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Ectopic expression of an *FT* homolog from *Citrus* confers an early flowering phenotype on trifoliate orange (*Poncirus trifoliata* L. Raf.)

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Abstract

Citrus FT (*CiFT*) cDNA, which promoted the transition from the vegetative to the reproductive phase in *Arabidopsis thaliana*, when constitutively expressed was introduced into trifoliate orange (*Poncirus trifoliata* L. Raf.). The transgenic plants in which *CiFT* was expressed constitutively showed early flowering, fruiting, and characteristic morphological changes. They started to flower as early as 12 weeks after transfer to a greenhouse, whereas wild-type plants usually have a long juvenile period of several years. Most of the transgenic flowers developed on leafy inflorescences, apparently in place of thorns; however, wild-type adult trifoliate orange usually develops solitary flowers in the axils of leaves. All of the transgenic lines accumulated *CiFT* mRNA in their shoots, but there were variations in the accumulation level. The transgenic lines showed variation in phenotypes, such as time to first flowering and tree shape. In F_1 progeny obtained by crossing 'Kiyomi' tangor (*C. unshiu* × *sinensis*) with the pollen of one transgenic line, extremely early flowering immediately after germination was observed. The transgene segregated in F_1 progeny in a Mendelian fashion, with complete co-segregation of the transgene and the early flowering phenotype. These results showed that constitutive expression of *CiFT* can reduce the generation time in trifoliate orange.

Introduction

Fruit and forest trees have a long juvenile period, during which no reproductive development occurs. In *Citrus*, the juvenile period ranges from 6 to 20 years and has hampered traditional breeding and genetic studies. In the past decade, efforts have been made to reduce the long juvenile period in woody perennials using flowering genes that were characterized in *Arabidopsis thaliana* (Martín-Trillo & Martínez-Zapater, 2002).

Weigel and Nilsson (1995) generated transgenic hybrid aspen, in which the *LEAFY* (*LFY*) gene of *Arabidopsis* was constitutively expressed. Flowering was induced on juvenile plants in less than 1 year, whereas wild type plants usually take 8–20 years to flower. However, the introduction of *LFY* failed to obtain progeny from the transgenic hybrid aspen due to the absence of pollen in abnormal male flowers. A subsequent experiment revealed that *APETALA1* (*AP1*) from *Arabidopsis* was not effective in hybrid aspen (Nilsson &

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Weigel, 1997). Rottmann et al. (2000) established that competence to respond to the constitutive expression of *LFY* varies widely among different interspecific *Populus* hybrids. They also reported that the overexpression of *PTLF*, an ortholog of *LFY* in poplar, was less effective than that of *LFY* when expressed constitutively in hybrid aspen. Peña et al. (2001) introduced either *AP1* or *LFY* into citrange, an intergeneric hybrid between sweet orange (*Citrus sinensis* Osbeck) and trifoliate orange (*Poncirus trifoliata* L. Raf.), and succeeded in reducing its generation time. The ectopic expression of either gene produced fertile flowers and fruits on transgenic citrange within one and a half years, and the early flowering and fruiting traits were transmitted to progeny. Peña et al. (2001) also reported that these two genes had different phenotypic effects on transgenic citranges; *LFY* triggered abnormality in vegetative organs, whereas *AP1* did not. These results have shown that the ectopic expression of *AP1* or *LFY* resulted in different effects on different woody species. A possible explanation was given, namely, that the inconsistent effects of these genes might be caused by some specific interactions between the introduced genes and the host plants (Rottmann et al., 2000; Peña et al., 2001; Martín-Trillo & Martínez-Zapater, 2002). The incorporation of other flowering genes into woody perennials could result in the reduction of the generation time. Such experiments could provide new clues for understanding the interactions between flowering genes and host plants as well as other ways to reduce the juvenile period.

FLOWERING LOCUS T (*FT*) is one of the flowering-time genes in *Arabidopsis* and is characterized as a floral pathway integrator (Araki, 2001; Simpson & Dean, 2002).

