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Identification of new mitochondrial genome organizations in wheat plants regenerated from somatic tissue cultures

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Summary. Plants have been regenerated from short- and long-term in vitro somatic tissue cultures made from immature embryos of the hexaploid wheat cultivar "Chinese Spring". The mitochondrial genome organization of each regenerated plantlet was studied, after one selfing, by probing Sal I-restricted total DNA with cloned Sal I fragments of wheat mitochondrial DNA derived from a segment of the genome, which displays marked structural changes in response to in vitro culture. Short-term in vitro cultures give rise to regenerated plants whose mitochondrial genome organization is either close to that of the parental cultivar or to that of embryogenic callus cultures, except for a single plant which has an organization resembling that of short-term non-embryogenic cultures. In contrast, all but one of the plants regenerated from long-term cultures exhibited a mitochondrial genome organization similar to that of long-term non-embryogenic cultures. In addition, extra labelled bands were detected in some of the regenerated plants with two of the probes used. These results emphasize the importance of the duration of the in vitro step preceding the regeneration process: the longer it is, the higher the probability is of obtaining mitochondrial DNA variability in regenerated plants. Furthermore, since increasing the duration of the in vitro stetp results in the production of regenerated plants with a mitochondrial genome organization resembling that of non-embryogenic tissue cultures, the question is thus raised as to whether regeneration from long-term cultures is suitable for use in plant breeding.

Key words: Mitochondrial DNA – Chondriome variability – In vitro culture – Plant regeneration – Wheat

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Introduction

The use of plant cell and tissue cultures has become important not only for plant propagation and haploid breeding but also in the application of the techniques of biotechnology (Rhodes et al. 1988). Growing cereal plants from either somatic (Maddock and Semple 1986) or haploid cells (De Buyser et al. 1987) has already provided new potential for plant improvement. In the future, the successful application of techniques such as protoplast fusion and transformation to wheat improvement will largely depend on our ability to routinely regenerate whole green plants from single cells or protoplasts.

In both gametic and somatic tissue cultures, the ability to regenerate is modified by the environmental growth conditions of the donor plant, by the type of explant and by the culture media used. Nevertheless, there is now considerable evidence that genetic factors are major contributors to the in vitro response of cereal tissue in culture. Indeed, several chromosomes seem to be involved in the control of regeneration in wheat (Zhang and Li 1984; Henry and De Buyser 1985). In addition, alien cytoplasm stimulates the androgenetic regeneration process (Picard et al. 1978). In the case of short-term somatic tissue cultures, shoot regeneration seems to be determined by a polygenic system (Galiba et al. 1986; Mathias and Fukui 1986; Higgins and Mathias 1987), and the capacity to organize shoot primordia is modified by the cytoplasmic background (Mathias et al. 1986). Furthermore, the lack of any significant correlation between regeneration frequencies in anther and in somatic tissue cultures has been demonstrated (Agache et al. 1988).

During the past few years, several studies have examined the rearrangements of the mitochondrial genome which occur either in in vitro cell and tissue cultures (McNay et al. 1984), or in plants regenerated from in

vitro cultures of calli (Gengenbach et al. 1981) or protoplasts (Kemble and Shepard 1984). We have recently shown that the mitochondrial genome of wheat embryogenic somatic tissue cultures initiated from immature embryos of the cultivar "Chinese Spring" underwent structural variations mainly characterized by the loss of two Sal I restriction fragments and also by either a relative decrease or a relative increase in the amounts of several other fragments (Hartmann et al. 1987, 1988). Moreover, it appeared obvious that the relative frequency of these variations increased as a function of time in culture, until an equilibrium was reached. The question was thus raised as to whether plants regenerated from short- and long-term cultures retained these rearrangements of the mitochondrial genome organization or whether new rearrangements were induced by the regeneration process itself.

In this paper, we report a molecular study of the mitochondrial genome organization of plants regenerated after in vitro culture of 4 and 14 months, using mitochondrial DNA probes for segments known to undergo changes during the in vitro step (Hartmann et al. 1987).

