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Molecular characterization of RAPD and SCAR markers linked to the *Tm-1* locus in tomato

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Abstract We have cloned and sequenced six RAPD fragments tightly linked to the *Tm-1* gene which confers tomato mosaic virus (ToMV) resistance in tomato. The terminal ten bases in each of these clones exactly matched the sequence of the primer for amplifying the corresponding RAPD marker, except for one in which the 5′-endmost two nucleotides were different from those of the primer. These RAPD clones did not cross-hybridize with each other, suggesting that they were derived from different loci. From Southern-hybridization experiments, five out of the six RAPD clones were estimated to be derived from middle- or high-repetitive sequences, but not from any parts of the ribosomal RNA genes (rDNA), which are known to be tightly linked with the *Tm-1* locus. The remaining clone appeared to be derived from a DNA family consisting of a few copies. These six RAPD fragments were converted to sequence characterized amplified region (SCAR) markers, each of which was detectable using a pair of primers having the same sequence as that at either end of the corresponding RAPD clone. All pairs of SCAR primers amplified distinct single bands whose sizes were the same as those of the RAPD clones. In four cases, the SCAR markers were present in the line with *Tm-1* but absent in the line without it, as were the corresponding RAPD markers. In the two other cases, the products of the same size were amplified in both lines. When these SCAR products were digested with different restriction endonucleases which recognize 4-bp sequences, however, polymorphisms in fragment length were found between the two lines. These co-dominant markers are useful for differentiating heterozygotes from both types of homozygote.

Key words Tomato · Random amplified polymorphic DNA (RAPD) · Sequence characterized amplified region (SCAR) · *Tm-1* gene · Tomato mosaic virus (ToMV) resistance

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Introduction

Mosaic disease caused by tomato mosaic virus (ToMV) is one of the most serious diseases in tomato (*Lycopersicon esculentum*). A ToMV-resistance gene, *Tm-1*, has been used, either independently or in combination with one of the other ToMV-resistance genes, *Tm-2* or *Tm-2a*, to breed resistant varieties. The *Tm-1* gene originated from *L. hirsutum*, a wild relative of *L. esculentum*, by interspecific crossing (Holmes 1957). This gene inhibits synthesis of viral RNA in isolated protoplasts as well as in intact plants (Motoyoshi and Oshima 1977; Watanabe et al. 1987), presumably by interacting with a viral RNA replicase (Meshi et al. 1988).

A number of DNA markers linked to the *Tm-1* locus have so far been identified. Using restriction fragment length polymorphism (RFLP) markers *Tm-1* was mapped to a position close to a ribosomal RNA gene (rDNA) which is located on the short arm of chromosome 2 (Tanksley et al. 1992). The length of the intergenic spacer sequence in the rDNA differs between tomato lines with and without *Tm-1* (Levesque et al. 1990). Therefore the rDNA itself can be used as an RFLP marker linked to *Tm-1*.

In addition to these DNA markers, we have detected eight random amplified polymorphic DNA (RAPD) bands present in a line carrying *Tm-1* but absent in its nearly isogenic line (NIL) without *Tm-1*. Six RAPD markers were arbitrary chosen out of the eight to examine whether they are linked to *Tm-1* (Ohmori et al. 1995 b). In 125 BC_1 plants, obtained by backcrossing an F_1 plant to the parent line without *Tm-1*, 63 ToMV-resistant plants carried five RAPD markers and 62 susceptible plants lacked them, suggesting tight linkage between *Tm-1* and each of these five RAPD markers. The remaining marker was also shown to be linked to *Tm-1*, because 63 ToMV-resistant plants carried this

marker, while 56 susceptible plants lacked it. In six ToMV-susceptible plants, however, the presence of the RAPD band could not be confirmed because one PCR product band was very close to the position of the RAPD band. As in the case of these six plants, RAPD primers sometimes amplify multiple non-specific DNA fragments, some of which cause difficulty in identifying specific RAPD bands.

Recently, sequence characterized amplified region (SCAR) markers converted from RAPD markers have proved to be useful (Paran and Michelmore 1993; Adam-Blondon et al. 1994; Maisonneuve et al. 1994). A SCAR marker represents a specified genomic region that is amplified by PCR using a pair of specific oligonucleotide primers. SCAR markers are advantageous over RAPD markers because they are identified as distinct single bands in agarose gels, and some of them show co-dominance which differentiates heterozygotes from both types of homozygote.

