# The tomato gene Sw5 is a member of the coiled coil, nucleotide binding, leucine-rich repeat class of plant resistance genes and confers resistance to TSWV in tobacco

Spassova, MI; Klein-Lankhorst, RM; Hille, Jacob; Goldbach, RW; Prins, M; Spassova, Mariana I.; Prins, Theo W.; Klein-Lankhorst, René M.; Goldbach, Rob W.

*Published in:*
Molecular Breeding

*DOI:*
10.1023/A:1011363119763



*Publication date:*
2001

*Molecular Breeding* **7:** 151–161, 2001.



# The tomato gene *Sw5* is a member of the coiled coil, nucleotide binding, leucine-rich repeat class of plant resistance genes and confers resistance to TSWV in tobacco

Mariana I. Spassova, Theo W. Prins[1], Rolf T. Folkertsma[1], René M. Klein-Lankhorst[2], Jacques Hille, Rob W. Goldbach[1] & Marcel Prins[1]*

*Department Molecular Biology of Plants, Groningen University, Kerklaan 30, 9751 NN Haren, Netherlands; [1]Laboratory of Virology, Wageningen University, Binnenhaven 11, 6709 PD Wageningen, Netherlands; [2]Greenomics, Plant Research International BV, Droevendaalsesteeg 1, 6708 PB Wageningen, Netherlands; *author for correspondence (fax: +31-317-484820; e-mail: marcel.prins@viro.dpw.wag-ur.nl)*

Received 20 September 2000; accepted in revised form 27 October 2000

*Key words:* Coiled coil, Leucine-rich repeat, NB-ARC, *Sw5*, TSWV, Virus resistance

**Abstract**

Tomato spotted wilt virus is an important threat to tomato production worldwide. A single dominant resistance gene locus, *Sw5*, originating from *Lycopersicon peruvianum*, has been identified and introgressed in cultivated tomato plants. Here we present the genomic organization of a 35 250 bp fragment of a BAC clone overlapping the *Sw5* locus. Two highly homologous (95%) resistance gene candidates were identified within 40 kb of the CT220 marker. The genes, tentatively named *Sw5-a* and *Sw5-b*, encode proteins of 1245 and 1246 amino acids, respectively, and are members of the coiled-coil, nucleotide-binding-ARC, leucine-rich repeat group of resistance gene candidates. Promoter and terminator regions of the genes are also highly homologous. Both genes significantly resemble the tomato nematode and aphid resistance gene *Mi* and, to a lesser extent, *Pseudomonas syringae* resistance gene *Prf*. Transformation of *Nicotiana tabacum* cv. SR1 plants revealed that the *Sw5-b* gene, but not the *Sw5-a* gene, is necessary and sufficient for conferring resistance against tomato spotted wilt virus.

## Introduction

Tomato spotted wilt virus (TSWV), the type species of the genus *Tospovirus* within the family *Bunyaviridae*, has become an increasing problem in many crops worldwide (Goldbach and Peters 1994; Prins and Goldbach 1998). Only limited numbers of TSWV resistance sources have become available over the years. Of these, the single dominant resistance gene *Sw5* introgressed from *Lycopersicon peruvianum* (Stevens 1964) has been used most prominently to date.

The *Sw5* gene has been introgressed in the fresh market tomato cultivar Stevens (Stevens 1964; Stevens et al. 1992) and shown not only to provide resistance to TSWV but also to two related tomato-infecting tospoviruses groundnut ringspot virus (GRSV) and tomato chlorotic spot virus (TCSV) (Boiteux and Giordano 1993). The *Sw5* gene has been mapped on the long arm of chromosome 9 (Chagué et al. 1996; Stevens et al. 1992, 1995). The region encompassing this resistance gene has been cloned and characterized to some extent (Brommonschenkel and Tanksley 1997). This revealed *Sw5* to be physically closely linked to the RFLP marker CT220 (Brommonschenkel and Tanksley 1997). Using YAC and cosmid clones Brommonschenkel and colleagues demonstrated that the *Sw5* locus is located on a chromosomal segment ca. 40 kb from RFLP marker CT220 (Brommonschenkel and Tanksley 1997; Brommonschenkel et al. 1998). Using a bacterial artificial chromosome (BAC) library of tomato cultivar Stevens, containing the *Sw5* locus, we have previously constructed a genomic DNA contig of 250 kb surrounding the RFLP marker CT220 that includes the *Sw5* locus (Folkertsma et al. 1999).

Although the *Sw5* locus has thus far been considered to consist of a single dominant resistance gene, the locus has been demonstrated to consist of at least three, but possibly five, related copies of resistance gene candidates (RGCs) (Folkertsma et al. 1999). Within the complete contig three distinct RGC sequences were identified, using degenerate primers to amplify the conserved NB-ARC box present in most plant R genes (Folkertsma et al. 1999).

A still increasing number of resistance genes has been cloned and sequenced since the description of the *Pto* gene seven years ago (Martin et al. 1993). As discussed in recent reviews (e.g. Ellis et al. 2000; Young 2000), the vast majority of these genes contain leucine-rich repeats (Jones and Jones 1997). The number of repeats can be highly variable, even within families (Dixon et al. 1996). In addition, many R genes also contain a highly conserved nucleotide binding site region, currently renamed NB-ARC, due to the sequence similarity of this resistance (R) gene domain with apoptosis-related proteins in nematodes (CED-4) and mammals (Apaf-1) (van der Biezen and Jones 1999). The members of the NB-ARC/LRR class are subdivided into groups I and II, based on the structure of their N terminal conserved domains (Pan et al. 2000). Genes belonging to the group I family of resistance genes carry an amino terminal TIR region, which has homology to Toll/Interleukin receptor domain (Whitham et al. 1994). Known resistance genes *N, M, L6, RPP1* and *RPP5* (Meyers et al. 1999; Pan et al. 2000 and references therein) conferring resistance to a wide range of pathogens including viruses, oomycetes and fungi, are members of this group. Interestingly, members of this group have not been found in Poaceae (Meyers et al. 1999), suggesting an early evolutionary separation from other classes of resistance genes (Pan et al. 2000). A second large group of NB-ARC containing R genes, like *RPS2, RPM1, I2, Mi, Dm3, Pi-B, Xa1, RPP8, RPS5* and *Prf* (Meyers et al. 1999; Pan et al. 2000 and references therein) encode another motif, the so-called coiled coil (CC) domain (Meyers et al. 1999; Pan et al. 2000 and references therein). This domain, of which leucine zippers (LZ) are a subclass, are thought to be involved into the higher order folding of interacting proteins into homo- or heteromultimerized helical supercoils (Lupas 1996).

Apart from the different amino terminal domains of the R genes of the NB-ARC-LRR groups I and II, a discerning tryptophan residue has been observed in the kinase-2 box (Pan et al. 2000). Group I (TIR) sequences are more abundant in the *Arabidopsis* genome and outnumber group II (non-TIR) sequences threefold (Meyers et al. 1999).

