Theor Appl Genet (1997) 95: 969–974

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Molecular mapping around the centromere of tomato chromosome 6 using irradiation-induced deletions

Received: 4 March 1997 / Accepted: 2 June 1997

Abstract Irradiation-induced deletion mapping was exploited to construct a detailed locus-order map around the centromere of tomato chromosome 6 (*CEN 6*). An F_1 hybrid heterozygous for the marker loci thiamineless (*tl*), yellow virescent (*yv*) and potato leaf (*c*), and homozygous recessive for the nematode resistance gene *mi*, was pollinated with γ-irradiated pollen from cultivar VFNT Cherry carrying the wild-type alleles at the corresponding loci. A dose of 100 Gy was found optimal for inducing mutants. By screening for pseudo-dominant plants showing the marker phenotypes and/or nematode susceptibility, 30 deletions encompassing one or more of the four loci were detected in the M_1 generation. Molecular-marker analysis revealed that 29 of these mutants included the *tl* and *mi* loci on the short arm and originated from terminal deletions of different sizes. Remarkably, the breakpoints of these deletions were not randomly distributed along the short arm but located within the centromeric heterochromatin. Only one *yv* interstitial deletion and no *c* mutations on the long arm of the chromosome were detected. Mapping of the various chromosomal breakpoints in the isolated mutants permitted the resolution of a cluster of molecular markers from the centromeric heterochromatin that was hitherto unresolvable by genetic linkage analysis. The usefulness of such a deletion-mapping approach for whole-genome mapping is discussed.

Key words Tomato · Irradiation · Mutagenesis · Deletions · Mapping · Centromere

Communicated by F. Salamini

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Introduction

As with most centromeric regions, there is little recombination close to the centromeres of the tomato (Tanksley et al. 1992). Consequently, to resolve the order of loci in such regions, very large segregating populations must be screened. Morphological markers closely flanking the region of interest can be helpful in pre-selecting for crossover events within the marker interval thereby rendering the number of plants for molecular analysis manageable (Koornneef et al. 1994; Van Wordragen et al. 1994; Dixon et al. 1995; Liharska et al. 1996).

In resolving the order of loci that can not be separated by genetic-linkage analysis, mapping the breakpoints of overlapping radiation-induced mutations by means of molecular markers provides an alternative that has been widely applied to the genome of *Drosophila*, mouse, and human. Similarly, in higher plants, irradiation-induced mutations have been utilized for many years in classical genetic studies (Khush and Rick 1968; McClintock 1984) and more recently to provide starting material for the isolation of genes (Sun et al. 1992; Okubara et al. 1994; Anderson et al. 1996). Ionizing radiation is highly energetic and causes chromosome breaks leading to deletions (as well as other types of chromosomal rearrangements) that are not correlated with the recombination frequency in a particular region and, hence, allow mapping of markers that remain difficult to resolve by genetic-linkage analysis.

On the other hand, deletion breakpoints may not be distributed randomly along the chromosome, as shown by Khush and Rick (1968). The availability at present of a rich source of molecular markers allows a much more detailed analysis of the distribution of breakpoints than was feasible in the days of Khush and Rick.

In the present study, we have investigated the potential of γ-irradiation to resolve the order of molecular markers around the centromere of chromosome 6 (*CEN 6*) using a combined genetic and morphological approach. This region is of great interest because it contains important disease resistance-genes such as *Mi-1*, *Cf-2* and *Cf-5*, *Meu-1* and the virus-tolerance gene *Ty-1* (Messeguer et al. 1991; Ho et al. 1992; Dickinson et al. 1993; Zamir et al. 1994; Kaloshian et al. 1995). Our previous attempts to construct a high-resolution molecular-linkage map of this region, which is flanked by the morphological markers *tl* (*thiamineless*) and *yv* (*yellow virescent*), have been hampered by the very low recombination frequency within this specific interval (Liharska et al. 1996). Cytogenetic analysis of *tl* and *yv* pseudo-dominant mutants by Khush and Rick (1968) showed that these two loci reside on different sides of the centromere. Here we show that deletion mapping provides an effective alternative to ordering markers in an otherwise recombinationally silent region of the chromosome.

