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ORIGINAL ARTICLE

Plant regeneration via direct and indirect adventitious shoot formation and chromosome-doubled somaclonal variation in *Titanotrichum oldhamii* (Hemsl.) Solereder

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Abstract The gesneriaceous perennial plant *Titanotrichum oldhamii* has beautiful foliage and attractive bright yellow flowers. However, breeding of *T. oldhamii* by conventional sexual hybridization may be difficult because sexual reproduction of this species is very rare. In the present study, plant regeneration systems via both direct and indirect formation of adventitious shoots from leaf explants were established as the first step toward breeding *T. oldhamii* by using biotechnological techniques. Adventitious shoots were formed efficiently on medium containing 0.1 mg l^{-1} benzyladenine. Histological observation showed that shoot formation on this medium occurred directly from leaf epidermal cells without callus formation. On the other hand, leaf explants formed calluses on medium containing 0.1 mg l^{-1} 2,4-dichlorophenoxyacetic acid. The calluses could be maintained by monthly subculturing to fresh medium of the same composition. When the calluses were transferred to plant growth regulator-free medium, they formed adventitious shoots. Directly and indirectly formed shoots rooted well on medium containing 0.1 mg l^{-1} indole-3-butyric acid. Plantlets thus obtained were successfully acclimatized and grew vigorously in the greenhouse. Flow cytometry analysis indicated that no variation in the ploidy level was observed in plants regenerated via direct shoot formation, whereas chromosome doubling occurred in several plants regenerated via indirect shoot formation. Regenerated plants with the same ploidy level as the mother plants showed almost the same phenotype as the mother plants, whereas chromosome-doubled plants showed apparent morphological alterations: they had small and crispate flowers, and round and deep green leaves.

Keywords Flow cytometry · Gesneriaceous plant · Histological observation · Plant regeneration · Ploidy level · Somaclonal variation

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Introduction

The gesneriaceous perennial plant *Titanotrichum oldhamii* is a monotypic species distributed in Taiwan, adjacent regions of China and the Iriomote Islands of Japan (Li and Kao 1998; Wang et al. 1998). In 1864, Richard Oldham first discovered this species in Taiwan during his collecting trip for the Royal Botanic Gardens, Kew. Since then, this species has been cultivated in Europe as an ornamental plant for its beautiful foliage and ability to grow in the shade (Wang and Cronk 2003). In addition, *T. oldhamii* has attractive flowers, bright yellow in color, which is unique among gesneriaceous plants. *T. oldhamii* has recently become popular in Japan for pot use.

In *T. oldhamii*, there appear to be few variations in horticultural traits, such as plant form, flower color and shape, and it is desirable to widen the variability. However, sexual reproduction of *T. oldhamii* is very rare and plants are generally propagated by vegetative bulbils (Wang et al.

2004), and thus breeding of this species by conventional sexual hybridization may be difficult. Biotechnological techniques, such as induction and selection of somaclonal variation, in vitro chromosome doubling and genetic transformation, offer alternative ways to widen the variability of *T. oldhamii*. In order to apply these biotechnological techniques, it is a prerequisite to develop an efficient plant regeneration system from cultured explants. Although efficient regeneration systems have already been established for several gesneriaceous species such as *Streptocarpus nobilis* (Handro 1983), *Kohleria* sp. (Geier 1988), *Saintpaulia ionantha* (Jungnickel and Zaid 1992; Lo 1997; Mithila et al. 2003; Mølgaard et al. 1991), *Sinningia speciosa* (Scaramuzzi et al. 1999), *Aeschynanthus radicans* (Jin et al. 2009) and *Chirita* spp. (Nakano et al. 2009), there have been no reports of in vitro plant regeneration in *T. oldhamii*.

In the present study, we examined the establishment of an efficient plant regeneration system in *T. oldhamii* as the first step toward breeding this species by using biotechnological techniques. Histological observation of direct and indirect shoot organogenesis from cultured leaf explants and flow cytometry (FCM) analysis of the ploidy level of regenerated plants were also carried out.

