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ORIGINAL PAPER

Regeneration of *Clivia miniata* and assessment of clonal fidelity of plantlets

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Abstract An efficient in vitro micropropagation system for *Clivia miniata* Regel was developed using basal tissues of young petals and young ovaries as explants. For callus induction, explants were incubated on Murashige and Skoog (MS) medium containing either 2.22 μM 6-benzyladenine (BA) and 4.52 μM 2,4-dichlorophenoxyacetic acid (2,4-D) or 4.44 μM BA, 5.37 μM α-naphthaleneacetic acid (NAA), and 9.05 μM 2,4-D. Moreover, callus was induced from young ovaries when these were incubated on MS medium containing 8.88 μM BA, 10.74 μM NAA, and 9.05 or 18.10 μM 2,4-D. Subsequently, callus was transferred to MS medium supplemented with kinetin (KT) and NAA for shoot organogenesis. Frequency of shoot regeneration from petal-derived callus was highest when callus was transferred to medium containing 2.69 μM NAA with either 9.29 or 13.94 μM KT. Shoot regeneration frequency from ovary-derived callus was highest when this callus was transferred to medium containing 9.29 μM KT and 10.74 μM NAA. Overall, different explant types exhibited different organogenic capacities wherein, young petals had higher shoot regeneration frequencies than young ovaries. The highest rooting frequency (98.25 ± 3.04%) was obtained when shoots were transferred to half-strength MS medium without plant growth regulators. Regenerated plantlets were transplanted to soil mix and acclimatized, yielding a 96.80% survival frequency. Only 0.6% of regenerated plantlets exhibited morphological changes. The diploid status ($2n = 22$) of regenerated plantlets was determined using chromosome counts of root-tips. Moreover, inter-simple sequence repeats were used to assess the genetic fidelity of regenerated plantlets. Overall, regenerated plants shared 90.5–100.0% genetic similarities with mother plants and 89.0–100.0% similarities with each other.

Keywords *Clivia miniata* · In vitro propagation · Inter-simple sequence repeats · Somaclonal variation

Abbreviations

MS	Murashige and Skoog
BA	6-benzyladenine
2,4-D	2,4-dichlorophenoxyacetic acid
NAA	α-naphthaleneacetic acid
KT	Kinetin
ISSRs	Inter-simple sequence repeats
RAPD	Randomly amplified polymorphic DNA
AFLPs	Amplified fragment length polymorphisms
LL	Low-light
HL	High-light
PGRs	Plant growth regulators
ANOVA	One-way analysis of variance

Introduction

The genus *Clivia* (Amaryllidaceae) is a group of perennial herbaceous plants (Ran et al. 2001a) native to southern Africa. *Clivia* has become the city flower of Changchun, China, and it is one of ten flowering plants that bloom during the highly anticipated traditional Chinese New Year

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celebration. The most commonly cultivated species is *Clivia miniata* Regel (Ran and Simpson 2005) which is cultivated in many parts of the world, especially in Europe, the USA, Japan, China, Australia, New Zealand and Belgium.

In addition to its ornamental value, *C. miniata* has also been reported to have medicinal value. Alkaloids isolated from the roots and leaves of *C. miniata* were found to have significant antiviral activities (Ieven et al. 1982). Also, boiling water extracts of *C. miniata* leaves were found to cause concentration-dependent contractions in both the isolated uterus and ileum (Veale et al. 1989). The supercritical fluid extracts from the roots of *C. miniata* were found responsible for uterotonic activity through cholinergic receptors (Sewram et al. 2001).

C. miniata is generally propagated through seeds and ramets; however, the former is time-consuming and the latter is inefficient. It takes 2.5–7 years for plants to mature from seeds (Finnie and van Staden 1999; Ran and Simpson 2005). Seed production is also not always true to type (Ran and Simpson 2005) due to a high degree of heterozygosity and the law of segregation. The phenotypic differences in the offspring are a disadvantage in the competitive flower market. Tissue culture of *C. miniata* might be an effective way to generate many plants with the same phenotype. Finnie and van Staden (1999) reported many tissues including meristems, fruit, embryos, ovaries, and pedicels of *C. miniata* could be used as explants, although plantlet regeneration was slow. Ran and Simpson (2005) demonstrated only 0–3.9 shoots could be regenerated from each explant of different hybrid lines, and concluded in vitro propagation was not commercially viable. In this study, we report the establishment of an efficient in vitro propagation system for *C. miniata* by using petal bases as explants.

In plant propagation, retaining the genetic integrity of explants with respect to the mother plants is crucial (Jin et al. 2008). Several strategies are available for detecting somaclonal variation including phenotypic identification (Kong et al. 2011), cytological analysis (Konieczny et al. 2010), DNA analysis techniques (Ai et al. 2011; Torres-Morán et al. 2010), and protein analysis (Northmore et al. 2011). Somaclonal variation analysis of regenerated plants of *C. miniata* has yet to be reported in cultivated material. Therefore, it is essential to confirm the degree of genetic stability of the in vitro tissue culture products.

