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Stress-induced intrachromosomal recombination in plant somatic cells

(homologous intrachromosomal recombination/*Nicotiana tabacum*/x-ray/mitomycin C/heat shock)

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ABSTRACT **Levels of induced homologous recombination between chromosomal repeats in plant somatic cells were examined. Transgenic plants of *Nicotiana tabacum* hemi- or homozygous for pairs of deletion derivatives of the neomycin phosphotransferase (*nptII*) marker gene integrated at a single genomic locus were produced. Homologous recombination within the overlapping parts of the *nptII* gene restored the function and the resulting kanamycin resistance was used for scoring recombination frequency. The recombination events were confirmed by the appearance of a characteristic 1245-base-pair *Eco*RV fragment detected in all kanamycin-resistant clones tested. The rate of spontaneous recombination was found to be related to the copy number of recombination substrates and was 9×10^{-5} and 19×10^{-5} for hemi- and homozygote strains, respectively. Ionizing radiation, mitomycin C, and heat shock markedly increased the frequency of intrachromosomal recombination. Low doses of x-rays (1.25 Gy) enhanced the relative recombination frequency to approximately twice the spontaneous value. The presence of mitomycin C increased the frequency of recombination 9-fold and exposure to an elevated temperature (50°C) increased it 6.5-fold. The x-ray and heat shock treatments reduced cell viability to 53% and 8%, respectively. Mitomycin C treatment had no effect on cell survival.**

Homologous reciprocal recombination between chromosomal repeats can result in deletion, duplication, inversion, or translocation of DNA. Recombination in mitotically active somatic tissues generates cell lineages different from the original genotype. Such alterations can remain silent or lead to a chimeric phenotype. It was shown in mammalian cells that the frequency of recombination can be increased in a dose-dependent manner by mutagenic treatments, including application of known carcinogens (1–3). The level of stimulation at low drug doses appeared to be inversely correlated with the DNA-repair capacity of particular cell lines (3, 4). This observation supported earlier data suggesting that in *Escherichia coli* and *Saccharomyces cerevisiae* induced recombinational repair circumvents unexcised DNA damage (for a review, see refs. 5–7).

In plants, the generation of chromosomal deletions by intrachromosomal recombination has been observed in mitotically active somatic cells cultured *in vitro* (8) and *in planta* (8, 9). In the latter case recombination events occurred in cotyledons, during embryo formation (8), and in leaves (8, 9). Plant somatic cells bearing genetic alterations may give rise to generative tissues since, in contrast to animals, cells contributing to the plant germ line differentiate only late in development. Thus, mitotic recombination events may contribute directly to genetic variability within plant populations and lead to a subpopulation with modified abilities for environmental adaptation. Therefore, we investigated whether changes in environmental conditions influenced the frequency of genetic recombination and, as a consequence, increased the variability.

We describe here an experimental approach that allows examination of the influence of specific external factors on the levels of mitotic intrachromosomal recombination in plant cells. We have produced transgenic *Nicotiana tabacum* (tobacco) plants containing pairs of deletion derivatives of the selectable marker gene neomycin phosphotransferase, *nptII*, integrated into a single chromosomal locus (8). Homologous intrachromosomal recombination between these deletion derivatives restores a functional *nptII* gene, confers kanamycin resistance, and thus provides a marker for such events. Recombination frequencies in cells derived from strains hemi- or homozygous for the recombination substrates were correlated with the copy number of substrates per cell. We have examined the influence of DNA-damaging agents such as x-rays and mitomycin C and of extreme heat shock on the level of intrachromosomal recombination. Our results show that plant cells are able to respond to such stress with markedly higher levels of induced reciprocal recombination compared to mammalian cells.

MATERIALS AND METHODS

Materials. Transgenic lines SR1hph2 and SR1hph5 were derived from *N. tabacum* cv. Petit Havana line SR1 (10) and have been described in detail (8).

Mitomycin C (MC; Sigma) stock solutions (400 μg/ml dissolved in water and filter sterilized) were always freshly prepared.