In this paper, we have characterized fragments cloned from the six RAPD markers linked to the *Tm-1* locus. We have also designed SCAR markers based on the sequences of the clones corresponding to the RAPD markers.

Materials and methods

Plant materials

GCR26 is a tomato variety 'Craigella' which is susceptible to ToMV. GCR237 is homozygous for *Tm-1* and has a common genetic background with 'Craigella' (Smith and Ritchie 1983). These NILs, originally bred at the Glasshouse Crops Research Institute (Littlehampton, UK), have been maintained through selfing from those used in previous studies (Motoyoshi and Oshima 1975, 1977).

DNA extraction

Total DNA was extracted by the method of Doyle and Doyle (1987) with minor modifications. Fresh leaf tissue (1.5 g) was frozen in liquid nitrogen and ground usig a mortar and pestle. The homogenate of the leaf tissue was placed in a test-tube together with 10 ml of DNA extraction buffer [2% (w/v) CTAB (hexadecyltrimethylammonium bromide), 1.4 M NaCl, 0.2% (v/v) 2-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0], and incubated in a water bath at 60 °C for 30 min with occasional swirling. After incubation, the lysate was extracted once with chloroform-isoamyl alcohol (24:1, v/v). The aqueous phase was mixed with a two-third volume of cold isopropanol. The precipitated DNA was spooled with a glass rod, and resuspended in TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA). The DNA was further purified by CsCl/ethidium bromide centrifugation (Sambrook et al. 1989).

Cloning and sequencing RAPD products

All the procedures for PCR and for the separation of amplified products were carried out as described previously (Ohmori et al. 1995 b). The RAPD bands were excised from agarose gels, and the DNA was purified using a Sephaglas™ Band Prep kit (Pharmacia). The terminal ends of the fragments were filled with T_4 DNA polymerase, and phosphorylated with T_4 polynucleotide kinase according to Sambrook et al. (1989). The fragments with blunt-ends were ligated into the *Sma*I site of a pBluescript II KS vector (Stratagene). The identity of these clones to the corresponding RAPD markers was examined by hybridization with them to Southern blots of PCR products amplified from the genomic DNAs of GCR26 (+/+) and GCR237 (*Tm-1/Tm-1*). The dideoxy nucleotide chain termination (Sanger et al. 1977) was carried out using oligonucleotide primers and fluorescent dye terminators (Applied Biosystems, Foster City, Calif., USA), and the products were analyzed by an automated DNA sequencer (373A, Applied Biosystems).

Southern hybridization

DNA samples from GCR237 (*Tm-1/Tm-1*) and GCR26 (+/+) were digested with *Dra*I, *Eco*RI, *Eco*RV or *Hin*dIII. Digested DNAs (5 μg/lane) were electrophoresed in 1.0% agarose gels, and transferred to nylon membranes (Hybond-N, Amersham) as described by Kreike et al. (1990). Hybridization was performed with digoxigenin (DIG)-labeled probes (Boehringer Mannheim) and chemical luminescent signals were detected according to the manufacture's instruction. The probe was labeled by PCR in a 25-μl solution containing 10 mM Tris-HCl, pH 8.3, 50 mM $MgCl_2$, 0.001% (w/v) gelatin, 200 μM each of dATP, dCTP and dGTP, 130 μM dTTP, 70 μM DIG-11-dUTP (Boehringer Mannheim), 1.0 μM primer (M13-20 or reverse primer), 150 ng linear DNA, and 1.25 units of *Taq* DNA polymerase (Perkin Elmer Cetus). The PCR was carried out in 30 cycles of denaturing for 1 min at 95 °C, annealing for 1 min at 55 °C, and primer extention for 1 min at 72 °C. A wheat-derived rDNA clone containing a single repeat unit of the 18*s*–26*s*, rRNA gene (pta71) (Gerlach and Bedbrook 1979) was similarly labeled with DIG, and used as a probe.

Design of primers and analysis of SCAR markers

A pair of 24-mer oligonuleotide primers, designed to have sequences identical to the 5'-endmost 24 bases of the cloned RAPD DNA, was synthesized by using an automated DNA synthesizer (391, Applied Biosystems). PCR was performed as described by Paran and Michelmore (1993) except that annealing was carried out at 65 °C. Amplified products were resolved by electrophoresis in a 1.4% agarose gel.

