

Overexpression of the Gibberellin 2-Oxidase Gene from *Camellia lipoensis* Induces Dwarfism and Smaller Flowers in *Nicotiana tabacum*

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Abstract Gibberellins (GAs) are plant hormones that control many aspects of plant growth and development. Gibberellin 2-oxidase plays an important role in determining the level of bioactive GAs. In this study, we isolated three *GA2ox* genes (*CIGA2ox1-3*) from *Camellia lipoensis* Chang et Xu. The results of a quantitative real-time reverse transcription polymerase chain reaction analysis indicated that *CIGA2ox1-3* may play a tissue-specific role in plant development. The transcript of *CIGA2ox1* was more abundant in the stem and apex, *CIGA2ox2* was highly expressed in mature leaves, and *CIGA2ox3* was more abundant in roots. We produced transgenic plants of *Nicotiana tabacum* L. by overexpressing the *CIGA2ox1-3* genes. Plants with overexpressed *CIGA2ox1* or *CIGA2ox3* genes exhibited dwarf phenotypes, including reduced growth, delayed flowering, and smaller, rounder, and darker green leaves. All of the transgenic plants overexpressing the *CIGA2ox1* gene bloomed normally, but their flowers were half the size of the control plants. Plants overexpressing

CIGA2ox3 could be categorized into two classes: moderately dwarfed and severely dwarfed. The *CIGA2ox2* gene had little effect on the morphological characterization of transgenic plants. Quantitative real-time PCR analysis showed that the *CIGA2ox3* expression level was generally correlated with the level of dwarfism. The endogenous level of bioactive GA₄ and GA₁ largely decreased in transgenic plants and was generally correlated with the degree of dwarfism in transgenic plants with the *CIGA2ox1* or *CIGA2ox3* gene. The application of GA₃ rescued the dwarf phenotype of transformants, indicating that the GA signaling pathway might function normally in transgenic plants. Therefore, morphological changes in transgenic plants may result from a decrease in the endogenous level of bioactive GAs. Additionally, the possibility of molecular breeding for plant form alternation in *Camellia* plants by genetically engineering the GA metabolic pathway is discussed.

Keywords *Camellia lipoensis* · Dwarf · Gibberellin 2-oxidase · Morphological alteration

Zheng Xiao and Ruipeng Fu contributed equally to this work.

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Introduction

Dwarfism and semi-dwarfism are key traits in orchard and cereal crop breeding. In fruit trees, dwarf and semi-dwarf varieties are often preferred. These cultivars allow for dense field cultivation, facilitate mechanized maintenance, increase the efficiency of fruit collection, and allow for more precise pesticide application by reducing spray drift (Busov et al. 2003). *Camellia* is a genus of flowering plants in the family Theaceae. This genus is composed of evergreen shrubs or small trees that are popular around the world for a variety of uses, especially as ornamental plants. Semi-dwarf or dwarf camellias are favored in landscape applications and potted flower production.

Many studies have demonstrated that dwarfism is commonly related to deficiencies in gibberellin (GA) levels or signaling (Spielmeyer et al. 2002; Olszewski et al. 2002). GAs are a class of phytohormones that are responsible for regulating many aspects of plant growth and development throughout the life cycle of plants, including leaf expansion, stem elongation, flowering, seed germination, and the transition from vegetative growth to flowering (Yamaguchi 2008; Peters 2013). Plants defective in GA biosynthesis display typical GA-deficient phenotypes, characterized by prolonged germination dormancy; small, dark green leaves; inhibited root growth; defective flowering; reduced seed production; and male sterility (Sun and Gubler 2004; Otani et al. 2013). Therefore, it is important to maintain optimal endogenous GA levels in plants. GA levels are precisely regulated by several mechanisms, including the transcriptional regulation of genes associated with synthesis and deactivation of the bioactive GA forms. The formation of C₁₉-GA, through the oxidation of C₂₀-GA, and the 3 β -hydroxylation of C₁₉-GA are important steps in the biosynthesis pathway of bioactive GA; GA20-oxidase and GA3-oxidase catalyze these respective steps in GA biosynthesis (Olszewski et al. 2002; Sun 2008). The deactivation of the bioactive GA forms GA₁ and GA₄ and their respective precursors, GA₉ and GA₂₀, are catalyzed by GA2-oxidases (GA2ox) (Thomas et al. 1999). Many genes encoding enzymes that catalyze GA biosynthesis and catabolism have been identified (Hedden and Thomas 2012; Plackett et al. 2012; Nishii et al. 2014). However, GA biosynthetic genes have received much more attention than catabolic genes. Functional analysis of GA2ox revealed that catabolic genes are also important in the control of GA levels (Zhou et al. 2010; El-Sharkawy et al. 2012).

GA 2-oxidase in *Arabidopsis*, rice (*Oryza sativa* L.), and poplar (*Populus trichocarpa* Torr. & Gray ex Hook.) are encoded by small gene families (Hedden and Thomas 2012; Daviere and Achard 2013). GA2oxs were first identified by screening cDNA expression libraries for 2 β -hydroxylase activity (Thomas et al. 1999; Martin et al. 1999). Five *Arabidopsis* GA2ox genes have been identified based on sequence homology, and their encoded protein activities have been confirmed (Hedden and Phillips 2000). The physiological functions of these genes have been studied in a variety of plant species, and it has been demonstrated that they are active against bioactive GAs and their immediate precursors. The overexpression of *AtGA2ox7* and *AtGA2ox8* in transgenic tobacco (*Nicotiana tabacum*) leads to a dwarf phenotype (Schomburg et al. 2003). Similar results have been obtained in other transgenic plants overexpressing GA 2-oxidases from rice (*O. sativa*) and poplar (*Populus spp.*) (Busov et al. 2003; Huang et al. 2010). *GA2ox7* and *GA2ox8* were identified from *Arabidopsis*, and they catabolize only the non-bioactive C₂₀-GAs (Schomburg et al. 2003). Phylogenetic analysis has shown that the GA 2-oxidases can be divided into three

classes (Yamaguchi 2008). Members of class III include *GA2ox7* and *GA2ox8*, distinguished by their preference for C₂₀-GAs. The biological-structural division between classes I and II remains less clear. To our knowledge, no *GA2ox* genes have yet been cloned from camellias, and their developmental functions and regulation during plant development are completely unknown.

