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Fine mapping of the tomato *I-3* gene for fusarium wilt resistance and elimination of a co-segregating resistance gene analogue as a candidate for *I-3*

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Abstract The *I-3* gene from the wild tomato species *Lycopersicon pennellii* confers resistance to race 3 of the devastating vascular wilt pathogen *Fusarium oxysporum* f. sp. *lycopersici*. As an initial step in a positional cloning strategy for the isolation of *I-3*, we converted restriction fragment length polymorphism and conserved orthologue set markers, known genes and a resistance gene analogue (RGA) mapping to the *I-3* region into PCR-based sequence characterised amplified region (SCAR) and cleaved amplified polymorphic sequence (CAPS) markers. Additional PCR-based markers in the *I-3* region were generated using the randomly amplified DNA fingerprinting (RAF) technique. SCAR, CAPS and RAF markers were used for high-resolution mapping around the *I-3* locus. The *I-3* gene was localised to a 0.3-cM region containing a RAF marker, eO6, and an RGA, RGA332. RGA332 was cloned and found to correspond to a putative pseudogene with at least two loss-of-function mutations. The predicted pseudogene belongs to the Toll interleukin-1 receptor-nucleotide-binding site-leucine-rich-repeat sub-class of plant disease resistance genes. Despite the presence of two RGA332 homologues in *L. esculentum*, DNA gel blot and PCR analysis suggests that no other homologues are present in lines carrying *I-3* that could be alternative candidates for the gene.

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Introduction

Fusarium oxysporum f. sp. *lycopersici* is the causal agent of vascular wilt in tomato, a disease important both economically and as a model system for vascular wilts. The fungus penetrates though the roots and proliferates in the vascular tissue, impeding water transport and causing spectacular wilting and rapid death of the plant. The interaction between *F. o. lycopersici* and tomato is race-cultivar specific, and three host-specific races of *F. o. lycopersici* have been described to date (Bohn and Tucker 1939; Alexander and Tucker 1945; Grattidge and O'Brien 1982; Volin and Jones 1982). Resistance to all three races has been identified in wild tomato species and incorporated into the cultivated tomato *Lycopersicon esculentum*.

Race-specific disease resistance in plants is determined by single dominant or semi-dominant resistance (*R*) genes that are involved in the recognition of the products of avirulence (*Avr*) genes from pathogens and the activation of plant defence responses (see Ellis et al. 2000; Dangl and Jones 2001; Van der Hoorn et al. 2002 for recent reviews). *R* genes conferring resistance to *F. o. lycopersici* race 1 have been identified and mapped to chromosomes 11 (Bohn and Tucker 1939; Paddock 1950) and 7 (Sarfatti et al. 1991) but have not yet been isolated. The *I-2* gene, conferring resistance to *F. o. lycopersici* race 2, has been isolated via a positional cloning strategy and is a member of the coiled coil (CC)-nucleotide-binding site (NBS)-leucine-rich repeat (LRR) class of resistance genes (Simons et al. 1998). *I-2* lies within a cluster of seven similar genes on the long arm of chromosome 11.

F. o. lycopersici race 3 was first reported in Australia in 1978 (Grattidge and O'Brien 1982). It has also been reported in Florida (Volin and Jones 1982) and then later in California (Davis et al. 1988). By the early 1980s, *F. o. lycopersici* race 3 was causing significant yield losses and putting land out of tomato cultivation in both continents. Resistance to race 3, controlled by a single dominant gene, was described in *L. pennellii* accessions PI414773 (McGrath et al. 1987) and LA716 (Scott and Jones 1989), and the genes responsible in both accessions were named *I-3*. We are pursuing isolation of the *I-3* gene from accession LA716, both because of its agronomic importance and also to determine the range of genes specifying resistance to vascular wilt in tomato.

The *I-3* locus was previously mapped to the long arm of chromosome 7 (Bournival et al. 1989), a region subsequently well populated with restriction fragment length polymorphism (RFLP) (Tanksley et al. 1992), amplified fragment length polymorphism (AFLP) (Haanstra et al. 1999) and conserved orthologue set (COS) markers (Fulton et al. 2002). In order to avoid the time and high-labour input inherent in RFLP and AFLP technology, we aimed to convert existing markers in the *I-3* region to PCR-based sequence characterised amplified region (SCAR) and cleaved amplified polymorphic sequence (CAPS) markers. The use of such markers allows large-scale, locus-specific screening experiments to be performed with a minimum of equipment and time. Existing map information was used to select sequences, including RFLP and COS markers, and known genes, that mapped to the *I-3* region and were suitable for conversion to PCR-based markers.

The majority of known plant resistance genes contain NBS and LRR domains. The conserved nature of motifs within these domains has been exploited to search for new resistance gene-like sequences or resistance gene analogues (RGAs) using a homology-dependent PCR-based approach (Kanazin et al. 1996; Yu et al. 1996; Aarts et al. 1998; Collins et al. 1998; Mago et al. 1999). Many RGAs have been mapped to genomic positions containing known resistance specificities, and RGAs have been shown to be derived from known resistance genes (Aarts et al. 1998; Collins et al. 1999). RGAs thus represent candidates for functional resistance genes.

Amplification products with homology to known resistance genes have been obtained from potato (*Solanum tuberosum*) using primers designed to conserved motifs within the NBS domain of *RPS2* and *N* (Leister et al. 1996). Two products, St332 and St334, were found to be linked to the *Gro1* locus for resistance to the root cyst nematode *Globodera rostochiensis*, located on potato chromosome 7 in a region syntenic to that containing *I-3* in tomato. Fragments of apparently orthologous genes have also been amplified from *L. esculentum* using similar primers (Ohmori et al. 1998; Pan et al. 2000a). These were mapped to bin 7-E on chromosome 7, and one, designated Q2 (orthologous to St334), was reported to be linked to *I-3* at a 2-cM resolution (Pan et al. 2000a). We found that a *L. pennellii* homologue of St332 (designated RGA332) co-segregated with *I-3*, and we cloned the corresponding single-copy gene from *L. pennellii* to determine if it was a candidate for the *I-3* gene.

