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Study of two different recombination events in maize cmsT-regenerated plants during reversion to fertility

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Summary. The change of phenotype from sterility to fertility for some cmsT callus tissue culture regenerated plants and their progenies has been correlated with changes in their mitochondrial genome. Those changes that have been analyzed here are the result of recombination events. Two different sets of repeated sequences have been found to be involved in those recombination events. The most common one is a recombination through a 127-bp repeat between various independently isolated revertants. The second one is a recombination through a 58-bp repeat. In every case the products of recombination containing the *urf13* gene have been deleted.

Key words: Maize – mtDNA – Male sterility – Recombination

Introduction

Maize plants with the T-cytoplasm are male sterile (unless the restorer nuclear genes are present), and are also highly susceptible to the T-toxin excreted by the fungal pathogen *Helminthosporium maydis*. The toxin alters the membrane permeability of the cmsT maize mitochondria. The cytoplasmic male-sterile phenotype is very stable as no reversion has been observed in the field, although the cmsT maize plants represented nine-tenths of the U.S. maize crop in the late 1960s. When plants were regenerated from tissue culture (either with or without the toxin selection) derived from cmsT immature embryos, a fair proportion of them were male fertile and toxin resistant (Gengenbach et al. 1977, 1981; Brettell et al. 1979, 1980). Only the two phenotypes, male fertile/toxin resistant or male sterile/toxin sensitive, out of the four possible combinations have been found. Studies of the restriction fragment profile of mtDNA from those regenerated plants have indicated that the phenotype change was also correlated to a change in the mitochondrial genome (Brettell et al. 1982; Umbeck and Gengenbach 1983). An XhoI 6.6-kb fragment unique to cmsT (Gengenbach et al. 1981; Brettell et al. 1982) has been replaced by a 6.2-kb XhoI fragment in the revertant (Fauron et al. 1987; Abbott and Fauron 1986; Wise et al. 1987), with the exception of a mutant T4 (Umbeck and Gengenbach 1983; Wise et al. 1987) and mutant V18 (this paper). In vitro protein synthesis using isolated cmsT mitochondria revealed the presence of a 13-K polypeptide unique to the T-cytoplasm (Forde et al. 1978; Forde and Leaver 1980). Fertility restoration of the cmsT plants in the presence or the restorer of fertility nuclear genes *Rf1* and *Rf2* suppresses the synthesis of this polypeptide (Forde and Leaver 1980).