In this study, we isolated three putative GA 2-oxidase genes (*CIGA2ox1*, *CIGA2ox2*, and *CIGA2ox3*) from *Camellia lipoensis* using reverse transcription PCR (RT-PCR) and the rapid amplification of cDNA ends (RACE) method. Based on the expression of *CIGA2ox1-3*, we suggest that the phenotypes might be largely attributed to individual GA 2-oxidases, in some cases with a specific expression pattern in plant development. To our knowledge, this is the first report of a GA 2-oxidase gene isolated from camellia plants. Moreover, the *CIGA2ox1* gene not only induces dwarf phenotypes but also affects flower size, suggesting that this gene might provide new tools for research and the genetic engineering of ornamental plant stature.

Materials and Methods

Plant Material

C. lipoensis Chang et Xu is an evergreen shrub and was cultivated in a greenhouse for this study. Tobacco (*N. tabacum*) leaves were harvested from greenhouse-grown plants, surface sterilized by soaking in 0.1 % (w/v) HgCl₂ for 5 min, and subsequently rinsed three times in sterile water. The leaves were cut into pieces through the midrib to obtain 0.5 × 0.5 cm² rectangular explants. The tobacco explants were placed on a solid regeneration medium, based on Murashige and Skoog (MS) medium (Murashige and Skoog 1962), supplemented with 30 g/L sucrose, 6.0 g/L plant agar, 0.5 mg/L 6-benzyladenine (BA), and 0.05 mg/L α -naphthaleneacetic acid (NAA). The pH of the medium was adjusted to 5.7 before autoclaving. In vitro shoot regeneration of tobacco was performed in 8-cm diameter Petri dishes. After 4 to 5 weeks, the tobacco shoots were harvested and transferred to a root-inducing MS medium containing half-strength MS microelements supplemented with 30 g/L sucrose and 6.0 g/L plant agar. Shoot regeneration and root induction were performed in a growth chamber at 25 °C with a 16-h photoperiod.

Agrobacterium tumefaciens Strains

A. tumefaciens strain EHA105 was used for transformation. The T-DNA region of the binary vector pCAMBIA1300 was under the control of the cauliflower mosaic virus (CaMV) 35S promoter, and the hygromycin phosphotransferase (HPT) gene was under the control of the CaMV 35S promoter.

Isolation and Sequencing of *Camellia* GA2ox cDNA Sequences

Total RNA was extracted from leaf of *C. lipoensis* using the RN38 EASYspin plus plant RNA Kit (Aidlab biotechnologies Co., Beijing, China) according to the manufacturer's protocol. The full-length ORF of the *C. lipoensis* GA2-oxidase gene was cloned through reverse transcription PCR (RT-PCR), and the rapid amplification of cDNA ends (RACE). Three pairs of degenerate primers (Table S1) were designed from the conserved regions of GA2ox orthologues. The isolated fragment was cloned in the vector pGEM-T Easy (Promega, WI, USA), sequenced, and analyzed using BLAST (Altschul et al. 1997). Extension of the partial cDNA clone was carried out using a SMARTER RACE cDNA Amplification Kit (Takara). Alignment of the camellia GA2ox predicted sequence, and the neighbor-joining tree construction were performed using the ClustalW program (Thompson et al. 1994).

Vector Construction and Plant Transformation

The *CIGA2ox1*, *CIGA2ox2*, and *CIGA2ox3* gene sequences were cloned separately in front of the 35S constitutive promoter within a pCambia1300 vector. The T-DNA contains a hygromycin gene for selection in plants under a duplicated 35S promoter. The vector also contains an *NPTII* gene for bacterial selection. The modified construct was cloned in the *Agrobacterium tumefaciens* strain EHA105.

A bacterial suspension was prepared from a 200- μ L frozen glycerol culture in 30 mL of LB medium (10 g/L bacto-tryptone, 5 g/L bacto-yeast extract, 10 g/L NaCl, pH 7.0) supplemented with 50 mg/L kanamycin and 25 mg/L rifampicin and cultured for 24 h at 28 °C. A bacterial suspension with an OD₆₀₀=0.5 was pelleted at 5000 rpm for 10 min at 4 °C. The supernatant was discarded, and the pellet was re-suspended in 20 mL of half-strength liquid MS medium; the subsequent solution was supplemented with 200 μ M acetosyringone. Young leaf explants from in vitro-grown tobacco plants were cut into 5×5 mm squares and submerged in the bacterial solution for 5 min. The explants were blotted dry on sterile filter paper and placed on MS medium supplemented with 100 μ M acetosyringone for 2 days in the dark. Then, the explants were transferred to a shoot-inducing medium supplemented with 0.5 mg/L 6-benzylaminopurine, 0.05 mg/L NAA, 20 mg/L hygromycin, and 200 mg/L Timentin. The explants were transferred to fresh media every 2 weeks. Regenerated shoots were cut from the explants and transferred to root-inducing medium (MS, 0.05 mg/L NAA, 20 mg/L hygromycin, and 200 mg/L Timentin). After rooting, plantlets were planted in a greenhouse.

