The Horticulture Journal *Preview*
doi: 10.2503/hortj.OKD-016

The Japanese Society for Horticultural Science
http://www.jshs.jp/

Gibberellin 2-Oxidase Genes from Satsuma Mandarin (*Citrus unshiu* Marc.) Caused Late Flowering and Dwarfism in Transgenic *Arabidopsis*

Nobuhiro Kotoda[1]*,, Satoshi Matsuo[2], Ichiro Honda[3], Kanako Yano[1] and Tokurou Shimizu[1]**

[1]*Institute of Fruit Tree and Tea Science, NARO, Shizuoka 424-0292, Japan*
[2]*Institute of Vegetable and Floriculture Science, NARO, Tsu 514-2392, Japan*
[3]*Department of Biotechnology, Maebashi Institute of Technology, Maebashi 371-0816, Japan*

Gibberellins (GAs) are involved in many aspects of plant growth and development in higher plants. In this study, we aimed to characterize Satsuma mandarin (*Citrus unshiu* Marc.) GA 2-oxidase genes encoding enzymes with GA inactivation activity because the accumulation of active GAs is regulated by the balance between their synthesis and inactivation. We showed that *CuGA2ox4*, *CuGA2ox2/3*, and *CuGA2ox8* were differentially expressed in various tissues in Satsuma mandarin and that these genes functioned like GA 2-oxidase genes in transgenic *Arabidopsis*. The expression pattern of *CuGA2ox4* resembled that of *CuGA20ox1*, which was reported previously as a Satsuma mandarin GA 20-oxidase gene, in the reproductive tissues of the adult trees in that both genes were expressed in juice sacs in November and December and peel in December, suggesting that *CuGA2ox4* and *CuGA20ox1* act in concert to regulate the accumulation of active GAs in fruit at the maturing stage. On the other hand, *CuGA2ox2/3* and *CuGA2ox8* were more highly expressed in seeds and flower buds, respectively. Further study of GA biosynthetic genes, including GA 2-oxidase genes, would provide insight into the mechanism of flowering, fruit development, seedlessness, biennial bearing, and peel puffins of citrus such as Satsuma mandarin.

Key Words: fruit tree, gene expression, parthenocarpy, plant growth, seedless fruit.

Introduction

Extensive studies over many years revealed that gibberellins (GAs) are involved in many aspects of plant growth and development, such as cell elongation, cell division, dormancy, flowering, and fruit development in higher plants. Researchers have also conducted biochemical and molecular studies to clarify the biosynthetic pathways of GAs in plants (Hedden and Kamiya, 1997; Hedden and Phillips, 2000; Yamaguchi, 2008). These studies revealed that there are 2 pathways for synthesizing active GAs; the early-13-hydroxylation pathway, where GAs such as GA_1 and GA_3, are synthesized sequentially through GA_{53}, GA_{44}, GA_{19}, and GA_{20} after the synthesis of GA_{12} through some steps from isopentenyl diphosphate and dimethylallyl diphosphate and the non-13-hydroxylation pathway where GAs, such as GA_4, are synthesized through GA_{15}, GA_{24}, and GA_9 after the synthesis of GA_{12}. In the biosynthetic pathway, GA 20- and GA 3-oxidase genes accelerate the synthesis of the active GAs, whereas GA 2-oxidase genes inactivate the active GAs through 2β-hydroxylation.

Citrus, such as Satsuma mandarin (*Citrus unshiu* Marc.), is thought to use the early-13-hydroxylation pathway because citrus accumulates 13-OH GAs such as GA_1, more abundantly than it accumulates 13-H GAs such as GA_4 (Goto et al., 1989; Poling and Maier, 1988; Talon et al., 1990a, b; Turnbull, 1989). In addition to the study in rice and *Arabidopsis*, much effort has been devoted to understanding the physiological role of GAs

Received; May 13, 2016. Accepted; August 1, 2016.
First Published Online in J-STAGE on September 27, 2016.
This study was supported by a Grant-in-Aid for Scientific Research (C) from the Ministry of Education, Culture, Sports, Science and Technology (No. 24580058) and a Research Project for Molecular Evaluation of Parthenocarpy on Horticultural Crops from NARO (No. 210).

* Corresponding author (E-mail: koto@cc.saga-u.ac.jp; nobu_fruits@yahoo.co.jp).
** Present address: Faculty of Agriculture, Saga University, Saga 840-8502, Japan

in woody plants, including fruit trees. It has been demonstrated that in apple (*Malus* × *domestica* Borkh.) and citrus trees, GAs function negatively in terms of flower induction (Luckwill and Silva, 1979; Monselise, 1979; Tromp, 1982). On the other hand, Nagao et al. (1989) reported that GAs play a positive role in the flowering of the Japanese cedar (*Cryptomeria japonica*). The rosette and dwarf seedlings with internodes of less than 1 mm obtained from the selfing and crossing of trifoliate orange (*Poncirus trifoliata* Raf.) strains, such as the 'Hiryu' ('Flying Dragon'), responded to GA_3 application with a significant elongation of the internodes, suggesting the importance of internal GAs for internode elongation in citrus (Yoshida, 1994). In several dwarf plum (*Prunus salicina* L.) genotypes, a markedly elevated level of a GA 2-oxidase gene (*PslGA2ox*) expression was responsible for the dwarfism, and *PslGA2ox* could be a marker for developing size-controlling rootstocks in prunus (El-Sharkawy et al., 2012). In grapes, a practical method for producing seedless berries by applying GA_3 was established as early as the late 1950s (Kishi and Tasaki, 1960).

The production of seedless fruit is also very important for breeders and growers to meet consumer demands in citriculture. Satsuma mandarins are considered to be absolutely seedless because it has both female and male sterility with parthenocarpy (Iwamasa, 1966; Vardi et al., 2008). Talon et al. (1990b) reported that in Satsuma mandarin, the development of ovaries preceded that in Clementine (*Citrus clementina* hort. ex Tanaka), which shows self-incompatibility and low parthenocarpy. In addition, active GAs and their precursors were more highly accumulated in the ovaries at around anthesis in Satsuma mandarin than in Clementine (Talon et al., 1990b, 1992). These results imply a relationship between the degree of seedlessness and the endogenous GAs. In recent years, GA biosynthetic genes have been isolated and analyzed to clarify their functions in citrus (Fagoaga et al., 2007; Huerta et al., 2009; Kotoda et al., 2016; Vidal et al., 2001, 2003). A previous study identified two GA 20-oxidase genes from Satsuma mandarin and confirmed their function using transgenic *Arabidopsis*. Consistent with the accumulation of active GAs in the ovaries at around anthesis, *CuGA20ox2* was specifically expressed in the flower bud just before anthesis in Satsuma mandarin (Kotoda et al., 2016). In this study, Satsuma mandarin GA 2-oxidase genes encoding enzymes with an inactivation activity of GAs were characterized because the accumulation of active GAs is regulated by the balance between their synthesis and inactivation. Molecular characterization of GA biosynthetic genes, including GA 2-oxidase genes, would be helpful in studying the mechanisms of important physiological events such as flowering and parthenocarpy in citrus.

Materials and Methods

Plant materials

Tissue samples of a Satsuma mandarin (*Citrus unshiu* Marc.) 'Silverhill' in the adult phase (age: 43 years) were collected from the experimental field at the Institute of Fruit Tree and Tea Science, NARO, in Okitsu, Shizuoka, Japan. Two-year-old nucellar seedlings of 'Silverhill', which were distinguished from hybrids by simple sequence repeat (SSR) genotyping analysis, were used for expression analysis in the juvenile phase. Seeds used in this study were derived from 'Silverhill' Satsuma mandarin harvested in January in 2009. Satsuma mandarins ('Silverhill' and 'Miyagawa wase'), 'Kiyomi' tangor (*C. unshiu* × *C. sinensis* Osbeck), Hyuganatsu (*C. tamurana* hort. ex Tanaka), Clementine, 'Clementine Vita' (*Citrus clementina* hort. ex Tanaka 'Clementine Vita'), sweet orange (*C. sinensis* Osbeck 'Trovita'), pummelo (*C. grandis* Osbeck 'Benimadoka' and 'Banpeiyu'), Sudachi (*C. sudachi* hort. ex Shirai), Yuzu (*C. junos* Sieb. ex Tanaka), Kunenbo (*C. nobilis* Lour.), and trifoliate orange were used for Southern blot analysis. Wild-type plants of the ecotype Columbia (Col) were used for *Arabidopsis* [*Arabidopsis thaliana* (L.) Heynh] transformation. *Arabidopsis* seeds were stratified for 3 to 4 days at 4°C and then grown on an agar plate containing 0.5× Murashige and Skoog (MS) medium (Wako Pure Chemicals Co. Ltd., Tokyo, Japan) in a growth chamber at 22°C. Ten days after incubation on the agar plate, seedlings were transferred to soil and grown in growth chambers under long day (LD) conditions (16 h light/8 h dark).

