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Overexpression of the gibberellin 2-oxidase gene from *Torenia fournieri* induces dwarf phenotypes in the liliaceous monocotyledon *Tricyrtis* sp.

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ABSTRACT

Gibberellins (GAs) are the plant hormones that control many aspects of plant growth and development, including stem elongation. Genes encoding enzymes related to the GA biosynthetic and metabolic pathway have been isolated and characterized in many plant species. Gibberellin 2-oxidase (GA2ox) catalyzes bioactive GAs or their immediate precursors to inactive forms; therefore, playing a direct role in determining the levels of bioactive GAs. In the present study, we produced transgenic plants of the liliaceous monocotyledon *Tricyrtis* sp. overexpressing the GA2ox gene from the linderniaceous dicotyledon *Torenia fournieri* (*TfGA2ox2*). All six transgenic plants exhibited dwarf phenotypes, and they could be classified into two classes according to the degree of dwarfism: three plants were moderately dwarf and three were severely dwarf. All of the transgenic plants had small or no flowers, and smaller, rounder and darker green leaves. Quantitative real-time reverse transcription-polymerase chain reaction (PCR) analysis showed that the *TfGA2ox2* expression level generally correlated with the degree of dwarfism. The endogenous levels of bioactive GAs, GA_1 and GA_4, largely decreased in transgenic plants as shown by liquid chromatography–mass spectrometry (LC–MS) analysis, and the level also correlated with the degree of dwarfism. Exogenous treatment of transgenic plants with gibberellic acid (GA_3) resulted in an increased shoot length, indicating that the GA signaling pathway might normally function in transgenic plants. Thus, morphological changes in transgenic plants may result from a decrease in the endogenous levels of bioactive GAs. Finally, a possibility of molecular breeding for plant form alteration in liliaceous ornamental plants by genetically engineering the GA metabolic pathway is discussed.

Introduction

The plant hormone gibberellins (GAs) play an important role in plant growth and development in both vegetative and reproductive phases, such as germination, stem elongation, leaf expansion, flower initiation and fruit development (Harberd et al., 1998). Various GAs have been found in plants, among which only GA_1 and GA_4 are major bioactive GAs. In higher plants, the late pathway of GA biosynthesis and the early pathway of GA metabolism are catalyzed by three kinds of enzyme, gibberellin 20-oxidase (GA20ox), gibberellin 3-oxidase (GA3ox), and gibberellin 2-oxidase (GA2ox) (Fig. 1; Hedden and Kamiya, 1997; Lange, 1998; Hedden and Phillips, 2000a). Among these enzymes, GA2ox functions to metabolize bioactive GAs (GA_1 and GA_4) and their immediate precursors (GA_9, GA_{12}, GA_{20} and GA_{53}) to inactive GAs by hydroxylating their 2-carbon position. Genes encoding GA2ox have been isolated and characterized in various plant species (Lester et al., 1999; Hedden and Phillips, 2000b; Solfanelli et al., 2005; Radi et al.,

Abbreviations: CaMV, cauliflower mosaic virus; FW, fresh weight; GA, gibberellin; GA_3, gibberellic acid; GA2ox, gibberellin 2-oxidase; GA3ox, gibberellin 3-oxidase; GA20ox, gibberellin 20-oxidase; HPT, hygromycin phosphotransferase; LC–MS, liquid chromatography–mass spectrometry; NOS, nopaline synthase; NPTII, neomycin phosphotransferase II; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; SEM, scanning electron microscope; SPAD, soil and plant analyzer development; UNI, uniconazol.

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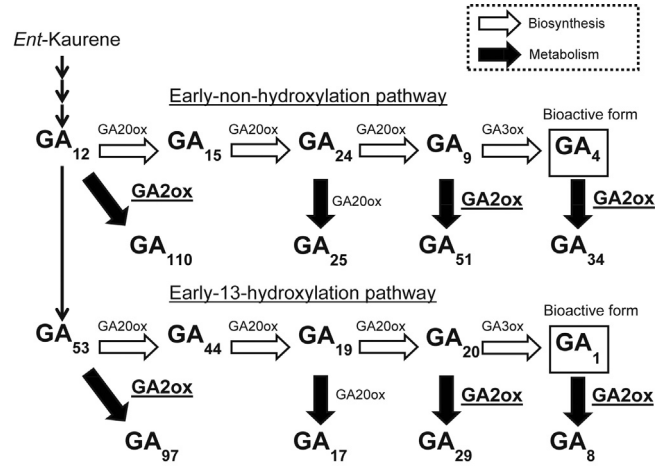

Fig. 1. GA biosynthetic and metabolic pathway in plants. White arrows indicate the GA biosynthetic pathway from precursors to bioactive forms catalyzed by GA20ox and GA3ox, and black arrows indicate the GA metabolic pathway catalyzed by GA2ox.

2006). In *Arabidopsis thaliana* and *Nicotiana tabacum*, overexpression of *AtGA2ox7* or *AtGA2ox8* from *A. thaliana* induced severely dwarf phenotypes (Schomburg et al., 2003). Furthermore, transgenic *Torenia fournieri* plants overexpressing *TfGA2ox* from the same species also showed dwarf phenotypes (Niki et al., 2006). On the other hand, a mutant for the *SLENDER* gene of *Pisum sativum*, which encodes GA2ox, exhibited increased plant height and accumulation of high levels of GA precursors in seeds (Martin et al., 1999). Hence, manipulation of the GA metabolic pathway by overexpression of the GA2ox gene may be an effective means for modifying plant height.