Protoplast Isolation and Estimation of Relative Recombination Frequency (RRF). Tobacco leaf protoplasts were prepared, cultured, and selected for kanamycin resistance as described (11, 12). For the determination of RRF, kanamycin-resistant (Kan^R) clones were scored after 6 weeks of culture, and the number of microcalli in control samples without selection was determined. These measurements were repeated 2 weeks later.

Southern Blot Analysis. DNA isolation and Southern blot hybridization were performed as described (8).

Induction of Recombination by X-Ray Radiation. Cultures of protoplasts embedded in agarose (10^6 protoplasts per sample) were subjected to x-ray radiation after 1 day of culture. The source of radiation was an Isovolt Seifert generator at 300 kV, 9 mA, equipped with a 1-mm aluminum filter. The radiation was measured at the level of the protoplasts to allow precise determination of radiation doses.

Induction of Recombination by MC. One million protoplasts per sample were resuspended in 1 ml of plating medium. MC

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Abbreviations: RRF, relative recombination frequency; MC, mitomycin C; Kan^R, kanamycin-resistant.

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was then added at 10 times the final concentration and the protoplasts were incubated for 15 min at room temperature. The protoplasts were then plated in 9 ml of agarose-solidified medium and cultured for 1 week. Thereafter, cultures were transferred to 50-ml bead-type cultures (12) containing 50 mg of kanamycin sulfate per liter but no MC. In some experiments, the initial 15-min incubation with concentrated MC was omitted without effects on protoplast survival or the level of recombination.

For *in planta* experiments, apical shoot segments (0.5–1.0 cm) were precultured for 2 weeks on medium containing 2.5–25 μg of MC per ml and then developed shoots were transferred to MC-free medium. After ≈6 weeks of further culture, leaf protoplasts were isolated and the RRF was determined.

Induction of Recombination at Elevated Temperature. Samples of one million freshly isolated protoplasts in 1-ml culture aliquots were distributed to 10-ml plastic tubes. The tubes were immersed in water at the desired temperature. Samples were gently mixed after 30 sec and the incubation was continued. Treatment was terminated by rapid cooling of the samples in an ice bath.

RESULTS

The recombination substrates consisted of pairs of deletion derivatives of the *nptII* marker gene separated by a second selectable hybrid gene coding for hygromycin phosphotransferase (*hph*) (Fig. 1). Transgenic tobacco plants containing chromosomal copies of the recombination substrates have been characterized in detail (8). Two strains were used in the present studies: strain SR1hph5 containing a single copy and strain SR1hph2 containing three copies at a single nuclear

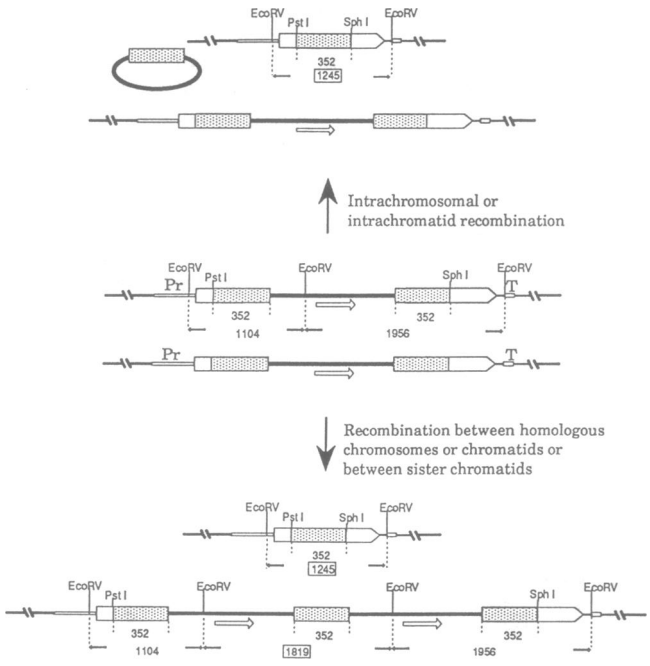

FIG. 1. Schematic drawing of the structure of the recombination substrate (middle) and the structures of possible recombination products. Broad boxes represent the *nptII* gene, the narrow open boxes represent the promoter and polyadenylylation signal of cauliflower mosaic virus, and the solid narrow box separating *nptII* deletion derivatives represents the *hph* marker gene. Homologous regions are shaded. Details of the construction and molecular characterization of plant strains have been described (8). The lengths of *Eco*RV fragments are indicated in base pairs, and the boxed numbers are the expected lengths of recombination products. Pr, promoter; T, poly(A) signal.

