Theor Appl Genet (2000) 101:918–924 © Springer-Verlag 2000

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Development of diagnostic PCR markers closely linked to the tomato powdery mildew resistance gene *Ol-1* on chromosome 6 of tomato

Received: 21 June 1999 / Accepted: 1 December 1999

Abstract *Lycopersicon hirsutum* G1.1560 is a wild accession of tomato that shows resistance to *Oidium lycopersicum*, a frequently occurring tomato powdery mildew. This resistance is largely controlled by an incompletely dominant gene *Ol-1* near the *Aps-1* locus in the vicinity of the resistance genes *Mi* and *Cf-2*/*Cf-5*. Using a new F_2 population (n=150) segregating for resistance, we mapped the *Ol-1* gene more accurately to a location between the RFLP markers TG153 and TG164. Furthermore, in saturating the *Ol-1* region with more molecular markers using bulked segregant analysis, we were able to identify five RAPDs associated with the resistance. These RAPDs were then sequenced and converted into SCAR markers: SCAB01 and SCAF10 were *L. hirsutum*-specific; SCAE16, SCAG11 and SCAK16 were *L. esculentum*-specific. By linkage analysis a dense integrated map comprising RFLP and SCAR markers near *Ol-1* was obtained. This will facilitate a map-based cloning approach for *Ol-1* and marker-assisted selection for powdery mildew resistance in tomato breeding.

Keywords Resistance · Tomato powdery mildew · Tomato · Mapping · *Oidium lycopersicum* · RFLP · Sequence characterised amplified region (SCAR)

Communicated by F. Salamini

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Introduction

Tomato powdery mildew caused by the fungus *Oidium lycopersicum* has become a serious disease in the Northern Hemisphere, especially in protected tomato cultivation. At present, almost all tomato cultivars appear to be susceptible, except for some newly developed commercial hybrids including DRW 4061 (Nunnink 1996), Belliro and Delito (De Ruiter Seeds catalogue 1998). Applying chemicals can control the disease but, nowadays, such an approach is undesirable in view of the growing need of an environmentally safe production. Moreover, as resistance genes to other pathogens have already been introgressed into tomato, *O. lycopersicum* remains as yet the only fungus to be controlled by fungicides in greenhouse culture in Northwest Europe.

Sources with resistance to *O. lycopersicum* have been identified in some wild tomato species, including *Lycopersicon hirsutum* (G1.1257, G1.1290, G1.1560, G1.1606=CPRO742208, Lindhout et al. 1994), LA1775, Kozik 1993; PI247087, Laterrot and Moretti 1993), *L. parviflorum* (G1.1601=CPRO731089) and *L. peruvianum* (LA2172) (Lindhout et al. 1994), *L. hirsutum* Gl.1560, among others, has been studied most extensively with respect to the inheritance of resistance to tomato powdery mildew (Van der Beek et al. 1994). The resistance was found to be controlled by an incompletely dominant gene, designated *Ol-1*, that was mapped on chromosome 6 near the *Aps-1* locus in the vicinity of the resistance genes *Mi* and *Cf-2*/*Cf-5* to *Meloidogyne* spp and *Cladosporium fulvum*, respectively (Van der Beek et al. 1994). Because of its monogenic and (incompletely) dominant nature, *Ol-1* can easily be incorporated into modern cultivars by classical breeding in five to nine backcrosses. However, with the help of molecular markers, the same goal would be reached in two to three

backcrosses. Therefore, marker-assisted selection (MAS) of *O. lycopersicum* (*Ol*) resistance would be of great help in developing new tomato cultivars carrying *Ol* resistance. As only a few linked markers have been identified so far, additional markers flanking the *Ol-1* region need to be developed to increase the efficiency of MAS. Such a saturated map should also facilitate map-based (positional) cloning of *Ol-1* in the near future.

