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

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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 GA1 and GA3, are synthesized sequentially through GA12 after the synthesis of GA12 through some steps from isopentenyl diphosphate and dimethylallyl diphosphate and the non-13-hydroxylation pathway where GAs, such as GA4, are synthesized through GA15, GA24, and GA9 after the synthesis of GA12. 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 GA1, more abundantly than it accumulates 13-H GAs such as GA4 (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...
in woody plants, including fruit trees. It has been demonstrated that in apple (Malus × domestica Borkh.) and citrus trees, GA_3 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 ‘Hiryū’ (‘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, CuGA2ox2 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 (Citrus unshiu × Citrus sinensis Osbeck), Hyuganatsu (Citrus tamurana Hort. ex Tanaka), Clementine, ‘Clementine Vita’ (Citrus clementina Hort. ex Tanaka ‘Clementine Vita’), sweet orange (Citrus sinensis Osbeck ‘Trovita’), pummelo (Citrus grandis Osbeck ‘Benimadoka’ and ‘Banpeiyu’), Sudachi (Citrus sudachi Hort. ex Shirai), Yuzu (Citrus junos Sieb. ex Tanaka), Kunenbo (Citrus 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-oxidase genes from Satsuma mandarin

To obtain genomic sequences of GA 2-oxidase genes from Satsuma mandarin, we screened a bacterial artificial chromosome (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
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 [AtGA20ox1 (AT1G78440), AtGA20ox2 (AT1G30040), AtGA20ox3 (AT2G34555), AtGA20ox4 (AT1G47990), AtGA20ox6 (AT1G02400), AtGA20ox7 (AT1G50960), AtGA20ox8 (AT4G21200), AtGA3ox1 (AT1G15550), AtGA3ox2 (AT1G80340), AtGA3ox3 (AT4G21690), AtGA3ox4 (AT1G80330), AtGA20ox1 (AT4G25420), AtGA20ox3 (AT5G07200), and AtKAO2 (AT2G32440)] and Satsuma mandarin [CuGA20ox1 (LC056054), CuGA20ox2 (LC149859), CuGA20ox2/3 (LC149860), and CuGA20ox8 (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 XbaI, 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 CuGA20ox4, CuGA20ox2/3, or CuGA20ox8 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.

<table>
<thead>
<tr>
<th>Gene Primer Oligonucleotide (5’ → 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cloning/probe labelling</td>
</tr>
<tr>
<td>CuGA20ox4 STR00402_F1 GTTCTGTATTTCTAGCAATTA</td>
</tr>
<tr>
<td>STR00402_R1 GATTCAGAAGATGCCTCAAAA</td>
</tr>
<tr>
<td>CuGA20ox2/3 STR00547_F1 AGAAATTAGAAGTAGGCC</td>
</tr>
<tr>
<td>STR00547_R1 ACTTCGACAAAAACCTTAAAT</td>
</tr>
<tr>
<td>CuGA20ox8 STR00577_F1 GTTCTTACGACAGAAGTCT</td>
</tr>
<tr>
<td>STR00577_R1 AAACAACTCAGTGAACATCTC</td>
</tr>
<tr>
<td>Real-time RT-PCR</td>
</tr>
<tr>
<td>CuGA20ox4 STR00402 (379 &gt; 402) GACCTTCCTAAATTTCCGGTGACAGC</td>
</tr>
<tr>
<td>(amplicon, 138 bp) STR00402 (491 &lt; 516) AAGGTCCTCTAGCATTTCTAGAAGT</td>
</tr>
<tr>
<td>CuGA20ox2/3 STR00547 (753 &gt; 775) TGCCTTTGCAGTAAAGCTAATG</td>
</tr>
<tr>
<td>(amplicon, 125 bp) STR00547 (851 &lt; 877) ATCTTTTCGGTCAAAGGTGGC</td>
</tr>
<tr>
<td>CuGA20ox8 STR00577 (902 &gt; 925) GAGACCTTATCCAGCGTCCAGA</td>
</tr>
<tr>
<td>(amplicon, 134 bp) STR00577 (1011 &lt; 1035) TGGACTTCTATACGATCAGTC</td>
</tr>
<tr>
<td>Vector construction</td>
</tr>
<tr>
<td>CuGA20ox4 STR00402_F1-XbaA AAtctagAGTCTCTGATATTCTAGCAATTA</td>
</tr>
<tr>
<td>STR00402_R1-SacI AAAGACGCTTACATTGCAATTTT</td>
</tr>
<tr>
<td>CuGA20ox2/3 STR00547_F1-XbaA AAtctagAAGAAATTAGAAGTAGTAGGCC</td>
</tr>
<tr>
<td>STR00547_R1-KpnI AAAGAGACCTTCGGACAAACCCTCAGT</td>
</tr>
<tr>
<td>CuGA20ox8 STR00577_F1-XbaA AAtctagAGTCTCTAACCAGCAGAAGTCT</td>
</tr>
<tr>
<td>STR00577_R1-SacI AAAGACGCTTACATTGCAATTTT</td>
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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 CuGA20ox4 [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 CuGA20ox2/3 [STR00547 (753 > 775) and STR00547 (851 < 877)] and CuGA20ox8 [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 of 2-year-old juvenile nucellar seedlings derived from ‘Silverhill’ Satsuma mandarin, as well as from 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<sub>2</sub>) 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 CuGA2ox<sub>4</sub>, CuGA2ox<sub>2/3</sub>, and CuGA2ox<sub>8</sub>, 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 CuGA2ox2/3, and STR00577_F1-XbaI and STR00577_R1-SacI for CuGA2ox8. Each amplified PCR product was subsequently digested with XbaI/SacI or XbaI/KpnI 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 μmol·m<sup>-2</sup>·s<sup>-1</sup>), 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<sub>2</sub>). 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<sub>2</sub> 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 (‘Miyanagawa 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-
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 bands for a single band of about 9.4 kb for *CuGA2ox2/3, CuGA2ox4*, and *CuGA2ox8*.

