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Allele-specific CAPS markers based on point mutations in resistance alleles at the *pvr1* locus encoding eIF4E in *Capsicum*

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Abstract Marker-assisted selection has been widely implemented in crop breeding and can be especially useful in cases where the traits of interest show recessive or polygenic inheritance and/or are difficult or impossible to select directly. Most indirect selection is based on DNA polymorphism linked to the target trait, resulting in error when the polymorphism recombines away from the mutation responsible for the trait and/or when the linkage between the mutation and the polymorphism is not conserved in all relevant genetic backgrounds. In this paper, we report the generation and use of molecular markers that define loci for selection using cleaved amplified polymorphic sequences (CAPS). These CAPS markers are based on nucleotide polymorphisms in the resistance gene that are perfectly correlated with disease resistance, the trait of interest. As a consequence, the possibility that the marker will not be linked to the trait in all backgrounds or that the marker will recombine away from the trait is eliminated. We have generated CAPS markers for three recessive viral resistance alleles used widely in pepper breeding, *pvr1*, *pvr1*^{1}, and *pvr1*^{2}. These markers are based on single nucleotide polymorphisms (SNPs) within the coding region of the *pvr1* locus encoding an eIF4E homolog on chromosome 3. These three markers define a system of indirect selection for potyvirus resistance in *Capsicum* based on genomic sequence. We demonstrate the utility of this marker system using commercially significant germplasm representing two *Capsicum* species. Application of these markers to *Capsicum* improvement is discussed.

Keywords Pepper · Potyvirus · eIF4E · Disease resistance · Marker-assisted selection

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Introduction

Marker-assisted selection (MAS) is an increasingly important tool in modern plant breeding programs. Indirect selection using molecular genotyping methods enables detection of desired alleles and haplotypes early in the plant life cycle and early in breeding line development and can reduce or eliminate the need for cycles of phenotypic assessment (Dubcovsky 2004; Frey et al. 2004). MAS is even more valuable when the trait is recessively inherited, polygenic or shows low heritability because selection based on phenotype is unclear or impossible. Using a co-dominant marker system, MAS eliminates the need for progeny testing, to identify desired genotypes carrying recessive alleles. In this paper, we report the development of a co-dominant PCR-based marker system for the selection of a series of recessive alleles for virus resistance in *Capsicum* using cleaved amplified polymorphic sequences (CAPS) analysis based on the SNPs implicated as causal for the phenotype of interest.

Cleaved amplified polymorphic sequences markers allow the detection of single nucleotide polymorphisms using polymerase chain reaction (PCR) and restriction endonuclease (Jarvis et al. 1994; Konieczny and Ausubel 1993; Michaels and Amasino 1998). The CAPS technique is a preferred marker system for MAS when SNP information is available. However, the development of CAPS markers is only possible where mutations disrupt or create a restriction enzyme recognition site. Derived-CAPS (dCAPS) markers eliminate the problems related with CAPS markers by generating mismatches in a PCR

primer, which are subsequently used to create a polymorphism based on the target mutation (Michaels and Amasino 1998; Neff et al. 1998).

Potyviruses, single stranded RNA plant viruses belonging to the Potyviridae family, are very destructive in agriculture and infect a wide range of hosts that include monocots and dicots. Potyvirus infection requires host factors for replication and subsequent systemic spread through the plant (Carrington et al. 1996). A deleted or defective host protein that is essential for viral infection but is dispensable for the host may therefore define a potyviral resistance factor. Genes showing recessive inheritance typically confer this type of resistance due to the fact that resistance occurs when a necessary host factor is impaired with respect to its function in viral infection or is absent (Kang et al. 2005b).

Recessive resistance is especially prevalent in potyviruses, comprising approximately 40% of all known potyviral resistance genes (Provvidenti and Hampton 1992). Several host genes whose mutations impair the infection cycle of plant viruses have been characterized, particularly in Arabidopsis (Kang et al. 2005b). The translation initiation factor *eIF4E* has been identified repeatedly in diverse hosts as a naturally occurring recessively inherited resistance locus. Mutations in this gene have been shown to produce resistance to potyviruses in several plant species including pepper at the *pvr1* locus recently shown to be identical to the *pvr2* locus (Kang et al. 2005a, Ruffel et al. 2002), lettuce at the *mo1* locus (Nicaise et al. 2003), and pea at the *sbm1* locus (Borgstrom and Johansen 2001; Gao et al. 2004). Recently, *eIF4E* was implicated in barley as *rym4/5* (Pellio et al. 2005; Stein et al. 2005; Wicker et al. 2005). The negative effects on the infectivity of various potyviral plant pathogens in diverse hosts of mutations resulting in amino acid substitutions in eIF4E imply that the requirement for eIF4E in potyviral infection is probably widely conserved.