This study addresses the needs outlined above in *C. miniata* using a simple novel micropropagation protocol. Chromosome counts (Jin et al. 2008) and inter-simple sequence repeats (ISSRs, Borchert and Hohe 2009; Hu et al. 2008; Souframanien and Gopalakrishna 2004; Fernández et al. 2002; Saini et al. 2004; Zhang et al. 2010) have been employed in detecting somaclonal variants.

Materials and methods

Plant materials

Inflorescences of *C. miniata* cultivar were collected from 5-year-old plants. The young inflorescences were thoroughly washed with distilled water for 10–20 min, decontaminated with 75% (v/v) alcohol for 30–60 s and a 0.1% (w/v) mercuric chloride solution for 3–5 min and then rinsed 4–5 times with sterile distilled water (Gao et al. 2010; Chen et al. 2007). Both young petals were cut, along their bases, and the young ovaries were cut into explants about 2–5 mm long. Five to seven explants were inoculated in a flask with 30–40 ml of callus induction medium. The basal callus induction medium was Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with 3.5% (w/v) sucrose, 0.45% (w/v) agar (Jinan Zhongtian Plant Tissueculture Center) and 400 mg l^{-1} casein hydrolysate (Promega). The callus induction media for petal bases contained either 2.22 μM 6-benzyladenine (BA) + 4.52 μM 2,4-dichlorophenoxyacetic acid (2,4-D) or 4.44 μM BA + 5.37 μM α-naphthaleneacetic acid (NAA) + 9.05 μM 2,4-D. The callus induction media for young ovaries contained 8.88 μM BA, 10.74 μM NAA combined with either 9.05 μM or 18.10 μM 2,4-D. The basal callus induction medium and the concentrations of BA, 2,4-D and NAA were chosen as our callus induction media because they have been shown in our own experiences to result in better callus induction than other basal medium and concentrations. For all the experiments pH of the medium was adjusted to 5.8 with NaOH prior to autoclaving at 121°C for 16 min. The temperature (25 ± 2°C) was maintained throughout the course of in vitro culture.

Callus induction

After inoculating the explants in flasks they were cultured under 0.2 μmol m^{-2} s^{-1} light (low-light, LL) provided by cool white fluorescent tubes (YZ18, Foshan Electrical and Lighting Co., Ltd) at a photoperiod of 12 h. To induce and maintain secondary callus, the yellow primary callus obtained after 3 months of culture were chopped to about 5 mm (diameter) with a scalpel blade then transferred onto the same callus induction medium described above. After 2 months of culture, the secondary callus grown from the chopped primary callus were used to induce shoots. After 30 days of culture, the percentage of explants producing callus out of the total number of explants was determined. Eighteen to 41 explants were used in each treatment and the experiment was repeated three times. All data were subjected to statistical analysis using Independent-Samples *T* tests ($P \leq 0.05$, SPSS ver.13.0) (Flores et al. 2011).

Shoot organogenesis and regeneration

The friable yellow callus from petal bases and compact yellow-green callus from ovary fragments were then transferred into a variety of regeneration media containing the basal regeneration medium, supplemented with NAA (10.74, 5.37, 2.69 or 0.00 μM) and kinetin (KT, 13.94 or 9.29 μM). The basal regeneration medium was the same as basal callus induction medium described above. The cultures were incubated under 40 μmol m^{-2} s^{-1} light (high-light, HL) provided by cool white fluorescent tubes ((YZ18, Foshan Electrical and Lighting Co., Ltd) at a photoperiod of 12 h. During the induction of shoots, the larger callus (diameter > 2 cm) were chopped into halves with a scalpel blade then transferred onto the same medium. After 4 months of culture, the percentage of callus producing shoots and the total number of shoots per callus was calculated. Ten to 30 callus were used in each treatment and the experiment was repeated two times. All data were subjected to statistical analysis using Duncan's Multiple Range test ($P \leq 0.05$, SPSS ver.13.0) (Guo et al. 2005; Sivanesan et al. 2011).

Rooting and acclimatization of plantlets

Shoots with 2–4 leaves were transferred onto half-strength MS medium (half-strength MS salts and vitamins), containing 2.0% (w/v) sucrose, 0.45% (w/v) agar, two concentrations (0.00 or 0.93 μM) of KT and three concentrations (0.00, 1.07 or 2.69 μM) of NAA. The cultures were incubated under HL provided by cool white fluorescent tubes at a photoperiod of 16 h. After 2 months of culture, the rooting rate (number of rooted shoots/number of shoots examined) was calculated. Thirty to 49 shoots were used in each treatment and the experiment was repeated three times. All data were subjected to statistical analysis using Duncan's Multiple Range test ($P \leq 0.05$, SPSS ver.13.0). The plantlets with 2–5 roots were transferred into sterilized (121°C, 30 min) decomposed oak leaves, and the pot was covered with plastic wrap film for 5–7 days in a growth room at 25 ± 5°C. After 1 month of culture in a growth room, the percentage of surviving plantlets was calculated.