Table 1. RRF of hemi- and homozygote strains for recombination substrates

Strain	Total no. of clones/10^6 pps	No. of Kan^R clones/10^6 pps	RRF
Hemizygote	527×10^3	47	9×10^{-5}
Homozygote	519×10^3	101	19×10^{-5}
Ratio*			2.1

The total number of clones and number of Kan^R clones are the means of values from four independent experiments. Each experiment involved three independent populations of 10^6 protoplasts (pps).
*Homozygote/hemizygote.

locus (8). Strain SR1hph2 gave on average three to five times more recombinant clones per sample in corresponding treatments and thus a significantly lower variability between repeated experiments. Therefore, this presentation of the results deals mainly with strain SR1hph2. The ratio of the number of Kan^R cell clones to the total number of clones recovered without selection is termed the RRF. The mean RRF values for particular treatments are shown in Tables 1–4. In addition, the data from individual experiments are presented in graphic form (Figs. 2 and 4) to allow direct assessment of experimental variability. Variation between sister plants and individual leaves of a single plant (data not shown) was negligible as compared to the variation between experimental repetitions. This allowed pooling of leaves from clonally propagated, genetically identical plants.

Frequency of Intrachromosomal Recombination in Homo- and Hemizygote Strains. The homozygous progeny produced after self-pollination of R0 plants was used for the present studies. The homozygocity of the R1 plants was determined from the segregation pattern of the *hph* gene in the R2. Hemizygotic strains were produced by backcrossing the homozygote to the nontransgenic SR1 strain (10). Hemizygotic and homozygotic plants showed no obvious differences in phenotypic characteristics or in the behavior of mesophyll protoplasts in culture. However, the RRF of homozygotes was approximately twice that of hemizygotes (Table 1 and Fig. 2). This suggests that the two loci containing recombination substrates act independently, increasing the probability of recombination in proportion to the number of additional copies. The lack of large somatic sectors of Kan^R cells in

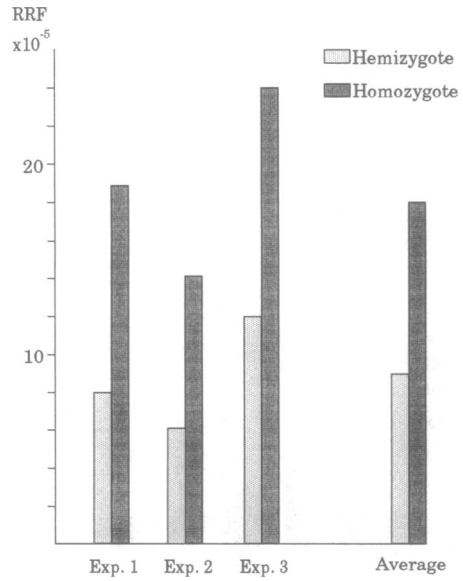

FIG. 2. Variation in RRF for hemi- and homozygous lines SR1hph2. Each bar represents the mean value from three populations of 10^6 protoplasts.

