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RFLP linkage analysis of the *Cf-4* and *Cf-9* genes for resistance to *Cladosporium fulvum* in tomato

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Abstract Four different populations segregating for one of the two closely linked (possibly allelic) tomato disease resistance genes to the fungus *Cladosporium fulvum*, *Cf-4* and *Cf-9*, were generated and analysed for recombination frequencies between the *Cf*-genes and restriction fragment length polymorphism (RFLP) loci. The population consisting of F_2 progeny from the interspecific cross *Lycopersicon esculentum* carrying *Cf-9* × *L. pennellii* was identified as the most useful for RFLP mapping of the *Cf-4/9* locus and an RFLP map around this locus was constructed mainly using this population. The two closest markers identified were CP46, 2.6 cM distal, and a group of 11 markers including TG236, 3.7 cM proximal to *Cf-4/9*. A polymerase chain reaction (PCR)-based procedure for the rapid identification of recombination events between these two markers was developed. The regions of foreign DNA introgression surrounding *Cf-4* and *Cf-9* in near-isogenic lines were delimited.

Key words *Lycopersicon esculentum* · Tomato leaf mould · Plant disease resistance gene · Restriction fragment length polymorphism (RFLP) analysis
Genetic linkage

Introduction

Both *Cf-4* and *Cf-9* were identified in wild species related to the cultivated tomato (*Lycopersicon esculentum*) as genes that conferred resistance to *Cladosporium fulvum*, the causal agent of tomato leaf mould. They were subsequently introgressed into *L. esculentum*; *Cf-4* from *L. hirsutum* and *Cf-9* from *L. pimpinellifolium* (Stevens and Rick 1988). Both interact with *C. fulvum* in a classical gene-for-gene manner in which dominant resistance genes specify the recognition of pathogen-derived factors which are the products of dominant avirulence genes. Both *Avr9* and *Avr4*, the genes encoding the polypeptides which interact with the *Cf-9* and *Cf-4* gene products respectively, have been cloned and shown to be dominant genes (van Kan et al. 1991; Joosten et al. 1994). This type of gene-for-gene resistance interaction is widespread throughout the plant kingdom (Sidhu 1987) but the molecular basis for recognition of the pathogen has not been elucidated for any such interaction. The cloning of a resistance gene involved in a gene-for-gene interaction would undoubtedly help characterise such recognition events and therefore be of fundamental interest for plant biology. The gene conferring resistance to bacterial speck in tomato has recently been cloned (Martin et al. 1993).

The isolation of genes whose product and mode of action are completely unknown, such as plant disease resistance genes, is a common problem in molecular biology. One solution is to use positional cloning, in which molecular markers linked to the gene are used to isolate neighbouring sequences which are even closer (Young 1990). In a series of such steps one can eventually isolate the DNA bearing the target gene. Positional cloning has been used to clone several genes from humans and other organisms (Rommens et al. 1989; Papp et al. 1991) as well as a few from plants (Arondel et al. 1992; Giraudat et al. 1992; Martin et al. 1993). This paper describes work aimed at the positional cloning of the *Cf-4* and *Cf-9* genes.

It has been previously reported that *Cf-4* and *Cf-9* are tightly linked or allelic (Jones et al. 1993) and *Cf-1* has been shown to map very close to *Cf-4* (Kerr and Bailey 1964). Thus, these three genes may constitute part of an allelic series or a complex locus (here designated the *Cf-4/9* locus), similar to other complex resistance loci identified in many plant species (Pryor 1987). It is thought that the widespread occurrence of these complex loci may in-

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dicate that new recognition specificities are generated by unequal crossing over between pre-existing resistance genes during meiosis (Sudupak et al. 1993). By cloning either *Cf-4* or *Cf-9* it may be possible to isolate the other genes at this locus by homology or due to the close proximity of their loci. The cloning of such a series of genes may provide valuable insights into how new specificity is generated.

Prerequisites for a successful positional cloning strategy are: (1) a start point which is physically very close to the gene, and (2) the identification of many individuals carrying recombination events near the gene. Using the recombination events it is possible to order probes with respect to the gene and determine when one has isolated DNA sequences which span the gene. This paper concerns efforts to satisfy these requirements for the *Cf-4/9* locus.

Initial RFLP mapping has placed *Cf-9* between the molecular markers TG236 and TG301 (Jones et al. 1993). The same report places *Cf-4* and *Cf-9* approximately 14 cM distal to the classical marker *aurea* on the short arm of chromosome 1. In the present paper a more detailed RFLP mapping of the *Cf-4/9* locus is provided and the regions of foreign DNA introgression associated with the introduction of *Cf-4* and *Cf-9* into *L. esculentum* from related wild species are investigated. The effect of genetic background on recombination rates around the *Cf-4/9* locus is documented and the development of PCR-based methods to facilitate the identification of recombination events in this region is reported. This work provides a basis for positional cloning of this locus.

