Theor Appl Genet (1993) 86:377–382

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T-DNA-tagged chromosome 12 in donor *Lycopersicon esculentum* × *L. pennellii* is retained in asymmetric somatic hybrids with recipient *Solanum lycopersicoides*

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Received September 1, 1992; Accepted October 7, 1992
Communicated by P. Maliga

Summary. Asymmetric somatic hybrid plants were recovered after fusing irradiated mesophyll protoplasts of donor *Lycopersicon esculentum* × *L. pennellii* (EP) interspecific hybrid with callus-derived protoplasts of recipient *Solanum lycopersicoides*. EP plant A54 had been previously transformed by an agrobacterium vector, and the T-DNA insert mapped to the *L. esculentum* chromosome 12. The T-DNA insert conferred kanamycin resistance to EP that was subsequently used to select cell fusion products and recover asymmetric hybrid plants that retained tagged chromosome 12. Doses of 50- and 100-Gy irradiation promoted the elimination of only a few donor chromosomes. At 200 Gy, the regenerated plants had ploidy levels higher than tetraploid. However, the T-DNA tagged chromosome 12 was always retained in the asymmetric hybrid plants tested. Likewise, all plants from the 100-Gy series, with the exception of number 160, were mixoploid in the root-tip cells. Such mixoploid asymmetric somatic hybrids could be stabilized by inducing adventitious shoots on leaf strips cultured on shoot regeneration medium containing kanamycin. The asymmetric hybrid plants did not produce viable seed when self-pollinated or backcrossed to tomato or *S. lycopersicoides*.

Key words: Asymmetric hybridization – Cell fusion – *Lycopersicon esculentum* × *L. pennellii* – *Solanum lycopersicoides* – T-DNA tag

Introduction

Somatic hybridization is a powerful technique toward genetically transcending sexual boundaries and, thereby, widening and enriching the gene pool available for breeding purposes. However, somatic cells are generally diploid, and the resulting hybrid genomes are an additive combination of the original diploid levels. Thus, the general problem of sterility is often incurred, making somatic hybrids unattractive to plant breeders (Larkin et al. 1990). Alternatively, the ability to transfer single chromosomes or chromosome fragments of a donor species to a recipient, thereby creating highly asymmetric hybrids, may offer a better potential in plant improvement. Partial genome transfer would appear to become particularly valuable as the taxonomic distance between the partners increases.

Asymmetric hybridization is generally achieved by irradiation of the donor cells, which results in fragmentation of the nuclear genome prior to fusion. The technique was originally developed by Zelcer et al. (1978) for directed transfer of organelles while eliminating nuclear input. However, Dudits et al. (1980) using a similar scheme discovered that asymmetric hybrids could be obtained if selection for nuclear-determined traits of the donor was made on the fusion products. To date, asymmetric hybridization has met with mixed results. While in general irradiation has indeed reduced the amount of donor DNA transferred, with certain species' combinations the reduction has been limited (Negrutiu et al. 1989). The degree of donor elimination has ranged from the transfer of single chromosomes to fusion combinations where the asymmetric cells or hybrid plants contained substantial amounts of the donor genome (Negrutiu et al. 1989).

An important post-fusion step is selection of the asymmetric hybrid cells and calluses. Several researchers have discriminated fusion products by utilizing transformed donor plants containing a selectable marker. For example, Bates et al. (1987) irradiated a kanamycin-resistant, nopaline-positive line of *Nicotiana plumbaginifolia*

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and fused these protoplasts with those of *N. tabacum*. Selection for hybrid cells on kanamycin-containing medium enabled the recovery of tobacco plants containing a single *N. plumbaginifolia* chromosome. Muller-Gensert und Schieder (1987) used a *Nicotiana paniculata* cell line transformed with a wild *Agrobacterium tumefaciens* strain; the cells were thus capable of hormone-independent growth. Such cells were fused with *N. tabacum* and asymmetric hybrids subsequently selected. Using a similar strategy, Sacristan et al. (1989) selected asymmetric hybrids by fusing an X-irradiated *Brassica nigra* containing an inserted hygromycin resistant gene with *B. napus*.

The logical progression of using selectable markers would be to use T-DNA inserts that are mapped or located to a specific chromosome and attempt to transfer only it, or fragments with the insert, by asymmetric hybridization. The interspecific tomato hybrid *Lycopersicon esculentum* × *L. pennellii* has been transformed with *Agrobacterium tumefaciens*, and the locations of the T-DNA inserts mapped for ten such individuals (Chyi et al. 1986). The report presented here contains the results of studies wherein protoplasts of two of these T-DNA mapped transgenic hybrids were irradiated and subsequently fused with *Solanum lycopersicoides*.

