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Locating the petunia *Rf* gene on a 650-kb DNA fragment

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Abstract A bulked segregant analysis was conducted in order to find RAPD and AFLP markers linked to the restorer of fertility (*Rf*) gene in petunia. One RAPD marker, OP704, and one AFLP marker, ECCA/MACT, were found to be closely linked to *Rf* (<1 cM) in our mapping population produced from an intraspecific *Petunia hybrida* cross. These two single-copy markers bracketing *Rf* were then mapped as RFLPs on the tomato map. Despite some rearrangement between the petunia and the tomato genomes, this synteny survey revealed two tomato markers, TG250 and CT24, closely linked to *Rf*. Physical mapping indicates that CT24, OP704 and ECCA/MACT lie on the same 650-kb *MluI* fragment. A physical to genetic distance ratio of 400 kb/cM around the *Rf* gene should make it feasible to identify markers physically very close to *Rf*.

Key words Petunia · *Rf* · Molecular marker · Mapping · Synteny

Introduction

The petunia *Rf* gene is a nuclear gene which restores fertility in cytoplasmic male-sterile (CMS) plants. The male sterility is encoded by a mitochondrial gene, termed *pcf*, which disrupts pollen development (Young and Hanson 1987). When present, *Rf* affects the abundance of transcripts and proteins encoded by the *pcf* gene (Pruitt and Hanson 1991). The restoration of normal pollen development is a dominant trait, with *rfrf* CMS plants being male-sterile, while *RfRf* and *Rfrf* CMS plants are both male-fertile. Although the phenotype of this trait is very easy to score in segregating populations, the molecular mechanism underlying the process of fertility restoration has yet to be described. In order to get some insight into the molecular interaction between the *Rf* and the *pcf* genes, we decided to clone *Rf* by a positional cloning strategy. In recent years, similar strategies have allowed the cloning of several genes in different species (Martin et al. 1993; Ori et al. 1997; Giraudat et al. 1992; Bent et al. 1994).

The first step in a map-based cloning project is to find tightly linked markers to the gene of interest. Since no extensive restriction fragment length polymorphism (RFLP) map is available in petunia, we decided to test the cosegregation of *Rf* with random amplified polymorphic DNA (RAPD) (Williams et al. 1990) and amplified fragment length polymorphism (AFLP) (Vos et al. 1995) markers by a bulked segregant analysis (BSA) (Michelmore et al. 1991). Once identified and mapped, petunia markers closest to *Rf* were then located on the tomato map. Despite some rearrangement between the tomato and petunia genomes, this synteny survey identified new markers in the vicinity of *Rf*. We report the finding of four molecular markers bracketing the *Rf* gene: OP704, CT24 and TG250 on one side and ECCA/MACT on the other side. Physical mapping indicates that the distance between ECCA/MACT and OP704 is at most 650 kb.

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Materials and methods

Plant material

The segregating population is a BC_2F_2 population. The original cross was intraspecific and involved two *P. hybrida*, 2423 CMS (*rfrf*)

and 7037 (*Rfrf*). After two backcrosses to 2423, a restored (*Rfrf*) BC_2 progeny was selfed to produce the segregating population. A subset of this population comprised of 62 sterile (*rfrf*) plants constituted our mapping population. Another *Rf* progenitor *P. hybrida*, 7984 (*RfRf*), was grown and used for high-molecular-weight DNA preparation. 2423, 7037, and 7984 were originally provided by Shamay Izhar (Bet Dagan, Israel).

Scoring of *Rf* phenotype

Plants were scored for the *Rf* phenotype simply by checking for the presence or the absence of pollen on mature anthers. Plants without any visible pollen shed by the anthers were scored *rfrf*, while plants with pollen were scored *Rf*. In order to ensure correct phenotyping, plants were scored three times.

Construction of the bulks

We decided to compare *RfRf* and *rfrf* bulks in order to find markers linked in *cis* with both *Rf* and *rf* alleles. Since *Rf* is dominant, this required genotyping *Rf* plants by progeny testing. Ninety-six *Rf* plants were genotyped in a greenhouse, with an average of 27 self progeny analyzed per *Rf* plant: 67 plants were found to be heterozygous (*Rfrf*) (at least 1 sterile plant in their progeny), while 29 were found homozygous (no sterile plants in their progeny). The average frequency of sterile progeny per *Rfrf* plant was found to be 0.19. This differs very significantly from the expected frequency of 0.25 ($P < 10^{-6}$). A gamete selection (*rf* pollen being less viable than *Rf* pollen) may explain this observation.

