Development of a CAPS marker for the Pvr4 locus: A tool for pyramiding potyvirus resistance genes in pepper

Carole Caranta, Arnaud Thabuis, and Alain Palloix

Abstract: The Pvr4 resistance gene in pepper confers a complete resistance to the three pathotypes of potato virus Y (PVY) and to pepper mottle virus (PepMoV). In order to use this gene in a marker-assisted selection (MAS) program and to permit the pyramiding of several potyvirus resistance genes in the same cultivar, tightly linked amplified fragment length polymorphism (AFLP) markers were obtained by the bulked segregant analysis method. Eight linked AFLP markers were mapped in an interval from 2.1 ± 0.8 to 13.8 ± 2.9 cM around this locus. The closest codominant AFLP marker was converted into a codominant CAPS (cleaved amplified polymorphic sequence) marker using data from the alignment of the two allele sequences. We have further characterized the relevance of the CAPS marker for MAS programs in different pepper breeding lines.

Key words: amplified fragment length polymorphism markers, bulked segregant analysis, Capsicum annuum, marker-assisted selection, potyvirus resistance.

Introduction

Potato Virus Y (PVY), the type member of the genus Potyvirus, is probably one of the most common viruses infecting pepper crops (Capsicum annuum L.). This aphid-transmitted virus occurs world wide, although it appears to be more frequent and more destructive in warmer areas (Green and Kim 1991). PVY isolates infecting pepper are highly variable in symptomatology (mosaic symptoms, vein banding, or foliar necrosis) and virulence. Their classification led to the definition of three pathotypes -0, -1, and -1,2, according to their virulence on pvr2+, pvr21, and pvr22 genotypes, respectively (Gebre-Selassié et al. 1985; Kyle and Palloix 1997). In France, Italy, and Spain, PVY isolates generally belong to the pathotype -0, but more virulent isolates were identified in most of the Southern Mediterranean countries (Luis Arteaga et al. 1993; Palloix et al. 1994) and in South America (Nagai 1989).

Owing to the losses caused by PVY infections and the difficulty to control the vector, much effort has been directed toward the identification of resistance sources (Cook and Anderson 1959). The pvr2 resistance system is effective against PVY-0, PVY-1, and also against common strains of tobacco etch virus (TEV) and is generally used by breeders. But, the occurrence of virulent PVY isolates motivates the use of other resistance genes. Among the seven potyvirus resistance genes (named pvr genes) described in pepper (for review, see Kyle and Palloix 1997), only one locus named Pvr4 has been shown to control all PVY pathotypes. This dominant gene from the Mexican C. annuum line, Criollo de Morelos 334, also controls a complete resistance to pepper mottle virus (PepMoV), a potyvirus restricted to the American continent (Dogimont et al. 1996).

The aim of the present study was to tag the Pvr4 locus using bulked segregant analysis and amplified fragment length polymorphism (AFLP) markers (Vos et al. 1995). To facili-
tate marker-assisted selection (MAS) and to permit the pyramiding of several pvr genes in the same cultivar, one of the closest AFLP markers linked to Pvr4 was transformed into a codominant PCR-based marker (i.e., cleaved amplified polymorphic sequence (CAPS) marker, Konieczny and Ausubel 1993). This marker was used to survey a set of pepper breeding lines for PCR polymorphism. It was proved to be usable for Pvr4 introgression in a large set of recipient genomes.

Materials and methods

Plant material and virus isolate

An F2 progeny of 151 plants was developed from the cross between the susceptible C. annuum L. cv. ‘Yolo Wonder’ (YW) and the potyvirus-resistant C. annuum accession Criollo de Morelos 334 (CM334) originating from Mexico. The 151 F2 plants were maintained in an insect-proof tunnel for DNA extractions and F3 progeny generation. The following pepper lines were checked for PCR polymorphism: 'H3', 'Vania', 'Perennial', 'Yolo Wonder', 'Yolo Y', 'CM334', 'Florida VR2', 'Lamu', 'Novi 3', 'SC81', 'Vat', PEI22719, 'Doux long des Landes', PEI201234, and 10 breeding lines supplied by different private companies. Among these lines, only 'CM334' was known to possess the Pvr4 locus, and is resistant to the three PVY pathotypes.

PVY isolate Son41 (isolated from Solanum nigrum in France, 1972) classified as pathotype -1,2 (Gebre-Selassie et al. 1985) was completely resistant to PVY -1,2 (thus Pvr4(+)Pvr4(_)), or heterozygous (Pvr4(+)Pvr4(_)) with an error risk \( \alpha = 7.5 \times 10^{-8} (\alpha = (3/4)\alpha + (1/4)\alpha \) with \( n = 25 \)).

