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Unravelling the transition from vegetative to reproductive stages using *Passiflora organensis* as a model plant

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To my brothers and my sister in law for all support and help.
To my friends, who are special and become essential in my life!

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“Science is the key to our future, and if you don’t believe in science, then you’re holding everybody back. If we educate a generation of people who don’t believe in science, that’s a recipe for disaster. Stand up for science!”

ABSTRACT

MORAES, T.S. **Unravelling the transition from vegetative to reproductive stages using *Passiflora organensis* as a model plant.** 2020. 170 p. Thesis (Doctorate in Science) – Center for Nuclear Energy in Agriculture, University of São Paulo, Piracicaba, 2020.

The genus *Passiflora* is an excellent model for phase transition studies, because there are obvious morphological differences between plants in the juvenile, adult vegetative, and adult reproductive stages. In almost all species of the genus *Passiflora*, plants in the juvenile stage produce leaves with different morphology from the adult plants, and do not produce tendrils. On the other hand, plants in the adult vegetative stage develop tendrils at the leaf axils, and in the adult reproductive stage plants produce from the axillary meristems, tendrils and flowers, simultaneously. The proteins belonging to the FT/TFL1 family are important regulators of the phase transition process and need to interact with specific transcription factors to perform their biological functions. The *basic region/leucine zipper* (*bZIP*) and the *teosinte branched1/ cycloidea/ proliferating cell factor* (*TCP*) transcription factor (TF) families, which contain key players of plant development, are two families of genes encoding proteins that form unique complexes with FT/TFL1. The product of the *FLOWERING LOCUS T* (*FT)* gene is considered as the florigen agent and interacts with the bZIP protein FLOWERING LOCUS D (FD), resulting in the induction of flowering by activating transcription of genes involved in floral meristem identity, such as *LEAFY* (*LFY*) and *APETALA1* (*AP1*). In addition, literature reports reveal that in some species, the proteins encoded by the *FT* and *bZIP* transcription factors interact with 14-3-3, a highly conserved scaffold protein, resulting in the formation of a hexameric protein complex. This complex plays a critical role in flowering time control, being designated as the 'florigen activation complex' (FAC). Moreover, some proteins belonging to the TCP family may interact with FT protein, as well as with the product of its paralog *TWIN SISTER OF FT* (*TSF*), modulating their activity in the axillary buds to repress the premature floral transition of axillary meristems. In *Passiflora* species the molecular mechanisms involved in the vegetative-reproductive phase transition are basically unknown. Thus, this work aims to elucidate the mechanisms involved in the phase transition process during the development of *Passiflora organensis*, focusing on the transition to flowering. Then, with the use of appropriate developmental study tools, including light and electron microscopy, associated with gene expression analysis and protein-protein interaction techniques, the present work (a) morphologically characterized the transition from vegetative to reproductive phases in *Passiflora organensis*, (b) identified and characterized the gene structure of *LFY* and *AP1* genes as well as *FT/TFL1*, *bZIP*, *TCP* and *14-3-3* gene families in *Passiflora organensis*, (c) characterized the expression pattern of the *FT/TFL1* gene family, *LFY*, and *AP1* by qRT-PCR and *in situ* hybridization analysis, (d) validated the activity of proteins encoded by genes belonging to *FT/TFL1*, *bZIP*, *TCP* and *14-3-3* families by yeast two-hybrid assay, and (e) performed heterologous functional analyzes by overexpression of the *Passiflora organensis* genes *PoFT, PoTSFa, PoTFL1, PoBFT, PoATC,* and *PoMFT* in the model plant *Arabidopsis thaliana*. The results of this work are fundamental to conclude the characterization of the activity of these genes in *Passiflora organensis* and will be important for selecting the right genes to focus on future research and for applications in studies of yield increase in *Passiflora* species with commercial interest, such as passionfruit.

Keywords: Branching. bZIP. Florigen. Flowering time. FT/TFL1. Passionfruit. Plant architecture. TCP. 14-3-3.

RESUMO

MORAES, T.S. **Desvendando a transição de fase vegetativa-reprodutiva usando *Passiflora organensis* como planta modelo.** 2020. 170 p. Tese (Doutorado em Ciências) - Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba, 2020.

O gênero *Passiflora* é um excelente modelo para estudos de transição de fase, pois há diferenças morfológicas evidentes entre as plantas nas fases juvenil, adulta vegetativa e adulta reprodutiva. Na quase totalidade das espécies do gênero *Passiflora*, as plantas na fase juvenil produzem folhas com morfologia diferente da fase adulta e não produzem gavinhas. Já as plantas na fase adulta vegetativa, produzem gavinhas nas axilas das folhas e na fase adulta reprodutiva produzem, a partir dos meristemas axilares, gavinhas e flores. As proteínas pertencentes à familia FT/TFL1 são importantes reguladores do processo de transição de fase e precisam interagir com fatores de transcrição específicos para desempenhar suas funções biológicas. Nesse sentido, as proteínas da família bZIP (basic region/leucine zipper) e TCP (teosinte branched1/ cycloidea/proliferating cell factor) são fatores de transcrição que formam complexos únicos com as proteínas da família FT/TFL1. O produto do gene *FLOWERING LOCUS T* (*FT*) é considerado como o agente florígeno e interage com a proteína FLOWERING LOCUS D (FD), pertencente à familia bZIP, resultando na indução do florescimento pela ativação da transcrição de genes envolvidos na identidade do meristema floral, como *LEAFY* (*LFY*) e *APETALA1* (*AP1*). Além disso, a literatura revela que as proteínas FT e bZIP em algumas espécies interagem com proteínas altamente conservadas chamadas 14-3-3, resultando na formação de um complexo proteico hexamérico. Este complexo desempenha um papel crítico no controle do tempo de floração e é designado como 'complexo de ativação do florigeno'. Ademais, algumas proteínas pertencentes à família TCP podem interagir com a proteína FT, assim como com o produto do seu parálogo *TWIN SISTER OF FT* (*TSF*), modulando a atividade dos meristemas axilares reprimindo a floração. Em *Passiflora* os mecanismos moleculares que controlam o desenvolvimento vegetativo-reprodutivo são praticamente desconhecidos. Dessa forma, o objetivo deste trabalho foi elucidar os mecanismos envolvidos no processo de transição de fases durante o desenvolvimento de *Passiflora organensis*, com foco maior na transição para o florescimento. Assim, com o uso de ferramentas apropriadas ao estudo do desenvolvimento, que incluem microscopia óptica e eletrônica, associadas a técnicas de análise de expressão gênica e interação proteína-proteína, o presente trabalho (a) caracterizou morfologicamente a transição das fases vegetativa-reprodutiva em *Passiflora organensis*, (b) identificou e caracterizou a estrutura gênica dos ortólogos das famílias de genes *FT/TFL1, bZIP, TCP* e *14-3-3,* além dos genes *LFY* e *AP1* em *Passiflora organensis*, (c) caracterizou o padrão de expressão dos genes da família *FT/TFL1* e dos genes *LFY* e *AP1* por qRT-PCR e hibridização *in situ*, (d) validou a atividade das proteínas codificadas pelos genes pertencentes à família *FT/TFL1, bZIP, TCP and 14-3-3* por analise de duplo híbrido, e (e) realizou análises funcionais heterólogas por superexpressão dos genes *PoFT*, *PoTSFa, PoTFL1*, *PoBFT* , *PoATC* e *PoMFT* de *Passiflora organensis* na planta modelo *Arabidopsis thaliana*. Os resultados desta pesquisa são fundamentais para concluir a caracterização da atividade desses genes em *Passiflora organensis* e serão importantes para selecionar os genes certos para focar em pesquisas futuras e para aplicações em estudos de incremento de produção em espécies do gênero *Passiflora* com interesse comercial, como o maracujazeiro.

Palavras-chave: Arquitetura da planta. bZIP. Florescimento. Florígeno. FT/TFL1. Maracujazeiro. Ramificação. TCP. 14-3-3.

SUMMARY

Unravelling the transition from vegetative to reproductive stages using *Passiflora organensis* as a model plant

Passiflora organensis illustration with the vines forming a DNA double helix
(made by Malene Gagnat - my dear norwegian friend)

1. INTRODUCTION

The *Passiflora* genus is represented by more than 500 species, being distributed in the tropical and subtropical regions. Most of them is found in Central or South America, however, some species occur in North America, Southeast Asia and Australia (Ulmer et al., 2004). The *Passiflora* species are organized into 4 subgenera (Muschner et al., 2003; Hansen et al., 2006) and two of the four subgenera are more numerous in terms of species: the *Passiflora* subgenus is composed of 250 species, among which *Passiflora edulis*, the yellow passionfruit. The subgenus *Decaloba* comprises 230 species, including *Passiflora organensis,* a small herbaceous vine, used in this work, while the subgenera *Astrophaea* and *Deidamiodes* include 60 and 14 species, respectively (Ulmer et al., 2004).

Passionfruit is an attractive, nutritious fruit crop and one important parameter for yield is the switch from vegetative to reproductive growth. This change requires the transition of the juvenile vegetative meristem to the adult vegetative meristem, followed by the differentiation of the inflorescence and floral meristems. Based on studies with model plants, such as *Arabidopsis thaliana*, it is known that the control of these transitions is regulated by environmental and genetic factors (Poethig, 2013).

In angiosperms, orthologous members of the *FLOWERING LOCUS T/ TERMINAL FLOWER* 1 (*FT/TFL1)* gene family have been shown to function both as promoters and suppressors of flowering. These proteins fulfil different functions and act as key regulators in the vegetative to reproductive phase transition, and in the control of various biological processes like flowering time, plant architecture, and seed germination (Jaeger et al., 2013; Park et al., 2014; Tao et al., 2014; Guo et al., 2015; Dave et al., 2016; Higuchi, 2018; Wu et al., 2019).

The FT/TFL1 proteins need to interact with specific transcription factors (TFs) in order to perform their biological functions in developmental phase transitions. The *basic region/ leucine zipper* (*bZIP*) and the *teosinte branched1/ cycloidea/ proliferating cell factor* (*TCP*) are two families of genes that encode TFs that form unique complexes with FT/TFL1 proteins (Jakoby et al., 2002; Li, 2015). Molecular studies in *Arabidopsis* have shown that *FLOWERING LOCUS D* (*FD*) and *FD PARALOGUE* (*FDP*) are key flowering genes encoding bZIP transcription factors that interact with FT resulting in the induction of flowering by activating genes involved in floral meristem identity, such as *LEAFY* (*LFY*) and *APETALA1* (*AP1*) (Jang et al., 2017). However, the literature reveals that the proteins

encoded by FT and bZIP transcription factors in some species interact with 14-3-3 proteins. In rice, for instance, it has been shown that the FT–FD interaction is mediated by 14-3-3 proteins forming the florigen activation complex (FAC) that promotes flowering in short-day conditions (Taoka et al., 2011). Moreover, studies have shown that TCP18 interacts with the florigen proteins FT and TWIN SISTER OF FT (TSF), modulating their activity in the axillary buds to repress the premature floral transition of axillary meristems (Niwa et al., 2013).

In this context, this thesis addresses the morphological and molecular mechanisms involved in plant phase transition. We subdivided the thesis into six chapters covering studies of morphology, gene expression, and protein-protein interactions during the vegetative to reproductive phase transition in *Passiflora organensis*. Additionally, we performed heterologous functional analyzes by overexpression of *PoFT*, *PoTSFa*, *PoTFL1*, *PoBFT*, *PoATC* and *PoMFT* genes of *P. organensis* in the plant model *A. thaliana*.

Chapter I, entitled "Morphological aspects of vegetative to reproductive phase transition in *Passiflora organensis*", explores the morphological characteristics of the phase transition and present this species as an excellent model for phase transition studies. A detailed understanding of the sequence of events related to the phase transition is essential to obtain solid results in the molecular analysis and to compare the different activities of the axillary meristems during plant development.

Chapter II, entitled "Characterization of *Passiflora organensis FT/TFL1* gene family and its putative role during phase transition and branching", aimed to identify the *FT/TFL1* orthologous genes in the *P. organensis* genome, to study their structure and their pattern of expression during the vegetative to reproductive development.

Chapter III, entitled "FT/TFL1: Calibrating Plant Architecture", presents a summarized review of plant architecture and primarily focus on the FT/TFL1 balance and its effect on plant form and development. We also propose passionfruit as a suitable model plant to study the effect of *FT/TFL1* genes on plant architecture. This article was published in Frontiers in Plant Science (Moraes et al., 2019).

Chapter IV, entitled "Identification of floral identity genes *LEAF* and *APETALA1* in *Passiflora organensis*", aimed to identify *LFY* and *AP1* orthologous genes in the *P. organensis* genome, to study their structure and their pattern of expression during the vegetative to reproductive development.

Chapter V, entitled “Protein-protein interactions involved in flowering control and branching in *Passiflora organensis*”, aimed to validate the activity of the proteins encoded by the genes belonging to the *Passiflora FT/TFL1* family by analyzing protein-protein interactions among the proteins encoded by the *bZIP*, *TCP*, and *14-3-3* gene families.

Finally, **Chapter VI,** entitled “Heterologous functional analyzes by overexpression of *Passiflora FT/TFL1* genes in the plant model system *Arabidopsis thaliana*”, aimed to explore the putative function of those genes in flowering regulation.

2. OBJECTIVES

Considering the very limited knowledge on the molecular mechanisms involved in phase transition studies in *Passiflora* species, the aim of this work was to elucidate the mechanisms involved in the transition from the vegetative to reproductive stages in *Passiflora organensis*. This species was chosen as a model *Passiflora* species because a draft of its genome sequence was produced by our group, thus making the molecular analyses more amenable. With this aim, the following specific objectives were defined:

1. To characterize the morphological modifications involved in phase transition in *Passiflora organensis;*
2. To identify ortholog genes in *Passiflora organensis* genome that may regulate the transition phase and branching formation during plant development;
3. To characterize the expression pattern of genes involved in phase transition and branching in *Passiflora organensis;*
4. To validate the activity of the selected genes by analyzing their protein-protein interaction;
5. To explore the putative function of *Passiflora FT/TFL1* genes in flowering regulation through the heterologous functional analyzes by overexpression of those genes in a plant model system *Arabidopsis thaliana.*

3. LITERATURE SUPPORT

3.1 Plant Phase Transitions

During their life cycle, higher plants develop through distinct growth phases: a juvenile phase, an adult vegetative phase, and an adult reproductive phase. The transitions between these phases are controlled by genetic factors that integrate endogenous and environmental cues **(**Huijser and Schmid, 2011; Cho et al., 2017).

The first transition from the juvenile to the adult vegetative stage is named vegetative phase change (VPC) and comprehends changes in a variety of characteristics, including changes in leaf size and shape, trichome distribution, plastochron and internode length, shoot physiology, and reproductive competence **(**Poethig, 2013; Fouracrea and Poethig, 2019). Plants in the juvenile stage are characterized by a ¨reproductive incompetent state¨, which means that the juvenile vegetative meristem is not competent to switch the plant to the reproductive stage. The VPC is regulated by the sequential activity of miR156 and miR172 and their respective targets, SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) and APETALA2 (AP2)-like transcription factors. In short, miR156 is expressed during the juvenile phase to control shoot development, while miR172 is expressed during the adult phase. Moreover, a condition that marks the transition from juvenile to adult phase occurs when the levels of miR156 start decreasing, and as a result the transcript level of its targeted *SPL* genes starts increasing. Then, SPL upregulate the transcription of miR172, resulting in *AP2-like* genes repression (Wu and Poethig, 2006; Wu et al., 2009; Wang et al., 2009; Zheng et al., 2019).

The reproductive phase change occurs when plants switch from vegetative to reproductive growth. This change requires the transition of the vegetative shoot apical meristem (SAM) to the inflorescence meristem (IM), then the transition of IM into a floral meristem (FM). In *Arabidopsis thaliana*, the molecular mechanisms involved in the phase transition from adult vegetative to adult reproductive stage are controlled by interactions of the products of genes, such as: *FT*, *TFL1*, *LFY*, *FRUITFULL* (*FUL*), *AP1*, and its paralog *CAULIFLOWER* (*CAL*). The product of *FT* is considered as the florigen agent that activates the entire flowering pathway, whereas *LFY*, *AP1*, *CAL*, and *FUL* promote the identity of the floral meristem, and *TFL1* is required to maintain the identity of the inflorescence meristem, regulating the expression pattern of *LFY* and *AP1* (Abe et al., 2005; Corbesier et al., 2007).

FT and *TFL1* encode proteins with similarity to phosphatidylethanolamine binding proteins (PEBP) which function as flowering promoters and repressors. Studies have shown that in the early stage of plant development, the ratio FT/TFL1 is low and will increase according to the development of the plant. The *FT/TFL1* gene family plays a key role in phase transition and organogenesis **(**Kobayashi et al., 1999; Wickland and Hanzawa, 2015; Moraes et al., 2019).

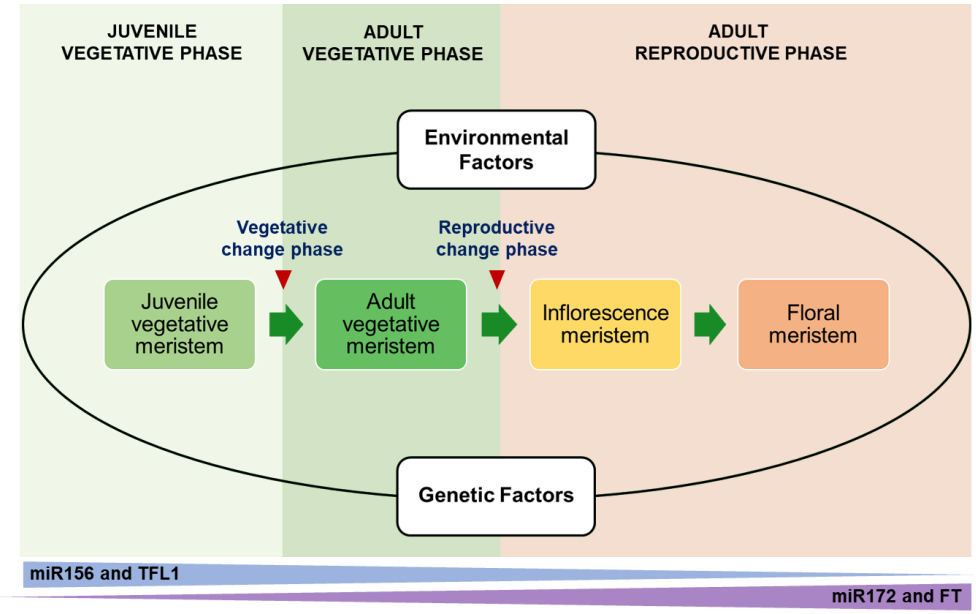

Figure 1 – **Plant phase changes.** Plants grow and go through distinct phases of development: juvenile vegetative, adult vegetative, and adult reproductive. During these processes the shoot apical meristem undergoes morphological modifications that are controlled by environmental and genetic factors. Early in development, the levels of miR156 and TFL1 are initially high, but these levels decrease, while the levels of miR172 and FT increase with time

3.2 The *FT/TFL1* gene Family

The *FT/TFL1* gene family members encode a small protein (~20 KDa) similar to phosphatidylethanolamine binding proteins (PEBP). PEBP play important roles in different processes during the life cycle and are conserved in most organisms, including bacteria, yeasts, plants and mammals (Banfield et al., 1998; Hengst et al., 2001; Chautaard et al., 2004; Wu et al., 2019).

In plants, members the *FT/TFL1* gene family are found in both gymnosperms and angiosperms. They are involved in several biological processes such as: flowering control, plant architecture, and seed germination (Jaeger et al., 2013; Park et al., 2014; Tao et al., 2014; Guo et al., 2015; Dave et al., 2016; Yu et al., 2019). In angiosperms, *FT/TFL1* genes are grouped in three major clades: *FT*-like, *TFL1*-like, and *MFT*-like subfamilies.

All these genes are regulated by a network of environmental and endogenous signals such as: the photoperiodic, vernalization, temperature, plant hormone, autonomous, and aging pathways (Kardailsky et al., 1999; Samach et al., 2000; Blázquez et al., 2003; Golembeski and Imaizumi, 2015).

Proteins encoded by these genes have been identified and studied in several plant species such as: grapes (Carmona et al., 2007), tomato (Carmel-Goren et al., 2003), jatropha (Li et al., 2015), rice (Tamaki et al., 2007), cotton (Zhang et al., 2016), corn (Danilevskaya et al., 2008), barley (Faure et al., 2007), tulip (Leeggangers et al., 2018), cucumber (Wen et al., 2019), among other species. In *A. thaliana,* six genes of this family have been identified: *FT* and *TSF*, involved in flowering promotion and belonging to the *FT*-like subfamily; *TFL1*, *BROTHER OF FT AND TFL1* (*BFT*) and *ARABIDOPSIS THALIANA CENTRORADIALIS HOMOLOG* (*ATC*), involved in flowering repression and belonging to the subfamily *TFL1*-like; and *MOTHER OF FT AND TFL1* (*MFT*), belonging to *MFT*-like subfamily and involved in the regulation of seed germination (Kobayashi et al., 1999; Xi et al., 2010; Wickland; Hanzawa, 2015).

FT and *TFL1* genes have opposing effects on flowering time; while FT activates the flowering pathway, being considered the florigen agent, TFL1 represses flowering, being responsible for the maintenance of the inflorescence meristem. Despite the opposite regulatory pathways and antagonistic functions, the FT and TFL1 proteins present high sequence similarity (Hanzawa et al., 2005; Ahn et al., 2006). Studies have shown the importance of the tyrosin residue at position 85 (Tyr-85) for FT to function as flowering inducer, and a histidine at position 88 (His-88) for TFL1 to function as a flowering repressor. A single base change of these amino acids may cause conversion of functions between FT and TFL1 (Hanzawa et al., 2005; Ahn et al., 2006; Ho and Weigel, 2014; Wang et al., 2017).

TSF has a similar function as FT. The expression of both genes is up-regulated in phloem cells and their encoded proteins move from leaves to the shoot apical meristem (SAM). However, TSF is less mobile than FT suggesting that it could have a major role in leaves. The *ft* mutant flowers late and presents indetermined growth, while *tsf* mutant delays flowering only in short-day conditions. The double mutant *tsf/ft* shows additive effects in both short- and long-day conditions. The overexpression of *FT* causes early flowering and conversion of the SAM into a terminal flower (Yamaguchi et al., 2005; Cobesier et al., 2007).

TFL1 expression occurs in the SAM, maintains the indeterminate growth and represses the floral meristem identity genes (Shannon and Meeks-Wagner, 1991; Hanano and Goto, 2011). *ATC* also represses the floral transition, but its expression occurs preferentially in short-day conditions (Huang et al., 2012). On the other hand, *BFT* has *TFL1*-like function, however, shows an expression pattern similar to *FT* in the leaves (Yoo et al., 2010; Ryu et al., 2014). In agreement with the observations above *tfl1* mutants flower early and their SAM is converted into a terminal flower (Conti and Bradley, 2007).

The *mft* mutant has no effect on flowering time, but *MFT* overexpression causes a slight reduction of flowering time suggesting that MFT may have a redundant role in flowering promotion. In addition, MFT has a role in seed germination and its expression is promoted by ABA signaling during this process (Yoo eta al., 2004; Yu et al., 2019).

3.3 bZIP transcription factor family

In plants, the *basic region/leucine zipper* (*bZIP*) family is among the largest families of transcription factors and it influences many aspects of plant growth and development. This gene family is characterized by a conserved domain, named as bZIP domain, which is composed of two motifs: a basic region responsible for specific binding of the transcription factor to its target DNA, and a leucine zipper required for dimerization. Plant bZIP proteins preferentially bind to DNA consensus motives which have a central core composed by ACGT sequence (Jakoby et al., 2002).

The *bZIP* gene family has been identified in several species with different numbers of members: 52 bZIP genes have been found in humans, 78 in *Arabidopsis,* 92 in rice, 125 in maize, and 247 in rapeseed **(**Corrêa et al., 2008; Rodriguez-Martinez et al., 2017; Zhou et al., 2017). In plants, the bZIP family is clustered in 10 groups (A-I and S) according to sequence similarities and functional features. They are master regulators of many biological processes including pathogen defense, morphogenesis, light and stress signaling, seed maturation, and flower development (Jakoby et al., 2002).

Molecular studies in *Arabidopsis* show that *FD* and *FDP* are key flowering genes encoding bZIP transcription factors that interact with FT, resulting in the induction of flowering by activating genes involved in floral meristem identity (Jang et al., 2017). *FD* and *FDP* genes are expressed in the SAM, both *fd* and *fdp* mutants flower late, however, *fdp* mutants show a slight flowering delay comparing to *fd* mutants. Moreover, the double mutant

fd/fdp shows additive effects that delay flowering, indicating that FD is truly an activator of the floral transition in *Arabidopsis* (Abe et al., 2005; Wigge et al., 2005).

In the C-terminal region of FD proteins there is a conserved SAP domain, which is necessary for the interaction between FD and FT (Abe et al., 2005; Collani et al., 2019). Studies have shown that FD directly interacts with other members of the FT/TFL1 family, such as TFL1, BFT, and ATC via its SAP domain, resulting in the repression of floral transition (Hanano and Goto, 2011; Huang et al., 2012; Ryu et al., 2014).

Like in *Arabidopsis,* it has been shown that rice FD plays an important role in flowering regulation. However, it was demonstrated that the FT–FD interaction is mediated by 14-3-3 proteins forming the florigen activation complex (FAC) that promotes flowering in short-day conditions. The FAC has a heteroexameric structure which is composed by two molecules of the rice florigen Hd3a (an ortholog of FT), two molecules of the FD homolog OsFD1, and two molecules of Gf14c, a 14-3-3 protein which acts as a molecular bridge between OsFD1 and Hd3a (Taoka et al., 2011).

3.4 14-3-3 family

The 14-3-3 proteins are highly conserved scaffold proteins of eukaryotic cells including animals, plants and yeast (Jaspert et al., 2011). These proteins are involved in a variety of plant regulatory pathways through protein–protein interactions. This association commonly requires phosphorylation of a serine or threonine residue within a specific sequence motif (De Boer et al., 2013; Wilson et al., 2016; Ormancey et al., 2017).

In plants, 14–3-3 proteins were identified in many species and have shown multiple paralogs that provide sequence and functional diversity (Taoka et al., 2011; Li et al., 2015; Cheng et al., 2018). In *A. thaliana* thirteen 14-3-3 proteins were identified, which are named as general regulatory factors (GRFs). These proteins were classified into two groups, namely, epsilon and non-epsilon groups. The 14-3-3 epsilon group is composed of five members: *mu*, *epsilon*, *pi*, *iota*, *omicron*, while eight members comprise the non-epsilon group: *kappa*, *lambda*, *psi*, *nu*, *upsilon*, *omega*, *phi*, and *chi*. Epsilon group members commonly contain more introns and motifs than non-epsilon group members (Wu et al., 1997; Diaz et al., 2011; Wilson et al., 2016). Previous studies revealed that 14–3-3 proteins belonging to the non-epsilon group play important roles in plant growth and development. In rice, for instance, it

has been shown that the FT–FD interaction is mediated by non-epsilon 14-3-3 proteins forming FAC that promotes flowering in short-day conditions (Taoka et al., 2011).

The rice FT ortholog Hd3a is produced in leaves and is transported through the phloem to the SAM. Once it enters the SAM cell, it initially binds to 14-3-3 proteins (Gf14c) in the cytoplasm. When the florigen-receptor complex enters the nucleus, it forms a complex with the OsFD1 transcription factor, which is retained in the nucleus and activates OsMADS15 transcription, leading to floral induction (Taoka et al., 2011).

3.5 TCP transcription factors

The TCP consists a plant-specific family of transcription factors, which name is derived from the first three characterized members of this family: TEOSINTE BRANCHED1 (TB1) in maize, CYCLOIDEA (CYC) in snapdragon, PROLIFERATING CELL NUCLEAR ANTIGEN FACTOR1 (PCF1) and PCF2 in rice. They are identified either by their functions in plant development or their DNA binding capacities (Li, 2015).

The TCP transcription factors have been demonstrated to be involved with circadian rhythms, host-pathogen interactions, plant hormone signaling and therefore controlling a large variety of developmental processes such as cell proliferation and growth, mainly in meristems and lateral organs (Martin-Trillo and Cubas, 2010; Li, 2015; Danisman, 2016).

Based on differences within their functional domains, two types of TCP proteins can be recognized: class I that contains PCF1 and PCF2 and class II that includes CIN and CYC/TB1 clades (Aggarwal et al., 2010). In *Arabidopsis* the subgroup CYC/TB1 consists of only 3 members, including TCP1, TCP12 (BRANCHED2), and TCP18 (BRANCHED1). The *BRANCHED* genes, *BRC1* and *BRC2*, are involved in regulating shoot branching that is an important mechanism for space occupancy and, therefore, plant survival. Studies have shown that BRC1 interacts with FT and TSF proteins, modulating their activity in the axillary meristems to repress the premature floral transition of axillary meristems (Niwa et al., 2013). This interaction is specific to FT/TSF and not to TFL1, enabling to functionally distinguish novel uncharacterized members of the FT/TFL1 family. Overexpression of *BRC1* in the SAM delays flowering, while in *brc1* mutants the level of expression of the meristem identity genes *AP1* and *FUL* increases, suggesting that BRC1 represses the induction of these genes by FT.

The CIN clade genes are key regulators of the timing of the transition from division to expansion in dicot leaves. In *Arabidopsis*, eight members are further distinguished into two smaller groups based on their post-transcriptional regulation. Either they possess

binding-site for the microRNA319 (miR319 or miRJAW) and hence are also referred to as JAW-TCPs or are called TCP5-like CIN-TCPs hence they lack miR319 binding site (Palatnik et al., 2003; Nicolas and Cubas, 2016).

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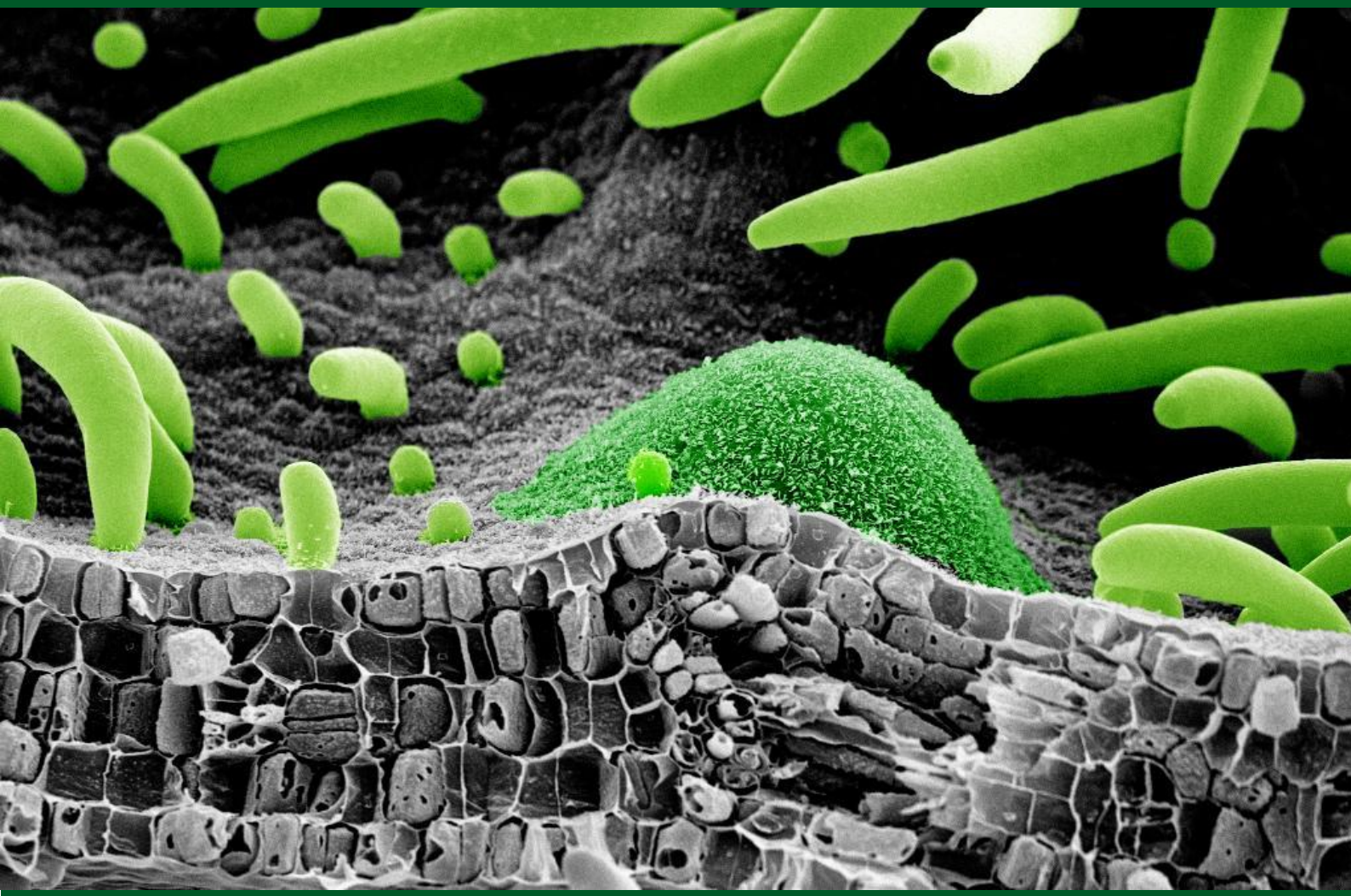
CHAPTER I

Morphological aspects of vegetative-reproductive phase transition in *Passiflora organensis*



Passiflora organensis - extrafloral nectaries in the leaf surface

4. Morphological aspects of vegetative-reproductive phase transition in *Passiflora organensis*

Abstract

Morphological and anatomical traits, which change in a coordinated fashion at predictable times during plant development, allow this process to be divided into phases. The transition from vegetative to reproductive phase has significant effects in plant yield, because the development of flowers, and consequently of fruits and seeds, depends on this process. Thus, the morphology and function of the organs that differentiate from the shoot apical meristem (SAM) are important in plant phase transitions. Considering that within the genus *Passiflora* the mechanism underlying the vegetative to reproductive transition remains largely unknown, the aim of this work was to characterize the morphological modifications occurring during phase transitions in *Passiflora organensis*. During the juvenile stage, *P. organensis* has variegated leaves and this is replaced by the presence of extrafloral nectaries on the abaxial side of adult leaves. The adult stage is also characterized by the presence of tendrils on the axil of leaves. At the reproductive stage, floral meristems are originated from the same axillary meristem producing tendril primordia. We present *P. organensis* as an excellent model for *Passiflora* development studies because this small herbaceous vine presents many important features that highlight it as a promising model plant.

Keywords: Axillary meristem. Extrafloral nectaries. Passionfruit. Shoot apical meristem. Tendril. Variegated leaves.

4.1 Introduction

The *Passiflora* genus is the largest within the Passifloraceae family, and it is composed by shrubs and herbaceous species, mostly climbers with axillary tendrils. *Passiflora* species are originated in ecosystems with dense vegetation and competition for light, thus the tendrils are an adaptative advantage that allowed plants to climb on hosts in order to reach areas with higher light intensity (Ulmer; MacDougal, 2004; Muschner et al., 2012).

Besides the tendrils, *Passiflora* species show a wide diversity of leaf and floral shapes, as well as flower sizes and colors. In addition, these species show floral organ innovations such as the corona, which is an additional floral whorl containing filaments and/or membranes between the perianth and the androgynophore. The androgynophore, is also a characteristic structure of this genus, that is responsible to elevate the gynoecium and the androecium above the perianth insertion. The function of both corona and androgynophore is related to the attraction of pollinators, allowing these animals to touch the stigma and anthers while foraging for nectar. Thus, an explanation for this floral diversity may be related to its coevolution with pollinators (MacDougal, 1994; Ulmer; MacDougal, 2004; Aizza; Dornelas, 2011).

The *Passiflora* genus is subdivided into four subgenera: *Passiflora, Decaloba, Astrophea*, and *Deidamioides*. *Decaloba* is the second largest subgenus (behind *Passiflora*), including approximately 230 species of vines with small-sized, bilobed variegated leaves and small flowers (Feuillet; MacDougal, 2007; Milward-De-Azevedo et al., 2010; Krosnick et al., 2013). *Passiflora organensis* is a small herbaceous vine belonging to the *Decaloba* subgenus, found in the Atlantic Forest of Brazil, around the borders of São Paulo, Minas Gerais, and Rio de Janeiro states. The genus comprehends long-day plants that need around 12 hours of light to bloom. Their vegetative period occurs from May to October, and the reproductive period from November to April. *P. organensis* has one of the smallest genome sizes (212 Mbp) and flower diameters (2,68 cm) among the *Passiflora* species. Most *Decaloba* species are considered diploid, with 2n = 12 (Yotoko et al., 2011).

Beyond the obvious morphological differences among the juvenile, adult vegetative, and adult reproductive stages, *P. organensis* has some characteristics that make it amenable as a model system. Typically, the choice of one species as a model involves practical reasons such as economic value or intrinsic properties that suit the species chosen for laboratory use. Accordingly, *P. organensis* has some advantages over the most commercially important

Passiflora species, such as *P. edulis*: i) smaller genome size and availability of a reference draft genome, produced by our group; ii) small plant size, making it easy to grow and to maintain plants in greenhouse conditions, iii) a life cycle that is representative of other *Passiflora* species, including the commercially important *P. edulis*.

The mechanisms underlying phase transition remain largely unknown in *Passiflora*, thus, the initial study of the morphological aspects involved in this process becomes important. In this chapter we will address the structural aspects involved in phase transitions in *P. organensis* with the use of appropriate developmental study tools, including optical and electron microscopy, energy-dispersive x-ray spectroscopy, and microcomputed tomography. Here, the aim was to explore the morphological modifications during phase transitions in *P. organensis* and present this species as an excellent model for phase transition studies. In addition, because the developmental phases are based on the morphological and functional characteristics of the organs that differentiate from the shoot apical meristem (SAM), it is essential to follow the dynamics involving the different components of the leaf axillary bud during phase transition.

4.2. Materials and Methods

4.2.1 Plant material

Plants of *P. organensis* were grown *in vitro* (Figure 1A) and at greenhouse conditions (Figure 1B) at the Center for Nuclear Energy in Agriculture, University of São Paulo - CENA / USP. The initial plant material was obtained from the *Passiflora* germplasm collection of the Institute of Biology, University of Campinas - UNICAMP.

