UNIVERSIDADE FEDERAL DE VIÇOSA

PEDRO ALEXANDRE SODRZEIESKI

CLONING AND SPATIO-TEMPORAL EXPRESSION ANALYSIS OF THE *CRABS CLAW* GENE INVOLVED IN EXTRAFLORAL PETIOLAR NECTARY DEVELOPMENT IN *Passiflora alata* CURTIS AND *Passiflora cincinnata* MASTERS

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Dissertation submitted to the Plant Physiology Graduate Program of the Universidade Federal de Viçosa as part of the requirements for the degree of *Magister Scientiae*.

Adviser: Wagner Campos Otoni

Co-advisers: Andréa Dias Koehler
Lorena Melo Vieira

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Wagner Campos Otoni
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ABSTRACT

SODRZEIESKI, Pedro Alexandre, M.Sc., Universidade Federal de Viçosa, December, 2022. **Cloning and spatio-temporal expression analysis of the *CRABS CLAW* gene involved in extrafloral petiolar nectary development in *Passiflora alata* Curtis and *Passiflora cincinnata* Masters**. Adviser: Wagner Campos Otoni. Co-advisers: Andréa Dias Koehler and Lorena Melo Vieira.

The genus *Passiflora* includes more than 500 species distributed in five subgenera, of which the subgenus *Passiflora* is the most representative. *Passiflora alata* and *P. cincinnata* are species belonging to this subgenus and are of economic and nutraceutical importance. Extrafloral nectaries are a striking feature in this botanical group, being responsible for several important processes in attracting natural enemies of predators of these species. In addition, extrafloral nectaries are also defense structures, mimicking predator eggs to avoid oviposition and possessing several compounds to reduce herbivory. The genetic mechanisms of nectary formation are still poorly understood; however, it is known that there are two genes that act as master regulators of this process: *CRABS CLAW* (*CRC*) and *STYLISH* (*STY*). *STY* is responsible for the formation of nectaries in basal eudicots, while CRC, in nuclear eudicots. In Arabidopsis floral nectaries, stimulated by *ARABIDOPSIS RESPONSIVE FACTORS 6/8*, *CRC*, belonging to the *YABBY* family of transcription factors, initiates its expression at the site where the nectary will be formed, and is limited to its interior. The spatial regulation of *CRC* expression is controlled by genes such as *LEAFY* (*LFY*) and *APETALLA1* (*AP1*) that will inhibit its expression outside the nectary tissues. Since Passifloras are species belonging to the nuclear eudicots and a *CRC* fragment was found in a transcriptome of leaf primordium in *P. cincinnata*, the aim of this work was to investigate morphological and anatomical aspects of petiolar nectary development and *CRABS CLAW* gene expression at three distinct developmental stages in *Passiflora alata* and *Passiflora cincinnata*. For this, petiolar nectaries were collected from both species using three different stages of leaf development as a guide: primordium, expanding and expanded. Analyses of morphology under stereomicroscope, photomicroscope and scanning electron microscope, besides *in situ* hybridization and quantitative real-time PCR (RT-qPCR) were performed. The petiolar nectaries of *P. cincinnata* have an oval shape while those of *P. alata* are calyx-shaped. The nectaries of both species have similar anatomical aspects presenting a division into three distinct tissues: epidermis and nectariferous and subnectariferous parenchyma. The epidermis is multiseriate only in the crater region, the nectariferous tissue is composed of smaller cells with dense cytoplasm and those of the subnectariferous by larger, less dense cells and the presence of vascular tissues. *CRC* expression was restricted to the interior of the nectaries and its expression level peaked at the expanding leaf stage, showing similar values between the primordium, and expanded leaf. Due to the inefficiency of the primers used, it was not possible to obtain the complete expressed sequence by means of RACE. There is strong evidence that *CRC* acts as a key regulator in petiolar nectary formation in *P. cincinnata* and *P. alata*.

Keywords: *CRABS CLAW*. Gene expression. Nectary Morphoanatomy. Passifloraceae. Secretory structures.

RESUMO

SODRZEIESKI, Pedro Alexandre, M.Sc., Universidade Federal de Viçosa, dezembro de 2022. **Clonagem e análise espaço-temporal da expressão do gene *CRABS CLAW* envolvido no desenvolvimento de nectários extraflorais peciolares em *Passiflora alata* Curtis e *Passiflora cincinnata* Masters**. Orientador: Wagner Campos Otoni. Coorientadores: Andréa Dias Koehler e Lorena Melo Vieira.

O gênero *Passiflora* abarca mais de 500 espécies distribuídas em cinco subgêneros no qual o subgênero *Passiflora* é o mais representativo. *Passiflora alata* e *P. cincinnata* são espécies pertencentes a este subgênero e de importância econômica e nutracêutica. Nectários extraflorais são uma característica marcante neste grupo botânico, sendo responsáveis por pela atração de inimigos naturais de predadores destas espécies. Além disso, os nectários extraflorais também são estruturas de defesa, mimetizando ovos de predadores para evitar oviposição e possuindo diversos compostos para redução da herbivoria. Os mecanismos genéticos de formação dos nectários ainda são pouco conhecidos. Entretanto, sabe-se da existência de dois genes que atuam como reguladores mestres deste processo: *CRABS CLAW* (*CRC*) e *STYLISH* (*STY*). *CRC* é responsável pela formação dos nectários em eudicotiledôneas nucleares, enquanto o *STY*, em eudicotiledôneas basais . O *CRC*, pertencente à família *YABBY* de fatores de transcrição, inicia sua expressão no local onde o nectário será formado, ficando limitado ao seu interior. A regulação espacial da expressão do *CRC* é controlada por genes como *LEAFY* (*LFY*) e *APETALLA1* (*AP1*) que irão inibir sua expressão fora dos tecidos nectaríferos. Como *Passiflora* constitui um gênero de eudicotiledôneas nucleares e um fragmento do *CRC* foi encontrado em um transcriptoma de primórdio foliar em *P. cincinnata*, o objetivo deste trabalho foi investigar aspectos morfológicos e anatômicos do desenvolvimento de nectários peciolares e a expressão gene *CRABS CLAW* em três estágios distintos de desenvolvimento em *Passiflora alata* e *Passiflora cincinnata.* Para isso, coletou-se nectários peciolares das duas espécies usando como guia três estágios diferentes de desenvolvimento da folha: primórdios foliares, folhas jovens em expansão e folhas adultas totalmente expandidas. Realizou-se análises morfo-anatômicas, hibridização *in situ* e RT-qPCR. Os nectários peciolares de *P. cincinnata* apresentam um formato ovalado enquanto os de *P. alata*, em forma de cálice. Os nectários de ambas as espécies possuem aspectos anatômicos semelhantes, apresentando uma divisão em três tecidos distintos: epiderme, tecido parenquimático nectarífero e subnectarífero. A epiderme é multisseriada apenas na região do polo secretor, o tecido nectarífero é composto por células menores de citoplasma denso e os do subnectarífero por células maiores, menos densas e pela presença de tecidos vasculares. A expressão do *CRC* ficou restrita ao interior dos nectários e o seu nível de expressão teve o pico na fase de folha em expansão, apresentando valores similares entre o primórdio e folha expandida. Devido à ineficiência dos primers utilizados, não foi possível obter a sequência expressa completa por meio da técnica de RACE. Há forte evidência de que o *CRC* atua como regulador chave na formação de nectários peciolares em *P. cincinnata* e *P. alata*.

Palavras-chave: *CRABS CLAW*. Estruturas secretoras. Expressão gênica. Morfoanatomia de nectários. Passifloraceae.

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LIST OF ACRONYMS AND ABBREVIATIONS

AG	*AGAMOUS*
AP	*APETALLA*
ARF	*AUXIN RESPONSIVE FACTOR*
BOP	*BLADE ON PETIOLE*
BTB	Broad-Complex, Tramtrack and Bric a brac
CRC	*CRABS CLAW*
EFN	Extrafloral nectaries
ERF	*ETHYLENE RESPONSIVE FACTOR*
JA	Jasmonic Acid
L-DOPA	l-3,4-Dihydroxyphenylalanine
LFY	*LEAFY*
NPR1	*NON-REPRESSOR OF PR1*
PAN	*TGA TF PERIANTHIA*
POZ	POxvirus and Zinc finger
RACE	Rapid amplification of cDNA ends
SEP	*SEPALLATA*
SHP	*SHATTERPROOF*
STY	*STYLISH*
TF	Transcription factor
TGA	TGACG-Binding
UFO	*UNUSUAL FLORAL ORGANS*
YAB	*YABBY*
YUC	*YUCCA*

SUMMARY

CHAPTER 1 – CLONING AND SPATIO-TEMPORAL EXPRESSION ANALYSIS OF THE *CRABS CLAW* GENE INVOLVED IN EXTRAFLORAL PETIOLAR NECTARY DEVELOPMENT IN *PASSIFLORA ALATA* CURTIS AND *PASSIFLORA CINCINNATA* MASTERS

GENERAL INTRODUCTION

Passionflower was first reported in 1529 by Spanish missionaries traveling in South America where they called it "*Flos Passionis*" by its flower centre resemblance to the symbol of crucifixion, a term latter latinized as *Passiflora* by Linnaeus (Linné, 1797; Killip, 1938; Vecchia, 2022). Since its discovery, the passionflower has long been appreciated by its fruit, commonly consumed *in natura*, processed, or for industrial purposes (Faleiro et al., 2019). The fruit contains great nutritional value with high levels of vitamins (A, B2 and C), carotenoids, polyphenols, minerals (K, P, Ca, Fe, Na, Mg, S, Cl) and proteins (Thokchom and Mandal, 2017). Also, its extracts are rich in tannins, coumarin alkaloids, flavonoids, tyrosine, and glycine; being used for cosmetics and as a phytotherapic for insomnia, menopause symptoms and as an anti-inflammatory, antioxidant, and sedative (Kim et al., 2017; Proaño et al., 2020; Rocha et al., 2020). Furthermore, passionflowers are highly appreciated by its climbing habit, appealing leaves, and dazzling and fragrant flowers, being widely used for ornamental purposes (Rezende et al., 2014).

Brazil is, today, the most important passionflower grower and consumer, detaining 80% of the annual 1 million tons production (ITITROPICALS, 2016; Faleiro et al., 2017). Nonetheless, its commercial importance is increasing, due to a higher demand for exotic and phytotherapic products (Carmona and Pereira, 2013; Faleiro et al., 2017). About 70 passionflowers species are known to produce edible fruits and 50 have commercial potential, even so, 90% of total production comes from a single species, *Passiflora edulis*, denoting an open space for cultivation and studies regarding other species within the genus (Faleiro et al., 2017; Faleiro et al., 2019; Lobato and Costa, 2022).

Today, the *Passiflora* genus, a member of the Passiforaceae family, has around 588 known species that are distributed in America, Southeast Asia, and Oceania, with about 147 species native of Brazil, making it the country of its biggest diversity (Ferreira, 2005; Pérez and d'Eeckenbrugge, 2017; Faleiro et al., 2019). This genus is composed by herbaceous or woody vines, with climbing tendrils, alternate leaves, axillary stipules, and floral and extrafloral nectaries (Pérez and d'Eeckenbrugge, 2017). Contrariwise, some *Passiflora* species have different growth habits, like *Passiflora macrophylla*, a weak tree up to 5 m height, or *Passiflora quelchii*, a shrub (Vanderplank et al., 2017; Mezzonato-Pires et al., 2018). Passionfruit flowers possess androgynophore, five petals, five sepals, a complex corona with one or many concentric filament rows and a limen-operculum system that restraint the nectary chamber access (Killip, 1938; Pérez and d'Eeckenbrugge, 2017; Rocha et al., 2020). The fruit is commonly a globose to ovoid berry with a colored parchment-like intertegument, and a mucilaginous pulp that covers each seed (Killip, 1938; Vecchia, 2022). Fruit size can vary greatly, being small as 6 -15 mm diameter, like *Passiflora suberosa*, or up to 1.5 – 3 kg fruit, commonly found in *Passiflora quadrangularis* (Fern, 2022; Vecchia, 2022). Distinctively, some species, like *Passiflora capsularis*, have a dried capsule fruit, which opens irregularly when its ripe (Faria and Stehmann, 2010). The seeds are usually flat, heart-shaped with a hard and wrinkled tegument (Killip, 1938; Vecchia, 2022).

The passionflowers populate a wide range of habitats, from arid regions to the tropical rainforests, which evolved the genus to a high morphological diversity between species (Pérez and d'Eeckenbrugge, 2017; Rocha et al., 2020). The leaf morphology can differentiate in color, number of lobes, margin, and many other features (Killip, 1938; Chitwood and Otoni, 2017). The flowers, also, present a great difference in terms of morphology and functionality, particularly due to coevolution with a wide range of pollinators, such as bats, birds, and insects, each one selecting the characteristics that attract the most (Pérez and d'Eeckenbrugge, 2017). Many attempts were made group all this diversity, the first made by Killip (1938) where the genus was divided in 22 subgroups. A latter attempt by Feuillet and MacDougal (2003) divided it in only four subgenera: *Astrophea*, *Decaloba*, *Deidamioides* and *Passiflora* in which later Krosnick et al. (2013) added the *Tetrapathea* subgenus. *Passiflora* subgenus *Passiflora* is the bigger and most representative subgenera of *Passiflora*. It is composed of lianas of large and showy flowers, a conspicuous calyx tube, complex filament corona, large fruits, entire or lobed leaves (3 to 7), and by presence of nectaries at the petiole (MacDougal and Ulmer, 2004; Cervi and Imig, 2013).