Materials and methods

Plant material and adventitious shoot induction

Potted plants of *T. oldhamii* were cultivated in the greenhouse without heating and used as plant material. Fully expanding leaves (5–10 cm in length) were harvested 2–3 months before anthesis. They were washed in running water for 1 min, surface-sterilized with a sodium hypochlorite solution containing 1% active chlorine for 10 min, rinsed 3 times with sterile, distilled water, and then cut into ca. 10 × 10 mm segments. Leaf explants were cultured on half-strength MS medium (Murashige and Skoog 1962) lacking plant growth regulators (PGRs), or containing 0.1 or 1 mg l^{-1} of α-naphthaleneacetic acid (NAA) or 2,4-dichlorophenoxyacetic acid (2,4-D) alone or in combination with 0.1 or 1 mg l^{-1} benzyladenine (BA), to examine the effect of PGRs on adventitious shoot induction (Table 1). All media used in the present study were supplemented with 30 g l^{-1} sucrose and 2 g l^{-1} gellan gum, and adjusted to pH 5.8 before autoclaving at 121°C under a pressure of 1.2 kg cm^{-2} for 15 min. Plastic Petri dishes (9 cm diameter) were filled with 30 ml of each medium, and 10 explants were cultured per dish. Cultures were incubated

Table 1 Effect of PGRs on formation of adventitious shoots, adventitious roots and calluses from leaf explants of *Titanotrichum oldhamii* 1 month after culture initiation

PGRs (mg l^{-1})			% of explants forming shoots[a]	No. of shoots per explant[a]	% of explants forming roots[a]	Degree of root formation[b]	% of explants forming calluses[a]	Degree of callus formation[c]
BA	NAA	2,4-D						
0	0	0	70.0 ± 17.0 ab	3.4 ± 1.0 c	6.6 ± 5.4 d	+	0 b	−
0	0.1	0	70.0 ± 17.0 ab	6.2 ± 2.6 bc	100 a	++	0 b	−
0	1	0	0 c	0 c	96.7 ± 2.7 a	+++	0 b	−
0	0	0.1	0 c	0 c	0 d	−	100 a	+++
0	0	1	0 c	0 c	0 d	−	96.7 ± 2.7 a	++
0.1	0	0	100 a	15.6 ± 0.7 b	43.3 ± 21.2 bc	+	0 b	−
0.1	0.1	0	90.0 ± 8.2 a	11.5 ± 2.6 b	56.7 ± 23.2 b	+	0 b	−
0.1	1	0	3.3 ± 2.7 c	0.1 ± 0.1 a	30.0 ± 14.1 c	+	0 b	−
0.1	0	0.1	0 c	0 c	0 d	−	6.7 ± 2.7 b	+
0.1	0	1	0 c	0 c	0 d	−	16.7 ± 7.2 b	+
1	0	0	93.3 ± 5.4 a	32.1 ± 3.4 a	3.3 ± 2.7 d	+	0 b	−
1	0.1	0	40.0 ± 4.7 b	3.1 ± 1.5 c	0 d	−	0 b	−
1	1	0	16.7 ± 9.8 bc	0.3 ± 0.2 c	0 d	−	0 b	−
1	0	0.1	0 c	0 c	0 d	−	3.3 ± 2.7 b	+
1	0	1	0 c	0 c	0 d	−	0 b	−

[a] Values represent the mean ± SE of 3 independent experiments each of which consisted of 10 explants. Different letters among the means in the same column indicate significant differences at the 0.05 level with LSD test

[b] Degree of root formation was expressed as follows: − no roots, + 1–20 roots per explants, ++ 20–50 roots per explants, +++ more than 50 roots per explant

[c] Degree of callus formation was expressed as follows: − no calluses, + small calluses were formed on the edge of the explant, ++ nearly half of the explant surface was covered with calluses, +++ explant surface were nearly completely covered with calluses

Table 2 Effect of BA on adventitious shoot formation from calluses of *Titanotrichum oldhamii*

BA (mg l^{-1})	% of calluses forming shoots	No. of shoots per 1 g FW of calluses
0	100 a	71.3 ± 29.5 a
0.1	100 a	16.0 ± 4.1 b
1	100 a	9.3 ± 1.5 b

Data were recorded 2 months after transfer of calluses from medium containing 0.1 mg l^{-1} 2,4-D to shoot induction medium. Values represent the mean ± SE of 3 independent experiments each of which consisted of 1 g FW of calluses. Different letters among the means in the same column indicate significant differences at the 0.05 level with LSD test

Table 3 Effect of IBA on rooting of adventitious shoots of *Titanotrichum oldhamii*

IBA (mg l^{-1})	% of shoots forming roots[a]	Degree of root formation[b]
0	90.0 ± 4.7 a	++
0.1	96.6 ± 2.7 b	+++
1	83.3 ± 7.2 a	++

Adventitious shoots more than 10 mm in length, that were regenerated directly from leaf explants cultured on medium containing 0.1 mg l^{-1} BA, were used. Data were recorded 1 month after transfer of shoots to rooting medium

[a] Values represent the mean ± SE of 3 independent experiments each of which consisted of 10 shoots. Different letters among the means indicate significant differences at the 0.05 level with LSD test

[b] Degree of root formation was expressed as follows: − no roots, + 1–5 roots per shoot, ++ 6–10 roots per shoot, +++ more than 11 roots per shoot

at 25°C under continuous illumination with fluorescent lighting (50 μmol m^{-2} s^{-1}). Data on formation of adventitious shoots, adventitious roots and calluses from leaf explants were recorded 1 month after culture initiation.