Morphological evaluation and ploidy determination in one year-old regenerated plantlets

One thousand regenerated plantlets derived from four explants (Fig. 1) of two mother plants were selected for morphological analysis just before transplanting. These plantlets were obtained via 15 months (nine subcultures) of in vitro culture procedure (Fig. 1). The culture procedure consisted of 5 months of callus induction on callus induction media (three subcultures), 8 months of shoot induction on regeneration media (five subcultures) and 2 months of root induction on root media (one subculture). After 15 months of in vitro culture, the plantlets were transplanted into pots in growth room. At 8–10 weeks after transplanting, we randomly selected 36 plantlets for chromosome counts. Among them, 21 plantlets were regenerated from young petal bases, and the remaining 15 plantlets were derived from young ovaries. The root tips were pretreated with distilled water for 24 h at 4°C (Ran et al. 2001a). Then they were fixed in freshly prepared Carnoy's

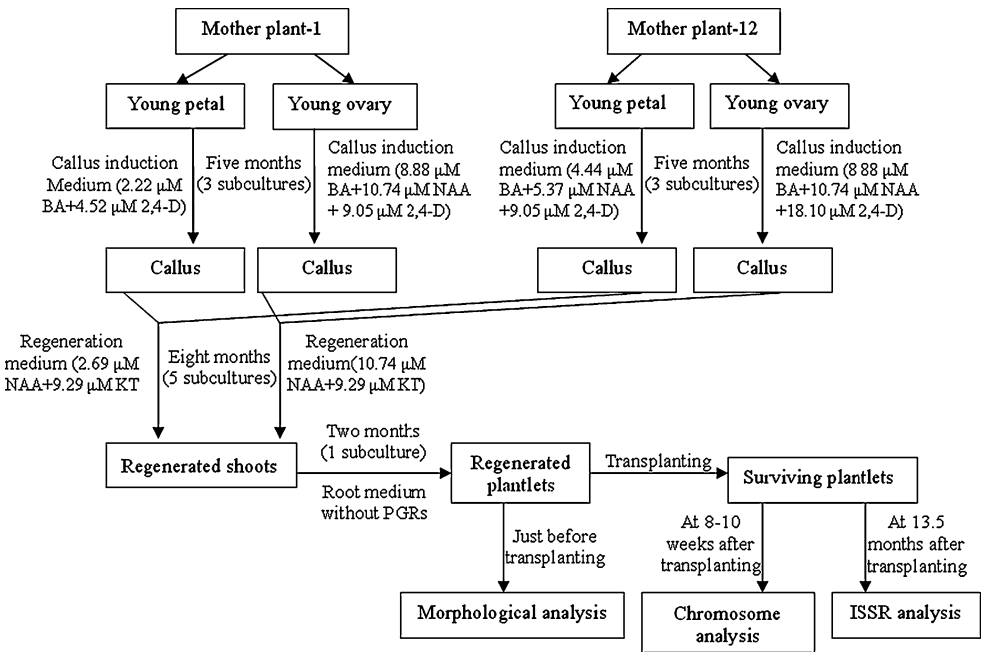

Fig. 1 Processes used to obtain the plantlets used in morphological, chromosome and ISSR analysis

Table 1 The nucleotide sequences of primers used for ISSR analysis

Primer	5′ to 3′	Primer	5′ to 3′
Primer-1	GGGTGGGGTGGGGTG	Primer-11	CSCGAGAGAGAGAGA
Primer-2	TCTCTCTCTCTCTCTCA	Primer-12	GCWGAGAGAGAGAGAG
Primer-3	TCTCTCTCTCTCTCTCC	Primer-13	CCAGTGGTGGTGGT
Primer-4	GAGAGAGAGAGAGAGAYT	Primer-14	SSWNGACAGACAGACA
Primer-5	ACACACACACACACACYA	Primer-15	AAGAGAGAGAGAGAGT
Primer-6	ACACACACACACACACYG	Primer-16	AGAGAGAGAGAGAGAGC
Primer-7	CTCTCTCTCTCTCTCTCTG	Primer-17	GAGAGAGAGAGAGAGAT
Primer-8	GAGAGAGAGAGAGAGAGAC	Primer-18	CTCTCTCTCTCTCTCTA
Primer-9	CTCTCTCTCTCTCTCTRG	Primer-19	AGAGAGAGAGAGAGAGYT
Primer-10	CTCTCTCTCTCTCTCTRC	Primer-20	AGAGAGAGAGAGAGAGYC

solution (alcohol:acetic acid = 3:1) for 24 h at room temperature and stored in 70% (v/v) ethanol at 4°C. Chromosome spreads were made using 0.1 M HCl for 20 min at 60°C (Wang et al. 1992) and then were stained in Carbol-fuchsin (100 mM D-glucitol, 18.88 mM phenol, 43.28% (v/v) acetic acid, 0.19% formaldehyde (v/v) and 0.28% (v/v) alcohol) for 20–30 min. Chromosomes were observed using a light microscope (BX51TF; Olympus Corp., Japan) at the 1000× magnification. Well spread chromosomes in metaphase were photographed using a camera (E-330; Olympus Corp. Japan).