AFLP analysis

Total DNA was extracted from approximately 1 g of fresh young leaves of F2 plants as described in Lefebvre et al. (1995) and Caranta et al. (1996). DNA samples from seven F2 plants that generated F3 families completely susceptible to PVY-1,2 (thus Pvr4(+)Pvr4(_)) and 20 F2 plants that generated F3 families completely resistant to PVY-1,2 (thus Pvr4(+)Pvr4(_)) were pooled for the bulked segregant analysis (Michelmore et al. 1991). For AFLP mapping, 250 ng of genomic DNA from the parental line, the two pools, and from individual F2 plants were digested with 2.5 U of the restriction enzymes MseI and EcoRI (Gibco-BRL) in a total volume of 20 \( \mu L \). The ligation of adapters was performed essentially as described by Vos et al. (1995). MseI (+3 selective nucleotides) and EcoRI (+3 selective nucleotides) primers were provided by Keygene N.V. (Wageningen, The Netherlands). EcoRI primers were labelled with \( ^{32}P \)-pyruvate using T4 polynucleotide kinase (Pharmacia, 0.24 U/\( \mu L \)). All amplification reactions were performed in a 9600 thermal cycler (Perkin-Elmer Cetus) with the conditions described by Vos et al. (1995). Amplification products were denatured and resolved by electrophoresis on a 4.5% polyacrylamide, 7.5 M urea, 0.5x TBE buffer gel at 130 W for 2.5 h in 1x TBE running buffer, 1.5 M NaAc bottom buffer. After electrophoresis, the gel was transferred to Whatman 3MM paper, dried 1.5 h, and exposed to X-ray film for 5 days.

CAPS design and analysis

The two alleles of the codominant AFLP marker E41/M49-645 (i.e., one 631-bp fragment from ‘Yolo Wonder’ and one 645-bp from ‘CM334’) were cloned and sequenced according to Meskem et al. (1995) in order to design specific primers for PCR. Polymorphic AFLP fragments were cut from the dried sequencing gel and rehydrated in TE buffer. To check that the correct fragments were eluted, an aliquot was reamplified with the primers used for AFLP reaction (E41 and M49) and the size of the reamplification product was compared with the expected size. Cloning was carried out in a TA vector (pCR2.1, Invitrogen, Netherlands). To check that the cloned DNA was the same size as the original AFLP fragment, an AFLP reaction was performed on the plasmid DNA. Three independent clones for the ‘Yolo Wonder’ fragment and one clone for the CM334 fragment were sequenced (Genome Express, Grenoble, France).

Specific primers of 15 nucleotides were defined using the oligo 4.0 software and synthesized by Oligo Express (Paris, France). Specific PCR amplification was performed in a total volume of 25 \( \mu L \) containing 30 ng of genomic DNA, Promega 1x buffer, 2 mM MgCl2 (Promega), 0.2 mM of each nucleotide, 0.15 \( \mu \)mol each of primers CSO-F (5'-CGAAGAGAGGATCC-3') and CSO-R (5'-TCGAGCTCTAGGTATTTATG-3') and 0.2 U of Taq polymerase (Promega). These primers led to the amplification of a 458-bp fragment in both ‘Yolo Wonder’ and ‘CM334’ using the following amplification program: 1 min at 93°C then 35 cycles 45 s at 93°C, 1 min at 47°C, and 2 min at 72°C.

Cleavage of the amplified fragment was performed at 37°C for 2 h using the following conditions: 15 \( \mu L \) of the PCR amplification, 1x buffer 2 (Gibco–BRL), 0.4 mM of spermidine, and 5 U of the restriction enzyme AluNI (Gibco–BRL) in a final volume of 20 \( \mu L \). Products were separated on a 2% agarose gel in 1x TAE buffer.

Linkage analysis

Marker order and genetic distances were estimated using MAPMAKER software v. 3.0 (Lander et al. 1987). Distances between markers were presented in Kosambi centimorgans (cM, Kosambi 1944).

Results

Pvr4 tagging using bulked segregant analysis and AFLP markers

As expected with previous results (Dogimont et al. 1996), the observed ratio (41 homozygous susceptible, 58 heterozygous resistant, 36 homozygous resistant, and 16 nongenotyped F3, because of germination problems) for resistance to PVY-1,2 in the F2 progeny ‘Yolo Wonder’ × ‘CM334’ fits with the segregation of a dominant gene in a completely classified F2 progeny (theoretical ratio 1:2:1, \( P = 21.8\% \)) with a slight deficiency of heterozygous F2 plants.