Primary calluses formed on medium containing 0.1 mg l^{-1} 2,4-D were subcultured monthly to fresh medium of the same composition under the same conditions. After 3 subcultures, calluses were transferred to half-strength MS medium lacking PGRs, or containing 0.1 or 1 mg l^{-1} BA, to examine the effect of BA on adventitious shoot induction from calluses (Table 2). Plastic Petri dishes (9 cm diameter) were filled with 30 ml of each medium, and 1 g fresh weight (FW) of calluses was cultured per dish. Cultures were incubated at 25°C under continuous illumination with fluorescent lighting (50 μmol m^{-2} s^{-1}). Data on formation of adventitious shoots and adventitious roots from calluses were recorded 2 months after transfer of calluses.

Plantlet regeneration and acclimatization

Adventitious shoots more than 10 mm in length, which were obtained from leaf explants cultured on medium containing 0.1 mg l^{-1} BA for 2 months, were excised from the explants and transferred to half-strength MS medium lacking PGRs, or containing 0.1 or 1 mg l^{-1} indole-3-butyric acid (IBA) to examine the effect of IBA on rooting of shoots (Table 3). For culture, test tubes (2.5 × 13 cm) containing 10 ml of each medium and capped with aluminum foil were used, and 1 shoot was cultured per tube. Cultures were incubated at 25°C under continuous illumination with fluorescent lighting (50 μmol m^{-2} s^{-1}). Data on rooting of shoots were recorded 1 month after transfer of shoots.

Regenerated plantlets with a well-established root system were washed carefully with tap water to remove gellan gum and transferred to pots containing moist vermiculite. They were acclimatized in a transparent plastic cabinet covered with a polyethylene sheet at 25°C under continuous illumination with fluorescent lighting (50 μmol m^{-2} s^{-1}). Small holes were punched in the sheet after 2 weeks, and then the sheet was gradually removed in a stepwise fashion. Acclimatized plants were then transplanted to pots containing soil and cultivated in the greenhouse under the same conditions as the mother plants.

Histological observation

Leaf explants cultured on medium containing 0.1 mg l^{-1} BA were sampled every 5 days after culture initiation. Leaf-derived calluses were also sampled 1 month after transfer to PGR-free medium. They were fixed for 24 h in FAA (70% ethanol:formalin:acetic acid = 90:5:5; v/v/v), dehydrated in a graded ethanol series, and embedded in paraffin (58°C) according to Nakano et al. (2000b). Sections (10 μm thick) were stained with Delafield's haematoxylin. Then, sections were observed under a light microscope.

FCM analysis and pollen viability

Ploidy level of regenerated plants was determined by measuring relative DNA content of isolated nuclei using a flow cytometer (Partec PA, Münster, Germany) as previously described (Saito et al. 2003). Pollen viability was evaluated by staining pollen grains with 1% (w/v) aceto-carmine and described as the percentage of pollen grains with deeply stained cytoplasm (Nakano and Mii 1993).

Results

Formation of adventitious shoots, adventitious roots and calluses from leaf explants

One month after culture initiation, leaf explants showed various responses to PGRs as shown in Table 1.

Adventitious shoots started to appear mainly on the leaf veins and at the cut end of leaf explants within 20 days of culture. On medium containing 0.1 or 1 mg l^{-1} BA alone, or containing 0.1 mg l^{-1} of both NAA and BA, 90% and more of leaf explants formed adventitious shoots. Although the maximum number of shoots per explant (32.1) was obtained on medium containing 1 mg l^{-1} BA alone, most shoots on this medium showed little elongation even after 2 months of culture initiation. Such small shoots were difficult to excise from the explant and prone to die after transfer to rooting medium. On medium containing 0.1 mg l^{-1} BA alone, all the explants formed adventitious shoots and 15.6 shoots were obtained per explant. In addition, shoot elongation occurred efficiently on this medium, and about 50% of the shoots elongated over 10 mm in length after 2 months of culture initiation (Fig. 1a).

