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X-ray irradiation promoted asymmetric somatic hybridisation and molecular analysis of the products

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Summary. Complementation of two metabolic deficiences – nitrate reductase and tryptophan synthase – was used to select for somatic fusion hybrids between tobacco (*Nicotiana tabacum*) and henbane (*Hyoscyamus muticus*) with prior X-irradiation of one partner. Using species specific, radioactively labelled DNA probes it could be shown that a) irradiation significantly reduced the amount of chromosomal DNA of the irradiated fusion partner in the somatic hybrid, b) irradiation with doses which completely inhibit protoplast division did not pevent transfer of substantial amounts of chromosomal DNA into the fusion hybrids (so called 'cybrids') and c) this method transfers functional nuclear genes together with the partial genome from the irradiated partner.

Key words: Protoplasts – Fusion hybrid – X-ray irradiation – Repetitive DNA – Species specific

Introduction

There has been considerable progress in recent years in the development of methods for somatic gene transfer in plants. Methods for the transfer of single isolated genes are now routine using *Agrobacterium* based systems (e.g. van Montagu and Schell 1982) and recently techniques for direct gene transfer into protoplasts (e.g. Paszkowski et al. 1984), liposome mediated transfer (Deshayes et al. 1985) and microinjection into protoplasts (Reich et al. 1986; Crossway et al. 1986) have been developed. However these methods rely on the availability of previously isolated genes and appear to be relatively limited in the amount of DNA which can be transferred. Therefore considerable attention has been given to the techniques of somatic cell fusion which may offer more possibilities for the transfer of the, as yet uncharacterised and probably complex, gene families thought to be important for the expression of many economically valuable characteritics.

The combination of complete genomes via plant protoplast fusion is now a routine technique (for review see Harms 1983). However unless the partners are closely related the final product of such a fusion is often an asymmetric combination of the two genomes, with parts of one or both genomes being lost during development (Pelletier et al. 1983; Gleba et al. 1984). The extent to which the two genomes are represented in a plant fusion product is largely unpredictable. In order to control the extent to which one genome is represented in a fusion product it is necessary to provoke and direct the loss of parts of one genome, such that one can recover asymmetric hybrids with small parts of this genome, in an otherwise unaltered host genome. To this end X-ray irradiation of one partner could be of use. X-ray irradiation is known to cause aberrations and fragmentation of chromosomes (Evans 1974). Such changes in a chromosome might be expected to lead to preferential loss from fusion hybrids. Experiments with mammalian cells have shown that such treatment can lead to extensive elimination of chromosomes and integration of small fragments into the "host" genome (Rodgers 1979).

The use of X-ray irradiation during plant cell fusion experiments, in order to limit the transfer of genetic material, has also been reported. X-ray irradiation has been used in several cases to promote the selective transfer of organelles in the production of "cybrids"

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(Galun and Aviv 1986). Carrot plants regenerated from fusion hybrids between X-ray irradiated parsley (*Petroselinum hortense*) and untreated carrot (*Daucus carota*) protoplasts contained a normal complement of carrot chromosomes together with an extra chromosome (Dudits et al. 1980). Fusion of tobacco (*Nicotiana tabacum* cv. Gatersleben) protoplasts with X-ray irradiated *Datura* or *Physalis* protoplasts led to the correction of the nitrate reductase deficiency of the tobacco cell line (Gupta et al. 1982). Recently Gupta *et al.* 1984 reported that plants regenerated from fusions between X-ray irradiated *Physalis* and untreated nuclear albino mutant *Datura* protoplasts contained 3 or 1 *Physalis* chromosomes in a background of a respectively tetraploid or octaploid *Datura* chromosome complement.

Chromosome rearrangements, changes in chromosome complement which occur spontaneously during cell culture and the difficulty often experienced in identifying chromosomes from the two parents mean that cytological analysis of fusion hybrids can be difficult and sometimes misleading. In paticular, small parts of a genome may be retained as integrated fragments or "mini-chromosomes" without altering the karyotype. In this report we have used a molecular technique to analyse the amount of donor DNA transferred and maintained in fusion hybrids between X-ray irradiated *Nicotiana tabacum* and untreated *Hyoscyamus muticus* protoplasts. The technique involves hybridisation of DNA from the fusion hybrids to species specific radioactively labelled DNA sequences as used previously for the identification of somatic hybrids between these two species (Saul and Potrykus 1984). We present measurements which show that X-ray irradiation prior to fusion does indeed lead to loss of DNA of the irradiated partner but that relatively large amounts of nuclear DNA from the irradiated partner are still retained in fusion hybrids.