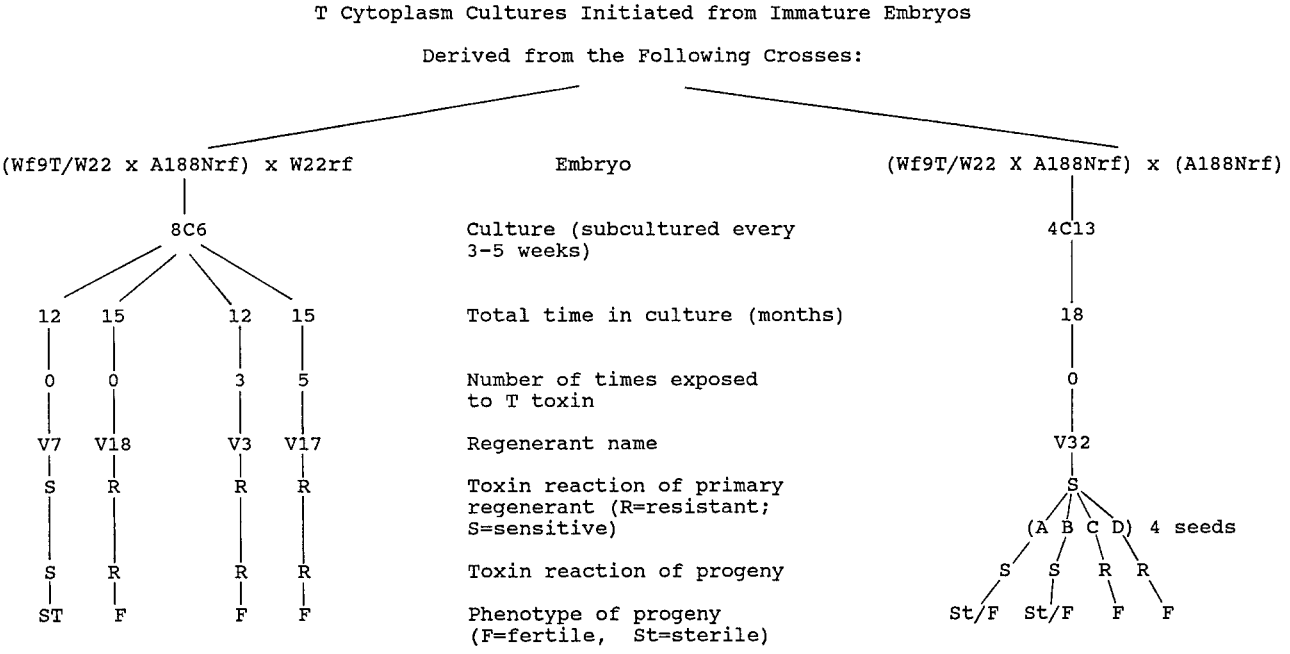
In 1986 Dewey et al. showed that a 345-bp open reading frame named *urf13*, unique to cmsT mtDNA, could encode a 13-K polypeptide. This locus is located on the 6.6-kb XhoI fragment just mentioned, and has been strongly correlated with the male-sterile phenotype (Dewey et al. 1986, 1987, 1988; Wise et al. 1987), it is either deleted (Fauron et al. 1987; Rottman et al. 1987) or truncated (Wise et al. 1987) in the male-fertile regenerated progenies. Recently, it has been shown that the deletion of *urf13* locus in the revertant V3 is the result of a recombination event involving two sets of repeats (Fauron et al. 1990). In order to better understand the recombination event, this region was studied in the progenies (three sterile and three fertile) of various cmsT tissue culture regenerated plants.

Materials and methods

Maize lines

Maize tissue culture and plant regeneration have been described previously (Brettell et al. 1979, 1980). Regenerated plants and

Table 1. Details of cmsT tissue culture regenerated plants and analysis of their progenies



their progenies were tested for toxin sensitivity and pollen development as described previously (Brettell et al. 1980). The genotypes used as a source of T-cytoplasm tissue culture were the backcross of a three-way hybrid. Details on the lines are given in Table 1. The fertile lines V3, V18, V32C, V32D were propagated by repeated self-fertilization. The sterile lines V32A, V32B were propagated by hybridization with V32D pollen, while V7 was fertilized with V3 pollen.

mtDNA purification

Mitochondria were purified from 50 to 100 g of seedlings grown in the dark by differential centrifugation as in Kemble et al. (1980). The pellets were lysed into a TRIS/EDTA/SDS buffer, then deproteinized with proteinase K and run into two CsCl gradients.

mtDNA libraries. Plasmid libraries of V18, V32A, V32B, V32D, V7, V17 were made into the BamHI site of vector puc8. Cosmid clone libraries using vector pwe15 (Wahl et al. 1987) or vector pHC79 (Hohn and Collins 1980) were also constructed for the regenerants V3, V18, V32A, V32B, V32D, V7.

DNA analysis. Miniprep or maxiprep DNA extraction, restriction enzyme digests, agarose gel electrophoresis, transfer of DNA to MSI membrane, labelling of DNA, and filter hybridization were as in Fauron et al. (1989).

Sequencing. The DNA was purified as minpreps. Double-strand DNA sequencing was done using the chain termination method as in Chen and Seeburg (1985) with the sequenase enzyme (United States Biochemical Corporation).

