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FINAL REPORT
PROJECT NO. IS-1631-89

**Asymmetric Hybridization in Crop Plants: Studies on
Cellular and Genetic Mechanisms and Transfer of Viral
Resistance**

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1995

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4447 0

Standard BARD Cover Page for Scientific Reports

DATE: JANUARY 1995

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Bet Dagan, ISRAEL

BARD project No. 15-1631-82

Title:

Asymmetric hybridization in crop plants:

Studies on cellular and genetic mechanisms and transfer of

viral resistance.

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Project's starting date: July 1990

Type of Report: 1st Annual ☐ 2nd Annual ☐ Final ☒

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630.72
BARD/ZEL
מחל
הספריה המרכזית
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בית דגן

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Abstract

Somatic hybridization is a well established technology for generating novel genomic combinations between sexually isolated species. Agricultural applications in this field are particularly promising for asymmetric hybrids, that result when chromosome elimination (ChE) is induced in one of the parental genotypes, prior to somatic fusion. Results from several laboratories indicate that ChE in asymmetric hybrids is often incomplete and unpredictable.

This project concentrated on studies aimed at providing an in-depth understanding of the cellular processes that lead to ChE and the formation of asymmetric hybrids. A model system was established for evaluation of ChE in Nicotiana asymmetric hybrids, based on a donor genotype carrying two unlinked marker genes (the "Sulphur" mutation, and a transgenic locus harboring NPT II and GUS), whose expression can be detected in somatic hybrid calli and plants. Following irradiation of the donor genotype and fusion, hybrid calli were selected based on expression of one of the markers. ChE was subsequently monitored by screening the hybrids for loss of expression of the unselected marker.

Intraspecific and interspecific asymmetric hybrids were produced, by using N. tabacum or N. plumbaginifolia as donor genotypes, respectively.

Intraspecific asymmetric hybrids: Following irradiation with 10, 30 or 100 kR, the GUS transgene was lost at frequencies of 71.5%, 86.7% and 88%, respectively. Three distinct classes of hybrid calli were observed: those regenerating exclusively GUS-positive shoots, those regenerating exclusively GUS-negative shoots, and chimeric calli that regenerated both positive and negative shoots at different ratios. The time in culture before onset of regeneration did not to affect chromosome elimination levels for the three irradiation doses under study.

Interspecific asymmetric hybrids: ChE was determined by marker segregation (as described above) and by dot blot hybridization using a dispersed, plumbaginifolia-specific repetitive DNA sequence as a probe. An exponential increase in the amount of ChE as the radiation dose is increased from 5 to 50 krad was detected, and ChE continued during extended callus culture. The radiation dose response is similar whether ChE is measured by dot blot hybridization or by loss of the Su allele.

Alternative treatments for ChE were evaluated. Preliminary results indicated that exposure of protoplasts to isopropyl-N(3-chlorophenyl) carbamate (CIPC) - a compound reported to interfere with organization of the mitotic spindle - induced a low but consistent elimination of the GUS marker in protocalli and regenerated plants.

With regards to an additional objective of the proposal (namely, the transfer from tobacco to tomato by asymmetric hybridization of the N gene for resistance to TMV), two preparatory studies were performed:

1. A N. tabacum line was identified in which the endogenous TMV-resistance gene and an introduced kanamycin-resistance gene are closely linked (4-5 map units apart)
2. Optimization of tomato genetic transformation was studied for a tomato genotype most suitable for in-vitro genetic manipulations, due to its compact growth habit and short generation time.

Objectives of the original research proposal

The long-term goal of this project is the development of reliable technology for introgression of chromosomes or chromosomal fragments between sexually isolated taxa. It is anticipated that this genetic enhancement will undoubtedly have a practical impact on breeding of crop plants.

The specific goals of this project are:

1. An in-depth study of the cytological determinants in the process of asymmetric hybridization.
2. A detailed biochemical assessment of alien chromosomal introgression in tomato.
3. The optimization of asymmetric hybridization in agricultural crops by means of an extensive evaluation of novel tools for chromosome elimination.
4. The asymmetric transfer of the *Nicotiana* N-locus, coding for hypersensitive resistance to TMV, into tomato cultivars.
5. An assessment of the expression and genetic stability of the introgressed N-locus.

The project was funded with the stipulation that the research concentrate on objective 1.