Conserved motifs within the RGA332 fragment suggested that, unlike the *I-2* gene, which is a member of the CC-NBS-LRR subclass (Simons et al. 1998), this gene belonged to the Toll interleukin-1 receptor homology (TIR)-NBS-LRR subclass (Meyers et al. 1999; Pan et al. 2000b). However, the TIR-NBS-LRR gene corresponding to RGA332 was found to be a putative pseudogene that contained at least two loss-of-function mutations. Moreover, no other homologues of RGA332 were found in tomato lines carrying *I-3*. These data eliminate RGA332 as a candidate for *I-3* and exclude the possibility that *I-3* might be a homologue of RGA332.

Materials and methods

Plant production and fungal inoculations

For general experimental work, plants were grown in heated glasshouses at a maximum day temperature of 30°C and a minimum night temperature of 18°C in a steam-sterilised soil mix. For inoculation with *Fusarium oxysporum* f. sp. *lycopersici*, plants were grown in tunnel houses at ambient temperature and inoculated with *F. o. lycopersici* race 3 isolate no. 1943, provided by the Queensland Department of Primary Industry, Plant Pathology Section. The fungus was maintained on potato dextrose agar, and inoculum was produced by washing conidia from plates with sterile distilled water. Tomato seedlings were germinated in University of California (UC) mix (50% peat, 50% sand). Three-week-old seedlings were removed from soil, dipped in a 10^6 ml^{-1} suspension of conidia and then replanted in UC mix. Symptoms were evaluated 3 weeks after inoculation and scored on a 0–5 severity scale with 0 = no reaction, 1 = localised vascular staining in the tip of the primary root, 2 = staining to cotyledonary node, 3 = staining beyond cotyledonary node but with no external symptoms, 4 = external symptoms and severe stunting, 5 = complete collapse, death. Classes 0–2 were classified as resistant, and classes 3–5 were classified as susceptible. At least 25 seedlings were scored for each line tested.

Tomato lines and markers used for mapping

Lines IL7-2, IL7-3 and IL7-4 contain overlapping introgressions of the *Lycopersicon pennellii* LA716 genome in an *L. esculentum* cv. M82 background (Eshed and Zamir 1994). The IL lines and M82 were provided by Dani Zamir, Hebrew University of Jerusalem, Rehovot, Israel. Fla. 7841 and Fla. 7547 are commercial release breeding lines from the University of Florida (Scott and Jones 1995) and were provided by Jay Scott, University of Florida, Bradenton, Fla. An F_2 population segregating for resistance to race 3 was obtained by crossing *L. esculentum* cv. M82 with the introgression line IL7-3. Recombinant plants from the F_2 generation were selfed, and the *I-3* genotype was determined by analysing segregation of resistance in the F_3 progeny. TG clones were obtained from Steven Tanksley, Department of Plant Breeding and Biometry, Cornell University, New York State. Sequences for all of the other markers were obtained from public databases.

General molecular methods

Total plant DNA was extracted for PCR-based genotyping using the method of Edwards et al. (1991) as modified by Giraudat et al. (2003) using a single cotyledon from a 1- to 2-week-old seedling. For the extraction of total plant DNA for gel blot analysis, 200 mg

of plant tissue was ground to a fine powder under liquid nitrogen and resuspended in 6 ml CTAB buffer (200 m*M* Tris-HCl pH 7.5, 50 m*M* EDTA, 2 *M* NaCl, 2% (w/v) CTAB, 0.8% (w/v) sodium bisulphite). A volume of 1.5 ml 1% (w/v) *N*-lauroylsarcosine was added and the sample incubated at 60°C for 20 min. The sample was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). DNA was precipitated from the aqueous phase with 0.6 vol. of isopropanol, collected by spooling, washed in 70% (v/v) ethanol and resuspended in TE pH 8.0 (100 m*M* Tris-HCl, 10 m*M* EDTA). PCR was performed using a PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, Mass.) in a reaction volume of 10 μl. Reactions contained 1× PCR buffer [10 m*M* Tris-HCl pH 8.3, 50 m*M* KCl, 1.1 m*M* $MgCl_2$, 0.01% (w/v) gelatin; Sigma, St. Louis, Mo.], 200 μM of each dNTP, 30 n*M* of each oligonucleotide, 0.05 U μl^{-1} REDTaq DNA polymerase (Sigma) and 50–100 ng template. The reaction conditions consisted of one cycle of 94°C for 2 min and 30 cycles of 92°C for 15 s, 50–55°C for 30 s, 72°C for 1 min per kilobase of expected product. PCR for the SCAR marker TG64 and for obtaining sequences from homologues of *ψLpHgr1-1* in M82 was performed using the Expand Long Template PCR System (Roche, Germany). Restriction digestion, gel electrophoresis and DNA gel blot analysis were carried out using standard protocols (Sambrook and Russell 2001). DIG probe labelling, hybridisation and detection were carried out using the DIG High Prime Labelling and Detection kit (Roche, Indianapolis, Ind.). PCR products were cloned into the pCR2.1 vector using the TA cloning kit (Invitrogen, Carlsbad, Calif.).

Randomly amplified DNA fingerprinting (RAF) analysis

The RAF protocol was performed essentially as described by Waldron et al. (2002). RAF reactions were prepared in a total volume of 10 μl containing 1× DAF buffer (10 m*M* Tris-HCl pH 8.0, 10 m*M* KCl, 5 m*M* $MgCl_2$), 1.5 U Amplitaq DNA polymerase Stoffel Fragment (Applied Biosystems, Foster City, Calif.), 20 μM dNTPs, 1 μCi α-labelled [^{33}P]-dATP and 5 μM of a single oligonucleotide primer of ten nucleotides obtained from Operon Technologies (Alameda, Calif.). PCR was performed in a thermal cycler with a hot-start (85°C), followed by a denaturation at 94°C for 5 min, then 30 cycles of 94°C for 30 s and 60 s at each of 57°C, 56°C, 55°C, 54°C and 53°C. The PCR was concluded with a final extension step at 72°C for 5 min. Radiolabelled PCR products were mixed with an equal volume of formamide dye [98% (w/v) formamide, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol, 10 m*M* EDTA pH 8.0], then denatured at 95°C for 5 min and immediately placed on ice prior to electrophoresis. Amplification products were separated on 4% (w/v) polyacrylamide-TBE gels containing 7.5 *M* urea. The gels were run at 100 W (50 mA) for 2 h 15 min, then transferred to filter paper (Whatman 3 MM), dried and exposed to Kodak Biomax-MX-ray film overnight at ambient temperature.