Results

Cloning and characterization of RAPDs linked to the *Tm-1* gene

PCR-amplified products corresponding to the six RAPDs linked to the *Tm-1* gene (Ohmori et al. 1995 b) were extracted from agarose gels after electrophoresis and cloned into a pBluescript II KS vector. These clones were found to be identical to their corresponding RAPDs by Southern hybridization.

At least 200 nucleotides were sequenced from both 5'-ends of each clone. In both ends of the clones from $OPB10_{1300}$, $G12_{800}$, $I10_{1100}$, $N09_{1100}$ and $N20_{1400}$ the terminal ten bases exactly matched the sequence of each primer for the corresponding RAPD. The proximal eight bases of the clone from $OPA15_{1000}$ also matched the eight bases of the primers, but the terminal two bases at either end of the clone were not identical to those of the primer. This result indicates that the primer annealed with the proximal eight bases of genomic DNA to generate $OPA15_{1000}$. These six clones did not cross-hybridize with each other (data not shown), suggesting that the six RAPD markers occupy independent loci close to *Tm-1*.

To determine whether each RAPD clone derived either from one of the multi-copied sequences or from the single-copied one, DNA samples prepared from GCR237 (*Tm-1/Tm-1*) and GCR26 (+/+) were digested with *Dra*I, *Eco*RI, *Eco*RV or *Hin*dIII, electrophoresed in agarose gels, and transferred to membrane filters. In both GCR237 (*Tm-1/Tm-1*) and GCR26 (+/+), the clones from $OPA15_{1000}$, and $I10_{1100}$ strongly hybridized to a large number of different DNA fragments, suggesting that they derived from highly repetitive DNA sequences (Fig. 1 A). The clones from $OPB10_{1300}$, $N09_{1100}$ and $N20_{1400}$ were also shown to hybridize to repetitive DNA fragments, but the numbers of the hybridized bands were less than those with $OPA15_{1000}$ or $OPI10_{1100}$ (Fig. 1 B). In contrast to these clones, that from $OPG12_{800}$ hybridized to at most three fragments, which were found only in GCR237 (*Tm-1/Tm-1*) (Fig. 1 C).

There is a possibility that at least some of the RAPD markers are parts of the rDNA, since the rDNA has been suggested to be linked to the *Tm-1* locus (Levesque et al. 1990; Tanksley et al. 1992). To examine this possibility, GCR237 (*Tm-1/Tm-1*) and GCR26 (+/+) DNAs digested with several restriction endonucleases were electrophoresed and their Southern blots were hybridized with a wheat rDNA as a probe. The rDNA hybridized with from one to six bands depending on the enzyme. *Eco*RV produced a single band whose size is approximately 10.5 kb in GCR237 (*Tm-1/Tm-1*) and 8.0 kb in GCR26 (+/+) (Fig. 2). These hybridization patterns with the rDNA clone were completely different from those of any of the RAPD clones, as demonstrated above (Fig. 1 A, B, C). None of the RAPD marker clones hybridized to the rDNA clone.

