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ORIGINAL PAPER

Callus-mediated and direct protocorm-like body formation of *Bletilla striata* and assessment of clonal fidelity using ISSR markers

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Abstract Two efficient morphogenetic pathways for micropropagation of *Bletilla striata* (Thunb.) Reichb. f. have been established through the callus-mediated and direct formation of protocorm-like bodies (PLBs) from protocorms and shoot tips. Green calli were induced from the basal surface of protocorms and the cut-end of shoot tips on Vacin and Went (VW) medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) or α-naphthalene acetic acid (NAA) after 3–5 weeks, with the highest frequency of explants forming callus (48.0 %) from protocorms at 1.0 mg l^{-1} 2,4-D. The calli obtained from all plant growth regulator (PGR) treatments could proliferate and differentiate PLBs on the PGR-free medium. NAA and 2,4-D significantly enhanced the growth of callus. The fastest growth rate of callus was achieved at the combination of 1.0 mg l^{-1} 2,4-D and 1.0 mg l^{-1} TDZ with 46.2-fold within 3 months. The regeneration of PLBs from callus was significantly improved by 6-benzyladenine (BA), and a mean number of 48.4 PLBs was produced from 100 mg calli at 1.0 mg l^{-1} BA within 3 months. BA and thidiazuron (TDZ) promoted the direct formation of PLBs from explants. The highest frequency of direct PLBs formation (76.0 %) and the highest mean number of PLBs per explant (30.2) were observed in protocorms cultured with 0.5 mg l^{-1} BA. Assessment of clonal fidelity by inter-simple sequence repeat (ISSR) markers revealed similarity ranges of 99.8–100.0 % between the regenerants and their mother plants and 99.5–100.0 % among the regenerants, which suggested the micropropagation protocols were genetically stable.

Keywords *Bletilla striata* · Callus · Micropropagation · Protocorm-like body (PLB) · ISSR marker

Abbreviations

BA	6-Benzyladenine
2,4-D	2,4-Dichlorophenoxyacetic acid
ISSR	Inter-simple sequence repeat
NAA	α-Naphthalene acetic acid
PGR	Plant growth regulator
PLB	Protocorm-like body
TDZ	Thidiazuron
VW	Vacin and Went medium

Introduction

Bletilla striata (Thunb.) Reichb. f. is a sympodial terrestrial orchid that is native to a few countries in eastern Asia. The plant has attractive purple-pink inflorescences with up to 12 flowers and rich green foliage, and is therefore widely used in landscaping. In addition, *B. striata* has been used for over 1500 years in traditional Chinese medicine. Recently, several important secondary metabolites have been isolated from *B. striata* (Wang et al. 2006; Feng et al. 2008), and evidence indicates that they have important roles in the treatment of liver tumors (Qian et al. 2003), breast cancer (Morita et al. 2005), gastric ulcers (Li et al. 2005) and wound healing (Wang et al. 2006; Diao et al. 2008). However, the number of wild plants is steadily declining to endangered status due to commercial over-

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collection from natural areas and change of habitats. Hence, there is an urgent need for conservation and cultivation of this species for future use.

B. striata is typically propagated by dividing pseudobulbs, while this method is time-consuming and not economically viable. Although this species also can be sexually propagated using seeds, the offspring is generally difficult to obtain in nature because of the low level of fruit set and low frequency of seed germination (Sugiura 1995; Chung and Chung 2005). Therefore, it is essential to establish efficient propagation methods for the large-scale commercial requirements and the germplasm conservation of this ornamentally and medicinally important plant.

Over the past few decades, tissue-culture techniques have been extensively exploited in the rapid and mass propagation of several commercially important orchids as well as the ex situ conservation of endangered orchids (Chugh et al. 2009). Different protocols have been developed using various tissues of orchids as explants, such as inflorescences, shoot tips, axillary buds, leaves, stems, roots, rhizomes, mature seeds, immature seeds and protocorms. These protocols could be classified into two types: shoot formation via organogenesis and protocorm-like body (PLB) formation through somatic embryogenesis (Zhao et al. 2008). PLB is analogous to somatic embryo (Lee et al. 2013) and could be induced from explants through callus-mediated and direct regeneration in orchids. An average of 90.7 PLBs was developed from callus of *Cymbidium* after 2 months (Huan et al. 2004). In *Oncidium* 'Gower Ramsey', a mean number of 134.2 PLBs was induced from each leaf explant after 8 weeks (Chen and Chang 2004a) and approximately 6,000 PLBs were generated in 2 months from an initial culture of 1 g callus fresh weight (Jheng et al. 2006). Because of the high frequency of somatic embryo formation and efficiency of somatic embryo conversion into plant, the mass propagation through PLBs formation was regarded as an efficient protocol for orchids.

Hitherto, rare protocols have been established for micropropagation of *B. striata*. Dhiman et al. (2007) reported in vitro regeneration through callus-mediated and direct shoot buds formation from leaf segments. However, to date, there were no reports on plant regeneration of *B. striata* via PLBs formation. In this paper, for the first time, we describe rapid, efficient and reproducible regeneration protocols for *B. striata* through callus-mediated and direct formation of PLBs from protocorms and shoot tips. The effects of plant growth regulators (PGRs) on the induction of callus and PLBs were examined, and the clonal fidelity of regenerants was assessed using analysis of inter-simple sequence repeat (ISSR) markers.

