

Somaclonal variation in ‘Red Flash’ caladium: morphological, cytological and molecular characterization

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Abstract Somaclonal variation is a major concern to many applications of plant cell and tissue culture techniques, yet it could be a useful source of variation for plant genetic improvement. High frequencies of somaclonal variants were reported in caladium, but little was known about changes in caladium somaclonal variants at the cellular and molecular levels. Twenty-four somaclonal variants were identified among ‘Red Flash’ caladium plants regenerated from two types of leaf explants and on two media containing different auxins. Results showed that the type of leaf explants and auxin affected the occurrence of somaclonal variants in ‘Red Flash’. The highest percentage of variants (25.0 %) was observed among plants regenerated from mature leaf explants cultured on the media containing 2,4-dichlorophenoxyacetic acid. These somaclonal variants exhibited considerable changes in leaf shape, coloring of the main veins, spots, margins, and leaf size, and could be separated into 10 somaclonal variant groups. Twelve variants contained 1.1–5.4 % less nuclear DNA and appeared to have lost one chromosome. Two

variants contained 5.4–9.2 % less nuclear DNA and appeared to have lost two chromosomes. One variant contained 95.0 % more nuclear DNA and $2n = 58$ chromosomes. Two SSR markers (CaM1 and CaM103) revealed DNA banding pattern changes in nine variants, including allele loss in eight variants and allele size change in one variant. These results suggested that several cytological and/or molecular causes were involved in the somaclonal variation in ‘Red Flash’ and chromosome number change was a frequent cause leading to a high frequency of aneuploids and marker allele loss. The CaM1-carrying chromosome seemed to be unstable and prone to loss during tissue culture.

Keywords Somaclonal variation · Aneuploid · Caladium · Morphological change · Chromosome number change · Nuclear DNA content · SSR marker analysis

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
6-BA	6-Benzyladenine
CTAB	Cetyl trimethylammonium bromide
FCM	Flow cytometry
NAA	1-Naphthyl acetic acid
MS	Murashige and Skoog’s medium
SD	Standard deviation
SSR	Simple sequence repeat
SV	Somaclonal variant or variation
SVT	Somaclonal variant type

Zhe Cao and Suizhao Sui have been contributed equally to this study.

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Introduction

Somaclonal variation has been a major concern to many applications of plant cell and tissue culture, yet it could be an important source of genetic variation for crop

improvement and cultivar development (Larkin 1998). Somaclonal variation may offer some advantages over spontaneous variation for genetic improvement, including higher mutation frequencies, potential to screen mutants in vitro, and identification of mutants with novel characteristics (Jain 2001). Somaclonal variations arise from multiple genetic causes, including chromosome number changes (polyploidy and aneuploidy), chromosomal structural rearrangements, gene mutations, gene copy number changes, and activation and transposition of transposable elements (Lee and Phillips 1987; Lee 1988; Bairu et al. 2011). Somatic variation could also result from epigenetic changes or pre-existing mutations in the explants used in cell and tissue culture (Alatzas and Foundouli 2006; Schellenbaum et al. 2008; Bairu et al. 2011).

Caladium (*Caladium × hortulanum* Birdsey), a member of the Araceae (aroid) family, is an important ornamental plant often grown in containers and planted in gardens. The ornamental value of caladiums comes from their dramatic leaves, which come in many different shapes, colors, and variegation patterns. Development of new cultivars with novel leaf traits has been the main goal of caladium breeding (Wilfret 1993; Deng 2012). The main approach for caladium cultivar development has been through sexual hybridization between existing cultivars and/or breeding lines (Wilfret 1993; Deng 2012). After decades of intense breeding and selection, it is now becoming increasingly difficult to find novel leaf traits in hybrid breeding populations, even in large breeding populations (Deng 2012). This bottleneck is probably due to the restricted variability in the available germplasm and gene pool. Therefore, it is important to explore other possible sources of genetic variation for caladium breeding. Somaclonal variation has proven valuable in ornamental plant breeding. Identification, evaluation and selection of somaclonal variation have resulted in the release of nearly 80 new cultivars in eight genera of the aroid family (*Aglaonema*, *Alocasia*, *Anthurium*, *Calathea*, *Dieffenbachia*, *Philodendron*, *Spathyllum*, and *Sygonium*) (Chen et al. 2003; Henny et al. 2003; Chen and Henny 2008).

Tissue culture has been used in caladium to produce virus-free stock, propagate new cultivars, and generate synthetic seeds (Hartman and Zettler 1974; Ahmed et al. 2002). In such efforts, somaclonal variation was an undesirable side effect. Since Chu and Yazawa (2001) reported somatic variation in caladium, several papers described the occurrence of somatic variation in caladium and the effects of explant and culture medium on these occurrences (Ahmed et al. 2002, 2004; Thepsithar et al. 2010). These reports indicated that somatic variation was common in caladium, especially in certain cultivars. Observed variations include changes in leaf shape, apex, base and margin, color of lamina, spotting, veins, and petiole attachment and

color (Ahmed et al. 2002, 2004; Thepsithar et al. 2010). In a previous study, we observed a number of caladium aneuploids, which prompted us to suspect that chromosome number changes might be part of the genetic causes of the observed variations (Cai et al. 2015).

Although there were a number of reports on the type and frequency of somaclonal variation in caladium (Ahmed et al. 2002, 2004, 2007; Somkanea et al. 2010; Thepsithar et al. 2010, 2011; Thongpukdee et al. 2010), little was known about somaclonal variation at the cellular and molecular levels. The objectives of this study were to (1) assess the type and frequency of somaclonal variation in a caladium cultivar that was widely grown and phenotypically stable, (2) evaluate the effect of two types of explant and auxin on somaclonal variation, and (3) characterize the nuclear DNA content, chromosome number, and molecular marker banding pattern of the obtained somaclonal variants. Experimental data on these aspects of somaclonal variation had been scarce in the aroid family (Raimondi et al. 2001; Pontaroli and Camadro 2005). Information gained from this study might also be valuable to other aroids and ornamental plants.

Materials and methods

Plant material

Caladium cultivar ‘Red Flash’ was chosen for this study. Prior to this study, the cultivar was asexually propagated by tuber division for over 60 years, and its plant and foliar characteristics were stable during asexual propagation and field production. Plants of ‘Red Flash’ were forced from tubers and grown in commercial soilless potting mix in plastic containers in a greenhouse. The temperature in the greenhouse was maintained between 21 °C (night) and 30 °C (day). Plants were fertilized with a controlled-release fertilizer (Osmocote®), approximately 15 grams per container. Both immature (about 15-days old and still furled) and mature leaves (completely unfurled, expanded, and approximately 40-days old) were collected from ‘Red Flash’ plants in early November 2013. All collected leaves were washed under running water for 20 min, surface-sterilized by soaking in 70 % (v/v) ethanol for 15 s and then in a 0.5 % (w/v) sodium dichloroisocyanuric acid solution with two drops of Tween 20 for 20 min, and finally rinsed three times with autoclaved water.

