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Development of a *CiFT* Co-expression System for Functional Analysis of Genes in Citrus Flowers and Fruit

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We have developed a *CiFT* co-expression system for the rapid evaluation of transgenic citrus flowers and fruit. In order to co-express a target gene and the *CiFT* gene, a *CiFT* co-expression vector was constructed by inserting the fragment containing a chimeric target gene into the binary vector harboring *P35S::CiFT*. This system was applied to metabolically engineer aroma components in trifoliate orange (*Poncirus trifoliata* L. Raf). In order to reduce the limonene level, a gene encoding limonene synthase (*CitMTSE1*) from satsuma mandarin (*Citrus unshiu* Marc.) was introduced into trifoliate orange in an anti-sense orientation with the *CiFT* co-expression vector. Transgenic plants flowered extremely early, and began to produce normal fruit within 2 years of *Agrobacterium* infection. Transcripts of transgenes were accumulated in reproductive tissues of transgenic plants, and endogenous transcripts for limonene synthase were reduced. The ratio of limonene to other monoterpenes was significantly decreased in flowers and fruit of transgenic plants. These results clearly indicated that the *CiFT* co-expression system is a useful tool for the functional analysis of genes in citrus flowers and fruit. Such analyses are important for several applications, including the metabolic engineering of aroma components in citrus.

Key Words: *CiFT*, citrus, co-expression, limonene, metabolic engineering.

Introduction

Citrus, one of the most commercially important fruit crops, has many unique characteristics, such as various secondary metabolites, parthenocarpy, seedlessness, apomixes, and sexual incompatibility. However, the long juvenile period, ranging from 6 to 20 years, has hampered genetic studies on these characteristics, as well as traditional breeding. For example, a recent Japanese citrus cultivar, ‘Harehime’, was released in 2001, originating from a cross in 1990. It took about 5 years to first fruiting in spite considerable efforts to shorten the juvenile period by top-grafting (Yoshida et al., 2005). Reduction of the generation time in citrus would accelerate studies on the complex regulatory mechanisms that underlie biological and agronomical traits, and lead to the manipulation of agronomically important traits, such as the nutritional value and fruit quality. Recently, a reduction of the long juvenile period of citrus has been achieved using flowering genes such as *APETALA1* (*AP1*), *LEAFY* (*LFY*), and the *Citrus* homolog of *FLOWERING LOCUS T* (*CiFT*) (Endo et al., 2005; Peña et al., 2001). The overexpression of either of these genes caused early flowering and fruiting within a few years of *Agrobacterium* infection. In recent years, the *Arabidopsis FT* gene and its orthologous gene in rice, *Hd3a*, have emerged as the most plausible candidates for the flower-inducing hormone “florigen”. FT and Hd3a proteins are produced in leaves, move to shoot apices, and then induce flowering (Abe et al., 2005; Corbesier et al., 2007; Takada and Goto, 2003; Tamaki

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et al., 2007). Therefore, *CiFT* is one of the most useful genes to develop new experimental systems to reduce the time until flowering and fruiting in citrus transgenic plants, and to rapidly evaluate the effects of introduced genes on these reproductive tissues. Such experimental tools will advance functional analyses of various genes related to morphogenesis and fruit quality in citrus.

The flavors in *Citrus* and the closely related genera *Poncirus* and *Fortunella* are characterized by a variety of monoterpenes and sesquiterpenes (Sawamura, 2000). A typical feature of the volatile substances in most citrus fruit is that *d*-limonene comprises more than 70% of the essential oils. The high proportion of *d*-limonene in volatile citrus substances is thought to result from the lower Km value of *d*-limonene synthase compared with other monoterpene synthases (Lücker et al., 2002). Several monoterpene synthases have been isolated from citrus species (Lücker et al., 2002; Shimada et al., 2004, 2005a, 2005b), and different volatile compositions in flowers and fruit were explained by different patterns of transcript accumulation of the corresponding monoterpene synthase genes (Shimada et al., 2005a). So far, metabolic engineering to modify monoterpene biosynthesis has been attempted in tomato (Lewinsohn et al., 2001), petunia (Lücker et al., 2001), and tobacco (Lücker et al., 2004a, 2004b; Ohara et al., 2003). However, their corresponding products in transgenic plants accumulated at very low levels, or were inactivated by glycosylation and hydroxylation. Overexpression of the geraniol synthase (GES) gene in mature tomato fruit caused marked changes in the volatile composition, along with unexpected increases of myrcene, limonene, and ocimene (Davidovich-Rikanati et al., 2007). Given these very different results in different plant species, it will be of interest to determine the effects of modifying the transcription of monoterpene synthase genes on aroma components in citrus flowers and fruit.

In this study, we developed a new experimental system—the *CiFT* co-expression system—in which a target gene was co-introduced and co-expressed with *CiFT* in transgenic plants. This system was applied to metabolic engineering for aroma components in trifoliate orange (*Poncirus trifoliata* L. Raf.). The anti-sense sequence of a gene encoding *d*-limonene synthase (*CitMTSE1*) was introduced along with *P35S::CiFT* to reduce limonene levels in the flowers and fruit of trifoliate orange. Transgenic plants which developed flowers and fruit were obtained within 2 years of *Agrobacterium* infection. Gene expression and the composition of volatiles were analyzed in transgenic plants, and we observed a significant reduction of limonene levels compared with control plants.

Materials and Methods

Plant materials and vector construction

Trifoliate orange (*Poncirus trifoliata* L. Raf), sour orange (*Citrus aurantium* L.), and rough lemon (*Citrus jambhiri* Lush.) were used in these experiments. Plants were cultivated at the National Institute of Fruit Tree Science (NIFTS), Okitsu Citrus Research Station, Shizuoka, Japan. Trifoliate orange was used as the source for transformation experiments. Sour orange was used as the first rootstock for the *in vitro* grafting of regenerated shoots. Rough lemon was used as the second rootstock for the second grafting of trifoliate orange on sour orange. Seeds from all species were germinated and grown as described previously (Endo et al., 2005) to obtain the materials required for the experiments.