In pepper, a series of resistance genes have been reported to confer resistance to several *Potyvirus* species including *Tobacco etch virus* (TEV), *Potato Y virus* (PVY), and *Pepper mottle virus* (PepMoV) (Watterson 1993). Beginning in the 1950 s, these loci were given various names based on the viruses and hosts involved (summarized in Greenleaf 1986). This nomenclature system, however, proved unwieldy due to mis-identification and reclassification of viral isolates and host taxa. In addition, the fact that several resistance genes appear to control viral isolates belonging to more than one viral species has resulted in multiple loci designations for true alleles. In 1997, according to a new nomenclature strategy for this system, each locus identified to date was re-designated *pvr* for potyvirus resistance, followed by a number reflecting the sequence in which it was reported (Kyle and Palloix 1997; Caranta et al. 1997). Each *pvr* allele shows a characteristic spectrum of resistance, controlling distinctive sets of viral pathotypes or strains (Greenleaf 1986; Murphy et al. 1998).

The *pvr1* locus was first described in relation to *Tobacco etch virus* (Greenleaf 1956) and has subsequently been mapped to chromosome 3 (Murphy et al. 1998). It is now known that the allele *pvr1* confers resistance against TEV-HAT, PVY (0), and PepMoV. In contrast to this relatively broad resistance spectrum, another gene eventually designated $pvr2^1$ conferred resistance to PVY (0) only (Caranta et al. 1997; Murphy et al. 1998). A third gene, originally designated $pvr2^2$, was allelic with $pvr2^1$, and conferred resistance to TEV-HAT and PVY (0, 1) (Greenleaf 1986). Based on this difference in resistance spectrum, it was incorrectly assumed that *pvr1* and *pvr2* defined distinct loci. Recently, however, genetic complementation analysis demonstrated allelism of *pvr1* and $pvr2^2$ (Kang et al. 2005a). Based upon this result and the guidelines for potyviral resistance gene nomenclature in *Capsicum* (Kyle and Palloix, 1997), $pvr2^1$ and $pvr2^2$ have been redesigned $pvr1^1$ and $pvr1^2$, respectively (Kang et al. 2005a). A third gene, *pvr5*, which shows linkage with *pvr1*, was designated for resistance to PVY (0) (Chaine-Dogimont et al. 1996). However, the absence of allelism data implies that it is possible that *pvr5* is also an allele at the *pvr1* locus.

The identity of the gene encoded at the locus *pvr1* was determined to be a homolog of the eukaryotic translation initiation factor *eIF4E* using the candidate gene approach (Kang et al. 2005a; Ruffel et al. 2002). Structural analysis using genomic sequence of the *eIF4E* gene in pepper showed that the *eIF4E* gene encoded by the *pvr1* locus consists of five exons and four introns (Ruffel et al. 2004). Sequence information for *eIF4E* encoded by the $Pvr1^+$, *pvr1*, $pvr1^1$, or $pvr1^2$ alleles showed that each resistance allele contained two to four nucleotide substitutions within the 687 bp coding sequence (Kang et al. 2005a). All these point mutations resulted in non-conservative amino acid substitutions in the respective predicted proteins.

To identify recessive resistance alleles at the *pvr1* locus in a breeding program, multiple screening with distinct isolates of several potyviruses must be performed. In this system, a reliable molecular marker system would substantially reduce the amount of time and effort involved in identifying these alleles. This paper describes the development of CAPS markers based on the SNPs within the coding region that are presumably responsible for resistance. These PCR-based markers can distinguish each known recessive resistance allele at the *pvr1* locus from each other and from the susceptible allele.

Materials and methods

Plant and virus materials

Capsicum annuum 'NuMex RNaky' (RN), 'Early Cal Wonder' (ECW), and 'Yolo Y' (YY) were obtained from Asgrow Seed Co. (San Juan Bautista, CA, USA). *C. annuum* 'Dempsey' (DEMP) was provided by M. Deom, University of Georgia (Athens, GA, USA); *C. annuum*

'Jupiter' (JP) was provided by Syngenta Seeds (Naples, FL, USA); *Capsicum chinense* PI 159234 (234), was obtained from the USDA Southern Regional Plant Introduction Station (Experiment, GA, USA); and *C. chinense* 'Habanero' (HAB) was obtained from Tomato Growers Supply Co. (Fort Myers, FL, USA). TEV-HAT cultures were obtained from T. Pirone, University of Kentucky (Lexington, KY, USA). PepMoV-FL and PVY (0) cultures were obtained from J.F. Murphy, Auburn University (Auburn, AL, USA). All viral cultures, were maintained on TMV-resistant *Nicotiana tabacum* 'Kentucky 14' and were transferred every 4–6 weeks. To test the applicability of allele-specific CAPS markers, 23 pepper lines from two breeding programs, eight genotypes from Cornell University (Ithaca, NY, USA) and 15 breeding lines from Enza Zaden (Enkhuizen, The Netherlands), were evaluated.