The expression of *FT* is regulated by the photoperiodic pathway (Kardailsky et al., 1999; Kobayashi et al., 1999; Samach et al., 2000; Suárez-López et al., 2001; Yanovsky & Kay, 2002), by the light-quality pathway (Cerdán & Chory, 2003; Halliday et al., 2003), and, in part, by the autonomous pathway (Samach et al., 2000) in *Arabidopsis*. *FT* orthologs in rice have a conserved role for the induction of flowering by the photoperiod (Izawa et al., 2002; Kojima et al., 2002; Hayama et al., 2003). *FT* consists of a small gene family with *TERMINAL FLOWER1* (*TFL1*)/*CENTRORADIALIS* (*CEN*) homologs in the angiosperms, and its function in floral transition is antagonistic with that of *TFL1* (Kardailsky et al., 1999; Kobayashi et al., 1999). Recently, Pillitteri et al. (2004) demonstrated that transcript accumulation of *CsTFL*, a homolog of *TFL1* in citrus, was positively correlated with juvenility in citrus. In addition to the sequence similarity of the *FT-TFL1* family proteins to the human phosphatidylethanolamine-binding protein (PEBP), the crystal structure of *CEN*, an ortholog of *Arabidopsis TFL1* in snapdragon, suggested its functional similarity to PEBP as a kinase regulator (Banfield & Brady, 2000). However, their endogenous biochemical functions have not been elucidated so far. An *FT* homolog *CiFT* was found in the EST catalogue of a cDNA library from the fruit of satsuma mandarin (*Citrus unshiu* Marc.) (Hisada et al., 1997). The deduced amino acid sequence of *CiFT* had high homology to FT and its rice ortholog Hd3a (Kojima et al., 2002), and the overexpression of *CiFT* was previously shown to induce an early flowering phenotype in *Arabidopsis* (Kobayashi et al., 1999).

In the present study, we incorporated *CiFT* into trifoliate orange. Transgenic trifoliate orange had a dramatically reduced period to flower. The effects were transmitted into their nucellar seedlings as well as into the intergeneric hybrid progeny of a monoembryonic *Citrus* cultivar crossed with transgenic pollen.

Materials and methods

Plant materials

All materials used in this study, including trifoliate orange (*Poncirus trifoliata* L. Raf.), 'Kiyomi' tangor (*C. unshiu* Marc. × *sinensis* Osbeck), sour orange (*C. aurantium* L.), and rough lemon (*C. jambhiri* Lush.), were cultivated at the National Institute of Fruit Tree Science (NIFTS), Okitsu (Shimizu-ku, Shizuoka, Japan). Trifoliate orange was used as the source for the transformation experiments. After the seed coats were peeled and sterilized, the embryos were placed on an MS medium that contained MS salts (Murashige & Skoog, 1962) supplemented with 100 mg/l *m*-inositol, 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine-HCl, 0.1 mg/l thiamine-HCl, 2 mg/l glycine, and 0.2% gelrite. The embryos were

incubated at 25°C in the dark for germination to obtain the materials required for the experiments. Two different types of rootstock were prepared for two-step grafting, which promotes the growth of the regenerated plants. Sour orange was used as the first rootstock for the *in vitro* grafting of regenerated plants. It was germinated and etiolated in the same way as trifoliate orange and used 2–4 weeks after seed planting. Rough lemon was used as the second rootstock for the second grafting of trifoliate orange on sour orange. It was germinated and grown in the greenhouse for about 1 year before use.

To investigate the transmission of the transgene progeny, seeds were obtained from fruit on both primary transgenic plants and the monoembryonic 'Kiyomi' tangor crossed using the pollen of a transgenic line (No. 3–10) and germinated on the same medium at 25°C.

Agrobacterium-mediated transformation

A construct of the *CiFT* gene, described in a previous study (Kobayashi et al., 1999) was used. A *CiFT* fragment of approximately 0.65 kb was excised from *CiFT* cDNA in pBluescript SK(−) with *Xba*I and *Eco*RV, whose recognition sites are in the cloning site and the 3′ untranslated region (at 648 of GenBank AB027456) of *CiFT*, respectively, and inserted between the 35S promoter of the Cauliflower Mosaic Virus (CaMV) and the nopaline synthase terminator in the pCGN1547 vector (Calgene, Davis, CA). The plasmid was transferred into the *Agrobacterium tumefaciens* strain LBA4404 by tri-parent mating. Epicotyl segments from 2-week-old etiolated seedlings of trifoliate orange were infected with *Agrobacterium* according to the method of Kaneyoshi et al. (1994). Explants after 3 days of co-cultivation were put on an MS medium supplemented with 3% sucrose, 2×10^{-5} M 6-benzylaminopurine (BA), 5×10^{-7} M α-naphthalene acetic acid (NAA), 100 mg/l kanamycin, and 500 mg/l Claforan for shoot regeneration and first selection. Explants were subcultured on an MS medium supplemented with 3% sucrose, 2×10^{-6} M BA, 200 mg/l kanamycin, and 500 mg/l Claforan for shoot elongation and second selection. The adventitious shoots that emerged on the segments were detached and individually grafted *in vitro* onto etiolated seedlings of sour orange, which had been previously germinated and prepared as rootstock. The second grafting was carried out to stimulate growth. *In vitro*-grafted plants were grafted again on one-year-old rough lemon rootstock. Control plants free of infection were concurrently regenerated and grown without kanamycin selection.