Materials and methods

Plant materials

Protocorms about 2–3 mm and shoot tips with two leaf primordia of *B. striata*, taken from in vitro asymbiotic seed germination, were used as the source of explants. In vitro asymbiotic seed germination was carried out as follows: artificial pollination was conducted on the blossomed wild plants at the beginning of May in the Administration Bureau of Tianmu Mount National Nature Reserve of Zhejiang Province. Mature pods about 6 months after artificial pollination were harvested and kept immersed in 2.5 % sodium hypochloride for 20 min, and then rinsed thoroughly three times with sterile distilled water. Seeds were removed from the pods and sown on 0.7 % agar-solidified VW medium (Vacin and Went 1949) supplemented with 30 g l^{-1} sucrose. The pH of the medium was adjusted to 5.8 prior to autoclaving at 121 °C for 20 min. Seeds were cultured at 25 ± 2 °C under a 16-h photoperiod illumination of approximately 40 μmol m^{-2} s^{-1} provided by white fluorescent lamps. For all the subsequent studies, the VW medium and culture conditions were the same as these described for seed germination.

Induction of callus and PLBs

Protocorms and shoot tips were inoculated onto 100 ml of VW basal medium supplemented with PGRs in 250 ml tissue-culture flasks, with the basal surface of protocorms and the cut-end of shoot tips in contact with the media. The PGR treatments consisted of 6-benzyladenine (BA, 0.5, 1.0, 2.0 and 4.0 mg l^{-1}), thidiazuron (TDZ, 0.5, 1.0, 2.0 and 4.0 mg l^{-1}), 2,4-dichlorophenoxyacetic acid (2,4-D, 0.2, 0.5, 1.0 and 2.0 mg l^{-1}), α-naphthalene acetic acid (NAA, 0.2, 0.5, 1.0 and 2.0 mg l^{-1}), as well as 1.0 mg l^{-1} 2,4-D combined with BA or TDZ (0.5, 1.0, and 2.0 mg l^{-1}). There were five replicates each containing 20 explants for each treatment. The cultures were maintained for 3 months to induce callus and PLBs. Then, the percentages of explants survived, forming callus and directly forming PLBs were evaluated. To distinguish between the direct and callus-mediated PLBs, calli were transferred to new flasks containing same media before calli differentiated PLBs. The morphology was observed by the naked eyes directly or using a stereomicroscope (SZ2-ILST, Olympus, Japan).

Subculture of callus and regeneration of PLBs from callus

Calli induced from all treatments were subcultured on the PGR-free medium to assess their ability to grow in the

absence of exogenous PGRs. Meanwhile, calli originally induced from protocorms with 1.0 mg l^{-1} 2,4-D were selected to examine the effect of exogenous PGRs on the growth and differentiation potential of callus. The selected calli were firstly subcultured on the PGR-free medium for 9 months with three subculture times to remove the effects of induction by 2,4-D, and were then transferred to VW basal medium supplemented with PGRs. The PGR treatments consisted of BA (0.5, 1.0 and 2.0 mg l^{-1}), TDZ (0.5, 1.0 and 2.0 mg l^{-1}), 2,4-D (0.2, 0.5 and 1.0 mg l^{-1}), NAA (0.2, 0.5 and 1.0 mg l^{-1}), as well as 1.0 mg l^{-1} TDZ combined with 2,4-D (0.5, 1.0 and 2.0 mg l^{-1}). There were five replicate flasks each culturing one undifferentiated callus mass weighing about 100 mg.

After 3 months of culture, the necrotic calli were separately weighed and given as a percentage of the total final callus weight. The growth rate of callus is expressed as $(W_f - W_p - W_i)/W_i$, where W_f is the final fresh weight of the entire tissue mass, W_p is the fresh weight of the regenerated PLBs and plantlets and W_i is the initial fresh weight of the callus (Roy et al. 2007). The PLBs derived from 100 mg calli comprised the regenerated PLBs and plantlets.

Ex vitro establishment of plantlets

Plantlets with 4–5 roots were carefully removed from the flasks and thoroughly rinsed in running water to remove agar media. After dipped in 0.2 % (v/v) carbendazim for 1 min, they were transplanted into plastic pots containing a 1:1 (v/v) mixture of vermiculite and humus soil. The potted plantlets were kept in a greenhouse under 60 % shading and 60 % relative humidity for further growth and development.

Fidelity analysis of regenerants using ISSR markers

A total of 54 plantlets each about 8 cm growing in vitro were used for the fidelity analysis. The first 18 plantlets were selected from the regenerants obtained from one protocorm through direct PLBs formation with 0.5 mg l^{-1} BA, of which 9 plantlets were directly formed from the mother protocorm and 9 plantlets were regenerated after 12 months of subculture with 0.5 mg l^{-1} BA. The second 18 plantlets, which produced from one protocorm through induction of callus with 1.0 mg l^{-1} 2,4-D and regeneration of PLBs with 1.0 mg l^{-1} BA, comprised 9 plantlets regenerated from the callus without subculture and 9 plantlets regenerated from the callus subcultured on the PGR-free medium for 12 months. The third 18 plantlets, which produced from one protocorm also through induction of callus with 1.0 mg l^{-1} 2,4-D and regeneration of PLBs with 1.0 mg l^{-1} BA, consisted of 9 plantlets regenerated from the callus without subculture and 9 plantlets from the callus subcultured with the combination of 1.0 mg l^{-1} 2,4-D and 1.0 mg l^{-1} TDZ for 12 months.

Genomic DNA was extracted from leaf tissue using a Plant Genomic DNA Kit (TIANDZ, China). The quality and quantity of DNA were inspected by both 0.8 % agarose gel electrophoresis and spectrophotometry (Unican UV300, Thermo, USA). To provide a common genetic background, DNA samples of 50 regenerants from one mother protocorm were mixed in equal concentration to generate a pool representing the mother plant DNA. Total 100 ISSR primers (UBC801–900, The University of British Columbia) were initially tested using 3 mother plant DNAs as templates to screen for suitable primers, out of which produced clear and reproducible bands were used in further fidelity analysis. The primers were synthesized by Sangon Biotech Co., Ltd., Shanghai.

