

# The morphological study and gene expression analysis in citrus hybrid with a short juvenile period

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**Abstract:** *Citrus plants have a long juvenile period, which has hampered the breeding progress of these species. In this sense, research focused on reducing the juvenile phase is needed to accelerate the breeding process. In this study, the morphology of the hybrid [(‘Rangpur’ lime) x YMCT (‘Yuma’ citrange) - 005] x (Microcitrus papuana) - 001} (H011), which has a short juvenile period, and the gene expression levels of the FLOWERING LOCUS T (FT) were evaluated. Morphological studies indicated early flowering and fruiting of the hybrid, characteristics that make it a promising candidate for citrus breeding. In addition, winter temperatures influenced the expression of the FT gene in stems and leaves.*

**Keywords:** Flower, *Citrus spp.*, floral induction, FT gene.

## INTRODUCTION


The life cycle of higher plants is characterized by two development stages, the vegetative and the reproductive. The former consists of the juvenile and adult vegetative stages. During the juvenile vegetative phase, the plants are unable to flower, even under inductive conditions. In certain perennial plants, this period can persist for several decades (Amasino 2010, Yamagishi et al. 2014). Many *Citrus* species with an extensive juvenile period will produce neither flowers nor fruits for many years, decades or even longer (Davies and Jackson 2009, Pajon et al. 2017). This long juvenile phase bars the use of genetic strategies to improve responses to environmental stress, fruit quality and disease resistance (Krajewski and Rabe 1995, Velázquez et al. 2016, Pajon et al. 2017).

The transition from the juvenile to the adult stage involves exogenous and endogenous factors, controlled by a pool of complex genes, and depends on the duration of the juvenile stage (Dornellas et al. 2007, Song et al. 2013, Ha 2014). Molecular studies suggested that the FLOWERING LOCUS T (FT) is an important part of the flowering hormone and the primary component of the flowering signal cascade (Lemmetynen et al. 2004, Corbesier et al. 2007, Kong et al. 2010, Imamura et al. 2011, Velázquez et al. 2016). The FT gene and homologous genes, reported in a wide variety of plants, play similar and important roles. Many studies have shown that an FT overexpression can promote early flowering

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in transgenic plants (Kojima et al. 2002, Endo et al. 2005, Bohlenius et al. 2006, Hsu et al. 2006, Lifschitz et al. 2006, Nakatsuka et al. 2009, Xu et al. 2012). Moreover, *FT* expression facilitates vernalization and flowering pathways and can induce flowering. (Huang et al. 2005, Corbesier et al. 2007, Xu et al. 2012).

Several studies have been performed to elucidate the molecular mechanisms involved in flower formation and differentiation in citrus. In transgenic plants of the trifoliolate orange (*Poncirus trifoliata* L. Raf.), the constitutive expression of the citrus *FT* gene (*CiFT*) induced flowering after 12 weeks, unlike the generally several-year-long juvenile phase in wild-type plants (Endo et al. 2005). The expression of flowering-related genes and the function of low temperatures for floral induction were studied in Satsuma mandarin (*Citrus unshiu* Marc.). Only in adult Satsuma mandarin trees, flowering is triggered by low temperatures, by activation of *CiFT* transcription, while in the juvenile plant, *CiFT* transcription is irresponsive to cold (Nishikawa et al. 2007, Nishikawa et al. 2009). The constitutive expression of the *FT* gene of *Arabidopsis thaliana* and *C. sinensis*, by means of a virus vector based on citrus leaf spot virus, was able to induce flowering in juvenile plants of a variety of citrus types (Velázquez et al. 2016). In a mature tree of pummelo (*C. grandis* Osbeck) and 'Pineapple' sweet orange (*C. sinensis* (L.) Osbeck), it was observed that the *CiFT* gene is probably involved with shoot apex differentiation and flower bud determination, but not associated with dormancy interruption and further flower bud development (Pajon et al. 2017).

Exploiting the wide genetic variability contained in the Active Germplasm Bank (BAG), the Citrus Breeding Program of Embrapa Cassava and Fruits (Cruz das Almas, Bahia, Brazil) selected hybrids with a short juvenile period. Among these citrus hybrids, {[('Rangpur' lime) x YMCT ('Yuma' citrange) - 005] x (*Microcitrus papuana*) - 001} (H011) is particularly outstanding, with a juvenile period of one year. Thus, this study addressed the morphological characterization of this hybrid [(LCR x CTYM - 005) x MCP - 011], named Microcitrangemonia 11, and investigated the effect of differentiated *FT* expression levels on the reduction of its juvenile phase.