A shuttle vector (Fig. 1a) was constructed as follows: The binary vector pBE2113 (Mitsuhara et al., 1996) was digested with *Xba*I and *Sac*I to remove the *uidA* fragment, and ligated with the linker fragment obtained by the digestion of pUC18 (Yanisch-Perron et al., 1985) with *Xba*I and *Sac*I. The resultant vector was used as a template for a PCR reaction with a set of primers: pBI101F2419: 5'-CTTCCGGCTCGTATGTTGTGT-3' and pBI101R4775H: 5'-AT<u>AAGCTT</u>GGCGAAAGGG GGATGTGCT-3'; *Hin*dIII site underlined. The amplified fragment contained the *El$_2$-35S* region, which comprises El$_2$ (duplicated 5'-upstream (–419 to –90) sequence of cauliflower mosaic virus (CaMV) 35S promoter), the 5'-upstream (–90 to –1) sequence of the CaMV 35S promoter and 5'-untranslated (Ω) sequence of tobacco mosaic virus (TMV), and the nopaline synthase terminator (*Tnos*). It was inserted into the *Hin*dIII site of a modified pUC18 that lacked the cloning sites from *Sph*I to *Eco*RI. The *CiFT* co-expression vector was constructed using this shuttle vector. The *d*-limonene synthase 1 (*CitMTSE1*) cDNA clone (AB110636) from satsuma mandarin (*C. unshiu* Marc.) (Shimada et al., 2004) was amplified with TAEASY1F-Xba (5'-AT <u>TCTAGA</u>TCATCCTTTGGTGACAGGTG-3'; *Xba*I site underlined) and TAEASY1R-Sma (5'-AT<u>CCCGGG</u> ATGTCTTCTTGCATTAATCC-3'; *Sma*I site underlined), digested, and inserted downstream of *El$_2$35S* on the shuttle vector with an anti-sense orientation (Fig. 1b). This construct was digested with *Hin*dIII, and the chimeric gene was inserted into the binary vector pCGN1547 (Calgene, USA) harboring *P35S::CiFT* (Kobayashi et al., 1999). The *CiFT* co-expression vector (Fig. 1c) was transferred into the *Agrobacterium tumefaciens* strain LBA4404 by triparental mating.

Agrobacterium-mediated transformation

The transformation method for trifoliate orange was as described previously (Endo et al., 2005). Adventitious shoots emerging from segments were detached and individually subcultured on MS medium supplemented with 3% sucrose, 10^{-7} M NAA, 10^{-8} M GA_3, and 0.2% gelrite (pH 5.6) for 1 to 3 months. Shoots were then grafted *in vitro* onto the etiolated seedlings of sour orange, which had been previously germinated and prepared as rootstock. *In vitro*-grafted plants were grafted again onto rough lemon rootstock and grown in

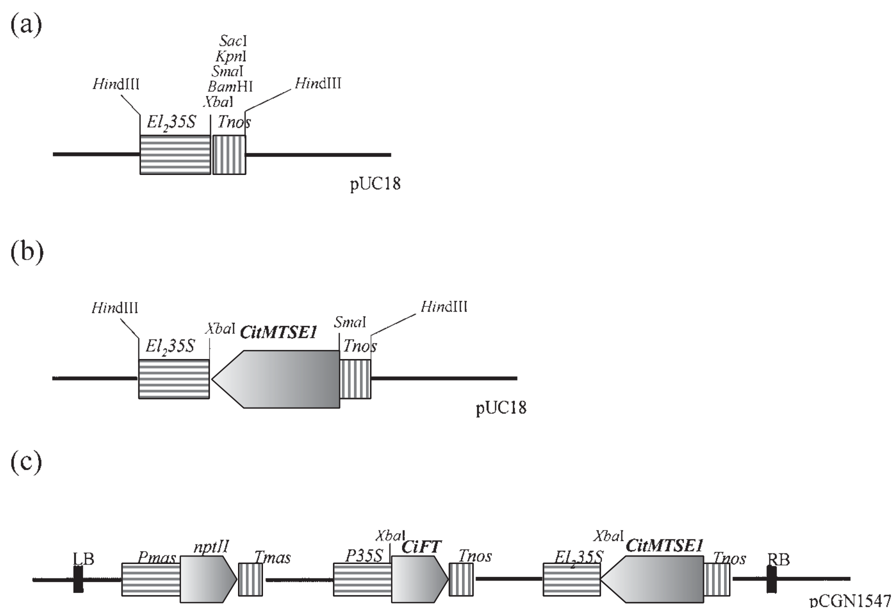


Fig. 1. Structures of shuttle vector (a), shuttle vector with anti-sense *CitMTSE1* (b), and *CiFT* co-expression vector (c). The target gene, in this case the anti-sense *CitMTSE1*, is finally inserted into the pCGN1547 binary vector harboring *P35S::CiFT* through the shuttle vector. LB, left border of T-DNA; RB, right border of T-DNA; *P35S*, Cauliflower mosaic virus 35S promoter; *El_2-35S*, El_2 (duplicated 5'-upstream (−419 to −90) sequence of *P35S*)-5'-upstream (−90 to −1) sequence of *P35S*—5'-untranslated (Ω) sequence of TMV from pBE2113; *Pmas*, mannopine synthase promoter; *Tnos*, nopaline synthase terminator; *Tmas*, mannopine synthase terminator; *npt II*, kanamycin resistance gene.

a greenhouse. Control plants free of infection were concurrently regenerated and grown without kanamycin selection.