4.2.2 Culture and growth conditions

Because the shoot apices taken from *Passiflora* plants in the adult vegetative or reproductive phase, show reversion to juvenile stage when cultivated under *in vitro* conditions (Dornelas; Vieira, 1994; Dornelas et al., 2006), we made a stock of *in vitro P. organensis* juvenile plants and this was the material used whenever we refer to juvenile plants.

The medium used to cultivate the plants *in vitro* was based on ½ MS salts and ½ MS vitamins (Murashige; Skoog, 1962), supplemented with 30 g l^{-1} sucrose, 2 g 1^{-l} phytagel (Sigma). *P. organensis* shoots were placed in 2.5 × 12.5 cm tubes containing 15 ml of

autoclaved medium. The pH was adjusted to 5.8 prior to adding phytagel. Cultures were maintained under a 16 h photoperiod under 23 µmol m^{-2} s^{-1} light radiation. The temperature of the culture room was maintained at 26 ± 2°C.

4.2.3 Microscopical analyses

Samples were fixed in a modified Karnovsky (1965) solution (2 % glutaraldehyde; 2 % paraformaldehyde, 0.001 M $CaCl_2$ in 0.05 M cacodylate buffer, at pH 7.2) initially under vacuum, then under refrigeration for 48 hours, dehydrated in an ethyl alcohol series (30 % - 70 % v/v) for 6 hours, and maintained under refrigeration until further use.

For light microscopy, samples in 70 % ethanol were further dehydrated through 100 % in an ethanol series, followed by propanol (100 %) and butanol (100 %), then infiltrated using butanol:infiltration medium (3:1, 1:1, 1:3), and embedded in Historesin (Leica, Heidelberg, Germany). The resin was polymerized at room temperature for 48 hours. Serial histological sections (4-5 µm) were obtained in a rotary microtome (Leica RM 2155, Nussloch, Germany). The sections were placed in water on histological slides and dried. Sections were stained with acid fuchsin (1%), followed by toluidine blue (0.05%) (Feder; O'Brien, 1968), covered with synthetic resin (Entellan®, Merck) and coverslipped. The sections were then analyzed and digital images obtained with an Axioskop 2 photomicroscope (Carl Zeiss, Jena, Germany).

For scanning electron microscopy (SEM), energy dispersive X-ray analysis (EDX), and micro-computed tomography (Micro-CT), samples maintained under refrigeration in ethanol (70%) were dehydrated through 100 % ethanol, critical point-dried through liquid CO_2 (Leica EM CPD 300, Balzers, Germany) and mounted on metal stubs with double sticky tape (SEM), or carbon tape (EDX). For SEM samples were sputter-coated with gold, at 80 nm thickness (Leica EM ACE600, Balzers, Germany), analyzed under a LEO 435 scanning electron microscope (Carl Zeiss, Jena, Germany), using a secondary electrons detector, and digital images recorded. For EDX, samples were sputter-coated with carbon and images obtained under the SEM using an energy dispersive X-ray detector (EDX, Oxford Instruments, Abingdon, Oxford shire, UK) coupled to a JEOL JSM-IT300LV (Tokyo, Japan) scanning electron microscope. For Micro-CT, critical point dried samples were scanned (SkyScan 1272, Bruker, at the LNNano Lab, CNPEM, Campinas, SP, Brazil) and 2D projection images were captured, while the segment was rotated over 360°. The 2D projection

images were reconstructed into a 3D data set using the VolViewer software (http://cmpdartsvr3.cmp.uea.ac.uk/wiki/ BanghamLab/index.php/VolViewer).

For ultrastructural analyses, samples fixed with the same modified Karnovsky solution, initially under vacuum, followed by 48 h under refrigeration, were then rinsed in cacodylate buffer (0.1 M) and post fixed in osmium tetroxide (1 % in 0.1 M sodium cacodylate buffer, pH 7.2), for 1 h, at room temperature. Samples were then dehydrated in a graded acetone series (30 – 100 %) and embedded in Spurr (1969) low viscosity resin (EMS, Electron Microscopy Sciences, Hatfield, PA, USA), for 48 h. Ultrathin sections (60 – 90 nm) were obtained using a diamond knife in an ultramicrotome (Porter Blum MT2, Dupont-Sorvall), collected on copper grids (300 mesh) and poststained with aqueous uranyl acetate (2.5%), followed by lead citrate (0.1%) (Reynolds, 1963). Sections were examined at 80 kV under a transmission electron microscope (JEM1400 JEOL, Tokyo, Japan), and the images digitalized.

4.3. Results

4.3.1 *Passiflora* plants change their morphology upon phase transition

In *P. organensis* juvenile plants, the leaf adaxial surface is green, presenting light green or silvery green irregular patches (Figure 1C), however, as plants grow and reach the adult stage, these patches are not present; adult leaves are green and develop extrafloral nectaries (EFN) (Figure 1D). The EFN attracted ants that are frequently observed on the adult leaves (Figure 1H). Moreover, plants in the juvenile vegetative stage grow slowly producing new leaves and branches (Figures 1E), while plants in the vegetative adult stage grow continuously, and besides new branches, also produce tendrils from the leaf axils (Figure 1F). At the adult reproductive stage, in the leaf axils, tendrils and flower buds are observed simultaneously (Figure 1G), two flower buds and one tendril per axil.

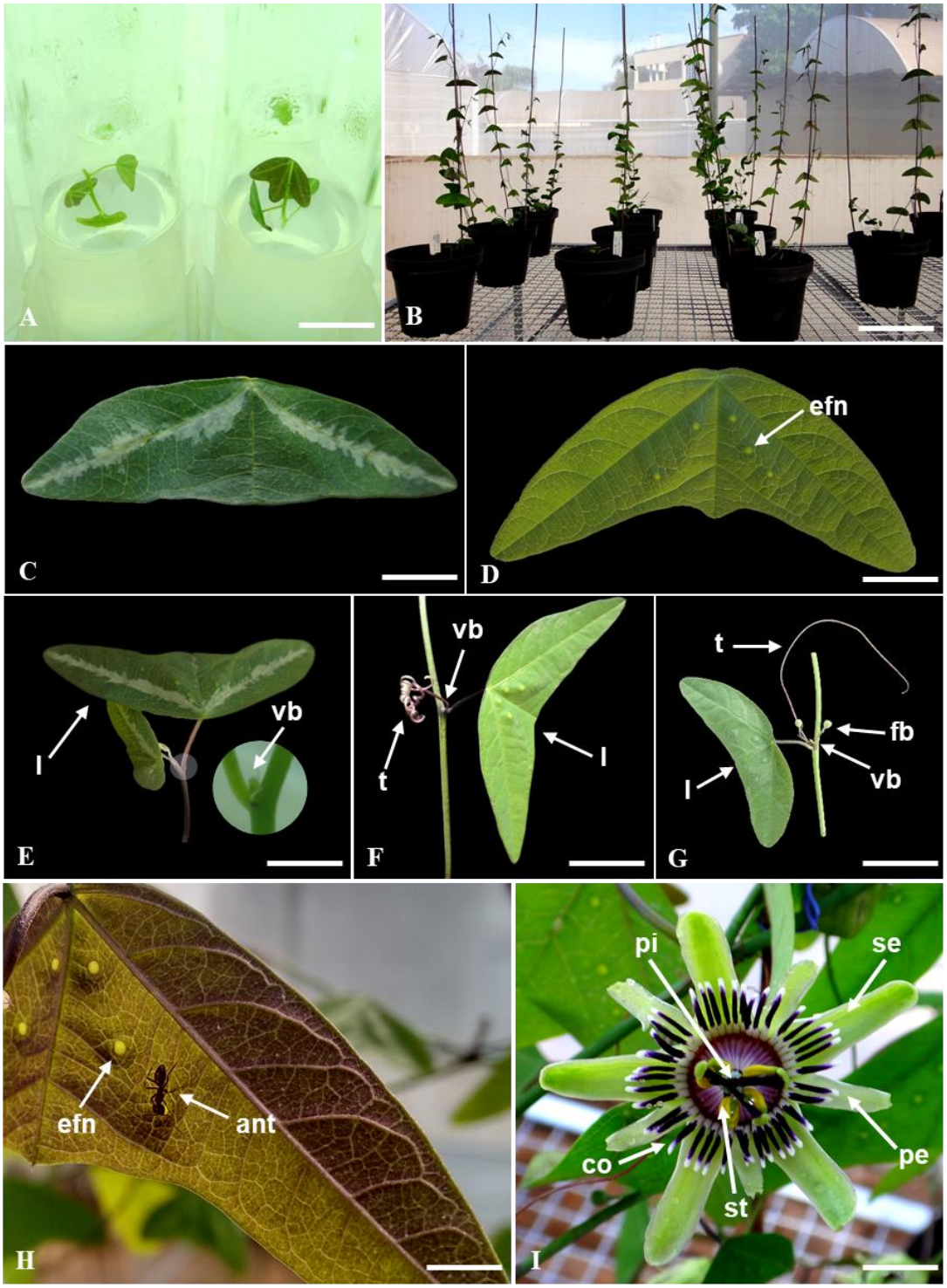


Figure 1 - ***Passifora organensis*** **morphology**. **A.** *In vitro* culture. **B.** Greenhouse-grown plants. **C.** Juvenile leaf. **D.** Adult leaf, the arrow indicates the extrafloral nectary (efn). **E.** Juvenile vegetative branch, with a vegetative bud (vb) in the leaf axil. **F.** Adult vegetative branch, with a vegetative bud and a tendril (t) in the leaf axil. **G.** Adult reproductive branch, with a vegetative bud, a tendril, and two flower buds (fb) in the leaf axil. **H.** Abaxial leaf surface showing the presence of extrafloral nectaries and an ant foraging for nectar. **I.** General morphology of the flower, with sepals (se), petals (pe), corona (co), stamens (st), and pistil (pi). Bars: A = 1 cm, B = 18 cm, C = 0.8 cm, D =1.4 cm, E = 1.3 cm, F = 2 cm, G = 2.5 cm, H = 1.8 cm, I = 0.6 cm

Besides the clear separation of the vegetative and reproductive phases during the development of *Passiflora* species, these transitions are not sudden, they occur gradually (Figure 2) as a result of the morphological and functional characteristics of the organs that differentiate from the shoot apical meristem. Thus, during phase transition, it is possible to find plants that still produce variegated leaves, but already produce tendrils.

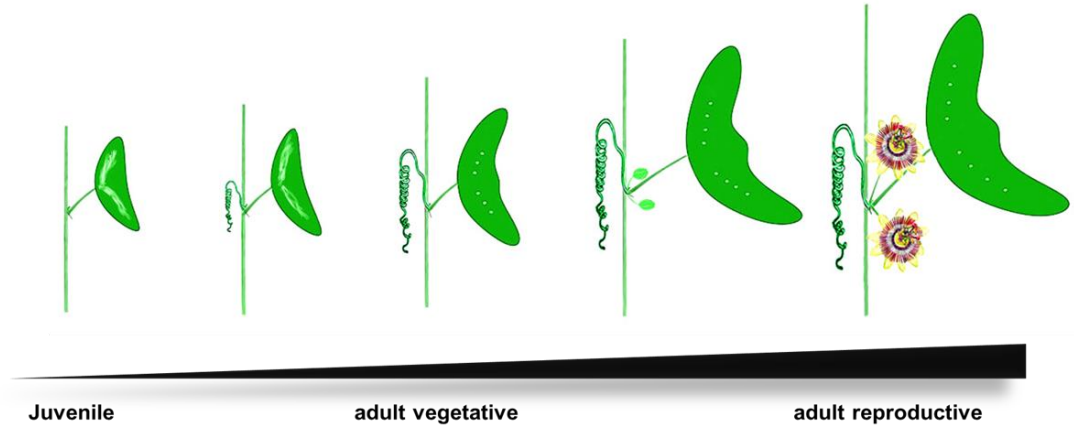


Figure 2 - **Morphological changes during phase transition in *Passiflora organensis***

4.3.2 *Passiflora organensis* juvenile leaf variegation

Detailed observations of *P. organensis* show that the patched light green or silvery green areas in juvenile variegated leaves usually follow the outline of the primary veins (Figure 3A-B), while the adaxial surface of adult leaves are not variegated (Figure 3C). Observations under SEM showed that the adaxial epidermal surface of juvenile leaves is similar in light green (Figure 3D) and green (Figure 3E) areas, as well as in adult leaves (Figure 3F). Moreover, anatomical observations showed that light green areas of the juvenile leaves present intercellular spaces between the epidermis and the palisade parenchyma (Figure 3G,J) at the adaxial surface, however, these intercellular spaces are either reduced, or absent, within the adjacent green area in juvenile leaves (Figure 3H,K), or in adult leaves (Figure 3I,L). Ultrastructural observations showed that chloroplasts have similar structure in both light green (Figure 3M) and green (Figure 3N) areas of the juvenile leaves, while in the adult leaves the structure is also similar, however more starch grains are observed in the chloroplasts, throughout the mesophyll (Figure 3O).

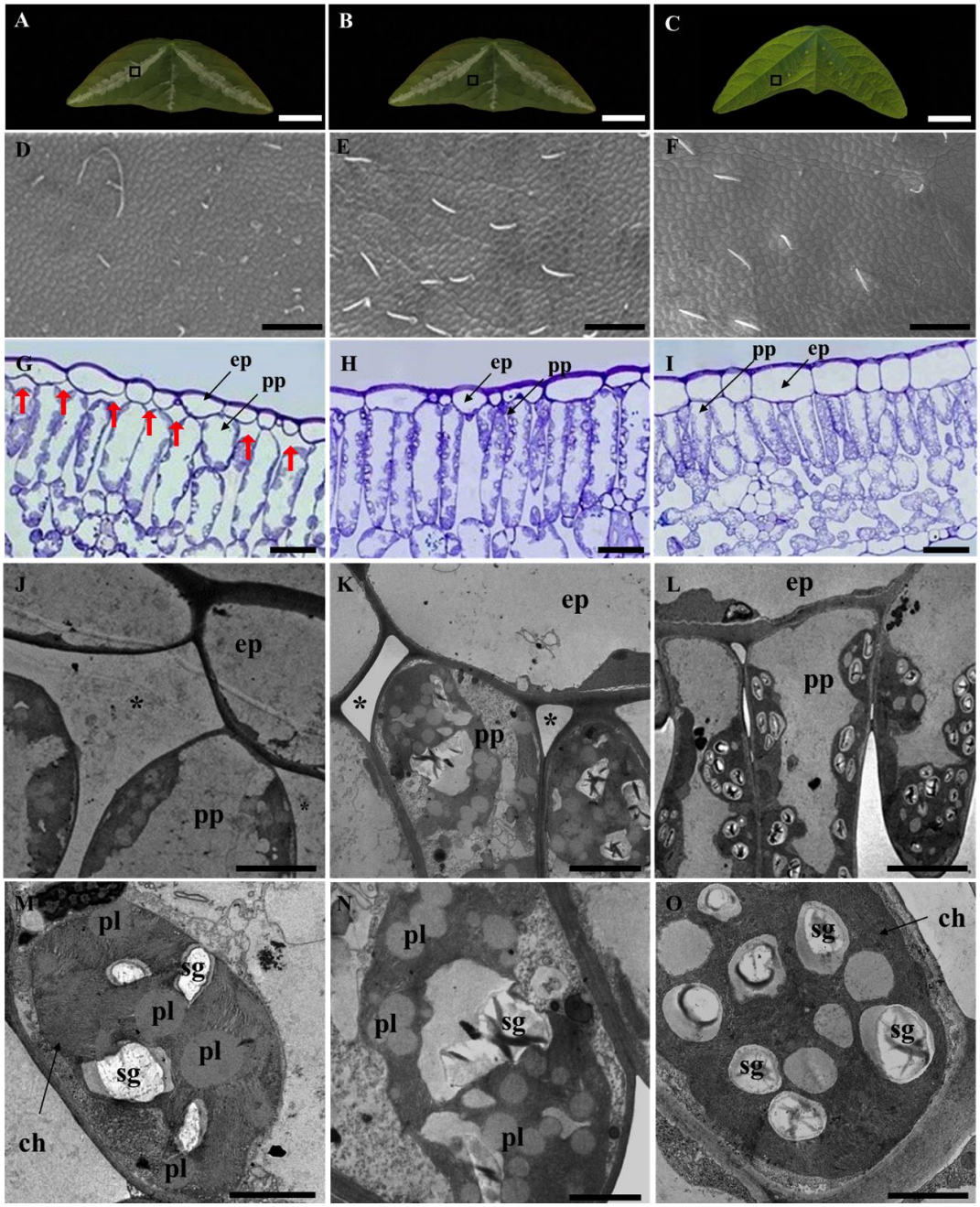


Figure 3 - **Leaf anatomy of variegated juvenile and adult leaf in *Passiflora organensis***. A-B. Juvenile leaf, the black boxes indicate the light green (A) and green area (B) used in the analysis. C. Adult leaf, the black boxes indicate the area used in the analysis. D-F. Surface views of the adaxial epidermal cells of the light green (D) and green (E) areas of a juvenile leaf, and green area of an adult leaf (F) observed by scanning electron microscopy. G-I. Transverse section of the light green (G) and green area (H) of the juvenile leaf, and green area of adult leaf (I) observed by light microscopy. The red arrows (G) indicate intercellular spaces between the adaxial epidermis and the palisade parenchyma. J-O. Ultrastructure observed under transmission electron microscopy. J. Intercellular spaces (*) between adaxial epidermis (e) and palisade parenchyma (p) in the light green area. K-L. Absence or reduced intercellular space between adaxial epidermis and palisade parenchyma in the green area of juvenile leaf (K) and adult leaf (L). M-O. Ultrastructure of chloroplasts in the light green (M) and green (N) area of a juvenile leaf, and green area of an adult leaf. Chloroplast (ch), intercellular space (*), adaxial epidermis (ep), plastoglobules (pl), palisade parenchyma (pp), starch grains (sg). Bars: A = 0.8 cm, B = 0.8 cm, C = 1,5 cm, D = 150 µm, E = 150 µm, F = 150 µm, G = 25 µm, H = 30 µm, I = 40 µm, J = 7,5 µm, L = 5 µm, K = 7,5 µm, M = 2 µm, N = 2 µm, O = 2 µm

4.3.3 Extrafloral nectaries in *Passiflora organensis* adult leaves

P. organensis plants bear an undefined number of EFNs in the leaf surface (4 to ~ 12), but more frequently six EFNs are observed. The EFNs are button shaped (Figure 4A) and anatomically the EFNs appear as discoid structures, somewhat concave surrounded by adjacent longer epidermal cells (Figure 4C). The secretory area of the nectary is located on the abaxial surface of the leaf, and it is noteworthy that there is vascular connection between the mesophyll tissue and the nectary cells (Figure 4B). The abaxial epidermis cells of the nectary (Figure 4E) secrete the nectar and the cells right next to them are prone to produce it (Figure 4D and F).

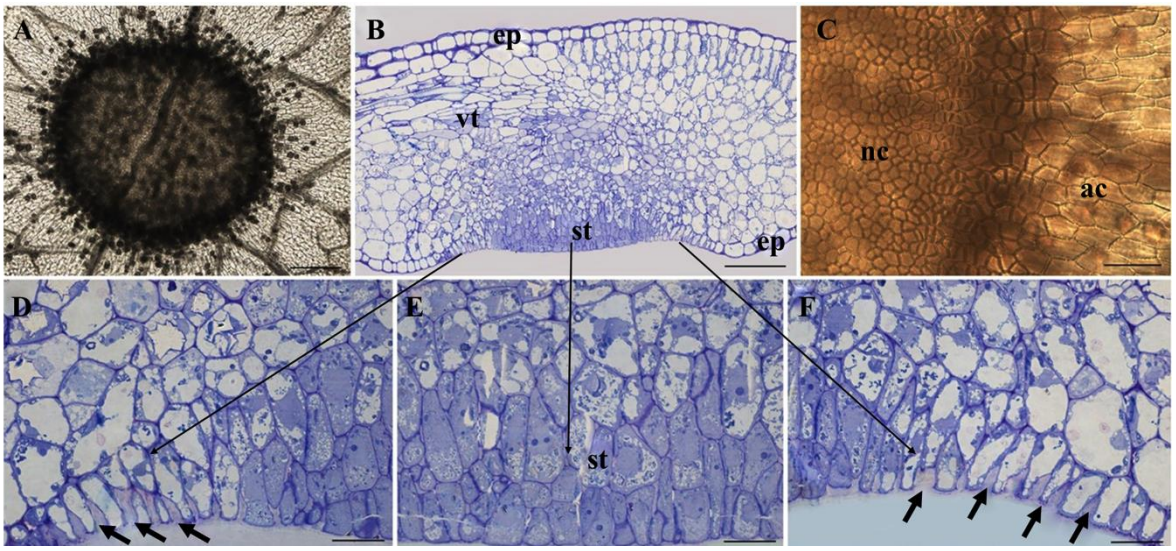


Figure 4 – **Extrafloral nectary of *Passiflora organensis***. **A.** General view of the extrafloral nectary observed in the stereomicroscope. **B** Transverse section of the leaf showing the secretory tissue (st), vascular tissue (vt), and cells adjacent to the nectary. **C.** Overview of the intercession between nectary cells and adjacent cells. **D-F.** Details of B: the adjacent cells of the secretory tissue (D, F) and the central region of the secretory tissue (E), Small arrows indicate the accumulation of fluid compound of the adjacent cells. Bars: A = 200 µm, B = 100 µm, C = 50 µm, D-F = 20 µm

Calcium oxalate crystals were frequently associated to the nectaries. In *P. organensis* EFNs an increasing concentration of calcium during leaf development and nectary differentiation were showed (Figure 5A-B). EDX imaging allowed the observation of the spatial distribution of calcium within leaf tissues, which correlated with the position of developing nectaries. These results are in agreement with calcium deposition in the form of calcium oxalate, during nectary maturation. Accordingly, through micro-computed

microtomography we observed that the glandular tissue of mature EFNs was surrounded by denser material, corresponding to druse crystals of calcium oxalate (Figure 5C).

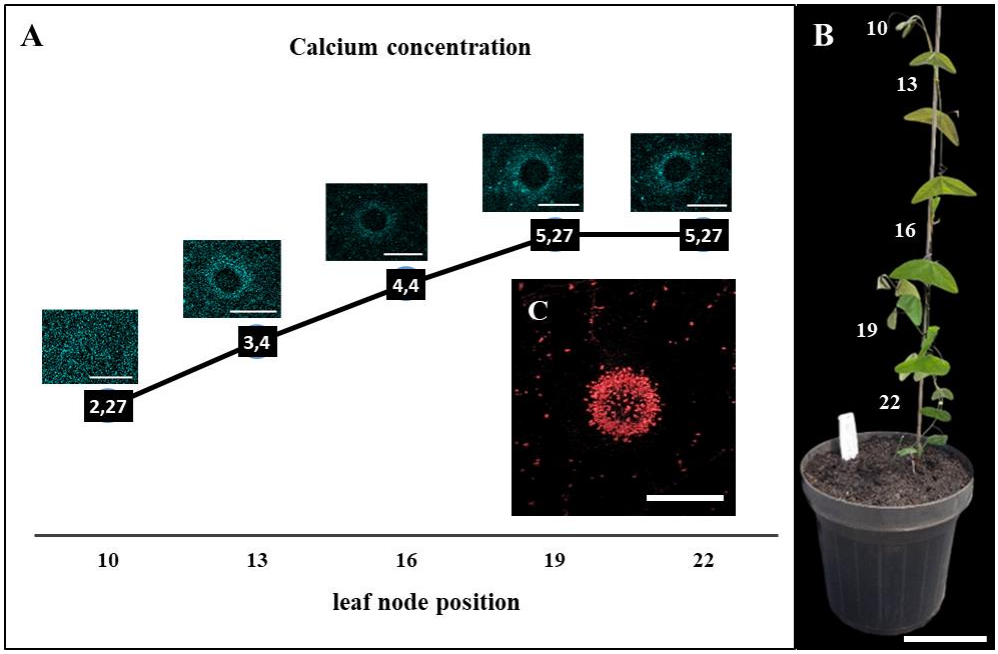


Figure 5 – **Calcium concentration in extrafloral nectaries of *Passiflora organensis* measured by energy-dispersive x-ray analysis in the scanning electron microscope. A.** Concentration of calcium in the EFN in different node positions. **B.** Leaf node positions in the plant. **C.** Micro-CT image shows calcium accumulation on the EFN. Bars: A: node 10 = 250 µm, node 13 = 500 µm, node 16 = 500 µm, node 19 = 500 µm, node 22 = 500 µm; B = 9 cm; C = 800 µm

4.3.4 Plasticity of *Passiflora organensis* leaf axillary meristems

The different morphologies of *P. organensis* axillary leaf meristems (AMs) were characterized by scanning electron microscopy. We also observed the morphological characteristics of the shoot apical meristem (SAM) during the juvenile (Figure 6A-E), adult vegetative (Figure 6F-J), and adult reproductive phases (Figure 6K-O).

The first leaf primordium was observed as a bump that produced two additional lateral structures which later developed into the stipules (Figure 6A, F, and K). In the axil of the third leaf primordium of juvenile and adult samples we observed a group of cells that, in the juvenile vegetative phase, gave rise to a vegetative bud (Figure 6 B-E). On the other hand, in the vegetative adult phase, this group of cells gave rise to the vegetative bud and a tendril primordium (Figure 6G-J). Subsequently, in the adult reproductive phase, this axillary meristem gave rise simultaneously to a tendril primordium and two flower meristems, in addition to the vegetative bud (Figure 6L-O). The size of this meristematic region was

different between the juvenile and adult vegetative phases (Figure 6B and 6G), suggesting a correlation between the size of the meristem and the structure(s) that will be formed.

After the transition to the reproductive stage, the axillary meristem started to produce two floral meristems, each of them positioned laterally, in relation to the tendril primordium (Figure 6L-M). In the reproductive phase, the vegetative bud continued to be formed between the insertion of the tendril and the stem (Figure 6N-O).

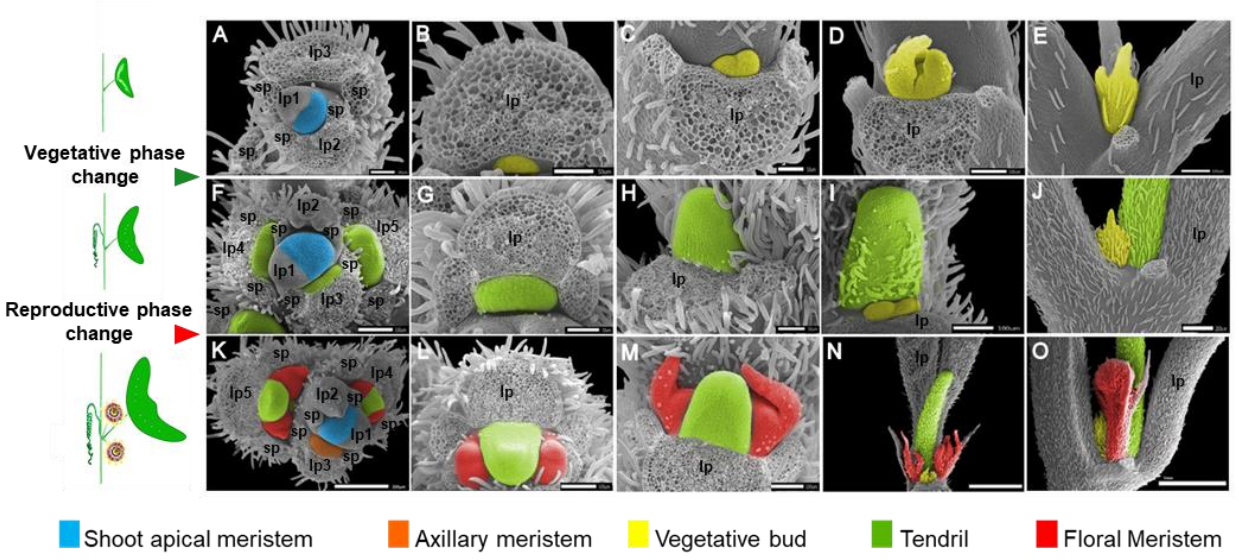


Figure 6 - ***Passiflora organensis*** **shoot apical meristem development observed under the scanning electron microscope**. **A - E.** Plant in the juvenile vegetative stage, producing only the vegetative bud in the leaf axil. **F - J.** Plant in the vegetative adult stage showing that from the axillary meristem both, a vegetative bud and a tendril are formed. **K - O.** Plant in the adult reproductive stage giving rise to a vegetative bud, a tendril and a floral bud from the axillary meristem. lp: leaf primordium, sp: stipule primordium. Bars: A-C, G-H = 50 µm; D-F, I, M-N = 100 µm; J, L = 200 µm; O-P = 1 mm

4.4 Discussion

The species of *Passiflora* present a clear juvenile period and in almost all the species of the genus *Passiflora*, the plants in the juvenile stage do not produce tendrils and produce leaves with a different morphology when compared to those from plants in the adult stage. For example, juvenile leaves are lanceolate in *P. edulis* while adult leaves are trilobed (Ulmer; Macdougal, 2004). We showed that *P. organensis* has variegated leaves in the juvenile stage due to an air space type mechanism, instead of a pigment-related mechanism. Pigment-related mechanisms of variegation are based on chlorophyll deficiency or the presence of specific pigments in the leaf tissues (Hara, 1957; Sheue et al., 2012). The air space mechanism involves intercellular spaces between the adaxial epidermis and palisade

parenchyma and the pale area is only visible when light is reflected from the adaxial surface of the leaf (Tsukaya et al., 2004). Thus, if we observe a *P. organensis* juvenile leaf under transmitted light, variegation is no longer observable. Studies have shown that variegation in plants is not just a color mutation but has some adaptive functions that may benefit the plants, such as: defense from enemies including aposematic coloration, mimicry of dead or infested plants, masquerade and camouflage, improved water or gas transport, mitigation of UV radiation and thermoregulation (Fooshee; Henny, 1990; Lev-Yadun et al., 2004; Roelfsema et al., 2006; Lev-Yadun, 2016; Lev-Yadun, 2017; Niu et al., 2018; Shelef et al., 2019).

Leaves of *P. organensis* in the adult phase do not display variegation, but in contrast produce EFNs in the abaxial surface of the leaf. The EFNs are nectar glands formed apart from the flower, which may be distributed on various plant parts providing an indirect defense against herbivores, through its mutualistic association with ants (Apple; Feener, 2001; Hossaert-McKey et al., 2001; Wirth; Leal, 2001; Marazzi, 2013). The EFNs have a particular tissue anatomy and composition for the production of extrafloral nectar. Here we showed that during EFN development, calcium concentration increases, in agreement with the deposition of calcium oxalate crystals throughout the EFN tissues. Studies have shown that calcium oxalate crystals are reported within or in the region of EFNs of a wide diversity of angiosperms (Schnell et al., 1963; Elias, 1983; Tilney; Van Wyk, 2004; Paiva et al., 2007) and they may correlate with the ecology of the plant, acting as a protection against herbivory (Rico-Gray; Oliveira, 2007; Walters, 2011; Yamawo et al., 2014).

Passiflora species in the adult vegetative stage develop tendrils at the leaf axils, and its presence is another precise parameter to distinguish if the plant is in its juvenile or adult stage of development. The ontogenesis and arrangement of this structure lead authors to consider the *Passiflora* tendril as part of the primary axis of a reduced inflorescence (Nave et al., 2010; Cutri et al., 2013). Differently, in pea, tendrils are considered modified leaves produced by the vegetative meristem (Gourlay et al., 2000), while in grapevine the tendrils are formed by the axillary meristems, which can originate either a tendril or an inflorescence (Calonje et al., 2004).

P. organensis plants in the adult reproductive stage produce tendrils and flowers simultaneously from the axillary meristems. Species belonging to subgenus *Passiflora*, like *P. edulis*, generally produce a solitary flower and one tendril per node. However, plants of *P. organenis* produce two flowers per node and they are positioned side by side with the tendril. This plasticity of flower numbers per node may allow manipulation of *P. edulis*

genotypes to produce more flowers, and therefore more fruits, either by hormonal treatment or breeding (Cutri et al., 2013).

The axillary meristems (AMs) of *P. organensis* are complex in comparison to AMs in other species, such as the model plant *Arabidopsis thaliana*. In the vegetative phase, the *Arabidopsis* primary shoot meristem gives rise to leaf primordia displayed as a rosette. After phase transition, the shoot apical meristem is converted into an inflorescence meristem that produces a few cauline leaves or bracts, followed by the formation of determinate floral meristems on the periphery of the inflorescence meristem (Bradley et al., 1997; Ratcliffe et al., 1998). The plasticity observed in the *Passiflora* AMs and other plants may be related to the altered expression patterns of genes involved in multiple genetic pathways, including those related to age, temperature and photoperiod perception, as well as hormone biosynthesis and perception (Boss et al., 2004; Jack, 2004; Amasino; Michaels, 2010).

Other plant species have a similar growth habit and AMs as complex as those observed in *Passiflora*. For example, cucumber plants also produce tendrils and after phase transition, unisexual flowers are produced from AMs. (Wen et al., 2019). In grapevine, adult plants have special AMs opposed to leaves giving rise to tendrils for a long period of time before the plant initiates flowering, and at the reproductive phase, inflorescences are formed in place of tendrils from the AMs (May, 2004; Carmona et al., 2008).

All the morphological characteristics that we have shown suggest that *Passiflora* AM behavior during phase transition is more complex in comparison with AMs from other plant species, indicating that *P. organensis* might be an important model species to understand how AM modulation gives rise to different structures.

4.5 Conclusions

Passiflora organensis is an excellent model for phase transition studies, because there are obvious morphological differences between the plants in the juvenile, adult vegetative and adult reproductive stages. The results from this study will help us to contextualize spatially and temporally mechanisms involved in phase transition, which we will access in the next chapters.

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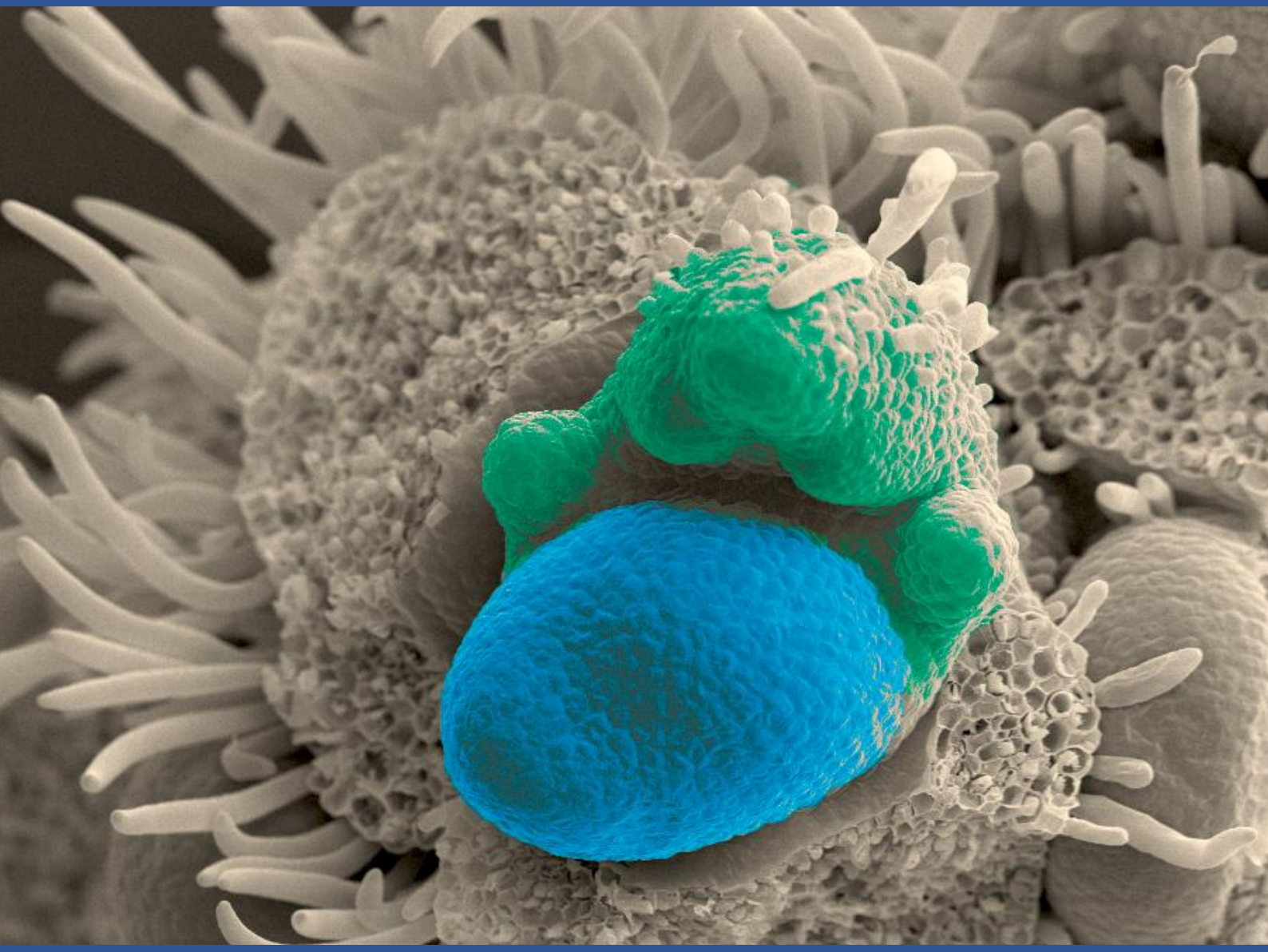
CHAPTER II

Characterization of *Passiflora organensis FT/TFL1 gene* family and its putative role during phase transition and branching



Passiflora organensis – shoot apical meristem

5. Characterization of *Passiflora organensis FT/TFL1* gene family and its putative role during phase transition and branching

Abstract

The *FT/TFL1* gene family regulates phase transition during plant development. Despite being studied in many plant species, there is hardly any knowledge about this gene family in *Passiflora*, which includes the passionfruit, an economically important crop cultivated in Brazil. We identified eight *FT/TFL1* genes in *Passiflora organensis*: *PoFT*, *PoTSFa*, *PoTSFb*, and *PoTSFc*, belonging to the *FT*-like subfamily; *PoTFL1*, *PoBFT*, and *PoATC*, belonging to the *TFL1*-like subfamily; and *PoMFT*, belonging to the *MFT*-like subfamily. We characterized the members of the *Passiflora FT/TFL1* family by analyzing their phylogeny, gene structure, and expression patterns. *Passiflora FT*-like genes were more expressed during the adult phase and they might have an important role in flowering activation. On the other hand, *Passiflora TFL1*-like genes were more expressed during the juvenile phase. We also showed that the proteins encoded by the *Passiflora FT/TFL1* members display the conserved amino acid residues important for their function, either as inducers or repressors of flowering, except for PoTSF. In addition, we demonstrated that *PoFT* and its paralog *PoTSF* displayed a bimodal expression pattern during day/night periods. The results of these studies provide insights into the potential functions of *FT/TFL1* gene family in *P. organensis*, and contribute to more focused studies on passionfruit reproduction.

