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# Identification of RAPD markers linked to the *Tm-2* locus in tomato

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**Abstract** *Tm-2* and *Tm-2a* are genes conferring resistance to tomato mosaic virus in *Lycopersicon esculentum*. They are allelic and originated from different lines of *L. peruvianum*, a wild relative of tomato. In this study, random amplified polymorphic DNA (RAPD) markers linked to these genes were screened in nearly isogenic lines (NILs). To detect RAPDs differentiating NILs, 220 different 10-base oligonucleotide primers were examined by the polymerase chain reaction (PCR), and 43 of them generated 53 consistent polymorphic fragments among the NILs. Out of these 53 fragments, 13 were arbitrarily chosen and examined in respect of whether they were linked to the netted virescent (*nv*) gene, since *nv* is tightly linked to the *Tm-2* locus and its phenotype is more easily distinguishable. As a result, all 13 markers were shown to be linked to *nv*, and hence to the *Tm-2* locus. Among them, two fragments specific to the NIL carrying *Tm-2* three specific to the NIL carrying *Tm-2a*, and four specific to both of these NILs were closely linked to *nv*.

**Key words** Polymerase chain reaction (PCR)
Random amplified polymorphic DNA (RAPD)
Disease resistance · Tomato mosaic virus
Nearly isogenic lines (NILs)

## Introduction

*Tm-2* and *Tm-2a* (synonymous with *Tm-2*$^2$) are important genes for breeding tomato [*Lycopersicon esculentum* (L.) Mill.], because they confer resistance to tomato mosaic virus (ToMV) (Cirulli and Alexander 1969; Soost 1963). These two genes originated from two different lines of *L. peruvianum* L., a wild relative of *L. esculentum*, and were introduced into *L. esculentum* in two independently conducted tomato breeding programs in the USA (Kikuta and Frazier 1947; Alexander 1963). The *Tm-2* and *Tm-2a* genes are allelic and are located on the long arm of chromosome 9 in a region close to the centromere (Khush et al. 1964; Pécaut 1965). Both genes express a similar resistance to ToMV, but can be differentiated in respect of their responses to some ToMV strains (Pelham 1972). A wild-type strain of ToMV was able to multiply in infected cells carrying either the *Tm-2* or the *Tm-2a* gene, but did not spread from cell to cell (Motoyoshi and Oshima 1975, 1977; Nishiguchi and Motoyoshi 1987). Meshi et al. (1989) suggested that the resistance expressed by the *Tm-2* gene involves an alteration in, or inhibition of the function of the virus-coded 30-kDa protein which is necessary for spreading the virus from cell to cell.

In order to elucidate the resistance mechanism of the *Tm-2* and *Tm-2a* genes in more detail, it may be a prerequisite to clone them and analyze their molecular structures. Since no mRNAs or the proteins encoded by these genes are known, a positional gene-cloning strategy may be the most suitable approach for cloning them. Before starting positional cloning, it is necessary to identify DNA markers tightly linked to the target gene. Using nearly isogenic lines (NILs) and restriction fragment length polymorphism (RFLP), Young et al. (1988) have isolated five different markers tightly linked to the *Tm-2a* locus. More recently, Tanksley et al. (1992) assigned 14 RFLP markers to the position of *Tm-2a* in the tomato genetic map.

Random amplified polymorphic DNA (RAPD) markers prepared by the polymerase chain reaction (PCR) are useful for mapping genes, because RAPD markers are more rapidly and more easily detectable than are RFLP markers (Welsh and McClelland 1990; Williams et al. 1990; Foolad et al. 1993). Using NILs of tomato, disease resistance genes on introgressed segments from wild relatives have been efficiently mapped with RAPD markers (Klein-Lankhorst et al. 1991; Martin et al. 1991; Williamson et al. 1994). The NILs carry-

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ing *Tm-2*, *Tm-2a*, or their common ToMV-susceptible allele, were expected to be useful the efficient detection of RAPD markers linked to these alleles. Using 10-base oligonucleotides as primers, we attempted to find RAPDs in three NILs for the *Tm-2* locus.

