UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

POSGRADO EN CIENCIAS BIOLÓGICAS

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BIOLOGÍA

Transcriptómica comparativa del desarrollo floral en especies del género *Disocactus* (Hylocereeae, Cactaceae)

TESIS

QUE PARA OPTAR POR EL GRADO DE:

DOCTORA EN CIENCIAS BIOLÓGICAS

PRESENTA:

M. en C. ISAURA ROSAS REINHOLD

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COORDINACIÓN DEL POSGRADO EN CIENCIAS BIOLÓGICAS

INSTITUTO DE BIOLOGÍA

OFICIO CPCB/1141/2022

ASUNTO: Oficio de Jurado

M. en C. Ivonne Ramírez Wence
Directora General de Administración Escolar, UNAM
P r e s e n t e

Me permito informar a usted que en la reunión ordinaria del Comité Académico del Posgrado en Ciencias Biológicas, celebrada el día **31 de octubre de 2022** se aprobó el siguiente jurado para el examen de grado de **DOCTORA EN CIENCIAS** de la estudiante **ROSAS REINHOLD ISAURA** con número de cuenta **304029426** con la tesis titulada **"TRANSCRIPTÓMICA COMPARATIVA DEL DESARROLLO FLORAL EN ESPECIES DEL GÉNERO *DISOCACTUS* (HYLOCEREEAE,CACTACEAE)",** realizada bajo la dirección del **DR. ÁNGEL SALVADOR ARIAS MONTES,** quedando integrado de la siguiente manera:

Presidente:	DRA. SONIA VÁZQUEZ SANTANA
Vocal:	DRA. TERESA MARGARITA TERRAZAS SALGADO
Vocal:	DRA. ALEJANDRA VÁZQUEZ LOBO YURÉN
Vocal:	DR. DANIEL SÁNCHEZ CARBAJAL
Secretario:	DRA. SVETLANA SHISHKOVA

Sin otro particular, me es grato enviarle un cordial saludo.

A T E N T A M E N T E
"POR MI RAZA HABLARÁ EL ESPÍRITU"
Ciudad Universitaria, Cd. Mx., a 06 de diciembre de 2022

COORDINADOR DEL PROGRAMA

DR. ADOLFO GERARDO NAVARRO SIGÜENZA

POSGRADO EN CIENCIAS BIOLOGICAS
COORDINACIÓN

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Dedicatoria

Este trabajo está dedicado a mis hermanos, Amaranta y Balam mis amigos de toda la vida.

A mi compañero de vida, amigo y confidente Alejandro.

Índice general

Resumen

Investigar a las cactáceas desde la perspectiva de la evolución del desarrollo (evo-devo) y estudiar las bases genéticas que subyacen en el desarrollo de los órganos que las componen, incluidos los de la flor, nos permite comprender el origen de las estructuras en la familia y además conocer las causas de la variación morfológica en el grupo. En esta investigación, obtuve el ensamble *de novo* del transcriptoma de dos especies del género *Disocactus* con morfología floral contrastante en diferentes estadios de desarrollo: *Disocactus speciosus* y *D. eichlamii*. Los dos transcriptomas, uno por especie, fueron de buena calidad, con estos se llevaron a cabo análisis de expresión diferencial que arrojaron variación de la expresión génica de transcritos asociados al tamaño y número de órganos. El Weigted Gene Co-expression Network Analysis (WGCNA) mostró módulos de co-expresión enriquecidos con procesos biológicos involucrados en el crecimiento y en el tamaño final de los órganos, además de los relacionados al desarrollo floral. También, medí el área de células epidérmicas de la cara adaxial de tépalos de ambas especies. Estos análisis de tamaño celular en los diferentes estadios de desarrollo en las dos especies mostraron que el tamaño de la flor es especie específica y se correlaciona con el tamaño celular. Con microscopía electrónica de barrido (MEB) observé que las células epidérmicas de tépalos no son como las descritas en especies modelo y en vez de ser cónicas son tabulares, sugiriendo que las células de epidermis en los tépalos de las especies estudiadas son diferentes a las reportadas en otras especies y que posiblemente el efecto que tiene la incidencia de la luz en estas flores es diferente a la que se observaría en flores con pétalos con células cónicas. Con los datos obtenidos podemos concluir que la diferencia en los patrones de expresión de genes específicos como son *BIG BROTHER* y *BIG PETAL* pueden ser responsables de la variación en el tamaño de la flor entre especies cercanamente relacionadas. También analicé la *rama-flor* de Cactaceae revisando la información sobre el desarrollo de éstas y de otros géneros del orden Caryophyllales. Además, revisé los estudios en *Arabidopsis* y en otras especies modelo para comprender las bases genéticas del desarrollo floral, principalmente el modelo ABCE de determinación de verticilos florales. Finalmente propuse tres hipótesis que explicarían el desarrollo floral en Cactaceae, en lo referente a la identidad genética de tépalos y del pericarpelo.

Palabras clave: Desarrollo floral, tépalos, RNA-seq, cactus, evo-devo, tamaño floral, crecimiento floral

Abstract

Studying cacti from the perspective of developmental evolution (evo-devo) and the genetic bases that underlie the development of the organs that compose them, including those of the flower, allows us to understand the origin of the structures in the cacti family and also to know the causes of morphological variation in the group.

In this investigation, the *de novo* transcriptome assemblies of two species of *Disocactus* genus with contrasting floral morphology at different stages of development were obtained: *Disocactus speciosus* and *D. eichlamii*. The two transcriptomes, one per species, were of good quality; differential expression analysis were carried out with these data, which revealed variation in the gene expression of transcripts associated with the size and number of organs. The Weigted Gene Co-expression Network Analysis (WGCNA) showed co-expression modules enriched with biological processes involved in growth and final organ size, in addition to those related to floral development. Also, I measured the tepal epidermal cell area of the adaxial face in both species. These analyzes of cell size in the different stages of development in the two species showed that flower size is species-specific and correlates with cell size. With scanning electron microscopy (SEM) I observed that the epidermal cells of tepals are not like those described in model species and instead of being conical they are tabular, suggesting that the epidermal cells in the tepals of the studied species are different from those reported in other species and that possibly the effect that the incidence of light has on these flowers is different from what would be observed in flowers with petals with conical cells. With the data obtained we can conclude that the difference in the expression patterns of specific genes such as *BIG BROTHER* and *BIG PETAL* may be responsible for the variation in flower size between closely related species. I also analyzed the *branch-flower* of Cactaceae reviewing the information on the development of these and other genera of the order Caryophyllales. In addition, I reviewed studies on *Arabidopsis* and other model species to understand the genetic basis of flower development, mainly the ABCE model of flower whorl determination. Finally, I propose three hypotheses that would explain the floral development in Cactaceae, in relation to the genetic identity of tepals and the pericarpel.

Key words: flower development, tepals, RNA-seq, cacti, evo-devo, flower size, floral growth.

Introducción general

La flor es una estructura compleja conformada generalmente por cuatro tipos de verticilos: cáliz (sépalos), corola (pétalos), gineceo (ovario, estilo y estigma) y androceo (filamentos y anteras). Debido a los avances en genética y biología del desarrollo hoy se sabe que la identidad de estos verticilos está determinada genéticamente por los genes descritos en el modelo ABCE en *Arabidopsis*/*Antirrhinum* (Bowman et al., 1989; Chanderbali et al., 2010; Coen & Meyerowitz, 1991). El modelo plantea que la expresión de estos genes homeóticos, que están agrupados en diferentes clases, dirigen el desarrollo de los cuatro verticilos que típicamente conforman una flor. La expresión de genes de función A (*APETALA1* [*AP1*] y *APETALA2* [*AP2*]), en la zona más externa del meristemo floral, dirige el desarrollo de los primordios que darán origen a los sépalos; la expresión simultánea de genes de función A y B (*APETALA3/DEFICIENS* [*AP3/DEF*] y *PISTILLATA/GLOBOSA* [*PI/GLO*]) determina el desarrollo de los pétalos; mientras que la expresión simultánea de genes de función B y C (*AGAMOUS/PLENA* [*AG/PLE*]) determina el desarrollo de los estambres. Finalmente, la expresión de genes de función C, determina el desarrollo de los carpelos. Los genes antes mencionados, con excepción de *AP2*, son factores de transcripción de la familia génica de los *MADS-box* tipo II (Bowman et al., 1989) (Bowman et al., 1989)También se sabe que variaciones en la morfología floral están determinadas por otras clases de genes como los relacionados a la simetría floral, por ejemplo: *CYCLOIDEA* (*CYC*), *DICHOTOMA* (*DICH*), *RADIALIS* (*RAD*) y *DIVARICATA* (*DIV*), y *RAD-and-DIV-Interacting-Factors* (*DRIFs*), observados en especies modelo con simetría bilateral como *Antirrhinum majus* (Corley et al., 2005). Los cambios en la expresión de genes involucrados en las rutas de síntesis de betalaínas y antocianinas han sido relacionados con cambios en patrones de pigmentación en flores de diferentes especies (Hatlestad et al., 2012). Por otro lado, genes como *REVOLUTA/INTERFASCICULAR FIBERLESS 1* (*REV/IFL1*), *ARGOS* (*ARG*), *AINTEGUMENTA* (*ANT*) y *BIG PETAL* (*BP*) han sido observados en modificaciones en el tamaño de la flor (Nishijima, 2012).

Aunado a esto, cambios de expresión en genes como *SUPERMAN* (*SUP*), *PERIANTHIA* (*PAN*) y *ETTIN* (*ETT*) (Cheng & Zhao, 2007; Sakai et al., 1995; Sessions et al., 1997*)* han sido relacionados a la merosidad, es decir, a la variación en el número de órganos florales como pétalos y estambres.

En Cactaceae existe una gran diversidad de formas y tamaños en las flores: hay flores pequeñas (5—7 mm de longitud) como las de *Blossfeldia liliputana* (Barthlott & Porembski, 1996*)* o gigantes (30—32 cm de longitud) como las de *Epiphyllum chrysocardium*. Las flores pueden ser de diversos colores: de blanco a magenta, amarillo y anaranjado. Algunas flores tienen aromas perfumados como las especies del género *Epiphyllum*, mientras que otras emiten olores desagradables como en algunas flores de especies columnares, generalmente polinizadas por murciélagos (Valiente-Banuet, 2002). En cactáceas hay flores zigomorfas como las de *Aporocactus flagelliformis* y actinomorfas observadas en la mayoría de los géneros. Finalmente es posible observar en las diferentes especies una gran variación en el número de segmentos del perianto.

Las cactáceas presentan patrones de desarrollo floral diferentes a los de sus hermanos dentro del orden Caryophyllales (Cuénoud et al., 2002). Mientras que en Anacampserotaceae se observan dos brácteas involucrales, cinco pétalos y 13—15 estambres que surgen de un anillo meristemático (Vanvinckenroye & Smets, 1999), en Cactaceae, se observan múltiples segmentos del perianto, el desarrollo espiralado y gradual desde estructuras que han sido denominadas como brácteas u hojas ya que portan "yemas" en sus axilas (Mauseth, 2016) a tépalos-petaloides, la presencia de un anillo meristemático que da origen a múltiples estambres y el pericarpelo el cual podría tener una identidad axial (Mauseth, 2006).

La variación morfológica a nivel floral descrita previamente y los patrones de desarrollo floral observados en la familia abren preguntas diversas como, por ejemplo, la identidad genética de los órganos y cómo es que, los genes del modelo ABCE están interactuando para formar los diferentes verticilos que componen a la flor de Cactaceae. También nos hace cuestionarnos cómo se regulan los genes que determinan la forma zigomorfa en esta familia y si son los mismos a los reportados en Orchidaceae

(Mondragón-Palomino & Theissen, 2008) o cómo se regulan los genes que controlan el tamaño final en las flores.

En disciplinas como taxonomía y sistemática, las flores de Cactaceae como de otros clados de angiospermas, tienen un papel importante porque son indispensables para la identificación de especies (Anderson, 2001). Pero, poco sabemos de los procesos de desarrollo de la flor (Payer, 1857) y prácticamente nada de los genes, menos aún de los mecanismos genéticos que están involucrados en la variación de la morfología floral en esta familia. Gracias a trabajos en especies no modelo podemos suponer que ortólogos de genes como los mencionados anteriormente pudieran estar jugando un papel en la variación observada en las flores de diferentes especies. Para poder determinarlo son necesarios estudios en genética del desarrollo y ontogenia, que permitan resolver preguntas como cuáles son los mecanismos genéticos que influyen en la diversidad morfológica floral en Cactaceae ó qué variaciones en sus patrones de expresión podrían correlacionarse con la variación morfológica en este grupo, y cómo los genes del modelo ABCE están determinando órganos como tépalos y pericarpelo, estructura formada por una mezcla de tejidos de origen floral y de origen axial. En este sentido los transcriptomas, herramientas que proporcionan información sobre la expresión génica, pueden ser una gran herramienta que permita comenzar a entender el control genético que subyace el origen de la variación de las flores en Cactaceae.

En este trabajo me enfoqué en *Disocactus*, que es un género pequeño de cactáceas principalmente epífitas, con 13 especies que se distribuyen en México y Centroamérica (Cruz et al., 2016) A diferencia de sus géneros hermanos que tienen flores nocturnas y blancas (*Epiphyllum* y *Selenicereus*), las especies del género *Disocactus* presentan flores diurnas principalmente (Britton & Rose, 1923; Cruz et al., 2016), aunque también pueden observarse flores con antesis diurna-nocturna y nocturna, blancas o de colores, con formas y tamaños diversos. Algunas especies, tienen flores pequeñas y color magenta, con los órganos reproductivos exsertos como las de *D. eichlamii*; algunas, tienen flores blancas, aromáticas como las de *D. macranthus*, y otras, las tienen rojas, con múltiples segmentos del perianto como las de *D. speciosus* (Britton & Rose, 1922; 1923).

El objetivo de esta investigación fue el de identificar patrones y comparar la expresión génica durante el desarrollo floral en *Disocactus speciosus* y *D. eichlamii*, dos

especies cercanamente relacionadas pero que presentan morfologías florales contrastantes, y la relación de estos patrones de expresión con la diversidad de la morfología floral observada en el género. Para ello se llevaron a cabo análisis transcriptómicos, complementados con análisis morfoanatómicos y estudios teóricos sobre conceptos morfológicos de la flor en Cactaceae.

Está tesis está dividida en dos capítulos. El capítulo I, está compuesto por un artículo de revisión donde además de analizar los trabajos realizados en Cactaceae sobre desarrollo floral, se proponen hipótesis genéticas que explican la posible identidad de los órganos florales. A partir de los trabajos en genética del desarrollo, principalmente realizados con base en el modelo ABCE de determinación de verticilos florales, propuesto en *Arabidopsis thaliana* y *Anthirrinum majus*, nosotros sugerimos tres hipótesis que explicarían el desarrollo y la identidad de los tépalos en las flores de esta familia. Por otro lado, en este artículo también proponemos hipótesis sobre el desarrollo del pericarpelo, tejido que recubre al ovario y que por sus características morfo anatómicas ha sido asociado a un tallo, sin embargo, la identidad ontogenética de este es incierta. Finalmente, proponemos diferentes enfoques que ayudarían a elucidar la identidad de estos órganos, incluyendo análisis moleculares y transcriptómicos, este primer artículo representa el artículo de requisito.

El capítulo II está compuesto por un artículo original, en el cual a partir del análisis de transcriptomas de dos especies cercanamente emparentadas del género *Disocactus*: *D. speciosus* y *D. eichlamii:* exploramos cambios de expresión en los genes involucrados en la variación de tamaño y número de órganos, los cuales han sido previamente identificados en otras especies. Además, realizamos un análisis llamado Weigthed Gene Co-expression Network Analysis (WGCNA, por sus siglas en inglés), un método no supervisado de agrupamiento. Este análisis permitió recuperar módulos de expresión relacionados a procesos biológicos involucrados en la determinación del tamaño de órganos, así como procesos relacionados al desarrollo floral. También, se llevó a cabo el estudio de la dinámica celular en tépalos en diferentes estadios de desarrollo, y se concluyó que las diferencias de tamaño en los tres estadios analizados podrían deberse a procesos de crecimiento celular, más que proliferación celular y que estos a su vez son especie específicos. Estos resultados, se complementaron con imágenes de microscopía electrónica

de barrido (MEB) que nos permitieron observar que las células epidérmicas de los tépalos de estas dos especies son tabulares, cuadrangulares, elongadas y con la pared anticlinal estriada, esta micromorfología es diferente a la observada en las células epidérmicas de pétalos en otras especies de angiospermas, las cuales, como se ha reportado, son principalmente cónicas (Whitney et al., 2011).

A partir de los resultados obtenidos de los análisis transcriptómicos y de la dinámica celular en los tépalos, puedo sugerir que las diferencias observadas en el tamaño floral entre especies cercanamente relacionadas de *Disocactus* podrían deberse a cambios en patrones de expresión génica de genes específicos. Por ejemplo, los transcritos de *BIG BROTHER* (*BB*) y *BIG PETAL* (*BP*) que se acumulan en mayor cantidad en *D. eichlamii*, especie con flor pequeña, mientras que en *D. speciosus*, que tiene la flor grande, detectamos una menor acumulación de transcrito de *BB* comparada con la de *D. eichlamii*, mientras que no pudimos detectar al transcrito de *BP* en ninguna de las muestras. Estos resultados permiten sugerir que las diferencias en la expresión de los dos genes en las especies analizadas pueden afectar procesos de crecimiento celular y posiblemente la proliferación celular, alterando el tamaño final de los órganos florales. El manuscrito que describe este análisis fue sometido a la revista *American Journal of Botany*.

Capítulo I

"Blurring the boundaries between a branch and a flower: potential developmental venues in Cactaceae"

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Review

Blurring the Boundaries between a Branch and a Flower: Potential Developmental Venues in CACTACEAE

Isaura Rosas-Reinhold [1,2], Alma Piñeyro-Nelson [3,4], Ulises Rosas [1] and Salvador Arias [1,*]

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Abstract: Flowers are defined as short shoots that carry reproductive organs. In Cactaceae, this term acquires another meaning, since the flower is interpreted as a branch with a perianth at the tip, with all reproductive organs embedded within the branch, thus giving way to a structure that has been called a "flower shoot". These organs have long attracted the attention of botanists and cactologists; however, the understanding of the morphogenetic processes during the development of these structures is far from clear. In this review, we present and discuss some classic flower concepts used to define floral structures in Cactaceae in the context of current advances in flower developmental genetics and evolution. Finally, we propose several hypotheses to explain the origin of these floral shoot structures in cacti, and we suggest future research approaches and methods that could be used to fill the gaps in our knowledge regarding the ontogenetic origin of the "flower" in the cactus family.

Keywords: flower development; floral shoot; flower evolution; cacti evolution; evo-devo; flower organ identity

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1. Introduction

Flowers, unlike other organs such as leaves, roots and stems, are composite structures made of a number of organs that form an ordered pattern [1]. The basic flower structure or floral ground plan is the result of key innovations in angiosperms that may have originated from coadaptations with pollinators [1,2]. Some of these key novelties are bilateral symmetry, the specialization of the perianth into sepals and petals [3] and the gynoecium composed of carpels [4]. Molecular clock dating studies have suggested that flowering plants originated in the late Triassic period, ~209 million years ago [5]. These studies also suggest that the core group of angiosperms appeared in the Jurassic, while the Cretaceous period observed the emergence of multiple other diversifications across flowering plants (~140–90 mya; [5]).

Most angiosperms have a conserved floral organization that consists of four whorls of concentric organs. This structure is commonly organized into two whorls of sterile organs, i.e., sepals and petals, arranged as the first and second whorls, respectively, and then a whorl of stamens and, in the innermost whorl, the carpel, which is a novel structure enclosing the ovules, not present in other seed plants [2]. Such an organization seems to be genetically determined by the interplay of a set of homeotic genes that interact in a whorl-specific manner. This was documented in floral mutants of *Arabidopsis thaliana* and *Antirrhinum majus*, which were used as model species in flower developmental genetics,

giving way to the so-called ABC model [6,7]. This model posits that the development of the four whorls that typically comprise a flower is directed by the spatiotemporal expression and interaction of three gene classes. The expression of class A genes in the outermost section of the flower meristem (*APETALA1* (*AP1*) and *APETALA2* (*AP2*)) gives way to sepals; the concerted expression of class A and B genes (*APETALA3* (*AP3*) and *PISTILLATA* (*PI*)) determines petal formation; the concerted expression of class B and C genes (*AGAMOUS* (*AG*)) determines the development of stamens in the third whorl; and, finally, the expression of the class C gene alone originates the carpels in the fourth whorl [6]. Furthermore, all genes except for *AP2* are part of the MADS-box type II gene family of transcription factors [6]. An additional category of genes, *SEPALLATA* (*SEP*) or E class genes, characterized years later, is expressed across the floral meristem, and while four paralogs have been documented in *A. thaliana*, *SEP3* is part of the whorl-specific protein tetramers that, together with the ABC class genes, underlie the differentiation of each whorl [8,9]. Due to their functional and sequence conservation across angiosperms, ABC genes have been studied from an evolutionary and developmental perspective, testing whether variations in the spatiotemporal expression of orthologs due to subfunctionalizations, neofunctionalizations or new protein–protein associations could underlie diverse floral morphologies [10,11]. Thus, ABCE genes have become a useful model to test hypotheses regarding how development has evolved, leading to morphological variations in flowers. For example, molecular evolution as well as spatiotemporal patterns of expression in developing flower primordia in non-model plants such as species within the Ranunculaceae [12], Triuridaceae [10,13,14], Aizoaceae [15] or Orchidaceae [16] have been used to analyze both the functional conservation of ABC class gene activity and its variants across angiosperms, broadening our knowledge of the genetic bases of floral organ diversification and opening new avenues of research regarding the molecular underpinnings of perianth evolution [15,17].

