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Note

Horticultural characterization of a tetraploid transgenic plant of *Tricyrtis* sp. carrying the gibberellin 2-oxidase gene

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Abstract The gibberellin 2-oxidase catalyzes the bioactive gibberellins or their immediate precursors to inactive forms. We have previously produced transgenic plants of the liliaceous plant *Tricyrtis* sp. containing the GA2ox gene from the linderniaceous plant *Torenia fournieri*. These transgenic plants showed dwarf phenotypes as expected but unfortunately had no flowers or only small, unopened flowers. Recently, one newly produced transgenic line (G2-55) formed fully opened flowers. G2-55 showed a moderately dwarf phenotype and the shoot length decreased to 63.4% of that of the control, non-transgenic plants. No significant differences in the number of flowers per shoot and in the flower size were observed between G2-55 and the control. Flow cytometry analysis and chromosome observation showed that G2-55 was tetraploid ($2n=4x=52$), whereas the other transgenic lines producing no or only small flowers were diploid ($2n=2x=26$) as the mother plant. Pollen fertility of G2-55 was 81.2% as determined by acetocarmine staining. The tetraploidy in G2-55 might be resulted from somaclonal variation of embryogenic calluses used as a target material for *Agrobacterium*-mediated transformation. The tetraploid transgenic plant G2-55 may be useable not only directly as a potted plant, but also as a material for further breeding of *Tricyrtis* spp.

Key words: Dwarf phenotype, GA metabolic pathway, Genetic transformation, Liliaceous ornamental plant, Somaclonal variation.

In ornamental plants, dwarfness or semi-dwarfness is one of the most important traits for pot or garden uses. Most of the dwarf and semi-dwarf cultivars of ornamental plants have so far been produced by selecting natural variations. Dwarf ornamental plants have recently been produced by genetic transformation with wild-type strains of *Agrobacterium rhizogenes* (Choi et al. 2004; Godo et al. 1997; Hoshino and Mii 1998; Koike et al. 2003; Mishiba et al. 2006) or by transformation with the *rol* genes of *A. rhizogenes* (Christensen et al. 2008; van der Salm et al. 1997; Winefield et al. 1999; Zuker et al. 2001). However, these transgenic plants exhibited undesirable morphologies known as the hairy root syndrome (Spena et al. 1987) in addition to dwarfness.

Gibberellins (GAs) play a key role in the regulation of many aspects of plant growth and development including internode elongation, leaf expansion and transition from vegetative growth to flowering (Koornneef and van der Veen 1980; Sun and Kamiya 1994; Wilson et al. 1992). In higher plants, GAs are catalyzed and metabolized by several different GA oxidases, which control the endogenous level of bioactive GAs (Hedden and Kamiya 1997; Hedden and Phillips 2000; Lange 1998). Among these enzymes, gibberellin 2-oxidase (GA2ox) functions to metabolize bioactive GAs and their immediate precursors to inactive GAs. In *Pisum sativum*, a mutant for the *SLENDER* gene, which encodes GA2ox, exhibited increased plant heights (Martin et al. 1999). On the other hand, overexpression of the GA2ox gene induced dwarf phenotypes in transgenic *Arabidopsis thaliana*, *Nicotiana tabacum* (Schomburg et al. 2003), *Torenia fournieri* (Niki et al. 2006), *Solanum* spp. (Dijkstra et

Abbreviations: CaMV, cauliflower mosaic virus; FCM, flow cytometry; GA, gibberellin; GA2ox, gibberellin 2-oxidase; HPT, hygromycin phosphotransferase; NOS, nopaline synthase; NPTII, neomycin phosphotransferase II; PCR, polymerase chain reaction; SEM, scanning electron microscope; SPAD, soil and plant analyzer development.

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al. 2008), *Brassica napus* (Zhou et al. 2012), *Kalanchoe blossfeldiana* and *Petunia hybrida* (Gargul et al. 2013). Thus genetic transformation with the GA2ox gene may be an alternative approach to transformation with wild-type *A. rhizogenes* or the *rol* genes for producing dwarf ornamental plants.