Reverse Transcription Polymerase Chain Reaction and Southern Blot

For reverse transcription polymerase chain reaction (RT-PCR) verification of CIGA2ox expression, total RNA was isolated from all transgenic lines of tobacco. The RNA was isolated from ground plant material using the RN38 EASYspin plus plant RNA Kit (Aidlab biotechnologies Co., Beijing, China) according to the manufacturer's protocol, which also removed the genomic DNA. The RNA concentration and quality were measured using a NanoDrop 2000 (Thermo Fisher Scientific Inc., Waltham, USA). Reverse transcription was performed using the First Strand cDNA Synthesis Kit (Fermentas) with 5 μ g of RNA. RT-PCRs were prepared using 10 ng of cDNA in a final volume of 25 μ L containing 0.25 μ M each CIGA2ox primers (Table S2), 0.15 mM each dNTP, 2 mM MgCl₂, and 2 U of rTaq polymerase (Takara). The reaction consisted of 3 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 58 °C for 30 s, and 80 s at 72 °C. A final 10-min extension step at 72 °C was applied. PCR amplification was conducted in a thermocycler (ABI PCR system 9700, USA).

Genomic DNA was isolated using the CTAB method (Stewart and Via 1993). For Southern blot hybridization, 25 μ g of DNA from the control and transgenic plants was digested using 50 units of *Bam*HI (Takara) for 24 h. The resulting DNA fragments were separated on agarose gels and transferred to a membrane (Srisankarajah et al. 2007). A DIG-labeled (digoxigenin) probe was prepared by PCR according to the manufacturer's protocol (Roche Applied Science Co., Germany) using the *CIGA2ox1*, *CIGA2ox2*, and *CIGA2ox3* gene primer pairs (Table S4). The hybridization and post-hybridization and an estimation of the visualized fragments were also performed following the protocol.

Quantitative Real-Time Polymerase Chain Reaction Analysis

Total RNA was isolated using the RN38 EASYspin plus plant RNA kit. The gene expression levels of various organs in *C. lipoensis* were determined by qRT-PCR using specific primer sets (Table S3), and *ClActin* (actin gene of *C. lipoensis*, GenBank accession number: KF366912) was used as an internal standard. Transcripts of transgenes in young leaves of transgenic plants were also quantified by quantitative real-time polymerase chain reaction (qRT-PCR) analysis using primer sets (Table S3), and *NicActin* (actin gene of *N. tabacum*, GenBank accession number: AB158612) was used as an internal control.

Quantitative real-time PCR analysis was performed using the Applied Biosystems 7300 Real Time PCR System. One microgram of total RNA was reverse transcribed using a PrimeScript RT reagent kit (Takara). The qRT-PCR reaction

was performed using a SYBR premix Ex Taq II kit (Takara). Each PCR was performed in three replicates under the following conditions: 30 s at 95 °C, 31 s at 60 °C, and plate read (detection of fluorescent product) for 40 cycles. To characterize the PCR products, a melting curve analysis was performed by slowly raising the temperature from 60 to 95 °C, with fluorescence data obtained at 0.5 °C intervals. Quantifications of each cDNA sample were made in triplicate to ensure consistent results from the three separately prepared RNA samples. The relative amounts of target gene transcripts were calculated using the comparative cycle threshold method, and the results were normalized to the internal control.

Phenotypic Characteristics of Transgenic Plants

Transgenic plants (generation 1) and non-transgenic plants were transplanted to pots and kept under 16 h photoperiod conditions at 24 °C/18 °C (day/night). After 2 months of cultivation, morphological characterizations (height, internode length, leaf length, and width) were performed during the flowering season. The mean plant development values of three randomly selected plants were measured.

Plant Treatments with GA₃ and Paclobutrazol

The transgenic lines (generation 1) were cultivated in MS medium containing 100 μM GA₃. Non-transgenic plant was treated with 2 μM of a GA biosynthesis inhibitor, paclobutrazol (PAC). Changes in growth were observed and photographed 2 weeks after treatment. Experiments were performed three times.

Analysis of the Endogenous GA Level

The procedures for extraction, purification, and quantification of endogenous GAs were performed as previously described (El-Sharkawy et al. 2012). Aliquots from apical shoots including the three youngest developed leaves from plants just before flowering were harvested and weighted for later analysis. The concentrations of GAs in the extracts were determined by high-performance liquid chromatography/mass spectrometry (HPLC/MS). All experiments were carried out in three independent replicates.

Results

Isolation of *CIGA2ox1-3*

Sequence analysis of the PCR product showed that the fragment encoded a part of the GA2ox gene family. Three full-length cDNAs of GA2ox were obtained, containing open reading frames of 999, 996, and 1002 bp, and the cloned genes were subsequently named *CIGA2ox1*, *CIGA2ox2*, and *CIGA2ox3*, respectively (GenBank accession numbers: KJ502290, KJ502289, and KJ502291, respectively). The *CIGA2ox* amino acid sequences were aligned with those of other reported GA2oxs. The deduced amino acid sequences of *CIGA2ox1-3* share 70, 59, and 54 % identity with *P. trichocarpa PtGA2ox1* (Niu et al. 2013), *A. thaliana AtGA2ox1* (Lee et al. 2014), and *O. sativa OsGA2ox3* (Sakai et al. 2003), respectively. An alignment of the deduced amino acid sequences of *CIGA2ox1-3* with other GA2ox sequences (Figure S1) showed that they contain characteristic sequences

