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Effect of radiation dose on the production of and the extent of asymmetry in tomato asymmetric somatic hybrids

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Summary. Asymmetric somatic hybrids were recovered following fusion of tomato leaf mesophyll protoplasts with irradiated protoplasts isolated from *Lycopersicon pennellii* suspension cells. The asymmetry was determined by scoring the regenerants at between 20 and 24 loci using isozymes and restriction fragment length polymorphisms. In addition, three quantitative traits, fruit size, leaf shape, and stigma exsertion, were measured in the regenerants. The recovery of asymmetric somatic hybrids was as high as 50% of the regenerants, and there was no requirement for the transfer of a selectable marker gene from the irradiated partner. The amount of nuclear DNA transferred from the irradiated protoplast fusion partner was found to be inversely proportional to the radiation dose. It was possible to recover tomato asymmetric somatic hybrids which were self-fertile and contained limited amounts of genetic information from *L. pennelli*.

Key words: Asymmetric somatic hybrids – Tomato – *Lycopersicon* – Gamma radiation

Introduction

Theoretically, somatic hybrids are tetraploid and contain complete nuclear genomes from both of the fusion partners. To be valuable in a breeding program, these plants must be reduced to diploid and backcrossed with the cultivar of choice. If irradiated protoplasts were used as fusion partners, then one could expect that the regenerated plants would be diploid and contain only small fragments of genetic information contributed by the irradiated protoplast from the wild species. Transformation rates are increased in frequency by treatment with DNA damaging agents, i.e., ionizing radiation (Kohler et al. 1989, 1990). By exploiting random recombination events between the fragmented genome of the wild species with the intact genome of the cultivar, one could introduce fragments of chromosomes from another plant species. Nitrate reductase mutants have been complemented with wild-type genes from irradiated protoplasts (Gupta et al. 1982; Somers et al. 1986). Gupta et al. (1984) have evidence of alien chromosome addition lines following the fusion of irradiated protoplasts with untreated protoplasts. Movement of selectable markers from irradiated protoplast donors has been achieved between species of *Brassica* (Sacristan et al. 1989; Yamashita et al. 1989), species of *Nicotiana* (Bates et al. 1987; Famelaer et al. 1989; Piastuch and Bates 1990), and from carrot to tobacco (Dudits et al. 1987). Asymmetric somatic hybrids between *Lycopersicon* species have also been described; these hybrids were recovered by requiring the transfer of genes controlling regeneration potential from the irradiated parent (Wijbrandi et al. 1990). Disease resistance traits from sexually incompatible species have been introduced into crop plants using somatic hybridization (Austin et al. 1985; Helgeson et al. 1986) or asymmetric somatic hybridization (Sjodin and Glimelius 1989).

We have constructed asymmetric somatic hybrids between tomato cv 'UC82' and the wild species, *Lycopersicon pennelli* LA716. We chose to use these particular genotypes for two reasons: first, the molecular map of tomato was constructed based on RFLPs between these two species; therefore, we had a large number of molecular markers to use to determine the extent of heterozygosity in the asymmetric somatic hybrids. Secondly, there are many valuable traits in *L. pennelli* which have not

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been exploited in commercial tomato cultivars: insect resistances (Goffreda and Mutschler 1989), disease resistances (Bournival et al. 1990), as well as abiotic stress tolerances like drought tolerance (Rick 1973), salt tolerance (Tal and Shannon 1983), and water use efficiency (Martin and Thorstenson 1988).

Materials and methods

Plant material, protoplast isolation and treatments

Seed of *Lycopersicon pennellii*, LA716, were generously provided by C. Rick, Tomato Genetics Stock Center, University of California, Davis; seed of *L. esculentum*, cv 'UC82', a widely grown processing tomato, were obtained from PetoSeed Co. Plants of 'UC82' were grown from seed in a growth chamber, 17 h light, 24 °C, 7 h dark, 19 °C, and watered daily with Nitsch salt solution (Nitsch 1965). Protoplasts were isolated from leaves of 4-week-old 'UC82' plants as described by Tan et al. (1987a). The leaf mesophyll protoplasts were treated with 3 m*M* iodoacetamide for 20 min at 4 °C, followed by three washes with W5 salts (Bonnema et al. 1990).

