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Increase in the rate of recombinants in tomato (*Lycopersicon esculentum* L.) after in vitro regeneration *

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Summary. Modification to the cross-over (C. O.) rate of tomato (*Lycopersicon esculentum*) was attempted by using in vitro plant regeneration. F1 hybrids with the same genetical homozygous background were compared at two loci: *"bs-ms32"* on chromosome I, and *"aa-d"* on chromosome II. For each, the genetic distance separating the two markers was about 20 to 30 map units. One cotyledon of each F2 hybrid seedling was used as in vitro tissue culture material, while the rest of the plantlet was grown as a control. Recombination rates of the selfed progenies from each regenerated and matched control couple were compared. For the first set of markers 59,000 seeds were analysed (5 controls' and 7 regenerated progenies), and for the second, 11,000 (5 controls' and 8 regenerated progenies). There were significant increases in the genetic distance between markers in about half the regenerated individuals. For the first set the increases ranged from 6.07 to 6.91 units out of a control distance of the 19.84 to 25.65, corresponding to lengthenings of 30.59 to 35.29%. For the second set they ranged from 4.92 to 6.04 out of a control distance of 25.05 to 26.57, representing increases of 19.64 to 22.75%. Such a phenomenon can be important either from a fundamental or practical viewpoint, regarding selection efficiency in plants, and potential for gene reassortment.

Key words: Tissue culture – Regeneration – *Lycopersicon esculentum* – Recombinant rate – Crossing-over

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Introduction

One of the most important genetic events is that of recombination and the resulting new organization of the genes. However, little is known about the regulation of this phenomenon. The possibility of increasing the rate of recombination is of fundamental interest in plant improvement when chromosome breakage and separation of close-linked genes is desired.

Culturing somatic tissues of diploid species in vitro has produced new phenotypes in several species apparently related to new gene regulation (De Mars 1974; Siminovitch 1976; Skokut and Filner 1980). These include homozygous lettuce (Sibi 1974, 1976), species like maize (Green and Phillips 1975), alfalfa (Reisch and Bingham 1979), red clover (Keyes et al. 1980) and homozygous tomato (Sibi 1979, 1981, 1982; Nsika-Mikoko 1982). In lettuce and tomato regenerated individuals, genetic analyses have shown heritable changes consistent with modifications occurring in cellular parts not associated with codantnuclear DNA (Sibi 1981, 1982).

Granted that regulatory mechanisms can affect the control of crossing-over (Barker et al. 1976) and that recombinant individuals are related to this phenomenon, could in vitro tissue culture and regeneration be used as tools for modifying the rate of recombination?

Materials and methods

Choice of markers in 'Monalbo' tomato cultivar

There were two independent experiments, each utilizing two different genes. Phenotypically distinguishable recessive marker genes were chosen to be in the 'Monalbo' homozygous genetic background.

For the first experiment, the two genes were located on chromosome I, namely *bs* (brown seeded and germinating inhibition) and *ms32* (male sterile), the genetic distance of which is 30 map units; for the second experiment, the marker genes were located on chromosome II, namely *aa* (anthocyanin absent) and *d* (dwarf), 20 map units apart.

The genetical composition of the former parents was *bs/bs* and *ms32/ms32* and for the latter *aa/aa* and *d/d*. The two expected hybrids were then respectively (*ms32/+*, *+/aa*) and (*aa/+*, *+/d*).

Genetic analysis was made on the selfings of these two types of hybrids according to whether they had or had not been regenerated after in vitro tissue culture.

Mating design

The female homozygous parent was (*ms32*) for the first set and (*d*) for the second.

After the young flowers were emasculated, they were pollinated two or three times. Seeds were extracted from the fruits and cleaned.

Cotyledons tissue culture of the hybrids

The regeneration medium was that described by Sibi (1981) supplemented by IAA (indol acetic acid) $2 \cdot 10^{-7}$ and zeatin 10^{-7} for the initiation of culture, and by IAA $5 \cdot 10^{-7}$ and zeatin 10^{-7} (all these are concentrations) for the transferred callus.