Isolation of GA 2-oxdase genes from Satsuma mandarin

To obtain genomic sequences of GA 2-oxidase genes from Satsuma mandarin, we screened a bacterial artificial clone (BAC) library of Satsuma mandarin ('Miyagawa wase') and sequenced three clones named STR00402, STR00547, and STR00577, which contained homologous genes of GA 2-oxidase. The 3 GA 2-oxidase-like genes in the clones STR00402, STR00547, and STR00577 were designated as *CuGA2ox4* (Accession No. LC149862), *CuGA2ox2/3* (Accession No. LC149863), and *CuGA2ox8* (Accession No. LC149864) based on the phylogenetic analysis described below. Leaves and ovaries of 'Silverhill' Satsuma mandarin were used to isolate the cDNA of *CuGA2ox4*, *CuGA2ox2/3*, and *CuGA2ox8*. cDNA from the leaves and ovaries was amplified by polymerase chain reaction (PCR) with the oligonucleotide primers STR00402_F1 and STR00402_R1 for *CuGA2ox4*, STR00547_F1 and STR00547_R1 for *CuGA2ox2/3*, and STR00577_F1 and STR00577_R1 for *CuGA2ox8*, designed based on the genomic sequences of *CuGA2ox4*, *CuGA2ox2/3*, and *CuGA2ox8*, respectively, using a taq polymerase, KOD-plus (Toyobo, Osaka, Japan). Each amplified product was cloned into the

*Eco*RV site of the pBlueScript II SK(+) vector (Agilent Technologies, Santa Clara, CA, USA) after phosphorylation of the products. All PCR products for isolation of the cDNA of *CuGA2ox4*, *CuGA2ox2/3*, and *CuGA2ox8* were subcloned and sequenced (ABI 3130xl DNA sequencer; Life Technologies, Carlsbad, CA, USA). The oligonucleotide primer sets used in gene cloning are listed in Table 1.

Construction of a phylogenetic tree

The amino acid sequences were aligned using the ClustalX multiple sequence alignment program ver. 2.0.5 (Larkin et al., 2007; Thompson et al., 1997), and the phylogenetic tree was constructed by the neighbor-joining (N-J) method for the deduced amino acid sequence of the GA biosynthetic genes from *Arabidopsis* [*AtGA2ox1* (AT1G78440), *AtGA2ox2* (AT1G30040), *AtGA2ox3* (AT2G34555), *AtGA2ox4* (AT1G47990), *AtGA2ox6* (AT1G02400), *AtGA2ox7* (AT1G50960), *AtGA2ox8* (AT4G21200), *AtGA3ox1* (AT1G15550), *AtGA3ox2* (AT1G80340), *AtGA3ox3* (AT4G21690), *AtGA3ox4* (AT1G80330), *AtGA20ox1* (AT4G25420), *AtGA20ox3* (AT5G07200), and *AtKAO2* (AT2G32440)] and Satsuma mandarin [*CuGA20ox1* (LC056054), *CuGA2ox4* (LC149859), *CuGA2ox2/3* (LC149860), and *CuGA2ox8* (LC149861)]. The unrooted tree was displayed using the NJplot program (Perrière and Gouy, 1996). The alphanumeric characters in parentheses represent the accession numbers used in TAIR (The Arabidopsis Information Resource, https://www.arabidopsis.org/) or GenBank/EMBL/DDBJ.

DNA extraction and hybridization analysis

Genomic DNA was isolated using a cetyltrimethylammonium bromide (CTAB)-based method as described in Kotoda et al. (2002). For Southern blot analysis, the genomic DNA (5 µg) was digested with *Xba*I, electrophoresed on a 0.8% agarose gel, and then blotted onto Hybond-N+ nylon membranes (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). Each blotted membrane was hybridized with digoxigenin (DIG; Roche Diagnostics, Mannheim, Germany)—labeled *CuGA2ox4*, *CuGA2ox2/3*, or *CuGA2ox8* cDNA, which was amplified by PCR with a pair of oligonucleotide primers used for gene cloning and a DIG mixture (Roche Diagnostics). Hybridization was performed in DIG Easy Hyb (Roche Diagnostics) at 42°C for 16 h, followed by 2 rinses in 2x saline sodium citrate (SSC) containing 0.1% (w/v) sodium dodecyl sulfate (SDS) at room temperature for 2 min and 2 washes in 0.5x containing 0.1% (w/v) SDS at 68°C for 20 min as described by Kotoda et al. (2006). Detection was

Table 1. Oligonucleotide primer sets used in cloning, quantitative real-time RT-PCR analysis, vector construction, and probe labelling.

Gene	Primer	Oligonucleotide (5′ → 3′)
Cloning/probe labelling		
CuGA2ox4	STR00402_F1	GTTCTGATATTTCATGCAATTA
	STR00402_R1	GATTCAGAAGATGCCTCAAAA
CuGA2ox2/3	STR00547_F1	AGAAATTAGAAGTAGTAGCC
	STR00547_R1	ACTTCGACAAAAACCCTAAT
CuGA2ox8	STR00577_F1	GTTCTTACCAGCAGAAGTCT
	STR00577_R1	AAACTAATCGTAGAAAACATTC
Real-time RT-PCR		
CuGA2ox4	STR00402 (379 > 402)	GACCCTTCAAAATTCGGGTCAGCG
(amplicon, 138 bp)	STR00402 (491 < 516)	AACGTCCCTGATCATCTTACTGAAGT
CuGA2ox2/3	STR00547 (753 > 775)	TGCCTTGCAG GTAATGACTAATG
(amplicon, 125 bp)	STR00547 (851 < 877)	ATCTTTTCGCTCAAAGGTGGCC
CuGA2ox8	STR00577 (902 > 925)	GAGACTTATTCCAGGCTTGGAGCA
(amplicon, 134 bp)	STR00577 (1011 < 1035)	TGAATTCTGAATCACAGTGTCGTAC
Vector construction		
CuGA2ox4	STR00402_F1-XbaI	AAtctagaGTTCTGATATTTCATGCAATTA
	STR00402_R1-SacI	AAgagctcGATTCAGAAGATGCCTCAAAA
CuGA2ox2/3	STR00547_F1-XbaI	AAtctagaAGAAATTAGAAGTAGTAGCC
	STR00547_R1-KpnI	AAggtaccACTTCGACAAAAACCCTAAT
CuGA2ox8	STR00577_F1-XbaI	AAtctagaGTTCTTACCAGCAGAAGTCT
	STR00577_R1-SacI	AAgagctcAAACTAATCGTAGAAAACATTC

The thermal cycle programs for real-time RT-PCR were as follows: 95°C for 10 min, followed by 40 cycles of at 95°C for 15 s, 62°C for 60 s for *CuGA2ox4* [STR00402 (379 > 402) and STR00402 (491 < 516)], 95°C for 10 min, followed by 40 cycles of at 95°C for 15 s, 60°C for 60 s for *CuGA2ox2/3* [STR00547 (753 > 775) and STR00547 (851 < 877)] and *CuGA2ox8* [STR00577 (902 > 925) and STR00577 (1011 < 1035)]. Characters in lowercase indicate the site of restriction enzymes used for vector construction. Oligonucleotide primer sequences and PCR conditions for *CuActin* and *AtTUB4* are described in Kotoda et al. (2016).

performed according to the manufacturer's protocol (Roche Diagnostics). Chemiluminescent signals were visualized using the NightOWL image analyzer (Berthold Technologies, Bad Wildbad, Germany). The oligonucleotide primer sets used for labelling are listed in Table 1.