In this paper, we review the current understanding of embryological, histological, and genetic data underlying flower development in Cactaceae, a family comprising approximately 1438 to 1870 species [18,19]. Some of these taxa are remarkable as crop species (i.e., *Opuntia ficus-indica*, *Selenicereus undatus*) or charismatic ornamental species; nonetheless, and despite their economic importance and botanical allure, the origins of their floral novelties and floral developmental patterns are far from fully understood. In this work, we will focus on two singular structures whose development has received little attention in this group of plants: (1) the pericarpel, a seemingly vegetative tissue that encloses the portion of the receptacle where the ovary and the stamens originate; and (2) the perianth, which shows a morphological gradation from bracts to petal-like structures in all cacti (see Figure 1).

Last, based on the current understanding of the molecular bases of reproductive structure induction and flower development in angiosperms, as well as our own observations in diverse cacti taxa, we propose several hypotheses that could explain the complex ontogenetic origin of the unique floral structures in this group, pointing to new research venues.

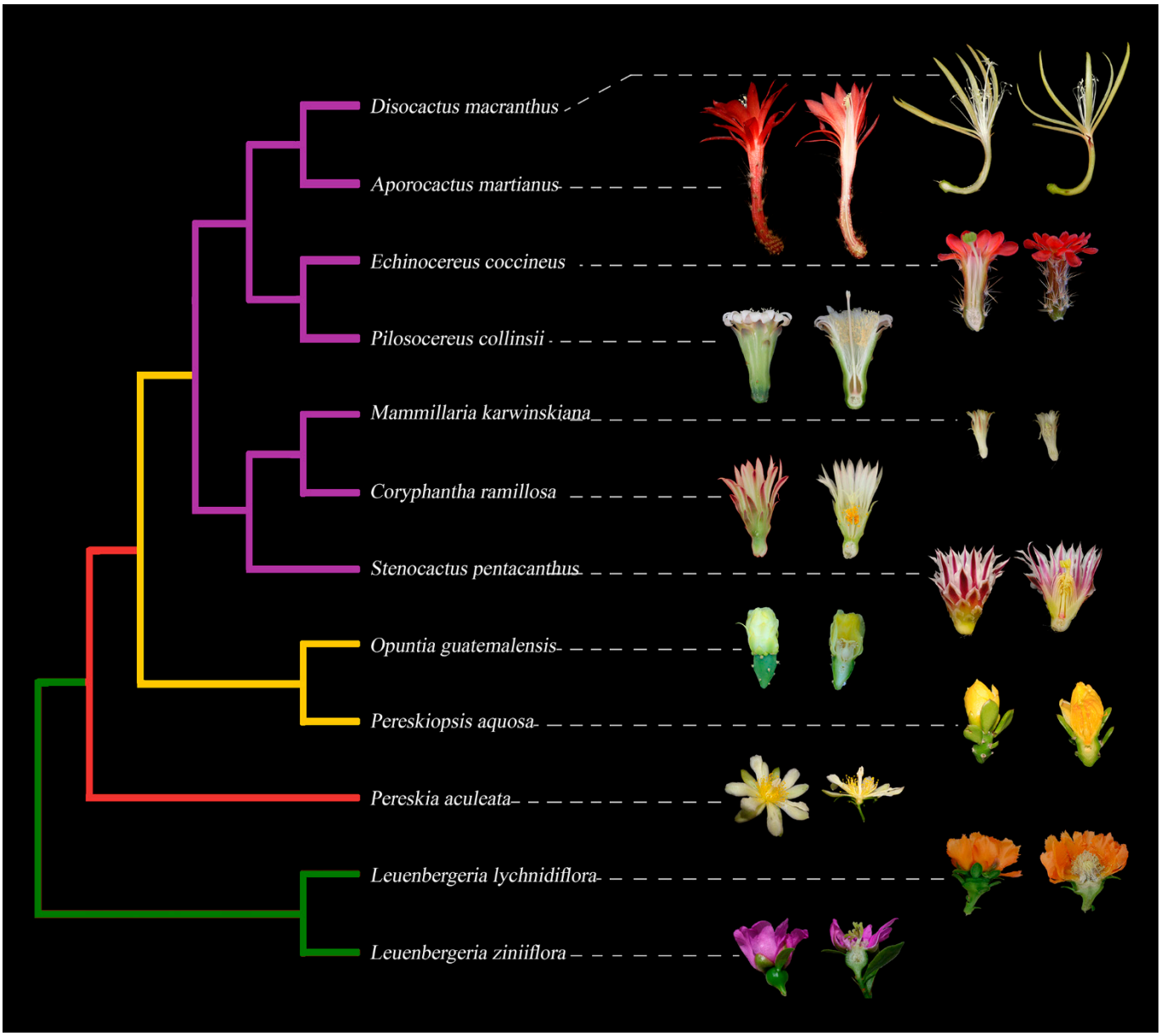

Figure 1. Simplified phylogeny of Cactaceae with representative flowers in longitudinal sections, exemplifying the diversity in floral morphology across the family. Leuenbergerioideae (green clade): *Leuenbergeria zinniiflora*, flowers with a hypanthium-like structure covering the ovary, with green sepal-like whorls and purple petal-like whorls; *L. lychnydiflora*, with green sepal-like whorls and orange petal-like whorls. Pereskioideae (red clade): *P. aculeata*, flowers with receptacle with areoles covering the superior ovary. Opuntioideae (yellow clade): *Pereskiopsis aquosa*, flowers with areoles and laminar leaves over the pericarpel; *Opuntia guatemalensis*, flowers with a succulent and thick pericarpel, foliar organs becoming tepaloid towards the apex. Cactoideae (purple clade): *S. pentacanthus*, *C. ramillosa* and *M. karwinskiana* showing a very reduced campanulate receptacle and a reduced pericarpel without areoles, spines and bracts; *P. collinsii*, showing a campanulate receptacle, without spines in the pericarpel and decurrent podaries; *E. coccineus*, showing a campanulate receptacle with a green spiny pericarpel and a red perianth; *A. martianus* showing a long tubular receptacle with bracts, spines and a red pericarpel, as well as a red perianth; *D. macranthus*, with a campanulate receptacle and a long tube, with a very reduced pericarpel and areoles, with a yellow perianth.

2. Evolutionary History and General Flower Structure in CACTACEAE

Cactaceae are a eudicot family within the Caryophyllales and have been organized into five subfamilies: Leuenbergerioideae, Pereskioideae, Maihuenioideae, Opuntioideae and Cactoideae [18,20,21]; see Figure 1 for a simplified phylogeny. The family diverged

quite recently, at approximately 35 Mya, placing Cactaceae as a late arrival in angiosperm history. It is thought that the majority of species diversification occurred during the late Miocene, ≈10–5 Mya [22].

Cactaceae are distributed across a wide variety of ecosystems, from deserts to rainforests, and have a highly specialized vegetative axis where the majority of species seem to lack leaves as these are highly reduced, and develop succulent photosynthetic stems that, in turn, exhibit a wide variety of shapes (cylinder, barrel shape, flattened) and sizes [23]. For example, the diameter of the body in *Blossfeldia liliputana* is 10 mm and is considered the smallest plant in the family [24]. In contrast, *Echinocactus platyacanthus* can reach a width of 1.5 m.

In addition to the attractiveness of their stems, their astonishing flowers are another distinctive feature of this family, possessing a number of important characters with taxonomic importance, such as color, size, shape, presence or absence of spines, leaves and bracts [23]. Most attention on cactus flowers has focused on analyzing traits from these organs from a systematic perspective, yet the developmental events leading to the formation of these organs have been largely understudied [25–35]. This is the case for the evolutionary and developmental origins of the perianth and the pericarpel, two puzzling structures that require contemporary approximations to fill in the gaps pertaining to floral evolution in Cactaceae.

From a phylogenetic perspective, Cactaceae is a member of the Portulacineae clade, sister to Anacampserotaceae, and is closely related to Portulacaceae, Talinaceae, Basellaceae, Halophytaceae and Montiaceae [21,36–38]. A common theme among members of these families is that they have a meristematic ring from where stamens develop. In *Anacampseros*, the multiplication of stamens is divided into two groups: the first group comprises the multistaminated species characterized by the presence of a well-defined ring meristem, and the second group includes species with a low stamen number [39]. *Talinum*, *Portulaca* and *Calandrinia* are in the first group. Unsurprisingly, Cactaceae species also have a meristematic ring corresponding to the first group [25–27,29], equivalent to the one described in *Anacampseros* [39]. While the meristematic ring is a common feature in this group of plants, the genetic mechanisms underlying its formation remain to be fully investigated [40].

Despite their close phylogenetic relationship, the morphology of the "cactus flower" deviates from some features found in several members of the *Portulacineae*. For example, a widespread feature in families within this clade is that flowers are generally inserted within an involucre of two median bract-derived phyllomes, having a sepaloid appearance [41]. This is the case for species of *Talinum*, *Claytonia*, *Anacampseros* and *Calandrinia* [39,42–44], in which each flower is subtended by two involucral bracts that protect the young floral buds, covering the floral apex and the five tepals [39,42–44]. Regarding the nature of the involucre as bracts or sepals, the topic has been extensively discussed, although evidence for a sepal identity is weak and is contradicted by the morphology of the petaloid organs that resemble true sepals [41]. The presence of two bracts associated with the flower as well as petaloid tepals appears to be a synapomorphy for the clade including Portulacaceae and Cactaceae [41,45]; nevertheless, in the latter, involucral bracts are absent [41]. Furthermore, phylogenetic reconstructions of perianth characteristics in Caryophyllales show that in Portulacineae, the perianth is biseriate with bract- and sepal-derived organs; in Cactaceae, the perianth is multiseriate and has been argued to be composed of bract- and sepal-derived organs [41]. It is worth mentioning that this type of perianth is unique to the Cactaceae [41]. In short, some of the evolutionary novelties found in Portulacineae flowers are not shared in Cactaceae.

Flowers in cacti are usually sessile (without a stalk), with exceptions in *Pereskia* and *Leuenbergeria* (Figure 2a). They come in a variety of sizes: *Selenicereus* has the largest flowers (35+ cm), while species of *Epithelantha* have very small flowers (6 to 18 mm) [23,46,47]. Most flowers are bisexual [23], but some dioecious species have been reported, such as *Opuntia stenopetala* [30,48], *Opuntia robusta* [34] and *Mammillaria dioica* [32]. Cacti flowers

are usually described as solitary [23], but a few species from *Pereskia* develop racemose inflorescences [49]. Furthermore, in *Myrtillocactus geometrizans*, a widespread columnar cactus from arid Mexican landscapes, it is common to find several flowers in clusters originating from a single vegetative areole [23,50,51].

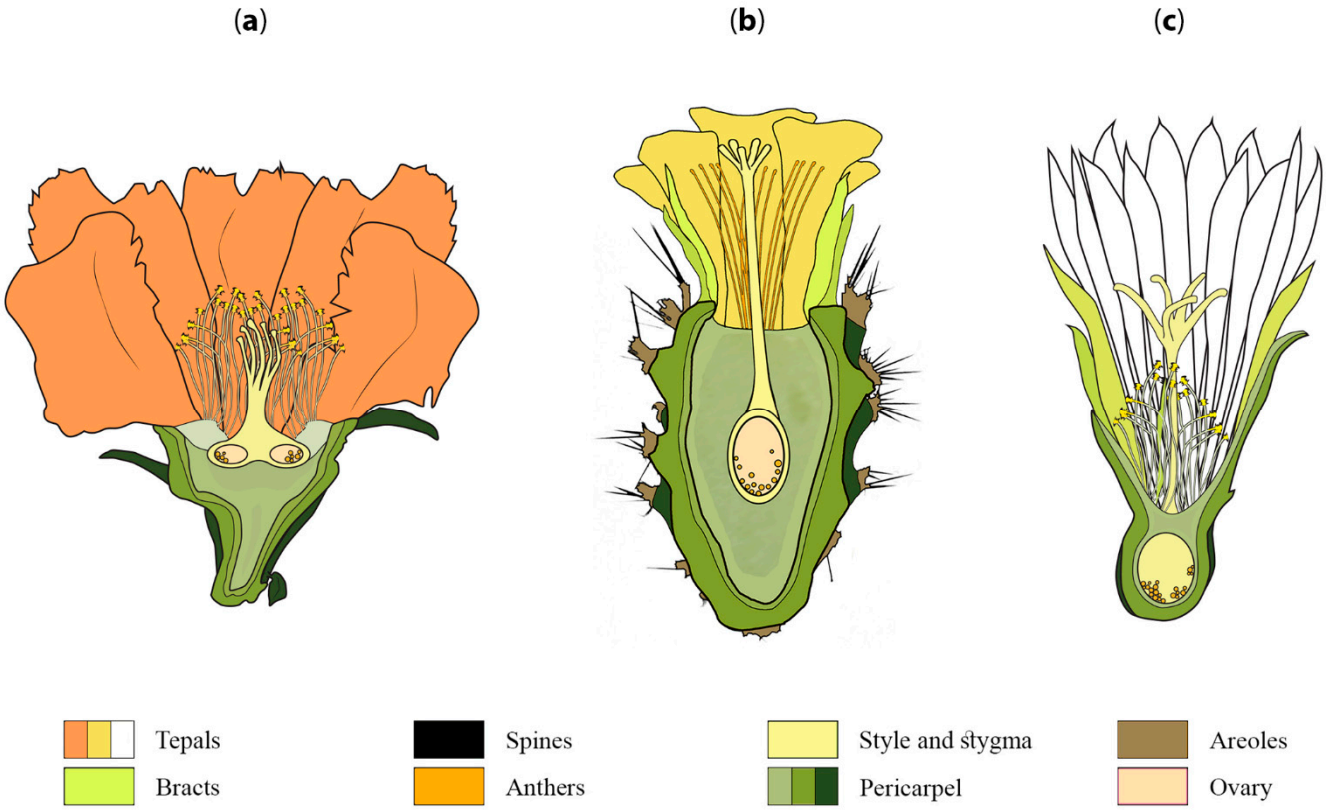

Figure 2. Schematic representation of three different flowers of Cactaceae. Longitudinal section through a flower of (**a**) *Leuenbergeria lychnidiflora* (Leuenbergerioideae), (**b**) *Opuntia guatemalensis* (Opuntioideae) and (**c**) *Coryphantha delicata* (Cactoideae) illustrating the variation in morphology found in cactus flowers.

Although cacti flowers are radially symmetrical, a few species have been described as asymmetrical [50], such as *Aporocactus flagelliformis* and *Zygocactus* species. This is supported by observations of the orientation of stamens and pistils, bending of the floral tube, the presence of an oblique tube throat or even the shape of the ovary, as reported in *Selenicereus spinulosus* [50]. Whether cacti species display true flower asymmetry remains to be further analyzed; however, the aforementioned observations are consistent with our studies in *Disocactus speciosus* and *D. kimnachii*, where preliminary data show that stamens are more abundant on the ventral side of the whorl rather than arranged as a homogeneous ring, and the same can be observed in the South American cacti genera *Trichocereus* and *Harrisia*. In contrast, in *Aporocactus flagelliformis*, there is a more prominent presence of stamens on the dorsal side of the whorl, while in its sister species, *A. martianus*, stamens have a ring disposition. Interestingly, variability between species in the same genus has also been observed in some *Selenicereus* species; for example, whereas stamens emerge as a ring in *Selenicereus undatus*, in *Selenicereus validus*, more stamens are present on the dorsal side. The abortion or prevalence of male organs on one side of the flower is part of the flower asymmetry syndrome documented in other angiosperms, such as the Lamiales [52]. These observations suggest that incipient flower asymmetry might be independently evolving in different clades of epiphytic cacti, but the underlying ontogenetic mechanisms have yet to be validated through developmental and genetic analyses.

The subfamilies Leuenbergerioideae and Pereskioideae (Figure 1, green and red clades, respectively) are the least "cacti-looking" lineages and are considered to contain the earliest

diverging group in Cactaceae [49,53–56]. Species in the genera *Pereskia* and *Leuenbergeria* possess broad leaves, internodes and a minimum of succulence. The spines associated with axillary buds (the areole) are the diagnostic feature that groups them together with the rest of the Cactaceae [57].

Another relevant feature in *Pereskia* and *Leuenbergeria* is that true inflorescences are produced only in these genera: both paniculate and cymose inflorescences [23,49]. Another salient feature is that some species, such as *P. aculeata*, *P. diaz-romeroana* and *L. lychnidiflora*, have a superior to half-inferior ovary [26–28,31]. Moreover, the ovary in *L. lychnidiflora* is also multilocular, with several separate carpels, resembling axile placentation with pocket-like locules [26] (Figure 2a). The superior position of the ovary is considered to be an ancestral feature in the family [23], while the inferior receptacular ovary in the other subfamilies is derived (Maihuenioideae, Opuntioideae and Cactoideae; Figure 2b and c). According to developmental studies in *Pereskia* and *Leuenbergeria*, the ovary is in a transition process; thus, the definition of the ovary position in these genera is complex [31,58]. Nevertheless, a short tube showing an early tendency to form a receptacle can be observed in *P. aculeata* [50], with areoles and leafy bracts emerging from it.

Flowers in the subfamily Opuntioideae (the prickly pear group) (Figure 1, yellow clade) are structurally more complex than those in Pereskioideae and Leuenbergerioideae because the ovary is embedded within the axial tissue denominated the pericarpel [50,59] (Figure 2b). In *Opuntia*, the pericarpel bears stomata and areoles (axillary buds) on its surface [59]. In addition, the presence of mucilaginous cells, druses and parenchyma cells resembles those present in the stem [60,61]. Based on this evidence, it has been suggested that the pericarpel has an axial origin; in other words, the pericarpel is a vegetative tissue originating from a vegetative meristem within the areole. Due to the axial–appendicular nature of the tissue, Mauseth [60,61] and most contemporary cacti specialists refer to this unique and remarkable structure as a "flower shoot".

These flower shoots are generally described as solitary, although in some exceptions, new flower shoots emerge from the areoles present on the pericarpel, forming a chain-like structure of flower shoots, a phenomenon that often occurs in *Cylindropuntia* species [60,62]. The axillary buds (areoles) on the flower shoots of *Cylindropuntia fulgida* ("chain fruit cholla"), *C. leptocaulis* and a few other species produce floral shoots that later become "fruits", whose axillary buds repeat the process [60,61]. Such arrangement of branch flowers growing over one another could be considered an inflorescence-like formation pattern, but this is an issue that has to be more thoroughly analyzed. An example of solitary flower shoots is those present in prickly pears (*Opuntia* spp.), which are born at the apex of flattened stems. Their perianth segments are generally yellow to red (due to betaxanthin pigmentation), and the pericarpel often presents a round shape, with spines and deciduous leaves [56,59].

Maihuenioideae is a monogeneric subfamily from the Patagonian Desert [23,63]. *Maihuenia* comprises two species: *M. patagonica* and *M. poeppigii* [23]. This genus is characterized by its small-leaved and dense, compact, mound-forming plants [63]. *Maihuenia* was initially included inside Opuntioideae because it shares vegetative and floral features with the latter, as *Maihuenia* flowers are diurnal, white and yellowish in color, very similar to *Opuntia* flowers [63]. More recent phylogenetic analyses showed that *Maihuenia* is an independent group from the Opuntioideae subfamily, giving rise to a new subfamily: Maihuenioideae [64]. Nevertheless, given the limited information on the morphology and anatomy of flower shoots from Maihuenioideae, we did not include them in Figure 1.

The general idea of a cactus comprises species such as peyote (*Lophophora williamsii*) or the saguaro cactus (*Carnegiea gigantea*), both belonging to the Cactoideae. This subfamily displays the largest variation observed in flower shoots. Thus, flower shoots within Cactoideae species can have podaria, ribs, tubercles that cause cortical succulence, bracts with a reduced laminar part, elongated receptacle tubes, thickness or rigidity in the pericarpel [50,56]. Flower shoots in some columnar cacti, such as *Cephalocereus* and *Pachycereus*, are large in size and have nocturnal anthesis, bearing a white perianth and,

in some cases, an unpleasant odor [65,66]. Large flower shoots can also be observed in the epiphytic species *Selenicereus undatus* (dragon fruit or pitahaya), which has one of the largest flowers in the family, reaching up to 29 cm in size [54]. In contrast, small flower shoots can be observed in different genera, such as *Mammillaria*, *Coryphantha*, *Lepismium* and *Rhipsalis*. These small flower shoots display a reduction in pericarpel tissue [50,56] as well as a reduction in or loss of axial characteristics of the flower shoot, such as the loss of areoles, scaly bracts and green cortex, in addition to the nonshowy color of the tube, as well as the transformation of bracts into petaloid structures [50] (Figure 2c).

3. Cactus Flower Development

According to Boke [57], the development of the so-called flower shoot in Cactaceae is similar to that in many other plants, and the most significant differences are observed in pistil and, in particular, carpel development. For this reason, most investigations on flower shoots have focused on the reproductive organs (androecium and gynoecium, [26–29]), their embryology [31] and the mechanisms underlying dioecy, such as those documented in *Opuntia stenopetala* [30,48], in *O. robusta* [34] and in *Mammillaria dioica* [32]. These studies have shown that dioecy in cacti is the product of programmed cell death and other developmental mechanisms, such as ovule abortion, rather than the lack of floral whorl determination.