BiBAC library screening and BAC DNA preparations

The *L. pennellii* LA716 BiBAC library filter set (TA 56) was obtained from the Texas A&M BAC Center, Crop Biotechnology Center, Texas A&M University, Texas. [^{32}P]-labelled probes were produced by random primer labelling using an oligolabelling kit (Amersham Pharmacia Biotech, UK). Unincorporated nucleotides were removed using a NICK column (Amersham Pharmacia Biotech). Hybridisation was carried out at 65°C in 0.5 *M* sodium phosphate pH 7.2, 7% (w/v) sodium dodecyl sulfate (SDS), 1% (w/v) bovine serum albumin (BSA), 1 m*M* EDTA with 10 mg sheared, denatured salmon sperm DNA. Filters were washed three times, 20 min each time, in 0.5× SSC, 0.1% (w/v) SDS at 65°C and exposed to Kodak X-OMAT X-ray film at −80°C. Large-scale, high-quality bacterial artificial chromosome (BAC) DNA for sequencing was prepared using the Large Construct Plasmid Purification kit (Qiagen, Valencia, Calif.).

DNA sequencing and sequence analysis

DNA sequencing was performed by the Australian Genomic Resource Facility, Brisbane, Queensland using ABI PRISM BigDye Terminator v3.0 chemistry (Applied Biosystems) and ABI 377 automatic DNA sequencers. Direct sequencing from BAC templates was performed using 4 μg high-quality template and 80 cycles of 96°C for 30 s, 50°C for 15 s, 60°C for 4 min. Windows 32 EDITSEQ 4.03 and SEQMAN 4.03 (DNASTAR, Lasergene) were used for editing sequence chromatograms, sequence manipulation and contig assembly. An average sequence coverage of 2.29× was obtained for the 9-kb genomic clone, with at least three clear reads, including both the forward and reverse direction, over predicted loss-of-function mutations. Primer design for both PCR and sequencing was performed using PRIMER3 (Rozen and Skaletsky 2000). Multiple alignments were performed manually using a combination of the CLUSTAL W algorithm (Thompson et al. 1994) as an accessory application to BIOEDIT SEQUENCE ALIGNMENT EDITOR V5.0.9 (Hall 1999) and pairwise BLAST alignments (Tatusova and Madden 1999). Intron-exon boundaries in genomic sequences were predicted using alignment with homologues and the GENSCAN web server at MIT (http://genes.mit.edu/GENSCAN).

Results

Generation of PCR-based markers at the *I-3* locus

The tomato *I-3* locus has been mapped to the long arm of chromosome 7 between the molecular markers TG183 and TG216 (Bournival et al. 1989; Tanksley and Costello 1991). Testing the introgression lines IL7-2, IL7-3 and IL7-4 for resistance to *F. o. lycopersici* race 3 we confirmed that *I-3* is located in bin 7-E, the region of overlap between the *L. pennellii* introgressions in these three lines. Sequences mapping to bin 7-E were identified as suitable for conversion to PCR-based markers, including RFLP markers, COS markers, a resistance gene analogue and previously described genes. Sequences were obtained from public databases or by the sequencing of tomato genomic (TG) clones from the M13 (-20) and M13 reverse priming sites. The clone end sequences were used to design oligonucleotides for PCR amplification of the corresponding genomic fragments. In the case of known or predicted genes, primers were designed where possible to amplify introns so as to take advantage of the greater sequence variation associated with non-coding regions. For non-gene sequences, primers were designed to amplify the largest fragment possible. PCR amplification was performed on the susceptible backcross parent *L. esculentum* cv. M82 and resistant introgression line IL7-3.

PCR products of different sizes were generated from resistant and susceptible genotypes for the sequences TG64, TG143, TG170, TG183, TG202 and 2A11, thus producing SCAR markers (Table 1). For markers TG64, TG143, TG183 and TG202, co-dominant SCAR markers were produced, for which it was possible to distinguish heterozygotes from homozygotes. However, for markers TG170 and 2A11, dominant SCAR markers were produced, for which preferential amplification of one sequence prevented heterozygotes being distinguished from one of the homozygotes. For the sequence cTOB903, a PCR product was amplified from M82 but not IL7-3. For

Table 1 Oligonucleotide primer sequences and marker characteristics for SCAR and CAPS markers on chromosome 7 of *Lycopersicon esculentum* and *L. pennellii*

Marker	Primer pair	Restriction enzyme	Product sizes (bp)	
			L. esculentum	*L. pennellii*
TG64[a]	ATTCAGACCACTTCAATCACTAC TTATCAAGGATTCATCAGTAACC	–	2,400	5,500
TG143[a]	AATCGGTGTGTATTTATCGCGACGC GTTCTCCCTACTGATCCCAATACGAC	–	900	1,100
TG170[a]	TCTGTGTCCAGTTTCTTAATATGGC CGTTGTGTCGTTAAATGGCG	–	390	410
TG183[a]	CTACTTGTCTGCCAAGGATTAC CGTGCCGTTCAAGAAGAGTG	–	1,200	800
TG202[a]	GCTAATGAATCTGATAGCAATGGAC TTTCTTCTCTCCCTGTGTAATCTTG	–	1,200	1,500
cTOB903[b]	ATCCTCATCACAATTAGTATTCACG CTACGGGGAACGCAAAGG	–	1,100	–
2A11[c]	CGTTGTTCTTTTGACGACCAC GTATGTCTCGAAGAGCCATCAC	–	791	Multiple weak
TG128[a]	TGGGTACTAGCTGATTTATCCACTC ACTAATCTGCTGTAGTGCATGAGTC	*Bcl*I	439	243, 196
TG190[a]	GCAGTACACTTCTCCTTATCATGTG AGTTTCAGTAGTTGTTCCAAATTCC	*Hha*I	773	598, 175
TG217[a]	CGGAGAATAGTGGATTTGCTCC GTCATAAATCAGCAGACCATTGC	*Nla*III	850, 200, <100	600, 400, <100
TG637[a]	GGAAATATACACTAAGCAAATAAG GCTGCAGTGAAGGAATAAAGAG	*Mnl*I	850, 450, <100	650, 450, <200
CT84[d]	GGTGGACTTCATTGCTGATTACTAC ACGGTAGCACAGAGGAAAAGTG	*Apo*I	500, 300, 200	500, 200
CT226[d]	GTGAAGGAGTGTCAAAGGCAAC GGAATGAACAATTTATATGCAGCAG	*Mae*III	287, 114	401
T1329[b]	ATGAAACAGTTGGAATGGTAGAGTC ATAACACTGGGATTGGATGTATTTG	*Dde*I	477, 189	666
RGA332[e]	CGATTCGTTTGAAGGAGCTAA TTGTTTAAAAAGTCGTAAGCTTTCAT	*Alu*I	221, 19, 17	124, 87, 19, 17
pTOM13[f]	CCATGGCCCATAAGCCATC GCAAGAAGAAAGTGCTTTCCC	*Dra*I	518, 174	892

