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Research Article

Genetic stability evaluation of caladium somaclonal variants by morphological, cytological, and SSR analysis in three successive generations

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Abstract

Somaclonal variants with valuable agronomic traits play a crucial role in crop breeding, provided that they are stably passed down in subsequent generations. Although numerous somaclonal variants have been identified and studied among *in vitro* regenerated plants in the foliage plant caladium (*Caladium* × *hortulanum* Birdsey), no research has been carried out to evaluate their genetic stability in subsequent generations. This study aimed to evaluate the genetic stability of three types of diploid caladium somaclonal variants, previously derived from *in vitro* callus cultures, over three successive tuber-propagated generations. The analysis of quantitative morphological characteristics revealed a greater degree of variation among the established plants in the first generation (G_1) compared to the second (G_2) and third generations (G_3). Seven plants exhibiting distinct leaf coloration changes were identified in the G1 generation and were found to be stably passed down to the subsequent G_2 and G_3 generations. These findings indicate a wide range of morphological variation in the G_1 generation, followed by relative stability in subsequent generations. A comprehensive cytological and molecular analysis revealed that five of the seven newly observed variants displayed notable differences in relative nuclear DNA content, chromosome number, or SSR (simple sequence repeat) banding patterns compared to their corresponding normal counterparts. The findings of this study will be instrumental in developing new cultivars with distinctive plant morphology through the exploitation of somaclonal variation in caladium.

Keywords: caladium breeding; chromosome number; nuclear DNA content; somaclonal variation; tuber generations

Introduction

Plant cell and tissue culture has become a fundamental and efficient tool in plant science research. It is a widely utilized technique for rapid propagation, germplasm conservation, and secondary metabolite production of plant resources (Abd Elaziem *et al*., 2022; Fazili *et al*., 2022). Nevertheless, it has been extensively documented those plants regenerated *in vitro* undergo changes in various aspects, including morphological

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features, chromosome number and structure, physiological-biochemical traits, DNA sequence, or epigenetic regulation, in most economically important plant species (Bairu *et al*., 2011; Eeckhaut *et al*., 2020; Zhang *et al*., 2021; Zhao *et al*., 2023). The variation among the regenerants produced through *in vitro* culture of cells or tissue is called somaclonal variation (Ferreira *et al*., 2023). Maintaining the phenotypic fidelity of regenerated plants in relation to the donor plant is essential, as this is a crucial aspect of commercial clonal propagation of plants. Consequently, somaclonal variation is generally regarded as unfavourable (Bairu *et al*., 2011; Zhang *et al*., 2023). Nevertheless, it has been extensively documented that somaclonal variation is pivotal in generating novel genetic resources for geneticists and breeders (Krishna *et al*., 2016; Ferreira *et al*., 2023). The process of somaclonal variation has the potential to generate lines with desirable agronomic traits while simultaneously minimizing the occurrence of other changes to the parental genomes. Examples of improved agronomic traits include enhanced disease resistance (Chakraborty *et al*., 2020), increased abiotic stress tolerance (Hannachi *et al*., 2021), high yield potential (Zeid *et al*., 2022), and improved ornamental characteristics (Cai *et al*., 2015; Cao and Deng, 2020; Parrish *et al*., 2023). Nowadays, the utilization of somaclonal variation as a valuable tool for genetic and cultivar improvement has been observed in numerous crop species, particularly in ornamental plants (Chen and Henny, 2006; Eeckhaut *et al*., 2020; Ferreira *et al*., 2023).

From a breeding perspective, it is important that the traits identified in somaclonal variants can be stably inherited and expressed in subsequent sexual or asexual generations. Nevertheless, the utilization of somaclonal variation in crop breeding has encountered several obstacles, including unpredictable variation, diminished regeneration capacity, and genetic instability (Krishna *et al*., 2016; Ferreira *et al*., 2023). DNA-related heritable and epigenetic mechanisms contribute to somaclonal variation (Bairu *et al*., 2011; Zhang *et al*., 2021). Heritable somaclonal variation in plants results from alterations to single or multiple genes, chromosomes, or entire sets of chromosomes. In most cases, it can be transmitted to the regenerated plants and their progeny through sexual or repeated asexual generations (Barpete *et al*., 2020; Ferreira *et al*., 2023). In contrast, epigenetic changes involving gene expression variations, such as gene silencing and gene activation effects, are typically transient, unstable, and non-heritable under normal conditions (Kaeppler *et al*., 2000). Consequently, their practical value in crop breeding is significantly diminished. In certain instances, epigenetic modifications, such as DNA methylation resulting from tissue culture, have been observed to be stable and heritable. It has been demonstrated by Stelpflug *et al*. (2014) in maize and Stroud *et al*. (2013) in rice. However, studies related to somaclonal variation typically concentrate on screening, selecting, and identifying regenerated somaclones during the *in vitro* phase. Information on the stability of somaclonal variants is lacking.

Caladium (*Caladium* × *hortulanum* Birdsey) is a perennial herbaceous aroid plant species in the Araceae family. The leaves of this plant exhibit a wide range of color patterns, sizes, and shapes, rendering it an intriguing ornamental species widely planted in containers and urban landscapes (Deng *et al*., 2023). At present, considerable efforts have been made to develop new caladium cultivars through conventional sexual hybridization (Deng and Harbaugh, 2006; Deng *et al*., 2021; Deng *et al*., 2023). In recent years, a number of studies have demonstrated that somaclonal variation is a common phenomenon in caladium. The occurrence of the variation is dependent on several factors, including the explant genotype and age, the concentration and type of plant growth regulators, and the number of subculture cycles (Cao *et al*., 2016; Cao and Deng, 2020; Chen *et al*., 2021; Yu *et al*., 2022; Parrish *et al*., 2023). To date, a considerable number of somaclonal variants in caladium have been identified and characterized. The causes of the novel leaf characteristics of these variants have been the subject of considerable research. Several factors have been identified as potential contributors, including aberrant chromosome number, changes in nuclear DNA content, and structural DNA changes as detected by DNA band profiles (Cao *et al*., 2016; Cao and Deng, 2020; Chen *et al*., 2021; Yu *et al*., 2022; Parrish *et al*., 2023). Among these somaclonal variants, a small proportion were found to have enhanced aesthetic qualities (Cao *et al*., 2016; Chen *et al*., 2021), suggesting potential for further genetic investigation and commercial enhancement of caladium. However, it is essential to note that no research has been conducted

on the potential value of somaclonal variation in caladium breeding, including the stability of variants across tuber propagation. This represents a significant research gap that needs to be addressed to fully understand and exploit the potential of somaclonal variation in caladium breeding.