In Cactaceae, the androecium has numerous stamens (multistaminated), which initiate in a uniform and prominent ring primordium, on which the stamens arise in a centrifugal succession [25,27,50,67]. This stamen ring is also observed in *Talinum* [43] and *Anacampseros* [39]. In the Caryophyllales, the multistaminate androecium presents morphological similarities to the Paeoniaceae subfamily and Dilleniidae subclass, and it is thus considered to be an ancestral feature in the Caryophyllales [27,67].

Generally, cacti ovaries develop multiple ovules [68], but *Pereskia aculeata* develops fewer than five ovules [27,49]. Ovule development in cacti exhibits similar characteristics to other members of the Caryophyllales, such as the hook-like shape of the carpels, which is also reported in *Phytolacca*, as well as the ovules' primordium at the base of the ovary, such as that seen in *Phytolacca* and *Tetragonia* [50]. Another feature is the secondary augmentation of the ovules along the cross-zone, as in *Trianthema* and *Mesembryanthemum* [50]. The structure that has no homologs in other members of Caryophyllales is the pericarpel, a tissue that covers the ovary and seemingly is ontogenetically related to the stem. The ontogeny of this structure, together with the perianth, deserves further attention due to its likely complex evolution. In the next section, we will summarize the current understanding of perianth development and discuss its relationship with the pericarpel.

4. Sepals or Petals as Cacti Perianth Organs, or Neither?

While a double perianth with outer green sepals and inner colored petals is a well-established feature in the core eudicots [69], perianths with only one whorl or with more than two whorls also occur [1], a feature that has been documented in species within the Caryophyllales. In this order, such a simple classification can be deceiving, as some organs with equivalent functions can have different developmental origins, and sepals and petals cannot be distinguished on the basis of the presence or absence of pigmentation [68]. In addition, a number of families in the Caryophyllales are characterized by a simple perianth, but contrary to expectations, the whorl that is missing corresponds to the petals, not the sepals [70,71].

The discussion on the origin of the perianth across angiosperms and core eudicots, and particularly in Caryophyllales, has received a great deal of attention [17,69,72–74], and multiple independent origins of sepals and petals have been reported. Ronse De Craene [69] suggested that petals have been independently lost and "reinvented" at least five times in Stegnospermataceae, Aizoaceae, Portulacaceae, Caryophyllaceae and Molluginaceae. Similarly, Brockington et al. [37] suggested at least nine independent origins of a differentiated perianth within the Caryophyllales. An example of this phenomenon can

be found in *Delosperma napiforme*, where the outer stamen primordia develop into sterile staminodes and become increasingly petaloid in a centrifugal pattern, resulting in many white and showy petal-like staminodes in the mature flower, which contrast with the green sepals [15].

Cactaceae represent a highly derived clade with an increased number of petaloid sepals [41], which are indeterminate and polymerous [68], developing in a centripetal order [50] with a spiral phyllotaxis [37]. Ronse De Craene [72] considered that the perianth in Cactaceae is formed by sepals and not by petals. Due to this, he denominated this structure as "petaloid sepals" and considered them nonhomologous to the petals present in species such as *A. thaliana* or *A. majus*. Thus, petaloidy of the sepal whorl is an important evolutionary phenomenon that has evolved either independently from modifications in the petal whorl or as a consequence of a reduction in the petal whorl [69]. As we mentioned above, the perianth in the cactus family has been considered to have a gradation from bracts to sepals to petals [23,50] or bracts to tepals [50,56]. This phenomenon has never been addressed in detail from a developmental or an ontogenetic perspective, but studying it would be instrumental to determine whether the perianth of a seemingly sepaloid origin present in this family is a product of a reduction in the petal whorl or if there is, in fact, a morphological gradation related to the differential expression of transcription factors directing perianth development that favors the interpretation of a transition from bracts to sepals and petals within a cactus flower (Figure 3). This last hypothesis, the loss of or reduction in petal primordia, would suggest different underlying morphogenetic scenarios and would also potentially shed light on the ontogeny of the flower shoot (Figure 4).

In many cacti, flower shoots exhibit a gradual development of flower-associated structures. For example, from the base of the flower shoot to its apex, some bracts as small as scales grow in size to the outer perianth segments. That is, these "outgrowths" go from the outside to the inside. While they are small, growing in the basal-most part of the pericarpel, they look like leaves, and they turn into sepal-like structures when they reach their maximum size, in the most distal part of the flower shoot [50]. Some examples of gradual modification of bracts into sepaloid or petaloid organs are seen in *Ferocactus*, *Selenicereus* or *Polaskia* (Figure 3). The "flower shoots" in these genera show green bract-like or scale structures in the outermost section of the perianth, gradually changing color and texture as subsequent whorls develop and ultimately developing a petal-like morphology in the innermost whorl. This gradation was interpreted by Buxbaum [50] as foliar organs becoming petaloid at the perianth section of the flower shoot. In contrast, in *Pereskia*, species such as *P. aculeata* have an abrupt transition from green bracts to petaloid perianth segments [27], akin to having a double perianth (with differentiated sepals and petals). This abrupt transition between green sepaloid and pigmented petaloid perianth organs is also observed in *L. ziniiflora* (Figure 1, green clade).

In cases where the vasculature has been analyzed, it has been documented that in *Opuntia*, tepals show multiple vascular bundles derived from a central bundle, which differs in size from the remaining bundles, with the central bundle being larger than the others. These vascular bundles resemble those seen in the leaves of some cacti and are arranged or located in a collateral manner. Such an arrangement can also be seen in vascular bundles of the cortical and medullary bundles in the stems of Cactoideae species [59]. In *P. aculeata* [27], bracts and tepals can be distinguished because the procambium distribution is different: the midvein is prominent in bracts, while in tepals, it is small. Vascular bundles have been used to distinguish the origin of petal-like structures in other species. Nevertheless, Ronse De Craene [41] argued that using the vasculature to distinguish any flower-associated laminar structure as either petal or sepal is far from accurate.

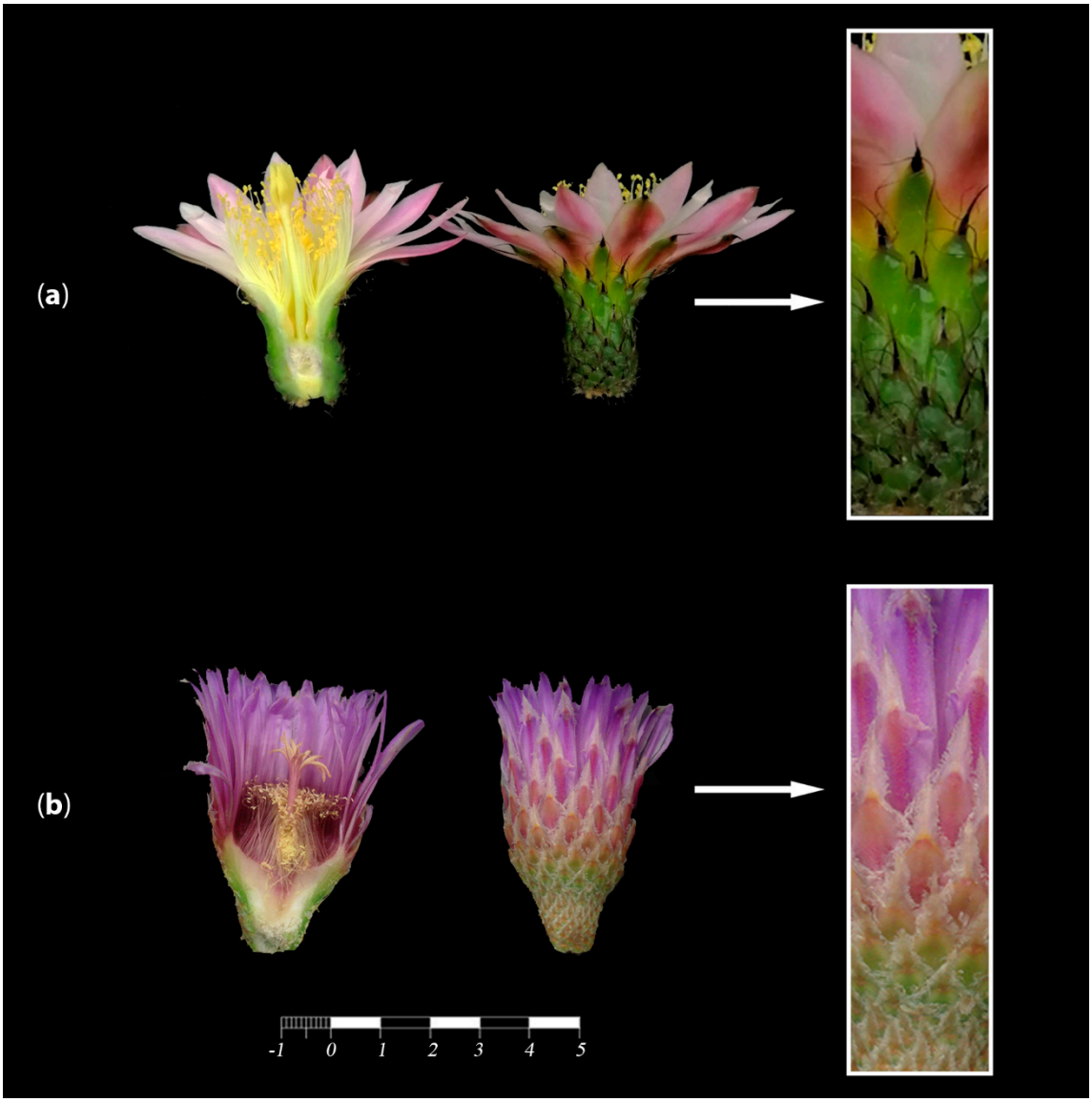

Figure 3. Development of bracteoid and perianth-like structures from the base to the apex of the flower shoot. (**a**) *Polaskia chende* with sepal-like structures that cover the branch flower, which abruptly become petal-like elements of the perianth. (**b**) *Ferocactus latispinus* with papery bracts at the base of the flower shoot that gradually become elements of the perianth (better referred to as perigonium, a term used when there is no clear differentiation between sepals and petals).

The lack of distinction between bract scales and sepaloid and petaloid structures described by several botanists, and therefore referred to as tepals, led us to consider whether the genetic mechanisms that control the identity of these organs can be used to better assign their type of floral whorl (Figure 4). It is likely that evolutionary modifications of the ABC model, which describes the mechanism of whorl identity in *A. thaliana* (described above), could be related to perianth morphology in the Cactaceae. In cacti, one possibility is that the orthologs of B class genes are not involved in the determination of tepals, therefore lacking petaloid identity, so tepals could, in fact, be modified sepals (Figure 4; Hypothesis I). In this scenario, class A genes could be the only ones acting in the determination of these organs, resulting in a reduction in or overall loss of petals in the perianth of cacti. This is the case for species in Ranunculales, where the loss of petals correlates with the decreased or overall lack of expression of the class B gene *AP3*-like [12,76]. A second possibility is that genes from classes A and B are expressed in an intergraded manner, consistent with the "fading borders" variation of the ABC model proposed for some species of *Nelumbo*, which also have a spiral arrangement of perianth organs [75,77]. In Cactaceae, it would

only apply to the sterile perianth-like organs preceding the staminodial ring meristem (Figure 4; Hypothesis II).

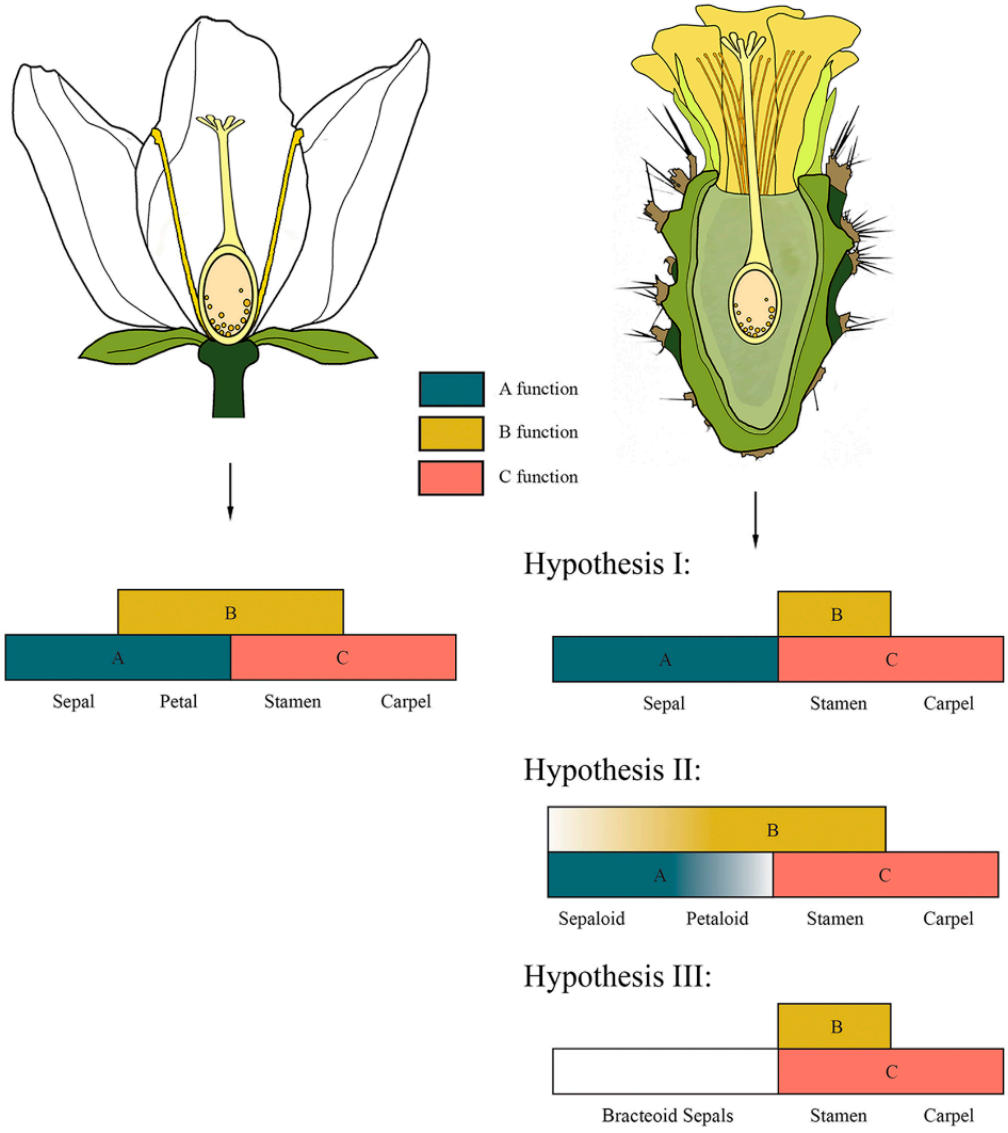

Figure 4. Canonical ABC model of organ identity vs. hypothesis of possible variations of the ABC model in a cactus flower. As the genetic identity of sterile structures in Cactaceae is not well resolved, we propose two hypotheses to explain the origin of these organs. Hypothesis I: Only genes from class A determine the perianth segments; thus, laminar sterile structures are modified sepals, while genes of class B are only expressed in stamen primordia (shifting boundaries model). Hypothesis II: As several authors have described the perianth segments in Cactaceae as intergraded structures that go from bracts to sepals to (petaloid) tepals [75] and are denominated sepaloid petals, we propose that the expression of class A and B genes is consistent with the "fading borders model" but applied only to sterile perianth organs. Hypothesis III: There is no expression of A-class genes and as such, the perianth is bracteoid.

Given that *Pereskia* and *Leuenbergeria* exhibit an abrupt transition from bracts to pigmented tepals not observed in other subfamilies, there is the possibility that the genetic control of perianth development in these genera differs from the mechanisms proposed above for clades such as Opuntioideae and Cactoideae. A third possibility is that components of the perianth are highly modified bracts, whose development would not involve the activity of class A or class B genes (Figure 4; Hypothesis III).

In this regard, comparative genetics studies highlight that in many angiosperms, the corolla does not need B or A class gene function to develop [78,79], while A class genes seem to exert a cadastral function that limits the expression of B and C class genes into the outer whorl(s) of many flower species rather than playing an important role in petal development, as was originally proposed based on the model species *A. thaliana* [78]. This could very well be the case in Cactaceae, as studies of the spatiotemporal expression of B class genes in developing flowers of closely related families show that their petaloid perianth develops by other means aside from B class gene function [15]. At the ordinal level, there are certain lines of evidence showing a diversity of genetic mechanisms that could determine the perianth in Caryophyllales. For example, Brockington et al. [15] analyzed B and C class gene expression in *Sesuvium portulacastrum* and *Delosperma napiforme* (Aizoaceae) using mRNA in situ hybridization of the orthologs of *AP3*, *PI* and *AG* to survey their role in determining petal identity. These authors reported no expression of *SpPI* and *SpAP3* in developing tepals; rather, these genes were expressed during the development of the androecium and gynoecium of *S. portulacastrum*. In addition, they found that in *D. napiforme*, a species with stamen-derived petals or andropetals, *DnPI* is expressed less intensely in stamen primordia that will give way to the innermost andropetals, while *DnAP3* is expressed more strongly in stamen primordia that will develop as outer andropetals. Furthermore, both *DnPI* and *DnAP 3* show only early and transient expression in andropetal primordia; thus, no heterotopic gene expression pattern of B class genes explains petaloid perianth development in these two species [15]. Hence, evidence of the homoplastic perianth origin across the Caryophyllales together with their experimental data led Brockington and colleagues [15] to propose that petaloid evolution in this order as well as in other angiosperms could occur by developmental mechanisms alternative to those proposed in the ABC model of floral development. In the case of cacti, gene expression studies of B and C class genes in the developing flower primordium could not only aid in analyzing whether B class genes play a role in the morphological gradient of petaloid organs observed on the outer surface of the pericarpel but also help to unravel the ontogeny of the "petal" primordia that can be observed developing on the outer rim of the ring primordium where stamens develop, as documented in some studies [57,80].

In this regard, it has been acknowledged by several authors that the perianth is the most plastic organ in flowering plants, with multiple independent examples of gains and losses, a phenomenon that is particularly acute in Caryophyllales [41,81]. Although petals are often topographically defined as the inner whorl in the perianth, this is clearly limited and unreliable, as it does not provide any information on homology [69]. In contrast, distinct evolutionary origins of a differentiated perianth, with contrasting petal derivations, do provide the necessary variation and evolutionary replicates to assess the role of variations in the canonical eudicot petal identity program in recurrent petal evolution [15].

5. The Flower Shoot

As mentioned above, the flower in Cactaceae is referred to as a flower shoot because of the apparent vegetative origin of the pericarpel. However, the ontogenetic origin of this tissue is still unclear. A hint towards comprehending this issue could lie in understanding the origin of the inferior ovary in Cactaceae. Two different hypotheses of the origin of the inferior ovary have been proposed. The first is the appendicular origin of the inferior ovary, which proposes that the nature of the external ovary wall originated from the fusion of the concrescence from the bases of the calyx, corolla, androecium and gynoecium. The second hypothesis is the receptacular origin of the inferior ovary, which posits that the external ovary wall has an axial-vegetative nature [25,82]. Both hypotheses have been discussed for different groups of plants, including Cactaceae, but only the second hypothesis might be able to explain the origin of the inferior ovary in this family [82]. Nevertheless, axial tissue covers the inferior ovary in Cactaceae, but stamens and tepals are also embedded

in it [60,61]. Such morphological arrangement explains why the floral structure in cacti is often called a "flower shoot" [60].

This concept of the flower shoot or the assembly between vegetative and floral tissues has been widely accepted in cacti [60,61,83]; however, no formal study has been conducted to determine the developmental and evolutionary origins of the pericarpel. The term pericarpel was proposed by Buxbaum [50] to define the receptacle tissue that surrounds the carpels. The pericarpel in some genera (i.e., *Selenicereus* and *Epiphyllum*) is prominently prolonged, forming a tube, but it is considered nonhomologous to a flower tube because it is an extension of the pericarpel, which is considered to be of a vegetative origin [50,84]. Although several authors often use the term pericarpel, mainly in Cactoideae [18,23,56,62], Leuenberger [49] argued that this distinction is not feasible in *Pereskia*, where the androecium and gynoecium are not hidden inside the axial tissue, nor are the sterile perianth whorls. Consequently, he continues to call this the receptacle or receptacle cup.

To date, multiple pieces of evidence support the so-called flower shoot concept: the presence of laminar leaves on the pericarpel [61], which have the same developmental pattern as in vegetative branches observed in *Opuntia* [59] and *Pereskiopsis* (Figure 5a); the presence of vegetative organs such as bracts or spines on the pericarpel (i.e., *Echinocereus*, Figure 5b); and the elongation of the "floral tube" in many species (i.e., *Aporocactus*, Figure 5c), which supports the idea that the "floral organs" are surrounded by "axial tissue" [26].