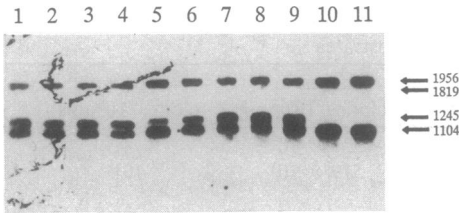

FIG. 3. Southern blot analysis of nine Kan^R lines resulting from recombination in a homozygous line, SR1hph2. DNA samples were digested with *Eco*RV and the *nptII* coding region was used as a probe for hybridization. Lanes 1–9, independent Kan^R recombinant clones; lane 10, control parental line SR1hph2; lane 11, plasmid pESPB (8) used for production of line SR1hph2. The arrows indicate 1104-bp and 1956-bp fragments characteristic of recombination substrates and the 1245-bp fragment diagnostic of predicted recombination products. The presence of an 1819-bp fragment would indicate a reciprocal recombination product.

hemi- and homozygote strains indicated that the recombination events occurred late in development for both genotypes.

The DNA of 44 randomly chosen Kan^R clones derived from homozygous lines (21 from SR1hph5 and 23 from SR1hph2) was analyzed. An additional band corresponding to the homologous recombination product (1245 bp, Fig. 1) was present in all Kan^R lines. Representative results for 9 Kan^R clones derived from line SR1hph2 are presented in Fig. 3. The persistence of the initial pattern for nonrecombined substrates (1956-bp and 1104-bp fragments in Fig. 3) demonstrated that not all copies of the substrates were involved in recombination. This was also the case in the homozygous line SR1hph5 (data not shown), which contained only a single copy of the recombination substrates on each homologous chromosome.

In homozygotes, in addition to intrachromosomal recombination, exchanges between homologous chromosomes might also take place (13). The absence of distinct chromosomal markers flanking the recombination substrates does not allow an unequivocal distinction between these two events. However, in the case of reciprocal exchanges between homologous chromosomes, there is an increased probability of the recovery of both reciprocal recombination products in Kan^R clones (the restored *nptII* gene and tandem repeats of the *hph* gene separated by the common region of *nptII* deletion derivatives, Fig. 1). In such cases, an additional *Eco*RV fragment should appear (1501 bp and 1819 bp for lines SR1hph5 and SR1hph2, respectively) (Figs. 1 and 3). Bands of these sizes were not detected among the 52 DNA samples of Kan^R lines derived from homozygous SR1hph2 and SR1hph5 strains. Thus, within our detection limits, there was no indication of frequent somatic exchange between homologous chromosomes.

Table 2. Influence of x-rays on RRF

Radiation dose, Gy	Total no. of clones/10^6 pps	No. of Kan^R clones/10^6 pps	RRF	% of control
0.00	384×10^3	130	33×10^{-5}	100
1.25	205×10^3	135	65×10^{-5}	192
2.50	235×10^3	118	50×10^{-5}	166
5.00	187×10^3	90	48×10^{-5}	145
10.00	102×10^3	26	25×10^{-5}	75
15.00	55×10^3	5	9×10^{-5}	27

For every radiation dose the RRF values are the means of four independent experiments, each including three populations of 10^6 protoplasts (pps).

Effect of X-Ray Radiation on the Recombination Frequency. We exposed populations of protoplasts isolated from a homozygous SR1hph2 strain to different doses of x-rays. Irradiated protoplasts and the corresponding controls were subjected to kanamycin selection after a further week of culture. Even the lowest radiation doses reduced cell viability, which decreased with increasing x-ray dose (Table 2). The absolute number of Kan^R clones per 10^6 treated protoplasts was constant at low radiation doses (1.25–2.5 Gy) and then decreased markedly. The mean RRF values were consistently higher at low doses of radiation and declined at higher doses (Table 2 and Fig. 4*A*). This surprising response may be due to two associated consequences of radiation—namely, stimulation of recombination and selection of a radiation-tolerant subpopulation.