In our previous study, various variant plants were obtained from the long-term subcultured callus of the 'Red Flash' caladium. These plants were confirmed as somaclonal variations through meticulous morphological, cytological, and pigment analysis (Chen *et al*., 2021). Evaluating the stability of these somaclonal variants in clonally propagated generations is crucial, as this will contribute to the development of new cultivars in caladium. The present study analyzed the genetic stability of three types of somaclonal variants of 'Red Flash' caladium over three successive clonally propagated generations using morphological, flow cytometric, chromosome number, and simple sequence repeat (SSR) analysis. The objective is to assess the potential of these variants in subsequent caladium breeding.

Materials and Methods

Plant materials

Three somaclonal variants (SVT1, SVT2, and SVT10) were obtained from the long-term subcultured callus of 'Red Flash' caladium (Chen *et al*., 2021) and employed in this study. The variants were confirmed to be diploid and exhibited the same chromosome number (2n = 2x = 30) as the wild caladium (Chen *et al*., 2021). Upon the temperature dropping below 15 °C, the tubers of the variant plants (G_0) were harvested and stored individually. In the middle of March of the following year, the stored tubers were divided and then buried in sterilized wet sand in an artificial climate box with a temperature of 25.0 ± 1.0 ℃ and relative humidity of approximately 75% for sprouting. Once the sprouted buds reached a height of approximately 1.0 cm, they were individually transplanted into plastic pots (14.5 cm × 17.0 cm) containing peat and perlite in a 3:1 volume ratio. The plants were cultivated under a 14-h photoperiod at 120 μmol m^{-2} s^{-1}, 26 ± 2 °C, and approximately 75% relative humidity. Totally 42 plants were established in the first tuber-propagated generation (G_1), comprising 20, 14, and 8 plants propagated from the tubers of the SVT1, SVT2, and SVT10, respectively. In the second (G_2) and third tuber (G_3) generations, the harvested tubers from the previous generation (G_1 or G_2) were divided, forced to sprout, and cultivated in pots. The plants were watered once a week. Each plant was designated a name based on its variation type and assigned a sequential number.

Plant establishment and morphological characterization

Following four months of cultivation in the G_1 generation, a series of morphological characteristics were evaluated, including leaf coloration patterns, plant height, leaf number, leaf size, and petiole diameter. The diameter of petioles was measured at the midpoint of each petiole using an electronic digital caliper. Three mature leaves per plant were evaluated for leaf length, leaf width, and petiole diameter. The plants were observed in detail over three successive generations of tubers. In the G_1 generation, plants exhibiting distinct and stable morphological changes were identified and subsequently examined in the G_2 and G_3 generations. Three plants exhibiting no morphological alterations from each somaclonal variant (normal SVT) and newly observed variants were subjected to a comprehensive genetic stability assessment in the G_3 generation, encompassing cytological and SSR analysis.

Flow cytometry analysis

A relative DNA content analysis was conducted using a Beckman CytoFLEX flow cytometer (Suzhou, China) equipped with a 488 nm argon laser. To prepare for flow cytometry, intact nuclei were released in woody plant buffer (WPB) as described by Loureiro *et al*. (2007) and fluorescently stained with propidium

iodide (PI) in accordance with the methodology outlined by Zhang *et al*. (2020). The relative fluorescence intensity of at least 3000 nuclei was measured for each plant sample, with three replicates per run.

Chromosome counting

Chromosome counting was conducted on actively growing root tips using conventional squash techniques to determine the chromosome number. The preparation of metaphase chromosomes was conducted following Cao *et al.* (2016). A minimum of five cells with well-spread chromosomes per slide and 5-10 root tips were observed and imaged using a Nikon Eclipse Ni-U microscope (Nikon, Tokyo, Japan) at 1000 times magnification.

SSR analysis of the nuclear genome

Eight pairs of caladium-specific simple sequence repeat (SSR) primers retrieved from Gong and Deng (2011), namely CaM5, CaM16, CaM24, CaM42, CaM78, CaM87, CaM101, and CaM106, were employed for the analysis of the fresh young leaves of the somaclones exhibiting color changes and their three corresponding normal counterparts. Approximately 100 mg of the plant samples were ground with liquid nitrogen, and genomic DNA was extracted using the modified cetyltrimethylammonium bromide (CTAB) method described by Fulton *et al*. (1995). The extracted DNA samples were quantified using a NanoDrop One C spectrophotometer (Thermo Fisher Scientific, Madison, WI, USA). Then they were diluted to a working concentration of 25 ng μl^{-1} for SSR-PCR analysis. Each 20 μl of reaction mixture contained 2 μl of template DNA, 0.4 μl of each forward and reverse primer (10 μM),10 μl of 2 × Taq PCR StarMix with loading dye (GenStar, Beijing, China), and 7.2 μl of deionized water. The genomic DNA was amplified using the protocol described by Zhao et al. (2023) on a T100 thermocycler (BIO-RAD, Singapore). The amplified products were subsequently analyzed on 8% non-denaturing acrylamide gels, as described by Yu *et al*. (2022).

Data analysis

All variables were expressed as mean ± standard deviation (SD). Independent sample t-tests were conducted using SPSS version 23.0 to ascertain statistically significant differences between the novel leaf color variants and their corresponding normal counterparts at $p < 0.05$. Correlations among the different parameters were analyzed using Pearson's correlation.