Materials and methods

Regeneration of plants

As described in a previous paper (Rode et al. 1988), a new nomenclature for callus cultures and regenerated plants has been adopted. For callus cultures, this nomenclature takes into account the number (x) of subcultures (SC) preceding their study and is denoted SC^x. For regenerated plants (R), the number of subcultures which the calli have undergone before regeneration is shown by a superscript suffix (^{x}) and the number of selfings before study by a subscript suffix ($_y$). For example, a plant regenerated after six subcultures ($x=6$) and having undergone three selfings ($y=3$) will be denoted $R^6{}_3$.

Somatic tissue cultures, initiated from immature embryos of the wheat cultivar "Chinese Spring", were subcultured as previously described (Rode et al. 1987a). Calli obtained after 4 months and 14 months of culture (SC^1 and SC^6 calli, respectively) were subdivided and transferred onto a regeneration medium [MS medium devoid of 2,4-D (Murashige and Skoog 1962)]. Regenerated plantlets were grown under greenhouse conditions and were selfed once before isolation of their total DNA.

Isolation of total DNA

Total DNA from the parental "Chinese Spring" cultivar and from individual plants regenerated after the first and after the sixth subculture and having undergone one selfing ($R^1{}_1$ and $R^6{}_1$ plants, respectively) was prepared as described by Dellaporta et al. (1983) with minor modifications (Rode et al. 1987b).

Electrophoresis and Southern blotting

Samples of total DNA and of control mt- and ctDNA were digested with Sal I and fractionated by electrophoresis in vertical 0.8% agarose gel slabs in TAE buffer (Maniatis et al. 1982). A typical restriction pattern of wheat Sal I mtDNA has been published elsewhere (Rode et al. 1987a). The nomenclature adopted for Sal I restriction fragments is that of Quétier et al. (1985). The nomenclature for Sal I ctDNA fragments is that of Bowman et al. (1981). DNA transfers (Southern 1975) were carried out using Hybond™-C Extra membranes (Amersham).

DNA-DNA hybridizations

Southern transfers were probed with Sal I restriction fragments derived from a library of wheat mtDNA clones (kindly supplied by Dr. B. Lejeune) as described by Rode et al. (1987a).

Results

We had previously shown (Rode et al. 1987a, 1988; Hartmann et al. 1987) that the region of the wheat mitochondrial genome encompassing the repeated sequences numbers 5 and 10 (RS5 and RS10) undergoes gross structural changes in non-embryogenic as well as in embryogenic somatic tissue cultures initiated from immature embryos. This particular region is shown schematically in Fig. 1. Briefly, Sal I restriction fragments J', K_1, K' and M on the one hand, and E_1, G_3, K_3 and N_3 on the other hand, arise through reciprocal recombination events which occur in RS5 and RS10, respectively. In addition, fragment X_2, which does not encompass any recombinogenic repeated sequence, connects either J' or K' to either N_3 or K_3, as shown by data derived from cosmid cloning (Falconet et al. 1984; Lejeune, personal communication). Under these conditions, probing Sal I-restricted DNA with, for example, K', K_3 and X_2 allows one to analyze the whole region. Our previous results had, in fact, shown that there was a loss of fragments E_1 and K_3 in

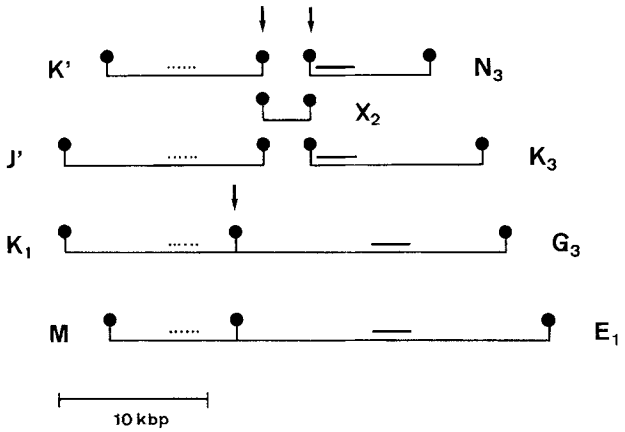

Fig. 1. Schematic representation of the fraction of the wheat mitochondrial genome undergoing gross variations in callus cultures. The Sal I restriction fragments are designated according to Quétier et al. (1985). ●: Sal I site. · · · · ·: recombinationally active repeated sequence no. 5. ——: recombinationally active repeated sequence no. 10. *Arrow:* location of Sal I sites where multiple possibilities of Sal I fragment arrangements can occur (according to Falconet et al. (1984) and Lejeune, personal communication)

long-term embryogenic somatic tissue cultures initiated from cv. "Chinese Spring", and of fragments E_1, K_3, J', K', N_3 and X_2 in long term non-embryogenic cultures initiated from cv. "Aquila". However, these fragments were consistently diminished – but still present – in corresponding short-term (SC^0 and SC^1) callus cultures (Rode et al. 1987a, 1988 and unpublished results; Hartmann et al. 1987).