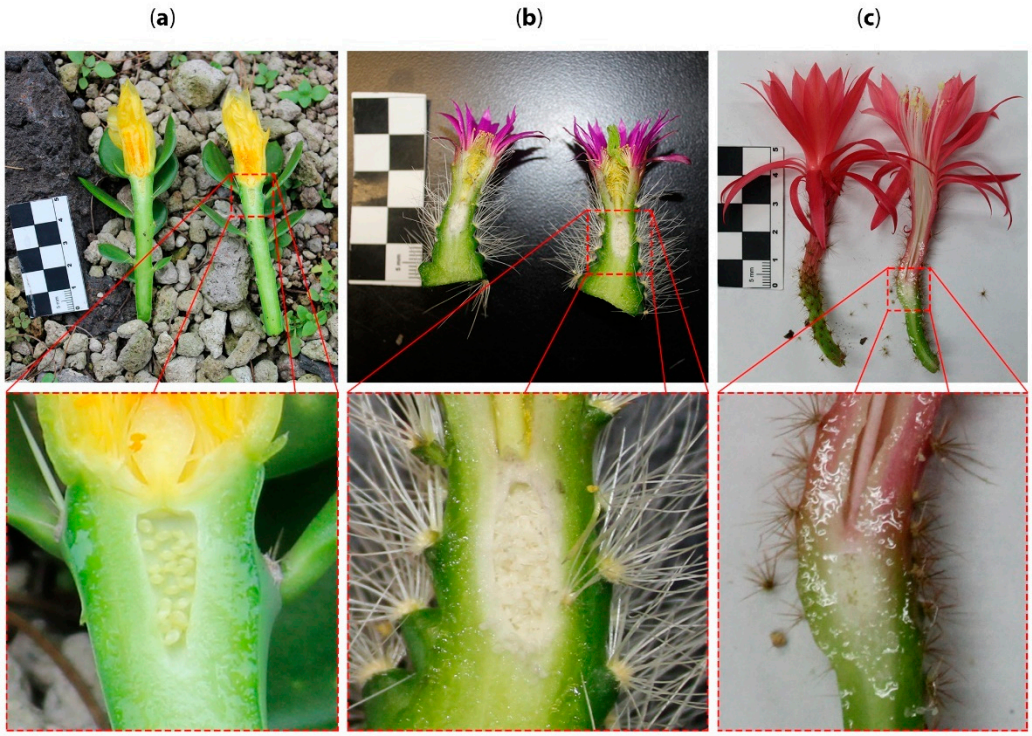

Figure 5. Flowers from different genera across Cactaceae where continuity between the stem and floral elements is evident. (**a**) *Pereskiopsis aquosa* (Opuntioideae, Cylindroputieae), stem looks like a branch with leaves, nodes and internodes topped by a flower. The ovary is sunken into the stem. In these examples, the flowers and the stem do not resemble distinct organs; rather, they display an intergradation of vegetative and flower-associated structures, with a sunken ovary. (**b**) *Echino-cereus parkerii* (Cactoideae, Pachycereeae), the stem narrows towards the apex where the flower develops. (**c**) *Aporocactus martianus* (Cactoideae, Hyloceareeae), the apex of the long stem is topped off with a red flower, and the ovary is sunken into the axial tissue.

The pericarpel can also bear areoles with woolly hairs (trichomes) and a few spines or bristles in their axils [23]. Furthermore, the areole is considered homologous to an axillary

bud [50,85,86] but not simply an axillary bud. Areoles are unique to cacti [23] and emerge in the axils of developing leaves or tubercles, and they are distinctive because they can originate spines, glochids (deciduous spines) and masses of trichomes that can be short or have long hair-like structures. Areoles also have the capability to originate flowers, branches and roots [57,87]. It is believed that areoles are complex axillary meristems that are composed of collapsed shoot nodes and internodes; therefore, the areole is not a single axillary meristem but rather a group of several axillary meristems [60]. Hence, the areole might have different functional meristematic domains, each of which could originate from different organs. The term areole is useful because the bud's spines persist even if the axillary bud meristem goes on to produce a flower and fruit [60]. Flowering in most angiosperms causes bud scale abscission; therefore, after the fruit is shed, the region is little more than a set of scars, but in cacti, the entire set of spines is still present [60]. Furthermore, some cacti produce spines for a prolonged period, longer than most axillary buds, which produce bud scales; therefore, these growing structures are more appropriately considered short shoots rather than merely buds [60]. Thus, the presence of areoles over the pericarpel indicates that this structure is more complex than just a receptacle.

The presence of podaria (an enlarged leaf pedicel), tubercles and ribs (fused enlarged leaf pedicels) in the flower shoot has been taken as additional evidence to support the axial origin of the pericarpel [50] because these structures are typical of stems in the Cactaceae family. For example, anatomical similarities were observed in the stem and the pericarpel of some *Opuntia* species and other members of the Opuntioideae subfamily, such as pericarpel epidermal tissue, hypodermis, cortex and vascular tissue [59,88]. While these observations are suggestive, we caution that mere morphological and anatomical observations might not provide robust evidence to argue for the developmental and evolutionary origins of the pericarpel.

In summary, the concept of flower shoots has been commonly used by most scholars to describe the singular reproductive units in Cactaceae; nevertheless, the ontogeny of this structure has been vastly understudied, leaving two key innovations to be further investigated in this unique plant family: first, the pericarpel proper, which we interpret as a unique structure developed from a process of synorganization between axial and reproductive structures (Figure 2); second, a spiral perianth where a continuous intergradation of bracts to sepaloid to petaloid structures takes place in an acropetal manner throughout the flower shoot, together forming a unique type of seemingly terminal flower in the majority of cacti species (Figures 4 and 5). These phenomena are likely intertwined and could be the product of the blurring of different kinds of boundaries: those related to the differentiation of floral organ whorls (as contemplated in the ABC model discussed above), likely enabled by the existence of an androecial ring primordium, and the unique ontogeny of an inferior ovary that apparently becomes fused with the underlying vegetative axis in what has been construed as a terminal flower. We posit that the close proximity and spatial disposition of axillary meristems within an areole, which is in itself a compressed branch, could be a proximal explanation for the apparent synorganization of the reproductive axes in cacti species. In the following sections, we review the available developmental data for each of these structures and propose developmental hypotheses of how flower shoots could be formed. Furthermore, we discuss different experimental approaches that could help test these ontogenetic hypotheses and help to unravel the ontogeny of this unique reproductive unit.

6. The Unique Pericarpel of Cactaceae

One of the few authors to articulate a developmental explanation for the origin of the flower shoot in cacti is Mauseth [61], who explained how the cactus flower ended up being a flower shoot through a metaphor of an elongated party balloon, in which the floral whorls are positioned at different levels outside the balloon: the lower half bears leaves; the middle part bears sepals, and "petals" are placed above this section, leaving stamens and carpels to develop on the top of the balloon. In other words, this could be interpreted as an elongated

flower primordium. Then, the author proposed that a force pushes all the elements of the flower inside the balloon (into the axis of the stem) until the entire flower ends up inside, externally covered by leaves. This proposal of the invagination of floral whorls into the floral tube of a cacti flower shoot is an interesting idea to explore; however, it should be noted that in some anatomical sections of *Echinocereus*, it appears that the floral meristem forms within the areole (vegetative meristem), giving way to carpels, stamens and petaloid organs while still below the areole surface, in the "inside" of the developing reproductive unit [35,57,80,89]. The invagination of the ovary, together with the differential growth of the style, takes place in later stages [80]. Further during development, the residing ovary seems to fuse with the surrounding stem tissue, forming the internal part of the pericarpel. Additional evidence of the integrated nature of this structure is the fact that the perianth vasculature arches beneath the carpel [80]; that is, the vascular supply to the placentae is derived from the recurrent receptacular system, which diverges downward after providing traces to the perianth and androecium and from which the dorsal and ventral carpellary bundles diverge at the level of the ovary roof [80]. While some authors suggest that a precondition for synorganization is the existence of floral organs organized in whorls [90], this particular case entails fusion with an organ (the carpel) that sits on top and becomes progressively embedded into the surrounding vegetative tissue (Figure 5).

Fusion between different structures has been considered an important macroevolutionary trend in angiosperms, with potential adaptive implications [91,92]. In the case of cacti, the protected nature of the inferior ovary, as well as other floral organs embedded and protected by the lateral organs that develop on the external part of the pericarpel (i.e., areoles, or bracts), has been considered an important adaptation to the extreme environments where many cacti species dwell [23,50,56]. The pericarpel thus entails the integration of axial and reproductive tissue, a phenomenon whose underlying genetic mechanisms could entail a blurring of boundaries between floral and axial meristems. This phenomenon could be related to the close proximity of several meristematic tissues within an areole: spine meristem, axial meristem and leaf meristem [50,89], as well as the identity of the areole, being either a vegetative meristem, an inflorescence meristem or a floral meristem, or a mixture of several types of meristems as described above.

During the reproductive transition, vegetative meristems receive cues for their developmental conversion into reproductive meristems. Some of these cues are sparked by so-called florigen genes, such as *CONSTANS* (*CO*), *FLOWERING LOCUS T* (*FT*) and *FLOWERING LOCUS D* (*FD*) [93]. When vegetative meristems become competent to produce reproductive structures, they turn into inflorescence meristems. In angiosperms, inflorescence meristems can have different degrees of vegetativeness [94], producing many floral meristems, as in indeterminate inflorescences (i.e., *Arabidopsis* and *Antirrhinum*), or a single floral meristem, as in solitary flowers (i.e., tulips or hibiscus). The degree of vegetativeness of an inflorescence seems to be controlled by a gene known in *Arabidopsis* as *TERMINAL FLOWER 1* or *TFL1* [14,95] and its ortholog in *Antirrhinum*, *CENTRORADIALIS* or *CEN* [96–98]. In both species, mutants in these genes cause premature exhaustion of the inflorescence meristem, giving way to a terminal flower. While TFL1/CEN maintain an undifferentiated inflorescence meristem, they antagonize the transcription factor *LEAFY* (*LFY*) in *Arabidopsis* [99] or *FLORICAULA* (*FLO*) in *Antirrhinum* [100], whose activity gives floral meristem identity to the inflorescence meristem, by activating floral whorl identity genes (mentioned in the ABC model). Loss of function in LFY/FLO results in plants possessing what seem to be inflorescences but are instead holding bracts or leaves instead of flowers. In other words, once the reproductive transition has occurred and the vegetative meristem transitions into an inflorescence meristem, *TLF1/CEN* maintain meristem vegetativeness, while *LFY/FLO* promote meristem determinacy towards a floral meristem. Although the activities of these genes have been primarily studied in model species, their function seems to be conserved across many angiosperms, and therefore these genes have often been used to determine, for example, whether some structures are flowers or inflorescences [101].

To ascertain what type of meristematic tissue is present in the pericarpel (and associated tissues in the mature flower shoot), we propose and discuss three alternative scenarios. Our first hypothesis is the simplest one, where the induction of a floral meristem within an areole could induce the growth of a nearby axial meristem that eventually fuses onto the basal part of the developing floral meristem, giving way to a compressed branch of nonreproductive origin, which would be the pericarpel (Figure 6a). This proposal surmises that the pericarpel has a purely vegetative origin, while the floral whorls are on top and embedded. In this scenario, *LFY/FLO* expression would be expected in the tissue that originates from the floral whorls but not in the pericarpelar tissue surrounding the ovary. *TFL1/CEN* expression would also be expected to transiently precede *LFY/FLO* expression but disappear afterwards as the inflorescence meristem is consumed. This also implies that the pericarpel has a vegetative origin and that gene activities in that tissue would resemble the stem. In the second scenario, the inflorescence meristem (IM) differentiates within the areole niche and protrudes to form the flower shoot, differentiating into a floral meristem in the distal part of the protruding tissue, while it remains somewhat undifferentiated in the proximal part of the meristem, where it develops into a highly modified inflorescence with a terminal flower, where a series of bract-like structures develop on the outside, and parenchymatous and vascular tissues develop in the inside, forming the pericarpel (Figure 6b). This would mean that the flower shoot is, in fact, the outcome of the inflorescence meristem, with a terminal flower and several axillary vegetative buds (areoles) constituting the pericarpel. Similar to the previous scenario, orthologs of LFY/FLO would be expected on the tissues that will originate the floral whorls; however, orthologs of TFL1/CEN would be expected to be active on the pericarpel tissue, maintaining the vegetativeness of axillary meristems (areoles). However, this hypothesis does not explain how the axillary buds remain dormant; therefore, they do not produce inflorescence branches, which might then be controlled by a different mechanism. In the third scenario, the entire flower shoot could originate directly from a floral meristem (FM), with a transient inflorescence meristem state, and thus the ontogenetic origin of the pericarpel would be floral (Figure 6c). This would mean that orthologs of *LFY/FLO* are active in the tissue that conforms to the pericarpel as well as the tissue that will originate from the floral whorls. This hypothesis implies that bracts on the pericarpel, as in *Ferocactus* (Figure 3b), have a floral whorl identity.

While, in advanced stages of development, the branch-like nature of the pericarpel manifests through the formation of areoles (Figure 5) on its surface, many cacti species show a morphological gradient of bracteoid–sepaloid–petaloid organs towards the distal part of the flower shoot, commonly organized into a structure that resembles a perianth (Figure 4). It is possible that concomitant with the internal synorganization taking place between the vascular tissue of the inferior ovary and the internal part of the pericarpel, on the outside, a basipetal hormonal and transcriptional gradient can induce the progressive transformation of bracts into petaloid lamina.

Another phenomenon that warrants attention and that could enable us to ascertain if the developing flower primordium becomes embedded into the pericarpel tissue or if the pericarpel tissue grows around the developing flower is analyzing the very early stages of flower development in several species. Nevertheless, this is not straightforward, as the cacti flower meristem is often difficult to recognize on the surface or even develops while initially hidden within the areole, as is the case of *Echinocereus* species, where the areole with the flower meristem is engulfed by the stem tissue [89]. Thus, two possible scenarios are proposed: in the first scenario, the flower meristem forms a bulge that protrudes from the surface of the stem, as is common in most flower meristems (Figure 7a), but the differential growth rate of the underlying surrounding tissue and the process of synorganization translates into a floral meristem that is embedded within the developing pericarpelar tissue. An alternative scenario would be one where the flower meristem invaginates into the areole and fuses with the surrounding meristem (light green, Figure 7b), then both meristem types (flower meristem and surrounding vegetative meristem) grow together and the floral meristem is thrust outwards by the surrounding tissue, which has a higher growth

rate, and eventually covers the developing flower. Both proposals have in common that we assume that the pericarpel tissue is axial in origin and that a process of synorganization takes place early on, with the surrounding tissue growing faster than the floral tissue, but in the first case, the floral meristem is covered by the axial tissue, while in the second case, it initially appears to be embedded into it.

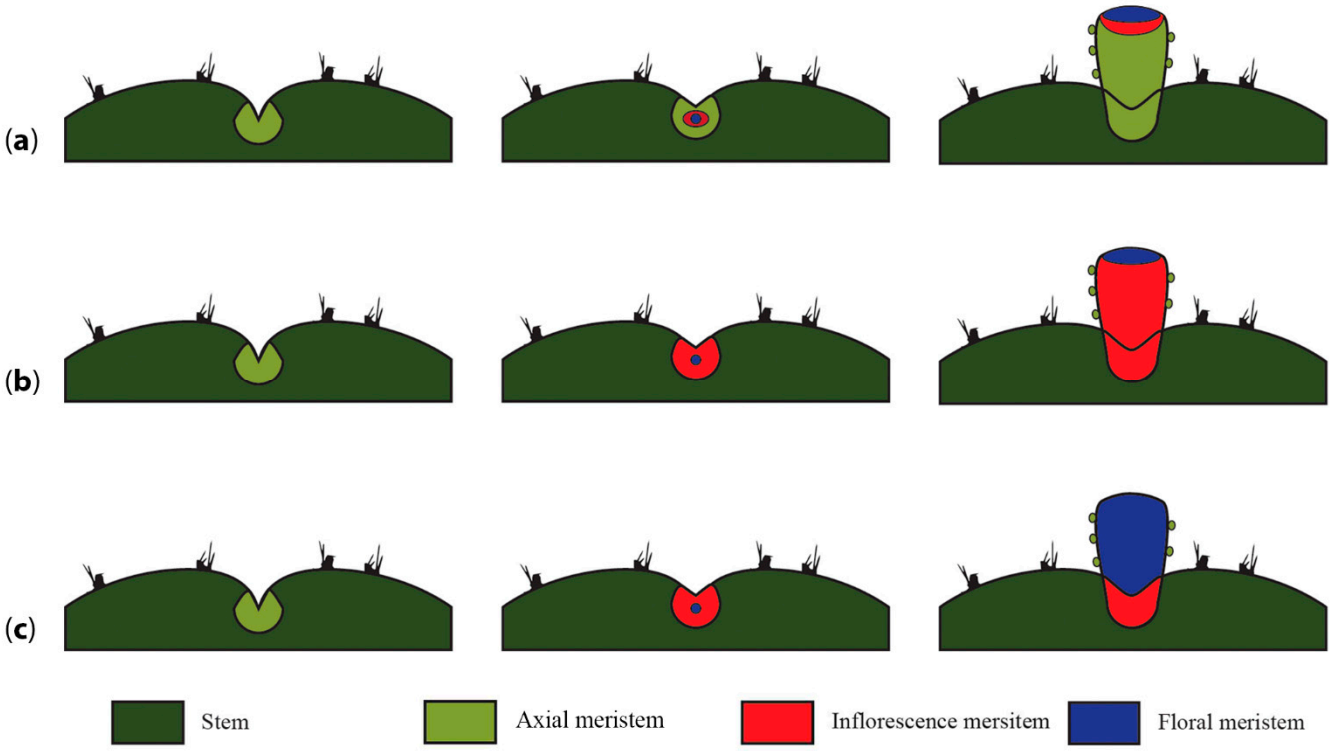

Figure 6. Alternative hypotheses of flower shoot inception and the origin of the pericarpel. (**a**) An axial meristem is induced to differentiate into an inflorescence meristem; this phenomenon induces a nearby vegetative meristem present in the same areole to grow and elongate around the basis and beneath the developing flower, eventually enclosing it and forming the vegetative part of the pedicel. (**b**) The inflorescence meristem gives way to the pericarpel, which will develop floral organs in the distal part of the flower branch and compacted branches (areoles) towards the proximal section where the reproductive unit inserts into the stem. (**c**) Once a meristem is induced to produce a flower, the inflorescence meristem gives way to a floral meristem that elongates with a peduncle that bears areoles instead of bracts.

At the core of our proposal is the notion of a particular case of synorganization between floral and axial tissue, where "floral identity" genes are likely necessary for reproductive organ formation but not for perianth development; thus, A class genes would be involved in boundary delimitation [85]. In this context, genes involved in organ fusion/boundary delimitation, such as the orthologs of *CUP-SHAPED COTYLEDON* and *NO APICAL MERISTEM* (*NAM*), which are part of an NAC-domain family of transcription factors that are present in all angiosperms analyzed to date, could be playing a role in floral fusion. *A. thaliana* mutants of *CUC* and *NAM* have shown partial to complete cotyledon fusion and reduced to complete meristematic inactivity [102]. Therefore, these genes have been shown to be involved in embryonic development, floral organ boundary specification [103,104], carpel development [105] and meristem delimitation [106]. Hence, they could be interesting candidates to analyze in Cactaceae and allied families. The spatiotemporal analysis of gene expression as well as the epigenetic regulation of orthologs of *CUC* and *NAM* genes [106] in all meristems present in an areole, as well as in developing flower shoots across select cacti species, could shed light on the ontogenetic mechanisms that underlie the formation of this unique reproductive unit, where synorganization seems to be taking place.

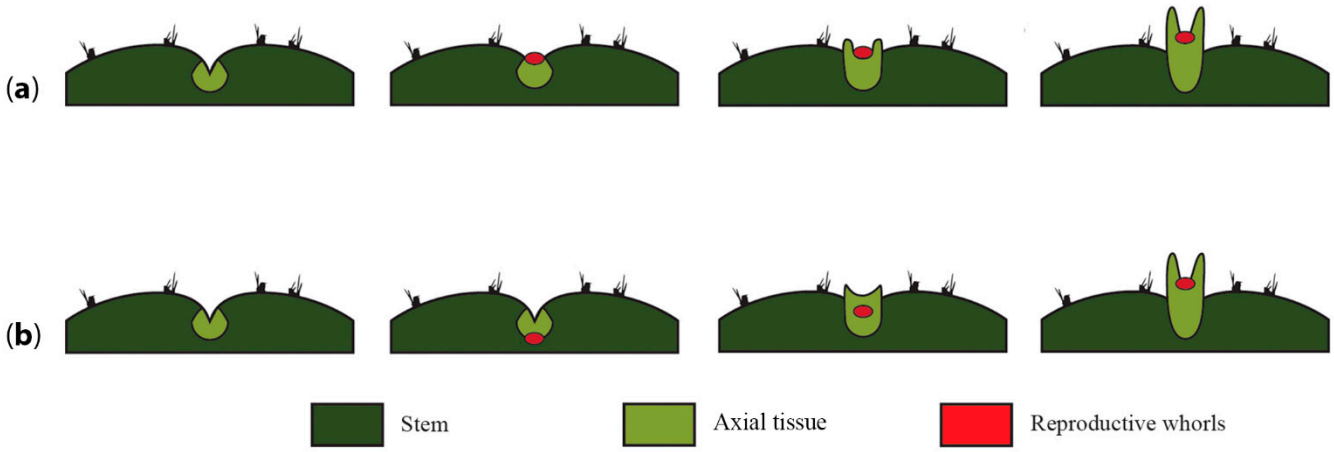

Figure 7. A flower sunken or covered by a shoot. (**a**) Once the axial meristem is induced to differentiate into a reproductive unit, the reproductive whorls (red dot) develop as a bulge protruding from the surface of the areole and induce the differentiation of lateral pericarpel tissue into a "branch" (light green structure). These two structures continue to develop, but accelerated growth of the axial tissue ends up surrounding and partially covering the developing floral meristem. (**b**) The reproductive whorls (red dot) invaginate into the areole and induce the development of lateral pericarpel tissue into a branch that eventually thrusts the inflorescence meristem outward, generating the flower shoot. Accelerated growth of the axial tissue ends up surrounding and partially covering the developing floral meristem.

