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IDENTIFICATION OF CDNA FRAGMENT TIGHTLY LINKED TO NV AND TM-2 LOCI IN TOMATO

(IDENTIFIKASI FRAGMENT CDNA YANG BERPAUTAN DENGAN LOKUS NV DAN TM-2 PADA TOMAT)

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Kata kunci: lokus *Tm-2*, differential display, fragmen cDNA, tomat
Key words: *Tm-2* locus, differential display, cDNA fragment, tomat

Abstract

Tm-2 is a resistance gene in tomato to Tomato Mosaic Virus (ToMV), located in heterochromatic region of chromosome nine. Since map based cloning difficult to perform for identify the gene on that region, we apply differential display approach by using two near-isogenic tomato lines (NILs), one without *Tm-2* and the other with *Tm-2* to identify cDNAs of the transcripts from the region surrounding the *Tm-2* locus. Among the 150 combinations of three anchor primers and fifty arbitrary primers, 10

combinations generated cDNA polymorphic bands. Out of them, only one combination of CA6, exhibited polymorphic band under southern blot analysis, subsequently a genetic experiment showed that the CA6 locus tightly linked to the *Tm-2* locus. The CA6 fragment also hybridized to genomic DNA fragments from a tomato line carrying *Tm-2a*, a line of *L. peruvianum* from which *Tm-2a* originated, and a tomato line carrying another *Tm-2*-like gene. A northern hybridization blotting result suggested that the gene corresponding to CA6 fragment was constitutively transcribed.

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Sari

Tm-2 adalah gen ketahanan terhadap *Tomato Mosaic Virus* (ToMV), yang berada pada daerah heterokromatik pada kromosom 9. Oleh karena pendekatan *map based cloning* sulit dilakukan pada daerah tersebut, maka kami melakukan analisis *Differential Display* terhadap dua NILs (*Near Isogenic Lines*), satu tanpa gen *Tm-2* dan yang lainnya membawa gen *Tm-2*, untuk mendapatkan cDNA yang berasal dari RNA yang ditranskripsikan dari daerah disekitar lokus *Tm-2*. Dari 150 kombinasi primer diperoleh 10 kombinasi primer yang menghasilkan pita polimorfis. Pada penapisan dengan analisis hibridisasi *southern blot*, satu kombinasi yaitu CA6 menunjukkan polimorfis tingkat DNA. Analisis pemetaan menunjukkan bahwa fragment CA6 berpautan erat dengan lokus *Tm-2*. Pengujian selanjutnya menunjukkan bahwa fragment CA6 terdapat pada genom tomat yang membawa gen *Tm-2a*, genom *L. peruvianum*, dan genom tomat lain yang membawa gen yang mirip dengan *Tm-2*. Analisis ekspresi dengan metode *northern blot* menunjukkan bahwa fragment CA6 ditranskripsikan secara terus menerus.

Introduction

Resistance in tomato to *Tomato Mosaic Virus* (ToMV) is conferred by *Tm-1*, *Tm-2* and *Tm-2(2)* (Pelham, 1966; Alexander, 1971). Clayberg (1960) found that *Tm-2* confer higher levels of resistance than *Tm-1*, some ToMV strains overcome the resistance conferred by either *Tm-1* or *Tm-2*, and multiply in the resistant varieties. The *Tm-2* varieties are usually resistant to more strains of ToMV than *Tm-1* varieties (Pelham 1966). *Tm-2* and *Tm-2(2)* are allelic (Pecaut, 1965), however, they interacts differentially with the *Tomato Mosaic Virus* 30 kDa movement protein (Weber *et al.*, 2004).

The *Tm-2* is located on the long arm of chromosome 9 in a heterochromatic re-

gion close to the centromere (Khush *et al.* 1964; Pecaut 1965). It has been known that *Tm-2* gene tightly links to a gene called *netted virescent* (*nv*), which causes stunting and yellowing (Pelham 1966). In the tomato chromosomes, typical large heterochromatic regions exist around the centromeres. These heterochromatic regions have been presumed to lack of gene activities (Snoad 1963) with some exceptions. As a rare case, Khush *et al.* (1964) demonstrated that *nv* and *Tm-2* reside near the centromere in the heterochromatic region of chromosome 9.