Passiflora alata (Fragrant Granadilla; "Maracujá-doce") and *Passiflora cincinnata* (Maracuja Mochila; "Maracujá-do-mato") are two commercial species of the *Passiflora* subgenus *Passiflora* (Masters, 1872a; Vanderplank, 2000). *Passiflora alata* is a perennial vine with four-angled stems that can reach up to 15 meters high (Fern, 2022; Lachelin, 2022). It has entire ovoid glabrous leaves with two to four petiolar nectaries and the bracts are lanceolate and usually shorter than flower tube (Masters, 1872a). Its flowers are very fragrant, large (100-120 mm), and showy having oblong dark crimson petals with a white margin inside and green outside; and a corona divided in 4 ranks with long and colored filaments (Masters, 1872a; Vanderplank, 2000). The fruit has a pyriform shape with size about 8 – 11 cm long and 5 – 8 wide, much appreciated by its sweetness that are usually consumed *in natura*, but that are also used for desserts, industrial and pharmaceutical purposes (Masters, 1872a; Vanderplank, 2000; Fern, 2022). Its abeyant potential is currently being explored by the EMBRAPA cultivar’s BRS Mel do Cerrado; Baiense and Dom Basílio from ISLA SEMENTES LTDA; Monique and Precioso from Feltrin Sementes LTDA; and Corupá-015 from Alvim Seidel Orquidário catarinense LTDA (EMBRAPA, 2022; MAPA, 2022).

Passiflora cincinnata is a perennial vine of teeter stems and membraneceous leaves that has three deeply divided lobes during the juvenile stage and five in the adult vegetative stage, a phenomena known as heteroblasty (Masters, 1872b; Vanderplank, 2000; Chitwood and Otoni, 2017). It has two petiole glands, and several in the leaf’s lobes (Masters, 1872b; Vanderplank, 2000). Its flowers are very fragrant, large (75 – 125 mm) of showy colors (violet or violet and blue) and several series of colorful (lower part: deep purple; middle: pinkish blue; upper: pale or deep blue) corona filaments that grows longer as it approaches the border (Vanderplank, 2000). The fruits are globose to ovoid, about 5 – 6 cm long and 3 – 4 cm wide, very fragrant, and often appreciated fresh, in juices, jams or desserts; with its seeds often used in cosmetics and skin care products (Vanderplank, 2000; Fern, 2022). These characteristics are now being explored by the BRS Sertão Forte EMBRAPA’s cultivar (EMBRAPA, 2022; MAPA, 2022).

Passiflora species have long been studied by its morphological variability (Pérez and d’Eeckenbrugge, 2017) and because of the presence of many interesting features, such as floral and extrafloral nectaries. The term nectary is surrounded by a lot of discussion and had many attempts to categorize it (Schmid, 1988). One usual definition is addressed by Schmid (1988), who define nectary as:

> “A more or less localized, often multicellular glandular structure that occurs on vegetative or reproductive organs and that regularly secretes nectar, a sweet solution containing mainly sugars and generally serving as a reward for pollinators or for protectors (e.g., ants) against herbivores, or, in carnivorous plants, as a lure for animal prey. Some nectaries are unicellular hairs.”

By this definition, for a tissue to be considered a nectary needs to match three pre-requisites (Schmid, 1988): I. it needs to have the anatomy of a secretory gland for the capacity to secrete a liquid; II. the secreted substance must be composed mainly by sugars, although many other compounds can be found; and III. it needs to have the function to attract animals that helps the plant in some sort of way, even though it can present many other functions. This definition, although excludes many structures that can be classified as nectaries, such as: non-structural nectaries, which are commonly a discontinuous group of cells scatter scattered in an area; and nectaries that do not present the function to attract animals (Nepi, 2007).

The great variability of nectaries showed by its definition can be explained by the convergent nature of its evolution which happened independently in different events in the angiosperm and, by its co-evolution with animals (Pérez and d'Eeckenbrugge, 2017). This provided the environment that gave rise to its great morphological, physiological, and anatomical variability (Pérez and d'Eeckenbrugge, 2017). This variability can be seen even in the same plant, as the nectaries can be positioned in different parts of the plant each one having their one morphology and functionality (Chatt et al., 2019). By the organ the nectary is positioned, they can be classified as floral or extrafloral; and, by their function they can be classified as nuptial, when it is related to the process of pollination, or extra nuptial, when they are not involved in pollination, even if located in the flower (Schmid, 1988).

Although floral nectaries are a more studied group, extrafloral nectaries have a great importance for plants, providing a plethora of services. The primary function of extrafloral nectaries is to attract ants and other predators to common pests and herbivores that attack the plant (Bentley, 1977). A common example is the ants *Myrmica* spp. and *Formica lemani* that are often visitors of extrafloral nectaries of *Pteridium aquilinum* in which they cause a several reduction in moth caterpillars' population (Heads and Lawton, 1985). In *Senna mexicana* not only ants are an important mutualist, but spiders, ladybugs, wasps, and hemipterans participate in the control of caterpillars, especially in the absence of ants (Koptur et al., 2015). This control can even extend to plants who do not have nectaries as the attraction to more predators to the area decreases these herbivores population (Rezende et al., 2014) Furthermore, extrafloral nectaries also helped to provide higher yields in *Cassia fasciculata* due to the promotion of lower population of seeds predators (*Phoebis sennae*) and herbivores by the attraction of predators (Barton, 1986). Besides that, extrafloral nectaries do much more other than attracting predators. Studying *Vicia faba*, it was reported that its nectaries have increased levels of L-DOPA, an aminoacid toxic to insects and precursor in the synthesis of many alkaloids, were found in the nectaries of (Soares et al., 2014; Gish et al., 2016). Also, increased levels of lignification and the formation of calcium oxalate druses, crystal deposition of calcium oxalate that commonly occurs below the secreting tissue, can be found in nectaries, and are probably related to protect the plant against chewing (Gish et al., 2016; Moraes et al., 2022). Furthermore, a study by Heil (2011) describes the presence of nectar proteins that has antimicrobial function in the nectar that, when exudated, protects it from microbes and possible infection through nectaries. Also, it was found many secondary compounds (such as alkaloids) that could act as repellent for non-mutualistic animals (Heil, 2011). Nectaries are structures with clearly defensive and ecological importance, making valuable to understand their ontogeny and development.

The most studied model of nectary development comes from floral nectaries of *Arabidopsis thaliana.* The nectaries of *Arabidopsis thaliana* are formed in the third whorl of the flower which is governed by the ABC(E) genes (Slavković et al., 2021). As a third whorl structure, nectaries formation will be affected by these genes, even though it is only an indirect effect, as it is the case of B- and E- (*SEPALLATA*) gene lineages (Slavković et al., 2021). Contrariwise, the C- class gene plays an important role in nectary formation, as no nectary is form in the C-lineages *ag/shp-1/shp-2* triple mutant, who seems to promote the expression of master regulators of nectary development (Slavković et al., 2021).

The development of nectaries is mainly controlled by three key genes: *CRABS CLAW* (*CRC*) and *BLADE ON PETIOLE* (*BOP*), or *STYLISH* (*STY*). *CRC* is one of the only two members of the *YABBY (YAB)* family that are expressed in the reproductive tissues (Bartholmes et al., 2012). There, the *CRC* will provide distinct functions: floral meristem termination, activation and promotion of nectary development, and carpel fusion (Slavković et al., 2021). For long has been observed the importance of CRC in nectary formation since the *crc* mutants show no nectary initiation, differently from other genes involved in nectaries development where, even though malformed, the nectaries are not absent (Bowman and Smyth, 1999; McKim et al., 2008; Slavković et al., 2021). To achieve the nectary formation, the *CRC* will initiate its expression prior to nectary emergence and will occupy all the cells that will be involved in nectary formation promoting their differentiation (Slavković et al., 2021). Its spatial limitation is provided by *APETALLA1* (*AP1*), *LEAFY* (*LFY*) and *UNUSUAL FLORAL ORGANS* (*UFO*), that will inhibit *CRC* expression outside the nectaries and the carpels (Slavković et al., 2021). Interestingly, the *YAB* protein family primary function relates to the determination of lateral organs abaxial-adaxial polarity (Meister et al., 2005). These proteins are characterized by the presence of a C2C2 - zinc finger motif, and a C-terminal Helix-Loop-Helix motif, also called the *YABBY* domain, which is similar to the High Mobility Group box (Meister et al., 2005; Bartholmes et al., 2012; Fourquin et al., 2014). Differently from its family members, *CRC* accumulated other functions rather than the polarity determination (Meister et al., 2005). This characteristic could be due to alterations in the adjacence of the zinc finger motifs, a diverse class of protein related to DNA-binding and protein – protein interaction (Meister et al., 2005; İnal et al., 2017). Also, *CRC* maintains distinct functions in flowering tissue. To fulfill this, the *YABBY* domain plays an important role as it serves as harbor to homo and heterodimers formation (Gross et al., 2018; Romanova et al., 2021). Depending on the protein interacting with the *CRC* it will alter the target DNA-binding sequence conferring its great capacity to regulate a plethora of genes, inhibiting some and promoting others (Gross et al., 2018; Romanova et al., 2021).

Although *CRC* plays a main role in nectary development, its expression is not sufficient to initiate it (Slavković et al., 2021). *BOP* is another gene that plays important role in nectary development. *BOP1* and *BOP2* are members of the *NON-REPRESSOR OF PR1* (*NPR1*) protein family which are characterized by several conserved cysteines and two protein–protein interaction domains, the Broad-Complex, Tramtrack and Bric a brac/POxvirus and Zinc finger (BTB/POZ) motif and four ankyrin repeats (Hepworth et al., 2005; McKim et al., 2008). These proteins play a role in cell differentiation and in the termination of indeterminate growth in meristematic cells of lateral organs (Hepworth et al., 2005; Chan et al., 2007; McKim et al., 2008; Slavković et al., 2021). Studying the *bop1/2* double mutant they that the nectaries exhibit a reduced size and malformed the nectariferous parenchyma and the secretory tissue (McKim et al., 2008). For this, it is believed that *BOP1/2* regulates nectary development alongside *CRC*, being expressed very early in the nectary development (McKim et al., 2008; Slavković et al., 2021). Although it is not known it way of action, *BOP* interacts with TGACG-Binding (*TGA*) *transcription factor* (*TF*) *PERIANTHIA* (*PAN*) coordinating the abaxial patterning of the floral meristem and possibly having a relation to nectary development (Yu, 2019; Slavković et al., 2021). Also, by the four ankyrin repeats, *BOP* could interact with other *TGA TFs* that possibly could be a pathway that regulates the nectary development (Hepworth et al., 2005).

Another known factor that regulates nectary development are hormones, especially auxins. The *AUXIN RESPONSIVE FACTOR 6* (*ARF6*) and *ARF8* are responsible to coordinate the nectary growth and function along with other flower organs (Reeves et al., 2012; Slavković et al., 2021). The *arf6/8* double mutant showed a decreased expression of the *CRC* gene and underdeveloped nectaries, whereas not absent (Reeves et al., 2012). The *STYLISH* gene is a coordinator of auxin homeostasis and targets many *YUCCA* (*YUC*) proteins, which are related to the auxin synthesis path and induces the activity of *ARF6*/8 (Ståldal et al., 2012). In this sense it was found that *YUC8*, a target of *STY*, is expressed during nectary formation, possibly tracing a relationship between it and *Arabidopsis* nectary development (Ståldal et al., 2012). Further, *ARF6/8* also promotes the Jasmonic acid (JA) synthesis pathways which are responsible for the flower termination, growth, and the production of volatile sesquiterpenes, but no relation to nectary development was found (Reeves et al., 2012).

Although this system works for floral nectaries of Arabidopsis, and probably for other nectaries in core eudicots, the *CRC* participation in nectary development was not registered outside them (Moyroud, 2019). In the study of Min et al. (2019) they showed that *CRC* is present in the plant genome of the basal eudicot *Aquilegia* and is responsible of flowering development, but do not take part in nectary development. The evidence points to be the *STY* is the key regulator coordinating the nectary development through the *ARF6/8* without *CRC* involvement (Min et al., 2019). Also, even though was reported by Lee et al. (2005) that *CRC* plays a role in the nectary development of *G. hirsutum* (core eudicot), Pei et al. (2021) found a new gene, the *AP2/Ethylene responsive factor* (*ERF*) that may promote floral and extrafloral nectary development independently of *CRC* or *STY*. This was confirmed by an RNA-seq analysis comparing a nectary tissue of *G. hirsutum* (cotton) with a nectariless leaf of *G. arboretum*, where they found 895 differently expressed genes but no one fell in the category of the *CRC* or *STY* class (Pei et al., 2021). Additionally, the authors claimed that the position where the nectary is may affect the genes that regulates them (Pei et al., 2021). *CRABS CLAW*, in *Arabidopsis*, is highly dependent of the ABC(E) system for its activation whereas *STYLISH*, in *Aquilegia*, regards on petal formatting genes for its activation, as the nectaries on this species are in the abaxial face of petals; at the same time *AP2/ERF* seems responsible for floral and extrafloral nectary development in *G. hirsutum* (Min et al., 2019; Pei et al., 2021; Slavković et al., 2021). A transcriptome analysis by Chatt et al. (2019) in four types of nectaries of *G. hirsutum*, floral, foliar, bracteal and circumbracteal showed that each nectary had a different program of development and rarely expressed the same genes in the same phase of development. The most similar development patterns were between bracteal and circumbracteal where they only share 17% in the pattern of differently expressed genes; whereas the least similar, floral and foliar nectaries shared only 0.02% of the differently expressed genes, having only one single gene that showed the same pattern across all nectaries, the Terpene synthase 21 (AT5G23960.2) (Chatt et al., 2019). The complexity of nectary regulation and its convergent evolutionary nature make it hard to pinpoint specific genes and pathways, although some key genes as *CRC* and *STY* were discovered little is known about its targets and how they mold the great morphological diversity of nectaries (Moyroud, 2019).