Report on research activities

INTRODUCTION

a brief description of the research activities

1. Model Systems for Evaluation of ChE.

Our approach uses plants carrying two marker genes, each on separate chromosomes, whose expression can be detected in somatic hybrid calli and plants. Following irradiation and fusion, ChE can be monitored by evaluating the hybrids for loss of expression of one or both markers. The markers are the β -glucuronidase (GUS) gene linked to kanamycin resistance (KmR) (introduced by *Agrobacterium*-mediated transformation), and the "sulphur" mutation of tobacco (and its corresponding wild type allele). The "sulphur" (Su) locus of *N. tabacum* is a semidominant nuclear trait that affects chlorophyll production in all aerial organs. Recessive genotypes are yellow, while heterozygous plants are pale green. Complementation of Su via somatic hybridization has been previously demonstrated for *Nicotiana* species. The experimental design is as follows: Recessive Su/Su plants are used as the source of recipient protoplasts. Normal green *Nicotiana* species, wt for the Su locus and transgenic for GUS, serve as the donor genotype. Donor protoplasts are irradiated and fused with the recipient genotype. Green colonies (putative heterokaryons) are identified on regeneration medium and checked for GUS expression. Treatments that enhance ChE should result in a greater fraction of green colonies that are negative for GUS. Alternatively, hybrids can be recovered by selection for KmR and scored for their ability to green; here,

treatments increasing ChE should result in a larger fraction of KmR calli that are sulphur colored. For the sake of a complementary approach, the Israeli and the American groups concentrated on intraspecific (N. tabacum + N. tabacum), or interspecific (N. tabacum + N. plumbaginifolia) somatic hybrids, respectively.

2. Intraspecific fusions between Su/Su tobacco and KmR-GUS tobacco (ARO)

A genetic linkage analysis in donor genotypes was performed, in order to confirm that the two markers (GUS and su) segregate independently. Following fusion, calli regenerating green shoots (putative hybrids) were identified, and the shoots assayed for GUS. Several shoots were usually regenerated from each callus. Based on the information gathered from 3278 regenerants (representing 279 individual calli), the following observations can be stated:

1. Irradiation with increasing doses resulted in higher levels of chromosome elimination. Following irradiation with 10, 30 or 100 kR, GUS-negative shoots were detected at frequencies of 71.5%, 86.7% and 88%, respectively.
2. Most of the hybrid calli gave rise to both green and yellowish shoots, suggesting the su (normal green) locus derived from the irradiated parent is also undergoing elimination.
3. Three distinct classes of hybrid calli were observed: calli regenerating exclusively GUS-positive shoots, calli regenerating exclusively GUS-negative shoots, and chimeric calli that regenerated both positive and negative shoots at different ratios.
4. The time in culture doesn't seem to affect chromosome elimination levels for the three irradiation doses under study.

The results are detailed in the following (unpublished work) and are being prepared for full publication.

3. Interspecific fusions between Su/Su tobacco and KmR-GUS N. plumbaginifolia (FSU)

Protoplasts of N. plumbaginifolia carrying a single KmR-GUS, T-DNA insertion were irradiated and fused with Su/Su N. tabacum. Hybrids were recovered by selection for kanamycin resistance. Two methods have been used to analyze the extent of ChE in the hybrids: 1) Dot blot hybridization using a dispersed, species-specific repetitive DNA sequence as a probe. This probe hybridizes to all of the chromosomes of the donor, N. plumbaginifolia, but does not hybridize to chromosomes or DNA of N. tabacum (Piastuch and Bates, 1990). With this probe, dot blots of genomic DNA isolated from the hybrids reveal the relative amount of N. plumbaginifolia DNA in the hybrids, from this data the extent of donor ChE can be calculated (Piastuch and Bates, 1990), 2) the fraction of yellow:green hybrid calli can also be used as a measure of donor chromosome elimination. We have found that: 1) there is an exponential increase in the amount of ChE as the radiation dose is increased from 5 to 50 krad. After a 50 krad dose the hybrids retain, on average, 20%-30% of the donor DNA six months after fusion. This is an amount equal to 4-5 donor chromosomes. However, there is considerable variation in the extent of ChE from hybrid to hybrid. Even after a 50 krad dose some hybrids retain

60% of the donor DNA. 2) The radiation dose response of ChE is similar whether ChE is measured by dot blot hybridization or by loss of the SU allele. This observation suggests that all donor chromosomes have an equal probability of being eliminated in Nt (+) Np asymmetric hybrids. 3) ChE continues during extended callus culture, even after 18 months ChE is still occurring. 4) plant regeneration is not associated with a significant change in donor DNA content, however, elimination of donor chromosomes continues after regeneration. These observations 1) - 4) were recently published (Trick et al., 1994)

4. Linkage between the TMV-Resistance Gene (N Gene) and the KmR Gene in Tobacco (FSU and ARO).

We have identified a N. tabacum line in which the endogenous TMV-resistance gene and an introduced kanamycin-resistance gene are linked. Prior to this project Bates' laboratory created a group of transformed N. tabacum plants, heterozygous for the N gene, which carried a single KmR (NPT II) gene. Preliminary backcrosses had identified a number of transformants in which KmR and the N gene were potentially linked. To verify linkage, we grew KmR/TMV-R, F1 progeny to maturity and backcrossed a second time. This second round of backcrosses has now led to the identification of one transformed plant line in which KmR and the N gene are closely linked, they are only 4-5 map units apart. This linkage has been confirmed independently in both Zelcer's and Bates' laboratories (Bates and Zelcer, 1992).

