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Cloning and characterization of the durable tomato mosaic virus resistance gene *Tm-2²* from *Lycopersicon esculentum*

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Abstract

In tomato, infections by tomato mosaic virus are controlled by durable *Tm-2²* resistance. In order to gain insight into the processes underlying disease resistance and its durability, we cloned and analysed the *Tm-2²* resistance gene and the susceptible allele, *tm-2*. The *Tm-2²* gene was isolated by transposon tagging using a screen in which plants with a destroyed *Tm-2²* gene survive. The *Tm-2²* locus consists of a single gene that encodes an 861 amino acid polypeptide, which belongs to the CC-NBS-LRR class of resistance proteins. The putative *tm-2* allele was cloned from susceptible tomato lines via PCR with primers based on the *Tm-2²* sequence. Interestingly, the *tm-2* gene has an open reading frame that is comparable to the *Tm-2²* allele. Between the tm-2 and the *Tm-2²* polypeptide 38 amino acid differences are present of which 26 are located in the second half of the LRR-domain. Susceptible tomato plants, which were transformed with the *Tm-2²* gene, displayed resistance against ToMV infection. In addition, virus specificity, displayed by the *Tm-2²* resistance was conserved in these transgenic lines. To explain the durability of this resistance, it is proposed that the *Tm-2²*-encoded resistance is aimed at the Achilles' heel of the virus.

Abbreviations: Avr, Avirulence; CaMV, cauliflower mosaic virus; CC domain, coiled coil domain; cM, centiMorgan; CP, coat protein; LRR, leucine-rich repeat; LZ, leucine zipper; MP, movement protein; NB-ARC domain, Nucleotide Binding site, Apoptosis, *R* gene products and CED-4 domain; NBS, nucleotide binding site; R, resistance; ORF, open reading frame; RACE, rapid amplification of cDNA ends; RdRP, RNA-dependent RNA polymerase; (s)Ac, (stabilized) Activator; TMV, tobacco mosaic virus; ToMV, tomato mosaic virus

Introduction

Many vegetable and ornamental crops can be infected by tobamoviruses. Infections usually give rise to characteristic mosaic symptoms and lead to considerable cosmetic damage and yield losses. Tobamoviruses, including tobacco and tomato mosaic virus (TMV and ToMV, respectively) belong to the α-like supergroup of viruses. They consist of a characteristic protein-aceous rod, made up of 2140 coat protein (CP) copies, which envelop the positive-stranded linear RNA genome. After infection of the plant cell the RNA genome is uncoated and the viral gene products, the RNA-dependent RNA polymerase (RdRP), the movement protein (MP) and the coat protein (CP), are produced. Infection of neighbouring cells commences with the movement of RNA-MP complexes through plasmodesmata with MP-induced altered size exclusion limits

(Citovsky and Zambryski, 2000). Long-distance transport of the virus proceeds through the vascular tissue and depends both on MP and CP. Tobamoviruses, especially TMV, are the classical model system for the study of virus infection in plants (Zaitlin and Palukaitis, 2000).

In cultivated tomato, ToMV infections are controlled by the introgressed *Tm-1*, *Tm-2* and *Tm-2^2* resistance (*R*) genes (Pelham, 1966; Hall, 1980). Among these resistances the *Tm-2^2* resistance has shown to be remarkably durable and, therefore, of ongoing practical importance. The few *Tm-2^2* resistance-breaking ToMV isolates ever obtained are crippled and less fit and virulent than wild-type isolates (Fraser *et al.*, 1989). Furthermore, infection with a *Tm-2^2*-overcoming virus strain results in visual distortion of the affected plant, which allows early preventive removal of this plant. Hence, the *Tm-2^2*-breaking virus strains are unable to establish themselves in the virus population and are not capable of becoming a threat to the durability of the *Tm-2^2* resistance. On the other hand, the often naturally occurring ToMV strains that are able to overcome *Tm-1* and *Tm-2* resistance do not show a reduction in virulence; thus, these two resistances are not durable and have lost their practical importance. The 'durability' concept describes the ability of a resistance to remain useful in crop cultivation for a long period of time without pathogen strains appearing which are able to overcome the resistance.

The *Tm-1 R* gene was introgressed from the wild tomato species *Lycopersicon hirsutum* and mapped to chromosome 5. Genetic analysis of ToMV strains capable of overcoming this resistance has shown that the matching *Avirulence* (*Avr*)-gene of ToMV is the *RdRP* gene (Meshi *et al.*, 1988). Both the *Tm-2* and the *Tm-2^2* resistances were introgressed from *L. peruvianum*. They are both located close to the centromer of chromosome 9 and are considered to be allelic (Khush *et al.*, 1964; Pelham, 1966; Schroeder *et al.*, 1967; Hall, 1980; Tanksley *et al.*, 1992). *Tm-2^2* resistance is typified by the absence of macroscopical local lesions during the resistance response. This absence is interpreted as *Tm-2^2* responding rapidly to the infection by ToMV so virus infection is restricted to only a few cells as is the hypersensitive response and lesion formation. Analysis of the nucleotide sequence of virus strains, which were capable of overcoming the *Tm-2^2* resistance, revealed that the MP of ToMV is the matching Avr protein (Calder and Palukaitis, 1992; Weber *et al.*, 1993; Weber and Pfitzner, 1998).

Molecular isolation of the *Tm-2^2* gene via map-based cloning has been shown to be difficult, especially due to the lack of recombination in the centromeric region (Tanksley *et al.*, 1992; Ohmori *et al.*, 1995, 1998; Motoyoshi *et al.*, 1996; Pillen *et al.*, 1996; Sobir *et al.*, 2000). Here, we report on the isolation of the *Tm-2^2* gene via transposon tagging and its characterization. We also isolated the allele from susceptible *L. esculentum* lines, *tm-2*, via PCR with primers developed on the basis of the sequence of the *Tm-2^2* gene.