In a previous study (Otani et al. 2013), we examined genetic transformation of the liliaceous ornamental plant *Tricyrtis* sp. with the GA2ox gene from the linderniaceous ornamental plant *Torenia fournieri* (*TfGA2ox2*) in order to induce dwarf phenotypes, and produced six transgenic plants overexpressing *TfGA2ox2*. Although all the six transgenic plants showed dwarf phenotypes as expected, they had unfortunately no flowers or only small, unopened flowers. After that, we newly produced several transgenic *Tricyrtis* sp. plants containing the same gene, among which one line formed fully-opened flowers. In the present study, we perform ploidy level analysis and horticultural characterization of this transgenic line.

Tricyrtis sp. 'Shinonome' ($2n=2x=26$) was used in the present study. Co-cultivation of embryogenic calli with *Agrobacterium tumefaciens* strain EHA101/pIG-*TfGA2ox2*, selection of transgenic cells and tissues, and regeneration of transgenic plants were performed as previously described (Otani et al. 2013). The T-DNA region of the binary vector pIG-*TfGA2ox2* contained *TfGA2ox2* (AB610639 in the GenBank/EMBL/DDBJ databases) under the control of the cauliflower mosaic virus (CaMV) 35S promoter, the neomycin phosphotransferase II (NPTII) gene under the control of the nopaline synthase (NOS) promoter, and the hygromycin phosphotransferase (HPT) gene under the control of the CaMV 35S promoter. Five independent hygromycin-resistant plantlets were obtained in the present study. Gene-specific polymerase chain reaction (PCR) analysis using the HPT gene primer set (Otani et al. 2013) showed all the five hygromycin-resistant plantlets to contain the expected insert of 290 bp (Figure 1). Morphological characterization of five transgenic plants was carried out three years after cultivation in pots during the flowering season (Table 1; Figure 2). Shoot length, number of nodes per shoot and stem diameter of the longest shoot, mean leaf length and width of randomly selected 3 expanded leaves, total number of flowers per plant, and mean flower length and diameter of randomly selected 3 flowers were recorded. Mean SPAD value of randomly selected 3 expanded leaves was measured using a chlorophyll meter (SPAD-502; Fujiwara Scientific Co., Tokyo, Japan) according to Koike et al. (2003). All the five transgenic plants exhibited dwarf phenotypes, and their shoot length and internode length significantly decreased to 6.5–63.4% and 7.7–57.7% of those of cutting-derived non-transgenic plants (control), respectively. Transgenic lines could be classified into the following three types according to their morphologies (Figure 2a). Type I transgenic lines (G2-21 and G2-22) were severely dwarf (shoots were less than 3.0 cm in length) and produced no visible flower buds. Type II transgenic lines (G2-32 and G2-34) were moderately dwarf (shoots were more than 5.0 cm in length) and produced only small, unopened flowers. Type III transgenic line (G2-55) also showed a moderately dwarf phenotype but produced

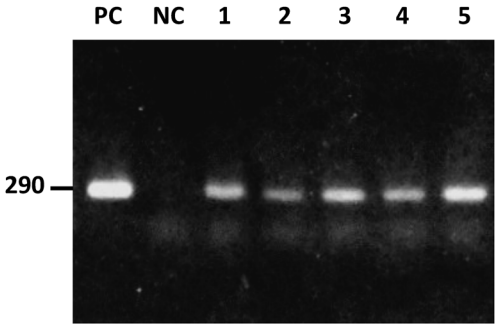

Figure 1. PCR analysis for detecting the HPT gene in hygromycin-resistant *Tricyrtis* sp.plantlets. Lane PC, binary plasmid pIG121Hm as a positive control; lane NC, non-transgenic plant as a negative control; lane 1, G2-21; lane 2, G2-22; lane 3, G2-32; lane 4, G2-34; lane 5, G2-55. Numeral on the left indicates the molecular size in bp.