Expression analysis by quantitative real-time RT-PCR

Using a phenol-SDS-based method as described by Ikoma et al. (1996), total RNA was extracted for analysis from seeds (derived from 'Silverhill' Satsuma mandarin harvested in January) and roots (whole roots including lateral ones), stems, new and old leaves, and nodes of 2-year-old juvenile nucellar seedlings derived from 'Silverhill' Satsuma mandarin, as well as from new and old leaves, shoot apexes in August, flower buds (just before anthesis), young fruit in June, juice sacs (in September, November, and December), and peel (in September and December) of adult 'Silverhill' Satsuma mandarin trees. For transgene expression analysis, whole plants of transgenic *Arabidopsis* in the second generation (T_2) and controls were collected 2 months after incubation, and total RNA was extracted from 2 individuals per line by using a CTAB-based method. The samples of total RNA were then purified using an RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). First-strand cDNA was synthesized from 1 μg of total RNA in 20 μL of a reaction mixture using a QuantiTect Reverse Transcription Kit (Qiagen). Subsequent PCR reactions were performed with 1 μL of the first-strand cDNA as a template in a total volume of 12.5 μL using the ABI PRISM® 7000 (Life Technologies). Transcripts of *CuGA2ox4*, *CuGA2ox2/3*, *CuGA2ox8*, *CuActin* (for reference, Phytozome Transcript Name Ciclev10025866m), and an *Arabidopsis* β tubulin gene (*AtTUB4*) were identified with the specific primers. The oligonucleotide primer sets used in the quantitative real-time RT-PCR (qRT-PCR) analysis and the PCR conditions are described in Table 1. A citrus *CuActin* gene and an *Arabidopsis AtTUB* gene were used as an internal control for analyzing Satsuma mandarin and *Arabidopsis*, respectively. qRT-PCR was performed three times (technical replicates) and transcript levels were normalized against each internal control.

Construction of the transformation vector

To construct vectors for the constitutive expression of *CuGA2ox4*, *CuGA2ox2/3*, and *CuGA2ox8*, the coding region of each gene was amplified by PCR with pairs of oligonucleotide primers STR00402_F1-XbaI and STR00402_R1-SacI for *CuGA2ox4*, STR00547_F1-XbaI and STR00547_R1-KpnI for *CuGA2ox4*, and STR00577_F1-XbaI and STR00577_R1-SacI for *CuGA2ox8*. Each amplified PCR product was subsequently digested with *Xba*I/*Sac*I or *Xba*I/*Kpn*I and then cloned into the corresponding site of the modified pSMAK193E (35SΩ/pSMAK193E; described in Kotoda et al., 2010), a kanamycin-resistant binary vector, to be placed between the 35SΩ and 3' regions of the *Arabidopsis* rbcS-2B gene (TrbcS).

Arabidopsis transformation

An *Agrobacterium tumefaciens* strain EHA101 harboring a binary vector with 35SΩ::*CuGA2ox4*, 35SΩ::*CuGA2ox2/3*, or 35SΩ::*CuGA2ox8* was used to transform *Arabidopsis thaliana* (Col) plants using the floral-dip method (Clough and Bent, 1998). After 2 to 5 adult leaves had developed on the agar plate with kanamycin in a growth chamber (Biotron; Nippon Medical and Chemical Instruments Co., Ltd., Tokyo, Japan) set at 22°C under LD conditions (16-h photoperiod; cool white fluorescent light, 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$), kanamycin-resistant transformants were transplanted from the plate to moistened potting soil composed of vermiculite and perlite (1:1) and further grown in the growth chamber under the same conditions. Morphological and expression analyses were performed on the second generation (T_2). For morphological analysis, the number of days to flowering and the number of rosette leaves at flowering were counted and the length of the 1st inflorescences (infl.) 1 month after the start of incubation was measured. Whole plants of T_2 and controls were collected 2 months after the start of incubation in the growth chamber for qRT-PCR analysis.

Statistical analysis

Data on the number of days to flowering, the number of rosette leaves, and the length of the 1st inflorescence shown in Table 2 were analyzed using one-way ANOVA, and the differences were contrasted using Tukey's multiple comparison test. All statistical analyses were performed at a significance level of $P < 0.05$ using R-3.2.0 (R Core Team, 2015).

Results

Isolation of CuGA2ox4, CuGA2ox2/3, and CuGA2ox8 cDNA from Satsuma mandarin

To investigate the function of GA 2-oxidase genes in Satsuma mandarin, we isolated the cDNA of these genes from Satsuma mandarin 'Silverhill' by PCR with oligonucleotide primer sets based on the genomic sequences of *GA2ox4*-, *GA2ox2/3*-, and *GA2ox8-like* genes, which were cloned from a Satsuma mandarin ('Miyagawa wase') BAC library. The coding sequences of *Citrus unshiu GA2ox4-like* (*CuGA2ox4*; Accession No. LC149859), *Citrus unshiu GA2ox2/3-like* (*CuGA2ox2/3*; Accession No. LC149860), and *Citrus unshiu GA2ox8-like* (*CuGA2ox8*; Accession No. LC149861) cDNA were 1005, 1038, and 1089 bp long including a stop codon and encoded 334, 345, and 362 amino acids, respectively. For the genomic clones of *CuGA2ox4*, *CuGA2ox2/3*, and *CuGA2ox8*, sequences including the coding region of each gene and the exon-

Table 2. Characteristics of transgenic lines ectopically expressing *CuGA2ox4*, *CuGA2ox2/3*, or *CuGA2ox8*.

Line	LD conditions (16 h light/8 h dark)			
	No. of plants	Days to flowering	No. of rosette leaves	Length of the 1st infl. (cm)
Controls				
Wt (Col)	12	28.17 ± 1.27^{ab}	6.58 ± 0.79^{a}	16.38 ± 3.84^{a}
Vector/wt (Col)	10	27.70 ± 1.77^{a}	7.10 ± 1.20^{ab}	16.60 ± 2.86^{a}
35SΩ: *CuGA2ox4*/wt (T_2)				
#7	13	27.15 ± 1.95^{a}	7.92 ± 1.55^{ab}	15.62 ± 3.28^{a}
#8	13	32.46 ± 3.38^{ab}	9.31 ± 1.38^{bc}	**6.15 ± 3.61^{bc}**
#9	13	**33.62 ± 3.01^{bc}**	8.85 ± 1.41^{ab}	**5.69 ± 1.84^{cd}**
#11	12	**34.92 ± 3.40^{d}**	9.50 ± 2.58^{bc}	**5.29 ± 2.35^{cd}**
35SΩ: *CuGA2ox2/3*/wt (T_2)				
#15	13	**41.54 ± 5.25^{e}**	**11.62 ± 1.39^{c}**	**0.23 ± 0.83^{d}**
#16	13	**36.46 ± 4.52^{d}**	**9.85 ± 1.86^{c}**	**3.19 ± 5.20^{cd}**
#17	13	29.92 ± 2.75^{ab}	9.15 ± 1.99^{b}	12.69 ± 4.78^{ab}
#19	12	**45.42 ± 1.38^{e}**	**11.17 ± 2.29^{c}**	**0.00 ± 0.00^{d}**
35SΩ: *CuGA2ox8*/wt (T_2)				
#15	12	**35.50 ± 4.38^{d}**	**9.92 ± 1.16^{c}**	**1.00 ± 1.26^{d}**
#16	13	**33.77 ± 3.49^{c}**	9.46 ± 0.88^{bc}	**1.38 ± 1.37^{cd}**
#17	12	**34.58 ± 5.07^{c}**	9.58 ± 1.44^{bc}	**3.17 ± 3.64^{cd}**
#20	12	**39.25 ± 4.14^{d}**	**10.08 ± 1.08^{c}**	**0.00 ± 0.00^{d}**

Plants in the second generation (T_2) and controls were grown under long-day (LD) conditions. Numbers of rosette leaves were counted on the day floral organs became visible. The length of the 1st inflorescences was measured 1 month after the start of incubation in a growth chamber. Values are means ± standard deviation (SD). Tukey's multiple comparisons were performed to compare the effects of the ectopic expression of the genes. Superscripts letters indicate a statistically significant difference from results with other letters, and values in bold indicate a statistically significant difference from those of a control plant (vector/wt) in the same column ($P < 0.05$).

intron structure were identified (Fig. 1A). To examine the evolutionary relationships among GA biosynthetic genes such as GA 2-, 3-, and 20-oxidase genes, we performed phylogenetic analysis of the amino acid sequences corresponding to these genes from *Arabidopsis* and Satsuma mandarin. N-J distance analysis showed that CuGA2ox4, CuGA2ox2/3, and CuGA2ox8 were grouped closely with AtGA2ox4, AtGA2ox2/3, and AtGA2ox8, respectively (Fig. 1B). Southern blots of the genomic DNA of 12 *Citrus* species/cultivars and a trifoliate orange standard strain digested with *Xba*I hybridized with the DIG-labeled cDNA of *CuGA2ox4*, *CuGA2ox2/3*, and *CuGA2ox8* showed polymorphic bands for *CuGA2ox4* and *CuGA2ox8* (Fig. 2A, C) and a single band of about 9.4 kb for *CuGA2ox2/3* (Fig. 2B).