In the research presented here, the *Sw5* resistance gene locus was analysed by genomic sequencing of BAC 18-16F, containing the CT220 marker sequence (Folkertsma et al. 1999). In a fragment of 35 250 bases containing the CT220 sequence on the telomeric side, two candidate resistance genes of the CC-(NB-ARC)-LRR type (group II) were identified within the previously determined 40 kb distance of CT220 (Brommonschenkel et al. 1998). Both genes were transformed in tandem and individually to *Nicotiana tabacum* cv. SR1 plants and analysed for their capability to confer TSWV resistance.

## Materials and methods

### *Plant material and vectors*

The TSWV strain BR-01 (Àvila et al. 1993) was maintained on the systemic host *Nicotiana benthamiana*. In order to prevent the generation of defective interfering particles (DIs) (Resende et al. 1991), virus was used only when no more than three mechanical passages followed transmission by the natural vector *Frankliniella occidentalis*. In addition, virus was only transmitted mechanically to *N. benthamiana* from infected *Emilia sonchifolia* plants, which have been observed incapable of generating DIs (Inoue-Nagata et al. 1997).

Recipient plants used in the transformation experiments were *N. tabacum* cv. SR1 plants. All manipulations with transgenic plant material were carried out under conditions (PKII) imposed by the Dutch authorities (VROM/COGEM).

### *Genomic sequencing of 35 kb of BAC 18-16F and analysis of putative gene products*

Tomato BAC clone 18-16F (Folkertsma et al. 1999) was partially sequenced to a 6-fold genomic coverage using a shotgun approach. The DNA was cesium chloride-purified and sheared by nebulization into fragments with an average size of 1200 bp. Blunt repair of the ends was performed with *Pfu* DNA polymerase (Stratagene) according to the manufacturer's directions. DNA fragments were size-fractionated by gel electrophoresis and cloned into the *Eco*RV site of pBluescriptSK (Stratagene). After transformation into XL2 blue competent cells (Stratagene) a thousand

recombinant colonies were picked randomly. DNA templates for sequencing were isolated using QIAprep Turbo kits (Qiagen) on a QIAGEN BioRobot 9600. Sequencing was performed using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready reaction kit with FS AmpliTaq DNA polymerase (Perkin Elmer) and analysed on an ABI 3700 DNA analyser.

Shotgun sequences were base-called by the PRED basecaller, and assembled with the PHRAP assembler (Ewing and Green 1998; Ewing et al. 1998). By means of the PREGAP4 interface, PHRAP-assembled data were stored in the GAP4 assembly database (Bonfield et al. 1995). The GAP4 interface and its features were then used for editing and sequence finishing. Consensus calculations with a quality cut-off value of 40 were performed from within GAP4 using a probabilistic consensus algorithm based on expected error rates output by PHRED. Sequencing PCR products bridging the ends of existing contigs filled remaining gaps in the sequence.

Genomic DNA composition, structure, repeats, and restriction enzyme pattern were analysed with the University of Wisconsin Genetics Computer Group programs (Devereux et al. 1984) and DNAstar (Lasergene, Madison, WI). Open reading frames (ORFs) encoding more than 50 amino acids (150 bp) were considered to be protein encoding and hence designated putative genes.

Sequences were analysed using DNA and protein comparisons with entries in the sequence databases and were performed with FASTA and BLAST programs (Gish and States 1993; Altschul et al. 1997). Motif searches were done against the Prosite release 14 database (Bairoch et al. 1997; Fabian et al. 1997). MAR finder software (http://www.ncgr.org/MarFinder/) (Singh et al. 1997) was used to predict putative matrix attachment regions (Stief et al. 1989; Paul and Ferl 1999). The COILS program (http://www.ch.embnet.org/software/COILS_form.html) was used to scan the resistance gene candidates for putative coiled coil regions using windows between 14 and 28, matrix MTIDK similar to (Pan et al. 2000).

*Cloning RGCs of the* Sw5 *locus*

BAC DNA of the clone 18-16F was digested either with the restriction enzyme *Nsi*I or *Pst*I and fractionated on a 0.7% agarose gel. DNA migrating between 8 and 12 kb and between 14 and 18 kb, respectively, was excised as gel slices, purified by QIAquick gel extraction kit (Qiagen) and ligated into the *Xba*I (*Nsi*I isoschizomer) or *Pst*I site of the plant transformation vector pCGN1578 (McBride and Summerfelt 1990) according to Sambrook et al. (1989). The ligation mixtures were transformed into *Escherichia coli* DH5$\alpha$ (Promega) by electroporation. Colony blot hybridization of the transformants with $^{32}$P-labelled NB domain-specific probes (Folkertsma et al. 1999) identified the positive clones. Restriction digests and comparison with the sequence data allowed verification of the clones which contain inserts with the RGCs of the *Sw5* locus plus their flanking sequences (Figure 1B).

*Plant transformation*

The pCGN1578-derived constructs containing the resistance gene candidates identified within the 35 kb BAC fragment telomeric of CT220: *Sw5-a*, *Sw5-b* and a fragment spanning both *Sw5-a* and *Sw5-b*, were introduced into *Agrobacterium tumefaciens* strain LBA4404 by triparental mating, with pRK2013 (Horsch et al. 1985) as a helper plasmid. Subsequently the purified transconjugants were checked for carrying unaltered gene constructs and used to transform *N. tabacum* cv. SR1 leaf explants as described by Horsch et al. (1985). The leaf explants were dipped in an *A. tumefaciens* suspension and co-cultivated for 48 h on medium containing Murashige and Skoog salts and vitamins (Duchefa, Netherlands), 30 g/l sucrose, 8 g/l agar, 10 mg/l NAA and 100 mg/l BAP. The explants were then transferred and cleared from *A. tumefaciens* with the same medium supplemented with 100 mg/l kanamycin and 250 mg/l carbenicillin. Subsequently, the developed shoots were transferred to rooting medium (Murashige and Skoog salts and vitamins, 30 g/l sucrose, 8 g/l agar, 100 mg/l kanamycin and 250 mg/l carbenicillin). Kanamycin-resistant plantlets were transferred to soil and grown under standard greenhouse conditions.

