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Genetic stability at nuclear and plastid DNA level in regenerated plants of *Solanum* species and hybrids

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Abstract In this work we detected the extent of variability at nuclear and cytoplasmic DNA level of regenerated plants belonging to *Solanum* genotypes with a different genetic background and somatic chromosome number. As for the nuclear characterization, a total of 66 (18.5%) polymorphic bands were scored using 13 ISSR primers on 45 randomly selected regenerants. Our results show that the regenerants obtained from clone cmm 1T and, at lower level, those from cph 1C are unstable under in vitro conditions or rather more prone to in vitro-induced stress leading to somaclonal variation than the other genotypes used. Two types of changes were observed: disappearance of parental ISSR fragments, termed "loss"; appearance of novel ISSR fragments, termed "gain". The most frequent event occurring in the regenerants was the loss of fragments (41 bands). Regenerated plants were analyzed with seven plastid universal primers to determine the cytoplasmic composition at chloroplast level. All cpDNA primer pairs tested produced amplicons of the same size in all genotypes analyzed and no polymorphic fragments were observed with any universal primers used. Our results show that under in vitro culture conditions genotype affects the integrity of the genome. In addition, the absence of polymorphism at plastid level confirms the greater genetic stability of cytoplasmic DNA.

Keywords AFLP · Molecular markers · Potato · Somaclonal variation · Tissue culture

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Introduction

Cultivated potato (*Solanum tuberosum* L.) and its wild relatives are considered model plants for cell and tissue culture. To date, different types of tissues have been successfully used as explants for various in vitro approaches. For example, meristem culture has become routine in potato seed certification schemes (Struik and Wiersema 1999), and anthers have been used to produce haploids for mapping studies and breeding strategies (Veilleux 2005). In vitro culture represents a powerful tool to overcome sexual barriers hampering the introgression of useful genes and allelic diversity into the cultivated gene pool (Romano et al. 2001; Orczyk et al. 2003). Potato cell and tissue culture has also become a way to generate new genotypes by exploiting somaclonal variation. Through this approach many traits can be altered, including plant height, yield, resistance to biotic and abiotic stresses, nutrition quality, and agronomic characteristics. Somaclones regenerated from *S. tuberosum* were found resistant to *Erwinia* spp. and

Verticillium dahliae by Taylor et al. (1993) and Sebastiani et al. (1994), respectively. Thieme and Griess (1996) produced thousands of somaclones from various potato cultivars and found variability for agronomic traits related to haulm growth, earliness and tuber yield. Moreover, improved nutritional quality was obtained by Langille et al. (1998) in protoplast-derived calli selected in presence of the amino acid analog, ethionine.

The genetic alterations involved in somaclonal variation generally occur at genomic, genic and chromosomic level. Genomic mutations affect the number of chromosomes (the ploidy) and can be detected by cytometry or chromosome counting. By contrast, both genic and chromosomic mutations like inversion, deletion or translocation can be detected by molecular markers. Since mutations could occur both on nuclear DNA and on mitochondrial (mt) or chloroplast (cp) DNA (Kawata et al. 1995; Rani et al. 2000), various DNA markers like RAPD (random amplified polymorphic DNA), ISSR (inter simple sequence repeat) for nuclear genome screening (Gostimsky et al. 2005), and RFLP (random fragment length polymorphism) for both nuclear and organellar genome screening (Devarumath et al. 2002) are required to assess the genetic stability of somaclones. The occurrence of somaclonal variation is influenced by several factors such as explant type, culture medium, age of donor plant, number of subcultures and tissue culture system itself. Among all, genotype is probably the most important factor leading to variation (Etienne and Bertrand 2003; Bordallo et al. 2004). In the literature, mixed results are given about the effect of tissue culture on the genetic integrity of regenerated plants. Binsfield et al. (1996), for example, found isozyme variation in plants regenerated from *S. tuberosum*. By contrast, Barandalla et al. (2006) did not detected AFLP polymorphism in regenerated plants of diploid *Solanum* genotypes. To shed additional information on the association between molecular stability and somaclonal variation, in this work we produced regenerants from 2×, 3×, 4× and 6× genotypes of *Solanum* species and hybrids using a regeneration method routinely employed in our laboratory (Iovene et al. 2002). The genotypes chosen carry noteworthy traits (Hanneman and Bamberg 1986; Savarese 2008) and are currently employed in our cell and tissue culture experiments. Our objectives were to establish the extent of genetic variation of regenerants obtained from five *Solanum* genotypes with different origin, pedigree as well as determine if genotypes influenced the extent of such variability. The assessment was carried out at nuclear level by ISSR markers, and at plastid DNA level by ''universal primers'' homologous to conserved sequences of cpDNA.