Virus screening procedures

All genotypes tested were sown in Cornell soilless planting mix in the greenhouse. Routine insect control procedures were followed to ensure absence of insect viral vectors. Seedlings were mechanically inoculated with virus at the five to six leaves stage of development. After lightly dusting with carborundum, the first and second true leaves (leaves 1 and 2) were mechanically inoculated with virus. Inoculum consisted of systemically infected tobacco tissue containing TEV-HAT, PVY (0), or PepMoV-FL ground in 50 mM potassium phosphate buffer, pH 7.5 (1 g tissue: 20 ml buffer). For each virus, eight to ten plants per genotype were inoculated, and four plants for each test were mock inoculated with buffer as controls. Plants were monitored daily for the presence and severity of viral symptoms. Leaf tissue was tested for the presence of viral antigen using indirect enzyme-linked immunosorbent assay (ELISA) as described by Murphy et al. (1998). Anti-viral immunoglobulins were obtained from Agdia Inc. (Elkhart, IN, USA) and used according to the manufacturer's instructions. Virus accumulation in inoculated leaves was tested 10 days post-inoculation (dpi) and in uninoculated leaves at 21 dpi. For each test, ELISA readings were made every 20 min using a Tecan Sunrise microtiter plate reader at 405 nm (Tecan Sunrise, Sunnyville, CA, USA). A sample was considered susceptible for virus when the ELISA absorbance value was greater than the threshold determined from the mean absorbance value of healthy control samples of each genotype plus three standard deviations.

DNA extraction

Total nucleic acid was extracted from freshly harvested leaves of each cultivar (Kang et al. 2001). The tissue (2–3 g) was ground in liquid nitrogen, and then incubated at 60°C for 45 min with gentle agitation in 25 ml of extraction buffer [50 mM Tris–HCl pH 7.5, 1.4 M NaCl, 20 mM EDTA pH 8.0, 0.5% SDS, 1% polyvinylpyrrolidone (insoluble), and 1% β-mercaptoethanol]. Chloroform (25 ml) was added to the mixture and homogenized for 30 min. The homogenate was centrifuged at 7,000 *g* for 10 min and the nucleic acid precipitated from the supernatant with ethanol was treated with RNase A. DNA concentration was adjusted to 0.1 μg/μl for PCR amplification.

Genome-walking and alignment of genomic sequence for *eIF4E* intron1

To obtain the sequence of *eIF4E* intron1, genome walking was performed using gene-specific primers and universal primers included in the Genome-Walking Kit following manufacturer's instructions (Clontech, Palo Alto, CA, USA). Gene-specific primers designed for genome walking are listed in Table 1. Gene-specific primers, Intron1F1 and Intron1F2 were used for the first walk; Intron1F3 and Intron1F4 were used for the second walk. The remainder of intron1 was spanned using primers, Intron1F5 and Intron1R1. The PCR products were gel purified using the Qiaquick gel purification kit (Qiagen, Valencia, CA, USA), and cloned into pGEM-T using the pGEM-T Easy kit (Promega, Madison, WI, USA). All the sequencing was performed by Biotechnology Resource Center, Cornell University (www.brc.cornell.edu) using the automated sequencer. A contig was created that spanned the *eIF4E* locus using overlapping PCR clones with Seqman software (DNASTAR Inc., Madison, WI, USA).

Table 1 Primer sequences used in this study

Primer name	Primer sequence
Intron1F1	5′-AGCTGAAATGGAGAAAACGACGA-3′
Intron1F2	5′-TTTGATGAAGCTGAGAAGGTGAAATTG-3′
Intron1F3	5′-CGGCTTGAAAGTTCAGTTCGTCAAC-3′
Intron1F4	5′-GCCAACTAACTTAGCTACTAAGATTTTCAG-3′
Intron1F5	5′-GCCATTCGACTAATCCTCAGCAAC-3′
Intron1R1	5′-CCTCCATTGGCACATACAGGATCT-3′
Pvr1-S F	5′-GCTAATGAGGCAGATGATGAAGTTG-3′
Pvr1-S R	5′-CAACCATAAATATACCCCGAGAAT-3′
Pvr1-R2 F	5′-GGGCTAAAATACGCTCATCTCCCTTC-3′
Pvr1-R2 R	5′-GGCTCAATTTTATGCTTGAAACAATGTAAGC-3′

Analysis of CAPS markers

PCR primers for allele-specific CAPS markers were generated using the sequences of exon1, exon2, and intron1 at the *Capsicum pvr1* locus (Genbank accession no. DQ066647). The CAPS differential cleavage sites were based on SNPs within the coding region of the $Pvr1^+$, *pvr1*, $pvr1^1$, and $pvr1^2$ alleles; restriction endonucleases were selected according to the CAPS differential cleavage sites. PCR was carried out in a 25 µl volume containing 50 ng genomic *Capsicum* DNA, 50 mM KCl, 10 mM Tris–HCl, 2 mM $MgCl_2$, 0.2 mM each dNTP, 0.4 µM each forward primer and reverse primer, and 1 unit *Taq* polymerase (Roche, Indianapolis, IN, USA). PCR was performed using a MJ research thermocycler model PTC-225 (MJ Research, Ramsey, MN, USA). Cycling conditions were 95°C 5 m, (95°C 1 m, 59°C 1 m, 72°C 1 m) × 30 cycles, 72°C 10 m. Aliquots of 10 µl PCR product were digested with 2.5 units of the appropriate restriction endonuclease and 2.5 µl, 10× reaction buffer provided by the manufacturer (total volume 25 µl) for 2 h at the temperature recommended by the manufacturer. The restriction endonucleases used for each CAPS marker were *Fnu*4HI (New England Biolabs, Beverly, MA, USA), *Hin*dIII (Roche), and *Bsr*I (New England Biolabs). After digestion, 10 µl of the reaction buffer was analyzed by gel electrophoresis on 2.2% agarose gel in 1× TAE buffer.