Materials and methods

Plant material

Two sexual hybrids of *Lycopersicon esculentum* (VF 36; 2n=2x=24) × *L. pennellii* (2n=2x=24) LA716, plants A54 and A181, herein termed EP, were obtained from Richard Jorgensen, DNA Plant Technology Corp, Oakland, Calif. These plants had been transformed by *Agrobacterium tumefaciens* strain C58C1/pGV3850, which conferred kanamycin resistance (Chyi et al. 1986). Chyi et al. (1986) mapped the single T-DNA inserts to *L. esculentum* chromosomes 12 and 2 in plants A54 and A181, respectively. Seeds of *Solanum lycopersicoides* (2n=2x=24) LA 1990 were kindly provided by C.M. Rick, University of California, Davis, Calif.

Protoplast isolation

Mesophyll protoplasts of EP were obtained from 7-month-old plants grown in a controlled environment chamber at 27 °C (day)/22 °C (night) under a light intensity of 200 μE m^{-2} s^{-1} (16 h/day) from cool-white fluorescent bulbs according to Guri et al. (1991). The leaves were sliced and incubated in enzyme solution containing 0.5% Cellulysin and 0.1% Macerase in CPW salts (Frearson et al. 1973) plus 0.4 *M* mannitol at pH 5.8.

S. lycopersicoides hypocotyl-derived callus was obtained according to Handley and Sink (1985). Protoplasts were isolated from 3 g of tissue incubated in 15 ml of enzyme solution consisting of 1% Cellulysin, 1% Driselase, 0.4% Macerase in CPW salts plus 0.6 *M* sorbitol at pH 5.6. The incubation time for both the leaves of EP and callus of *S. lycopersicoides* was for 12–16 h in the dark at 27 °C on a gyratory shaker at 30 rpm.

Protoplast fusion and culture

Freshly isolated protoplasts of donor EP were irradiated with 50-(A54), 100-(A54) and 200-(A181) Gy of [^{60}Co]-gamma rays. Irradiated donor protoplasts were immediately fused with those of *S. lycopersicoides* using the fusion and culture methods of Guri et al. (1991). Plating efficiency was calculated as the percentage of total protoplasts undergoing divisions 14 days post-fusion. To select for hybrid calli, 25 μg/ml of kanamycin was added to the culture medium 16 days after fusion. Hybrid shoots were regenerated and rooted according to Guri et al. (1991). Somatic hybrid plants were retested for resistance to kanamycin by placing leaf strips on shoot regeneration medium containing the antibiotic and by spraying greenhouse plants with an aqueous kanamycin solution (400 mg l^{-1}) that bleaches sensitive plants (Weide et al. 1989).

Isozyme analysis

Plants of *L. esculentum* VF36 and *L. pennellii* LA716, A54 and A181, *S. lycopersicoides*, and selected asymmetric hybrids were analyzed. Young leaf material (100 mg) was homogenized with liquid nitrogen and extracted in 0.1 *M* TRIS-HCl, pH 5.6, containing 4% PEG (MW 3,350) and 0.15% 2-mercaptoethanol. *Skd-1*, *Pgm-2*, *Pgi-1* and *Got-2* were separated on 12% starch gels (Scandalios 1969) and stained according to Vallejos (1983). These four allozymes are located on *L. esculentum* chromosomes 1, 4, 7 and 12, respectively. *Skd1*, *Got-2* and *Pgi-1* are polymorphic with *L. pennellii* and *S. lycopersicoides* whereas *Pgm-2* is only polymorphic with the latter species.

Cytology and pollen viability

Actively growing root tips (8–10 mm) were removed from isozyme-confirmed hybrid plants and placed in water at 4 °C for 24 h. After pretreatment, the roots were transferred to a fixative solution, 3:1 ethanol:glacial acetic acid, for a further 48 h at 4 °C. Following fixation, roots were placed in 1 *N* HCl at 55 °C for 30 min. Washed root tips were squashed, stained with aceto-carmine, and the mitotic cells viewed with a light microscope. Pollen viability was assessed in 1% aniline blue solution.

Results

Selection of hybrid calluses and regeneration of plants

The three irradiation levels arrested division of A54 and A181 protoplasts. The addition of kanamycin on day 16 to the fusion plates inhibited the growth of *S. lycopersicoides* cells. These respective selection pressures on each fusion partner effectively ensured the growth of only hybrid calli.