Materials and methods

Protoplast isolation

The *Nicotiana tabacum* nia 115, nitrate reductase deficient cell suspension (Mendel et al. 1981) was used as the source of protoplasts for one partner in the fusion. The suspension cultures were sub-cultured every 7 days in AA-medium (Müller and Grafe 1978). For protoplast production cells were taken 4 days after sub-culture, the medium was removed after allowing the cells to settle, and replaced with an equal volume of enzyme solution (2% cellulase-R10, 1% Macerozyme-R10, 0.5% Driselase dissolved in 0.3 M Mannitol, 0.04 M $CaCl_2$, 0.5% MES pH 5.6). After 3 h incubation at room temperature the digestion mixture was passed through a 100 µm stainless steel sieve. An equal volume of 0.6 M sucrose, 0.5% MES pH 5.6 was added to the filtrate and the resulting mixture was distributed into 13 ml plastic centrifuge tubes. 1 ml of 0.16 M $CaCl_2$ solution was carefully overlaid in each tube and the tubes were then centrifuged for 10 min at 100 g. The protoplasts collected at the interface were removed and washed two times in wash solution (0.25 M Mannitol, 0.1 M $CaCl_2$, 0.5% MES pH 5.6).

The other partner in the fusion was the *Hyoscyamus muticus* VIIIB9 tryptophan synthase deficient cell line (Gebhardt et al. 1981) which was kindly provided by Dr. P. J. King of the Friedrich Miescher Institute. The cell suspension was maintained with weekly sub-culture in P2 medium (Potrykus et al. 1979) supplemented with tryptophan at 0.5 mM. Protoplasts were isolated from cultures 3 days after sub-culture in a manner identical to that described for the nia-115 culture except that digestion was in a mixture of 4% Cellulase R-10, 2% Macerozyme R-10 in 0.25 M Sorbitol, 0.025 M $CaCl_2$, 0.5% MES pH 5.6 for 16 h at room temperature. Protoplasts were adjusted to a density of 4×10^5/ml in wash solution.

For X-ray irradiation, which lasted for several hours when applying large doses, the nia-115 protoplasts were taken up at a density of approx. 1×10^5 cells/ml in wash solution containing 1/10 concentation enzyme mix in order to prevent synthesis of a new cell wall. The protoplasts were irradiated with either 20 or 40 Krad at a rate of 350 Roentgen/min. After irradiation the protoplasts were washed twice in washing solution and adjusted to a density of 4×10^5/ml in wash solution.

Fusion

The two protoplast populations were mixed in equal proportions to give a total population density of 4×10^5/ml. Four droplets of 125 µl each were pipetted onto the base of a 6 cm petri dish. 75 µl of PEG solution (40% PEG 6000 in solution A (0.13 M $CaCl_2$, 0.185 M sorbitol, 0.5% MES pH 7.0)) was added to each protoplast droplet to give a final concentration of 15% PEG. After 20 min 0.4 ml of solution A was gently added to the dish, followed at 5 min intervals by 0.5 ml and 1.5 ml. The liquid was then removed from the dish, the protoplasts remained adhering to the bottom, and replaced with 3 ml of AA-CH medium supplemented with 0.5 mM tryptophan. AA-CH is the AA medium of Müller and Grafe (1978) with xylose and arabinose at 150 mg/l, glucose at 180 mg/l, inositol at 100 mg/l and sucrose at 34 g/l. The protoplasts were cultured for two weeks in this non-selective medium and then 1 ml of the selection medium (Glimelius et al. 1978, AA medium containing 3.8 g/l KNO_3 as the sole nitrogen source, but modified by omitting nicotinic acid and containing 1 mg/l 2,4 D, 0.2 mg/l kinetin and 0.1 mg/l gibberelic acid) was added to each dish. After three to four weeks the contents of the dish were plated onto selection media solidified with 0.8% agar in a 9 cm petri dish, for further culture. All cultures were kept at 26 ° in the dark.

DNA Isolation

DNA to be used in mixtures for calibration of the dot blot method was isolated from each of the two parental lines by the method of Murray and Thompson (1980) and the concentration mesured spectrophotometrically.

DNA from the calli resulting from the fusion was isolated by a modification of that described in Paszkowski et al. (1984). The method was followed exactly until the ethanol precipitation step after which, instead of a cesium chloride gradient, the DNA was dissolved in TE (10 mM Tris, 1 mM EDTA, pH 7.5), RNA'se was added to 10 µg/ml and incubated at room temperature for one hour followed by a further ethanol precipitation. The DNA from approx. 0.5 g callus was dissolved in 50 µl of TE. The DNA concentration was estimated by comparison with standards after agarose gel electrophoresis.

