Isolation and Characterization of a *Citrus FT/TFL1* Homologue (*CuMFT1*), Which Shows Quantitatively Preferential Expression in *Citrus* Seeds

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A *Citrus FT/TFL1* homologue (*CuMFT1*) was isolated from Satsuma mandarin (*Citrus unshiu* Marc.), and a high degree of expression was detected in mature seeds, indicating that its promoter is expected to induce the expression of a target gene in the late embryonic stage of *Citrus* seed. To obtain a seed-specific promoter useful for *Citrus* transgenic research, the 5’ upstream region of *CuMFT1* was isolated from the BAC library, and its promoter activity was characterized using particle bombardment and transgenic *Arabidopsis*. The 2.4 kbp in the 5’ upstream region (*CuMFT1* promoter) contained RY (CATGCAT), E-box (CANNTG), and distant B-box (GCCACTTGTC) cis-elements, which have been reported to promote seed-specific gene expression in plants. The *CuMFT1* promoter fused to the *uidA* gene was directly incorporated into tissues from *Citrus* and its relatives using particle bombardment. The results showed that the *CuMFT1* promoter conferred high β-glucuronidase (GUS) activity in seed. The *CuMFT1* promoter-GUS fusion construct was also incorporated into *Arabidopsis*, and transgenic *Arabidopsis* was evaluated by histochemical staining and fluorometric GUS analysis. The experiments revealed that the *CuMFT1* promoter conferred quantitatively preferential expression in *Arabidopsis* seeds; thus, it was suggested that the cis-elements in the *CuMFT1* promoter required for expression in *Citrus* seed were functionally conserved in the heterologous *Arabidopsis* plant. The *CuMFT1* promoter could be utilized as a promoter regulating the quantitatively preferential expression in seed, and is useful for studies of seed development and manipulation by genetic engineering in *Citrus* and *Arabidopsis*.

Key Words: *Citrus*, MOTHER OF FT AND TFL1 (MFT), particle bombardment, promoter, seed.

Introduction

*Citrus* is a commercially important fruit crop and has various unique characteristics, such as complex secondary metabolites of terpenoids and flavonoids, seedlessness, apomixis, sexual incompatibility, and long juvenility. With the use of genome-wide EST collection and microarray analysis, the study of functional genomics has developed and is close to achieving the molecular characterization of these biological and agronomical traits in *Citrus* (Cercós et al., 2006; Forment et al., 2005; Shimada et al., 2005a). Transgenic research is an important aspect of functional genomics of *Citrus*, and recent efforts have made it possible to reduce the long juvenile period in perennial *Citrus* using flowering genes, such as *FLOWERING LOCUS T* (*FT*), *LEAFY* (*LFY*), or *APETALA1* (*AP1*) (Endo et al., 2005; Peña et al., 2001). The reduction of generation time by genetic engineering could accelerate research to understand the complex regulatory mechanism of biological and agronomical traits as well as the manipulation of the nutritional value and quality of fruits. Next, efforts should be made to develop tissue-specific and inducible promoters for the control of incorporated gene expression. A number of promoters of fruit maturation or ripening-related genes have been reported in plants (Giovannoni, 2001), but few have been isolated and characterized in *Citrus* species, except for promoter analysis of a metallothionein-like gene, *CitMT45* (Endo et al., 2007). The *CitMT45* promoter conferred the
quantitatively preferential expression in *Citrus* juice sacs and siliques of transgenic *Arabidopsis* and is able to manipulate fruit quality by genetic engineering in *Citrus* and *Arabidopsis*.

Generally, consumers consider seeds in *Citrus* fruits to be unfavorable, and they reduce the commercial value of the fruit. Understanding the molecular mechanism of seed development is extremely important for the species will be very valuable in the progress of this study as well as in the manipulation of embryogenetic traits. During a cDNA-cataloguing project for *Citrus*, the homologue of an *FT/TFL1* family gene, *MFT*, was obtained in the library from seed (Hisada et al., 1996). MOTHER OF *FT* AND TFL1 (*MFT*) is a member of the phosphatidylethanolamine-binding protein family in *Arabidopsis* and has a redundant function with FT in flowering promotion (Kobayashi et al., 1999; Yoo et al., 2004). In this study, a full-length cDNA clone homologous to *MFT*, *CuMFT1*, was isolated from Satsuma mandarin, and the gene expression pattern was analyzed. Since *CuMFT1* showed quantitatively preferential expression in seed, we isolated the genomic region of *CuMFT1* to obtain a seed-specific promoter in *Citrus*, and its promoter activity was investigated using particle bombardment and transgenic *Arabidopsis*. The *CuMFT1* promoter contained several known *cis*-elements for seed-specific expression in plants, and our results suggested that the *CuMFT1* promoter is useful to regulate the quantitatively preferential expression of a target gene in *Citrus* and *Arabidopsis* seeds.