Materials and methods

Plant materials

Table 1 lists plant materials used to produce regenerants analyzed in this study. In particular, one clone each of *S. cardiophyllum*, *S. commersonii* and *S. nigrum*, one sexual hybrid (*S. fendleri* × *S. phureja*) and one somatic hybrid [*S. tuberosum* (+) *S. bulbocastanum*] were included in this study.

Clones cmm 1T (PI 243503) and cph 1C (PI 347759), derived from a single seed, were provided by Dr. J. Bamberg, UW-Madison. Ngr 2 and the hybrids were from our breeding programs. In particular, the sexual hybrid was obtained from 4× × 2× crosses between 4× *S. fendleri* and 2× *S. phureja*. The somatic hybrid was regenerated from protoplast fusion between clone blb 1C of *S. bulbocastanum* (PI 275188) and *S. tuberosum* haploid V2I-59 (Iovene et al. 2007). Clonal copies of the original plant material were obtained by excising nodes with an axillary bud from in vitro grown plantlets of each

Table 1 Plant materials used for the production of regenerants analyzed in this study

Plant material	Clone code	Ploidy	Origin-accession
Wild species			
S. commersonii	cmm 1T	2×	PI 243503
S. cardiophyllum	cph 1C	2×	PI 347759
S. nigrum	ngr 2	6×	
Triploidy hybrid			
	fenph 1	3×	*S. fendleri* × *S. phureja*
Somatic hybrid			
	HF 5A	4×	*S. tuberosum* haploids (+) *S. bulbocastanum*

genotype. They were cultured on Murashige and Skoog (MS) medium (1962) supplemented with 30 g l^{-1} sucrose, 0.9 g l^{-1} agar; pH 5.8. The genotypes were incubated in a growth chamber at 24°C, 16 h cool white light, 4,000 lux and maintained by means of monthly subcultures.

Shoot regeneration

Leaf explants were excised from 4-week-old plants grown in vitro as described above. To achieve 0.25 cm^2 leaf squares, leaflets were divided longitudinally into two pieces or cultured entirely, always with the abaxial surface in contact with the medium. Explants were cultured for 2 weeks on MS medium supplemented with 30 g l^{-1} sucrose, 0.186 mg l^{-1} NAA, 2.25 mg l^{-1} BAP, 0.9 g l^{-1} agar, pH 5.8 for callus induction, and then transferred for shoot induction/elongation to the same medium without NAA, but with 5 mg l^{-1} GA_3, for 5 weeks (Karp et al. 1984). GA_3 was filter-sterilized and added to autoclaved medium. Explants were incubated in 9 cm plastic Petri dishes at a constant 24°C, 16 h cool white light, 4,000 lux. The frequency of explants with shoots (at least 0.5–1 cm in length) and the number of shoots per explant were recorded.

Nuclear DNA analysis

DNA from parents and 45 regenerants was extracted from leaves of in vitro-grown plants, using QIAGEN DNeasy Plant Mini Kit (Missisauga, ON) according to the manufacturer's instructions. Fourteen regenerants from cmm 1T, 10 from cph 1C, 7 from ngr 2, 4 from fenph 1 and 10 from HF 5A, all coming from different calli were analyzed. Thirteen ISSR primers (Table 2) were used to evaluate the extent of somaclonal variation by comparing the ISSR profile of each regenerant with that of its parent. Twenty-five nanograms of template DNA were used in PCR amplifications. Amplification reactions (25 µl) consisted of 1.2 µM of each primer, 200 µM of each dNTP (Invitrogen, Carlsbad, CA), 1 units of *Taq* DNA polymerase (Invitrogen) and 1× reaction buffer supplied by the manufacturer (Perkin Elmer Cetus). Amplifications were performed in a DNA Thermal Cycler (Applied Biosystem, Toronto, ON). PCR parameters were drawn up according to Scarano et al. (2002). PCR products were separated on a 1.5% agarose gel and visualized by ethidium bromide staining. To have reproducible and clear banding patterns, each amplification was repeated at least three times separately. Only bands showing consistent amplification were considered; smeared and weak bands were excluded. Fragments differing between parents and the regenerants were considered polymorphic. Profiles for each somaclone were constructed by scoring 0 or 1 for absence or presence of fragments, respectively.