Adventitious roots were formed mainly on the adaxial side of leaf explants. On medium containing 0.1 or 1 mg l^{-1} NAA alone, more than 95% of leaf explants formed adventitious roots, and the degree of root formation was very high (Table 1). However, leaf explants cultured on medium containing NAA alone turned brown and died within 2 months after culture initiation.

Calluses were formed only on medium containing 2,4-D alone. On medium containing 0.1 mg l^{-1} 2,4-D alone, all the explants formed calluses, and the degree of callus formation was highest. Leaf explants cultured on this medium swelled within 15 days of culture, and watery and greenish yellow calluses started to appear around the cut end of the explants about 1 month after culture initiation (Fig. 1b). The calluses on medium containing 0.1 mg l^{-1} 2,4-D alone could be maintained by monthly subculturing to fresh medium of the same composition, and about a 1.5-fold increase in callus FW was obtained 1 month after subculture. On medium containing 1 mg l^{-1} 2,4-D alone, watery and dark greenish calluses were formed about 1 month after culture initiation, but these calluses showed little proliferation, turned brown, and ultimately died within 2 months after culture initiation.

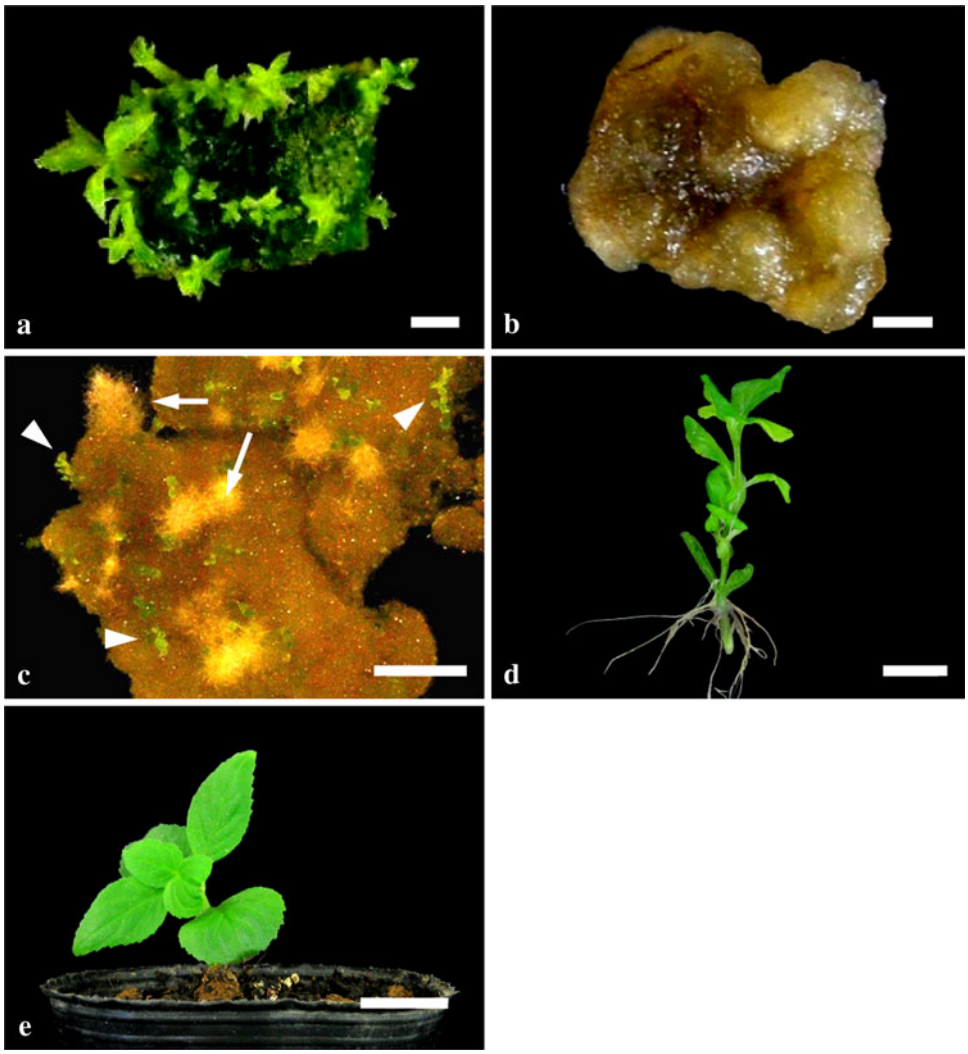

Fig. 1 Adventitious shoot formation and plant regeneration in *Titanotrichum oldhamii*. **a** Adventitious shoot formation from a leaf explant 1 month after culture on medium containing 0.1 mg l^{-1} BA. *Bar* 2 mm. **b** Callus formation from a leaf explant 2 months after culture on medium containing 0.1 mg l^{-1} 2,4-D. *Bar* 2 mm. **c** Formation of adventitious shoots (*arrowheads*) and roots (*arrows*) from leaf-derived calluses 2 months after transfer from medium containing 