*Virus resistance assays*

Inoculation of transgenic *N. tabacum* cv. SR1 plants was carried out with plant sap of TSWV (BR-01)-infected *N. benthamiana* plants 1:10 diluted in 10 mM sodium phosphate pH 7.0 buffer containing 1% $Na_2SO_3$. Untransformed plants were used as controls for virus inoculation. Plants were all inoculated twice with a four day interval to rule out random escape of inoculation. Virus symptoms were monitored on a daily basis for the duration of the experiment (17 days). Plants remaining symptomless were checked

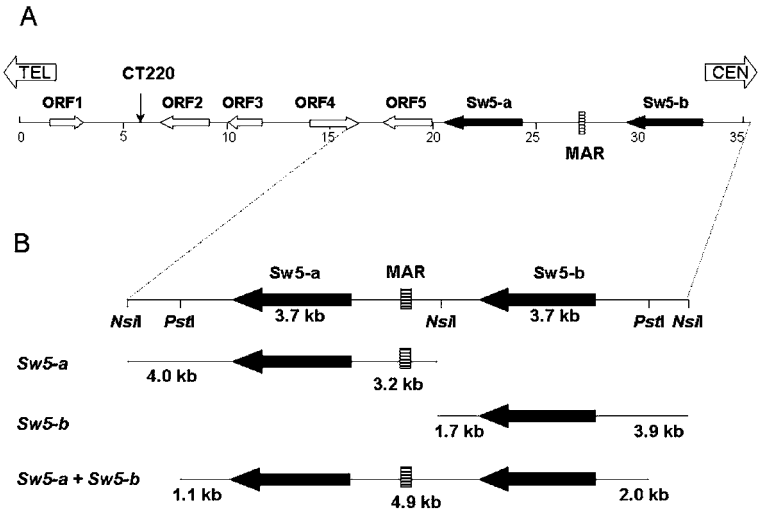


*Figure 1.* A. Genetic organisation of the 35 kb fragment of BAC 18-16F containing CT220 marker on the telomeric side. Seven ORFs are indicated. Homologies to (putative) proteins in the database are discussed in the text. *Sw5-a* and *Sw5-b* indicate the positions of two resistance gene candidates highly homologous to *Mi* that were used in complementation assays. MAR indicates the position of the putative matrix association region. B. Close-up of the *Sw5-a* and *-b* region indicating the restriction sites that were used for subcloning of the respective genes into the binary vector used for transformation of *N. tabacum* cv. SR1 plants, and resulting size of 5′- and 3′-untranslated sequences containing promoter and terminator elements.

for the presence of virus in systemic leaves by means of ELISA with the use of polyclonal antiserum against the viral nucleoprotein (Àvila et al. 1993).

## Results

### *Genetic organisation of the* Sw5 *locus*

Analyses of the *Sw5* locus in tomato (Brommonschenkel and Tanksley 1997; Folkertsma et al. 1999) revealed the presence of several resistance gene candidates in the vicinity of the CT220 marker. The BAC clone 18-16F, containing an insert of ca. 100 kb of tomato DNA, nearly completely maps to the centromeric side of CT220 (Folkertsma et al. 1999). The *Sw5* locus was therefore predicted to be located on the telomeric half of this clone. We sequenced 35 250 nucleotides containing the CT220 sequence, which was identified between 5354 and 5567 nucleotides from the telomeric side of the insert (Folkertsma et al. 1999) (Figure 1B). This tomato DNA sequence derived from BAC 18-16F has been submitted to the GenBank database with accession number AY007366. The organization of the *Sw5* locus in the vicinity of the CT220 marker (Figure 1) contains 7 open reading frames encoding putative proteins larger than 50 amino acids. ORF 1 encodes a protein with homology to a putative pirin protein of *Arabidopsis thaliana*, ORF 2 resembles a tobacco GTP-binding protein, ORF 3 a human pirin gene, ORF 4 a latex-abundant protein from the rubber tree (*Hevea brasiliensis*) and ORF 5 has sequence similarity to an *Arabidopsis* EST of unknown function. Open reading frames 6 and 7, located between 15 276 and 19 011 and between 23 965 and 27 703 respectively from CT220 are very homologous to each other, both in coding sequences as in 5′- and 3′-flanking sequences. The genes, tentatively designated *Sw5-a* and *Sw5-b*, display all characteristics of typical resistance genes. When using the MAR-finder program (see Materials and methods) a prominent matrix attachment region (MAR) (Stief et al. 1989; Paul and Ferl 1999) localizes in between both RGCs. MAR elements are thought to greatly influence the expression of genes by attachment of DNA loops to proteins scaffolding, thereby creating loops and euchromatin with increased accessibility for the transcription machinery of the cell nucleus (Stief et al. 1989).

No resistance gene candidates were found in 25 kb of sequence distal (centromeric) to *Sw5-b* with respect to CT220 (unpublished results). Based on our previous observations (Folkertsma et al. 1999), it seems likely that more RGCs similar to *Sw5-a* and *Sw5-b* are present further away from CT220. These genes, however, do not map within the previously determined genetic distance of *Sw5* to CT220 (Brom-

monschenkel et al. 1998) and were not included in our complementation studies.

*Analysis of the* Sw5-a Sw5-b *genes*

Both *Sw5-a* and *Sw5-b*, are of the coiled-coil (CC) / nucleotide-binding (NB-ARC) / leucine-rich repeat type of resistance gene candidates (Pan et al. 2000 and references therein) and closely resemble known resistance genes from tomato, of which the nematode resistance gene product Mi (51% amino acid similarity) (Milligan et al. 1998) and Prf (43%), involved in *Pseudomonas syringae* resistance (Salmeron et al. 1994), are most closely related (Figure 2). Three stretches of conserved amino acids that are only found in group II NB-ARC domains were also observed in *Sw5-a* and *Sw5-b* and are boxed in Figure 2B. As is the highly conserved tryptophan in the kinase 2 domain (Pan et al. 2000; Young 2000). Although we did not analyse the cDNA sequences of either gene, no introns are predicted from the genomic sequence of *Sw5-a* nor *Sw5-b*, in contrast to what was observed for both *Mi* copies. Around −100 from the start codon a TATA box sequence was found in both homologues. Overall the homology in the 5′-untranslated region was >98% over a distance of 1000 nucleotides, which may suggest co-ordinate expression of these genes.

*Mi* gene homologues 1.1 and 1.2 (Milligan et al. 1998) are not only highly homologous to *Sw5-a* and *Sw5-b* in sequence composition, the sizes of their encoded proteins are also nearly identical (1257 amino acids for Mi-1.1, 1255 for Mi-1.2, 1245 for Sw5-a and 1246 for Sw5-b), while Prf (1824 amino acids) has an extensive amino-terminal extension (Salmeron et al. 1994). The length of the LRR region of the Sw5-a and Sw5-b proteins is very similar to the Mi homologues as well as Prf (Figure 2C). A putative leucine zipper was observed in both Sw5 homologues at a position that mapped close to the predicted LZs of Mi and Prf (Figure 2A, lower panel). Strikingly, the *Sw5-b* gene encoded an additional leucine zipper further to its amino-terminal end (Figure 2A, upper panel), that was absent in *Sw5-a*. Nonetheless, both areas in both genes were predicted to form putative coiled-coil regions according to the COILS program with window 14 (see Materials and methods). A PCI domain (Hofmann and Bucher 1998) proposed by Vos et al. (1998) to be specific for the *Mi* class of NB-LRR genes was not observed for the *Sw5* sequences.