Organ-specific expression patterns of CuGA2ox4, CuGA2ox2/3, and CuGA2ox8

To clarify the expression patterns of *CuGA2ox4*, *CuGA2ox2/3*, and *CuGA2ox8*, qRT-PCR was performed on various tissues in Satsuma mandarin in the juvenile and adult phases using oligonucleotide primers specific to each gene (Table 1). The transcript of *CuGA2ox4* accumulated predominantly in reproductive tissues, such as juice sacs (JS) in November and December and peel (PL) in December, but only faintly in roots, seeds, and flower buds (FB) (Fig. 3A). In contrast, the transcript level of *CuGA2ox2/3* was extremely low compared with those of the other 2 genes and accumulated mainly in seeds from mature fruit and vegetative tissues, such as roots, new leaves (NL), old leaves (OL), and nodes of seedlings in the juvenile phase, and shoot apices (SA) in the adult phase. *CuGA2ox2/3* was also expressed in flower buds and juice sacs in November (Fig. 3B). The transcript of *CuGA2ox8* accumulated mainly in new leaves and flower buds in the adult phase and stems in the juvenile phase, with low expression in all tissues except seeds, roots, and juice sacs in December.

Phenotypes of transgenic Arabidopsis ectopically expressing CuGA2ox4, CuGA2ox2/3, or CuGA2ox8

To elucidate the function of GA 2-oxidase-like genes of Satsuma mandarin, *CuGA2ox4*, *CuGA2ox2/3*, or *CuGA2ox8*, we introduced the coding region of each gene under the control of the cauliflower mosaic virus (CaMV) 35S promoter fused with an Ω sequence (35SΩ) into *Arabidopsis* (Fig. 4A). We analyzed four independent lines with 12–13 individuals (n = 12–13) in the T_2 generation for phenotypes, including the number of days to flowering, the number of rosette leaves at flowering, and the length of the 1st infl. 1 month after the start of incubation in a growth chamber (Fig. 4; Table 2). For the number of days to flowering and the length of the 1st infl. of plants, there were significant differences between the transgenic lines with

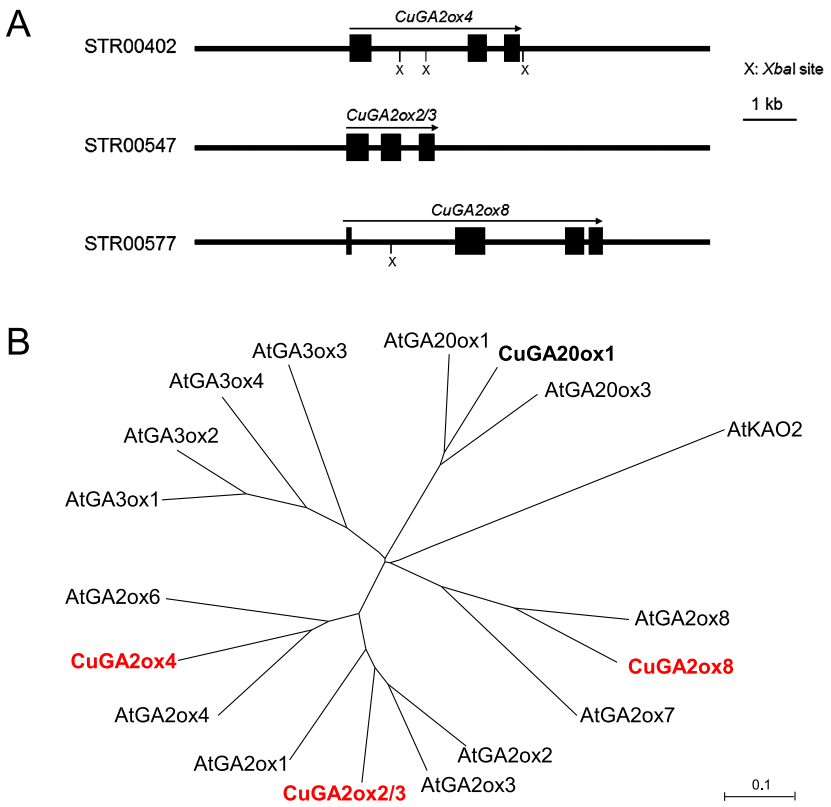

Fig. 1. (A) Schematic representation of the genomic organization and (B) phylogenetic analysis of *CuGA2ox4*, *CuGA2ox2/3*, and *CuGA2ox8*, together with other GA biosynthetic genes. The tree was constructed by the neighbor-joining (N-J) method for the deduced amino acid sequence of the genes from *Arabidopsis* [*AtGA2ox1* (AT1G78440), *AtGA2ox2* (AT1G30040), *AtGA2ox3* (AT2G34555), *AtGA2ox4* (AT1G47990), *AtGA2ox6* (AT1G02400), *AtGA2ox7* (AT1G50960), *AtGA2ox8* (AT4G21200), *AtGA3ox1* (AT1G15550), *AtGA3ox2* (AT1G80340), *AtGA3ox3* (AT4G21690), *AtGA3ox4* (AT1G80330), *AtGA20ox1* (AT4G25420), *AtGA20ox3* (AT5G07200), and *AtKAO2* (AT2G32440)] and Satsuma mandarin [*CuGA20ox1* (LC056054), *CuGA2ox4* (LC149859), *CuGA2ox2/3* (LC149860), and *CuGA2ox8* (LC149861)]. The alphanumeric characters in parentheses represent the accession numbers in TAIR or GenBank/EMBL/DDBJ. Black boxes represent exons and lines represent introns. Vertical bars with x represent the site of the restriction enzyme *Xba*I. The scale bar on the map of the genomic organization represents approximately 1 kb.

35SΩ::*CuGA2ox4*, 35SΩ::*CuGA2ox2/3*, and 35SΩ::*CuGA2ox8* and the control plants [Vector/wt(Col): transgenic lines with an empty vector]. A wild-type *Arabidopsis* plants (wt) was also included in the experiment for reference. The transgenic lines of 35SΩ::*CuGA2ox2/3* and 35SΩ::*CuGA2ox8* showed a late-flowering phenotype and shorter inflorescences compared with the control plants. Notably, all 4 lines of 35SΩ::*CuGA2ox8* showed statistically siginificant differences from the control plant in days to floweirng and plant height 1 month after the start of incubation in the growth chamber. The transgenic lines with 35SΩ::*CuGA2ox4* showed a significant late flowering for line 9 and 11 (Table 2). As for the number of rosette leaves, on the other hand, there were significant differences in 35SΩ::*CuGA2ox2/3* and 35SΩ::*CuGA2ox8* as compared with the control plants. Three transgenic lines of 35SΩ::*CuGA2ox4* showed significant shorter inflorescences than the control plants, but the flowering time of one of them and the number of rosette leaves of all three lines did not differ from those of the control. The typical appearance of each transgenic line and the control plants 24 days after incubation is shown in Figure 4B. The transgenic lines with 35SΩ::*CuGA2ox4*, 35SΩ::*CuGA2ox2/3*, and 35SΩ::*CuGA2ox8* had a shorter inflorescence and a tendency to flower later than the control plant, though to varying degrees. The appearance of inflorescence in each line looked similar to that of the control except for the 1st infl. length (Fig. 4C–F), but there were some defects in the floral oragans of the transgenic lines (Fig. 4H–J). In line 8#4 of *CuGA2ox4*, for example, the stamens were degenerated (Fig. 4H), while, in line 15#5 of 35SΩ::*CuGA2ox2/3*, aberrant forms of petals and stamens were observed (Fig. 4I). In line 15#3 of 35SΩ::*CuGA2ox8*, sepals and petals were slightly degenerated (Fig. 4J). Typically, the transgenic lines with 35SΩ::*CuGA2ox2/3* and 35SΩ::*CuGA2ox8* showed a strong phenotype of dwarfism and those with 35SΩ::*CuGA2ox4* showed a weak phenotype (Fig. 4K–M).