Tissue culture and plant regeneration

Disinfected caladium leaves were trimmed off the margins using a scalpel blade, leaving leaf strips 3–5 cm wide. The trimmed leaves were cut along the main veins into small

segments (approximately 0.5 cm × 0.5 cm) using a scalpel under a biological safety cabinet (Nuair, MN, USA). Leaf segments were cultured in disposable plastic Petri dishes (15 × 100 mm), with ten segments per dish. Each Petri dish contained approximately 30 mL of the C2 or C4 medium. Each medium contained 4.43 g L⁻¹ commercial Murashige and Skoog (MS) (1962) basal medium (PhytoTechnology Laboratories, KS, USA), 20 g L⁻¹ sucrose, 7.5 g L⁻¹ agar (pH = 5.8), and two plant growth regulators (PGRs). The C2 medium contained 1 mg L⁻¹ 6-BA and 0.1 mg L⁻¹ NAA, while the C4 medium contained 1 mg L⁻¹ 6-BA and 0.1 mg L⁻¹ 2,4-D. The leaf explants were cultured in an incubator at temperatures of 26 ± 1 °C and light intensity of 110 ± 10 μmol m⁻² s⁻¹. In early January 2014, newly formed shoots were rooted in a solid medium consisting of ½MS basal medium (PhytoTechnology Laboratories), 20 g L⁻¹ sucrose, and 8.0 g L⁻¹ agar (pH 5.8), supplemented with 2 mg L⁻¹ IBA, 1 mg L⁻¹ NAA, and 0.5 g L⁻¹ activated charcoal. Figure 1 shows the overall timeline and plant regeneration process. Both types of caladium leaf explants were subject to the same tissue culture process as described above.

Acclimatization and growth in the greenhouse

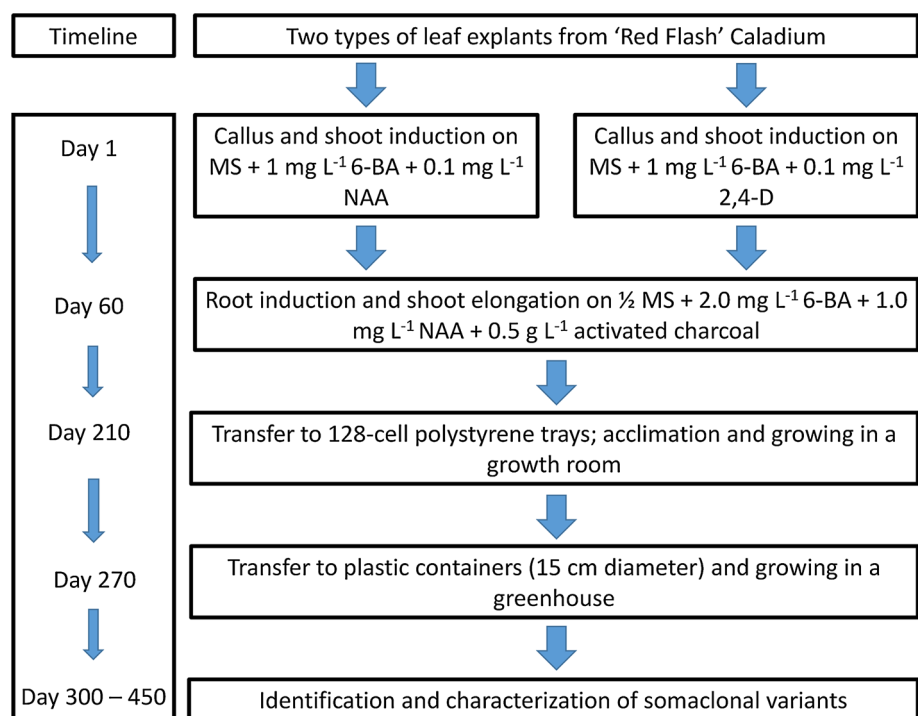
Well-rooted plantlets were transplanted individually to 128-cell polystyrene trays filled with the commercial potting mix. A transparent plastic dome was placed on each tray to maintain humidity and facilitate the acclimation of

caladium plantlets. Plantlets in the trays were grown in a growth room at 27 °C and light intensity at 150 μmol m⁻² s⁻¹; they were watered twice a day and fertilized twice a week with a commercial water-soluble fertilizer containing 1.1 % (w/w) ammonia nitrogen, 11.8 % (w/w) nitrate nitrogen, 2.1 % (w/w) urea nitrogen, 5 % (w/w) phosphate (P₂O₅), and 15 % soluble potassium (K₂O) (Peters® Excel). After 3 weeks, the domes were removed. Two months after transplanting (September 2014), the established young plants were transferred into plastic containers (15 cm diameter) filled with a soilless commercial potting mix. Five grams of the controlled-released fertilizer Osmocote® were applied to each container. Plants in the containers were moved to a greenhouse and grown there for identification and characterization of somaclonal variants. Plants were hand-watered daily.

Identification of somaclonal variants

The regenerated plants were grown side-by-side with the wildtype plants of ‘Red Flash’ on the same bench in the same greenhouse under the same conditions. The wildtype plants were forced from ‘Red Flash’ tubers. Regenerated plants as well as the wildtype plants were visually examined weekly between November 2014 and April 2015. When the regenerated plants were about 4 months old, the occurrences of somaclonal variants were recorded, and their leaf lengths and widths were measured and the color of various leaf parts was determined according to the Royal

Fig. 1 An overall process and timeline for caladium tissue culture, plant regeneration, acclimation, and variant identification



Horticultural Society (RHS) Colour Chart (RHS 1986) following the manufacturer's recommendations. For each putative somaclonal variant, at least three fully mature leaves were sampled for morphological characterization.

Determination of nuclear DNA contents

Nuclear DNA contents of 'Red Flash' wildtype and somaclonal variants were determined on an Accuri C6 flow cytometer (Accuri, Ann Arbor, MI, USA) using the protocol recommended by Doležel et al. (2007) and modified by Cao et al. (2014). Approximately 30 mg of fresh leaf tissues were co-chopped with a similar amount of leaf tissues of 'Daňkovské' rye (*Secale cereal*) in a Petri dish in 1 mL of cold HB01 lysis buffer (Doležel et al. 2007). The rye plants were obtained by germinating seeds from Dr. Doležel and grown in plastic containers in the same greenhouse where 'Red Flash' plants were grown. The homogenate containing caladium and rye nuclei was filtered through a nylon mesh (50 µm pore size) into a sample loading tube with 50 µL of a stock solution containing DNA fluorochrome propidium iodide (Sigma-Aldrich; 1 mg mL⁻¹) and RNase (Sigma-Aldrich; 1 mg mL⁻¹); it was then analyzed on the flow cytometer. At least three runs were performed for each wildtype or variant plant, and a minimum of 3000 nuclei were counted in each run. The nuclear DNA contents of caladium samples were calculated as recommended by Doležel et al. (2007): nuclear DNA content of caladium samples = nuclear DNA content of 'Daňkovské' rye (the internal standard; 16.19 pg/2C) × (mean fluorescence value of caladium samples ÷ mean fluorescence value of the internal standard).