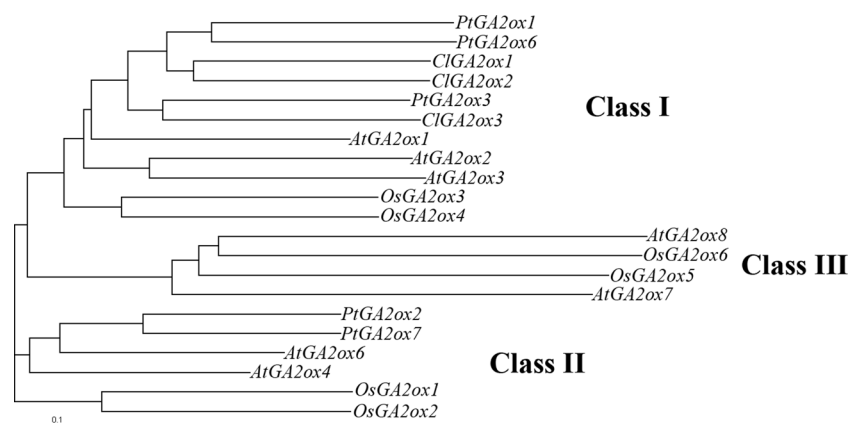


Fig. 1 Phylogenetic relationship of *CIGA2ox1-3* with other plant GA2oxs based on the full-length amino acid sequence. The GenBank accession numbers for each protein are *CIGA2ox1* (KJ502290), *CIGA2ox2* (KJ502289), *CIGA2ox3* (KJ502291), *Populus trichocarpa PtGA2ox1* (EEE85235), *PtGA2ox2* (EEE80803), *PtGA2ox3* (EEE86215), *PtGA2ox6* (EEE97401), *PtGA2ox7* (EEE99275), *Arabidopsis thaliana AtGA2ox1* (CAB41007), *AtGA2ox2* (CAB41008),

AtGA2ox3 (CAB41009), *AtGA2ox4* (AEE32235), *AtGAox6* (AEE27425), *AtGAox7* (AEE32606), *AtGAox8* (AEE84420), *Oryza sativa OsGAox1* (BAB40934), *OsGA2ox2* (BAC167451), *OsGA2ox3* (BAC16752), *OsGAox4* (BAF17950), *OsGAox5* (BAF20604), and *OsGAox6* (BAF15255). The scale value of 0.1 indicates 0.1 amino acid substitutions per site

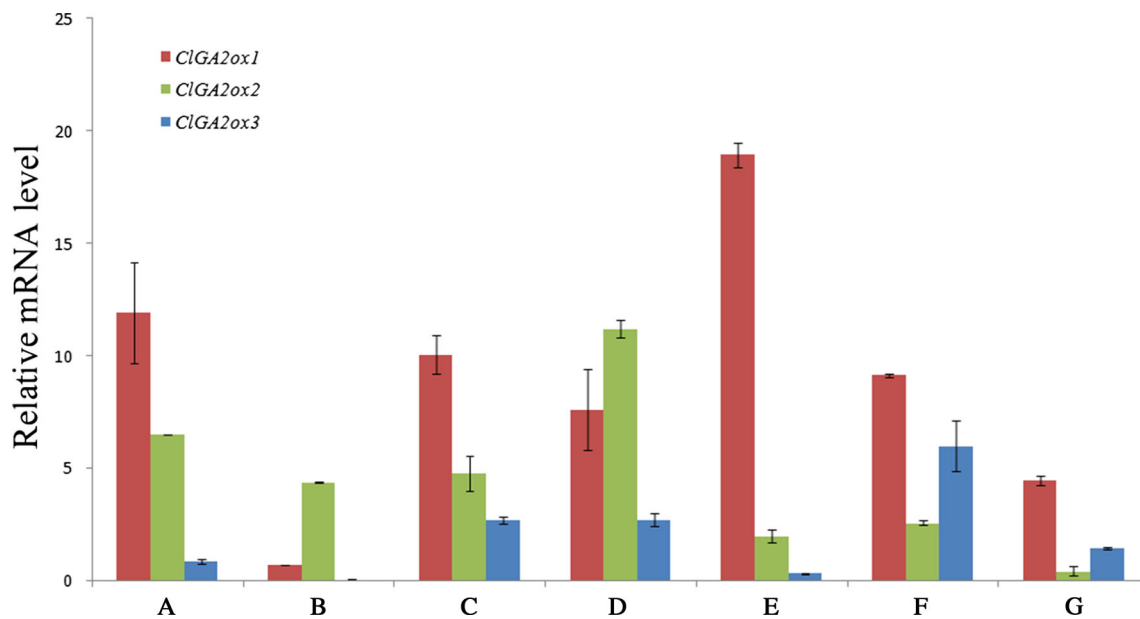


Fig. 2 Relative expression levels of the *CIGA2ox1-3* genes measured by qRT-PCR. Expression levels in the following organs from 3-year-old *Camellia lipoensis*. *A* apex, *B* young leaf, *C* medium-aged leaf (fully

elongated), *D* mature leaf, *E* internode, *F* roots, *G* seed. Relative amounts of each gene are normalized to *ClActin*. Values are means \pm SE of three technical replicates

that are conserved within the GA 2-oxidase class, including the 2-oxoglutarate-binding and the iron-binding sites (Thomas et al. 1999), confirming that *CIGA2ox1-3* belongs to this class of enzymes. A phylogenetic tree was constructed using the deduced amino acid sequences of *CIGA2oxs* with other plant GA2oxs (Fig. 1). The GA 2-oxidase family was distributed among three different clades.

To elucidate the roles of each *GA2ox* gene in various organs of *C. lipoensis*, total RNA was extracted from the apex, leaves, stems, roots, and seeds. To assess the function of each of the *CIGA2ox1-3* genes in plant development, we analyzed their developmental expression profiles using qRT-PCR. Figure 2 shows that *CIGA2ox1-3* transcripts could be detected at all developmental stages analyzed, indicating GA activity throughout plants during many developmental processes (Fleet and Sun 2005). *CIGA2ox1* was the most highly expressed gene in most samples, with the exception of young

and mature leaves. The expression levels of the other two genes increased with leaf development. *CIGA2ox3* was dominant early in development, with high levels found in roots, and *CIGA2ox2* transcripts reached the highest levels in mature leaves. This data set showed that the *CIGA2ox1* transcript was more abundant in the stem and shoot apex than in other tissues, *CIGA2ox2* was more abundant in mature leaves, and *CIGA2ox3* was more abundant in roots. Additionally, *CIGA2ox1* was the most highly expressed gene at most developmental stages.