To identify markers linked to Pvr4, we used AFLP technology (Vos et al. 1995) in combination with a bulked segregant analysis (Michelmore et al. 1991) using DNA pools of 20 homozygous-resistant plants and 7 homozygous-susceptible plants from the F2 progeny. Five EcoRI–MseI primer combinations out of 145 enabled us to identify eight AFLP markers as polymorphic between the two DNA pools. The five combinations were tested on the 151 F2 plants and
led to the detection of eight markers linked to \textit{Pvr4} (Table 1). These markers span a pepper genomic region of 20.0 cM. The resistance locus was flanked on both sides by AFLP markers, and the closest markers were located 2.1 cM from \textit{Pvr4} (Fig. 1).

Table 1. Characteristics of the AFLP markers linked to \textit{Pvr4} and detected using bulked segregant analysis.

<table>
<thead>
<tr>
<th>Primer combination</th>
<th>Sequence</th>
<th>Size</th>
<th>Phase</th>
<th>Distance from \textit{Pvr4} (± s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E33–M54</td>
<td>E-AAG/M-CCT</td>
<td>126</td>
<td>‘CM334’</td>
<td>6.2 ± 2.0</td>
</tr>
<tr>
<td>E38–M61</td>
<td>E- ACT/M-CTG</td>
<td>403</td>
<td>‘CM334’</td>
<td>2.1 ± 1.2</td>
</tr>
<tr>
<td>E41–M49</td>
<td>E-AGG/M-CAG</td>
<td>296</td>
<td>‘CM334’</td>
<td>2.1 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>645/631</td>
<td></td>
<td>‘Yolo Wonder’</td>
<td>9.0 ± 2.4</td>
</tr>
<tr>
<td>E41–M54</td>
<td>E-AGG/M-CCT</td>
<td>138</td>
<td>‘CM334’</td>
<td>2.1 ± 1.2</td>
</tr>
<tr>
<td>E41–M55</td>
<td>E-AGG/M- CGA</td>
<td>102</td>
<td>‘CM334’</td>
<td>2.1 ± 1.2</td>
</tr>
</tbody>
</table>

\textit{E}. \textit{EcoRI}; \textit{M}. \textit{MseI}.

\textit{b} Size of the polymorphic fragments are indicated in base pairs.

\textit{c} Indicates the origin of the polymorphic AFLP fragment.

\textit{d} Distances in cM (± standard error) and calculated using the Kosambi function.

Transformation of E41/M49-645 into CAPS marker

In order to develop markers easy to use in MAS programs, the two alleles of the codominant marker E41/M49-645 (one from ‘Yolo Wonder’ and the other from ‘CM334’, Fig. 2A) were cloned and sequenced. The two sequences were aligned. The restriction sites (\textit{EcoRI} and \textit{MseI}), the two adapter sequences and the deletion responsible for the molecular weight difference between the two alleles were identified. The 14-bp deletion was localized at the 3’ end of the amplified fragment just adjacent to the \textit{EcoRI} adapter, making it impossible to define specific primers flanking the deletion. Finally, two specific primers flanking a unique restriction site specific from the ‘CM334’ allele (revealed by the restriction enzyme \textit{AlwNI}) were defined. These primers did amplify a 458-bp fragment both in the ‘Yolo Wonder’ and ‘CM334’ pepper lines. To detect a codominant \textit{Pvr4}-associated sequence polymorphism, the PCR fragments were digested with \textit{AlwNI} (Fig. 2B) and led to the identification of a CAPS marker (Konieczny and Ausubel 1993).

PCR analysis of DNA from individual \textit{F2} progeny (151 plants) revealed that the CAPS marker cosegregated with the originating AFLP marker E41–M49–645 (Fig. 2). The distance between the CAPS marker and \textit{Pvr4} was estimated at 2.1 ± 0.8 cM. The complete cosegregation confirmed that the region amplified was homologous to that found in the AFLP products amplified using primers E41 and M49. The CAPS marker, as did the originating AFLP, allows one to distinguish homozygous-resistant, heterozygous-resistant, and homozygous-susceptible \textit{F2} plants.

Evaluation of the CAPS polymorphism in a set of pepper breeding lines

To assess whether the CAPS marker might be present in genotypes that do not possess the \textit{Pvr4} resistance allele, and thus decrease the interest of this marker in MAS programs, a set of 23 pepper breeding lines susceptible to PVY-1,2 was checked for CAPS polymorphism. After \textit{AlwNI} restriction of the amplification product, all the pepper lines without the \textit{Pvr4} allele presented the CAPS pattern associated with susceptibility to PVY-1,2. The restriction site appears to be specific to ‘CM334’ and consequently, the CAPS marker is useful to assist \textit{Pvr4} introgression into several susceptible backgrounds.