Furthermore, the interplay between these genes and others that are involved in maintaining the identity of the shoot apical meristem *SHOOT-MERISTEMLESS* (*STM*) [107], the transition from an inflorescence meristem to a single terminal flower meristem (*TERMINAL FLOWER1* (*TFL1*); [95]) and their interaction with the plant-specific *LEAFY* (*LFY*) gene, which is fundamental for flower meristem determination and organ boundary formation in *Arabidopsis* [99], through interaction with ABC class genes [14], would also be informative, as it is pivotal in floral development [14]. In addition, *LFY* has diverse roles in different angiosperms [108] and has been proposed to be a useful marker for inflorescence/flower boundary determination in other angiosperms, where it appears to play a central role in the initiation of angiosperm flowers, although other factors can be responsible for detailed floral patterning [109]. In the case of Cactaceae, the analysis of gene expression and protein interactions of the LFY ortholog could also help shed light on the morphogenetic identity of the seemingly compressed flower shoot. However, LFY expression must be considered with caution, as its expression does not always correlate with floral meristems [110].

7. Perspectives

The conspicuous characteristics observed in flower shoots of cacti, such as the presence of multiple perianth series with a possible sepaloid/bracteoid origin and the existence of a unique structure termed the pericarpel, make cacti flower shoots an exciting model to further our insights into the different developmental venues that underlie flower diversification in angiosperms.

The ABC model proposed a generic mechanism for flower determination that has been a useful conceptual framework to test for the genetic basis of homologous organs in many angiosperms. Recent studies in a diverse set of flowering plants have suggested that several variations of the *Arabidopsis*/*Antirrhinum* model exist, particularly with respect to the perianth whorls [111–114]. In Cactaceae, the apparent lack of homology of perianth structures with respect to *Arabidopsis*, as well as the unique pericarpel structure, suggests that cacti could represent an additional variation on a theme involving synorganization between reproductive and vegetative tissues; this must be further investigated through comparative gene expression and developmental genetic studies that could help ascertain the conserved functions and unique variations present in a structure whose morphological and histological identities need further study. In this regard, the pericarpel deserves further

attention within its phylogenetic context, as we do not know whether it has homologous structures in Caryophyllales or in other angiosperm families. Different techniques can help us test the four main sets of hypotheses presented here, namely, the ontogenetic identity of the perianth (Figure 5), the identity of the pericarpel (Figure 6), how/if the flower became embedded (Figure 7) and the mode of synorganization between axial and flower structures. In situ hybridizations of orthologs of ABC class genes in different stages of reproductive unit development, as well as *CUC*, *STM*, *LFY* and *TFL1*, could yield information regarding their spatiotemporal expression and possible interactions; yeast one- and two-hybrid experiments could provide information pertaining to protein–protein interactions, while transcriptome analyses of different tissues, such as bracts, leaves, spines, tepals, the ring primordium, the carpel and the pericarpel, could help unravel the genetic mechanisms important in cactus flower development. Additionally, complementation studies using candidate genes in cacti expressed in plant model species (i.e., *Arabidopsis* or *Solanum*) could shed light on the functional diversification of candidate genes. Mutagenesis in cacti has not been implemented; however, some species with aberrant flowers can be found, sometimes in natural populations [48] or in artificially obtained hybrids. This is the case for *Astrophytum* or *Epiphyllum* hybrids, where the perianth displays a wide range of phenotypes, adding functional data to shed light on the molecular basis of the perianth and the pericarpel.

Despite the popularity of cacti as ornamentals due to their charismatic features, as well as the economic value of products from the *Opuntia* genus, knowledge of their preanthetic developmental patterns is still incipient. Although considerable efforts have been made to resolve the phylogenetic relationships between different genera and species, with the objective of understanding the evolution of these spectacular plants, these efforts are still far from providing a full comprehension of their floral development.

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Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Capítulo II

"A tale of giants and dwarfs: transcriptomic analyses of different flower size in two closelyrelated *Disocactus* species"

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Abstract

Premise: Floral variation is a main driver of angiosperm evolution, as changes in perianth pigmentation, floral organ number and flower size have been related to diversification and speciation. Developmental genetic studies in model species unveiled genes that direct floral organ specification, delimit organ number and boundaries between adjacent whorls, and control organ size. The roles of homologs of these genes in angiosperms with complex ontogenies such as the "flower shoots" of Cactaceae have not been analyzed.

Methods: We present a comparative transcriptomic analysis across different developmental stages of flower buds from two related cacti species with contrasting organ numbers and flower size: *Disocactus speciosus* and *Disocactus eichlamii*.

Key results: Differential Gene Expression analyses show that homologs of genes associated with flower size and organ number, have different expression patterns, while Weighted Gene Co-expression Network Analysis (WGCNA) modules are differentially enriched processes related to organ and flower growth in each species. Complementarily, scanning electron microscope photographs of outer and inner tepals of developing flower buds suggest that different cellular dynamics underlie final perianth size.

Conclusions: Our data suggest that heterochronicity could play a major role in defining flower size, a common phenomenon in cacti floral diversification. Furthermore, the epidermal structure of petaloid perianth organs in cacti is different than previously documented in model species.

Keywords: Cactaceae, flower development, growth genes, merosity, organ growth, petal, tepal

INTRODUCTION

The ontogenetic processes governing overall growth and body size have been subject of study in both animals and plants. In plants, growth is defined as an irreversible increase in the volume and/or mass of an organ or its parts, regardless of the formation of new structures such as organs, tissues, cells, or organelles (Brukhin and Morozova, 2011). Flowers exhibit extraordinary variation in structure, organ number, pigmentation, shape, and size; these features give rise to the great diversity documented in angiosperms and affect the fitness and evolution of particular lineages (Moyroud and Glover, 2017). Whether flower size evolution at the extremes of a range is governed by the same rules that act upon average-sized flowers is currently unknown (Davis *et al.*, 2008). For example, while *Rafflesia* represents one of the extremes in flower size, with flowers reaching up to one meter in diameter (Davis, 2008), Rafflesiaceae are embedded within the spurge family (Euphorbiaceae), whose flowers typically measure just a few millimeters in diameter. Other examples of extreme variation in organ size are present in Araceae, where the inflorescence of *Amorphophallus titanum* can reach 3 m tall (Sedayu *et al.*, 2010; Iwashina *et al.*, 2020), while *Wolffia* exhibits extreme organ reduction with microscopic inflorescences (Bown, 2008). Cactaceae species have undergone extreme developmental modifications of stems and flower organs, and probably no other plant family exceeds cacti in terms of structural diversity (Mauseth, 2006). Its members display wide diversity in growth habit including trees, bushes, and geophytes (Vázquez-Sánchez *et al.*, 2012), as well as variation in shoot size (Mauseth, 2006). In this family, dwarfism and gigantism are common not only in shoots (Mauseth, 2006), but also in other structures, such as leaves, spines, flowers, and fruits. Size variation can be observed even among closely related species. For example, *Selenicereus* has species with flowers ranging from 3-3.5 cm in *S. minutiflorus*, to approximately 30 cm in length in *S. undatus* (Britton and Rose, 1920). Furthermore, the genus *Disocactus* harbors species that exhibit contrasting flower morphological variation, in terms of flower size as well as color and organ number (Cruz *et al.*, 2016; Korotkova *et al.*, 2017), making *Disocactus* an interesting group for studying the genetic and ontogenetic differences underlying extreme morphological variation (Fig. 1).

A central conundrum in plant evolutionary developmental biology is how the genetic control of organ size is determined and whether the mechanisms underlying organ growth are conserved among land plants (Hong and Roeder, 2017). Flower size results from the balance between pollination efficiency and the energetic cost of building floral structures (Davis *et al.*, 2008). Although organ size in plants is influenced by external environmental signals,

strong evidence suggests that organ size is primarily controlled by gene regulatory networks (Mizukami, 2001).

The control of floral size can be separated into two different aspects: the number of organs in a whorl or/and the number of whorls per type (in particular, petals and stamens) as well as the size of each organ within a flower (Weiss *et al.*, 2005). In *Arabidopsis thaliana*, the duration and rate of cell proliferation are positively regulated by *AINTEGUMENTA* (*ANT*), the *ARGOS* gene family, *ORGAN SIZE RELATED1* (*OSR1*), and *KLUH* (*KLU*) (Anastasiou *et al.*, 2007) but negatively regulated by *BIG BROTHER* (*BB*), *DA1* and *TEOSINTE BRANCHED–CYCLOIDEA–PCF 4 (TCP4)* (Anastasiou *et al.*, 2007; Shan *et al.*, 2019). *JAGGED* (*JAG*) encodes a C2H2 zinc-finger transcription factor that plays a critical role in controlling primordium initiation, distal growth of floral organs, and laminar development of leaflets (Min and Kramer, 2017). Some members of the *ERECTA* (*ER*) gene family acts as redundant receptors that link cell proliferation to organ growth and patterning (Shpak *et al.*, 2004). *TARGET OF RAPAMYCIN* (*TOR*) *KINASE* integrates nutrient and energy signaling to promote cell proliferation and growth (Xiong and Sheen, 2014), while *BIG PETAL* (*BPEp*) (Szécsi *et al.*, 2006) controls cell expansion in petals.

Another component often associated with organ size variation is merosity, *i.e.*, the number of organs of each whorl within the flower structure (Specht and Bartlett, 2009; Pieper *et al.*, 2016). Merosity can be altered in two main ways: by enlarging/reducing the overall size of the floral meristem, or by homeotic conversion of one type of floral organ into another of a different class (Moyroud and Glover, 2017). While most eudicot orders exhibit pentamerous merosity (Hofmann, 1994; Ronse De Craene *et al.*, 1998), the order Caryophyllales, which includes Cactaceae, is distinguished from other eudicot by unstable merosity. In Cactaceae, the stamens have centrifugal development, and tepals have a spiral arrangement (Hofmann, 1994) with indeterminate merosity. Although some aspects of flower development, such as whorl determination, are well represented in the ABC model (Bowman *et al.*, 1989; Coen and Meyerowitz, 1991), little is known about the genes that control the specific number of whorls per organ class, or the number of different floral organs per whorl (Pieper *et al.*, 2016). Nevertheless, some genes that modify the number of floral organs within particular whorls have been identified. Mutations in *PERIANTHIA* (*PAN*) alter the numbers of sepals and petals (Running and Meyerowitz, 1996). *ETTIN* (*ETT*) and *PAN* (Sessions *et al.*, 1997) control floral organ number by acting independently of *CLAVATA*; the latter can also be correlated with an increase in floral meristem size at the time of organ initiation (Clark et al., 1996). *SUPERMAN* (*SUP*), which regulates floral homeotic genes (Bowman *et al.*, 1992),

interacts with components of the polycomb-repressive complex 2 (*PRC2)* and fine-tunes local auxin signaling by negatively regulating the auxin biosynthesis genes *YUCCA1/4* (*YUC1/4*). In *sup* mutants, *YUC1/4* activity increases auxin levels at the boundary between whorls 3 and 4, which leads to an increase in the number of stem cells and their prolonged maintenance, therefore increasing the number of reproductive organs, particularly stamens (Kocyan, 2007; Xu *et al.*, 2018). Several mutations that affect floral organ number without affecting floral organ identity or floral meristem identity have been isolated (Running and Meyerowitz, 1996). The mechanisms controlling flower size and organ number have been addressed in some crops, often revealing that such processes are species specific (Nelissen and Gonzalez, 2020).

In this work, we investigate the genetic bases of the establishment of floral organ size and organ number in cacti through a comparative transcriptome analysis of two closely related species within the epiphytic cactus genus *Disocactus,* a recently evolved genus with species in North and Central America that dwell in diverse habitats and exhibit contrasting flowers and pollination syndromes (Hernández-Hernández *et al*., 2014; Cruz *et al*., 2016). The species selected, *Disocactus speciosus* and *D. eichlamii*, bear contrasting floral morphologies, including variation in floral organ size and indefinite merosity (Fig. 1). *D. speciosus* has 10-cm-long flowers with 30-40 tepals, multiple stamens (~ 200), and 10 stigmas while *D. eichlamii* develops 5 cm-long flowers with 9-12 tepals, few stamens (~ 20) and five stigmas. We complemented RNA-seq data analyses at different developmental stages of floral bud formation with measurements of tepal epidermal cell size and structure using scanning electron microscopy (SEM) of tepal epidermal cells.

Here, we identify significant differences in tepal epidermal cell sizes in both species during flower development that correlate with final flower size. We found that epidermal cells are not conical, in contrast with what has been documented for petals in other species (Glover and Martin, 1998; Whitney *et al.*, 2011; Cavallini-Speisser *et al.*, 2021). Additionally, differential expression analyses show that genes associated with flower size and organ number have different expression patterns in *D. eichlamii* in both types of tissues evaluated; in contrast, in *D. speciosus* there are no differences in expression for the same transcripts. WGCNA modules are highly enriched in growth and proliferation processes related to organ and flower growth. We also detected differential regulation of homologs of *BPEp, BB, ER, SUP* and *PAN* in both species, which likely play specific roles in final organ size and organ number in the contrasting flowers of *D. speciosus* and *D. eichlamii*.

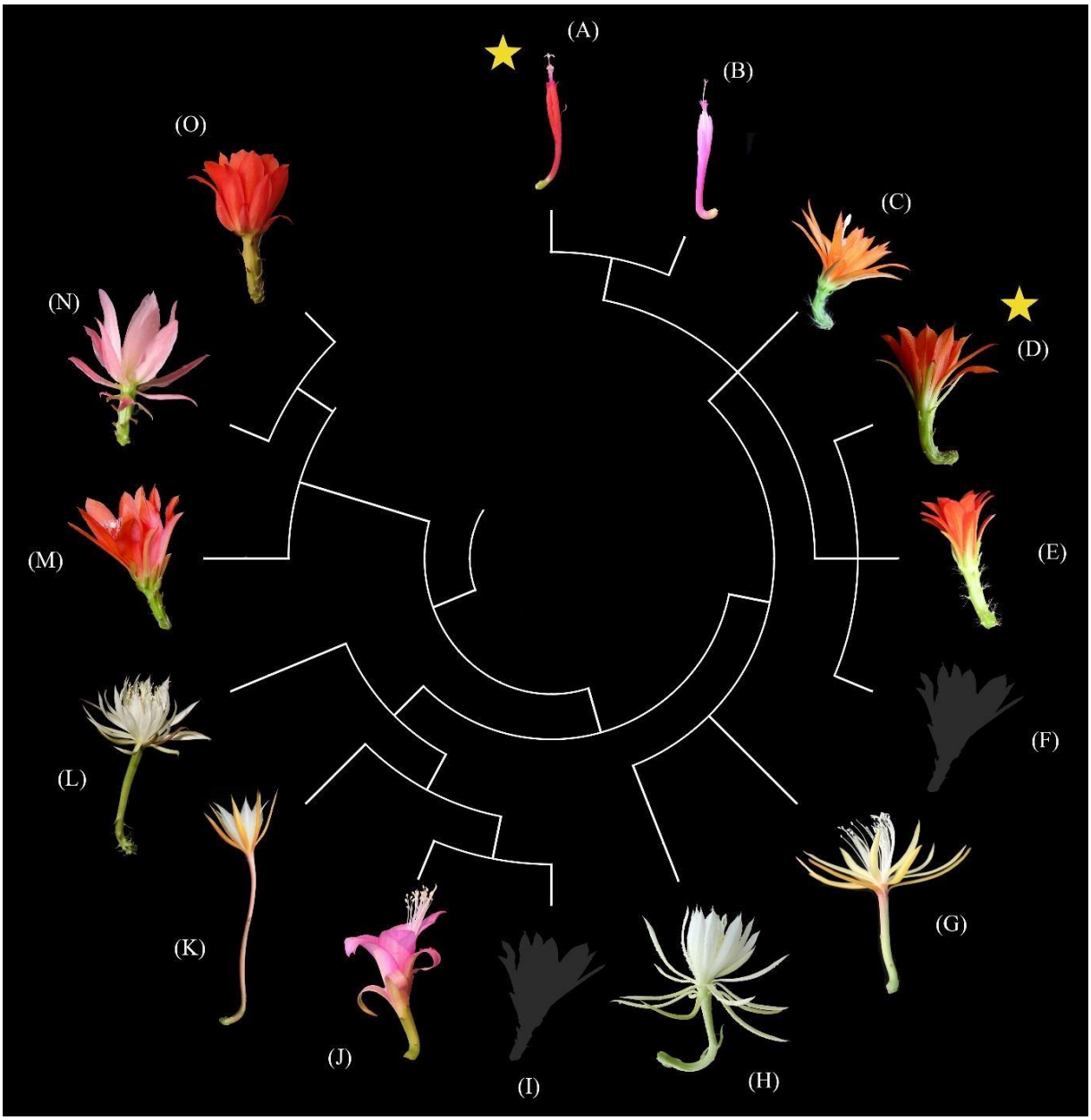


FIGURE 1. The *Disocactus* genus exhibits a wide variety of flower morphologies. This simplified phylogeny, modified from the strict consensus tree from (Cruz *et al.*, 2016), includes: (A) *Disocactus eichlamii*, (B) *D. quezaltecus*, (C) *D. aurantiacus*, (D) *D. speciosus*, (E) *D. cinnabarinus* (F) *D. bierianus* (no photographic record available), (G) *D. macranthus*, (H) *D. crenatus*, (I) *D. biformis* (no photographic record available), (J) *D. nelsonii*, (K) *D. anguliger*, (L) *D. lepidocarpus*, (M) *D. ackermannii*, (N) *D. phyllanthoides*, and (O) *D. kimnachii*. The stars indicate *D. speciosus* and *D. eichlamii*, the two species included in this study. Flowers are not to scale.

MATERIALS AND METHODS

Plant material—

We collected flower buds of *D. speciosus* and *D. eichlamii* corresponding to three developmental stages between January and March 2019 from 12 to 1 PM UTC-6 (see

description in Results section, Fig. 2). *D. eichlamii* (DE) flower buds were collected in the greenhouse of the Epiphytic Cacti Collection from the Botanical Garden of the Biology Institute at the Universidad Nacional Autónoma de México (UNAM), while *D. speciosus* (DS) flower buds were collected from wild specimens from the Reserva Ecológica del Pedregal de San Ángel at UNAM (REPSA).

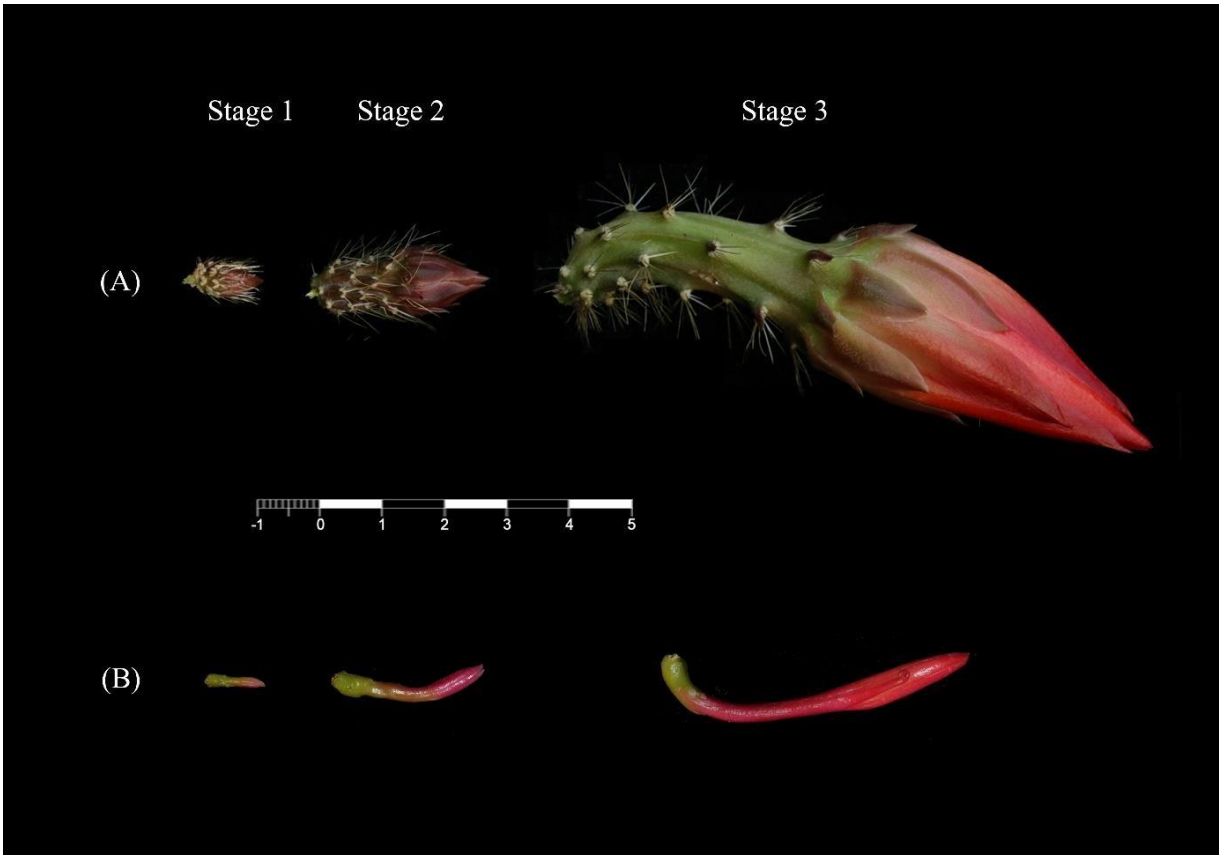


FIGURE 2. Flower development of *Disocactus speciosus* (A) and *D. eichlamii* (B). In stage 3 *D. speciosus* shows a very large green pericarpel covered by areoles with trichomes and spines, as well as numerous tepals. In contrast, *D. eichlamii* flower buds show a reduced pericarpel without areoles and spines and few tepals.

