leaves, and later, in the stage of six to eight true leaves, on the four intermediate leaves. At each inoculation, the differential host species and the pepper differential varieties described above were inoculated as well. Besides the two parental lines and the six pepper differential varieties, plants of ten additional pepper varieties from different origins and with different responses against PVY were also inoculated following the same procedure, except that they were inoculated only in the six to eight true leaves stage.

Plants were maintained in a greenhouse with a temperature regime that ranged between 15 °C and 25 °C. Symptoms were evaluated up to 2 months after inoculation, observing local lesions and systemic symptoms in the inoculated plants. The presence of viruses in the plants was also assessed by the DAS-ELISA serological test (Clark and Adams 1977) using the commercial PVY-10E3 monoclonal antibody (Ingenasa, Spain). ELISA absorbances were considered to be positive if they were three-times higher than those corresponding to non-inoculated pepper plants, used as healthy controls.

DNA extraction, bulked DNA preparation and RAPD analysis

Total DNA was extracted before inoculation by the method of Doyle and Doyle (1987) with minor modifications and adapted to small tissue quantities (Hormaza 1999). Fresh young leaves (0.2 g) were ground in a 1.5-ml microfuge tube with 800 µl of extraction buffer [(2% w/v) CTAB (hexadecyltrimethylammonium bromide), 1.4 M NaCl, 20 mM EDTA, 100 mM Tris–HCl, pH 8.0, 1% PVP, 0.1% sodium bisulfite and 0.2% (v/v) 2-mercaptoethanol] and incubated in a water bath at 60 °C for 30 min with occasional swirling, mixed with an equal volume of chloroform:isoamyl alcohol (24:1, v/v) and centrifuged at 13,000 g for 15 min. The aqueous phase was transferred to a new tube and mixed with 2/3 vol of iced isopropanol. The DNA precipitate was centrifuged at 13,000 g for 15 min, the pellet washed with 10 mM of ammonium acetate in 76% ethanol, dried overnight at room temperature and resuspended in 500 µl of MTE buffer (10 mM Tris–HCl pH 8.0, 0.1 mM EDTA). Finally it was centrifuged at 13,000 g for 5 min and the aqueous phase with the dissolved DNA was quantified in a spectrophotometer (GeneQuant, Pharmacia Biotech) and diluted to 10 ng/µl to be used for amplification.

For the bulks, the DNA was extracted separately from each individual of the progeny. Initially, DNA from ten resistant and ten susceptible F_2 individuals was pooled in equal concentrations to

constitute the resistant and susceptible bulks respectively. In a second screening for molecular markers, two bulks with five plants each were used.

PCR amplifications were carried out according to the method described in Williams et al. (1990) with minor modifications (Hormaza et al. 1994). The final reaction volume was 20 µl and contained: 40 ng of genomic DNA, 20 mM of Tris–HCl (pH 8.4), 50 mM of KCl, 2 mM of $MgCl_2$, 100 µM each of dATP, dGTP, dCTP and dTTP (Gibco BRL), 0.4 µM of primer (obtained from Operon Technologies, and the University of British Columbia, Vancouver, Canada) and 0.6 units of *Taq* DNA Polymerase (Gibco BRL). The samples were overlaid with 25 µl of mineral oil and briefly centrifuged before amplification. The reactions were performed in a MJ PTC 100 thermocycler, using the following procedure: 1 cycle of 2 min at 94 °C, followed by 35 cycles of 45 s at 94 °C, 1 min at 36 °C and 2 min at 72 °C. Finally the samples were incubated for 5 min at 72 °C. Amplification products were separated by gel-electrophoresis in 1.7% agarose in 1 × TBE buffer, stained with ethidium bromide at 50 ng/µl and visualised under UV light with an image analysis system (GelDoc2000, BioRad, Hercules, Calif.).