Plants regenerated from short-term cultures

Sal I-restricted total DNA, prepared from the parental cultivar and from six individual plantlets regenerated after the first subculture, was probed, after gel electrophoresis and Southern transfer, with the labelled cloned Sal I mtDNA fragments K', K_3, N_3 and X_2 (Fig. 2). Sal I-restricted mtDNA and ctDNA, prepared from the parental cultivar, were used as controls (not shown here). Indeed, the detection of a hybridization signal corresponding to a Sal I ctDNA fragment reveals a ctDNA sequence with homology to the Sal I mtDNA fragment used as a probe, which allows us to assign either a mitochondrial or a chloroplastic origin to hybridization signals detected in Sal I-restricted total DNA samples.

Probing with K' (Fig. 2) reveals the presence, in each regenerated plant, of the four fragments encompassing the RS5. However, fragments J' and K' display lower relative stoichiometries in the regenerated plant no. 5. Note that the relative amounts of both fragments J' and K' are likewise consistently diminished in non-embryogenic short-term callus cultures initiated from various wheat cultivars (Rode et al. 1988 and unpublished results).

Mitochondrial Sal I fragments K_3 and N_3 were used to study the variability which can occur in fragments encompassing the RS10. Indeed, fragment K_3, whose length is close to that of the ctDNA Sal 4 fragment, shares a common sequence with the latter, whereas fragment N_3 does not. It is thus difficult to distinguish K_3 from ctDNA Sal4 fragment when probing Sal I-restricted total DNA with K_3. However, hybridization with K_3 was found to be essential: N_3 shares only the RS10 in common with E_1 whereas homology between K_3 and E_1 extends from the left border of RS10 to the right Sal I site (Fi. 1). Under these conditions, the detection of fragment E_1, if it is present, is made easier when using K_3 as a probe. However, the use of fragment N_3 as a probe demonstrates, in the parental cultivar as well as in regenerated plant total DNA, the presence of a hybridization signal from a fragment close in size to that of fragment E_1 (homology to ctDNA Sal 2 fragment), which is not the case when probing with K_3. Probing with K_3 and N_3 (Fig. 2) clearly shows that the relative amount of N_3 is diminished in regenerated plant no. 5. This result is consistent with the hybridization pattern obtained when

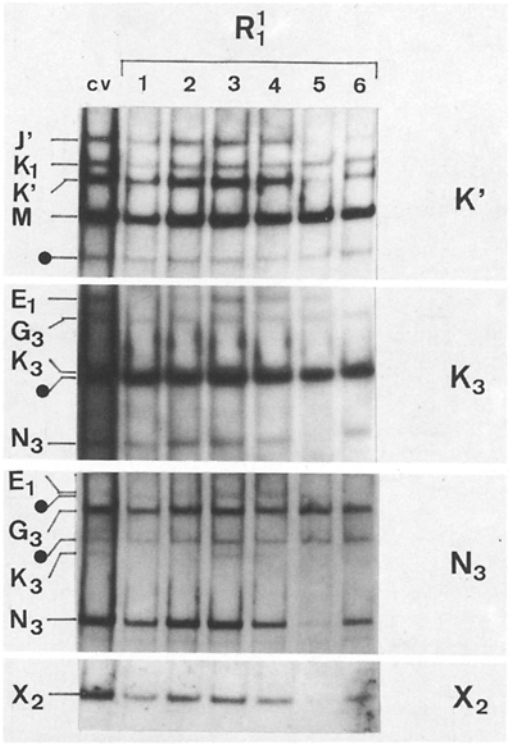

Fig. 2. DNA-DNA hybridization between Sal I-restricted total DNA isolated from the "Chinese Spring" parental cultivar (cv.) and plants regenerated from SC^1 calli (R_1^1 plants numbered 1–6) with the labelled Sal I-cloned mtDNA probes K', K_3, N_3 and X_2. The labelled probes used are indicated on the right of each hybridization pattern. The nomenclature for Sal I fragments is that of Quétier et al. (1985). *Black spots* (•) correspond to the ctDNA Sal I restriction fragments with homology to the mtDNA probe used (ctDNA Sal 5 fragment, internal to the inverted repeat, when probing with K'; ctDNA Sal 4 fragment, when probing with K_3; ctDNA Sal 2 and Sal 3a fragments, when probing with N_3