Gene incorporation was confirmed in transgenic plants by PCR, using three sets of primers: P35S (5'-ATCTCCACTGACGTAAGGGATGACG-3') and Tnos (5'-ACAGGATTCAATCTTAAGAAACTTT-3'); P35S and CiFTgR4 (5'-AAAGCTGGCCCCTGTGGTTGC-3'); P35S and TAE1-R1 (5'-AGAAACTGAGCTTTGC GAGGGAC-3').

Southern blot analysis

Transgenic trifoliate orange genomic DNA was extracted from fresh, fully expanded leaves according to Dellaporta et al. (1983). DNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA). To estimate the number of transferred genes in transgenic plants, 10 μg of total DNA was completely digested with *Xba*I and analyzed by Southern blot. A 0.87 kb fragment obtained from the digestion of pBI221 (BD Biosciences, USA) with *Eco*RV and *Hin*dIII, which contained the *P35S* enhancer region, was used as a probe. Digested DNA was separated by electrophoresis on a 1.0% (v/v) agarose gel and blotted onto a nylon membrane (Hybond-NX, GE Healthcare, UK). Probe labeling by digoxigenin (DIG), hybridization, and detection were conducted according to the manufacturer's instructions (Roche Diagnostics GmbH, Germany). Washing was carried out twice in 0.1 × SSC and 0.1% SDS at 68°C for 15 min, and the membrane was then exposed to X-ray film.

Gene expression analysis by reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the leaves, flowers, juice sacs, and peel of mature fruit of transgenic plants using the methods described by Ikoma et al. (1996). RNA was quantified using a NanoDrop ND-1000 spectrophotometer. For RT-PCR, the first strand cDNA was synthesized from 1 μg of total RNA by reverse transcriptase with Oligo-(dT) primers according to the instructions of the Ready-To-Go You-Primed First Strand Kit (GE Healthcare). The first strand cDNA was diluted to 60 μL in water, and aliquots of 1 μL were used in each RT-PCR reaction (total volume: 20 μL). PCR reactions were performed in a thermal cycler under the following conditions: 24 cycles of 10 s at 94°C, 15 s at 58°C, and 90 sec at 72°C. The gene expressions of *CiFT*, the endogenous limonene synthase gene in trifoliate orange (*PtLim1*), and total limonene synthase genes (introduced *CitMTSE1* and endogenous *PtLim1*) were analyzed with the following primer sets: *CiFT* (5'-GCTTAGTGTTGTTGAGTGTTTGTTTG-3' and 5'-AAAGCTGGCCCCTGTGGTTGC-3'); *PtLim1* (5'-TGTTTTCTTGCGCTTTAC-3' and 5'-GTCGAGGCCA ACATCAATCGAAACTG-3'); total limonene synthase genes (5'-ATGTCTTCTTGCATTAATCCCTCA-3' and 5'-ATGCTACCAAGCTGTCCCTCGCA-3'). The full length of *PtLim1* was isolated using the SMART RACE cDNA Amplification Kit (Clontech, USA) and the sequence of its 3' UTR region was utilized to design specific primers for *PtLim1*. The accession number of *PtLim1* is AB363936. The primers designed to amplify the alpha subunit of the elongation factor 1 (EF1-α) gene

(5'-AAGGCTGAGCGTGAACGTGG-3' and 5'-ACG GCAATGTGGGAGGTGTG-3') were used for internal control reactions. The PCR products of each reaction were analyzed by electrophoresis on 1.5% (v/v) agarose gels.

Extraction of volatile components and GC-MS analysis

Flowers at anthesis and the peel of mature fruit were collected from transgenic plants and prepared for the analysis of their volatile components according to the method of Hara et al. (1999). The instrument and assay conditions for GC-MS analysis were as follows: instrument, MSD 5890 (Hewlett Packard, USA); HP-1 capillary column, 30 m × 0.25 mm internal diameter; film thickness, 2.5 μm, (Agilent Technologies, USA); carrier gas, He (1.0 ml·min^{-1}); injector temperature, 100°C; ionization voltage, EI 70 eV; oven program, started at 50°C (5-min hold), 5°C·min^{-1} to 200°C.

Results

Construction of CiFT co-expression vector and production of transgenic plants

In order to drive a target gene with the *CiFT* gene, we developed a *CiFT* co-expression system (Fig. 1). The target gene was inserted into the binary vector harboring *P35S::CiFT* (Kobayashi et al., 1999) through the shuttle vector. The shuttle vector has the *El$_2$-35S* region (see Materials and Methods) and the nopaline synthase terminator (*Tnos*) of pBE2113 (Mitsuhara et al., 1996) (Fig. 1a). The *d*-limonene synthase 1 gene (*CitMTSE1*) was inserted with an antisense orientation between the promoter and terminator of this shuttle vector (Fig. 1b). The chimeric gene (*El$_2$-35S::anti-CitMTSE1::Tnos*) was inserted into the *Hin*dIII site of the binary vector harboring *P35S::CiFT* (Kobayashi et al., 1999; Fig. 1c). The binary vector, which had *P35S::CiFT* and a target gene, was designated as the *CiFT* co-expression vector.

A total of 494 epicotyl segments from etiolated seedlings of trifoliate orange were infected with *A. tumefaciens* LBA4404 harboring the *CiFT* co-expression vector. Adventitious shoots that regenerated from the segments on the selection medium were excised individually and grafted twice to promote growth (see Materials and Methods). After the second grafting, regenerated plants were transferred to the greenhouse. PCR analysis showed the presence of the transgene in 16 regenerated plants.