Data analysis

A completely randomized block design with four replicates was used to assess the effect of leaf explants and auxin on caladium callus induction, plant regeneration, and occurrence of somaclonal variants. One-way analysis of variance (ANOVA) was conducted using the JMP 12.0 software (SAS Institute Inc., Cary, NC, USA) to determine the significance of differences among the four treatments (two types of leaf explants and two types of auxin) in callus or shoot induction and somaclonal variation. Tukey's HSD test (P value <0.05) was used for mean comparisons among the four treatments. Nuclear DNA content data were analyzed using multiple T tests with a Bonferroni corrected P value <0.0002.

Chromosome counting

Vigorously growing root tips (about 1 cm long) were excised from 'Red Flash' wildtype and somaclonal variant

plants grown in containers, immediately immersed in a 0.002 M 8-hydroxyquinoline solution, and treated in this solution in the dark for 3 h. Pre-treated root tips were fixed in a fixative solution (3 methanol: 1 acetic acid, v/v) overnight. After fixation, the roots tips were rinsed in tap water three times and then digested in hydrochloric acid (1 N HCl) at ambient temperature (approximately 24 °C) for 23 min. Thereafter, the softened root tips were rinsed in deionized water three times and stained in an acetocarmine staining solution (Carolina Biology Supply Company, Burlington, NC, USA) for at least 3 h. A sharp scalpel was used to remove the root caps and transfer the meristematic tissue onto a glass slide. The meristematic tissue was covered with a coverslip and gently pressed manually to spread the stained cells. The prepared slides were observed under a BX41 microscope with an Olympus Q-color 5 camera (Olympus America Inc., Melville, NY, USA). Root tip cells with well-spread and darkly stained chromosomes were photographed at 1000 times magnification.

SSR marker analysis

Total genomic DNA was extracted from 100 to 150 mg of fresh caladium leaves using the modified CTAB method of Fulton et al. (1995). DNA concentrations were determined on a NanodropTM 1000 spectrophotometer (Thermo Scientific, Odessa, TX, USA) and diluted to a concentration of 8 ng µL⁻¹ in autoclaved water. The SSR primers developed by Gong and Deng (2011) were used. They were synthesized by Eurofins Scientific LLC (Huntsville, AL, USA) with an M13 tail (5'-CCCAGT-CACGACGTTG-3') added to each forward primer at the 5' end (Supplementary Table 1). Each PCR reaction contained 32 ng of DNA template, 2 mM dNTPs, 0.25 units of *Taq* DNA polymerase, 1× PCR reaction buffer, 1.5 mM MgCl₂ (New England Biolab, Ipswich, MA, USA), 0.2 pmol of the respective forward primer with an M13 tail, 2.0 pmol of the respective reverse primer, and 1.8 pmol IRD700-labeled M13 tail primer (MWG, Highpoint, NC, USA). PCR amplification was performed on a MasterCycler (Eppendorf, AG, Hamburg, Germany) thermal cycler using a touchdown program: initial denaturation at 94 °C for 2 min, seven cycles of 45 s at 94 °C, 45 s at 68 °C (decreasing 2 °C progressively each cycle), and 60 s at 72 °C, then 30 cycles of 45 s at 94 °C, 45 s at 54 °C, and 60 s for 72 °C, and a final extension at 72 °C for 5 min. PCR products were separated and visualized on a LI-COR 4300 DNA analyzer (LI-COR, Lincoln, NE, USA) and 6.5 % polyacrylamide gels. Gel running conditions were the same as described by Gong and Deng (2011).

Results

Effect of PGR and explant type on plant regeneration and occurrence of somaclonal variation

Calluses appeared from leaf segments along cut surfaces about 1 month after culture. Some roots began to appear approximately one and a half months after culture. Some of the calluses began to produce shoots about 2 months after culture. At this time, data were taken on the percentage of leaf explants forming calluses or roots, and the percentage of callus-forming shoots. There were no significant differences between the C2 and the C4 media in the percentage of leaf explants forming calluses or roots, nor in the percentage of callus-regenerating shoots (Table 1). There were, however, significant differences between the two types of leaf explants in callus induction, root formation, and shoot regeneration (Table 1). More than 90 % of the immature leaf explants had produced calluses 2 months after culture, regardless of tissue culture medium, while 74.5–78.8 % of mature leaf explants formed calluses (Table 1). More immature leaf segments produced roots (47.2–54.9 %) compared to mature leaf segments (9.1–11.4 %; Table 1). Calluses induced from immature leaf segments exhibited a greater capacity to regenerate new shoots than those from mature leaf segments (Table 1). After being cultured on the C2 or C4 medium for 2 months, 21.4–22.5 % of the calluses from immature leaf explants regenerated new shoots, while only 5.7–6.1 % of the calluses from mature leaf explants formed new shoots.

A total of 208 plants regenerated from the cultured leaf segments were established in containers. The plants were closely observed for 3 months to identify morphological differences among them. The lowest percentage of somaclonal variants (6.9 %) was observed for the immature leaf segments cultured on the C2 medium, while the highest rate of somaclonal variation (25.0 %) was recorded for mature leaf explants cultured on the C4 medium. Moderate

rates of somaclonal variants (11.7–12.5 %) were observed for immature leaf segments cultured on the C4 medium and mature leaf segments cultured on the C2 medium resulted.

Somaclonal variation and variant types

The ‘Red Flash’ wildtype was characterized by red leaf main veins, numerous white and pink spots, and green leaf margins (Fig. 2). Twenty-four somaclonal variants showed obvious differences from the wildtype. The most frequent type of variation was related to leaf spotting, absence of white spots and/or presence of deeper red spots. These changes were observed on 15 variants. The second most common type of variation (eleven variants) was the change of the leaf’s main veins from red to green, reddish purple, or lighter red (RHS 34A, 46A). The third common variation (seven variants) was more rounded leaves with smaller leaf length-to-width ratios. Changes of leaf margin colors were the least common (three variants).