A total number of 800 decamer primers were screened and each amplification reaction was repeated at least once when either polymorphism or no amplification was observed. When a polymorphism was observed between the bulks, each DNA sample included in the bulks was amplified separately with the corresponding primers. The *Pvr4* linked marker obtained was also analysed on the parental lines, the F_1, the F_2 and BC_1 generations, and on the additional 16 pepper varieties studied.

SCAR design

After ethidium-bromide staining, and using 1 × TAE buffer for the electrophoresis, the amplified fragment of interest was excised from the agarose gel and purified with the Wizard DNA Purification System (Promega) according to the manufacturer's instructions. The fragment was reamplified as described above and the standard tailing procedure was performed to ensure that a single deoxyadenosine was added to the 3′-end of the reamplified fragment. The fragment was then cloned into the pGEM-T vector (Promega) with the ratio 3:1 (insert:vector), and the sticky-end ligated into the multiple cloning site (MCS) according to the manufacturer's instructions. The JM109 High Efficiency Competent Cells (Promega) were transformed with these new plasmid vectors (Sambrook et al. 1989) and the plasmids were purified with FlexiPrep Kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Sequencing of the cloned fragments was carried out by the dideoxy nucleotide-chain termination (Sanger et al. 1977) using the T7 and SP6 primers by the Sequencing Department at the Universidad Autónoma de Madrid (Spain).

Two specific oligonucleotides were then designed using the Netprimer software (Premier Biosoft International). The forward primer (SCUBC19-1) contains the ten bases of the RAPD primer plus the 15 adjacent bases. The reverse primer (SCUBC19-2) contains the last 3′ end base of the RAPD primer plus the 23 adjacent bases. Amplification of genomic DNA with the SCAR primers was carried out under the same conditions as the RAPD reaction described above, except for the $MgCl_2$ concentration that was changed to 3 mM, the annealing temperature that was raised to 62 °C and the number of amplification cycles was reduced to 30. The SCAR primers designed were used to amplify DNA obtained from the 'SCM-334' and 'Yolo Wonder' parental lines, as well as the F_1, BC_1, F_2 ('SCM-334' × 'Yolo Wonder') and the 16 additional pepper varieties.

Linkage analysis

The putative RAPD markers linked to PVY were analysed on every individual of the F_2 population. The chi-square test was used to study the inheritance of the genetic resistance present in the evaluated crosses. The genetic distance between markers and the *Pvr4* locus was assessed using the Mapmaker 3.0 software (Lander et al. 1987). Recombination fractions were converted into Kosambi centimorgans (cM) (Kosambi 1944) and the standard error calculated.

Localisation of markers on a pepper genetic map

The UBC19 primer that produced the marker linked to *Pvr4* was used to amplify genomic DNA obtained from 44 plants of the *C. annuum* L. 'NuMex RNaky' × *C. chinense* PI159234 F_2 mapping population (Livingstone et al. 1999) and the parental lines. The amplification products were resolved on a 3.5% polyacrylamide gel and visualised after silver staining. Linkage analysis was performed using Mapmaker 3.0 software establishing a LOD score of 3.0. The distances shown on the genetic map were derived by multipoint analysis.

Results

Inoculation results

The reactions of the differential host plants and the pepper differential varieties confirmed that the inoculations were carried out with the PVY pathotype 1-2.

The resistant parent 'SCM-334' usually developed pinpoint necrotic local lesions on the inoculated leaves and was free of systemic infection monitored by both ELISA and symptom observation (Table 1). Plants of the PVY-susceptible cultivar 'YW' showed systemic vein banding and all the plants contained antigen levels above the threshold.

The two F_1 populations (SCM-334 × YW and YW × SCM-334) and the backcross to 'SCM-334' as recurrent parent were free of systemic infection monitored by both ELISA and symptom observation. On the other hand, the backcross with 'YW' as recurrent parent was consistent with the 1 resistant:1 susceptible ratio and the F_2 (SCM-334 × YW) progeny was not significantly different from a 3 resistant:1 susceptible ratio. All the susceptible plants of these generations showed systemic vein banding and positive ELISA test results, while the resistant plants showed small local lesions on the inoculated leaves and did not show any systemic symptoms (Table 1).