probing with K_3. Note that fragment N_3 is notably under-represented in short-term non-embryogenic callus cultures (as are fragments J' and K'). Fragments E_1 (using K_3 as a probe) and K_3 (using N_3 as a probe) seem to be consistently less abundant in regenerated plants no. 1, no. 2, no. 5 and no. 6 than in regenerated plants no. 3 and no. 4. This relative decrease in amounts of fragments E_1 and K_3 has been observed in both embryogenic and non-embryogenic short-term callus cultures. Fragment G_3 (using N_3 as a probe) is present in all regenerated plants, as it is present in both embryogenic and non-embryogenic short-term somatic tissue cultures.

Probing with X_2 (Fig. 2) shows that the relative amount of this fragment is considerably diminished only in the regenerated plant no. 5.

In Fig. 4, the data are collected concerning the restriction fragments whose relative amounts can be diminished in plants regenerated from short-term callus cultures as compared to parental cultivar. The equivalent data con-

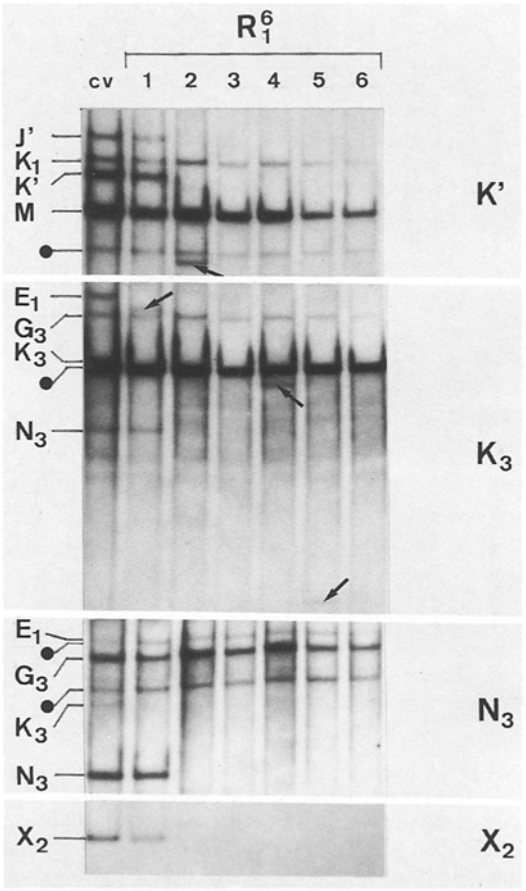

Fig. 3. DNA-DNA hybridization between Sal I-restricted total DNA isolated from the Chinese Spring parental cultivar (cv.) and plants regenerated from SC^6 calli (R_1^6 plants, numbered 1–6) with the labelled Sal I-cloned mtDNA probes K′, K_3, N_3 and X_2. *Arrows* indicate the position of extra hybridization bands. Other details as in Fig. 2

cerning embryogenic and non-embryogenic cultures have been added for comparison. Two regenerated plants (no. 3 and no. 4) are like the parental cultivar, whereas three other ones (no. 1, no. 2 and no. 6) display an organization close to that of embryogenic short-term cultures. Surprisingly, the mitochondrial genome organization of one regenerated plant (no. 5) was similar to that of non-embryogenic short-term cultures.

Plants regenerated from long-term cultures

Taking into account the probable importance of the duration of the in vitro culture period with respect to the organization of the mitochondrial genome of regenerated plants, we performed a series of experiments using plants regenerated from long-term callus cultures. For this purpose, we used six individuals (numbered 1–6) regenerated after a sixth subculture ($R^6{}_1$ plants).

Figure 3 shows the hybridization patterns obtained after probing Sal I-restricted total DNA from the parental cultivar and from regenerated plants with the labelled cloned Sal I mtDNA fragments K′, K_3, N_3 and X_2. Sal I-restricted mtDNA and ctDNA were loaded on gels as controls (not shown here).