**Materials and Methods**

1. **Plant materials**

Trifoliate orange (*Poncirus trifoliata* L. Raf.), Satsuma mandarin (*Citrus unshiu* Marc. ‘Miyagawa-wase’ and ‘Aoshima’), and Kishu-mikan (*Citrus kinokuni* Hort. ex Tanaka), which were cultivated at the National institute of Fruit Tree Science (NIFTS), Shizouka, Japan, were used in this study. For the investigation of developmental changes in mRNA levels, leaves, stems, flowers at anthesis, whole fruits at 30 days after flowering (DAF), and peels and juice sacs at 60 DAF, 120 DAF, and 180 DAF were collected from ‘Miyagawa-wase’ Satsuma mandarin. In addition, seeds and four-month-old seedlings of ‘Aoshima’ Satsuma mandarin were collected. Embryos at 0, 4, and 8 weeks after flowering were collected from Kishu-mikan.

2. **DNA blot analysis**

The genomic DNA of ‘Miyagawa-wase’ Satsuma mandarin was isolated from leaves by the method of Dellaporta et al. (1983). Ten µg of total DNA of Satsuma mandarin was completely digested with *DraI*, *EcoRI*, or *HindIII*. A 0.54 kb fragment of *CuMFT1* cDNA was used as a probe. Digested DNA was electrophoresed on a 1.0% agarose gel and blotted onto a nylon membrane (Hybond-NX, GE Healthcare, Little Chalfont, UK). Probe labeling by DIG, hybridization, and detection was conducted according to the manufacturer’s instructions (Roche Diagnostics GmbH, Mannheim, Germany).

3. **RNA extraction, reverse transcription-polymerase chain reaction (RT-PCR), and RT-PCR Southern blot analysis**

Total RNA was extracted by the methods of Ikoma et al. (1996). For RT-PCR, first-strand cDNA was synthesized from 1 µg of total RNA by reverse transcriptase with an oligo-(dT) primer according to the instructions of the Ready-To-Go You-Primed First-Strand Kit (GE Healthcare). For isolation of the *MFT* homologue from Satsuma mandarin, the RT-PCR reaction was performed with *CuMFT1*-F and *CuMFT1*-R primers (Table 1), which were designed on the basis of the nucleotide sequence from the *Citrus MFT* homologue found in the EST catalogue and cDNA derived from the seed of Satsuma mandarin. PCR products were cloned into the pCR-TOPO vector with a TOPO TA Cloning Kit (Invitrogen, San Diego, CA, USA). Sequences were determined with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) using the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

For RT-PCR Southern analysis, the first-strand cDNA produced as described above was diluted up to 60 µL in water, and aliquots of 1 µL were used in the RT-PCR reaction (20 µL final volume) in the presence of 0.5 µM gene-specific primers, *CuMFT1*-F and *CuMFT1*-R (Table 1). RT-PCR was conducted using 25 PCR cycles. Seven µL of the PCR product in each reaction was analyzed by electrophoresis in 1.5% (w/v) agarose gels, which were transferred to Hybond-N (Amersham Bioscience) with 20 × SSC, and the blots were exposed to UV light for 2 min. The membranes were prehybridized for more than 2 h in DIG Easy Hyb (GE Healthcare) and hybridized with DIG-labeled DNA probes overnight at 45°C. Probes were prepared using a PCR DIG Probe Synthesis Kit (GE Healthcare). Detection after hybridization was performed by chemiluminescence with CDP-Star (Roche Diagnostics). Chemiluminescent images of the blots were acquired using a CCD camera (Night Owl LB 981, Berthold Technologies, Bad Wildbad, Germany). EF1-α was used as a constitutive control.