RfRf bulk

The *RfRf* bulk for the RAPD screening consisted of 14 plants for which the number of progeny analyzed was at least 30. (The probability of an *Rfrf* plant occurring in this bulk is about 0.02). A subset of 10 plants from this bulk was used for the AFLP screening.

rfrf bulk

D165, a marker previously shown to map 40 cM from *Rf* in our population (Calfee 1996), was used to select 15 sterile individuals recombined for D165 as the members of the *rfrf* bulk for the RAPD screening. This should confine any new polymorphic markers to be closer to *Rf* than D165. A subset of 10 plants from this bulk was used for the AFLP screening.

RFLP analysis

Genomic DNA from the parents and the BC_2F_2 progeny was extracted according to Bernatzky and Tanksley (1986). DNA was digested with the appropriate enzyme, separated by electrophoresis through 1% agarose and blotted onto Hybond N + membrane (Amersham). Probes were radiolabeled with [^{32}P]-dCTP using the decaprime random priming kit (Ambion). Hybridization and washing were performed as described in Bernatzky and Tanksley (1986).

RAPD analysis

A total of 636 decanucleotide primers (Operon Technologies, Alameda, Calif.) were used to amplify 50 ng of DNA from both restored (*RfRf*) and sterile (*rfrf*) bulks as described in Martin et al. (1991). RAPD reactions were performed with a MJ Research PTC100 Programmable Thermal Controller. Amplified products were visualized after electrophoresis through 2% agarose and ethidium bromide staining. Polymorphic RAPD products were picked out with a pasteur pipette and re-amplified using the same primers and conditions. The re-amplified products were excised from an agarose gel with a clean razor blade and purified using the Geneclean kit (Bio 101, Vista, Calif.). The purified products were then cloned into the PCRII vector using the TA cloning kit (Invitrogen).

Conversion of RAPD marker to sequence-characterized amplified region (SCAR) marker

Some of the RAPD markers linked to *Rf* were converted to SCAR markers using the procedures previously described by Paran and Michelmore (1993). The only minor difference is in the design of the SCAR primers. Instead of including the original 10 bases of the RAPD primer plus the next 14 internal bases, the SCAR primers were more internal and from various lengths (24–25 nucleotides) as a result of MacVector primer designing (Kodak, Int Biotechnologies, New Haven, Conn.). The exclusion of the original 10 bases of the RAPD primer from the SCAR primer was imposed to increase the chance of amplifying both parental alleles. For all the SCAR markers used in this study, the discrimination between parental alleles was possible by finding a frequent cutter (endonuclease with a 4- to 5-bp recognition site) which cut one allele but not the other one. As for RAPD reactions, the SCAR reactions were performed with a MJ Research PTC100 Programmable Thermal Controller. Sequencing of the ends of the RAPD clones and synthesis of SCAR primers were done in the Cornell Sequencing and Oligosynthesis Facility.

AFLP analysis

The AFLP protocol was essentially as described in Vos et al. (1995) with the following slight modification. The *Eco*RI adaptor was 5′-biotinylated; this allowed the selection of the ligation products by using Streptavidine beads (M280, Dynal).

Cloning of the AFLP bands

Isolation of DNA fragments from sequencing gels and re-amplification

The DNA fragments to be isolated were sliced out of the dried gel and eluted into 100 µl H_2O at 4°C, for 1 h (the tube was vortexed periodically). The acrylamide was pelleted by centrifugation, and 2 µl of the resulting supernatant was used for re-amplification under the same conditions as for the pre-amplification except that the number of cycles was increased to 30. The resulting polymerase chain reaction (PCR) product(s) was checked by electrophoresing 5 µl on a 1.5% agarose gel. The remaining DNA was precipitated and dissolved in 10 µl of H_2O.

Cloning of the re-amplified DNA fragments in pGEM-T

Of the re-amplified DNA fragment 3–5 µl was cloned into the pGEM-T vector (Promega) according to standard protocols and (heat-shock) transformed to MC 1061.

Mapping

Linkage maps were constructed using the Macintosh V2.0 version of MAPMAKER (Lander et al. 1987). Map distances in centiMorgans (cM) were calculated from recombination frequencies using the Kosambi (1944) function. All the orders presented have been obtained with LOD scores greater than 3 unless otherwise noted.

Physical mapping

High-molecular-weight (HMW) DNA was isolated from 7984, an *RfRf P. hybrida*, using a nuclei preparation procedure (Liu and Whittier 1994). HMW DNA was embeded in agarose plugs and digested according to Liu and Whittier (1994), except that 100 units of restriction enzyme were used instead of 50 units. The digested HMW DNA was then fractionated in an 1% agarose gel and 0.5 × TBE (1x : 90 mM Tris-borate, 2 mM EDTA, pH 8.0) using the CHEF Mapper pulsed field electrophoresis system (Bio-Rad) for 28 h at 14°C with the following conditions : 6V/cm, $\alpha = 120$, initial switch time = 12.55 s, final switch time = 33.69 s. These conditions are determined to separate HMW DNA in a 100 kb to 1 Mb range.