Suspension cell cultures derived from callus initiated from hypocotyls of *L. pennellii* were maintained on UM1a (Uchimaya and Murashige 1974) and subcultured weekly. New callus cultures were initiated throughout these experiments such that all of the fusion experiments were performed with a cell culture 21–22 weeks old. Radiation treatments and/or protoplast isolations were performed 4 days after subculture. Protoplasts were isolated by digestion with 6 vol of enzyme solution [CPW salts (Bonnema et al. 1990), 9% mannitol, 1% cellulysin, 0.5% pectinol, 0.25% rhozyme, 0.05% pectolyase, 12.5 mg/ml fluorescein-isothiocyanate, 5 m*M* 2-(*N*-morpholino) ethanesulfonic acid (MES), pH 5.8] for 16 h in the dark at room temperature with shaking. Further isolation was as described by Tan et al. (1987b). Radiation treatments were conducted on freshly isolated protoplasts or intact suspension culture cells. For radiation treatments, protoplasts were resuspended in W5 plus 5 m*M* MES; suspension cell cultures were harvested and then resuspended in approximately 2 vol of fresh UM1a. Samples were placed on ice and subjected to 5–100 krads ^{60}Co gamma radiation at dose rates between 1.9 and 35.4 rads/sec (Table 1). Irradiated protoplasts were immediately used in fusion experiments, while irradiated cells were digested overnight and used in fusion experiments the next day.

Protoplast fusion, culture and regeneration

Protoplasts of 'UC82' and *L. pennellii*, treated as described, were mixed in a 1 : 1 ratio at a final concentration of 4×10^6 protoplasts/ml in W5 salts. Fusion was performed as described by Menczel and Wolfe (1984) with the modification that the fusion solution contained 2 m*M* mannitol and 75 m*M* $CaCl_2$. After fusion, protoplasts were pelleted, washed in CPW salts plus 15% sucrose, and the fraction which floated was collected and washed with CPW salts plus 2% KCl. The pelleted protoplasts were resuspended in protoplast culture medium. Protoplasts were plated at a density of 1×10^5 cells/ml and cultured and plants regenerated as described earlier (Bonnema et al. 1990; O'Connell and Hanson 1985).

Isozyme analysis and cytology

Approximately 0.5 g (wet weight) of leaf tissue was ground in 0.12 *M* reduced glutathione and adjusted to pH 7.6 with 1 *M* TRIS. Samples were absorbed into paper wicks and electrophoresed on starch gels and then stained for phosphoglucomutase (Pgm) and phosphoglucoisomerase (Pgi) activities as described by Vallejos (1983). Root tips were collected and incubated for 4 h at 18 °C in 2 m*M* 8-hydroxyquinoline. The tissue was hydrolyzed for 20 min in 4 *N* HCl at room temperature, rinsed with water and kept at 4 °C in water overnight, stained with acetocarmine, and squashed. Viable pollen was determined after staining as described by Alexander (1969).

Table 1. Description of the fusion experiments. In all of the experiments freshly isolated leaf mesophyll protoplasts from UC82 were fused with protoplasts isolated from a suspension culture of *L. pennellii*. The radiation dose received by the cells and the cell type irradiated, as protoplasts or as suspension cells, is indicated below. Irradiated protoplasts were fused on the day of irradiation; irradiated suspension cells were digested overnight and fused the day after irradiation

Dose (krads)	Dose rate (rads/sec)	Cell type irradiated	Number of calli recovered	Regeneration frequency (%)
5	16.1	Cell	>200	38
10	16.1	Protoplasts	23	9
10	16.1	Cells	>200	>50
15	16.1	Protoplasts	28	14
15	16.1	Cells	>200	48
25	16.1	Protoplasts	35	17
25	16.1	Cells	117	34
50	35.4	Protoplasts	52	15
100	1.9	Protoplasts	>200	>50

DNA isolation, and Southern analysis

Total DNA was extracted from fresh leaf tissue as described by Doyle and Doyle (1989) with the addition of a single CsCl centrifugation after the isopropanol precipitation. Restriction enzyme digests were of 10 μg of total DNA for at least 4 h at 37 °C. The methods for electrophoresis, Southern transfer, hybridization, and washes have been previously described (Melzer et al. 1989). Blots containing DNA isolated from 16 individual regenerants from each of the five fusion combinations and digested with either EcoRI, EcoRV, DraI, or HindIII were prepared. Each blot also contained DNA from the two parents and the interspecific sexual hybrid. The nuclear DNA composition of regenerants was analyzed using gel-purified fragments of mapped tomato cDNA (Bernatzky and Tanksley 1986) and total genomic clones (Mutschler et al. 1987; Zamir and Tanksley 1988) as probes. Probes were oligolabelled with α^{32}P-dCTP as described by Feinberg and Vogelstein (1983).