Dwarfness or semi-dwarfness is one of the most important traits in ornamental plants for pot or garden uses. In ornamental plants, most of the dwarf or semi-dwarf cultivars have been derived from natural variations. Recently, production of dwarf plants by genetic transformation has been examined in some ornamental plants. In *Nierembergia scoparia* (Godo et al., 1997), *Antirrhinum majus* (Hoshino and Mii, 1998), *Angelonia salicariifolia* (Koike et al., 2003), *Catharanthus roseus* (Choi et al., 2004), and *Gentiana* sp. (Mishiba et al., 2006), hairy root-derived dwarf plants have been obtained by transformation with wild-type strains of *Agrobacterium rhizogenes*. Dwarf plants have also been produced by transformation with the *rol* genes of *A. rhizogenes* in *Rosa hybrida* (Van der Salm et al., 1997), *Petunia hybrida* (Winefield et al., 1999), *Dianthus caryophyllus* (Zuker et al., 2001), and *Kalanchoe blossfeldiana* (Christensen et al., 2008). However, most of these transformants displayed the hairy root syndrome (Spena et al., 1987) and exhibited undesirable morphologies in addition to dwarfness, such as decreased apical dominance and curly and/or wrinkled leaves. Thus, alternative approaches are desired for producing transgenic dwarf ornamental plants.

Tricyrtis spp., liliaceous geophytes native to Japan, have recently become popular as ornamental plants. They are mainly cultivated for pot or garden uses. An efficient and reproducible system of *Agrobacterium*-mediated genetic transformation has already been established in *Tricyrtis* sp. (Adachi et al., 2005; Mori et al., 2008). Furthermore, unlike most liliaceous species, *Tricyrtis* sp. requires only one year from *in vitro* regeneration to flowering (Nakano et al., 2006a). Therefore, this plant seems to be suitable as a model for genetic transformation studies in liliaceous ornamental plants.

In the present study, we produced and characterized transgenic *Tricyrtis* sp. plants overexpressing the GA2ox gene from the lindernaceous dicotyledon *T. fournieri* (*TfGA2ox2*). The results obtained in the present study showed a possibility of molecular breeding for plant form alteration in liliaceous ornamental plants by genetically engineering the GA metabolic pathway.

Materials and methods

Plant material and embryogenic callus induction

Potted plants of *Tricyrtis* Wall. sp. 'Shinonome', sometimes called 'Japanese toad lily', were cultivated in the greenhouse without heating. Tepal-derived embryogenic callus cultures of this cultivar were induced and maintained as previously described (Nakano et al., 2004).

Agrobacterium tumefaciens strains

A. tumefaciens strain EHA101/pIG-*TfGA2ox2* was used for transformation. 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 (Fig. 2).

Production and morphological characterization of transgenic plants

Co-cultivation of embryogenic calli with *Agrobacterium*, selection of transgenic cells and tissues, and regeneration of transgenic plants were performed as previously described (Adachi et al., 2005). The presence of the transgene was confirmed using polymerase chain reaction (PCR) analysis with the primer set hpt290-F (5′-GTG CTT TCA GCT TCG ATG TAG G-3′) and hpt290-R (5′-GCT CGT CTG GCT AAG ATC GG-3′). Transgenic plants and non-transgenic embryogenic callus-derived plants (control) were transplanted to pots and cultivated in a growth chamber according to Mori et al. (2008). After three years of cultivation, morphological characterization was performed during the flowering season according to Nakano et al. (2006b). The mean soil and plant analyzer development (SPAD) value of three randomly selected expanded leaves was measured using a chlorophyll meter (SPAD-502; Fujiwara Scientific Co., Tokyo, Japan) according to Koike et al. (2003).

Scanning electron microscope (SEM) observation

SEM observation of the surface of stems was performed according to Nonaka et al. (2011). Samples were harvested from plants

Fig. 2. T-DNA region of the binary vector pIG-*TfGA2ox2*. *HPT*, hygromycin phosphotransferase; LB, left border; *NPTII*, neomycin phosphotransferase II; P-NOS, nopaline synthase promoter; P-35S, cauliflower mosaic virus (CaMV) 35S promoter; RB, right border; *TfGA2ox2*, GA2ox gene from *Torenia fournieri*; T-NOS, nopaline synthase terminator.

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during the flowering season. The size of ten epidermal cells per sample was measured in three replicates.

Inverse PCR analysis

To estimate the transgene copy number in transgenic plants, inverse PCR analysis was carried out according to Mori et al. (2008). Genomic DNA was digested with *Eco*RI (Roche, Basel, Switzerland), which cleaves at a site within *HPT*, and ligated to generate circular molecules using the DNA Ligation Kit (Takara Bio, Shiga, Japan). The primer set hpt-P1 (5′-GGC CGT CTG GAC CGA TGG CTG TGT AGA AGT ACT CG-3′) and hpt-P2 (5′-TGC AGA ACA GCG GGC AGT TCG GTT TCA GGC AGG TC-3′) was used for the first PCR, and the primer set hpt-P3 (5′-GAC GCC CCA GCA CTC GTC CGA GGG CAA AGG AAT AG-3′) and hpt-P4 (5′-ACA CCC TGT GCA CGG CGG GAG ATG CAA TAG GTC AG-3′) was used for the second PCR. Each PCR was performed under the following conditions: 3 min at 94 °C followed by 28 cycles of 10 s at 98 °C and 10 min at 68 °C. Inverse PCR analysis was repeated three times for each transgenic line.