Results

RAPD screening

Of the 636 primers used with both restored (*RfRf*) and sterile (*rfrf*) bulks, 48 (8%) did not give any amplification products, while the remaining 588 primers produced 3225 bands. The average of bands per primer was 5, which is in good agreement with previous reports on this technique (Martin et al. 1991; Ballvora et al. 1995). Four primers gave 5 reproducible polymorphic bands between the bulks (Fig. 1). Of these 5 polymorphic bands 3 were present in the sterile bulk, and absent (or at least much less intense) in the restored bulk, and vice versa for the 2 remaining polymorphic bands. Information on the primer sequences and the origin of the corresponding RAPDs is given in Table 1. In order to validate the results of the BSA, we carried out PCR reactions with the individual DNAs constituting the bulks and the parental DNAs, and the products were run on an agarose gel (Fig. 2A). This preliminary mapping confirmed the results of the BSA and indicated that the 2 polymorphic bands amplified with OP605 were likely to be allelic (Fig. 2A). To further support the linkage between these RAPDs and the *Rf* locus and to estimate more accurately the distance between them, we decided to test their cosegregation on a bigger population. Our choice to use only the sterile plants for the mapping population was dictated by the dominance of *Rf*. Indeed, only the genotype of a sterile plant (*rfrf*) is known without any ambiguity. A fertile plant, even if the progeny testing has shown it to be *RfRf*, still presents the risk of being misgenotyped. For instance, examination of the segregation of OP605 in the *RfRf* plants belonging to the *RfRf* bulk shows 4 heterozygous plants (Fig. 2A). Are those plants recombinants between *Rf* and OP605 or heterozygous *Rfrf*? Despite the care exercised to genotype these plants by testing at least 30 progeny per plant (cf. Materials and methods) the possibility of *Rfrf* plants occurring in the *RfRf* bulk cannot be ruled out.

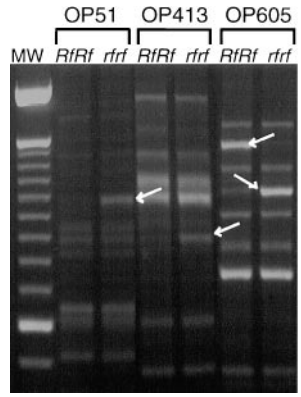

Fig. 1 RAPDs detected between *RfRf* and *rfrf* bulks. Pairs of lanes are loaded with PCR products produced with different 10-mer oligonucleotide primers : OP51, OP413 or OP605. In each pair, the *first lane* contains PCR products amplified from the homozygous restored (*RfRf*) bulked DNA, and the *second lane* contains PCR products amplified from the homozygous sterile (*rfrf*) bulked DNA. The polymorphic bands distinguishing the bulks are indicated by a *white arrow*. Three of the bands shown are associated with the *rf* allele (OP51, OP413, OP605), while one is associated with the *Rf* allele (OP605). *MW* Molecular-weight ladder (100 bp, Gibco-BRL)

Table 1 RAPD markers identified by the BSA with the operon code of the corresponding primers, their sequences and the origin of the RAPD marker

Marker	Operon code	Primer sequences	Origin of the RAPD
OP51	OPY-11	AGACGATGGG	*rf*
OP413	OPQ-13	GGAGTGGACA	*rf*
OP605	OPG-05	CTGAGACGGA	*rf* and *Rf*
OP704	OPN-04	GACCGACCCA	*Rf*

Mapping the RAPD markers with 62 sterile plants

Of all the RAPDs identified by the BSA, only OP605, because it is codominant and nicely amplified, could be mapped as a RAPD marker on our mapping population. OP51 and OP413, both linked in *cis* with the *rf* allele could not be used as RAPD markers; OP704, although linked in *cis* with the *Rf* allele, was difficult to score (data not shown). We decided to convert these three RAPD markers into reliable codominant markers, either SCAR or RFLP markers depending on their copy number. The polymorphic bands corresponding to OP51, OP413 and OP704 were cloned and then used as probes against Southern blots of petunia genomic DNA in order to determine their genomic copy number. OP704 turned out to be single copy,