Instead of the *Tm-2* or *Tm-2a* gene itself, we used a recessive allele 'netted virescent' (*nv*) to map the RAPDs, since it was known that there is a tight linkage between the *Tm-2* and the *nv* loci (Soost 1958, 1963; Clayberg 1959). In test inoculations of 146 families at various backcrossed and self-pollinated generations segregating resistant and susceptible plants, Soost (1963) confirmed that all virescent plants were resistant and that resistance only occurred in lines which were also segregating for virescence. The seedlings homozygous for the *nv* allele are stunted and express pale interveinal chlorosis. Differentiating virescence from the wild-type is thus easier and more rapid than screening resistance to the virus.

## Materials and methods

### Plant material and segregation analysis

GCR26 is a line of the tomato variety 'Craigella' which is susceptible to ToMV. GCR236 and GCR267 also have a common genetic background of 'Craigella', but the former carries *Tm-2* and *nv*, whereas the latter carries *Tm-2a* (Smith and Ritchie 1983). These three GCR lines, originally bred at the Glasshouse Crops Research Institute (Littlehampton, UK), have been maintained through the selfing of those used in previous studies (Motoyoshi and Oshima 1975, 1977).

Two $F_2$ segregating populations were employed to analyze the linkage between the RAPD markers and the *nv* gene. One consisted of 133 $F_2$ individuals derived from a cross between GCR236 (*Tm-2 nv*) and GCR26 (+ +). The other consisted of 108 $F_2$ individuals from a cross between GCR267 (*Tm-2a* +) and GCR236 (*Tm-2 nv*).

Linkage values and map distances between RAPD markers were calculated based on Kosambi's mapping function (Kosambi 1944) using MAPL Ver3.0 programmed by Dr. Y. Ukai, University of Tokyo.

### DNA isolation

Total DNA was extracted from approximately 0.1 mg of fresh leaf tissue of each plant by the method of Murray and Thompson (1980) with minor modifications. The tissue was frozen in liquid nitrogen and pulverized with a plastic pipette tip in a 1.5-ml Eppendorf tube, and 400 µl of extraction buffer [100 mM Tris-HCl pH 8.0, 0.35 M sorbitol, 5 mM EDTA and 1.0% (v/v) 2-mercaptoethnol] was added to each of the powdered samples. The samples were centrifuged for 15 min at 4000 rpm in a microcentrifuge. The pellet was resuspended in 200 µl of extraction buffer containing 1.0% (w/v) CTAB (hexadecyltrimethylammonium bromide), 1 M NaCl, 25 mM EDTA and 1.0% (w/v) lauroylsarcosine at final concentrations, and incubated at 60 °C for 20 min. The lysate was extracted once with chloroform/isoamylalchol (24:1) and the aqueous phase was mixed with an equal volume of isopropanol to precipitate DNA. The precipitate was washed with 70% (v/v) ethanol, and dissolved in the appropriate amount of TE (10 mM Tris-HCl, 1 mM EDTA pH 8.0) containing RNase A (Sigma) at 10 µg/ml.

### RAPD analysis

For RAPD analysis of the NILs, 220 different 10-base oligonucleotide primers were used, all of which were obtained from Operon™ Technologies (Alamede, California).

The protocol described by Williams et al. (1990) was employed for DNA amplification but with a slight modification. PCR was carried out in a 25-µl solution containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 100 µM each of dATP, dCTP, dGTP and dTTP, 5 pmol of an oligonucleotide primer, 10 ng of DNA, and 0.5 units of *Taq* DNA polymerase (Perkin Elmer Cetus). One drop of pure mineral oil was overlaid onto the sample prior to the amplification. DNA was amplified in a Perkin Elmer Cetus DNA Thermal Cycler 480 by 45 cycles of 1 min at 94 °C for denaturing, 1 min at 37 °C for annealing, and 2 min at 72 °C for primer extension. Amplification products were resolved by electrophoresis in 1.4% (w/v) agarose gels and stained with ethidium bromide.