Materials and methods

Transposon tagging experiment

The two-component Ac/Ds transposon system was utilized to isolate the *Tm-2^2* gene of tomato. For this purpose a tomato line *Ds$_{13\text{-}15}$* was available which has a *Ds$_{13\text{-}15}$* transposon on chromosome 9 (Gidoni *et al.*, 2003; Table 1) that also harbours the *Tm-2^2* gene. The distance between the transposon and the resistance gene was approximated to be 2 cM by back-crossing tomato genotype *Ds$_{13\text{-}15}$* twice with tomato genotype ATV840 homozygous for *tm-2* (Table 1).

In order to obtain an activated transposon it was necessary to introduce a stabilized Activator (*sAc*) in the tomato genotype *Ds$_{13\text{-}15}$*. For this purpose genotype ATV847 (homozygous *Tm-2^2*; Table 1) was crossed with genotype MMSLJ10512 (homozygous for *sAc* (Takken *et al.*, 1998; Table 1). Selfings from the progeny of this cross were selected for homozygosity of both *sAc* and *Tm-2^2* via PCR (Table 2). One of these plants that was designated *TmSLJ*, was subsequently used in a cross with genotype *Ds$_{13\text{-}15}$*. Progeny of this cross was selected via PCR for the presence of *Ds$_{13\text{-}15}$*. Finally, about 100 independent plants with the genotype *Ds*, –; *sAc*, –; *Tm-2^2*, *Tm-2^2* were selected and used as males and females in a cross with transgenic tomato line ATV 840-4230-2, which is homozygous for the ToMV *MP* gene (Table 1) for a large-scale tagging experiment.

DNA isolation, DNA blot analysis, gene rescue, and cloning

Tomato DNA was isolated according to van der Beek *et al.* (1992) and digested with the indicated restriction enzymes. DNA blot hybridization and labelling of the DNA probes were performed according to standard procedures (Sambrook *et al.*, 1989). As

Table 1. Tomato accessions used in this study.

Accession	Genotype
MoneyMaker-vir	*Tm-2²*, *Tm-2²*
Ds_{13-15} (MoneyMaker-vir; (Gidoni *et al.*, 2003))	*Ds*, *Ds*; *Tm-2²*, *Tm-2²*
ATV840 (Syngenta Seeds)	*tm-2*, *tm-2*
ATV847 (Syngenta Seeds)	*Tm-2²*, *Tm-2²*
ATV 840-4230-2 (Syngenta Seeds)	*ToMV-MP*, *ToMV-MP*; *tm-2*, *tm-2*
MMSLJ10512 (Takken *et al.*, 1998)	*sAc*, *sAc*; *tm-2*, *tm-2*
TmSLJ	*sAc*, *sAc*; *Tm-2²*, *Tm-2²*
GCR26 (Craigella)	*tm-2*, *tm-2*
GCR267 (Craigella)	*Tm-2²*, *Tm-2²*
Stevens	*Sw5*, *Sw5*; *tm-2*, *tm-2*

Table 2. PCR primers used in this study and their target sequences.

Target	Name	Primer	Direction
sAc	PrRuG003	5′-CGTCCTGTAGAAACCCCAACC-3′	F
	PrRuG004	5′-CGGCGTGGTGTAGAGCATTAC-3′	R
Tm-2²	PrRuG024	5′-GTCAAGTAAGATTTCACTCGCTCTTGGC-3′	R
	PrRuG053	5′-GTAACTGAGAGCCAATACCTTAGCACATCC-3′	R
	PrRuG084	5′-CTTGACAAGACTGCAGCGAGTGATTGTC-3′	F
	PrRuG086	5′-CTACTACACTCACGTTGCTGTGATGCAC-3′	R
	PrRuG151	5′-GAGTTCTTCCGTTCAAATCCTAAGCTTG AGAAG-3′	F
	PrRuG325	5′-CTCATCAAGCTTACTCTAGCCTAC-3′	F
*Nco*I-introducing	PrRuG097	5′-TTTTCCATGGCTGAAATTCTTCTTACATCAGTAATCAATAAATCTG-3′	F
*Nco*I-introducing	PrRuG102	5′-CTGACCTGCCATGGTGTTCATTTACTCAGCTTTTTAAGCC-3′R	

Underlined residues indicate introduced mutations.

probes were used an Ori probe specific for the origin of replication, which is present in the *Ds_{13-15}* transposon (Takken *et al.*, 1998), and a *Tm-2²*-specific probe generated from a *Hin*dIII/*Hin*dIII fragment from the *Tm-2²* gene (Figure 1B). Hybridization was under stringent conditions: in 7% w/v sodium dodecyl sulfate (SDS), 0.5 M sodium phosphate buffer pH 7.2, 1 mM Na_2EDTA and 1% w/v bovine serum albumin, at 60 °C. After a short rinse in 2× SSC (0.3 M NaCl, 0.03 M sodium citrate.$2H_2O$) membranes were washed for 10 min in 2× SSC followed by 5 min in 0.1% w/v SDS and 0.1× SSC at 60 °C. Hybridizing bands were visualised with an Optiquant phospho-imager.

The flanking plant DNA sequences of the transposed Ds_{13-15} element from mutant plants were rescued according to Rommens *et al.* (1992). Genomic tomato DNA of the putative mutant plants was digested with either *Bam*HI or *Sac*I, which allows the isolation of plant DNA from both sides of the transposon. The digestion products were circularised with T4 ligase and then chemically transformed into Epicurian Coli XL-10 Gold Ultracompetent Cells (Stratagene) following the instructions of the supplier's manual.

Isolation of the alleles tm-2 *and* Tm-2^2 *from other* Lycopersicon *accessions*

The additional *Tm-2²* and *tm-2* alleles were isolated by PCR from the tomato lines ATV840 (*tm-2*), ATV847 (*Tm-2²*), Stevens (*tm-2*), GCR26 (*tm-2*), and GCR267 (*Tm-2²*) with gene-specific primers (PrRuG84 and PrRuG86; Table 2), which were developed with the sequence of *Tm-2²* from *L. esculentum* genotype Ds_{13-15}. The recessive wild-type allele that does not confer resistance was named *tm-2*, based on the presumed allelicity of *Tm-2* and *Tm-2²*, the absence of differences between this allele isolated from different sources (see below) and the presence of a single

copy of the *Tm-2²* gene in the tomato genome (see below). PCR was performed on genomic DNA with Platinum Taq (Life Technologies) or Ex Taq (TaKaRa Bio). Three independent PCR products for each allele were cloned into the pGEM-T-Easy Vector (Promega) and their nucleotide sequences determined.