Table 1. Morphological characteristics of transgenic *Tricyrtis* sp. containing *TfGA2ox2* investigated during the flowering season.[1]

Plant strain	Shoot length (cm)[2]	No. of nodes per shoot[2]	Internode length (mm)[3]	Stem diameter (cm)[4]	Leaf length (cm)[5]	Leaf width (cm)[5]	Leaf length/ width[5]	Leaf SPAD value[5]	No. of flowers per plant	Flower diameter (cm)[6]	Flower length (cm)[6]
Control	35.2±1.1 a	13.6±0.3 abc	2.6±0.1 a	3.2±0.1 abc	8.7±0.3 a	2.8±0.1 bc	3.1±0.0 a	27.2±0.5 d	4.7±0.3 ab	3.9±0.2 a	2.7±0.0 a
G2-21	2.3±0.1 de	11.4±0.9 bc	0.2±0.0 de	2.8±0.2 bc	4.1±0.1 c	2.0±0.1 d	2.1±0.1 b	42.1±0.3 ab	0.0±0.0 d	nd[7]	nd
G2-22	2.7±0.3 de	11.6±0.3 bc	0.2±0.0 de	3.1±0.1 abc	4.3±0.1 c	2.2±0.1 d	2.0±0.0 b	41.0±0.6 abc	0.0±0.0 d	nd	nd
G2-32	6.8±0.4 cde	14.6±0.3 ab	0.5±0.0 cde	3.4±0.0 ab	6.1±0.1 b	2.8±0.1 abc	2.2±0.0 b	38.6±0.6 bc	2.7±0.3 bc	0.5±0.0 b	0.9±0.0 b
G2-34	10.3±0.9 cd	14.4±0.3 ab	0.7±0.0 cd	3.4±0.1 abc	6.4±0.1 b	3.2±0.1 abc	2.0±0.0 b	38.9±0.8 bc	3.0±0.0 bc	0.5±0.0 b	0.8±0.0 b
G2-55	22.3±2.2 b	14.8±0.5 ab	1.5±0.1 b	3.3±0.1 abc	6.7±0.3 b	3.4±0.2 a	2.0±0.1 b	39.1±0.7 bc	3.3±0.3 abc	3.8±0.2 a	2.9±0.1 a

[1] Diploid, non-transgenic plants were used as the control. Among five transgenic lines, G2-21 and G2-22 showed a severely dwarf phenotype, whereas G2-32, G2-34 and G2-55 showed moderately dwarf phenotypes. G2-21, G2-22, G2-32 and G2-34 are diploid, whereas G2-55 is tetraploid. Values represent the mean±standard error of 3 plants for each plant strain. Values within the same column followed by different letters are significantly different at the 0.05 level with the Tukey–Kramer's test. [2] The longest three shoots were investigated for each plant. [3] The most basal node of the longest three shoots were measured for each plant. [4] Middle position of the longest three shoots was measured for each plant. [5] Randomly selected three expanded leaves from each of the longest three shoots were investigated for each plant. [6] Randomly selected three flowers were investigated for each plant. [7] Not determined.