## MATERIAL AND METHODS

### Plant material and morphological characterization

The experiment was carried out on an experimental field of Embrapa Cassava and Fruits (lat 12° 40' 19" S, long 39° 06' 22" W, and alt 220 m asl), in Cruz das Almas, Bahia, Brazil. Three approximately 4-year-old plants of the hybrid {[('Rangpur' lime) x YMCT ('Yuma' citrange) - 005] x (*Microcitrus papuana*) - 001} (H011), grafted onto (*Citrus sunki* x *Poncirus trifoliata*), were evaluated. In addition, the female parent {[('Rangpur' lime) x YMCT ('Yuma' citrange) - 005] and male parent (*Microcitrus papuana*) - 001 were grafted on the same rootstock as the hybrid.

Thirty mature leaves of each plant were collected and analyzed. Ten fruits and 10 flowers were also evaluated. Morphologic traits were chosen from among the Descriptors for Citrus of the International Board for Plant Genetic Resources (IBPGR 1999). Among the 28 selected descriptors, 17 were qualitative and 11 quantitative. Of the qualitative descriptors, seven were related to fruit appearance [fruit shape, fruit apex shape, pulp texture, seed shape, seed surface, and seed and pulp color], five to leaf aspects [leaf shape, leaf blade margin, petiole type, intensity of green color of the leaf blade] and five to flower traits [flower type, flower arrangement, flower, anther color, color of open flower]. In turn, of the quantitative descriptors, three were related to fruit traits [fruit diameter, number of seeds per fruit, fruit weight], five to flower traits [number of petals per flower, petal width, calyx diameter, pedicel length, anther length relative to stigma] and three leaf traits [leaf length, leaf width, leaf thickness].

### Confirmation of the nucellar clones

To confirm the nucellar origin, genomic DNA was extracted from the leaves of Microcitrangemonia 11, as proposed by Murray and Thompson (1980). Microsatellite markers were amplified from the following pairs of primers: miCrCIR01E02, IC06A05b, CMS14, OT4S, CMS26 [Supplementary Table 1] (Yong et al. 2006, Froelicher et al. 2008, Cristofani-Yaly et al. 2011). The amplification reactions were prepared for a final volume of 25 µL, containing 5.0 ng µL<sup>-1</sup> DNA, 1.0 U Taq DNA polymerase, 10X buffer (500 mM KCl, 100 mM Tris-HCl, pH 8 Invitrogen), 1.0 mM MgCl<sub>2</sub>, dNTP (0.2 mM), and 0.2 µM of each primer. The PCR protocol consisted of: after initial denaturation, 35 cycles of 30 s at 94 °C, 30 s at 52 °C and a final elongation step of 45 s at 72 °C. The annealing temperature was 65 °C, decreasing 0.3 °C per cycle, followed by three annealing cycles at 56 °C. The PCR products were visualized on 3% agarose gel stained with ethidium bromide (0.5 ng mL<sup>-1</sup>).

### RNA extraction and cDNA synthesis

After confirming the nucellar clones, they were divided into two groups: i) plants with early flowering; ii) plants without flowering. The samples were collected in two different seasons: winter (August/2015) and summer (February/2016). Total RNA was isolated with the kit RNAqueous® (Applied Biosystems, Ambion), according to the manufacturer's recommendations. Three independent biological replicates with 10 samples of leaves, buds and stems were collected, frozen in liquid nitrogen and stored at -80 °C.

The RNA samples were treated with TURBO DNA-free (Applied Biosystems, Ambion) prior to reverse transcription. cDNA synthesis was performed with 20 ng  $\mu\text{L}^{-1}$  RNA, using the High Capacity RNA-to-cDNA kit (Applied Biosystems), according to the manufacturer's instructions.

### Gene expression analysis

Gene expression levels were measured with reverse - transcription quantitative real-time PCR (RT-PCR), using ABI 7500 FAST Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed in triplicate, in 20  $\mu\text{L}$  reaction volumes; each reaction contained 100 ng cDNA, 0.6  $\mu\text{L}$  of each primer (10  $\mu\text{M}$ ), 6.0  $\mu\text{L}$  of the Power SYBR® Green Master Mix (Thermo Fisher Scientific) and 0.8  $\mu\text{L}$  sterile Milli-Q water. The reaction parameters were set as follows: 1) activation of Taq DNA Polymerase at 95 °C for 2 min; 2) denaturation at 94 °C for 45 s; 3) annealing at 60 °C for 45s; and 4) extension at 72 °C for 1 min. Steps 3-4 were repeated for 40 cycles.