5′ and 3′ RACE

Total RNA was isolated from tomato genotype ATV847 with the RNeasy Plant mini kit from Qiagen, according to the supplier's manual. The 5′ and 3′ rapid amplification of cDNA ends (RACE) were performed with relevant products from Life Technologies, 5′ and 3′ systems for RACE as described by the supplier's manual. Next to the primers supplied with the system the following gene-specific primers were used: PrRuG86 and the nested primers PrRuG53 and PrRuG24 (Table 2). For the 3′ RACE cDNAs were synthesized with Superscript II RT and oligo(dT)-containing Adapter Primer (Life Technologies). Next the cDNA was PCR-amplified with, next to the primers supplied with the system, the gene-specific primer PrRuG151 and the nested gene-specific primer PrRuG325 (Table 2). The amplified PCR products of both 5′ and 3′ RACE were ligated into the pGEM-T-easy vector and their nucleotide sequences determined.

Introduction of the Tm-2² *resistance gene into a susceptible* L. esculentum

Two types of recombinant binary vectors were constructed to transfer the *Tm-2²* resistance gene into the genome of susceptible ATV840 plants: a binary vector in which the *Tm-2²* gene was under the control of its own promoter and polyadenylation signal and a binary vector in which the *Tm-2²* gene is under the control of the CaMV 35S promoter and the NOS polyadenylation signal.

The binary vector with the *Tm-2²* gene under the control of its own promoter and polyadenylation signal sequences (the native gene) was constructed with the rescued plasmids. A fragment carrying the 3′ end of the *Tm-2²* gene was excised from the plasmid pS1 by digesting with *Xho*I and *Sac*I and subsequently cloned into pBluescript (Stratagene) resulting in the plasmid pBlueCterm. Subsequently, both pBlueCterm and the plasmid pB1 were digested with *Xho*I and the *Xho*I fragment from pB68 carrying the 5′ end of *Tm-2²* gene was ligated into pBlueCterm. The relative orientation of the 5′ end and the 3′ end of the *Tm-2²* gene were checked by PCR and digestion analysis. The resulting plasmid, named pTm2²:Ds, still comprises sequences originating from the *Ds* transposon in the *Tm-2²* gene. With primers PrRuG84 and PrRug86 (Table 2) a PCR product was amplified from genomic DNA of the tomato line $Ds_{13\text{-}15}$. The obtained PCR product and pTm2²:Ds were digested with *Aat*II and *Nhe*I and followed by the replacement of the *Aat*II/*Nhe*I fragment of pTm2²:Ds with the fragment of the PCR fragment, resulting in plasmid pTM7. In this plasmid the complete and intact *Tm-2²* ORF with 750 bp of the *Tm-2²* promoter and 1000 bp of the *Tm-2²* polyadenylation signal are present. Plasmid pTM7 was digested with *Sac*I and *Xho*I and the *Tm-2²* gene was cloned into pZO1560, a pBluescript derivative in which the original multiple cloning site was replaced by the AGLINK multicloning site (Syngenta Seeds) with its *Sac*I and *Sal*I sites. The resulting plasmid pTM9 was digested with *Pac*I and *Asc*I and the *Tm-2²* gene was cloned into the binary vector pVictorHiNK (Syngenta Seeds) resulting in plasmid pTM35. This binary vector contains resistances against kanamycin and spectinomycin as selective markers.

The binary vector with the *Tm-2²* gene under the control of the CaMV 35S promoter and the NOS polyadenylation signal (the chimeric gene) was constructed with a PCR-amplified ORF in which two *Nco*I sites were introduced. First, the NOS polyadenylation signal from plasmid pZU442 (Syngenta Seeds) was transferred to plasmid pZO1560 with *Pst*I and *Hin*dIII, resulting in plasmid pTM1. Next the CaMV 35S promoter from pZU442 was transferred into this plasmid with *Bam*HI and *Nco*I which resulted in plasmid pTM6. With primer PrRuG97 and PrRuG102 (Table 2) a PCR product containing the complete ORF of *Tm-2²* with an introduced *Nco*I site at the ATG and an introduced *Nco*I site 11 bp downstream of the TGA, was amplified from genomic DNA of tomato line ATV847. The PCR product was digested with *Nco*I and this fragment was introduced into the *Nco*I site of pTM6. The orientation of the ORF relative to the promoter and polyadenylation signal was checked by digestion and the plasmid was named pTM40. pTM40 was digested with *Bam*HI and *Xba*I and the chimeric *Tm-2²* gene was cloned into the binary vector pVictorHiNK, resulting in plasmid pTM42.

The plasmids pTM35 and pTM42 were introduced into *Agrobacterium tumefaciens* strain LBA4404 by electroporation. Transformants were selected on L-broth plates. Subsequently, the transformants were checked for carrying unaltered gene constructs and

used to transform tomato line ATV840 leaf explants as described essentially by Horsch *et al.* (1985).

Virus resistance assays

Individual plants or three 30-day old cuttings of the independent transgenic plants of tomato line ATV840 were inoculated with leaf homogenates of *Nicotiana tabacum* SR1 plants infected with the various isolates of ToMV (see legend to Table 4), which were diluted in 10 mM sodium phosphate buffer pH 7.0 containing 1% Na_2SO_3. For the transformants untransformed plants were used as controls for virus inoculation. The plants were all inoculated twice at a four-day interval to rule out random escape from inoculation. Virus symptoms were monitored on a daily basis for the duration of the experiment (21 days). After 21 days a leaf homogenate of a non-inoculated leaf of tomato was inoculated onto *N. glutinosa* and lesions were scored after 3 to 4 days.

Analysis software

Sequences were analysed with ClustalW (Thompson *et al.*, 1994), Clone Manager Software (Scientific and Educational Software) and Blast (Altschul *et al.*, 1990) software.