Trying to fill this gap in the *Passiflora* genus Krosnick (2006) worked with three *Passiflora* species from the subgenus *Decaloba* (*Passiflora tricuspis*, *P. morifolia* and *P. perakensis*). In the study it was found that *CRABS CLAW* is readily accessible in genomic DNA and is expressed in floral nectaries potentially playing its role of a master regulator of nectary development (Krosnick, 2006). However, the amplification of *CRC* sequence from cDNA of extrafloral nectaries failed, and no expression was found (Krosnick, 2006). As this is the only report, as far as we know, of *CRABS CLAW* expression in extrafloral nectaries of *Passiflora*, it may lead to a conclusion that *CRC* although participate in floral nectary development it is not responsible for the extrafloral. Interestingly, a leaf primordia-based transcriptome of *P. cincinnata* (unpublished data) revealed the presence of the *CRABS CLAW* gene. Considering that *CRC*, apart from some exceptions, it is not expressed outside flowering tissue (Bartholmes et al., 2012) and that one of these exceptions are in extrafloral nectaries of core eudicots (*G. hirsutum* and *Capparis flexuosa*) (Lee et al., 2005), like the *Passiflora* genus. Although contradictory, the *Passiflora* genus is known for its great variability (Pérez and d'Eeckenbrugge, 2017) and *Passiflora alata* and *Passiflora cincinnata* are species from the *Passiflora* subgroup in a way that this divergent, in a tissue that presents a lot of variability, could be expected.

Nectaries are a complex tissue and proved itself to hold a great variability, presenting three different pathways known to date that promote its development (Moyroud, 2019; Pei et al., 2021; Slavković et al., 2021). Also, each type of nectary of the same plant holds its own pattern and development schedule (Chatt et al., 2019). Due to the importance of the *Passiflora* genus and the environmental and commercial value of *P. alata* and *P. cincinnata* we aimed to investigate the role of the *CRABS CLAW* in the development of extrafloral nectaries in this tissue.

REFERENCES

Bartholmes C, Hidalgo O, Gleissberg S (2012) Evolution of the *YABBY* gene family with emphasis on the basal eudicot *Eschscholzia californica* (Papaveraceae). Plant Biol **14**: 11–23

Barton AM (1986) Spatial Variation in the Effect of Ants on Extrafloral Nectary Plant. Ecology **67**: 495–504

Bentley BL (1977) Extrafloral Nectaries and Protection by Pugnacious Bodyguards. Annu Rev Ecol Syst **8**: 407–427

Bowman JL, Smyth DR (1999) *CRABS CLAW*, a gene that regulates carpel and nectary development in *Arabidopsis*, encodes a novel protein with zinc finger and helix-loop-helix domains. Development **126**: 2387–2396

Carmona F, Pereira AMS (2013) Herbal medicines: old and new concepts, truths, and misunderstandings. Rev Bras Farmacogn **23**: 379–385

Cervi AC, Imig DC (2013) A new species of *Passiflora* (Passifloraceae) from Mato Grosso do Sul, Brazil. Phytotaxa **103**: 46–50

Chan MH, Ji HJ, Hong GN, Fletcher JC (2007) *BLADE-ON-PETIOLE* 1 and 2 control *Arabidopsis* lateral organ fate through regulation of LOB domain and adaxial-abaxial polarity genes. Plant Cell **19**: 1809–1825

Chatt EC, Mahalim SN, Mohd-Fadzil NA, Roy R, Klinkenberg PM, Horner HT, Hampton M, Carter CJ, Nikolau BJ (2019) Systems analyses of key metabolic modules of floral and extrafloral nectaries of cotton. bioRxiv. https://doi.org/10.1101/857771

Chitwood DH, Otoni WC (2017) Divergent leaf shapes among *Passiflora* species arise from a shared juvenile morphology. Plant Direct **1**: 1–15

EMBRAPA (2022) Embrapa's passion fruit cultivars. EMBRAPA, https://www.embrapa.br/en/cultivar/maracuja. Access: 10/03/2023

Faleiro FG, Junqueira NTV, Jesus ON de, Costa AM, Machado C de F, Junqueira KP, Araújo FP de, Junghans TG (2017) Espécies de maracujazeiro no mercado internacional. *In* TG Junghans, ON de Jesus, eds, Maracujá: do cultivo à comercialização, Embrapa, Brasília, BR, pp 15–38

Faleiro FG, Oliveira J da S, Junqueira NTV, Santos RS dos (2019) Banco de germoplasma de *Passiflora* L. "Flor da Paixão." Embrapa Cerrados, Planaltina, BR

Faria FS, Stehmann JR (2010) Biologia reprodutiva de *Passiflora capsularis* L. e *P. pohlii* Mast. (*Decaloba*, Passifloraceae). Acta Bot Bras **24**: 262–269

Fern K (2022) Tropical plants data base. Topical.theferns, https://tropical.theferns.info/viewtropical.php?id=Passiflora+suberosa. Access: 10/03/2023

Ferreira FR (2005) Recursos Genéticos em Passiflora. *In* FG Faleiro; NTV Junqueira; MF Braga. EMBRAPA, Planaltina, BR, pp 41–51

Feuillet C, Macdougal JM (2003) A new infrageneric classification of *Passiflora* L. (Passifloraceae). Passiflora **13**: 34–38

Fourquin C, Primo A, Martínez-Fernández I, Huet-Trujillo E, Ferrándiz C (2014) The CRC orthologue from *Pisum sativum* shows conserved functions in carpel morphogenesis and vascular development. Ann Bot **114**: 1535–1544

Gish M, Mescher MC, de Moraes CM (2016) Mechanical defenses of plant extrafloral nectaries against herbivory. Commun Integr Biol **9**: e1178431

Gross T, Broholm S, Becker A (2018) *CRABS CLAW* acts as a bifunctional transcription factor in flower development. Front Plant Sci. **9**: 835

Heads PA, Lawton JH (1985) Bracken, ants and extrafloral nectaries. III. How insect herbivores avoid ant predation. Ecol Entomol **10**: 29–42

Heil M (2011) Nectar: generation, regulation, and ecological functions. Trends Plant Sci **16**: 191–200

Hepworth SR, Zhang Y, McKim S, Li X, Haughn GW (2005) BLADE-ON-PETIOLE–Dependent Signaling Controls Leaf and Floral Patterning in *Arabidopsis*. Plant Cell **17**: 1434–1448

İnal B, Büyük İ, İlhan E, Aras S (2017) Genome-wide analysis of *Phaseolus vulgaris* C2C2-YABBY transcription factors under salt stress conditions. Biotech **7**: 1–10

ITITROPICALS (2016) Global production of passionfruit. ITITROPICALS, Lawrenceville, US.

Killip EP (1938) The American species of Passifloraceae. doi: 10.5962/bhl.title.2269

Kim M, Lim HS, Lee H-H, Kim TH (2017) Role Identification of *Passiflora incarnata* Linnaeus: A Mini Review. J Menopausal Med **23**: 156–159

Koptur S, Jones IM, Peña JE (2015) The Influence of Host Plant Extrafloral Nectaries on Multitrophic Interactions: an Experimental Investigation. PLoS One **10**: e0138157

Krosnick SE (2006) Phylogenetic relationships and patterns of morphological evolution in the Old World Species of *Passiflora* (subgenus *Decaloba:* Supersection *Disemma* and subgenus *Tetrapathaea*). Thesis. Ohio State University, Columbus, Ohio

Krosnick SE, Porter-Utley KE, Macdougal JM, Jørgensen PM, McDade LA (2013) New insights into the evolution of *Passiflora* subgenus *Decaloba* (Passifloraceae): Phylogenetic relationships and morphological synapomorphies. Syst Bot **38**: 692–713

Lachelin L (2022) *Passiflora alata* Curtis (Passifloraceae): Wing-stemmed passionflower. University of Oxford. https://herbaria.plants.ox.ac.uk/bol/plants400/Profiles/OP/Passiflora Access: 10/03/2023

Lee JY, Baum SF, Oh SH, Jiang CZ, Chen JC, Bowman JL (2005) Recruitment of CRABS CLAW to promote nectary development within the eudicot clade. Development **132**: 5021–5032

Linné CV (1797) *Species plantarum*. Laurentius Salvius, Stockholm, SE.

Lobato B, Costa V (2022) Pesquisadores preparam dez novas cultivares de maracujá. Embrapa Produtos e Mercados, https://www.embrapa.br/en/busca-de-noticias/-/noticia/1961787/pesquisadores-preparam-dez-novas-cultivares-de-maracuja Access: 10/03/2023

MacDougal J, Ulmer T (2004) *Passiflora*: Passionflowers of the World. Timber Press, Portland, US.

MAPA (2022) Registro Nacional de Cultivares. MAPA, https://sistemas.agricultura.gov.br/snpc/cultivarweb/cultivares_registradas.php. Access: 10/03/2023

Masters MT (1872a) Passifloraceae. *In* CFP Martius, AW Eichler, I Urban, eds, Flora brasiliensis. F. Fleischer, Munique, DE, pp 596–597

Masters MT (1872b) Passifloraceae. *In* CFP Martius, AW Eichler, I Urban, eds, Flora brasiliensis. F. Fleischer, Munique, DE, pp 609–610

McKim SM, Stenvik GE, Butenko MA, Kristiansen W, Cho SK, Hepworth SR, Aalen RB, Haughn GW (2008) The BLADE-ON-PETIOLE genes are essential for abscission zone formation in *Arabidopsis*. Development **135**: 1537–1546

Meister RJ, Oldenhof H, Bowman JL, Gasser CS (2005) Multiple Protein Regions Contribute to Differential Activities of YABBY Proteins in Reproductive Development. Plant Physiol **137**: 651–662

Mezzonato-Pires AC, Milward-de-Azevedo MA, Mendonça CBF, Gonçalves-Esteves V (2018) Taxonomy, palynology, and distribution notes of seven species of Passiflora L. (Passifloraceae s.s.) newly recorded from Brazil. PhytoKeys **95**: 1–14

Min Y, Bunn JI, Kramer EM (2019) Homologs of the STYLISH gene family control nectary development in *Aquilegia*. New Phytol **221**: 1090–1100

Moraes TS, Rossi ML, Martinelli AP, Dornelas MC (2022) Morphological and anatomical traits during development: highlighting extrafloral nectaries in *Passiflora organensis*. Microsc Res Tech **85**: 2784–2794

Moyroud E (2019) How to be STYLISH: columbine study sheds new light on the obscure mechanisms of nectary formation. New Phytol **221**: 614–617

Nepi M (2007) Nectary structure and ultrastructure. *In* SW Nicolson, M Nepi, E Pacini, eds, Nectaries and Nectar. Springer Netherlands, Dordrecht, pp 129–166

Pei Y, Zhang J, Wu P, Ye L, Yang D, Chen J, Li J, Hu Y, Zhu X, Guo X, et al (2021) GoNe encoding a class VIIIb AP2/ERF is required for both extrafloral and floral nectary development in *Gossypium*. Plant J **106**: 1116–1127

Pérez JO, d'Eeckenbrugge GC (2017) Morphological characterization in the genus *Passiflora* L.: an approach to understanding its complex variability. Plant Syst Evol **303**: 531–558

Proaño J, Rivadeneira E, Moncayo P, Mosquera E (2020) Passion fruit oil (*Passiflora edulis*): Use of the seeds in cosmetic products. Enfoque UTE **11**: 119–129

Reeves PH, Ellis CM, Ploense SE, Wu MF, Yadav V, Tholl D, Chételat A, Haupt I, Kennerley BJ, Hodgens C, et al (2012) A Regulatory Network for Coordinated Flower Maturation. PLoS Genet **8**: e1002506

Rezende MQ, Venzon M, Perez AL, Cardoso IM, Janssen A (2014) Extrafloral nectaries of associated trees can enhance natural pest control. Agric Ecosyst Environ **188**: 198–203

Rocha DI, Batista DS, Faleiro FG, Rogalski M, Ribeiro LM, Mercadante-Simões MO, Yockteng R, Silva ML da, Soares WS, Pinheiro MVM, et al (2020) *Passiflora* spp. Passionfruit. *In* RE Litz, F Pliego-Alfaro, JI Hormaza, eds, Biotechnology of Fruit and Nut Crops, 2nd ed. CABI, Boston, pp 381–408

Romanova MA, Maksimova AI, Pawlowski K, Voitsekhovskaja OV (2021) YABBY Genes in the Development and Evolution of Land Plants. Int J Mol Sci **22**: 4139

Schmid R (1988) Reproductive versus extra-reproductive nectaries—historical perspective and terminological recommendations. Bot Rev **54**: 179–227

Slavković F, Dogimont C, Morin H, Boualem A, Bendahmane A (2021) The Genetic Control of Nectary Development. Trends Plant Sci **26**: 260–271

Soares AR, Marchiosi R, de Cássia Siqueira-Soares R, de Lima RB, dos Santos WD, Ferrarese-Filho O (2014) The role of L-DOPA in plants. Plant Signal Behav **9**: e28275

Ståldal V, Cierlik I, Landberg K, Myrenås M, Sundström JF, Eklund DM, Chen S, Baylis T, Ljung K, Sundberg E (2012) The *Arabidopsis thaliana* transcriptional activator STYLISH1 regulates genes affecting stamen development, cell expansion and timing of flowering. Plant Mol Biol **78**: 545–559

Thokchom R, Mandal G (2017) Production Preference and Importance of Passion Fruit (*Passiflora edulis*): A Review. Journal of Agricultural Engineering and Food Technology **4**: 27–30