The comparative Ct ( $\Delta\Delta\text{CT}$ ) method was used to quantify the gene expression, and the data were derived from three biological replicates. The Dissociation Curve software 1.0 (Applied Biosystems, EUA) detected that only one PCR product was generated by amplification (Additional file: 2). The glyceraldehyde-3-phosphate dehydrogenase C2 (*GAPC2*) [Supplementary Table 2] (Mafra et al. 2012) was used as endogenous. The *FLOWERING LOCUS T (FT)* primer [Supplementary Table 2] was designed based on the analysis of the sequences of *Citrus sinensis* (gi|568875304|ref|XM\_006490681.1|) and orange1.1g037140m) and *Citrus clementina* (Ciclev10009697m) available at the National Center for Biotechnology Information (NCBI) and Phytozome v.9.1. Samples of early flowering plants of this study were used in further studies of gene expression.

The statistical analysis of gene expression in the different seasons was based on mean comparison by the Tukey test at  $p < 0.05$  in all cases.

## RESULTS AND DISCUSSION

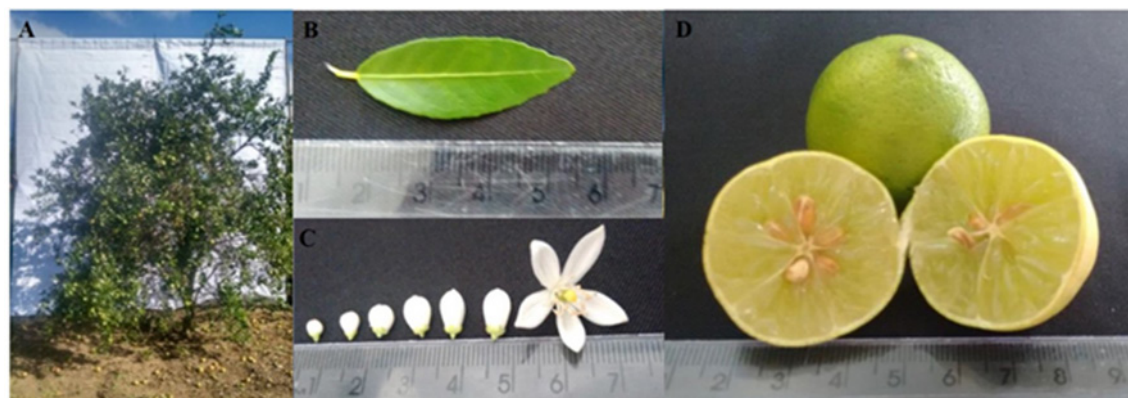
### Morphological characterization

The hybrid *Microcitrangemonia 11* is a medium-sized plant (250 cm, 4 years old), with a tree trunk diameter of approximately 200 cm (Figure 1A). The female parent (LCR X CTYM- 005) reaches a mean height of 280 cm, while the male parent *M. papuana* is short, approximately 140 cm (Santos et al. 2015). The tree canopy is very dense and has an ellipsoid shape, with the presence of thorns on the branches, similar to the male parent.

The leaves are dark green, with simple division and sessile leaf attachment (Figure 1B). the leaf shape was classified as lanceolate, with dentate leaf margin and acute leaf apex. Moreover, the leaves have a single and continuous blade, similar to the male parent. The mean leaf length, width and thickness are listed in Table 1.

The juvenile phase of hybrid *Microcitrangemonia 11* is short (approximately 1 year) and flowering is possible as of the second month of growth. Moreover, adult plants produce flowers several times in the same year, independently of environmental stimuli, and can even bear flowers and fruits all year long. These characteristics were not observed in the parents (Santos et al. 2015) and are not common in other citrus genotypes. In a sweet orange spontaneous early-flowering mutant, named 'x11', flowering occurs repeatedly in all seasons, without requiring environmental stimuli, except for pruning (Pinheiro et al. 2014). The flowers of *Microcitrangemonia 11* are hermaphrodite, have a superior ovary and on average five petals per flower (Figure 1C). The anthers are yellow and relatively short in relation to the stigma. The quantitative characteristics such as calyx diameter, pedicel length and petal width and length are shown in Table 1.