Keywords: Flowering. FT-like. MFT-like. Passionfruit. PEBP. TFL1-like.

5.1 Introduction

For distinct developmental phases the shoot apical meristem produces different types of organs, such as leaves, branches, and flowers (Huijser; Schmid, 2011; Poethig, 2013). Transitions between these developmental phases depend on the physiological state of plants that are regulated through the integration of multiple environmental and endogenous signals (Imamura et al., 2011).

At the molecular level, we can highlight the *FT/TFL1* gene family, which encodes proteins with high similarity to phosphatidylethanolamine binding proteins that plays a key role in phase transition and function as flowering promoters or repressors (Wickland; Hanzawa, 2015).

In *Arabidopsis*, CONSTANS (CO) senses photoperiod signals in leaves, where it promotes the expression of the major component of florigen: *FLOWERING LOCUS T (FT)*. The FT protein is transported to the shoot apical meristem, where it associates with the transcription factor FD to activate the floral identity genes, such as *LFY* and *AP1* (Abe et al., 2005; Turck et al., 2008). On the other hand, TERMINAL FLOWER 1 (TFL1) have the antagonist function of FT, repressing flowering by competing for the interaction with FD and repressing the expression of floral identity genes (Mach, 2011). In rice, the proteins encoded by the FT and bZIP transcription factors interact with each other via a 14-3-3 bridging protein. This protein forms a homodimer and interacts with homodimers of FT and FD, forming a hexameric protein complex that plays a critical role in flowering (Taoka et al., 2011).

Besides flowering, studies have shown other roles for FT-like proteins in plant development, such as: leaf complexity and curling, stomatal opening, tuberization, inflorescence architecture, and branching (Krieger et al., 2010; Kinoshita et al., 2011; Gonzalez-Schain et al., 2012; Niwa et al., 2013; Moraes et al., 2019). In *Arabidopsis*, BRANCHED1 (BRC1) interacts with FT and interferes with the control of flower differentiation in axillary meristems (Niwa et al., 2013). The diversified functions of the FT-like proteins show the importance of the *FT/TFL1* gene family in plant developmental processes. Moreover, this family is characterized by gene duplication events and these variable roles of FT-like proteins may be a result of sub- or neo-functionalization (Flagel; Wendel, 2009; Wickland; Hanzawa, 2015).

The information provided by this study will help elucidate the biological functions of this gene family in *Passiflora organensis,* a species belonging to the same genus of the

passionfruit, an economically important fruit crop cultivated in Brazil. The passionfruit plant is a liana with solitary flowers being produced seasonally at the axillary meristems, after the transition from the vegetative to the reproductive phase (Cutri et al., 2013). Nevertheless, the genus *Passiflora* shows enough plasticity to present branched inflorescences producing two or more flowers (Cutri et al., 2013; Nave et al., 2010). Therefore, understanding the regulation of the transition to the reproductive phase and axillary meristem activation, may contribute to breeding studies aiming at higher passionfruit yields.

5.2 Materials and Methods

5.2.1 Multiple sequence alignment and phylogenetic analysis

The search for the orthologous genes was performed using the genome sequencing database of *P. organensis*. The protein sequences of *Arabidopsis thaliana* were used as query sequences. The BioEdit software was used to find candidate genes in each contig obtained at the *P. organensis* database. The identification of exons/introns boundaries was done with the NetPlantGene software (Pertea et al., 2000).

Identified proteins in *Passiflora* were aligned with sequences of other species using the ClustalX program (Thompson et al., 1997). Phylogenetic analysis of these proteins was performed using full-length proteins. A neighbor-joining tree (Saitou; Nei, 1987) was constructed using the MEGA 7.0 software (Kumar et al., 2016). Bootstrap confidence values were calculated based on 1000 replications. Protein sequences of other plant species used in this study were obtained from the NCBI protein database (http://www.ncbi.nlm.nih.gov/).

5.2.2 Gene expression pattern analyses by qRT-PCR

To investigate the expression patterns of each homolog, qRT-PCR experiments were performed using shoot apices or leaves of *P. organensis*. Total RNA was extracted from samples harvested of plants in juvenile, adult vegetative, and adult reproductive stages, using RNeasy® Plant Mini Kit (50) - QIAGEN. The first-strand cDNA was synthesized with a SuperScript® III First-Strand Synthesis kit - Invitrogen™, according to the manufacturer's instructions. qRT-PCR was performed with Fast SYBR Green Master Mix (Applied Biosystems) on the StepOne Real-Time PCR System (Applied Biosystems). Primers used for the qRT-PCR are listed in Table S1 and the melting curve with their specificity is showed in Figure S2. qRT-PCR was performed with three independent biological replicates and three

technical replicates for each sample. Data was analyzed using the mathematical model for relative quantification in real-time RT-PCR, as described by Pfaffl (2001). Expression levels of specific genes were normalized using *Passiflora CLATHRIN ADAPTOR COMPLEX* (*CAC*) and *MONENSIN SENSIVITY 1/SAND* family (*SAND*) (Scorza, 2015).

5.2.3 *In situ* hybridization

P. organensis shoot apices of plants in the juvenile, adult vegetative, and adult reproductive stages were fixed in paraformaldehyde (4%) for 24h at 4˚C and dehydrated in an ethanol series; embedded in paraplast xtra, sectioned (6 µm) in a rotary microscope (Leica, Heidelberg, Germany), and attached to silanized glass slides. Prior to hybridization, the paraplast xtra was removed from the sections by quickly rinsing the slides in xylol. Nonradioactive probes were labeled with digoxygenin (DIG-dUTP) following the instructions of the manufacturer (Roche). The prehybridization and hybridization conditions were described elsewhere (Dornelas et al., 1999; Dornelas et al., 2000). Hybridization signal was visualized using anti-DIG antibodies conjugated to alkaline phosphatase and a NBT/BCIP solution as a substrate. Hybridized slides were observed and documented in a Zeiss Axioskop 2 microscope (Carl Zeiss, Jena, Germany).

5.3. Results

5.3.1 Identification of *FT/TFL1* family members in *Passiflora organensis*

To identify potential *FT/TFL1* genes in *Passiflora*, protein sequences encoded by *Arabidopsis* members of the *FT/TFL1* gene family were used to perform a BLAST survey (Altschul et al., 1990) against the genome database of *P. organensis*. This database has genome data of three different genotypes and it was stored in different libraries named as: LIB042, LIB043, and LIB044.

We identified eight *FT/TFL1* putative genes in the *P. organensis* genome and we named them: *PoFT*, *PoTSFa*, *PoTSFb*, *PoTSFc*, *PoTFL1*, *PoBFT*, *PoATC*, *PoMFT*, according to their closest homologs in *Arabidopsis*. All the genes identified in *P. organensis* have four exons and three introns. This genomic organization is conserved in other *FT/TFL1* genes found in other species (Li et al., 2015). The length of exons 2 and 3 were conserved when compared to their *Arabidopsis* putative orthologs, with 62 and 41bp, respectively (Figure 1).

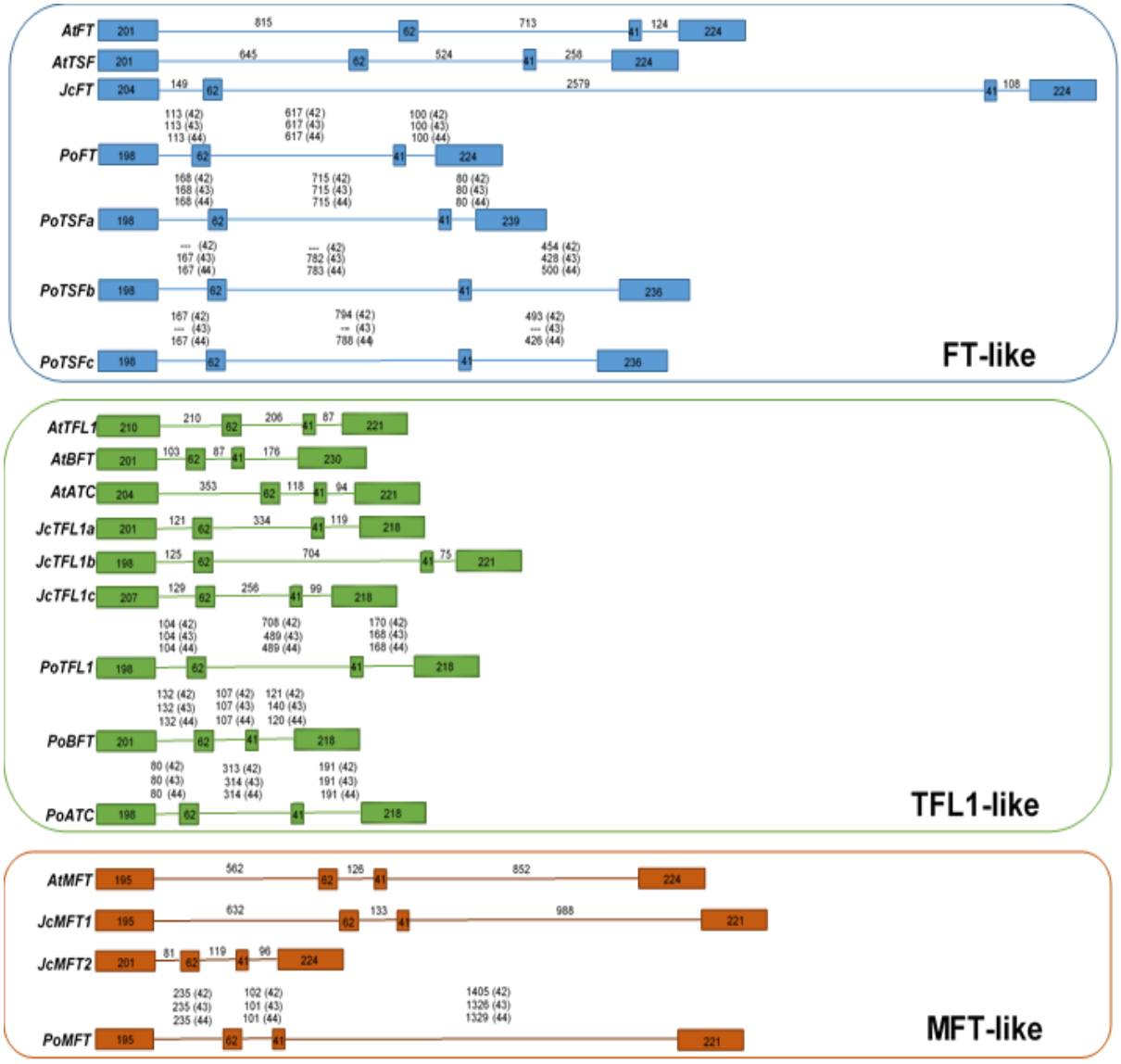


Figure 1 – **Genomic organization of *FT/TFL1* gene family in *Passiflora organensis*, *Arabidopsis thaliana*, and *Jatropha curcas***. Boxes represent exons and lines represent introns. Numbers indicate the lengths of exons and introns in base pairs. Numbers in parentheses indicate the libraries: LIB042, LIB043, and LIB044

5.3.2 Phylogenetic analysis of *Passiflora organensis FT/TFL1* genes

To analyze the phylogenetic relationships among putative orthologs of the *FT/TFL1* family, we performed phylogenetic analysis using protein sequences of *P. organensis* and other angiosperms. A neighbor-joining phylogenetic tree was generated and three major subfamilies were apparent, placing *PoFT*, *PoTSFa*, *PoTSFb*, and *PoTSFc* in the *FT*-like subfamily; *PoTFL1*, *PoBFT*, and *PoATC* in the *TFL1*-like subfamily; and *PoMFT* in the *MFT*-like subfamily (Figure 2).

We observed that the *Passiflora FT/TFL1* proteins (Figure 2) were more closely related to those from perennial plants, such as *Jatropha curcas* and *Vitis vinifera*. PoFT, the putative homolog of *Arabidopsis* FT, displayed all of the characteristic features of the FT-like protein subfamily (Ahn et al., 2006). This includes the conservation of Tyr85 and Gln140 (Tyr84 and Gln139 in PoFT, respectively) and the highly conserved amino acid sequences LGRQTVYAPGWRQN and LYN, corresponding to the binding regions of FT with FD coded by exon IV (Abe et al., 2005; Wigge et al., 2005). However, the paralogs of PoFT: PoTSFa, PoTSFb, and PoTSFc displayed different amino acid residues that are known to be important for the florigen (flower induction) function: they display His139 instead of Gln139 (Figure 3).

PoTFL1, PoBFT and PoATC bear the conserved residues His88 and Asp144 in similar positions to *Arabidopsis* TFL1 (Hanzawa et al., 2005) (His84 and Asp138 in PoTFL1 and PoATC, and His84 and Asp139 in PoBFT) (Figure 3). The PoMFT contains a critical amino acid residue (Trp) that differs from Tyr and His in FT and TFL1, respectively, and a conserved Pro in the C-terminal region (Figure 3), which was not found in the *Arabidopsis* FT-like nor TFL-like subfamilies (Hedman et al., 2009).

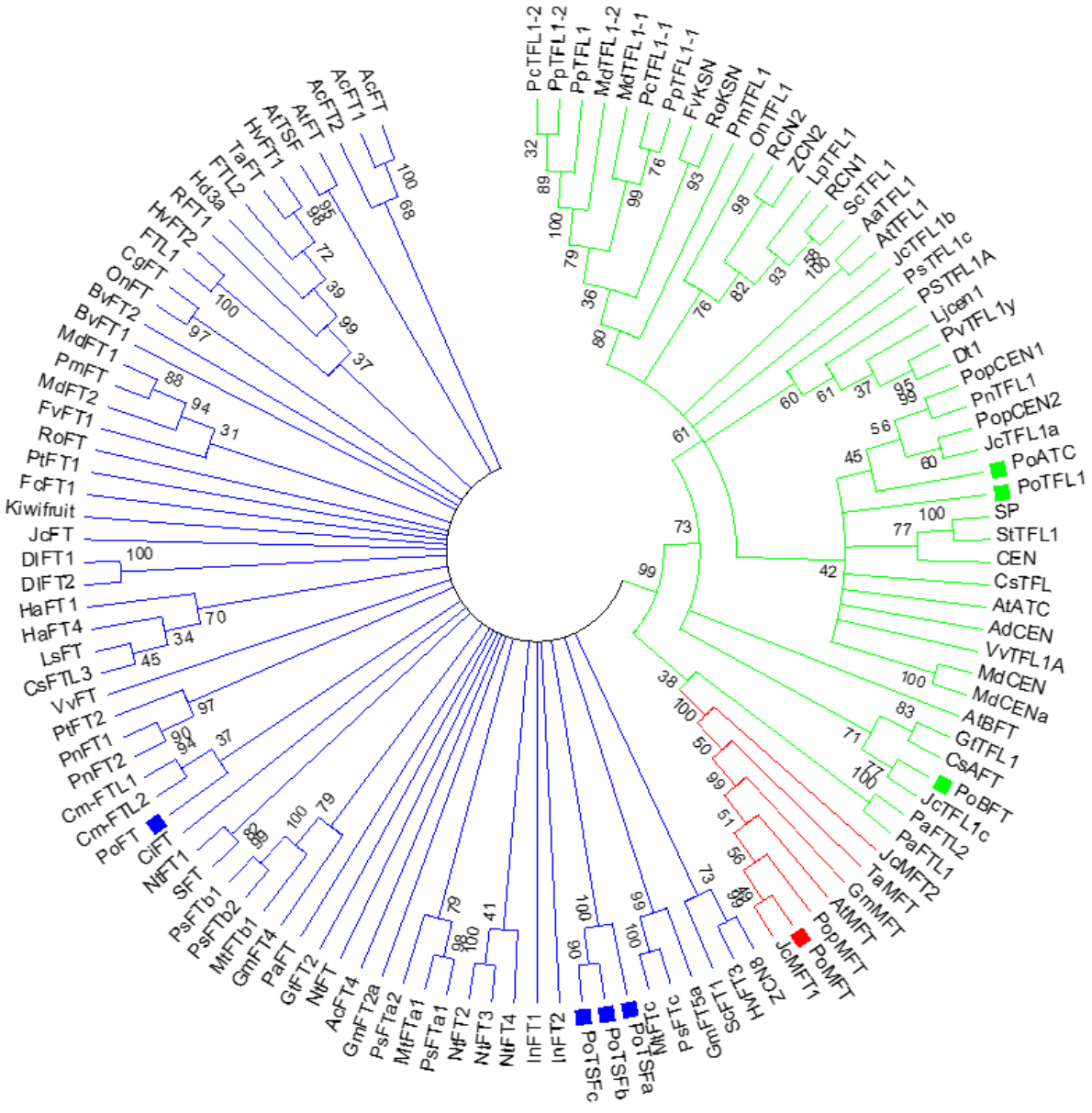


Figure 2 – **Phylogenetic analysis of the *FT/TFL1* gene family members in *Passiflora organensis* and other angiosperms.** The tree was constructed using the neighbor-joing method and MEGA7 software. Only bootstrap values > 30% (1000 resamples) are shown in the branches. Blue indicate members of the FT-like subfamily, green the TFL1-like subfamily and red the MFT-like subfamily. The colored boxes highlight the members of the FT/TFL1 family in *Passiflora organensis.* The species used in this analysis are listed in Table S3

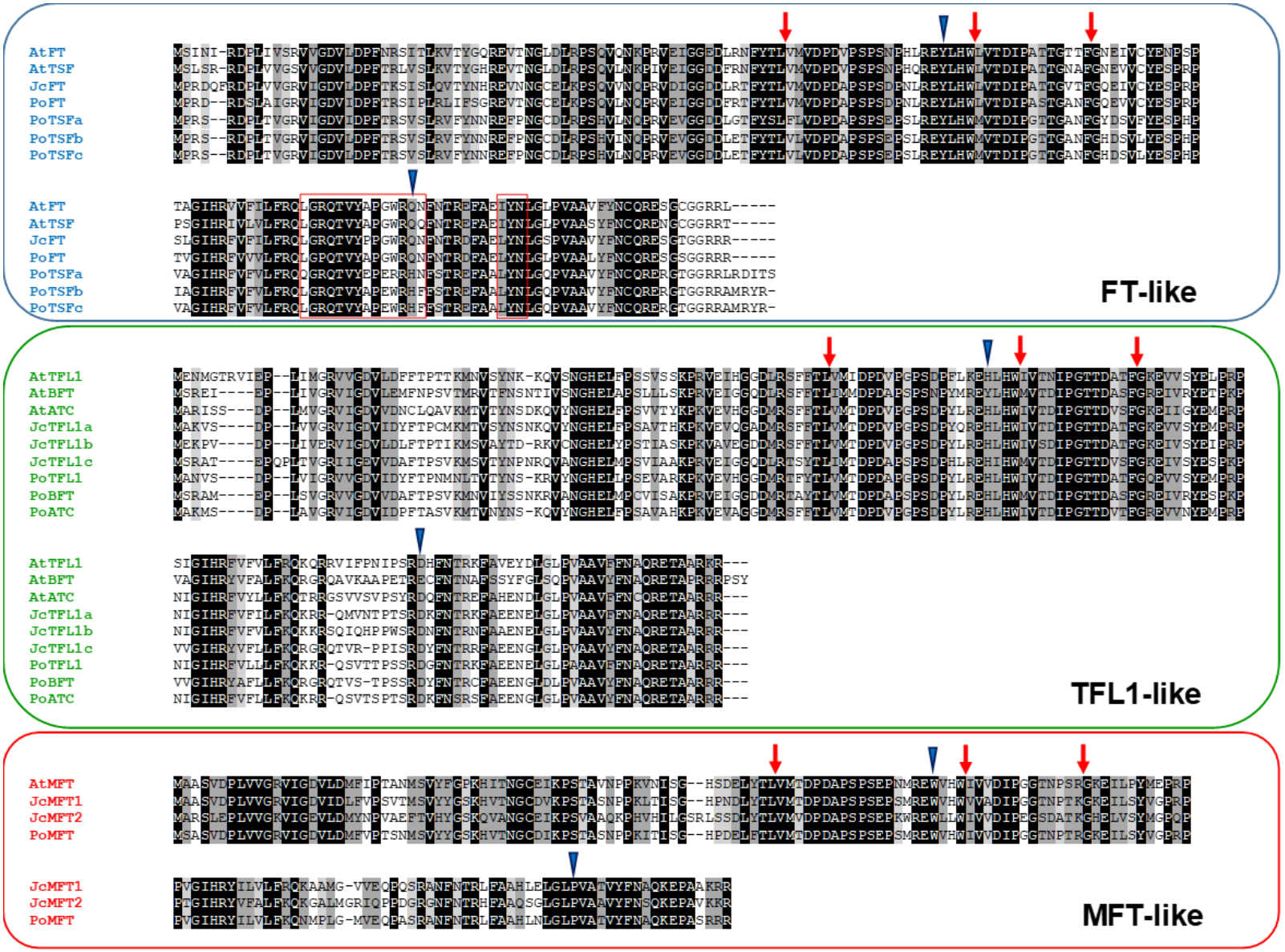


Figure 3 - **Amino acid sequence alignments of the FT/TFL1 proteins in *Passiflora organensis*, *Arabidopsis thaliana*, and *Jatropha curcas*.** A black background indicates a similarity level of 100% and a grey background indicates a conserved residue with a similar charge. Intron positions are indicated by red arrows above sequences. Blue arrowheads indicate amino acids that are critical to define FT, TFL1, or MFT-like proteins. The two red boxes indicate the important amino acid sequences in exon IV of FT-like proteins.

5.3.4 Expression patterns of *Passiflora organensis FT/TFL1* genes

Our results of gene expression analysis by real time quantitative PCR showed a higher level of *PoFT* expression in the adult vegetative phase (Figure 4A), while its paralog *PoTSFa* showed a higher level of expression in the adult reproductive phase (Figure 4B). In *P. organensis* there are three *TSF* paralogous genes: *PoTSFa*, *PoTSFb* and *PoTSFc*. Their sequences have a high level of identity (Figure S4) and it was not possible to design primers specific for *PoTSFb* and *PoTSFc.* Thus, *PoTSFs* indicates the gene expression of all paralogs together, with the highest level of expression observed in the adult reproductive phase (Figure 4C).

The expression of *TFL1*-like genes was higher in the juvenile phase. *PoTFL1*, *PoBFT* and *PoATC* presented, respectively, approximately 70, 30, and 5-fold increase in expression in the juvenile stage when compared to the adult stage (Figure 4D-F). Finally, *PoMFT* showed a higher expression in the juvenile phase, decreasing during further development (Figure 4G).

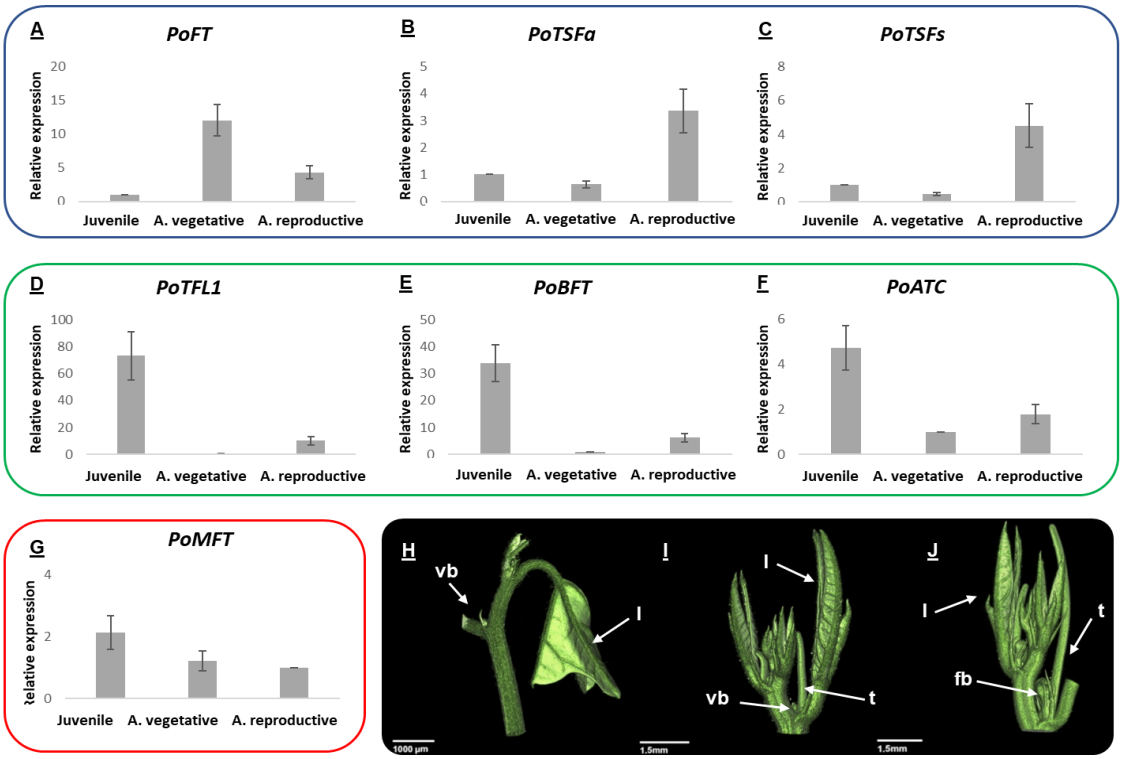


Figure 4 – **Relative expression of *FT/TFL1* genes in shoot apices of *Passiflora organensis*. A-G.** Relative expression of *FT*-like genes (A-C), *TFL1*-like genes (D-F), *PoMFT* (G). **H-J.** Samples used in the RT-qPCR: shoot apex in the juvenile vegetative stage (H), shoot apex in the adult vegetative stage (I), and shoot apex in the adult reproductive stage (J). The arrows indicate: flower bud (fb), leaf (l), vegetative bud (vb), and tendril (t). In each graphic the sample with lowest Ct value was used as calibrator. Error bars indicate the standard deviation

Considering that the photoperiod was shown to have an important impact in the expression of the *FT/TFL1* genes in model plants such as *Arabidopsis* (Song et al., 2018), A comparative analysis of the expression patterns of the *FT/TFL1* genes was performed (in October of 2016) during a 24h-period (Figure 6), with samples harvested every 2h. Moreover, a comparative pre-analysis of the expression pattern of these genes in leaves of different ages (positioned in different nodes or plastochrons) was performed to standardize the samples (Figure 5). In the two analyzes mentioned, we did not detect the expression of *PoTFL1*, *PoATC*, and *PoMFT* in leaf tissues in agreement with what is reported in the literature for *Arabidopsis* (Mimida et al., 2001; Tao et al., 2014). Older leaves (i.e. nodes 16-17), presented higher levels of *PoFT*, *PoTSFs* and *PoBFT* (Figure 5). *PoFT* and *PoTSFs* showed an increase of transcript production at dawn and at dusk, with the expression of *PoFT* increasing slightly earlier than that of *PoTSFs* (Figure 6A-B).

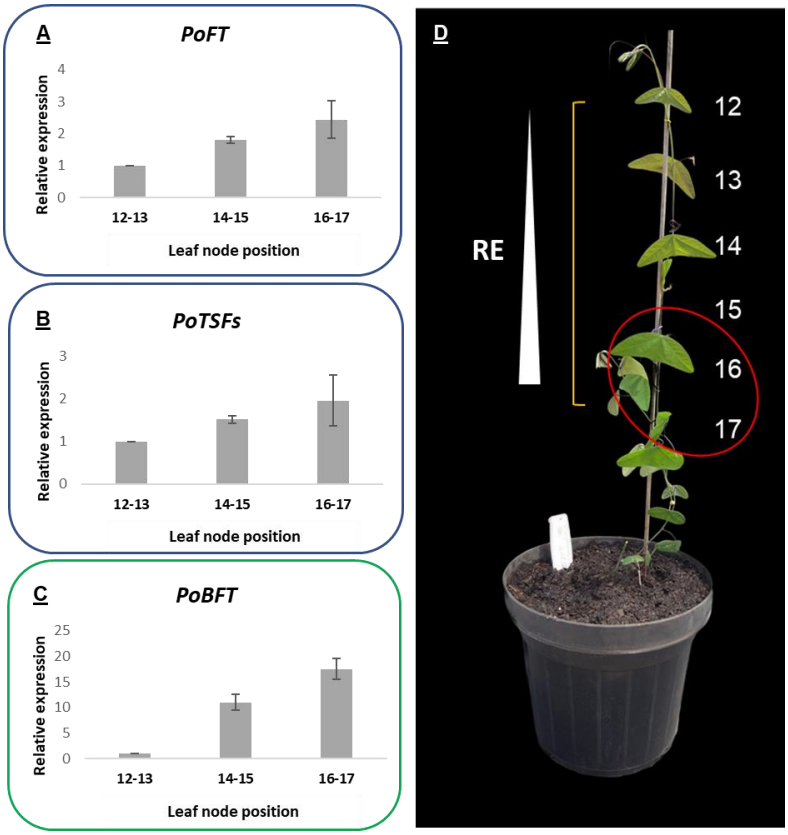


Figure 5 – **Relative expression of *PoFT*, *PoTSFs*, and *PoBFT* in leaves positioned in different nodes of *Passiflora organensis* in the adult vegetative phase. A-B.** Relative expression of genes belonging to the *FT*-like subfamily. **C.** Relative expression of genes belonging to the *TFL1*-like subfamily. **D**. Leaf node position in *P. organensis*: the relative expression (RE) increases as the leaf matures. The red circle indicates the positions of leaf nodes selected for the photoperiodic expression analyses. In each figure the sample with lowest Ct value was used as the calibrator. Error bars indicate the standard deviation

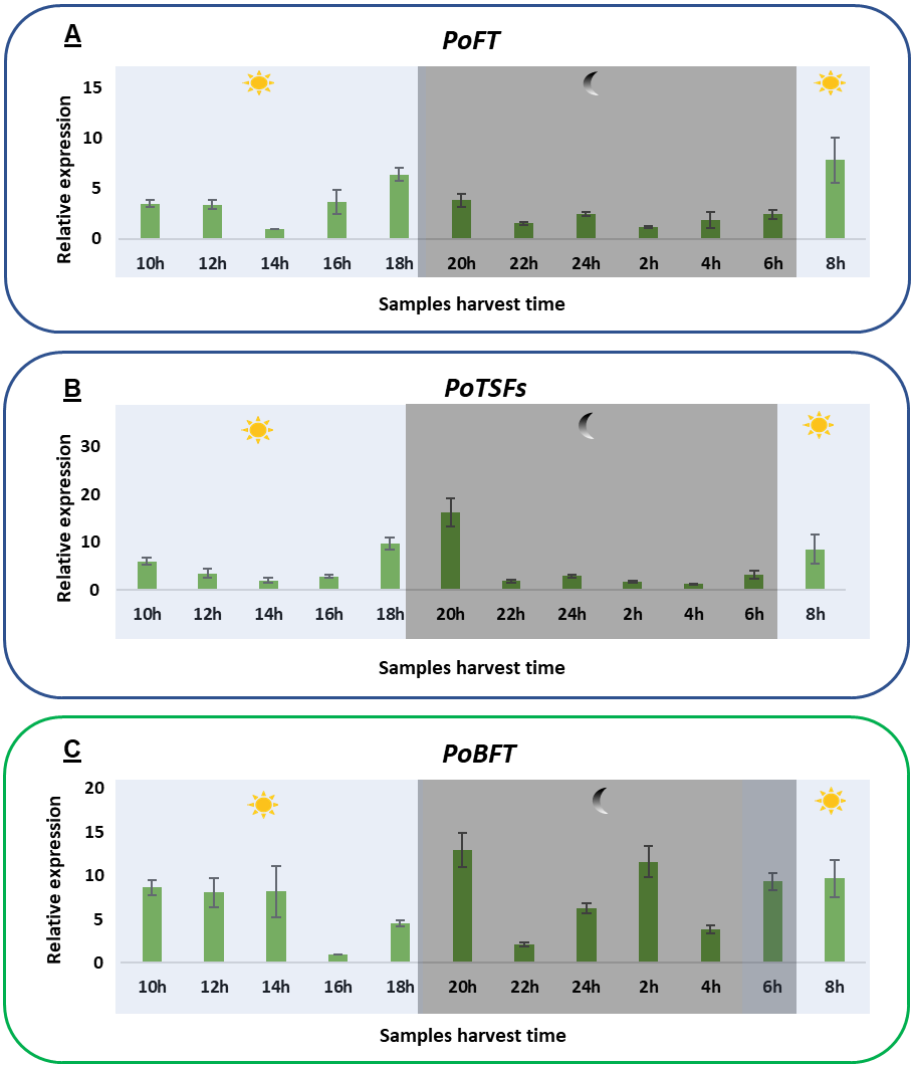


Figure 6 – **Relative expression of *PoFT*, *PoTSFs*, and *PoBFT* in leaves (nodes 16-17) and shoot apices of *Passiflora organensis*, during the Spring of 2016.** The samples were harvested during a period of 24h. In each figure the sample with lowest Ct value was used as the calibrator. Error bars indicate the standard deviation

Next, *in situ* hybridization was applied to detect the spatial and temporal expression patterns of *Passiflora FT/TFL1* genes. Almost no hybridization signal was detected for *PoFT* in the shoot apical meristems of juvenile plants (Figure 7A). *PoFT* transcripts were detected in the provascular tissues of the tendril primordium in the adult vegetative stage (Figure 7D). During reproductive development, stronger *PoFT* expression was detected in meristems (7 B;C;E) and in the SAM (Figure 7E). The transcripts of *PoTSFa* were detected very clearly in the SAM, axillary buds, and in the adaxial surface of the leaves of plants in the juvenile stage (Figure 7F-G). During adult development, expression was detected in the axillary buds and in the leaf primordia and young leaves (Figure 7I). In addition, the expression of *PoTSFa*

was absent in the center of the SAM (Figure 7I). In the reproductive stage, *PoTSFa* expression remained evident in the adaxial surface of the leaves and in the floral meristems (Figure 7J).

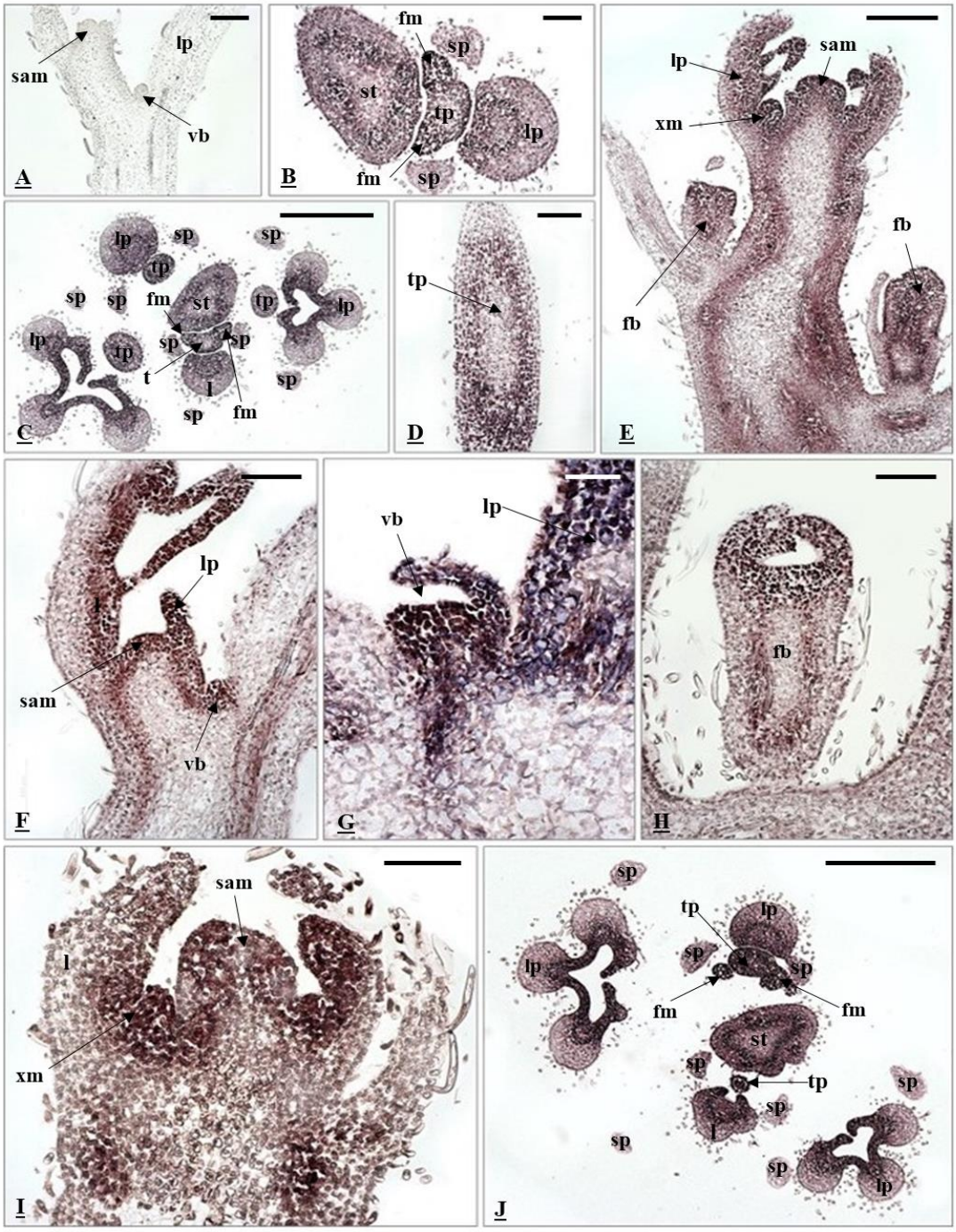


Figure 7 - ***In situ*** **hybridization patterns of *FT*-like genes in *Passiflora organensis*. A – E.** Expression patterns of *PoFT*. **F - J.** Expression patterns of *PoTSFa.* **A;D;E;F-I.** Longitudinal sections. **B-C;J.** Transverse sections. Axillary meristem (xm); floral bud (fb); floral meristem (fm); leaf primordium (lp); shoot apical meristem (sam); stem (st); stipule primordium (sp); tendril primordium (tp); vegetative bud (vb). Bars: A;E = 200 µm; B;D;F;H;I = 100 µm; C;J = 500 µm; G = 50 µm

The *TFL1*-like genes showed strong expression in the juvenile stage compared to the adult stage (Figure 8A-I). During juvenile development, *PoTFL1* transcripts were detected in the adaxial part of the leaves, axillary meristems and in the SAM (Figure 8A). However, during adult development, the level of *PoTFL1* transcripts decreased considerably in the central zone of the SAM (Figure 8B), and was almost undetectable in the reproductive stage (Figure 8C). *PoBFT* expression was detected in all analyzed tissues, mainly in the adaxial leaf primordium surface, axillary meristems and provascular tissue (Figure 8D). Then, there was a drastic reduction in hybridization signal in the adult tissues. It was restricted to the adaxial portion of the leaves and in the SAM, with a weak expression in tendril primordia (Figure 8E-F). Furthermore, *PoATC* transcripts were detected in the axillary buds and leaf primordium (Figure 8G) during the juvenile development. On the other hand, in the adult vegetative stage, its expression was restricted to provascular tissues and cortex, tendril primordium, and older leaves (Figure 8H). Afterwards, in the reproductive development, *PoATC* expression was extended to the adaxial surface of young leaves (Figure 8I).

