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ORIGINAL ARTICLE

AFLP analysis to assess genomic stability in *Solanum* regenerants derived from wild and cultivated species

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Abstract The cultivated potato as well as its tuber-bearing relatives are considered model plants for cell and tissue culture, and therefore for exploiting the genetic variation induced by in vitro culture. The association between molecular stability and tissue culture in different genetic backgrounds and ploidy levels has already been explored. However, it still remains to be ascertained whether somaclonal variation differs between callus-derived chromosome-doubled and undoubled regenerants. Our research aimed at investigating, through amplified fragment length polymorphism (AFLP) markers, the genetic changes in marker-banding patterns of diploid and tetraploid regenerants obtained from one clone each of *Solanum bulbocastanum* Dunal and *S. cardiophyllum* Lindl (both $2n = 2x = 24$) and tetraploids from cultivated *S. tuberosum* L. ($2n = 4x = 48$). Pairwise comparisons between the banding patterns of regenerants and parents allowed detecting considerable changes associated to in vitro culture both at diploid and tetraploid level. The percentages of polymorphic bands between diploid and tetraploid regenerants were, respectively, 57 and 69% in *S. bulbocastanum* and 58 and 63% in *S. cardiophyllum*. On average, the frequencies of lost parental fragments in regenerants were significantly higher than novel bands both in *S. bulbocastanum* (48 vs. 22%) and *S. tuberosum* (36 vs. 18%) regenerants. By contrast, in *S. cardiophyllum*, a similar incidence of the two events was detected (32 vs. 29%). Our results revealed that structural changes after tissue culture process strongly affected the genome of the species studied, but diploid and tetraploids regenerated plants responded equally.

Keywords Potato · Somaclonal variation · Tissue culture · Genetic variability · Polyploidy

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Introduction

The diploid ($2n = 2x = 24$) relatives of the potato provide an inestimable resource of genes that can potentially be transferred into the cultivated gene pool (Jansky and Peloquin 2006). However, due to incompatibility barriers, some species are sexually isolated from the tetraploid ($2n = 4x = 48$) cultivated *Solanum tuberosum* L. The mechanisms beyond are still an open question, but there is evidence that interspecific pollen–pistil incompatibility, nuclear–cytoplasmatic male sterility, and the endosperm are major forces that strengthen the external hybridization barriers (Camadro et al. 2004). Early studies reported that chromosome doubling might represent a possible strategy to improve the sexual compatibility between incongruent wild species and the cultivated potato (Carputo et al. 1997). In vitro culture systems provide a tool to easily manipulate ploidy through adventitious shoot regeneration (Cardi et al. 1992). Indeed, it has been suggested that the instability in the regulation of the mitotic process during dedifferentiation into calli and regeneration into shoots often results in chromosome doubling due to endopolyploidization or nuclear fusion (Bayliss 1980). Besides overcoming sexual barriers, regenerated polyploids can offer the opportunity to break down the gametophytic self-incompatibility (GSI) through competitive interactions (Golz et al. 2001) between pollen-S alleles and the S-RNase present in the style (Luu et al. 2000). Together with the advantages discussed

above, the use of in vitro systems to double chromosome numbers may generate cryptic genetic defects with undesired effects on the phenotype that can seriously limit the broader utility of regenerated polyploids. Tissue culture system itself, in fact, acts as a mutagenic system, which leads to the occurrence of somaclonal variation associated with point mutations, chromosome aberrations, and epigenetic changes (for a review, see Jain 2001). For these reasons, the need to ascertain the level of genetic and phenotypic changes associated to tissue culture is now widely followed among investigators, in order to understand its advantages and disadvantages, as discussed by Veilleux and Johnson (1998).

Cytogenetic, biochemical and molecular methods can be used to investigate somaclonal variation (Vazquez 2001). In particular, DNA-based marker systems, such as RAPD, RFLP and ISSR have been extensively employed. Amplified fragment length polymorphism (AFLP) is probably the most robust molecular technique for variability analysis (Hale and Miller 2005). Indeed, its high reproducibility, and the possibility to multiplex 50–100 loci per primer evenly distributed throughout the genome, make AFLP a powerful tool to detect tissue culture-induced changes. Over the last decade, several studies have investigated somaclonal variation by this technique, highlighting either stability or instability of the genomes analyzed (Hashmi et al. 1997; Arencibia et al. 1999; Vendrame et al. 1999; Wilhelm 2000; Polanco and Ruiz 2002; Labra et al. 2004; Li et al. 2007; Martelotto et al. 2007). However, most observations revealed that the genome integrity is decidedly affected among plantlets regenerated from callus tissue, and clonal uniformity is recognized to be the exception rather than the rule (Jain 2001).