In order to rapidly obtain markers linked to resistance genes for genetic analysis and for physically characterising the respective regions, Paran and Michelmore (1993) developed the sequence-characterised amplified region (SCAR) marker that is most suitable for standard polymerase chain reaction (PCR) analysis. They successfully derived eight SCARs from RAPDs linked to downy mildew resistance gene in lettuce, with three of them being codominent. Since then, SCAR analysis has been widely applied, for instance, to localise genes controlling resistance (e.g. Geffroy et al. 1998; Deng et al. 1997), fruit quality (Fang et al. 1997) and plant development (Jiang and Sink 1997). It has also been employed in taxonomic studies of plants (Parent and Page 1998; Bodenes et al. 1996; Roose et al. 1993) and fungi (Francis et al. 1994; McDermott et al. 1994). SCAR analysis has also become a useful technique in practical breeding of various crop species; for example, banana (Damasco et al. 1998), grapevine (Lahogue et al. 1998), hemp (Mandolino et al. 1999), kiwifruit (Harvey et al. 1998) and orchid (Handa et al. 1998).

In the study reported here, we analysed a new F_2 population (n=150) of Moneymaker×*L. hirsutum* (G1.1560 to identify 15 additional restriction fragment length polymorphism) (RFLP) markers which co-segregate with the resistance gene *Ol-1*. We also applied bulked segregant analysis (Michelmore et al. 1991) with random amplified polymorphic DNAs (RAPDs) as a bridge to develop SCARs (Paran and Michelmore 1993) that will serve as convenient PCR markers in commercial breeding programmes. Two *L. hirsutum*-specific and three *L. esculentum*-specific SCAR markers were designed based on the sequences of the RAPDs that co-segregated with *Ol-1*. These SCARs will provide a major tool in rapidly detecting the resistance locus in practical breeding and future research.

Materials and methods

Plant and fungus materials

An F_2 population of 150 plants derived from an interspecific cross between individual plants of the susceptible *L. esculentum* cv. Moneymaker and the resistant accession *L. hirsutum* G1.1560 (Lindhout et al. 1994) was used for mapping the *Ol-1* gene. The F_2 plants as well as the F_1 and their parents were obtained from CPRO-DLO, Wageningen, The Netherlands. F_2 plants were selfed to generate F_3 lines.

The pathogenic fungus *O. lycopersicum*, which originated from infected commercial tomato plants (Lindhout et al. 1994), was maintained on Moneymaker plants in a greenhouse compartment at 20°±3°C with 70±15% relative humidity.

Disease test

A disease test was performed by spraying 27-days-old tomato plants with a spore suspension of 2×10^4 conidia ml^{-1}. The inoculum was prepared by washing conidial spores from the freshly sporulating leaves of heavily infected Moneymaker plants in tap water and used immediately. The experimental set-up of the disease test was according to a completely randomised block design with 15 blocks, each containing 2 plants of each parent, 2 F_1 plants and 10 F_2 plants. The inoculated plants were grown in a greenhouse at 20°±3°C with 30–70% relative humidity.

The disease symptoms were evaluated at 10, 14, 17, 21, 24 and 28 days post-inoculation (dpi). The evaluation was made on the basis of two categories of disease index (DI). DI 0–3 indicates the size of the infected areas per inoculated leaf: 0=no infection, 1=less than 10% infected leaf area, 2=10–30% infected leaf area and 3=more than 30% infected leaf area. DI a, b and c refer to the disease severity of the infected leaf areas: a=mycelium faintly visible, often with only some yellow spots; b=obvious presence of mycelium but only with slight sporulation, c=abundant sporulation.

RFLP analysis

Total DNA was extracted from newly grown leaves without fungal infection, and RFLP analysis was carried out as described by Van der Beek et al. (1992). Fifteen RFLP markers were used: eight TG markers, TG25, TG153, TG162, TG164, TG215, TG240, TG253 and TG298 (*Pst*I or *Eco*RI size-selected tomato genomic fragments; Miller and Tanksley 1990; Tanksley et al. 1992), two GP markers, GP79 and GP164 (*Pst*I size-selected potato genomic fragments; Gebhardt et al. 1989); three H markers, H2Cl, H8C4 and H9A11 (tomato genomic clones from a *Hin*dIII library in plasmid *pUC*18; Klein-Lankhorst et al. 1991a); and two cDNA clones, Adh-2 (Wisman et al. 1991) and Aps-1 (Aarts et al. 1991). The TG and GP markers were provided by S.D. Tanksley, Cornell University, New York, USA, and C. Gebhardt, Max Planck Institut für Züchtungsforschung, Köln, Germany, respectively. The cDNA clones were developed at the Laboratory of Molecular Biology, WAU, Wageningen, The Netherlands (Aarts et al. 1991; Wisman et al. 1991).