Southern blot analysis was carried out to confirm the integration of the transgene into the trifoliate orange genome, and to estimate the transgene copy number. Genomic DNA of each transgenic plant was digested with *Xba*I and subjected to hybridization with a DIG-labeled *P35S* probe. All plants had the expected 1.7 kb fragment derived from the region between two *Xba*I sites in the T-DNA region (Figs. 1c and Fig. 2). The signals except for 1.7 kb were thought to show line-dependent fragments between the *Xba*I site downstream of *P35S* in the T-DNA and the *Xba*I site in the genomic sequence flanked with the left border. The fragment pattern showed that 16 transgenic plants were derived from 14 independent transgenic lines. A single copy of the transgene was detected in 9 lines, and two copies were detected in 5 lines.

Transgenic plants began to flower as early as 30 weeks after *Agrobacterium* infection, just after transfer to the greenhouse. Corresponding to the previous observation (Endo et al., 2005), the flowering of transgenic plants occurred continually. Some transgenic plants started fruit

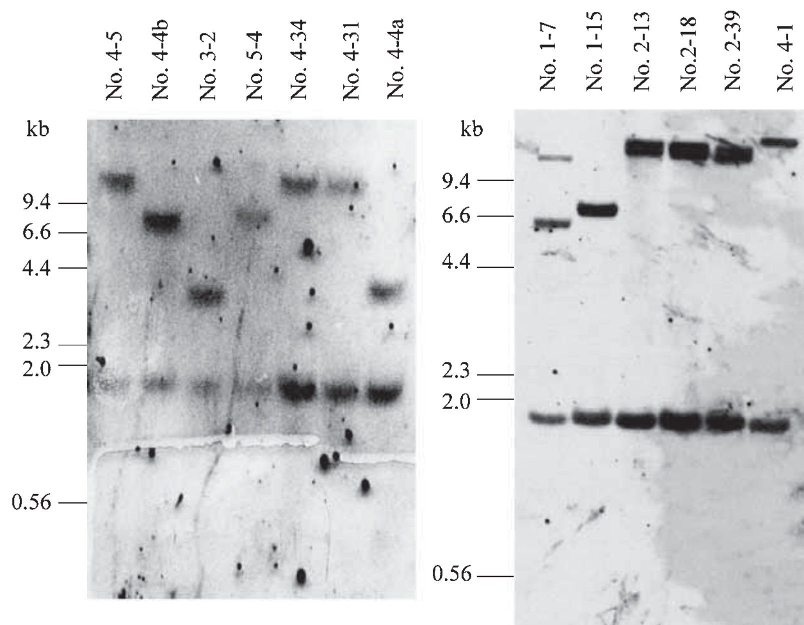


Fig. 2. Southern blot analysis of transgenic plants. Genomic DNA (10 μg) from each transgenic plant was digested with *Xba*I and hybridized with a fragment of the *P35S* enhancer region.

development as early as 1 year after *Agrobacterium* infection. Within 2 years of *Agrobacterium* infection, all transgenic lines developed morphologically normal flowers (Fig. 3a) and six lines began to develop morphologically normal fruit (Fig. 3b). This was the same phenotype as the transgenic trifoliate orange with *P35S::CiFT* in the previous report (Endo et al., 2005). The efficiency of obtaining transgenic lines with flowers or fruit was approximately 2.8 or 1.2%, respectively, based on the number of epicotyl segments infected. Six independent transgenic lines (1-7, 1-15, 2-39, 4-1, 4-34, and 5-4) were used for further analyses (Table 1).

Gene expression analysis in transgenic plants

Prior to gene expression analysis in transgenic plants, we isolated the endogenous limonene synthase gene in trifoliate orange and the full-length nucleotide and amino acid sequences were registered as *PtLim1* in DDBJ (AB363936). *PtLim1* consists of 2,133 bp with an ORF of 1,824 bp. It shows 96.0% sequence homology with the ORF of *CitMTSE1* at the nucleotide sequence level. In order to discriminate endogenous gene expression from that of the transgene, a *PtLim1*-specific primer set was designed from the 3' UTR sequence, which showed a low-level similarity with the introduced *CitMTSE1*. In the wild-type trifoliate orange, the transcript of *PtLim1* accumulated in the flowers and peel, but did not accumulate in leaves, stems, and juice sacs (Fig. 4). This is similar to the gene expression pattern of *CitMTSE1* in satsuma mandarin, except that satsuma mandarin accumulates transcripts in juice sacs at a mature stage (Shimada et al., 2004, 2005b).

For transgenic plants, transcript levels of *CiFT*, total limonene synthase genes (introduced *CitMTSE1* and endogenous *PtLim1*), and endogenous *PtLim1* were investigated in leaves, flowers, and mature fruit by RT-PCR. Since the fruit of transgenic Nos. 4-1 and 1-15 dropped before maturation, gene expression analysis of fruit was not carried out for these two lines. Since the primer set for *CitMTSE1* amplified both the transgene and endogenous genes, gene expression of the introduced *CitMTSE1* was deduced from the difference between the expression patterns of *PtLim1* and total limonene synthase genes (introduced *CitMTSE1* and endogenous *PtLim1*). The *CiFT* transcript was detected in all tissues examined (Fig. 5). Transcripts of total limonene synthase genes (introduced *CitMTSE1* and endogenous *PtLim1*) were also detected in all tissues examined (Fig. 5). The *PtLim1* transcript was not detected in leaves of any transgenic lines (Fig. 5a). This agreed with the wild-type result (Fig. 4) and indicated that all the transcripts for total limonene synthase genes in transgenic leaves were attributable to the transgene. The *PtLim1* transcript was detected in flowers of transgenic lines Nos. 1-7 and 2-39, and slightly in No. 4-1, but barely in Nos. 4-34, 1-15, and 5-4 (Fig. 5b). In fruit, the *PtLim1* transcript was detected in peel of Nos. 1-7 and 2-39 but barely in Nos. 4-34 and 5-4 (Fig. 5c). These results indicated that, at least in some transgenic lines such as Nos. 4-34 and 5-4, anti-sense transcripts for the introduced *CitMTSE1* accumulated in all tissues, and expression levels of the endogenous limonene synthase gene in flowers and fruit were reduced. Therefore, it was confirmed that the introduction of the *CiFT* co-expression vector caused ectopic expression both of *CiFT* and anti-sense *CitMTSE1* in tissues including flowers and fruit of transgenic plants.