The typical tryptophan within the kinase 2 domain as well as the other two discerning features of group II RGCs were also found in both homologues (Figure 2B), clearly demonstrating both *Sw5* homologues belong to the CC-(NB-ARC)-LRR class of resistance genes.

*Subcloning of* Sw5-a *and* Sw5-b *ORFs in binary vectors*

On the basis of the established DNA sequence a strategy was developed to subclone the two *Sw5* resistance gene candidates. Both ORFs were subcloned individually as well as together in a binary vector in order to assess the contribution of the individual ORFs in conferring resistance to the pathogen. With the restriction enzyme *Nsi*I (Figure 1B) both ORF *Sw5-a* and *Sw5-b* could be individually subcloned into the binary vector pCGN1578 as 10.9 kb and 9.3 kb fragments, respectively. In this way the binary vector with *Sw5-a* retains the putative MAR sequence, 3.2 kb upstream of the translation start and 4.0 kb 3′ of the coding sequence. The vectors with *Sw5-b* contain 3.9 kb 5′-untranslated sequences and 1.7 kb downstream of the gene. In addition, the combination of *Sw5-a*, the putative MAR and *Sw5-b* was subcloned as a 15.6 kb *Pst*I fragment in the same binary vector pCGN1578. In this case, 2.0 kb of flanking sequences preceding *Sw5-a* were maintained and 1.1 kb 3′ downstream of *Sw5-b* (Figure 1B). In all cases both orientations of the inserts were cloned in order to make sure the relative position of the *nos* promoter driving the *NPTII* gene would not adversely influence the expression of the *Sw5* transgenes.

*Introduction of* Sw5-a *and* Sw5-b *in* N. tabacum

The six binary constructs, containing respectively *Sw5-a*, *Sw5-b* and the combination of *Sw5-a* and *Sw5-b* in both tandem and opposite orientation to the *NPTII* gene, were transferred to *A. tumefaciens* strain LBA4404 by triparental mating. After purification of individual colonies the integrity of the constructs in *A. tumefaciens* was verified. Subsequently, *N. tabacum* cv. SR1 leaf explants were transformed with the different *Agrobacterium* strains. In each case independent *N. tabacum* transformants were regenerated to shoots, the shoots were rooted and the *in vitro* grown plantlets were transferred to soil and further grown in the greenhouse. A total of 57 transgenic *N. tabacum* plants were generated expressing *Sw5-a*, *Sw5-b* or a combination of both genes (Figure 3).