Since the physical distance corresponding to 1 cM in the region surrounding *Tm-2* locus has been predicted to be as long as 4000 kb (Ganal *et al.*, 1989) and the region appears to be rich in repetitive sequences (Motoyoshi *et al.*, 1996), it may be difficult to isolate by map-based cloning. Instead, it may be feasible to screen a cDNA candidate corresponding to the target gene from a pool of cDNAs of transcripts from active genes in this specific region.

The differential display of transcripts from different tissues has been recently described as a powerful technique for isolating transcripts of differentially expressing genes between different genotypes, or of stage-specific genes (Liang and Pardee 1992; Liang *et al.*, 1993; Bauer *et al.*, 1993), and has been successfully used for isolating several genes of tomato (Kadyrzhanova *et al.*, 1998). This technique is based on the amplification of fragments from a first-strand cDNA using a combination of a poly-dT anchor primer and a random decamer primer (Liang and Pardee 1992).

In this study, differential display was conducted between two tomato lines GCR26 (+/+) and GCR236 (*Tm-2/Tm-2*), that originated from common genetic background of 'Craigella'. More-

over, southern blot analysis, mapping and northern blot analysis were performed to elucidate genetic properties of polymorphic fragments exhibited in differential display. The study aimed at revealing cDNA fragment linked to *Tm-2* loci in tomato and develop them as genetic marker for resistance to ToMV.

Materials and Methods

GCR26 is a tomato variety 'Craigella' that is susceptible to ToMV. GCR236 and GCR267 are homozygous for *Tm-2* and *Tm-2a*, respectively, and have a common genetic background with 'Craigella' (Smith and Ritchie, 1983). Perou2 is a line bred in France and carries a *Tm-2*-like gene that is derived from a line of *L. peruvianum* and allelic to *Tm-2* (Laterrot and Pecaat, 1969; Provvidenti and Schroeder, 1969). TMJ54-28, a line bred in Japan, also has a *Tm-2*-like gene allelic to *Tm-2* that has originated from a line of *L. peruvianum* (Yamakawa *et al.*, 1987). *L. peruvianum* P.I. 128650 is the source of *Tm-2a* that has been introgressed into tomato (Alexander, 1971).

Total RNA was isolated from fully developed leaves of 40 days old tomato seedlings by guanidium thiocyanate-phenol-chloroform extraction method (Chomeczynski and Sacchi, 1987). One gram of fresh leaf tissue was ground in liquid nitrogen with a mortar and pestle, and homogenized in 10 ml RNA isolation buffer containing: 4 M guanidium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% lauroylsarcosine and 0.1 M mercaptoethanol. One ml 2 M sodium acetate pH 4.0, 10 ml water-saturated phenol, and 2 ml chloroform: isoamyl-alcohol (24:1) was added to the homogenate successively. The mixture was vortexed and centrifuged at 10 000 g for 20 min. The lower phase was discarded, and 10 ml isopropanol was

added to the upper phase to precipitate the RNA. The RNA was pelleted by centrifugation at 3000 rpm for 10 min. The pellet was resuspended by vigorous mixing with the maximum speed of a vortex mixer in 2 ml of 4 M LiCl to solubilize polysaccharides. The insolubilized RNA was pelleted by centrifugation at 3000 rpm for 10 min, and the supernatant was discarded. The resulting pellet was redissolved in 0.6 ml 10 mM Tris, pH 7.5, 1 M EDTA, 0.5% sodium dodecyl sulfate (SDS), and 0.6 ml chloroform:isoamyl-alcohol (24:1) was added and mixed with the water phase by vortexing. After centrifugation at 15 000 rpm for 15 min, the upper phase was collected and precipitated by adding a mixture of 0.6 ml isopropanol and 60 μ l 2.0 M of sodium acetate pH 5.0.

Using 2.5 μ g of total RNA, first strand cDNA was synthesized using GT15N (N=G, A, C) as an anchor primer at a concentration of 2.5 μ M. Each reaction was performed in the presence of the four dNTPs of 0.5 μ M and 200 units MMLV reverse transcriptase (Gibco BRL) in a volume of 20 μ l at 25°C for 10 min and 42°C for 50 min. The reaction was stopped by heating at 72°C for 15 min. The reaction mixture was meshed up to 100 μ l.