Materials and methods

Plant material and segregating populations

Near-isogenic lines (NILs) of the susceptible *L. esculentum* cultivar Moneymaker carrying *Cf-4* or *Cf-9* (and designated Cf4 and Cf9, respectively), were obtained from R. Oliver (University of East Anglia, Norwich) and their authenticity confirmed as described in Jones et al. (1993). These are true-breeding NILs developed by I. Boukema at the Centre of Genetic Resources (CPRO, the Netherlands) by backcrossing the donor parents to Moneymaker (designated Cf0) five times as the recurrent parent and thereafter selfing for three generations (Tigchelaar 1984; I. Boukema personal communication). *L. pennellii* LA716 was provided by C. Rick (Tomato Genetic Resource Centre, Davis, California). The *L. pimpinellifolium* line PI126933 was provided by USDA ARS Plant Genetic resources Unit, Cornell University, Ithaca. Populations from four different crosses were used in the mapping experiments: an F_2 population from a Cf9 × *L. pennellii* cross ($F_2$9P), an F_2 population from a Cf4 × *L. pennellii* cross ($F_2$4P), an F_2 population from a Cf9 × *L. esculentum* cross ($F_2$9E), and an F_2 population from a Cf4 × *L. esculentum* cross ($F_2$4E). A population from the test cross (Cf9 × *L. pennellii*) × Cf0 (Jones et al. 1993) is referred to as TC9P. RFLP analysis was only performed on susceptible F_2 plants to facilitate linkage analysis (in contrast to the TC9P population in which all individuals were subjected to RFLP analysis (Jones et al. 1993).

Testing resistance by inoculation with *C. fulvum*

A pure culture of *C. fulvum* race 5 was obtained from R. Oliver (University of East Anglia, Norwich) and its authenticity confirmed as described in Jones et al. (1993). Fungal-spore suspensions were obtained from leaves of infected tomato plants. Spores were washed off the leaves 3 weeks after inoculation at which point heavy sporulation and the formation of brown conidiophores was observed. Heavy spore suspensions (approximately 10^6 spores/ml) were used for inoculation. Plants for inoculation were treated with the plant growth inhibitor, paclobutrazol (ICI). This prevents etiolation of the seedlings under the conditions of high humidity required for fungal development. The paclobutrazol was applied to the compost in one treatment of 50 ml 10^{-5} M solution/500 ml compost at the cotyledon stage. Plants were inoculated by dipping in the spore suspension at the 4-leaf stage. The plants were maintained at 100% humidity in propagators for 3 days after which the humidity was lowered to about 80% for 11 days. Symptoms of mycelial growth and profuse sporulation (present in compatible but not incompatible reactions) were scorable 14 days after inoculation.

Scoring segregation of resistance genes by injection with intercellular fluids (IF) containing avirulence peptides

The production of avirulence-gene-encoded peptides in the apoplastic fluid of susceptible tomato plants supporting the growth of *C. fulvum*, and their use in the induction of necrotic or chlorotic responses following injection into resistant tomato plants, has been well documented (De Wit and Spikman 1982; Scholtens-Toma and De Wit 1988). Intercellular fluids containing the *Cf-9*-specific elicitor were isolated as described by De Wit and Spikman (1982) from Cf0 plants 14 days after inoculation with race 5 and injected via the underside into the leaves of plants segregating for *Cf-9*, using a 1-ml disposable syringe without a needle. The plants were kept in the greenhouse, under supplementary lighting when necessary, until symptoms (necrosis for plants carrying *Cf-9*) were observed, usually 1–2 days after injection.

DNA extraction/RFLP analysis

Nuclear DNA was extracted by a method based on that described by Bernatzky and Tanksley (1986) and modified by V. Klimyuk (personal communication). Approximately 3 g of young leaf material from each plant was collected on ice and ground in a sap extractor (Wenig and Koch, Hannover, Germany) while the rollers were washed with 3 ml of 115 mM Tris/HCl pH 7.5, 29 mM EDTA pH 8.0, 1.17 M NaCl, 1.17% CTAB, 12 mM Na bisulphite (added just before use) and with 0.6 ml of 5% N-lauryl sarkosine. The washing fluid was collected in a 15-ml capped tube (falcon) and incubated at 65°C for 15 min. Four milliliters of phenol/chloroform (1:1) was added to each tube followed by vortexing. The tubes were centrifuged at 1500 *g* for 10 minutes. The aqueous phase was taken and DNA was precipitated by addition of 3 ml of isopropanol. The tubes were centrifuged as above, the supernatant discarded and the DNA pellets washed for 30 min with 70% ethanol. The tubes were centrifuged briefly at 1500 *g* and the supernatant discarded. The pellets were allowed to dry and dissolved in sterile distilled water. About 100 μg of DNA was obtainable by this method.

For Southern blots, 10 μg of DNA was digested with the appropriate restriction endonuclease according to the supplier's, directions (Northumbria Biologicals Limited; Bethesda Research Laboratories). Electrophoresis was performed on 1% agarose gels and blotted onto Hybond membranes. The blots were probed by the Church and Gilbert (1984) method with DNA labelled with ^{32}P-dCTP (Du Pont) by the random hexamer method (Sambrook et al. 1989).

The sources of the probes used in this study are listed in Table 1. Inserts for radiolabelling were generally prepared by performing PCR on the plasmids carrying the original clones. Universal (M13) forward and reverse primers at 0.5 μM were used for the PCR. The amplified product was purified on a prepacked NICK column (Pharmacia) containing Sephadex G-50

Preparation of plant tissue for PCR

The preparation of plant tissue for use as a template in PCR was similar to that described in Klimyuk et al. (1993) . This technique is more

difficult to perform on tissue from plants derived from *L. esculentum* × *L.pennellii* crosses than on material from pure *L. esculentum* lines. Vacuum infiltration of the plant tissue with 0.25 M NaOH prior to the first boiling was found to increase the efficiency of the procedure.

DNA sequencing

DNA sequence analysis was performed with a Sequenase sequencing kit (USB) according to the manufacturer's instructions.