Results

The design of the construction of tomato asymmetric somatic hybrids utilized a double inactivation to prevent the growth of both unfused cells and of fusion products forming homokaryons. The protoplasts isolated from 'UC82' were treated with iodoacetamide, usually 3 m*M*, prior to fusion. In earlier experiments we had established that exposure of *L. pennellii* protoplasts to doses above 5 krads prevented growth of cells to a callus stage (O'Connell and Hanson 1985, 1987). Cultures of

Table 2. Description of the fusion products recovered in unselected populations of fusion products as a function of radiation dose received by the fusion partner *L. pennellii*. Leaflet ratio is expressed as length/width, and fruit size is the length in the longest dimension. The values of these traits for the parents and the interspecific sexual hybrid are also listed. Plants were named asymmetric somatic hybrids (aSH), full somatic hybrids (SD), *L. esculentum* (E), or as *L. pennellii* (P) following scoring of between 20 and 24 loci as described in the text. The number of plants characterized are indicated in parentheses

Dose (krads)	Stigma exsertion (mm)	Leaflet ratio	Fruit size (cm)	%SH	%aSH	%E	%P
5 (16)	1.33±1.19	1.04±0.13	1.53±0.58	94	6	0	0
10 (16)	1.20±1.68	1.03±0.12	1.65±0.71	100	0	0	0
15 (17)	2.16±3.05	1.17±0.12	1.61±0.72	70	30	0	0
25 (17)	0.23±0.61	1.29±0.30	1.87±0.96	53	47	0	0
50 (4)	0.00	1.97±0.45	3.57±0.40	0	50	50	0
100 (20)	0.30±0.48	1.61±0.15	4.21±0.60	0	30	70	0
UC82	0.00	2.10±0.08	4.70±0.34				
F_1	1.50±0.08	1.35±0.05	2.20±0.16				
L. pennellii	2.50±0.14	0.98±0.09	1.50±0.14				

parental protoplasts treated in this manner are unable to grow; following fusion of these treated protoplasts, however, heterokaryons should be able to grow and form callus and eventually regenerate. In order to determine the dose of radiation that was optimal for the recovery of nuclear asymmetric somatic hybrids we fused iodoacetamide-treated leaf mesophyll protoplasts from 'UC82' with *L. pennellii* suspension cell protoplasts that had been irradiated with either 5, 10, 15, 25, 50, or 100 krads of gamma radiation from ^{60}Co. We recovered calli and regenerated plants from all the fusion combinations described (Table 1). We were able to recover reasonably large numbers of calli with equally high frequencies of regeneration. Therefore, we expect that the 16–20 plants selected randomly for detailed genetic analysis represent the range of effects caused by the various doses of radiation.

Characterization of the regenerated fusion products

To determine the extent of hybridity in the regenerated plants, three types of analyses were performed. Initially, as the plants first regenerated, they were scored at two loci using isozyme activities, Pgm-2 and Pgi-1 (Tanksley et al. 1982). As the plants grew large enough for transfer to the greenhouse, three quantitative traits were measured – leaflet ratio, the length of the exserted stigma, and the size of the fruit – and compared to these characters in the parents, the sexual hybrid (Tal 1967; Tanksley et al. 1982), and in well-characterized somatic hybrids (O'Connell and Hanson 1987; O'Connell et al. 1986). And finally, on 16–20 selected individuals for each fusion combination, total DNA was isolated and the genotype at between 20 and 24 loci was scored using restriction fragment length polymorphisms around cDNA and cloned genomic DNA loci (Bernatzky and Tanksley 1986; Mutschler et al. 1987). Using this type of an approach we have been able to demonstrate the extent of asymmetry in two somatic hybrids which had lost *L. esculentum* chromosomes (Melzer and O'Connell 1990).