A 2 μ l aliquot of the mixture containing the reverse transcript was used for PCR. The final reaction mixture of 25 μ l contained the reverse transcript as a template, 100 μ M of each dNTPs, 0.4 mM of each GT₁₅N (N=G, A, or C) primer, 0.4 mM arbitrary decamer primer (Nippon Gene) and 1 U of Gene Taq Polymerase (Nippon Gene). The reaction was carried out using a Perkin Elmer Cetus DNA thermal cycler 2400, for one cycle of 3 min at 95°C, 5 min at 40°C and 5 min at 72°C, and then for 24 cycles of 15 seconds at 95°C, 1 minute at 40°C and 2 min at 72°C, followed by a final extension step of 10 min at 72°C.

PCR amplification products were resolved in 12% denaturing polyacrylamide gels by electrophoresis and stained with ethidium bromide.

Cloning of polymorphic bands. The cDNA bands that were confirmed to be polymorphic in two trials using independently prepared RNA samples were selected for cloning. Each band was excised from a gel and placed at -20°C in a refrigerator until use. The excised band was pulverized with a micropipette tip in a 0.5 ml PCR tube, diluted with 38 μl H_2O , and vortexed vigorously. The cDNA in the diluted gel extract was further amplified by PCR using the same primer combination. The PCR was carried out in a final volume of 50 μl containing 38 μl of the diluted gel extract, 5 μl PCR buffer, 5 μl of 10 mM each dNTPs mixture, 1 μl of 10 mM anchor primer, 1 μl of 10 mM arbitrary primer, and 2 U Taq Polymerase (Perkin Elmer) by mean of a Perkin Elmer Cetus DNA thermal cycler 2400, for one cycle of 2 min pre-PCR at 95°C , and for 20 cycles of 15 sec at 95°C , 1 min at 40°C and 2 min at 72°C , followed by the final extension step of 7 min at 72°C .

The terminal ends of the amplified fragment were filled with T4 DNA polymerase (Toyobo), and phosphorylated with T4 polynucleotide kinase (Toyobo). The blunt ends of the fragments were ligated to the *Sma*I site of a pBluescript II SK vector (Stratagene).

Southern hybridization analysis. Extraction of genomic DNA for Southern hybridization was performed by using method of Doyle and Doyle (1987) with slight modification. A total genomic DNA sample of 3 μg was digested with a restriction enzyme for overnight, and electrophoresed in a 1.0% agarose gel. The gel was soaked for 30 min in 0.5 N NaOH containing NaCl 1.5 M at with constant and gentle agitation for DNA

denaturation, followed by blotting DNA bands to nylon membranes (Hybond-N, Amersham) by capillary transfer with alkaline solution (1.0 M NH_4COOH , 20 mM NaOH) overnight as described by Kreike *et al.* (1990). After blotting, the DNAs were fixed by baking at 80°C for 2 hours.

The membrane was prehybridized in hybridization solution containing 50% (v/v) formamide, 5XSSC, 0.05 M Naphosphate pH 7.0, 0.1% (w/v) lauroyl-sarcosine, (w/v) 2% blocking reagent, 0.5% (w/v) yeast RNA, and 7% (w/v) SDS at 42°C for 1 hour. After boiling for denaturation, the DIG-labeled (Boehringer Mannheim) probe was added into the hybridization buffer and the blots were hybridized overnight at 42°C . The membrane was washed twice for every 5 min at room temperature with 2XSSC and 0.1%SDS, and twice for every 15 min at 50°C with 0.5X SSC containing 0.1%SDS. After washing, the membrane was equilibrated in buffer I [0.1 M maleic acid, 0.15 M NaCl and 0.3% (v/v) tween] for 5 min, and submerged in anti-DIG solution [1% (w/v) blocking reagent and 0.01% (v/v) anti-Digoxigenin-AP (Boehringer Mannheim) in buffer I] for 1 hour at room temperature. The membrane was washed twice with buffer I. To develop chemical luminescent signals, the membrane was submerged in CSPD (Boehringer Mannheim) solution and exposed to an X-ray film for 2 hours.