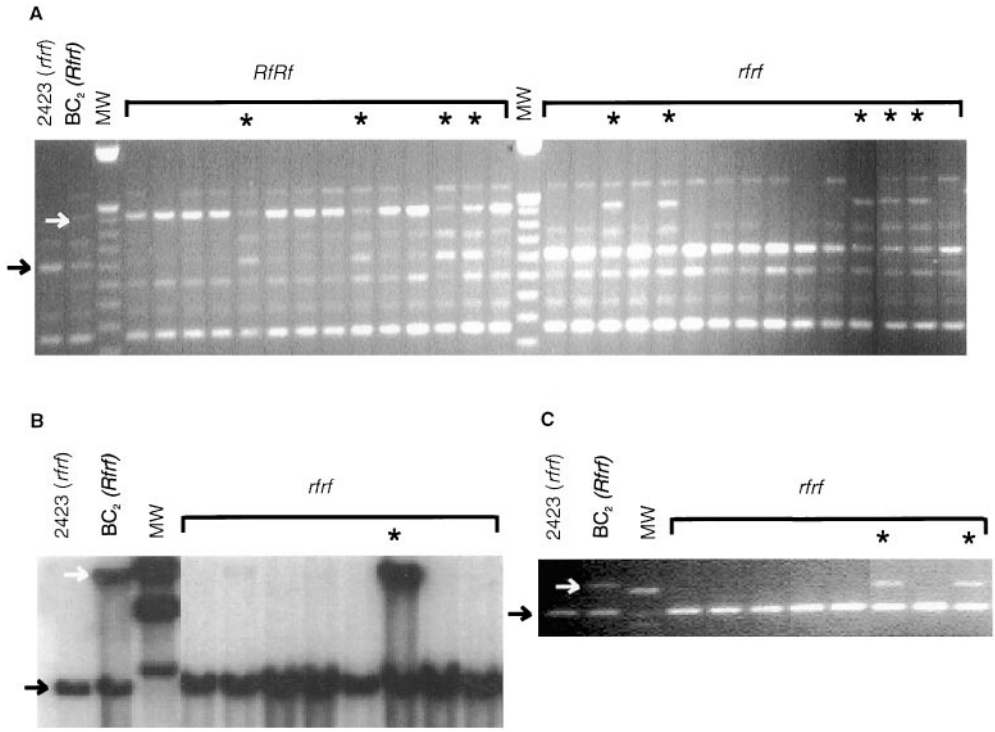

Fig. 2 A–C Segregation of molecular markers linked to *Rf*. In the three panels **A, B** and **C,** 2423 (*rfrf*) CMS is the parent used in the original cross and as a recurrent parent in our crossing scheme, BC_2 is the parent of the mapping population (cf. Materials and methods for more details). The *white arrow* indicates the allele linked to the *Rf* allele while the *black arrow* indicates the allele linked to the *rf* allele. The *asterisks* (*) indicate the heterozygous progeny. **A** Segregation of OP605 as a RAPD marker on individual plants constituting the bulks. *Below* the *RfRf bar* are the progeny constituting the *RfRf* bulk. *Below* the *rfrf bar* are the progeny constituting the *rfrf* bulk. *MW* Molecular-weight ladder (100 bp, Gibco-BRL). **B** Segregation of OP704 as an RFLP marker on a subset of the mapping population. *Under* the *rfrf bar* are sterile progeny produced from the selfing of BC_2. The *asterisk* (*) indicates the only recombinant plant found in our mapping population. *MW* Molecular-weight ladder (*λ-Hin*dIII, Gibco-BRL). **C** Segregation of OP51 as a SCAR marker on a subset of the mapping population. *Under* the *rfrf* bar are sterile progeny produced from the selfing of BC_2. *MW* Molecular-weight ladder (1 kb, Gibco-BRL)

Table 2 Codominant SCAR markers converted from RAPD markers linked to the *Rf* gene

Marker	Primer sequences	Enzyme used
OP51	GGACCAACCTTTTAATGATAGCAAC CATCGAGTAGTGCAAAACTTGTAC	*Hin*fI
OP413	TGGATGTTTGTGTGGTTGTAACTC CCTTTCTAGGTTGATTAACATGCAC	*Rsa*I

while OP51 and OP413 showed smeared hybridization signals characteristic of repetitive sequences (data not shown). After the identification of a restriction enzyme differentiating the parental alleles, OP704 was mapped as an RFLP marker (Fig. 2B). Only one recombinant out of 62 BC_2F_2 plants was found between OP704 and the *Rf* locus, resulting in an estimated distance of 0.8 cM between these 2 loci. OP51 and OP413 were converted to codominant SCAR markers by first designing primers amplifying both parental alleles and then finding a restriction enzyme cutting one parental allele but not the other one. Information on the SCAR primers and the enzymes used is given in Table 2. Segregation of OP51 as a SCAR marker is presented on a subset of the mapping population (Fig. 2C). Mapping of OP51 and OP413 as SCAR markers locates them farther away from the *Rf* locus than OP704, both of them at 7.3 cM but on opposite sides of *Rf*.