Dot blot analysis

Estimation of the contents of the two genomes in the fusion hybrids was obtained by comparison with calibration mixtures in a dot blot analysis with species specific middle repetitive probes. The DNA to be applied to the filter (0.2–0.6 μg per dot) was denatured by the addition of an equal volume of 0.4 M NaOH followed by incubation at room temperature for 10 mins. The sample was then chilled on ice and an equal volume of 4 M NH_4Ac was added. The samples were then applied to a nitrocellulose filter (Schleicher and Schuell, BA 85) using a BRL dot hybridisation system. After the addition of the sample 200 μl of 1 M NH_4Ac was passed through each well, followed, after a 5 min incubation period, by 200 μl of 5× SSC (1× SSC is 15 mM Na Citrate, 150 mM NaCl). Finally the filter was baked at 80 °C for 2 h. Two identical filters were prepared along with calibration mixes of *N. tabacum* and *H. muticus* and each was probed with either a *N. tabacum* specific probe (H10) or a *H. muticus* specific probe (F6) as described in Saul and Potrykus (1984). The probes were labelled to a specific activity of approx 1×10^8 c.p.m. per μg by nick translation (Rigby et al. 1977). Hybridisation, washing etc. were carried as described for "Southern" analysis in Paszkowski et al. (1984).

For a more accurate estimation of the radioactivity bound to each applied sample, the piece of filter corresponding to each dot was cut out and the radioactivity bound was counted in a scintillation counter.

Results

In order to investigate the effects of X-ray irradiation of one partner in a fusion on the amount of DNA retained from this partner in the fusion hybrids, we carried out fusion experiments between *N. tabacum* and *H. muticus* protoplasts with prior irradiation of the *N. tabacum* protoplasts. A preliminary experiment on the effects of irradiation showed that an exposure of 5 or 10 Krad led to a significant decrease in the plating efficiency of the *N. tabacum* protoplasts on a non-selective medium. At levels of 15–40 Krad no colonies were formed on non-selective media although even at 40 Krad some divisions were observed. Therefore we chose to irradiate the *N. tabacum nia-115* protoplasts with 0, 20 and 40 Krad prior to fusion with *H. muticus*. Table 1 shows the effects of such irradiation on the number of fusion products, from identical fusion treatments, growing on selective medium 3 months after the fusion treatment. It can be seen that irradiation reduces the number of hybrid calli produced and that a stronger irradiation leads to a lower complementation frequency. Control experiments of self fusion of *N. tabacum* protoplasts with or without prior irradiation and self fusion of *H. muticus* protoplasts were always carried out in parallel and never yielded growing colonies on selective medium.

In order to estimate the amount of DNA from each parent in the fusion hybrids produced, DNA was isolated from 19, 4 and 3 separate calli from experiments with 0, 20 and 40 Krad prior irradiation respectively, after approx. eight months of growth. The DNA from each hybrid was loaded onto two identical dot blot filters as described in the "Materials and methods" section. In addition, for calibration purposes, a series of mixtures of purified DNA from each of the two parents was applied to each filter. Each filter was then hybridised with a probe consisting of middle repetitive DNA specific for either *N. tabacum* or *H. muticus* (H10

Table 1. Effects of X-irradiation on complementation frequency in the nia + trp combination

Dose (Krad)	Independent treatments	No. of selected colonies[a]	Complementation frequency[b]
0	7	115	2×10^{-5}–1×10^{-4}
20	9	23	0–5×10^{-5}
40	10	25	0–4×10^{-5}

[a] The number of colonies on the selective medium was scored 3 months after the fusion treatments

[b] Based on total number of protoplasts (6×10^5 in each fusion treatment)

Table 2. Relative genome contents in *N. tabacum*/*H. muticus* somatic hybrids measured by dot hybridisation. Samples 1–19 derived from treatments with 0Krad, 20–23 from treatments with 20Krad and 24–26 from treatments with 40Krad. The DNA content corresponding to each species was estimated from the calibration curves in Fig. 2