[a] Original map position from Tanksley et al. (1992) and *L. esculentum* sequence obtained by sequencing the TG clone
[b] Original map position and sequence from the Solanaceae Genomics Network (SGN)
[c] Original map position and sequence from Pear et al. (1989) and Alpert et al. (1990)
[d] Original map position from Tanksley et al. (1992) and *L. esculentum* sequence from Ganal et al. (1998)
[e] Original map position and sequence from Leister et al. (1996)
[f] Original map positon and sequence from Holdsworth et al. (1987) and Giovannoni et al. (1999)

those markers where monomorphic PCR products were produced, sequences were obtained from the PCR products derived from IL7-3, either by direct sequencing of the PCR product using the forward and reverse primers used to amplify the fragment or by sequencing the cloned PCR product using M13 (–20) and M13 reverse primers. Restriction site polymorphisms were identified in nine sequences—pTOM13, TG637, T1329, TG217, TG128, TG190, CT226, CT84 and RGA332—thus generating CAPS markers (Table 1).

In order to saturate the *I-3* region with molecular markers, we employed RAF (Waldron et al. 2002) to generate new markers. Ninety-five random oligonucleotides were used to amplify about 7,000 RAF markers, and these were screened on lines M82, IL7-2, IL7-3 and IL7-4 and the breeding lines Fla. 7481 and Fla. 7547. In total, 475 DNA fragments were identified that were polymorphic between M82 and the introgression lines. An average of five polymorphic DNA fragments were generated per oligonucleotide. Twelve RAF markers were generated that mapped to bin 7-E, the region of overlap between the *L. pennellii* introgressions in lines IL7-2, IL7-3 and IL7-4.

Resistance to race 3 co-segregates with a resistance gene analogue and a RAF marker

The introgression lines IL7-2, IL7-3 and IL7-4 and the breeding lines Fla. 7481 and Fla. 7547 were screened with all SCAR, CAPS and RAF markers. Together these five lines divide chromosome 7 into five 'bins' or regions of unique genome coverage (Liu and Zamir 1999) (I–V, Fig. 1). Markers were located in three of these bins—a central bin containing the *I-3* gene (Bin III, common to all five resistant lines) and two bins flanking this region (II and IV) defined by the north- and southwards extent of the introgressed segments in lines Fla. 7547 and IL7-4, respectively. The bin-map allowed markers to be identified that lay within the IL7-3 introgression but flanked the *I-3* gene.