Linkage analysis

Recombination values (r) were measured as the proportion of gametes recombinant between the markers analysed from the total F_1 gametes contributing to the test cross or susceptible F_2 progeny. The standard error was calculated assuming a binomial distribution of r. For recombination values of zero the upper limit of recombination at $P = 0.05$ for n gametes screened was calculated according to the formula $1\text{-}p^{1/n}$ (see Table VIII, Fisher and Yates 1963). Differences in recombination rates between different populations were tested by the construction of contingency tables and use of the χ^2 test. The Haldane function was used for the calculation of genetic distances in centiMorgans (cM).

Results

Southern hybridisation analysis of near-isogenic lines

Probes previously mapped to the short arm of chromosome 1 of tomato and potato, which is largely colinear with tomato (Gebhardt et al. 1991), were obtained from various colleagues. A total of 31 of these probes were used for Southern hybridisation analysis of *L. pennellii* and the NILs Cf0, Cf4 and Cf9 in order to identify restriction enzymes that reveal RFLPs between *L. pennellii* and *L. esculentum* or between the NILs (Table 1). The detection of a polymorphism between the NILs was consistent with the probe hybridising to the introgressed region of foreign DNA around the resistance gene (see below). Five probes were identified that showed polymorphisms distinguishing the NILs Cf0, Cf9 and Cf4 from each other: TG301, CT2, CT197, CP46 and GP264 (Fig. 1). GP264 and TG301 probably hybridise to identical restriction fragments of tomato DNA as they co-segregate and show identical RFLP patterns when analysed on Southern blots of Cf0, Cf4, Cf9 and *L. pennellii* DNA cut with four different restriction enzymes. TG301 has been reported previously to detect a polymorphism between Cf0 and Cf9 (van der Beek et al. 1992) but not between Cf0 and Cf4. We have observed a TG301 polymorphism between Cf0 and Cf4 using the enzyme *Sac*I (Fig. 1). One probe, 721AR2, revealed polymorphisms between Cf0 and Cf4 but not between Cf0 and Cf9. Six enzymes were tested of which three, *Bst*YI, *Eco*RV and *Dra*I, revealed clear RFLPs between Cf0 and Cf4.

Delimiting regions of foreign DNA introgression around the resistance genes

Figure 2 shows the regions on the short arm of chromosome 1 in the NILs Cf4 and Cf9 revealed by RFLP analysis to differ from the Moneymaker (Cf0) recurrent parent. This indicates the minimal regions of foreign DNA introgression from the species from which *Cf-4* and *Cf-9* were originally introduced into *L. esculentum*. The fact that RFLP markers outside these regions do not show polymorphisms between the NILs is consistent with them lying outside the region of introgression. It is possible that the DNA in these regions is highly conserved between *L. esculentum* and *L. hirsutum* or *L. pimpinellifolium* in which case RFLPs may not be detectable despite the fact that the locus still

Fig. 1 RFLPs between *L. pennellii* (*P*) and the NILs Cf0 (*0*), Cf4 (*4*) and Cf9 (*9*) detected by Southern-blot analysis using the probe and enzymes indicated

Table 1 A summary of the RFLP probes used in this study. All probes had been previously mapped to the short arm of chromosome 1 of tomato or potato. The sources of the probes were S. Tanksley (ST) (Tanksley et al. 1992), C. Gebhardt (CG) (Gebhardt et al. 1991), J. Hille (JH) (Rommens et al. 1992) (original name of probe FT33 was AAT6514-30), D. Grierson (DG) (Slater et al. 1985), J. English (JE) (PhD. thesis, UEA Norwich, UK), and C. Thomas (SLJ) (Thomas et al. 1993). In each case at least four of the following different restriction enzymes were used to try to identify RFLPs: *Eco*RI, *Eco*RV, *Dra*I. *Hin*dIII, *Hae*III. If an RFLP was detected, the enzyme showing the clearest polymorphism is indicated. A cross indicates that no RFLP was detected. A dash indicates that no RFLP analysis was carried out

Probe	Source	*Polymorphism*		
		L. pennellii/Cf9	Cf0/Cf4	Cf0/Cf9
721AR2	SLJ	*Eco*RV	*Eco*RV	×
CP45	CG	*Eco*RI	×	×
CP46	CG	*Hae*III	*Hae*III	*Hae*III
CP100	CG	*Hin*dIII	×	×
CP108	CG	*Hae*III	×	×
CT2	ST	*Hae*III	*Eco*RI	*Eco*RI
CT25	ST	*Hin*dIII	×	×
CT87	ST	*Eco*RI	×	×
CT116	ST	*Eco*RI	–	×
CT122	ST	*Eco*RV	×	×
CT197	ST	*Hin*dIII	*Dra*I	*Hin*dIII
CT209	ST	*Eco*RI	×	×
CT233	ST	*Hin*dIII	×	×
CT268	ST	*Hin*dIII	×	×
GP264	CG	*Hin*dIII	*Sac*I	*Hin*dIII
GP519	CG	*Eco*RI	–	×
TG24	ST	*Hin*dIII	×	×
TG51	ST	*Hin*dIII	×	×
TG58	ST	×	×	×
TG59	ST	*Dra*I	×	×
TG67	ST	*Hin*dIII	-	×
TG125	ST	*Hin*dIII	×	×
TG184	ST	*Hae*III	×	×
TG224	ST	*Hin*dIII	×	×
TG236	ST	*Hin*dIII	×	×
TG301	ST	*Hin*dIII	*Sac*I	*Hin*dIII
TG410	ST	*Hae*III	×	×
pTOM31	DG	*Eco*RV	×	×
pTOM41	DG	*Eco*RI	–	×
FT33	JH	*Hin*dIII	×	×
13715V	JE	*Hin*dIII	×	×