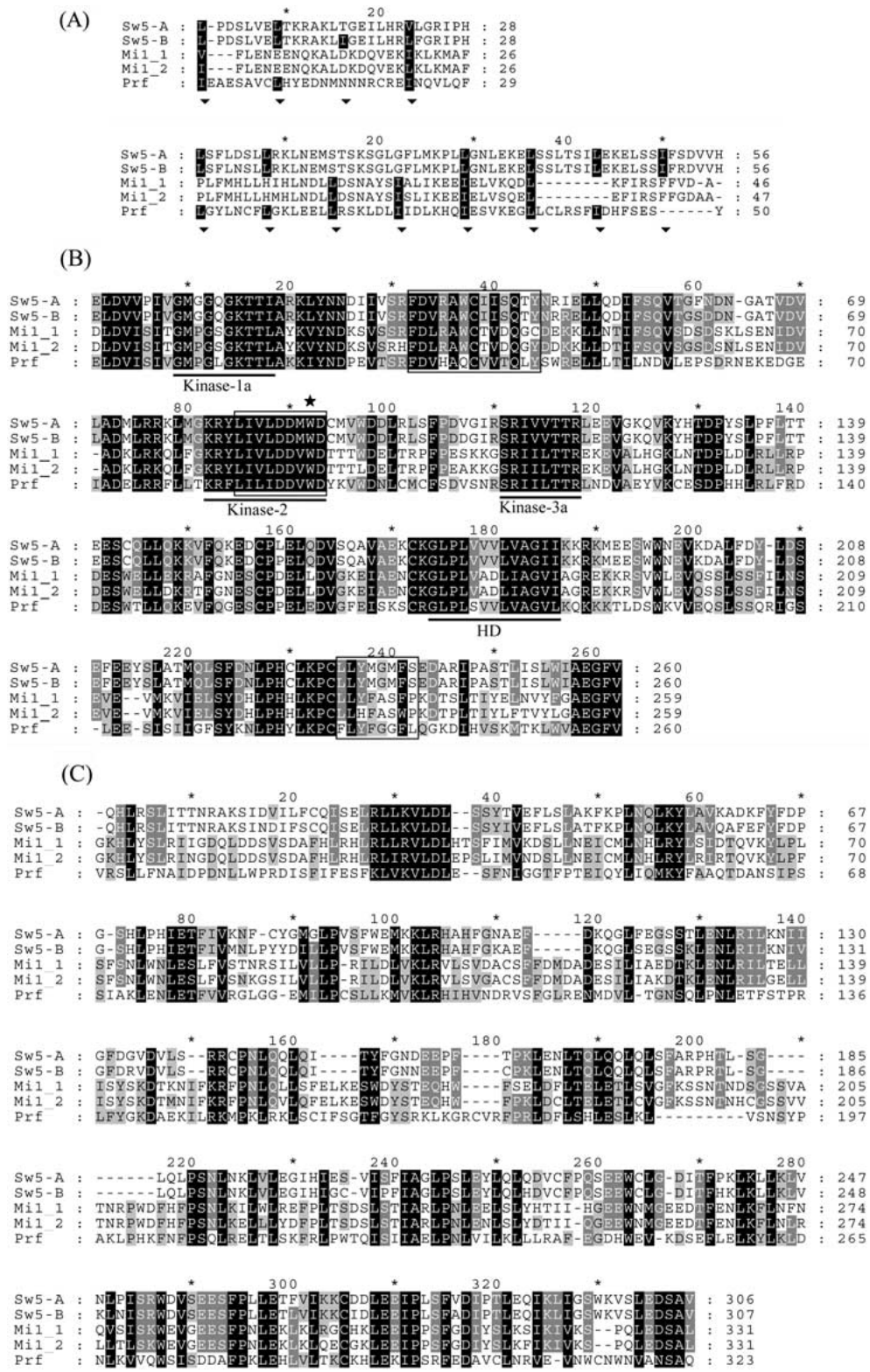


*Figure 2.* Representation of a homology analysis of the deduced amino acid sequences of *Sw5-a*, *Sw5-b*, *Mi-1.1*, *Mi-1.2* and *Prf* in protein coding regions. Sequences from the GenBank database (*Prf* accession number U65391, *Mi-1.1* AF039681, *Mi-1.2* AF039682). A. Comparison of the possible leucine zipper regions of Sw5-a, Sw5-b, Mi-1.1, Mi-1.2 and Prf. The putative leucine zipper in the first part of the figure is only applicable for Sw5-b. The positions of the leucines and isoleucines in the zipper are indicated by black triangles. Displayed in the upper panel are amino acids 51–78 of Sw5-a in the lower panel 398–444. B. Comparison of the central conserved regions. Kinase-1a, kinase-2 and possible kinase-3a motifs are indicated. HD indicates the position of the conserved hydrophobic domain. Boxed amino acids are conserved residues typical for group II RGCs. The larger ⋆ indicates the conserved tryptophan typically found in the kinase-2 domain of all group II members. Displayed are amino acids 554–813 of Sw5-a. C. Comparison of the LRR domains of Sw5-a, Sw5-b, Mi-1.1, Mi-1.2 and Prf. Displayed are amino acids 917–1222 of Sw5-a. Black boxes indicate that all residues in a column are identical or conserved substitutions. Sequence gaps inserted to maintain alignment are shown in dashes. Sequences were aligned with UW-GCG program PileUp. The GeneDoc program version 2.6.01 (http://www.psc.edu/biomed/genedoc) was used to display the pileup.

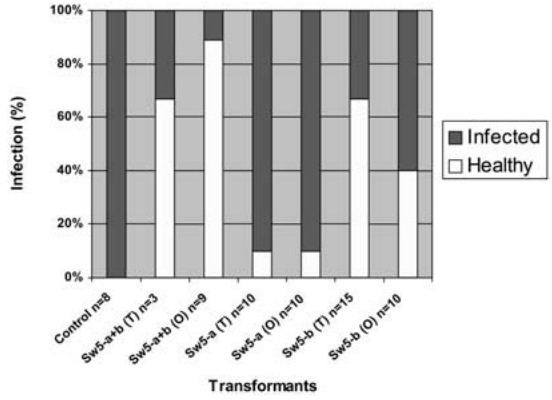


*Figure 3.* Frequency of resistance against TSWV 17 days after inoculation. Bars indicate percentage of infected plants. Numbers of plants tested per transformation construct are indicated at the bottom of each bar. BAC subclone constructs are as indicated in Figure 1B. T indicates tandem orientation with respect to the NPTII gene, O implies opposite orientation.

### *Resistance in transgenic* N. tabacum *SR1 plants correlates to the presence of* Sw5-b

To rule out the possible interference on the expression of the resistance gene candidates, both tandem and opposite orientations with respect to the *nos* promoter driven *NPTII* gene were used (Figure 3). All transformed plants were inoculated twice with tomato spotted wilt virus (TSWV) and monitored in time for the development of virus symptoms. Figure 3 indicates that all control plants are susceptible to the virus, whereas plants transformed with constructs containing *Sw5-a* and *Sw5-b* together were protected from the virus. Subsequently, plants expressing only the *Sw5-a* homologue displayed no resistance to the virus, whereas a high incidence of plants expressing *Sw5-b* were capable of resisting TSWV infection.

The orientation of the transgene with respect to the *NPTII* gene had no influence on the level of resistance, irrespective of whether they were constructed in tandem or in opposite direction relative to the *NPTII* gene. Clearly, expression of *Sw5-b* by itself is sufficient for TSWV resistance, judging the high resistance scores of plants transformed with constructs Sw5-b (T) and Sw5-b (O). The effect of the expression of the two *Sw5* homologues on TSWV symptoms in transgenic plants is shown in Figure 4.

## Discussion

Genomic sequence determination of a 35 kb region of BAC clone 18-16F, containing RFLP marker CT220 (Folkertsma et al. 1999), has revealed the presence of seven open reading frames. Open reading frames one through five do not appear to be involved in TSWV resistance. However, the other two ORFs, denoted *Sw5-a* and *Sw5-b*, were highly homologous to previously identified resistance genes of the coiled-coil / nucleotide-binding ARC / leucine-rich repeat class such as *Mi* and *Prf* and were therefore studied in detail. Interestingly, the *Sw5-a* gene encodes only one putative leucine zipper, whereas *Sw5-b* potentially forms two of these structures. As leucine zippers are thought to be involved in multimerization of proteins and therefore also in binding to other proteins, this could imply a specific difference reflected in the functionality of gene *Sw5-b* and not gene *Sw5-a* to operate as resistance gene against TSWV. On the other hand, the Sw5-a protein is predicted to have a potential coiled coil structure in the N terminal domain, despite the absence of a second leucine zipper motif and may thus interact with other proteins all the same. A characteristic group II amino acid composition of parts of the nucleotide binding region was also observed on both homologues, including the tryptophan at the end of the kinase-2 domain (Figure 2B). High homology was observed to the tomato nematode and aphid resistance gene product Mi, both in length and in amino acid composition. Both *Sw5* genes clearly resemble *Mi* subgroup members of the LZ-NBS-LRR resistance gene class (Milligan et al. 1998; Vos et al. 1998), albeit the PCI domain, present in several proteins involved in multi protein complexes (Hofmann and Bucher 1998), could not be identified in the *Sw5* gene homologues. This domain was proposed by Vos et al. (1998) to be specific for the *Mi* subclass. As both *Mi* and *Sw5* are originally introgressed from *L. peruvianum* it is tempting to speculate a recent common origin of these gene families within that plant species, even though both clusters are present on separate chromosomes, i.e. 6 (Klein-Lankhorst et al. 1991) and 9 (Stevens et al. 1995), respectively.

Of the tomato resistance genes sequenced to date, *Pto* (Martin et al. 1993) is the only member of a subclass encoding a cytosolic protein kinase. The *Cf* genes contain leucine-rich repeats and a membrane anchor region, but no NB-ARC domain (Jones et al. 1994; Dixon et al. 1996, 1998; Thomas et al. 1997). Four tomato genes containing an NB-ARC motif have been sequenced to date: *Prf* (Salmeron et al. 1994), *I2c* (Ori et al 1997), *Mi* (Milligan et al. 1998; Vos et al. 1998) and *Sw5* (this report). All these genes belong to group II of the (NB-ARC)-LRR class. Despite