Accession numbers

The accession numbers for the *Lycopersicon tm-2* gene and the *Tm-2*^{2} locus are AF536199 and AF536201, respectively.

Results

Acquisition of transposon tagged Tm-2^2 *mutant plants and their characterization*

For selection for a transposon tagged *Tm-2*^{2} gene a stringent selection procedure was necessary. The observation of Weber and Pfitzner (1998), who showed that the cross between tomato plants containing the *Tm-2*^{2} gene and transgenic tomato plants expressing the *ToMV-MP* gene results in a progeny which dies in the seedling stage, was used to develop a selection method. The rationale behind the selection method is that when the *Tm-2*^{2} gene is disrupted by a transposon insertion event, seedlings containing the otherwise lethal combination of the *Tm-2*^{2} gene and the *ToMV-MP* transgene will survive. In these survivors the resistance gene will be tagged by the transposon and isolation of the disrupted *R* gene will become possible.

In the tagging experiment 100 independent plants with the genotype *Ds*, –; *sAc*, –; *Tm-2*^{2},*Tm-2*^{2} were crossed with line ATV 840-4230-2 (Table 1; see Materials and methods). From about 30 000 seeds obtained from these crosses, 5 putative mutants were obtained. All contained the *Ds* element and the *MP* gene and only mutant plant 108 still contained *sAc.* To test whether these 5 putative mutants were really mutants in the *Tm-2*^{2} gene, cuttings of these plants were inoculated with ToMV. All the 5 putative mutants were susceptible for ToMV infection and further analysed.

In the surviving mutant plants, excision and reinsertion of the Ds_{13-15} could be demonstrated by DNA blot analysis with a radioactively labelled DNA fragment corresponding with the origin of replication, which is present in the transposon, as a probe (Figure 1A). As expected transposon-specific DNA fragments were absent in the tomato plants without the $DS_{13\text{-}15}$ transposon (exemplified by TmSLJ), whereas the tomato accession Ds_{13-15} shows the original position of the *Ds* transposon. In the surviving mutant plants two different hybridization patterns were observed on the DNA blots (Figure 1A). Putative mutant plants 65, 68, 107 and 108 (group 1 mutants) displayed all a similar pattern that was indicative for having a transposon at its original position and another one at a new position. Putative mutant plant 144 (a group 2 mutant) has only one copy of the transposon at a position different from both the original and the new position of the group 1 mutants.

Nucleotide sequence of the transposon flanking plant DNA

Plasmid rescue was used to obtain transposon-flanking plant DNA from the mutant plants. The plasmids pB1 and pS1, which were isolated with *Bam*HI and *Sac*I from group 1 mutants, yielded 7.5 kb and 2.3 kb of plant DNA, respectively. The plasmids pB2 and pS2, which were rescued from the group 2 mutant plant, yielded 6.9 kb and 1.5 kb of plant DNA respectively. All four plasmids were sequenced and it became evident that plasmids pB1, pS1 and pS2 were mutually related. An 8 bp repeat typical of the insertion of a *Ds*-type transposon could be recognized in pB1 and pS1, while pS1 and pS2 contained identical plant-derived sequences. Using the 8 bp repeat and the sequences of the pB1, pS1 and pS2 plasmids a strand of 9.8 kb