## Results

### Screening NILs with random primers

The 220 primers used produced 1060 discrete PCR products in total (an average of 4.7 products per primer). Their size ranged from 0.2 to 4.0 kilobases (kb), with a majority of 0.5–2.0 kb. Most of the products were common among GCR26 (+ +), GCR236 (*Tm-2 nv*) and GCR267 (*Tm-2a* +), while 53 products (5.0%) amplified by 43 primers (19%) were polymorphic among the NILs. Out of these 53 products, 38 were present in one NIL but not in others (class I, Table 1). Among them, ten products appeared only in GCR236 (*Tm-2 nv*). GCR267 (*Tm-2a* +) and GCR26 (+ +) also had ten and 18 specific fragments, respectively. Fifteen products were present in two NILs but not in the other (class II, Table 1). Ten products appeared in both GCR236 (*Tm-2 nv*) and GCR267 (*Tm-2a* +), four in both GCR236 (*Tm-2 nv*) and GCR26 (+ +), and one in both GCR267 (*Tm-2a* +) and GCR26 (+ +).

### Linkage of RAPD markers to the *nv* locus

To determine the linkage between the *nv* locus and the RAPD markers which were present in GCR236 and/or GCR267, but not in GCR26, 13 primers were arbitrarily chosen from the 43 that generated polymorphic

**Table 1** Number of polymorphic fragments detected by RAPD analysis in the nearly isogenic lines

| Class | Nearly isogenic lines | | | No. of polymorphic fragments |
|---|---|---|---|---|
| | GCR26 (+ +) | GCR236 (*Tm-2 nv*) | GCR267 (*Tm-2a* +) | |
| I | +[a] | − | − | 18 |
| | − | + | − | 10 |
| | − | − | + | 10 |
| II | + | + | − | 4 |
| | + | − | + | 1 |
| | − | + | + | 10 |
| Total | | | | 53 |

[a] + and −: presence and absence of a fragment giving polymorphism, respectively

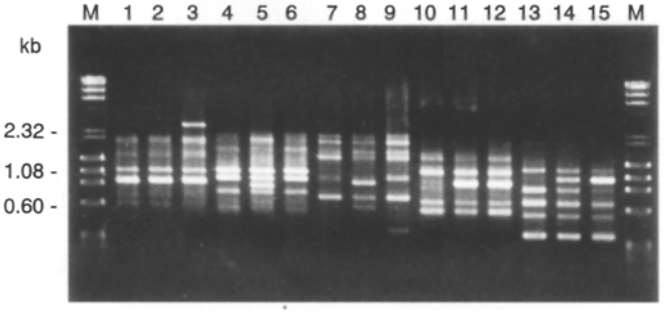


**Fig. 1** Electrophoretic patterns of PCR-amplified DNA products from genomic DNAs of the NILs using 10-base oligonucleotide primers: OPF02 (*lanes 1–3*), OPG10 (*lane 4–6*), OPI18 (*lanes 7–9*), OPE 16 (*lanes 10–12*), and OPN13 (*lanes 13–15*). *Lanes 1, 4, 7, 10, and 13*; GCR26 (+ +); *lanes 2, 5, 8, 11, and 14*; GCR236 (*Tm-2 nv*); *lanes 3, 6, 9, 12, and 15*; GCR267 (*Tm-2a* +); *M*; molecular size markers (λ/*Hind* III-digest–ΦX174/*Hae*III-digest)

fragments. Among the 13 primers selected, five (OPB12, D20, G10, H01, and I18) produced fragments specific to GCR236 (*Tm-2 nv*), three (OPF02, H11, and M09) to GCR267 (*Tm-2* +), and five (OPE16, G09, H09, I15, and N13) to both GCR236 (*Tm-2 nv*) and GCR267 (*Tm-2a* +) (Fig. 1).