Results

As already shown (Fauron et al. 1987) there has been a shift in the 6.6-kb XhoI fragment of cmsT mtDNA to a fragment of 6.2-kb in various fertile revertants. The change has been more precisely mapped onto a 1.5-kb AvaI fragment in cmsT that is replaced by a 2.1-kb AvaI fragment in the revertant. This 2.1-kb AvaI fragment is very similar to one present in the normal fertile cytoplasm. This shift is the result of a recombination event (Fauron et al. 1987) involving a 127-bp repeat (Rottman et al. 1987): one copy of the repeat starts 4 bp away from the 3′ end of *urf13* and ends within the *urf25* reading frame; the other copy is located 248 kb away (coordinate 153 on the cmsT map; Fauron et al. 1989). The sequences of the cmsT DNA 1.5-kb AvaI fragment were compared to the sequences homologous in N and V3 mtDNA; they were found to diverge between the *urf13* and *urf25* coding regions (Qin et al. 1987), resulting in the deletion of the *urf13* locus (Fauron et al. 1987; Rottman et al. 1987). Recently, it was shown in the V3 genome that the *urf13* gene is lost in a recombination involving two sets of repeats (Fauron et al. 1990: the 127-bp repeat (R2) and the 4.6-kb repeat (R1). The latter has one copy located 70 bp 5′ of the *urf13* locus and the other copy 83 kb away (coordinates 523 and 440 on the cmsT map; Fauron et al. 1989). A combined analysis of the mapping and sequencing data shows that 0.423 kb of DNA including the *urf13* gene have been eliminated on the V3 genome.

Regenerants used in this study

In order to understand the recombination mechanism responsible for the phenotypic change (i.e., sterile → fertile), various revertants originating from different tissue

Table 2. Results of the test for toxin sensitivity and pollen development for the four linese derivded from the V32 primary regenerated plant originating from tissue culture 4C13 (see also Dixon et al. 1982)

	Regenerant V32			
	Scored as fertile/sensitive			
	Self pollinated			
	4 seeds			
	A	B	C	D
	sensitive	sensitive	resistant fertile (>90% anthers extruded and shedding pollen)	resistant fertile (>90% anthers extruded and shedding pollen)
	~55% anthers extruded and shedding pollen	~70% anthers extruded and shedding pollen		
	Self pollinated	Self pollinated	Self pollinated	Self pollinated
	32A	32B	32C	32D
Toxin reaction (uniform in each case)	sensitive	sensitive	resistant	resistant
Progeny tested	16	16	18	18
Completely sterile	14	6	0	0
Partly fertile	2	6	5	0
Fully fertile	0	4	11	18
			2 tassels aborted	

cultures grown in the presence or absence of the T-toxin were analyzed. Some sterile regenerants obtained from the same callus were used as controls for this study. The two cultures, 8C6 and 4C13, were initiated from immature embryos derived from the three-way hybrid (Wf9T/W22 × A188Nrf) × W22rf and (Wf9T/W22 × A188Nrf) × $(A188rf)^2$, respectively (Table 1). Plant regeneration was induced from sublines after a period of 12 months (V3, V7), 15 months (V17, V18), and 18 months (V32). V3 and V17 were the only regenerants studied in this paper that were exposed to the T-toxin during the culture phase C. V32 regenerated from culture 4C13 produced two apical inflorescences with spikelets containing some well-developed fertile anthers. A leaf assay classified this plant as sensitive to T-toxin (Brettell et al. 1980). Self-pollination resulted in the recovery of four seeds – A, B, C, D – that gave the lines V32A, V32B, V32C, V32D. Plants obtained from V32A and V32B were toxin sensitive; plants from V32C and V32D were toxin resistant. They were also scored for pollen fertility (Dixon et al. 1982 and Table 2).

Analysis of mtDNA

Southern blot hybridization analysis of AvaI-digested mtDNAs from those various regenerant plants were probed with the cmsT AvaI 1.5-kb fragment (Fig. 1). V7, V32A, V32B that are male sterile/toxin sensitive still contain the 1.5-kb AvaI fragment as in cmsT mtDNA, while V3, V17, V32C, V32D that are male fertile/toxin resistant have a 2.1-kb AvaI fragment replacing the 1.5-kb fragment. V18, which is also fertile/toxin resistant, differs in that it contains a 1.25-kb AvaI fragment instead of the 2.1-kb fragment. Therefore, in five independently isolated revertant lines, four (V17, V18, V32C, V32D) were assumed to have undergone the same recombinational event as identified for V3, while the same region in V18 has been rearranged in a different manner and will be analyzed in the last paragraph of this paper. The three other regenerants (V7, V32A, V32B) that have remained sterile do not seem to differ from their progenitor cmsT. The other AvaI fragments of 1.0 kb and 3.7 kb that also hybridize with the probe have been identified previously (Fauron et al. 1987) and are the same in all plants examined here.