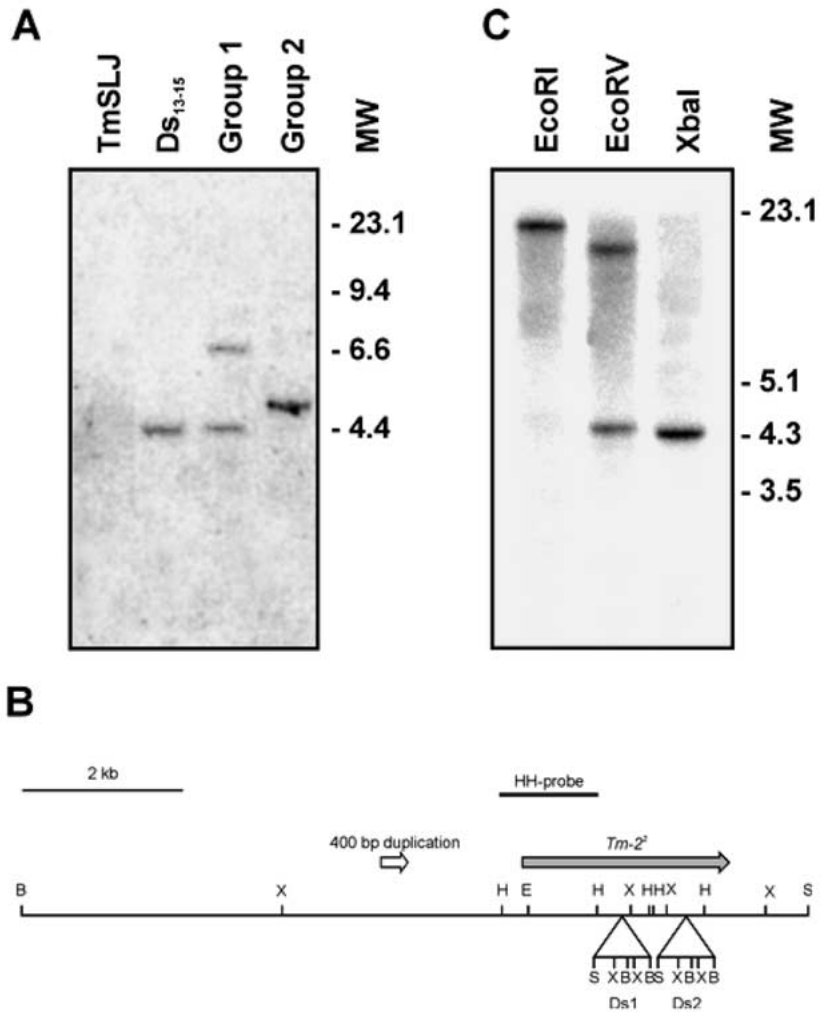


Figure 1. Characterization of plants used to isolate the *Tm-2*^{2} gene. A. DNA blot analysis of the parental tomato lines, *Ds*$_{13\text{-}15}$ and TmSLJ, with and without the transposon, respectively, and representatives of the Group 1 and Group 2 mutant plants. Genomic DNA was digested with *Xba*I and separated on a 0.7% agarose gel. After transfer onto a membrane DNA was probed with an Ori-probe (Takken *et al.*, 1998). Molecular weights of the marker are indicated on the right. B. Schematic representation of the 9.8 kb of rescued plant DNA. The grey arrow indicates the observed ORFs and its direction of transcription. The white arrow indicates the location of the duplication. The triangles, marked Ds1 and Ds2, indicate the positions of the transposon insertion sites of the group 1 and 2 mutants, respectively. The transposons are not drawn on scale (size of the *Ds*$_{13\text{-}15}$ transposon is 6.9 kb). Locations of restriction sites are indicated by their first letter: H, *Hin*dIII; B, *Bam*HI; E, *Eco*RV; S, *Sac*I; X, *Xba*I. The bar marked HH-probe indicates the *Hin*dIII/*Hin*dIII fragment used as probe in DNA blot analysis (C). C. DNA blot analysis of the tomato accession ATV847 with the genotype *Tm-2*^{2}, *Tm-2*^{2}. Genomic DNA was digested with the indicated restriction enzymes and separated on a 0.7% agarose gel. After transfer to a membrane DNA was probed with a HH probe (see B). Molecular weights of the marker are indicated on the right.

of continuous plant-derived DNA could be created (Figure 1B).

Analysis and sequencing of the pB2 plasmid revealed that the plant DNA rescued in this plasmid had no obvious relation with the other three rescued plasmids. Also, no relation with other sequences in the databases was observed. However, because only one *Ds*-type transposon was present in the genome of the group 2 mutant plant it was assumed that the pB2 plasmid represented one of the flanking regions of the inserted transposon in the group 2 mutant plant and that in this mutant plant a deletion or re-arrangement of unknown size has occurred that resulted in the loss of the 75% of the 5′ end of the *Tm-2*^{2} gene.

The 9.8 kb of continuous plant DNA contains an open reading frame (ORF) of 2586 bp (Figure 1B),

which corresponds with a polypeptide of 861 amino acids. *In silico* analysis of the ORF did not predict the presence of introns. The polypeptide encoded by the 2586 bp ORF shows the characteristics of the CC-NBS-LRR class of R proteins (see below). This, in combination with the facts that this ORF was disrupted in both groups of mutant plants and that in both groups of mutant plants resistance was lost, strongly suggests that the *Tm-2*^{2} gene has been identified. It also became clear that ca. 1700 bp upstream of the *Tm-2*^{2} gene a partial duplication of the gene is present (Figure 1B). This duplication has a size of ca. 400 bp and showed homology with part of the promoter region and the first 245 bp of the *Tm-2*^{2} ORF. It contains the translation initiation codon but, due to the presence of a translation stop, it will, if translated, only give rise to a polypeptide of 49 amino acids. DNA blot analysis of genomic DNA digested with the restriction enzymes *Eco*RI, *Eco*RV and *Xba*I, with as probe a *Hin*dIII fragment of the rescued plant DNA (Figure 1C), showed only one to two intensely hybridizing fragments. The sizes of these fragments were in accordance with the obtained sequence of the *Tm-2*^{2}-containing rescued plant DNA (Figure 1B) and demonstrated that the tagged putative *Tm-2*^{2} gene is present as a single copy in tomato line ATV847 (Figure 1C).

In order to confirm the assignment and functionality of the predicted *Tm-2*^{2}-coding domain total RNA was isolated from ATV847 plants and 5′-RACE and 3′-RACE analyses were performed. The 5′-RACE experiments revealed mRNAs of the *Tm-2*^{2} gene with two mRNA initiation sites: in one experiment mRNA started at −26 relative to the ATG, whereas in a second independent experiment the mRNA started at −35. The 3′-RACE experiments revealed three polyadenylation sites in the mRNA, namely at 234, 236 and 259 downstream of the TGA stop codon. Moreover, PCR products amplified from cDNA with *Tm-2*^{2} 5′- and 3′-end-specific primers had sizes similar to the products obtained from genomic DNA. Thus, an mRNA of the *Tm-2*^{2} gene could be detected and the 5′- and 3′-RACE experiments confirmed the annotation of the ORF. Combining the RACE and PCR results indicates that the mRNAs of the *Tm-2*^{2} gene are ca. 2860 nucleotides long, excluding the polyA-tail. Moreover, no post-transscriptional processing events were observed by these experiments, which suggests the absence of introns.

Introduction of the Tm-2^2 *open reading frame in an* L. esculentum *line susceptible to ToMV infection*

Final confirmation that the *Tm-2*^{2} gene had been identified came from transformation of tomato line ATV840, which is susceptible to ToMV infection, with the native gene (pTM35) or with a chimeric gene with the cauliflower mosaic virus (CaMV) 35S promoter (pTM42). Primary transformants were grown in the greenhouse and three cuttings were taken from each individual plant. The cuttings were inoculated twice with a Dutch wt-ToMV isolate (ToMV-GdK, see legend to Table 4) with diluted leaf sap from infected *N. tabacum* SR1 plants.

Three phenotypes could be observed in the cuttings of the primary transgenic plants when they were infected with ToMV. These phenotypes were observed on both the plants transformed with the native (pTM35) and chimeric gene (pTM42) construct (Table 3). The first phenotype was indicative for virus susceptibility and was comparable to the phenotype of non-transgenic ATV840 plants: small deformations of the developing leaves and *N. glutinosa* leaves inoculated with leaf sap from these plants developed local lesions. The second phenotype was indicative of virus resistance and was comparable to the phenotype of ATV 847 plants, harbouring *Tm-2*^{2}: no visible symptoms on tomato and *N. glutinosa* leaves inoculated with leaf sap from these plants did not develop local lesions. The third phenotype is characterized by the appearance of a spreading necrosis on both inoculated and non-inoculated leaves after the tomato plants had been infected with ToMV. The spreading necrosis was characterized by the appearance of expanding black spots on all developing leaves, while the surroundings of the spots and finally the whole leaf became chlorotic (Figure 2). *N. glutinosa* leaves infected with leaf sap from these plants developed local lesions, which indicated that these transformants were systemically infected. In the light of the known variability of expression of resistance in different genetic backgrounds and different growth temperatures (Hall, 1980), phenotype 3 was also considered to be the result of expression of the *Tm-2*^{2} transgene in the primary transformants.