Vanderplank J (2000) Passion Flowers, MIT press, Cambridge, MA

Vanderplank J, Edwards S, Hilgenhof R, Rodriguez CM (2017) *Passiflora macrophylla*. Curtis's Botanical Magazine **34**: 123–132

Vecchia M (2022) The *Passiflora* genus. National Italian Passiflora Collection, https://www.passiflora.it/the-passiflora-genus/. Access: 10/03/2023

Yu Y (2019) New Interacting Partners of *BLADE-ON-PETIOLE* in Regulation of Plant Development. Plant Physiol **180**: 697–698

CHAPTER 1 – CLONING AND SPATIO-TEMPORAL EXPRESSION ANALYSIS OF THE *CRABS CLAW* GENE INVOLVED IN EXTRAFLORAL PETIOLAR NECTARY DEVELOPMENT IN *PASSIFLORA ALATA* CURTIS AND *PASSIFLORA CINCINNATA* MASTERS

ABSTRACT

Extrafloral nectaries are defensive structures that, through nectar, attract predators that protect the plants against herbivores. The *Passiflora* genus, known for its economical and nutraceutical importance, has a remarkable presence of extrafloral nectaries. Although it is still little known, there are two master regulators of nectary development: *STYLISH* (*STY*) and *CRABS CLAW* (*CRC*). *STY* act as a key regulator in basal eudicots through the auxin pathway. *CRC* is a member of the *YABBY* family of transcription factor that seems to regulate floral and extrafloral nectary development in many core eudicots. The *Passiflora* genus belongs to the Rosids clade that is within the core eudicot group. Also, a fragment of the *CRC* transcript was found in a leaf primordium transcriptome of *Passiflora cincinnata*, being possible that *CRC* might act as a master regulator of nectary formation in the *Passiflora* genus. Therefore, here we propose to investigate the development of petiolar nectaries under the morphological and anatomical aspects and expression of *CRC* gene in distinct stages of nectary development of *Passiflora alata* and *Passiflora cincinnata*. Petiolar nectaries of *P. alata* and *P. cincinnata* were collected in three distinct stages using as guide the leaf development stages: primordium, in expansion, and expanded. The samples were processed for macromorphology by stereomicroscopy, micromorphology by scanning electron microscopy, anatomy by *in situ* hybridization, RT-qPCR and full-length cDNA by RACE. *P. cincinnata* nectaries are oval-shaped nectary while *P. alata* is calyx-shaped. The nectaries of both are divided into three distinct tissues: epidermis, nectariferous and subnectariferous tissue. The epidermis is multiseriate only in the crater region, whereas the nectariferous tissue is composed of smaller cells with dense cytoplasm. The subnectariferous tissue, by its turn, presents larger, less dense cells and the presence of vascular bundles, that terminate before the nectariferous tissue. *CRC* expression was restricted to the interior of the nectaries and its expression level peaked at the expanding leaf stage; primordia and expanded leaf stage showed similar values. Due to the inefficiency of the primers used, it was not possible to obtain the complete expressed sequence by RACE. There is strong evidence that *CRC* acts as a key regulator in petiolar nectary formation in *P. cincinnata* and *P. alata.*

1. INTRODUCTION

Angiosperms developed many ways to attract animals: juicy nutritional fruits, aromatic compounds, pheromones, sugary substances, garish colors, dressy forms, and many others (Owen, 1980). One of the most common are nectaries, that are described by Schmid (1988) as a specialized set of multicellular glandular structure that secretes nectar, a solution constituted mainly of sugars, used as a reward for pollinators and natural enemies. This feature is spread among angiosperms and convergently evolved multiple times in angiosperms (Antón et al., 2017; Solís et al., 2017). They can be denominated as floral nectaries or as extrafloral nectaries, located in floral or vegetative tissues, when it has other functions instead of attracting pollinators (Solís et al., 2017). Although they are less studied, extrafloral nectaries plays an important role in plant survivability which is often attributed to the attraction of ants and carnivore insects to protect the plant from herbivory and predatory insects (Gish et al., 2016). Further, extrafloral nectaries can function as egg mimics, discouraging oviposition from common predators, as described in Cardoso-Gustavson et al. (2013a) about *Passiflora* species and it most common predator, the *Heliconiini caterpillars*. Still, extrafloral nectaries alone are a defensive structure, since it developed strategies, such as (A) the deposition of calcium crystals in the form of druses in the tissues below the epidermis, which causes difficulty for animals to chew the tissue; (B) increased lignification; (C) prominent levels of L-DOPA, an aminoacid toxic to insects and precursor to many alkaloids, and (D) secondary compounds (Cardoso-Gustavson et al., 2013; Soares et al., 2014; Gish et al., 2016; Moraes et al., 2022), making it a great evolutionary feature as a defensive organ.

Passionflowers are usually herbaceous or woody vines with alternate leaves, axillary stipples, climbing tendrils and have petiolar or/and laminar nectaries (Pérez and d'Eeckenbrugge, 2017). The *Passiflora* genus belong to the Rosids clade (core eudicot) and are the most representative of the Passiforaceae family that has around 588 species native in America, Southeast Asia, and Oceania from which 147 species are native from Brazil (Rome and Coppens d'Eeckenbrugge, 2017; Bruckner et al., 2021). Worldwide passion fruit annual production is about one million tons, from where 80% come from Brazil, with increasing statics due to a higher demand for premium quality and exotic products (ITITROPICALS, 2016; Faleiro et al., 2017). *Passiflora cincinnata* and *Passiflora alata* are two economically relevant species in this genus (Faleiro et al., 2017). The first known as "Maracujá-azedo" has great industrial uses and the second, "Maracujá-doce" is very appreciated for *in natura* consumption (Klein and Ullmann, 2002; CEAGESP, 2018). Both have a very characteristic presence of extrafloral nectaries in the petiole having contributions to its taxonomy (Pérez and d'Eeckenbrugge, 2017; Miranda et al., 2022).

Floral nectary development of *Arabidopsis thaliana*, the most studied model, is coordinated by three key genes: *STYLISH* (*STY*), *CRABS CLAW* (*CRC*) and *BLADE ON PETIOLE* (*BOP*; Slavković et al., 2021). The *STY* gene is mainly responsible for auxin homeostasis which promotes the *AUXIN RESPONSE FACTOR* 6/8 (ARF6/8) that induces the expression of *CRC* (Min et al., 2019). The *BOP* gene is induced by the expression of *TGA TF PERIANTHIA* (*PAN*) and promotes nectary formation alongside *CRC* by acting in the repression of cell division and inducing lateral organ differentiation (Magne et al., 2018). *CRABS CLAW*, distinctively, acts as the master regulator of the process, directly inducing the nectary formation and participate in a regulatory loop with *SHATTERPROOF* (*SHP*), *AGAMOUS* (*AG*), *LEAFY* (LFY), *APETTALA* (*AP*) and *SEPALLATA* (*SEP*), playing an essential role in nectary development (Morel et al., 2018; Slavković et al., 2021). The *CRC* is a transcription factor member of the YABBY family, which is highly conserved in core eudicots, including Asterids and Rosids, two of the biggest clades of the order (Morel et al., 2018). This gene is thought to be originated once from an ancestral *YABBY* gene that was expressed in all lateral organs during flower development, having many orthologs that diversified by speciation (Lee et al., 2005). As a member of the YABBY family, *CRC* has a conserved N-Terminal Zinc Finger domain and a C-terminal Helix-Loop-Helix motif, also called as the YABBY domain, which is similar to the High Mobility Group box (HMG-box) according to Bartholmes et al. (2012) and Fourquin et al. (2014).

Although this may be true to flower nectaries on core eudicots, the extrafloral nectaries development is still uncertain. A transcriptome analysis comparing four types of cotton nectaries by Chatt et al. (2019) revealed that each nectary has its own program of development, rarely sharing the same expression pattern in each developmental stage, in which floral and petiolar nectaries had in common only 0.02% of the differentially expressed genes. Also, a study performed by Krosnick (2006) with three species of the *Passiflora* genus subgenus *Decaloba* (*Passiflora tricuspis*, *Passiflora morifolia* and *Passiflora perakensis*) showed that the *CRC* gene is readily accessible in genomic DNA and is expressed in the floral nectaries but was found no expression in extrafloral nectaries in those species. Further, due to its convergent evolution nature, many species developed other pathways for its development, as is observed in *Aquilegia* (basal eudicot), where compelling evidence support the idea the *STY* gene promotes the nectary development without the *CRC* involvement (Min et al., 2019). Unexpectedly, a transcriptome analysis of *P. cincinnata* primordium (unpublished data) showed the expression of the *CRC* in its tissue, tracing a possible relation between extrafloral nectary development and *CRC* in this species. Although *CRC* is rarely expressed outside flower tissue (Bartholmes et al., 2012) it is reported its presence in extrafloral nectaries and leaves of *Gossypium hirsutum* (cotton), a Rosid (Lee et al., 2005; Hu et al., 2020b). In addition, the *Passiflora* genus exhibit a high and complex variability, having three major clades (*Decaloba*, *Astrophea* and *Passiflora*) in its genus, that could share many genetic differences not yet understood (Pérez and d'Eeckenbrugge, 2017).

Species within *Passiflora* are interesting models for nectary development studies due to the marked presence of extrafloral nectaries and high variability of such secretory structures. In addition, the presence of transcripts of *CRC* gene in leaf primordia of *P. cincinnata* revealed in transcriptome data suggests that the *CRC* expression may play a role in extrafloral nectaries of the petiole in *Passiflora* genus. Thus, in this work we propose to investigate the development of petiolar nectaries under the morphological and anatomical aspects and expression of *CRC* gene in distinct stages of nectary development of *Passiflora alata* and *Passiflora cincinnata*.

2. MATERIALS AND METHODS

Plant material

Seeds of *P. cincinnata* ('BRS- Sertão Forte') and *P. alata* ('BRS-Mel-do-Cerrado'), kindly gifted by Dr. Fábio G. Faleiro (Brazilian Agricultural Research Corporation - EMBRAPA - Cerrados), were sown in 280 cm^3 capacity polypropylene tubes (T-280P, MECPLANT, Paraná, Brazil) containing commercial soil conditioner substrate (MECPREC Florestal 1) with 3 gL^{-1} of Osmocote® Plus 16-08-12 5-6M (ICL, USA), and grown in a greenhouse of the Microbiology Department at the Universidade Federal de Viçosa (UFV), Viçosa, Minas Gerais, Brazil. Plants were grown under natural light conditions (around 12 h photoperiod), with temperatures ranging from 35 - 18 °C during the day and 21 - 11 °C at night (INMET, 2022). When the plants have about twelve leaves, they were transferred to 6 L vases containing the same substrate. Plants were always kept moist and fortnightly.

Nectaries number and position

To determine the number of nectaries, their position and transition, the nectaries of 50 different plants of our population for each species were observed.

Macro-morphology of petiolar nectaries

Petiolar nectaries were sampled from three stages of leaf development: (A) leaf primordium (approximately 1 cm long); (B) expanding leaves (leaves with 15 days old counted from the primordia appearance); and (C) fully expanded leaves (30 days old leaves). The samples were kept moist while observed with the Stereomicroscope (Zeiss) coupled with the Zeiss AxioCam ERc 5s1 camera and photographed.

Nectaries micromorphology

The samples were fixed in Karnovsky solution (Karnovsky, 1965) and submitted to vacuum (-33,3 kPa) for 1 h. Samples were dehydrated in a graded ethanolic series, desiccated in the critical point dryer (Balzers CPD 030) and covered with gold on rotary pumped coater (Quorum Q150R Plus). The material was observed under scanning electron microscope (Zeiss Leo 1430VP) with LEO 32 Software and photo documented.

Nectary anatomy

Petiolar nectaries, of all stages, were fixed in Karnovsky solution (Karnovsky, 1965) and submitted to vacuum (-33,3 kPa) for 1 h. Then, they were dehydrated in a graded ethanolic series and embedded in glycol methacrylate medium (Historesin®, Leica, Wetzlar, DE). Serial sections (5 µm) were obtained in a rotary microtome (RM2265, Leica, Wetzlar, DE) and stained with 1% toluidine blue solution (O'Brien and McCully, 1981). The slides were mounted in Permount™ (Fisher Scientific, Waltham, EUA). Photos were taken in the Zeiss Olympus AX70 photomicroscope coupled with the Zeiss AxioCam HRc camera.

In situ hybridization

Sample preparation

All stages samples were gathered and immediately put in 1,5 ml Eppendorf containing 1.5 ml of fixate solution (Paraformaldehyde 4.0 %; Glutaraldehyde 0.25 %; Phosphate buffer 0.01 M) submitted to 1 h vacuum (-33.3 kPa) and stored for 6 h at 4 °C. The samples were then dehydrated in an ethanolic series (30%, 50%, 70%, 80%, 90%, 100%). The ethanol solution was substituted for xylene (25% xylol: ethanol; 50%; 100%) and then included with increasing concentrations of Histosec® (Leica, Wetzlar, DE) at 65 °C during a one-week period. Then, they were assembled in wood blocks, cut in 8 µm by a rotary microtome with steel microtome blades (Leica, Wetzlar, DE), prepared in silane-prep glass slides (FisherBrand™ Probe On Plus™, Waltham, USA) and stored at 4 °C.