PCR was carried out in 20 μl volumes containing 50 ng genomic DNA, 1.0 μM primer, 1× PCR buffer, 0.2 mM dNTPs, 2.0 mM $MgCl_2$ and 1 U *Taq* DNA polymerase (Sangon Biotech, China). The reaction was performed in a thermal cycler (C1000, Bio-Rad, USA) programmed for an initial denaturing at 94 °C for 5 min, followed by 40 cycles of 1 min denaturation at 94 °C, 1 min annealing (temperature specific to the primer) and 2 min extension at 72 °C, with a final extension at 72 °C for 10 min. PCR amplification products were separated by electrophoresis on an 8 % polyacrylamide gel in 1× TBE buffer. After stained with silver nitrate, the banding profiles were documented using a gel documentation system (FR-200A, Furi, China).

Statistical analysis

All culture experiments were performed according to a completely randomized design. The data were recorded and analyzed statistically using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test at 5 % level. For fidelity analysis, all PCR reactions were repeated at least twice. Only distinct, consistently reproducible and well-resolved bands were scored as 1 for the presence and 0 for the absence across the regenerated plantlets and their mother plants. The similarity coefficients were determined using the NTSYSpc 2.10e software package (Rohlf 2000).

Results and discussion

Induction of callus

Green calli were induced from the basal surface of protocorms and the cut-end of shoot tips after 3–5 weeks of inoculation (Fig. 1a). Better response toward callus

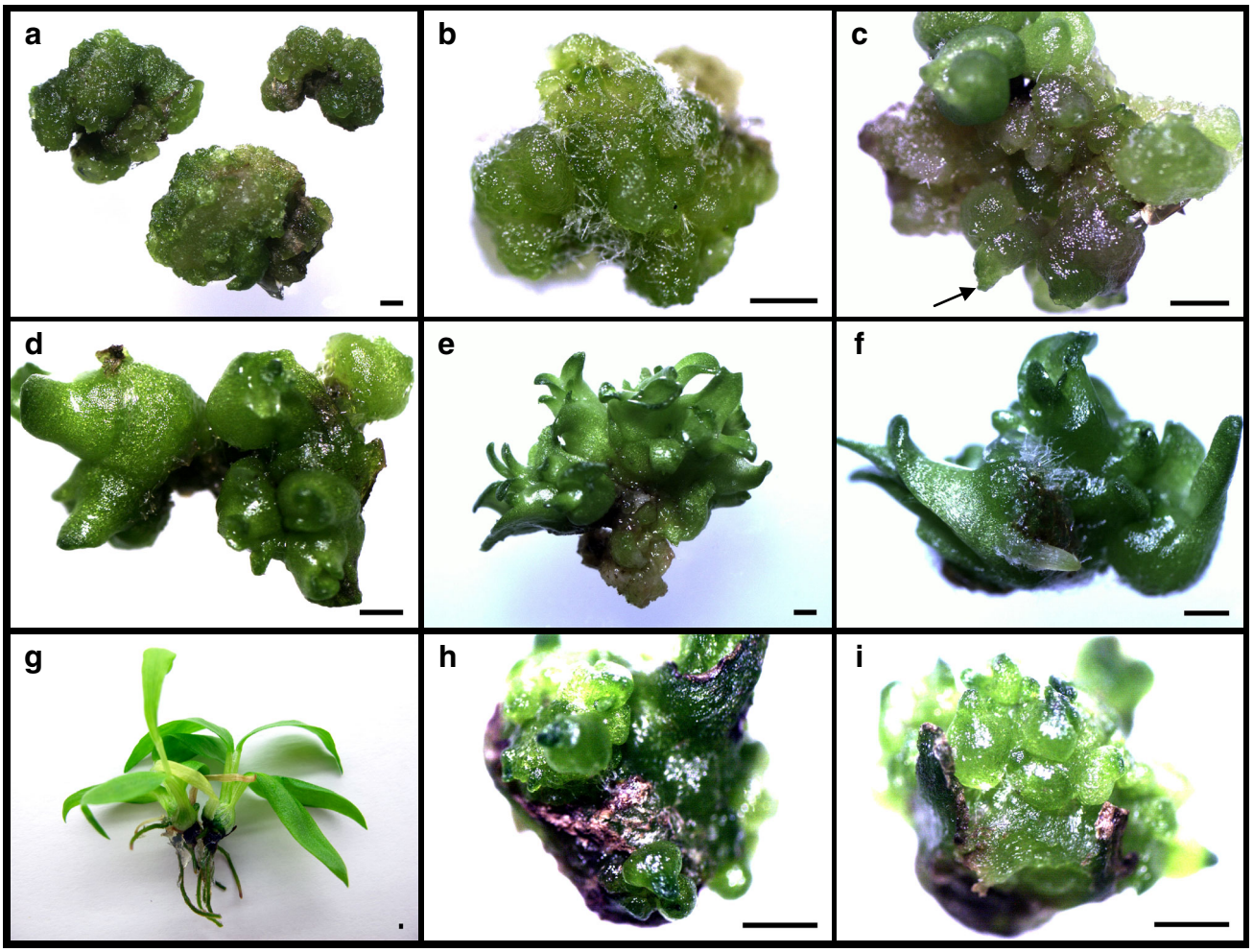

Fig. 1 Morphogenesis of callus-mediated and direct formation of PLBs in *Bletilla striata*. **a** Calli induced from protocorms and shoot tips. **b** Globular PLBs emerged from callus. **c** PLBs elongated and some formed a protrusion (*arrow*). **d** Leaves developed from PLBs. **e** Leafy plantlets with callus. **f** Roots developed from PLBs. **g** Well-developed plantlets with roots. PLBs regenerated directly from the surface of protocorm (**h**) and the axil (**i**). *Scale bars* 1 mm

induction was observed from protocorms (6.0–48.0 %) than shoot tips (1.0–18.0 %) because more severe necrosis existed in the later (Table 1). This may be the result of wounding in shoot tips, which caused the ensuing browning by oxidation of phenolic substances as described in the bisected PLBs (Teixeira da Silva et al. 2006).