Stimulation of Intrachromosomal Recombination by MC. The effect of MC on the frequency of intrachromosomal recombination was examined using homozygous and hemizygous SR1hph2 lines. Plant cells appeared to be rather resistant to MC in comparison to mammalian cells (1). Low MC concentrations had a slight stimulatory effect on protoplast plating efficiency (Table 3). High doses of up to 50 μg/ml applied as pulse treatments (15–90 min) did not affect the survival of cells. Only the continued presence of MC

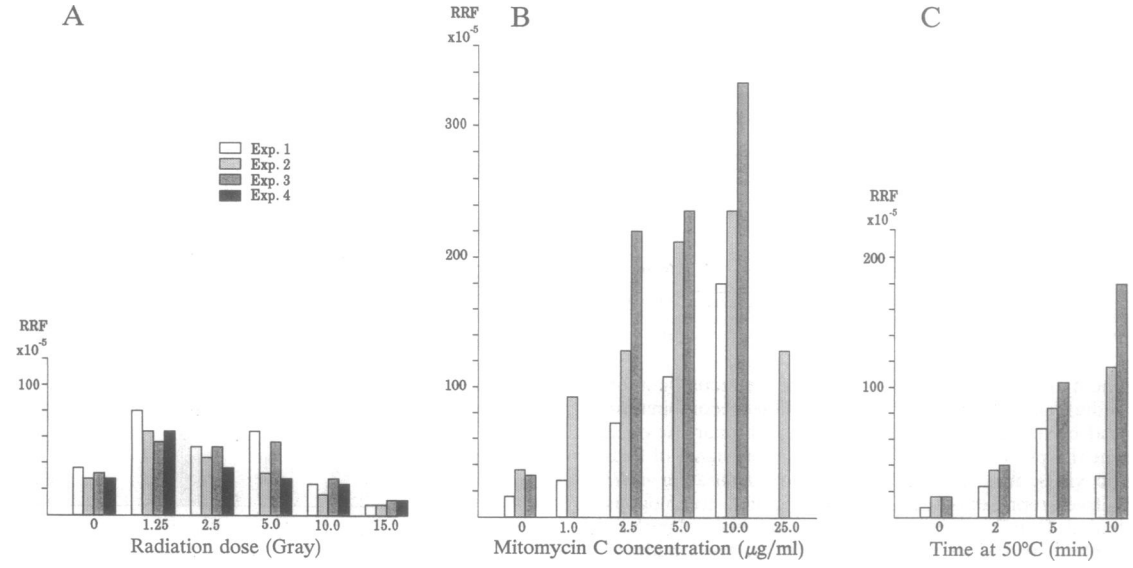

FIG. 4. Variation in relative recombination frequency after x-ray (*A*), mitomycin C (*B*), and heat (*C*) treatment. Each bar represents the mean values from three populations of 10^6 protoplasts.

Table 3. Influence of MC on RRF

MC, μg/ml	Total no. of clones/10^6 pps	No. of Kan^R clones/10^6 pps	RRF	% of control
0	319×10^3	87	27×10^{-5}	100
1.0	432×10^3	253	59×10^{-5}	219
2.5	395×10^3	558	141×10^{-5}	522
5.0	362×10^3	676	187×10^{-5}	693
10.0	293×10^3	726	248×10^{-5}	919
25.0	139×10^3	178	128×10^{-5}	474

The values of RRF for each MC concentration are the means of three independent experiments, each including three populations of 10^6 protoplasts (pps).

during initial culture periods caused a dose-dependent inhibition of microcalli formation. Even then, doses of 25 μg/ml only decreased the number of recovered cell clones to 44% (Table 3) and doses as high as 50 μg/ml still permitted growth of 0.08% clones compared to the untreated control. Calli derived from MC-treated protoplasts showed no growth aberrations and the ability to regenerate phenotypically normal, fertile plants was retained.

The selection of recombinant Kan^R clones derived from a homozygous SR1hph2 line revealed a significant, dose-dependent stimulation of intrachromosomal recombination (Table 3 and Fig. 4*B*). At the most effective dose (10 μg/ml), the RRF was 9-fold higher than in nontreated controls. Cell survival was comparable to that of untreated cells. Similar results were obtained with MC-treated protoplasts of a hemizygous SR1hph2 line (data not shown).