AFLP screening

Forty-nine primer combinations (PC) were used in a BSA against subsets of *RfRf* and *rfrf* bulks (cf. Materials and methods for more details). This analysis surveyed approximately 2700 bands (55 bands per PC), and identified 7 polymorphic bands between the bulks: 6 present in the *RfRf* bulk and 1 present in the *rfrf* bulk. Two of these bands were amplified with the same PC (ECCA/MACT). When tested with the individual DNAs constituting the pools, only these 2 bands appeared completely linked with the *Rf* locus (data not shown). Since these 2 bands were amplified with the same PC, were linked in repulsion (one present in the

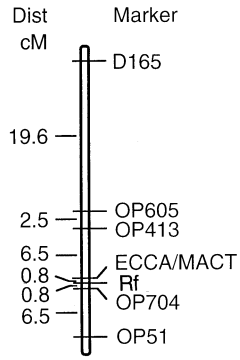

Fig. 3 Genetic map of the *Rf* region. OP markers were first identified as RAPD markers, and ECCA/MACT as an AFLP marker. D165 was found in a previous attempt to identify markers linked to *Rf* (Calfee 1996)

RfRf individuals, the other one in the *rfrf* individuals) and were very close on the polyacrylamide gel (data not shown), there was a strong likelihood that these 2 bands were allelic. This was demonstrated by cloning and sequencing these 2 bands: the allele linked to *Rf* possessed only one more nucleotide than the allele linked to *rf* and diverged from it by three substitutions. When used as a probe against a Southern blot containing petunia genomic DNA, this AFLP band appeared to be single copy. This AFLP marker designated by its PC, ECCA/MACT, was furthermore mapped as an RFLP marker on the 62 sterile plants constituting our mapping population. Only 1 recombinant was found between ECCA/MACT and the *Rf* locus. This recombinant differed from the one found between OP704 and *Rf*, thus placing *Rf* in between ECCA/MACT and OP704. Linkage analysis of the segregation data obtained with RAPD and AFLP markers resulted in the map shown in Fig. 3. The order shown on the map with *Rf* bracketed by ECCA/MACT and OP704 is the most likely order as a result of MAPMAKER computation. This order is 575 times more likely than the second possible order with *Rf* being excluded from the ECCA/MACT-OP704 interval (again a result of MAPMAKER computation).

Synteny with the tomato genome

Since tomato and petunia belong to the same family, the Solanaceae, it was worthwhile to try to map ECCA/MACT and OP704 on the tomato map. First we checked that these petunia probes could give good hybridization signals on a tomato DNA blot; then we mapped ECCA/MACT and OP704 as RFLPs on the tomato map (the tomato mapping filters were kindly provided by Steve Tanksley). ECCA/MACT and OP704 both mapped to chromosome 2 of tomato but at very different positions; ECCA/MACT was mapped to the top of chromosome 2, while OP704 was mapped to the bottom of chromosome 2 (Fig. 4). We then followed the segregation of the tomato markers in the vicinity of ECCA/MACT and OP704 on the petunia mapping population. Tomato markers close to ECCA/MACT were either monomorphic as TG31, TG189, CT251 or unlinked to *Rf* as CT106A (Fig. 4). However, some of the tomato markers linked to OP704 in the tomato genome, e.g. TG620, still showed linkage in the petunia genome (Fig. 4). Two of the tomato markers, CT24 and TG250, cosegregated with OP704 on our petunia mapping population and hence are located 0.8 cM from the *Rf* locus (Fig. 4).

Physical mapping

The relationship of genetic to physical distance was determined by digesting HMW DNA extracted from nuclei of an *Rf* progenitor with infrequent cutters and then separating the digestion products by pulsed-field gel electrophoresis (PFGE) and blotting them onto Hybond N + membrane. The same blot was hybridized with 3 of the 4 markers bracketing *Rf*; namely OP704, CT24 and ECCA/MACT (Fig. 4). TG250 was not used because of its complex hybridization pattern on petunia genomic DNA. OP704, CT24 and ECCA/MACT all recognized the same *MluI* fragment approximately 650 kb in size (Fig. 5). Given an estimated distance of 1.6 cM between OP704, CT24 and ECCA/MACT, this results in a ratio of about 400 kb/cM in the petunia genome surrounding *Rf*. OP704 and CT24, which cosegregate and map to the same side of the *Rf* locus, hybridized to the same two *Sal*I fragments approximately 200 and 150 kb in size (Fig. 5). Since the *Rf* progenitor is *RfRf* and there is no *Sal*I site in the sequences of OP704 and CT24 (data not shown), methylation sensitivity of *Sal*I could account for the presence of 2 bands. Sharing of the same 150-kb *Sal*I fragment indicates that OP704 and CT24 are at most 150 kb apart.