Southern Blot Analysis and qRT-PCR Analysis for *CIGA2ox1-3* Gene Expression

RT-PCR analysis revealed that *CIGA2ox1-3* was overexpressed in transgenic tobacco plants (Fig. 3). Moreover, Southern blot analysis showed that the nine transgenic lines were derived

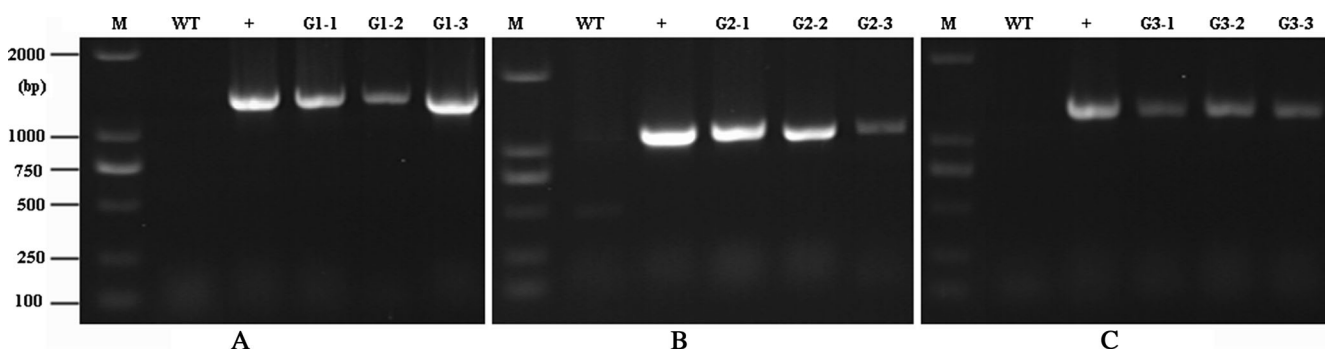


Fig. 3 RT-PCR of the transgenic lines using *CIGA2ox1-3* specific sequence as the forward primer and the reverse primer (Table S2). **a** Transgenic lines with the *CIGA2ox1* gene. **b** Transgenic lines with the

CIGA2ox2 gene. **c** Transgenic lines with the *CIGA2ox3* gene. *M* DNA molecular marker, + positive control amplifying plasmid, *WT* non-transgenic plants

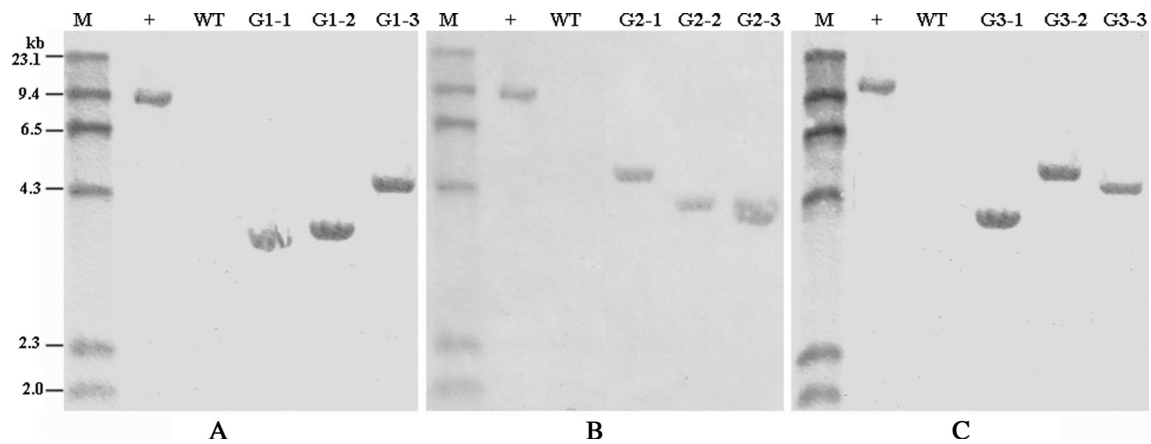


Fig. 4 Southern blot of transgenic plants. **a** Transgenic lines with the *CIGA2ox1* gene. **b** Transgenic lines with the *CIGA2ox2* gene. **c** Transgenic lines with the *CIGA2ox3* gene. The genomic DNA was

digested with *Bam*HI, and a DIG-labeled probe was formed using the primer sets (Table S3). *M* DNA molecular weight marker, + positive plasmid, *WT* represents the non-transgenic plant

from independent plants (Fig. 4). All of the transgenic lines had one copy of the transgene. The control plants yielded no fragments. *CIGA2ox1-3* transcripts were detected in all of the transgenic lines, and no transcripts were detected in non-transgenic plants according to qRT-PCR analysis (Fig. 5). Among the three transgenic lines with the *CIGA2ox3* gene, two severely dwarfed lines (G3-1 and G3-3) showed relatively high expression levels, whereas the moderately dwarfed line (G3-2) showed a relatively low expression level. The transgenic lines with the *CIGA2ox1* or *CIGA2ox3* gene showed relatively higher expression levels than the *CIGA2ox2* gene, inducing dwarfism.