Real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis

Transgene transcripts in young leaves of transgenic plants were quantified by real-time RT-PCR analysis using the DNA Engine Opticon System (MJ Research, Waltham, MA, USA) as previously described (Mori et al., 2007; Kamiishi et al., 2012). The primer sets used were a *TfGA2ox2*-specific primer set, Tore2ox2 RT-F1 (5′-CAG AAT CCA GAA GCT GTT GTT CC-3′) and Tore2ox2 RT-R2 (5′-TTG GCT CGA TTT TCA TTT CG-3′); and a *Thact2* (actin gene of *Tricyrtis* sp.; accession number AB196261 in the GenBank/EMBL/DDBJ databases)-specific primer set, Thact1-F (5′-CCG ACT CCC TCA TGA AAA TCC-3′) and Thact2-R (5′-CTC GAG CTC CTG TTC GTA GTC A-3′). Each PCR was performed in three replicates under the following conditions: 5 s at 95 °C, 20 s at 60 °C, and plate read (detection of fluorescent product) for 45 cycles. To characterize the PCR products, a melting curve analysis was performed by slowly raising the temperature from 70 to 95 °C, with fluorescence data at 0.5 °C intervals (Ririe et al., 1997). The relative amount of *TfGA2ox2* transcripts was calculated using the comparative cycle threshold method, and the results were normalized to *Thact2*.

Analysis of the endogenous GA level

GA analysis was carried out according to Katsumata et al. (2011) using the 6410 Triple Quad liquid chromatography-mass spectrometry (LC-MS) instrument (Agilent Technologies Inc., Santa Clara, CA, USA) including the Agilent 1200 series rapid resolution liquid chromatography system fitted with the Zorbax Eclipse XDB-C18 column (1.8 μm, 2.1 mm × 50 mm; Agilent Technologies Inc., Santa Clara, CA, USA). The internal standards were d2-GAs (OlChemIm Ltd., Olomouc, Czech Republic). After the extraction and pre-purification of samples, acidic fractions were subjected to LC–MS analysis. Exactly 1 g fresh weight (FW) of young leaves was used for each measurement.

Exogenous treatments with gibberellic acid (GA_3) and uniconazol (UNI)

Some transgenic plants and the control, non-transgenic plants were treated with 50 mg L^{-1} of GA_3 (Meiji Holdings, Tokyo, Japan) or 10 mg L^{-1} of a GA biosynthesis inhibitor, UNI [(E)-(S)-1-(4-Chlorophenyl)-4,4-dimethyl-2-(1H-1,2,4-triazol-1-yl)-pent-1-en-3-ol; Sumitomo Chemical, Tokyo, Japan]. Every two weeks from mid-May to early September, 1 mL of GA_3 or UNI solution per plant was sprayed on leaves, for a total of nine times per plant.

Results

Production and morphological characterization of transgenic plants

In total, six independent transgenic plants containing *TfGA2ox2* were shown by gene-specific PCR analysis to contain the desired insert (data not shown). Some produced flowers 1–2 years after cultivation in a growth chamber. Three years after cultivation, they were subjected to morphological characterization during the flowering season (Fig. 3 and Table 1). All of the transgenic plants were morphologically distinguishable from the control, non-transgenic plants. They exhibited dwarf phenotypes due to decreased internode lengths. Both shoot and internode lengths in all of the transgenic lines were significantly shorter than the control. Transgenic lines could be classified into two classes according to the degree of dwarfism: three lines (G2-3, G2-4 and G2-6) were moderately dwarf (shoots were more than 5.0 cm in length) and the other three lines (G2-1, G2-2 and G2-10) were severely dwarf (shoots were less than 3.0 cm in length, Fig. 3a and Table 1). All of the transgenic lines produced smaller, rounder and darker green leaves compared with the control plants (Fig. 3b). Leaves of severely dwarf transgenic lines were much smaller than those of moderately dwarf lines. Moderately dwarf lines produced small flowers that did not open fully (Fig. 3c), and severely dwarf lines produced no visible flower buds.

SEM observation of stem epidermal cells

In all of the transgenic lines, the length of stem epidermal cells significantly decreased to 10–46% of that of the control plants (Fig. 3d–f and Table 2). The widths of stem epidermal cells in G2-4 and G2-10 were significantly larger and smaller than the control, respectively. No significant differences in the widths were observed between the other transgenic lines and the control. The ratio of cell length to width significantly decreased in all of the transgenic lines. Among the six transgenic lines, G2-1 and G2-10 showed much smaller ratios of cell length to width (0.9–1.2), and the cell shapes in these strains became rounded compared with the control. No apparent correlations were observed between the degree of dwarfism and the length, width, and ratio of cell length to width of stem epidermal cells.