The mean fruit weight of the hybrid *Microcitrangemonia 11* was 15.39 g. The fruit (Figure 1D) shape is spheroid,



**Figure 1.** Hybrid {[(‘Rangpur’ lime) x YMCT (‘Yuma’ citrange) - 005] x (*Microcitrus papuana*) - 001} (H011) of the Citrus Germplasm Collection of Embrapa Cassava and Fruits in Cruz das Almas, BA. Plant with approximately four years old (A), fruits (B), flower buds and flower (C) and leaf blade (D).

the fruit base convex and the apex shape truncate, similar to that of the female parent. In contrast, the male parent had small elongated fruits and a crispy pulp texture (Santos et al. 2015). The mean fruit diameter and length of the studied hybrid and its parents are shown in Table 2. The mature fruit is yellowish green and has a smooth surface texture; fruits are produced in large amounts and have a strong fragrance and this trait makes them more attractive. These characteristics may be useful for the production of essential oils and with regard to the ornamental potential. The fruit pulp is light green and soft. In relation to seed, the hybrid produced an average of 10 viable seeds per fruit, with a medium size of 6.74 mm and spheroid shape. The seeds produced between one and five embryos and 50% polyembryony. Molecular analysis by microsatellite markers (SSRs) of 300 plants grown in a greenhouse showed that 132 plants were nucellar and 168 zygotic (data not shown).

### Gene expression of the FT gene

Gene expression analysis demonstrated a major influence of low temperatures on *FT* expression in leaves and stems of hybrid *Microcitrangemonia* 11 (Figure 2). In the leaves, *FT* gene expression was upregulated in the winter, while in the stem down-regulation was observed. The opposite was observed in the summer period, when there was a low regulation of the *FT* gene in leaves and increased expression in the stem. Among the environmental factors, the most important is temperature, followed by photoperiod and exposure to prolonged cold periods that induce vernalization (Amasino 2010, Srikanth and Schmid 2011, Huijter and Schmid 2011). Low temperatures induce *FT* gene expression, promoting floral induction in adult citrus trees (Valiente and Albrigo 2004, Nishikawa et al. 2007, Pajon et al. 2017). According to Nishikawa et al. (2007), a temperature of 15 °C is effective for floral induction in Satsuma mandarin, by means of an increase in the *FT* expression. In *Poncirus trifoliata*, floral development was observed in the winter when *FT* gene expression increased (Nishikawa et al. 2009).

Some studies indicate that in citrus, temperatures below 20 °C can induce flowering (Valiente and Albrigo 2004, Nishikawa et al. 2007, Nishikawa et al. 2009). Our study showed that the mean winter temperature influenced the

**Table 1.** Morphological leaf and flower traits of hybrid {[(‘Rangpur’ lime) x YMCT (‘Yuma’ citrange) - 005] x (*Microcitrus papuana*) - 001} (H011)

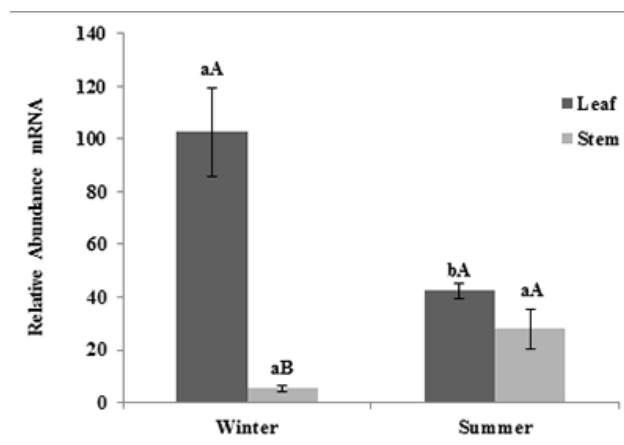
Morphological traits	Mean (mm)*
Leaf	
Length	31.5 ± 2.63
Width	12.7 ± 1.70
Thickness	0.31 ± 0.02
Flower	
Petal length	11.0 ± 0.73
Petal width	4.4 ± 0.49
Calyx diameter	5.0 ± 0.33
Pedicle length	1.3 ± 0.41

\* Mean values ± Standard Error.