Scanning electron microscopy (SEM) and statistical analysis of tepal epidermal cells— The innermost and outermost tepals per developmental stage were analyzed, dissecting five flowers per stage of *D. speciosus* and *D. eichlamii*. Selected tepals were fixed in FAA (formaldehyde-alcohol-acetic acid). Five tepals per position and developmental stage were dehydrated in an ethanol series (70-100%), critical point-dried with CO_2 (EMITECH K850), mounted on aluminum stubs, and sputter coated with gold (QUORUM Q15OR). Images were taken using a scanning electron microscope (Hitachi SU1510) with a digital camera attachment. The statistical analysis of tepal epidermal cells was made using five transparent tepals (chlorine + sodium hydroxide) per position and developmental stage. Adaxial paradermal

micrographs were taken at 40x magnification using a light microscope and FlyCapture software. A total of 150 cells from the median zone, per tepal position and per developmental stage were measured in each species. Cell area was calculated using ImageJ 1.x (Schneider *et al.*, 2012). Data from cell area measurements were used to perform a three-way ANOVA with interaction and a Tukey test using R (https://cran.r-project.org/).

RNA extraction and RNA-seq library construction—

Based on information regarding cacti flower ontogeny, two types of tissues were dissected and processed separately: a) floral tissue including stamens, stigma and tepals (together referred to as the perianth = PA), and another section including vegetative tissue and the enclosed ovary (together referred to as the pericarpel = PC).

Nine flowers of *D. speciosus* (3 per developmental stage, Fig. 2) and nine of *D. eichlamii* (3 per developmental stage, Fig. 2) were collected. The dissected tissue was immediately frozen in liquid nitrogen and stored at -80 ºC until use.

Total RNA extraction was performed on 18 samples per species (9 from PC and 9 from PA) with the Spectrum™ Plant Total RNA Kit for Difficult Plant Tissues (Sigma–Aldrich) following the manufacturer's instructions. Purified RNA was quantified using a NanoDrop 2000 (Thermo Fisher Scientific, USA) and Qubit 4 (Thermo Fisher Scientific, USA) in RNA High Sensitivity mode. RNA integrity was assessed using a Bioanalyzer (Agilent) and visually inspected by electrophoresis in gel-chlorine (Aranda *et al.*, 2012). Library preparation and sequencing were performed by the Beijing Genomic Institute (BGI), Hong Kong, China, with the Illumina HiSeq™ 4000 platform, paired-end (PE) reads (2x150 nt) and approximately 20 M reads per sample.

Transcriptome de novo assembly and functional annotation—

The raw reads were analyzed with FastQC v.0.11.9 (Andrews, 2010) and multiQC (Ewels *et al.*, 2016). Adapters, duplicated sequences and low quality reads were eliminated with Trimmomatic v.032 (Bolger and Giorgi, 2014) using the following parameters: *ILLUMINACLIP:TruSeq3-PE-2.PA:2:30:10 LEADING:25 TRAILING:25 MINLEN:75 SLIDINGWINDOW:4:25*.

The transcriptome was *de novo* assembled for each species using Trinity 2.4.0 (Grabherr *et al.*, 2011) in paired-end mode (--Paired-end); other parameters were set to the default. The reads were aligned against the *Selenicereus undatus* genome (Zheng *et al.*, 2021) using Bowtie2 (Langmead and Salzberg, 2012) through Galaxy (https://usegalaxy.org/). All reads unmapped to the *S. undatus* genome were reassembled using Trinity (Grabherr *et al.*, 2011). The transcriptome was annotated using Pannzer (Koskinen *et al.*, 2015; Törönen *et al.*, 2018)

against the Viridiplantae database. Completeness of the assemblies was evaluated with BUSCO v4 with the Viridiplantae gene set through gVolante (Nishimura *et al.*, 2017) (Appendix S1; see Supplemental Data with this article).

Transcripts quantification and differential expression (DE) analyses—

Transcript abundance was assessed by aligning the RNA-seq reads to the corresponding assembly and quantified with Salmon 1.5.2 (Patro *et al.*, 2017). Count matrices were created using the R package tximport (Soneson *et al.*, 2015). Differential gene expression was analyzed using the edgeR package (Robinson *et al.*, 2010; McCarthy *et al.*, 2012). The counts were normalized using the Trimmed Mean of M values (TMM). Principal component analysis (PCA) was performed for each species, and the results were plotted together. Transcripts were considered differentially expressed when the false discovery rate (FDR) and the log fold change were between the intervals FDR < 0.05 and log2FC > 1.5 or FDR < 0.05 and log2FC < -1.5.

WGCNA—

A weighted gene correlation network analysis (WGCNA) was performed for each species using the R package WGCNA v1.68 (Langfelder and Horvath, 2008). All transcripts with read counts >5 counts per million (cpm) in fewer than 2 samples were removed. The resulting datasets were analyzed to identify gene expression modules. The adjacency matrix was calculated using a soft threshold power $\beta = 9$, and the minimum module size was set to 30. Correlated modules ($r > 0.5$; correlation p value < 0.01) were annotated using the transcriptome annotation database; the GO term enrichment analysis for every cluster was performed using PANTHER (Thomas *et al.*, 2003) as implemented in TAIR (Berardini *et al.*, 2015).

RESULTS

Epidermal cell size correlates with tepal size variation in Disocactus—

Three developmental stages for each species were selected based on flower bud size and morphological characteristics (Fig. 2). In *D. speciosus*, buds in stage 1 have a brownish color and are ~1 cm in length, the floral tube has not yet developed, and the pericarpel is small and covered by bracts and spines. In stage 2, buds are still brown, but the flower tube has begun to develop, and the buds are ~ 3 cm in length. In stage 3, the flower buds are preanthetic and fully developed, reaching 10 cm in length. The pericarpel, bracts and flower tube are green/photosynthetic, while the tepals are red. *D. eichlamii* flower buds in stage 1 are approximately 1 cm long, the flower tube has not yet developed, the pericarpel is green with

extremely reduced bracts and spines, and the tepals have a pinkish color. In stage 2 flower buds are 2.5 cm in length, the flower tube has begun to develop, and the tepals have acquireda magenta color, while the pericarpel remains green. In stage 3, the flower bud is preanthetic and reaches 5 cm in length. In this stage, the tepals have a vivid magenta color and the pericarpel is green without bracts and spines (Fig. 2).

A three-way ANOVA with interaction was conducted to test for effects of species, stage and tepal position as independent variables (see Methods). All effects were statistically significant at the 0.05 significance level: species F1, 2999 = 707.9, P less than 0.001, stage F2, 2999 = 4274.5, P less than 0.001, and tepal position F3, 2999 = 49.6, P less than 0.001. These results show that cell size depends on the species, developmental stage, and whether the tepal is in the inner or outer position in the flower.

In both species, tepal cells are similar in size in stage 1, and increase in size from stage 1 to 3 (Fig. 3) in a longitudinal manner. *D. eichlamii* in stage 2 shows outer tepal cells that are slightly larger than the inner cells, while in *D. specious* in stage 2, the size of the outer tepal cells is significantly larger than that of the inner ones. This differential growth is inverted for *D. speciosus* in stage 3, where the inner cells grow larger than the outer tepal cells. In contrast, in *D. eichlamii* in stage 3, the size of the inner tepal cells is comparable to that of the outer ones (Fig. 3A). Although the initial tepal cell size is the same for both species, in the third stage, the cells of *D. speciosus* are four times larger than the equivalent cells in *D. eichlamii*. This can also be observed in the SEM micrographs, where epidermal cells are comparable at the first developmental stage for both species, while in the second and third stages, the tepal cells of *D. eichlamii* remain smaller (~ 200 µm) than those of *D. speciosus* (~ 800-1000 µm) (Fig. 3B). Furthermore, the tepal epidermal cells in *D. speciosus* are tabular, rugose, striate and isodiametric in the first two developmental stages. Then the cells become tabular, rugose and quadrangular with a striate surface in the internal tepals but a smooth surface in the external ones. In contrast, in *D. eichlamii* in the stage 1 cells are already tabular, flat, quadrangular and slightly elongated, and from the second stage onward, the cells remain quadrangular-elongated with striations on the surface. However, the cell surface remained smooth in the internal tepals in the third stage.

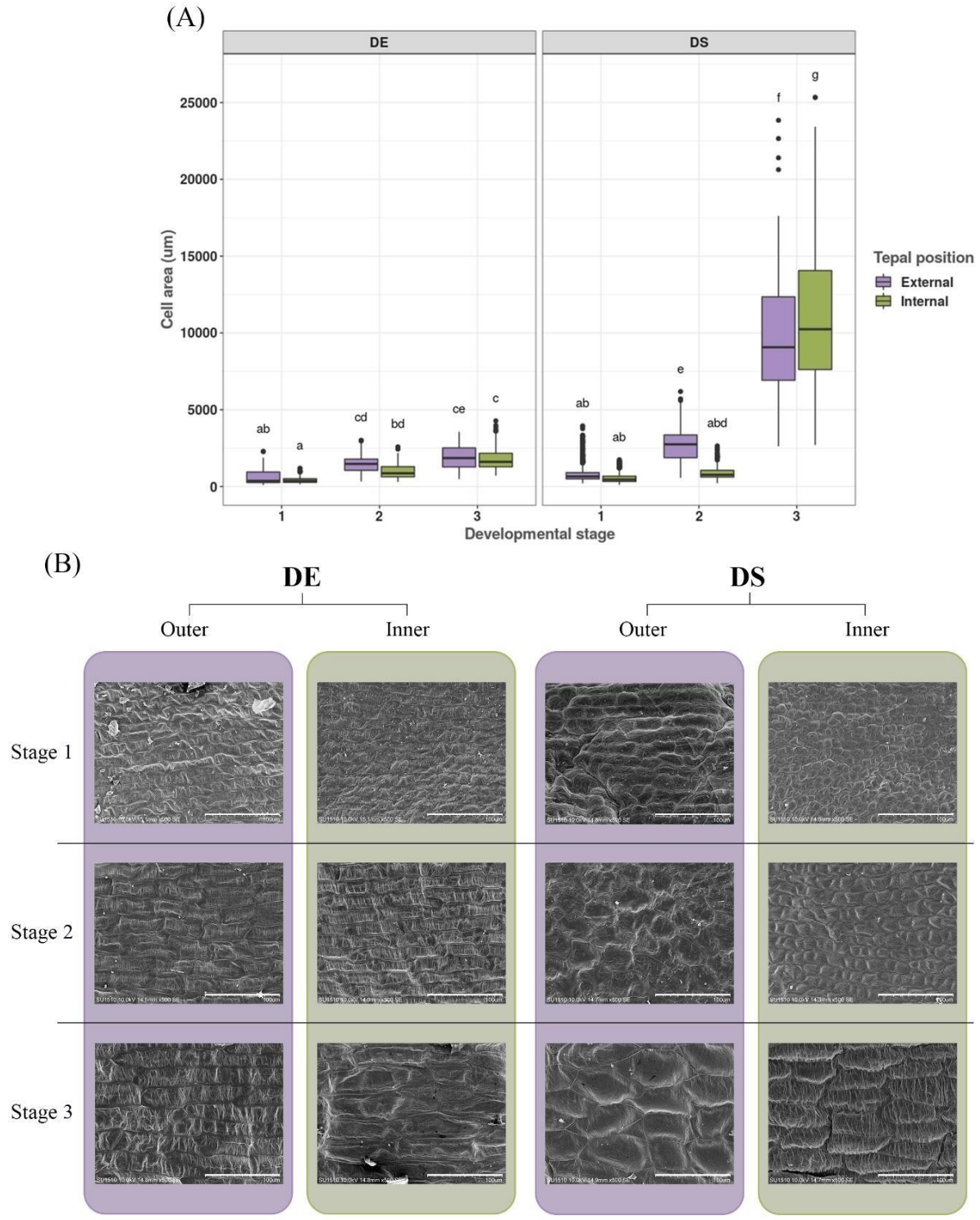


FIGURE 3. Cell area estimation and SEM micrographs of the epidermis in developing tepals in *D. eichlamii* (DE) and *D. speciosus* (DS), corresponding to the three selected developmental stages. (A) Area of tepal epidermal cells in three developmental stages. Different letters in the same box indicate that the difference is significant at the 0.05 level after Tukey's test. The mean of the boxes within a stage with different letters above them are statistically different according to the Tukey *a posteriori* test. (B) Exemplary SEM micrographs of the medial zone and adaxial

face of the three developmental stages in each species. Colors indicate tepal position: purple indicates outer tepals, and green indicates inner tepals. Scale bar = 100 μm.

Similar transcriptomes, but different expression profiles between Disocactus species— To characterize the gene expression profiles of flower development in our selected *Disocactus* species, we sequenced, and *de novo* assembled their transcriptomes. We obtained two transcriptomes of 99,167 and 102,259 transcripts for *D. speciosus* and *D. eichlamii*, respectively (Table 1); both transcriptomes have an average completeness of 90% as assessed by Bench-marking Universal Single-Copy Orthologs (BUSCO) v4 with the Viridiplantae gene set (Appendix S1).

Table 1. *De novo* transcriptome assembly results

	D. speciosus (transcripts)	***D. eichlamii*** (transcripts)
Number of sequences	99,167	102,259
Total length (nt)	131,595,696	135,694,494
Mean length (nt)	1,327	1,327
N50 (nt)	1,953	1,872
%GC	41.43	43.07

The annotation reveals that 64.66% and 65.93% (64,131 and 67,427 transcripts) of the assembled contigs were annotated for *D. speciosus* and *D. eichlamii*, respectively. The ten principal gene ontology categories (GO terms) in the two species are similar, showing only differences in the biological process (BP) ontology, where two processes are not shared between the two species: protein transport is only detected in *D. eichlamii*, while transcripts related with defense response are only detected in *D. speciosus*. In the other two categories (CC and MF), the two species share the 10 main categories (Fig. 4A). The genome of *Selenicereus undatus*, an epiphytic cactus from the same subfamily and tribe as *Disocactus*, was recently released (Chen *et al.*, 2021). A comparative analysis shows that 15,436

transcripts in *D. speciosus* and 15,903 transcripts in *D. eichlamii* are homologous to *S. undatus* genes.

Principal component analysis (PCA) showed that the two first principal components explained >50% of the variance, with PC1 accounting for 39.1% and 31.59% and PC2 accounting for 12.57% and 17.17% in *D. speciosus* and *D. eichlamii*, respectively (Appendix S2). The PCA (Fig. 4B) in both species shows that the perianth tissue (PA) in stages 1 and 3 groups species together, suggesting that the general transcriptional landscape of the PA in the two species is very similar at least for stages 1 and 3, which correspond to the early development of the flower bud and the preanthesis stage, respectively. The PCA also suggests that transcriptomes from the pericarpel (PC) tissue in stages 2 and 3 in both species have similar expression profiles. For *D. eichlamii* the PA and PC transcriptional landscapes in stages 1 and 2 are similar, and only in stage 3 (preanthesis) do they exhibit clear differences that separate the two types of tissues. In *D. speciosu*s the PA and PC are transcriptionally comparable in stage 1 but become clearly separated across stages 2 and 3 (Fig. 4B). These results show that independent of final flower size, the general patterns of gene expression in the two species are very similar in both types of tissues and that differences in organ size and number might be caused by very specific genes and differences in their expression.

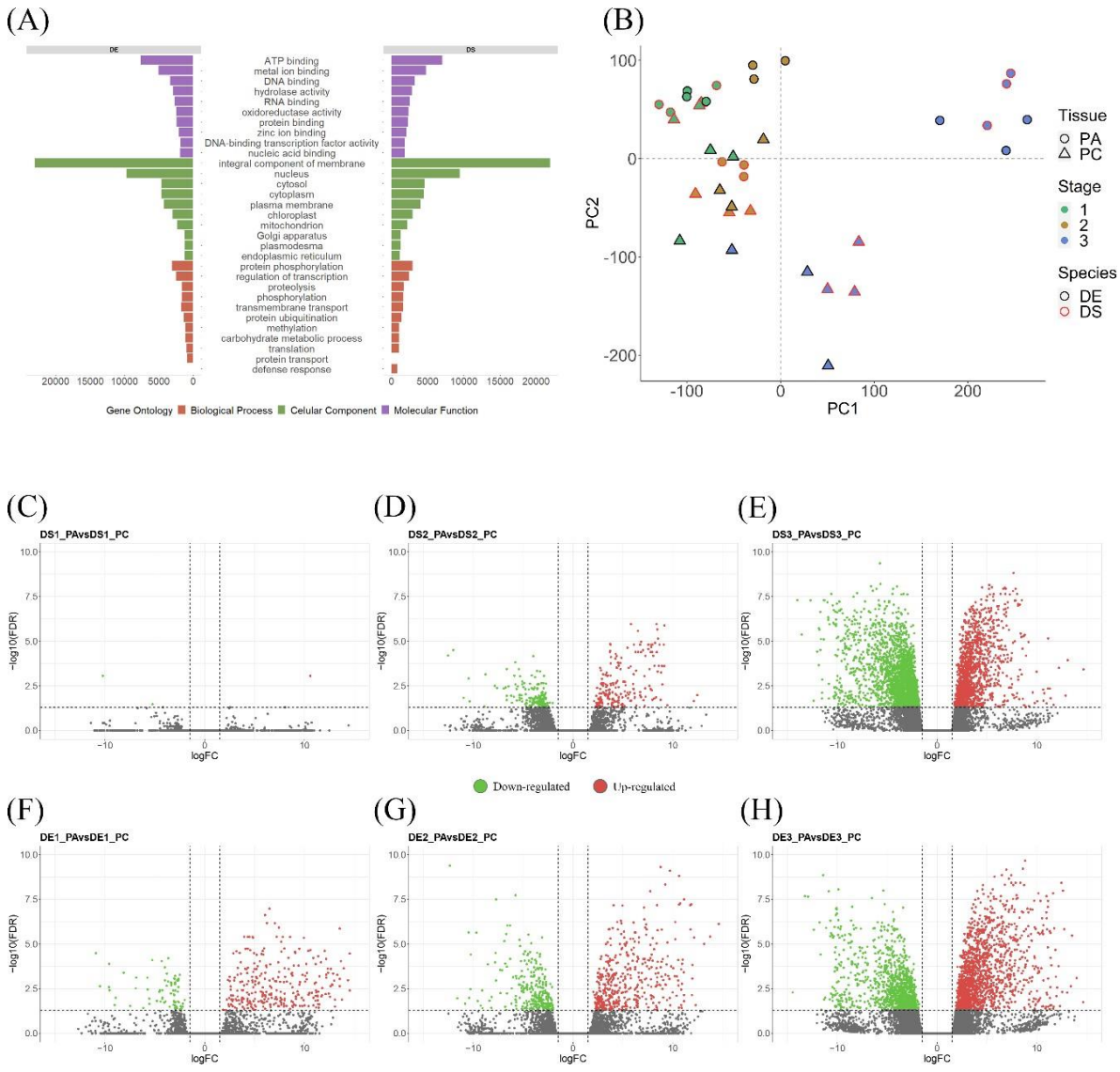


FIGURE 4. Gene ontology (GO) enrichment, principal component analyses of floral tissue (PA) and pericarpel tissue (PC) in three different developmental stages and volcano plots from differential expression analyses comparing PA vs. PC in *D. speciosus* (DS) and *D. eichlamii* (DE). (A) Top 10 enriched GO terms in the *D. eichlamii* (DE) and *D. speciosus* (DS) transcriptomes. (B) PCA based on TMM values from 3 replicates of each tissue/developmental stage in *D. eichlamii* (DE) and *D. speciosus* (DS). (C-E) *D. speciosus*, (C) volcano plot DS1_PA vs. DS1_PC, (D) volcano plot DS2_PA vs. DS2_PC, (E) volcano plot DS3_PA vs. DS3_PC, (F-H) *D. eichlamii*, (F) volcano plot DE1_PA vs. DE1_PC, (G) volcano plot DE2_PA vs DE2_PC, and (H) volcano plot DE3_PA vs DE3_PC. Colored dots correspond to differentially expressed transcripts (FDR < 0.05 and log2FC > 1.5 | FDR < 0.05 and log2FC < -1.5). The number of differentially expressed transcripts (DEGs) is shown at the bottom of each plot. The red dots indicate up-regulated transcripts, the green dots indicate down-regulated transcripts, and the gray dots indicate non significantly differentially expressed transcripts.