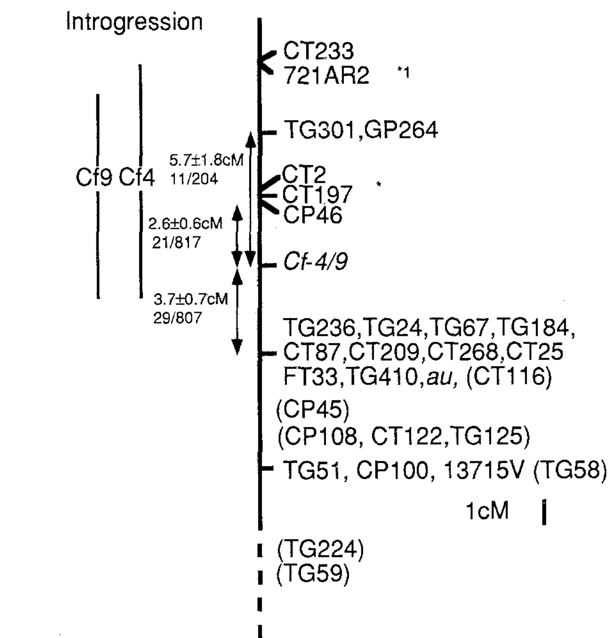

Fig. 2 An RFLP map of the short arm of chromosome 1 of tomato. Recombination distances are based upon analysis of susceptible plants from an $F_2$9P population. No recombination event has been found between markers separated by a comma in any population analysed. The exact recombination values of those intervals most intensively studied are shown on the left of the map. The actual recombination fractions (number of recombination events/number of F_1 gametes screened) are shown under the recombination values in each case. The minimal regions of foreign DNA introgression associated with introduction of *Cf-4* and *Cf-9* into the NILs Cf4 and Cf9 respectively are shown on the left of the figure. Markers in *brackets* were not mapped in this study but were examined to see whether they revealed an RFLP between the NILs. They have been assigned an approximate map position by reference to other studies (Gebhardt et al. 1991; Tanksley et al. 1992). Too-little information is available to assign positions to pTOM31, pTOM41 and GP519.*, the order of these markers, as shown by their vertical sequence, was determined using an $F_2$9E population. Limited analysis in the $F_2$9P population of their relative positions failed to separate them. *1, the order of these markers is assumed from analysis of the Cf0 and Cf4 NILs. 721AR2 gives an RFLP between the NILs but CT233 does not

lies in the region of introgression. This is more likely in the case of the Cf9 NIL as *L. pimpinellifolium* (the species from which *Cf-9* originates) has been shown, by RFLP analysis, to be the most-closely related to *L. esculentum* of all the *Lycopersicon* species. *L. hirsutum*, by contrast, is among the least-closely related (Miller and Tanksley 1990).

To address this possibility, DNA was analysed from the original *L. pimpinellifolium* line (PI126933) in which *Cf-9* was identified. The probe 721AR2, distal to the presumed region of introgression, shows a *Hin*dIII RFLP between Cf0 and PI126933 DNA and between Cf9 and PI126933 DNA while CP46, which lies within the region of introgression, shows an RFLP between Cf0 and PI126933 but not between Cf9 and PI126933, as expected (data not shown). These data are consistent with 721AR2 lying distal to the region of introgression in Cf9 and CP46 lying inside it. However, four probes proximal to the region of introgression, CT116, TG24, CT268 and TG236, detect no RFLP between PI126933 and Cf0 with five different restriction enzymes (data not shown). The region of foreign DNA introgression in Cf9 may therefore extend further proximal than is shown in Fig. 2. The lack of identified polymorphism proximal to *Cf-4* in the Cf4 NIL and proximal to *Cf-9* in the Cf9 NIL meant that the $F_2$9E and $F_2$4E populations could not be used for the mapping of RFLP markers or the identification of recombination events proximal to *Cf-4/9*.

Molecular mapping of the *Cf-9* locus

Of the four populations ($F_2$9P, $F_2$4P, $F_2$9E and $F_2$4E) the $F_2$9P population was identified as the most useful for gen-

eral mapping purposes (see below) and was used for the construction of a map at the *Cf-4/9* locus. The mapping of four probes relative to *Cf-9* has already been reported (Jones et al. 1993). In the present study a population of 107 susceptible F_2 plants from an $F_2$9P population of 336 progeny screened for resistance conferred by *Cf-9* was used to map 26 markers around *Cf-9* (Fig. 2). The two closest markers flanking the gene were found to be CP46 distal to *Cf-9* and the 'TG236 cluster' proximal to the gene (Fig. 2). These markers were used to score a further 418 $F_2$9P progeny (resistant and susceptible) using the PCR-based method detailed below. Figure 2 summarises all these data. The markers CT197, CT2 and CP46 were not separated by analysis of the $F_2$9P population but recombination events between these markers were identified during analysis of 309 susceptible plants from a $F_2$9E population which enabled their order to be established, CP46 being closest to *Cf-4/9* and CT2 furthest away (see Fig. 2). The determined order of the markers was consistent with that reported by Gebhardt et al. (1991) and Tanksley et al. (1992). CT2, CT197 and TG301 were previously reported to co-segregate (Tanksley et al. 1992); however, recombination events between them were identified here due to either the greater number of plants analysed or to a higher recombination in this region than observed in the previously analysed population (see Discussion).

The initial $F_2$9P population was made up of 107 susceptible plants from a total of 336 plants screened for susceptibility. The ratio 229:107 is significantly different at the 1% level from a 3:1 ratio. This segregation distortion is consistent with that previously reported for *L. esculentum* × *L. pennellii* crosses (Chetelat and De Verna 1991; de Vicente and Tanksley 1993).