The *PoMFT* expression was weak, and showed uniform distribution in all tissues during juvenile and adult stages (Figure 8J-L).

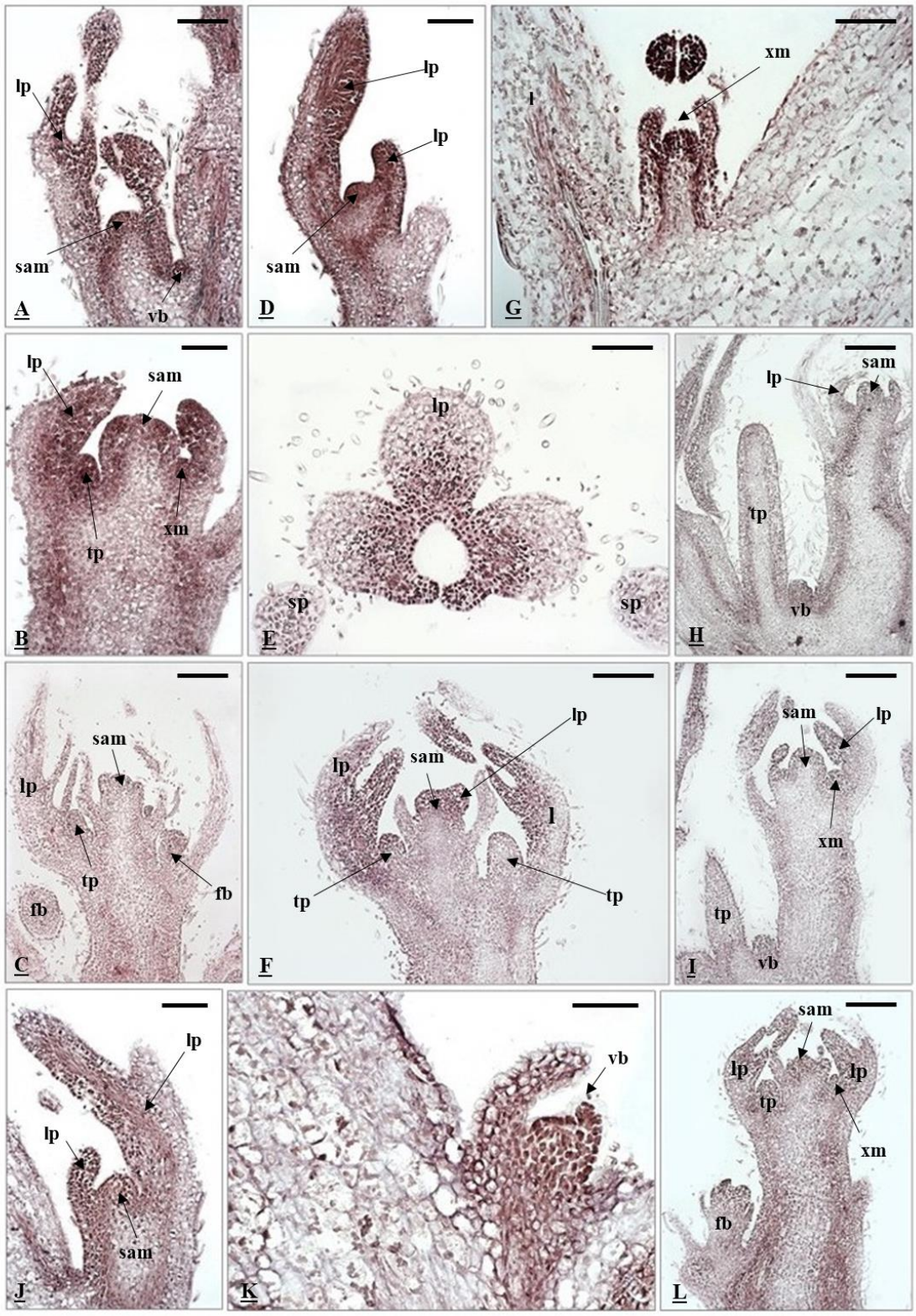


Figure 8 - ***In situ*** **hybridization patterns of *TL1*-like and *MFT*-like genes in *Passiflora organensis*.** **A – C.** Expression patterns of *PoTFL1*. **D - F.** Expression patterns of *PoBFT*. **G – I.** Expression patterns of *PoATC*. **J - L.** Expression patterns of *PoMFT*. **A-D;E;F-L.** Longitudinal sections. **E.** Transverse sections. Axillary meristem (xm); floral bud (fb); leaf primordium (lp); shoot apical meristem (sam); stipule primordium (sp); tendril primordium (tp); vegetative bud (vb). Bars: A-B;D-E;G;J = 100 µm; C;F;H;I;L = 200 µm; K = 50 µm

5.4 Discussion

In plants, *FT/TFL1* gene family acts as potential regulators of the transition from the vegetative to the reproductive phases and play important roles in plant architecture (Henrik et al., 2006; Corbesier et al., 2007; Higuchi, 2018; Moraes et al., 2019). Proteins encoded by these genes have been identified and studied in several plant species like grapevine, tomato, physic nut, rice, cotton, corn, barley, lily, tulip, among others (Carmona et al., 2007; Faure et al., 2007; Carmel-Goren et al., 2003; Tamaki et al., 2007; Danilevskaya et al., 2008; Li et al., 2015; Zhang et al., 2016; Leeggangers et al., 2018; Wu et al., 2019).

In this study, we identified and characterized eight members of the *FT/TFL1* gene family in *Passiflora organensis* and compared them to their putative orthologs in *Jatropha curcas,* which belongs to the Euphorbiaceae family, closely related to Passifloraceae within the Malpighialles, and in *Arabidopsis thaliana.*

The *P. organensis FT/TFL1* genes shared conserved primary structures (four exons and three introns) when compared to their *Arabidopsis* counterparts (Bradley et al., 1997). We further verified that this same structure is also observed in *Jatropha curcas* (Li et al., 2015), grapevine (Carmona et al., 2007), cucumber (Sato et al., 2009), *Lombardy poplar* (Igasaki et al., 2008), *Gentian* (Imamura et al., 2011), tobacco (Harig et al., 2012), and *Petunia* (Wu et al., 2019). This indicates that the structure of *FT/TFL1* family genes has been highly conserved during evolution. Additionally, their encoded putative proteins showed high amino acid sequence similarity with their putative orthologs in other species, suggesting a putative conservation of their biological roles as inducers and repressors of flowering.

Our results revealed that there are three *Passiflora FT* paralogs: *PoTSFa*, *PoTSFb*, and *PoTSFc*. Gene duplication generally leads to the retention of the original function in both copies, degeneration of one of the copies to form a pseudogene, or more frequently, to either neo- or subfunctionalization (Conant; Wolfe, 2008; Innan; Kondrashov, 2010; Kaessmann, 2010). Taking into account that the expression patterns of the *PoFT*, *PoTSFa,* and *PoTSFs* were different during *Passiflora* development, it appears that, in this case, the gene duplication resulted in either neo- or subfunctionalization. Further functional studies are necessary to further discriminate between these alternatives. According to the expression pattern results, *PoFT* acts during the adult vegetative phase, while *PoTSFs* during the adult reproductive phase, thus together they would play an important role in flowering time. Furthermore, these observations, together with the *in situ* hybridization data, provide

evidence that *PoFT-like* expression could be involved in the regulation of phase transition and flower development in *Passiflora*.

However, unlike the PoFT protein, that displayed the conserved amino acids residues, Tyr84 and Gln139, important for the function of an inducer of flowering, the three *Passiflora* TSF paralog proteins did not display the conserved amino acid Gln139. Instead, they displayed His139. It is not yet known whether a single base change (Gln139 to His139) could cause a change in the function of *Passiflora* TSF proteins. Moreover, the evolution of FT paralogs might represent a common strategy in plants to refine floral initiation according to multiple environmental and endogenous pathways intrinsic to each individual. Some examples of how *FT/TFL1* gene duplication might contribute to plant evolution will be described in the next chapter.

The three members of the *Passiflora TFL1*-like subfamily exhibited the same expression patterns: they were highly expressed in the juvenile vegetative phase. The qRT-PCR data agreed with the *in situ* hybridization results. FT and TFL1 have antagonistic functions in plant development. While FT activates the flowering pathway, being considered the florigen agent, TFL1 represses flowering, being responsible for the maintenance of the inflorescence meristem (Wickland; Hanzawa, 2015). Accordingly, our results show that when *PoFT* expression is the highest, *PoTFL1* expression is the lowest, and vice versa, confirming their putative antagonistic roles. Furthermore, Hanzawa and collaborators (2005) showed that in *Arabidopsis* a single base change in specific amino acid residues (Tyr85 and Gln140 in FT protein and His88 and Asp144 in TFL1 protein), could cause conversion of FT to TFL1 functions and vice versa. In *P. organensis*, PoTFL1, PoBFT and PoATC bear the conserved His and Asp residues in similar positions to the *Arabidopsis* TFL1, suggesting that the *P. organensis* orthologs would perform anti-florigen functions, thus repressing flowering.

In *Arabidopsis,* it has been recently reported that MFT mediates seed germination (Xi et al., 2010). However, it has also been reported that MFT has a weak flowering-promoting activity in *Arabidopsis* (Yoo et al., 2004). PoMFT displayed the conserved amino acid residues tryptophan and proline that are important for classifying the protein as a member of the MFT-like subfamily. On the other hand, *P. organensis MFT* showed the highest expression levels during the juvenile phase with a decrease in its transcripts during phase transition. In addition, our *in situ* hybridization data showed that the level of *PoMFT* transcripts were low and slightly constant in all analyzed tissues during juvenile and adult stages. Therefore, a flowering inducing role for PoMFT seems unlikely due to a weak data support.

Another important aspect is the fact that plants sense light and temperature changes to regulate flowering time. In *Arabidopsis* the main player in photoperiod perception is CONSTANS (CO) and under long-day conditions the CO protein is stabilized and promotes the expression of *FT* mRNA resulting in floral transition. With this in mind, we analyzed the expression patterns of *PoFT*, *PoTSFs*, and *PoBFT* during a period of 24h. *PoTFL1*, *PoATC*, and *PoMFT* were not included in this analysis because they were not expressed in leaf tissues. In *Arabidospis, FT* and its paralog *TSF* showed a bimodal expression pattern, similar to what was observed in *Passiflora*, with peaks in the morning and around dusk (Song et al., 2018). It was shown in *Arabidopsis* that key light signaling components such as PHYTOCROME A and EARLY FLOWERING 3, play important roles in morning FT expression. These conditions stabilize CONSTANS protein, a major FT activator, in the morning, which is probably a critical mechanism for photoperiodic flowering in nature (Song et al., 2018).

Besides that, to perform their biological functions, FT/TFL1 proteins may need to interact with other proteins. The bZIP transcription factors and 14-3-3 proteins may be important interactors during the flowering transition, and TCP transcription factors may be important to regulate plant architecture and branching (Niwa et al., 2013; Jang et al., 2017). Taking this into account, we will access the protein-protein interactions among these elements in Chapter V.

The results showed here demonstrate that the *P. organensis FT/TFL1* family members characterized present conserved structural and sequence features. This indicates that the *Passiflora* orthologs of important molecular players controlling plant phase transition and flowering, such as FT and TFL1 were uncovered. Addionally, their expression patterns help us to elucidate the biological functions of FT/TFL1 family members in *P. organensis* and might provide useful information on how to improve passionfruit production in the future.

5.5 Conclusions

In summary, we isolated eight members of the *FT/TFL1* family, and analyzed their expression patterns during the juvenile, adult vegetative and adult reproductive phases. These genes were classified into *FT*-like, *TFL1*-like and *MFT*-like subfamilies. Then, we found that *TSF* has a duplication event in *P. organensis* genome. We showed that *Passiflora FT*-like genes have higher expression during the adult stage, while *TFL1*-like genes showed higher expression in the juvenile stage. We also showed that the proteins encoded by these genes diplay the important conserved amino acids residues for the biological function of flowering

inducers (FT-like subfamily, except PoTSF) and repressors (TFL1-like subfamily). Moreover, this study demonstrated that *Passiflora FT*-like genes, such as *FT* and *TSF*, are able to respond to photoperiod. Our observations on the expression patterns of *FT/TFL1* family members in *Passiflora* provide a new insight into the functional evolution of this gene family. Moreover, the identification and characterization of all these genes will be important for analyzing the protein-protein interactions of FT/TFL1, bZIP, 14-3-3, and TCP proteins, which we will discuss in the Chapter V.

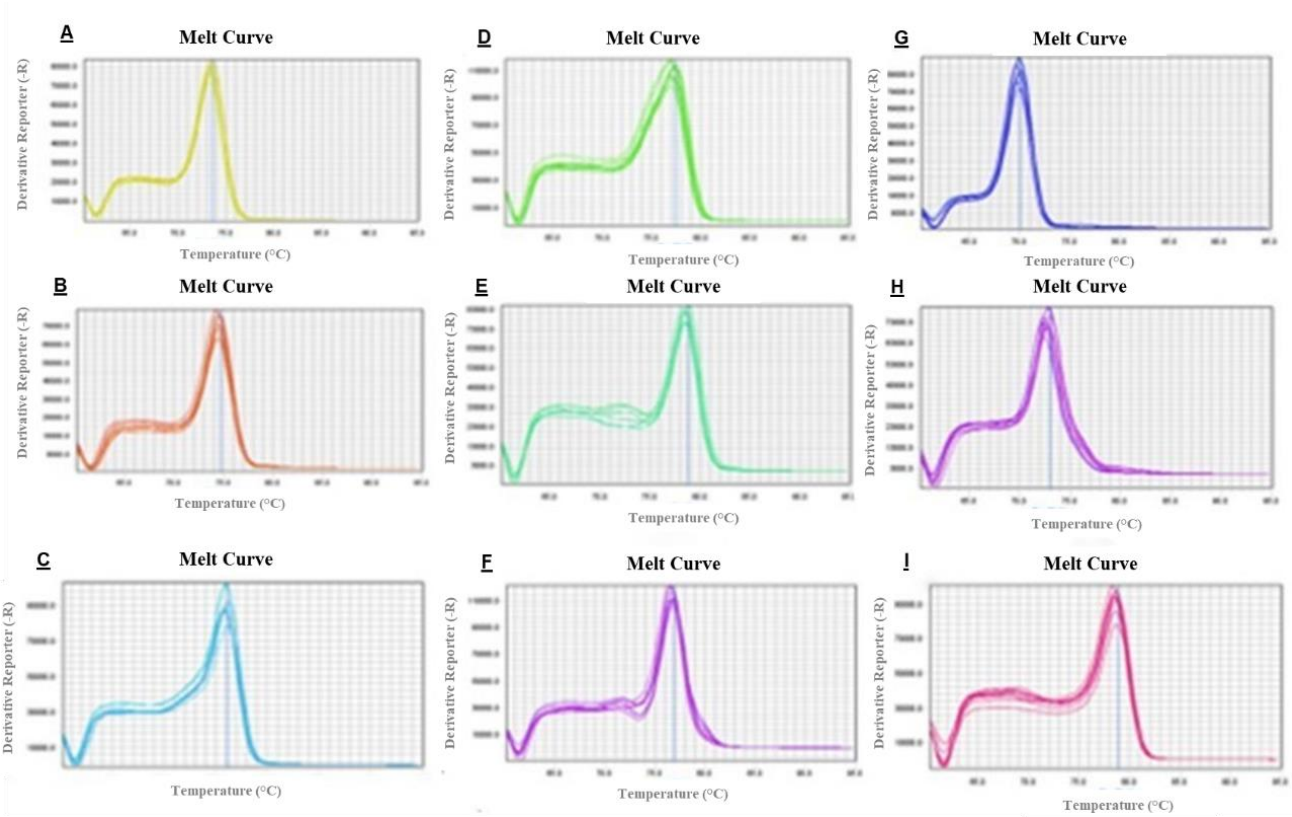
5.6 Supplementary Material

S1 - Primers used for qRT-PCR analyses

Primer	**Sequence**	**Amplicon (pb)**	**Annealing Temperature (°C)**
PoFT - F	GCCTGTGGCTGCACTTTACT	139	60
PoFT - R	GCGCTTCATCTTCGGGAAT		
PoTSFa - F	TGGCCGCTGTTTACTTCAAT	95	60
PoTSFa - R	GAGGCTGGCTGGCTGGTT		
PoTSFs - F	GCCCGCATCCAGTAGCTG	167	60
PoTSFs - R	CTGGCAATTGAAGTAAACAGCG		
PoTFL1 - F	GTGACCACACCATCGTCAAG	168	60
PoTFL1 - R	CAACCCTGCTGCTGATTTTT		
PoBFT - F	CTGCTGCGAGAAGGAGATGA	109	60
PoBFT- R	AACCCATACCCAACCCTAGC		
PoATC - F	CGTTGAGCTAGCAACGAACA	83	60
PoATC - R	TTGATGGACAACGCAAAGAG		
PoMFT - F	AGGCTCTTTGCTGCACATCT	119	60
PoMFT - R	CCCAATCCCATCGTCAATTACT		
PeCAC - F	TCAAGAGGGAGTGCGTTGAC	90	60
PeCAC - R	CAACCAACAGCGCCTGTAAC		
PeSAND - F	GGAGCTGCTTCTCCCCATTT	78	60
PeSAND -R	AGGGCCACCAATTCCAATGA		

F = *forward;* R = *reverse*

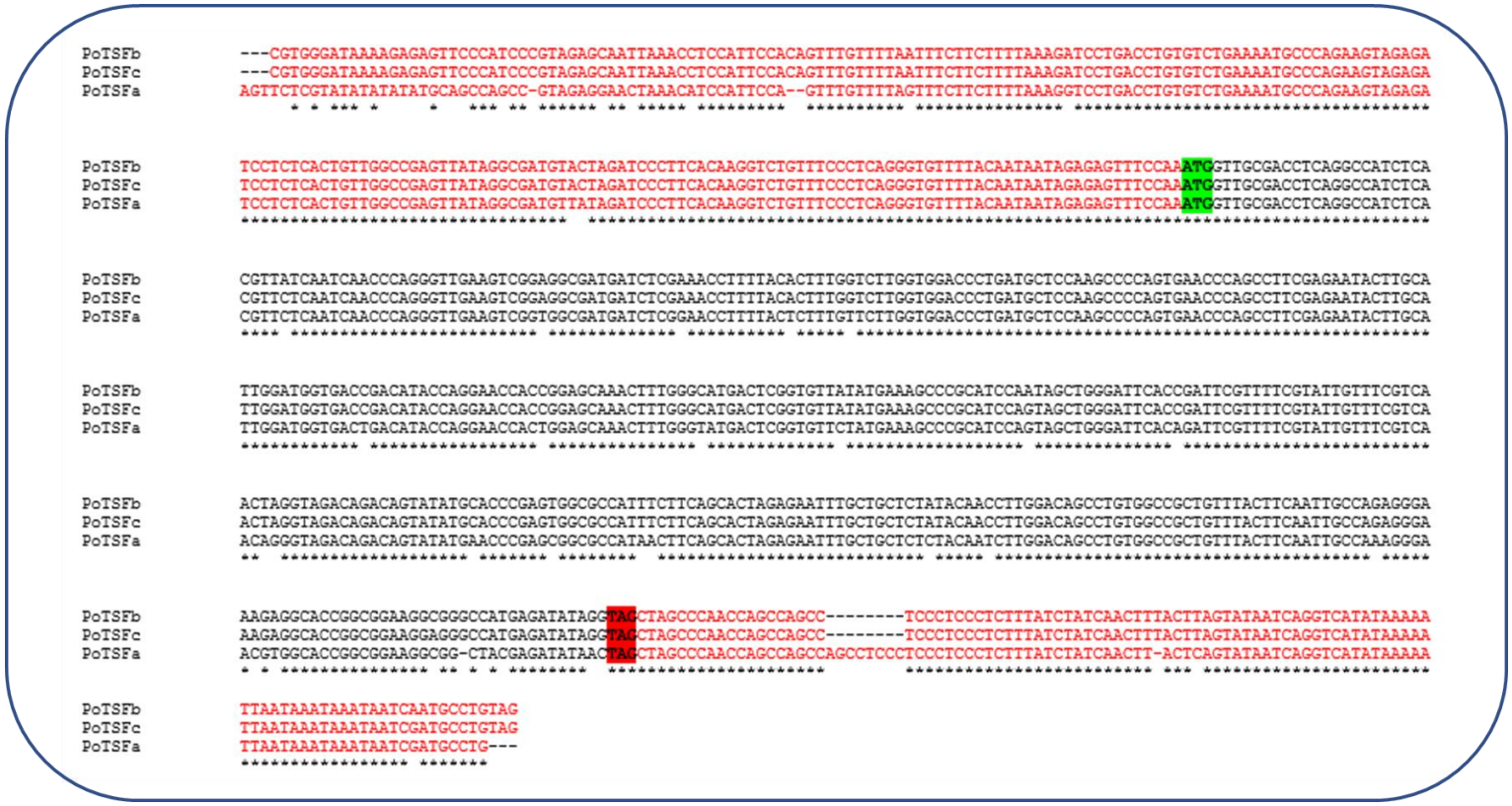
S2 - Melting curve in the RT-qPCR reaction. (A) PeSAND; (B) PeCAC; (C) PoTFL1, (D) PoFT; (E) PoTSFa; (F) PoTSFs; (G) PoBFT; (H) PoATC; (I) PoBFT; and (J) PoMFT



S3 – Species used to build the FT/TFL1 phylogenetic tree

AaTFL1: *Arabis alpina*
AcFT: *Allium cepa*
AcFT1*: Allium cepa*
AcFT2*: Allium cepa*
AcFT4*: Allium cepa*
AdCEN: *Actinidia deliciosa*
AtATC: *Arabidopsis thaliana*
AtBFT: *Arabidopsis thaliana*
AtFT: *Arabidopsis thaliana*
AtMFT: *Arabidopsis thaliana*
AtTFL1: *Arabidopsis thaliana*
AtTSF: *Arabidopsis thaliana*
BvFT1: *Beta vulgaris*
BvFT2: *Beta vulgaris*
CEN: *Antirrhinum*
CgFT: *Cymbidium goeringii*
CiFT: *Citrus unshiu*
Cm-FTL1: *Cucurbita moschata*
Cm-FTL2: *Cucurbita moschata*
CsAFT: *Chrysanthemum seticuspe*
CsFTL3: *Chrysanthemum seticuspe*
CsTFL: *Chrysanthemum seticuspe*
DIFT1: *Dimocarpus longan*
DIFT2: *Dimocarpus longan*
Dt1: *Glycine max*
FcFT1: *Ficus carica*
FTL1: *Brachypodium distachyon*
FTL2: *Brachypodium distachyon*
HvFT2: *Hordeum vulgare*
HvFT3: *Hordeum vulgare*
InFT1: *Ipomoea nil*
InFT2: *Ipomoea nil*
JcFT: *Jatropha curcas*
JcMFT1: *Jatropha curcas*
JcMFT2: *Jatropha curcas*
JcTFL1a: *Jatropha curcas*
JcTFL1b: *Jatropha curcas*
JcTFL1c: *Jatropha curcas*
Kiwifruit FT: *Actinidia chinensis*
Ljcen1: *Lotus japonicus*
LpTFL1: *Lolium perenne*
LsFT: *Lactuca sativa*
MdCEN: *Malus domestica*
MdCENa: *Malus domestica*
MdFT1: *Malus domestica*
MdFT2: *Malus domestica*
MdTFL1-1: *Malus domestica*
MdTFL1-2: *Malus domestica*
MtFTa1: *Medicago truncatula*
MtFTb1: *Medicago truncatula*
MtFTc: *Medicago truncatula*
NtFT: *Narcissus tazetta*
NtFT1: *Nicotiana tabacum*
NtFT2: *Nicotiana tabacum*
NtFT3: *Nicotiana tabacum*
NtFT4: *Nicotiana tabacum*
PoATC: *Passiflora organensis*
PoBFT: *Passiflora organensis*
PoFT: *Passiflora organensis*
PoMFT: *Passiflora organensis*
PopCEN1: *Populus trichocarpa*
PopCEN2: *Populus euphratica*
PopMFT: *Populus trichocarpa*
PoTFL1: *Passiflora organensis*
PoTSFa: *Passiflora organensis*
PoTSFb: *Passiflora organensis*
PoTSFc: *Passiflora organensis*
PpTFL1: *Pyrus pyrifolia*
PpTFL1-1: *Pyrus pyrifolia*
PpTFL1-2: *Pyrus pyrifolia*
PsFTa1: *Pisum sativum*
PsFTa2: *Pisum sativum*
PsFTb1: *Pisum sativum*
PsFTb2: *Pisum sativum*
PsFTc: *Pisum sativum*
PSTFL1A: *Pisum sativum*
PsTFL1c: *Pisum sativum*
PtFT1: *Populus tomentosa*
PtFT2: *Populus tomentosa*
PvTFL1y: *Phaseolus vulgaris*
RCN1: *Oryza sativa*
RCN2: *Oryza sativa*
RFT1: *Oryza sativa*
RoFT: *Rosa chinensis*

S4 - Nucleotide sequences alignment of *PoTSFa*, *PoTSFb*, and *PoTSFc* genes. The red sequences indicate the 3' and 5' UTR regions. The start and stop codons are indicated by the green and red boxes, respectively. The coding region is represented between the start and stop codons



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CHAPTER III

FT/TFL1: Calibrating Plant Architecture

Passiflora organensis – juvenile stage

6. FT/TFL1: Calibrating Plant Architecture[1]

Abstract

There is a very large diversity of plant architecture in nature. Over the past few years novel theoretical concepts and analysis methods have proven to be powerful tools to reveal important aspects of plant architecture. Plant architecture basically depends on the relative arrangement of three types of organs: leaves, shoots and flowers. In plant development the architecture is modulated by the balance of two homologous proteins: FLOWERING LOCUS T (FT) and TERMINAL FLOWER 1 (TFL1). The FT/TFL1 balance contributes to define the plant growth (indeterminate or determinate), modulating the pattern of formation of vegetative and reproductive structures in apical and axillary meristems. Here, we present a summarized view about plant architecture, mainly focusing on the *FT/TFL1* balance and suggest that species of *Passiflora* can be a valuable model to study *FT/TFL1* genes.

Keywords: Axillary meristems. Branching. Flowering. Gene duplication. Passionfruit. PEBP.

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6.1 Introduction

Advances in our understanding of plant architecture has increased in the last few decades and research in this field has given rise to innovations in various aspects of plant science. High-performance computers have become available for plant growth data analysis and simulation, triggering the development of various representations of plant architecture (Kuchen et al., 2012; Coen et al., 2017; Whitewoods; Coen, 2017).

Plant architecture is based on the number and arrangement of organs that are formed from the shoot apical meristem (SAM) (Benlloch et al., 2007). During the vegetative stage the SAM gives rise to shoots and leaves, however, after floral transition, in the reproductive stage, plants produce flowers (Benlloch et al., 2007).

In the annual model plant *Arabidopsis thaliana* the growth habit is monopodial, as the apical meristem is indeterminate and active throughout the plant's lifetime (Bowman et al., 1994). Thus, the formation of branches, leaves, and flowers are clearly lateral, resulting in a clear distinction between the vegetative and reproductive stages (Bradley et al., 1997).

Perennial plants differ from annual herbaceous plants such as *Arabidopsis* because they have different characteristics, which influence the growth pattern and consequently the plant architecture. The branching habit of a perennial plant is even more complex because axillary meristems can have multiple fates, either forming a shoot without a dormant period or, after a dormant period, developing into a floral bud that opens the following spring, or remaining dormant indefinitely. In addition, in perennial plants the SAM is maintained with a high vegetative level, or "vegetativeness" (Prusinkiewicz et al., 2007). According to these authors, depending on its "vegetativeness" the meristem will form a flower or a branch, while a high "vegetativeness" will correspond to a indeterminate shoot meristem identity, a determinate floral meristem identity corresponds to low levels of "vegetativeness" (Prusinkiewicz et al., 2007).

Plant architecture is controlled by genetic mechanisms associated to environmental factors, and it largely depends on meristem identity, which in turn establishes the development of shoots or flowers. Genetic mechanisms controling meristem identity have been studied in detail in *Arabidopsis* and it is known that plant architecture is controlled by a few groups of genes (Bradley et al., 1997; Conti; Bradley, 2007; Ho; Weigel, 2014). Among these, we can highlight *FLOWERING LOCUS T* (*FT*) and *TERMINAL FLOWER 1* (*TFL1*) that belong to the *FT/TFL1* gene family and encode proteins with similarities to phosphatidylethanolamide binding proteins (PEBP) (Wickland; Hanzawa, 2015). A balance

between these two homologous proteins, FT and TFL1, involved in controlling indeterminate and determinate plant growth, modulates plant architecture, reflecting the pattern of production of vegetative and reproductive organs in the apical meristem (Park et al., 2014).

In this way, here we aim to present an updated view on plant architecture and modulation of axillary meristems, mainly focusing on *FT/TFL1* genes, presenting new discussions about the current knowledge in this field and the possible implications and perspectives concerning plant architecture in plant developmental studies.

6.2 Effects of *FT/TFL1* balance in annual plants (*Arabidopsis*)

In *Arabidopsis* six genes of the *FT/TFL1* family have been identified*: FLOWERING LOCUS T* (*FT*) and *TWIN SISTER OF FT* (*TSF*), involved in flowering promotion and belonging to the *FT*-like subfamily; *TERMINAL FLOWER 1* (*TFL1*), *BROTHER OF FT AND TFL1* (*BFT*) and *ARABIDOPSIS THALIANA CENTRORADIALIS HOMOLOG* (*ATC*), involved in flowering repression and belonging to the subfamily *TFL1*-like; and *MOTHER OF FT AND TFL1* (*MFT*), belonging to *MFT*-like subfamily and involved in regulation of seed germination (Kobayashi et al., 1999; Xi et al., 2010; Wickland; Hanzawa, 2015).

FT and TFL1 have antagonistic functions in plant development. While FT activates the flowering pathway, being considered the florigen agent, TFL1 represses flowering, being responsible for the maintenance of the inflorescence meristem. The FT/TFL1 balance modulates the architecture of the plant because they are involved in the control of the indeterminate versus determinate plant growth habit, which is essentially based on the pattern of production of vegetative versus reproductive organs by the apical meristem (Matsoukas et al., 2012; Xu et al., 2012; Jaeger et al., 2013; Nakano et al., 2015; Patil et al., 2018).

In *Arabidopsis*, the transcription factor CONSTANS (CO) activates *FT* in the leaves, where it is transcribed and translated, and its protein is then transported via phloem to the vegetative apex. At the apex the FT protein forms a complex with a bZIP protein, FLOWERING LOCUS D (FD), resulting in the induction of flowering by activating genes involved in floral meristem identity, such as *LFY* and *APETALA1* (Abe et al., 2005). The *ft* mutants flower late and present indetermined growth, whereas the overexpression of *FT* causes early flowering and conversion of the SAM into a terminal flower (Cobesier et al., 2007). In contrast, *TFL1* is expressed in the SAM maintaining the indeterminate growth and repressing the floral meristem identity genes (the TFL1 protein is also capable of interacting with the FD transcription factor). Thus, *tfl1* mutants flower early and their SAM is converted

into a terminal flower. On the other hand, overexpression of *TFL1* causes late flowering and prevention of terminal flower formation (Bradley et al., 1997).

6.3 Effects of *FT/TFL1* balance in perennials (tomato)

In tomato (*Solanum lycopersicum)* the balance between *FT* and *TFL1* orthologs *SINGLE FLOWER TRUSS* (*SFT*) and *SELF-PRUNING* (*SP*), respectively, coordinate primary growth with regular sympodial cycles. A high *SFT/SP* ratio in the meristem promotes determinate growth with an eventual conversion of the SAM into a flower, while a low *SFT/SP* balance promotes indeterminate plant growth (Pnueli et al., 1998; 2001; Lifschitz et al., 2014).

Studies have shown that *sft* mutations may increase the productivity of tomato plants through a determinate growth habit (Park et al., 2014). In *sft* mutants, loss of florigen activity results in highly vegetative plants with few flowers and fruits. However, when plants with determinate growth are heterozygous for the *sft* mutation, there is a partial reduction of florigen activity, with a slight supression of *SP*, resulting in more sympodial branches and inflorescences. On the other hand, when *SP* is present as a dominant allele, plants show indeterminate growth and continuous production of inflorescences and fruits. However, when the tomato plants have a recessive allele for this gene, they exhibit a specific architecture characterized by an early interruption of inflorescence production and shorter plant stature (Pnueli et al., 1998; 2001; Jiang et al., 2013). These results suggest that *sft* and *sp* mutations combined with heterozygous dosage effects, should be further explored to modulate flowering and plant architecture to optimize tomato yields.

6.4 How *FT/TFL1* gene duplication contributes to the evolution of plant architecture

Gene duplication, that gives rise to paralogous genes, is a very common phenomenon in plants and an important source of new adaptive functions prone to selection during evolution (Kondrashov et al., 2002). Some gene pairs formed by duplication might have a short lifetime, only one copy might be kept functional while the other copy becomes pseudogenized. However, some gene pairs might persist after duplication. Paralogous proteins may give rise to new functions through mutations that affect, for example, gene expression or amino acid sequences, which may result in different phenotypes that arise through adaptive evolution of new protein functions (Linch; Conely, 2000).

Apparently, during evolution some *FT* homologous genes acquired the function of suppressing flowering. In some species, there is an *FT* with a repression function that antagonizes the flowering induction function of its paralog (Pin et al., 2010; Kotoda et al., 2010; Hsu et al., 2011; Harig et al., 2012). It is of great significance that the evolution of FT paralogs might represent a common strategy in plants to refine floral initiation according to multiple environmental and endogenous pathways intrinsic to each individual.

In *Beta vulgaris*, the regulation of flowering time is controlled by BvFT1 and BvFT2, which are orthologous to the *Arabidopsis* FT protein (AtFT). These genes are involved in response to flowering at low temperatures during winter, associated with the phenomenon of vernalization. However, these two paralogous genes in beet have antagonistic functions. While BvFT2 is functionally conserved with AtFT, being essential for flowering (expressed at late afternoon, in long days), BvFT1 represses flowering (preferentially expressed early in the morning, in short days) (Pin et al., 2010). Pin *et al* observed that both proteins, BvFT1 and BvFT2, contain amino acids that determine the FT function (Tyr85 and Gln140). However, the binding of specific residues at the external loop of the tertiary structure showed differences between the two proteins. Thus, these authors suggest that BvFT1 was initially a promoter of flowering, but that mutations within the outer loop of the protein resulted in a change in function towards the repression of flowering.

Similarly, in *Populus trichocarpa* two FT homologs proteins are required to coordinate the recurrent seasonal flowering cycle in response to temperature (Hsu et al., 2011). PtFT2 is involved in the vegetative growth. It is activated by high temperatures and long photoperiods during spring and summer. On the other hand, PtFT1, which activates reproductive growth, is repressed by high temperatures and induced by low temperatures during the winter.

Again, in *Nicotiana tabacum*, three out of four FT homologs repress flowering. Harig et al. (2012) found out that all four genes were expressed in leaves under short-day conditions, and at least *NtFT3* expression was restricted to phloem companion cells. Nevertheless NtFT1, NtFT2, and NtFT3 proteins are floral inhibitors, whereas only NtFT4 is a floral inducer (Harig et al., 2012).

In contrast, although *TFL1* gene duplications have also been described in the literature (Carmona et al., 2007; Li et al., 2015), it is still unclear which would be the specific function of each paralog, with no reports on *TFL1* paralogs possessing an antoganistic function, such as the activation of flowering (Carmona et al., 2007; Li et al., 2015).

6.5 Modulation and complexity of axillary meristems

The axillary meristems (AMs) are important elements in establishing plant architecture and their reproductive success (Wang; Jiao, 2018). The flexibility of the AM activity is directly related to the FT/TFL1 balance (McGarry; Ayre, 2012).

In summary, a plant with a high FT/TFL1 ratio flowers early and presents a short stature, as its apical meristem is converted into a terminal flower. As this ratio decreases, the vegetative level, or "vegetativeness", increases and the plants produce fewer flowers, and as *FT* is repressed the plant considerably increases its vegetative growth (Figure 1).

In most annual plants, the SAM remains indeterminate while the axillary meristems are determinate. Thus, the SAM gives rise to the vegetative meristem, when the FT/TFL1 ratio is low. As the plant ages, the FT transport increases because there are more leaves contributing to the FT pool and, at the apex, the effects of accumulated FT exceed the TFL1 function. As a result, a transition from a vegetative to a reproductive meristem is observed and, subsequently, the plant life cycle is completed. In contrast, perennial plants present high levels of TFL1 at the SAM, which remains vegetative, while in the axillary meristems the FT level prevails, activating genes involved in floral meristem identity (McGarry; Ayre, 2012).