5. A compact tomato genotype suitable for in-vitro genetic manipulations (ARO).

In vitro genetic manipulations require genotypes that perform well in tissue culture. In addition, genetic analyses of novel traits are facilitated by use of genotypes with short generation times. MicroTom (Royal Sluis Co.), a "basket-type" tomato with outstanding short generation time (60-70 days from seeding till harvesting of first fruits) was evaluated for this purpose. MicroTom has the additional convenience of having a very compact growth habit, making it appropriate for growth under in vitro or growth chamber conditions. Adventitious regeneration from leaf, hypocotyl or cotyledon explants was attempted in MS based media, containing various levels of hormones. Best shoot regeneration was obtained in leaf explants cultured in MS medium containing the following hormone combinations (in mg/l): zeatin (0.5) and IAA (0.05), or BAP (3.0) and IAA (0.1).

Best development of callus tissue was observed in MS medium supplemented with 2,4-D (1.0) and kinetin (1.0). In vitro grown seedlings served as source for mesophyll protoplasts. Isolation, culture and regeneration of protoplasts was performed basically as described by Shahin (Theor. Appl. Genet. 69:235). Good plating efficiency (>20%) and efficient regeneration was observed with MicroTom protoplasts. Leaf disk transformation with *Agrobacterium tumefaciens* harboring a disarmed binary Ti construct (NPT II-GUS) was optimized for MicroTom explants. (Zelcer, et al., manuscript in preparation).

Report on research activities Unpublished work

A model system for evaluation of chromosome elimination in intraspecific somatic hybrids between Su/Su tobacco and KmR-GUS tobacco

D. Wolf, G. Bates, A. Zelcer (full manuscript in preparation)

1. Segregation of the Sulfur and the GUS loci in donor tobacco plants.

A prerequisite for the validity of the model proposed in studies of chromosome elimination (see Introduction), is that both loci should be located on different chromosomes. At present, no genetic map has been constructed for tobacco, and therefore the absolute position of the loci cannot be determined. However, a classic linkage analysis could provide the needed information. We therefore produced specific crosses to check for genetic linkage of the traits.

A cross was made between a tobacco line heterozygous for Su (Su/su) and the tobacco line hemizygous for the transgenic GUS locus (G/-) that we intended to use in our studies. The observed segregation of the expected phenotypes in the progeny of this cross are detailed in table 1.

Table 1: Segregation of the Su and the GUS loci in progeny
of the cross Su/su, -/- X su/su, G/-

Genotypes (phenotypes)	su/su (green)	Su/su (yellow-green)
G/- (GUS positive)	35	40
-/- (GUS negative)	39	46

The segregation data is not significantly different from a 1:1 ratio for each trait, confirming thus their monogenic nature. In the next step, plants with the genotype Su/su, G/- were test-crossed with a wild type tobacco, in order to assay for genetic linkage among the traits. The segregation of the expected phenotypes in the progeny of this cross are detailed in table 2.

The observed ratios agree very significantly with the expected segregation ratios for two independent and unlinked loci. We concluded that the donor genotype is an appropriate model for evaluating chromosome elimination.

Table 2: Segregation of the Su and the GUS loci in progeny of the cross Su/su, G/- X su/su, -/-

Genotypes (phenotypes)	su/su (green)	Su/su (yellow-green)
G/- (GUS positive)	27	21
-/- (GUS negative)	23	23

2. Studies on evaluation of chromosome elimination

The rationale and experimental design of these experiments were already discussed in the Introduction. In the following, a brief description of the model system is included.

Table 3: Fusion frequencies and phenotype complementation in asymmetric hybrids between sulphur and irradiated wt tobacco protoplasts.