Probe synthesis

Initially, a cDNA contig corresponding to a partial sequence of the *CRC* gene was rescued from transcriptome dataset of *P. cincinnata* (unpublished data). From this 495 pb sequence, a primer pair (Table 1) was designed and used to the gene fragment amplification in both species. *P. cincinnata* and *P. alata* cDNA samples were synthetized from an RNA pool of the three stages of petiolar nectaries. For the reactions were used 5 ng of cDNA, 200 nM of each primer and Platinum II Taq Hot-Start DNA polymerase (Invitrogen, Waltham, USA), according to manufacturer's recommendations. The PCR products were purified with the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, USA), linked on pGEM®-T easy vector (Promega, Madison, USA) and inserted in competent cells of *E. coli* (DH5α). Plasmid DNA of positive clones were isolated, and the cloning confirmed by sequencing using the universal primer M13. Chromatograms were analyzed and the sequences were aligned to the initial sequence using the BioEdit software (full-length obtained sequence are available in SUPPLEMENTARY MATERIAL). The purified plasmid of the confirmed clones for each species was utilized for the anti-sense and sense (negative control) probes using the DIG RNA labeling kit (Roche Applied Science, Indianapolis, USA).

Hybridization reaction and immunological detection

The slides were deparaffinated in Xylol and gradually rehydrated in 0.1% DEPC-treated water. After, a Proteinase K treatment (1µg ml^{-1}) was applied for 30s at 37 °C followed by two DEPC-treated water washes. It was utilized 60 ng of the probe per slide for the hybridization reaction and it occurred at 42 °C for 16 h. Then the slides were washed in decreasing concentrations of SSC buffer at 42 °C and the reaction blocked with a 2% Bovine Serum Albumin (BSA) solution at room temperature for 30 min. For the immunological detection, 150 µL of a 1.5 U ml^{-1} Anti-Digoxigenin-AP anti-bodies solution (Roche Applied Science, Indianapolis, USA) was applied and the slides were kept in the dark for 1h at room temperature. It was used the NBT – BCIP color development substrate for alkaline phosphatase (Promega, Madison, USA) and the reaction occurred in dark, at room temperature, for about 2 h. The slides were washed and prepared in 50% glycerol solution. Sample processing and hybridization protocol were carried according to Dusi (2015). Photos were taken in the Zeiss Olympus AX70 photomicroscope coupled with the Zeiss AxioCam HRc camera.

Rapid amplification of cDNA ends (RACE) for complete CRC sequence

RNA poly A+ was isolated with Dynabeads® mRNA purification Kit (Invitrogen, Waltham, USA) using 75µg of starting total RNA pool from petiolar nectaries for each species. The mRNA was used for the double strand cDNA library construction with the Marathon® cDNA Amplification kit (Takara, San Jose, CA, EUA). Targets 5’ and 3’ for gene CRC were amplified by PCR with the Advantage 2 polymerase mix (Takara, San Jose, CA, EUA). For this, specific primers forward and reverse were combined with Adapter Primers, AP1 and AP2. The amplification product was cloned and sequenced as previously described.

CRC expression levels in petiolar nectaries by quantitative real time PCR (qRT-PCR)

Sample of all stages were collected and immediately frozen in liquid nitrogen. Total RNA was extracted with TRIZOL reagent (Ambion® Life Sciences, Austin, TX, USA). The cDNA synthesis was performed from 600 ng of total RNAs and 5 pmol of Oligo-$(dT)_{18}$ using M-MMLV reverse transcriptase (Promega, Madison, WI, USA). CRABS CLAW gene specific primers were used with the SYBR Green I (Bio-Rad Laboratories, Hercules, CA) in the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). Actin was used as the housekeeping control gene and the expression was calculated by the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen, 2001). Five biological and two technical replicates were utilized for each treatment. The primers used are listed in the Table 1.

Table 1. List of primers used in the experiments.

Target Gene	Primer name	Primer sequence (5' to 3')	Purpose
Crabs claw	PcCRC_F	CTTGGCTTCACCATCCGAAC	Cloning and probe synthesis
Crabs claw	PcCRC_R	GCTTCTAGAGGCAGACCCAG	Cloning and probe synthesis
Actin	PeACT_F	CCAGGCATTGCTGATAGG	RT-qPCR
Actin	PeACT_R	ATTCTGCCTTTGCAATCCAC	RT-qPCR
Crabs claw	PcaCRCqF	CAGCACTGTTCTTGCGGTTG	RT-qPCR
Crabs claw	PcaCRCqR	AGACATTGACCCTGGACGGTA	RT-qPCR

3. RESULTS

Nectaries description

Passiflora cincinnata nectaries do not develop until around the fourth phytomer, when a pair of parallel disposed nectaries appears at the adaxial face the petiole (Figure 1). Moreover, *P. cincinnata* does not develop another pair of nectaries during its lifespan (Figure 2). At the early stages, the nectary initiates as a globular protuberance and then starts to flatten on its top surface changing to an elliptic shape while acquiring a small concavity on later stages from where the secretion flows (Figure 3).

In contrast, *Passiflora alata* Curtis has one to two pairs of nectaries disposed in the adaxial face of the leaf petiole (Figure 1). The first pair of nectaries starts to appear around the third phytomer and a second pair appears around the fifth phytomer. Sometimes, this transition occurs gradually, where a pair plus one nectary develops. The basal pair is the first to develop and tend to be greater in size than the apical pair (Figure 2). The nectary formation starts with a globular protuberance at the base of the leaf primordium. Then, the peripherical portion starts to grow, flattening the top surface of the nectary and protruding it (Figure 3). As the marginal portions continually grow, the depth of the crater increases until it acquires a chalice-like shape, which serves as a nectar reservoir.

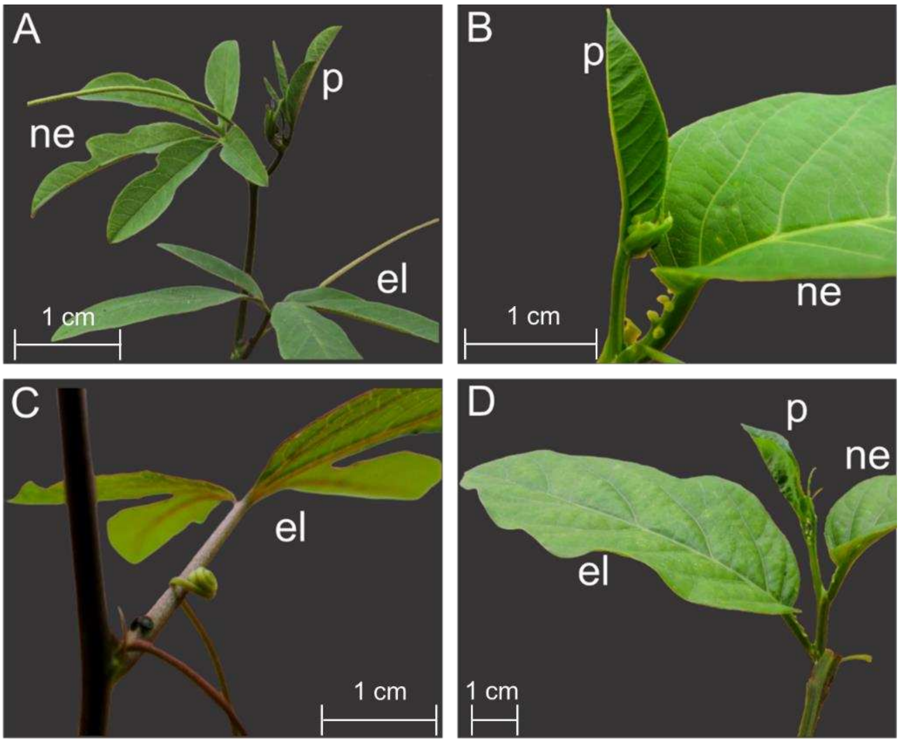

Figure 1. Macro-morphology of *Passiflora cincinnata* (A; C) and *P. alata* (B; D) petiolar nectaries. p: primordia leaf; ne: non-expanded leaf; el: expanded leaf.

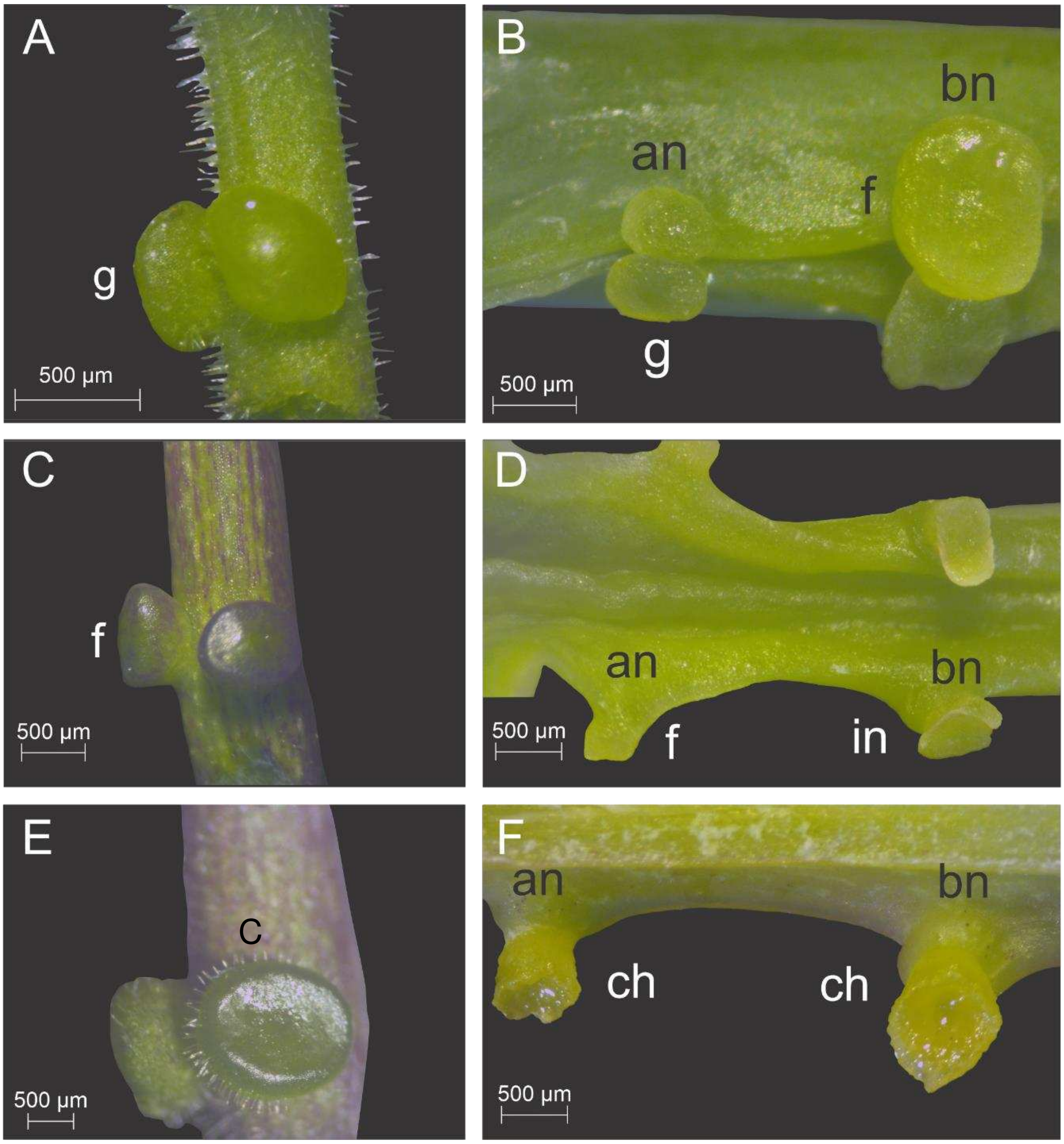

Figure 2. Details of *Passiflora cincinnata* (A; C; E) and *P. alata* (B; D; F) petiolar nectaries under stereomicroscope. Photos were taken of different stages of leaf development: (A-B) leaf primordia; (C-D) non-expanded leaf; and (E-F) expanded leaf. g: globular shape nectary; f: flattening of nectary top surface; c: concavity acquisition of late-stage nectary development; an: apical pair of nectary; bn: basal pair of nectary, in *P. alata*, the basal pair is bigger and develops first than the apical; in: inwards protrusion of nectary top surface; ch: chalice-like shape.

Nectaries anatomy

The two species display a similar anatomy, with an evident cell zoning, which can be seen even in early stages of development. *P. alata* and *P. cincinnata* epidermis have two layers of stratified palisade cells at the crater region, characterizing a multiseriate epidermis (Figure 4). As the epidermis departs from the crater, it converts to a single layer of continuous isodiametric shape cells until it links to the petiole epidermis. At this region, the nucleus is evident at the center of the cell and a thin cuticle covers its surface which protrudes when exudates (Figure 4)

Bellow the epidermis, the nectariferous parenchyma is composed of compact cells with thin walls, small volume and more vacuolated. At the primordia and non-expanded leaf stage, highly meristematic activity can be seen, especially at the in-formation crater region, characterizing the ongoing growth of the nectary whereas this activity is little at the expanded leaf stage, representing a mature nectary (Figure 4c/f).