Quantitative traits

Within each group of regenerants for a particular fusion combination there was a large range of expression for each of the three quantitative traits measured as evidenced by the large standard deviations for these measurements (Table 2). However, there is still a trend for the quality of the trait to resemble the 'UC82' parent more strongly as the dose of radiation received by the *L. pennellii* protoplast fusion partner increases. Within each population, there are individuals with relatively large fruit and an exserted stigma, or large fruit and more rounded leaflets, indicating that these traits are transferred following fusion independently of one another.

Analysis of molecular markers

The molecular map of the tomato genome has enabled us to readily score the extent of hybridity in the regenerants. The 35 molecular loci used in this study were: chromosome 1, CD15, CD24, CD28; chromosome 2, CD35, CD37, Rbcs-1, Cab-1; chromosome 3, Rbcs-2, CD6, CD13, CD31; chromosome 4, CD39, TG15, Pgm-2; chromosome 5, CD31, CD41, TG69; chromosome 6, TG54, TG115, CD13, CD14; chromosome 7, TG13A; chromosome 8, CD40; chromosome 9, CD8, CD32B, TG18; chromosome 10, CD32A, CD38A, TG63; chromosome 11, TG30, TG46; chromosome 12, CD2, CD6, CD19, CD27, Pgi-1 (Bernatzky and Tanksley 1986; Mutschler et al. 1987; Zamir and Tanksley 1988). Not all of the regenerants were scored at all of these loci; however, all of the regenerants were scored at between 20 and 24 of these loci. An example of the analysis at one of these loci is shown in Fig. 1. An EcoRV digestion of

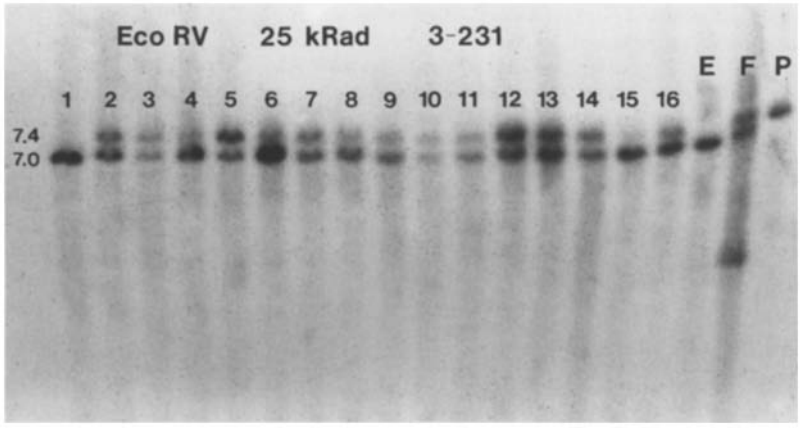

Fig. 1. Analysis of genotype at locus CD35 of regenerants following fusion of 25 krad-treated *L. pennellii* protoplasts with tomato. *Lanes 1–16* contain DNA isolated from individual regenerants; *lanes E, F, and P* contain DNA isolated from tomato, the sexual interspecific hybrid, and *L. pennellii*, respectively. The DNA was restricted with EcoRV and probed with the cDNA clone, 3-231. The sizes of the hybridizing fragments are indicated in kb

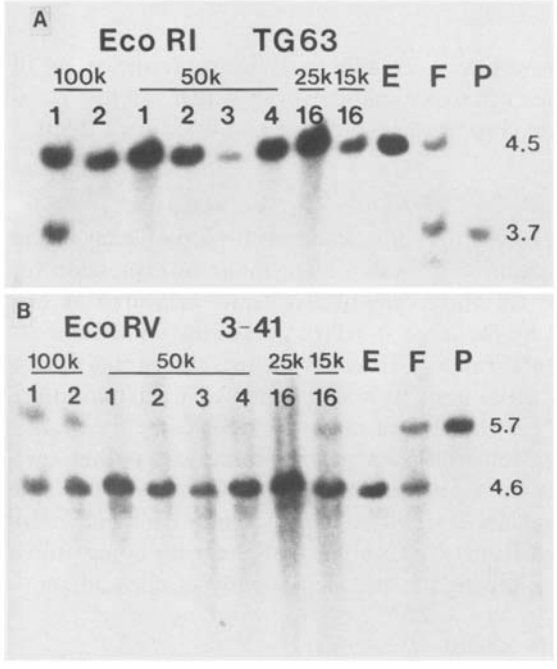

Fig. 2 A and B. Analysis of the genotype at loci TG63 (**A**) and CD14 (**B**) of regenerants following fusion of 15k, 25, 50 or 100 krad-treated *L. pennellii* protoplasts with tomato. The *first eight lanes* contain DNA isolated from the individual regenerants named; *lanes E, F, and P* contain DNA isolated from tomato, the sexual interspecific hybrid, and *L. pennellii*, respectively. The DNA was restricted with EcoRI (**A**) or EcoRV (**B**), and probed with either the genomic clone TG63 (**A**) or the cDNA clone, 3-41 (**B**). The sizes of the hybridizing fragments are indicated in kb