Mapping. An F₂ population consisting of 108 plants derived from a cross between GCR236 (*Tm-2/Tm-2, nv/nv*) and GCR26 (+/+, +/+) was analyzed for the linkage relationship of the locus corresponding to a cDNA fragment CA6 to the *nv* locus and three SCAR markers that tightly linked to the *Tm-2* (Sobir *et al.* 2000). Genomic DNA was extracted by the method of Murray and Thompson (1980) with slight modifications. Approximately 0.1 mg fresh

leaf tissue was frozen in liquid nitrogen, and pulverized with a plastic micro-pipette tip in a 1.5 ml Eppendorf tube, and 400 μ l of the extraction buffer [100 mM Tris-HCl pH 8.0, 0.35 M sorbitol, 5 mM EDTA and 10% (v/v) 2-mercapto-ethanol] was added to each of the sample. The samples were centrifuged for 15 min at 4000 rpm at room temperature. The pellet was resuspended in 200 μ l of extraction buffer containing 1.0% (w/v) CTAB (hexadecyltrimethylammonium bromide), 1 M NaCl, 25 mM EDTA, and 1.0% (w/v) lauroyl-sarcosine, and incubated at 60°C for 20 min. The lysate was extracted once with chloroform/isoamylalcohol (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 50 μ l TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA) containing RNase A at 10 μ g/ml.

Northern hybridization analysis. For RNA gel blot hybridization, an aliquot of total RNA was denatured and electrophoresed (10 μ g per lane) in a denaturing gel (1.2% agarose, 0.2 M formaldehyde). The gel was blotted onto Hybrid-N⁺ nylon membranes (Amersham) with 20XSSC, and then the RNA was fixed to the membrane by baking at 80°C for 2 hours. The membrane was prehybridized for 1 hour in hybridization buffer containing 0.25 M Na₂HPO₄, 1 mM EDTA, 20% SDS and 0.5% blocking reagent (Boehringer Mannheim). After denaturation by boiling, the DIG-labeled (Boehringer Mannheim) probe was added to the hybridization buffer and the blot was hybridized overnight at 42°C. After hybridization, the membrane was washed twice with 20 mM Na₂HPO₄, 1 mM EDTA and 1% SDS at 65°C for 15 min. After washing, the membrane was submerged in anti-DIG solution for 1 hour at room temperature, washed twice

with a solution containing 0.1 M maleic acid, 3 M NaCl and 0.3% tween. To develop chemical-luminescent signals, the membrane was submerged in CSPD solution and exposed for to an X-ray film for 2 hours.

Results

Differential display. The 150 combinations of 3 anchor primers and 50 arbitrary primers produced 2742 discrete PCR products, with an average of 18.28 products per primer combination. The size of amplified fragments ranged from 100 to 1000 bp, with majority of 150–300 bp. Most of the products were common between GCR26 (+/+) and GCR236 (*Tm-2/Tm-2*), while 41 primer combinations (27%) produced polymorphic bands between NILs. Out of them, some fragments amplified by 39 combinations were apparently specific to GCR236, while those by two combinations were to GCR26 (Table 1).

Since the reaction for differential display was reported often to produce artifacts (Liang *et al.*, 1993), we did the differential display again using independently prepared RNA samples and the primer combinations that generated polymorphism in the first trial. In this step, we found that 10 primer combinations (6.7%) reproduced polymorphic bands, which appeared to be identical to those produced in the first trial. From those 10 combinations, the bands produced with the nine combinations were specific to GCR236 and that with one combination was specific to GCR 26 (Table 1). The fragment size ranged from 150 to 300 bp. To verify specificity of these cloned cDNA fragments to GCR236, cross hybridization of blots of the cDNA clones with the DIG-labeled GCR26 RNA was performed. The clones that were not hybridizes with the GCR26 RNA were chosen for genomic Southern analysis.