In addition to the type of explants, the type and concentration of PGRs also influenced the frequency of explants forming callus (Table 1). Calli could be induced from protocorms and shoot tips in most of the 2,4-D and NAA treatments with different frequencies from 1.0 to 48.0 %. 2,4-D was found to be more effective than NAA in inducing callus from protocorms, and the best response (48.0 %) was obtained in VW medium supplemented with 1.0 mg l^{-1} 2,4-D. NAA also could induce calli from protocorms and shoot tips, but the frequencies of explants forming callus were just 5.0–18.0 %. In contrast, no callus formed from explants with the PGR-free medium and cytokinin treatments (BA or TDZ). The combinations of 2,4-D with BA or TDZ induced calli from protocorms and shoot tips, while they were no more efficient than 2,4-D used alone.

Although a few studies indicated the induction of callus could occur in the absence of exogenous PGRs in some orchids (Roy et al. 2007), the presence of PGRs was critical for the formation of callus in most cases of orchids. Earlier observations demonstrated that the development of callus from different types of explants primarily depended upon exogenous auxins. Cytokinins incorporated along with auxins showed better responses, but cytokinins had little effect on the induction of callus by themselves (Lin et al. 2000; Huan et al. 2004). However, calli were induced from explants of *Dendrobium chrysotoxum* (Roy et al. 2007), *Dendrobium candidum* (Zhao et al. 2008) and *Lycaste*

Table 1 Effects of plant growth regulators on protocorms and shoot tips of *Bletilla striata* after 3 months of culture on VW medium

Plant growth regulators (mg l^{-1})	Explants forming callus (%)		Explants forming direct PLBs (%)		Direct PLBs per explant	
	Protocorm	Shoot tip	Protocorm	Shoot tip	Protocorm	Shoot tip
PGR-free	0 ± 0^{g}	0 ± 0^{g}	17.0 ± 5.7^{ghi}	15.0 ± 3.5^{e}	8.1 ± 1.0^{fg}	5.9 ± 1.0^{e}
BA 0.5	0 ± 0^{g}	0 ± 0^{g}	76.0 ± 6.5^{a}	44.0 ± 4.2^{a}	30.2 ± 6.3^{a}	14.8 ± 1.0^{a}
BA 1.0	0 ± 0^{g}	0 ± 0^{g}	60.0 ± 7.9^{b}	36.0 ± 4.2^{b}	24.9 ± 4.3^{b}	8.7 ± 0.8^{b}
BA 2.0	0 ± 0^{g}	0 ± 0^{g}	54.0 ± 6.5^{c}	33.0 ± 5.7^{b}	23.2 ± 2.9^{b}	$8.1 \pm 1.1b^{c}$
BA 4.0	0 ± 0^{g}	0 ± 0^{g}	27.0 ± 5.7^{f}	19.0 ± 4.2^{d}	15.7 ± 4.0^{c}	$7.9 \pm 0.8b^{c}$
TDZ 0.5	0 ± 0^{g}	0 ± 0^{g}	46.0 ± 4.2^{d}	24.0 ± 4.2^{c}	15.7 ± 2.6^{c}	7.6 ± 1.1^{c}
TDZ 1.0	0 ± 0^{g}	0 ± 0^{g}	40.0 ± 5.0^{e}	20.0 ± 3.5^{d}	13.5 ± 1.1^{cd}	6.7 ± 1.2^{d}
TDZ 2.0	0 ± 0^{g}	0 ± 0^{g}	25.0 ± 3.5^{f}	13.0 ± 2.7^{e}	11.0 ± 1.7^{def}	5.4 ± 0.7^{e}
TDZ 4.0	0 ± 0^{g}	0 ± 0^{g}	22.0 ± 2.7^{fg}	12.0 ± 2.7^{e}	9.8 ± 0.9^{ef}	5.7 ± 0.5^{e}
2,4-D 0.2	35.0 ± 7.1^{cd}	8.0 ± 5.1^{cd}	0 ± 0^{k}	0 ± 0^{f}	0 ± 0^{h}	0 ± 0^{h}
2,4-D 0.5	31.0 ± 6.5^{d}	4.0 ± 3.7^{efg}	0 ± 0^{k}	0 ± 0^{f}	0 ± 0^{h}	0 ± 0^{h}
2,4-D 1.0	48.0 ± 5.7^{a}	0 ± 0^{g}	0 ± 0^{k}	0 ± 0^{f}	0 ± 0^{h}	0 ± 0^{h}
2,4-D 2.0	39.0 ± 4.2^{bc}	0 ± 0^{g}	0 ± 0^{k}	0 ± 0^{f}	0 ± 0^{h}	0 ± 0^{h}
NAA 0.2	6.0 ± 4.2^{fg}	10.0 ± 4.5^{c}	0 ± 0^{k}	0 ± 0^{f}	0 ± 0^{h}	0 ± 0^{h}
NAA 0.5	17.0 ± 5.7^{e}	18.0 ± 2.4^{a}	0 ± 0^{k}	0 ± 0^{f}	0 ± 0^{h}	0 ± 0^{h}
NAA 1.0	15.0 ± 3.5^{e}	14.0 ± 3.7^{b}	0 ± 0^{k}	0 ± 0^{f}	0 ± 0^{h}	0 ± 0^{h}
NAA 2.0	16.0 ± 4.2^{e}	5.0 ± 4.5^{de}	0 ± 0^{k}	0 ± 0^{f}	0 ± 0^{h}	0 ± 0^{h}
2,4-D 1.0 + BA 0.5	43.0 ± 4.5^{ab}	0 ± 0^{g}	12.0 ± 2.7^{ij}	0 ± 0^{f}	10.5 ± 1.2^{def}	3.4 ± 0.4^{fg}
2,4-D 1.0 + BA 1.0	42.0 ± 5.7^{ab}	1.0 ± 2.0^{fg}	18.0 ± 2.7^{gh}	1.0 ± 2.2^{f}	11.7 ± 1.0^{de}	2.7 ± 0.7^{g}
2,4-D 1.0 + BA 2.0	42.0 ± 4.5^{ab}	2.0 ± 2.4^{efg}	15.0 ± 3.5^{hij}	2.0 ± 2.7^{f}	10.8 ± 2.3^{def}	3.7 ± 0.7^{f}
2,4-D 1.0 + TDZ 0.5	44.0 ± 7.4^{ab}	1.0 ± 2.0^{fg}	11.0 ± 4.2^{j}	1.0 ± 2.2^{f}	6.5 ± 2.0^{g}	2.6 ± 0.4^{g}
2,4-D 1.0 + TDZ 1.0	44.0 ± 6.5^{ab}	2.0 ± 2.4^{efg}	16.0 ± 4.2^{hij}	1.0 ± 2.2^{f}	5.2 ± 2.8^{g}	2.8 ± 0.6^{g}
2,4-D 1.0 + TDZ 2.0	46.0 ± 4.2^{a}	5.0 ± 3.2^{de}	17.0 ± 4.5^{ghi}	3.0 ± 2.7^{f}	7.9 ± 1.5^{fg}	3.0 ± 0.4^{fg}