Gene incorporation was confirmed in both the primary transgenic plants and their offspring by the standard PCR amplification technique with a primer set of the 35S promoter (5′-ATCTCCACTGACGTAAGGGATGACG-3′) and *CiFT* (5′-AAAGCTGGCCCCTGTGGTTGC-3′) sequences. The thermal condition was 30 cycles of 1 min at 94°C, 1 min at 57°C, and 1 min at 72°C.

Southern blot analysis

Ten microgram of total DNA of the transgenic plants was completely digested with *Xba*I, whose recognition site is located between the 35S promoter and *CiFT* cDNA of the construct. A 0.65 kb fragment of the CaMV P35S enhancer region obtained from the digestion of pBI221 (Clontech Laboratories, CA, USA) with *Eco*RV and *Hin*dIII was used as a probe.

Digested DNA was electrophoresed on a 1.0% agarose gel and blotted onto a nylon membrane (Hybond-NX, Amersham-Pharmacia Biotech, Little Chalfont, UK). Probe labeling by Digoxigenin (DIG), hybridization, and detection were conducted according to manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany).

Northern blot analysis

For the analysis of *CiFT* expression, shoots including leaves, nodes, and internodal segments were collected from the transgenic lines. Total RNA was extracted by the methods of Ikoma et al. (1996). Ten microgram of a sample was subjected to electrophoresis on a 1.2% agarose gel containing 8% (v/v) formaldehyde, stained with ethidium bromide (EtBr), and transferred to a nylon membrane. A 0.65 kb fragment of the *CiFT*, which extends from the 5′ end to the *Eco*RV site in the 3′ untranslated region, was used as a probe. The experiment was carried out according to manufacturer's instructions (Roche Diagnostics GmbH).

Results

Early flowering and fruiting on trifoliate orange with 35S:CiFT

To investigate the effect of the overexpression of *CiFT*, a construct driven by the 35S CaMV promoter (Kobayashi et al., 1999) was introduced into trifoliate orange via *Agrobacterium tumefaciens*. Adventitious shoots that developed from epicotyl segments on the selection medium were detached and individually grafted on a rootstock (see Materials and methods). After the second grafting, regenerated plants were transferred to the greenhouse. The timing of transfer to the greenhouse was dependent on the plants and corresponded to 16–24 weeks after epicotyl segments had been infected. Among six independent transgenic lines grown in the greenhouse, five developed flowers in less than 8 months, as early as 12 weeks in line No. 2–41, after transfer to the greenhouse (Table 1, Figure 1(a)). Line No. 3–21 required the longest time, approximately 20 months, to have the first flower after transfer to the greenhouse. Most flowers were morphologically normal (Figure 1(d) and (e)) and contained fertile pollen. Four transgenic lines developed morphologically normal fruits with intact seeds (Table 1, Figure 1(b), (f) and (g)). Flowering and fruiting of these transgenic lines occurred continually. Seeds from the fruits of lines Nos. 2–11 and 3–10 were germinated *in vitro*, and seedlings from nucellar embryos due to polyembryony were obtained. Eleven of 11 (line No. 2–11) and 1 of 3 (line No. 3–10) nucellar seedlings developed floral bud(s) with one or two pairs of bract-like leaves (Figure 1(c)), or primary shoot apices fell immediately upon germination. In contrast, no flower development occurred in control trifoliate orange plants.

Morphology of the CiFT-introduced trifoliate orange

Morphological differences were observed between the transgenic trifoliate orange and wild-type plants. Wild-type trifoliate oranges usually develop solitary flowers in the axils of leaves. Differentiation of floral buds starts in early summer and is completed prior to the onset of winter dormancy (Spiegel-Roy & Goldschmidt, 1996). In the subsequent spring, flowers develop on leafless branches (Figure 2(a)). Wild-type vegetative shoots develop thorns concurrently with leaves (Figure 2(c)). Thorns are obvious even on adult trees. As a result, flowers can often be observed on the flanks of thorns (Figure 2(d)). On the other hand, all lines of the *35S:CiFT* plants had a leafy inflorescence architecture, in which flowers and leaves concurrently developed (Figure 2(b)). The development of flowers was not interrupted by a dormant period. Since flowers developed repeatedly along with lateral shoot growth from the axils of leaves, most of the flowers in the transgenic plant were on the inflorescence. After the repetition of lateral shoot growth, only a few axillary buds on the transgenic plants developed solitary flowers (Figure 2(e)). The tree shape of transgenic plants was dwarfed and branched in