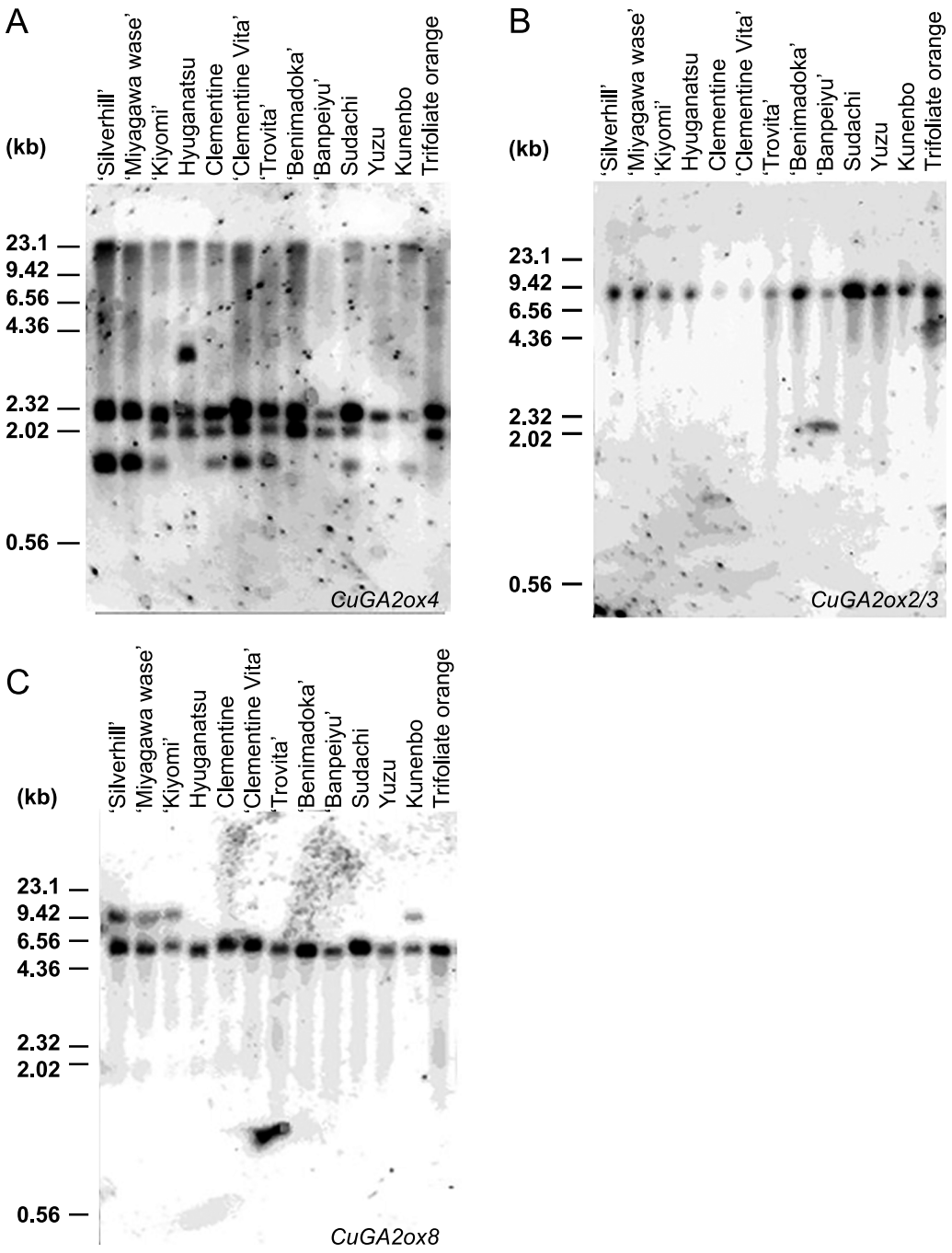

Fig. 2. Southern blot analysis of *CuGA2ox4* (A), *CuGA2ox2/3* (B), and *CuGA2ox8* (C). The genomic DNA (5 μg) of 'Silverhill' and 'Miyagawa-wase' Satsuma mandarin cultivars and other citrus/trifoliate orange species/cultivars, 'Kiyomi' tangor, Hyuganatsu, Clementine, 'Clementine Vita,' 'Trovita' sweet orange, 'Benimadoka' and 'Banpeiyu' pummelo, Sudachi, Yuzu, Kunenbo, and trifoliate orange, were digested with *Xba*I and then separated on a 0.8% agarose gel. Molecular size markers are shown in kb on the left.

Expression analysis for transgenes in whole plants of transgenic Arabidopsis

In addition to the phenotypic analysis, the transgene expression in four independent lines per construct was confirmed by qRT-PCR. Of the transgenic lines with 35SΩ::*CuGA2ox4*, lines 9 and 11 showed higher expression, followed by lines 8 and 7, in decreasing order (Fig. 5A). Of the transgenic lines with *CuGA2ox2/3*, line 16 showed the highest expression on average, followed by lines 17, 15, and 19, in decreasing order (Fig. 5B). The transgenic lines with *CuGA2ox8* showed that lines 15 and 16 had the highest expression on average, followed by lines 17 and 20, in decreasing order (Fig. 5C). The lines with a higher expression of each transgene (over five times the expression of *AtTUB4*), such as the lines indicated by asterisks in Figure 5, showed a phenotype of significantly late flowering and/or short inflorescence, although line 17 of