Probing with K′ (Fig. 3) reveals that the regenerated plant no. 1 possesses the same hybridization pattern as the parental cultivar and the embryogenic callus cultures. The situation is strikingly different as far as regenerated plants no. 2–no. 6 are concerned: both fragments J′ and K′ are lost, as found in non-embryogenic callus cultures derived from cv. "Aquila" (Rode et al. 1987a). In addition, an extra hybridizing band (6.2 kbp long) appears in the regenerated plant no. 2.

Probing with K_3 and N_3 (Fig. 3) shows, firstly, that fragment N_3 is present only in the regenerated plant no. 1 and, secondly, that K_3 and E_1 are lost in all the regenerated plants. Fragment G_3 is present in each regenerated plant. Furthermore, several additional hybridization signals are detected when probing with K_3: one of about 19 kbp in regenerated plant no. 1, another of about 11 kbp in regenerated plant no. 4 and a third of about 3 kbp in regenerated plant no. 5.

Probing with X_2 (Fig. 3) reveals that this fragment is present only in regenerated plant no. 1.

It is clear, as shown in Fig. 4, that the organization of this variable region of the mitochondrial genome in regenerated plant no. 1 and in regenerated plants no. 2–no. 6 is close to that of embryogenic and non-embryogenic cultures, respectively. However, it must be kept in mind that extra hybridization signals are detected in some of the regenerated plants when using probes corresponding to mitochondrial fragments which have disappeared from the latter, thus indicating that at least a part of a lost fragment is in a new configuration.

Discussion

Some of our previous results had shown the occurrence of changes, in comparison with the parental cultivar, of the organization of the mitochondrial genome in embryogenic (Hartmann et al. 1987) and non-embryogenic (Rode et al. 1987a) wheat somatic tissue cultures. The study presented in this paper dealing with regenerated plants now allows us to follow, for the first time, the evolution of the organization of a plant mitochondrial genome through a complete in vitro culture process from that in the parental cultivar to that in short- and long-term embryogenic somatic tissue cultures and corresponding regenerated plants. Our results show that lengthening the duration of the culture process increases the probability of obtaining regenerated plants displaying modifications of their mitochondrial genome. In fact, plants regenerated from short- and long-term cultures can be put into one of three classes with respect to the organization of their mitochondrial genome.

		J'	K'	X_2	E_1	K_3	N_3
$R^1{}_1$	1				○	○	
	2				○	○	
	3						
	4						
	5	○	○	○	○	○	○
	6				○	○	
SC^1	E(a)				○	○	
	NE(b)	○	○	○	○	○	○
$R^6{}_1$	1				●	●	
	2	●	●	●	●	●	●
	3	●	●	●	●	●	●
	4	●	●	●	●	●	●
	5	●	●	●	●	●	●
	6	●	●	●	●	●	●
SC^6	E(a)				●	●	
	NE(b)	●	●	●	●	●	●

Fig. 4. Evolution, in plants numbered 1–6 and regenerated from SC^1 and SC^6 calli (R^1_1 and R^6_1 plants, respectively), of the Sal I restriction fragments which are lost in long-term embryogenic (E_1 and K_3) and non-embryogenic (J', K', X_2, E_1, K_3 and N_3) cultures. (a): Sal I fragments lost or diminished in embryogenic (E) cultures. Data from Hartmann et al. (1987). (b): Sal I fragments lost or diminished in non-embryogenic (NE) cultures. Data from Rode et al. (1987 a) and Rode et al. (1988) ○: stoichiometric decrease (R^1_1 plants). ●: loss (R^6_1 plants)

One class, similar in the mitochondrial genome organization to the parental cultivar, is found only in plants regenerated from short-term cultures. This feature is in agreement with the fact that the Sal I fragments (E_1 and K_3), which are lost when lengthening the in vitro culture duration, are still present, although in much decreased amounts, in short-term somatic tissue cultures. We speculate that the early passage out of in vitro culture would allow the replication process of the subgenomic molecule(s) encompassing these fragments to be reinitiated after they had been stopped on passage to in vitro culture.