Table 2 ISSR primers used to characterize the regenerants of five *Solanum* genotypes

Primer pair (code)	Sequence[a]	Annealing temperature (°C)	Reference for sequence
ISSR			
1	$HVH(TG)_7T$	54	1
2	$HVH(TCC)_5$	54	1
5	$BDB(TCC)_5$	54	1
10	$(AG)_8YG$	54	1
16	$(AC)_8$	54	2
19	$(GCC)_5$	64	2
1425	$BDV(CAG)_5$	55	3
2102	$HVH(CTT)_5$	54	3
2103	$HVH(GTC)_5$	55	3
2104	$BDB(GAT)_5$	52	3
2105	$BDB(GAC)_5$	55	3
MC 1	$VHV(GT)_7G$	55	4
MC 2	$DHB(CGA)_5$	55	4

For each primer pair the sequence and the annealing temperature are given

[a] B = T or C; D = G, A or T; H = A, T or C; V = G, A or C

1 = Scarano et al. (2002), 2 = Bornet et al. (2002), 3 = Matthews et al. (1999), and 4 = McGregor et al. (2000)

Cytoplasmic DNA analysis

DNA from parents and 45 regenerants was extracted from young leaves of greenhouse-grown plants, using QIAGEN DNeasy Plant Mini Kit (Missisauga, ON) according to the manufacturer's instructions. Fourteen regenerants from cmm 1T, 10 from cph 1C, 7 from ngr 2, 4 from fenph 1 and 10 from HF 5A were analyzed. Fifty-five nanograms of genomic DNA were used in PCR amplifications with gene specific primers for cp (seven primer pairs, Table 3). Primer pairs were first used to characterize intergenic regions

Table 3 Primer pairs specific to cpDNA regions used to study regenerants of five *Solanum* genotypes. For each primer pair, the amplified genomic region and the annealing temperature are given

Primer pair (code)[a]	Amplified genomic region	Annealing temperature (°C)	Reference for sequence[b]
pucA	*trn*H-*trn*K	55	1
pucE	*trn*S-*trn*T	57.5	1
pucG	*trnK*	55	1
pucH	*trn*C-*trn*D	55	1
pucJ	*trn*F-*trn*V	57.5	2
pucN	*rbc*L-*aac*D	55	3
pucQ	*rpo*C2-*rpo*C1	55	3

[a] Primer codes as in Bastia et al. (2001) and Scotti et al. (2003)

[b] 1 = Demesure et al. (1995), 2 = Dumolin-Lapégue et al. (1996), and 3 = Petit et al. (1998)

of the parents used. Amplification reactions (25 µl) consisted of 0.4 µM of each primer, 200 µM of each dNTP (Invitrogen, Carlsbad, CA), 0.2 units of *Taq* DNA polymerase (Invitrogen,) and 1× reaction buffer supplied by the manufacturer (Perkin Elmer Cetus). Amplifications were performed in a DNA Thermal Cycler (Applied Biosystem, Toronto, ON), programmed as follows: 4 min at 94°C, 30 cycles of 45 s at 94°C/1 min at the annealing temperature/3 min at 72°C, and a final extension of 10 min at 72°C. Amplification products were directly analyzed by electrophoresis in 1% agarose gel and stained with ethidium bromide. To have reproducible and clear banding patterns, each amplification was repeated at least three times separately. Fragments differing between parents and the regenerants were considered polymorphic. For each genotype, polymorphic fragments were recorded as present (1) or absent (0).

Data analysis

The statistical significance of differences between genotypes for regeneration frequencies and frequency of polymorphic bands was tested by χ^2 analysis. In order to determine significance of differences between genotypes and ploidy level in terms of molecular data, analysis of variance was carried out. Genetic similarity (GS) between the samples was calculated by simple matching coefficient (Kosman and Leonard 2005) $SM = (a + d)/(a + b + c + d)$, where a and d are the cases where the values of both variables agree (i.e. the same band is either present or absent in two genotypes, respectively), and b and c those in which they disagree. The genetic similarities were graphically represented by a dendrogram constructed using the UPGMA (unweighted pairgroup method, arithmetic average) clustering algorithm. The genetic distance calculations and dendrogram construction were performed using NTSYS-pc package (Rohlf 1998).