Inverse PCR analysis and real time RT-PCR analysis for TfGA2ox2 gene expression

Inverse PCR analysis of the six transgenic lines showed that they were derived from independent transgenic events (Fig. 4). Three transgenic lines (G2-1, G2-2 and G2-10) had a single copy of the transgene, two (G2-4 and G2-6) had three copies, and one (G2-3) had four copies of the transgene. The control, non-transgenic plants yielded no amplified fragments.

Fig. 5 shows the relative amounts of *TfGA2ox2* transcripts in young leaves of the control, non-transgenic plants and the six transgenic lines. *TfGA2ox2* transcripts were detected in all of the transgenic lines, but not in the control. Among the six transgenic lines, two severely dwarf lines (G2-1 and G2-10) showed relatively high expression levels, whereas one moderately dwarf line (G2-3) showed a relatively low expression level.

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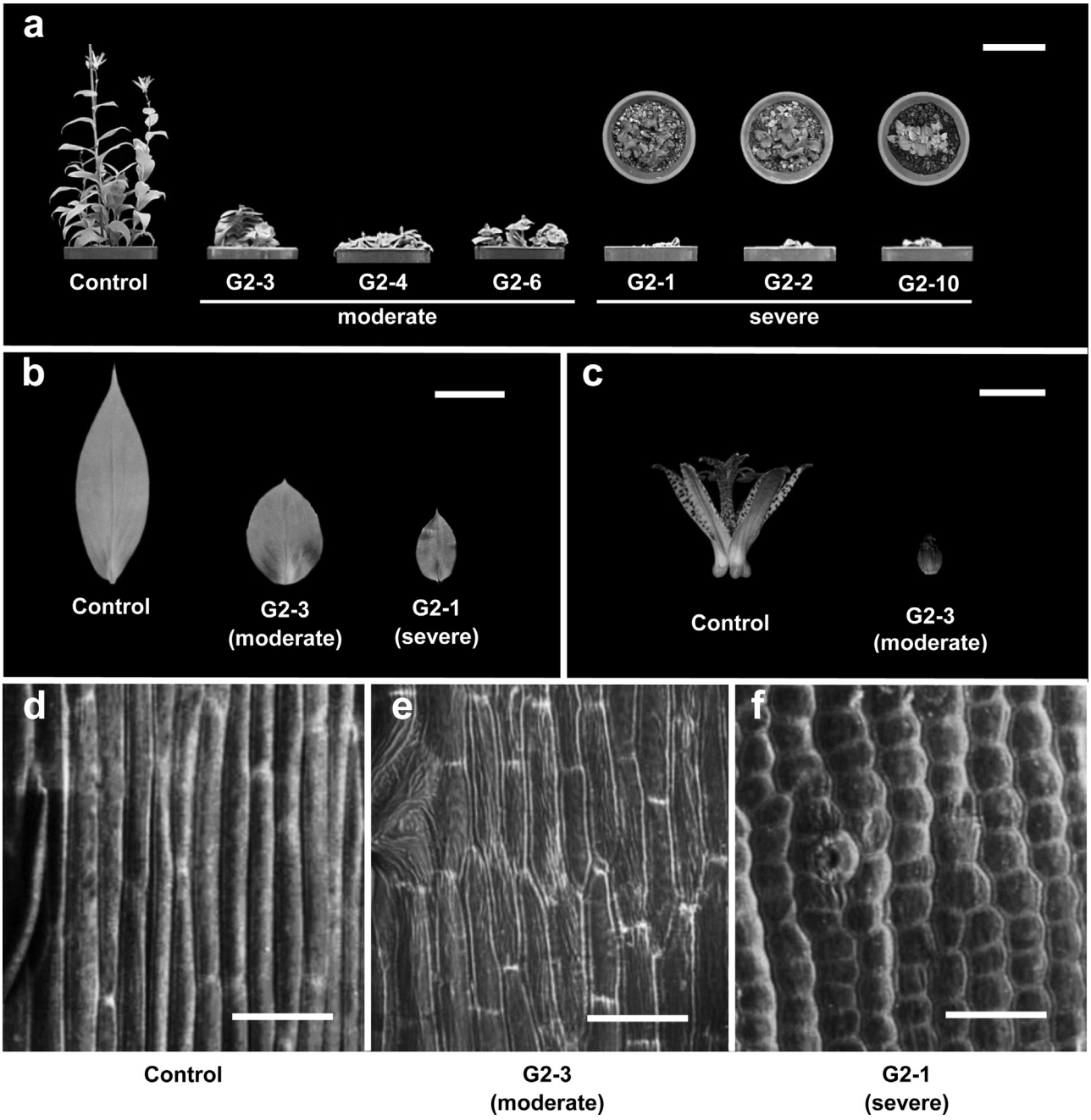

Fig. 3. Morphological characterization of the control, non-transgenic plants and transgenic plants containing *TfGA2ox2* of *Tricyrtis* sp. Three transgenic lines (G2-3, G2-4 and G2-6) showed moderately dwarf phenotypes and the other three lines (G2-1, G2-2 and G2-10) showed severely dwarf phenotypes. (a) Plants during the flowering season. Bar = 10 cm. (b) Leaves. Bar = 3 cm. (c) Flowers. Bar = 2 cm. (d–f) SEM observation of stem epidermal cells. Bar = 100 μm.