Discussion

In order to find markers linked to the *Rf* locus, we performed bulked segregant analysis with RAPD and AFLP markers. This strategy has already been successfully applied in numerous cases (e.g. Ballvora et al. 1995; Cnops et al. 1996). We report the identification of 2 markers, one AFLP (ECCA/MACT) and one RAPD (OP704), closely linked to the *Rf* gene. The general agreement for validating an order in mapping studies has been a LOD score of 3 (e.g. Tanksley et al. 1992). In other words, an order is definitely accepted only if it is at least 1000 times more likely than any other alternative order. The order presented in this paper with *Rf* being bracketed by ECCA/MACT and OP704 is slightly under the threshold of 3 with a LOD score of 2.76 over the alternative order (*Rf* being excluded from the ECCA/MACT-OP704 interval). A LOD score of 2.76

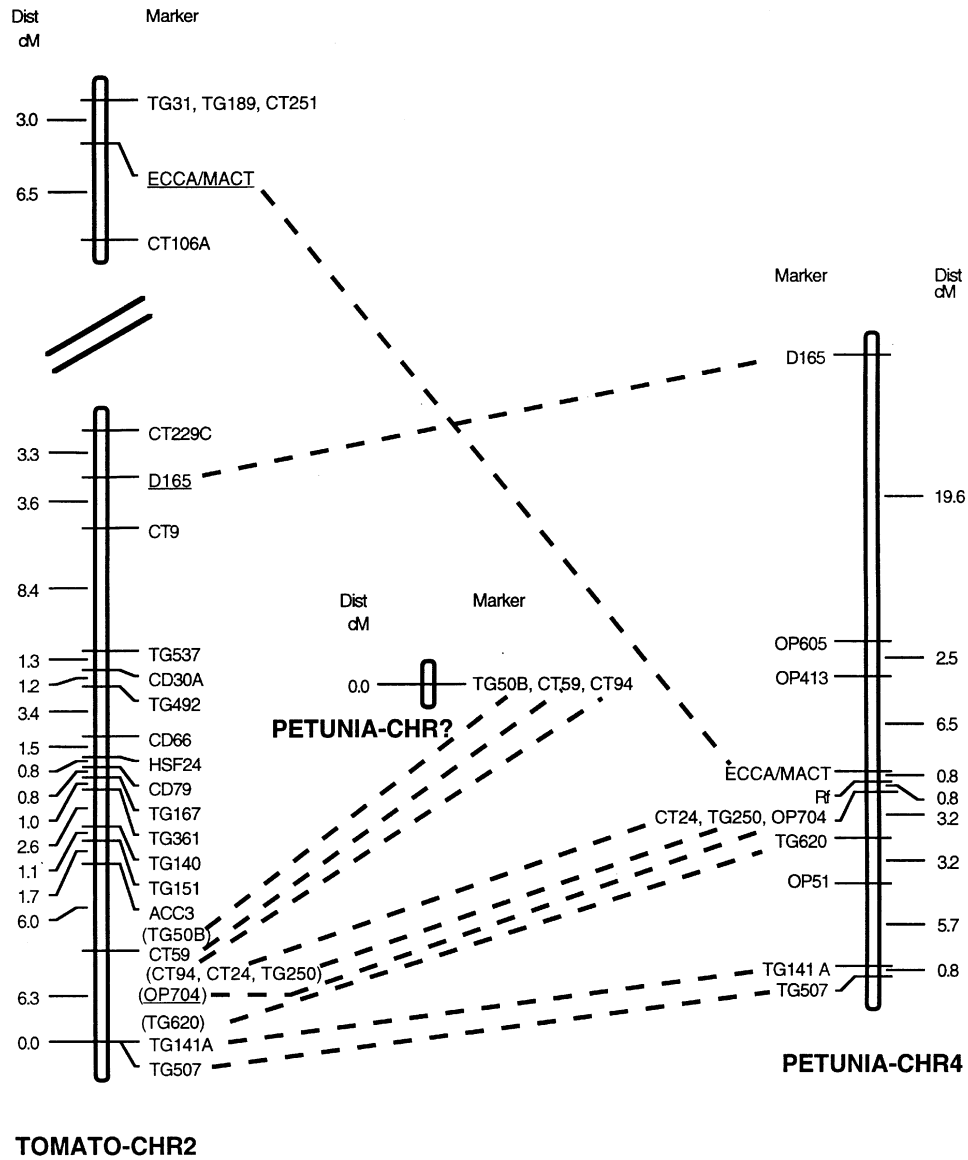

Fig. 4 Comparison of the molecular linkage maps of part of the tomato chromosome 2 (*left*, Tanksley et al. 1992), of the petunia chromosome 4 around the *Rf* locus (*right*) and of a portion of an unassigned petunia chromosome (*center*). There is a slight modification on the tomato map as shown here with the map previously published (Tanksley et al. 1992); (CT94, CT24, TG250) has been moved from the bottom of the chromosome to below CT59 (see text for more details). Markers located with LOD < 3 are enclosed in parentheses, except for *Rf* (discussed in Results and in Discussion). Markers separated by *commas* cosegregate. Petunia markers which have been added to the tomato map as a result of this study are *underlined*. *Dashed lines* bridge orthologous loci in petunia and tomato

still means that the order ECCA/MACT-*Rf*-OP704 is at least 575 times more likely than *Rf*-ECCA/MACT-OP704.