Molecular analysis of plantlets using inter-simple sequence repeats (ISSRs)

At 13.5 months after transplanting, we randomly selected 20 plantlets from the foregoing regenerated plantlets derived from the four explants (Fig. 1). Genetic fidelity between the mother plants and the 20 regenerated plantlets was assessed by PCR-based ISSR analysis. Genomic DNA of the test samples was obtained by using a small-scale DNA isolation method (AxyPrep™ Multisource Genomic DNA Miniprep Kit). DNA was extracted from leaves from each plant and quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific). The samples were marked from 1 to 22. Sample 1 and sample 12 represent mother plants. Samples 2–11 and 13–22 were randomly selected transplanted plantlets and were derived from mother plants 1 and 12, respectively. Samples 2, 6, 14, 17 and 21 were regenerated plantlets derived from young ovaries; 3–5, 7–11, 13, 15, 16, 18–20 and 22 from young petals.

A total of 20 ISSR primers were selected according to the number and consistency of amplified fragments. The 5′- and 3′-anchored primers were included in the 20 primers. The primers were custom synthesized from Sangon Biotech Co., Ltd., Shanghai. Table 1 shows the nucleotide sequences of these primers.

PCR mixtures (25 µl) contained 50 ng genomic DNA, 12.5 µl Premix Taq (Takara company, China) and 0.4 µM of each primer. Amplification was performed in an Eppendorf Master cycler gradient. Amplification conditions were: 1) one cycle at 94°C for 4 min, then 2) five cycles in this sequence: 94°C for 30 s, 55°C for 45 s but with a stepwise reduction of 1°C after each cycle, and 72°C for 2 min, In the subsequent 35 cycles, the annealing temperature was maintained at 50°C, followed by one cycle of 10 min at 72°C. PCR products were separated by electrophoresis in 1% agarose gels together with 3 µl DNA Marker-DL2000 (Takara) at 90 V. The gels were visualized under ultra-violet light after staining with 0.001% (w/v) ethidium bromide and documented using a gel documentation system (Uvitec Cambridge).

Only clear and reproducible bands on gels were scored, based on presence (1) and absence (0). Two independent amplification reactions were performed with all the 20 ISSR primers. Software SPSS ver.13.0 was used to perform cluster analysis of the complete data set. Similarity estimates calculated by using proximity coefficients and cluster analysis were carried out by the Ochiai method and represented as a dendrogram (Goel et al. 2009; Souframanien and Gopalakrishna 2004).

Results and discussion

Callus induction

The primary callus were induced from the young petal and ovary explants after 7–14 days of culture on the basal callus induction medium containing BA, 2,4-D with or without NAA. Callus were initiated at the cut ends of the explants. When the yellow primary callus were chopped with a scalpel blade and cultivated on the same callus induction medium, they divided actively and gave rise to friable yellow callus from petals and compact

