The Broad-Spectrum Tospovirus Resistance Gene *Sw-5* of Tomato Is a Homolog of the Root-Knot Nematode Resistance Gene *Mi*

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We used a positional cloning approach to isolate the *Sw-5* disease resistance locus of tomato. Complementation experiments with overlapping cosmid clones enabled us to demonstrate that *Sw-5* is a single gene locus capable of recognizing several tospovirus isolates and species. Analysis of the predicted *Sw-5* protein suggests that it is a cytoplasmic protein, with a potential nucleotide binding site (NBS) domain and a C-terminal end consisting of leucine-rich repeats (LRRs). Based on its structural features, *Sw-5* belongs to the class of NBS-LRR resistance genes that includes the tomato *Mi*, *I2*, and *Prf* genes; the Arabidopsis *RPM1* gene; and the plant potato virus X resistance gene *Rx*. The overall similarity between the *Sw-5* and *Mi* proteins of tomato suggests that a shared or comparable signal transduction pathway leads to both virus and nematode resistance in tomato. The similarity also supports the hypothesis that *Sw-5* provides resistance via a hypersensitive response. *Sw-5* is a member of a loosely clustered gene family in the telomeric region of chromosome 9. Members of this family map to other regions of chromosome 9 and also to chromosome 12, where several fungal, virus, and nematode genes have been mapped, suggesting that paralogs of *Sw-5* may have evolved to provide different resistance specificities.

Additional keywords: *Lycopersicon esculentum*, map-based cloning, *Tomato spotted wilt virus*, TSWV.

Tospoviruses are thrips-transmitted viruses that belong to the genus *Tospovirus* (family *Bunyaviridae*) and cause substantial yield losses to ornamental and vegetable crops in several areas of the world (German et al. 1992; Peters et al. 1996). The members of this genus are characterized by a genome comprising three separate, single-stranded RNAs (S, M, and L RNAs) enveloped in a lipidic membrane and associated with two glycoproteins, G1 and G2 (German et al. 1992; van Poelwijk et al. 1996). Based on serological properties, vector specificity, host range, and nucleotide and amino acid sequences of the nucleoprotein gene, several species have been established in the genus *Tospovirus* (de Ávila et al. 1993;

Goldbach and Kuo 1996; Peters et al. 1996). *Tomato spotted wilt virus* (TSWV), *Tomato chlorotic spot virus* (TCSV), and *Groundnut ringspot virus* (GRSV) are the *Tospovirus* species commonly associated with important yield losses in the tomato crop. TSWV, the type species of the genus, has a worldwide distribution, whereas GRSV and TCSV have thus far only been identified on tomatoes grown in Brazil and Argentina (Dewey et al. 1996; Peters et al. 1996; Resende et al. 1996). In Brazil, new *Tospovirus* species that infect tomato are also being characterized (Resende et al. 1996).

Because genetic resistance offers the best means of protecting crop plants against tospovirus infection, considerable effort has been devoted to the development of resistant varieties (Cho et al. 1996; Roselló et al. 1998). Naturally occurring host resistance to tospoviruses has been reported in many *Lycopersicon* species (Cho et al. 1996; Finlay 1953; Krishna Kumar et al. 1993; Paterson et al. 1989; Roselló et al. 1996; Stevens et al. 1994). However, in many cases, this resistance is species and/or isolate specific and therefore not very useful for breeding purposes. Broad-spectrum tospovirus resistance was found in the *L. esculentum* cv. Stevens (van Zijl et al. 1986). Stevens et al. (1992) showed that the TSWV resistance of this cultivar, introgressed from an unknown *L. peruvianum* accession, is conferred by a single gene, *Sw-5*, that is incompletely dominant. This gene also seems to provide resistance to TCSV and GRSV (Boiteux and Giordano 1993).