Table 1. Summary of the *35S:CiFT* transgenic and regenerated wild type trifoliate oranges

Line	Time to flowering (weeks)[a]	Fruit development	Estimated copy No. of the transgene[c]	Tree shape
1–14	16	–[b]	1	Semi-dwarfed & highly branched in the distal part
1–26	33	–	1	Semi-dwarfed & branched in the distal part
2–11	28	+	1	Semi-dwarfed & branched
2–41	12	+	2	Dwarfed & highly branched
3–10	14	+	2	Semi-dwarfed & highly branched
3–21	86	+	1	Semi-dwarfed & branched
Control	–	–	0	Upright

[a]Observation after transfer to the greenhouse.
[b]Presence (+) or absence (–) of the development and maturation of fruits on a plant.
[c]Refer to Materials and methods.

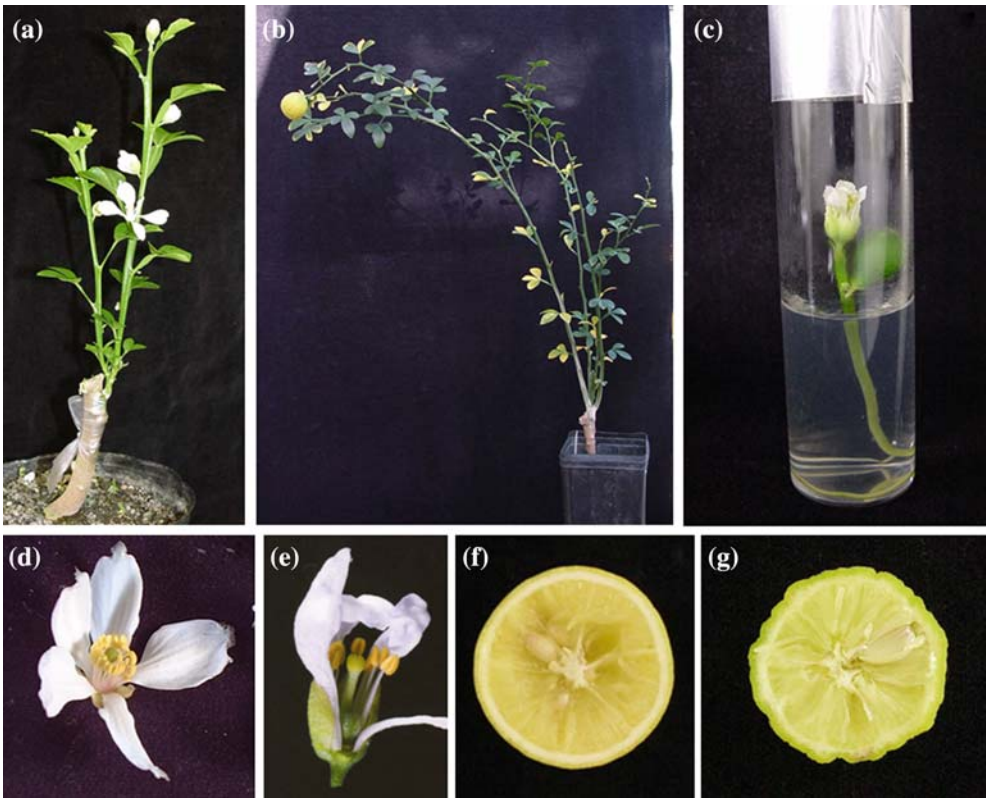

Figure 1. Reproductive development in transgenic trifoliate oranges with *35S:CiFT*. (a) Early flowering on line No. 2–41 12 weeks after transfer to the greenhouse. (b) Maturation of a fruit on line No. 2–11 46 weeks (approximately 10 months) after transfer to the greenhouse. (c) Flowering in a nucellar seedling of line No. 2–11 3 weeks after seed planting. (d) and (e) Flower picked on wild-type trifoliate orange (d) or transgenic line No. 3–10 (e). (f) and (g) Cross-section of ripened fruit on wild-type trifoliate orange (f) or transgenic line No. 3–10 (g).

comparison with that of the controls, and the leaf shape was morphologically altered in the transgenic plant (Table 1, Figure 2(i)). A typical leaf is shown in Figure 2(g), right; this leaf was small, lacked color on the margin, and had a leaflet at the center of a trifoliate leaf that was smaller than the other leaflets. This type of leaf appeared in the inflorescence structure prior to flower development and was next to the flowers.