Identification of RAPD markers

Bulked segregant analysis (BSA; Michelmore et al. 1991) was applied to identify RAPD markers associated with resistance to *O. lycopersicum.* Based on the disease test and RFLP analysis, 7 resistant F_2 plants homozygous for *L. hirsutum* in the TG153–TG163 interval (10.9 cM) spanning *Ol-1* and 7 susceptible F_2 plants homozygous for *L. esculentum* in this interval were selected to constitute the resistant and susceptible pool, respectively. Three hundred arbitrary decamer primers (Operon) were used for RAPD analysis as described by Klein-Lankhorst et al. (1991b). RAPDs associated with the resistance were used to identify the rest of the F_2 individuals.

Cloning and sequencing RAPD products

Diagnostic RAPD bands were excised from agarose gels, and the DNA was purified using a ‘Gene Clean’ kit (Bio 101). The purified DNA was reamplified using the same primer that generated the RAPD polymorphism. The reamplified products were resolved on a 1.5% agarose gel, excised from the gel, purified by ‘Gene Clean’ kit and blunt-end ligated into the *Sma*I site of pBluescript KS$^+$ vector. Before ligation, the linearised vector was tailed with T′ at 72°C for 2 h in 100 µl of a reaction mixture containing 10 m*M* TRIS-HCl, 1.5 m*M* $MgCl_2$, 50 m*M* KCl, 0.01% gelatine, 0.1% Triton X-100, 0.2m*M* dTTP, 2 µg vector DNA (digested with *Sma*I and purified with ‘Gene Clean’ kit) and 1 U Super*Taq*. The identi-

ty of the cloned RAPD products was verified by comparing the size of the digested plasmids using the corresponding enzymes and by hybridising the cloned fragments to Southern blots of resistant and susceptible pools. The plasmid DNA was isolated by alkali mini-preparation (Maniatis et al. 1989) and purified using a QIAGEN-tip20 column (QIAGEN). Sequencing was carried out by the Pharmacia Biotech Company.

Design of primers and analysis of SCAR markers

For each cloned RAPD amplification product, a pair of 21- to 25-mer oligonucleotide primers was designed to be used as SCAR primers, and synthesis of these was by Pharmacia. Two SCAR primers, SCAE16 and SCAF10, were designed by extending the original 10-mer RAPD primer plus the next 14 nucleotides at the 3′ end (Paran and Michelmore 1993). The other three SCAR primers (SCAB01, SCAG11 and SCAK16) were designed using the computer programme PRIMER based on their corresponding RAPD sequences. Amplification of genomic DNA of F_2 plants was executed in 50 μl of the same reaction mixture as applied in the RAPD reaction but with 100 ng of SCAR forward and reverse primers each. Each PCR consisted of 30 cycles of 1 min at 94°C, 2 min at 60°C (for SCAE16) and 2 min at 72°C. The annealing temperature for SCAF10, SCAB01, SCAG11 and SCAK16 was 65°C, 62°C, 60°C and 58°C respectively. The amplified products were separated by electrophoresis on a 1.5% agarose gel.

Linkage analysis

JOINMAP (Stam 1993) was used to perform linkage analysis and to generate a genetic map, which was drawn by using DRAWMAP 2.0 (Van Ooijen 1994).

Results

Disease test

A disease test on an F_2 of *L. esculentum* cv Moneymaker×*L. hirsutum* G1.1560 was performed to confirm the inheritance of resistance in *L. hirsutum* (G1.1560. The resistance classification was carried out by using two categories of disease index (DI). A DI of 0–3 refers to the size of the infection areas on inoculated leaves, and index a–c to the severity of infection. As expected, all Moneymaker plants were scored as DI-3 (Fig. 1). Most individuals of the resistant parent were scored as DI-1, and a few as DI-2. Therefore, plants with DI-3 were considered to be susceptible and plants with DI 0–2 as resistant. By using this index, we were able to unambiguously identify most F_2 plants as either resistant or susceptible (Fig. 1). Segregation of resistance was in accordance with a monogenic, dominant trait (Van der Beek et al. 1994). Some F_1 plants were evaluated as susceptible, probably as a result of the combination of an intermediate expression of the resistance of the heterozygote and a large experimental error that is also manifest in the resistant control *L. hirsutum* (G1.1560 (Fig. 1). In order to minimise misinterpretation of F_2 plants, the appearance of mycelia and sporulation was also taken into account. Thus, nearly all F_2 plants could be clearly classified as either resistant or susceptible.

