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Construction of a high-resolution genetic map and YAC-contigs in the tomato Tm-2a region

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Abstract With the ultimate goal of cloning the Tobacco Mosaic Virus (TMV) resistance gene *Tm-2a* from tomato by means of positional cloning, a high-resolution map of a 4.3-cM region surrounding the *Tm-2a* gene has been constructed. In total, 13 RFLP and RAPD markers were mapped in close proximity to *Tm-2a* using 2112 individuals from an intraspecific *Lycopersicon peruvianum* backcross. The closest flanking markers were separated from *Tm-2a* by 0.05 cM on each side. Only one marker, the cDNA clone R12, co-segregated with *Tm-2a*. In order to physically cover the *Tm-2a* region, R12 and the flanking DNA marker TG207 were used to select homologous YAC clones. To-date, two YAC-contigs spanning approximately 340 kb and 360 kb have been constructed. The data obtained from these experiments indicate that recombination around the centromere of chromosome 9 is extremely suppressed.

Key words Tomato · TMV resistance · RFLP · High resolution map · YAC contig

Introduction

The Tobacco Mosaic Virus (TMV) causes severe damage in tomato cultivation resulting in stunted plants with mottled, deformed leaves and reduced fruit yield and fruit quality (Cirulli and Alexander 1969). Three genes conferring resistance to TMV, *Tm-1*, *Tm-2* and *Tm-2a*, have been identified in green-fruited wild species of the genus *Lycopersicon* and have been introgressed into tomato cultivars (Hall 1980; Young et al. 1989). The *Tm-1* gene is located on tomato chromosome 2 and confers resistance to TMV due to an interaction with the viral replicase (Meshi et al. 1988). In contrast, *Tm-2* and its allele *Tm-2a* act on the viral movement protein, suppressing the cell-to-cell spread of the virus (Meshi et al. 1989; Citovsky et al. 1993; Weber et al. 1993). *Tm-2a* has proven to be the most stable gene and confers resistance to all known strains of TMV (Hall 1980). *Tm-2a* was introgressed into tomato cultivars from *L. peruvianum* (Alexander 1971) and is located near the centromere of chromosome 9 (Clayberg 1961; Khush and Rick 1968; Tanksley et al. 1992).

Since the introduction of positional cloning as a means to isolate genes based on their map position, more than 40 human target genes have been isolated (Collins 1995). The power of molecular cloning has been demonstrated in plant molecular biology where the first plant resistance genes have been cloned by means of either positional cloning (Martin et al. 1993; Bent et al. 1994) or transposon tagging (Jones et al. 1994; Whitham et al. 1994). In order to facilitate the positional cloning approach, Tanksley et al. (1995) introduced the concept of chromosome landing. To reduce the effort required for chromosome walking, chromosome landing places more emphasis on the selective enrichment of DNA markers within a sub-cM region around the target gene. The pool of useful marker types for this purpose includes classical RFLP markers (Botstein et al. 1980), PCR-based markers like RAPDs (Rafalski and Tingey 1993), microsatellites (Weissenbach et al. 1992) and AFLPs (Vos et al. 1996) and, in addition, expressed sequences selected by differential display (Liang and Pardee 1992; Hannappel et al. 1995). So far, the chromosome-landing strategy has led to the construction of a variety of high-resolution maps, each with molecular markers within a sub-cM distance of a target gene (Zhang et al. 1994; Alpert et al. 1995; Dixon et al. 1995; Giovannoni et al. 1995; Schumacher et al. 1995; Brommonschenkel et al. 1996).

In the present paper, we report the construction of a high-resolution map around the *Tm-2a* locus and the selec-

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tion of two YAC-contigs, together representing approximately 700 kb of the *Tm-2a* region. In future, the high-resolution map and the YAC-contigs can be utilized to ultimately clone the *Tm-2a* gene.

Materials and methods

Plant material

Two *L. peruvianum* accessions, PI 128650 and PI 128657, were crossed to generate a BC_1-population segregating for TMV resistance. A plant from accession PI 128650 was selected as the heterozygous resistant female parent (R/r) and a plant from accession PI 128657 served as the homozygous susceptible pollinator (r/r).