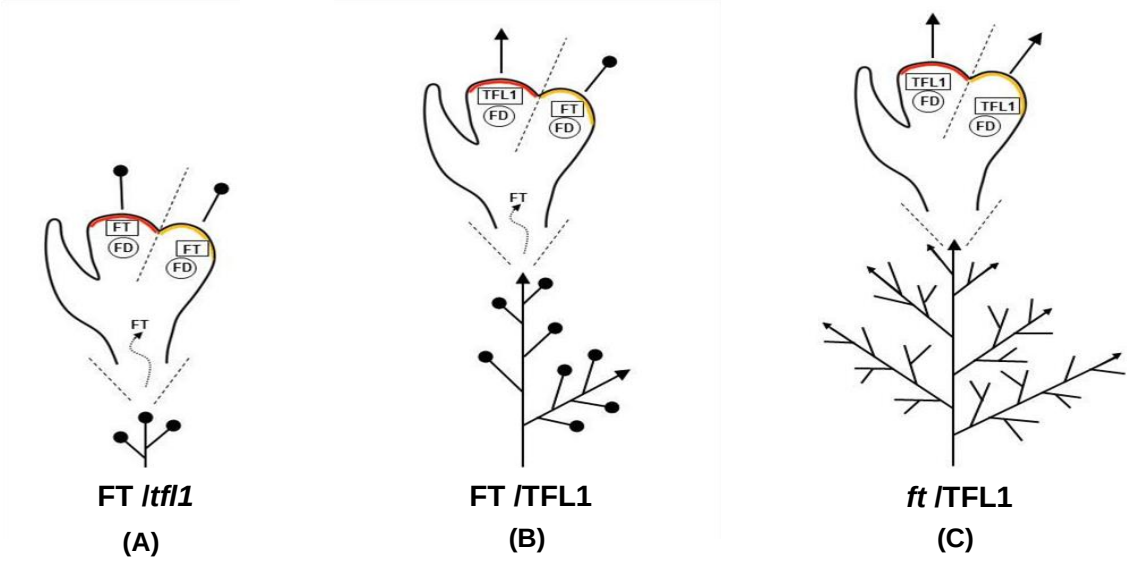

Figure 1 – **Representation of changes in plant architecture according to FT/TFL1 balance**. FT and TFL1 can compete for FD binding. (A) Plant with high FT/TFL1 ratio flowers early and with a short stature, because its apical and axillary meristems are each converted into a flower. (B) Moderate ratio of FT/TFL1 allows the balance between shoots and flowers through the axes of the plant. (C) A low FT/TFL1 ratio increases the plant vegetative growth and its apical and axillary meristems give rise to shoots. A red region represents the shoot apical meristem (SAM), an orange region represents the axillary meristem (AM), arrows represent indeterminate meristems and circles represent flowers

In *Arabidopsis* the protein encoded by the gene *BRANCHED1* (*BRC1*) interacts with FT modulating its activity in the axillary buds to repress the premature floral transition of axillary meristems (Hiraoka et al., 2013; Niwa et al., 2013). BRC1 also known as TCP18 is a member of TCP family, a plant-specific family of transcription factors, that is involved in a large variety of developmental processes like cell proliferation and growth, manly in meristems and lateral organs, through these processes, it is involved in the establishment of plant form and architecture (Aggarwal et al., 2010; Manassero et al., 2013).

In perennial plants, for example lianas, a woody climbing vines abundant in tropical forests, the growth habit might be different. The acquisition of the climbing habit constitutes a innovation and its successful in climbers is correlated with the development of specialized structures, such as tendrils. Lianas begin their life on the floor but depend on trees for support as their climb upwards the sunlight, required for light competition and survival. Thus, their SAM have indeterminate vegetative growth and repressing the development of AMs making it possible to reach the forest canopy (Rodriguez-Ronderos et al., 2016; Sousa-Baena et al., 2018).

The *Arabidopsis* AMs are simple in comparison to AMs in other families, such as *Vitaceae* and *Passifloraceae*. The presence of additional accessory meristems that give rise to tendrils or inflorescences are a special feature of *Vitaceae*. In grapevine, *Vitis* spp., a woody perennial vine, adult plants have special AMs called uncommitted lateral meristems (UCMs). The UCMs become opposed to leaves in the expanded shoot giving rise to tendrils for a long period of time before the plant initiates flowering. However, upon flowering induction, the Inflorescences are formed in place of tendrils from the same UCMs (May, 2004; Carmona et al., 2008). In *Passiflora* species AMs acquire different features during life stages. Taking the passinfruit (*P. edulis*) as an example, the AMs of juvenile plants will give rise to a vegetative meristem. Adult vegetative plants will have AMs producing a tendril next to a vegetative meristem and, finally, adult reprodutive plants will have besides the vegetative meristem an AMs that will divide in two domes to form tendrils and flowers, simultaneously (Ulmer; Macdougal, 2004; Dornelas et al., 2006; Cutri et al., 2013).

We therefore suggest that species of *Passiflora* might be a valuable model to study *FT/TFL1* balance in order to understand how AM modulation gives rise to different structures, considering that passionfruit AMs are predicted to be more complex in comparison with AMs from another species.

6.6 Conclusion

The balance between *FT/TFL1* orthologous genes is important for the adaptation of plants to diverse environmental conditions. It is notable that domestication of several wild and exotic species into agronomic cultures with specific growth habits results from a selection of a differential balance between *FT/TFL1*. Thus, studies characterizing the interaction between these genes becomes an important tool for plant breeding programs of plants of commercial interest, since the ability to modulate plant size might allow increasing planting density, facilitate fruit harvest, raise crop productivity, among other agronomic benefits.

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CHAPTER IV

Identification of floral identity genes

LEAFY and *APETALA1* in *Passiflora organensis*

Passiflora organensis – flower

7. Identification of floral identity genes *LEAFY* and *APETALA1* in *Passiflora organensis*

Abstract

Flowering is a vital component of the plant life cycle and fundamental for reproductive fitness and adaptation, as well as for crop production. The growth of shoots and the initiation of flowering depend on the status of stem cells in a region of the shoot apical meristem. Various endogenous and exogenous cues are integrated by a complex molecular network responsible for the transition from vegetative to reproductive development. Many floral integrator genes are involved in this molecular network, which includes the floral meristem identity genes *LEAFY* (*LFY*) and *APETALA1* (*AP1*), to initiate the growth of floral meristems. Here, we identified and characterized the putative *LFY* and *AP1* orthologous genes of *Passiflora organensis* (*PoLFY* and *PoAP1*, respectively), a small herbaceous vine, from the same genus as the passionfruit, and studied their pattern of expression during plant development. We characterized the *PoLFY* and *PoAP1* gene structures and their phylogeny based on their expected encoded protein sequences. The expression patterns of *PoLFY* and *PoAP1* in juvenile, adult vegetative, and adult reproductive phases were examined by quantitative real-time PCR (qRT-PCR). We showed that the expression of the *PoLFY* gene increased during the plant lifetime, while the *PoAP1* gene increased its expression in the adult vegetative phase, however, with a considerable decrease in its level of expression during the adult reproductive phase. The results of this study will contribute to understand the potential molecular roles of *PoLFY* and *PoAP1* during the transition to the reproductive development in *P. organensis*. The identification of the molecular mechanisms underlying flower development might additionally help breeding more productive passionfruit.

Keywords: Flowering. Floral meristem. MADS-box. Passionfruit. Transcription factor.

7.1 Introduction

During the transition from vegetative to reproductive development the meristems are affected by external and endogenous factors (Wellmer and Riechmann, 2010). Many floral integrators, such as FLOWERING LOCUS T (FT) and SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1), are involved in the molecular mechanisms that control flowering in plants (Wils; Kaufmann, 2017). They activate directly or indirectly the floral meristem identity genes *LEAFY* (*LFY*) and *APETALA1* (*AP1*), to initiate the growth of floral meristems (Lee; Lee, 2010).

LFY is a plant-specific transcription factor (TF) that plays important roles in flower development and activates the transcription of the *AP1* gene (Coen et al., 1990; Weigel et al., 1992). In addition, AP1 is a MADS-box TF that functions as an A-class gene involved in the identity of sepals and petals (Litt and Kramer, 2010). Constitutive expression of *LFY* is sufficient to convert both apical and lateral meristems into terminal flowers (Weigel; Nilsson, 1995), while *lfy* mutant shows partial loss of flower meristem identity, partially converting flowers into shoots (Weigel et al., 1992). In *ap1* mutants, petals are not formed, and sepals are transformed into bract-like organs. In the axil of these bract-like organs, axillary floral meristems appear producing secondary flowers, partially converting flowers into inflorescences (Irish; Sussex, 1990; Bowman et al., 1993). Genetic evidence suggests that both *LFY* and *AP1* confer floral identity to the meristem. The *lfy ap1* double mutants showed a much more severe effect in flower formation than in either of the single mutants (Weigel et al., 1992).

Some information is already available for partially sequenced *Passiflora LFY* and *AP1* putative ortologs. Cutri (2009) showed that the putative ortholog of the *Arabidopsis thaliana* gene *LEAFY* is expressed in axillary meristems and tendril primordia in both *Passiflora edulis* and *Passiflora suberosa*, Additionally, Hernandes-Lopes et al. (2019) showed that *P. edulis* and *P. suberosa*, *AP1* homologs, are expressed during tendril development. Likewise, Scorza et al. (2017) showed that a putative *P. edulis* ortolog of *AP1* was expressed during tendril development and also detected in meristematic regions, in sepals, and in petals.

Assuming *Passiflora organensis* as a model species for *Passiflora* developmental studies, here we identified and characterized *PoLFY* and *PoAP1* genes and investigated their expression patterns throughout the juvenile, adult vegetative and adult reproductive developmental stages. The information provided by this study will contribute to shed light

into the molecular mechanisms involving the putative *Passiflora* ortologs of *LFY* and *AP1* and their potential role in regulating floral development. Furthermore, since some proteins of the FT/TFL1 family activate or repress the transcription of the genes involved in the identity of the floral meristems, these results will be helpful to connect the *FT/TFL1* expression patterns (see Chapter II) to their putative biological functions during vegetative to reproductive phase transition in passionfruit.

7.2 Materials and Methods

7.2.1 Multiple sequence alignment and phylogenetic analysis

The search for the *Passiflora* orthologs of *LEAFY* and *APETALA1* was performed using the genome sequence database of *P. organensis.* The corresponding protein sequences of *Arabidopsis* were used as query sequences. The BioEdit software was used to find candidate genes in each contig obtained from the *P. organensis* database. Exons/introns boundaries were predicted with the NetPlantGene software.

Once the genes were identified in *P. organensis*, their corresponding encoding proteins were aligned with sequences of other species using ClustalX program. Phylogenetic analysis of these proteins was performed using full-length proteins. A neighbor-joining tree (Saitou; Nei, 1987) was constructed using the program MEGA 7.0 (Kumar et al., 2016). Bootstrap confidence values were calculated based on 1000 replications. Protein sequences of other species used in this study were obtained from NCBI (http://www.ncbi.nlm.nih.gov/).

7.2.2 Gene Expression Pattern Analyzed by qRT-PCR

To investigate the expression patterns of *PoLFY* and *PoAP1*, qRT-PCR experiments were performed using samples from shoot apices of plants in juvenile, adult vegetative, and adult reproductive stages of *P. organensis*. Total RNA was extracted using RNeasy® Plant Mini Kit (50) (QIAGEN). The first-strand cDNA was synthesized with a SuperScript® III First-Strand Synthesis kit (Invitrogen, San Diego, CA), according to the manufacturer's instructions. qRT-PCR was performed with Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA) on the StepOne Real-Time PCR System (Applied Biosystems). Primers used and the melting curve with their specificity are presented in Table S1 and Figure S2, respectively. qRT-PCR was performed with three independent biological replicates and

three technical replicates for each sample. Data was analyzed using the mathematical model for relative quantification in real-time RT-PCR as described by Pfaffl (2001). Expression levels of specific genes were normalized using *Passiflora CLATHRIN ADAPTOR COMPLEX* (*CAC*) e *MONENSIN SENSIVITY 1/SAND* family (*SAND*) (Scorza, 2015).

7.3 Results

7.3.1 Identification and phylogenetic analysis of a putative *P. organensis LEAFY* ortholog

A putative ortholog of the *Arabidopsis LFY* gene was identified in the *P. organensis* genome database, and named it *PoLFY*. The complete gene sequence showed three exons and two introns, and the genomic organization was conserved when compared to *LFY* genes from other species (Figure 1).

PoLFY encodes a putative protein of 384 amino acids and displays a conserved domain at the C-terminal region. The conserved domain of LFY from other plant species is known to be similar to the DNA-binding domain of helix–turn–helix proteins (Hame´s et al. 2008). The deduced amino acid sequence of PoLFY was compared with amino acid sequences of LFY in other species (Figure 2). The comparison revealed that PoLFY shows similarities of 74% to *Arabidopsis thaliana* (AtLFY), 79% to *Malus domestica* (MdLFY), 80% to *Nicotiana tabacum* (NtLFY), 84% to *Gossypium hirsutum* (GhLFY)), 59% to *Oryza sativa* (OsLFY), and 53% to *Ginkgo biloba* (GbLFY), suggesting that LEAFY proteins are highly conserved among different plant taxa, mainly within closest clades, such as eudicots, presenting sequence similarities above 70% (Figure 2).

The evolutionary relationships among the PoLFY and its putative orthologs from 42 other species were analyzed by building a phylogenetic tree (Figure 3). The tree was constructed using the neighbor-Joing method, generating several distinct groups. These groups reflected the phylogenetic relationships among the plant taxa studied such as: pteridophytes, gymnosperms, and angiosperms (with monocots and eudicots separated in strongly supported branches), suggesting that the LEAFY protein sequence is highly conserved among phylogenetically distant plants.

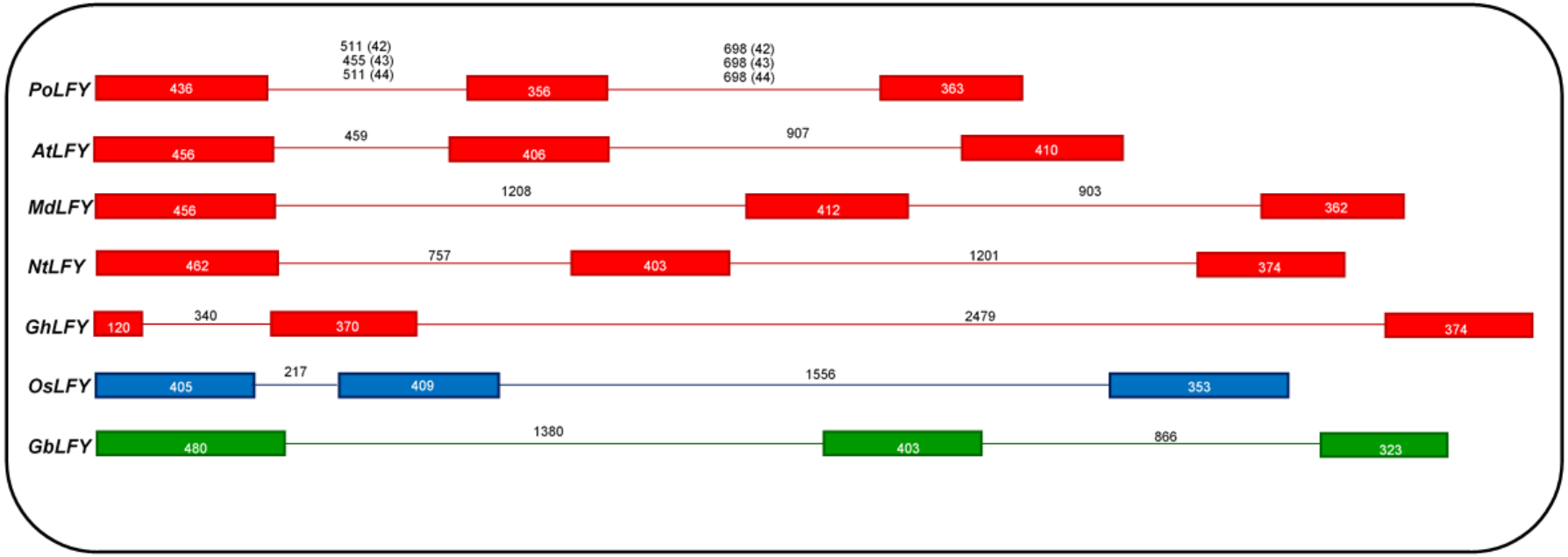


Figure 1 - **Genomic organization of *LEAFY* gene in *Passiflora organensis* (*PoLFY*), *Arabidopsis thaliana* (*AtLFY*), *Malus domestica* (*MdLFY*), *Nicotiana tabacum* (*NtLFY*), *Gossypium hirsutum* (*GhLFY*), *Oryza sativa* (*OsLFY*), and *Ginkgo biloba* (*GbLFY*)**. Boxes represent exons and lines represent introns. Numbers indicate the lengths of exons and introns in base pairs. In red, blue, and green are angiosperms (eudicots), angiosperm (monocot) and gymnosperm, respectively. Numbers in parentheses indicate the libraries: LIB042, LIB043, and LIB044.

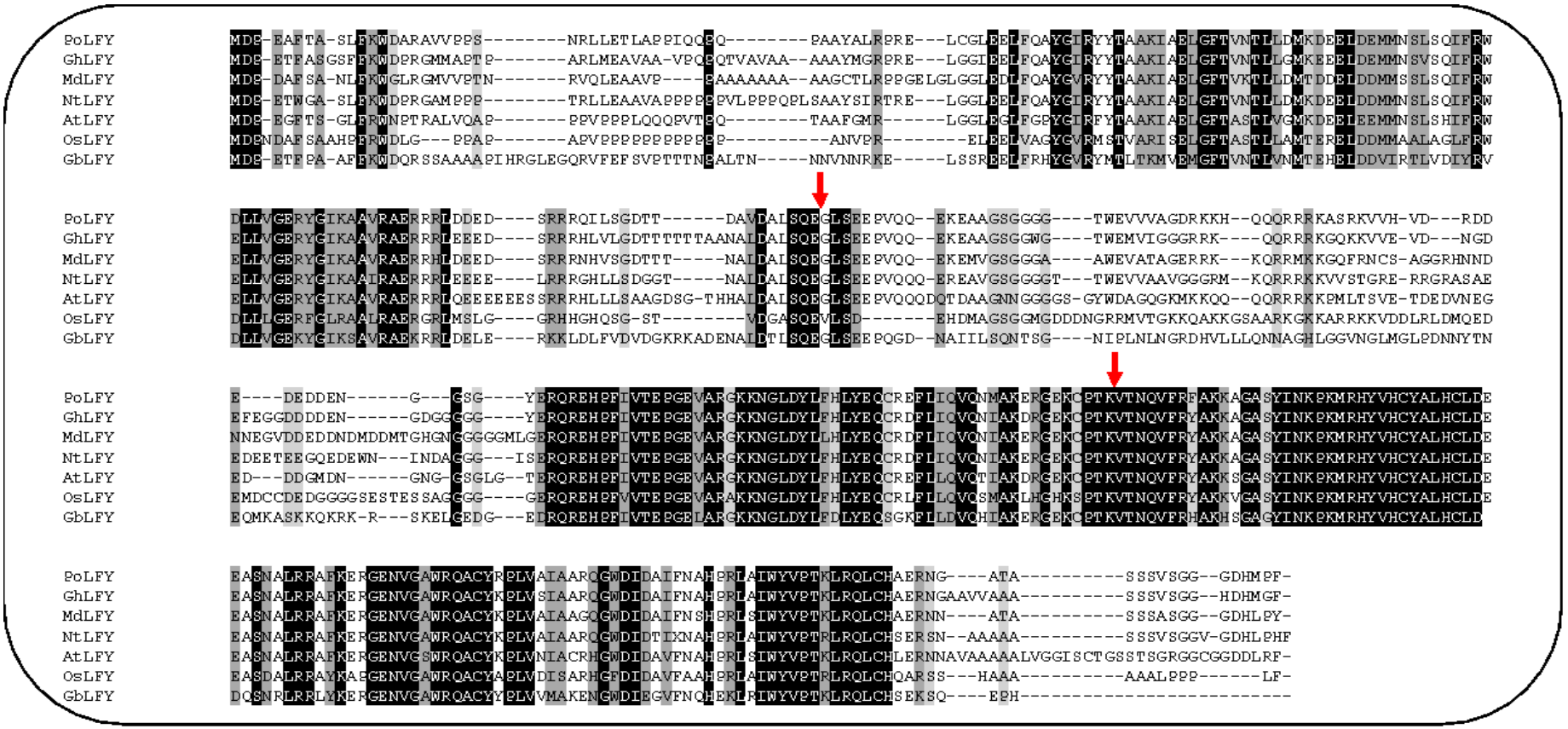


Figure 2 - **Amino acid sequence alignment of ortologous LEAFY proteins from *Passiflora organensis, Gossypium hirsutum, Malus domestica, Nicotiana tabacum, Arabidopsis thaliana, Oryza sativa, and Ginkgo biloba*.** A black background indicates a similarity level of 100% and a grey background indicates a conserved amino acid with a similar charge. Intron positions are indicated by red arrows above sequences.

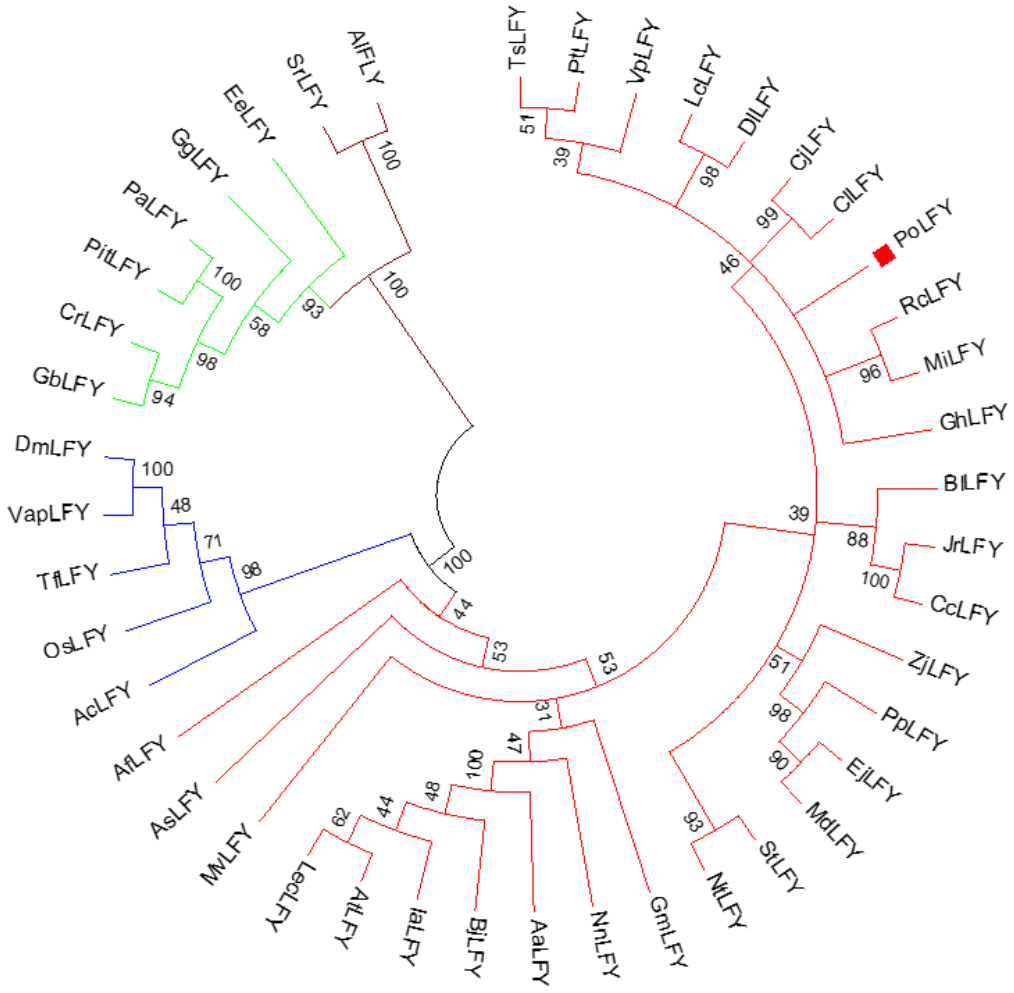


Figure 3 – **Phylogenetic analysis of LEAFY proteins in *Passiflora organensis* and other plants.** The tree was constructed using the neighbor-joing method and MEGA7 software. Only bootstrap values > 30% (1000 resamples) are shown in the branches. The brown and green branches indicate, LFY proteins in pteridophyte and gymnosperm classes, respectively. The blue and red branches indicate LFY proteins in plants of monocots and dicots subclass, within angiosperm class, respectively. The red square highlights PoLFY. The name of all species used in this analysis are listed in Table S3.

7.3.2 Identification and phylogenetic analysis of a putative *P. organensis APETALA1* ortholog

The complete gene sequence of the putative *P. organensis* ortholog of the *AP1* gene, named *PoAP1*, shows eight exons and seven introns. This genomic organization was also observed in other species, as shown in Figure 4.

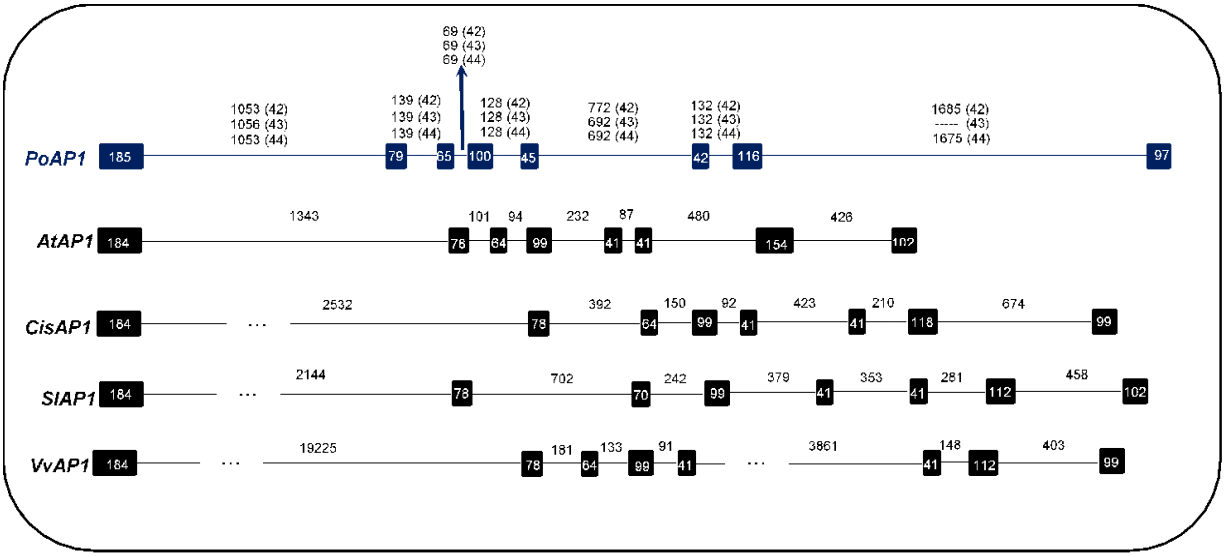


Figure 4 - **Genomic organization of putative orthologs of *APETALA1* gene in *Passiflora organensis* (*PoAP1*), *Arabidopsis thaliana* (*AtAP1*), *Citrus sinensis* (*CisAP1*), *Solanum lycopersicum* (*SlAP1*), and *Vitis vinifera* (*VvAP1*).** Rectangles represent exons and lines represent introns. Numbers indicate the lengths of exons and introns in base pairs. Numbers in parenthesis indicate the genomic libraries from which the sequences originated: LIB042, LIB043, and LIB044.

The *PoAP1* gene encodes a putative protein of 242 amino acids. This deduced protein sequence was compared with those from putative AP1 orthologs in other species (Figure 5). Sequence comparison revealed that PoAP1 shows similarities of 73.5% to *Arabidopsis thaliana* (AtAP1), 72% to *Malus domestica* (MdAP1), 78.5% to *Jatropha curcas* (JcAP1), 73% to *Citrus sinensis* (CisAP1), 78.5% to *Vitis vinifera* (VvAP1), and 70% to *Nicotiana tabacum* (NtAP1) orthologs (Figure 5). Additionally, we were able to identify the MADS domain, as well as the intervening (I) and the keratin-like (K) regions. The C-terminal region of the analyzed sequences contains a motif with conserved amino acid residues that allows the discrimination between the euAP1 and the euFUL (FRUITFUL) clades. The euFUL clade generally contains proteins similar in sequence to the euAP1 members, but with distinct biological functions (Litt and Irish, 2003).

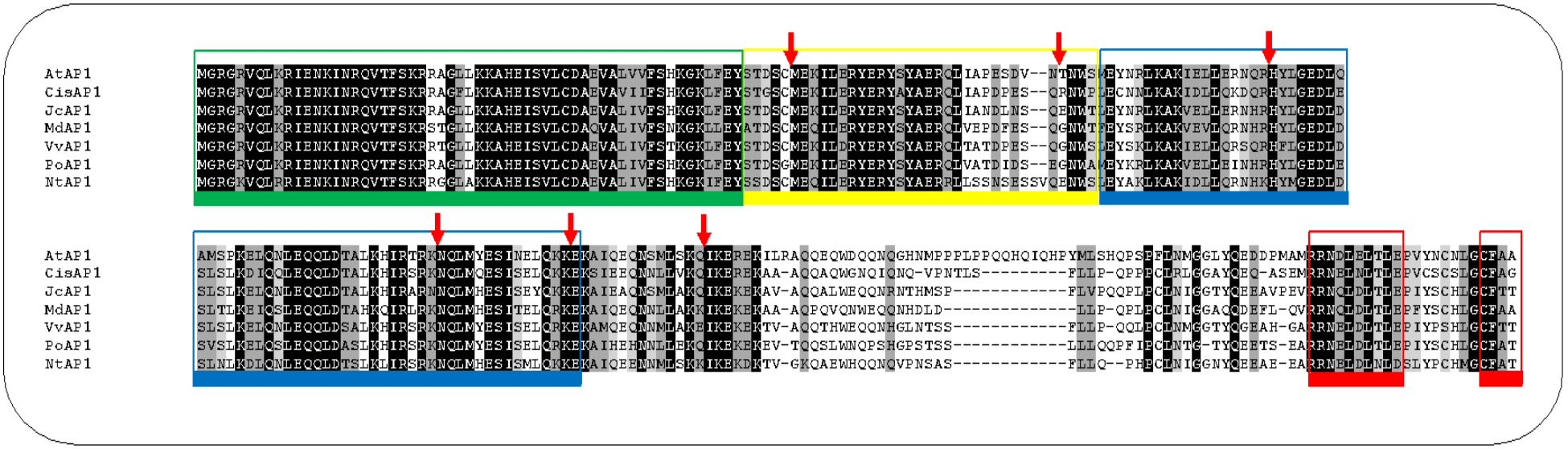


Figure 5 - **Amino acid sequence alignment of putative APETALA1 orthologs from *Passiflora organensis* and other angiosperms.** A black background indicates a similarity level of 100% and a grey background indicates conserved amino acids with similar charges. Intron positions in *P. organensis* are indicated by red arrows above the sequences. The green, yellow, and blue boxes indicate the MADS-box, I, and K-box domains, respectively. The two red boxes indicate conserved euAP1 motif sequences located in the C-terminal portion of the protein.

A phylogenetic tree was built to analyze the evolutionary relationships among the AP1 ortholog in *P. organensis* and AP1 and FUL proteins from other plant species. (Figure 6). The tree was constructed using the neighbor-joing method, generating two distinct groups. These groups represent sequences of amino acids encoded by the euAP1 and the euFUL clades. The identified PoAP1 protein sequence is in the same clade as the sequences considered to belong to the euAP1 group (Figure 6).

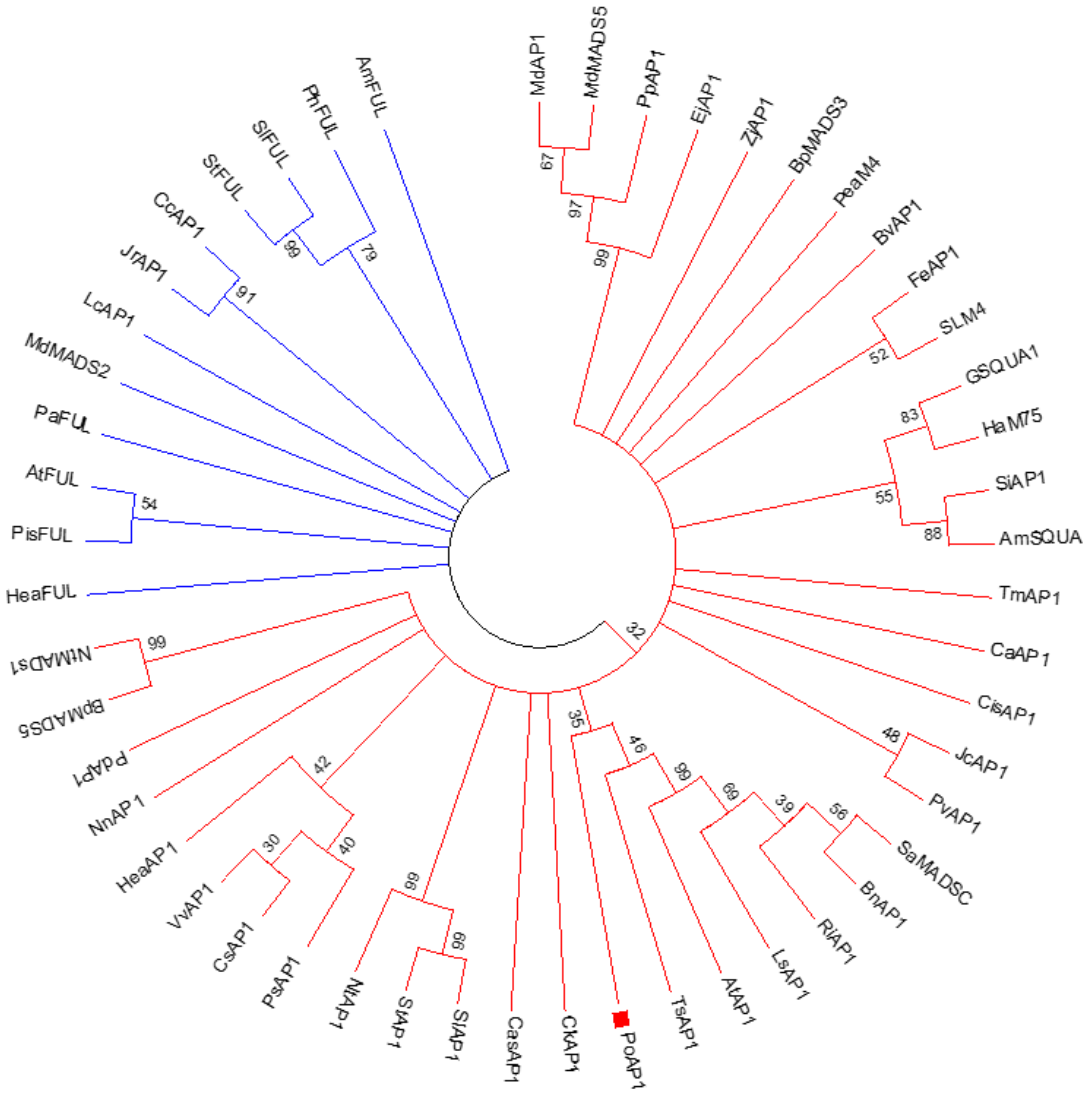


Figure 6 – **Phylogenetic analysis of the *Passiflora organensis* APETALA1 ortholog and AP1 and FUL proteins from other plants.** The tree was constructed using the neighbor-joing method and MEGA7 software. Only bootstrap values above 30% (1000 resamples) are shown. The blue and red branches indicate FUL and AP1 proteins, respectively. The red square highlights PoAP1. The name of all plant species used in this analysis are listed in Table S4.

7.3.3 Gene expression patterns of *P. organensis* putative orthologs of *LEAFY* and *APETALA1*

To help understand the role of the *PoLFY* and *PoAP1* genes during phase transition in *P. organensis*, we studied the expression patterns of these genes during the juvenile (Figure 7A), adult vegetative (Figure 7B) and adult reproductive (Figure 7C) developmental stages.

We observed that the expression of *PoLFY* increased approximately 2-fold during the adult reproductive phase in comparison to the juvenile and adult vegetative phases (Figure 7D). On the other hand, *PoAP1* showed the highest level of expression during the adult vegetative phase, corresponding to approximately 10-fold the expression level observed during the juvenile phase, whereas in the adult reproductive phase, the expression of this gene showed a 7-fold increase in comparison to the juvenile phase (Figure 7E).

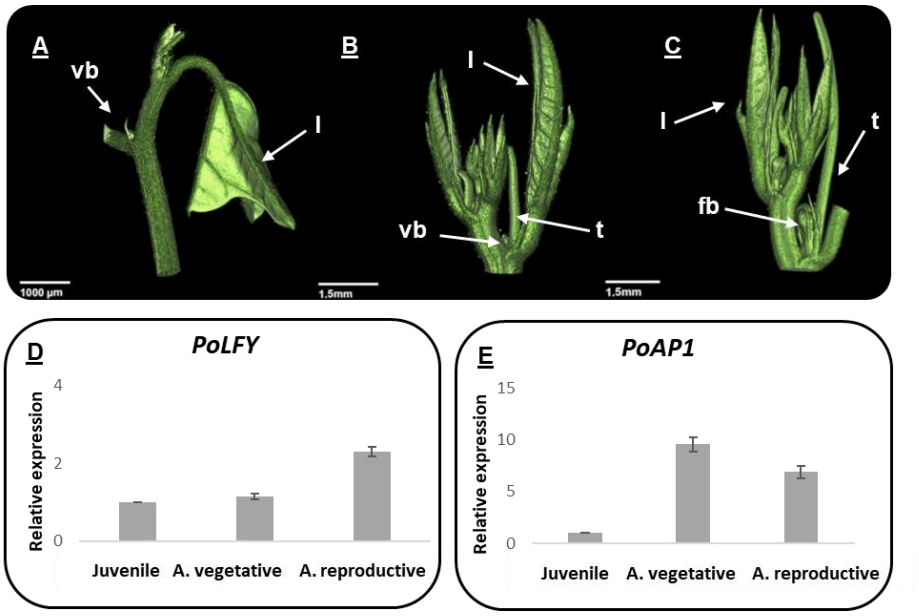


Figure 7 – **Relative expression of *PoLFY* and *PoAP1* genes in shoot apices of *Passiflora organensis***. **A-C.** Shoot apices in the juvenile stage (A), adult vegetative stage (B), and adult reproductive stage (C). The arrows indicate: flower bud (fb), leaf (l), tendril (t), and vegetative bud. (**D-E).** Relative expressions of *PoLFY* (D) and of *PoAP1* (E). In each graphic the sample with lowest Ct value was used as a calibrator. Error bars indicate the standard deviation.

7.4 Discussion

Transcription factors such as LFY and AP1 act as central regulators in key developmental processes of plant reproduction, including the floral transition and the formation of floral organs (Goslin et al., 2017).