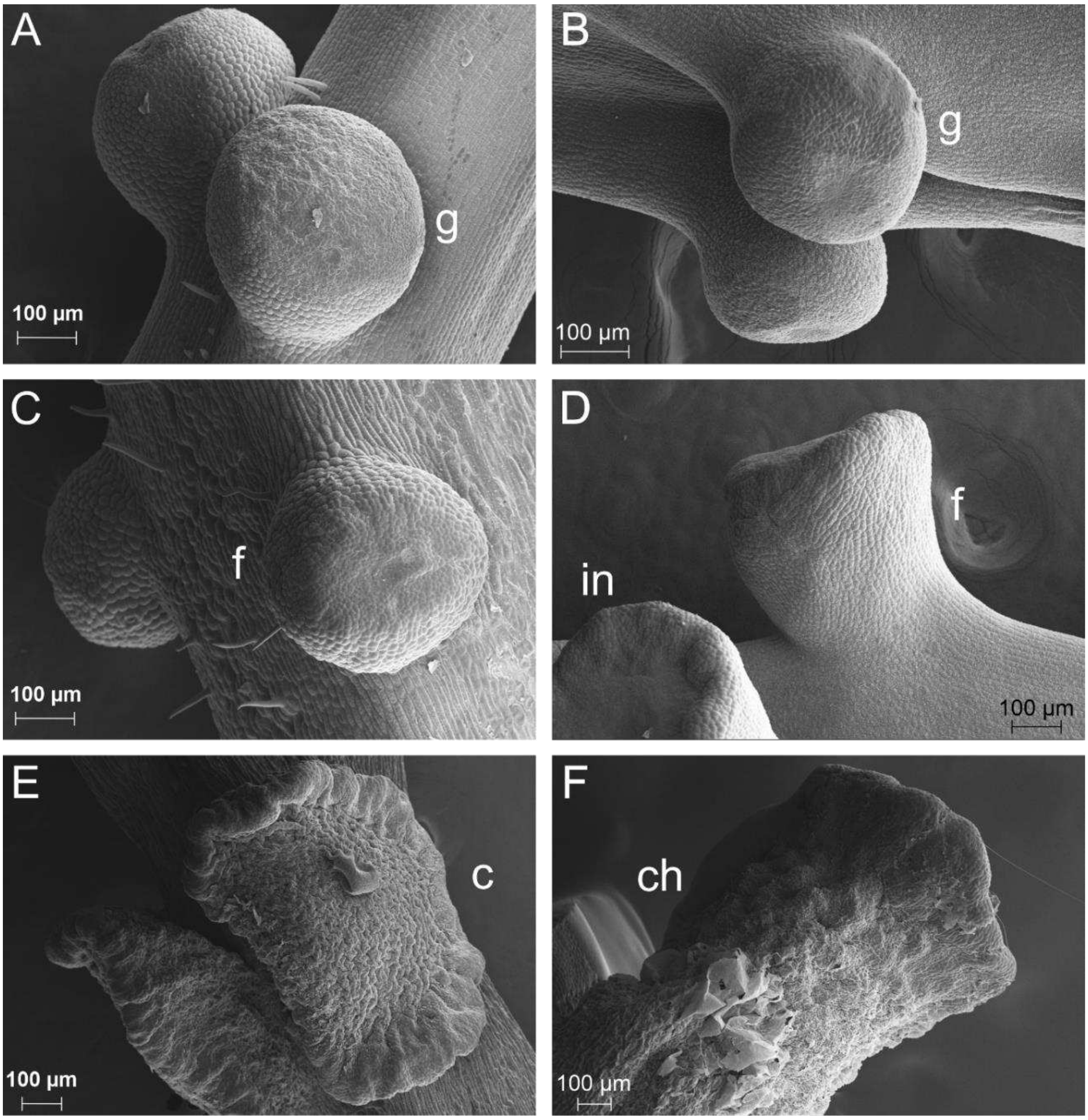

Figure 3. Details *P. cincinnata* (A; C; E) and *P. alata* (B; D; F) petiolar nectaries in Scanning Electron Microscopy (SEM). Photos were taken of different stages of leaf development: (A-B) leaf primordia; (C-D) non-expanded leaf; and (E-F) expanded leaf. g: globular shape nectary; f: flattening of nectary top surface; c: concavity acquisition of late-stage nectary development; in: inwards protrusion of nectary top surface; ch: chalice-like shape.

Closer to the petiole is the subnectariferous parenchyma, characterized by its greater cells, less dense vacuole content and by the presence of vascular bundles. The vascular tissue comes from the nearest bundle of the petiole which branches at base of the nectary (Figure 4c/f). The terminations seem to be composed of phloem only in *P. alata*, and phloem and xylem in *P. cincinnata* (Figure 4c/e). In this tissue, are also found the formation of calcium oxalate druses since the expanding stage (Figure 4**Erro! Fonte de referência não encontrada.**c/e). Little meristematic activity can be seen in this tissue even in the primordia stage, demonstrating the early differentiation of these cells (Figure 4).

CRC gene expression and localization

Analysis of RT-qPCR demonstrated a lower expression of *CRC* in extrafloral nectaries in both species, especially in early stages of development, having its peak during the leaf expansion stage, and then decreases in the expanded leaf stage. In *P. cincinnata*, the expression levels were 3.5-fold superior from the primordia stage to the in-expansion stage, which then decrease to an intermediate level at the expanded leaf stage (Figure 6). On the other hand*,* *P. alata* showed a 13-fold increase when comparing to the primordium stage, decreasing to the levels of the primordium at the expanded leaf stage (Figure 7).

The spatial expression of *CRC* in *Passiflora alata* and *P. cincinnata* occurs only in the interior of the nectary and at all stages of development, showing a distinguishable line that divides the nectary from the petiole tissue (Figure 5). A stronger signal also appears in the borders of nectary, which could indicate that the gene is expressed more in this region of the tissue. Contrariwise, in this same region appears signs of hybridization with the sense probes. (Figure 5).

Unfortunately, we could not obtain the full-length cDNA of CRC by the RACE technique probably due to the short disponible sequence to work on, where we could not design an efficient primer.

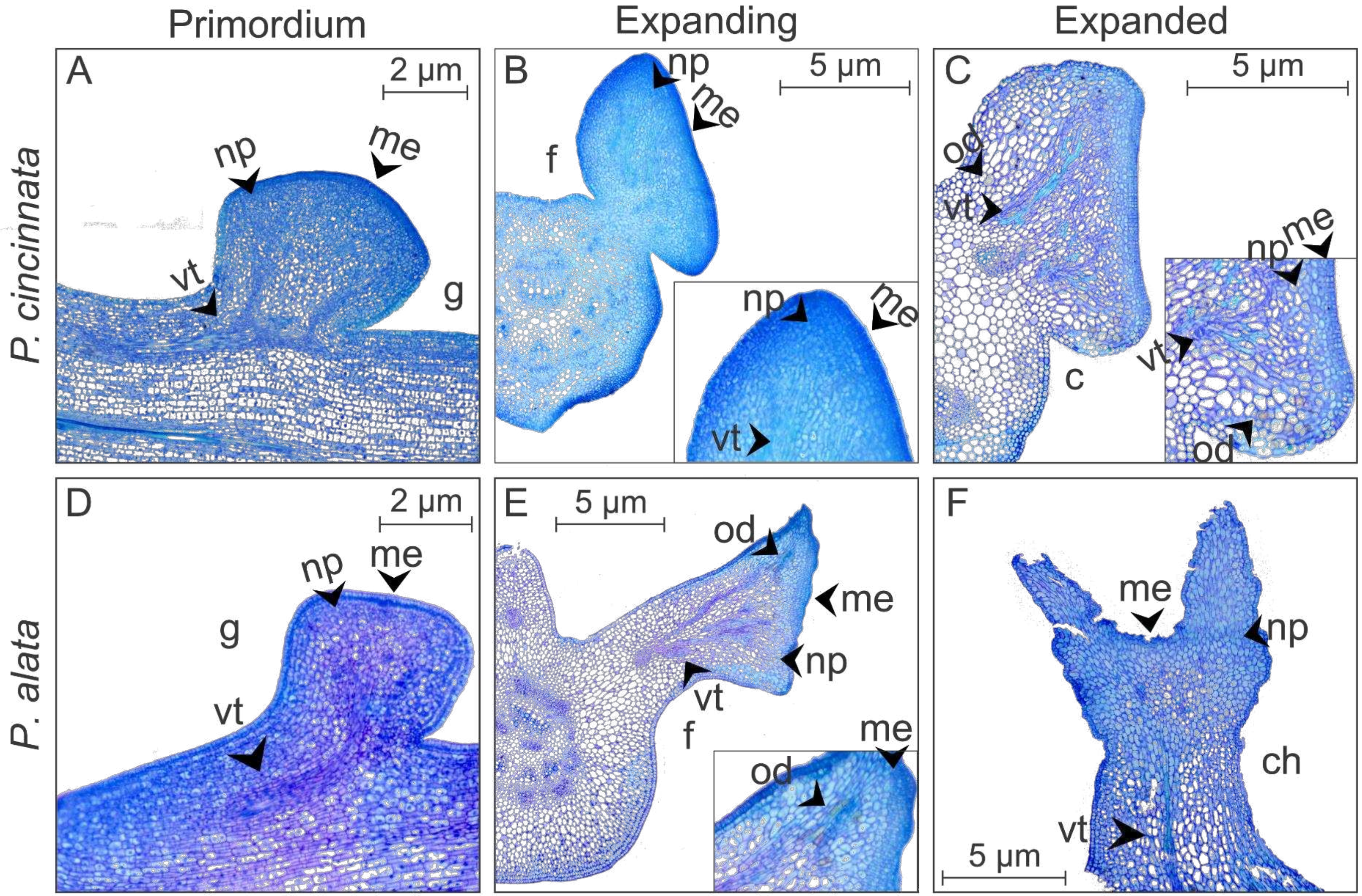

Figure 4. Details of *Passiflora cincinnata* petiolar nectaries in stereo microscope and SEM. Photos were taken of different stages of leaf development: (A-B) leaf primordia; (C-D) non-expanded leaf; and (E-F) expanded leaf. me: multiseriate epidermis; np: nectariferous parenchyma; vt: vascular tissue, the irrigation system of the nectary comes from the nearest vascular bundle and irrigates the subnectariferous parenchyma, but without reaching the nectariferous parenchyma; od: calcium oxalate druses; g: globular shape nectary; f: flattening of nectary top surface; c: concavity acquisition of late-stage nectary development; ch: chalice-like shape.

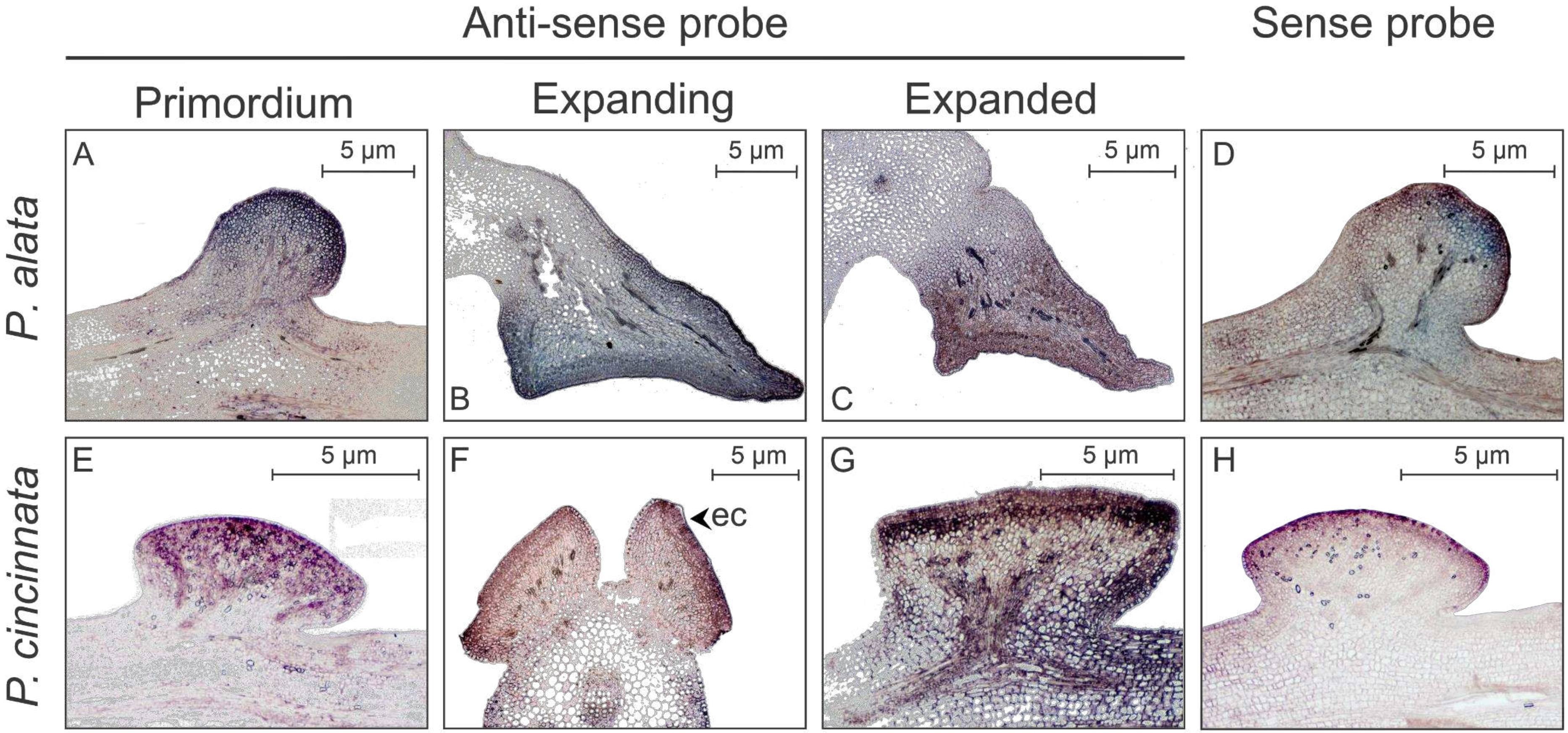

Figure 5. *Passiflora alata* and *Passiflora cincinnata in situ* hybridization for the *CRABS CLAW* (*CRC*) gene anti-sense (A – C and E - F) and sense (D; H) probes taken in different stages of leaf development: (A; E) leaf primordia; (B; F) expanding leaf; (C; G) expanded leaf. ec: erupt cuticle.