In the perspective of effectively capturing genetic diversity of two incongruent late blight-resistant diploid potato species (*S. bulbocastanum* Dunal and *S. cardiophyllum* Lindl) and put it in an adapted form, we produced synthetic tetraploids through tissue culture. The objective of this research was to genotype (through AFLP markers) the regenerants to investigate the effect of in vitro culture on callus-derived chromosome-doubled and undoubled regenerants. Since the regenerants did not experience any sexual event, the genetic changes they went through can be mainly attributed to tissue culture.

Materials and methods

Production of 2x and 4x regenerants

The following clones were used in this study: blb1C of *S. bulbocastanum* Dunal ssp. *bulbocastanum* (PI 275190) and cph1C of *S. cardiophyllum* Lindl ssp. *cardiophyllum* (PI 283062), both diploid ($2n = 2x = 24$) species originating from Mexico and displaying several resistance traits. PIs were provided by the IR-1 Potato Introduction Project, Sturgeon Bay, WI, USA. *S. tuberosum* cultivar Désirée ($2n = 4x = 48$) was used as control in all performed assays. Plantlets of each genotype were grown in vitro and cultured on Murashige and Skoog (1962) (MS) medium supplemented with 3% sucrose, 0.9% agar; pH 5.8. They were incubated in a growth chamber at 24°C, 16 h cool white light, 4,000 lux and maintained by means of monthly subcultures. For each genotype, explants were excised from 10 different 4-week-old seedlings obtained through axillary buds. Plants were grown in vitro as described above. In order to promote callus differentiation, explants were cultured for 2 weeks in 9-cm Petri dishes on MS medium supplemented with 3% sucrose, 0.9% agar, 0.186 mg l^{-1} 1-naphthaleneacetic acid (NAA), and 2.25 mg l^{-1} 6-benzylaminopurine (BAP); pH 5.8. All cultures were incubated at 24°C, 16 h cool white light, 4,000 lux. Then, all the explants were transferred for 5 weeks onto the same medium containing 5 mg l^{-1} filter-sterilized Gibberellic acid (GA_3), instead of NAA, for shoot induction/elongation (Karp et al. 1984). The frequency of regenerating explants and the number of shoots per explant were evaluated. Regenerated shoots were transferred to MS medium and incubated as described above. Four-week-old rooted plantlets were transferred into styrofoam trays filled with sterile soil and acclimated to ex vitro conditions in a growth chamber at 22/18°C day/night, 16 h light. For each regenerant, the chloroplast number in stomata guard cells of epidermal strip leaves and the number of somatic chromosomes in root tips was used for determining the ploidy level (Iovene et al. 2002).

AFLP analysis

We deployed 19 *S. bulbocastanum* (5 diploids and 14 tetraploids) and 20 *S. cardiophyllum* (5 diploids and 15 tetraploids) in vitro-regenerated genotypes. *S. tuberosum* (cv. Désirée) and five of its regenerants were also analyzed. DNA was isolated using the Qiagen Plant DNeasy Kit according to the manufacturer's instructions (Qiagen, Valencia, CA, USA). AFLP fingerprints (Vos et al. 1995) were generated using the commercially available AFLP kit and protocol (Gibco-BRL AFLP analysis System I; Life Technologies, Gaithersburg, MD, USA), which employs *Eco*RI and *Mse*I as restriction enzymes. For selective amplification, five combinations of primers were used: E-AGG + M-CAA, E-ACC + M-CTA, E-AGG + M-CTA, E-ACT + M-CTG, and E-AGC + MCAA. Technical and biological replicates were performed twice and only reproducible banding patterns were considered for analysis.