These variants could be grouped into 10 somaclonal variant types (SVT1 to SVT10) based on the color of main leaf veins, leaf margins, and spots, and the leaf length-to-width ratio (Table 2; Fig. 2). Plants in the SVT1 (M2 and M3) were different from the wildtype because they had green main veins and red leaf spots. Plants in the SVT2 (M1, M8, M9, M14, and M44) had deeper red main veins. The SVT3 (M75) differed from the wildtype because they had solid red leaf spots and yellow-green leaf margins. The leaves of the SVT4 (M23 and M36) were markedly different from the wildtype in that they had bright pink overtones across the entire leaf. Five variant plants in the SVT5 (M24, M46, M50, M52, and M187) were different from the wildtype but similar to the SVT4, except for their green leaf margins. Plants of the SVT6 (M45 and M68) through SVT9 (M4) had more rounded leaves with smaller leaf length-to-width ratios (1.27–1.31 vs. 1.69 in wildtype; Table 2). Plants in the SVT6 (M5 and M6) also exhibited darker green margins and deep red main veins, while the plants of the SVT7 had pale red main veins. Plants of the SVT8 (M7 and M33) and SVT9 showed similar leaf

Table 1 Callus induction, organogenesis, and occurrence of somaclonal variation from two types of ‘Red Flash’ caladium leaf segments after being cultured on two media for 2 months

Medium	6-BA ^x	NAA ^x	2,4-D ^x	Leaf type	Explants forming callus (%) (± SD) ^y	Explants forming roots (%) (± SD) ^y	Callus regenerating shoots (%) (± SD) ^y	Regenerated plants (no.)	Somaclonal variants (%) ^z
C2	1	0.1		Immature	94.4 ± 4.9 ^a	47.2 ± 2.45 ^a	22.5 ± 6.3 ^a	86	6.9
	1	0.1		Mature	74.5 ± 7.0 ^b	11.4 ± 2.5 ^b	6.1 ± 5.4 ^b	34	11.7
C4	1		0.1	Immature	92.6 ± 5.9 ^a	54.9 ± 17.36 ^a	21.4 ± 3.4 ^a	64	12.5
	1		0.1	Mature	78.8 ± 8.9 ^b	9.1 ± 0.8 ^b	5.7 ± 5.2 ^b	24	25.0

^x These plant growth regulators were added to the medium at mg L⁻¹

^y Means followed by different letters within the same columns were statistically different according to Tukey’s HSD test at $P = 0.05$

^z Occurrence of somaclonal variants (%) was determined by dividing the number of somaclonal variants by the number of regenerated plants

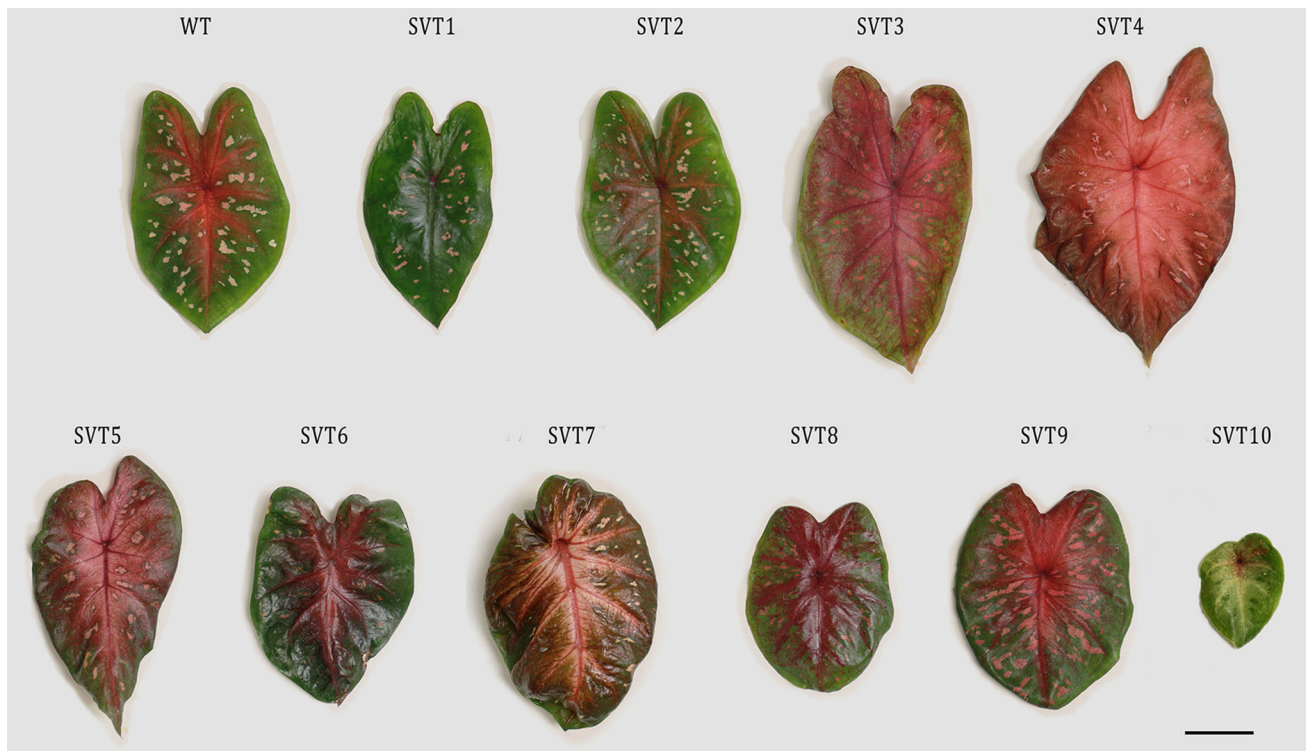


Fig. 2 Leaf morphology of the wildtype (WT) and ten somaclonal variant types (SVT) of 'Red Flash' caladium. For the wildtype and each SVT, the photo of the leaf was taken from: SVT1 from Plant M2, SVT2 from Plant M1, SVT3 from Plant M5, SVT4 from Plant M36,

SVT5 from Plant M187, SVT6 from Plant M45, SVT7 from Plant M75, SVT8 from Plant M7, SVT9 from Plant M4, and SVT10 from Plant M203. Scale bar 3 cm

characteristics, but the former had reddish purple main veins and the latter had red main veins like the wildtype. Plants of the SVT9 displayed larger leaf spots and thicker leaves (data not shown) than plants of the other SVTs and the wildtype. The two plants in the SVT10 (M199 and M203) were weak and few of their leaves were available for detailed characterization and comparison.

Nuclear DNA contents

The wildtype 'Red Flash' plants had a nuclear DNA content of 9.36 pg/2C (Table 3). The two variants in SVT1 (M2 and M3) shared very similar nuclear DNA content (9.35 and 9.30 pg/2C, respectively) with the wildtype. Three variants (M1, M8, and M9) in the SVT2 group had similar nuclear DNA content to the wildtype, but two other variants (M14 and M44) had 5.4 and 3.1 % lower DNA content, respectively. Lower DNA content (2.6–5.4 %) were observed in variant M75 (SVT3) and M23 and M36 (SVT4). Out of the five variants in SVT5, two (M24 and M46) showed similar DNA content to the wildtype, while the other three (M50, M52, and M187) had 2.6–4.5 % lower DNA content. Lower DNA content (1.9–9.2 %) were also observed in SVT6 (M45 and M68), SVT7 (M5 and

M6), and SVT10 (M199 and M203). On the other hand, SVT8 (M7 and M33) showed increased DNA content by 5.6 and 4.5 %, respectively. Surprisingly, the variant M4 in the SVT9 contained 95 % more nuclear DNA than the wildtype.