To identify differentially expressed transcripts during flower development, we compared transcript expression levels between developmental stages and between tissues (Fig. 4, Appendix S3, S4). The comparisons of early developmental stages in both species showed that the expression profiles are similar both between tissue types and stages, while differences among tissue types increase as flower buds develop (stages 2 and 3). It should be noted that for both species, the number of transcripts increases from stage 1 to stage 3. This holds true at every level of comparison, indicating that most of the differentially regulated gene during flower development had a late onset.

In the differential expression analysis performed across the different tissues and developmental stages examined, 23,881 DEGs were identified in *D. speciosus,* while 12,239 DEGs were identified in *D. eichlamii*. The results for *D. speciosus* in the same developmental stage indicate that the two types of tissue (PA and PC) have very similar transcriptomes in stages 1 and 2 (Figs. 4C, D), but become different in stage 3 (Fig. 4E). These results agree with the PCA (Fig. 4B). In *D. eichlamii,* expression profiles for the two types of tissue analyzed show slight differences in the transcriptomes during stages 1 and 2 (Figs. 4F, G), but in stage 3 (Fig. 4H), they become completely different, as in *D. speciosus* (Fig. 4E). This pattern is maintained among all comparisons, regardless of whether PA or PC tissue is analyzed (Appendix S3, S4).

Cell measurements of epidermal tissue in tepals suggest that in the developmental stages evaluated, the differences in final organ size are related to cell growth and elongation (Fig. 3A); for this reason, we searched within the DEGs for transcripts involving those processes, as well as transcripts related to organ number specification. In *D. eichlamii* we found that across tissue types and stages, some transcripts related to the aforementioned processes had differential expression (FDR < 0.05 and log2FC > 1.5). This is the case for the putative orthologs of *BB*, *BPEp*, *ER*, *ANT*, *ARF2, AGAMOUS-Like* and *KLUH*, which are down-regulated in PA comparisons, but overexpressed in PA vs. PC comparisons.

In *D. speciosus*, *ER* is up-regulated in PA comparisons but down-regulated in PA vs. PC. *ANT* and *JAG* are down-regulated in PA comparisons, but the latter is up-regulated in PA vs. PC. There is no evidence of changes in the expression of *BB*, *ARF2*, *AG-LIKE* and *KLUH* in this species. In this species *BPEp* homolog was not found, it is possible that we were unable to assemble the ortholog, but other possibilities include a low expression on the transcript, making undetectable to the analysis, or maybe the *BIG PETAL* is not expressed in this species at the collected stages.

Given that some transcripts encode transcription factors (TFs), small changes in expression could have a magnifying effect on final flower morphology, regarding overall size and organ number (Appendix S6). Specifically in *D. speciosus*, we identified genes whose function has been related to an increase in organ number, such as *SUP*, *PAN* and *ETT*. Although these transcripts were not differentially expressed based on our established thresholds (see Methods), when we lowered the threshold in the DEG analysis (FDR < 0.05 and log2FC > 1), we found that they were differentially expressed. Complementally, gene abundance between species is different, which is probably related to the contrasting sizes and number of organs observed in preanthetic flowers (Appendix S6). For example, the *BB* gene, which is a negative regulator of cell proliferation, shows a gradual increase in count numbers in *D. eichlamii*, mainly in PA_1 to PA_3, suggesting a possible role in the reduction of cell proliferation across development, while in *D. speciosus BB* expression is low throughout flower development in both tissues, allowing a longer period for cell proliferation. Transcripts of *SUP, PAN* and *ETT* were only found in *D. speciosus*. Specifically, *SUP* and *ETT* show a decrease in count number throughout flower development, while *PAN* does not show changes across developmental stages. Pertaining to genes involved in floral organ determination, we found, orthologs of *SEPALLATA* (*SEP*), *PISTILLATA* (*PI*), *AGAMOUS* (*AG*), *APETALA1* (*AP1*) and *APETALA3* (*AP3*). These transcripts were identified in PA and PC tissues in both species across different developmental stages.

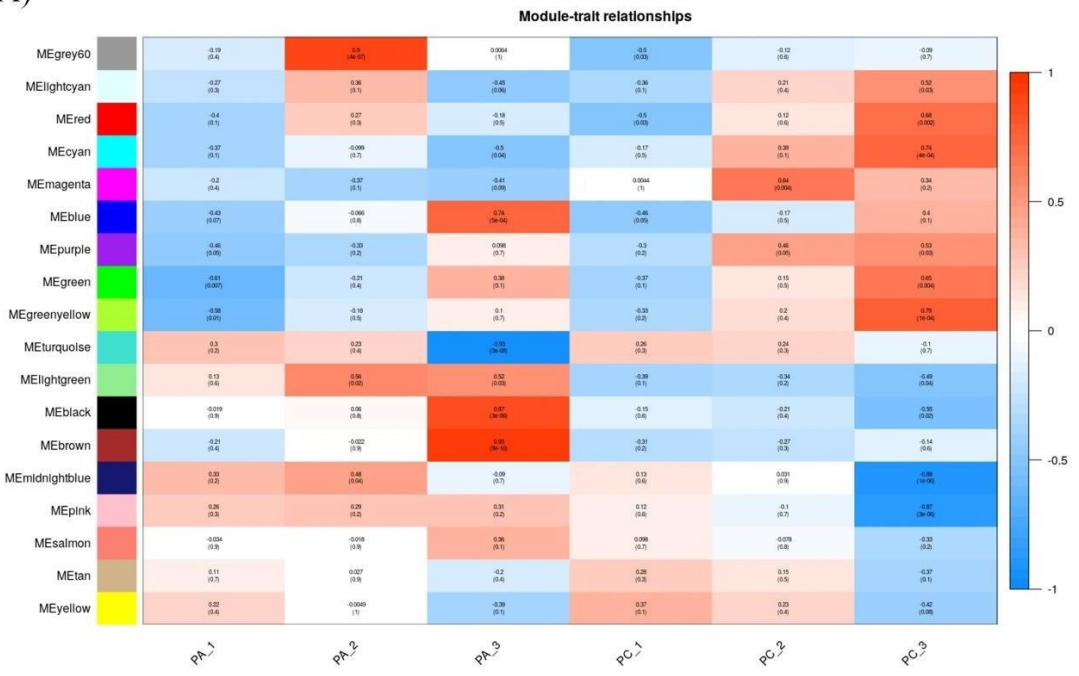
WGCNA recovers co-expressed modules related to organ size control pathways during flower development of Disocactus—

A Weighted gene coexpression network analysis (WGCNA) was performed to identify discrete modules related to developmental stage and tissue type in each species. To explore key genes and coexpression networks that might play important roles during the growth and floral development in *Disocactus,* a separate WGCNA was performed for each species using only transcripts with more than 5 CPM in at least two samples; therefore, WGCNA was performed with 39,2323 transcripts for *D. speciosus* and 40,465 transcripts for *D. eichlamii*. 18 modules and 53 modules were identified for *D. speciosus* and *D. eichlamii* (Figs**.** 5A, B, Appendix S5).

In *D. speciosus,* for PA and PC tissues from stages 2 and 3, at least one highly specific module was identified ($r > 0.7$; correlation p value < 0.01), although in some cases, multiple modules showed significant correlations with the same tissue (Fig. 5A). In PA and PC tissues for stage 1 in *D. speciosus* the module correlation values are $r > 0.5$, showing a weak correlation between modules and features. In contrast, in *D. eichlamii* in each tissue type and

developmental stage at least one module was highly correlated with the same tissue and/or more tissues showed correlations with the same module ($r > 0.7$; correlation p-value < 0.01) except for PC_1, where the module correlation value was $r > 0.5$ (Fig. 5B).

(A)



(B)

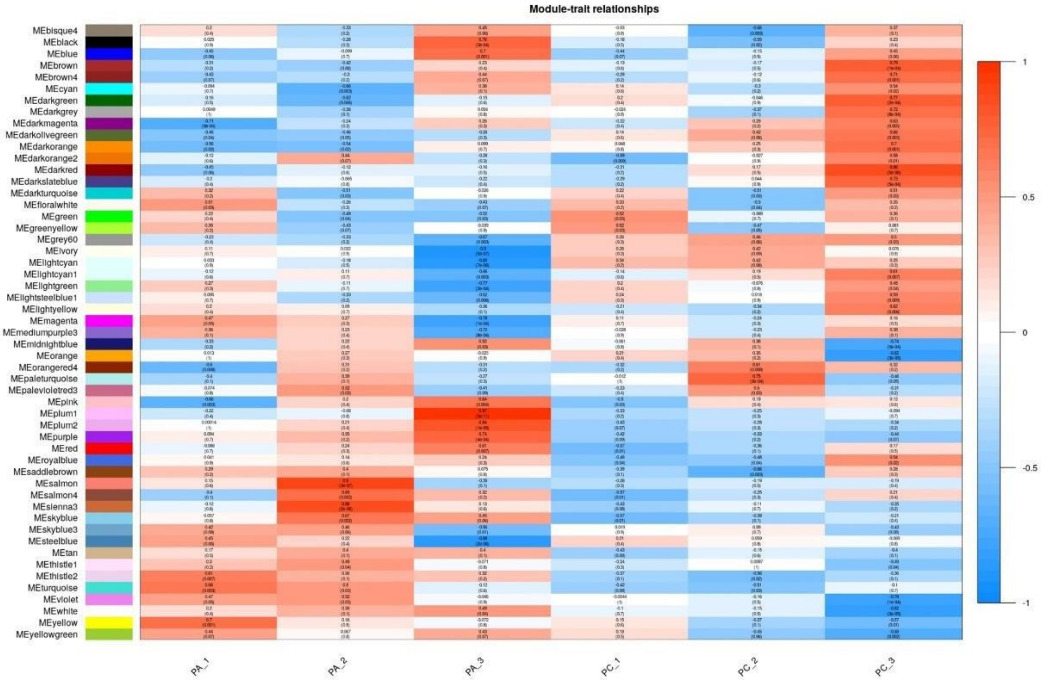


FIGURE 5. Clustering of module eigengenes from weighted gene coexpression network analysis (WGCNA) in (A) *D. speciosus* and (B) *D. eichlamii*. The corresponding correlation (top) and p value (bottom) are displayed in each cell. The table is color-coded by correlation according to the color key.

To obtain a broad overview of genes in the modules of interest, we performed a GO term enrichment analysis in both species for modules with high positive correlation values with stage and tissue (Fig. 6).

Interestingly, in *D. speciosus* in PA_1, all modules have low correlation values, although the midnight blue module was enriched in processes corresponding to flower development and organ growth. In PA_2, module grey60 has the highest correlation values, with an overrepresentation of processes related to flower pigmentation. In PA_3, modules brown and black were the highest correlated modules which show overrepresentation of processes related to maturation, growth, cell elongation, whorl and flower development which are consistent with the developmental stage of the *D. speciosus* flower. In the pericarpel tissue, in the PC_1 yellow module, in the PC_2 magenta and in the PC_3 green-yellow and cyan modules is overrepresented processes related with reproduction, growth, leaf development and cell morphogenesis, which is consistent with the fact that the pericarpel tissue collected contains the ovary and ovules, and probably some axial photosynthetic tissue (Fig. 6A).

In *D. eichlamii* the PA_1 yellow module encompasses processes related to the bud developmental stage, especially those related to growth. In PA_2 the salmon, and in PA_3 the black module, have an overrepresentation of processes consistent with the organs developing within and with the developmental stage of the collected bud, namely increase in organ size and changes in pigmentation and floral organ development (Fig. 3). In the PC_1 green-yellow module, in the PC_2 pale-turquoise and orangered4, modules and finally in the PC_3 brown and dark_green, modules there is enrichment of processes related to the bud developmental stage and tissue type, such as phylome development, ovule development and growth (Fig. 6B).

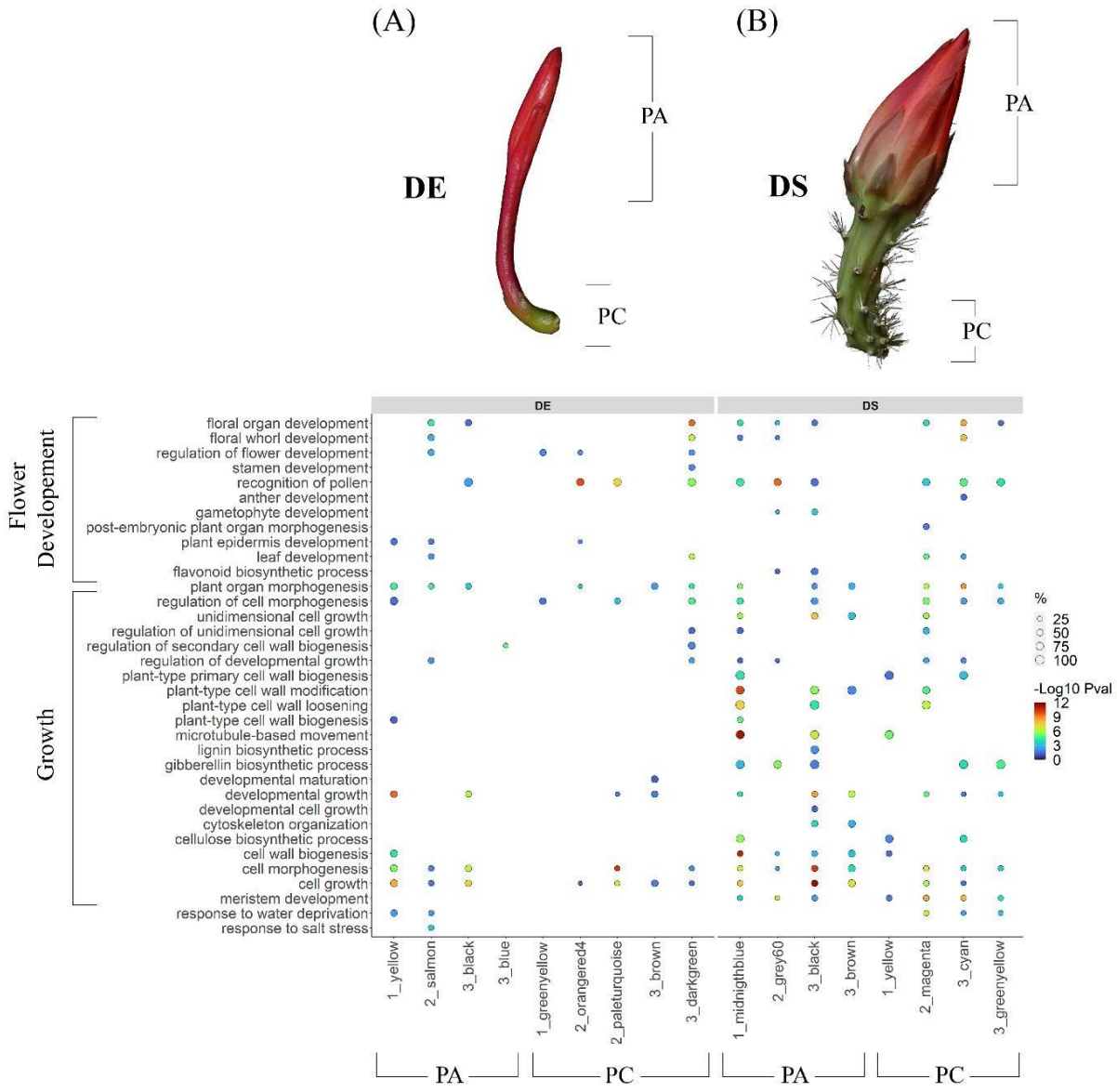


FIGURE 6. Selected GO terms enriched from modules of interest per tissue type in the three developmental stages studied in *D. speciosus* and *D. eichlamii.* (A) In *D. eichlamii* 9 out of 53 modules found in the analysis in the three developmental stages are positively correlated with the PA or PC tissue. (B) In *D. speciosus* only 8 out of 18 modules detected in the three developmental stages are correlated with PA or PC tissue.

DISCUSSION

Our study explores the relationships between variation in gene expression and differences in growth rate and final floral morphology, as related to tepal and stamen number and overall flower size in two closely related species: *Disocactus speciosus* and *D. eichlamii.* We used SEM photography to explore the characteristics of inner and outer tepals, as well as a comparative transcriptomics approach during floral bud development. While the processes of floral organ determination, organ boundary delimitation and organ growth have been

extensively studied in model systems such as *Arabidopsis* (Bowman *et al.*, 1989; Coen and Meyerowitz, 1991; Sessions *et al.*, 1997; Breuil-Broyer *et al*., 2004; Alvarez-Buylla *et al.*, 2010; Xu *et al.*, 2018) and specific genes and gene regulatory networks have been proposed to underlie such processes (Bowman *et al.*, 1989; Coen and Meyerowitz, 1991; Espinosa-Soto *et al.*, 2004), research on non-model organisms has painted a more nuanced picture regarding the universality of inferences made in *Arabidopsis*. On the other hand, knowledge about the genetic control of organ number is limited (Pieper *et al.*, 2016), although some genes such as *SUP*, *PAN*/*ETT*, and *CLV*/*WUS* have been identified as regulators of merosity (Moyroud and Glover, 2017). It is known that plant organ growth is a complex and multifactorial process that can be explored at multiple levels (Nelissen and Gonzalez, 2020), but most quantitative genetic analyses have been focused on unraveling QTLs implicated in flowering time and size differences in fruits, rather than flowers, among populations within a single species and heavily focusing on crops and closely related species (for a recent review, see: Kulwal, 2018).

Regarding cellular growth dynamics related to perianth development, our measurement of tepal epidermal cells in *D. eichlamii*, shows that the cells remain small until preanthesis, while in *D. speciosus*, preanthetic buds are 3 times the size of equivalent cells in *D. eichlamii*, suggesting that final size in the tepals in this species is the result of cellular elongation; however, the role of cell proliferation needs to be further evaluated through dynamic studies addressing the cell cycle. In both species, external and internal epidermal cells in stage 1 have similar sizes in both internal and external tepals, and this period may correspond to growth by proliferation, when cell size remains largely constant and the cells stay densely cytoplasmic (Powell and Lenhard, 2012). In stage 2, external epidermal cells are larger than internal cells, but the difference is greater in *D. speciosus* (Fig. 3), suggesting that the external cells begin their expansion, but internal cells might still be in a proliferative phase. Finally, in stage 3 in *D. speciosus,* the size relation is inverted, where internal epidermal cells are larger than external cells and both are expanded. In *D. eichlamii*, external cells remain larger than the internal ones but not significantly, as cell expansion on both sides remains low. In early developmental stages, the external tepal-bracts grow to protect the developing organs (androecium and gynoecium) within the flower; afterward, the internal tepals grow to finally open. It is worth mentioning that differences in size and cell morphology, especially in stage 1, in both species could be due to these species being heterochronic (not being in the same stage of development), as our observations suggest that *D. eichlamii* has a faster development than *D. speciosus*. One possibility is that slower flower development in *D. speciosus* allows

for more rounds of cell proliferation and/or cell expansion, yielding larger flowers; such hypotheses need to be tested.

Our SEM micrographs also showed that the epidermal cells were not conic and developed striations in the surface, in accordance with recent studies that show a more diverse set of cellular shapes in petaloid organs than previously acknowledged (Glover and Martin, 1998; Whitney *et al.*, 2011; Cavallini-Speisser *et al.*, 2021). These striations of the epidermal cells could be ornamentations of the cuticle, which probably have a role in light refraction (Whitney *et al.*, 2009) and interspecies communication (Kourounioti *et al.*, 2013). Nevertheless, little is known about the functional implications of these patterns in Cactaceae, especially in epiphytic cacti, where little is known about pollinator syndromes (Fig. 3).

The differential gene expression analyses showed changes in expression of key regulators in final organ size (Krizek, 2009; Powell and Lenhard, 2012) as well as genes from gene regulatory networks regulating flower patterning and morphogenesis, including those involved in meristem maintenance such as *WUS/CLV3* and, *CUCs*; organ identity such as *LFY*, *AP1*, *AP2*, *AP3*, *PI*, *SEP3*, and *AG*; and polarity such as *YABBY*, *KAN*, *REV*, *PHB*, and *PHV* (Refahi *et al.*, 2021). Regulatory genes that play a role in the control of cell proliferation were identified in both species (*KLUH*, *ARF2*, *JAG*, *BB* and *ANT)*, as well as genes involved in cell expansion (*TOR* and *ER)*. The different patterns of expression of these genes can explain the contrasting final organ size and organ number (tepals and stamens) in each species. For example, the absence of *BPEp* in the transcriptome of *D. speciosus*, which is a TF that regulates petal growth through restriction of cell expansion (Szécsi *et al.*, 2006), could be related to a longer period of cell expansion compared to that in *D. eichlamii*, where *BPEp* is differentially expressed during flower development, with a gradual increase, especially in PA tissue. Plants that lack the petal-expressed variant BPEp have larger petals as a result of increased cell size, showing that BPEp interferes with postmitotic cell expansion (Szécsi *et al.*, 2006). On the other hand, the low expression of *BB* in *D. speciosus*, which negatively regulates cell proliferation can also explain the large size of its flowers; thus, it is possible that *D. speciosus* not only has larger cells but also has a greater number of cells per area, translating into a large flower. In contrast, *D. eichlamii* buds have higher expression of *BB* and *BPEp* homologs, suggesting that the cell proliferation rate and cell expansion could be limited by these genes; thus, it is possible that this species not only has small cells, but also fewer cells per area, resulting in small flowers.