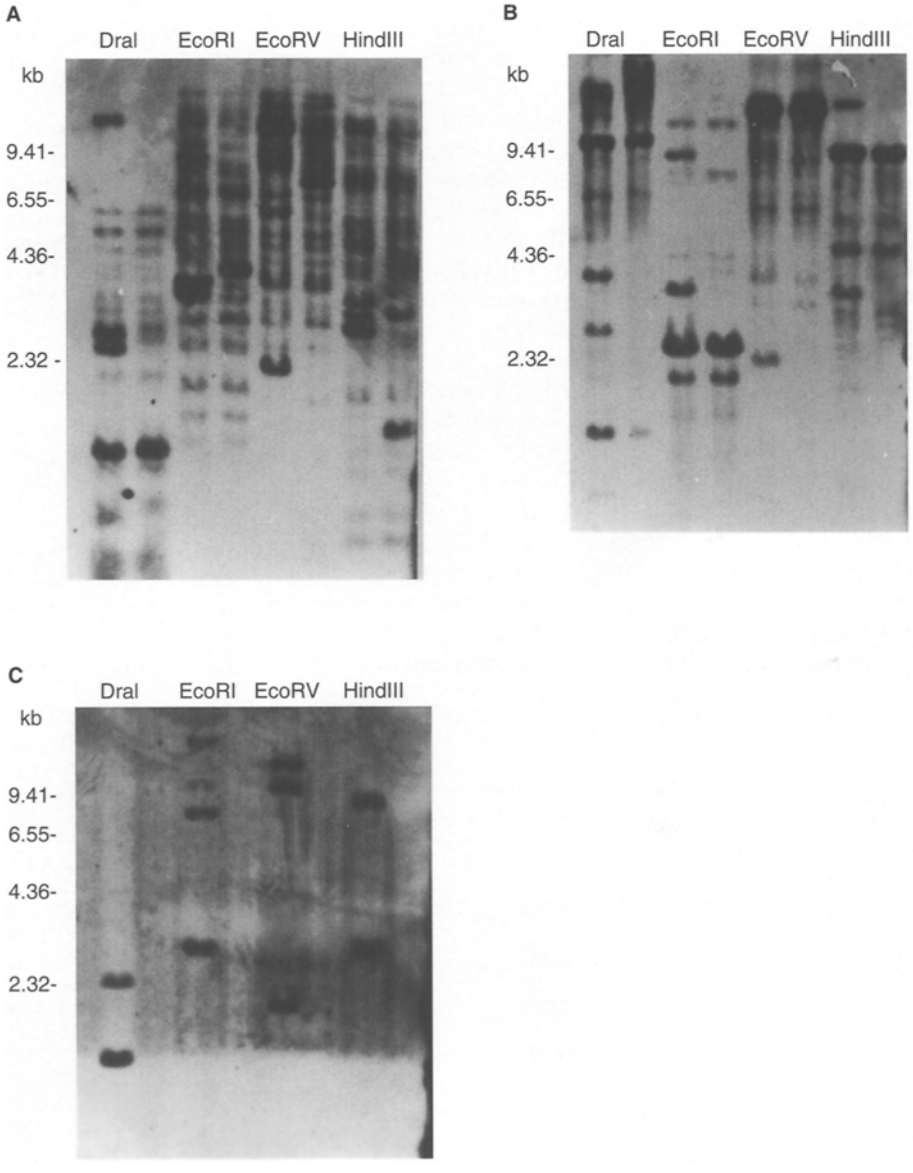

Fig. 1 A–C Southern hybridization with DNA fragments cloned from the RAPD markers $OPA15_{1000}$ (**A**), $OPN09_{1100}$ (**B**) and $OPG12_{800}$ (**C**) to DNAs from GCR237 (*Tm-1/Tm-1*) (left lane in each pair) and GCR26 (+/+) (right lane in each pair). The restriction endonucleases used are indicated above the lanes

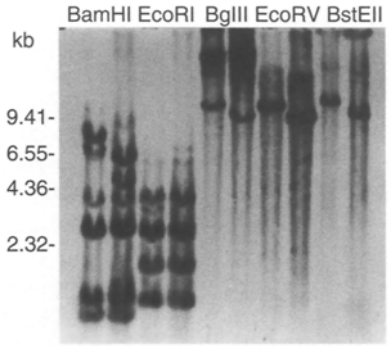

Fig. 2 Southern hybridization with a wheat rDNA to DNAs from GCR237 (*Tm-1/Tm-1*) (left lane in each pair) and GCR26 (+/+) (right lane in each pair). The restriction endonucleases used are indicated above the lanes

Identification of SCAR markers and their characteristics

A pair of 24-mer oligonucleotide primers were synthesized based on the sequences at both ends of each RAPD marker clone (Table 1). PCR was carried out using each pair of primer and genomic DNAs from GCR26 (+/+) and GCR237 (*Tm-1/Tm-1*) as templates. With four pairs of primers, bands appeared in GCR237 (*Tm-1/Tm-1*) but not in GCR26 (+/+). Their sizes were much the same as those of the RAPD markers OPA_{1000}, $OPG12_{800}$, $OPN09_{1100}$ and $OPN20_{1400}$ (Fig. 3 A). These new markers, designated as $SCA15_{1000}$, $SCG12_{800}$, $SCN09_{1100}$ and $SCN20_{1400}$, are expressed dominantly as are the corresponding RAPD markers.