Tight linkages between the wild-type allele of *nv* and three RAPD markers specific to GCR267 (*Tm-2a* +) were shown by co-segregation of these markers in 108 $F_2$ individuals that were derived from a cross between GCR267 (*Tm-2a* +) and GCR236 (*Tm-2 nv*). No recombinants were observed in any of the combinations (Table 2).

In 133 $F_2$ individuals derived from a cross between GCR236 (*Tm-2 nv*) and GCR26 (+ +), segragations involving the *nv* and RAPD markers deviated markedly ($P < 0.001$) from a 9:3:3:1 ratio. This was due to the linkage between the *nv* and RAPD markers as well as the deviation ($0.05 < P < 0.01$) of segregation ratios from 3:1 in the phenotypes involving single RAPDs (Table 2). To determine which RAPD markers are closer to *nv*, co-segregating markers with *nv* were checked for eight recombinants detected in the $F_2$ population (Table 3). One of the recombinants was homozygous for *nv*, and the seven others were all shown to be heterozygotes (*nv*/+) as examined in the $F_3$ progeny. One plant homozygous for *nv* and three heterozygous plants were missing a RAPD marker, $OPI15_{2500}$, but carried all nine other RAPDs. The $OPH01_{3000}$ marker was missing in three heterozygotes carrying the other nine RAPDs and, in addition, one heterozygote had lost

**Table 3** RAPD-recombinants found in $F_2$ populations (133 plants) derived from a cross between GCR236 and GCR26

| Genotype | Locus | | | | | Number of plants |
|---|---|---|---|---|---|---|
| | $OPH01_{3000}$ | $OPB12_{1200}$ | $OPI18_{900}$ | $OPI15_{2500}$ | Other six[a] loci | |
| *nv*/+ | –[b] | + | + | + | + | 3 |
| | – | – | – | + | + | 1 |
| | + | + | + | – | + | 3 |
| *nv*/*nv* | + | + | + | – | + | 1 |

[a] $OPD20_{1800}$, $OPG10_{800}$, $OPE16_{900}$, $OPG09_{700}$, $OPG09_{1000}$, $OPN13_{1000}$

[b] + and –: presence and absence of the RAPD marker, respectively

**Table 2** Phenotypic frequencies and $\chi^2$ values for goodness of fit to 3:1 (single point; $\chi^2$A, $\chi^2$B), linkage ($\chi^2$L) and 9:3:3:1 (two-point; $\chi^2$AB) expected ratios in pairwise combinations of the *nv* and RAPD markers

| Locus | | Phenotypes | | | | $\chi^2$A | $\chi^2$B | $\chi^2$L | $\chi^2$AB |
|---|---|---|---|---|---|---|---|---|---|
| A | B | AB | Ab | aB | ab | | | | |
| $OPF02_{2500}$ | *nv* | 84 | 0 | 0 | 24 | 0.44 | 0.44 | 92.59** | 93.48** |
| $OPH11_{1500}$ | *nv* | 84 | 0 | 0 | 24 | 0.44 | 0.44 | 92.59** | 93.48** |
| $OPM09_{800}$ | *nv* | 84 | 0 | 0 | 24 | 0.44 | 0.44 | 92.59** | 93.48** |
| $OPH01_{3000}$ | *nv* | 59 | 26 | 48 | 0 | 8.72** | 2.11 | 22.20** | 33.05** |
| $OPB12_{1200}$ | *nv* | 62 | 26 | 45 | 0 | 5.54* | 2.11 | 19.05** | 26.69** |
| $OPI18_{900}$ | *nv* | 62 | 26 | 45 | 0 | 5.54* | 2.11 | 19.05** | 26.69** |
| $OPI15_{2500}$ | *nv* | 60 | 25 | 47 | 1 | 8.72** | 2.11 | 18.05** | 28.88** |
| Other loci[a] | *nv* | 63 | 26 | 44 | 0 | 4.63* | 2.11 | 18.05** | 24.79** |
| $OPH01_{3000}$ | $OPB12_{1200}$ | 85 | 0 | 3 | 45 | 8.72** | 5.54* | 193.28** | 207.54** |
| $OPH01_{3000}$ | $OPI18_{900}$ | 85 | 0 | 3 | 45 | 8.72** | 5.54* | 193.28** | 207.54** |
| $OPH01_{3000}$ | Other loci | 85 | 0 | 4 | 44 | 8.72** | 4.63* | 183.76** | 196.72** |
| $OPH01_{3000}$ | $OPI15_{2500}$ | 81 | 4 | 4 | 44 | 8.72** | 8.72** | 171.44** | 188.48** |
| $OPB12_{1200}$ | $OPI18_{900}$ | 88 | 0 | 0 | 45 | 5.54* | 5.54* | 203.05** | 214.12** |
| $OPB12_{1200}$ | Other loci | 88 | 0 | 1 | 44 | 5.54* | 4.63* | 193.28** | 203.45** |
| $OPB12_{1200}$ | $OPI15_{2500}$ | 84 | 4 | 1 | 44 | 5.54* | 8.72** | 180.64** | 194.90** |
| $OPI18_{900}$ | $OPI15_{2500}$ | 84 | 4 | 1 | 44 | 5.54* | 8.72** | 180.64** | 194.90** |
| $OPI18_{900}$ | Other loci | 88 | 0 | 1 | 44 | 5.54* | 4.63* | 193.28** | 203.45** |