Terminology

The standard, regenerated or total of the progenies are indicated by "Stan", "Reg" or "Tot", respectively.

The standards are named with a capital letter (i.e. = B) and the respective regenerated plants, with a small letter supplemented by a number corresponding to the number of transfers before the callus regenerated the plant (i.e. = for the B control, the "b1" plant emerged from the first transfer of the in vitro tissue culture, the "b2" plant from the second transfer, etc.). For the second experiment a dash is added (i.e. = B′, b′1, b′2).

Description of the experimental design

As 42,600 individuals were required for analysis, successive identical trials were conducted during the year, each being composed of several randomized blocks with the same number of individuals, growing in an heated greenhouse in winter. Thus, greenhouse trials comprised 31,600 plants analyzed in 6 blocks while outdoor trials contained 11,000 plants analyzed in 2 blocks.

Each plant was grown in a peat "giffty-pot" ($\varnothing = 8$ cm); so the distance between two plants was 8 cm.

The data were taken when plants were young as to avoid competitive effects.

Characteristics observed

For the selfed progenies, the characters analyzed in situ were those resulting from the marker genes.

Phenotypically, the first set *bs-ms32* belonging to chromosome I gives two parental categories *+ ms32*, *bs +* and two recombinant ones *+ +* and *bs ms32*. Of these, the *bs* phenotype corresponds to the homozygous recessive situation; it can be observed in the fruit as soon as seeds are mature (endosperm coloration) but as it hinders germination, analysis of *bs +* and especially of *bs ms32* progenies is not possible. Data were therefore first obtained from the number of brown seeds resulting from selfing of hybrid flower, and then from the number of (*ms32*) phenotypes shown by the individuals after germination of the yellow seeds.

For the second set *aa-d* belonging to chromosome II all individuals, either homologous or heterozygous, could be analysed.

Statistical analysis

The statistical studies were made by comparing the rate of recombinants for the progeny of each standard against that of each corresponding regenerated plant.

The rate of recombined genotypes was obtained by the maximum likelihood method (Mather 1965).

A "one way" analysis of variance was first made on the total of the progenies (Tot) and then separately for the two following groups: the standards (Stan) and that of regenerated plants (Reg).

A two means comparison was made for these two groups using Student's *t*-test for equal variances then for unequal variances and finally by the pairing method (Dagnelie 1969).

Duncan's multiple range test was used to verify the significance of differences between means (Dagnelie 1969).

Results

Morphological characteristics of the standard and regenerated F_1 plants

Regeneration of one or two plantlets was obtained for a series of five controls for either (*bs/+*, *+/ms32*) or (*aa/+*, *+/d*) hybrids: this represents eight regenerated plants for the former and seven for the latter.

At this stage of the experiment the controls were true to type, while one to three of the observed characteristics were affected in each regenerated plant. These were mainly internode length and shape of the leaves. These results agree with those previously reported for tomato epigenic variants (Sibi 1974, 1980, 1981, 1982; Nsika-Mikoko 1982).

Segregation of the markers in the F_2 generation

It must be pointed out that the number of seedlings to be analysed increases as the genetic distance between two chosen markers diminishes. The statistically significant difference revealed is thus smaller. For example, to be significant at the 5% level, a variation of one tenth out of 30 C.O.u. (Crossing Over units) requires observation of 8,000 seedlings and for markers separated by 1 C.O.u., 38,400 individuals should be analysed. Thus, in the present experiment, the choice of markers was based on the medium proximal distance on the same arm of a chromosome, and on the possibility of obtaining parents with the same genetical background.

Some problems arose from the first set *bs-ms32*, as the brown seeds (*bs/bs*) did not germinate and direct observation of the double recombinant *bs ms32* category was therefore not possible. Because of a lack of time and greenhouse space, the backcrosses could not be made, and estimated values were obtained from the other categories as previously mentioned. Thus, out of the 59,000 F_2 seeds which were analysed to detect *bs* homozygosity after segregation, more than 31,600 seedlings were grown to check the rest of the categories.