tomato DNA reveals a species-specific polymorphism for locus CD35; a single 7.4-kb fragment is observed in DNA isolated from *L. pennellii*, while a 7.0-kb fragment is observed in DNA isolated from *L. esculentum* following probing with this cDNA clone. The 16 individual regenerants following fusion of 25 krad-irradiated *L. pennellii* protoplasts with 'UC82' protoplasts all have the 7.0-kb EcoRV fragment. Most, individuals numbers 2–14 and 16, are hybrid at this locus and display both fragments. Two, individuals numbers 1 and 15, are scored as having only the *L. esculentum* allele. We did not consider the intensity of the bands in the autoradiogram when we scored for asymmetry. However, visual inspection of these signals indicates that among the individuals scored as hybrid some appear to have equivalent amounts of the two types of alleles – individuals numbers 3, 12, and 13 – while others appear to have less of the *L. pennellii* allele – individuals numbers 4, and 6. The intensity of the two bands in the interspecific hybrid appear to be equal, so the reduction in hybridization signal in some of the *L. pennellii* specific fragments is not due to a lack of homology between the probe and the heterologous allele.

Determination of asymmetry

Individuals were considered asymmetric if they scored as hybrid at one locus and as *L. esculentum* at another locus. An example of this type of determination is presented in Fig. 2. Panels A and B contain DNA from the same plants, restricted with either EcoRI or EcoRV and then probed with TG63 or CD14. From this analysis individuals 15k-16 and 100k-2 would be considered asymmetric somatic hybrids since each scored as hybrid at locus CD14 and as *L. esculentum* at locus TG63. A summary of these analyses, is presented in Table 2. The regenerants which scored as hybrid at all loci tested are considered full somatic hybrids; those regenerants which scored as hybrid at some loci and as *L. esculentum* at other loci are considered to be asymmetric somatic hybrids. Individuals which scored as *L. esculentum* at all loci tested are considered to be non-hybrid. Two individuals contained only the *L. pennellii* allele at some of the loci, these individuals were also scored as asymmetric somatic hybrids. The frequency of asymmetric somatic hybrids in the population of regenerants for each fusion combination is listed in Table 2.