Results

Shoot regeneration was induced in all genotypes analyzed (Table 4). Ngr 2 gave the best response both in regeneration frequency and in the average number of shoots per leaf explant (72.5% and 6.7%, respectively). Among 2× genotypes, cph 1C showed the highest regeneration frequency (52% with 2.4 shoots per explant). Fenph 1 showed the lowest regeneration frequency (2.3%) and the lowest number of shoots per explant (2.3). Differences between the five genotypes were highly significant in terms of regeneration ability ($\chi^2 = 88.2$, $P < 0.01$).

The results of ISSR analysis on 45 randomly selected regenerants are shown in Table 5. For each

Table 4 Regeneration frequency and mean number of shoots/callus of five *Solanum* genotypes. For pedigree see Table 1

Genotypes	Cultured explants (no.)	Regeneration frequency (%)	Shoots per callus no. ± SE	Total shoots (no.)
cmm 1T	132	27.3	2.5 ± 0.2	92
cph 1C	72	52.0	2.4 ± 0.5	121
ngr 2	40	72.5	6.7 ± 0.8	198
fenph 1	131	2.3	2.3 ± 1.6	7
HF 5A	120	37.5	3.9 ± 0.5	177
Mean	99.0	38.3	3.6 ± 0.7	119.0

Table 5 Synthesis of results from ISSR and universal primer PCR fingerprinting of nuclear and plastid genomes of regenerants obtained from five *Solanum* genotypes (for pedigree see Table 1). Polymorphic were considered parental bands present/absent in the regenerants

	No. of analyzed regenerants from				
	cmm 1T	cph 1C	ngr 2	fenph 1	HF 5A
	14	10	7	4	10
Nuclear DNA (ISSR)					
Primers used, no.[a]	9	8	7	10	9
Total bands, no.	88	65	52	70	82
Polymorphic bands, no. (%)	39 (44.3)	15 (23.1)	5 (9.6)	5 (7.1)	2 (2.4)
Loss fragments, no.	19	15	5	0	2
Gain fragments, no.	20	0	0	5	0
Plastid DNA (universal primer)					
Primers used, no.	7	7	7	7	7
Total bands, no.	98	70	49	28	70
Polymorphic bands, no. (%)	0	0	0	0	0
Loss fragments, no.	0	0	0	0	0
Gain fragments, no.	0	0	0	0	0

[a] Thirteen primers were used. For each parental genotype, only ISSRs producing clear and scorable profile in all regenerants tested were taken into account

genotype, only ISSR producing clear and scorable profiles in all regenerants tested were taken into account. Nine ISSR allowed the detection of 88 bands in cmm 1T regenerants, 8 ISSR detected 65 bands in cph 1C regenerants, 7 ISSR gave 52 bands in ngr 2 regenerants, 10 ISSR distinguished 70 bands in fenph 1 regenerants, and 9 ISSR detected 82 bands in HF 5A regenerants. A total of 66 (18.5%) polymorphic bands were scored. Regenerants from cmm 1T showed the highest frequency of polymorphism (44.3%), those from HF 5A the lowest one (2.4%). Differences among genotypes in terms of frequency of polymorphic bands were significant ($F = 15.2$, $P < 0.01$). The analysis of data according to the ploidy of parental genotypes provided evidence that HF 5A ($2n = 4\times = 48$), fenph 1 ($2n = 3\times = 36$) and ngr 2 ($2n = 6\times = 72$) displayed a lower polymorphism (2.4%, 7.1%, and 9.6%, respectively) compared to that scored in diploid genotypes (44.3% in cmm 1T and 23.1% in cph 1C). These differences were statistically significant ($F = 5.13$; $P < 0.01$). Cmm 1T regenerants analyzed showed the most variable number of polymorphic bands, ranging from 0 (profile with one band obtained with ISSR 2105 primer) to 16 (profile with 20 bands, with MC2 primer, Fig. 1a). Two types of changes were observed: disappearance of parental ISSR fragments, termed "loss"; appearance of novel ISSR fragments, termed "gain". The most frequent event occurring in the regenerants was the loss of fragments (41 bands). Only cmm 1T regenerants showed a similar high number of "loss" (19 fragments) and "gain" (20