The transgenic trifoliate orange sometimes showed polarity in the morphological changes of flowers, leaves, and thorns within a shoot. A terminal flower was large and had a well-developed pistil, whereas lateral flowers, especially at the proximal position, were small with tiny pistils. The leaf shape in the proximal part of a shoot was almost identical to that of non-transformed plants (Figure 2(g, left) and (h)), whereas leaves characteristic of transgenic plants appeared prior to flower development and became small and, in some cases, nearly invisible in the distal part of a shoot (Figure 2(b)). A comparison between an inflorescence on the transgenic plants (Figure 2(b)) and a vegetative shoot of the untransformed controls (Figure 2(c)) indicated that flowers on the transgenic plants took the place of thorns. A node on the transgenic inflorescence consists of a leaf, a flower, and axillary buds, whereas a thorn, instead of a flower, is contrastively a constituent of a node on a wild-type vegetative shoot. In addition, a small flower-like organ at the tip of a thorn was observed on the transgenic shoot, as shown in Figure 2(f). Such organs were often present in the proximal part of an inflorescence shoot, followed by the development of flowers in the distal part. These observations suggest that thorns were converted into flowers in the *35S:CiFT* transgenic trifoliate

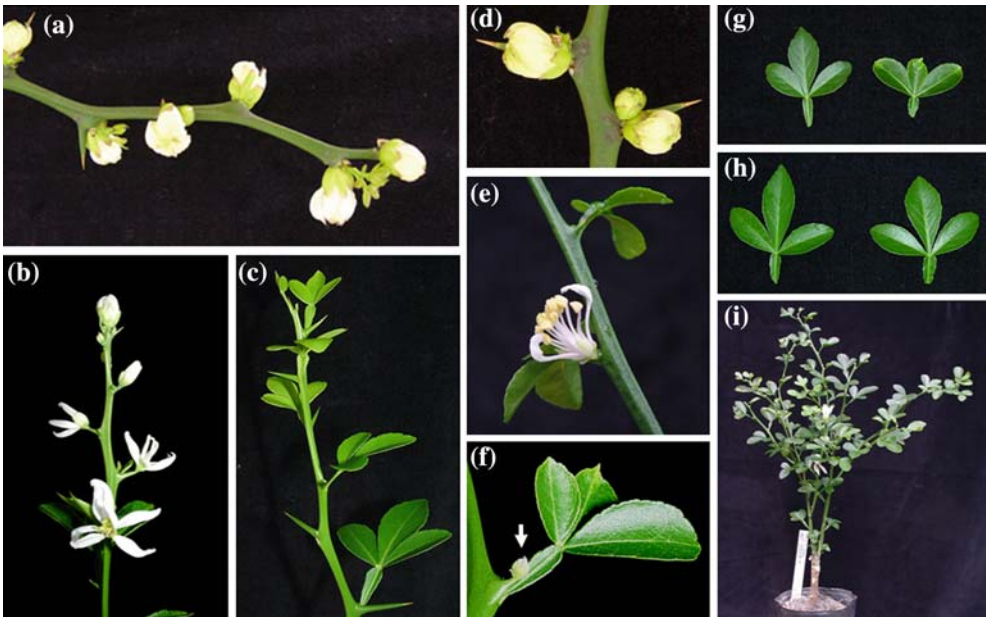

Figure 2. Flowering and vegetative architectures in wild-type and transgenic plants. (a) Flowering on wild-type trifoliate orange. Solitary flowers develop on previous year's shoot. Flower and foliage spur from a node are visible. (b) Inflorescence structure on line No. 1–14. Flowers took the place of thorns that were conspicuous on a vegetative shoot of a control plant (c). (d) Solitary flowers on wild-type plant. These flowers are on the flanks of thorns. (e) Solitary flower developed on line No. 2–41 in the axils of the leaf. (f) Arrow indicates a small flower-like organ at the tip of a thorn on line No. 1–14. (g) and (h) Leaves from the proximal and distal parts of line No. 2–11 (g) or control plants (h) were aligned from left to right. (g) and (h) are in the same scale. (i) Highly branched tree shape of line No. 2–41.

orange. The leafy inflorescence was often accompanied with gradual changes of thorns into flowers and observed specifically on the transgenic plant. Polarity changes were also observed in the transgenic citrange harboring either *35S:AP1* or *35S:LFY*, in which thorn development was suppressed as shoots grew, although the conversion of thorns into flowers was not described (Peña et al., 2001).