Mapping and sequencing

The 2.1-kb AvaI fragment from V3 mtDNA used as a probe against the revertant cosmid libraries identified for each revertant the expected two sets of cosmids. The set b_1 (Fig. 2) contained the 1.7-kb AvaI fragment as found

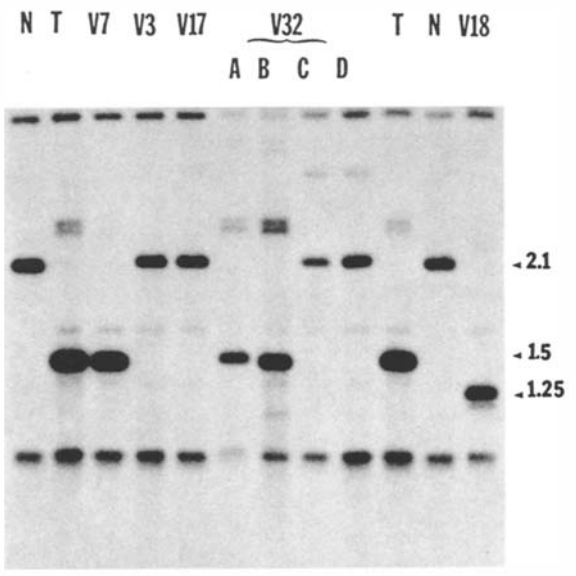


Fig. 1. Southern blot hybridization of AvaI-digested mtDNA from various maize lines (as marked above the figure) probed with the V3 1.5-kb AvaI fragment. The 1.5-kb AvaI fragment in T, V32A, V32B has been replaced by a 2.1-kb AvaI fragment in the revertant V3, V17, V32C, V32D and a 1.25-kb AvaI fragment in revertant V18. Hybridization to other bands common to all genomes has been described in Fauron et al. (1987)