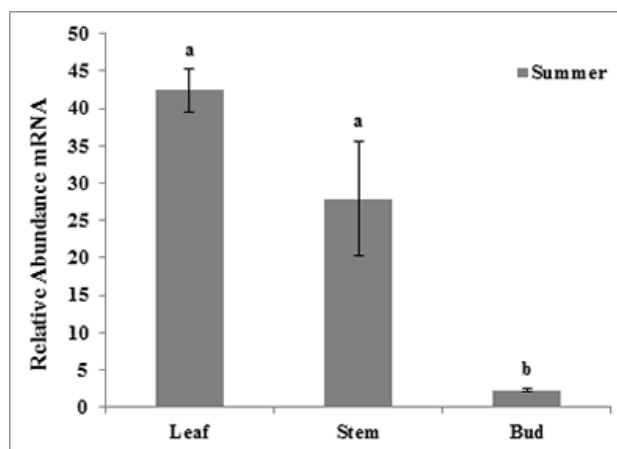
**Table 2.** Morphological fruit traits (in mm) of the hybrid {[(‘Rangpur’ lime) x YMCT (‘Yuma’ citrange) - 005] x (*Microcitrus papuana*) - 001} (H011) and its parents

Trait	Mean (mm)*		
	Hybrid	Female parent	Male parent
Fruit diameter	28.9 ± 2.71	38 ± 0.10	19 ± 0.08
Fruit length	32 ± 3.62	41 ± 0.25	54.5 ± 0.38

\* Mean value ± Standard Error.



**Figure 2.** Expression of the *FT* gene in leaves and stem of hybrid *Microcitrangemonia* 11 in winter and summer. Vertical bars indicate the mean  $\pm$  SE (n=3). Lowercase letters show differences between plant tissues in winter and summer; uppercase letters show differences between winter and summer ( $P < 0.05$ ).



**Figure 3.** Expression of *FT* gene in leaves, stem and bud of hybrid *Microcitrangemonia* 11 in the summer. Vertical bars indicate the mean  $\pm$  SE (n=3). Lowercase letters show differences between plant tissues in the summer.

*FT* expression, whereas the temperature pattern depends on the climatic conditions of the cultivation region (Davies and Albrigo 1994) and that this pattern varies according to the species (Ha and Johnston 2013). The stimulating effect of low temperatures on flowering is observed in both leaves and stem (Hall et al. 1977), however the upregulation in *FT* expression in citrus in winter can be explained by the capacity of the leaves to respond primarily to environmental stimuli such as temperature.

In the summer period, *FT* expression in the buds was also evaluated (Figure 3). The *FT* levels were significant but down-regulated, compared to leaves and stem. In transgenic citrus (Kobayashi 1999) and apple plants (Kotoda et al. 2010), *FT* expression in buds was confirmed, although higher *FT* levels were observed in leaves and stems, corroborating the results found in this study.

The flowering period of hybrid *Microcitrangemonia* 11 occurs a few months after the summer. The results found here suggest that the juvenile period was shortened and that the *FT* gene induced flowering in the studied plants. This confirms the findings of Endo et al. (2005), that the constitutive *FT* expression in *P. trifoliata* was able to significantly reduce the time of flowering induction in transgenic plants, highlighting the role of the *FT* gene as an important regulator of floral induction in citrus.

In grafting experiments with *Arabidopsis thaliana* mutants without the *FT* gene, the *FT* protein was transported from the rootstock to the scion where it promoted flowering (Xu et al. 2012). Moreover, the *FT* protein is transported from the leaves to the apical meristem, where floral differentiation occurs (Corbesier et al. 2007). In this sense, Nocker and Gardiner (2014) suggested that a stable transgenic line with constitutive *FT* expression could be used as rootstock to drive flowering of a transplanted graft. However, in the case of hybrid *Microcitrangemonia* 11, this is a natural characteristic, which is much more interesting for citrus breeding.

## CONCLUSION

The evaluation of the morphological data indicated differences, mainly related to flowering, among different *Microcitrangemonia* 11 plants. Some seedlings produced flowers as of the second month of cultivation, and on adult trees, flowers and fruits were observed simultaneously throughout the year.

The levels of *FT* expression found in the different tissues of genotype *Microcitrangemonia* 11 confirmed an early juvenile period. It was also observed that the *FT* gene induced flowering in these plants and that low temperatures influenced the *FT* expression levels.

This study provides insights into the effects of *FT* expression and low temperatures on hybrid *Microcitrangemonia* 11. This genotype is a promising candidate for citrus breeding, although further analyses are needed to elucidate the molecular basis of its short juvenile period.

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