Materials and methods

Plant material

The female parent (*Tl*/*tl*, *Yv*/*yv*, *C*/*c*, *mi*/*mi*) used in crosses with irradiated pollen was a hybrid between W607 and the cultivar Moneymaker. The homozygous marker line W607 (*tl*/*tl*, *yv*/*yv*, *c*/*c*, *mi*/*mi*) could not be used for the present experiments because it is a poorly growing plant with a low seed setting (Liharska et al. 1996). Tomato line VFNT Cherry (LA1221) carrying dominant alleles at the tester loci *tl*, *yv*, *c* and *mi* was used as a pollen donor. This line carries a *Lycopersicon peruvianum* introgression covering the short arm, CEN 6, and the centromere-proximal part of the long arm (Fig. 2; Messeguer et al. 1991; Ho et al. 1992) . The parents and the offspring were grown in a greenhouse in Wageningen during the years 1994 and 1995.

Mutagenesis

Using an "electric bee", mature pollen from VFNT Cherry plants was collected in a glass container, which was then sealed with parafilm and exposed to a γ-radiation (Co^{60}) source at the Institute for Agrotechnological Research in Wageningen, with a dose rate of 14.8 Gy/s. The duration of the exposure and the distance from the radiation source were varied in different experiments to provide four different total doses of radiation. The pollen was applied immediately after irradiation to stigmata of flowers emasculated 0–2 days before.

Phenotypic selection for mutants in M_1

M_1 seeds from each fruit were sown separately in trays and 3–4 weeks later the seedlings were scored for the *tl*, *yv* and *c* phenotypes. At this stage, the M_1 plants were transferred to pots with sandy soil and tested for resistance/susceptibility to root-knot nematodes.

Nematode test

Second-stage juveniles (J2), from a *Meloidogyne incognita* population grown on susceptible tomato plants, were kindly provided by Mr. Jan van Bezooyen (Department of Nematology, WAU). Between 1500 and 2000 J2s were applied per pot in holes made around the roots of each M_1 plant and several parental-line plants (VFNT Cherry and the W607 × Moneymaker hybrid) that served as controls. Each time the nematode test was performed care was taken, at least during the first 3 days after inoculation, to keep the soil temperature below 28°C and the moisture content optimal.

Six weeks after inoculation, the roots of the plants were inspected for the presence of galls. In our hands the root-knot nematode-resistant test always gave clear plus/minus results in that the roots of the susceptible plants were studded with galls and the roots of resistant plants were clean.

DNA isolation and molecular markers

For Southern analysis, plant DNA was isolated from leaves as previously described (Van der Beek et al. 1992). PCR analysis was carried out on DNA samples prepared as described by Wang and Cutler (1993). The SCAR marker REX-1 was developed by Williamson et al. (1994); SCAH13 is a SCAR marker developed by R. Weide (personal communication). For the *Aps-1* marker primers were designed using the sequence information from a genomic *Aps-1* clone (Aarts et al. 1991); forward primer: 5′-ATGGTGGGTCCAG-GTTATAAG-3′ reverse primer: 5′-GATTGGCACAAGCTCAT TCTG-3′. The amplified fragments were restricted with *Taq*I to reveal a *L. esculentum*/*L. peruvianum* polymorphism. The sources of the RFLP markers CD14, GP79, GP164, H4H10, H5G4, H6A2c2, LC216, RC8 and TG231, used in this work, were described by Weide et al. (1993).

Results and discussion

The root-knot nematode-susceptible (*mi*/*mi*) hybrid (W607 × Moneymaker), which is heterozygous for the morphological markers *tl*, *yv* and *c* on chromosome 6, was pollinated with γ-irradiated pollen from the nematode-resistant (*Mi*/*Mi*) line VFNT Cherry carrying the wild-type alleles of the marker genes. Gamma irradiation-induced mutations affecting the wild-type alleles were identified on the basis of pseudo-dominance of one or more of the four phenotypic markers *tl*, *yv*, *c*, and *mi*.

Effect of the dose of irradiation on the yield of mutants

To establish an irradiation dose with a high mutagenic effect, but without deleterious effects on the M_1 seed set and seed germination, we varied the irradiation dose around the effective dose of 50 Gy reported in the literature (Khush et al. 1964; Van Wordragen et al. 1994). Furthermore, different doses of radiation can be expected to induce different spectra of chromosomal

Table 1 Number of pseudo-dominant, γ irradiation-induced mutants induced at the chromosome-6 loci *tl*, *yv*, *c* and *Mi* in two M_1 populations. In experiment I only the *tl* plants were tested for nematode resistance, as were all plants in experiment II. nd = not done