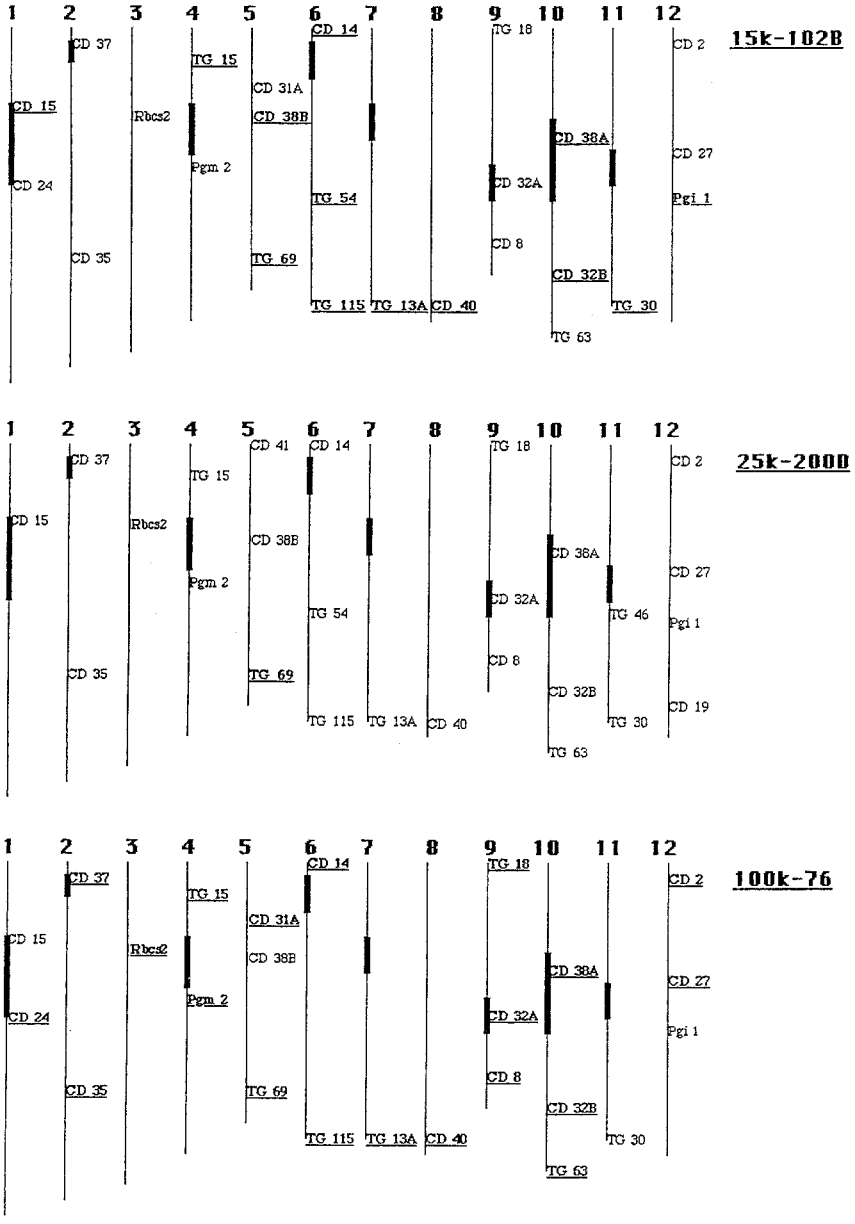

Fig. 3. The molecular map of three asymmetric somatic hybrids. The chromosomal location of the loci are based entirely on the published molecular map of the tomato nuclear genome (Bernatzky and Tanksley 1986; Mutschler et al. 1987; Zamir and Tanksley 1988). The genotype of all the loci tested for regenerants 15k-102B, 25k-200D, and 100k-76 are indicated: hybrid loci are indicated in *bold, underlined type*, *esculentum* loci are indicated in *plain type*

The regenerants from the fusion cultures using low dose treatments demonstrate that our double inactivation process, irradiation and iodoacetamide treatment, was sufficient to prevent the growth of non-fused protoplasts, as no parental genotypes were recovered in the low dose combinations (Table 2). Therefore, the regenerants recovered in these studies are the products of heterokaryon fusion. As the initial fusion product divides, genetic information initially present may be lost to varying degrees depending on the radiation treatment. The higher frequency of regenerants with *L. esculentum* genotypes recovered in fusion combinations with 50 and 100 krad-treated *L. pennellii* protoplasts reflects the loss of *L. pennellii* information, not that these are regenerants from unfused protoplasts.

The results in Table 2 reveal a dose reponse in the production of asymmetric somatic hybrids. Irradiation of *L. pennellii* protoplasts with 5 or 10 krads is sufficient to prevent cell division (data not shown), but following fusion with 'UC82' the irradiated genome is apparently rescued entirely in the fusion product. At these doses almost all of the regenerants are full somatic hybrids. At doses of 15 and 25 krads we detected an increased frequency of asymmetric somatic hybrids, regenerants which have lost some but not all of the *L. pennellii* genetic information. The frequency of asymmetry in the 50 and 100 krad population is about the same as at slightly lower doses, but no full somatic hybrids are recovered; instead, most of the regenerants display only *L. esculentum* alleles.