A fluorometric method was used for detecting AFLP fragments with higher resolution than conventional radio-detection techniques (Schwarz et al. 2000). *Eco*RI primers were labeled with 6-carbozy-fluorescein (6-FAM), and the *Mse*I primers were unlabeled. AFLP fragments were electrophoretically separated on 6% denaturing polyacrylamide gels and the fluorescence detected with a Typhoon 9210 scanner (Amersham Biosciences). AFLP images were analyzed with ImageQuant TE, v2002.01 (Amersham Biosciences), and by visual inspection. For each genotype, polymorphic fragments were recorded as 1/0 binary matrices, where 1 indicates the presence and 0 the absence of a given fragment. The coding generated from the analysis of the AFLP profiles described the presence/absence of each fragment in the parent, in 2x and in 4x regenerants. We took into consideration the following patterns: (111) referring to fragments unaffected by tissue culture; (011) fragments present both in the 2x and the 4x regenerants, but not in the parent; (001) fragments present in the 4x regenerants, but not in the parent and in the 2x regenerants; (010) fragments present in the 2x regenerants, but not in the parent and in the 4x regenerants; (100) parental fragments absent both in the 2x and in the 4x regenerants; (110) parental fragments absent only in the 4x regenerants; (101) parental fragments absent only in the 2x regenerants. Overall, the changes observed were assessed and classified into two main categories: "gain" [(011), (001) and (010)], where the appearance of ex novo AFLP fragments occurred in the regenerants, and "loss" [(100), (110) and (101)], where the disappearance of parental AFLP fragments was observed in the regenerants. It must be pointed out that the (100)-pattern does not refer to bands absent in all regenerants, but to those parental bands which disappeared at the same time in at least one 2x and one 4x regenerant.

Data analysis

The statistical significance of differences between genotypes for regeneration and doubling frequencies was tested by χ^2 analysis. This analysis was also used to determine the significance of differences between genotypes, species and ploidy level in terms of molecular data. Yates's correction for continuity was employed. Genetic similarities among clones were calculated as described by Aversano et al. (2009).

Results

The production of tissue culture-derived regenerants is reported in Table 1. All genotypes produced plantlets, with higher regeneration frequencies in *S. cardiophyllum* (62%). As for *S. bulbocastanum*, even though regeneration frequency was lower (34%) than that displayed by *S. cardiophyllum*, the number of shoots per explant was higher (2.5). The analysis of chromosomes in root tips revealed that plants considered 2x on the basis of chloroplast count always had 24 chromosomes; those considered 4x always showed 48 chromosomes. This analysis allowed the detection of 19 diploid regenerants (31%) and 42 tetraploid regenerants (69%). Comparing data obtained from *S. bulbocastanum* and *S. cardiophyllum*, doubling frequencies were not significantly different ($p < 0.05$). *S. tuberosum* regenerated only 4*x* shoots.

We used five AFLP primer pairs to examine the effect of in vitro regeneration at whole genome level both in 2x and in 4x derivatives. To better appreciate the response of regenerants according to ploidy, we examined separately the diploid and the tetraploid derivatives for each species. All bands were scored as a binary character for absence (0) and presence (1). Total polymorphism scored is given in Table 2. In *S. bulbocastanum*, the assay generated a total of 2,887 bands, representing 241 distinguishable genetic loci (data not shown). A pairwise comparison between loci detected in the parent with those in 2x regenerants showed that 79 sites out of 139 were changed, of which 45% (63/139) were lost in 2x regenerants and 12% (16/139) were gained. Differences between "loss" and "gain" were statistically significant based on an expectation of equal occurrence ($\chi^2 = 27.9$, $p < 0.01$). In 4x regenerants, we found the "loss" sites predominant as well. In particular, 50% (80/160) of polymorphic sites disappeared in 4x regenerants and 19% (31/160) appeared ex novo. These differences were statistically significant ($\chi^2 = 21.6$, $p < 0.01$). As for *S. cardiophyllum*, we scored a total of 4,357 bands representing 303 loci. A pairwise comparison between cph1C and 2 regenerants indicated that in 2x

Table 1 Regeneration frequency, mean number (±SE) of shoots and ploidy level of genotypes regenerated from *S. bulbocastanum* (*blb1C*), *S. cardiophyllum* (*cph1C*), and *S. tuberosum* (Désirée)

Genotype	Cultured explants (*n*)	Regeneration (%)	Shoots/explant ($n \pm$ SE)	Regenerants [*n* (%)]	
				2x	4x
blb1C	64	34.4	2.5 ± 1.5	12 (37.5)	20 (62.5)
cph1C	45	62.2	1.9 ± 1.1	7 (29.2)	17 (70.8)
Désirée	16	28.1	1.6 ± 0.5	0	5 (100)
Total				19 (31.1)	42 (68.9)