0.1 mg l^{-1} 2,4-D to PGR-free medium. *Bar* 10 mm. **d** Plantlet with a well-established root system obtained by culturing leaf-derived shoots on medium containing 0.1 mg l^{-1} IBA for 1 month. *Bar* 10 mm. **e** Regenerated plant 5 months after cultivation in the greenhouse. *Bar* 2 cm

Formation of adventitious shoots from calluses

Calluses, which were formed on medium containing 0.1 mg l^{-1} 2,4-D alone and subcultured 3 times, were transferred to PGR-free medium or medium containing 0.1 or 1 mg l^{-1} BA alone. Although adventitious shoots were formed on all media tested, the maximum number of shoots (71.3) per 1 g FW of calluses was obtained on PGR-free medium 2 months after transfer (Table 2). The number of shoots decreased as BA concentration increased. Calluses transferred to PGR-free medium changed from watery and greenish yellow to hard and deep green within 10 days, and adventitious shoots started to appear on the surface of these calluses after 1 month of transfer (Fig. 1c).

Histological observation of adventitious shoot formation from leaf explants and calluses

Figure 2a–d show adventitious shoot formation from leaf explants on medium containing 0.1 mg l^{-1} BA alone. Leaf explants of *T. oldhamii* had a single-layered epidermis (Fig. 2a). Five days after culture initiation, adaxial epidermal cells of the explants exhibited an early stage of cell division (Fig. 2b). Continuous cell division led to groups of small cells with high cytoplasmic content and prominent nuclei (Fig. 2c). At later stages of development, adventitious shoots with leaf primordia were observed (Fig. 2d). Callus formation was never observed throughout the processes of adventitious shoot formation, and shoots were formed directly from leaf epidermal cells.