To examine the stimulation of intrachromosomal recombination *in planta*, we precultured apical shoot segments on medium containing MC at concentrations of 2.5–25 μg/ml (see *Materials and Methods*). Only the apical shoot segments precultured on MC concentrations of 2.5 and 5 μg/ml formed roots. The resulting plants were used for preparation of protoplasts. Protoplast plating efficiencies did not differ from those of untreated control plants—i.e., cell survival was not affected by the MC doses used for shoot pretreatment. Cell clones derived from protoplasts were selected for kanamycin resistance. The mean number of intrachromosomal recombination events observed at concentrations of 2.5 and 5.0 μg/ml was 1.5- and 1.6-fold that found in control plants, respectively. The frequencies were calculated as the mean RRF values of two independently treated plants; two independent populations from each plant, each of one million protoplasts, were assayed for RRF. One possible explanation for the relatively low RRF increase *in planta* is limited penetration of MC throughout the plant tissues and thus lower uptake. It is also possible that the presence of MC in the medium stimulates recombination events early in development that are reflected in the increased number of Kan^R clones after protoplasting.

Effect of Heat Shock on Recombination. The direct damage of DNA by x-rays or MC may explain the activation of recombinational repair. It was of interest to ask whether other drastic stresses that threaten cell survival could induce a recombination response.

Protoplasts of a homozygous line of SR1hph2 were treated for various time periods at two elevated temperatures (45°C and 50°C) (Table 4). Survival of cells was affected only at the higher temperature. The absolute number of Kan^R clones decreased with increasing time of treatment but significantly less than cell viability, as shown by the number of surviving clones. Hence there was a significant temperature-dependent increase of RRF values (Table 4 and Fig. 4*C*). However, it may be that the apparent stimulation of intrachromosomal recombination is a result of the selection of recombination-proficient clones during harsh heat shock conditions.

Molecular Analysis of the Recombined Clones. Five to 10 randomly chosen Kan^R clones from each stimulatory treatment (in total 60 clones) were analyzed by Southern blot hybridization. The DNA of all analyzed clones contained the diagnostic 1245-bp *Eco*RV fragment (Fig. 1) resulting from homologous recombination but no 1815-bp fragment (Fig. 3 and further data not shown).

DISCUSSION

We have examined the frequencies of intrachromosomal homologous recombination between close genomic repeats in plants and addressed the following questions. (*i*) Is the recombination frequency correlated with the number of copies of recombination substrates present in the genome? (*ii*) Do mutagenic treatments or stresses applied to plant cells influence the frequency of homologous recombination?

The RRF of hemi- and homozygote lines containing three copies of the recombination substrate per locus (8) was compared. The presence of recombination substrates on both homologous chromosomes at the same location eliminated a possible position effect on the recombination frequency since both sets of recombination substrates are placed in a similar chromosomal environment (14–16). RRF values were proportional to the number of copies of recombination substrates in hemi- and homozygotes. This agrees with results from a mammalian system (15) and with recent data from plants (17, 18) where a different method for scoring recombination frequencies was used (9, 17).

The stability of the cell genome is achieved by high-fidelity replication and the efficient repair of accidental damage. However, when the amount of damage exceeds the excision repair capability, recombinational repair can be induced (3–7). Recombination repair may itself influence genomic stability and it is, therefore, of interest to determine levels of recombination induced in plant cells and compare them to other higher eukaryotes.

Ionizing radiation causes the biochemical modification of DNA bases and double-strand breaks in the DNA (19, 20). Since damaged DNA needs to be repaired before replication, the activity of the repair–recombination system is stimulated under these conditions. This has been well documented for prokaryotes and yeast (6, 21). Mammalian cells, however, do not react to ionizing radiation by increased recombination frequency (1). In contrast, in the present experiments with plant cells, the lower radiation doses applied (1.25 and 2.5

Table 4. Influence of elevated temperature on RRF

Duration of treatment, min	45°C				50°C			
	Total no. of clones/10^6 pps	No. of Kan^R clones/10^6 pps	RRF	% of control	Total no. of clones/10^6 pps	No. of Kan^R clones/10^6 pps	RRF	% of control
0	634×10^3	90	14×10^{-5}	100				
2	469×10^3	87	18×10^{-5}	128	373×10^3	113	30×10^{-5}	214
5	658×10^3	78	12×10^{-5}	85	131×10^3	93	71×10^{-5}	507
10	484×10^3	74	15×10^{-5}	107	53×10^3	48	91×10^{-5}	650

The RRF values for each temperature step are the means of three independent experiments, each including three populations of 10^6 protoplasts (pps).