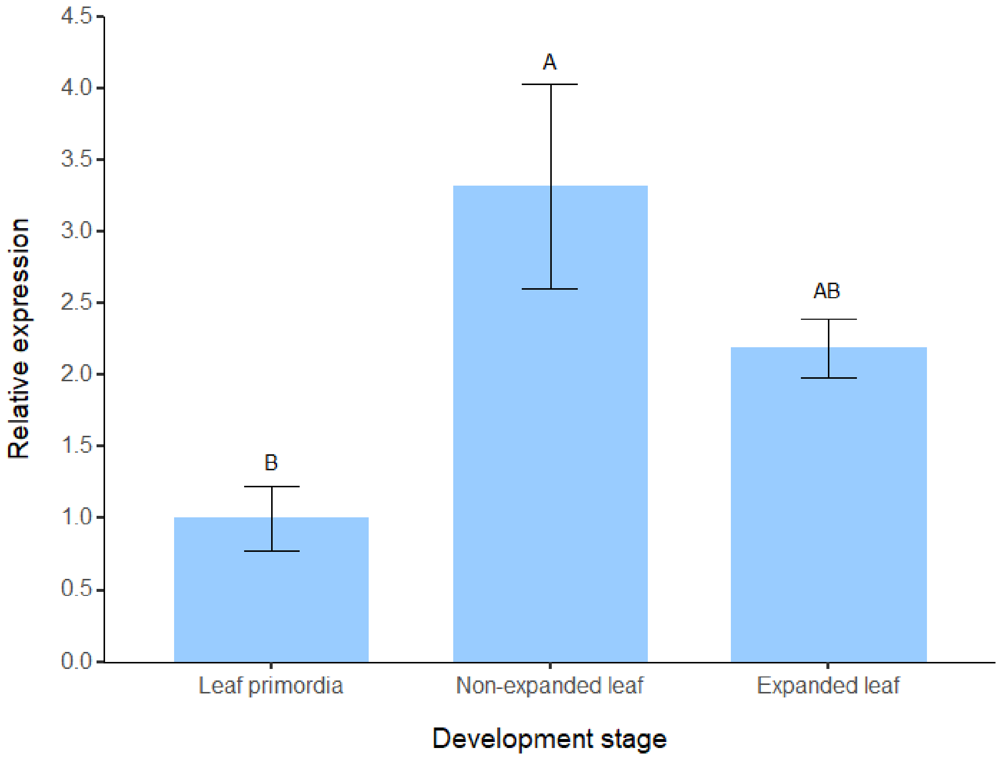

Figure 6. Relative *CRABS CLAW* gene expression in three stages of leaf development of *Passiflora cincinnata*. Same letters do not differ by Tukey's test (P<0.5). Actin was used as the housekeeping gene.

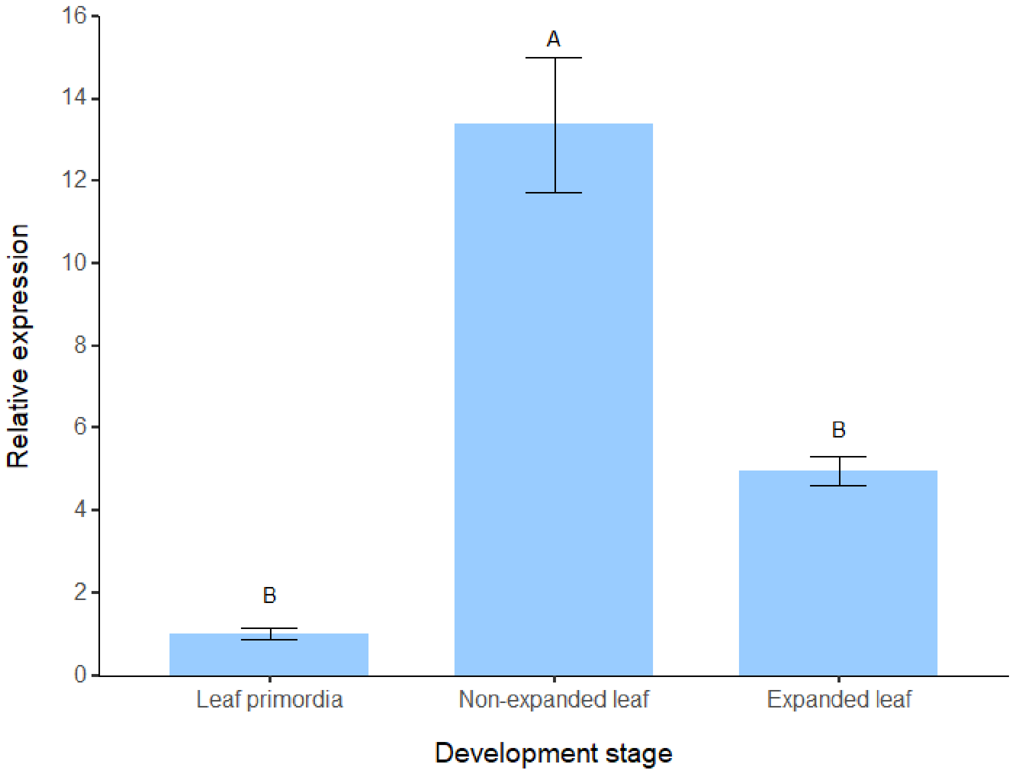

Figure 7. Relative *CRABS CLAW* gene expression in three stages of leaf development of *Passiflora alata*. Same letters do not differ by Tukey's test (P<0.5). Actin was used as the housekeeping gene.

4. DISCUSSION

Macro and micromorphology

The data obtained in this study in what concerns the characteristics of the macro and micromorphology of the nectaries matches with previous description for these species (Nascimento and Barbosa, 2014; Vargas-Silva et al., 2018). The features presented in *P. alata*, led it to be described as a structural elevated nectary with a concave secretory surface (Nascimento and Barbosa, 2014). This type of nectaries is highly specialized, whereupon its size and complex morphology denotes a high investment in maintenance and secretion (Gonzalez and Marazzi, 2018; Jáuregui et al., 2001; Jáuregui et al., 2002; Marazzi et al., 2019; Nascimento and Barbosa, 2014; Rocha et al., 2009). Even though smaller, *P. cincinnata* petiolar nectaries can also be described as structural elevated nectaries, having its secretory surface localized at a concavity at medium-top region (Vargas-Silva et al., 2018).

The three-tissue stratification are a common feature in *Passiflora* and in elevated extrafloral nectaries (EFN), having it divided in epidermis, nectariferous and subnectariferous parenchyma (Silva et al., 2017; Gonzalez and Marazzi, 2018). The epidermis of both species is characterized as multiseriate (Nascimento and Barbosa, 2014; Vargas-Silva et al., 2018), being a common feature seen in other *Passiflora* species, such as, *P. organensis* (Moraes et al., 2022), *P. caerulea*, and *P. biflora* (Durkee, 1982). Other characteristic is the thin cuticle that covers the epidermis and is broke in the moment of the exudation, being commonly seen in many species of the *Passiflora* genus (Durkee, 1982; Nascimento and Barbosa, 2014; Vargas-Silva et al., 2018; Moraes et al., 2022)

The nectariferous parenchyma is commonly attributed to be responsible for nectary production and, in all nectary types, is composed by isodiametric cells with dense vacuole (Nascimento and Barbosa, 2014; Chatt et al., 2021). In *P. alata* these cells commonly present a disform format, mainly because many division planes happen in this tissue, whereas in *P. cincinnata* they are more uniform (Nascimento and Barbosa, 2014; Gonzalez and Marazzi, 2018; Vargas-Silva et al., 2018). On the other hand, the subnectariferous parenchyma commonly present cells with less dense vacuole and larger cells (Chatt et al., 2021). This tissue usually differentiates from the nectariferous parenchyma by the presence of vascular vessels, which comes from the nearest bundle, who terminates without reaching the nectariferous parenchyma (Durkee, 1982; Cardoso-Gustavson et al., 2013; Silva et al., 2017). Interestingly, this termination is different between the species: *P. cincinnata* have vascular bundles that terminates with phloem and xylem termination, whereas *P. alata* have only phloem ones (Nascimento and Barbosa, 2014; Vargas-Silva et al., 2018). The conjunction of nectariferous and subnectariferous parenchyma probably forms a unit that is responsible for nectar production, as occurs in *P. organensis* (Moraes et al., 2022). Another feature common in this tissue is the presence of deposition of calcium oxalate in the form of druses. This hardened deposition occurs in the interior of the cell and confers a mechanical hindrance for herbivores with chewing mouths, helping to prevent that they predate the nectary (Gish et al., 2016).

CRC expression

Krosnick (2006) found no expression of *CRC* in the extrafloral nectaries of *Passiflora tricuspis*, *Passiflora morifolia* and *Passiflora perakensis*, even though they were expressed in floral nectaries. Conversely, we proved, through in situ hybridization and RT-qPCR, that the *CRC* is expressed in the interior of extrafloral nectaries of *Passiflora alata* and *Passiflora cincinnata* and that its rate varies in the different stages of nectary development. Nectaries evolved multiple times in the angiosperms (Marazzi et al., 2013) and species used in Krosnick (2006) work were from the *Passiflora* subgenus *Decaloba* whereas the species in this study comes from *Passiflora* subgenus *Passiflora*. This could explain the divergent results as the different subgenus could had developed its extrafloral nectaries independently, through different developmental mechanism (Marazzi et al., 2013).

The expression of CRC in floral tissue of *Arabidopsis thaliana* begins early, starting in the areas that will further develops on nectaries and then occupying the whole interior of the nectary (Bowman and Smyth, 1999). Also, a stronger signal is commonly verified in the secretory epidermis and its nearby tissues (Bowman and Smyth, 1999). This pattern was also observed in extrafloral nectaries of *Capparis flexuosa* and *Gossypium hirsutum* where the *CRC* was highly expressed in the inner parts of developing extrafloral nectaries (Lee et al., 2005). From where, through RNA analysis, the authors could establish a relation between extrafloral nectaries development in those species and the expression of *CRC* (Lee et al., 2005). By this comparative analysis between *P. cincinnata*, *P. alata*, *G. hirsutum* and *A. thaliana*, we could trace a relation that infers that *CRC* also participate in extrafloral nectaries formation in these species.

In flowering tissue, the mechanisms that underlying the spatial expression of the *CRC* are known to be the *LFY* and *AP3/PI* genes (Slavković et al., 2021). *LFY* is expressed in the early stages of leaf development, albeit not in older leaves, due to the repression of this transcription factor by the floral inhibitor *TERMINAL FLOWER 1* (*TFL1*) (Blázquez et al., 1997; Yamaguchi, 2021). Other researchers also found that *LFY* is expressed in leaf primordia, before it is repressed during its development and in species like orchids and hickory, it was highly expressed in young leaf tissue (Blázquez et al., 1997; Hu et al., 2020a). The *AP1* gene, also, have reports of being expressed in vegetative tissue (Tsaftaris et al., 2004). By this, it is still to be determined in future studies whether it is possible that the same mechanism of flower nectaries, through *LFY* and *AP1* homologs, can exert this function of inhibition of *CRC* expression outside the nectariferous tissue at least during the early stages of leaf development.

The unspecific hybridization with the sense probe was a problem in all stages of leaf development, with the signal being stronger, when not only, in the epidermis and its nearby tissue. DIG-RNA labeled probes unspecific ligation should be very small background and the anti-digoxigenin antibody-AP inaccurate ligation are an extremely rare phenomenon (Roche Diagnostics, 2012; Weil et al., 2012). Anti-sense RNA are transcripts that are naturally synthesized with complementary strand of DNA, forming a mRNA constructed in the 3' – 5' direction (Reis and Poirier, 2021). Although not well established, natural anti-sense transcripts are very common, making 30% of total transcripts of *A. thaliana*, and they are probably related to regulatory process (Wang et al., 2014; Reis and Poirier, 2021). So, the hybridization could have occurred with anti-sense mRNA causing the background seen in the sense probe treatment.

The expression patterns of *CRC* in extrafloral nectaries of *P. alata* and *P. cincinnata* was very similar with the floral nectaries of *Arabidopsis thaliana*. In them, the *CRC* expression was lower at the beginning of the nectary development, and it increases until its peak close to the anthesis, when it starts to decrease to a basal state (Bowman and Smyth, 1999). In the case of *P. cincinnata*, specifically, the *CRC* levels did not decrease in the expanded leaf stage to the same levels of the primordium, like happened in *P. alata*. As leaves are organ with long life cycle and maturation period, *P. cincinnata* levels of *CRC* could still be lowering by the time of collection. Other species like *Petunia hybrida*, *Gossypium hirsutum* and *Capparis flexuosa*, both core eudicots, also present similar patterns of *CRC* spatio-temporal expression providing a high correlation between *CRC* expression and development of extrafloral nectaries (Lee et al., 2005).

Extrafloral nectaries development genetics are still a mystery, having only few studies published yet. By this time, two mechanisms that act as master regulators of nectary development were found: *CRC* and *STY* (Bowman and Smyth, 1999; Min et al., 2019). Future studies with different species could prove that these number can be greater. In this work was found a strong relation between the *CRC* expression and nectary development. When its expression pattern is compared to other species where *CRC* act as master regulator of nectary development it showed similar results, proving strong evidence of its importance to the *P. alata* and *P. cincinnata* extrafloral nectary development.

5. CONCLUSION

The *CRC* is expressed in the interior of the petiolar nectaries of *P. alata* and *P. cincinnata* and have its expression peak during the expansion process of the leaf. These results suggest that *CRC* participate in the extrafloral nectary development of these two species being, probably, its master regulator. Further studies are needed to determine which other genes participate in extrafloral nectary development in the *Passiflora* genus and their targets.