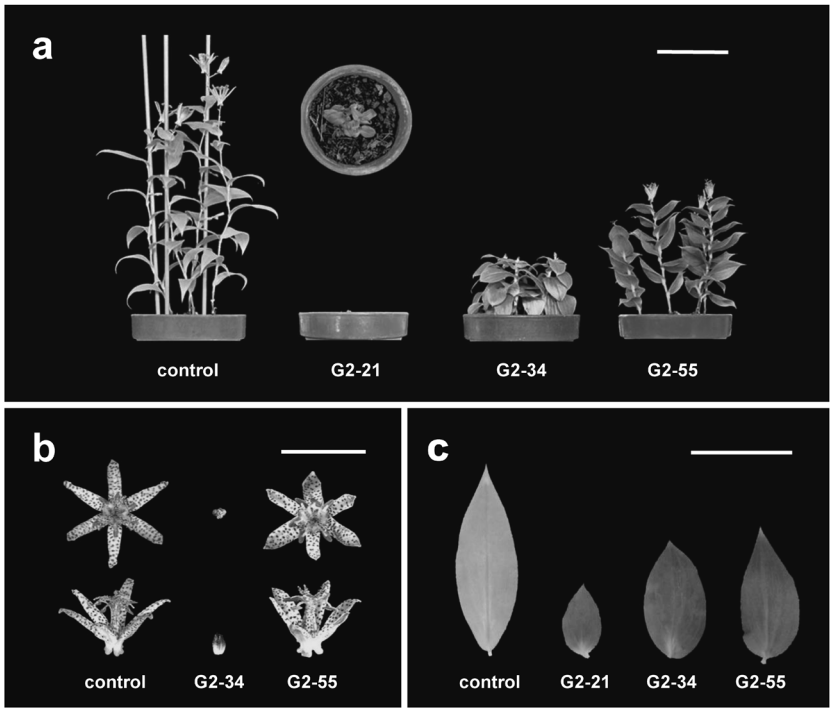

Figure 2. Morphological characteristics of transgenic *Tricyrtis* sp. containing *TfGA2ox2*. Diploid, non-transgenic plants were used as the control. Among three transgenic lines, G2-21 showed a severely dwarf phenotype, whereas G2-34 and G2-55 showed moderately dwarf phenotypes. G2-21 and G2-34 were diploid, whereas G2-55 was tetraploid. a Plants during the flowering season. Bar=10 cm. b Flowers. Bar=3 cm. c Leaves. Bar=5 cm.

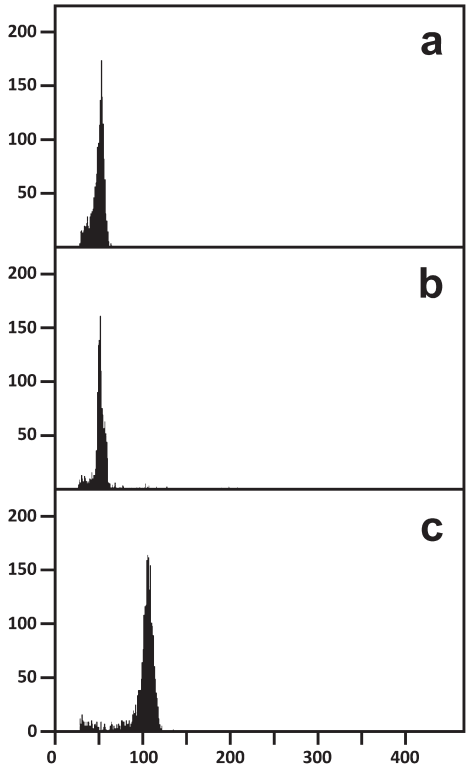

Figure 3. Histograms from FCM analysis of nuclear DNA content of transgenic *Tricyrtis* sp. containing *TfGA2ox2*. DNA content is expressed as the relative fluorescence intensity on the horizontal axis, and the number of nuclei on the vertical axis. a Diploid, non-transgenic plants used as the control. b Severely dwarf, diploid transgenic line G2-21. c Moderately dwarf, tetraploid transgenic line G2-55.

fully-opened flowers. All the transgenic lines produced smaller and rounder leaves compared with the control (Table 1; Figure 2c). In addition, Leaves of the transgenic plants were darker green due to increased chlorophyll contents (Table 1). Similar characteristics have already been observed in transgenic plants of various species overexpressing the GA2ox gene (Dijkstra et al. 2008; Gargul et al. 2013; Schomburg et al. 2003; Zhou et al. 2012). Type I and Type II have already been obtained in our previous study (Otani et al. 2013). Type I and Type II transgenic plants unfortunately produced only abnormal or no flowers, which largely decreases the ornamental value. On the other hand, Type III transgenic line G2-55 produced fully-opened flowers, even though this line showed a moderately dwarf phenotype as Type II transgenic lines (Figure 2). There were no significant differences in the number of flowers per plant and in the flower size between G2-55 and the control (Table 1; Figure 2b). Therefore, G2-55 may be horticulturally attractive.