Overall a wide range of nuclear DNA contents was observed in the 24 variant plants, from 8.51 pg/2C in M45 to 18.25 pg/2C in M4, corresponding to 9.2 % decrease and 95 % increase in nuclear DNA content compared to that of the wildtype. Seven variants (M1, M2, M3, M8, M9, M24, and M46) had similar nuclear DNA content to the wildtype. Fourteen variants (M5, M6, M14, M23, M36, M44, M45, M50, M52, M68, M75, M187, M199, and M203) contained 1.9–9.2 % less nuclear DNA than the wildtype, while two variants (M7 and M33) contained 4.5–5.6 % more nuclear DNA than the wildtype, and one variant (M4) had nearly twice the nuclear DNA content of the wildtype.

Chromosome counts

Multiple root tips were collected from each of the 24 variants, then stained and squashed, resulting in the identification of 305 cells with well-spread chromosomes. For

Table 2 Main foliar characteristics of the wildtype and 10 somaclonal variant types (SVT) of ‘Red Flash’ caladium when they were grown in the container in a greenhouse in Wimauma, Florida, USA between November 2014 and March 2015

Somaclonal variant types	Main veins ^y	Leaf spots ^y	Leaf margins ^y	Leaf length–width ratio (mean ± SD) ^z	Variant plants
Wildtype	Red (50A)	White (155C) and red (38A)	Green (137B)	1.69 ± 0.09 ^a	
SVT1	Green (137A)	Red (38A)	Green (137A)	1.77 ± 0.05 ^a	M2, M3
SVT2	Red (34A)	White (155C) and red (38A)	Green (137B)	1.61 ± 0.10 ^a	M1, M8, M9, M14, M44
SVT3	Red (50A)	Red (49A)	Yellow green (146D)	1.70 ± 0.18 ^a	M75
SVT4	Red (50A)	Red (51C)	Red (51B)	1.67 ± 0.07 ^a	M23, M36
SVT5	Red (50A)	Red (51C)	Green (137B)	1.76 ± 0.11 ^a	M24, M46, M50, M52, M187
SVT6	Red (46A)	Red (49C)	Green (137A)	1.27 ± 0.09 ^b	M45, M68
SVT7	Pale Red (50C)	White (155C) and red (38A)	Green (137A)	1.26 ± 0.09 ^b	M5, M6
SVT8	Red purple (60A)	Red (51C)	Green (137B)	1.31 ± 0.03 ^b	M7, M33
SVT9	Red (50A)	Red (38A)	Green (137B)	1.29 ± 0.08 ^b	M4
SVT10	Light Green (157D)	No spots	Green (137B)	1.30 ± 0.02 ^b	M199, M203

^y Color of leaf main veins, spots, and margins was determined by comparing the color of the various leaf parts to the Royal Horticultural Society Colour Chart (RHS 1986). Numbers and letters (in parenthesis) following color categories show the closest match in the RHS Colour Chart. Sampled caladium plants were about 5-month old

^z Leaf length–width ratios were calculated by measuring the leaf length and width of at least three fully expanded, mature leaves per plant. Means followed by different letters within the same columns were statistically different according to Tukey’s HSD test at $P = 0.05$

18 of the variants, 10–21 cells with well-spread chromosomes were photographed per variant; for the remaining six variants, six to nine cells with well-spread chromosomes were counted for chromosomes per variant. The wildtype plants of ‘Red Flash’ had 30 chromosomes as previously reported by Cao et al. (2014; Table 3; Fig. 3). The seven variants (M2 and M3 in the SVT1, M1, M8, and M9 in the SVT2, and M24 and M46 in the SVT5) that showed similar DNA contents to the wildtype had the same chromosome number ($2n = 2x = 30$) as the wildtype. Two variants in the SVT8 (M7 and M33) had the same chromosome number as the wildtype, although they contained 4.5–5.6 % more nuclear DNA content. The 12 variants (M14 and M44 in the SVT2, M23 and M36 in the SVT4, M50, M52 and M187 in the SVT5, M68 in the SVT6, M5 and M6 in the SVT7 and M199 and M203 in the SVT10) that contained 1.1–5.4 % less nuclear DNA lost one chromosome ($2n = 2x - 1 = 29$, monosomics). Two variants (M75 in the SVT3 and M45 in the SVT6) that contained 5.4–9.2 % less nuclear DNA lost two chromosomes. These two variants could be either nullisomics ($2n = 2x - 2 = 28$) or double monosomics ($2x - 1 - 1 = 28$). The variant M4 in the SVT9 group contained $2n = 58$. Theoretically, the loss of the two chromosomes could have occurred before chromosome doubling (i.e. from $2n = 30$ to $2n = 29$, then to $2n = 58$) or after chromosome doubling (from $2n = 30$ to $2n = 60$, then to $2n = 58$). Should the loss occur before chromosome doubling, the variant would be a disomics ($4x - 2 = 58$); otherwise, it could be a double trisomics ($4x - 1 - 1$).

SSR marker profiling

Forty samples, including the wildtype, 24 somaclonal variants, and 15 randomly selected, normal-looking regenerated plants, were analyzed with 20 SSR markers. All markers produced one or two reproducible, scorable bands on 6.5 % polyacrylamide gels. Together these markers amplified 37 DNA bands (alleles) in wildtype ‘Red Flash’ caladium. Eighteen markers (CaM10, CaM11, CaM14, CaM18, CaM24, CaM32, CaM35, CaM42, CaM48, CaM61, CaM62, CaM66, CaM67, CaM71, CaM87, CaM93, CaM101 and CaM106) did not detect any banding pattern changes among the 40 samples, but two SSR markers, CaM1 and CaM103, revealed changes in DNA banding patterns in nine variants (Fig. 4). Marker CaM1 amplified two DNA bands (two alleles) in the wildtype, all 14 normal-looking regenerants, and 17 somaclonal variants, but only one DNA band in seven variants (Fig. 4a). Three variants (M14, M23, and M24) lost the large fragment (large allele) and four variants (M68, M187, M199, and M203) lost the small fragment (small allele; Fig. 4a). Marker CaM103 revealed DNA banding pattern changes in two variants, M36 and M75 (Fig. 4b). Variant M36 lost the large allele but showed a new DNA band of an intermediate size (medium allele), and variant M75 lost the small allele.