Selfings from resistant primary transformants (both with pTM47 and pTM49) were also tested for kanamycin resistance, for the presence of the constructs with the use of construct-specific primers and virus-resistance. Of each construct at least five lines were obtained which displayed segregation in the F_1 of both the resistant phenotype and presence of the

Table 3. Number of primary transformants obtained and their phenotypes upon challenging the plants with ToMV-0 (see Table 4).

Construct	Number with phenotype 1 (%)	Number with phenotype 2 (%)	Number with phenotype 3 (%)	Total number of transformants tested
pTM35	4 (36)	2 (18)	5 (46)	11
pTM42	10 (42)	4 (17)	10 (42)	24

Phenotype 1: *L. esculentum* with an infected appearance giving lesions on *N. glutinosa*; phenotype 2: healthy looking *L. esculentum* giving no lesions on *N. glutinosa*.; phenotype 3: *L. esculentum* with systemic necrosis giving lesions on *N. glutinosa.*

Table 4. Virus specificity of the transgenic tomato lines, assessed by inoculation with various tobamovirus isolates.

Tomato accession	Introduced construct	Tobamovirus isolate[a]					
		TMV[a]	Cg	0	1	2	2A
ATV840		+[b]	+	+	+	+	+
ATV847		−	−	−	−	−	+
ATV840	pTM35[c]	−	−	−	−	−	+
ATV840	pTM42[c]	−	−	−	−	−	+

[a]Virus isolates: TMV, tobacco mosaic virus U isolate; Cg, TMV-Cg isolate (an *Arabidopsis*- and tobacco-infecting tobamovirus); 0, ToMV-GdK (wild-type tomato mosaic virus); 1, ToMV-SPS (*Tm-1*-breaking isolate); 2, ToMV-GeRo (*Tm-2*-breaking isolate); 2A, ToMV-GM65 (*Tm-2*^{2}-breaking isolate). Virus isolates were obtained from the Plant Research International, Wageningen, Netherlands, except for TMV-Cg which was obtained from Dr Masayuki of the Hokkaido University, Japan.
[b]+ indicates infection, − indicates no infection.
[c]of each construct at least 3 lines were tested and all displayed the same virus specificity.

binary vector. No obvious differences between the phenotypes were observed between the two different constructs.

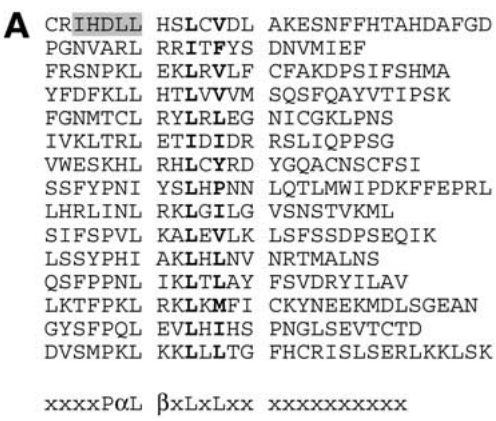
Homozygous F_2 plants of the lines with pTM47 and pTM49 were analysed for their virus specificity. For these experiments 6 tobamoviruses were used: TMV (tobacco mosaic virus), ToMV-GdK (wild-type tomato mosaic virus); Isolate 1, ToMV-SPS (*Tm-1*-breaking isolate); Isolate 2, ToMV-GeRo (*Tm-2*-breaking isolate); Isolate 2A, ToMV-GM65 (*Tm-2*^{2}-breaking isolate); and TMV-Cg (an *Arabidopsis*- and tobacco-infecting tobamovirus). No difference in resistance/susceptibility was observed between the transformants (both with the pTM47 and pTM49) and ATV847 (Table 4). They were all resistant to TMV, ToMV-GdK, ToMV-SPS, ToMV-GeRo and TMV-Cg. Both transgenic lines from both constructs and ATV847 could be infected by ToMV-GM65, the *Tm-2*^{2}-breaking ToMV isolate. These observations indicate that, next to the preservation of the ability of conferring resistance, also the characteristics of the *Tm-2*^{2} resistance gene are conserved after transformation of the *Tm-2*^{2} gene into a susceptible tomato background.

Also, in the progeny of primary transformants, both with phenotype 2 and phenotype 3, spreading necrosis was sometimes observed. However, although preliminary observations indicate influence of inoculation dosage and climatalogical variations no attempts were made to further study this phenomenon.

Predicted structure of the Tm-2^2 *gene product*

Translation of the 2586 bp ORF resulted in a predicted polypeptide of 861 amino acids with a calculated molecular mass of 98.8 kDa and a pI of 8.3. With the clustalX program the predicted *Tm-2*^{2} protein was aligned with several R proteins. The protein shows the highest homology with a CC-NBS-LRR protein

Figure 2. Development of spreading necrosis on *Tm-2*^{2} transgenic plants upon ToMV infection. Sequential photographs of the leaf above the inoculated leaf, which were taken with an interval of one day and starting 3 days after inoculation of the transgenic plant with ToMV.