**Table 1.** Primer sequences

<table>
<thead>
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<th>Sequence</th>
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<tr>
<td><em>CuMFT1</em>-F</td>
<td>5’-GACACCAGAACTTTACTCATC-3’</td>
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<tr>
<td><em>CuMFT1</em>-R</td>
<td>5’-ACAAAGCCAACACCTACATGCT-3’</td>
</tr>
<tr>
<td><em>CuMFT1</em>-2396-5’</td>
<td>5’-CAACATGAAGCTTATGGCCG-3’</td>
</tr>
<tr>
<td><em>CuMFT1</em>-2396-3’</td>
<td>5’-ATCCCGGGTAAATTTGCGTG-3’</td>
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4. Isolation of a CuMFT1 genomic clone
To isolate the genomic clone from the Satsuma mandarin BAC library (Shimada et al., 2005b), PCR screening was carried out with primers CuMFT1-F and CuMFT1-R, shown in Table 1. The PCR-positive BAC clone was subjected to sequencing analysis, and the genomic DNA sequence in the upstream region of CuMFT1 was determined using the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The primer-walking method was carried out with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

5. Transient assay by particle bombardment
In order to make the promoter-β-glucuronidase (GUS) fusion constructs for the transient assay (pTPMFT1; Fig. 1B), the promoter was amplified using a high-fidelity Platinum Pfx DNA polymerase (Invitrogen) with the CuMFT1-2396-5' primer and the CuMFT1-2396-3' primer (Table 1), in which a HindIII or an SmaI cloning site was artificially inserted, respectively. The amplified PCR products were digested with HindIII and SmaI and inserted into the modified pUC18 vector for the transient assay, which contained a promoter-less GUS gene with a NOS termination signal. pE2113, which is a constitutive strong promoter constructed by El2-CaMV35S-Ω, was used as a positive control (Mitsuhara et al., 1996). For particle bombardment, three tissues (seeds and leaves from trifoliate orange and juice sacs from Satsuma mandarin) were used. The skin of mature fruits was removed, and juice sacs were detached and placed separately in MS medium containing 0.2% gelrite in a Petri dish. Leaves from 1-month-old seedlings were placed in the same medium with the abaxial side downward. Mature seeds with their coat removed were placed into the same medium. The Biolistic Particle Delivery System, Model PDS-1000/He from BioRad (Richmond, CA, USA), was used for the acceleration of DNA-coated gold particles. Gold particles (1.0 μm) were coated with the respective plasmids, and a helium pressure of 9.3 MPa was employed. We used 500 μg of gold particles coated with 1 μg of each plasmid in one shot per Petri dish. The target distance between the stop screen and the Petri dish was set at about 3 cm. After bombardment, tissues in the Petri dish were kept at 25°C overnight and then subjected to histochemical staining of GUS activity. Each tissue was soaked in the substrate solution for several days, and the spot number was then counted under the stereomicroscope.

Promoter activity of the CuMFT1 5' flanking region was evaluated by the ratio of the number of GUS spots of the CuMFT1 promoter to pE2113 (CuMFT1/pE2113) for each Petri dish. Each experiment was repeated more than six times.

6. Arabidopsis transformation
In order to make the promoter-GUS fusion constructs for Arabidopsis transformation (pSPMFT1) (Fig. 1C), the same promoter region as described above was inserted upstream of the promoter-less GUS gene in a modified pSMAK311 binary vector (kindly provided by H. Ichikawa, National Institute of Agrobiological Sciences, Japan), which is a derivative of pSMAK251 (Yamashita et al., 1995). As a control, pSMAK311, which contains the CaMV35S promoter fused to the GUS gene, was used. The recombinant constructs were introduced into the Agrobacterium tumefaciens strain LBA4404 by tri-parent mating and selected for resistance to rifampicin and spectinomycin.