When both chromosome number and SSR marker data were considered, the 15 loss-of-chromosome variants could be separated into three groups. Group 1 would consist of six variants (M14, M23, M68, M187, M199, and M203)

Table 3 Nuclear DNA contents, chromosome numbers, and simple sequence repeat (SSR) marker analysis of the wildtype and 24 somaclonal variants of ‘Red Flash’ caladium

Variant types	Plants	DNA content (pg/2C) (\pm SD)	Nuclear DNA content change compared to wildtype (%)	Root tip cells observed (no.)	Somatic chromosome numbers (2n)	Chromosome number changes	SSR banding pattern changes	
							CaM1	CaM103
Wildtype	Red Flash	9.36 \pm 0.03 ^{cde}	–	8	30	2x	–	–
SVT1	M2	9.35 \pm 0.02 ^{cde}	0.1	11	30	2x	No	No
	M3	9.30 \pm 0.04 ^{def}	–0.6	13	30	2x	No	No
SVT2	M1	9.41 \pm 0.04 ^{cd}	0.5	16	30	2x	No	No
	M8	9.45 \pm 0.02 ^c	0.9	11	30	2x	No	No
	M9	9.42 \pm 0.03 ^{cd}	0.6	10	30	2x	No	No
	M14	8.85 \pm 0.02 ^m	–5.4	19	29	2x – 1	Loss of upper band	No
	M44	9.07 \pm 0.03 ^{ijk}	–3.1	9	29	2x – 1	No	No
SVT3	M75	8.85 \pm 0.04 ^m	–5.4	12	28	2x – 2	No	Loss of lower band
SVT4	M23	9.12 \pm 0.05 ^{hij}	–2.6	8	29	2x – 1	Loss of upper band	No
	M36	9.00 \pm 0.04 ^{kl}	–3.8	15	29	2x – 1	No	Band change
SVT5	M24	9.25 \pm 0.06 ^{efg}	–1.1	15	30	2x	Loss of upper band	No
	M46	9.26 \pm 0.04 ^{efg}	–1.0	7	30	2x	No	No
	M50	9.10 \pm 0.02 ^{hij}	–2.8	10	29	2x – 1	No	No
	M52	9.12 \pm 0.02 ^{hi}	–2.6	8	29	2x – 1	No	No
	M187	8.94 \pm 0.06 ^{lm}	–4.5	13	29	2x – 1	Loss of lower band	No
SVT6	M45	8.51 \pm 0.04 ⁿ	–9.2	14	28	2x – 2	No	No
	M68	9.14 \pm 0.01 ^{ghi}	–2.3	6	29	2x – 1	Loss of lower band	No
SVT7	M5	9.18 \pm 0.03 ^{fgh}	–1.9	21	29	2x – 1	No	No
	M6	9.10 \pm 0.02 ^{hij}	–2.7	11	29	2x – 1	No	No
SVT8	M7	9.89 \pm 0.03 ^b	5.6	18	30	2x	No	No
	M33	9.78 \pm 0.08 ^b	4.5	12	30	2x	No	No
SVT9	M4	18.25 \pm 0.02 ^a	95	18	58	4x – 2	No	No
SVT10	M199	8.96 \pm 0.04 ^{klm}	–4.3	12	29	2x – 1	Loss of lower band	No
	M203	9.12 \pm 0.06 ^{hij}	–2.6	8	29	2x – 1	Loss of lower band	No
Total	24	8.51 to 18.25	–9.2 to 95.0	305	28, 29, 30, 31, 58		Band loss in 7 variants	Band loss in 1 variant, band change in 1 variant

Nuclear DNA contents were determined using an Accuri C6 flow cytometer and the rye (*Secale cereale*) ‘Daňkovské’ as an internal reference (Doležel et al. 2007). Mean nuclear contents followed by different letters were statistically different according to *T* test with the Bonferroni adjusted *P* value = 0.0002. Nuclear DNA content changes in the somaclonal variants were calculated as described by Cai et al. (2015), by assuming 100 % in the wildtype and then subtracting 100 from the percent DNA content increase (+) or decrease (–) of the variants. Chromosome numbers were determined as described by Cao et al. (2014), by squashing root tip cells, staining them with aceto-carmin, and photographing at \times 1000 magnification

that had lost one chromosome, the one that carries one of the CaM1 alleles. Group 2 would consist of one variant (M75) that lost two chromosomes and lost one of the CaM103 alleles. One of the two lost chromosomes carries the CaM103 allele. Group 3 would consist of the remaining eight variants (M4, M5, M6, M36, M44, M45, M50, and M52) that lost one or two chromosomes but did not lose any alleles of CaM1 and CaM103 or any alleles of the other 18 SSR markers.

Discussion

This study represented the first cytological and molecular characterization of caladium somaclonal variation. A majority of the ‘Red Flash’ somaclonal variants (14 out of 24 = 58.33 %) lost one or two chromosomes, indicating that chromosome number changes, particularly aneuploidy, is a common cause of somaclonal variation in caladium. When the variant with 58 chromosomes was included, the

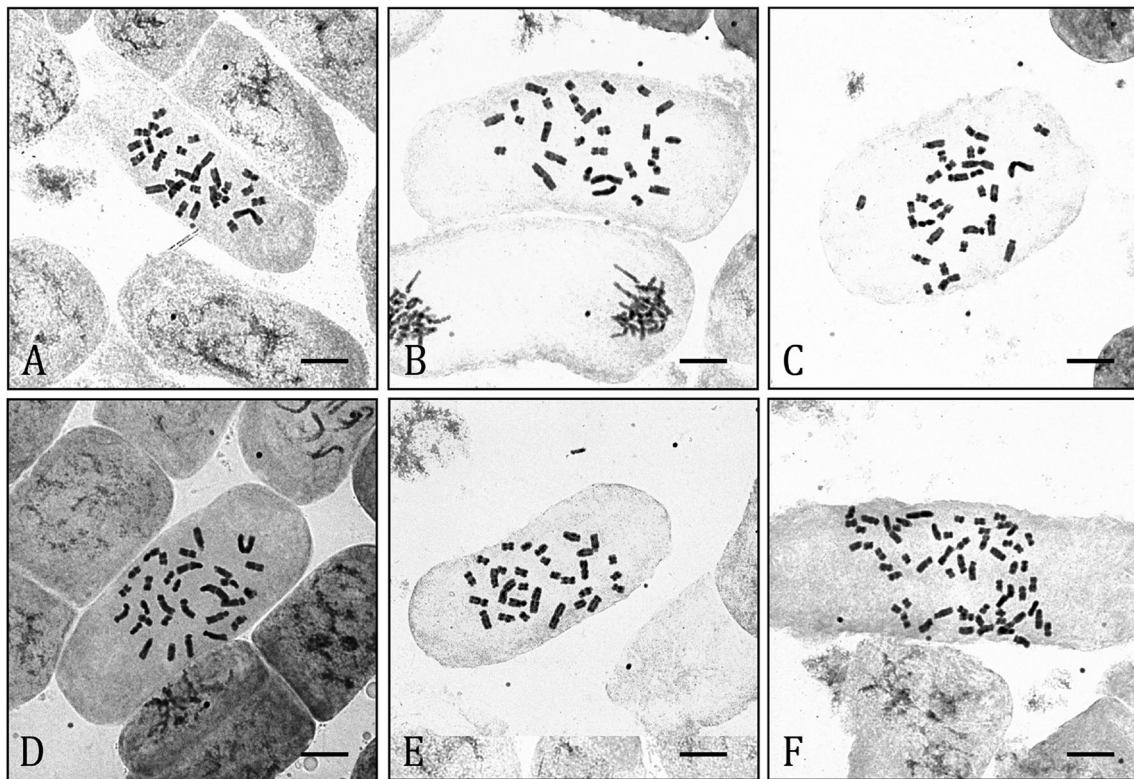


Fig. 3 Micrographs of somatic chromosomes in the root tips of the wildtype and five somaclonal variants (M25, M187, M3, M7, and M4) of ‘Red Flash’ caladium obtained from *in vitro* cultured young or mature leaf segments. Photographs were taken under a bright field