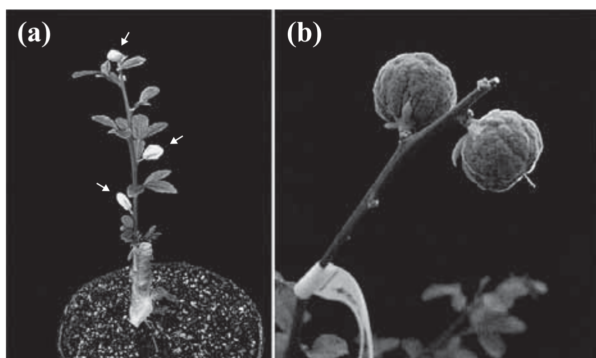


Fig. 3. Flowers (a: arrow) and fruit (b) of transgenic trifoliate orange (line No. 5-4).

Table 1. Summary of transgenic plants further analyzed.

Line	Time to flowering (month)[z]	Fruit development	Estimated copy No. of transgenes
1-7	8	+[y]	2
1-15	7	−	1
2-39	12	+	2
4-1	9	−	1
4-34	14	+	1
5-4	7	+	1

[z] Observation after *Agrobacterium* infection.
[y] Presence (+) or absence (−) of fruit development and maturation on the plant.

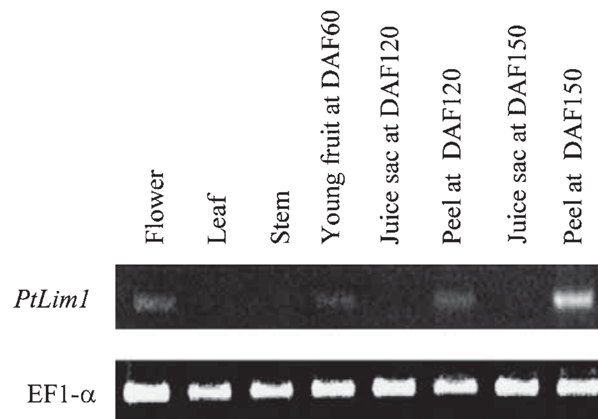


Fig. 4. Gene expression analysis of limonene synthase 1 (*PtLim1*) in various tissues of wild-type trifoliate orange by RT-PCR. EF1-α was used as the control.

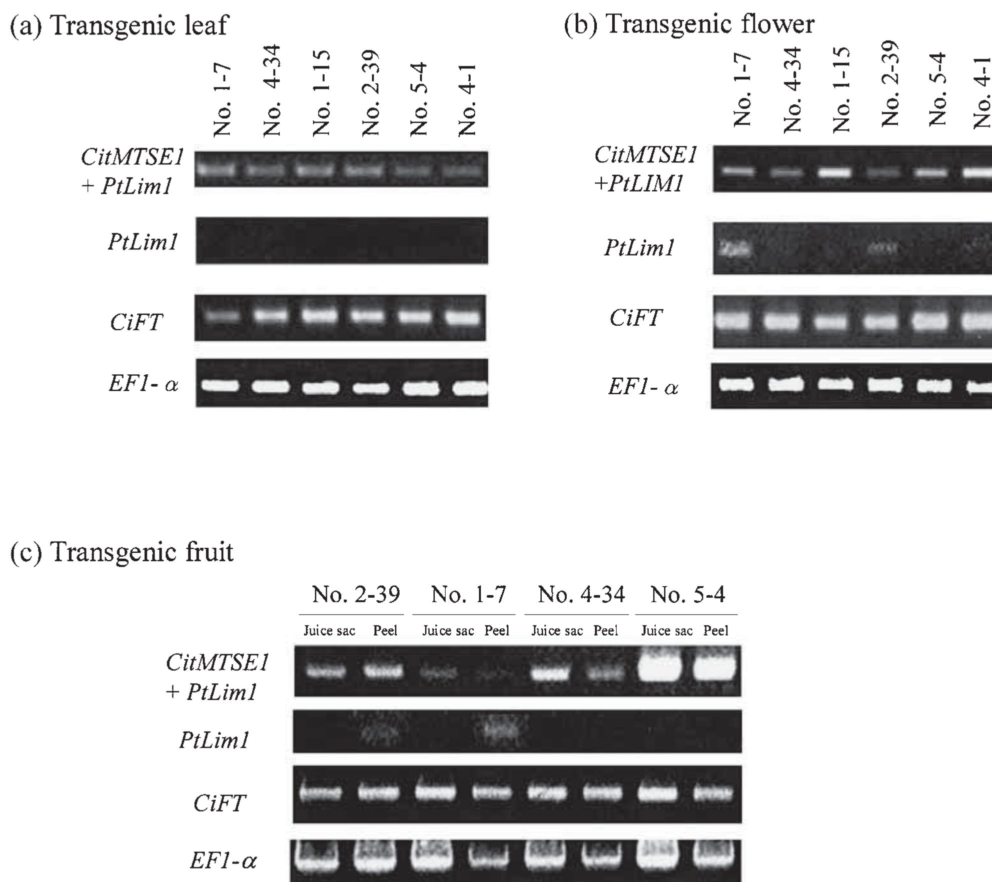
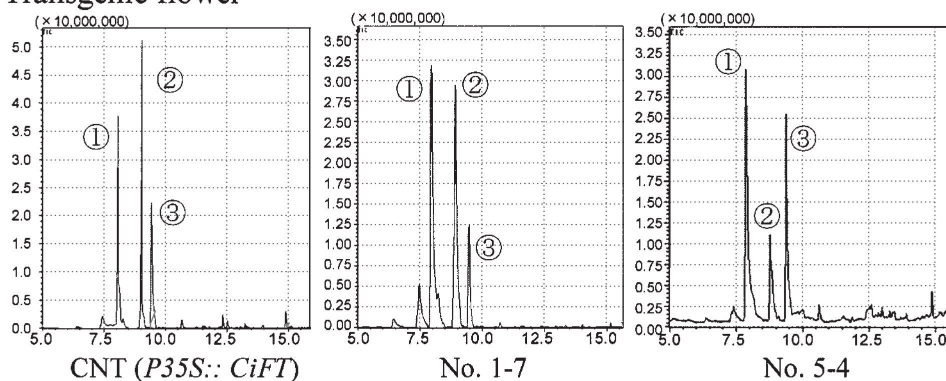