High-resolution mapping

Approximately 2500 BC_1 offspring seeds were germinated in small pots in the greenhouse. After 3 weeks, 2–3 leaflets per individual were collected in an Eppendorf-tube and DNA was isolated following a microprep procedure (Fulton et al. 1995). The DNA yield per individual was sufficient for 1–2 restriction-enzyme digests. Since the two markers CT215C and CD3, which were used to select recombinants in the *Tm-2a* region, could be mapped with the same restriction enzyme, only one Hybond-N^+ filter containing *Eco*RV-digested DNA of BC_1 individuals had to be produced. For filter production, probe labeling and hybridization procedure see Bernatzky and Tanksley (1986).

Individuals showing a crossing-over event between loci CT215C and CD3 were used for further fine mapping of markers placed within the *Tm-2a* region. DNA from recombinant plants was extracted and used to produce additional *Bgl*II, *Bst*NI, *Dra*I, *Eco*RI, *Eco*RV *Hae*III and *Ssp*I filters. RFLP and RAPD markers which had been previously associated with *Tm-2a* using nearly isogenic lines (Young et al. 1988; Pillen, unpublished data) were mapped after hybridization with the suitable filter containing digested DNA of the selected recombinants. RAPD markers were transformed into DNA probes prior to mapping. For this purpose, polymorphic PCR fragments were recovered from electrophoresis gels, purified in Sephadex G-50 columns, labeled and subsequently hybridized to the same filters as those used with the RFLP probes (Chunwongse et al. 1994). The primer sequences used to amplify RAPD markers are as follows (5′-3′): RAP50: CTCGGTGTAC; RAP62: TGAGCCTACA; RAP101: AGATCGCATG. The genetic distance between loci was calculated using the software program Linkage-1 (Suiter et al. 1983). Recombination frequencies were transformed into centi Morgans (cMs) after Kosambi (1944). Taking into account that each recombinant plant was selected out of 2112 BC_1 individuals, one recombination event represents a genetic distance of approximately 0.05 cM.

Test for TMV resistance

In order to place the *Tm-2a* gene within the high-resolution map, all selected recombinant BC_1 individuals were inoculated with the TMV strain U1 (Siegel and Wildman 1954) which was kindly provided by Dr. M. Zaitlin (Cornell University, Ithaca, N.Y., USA). Young leaves of 4–6-week-old plants were twice inoculated mechanically with the U1 strain (50 mg/ml) and the plants were further grown in the greenhouse with temperatures below 20°C. After 3–5 weeks, symptoms of systemic TMV infection were evaluated visually. Resistant individuals (R/r) revealed no symptoms whereas susceptible plants (r/r) were stunted and showed mottled leaflets. The presence or absence of virus in recombinant BC_1 individuals was further confirmed by a tobacco bioassay. Two- to three-weeks post-inoculation, extracts from young *L. peruvianum* leaves were applied to the TMV-resistant tobacco cultivar Samsun NN. The presence of TMV particles in the tomato leaf extract and, consequently, the susceptibility of the tomato plant, could be detected after 4 days by the occurrence of typical hypersensitive response (HR) lesions on Samsun NN (Cirulli and Alexander 1969).

YAC library screen

A tomato YAC library (Martin et al. 1992) was screened to select YAC clones homologous to DNA markers located within the *Tm-2a* region. The library contains approximately 40000 individual YACs with an average insert size of 220 kb (unpublished results). YAC clones were selected using a conventional colony hybridization protocol (Martin et al. 1992) or a PCR-based YAC selection procedure (Pillen et al., 1996). The presence of DNA markers on single YAC clones was confirmed by hybridization with intact YAC chromosomes separated by pulsed-field gel electrophoresis (PFGE) and with *Eco*RI-digested total yeast DNA, blotted onto Hybond-N^+ filters. In order to compare the genetic and physical maps around the *Tm-2a* locus, YAC end-sequences were isolated by inverse PCR (Ochman et al. 1988) and subsequently located on the high-resolution map. After sequencing and designing appropriate PCR primers, the YAC end-sequences were used to select new, overlapping YAC clones to extend the original YAC-contig.

Results and discussion

High resolution mapping

A new screening strategy for recombination events which reduces the effort of high-resolution mapping was previously described by Balint-Kurti et al. (1994). The authors used two flanking markers in a PCR-based screen to select recombinants near the tomato *Cf4/Cf9* locus. A similar, RFLP-based screen was used to construct a high-resolution genetic map around the *Tm-2a* locus on tomato chromosome 9. Recombination events near *Tm-2a* were identified from an intraspecific *L. peruvianum* backcross population which segregated for TMV resistance. The complete population was screened with two RFLP markers (CT215C and CD3) known to flank *Tm-2a*. The RFLP screen required only a minimal amount of DNA from each individual which allowed us to apply a simple and fast DNA microprep procedure (Fulton et al. 1995). Out of 2112 BC_1 individuals surveyed, 91 plants revealed a recombination event between loci CT215C and CD3. Applying the software package Linkage-1 (Suiter et al. 1983) the recombination frequency between CT215C and CD3 was calculated to be 4.3±0.4 cM.