Table 2 Number of total and polymorphic AFLP bands scored among 2x and 4x regenerants of *Solanum bulbocastanum*, *S. cardiophyllum* and *S. tuberosum*

	Total bands (*n*)	Polymorphic bands [*n* (%)]	Loss [*n* (%)]	Gain [*n* (%)]	$\chi^2_{\text{loss vs. gain}}$
S. bulbocastanum					
2x	139	79 (57)	63 (45)	16 (12)	27.9*
4x	160	111 (69)	80 (50)	31 (19)	21.6*
$\chi^2_{\text{2x vs. 4x}}$		5.3 ns	2.0 ns	4.7 ns	
S. cardiophyllum					
2x	297	172 (58)	89 (30)	83 (28)	0.2 ns
4x	303	190 (63)	101 (33)	89 (30)	0.7 ns
$\chi^2_{\text{2x vs. 4x}}$		0.9 ns	0.7 ns	0.2 ns	
S. tuberosum					
4x	207	111 (54)	74 (36)	37 (18)	12.3*

For each ploidy level, the frequency of loss-type and gain-type bands is reported

ns Not significant

* Significant at $p < 0.01$

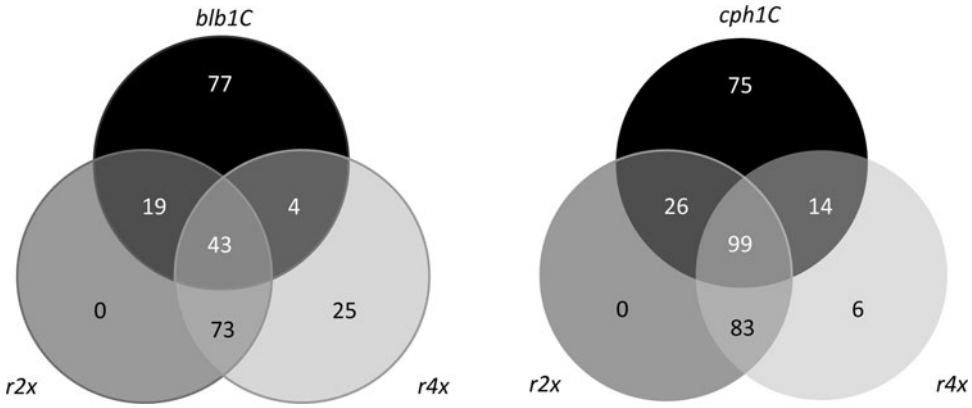

Fig. 1 Venn diagram showing unique and shared fragments among *S. bulbocastanum* and *S. cardiophyllum* diploid (*r2x*) and tetraploid (*r4x*) regenerants and the parents (*blb1C* and *cph1C*, respectively) they derived from

regenerants, the frequency of "loss" loci was slightly higher (30%) than the "gain" (28%). Similar results were obtained in the 4x regenerants (33 vs. 30%). In *S. tuberosum*, the frequency of "loss" fragments (36%) and that of "gain" (18%) were statistically significant ($\chi^2 = 12.3$, $p < 0.01$). Overall, we did not find significant differences between 2x and 4x in terms of loss and gain.

A broad comparison among parents, 2x and 4x regenerants was carried out. Seven types of banding patterns were observed as described in "Materials and methods". We used the Venn diagram to compare them (Fig. 1). As for *S. bulbocastanum*, we found that both in 2x and 4x regenerants, 73 fragments were gained and 77 parental fragments disappeared. We considered these bands as an effect of tissue culture-induced variation (classes 011 and 100, respectively). Twenty-five loci were de novo observed only in the 4x derivates, whereas 19 were lost only in the tetraploid ones (classes 001 and 110, respectively). We called these bands "diagnostic fragments" because they may be ploidy-associated. Among the diploid regenerants no de novo loci (010) were scored, whereas four parental fragments disappeared (101). As for *S. cardiophyllum*, 83 bands belonged to 011 class and 75–100 class. Six loci were de novo observed only in the 4x derivates, whereas 26 were lost only in the tetraploids (classes 001 and 110, respectively). No de novo loci were found in the diploid regenerants (010), whereas 14 were lost only in 2x regenerants.