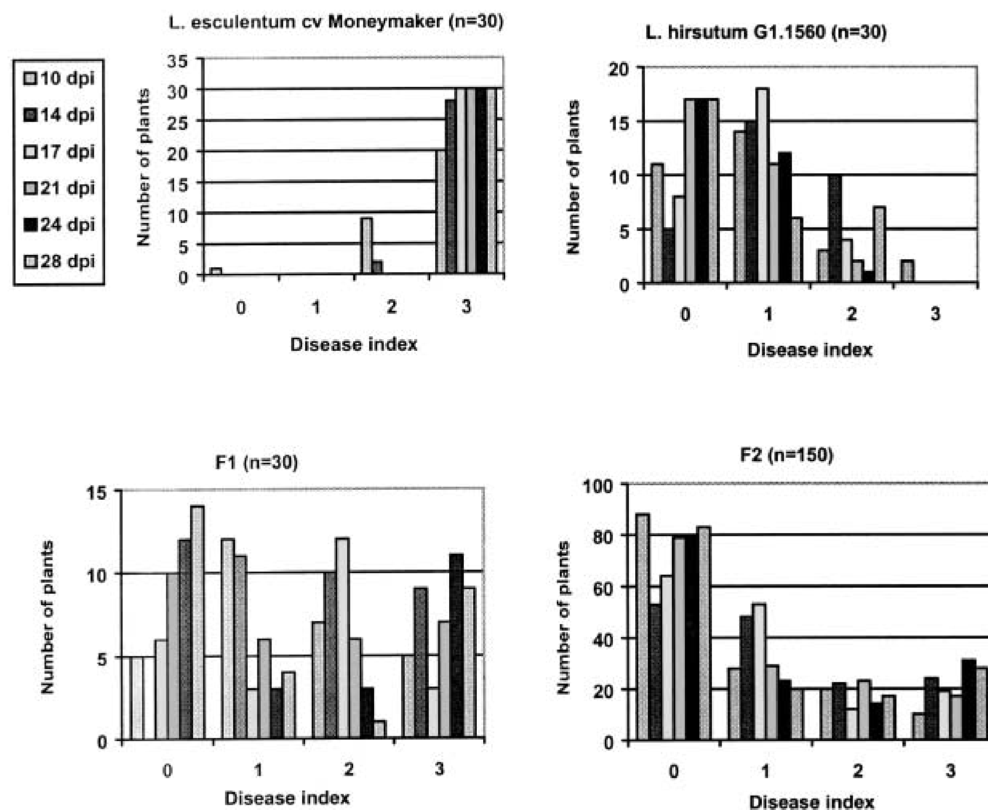

Fig. 1 Frequently distribution of resistance to *O. lycopersicum* in *L. hirsutum* G1.1560, *L. esculentum* cv. Moneymaker and their F_1 and F_2 progenies. Population size is indicated *above* the panels *in parenthesis.* The disease index was defined as: *O*=no infection; *1*=less than 10% of the foliar area affected; *2*=10–30% foliar area affected; *3*=more than 30% foliar area affected. Evaluations were clone at 10, 14, 17, 21, 24 and 28 days post-inoculation (*dpi*)

Table 1 SCAR markers converted from RAPD markers closely linked to the *Ol-1* gene

SCAR	Primer	Sequence (′5-→3′)[a]	Annealing temperature (°C)[b]
SCAE16	$OPAE16_{778}$ forward Reverse	*TCCGTGCTGA*ATGAAGATTCAAAC *TCCGTGCTGA*TAAAACTGTTAGAC	60.0
SCAF10	$OPAF10_{464\ forward}$ Reverse	*GGTTGGAGAC*GAATGGAAAGATGC *GGTTGGAGAC*AATAGACTCGAGAT	65.0
SCAB01	$OPAB01_{866}$foward Reverse	GCTTCTAGATGCAGAAAGTTGGCG CGCCCATTCCCGCATATACAG	62.0
SCAG11	$OPAG11_{1400}$ forward Reverse	TGGGATCACAGATTAACAAATGCG ATGTGTGCGATGAGAAACGTGG	60.0
SCAK16	$OPAK16_{1200}$ forward Reverse	CAAACAAAGCAGTGGATTTTTTTCG TAAAAGCCTTAGTGGGACAGGGC	58.0