The 2 linked markers reported in this study have been identified after analyzing a total of 5925 bands. Given a genomic size of 1200 Mb for petunia (Arumuganthan and Earle 1991) and a maximum size of 650 kb between the targeting markers and assuming a random distribution of the bands analyzed, the expected number of bands falling in this interval should be about 3 ($5925 \times 0.65/1200$). The identification of 2 polymorphic bands points to an acceptable level of polymorphism around the *Rf* locus between the parental lines we used to generate our segregating population. This is fortunate because the precise origin of the restorer gene was never recorded (discussed in Izhar 1984). Petunia breeders often used wild material collected in South America. It is thus not known whether the restorer gene came from a genotype that would be recognized as a different species than the horticultural petunia, i.e. *P. hybrida*. This is the reason why we decided to maximize our chance of finding markers linked to *Rf* by comparing *RfRf* and *rfrf* bulks. This proved to be unnecessary *a posteriori* since the 2 targeting markers are both linked in *cis* with the *Rf* allele. In other words, these 2 markers would have been identified by using *Rf* and *rfrf* bulks.

The existence of linkage conservation or synteny across species has been well documented in the recent years both in monocots (Ahn and Tanksley 1993; Kilian et al. 1995) and in dicots (Tanksley et al. 1992; Kowalski et al. 1994). In the Solanaceae, tomato and potato show a very high synteny since their maps differ only by five paracentric inversions (Tanksley et al.

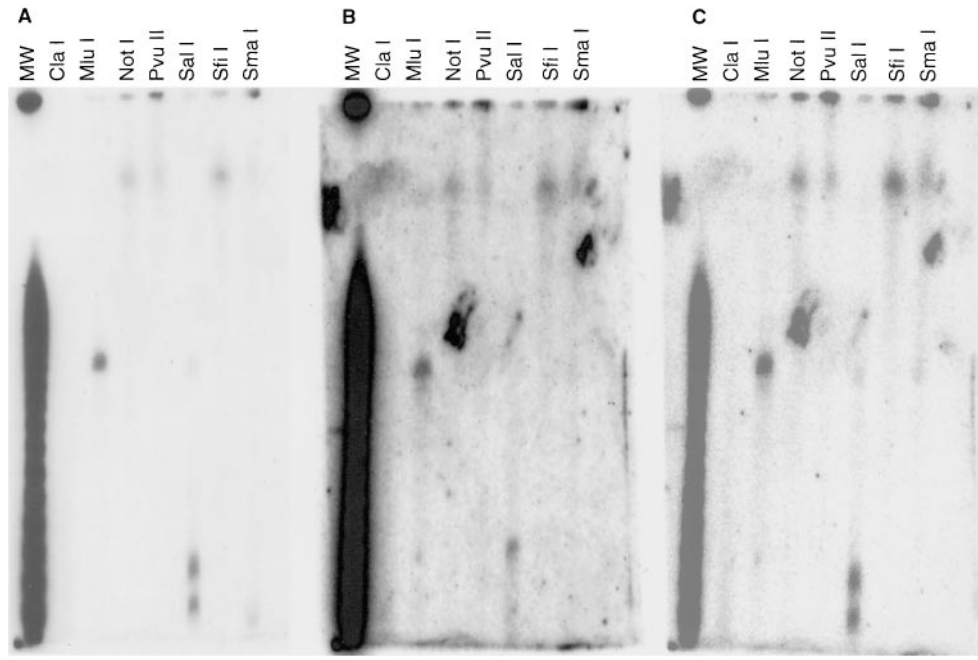

Fig. 5A–C Physical mapping of the markers tightly linked to the *Rf* gene. Three autoradiographs of the same high-molecular-weight (HMW) DNA blot hybridized with the three single-copy clones **A** OP704, **B** ECCA/MACT and **C** CT24. The HMW DNA was digested by the following restriction enzymes: *Cla*I, *Mlu*I, *Not*I, *Pvu*II, *Sal*I, *Sfi*I and *Sma*I. *MW*: λ concatemer ladder (New England Biolabs)

1992). On the other end of the synteny spectrum among Solanaceae, tomato and pepper have undergone extensive rearrangement (Tanksley et al. 1988). From the study of a small chromosomal region presented here, it seems that this applies also to tomato and petunia. An inversion of the fragment bracketed by D165 and ECCA/MACT has occurred since petunia and tomato diverged (Fig. 4). A translocation of the tomato fragment above OP704 is likely to have occurred in petunia since CT59, TG50B and CT94, although still linked together, do not show any linkage to OP704 in petunia (Fig. 4). The location of OP704 on the tomato map is not definitive. Two locations almost equally likely were found for this marker, either above TG620 (as shown in Fig. 4) or on the bottom of the chromosome. We chose to represent OP704 above TG620 because this order is also found in petunia. We also chose to move the group of cosegregating markers (CT94, CT24, TG250) from their putative location at the bottom of chromosome 2, which was acknowledged to not be definitive (Tanksley et al. 1992). Comparison with their location on the petunia genome suggests that these markers may actually reside near to CT59 and OP704 on chromosome 2 (Fig. 4). This is supported by the cosegregation in our petunia mapping population of TG250 and CT24 with OP704 and of CT94 with CT59 and TG50B.