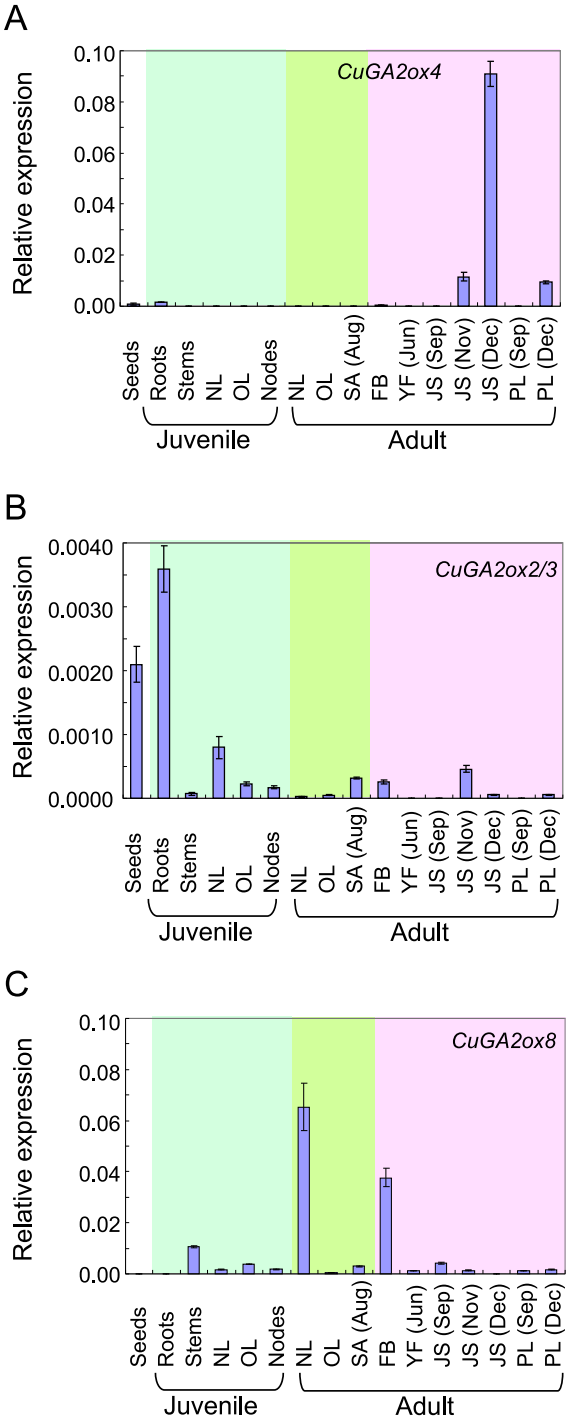

Fig. 3. Expression patterns of *CuGA2ox4* (A), *CuGA2ox2/3* (B), and *CuGA2ox8* (C) in various Satsuma mandarin tissues analyzed by quantitative real-time RT-PCR. The samples for (A) and (B), from left to right, are as follows: seeds of 'Silverhill' Satsuma mandarin harvested in January, roots, stems, new leaves (NL), old leaves (OL), and nodes of 2-year-old nucellar seedlings of the cultivar 'Silverhill' in the juvenile phase; NL, OL, and shoot apices (SA) in the adult phase; and flower buds (FB), young fruit (YF) in June, juice sacs (JS) in September, November, and December, and peel (PL) in September and December in the reproductive phase. Levels of detected amplicons were normalized by reference to *CuActin* (*Citrus unshiu* actin gene). Values are means ± SD of the results from three technical replicates. The primer sets used in this experiment and the PCR conditions are described in Table 1.

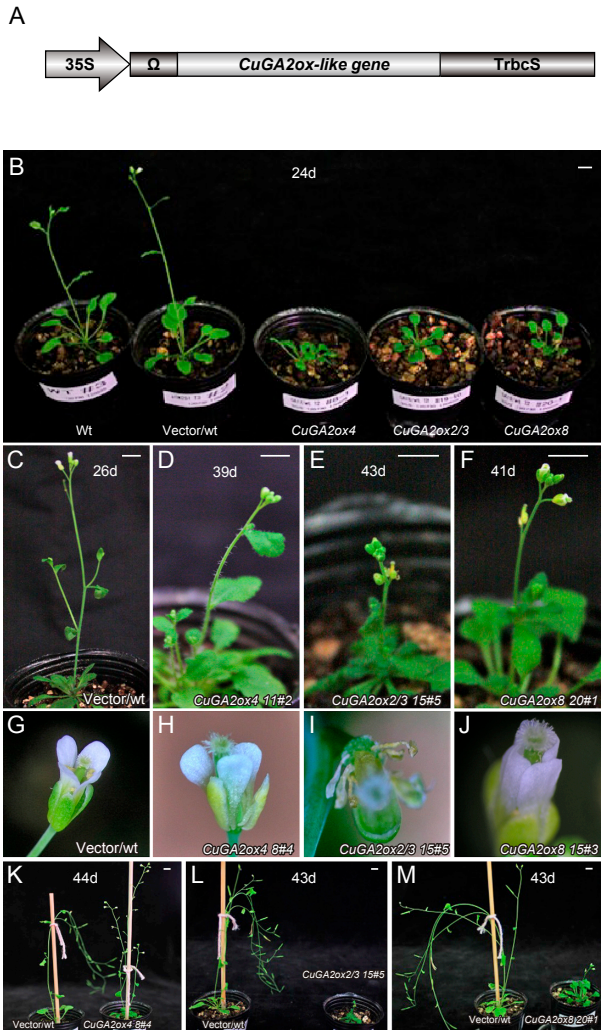

Fig. 4. (A) Schematic representation of transformation vectors. (B) Typical phenotypes of transgenic *Arabidopsis* with Satsuma mandarin *CuGA2ox4* (line 8), *CuGA2ox2/3* (line 19), and *CuGA2ox8* (line 20) 24 days (24d) after the start of incubation in a growth chamber under long-day conditions. (C–F) The appearance of the inflorescence of the control (C, 26d) and the transgenic lines with *CuGA2ox4* (D, 39d), *CuGA2ox2/3* (E, 43d), and *CuGA2ox8* (F, 41d). (G–J) A close-up view of the flower of the control (G) and the transgenic lines with *CuGA2ox4* (H), *CuGA2ox2/3* (I), and *CuGA2ox8* (J). (K–L) The appearance of the whole plant of the control (vector/wt) and transgenic lines with *CuGA2ox4* (K, 44d), *CuGA2ox2/3* (L, 43d), and *CuGA2ox8* (M, 43d). Wt: wild-type plants; vector/wt: transgenic control plants with an empty vector. White bars in the upper right corner of the panels represent 5 mm. Numbers with 'd' in the upper center of the panels represent the days after the start of the incubation in a growth chamber.

CuGA2ox2/3, which expressed *CuGA2ox2/3* at a higher level than line 19, did not show any significant differences in the number of days to flowering, the number of rosette leaves, or the length of the first inflorescence (Fig. 5B).

Discussion

We cloned 3 GA 2-oxidase-like genes, *CuGA2ox4*,

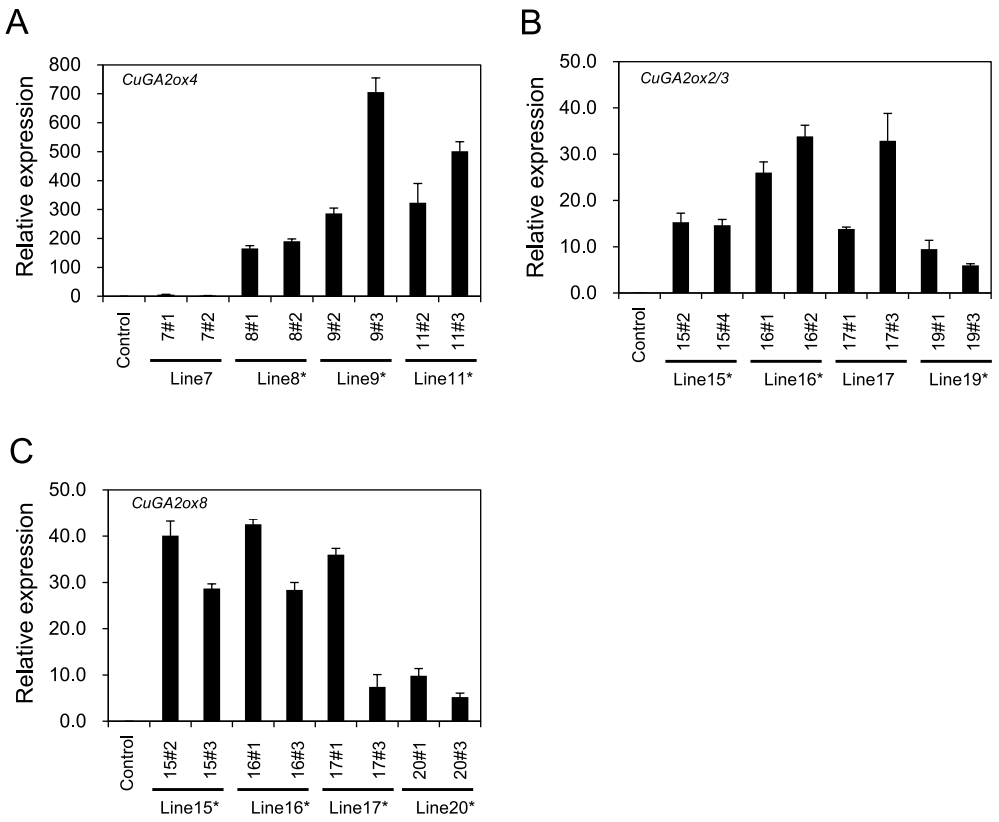

Fig. 5. Expression analysis for transgenes in whole plants of transgenic *Arabidopsis*. Gene expression of *CuGA2ox4* (A), *CuGA2ox2/3* (B), and *CuGA2ox8* (C) in transgenic lines collected 2 months after the start of incubation in a growth chamber. Levels of detected amplicons were normalized by reference to *AtTUB4*. Values are means ± SD of the results from three technical replicates per line. Asterisks represent the transgenic lines that exhibit a late flowering and/or a short inflorescence phenotype with a statistically significant difference from the control shown in Table 2.