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 (35SSΩ) into *Arabidopsis* (Fig. 4A). We analyzed four independent lines with 12–13 individuals (n = 12–13) in the T2 generation for phenotypes, including the number of days to flowering, the number of rosette leaves at 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
35S::CuGA2ox4, 35S::CuGA2ox2/3, and 35S::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 35S::CuGA2ox2/3 and 35S::CuGA2ox8 showed a late-flowering phenotype and shorter inflorescences compared with the control plants. Notably, all 4 lines of 35S::CuGA2ox8 showed statistically significant differences from the control plant in days to flowering and plant height 1 month after the start of incubation in the growth chamber. The transgenic lines with 35S::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 35S::CuGA2ox2/3 and 35S::CuGA2ox8 as compared with the control plants. Three transgenic lines of 35S::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 35S::CuGA2ox4, 35S::CuGA2ox2/3, and 35S::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 organs 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 35S::CuGA2ox2/3, aberrant forms of petals and stamens were observed (Fig. 4I). In line 15#3 of 35S::CuGA2ox8, sepals and petals were slightly degenerated (Fig. 4J). Typically, the transgenic lines with 35S::CuGA2ox2/3 and 35S::CuGA2ox8 showed a strong phenotype of dwarfism and those with 35S::CuGA2ox4 showed a weak phenotype (Fig. 4K–M).
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 35Ω::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. 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,’ ‘Trovia’ sweet orange, ‘Benimadoka’ and ‘Banpeiyu’ pummelo, Sudachi, Yuzu, Kunenbo, and trifoliate orange, were digested with XbaI and then separated on a 0.8% agarose gel. Molecular size markers are shown in kb on the left.
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.

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,
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 XbaI 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 XbaI were present in the first intron and one site was present right after the third exon for CuGA2ox4, and only one site of XbaI 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 CuGA2ox1 in the reproductive tissues of the adult trees in that both genes were expressed in juice sacs in November and December (Fig. 3A; Kotoda et al., 2016), suggesting that CuGA2ox4 and CuGA2ox1 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 puffs to some extent. As reported earlier, CuGA2ox1 and CuGA2ox2 were independently expressed in the new leaves and flower buds, respectively, whereas CuGA2ox8 was highly expressed in both tissues.
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 phenomena 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.

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