Seed stock (ecotype Columbia) was obtained from the Arabidopsis Biological Resource Center (ABRC) at Ohio State University, USA. All seeds were washed in

Fig. 1. Structure of constructs used in this study. (A) pE2113: Construct of El2-CaMV35S-Ω fused to GUS gene (Mitsuhara et al., 1996). (B) pTPMFT1: Construct of the CuMFT1 promoter region (−2396 to −37 from translation start site) fused to the GUS gene in pUC18. (C) pSPMFT1: Construct of the CuMFT1 promoter region (−2396 to −37 from translation start site) fused to the GUS gene in the binary vector, pSMAK311.
70% ethanol and 5% (v/v) dehydrochloric acid and rinsed three times with distilled water. Seeds were kept in 0.1% (w/v) agarose gel in MS medium at 4°C for up to four days prior to transfer to 21°C. When plants produced more than four leaves, seedlings were planted in soil. Arabidopsis plants were transformed with the A. tumefaciens strain LBA4404 using the floral-dip method (Clough and Bent, 1998). Transformed seeds were selected and assayed for GUS activity as described by Endo et al. (2007). The following tissues were subjected to measure GUS enzyme activity: roots, rosette leaves, flowers, immature siliques, including immature seeds, which develop before degreening, mature siliques without seeds, which occur after degreening, and mature seeds. The GUS histochemical assay was also performed as described by Endo et al. (2007).

**Results and Discussion**

1. **Structure and expression of the CuMFT1 gene**

A partial nucleotide sequence for the MFT homologue was obtained in an EST library derived from immature seeds of 'Valencia' orange (C. sinensis Osbeck) in the process of a cDNA-cataloguing project in Citrus (Hisada et al., 1996). To estimate the number of Citrus MFT copies in the genome, genomic hybridization was performed. Digestion by the three restriction enzymes estimated that MFT is a single or low copy in Satsuma mandarin (Fig. 2). On the basis of the EST nucleotide sequence, we designed the primer for RT-PCR to determine the nucleotide sequence of the MFT homologue in Satsuma mandarin. The MFT homologue obtained from Satsuma mandarin was designated as CuMFT1 (accession no. AB304142). It encoded 172 amino acid residues, which showed the highest identity (58.0%) with that of Arabidopsis MFT and high identity with those of GFT (47.9%) (Kobayashi et al., 1999) and CsTFL (47.9%) (Pillitteri et al., 2004) (Fig. 3). Hanzawa et al. (2005) showed that swapping a single amino acid was sufficient to convert TFL1 to antagonistic FT function. In the deduced amino acid sequence of CuMFT1, the key residue contributing FT and TFL1 functions, which is Tyr-85, His-88, and Typ-83 in Arabidopsis FT, TFL1, and MFT respectively (Hanzawa et al., 2005), was consistent with that of Arabidopsis MFT (Fig. 3A). In addition, phylogenic tree analysis revealed that CuMFT1 is a member of the FT/TFL1 family and that the function of CuMFT1 might be more similar to that of Arabidopsis MFT than to that of other family members (Fig. 3B).

To investigate the expression of CuMFT1 in several Citrus tissues, we performed RT-PCR Southern blot analysis, which is sensitive and can confirm that the amplified products of RT-PCR result from CuMFT1. CuMFT1 mRNA accumulation was detected distinctly in both seeds and flowers, but was much higher in seeds (Fig. 4A). In RT-PCR Southern blot analysis, CuMFT1 expression was not detected in embryos at 0, 4, and 8 weeks after flowering (Fig. 4B). Thus, the mRNA of CuMFT1 was accumulated largely in mature seeds; however, this preferential expression in seed made it difficult to predict the function of CuMFT1. In Arabidopsis, over-expression of Arabidopsis MFT led to slightly early flowering, and MFT was considered to function as a floral inducer (Yoo et al., 2004). CuMFT1 also has a similar function, caused a slightly early flowering phenotype, as determined from the results of transgenic Arabidopsis, which over-expressed CuMFT1 (unpublished data). Our results showed a small but distinct expression of CuMFT1 in flowers, suggesting that CuMFT1 may play a role in flowering in Citrus. On the other hand, in Citrus MADS-box genes, the regulatory relationships involved in Arabidopsis flower and fruit development were not directly applicable to Citrus, and different gene functions might be considered in fruit development (Endo et al., 2006). Considering these results, CuMFT1 might play a role in seed maturation as well as floral induction in Citrus. From the preferential expression of CuMFT1 in seeds, we considered that the 5' upstream region of CuMFT1 could be utilized as a useful seed-specific promoter.
Fig. 3. (A) Amino acid sequences of AtFT (accession no. AB027504), AtTFL1 (U77674), AtMFT (NM101672), CiFT (AB027456), CsTFL (AY344244), and CuMFT1. Identical residues of at least four alignments are represented by the framed area. The asterisk indicates the key amino acid residue contributing to FT and TFL1 functions (Hanzawa et al., 2005). (B) Phylogenetic tree of AtFT (accession no. AB027504), AtTFL1 (U77674), AtMFT (NM101672), CiFT (AB027456), CsTFL (AY344244), and CuMFT1. The NJ program was used to align 6 complete protein sequences. The numbers refer to the percentage in bootstrap analysis between the compared sequences.