Expression of *Cf-9* and *Cf-4* in *L. esculentum* × *L. pennellii* interspecific crosses

It is clear that the reliable scoring of resistance conferred by *Cf-4* or *Cf-9* is an important criterion for deciding which population to use for mapping. RFLP and genetic analysis of an $F_2$4P population (data not shown) revealed that the scoring of the phenotype conferred by *Cf-4* was very unreliable in a 50% *L. pennellii* background. Seventeen out of sixty-seven plants identified as sensitive were heterozygous or homozygous Cf4-like for RFLP markers immediately proximal and distal to *Cf-9*. Assuming that *Cf-4* is in the same RFLP interval as *Cf-9*, this means that these 17 plants actually carried *Cf-4*. Ten plants from the $F_2$4P population were completely genotyped for *Cf-4* by crossing them to Cf0 and scoring the progeny for resistance conferred by *Cf-4*. Analysis of these plants showed that *Cf-4* co-segregated with CP46 and was distal to TG236. This is consistent with a similar location for *Cf-4* on the RFLP map to that of *Cf-9*, which would be expected from the allelism tests conducted previously (Jones et al. 1993). For these reasons, *Cf-4* and *Cf-9* have been assumed to occupy the same locus, referred to here as *Cf-4/9*. These data effectively rule out the use of an $F_2$4P population for mapping of the *Cf-4/9* locus, but mean that results obtained using a $F_2$9P population can be extrapolated to *Cf-4*.

Other experiments showed that *Cf-4* is much easier to score accurately when it is segregating in a solely *L. esculentum* background; from a population of 72 plants of known *Cf-4* genotype (12 *Cf-4* absent, 52 *Cf-4* heterozygote, 8 *Cf-4* homozygote) no plants were mis-scored as susceptible and only two plants without *Cf-4* were mis-scored as resistant after inoculation with *C. fulvum* race 5.

Some difficulties were also encountered in the scoring of *Cf-9* in $F_2$9P populations. From the first population screened, four plants from a total of 36 scored as sensitive were later inferred, by RFLP analysis, to probably carry *Cf-9* (as they were heterozygous for both the closest markers flanking the gene). In a similar population screened subsequently, susceptible plants identified after inoculation with the race-5 fungus were re-checked by injection of intercellular fluid containing the AVR9 elicitor which is specific for plants carrying *Cf-9* (see Materials and methods). This procedure improved the accuracy of the scoring with only one probable mis-score from 71 plants scored as susceptible (based upon RFLP analysis). As with *Cf-4* it has been shown that resistance conferred by *Cf-9* in a wholly *L. esculentum* background is stronger than in one which is partially *L. pennellii* (Hammond-Kosack and Jones 1993).

Analysis of the "TG236 cluster"

On the tomato RFLP map reported by Tanksley et al. (1992), which was constructed by analysis of 67 F_2 plants derived from a *L. esculentum* × *L. pennellii* cross, TG236 is shown to co-segregate with 11 other RFLP markers. In this study the two markers from this cluster most frequently used, TG236 and TG24, co-segregated in 52 F_2 individuals analysed from $F_2$9P and $F_2$4P populations. For convenience we have designated this group collectively as the TG236 cluster. We have also noted co-segregation with TG236 of the recessive mutant *aurea* (0% recombination with an upper limit of 4.1% at $P = 0.05$) (Balint-Kurti et al. 1994; Jones et al. 1993) and of another DNA marker, FT33, in similar crosses. As these co-segregating markers represent the closest loci identified proximal to the gene, an attempt was made to determine the order of the markers relative to *Cf-9* and to each other, in order to determine which one was closest to the resistance gene. Eight $F_2$9P plants known to carry recombination events between TG236 and *Cf-9*, and seven plants from other *L. esculentum* × *L. pennellii* crosses which carried recombination events between TG301 and TG236, were subjected to RFLP analysis with ten of the markers in the cluster (all the RFLP markers shown in the TG236 cluster on Fig. 2 except CT116). No recombination events between these ten markers were identified. As all these plants carried recombination events distal to TG236, it is possible that recombination events were not observed because TG236 is the most distal of the markers in the cluster. However de Vicente and Tanksley (1993) present data that TG24 is distal to

TG236 based on the analysis of 432 plants from a *L. esculentum* × *L. pennellii* population, while no recombination was detected between TG236 and TG184. It is more likely, therefore, that the region of the genome spanning all these loci undergoes very little recombination in the *L. esculentum* × *L. pennellii* cross. This conclusion is further supported by the fact that no recombination was observed between TG24, TG236 and TG184 (three of the markers in the cluster) among 84 F_2 individuals from a *L. esculentum* × *L. pennellii* cross (P. Lindhout personal communication) or between FT33 and TG236 amongst 87 F_2 plants from a similar cross (J. Hille personal communication).

The effect of genetic background upon recombination in the *Cf-4/9* region

Due to the difficulty of scoring resistance conferred by *Cf-4* in the $F_2$4P population and the absence of markers which revealed differences between Cf0 and Cf4 or Cf9 proximal to the *Cf-4/9* locus, only plants from $F_2$9P populations were useful for RFLP analysis on both sides of the *Cf-4/9* locus. However, some analysis of other populations was carried out to compare, where possible, recombination rates in this region. These data are reported in Table 2. The difference in recombination fraction between $F_2$9P and $F_2$4E in the *Cf-4/9*-TG301 interval is significant at the 5% level. No other differences between populations are significant at this level although those between the $F_2$4E and $F_2$9E and between the $F_2$9E and $F_2$9P populations in the *Cf-4/9*-TG301 interval are significant at the 10% level. From these results the $F_2$9P population does not show less recombination than any of the other populations examined in any of the intervals examined and, as such, is the best choice for use as a population for mapping and for the identification of recombination events either side of the *Cf-4/9* locus, as it shows identifiable polymorphisms throughout the region of interest.