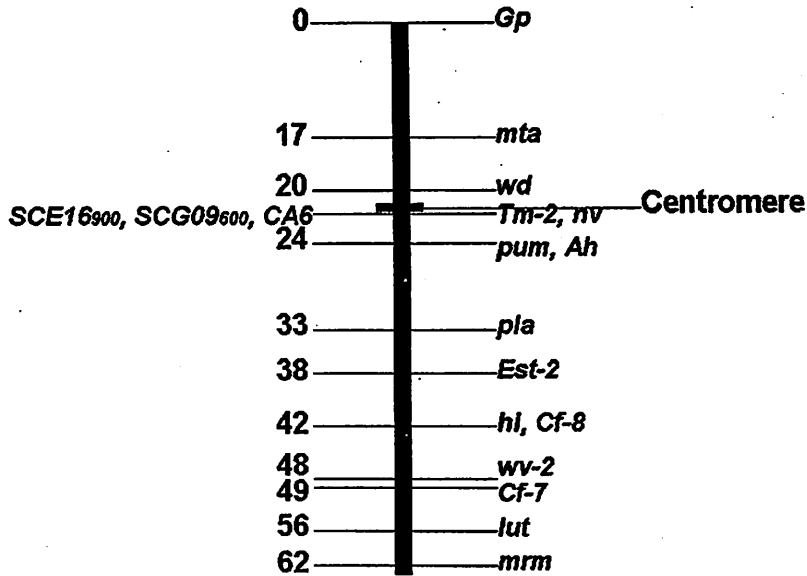


Figure 1. Mapping of the site corresponding to the CA6 clone to tomato chromosome 9.

Table 1. Primer combinations produced polymorphic bands in two trials of differential displays.

Anchor primer	Arbitrary primer	
	First trial (RNA Sample I)	Second trial (RNA Sample II)
GT15A	A2, A3, A5, A6, A7, A10, A13, A20, A25, B2, B8, B18	A2, A10, A13
GT15C	A6, A7, A11, A14, A16, A19, A20, A25, B1, B2, B6, B7, B10, B15, B17, B21, B23, B25	A6, A7, A11 B6
GT15G	A2, A10, A11, A13, A18, A24, B6, B14, B23	A2, A7 A10

Southern analysis. In order to examine whether the polymorphic fragments

exhibited in the differential display are products from genes in the introgressed segment on chromosome 9, the 10 clones were labeled with DIG, and were hybridized to blots of restriction-digested genomic DNAs from GCR26 and GCR236.

The Southern hybridization revealed that there were one to four copies of the sequences homologous to the cDNA fragments in both of the NILs. Out of them, three clones, AA13, CA6 and GA10, hybridized to one copy, five clones, AA2, AA10, CB6, CA7 and GA2, to two copies, one clone, CA11, to three copies, and one clone, GA7, to four copies of homologous sequences. The CA6 clone hybridized to restriction enzyme-digested fragments of different sizes, one specific to GCR236 and the other to GCR26, i.e. this clone exhibited a restriction fragment length polymorphism (RFLP) between the two tomato lines. With the other clones, no RFLPs were detected between the NILs.

Mapping of a sequence corresponding to CA6 cDNA fragment. For further confirmation that the CA6 fragment was derived from the introgressed region surrounding the *Tm-2* locus, a pair of 24-mer primers specific to CA6 fragment was designated. The sequences are:

CTTTCGTCTGTGTCA
ATCTGTAGT (CA6A) and
CACCCGACCCTGAGCCTAC
ATTAC (CA6B).

This primer pair produced a single band specific to GCR236. In linkage analysis, the locus corresponding to the CA6 fragment was proved to be tightly linked to the *nv* locus and the three SCAR markers SCE16900, SCG09700 and SCN131000 (Sobir *et al.*, 2000), suggesting that the locus corresponding to the CA6 fragment (the CA6 locus) resides in the introgressed region surrounding the *Tm-2* locus (Table 2, Figure 1).

Detection of loci homologous for the CA6 locus. To know whether the CA6 locus is conserved in the other tomato lines, TMJ54-28 (homozygous for *Tm-2* like gene), GCR267 (*Tm-2a/Tm-2a*) and *L. peruvianum* P.I.128650 (*Tm-2a/Tm-2a*), Southern hybridization analysis was carried out. Their genomic DNAs were digested with *EcoRV* that generated a distinct polymorphic band specific to GCR236. The DNA blots

were hybridized with DIG-labeled CA6 fragment. As a result, GCR267 and *L. peruvianum* P.I.128650 showed a signal of the same size as that of GCR236, while TMJ54-28 showed a single signal that was polymorphic to those in GCR26 and GCR236 (Figure 2).

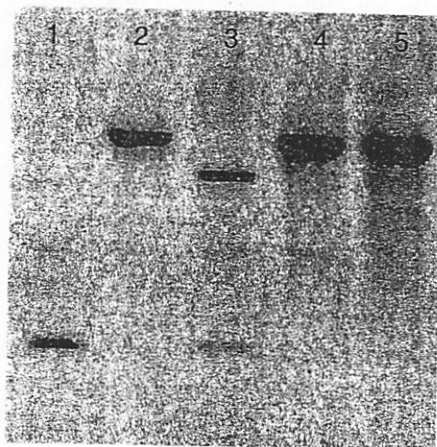


Figure 2. Southern hybridization to genomic DNA of tomato GCR26 (1), GCR236 (2), TMJ54-28 (3), GCR267 (4) and *L. peruvianum* P.I. 128650 (5), digested with *EcoRV* and hybridized to CA6 mRNA fragment.