microscope at 1000 magnifications. **a** M45 ($2n = 2x - 2 = 28$), **b** M187 ($2n = 2x - 1 = 29$), **c** wildtype ($2n = 2x = 30$), **d** M3 ($2n = 2x = 30$), **e** M7 ($2n = 2x = 30$), and **f** M4 ($2n = 4x - 2 = 58$). Scale bars 10 μ m

percentage of aneuploid variants reached 62.5 %. This percentage was 77.1 % higher than the rate of aneuploids (35.7 %) in ‘Tapestry’ caladium plants regenerated from colchicine-treated leaf explants (Cai et al. 2015). The ‘Tapestry’ leaf explants used in the previous study (Cai et al. 2015) were from immature, furred leaves cultured on a medium containing NAA. These conditions (immature leaf explants + NAA) also resulted in less somaclonal variation compared to the mature leaf explants cultured on 2,4-D-containing media (Table 1).

One SSR marker (CaM1) detected loss of one allele in seven somaclonal variants; another marker (CaM103) revealed loss of one allele in one variant (M75) and change of a different allele (size change) in another variant (M36). Seven of the eight somaclonal variants showing an SSR marker allele loss were aneuploids that had lost one or two chromosomes. If the SSR markers (CaM1 and CaM103) were located on these chromosomes, the marker alleles would be lost along with the chromosome. Therefore, the chromosome counting and SSR marker analysis data lead us to believe that markers CaM1 and CaM103 are located on two different caladium chromosomes, and their allele losses in six variants (M14, M23, M68, M187, M199, and

M203) were most likely due to the loss of these chromosomes.

Variant M36 showed an allele size change at the CaM103 locus. Experimental data from model organisms have shown that SSR marker loci tend to gain or lose units of repeat sequences during DNA synthesis due to the DNA slippage by DNA polymerases (Tautz and Schlötterer 1994). Thus, we suspect that the new CaM103 allele in M36 might originate from the loss of one or two units of the repeat motif (AG) from an existing allele during DNA synthesis.

The ‘Red Flash’ wildtype has 15 pairs of chromosomes. Should the loss of any specific chromosome in ‘Red Flash’ be random, the expected frequency for the loss of a specific chromosome would be 6.7 % (1/15). The CaM103-carrying chromosome was observed to be lost in one (M75) out of 15 chromosome-loss variants, or 6.7 %, the expected frequency. The CaM1-carrying chromosome was lost in six variants (Group 1), for a frequency of 40.0 % (6/15). This was much higher than expected, and may indicate that the CaM1-carrying chromosome was unstable and prone to getting lost in caladium.

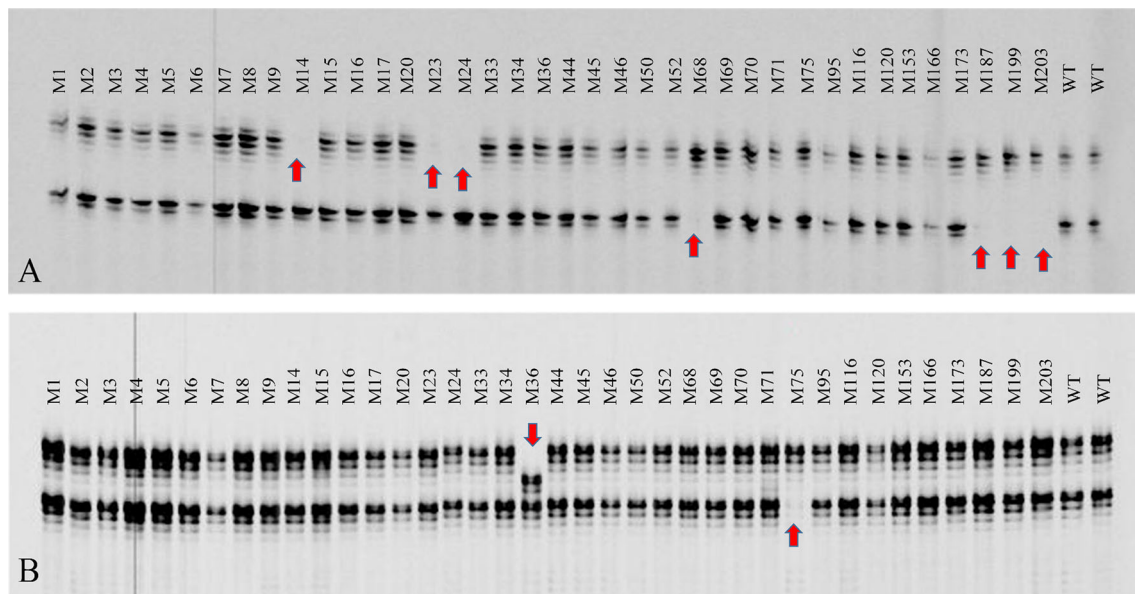


Fig. 4 Molecular marker banding profiles of two wildtypes (WT), 24 somaclonal variants (M1, M2, M3, M4, M5, M6, M7, M8, M9, M14, M23, M24, M33, M36, M44, M45, M46, M50, M52, M68, M75, M187, M199, M203), and 14 normal-looking, regenerated plants (M15, M16, M17, M20, M34, M69, M70, M71, M95, M116, M120, M153, M166, M173) of ‘Red Flash’ caladium for two simple

sequence repeat (SSR) markers CaM1 and CaM103. **a** Banding profile for CaM1. The two major bands were 199 bp (*top*) and 209 bp (*bottom*). **b** Banding profile for CaM103. The *top* and *bottom* major bands were 189 and 207 bp, respectively. *Arrows* point to the SSR bands that were missing or changed in somaclonal variants

The majority of ‘Red Flash’ somaclonal variants showed reduced ornamental/aesthetic values. The occurrence of these variants in caladium tissue culture-derived plants will be a serious concern for the practical applications of tissue culture in caladium. On the other hand, a number of the somaclonal variants showed novel characteristics and much improved ornamental value. For example, two variants (M23 and M36) exhibited pink leaves and were distinct from the wildtype. The variant M4 had thicker leaves and petioles and larger pink spots on leaves; this combination of characters was unique compared to existing commercial cultivars. The 12 monosomics obtained represent the first group of monosomics in caladium. Use of monosomics facilitated localization of genes to specific chromosomes in agronomic crops (Yang et al. 1990). These caladium monosomics may play similar roles in caladium genetic studies.