An intraspecific *L. peruvianum* population was selected for mapping since the recombination frequency is often significantly reduced in wide tomato crosses (Rick 1969; Paterson et al. 1988; Messeguer et al. 1991; Ganal and Tanksley 1996). The suppression of recombination has also been observed in crosses which involve *Tm-2a* introgression lines (Ganal et al. 1989). As expected, the genetic size of the interval CT215C–CD3 as measured in our intraspecific *L. peruvianum* population (4.3 cM) is larger than the same interval mapped within the interspecific tomato mapping population (2.0 cM, Tanksley et al. 1992). Since the *L. esculentum* parent of the latter population included a *Tm-2a* introgression on chromosome 9, it represents an

interspecific *L. peruvianum*×*L. pennellii* cross with regard to the *Tm*-2a region. However, Frary et al. (1996) reported a distance of 7.1 cM for the CT215C–CD3 interval obtained from an *L. esculentum*×*L. pennellii* population (see Table 2). The reason for the enhanced recombination frequency in the latter population remains unclear.

In order to determine the position of the crossover events relative to *Tm-2a*, the 91 recombinants were inoculated with TMV and their resistance response was assessed visually and via a tobacco bioassay. Screening for TMV resistance in tomato is more difficult than in tobacco since homozygous resistant tomatoes show no symptoms whereas resistant tobacco plants reveal a typical hypersensitive response (HR). Furthermore, the TMV response in tomato is temperature-dependent (Schroeder et al. 1967; Cirulli and Alexander 1969). At elevated temperatures (26–28°C), tomato plants heterozygous for *Tm-2a* show necrotic leaflets which are sometimes difficult to distinguish from the mottled phenotype of homozygous susceptible plants. In order to avoid a mis-scoring of the *Tm-2a* genotype, a tobacco bioassay was performed. The genetic distances CT215C–*Tm-2a* and *Tm-2a*–CD3 were calculated as 3.0±0.4 and 1.3±0.2 cM, respectively.

Subsequently, a set of 13 *Tm-2a*-linked RFLP and RAPD markers (Table 1) were genetically separated by utilizing the 91 recombinants of the *Tm-2a* region. Figure 1 exhibits the high-resolution map resulting from the described mapping strategy. Only one marker, the 3.2 kb cDNA clone R12, co-segregated with *Tm-2a*. The adjacent markers, GP125A and a cluster of eight co-segregating markers, flank *Tm-2a* with a distance of 0.05 cM on either side. The recombination values reported here are in accordance with previous mapping results obtained within the *Tm-2a* region (Table 2).

The R12 clone was originally selected from a tomato cDNA library by low-stringency colony hybridization with the *Pto* resistance gene. The *Pto* gene product is a member of the serine-threonine protein kinase family and confers resistance to *Pseudomonas syringae* pv *tomato* (Martin et al. 1993). The map position of R12 and its similarity with the *Pto* resistance gene made R12 a possible candidate for the *Tm-2a* gene. Consequently, we tested this hypothesis by conducting complementation experiments with full-length cDNA clones of R12 and genomic clones containing R12 under the control of its endogenous promoter (Ganal et al., in preparation). However, TMV-susceptible transgenic tomato and tobacco plants containing these constructs remained susceptible. This finding shows that the R12 gene lacks the ability to induce TMV resistance in susceptible plants. Accordingly,