6. REFERENCES

Antoń S, Komoń-Janczara E, Denisow B (2017) Floral nectary, nectar production dynamics and chemical composition in five nocturnal *Oenothera* species (Onagraceae) in relation to floral visitors. Planta **246**: 1051–1067

Bartholmes C, Hidalgo O, Gleissberg S (2012) Evolution of the *YABBY* gene family with emphasis on the basal eudicot *Eschscholzia californica* (Papaveraceae). Plant Biol **14**: 11–23

Blázquez MA, Soowal LN, Lee I, Weigel D (1997) *LEAFY* expression and flower initiation in *Arabidopsis*. Development **124**: 3835–3844

Bowman JL, Smyth DR (1999) *CRABS CLAW*, a gene that regulates carpel and nectary development in *Arabidopsis*, encodes a novel protein with zinc finger and helix-loop-helix domains. Development **126**: 2387–2396

Bruckner CH, Santos CEM, Borém A (2021) Maracujá do plantio à colheita. Editora UFV, Viçosa, BR

Cardoso-Gustavson P, Andreazza NL, Sawaya ACHF, Castro MM, Cardoso-Gustavson P, Andreazza NL, Sawaya ACHF, Castro MM (2013) Only Attract Ants? The Versatility of Petiolar Extrafloral Nectaries in *Passiflora*. Am J Plant Sci **4**: 460–469

CEAGESP (2018) Saiba mais sobre o Maracujá Azedo, o produto destaque da semana no ETSP. https://ceagesp.gov.br/comunicacao/noticias/saiba-mais-sobre-o-maracuja-azedo-o-produto-destaque-da-semana-no-etsp/. Access: 10/03/2023

Chatt EC, Mahalim SN, Mohd-Fadzil NA, Roy R, Klinkenberg PM, Horner HT, Hampton M, Carter CJ, Nikolau BJ (2019) Systems analyses of key metabolic modules of floral and extrafloral nectaries of cotton. bioRxiv. https://doi.org/10.1101/857771

Chatt EC, Mahalim SN, Mohd-Fadzil NA, Roy R, Klinkenberg PM, Horner HT, Hampton M, Carter CJ, Nikolau BJ (2021) Nectar biosynthesis is conserved among floral and extrafloral nectaries. Plant Physiol **185**: 1595–1616

Durkee LT (1982) The floral and extra-floral nectaries of *Passiflora* II: The extra-floral nectary. Am J Bot **69**: 1420–1428

Dusi DM de A (2015) Hibridização *in situ* para detecção da expressão de genes em tecidos vegetais. *In* ACM Brasileiro, VTC Carneiro, eds, Manual de transformação genética de plantas, EMBRAPA, Brasília, BR, pp 303–328

Faleiro FG, Junqueira NTV, Jesus ON de, Costa AM, Machado C de F, Junqueira KP, Araújo FP de, Junghans TG (2017) Espécies de maracujazeiro no mercado internacional. *In* TG Junghans, ON de Jesus, eds, Maracujá: do cultivo à comercialização, Embrapa, Brasília, BR, pp 15–38

Fourquin C, Primo A, Martínez-Fernández I, Huet-Trujillo E, Ferrándiz C (2014) The *CRC* orthologue from *Pisum sativum* shows conserved functions in carpel morphogenesis and vascular development. Ann Bot **114**: 1535–1544

Gish M, Mescher MC, de Moraes CM (2016) Mechanical defenses of plant extrafloral nectaries against herbivory. Commun Integr Biol **9**: e1178431

Gonzalez AM, Marazzi B (2018) Extrafloral nectaries in Fabaceae: filling gaps in structural and anatomical diversity in the family. Bot J Linnean Soc **187**: 26–45

Hu J, Jin Q, Ma Y (2020a) *AfLFY*, a *LEAFY* homolog in *Argyranthemum frutescens*, controls flowering time and leaf development. Sci Rep 2020 10:1 **10**: 1–11

Hu W, Qin W, Jin Y, Wang P, Yan Q, Li F, Yang Z (2020b) Genetic and evolution analysis of extrafloral nectary in cotton. Plant Biotechnol J **18**: 2081–2095

INMET (2022) Mapa de estações meteorológicas. Portal INMET, https://mapas.inmet.gov.br. Access: 10/03/2023

ITITROPICALS (2016) Global production of passionfruit. ITITROPICALS, Lawrenceville, US.

Jáuregui D, García M, Pérez D (2001) Morfoanatomia de las glándulas secretoras de *Passiflora guazumaefolia* Juss. y *Passiflora aff. P. tiliaefolia* L. (Passifloraceae) presents en Venezuela. Rev Int Bot Exp **70**, 229–235.

Jáuregui D, García M, Pérez D (2002) Morfoanatomía de las glándulas em cuatro espécies de Passiflora L. (Passifloraceae) de Venezuela. Caldasia **24**, 33–40.

Karnovsky MJ (1965) A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. J Cell Biol **27**: 137

Klein D, Ullmann S (2002) Maracujá: variedades para industrialização. UFRGS, https://www.ufrgs.br/alimentus1/feira/mpfruta/maracuja/vari.htm. Access: 10/03/2023

Krosnick SE (2006) Phylogenetic relationships and patterns of morphological evolution in the Old World Species of *Passiflora* (subgenus *Decaloba:* Supersection *Disemma* and subgenus *Tetrapathaea*). Thesis. Ohio State University, Columbus, Ohio

Lee JY, Baum SF, Oh SH, Jiang CZ, Chen JC, Bowman JL (2005) Recruitment of *CRABS CLAW* to promote nectary development within the eudicot clade. Development **132**: 5021–5032

Livak KJ, Schmittgen TD (2001) Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. Methods **25**: 402–408

Magne K, George J, Berbel Tornero A, Broquet B, Madueño F, Andersen SU, Ratet P (2018) *Lotus japonicus NOOT-BOP-COCH-LIKE1* is essential for nodule, nectary, leaf, and flower development. Plant J **94**: 880–894

Marazzi B, Bronstein JL, Koptur S (2013) The diversity, ecology and evolution of extrafloral nectaries: current perspectives and future challenges. Ann Bot **111**: 1243–1250

Marazzi B, Gonzalez AM, Delgado-Salinas A, Luckow MA, Ringelberg JJ, Hughes CE (2019) Extrafloral nectaries in Leguminosae: phylogenetic distribution, morphological diversity and evolution. Aust Syst Bot **32**: 409–458

Min Y, Bunn JI, Kramer EM (2019) Homologs of the *STYLISH* gene family control nectary development in *Aquilegia*. New Phytol **221**: 1090–1100

Miranda VS, Gütler Rodrigues L, Dutra SC, G. Sobrinho T, Alves-Araújo A (2022) Extrafloral nectaries of an Atlantic Forest conservation area in Southeastern Brazil. Acta Bot Bras **36**: e2021abb0187

Moraes TS, Rossi ML, Martinelli AP, Dornelas MC (2022) Morphological and anatomical traits during development: highlighting extrafloral nectaries in *Passiflora organensis*. Microsc Res Tech **85**: 2784–2794

Morel P, Heijmans K, Ament K, Chopy M, Trehin C, Chambrier P, Bento SR, Bimbo A, Vandenbussche M (2018) The Floral C-Lineage Genes Trigger Nectary Development in *Petunia* and *Arabidopsis*. Plant Cell **30**: 2020–2037

Nascimento KC, Barbosa JF (2014) Caracterização morfoanatômica de nectários extraflorais de *Passiflora alata*, Passifloraceae. Rev UNINGÁ **20**: 45–50

O'Brien TP, McCully ME (1981) The study of plant structure: principles and selected methods. Blackwell Scientific Publications, Oxford, UK.

Owen DF (1980) How Plants May Benefit from the Animals That Eat Them. Oikos **35**: 230

Pérez JO, d'Eeckenbrugge GC (2017) Morphological characterization in the genus *Passiflora* L.: an approach to understanding its complex variability. Plant Syst Evol **303**: 531–558

Reis RS, Poirier Y (2021) Making sense of the natural antisense transcript puzzle. Trends Plant Sci **26**: 1104–1115

Roche Diagnostics (2012) DIG System Sensitivity and Specificity. Roche Diagnostics, Mannheim, DE.

Rome M, Coppens d'Eeckenbrugge G (2017) Delimitation of the series Laurifoliae in the genus *Passiflora* (Passifloraceae). Phytotaxa **309**: 245–252

Rocha DI, Campos Da Silva L, Valente VMM, Francino DMT, Meira RMSA (2009) Morphoanatomy and development of leaf secretory structures in *Passiflora amethystina* Mikan (Passifloraceae). Aust J Bot **57**: 619

Schmid R (1988) Reproductive versus extra-reproductive nectaries—historical perspective and terminological recommendations. Bot Rev **54**: 179–227

Silva EO, Feio AC, Cardoso-Gustavson P, Milward-De-Azevedo MA, dos Santos JUM, de Aguiar Dias ACA (2017) Extrafloral nectaries and plant–insect interactions in *Passiflora* L. (Passifloraceae). Rev Bras Bot **40**: 331–340

Slavković F, Dogimont C, Morin H, Boualem A, Bendahmane A (2021) The Genetic Control of Nectary Development. Trends Plant Sci **26**: 260–271

Soares AR, Marchiosi R, de Cássia Siqueira-Soares R, de Lima RB, dos Santos WD, Ferrarese-Filho O (2014) The role of L-DOPA in plants. Plant Signal Behav **9**: e28275

Solís SM, Zini LM, González VV, Ferrucci MS (2017) Floral nectaries in Sapindaceae s.s.: morphological and structural diversity, and their systematic implications. Protoplasma **254**: 2169–2188

Tsaftaris AS, Pasentsis K, Iliopoulos I, Polidoros AN (2004) Isolation of three homologous AP1-like MADS-box genes in crocus (*Crocus sativus* L.) and characterization of their expression. Plant Sci **166**: 1235–1243

Vargas-Silva VM, López-Meruvia MY, Quevedo AW (2018) Morfo-anatomía de nectarios extraflorales de *Passiflora cincinnata* Mast. y *Passiflora tricuspis* Mast. (Passifloraceae). Rev Soc Boliv Bot **10**: 4–14

Wang H, Chung PJ, Liu J, Jang I-C, Kean MJ, Xu J, Chua N-H (2014) Genome-wide identification of long noncoding natural antisense transcripts and their responses to light in *Arabidopsis*. Genome Res **24**: 444–453

Weil R, Wolf C, Farfsing A, Tiantom J, Heller A, Bergauer T, Day W, Rueger B (2012) A Method for High Quality Digoxigenin-Labeled RNA Probes for In Situ Hybridization. Roche Diagnostics, Mannheim, DE.

Yamaguchi N (2021) *LEAFY*, a Pioneer Transcription Factor in Plants: A Mini-Review. Front Plant Sci **12**: 701406

FINAL CONSIDERATIONS

This work suggests strong evidence that *CRABS CLAW* plays an important role in the development of extrafloral nectaries of *P. alata* and *P. cincinnata*. Its expression is limited to the nectariferous tissue and has its peak during the leaf expansion phase. Although an attempt to clone the complete expressed sequence of the *CRC* was made, it was frustrated by the inadequacy of the specific primer. A novel trial will be made with a primer designed from the *CRC* sequence recently obtained from the *Passiflora organensis* genome. This work will serve as a base for prospects on the development and role of the *CRC* on extrafloral nectaries of the *Passiflora* genus.

SUPPLEMENTARY MATERIAL

CLONED *CRABS CLAW* PCR PRODUCT USED FOR SYNTHESIS OF *IN SITU* HYBRIDIZATION PROBES

Passiflora cincinnata
ATGGACTTGGCTTCACCATCCGAACATCTCTGCTATGTCCGCTGCAACTTCTGC
AGCACTGTTCTTGCGGTTGGGATTCCGTGCAAGCGCTTGCTGGAAACTGTGACC
GTCAAATGTGGCCACTGCAACAACTTATCCTTTCTCAGCAGCAGACCTACCGTC
CAGGGTCAATGTCTTGATCTTCATCAATTGAATCTTCATGAAAAGCATGGATTTT
GCAGTGAATTAAGGAAGGGTCAGTCATCATCTTCATCATCATCCACTTCCAGTGA
GCCATTGTCACCCAAGGCACCTTTTGTCGTGAAACCTCCAGAGAAGAAACACCG
GCTTCCATCTGCTTACAACAGATTTATGAAGGAGGAGATACAGCGCATCAAAGC
TGCCAATCCTGAGATACCACATCGGGAAGCTTTTAGCACTGCAGCAAAAAATTG
GGCTAGGTACATCCCAAACTCCTCCTCTGGGTCTGCCTCTAGAAGCAGTAACAA
TACTTGA

Passiflora alata
CTTGGCTTCACCATCCGAACATCTCTGCTATGTCCGCTGCAACTTCTGCAGCAC
TGTTCTTGCGGTTGGGATTCCGTGCAAGCGCTTGCTGGAAACTGTGACTGTCAA
ATGTGGCCACTGCAACAACTTATCCTTTCTCAGCAGCAGACCTACCGTCCAGGG
TCAATGTCTTGATCTTCATCAATTGAATCTTCATGAARAGCATGGATTTTGCAGT
GAATTAAGGAAGGGTCAGTCATCATCTTCATCSTCATCAACTTCCAGTGAGCCAT
TGTCACCCAAGGCACCTTTTGTCGTGAAACCTCCAGAGAAGAAACACCGGCTTC
CATCTGCTTACAACAGATTTATGAAGGAGGAGATACAGCGCATCAAAGCTGCCA
ATCCTGAGATACCACATCGGGAAGCTTTTAGCACAGCAGCCAAAAATTGGGCTA
GGTACATCCCAAACTCCTCCTCTGGGTCTGCCTCTAGAAGC