Variations of CiFT mRNA levels and phenotypes in transgenic lines

The level of the *CiFT* transcript was evaluated by northern blot analysis (Figure 3). No signal was observed in the control wild-type trifoliate orange, although the same heterologous probe could detect the endogenous *CiFT* genes of the wild-type genome by Southern blot analysis (data not shown). We concluded that the endogenous *CiFT* mRNA of the control plant was below the detection level in northern blot analysis because RT-PCR analysis detected the *CiFT* transcript in shoots of the wild-type plant (data not shown). All of the transgenic lines accumulated *CiFT* mRNA in their shoots, but there were variations in the accumulation level. Line No. 2–41, with the highest *CiFT* mRNA level, developed flowers the earliest (Table 1) and had a heavily branched and dwarfed tree shape (Figure 2(i)). Most shoots on line No. 2–41 were flower bearing, and the earliest solitary flower developed on this line within 1 year of transfer to the greenhouse. In contrast, line No. 3–21, with an obviously lower mRNA accumulation level than other transgenic lines, took the longest time to flower (Table 1) and developed fewer branches than other transgenic lines (data not shown). The other four lines had different levels of *CiFT* mRNA and showed variation in phenotypes, such as time to first flowering, tree height, and branch pattern (Table 1). The lack of fruit development for lines Nos. 1–14 and 1–26 could be explained by the differences in growth conditions and expression of other flowering genes, although these explanations have not been experimentally verified.

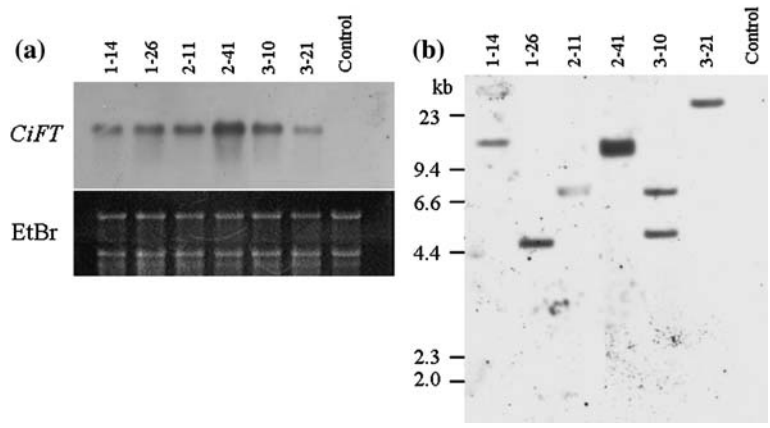

Figure 3. Northern and Southern blot analysis of transgenic plants. (a) Northern analysis for *CiFT* in shoots. Ten microgram of total RNA of the transgenic (line Nos. 1–14, 1–26, 2–11, 2–41, 3–10, and 3–21) or the regenerated control plant was electrophoresed, stained with ethidium bromide (EtBr, lower panel), transferred to a membrane, and hybridized with *CiFT* cDNA as described in Materials and methods. 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 (upper panel). (b) Southern analysis for the confirmation of gene integration, and estimation of copy number. Ten microgram of genomic DNA of transgenic or the regenerated control plant was digested with *Xba*I, electrophoresed, stained with EtBr, transferred to a membrane, and hybridized with a fragment of the CaMV P35S enhancer region as described in Materials and methods. 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.

Parallel relationships of the transgene expression level and extent of its causal phenotypes have been observed in *Arabidopsis* with *35S:FT* (Kardailsky et al., 1999; Kobayashi et al.,1999), rice overexpressing *Hd3a* (Kojima et al., 2002), and citrange with *35S:LFY* (Peña et al., 2001). In the present study, a parallel relationship was observed between lines Nos. 2–41 and 3–21 with the extreme mRNA accumulations and phenotypes, but it was inapplicable to the other lines with intermediate levels of *CiFT* mRNA and various phenotypes. We believe that these various phenotypes observed in the lines with intermediate levels of *CiFT* mRNA might be caused by environmental conditions during plant growth and interactions between ectopically expressed *CiFT* and endogenous genes related to development.

The insertion of the transgene was examined by Southern blot analysis (Figure 3(b)). The copy number of the gene insertion was estimated (Table 1) using the 35S CaMV promoter as a probe. The copy number was not related to the mRNA level or the phenotypes in this study.