Genes conferring resistance to different isolates of the same pathogen or different pathogens are often organized in the plant genome as tandem arrays of homologs (Hammond-Kosack and Jones 1997). Therefore, the multiple resistances conferred by *Sw-5* raise the question of whether the *Sw-5* locus consists of a cluster of tightly linked genes, each gene having its own virus specificity, or whether it encodes a single gene product capable of recognizing several *Tospovirus* isolates and species. In addition, the physiological mechanism by which *Sw-5* provides resistance is unclear. In tomato plants carrying *Sw-5*, the virus cannot spread systemically throughout the plant, and in many cases, the inoculated leaves do not show any macroscopic symptoms. However, for some *Tospovirus* isolates and/or under high inoculum pressure, local and systemic necrotic lesions appear on the inoculated plants, suggesting that *Sw-5* resistance can elicit a hypersensitive response (Roselló et al. 1998; Stevens et al. 1992; S. H. Brommonschenkel, un-

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published data). *Sw-5* may also be developmentally regulated, as the resistance gene does not appear to provide tospovirus resistance in tomato fruits (de Haan et al. 1996).

These unusual characteristics of *Sw-5* and its tight linkage to restriction fragment length polymorphism (RFLP) marker CT220 on the long arm of tomato chromosome 9 prompted us to isolate this gene via map-based cloning. By high resolution-genetic linkage analysis of *Sw-5* recombinant plants and chromosome walking, we previously demonstrated that the *Sw-5* locus resides in a 100-kilobase (kb) genomic region spanned by 10 overlapping plant transformation-competent cosmid clones (Brommonschenkel and Tanksley 1997). In this paper, we describe the characterization of the *Sw-5* locus and show that it contains a single gene capable of providing resistance to different *Tospovirus* species and that it belongs to the nucleotide binding site (NBS) domain-leucine-rich repeat (LRR) class of plant disease resistance genes.

RESULTS AND DISCUSSION

Complementation experiments reveal that the *Sw-5* locus is located within a 20-kb cosmid clone.

Our earlier work narrowed down the location of *Sw-5* to a 100-kb segment of tomato chromosome 9 spanned by 10 over-

lapping plant transformation-competent cosmid clones (Fig. 1A). To further refine the position of *Sw-5* within this contig, the cosmid clones were transferred into *Agrobacterium tumefaciens* and used to transform the TSWV susceptible tomato cv. Moneymaker. For each cosmid, three to 10 independent transformants were generated. The primary transformants were selfed and 20 to 30 progeny of each T₁ plant were screened separately for resistance to TSWV, GRSV, and TCSV isolates in the greenhouse. All of the progeny plants, except those derived from transgenic plants carrying cosmid TC134 (insert approximately 20 kb), were completely susceptible to the *Tospovirus* species and isolates. In contrast, progeny plants derived from 'Moneymaker':TC134 transformants showed a hypersensitive-like response to TSWV and no symptoms to GRSV and TCSV on inoculated leaves. These transgenic plants resembled the control plants carrying *Sw-5* in that systemic invasion did not occur (Fig. 2). Double-antibody sandwich-enzyme-linked immunosorbent assays (DAS-ELISAs) with uninoculated apical leaf samples from these plants confirmed the absence of the viruses. Polymerase chain reaction (PCR) analysis indicated a perfect correlation between tospovirus resistance and the presence of TC134 cosmid sequences. Progeny plants that were not carrying TC134 (due to normal segregation) showed typical tospovirus symptoms such as stunting and general

Fig. 1. Positional cloning of the *Sw-5* tospovirus resistance locus. **A**, Diagram showing alignment of cosmid clones in the chromosome region around the restriction fragment length polymorphism marker CT220 that contains *Sw-5*, based on high resolution genetic linkage analysis (Brommonschenkel and Tanksley 1997). Horizontal lines: cosmid inserts contained within 10 overlapping cosmid clones. Circled letters R and S identify cosmid clones that either confer (R) or fail to confer (S) resistance to tospoviruses upon mechanical inoculation. **B**, Fine mapping of *Sw-5* inside cosmid TC134 by complementation analysis. Cosmid clone TC53 that overlaps with TC134 and lacks the TCD1-homologous region (horizontal line) fails to complement the susceptible phenotype of 'Moneymaker'. Later sequence analysis showed that the genomic region containing *Sw-5* (shown as diagonal lines) is truncated in both noncomplementing cosmids TC53 and TC55.

necrosis. Overall, these results indicate that all *Sw-5* specificity resides on the cosmid TC134.