Two UPGMA dendrograms are presented in Fig. 2a, b. Due to the low similarity values, the *S. bulbocastanum* and *S. cardiophyllum* dendrograms lacked a well-supported grouping, suggesting that regenerants have diverged significantly from each other and from the parents. Several clusters were found with both 2x and 4x regenerants clustered together.

Discussion

Recently, the association between molecular stability and tissue culture in various potato species has been explored (Aversano et al. 2009; Sharma et al. 2007; Barandalla et al. 2006). However, so far, it remains to be ascertained whether somaclonal variation differs between callus-derived chromosome-doubled and undoubled regenerants. To address this issue, we have firstly produced diploid and tetraploid regenerants from wild *S. bulbocastanum* and *S. cardiophyllum* (both $2n = 2x = 24$) and tetraploids from

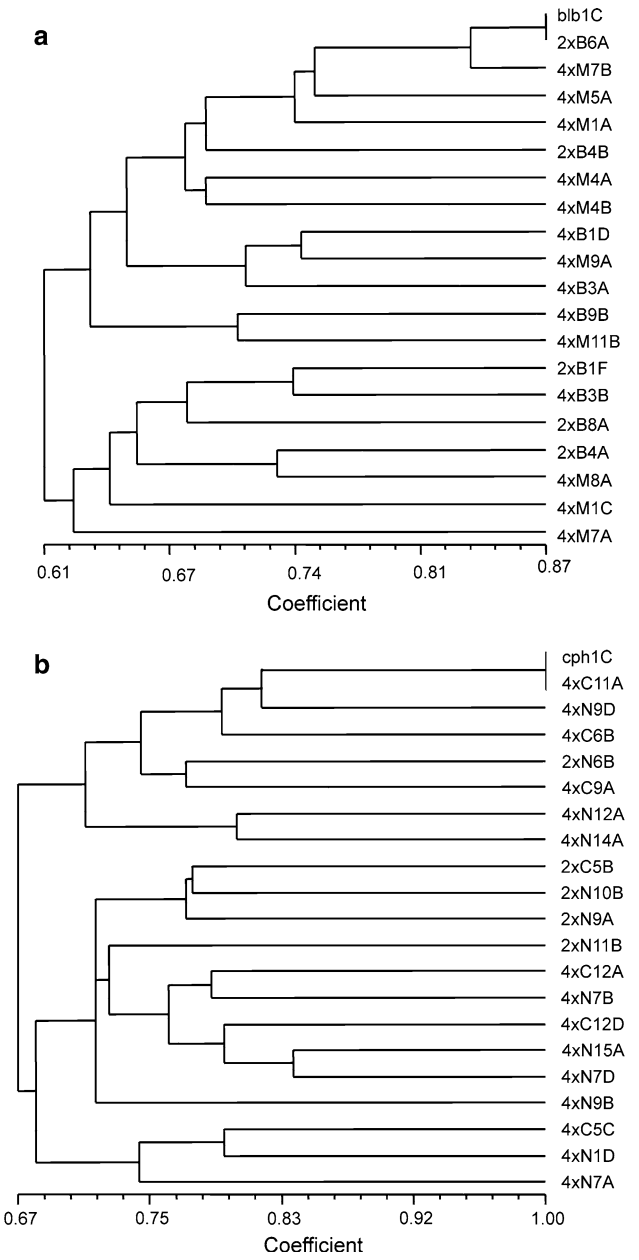

Fig. 2 UPGMA dendrograms of *S. bulbocastanum* (**a**), *S. cardiophyllum* (**b**) 2x and 4x regenerants obtained through in vitro tissue culture. The parental genotypes blb 1C and cph 1C are also included