Results

Evaluation of the resistance spectra of *pvr1*, $pvr1^1$, or $pvr1^2$ alleles in *Capsicum*

To confirm the reported resistance spectra and extend this analysis to all the viral isolates and resistance alleles used in this study, pepper varieties or lines known to be homozygous for each *pvr1* allele were screened with TEV-HAT, PepMoV-FL, and PVY (0). The response to the virus was determined visually, symptoms were recorded and responses were confirmed by ELISA 2 weeks post-inoculation. $Pvr1^+/Pvr1^+$ genotypes were used as susceptible controls. A sample was considered susceptible when the ELISA absorbance value was greater than the threshold determined from the mean absorbance value of healthy samples plus three standard deviations. Our results confirmed that the three recessive resistance alleles at the *pvr1* locus displayed different resistance spectra when screened with TEV-HAT, PVY (0), and PepMoV-FL. The resistance spectra of these alleles are summarized in Table 2. The *pvr1*/*pvr1* genotype showed resistance to TEV-HAT, PVY (0), and PepMoV-FL; the $pvr1^1/pvr1^1$ genotype was resistant to only PVY (0); the $pvr1^2/pvr1^2$ genotype was resistant to TEV-HAT and PVY (0), but developed infection with PepMoV. However, delayed susceptibility was observed in $pvr1^1/pvr1^1$ genotypes infected with TEV-HAT and $pvr1^1/pvr1^1$ and $pvr1^2/pvr1^2$ genotypes infected with PepMoV-FL. These genotypes showed ELISA absorbance values, which were not greater than the threshold determined from the mean absorbance value of healthy samples plus three standard deviations at 3 dpi. Previous work has shown that during the early stages of TEV-HAT infection (3 dpi) in $pvr1^1/pvr1^1$ genotype low ELISA values were typically observed (Kang et al. 2005a). When susceptible $Pvr1^+/Pvr1^+$ genotypes were compared to $pvr1^1/pvr1^1$ and $pvr1^2/pvr1^2$ genotypes after inoculation with PepMoV-FL, much more severe symptoms were apparent, although all plants eventually became infected systemically (data not shown).

Table 2 Resistance spectra of *Capsicum pvr1* alleles after inoculation with three *Potyvirus* species, *Tobacco etch virus* (TEV-HAT), *Potato virus Y* (PVY(0)) and *Pepper mottle virus* (PepMoV-FL). ELISA was performed 2 weeks post-inoculation

Genotypes	Response to three potyvirus species		
	TEV-HAT	PVY(0)	PepMoV-FL
$Pvr1^+/Pvr1^+$	S	S	S
pvr1/*pvr1*	R	R	R
$pvr1^1/pvr1^1$	S*	R	S*
$pvr1^2/pvr1^2$	R	R	S*

S susceptible phenotype, *R* resistant phenotype, *S** susceptible phenotype showing delayed onset of mild symptoms

Sequence comparison between alleles at the *pvr1* locus

Previously, full-length coding sequences for *eIF4E* have been obtained for 13 *Capsicum* genotypes known to be homozygous for each of the four *pvr1* alleles; $Pvr1^+$, the allele for susceptibility, and the three resistance alleles, *pvr1*, $pvr1^1$ or $pvr1^2$ (Kang et al. 2005a). Each recessive resistance allele showed consistent, specific, and distinctive nucleotide substitutions within the 687 bp coding regions when compared with the dominant susceptible allele, $Pvr1^+$. To develop allele-specific CAPS markers based on these SNPs, we compared the positions and identified the point mutations (Table 3). The *pvr1* allele was defined by three substitutions relative to susceptible *C. annuum* $Pvr1^+/Pvr1^+$ genotypes: A to G at position 151 (abbreviated A151G), C196A, and G319A. The $pvr1^2$ allele carries three distinct substitutions relative to the susceptible allele, T200A, T236G, and G325A while $pvr1^1$ shared only T200A and T236G.

Sequence analysis of intron1 in *eIF4E*

The A151G and C196A substitutions in *pvr1* and the T200A and T236G substitutions in $pvr1^1$ and $pvr1^2$ were all located in exon1, while the G319A substitution in *pvr1* and the G325A substitution in $pvr1^2$ were located in exon2 (Table 3, Fig. 1), therefore, the sequence of intron1 was required to design CAPS markers. The genomic sequence of intron1 in *eIF4E* was obtained by

Table 3 Single nucleotide polymorphism (SNPs) within the coding region of four alleles at the *pvr1* locus upon which the allele-specific markers reported in this study are based

eIF4E allele	Location of mutated nucleotides in *eIF4E* alleles					
	151	196	200	236	319	325
$Pvr1^+$	A	C	T	T	G	G
pvr1	G[a]	A[a]	T	T	A	G
$pvr1^1$	A	C	A[a]	G	G	G
$pvr1^2$	A	C	A[a]	G	G	A[a]

[a]SNPs used for CAPS marker development

genome-walking and PCR using genomic DNA from *C. chinense* PI 159234 (*pvr1*/*pvr1*) (GenBank accession no. DQ066647). Two sets of genome walking were required to obtain the complete sequence of intron1 (3,577 bp), which was used to design primers for CAPS markers that allowed amplification of PCR products of the optimal size for analysis on agarose gel.