The LFY protein is known as a plant-specific transcription factor (TF) and unlike many other TFs that belong to multigene families, it exists as a single-copy gene in most angiosperm genomes, which makes it unusual among TFs in plants (Weigel et al., 1992). Homologs of *LFY* have been identified among distantly related species, such as grapevine (Carmona et al., 2002), chrysanthemum (Ma et al., 2008; Ma et al., 2013), alfalfa (Zhang et al., 2013), carrot (Zhang et al., 2016), isoetes (Yang et al., 2017), litchi (Ding et al., 2018), *Prunus mume* (Ahmad et al., 2019), among others. The *PoLFY* gene showed three exons and two introns, and this genomic organization is conserved among other *LFY* orthologs from other species. Our phylogenetic tree obtained by amino acid comparisons showed that PoLFY is a member of the dicotiledoneous LFY clade which contains other angiosperm species including *Gossypium hirsutum, Litchi chinensis, Mangifera indica, Populus tremula, Viola pubescens, among others*. These proteins share a conserved DNA-binding domain at the C-terminal region that is structurally related to the helix–turn–helix (HTH) domains (Hame et al., 2008).

The Arabidopsis AP1 is an identity gene that controls the formation of flower organs along with other homoeotic genes (Liu et al., 2007). Sequence analyses and multiple sequence alignments confirmed that *PoAP1* is similar to other *AP1* genes from different species. These genes carry conserved sequences encoding the MADS-box domain. The presence of the MADS-box domain implies that the protein can bind to DNA and function as a transcription factor (Messenguy; Dubois, 2003). *PoAP1* also presents an intervening region (I) that, in *Arabidopsis*, is thought to contribute to the specificity of dimerization, and also a keratin-like region (K), involved in protein-protein interactions (Melzer et al., 2010). Moreover, phylogenetic analysis among PoAP1 and AP1 and FRUITFULL (FUL) from different species generated a tree containing two main groups that coincide with the euAP1 and euFUL clades, described by Litt and Irish (2003). The euAP1 and euFUL clades are the result of a gene duplication event that coincided with the origin of the core eudicots. Although their sequences are similar, as is expected from paralogs, the AP1 and FUL proteins have diverging conserved motifs in their C-terminal domains, which probably reflect differences

in their abilities to interact with their respective molecular partners and, therefore, differences in their biological roles (Litt; Irish, 2003).

The regulatory relationships between LFY and AP1 has become a case example for gene interactions in the control of developmental transitions. LFY has been shown to directly activate AP1 expression in floral primordia (Wagner et al., 1999; William et al., 2004), while AP1 then acts on LFY to reinforce its expression (Kaufmann et al., 2010). Thus, LFY and AP1 are part of a positive feedback loop, which ensures that these floral meristem identity factors are expressed at high levels in early stage floral primordia.

Our study of gene expression during *P. organensis* development showed that the level of *PoLFY* transcripts increased as the plant progresses through its different phases. *PoAP1* showed the highest level of expression during the adult vegetative stage, followed by a decrease in the level of expression at the adult reproductive stage. The acquisition of flower identity is determined by the balance of the expression of inflorescence identity genes such as *TERMINAL FLOWER 1* (*TFL1*) and flower meristem identity genes such as *LFY* and *AP1* (Bradley et al., 1997). Thus, a potential hypothesis for the decrease of *PoAP1* transcripts during the adult reproductive phase can be explained by the small increase in the expression of *PoTFL1* (see Chapter II). Besides being important for the maintenance of the indeterminate growth of *Passiflora*, the balance between these two genes could be involved in the number of flowers formed, however, a more detailed investigation is needed to confirm this hypothesis. Furthermore, a reduction of *AP1* expression in *Arabidopsis ap1* mutants resulted in the activation of cryptic axillary meristems in the axil of the perianth floral organs (Han et al, 2014). It is noteworthy that in *Passiflora*, the production of the filaments of the corona, a synapomorphy among *Passiflora* species, is thought to be originated by the activation of an ectopic meristem (Claßen-Bockhoff; Meyer, 2016). It will be interesting to investigate the spatial expression patterns of *PoAP1* during flower development and to establish whether this gene is expressed during corona primordium development, as observed for PeAP1 (Scorza et al., 2017).

7.5 Conclusions

In this study we identified and characterized *PoLFY* and *PoAP1* and we showed the expression patterns of these genes during the juvenile, adult vegetative, and adult reproductive phases in *Passiflora organensis*. Both genes showed high expression during the adult stage, suggesting that they are both important for establishing the floral identity during

the vegetative to reproductive transition in *Passiflora*, like it is observed for model species such as *Arabidopsis*. Nonetheless, future studies on these genes are needed to have a better understanding of their biological function in this phase transition.

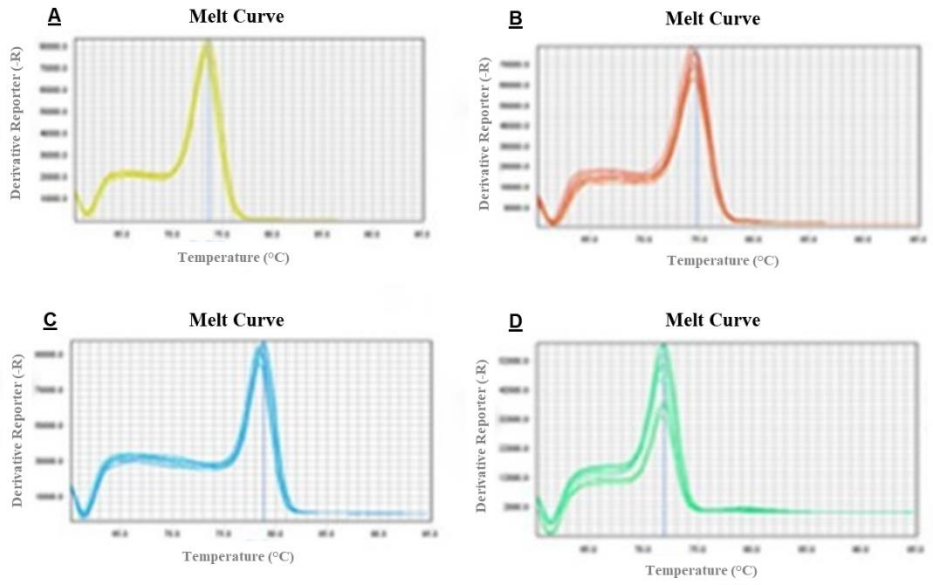
7.6 Supplementary Material

S1 - Primers used for the qRT-PCR analysis

Primer	**Sequence**	**Amplicon (pb)**	**Annealing Temperature (°C)**
PoLFY - F	ATGCACTTCCATTGCCTCGAC	166	60
PoLFY - R	CGAGGATGTGCGTTGAAAAT		
PoAP1 - F	GGAACGAGCTTGACCTTACG	154	60
PoAP1 - R	TTTCTCATTTCATGGGCATTT		
PeCAC - F	TCAAGAGGGAGTGCGTTGAC	90	60
PeCAC - R	CAACCAACAGCGCCTGTAAC		
PeSAND - F	GGAGCTGCTTCTCCCCATTT	78	60
PeSAND -R	AGGGCCACCAATTCCAATGA		

F = *forward;* R = *reverse*

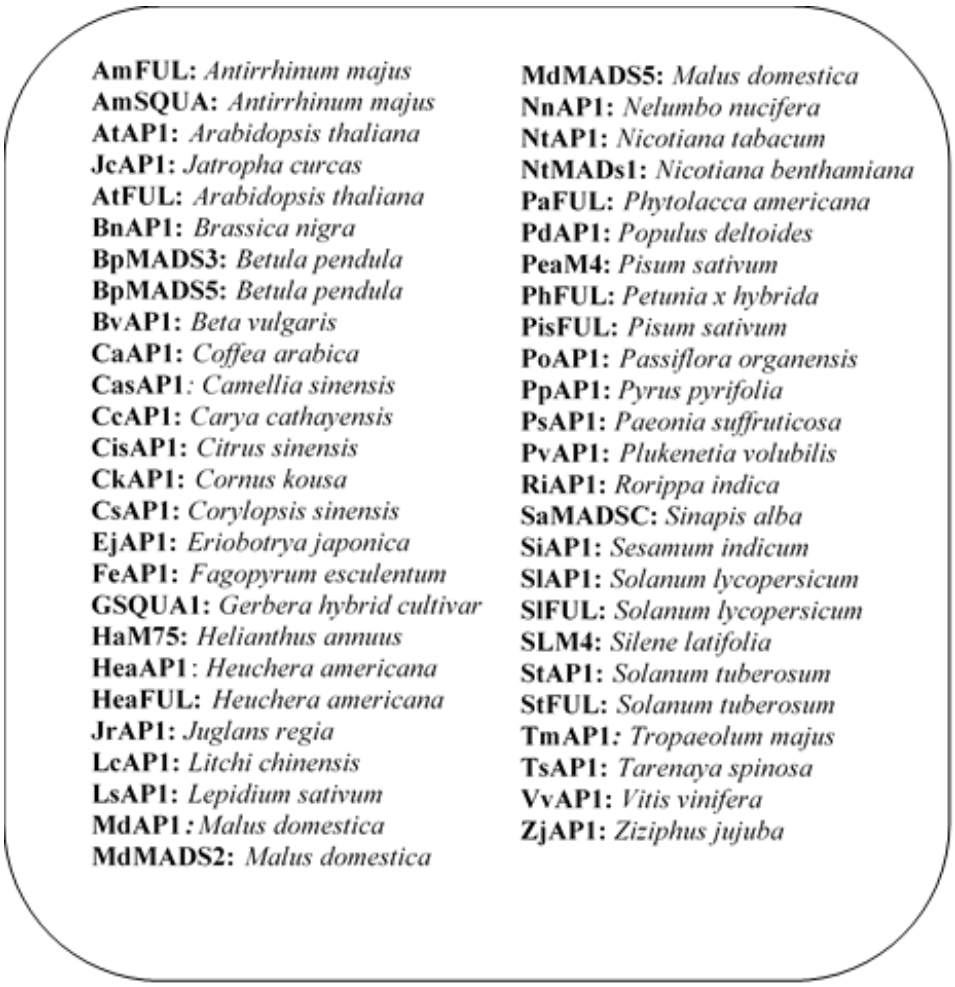
S2 - Melting curve in the RT-qPCR reaction. (A) *PeSAND*; (B) *PeCAC*; (C) *PoLFY*; and (D) *PoAP1*



S3 – Species used to build the LFY phylogenetic tree

AaLFY: *Arabis alpina*
AcLFY: *Allium cepa*
AfLFY: *Aquilegia formosa*
AlFLY: *Angiopteris lygodiifolia*
AsLFY: *Acca sellowiana*
AtLFY: *Arabidopsis thaliana*
BjLFY: *Brassica juncea*
BlLFY: *Betula luminifera*
CcLFY: *Carya cathayensis*
CjLFY: *Citrus japonica*
ClLFY: *Clausena lansium*
CrLFY: *Cycas revoluta*
DlLFY: *Dimocarpus longan*
DmLFY: *Dendrobium moniliforme*
EeLFY: *Ephedra equisetina*
EjLFY: *Eriobotrya japonica*
GbLFY: *Ginkgo biloba*
GgLFY: *Gnetum gnemon*
GhLFY: *Gossypium hirsutum*
LcLFY: *Litchi chinensis*
LecLFY: *Leavenworthia crassa*
MdLFY: *Malus domestica*
MiLFY: *Mangifera indica*
MvLFY: *Magnolia virginiana*
NnLFY: *Nelumbo nucifera*
NtLFY: *Nicotiana tabacum*
OsLFY: *Oryza sativa*
PaLFY: *Picea abies*
PitLFY: *Pinus tabuliformis*
PoLFY: *Passiflora organensis*
PpLFY: *Prunus persica*
PtLFY: *Populus tremula*
RcLFY: *Rhus chinensis*
SrLFY: *Sceptridium robustum*
StLFY: *Solanum tuberosum*
TfLFY: *Tricyrtis formosana*
TsLFY: *Triadica sebifera*
VapLFY: *Vanilla planifolia*
VpLFY: *Viola pubescens*
ZjLFY: *Ziziphus jujube*

S4 – Species used to build the AP1 phylogenetic tree



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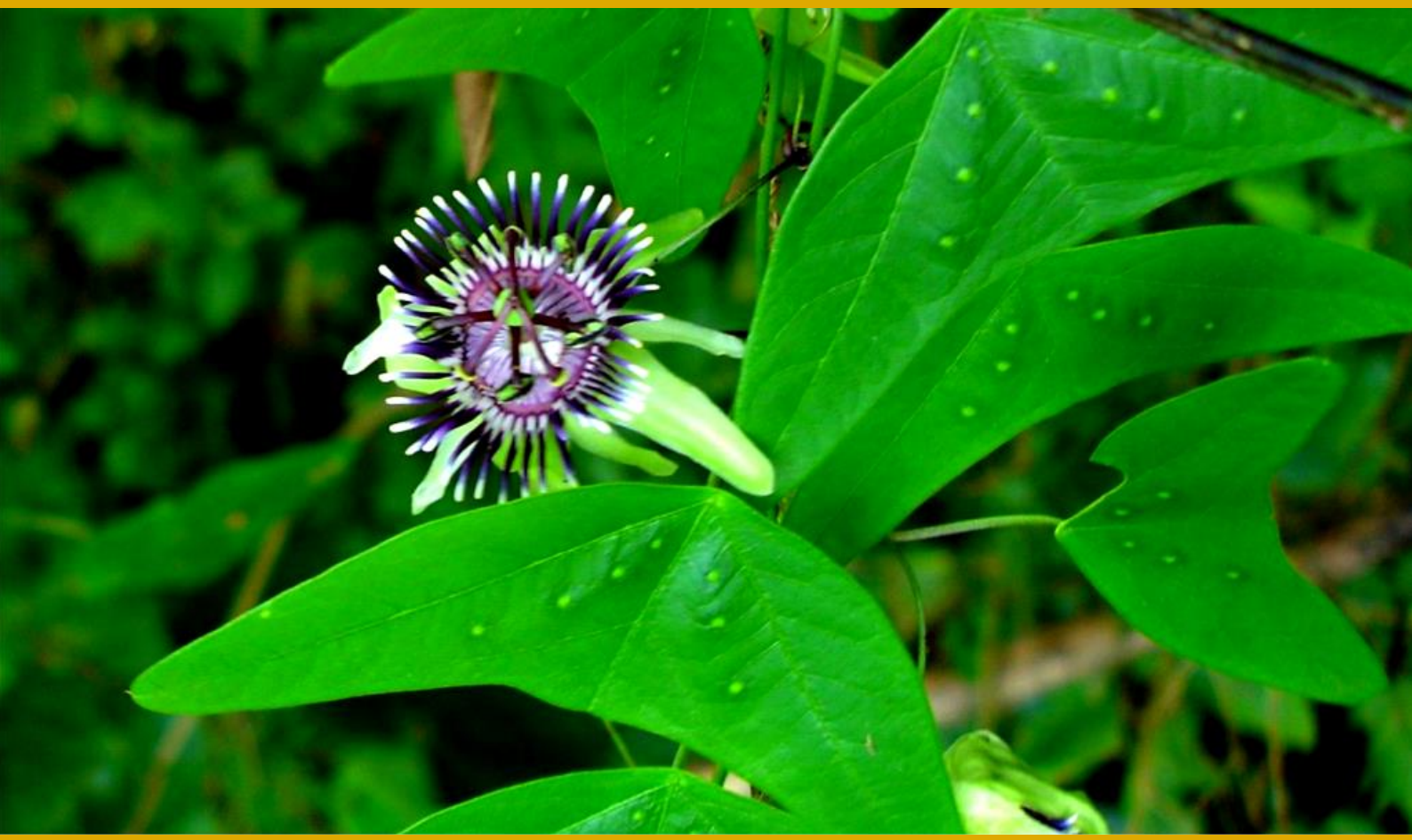
CHAPTER V

Protein-protein interactions involved in flowering control and branching in *Passiflora organensis*



Passiflora organensis – adult stage

8. Protein-protein interactions involved in flowering control and branching in *Passiflora organensis*

Abstract

Studies on protein–protein interactions can bring clues about the biological roles of the proteins in question and their underlying molecular mechanisms. In model plants, FT/TFL1 proteins are members of the PEBP (phosphatidyl ethanolamine-binding protein) family and need to interact with specific transcription factors in order to perform their biological functions in developmental phase transitions. The basic region/leucine zipper (bZIP) and the Teosinte branched1/Cycloidea/Proliferating cell factor (TCP) families, which contain key players of plant development, encode proteins that form unique complexes with FT/TFL1 family members. Moreover, proteins encoded by the FT and bZIP transcription factors might interact with 14-3-3 proteins which are highly conserved scaffold elements, resulting in the formation of hexameric complexes. These complexes play critical roles in flowering time control. We are interested in uncovering the molecular mechanisms involved in the vegetative to reproductive phase transition in *Passiflora organensis*, a small herbaceous vine, from the same genus of the economically important passionfruit. In this study our aim was to access the protein-protein interactions and the putative complexes formed by proteins encoded by the *Passiflora FT/TFL1*, *bZIP*, *TCP*, and *14-3-3* gene families. We show that PoFT interacts with PobZIPs, and PobZIPs interact with some of the Po14-3-3 proteins. However, no direct interactions were detected between PoFT and 14-3-3 proteins. These results suggest that the flowering activation complexes in *Passiflora* might have a different composition from what has been recently proposed for other plant taxa. Furthermore, the *Passiflora* FT-like proteins can interact with multiple *Passiflora* TCP proteins. The results of this research are fundamental to complete the *FT/TFL1* gene activity characterization in *Passiflora organensis* and to select members of this family for future studies.

Keywords: bZIP. FAC. Florigen. Flowering time. FT/TFL1. Passionfruit. Plant architecture. TCP. 14-3-3.

8.1 Introduction

The FLOWERING LOCUS T/TERMINAL FLOWER 1 proteins play a key role in the control of flowering in plants (Wickland and Hanzawa, 2015). The FLOWERING LOCUS T (FT) protein interacts with transcription factors such as FLOWERING LOCUS D (FD) and BRANCHED1 (BRC1) and these interactions are essential for the formation of protein complexes to control flowering time (Abe et al., 2005) and branching processes (Niwa et al., 2013). The BRC1 protein belongs to the Theosinte branched1/Cycloidea/Proliferating cell factor (TCP) family, whose members are involved in key aspects of plant development (Martín-Trillo and Cubas, 2010). The interaction between FT and FD form a complex at the shoot apical meristem that promotes flowering (Abe et al., 2005), while the interaction of FT and BRC1 appears to delay floral transition at the axillary meristems (Niwa et al., 2013). Thus, the interactions of FT/TFL1 proteins with different partners specify the distinct roles of these proteins during plant development.

Studies have shown that the FT protein is the main component of the ¨florigen¨, a systemic signal that induces flowering in plants (Wickland; Hanzawa, 2015; Hiroyuki, 2017). In *Arabidopsis* and rice, the respective FT protein ortholog is synthesized in leaves and transported to the shoot apical meristem (SAM) through the phloem (Bradley et al., 1997; Tamaki et al., 2007). Whereas in *Arabidopsis,* FT promotes floral transition by the interaction with the FD protein; in rice the interaction between FT and FD is mediated by 14-3-3 proteins. Taoka et al. (2011) demonstrated that HD3A (the rice ortholog of FT) interacts firstly with 14-3-3 proteins in the cytoplasm, and the resulting protein complex then translocates to the nucleus where it binds to OsFD1. The resultant protein complex, named as ¨florigen activation complex¨ (FAC), interacts with the promoter of the meristem identity gene OsMADS15 inducing its transcription (Taoka et al., 2011). In addition, Pnueli et al. (2001) suggested that *FT/TFL1* (or *CETs*) genes in tomato encode a family of modulator proteins with the potential to interact with a variety of signaling proteins in a manner analogous to that of 14-3-3 proteins.

In this study we are interested in understanding how *Passiflora* putative orthologs of FT, FD, 14-3-3, and TCP proteins interact, aiming to provide insights into the regulation of phase transition and branching in *Passiflora organensis,* a small herbaceous vine, from the same genus of the economically important passionfruit.

8.2 Materials and Methods

8.2.1 Multiple sequence alignment and phylogenetic analysis

The search for the orthologous genes was performed using the genome sequencing database of *P. organensis*. The protein sequences of *Arabidopsis thaliana* were used as query sequences. The BioEdit software was used to find candidate genes in each contig obtained from the *P. organensis* database. The identification of exons/introns boundaries was done using the NetPlantGene software (Pertea et al., 2000).

Identified proteins in *Passiflora* were aligned with sequences of other species using Clustal X (Thompson et al., 1997). Phylogenetic analysis of these proteins was performed using full-length proteins. A neighbor-joining tree (Saitou; Nei, 1987) was constructed using the program MEGA 7.0 (Kumar et al., 2016). Bootstrap confidence values were calculated based on 1000 replications. Protein sequences of other plant species used in this study were obtained from NCBI (http://www.ncbi.nlm.nih.gov/) and IPK Barley Blast Server (https://webblast.ipk-gatersleben.de/barley_ibsc/viroblast.php).

8.2.2 Yeast two-hybrid assay

Yeast vectors pDEST22 (prey vector, AD; Trp selection marker gene for yeast and ampicillin for bacteria; Invitrogen), pDEST32 (bait vector, BD; Leu selection marker gene for yeast and gentamycin for bacteria; Invitrogen), pARC352 (Gateway-compatible version of pTFT1; for Y3H; Ade2 selection marker gene for yeast and ampicillin for bacteria), and yeast strain PJ69-4A and PJ69-4α were used in the yeast two-hybrid and tree-hybrid assays. For all Gateway compatible clonings, pDONR201 cloning vector (Invitrogen, Carlsbad, CA, USA), with kanamycin selection for bacteria, was used to generate the entry vectors. Primers sequences with two recombination sites, attB1 and attB2, for recombinational cloning are listed in Table S1. All constructs were verified by sequencing.

The protein–protein interaction analyses were performed by yeast two and tree hybrid assays, as described by Folter and Immink (2011). Auto-activation was determined for all bait vectors used in this study, prior to the interaction assays. Transformants were selected on SD-glu medium lacking leucine (-L), tryptophan (-W), histidine (-H), adenine (-A), and/or supplemented with 3AT (3-amino-1,2,4-triazole) to test the interactions.

8.3 Results

8.3.1 Identification and phylogenetic analysis of *Passiflora bZIP* genes

We identified seventeen members of the bZIP family (group A) in the *P. organensis* genome and initially we named them according to the contig number of the *P. organensis* database. To investigate the phylogenetic relationships among the *Passiflora* bZIP we included protein sequences from *Arabidopsis thaliana*, *Jatropha curcas*, *Vitis vinifera*, and *Hordeum vulgare* and we constructed a phylogenetic tree (Figure 1).

The selected proteins PoBZIP1 (PobZIP159), PobZIP2 (PobZIP10547), PobZIP3 (PobZIP12122), PobZIP4 (PobZIP10155), PobZIP5 (PobZIP14), and PobZIP6 (PobZIP607) shared high sequence identity within the bZIP domain with other characterized flowering related bZIP proteins, including the orthologs from *Arabidopsis* FD (AtbZIP14) and FDP (AtbZIP27) (Figure 2). The bZIP domain consists of two structural features located on a contiguous α-helix (Abe et al., 2005): a basic region responsible for sequence-specific DNA binding, and a leucine zipper domain required for homo/hetero-dimerization (Figure 2).

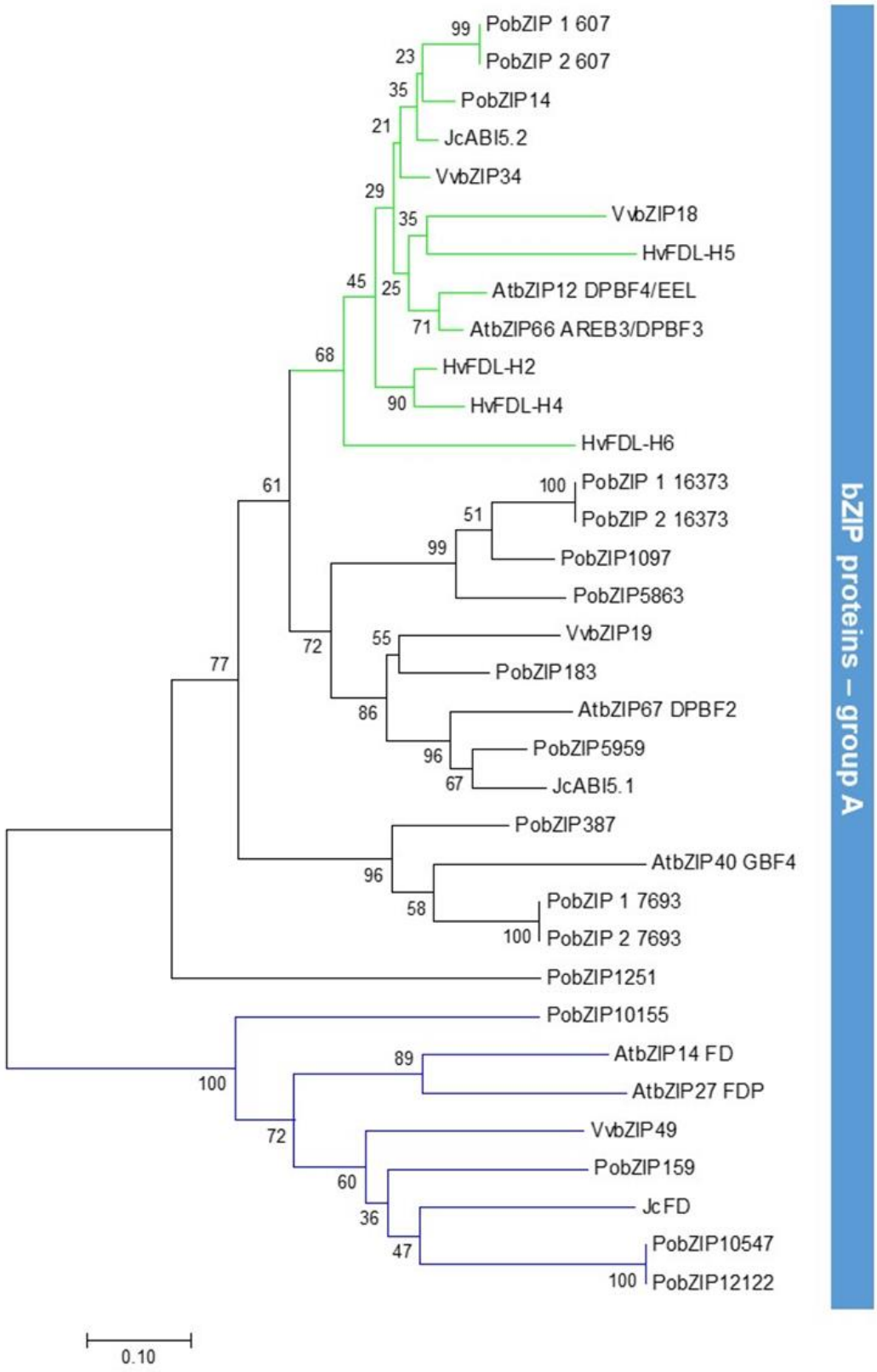


Figure 1 - **Phylogenetic analysis of the bZIP family members from *Passiflora organensis*, *Arabidopsis thaliana*, *Jatropha curcas*, *Vitis vinifera*, and *Hordeum vulgare*.** The tree was constructed using the neighbor-joining method and MEGA7 software. Bootstrap values for 1000 re-samplings are shown on branches. Blue and green branches indicate the closest FD and FDP homologs of *Arabidopsis* and barley, respectively.

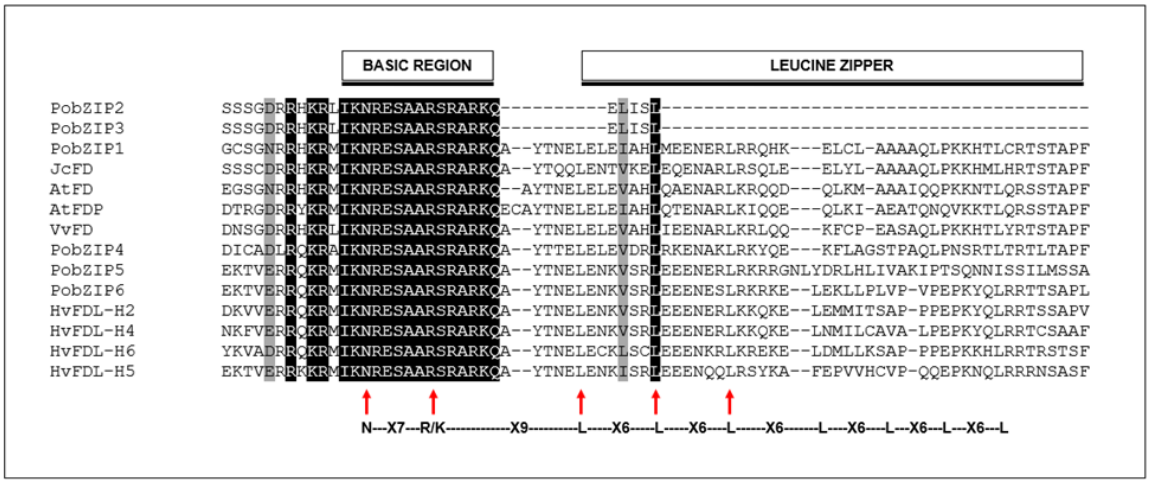


Figure 2 - **Sequence alignment of the bZIP domain from putative orthologous proteins of *Passiflora organensis*, *Arabidopsis thaliana*, *Jatropha curcas*, *Vitis vinifera*, and *Hordeum vulgare*.** Identical amino acids are shown in black boxes, while amino acids with similar charge or hydrophobicity are shown in grey. The highly conserved residues are highlighted with red arrows. Amino acid sequences were aligned with Clustal X.

8.3.2 Identification and phylogenetic analysis of *Passiflora* 14-3-3 genes

In *Passiflora organensis* we identified ten 14-3-3 genes and then a multiple sequence alignment was performed using 14-3-3 protein sequences from different plant species (Figure 3). The results showed that 14-3-3 proteins from *Passiflora organensis*, *Arabidopsis thaliana*, *Jatropha curcas*, *Vitis vinifera*, and *Hordeum vulgare* contained several conserved amino acids in their aligned sequences.

Based on the phylogenetic analysis (Figure 4), 14-3-3 proteins were classified into two distinct evolutionary groups, namely, non-epsilon and epsilon groups. Six of the *Passiflora* 14-3-3 proteins belong to the non-epsilon group: Po14-3-3A, Po14-3-3C, Po14-3-3E, Po14-3-3F, Po14-3-3G, Po14-3-3I, while four *Passiflora* 14-3-3 proteins belong to the epsilon group: Po14-3-3B1, Po14-3-3B2, Po14-3-3D, Po14-3-3H.

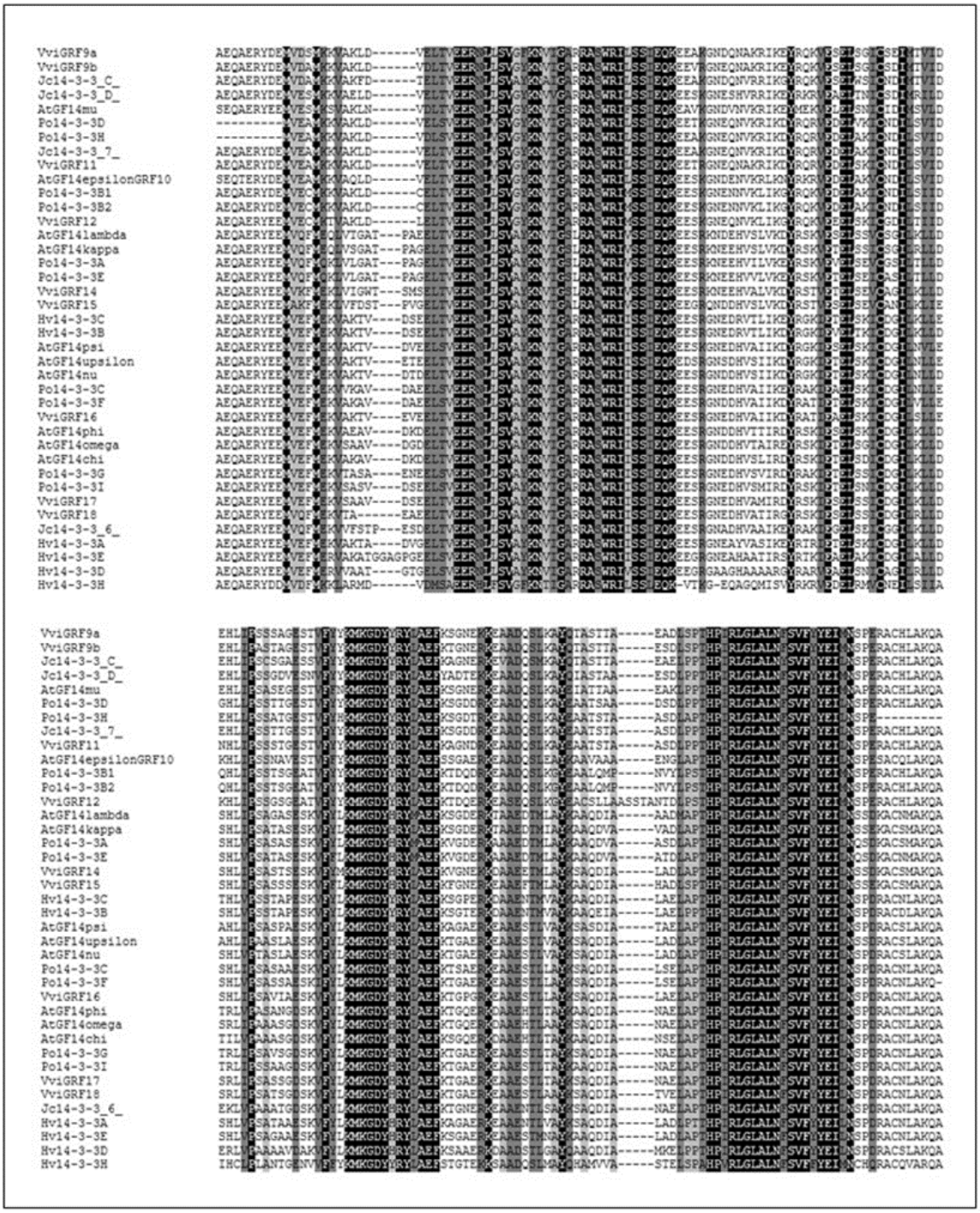


Figure 3 - **Sequence alignment of the 14-3-3 putative orthologous proteins from *Passiflora organensis*, *Arabidopsis thaliana*, *Jatropha curcas*, *Vitis vinifera*, and *Hordeum vulgare***. Only the conserved regions of the proteins are shown. Identical amino acids are shown in black boxes, while amino acids with similar charge or hydrophobicity are shown in grey. Amino acid sequences were aligned with Clustal X.

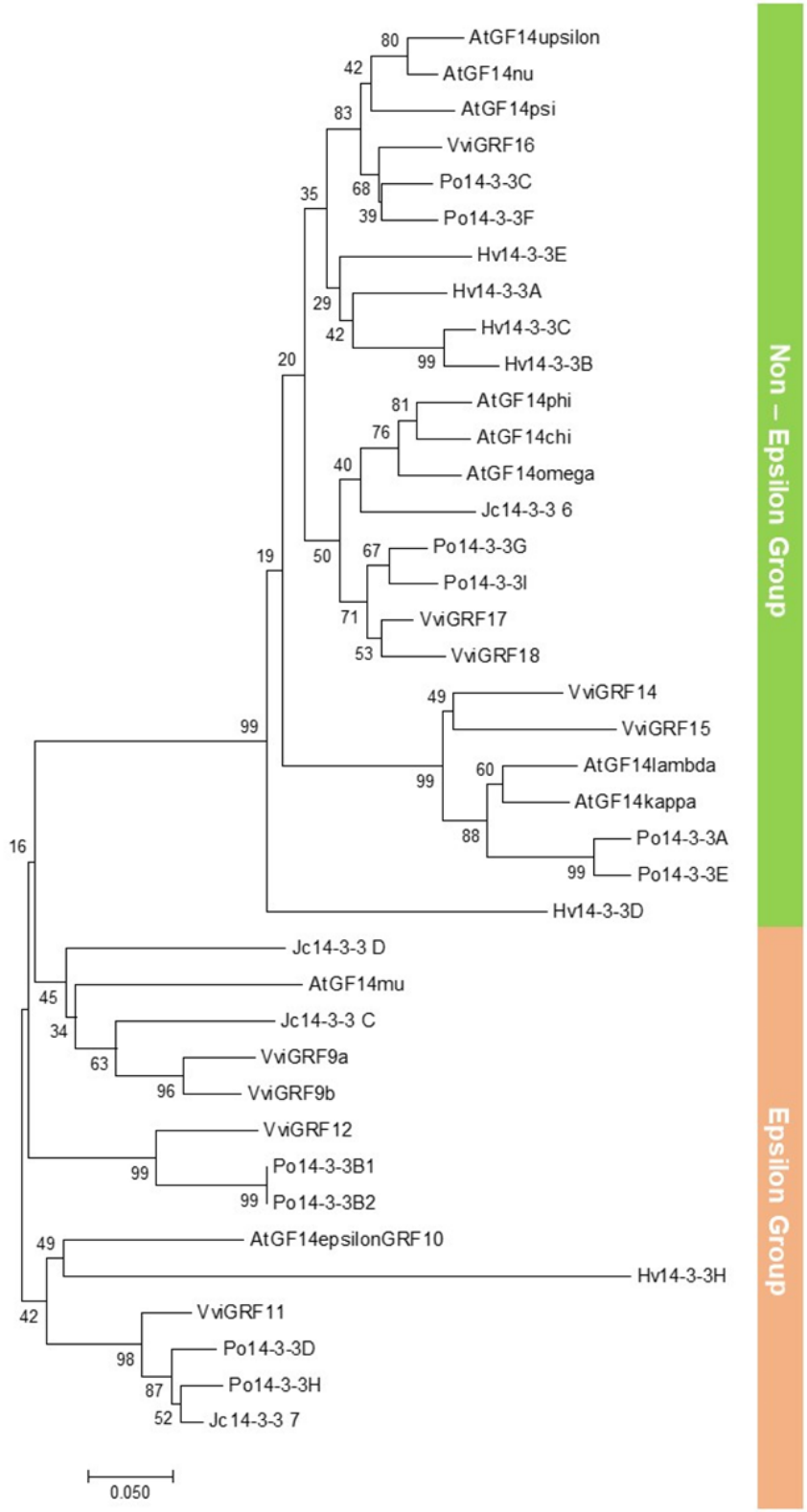


Figure 4 - **Phylogenetic analysis of 14-3-3 members from *Passiflora organensis*, *Arabidopsis thaliana*, *Jatropha curcas*, *Vitis vinifera*, and *Hordeum vulgare***. The tree was constructed using the neighbor-joining method and MEGA7 software. Bootstrap values for 1000 re-samplings are shown on branches.