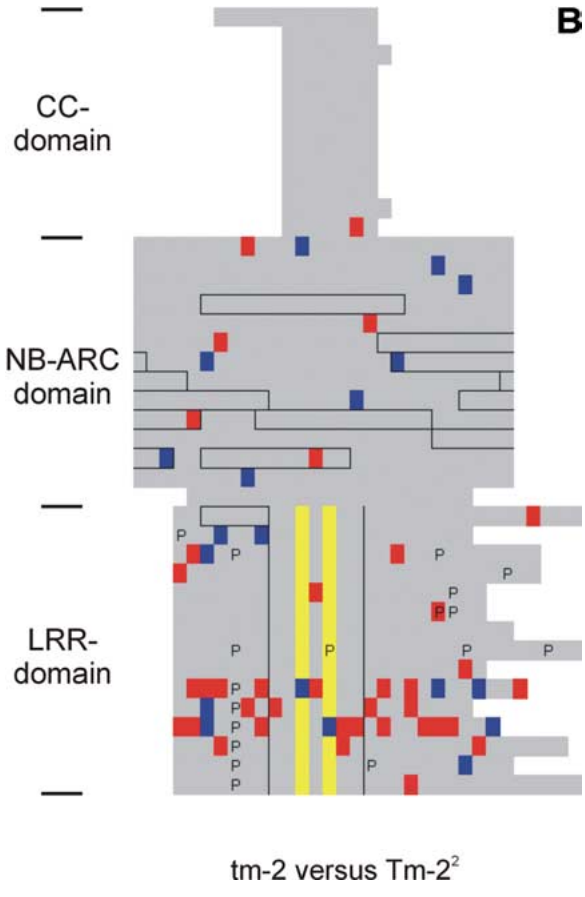


Figure 3. Structure of the *Tm-2*^{2} protein. A. Amino acid composition and the putative structure of the leucine-rich repeat domain of the *Tm-2*^{2} protein. Leucine-rich repeats (LRR) are aligned with the use of the core sequence of the LRR. This core sequence comprises the β-strand/β-turn motif conserved in all LRR proteins and is implied in ligand binding, is demarked by spaces between the amino acid residues. Deduced Tm-2^2 LRR consensus sequence is shown at the bottom, in which L represents L, I, M, V, Y or F; α represents K, Q, N or H; and β represents K, R, E, or H. Boxed in grey is motif 5 of the NB-Arc domain. B. Map of the differences of the putative *tm-2* protein with *Tm-2*^{2} protein. The 11 leucine zippers (LZ) of the coiled-coil domain are aligned by LZ consensus sequence (LXXLXXX; in which L represents L, I, M or V). The LRRs are aligned according to A. Synonymous and non-synonymous differences in the codons of the *tm-2* ORF are indicated in dark blue and red, respectively. In yellow are indicated the codons encoding the conserved aliphatic residues of the LRR core sequence. Boxes indicate positions of the nine NB-ARC motifs (see text). P indicates a proline residue.

from *Arabidopsis thaliana* (highest shared identity of 25% with RPP13; Bittner-Eddy *et al.*, 2000). Homology with characterized R proteins from *L. esculentum* (Mi-1.2, I-2, Prf and Sw-5b) was considerably lower: 10–15% identical residues (Salmeron *et al.*, 1996; Ori *et al.*, 1997; Milligan *et al.*, 1998; Brommonschenkel *et al.*, 2000; Spassova *et al.*, 2001).

The predicted *Tm-2*^{2} polypeptide contains all the features of the CC-NBS-LRR class of R proteins (Hammond-Kosack and Jones, 1997). In the first 90 N-terminal amino acids 11 putative heptad leucine zipper motifs (LZ) could be recognized. A NB-ARC (nucleotide-binding site, apoptosis, *R* gene products, CED-4) domain could be recognized in the amino acid stretch between amino acids 91 and 483. All nine NB-ARC motifs are present (Hammond-Kosack and Jones, 1997; van der Biezen and Jones, 1998). With the core of the LRR consensus, xxLxLxx (L represents mainly L, I, but also sometimes M, V, F, and Y), which includes the β-loop/β-strand part of a single LRR motif (Jones and Jones, 1997), fifteen imperfect LRRs could be discriminated in the polypeptide stretch ranging from amino acids 477 to 861 (Figure 3A). The consensus sequence for the complete cytoplasmic LRR, as defined by Jones and Jones (1997), LxxLxxLxxLxLxx(N/C/T)x(x)LxxIPxx, was difficult to recognize in most of the repeats because both the N/C/T residues and P residues are poorly conserved in the Tm-2^2 protein. An alternative consensus could be deduced from the alignment of the LRRs of the Tm-2^2 protein (Figure 3A): xxxxPαLβxLxLxxxx..... (in which L represents L, I, M, V, F or Y; α represents K, Q, N or H, and β represents K, R, E or H). This consensus is comparable to the consensus obtained for RPP13 by Bittner-Eddy *et al.* (2000) with the exception of the proline at position 5 of the consensus, which is absent in most of the LRRs of RPP13. The position of this proline is such that it will disrupt the α helix at the concave site of the LRR domain and initiates the β loop preceding the β strand of the LRR (Jones and Jones, 1997). The sizes of the repeats varied between 21 and 30 residues. According to these assignments motif 5 of the NB-ARC (sequence IDHLL) is located in or close to the first LRR.

No targeting sequences were observed in the Tm-2^2 protein, suggesting a cytoplasmic location of this protein.

Molecular cloning of homologous alleles from tm-2- *and* Tm-2^2*-containing tomato accessions*

The *Tm-2*^{2} locus contained a single gene (see above), which simplified the cloning of the homologous *tm-2* allele with the *Tm-2*^{2}-specific primers PrRuG84 and 86 (Table 2). Analysis and comparison of the sequence of *tm-2* revealed that the susceptible allele has an intact ORF of a size identical to *Tm-2*^{2}. At the DNA-level no differences could be observed between the *tm-2* alleles from either line Stevens, line ATV840 or line GCR26. This was also the case for the *Tm-2*^{2} allele from tomato genotype Ds_{13-15} and *Tm-2*^{2} alleles from resistant tomato lines ATV847 and GCR267. This suggests that all *Tm-2*^{2} alleles and all *tm-2* alleles tested have common origins and were separated relatively recently. The differences between *Tm-2*^{2} and *tm-2* are considerable: at the DNA level 62 differences (2.3%) were observed, which results on a protein level in 38 differences (4.3%; Figure 3B). In total, 38 non-synonymous and 23 synonymous differences were observed when *tm-2* was compared with the *Tm-2*^{2} gene. In one codon two base changes were present. Like for the *Tm-2*^{2} protein, no signal sequences were observed in the tm-2 protein, suggesting a similar and hence cytoplasmic location of this tm-2 protein (if produced).