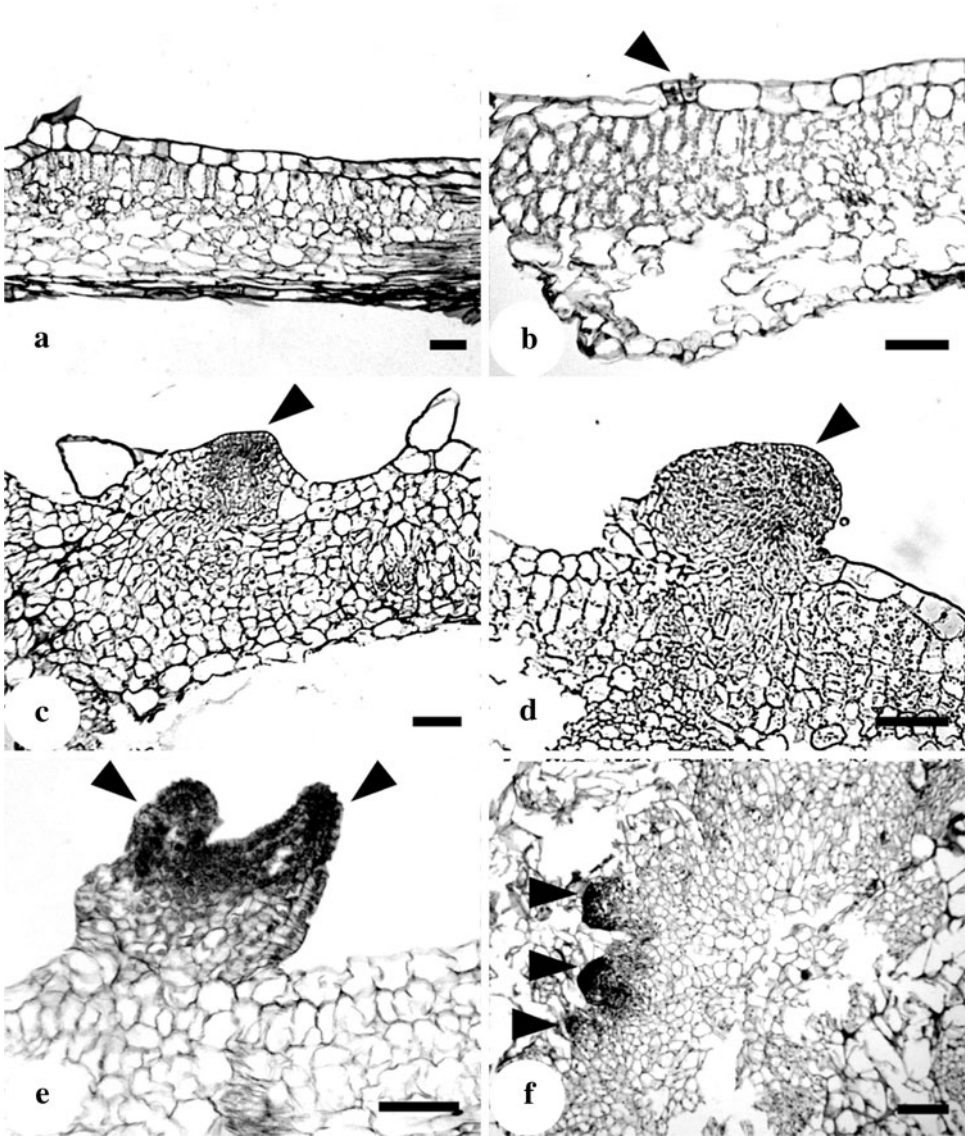

Fig. 2 Histological observation of adventitious shoot formation from leaf explants or calluses of *Titanotrichum oldhamii*. **a** Leaf explant before culture initiation. **b** Cell division in the adaxial leaf epidermis (*arrowhead*) 10 days after culture initiation on medium containing 0.1 mg l^{-1} BA. **c** Cell division to form a meristematic zone (*arrowhead*) 15 days after culture initiation on medium containing 0.1 mg l^{-1} BA. **d** Adventitious shoot primordium (*arrowhead*) formed 20 days after culture initiation on medium containing 0.1 mg l^{-1} BA. **e** Adventitious shoot with leaf primordia (*arrowheads*) formed 25 days after culture initiation on medium containing 0.1 mg l^{-1} BA. **f** Cell division to form meristematic zones (*arrowheads*) in leaf-derived calluses 20 days after transfer from medium containing 0.1 mg l^{-1} 2,4-D to PGR-free medium. *Bars* 100 μm

Figure 2e shows leaf-derived calluses 20 days after their transfer to PGR-free medium. Meristematic regions, composed of small, tightly packed cells, were observed within the calluses. These meristematic regions developed into adventitious buds thereafter.

Plantlet regeneration and acclimatization

When adventitious shoots formed directly from leaf explants were transferred to rooting medium, the highest percentage of shoots forming roots (96%) and the highest degree of root formation were obtained on medium containing 0.1 mg l^{-1} IBA (Table 3). On medium containing 1 mg l^{-1} IBA, not only roots but also calluses were formed at the cut end of adventitious shoots. Most of leaf-derived adventitious shoots developed into plantlets with a well-established root system on medium containing 0.1 mg l^{-1} IBA (Fig. 1d). However, many plantlets died during acclimatization, and only about 50% of the plantlets could be transplanted to the greenhouse. A total of 24 plants regenerated via direct shoot formation from leaf explants on a medium containing 0.1 mg l^{-1} BA alone were cultivated in the greenhouse.

Adventitious shoots that were formed from leaf-derived calluses on PGR-free medium also efficiently developed into plantlets on medium containing 0.1 mg l^{-1} IBA. However, only about 50% of these plantlets could be acclimatized and transplanted to the greenhouse as in the case of plantlets regenerated via direct shoot formation. A total of 20 plants regenerated via indirect shoot formation from calluses on PGR-free medium were cultivated in the greenhouse.

FCM analysis of regenerated plants

Regenerated plants cultivated in the greenhouse were subjected to FCM analysis in order to determine their ploidy level (Fig. 3). All the 24 plants regenerated via direct shoot formation showed the same ploidy level as the mother plants: their FCM histograms had a single 2C peak (Fig. 3b) as those of the mother plants (Fig. 3a). On the other hand, chromosome doubling occurred in 4 out of 20 plants regenerated via indirect shoot formation: FCM histograms of these 4 plants had a single peak corresponding to 4C DNA contents (Fig. 3c). The remaining 16 plants showed the same ploidy level as the mother plants.