Molecular cloning of the *Sw-5* gene.

To identify candidates for the *Sw-5* gene from the TC134 genomic region, a leaf cDNA library constructed from the susceptible cv. Monegion was probed with TC134 subclones. Two different cDNAs were obtained, TCD1 (approximately 2.2 kb) and TCD11 (approximately 1.2 kb). Sequence analysis revealed that the predicted TCD1 protein product has regions of homology with known plant disease resistance proteins, showing the greatest similarity to the tomato gene *Prf* (Salmeron et al. 1996). In contrast, TCD11 showed no homology with known plant resistance (*R*) genes. DNA gel blot analysis demonstrated that, while TCD11 is single copy, numerous TCD1-related sequences are present in the tomato genome (Fig. 3). This result suggested that TCD1 was a member of a multigene family. This finding correlates well with previous studies that indicate that plant *R* genes often occur in gene families (Hammond-Kosack and Jones 1997; Staskawicz et al. 1995). From these two lines of evidence, TCD1 appeared to be the most likely candidate for *Sw-5*. To prove that TCD1 was indeed derived from the *Sw-5* locus, additional transformation experiments were performed with another cosmid clone, TC53, that does not cross hybridize to TCD1 but does contain the remainder of the genomic DNA in TC134 (Fig. 1B). Three independent transgenic 'Moneymaker' plants were obtained and

selfed, and their progenies were analyzed for tospovirus resistance. All plants were found to be completely susceptible, leading us to conclude that *Sw-5* lies in the region corresponding to TCD1.

Expression and structure of the *Sw-5* gene.

Gel blots containing poly(A)⁺ RNA isolated from the leaves of susceptible and resistant tomato lines were hybridized with TCD1. A weakly expressed transcript of approximately 4.4 kb was detected in both resistant and susceptible plants (Fig. 4A). Given that the TCD1 cDNA is only 2.2 kb, this result suggests that TCD1 is derived from a truncated *Sw-5* transcript. Sequencing of the region of the TC134 cosmid that hybridizes to TCD1 and an additional approximately 4.0 kb upstream of the 5' end of this cDNA identified only one large, intronless, open reading frame of 3,738 base pairs (bp), encoding a putative hydrophilic polypeptide of 1,246 amino acid residues with a molecular mass of 144 kDa (Fig. 4B).

The complete structure of *Sw-5* was determined by characterizing additional cDNAs derived from a size-selected unidirectional cDNA library constructed from leaf tissue of the *Sw-5* homozygous line SW99-1. After screening 3×10^6 primary cDNA clones with TCD1 as a probe, 16 hybridizing cDNA clones were identified. Isoline DNA blotting analysis, restriction mapping, and sequence analysis revealed that six clones corresponded to *Sw-5*, whereas the other 10 corresponded to at least three additional genes with a similar sequence. The long-

Fig. 2. Genetic complementation with cosmid clone TC134. Symptoms of a systemic infection in an untransformed 'Moneymaker' plant and resistance response of a 'Moneymaker' plant transformed with cosmid TC134.