Dose (Kr)	Total No. protoplasts ($\times 10^6$)	% fusion	Estimate No. calli ($\times 10^3$)	No. green calli	Complementation frequency ($\times 10^2$)
10	2.5	nt	3.0	20	0.6
	1.9	3.7	1.0	19	1.9
	1.8	6.8	1.0	15	1.5
	1.8	4.5	1.0	20	2.0
	1.8	6.0	1.0	10	1.0
	2.3	4.6	1.0	17	1.7
30	2.0	nt	1.5	24	1.6
	4.0	nt	2.5	41	1.6
	2.0	nt	1.0	23	2.3
100	2.0	nt	nt	18	nt
	1.2	0.8	1.0	14	1.4
	1.2	3.7	1.0	14	1.4
	1.4	4.3	1.5	15	1.0
	2.5	2.4	2.0	29	1.4

Fourteen electrofusion between protoplasts from a recipient tobacco genotype (Su/Su, -/-) and a donor genotype (su/su, G/-) were performed. Prior to fusion, donor protoplasts were irradiated with doses denoted in table. Percentage of cell fusion was estimated by microscopic examination, aid by the distinct phenotype of parental protoplasts. An estimate of the number of developing calli was made at transfer time. Complementation frequency is defined as the percentage of green calli (resulting from correction of the sulfur mutation by the donor su locus) among the total calli population.

A fusion was made between protoplasts of a recipient tobacco line and a donor line. The donor genotype is a tobacco line carrying a homozygous GUS locus, introduced by genetic transformation, and is wild type for the sulfur locus (normal green color of leaves). The recipient tobacco line is homozygous for a sulfur mutation, that induces chlorophyll deficiency. Protoplasts from the donor line were irradiated with several doses of x-rays prior to fusion, in order to study the effect of irradiation on the extent of chromosome elimination. Fusion products were identified based on the correction of the sulfur mutation of the recipient. Chromosome elimination in fusion products was estimated by assaying for activity of a second locus of the donor-the transgenic GUS gene.

Table 3 summarizes the data obtained from several fusion experiments.

Green calli (putative fusion products) were subsequently transferred to regeneration medium. From the numerous regenerants, 6-12 shoots from each individual callus were selected for assay of GUS activity. A total of 3278 shoots representing 279 individual calli were assayed. The results of this screening are shown in table 4.

Table 4: The effect of irradiation dose on the elimination of the GUS locus in regenerants of fusion products

Experiment	Dose (kR)	Total No. shoots	GUS ⁻ shoots	% elimination
1	10	246	192	78.05
2	10	225	172	76.44
3	10	180	130	72.23
4	10	234	170	72.65
5	10	120	86	71.66
6	10	204	118	57.85
Total		<u>1209</u>	<u>868</u>	aver.= 71.55±2.9 a
7	30	258	221	85.66
8	30	497	431	86.73
9	30	254	223	87.79
Total		<u>1009</u>	<u>875</u>	aver.= 86.73±0.6 b
10	100	222	198	89.19
11	100	168	152	90.48
12	100	156	142	91.03
13	100	172	148	86.05
14	100	342	285	83.33
Total		<u>1060</u>	<u>925</u>	aver.= 88.00±1.5 b

Green calli (putative fusion products) obtained as described in table 1 were transferred to regeneration medium. From each individual callus, 6-12 shoots were assayed for GUS activity with the MUG substrate (fluorimetric assay). Percentage of elimination is defined as the proportion of GUS negative shoots from the total shoot population. Statistical significance between averages is denoted by small letters.

A statistically significant difference was detected in the marker elimination frequency obtained after a 10 kR irradiation, as compared with the higher doses (30 and 100 kR). No significant differences were detected between the two highest irradiation doses.

On the level of individual calli resulting from fusion products, three distinct groups were detected: calli regenerating exclusively GUS⁺ shoots ("All positive" calli), calli regenerating exclusively GUS⁻ shoots ("All negative" calli), and calli regenerating both positive and negative shoots ("chimeric calli"). The distribution of these three classes among the different radiation doses is summarized in table 5

Table 5: The distribution of calli regenerating all GUS⁺ or all GUS⁻ shoots, or a mixture ("chimeric calli") among the different radiation doses.

Dose (kR)	Total No. hybr. calli	All negative hybr. calli	All positive hybr. calli	Chimeric hybr. calli
10	101 (100%)	27 (26.7%)	8 (7.9%)	66 (65.3%)
30	88 (100%)	34 (38.6%)	0 (0%)	54 (61.4%)
100	90 (100%)	35 (38.9%)	1 (1.1%)	64 (71.1%)

In all cases, the chimeric calli represent the most abundant class. A more informative picture is obtained when data is plotted as calli distributed by the percentage of GUS elimination in shoots arising from each individual callus, for the different radiation doses (fig 1). For higher doses (30, 100 kR) most calli concentrate in the 60 to 100% elimination bracket, while in the low dose (10kR), a substantial proportion of the calli population show elimination percentage <60%. Individual calli differed in the timetable for shoot regeneration. Some minicalli regenerated soon after transfer to regeneration medium, while others required a longer callus phase and additional transfers before the onset of shoot regeneration. This situation enabled us to evaluate whether the length of the callus phase has any effect on the eventual level of chromosome elimination. Table 6 summarizes the data for two duration times: a fast regeneration response (up to 75 days from plating date), and a longer callus phase (longer than 75 days, and up to 150 days).