CuGA2ox2/3, and *CuGA2ox8*, from Satsuma mandarin. Phylogenetic analysis using putative amino acid sequences revealed that CuGA2ox4, CuGA2ox2/3, and CuGA2ox8 were grouped with AtGA2ox4, AtGA2ox2/3, and AtGA2ox8, respectively (Fig. 1B). The exon-intron structures of *CuGA2ox4* and *CuGA2ox2/3*, each of which has three exons, were comparable to those of *AtGA2ox4* and *AtGA2ox2/3* in *Arabidopsis* genome. However, *CuGA2ox8* consisted of four exons, whereas *AtGA2ox8* consists of three exons (Fig. 1B). Southern blot analysis revealed that *CuGA2ox4*, *CuGA2ox2/3*, and *CuGA2ox8* genes would be single copy genes and that there would be some variations in the non-coding sequences of *CuGA2ox4* and *CuGA2ox8* genes. The blot probed with *CuGA2ox4* and *CuGA2ox8* showed polymorphic bands, indicating diversity around the *Xba*I site in citrus *CuGA2ox4* and *CuGA2ox8* genes. There were 2 bands found for Satsuma mandarin (‘Silverhill’ and ‘Miyagawa wase’) in both blots because two sites of *Xba*I were present in the first intron and one site was present right after the third exon for *CuGA2ox4*, and only one site of *Xba*I was present in the first intron for *CuGA2ox8* (Fig. 1A). The reason the blot probed with *CuGA2ox2/3* showed a single band is that the *CuGA2ox2/3* gene has shorter introns than the others do. Considering that the genealogy of Satsuma mandarin and Kunenbo may be related, it is interesting that the band patterns in the blot probed with *CuGA2ox4* and *CuGA2ox8* were purely coincidental (Fig. 2A, C).

CuGA2ox4, *CuGA2ox2/3*, and *CuGA2ox8* were differentially expressed in various tissues, as shown in Figure 3. *CuGA2ox4* was mainly expressed in the fruit parts, such as juice sacs and peel, and *CuGA2ox8* was expressed in the new leaves and flower buds of adult trees and in the stems of seedlings in the juvenile phase, with lower expression in other tissues. The expression pattern of *CuGA2ox4* resembled that of *CuGA20ox1* in the reproductive tissues of the adult trees in that both genes were expressed in juice sacs in November and December and in peel in December (Fig. 3A; Kotoda et al., 2016), suggesting that *CuGA2ox4* and *CuGA20ox1* act in concert to regulate the accumulation of active GAs in fruit at the maturing stage. Because these genes are expressed in both juice sacs and peel, they might be involved in the occurrence of peel puffins to some extent. As reported earlier, *CuGA20ox1* and *CuGA20ox2* were independently expressed in the new leaves and flower buds, respectively, whereas *CuGA2ox8* was highly expressed in both tissues

(Fig. 3C; Kotoda et al., 2016). These results suggest that *CuGA2ox8* and *CuGA20ox1* act in new leaves, whereas *CuGA2ox8* and *CuGA20ox2* act in flower buds to control the level of active GAs. To clarify the process of the fruit development of citrus such as Satsuma mandarin at the early stage, the expression and function of *CuGA2ox8* and *CuGA20ox2* in the ovaries around anthesis should be studied in the future. On the other hand, the expression level of *CuGA2ox2/3* was lower than that of the other two genes, although it was mainly expressed in seeds and in the roots of seedlings, suggesting a lower contribution of *CuGA2ox2/3* to the development of organs/tissues except for seeds and the roots of seedlings (Fig. 3B).

Transgenic *Arabidopsis* plants ectopically expressing *CuGA2ox4*, *CuGA2ox2/3*, or *CuGA2ox8* were examined to elucidate the function of these 3 genes in Satsuma mandarin. Phenotypic analysis revealed that *CuGA2ox4*, *CuGA2ox2/3*, and *CuGA2ox8* caused reduced inflorescence in transgenic *Arabidopsis* as compared with the control (Fig. 4; Table 2). As expected, the lines with higher expression of *CuGA2ox4*, *CuGA2ox2/3*, or *CuGA2ox8* showed a late-flowering and/or a short inflorescence phenotype, with a statistically significant difference except for line 17 of 35S:: *CuGA2ox2/3* (Fig. 5). The transgenic plants with *CuGA2ox2/3* or *CuGA2ox8* showed a severe dwarf phenotype (Fig. 4L, M). For the timing of flowering, all 4 lines of *CuGA2ox8* and 3 of 4 lines of *CuGA2ox2/3* showed significantly delayed flowering as compared to the control plants, although only 2 lines of *CuGA2ox4* flowered significantly later than the control. These results demonstrated that *CuGA2ox2/3* and *CuGA2ox8* functioned like GA 2-oxidase genes and worked more effectively than *CuGA2ox4*. Similarly, dwarf and late-flowering phenotypes were reported in transgenic *Arabidopsis* and tobacco plants ectopically expressing *AtGA2ox7* and *AtGA2ox8* (Schomburg et al., 2003). In the hybrid aspen, dwarf mutant trees (*stumpy*) were obtained with an increased expression of *PtaGA2ox1*, a GA 2-oxidase gene from the same family as *AtGA2ox4*, by activation tagging (Busov et al., 2003). In addition, transgenic aspen plants tissue-specifically expressing *AtGA2ox2* or *AtGA2ox8* exhibited a lower growth rate than the wild-type plants, resulting in reduced shoot elongation (Eriksson et al., 2015; Mauriat et al., 2011). In the transgenic lines showing a severe dwarf phenotype, some defects were observed in the floral organs such as the petals and stamens (Fig. 4H–J). Similar defects in floral organs were reported in the transgenic *Arabidopsis* ectopically expressing *PslGA2ox*, a plum GA 2-oxidase gene classified in the same group as *CuGA2ox2/3* (El-Sharkawy et al., 2012). These aberrant organs would be produced due to a disturbance in the distribution and/or concentration of GAs caused by the ectopic expression of GA 2-oxidase genes introduced.

Based on our results and reports on other species, *CuGA2ox4*, *CuGA2ox2/3*, and *CuGA2ox8* would function as GA 2-oxidase genes and reduce the concentration of active GAs in specific tissues in Satsuma mandarin. In citriculture, it is also important to study the phenomina called biennial bearing because it affects the annual production of fruit, causing annual price fluctuations. In apple, 3 quantitative trait loci (QTLs) for biennial bearing were detected, and *MdGA2ox2b* (an apple homolog of *CuGA2ox2/3*) and *MdGA2ox8a* (an apple homolog of *CuGA2ox8*) were located near a biennial bearing QTL on the south end of linkage group 10 (Guitton et al., 2012). Because *CuGA2ox2/3* and *CuGA2ox8* were relatively highly expressed in seeds and flower buds, respectively, *CuGA2ox2/3* could be involved in biennial bearing and *CuGA2ox8* might be involved in fruit set in Satsuma mandarin.

We conclude that *CuGA2ox4*, *CuGA2ox2/3*, and *CuGA2ox8* were differentially expressed in various tissues in Satsuma mandarin and that these genes functioned like GA 2-oxidase genes in transgenic *Arabidopsis*. Further study of GA biosynthetic genes including GA 2-oxidase genes will provide insight into the mechanism of flowering, fruit development, seedlessness, biennial bearing, and the peel puffins of citrus such as Satsuma mandarin.

Acknowledgments

We thank Dr. H. Ichikawa for providing the binary vector pSMAK193E, Dr. E. E. Hood for providing *Agrobacterium tumefaciens* EHA101, and Ms. C. Hirano and Ms. Y. Uchida for their technical assistance.

Literature Cited

Busov, V. B., R. Meilan, D. W. Pearce, C. Ma, S. B. Rood and S. H. Strauss. 2003. Activation tagging of a dominant gibberellin catabolism gene (GA 2-oxidase) from poplar that regulates tree stature. Plant Physiol. 132: 1283–1291.