LC–MS analysis of the endogenous GA level

Fig. 6 shows the concentrations of bioactive GAs (GA_1 and GA_4), precursor of GA_4 (GA_{24}) and metabolic product of GA_4 (GA_{34}) in young leaves of the control, non-transgenic plants, moderately dwarf transgenic line (G2-3), and severely dwarf transgenic line (G2-1). Because the GA_4 concentration was much higher than the GA_1 concentration in the control, GA_4 may function as the major bioactive GA at least in leaves of *Tricyrtis* sp. The GA_4 concentration significantly decreased in both transgenic lines compared with the control, and the concentration in G2-1 was lower than in G2-3. The GA_4 concentration in G2-3 decreased to 5.1% of that of the control, and G2-1 contained no detectable amount of GA_4. Although no significant differences in GA_1, GA_{24} and GA_{34} concentrations were observed between the control and both transgenic lines, GA_1 and GA_{24} concentrations in both transgenic lines were lower than the control.

Exogenous treatments with GA_3 and UNI

Moderately dwarf transgenic line (G2-3) and severely dwarf transgenic line (G2-1) were treated with GA_3, whereas the control, non-transgenic plants were treated with UNI (Fig. 7). The exogenous GA_3 treatment largely increased the shoot length and leaf size of both transgenic lines. The GA_3-treated transgenic lines were similar in plant form, leaf shape, and leaf color to the control, and they produced flowers normally. The exogenous UNI treatment induced a dwarf phenotype in the control. Plant form, leaf shape, and leaf

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

Plant strain	Shoot length (cm)[b]	No. of nodes per shoot[b]	Internode length (mm)[c]	Stem diameter (cm)[d]	Leaf length (cm)[e]	Leaf width (cm)[e]	Leaf length/width[e]	Leaf SPAD value[e]
Control plants[f]	34.6 ± 1.8 a	13.7 ± 0.2 ab	2.5 ± 0.1 a	3.2 ± 0.2 bcd	8.9 ± 0.6 a	2.8 ± 0.2 b	3.2 ± 0.0 a	27.6 ± 0.9 c
Transgenic plants								
G2-1	2.4 ± 0.2 de	12.6 ± 1.9 abc	0.2 ± 0.0 c	2.8 ± 0.1 cd	4.5 ± 0.1 def	2.0 ± 0.1 c	2.2 ± 0.1 bc	40.2 ± 0.7 b
G2-2	2.4 ± 0.2 de	11.9 ± 0.8 abc	0.2 ± 0.0 c	2.4 ± 0.1 cd	4.1 ± 0.0 ef	2.0 ± 0.1 c	2.1 ± 0.1 bc	35.7 ± 1.3 b
G2-3	9.8 ± 0.5 b	15.6 ± 1.3 ab	0.6 ± 0.0 b	4.1 ± 0.5 ab	6.2 ± 0.2 bc	3.5 ± 0.1 a	1.8 ± 0.0 cd	40.7 ± 1.4 b
G2-4	5.5 ± 0.5 c	11.8 ± 0.6 abc	0.5 ± 0.1 b	4.5 ± 0.2 abc	5.5 ± 0.1 bcd	3.4 ± 0.3 a	1.6 ± 0.0 de	40.4 ± 1.8 b
G2-6	6.3 ± 0.6 bc	9.7 ± 0.5 bc	0.9 ± 0.2 b	3.0 ± 0.1 bcd	5.3 ± 0.5 cde	3.7 ± 0.2 a	1.4 ± 0.1 de	50.5 ± 0.7 a
G2-10	2.5 ± 0.3 de	11.7 ± 1.1 abc	0.2 ± 0.0 c	2.6 ± 0.2 cd	4.2 ± 0.1 ef	2.1 ± 0.1 c	2.0 ± 0.2 bcd	40.9 ± 1.6 b

[a] Values represent the mean ± standard error of three plants for each strain. Values within the same column followed by different letters are significantly different at the 0.01 level with the Tukey–Kramer's test.
[b] The longest three shoots were investigated for each plant.
[c] The most basal node of the longest three shoots was measured for each plant.
[d] Middle position of the longest three shoots was measured for each plant.
[e] Randomly selected three expanded leaves from each of the longest three shoots were investigated for each plant.
[f] Embryogenic callus-derived plants without transformation.

Table 2
Morphological characteristics of stem epidermal cells of transgenic *Tricyrtis* sp. plants containing *TfGA2ox2*.[a]

Plant strain	Cell size (μm)		Cell length/width
	Length	Width	
Control[b]	238.9 ± 8.0 a	25.1 ± 0.9 cd	9.8 ± 0.5 a
Transgenic plants			
G2-1	29.0 ± 1.1 d	33.1 ± 1.0 abc	0.9 ± 0.0 de
G2-2	65.2 ± 5.1 c	29.6 ± 0.9 bcd	2.2 ± 0.1 cd
G2-3	65.4 ± 2.2 c	26.2 ± 0.7 cd	2.5 ± 0.1 cd
G2-4	56.4 ± 2.8 c	36.9 ± 1.6 ab	1.6 ± 0.1 cde
G2-6	110.6 ± 4.4 b	28.8 ± 1.2 cd	4.0 ± 0.2 b
G2-10	24.7 ± 1.1 d	19.5 ± 0.5 e	1.2 ± 0.0 de

[a] Values represent the mean ± standard error of triplicate. Values within the same column followed by different letters are significantly different at the 0.01 level with the Tukey–Kramer's test.
[b] Embryogenic callus-derived plants without transformation.