Table 1. Recombinant frequencies from F1 progenies

a *bs–ms* Stan	Reg	Difference	Significance
A = 0.1893	$a_1 = 0.2205$	0.0312	0
	$a_2 = 0.1228$	0.0445	0
B = 0.2085	$b_1 = 0.2591$	0.0607	+
C = 0.2085	$c_1 = 0.2390$	0.0305	0
D = 0.1816	$d_1 = 0.1457$	0.0641	+
	$d_1 = 0.1730$	−0.0086	0
E = 0.2065	$e_1 = 0.2249$	0.0691	+
	$e_2 = 0.2756$	0.0691	+
$\bar{x}_s = 0.1969$	$\bar{x}_r = 0.2340$	d = 0.0387	
b *aa–d* Stan	Reg	Difference	Significance
A′ = 0.2449	$a'_1 = 0.2482$	0.0033	0
B′ = 0.2657	$b'_1 = 0.2916$	0.0259	0
	$b'_2 = 0.3261$	0.064	+
C′ = 0.2505	$c'_1 = 0.3154$	0.0558	+
D′ = 0.2505	$d'_1 = 0.2997$	0.0492	+
E′ = 0.2741	$e'_1 = 0.2587$	−0.0154	0
	$e'_2 = 0.2873$	0.0132	0
$\bar{x}_s = 0.2589$	$\bar{x}_r = 0.2854$	$\bar{d} = 0.0275$	

The frequencies are calculated by maximum likelihood method The significance is given according Duncan's multiple range test at the 5% level: + significant; 0 non significant

For the second set, *aa-d,* all categories were represented in the 11,000 seeds that were sown.

In each case, the genetic distance observed between the two markers for the Reg and Stan progenies, and the respective modifications, are listed in Table 1 a and b. Table 3 shows the higher values and the respective percent of lengthening for both experiments.

These increases in rates of recombinants are respective to the short genetic space included between the chosen markers. For the first set they ranged from 6.07 to 6.91 u., respectively, to a standard distance of 19.84 to 25.65 u. This corresponds to an increase of 30.59% to 35.29%. For the second set, the increase ranged from 4.92 to 6.04 u. out of the standard distance of 25.05 to 26.57 u., representing a relative genetic distance lengthening of 19.64% to 22.73%.

The analysis of variance (Table 2) revealed no significant differences between the Stan group progenies; on the contrary, Tot and Reg progenies contained significant (5%) differences.

The recombinant rate of the Stan and of the Reg group (respectively 0.1969 and 0.2340 on the one hand, 0.2589 and 0.2854 on the other) were significantly (< 5% level) different when tested by any of the three statistical methods. The mean genetic distance of each pair of markers was therefore less for the Stan than for the Reg progenies.

The means comparison were made by the multiple range test of Duncan and in neither case did the controls differ from one another significantly. Tables 1 a

Table 2. "One way" analysis of variance

Group	Source	d.f.	Square sum	Mean square	F calculated
bs – ms32 Stan	progeny	4	0.00314	0.000785	0.983
	block	5	0.02350	0.0047	5.886**
	error	20	0.01597	0.0007985	
	total	29	0.04261		
bs – ms32 Reg	progeny	7	0.03905	0.0055786	3.094*
	block	5	0.10093	0.020186	11.196***
	error	35	0.0631	0.0018029	
	total	47	0.20308		
bs – ms32 Tot	progeny	12	0.06759	0.0056325	3.531***
	block	5	0.107779	0.0215580	13.513***
	error	60	0.09572	0.0015953	
	total	77	0.27110		
aa – d Stan	progeny	4	0.00109	0.0002725	0.306
	block	1	0.00001	0.00001	0.0112
	error	4	0.00356	0.00089	
	total	9	0.00466		
aa – d Reg	progeny	6	0.00956	0.0015933	20.77***
	block	1	0.00033	0.000333	4.30
	error	6	0.00	0.0000767	
	total	13	0.0035		
aa – d Tot	progeny	11	0.01610	0.0014636	3.93*
	block	1	0.00027	0.00027	0.72
	error	11	0.0041	0.0003727	
	total	23	0.02047		

