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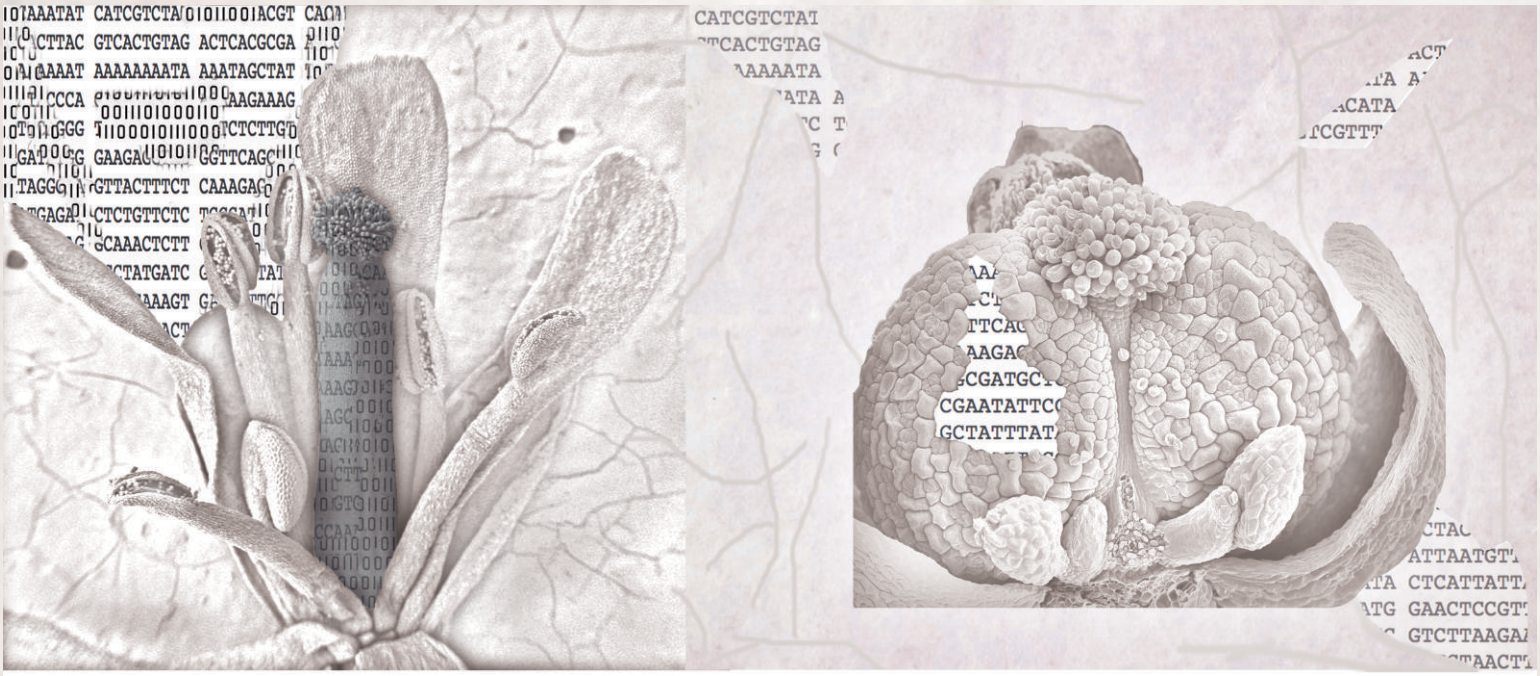

Programa de Doctorado en Biomedicina y Biotecnología

Generation of a toolbox for the study of carpel and fruit development in Brassicaceae

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Como directora de la tesis doctoral "Generation of a toolbox for the study of carpel and fruit development in Brassicaceae", realizada por José Moya Cuevas dentro del Programa de Biomedicina y Biotecnología de la Universitat de València, doy el visto bueno y autorizo la solicitud de depósito de la misma. La tesis consta de tres capítulos, todos ellos centrados en la generación de herramientas moleculares y modelos teóricos para dirigir el estudio de la formación de los gineceos y frutos de especies de la familia de las Brasicáceas desde el análisis genético.

José Moya Cuevas ha realizado un trabajo muy completo, abarcando un gran número y diversidad de metodologías y abordajes, obteniendo resultados muy interesantes que darán lugar, al menos, a tres publicaciones en las que figura como primer autor. Su trabajo tiene la calidad y cantidad necesarias para defender una tesis doctoral.

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“si algún mérito tiene esta obra, a vosotros se debe; en cuanto a los errores, yo soy el único responsable”

Desesperación

Stephen King

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RESUMEN

Las angiospermas son el grupo más grande y diverso de plantas terrestres y, a diferencia de las gimnospermas, el grupo más antiguo de plantas con semillas, sus óvulos están alojados y protegidos durante su desarrollo dentro del gineceo, en lugar de exhibir estructuras generalmente al descubierto. Los carpelos y los frutos son innovaciones evolutivas cruciales de las angiospermas. Estos carpelos facilitan la polinización y, después de la fertilización de los mismos, se transforman en frutos que protegen las semillas en desarrollo y ayudan a su dispersión. Para cumplir estas funciones, los carpelos y los frutos desarrollan tejidos altamente especializados que forman órganos complejos, los cuales se han diversificado enormemente en las plantas con flores. En la flor, podemos encontrar gineceos formados por un solo carpelo, por varios carpelos no fusionados o por una estructura sincárpica de múltiples carpelos fusionados. A pesar de la diversidad morfológica de los gineceos a lo largo de las angiospermas, todas comparten un plan estructural básico, donde *Arabidopsis thaliana*, una pequeña angiosperma dicotiledónea, se ha convertido en el organismo modelo más utilizado para la investigación en biología vegetal tanto básica como aplicada.