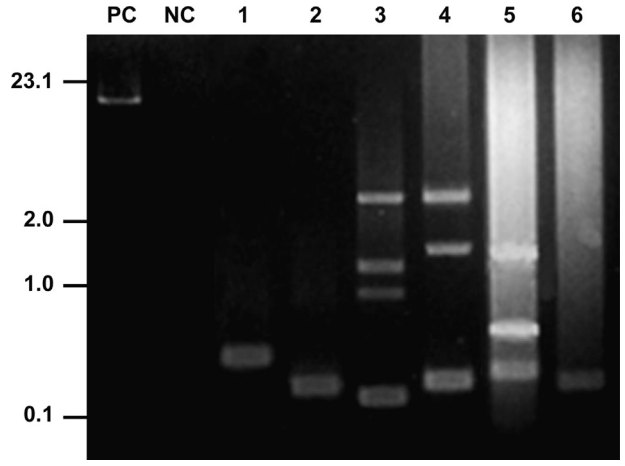

Fig. 4. Inverse PCR analysis of transgenic *Tricyrtis* sp. plants. Lane PC, plasmid pIG121Hm as a positive control; lane NC, non-transgenic plant as a negative control; lane 1, G2-1; lane 2, G2-2; lane 3, G2-3; lane 4, G2-4; lane 5, G2-6; lane 6, G2-10. Numerals on the left indicate molecular sizes in kbp.

color of the UNI-treated control plants were similar to those of G2-3, but they produced flowers normally.

Discussion

In the present study, we produced and characterized transgenic *Tricyrtis* sp. plants overexpressing the GA2ox gene from *T. fournieri*.

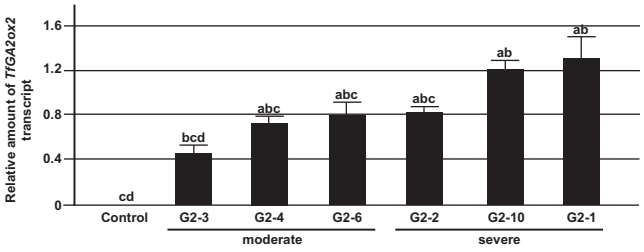

Fig. 5. Real-time RT-PCR analysis of *TfGA2ox2* transcripts in young leaves of the control, non-transgenic plants and transgenic plants containing *TfGA2ox2* of *Tricyrtis* sp. Relative amount of *TfGA2ox2* transcripts was normalized to *Thact2*. Values with different letters are significantly different at the 0.01 level with the Tukey–Kramer's test.

All of the transgenic plants exhibited reduced shoot, internode and leaf lengths, and they produced darker green leaves compared with the control, non-transgenic plants (Fig. 3 and Table 1). Morphological alterations by overexpression of the GA2ox gene have already been reported for various plant species: transgenic *A. thaliana* and *N. tabacum* plants containing *AtGA2ox7* or *AtGA2ox8* from *A. thaliana* exhibited dwarf phenotypes (Schomburg et al., 2003); transgenic *Solanum melanocerasum* and *S. nigrum* plants expressing *PcGA2ox* from *Phaseolus coccineus* showed dwarf phenotypes and produced darker green leaves with higher concentrations of chlorophylls (Dijkstra et al., 2008); and transgenic *Brassica napus* plants containing *AtGA2ox8* showed reduced plant heights and internode lengths, and produced small and darker green leaves (Zhou et al., 2012). Similar morphological alterations were also observed in transgenic *Tricyrtis* sp. plants overexpressing *TfGA2ox2*.

In *A. thaliana*, a GA biosynthesis mutant, *ga-1*, contained low levels of bioactive GAs and it exhibited late flowering and abnormal flower morphologies as well as dwarfism (Koornneef and van der Veen, 1980; Sun and Kamiya, 1994; Silverstone et al., 2001). In addition, transgenic *A. thaliana* plants overexpressing *AtGA2ox7* or *AtGA2ox8* showed delayed floral induction (Schomburg et al., 2003). In the present study, moderately dwarf transgenic plants produced abnormal flowers but no apparent alteration in the flowering time was observed (data not shown). On the other hand, severely dwarf transgenic plants produced no visible flower buds. Histological studies are necessary to clarify the effects of overexpressing the GA2ox gene on flower bud formation in *Tricyrtis* sp.