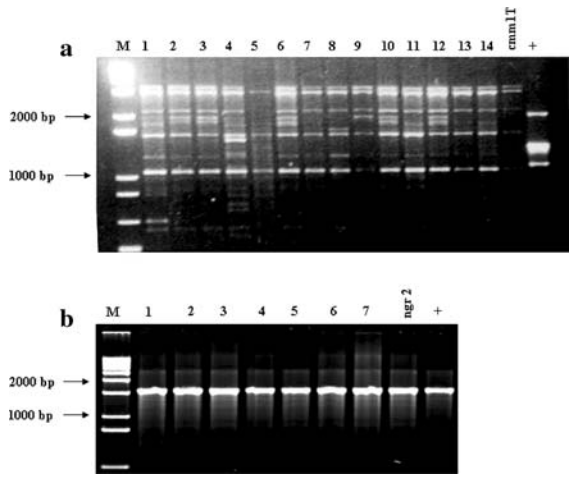

Fig. 1 Molecular patterns generated on: (**a**) nuclear DNA of *S. commersonii* (cmm 1T) and its regenerants (1–14) by using ISSR MC2 and (**b**) plastid DNA of *S. nigrum* (ngr 2) and its regenerants (1–7) using universal primer PUCG (**b**). M: 1 kb *plus* marker; +: positive control *S. tuberosum*

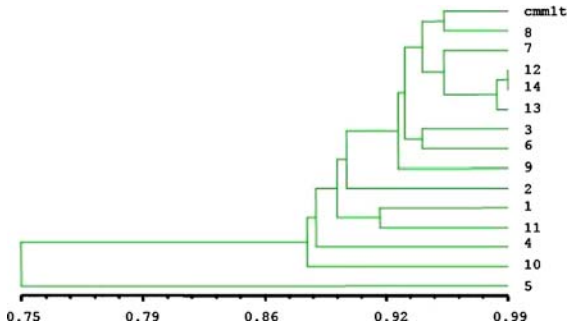

Fig. 2 Dendrogram illustrating the coefficient of similarity among 14 regenerants (coded 1–14) and their parent *S. commersonii* (cmm 1T), obtained by UPGMA cluster analysis based on ISSR molecular markers

fragments). The UPGMA dendrogram for cmm 1T and its regenerants is presented in Fig. 2. The tree reflects the degrees of differences among the *S. commersonii* regenerants analyzed. The divergence between the somaclones originating from cmm 1T estimated by Jaccard's similarity ranged from 0.72 to 0.99. As for fenph 1, HF 5A, cph 1C and ngr 2 regenerants, the dendrograms lack well-supported groupings due to the high similarity values detected for all regenerants (ranging from 0.91 to 1.00). Therefore, they all clustered with the genotypes they derived from (data not shown).

Regenerated plants were analyzed with seven plastid universal primers to determine the DNA composition at chloroplast level. All seven cpDNA primer pairs tested gave clear and repeatable amplifications. Each primer tested produced amplicons of the same size in all genotypes analyzed. In particular, the size of PCR fragments amplified was 3 kb for puc J; 1.2 kb for puc E; 3 kb for puc H; 2.7 kb for puc G; 1.8 kb for puc A; 3 kb for puc N; 3.5 kb for puc Q (data not shown). No polymorphic fragments were observed with universal primers used (Fig. 1b).

Discussion

In recent years a number of studies have measured, through molecular markers, the extent of somaclonal variation in plants. Lack of polymorphisms associated with in vitro regeneration was reported in tomato (Smulders et al. 1995), Norway spruce (Fourré et al. 1997), oil palm (Rival et al. 1998), begonia (Bouman and De Klerk 2001), almond (Martins et al. 2004), and potato (Barandalla et al. 2006; Sharma et al. 2007) using RAPD, ISSR and AFLP markers. By contrast, major differences were found in alfalfa (Piccioni et al. 1997), in *Codonopsis lanceolata* (Guo et al. 2006), wild pear (Palombi et al. 2007) using RAPD and ISSR markers. We wanted to establish the extent of genetic variation of regenerants obtained from five *Solanum* genotypes with different origin and pedigree. We used ISSR and plastid universal primers for studying variation at nuclear and plastid DNA level, respectively. ISSR markers are known to be a fast, highly reproducible and economic method to generate informative genetic markers. Simple sequence repeats showed increased levels of instability in tissue culture, giving rise to clonal regenerants containing variable copy number (Phillips et al. 1994). Therefore, ISSR markers represent a suitable system for the analysis we performed in this study.