Data shown are the mean of five replicates ± standard error (SE). Different letters within a column represent significant difference at P 0.05 with Duncan's multiple range tests

(Huang and Chung 2011) by cytokinins. Dhiman et al. (2007) reported that callus formation from leaf segments of *B. striata* depended upon NAA, and 1 mg l^{-1} each of NAA and BAP was the most beneficial (25 %). Our present study also indicated that auxins were indispensable for the induction of callus from protoroms and shoot tips of *B. striata*, while the most effective treatment was 1.0 mg l^{-1} 2,4-D alone. The different responses toward PGRs might be dependent on explants.

Subculture of callus and regeneration of PLBs from callus

A large number of PLBs emerged from callus after 5–6 weeks of subculture on the optimal media. At the early developmental stage, a mass of globular granules differentiated from callus and these globular granules are PLBs (Fig. 1b). The PLBs elongated and formed a protrusion (Fig. 1c), and then succeeding leaves developed in an alternating sequence (Fig. 1d, e). Finally, roots generated from the base of PLBs (Fig. 1f) to form complete plantlets (Fig. 1g). About 6–7 weeks were needed for PLBs to develop into complete plantlets.

The calli obtained from all PGR treatments could proliferate and differentiate PLBs in the absence of exogenous PGRs. When the calli originally induced from protocorms with 1.0 mg l^{-1} 2,4-D were investigated, although 13.3 % of calli turned necrotic, the fresh weight of callus increased 21.3-fold compared with the initial weight, and averagely 26.2 PLBs regenerated from 100 mg calli on the PGR-free medium within 3 months (Table 2). This phenomenon was also described in other orchid species, among of which some results showed high frequency of proliferation and differentiation. An average of 90.7 PLBs formation per 40 mg calli was observed after 4 weeks of culture on the medium without PGRs in *Cymbidium* (Huan et al. 2004). In *D. chrysotoxum*, the fresh weight of callus increased 18-fold, and 25 PLBs formed from 100 mg calli in the absence of exogenous PGRs after 3 months of culture (Roy et al. 2007). Because it has been proved that PGRs are one of the factors which may cause somaclonal variation in micropropagation of plants (Bairu et al. 2011), the efficient

Table 2 Effects of plant growth regulators on growth and differentiation of protocorm-derived callus of *Bletilla striata* after 3 months of culture on VW medium

Plant growth regulators (mg l^{-1})	Necrosis of callus (%)	Growth rate of callus	Number of PLBs derived from 100 mg calli
PGR-free	13.3 ± 1.9^{ghij}	21.3 ± 2.5^{f}	26.2 ± 7.0^{cd}
BA 0.5	12.1 ± 1.1^{ij}	9.8 ± 1.5^{h}	36.0 ± 7.5^{b}
BA 1.0	11.6 ± 2.0^{j}	5.9 ± 1.1^{i}	48.4 ± 4.4^{a}
BA 2.0	13.4 ± 1.0^{ghij}	5.7 ± 0.9^{i}	43.2 ± 4.0^{a}
TDZ 0.5	12.2 ± 1.0^{hij}	15.7 ± 1.5^{g}	28.2 ± 5.2^{c}
TDZ 1.0	13.0 ± 0.3^{ghij}	14.0 ± 1.3^{g}	20.8 ± 6.2^{cd}
TDZ 2.0	13.8 ± 0.6^{ghi}	9.5 ± 1.3^{h}	22.8 ± 5.4^{cd}
NAA 0.2	14.1 ± 1.2^{fghi}	26.7 ± 1.7^{e}	24.2 ± 8.0^{cd}
NAA 0.5	14.7 ± 1.1^{fg}	27.6 ± 1.6^{e}	13.6 ± 4.7^{ef}
NAA 1.0	16.1 ± 1.1^{ef}	28.0 ± 2.0^{e}	11.6 ± 5.1^{fg}
2,4-D 0.2	14.3 ± 0.7^{fgh}	38.6 ± 2.1^{c}	23.0 ± 7.6^{cd}
2,4-D 0.5	19.4 ± 1.8^{bc}	42.8 ± 3.9^{b}	12.2 ± 4.3^{fg}
2,4-D 1.0	21.0 ± 1.9^{b}	37.8 ± 1.4^{c}	7.8 ± 4.1^{fg}
2,4-D 0.5 + TDZ 1.0	18.6 ± 2.3^{cd}	42.9 ± 3.0^{b}	19.4 ± 2.5^{de}
2,4-D 1.0 + TDZ 1.0	17.4 ± 1.6^{de}	46.2 ± 1.2^{a}	10.8 ± 1.9^{fg}
2,4-D 2.0 + TDZ 1.0	32.7 ± 2.1^{a}	32.4 ± 3.4^{d}	5.0 ± 1.6^{g}