est *Sw-5* cDNA clone was 3.9 kb. The longest insert in the other five *Sw-5* homologous cDNA clones was only 2.6 kb. However, when organized together, these cDNA clones produce a deduced transcribed sequence of approximately 4,172 bp, which is close to the size of the *Sw-5* mRNA as determined by RNA blot analysis (Fig. 4A). Comparison of the cDNA and genomic sequences revealed the presence of an intron (approximately 1,075 nucleotides) in the 5' untranslated region of *Sw-5* (Fig. 4B). Thus, the processed transcript has a 5' untranslated region of approximately 234 nucleotides. The length of the 3' untranslated region is 160 nucleotides. The nucleotides flanking the putative *Sw-5* translation start site (GAAAATGGC) are consistent with the consensus sequence for translation initiation in plants (AACAAUGGC) (Lütcke et al. 1987), and several in-frame stop codons are located upstream of the start codon. A typical TATA box sequence is present 1,523 bases upstream of the start codon. The polyadenylation signal is located 135 nucleotides downstream of the termination codon.

The deduced amino acid sequence of *Sw-5* is shown in Figure 4C. The *Sw-5* sequence shows the greatest similarity to the amino acid sequence of the nematode resistance protein *Mi* (30% identity) and the protein required for *Pseudomonas* resistance and fenthion sensitivity, *Prf* (35% identity) (Fig. 5). The *Sw-5* protein contains many motifs found in previously cloned *R* genes: a putative NBS composed of kinase-1a (or P loop, amino acids 562 to 571), kinase-2a (amino acids 633 to 644), and putative kinase-3a (amino acids 663 to 674) domains (Grant et al. 1995; Hammond-Kosack and Jones 1997). An additional motif of unknown function is present (amino acids 781 to 787) that is well conserved in the products of other *R* genes (Grant et al. 1995; Hammond-Kosack and Jones 1997). The C-terminal region of *Sw-5* is composed of 14 imperfect LRRs, beginning at amino acid 916 (Fig. 4C).

Mechanism of TSWV resistance.

The NBS-LRR class of *R* proteins have one common feature: they mediate the development of cell death at sites of infection. Thus, the similarity of *Sw-5* to these proteins supports the possibility that the mechanism of TSWV resistance involves the elicitation of a hypersensitive response. For example, the resistance to root-knot nematodes provided by the *Mi* gene, which is the *R* gene with the greatest similarity to *Sw-5*, is characterized by a localized necrosis near the site where feeding cells would normally be initiated (Dropkin 1969). The fact that the hypersensitive response is not always observed in a *Sw-5* background may be due to differences in the affinity of the tospovirus elicitor to *Sw-5* and/or differences in the timing of the interaction.

The *Sw-5* protein is remarkably similar to the *Mi* protein. With the exception of the four heptad amphipathic leucine zippers at the N terminus that are not evident in *Sw-5*, the similarity is dispersed over the entire molecule. It seems reasonable to hypothesize that the functionally and structurally related *Sw-5* and *Mi* proteins may act through a common signal transduction pathway. As root-knot nematodes (Williamson and Hussey 1996) and viruses probably secrete/produce several proteins inside the plant cell as part of their life cycles/pathogenic strategies, the similarity of *Sw-5* to *Mi* is not surprising and probably reflects the remarkable epitope-recognition ability and signal transduction activation of NBS-LRR proteins.

Genomic distribution of the *Sw-5* gene family.

Cosmid DNA blot analysis did not reveal any additional copies of *Sw-5* in the 150-kb cosmid contig around the *Sw-5* locus (Brommonschenkel and Tanksley 1997; data not shown). Because the cDNA screening and isolate DNA blot analysis suggested that *Sw-5* is a member of a multigene family, the chromosomal positions of the homologous sequences were assessed by RFLP mapping in an *Lycopersicon esculentum* × *L. pennellii* F₂ mapping population (Tanksley et al. 1992). Most of the *Sw-5* homolog sequences are dispersed on tomato chromosome 9 (Fig. 6). Four *Sw-5* homolog sequences map to the telomeric region of chromosome 9: *Sw-5.1*, *Sw-5.2*, and *Sw-5.3* map 3.6, 5.7, and 6.3 cM below CT220, respectively. This is the site of the *Globodera pallida* resistance gene *Gpa6* in the homeologous potato genome (van der Voort 1998). The fourth homolog, *Sw-5.4*, cosegregates with CT71, located 2.7 cM above CT220. Two other two homologs map on chromosome 9 in the vicinity of the *Tm2a R* gene of *Tobacco mosaic virus* (Pillen et al. 1996): *Sw-5.5* cosegregates with CD3, just below the centromeric region, and *Sw-5.6* maps with TG551 further down the long arm of chromosome 9. The *Sw-5.7* homolog maps to chromosome 12, cosegregating with TG360, the same region where the powdery mildew resistance gene *Lv* has been mapped (Chunwongse et al. 1997). Clearly, *Sw-5* is a member of a multigene family whose members are dispersed throughout the tomato genome and may be conferring resistance to a variety of pathogens.