Table 2 A comparison of recombination frequencies detected for three intervals in populations from five different crosses. The actual recombination fractions (number of recombination events/number of F_1 gametes screened) are shown under the genetic distance in each case. The number of plants analysed is different between intervals within the $F_2$9P population as not all plants were analysed for all the intervals. TG24 rather than TG236 was used to analyse the TC9P population. The co-segregation of these two markers has been well documented (This paper; Tanksley et al. 1992)

Population	Interval		
	TG301-*Cf-4/9*	*Cf-4/9* TG236	TG301-TG236
$F_2$9P	5.7±1.8 cM 11/204	3.7±0.7 cM 29/807	11.9±2.9 cM 19/180
$F_2$4P	–	–	7.6±2.8 cM 8/114
$F_2$9E	2.7±0.7 cM 16/618	– –	–
$F_2$4E	0 + 4.4%* 0/66	–	–
TC9P	–	1.6±1.6 cM 1/65	–

* upper limit at $P = 0.05$

A PCR-based strategy for detection of recombination events without the need for genomic DNA extraction

Due to the considerable genetic distance between *Cf-4/9* and the closest markers identified (Fig. 2), the isolation of molecular markers closer to *Cf-4/9* than those described here is being pursued (C. M. Thomas in preparation). In order to identify sufficient recombination events around *Cf-4/9* to order these anticipated markers, a more efficient method than RFLP analysis was developed.

The DNA sequence of both ends of the probes TG236 and CP46 was determined. From these sequences PCR primers were designed (Fig. 3 a) to enable amplification of the loci corresponding to CP46 or TG236 from Cf9 and *L. pennellii*. Neither amplification product is polymorphic in size; however, digestion of the TG236 Cf9 and *L. pennellii* amplification products with 17 restriction enzymes identified three which revealed differences between them (*BstU*I, *Hinf*I and *Dra*I). Three restriction enzymes from 12 tested revealed differences between the CP46 Cf9 and *L. pennellii* amplification products (*Mae*II, *Rsa*I and *Taq*I). Restriction digestion was also performed upon the products from the NILs to try and detect differences between them. No differences were detected between the TG236 amplification products from Cf0, Cf4 and Cf9 with the nine enzymes tested. Three restriction enzymes (*Mae*II, *Taq*I and *Rsa*I) revealed differences between the Cf0 and Cf4 CP46 amplification products while none of the 22 enzymes tested revealed differences between the Cf0 and Cf9 CP46 amplification products. This is consistent with there being a greater DNA sequence divergence between the genomes of *L. esculentum* and *L. hirsutum* than between those of *L. esculentum* and *L. pimpinellifolium*.

When the PCR followed by digestion with the appropriate enzyme was performed on DNA from F_1 plants from the cross Cf9 × *L. pennellii*, a combination of both restriction fragment patterns was observed, as expected, for both amplification products. Thus, by using this PCR-based technique, it was possible to characterise $F_2$9P plants completely for the two closest markers identified flanking *Cf-9*.

A recently published protocol describing the use of intact plant tissue as a source of template DNA for PCR (Klimyuk et al. 1993) was modified for use on $F_2$9P plants (see Materials and methods). About 80% of the plants could be scored at both loci using this procedure (Fig. 3 c). Five hundred and thirteen F_2 plants have, so far, been characterised at both the TG236 and CP46 loci and have been scored for the presence of *Cf-9* by injection with IF containing AVR9 elicitor. Several of the recombination events identified by PCR have been confirmed by RFLP analysis. Thus plants recombinant between CP46 and TG236 can be