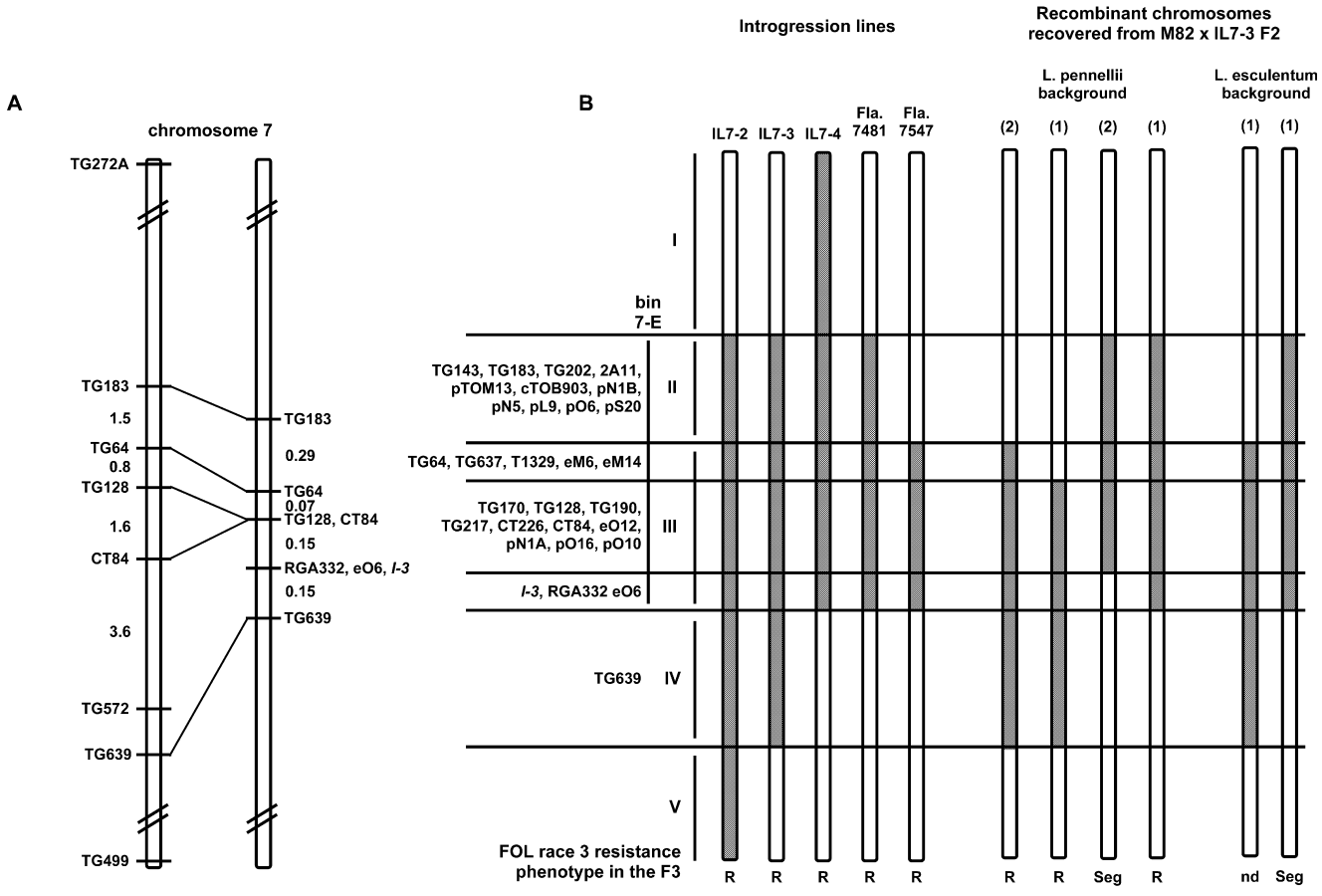

Fig. 1 **A** Relationship between the linkage map generated by this study and the composite Solanaceae Genomics Network (SGN) map. Only one selected marker from each co-segregating group of markers is shown. **B** Extent of introgressed *Lycopersicon pennellii* DNA present on chromosome 7 of the introgression lines and recombinant chromosomes recovered from a M82×IL7-3 F_2 mapping population. Introgressed segments of the *L. pennellii* genome are *shaded*. The introgression lines divide chromosome 7 into five bins *I–V*. The number of recombinant chromosomes in each class is given *in parenthesis above* each recombinant chromosome. The *Fusarium oxysporum* f. sp. *lycopersici* (FOL) race 3 resistance phenotype of the F_3 progeny is indicated *below* each recombinant chromosome. *R* Resistant, *S* susceptible, *Seg* segregating for resistance, *nd* not determined

An F_2 population segregating for resistance to *F. o. lycopersici* race 3 was produced by crossing the *L. esculentum* line M82 with the introgression line IL7-3. We subsequently used the SCAR marker TG183 and the CAPS marker TG639 (Burbidge et al. 2001) to screen 688 F_2 plants to identify recombination events in the vicinity of the *I-3* gene. Eight plants were identified with recombination breakpoints between TG183 and TG639 of which six carried recombination breakpoints north of *I-3* and within the central bin. *I-3* was separated from all of the markers in the central bin except RGA332 and the RAF marker eO6 by two of these recombination breakpoints (0.15 cM). The two remaining plants carried recombination breakpoints south of *I-3*. We therefore concluded that the *I-3* gene is contained within a 0.3-cM region flanked by the markers CT226 and TG572, the RFLP marker immediately south of the introgressed region in IL7-4 (Liu and Zamir 1999).

Isolation of a resistance gene analogue sequence co-segregating with the *I-3* gene

Using the 387-bp RGA332 fragment as a probe, we identified a BiBAC clone containing the corresponding gene from a *L. pennellii* LA716 BiBAC library (Hamilton et al. 1999) and, by means of restriction mapping (data not shown), determined that this BiBAC insert was approximately 50 kb. A 9-kb *Bam*HI fragment containing the RGA was completely sequenced. This fragment contains the entire gene that corresponds to RGA332 and conceptually encodes a protein containing distinct TIR, NBS and LRR domains. A portion of a second gene lies in the opposite orientation, 536 bp downstream of the TIR-NBS-LRR gene, and encodes a protein with similarity to the S-locus receptor-like kinase (SRLK) family.

The predicted protein sequence encoded by the RGA332 gene contains a TIR domain and an NBS domain with the highly conserved P-loop, kinase 2, kinase 3a and GLPL motifs (Fig. 2) (Traut 1994; Meyers et al. 1999; Pan et al. 2000b). The LRR domain consists of

MNQESSLLPSPEII
*RWS*DVFLSFRGEDVRKTFVDHLYLALQQKCINTFKDDEK*
LEKGKFISPELESSIEESRIALIIFSRNYANSTWCLDELT
KIMECKNVKGQIVVPVFYDVDPSTVRKQKNMFGEAFSKHE
ARFQEDKVQKWRAALVEAAN
ISGWDLPNTSNG^HEARVIEKIVEDIMARLGSQRHASNAR
N
LVGMESHMHQVYKMLGIGSGGVRFLGILGMSGVGKTTLAK
VIYDNLRSRFQGACFLHEVRDRSAKQGLEHLQEILLSEIL
VVKKLRINDSFEGANMQKQRLQYKKVLLVLDDVDHIDQLD
ALAGKREWFGDGSRIIITTKDKHLLVKYETEKIYRMKTLD
KYESLRLFKQHAFKKNYPTKEFEDLSAQVIEHTGGLPLAL
KVLGSFLYGRGLDEWISQVERLKQIPENEILKKLELSFTG
LHNTEQKIFLDIACFFSGKR
KDSVTRILESFHFSPVIGIKVLMEKCLITILKGRVIIHQL
IQEMGWHIVRREASYNPRICSRLWKRKDICPVLERNL^GT
DKIEGILLHLTNEEEVNFGGKAFMQMTSLRFLKFRNAYVC
QG
PEF**L**PDE**L**RW**L**DWHGYPSKS**LP**N
S**F**KGDQ**L**VS**L**K**L**KKSRIIQ **L***RT
SKD**L**GK**L**KY**M**N**L**SHSQK**L**IR**MP**D
FS**V**TPN**L**ER**L**V**L**EE**C**TS**L**VE**I**NFS
IGD**L**GK**L**VF**L**N**L**KN**C**RN**L**KT**LP**KS
I R**L**EK**L**EI**L**VL**S**G**C**SK**L**RT**FP**E**I**
EEK**M**NR**L**AE**L**YL**G**A**T**A **L**SE**LP**AS
VEN**F**PG**V**GV**I**N**L**SY**C**KH**L**ES**LP**SS
IFR**L**KC**L**KT**L**D**V**SG**C**SK**L**KN**LP**DD
LGL**L**VG**L**EE**L**H**F**TH**T**A **I**QT**IP**SS
MSL**L**KN**L**KH**L**YL**R**G**C**TA**L**SSQVSSSSRGQKSMGVNFQN
LSG**L**CS**L**IM**L**D**L**SD**C**N **I**SDGG**I**LSN
LGF**L**PS**L**EG**L**VL**D**G**N**N **F**SN**I**AAAN
ISR**L**TN**L**KF**L**AL**A**G**C**RR**L** E**LP**E
LPP SI**T**G**L**YADE**C**TS**L**MS**I**DQL
TKYPMLLEVSFTKCHQLVKNKQHASMVDSLLKQMDKV^ST
LLFMNGLRMYVPGVEIPEWFTYKNSGTGSISVALPKNWFT
PTFRGFAVCAVFDMMNPFILRKFKWDGPMNFHTDKFLRNS
RGPAAWFKFTSHDGLRRESWFSFSSIGSEKAVGLGDTFLA
HLPVHPYWTSKDVNYNNYIQLEVGFHDEVHKDVVVKGMGV
RLVYEN