The data presented in Table 1 suggest that the resistance carried by the variety 'SCM-334' against the PVY pathotype 1-2 is due to a unique and dominant gene described as *Pvr4* (Dogimont et al. 1996).

Identification of a RAPD marker linked to the *Pvr4 locus*

A total of 500 random decamer primers were used for screening the initial bulks containing ten plants each. The average band number per primer was between 6 and 7. Only two primers produced polymorphisms between the bulks, but when each individual plant was analysed, the polymorphic bands were amplified in only one out of the

Table 1 Response to the PVY-1-2 (P-22-88) isolate of the pepper varieties 'SCM-334', 'Yolo Wonder' and several progenies. Expected frequencies, χ^2, and probabilities were calculated according to the one dominant resistance-gene hypothesis. R resistant; S susceptible

Genotype	No. resistant	No. susceptible	Expected ratio (R:S)	χ^2	*P*
SCM-334	19	0	0:1		
Yolo Wonder	0	20	0:1		
F_1 (SCM-334 × YW)	20	0	1:0		
F_1 (YW × SCM-334)	20	0	1:0		
BC_1 (SCM-334 × YW) × YW	12	11	1:1	0.043	0.83
BC_1 (SCM-334 × YW) × SCM-334	20	0	1:0		
BC_1 YW × (SCM-334 × YW)	5	7	1:1	0.33	0.56
F_2 (SCM-334 × YW)	81	29	3:1	0.11	0.74

ten plants included in the susceptible bulk and, consequently, those markers were discarded.

A second screening for polymorphism was then made, reducing the number of plants down to five plants per bulk. In this screening, the 500 initial primers and 300 additional primers were analysed, and the average band number per primer was again between 6 and 7. The primer UBC19 5′-GCCCGGTTTA-3′ generated a 1,432-bp DNA fragment which was present in both bulks but with a consistent difference in intensity. When analysing the individuals used to make the bulks, the $UBC19_{1432}$ fragment was amplified in the DNA obtained from each of the five plants included in the susceptible bulk but only in one out of the five plants included in the resistant bulk.

When the DNA obtained from the genotypes of the F_2 population was amplified with UBC19, the $UBC19_{1432}$ fragment was detected in 28 out of the 29 susceptible plants and in 56 out of the 81 resistant plants. These numbers are consistent with the hypothesis that the $UBC19_{1432}$ fragment is linked in repulsion to the *Pvr4* resistance gene and, consequently, the susceptible plants which do not show amplification of the $UBC19_{1432}$ fragment can be considered as recombinants between the marker and the *Pvr4* locus.

To confirm this hypothesis, 96 F_3 lines obtained after selfing 96 F_2 plants were inoculated with the PVY-1-2 (P-22-88) isolate. Most of the F_3 plants showed necrotic local reactions and the systemic reaction included vein banding mosaic and necrotic veins. All the F_3 plants which showed systemic necrotic reactions and not very clear symptoms caused by PVY were analysed by ELISA to confirm virus infection. Of the 96 F_3 lines analysed, 21 were homogeneously resistant, 24 homogeneously susceptible and 51 segregated for resistance. These results also fit into the hypothesis of a unique dominant gene (probability 0.755) conferring resistance against PVY 1-2.

The UBC19 primer was also tested on the F_3 lines to calculate the number of recombinant individuals between the RAPD fragment and the *Pvr4 locus*. $UBC19_{1432}$ was amplified in DNA from just two of the 21 homogenous resistant lines, and from 23 of the 24 homogeneous susceptible lines. Finally, within the F_3 segregating lines, three recombinant lines were observed. Thus, after analysing the 110 F_2 plants and the 96 F_3 families, eight recombination events were detected. Therefore, considering 96 F_2 plants, the estimated genetic distance between the $UBC19_{1432}$ marker and the locus *Pvr4* in this population was 4.3 cM with a standard error of 2.1 cM.