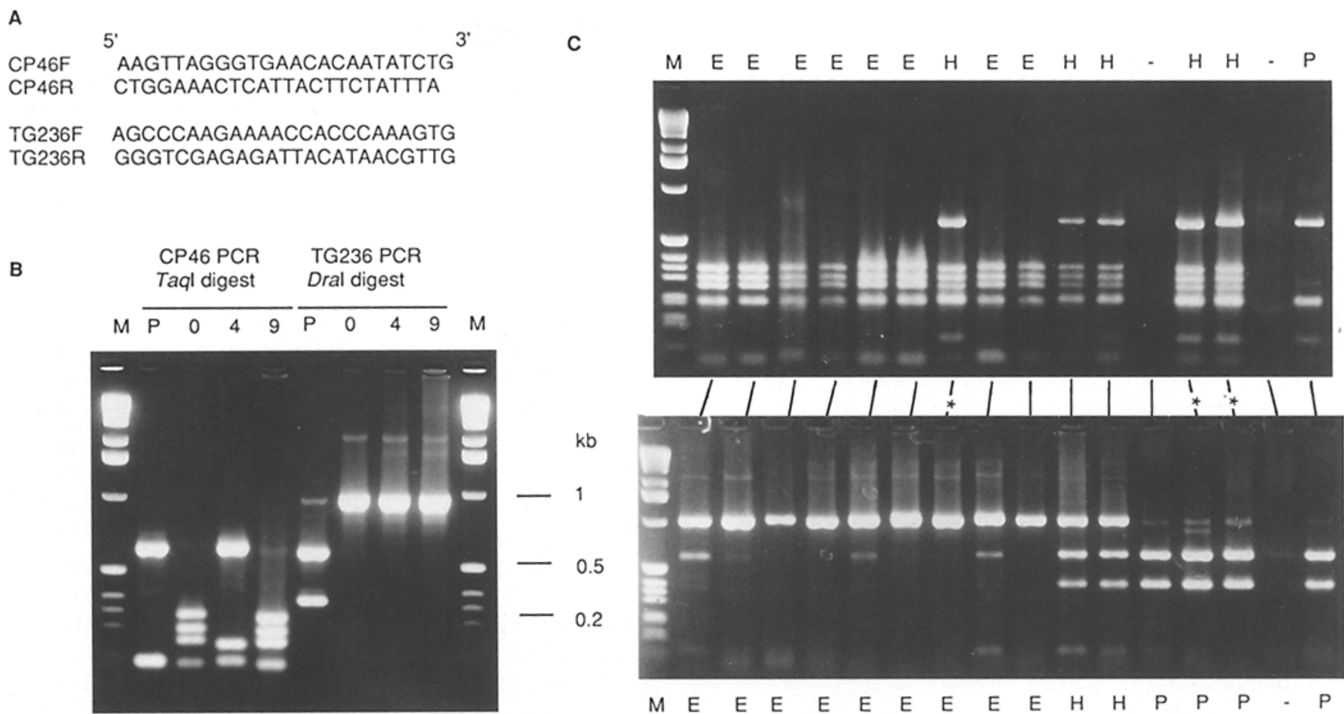

Fig. 3 A The sequences of the PCR primers designed for amplification of the CP46 and TG236 loci. **B** the products from CP46 and TG236 PCRs using *L. pennellii* (P), Cf0 (0), Cf4 (4) or Cf9 (9) DNA as template, digested with the restriction enzymes indicated and electrophoresed in a 2% agarose gel. The pattern given by plants heterozygous for both markers is shown in **C**. **C** an example of the use of PCR to characterise 16 F_2 progeny from a *L. esculentum* × *L. pennellii* cross for both the CP46 and TG236 loci (CP46 on the upper figure). Lines between the pictures connect PCRs performed on the same individual. Homozygous *L. esculentum* (E), heterozygous (H), and homozygous *L. pennellii* (P) individuals are indicated. Individuals carrying recombination events in the TG236-CP46 interval are indicated (*). Those lanes marked with a dash (–) indicate PCRs which did not work

identified in a fraction of the time it would take using conventional RFLP analysis. The results of this analysis are included in Fig. 2.

Discussion

A detailed RFLP map of the *Cf-4/9* locus

The order of the markers reported here is consistent with maps published previously (Gebhardt et al. 1991; Tanksley et al. 1992). TG301, CT2 and CT197 were previously reported to co-segregate (Tanksley et al. 1992) but have been separated in the present study. This may be due to the greater number of individuals analysed with these markers. However, it is interesting to note that the distance between TG301 and TG236 was reported as 3.4 cM based on 67 F_2 plants from a *L. esculentum* cv VF36-Tm2a × *L. pennellii* cross (F_2PE) (Tanksley et al. 1992) while the same interval was found to be 11.9 cM in the $F_2$9P cross based on 90 individuals (180 meioses). It seems therefore that recombination in the $F_2$9P population in the TG236-TG301 interval may be greater than that in the F_2PE population. The major difference between these two crosses is the presence of the introgressed *L. pimpinellifolium* DNA in Cf9 so this may be the cause of the discrepancy. The TG301-*Cf-9* distance of 5.7 ± 1.8 cM based on an analysis of the $F_2$9P population and that of 2.7 ± 0.7 cM from the $F_2$9E population are not significantly different from the value of 5.5 ± 2.3 cM previously reported for the same interval based on an analysis of F_2 progeny from the *L. esculentum* F_1 hybrid cv 'Sonatine' which is heterozygous for *Cf-9* (van der Beek et al. 1992). These results also confirm the previously reported co-linearity of the tomato and potato genomes in this region (Gebhardt et al. 1991; Tanksley et al. 1992) as the three potato markers mapped in this study, CP46, GP264 and CP100, are located in the same order relative to each other as they are in potato (Gebhardt et al. 1991).

The RFLP map around *Cf-4/9* was constructed solely from data from populations segregating for *Cf-9*. The difficulty of scoring *Cf-4* in the $F_2$4P population and the lack of detectable recombination events in the $F_2$4E population meant that no useful data was obtained using these populations. However, sufficient data exists (see Results) to suggest that *Cf-4* is situated at an identical locus to *Cf-9* on the RFLP map as it stands.

The unreliability of the scoring of *Cf-4* in the $F_2$4P population made the use of this population impractical for mapping purposes. The lack of detectable RFLPs between Cf0 and either Cf9 or Cf4 proximal to *Cf-4/9* meant that the $F_2$9E and $F_2$4E populations were only of use for mapping and the detection of recombination events distal to *Cf-4/9*. The $F_2$9P population showed recombination rates around *Cf-4/9* similar to those of the other populations examined.

This, combined with the relative reliability of resistance scoring and the ability to detect polymorphisms either side of *Cf-4/9*, made the $F_2$9P population the most useful for mapping studies. The use of the AVR9 elicitor, specific to *Cf-9*, facilitated scoring for this gene.