Primer design and restriction enzyme selection for CAPS markers

Three allele-specific CAPS markers, Pvr1-S, pvr1-R1, and pvr1-R2, were generated for recessive resistance alleles at the *pvr1* locus. The Pvr1-S marker is based on the C196A substitution in the *pvr1* allele and on the T200A in $pvr1^1$ and $pvr1^2$ alleles (Tables 3, 4). These nucleotide substitutions, C196A and T200A, eliminate the *Bsr*I restriction site ACTGGn, which existed only in $Pvr1^+$ allele. The pvr1-R1 marker uses the same primers as Pvr1-S and distinguishes *pvr1* from $Pvr1^+$, $pvr1^1$, and $pvr1^2$ based on the A151G substitution because *Fnu*4HI digests the sequence GCnGC found only in *pvr1* (Tables 3, 4). The single SNP specific to $pvr1^2$ allele (G325A) does not generate a restriction-site. To distinguish $pvr1^2$ allele from $Pvr1^+$, *pvr1*, and $pvr1^1$, we designed a dCAPS marker, pvr1-R2. The forward primer for pvr1-R2 is based on intron1 sequence; the reverse primer is based on the sequences of exon2. The reverse primer is a mismatch, which creates the *Hin*dIII recognition site (AAGCTT) only in the presence of the $pvr1^2$ allele (Tables 3, 4).

Allele-specific CAPS markers discriminate *pvr1*, $pvr1^1$, $pvr1^2$, and $Pvr1^+$ alleles

Results of the application of the marker system developed are described in Table 4 and Fig. 2.

The Pvr1-S marker distinguished $Pvr1^+$ from *pvr1*, $pvr1^1$, and $pvr1^2$. The $Pvr1^+$ genotype was indicated when the Pvr1-S marker gave the genotyping result, $r1^+$ (Table 4). After amplification of the 711 bp product using the Pvr1-S primer set, products were digested with *Bsr*I generating two fragments, 133 and 578 bp, only in $Pvr1^+/Pvr1^+$ genotypes. All $Pvr1^+/Pvr1^+$ genotypes tested, ECW, HAB, JP, and RN, a diverse panel representing two *Capsicum* species, showed these two fragments after restriction (Fig. 2a). In contrast, all *pvr1*/*pvr1*, $pvr1^1/pvr1^1$, and $pvr1^2/pvr1^2$ genotypes showed only the 711 bp band after *Bsr*I treatment.

The pvr1-R1 marker distinguished *pvr1* from $Pvr1^+$, $pvr1^1$, and $pvr1^2$. The *pvr1* genotype was indicated when the pvr1-R1 marker gave the genotyping result, r1 (Table 4). In DNA samples extracted from all genotypes known to be homozygous for the *pvr1* allele, digestion of the PCR product (556 bp) with *Fnu*4HI resulted in three fragments, 556, 86, and 69 bp. The two smaller fragments, 86 and 69 bp were visible, but could not be distinguished from each other in 2.2% agarose gel resulting in a smeared lower band in the *pvr1*/*pvr1* genotypes, *C. annuum* 5502 and *C. chinense* 234 (Fig. 2b). In contrast, amplification products from DNA samples extracted from *Capsicum* genotypes homozygous for the alleles, $Pvr1^+$, $pvr1^1$, and $pvr1^2$ showed the 556 bp fragment and a 155 bp restriction product (Fig. 2b). Although discrimination of the 86 and 69 bp fragments was impossible on 2.2% agarose gels, the presence and absence of 155 bp band clearly allowed the determination of genotype based on the pvr1-R1 marker.

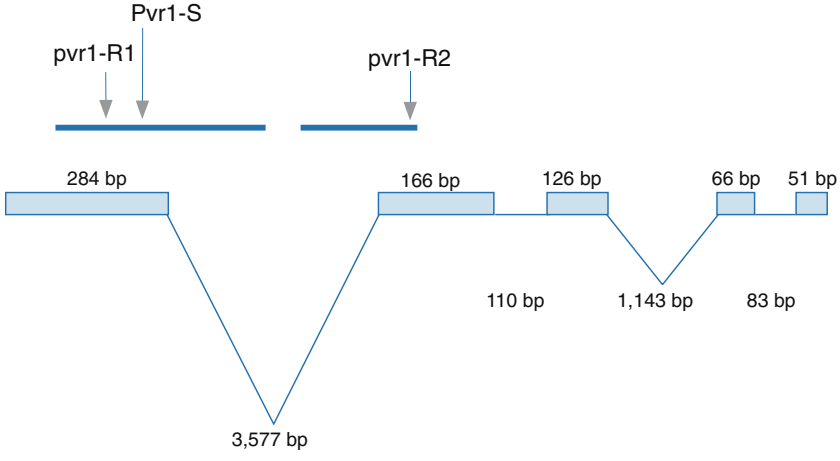

Fig. 1 The genomic structure of the *eIF4E* locus. *Boxes* represent exons, and the *horizontal lines* represent introns (Ruffel et al. 2002). Locations of sequence-based CAPS markers pvr1-R1, pvr1-R2, and Pvr1-S are depicted