Expression pattern of the CA6 locus. In order to identify whether the CA6 locus is constitutively transcribed or alternatively its transcription is induced by ToMV infection, cotyledons of GCR267 (*Tm-2a/Tm-2a*) which is

Table 2. Phenotype frequencies and χ^2 values for goodness of fit to expected ratios 3:1 (χ^2), 9:3:3:1 (χ^2_{AB}), and linkage (χ^2_L) between the CA6 locus and *nv* or each SCAR marker.

Locus		Phenotype				χ^2	χ^2_{AB}	χ^2_L
A	B	AB	Ab	aB	ab			
CA6	<i>nv</i>	74	0	0	30	0.82	128.07**	99.18**
CA6	SCE16900	74	0	0	30	0.82	128.07**	99.18**
CA6	SCG09700	74	0	0	30	0.82	128.07**	99.18**
CA6	SCN131000	74	0	0	30	0.82	128.07**	99.18**

resistant to Ltb1, a ToMV strain, were inoculated with this strain or mock-inoculated with 50 mM phosphate buffer without ToMV. The inoculated and mock-inoculated plants were maintained at 25°C. The cotyledons were harvested 48 hours after inoculation, and total RNAs were extracted from them. The Northern hybridization of the RNAs with the DIG labeled CA6 fragment as a probe (Figure 3) showed that the levels from the gene corresponding to the CA6 locus were similar in the inoculated and mock-inoculated cotyledons, suggesting that the CA6 locus is constitutively transcribed.

ToMV Mock

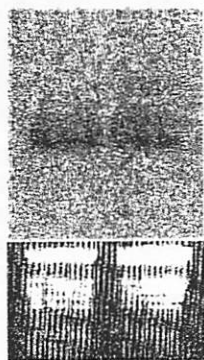


Figure 3. Northern analysis of CA6 mRNA fragment into total RNAs from ToMV-inoculated and mock-inoculated cotyledons 48 hours after inoculation.

Discussion

In differential display, 10 combinations of random and anchor primers amplified reproducible polymorphic fragments, but Southern hybridization of genomic DNAs with one fragment (CA6) only produced RFLP between GCR236 and GCR26. These results suggested that the CA6 locus is present in the region surrounding the *Tm-2* locus. The loci corresponding to the other nine cDNA fragments might locate also in this region, although they showed no RFLPs.

The region surrounding the *Tm-2* locus in GCR236 might be less heterochromatic than the homologous region in GCR26, since nine of the ten cDNAs differentially displayed is specific to GCR236 and only one is to GCR26. Khush *et al.* (1964) speculated that the loci themselves of active genes are not heterochromatic, though they exist in a heterochromatin. These 10 cDNA fragments however do not necessarily correspond to all 10 active genes; i.e. more than one fragment might be amplified from different parts of one locus as indicated by Habu *et al.* (1997).

The CA6 fragment corresponds to a single copy locus. The proposal of Khush *et al.* (1964) suggests a possibility that the CA6 cDNA fragment is available for a specific marker to represent an euchromatic part in a heterochromatic region. In addition the CA6 cDNA fragment can be used as a co-dominant RFLP marker, especially for differentiating the *Tm-2*-like gene in TMJ54-28 from *Tm-2* and *Tm-2a*.

Conclusions

Differential Display technique on two near isogenic lines of GCR26 (+/+) and GCR236 (*Tm-2/Tm-2*) of tomato, resulted in a CA6 cDNA fragment that exhibited a polymorphic band under Southern blot analysis, and tightly linked to the *nv* and *Tm-2* locus.

The CA6 fragment hybridized to genomic DNA fragments from a tomato line carrying *Tm-2a*, a line of *L. peruvianum* from which *Tm-2a* originated, and a tomato line carrying another *Tm-2*-like gene.

Northern hybridization analysis results suggested that the gene corresponding to the CA6 fragment was constitutively transcribed.

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