In summary, several cytological and/or molecular causes seemed to be involved in the origin of somaclonal variation in ‘Red Flash’ caladium, including chromosome losses, chromosome gains, polyploidization, and alterations at the DNA level. Aneuploidy resulting from chromosome losses or gains was common; the chromosome carrying the CaM1 marker seemed to be especially unstable and prone to getting lost during cell and tissue culture. A number of the somaclonal variants obtained in this study appeared to be very valuable for future genetic studies and cultivar

development in caladium. In the meantime, the observed somaclonal variation warrants careful selection of caladium explants and culture conditions to minimize the occurrence of somaclonal variation and constant efforts to rouge out variants so that the genetic integrity or trueness-to-type of the original caladium cultivars or germplasm accessions can be maintained during tissue culture.

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Author contributions Z. Cao and S. Sui identified caladium somaclonal variants, conducted morphological characterization, counted chromosomes, determined nuclear DNA contents, and ran SSR marker analysis. Z. Cao conducted statistical analyses and drafted the manuscript. Z. Cao and S. Sui contributed equally to this manuscript. X. Cai initiated leaf segment cultures and produced caladium plantlets. Q. Yang participated in flow cytometry analysis.

Z. Deng supervised the experiments and revised and finalized the manuscript.

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References

- Ahmed E, Hayashi T, Zhu Y, Hosokawa M, Yazawa S (2002) Lower incidence of variants in *Caladium bicolor* Ait. plants propagated by culture of explants from younger tissue. *Sci Hortic-Amst* 96:187–194
- Ahmed E, Hayashi T, Yazawa S (2004) Auxins increase the occurrence of leaf-color variants in caladium regenerated from leaf explants. *Sci Hort-Amst* 100:153–159
- Ahmed E, Hayashi T, Yazawa S (2007) Shading and reducing tuber provide young green leaf explants for the propagation of true-to-type *Caladium bicolor* Ait. plants. *Acta Hortic* 755:87–91
- Alatzas A, Foundouli A (2006) Distribution of ubiquitinated histone H2A during plant cell differentiation in maize root and dedifferentiation in callus culture. *Plant Sci* 171:481–487
- Bairu M, Aremu A, Staden J (2011) Somaclonal variation in plants: causes and detection methods. *Plant Growth Regul* 63:147–173
- Cai X, Cao Z, Xu S, Deng Z (2015) Induction, regeneration and characterization of tetraploids and variants in ‘Tapestry’ caladium. *Plant Cell Tissue Org Cult* 120:689–700
- Cao Z, Deng Z, McLaughlin M (2014) Interspecific genome size and chromosome number variation sheds new light on species classification and evolution in *Caladium* (Araceae). *J Am Soc Hort Sci* 49:449–459
- Chen J, Henny RJ (2008) Ornamental foliage plants: improvement through biotechnology. In: Kumar A, Sopory SK (eds) Recent advances in plant biotechnology and its applications. I.K. International Publishing House Pvt. Ltd., New Delhi, pp 140–156
- Chen J, Henny RJ, Chao TC (2003) Somaclonal variation as a source for cultivar development of ornamental aroids. In: Pandalai SG (ed) Recent research development in plant science, vol 1. Research Signpost, Kerala, pp 31–43
- Chu Y, Yazawa S (2001) The variation and the hereditary stability on leaf character of plantlets regenerated from micropropagation in caladium. *J Chin Soc Hort Sci* 47:59–67
- Deng Z (2012) Caladium genetics and breeding: recent advances. *Floriculture Biotechnol* 6(Special issue 1):53–61
- Doležel J, Greilhuber J, Suda J (2007) Estimation of nuclear DNA content in plants using flow cytometry. *Nat Protoc* 2:2233–2244
- Fulton TM, Chunwongse J, Tankley SD (1995) Microprep protocol for extraction of DNA from tomato and other herbaceous plants. *Plant Mol Biol Rep* 13:207–209
- Gong L, Deng Z (2011) Development and characterization of microsatellite markers for caladiums (*Caladium Vent.*). *Plant Breed* 130:591–595
- Hartman RD, Zettler FW (1974) Effects of dasheen mosaic virus on yields of *Caladium*, *Dieffenbachia*, and *Philodendron*. *Phytopathol* 64:768
- Henny R, Chen J, Norman D (2003) ‘Diamond bay’ and ‘emerald bay’ *Aglaonema*. *HortScience* 38:1446–1447
- Jain SM (2001) Tissue culture-derived variation in crop improvement. *Euphytica* 118:153–166
- Larkin P (1998) Introduction. In: Jain SM, Brar DS, Ahloowalia BS (eds) Somaclonal variation and induced mutations in crop improvement. Kluwer, Dordrecht, pp 3–13
- Lee M (1988) The chromosomal basis of somaclonal variation. *Ann Rev Plant Physiol Plant Mol Biol* 39:413–437
- Lee M, Phillips RL (1987) Genomic rearrangements in maize induced by tissue culture. *Genome* 29:122–128
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 15:473–497
- Pontaroli AC, Camadro EL (2005) Somaclonal variation in *Asparagus officinalis* plants regenerated by organogenesis from long-term callus cultures. *Genet Mol Biol* 28:423–430
- Raimondi J, Masuelli, Camadro E (2001) Assessment of somaclonal variation in asparagus by RAPD fingerprinting and cytogenetic analyses. *Sci Hort-Amst* 90:19–29
- Royal Horticultural Society (1986) RHS Colour Chart. Royal Horticultural Society, London
- Schellenbaum P, Mohler V, Wenzel G, Walter B (2008) Variation in DNA methylation patterns of grapevine somaclones (*Vitis vinifera* L.). *BMC Plant Biol* 8:78
- Somkanea A, Thepsithar C, Thongpukdee A (2010) Somaclonal variation of caladium [*Caladium bicolor* (Ait.) Vent.] in vitro culture propagation. *Acta Hortic* 55:281–288
- Tautz D, Schlötterer C (1994) Simple sequences. *Curr Opin Genet Dev* 4:834–837
- Thepsithar C, Thongpukdee A, Chiensil P (2010) Micropropagation of *Caladium bicolor* (Ait.) Vent. ‘Thep Songil’ and incidence of somaclonal variants. *Acta Hortic* 855:273–279
- Thepsithar C, Thongpukdee A, Sugaram R, Somkanea U (2011) Mutated clones of *Caladium humboldtii* ‘Phraya Savet’ from in vitro culture and occurrence of variants from somatic hybridization between two caladium species. *J Life Sci* 5:352–359
- Thongpukdee A, Thepsithar C, Chiensil P (2010) Somaclonal variation of *Caladium bicolor* (Ait.) Vent. ‘Jao Ying’ after in vitro culture propagation. *Acta Hortic* 855:281–287
- Wilfret GJ (1993) Caladium. In: de Hertogh A, LeNard M (eds) The physiology of flower bulbs. Elsevier, Amsterdam, pp 239–247
- Yang T, Shu W, Shen K (1990) Monosomic analyses of stripe rust and leaf rust resistance genes in winter wheat varieties Lüquyu and Yantar. *Euphytica* 48:83–86