Table 4 Primers and restriction enzymes used for *pvr1* allele-specific CAPS markers in *Capsicum*

Marker name	PCR product size (bp)	Restriction enzyme for SNPs detection	Fragments size after digestion (bp)	Expected[a] genotype	Genotyping code
Pvr1-S	711	*Bsr*I (ACTGGn)	133 + 578	$Pvr1^+/Pvr1^+$	$r1^+$
			711	Not $Pvr1^+/Pvr1^+$	$nr1^+$
pvr1-R1	711	*Fnu*4HI (GCnGC)	69 + 86 + 556	*pvr1*/*pvr1*	r1
			155 + 556	Not *pvr1*/*pvr1*	nr1
pvr1-R2	412	*Hin*dIII (AAGCTT)	32 + 380	$pvr1^2/pvr1^2$	$r1^2$
			412	Not $pvr1^2/pvr1^2$	$nr1^2$

[a] *Underlines* indicate letters used for genotyping codes

The dCAPS marker, pvr1-R2 distinguished genotypes homozygous for $pvr1^2$ from those carrying $Pvr1^+$, *pvr1*, or $pvr1^1$ (Fig. 2c). The $pvr1^2$ genotype could be determined when the pvr1-R2 marker gave the genotyping result, $r1^2$ (Table 4). The pvr1-R2 primer set produced a product of 412 bp in all genotypes, however *Hin*dIII digestion produced a 380 bp fragment only in the $pvr1^2$ genotype, DEMP (Fig. 2c). The difference in size caused by the mismatch primer (32 bp) allowed differentiation of $pvr1^2$ from the other genotypes using a 2.2% agarose gel, although the 32 bp fragment was not resolved in this system.

Evaluation of CAPS markers for use in plant breeding programs

Evaluation of the marker system described above was performed using DNA samples prepared from 15 – pepper-breeding lines derived from a commercial breeding program at Enza Zaden (Enkhuizen, The Netherlands) and in a public sector breeding program at Cornell University. DNA from the commercial breeding program was scored genotypically without knowledge of results from previous phenotypic evaluations. The genotyping results obtained for the pvr1-R1, pvr1-R2, and Pvr1-S markers are summarized in Table 5. Because the markers developed in this study are co-dominant, homozygosity in addition to allele identity was detected. All genotype determinations were based on the Pvr1-S, pvr1-R1, and pvr1-R2 marker system. The presence of the *pvr1* allele could be confirmed when the pvr1-R1 marker gave the genotyping result r1. The presence of the $pvr1^2$ allele was indicated when the pvr1-R2 marker gave the genotyping result $r1^2$. The presence of the $pvr1^1$ allele required coordinated genotyping results from all three markers as follows: result nr1 from pvr1-R1 marker, result $nr1^2$ from pvr1-R2 marker, and result $nr1^+$ from Pvr1-S marker. These scores indicate the $pvr1^1$ genotype as shown with ENZA13 and Crusader.

When genotype scores based on allele-specific CAPS markers were compared with predictions based on phenotypic assessment in a commercial setting, agreement was almost always observed (Table 5). One exception was noted in the case of the breeding line ENZA12. In this case, the $pvr1^1$ genotype was expected, but it genotyped as $pvr1^2$, prompting re-assessment of phenotypes.

In cases of Crusader and Cornago, which show resistance to PepMoV, these varieties were determined to be $pvr1^1/pvr1^1$ and $Pvr1^+/Pvr1^+$, respectively. We therefore conclude that resistance to PepMoV in the absence of the *pvr1* allele in both cases is a consequence of resistance conferred at other loci.

Discussion

In this paper, we report a marker system that successfully distinguished the allelic series at the potyvirus resistance locus *pvr1* in *Capsicum* that includes two alleles previously mis-designated $pvr2^1$ and $pvr2^2$. We have shown that a rapid and convenient PCR-based seedling screen reliably predicts genotype, which has previously been determined using lengthy and complex phenotypic evaluations of response to a series of viral pathotypes. The indirect selection provided by this method based on combined information from the allele-specific markers for the locus *pvr1* is a promising tool for efficient and cost-effective assessment of the potyviral resistant genotypes at this locus. Resistance alleles at this locus have been deployed globally in resistant varieties against potyviruses for more than 50 years; therefore, this tool is likely to be widely relevant in public and private sector pepper-breeding programs. The multi-allelic series at *pvr1* can only be determined by sequential screening with a range of viral species or isolates. Therefore, phenotype-based determinations in this system either require controlled inoculation of large parallel tests with each pathogen or screening of multiple generations. Molecular marker-based genotyping at the seedling stage represents a major improvement in selection, particularly if there is strong agreement with phenotype-based evaluations as demonstrated in this study.