Fig. 5. Gene expression analysis of *CiFT*, total limonene synthase genes (introduced *CitMTSE1* and endogenous *PtLim1*) and *PtLim1* in the leaf (a), flower (b), and fruit (c) of transgenic trifoliate orange by RT-PCR. EF1-α was used as the control.

GC-MS analysis of transgenic plants

The flowers and peel of mature fruit from transgenic plants were analyzed using GC-MS to determine the composition of volatile components. *P35S::CiFT* (CNT; Endo et al., 2005) and wild-type trifoliate orange (WT) were used as controls. In flowers and peel of CNT and WT, the major monoterpenes were β-myrcene, *d*-limonene, and (*E*)-β-ocimene (Fig. 6 and Table 2). Therefore, the levels of these three monoterpenes were investigated in transgenic plants. In WT flowers, the peak area percentages of β-myrcene, *d*-limonene, and (*E*)-β-ocimene were 29.06, 26.05, and 17.07%, respectively (Table 2), which were similar to those reported previously for flowers and fruit of trifoliate orange (Sawamura, 2000). In flowers of CNT, the peak area percentages of *d*-limonene and β-myrcene were higher than in the WT, but the ratio of *d*-limonene/β-myrcene was similar to that of the WT. Flowers of the transgenic line No. 1-7 showed values of the three major monoterpenes similar to CNT. Transgenic line Nos. 1-15, 4-1, 4-34, and 5-4 showed reduced ratios of *d*-limonene/β-myrcene in their flowers (approximately 0.1 to 0.4). Transgenic line No. 2-39 showed an intermediate ratio of *d*-limonene/β-myrcene in flowers. In the peel of CNT and WT, the ratio of (*E*)-β-ocimene/β-myrcene was low compared to that in the flowers. A difference in the (*E*)-β-ocimene content was also observed in satsuma mandarin, where (*E*)-β-ocimene was comparatively abundant in flowers and then decreased as the fruit developed (Shimada et al., 2005a). This result was in accordance with the transcriptional repression of *CitMTSL4* in fruit. Since mature fruit of transgenic line Nos. 1-15 and 4-1 were not available, the remaining four lines were analyzed. In peel of transgenic line Nos. 1-7 and 2-39, values of the three monoterpenes was similar to those in CNT and WT. In contrast, the ratio of *d*-limonene/β-myrcene in fruit was successfully reduced to approximately 0.4 in transgenic line Nos. 4-34 and 5-4. The content of other monoterpenes, except limonene, was not significantly different in any transgenic lines, both in the flowers and fruit, when compared to CNT or the WT (data not shown).

The relative limonene content in transgenic flowers and fruit was associated with the accumulation of endogenous *PtLim1* transcripts in each line (Fig. 5b, 5c, and Table 2). Flowers and peel of line No. 1-7 accumulated transcripts of *PtLim1* and had a similar limonene content relative to controls. Transgenic plant Nos. 1-15, 4-34, and 5-4, all of which showed repressed endogenous *PtLim1* expression, had a reduced limonene



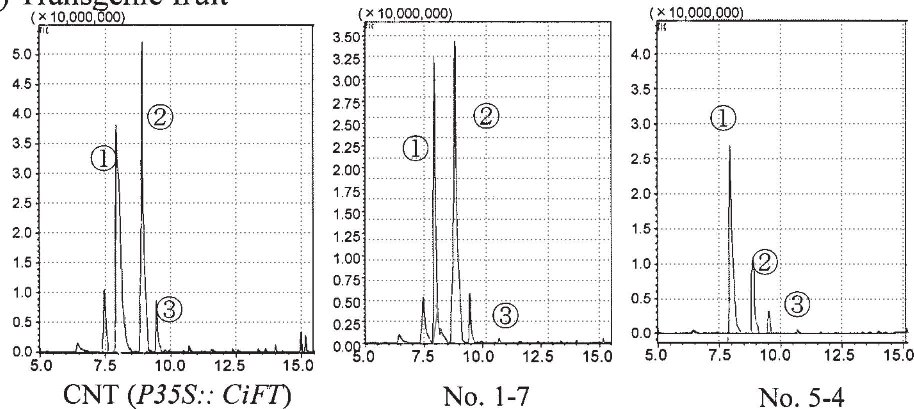


Fig. 6. GC-MS charts of aroma components in flowers (a), and fruit (b) of transgenic trifoliate orange and controls. Numbers in charts indicate ① β-myrcene; ② *d*-limonene; ③ (*E*)-β-ocimene. Relative *d*-limonene contents in flowers and fruit of transgenic line No. 5-4 were markedly lower than those in CNT (*P35S::CiFT*) and transgenic line No. 1-7.