Data shown are the mean of five replicates ± standard error (SE). Different letters within a column represent significant difference at P 0.05 with Duncan's multiple range tests

proliferation and differentiation of callus without PGRs were proposed to have considerable practical implication for mass propagation of orchids with least possibility of somaclonal variation (Roy et al. 2007).

Meanwhile, exogenous PGRs had different effects on the growth of callus and the regeneration of PLBs from callus (Table 2). In the presence of BA or TDZ, the percentage of necrotic calli was not significantly different from that on the PGR-free medium, while the growth rate of callus decreased significantly. The application of BA significantly enhanced the regeneration of PLBs from callus, with the most effective concentration at 1.0 mg l^{-1} which produced 48.4 PLBs from 100 mg calli within 3 months. All TDZ treatments showed neither improvement nor inhibition for the regeneration of PLBs from callus. Among the concentrations of NAA and 2,4-D tested, necrosis of callus significantly increased when the concentration of NAA and 2,4-D reached 1.0 mg l^{-1} and 0.5 mg l^{-1}, respectively. NAA and 2,4-D improved the growth of callus, while they failed to improve the regeneration of PLBs from callus and even prevented it when the concentration reached 0.5 mg l^{-1}. When the combinations of 2,4-D and TDZ were applied, the callus showed a significantly aggravated necrosis and the number of PLBs derived from 100 mg calli significantly decreased compared with that on the PGR-free medium. However, significant growth enhancements of callus were obtained in these combinations, and the fastest growth rate was achieved at the combination of 1.0 mg l^{-1} 2,4-D and 1.0 mg l^{-1} TDZ with 46.2-fold within 3 months.

Although it is efficient for callus to proliferate and differentiate without PGRs in some orchids, different types and concentrations of PGRs were required for callus in other orchids. The combinations of 2,4-D and TDZ were often regarded as effective for proliferation of callus. For callus induced from seed-derived protocorms, the best responses were, respectively, obtained at 5 mg l^{-1} 2,4-D and 1 mg l^{-1} TDZ in *Paphiopedilum* hybrid (Lin et al. 2000), and 4.52 μM 2,4-D and 4.54 μM TDZ in *Cypripedium formosanum* (Lee and Lee 2003). Callus from root tips of *Oncidium* 'Gower Ramsey' also proliferated 2–4 times in 1 month in the presence of 3–10 mg l^{-1} 2,4-D and 0.1–3 mg l^{-1} TDZ (Chen and Chang 2000). For regeneration of PLBs from callus, different responses toward PGRs were observed in earlier investigations. The best effects were, respectively, obtained at 4.44 μM BA in *C. formosanum* (Lee and Lee 2003), and the combination of 3 mg l^{-1} TDZ and 0.1 mg l^{-1} NAA in *Oncidium* 'Gower Ramsey' (Chen and Chang 2000). Wu et al. (2004) reported PLBs could be induced from callus by both cytokinins (2ip, BA, kinetin, TDZ and zeatin alone) and auxins (IAA, IBA, NAA and picloram alone) in *Oncidium* 'Gower Ramsey', and the best responses were, respectively, obtained at 0.5 mg l^{-1} zeatin and 2 mg l^{-1} IAA. Furthermore, contrary responses were observed at different concentrations of NAA in *D. candidum*, which showed the regeneration of PLBs from callus was promoted at low concentrations but reduced at high concentrations (Zhao et al. 2008). In addition, Roy et al. (2007) reported that BAP or NAA alone improved both the growth of callus and the regeneration of PLBs. The present investigation demonstrated that the growth of callus was improved in the presence of NAA or 2,4-D, and the best effect was obtained at the combination of 1.0 mg l^{-1} 2,4-D and 1.0 mg l^{-1} TDZ, which was similar to the previous reports (Chen and Chang 2000; Lin et al. 2000; Lee and Lee 2003). At the same time, our result indicated that only BA enhanced the regeneration of PLBs from callus significantly among all the PGR treatments tested.

Direct formation of PLBs

Apart from the callus-mediated pathway, direct formation of PLBs from explants without an intermediary callus phase was also observed in some treatments (Table 1). Such regeneration of PLBs occurred from both the surface of protocorms (Fig. 1h) and the axils (Fig. 1i) after 7–9 weeks of culture. The directly formed PLBs grew into

plantlets when cultured on the induction media or transferred to the PGR-free medium. Meanwhile, these PLBs could further produce secondary PLBs on the induction media continuously.