The plating efficiency of the non-irradiated (0 Gy) control fusion of the parents was five- to six-fold higher than that of the respective parents self-fused (Table 1). However, irradiation diminished plating efficiency, with 50-Gy-irradiated protoplasts showing a 50% decrease, 100-Gy-irradiated protoplasts, a further 50% decrease, and 200-Gy-irradiated protoplasts, a still further 75% decline over the non-irradiated control. Increased irradiation doses also decreased the total number of calli produced.

Irradiation also had a marked effect on the frequency of regeneration of plants from calli (Table 1). An approximate 25% reduction in the number of plants recovered from calli occurred each time the irradiation dose was

Table 1. Efficiency of asymmetric hybridization between *Lycopersion esculentum* × *L. pennellii* (EP) (+) *Solanum lycopersicoides* (SL)

Irradiation dose (Gy)	Protoplasts fused ($\times 10^6$)		Plating efficiency (%)	Kan^r calli (total number)	Calli-attempted regeneration (number)	Calli-regenerated somatic hybrid plants (number)
	EP	SL				
0	0.5	–	6	0	0	0
0	–	0.5	5	0	0	0
0	1.4	4.3	31	1610	128	128
50	1.5	4.5	16	1120	150	60
100	1.0	3.0	8	560	150	45
200	0.5	1.5	2	280	150	32

Table 2. Isozyme patterns and chromosome numbers of donor EP (+) recipient SL asymmetric somatic hybrid plants

Plant number	SKDH (1)[a]		PGM (4)	GOT (7)		PGI (12)		Chromosome range
	E	P	E	E	P	E	P	
50-Gy donor A54								
19	+[b]	–	+	+	+	+	+	45–47
33	+	–	+	+	+	+	+	45–48
45	+	+	+	+	+	+	+	48–50
110	+	+	+	+	+	+	+	46–48
136	+	+	+	+	+	+	–	46–48
270	+	+	+	+	+	+	–	46–62
100-Gy donor A54								
148	+	+	+	+	+	+	+	46–48
160	+	+	+	+	+	+	+	48
231	–	+	+	+	–	+	+	41–44
239	–	+	+	+	+	+	+	NC[c]
301	+	–	+	+	+	+	+	44–46

[a] Chromosome location in *L. esculentum*
[b] +, Present; –, missing
[c] Not counted

doubled. Regeneration of plants was also delayed by successively longer time increments as the irradiation level increased. Also, regenerated shoots from the higher irradiation exposures were more difficult to root.

Elimination of donor chromosomes

The chromosome number in root-tip cells was determined for six 50-Gy-, four 100-Gy-, and five 200-Gy-derived plants. The chromosome counts revealed that all asymmetric somatic hybrids were mixoploid except for hybrid 160, which was $2n=4x=48$ (Table 2). Chromosome loss in the six 50 Gy plants was minimal. Plants 19 and 33 had lost three chromosomes, the most observed for the 50-Gy donor. Chromosome loss in the 100-Gy hybrids was greater than what had occurred at the lowest irradiation level, but was still not extensive (Table 2). Plant 231 lost seven chromosomes; otherwise the loss was only two to four chromosomes. For both 50- and 100-Gy-derived plants, only intact chromosomes were observed, and no evidence of centric or acentric fragments was found. The isozyme analysis for *Pgi-1*, located 7 cM from the T-DNA insert in *L. esculentum* of donor A54 (Chyi et al. 1986), revealed the presence of chromosome 12 in all of the plants tested (Fig. 1 and Table 2). Other isozyme patterns marking chromosomes 1, 4, and 7 indicated that the respective chromosome was either present or missing (Table 2). Of the five 200-Gy-derived plants studied, all had more than 48 chromosomes and were polyploid: four penta- and one hexaploid (data not shown). The leaf morphology of the asymmetric hybrids was intermediate between the parental types. However, there also was considerable phenotypic variation between individual plants derived from each irradiation level (Fig. 2). The observed variation is apparently not strictly due to somaclonal variation, as the same range in leaf morphology was not present in symmetric hybrids. For the 200-Gy-derived plants, morphology could not be used as an indication of chromosome loss as the plants which most resembled *S. lycopersicoides* tended to be pentaploids, in which case the polyploidy probably resulted from the presence of several recipient genomes.