Table 2. Peak area percentages of β-myrcene, *d*-limonene, and (*E*)-β-ocimene in flowers and fruit of transgenic plants and controls.

	Plant material	β-myrcene (Rt = 7.95)	*d*-limonene (Rt = 8.95)	(*E*)-β-ocimene (Rt = 9.46)	
Flower			Peak area (%)		Total
	1-7	35.5 (1.00)	33.08 (0.93)	15.32 (0.43)	83.9
	1-15	37.07 (1.00)	3.45 (0.09)	21.82 (0.59)	62.34
	2-39	34.23 (1.00)	24.07 (0.70)	17.41 (0.51)	75.71
	4-1	33.63 (1.00)	10.56 (0.31)	19.75 (0.59)	63.94
	4-34	35.72 (1.00)	10.77 (0.30)	19.21 (0.54)	65.7
	5-4	36.27 (1.00)	15.84 (0.44)	25.7 (0.71)	77.81
	P. trifoliata (WT)	29.06 (1.00)	26.05 (0.90)	17.07 (0.59)	72.18
	P35S::CiFT (CNT)	34.58 (1.00)	38.21 (0.94)	14.26 (0.41)	87.05
Peel			Peak area (%)		Total
	1-7	37.9 (1.00)	36.91 (0.97)	3.58 (0.09)	78.39
	2-39	30.5 (1.00)	33.51 (1.10)	2.97 (0.10)	66.98
	4-34	44.25 (1.00)	16.35 (0.37)	2.32 (0.05)	62.92
	5-4	46.1 (1.00)	20.06 (0.44)	3.41 (0.07)	69.57
	P. trifoliata (WT)	40.25 (1.00)	39.74 (0.99)	7.38 (0.18)	87.37
	P35S::CiFT (CNT)	36.74 (1.00)	34.55 (0.94)	4.66 (0.13)	75.95

Values are averages of two experimental replications.
Numbers in parentheses indicate the ratio to peak area of β-myrcene.

content in their flowers. A reduced limonene content was also observed in the fruit of Nos. 4-34 and 5-4. Fruit of No. 1-15 were not analyzed. In line No. 2-39, *PtLim1* transcripts were detected in flowers and fruit, and the relative limonene content was similar to controls in fruit. However, in flowers, the ratio of limonene was reduced

to some extent. In this case, the transcript accumulation level in flowers of line No. 2-39 was slightly lower than that of line No. 1-7, and this repression of *PtLim1* expression was attributed to a small reduction in the relative limonene content. Similarly, it is plausible that flowers of line No. 4-1 also showed a lower accumulation of the transcript, which resulted in the reduced limonene ratio. That is, the repression of endogenous limonene synthase gene expression, which was probably caused by the ectopic expression of antisense *CitMTSE1*, was consistent with the reduction of the relative *d*-limonene content.

Discussion

Our results clearly indicated that the *CiFT* co-expression system could reduce the time to obtain flowers and fruit of transgenic trifoliate orange, thus enabling the rapid evaluation of the effects of gene introduction into reproductive tissues. Indeed, this is the first successful alteration of metabolic components in flowers and fruit of transgenic citrus plants. The long juvenile period of citrus, ranging from 6 to 20 years, has hampered the genetic improvement of reproductive tissues in transgenic plants. Although there have been some reports on gene introduction aimed at the improvement of citrus fruit quality (Guo et al., 2005; Li et al., 2002), only vegetative tissues in transgenic plants have been investigated so far. The introduction of the *CiFT* co-expression vector caused early flowering and fruiting in transgenic trifoliate orange. Transgenic plants producing flowers and fruit were obtained within 2 years of *Agrobacterium* infection, which is almost the same as that reported previously (Endo et al., 2005). Although all 14 transgenic lines obtained in this study flowered, fruit development was observed only in 6 lines within 2 years of *Agrobacterium* infection. Since most of plants without fruiting had a dwarfed and heavily branched tree shape and small leaves, similar observations to a previous report (Endo et al., 2005), nutritional problems might prevent fruit development in these transgenic plants. The frequent obtaining of fruit-bearing transgenic plants is important to improve this system. This system can be used to alter expression of the target gene, limonene synthase, in reproductive organs of flowers and fruit, resulting in a significant reduction in the relative limonene content in transgenic plants. Therefore, this system will make a useful contribution to advancing the metabolic engineering of citrus. There have been several attempts to alter plant aroma using monoterpene synthase genes. Transgenic tobacco that over-expressed monoterpene synthase genes showed a small accumulation of their corresponding products, or products that were inactivated by glycosylation or hydroxylation (Lücker et al., 2004a, 2004b; Ohara et al., 2003). Transgenic tomato that expressed the geraniol synthase (GES) gene in mature fruit showed marked changes in the composition of volatiles, along with unexpected increases of myrcene, limonene, and ocimene (Davidovich-Rikanati et al., 2007). In citrus, peels are especially rich in oil glands containing essential oils. These essential oils consist of various terpenoids, such as hemiterpenes, monoterpenes, and sesquiterpenes, and their derivatives, such as alcohols, esters, and acetates. They are known to be involved in ecological interactions with insects, pathogens, and other plants and are commercially valuable in terms of fragrances, pharmaceuticals, and agrochemicals. For example, *d*-limonene and 1,8-cineole have insecticide and anti-fungal activities (Byun-McKay et al., 2006; Pattnaik et al., 1997). Since this study showed that endogenous monoterpene metabolism in citrus fruit can be modified in transgenic plants, our approach will potentially lead to new insights into the biological functions of various monoterpenes and the regulation of monoterpene metabolism.