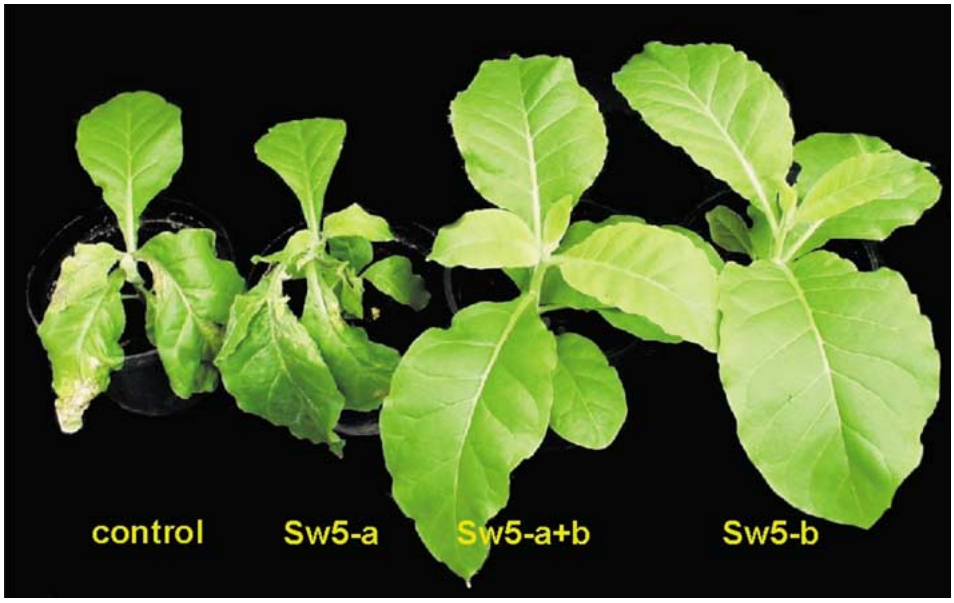


*Figure 4.* Phenotype of inoculated tobacco plants two weeks after inoculation with TSWV strain BR-01. Control indicates untransformed plants, *Sw5-a* and *Sw5-b* indicate plants transformed with the respective single open reading frames plus surrounding genomic DNA. *Sw5-a+b* designates a plant transformed with the 15.6 kb *Pst*I fragment containing both resistance gene candidates (see Figure 1).

the rather small sample it is typical that all tomato resistance genes containing a nucleotide binding box are of the coiled-coil type and none of them has been shown to contain a group I TIR domain. For *Arabidopsis* two thirds of the NB-ARC-containing resistance gene candidates fall into the TIR-containing group I, based on genetic sequence (Meyers et al. 1999). The pepper resistance gene *Bs2* encodes neither a TIR nor LZ (Tai et al. 1999), but has typical group II properties in the NB box. The sequenced potato resistance genes *Rx* (Bendahmane et al. 2000) and *Gpa2* (van der Vossen et al. 2000) are also of the CC/LZ type. Others, however, have found evidence for TIR-containing NB resistance genes in potato (Hehl et al. 1999; Sorri et al. 1999), indicating that, unlike cereal plants (Pan et al. 2000), *Solanaceae* encode TIR-containing NB resistance genes in their genome despite the fact that most of the sequenced genes are of the CC/LZ containing NB group II resistance genes. This suggests both the 'EDS1'- and 'NDR1'-related pathways defined in *Arabidopsis* (Aarts et al. 1998) are present even though the 'NDR1 route' seems to be used more frequently.

Two closely related homologues of *Mi* as well as two of *Sw5* both seem to have arisen from recent evolutionary events judging their high levels of homology, indicating an active strategy of gene duplication in *L. peruvianum*, the common origin of both types of resistance genes (Smith 1944; Stevens 1964). Detailed analysis of the *Cf4* and *Cf9* loci had already indicated the same for tomato species *L. hirsutum* and *L. pimpinellifolium* (Parniske et al. 1997). Interestingly, despite high homology, both the RGCs on the *Mi* and the *Sw5* locus have one active and one inactive gene in terms of observed pathogen resistances (Milligan et al. 1998; this paper). Similarities between *Sw5-a* and *Sw5-b* are not restricted to the coding regions, also the promoter and terminator regions are highly homologous over considerable lengths of sequence. The promoter region of both genes differs less than 2% over 1000 bp preceding the start codons. As no putative splice sites have been observed, this may indicate a co-ordinate expression of *Sw5-a* and *Sw5-b*, with the possibility of mandatory co-operative action of the expressed proteins. However, our experiments show that *Sw5-b* by itself is capable of conferring resistance at least to tomato spotted wilt virus, although the effect of *Sw5-a* on the action of *Sw5-b* cannot be fully excluded, considering the increased resistance rates in plants transformed with both genes at the same time. The latter, however, may be caused by the presence of a prominent MAR region in the *Sw5-a+b* constructs. A high resistance frequency has also been found for complementation experiments with other resistance genes using their adjacent MAR elements (E. van der Vossen and R. Klein-Lankhorst, personal communication). An effect of the MAR region could be the

higher frequency of resistance due to a more stable and higher level of expression of the resistance transgene (Mlynarova et al. 1995).

The *Sw5* locus as a whole has been demonstrated to confer resistance to a broad range of tospoviruses (Boiteux and Giordano 1993). In our previous work (Folkertsma et al. 1999) we suggested that different homologues could be responsible for resistance against the different tospoviruses. As we have presently not tested the resistance-conferring capability of *Sw5-b* to other tospoviruses, the importance of other resistance gene candidates in the region can not be ruled out. The fact that a single resistance gene can be responsible for resistance against two completely different pathogens as different as nematodes and aphids (Cook 1998; Jung 1998; Milligan et al. 1998; Rossi et al. 1998; Vos et al. 1998) would suggest the *Sw5-b* gene could single-handedly be responsible for this unusually broad tospovirus resistance. On the other hand, work by Takken et al. (1999) suggests other resistance gene copies within resistance loci of tomato can individually contribute to resistance, albeit by recognizing different avirulence gene products. Similarly, the *Peronospora parasitica* resistance gene *RPP8* and its paralogue *HRT*, a virus resistance gene, are located at the same locus in *Arabidopsis* (Cooley et al. 2000) like the nematode resistance gene *Gpa2* is distinct yet tightly linked to potato virus X resistance gene *Rx* (Bendahmane et al. 2000; van der Vossen et al. 2000). Based on our previous results (Folkertsma et al. 1999) we must conclude the *Sw5* locus is more complex than the two genes presented in this research. Indeed, our preliminary studies have shown three additional RGCs with similarity to *Sw5-a* and *Sw5-b* that are present further away from CT220, closer to the centromere of chromosome 9, and represent additional members of this resistance locus.

As the avirulence gene of *Sw5* has been assigned by viral genetic reassortants to the TSWV M RNA segment (Qiu et al. 1998), this does not indicate the avirulence product must also be defined by the M RNA segments of other tospoviruses, even more so when other *Sw5* homologues would be involved in broad tospovirus resistance. Further testing of (additional) *Sw5* homologues for their contribution to broad tospovirus resistance will be necessary for that purpose.

**Acknowledgements**

We gratefully acknowledge the financial support of the Netherlands Organization for Scientific Research (NWO) by its foundations ALW and STW (project 850.22.771). Marleen Henkens, Marjo van Staveren and Sander Peters are thanked for their involvement in sequencing the BAC clone and Hans Sandbrink for bio-informatics.

## References

Aarts N., Metz M., Holub E., Staskawitz B.J., Daniels M.J. and Parker J.E. 1998. Different requirement for EDS1 and NDR1 by disease resistance genes define at least two different signalling pathways in *Arabidopsis*. Proc. Natl. Acad. Sci. USA 95: 10306–10311.