Protocorms were more effective than shoot tips for the direct formation of PLBs. On the PGR-free medium, 17.0 % of protocorms and 15.0 % of shoot tips directly regenerated PLBs after 3 months, and an average of 8.1 and 5.9 PLBs induced from one protocorm and one shoot tip, respectively. The application of cytokinins was beneficial for the direct formation of PLBs. Of the all PGRs tested, BA was found to be the most effective. In the presence of 0.5 mg l^{-1} BA, not only the highest frequencies of explants directly forming PLBs were observed in protocorms (76.0 %) and shoot tips (44.0 %), but also the highest mean numbers of PLBs were induced from one protocorm (30.2) and one shoot tip (14.8), respectively. Further increase in concentration of BA yielded no significant enhancements and even caused declines in both the frequency of explants regenerating PLBs and the mean number of PLBs per explant. In a manner similar to BA, TDZ was most effective at 0.5 mg l^{-1}. At this concentration, 46.0 % of protocorms directly regenerated a mean number of 15.7 PLBs per explant, and 24.0 % of shoot tips directly formed a mean value of 7.6 PLBs per explant. In contrast, no PLB regenerated from protocorms and shoot tips cultured with NAA or 2,4-D even after 3 months. Moreover, BA or TDZ combined with 2,4-D also induced the direct regeneration of PLBs from protocorms and shoot tips, but was not as effective as BA or TDZ used alone.

Previous investigations have indicated that PLBs could be directly induced from explants in the presence of exogenous cytokinins, auxins and cytokinin–auxin combinations. Chen and Chang (2006) reported 0.03 mg l^{-1} TDZ combined with 0.01 mg l^{-1} NAA was effective on PLBs induction from leaf explants of *Phalaenopsis amabilis*. PLBs were developed from young leaf segments of *P. amabilis* on media supplemented with auxins and cytokinins alone or in combination, and 3 mg l^{-1} TDZ was the most effective (Khoddamzadeh et al. 2011). TDZ was considered as a more potent cytokinin for the direct induction of PLBs in many orchids. For example, the best responses were also obtained from seed-derived protocorms of *P. amabilis* var. *Formosa* Shimadze at 13.62 μM TDZ (Chen and Chang 2004b) and shoot tips of *D. chrysotoxum* at 1 μM TDZ (Roy et al. 2007). Here, we showed that cytokinins (BA and TDZ) improved the direct formation of PLBs in *B. striata*, but the best response was achieved at 0.5 mg l^{-1} BA. Meanwhile, auxins (NAA and 2,4-D) alone could not induce PLBs from protocorms and shoot tips of *B. striata* directly.

Ex vitro establishment of plantlets

More than 90 % of the regenerated plantlets survived and resumed growth after 3–4 weeks in greenhouse. There were no variations of morphological and growth characteristics among the potted regenerants.

Fidelity analysis of regenerants using ISSR markers

Out of the 100 ISSR primers that were initially screened, 42 primers which produced distinct, consistently reproducible and well-resolved banding patterns were used for subsequent analysis of fidelity. A total of 406 bands ranging from 200 to 2,000 bp in size were amplified from the regenerated plantlets and their mother plants using the 42 primers. The number of bands scored in each primer varied from 4 (UBC836, UBC844 and UBC856) to 21 (UBC891 and UBC899) with an average of 9.67 bands per primer.

As amplification bands of UBC834 showed in Fig. 2a, all banding profiles of the 18 plantlets regenerated from one protocorm through direct formation of PLBs, regardless of whether they were directly regenerated from the mother protocorm or regenerated after 12 months of subculture, were monomorphic and similar to those of their mother plant. With respect to the regeneration protocols involving callus-mediated formation of PLBs, no polymorphism was detected among the plantlets regenerated from callus without subculture compared with their mother plants (Fig. 2b, c). Similarly, no polymorphic band existed in nine plantlets which regenerated from callus with 12 months of subculture on the PGR-free medium. However, two polymorphic bands were observed among the plantlets obtained from callus subcultured with the combination of 1.0 mg l^{-1} 2,4-D and 1.0 mg l^{-1} TDZ for 12 months. The polymorphisms included the presence of a novel band about 400 bp generated by primer UBC818 in regenerant plantlet no. 12 (Fig. 2b) and the absence of a fragment about 600 bp produced by primer UBC888 in regenerant plantlet no. 15 (Fig. 2c). Analysis of similarity coefficient values revealed similarity ranges of 99.8–100.0 % between the regenerants and their mother plants and 99.5–100.0 % among the regenerants, which suggested the conditions for in vitro propagation of *B. striata* applied in this work provided relatively high genetic stability of the species upon the callus-mediated and direct PLBs formation.

Somaclonal variation is influenced by a range of factors such as the regeneration protocol, genotype, type of explant, type and concentration of PGRs, and number and duration of subculture (Bairu et al. 2011; Neelakandan and Wang 2012). Generally, the more the structure of plant is disrupted, the greater the chance of mutations occurred (Cooper et al. 2006). It is obvious from the results that all the regenerants obtained through the direct formation of