Dose (Gy)	Experiment I					Experiment II						Total number of mutants
	Number of M_1 plants	Mutants				Number of M_1 plants	Mutants					
		tl	*yv*	*c*	*tl*, *mi*		*tl*	*yv*	*c*	*mi*	*tl*, *mi*	
50	2277	0	0	0	5	1359	1	1	0	5	2	14
75	nd	–	–	–	–	515	0	0	0	1	2	3
100	330	0	0	0	4	559	1	0	0	3	5	13
125	nd	–	–	–	–	12	0	0	0	0	0	0
Total	2607	0	0	0	9	2445	2	1	0	9	9	30

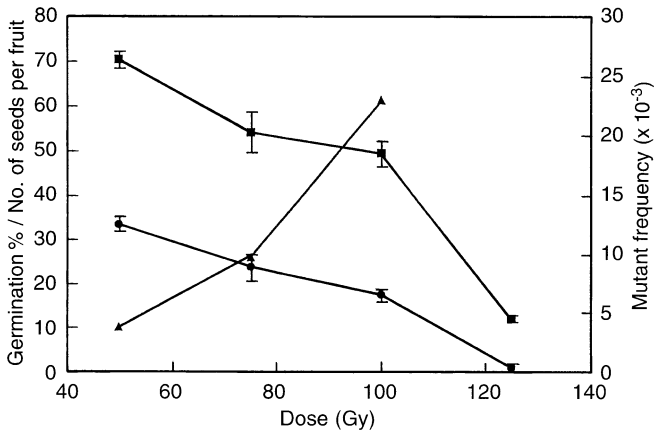

Fig. 1 Effect of the dose of γ-irradiation (*Gy*) on the seed setting (number of seed per fruit, *circles*), germination rate (%, *squares*) and on the frequency of mutation for the *tl* locus (per total number of scored M_1 plants, *triangles*). *Vertical bars* represent standard errors of the means

mutations. For example, at higher doses the probability of inducing double breakpoints by two hits within a small distance on the chromosome will be higher. As a result, at high doses a higher number of interstitial deletions is expected compared to terminal deletions. Four different doses of γ-irradiation varying from 50 Gy to 125 Gy were applied (Table 1, Fig. 1). An increase of the dose was associated with a consequent drop in the number of M_1 seeds per fruit and the germination percentage of the seeds (Fig. 1). Pollination with VFNT Cherry pollen that had been treated with 125 Gy resulted in such a low seed setting and germination that only 12 M_1 plants were obtained (Table 1). A comparison between the effects of radiation doses of 50 Gy and 100 Gy revealed that, although the same number of flowers were pollinated and a similar number of mutants was obtained, the total number of M_1 plants to be screened differed significantly (Table 1). As a result, the calculated mutation frequency was six-times higher at 100 Gy than at 50 Gy. With the highest mutation frequency at the investigated loci and relatively good seed set and germination rate (Fig. 1), a dose of 100 Gy appeared optimal for producing pseudo-dominant mutants.

Isolation of the mutants

A total of 5052 M_1 plants from the cross of F_1 (W607 × Moneymaker) × VFNT Cherry (pollen-irradiated) were examined for pseudo-dominance of the morphological markers *tl*, *yv* and *c*, amongst which 20 phenotypically *tl* individuals (subsequently referred to as "mutants") and one *yv* mutant were recovered (Table 1). No plants showing the *c* phenotype nor *tl*, *yv* double mutants, were found. Of the same M_1 plants, 2445 individuals were subjected to a nematode-resistance test yielding nine nematode susceptible mutants. No phenotypes with intermediate nematode susceptibility were observed in this M_1 generation.

The closely linked loci *tl* and *Mi* on the short arm of chromosome 6 (Van Wordragen et al. 1994, 1996) showed similar mutant frequencies per chromosome, 7.9×10^{-3} and 7.4×10^{-3} respectively. When the *tl* mutants were subjected to a nematode-resistance test, all but two turned out to be nematode-susceptible indicating that the mutation included both the *tl* and *Mi* loci. This was later confirmed by molecular-marker analysis.

For the *yv* locus, a relatively low mutation frequency of 0.4×10^{-3} was found, suggesting that only rather small interstitial deletions of the long arm of chromosome 6 are viable or had been transmitted through the pollen (Khush and Rick 1968). The total absence of *c* mutants in our M_1 population is difficult to explain. The *c* gene is located on the distal end of the long arm of the chromosome (see Fig. 2 A) and has been shown to be encompassed by terminal deletions (Khush and Rick 1968) though at a low frequency (Van Wordragen et al. 1996).