Production and Morphological Characterization of Transgenic Plants

All of the transgenic plants were morphologically distinguished from the control plants (Table 1). The transgenic plants with *CIGA2ox1* or *CIGA2ox3* genes exhibited dwarf phenotypes due to their decreased internode length. Both leaves and internode lengths in the transgenic lines were significantly shorter than the control, producing smaller, darker green, and rounder leaves compared with the control. Transgenic lines with the *CIGA2ox3* gene could be classified into two classes according to the

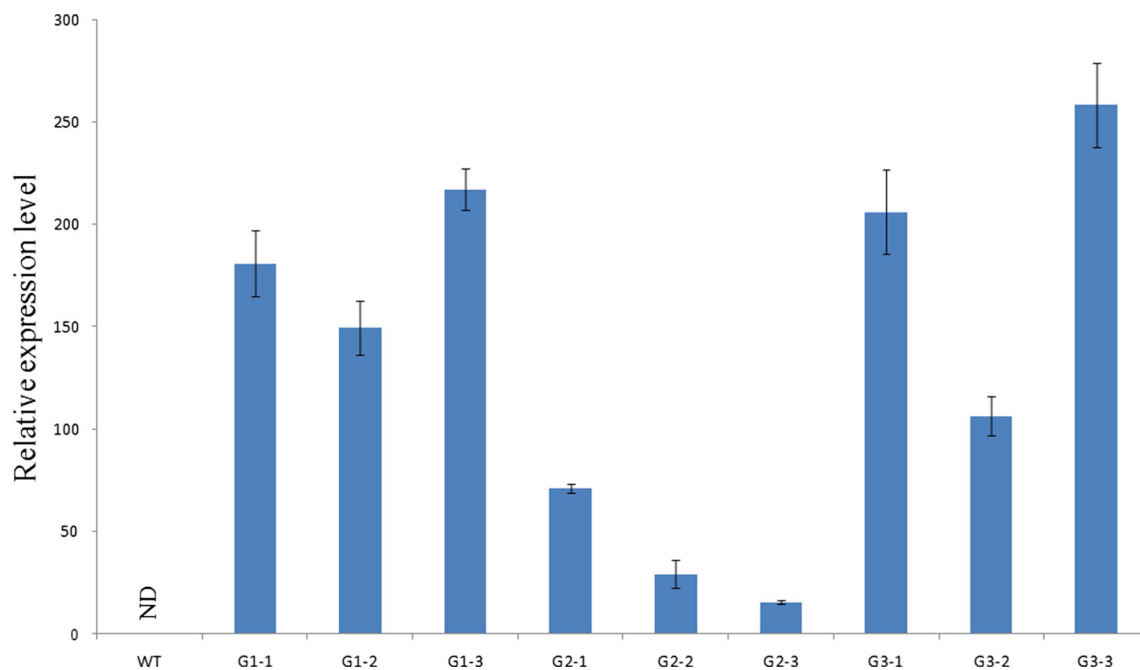


Fig. 5 Quantitative real-time PCR analysis of *CIGA2ox1-3* transcripts in young leaves of the control plant and transgenic plants. The expression levels of each gene were relative to *NicActin*. *WT* non-transgenic plant. No expression of *CIGA2ox1-3* gene was detected in *WT* (*ND* not

detected). *G1-1~G1-3* expression level of the *CIGA2ox1* gene in different transgenic lines. *G2-1~G2-3* expression level of the *CIGA2ox2* gene in different transgenic lines. *G3-1~G3-3* expression level of the *CIGA2ox3* gene in different transgenic lines

Table 1 Characterization of transgenic tobacco overexpressing *CIG2ox1-3*

Traits	WT	35S: <i>CIG2ox1</i>	35S: <i>CIG2ox2</i>	35S: <i>CIG2ox3</i>
Height (cm)	51.0±1.7a	5.5±0.9b	49.1±1.0a	3.1±0.6c
Leaf length (cm) ^a	23.4±0.2a	7.5±0.2b	24.4±0.8a	5.3±0.2c
Leaf width (cm) ^a	13.1±0.9a	5.1±0.1b	13.9±0.3a	4.2±0.2b
Internode length (cm) ^b	3.4±0.4a	0.3±0.1b	3.5±0.3a	0.2±0.1b
Petal length (cm) ^c	4.9±0.2a	2.7±0.1b	4.8±0.1a	4.7±0.1a
Days to flower bud (day) ^d	49.0±2.0c	68.7±4.0b	54.7±3.5bc	89.0±6.6a
Number of fruit	41.7±5.1a	10.3±0.6b	20.3±1.5b	15.7±2.1b

Values represent the mean ± standard error of three plants for each line. Letters (a, b, c) represent significant differences at the 0.01 level according to the Tukey-Kramer test

^a Randomly selected, the three largest leaves were measured for each plant

^b The sixth internode counting from the apex was measured for each plant

^c Three randomly selected flowers were measured for each plant

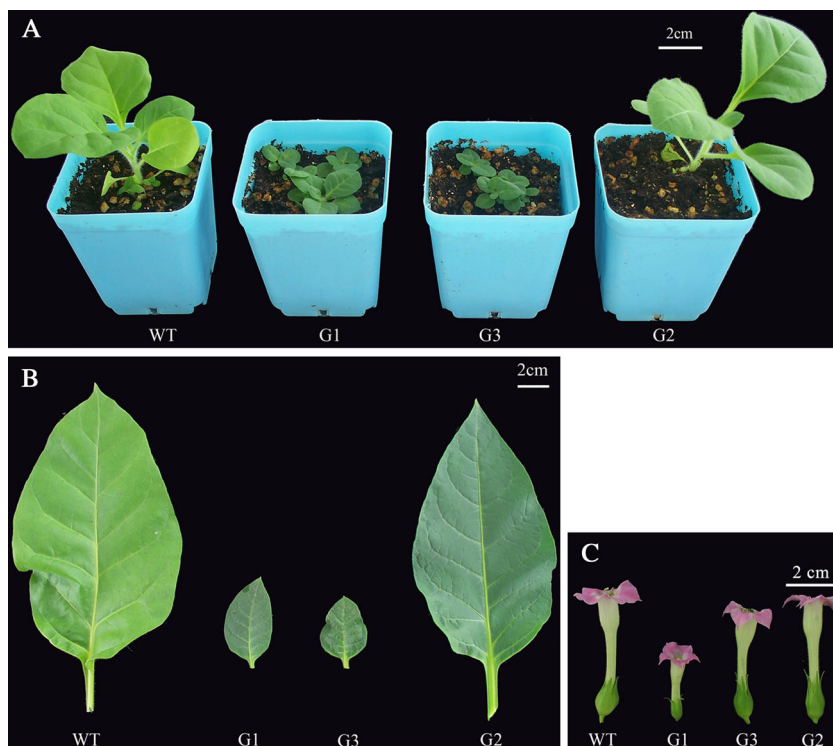
^d Mean number of days until the first open flower