Table 6: The effect of culture time before shoot regeneration on the percentage of chromosome elimination

Irradiation dose (kR)	Short culture time		Long culture time	
	Total assayed shoots	% GUS ⁻ shoots	Total assayed shoots	% GUS ⁻ shoots
10	288	77.1	921	70.7
30	258	86.1	751	87.6
100	622	87.2	438	87.9

No significant differences were observed between these two classes (short or long culture time), for the different irradiation doses.

Achievements regarding the proposed objectives of the ongoing project.

Our studies have focused on developing model genetic systems for monitoring chromosome elimination (ChE) during asymmetric hybridization. We have used these models to study the timing of ChE, how it is affected by radiation dose, culture conditions, and plant regeneration. A preliminary evaluation was made of alternative approaches for the induction of ChE, particularly UV irradiation and the use of microtubule depolymerizing agents.

With respect to our second original objective, the transfer of TMV resistance from tobacco to tomato, we have carried out two studies: the first was a genetic characterization of tobacco plants in which the TMV resistance gene and the KmR gene are closely linked. The second study documents the utility of a tomato genotype - the cultivar MicroTom - for cell culture, protoplast fusion, and transformation experiments.

Our studies contributed to a better understanding of the process of ChE throughout the development of asymmetric somatic hybrids, and have revealed the dynamics of elimination in intraspecific as well as interspecific fusion products. Our preparatory studies for the fusion-mediated transfer of the N gene may be used in the future for the intergeneric transfer of a valuable trait that may not be directly selected for in hybrids.

Cooperation

The Israeli PI visited the FSU lab, for coordination of activities. A close cooperation between the participating laboratories was maintained throughout the different phases of the project: preparation of the proposal, designing of the experimental schemes, exchange of complementary biological material, analyses of results and preparation of joint publications. The body of the report describes in detail complementary approaches that were undertaken for fulfilling the experimental goals. Two joint articles were published, and one additional joint manuscript is being prepared for publication.

Publications

Journal articles

- G. W. Bates (1992) Molecular analysis of nuclear genes in somatic hybrids. *Physiol. Pl.* 85: 308-314.
- G. W. Bates and A. Zelcer (1992) Molecular tagging of the tobacco chromosome carrying the TMV-resistance gene (N gene) by Agrobacterium-mediated transformation. *Theor. Appl. Gen.* 83: 981-986
- H. Trick, A. Zelcer, and G. W. Bates (1994) Chromosome elimination in asymmetric somatic hybrids: effects of gamma dose and time in culture. *Theor. Appl. Genet.* 88: 965-972
- A.M.A. Wolters, H.C.H. Schoenmakers, J.J.M. van der Meulen-Muisers, E. van der Knaap, F.H.M. Derks, M. Koornneef and A. Zelcer (1991) Limited DNA elimination from the irradiated potato parent in fusion products of albino Lycopersicon esculentum and Solanum tuberosum. *Theor. Appl. Gen.* 83: 225-232

Abstracts of lectures in scientific meetings

- A. Zelcer, D. Wolf and G.W. Bates (1993) Protoplast fusion for crop improvement. In: Abst. Israel-Brazil Symp. on Agricultural Biotechnology, Feb. 22-23, NCRD, Jerusalem.
- A. Zelcer, D. Wolf and G.W. Bates (1993) Asymmetric somatic hybridization as a tool for the transfer of virus resistance in agricultural crops. In: Abst. Israel-France Binat. Symp. of Plant Virology, Apr. 14-15, INRA, Paris.
- D. Wolf, G.W. Bates, and A. Zelcer (1994) The use of marker genes as a tool for the quantitative evaluation of chromosome elimination in asymmetric somatic hybrids. Abst. VIIIth Int. Congr. of Plant Tissue & Cell Culture, June 12-17, IAPTC, Firenze

Manuscripts in preparation

- A. Zelcer, D. Wolf and C. Weksler. A compact tomato genotype suitable for in-vitro genetic manipulations (in preparation)
- D. Wolf, G. W. Bates and A. Zelcer. A model system for evaluation of chromosome elimination in intraspecific somatic hybrids between Su/Su tobacco and KmR-GUS tobacco (in preparation)