Clone no.	*N. tabacum* DNA (μg)	*H. muticus* DNA (μg)	Ratio *N. tabacum* to *H. muticus* (Av = sample average)
1	0.35	0.14	2.5
2	0.15	0.10	1.5
3	0.16	0.13	1.2
4	0.13	0.16	0.8
5	0.14	0.10	1.4
6	0.16	0.08	2.0
7	0.18	0.10	1.8
8	0.16	0.07	2.3
9	0.17	0.05	3.4
10	0.14	0.14	1.0
11	0.19	0.12	1.6
12	0.20	0.20	1.0
13	0.15	0.20	0.8
14	0.11	0.08	1.4
15	0.15	0.14	1.1
16	0.16	0.07	2.3
17	0.35	0.21	1.7
18	0.20	0.15	1.3
19	0.23	0.15	1.5 Av. 1.6
20	0.06	0.24	0.25
21	0.06	0.20	0.30
22	0.08	0.24	0.33
23	0.10	0.32	0.31 Av. 0.30
24	0.16	0.36	0.44
25	0.13	0.34	0.38
26	0.26	0.48	0.54 Av. 0.45

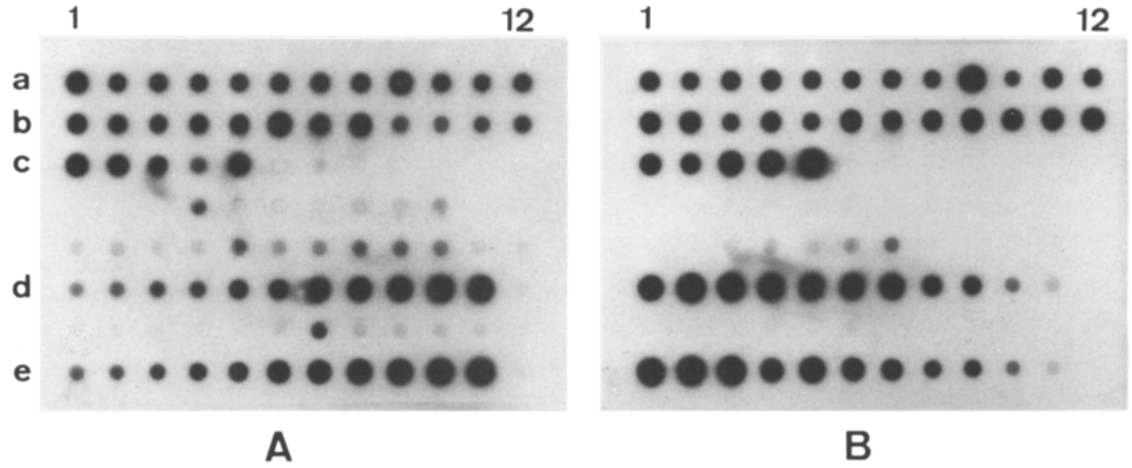

Fig. 1 A, B. Dot blot analysis of DNA samples from fusion hybrids and calibration mixes. **A** Probed with *N. tabacum* specific probe; **B** Probed with *H. muticus* specific probe. On each filter, a3-b10 hybrids from 0Krad treatment, b11-c2 hybrids from 20Krad treatment, c3-c5 hybrids from 40Krad treatment and d,e 1–12 calibration mixes (0.6 µg total) with a portion 0 µg (d,e 1) to 0.6 µg (d,e 12) of *N. tabacum* DNA

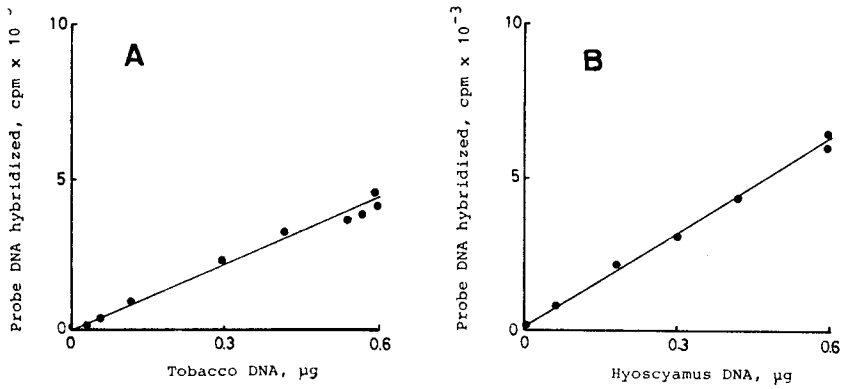

Fig. 2 A, B. Calibration plots derived from the dots in d,e 1–12 in Fig. 1. **A** Probed with *N. tabacum* specific probe; **B** Probed with *H. muticus* specific probe