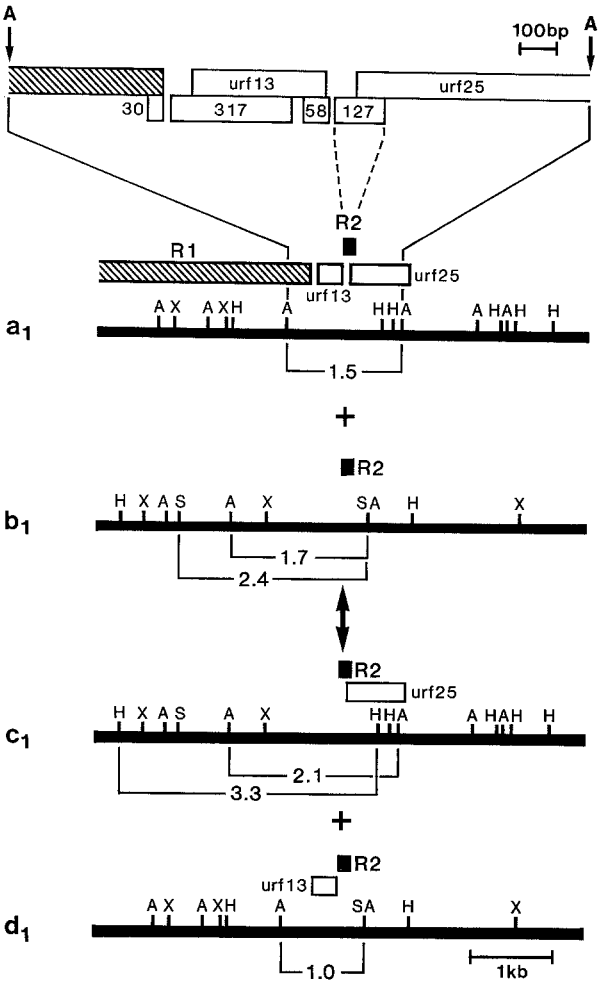


Fig. 2. AvaI (*A*), HindIII (*H*), XhoI (*X*), SmaI (*S*) restriction map of the four products of recombination (a1, b1, c1, d1) through the repeat R2 of 127 bp. A precise location of the repeat located 3′ of *urf13* is shown on a diagram (amplification 5 ×) at the top of the figure. Located are also the 58-bp repeat with the other copy located within the coding region of the *rrn26* gene (Dewey et al. 1986) and a 317-bp repeat also found on the 3′ flanking region of the *rrn26* gene. Hybridization studies have shown that only the forms b1 with the AvaI 1.7-kb fragment and C1 containing the AvaI 2.1-kb fragment are present in the revertants mtDNA. a1 and d1, characterized by the fragments AvaI 1.5-kb and AvaI 1.0-kb, have been eliminated

in cmsT and the set C_1 contained the 2.1-kb AvaI fragment itself. The 2.1-kb AvaI represents the junction fragment obtained from recombination via the 127-bp repeat between the AvaI-1.5 kb and AvaI-1.7 kb fragments of cmsT (Fig. 2).

In order to understand the details of the recombination event that appears to be identical in four different regenerated plants and responsible for the sterility → fertility reversion, we decided to sequence this novel junction containing a copy of the 127-bp repeat. The 3.3-kb HindIII and 2.4-kb SmaI fragments covering almost entirely the AvaI 2.1-kb fragment and entirely the AvaI 1.7 kb (Fig. 2), were subcloned into the vector puc18 and sequenced with the dideoxy method. They do not contain any open reading frame and will be published elsewhere. Synthetic primers located on each side of the 127-bp repeat near the *urf13* gene were used for sequencing the repeat and to compare with V3 the crossover point in the other revertants. The sequences of the various versions of the R2 repeat are aligned in Fig. 3. The sequence is identical for V3, V17, and V32D. As already mentioned by Rottman et al. (1987), the recombination has taken place within the 54-bp core sequence [bp 45 to 98 of the repeat or bp 1,569 to 1,695, using Dewey et al. (1986) notation].

Comparison of the sequence of the 127-bp repeat of the sterile regenerants V7, V32A, V32B shows 100% identity with cmsT.

Analysis of V18 revertant

V18 is fertile like V3 or V32D, but differs in that it does not contain the AvaI 2.1-kb fragment characteristic of the recombination event through the 127-bp repeat. A library of pwe15 cosmid clones from V18 mtDNA was probed with the Ava 1.5-kb fragment. Positive clones were mapped with the three restriction endonucleases BamHI, XhoI, and SmaI. This led to the identification of three different sets of cosmid clones. It identifies the ATPase 6 gene region and the *rrn26* gene region, as in the other revertant and already explained in Fauron et al.

Fig. 3. Nucleotide sequence of the repeat R2 and its immediate surroundings. The repeat R2 itself has been *underlined*. The base pair numbering is as in Dewey et al. (1986). The sequence differs from Dewey et al. at position 1565 just before the beginning of the repeat, where a G resides is deleted in our sequence, and at position 1603–1604, where we have the sequence CG instead of GC. These differences are consistent in our results, as they have been found in all the maize line studied here. As already stated by Rottman et al. (1987), R2copy1 and R2copy2 have a completely homologous sequence of 55 bp central to the repeat, while mismatches are observed in each side. The repeats in V3, V32D, V17 recombinants are identical to each other. Their left side is identical to cmsTcopy2 and their right side to copy 1. * Located 3′ end of *urf13* locus. Identical in sterile regenerant V7), V32A, V32B. + Located coordinate 153 on cmsT map (Fauron et al. 1989). # From recombinant c1 on Fig. 2. Identical in the fertile revertant V3-V32D-V17