[a] Primers for SCAE16 and SCAF10 were designed by extending the original 10-mer RAPD primer (underlined) with 14 bases at the 3′ end. Primers for SCAB01, SCAG11 and SCAK16 were designed by using computer programme PRIMER. The sequences of the original 10-mer RAPD primers OPAB01, OPAG11 and OPAK16 were CCGTCGGTAG, TTACGGTGGG and CTGCGTGCTC respectively

b The annealing temperature for RAPD was ±35°C

Mapping of *Ol-1* with RFLP markers

Though *Ol-1* has been mapped on chromosome 6 of tomato (Van der Beck et al. 1994) using 3 RFLP markers (GP79, TG153 and TG178), the linkage map of the *Ol-1* region was still far from saturated. In order to add more markers to the map, we analysed the F_2 using 12 additional RFLP markers that were well-distributed on chromosome 6 (Tanksley et al. 1992). All 15 markers showed a polymorphism between the two parents applying the restriction enzymes *Eco*RI, *Hae*III and *Taq*I. Based on the segregation of the F_2, an RFLP map around the *Ol-1* region was constructed showing *Ol-1* to be located between TG153 and TG164 with an accuracy of about 3 cM (Fig. 3).

Identification of diagnostic RAPD markers

Saturation of the TG153-TG164 interval encompassing *Ol-1* was accomplished by means of BSA with RAPDs on DNA from the resistant and susceptible pools as templates. With 300 decamer random primers, five diagnostic RAPDs were identified: two *L. hirsutum*-specific, $OPAB01_{866}$ and $OPAF10_{464}$, with a length of 866 bp and 464 bp, respectively, three *L. esculentum*-specific, $OPAE16_{778}$, $OPAG11_{1400}$ and $OPAK16_{1200}$, 778 bp, 1400 bp and 1200 bp in length, respectively. By analysing 27 recombinants in the TG153-TG164 interval, we mapped OPAB01, OPAE16, OPAF10, OPAK16 and OPAG11 near *Ol-1* (data not shown), confirming a tight linkage between these RAPDs and *Ol-1*.

Cloning and sequencing of the diagnostic RAPD markers

Since SCAR markers are more reliable, reproducible and locus-specific than RAPD markers (Paran and Michelmore 1993), the newly identified RAPDs were converted into SCARs. The amplified products $OPAF10_{464}$, $OPAB01_{866}$, $OPAE16_{778}$, $OPAG11_{1400}$ and $OPAK16_{1200}$ were extracted from the gel and cloned into the *Sma*I site of a Bluescript vector. Cloned fragments were shown to be derived from and identical to their corresponding RAPDs by Southern analysis. To determine whether each cloned fragment corresponded to multi-copy sequence family or to a single locus, we conducted Southern analysis with DNA of both parents following digestion with *Bam*HI, *Bgl*II, *Bst*NI, *Dra*I, *Eco*RI, *Eco*RV, *Hae*III, *Hin*dIII and *Taq*I. $OPAF10_{464}$ and $OPAG11_{1400}$ appeared to represent members of a repeat family (not shown); the others were derived from a single locus. $OPAF10_{464}$, $OPAB01_{866}$ and $OPAE16_{778}$ were completely sequenced, although only two ends (500–600 bases) of $OPAG11_{1400}$ and $OPAK16_{1200}$ were analysed. At both ends, the terminal 10 bases corresponding to the original RAPD primers were recovered.

SCAR analysis

For SCAR analysis, a pair of primers for each cloned RAPD product was designed and synthesised as mentioned in the Materials and methods (Table 1). PCR reactions were performed using genomic DNA of resistant and susceptible pools as templates. As expected, a unique band was detected in the susceptible pool when using *L. esculentum*-specific SCAR primers, as was one found in the resistant pool when using *L. hirsutum*-specific SCAR primers (Fig. 2). The sizes of the SCAR amplification products were identical to those of the corresponding RAPD amplification products (data not shown). SCAR analysis for individual plants of the resistant and susceptible pools was carried out to confirm the alleles of these marker loci. As expected, a susceptible allele was detected in the 7 susceptible plants when using *L. esculentum*-specific SCAR primers, as was a resistant allele found in the resistant plants using *L. hirsutum*-specific SCAR