An extremely early flowering phenotype in the F_1 progeny of the transgenic plant

Trifoliate orange generally shows polyembryony, as most *Citrus* species; a mature seed contains sexual and apomictic embryos (Spiegel-Roy & Goldschmidt, 1996). Since supernumerary nucellar embryos make it difficult to obtain a self-pollinated progeny of *35S:CiFT* trifoliate orange, monoembryonic Kiyomi tangor was pollinated with the pollen from one transgenic line, No. 3–10. Among 32 intergeneric F_1 hybrids obtained, 14 seedlings developed flowers *in vitro* immediately after germination (Figure 4(a)). Flower buds on all 14 seedlings were visible within 10 days of *in vitro* seed planting without any grafting procedure. They developed roots at first and then the primary shoot, which was terminated in a flower after the formation of a few nodes. The architectures of these hybrids were very similar to that of the nucellar seedlings (Figure 1(c)). The remaining 18 F_1 seedlings grew normally without floral development. To confirm the inheritance of the transgene in the progeny, PCR was conducted using DNA from roots of individual seedlings. All 14 seedlings with precocious flowers had the transgene, and the remainder without flowering lacked it. The segregation of the transgene took place in a Mendelian ratio (1:1) with complete co-segregation of the transgene and the early flowering phenotype. The F_1 seedlings with the transgene could be further grown by the rescue of lateral shoots that emerged from the basal part of the cotyledon (Figure 4(b)). Seven F_1 seedlings with the transgene were grown

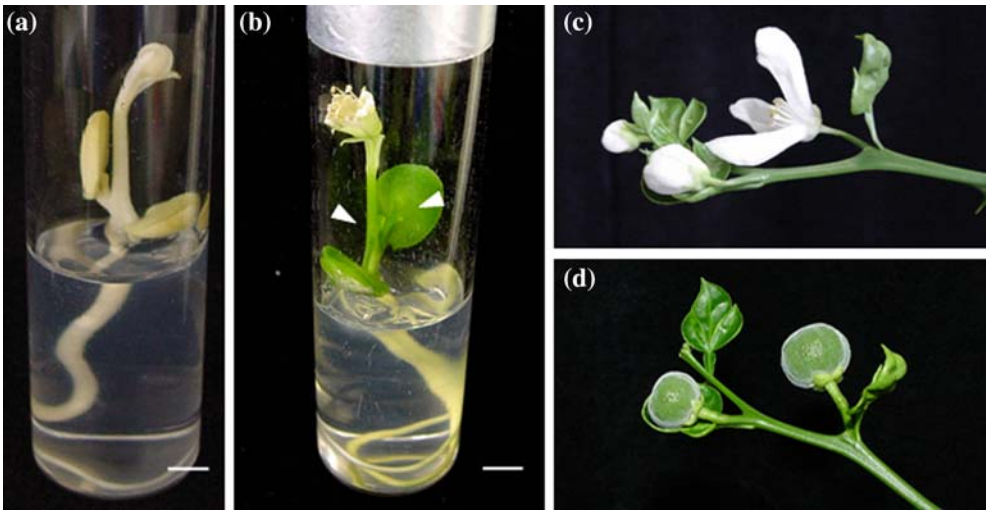

Figure 4. Flowering phenotypes of the F_1 progeny with the transgene obtained through crossing with Kiyomi tangor. (a) Flowering *in vitro* on ethiolated seedling 11 days after seed planting. The scale bar indicates 0.5 cm. (b) Flowering and lateral shoot growth from the basal part of the cotyledon (arrowheads) 19 days after seed planting. The scale bar indicates 0.5 cm. (c) and (d) Flowers (c) and fruits (d) on a rescued plant containing the transgene.

in the greenhouse using the same method used for transformed regenerated shoots. Two developed flowers within 2 years, and one had a fruit set (Figure 4(c) and (d)). Their inflorescence structures were identical to those of the parent *35S:CiFT* trifoliate orange, whereas the shape of the leaf was more altered (Figure 4(c) and (d)). Thirteen siblings without the transgene did not flower. The difference in the degree of phenotype between parents and the F_1 hybrid appears to be due to the genetic background. However, as the segregation of *35S:CiFT* produced, qualitatively, identical phenotypic effects on F_1 hybrids, we concluded that the early flowering phenotype caused by the overexpression of *CiFT* was transmitted to the zygotic progeny through the meiotic process.

Discussion

CiFT promoted early flowering and fruiting in trifoliate orange

This study demonstrated that a citrus homolog of the *FT* gene is capable of inducing extremely early flowering and fruiting in trifoliate orange by ectopic expression. The flowering features of transgenic trifoliate oranges with *35S:CiFT* were clearly different from the 'precocious flowering' of pummelo (*C. grandis* Osbeck) or grapefruit (*C. paradisi* Macf.), which developed flowers on one-year seedlings but did not flower in subsequent years (Yamada et al., 1991). In these species, no fruit was developed from the precocious flower. Furthermore, extremely precocious flowering in the progeny of the transgenic plant happened obviously earlier than in the seedlings of pummelo and grapefruit.