Table 3. Description of the asymmetric somatic hybrid plants regenerated. The number of loci which scored as *L. esculentum* (E), *L. pennellii* (P), or hybrid (H), the number of chromosomes, and whether seed has been obtained following self-fertilization are listed

Name	Number of E loci	Number of H loci	Number of P loci	Chromosome number	Seed
5k-3	0	3	18	30	No
15k-3	14	5	1	52±4	No
15k-5	1	19	0	48	No
15k-10	1	18	0	48	No
15k-14	1	20	0	48	No
15k-16	13	10	0	48	Yes
25k-1	22	1	0	30–34	Yes
25k-4	1	24	0	48	No
25k-6	1	24	0	46-48	No
25k-9	1	24	0	48±2	No
25k-14	1	24	0	48±2	No
25k-15	1	23	0	48±2	No
25k-16	2	22	0	48±2	No
25k-17	21	2	0	–	No
50k-1	17	1	0	24±2	Yes
50k-2	22	1	0	24	Yes
100k-1	4	19	0	–	No
100k-2	4	17	0	–	No
100k-81	18	1	0	–	Yes
100k-100D	24	1	0	24	Yes
100k-100E	14	1	0	24	Yes
100k-121	21	1	0	24	Yes

A description of the number of hybrid loci and the genotype of the non-hybrid loci for the asymmetric somatic hybrids is presented in Table 3. In general the number of hybrid loci in the individual regenerants is decreased in fusion products where the *L. pennellii* parent received higher doses of radiation. However, examples of individuals that are hybrid at 5 or fewer loci are found in 15, 25, 50, and 100 krad fusion combinations. There is a dose effect for the frequency of asymmetric regenerants in the population of regenerants, and there is a trend for a decrease in the extent of hybridity per individual as a function of increasing radiation dose.

Curiously, we recovered individuals that had lost *L. esculentum* alleles. Two of these types of regenerants are listed in Table 3, 5k-3 and 15k-3. These regenerants scored as hybrid or *L. esculentum* at several loci and as *L. pennellii* at other loci. We also recovered two other regenerants that have scored as *L. esculentum* at all loci except for one, TG69, at which they are null. They have apparently lost this region of the *L. esculentum* chromosome.

Several of the asymmetric somatic hybrids are hybrid at only limited regions of the genome: 25k-1, 25k-17, 50k-1, 50k-2, 100k-81, 100k-100D. 100k-100E, 100k-121; all except 25k-17 are self fertile. These individuals are likely candidates for further screening in tomato breeding programs.

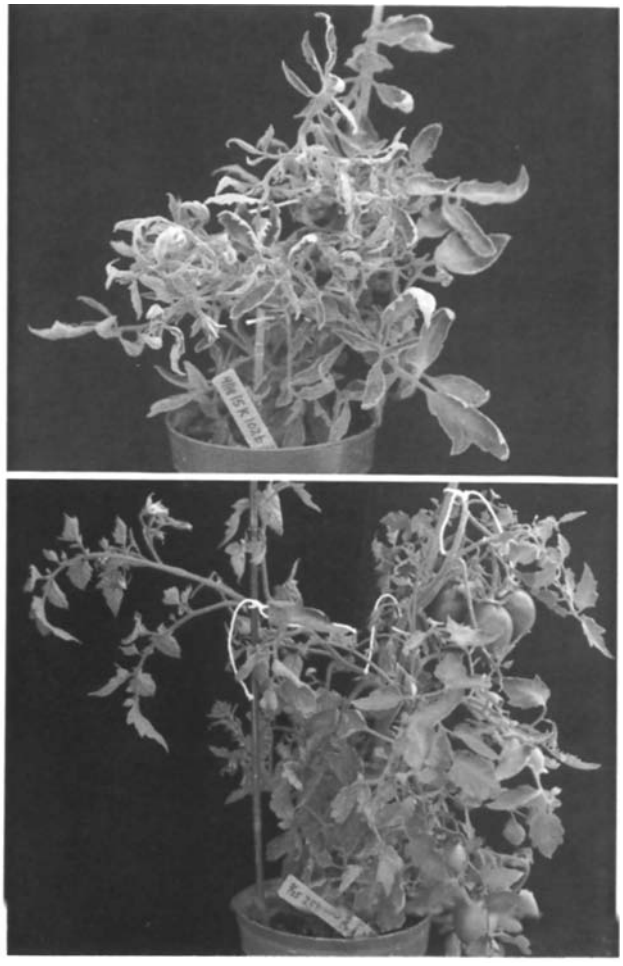

Fig. 4. Regenerants 15k-102B and 25k-200D

Three examples of the genetic maps generated in the analysis of the extent of asymmetry are provided in Fig. 3. In one case, 15k-16, the regenerant was hybrid at about half of the loci; in the second case, 25k-17, the regenerant was hybrid at only 1 locus, and in the last case, 100k-1, the regenerant was hybrid at most of the loci. The appearance of two regenerants is shown in Fig. 4.