Morphological characterization and pollen viability of regenerated plants

Plants regenerated via both direct and indirect shoot formation grew well in the greenhouse, although chromosome-doubled plants showed somewhat slow growth. Two years after acclimatization, all the regenerated plants except for 2 out of 4 chromosome-doubled plants produced flowers. Preliminary characterization of the regenerated plants showed that all the 24 plants regenerated via direct

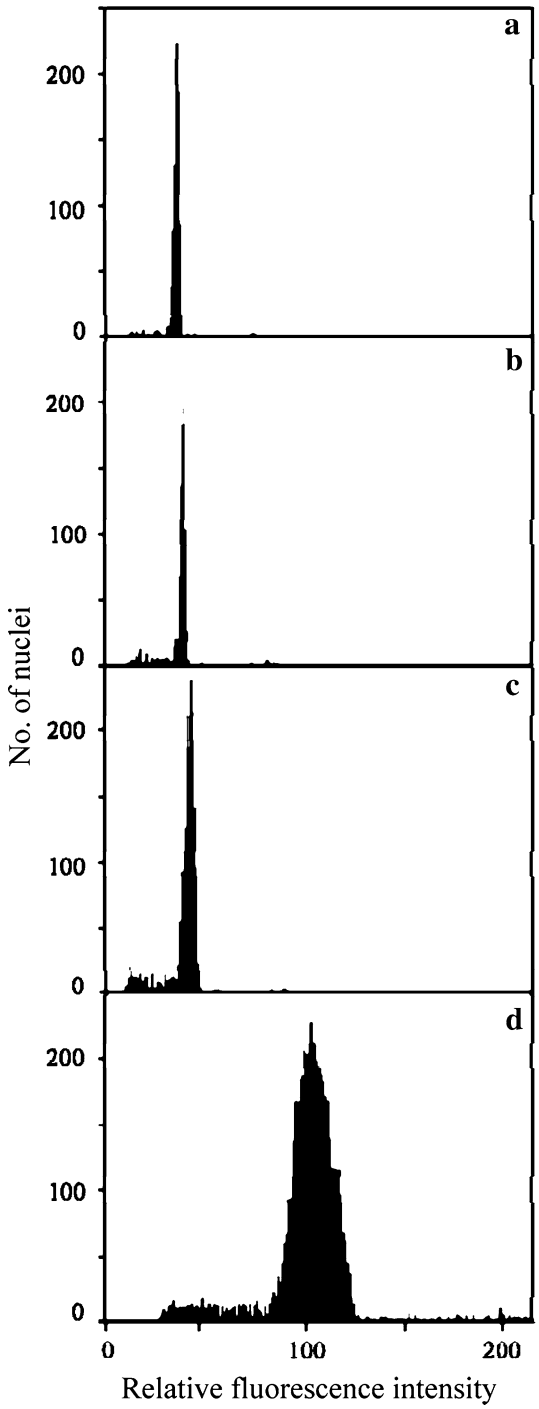

Fig. 3 Histograms from flow cytometry analysis of nuclear DNA content of *Titanotrichum oldhamii* plants. **a** Leaves of the donor plant. **b** Leaves of a plant regenerated via direct shoot formation without chromosome doubling. **c** Leaves of a plant regenerated from leaf-derived calluses without chromosome doubling. **d** Leaves of a chromosome-doubled plant regenerated from leaf-derived calluses

Fig. 4 Flowers (**a**–**d**) and leaves (**e**–**h**) of regenerated plants of *Titanotrichum oldhamii*. The most basal flowers in the inflorescence and leaves just below the bract were harvested. **a**, **e** Mother plant. **b**, **f** Plant regenerated via direct shoot formation without chromosome doubling. **c**, **g** Plant regenerated via indirect shoot formation from leaf-derived calluses without chromosome doubling. **d**, **h** Chromosome-doubled plant regenerated via indirect shoot formation from leaf-derived calluses. *Bar* 1 cm (**a**–**d**), 5 cm (**e**–**h**)

shoot formation showed almost the same phenotype as the mother plants with respect to flower and leaf morphology (Fig. 4b, f). Among the plants regenerated via indirect shoot formation, those with the same ploidy level as the mother plants also showed almost the same phenotype as the mother plants (Fig. 4c, g). However, chromosome-doubled plants showed apparent morphological alterations: they had small and crispate flowers, and round and deep green leaves compared with the mother plants (Fig. 4d, h).

Pollen viability of over 90% was obtained for mother plants and regenerated plants without chromosome doubling. On the other hand, chromosome-doubled plants showed the pollen viability of about 60%, and their pollen grains were slightly larger than those of the mother plants (data not shown).