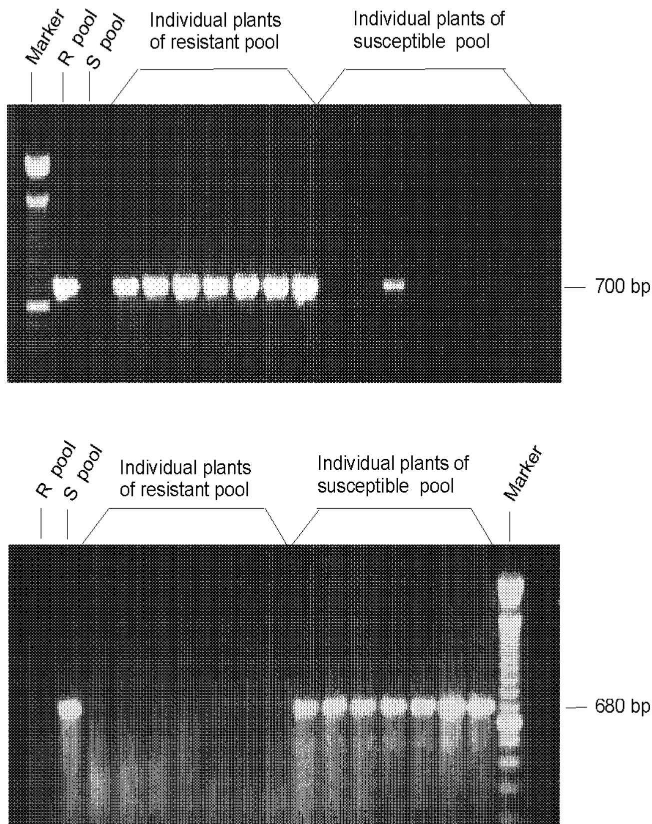

Fig. 2 SCAR analysis of genomic DNA of individual plants of either the resistant (*R*) or susceptible (*S*) pool using *L. hirsutum* (resistant allele)-specific SCAR primer SCAB01 (*upper panel*) and *L. esculentum* (susceptible allele)-specific SCAR primer SCAG11 (*lower panel*)

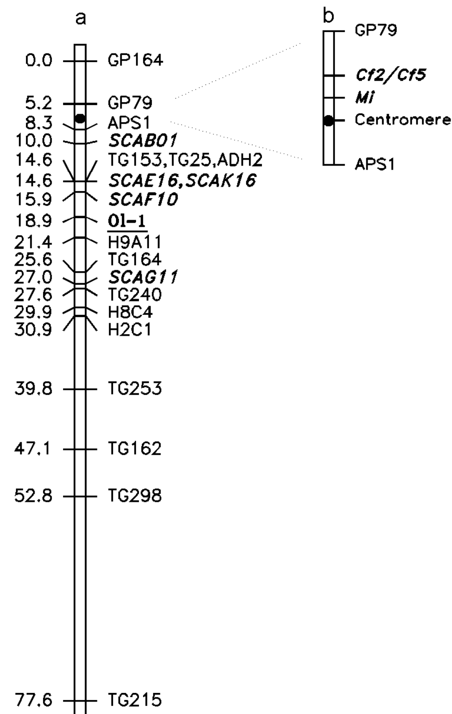

Fig. 3 a Genetic map of part of chromosome 6 of tomato showing the map position of *Ol-1*, which confers resistance to *Oidium lycopersicum* in tomato. Markers in *normal print* are RFLPs; those in *bold* and *italics* are SCARs. **b** Map position of centromere and *Cladosporium fulvum* resistance genes *Cf2*/*Cf5* and *Meloidogyne incognita* resistance gene *Mi* on chromosome 6 (Dixon et al. 1996, 1998; Milligan et al. 1998; Van Wordragen et al. 1996; Zhong et al. 1999)

primers (Fig. 2). However, an *L. hirsutum*-specific allele for SCAB01 was detected in 1 plant of the susceptible bulk (Fig. 2, upper panel), indicating the occurrence of recombination between *Ol-1* and SCAB01, or heterozygosity between *Ol-1* and SCAB01. The absence of this *L. hirsutum*-specific allele in the susceptible pool is probably due to the low relative concentration of the corresponding DNA (template) in that pool.