degree of dwarfism: one line (G3-2) was moderately dwarfed, and two lines (G3-1 and G3-3) were severely dwarfed. G3-2 produced normal flowers, but the severely dwarfed lines (G3-1 and G3-3) produced no visible flower buds. All of the transgenic lines with the *CIG2ox1* gene produced smaller flowers compared with the control (approximately half the size of the control flowers). The *CIG2ox2* gene had little effect on the morphological characterization of transgenic plants but did result in darker green leaves (Fig. 6).

Dwarfism is Reversed by GA₃ Application

GA₃ is a bioactive form of GA, which cannot be metabolized by GA2ox (Sakamoto et al. 2001). Therefore, if the transformants have low levels of bioactive GA due to a high rate of inactivation by GA2ox, the application of GA₃ should be able to rescue the dwarf phenotype. The exogenous GA₃ treatment increased the shoot length and leaf size of both transgenic lines. Two weeks after GA₃ application, the phenotypes of the transgenic lines were restored, similar to the

Fig. 6 The phenotypes of transgenic plants. **a** Phenotypes of the second generation transgenic plants grown for 4 weeks. **b** Leaves from transgenic plants grown for 50 days. **c** Flowers from transgenic plants. *G1*, *G2*, and *G3* represent transgenic plants with the *CIG2ox1*, *CIG2ox2*, and *CIG2ox3* genes, respectively. *WT* represents the non-transgenic plant



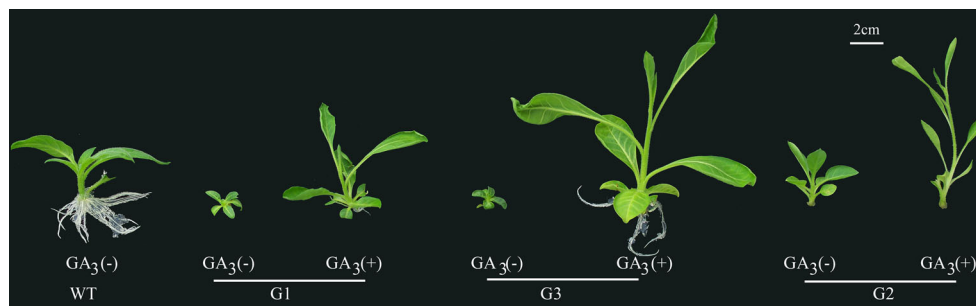


Fig. 7 Effects of GA_3 on plant phenotype of transgenic plants. Two-week-old plants cultivated in MS medium containing 100 μM GA_3 . GA_3 (+) and GA_3 (-) represent treatments with and without GA_3 ,

respectively. *G1*, *G2*, and *G3* represent transgenic plants with the *CIGA2ox1*, *CIGA2ox2*, and *CIGA2ox3* genes, respectively. *WT* represents the non-transgenic plant. *Bar* represents 2 cm

control (Fig. 7). The results demonstrated that the GA signaling pathway might function normally in transgenic plants. Therefore, morphological changes in transgenic plants may result from a decrease in the endogenous level of bioactive GA.

Analysis of the Endogenous GA Level

The level of bioactive GA_4 and GA_1 decreased in both transgenic plants and control plants treated with PAC (Fig. 8). The GA_4 concentration in the transgenic plants overexpressing the *CIGA2ox3* gene reduced to 42.9 % of the controls, similar to that of control plants treated with PAC, and the GA_1 content reduced to 38.7 %. The level of GA_{34} increased in the transgenic plants. No metabolism of GA_8 was observed in the transgenic plants. There were no significant changes in the GA_3 concentration between transgenic plants and controls.

Discussion

In this study, we isolated three full-length cDNA clones encoding GA 2-oxidase from *Camellia*. The predicted proteins of these cDNAs contain the regions typically conserved in plant 2-oxoglutarate-dependent dioxygenases. The sequences are closely related to other known GA 2-oxidases

and share 70 % amino acid identity with *PtGA2ox1*. A phylogenetic tree was constructed, and the GA 2-oxidase family was distributed among three different clades. The class I and class II enzymes catabolize C_{19} -GAs, and class III enzymes catabolize C_{20} -GAs (Yamaguchi 2008). The phylogenetic tree revealed that *CIGA2oxs* belong to class I, with *AtGA2ox1*, *AtGA2ox2*, *AtGA2ox3*, *OsGA2ox3*, and *OsGA2ox4*.

GA 2-oxidases are encoded by multigene families. In contrast to the 20-oxidase and 3 β -hydroxylase genes, which show tissue-specific expression (Phillips et al. 1995), *CIGA2ox1-3* showed different patterns of expression. The transcript abundance of the *CIGA2ox1* gene in the internode and apex was higher than that of *CIGA2ox2-3* genes. The *CIGA2ox2* gene was found to be more abundant in mature leaves, and *CIGA2ox3* was more abundant in roots. Thus, future characterization of the camellia GA2ox gene family and its regulatory mechanisms and functional roles should enable more specific manipulation of GA-related traits.