Naturally existing SNPs, the most abundant and widely available type of DNA polymorphism throughout the genome when sequence information is available, provide an excellent foundation for molecular markers (Henikoff and Comai 2003; Mooney 2005; See et al. 2000; Syvanen 2001; Torjek et al. 2003). If the SNP is not responsible for the phenotype of interest, however, the risk of recombination or absence in some relevant genetic backgrounds is still present (Hinds et al. 2005; Mooney 2005; See et al. 2000; Thiel et al. 2004; Torjek et al. 2003). Detailed studies focusing on markers based on SNPs in the intragenic region of targeted genes

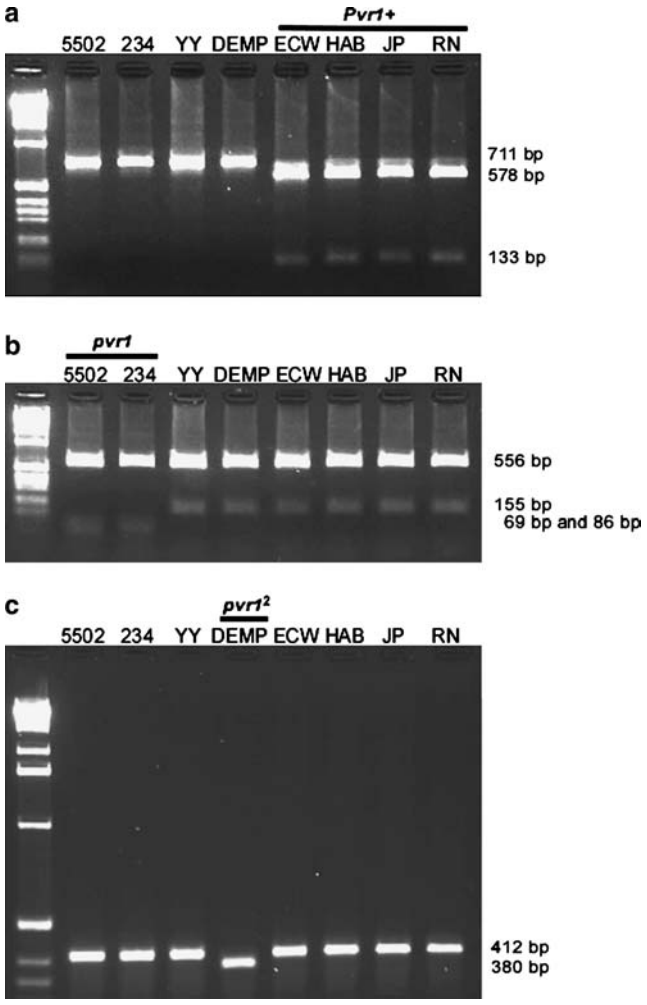

Fig. 2 Application of allele-specific CAPS markers for recessive resistance alleles at the *pvr1* locus. **a** DNA analysis of Pvr1-S marker. Pvr1-S marker can distinguish $Pvr1^+$ allele from *pvr1*, $pvr1^1$, and $pvr1^2$. *Bsr*1 digest generates 133 and 578 bp products for $Pvr1^+$ genotype and 711 bp product for the other genotypes. **b** DNA analysis of pvr1-R1 marker. A restriction enzyme, *Fnu*4HI, will recognize the sequence GCnGC, in only *pvr1*genotypes. The treatment with *Fnu*4HI generates 69, 86, and 556 bp products in *pvr1* genotype and 155 and 556 bp products in the other genotypes. **c** DNA analysis of pvr1-R2 marker. pvr1-R2 marker is a derived-CAPS (dCAPS) marker and can distinguish $pvr1^2$ allele from $Pvr1^+$, $pvr1^1$, and $pvr1^1$ alleles. Treatment with *Hind*III will generate 380 and 32 bp products only in $pvr1^2$ genotype. *C. annuum* 'NuMex RNaky' (RN), 'Jupiter' (JP), 'Early Cal Wonder' (ECW), and *C. chinense* 'Habanero' (HAB) were used as $Pvr1^+/Pvr1^+$ genotypes. *C. chinense* PI 159234 (234) and *C. annuum* '5502' were used as *pvr1*/*pvr1* genotypes. *C. annuum* 'YoloY' (YY) was used as $pvr1^1/pvr1^1$ genotype. *C. annuum* 'Dempsey' (DEMP) was used as $pvr1^2/pvr1^2$ genotype

including promoter, exons and introns have also been published. A single nucleotide length polymorphism (SNLP) marker was reported for the *Pi-ta* rice blast resistance gene based on the sequence information from the intron (Jia et al. 2004). The single-strand conformational polymorphism (SSCP)-SNP marker system developed in pearl millet detected SNPs located at the intron–exon borders (Bertin et al. 2005). Recently, another marker based on the SNP responsible for the trait of interest has also been reported in *Glycine max*. A single nucleotide amplified polymorphism (SNAP) marker for nodule autoregulation receptor kinase (*Gm*NAPK) was developed based on a SNP that results in an early stop codon (Kim et al. 2005).