Results

Variation in quantitative traits in different generations

Quantitative morphological traits, including plant height, leaf number, leaf length, leaf width, and petiole diameter, were measured in the progeny of the three caladium somaclonal variants (SVT1, SVT2, and SVT10) over three consecutive generations. As illustrated in Table 1, the coefficient of variation (CV) for each morphological trait exhibited a higher value in the G_1 generation relative to the G_2 and G_3 generations. Among the three generations, the three types of variants consistently exhibited the lowest CV values for all measured parameters in the G_3 generation. Table 1 indicates that the measured phenotypic parameters of all somaclones exhibited the most tremendous variation in the G_1 generation, followed by the G_2 and G_3 generations. Furthermore, it was observed that the different variation types exerted a significant influence on the CV values of the morphological parameters of their tuber-propagated progeny, particularly in the G_1 generation. For example, the SVT1 exhibited the highest CV values for plant height (35.12%), leaf number (21.25%), leaf length (27.65%), and leaf width (31.28), but the lowest CV value for petiole diameter (19.38%) among the three types of plants in the G_1 generation.

Table 1. Descriptive statistics for phenotypic parameters of the tuber progeny derived from three types of caladium somaclonal variants (SVT1, SVT2, and SVT10) in the first (G_1), second (G_2), and third (G_3) generations

Plants	Gene-rations	Plant height (cm)			No. of leaves per plant			Leaf length (cm)			Leaf width (cm)			Petiole diameter (mm)		
		Min	Max	CV (%)	Min	Max	CV (%)	Min	Max	CV (%)	Min	Max	CV (%)	Min	Max	CV (%)
SVT1	G_1	25.0	52.0	35.12	2.0	4.0	21.25	15.3	42.3	27.65	8.5	24.1	31.28	2.8	6.9	19.38
	G_2	35.5	49.2	21.55	3.0	5.0	16.53	15.8	40.2	22.81	8.6	24.0	28.25	3.7	6.7	15.40
	G_3	40.6	55.5	18.06	3.0	6.0	13.95	17.6	41.0	17.43	9.6	24.8	21.13	4.0	8.7	15.01
SVT2	G_1	31.8	58.0	25.65	2.0	5.0	20.83	16.5	34.0	19.30	10.7	21.2	21.62	2.6	6.6	25.25
	G_2	44.2	58.5	18.82	4.0	5.0	15.24	18.0	36.6	18.32	13.2	24.1	18.50	3.6	6.8	18.77
	G_3	46.2	54.7	15.80	4.0	5.0	8.33	26.3	42.0	18.22	15.5	25.2	17.45	4.4	8.0	17.92
SVT10	G_1	31.2	50.3	21.71	3.0	4.0	20.42	15.2	31.3	19.91	8.9	22.2	26.89	2.6	6.7	26.92
	G_2	32.8	57.5	17.54	3.0	4.0	17.94	16.8	31.6	17.33	9.5	22.5	22.99	3.0	6.4	24.91
	G_3	34.3	54.8	11.42	3.0	4.0	16.22	16.5	31.0	12.30	10.7	21.2	18.84	3.2	7.1	20.16

Changes in leaf coloration in three successive generations

The leaf colorations of these variants were observed in three successive generations at the maturation stage of the plants. Most plants belonging to the same variation type exhibited no discernible differences in the coloration of the main veins, interveinal areas, leaf margins, and leaf spots. The plants were classified as belonging to the standard category: normal SVT1, normal SVT2, and normal SVT10. Upon close observation, a total of seven plants from the three types of variants exhibited distinct and stable morphological changes compared to their normal counterparts (Table 2). The frequency of observed leaf color changes was found to vary from 12.5% to 20.0% in the G_1 generation, with the SVT1 plants exhibiting the highest frequency (20.0%), followed by the SVT2 (14.3%) and the SVT10 (12.5%). No alterations in leaf coloration were discerned in the G_2 and G_3 generations, and all somaclones exhibited identical leaf coloration as observed in the G_1 generation (Table 2).

Table 2. Number of plants exhibiting leaf color changes and frequency of leaf color changes in the tuber-propagated progeny derived from three types of caladium somaclonal variants in three successive generations

Plants	G_1		G_2		G_3	
	No. of plants with leaf color changes [a]	Frequency of leaf color changes (%) [b]	No. of plants with leaf color changes	Frequency of leaf color changes (%)	No. of plants with leaf color changes	Frequency of leaf color changes (%)
SVT1	4	20.0	0	0.0	0	0.0
SVT2	2	14.3	0	0.0	0	0.0
SVT10	1	12.5	0	0.0	0	0.0

[a] Leaf color changes were identified based on distinct and stable alterations in the color of main veins, interveinal areas, leaf margins, and leaf spots

[b] Frequency of leaf color changes (%) = the number of plants exhibiting leaf color changes/the total number of established plants × 100

Morphological characterization of the somaclones in the G_3 generation

In the G_3 generation, changes in leaf color were observed in four plants (SVT1-4, SVT1-8, SVT1-12, and SVT1-19) derived from the SVT1, two plants (SVT2-5 and SVT2-8) from the SVT2, and one plant

(SVT10-1) from the SVT10 (Figure 1 and Table 3). The normal SVT1 exhibited heart-shaped (fancy) leaves with green main veins, several white or pinkish-red spots, and purple-red or green interveinal areas. Compared to the normal SVT1, the leaves of SVT1-4, SVT1-12, and SVT1-19 exhibited notable differences, displaying red main veins and extensive red interveinal areas. The SVT1-8, on the other hand, had larger leaves with pinkish-red main veins (Figure 1 and Table 3). The main veins and interveinal areas of the normal SVT2 were green, while the two plants with color changes exhibited purple-red (SVT2-5) or pinkish-red (SVT2-8) main veins and dark-red (SVT2-5) or pinkish-red interveinal areas (SVT2-8) (Figure 1 and Table 3). The distinction between the normal SVT10 and the SVT10-1 was merely a matter of color tone. The SVT10 exhibited a more pronounced hue in the main veins, interveinal areas, and leaf margins (Figure 1 and Table 3).