* Significant at the 0.05 probability level
** Significant at the 0.01 probability level

[a] $OPD20_{1800}$, $OPG10_{800}$, $OPE16_{900}$, $OPG09_{700}$, $OPH09_{1000}$, $OPN13_{1000}$

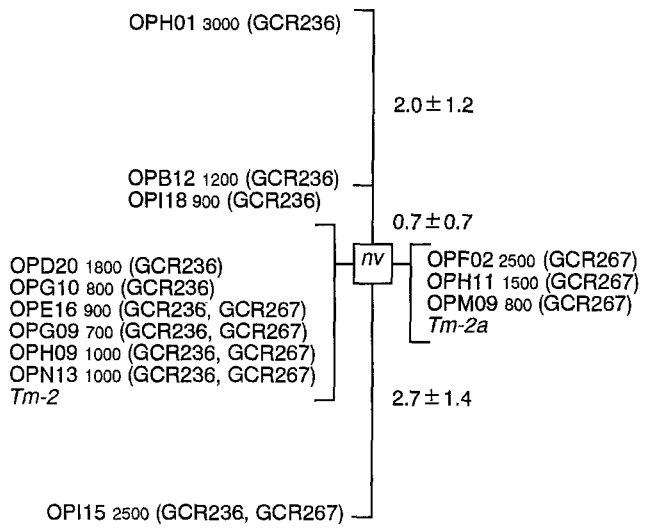


**Fig. 2** A genetic map of RAPD markers close to the *nv* locus on chromosome 9. Genetic distances are given (cM) to the right of the vertical lines. The tomato lines which RAPD markers are specific to are indicated in *parenthesis*

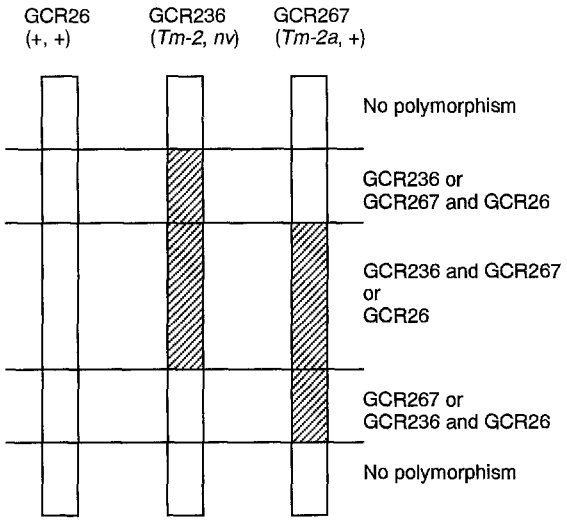


**Fig. 3** A model explaining the polymorphic pattern by RAPDs among tomato lines GCR26, GCR236 and GCR267. The *columns* illustrate portions surroundings the *nv* locus in chromosome 9. The *hatched areas* represent the introgressed segments from *L. peruvianum* and the *white areas* are parts from chromosome 9 of *L. esculentum*