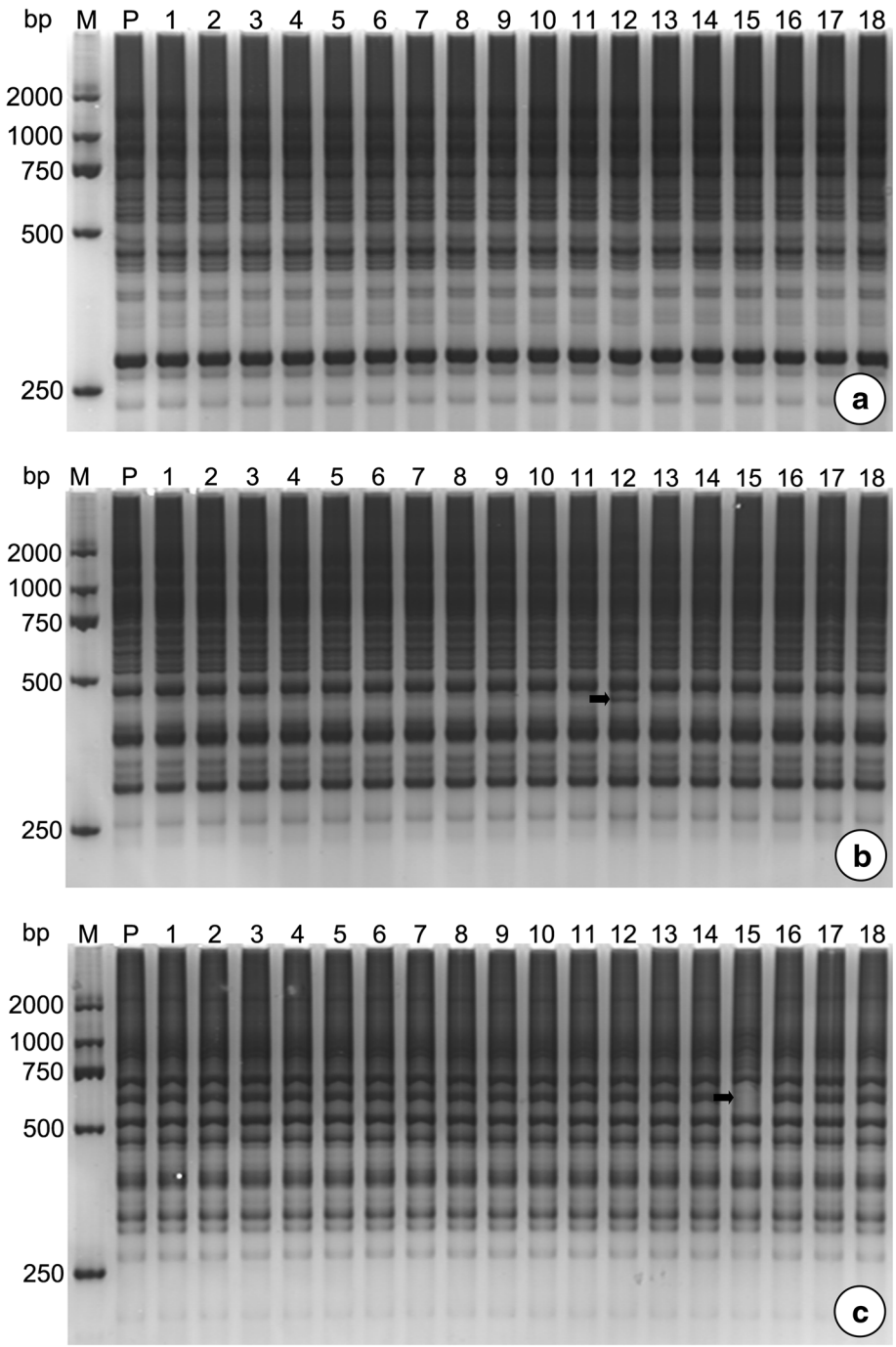

Fig. 2 ISSR profiles of the regenerants (*lanes 1–18*) and their mother plants (P). **a** UBC834, 18 plantlets produced from one mother protocorm by direct formation of PLBs; plantlets no. *1–9* were directly formed from the mother protocorm, and plantlets no. *10–18* were regenerated after 12 months of subculture with 0.5 mg l^{-1} BA. **b** (UBC818) and **c** (UBC888), 18 plantlets produced from one mother protocorm through induction of callus with 1.0 mg l^{-1} 2,4-D and regeneration of PLBs with 1.0 mg l^{-1} BA; plantlets no. *1–9* regenerated from callus without subculture, and plantlets no. *10–18* from callus subcultured with the combination of 1.0 mg l^{-1} 2,4-D and 1.0 mg l^{-1} TDZ for 12 months. Polymorphisms included the presence of a novel band (**b**, *lane 12*, *black arrow*) and the absence of an original band (**c**, *lane 15*, *black arrow*). M, DL 2000 marker

PLBs, regardless of whether they were directly regenerated from the mother protocorm or regenerated after 12 months of subculture, were genetically stable and absolutely true-to-type to the mother plant. On the other hand, a low frequency of somaclonal variations occurred in the plantlets regenerated from the callus subcultured with the combination of 1.0 mg l^{-1} 2,4-D and 1.0 mg l^{-1} TDZ for 12 months. Similar results were observed in plantlets of *Aloe vera* L. (Rathore et al. 2011) and garlic (Al-Zahim et al. 1999) regenerated from callus. These variations are not desirable for generating true-to-type plants in commercial micropropagation, but can be exploited in germplasm improvement of the species. To be specially mentioned, the present study demonstrated that the plantlets regenerated from callus with 12 months of subculture on the PGR-free medium were absolutely true-to-type clones. The result firstly at molecular level supports the view proposed by Roy et al. (2007), i.e., in vitro culture without PGRs can reduce the probability of somaclonal variation in micropropagation of orchids.

Conclusion

This is the first report describing PLB regeneration through callus-mediated and direct formation in *B. striata*. Efficient protocols have been established to obtain large numbers of true-to-type plantlets within a short period. For the callus-mediated formation of PLBs, callus could be initiated most efficiently with 1.0 mg l^{-1} 2,4-D and proliferated fastest (46.2-fold) with the combination of 1.0 mg l^{-1} 2,4-D and 1.0 mg l^{-1} TDZ within 3 months. Averagely 48.4 PLBs were produced from 100 mg calli in the presence of 1.0 mg l^{-1} BA. The callus also maintained growth and differentiation potential in the absence of PGRs, and the plantlets regenerated from callus subcultured without PGRs for 12 months showed no detectable somaclonal variation. Direct formation of PLBs without detectable somaclonal variation was also induced from explants, and a mean number of 30.2 PLBs was produced from one protocorm in the presence of 0.5 mg l^{-1} BA within 3 months. These protocols not only could be used for accelerated propagation of this ornamentally and medically important plant and subsequent conservation of its natural resources, but also is potentially useful for long-term in vitro germplasm preserving and genetic transformation of the species.

Author contribution C. X. Wang designed and carried out the experiments, and wrote the manuscript. M. Tian helped in data collection and statistical analysis.

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