Conversion of $UBC19_{1432}$ into a SCAR marker

The complete $UBC19_{1432}$ fragment was sequenced, and at both ends of the sequence the terminal ten bases exactly matched the sequence of the UBC19 primer. No significant homology was found between the sequenced RAPD fragment and known sequences in the databases using Blast (Altschul et al. 1997). From that complete sequence, two specific SCAR primers, named SCUBC19-1 (5′-GCCCGGTTTATATATTACGAAAAG-A-3′) and SCUBC19-2 (5′-AATGGAGAAGCATAATG-ACGGAGA-3′), were designed specifically lacking palindromic regions and containing between 35 and 60% G + C. Since the first base of SCUBC19-2 overlapped with the last base of the RAPD primer, the fragment produced after amplification with the SCAR primers was 1,423-bp long, 9-bp shorter than the original RAPD fragment. The SCAR primers amplified a single 1,423-bp band in all the susceptible plants of the F_2 generation, except for the genotype that did not show amplification of the $UBC19_{1432}$ fragment. The $SCUBC19_{1423}$ fragment was also present in all the F_2 resistant plants that also showed amplification of the $UBC19_{1432}$ fragment and was absent in the rest of the resistant individuals. The parental lines, the F_1 made in both directions and the backcross generations also showed the same amplification pattern as revealed with the UBC19 primer. The $SCUBC19_{1423}$ marker showed a dominant polymorphism, associated to the recessive *Pvr4*$^+$ allele and, consequently, it was not possible to distinguish between the susceptible and heterozygous resistant plants.

Analysis of PVY inoculations and PCR-markers in several pepper varieties

The results obtained after the inoculation of 18 pepper varieties with the PVY-1-2 isolate (P-22-88) are presented in Table 2. The results obtained after analysing all the inoculated plants by ELISA confirmed the observed symptoms and, consequently, the studied genotypes were classified as resistant or susceptible against PVY inoculation.

Table 2 Inoculation results of several pepper genotypes with the P-22-88 (PVY 1-2) isolate and their amplification with the RAPD primer UBC19 and the SCAR primers, SCUBC19-1 and SCUBC19-2

Varieties (number of inoculated plants)	P-22-88 (PVY 1-2)[a]	*Pvr4*	$UBC19_{1432}$[b]	$SCUBC19_{1423}$[b]
Doux des Landes (5)	vn/vn Tn vb	Absent	+	+
NuMex R-Naky (5)	vn/vn Tn vb	Absent	+	+
Jupiter (5)	vn/vn Tn vb	Absent	+	+
Yolo Wonder (5)	0/vb	Absent	+	+
Yolo Y (5)	0/vb	Absent	+	+
Florida VR2 (5)	0/vb	Absent	+	+
Puerto Rico Wonder (5)	0/0	Absent	+	+
Serrano Veracruz (5)	0/0	Absent	+	+
Avelar (5)	0/vb	Absent	+	+
Perennial (5)	vn/vn vb	Absent	+	+
SCM-229 (5)	0/0	Present?	+	+
SCM-330 (7)	2 lln/1 vn vb	Present?	+	+
SCM-334-11 (5)	0/0	Present	–	–
SCM-334-25 (5)	lln/vn vb	Absent	+	+
S.118.2 (5)	0/2 vb	Present?	+	+
S.20.1 (4)	0/1 vb	Present?	+	+
S.20.1 bis (3)	0/2 vn vb	Present?	+	+
PI159236-90939093 (5)	0/0	Absent	+	+

[a] local reaction/systemic reaction. vn: veinal necrosis; Tn: necrotic stem; vb: mosaic 'vein banding; lln: local necrotic lesions; 0: no symptoms
[b] +: present; –: absent