Discussion

We describe the isolation of the *Tm-2*^{2} gene from *L. esculentum*. The *Tm-2*^{2} gene is the second *R* gene isolated that confers resistance to a tobamovirus. The first was the *N* gene (Dinesh-Kumar *et al.*, 1995). By transposon tagging and by a screen based on the survival of plants in which the *Tm-2*^{2} gene is disrupted, 5 mutant plants out of about 30 000 seeds were obtained. These mutant plants represent two independent insertion events, which disrupt the same *R* gene. The intact gene conferred resistance to ToMV infections in the susceptible tomato line ATV840 when these plants were transformed with gene constructs containing the ORF under the control of its own promoter and with its own polyadenylation signal or under the control of the CaMV 35S promoter and the nopaline synthase (NOS) polyadenylation signal. Next to the resistant phenotype, primary transformants were obtained that demonstrated systemic necrosis upon infection with ToMV. This phenotype is also considered to be an expression of the presence of the *Tm-2*^{2} transgene in the susceptible lines, because this type of response was

absent in untransformed susceptible plants and has already been observed in tomato lines in which *Tm-2²* was introduced via breeding (Hall, 1980). The appearance and intensity of systemic necrosis in these classic tomato lines depends on the genetic background, temperature, virus dose and zygosity of the plants (Hall, 1980; Pilowsky *et al.*, 1981). Moreover Brading *et al.* (2000) observed a similar phenotype upon inoculation with ToMV when the *Tm-2²* resistance was present in tomato plants expressing the *NahG* gene. This bacterial gene encodes a salicylate hydroxylase, which make the plants unable to accumulate salicylic acid. Apparently, this slows down the hypersensitive response, which enables the virus to infect surrounding cells.

In transgenic resistant tomato lines the expression of *Tm-2²* resistance is similar to the resistance in the tomato accession from which the gene originates: virus specificity is conserved, as is the fast response.

Isolation of the *Tm-2²* gene raises the possibility to introduce this gene and, thus, the durable ToMV resistance in susceptible solanaceous species such as *Capsicum annuum* (sweet pepper) and *N. tabacum* SR1. Indeed, we were recently able to transfer the *Tm-2²* gene to the latter plant with the use of both the native (pTM35) and the chimeric gene constructs (pTM42), that resulted in resistance against TMV infections (Lanfermeijer *et al.*, in preparation).

Structure of the predicted Tm-2² *protein*

The *Tm-2²* protein is a CC-NBS-LRR R protein (Hammond-Kosack and Jones, 1997). It contains a CC domain that consists of eleven heptad LZ motifs. It contains all nine NB-ARC motifs (van der Biezen and Jones, 1998) and it contains 15 imperfect LRRs. However, the assignment of LRR motifs to R proteins is an arbitrary procedure because the LRRs of cytoplasmic R proteins show various levels of imperfectness and of conformity with the consensus for the cytoplasmic LRR motifs (Jones and Jones, 1997). Difficulties in assigning LRR motifs in the Tm-2² protein arose due to low conservation of Asn, Cys or Thr at position 15 and Pro at position 21/22 of this consensus sequence in the Tm-2² protein.

The tm-2 and Tm-2² alleles

Because *tm-2* translates into a complete protein with all the characteristics of a CC-NBS-LRR protein, susceptibility might be determined by the conformation of the encoded protein. Mechanistically, the reasons for absence of resistance can either result from defects in the recognition of the pathogen or from defects in transmission of the signal down to the signal transduction pathway. However, within the limits of our knowledge of the function and importance of amino acid residues in R proteins, no amino acids in the tm-2 protein, different from those in the Tm-2² protein, could be pointed out at as the reason for a non-functional resistance protein.

On the other hand, the distribution of the differences between the two alleles is not at random. They concentrate in the C-terminal half of the LRR-domain (26 of the 38 differences). This, in combination with the fact that the susceptible allele contains a complete ORF, suggests that this gene is still subjected to selective pressure and, thus, is expressed and functional. Two possible explanations come in view. Firstly, the susceptible alleles are responsible for resistance against ToMV strains not detected in present tomato cultivation. The concentration of the differences in the LRR domain, which is suggested to play a role in the detection of the Avr protein, agrees with this view. Secondly, in the context of the 'guardee' hypothesis, which proposes that R proteins function as proteins, which monitor an affected functionality or state of a host protein, i.e. the 'guardee' or the virulence target in the presence of the pathogen (Ellis *et al.*, 2000; Glazebrook, 2001; van der Hoorn *et al.*, 2002), one could imagine that R proteins function not only in detecting deleterious modifications induced by pathogens but also function in detecting detrimental changes in their 'guardee' which are induced by other phenomena. In this sense, R proteins and their homologues could have a broader function in the biology of the cell. However, analysis of selfings of mutant plant 144 revealed that plants homozygous for the disrupted *Tm-2²* gene are viable (data not shown). Several other examples of alleles encoding intact R proteins with no apparent pathogen specificity have been observed at simple resistance loci in susceptible plant varieties (Bittner-Eddy *et al.*, 2000; Bryan *et al.*, 2000).

Our observations show that that durable *Tm-2²* resistance is conferred by a gene encoding a regular resistance protein with no extraordinary characteristics. The durability of *Tm-2²* resistance might, therefore, be explained by the reduced virulence of the virus (Fraser *et al.*, 1989) caused by the mutations in the coding region of the C terminus of MP, which are necessary to overcome the resistance either because the mutations affect a function of the MP or because the

mutations affect expression of the CP or because the 5′-untranslated region of *CP* gene coincides with the part of the *MP* ORF that encodes the C terminus of MP (Lartey *et al.*, 1996).

The Tm-2^2 resistance protein seems to have targeted a domain of MP, which does not have much freedom to change its amino acid composition, either because of the role of the MP or due to the second function of the coding region of the *MP* gene. In other words, the Tm-2^2 protein targets a weak spot or the Achilles' heel of the ToMV virus.

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