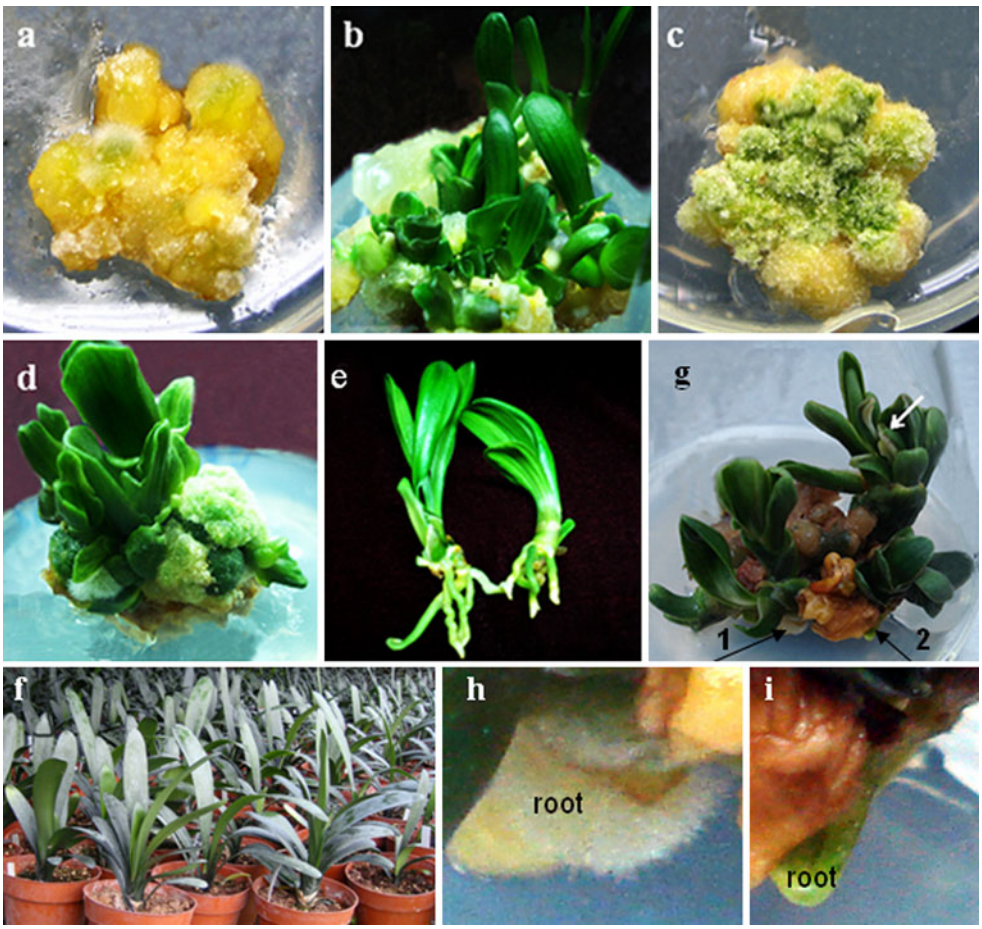

Fig. 2 Plant regeneration of *C. miniata* from young petal bases and young ovaries. **a** Secondary yellow callus derived from petal bases after 4-month culture. The callus was relatively friable. **b** Rosette shoots from petal bases. **c** Secondary yellow–green callus from young ovaries after 4 months of culture. **d** Regenerated shoots from young ovaries. **e** Rooting in half-strength MS hormone-free medium. **f** Rooted plantlets transferred to pots 23–24.5 months ago. **g** Regenerated variant plantlets with abnormal coiled leaves and white leaf edges (*white arrow*). *Black arrows* 1 and 2 indicate roots. Roots 1 and 2 are magnified in (**h** and **i**), respectively

yellow-green callus from ovaries after 30–40 days of culture (Fig. 2a, c). Sané et al. (2006) achieved similar results with date palm (*Phoenix dactylifera* L.). In the context of this article, both friable yellow callus from petals and compact yellow–green callus from ovaries are termed as ideal callus.

In the present study, the two plant growth regulators (PGRs) combinations (Table 2) gave similar efficiency for callus induction (76.85 ± 5.78 vs. 73.61 ± 6.36%) from young petal explants. The basal callus induction medium with 8.88 μM BA, 10.74 μM NAA combined with 9.05 or 18.10 μM 2,4-D also had similar levels of efficiency (88.80 ± 8.17 vs. 89.51 ± 8.52%) in inducing callus from young ovaries. The results of the present study show ovaries used to start explants need a higher level of PGRs and provided a higher frequency of callus development than the petal bases. We speculate that ovaries need higher levels of exogenous PGRs for dedifferentiation to counter the effects of endogenous hormones, which stimulate differentiation because relatively higher endogenous hormone levels exist in ovaries when compared with levels in petals.

Table 2 Effect of PGRs on callus induction in *C. miniata* young petal explants cultured in vitro for 30 days

PGRs treatments (μM)	Frequency of callus development[a] (%)
2.22 BA + 4.52 2,4-D	76.85 ± 5.78
4.44 BA + 5.37 NAA + 9.05 2,4-D	73.61 ± 6.36

[a] Each value represents mean ± SD of three replicates. Means are not significantly different at $P \leq 0.05$ (Independent-Samples *t* test)

The surface of about 90% of the explants and nearly 80% of the primary callus turned dull red when they were cultured under HL instead of LL. Pigmented callus grew slowly, could not become ideal secondary callus after being chopped with a scalpel blade and produced significantly fewer shoots (0–2 shoots per callus). Empirical evidence indicated that anthocyanin levels varied response to biotic

Table 3 The effect of different KT combinations with NAA on shoot regeneration of callus after 4 months of culture

PGRs treatments (μM)		Percent of callus producing shoots[a] (%)		No. of shoots per callus[b]	
NAA	KT	Petal-derived callus	Ovary-derived callus	Petal-derived callus	Ovary-derived callus
10.74	13.94	72.93^{b} ± 4.14	30.95^{b} ± 3.37	4.94^{b} ± 0.59	2.54^{c} ± 0.30
5.37	13.94	75.43^{b} ± 0.61	19.09 ^{cd} ± 1.29	6.87^{a} ± 1.22	5.00^{a} ± 0.71
2.69	13.94	77.66^{ab} ± 2.34	16.78 ^{cd} ± 1.98	7.99^{a} ± 0.16	3.25^{c} ± 1.06
0	13.94	30.67^{d} ± 3.77	14.55^{de} ± 0. 37	3.39^{b} ± 0.35	2.75^{c} ± 0.35
10.74	9.29	43.08^{c} ± 4.35	37.26^{a} ± 5.55	5.09^{b} ± 1.29	4.72^{ab} ± 0.40
5.37	9.29	41.43^{c} ± 2.02	29.29^{b} ± 1.01	3.75^{b} ± 0.35	3.50^{abc} ± 0.71
2.69	9.29	84.3^{a} ± 0.44	21.83^{c} ± 0.56	7.74^{a} ± 0.17	4.75^{ab} ± 0.35
0	9.29	29.71^{d} ± 5.12	10.32^{e} ± 1.12	3.38^{b} ± 0.18	3.09^{c} ± 0.59