Gy) induced reciprocal recombination to approximately twice the control level. Therefore the rate of stimulation was similar to that of nonreciprocal conversion events (17). With higher doses (10 and 20 Gy), cell survival decreased and the RRF dropped below the level of untreated samples. The repression of RRF at high doses can be explained by the "δ effect" described in yeast (22). The δ effect postulates different probabilities of clone formation by recombinant and nonrecombinant cells. Since we only scored recombination of a particular set of repeats (recombination substrates), other events caused by radiation (including recombination within other chromosomal segments) remained undetected. It is, therefore, possible that recombination-proficient clones have increased genomic instability and thus reduced survival.

In contrast, MC had no lethal effects within the dose range optimal for stimulation of RRF. MC is a bifunctional alkylating agent that crosslinks DNA strands (23). Besides its mutagenic activity (24), MC activates mitotic recombination in *Ustilago maydis* and *S. cerevisiae* (25). It also increases the frequency of crossovers in *Drosophila melanogaster* (26) and interchanges within chromosomes of human leukocytes (27). More recently, it was shown that low doses of MC stimulate homologous recombination between chromosomal repeats in mouse L cells (1). In plant cells, the dose-dependent enhancement of RRF by MC reached a maximum of 919% of the control with 92% survival at a MC concentration of 10 μg/ml. This most effective dose for plant cells is $\approx$10-fold higher than that used for mammalian cells. Different cell types and experimental setups cannot easily be compared. It is, however, possible to relate the increase in RRF to cell survival in both systems. In mouse L cells, MC at 1.5 μg/ml reduced survival to 71% with a maximal increase in RRF to only 278% of the control (at 2 μg/ml, RRF = 243% and survival = 59%) (1). Since plant cells react to MC with a higher frequency of recombination accompanied by almost no reduction in survival, this may indicate the efficient repair of DNA damage largely by induced recombinational repair. As a consequence, the degree of genomic instability under stress conditions would be different in plants and animals due to the different levels of induced recombination.

It is known that other stresses provoke alteration of chromosomal information in plant somatic cells (28, 29). Plants are often naturally exposed to thermal stress, with temperatures in the range of 40–45°C causing the well-studied cascade of heat shock responses (30). It has also been suggested that elevated temperatures influence the efficiency and nature of chromosomal repair and recombination events in *U. maydis* (31). Furthermore, raising the temperature to 40°C for 10 min has been reported to protect against chromatid aberrations induced by chemical mutagenesis and x-rays in root tips of *Vicia faba* (32). Data from several other systems also suggest either inhibition or stimulation of DNA repair processes. These somewhat contradictory results may be explained by the different degrees of heat stress used in particular experiments (for a review, see ref. 33).

Temperatures known to induce the heat shock reaction (45°C) (32) had no influence on either RRF or survival. However, incubation at 50°C for 5 or 10 min increased the RRF severalfold. This increase was accompanied by reduction in survival to $\approx$8%. This could mean that recombination reactions are activated only during extreme environmental threat, inducing a kind of SOS response (for a review, see ref. 7). The observed RRF increase could further be due to the preferential survival of recombination-proficient clones during or after heat shock.

Hemi- and homozygote strains showed similar levels of induced recombination. Thus each copy of the recombination substrate has a similar probability of recombination and this increases in a similar way upon stress exposure.

In summary, it may be concluded that the level of homologous intrachromosomal recombination in plant somatic cells is not constant but can be influenced by various extracellular stimuli. The nature and extent of the interaction between chromosomal repeats seem to be markedly different between plants and mammalian cells. Since, in plants, somatic progeny of genetically altered cells can participate in the formation of generative tissues, high levels of induced recombination may be an important factor in the adaptation of plant populations to modified environments.

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