Tot: total of the progenies
Stan: progenies from the standards
Reg: progenies from the regenerated plants
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Table 3. Summary of the lengthenings of the genetic distance, statistically significant (< 5%) according to the multiple range test of Duncan

Pair of markers	Genetic distance		Lengthening	
	Stan	Reg	L.	L. Stan %
bs – ms	B = 19.84 u	$b_1 = 25.91$ u	6.07 u	30.59%
	D = 18.16 u	$d_1 = 24.57$ u	6.41 u	35.29%
	E = 20.65 u	$e_2 = 27.56$ u	6.91 u	33.46%
aa – d	D′ = 25.05 u	$d'_1 = 29.97$ u	4.92 u	19.64%
	C′ = 25.05 u	$c'_1 = 21.54$ u	5.58 u	22.27%
	B′ = 26.57 u	$b'_2 = 32.61$ u	6.04 u	22.73%

u = crossing-over units (C.O.u.), or % of recombinants
Stan = standard progenies
Reg = Regenerated plants progenies
L = Lengthening of the genetic distance

and b show that the Reg progenies were either grouped with the Stan ones, or located above. Thus, the progenies e2, b1 and d1 on the one hand, and b'2, c'1 and d'1 on the other, were therefore either significantly superior to the Stan or identical, but they were never inferior.

Discussion

According to previous reports *bs* is 17 maps units and *ms32* 47 map units from the extremity of chromosome I, so the two markers *bs-ms32* should be expected to be 30 map units apart. Under the present conditions, the genetic distance measured between the markers for the standards ranged from 18.98 to 20.65 C.O.u. with a mean value of 19.68 C.O.u. Thus, the actual proportion of recombinants observed was less than expected. This stresses the importance of using a real standard as a comparative means of recording the "cross-over × environment" interaction. This is confirmed by the "block" effect observed in the present analysis of variance (the "one way" model used does not express the "progeny-block" interactions). In fact, this may be due to the *ms32* gene, as the expression of male sterility gene often seems to depend on the environmental conditions.

For the second set of markers, *aa-d,* the genetic distance for the standards ranged from 24.49 to 27.41 C.O.u. with a mean value of 25.89 and this is slightly higher than the expected 20 C.O.u. value calculated from the map.

However, since each (group) of regenerated plant(s) arose from the aseptic in vitro culture of one cotyledon while the rest of the young plant was grown as control, all the present values are standardized by comparison with the matched control.

Among the progenies of the seven regenerated plants in the first experiment or the eight in the second (compared to the five respective controls) three in each case showed a significant increase in the genetic distance between the two markers while the other progenies were similar to the controls.

Of the six Reg progenies exhibiting this increase, only two (e2 and b'2, e.g. one in each case) were regenerated after a second in vitro cycle; the others were regenerated during the first cycle. Because of the few "late-regenerated" individuals analysed, we can only conclude that a statistically significant higher number of recombinants can be obtained for plants even regenerated after a short period of in vitro tissue culture.

Therefore, the present experiment provides evidence that in vitro tissue culture can act on C.O. regulation and modify its local (Pandey 1972) control.

Even so, it is relevant to ask whether the present genetic lengthening of 19.64% to 20.77% (*d-aa*) and of 30.59% to 35.29% (*ms32-bs*) occurred only for the distance between the respective markers or also on the whole genome, or even if, in the other parts of the genome there was a decrease of recombinant rate in counterparts. Moreover, it might be possible to determine whether the enhanced values correspond to some physical modifications (DNA amplification, rearrangements, etc.) or to more frequent breakages related to new types of regulation either on C.O. or meiosis.

To conclude, the present phenomenon could well be exploited in selection processes such as elimination or insertion of genetic segments which may be close-linked to important characters (Demarly 1979), in the creation of new gene combinations after crossing, from which either parents or hybrids with high potentialities might be obtained. This type of experimentation of in vitro tissue culture could also be used in the fundamental study of C.O. which has to be modified to be more closely understood.

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