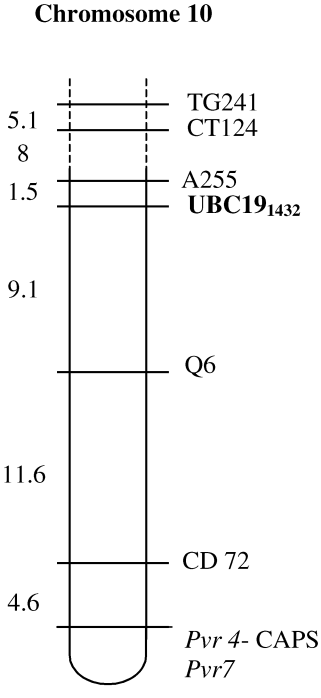

Fig. 1 Position of the RAPD $UBC19_{1432}$ *Pvr4* linked marker on the pepper map (Livingstone et al. 1999), including the mapping position of the *Pvr4*-CAPS marker and *Pvr7* (Grube et al. 2000). *Numbers to the left* indicate genetic distances between markers (cM). The markers included are: A255, an AFLP marker; Q6, a RAPD marker linked to the *Tsw* locus, the tomato cDNA clones CD72 and CT124 and the tomato genomic RFLP TG241. These markers were located according to Livingstone et al. (1999)

The pepper varieties included to distinguish the PVY pathotypes (Gebre-Selassie et al. 1985; Arnedo-Andrés et al. 1998), 'Doux des Landes', 'Yolo Wonder', 'Yolo Y', 'Florida VR2', 'Puerto Rico Wonder' and 'Serrano Veracruz', showed local and systemic symptoms, and all of these lines showed amplification of the $UBC19_{1432}$ and $SCUBC19_{1423}$ fragments as well. The pepper varieties 'Avelar', 'Perennial', and some plants of 'S.118.2', 'S.20.1' and 'S.20.1. bis' showed vein banding mosaic caused by PVY infection. While 'SCM-229' was asymptomatic, the other Mexican line, 'SCM-330', showed resistant and susceptible plants after PVY inoculation. The susceptible line 'SCM-334 (25)' showed vein banding mosaic after inoculation, while the resistant line 'SCM-334 (11)' did not develop any systemic symptoms and it was the only line included in this study which did not show amplification of the markers linked to the *Pvr4 locus*. Finally, the 'PI 159236-9093' line was resistant against the PVY-1-2 isolate.

Mapping of the *Pvr4*-linked marker

The marker associated with the *Pvr4 locus* was amplified in the *C. annuum* L. Numex RNaky parent and absent in *C. chinense* PI159234. $UBC19_{1432}$ mapped on linkage group 10 (Livingstone et al. 1999) at 1.5 cM (LOD 6.2) from the nearest marker A255 (Fig. 1). Other close markers were the RFLPs, CT124 at 7.6 cM (LOD 8.4), TG241 at 13.6 cM (LOD 5.4) and CD72 at 28.2 cM (LOD 1.2). Moreover, the $UBC19_{1432}$ marker was located at 9.1 cM to the Q6 RAPD marker linked to the *Tsw* locus (Jahn et al. 2000).

Discussion

In this work, BSA has been used to find a marker linked in repulsion to the *Pvr4* locus in pepper after screening 800 RAPD primers. This marker was found when five individual plants per bulk were used, but was not detected when the bulks included ten plants each. The absence of polymorphism in the initial bulks was due to its repulsion-phase status with the resistance allele and was explained through the dominant nature of the RAPD markers. When the first screening was made, four out of the ten resistant plants pooled in the resistant bulk were

heterozygous (*Pvr4 Pvr4*$^+$) and the other six were homozygous (*Pvr4 Pvr4*) for the resistance locus. Since the four heterozygous individuals showed amplification of the $UBC19_{1432}$ fragment within the resistant bulk, it was not possible to quantitatively distinguish the fragment between the bulks. In the second screening, when pooling five plants per bulk, only one heterozygous plant was included within the resistant bulk and this allowed us to obtain a quantitative fragment distinction between the bulks with the UBC19 primer. Michelmore et al. (1991) reported that the ability of BSA to detect different alleles between bulks in lettuce was between 0.2 and 0.4, a range that is similar to that observed in our work. A possible drawback of using a low number of plants per bulk could be the appearance of false polymorphisms. However, the reduction in the number of plants to five per bulk during the second screening resulted in few false positives and, therefore, that number seems to be appropriate in pepper, increasing the probability to detect polymorphisms not observable when a higher number of plants per bulk is used.