[ab] Each value represents mean ± SD of two replicates. Data within columns followed by the same letter are not significantly different at the 0.05 level by Duncan's Multiple Range test. Diameter of callus was 1–2 cm

or abiotic stress in plant cells (Cai et al. 2011). In this study, we speculate that HL is likely the leading factor for anthocyanin accumulation in explants and callus. Increasing light irradiation has been shown to induce anthocyanin synthesis in callus (Simões et al. 2009). It was also reported light and ultra-violet light were capable of inducing or increasing expression of some regulatory genes (Cone et al. 1993; Hollick et al. 2000) and structural genes (Contessotto et al. 2001; Gong et al. 1997; Lu and Yang 2006) of the anthocyanin biosynthetic pathway.

Shoot regeneration and proliferation from organogenic callus

Regeneration of yellow–green shoots occurred in ideal callus after 30 days of culture in the regeneration medium at HL. More shoots were differentiated in the callus than on the callus surface. A large number of shoots appeared when callus cleaved spontaneously (Fig. 2b, d). Table 3 presents the effect of KT in combinations with NAA on shoot regeneration from ideal callus. When using 2.69 μM NAA, the use of 13.94 and 9.29 μM KT proved to be a better concentration for shoot regeneration from the ideal callus derived from petal bases. With 13.94 μM KT, the frequency and numbers of shoots per callus produced were 77.66 and 7.99%, respectively. With 9.29 μM KT, the measurements were 84.31 and 7.74%, respectively. Six hundred and seventeen shoots were produced when the callus from one young petal base were cultivated on basal regeneration media with 2.69 μM NAA combined with 9.29 μM KT for 8 months after five subcultures. Basal regeneration medium containing 10.74 μM NAA combined with 9.29 μM KT was the optimal medium for shoot regeneration from ideal ovary-derived callus; the frequency of plantlet producing callus was 37.26% with 4.72 shoots produced per callus. Table 3 shows the explant types had a different regenerative capacity; young petals in all cases had high shoot regeneration frequency and regenerated many more shoots than young ovaries.

Tissue from fruit walls, peduncle-pedicel junctions and seedlings was successfully used as sources of explants for in vitro multiplication of *Clivia* but plantlet regeneration was very slow compared with regeneration in other plant species (Finnie and van Staden 1999; Ran and Simpson 2005). This study reports 617 shoots derived from one young petal base in contrast to two shoots derived from one peduncle-pedicel junction and about four shoots from one seedling explant (Ran and Simpson 2005). Whether this is caused by different characteristics of the explants, inducing ideal callus under LL or by transferring ideal callus into a medium specific for shoot induction is still a debatable issue. Moreover, it was reported that most plant species exhibited inherent variation which was translated to their varied responses to the different cytokinins (Aremu et al. 2011). Therefore, we speculate that using KT instead of BA for shoot induction may account for higher frequency regeneration in this study. However, this protocol is more effective than a previous method discussed by Ran and Simpson (2005) and may indeed be commercially viable considering the low frequency of success in traditional propagation techniques used with *C. miniata* and the large demand for it in the international market.

Table 4 The effect of PGRs on root formation of shoots after cultured in vitro for 60 days

PGRs treatments (μM)		Rooting rate[a] (%)
NAA	KT	
2.69	0.93	81.55 ^{cd} ± 4.23
1.07	0.93	88.89^{bc} ± 2.48
2.69	0	79.52^{d} ± 7.53
1.07	0	91.31^{ab} ± 2.44
0	0	98.25^{a} ± 3.04

[a] Each value represents mean ± SD of three replicates. Data within columns followed by the same letter are not significantly different at the 0.05 level by Duncan's Multiple Range test