Another factor that can impact flower size is the multiplication of floral whorls, which has been related to two different reasons: meristem size, where in species of *Eucryphia*, meristem size has been correlated with whorl number (Bull-Hereñu *et al.*, 2018), or meristem maintenance,

as changes in the expression of the TFs *PAN*, *ETTIN* and *SUP*, which act independently of the *CLV/WUS* pathway (Running and Meyerowitz, 1996) have been related to changes in merosity and the determination of stamens and carpel number (Sessions *et al.*, 1997; Bowman *et al.*, 1992; Xu *et al.*, 2018). In *D. speciosus*, we found expression of *PAN* and *ETT*, as well as *SUP***.** Polyandry in Cactaceae flowers as in other Caryophyllales has been suggested to be the outcome of a meristematic ring (Hofmann, 1994; Ronse De Craene, 2013). The meristematic ring is closely connected with the gynoecium, as loss of carpels can lead to stamen loss, while an increase in carpel number is correlated with an increase in stamens (Ronse De Craene, 2013). Meristematic rings have arisen multiple times in flowering plants; however, their genetic bases are still unknown (Kong and Becker, 2021). In the case of our species, *D. specious* has more than 200 stamens and 10 carpels, while in *D. eichlamii*, the number of stamens is nearly 20 and there are only five carpels. Further comparative studies of flower meristem size could explain whether the multiplication of floral organs is related to meristem size or to spatiotemporal changes in the expression of *PAN/ETT*.

WGCNA recovered coexpression modules with enriched pathways related to growth, final organ size and flower development. In *D. speciosus* we found modules in PA and PC samples related to cellular processes that underlie plant growth and development (Chávez Montes *et al.*, 2008), such as those linked to cell wall loosening related to growth by cell elongation, cell wall modification and cell wall organization. We also retrieved tissue-specific modules that can be related to the function and identity of particular structures, such as the response to water deprivation in PC tissue of *D. speciosus* (Figs. 5 and 6).

As expected, we found genetic modules related to flower development, with some noteworthy variations between species. For instance, a module of flower development pathway is present in all tissues in both species except for stage 2 PC in *D. eichlamii*. While we documented differential enrichment of certain modules containing particular developmental pathways in PC and PA tissues, suggesting that different biological processes were happening, they can also suggest different ontogenetic identities as has been proposed (Mauseth, 2006, 2016; Rosas-Reinhold *et al.*, 2021). Overall, more tissue types and developmental stages in *D. speciosus* had coexpression modules related to floral development than those in *D. eichlamii*. This phenomenon could be due to an experimental shortfall, namely, the establishment of developmental stages based on flower bud size and external morphological features that could have translated into collecting buds for each species that were not in equivalent developmental stages (Fig. 2). Thus, *D. eichlamii* floral buds in stages 1 and 2 might have been in more advanced developmental stages than their equivalents in *D.*

speciosus due to differences in developmental timing (Fig. 6). Future studies with these species will benefit from the establishment of developmental series for each that will allow for more precise timing in floral bud collection at equivalent stages, as well as to address the possibility of heterochrony. The heterochrony hypothesis is supported by our DEG analysis, where we observed a smaller number of transcripts differentially expressed in *D. eichlamii* than in *D. speciosus* (Fig. 4); however, genes related to final organ size showed major changes in the former, not the latter.

Finally, despite being closely related species with very similar transcriptome sizes and features, the two species show differential expression of unique genes related to organ number and the control of organ size, which correspond with the contrasting floral morphology in the two species. The WGCNA results suggest that despite using composite tissues (PC and PA comprise several organ types as well as reproductive and vegetative tissue), it is still possible to identify groups of transcripts and biological pathways that are tissue specific, and these results could help unravel the ontogenetic identity of those organs. On a broader scale, our study experimentally addresses some of the ontogenetic changes that could underlie the development of contrasting flowers found in closely related taxa, which might impact the formation of diverse pollination syndromes, a phenomenon that has been considered an integral part of the adaptive explanations underlying the rapid speciation events documented in numerous angiosperm lineages (Moyroud and Glover, 2017). In this context, the Cactaceae are an interesting model to further explore the relationship between historical patterns of climate change (aridification) that enabled rapid range expansion and high speciation rates in many genera and pollination syndrome diversification (Rowley, 1980; Hernández-Hernández *et al.*, 2014; Cruz *et al.*, 2016). *Disocactus*, under its current circumscription (Cruz *et al.*, 2016), includes species with very divergent flower morphologies, sizes and tepal pigmentations (Fig. 1), rendering it an interesting case for exploring the evolution of diverse pollination syndromes in a recently derived clade.

CONCLUSIONS

Our study is one of the first investigations addressing the role of differential gene expression on contrasting floral development in two closely related angiosperm species. We show that changes in the temporal expression of genes previously characterized as important in differential organ growth as well as co-expression modules, can help explain the evolution of distinct flower phenotypes, which in turn could allow for the emergence of new pollination syndromes. Furthermore, the analysis of the size and cellular structure of petaloid perianth

organs in *D. speciosus* and *D. eichlamii*, support the notion that petaloid-like structures are more diverse than previously thought based on studies from model species. These results should be followed by further experiments addressing the specific spatio-temporal expression of *BB* y *BPEp* during the development of the flower shoot in *Disocactus*.

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AUTHORS CONTRIBUTIONS

IRR, UR and SA original idea. IRR, UR, SA and GRA experimental design. IRR collected the biological material, performed RNA extractions, and SEM micrographs. IRR and CC performed the statistical analysis. IRR, CC and GRA performed bioinformatic analysis. IRR, APN and GRA wrote the first draft. SA, UR, and APN critically reviewed the manuscript. All authors agree with the final version of the manuscript.

DATA AVAILABILITY STATEMENT

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE209808 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE209808).

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SUPORTING INFORMATION

“Additional supporting information may be found online in the Supporting Information

section at the end of the article”

Appendix S1. Transcriptome completeness of *de novo* assembled

Appendix S2. Principal component analysis

Appendix S3. Differential expression analysis of *D. eichlamii*

Appendix S4. Differential expression analysis of *D. speiciosus*

Appendix S5. WGCNA soft-threshold identification and hierarchical clustering

Appendix S6. Comparison of specific counts of transcripts related to organ size and organ number

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Discusión general y conclusiones

Cactaceae es un grupo que tiene estructuras y órganos tanto vegetativos como reproductivos altamente modificados, por ejemplo, brotes con nudos y entrenudos acortados, tallos suculentos y fotosintéticos, hojas de tamaño reducido y posiblemente convertidas en espinas, areolas y flores con tejido de brote cubriéndolas. Estas modificaciones en los órganos y los límites difusos entre ellos complican el reconocimiento de la homología de las estructuras presentes en estas plantas. Gracias a los trabajos realizados en desarrollo floral en Cactaceae en la década de 1960 (Boke, 1964)y todos los realizados por Ronse De Craene en el orden Caryophyllales (Ronse De Craene, 2013), sabemos que las flores en la familia Cactaceae presentan patrones de desarrollo complejos y diferentes a los de sus familias hermanas (Anacampserotaceae y Portulacaceae). Estas diferencias y complejidades observadas lejos de ser un obstáculo en el estudio de las cactáceas nos permiten plantear hipótesis sobre las bases genéticas que subyacen a la morfología tan particular de esta familia.
En esta investigación abordamos diferentes preguntas relacionadas con el origen de la variación morfológica en Cactaceae desde una perspectiva teórica, desde una perspectiva transcriptómica y desde una perspectiva micromorfológica.

El análisis conceptual (Capítulo I), nos permitió reconocer que la *rama-flor*, a pesar de tener un papel muy relevante en la taxonomía y sistemática de Cactaceae, ha sido una estructura poco estudiada desde la perspectiva del desarrollo y menos aún desde la genética del desarrollo. Esto se debe en parte a que estas flores presentan diferentes niveles de complejidad, convirtiéndose en modelos difíciles de estudiar. Una de las complicaciones principales es el tamaño de los botones florales, ya que en los primeros estadios de desarrollo estos son muy pequeños y, por lo tanto, disectar y separar los diferentes tejidos se vuelve complejo; otra dificultad que presentan estas flores son los límites entre los órganos florales, que no siempre están bien definidos, por lo tanto, se vuelve complicado diferenciarlos y definir su homología. A pesar de esta complejidad pudimos plantearnos preguntas sobre la evolución del desarrollo de la *rama-flor* y sobre la identidad de los verticilos que componen a las flores de Cactaceae, y con la información que existe sobre los genes homeóticos y cómo estos interactúan para dar identidad a los verticilos, nosotros planteamos tres hipótesis que podrían explicar los mecanismos genéticos del desarrollo de flores de cactáceas: la primera hipótesis considera que los tépalos están determinados por genes de función

B, la segunda implica gradientes de expresión de genes de función A y genes de función B, similar al modelo propuesto para *Nuphar* y *Persea* (Chanderbali et al., 2010) y finalmente, en la tercera hipótesis los tépalos en Cactaceae podrían ser brácteas pigmentadas, es decir, su desarrollo no se regula por la expresión de genes de función A o de función B. Estas tres hipótesis deberán ser corroboradas con estudios experimentales.

Reconocer la homología de una estructura u órgano es esencial para entender la evolución de un organismo o de un grupo, es por eso, que considero importante el uso de herramientas transcriptómicas, así como el uso de análisis experimentales como los de la acumulación de transcrito por RT-qPCR e hibridación *in situ* de genes que participan en la determinación de identidad de órganos tanto vegetativos como florales, *virus induced gene silencing* (VIGS), y modificación de expresión de genes candidatos en plantas transformadas, las cuales son herramientas que se han sido utilizadas con éxito en especies modelo y que podrían ser útiles en la determinación de homologías en las estructuras que componen a las cactáceas.

Por otro lado, a partir de análisis transcriptómicos, que es el segundo enfoque de este trabajo (Capítulo II), buscamos comparar cambios en patrones de expresión que permitan entender la variación de caracteres morfológicos en las flores de Cactaceae, enfocándonos en el tamaño floral y el número de órganos. El tamaño y el número de órganos en esta familia es un carácter altamente diverso, es posible observar flores pequeñas y grandes distribuidas a lo largo de la familia, sin patrones específicos a nivel filogenético; uno de los ejemplos más contrastantes es el de *Selenicereus*, que puede tener flores pequeñas como las de *S. minutiflorus* que no rebasan los 6 cm de largo (Britton & Rose, 1920) o gigantes como las de *S. undatus* que alcanzan los 30 cm de largo (Britton & Rose, 1920). Las bases genéticas del tamaño de la flor han sido exploradas en especies modelo como *Arabidopsis*, sin embargo, poco se ha estudiado en especies no modelo. Gracias al desarrollo de herramientas genómicas como el RNA-seq podemos hoy estudiar patrones de expresión de genes previamente identificados en especies modelo, y que son relevantes en la determinación del tamaño en plantas no modelo, como las cactáceas. *Disocactus* nos permite explorar patrones de expresión relacionados a la variación morfológica floral porque es un género relativamente pequeño y monofilético que presenta una gran diversidad morfológica floral por ejemplo en colores, en tamaños, en números de verticilos, en periodos de antesis, entre otros (Cruz et al., 2016). El tamaño en flores y en otros órganos vegetales

se ha asociado a procesos multifactoriales: factores intrínsecos, como los genéticos, y factores extrínsecos, como el efecto de las variables climáticas (Powell & Lenhard, 2012), en conjunto estos factores afectan la proliferación y la elongación celular y por lo tanto el tamaño de los órganos vegetales. Estos factores ambientales e intrínsecos son denominados *organ size control checkpoints*, estos pueden actuar tanto en la fase de crecimiento incrementando o decreciendo la capacidad proliferativa y el crecimiento celular, o determinando el tiempo en el cual las células salen de la fase proliferativa y pasan a diferenciación y elongación celular (Bögre et al., 2008). Los análisis transcriptómicos comparativos en flores se han realizado en diferentes especies no modelo (Roberts & Roalson, 2017) (Roberts & Roalson, 2017), sin embargo, en cactáceas nunca se había realizado. *Disocactus speciosus* y *D. eichlamii* fueron usadas en esta investigación para analizar los patrones de expresión involucrados en las diferencias en el tamaño y en el número de verticilos (tépalos y estambres). Realizamos un análisis comparativo a diferentes niveles; en el primer nivel, se compararon los perfiles de expresión de los tejidos que componen al pericarpelo, estructura que surge a partir de un meristemo vegetativo (SAM) (Ramírez-Castro, 2022) y que según los resultados obtenidos por Ramírez-Castro (2022) tiene una identidad caulinar, vs. los órganos florales (tépalos, androceo y gineceo), derivados de un meristemo floral (FM). En el segundo nivel, se compararon los cambios de expresión entre estadios de desarrollo y finalmente, en el tercer nivel, se compararon las diferencias de genes específicos entre las especies. Con nuestros resultados demostramos que los perfiles transcripcionales del perianto y pericarpelo son distintos. Estas variaciones podrían deberse a sus diferentes identidades ontogenéticas ya mencionadas, que han sido discutidas por diversos autores, y que sugieren, por ejemplo, que el pericarpelo es una estructura morfológicamente muy similar a un brote que posiblemente surge desde un SAM vegetativo, mientras que el perianto, así como el androceo y gineceo son órganos florales que surgen del MF y que deben estar determinados por los ortólogos de los genes homeóticos descritos en el modelo ABCE (Coen & Meyerowitz, 1991)Esto se ve reflejado en los perfiles de expresión que son consistentes en las dos especies (Fig. 4B, Capítulo II), porque las muestras de los órganos que surgirían de un FM se agrupan mientras que las del pericarpelo que surge del SAM vegetativo se separan de las primeras. El análisis de expresión diferencial entre estadios de desarrollo también mostró cambios en el número de genes expresados diferencialmente. Sobre todo, se observó que en el

estadio 1 vs. el estadio 2, y en el estadio 2 vs. el estadio 3, el número de transcritos expresados diferencialmente también era mayor y esto fue consistente en las dos especies (Fig. 4, Capítulo II). Los caracteres morfológicos más contrastantes entre las dos especies estudiadas son el tamaño y número de órganos florales (tépalos y estambres). *D. speciosus* tiene una flor que alcanza los 10 cm de largo con 30 a 40 tépalos y cerca de 200 estambres, mientras que *D. eichlamii* alcanza los 5 cm de largo, con 9-12 tépalos y solo 20 estambres. Analizando sus transcriptomas, encontramos que está variación podría deberse a cambios de expresión en factores de transcripción involucrados en la elongación y proliferación celular: los posibles ortólogos de genes como *BIG BROTHER* y *BIG PETAL*, descritos como reguladores negativos de proliferación celular y elongación celular en *A. thaliana*, estarían regulados a la alza en las flores pequeñas de *D. eichlamii*, mientras que en las flores grandes de *D. speciosus BIG BROTHER* presenta valores de expresión más bajos que en la especie de flor pequeña y *BIG PETAL* no se expresa. Aunque resultados similares ya fueron reportados para otras plantas, es la primera vez que se exploran como posibles factores involucrados en el tamaño en flores de cactáceas. Además, el análisis de enriquecimiento mostró que procesos biológicos involucrados en la definición del tamaño celular están sobrerrepresentados en *D. speciosus* que tiene flor grande, mientras que en *D. eichlamii*, especie con flor pequeña, encontramos menos enriquecimiento relacionado a estos procesos (Fig. 5 y 6). Aunado a los análisis transcriptómicos se analizó el tamaño de células epidérmicas de la región media y de la cara adaxial de tépalos en los tres estadios de desarrollo estudiados. Los datos obtenidos sugieren que la variación del tamaño de la flor en estas dos especies se debe a la diferencia en el tamaño de las células, pero también podría deberse a diferencias en el número de las células, el cual no fue evaluado. Las mediciones mostraron que, durante su desarrollo, en la especie con flores pequeñas (5 cm de longitud) las células epidérmicas de tépalos se mantienen en menor tamaño, comparadas con la especie con flores grandes (10 cm de longitud), la cual tiene células de mayor tamaño a partir del segundo estadio del desarrollo. Este resultado coincide con la expresión del posible ortólogo de *BIG PETAL* en *D. eichlamii* el cual codifica un factor de transcripción que en *Arabidopsis* regula de manera negativa el tamaño de las células de los pétalos (Fig. 3). Nuestros resultados sugieren que la expresión diferencial de factores de transcripción relacionados con la proliferación y elongación celular podrían contribuir a la determinación del tamaño de la flor en las especies estudiadas de *Disocactus*. Por

el otro lado, la diferencia del tamaño de la flor podría deberse a la heterocronía entre las dos especies estudiadas. La heterocronía, puede definirse como el cambio en el momento o la velocidad de los eventos de desarrollo, en relación con los mismos eventos en el ancestro (McNamara, 2012). En otras palabras, la heterocronía se puede definir como la diferencia en la tasa de crecimiento y el tiempo de crecimiento, estas diferencias pueden observarse entre organismos de una misma especie, entre especies cercanamente emparentadas o incluso entre partes del mismo organismo (McNamara, 2012). Es por eso por lo que la heterocronía podría explicar las diferencias de tamaño entre *D. eichlamii* y *D. speciosus*, ya que las variaciones en la tasa y duración del crecimiento podrían ser las responsables del contraste en el tamaño final de los órganos florales.

En esta investigación también analizamos la expresión de algunos genes específicos, los cuales en las especies modelo están involucrados en los procesos de desarrollo floral. Sin embargo, las redes de regulación que controlan el tamaño y número de órganos no están resueltas en la familia Cactaceae y es un tema que aún queda por investigar. Por otro lado, tampoco exploramos el papel de las hormonas que han sido estudiadas en modelos como *Arabidopsis* y han mostrado ser relevantes para definir el tamaño final de los órganos; los transcriptomas que ensamblamos *de novo* permitirán realizar este tipo de estudios en el futuro. Una herramienta que considero será de gran utilidad en la comprensión de la identidad de los órganos florales en Cactaceae es la anatomía de flores en estadios tempranos de desarrollo. Esto lo pudimos comprobar con el trabajo "Análisis anatómico del desarrollo del pericarpelo en *Disocactus speciosus* ssp. *speciosus* implicaciones en el desarrollo floral de las cactáceas", proyecto de tesis de licenciatura de Genaro Ramírez Castro (Facultad de Ciencias, UNAM). En este trabajo realizado con técnicas de histología vegetal, demostramos que a partir de una serie de desarrollo en estadios muy tempranos (botones de 1 mm hasta 1 cm de longitud), el pericarpelo de *D. speciosus* se desarrolla a partir de un meristemo apical del brote vegetativo (SAM vegetativo) y por lo tanto su identidad es caulinar, además logramos observar que el tejido más externo del pericarpelo tiene características anatómicas de un parénquima en empalizada, posiblemente originado de las brácteas que recubren a la flor. Además, se demostró que a diferencia de lo que se pensaba antes, la flor en las cactáceas no está hundida en un tallo, sino que está recubierta por este como consecuencia de un desarrollo asincrónico y un crecimiento diferencial de los tejidos que provienen de un SAM de brote vegetativo, del meristemo intercalar, del receptáculo y de los verticilos que se desarrollan del meristemo floral (MF).

Cabe mencionar que a la fecha se han publicados transcriptomas de varias especies de Cactaceae como: el de *Lophophora williamsii* donde se analizaron datos de raíz y tallo, el de *Pachycereus pringlei* que incluye dato de raíz y el de *Selenicereus undatus* que incluyó datos de fruto (Ibarra-Laclette et al., 2015; Li et al., 2022; Rodríguez-Alonso et al., n.d.; Xie et al., 2020). Además, del transcriptoma de *Selenicereus megalanthus* (Xie et al., 2020) que incluye tejido proveniente de

tépalos, y que de acuerdo a nuestro conocimiento ha sido el único que ha incluido datos de flores, por lo tanto, los transcriptomas de esta tesis, que incluyen datos provenientes de los diferentes órganos que componen a la flor en *Disocactus*, se incorporan a los escasos transcriptomas de flores de Cactaceae, dando un total hasta ahora de tres especies analizadas, incluyendo las dos presentadas en esta tesis. Aunque hubiera sido deseable que los transcriptomas ensamblados en este proyecto de investigación fueran realizados a partir de órganos por separado, siguen siendo un gran aporte a la exploración de los perfiles de la expresión génica y su relación con la variación morfológica en flores de esta familia. Además, considero que es necesaria la generación de más datos provenientes de RNA-seq de otras especies de diferentes subfamilias y géneros, así como de múltiples tejidos, órganos y estructuras, incluyendo flores, tallos, areolas y raíces; esto para poder hacer estudios comparativos y generar hipótesis más robustas.

Por otra parte, es necesaria la producción de datos genómicos y sus anotaciones, ya que estos permitirán reconstruir los transcritos considerando el *splicing* alternativo, además de facilitar los análisis transcriptómicos. Aunque las cactáceas son un grupo icónico en México, los estudios genómicos y transcriptómicos en esta familia son escasos, sin embargo son muy importantes no solo para entender la genética del desarrollo y la evolución del grupo, sino también para la conservación de estas plantas (McMahon et al., 2014), por eso considero que sería deseable generar más datos de este tipo y sumarlos a las diferentes investigaciones para complementar el conocimiento en esta familia.

Finalmente, en esta tesis se proponen nuevas preguntas e hipótesis sobre el origen ontogenético de algunos órganos florales en Cactaceae, también se sugiere el papel de genes específicos en la variación en el tamaño y número de órganos. Además, propongo una serie de herramientas que permitirían estudiar a la flor de Cactaceae desde el punto de vista de la evolución del desarrollo con el fin de comprender el origen de la variación morfológica en la familia y a su vez el origen de la diversidad morfológica en angiospermas.

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