MATERIALS AND METHODS

Tospovirus resistance evaluation.

Tospovirus resistance was evaluated by mechanical inoculations. The *Tospovirus* strains used for the inoculation tests

Fig. 3. Southern blot analysis of cDNA clone TCD1. Genomic DNA from tomato cvs. SW99-1 (R) and Piedmont (S) was isolated from leaves. Aliquots of 3 µg were digested with the indicated enzymes, separated by electrophoresis on a 1% agarose gel, blotted onto Hybond N⁺ membrane and hybridized with radiolabeled TCD1 insert. The first line (M) represents the molecular length standard λ *Hind*III.

were as follows: BR-01, 'HR' (TSWV), V1-3 and BR-03 (TCSV), and SA-05 (GRSV). The original inoculum was kept at -80°C and thawed prior to multiplication in *Nicotiana*

tabacum cv. Havana 425. Inoculum was prepared by macerating young, newly infected 'Havana 425' leaves with a mortar and pestle in 0.1 M potassium phosphate buffer, pH 7.0,

Fig. 4. Expression and structure of the *Sw-5* gene. **A**, Blot analysis of *Sw-5* RNA. Polyadenylated RNA from leaves of the cultivars SW-99-1 (R, 10 μg) and Piedmont (S, 10 μg) were fractionated on 1.3% agarose gel containing formaldehyde, blotted onto Hybond N⁺ membrane, hybridized with radiolabeled TCD1 insert and exposed to Kodak X-AR film for 7 days. **B**, *Sw-5* homologous genomic region of cosmid TC134 and transcript are represented as solid black boxes. Angled lines indicate intron. **C**, Deduced *Sw-5* protein sequence. Boundaries of the nucleotide binding site conserved region are indicated. Polypeptide regions corresponding to kinase-1a, kinase-2, and kinase-3 motifs and two other conserved domains discussed in text are in bold. The leucine-rich repeat region is represented in italics.

containing 0.01 M sodium sulfite. The leaves of tomato plants at the three- to five-leaf stage (approximately 30 to 37 days after germination) were dusted with 600-mesh Carborundum powder and the inoculum was applied with cheesecloth. A second

inoculation was done 2 to 3 days after the first. Plants were evaluated weekly for symptoms and held in the greenhouse for at least 6 weeks after infection. Inoculations consistently resulted in 100% infection of the susceptible tomato control cv. Piedmont.

Fig. 5. Detailed analysis of the Sw-5 deduced protein structure. **A**, Amphiphilicity profile of the Sw-5 protein. **B**, Comparison of the conserved centrally located Sw-5 nucleotide binding site domain: RPM1 (Grant et al. 1995), Prf (Salmeron et al. 1996), Mi-1.2 (Milligan et al. 1998), Rx (van der Voort et al. 1999), and I2C (Ori et al. 1997). Conserved kinase-1a, kinase-2, and kinase-3a, and conserved hydrophobic domain are indicated. **C**, Comparisons of the leucine-rich repeat domains of Sw-5, Mi-1.2, and Prf. Black boxes in a column indicate identical or similar amino acids.