Clough, S. J. and A. F. Bent. 1998. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J. 16: 735–743.

El-Sharkawy, I., W. El Kayal, D. Prasath, H. Fernández, M. Bouzayen, A. M. Svircev and S. Jayasankar. 2012. Identification and genetic characterization of a gibberellin 2-oxidase gene that controls tree stature and reproductive growth in plum. J. Exp. Bot. 63: 1225–1239.

Eriksson, M. E., D. Hoffman, M. Kaduk, M. Mauriat and T. Moritz. 2015. Transgenic hybrid aspen trees with increased gibberellin (GA) concentrations suggest that GA acts in parallel with FLOWERING LOCUS T2 to control shoot elongation. New Phytologist 205: 1288–1295.

Fagoaga, C., F. R. Tadeo, D. J. Iglesias, L. Huerta, T. Lliso, A. M. Vidal, M. Talon, L. Navarro, J. L. García-Martínez and L. Peña. 2007. Engineering of gibberellin levels in citrus by sense and antisense overexpression of a GA 20-oxidase gene modifies plant architecture. J. Exp. Bot. 58: 1407–1420.

Goto, A., H. Yamane, N. Takahashi and K. Hirose. 1989. Identification of nine gibberellins from young fruit of Satsuma mandarin (*Citrus unshiu* Marc.). Agric. Biol. Chem. 53: 2817–2818.

Guitton, B., J.-J. Kelner, R. Velasco, S. E. Gardiner, D. Chagne

and E. Costes. 2012. Genetic control of biennial bearing in apple. J. Exp. Bot. 63: 131–149.
Hedden, P. and Y. Kamiya. 1997. Gibberellin biosynthesis: enzymes, genes, and their regulation. Annu. Rev. Plant Physiol. Plant Mol. Biol. 48: 431–460.
Hedden, P. and A. L. Phillips. 2000. Gibberellin metabolism: new insights revealed by the genes. Trends Plant Sci. 5: 523–530.
Huerta, L., A. Garcia-Lor and J. L. Garcia-Martinez. 2009. Characterization of gibberellin 20-oxidases in the citrus hybrid Carrizo citrange. Tree Physiol. 29: 569–577.
Ikoma, Y., M. Yano, K. Ogawa, T. Yoshioka, Z. C. Xu, S. Hisada, M. Omura and T. Moriguchi. 1996. Isolation and evaluation of RNA from polysaccharide-rich tissues in fruit for quality by cDNA library construction and RT-PCR. J. Japan. Soc. Hort. Sci. 64: 809–814.
Iwamasa, M. 1966. Studies on the sterility in genus *Citrus* with special reference to the seedlessness. Bull. Hort. Res. Sta. Japan Ser. (B) 6: 1–81.
Kishi, T. and M. Tasaki. 1960. Budou ni taisuru Gibberellin riyou shiken [Dai ippou] Delaware ni tsuite. Agric. Hortic. (Nogyo-oyobi-Engei) 35: 381–384 (In Japanese).
Kotoda, N., H. Hayashi, M. Suzuki, M. Igarashi, Y. Hatsuyama, S. Kidou, T. Igasaki, M. Nishiguchi, K. Yano, T. Shimizu, S. Takahashi, H. Iwanami, S. Moriya and K. Abe. 2010. Molecular characterization of *FLOWERING LOCUS T*-like genes of apple (*Malus* × *domestica* Borkh.). Plant Cell Physiol. 51: 561–575.
Kotoda, N., H. Iwanami, S. Takahashi and K. Abe. 2006. Antisense expression of *MdTFL1*, a *TFL1*-like gene, reduces the juvenile phase in apple. J. Amer. Soc. Hort. Sci. 131: 74–81.
Kotoda, N., S. Matsuo, I. Honda, K. Yano and T. Shimizu. 2016. Isolation and functional analysis of two gibberellin 20-oxidase genes from Satsuma mandarin (*Citrus unshiu* Marc.). Hort. J. 85: 128–140.
Kotoda, N., M. Wada, S. Kusaba, Y. Kano-Murakami, T. Masuda and J. Soejima. 2002. Overexpression of *MdMADS5*, an *APETALA1*-like gene of apple, causes early flowering in transgenic *Arabidopsis*. Plant Sci. 162: 679–687.
Larkin, M. A., G. Blackshields, N. P. Brown, R. Chenna, P. A. McGettigan, H. McWilliam, F. Valentin, I. M. Wallace, A. Wilm, R. Lopez, J. D. Thompson, T. J. Gibson and D. G. Higgins. 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23: 2947–2948.
Luckwill, L. C. and J. M. Silva. 1979. The effects of daminozide and gibberellic acid on flower initiation, growth and fruiting of apple cv Golden Delicious. J. Hort. Sci. 54: 217–223.
Mauriat, M., L. G. Sandberg and T. Moritz. 2011. Proper gibberellin localization in vascular tissue is required to control auxin-dependent leaf development and bud outgrowth in hybrid aspen. Plant J. 67: 805–816.
Monselise, S. P. 1979. The use of growth regulators in citriculture; A review. Sci. Hortic. 11: 151–162.
Nagao, A., S. Sasaki and R. P. Pharis. 1989. Cryptomeria japonica. p. 247–269. In: A. H. Halevy (ed.) Handbook of flowering. vol VI. CRC, Boca Raton, Fla.
Perrière, G. and M. Gouy. 1996. WWW-Query: An on-line retrieval system for biological sequence banks. Biochimie 78: 364–369.
Poling, S. M. and V. P. Maier. 1988. Identification of endogenous gibberellins in Navel orange shoots. Plant Physiol. 88: 639–642.
R Core Team. 2015. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. <http://www.R-project.org/>.
Schomburg, F. M., C. M. Bizzell, D. J. Lee, J. A. D. Zeevaart and R. M. Amasino. 2003. Overexpression of a novel class of gibberellin 2-oxidases decreases gibberellin levels and creates dwarf plants. Plant Cell 15: 151–163.
Talon, M., P. Hedden and E. Primo-Millo. 1990a. Gibberellins in *Citrus sinensis*: A comparison between seeded and seedless varieties. J. Plant Growth Regul. 9: 201–206.
Talon, M., L. Zacarias and E. Primo-Millo. 1990b. Hormonal changes associated with fruit set and development in mandarins differing in their parthenocarpic ability. Physiol. Plant. 79: 400–406.
Talon, M., L. Zacarias and E. Primo-Millo. 1992. Gibberellins and parthenocarpic ability in developing ovaries of seedless mandarins. Plant Physiol. 99: 1575–1581.
Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin and D. G. Higgins. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucl. Acids Res. 25: 4876–4882.
Tromp, J. 1982. Flower-bud formation in apple as affected by various gibberellins. J. Hort. Sci. 57: 277–282.
Turnbull, C. G. N. 1989. Identification and quantitative analysis of gibberellins in *Citrus*. J. Plant Growth Regul. 8: 273–282.
Vardi, A., I. Levin and M. Carmi. 2008. Induction of seedlessness in citrus: From classical techniques to emerging biotechnological approaches. J. Amer. Soc. Hort. Sci. 133: 117–126.
Vidal, A. M., W. Ben-Cheikh, M. Talon and J. L. García-Martínez. 2003. Regulation of gibberellin 20-oxidase gene expression and gibberellin content in citrus by temperature and citrus exocortis viroid. Planta 217: 442–448.
Vidal, A. M., C. Gisbert, M. Talon, E. Primo-Millo and I. Lopez-Diaz. 2001. The ectopic expression of a citrus gibberellin 20-oxidase enhances the non-13-hydroxylation pathway of gibberellin biosynthesis and induces an extremely elongated phenotype in tobacco. Physiol. Plant. 112: 251–260.
Yamaguchi, S. 2008. Gibberellin metabolism and its regulation. Annu. Rev. Plant Biol. 59: 225–251.
Yoshida, T. 1994. Dwarfism in trifoliate orange (*Poncirus trifoliata* Raf.), its inheritance and interaction with GA_3. J. Japan. Soc. Hort. Sci. 63: 23–30.