Fig. 4. RT-PCR Southern blot analysis. (A) PCR for CuMFT1 was performed on cDNAs obtained from various tissues: seed collected at 180 days after flowering (DAF), root of seedling (r), aerial part of seedling (a), adult stem (s), adult leaf (l), flower at bloom (f), fruit at 30 DAF, juice sac at 60, 120, and 180 DAF, and peel at 60, 120, and 180 DAF. The amplified PCR products were separated on a 1.5% (v/v) agarose gel. PCR products on the gel were blotted onto a nylon membrane and hybridized with the DNA probe for CuMFT1. Hybridization signals are detected based on chemiluminescent images acquired with a CCD camera. The bottom panel indicated is an RT-PCR result of EF1-α as a control.
2. Structure of the 5’ promoter region of CuMFT1

The CuMFT1 expressed preferentially in seed is interesting because various promoter menus are necessary in transgenic research. To isolate the 5’ flanking region of CuMFT1, PCR screening was carried out in the Satsuma mandarin BAC library (Shimada et al., 2005b), and a PCR-positive BAC clone was obtained. The genomic sequence of 2396 bps from the translation start site (TSS) in the 5’ flanking region of CuMFT1 was determined (accession no. AB329714) and subjected to a database search to find motif sequences for potential cis-acting transcription factor binding sites using the PLACE program (Higo et al., 1999) (Fig. 5). Figure 5 shows a schematic representation of the 5’ flanking region of CuMFT1. The putative TATA box was located about −79 bps upstream from TSS. Several cis-motifs, which regulate seed-specific gene expression, were found in the 5’ flanking region of CuMFT1. The single RY cis-motif (CATGCGAT) was found between −1279 and −1288 bps from TSS, and one ABRE-like distB element (GCCACTGGTC) was located between −548 and −557 bps from TSS. In addition to these cis-motifs, nine E-box (CANNTG) was also found in the 5’ flanking region of CuMFT1. To date, numerous studies on seed-specific gene expression have been reported. The RY cis-motif is required to maintain seed-specific promoter activity (Reidt et al., 2000) and acts as a key regulator during late embryogenesis. This motif is widely distributed in seed-specific gene promoters of dicots and monocots, such as the USP gene (Fiedler et al., 1993) and the napin gene (Ellerström et al., 1996). The RY motif is considered to play an alternative role as a negative element repressing expression in non-seed tissues (Morton et al., 1995). The ABRE-like distB is an essential element responding to ABA through ABI3, which is a major transcriptional activator of seed maturation in Arabidopsis (Ezcurra et al., 2000). E-box, which is overlapped by and similar to ABRE-like distB, was reported to be required for embryo- and endosperm-specific transcription in storage protein gene napA (Ellerström et al., 1996). It was also demonstrated that seed-specific regulation required several ABA-responsive complexes, consisting of an RY/G- and a B-box (Ezcurra et al., 1999; Reidt et al., 2000). Most of the cis-motifs found in the 5’ flanking region of CuMFT1 were related to the ABA response in seed development and maturation. Considering the expression feature that CuMFT1 was induced in a late stage of embryogenesis, quantitatively preferential expression of CuMFT1 in

![Fig. 5](image_url)

Diagram of the upstream region of the CuMFT1 gene, which was fused to the GUS gene. The nucleotide position is shown relative to the translation start site (+1). Potential cis-motifs as well as the TATA box are shown.