When working with ten plants per bulk, two additional polymorphic fragments between the bulks were observed, but when the DNA of each individual contained in the bulk was analysed separately, we observed that the polymorphism was due to the amplification of only one individual out of the ten included. Also in lettuce, Michelmore et al. (1991) concluded that such polymorphisms, due to a proportion around 0.1 within a bulk, are very rare and below this proportion is not detectable. In our case, the observation of two cases out of 500 primers tested further shows how rare this detection is.

Besides the number of plants per bulk, the plant material is an important factor to take into account when targeting a gene using BSA. The success of this approach depends on the degree of polymorphism around the gene of interest, and the probability of success increases as the divergence between the parental lines enlarges. Previous studies (Prince et al. 1992; Lefebvre et al. 1995) have shown that enough polymorphism is present within pepper to work with intraspecific crosses which have even been used to construct molecular linkage maps (Lefebvre et al. 1995). However, the level of polymorphism present in pepper is not always sufficient to detect polymorphic markers linked to genes of interest. This explains the different degree of success described in the literature. Thus, while several markers were found around the *Tsw* locus (resistance to tomato spotted wilt virus) using an interspecific cross (Moury et al. 2000), we could locate only one marker linked to the *Pvr4* locus using 800 primers, and no markers were detected in the vicinity of the *Bs3* (resistance to *Xanthomonas campestris* pv *vesicatoria*) with 750 primers (Pierre et al. 2000), and of *Me3* (resistance to *Meloidogyne* spp.) using 800 primers (Djian-Caporalino et al. 1998).

In order to increase the specificity of the reaction and to simplify the use of markers linked to PVY resistance in pepper breeding programmes, a SCAR marker was derived from $UBC19_{1432}$. One of the advantages of SCAR, compared to RAPD, markers (Paran and Michelmore 1993) is that in some cases SCARs can be transformed into codominant markers. In our case, that was not possible, probably because the differences around the *Pvr4* locus between the two genotypes studied are due to structural changes in the area of the primer union rather than to one or a few base changes. Paran and Michelmore (1993) suggested that those changes could produce reorientation on the primer union sites, or a longer distance between those union sites that make the amplification of the targeted sequence impossible.