As not a single pseudo-dominant *tl*, *yv* double mutant was found among the 5052 M_1 individuals,

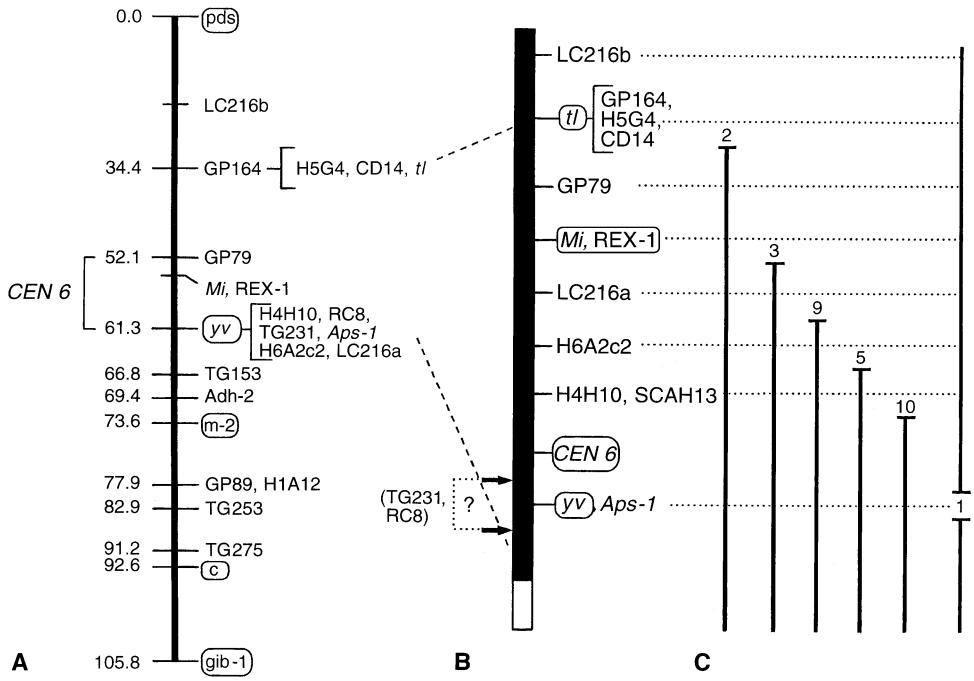

Fig. 2 **A** Integrated classical and molecular map of chromosome 6 after Weide et al. (1993). **B** Molecular marker order as established by mapping of deletions induced on VFNT Cherry pollen. The *black bar* indicates the extent of *L. peruvianum* introgression in VFNT Cherry. **C** The *ciphers* indicate the number of individual mutants of this kind

deletions including *CEN 6* are clearly not viable (Khush and Rick 1968).

Molecular-marker analysis of the mutants

Molecular markers polymorphic for *L. esculentum*/*L. peruvianum* were used to verify the genetic background of each of the mutant plants and to define the extent of the deletions. All 29 *tl* and *mi* pseudo-dominants were found by PCR analysis to contain the *L. peruvianum Aps-1*[1] allele, which resides on the long arm of chromosome 6 in tight linkage to the *yv* locus (Fig. 2A) (Khush and Rick 1968; Van Wordragen et al. 1996). The selected *yv* mutant was tested with markers REX-1, LC216 and H5G4 that are located on the short arm of chromosome 6. Indeed, the *yv*-deletion plant showed the presence of *L. peruvianum* alleles of these loci (Fig. 2 B), indicating that this plant carries an interstitial deletion.

The nature and the extent of the mutations was then determined by analyzing the 29 *tl* and/or *mi* plants with appropriate RFLPs and SCARs from the short arm of chromosome 6 (Fig. 2 B). None of the mutant plants carried the *L. peruvianum* allele of the most distal marker LC216b and of the *tl*-linked marker H5G4, suggesting that they all contain terminal deletions of the short arm of chromosome 6. The two *tl* plants which were nematode-resistant displayed the *L. peruvianum* alleles for the other tested molecular markers on the short arm. Analysis of the *tl* and *mi* plants with the markers located proximal to the *CEN 6* revealed deletions of various sizes, with most of the breakpoints (ten) near *CEN 6*. The markers LC216a and H4H10 have hitherto been found linked to the *yv* cluster (Ho et al. 1992; Van Wordragen et al. 1996) suggesting a location below *CEN 6* on the long arm (Van Wordragen et al. 1994). Since a number of *tl* and *mi* deletion plants from our study were lacking the *L. peruvianum* allele of LC216a and H4H10 (Fig. 2B), these markers are actually located on the short arm and not on the long arm as suggested by linkage analysis. Clearly, the deletion- mapping approach was successful in resolving the order of markers that were refractory to mapping by genetic-linkage analysis. The RFLP marker H6A2c2 was also resolved from the *yv* cluster (Weide et al. 1993) and found to be located on the short arm of chromosome 6, in agreement with recent data from Van Wordragen et al. (1996). The marker SCAH13b, that was originally identified as centromere-linked (Rob Weide, in preparation), was deleted in the same plants in which H4H10 was deleted.