Delimitation of minimal regions of introgression in NILs

We have delimited the minimum regions of foreign DNA associated with the introgression of *Cf-4* and *Cf-9* into the NILs Cf4 and Cf9 respectively. Five probes were identified that showed polymorphisms between the NILs Cf0, Cf9 and Cf4 :TG301, CT2, CT197, CP46 and GP264 (see Figs. 1 and 2). Subsequent analysis suggests that GP264 and TG301 hybridise to identical restriction fragments of tomato DNA. One probe, 721AR2, revealed polymorphisms between Cf0 and Cf4 but not between Cf0 and Cf9. Due to the high level of homology between *L. esculentum* and *L. pimpinellifolium* proximal to *Cf-9* it is not possible to define the proximal boundary of the region of introgression in Cf9. Kinzer et al. (1990) also reported difficulty in the identification of RFLPs between *L. pimpinellifolium* and *L. esculentum*. The greater divergence between the genomes of *L. esculentum* and *L. hirsutum* (from which *Cf-4* was introgressed) (Miller and Tanksley 1990) make the delimitation of the introgressed region in Cf4 more reliable.

Comparison of recombination rates in different crosses

A comparison of the levels of recombination in five different crosses, including four analysed in this study together with one (TC9P) analysed previously (Jones et al. 1993), revealed a difference between the populations $F_2$9P and $F_2$4E in the *Cf-4/9*-TG301 interval that was significant at the 5% level (Table 2). The differences between the $F_2$4E and $F_2$9E and between the $F_2$9E and $F_2$9P populations in the *Cf-4/9*-TG301 interval are significant at the 10% level. These data suggest suppression of recombination associated with the interaction of the *L. hirsutum* and *L. esculentum* genomes in this region. This argument is strengthened by the fact that no recombination was found in the *Cf-4*-721AR2 interval in the $F_2$4E population. This interval was not measurable in the $F_2$9E population as no 721AR2 RFLP exists between Cf0 and Cf9.

From these data it can be seen that there is no obvious correlation between the relatedness of *L. esculentum*, *L. pennellii*, *L. pimpinellifolium* and *L. hirsutum* and the level of recombination between their genomes in the region of *Cf-4/9*. Miller and Tanksley (1990) show that, of the four species listed above, the two most closely related are *L. esculentum* and *L. pimpinellifolium*. If relatedness of species were correlated to levels of recombination between their genomes it would be expected that the $F_2$9E population would show the highest levels of recombination around *Cf-4/9*. This is clearly not the case.

Several workers have cited the evolutionary distance between genomes as a possible explanation for differing recombination levels in different crosses, for example Messeguer et al. (1991) proposed this as an explanation for decreased recombination around the *Mi* gene for resistance to the root knot nematode from *L. peruvianum*, in crosses between *Mi*-containing lines and *L. esculentum* as compared to a cross between *L. esculentum* and *L. pennellii*. In the same paper it was observed that the *L. esculentum* × *L. pennellii* cross showed greater recombination than the *L. esculentum* × *L. peruvianum* cross, despite the divergence between the genomes being similar in each case. Dickinson et al. (1993) also report data which argue against this simplistic explanation for the suppression of recombination; no enhancement of recombination in a region on chromosome 6 was observed in a cross within *L. esculentum* compared with a *L. esculentum* × *L. pennellii* cross. Studies in barley have also shown that recombination rates do not vary consistently depending on the degree of divergence between the two parental species (Gorg et al. 1993). It seems, therefore, that it may be impossible to make predictions as to which cross would give the most recombination and that it depends upon the particular genomic region being analysed and the genotype of the parents in the cross. To optimise recombination frequencies in a region of interest several different populations should be generated and empirically tested.

Clustering of markers proximal to *Cf-4/9* is due to suppression of recombination in this region in the $F_2$9P population

Clear suppression of recombination around the TG236 RFLP marker in an *L. esculentum* × *L. pennellii* cross is reported in this paper and by other workers (Tanksley et al. 1992; P. Lindhout and J. Hille personal communication). Segregation evidence suggests that recombination is not suppressed in this region in crosses involving only *L. esculentum*. FT33 and *au* are both situated in the TG236 cluster and are both scorable in solely *L. esculentum* crosses; *au* is a visible, recessive mutation and the FT33 probe is from a sequence flanking a T-DNA which carries scorable marker genes in a transgenic tomato line. In separate, but essentially similar, crosses *au* was positioned 14 cM from *Cf-9* and FT33 3 cM from *Cf-9*, while in a cross in which FT33 and *au* were segregating in coupling they were positioned 15 cM from one another (D. Jones, unpublished data). All these crosses were in a wholly *L. esculentum* background. This implies that there is a reasonable degree of recombination between markers in the TG236 cluster in intraspecific *L. esculentum* crosses and suggests, therefore, that the suppression of recombination observed is due to the interaction of the two different genomes. A similar phenomenon has been observed by Martin et al. (1993): 19 markers were shown to co-segregate on chromosome 5 in a *L. esculentum* × *L. pennellii* cross, but when these markers were mapped in a cross between *L. esculentum* and a line with *L. pimpinellifolium* DNA at this locus they were found to cover a region of 14.8 cM.

Rapid identification of recombination events around *Cf-4/9* using PCR

The use of PCR considerably reduces the labour necessary to identify recombination events in the TG236-CP46 interval. The method of using RFLP markers to design PCR primers which amplify polymorphic products from the two species between which the RFLP was observed has been reported in rice (Williams et al. 1991). Konieczny and Ausubel (1993) report a similar procedure using the sequence of mapped *Arabidopsis* genes to design the PCR primers. The development of PCR-based markers linked to disease resistance genes in lettuce using primers designed from sequences of RAPD products has also been reported recently (Paran and Michelmore 1993). The use of intact plant tissue as the template material for the PCR reactions further eases the identification of recombinants. To date, 67 recombination events in the CP46-TG236 interval have been identified by either PCR or RFLP analysis.

Using this work as a basis, the positional cloning of the *Cf-4/9* locus seems possible. The isolation of more molecular markers around *Cf-4/9* is the next priority and is in progress. (C. M. Thomas in preparation).

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