Fig. 2 The predicted protein sequence of *ψLpHgr1-1*. The predicted TIR domain is in *italics*; the predicted NBS domain is *underlined*. The P-loop, RNBS-A, kinase-2, RNBS-B, GLPL and RNBS-D motifs of the NBS, as defined by Meyers et al. (1999), are *shaded*. Predicted leucine-rich repeats (LRR) are illustrated, with the LRR consensus shown in *bold*, according to the cytoplasmic consensus **L**xx**L**xx**L**xx**L**x**L**xx(**N/C/T**)x(x)**L**xx**IP**xx. Three sequence motifs with homology to conserved regions of the C-terminal non-LRR domain in other plant resistance proteins are also *shaded*. *Asterisk* (*) Stop codons, ^ position of predicted introns in the corresponding coding sequence

15 imperfect leucine-rich repeats of 22–25 amino acids, which match the consensus for cytoplasmic LRR proteins (Jones and Jones 1997). A region of low homology to other known proteins, termed the C-terminal non-LRR domain (Dodds et al. 2001), follows the LRR domain. This region contains short conserved sections common to the C-terminal non-LRR domain of other TIR-NBS-LRR proteins (Fig. 2). The characterised resistance gene to which the RGA332 gene shows the highest degree of homology is the potato *Gro1* gene for resistance to root cyst nematode *Globodera. rostochiensis* (Paal et al. 2004; gi37781266 gbAAP44390.1). The predicted RGA332 protein and Gro1 have 85% amino acid identity and 89% amino acid similarity and have the same domain structure and number of repeats within the LRR domain. The high degree of similarity between the two genes and their syntenic position in the potato and tomato genomes suggests that the RGA332 gene is an orthologue of *Gro1*. Consequently, we have named the gene corresponding to RGA332 *ψLpHgr1-1* (for *L. pennellii* Homologue of *G. rostochiensis* resistance gene *Gro1*). *ψLpHgr1-1* does not appear to be a functional gene. Two pre-mature stop codons interrupt the coding sequence, one early in the TIR domain and the other in the second predicted LRR, in a region where a conserved intron is normally found in TIR-NBS-LRR genes (Fig. 2). The sequences flanking both premature stop codons were analysed for the presence of conserved recoding signals associated with codon read-through in plant viruses (Beier and Grimm 2001; Harrell et al. 2002). No known recoding signals were identified, suggesting that neither stop codon is likely to be read-through. The presence of at least two mutations suggests that *ψLpHgr1-1* is a pseudogene derived from an ancient loss of function. Despite apparent loss of function, *ψLpHgr1-1* is still transcribed, as Reverse Transcriptase-PCR detects a transcript in root tissue of IL7-3 (B. Baillie, personal communication).

DNA gel blotting showed that the gene corresponding to RGA332 exists as a single copy in the introgression line IL7-3 (data not shown) while, as shown previously, two copies are present in *L. esculentum* cv. M82 (which must therefore both lie in the corresponding region of the *L. esculentum* genome) (Ohmori et al. 1998). Primers designed to the *ψLpHgr1-1* sequence were used to amplify fragments of the homologous genes from M82 to determine whether these genes also contain the same loss of function mutations. The sequence from M82 suggests that an orthologue of *ψLpHgr1-1*, designated *ψLeHgr1-1*, containing both premature stop codons, is present in the *L. esculentum* genome. A sequence was also obtained from a second gene, designated *LeHgr1-2*, which is divergent from *ψLpHgr1-1* at the nucleotide level (32 nucleotide differences in 1,300 bp of coding sequence), and does not contain either of the premature stop codons present in *ψLpHgr1-1*. At both positions, the amino acid residue encoded is the same as that found in the homologous region of Gro1. However, *LeHgr1-2* may also be non-functional, as a frameshift immediately after the last predicted LRR introduces a premature stop codon.

A partial sequence was also obtained from the NBS domain for *ψLeHgr1-1* and *LeHgr1-2*. Alignment of the predicted nucleotide sequences with those from potato and tomato suggests that the tomato RGAs TC11.2 (Ohmori et al. 1998), Q112 (Pan et al. 2000a) and *ψLeHgr1-1* represent alleles of the same gene and are orthologous to *ψLpHgr1-1* but have no obvious orthologue in potato, while the tomato RGAs Q2 (Pan et al. 2000a) and *LeHgr1-2* represent alleles of a second gene and are orthologous to the potato RGA St334 (corresponding to the *Gro1-3* gene) (Leister et al. 1996; Paal et al. 2004).

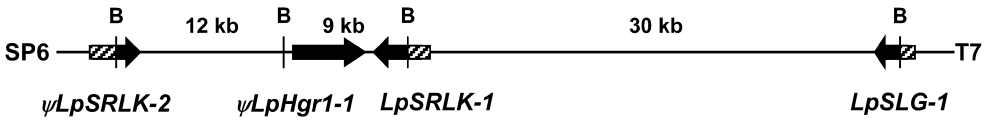

Fig. 3 Relative position and orientation of *ψLpHgr1-1*, *LpSLG-1*, *LpSRLK-1* and *ψLpSRLK-2* within the approximately 50-kb BiBAC insert. Approximate distances in kilobases between sequenced regions are indicated. *Filled arrows* indicate sequenced portions of genes or pseudogenes, *diagonally striped regions* indicate unsequenced portions of genes or pseudogenes, *arrowheads* indicate direction of transcription. *B* *Bam*HI

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LeSLG-1     MALCNIFPFAFIILSCSCYLISGQRFDYPTANLSTTWINSVSAPHSVDFTDG
            SRVRAILLRGTFGPKYACGFYCNGKCDTYLFAIFIVQTNSASQITSPSIGFP
            QVVWSANRNNPVKINSTLQFTAQGDLVLRNADGSLAWSTNTAGKSVAGLSLT
            DEGNLVLFDSKNATVWQSFDHPTDALVPGQKLVSGMKLTASVSTTNWGSNQS
LpSRLK-1                                                GSNQS