In the *yv*-deletion mutant, only one marker (*Aps-1*) from the *yv* cluster did not show the *L. peruvianum* allele. Apparently, this plant carries a small interstitial deletion on the long arm of chromosome 6. The other markers from the cluster that were tested, RC8 and TG231, can be located on either side of the *yv-Aps*-1 loci.

In summary, most of the mutants obtained by pollen irradiation have originated from terminal deletions of various size with breakpoints not being randomly distributed (Fig. 2 C). No correlation was found between the applied dose of radiation and the type of mutation

and/or the location of the deletion breakpoints. The majority of deletions included both loci, *tl* and *Mi*, and the *Mi*-linked marker REX-1. Recent cytogenetic studies in our laboratory indicate that REX-1 is located on the short arm of chromosome 6 within the euchromatin, but very close to the border of the centromeric heterochromatin (Zhong et al., in preparation). This finding, in combination with the low recombination frequency encountered between REX-1 and *CEN 6* (Liharska et al. 1996), suggests that the markers LC216a, H6A2c2, SCAH13b and H4H10 must be located in the heterochromatin. Thus, most of the induced deletions in chromosome 6 are the result of breakpoints within the heterochromatin on the short arm. This non-random distribution of viable breaks has been earlier described for X-ray mutagenesis (Khush and Rick 1968). The apparent preference for breaks within the centromere-proximal heterochromatin should be viewed in context with the question as to how chromosomes with terminal deletions can survive without a proper end, the telomere (Blackburn 1991). Possibly, the terminal deletions originate from two breaks, one near but not including the telomere. Alternatively, a mechanism may exist to heal the broken ends of the chromosome by the *de novo* addition of telomeric sequences, as shown for wheat (Werner et al. 1992). Heterochromatin might be the place where such a mechanism operates most efficiently.

Transmission of mutations

All 30 deletion-containing plants from this study produced flowers, with the single *yv* mutant as well as 18 of the 29 *tl* and *mi* plants providing viable seeds. M_2 generations of these mutants were raised, consisting of 40–100 individuals. In the progenies of the *tl* mutants only plants with a *tl*/*yv* phenotype were found, indicating that none of the deletions had been transmitted. In the M_2 generations of four *mi* mutants 6–10 plants were selected for molecular marker analysis. Tests with the PCR markers REX-1, SCAH13b and *Aps-1* revealed that in all plants only the *L. esculentum* alleles of these markers were present, indicating the absence of the mutated chromosome 6. These results support previous findings in maize, tomato, *Petunia* and *Arabidopsis* showing that the transmission of chromosomes with large terminal deletions is very low (Stadler and Roman 1948; Khush and Rick 1968; Gerats et al. 1984; Timpte et al. 1994; Vizir et al. 1994).

The M_2 progeny (146 individuals) of the *yv* mutant consisted of 109 *yv* and 37 *yv*, *tl* plants indicating a normal 3:1 segregation ratio ($\chi^2_{3:1} = 0.01$, $P > 0.05$) and thus a normal transmission of the chromosome carrying the deletion. Ten randomly selected *yv*-progeny plants tested with the *Aps-1* marker appeared to contain the *L. esculentum* allele of *Aps-1*, showing they were heterozygous for the *Yv* deletion. However, due to the relatively small sample tested, we can not exclude the possibility that genotypes homozygous for the *yv-Aps-1*^{1} deletion are viable.

Conclusions

This report describes the induction of deletions in a specific region of the tomato genome and their use for fine genetic mapping with molecular markers. The application of different doses of γ-radiation resulted in the same type of mutations; that is mainly large terminal deletions with breakpoints predominantly located in the pericentromeric heterochromatic region. The number of deletion mutants isolated in this study was helpful in resolving a cluster of molecular markers that may serve in the molecular cloning of *CEN 6*. On the other hand, the highly non-random distribution of the deletion's breakpoints will be a serious limitation in constructing a detailed locus-order map in other regions of the plant genome.

Acknowledgments We thank Jan van Bezooyen for supplying the nematodes and Henk Kuiper and his greenhouse staff for their concern and help which was instrumental to these experiments.

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