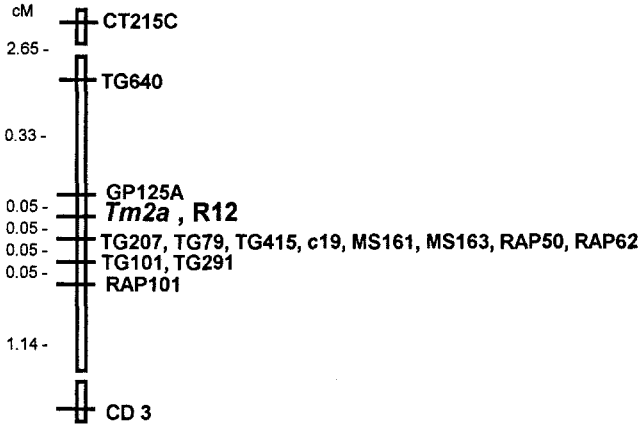

Fig. 1 High resolution genetic map of the *Tm-2a* region. Thirteen RFLP and RAPD markers were mapped close to the *Tm-2a* locus on tomato chromosome 9 (Table 1). Out of 2112 BC_1 individuals screened, only the cDNA clone R12 co-segregates with the resistance gene. The adjacent marker GP125A and the marker cluster TG207 are separated from *Tm-2a* by only one recombination event, which is equivalent to a genetic distance of 0.05 cM

Table 1 List of DNA markers mapped near *Tm-2a*

Marker	Clone type	Source	Reference
CD3	cDNA	tomato leave	Young et al. 1988
c19	cDNA	YAC yTG207.1	This report
CT215C	cDNA	tomato epidermis	This report, Frary et al. 1996
R12	cDNA	VFNT-cherry root	This report, Ganal et al. in prep.
GP125A	genomic	potato	Tanksley et al. 1992
TG79	genomic	tomato	Young et al. 1988
TG101	genomic	tomato	Young et al. 1988
TG207	genomic	tomato	Tanksley et al. 1992; Martin et al. 1992
TG291	genomic	tomato	Tanksley et al. 1992; Martin et al. 1992
TG415	genomic	tomato	This report
TG640	genomic	tomato	Tanksley et al. 1992
MS161	genomic	physically linked to tomato microsatellite	Broun and Tanksley 1996
MS163	genomic	physically linked to tomato microsatellite	Broun and Tanksley 1996
RAP50	RAPD	1.0 kb PCR fragment from Vendor-Tm2a	This report
RAP62	RAPD	0.7 kb PCR fragment from Vendor-Tm2a	This report
RAP101	RAPD	0.9 kb PCR fragment from Vendor-VFT	This report

Table 2 Comparison of genetic distances between DNA markers of the *Tm-2a* region

Marker[a]	Population[b]	Distance (cM)	Reference
CT215C-CD3	2000 BC_1 *(peru × peru)*	4.3	This report
CT215C-CD3	1600 F_2 *(esc × penn)*	7.1	Frary et al. 1996
CT215C-CD3	67 F_2 *(esc × penn)*	2.0	Tanksley et al. 1992
CT215C-TG79	2000 BC_1 *(peru × peru)*	3.1	This report
CT215C-TG79	1600 F_2 *(esc × penn)*	5.8	Frary et al. 1996
CT215C-TG79	1600 F_2 *(esc × pimp)*	3.5	Frary et al. 1996
TG79-CD3	2000 BC_1 *(peru × peru)*	1.2	This report
TG79-CD3	1600 F_2 *(esc × penn)*	1.3	Frary et al. 1996
TG79-CD3	140 F_2 *(esc × peru)*	0.9	Young et al. 1988
R12-TG79	2000 BC_1 *(peru × peru)*	0.05	This report
R12-TG79	1600 F_2 *(esc × penn)*	0.00	Frary et al. 1996

[a] Compare map positions with Fig. 1
[b] Species involved in construction of populations are: *L. esculentum (esc), L. pennellii (penn), L. peruvianum (peru), L. pimpinellifolium (pimp)*

the R12 gene is most likely not identical with the *Tm-2a* gene.

Construction of YAC-contigs

In order to cover the *Tm-2a* region with large insert clones, two YAC-contigs corresponding to markers R12 and TG207 were established (Fig. 2). An attempt to initiate a third YAC-contig with marker GP125A, which also flanks *Tm-2a*, failed to yield any homologous YAC clones. This can be explained by the existence of a predominant second locus, GP125B, on chromosome 11, for which two YAC clones were isolated. In addition, the probe GP125 is derived from a potato genomic library which might explain its reduced homology to tomato YAC clones.

The R12-contig includes three YACs (yR12-1, yR12-2 and yR12-3) and extends over approximately 340 kb. The TG207-contig contains the YACs yTG207-1 and yTG207-2 and represents a DNA segment of approximately 360 kb. The YAC end-sequences were isolated by inverse PCR (Ochman et al. 1988) and thereafter used for cross-hybridization experiments and high-resolution mapping. The two YAC-contigs could not be physically linked to one another. Moreover, both YAC-contig end-sequences co-segregated with the R12 and TG207 loci, respectively (Fig. 2). Since the R12 YAC-contig represents only a portion of the 0.05-cM DNA segment defined by the two recombination events adjacent to *Tm-2a*, it is unclear whether the *Tm-2a* gene is present on the current R12 YAC-contig (Fig. 2).