LeSLG-1     FSLSAMDNGLVAFIESNPTQTYFDATIGGLNPSGGSNYVKYLNGSLTLFTNS
LpSLG-1                                                 LFTNS
LpSRLK-1    FSFTVLDGSLVSSIDTNPPQ-YYIASSGG--DAQNSPFYNFDGRTFTALQDP

LeSLG-1     SSSPEMVLVSITPASSAQYMKLESNGHLKVYEWRSR-WREVDDLLTGFRGEC
LpSLG-1     SSTPELVLVSITPASSAQYMKLESNGHLKVYEWKSR-WREVDDLLTGFRGEC
LpSRLK-1    YTS--------------QFIKLGADGHLRLYQSDAYDWKQFDEVMNSDLGNC

LeSLG-1     NYPTVCGRYGICTM-GQCSCPISSNSTTYFRPINVRLPNLGCSEAKKLSCNN
LpSLG-1     NYPTVCGRYGICTM-GQCSCPISSNSTTYFRPINVRLPNLGCSEAKKLSCNN
LpSRLK-1    GYPMVCGRYSICTNDGQCNCPVEGN---FFRPIN-RNPDLGCSQLTSISCNS

LeSLG-1     LKKHRLLEVEDVDYFAFTAD--------ISNTDVSTCKRACLDKCSCKAAFF
LpSLG-1     LKKHRLLEVEDVDYFAFTAD--------ISNTDVNTCKRACLDKCSCKAAFF
LpSRLK-1    SQYHSLIELSDTTYFAFEINFYASSNMWFEGTKMENCKAACLSNCSCKAAVW

LeSLG-1     RSGLNSSRAIGECYLPTEIFSLMNNEKDKTRYDSVAFIKVQV*
LpSLG-1     RSGLNSSRAIGECYLPTEIFSLMNNEKDKTRYDSVAFIKVQV*
LpSRLK-1    SKTLR-----KNCLLLNEVFSLQDNWYGTDKTK-V-FLKVQNFAKAQYQPPI
            VSQRKQSRPLKVIVAFALAALVGIILSISAWFVLFKKRTLSVKAGDLLDLAP
ψLpSRLK-2                GSTIGASFGLLLMVLTCFAYIFRRRKGIEE-DEEEFLD

LpSRLK-1    ILPGILTRFSYDELKIITQDFSRKLGEGGFGSVYEGTLGNGNKIAVKRLNGV
ψLpSRLK-2   QIPGMRTRFSYEELTVMTENFNEKLGEGGFWCCI*RNNE*WHQKSGEASARF

LpSRLK-1    GQVKDSFLIEVKIVGSIHHVNLVKLIGFCAEKDHRVLIYEYMVNGSLDRWLS
ψLpSRLK-2   GNVKKSFLAEVATIGSIQHVYLVKLIGFCAEKSHRLLAYEYMANGSLDRWIF

LpSRLK-1    HENQENGLTWITRQKIISDIAKGLAYLHDECNQKIIHLDIKPHNILLDENFN
ψLpSRLK-2   HGTWEKSLTWDMRKKIISDIAKGLAYLHEDCNNKIIHLDIKPQNILLDHNLN

LpSRLK-1    AKISDFGLSKLIEKDKSKVVTRMRGTPGYLAPEWLRSVITEKVDVYAFGIVL
ψLpSRLK-2   AKVSDFGLSKLVGKDESKIARTMRGTSGYLAPEWLNEVITEKVDVNSFGVVI

LpSRLK-1    LEVICGRKNLDWSQADEDDVHLLSVFRRKVEQEKLIDMVDKNNEDMQLHREA
ψLpSRLK-2   LEIICVWKNLDRHQ-DEDDMHLLSLFMRKAGERQLLEMVDKKSENMHLHKKE

LpSRLK-1    VTEMMSLAAWCVQGDFNKRPSMPLVVKVLEGLVSVETNLDFNSTNLTDNQQM
ψLpSRLK-2   AVEMK-IAAWCLQSDYTKRPSMSLVVKVLQ-LVAAETDLDYSFTFPTMTRRV

LpSRLK-1    ---------EVPISSILPSILSGPR*
ψLpSRLK-2   AGTNQERESVVGISLPLPSQLSGPR*
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Fig. 4 Alignment of predicted protein sequence for SGN UNIGENE59624 (*LeSLG-1*) with partial sequence for *LpSLG-1*, *LpSRLK-1* and *ψLpSRLK-2*. Identical residues are shown in *bold*. The conserved residues of the predicted agglutinin, lectin-binding domain and S-locus glycoprotein domain are *shaded* in *LeSLG-1*. The twelve invariant cysteines characteristic of the S-locus receptor-like kinase (SRLK) family are *shaded* in *LeSLG-1*, *LpSLG-1* and *LpSRLK-1*. A possible transmembrane domain and the conserved residues of the predicted kinase domain are *shaded* in *LpSRLK-1*

Analysis of sequences from an S-locus cluster in the vicinity of the *I-3* locus

A gene with homology to SRLKs lies downstream of *ψLpHgr1-1* (RGA332). One end of the 9-kb fragment for which a complete sequence was obtained encodes 617 amino acids of the C-terminal portion of the protein, including the 12 invariant cysteines of the S-ectodomain, and predicted transmembrane and kinase domains. The predicted protein sequence shows high homology to predicted and characterised SRLK proteins from *Arabidopsis*, rice and other plant genomes, with the highest homology being to predicted (gbCAE05333, gbCAE05355) and characterised (gbS50767; Zhao et al. 1994) SRLKs from rice.

Sequences were obtained by direct sequencing of the ends of the BiBAC insert using priming sites on the BAC arms. The partial sequence from the T7 arm shows 96% nucleotide identity to *L. esculentum* expressed sequence tag (EST) contigs in the Solanaceae Genomics Network (SGN) database (http://sgn.cornell.edu) (SGN U143770 Build 2; SGN U55077, Build 3). Both contigs contain a single, complete open reading frame (ORF) encoding an S-locus glycoprotein (SLG) with predicted agglutinin, lectin binding and SLG domains. The *L. esculentum* gene represented in the SGN EST database is designated here as *LeSLG-1* (for *L. esculentum* S-locus glycoprotein), and the corresponding *L. pennellii* gene is designated *LpSLG-1*. Sequencing from the SP6 BAC arm identified yet another SRLK-like gene, which may be non-functional, due to the presence of a frameshift that introduces premature stop codons. This gene was designated *ψLpSRLK-2* (Fig. 3).