In order to map these SCARs, the whole F_2 population was screened for the presence of absence of corresponding polymorphic DNA bands by using each pair of the SCAR primers. All five SCARs identified only rare recombinants with *Ol-1*, indicating that they were tightly linked to this gene. The segregation data were used to construct an integrated genetic map including both RFLP and SCAR markers (Fig. 3).

Discussion

In the present study we identified 15 RFLP markers and 5 SCAR markers closely linked to the powdery mildew resistance gene *Ol-1*, which had been previously mapped on chromosome 6 of tomato (Van der Beek et al. 1994). A genetic linkage map comprising these 20 markers was constructed showing *Ol-1* to be flanked by markers SCAF10 and H9A11. Since the centromere of chromosome 6 is located between GP79 and APS1 (Zhong et al. 1999), *Ol-1* is apparently located on the long arm of chromosome 6 (Fig. 3), 10–13 cM, distant from the recently cloned resistance genes *Mi* and *Cf2*/*Cf5* on the short arm of chromosome 6 (e.g. Van Wordragen et al. 1996; Zhong et al. 1999; Dixon et al. 1996, 1998; Milligan et al. 1998). Resistance genes are often organised in clusters of homologous genes that may span from 36 kb up to several megabases in plant genomes (Meyers et al. 1998; Thomas et al. 1997). For instance, *Cf-4* and *Cf-9* are part of the so-called "Milky way" cluster that contains five very homologous genes and spans 36 kb in the tomato genome (Thomas et al. 1997), while at least 24 *Dm* gene homologues span about 4 Mb in the lettuce genome (Meyers et al. 1998). The distance between *Ol-1* and *Cf2*/*Cf5*/*Mi* suggests that these genes may be part of a *Dm*-gene like cluster of more than 1 Mb. If so, *Ol-1* may be homologous to *Cf2*/*Cf5* or *Mi*. However, this is not very likely as *Cf2*/*Cf5* are not homologous to *Mi*, and these genes are separated from *Ol-1* by the centromere.

The present map is based on an F_2 of *L. esculentum*×*L. hirsutum* and is similar to the genetic linkage map of *L. esculentum*×*L. pennellii* (Tanksley et al. 1992). The distance between GP79 and TG215 in the present map is 72 cM (Fig. 3) and 89 cM in the Tanksley map (Tanksley et al.

1992). However, some hitherto unresolvable markers (for example TG240 and TG298) became resolved in our map, probably as a result of the larger F_2 population applied.

By using BSA with 300 RAPD primers, five RAPDs were identified that were closely linked to *Ol-1*. This confirms the success of BSA for identifying RAPDs that are closely linked to a gene of interest (Michelmore et al. 1991; Giovannoni et al. 1991). Giovannoni et al. (1991) suggested an optimal pool size of more than 5 but fewer than 10 individual plants for a target interval of 10 cM as the probability of a plant having maximally one double crossover in such a pool would then be less than 10%. In our study, an interval of 11 cM spanning the *Ol-1* gene between TG153 and TG164 was targeted, and 7 plants were chosen for both pools. Among the five RAPDs identified, $OPAF10_{464}$ was the RAPD most closely linked to *Ol-1* (3 cM, shown as SCAF10 in Fig. 3).

Some disadvantages of the RAPD markers, including sensitivity to reaction conditions and amplification of multiple locis (Paran and Michelmore 1993) can be overcome by converting RAPDs into SCAR markers. In the present study, all primer pairs deduced from the sequences of the RAPD amplification products indeed generated locus-specific SCAR markers. SCAB01 and SCAF10 were *L. hirsutum*-specific and SCAE16, SCAG11 and SCAK16 were *L.-esculentum* specific. All five SCARs, but especially SCAF10 and SCAG11, should be highly suited for commercial breeding programmes as they are diagnostic for the introgression fragment containing the *Ol-1* gene and easy to handle as PCR markers. This would speed up breeding programmes for resistance to *O. lycopersicum* without disease tests or laborious RFLP analyses. However, since H9A11 is more closely linked to *Ol-1* than SCAG11, a corresponding SCAR marker of this RFLP marker would be desirable.

The genetic linkage map reported in this paper contains 20 markers in a region of about 80 cM, i.e. 1 marker per 4 cM. This dense map thus provides an essential framework for any future map-based cloning of *Ol-1*, the more so considering the availability of YAC and BAC libraries and AFLP technology to identify even more closely linked markers.

Acknowledgements This project is co-sponsored by Dutch breeding companies.

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