In all of the transgenic plants, the length of stem epidermal cells largely decreased compared with the control plants (Fig. 3 and Table 2). Cell lengths in the severely dwarf transgenic lines, G2-1 and G2-10 were much smaller than in moderately dwarf transgenic lines, whereas the other severely dwarf transgenic line G2-2

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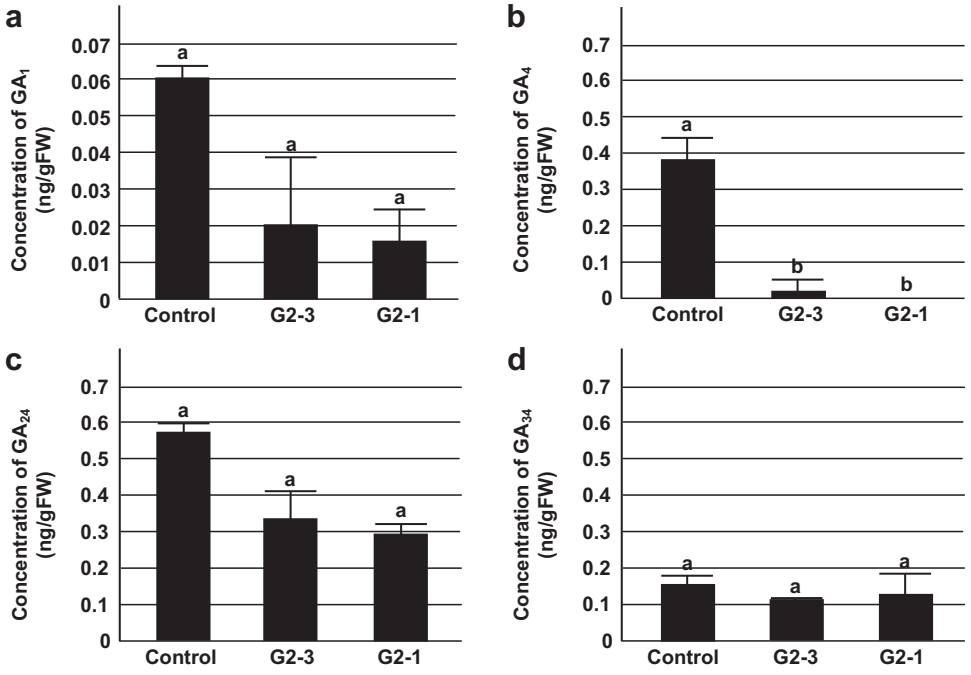

Fig. 6. Concentration of endogenous bioactive GAs, GA_1 (a) and GA_4 (b), a precursor of GA_4, GA_{24} (c), and a metabolic product of GA_4, GA_{34} (d) in young leaves of the control, non-transgenic plants and transgenic plants containing *TfGA2ox2* of *Tricyrtis* sp. Transgenic lines G2-3 and G2-1 showed moderately and severely dwarf phenotypes, respectively. Values represent the mean ± standard error of three replicates. Values with different letters are significantly different at the 0.01 level with the Tukey–Kramer's test.

showed a similar cell length to the moderately dwarf transgenic lines. Thus, there is not a complete correlation between the degree of dwarfism and cell length in transgenic plants. There were no significant differences in the number of nodes and the internode length among the three severely dwarf transgenic lines, G2-1, G2-2, and G2-10 (Table 1), indicating that dwarf phenotypes in transgenic plants may result not only from a decrease in the cell length but also from a decrease in the cell number per internode.