Table 3. Leaf coloration patterns of the tuber progeny from three types of caladium somaclonal variants (SVT1, SVT2, and SVT10) observed in the G_3 generation

Plants	Main veins	Interveinal areas	Leaf margins	Leaf spots
Normal SVT1	Green	Purple-red/Green	Green	White/Pinkish-red
SVT1-4	Red	Red	Light-green	Pinkish-red
SVT1-8	Purple-red	Pinkish-red	Green	White/Pinkish-red
SVT1-12	Dark-red	Red	Light-green	White/Pinkish-Red
SVT1-19	Red	Red	Light-green	Pinkish-red
Normal SVT2	Green	Green	Light-green	White
SVT2-5	Purple-red	Purple-red	Light-green	White/Pinkish-red
SVT2-8	Pinkish-red	Pinkish-red	Light-green	White/Pinkish-red
Normal SVT10	Dark-red	Dark-red	Green	Pinkish-red
SVT10-1	Red	Red	Light-green	Pinkish-red

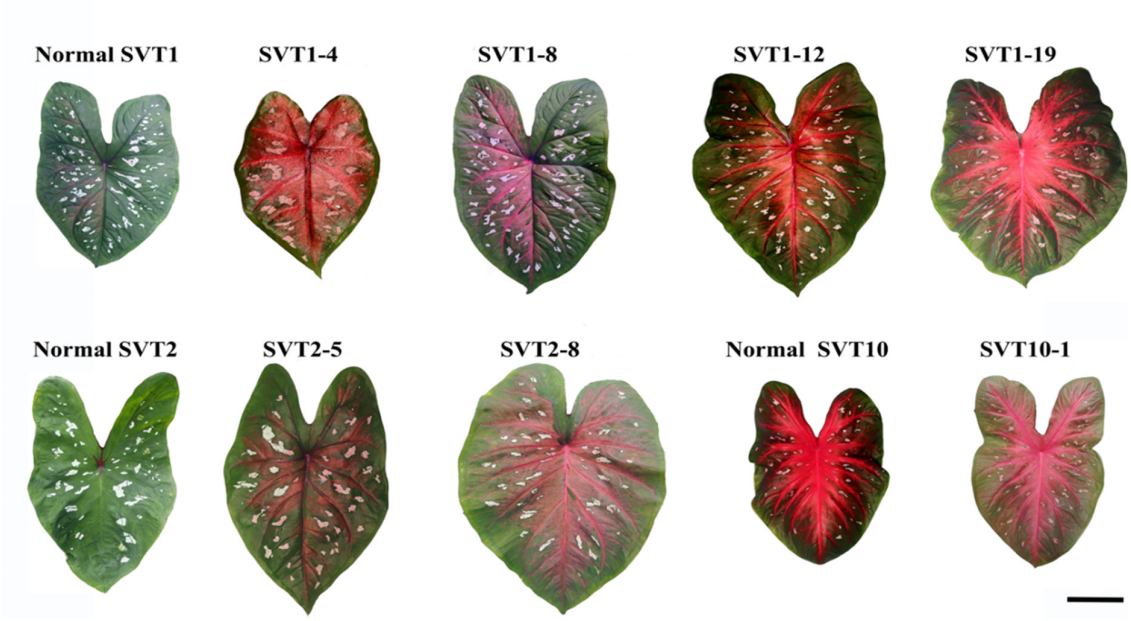

Figure 1. Representative leaves of the tuber progeny from three types of caladium somaclonal variants (SVT1, SVT2, and SVT10) observed in the G_3 generation
Scale bar = 5 cm

As demonstrated in Table 4, the morphological characteristics of the three types of variants were measured in the G_3 generation. In the SVT1 group, the SVT1-19 plant exhibited the greatest height (49.2 cm) and the lowest number of leaves (3.0). Both SVT1-4 and SVT1-12 exhibited no significant differences from the normal SVT1 in terms of leaf length, leaf width, leaf length/width ratio, and petiole diameter. The SVT1-8 plant had larger leaves and a significantly thinner petiole. In comparison to the normal SVT1, the SVT1-19 exhibited leaves with a more rounded appearance, accompanied by a significantly larger leaf width (22.0 cm) and a significantly lower leaf length/width ratio (1.31). With regard to the SVT2 and SVT10 groups, no significant differences were identified in the examined parameters between the newly observed leaf color variants and their corresponding normal counterparts.

Table 4. Morphological comparisons of the tuber progeny from three types of caladium somaclonal variants (SVT1, SVT2, and SVT10) in the G_3 generation

Plants	Plant height (cm)	No. of leaves per plant	Leaf length (cm)	Leaf width (cm)	Leaf length/width ratio	Petiole diameter (mm)
Normal SVT1	45.2 ± 5.0	4.3 ± 0.6	27.0 ± 4.7	17.0 ± 3.6	1.58 ± 0.17	5.3 ± 0.8
SVT1-4	37.6	4.0	23.8 ± 5.7 ^{ns}	15.3 ± 4.0 ^{ns}	1.56 ± 0.05 ^{ns}	4.5 ± 0.5 ^{ns}
SVT1-8	45.7	5.0	30.0 ± 9.0 ^{ns}	18.2 ± 4.2 ^{ns}	1.64 ± 0.27 ^{ns}	4.3 ± 0.6 *
SVT1-12	46.9	4.0	30.3 ± 1.8 ^{ns}	21.2 ± 1.7 ^{ns}	1.43 ± 0. 03 ^{ns}	5.6 ± 1.2 ^{ns}
SVT1-19	49.2	3.0	29.0 ± 4.2 ^{ns}	22.0 ± 2.4 *	1.31 ± 0.07 *	5.9 ± 0.8 ^{ns}
Normal SVT2	51.7 ± 6.1	4.8 ± 0.4	28.9 ± 5.3	18.4 ± 3.2	1.60 ± 0.27	5.2 ± 0.9
SVT2-5	57.2	5.0	31.9 ± 4.0 ^{ns}	19.7 ± 4.3 ^{ns}	1.65 ± 0.23 ^{ns}	6.0 ± 0.3 ^{ns}
SVT2-8	58.0	5.0	33.1 ± 1.3 ^{ns}	21.3 ± 0.1 ^{ns}	1.55 ± 0.07 ^{ns}	5.8 ± 0.8 ^{ns}
Normal SVT10	47.3 ± 5.4	3.7 ± 0.6	24.2 ± 4.7	14.1 ± 2. 9	1.72 ± 0.07	5.6 ± 0.8
SVT10-1	51.2	3.0	25.9 ± 3.2 ^{ns}	16.1 ± 3.0 ^{ns}	1.63 ± 0.16 ^{ns}	4.9 ± 1.0 ^{ns}