Another interesting outcome of synteny studies is the comparison of recombination frequency for conserved intervals. Prince et al. (1993) reported an overall higher rate of recombination in a tomato interspecific F_2 cross than in a pepper interspecific F_2 cross. Our results, because they are confined to only two conserved intervals, are much less conclusive. On one hand, OP704-TG507 exhibits a higher recombination frequency in petunia than in tomato (13.1 cM vs. 4.7 cM). On the other hand, the genetic distance of D165-ECCA/MACT is smaller in petunia than in tomato (28.6 cM vs. 80 cM). The difference found in this latter comparison is likely due to further rearrangements, such as translocation or deletion between the two genomes; none of the 3 loci detected by CT106 in petunia were linked to ECCA/MACT. The comparisons made here might also be biased by the nature of the crosses performed to generate the mapping populations, intraspecific in petunia vs. interspecific in tomato. In rice, the genetic map produced from an intraspecific cross is longer than the one obtained from an interspecific cross (Kurata et al. 1994; Causse et al. 1994). Finally, the occurrence of an inversion very close to the *Rf* locus between the petunia and the tomato genomes raises the question whether this rearrangement is fortuitous or not. The absence of a CMS/restorer system in tomato might have been caused in part by a rearrangement in the vicinity of *Rf* causing the loss of this gene or of its function during speciation in tomato. This can ultimately be addressed by cloning the *Rf* gene in petunia and examining the tomato genome for an homologous sequence.

If the signal detected at 650 kb with ECCA/MACT and OP704 is not the same DNA fragment but represents a coincidental hybridization to different 650-kb *Mlu*I fragments, then our calculation of physical distance between ECCA/MACT and OP704 would be incorrect. However, CT24, a completely different

marker from OP704 and ECCA/MACT, also hybridizes to a 650-kb *Mlu*I fragment. We believe it unlikely that three different markers would by chance hybridize to different 650-kb fragments.

Positional cloning is greatly facilitated if the ratio of physical to genetic distance in the targeted area is as low as possible. Some genomic areas such as centromeres show a very suppressed recombination (Tanksley et al. 1992). Pillen et al. (1996) reported a ratio of 6800 kb/cM in the tomato *Tm-2a* region containing the centromere of chromosome 9, making the positional cloning of *Tm-2a* a very tedious and difficult task. On the other hand, extremely low ratios have also been reported for other genes, such as 43 kb/cM around the *I-2* locus in tomato (Segal et al. 1992). With an estimated ratio of 400 kb/cM in the *Rf* area, it should be feasible to select enough recombinants to identify unequivocally a large insert clone containing *Rf*. Since recombination can be affected by the type of cross used to generate the mapping population (Ganal and Tanksley 1996), it would be worthwhile to investigate the recombination frequency around *Rf* in different crosses, as the 400 kb/cM has been estimated on a relatively small segregating population produced from one cross. Not only is the ratio of physical to genetic distance relatively low around *Rf*, but our data also suggest that this area is low in repetitive DNA; the two targeting markers identified by the BSA are single-copy, while more distant markers from *Rf* (OP51, OP413, OP605) appear to contain repetitive DNA. The *Rf* gene will be easier to clone than a gene surrounded by repetitive sequence, which impairs chromosome walking.

Because no petunia YAC library is available, an alternative would be to screen the tomato YAC library (Martin et al. 1992) with CT24. This marker, like OP704, is at most 650 kb from ECCA/MACT. We could use CT24 to walk on the tomato genome towards the *Rf* homolog. The risk associated with this strategy is the inversion which took place between CT24 and ECCA/MACT (Fig. 4). Depending on where the breakage point occurred, the *Rf* homolog in tomato can either be on the top of chromosome 2 near ECCA/MACT (in this case this strategy will fail) or on the bottom of chromosome 2 still linked to CT24 (in this latter case this strategy will succeed). We therefore plan to focus future work on saturating the *Rf* region with AFLP markers by a BSA using bulks of recombinants between ECCA/MACT and OP704. Bulks developed from plants selected for recombination around the *Cf*-9 gene in tomato were used by Thomas et al. (1995) to demonstrate that this strategy could identify AFLP markers 15.5 kb apart from the targeted gene.

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