At present, there is strong circumstantial evidence that the interaction between *eIF4E* and viral protein VPg is crucial for determining the outcome of potyvirus–host interactions. A recent paper (Kang et al. 2005a) showed that the mutations in eIF4E found in the *pvr1*, $pvr1^1$ and $pvr1^2$ resulted in gene products that failed to interact with the viral protein VPg. It is therefore likely that the CAPS markers reported in this study are based on biologically meaningful SNPs that are causal to the phenotypic difference of interest, in this case susceptibility or resistance. This type of marker will be more reliable and consistent than markers that rely on neutral polymorphism in non-coding DNA either within or outside the gene. Clearly markers based on coding sequences responsible for the trait of interest eliminate the possibility of a recombination event between the markers and the trait. Similar systems to that described in this paper will be straightforward to develop in the other crops for which eIF4E is involved, e.g., lettuce, pea, etc., and also perhaps tomato because of *pot1* (Parella et al. 2002; Ruffel et al. 2005).

One of the complexities of this system is the wide array of genetic resources spanning several species that are used in breeding programs to control these viruses in agriculture. The *pvr1* locus was the first, but is by no means the only locus involved in potyviral resistance in pepper (Kyle and Palloix 1997), demonstrated by the results obtained with the varieties Crusader and Cornago. Crusader and Cornago likely contain potyvirus resistance genes other than *pvr1* because they show resistance to PepMoV in the absence of the *pvr1* allele. These are thought to contain one or more of the resistance genes, *pvr3*, *Pvr4*, and/or *Pvr7*, which are unlinked to the *pvr1* locus and confer resistance to potyviruses, or they may contain other as yet unidentified resistance genes. Two dominant genes, *Pvr4* (a dominant allele from *C. annuum*) Criollo de Morelos 334 (CdM334) and *Pvr7* (*C. chinense* Jacq. PI159236), are tightly linked to each other (0.012–0.016 cM) but clearly distinct and located on pepper chromosome 10 (Chaine-Dogimont et al. 1996; Grube et al. 2000a, b). Both of these dominant resistance genes confer resistance to all PVY isolates and PepMoV (Chaine-Dogimont et al. 1996; Grube et al. 2000b). A recessive resistance gene called *pvr3*, first identified in *C. annuum* 'Avelar', confers resistance against PepMoV and is not linked with other known *pvr* loci (Murphy et al. 1998; Zitter and Cook 1973). Further molecular marker development for these *pvr* loci will provide more complete and accurate genotype evaluation.

It is evident that using these allele-specific CAPS markers for *pvr1* locus will reduce time, cost and considerable effort and will likely improve accuracy. The

Table 5 Genotyping and phenotyping results using homozygous breeding lines provided by Enza Zaden

Line number	Phenotyping				CAPS screening			
	PepMoV	PVY 0	TEV	Expected genotype based on phenotype	Genotyping codes			Determined genotype
					Pvr1-S	pvr1-R1	pvr1-R2	
ENZA1	NA	R	R	*pvr1*	$nr1^+$	r1	$nr1^2$	*pvr1*
ENZA2	NA	R	R	*pvr1*	$nr1^+$	r1	$nr1^2$	*pvr1*
ENZA3	S	R	R	$pvr1^1$ or $pvr1^2$	$nr1^+$	nr1	$r1^2$	$pvr1^2$
ENZA4	S	R	R	$pvr1^1$ or $pvr1^2$	$nr1^+$	nr1	$r1^2$	$pvr1^2$
ENZA5	S	R	R	$pvr1^1$ or $pvr1^2$	$nr1^+$	nr1	$r1^2$	$pvr1^2$
ENZA6	S	R	R	$pvr1^1$ or $pvr1^2$	$nr1^+$	nr1	$r1^2$	$pvr1^2$
ENZA7	S	R	NA	$pvr1^1$ or $pvr1^2$	$nr1^+$	nr1	$r1^2$	$pvr1^2$
ENZA8	S	R	NA	$pvr1^1$ or $pvr1^2$	$nr1^+$	NA	$r1^2$	$pvr1^2$
ENZA9	S	R	R	$pvr1^2$	$nr1^+$	nr1	$r1^2$	$pvr1^2$
ENZA10	S	R	R	$pvr1^2$	$nr1^+$	nr1	$r1^2$	$pvr1^1$
ENZA11	S	R	R	$pvr1^2$	$nr1^+$	nr1	$r1^2$	$pvr1^1$
ENZA12	S	R	S	$pvr1^1$	$nr1^+$	nr1	$r1^2$	$pvr1^1$
ENZA13	S	R	S	$pvr1^1$	$nr1^+$	nr1	$nr1^2$	$pvr1^1$
Crusader	R	R	NA	$pvr1^1$ or $pvr1^2$	$nr1^+$	nr1	$nr1^2$	$pvr1^1$
Cornago	R	R	NA	*Pvr4* or *Pvr7*	$r1^+$	nr1	$nr1^2$	$Pvr1^+$

NA not available, *R* resistance, *S* susceptible

result from screening the various pepper lines (Table 5) clearly demonstrated the application of these allele-specific CAPS markers in the MAS for pepper breeding. Definitive evaluation of the genotype at the *pvr1* will improve our ability to identify additional loci for resistance to this large and extremely destructive family of plant viruses and will accelerate future efforts to identify similar tools for other potyvirus resistance loci important to plant breeding programs.

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