^{ns} and * represent no significant and significant difference, respectively, between plants with leaf color changes and their corresponding normal counterparts according to independent-sample T test at $p < 0.05$

Changes in relative nuclear DNA content and chromosome number in the G_3 generation

As shown in Figure 2 and Table 5, variations in mean fluorescence intensity (MFI) were recorded between the seven plants with leaf color changes and their corresponding normal-type plants in the G_3 generation. No significant differences in MFI were observed for the SVT1-4, SVT1-8, SVT1-12, and SVT1-19 compared to the normal SVT1. In the SVT2 group, a considerable range in MFI was observed, with values spanning from 362437.9/2C (SVT2-8) to 404295.3/2C (SVT2-5). This represented a 9.06% decrease and a 1.45% increase, respectively, compared to the normal SVT2 (Table 5). The results of the independent-sample t-test indicated that the MFI of SVT2-8 was significantly lower than that of the normal SVT2. In contrast, no significant difference was observed between the SVT2-5 and the normal SVT2 (Table 5). In the SVT10 group, the SVT10-1 exhibited a statistically significant increase (12.87%) in the MFI compared to the normal SVT10 plants (Table 5).

Figure 3 and Table 5 present a detailed comparison of the chromosome number between the seven leaf-color-changing plants and their corresponding normal types in the G_3 generation. In the SVT1 group, all the somaclones, including the four-leaf color-changing plants (SVT1-4, SVT1-8, SVT1-12, and SVT1-19), were found to have $2n = 2x = 30$ chromosomes. The chromosome number of the four plants was identical to that of the normal SVT2 plants. In the case of the SVT2 somaclones, the SVT2-5 exhibited a comparable chromosome number ($2n = 2x = 30$) to that observed in the normal SVT2 plants. In contrast, the SVT2-8,

which displayed a 9.06% reduction in MFI, exhibited a loss of two chromosomes ($2n = 2x - 2 = 28$) compared to the normal SVT2. Concerning the SVT10, the chromosome number of the normal plants was $2n = 2x = 30$, whereas the SVT10-1, which exhibited a significantly increased MFI in comparison to its normal counterpart, displayed $2n = 2x + 2 = 32$ chromosomes.

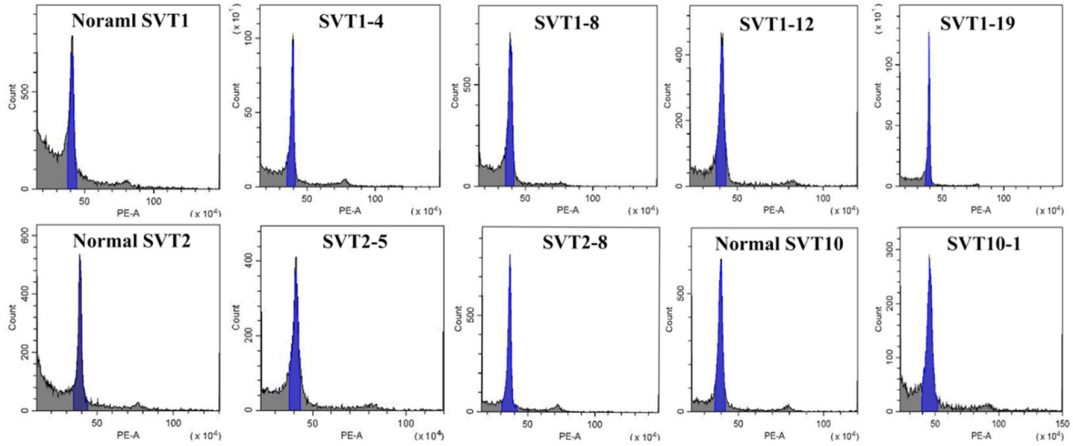

Figure 2. Histograms of the relative 2C DNA content in nuclei isolated from the tuber progeny of three types of caladium somaclonal variants (SVT1, SVT2, and SVT10) observed in the G_3 generation

Table 5. Changes in relative nuclear DNA content and chromosome number of the tuber progeny from three types of caladium somaclonal variants (SVT1, SVT2, and SVT10) in the G_3 generation

Plants	Mean fluorescence intensity (MFI)	MFI change compared to the corresponding normal counterpart (%) [a]	Chromosome number ($2n$)
Normal SVT1	399368.9 ± 7271.4	–	$2x = 30$
SVT1-4	391138.3 ± 7347.1 ns	-2.06	$2x = 30$
SVT1-8	391831.7 ± 7429.1 ns	-1.89	$2x = 30$
SVT1-12	401585.2 ± 8654.8 ns	0.55	$2x = 30$
SVT1-19	397852.1 ± 8585. 4 ns	0.38	$2x = 30$
Normal SVT2	398524.5 ± 5042.4	–	$2x = 30$
SVT2-5	404295.3 ± 14953.1 ns	1.45	$2x = 30$
SVT2-8	362437.9 ± 2987.4 *	-9.06	$2x - 2 = 28$
Normal SVT10	391485.9 ± 8029.9	–	$2x = 30$
SVT10-1	441866.1 ± 5019.4 *	12.87	$2x + 2 = 32$