To stabilize the mixoploid chromosome count, leaf strips of hybrid 231 ($2n=41-44$) were placed on MSOT medium containing 100 mg^{-1} kanamycin to ensure retention of chromosome 12, and adventitious shoots were regenerated. Of the ten adventitious shoots removed, five did not root. Of three whole plants studied cytologically, two had root-tip cell chromosome counts of 44 and the third had 42.

Transfer and stability of the T-DNA marked chromosome

At all irradiation levels, the initial addition of kanamycin to the protoplast culture medium not only ensured the selection of hybrid calli but ensured the specific selection of those which had retained donor chromosome 12. Regenerated shoots were later rooted on kanamycin, and leaf strips were removed again and tested on shoot regeneration medium containing the antibiotic (data not shown). As well, asymmetric hybrid plants transferred to

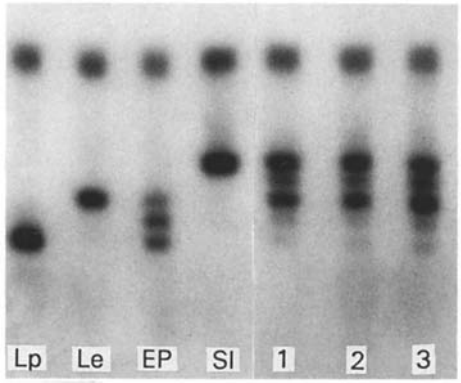

Fig. 1. *Lanes, left to right*, are isozyme patterns for PGI for leaf tissue extracts of *L. pennellii* (*Lp*), *L. esculentum* (*Le*), *L. pennellii* × *L. esculentum* (*EP*), *S. lycopersicoides* (*Sl*), and asymmetric hybrid representatives of the 50-, 100- and 200-Gy donor irradiations *1*, *2*, and *3*, respectively

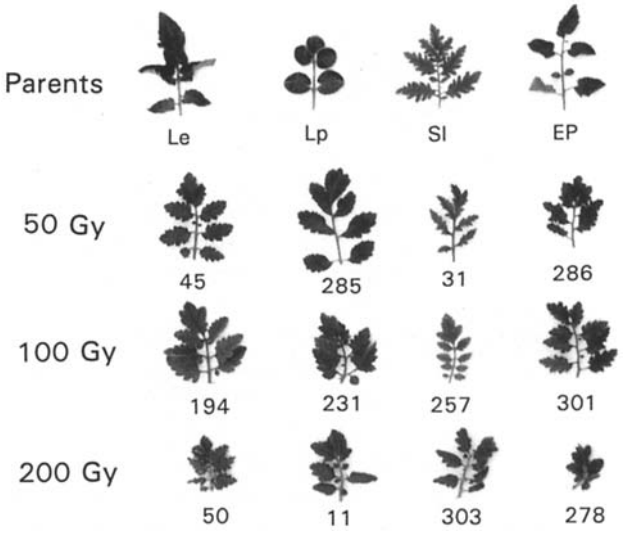

Fig. 2. Leaves of *Lycopersicon esculentum* (*Le*), *Lycopersicon pennellii* (*Lp*), *Solanum lycopersicoides* (*Sl*), the sexual hybrid (*EP*), and of a random selection of asymmetric hybrids from 50-, 100-, and 200-Gy donor irradiations

the greenhouse were confirmed for the incorporation of chromosome 12 by spraying their leaves with a solution containing kanamycin. Plants were retested every 6 months and were found to be consistently resistant to kanamycin.

Hybrid fertility

The percentage of hybrids which flowered decreased as the irradiation dosage increased. Whereas 80% of the 50-Gy hybrids flowered, only 50% of the 100-Gy did, and only one of the 200-Gy hybrids produced buds, which died soon after opening. Floral structure, unlike leaf morphology, was remarkably uniform and resembled symmetric hybrid flowers (Guri et al. 1991). Of the 21 plants that flowered, 13 produced no pollen, while 7 produced normal amounts of pollen but the viability was low, ranging from 0.7–2%. One of the hybrids, 110, had a pollen viability of 41.5%, however reciprocal pollinations to either parent did not set seed. Three 50-Gy plants produced fruit after self-pollination that contained several aborted seeds.