(1987), and a novel set of sequence rearrangements containing the *urf25* gene and the 5′ end of the ribosomal *rrn26* gene. As shown in Fig. 4, this represents a recombinant molecule that arose from a recombination event taking place within the 58-bp repeat common to the *rrn26* gene and *urf13* gene. Sequencing through this repeat confirms the results (Fig. 5). The 58-bp repeat within the *rrn26* gene (position 1,055–1,110, Dale et al. 1984) and the *urf13* locus (position 1,507–1,564, Dewey et al. 1986) have three mismatches (1 substitution, 2 bp deletions) within the first 11 bp, then a perfect identitiy for the remaining 47 bp. The recombination in V18 has taken place within the perfect homology of the two repeats as V18 match the 26S region on the left (Fig. 5). This arrangement is accompanied by the elimination of the *urf13* – containing parental and recombinant type.

Conclusion

The presence of the *urf13* gene that codes for the 13-K polypeptide has been shown to be responsible for the male-sterile phenotype (Dewey et al. 1986, 1987, 1988; Wise et al. 1987). No reversion to fertility has been observed in the natural population in the open field. However, callus tissue culture from which are derived the regenerant progenies analyzed in this study appears to induce the loss or the truncation of the *urf13* gene that is associated with the reversion to fertility. The mechanism by which various recombination events change the organization of the mitochondrial genome resulting in the loss of the *urf13* locus has been described in a different paper (Fauron et al. 1990).

Two different recombinational events have been identified here. The most common one occurs within the 127-bp repeat as described for V3 (Fig. 2, Qin et al. 1987), giving rise to a 2.1-kb AvaI fragment. It has now been identified in ten different independently isolated revertants (data not shown). The other recombination event involving the 58-bp repeat located within the coding regions of the *rrn26* gene and *urf13* gene has been found in only one instance: the V18 revertant. It would not be surprising that the analysis of more revertants would reveal recombination events taking place within

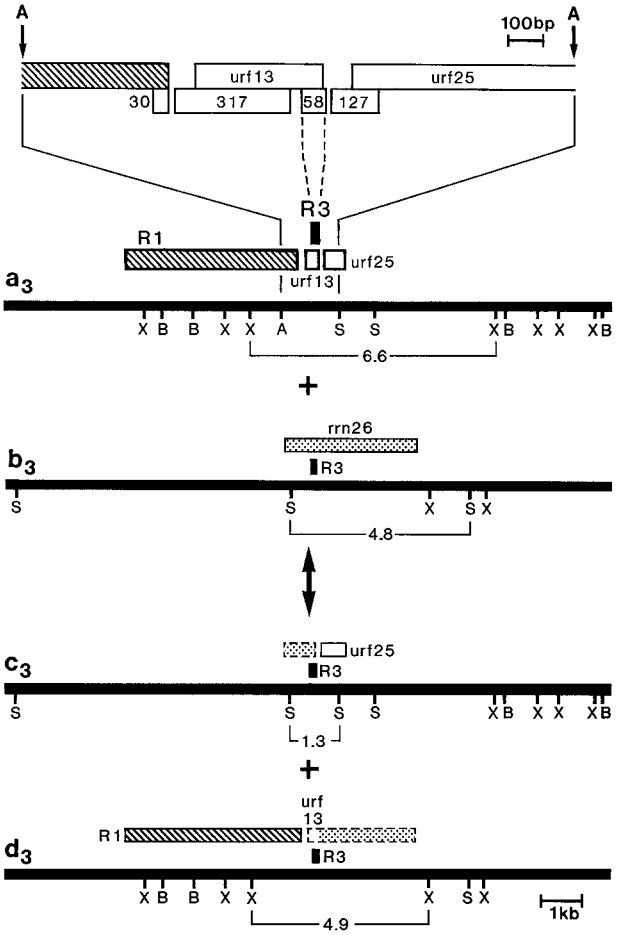


Fig. 4. Recombination in the revertant V18 mtDNA. BamHI (*B*), XhoI (*X*), SmaI (*S*) restriction enzyme map of the four recombination products (a3, b3, c3, d3) through the repeat R3 of 58 bp. A precise location of the repeat is shown at the top of the figure with a tenfold increased scale. Hybridization studies have shown that only the forms b3 and c3 are identified by SmaI fragment 4.8-kb and 1.3-kb. Forms a3 and d3 characterized by the fragments XhoI 6.6-kb and XhoI 4.9-kb have been deleted

the 317-bp repeat (Fig. 2). Or is it surprising that no such recombination was observed although the repeat is larger?