Altschul S.F., Madden T.L., Schaffer A.A., Zhang J.H., Zhang Z., Miller W. and Lipman D.J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucl. Acids Res. 25: 3389–3402.

Àvila A.C., de Haan P., Kormelink R., Resende R., Goldbach R.W. and Peters D. 1993. Classification of tospoviruses based on phylogeny of nucleoprotein gene sequences. J. Gen. Virol. 74: 153–159.

Bairoch A., Bucher P. and Hoffmann K. 1997. The PROSITE database, its status in 1997. Nucl. Acids. Res. 25: 217–221.

Bendahmane A., Querci M., Kanyuka K. and Baulcombe D.C. 2000. *Agrobacterium* transient expression system as a tool for the isolation of disease resistance genes: application to the *Rx2* locus in potato. Plant J. 21: 73–81.

Boiteux L.S. and Giordano L de B. 1993. Genetic basis of resistance against two Tospovirus species in tomato (*Lycopersicon esculentum*). Euphytica 71: 151–154.

Bonfield J.K., Smith K.F. and Staden R. 1995. A new DNA sequence assembly program. Nucl. Acids Res. 24: 4992–4999.

Brommonschenkel S. and Tanksley S. 1997. Map-based cloning of the tomato genomic region that spans the *Sw-5* tospovirus resistance gene in tomato. Mol. Gen. Genet. 256: 121–126.

Brommonschenkel S.H., Tanksley S.D., Frary A., Otoni W.C. and Cheavegatti A. 1998. Positional cloning, molecular characterization and heterologous expression of the tospovirus resistance gene *Sw-5*. Abstract 5.4.7, 7th International Congress of Plant Pathology (Edinburgh, UK, 9–16 August 1998).

Chagué V., Mercier J.C., Guénard M., de Courcel A. and Vedel F. 1996. Identification and mapping on chromosome 9 of RAPD markers linked to *Sw-5* in tomato by bulked segregant analysis. Theor. Appl. Genet. 92: 1045–1051.

Cooley M.B., Pathirana S., Wu H.J., Kachroo P. and Klessig D.F. 2000. Members of the *Arabidopsis HRT/RPP8* family of resistance genes confer resistance to both viral and oomycete pathogens. Plant Cell 12: 663–676.

Cook R.J. 1998. The molecular mechanisms responsible for resistance in plant-pathogen interactions of the gene-for-gene type function more broadly than previously imagined. Proc. Natl. Acad. Sci. USA 95: 9711–9712.

Devereux J., Haeberli P. and Smithies O. A comprehensive set of sequence analysis programs for the VAX. Nucl. Acids Res. 12: 387–395.

Dixon M.S., Jones D.A., Keddie J.S., Thomas C.M., Harrison K. and Jones J.D.G. 1996. The tomato *Cf-2* disease resistance locus comprises two functional genes encoding leucine-rich repeat proteins. Cell 84: 451–459.

Dixon M.S., Hatzixanthis K., Jones D.A., Harrison K. and Jones J.D.G. 1998. The tomato *Cf-5* disease resistance gene and six homologs show pronounced allelic variation in leucine-rich repeat copy number. Plant Cell 10: 1915–1925.

Ellis J., Dodds P. and Pryor T. 2000. Structure function and evolution of plant resistance genes. Curr. Opin. Plant Biol. 3: 278–284.

Ewing B. and Green P. 1998. Basecalling of automated sequencer traces using PHRED. II. Error probabilities. Genome Res. 8: 186–194.

Ewing B., Hillier L., Wendl M.C. and Green P. 1998. Basecalling of automated sequencer traces using PHRED. I. Accuracy assessment. Genome Res. 8: 175–185.

Fabian P., Murvai J., Hatsagi Z., Vlahovicek K., Hegyi H. and Pongor S. 1997. The SBASE protein domain library, release 5.0: a collection of annotated protein sequence segments. Nucl. Acids Res. 25: 240–243.

Folkertsma R.T., Spassova M.I., Prins M., Stevens M.R., Hille J. and Goldbach R.W. 1999. Construction of a bacterial artificial chromosome (BAC) library of *Lycopersicon esculentum* cv. Stevens and its application to physically map the *Sw-5* locus. Mol. Breed. 5: 197–207.

Gish W. and States D.J. 1993. Identification of protein coding regions by database similarity search. Nature Genet. 3: 266–272.

Goldbach R. and Peters D. 1994. Possible causes of the emergence of tospovirus diseases. Sem. Virol. 5: 113–120.

Hehl R., Faurie E., Hesselbach J., Salamini F., Whitham S., Baker B. and Gebhardt C. 1999. TMV resistance gene *N* homologues are linked to *Synchytrium endobioticum* resistance in potato. Theor. Appl. Genet. 98: 379–386.

Hofmann K. and Bucher P. 1998. The PCI domain: a common theme in three multiprotein complexes. Trends Biochem. Sci. 23: 204–205.

Horsch R.B., Fry J.E., Hoffmann N.L., Eichholtz D., Rogers S.G. and Fraley R.T. 1985. A simple method for transferring genes into plants. Science 227: 1229–1231.

Inoue-Nagata A.K., Kormelink R., Nagata T., Kitajima E.W., Goldbach R. and Peters D. 1997. Effects of temperature and host on the generation of tomato spotted wilt virus defective interfering RNAs. Phytopathology 87: 1168–1173.

Jones D.A. and Jones J.D.G. 1997. The role of leucine-rich repeats in plant defences. Adv. Bot. Res. 24: 90–167.

Jones D.A., Thomas C.M., Hammond-Kosack K.E., Balint-Kurti P.J. and Jones J.D.G. 1994. Isolation of the tomato *Cf-9* gene for resistance to *Cladosporium fulvum* by transposon tagging. Science 266: 789–793.

Jung C. 1998. A singular gene doubles up pest resistance. Nature Biotechnol. 16: 1315–1316.

Klein-Lankhorst R.M., Rietveld P., Machiels B., Verkerk R., Weide R., Gebhardt C., Koornneef M. and Zabel P. 1991. RFLP markers linked to the root knot nematode resistance gene *Mi* in tomato. Theor. Appl. Genet. 81: 661–667.

Lupas A. 1996. Coiled coils: new structures and new functions. Trends Biochem. Sci. 21: 375–382.

Martin G.B., Brommonschenkel S.H., Chunwongse J., Frary A., Ganal M.W., Spivey R., Wu T., Earle E.D. and Tanksley S.D. 1993. Map-based cloning of a protein kinase gene conferring disease resistance in tomato. Science 262: 1432–1436.

McBride K.E. and Summerfelt K.R. 1990. Improved binary vectors for *Agrobacterium*-mediated plant transformation. Plant Mol. Biol. 14: 269–276.