8.3.3 Identification and phylogenetic analysis of *Passiflora* TCP genes

In *Passiflora organensis* we identified twenty-seven members of TCP family and constructed a phylogenetic tree, using the neighbor-joining method. The phylogenetic tree consisted of the 24 members of the TCP family from *Arabidopsis thaliana* and all the identified *Passiflora organensis* TCP putative orthologs. The phylogenetic analysis distinguished the TCPs into two classes: Class I and Class II. Class II was then differentiated into two subclasses: CIN/JAW and CYC/TB1 (Figure 5).

We selected ten *Passiflora* Class II TCPs for further analyses. Six of these proteins belong to the subgroup CYC/TB1: PoBRCa, PoBRCb, PoBRCc, PoBRCd, PoBRCe, and PoBRCf. While four proteins belong to the subgroup CINCINNATA (CIN)/JAW: PoJAWa, PoJAWb, PoJAWc, and PoJAWd.

The length of the *Passiflora* TCP proteins selected for the subsequent studies varied from 347 (PoJAWc) to 580 amino acids (PoJAWd), with an average of 447 amino acids. We performed a sequence alignment of the conserved TCP domain of *Passiflora* and *Arabidopsis* proteins. The TCP domain is composed of 59 amino acid residues, forming a basic helix-loop-helix (bHLH) type of DNA-binding domain non-canonical to regular bHLH TFs (Martín-Trillo and Cubas, 2010) (Figure 6).

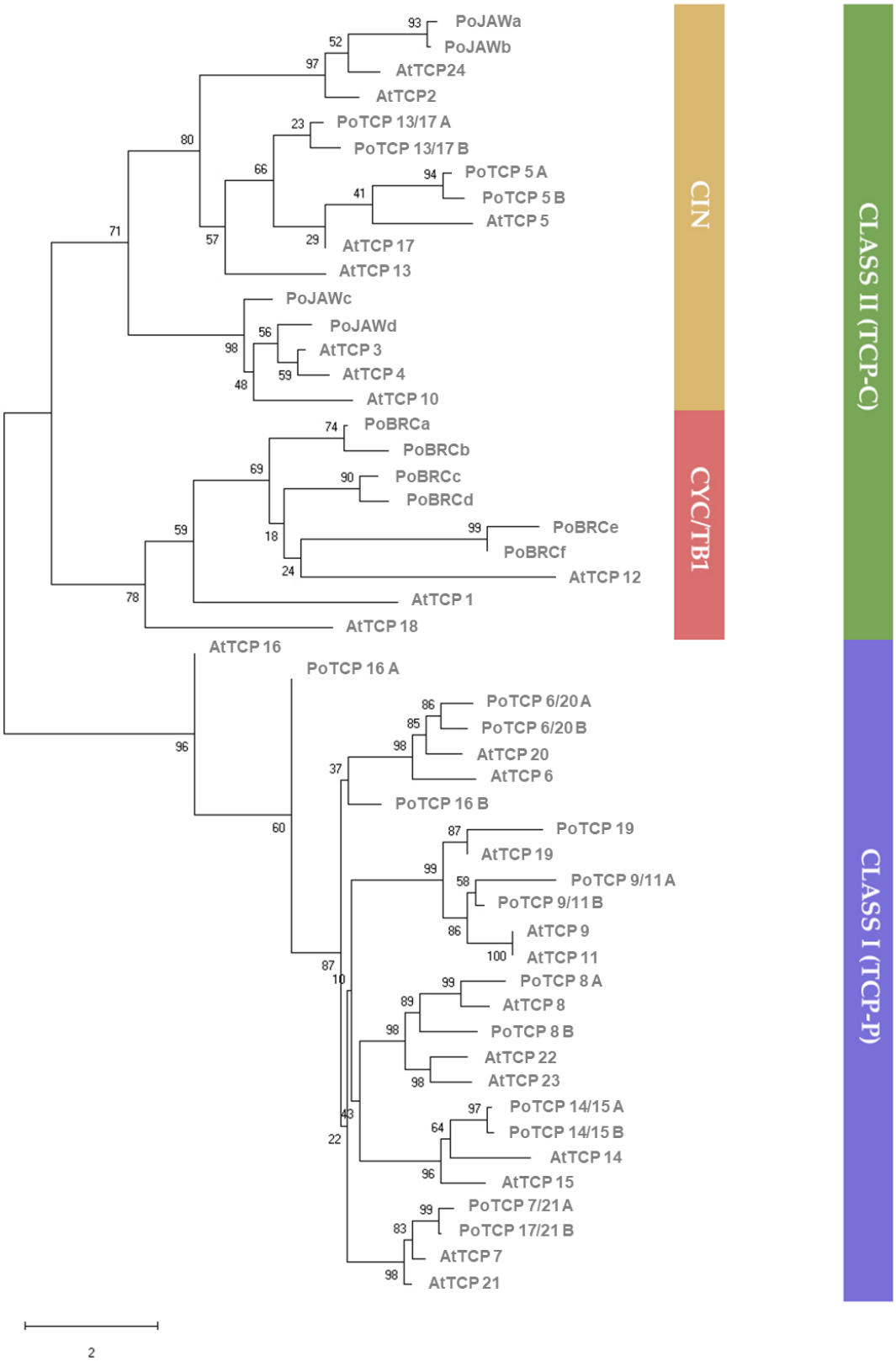


Figure 5 - **Phylogenetic analysis of the *TCP* family members from *Passiflora organensis* and *Arabidopsis thaliana*.** The tree was constructed using the neighbor-joining method and MEGA7 software. Bootstrap values for 1000 re-samplings are shown on branches

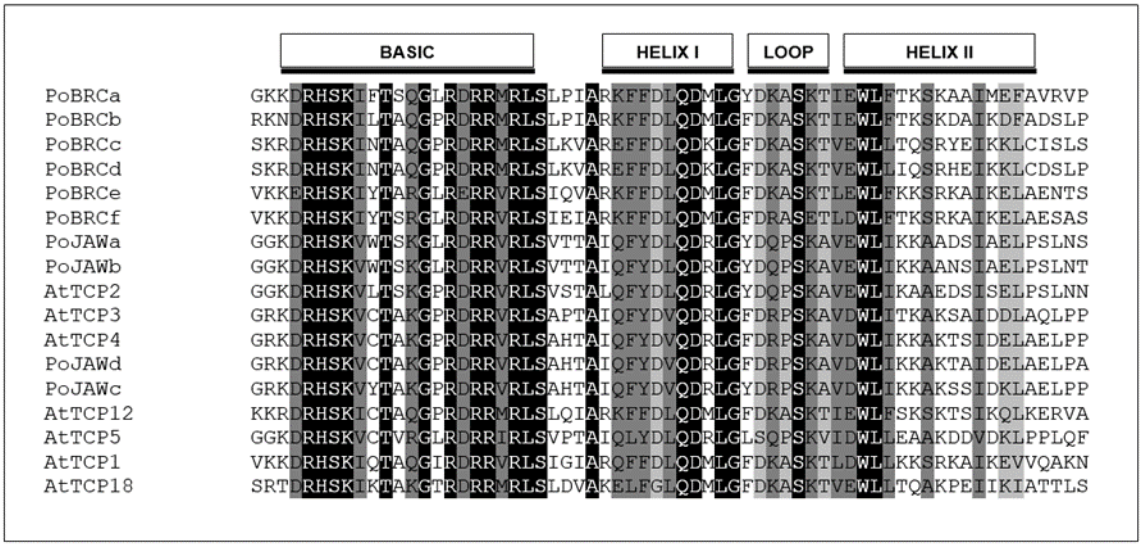


Figure 6 – Sequence alignment of the TCP domain of *Passiflora organensis* and *Arabidopsis thaliana*. Identical amino acids are shown in black boxes, while amino acids with similar charge or hydrophobicity are shown in grey. Amino acid sequences were aligned with Clustal X.

8.3.4 Interactions between *Passiflora* FT/TFL1 and bZIP proteins

To test whether *Passiflora organensis* bZIPs proteins are able to interact with their respective FT/TFL1 putative partners, we performed yeast two-hybrid (Y2H) assays (Figure 7) and obtained positive results for the interaction between FT-like proteins, such as: PoFT and PoTSFa, and bZIP TFs, such as PobZIP1, PobZIP5, and PobZIP6. Furthermore, PoTSFa had a stronger interaction with PoATC. In addition, the TFL1-like proteins: PoTFL1, PoBFT, an PoATC were also able to interact with the PobZIP proteins (Figure 7).

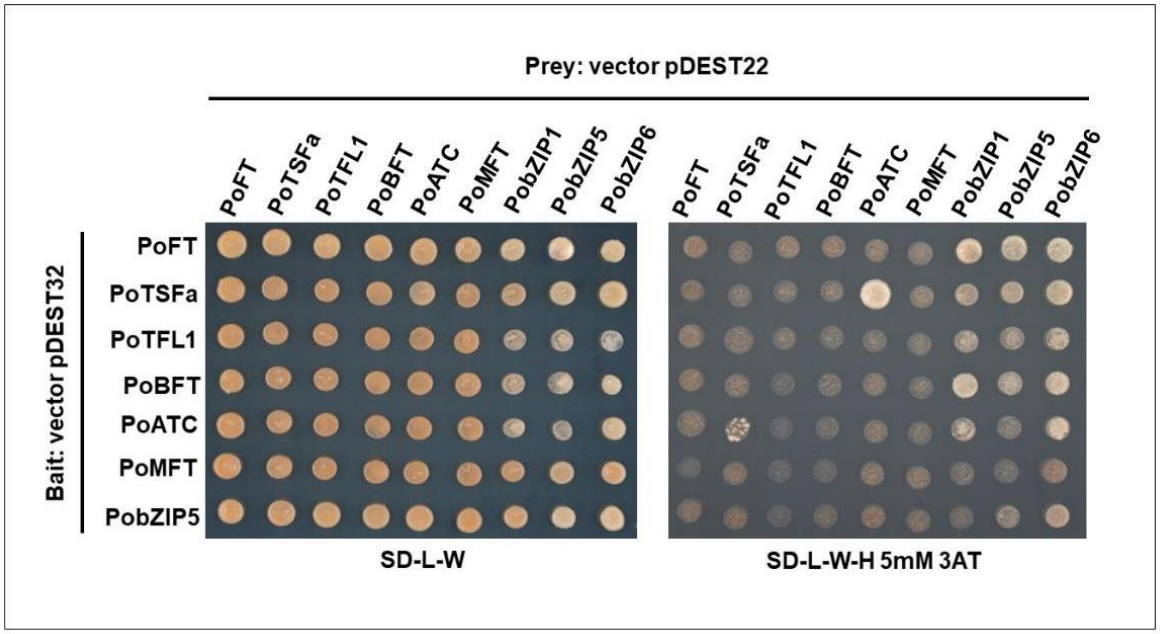


Figure 7 - **Yeast two-hybrid assays to test the interactions between *Passiflora organensis* FT/TFL1 and bZIP proteins**. SD-glu medium lacking leucine and tryptophan (-L-W) was used to select for yeast transformants containing both bait and prey vectors. Potential interaction capacity was tested on SD-glu medium lacking leucine, tryptophan, and histidine and supplemented with 5 mM of 3AT (3-amino-1,2,4-triazole).

8.3.5 *Passiflora* bZIP protein interact with Po14-3-3 while PoFT showed no interaction

There are evidences in the literature indicating that the proteins encoded by FT and bZIP transcription factors can interact with 14-3-3 proteins in grasses (Taoka et al. 2011; Li et al., 2015). To evaluate whether these *P. organensis* proteins can also interact with Po14-3-3, we performed Y2H analyses (Figure 8-9). We observed that among these proteins, only PobZIP5 protein was capable of interaction, and this occurred with almost all members of the 14-3-3 family, except for Po14-3-3D (Figure 8). However, no direct interactions were detected between *P. organensis* FT/TFL1 and 14-3-3 proteins.

We also tested the same matrix in the opposite direction (Figure 9), since PobZIP1 and PobZIP6 in the bait vector (BD; pDEST32) showed an intrinsic transcriptional activation activity, which caused the yeast to grow on selection medium, even without any protein interaction. Note that we used 20 mM of 3AT (3-amino-1,2,4-triazole) to inhibit the transcriptional auto-activation present also in the 14-3-3 proteins, and consequently preventing the yeast to grow on selection medium without any protein interaction. The results show that PobZIP1 and PobZIP6 have a stronger interaction with Po14-3-3C, Po14-3-3E, and Po14-3-3I (Figure 9).

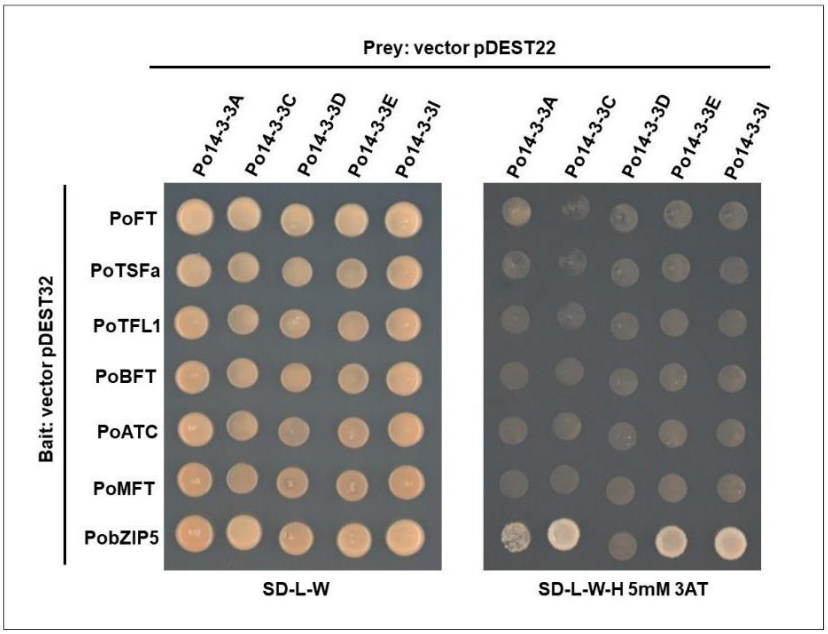


Figure 8 - **Yeast two-hybrid assays to test the interactions between *Passiflora organensis* FT/TFL1, bZIP, and 14-3-3**. SD-glu medium lacking leucine and tryptophan (-L-W) was used to select for yeast transformants containing both bait and prey vectors. Potential interactions were tested on SD-glu medium lacking leucine, tryptophan, and histidine and supplemented with 5 mM of 3AT (3-amino-1,2,4-triazole).

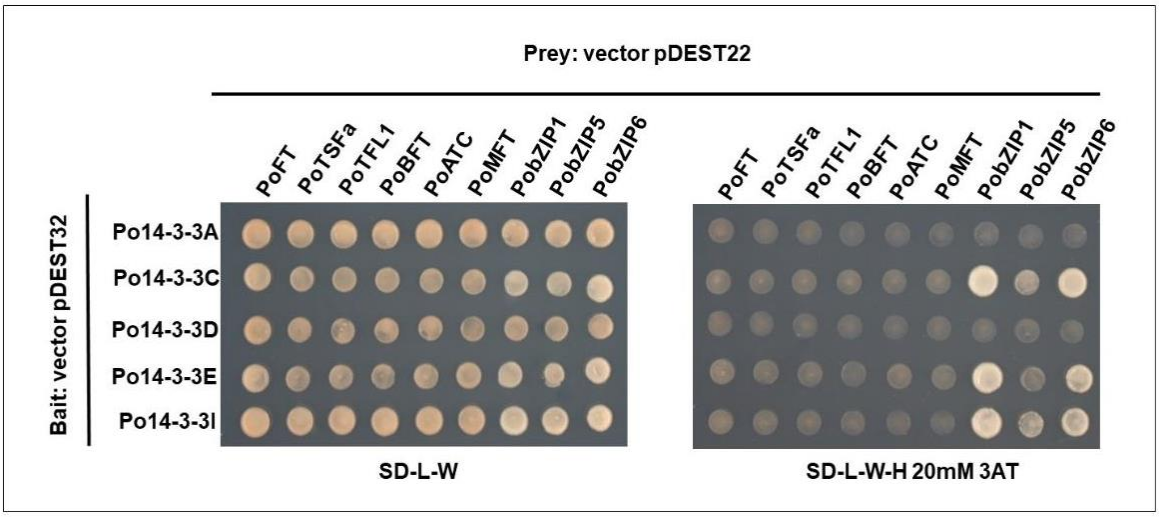


Figure 9 - **Yeast two-hybrid assays to test the interactions between *Passiflora organensis* FT/TFL1, bZIP, and 14-3-3**. SD-glu medium lacking leucine and tryptophan (-L-W) was used to select for yeast transformants containing both bait and prey vectors. Interactions strength were tested on SD-glu medium lacking leucine, tryptophan, and histidine and 20 mM of 3AT (3-amino-1,2,4-triazole).

As described in the literature, the flowering activation complex (FAC) is a hexameric protein complex formed by two FT monomers and two FD transcription factors that interact with a dimeric 14-3-3 protein bridge (Taoka et al., 2011). To observe how the *P. organensis* 14-3-3 proteins interact with each other we performed a Y2H matrix (figure 10). The results showed that Po14-3-3C, Po14-3-3E, and Po14-3-3I can interact with themselves to form homodimers and with each other to form heterodimers, including heterocomplexes with Po14-3-3A.

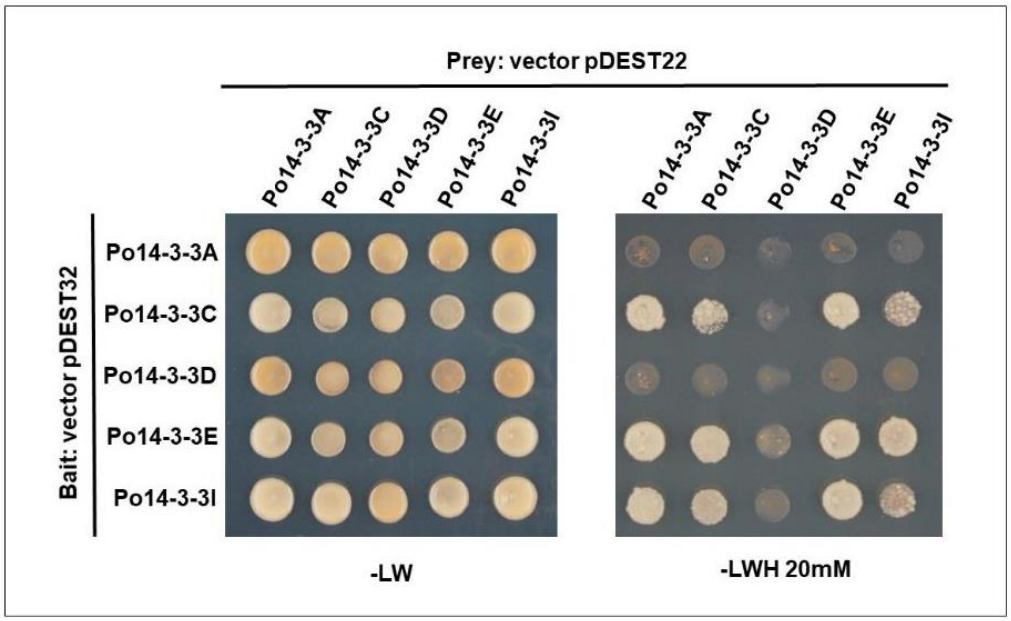


Figure 10 - **Yeast two-hybrid assays to test the interactions between *Passiflora organensis* 14-3-3s**. SD-glu medium lacking leucine and tryptophan (-L-W) was used to select for yeast transformants containing both bait and prey vectors. Potential interaction events were tested on SD-glu medium lacking leucine, tryptophan, and histidine and supplemented with 20 mM of 3AT (3-amino-1,2,4-triazole).

8.3.6 *Passiflora* FT:bZIP:14-3-3 proteins showed no interaction

Because our results suggested that the interaction patterns among PoFT and Po14-3-3 proteins differ from what have been proposed for other species, we decided to investigate whether a traditional FAC exists in *Passiflora.* For this, we performed a yeast three-hybrid (YH3) analysis using a bZIP protein as a linker between FT and 14-3-3 putative orthologs from *P. organensis*. Because PobZIP1 and PobZIP6 showed an intrinsic activation domain, we used PoBZIP5 as a potential linker between the *P. organensis* FT-like and 14-3-3 proteins. Our results showed no complex formation between these proteins (Figure 11).

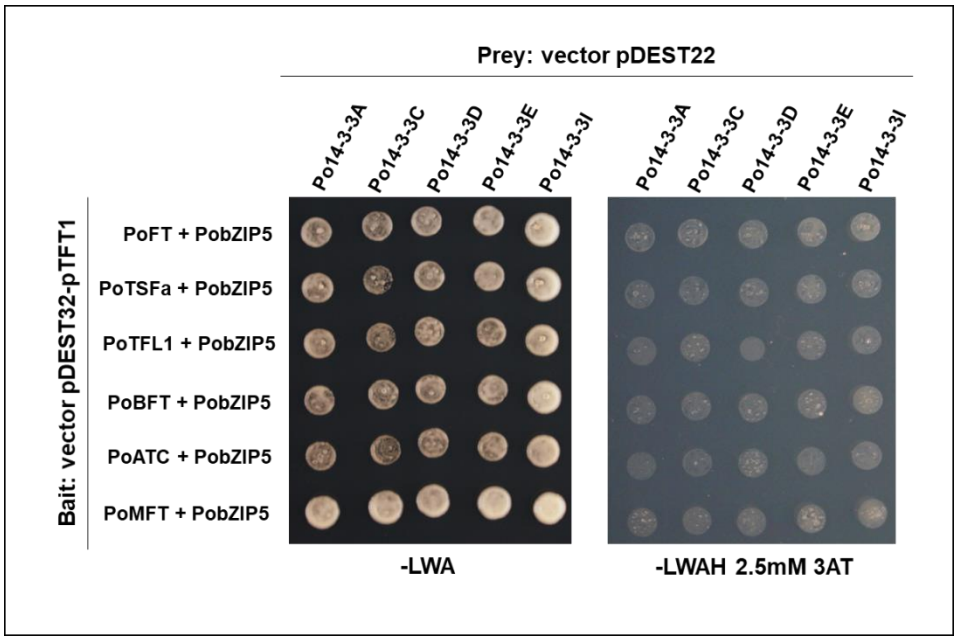


Figure 11 - **Yeast three-hybrid assays showing no interactions between *Passiflora organensis* FT/TFL1:bZIP5:14-3-3 proteins**. SD-glu medium lacking leucine and tryptophan (-L-WA) was used to select for yeast transformants containing both bait and prey vectors. Potential interaction events were tested on SD-glu medium lacking leucine, tryptophan, adenine, and histidine and supplemented with 2.5 mM of 3AT (3-amino-1,2,4-triazole).

8.3.7 *Passiflora* FT and TSFa proteins interact with multiple TCP proteins

In order to test whether *Passiflora* FT-like and CYC/TB1 class II TCP proteins interact, being able to have an important role in controlling development of axillary buds, and consequently may be involved in the formation of tendrils, lateral branches or flowers in *Passiflora* species we performed a Y2H analysis. PoFT was able to interact with almost all members of the TCP family in the matrix, except for PoBRCe, while PoTSFa was able to interact only with PoBRCf. The other *Passiflora* members of the FT/TFL1 family did not show any interaction with the TCP proteins tested (Figure 12).

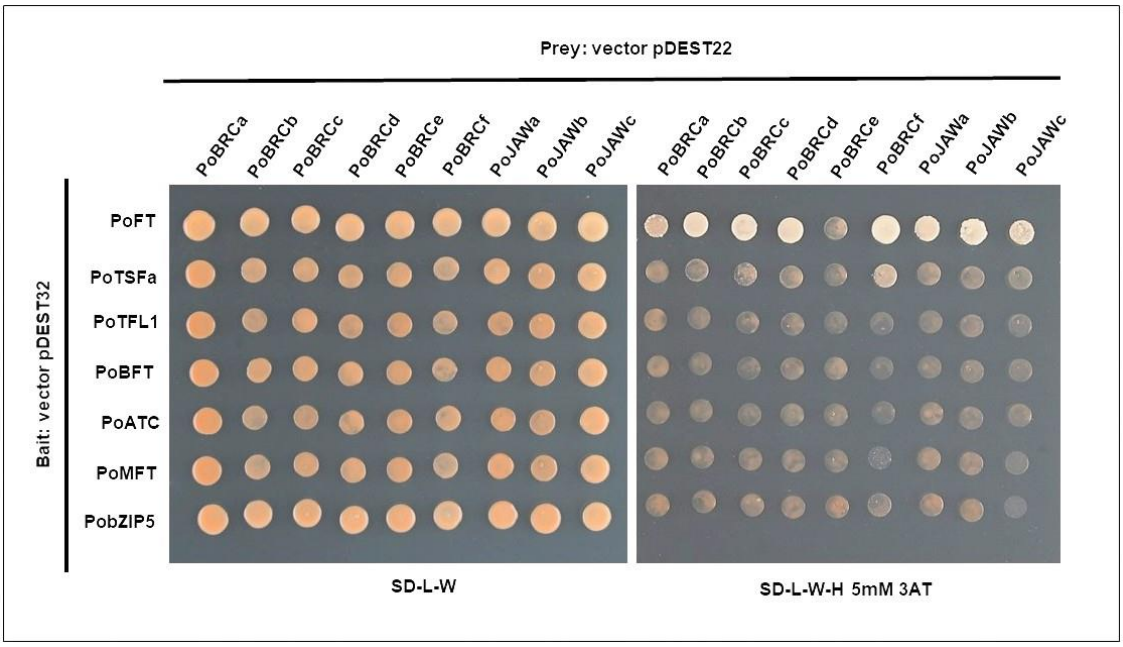


Figure 12 - **Yeast two-hybrid assays to test the interactions between *Passiflora organensis* FT/TFL1, bZIP, and TCP proteins.** SD-glu medium lacking leucine and tryptophan (-L-W) was used to select for yeast transformants containing both bait and prey vectors. Interactions strength were tested on SD-glu medium lacking leucine, tryptophan, and histidine and supplemented with 5 mM of 3AT (3-amino-1,2,4-triazole).

8.4 Discussion

The basic leucine zipper transcription factor gene family is one of the largest and most diverse families in plants (Abe et al., 2005). In our phylogenetic analysis, we included only the proteins belonging to the group A of bZIP proteins. In *Arabidopsis* there are 75 *bZIP* genes clustered into 10 groups (groups A-I and S; Jakoby et al, 2002). FD (AtbZIP14) and FDP (AtbZIP27) belong to group A and they are key genes for the flowering process, whose encoded proteins interact with FT, resulting in the induction of flowering (Abe et al., 2005). Therefore, we selected four *P. organensis* bZIP genes that were the closest putative orthologs of FD and FDP in *Arabidopsis*. We named them: PoBZIP1 (PobZIP159), PobZIP2 (PobZIP10547), PobZIP3 (PobZIP12122), PobZIP4 (PobZIP10155). We then also selected PobZIP5 (PobZIP14) and PobZIP6 (PobZIP607) that were in the same branch of the barley FD. Studies have shown that the rice, wheat, and barley orthologs of FT, FD and 14-3-3 proteins interact to form a florigen activation complex (Li et al., 2015).

We observed that *P. organensis* FT-like and TFL1-like proteins interact with bZIP proteins. These observations suggest the possibility of competition among the proteins that would be involved in flowering activation (such as PoFT and PoTSFa), with the proteins that

would be involved in flowering repression (such as PoTFL1, PoBFT, and PoATC), for the interaction with the bZIP proteins. Thus, this competition would be an important factor balancing determinate and indeterminate growth, which in turn would cause an impact in inflorescence development and terminal flower formation.

Although many studies have shown the importance of the interaction between FT-like proteins and FD during plant development (Abe et al., 2005; Wigge et al., 2005, among others), the literature has also shown TFL1-like proteins which function through interactions with bZIP proteins. For example, the cotton FD could interact with the cotton TFL1-like protein, and by antagonizing the FT activity in the apex, act as transcriptional repressor of flowering transition, leading to prolonged vegetative growth (Prewitt et all, 2018). Hanano and Goto (2011) showed that the *Arabidopsis* TFL1 negatively modulates the FD-dependent transcription of target genes to adjust flowering time and the development of the inflorescence meristem. In addition, *Chrysanthemum* antiflorigenic CsAFT (TFL1-like protein) inhibits flowering by direct competition with FT for FD binding (Higuchi et al., 2013). Finally, the *Arabidopsis* BFT protein have been shown to delay flowering under high salinity by competing with FT for binding to the FD transcription factor (Ryu et al., 2014). Furthermore, our results show that PoTSFa interacts strongly with PoATC, suggesting a potential competition during floral initiation, since ATC may contribute with an antiflorigenic activity in *Arabidopsis* (Huang et al., 2012).

There are evidences in the literature that the proteins encoded by the FT and bZIP transcription factors can interact with 14-3-3 proteins (Taoka et al. 2011; Li et al., 2015). In tomato, Pnueli et al. (2001), showed evidences that the SELF-PRUNING (SP), an ortholog of Arabidopsis FT, could interact with a diverse classis of regulatory proteins, e.g 14-3-3, to regulate shoot architecture and flowering. In rice, for instance, it has been shown that the FT–FD interaction is mediated by 14-3-3 proteins forming the florigen activation complex (FAC) that promotes flowering in short-day conditions (Taoka et al. 2011), in which complex formation can be largely combinatorial (Cerise et al., 2020). In contrast, a recent study (Wen et al., 2019) showed some evidences that the FAC in Cucumber is probably not conserved like in rice. Thus, although studies have shown that 14-3-3 proteins can interact with FT-like and FD-like proteins, the evidence for the bridging function is sparse and limited.

To test the existence of a *Passiflora* FAC, we identified 14-3-3 genes in the *P. organensis* genome. Based in our phylogenetic results, the *Passiflora* 14-3-3 proteins were clustered into two distinct evolutionary groups, namely, non-epsilon and epsilon groups.

Epsilon group members commonly contained more introns and motifs than the non-epsilon group (Pallucca et al., 2014). In *Arabidopsis,* there are five 14-3-3 epsilon proteins: mu, epsilon, pi, iota, and omicron and eight 14-3-3 non-epsilon proteins: kappa, lambda, psi, nu, upsilon, omega, phi, and chi. Studies have shown the expression of 14-3-3 χ (chi) in multiple floral organs, such as petals, pistils, and stems, while 14-3-3 ν (nu) knockout lines result in a flowering delay (Mayfield et al., 2007). In addition, the wheat FT1 protein interacts with multiple 14-3-3 proteins from the non-epsilon group (Li et al., 2015). These results suggested that most of 14-3-3 proteins acting as positive regulators of floral development belong to the non-epsilon group. Following up on this, we selected five *P. organensis* 14-3-3 proteins for further analysis: Po13-3-3A, Po14-3-3C, Po14-3-3E, and 14-3-3I, belonging to the non-epsilon group, and Po14-3-3D belonging to the epsilon group (used as a control).

We showed that Po14-3-3C, Po14-3-3E, and Po14-3-3I can form homodimers and interact with one another to form heterodimers, including heterocomplexes with Po14-3-3A. The ability of 14-3-3 proteins to form both homo- and hetero-dimers increases the combinatorial possibilities for potential specialized roles, increasing their potential functional diversity (Wilson et al., 2016).

We have also demonstrated that *P. organensis* bZIP proteins interact with Po14-3-3 proteins while the FT/TFL1 family members show no interaction with the Po14-3-3 proteins tested. These results were similar to those obtained with cucumber, where CsFT interacts with CsFD and CsFDP, and CsFD interacts with a cucumber 14-3-3 protein, however, no direct interactions are observed among CsFT and 14-3-3 proteins (Wen et al., 2019). Taken together Wen et al (2019) and our results, we hypothesize that FACs are likely not conserved and the formation of such complexes may be specific to monocots.

Furthermore, previous studies demonstrated that TCP proteins play vital roles in plant growth and development being able to interact with FT-like proteins (Manassero et al., 2013; Niwa et al., 2013; Li, 2015). We showed that there are six *P. organensis* proteins belonging to the CYC/TB1 subclade of TCP class II, while in *Arabidopsis* there are only three proteins. PoBRCa, PoBRCb, PoBRCc, PoBRCd, PoBRCe, and PoBRCf are the closest homologs to *Arabidopsis* TCP1, TCP12, and TCP18, that have been shown to be the central integrators for multiple environmental and developmental factors, regulating shoot branching (Finlayson, 2007; Aguilar-Martinez et al., 2007; Niwa et al., 2013). Niwa and collaborators (2013) showed that TCP18 (BRANCHED1) interacts with FT and interferes with its activity to suppress flowering in axillary meristems. *P. organensis* axillary meristems are more complex than those formed in *Arabidopsis* (Nave et al., 2010; Cutri et al., 2013). A possible

hypothesis for this observed complexity could be related to the gene duplication events observed in the *P. organensis* CYC/TB1 subclade. The TCPs may modulate the activity of FT-like proteins in the axillary buds to repress the premature floral transition of axillary meristems and, as a result, regulate shoot flowering and branching (Niwa et al, 2013).

In *Arabidopsis* CIN-like TCP factors seem to crosstalk with multiple different cellular pathways to control leaf and floral organ development (Palatnik et al., 2003; Koyama et al., 2007; Van Es et al, 2018). We showed that PoFT was able to interact with almost all members of the PoTCP family in the matrix, except for PoBRCe, while PoTSFa was able to interact only with PoBRCf. The other members of the *P. organensis* FT/TFL1 family did not show any interaction with the tested *P. organensis* TCP proteins. The evidence of these interactions between FT-like and TCPs proteins is an important start for future studies to investigate the balance of genes in order to understand the modulation of AMs giving rise to different structures, such as the tendrils and flowers in *Passiflora* species.

8.5 Conclusions

The regulation of flowering time and branching is an important target for plant breeding. We showed that PoFT interacts with PobZIP proteins, and PobZIP proteins interact with some of the Po14-3-3 proteins. However, no direct interactions were detected between PoFT and 14-3-3 proteins. These results suggest that the flowering activation complex in *Passiflora organensis* is different from the proposed current model systems, and apparently the differences in the FAC might occur between species. Furthermore, the *P. organensis* FT-like proteins can interact with multiple *P. organensis* TCP proteins. Thus, the results from this study expands the understanding of the mechanisms by which the putative *P. organensis* FT/TFL1 orthologs interact with their putative molecular partners and the mechanisms that lead to their functions during the flowering and branching processes. The control of flowering time and the control of plant architecture can provide important resources for passionfruit breeding in the future.

8.6 Supplementary Material

Primer	Sequence	Amplicon (pb)	Annealing Temperature (°C)
PoFT - F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGCCTAGGGATAGAGACTCCCTC	600	58
PoFT - R	GGGGACCACTTTGTACAAGAAAGCTGGGTAGCGCTTCATCTTCGGGAAT		
PoTSFa - F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGCCCAGAAGTAGAGATCC	561	58
PoTSFa - R	GGGGACCACTTTGTACAAGAAAGCTGGGTAGAGGCTGGCTGGCTGGTT		
PoTFL1 - F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGCAAATGTGTCGGATCCG	561	58
PoTFL1 - R	GGGGACCACTTTGTACAAGAAAGCTGGGTACAACCCTGCTGCTGATTTTT		
PoBFT - F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTCAAGAGCCATGGAACC	611	58
PoBFT- R	GGGGACCACTTTGTACAAGAAAGCTGGGTAAACCCATACCCAACCCTAGC		
PoATC - F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGCAAAAATGTCTGATCC	723	58
PoATC - R	GGGGACCACTTTGTACAAGAAAGCTGGGTATTGATGGACAACGCAAAGAG		
PoMFT - F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTCTGCCTCGGTTGATCC	548	58
PoMFT - R	GGGGACCACTTTGTACAAGAAAGCTGGGTACCCAATCCCATCGTCAATTACT		
PobZIP1 - F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTTGTCATCAACAGGTG	833	58
PobZIP1 - R	GGGGACCACTTTGTACAAGAAAGCTGGGTAGAACGGAACACTAATACTCA		
PobZIP5 – F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAGAGAAATTTGTTGAATGGG	1230	58
PobZIP5 - R	GGGGACCACTTTGTACAAGAAAGCTGGGTATCACATGGGAGATGCAAG		
PobZIP6 – F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGCTGAGAATGGGATCTC	1034	58
PobZIP6 - R	GGGGACCACTTTGTACAAGAAAGCTGGGTACCATTGGCATTGGATTGC		

F = *forward;* R = *reverse*

S1 - Primers used for Y2H and Y3H analyses

Primer	Sequence	Amplicon (pb)	Annealing Temperature (°C)
PoBRCa – F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTTTTCATCCTCAGGTAGCA	1501	58
PoBRCa - R	GGGGACCACTTTGTACAAGAAAGCTGGGTAGCCATTCGGGAAACTATATC		
PoBRCb – F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTTTTCTTCTGGCAGC	1637	58
PoBRCb - R	GGGGACCACTTTGTACAAGAAAGCTGGGTATAGGCTTCAACTGTTGTG		
PoBRCc – F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCCATGTTTCCGTCTACCAAC	1254	58
PoBRCc - R	GGGGACCACTTTGTACAAGAAAGCTGGGTAGAACTAGACCAGAGTTTGGATG		
PoBRCd – F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTTTCCGTTTGCCAAC	1309	58
PoBRCd - R	GGGGACCACTTTGTACAAGAAAGCTGGGTAGTCATCTTGGTGTATCGAGC		
PoBRCe – F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTTCTCTTCCAATGGTTC	1181	58
PoBRCe - R	GGGGACCACTTTGTACAAGAAAGCTGGGTATCAGTGTGGACACTGATTC		
PoBRCf – F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTTCTCTACCACTAACTCTTC	1179	58
PoBRCf - R	GGGGACCACTTTGTACAAGAAAGCTGGGTATTAACGTGGACATCGATTCC		
PoJAWa – F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGAGGCGGACGATATTC	1383	58
PoJAWa - R	GGGGACCACTTTGTACAAGAAAGCTGGGTACAGTTCTTTCCCTTTCCTTTC		
PoJAWb – F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCCGAAAGGCATGAACTTGC	1476	58
PoJAWb - R	GGGGACCACTTTGTACAAGAAAGCTGGGTAGTTCTTCCCTTTGCCTTTATG		
PoJAWc – F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAATGAACAGCTCAGGAGG	1244	58
PoJAWc - R	GGGGACCACTTTGTACAAGAAAGCTGGGTATCAATGCTGAGAATTGGGAG		

F = *forward;* R = *reverse*

Primer	Sequence	Amplicon (pb)	Annealing Temperature (°C)
Po14-3-3A – F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCCAAACAATGGCCGTGACCAC	795	58
Po14-3-3A - R	GGGGACCACTTTGTACAAGAAAGCTGGGTAGCTTATGGTGCCCTTTCA		
Po14-3-3C – F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTCGCCCACAGAATCATC	799	58
Po14-3-3C - R	GGGGACCACTTTGTACAAGAAAGCTGGGTACCTACTGAACCCGTCATTA		
Po14-3-3D – F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCTGTGAAGAGATGGTTGAAGC	827	58
Po14-3-3D- R	GGGGACCACTTTGTACAAGAAAGCTGGGTAGCAGATCACAAATCGCAACC		
Po14-3-3E – F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGCTGTAACCACCGTC	827	58
Po14-3-3E - R	GGGGACCACTTTGTACAAGAAAGCTGGGTACTCCTAGGGTAACTACTATCC		
Po14-3-3I – F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCTTCGTGTCTCGCAAAATGGCAG	814	58
Po14-3-3I - R	GGGGACCACTTTGTACAAGAAAGCTGGGTACAGCGGATGGTACTCACTG		

F = *forward;* R = *reverse*

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CHAPTER VI

Heterologous functional analyzes by overexpression of *Passiflora organensis FT/TFL1* genes in the plant model system *Arabidopsis thaliana*

Passiflora organensis FT/TFL1 overexpression T1 plants

9. Heterologous functional analyzes by overexpression of *Passiflora organensis FT/TFL1* genes in the plant model system *Arabidopsis thaliana*

Abstract

The plant-specific *FT/TFL1* gene family comprises three subfamilies: *FLOWERING LOCUS T*-like (*FT*-like), *TERMINAL FLOWER 1*-like (*TFL1*-like), and *MOTHER OF FT AND TFL1*-like (*MFT*-like). The members of this family are known to be involved in the transition from vegetative to reproductive phase, regulating the identities of determinate versus indeterminate meristems, flowering time, and plant architecture. Considering the very limited knowledge about *FT/TFL1 Passiflora* orthologs and their role in regulating flowering in this genus, our aim was to validate and explore the putative function of *Passiflora organensis FT/TFL1* genes in flowering regulation using *Arabidopsis thaliana* as an heterologous system. Ectopic expression of *PoFT* in transgenic *Arabidopsis* plants induced early flowering and inflorescence determinacy. However, ectopic expression of its paralog *PoTSFa* caused a delay in flowering. *PoTFL1* and *PoATC* ectopic expression caused delayed flowering in *Arabidopsis*, as it would be expected for a gene related to the *TFL1*-like subfamily, however the superexpression of *PoBFT* showed no significant change in the phenotype when compared to *Arabidopsis* Col-0 wild-type plants. Furthermore, *PoMFT* ectopic expression causes a slight reduction of flowering time suggesting a possible redundant role in flowering promotion. Elucidation of the flowering mechanism in *Passiflora* would be helpful for breeding studies aiming to enhance passionfruit yield.