[a] MFI change compared to the corresponding normal counterpart = [(MFI of the leaf color variant – MFI of its corresponding normal counterpart) ÷ MFI of the corresponding normal counterpart] × 100%. [ns] and * represent no significant and significant difference, respectively, between plants with leaf color changes and their corresponding normal counterparts according to independent-sample T test at $p < 0.05$

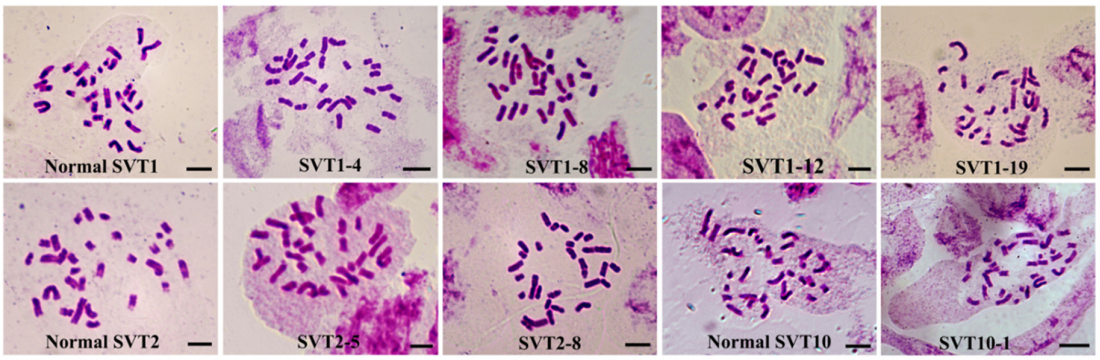

Figure 3. Micrographs (×1000) of somatic chromosomes in the root tips of the tuber progeny from three types of caladium somaclonal variants (SVT1, SVT2, and SVT10) in the G_3 generation
Bars = 10 μm

Correlations among morphological traits, relative nuclear DNA content, and chromosome number

The results of the correlation analysis among the morphological traits, relative nuclear DNA content, and chromosome number of the tuber progeny from the three variation types in the G_3 generation are presented in Table 6. Among the measured morphological traits, the plant height exhibited a significant ($p < 0.05$) correlation with the leaf length (r = 0.727) and petiole diameter (r = 0.649). The leaf width was found to be significantly ($p < 0.01$) positively correlated with the leaf length (r = 0.856) and significantly ($p < 0.05$) negatively correlated with the leaf length/width ratio (r = -0.714). No significant correlations were identified between the morphological traits and the mean fluorescence intensity or the chromosome number. Nevertheless, a significant correlation ($p < 0.01$) was identified between the mean fluorescence intensity and the chromosome number (r = 0.967).

Table 6. Correlations among morphological traits, relative nuclear DNA content, and chromosome number of the tuber progeny from the three types of caladium somaclonal variants in the G_3 generation

Correlated traits	Plant height	No. of leaves per plant	Leaf length	Leaf width	Leaf length/width ratio	Petiole diameter	Mean fluorescence intensity (MFI)
No. of leaves per plant	0.309						
Leaf length	0.727*	0.587					
Leaf width	0.514	0.194	0.856**				
Leaf length/width ratio	0.082	0.381	-0.257	-0.714*			
Petiole diameter	0.649*	-0.046	0.455	0.537	-0.314		
Mean fluorescence intensity (MFI)	-0.071	-0.562	-0.368	-0.291	0.101	-0.199	
Chromosome number	-0.268	-0.603	-0.542	-0.452	0.159	-0.362	0.967**

* and ** stands for significant correlation at the 0.05 and 0.01 level, respectively.

Verification of genetic stability using SSR markers in the G_3 generation

A genetic stability assessment was conducted using SSR analysis on the established plants in the G_3 generation, with three randomly selected normal plants serving as controls. Among the eight pairs of SSR primers utilized, five primer combinations, including CaM5 (Figure 4A), CaM24 (Figure 4B), CaM42 (Figure 4C), CaM87 (Figure 4D), and CaM101 (Figure 4E), were able to detect polymorphisms among these plant samples. In particular, the lower band of the SVT1-4 was observed to have a lower molecular weight than that of the normal SVT1 with primer CaM5 (Figure 4A). Additionally, a lower molecular weight DNA fragment loss was also observed in the SVT1-12 and SVT10-1 with primer CaM24 (Figure 4B). The loss of a lower and

an upper band was observed in SVT10-1 using primers CaM42 (Figure 4C) and CaM101 (Figure 4E), respectively. The amplification of the target sequence with primer CaM87 revealed that three somaclones including the SVT2-5, SVT2-8, and SVT10-1, exhibited a loss of a lower band (Figure 4D). In conclusion, alterations in the banding pattern were observed in the five new observed variants with the five primer pairs, including the SVT1-4, SVT1-12, SVT2-5, SVT2-8, and SVT10-1. The two plants exhibiting altered leaf coloration designated the SVT1-8 and SVT1-19, did not display any band alterations consistent with those observed in the SVT1.