$OPH01_{3000}$ together with two other markers, $OPB12_{1200}$ and $OPI18_{900}$, although it carried seven other markers. In all of these recombinants, the six other markers co-segregated with *nv*, indicating that *nv* was linked most closely to these markers. Genetic distances between every pair of these ten RAPD markers were calculated and they were mapped together with the markers specific to GCR267 (Fig. 2).

## Discussion

In 1060 discrete PCR products, 39 polymorphic fragments (3.7%) were detected between GCR26 (+ +) and GCR236 (*Tm-2 nv*), and 42 (4.0%) between GCR26 (+ +) and GCR267 (*Tm-2a* +), while 26 (2.5%) were polymorphic between GCR236 (*Tm-2 nv*) and GCR267 (*Tm-2a* +) (Table 1). This result indicates that GCR236 and GCR267 carry almost similar amounts of *L. peruvianum* genomic DNA. Thirteen RAPD markers arbitrarily selected from the 53 polymorphic products were all found to be linked to *nv* or its wild-type allele. It is thus probable that the majority of the polymorphic DNAs are linked to *nv* on chromosome 9. From such a pattern of polymorphism, we can assume that each of the chromosomal segments introgressed from *L. peruvianum* into GCR236 and GCR267 has an overlapping (homologous) region and a non-overlapping region as illustrated in Fig. 3. This figure explains those cases in which polymorphisms are found in every combination of GCR26, GCR236 and GCR267. It also indicates that the considerably high level of polymorphisms between GCR236 and GCR267 is mainly due to the presence of the non-overlapping areas of the introgressed segments. In addition to the polymorphisms explained as above, it is also possible that a part of the polymorphisms originated from some heterogeneity in the overlapping regions between GCR236 and GCR267 since their introgressed segments were derived from different lines of *L. peruvianum* (P. I. 126926 and P. I. 128650). The presence of heterogeneity in the introgressed segments was suggested by our recent observation that the *L. peruvianum* line P. I. 128650, which was the source of the introgressed segments in GCR267, showed a polymorphic pattern more similar to that of GCR267 than to that of GCR236 when the 13 markers were tested (Ohmori, Murata and Motoyoshi, unpublished data).

The RAPD markers tightly linked to the *Tm-2* locus are useful for identification of *Tm-2* or *Tm-2a* as well as for the selection of ToMV-resistant lines. They may also be useful for the selection of recombinant individuals to remove much of the long introgressed segments from *L. peruvianum*, as demonstrated by Young and Tanksley (1989) with their RFLP markers. RFLP as well as RAPD markers are also important for the positional cloning of genes of interest. In the case of the *Tm-2* and *nv* genes, however, it has been shown that they are located in a heterochromatic area near the centromere of chromosome 9 (Khush et al. 1964) where recombination between markers is highly suppressed (Rick 1971). Based on their physical mapping study Ganal et al. (1989) demonstrated that five RFLP markers closely linked to the *Tm-2a* gene are distributed over at least 4 megabases, although they cover a genetic distance of only about 1.2 cM. Three RFLP markers more tightly linked to *Tm-2a* were reported by Martin et al. (1992), out of which two were identified separately in two YAC clones of comparatively small insert sizes of 50 kb and 45 kb, respectively. Preparation of larger YAC clones and the selection of RFLP and RAPD markers most close to the *Tm-2* locus are necessary for chromosome

walking to the ToMV resistance genes. Out of the 13 RAPD markers selected in the present report, nine were identified to have tight linkages to the *Tm-2* locus, and the remaining untested RAPDs may be also an important source for selecting suitable markers.

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