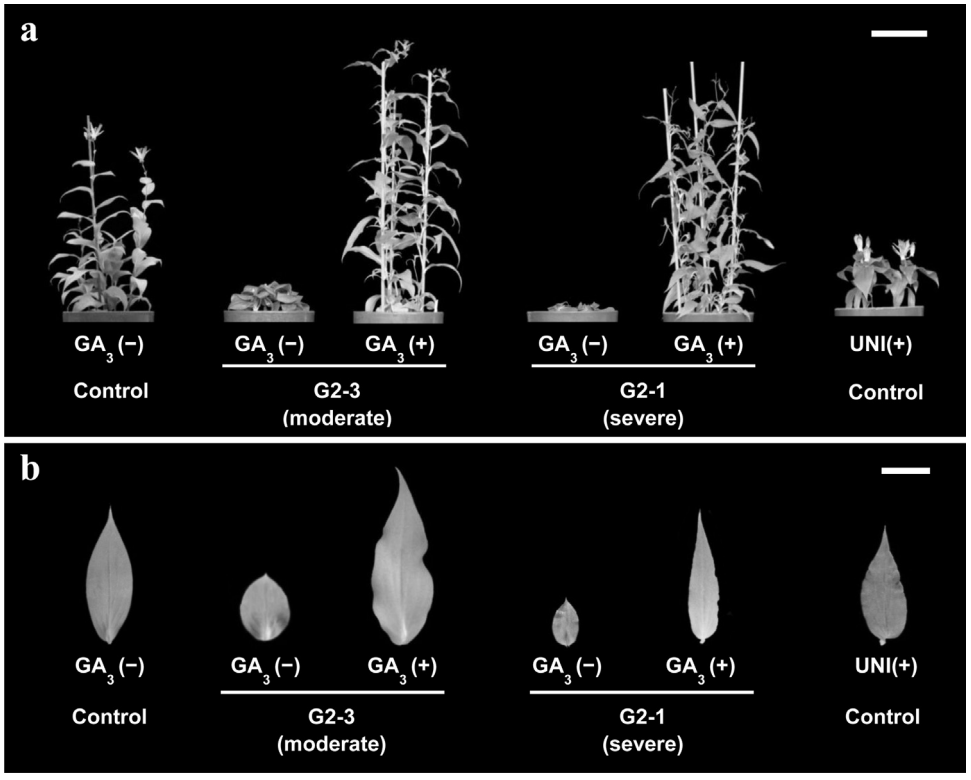

Fig. 7. Effect of exogenous GA_3 or UNI treatments on plant (a) and leaf (b) morphologies of the control, non-transgenic plants and transgenic plants containing *TfGA2ox2* of *Tricyrtis* sp. Transgenic lines G2-3 and G2-1 showed moderately and severely dwarf phenotypes, respectively. $GA_3(+)$ and $GA_3(-)$ represent treatments with and without 50 mg L^{-1} GA_3, respectively. UNI(+) represent a treatment with 50 mg L^{-1} UNI. Bars in (a) and (b) represent 10 and 3 cm, respectively.

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Among the six transgenic lines, three severely dwarf lines had a single copy of the transgene, whereas three moderately dwarf lines had multiple copies of the transgene as indicated by inverse PCR analysis (Fig. 4). Quantitative real-time RT-PCR analysis showed that the *TfGA2ox2* expression level generally correlated with the degree of dwarfism in transgenic plants (Fig. 5). Thus, the transgene copy number was negatively associated with the transgene expression level and the degree of phenotypic alteration in the present study. It is generally known that multiple gene copies frequently lead to co-suppression and gene silencing (Vaucheret et al., 1998).

In transgenic *A. thaliana* overexpressing the GA2ox gene from *Cucurbita maxima* (*CmGA2ox1*), dwarf phenotypes were accompanied not only by a severe reduction in the endogenous level of GA_4 but also by an increase in the endogenous level of GA_{34}, a metabolic product of GA_4 (Radi et al., 2006). In the present study, LC–MS analysis showed that the GA_4 level significantly decreased in transgenic plants, and the level also correlated with the degree of dwarfism (Table 1 and Fig. 6). However, there was no significant difference in the GA_{34} level between the control and transgenic plants. Thus, *TfGA2ox* might primarily catalyze intermediate precursors of bioactive GAs, such as GA_{12} and GA_9, and large parts of these precursors were consumed before reaching GA_4 in transgenic *Tricyrtis* sp. plants. Quantification of the endogenous level of GA_{12} and GA_9, and their corresponding metabolic products, GA_{110} and GA_{51}, is necessary.

Besides the reduction in the endogenous level of bioactive GAs, mutations in the GA signaling pathway also cause dwarf phenotypes (Ueguchi-Tanaka et al., 2000; Sasaki et al., 2003). Mutants in the GA signaling pathway cannot respond to endogenous as well as exogenous, bioactive GAs (Ueguchi-Tanaka et al., 2000). In the present study, dwarf transgenic plants clearly responded to an exogenous bioactive GA, and shoot and leaf lengths were increased by GA_3 treatment (Fig. 7), indicating that the GA signaling pathway might function normally in transgenic *Tricyrtis* sp. plants. GA_3-treated transgenic plants showed similar morphologies to the control plants, whereas the plant form, leaf shape, and leaf color of UNI-treated control plants were similar to those of transgenic plants. All of these results indicate that decreased levels of endogenous GAs, which resulted from overexpression of *TfGA2ox2*, were responsible for the dwarf phenotypes in the transgenic *Tricyrtis* sp. plants.

In ornamental plants, transformation with wild-type strains of *A. rhizogenes* (Godo et al., 1997; Hoshino and Mii, 1998; Koike et al., 2003; Choi et al., 2004; Mishiba et al., 2006) or with the *rol* genes of *A. rhizogenes* (Van der Salm et al., 1997; Winefield et al., 1999; Zuker et al., 2001; Christensen et al., 2008) has been performed to introduce dwarf phenotypes. In these studies, dwarf transgenic plants were successfully obtained, but they exhibited undesirable morphologies, such as decreased apical dominance and curly and/or wrinkled leaves. In the present study, such morphologies were not observed in the transgenic *Tricyrtis* sp. overexpressing *TfGA2ox2*. However, our transgenic plants unfortunately showed different undesirable morphologies, namely the production of only small or no flowers (Fig. 3), which largely decreases the ornamental value. The degree of morphological alterations in flowers of transgenic *Tricyrtis* sp. correlated with the degree of dwarfness, the *TfGA2ox2* expression level, and the endogenous level of bioactive GAs, indicating that the overexpression of *TfGA2ox2* is responsible for the morphological alterations in flowers. Sakamoto et al. (2003) reported that transgenic *Oryza sativa* overexpressing *OsGA2ox1* under the control of the *OsGA3ox2* promoter exhibited dwarf phenotypes without alterations in the flower morphology. Therefore, it may be possible to produce dwarf or semi-dwarf transgenic *Tricyrtis* sp. with normal flowers by using promoters with weak expression activities or tissue-specific promoters.

In the present study, dwarf transgenic plants were successfully obtained in the liliaceous ornamental plant *Tricyrtis* sp. by heterologous expression of the *GA2ox* gene from the linderniaceous plant *T. fournieri*. To the best of our knowledge, this is the first report of a plant form alteration in a monocotyledonous ornamental plant by genetic transformation using a GA metabolic gene from a dicotyledonous plant.

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