The qRT-PCR analysis showed that *CIGA2ox1* was expressed at a high level in the internodes and apex, *CIGA2ox2* was expressed at a high level in the mature leaves, and *CIGA2ox3* was highly expressed in roots and at relatively low levels in other organs compared with *CIGA2ox1-2*. It can be concluded that diverse GA2oxs may function quite differently throughout a plant's growth and development. The overexpression of a GA2ox that mainly functions in vegetative

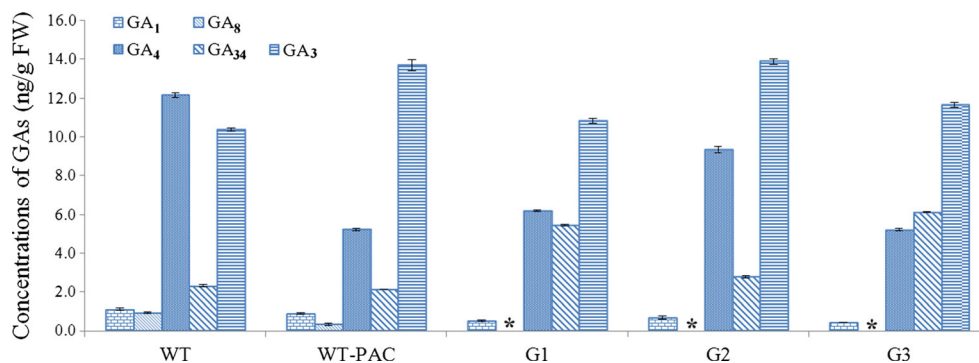


Fig. 8 Concentration of endogenous GAs in apical shoots. *WT* represents the non-transgenic plant. *WT-PAC* represents the control plant treated with paclobutrazol. *G1*, *G2*, and *G3* represent transgenic plants with the

CIGA2ox1, *CIGA2ox2*, and *CIGA2ox3* genes, respectively. Values represent the mean \pm standard error of three replicates. Asterisks undetectable GAs

production may cause dwarf plants with normal flowering and seed production, such as with *CIGA2ox1*. The overexpression of a GA2ox that mainly functions in old organ metabolism may have little effect on dwarfism induction, such as with *CIGA2ox2*. The overexpression of a GA2ox that is expressed at a low level throughout plant growth may cause severe dwarfism, such as with *CIGA2ox3*.

GA is known to stimulate stem elongation in many plant species, and GA treatment causes an increase in the number of vegetative stem internodes that elongate upon bolting and in the number of flowers, resulting in an overall increase in plant height (Rieu et al. 2008). The overexpression of *CIGA2ox1* or *CIGA2ox3* in *N. tabacum* had a profound effect on plant growth and development, and resulted in reduced growth and later flowering (Table 1). GA₄ and GA₁ are thought to be the major bioactive GAs that existed in most species. It has been confirmed that GA2ox can catalyze GA₄ and GA₁ to GA₃₄ and GA₈, respectively. The analysis of the endogenous GAs showed a reduced GA₄ and GA₁ level in transgenic plants; the GA₃₄ content increased compared with the wild plants. A similar severe dwarf phenotype was produced when the control tobacco was treated with the GA biosynthesis inhibitor PAC. The endogenous GA₄ and GA₁ level was reduced and was similar to that of transgenic plants. The GA-deficient phenotype of transgenic plants could be restored by applying GA₃ (Fig. 7). This result strongly supports the hypothesis that dwarf phenotypes are a result of the deficiency of bioactive GAs. Our findings also provide a potential method for controlling the growth of transgenic plants overexpressing GA2ox genes via the exogenous application of GA₃ during horticultural manipulation. For example, managers might speed early growth using GA application to reach a desired stature. Once the GA application ceases, the transgenic plant overexpressing GA2ox will resume slower growth.

Semi-dwarfism is one of the most valuable traits in horticulture breeding because semi-dwarf plants are more resistant to damage by wind and rain. The overexpression of GA 2-oxidase is an easy way to reduce the bioactive GA levels in transgenic plants; however, the ectopic expression of most GA2oxs caused severe dwarfism and seed abortion in various plant species (Singh et al. 2002; Biemelt et al. 2004; Hedden and Thomas 2012). *CIGA2ox1* and *CIGA2ox3* in *N. tabacum* caused dwarf plants to flower late and produce seeds normally. Notably, flowers from transformants overexpressing *CIGA2ox1* were half the size of the controls (Fig. 6c), and transgenic plants with *CIGA2ox2* or *CIGA2ox3* retained normal flower size; thus, *CIGA2ox1* may play a specific role in flower development. Few studies regarding GA2ox affecting flower size have been reported. This gene is very useful in horticulture breeding and not only induces dwarfism but also regulates flower development.

The manipulation of ornamental plant stature has long been a major goal in horticulture. Camellias are typically taller in

stature than is desirable in potted flower production. Consequently, plant growth retardants are widely applied to inhibit plant elongation, often acting by disrupting the synthesis of GAs (Rademacher 2000). However, plant retardants require repeated applications of chemicals that are not only expensive but also hazardous to workers and the environment. Genetic manipulation may provide an alternative approach to the use of plant growth retardants because plant dwarf phenotypes can be achieved by upregulating genes encoding enzymes involved in GA catabolism (Hedden and Phillips 2000).

In conclusion, the present study shows that the overexpression of *CIGA2ox1* or *CIGA2ox3* in transgenic tobacco results in a dwarf phenotype. Whereas flowers from transgenic plants with 35S:*CIGA2ox1* were approximately half the size of the control plants, and produced seeds normally. Flowers were not observed in transgenic lines with a high expression of *CIGA2ox3*, whereas the transgenic plants with lowly expressed *CIGA2ox3* flowered normally. These results demonstrate that the overexpression of *CIGA2ox1* may be a useful method for obtaining compact growth and small flower ornamental plants. The compact ornamental plants with normal flowers may be induced by a low expression of *CIGA2ox3*. The effects of transformations using constructs in which *CIGA2ox3* is controlled by a weak expression activity or tissue-specific promoter will be investigated in future studies.

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