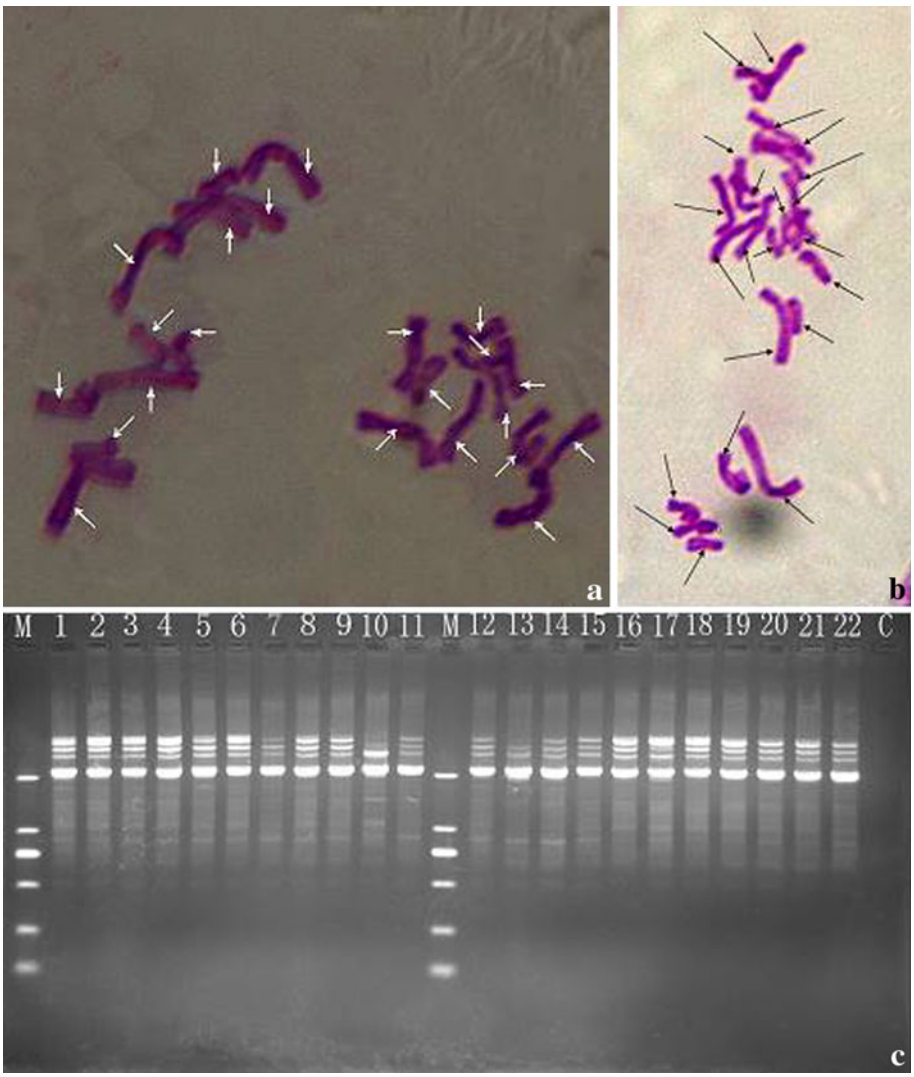

Fig. 3 The detection of somaclonal variation using chromosome counts and ISSRs. **a** Twenty-two chromosomes (*white arrows*) in a root tip cell of the regenerated plant from a young petal base. **b** Twenty-two chromosomes (*black arrows*) in a root tip cell of the regenerated plant from a young ovary. **c** ISSR profile of in-vitro plantlets and mother plants as shown by primer-3, where M, marker; 1 and 12, mother plants; 2–11, regenerated plants derived from mother plant-1; 13–22, regenerated plants derived from mother plant-12; C: water blank

Root induction from shoots and acclimatization of plantlets

About 7% of the developing shoots spontaneously formed roots in regeneration media. For non-rooted shoots, root formation was successfully induced after 7–30 days following transfer onto a root medium (Fig. 2e). One-way analysis of variance (ANOVA) revealed the rooting rate tended to decline as the concentrations of PGRs increased in the media and the half-strength MS medium without any PGRs was optimal for rooting (Table 4). In the present study, the presence of exogenous PGRs suppressed the rate of root formation in almost all cases, but root morphology was not affected. This is similar to observations of stem mustard (Guo et al. 2005) but in contrast to a previous finding in pumpkin ash (Stevens and Pijut 2011). We speculate endogenous hormones in *C. miniata* are adequate to induce root production because *C. miniata* plants with all the roots cut off would root in pots without any exogenous PGRs. Moreover, the transplanted plantlets were easy to harden off (Fig. 2f); 393 of 406 transplanted plantlets survived with a survival rate of 96.80%. This result agrees with a published report (Ran and Simpson 2005). This regeneration protocol may be applicable to the improvement of this plant by genetic engineering in the future. More studies may still be required to adopt the current protocol in genetic engineering.

Assessment of clonal fidelity of regenerated plantlets

A total of 1000 regenerated plantlets were analyzed, of which only six (0.6%) exhibited some form of abnormal phenotype when compared with the mother plants. All of the variants were from a young ovary of mother plant-12, had similar coiled leaves, and some had white leaf edges

(Fig. 2g–i). Unfortunately, all the six variants did not survive the acclimatization process.

Chromosome counts of regenerated plantlets from young petal bases and ovaries showed the number of chromosomes of root tip cells was still 22 (Fig. 3a, b).