Discussion

Type and concentration of PGRs were critical factors influencing organogenesis and callus formation from leaf explants of *T. oldhamii*. For inducing adventitious shoots from leaf explants, medium containing 1 mg l^{-1} BA alone was most effective. However, this concentration of BA inhibited shoot elongation, and adventitious shoots elongating over 10 mm in length, which were easy to handle and suitable for transferring to rooting medium, were obtained most efficiently by a lower concentration (0.1 mg l^{-1}) of BA. Similar results have also been reported for *Begonia* spp. (Simmonds and Werry 1987; Nakano et al. 1999) and *Chirita* spp. (Nakano et al. 2009). On the other hand, 2 types of auxin-like PGRs differently affected the development of leaf explants of *T. oldhamii*: NAA promoted adventitious root formation but had no promotive effect on callus induction, whereas 2,4-D promoted callus formation but inhibited adventitious root formation. Similar observation has also been reported for cotyledon segment culture of *Brassica* spp. (Murata and Orton 1987).

Leaf-derived calluses of *T. oldhamii* subcultured at least 3 times on medium containing 0.1 mg l^{-1} 2,4-D could produce adventitious shoots after transfer to shoot induction medium. However, a decline in the regeneration potential of cell and callus cultures with increasing age of the cultures has been demonstrated for a wide range of plant species (Vasil and Vasil 1985; Nakano et al. 2000a). Therefore, further studies are needed to examine the regeneration potential of the callus cultures established in the present study after long-term subcultures.

Histological observation in the present study showed that adventitious shoots were formed directly from leaf epidermal cells without an intervening callus phase on medium containing 0.1 mg l^{-1} BA. Direct formation of adventitious shoots has also been reported for other gesneriaceous spices including *Saintpaulia ionantha* (Ohki

1994), *Saintpaulia ionantha* × *confuse* hybrid (Lo et al. 1997) and *Chirita* spp. (Nakano et al. 2009). It has generally been reported that direct regeneration from explants results in plants genetically more stable than indirect regeneration from calluses (Lee and Phillips 1988; Małgorzata 2001). Similar results were obtained in the present study: no variation in the ploidy level was observed in any of the 24 plants regenerated via direct shoot formation, whereas chromosome doubling occurred in 4 out of 20 plants regenerated via indirect shoot formation.

For several plant species, somaclonal variation including chromosome doubling has often been detected in plants indirectly regenerated from cell or callus cultures, especially when long-term cultures were used (Kaeppler et al. 2000; Nakano et al. 2006; Mohanty et al. 2008; Jin et al. 2008). Although somaclonal variation is not acceptable for micropropagation, ex situ conservation of plant diversity and genetic transformation, it may be of value as a tool of plant breeding (Larkin and Scowcroft 1981; Karp 1995; Nakano et al. 2006). In the present study, 4 chromosome-doubled somaclonal variants were obtained via indirect shoot formation from calluses. These chromosome-doubled plants grew slowly compared with non-chromosome-doubled plants and produced small and crispate flowers. Wang et al. (2004) suggested a possibility of *T. oldhamii* being polyploid by nature since this species is bulbiliferous and has a high chromosome number ($2n = 40$) for Gesneriaceae. Thus, high polyploidy may be responsible for a reduced growth in the chromosome-doubled somaclonal variants. Although the chromosome-doubled *T. oldhamii* has poor ornamental value, horticulturally attractive variants may be obtained by mutation treatments of the callus cultures as in the case of *Tricyrtis hirta* (Nakano et al. 2006).

In conclusion, we have established plant regeneration systems via both direct and indirect adventitious shoot formation from leaf explants of *T. oldhamii*. The regeneration system via direct shoot formation consisted of the following tissue culture steps: (1) leaf explants were cultured for adventitious shoot formation on half-strength MS medium containing 0.1 mg l^{-1} BA for 1–2 months; and (2) adventitious shoots elongating over 10 mm in length were transferred for rooting to half-strength MS medium containing 0.1 mg l^{-1} IBA and cultured for 1 month. This system may be favorable for genetic transformation and micropropagation of valuable strains in *T. oldhamii*, since direct regeneration may suppress the occurrence of somaclonal variation. On the other hand, the regeneration system via indirect shoot formation consisted of the following steps: (1) leaf explants were cultured for callus formation on half-strength MS medium containing 0.1 mg l^{-1} 2,4-D for 2 months; (2) calluses were transferred for adventitious shoot formation to half-strength MS medium without PGRs and cultured for 2 months; and (3) adventitious shoots elongating over 10 mm in length were transferred for rooting to half-strength MS medium containing 0.1 mg l^{-1} IBA and cultured for 1 month. This system may be favorable for inducing horticulturally valuable somaclonal variants in *T. oldhamii*. For both regeneration systems, only about 50% of regenerated plantlets could be acclimatized, and, therefore, further experiments are necessary to increase the survival rate of *T. oldhamii* plantlets during acclimatization.

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