cultivated *S. tuberosum* (2x = 4x = 48). Under identical regeneration conditions (such as the in vitro process and its duration, hormone and nutrient balance, explant type and age of donor plant, etc.), the genotypes behaved differently in their ability to polyploidize. Indeed, diploid wild species doubled their chromosome complements, whereas tetraploid *S. tuberosum* did not produce regenerants with higher ploidy level. These data are in agreement with early reports demonstrating that cytological stability increases with ploidy level (Cardi et al. 1992; Pijnacker et al. 1989; Sree Ramulu 1987). The extent of genetic variation of regenerants obtained was ascertained through molecular markers. AFLP analysis revealed that structural changes due to the regeneration process strongly affected the genome of the species studied. We hypothesize that changes observed in our genotypes were due to the regeneration process accomplished through a callus phase rather than in vitro micropropagation through axillary buds. Indeed, in the potato, Sharma et al. (2007) demonstrated that no polymorphisms are detected among AFLP profiles of in vitro propagated plants obtained through axillary buds. In our study, the occurrence of novel bands as well as the disappearance of some parental fragments in the regenerants were detected at high rates. "Loss" fragments were significantly higher than "gain" fragments in *S. bulbocastanum* and in *S. tuberosum* but not in *S. cardiophyllum*, where a similar incidence of the two events was detected. It is well known that the duration of tissue culture strongly challenges genome integrity (for a recent review, see Bairu et al. 2011). Although our regenerants experienced a relatively short time exposure to tissue culture conditions, they still showed genomic variation. In the literature, it is reported that short time tissue culture may affect genome integrity. For instance, Guo et al. (2006) were able to detect genomic variation in 63 phenotypically-normal regenerants of *Codonopsis lanceolata* obtained by 2 weeks of culture onto a callus induction medium followed by a 4-week period for shoot regeneration from calli. Sultana et al. (2005) detected genome instability in Indica Basmati rice cultured for 2 weeks on callus induction medium. Such evidence reasonably suggests that the somaclonal variation observed in the regenerants analyzed here is brought about by dedifferention stress rather than the short time exposure to tissue culture. Its extent may also depend on the genotype, as already reported in several species including *Camellia* spp. (Devarumath et al. 2002), *Capsicum annuum* (Hossain et al. 2003), *Codonopsis lanceolata* (Guo et al. 2006) and *Solanum* (Aversano et al. 2009; Sharma et al. 2007). Moreover, to explain such a different response to tissue culture, we might hypothesize that activation of a cryptic mutator system like transposition of mobile elements (Kaeppler et al. 2000) and/or malfunction of a normally functional anti-mutational system rather than point mutations may vary from one genotype to another.

After whole genome integrity assessment, we compared the effects of in vitro culture between callus-derived chromosome-doubled and undoubled regenerants. Towards this goal, we considered only those AFLP bands present in 4x regenerants but missing in the 2x ones (parents and derivatives) and vice versa (001- and 110-types, respectively). Although these banding patterns totaled 18 and 10% in *S. bulbocastanum* and *S. cardiophyllum*, respectively, cluster analysis failed to reveal significant correlations between ploidy and observed changes. These results suggest that diploid and tetraploids regenerated plants

respond randomly to tissue culture conditions. Similarly, in a study by Stupar et al. (2007) on non-additive gene regulation in tetraploids of diploid *S. phureja* obtained by somatic embriogenesis, RAPD and AFLP analyses displayed no significant polymorphisms within the ploidy series. It might be argued that diagnostic fragments could be related to genome duplication per se rather than somaclonal variation. However, the genetic material produced in this study does not allow investigations on the effect of genome doubling. Such a hypothesis remains to be proved once any source of variability (besides polyploidization) has been ruled out. In *Solanum*, various authors have reported that somaclonal variation affects noteworthy traits such as resistance to tuber soft rot and root-knot nematodes (Taylor et al. 1993; Grammatikaki et al. 1999), nutrition quality (Langille et al. 1998) and agronomic traits (Thieme and Griess 1996). Despite the great plasticity of the potato genome (Hasim et al. 1990; Abe et al. 1999; Jain 2001), we did not observe shifts in terms of resistance to late blight in any of the regenerated plants tested (data not shown), and all genotypes showed the same high resistance level as their parents, revealing for this trait a "silent nature" of the changes observed. However, some gene changes might not have been observed at a morphological or physiological level because the structural differences in the gene product may not have altered its biological activity sufficiently to result in a modified phenotype. Since we found our species to be prone to genomic changes after in vitro regeneration, we cannot exclude that other phenotypic traits were affected by somaclonal variation.

In conclusion, our study provided evidence that the genome integrity of callus-derived regenerants is strongly affected by tissue culture. We believe that, when the aim of in vitro regeneration is not to generate genetic variability but rather offer tools for other purposes (e.g., overcome sexual barriers or self-incompatibility), breeders should bear in mind that plants derived even through organized meristem culture may not always be genetically true-to-type due to the unpredictable and uncontrollable nature of somaclonal variation. Our study also provided evidence that chromosome-doubled and undoubled regenerants are evenly affected by somaclonal variation.

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