To estimate the ploidy level of transgenic plants, flow cytometry (FCM) analysis of leaf tissues was performed using a flow cytometer PA (Partec, GmbH-Münster, Germany) as previously described (Saito et al. 2003). At least 1,500 nuclei were examined for each plant. FCM histogram of the control had a single peak corresponding to 2C DNA content (Figure 3a). Histograms of four out of five transgenic lines, G2-21, G2-22, G2-32 and G2-34, also had a single 2C peak (Figure 3b), indicating that

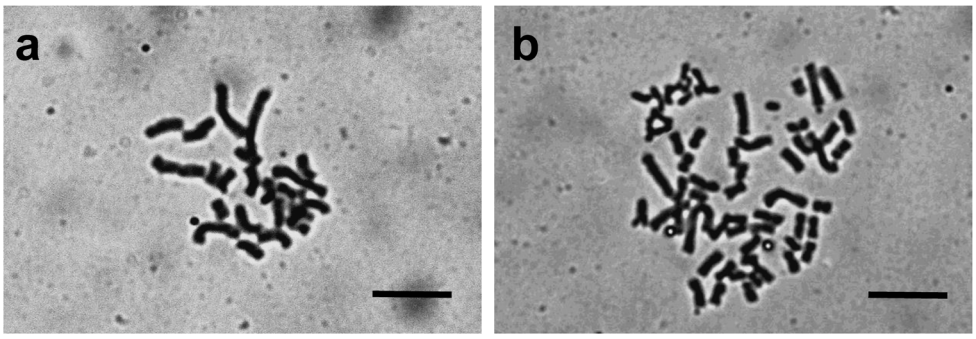

Figure 4. Chromosome observation in root-tip cells of transgenic *Tricyrtis* sp. containing *TfGA2ox2*. a Diploid, non-transgenic plants used as the control ($2n=2x=26$). b Moderately dwarf, tetraploid transgenic line G2-55 ($2n=2x=52$). Bars=10 μm.

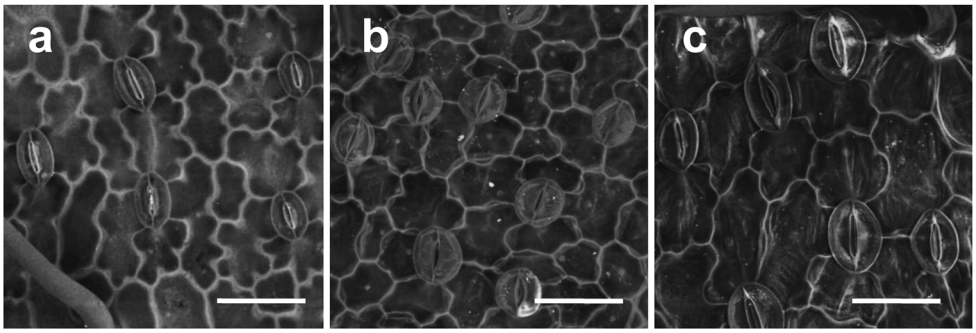

Figure 5. SEM observation of the leaf abaxial surface of transgenic *Tricyrtis* sp. containing *TfGA2ox2*. a Diploid, non-transgenic plants used as the control. b Severely dwarf, diploid transgenic line G2-21. c Moderately dwarf, tetraploid transgenic line G2-55. Bars=100 μm.

these four lines kept the diploid level. On the other hand, the histogram of G2-55 had a single 4C peak (Figure 3c), indicating that this line is tetraploid. In order to confirm the ploidy level, chromosome observation in root tip cells was carried out according to Nakamura et al. (2005) and Nakano et al. (2006). Four transgenic lines, G2-21, G2-22, G2-32 and G2-34, had $2n=26$ chromosomes as the control plants (Figure 4a). On the other hand, G2-55 had $2n=52$ chromosomes (Figure 4b). These results showed that Type III transgenic line G2-55 was tetraploid, whereas Type I and Type II transgenic lines were diploid as the control plants.