Under the same set of tissue culture conditions (such as in vitro process and its duration, hormone and nutrient balance, etc.), the five genotypes tested behaved differently in terms of mean polymorphism of regenerants. Therefore, it can be inferred that the genetic constitution of each genotype strongly determines the ability to change its genome.

Our results suggest that the regenerants obtained from clone cmm 1T and, at lower level, those from cph 1C are more unstable under in vitro conditions or rather more prone to in vitro-induced stress leading to somaclonal variation than the other genotypes used. Additional genotypes per each species are needed to drawn broad conclusions regarding the species stability under in vitro conditions. Whether the genetic changes observed in the present study influence traits of interest or not remains to be verified. The frequency of polymorphism detected in the *Solanum* regenerants is consistent with the data of other authors. Guo et al. (2006) using ISSR markers found a 16% polymorphism frequency in 63 regenerants of *Codonopsis lanceolata* obtained through androgenesis. Kuznetsova et al. (2005), performing ISSR analysis on *Pisum sativum* regenerants, found that the percentage of polymorphism ranged from 0.7% to 15%. In *Camellia* somaclones, ISSR markers allowed the detection of 12% polymorphism (Devarumath et al. 2002). Interestingly in *S. tuberosum* Sharma et al. (2007) found a very low level of AFLP marker profile variation amongst plants regenerated

by direct and indirect somatic embryogenesis. Similar results were reported in *Foeniculum vulgare* regenerated plants obtained through somatic embryogenesis (Bennici et al. 2004). As suggested by Swedlund and Vasil (1985) and Karlsson and Vasil (1986), lack of variation in somatic embryos may be due to strong selection against variant cells during somatic embryogenesis. In previous reports for dicot species *Arabidopsis thaliana*, *Medicago truncatula* and *Lotus japonica* (Mun et al. 2006), SSRs were found to reside in a non-transcribed fraction of gene-rich regions or within the untranslated portions of transcripts (i.e., UTRs and introns). Therefore, the somaclonal variation we observed may suggest that genomic mutational rates occurred mostly in such regions.

The occurrence of novel bands as well as the disappearance of parental fragments was detected in the regenerants analyzed. These bands are the footprint of restructuring events arising after in vitro culture. Indeed, the genome is abnormally reprogrammed or decidedly restructured when cells face trauma of dedifferentiation in calli, with a dramatic increase in genetic variation. That occurring at microsatellite regions is explained mostly by slipped strand mispairing (slippage) during replication, even though it could result from several other phenomena, as insertions or deletions. These mechanisms can modify the number of repeated units generating an ISSR marker loss and, at the same time, an ISSR marker gain. The polymorphic fragments detected in our *Solanum* regenerants were mostly of loss-type. They might appear as consequence of amplification failures, for example caused by whole chromosome block elimination or large genomic rearrangements. They can also be due to the fact that repeated regions are exposed to transposition, according to the molecular drive mechanisms described by Dover (1982). There are more common tissue culture-associated lesions than point mutation in the primer-annealing site.

With regard to plastid DNA characterization, we did not observe any change. Similarly, Devarumath et al. (2002) did not detect any polymorphism in plastid genome of the micropropagated plants of three tea clones. The chloroplast genome has been found to remain stable also in regenerants of *Coffea arabica* (Rani et al. 2000) and *F. vulgare* (Bennici et al. 2004). Only few studies have detected structural changes in the plastid genome. Jahne et al. (1991) in barley and Shihron Abarbanell and Breiman (1991) in *Hordeum marianum* found that long-term culture causes deletions in plastid genome. Kawata et al. (1995) analyzed plastid DNA of rice regenerants maintained in vitro for several years. They found a deletion that covered a large region of the genome, confirming that long-term culture causes mutations at plastid DNA level.

We believe that two main factors emerged from this work. First, the genetic background strongly affects genetic changes occurring at nuclear DNA level after in vitro culture. By contrast, the plastid DNA remains stable independently from the genotype. Second, changes mainly occurred as loss of fragments and this might be explained as consequence of large genomic rearrangements or whole chromosome block elimination.

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