Discussion

We have demonstrated the effectiveness of T-DNA, and specifically the *NPTII* gene, in having a dual use in synthesizing asymmetric plant hybrids. The ability to select at the cell level enabled the easy recovery of asymmetric cell hybrids for a specific chromosome. As well, the T-DNA-tagged chromosome 12 of donor *L. esculentum* was always retained in the asymmetric hybrid plants. The T-DNA tagging scheme has the potential to be applied to systems where highly asymmetric hybrids may be obtained; for example, with *Nicotiana* (Bates et al. 1987) or when there is a large phylogenetic distance between the fusion partners (Dudits et al. 1980). Likewise, it may be useful in combination with other gene transfer systems that have been developed to transfer single chromosomes. For example, Verhoeven and Sree Ramulu (1991) recently published a technique whereby using AMP, amiprophos-methyl, an anti-microtubule drug, and centrifugation enabled the isolation of sub-protoplasts containing an amount of DNA equivalent to one or a few chromosomes. If such systems can effectively ensure limited gene transfer, then T-DNA may also be used to trace integration into the host genome, as it has been shown that tissue culture actively induces mitotic recombination between non-homeologous chromosomes at high frequencies (Larkin et al. 1990).

This study also provides additional evidence that *Lycopersicon* species do not seem to regenerate highly asymmetric plants by using the present "donor-recipient" technique. Our results concur with those of Wijbrandi et al. (1990a, b); namely, that low dosage irradiation (50 Gy) results in very limited chromosome loss. The elimination of donor chromosomes can be increased at 100 Gy, but the loss is still minimal. It also seems that doses higher than 100 Gy do not increase chromosome loss but rather foster the regeneration of penta- and higher ploidy levels. However, it is possible that the levels of irradiation needed to eliminate *Lycopersicon* chromosomes may be much, much higher than those needed to eliminate chromosomes in such species as *Nicotiana*. Melzer and O'Connell (1992) were able to recover highly asymmetric hybrids of *Lycopersicon esculentum* (+) *L. pennellii* using irradiation levels of 500 and 1000 Gy.

Our results indicate that increasing the phylogenetic distance between the fusion partners does not necessarily promote the loss of donor chromosomes. In another *Lycopersicon* (+) *Solanum* fusion, Wolters et al. (1991) also found only limited donor elimination after they fused irradiated (500 Gy) *Solanum tuberosum* with *Lycopersicon esculentum*.

Obviously, before asymmetric hybridization can be usefully employed in single or fragmented chromosome transfers, the efficiency of donor genome elimination must be optimized. Also, the question as to why the more highly asymmetric hybrids cannot be regenerated must be answered. It is possible that when the donor chromosomes are damaged or fragmented they are being repaired by a mechanism inherent in the recipient and *Lycopersicon* species instead of being rejected. Alternatively, it is also possible that during tissue culture selection occurs for cells and/or plants with tetraploid or near tetraploid numbers, which may have a selective regeneration advantage. If this is the case, it could be speculated that after having received 50 or 100 Gy of irradiation the hybrid population may be composed of many symmetric cells that would regenerate quickly and override any highly asymmetric cell hybrids. At higher irradiation (200 Gy), the tetraploids and near-tetraploids may be eliminated, which would allow the regeneration of penta- and higher ploidy cells that are normally suppressed. Through these regeneration scenarios, highly asymmetric cells would always be lowest in the order of selective advantage; in effect, their regeneration would be suppressed. This would agree with the findings from Koornneef et al.'s (1989) investigation on the chromosome stability of tomato haploids and diploids in which the majority of regenerated plants from such protoplast and callus cultures were tetraploid. This also indicates that undifferentiated tissue cultures either cause tetraploidization or that tetraploids have a selective advantage in regeneration.

Irradiation obviously diminishes the frequency of potential regenerants since the plating efficiency drops and fewer calli regenerate shoots. In addition, as was also observed herein, the somatic calli and plants are more difficult to handle; they are increasingly slower to regenerate and more difficult to root as the irradiation level increases. Highly asymmetric plants regenerated with difficulty. About 50% of the 231 plants regenerated from leaf strips would not root, although it is possible that these lines had a low chromosome number. That those that were counted were not mixoploid attests to the stability of the donor chromosomes in the hybrid. The fidelity of ploidy levels when regenerating from leaf strips is an attribute in tomato that has already been observed in tissue culture (Koornneef et al. 1989).

In conclusion, the results presented in this paper demonstrate the usefulness of T-DNA, not only as a hybrid calli selection system, but also a selectable genetic marker for the transfer of a specific chromosome or fragment. Undoubtedly, it will have an application in gene transfer studies and in the study of specific gene functions, however, apparently not with asymmetric hybridization of tomato until the efficiency of donor chromosome rejection and exclusion has been improved.

Acknowledgement. This research was carried out under USDA Grant No. 87-CRCR-1-2290 to K.C. Sink.

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