Inter-simple sequence repeats have a high capacity to reveal polymorphism and offer great potential to determine intra- and intergenomic diversity when compared with other arbitrary primers like randomly amplified polymorphic DNA (RAPD, Souframanien and Gopalakrishna 2004). It was found that ISSR primers could produce more reliable and reproducible bands, when compared with RAPD primers (Mattioni et al. 2002). Also, ISSRs are easily established and facilitate the detection of polymorphisms with efficiency comparable to that of amplified fragment length polymorphisms (AFLPs, Borchert and Hohe 2009). Thus, the ISSR method was used to determine genomic-based variation in this study.

Twenty regenerated plants from two mother plants were analyzed; twenty primers yielded 137 clearly identifiable bands. All primers but primers 7 and 11 gave polymorphic bands. The total number of identifiable bands of these 18 primers was 128, of which 57 were polymorphic (44.53%), and the number of bands produced per primer ranged from three to 11 (Fig. 3c). Different bands were displayed in 19 out of 20 regenerated plants from the mother plants.

Cluster analysis represented as a dendrogram on the base of the similarity coefficient generated from ISSR data of 137 bands exhibited a similarity range of 90.5–100.0% between regenerated plantlets and their own mother plant, and 89.0–100.0% among plantlets regenerated from the same mother plant. Mother plant-1 and the nine plantlets regenerated from it (excepting regenerated plantlet 10) were grouped into one major cluster at 96.5% level, while mother plant-12 and the eight plantlets regenerated from it (excepting plantlets 13 and 17) were grouped into a second major cluster at 96.9% level (Fig. 4).

From the present study, it is clear that variability in genomic DNA is present in the micropropagated plants of *C. miniata*. However, only 0.6% of regenerated plantlets differed phenotypically from the parental clones. Chromosome counts revealed no variations in the number of chromosomes in root tip cells of regenerated plantlets when compared with the mother plants in our case. Chromosome counts also revealed that in vitro plantlets (from ovaries) were not regenerated from haploid ovules in young ovaries. This result might also mean that any callus with variations in chromosome numbers were unable to regenerate into plantlets (Jin et al. 2008).

The in vitro raised plants from the same mother plant exhibited 89.0–100.0% similarity to each other and 90.5–100.0% similarity compared with the mother plants (Fig. 4) through ISSRs. Plantlets 10, 13 and 17 were not

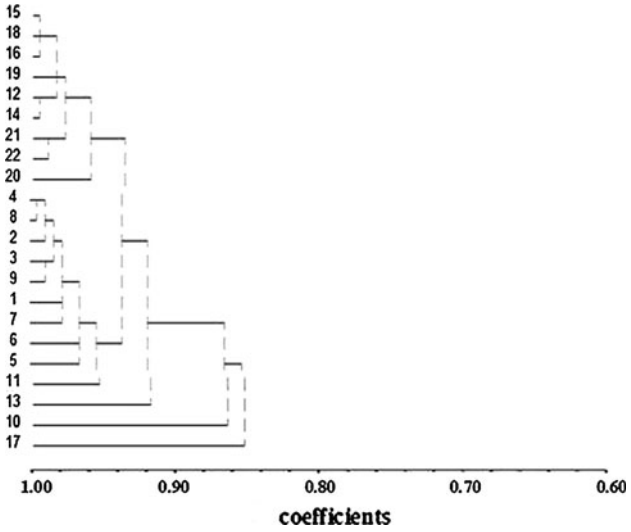

Fig. 4 Ochiai based dendrogram showing similarity between regenerated plantlets and mother plants. 1, 12, mother plants; 2–11, regenerated plantlets derived from mother plant-1; 13–22, regenerated plantlets derived from mother plant-12. 2, 6, 14, 17 and 21, regenerated plantlets derived from young ovaries; 3–5, 7–11, 13, 15, 16, 18–20 and 22, regenerated plantlets derived from young petals

grouped into their own mother's major cluster because of the lower similarity coefficient (90.9, 94.8 and 90.2%) to the mothers, but we did not find phenotypic variation in these plants. This could be explained in four ways. First, perhaps there were many sequences in the genome which were not expressed or have anything to do with genes; for example, pseudogenes could have been rearranged due to the cultivation stress in vitro. Second, perhaps synonymous mutations and some of the near-sense mutations of genes did not change the phenotype. Third, there may be some alternative genes and/or metabolic pathways in plant organisms, which compensate for the variation in the genome (Wu et al. 1999; Yu et al. 2004). Fourth, all the tested plants were in a juvenile phase during which morphological variations may not be observed (Ran et al. 2001b).

To best of our knowledge, this is the first report of successfully inducing plantlets from young petal bases of *C. miniata* and the first analysis of clonal fidelity of regenerated *C. miniata* plants. This protocol may indeed be commercially viable considering the relatively high regeneration frequency and low frequency of phenotypic variation in the regenerated plants.

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