The V32 primary regenerant represents a mutant where the somatic assortment has not as yet been completed in the germ line (mother cell that give the progeny seeds). It is a heteroplasmic situation as defined by Pring et al. (1988), where a single cell harbors more than one mitochondrion type or a mitochondrion more than one genome type. When segregation is accomplished, the plants that have reverted to fertile phenotype are no longer sensitive to the T-toxin (Table 2), fail to synthesize significant levels of a 13-K polypeptide specific to cmsT plants (Dixon et al. 1982), and have lost their *urf13* locus.

In order to define the precise location at which the recombination function is resolved and to know if it is identical for various revertants, we sequenced three junctions within the 2.1-kb AvaI fragment from three independently isolated revertants (V3, V17, V32D) from two different crosses. The three sequences were identical (Fig. 3). The recombination has operated on a 55-bp stretch of DNA where there is complete homology between the two repeats. The mechanism of genetic exchange between the two parental DNA stretches where recombination takes place does not induce any new mutation or reassortment of nucleotide that is different from the parental strand except for the crossing-over.

In order to know if tissue culture is likely to induce mutation in a region that seems favorable to recombinational changes, three regenerated sterile plants were also sequenced throughout their 127-bp repeat located 3′ of the *urf13* locus. No changes were observed when compared to the cmsT parental cultivar.

Although tissue culture does not always induce changes in the *urf13* region, this study allowed us to state that the *urf13* changes, when they do occur, are caused by

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                                                    1507
                                                     |
          gly arg ser asn asp gly ser thr leu ser lys pro lys tyr
urf13     GGT CGA AGC AAC GAC CAA TCC ACT CTC TCT AAG CCT AAG TAT
                                  .. ... ... .  ... ... ... ...
V18*      TTT ACG CGA GAA AGG GGC ACC ACC CTC T-- AAG CCT AAG TAT
          ... ... ... ... ... ...  .. ... ... ... ... ... ... ...
rrn26     TTT ACG CGA GAA AGG GGG ACC ACC CTC T-- AAG CCT AAG TAT
                                    |
                                  1055
                                                          1564
                                                           |
          ser ser met thr asp ser val gln val pro           |
urf13     TCC TCA ATG ACC GAT AGC GTA CAA GTA CCG TGA GGGACCAAA
          ... ... ... ... ... ... ... ... ... ... .. ......
V18       TCC TCA ATG ACC GAT AGC GTA CAA GTA CCG TGA GGACCAAA
          ... ... ... ... ... ... ... ... ... ... ... ... ..
rrn26     TCC TCA ATG ACC GAT AGC GTA CAA GTA CCG TGA GGAAAGGTG
                                                     |
                                                   1110
urf13     GTCTCCCTTTCTCTTT
          ................
V18       GTCTCCCTTTCTCTTT
rrn26     AAAAGAACCCCAATCG
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Fig. 5. Nucleotide sequence of the repeat R3 and its surrounding sequences. The base pair numbering is as in Dewey et al. (1986) for *urf13* and *rrn26*. The R3 repeats between the *urf13* and *rrn26* gene differ only at position 1516 and 1517. The repeat in recombinant molecule of V18 is identical to the *rrn26*-R3 repeat to the left side and identical to *urf13*-R3 on the right side. * From recombinant C3 on Fig. 4

the tissue culture process alone rather than toxin selection, since the V3 and V32D plants, one grown in the presence of toxin, the other without, show exactly the same DNA rearrangement, resulting in the formation of the 2.1-kb AvaI fragment. The variations analyzed here are part of a more complex rearrangement of the master chromosome, resulting in a deletion and duplication of a discrete part of the genome aimed at eliminating the deleterious *urf13* gene. However, as mentioned by Kemble et al. (1982) and also from our observations, a few other discrete sequence alterations have been observed in some of the revertants.

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