The genetic and physical data confirm that recombination in the *Tm-2a* region is highly suppressed. Based on the finding that the 340-kb R12 YAC-contig is still within the 0.05-cM genetic segment of the R12 locus, we calculated that, around the *Tm-2a* gene on chromosome 9, one cM represents a DNA segment of more than 6800 kb (=340 kb/0.05 cM). This value is in accordance with earlier cytological observations and physical mapping results from the *Tm-2a* region (Khush et al. 1964; Ganal et al. 1989). The suppressed recombination around the *Tm-2a* locus is, however, contrasted by other reports in tomato. The theoretical relation between physical and genetic distance in tomato is 750 kb/cM (Tanksley et al. 1992). Nonetheless, the actual value appears to vary considerably depending on the chromosomal region under study. Extremely low ratios are reported for the *rin* (200–300 kb/cM) and *nor* (200 kb/cM) regions on chromosomes 5 and 10, respectively, and for the *I-2* locus (43 kb/cM) on chromosome 11 (Segal et al. 1992; Giovannoni et al. 1995). To our knowledge, the ratio between physical and genetic distance in the *Tm*-2a region (>6800 kb/cM) represents the strongest suppression of recombination so far described in tomato.

Recently, DNA markers around the *Tm-2a* locus have been hybridized to a set of tomato secondary and tertiary trisomic lines in order to precisely map tomato centromeres

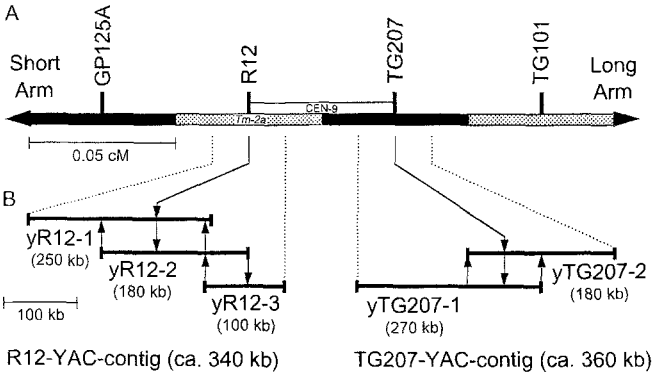

Fig. 2A, B Placement of YAC-contigs R12 and TG207 relative to the *Tm-2a* genetic high-resolution map. (**A**) Detail of the genetic high resolution map. *Gray and black* boxes represent genetic loci which are confined by recombination events and span over 0.05 cM. The centromere of chromosome 9 is located between markers TG207 and R12 (Frary et al. 1996) as indicated by the *empty box* labeled CEN-9. The possible location of the *Tm-2a* gene is implied by the *gray box* labeled *Tm-2a*. (**B**) Placement of the YAC-contigs. *Solid lines with arrows* indicate presence of RFLP markers or YAC end-sequences on YACs. *Dotted lines* indicate the approximate genetic location of both YAC-contigs. Note that, based on our genetic and physical data, it is still undecided whether or not the *Tm-2a* gene is located within the R12 YAC-contig

(Frary et al. 1996). The results indicated that the centromere of chromosome 9 is located within the 0.05-cM interval delimited by marker R12 and the TG207 cluster (Fig. 2). It is further estimated that a 5–10-fold reduction of recombination occurs near the centromeres of tomato (Tanksley et al. 1992). Thus, the severe reduction of the recombination frequency around the *Tm-2a*-R12 locus might be the result of its genomic position extremely close to the centromere.

Future work will be focused on three strategies to characterize the *Tm-2a*-bearing DNA segment and to clone the *Tm-2a* gene. First, we will extend the current R12-YAC-contig by chromosome walking until we pass the adjacent recombination events. Second, we will select additional recombinants within the GP125A–TG207 interval. The latter approach should yield recombination events which will further decrease the size of the *Tm-2a*-bearing interval and, thus, help to reduce the tedious and erroneous chromosome-walking effort. Third, the selection of new cDNA clones from the *Tm-2a*-bearing YAC-contig and the complementation results will finally verify which cDNA clone is the *Tm-2a* gene.

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