Discussion

We have undertaken a carefully detailed analysis of the relationship between the extent of the genome transferred and the dose of irradiation received by the protoplasts prior to fusion. The underlying premise in the use of radiation to construct asymmetric somatic hybrids and cybrids is that the amount of damage to the chromatin increases as the dose of radiation increases. From this premise one would expect that the amount of genetic information transferred following protoplast fusion

would decrease with increasing dose of radiation. We have presented evidence that this trend is observed in the specific combination of tomato species, *L. esculentum* and *L. pennellii*. Wijbrandi et al. (1990) have constructed asymmetric somatic hybrids between tomato and *L. peruvianum*. They used two doses of gamma radiation, and suggested that the amount of genetic information transferred from *L. peruvianum* was dose dependent.

Several groups have not observed a relationship between dose of radiation and the extent of hybridity in the regenerants. In some instances, the studies were performed between very diverged species, carrot and tobacco (Dudits et al. 1987) and *Nicotiana* and *Atropa* (Gleba et al. 1988). While in both of these studies asymmetric somatic hybrids were recovered, the frequency of asymmetry did not change with increasing doses of radiation. These combinations of diverged species can be expected to be unstable and may tend to undergo chromosome elimination in the absence of radiation (Harms 1983). In addition, the analysis of these asymmetric somatic hybrids was difficult since very few chromosomal markers were available. Recently, Piastuch and Bates (1990) described the chromosomal organization of asymmetric somatic hybrids constructed in *Nicotiana*. Using clones containing species-specific repetitive sequences, they were unable to demonstrate a dose dependence in the production of asymmetric somatic hybrids. The recovery of asymmetric somatic hybrids in their case required the transfer of a neomycin phosphotransferase gene from the irradiated donor fusion partner. Based on in situ hybridization of chromosomal spreads of the asymmetric somatic hybrids, again using species-specific repetitive sequences, Piastuch and Bates (1990) were able to determine the number of chromosomes transferred from the irradiated donor.

Working with closely related species with an abundance of chromosomal markers, we did observe a relationship between dose of radiation and the extent of hybridity in the regenerants. Less genetic information from the irradiated fusion partner was detected in regenerants derived from fusions in which the irradiated partner received a higher radiation dose. Famelaer et al. (1989) studying intrageneric fusion constructs in *Nicotiana* also observed a relationship between radiation dose and the asymmetry of the regenerants.

We also observed a relationship between the dose of radiation and the frequency of asymmetric somatic hybrids in the population of regenerants. Increasing radiation doses from 5 to 50 krads resulted in a higher frequency of asymmetric somatic hybrids among the regenerants. However, fewer asymmetric somatic hybrids were detected at very high doses (100 krads). Two possible explanations can be offered. One, the higher dose causes such extensive DNA fragmentation that rescue of such genetic material is difficult and most information is lost. Two, the greater fragmentation results in smaller asymmetric regions (i.e., insertions and translocations) that were not detected by the number of loci analyzed in this study.

While the presence of both parental alleles is indicated by a hybrid score in the RFLP analysis, the physical location of the *L. pennellii* allele in the genome is not known. There are two possibilities: one is that the *L. pennellii* allele is carried on an *L. esculentum* chromosome; the second is that the *L. pennellii* allele is present in the genome on an *L. pennellii* chromosome or mini-chromosome. In Fig. 3, regenerants 15k-16 and 100k-1 contain chromosomes in which all loci tested scored as hybrid (i.e., chromosome 6 in 15k-16 and chromosomes 2, 4, 6 and 9 in 100k-1). In these cases, the most likely explanation is that the regenerant contains at least one intact copy of the *L. pennellii* chromosome in addition to the *L. esculentum* homologs. In some cases, only the region near the centromere scores as hybrid for a particular chromosome, with the flanking regions scoring as *L. esculentum*, i.e., chromosome 10 in 15k-16. In these cases, it is possible that a minichromosome of chromosome 10 from *L. pennellii* is present in the regenerant. And finally, there are cases where the centromere region of the chromosome scores as *L. esculentum* and the flanking regions score as hybrid, i.e., chromosome 4 in 15k-16 and chromosome 12 in 100k-1. In these cases, the *L. pennellii* allele has probably recombined with an *L. esculentum* chromosome, although which chromosome and where on the chromosome is not known.

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