and F6 respectively, Saul and Potrykus 1984). After hybridisation and washing, the filters were exposed with X-ray film (Fig. 1) and then the radioactivity in each of the dots was quantified by counting in a scintillation counter. Figure 2 shows the relationship between the amount of DNA from one species in the calibration mixes and the amount of bound radioactivity. It can be seen that with both probes the relationship between the ratio of the different DNA's in the mixture and the amount of probe bound is linear. Using such calibration curves we were able to estimate the ratio of parental DNA's in the various fusion hybrids (Table 2). The ratio of *N. tabacum* to *H. muticus* DNA in experiments done without prior irradiation of the *N. tabacum* was on average 1.6 with values ranging between 3.4 and 0.8. After prior irradiation considerably lower ratios were obtained, with 20 Krad an average of 0.3 and with 40 Krad an average of 0.45.

The accuracy of the estimation, using this method, depends on the copy number and distribution of the repetitive sequences used to represent each genome. We estimate that the copy number of the repetitive DNA represented in the probes used here to be approx. 3,000 per haploid genome (data not shown). The pattern of hybridisation produced when the clones were used to probe digests of plant DNA in a Southern analyis, was a "smear" with some distinct bands (Saul and Potrykus 1984), which is typical for a middle repetitive DNA dispersed througout the genome (Flavell et al. 1981). Furthermore results from in-situ hybridisation of the same repetitive probes, labelled with ^{3}H, with chromosome spreads also support this interpretation (Mouras et al. 1987). Thus a measurement of the ratios of the repetitive DNA's represented by the probes should give a realistic estimate of the amount of DNA from each parent present in the hybrids.

Discussion

The data presented here show that X-ray irradiation of one partner in a somatic fusion can result in the transfer of a partial genome including a functional gene. An estimate of the extent of the reduction in transfer as an effect of the X-ray irradiation was provided by the use of species specific repetitive DNA clones. The mechanism underlying such a limited genome transfer is probably the preferential loss of the irradiated DNA during initial proliferation of the hybrid cell clones.

The accuracy of the method of measurement of the genomes in the fusion product depends on the characteristics of the probe used. As the linearity of the graphs, derived from the calibration mixes of both parents, shows, the technique is capable of measuring accurately the presence of the repetitive DNA segment down to a ratio of appox. 0.5%. However as discussed in the Results section the relationship between the amount of such a repetitive fragment and the total amount of DNA from this parent in a fusion hybrid depends on the copy number and distribution of this repetitive DNA. For distantly related species it has been shown that total genomic DNA, without prior cloning, can also be used as a probe to differentiate between genomes in a fusion hybrid (Sala et al. 1985).

The convenience of the methods presented mean that 50–100 callus samples of as little as 300 mg can be handled in one experiment and therefore hybrid callus can be identified and analysed at an early stage of culture. Calli showing the selected trait but which have received only a small part of the incoming, irradiated genome can therefore be easily detected and further analysed. The method should also allow the detection of hybrids without previous selection.

The variation in the ratio of DNA's found in the hybrids even with unirradiated parents is probably a true reflection of variation found in the loss of DNA from such hybrids. However since the two parents involved were grown as cell suspenions, variation in the DNA content in the individual protoplasts fused cannot be ruled out. Although the data show that X-ray irradiation leads to a highly significant reduction in the amount of DNA retained in a hybrid, there is no evidence for a difference between 20 and 40 Krad treatments. This could either be due to the small sample sizes analysed or to a levelling off of effects of X-ray irradiation in this region.

Although X-ray irradiation led to a clear reduction in the amount of *N. tabacum* DNA finally present in hybrid calli, relatively large amounts of DNA were still retained. We were unable to regenerate plants from the hybrids produced and it is therefore possible that further reduction in DNA content would have ocurred during development. However the calli tested had been growing for eight months under selective conditions before testing and thus the genomes were likely to have stabilised. We therefore suggest that the possibility of nuclear DNA transfer should be considered when using X-ray irradiation in order to promote the selective transfer of organelles in the production of 'cybrids'.

We have shown directly that X-ray irradiation prior to somatic fusion leads to a clear reduction in the amount of DNA retained from this partner in a fusion. We have used such partial genome transfer to complement an auxotrophic mutant. The method should however be capable of transferring complex characters which are based on multiple genes and are therefore not easily acessible to methods such as *A. tumefaciens* mediated transfer or 'direct gene transfer' which transfer relatively small amounts of DNA, usually in a cloned form. The reduction in the amount of DNA transferred combined with selection for a desired trait could also eventually lead to identification and isolation of the DNA responsible.

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