Keywords: Florigen. Flowering time. *FT*-like. *MFT*-like. Passionfruit. *TFL1*-like.

9.1 Introduction

Flowering is considered a key event in the plant life. In the model plant *Arabidospis thaliana*, the transition from vegetative to reproductive phase is mainly controlled by four major pathways, including photoperiod, vernalization, hormone and autonomous regulation (Kardailsky et al., 1999; Samach et al., 2000; Blázquez et al., 2003; Golembeski and Imaizumi, 2015). These pathways converge to regulate the floral integrator genes such as: *FLOWERING LOCUS T* (*FT*; Turck et al. 2008), *SUPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*; Lee and Lee, 2010), and the floral identity genes *LEAFY* (*LFY*; Maizel et al., 2005), *APETALA1* (*AP1*; Litt and Kramer, 2010), its paralog *CAULIFLOWER* (*CAL*; Alvarez-Buylla et al., 2006), and *FRUITFULL* (*FUL*; Pabon-Mora et al., 2012).

The *FT/TFL1* gene family controls important aspects of plant development: *FT*-like genes are floral activators, *TFL1*-like genes act as floral inhibitors, and *MFT*-like genes affect germination (Wickland and Hanzawa, 2015). The product of the *Arabidopsis* gene *FLOWERING LOCUS T* (*FT*) induces flowering in a photoperiod-dependent manner, whereas the product of another member of the *FT/TFL1* gene family, *TERMINAL FLOWER 1* (*TFL1*) delays flowering and is responsible for the maintenance of the inflorescence meristem (Wickland and Hanzawa, 2015). Despite the opposite effects on flowering time, their protein sequences present high similarity (Hanzawa et al., 2005; Ahn et al., 2006). Studies have shown the importance of the tyrosin residue at position 85 (Tyr-85) for FT to function as a flowering inducer, and a histidine at position 88 (His-88) for TFL1 to function as a flowering repressor. A single base change of these amino acids may cause conversion of functions between FT and TFL1 (Hanzawa et al., 2005; Ahn et al., 2006; Ho; Weigel, 2014; Wang et al., 2017).

During evolution, some FT homologous genes acquired the function of suppressing flowering. In some species, paralogs of FT assumed a repressive function, whether others maintained a flowering induction function (Kotoda et al., 2010; Pin et al., 2010; Hsu et al., 2011; Harig et al., 2012). For example, in *Dimocarpus longan*, a subtropical fruit tree cultivated mainly in Asia, ectopic expression of the *D. longan* FT putative ortholog genes in transgenic *Arabidopsis* plants, resulted in different flowering time phenotypes. The analysis of the expression patterns of these *D. longan* genes revealed that DlFT1 acts as a flowering promoter, while DlFT2 acts as a flowering inhibitor (Winterhagen et al., 2013). In cultivated beet (*Beta vulgaris*), flowering only occurs when plants are vernalized, and they are unable to form reproductive shoots during the first year of their life cycle. In beets, Pin et al. (2010)

showed the regulation of flowering time being controlled by the interplay of two FT paralogs, BvFT1 and BvFT2, acting with antagonistic functions: BvFT2 is essential for flowering induction and shows a conserved function with FT , on the other hand, BvFT1 represses flowering and the vernalization response in *B. vulgaris* is dependent on the down-regulation of BvFT1. In *Nicotiana tabacum,* four *FT*-like genes have been isolated and all of them are expressed in leaves under short-day conditions. NtFT1, NtFT2, and NtFT3 proteins are floral inhibitors, whereas only NtFT4 is a floral inducer (Harig et al., 2012). The ectopic expression of GmFT4, one of the soybean orthologs of FT, delay flowering time in transgenic *Arabidopsis,* whereas GmFT2a and GmFT5a act as flowering activators (Zhai et al., 2014). In sunflower, *Helianthus annuus,* there are four FT homologs, one of which resulted from a mutation that yielded a novel repressor function (Blackman et al., 2010).

TWIN SISTER OF FT (TSF) is the closest paralog to FT in *Arabidopsis* and has a similar function (Yamaguchi et al. 2005). Upon the induction of flowering, the expression of both genes is up-regulated in phloem cells and their encoded proteins move from leaves to the shoot apical meristem (SAM). However, TSF is less mobile than FT, suggesting that it could have a major role in leaves. The overexpression of *FT* causes early flowering and conversion of the SAM into a terminal flower, while the *ft* mutant flowers late and presents indetermined growth (Yamaguchi et al., 2005; Cobesier et al., 2007). Moreover, *TSF* overexpressing plants flower extremely early and the *tsf* mutant delays flowering only in short-day conditions. In contrast, under long-day conditions the effect of *tsf* loss of function is very weak or almost undetectable. The double mutant *tsf/ft* shows additive effects in both short- and long-day conditions (Yamaguchi et al., 2005).

TFL1 expression is detected in the SAM and it maintains the indeterminate growth and represses the floral meristem identity genes (Shannon; Meeks-Wagner, 1991; Hanano; Goto, 2011). Thus, *tfl1* mutants flower early and their SAM is converted into a terminal flower (Conti; Bradley, 2007). The other *TFL1*-like genes *ARABIDOPSIS THALIANA CENTRORADIALIS HOMOLOG (ATC) and BROTHER OF FT AND TFL1 (BFT)* also represses floral transition, but *ATC* expression occurs preferentially in short-day conditions (Huang et al., 2012), while *BFT* has shown an expression pattern similar to *FT* in the leaves (Yoo et al., 2010; Ryu et al, 2014).

MOTHER OF FT AND TFL1 (*MFT*) belongs to the *MFT*-like subfamily which is the evolutionary ancestor of *FT*-like and *TFL1*-like subfamilies **(**Hedman et al., 2009; Wang et al., 2015). Compared to FT and TFL1, which functions in the control of flowering time and plant architecture (Moraes et al., 2019), the exact biological function of MFT is not well understood. The *mft* mutant has no effect in flowering time, however *MFT* overexpression causes a slight reduction in flowering time suggesting that MFT may have a redundant role in promoting flowering (Yoo et al., 2004). In addition, MFT has a role in seed germination and its expression is promoted by ABA signaling during this process (Yu et al., 2019).

In *Passifora* there is hardly any knowledge about the molecular mechanisms involved in the vegetative to reproductive phase transition. Little research has been reported investigating the functions of *Passiflora* flowering time genes (Cutri; Dornelas, 2012; Scorza et al., 2017). Here, we report the functional analysis of orthologs for *FT/TFL1* genes from *Passiflora organensis*, a small herbaceous vine, from the same genus of the economically important passionfruit. The ectopic expression of these genes, that either promote or delay flowering in *Arabidopsis,* were demonstrated. Furthermore, taking into account what is already reported in the literature, we provide evidence that the alteration in the flowering time in transgenic *Arabidopsis* plants with ectopic expression of *PoTSFa* could be due a single base change in the *P. organensis* protein.

9.2 Materials and Methods

9.2.1 Plant material and growth conditions

Arabidopsis seeds were stratified for 2–3 days, at 4°C, and plants were grown under long-day conditions (16h light), at 20–23°C in 5x5 cm pots with soil, or on rockwool (Grodan Delta). Three independent segregating *Arabidopsis* lines transformed with overexpression constructs containing *Passiflora organensis FT/TFL1* genes were selected and approximately 30 plants of each line (T2 generation plants) were used for phenotyping. Flowering time was scored by counting the number of rosette leaves until bolt (inflorescence length of 1 mm). The number of rosette leaves were analyzed the using t-test through the Sigma Plot 12.0 software. All data were submitted and passed normality test (Shapiro-Wilk) and equal variance test. The input groups differedsignificantly ($P = < 0.05$; $P = <0.001$).

9.2.2 Cloning of the *FT/TFL1 Passiflora* genes

Primers containing attP1 and attP2 sites for recombinational cloning (see Table S1) were designed for the full-length coding sequences of *P. organensis FT/TFL1* genes identified previously (see Chapter II). Total RNA was extracted from *P. organensis* shoot apices, using RNeasy® Plant Mini Kit (50) - QIAGEN. The first-strand cDNA was synthesized with a SuperScript® III First-Strand Synthesis kit - InvitrogenTM, according to the manufacturer's instructions. PCR was performed with Q5® High-Fidelity DNA Polymerase (NEB) on the StepOne Real-Time PCR System (Applied Biosystems). PCR-amplified fragments were cloned into pDONR201 entry clone (Invitrogen, Carlsbad, CA, USA), and subsequently transferred to pK2GW7 (Invitrogen, Carlsbad, CA, USA) destination vector, downstream of the cauliflower mosaic virus 35S promoter (CaMV 35S), using the GATEWAY Cloning System (Life Technologies, Grand Island, NY, USA). All constructs were verified by sequencing.

9.2.4 Overexpression of *Passiflora FT/TFL1* genes in *Arabidopsis*

The resulting overexpression constructs were transferred into *Agrobacterium* C58C1 by electroporation and then transformed by floral dip (Clough and Bent, 1998) into *A. thaliana* Columbia C0 wild-type (*Col* or WT) plants. Putative transgenic plants were screened *in vitro* on MS medium (Murashige and Skoog, 1962) supplemented with kanamycin (25 $\mu g.ml^{-1}$) and timentin (50 $\mu g.ml^{-1}$), as described elsewhere (Ferrario et al., 2004).

9.2.5 Multiple protein sequence alignment

Identified proteins in *Passiflora organensis* (see Chapter II) were aligned with sequences of other species using the Clustal X program (Thompson et al., 1997). Protein sequences of other plant species used in this study were obtained from the NCBI protein database (http://www.ncbi.nlm.nih.gov/).

9.3 Results

Considering that there are conserved amino acid residues important for the function of inducing or repressing flowering (Hanzawa et al., 2005; Wickland; Hanzawa, 2015), we performed comparisons of deduced *P. organensis* FT/TFL1 protein sequences with those from *Arabidopsis thaliana*, *Allium cepa*, *Beta vulgaris*, *Glycine max*, *Helianthus annuus*, *Nicotiana tabacum*, *Saccharum officinarum*, and *Picea abies* (Figure 1). We showed that *P. organensis* FT protein contains a tyrosine residue at position 134 and a tryptophan residue at position 138 (based on the *Arabidopsis* FT protein sequence). In contrast, PoTSFa displayed a tyrosine at position 134 and an arginine at position 138. Other members of *P. organensis* FT/TFL1 family, such as PoTFL1, PoBFT, PoATC, and PoMFT contained amino acids other than tyrosine and tryptophan at positions 134 and 138, respectively.

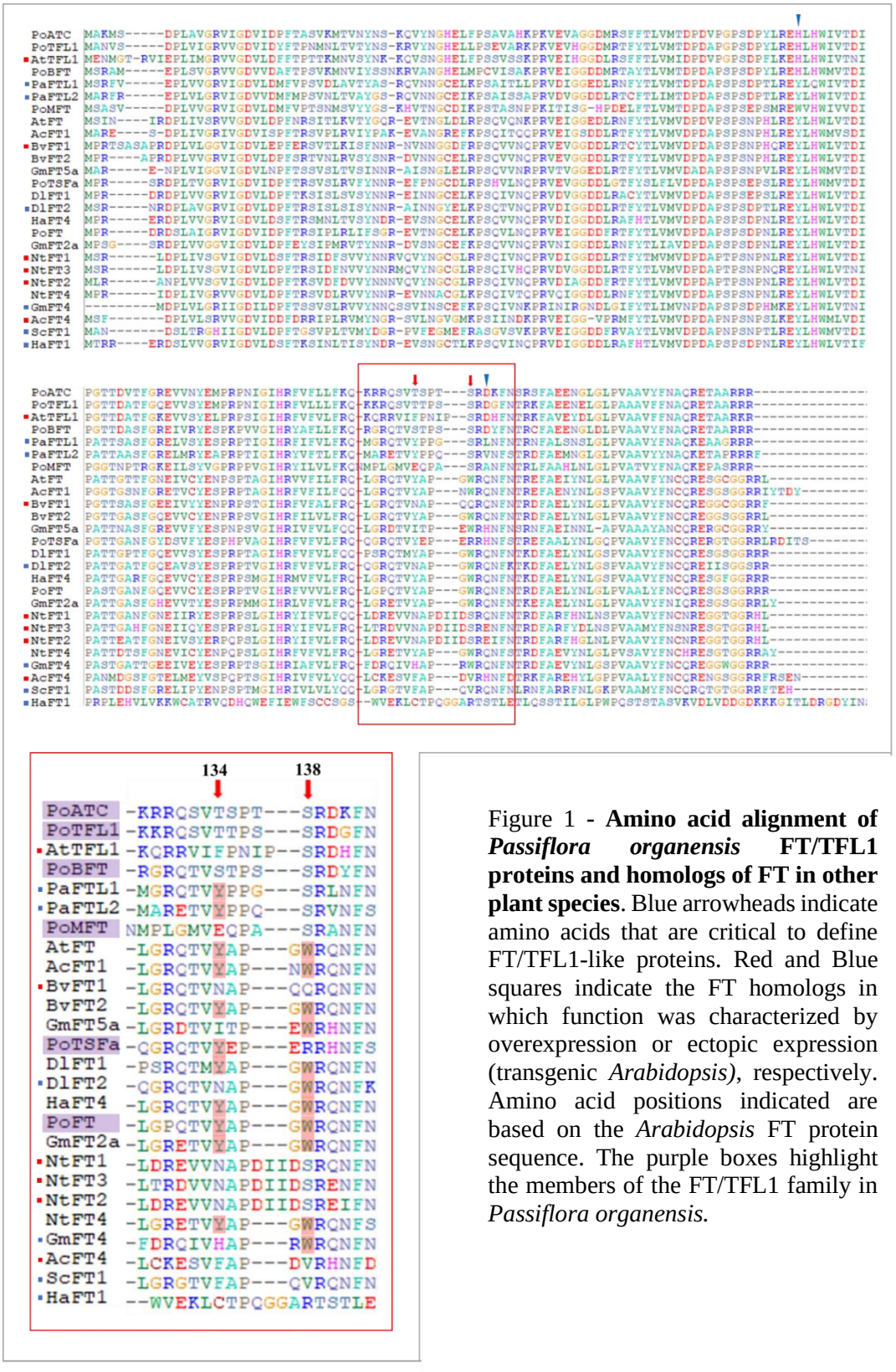

Figure 1 - **Amino acid alignment of *Passiflora organensis* FT/TFL1 proteins and homologs of FT in other plant species**. Blue arrowheads indicate amino acids that are critical to define FT/TFL1-like proteins. Red and Blue squares indicate the FT homologs in which function was characterized by overexpression or ectopic expression (transgenic *Arabidopsis)*, respectively. Amino acid positions indicated are based on the *Arabidopsis* FT protein sequence. The purple boxes highlight the members of the FT/TFL1 family in *Passiflora organensis.*

To explore the putative functions of *Passiflora FT/TFL1* genes in flowering regulation, the functional analyses of *PoFT*, *PoTSFa*, *PoTFL1*, *PoBFT*, *PoATC*, and *PoMFT* were performed in *Arabidopsis*, under long-day conditions. Flowering time measurements indicated that *35S::PoFT/Col* plants presented early flowering (Figure 2A). The average number of leaves in the three lines studied was 9.9 ± 2.5 (Table 1; Figure 3). In contrast, *35S::TSFa/Col* plants showed late flowering compared to the control non-transgenic plants (Figure 1), with an average of 21.4 ± 4.0 leaves per plant (Table 1; Figure 3).

Ectopic expression of the *TFL1*-like genes induced late flowering (Figure 2C), except for the plants overexpressing *PoBFT*, that showed no significant difference compared to non-transgenic plants. The average number of leaves was 20.7± 6.0 (Table 1; Figure 3). *35S::TFL1/Col* plants had an average of 21.0 ± 3.8 leaves for the three lines evaluated (Table 1; Figure 3), while *35S::ATC/Col* plants showed an average of 23.8 ± 3.9 leaves (Table 1; Figure 3). In addition, around 50% of *35S::TFL1/Col* and *35S::ATC/Col* plants exhibited a strong phenotype with defects in flower organ identity (Figure 2D). Moreover, flowering time measurements indicated that *35S::PoMFT/Col* plants showed a slight reduction of flowering time and serrated leaves (Figure 2B); the average number of leaves was 17.1 ± 1.9 (Table 1; Figure 3).

Table 1 - **Flowering characteristics of the 35S::*PoFT/TFL1* independent transgenic lines**

Plant genotype	**Number of leaves**	**Number of plants**
Col	18.4 ± 1.7	12
PoFT - line 8	9.6 ± 1.7	31
PoFT - line 13	9.8 ± 3.4	24
PoFT - line 15	10.1 ± 2.3	34
Total	**9.9 ± 2.5**	**89**
PoTSFa - line 9	21.2 ± 2.6	22
PoTSFa - line 11	20.3 ± 3.7	27
PoTSFa - line 12	22.9 ± 4.7	25
Total	**21.4± 4.0**	**74**
PoTFL1 - line 3	22.0 ± 5.1	22
PoTFL1 - line 4	19.4 ± 1.9	25
PoTFL1 - line 6	21.8 ± 3.5	27
Total	**21.0± 3.8**	**74**
PoBFT - line 4	21.4 ± 2.7	20
PoBFT - line 8	16.5 ± 2.7	12
PoBFT - line 9	23.9 ± 7.9	19
Total	**20.7 ± 6.0**	**51**
PoATC - line 9	24.4 ± 4.9	23
PoATC - line 10	24.7 ± 2.8	12
PoATC - line 13	23.0 ± 3.0	27
Total	**23.8 ± 3.9**	**62**
PoMFT - line 9	15.2 ± 2.5	12
PoMFT - line 11	17.8 ± 1.2	16
PoMFT - line 14	16.9 ± 1.9	14
Total	**17.1 ± 1.9**	**42**

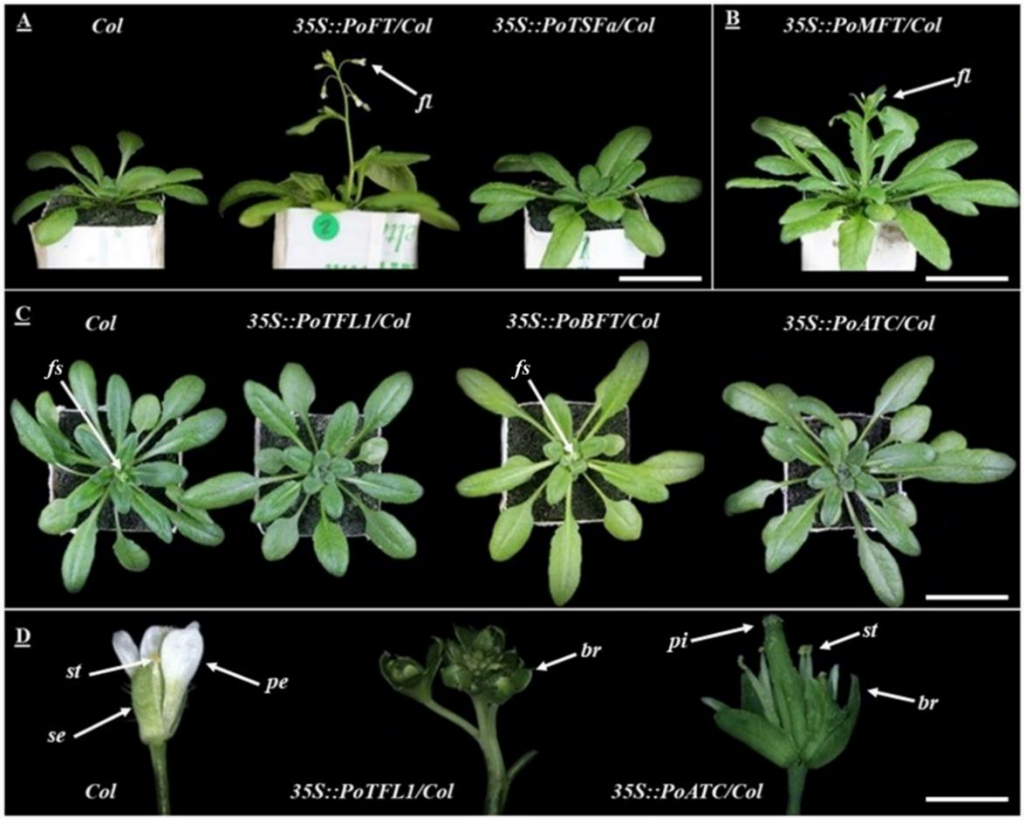

Figure 2 – **Ectopic expression of *Passiflora FT/TFL1* genes in *Arabdopsis* T2 plants grown under ambient LD conditions**. **A.** *Col* and *FT*-like overexpression lines. **B.** *PoMFT* overexpression lines. **C.** *Col* and *TFL1*-like overexpression lines. **D.** Flowers of *Col* and *TFL1*-like overexpression lines. (br) = bract-like organ; (fl) = flower; (fs) = flowering stem; (pe) = petal; (pi) = pistil; (se) = sepal; (st) = stamen. Bars: A = 4 cm, B = 4 cm, C = 4 cm, D = 0.5 cm.

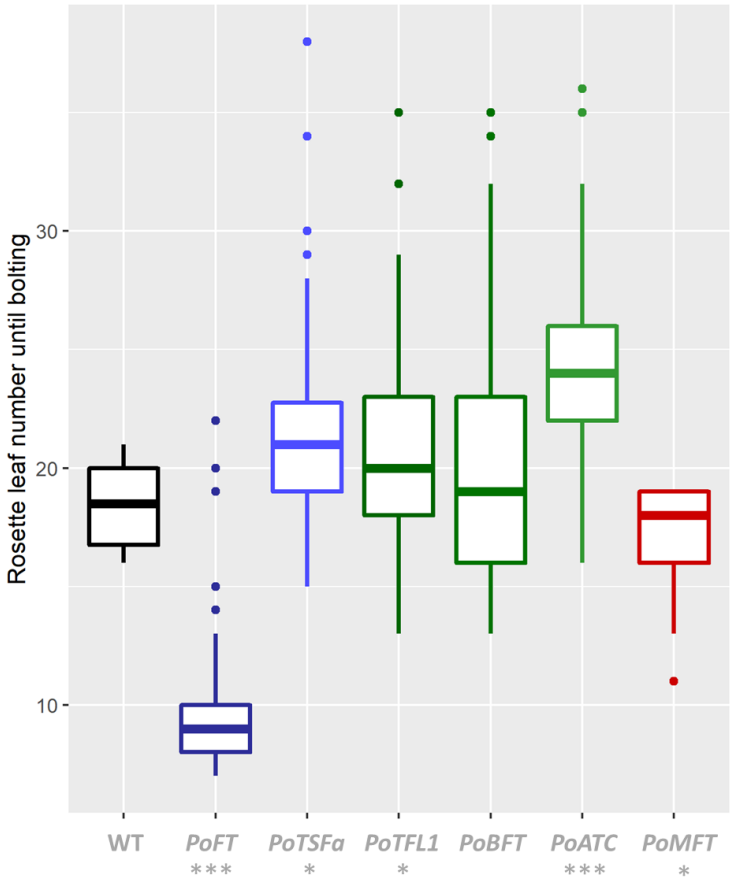

Figure 3 – **Boxplots showing number of rosette leaves until bolting of *Arabidopsis* T2 lines overexpressing *Passiflora FT/TFL1* genes grown under long day conditions**. A single asterisk indicates a significant difference with a P-value of ≤ 0.05 and triple asterisks a P-value of ≤0.001 (Student t-test relative to wild type).

9.4 Discussion

In *Arabidopsis thaliana*, six genes belonging to the *FT/TFL1* family have been identified: *FT* and *TSF*, involved in flowering promotion and belonging to the *FT*-like subfamily; *TFL1*, *BFT*, and *ATC* involved in flowering repression and belonging to the *TFL1*-like subfamily; and *MFT*, belonging to *MFT*-like subfamily and involved in the regulation of seed germination (Kobayashi et al., 1999; Xi et al., 2010; Wickland; Hanzawa, 2015). In *P. organensis*, we identified and characterized eight genes belonging to the *FT/TFL1* family (see chapter II). Here, we explored the putative functions of six of those genes in flowering regulation using *Arabidopsis* as a heterologous system. As we mentioned in Chapter II, there are three *P. organensis TSF* paralogous genes: *PoTSFa*, *PoTSFb* and

PoTSFc. Their sequences have a high level of identity and it was not possible to design specific primers for PoTSFb and PoTSFc. Therefore, we selected the *PoTSFa* paralog for this study.

In *Arabidopsis*, *FT* and its paralog *TSF* are almost identical to each other at the amino acid sequence level. *TSF* promotes flowering redundantly with *FT*, and both genes are regulated by CONSTANS (CO), and interact with FLOWERING LOCUS D (FD) to activate the transcription of floral meristem identity genes such as *AP1*, *FUL* and *LFY*, leading to the promotion of flowering (Abe et al., 2005; Wigge et al., 2005; Michaels et al., 2005; Yamaguchi et al., 2005; D'Aloia et al., 2011). We observed that *35S::PoFT/Col* plants showed an early flowering phenotype based on the number of rosette leaves until bolting. However, *35S::TSFa/Col* plants showed a later flowering phenotype. As we discussed before (see chapter II), unlike the PoFT protein, that displayed the conserved amino acid residues Tyr84 and Gln139, important for the function of inducing flowering, PoTSFa protein did not display the conserved amino acid Gln139. Instead, it displayed His139. In *Arabidopsis*, FT and TSF are both acting as inducers of flowering and also acting later in development, in the inflorescence meristem, to maintain its reproductive fate (Yamaguchi et al., 2005).

Moreover, despite the widespread conservation of these flowering regulators across flowering plants, recent studies have unveiled several examples of the swich from a function of FT as an inducer of flowering to that of a repressor of flowering. As examples we have sugar beet (Pin et al., 2010), sunflower (Blackman et al., 2010), tobacco (Harig et al., 2012), longan (Winterhagen et al., 2013), soybean (Kong et al., 2010; Nan et al., 2014; Zhai et al., 2014), among others.

Thus, in our protein sequence alignment, besides the *P. organensis* FT/TFL1 sequences we included the sequences of these above mentioned species. Our alignment showed that all orthologs of FT displayed a tyrosine residue at the conserved position 85 (according to the *Arabidopsis* FT protein sequence numbering of residues). Hanzawa et al. (2005) showed the importance of the tyrosin residue at position 85 (Tyr-85) for FT to function as a flowering inducer. Furthermore, our alignment showed that all FT proteins that had a function of an inducer of flowering contained a Tyr-134 (except GmFT5a) and a tryptophan at position 138, which was also observed in the *P. organensis* FT protein. Similarly, PoTSFa displayed a conserved tyrosine at position 134, but in contrast, it displayed an arginine instead of a tryptophan residue at position 138. On the other hand, the putative homologs of FT that had a repressor flowering function, contained a non-tyrosine residue at position 134

(except PaFTL1 and PaFTL2), and a non-tryptophan residue at position 138 (except DlFT2 and GmFT4).

Taking into account all these results, we suggest that PoTSFa functions as a flowering repressor, and a probable hypothesis for this is the absence of a tryptophan at position 138 and/or glutamine at position 140 (137 and 139 at *Passiflora* protein sequence). It is of great significance that some FT homologs apparently have acquired a repressor function during recent evolution. It might represent a common strategy in plants to refine floral initiation according to multiple environmental cues and endogenous pathways intrinsic to each individual.

Ectopic expression of *PoTFL1* in *35S::PoTFL1/Col* transgenic *Arabidopsis* plants caused a significant delay in flowering and a change in inflorescence structure. This suggests that the *PoTFL1* gene might have a role in the maintenance of vegetative growth and in delaying phase transition from vegetative to reproductive growth. The *35S::PoATC/Col* transgenic *Arabidopsis* plants flowered much later and showed increasing branched inflorescence architectures. A similar phenotype was observed for *35S::TFL1* transgenic *Arabidopsis* (Nakagawa et al., 2002; Zhang et al., 2005). The *35S::PoBFT/Col* plants showed no significant phenotype.

Arabidopsis TFL1 has been proposed to be antagonistic to both LFY and AP1. The expression of floral meristem identity genes can be inhibited by TFL1 resulting in a delay of flowering (Shannon; Meeks-Wagner, 1991; Schultz; Haughn, 1993). Likewise, the activity of TFL1 can be suppressed by LFY, and AP1. Thus, this antagonism among these genes is manifested in their complementary expression patterns and phenotypic effects (Bowman et al., 1993; Parcy et al., 2002). In *P. organensis*, *35S::TFL1/Col* and *35S::ATC/Col* plants exhibited a strong phenotype with defects in flower formation: petals were absent and sepals were converted into bract-like organs. This phenotype is similar to that of *Arabidopsis ap1* mutants where floral meristems appear in the axil of these bract-like organs, producing additional flowers with the same morphology, partially converting flowers into inflorescence-like structures (Irish; Sussex, 1990; Bowman et al., 1993).

Moreover, the flowering time measurements indicated that *35S::PoMFT/Col* plants showed a slight reduction in flowering time. Yoo et al. (2004) showed that the constitutive expression of *Arabidopsis* MFT led to slightly early flowering under long days, while a *mft* mutant did not show any obvious phenotype. Therefore these authors suggested that MFT functions as a floral inducer in *Arabidopsis* and that it may act redundantly in determining

flowering time. In addition, Xi et al. (2019) demonstrated that MFT regulates seed germination via ABA and GA signaling pathways in *Arabidopsis.*

Studies indicated that *MFT* genes are ancestral to *FT* and *TFL1* genes. For example, basal plant species such as mosses possess homologs of *MFT* but not of either *FT* or *TFL1* (Hedman et al., 2009, Karlgren et al., 2011). Data from gymnosperms suggest that the ancestor of FT and TFL1 functioned in a *TFL1*-like manner and that FT and TFL1 functions diverged after the evolutionary separation of gymnosperms and angiosperms (Karlgren et al., 2011, Klintenäs et al., 2012).

9.5 Conclusions

Our analysis exploring the putative functions of *P. organensis FT/TFL1* family members suggests that their roles in phase transition are similar to what was previously observed for *FT/TFL1* family members in another plant species (except for PoTSFa). Additional studies to further investigate the functions of these genes by overexpressing or knocking them out in transgenic *Passiflora* plants are still neeed. Amino acid swaps in strategic positions within the conserved domains that have been shown to modulate flowering induction or flowering repression functions might provide better information about the molecular mechanisms involved in modulating floral transition and plant architecture. Elucidation of the flowering mechanism in *Passiflora* would be helpful for breeding studies aiming to enhance passionfruit yield.

9.6 Supplementary Material

Primer	Sequence	Amplicon (pb)	Annealing Temperature (°C)
PoFT - F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGCCTAGGGATAGAGACTCCCTC	600	58
PoFT - R	GGGGACCACTTTGTACAAGAAAGCTGGGTAGCGCTTCATCTTCGGGAAT		
PoTSFa - F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGCCCAGAAGTAGAGATCC	561	58
PoTSFa - R	GGGGACCACTTTGTACAAGAAAGCTGGGTAGAGGCTGGCTGGCTGGTT		
PoTFL1 - F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGCAAATGTGTCGGATCCG	561	58
PoTFL1 - R	GGGGACCACTTTGTACAAGAAAGCTGGGTACAACCCTGCTGCTGATTTTT		
PoBFT - F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTCAAGAGCCATGGAACC	611	58
PoBFT- R	GGGGACCACTTTGTACAAGAAAGCTGGGTAAACCCATACCCAACCCTAGC		
PoATC - F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGCAAAAATGTCTGATCC	723	58
PoATC - R	GGGGACCACTTTGTACAAGAAAGCTGGGTATTGATGGACAACGCAAAGAG		
PoMFT - F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTCTGCCTCGGTTGATCC	548	58
PoMFT - R	GGGGACCACTTTGTACAAGAAAGCTGGGTACCCAATCCCATCGTCAATTACT		

F = *forward;* R = *reverse*

S1 - Primers used for cloning *FT/TFL1 Passiflora* gene

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10. GENERAL CONCLUSIONS

This work aimed to unravel the mechanisms involved in the transition from the vegetative to the reproductive stages in *Passiflora organensis*. We firstly seek to understand the morphological aspects involved in phase transition, since there were no data available in the literature in this regard. The detailed understanding of the sequence of events related to the phase transition was essential to obtain solid results with the molecular analysis of *Passiflora* phase transitions. Thus, in the first chapter we showed that *P. organensis* is an excellent model for phase transition studies, because there are obvious morphological differences between the plants in the juvenile, adult vegetative and adult reproductive stages. In addition, we showed the complexity of *Passiflora* axillary meristems (AMs): these are more complex when compared to AMs from other species. It suggests that species of *Passiflora* might be a valuable model to study *FT/TFL1* balance in order to understand how AM modulation gives rise to different structures, and how some *TCP* gene duplication can be related to this complexity.

To study the molecular mechanisms involved in *Passiflora* phase transition, we identified eight *FT/TFL1* genes in *Passiflora organensis*: *PoFT, PoTSFa, PoTSFb*, and *PoTSFc*, belonging to the *FT*-like subfamily; *PoTFL1, PoBFT*, and *PoATC*, belonging to the *TFL1*-like subfamily; and *PoMFT*, belonging to the *MFT*-like subfamily. We demonstrated that *P. organensis FT/TFL1* family members displayed conserved structural and sequence features indicating that the *P. organensis* orthologs, important molecular players controlling plant phase transition and flowering, such as FT and TFL1, were uncovered. We showed that *Passiflora FT*-like genes have higher expression during the adult stage, while *TFL1*-like genes showed higher expression in the juvenile stage. Addionally, ectopic expression of *PoFT* in transgenic *Arabidopsis* plants induced early flowering and inflorescence determinacy, while *PoTFL1* ectopic expression caused delayed flowering. It suggests that their roles in phase transition are conserved when compared to other plant species.

The *FT/TFL1* family is characterized by gene duplication events and these may result in sub- or neo-functionalization. Our study revealed that there are three *P. organensis FT* paralogs: *PoTSFa, PoTSFb*, and *PoTSFc*. Despite the fact that their higher transcript levels were detected during the adult reproductive stage, ectopic expression of *PoTSFa* in *Arabidopsis* caused a delay in flowering. We suggest that PoTSFa functions as a flowering repressor probably because of the absence of a tryptophan at position 137 and/or glutamine

at position 139. Thus, future experiments with amino acid swap in strategic positions might provide better information about the molecular mechanisms involved in flowering control.

As observed in model species, FT/TFL1 proteins may need to interact with other proteins to perform their biological functions. The bZIP transcription factors and 14-3-3 proteins may be important interactors during the flowering transition, and TCP transcription factors may be important to regulate plant architecture and branching. We showed that PoFT interacts with PobZIPs, and PobZIPs interact with some of the Po14-3-3 proteins. However, no direct interactions were detected between PoFT and 14-3-3 proteins. These results suggest that the flowering activation complex in *P. organensis* is different from the complex proposed for grasses. Furthermore, the *Passiflora* FT-like proteins can interact with multiple *Passiflora* TCP proteins. The PoTCPs may modulate the activity of PoFT-like proteins in the axillary buds to repress the premature floral transition of axillary meristems and, as a result, regulate flowering and branching in *Passiflora*.

The results of this work contribute to understanding the morphological aspects of the transition from vegetative to reproductive phase in *Passiflora organensis*, as well as to characterize the activity of *FT/TFL1* gene family and some of their interactors during this process. These results will be important for selecting the right genes to focus on future research and for applications in studies of yield increase in *Passiflora* species with commercial interest, such as the passionfruit.