A second class of mitochondrial genome organization is found in plants regenerated from both short- and long-term cultures. This organization is close to that of either short-term embryogenic cultures (three $R^1{}_1$ plants) or long-term embryogenic cultures (one $R^6{}_1$ plant). In both cases, the organization of regenerated plants reflects that of somatic tissue cultures from which they arose. In other words, this organization corresponds to a class for which passage out of in vitro culture does not seem to induce variability with respect to callus cultures.

The third class of mitochondrial genome organization is also found in plants regenerated from both short- and long-term cultures, with a greater frequency in the latter (five out of six, compared to one out of six for the former). In this case, the organization found in the plant regenerated from short-term culture is close to that found in short-term non-embryogenic cultures, whereas that found in plants regenerated from long-term cultures is similar to that found in long-term non-embryogenic cultures. However, if an identity between embryogenic cultures and regenerated plant mtDNA organization may be readily understood, this is not so in the case of regenerated plants with an organization close to that of non-embryogenic cultures. Indeed, all soft wheat cultivars checked to date are isocytoplasmic. We have obtained, especially with plants regenerated from long-term cultures, fertile wheat lines with a type of cytoplasm found, to date, only in somatic tissue cultures initiated from various wheat cultivars devoid of embryogenic ability (Rode et al. 1988). Of course, it will be of interest to check the regenerability of somatic tissue cultures initiated from these regenerated wheat lines. This work is currently underway.

In a general way, our present results seem to indicate that the mtDNA variability we have previously shown in embryogenic and non-embryogenic cultures (Rode et al. 1987; Hartmann et al. 1987) is not randomly generated, especially because of the discovery in some regenerated plants of a mtDNA organization similar to that of non-embryogenic cultures.

Another feature exhibited by the mitochondrial genome of regenerated plants is the appearance of novel restriction fragments, with respect to both parental cultivar and somatic tissue cultures from which plants were regenerated. In this case, the phenomenon seems to result from a random process: among the $R^6{}_1$ plants, only the individual no. 2 exhibits an extra hybridization band when probed with K', whereas probing with K_3 allows one to detect three differently sized extra hybridization bands in three different plants. In contrast, probing with N_3 and X_2 gives a negative result with respect to the presence of additional hybridization signals. It must be pointed out that no new hybridization signal was detected when probing $R^1{}_1$ plants, suggesting that the longer the in vitro step, the higher the probability is of obtaining new hybridization signals.

The appearance of novel restriction fragments with homology to fragments K' and K_3 could be, a priori, correlated with the disappearance of the original fragments. Indeed, the new arrangement found in regenerated plants could be due to the induction, mediated by the passage out of in vitro culture, of new recombination

phenomena. In order to check this hypothesis, we have recently made a Sal I genomic library, starting with mtDNA isolated from regenerated plants obtained after two selfings of the R^6_1 plant. The new 19.5 kbp Sal I fragment was obtained in a recombinant cosmid and a study of its molecular structure is currently underway. An alternative hypothesis to explain the presence of additional hybridization signals would be that there is an amplification of subgenomic molecules present in the parental cultivar in amounts too low to be detected (Small et al. 1987).

It is thus obvious that, in our plant system, both the first transition (passage from scutellum to somatic tissue culture) and the second transition (passage from embryogenic tissue culture to regenerated plants) induce some structural rearrangements of the mitochondrial genome. The qualitative and quantitative features of these rearrangements seem to be a function of the duration of the dedifferentiated state, providing further evidence on the plasticity of the plant mitochondrial genome. In a previous series of experiments dealing with the organization of the mitochondrial genome from doubled haploid wheat plants obtained through androgenesis in vitro (Rode et al. 1985), no rearrangement was found specific to the cultivar used as an anther donor. It should be emphasized that, in this case, the duration of in vitro culture was about 1 month, a period which is probably too short to induce irreversible rearrangements of the mitochondrial genome.

In conclusion, and in addition to the fact that cell and tissue culture techniques allow higher plants to be studied and manipulated at the cellular level, there is now an increasing amount of evidence to show that passage through somatic tissue culture can generate mtDNA rearrangements, so that plants arising from embryogenic tissue cultures are not exact copies of the donor plants. Moreover, it is likely that such investigations will provide an insight into the mechanisms governing the regulation of the genes involved in the ability of calli to regenerate and perhaps also into the molecular basis of developmental processes.

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