Discussion

Bulked segregant analysis in combination with AFLP marker technology allowed us to rapidly find markers linked to the \textit{Pvr4} locus involved in complete resistance to all known pathotypes of PVY and PepMoV. A genomic region of 20 cM around \textit{Pvr4} was spanned with eight AFLP markers. In a previous study, we attempted to tag the \textit{Pvr4} locus using the same strategy (i.e., bulked segregant analysis) but in combination with RAPD markers. More than 300 RAPD primers were tested on the same bulks but never amplified any polymorphisms between the bulks (unpublished results). AFLP markers were more efficient than RAPDs, probably...
because the number of loci screened using a single AFLP primer combination is much higher than using one RAPD primer combination (in pepper, an average of 85 loci per AFLP primer combination vs. 5 per RAPD primer combination, J.C. Chauvet, personal communication). Both markers were shown not to be uniformly distributed on the genome: more than 50% are clustered in centromeric regions of pepper chromosomes (Caranta et al. 1997a; V. Lefebvre, personal communication). However, this disadvantage for gene tagging is compensated in the case of AFLPs by the high number of loci revealed per primer combination.

The codominant AFLP marker E41/M49-645 located 2.1 cM from Pvr4 was transformed into a CAPS. CAPS markers are more reproducible and easier to manipulate in MAS programs. We chose this marker because of its codominant nature; indeed obtaining a codominant PCR-specific marker using the sequence alignment of the two alleles is easier because of the information obtained about both alleles. Our survey for CAPS polymorphism on a set of pepper breeding lines suggests that the CAPS generated in this study is specific from the Pvr4-genomic region and thus can be used in many susceptible genetic backgrounds.

Breeding for potyvirus resistance is a major challenge of pepper breeders. Although numerous pvr genes control more than one potyvirus (for example pvr2-2 for PVY pathotype -0 and -1 and common strains of TEV and Pvr4 for both PVY and PepMoV), the diversity of potyviruses infecting pepper in several major production areas (TEV, PepMoV, and PVY in America, PVY and PepMoV in Africa, or CVMV in South East Asia) requires the association of several potyvirus resistance genes. In some cases, combination of pvr genes led to an unexpected broadening of the spectrum of Fig. 2. DNA from the parents, the bulks, and the segregating F2 individuals scored for the AFLP marker E41–M49-645 (A) and for the corresponding CAPS marker (B). The AFLP and CAPS markers are indicated by arrows. Lane CM, resistant parent 'CM334'; lane YW, susceptible parent 'Yolo Wonder'; lane B1, resistant F2 bulk; lane B2, susceptible F2 bulk; and lanes 1–16 represent segregating F2 individuals. At the bottom of each AFLP lane, the genotype of the corresponding F2 plants are indicated, as checked in the F3 families: S, homozygous susceptible; R, homozygous resistant; and Ht, heterozygous resistant.
action against potyviruses. We have recently demonstrated that a complementation between the \textit{pvr2} (\textit{pvr2}1 or \textit{pvr2}2) and the \textit{pvr6} locus confers a new and complete resistance to pepper veinal mottle potyvirus (PVMV), one of the most damaging pepper diseases in West Africa, whereas both genes originate from PVMV-susceptible pepper lines (Caranta et al. 1996). The pyramiding of \textit{pvr} genes based on the complementarity for their spectrum of action is presently underway to control the main potyviruses in tropical countries (Palloix et al. 1998). Moreover, pyramiding of \textit{pvr} genes can also be a way to avoid the breakdown of potyvirus resistance. We hypothesize that the combination of \textit{pvr} genes with distinct modes of action can increase the durability of the resistance. For example, the combination of a locus like \textit{Pvr4} that controls an inhibition of PVY and PepMoV replication in host cells (Caranta et al. 1998) with a locus involved in restriction of cell-to-cell movement (e.g., \textit{pvr3} against PVY, Arroyo et al. 1996) or long distance movement of the virus (e.g., \textit{pvr3} against PepMoV, Murphy and Kyle 1995) should strengthen the resistance to PVY and PepMoV.

Combination of several \textit{pvr} genes can, however, be very difficult or impossible using phenotypic screening because of the masking effect of genes and (or) the close interaction between distinct potyviruses inoculated to the same plant (Marchoux et al. 1993). When a breeding line already has a gene, for example \textit{Pvr4}, which controls resistance to all PVY pathotypes, it cannot be distinguished from other lines with \textit{Pvr4} plus other genes involved in PVY resistance except if DNA markers are available for each resistance gene. Up to now, only PCR markers were available for the \textit{pvr2} locus (Caranta et al. 1997b). In this study, we presented the development of markers for the \textit{Pvr4} locus. Work is also underway for other major genes like \textit{pvr5}, and also for quantitative trait loci involved in broad-spectrum potyvirus resistance.

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References