![Fig. 6](image_url)

Promoter activity of the CuMFT1 promoter region measured by bombardment analysis. (A) The appearance of GUS-stained spots caused by pTPMFT1 on bombarded seed. (B) Number of GUS spots caused by pTPMFT1 in seeds, leaves and juice sacs (n = 6). (C) Relative number of GUS spots. The number of GUS spots caused by pTPMFT1 was divided by that caused by pE2113 (El2-CaMV35S-Ω promoter) (CuMFT1 promoter/pE2113). Means with the same letter were not significantly different at the 5% level by Tukey’s multiple range test (n = 6).
Citrus seed might be controlled by the ABA-dependent regulatory system.

3. Promoter activity of the 5′ flanking region of CuMFT1

Promoter analysis by particle bombardment was applied to evaluate CuMFT1 promoter activity in tissues from Satsuma mandarin and trifoliate orange because the transgenic approach is intensive labor and requires a long time to obtain seeds in Citrus. Two constructs of pE2113 and pTPMFT1 (Fig. 1A, B) were delivered into three tissues, seeds, leaves, and juice sacs. The introduced pE2113 caused visible GUS spots on the surface of all the target tissues (data not shown). The pTPMFT1 showed many GUS spots in seeds (Fig. 6A, B) but few in leaves and juice sacs (Fig. 6B). In this experiment, the promoter activity of CuMFT1 was evaluated using the CuMFT1/pE2113 spot number ratio. Based on these values, it was confirmed that the promoter activity of CuMFT1 in seeds was statistically ($P < 0.05$) higher than those in leaves and juice sacs (Fig. 6C). The relative number of GUS spots was more than 15-fold higher in seeds than in leaves and juice sacs. Thus, a transient promoter assay revealed that the CuMFT1 promoter regulated the quantitatively preferential expression in seed.

A transgenic promoter assay of CuMFT1 was carried out using Arabidopsis in order to evaluate the regulation of gene expression in stable transgenic plants and the applicability of the promoter in other plants. Two constructs of pSMASK311 (see Materials and Methods) and pSTMFT1 (Fig. 1C) were incorporated into Arabidopsis. Transgenic plants were confirmed by PCR amplification (data not shown). Histochemical staining was carried out using seven independent transgenic plants. Distinct GUS staining was observed only in seeds, and no staining was detected in leaves, stems (Fig. 7A–D), and roots (data not shown). Slight staining was observed in the style of flowers after bloom (Fig. 7E). For fluorometric analysis of GUS activity, five independent transgenic plants were used. Mature seeds were separated from mature siliques and were independently analyzed to identify tissue-specific GUS activities. Mature seeds exhibited remarkably higher GUS activity than the other examined tissues, at least seven-fold higher activity on average (Fig. 8). Thus, the CuMFT1 promoter conferred quantitatively preferential GUS activity in the seeds of transgenic Arabidopsis. These results of particle bombardment and transgenic Arabidopsis were consistent with the original gene

Fig. 7. GUS histochemical staining of transgenic Arabidopsis plants carrying the CuMFT1 promoter region (−2396 to −37 from the translation start site) fused to uidA gene. (A) and (B), Seed and silique; (C), rosette leaf; (D), cauline leaf and florets; (E), flower.

Fig. 8. Quantitative measurement of GUS enzyme activity in various tissues of at least five transgenic lines carrying the CuMFT1 promoter fused to the uidA gene. R, L, F, IS, MS, and S represent the following tissues: root, leaf, floret, immature silique including immature seed, mature silique without seed, and mature seed, respectively. The columns and bars represent the means and SD obtained from five individual lines.
expression and the seed-specific cis-elements found in the CuMFT1 promoter. Moreover, it was suggested that transcriptional regulation by the CuMFT1 promoter was generally conserved between Citrus and Arabidopsis.

In conclusion, it was demonstrated that the CuMFT1 promoter would be useful to regulate the quantitatively preferential expression in seeds of transgenic Citrus and Arabidopsis plants. This promoter is expected to induce the target gene in the late embryonic stage. In Citrus, there is great demand to generate seedless fruits (Yamashita, 1976) and to alter the composition of the seed. We consider that the CuMFT1 promoter would be useful in these studies as well as in the manipulation of embryogenic traits.

**Literature Cited**