We deduced that *35S:CiFT* primarily caused the conversion of vegetative shoots into leafy inflorescences rather than the transition from the juvenile to adult phases. In the transgenic trifoliate orange, most of the flowers were on the inflorescence in place of thorns, and the development of solitary flowers was infrequently observed at the later growth stage. Flowering on a leafy inflorescence is not observed in wild-type trifoliate orange, but solitary flowers develop in the axils of leaves during the adult phase. The solitary flower development of the wild-type is similar to that of the transgenic plant at the later growth stage. According to the results in *35S:FT Arabidopsis* (Kardailsky et al., 1999; Kobayashi et al., 1999), it is plausible that the ectopic expression of *CiFT* converted vegetative shoot apical meristems of trifoliate orange into inflorescence meristems. Meristem changes might not occur suddenly, and a gradual change in the meristem could result in the

characteristic inflorescence with polarized effects on lateral organs. Peña et al. (2001) also concluded that *35S:LFY* could result in thorn reduction in the transgenic citrange by a side effect of *LFY* rather than by the phase transition from juvenile to adult. *35S:AP1*, in contrast, was thought to confer the rapid progression of the growth phases in the transgenic citrange through the interaction with other endogenous MADS-box proteins (Peña et al., 2001; Martín-Trillo & Martínez-Zapater, 2002).

Distinctive phenotypes of 35S:CiFT trifoliate orange and 35S:LFY and 35S:AP1 citranges

The analogies between the phenotypes of *35S:CiFT* trifoliate orange and *35S:LFY* citrange suggest similar functions of *CiFT* and *LFY*. However, these transgenic plants have some distinctive phenotypes. *35S:CiFT* converted thorns into flowers in the inflorescence. Such a phenomenon has not been observed in the *35S:LFY* citrange. Peña et al. (2001) reported that flowering of transgenic citranges with *35S:LFY*, as well as *35S:AP1*, was still under environmental control, showing seasonal periodicity. In contrast, the transgenic trifoliate orange with *35S:CiFT* developed flowers repeatedly in the course of shoot growth. Furthermore, extremely early flowering immediately after germination from seeds in nucellar and hybrid seedlings of the *35S:CiFT* trifoliate orange is a novel phenotype, which was not observed in the case of the offspring from *35S:LFY* or *35S:AP1* citranges. The morphology of extremely early flowering on the primary shoot apex of the transgenic seedlings is reminiscent of the immediate floral induction of the *Arabidopsis* seedling with *35S:FT* and *35S:LFY* (Kardailsky et al., 1999; Kobayashi et al., 1999). This is perhaps due to the expression of the endogenous *LFY* homolog in citrus upon germination. It might be active in vegetative tissues, as it is in other tree species (Mouradov et al., 1998; Rottman et al., 2000). These phenotypic variations in these transgenic plants may reflect the different roles of endogenous orthologs of *FT* and *LFY* in the regulation of flowering, although the different genetic background between trifoliate orange and citrange should be taken into consideration.

Application of CiFT to the induction of early flowering in Citrus species

This study showed that transgenic trifoliate oranges with ectopic expression of *CiFT* have a reduced generation time. Although *Poncirus*, to which trifoliate orange belongs, and *Citrus* are sexually compatible and belong to the same family, there are critical differences in the development of these two genera: *Poncirus* is deciduous, and *Citrus* is evergreen. Meiotic transmission of *35S:CiFT* caused early flowering in the intergeneric hybrid between the *Poncirus* and *Citrus* species, suggesting that the overexpression of *CiFT* could reduce the generation time in *Citrus* species. Since differences of the genetic background seemed to affect phenotypes by the introduced *CiFT* gene, further investigations are required to verify the effect of *CiFT* in *Citrus*. In addition, *CiFT* overexpression in trifoliate orange resulted in vegetative growth and a flowering habit that had never been observed in the wild-type plant, which should be taken into consideration before any application is attempted. Nevertheless, *CiFT*-transgenic trifoliate orange plants could be a helpful tool for functional genomic studies in *Citrus*.

Martín-Trillo and Martínez-Zapater (2002) have suggested that the transition from the juvenile to the adult phase could be controlled by genetic and environmental pathways that are common to the annual induction of flowering in adult trees. How this regulation takes place in woody perennials and how *FT* homologs relate to this regulation are issues of considerable interest. Further research would be required to answer these questions as well as to reduce the generation time for the genetic study and breeding of woody perennials.

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