The results obtained after amplifications of DNA from several pepper genotypes (Table 2) with both markers confirmed some previous results and opened new questions. All the pepper genotypes included among the pepper differential varieties (Gebre-Selassie et al. 1985; Arnedo-Andrés et al. 1998) showed the expected response, according to the last revision of *potyvirus* resistance genes in *Capsicum* (Kyle and Palloix 1997), and all the lines included in this study showed amplification of both $UBC19_{1432}$ and $SCUBC19_{1423}$. Previous studies showed a partial resistance against PVY-1-2 in the 'Perennial' line (Caranta and Palloix 1996) since, although no symptoms were observed on inoculated plants, viral particles were detected on noninoculated leaves and their ELISA absorbances were significantly lower than the positive controls. In our case, we observed typical symptoms caused by PVY infection on the 'Perennial' plants and the absorbance values did not differ from the positive controls. Regarding the SCM lines, all of them were obtained from Dr. Guerrero (Mexico), and they were first studied for their reported resistance against the fungus *Phytophthora capsici* Leon. (Guerrero-Moreno and Laborde 1980). The results obtained could be due to several causes: the resistant plants could be heterozygous for the *Pvr4* locus or, alternatively, recombination between the markers and the *Pvr4* locus could have occurred. The presence of one or several resistance genes against PVY, different from *Pvr4*, could also be possible since these Mexican pepper lines have been less studied than 'SCM-334'. These explanations are also valid for the data obtained with the lines originated from India, 'S.118.2', 'S.20.1' and 'S.20.1. bis'. Finally, 'PI 159236-9093' was derived after several backcrosses to a recurrent *C. annuum* parent of a single selected *C. chinense* 'PI 159236' plant resistant to PepMoV (Grube et al. 2000). The resistance gene present in this line has been recently reported and named *Pvr7* (Grube et al. 2000). In these studies, *C. chinense* 'PI 159236-9093' showed resistance against all the PVY-pathotypes and it did not segregate independently from *Pvr7*, although the PVY resistance genetic control remains unclear and it might be due to *Pvr7*, *Pvr4* or another linked gene. According to the amplification pattern obtained in our studies, the putative presence of *Pvr4* in 'PI 159236-9093' would have a different origin than the allele present in SCM-334. However, the results obtained with *C. chinense* 'PI 159234', suggest that the small size difference between the $UBC19_{1432}$ fragment linked to *Pvr4*$^+$ and the

fragment obtained after amplification of DNA from 'PI 159234' could be due to differences between species, and that the fragment present in that *C. chinense* accession could have a deletion inside the amplified fragment.

The marker obtained in this study has also been mapped into a highly saturated *Capsicum* genetic map developed from an interspecific cross between *C. annuum* and *C. chinense* (Livingstone et al. 1999). Several resistance genes have already been located on that map, including genes against potyviruses (Grube et al. 2000) and tomato spotted wilt virus (Jahn et al. 2000). A CAPS marker linked to *Pvr4* (Caranta et al. 1999) has been mapped on linkage group 10 (Livingstone et al. 1999) at 4.6 cM from CD72, and the distance between this nearest marker and *Pvr4* was estimated to be 4.6 ± 2.9 cM (Grube et al. 2000). The marker described in our study is located in the same area on pepper linkage group 10, although some points should be considered. The mapping population consists of a small number of plants, and more accurate data could perhaps have been obtained from a larger mapping population. Moreover, the localisation of the *Pvr4* markers and their mapping positions have been obtained using different intra- and inter-specific populations, which may result in different distance estimates (Paterson et al. 1988; Messeguer et al. 1991). The distance between the RAPD marker described and another RAPD marker linked to *Tsw*, which confers a dominant resistance to tomato spotted wilt virus (Jahn et al. 2000), is estimated to be approximately 10 cM suggesting the existence of a dominant resistance gene cluster in pepper, as proposed by Grube et al. (2000).

The SCAR marker identified in this study, combined with other molecular markers linked in coupling-phase to the resistant allele, should allow routine MAS for resistance to PVY in pepper breeding programs, permitting an early selection of resistant genotypes without the cumbersome steps of inoculation, symptom detection and ELISA tests, and avoiding the problem of PVY changes from one pathotype to another. While coupling-phase markers linked to dominant resistance genes are more useful in the successive backcrosses to breed a pepper line carrying a resistance gene, the last selfing generation could be accelerated and improved by using a repulsion-phase marker. This higher efficiency is due to the low risk of selecting heterozygous-resistant or homozygous-susceptible plants instead of the required homozygous-resistant plants. One example was described by Haley et al. (1994) who compared the selection efficiency between a repulsion-phase marker (7.1 cM) and a coupling-phase marker (1.9 cM) both linked to the *bc-3* resistance gene in bean. Despite the higher genetic distance of the repulsion-phase marker, the selection was more efficient than with the coupling-phase marker. Consequently, a combination of coupling- and repulsion-phase markers could be desirable to develop highly efficient breeding programmes. These markers can be combined with markers linked to other resistance genes, allowing the possibility of using MAS to pyramid several resistance genes in a single pepper line.

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