Meyers B.C., Dickermann A.W., Michelmore R.W., Sivaramakrishnan S., Sobral B.W. and Young N.D. 1999. Plant disease resistance genes encode members of an ancient and divers protein family within the nucleotide binding superfamily. Plant J. 20: 317–332.

Milligan S.B., Bodeau J., Yaghoobi J., Kaloshian I., Zabel P. and Williamson V.M. 1998. The root knot nematode resistance gene *Mi* from tomato is a member of the leucine zipper, nucleotide binding, leucine-rich repeat family of plant genes. Plant Cell 10: 1307–1319.

Mlynarova L., Jansen R.C., Conner A.J., Stiekema W.J. and Nap J.P. 1995. The MAR-mediated reduction in position effect can be uncoupled from copy number-dependent expression in transgenic plants. Plant Cell 7: 599–609.

Ori N., Eshed Y., Paran I., Presting G., Aviv D., Tanksley S., Zamir D. and Fluhr R. 1997. The I2C family from the wilt disease resistance locus *I2* belongs to the nucleotide binding, leucine-rich repeat superfamily of plant resistance genes. Plant Cell 9: 521–532.

Pan Q., Wendel J. and Fluhr R. 2000. Divergent evolution of plant NBS-LRR resistance gene homologues in dicot and cereal genomes. J. Mol. Evol. 50: 203–213.

Parniske M., Hammond-Kosack K.E., Golstein C., Thomas C.M., Jones D.A., Harrison K., Wulff B.B.H. and Jones J.D.G. 1997. Novel disease resistance specificities result from sequence exchange between tandemly repeated genes at the *Cf-4/9* locus of tomato. Cell 91: 821–832.

Paul A.L. and Ferl R.J. 1999. Higher-order chromatin structure: looping molecules. Plant Mol. Biol. 41: 713–720.

Prins M. and Goldbach R. 1998. The emerging problem of tospovirus infection and nonconventional methods of control. Trends Microbiol. 6: 31–35.

Qiu W.P., Geske S.M., Hickey C.M. and Moyer J.W. 1998. Tomato spotted wilt Tospovirus genome reassortment and genome segment-specific adaptation. Virology 244: 186–194.

Resende R. de O., de Haan P., de Àvila A.C., Kitajima E.W., Kormelink R., Goldbach R. and Peters D. 1991. Generation of envelope and defective interfering RNA mutants of tomato spotted wilt virus by mechanical passage. J. Gen. Virol. 72: 2375–2383.

Rossi M., Goggin F.L., Milligan S.B., Kaloshian I., Ullman D.E. and Williamson V.M. 1998. The nematode resistance gene *Mi* of tomato confers resistance against the potato aphid. Proc. Natl. Acad. Sci. USA 95: 9750–9754.

Salmeron J.M., Oldroyd G.E.D., Rommens C.M.T., Scofield S.R., Kim H.-S., Lavelle D.T., Dahlbeck D. and Staskawicz B.J. 1994. Tomato *Prf* is a member of the leucine-rich repeat class of plant disease resistance genes and lies embedded within the *Pto* kinase gene cluster. Cell 86: 123–133.

Sambrook J., Fritsch E.F. and Maniatis T. 1989. Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Plainview, NY.

Singh G.B., Kramer J.A. and Krawetz S.A. 1997. Mathematical model to predict regions of chromatin attachment to the nuclear matrix. Nucl. Acids Res. 25: 1419–1425.

Smith P.G. 1944. Embryo culture of a tomato species hybrid. Proc. Am. Soc. Hort. Sci. 44: 413–416.

Sorri V.A., Watanabe K.N. and Valkonen J.P.T. 1999. Predicted kinase-3a motif of a resistance gene analogue as a marker for virus resistance. Theor. Appl. Genet. 99: 164–170.

Stevens J.M. 1964. Tomato Breeding. Project report W-Vv1, Department of Agricultural Technical Services, Republic of South Africa.

Stevens M.R., Scott S.J. and Gergerich R.C. 1992. Inheritance of a gene for resistance to tomato spotted wilt virus (TSWV) from *Lycopersicon peruvianum* Mill. Euphytica 59: 9–17.

Stevens M.R., Lamb E.M. and Rhoads D.D. 1995. Mapping the *Sw-5* locus for tomato spotted wilt virus resistance in tomatoes using RAPD and RFLP analyses. Theor. Appl. Genet. 90: 451–456.

Stief A., Winter D.M., Strätling W.H. and Sippel A.E. 1989. A nuclear DNA attachment element mediates elevated and position-independent gene activity. Nature 341: 343–345.

Tai T.H., Dahlbeck D., Clark E.T., Gajiwala P., Pasion R., Whalen M.C., Stall R.E. and Staskawicz B.J. 1999. Expression of the *Bs2* pepper gene confers resistance to bacterial spot disease in tomato. Proc. Natl. Acad. Sci. USA 96: 14153–14158.

Takken F.L.W., Thomas C.M., Joosten M.H.A.J., Golstein C., Westerink N., Hille J., Nijkamp H.J.J., de Wit P.J.G.M. and Jones J.D.G. 1999. A second gene at the tomato *Cf-4* locus confers resistance to *Cladosporium fulvum* through recognition of a novel avirulence determinant. Plant J. 20: 279–288.

Thomas C.M., Jones D.A., Parniske M., Golstein C. and Jones J.D.G. 1997. Characterization of the tomato *Cf-4* gene for resistance to *Cladosporium fulvum* identifies sequences that determine recognitional specificity in Cf-4 and Cf-9. Plant Cell 9: 2209–2224.

van der Biezen E.A. and Jones J.D.G. 1999. The NB-ARC domain: a novel signalling motif shared by plant resistance gene products and regulators of cell death in animals. Curr. Biol. 8: 226–227.

van der Vossen E.A.G., Rouppe van der Voort J.N.A.M., Kanyuka K., Bendahmane A., Sandbrink H., Baulcombe D.C., Bakker J., Stiekema W.J. and Klein-Lankhorst R.M. 2000. Homologues of a single resistance-gene cluster in potato confer resistance to distinct pathogens: a virus and a nematode. Plant J. 23: 567–576.

Vos P., Simons G., Jesse T., Wijbrandi J., Heinen L., Hogers R., Frijters A., Groenendijk J., Diergaarde P., Reijans M., Fierens-Onstenk J., de Both M., Peleman J., Liharska T., Hontelez J. and Zabeau M. 1998. The tomato *Mi-1* gene confers resistance to both root-knot nematodes and potato aphids. Nature Biotechnol. 16: 1365–1369.

Whitham S., Dinesh-Kumar S.P., Choi D., Hehl R., Corr C. and Baker B. 1994. The product of the tobacco mosaic virus resistance gene *N*: similarity to toll and the interleukin-1 receptor. Cell 78: 1101–1115.

Young N.D. 2000. The genetic architecture of resistance. Curr. Opin. Plant Biol. 3: 285–290.