In transgenic plants, the reduced levels of *d*-limonene in flowers and fruit were consistent with the transcriptional repression of the endogenous gene. This suggests that monoterpene biosynthesis is regulated at a transcriptional level. The same hypothesis was generated by previous results, in that a different volatile composition in each tissue was explained by transcriptional accumulation of the corresponding monoterpene synthase genes (Shimada et al., 2005a). It was reported that limonene is the predominant component of citrus aroma, since the Km value of limonene synthase was 4.5-fold lower than the Km values of β-pinene and γ-terpinene synthases (Lücker et al., 2002). However, in this study, the composition of other monoterpenes was not significantly altered in transgenic plants in spite of the reduction of the transcripts for the endogenous limonene synthase gene and relative *d*-limonene level. Limonene synthase forms *d*-limonene directly from geranyl pyrophosphate (GPP), a common C_{10} intermediate of isoprenoid biosynthesis, through the initial isomerization of GPP to the tertiary allylic isomer, linalyl pyrophosphate, and subsequent cyclization of this intermediate to the α-terpinyl carbocation (Fig. 7, McGarvey and Croteau, 1995). GPP is generated by the condensation of isopentenyl diphosphate (IPP) and its allylic isomer dimethylallyl diphosphate (DMAPP) in the plastidial 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway (Fig. 7, Rodriguez-Concepción and Boronat, 2002). IPP and DMAPP in the plastidial MEP pathway are responsible for the production of isoprene (C5), monoterpenes, diterpenes (C20; e.g., gibberellins), and tetraterpenes (C40; e.g., carotenoids). Therefore, we assumed that the GPP or IPP pool in transgenic flowers and fruit was consumed by other biosynthesis pathways, such as that involving gibberellins (van Schie et al., 2007), and the transcriptional reduction of the limonene synthase gene did not lead to a significant change in the levels of other monoterpenes.

In conclusion, the *CiFT* co-expression system is useful for the rapid evaluation of transgenic flowers and fruit,

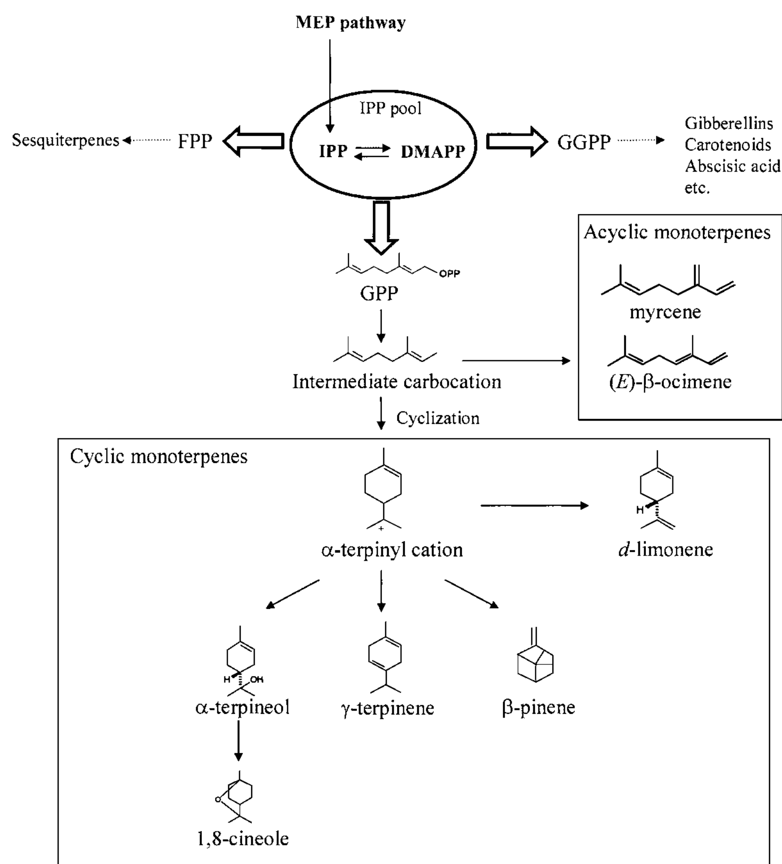


Fig. 7. Schematic diagram of the biosynthesis of monoterpenes discussed in this experiment. Isopentenyl pyrophosphate (IPP) and its allylic isomer dimethylallyl diphosphate (DMAPP) are used for the synthesis of GPP, FPP, and GGPP, which are precursors of higher terpenoids such as monoterpenes, sesquiterpenes, carotenoids etc. The formation of monoterpenes occurs through the intermediate carbocation. The decisive difference in the biosynthesis of acyclic and cyclic monotepenes depends on the cyclization of the intermediate carbocation.

and to study metabolic improvement in citrus. Citrus fruit is abundant in secondary metabolites, in particular, various terpenoids such as monoterpenes, sesquiterpenes, limonoids, and carotenoids, some of which could be utilized in pharmaceuticals and agrochemicals. Several studies involving citrus have suggested that the levels of secondary metabolites were directly affected by their transcriptional levels (Jacob-Wilk et al., 1999; Kato et al., 2004; Shimada et al., 2005a). The *CiFT* co-expression system can be applied to such secondary metabolites, and, thus, advance metabolic engineering. Recently, citrus promoters were isolated from satsuma mandarin and characterized, such as *CuMFT1* (Nishikawa et al., 2008) and *CitMT45* (Endo et al., 2007) promoters, which induce preferential expressions in seeds and fruit, respectively. The use of these promoters in the *CiFT* co-expression system will advance citrus transgenic studies, and contribute to the production of commercially desirable transgenic plants for use in pharmaceutical and agrochemical industries.

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