A pair of primers based on the sequence of $OPB10_{1300}$ amplified a fragment ($SCB10_{1300}$) of the same size as that of the RAPD marker specific to GCR237 (*Tm-1/Tm-1*). In GCR26 (+/+), however, the same pair of primers also amplified a fragment whose size was similar to that found in GCR237 (*Tm-1/Tm-1*). Similarly, a band was found in GCR26 (+/+) when a pair of primers designed from the sequence of $OPI10_{1100}$ was used, and the fragment size was also much the same as that ($SCI10_{1100}$) fragment in GCR237 (*Tm-1/Tm-1*). However, when the $SCB10_{1300}$ fragment in GCR237 (*Tm-1/Tm-1*) and the product of the same size in GCR26 (+/+) were digested with *Hae*III, *Hap*II, or the $SCI10_{1100}$ fragment in GCR237 (*Tm-1/Tm-1*) and the product in GCR26 (+/+) were treated with *Hae*III, *Hap*II or *Mbo*I, they revealed polymorphism (Fig. 3 B). Since the $SCB10_{1300}$ fragment in GCR237 (*Tm-1/Tm-1*) strongly cross-hybridized to the product in GCR26 (+/+) (data not shown), there is a high homology between the two products. The homology between $SCI10_{1100}$ in GCR237 (*Tm-1/Tm-1*) and the product in GCR26 (+/+) appeared to be lower than that found with $SCB10_{1300}$.

Discussion

PCR, using whole genomic DNA as a template and an oligonucleotide primer, tends to amplify middle- or high-repetitive sequences predominantly (Williams et al. 1990; Reiter et al. 1992; Delourme et al. 1994). In a previous study (Ohmori et al. 1995 a) we detected 53 RAPDs among NILs which carry *Tm-2*, *Tm-2a* or its ToMV-susceptible allele. All of the 13 RAPDs arbitrarily chosen among the 53 proved to be close to the *Tm-2* locus. Five out of six clones prepared from some of these 13 markers hybridized to middle- or high-repetitive sequences in Southern blots, indicating that the locus is associated with repetitive sequences (Ohmori et al., unpublished data). In the present study, six RAPD markers among 12 RAPDs linked to *Tm-1* (Ohmori et al. 1995 b) were arbitrarily chosen and cloned. In genomic Southern hybridization with these six clones as probes, five clones hybridized to middle- or high-repetitive sequences, and only one hybridized to low-copy DNA sequences. Therefore, the *Tm-1* locus was also closely associated with repetitive sequences.

A RAPD marker that is amplified with a 10-mer oligonucleotide primer is usually accompanied by several non-specific PCR products, whereas a SCAR marker amplified with a pair of 24-mer oligonucleotide primers expresses itself as a distant single band in a gel. In the present study, four out of six RAPD markers were

Table 1 SCAR markers converted from RAPD markers linked to the *Tm-1* gene

SCAR marker	Primer	Sequence[a]	Phenotype
$SCA15_{1000}$	$OPA15U_{1000}$	CCGAACCCCTTAAAAATAGTTTCA	Dominant
	$OPA15T_{1000}$	CCGAACCCAATCAGGAGGCTCATA	
$SCB10_{1300}$	$OPB10U_{1300}$	CTGCTGGGACAATGCAGAAAAGCA	Co-dominant with
	$OPB10T_{1300}$	CTGCTGGGACTATTGTAATTAGTG	*Hae*III or *Hap*II
$SCG12_{900}$	$OPG12U_{900}$	CAGCTCACGAACATTGAAGTTGAT	Dominant
	$OPG12T_{900}$	CAGCTCACGATAATCATTAAATTG	
$SCI10_{1100}$	$OPI10U_{1100}$	ACAACGCGAGGCCAAATCCCATCA	Co-dominant with
	$OPI10T_{1100}$	ACAACGCGAGTAGGTTTAGGGTGT	*Hae*III, *Hap*II or *Mbo*I
$SCN09_{1100}$	$OPN09U_{1100}$	TGCCGGCTTGCCTCACTCTCAGGT	Dominant
	$OPN09T_{1100}$	TGCCGGCTTGATCAATAGAATTGA	
$SCN20_{1400}$	$OPN20U_{1400}$	GGTGCTCCGTCGATGCAAAGTGCA	Dominant
	$OPN20T_{1400}$	GGTGCTCCGTAGACATAAAATCTA	