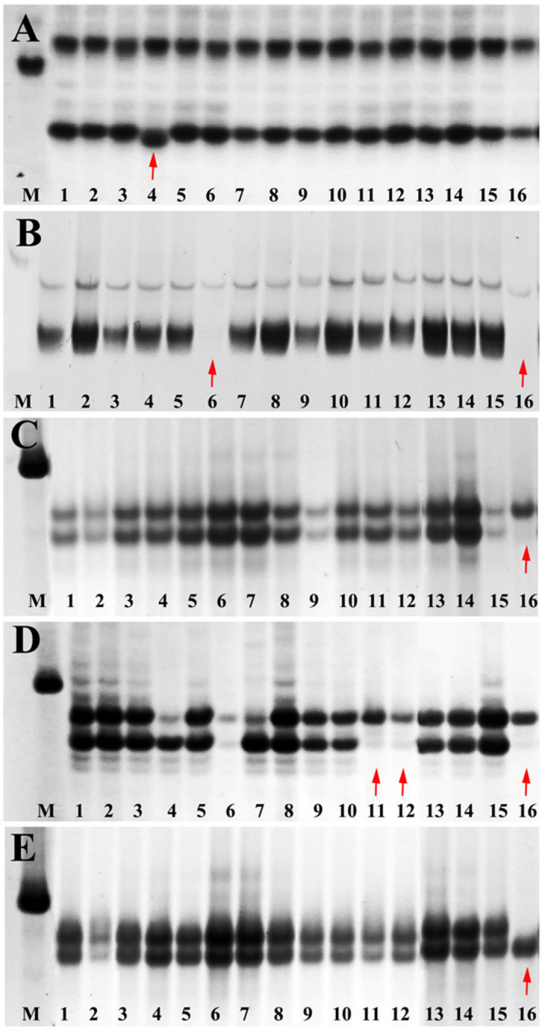

Figure 4. Polyacrylamide gel electrophoresis results of tuber progeny from three types of caladium somaclonal variants (SVT1, SVT2, and SVT10) in the G_3 generation by using five SSR primers, namely CaM5 (A), CaM24 (B), CaM42 (C), CaM87 (D), and CaM101 (E). M: 100 bp DNA marker; 1–3: Three normal SVT1; 4: SVT1-4; 5: SVT1-8; 6: SVT1-12; 7: SVT1-19; 8–10: Three normal SVT2; 11: SVT2-5; 12: SVT2-8; 13–15: Three normal SVT10; 16: SVT10-1. Red arrows indicate the presence or absence of SSR bands

Discussion

Somaclonal variation is regarded as a promising avenue for generating novel genetic resources for crop cultivar improvement and genetic research (Bairu *et al*., 2011; Krishna *et al*., 2016; Ferreira *et al*., 2023). However, the variation in qualitative and quantitative characteristics identified among *in vitro* regenerated plants may not be stably inherited by the progeny due to physiological disturbances, environmental effects, and epigenetic causes (Kaeppler *et al*., 2000; Barpete *et al*., 2020; Ferreira *et al*., 2023). Consequently, the successful application of somaclonal variation in crop improvement is largely contingent upon the stable inheritance of superior traits that have been identified in variants in subsequent generations. A relatively high percentage of somaclonal variation has been widely documented in caladium, with some variants exhibiting enhanced ornamental characteristics (Cao *et al*., 2016; Cao and Deng, 2020; Chen *et al*., 2021; Yu *et al*., 2022; Parrish *et al*., 2023). Nevertheless, no research has been conducted to assess the genetic stability of these caladium somaclonal variants for genetic improvement. Consequently, this investigation aims to estimate the genetic stability of the somaclonal variants of the 'Red Flash' caladium. It is a prerequisite for the widespread utilization of somaclonal variation in the development of caladium varieties.

Genetic variation can be detected at various levels, including morphological, cytological, physiological, biochemical, and molecular markers (Bairu *et al*., 2011; Zhang *et al*., 2021; Zhao *et al*., 2023). However, the primary selection of caladium somaclonal variants has predominantly relied on visual screening, despite the susceptibility of morphological traits to environmental factors (Cao *et al*., 2016; Chen *et al*., 2021; Yu *et al*., 2022; Parrish *et al*., 2023). This study evaluated the leaf coloration patterns and other phenotypic parameters of tuber-propagated progeny from the three types of caladium somaclonal variants over three consecutive generations. The quantitative traits of the caladium variants exhibited significant differences in the G_1 generation, becoming relatively stable only in the G_3 generation. Moreover, the caladium somaclonal variants demonstrated a frequency of leaf color alterations in the G_1 generation, yet these traits were stably transmitted to subsequent generations. Similarly, morphological instability of the somaclonal variants was also observed in Patchouli (*Pogostemon patchouli*) by Ravindra *et al*. (2012) and in sugarcane (*Saccharum officinarum*) by Akhter *et al*. (2021). This phenomenon may be attributed to the asynchronous development of regenerated plants during tissue culture (Akhter *et al*., 2021; Ravindra *et al*., 2012), which could have a significant impact on the growth of their progeny in the subsequent generation.

Cytological causes, including chromosome loss, gain, or duplication, have been identified as contributors to somaclonal variation in plants (Bairu *et al*., 2011; Eeckhaut *et al*., 2020; Ferreira *et al*., 2023). In this study, two somaclones, SVT2-8 and SVT10-1, exhibited significant changes in MFI compared to their corresponding normal counterparts. Chromosome counting revealed that the changes in chromosome number of the two plants were consistent with their measured MFI. A significant correlation was observed between the mean fluorescence intensity and the chromosome number. In previous studies, caladium somaclonal variants have also demonstrated a robust correlation between alterations in nuclear DNA content and changes in chromosome number in numerous instances (Cao *et al*., 2016; Chen *et al*., 2021; Cao and Deng, 2020; Yu *et al*., 2022). The remaining five plants exhibiting alterations in leaf color, including the four plants from the SVT1 (SVT1-4, SVT1-8, SVT1-12, and SVT1-19) and one plant from the SVT2 (SVT2-5), demonstrated no significant variation in MFI and no changes in chromosome number. Further investigation is required to determine the underlying causes.

Molecular markers, such as SSR, have been extensively utilized to assess the genetic stability of *in vitro* regenerated plants (Bairu *et al*., 2011; Cao and Deng 2020; Yu *et al*., 2022; Zhao *et al*., 2023). Therefore, an SSR analysis was conducted to elucidate the genetic causes underlying the alterations in leaf coloration observed in the somaclones derived from the three caladium variants in the G_3 generation. A total of five plants exhibited differences in DNA banding patterns, including the SVT1-4, SVT1-12, SVT2-5, SVT2-8, and SVT10-1. This may provide insight into the underlying causes of the observed changes in leaf coloration of the five plants.