Stomata on the abaxial surface of leaves were observed in different types of transgenic plants using scanning electron microscope (SEM) (Table 2; Figure 5). As a result, no significant differences in the major axis of stomata were observed among the control, Type I and Type II transgenic lines (Table 2). On the other hand, the major axis of stomata in Type III transgenic line G2-55 was significantly longer than the control. The minor axis of stomata in all the transgenic plants was significantly larger than the control. Thus, the stomatal shape of Type I and Type II transgenic lines was rounder compared with that of the control (Table 2; Figure 5). Increased stomatal size were reported in chromosome-doubled plants of various species (Chen et al. 2011; Gantait et al. 2011; Yang et al. 2006). In our previous study, chromosome-doubled somaclonal variants were regenerated from one-year-old embryogenic callus cultures of *Tricyrtis* sp., and it was concluded that the chromosome-doubled plants originated from chromosome-doubled callus cells already existing in the embryogenic callus cultures (Nakano et al. 2006). A preliminary study indicated that a few tetraploid plants were also regenerated from embryogenic callus cultures used in the present study without co-cultivation with *Agrobacterium* (data not shown). Thus tetraploid transgenic line G2-55 might be derived from chromosome-doubled callus cells in the

Table 2. Leaf stomatal size of transgenic *Tricyrtis* sp. containing *TfGA2ox2*.[1]

Plant strain	Major axis (μm)	Minor axis (μm)	Major axis/ minor axis
Control	54.5±0.9 bc	33.4±0.6 e	1.6±0.0 a
G2-21	49.4±1.0 cd	38.4±0.7 cd	1.3±0.0 c
G2-22	51.0±0.5 cd	42.9±0.5 b	1.2±0.0 d
G2-32	52.0±0.8 bcb	39.1±0.6 c	1.3±0.0 c
G2-34	52.4±0.7 bcb	36.5±0.6 d	1.4±0.0 b
G2-55	77.1±1.1 a	49.4±0.7 a	1.6±0.0 a

[1] Diploid, non-transgenic plants were used as the control. Among five transgenic lines, G2-21 and G2-22 showed a severely dwarf phenotype, whereas G2-32, G2-34 and G2-55 showed moderately dwarf phenotypes. G2-21, G2-22, G2-32 and G2-34 are diploid, whereas G2-55 is tetraploid. Values represent the mean±standard error. Values within the same column followed by different letters are significantly different at the 0.05 level with the Tukey–Kramer's test.

embryogenic callus cultures used as a target material for transformation.

Nakano et al. (2006) reported that tetraploid somaclonal variants of *Tricyrtis* sp. had longer shoots, larger leaves, larger flowers and higher numbers of flowers per shoot compared with the diploid mother plants. In the present study, the tetraploid Type III transgenic line G2-55 had longer shoots, larger flowers and higher numbers of flowers per shoot compared with the diploid Type I and Type II transgenic lines (Table 1; Figure 2). Thus it may be possible that the tetraploid nature counteracted the phenotypic alterations caused by heterologous expression of *TfGA2ox2* in G2-55. On the other hand, in our previous study, the degree of dwarfism and other phenotypic alterations in transgenic *Tricyrtis* sp. plants generally correlated with the *TfGA2ox2* expression level and the endogenous level of bioactive GAs (Otani et al. 2013). Finn et al. (2011) reported for *A. thaliana* that tetraploid transgenic plants showed reduced expression levels and phenotypic effects of transgenes driven by the CaMV 35S promoter compared with diploid ones. Likewise, the expression level of *TfGA2ox2* under the control of the CaMV 35S promoter might be reduced in G2-55. Analyses of the *TfGA2ox2* expression level and the endogenous level of bioactive GAs in transgenic plants are necessary.

Type III transgenic line G2-55 had normally-opened flowers as the control, and no apparent differences in the flower size and shape were observed between G2-55 and the control (Table 1; Figure 2b). Anthers of G2-55 dehisced normally at anthesis and contained morphologically normal pollen grains. Pollen viability of G2-55 was 81.2% as assessed by acetocarmine staining. High pollen fertility in G2-55 offers a possibility of using this transgenic line as a cross-breeding material. In our previous study, several horticulturally attractive interspecific hybrids have been produced in *Tricyrtis* via ovule culture (Tasaki et al. 2014). Production of hybrid plants showing not only transgene-induced dwarfness but also other novel morphological characteristics is now in progress using G2-55 as one parent.

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