[a] The underlines indicate the sequences based on the RAPD primers

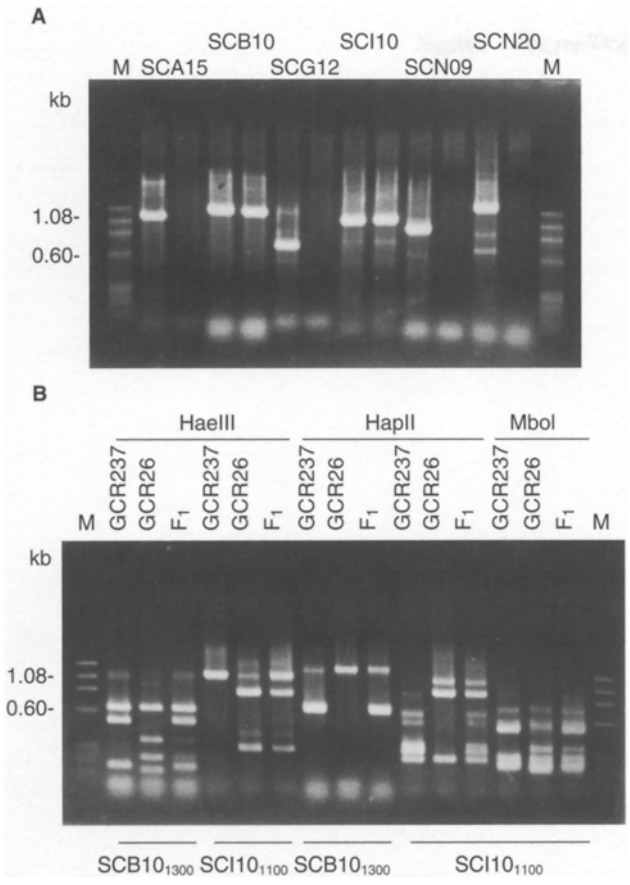

Fig. 3 A, B Agarose-gel electrophoresis of SCAR markers converted from RAPD markers. The SCAR primers were designed based on the sequences of the RAPD markers. **A** Products amplified from genomic DNA using SCAR primers. The amplified products were designated $SCA15_{1000}$, $SCB10_{1300}$, $SCG12_{800}$, $SCI10_{1100}$, $SCN09_{1100}$, and $SCN20_{1400}$ (from left to right). The PCR products from DNA samples of GCR237 (*Tm-1/Tm-1*) and GCR26 (+/+) are shown in the left and right lanes of each pair, respectively. *M* molecular-size markers (ϕX174/*Hae*III digest). **B** Polymorphism in restriction endonuclease-digests of the SCARs of present in both GCR237 (*Tm-1/Tm-1*) and GCR26 (+/+) ($SCB10_{1300}$ and $SCI10_{1100}$). The SCARs were amplified from genomic DNAs of GCR237, GCR26 and F_1, digested with *Hae*III, *Hap*II or *Mbo*I, and electrophoresed. *M* molecular-size markers (ϕX174/*Hae*III digest)

converted to SCAR markers specific to the tomato line carrying *Tm-1*. The remaining two primer pairs amplified single fragments not only in GCR237 (*Tm-1/Tm-1*) but also in GCR26 (+/+); but the size of fragments was different. In these cases, 10-mer primers may have failed to amplfiy fragments from the GCR26 (+/+) genome due to mismatches in the nucleotides as explained by Paran and Michelmore (1993). The addition of 14 nucleotides to the original 10-mers of a RAPD primer should have increased homology to the target site in the GCR26 (+/+) genome, so that fragments were amplified to the same level as that of the PCR products in GCR237 (*Tm-1/Tm-1*).

Both RAPD and SCAR markers are more rapidly and more easily detectable than RFLP markers (Welsh and McClelland 1990; Williams et al. 1990; Foolad et al. 1993). One of the disadvantages fo a RAPD marker is that it is usually dominant, and so is useless for differentiating heterozygotes from either homozygote. However, $SCB10_{1300}$ and $SCI10_{1100}$ revealed co-dominance between GCR237 (*Tm-1/Tm-1*) and GCR26 (+/+) when they were digested with specific restriction endonucleases recognizing 4 bp. Therefore, these markers may be useful as co-dominance markers for the efficient breeding of ToMV-resistant lines.

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