Figure 4 shows an alignment of the predicted protein sequence for LeSLG-1, LpSLG-1, LpSRLK-1 and *ψLpLK-2*. Although *LeSLG-1* is clearly an expressed gene, none of the *L. pennellii* genes nor the *L. esculentum* alleles of *LpSRLK-1* and *ψLpSRLK-2* were retrieved from the SGN EST database. This may result from the limited number of EST clones derived from *L. pennellii* and the higher expression of SLGs relative to SRLKs.

Discussion

Twenty-eight PCR-based SCAR, CAPS and RAF markers were generated that mapped to bin 7-E of tomato chromosome 7. Our map, which we generated using these 28 markers, places the *I-3* gene between the molecular markers CT226 and TG572 and is in agreement with other reports (Burbidge et al. 2001; Sela-Buurlage et al. 2001). From our mapping population, we estimate *I-3* to be contained within a 0.3-cM interval between these two markers, although greater distances have been reported from other mapping populations (Fig. 1) (Tanksley et al. 1992; Burbidge et al. 2001). The large differences in map distances that have been reported could arise from a repression of recombination in the region of chromosome 7

containing the *L. pennellii* introgression in the M82 × IL7-3 F_2 population. Repression of recombination, relative to that observed in an interspecific cross involving whole foreign chromosomes, has previously been observed in crosses involving tomato isolines carrying chromosomes with small segments of a foreign genome (Messeguer et al. 1991; Ho et al. 1992; Ganal and Tanksley 1996; Liharska et al. 1996). However, the larger map distances reported by Burbidge et al. (2001) were also obtained from a mapping population that could be expected to contain a relatively small introgression of *L. pennellii* DNA. Alternatively, it is possible that not all recombinants were recovered from our mapping population due to preferential amplification of one sequence and consequent incorrect scoring of markers used to select plants carrying recombinant chromosomes.

I-3 co-segregates in our mapping population with a predicted pseudogene belonging to the TIR-NBS-LRR resistance gene sub-class (*ψLpHgr1-1*). Putative pseudogenes have been identified at many resistance gene loci (see Lagudah et al. 1997; Meyers et al. 1998; Milligan et al. 1998; Creusot et al. 1999). Analysis of the complete set of NBS-LRR genes in the Col-0 *Arabidopsis* genome suggests as many as 10% of these may be pseudogenes (Meyers et al. 2003). It has been proposed that the retention of pseudogenes in genomes may provide a reservoir of genetic variation that is able to diverge unconstrained by functionality (Harrison and Gerstein 2002). Such variation adjacent to functional resistance genes might be re-integrated into the functional genes in response to changing pathogen pressures. However, it is also possible that the retention of pseudogenes close to functional resistance genes simply results from the rapid 'birth and death' evolution of these gene families and only reflects the accumulated detritus of multiple gene duplication and mutation events. *ψLpHgr1-1* exists as a single gene in the *L. pennellii* genome with no functional homologue, and the partial sequence from M82 suggests that *L. esculentum* may also lack a functional homologue. These data suggest that the original functional gene has become redundant and may indicate that *L. esculentum* and *L. pennellii* have been evolving in an environment where a pathogen once recognised by the ancestral gene is no longer a threat.

The predicted pseudogene *ψLpHgr1-1* lies within a cluster of genes belonging to the S-receptor gene family. S-receptor genes are involved in the well-characterised *Brassica* self-incompatibility (SI) system, which functions to prevent self-fertilisation via the rejection of self-pollen at the stigma surface. In this system, SI is determined by the direct interaction of two components—the S-locus receptor protein kinase (SRK) and the S-locus cysteine-rich protein (SCR) (Kachroo et al. 2001; Takayama et al. 2001). SRK is a single-pass transmembrane serine-threonine kinase located on the stigma epidermis, consisting of a cytoplasmic kinase domain and a highly polymorphic extracellular S-domain (Stein et al. 1991), while SCR is a small, secreted, cysteine-rich protein found in the pollen coat (Schopfer et al. 1999). A third component of SI in the S-receptor system is the SLG, a secreted stigma protein with similarity to the S-ectodomain of SRK, also encoded by the S-locus (Nasrallah et al. 1985). The function of SLG is not understood, although it appears to be a dispensable component of some incompatible interactions (Takasaki et al. 2000; Kusaba et al. 2001). The presence of genes with homology to both SRK and SLG at a single locus in tomato suggests that a SCR gene may also be present in the vicinity.

Retrieval of *LeSLG-1* as a complete cDNA from the EST database suggests that at least this gene, and likely other members of the cluster, are functional in *L. esculentum.* However, the potential function of these genes is unknown. *L. esculentum* is a self-compatible species, and SI in self-incompatible members of the Solanaceae operates via a ribonuclease-based system in which an S-locus-encoded ribonuclease is expressed in the pistil and acts to prevent pollen-tube development in incompatible interactions (see Wang et al. 2003). The involvement of the Solanaceous S-locus, or similar loci, in unilateral incompatibility has been proposed (Hancock et al. 2003). However, loci responsible for incompatibility of *L. esculentum* pollen with the *L. pennellii* stigma have not been mapped to chromosome 7 (Chetelat and DeVerna 1991).

The function of the S-receptor family is likely to extend beyond that of pollen incompatibility. Members of the SRLK superfamily display diverse expression patterns and are active in self-compatible species (Dwyer et al. 1994; Shiu and Bleecker 2001). The expression patterns of some family members suggest roles in plant growth and development, and SRLK genes have also been shown to be upregulated in response to wounding, salicylic acid and both pathogenic and non-pathogenic bacteria (Pastuglia et al. 1997; 2002). The isolation of *LeSLG-1* ESTs from vegetative plant tissues is consistent with such potential roles.

RGAs represent good candidates for functional resistance genes, and the identification of RGAs mapping to resistance loci can potentially accelerate positional cloning strategies for *R* gene isolation. We have eliminated the gene corresponding to an RGA that co-segregates with resistance to *F. o. lycopersici* race 3 as a candidate for the *I-3* gene and have identified a co-segregating RAF marker as well as molecular markers closely flanking the gene that will facilitate the identification of more informative recombination events. The identification of markers cosegregating with *I-3* will facilitate the construction of a physical contig containing the *I-3* gene. Isolation of the *I-3* gene will require further fine mapping and functional testing of candidate sequences from the *I-3* region.

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