All inoculated plants were assayed for the presence of the virus by DAS-ELISA (Cho et al. 1988) and species-specific polyclonal antisera. Symptomatic tissues from plants were sampled and tested for tospovirus. Plants not exhibiting systemic infection were held in the greenhouse for 6 weeks, after which time leaf samples from the middle and top of the plants were removed and tested for tospovirus infection.

RFLP analysis.

The standard F₂ mapping population (VF36Tm2a × *L. pennellii*) was employed in determining the map location of TCD1 homologous sequences essentially as described by Tanksley et al. (1992).

DNA sequencing and computer analysis.

DNA sequences were determined from double-stranded plasmid DNA by the dideoxy chain termination method. Sequencing reaction products were run on an automated sequencer. The DNA sequences were assembled with MacVector (Oxford Molecular, Oxford, U.K.). Homology searches of the

GenBank database were performed with the BLAST 2.0 algorithm (Altschul et al. 1997). Alignments with other sequences were performed with the MegaAlign program (DNA-STAR, Madison, WI, U.S.A.).

Plant transformation.

Cosmid clones were introduced into *Agrobacterium tumefaciens* LBA4404 by triparental mating or electroporation. Transformation of excised cotyledons from susceptible tomato cv. Moneymaker was conducted essentially as described by Frary and Earle (1996). Transgenic plants were identified by resistance to kanamycin and confirmed by PCR assays with specific primers that distinguished between the transgenic cosmid insert and endogenous sequences. Positive transformants were transferred to the greenhouse and selfed. The T₁ progeny were analyzed for virus resistance by mechanical inoculation.

cDNA library construction and screening.

Total RNA was isolated from unchallenged leaves of the Sw99-1 line and poly(A)⁺ RNA was purified with the Oligotex

Fig. 6. Linkage maps of chromosomes 9 and 12 showing the distribution of Sw-5 homologs in the tomato genome. Linkage maps were adapted from Tanksley et al. (1992). Approximate location of relevant tomato (*Tm2a* and *Lv*) and potato (*Rx*, *Gpa2*, and *Gpa6*) resistance genes indicated are also based on previous studies (Pillen et al. 1996; Chunwongse et al. 1997; van der Voort 1998, 1999).

mRNA maxi kit (Qiagen, Valencia, CA, U.S.A.). Double-stranded cDNA was synthesized with oligo(deoxyribosylthymine) as primer. After addition of *Eco*RI linkers, the cDNA molecules were size fractionated. Pools that contained molecules longer than 2.0 kb were combined and ligated to λ ZapII vector linearized with *Eco*RI and *Xho*I, encapsidated in vitro and amplified in the *Escherichia coli* strain XL1-Blue according to the supplier's instructions (Stratagene, La Jolla, CA, U.S.A.).

Approximately 3×10^6 primary recombinants were screened with the cDNA clone TCD1 as a probe. Hybridizations were performed at 65°C according to the method of Church and Gilbert (1984). Filters were washed three times at 65°C, once with $2 \times$ SSC ($1 \times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate (SDS), once with $1 \times$ SSC and 0.1% SDS, and once with 0.5% SSC and 0.1% SDS for 20 min each. After plaque purification, selected positive phage clones were converted to pBluescript SK⁻ phagemid clones with helper phage by following the supplier's instructions (Stratagene).

RNA gel blot analysis.

RNA was fractionated on a 1% agarose-0.66 M formaldehyde gel. RNA species were transferred to nylon membranes (Hybond-N⁺; Amersham, Buckinghamshire, U.K.) by capillary action with $10 \times$ SSC. After NaOH fixation, filters were hybridized at 65°C according to the method of Church and Gilbert (1984). Filters were washed three times at 65°C, once with $2 \times$ SSC and 0.1% SDS, once with $1 \times$ SSC and 0.1% SDS, and once with 0.5% SSC and 0.1% SDS for 20 min each. A 0.24- to 9.5-kb RNA ladder was used as the size reference.

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