However, the SVT1-8 and SVT1-19 did not exhibit any banding changes from SVT1 despite displaying leaf color changes. This discrepancy may be attributed to the fact that the five primer pairs employed, including CaM5, CaM24, CaM42, CaM87, and CaM101, could not provide comprehensive genome information for the analyzed plants. Previous studies have demonstrated that somaclonal variation can be caused by both heritable and epigenetic mechanisms (Bairu *et al*., 2011; Zhang *et al*., 2021). Epigenetic variation, such as alterations in DNA methylation patterns, can significantly affect gene expression, resulting in the plant phenotypic variation (Zhang *et al*., 2021; Ferreira *et al*., 2023). It is recommended that, in future studies, additional SSR primers, DNA-based molecular markers, and DNA methylation variation analysis be employed in order to provide more molecular information about the caladium variants.

Caladium displays considerable ornamental values, mainly due to the diversity of its leaf coloration patterns, which encompass the hue of the main vein. The color of the main veins of caladium is determined by a single locus, designated *V*, which has three alleles: V^r, V^w, and V^g. The three alleles, V^r, V^w, and V^g, confer red, white and green colors, respectively. The dominance order is $V^r > V^w > V^g$ (Deng and Harbaugh, 2006). The wild 'Red Flash' caladium has the genotype V^rV^g, which results in the formation of red main veins (Deng and Harbaugh 2006). Nevertheless, Chen et al. (2021) demonstrated that plants with green main veins could be regenerated *in vitro* from 'Red Flash' caladium callus. Chen et al. (2021) proposed that the observed changes in leaf main vein coloration may be attributed to the loss of the V^r allele or a mutation from V^r to V^g. This study identified the presence of pinkish-red, red, and purple-red main veins in the four newly observed variants of the SVT1 (SVT1-4, SVT1-8, SVT1-12, and SVT1-19), as well as in two leaf color-changed plants derived from the SVT2 (SVT2-5 and SVT2-8). This phenotype is analogous to that observed in the wild 'Red Flash' caladium. It is hypothesized that a reverse mutation from V^g to V^r may have occurred in these plants in the G_1 generation. Phenotypic reversion has also been documented in somaclonal variation in pineapple (*Ananas comosus*) (Halim *et al*., 2018) and date palm (*Phoenix dactylifera*) (Mirani *et al*., 2022). The phenomenon of somaclonal variation is typically associated with dynamic and reversible epigenetic alterations. These alterations are postulated to underpin the observed morphological transformations observed in somaclones across generations (Zhang *et al*., 2021; Ferreira *et al*., 2023). For instance, Biswas *et al*. (2009) observed that agronomical traits of 36% of somaclones reverted to their original phenotype within 2-3 generations in strawberry (*Fragaria* × *ananassa*). It can therefore be postulated that the reversion of the main vein color of these plants to the wild 'Red Flash' caladium may also be related to the DNA methylation of the V^g allele in this study. Further research is required to elucidate these alterations.

Moreover, previous studies have demonstrated that the majority of somaclonal variants are aneuploid, exhibiting an abnormal number of chromosomes (Cao *et al*., 2016; Cao and Deng, 2020; Chen *et al*., 2021; Yu *et al*., 2022). Aneuploidy can frequently result in aberrant growth or even lethality due to the genomic imbalance caused by incomplete chromosome sets (Henry *et al*., 2010; Dang *et al*., 2019). Nevertheless, some aneuploid plants have been observed to withstand the detrimental effects of aneuploidy, particularly in the case of polyploid genomes (Li *et al*., 2023). In this study, two plants with altered leaf coloration were identified as aneuploids: SVT2-8 ($2n = 2x - 2 = 28$) and SVT10-1 ($2n = 2x + 2 = 32$). The two plants exhibited normal growth and vigor in the three consecutive generations. Aneuploidy resulting from somaclonal variation can significantly affect genetic stability and induce remarkable phenotypic changes, which may be helpful to plant breeding and genetic research (Henry *et al*., 2010; Parrish and Deng 2022). It can be reasonably deduced that these two aneuploids (SVT2-8 and SVT10-1) may prove invaluable in the enhancement of caladium cultivars.

The combination of the results mentioned above revealed that a total of seven plants (SVT1-4, SVT1-8, SVT1-12, SVT1-19, SVT2-5, SVT2-8, and SVT10-1) from the three types of caladium variants (SVT1, SVT2, and SVT10) exhibited distinct changes in leaf color, chromosome number, or SSR banding pattern when compared to their corresponding normal counterparts. Although the established somaclones exhibited some variation in the G_1 generation, their leaf color traits were observed to be stably passed down to subsequent

generations in this study. As with numerous other floriculture plants, such as caladium, the screened and identified somaclonal variants can be vegetatively propagated, provided they are heritable and genetically stable in subsequent generations (Chen and Henny, 2006). Therefore, the selection of somaclonal variation in caladium is recommended to focus on the G_2 generation, with propagation of the screened variants conducted through tubers. This will permit further assessment of their agronomic characteristics, including tuber yield, disease resistance, and cold tolerance, which can be employed in developing novel cultivars.

Conclusions

Genetically stable somaclonal variants exhibiting desirable traits represent a valuable source for the development of crop cultivars. The study sheds light on the genetic stability of caladium somaclonal variants over three generations of tuber propagation. In the first generation (G_1), the progeny of these variants exhibited various quantitative morphological changes, while seven new leaf color variants were identified and consistently transmitted from the second (G_2) to the third (G_3) generation. Most of the newly observed variants exhibited genetic instability, as evidenced by variations in chromosome number and SSR banding patterns. Although most of the newly observed variants displayed genetic instability, two plants with altered leaf color showed no differences in chromosome number and SSR profiles compared to their normal counterparts, indicating potential involvement of epigenetic factors. To develop new caladium cultivars with unique leaf color characteristics, it is advisable to select somaclonal variants in the G_2 generation.

Authors' Contributions

Conceptualization: LH and XC; Data curation: YW and LH; Formal analysis: YW and LH; Investigation: YW, LH, and SY; Methodology: YW and SL; Software, YW and SY; Supervision, XC and SL; Writing–original draft: LH and YW; Writing–review & editing: LH, XC and SL. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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