Los meristemas florales surgen en la periferia del meristema apical del tallo (SAM) y, posteriormente, los primordios de los órganos florales comienzan a diferenciarse en verticilos. En el centro del meristema floral de Arabidopsis, la fusión congénita de dos carpelos origina finalmente el pistilo o gineceo, el órgano reproductor femenino. Ya en estadio de antesis, los diferentes módulos funcionales están claramente diferenciados. Siguiendo el eje apical-basal, encontramos el estigma, seguido de un estilo relativamente corto, un ovario bilocular y la posición basal está ocupada por un ginóforo de longitud reducida.

El estigma es una capa unicelular de células epidérmicas especializadas con morfología alargada, las cuales forman las papilas estigmáticas; Durante la fertilización, estas papilas están implicadas en la adhesión y germinación del polen. El estigma también representa el comienzo del tracto de transmisión, un tejido que se organiza en células también alargadas y conectadas por plasmodesmos, el cual segrega una matriz extracelular rica en polisacáridos (ECM) y especializada en guiar el crecimiento de los tubos polínicos a través del estilo y el ovario en dirección a los óvulos.

El estilo es un cilindro corto y compacto cuyas células epidérmicas están dispuestas en filas cortas y exhiben depósitos de cera; la parte central consiste en células elongadas axialmente pertenecientes al tejido del tracto de transmisión, a lo largo de las cuales crecen los tubos polínicos, y entre este núcleo central y las células epidérmicas hay un anillo de tejido vascular y tres filas de células de parénquima con clorofila. El origen de los tejidos apicales (estilo y estigma) son mediales, aunque solo ocupan posiciones apicales y muestran simetría radial.

El ovario ocupa la mayor parte de la longitud del gineceo. Externamente, observamos dos valvas que terminan en dos o tres filas de células de menor tamaño, las cuales forman un surco longitudinal llamado margen de valva y son adyacentes al replum en posición medial. En una sección transversal del ovario podemos ver la distribución de los tejidos a lo largo del eje medio-lateral: las valvas, en una posición lateral, corresponden a las dos paredes de los carpelos,

donde se distinguen varias capas de células. El septum se encuentra en una posición central o medial, donde los carpelos se fusionan a lo largo del tracto de transmisión, y junto con la placenta, ambos dividen el ovario internamente en dos lóculos que encierran los óvulos. Estos óvulos son estructuras complejas formadas por un núcleo central que alberga el saco embrionario, dos tegumentos, que envuelven al núcleo y un funículo que los conecta con el tejido de la placenta. Todos los tejidos ováricos muestran polaridad adaxial-abaxial. Por lo tanto, en la zona medial del ovario, el replum se encuentra en posición abaxial, formando una capa estrecha entre los márgenes de valva, mientras que el septum, el tracto de transmisión, la placenta y los óvulos son adaxiales. Las valvas, en posiciones laterales, también muestran polaridad abaxial-adaxial, tal y como se reflejan las diferentes capas celulares que las constituyen.

Por último, el ginóforo, una estructura corta similar a un pedicelo, une el ovario a la base de la flor.

Tras la fertilización de los óvulos, las células del ovario se dividen y expanden, el fruto se alarga y se diferencian una serie de tipos celulares, los cuales son esenciales para una correcta maduración y dispersión óptima de las semillas. El fruto seco y dehiscente de *A. thaliana*, también llamada silicua, es el representante de más de tres mil especies de la familia de las brasicáceas. Esta silicua alargada y cilíndrica, de uno a dos centímetros de largo y un milímetro de ancho, alberga unas cincuenta semillas desarrolladas. Tanto el desarrollo adecuado de las semillas dentro de la silicua, como la diferenciación de los tejidos necesarios para la dehiscencia del fruto, son los principales procesos de desarrollo que se producen después de la fructificación. Esta diferenciación tisular involucra principalmente el margen de la valva y el endocarpio b, que constituye la capa celular interna de las valvas. El margen de valva está entre las valvas y el replum y, una vez que está maduro, se convierte en la zona de dehiscencia (DZ). Esta zona comprende una capa de pequeñas células que forman la capa de separación, y define un plano longitudinal de ruptura a ambos lados del replum, junto con un área adyacente de células lignificadas que se extienden hacia la valva. El endocarpio b también está lignificado y estas dos regiones de células lignificadas proporcionan, cuando el fruto madura se seca, tensiones mecánicas que facilitan la apertura de la silicua.

En los últimos años, la gran diversidad y el potencial de las herramientas genéticas disponibles para Arabidopsis ha hecho posible la identificación de diversos genes implicados en la correcta diferenciación de los tejidos y estructuras que conforman el gineceo, siendo en su mayoría elementos que codifican factores de transcripción (FT). El trabajo de varios grupos de investigación, incluido el nuestro, ha permitido comenzar a esclarecer las GRN que participan en la diferenciación y coordinación de los procesos de desarrollo del carpelo y del fruto. De esta forma, son numerosos los estudios que durante las últimas décadas han centrado el interés de su trabajo tanto en el origen como en la conservación funcional de los módulos de identidad del gineceo. Gracias a la creciente disponibilidad y el desarrollo de nuevas tecnologías de secuenciación de próxima generación (NGS), junto con los enfoques pioneros de la biología de sistemas, se han podido reconstruir filogenias robustas que incluyen un mayor muestreo taxonómico, con el objetivo de proponer nuevas hipótesis evolutivas, así como arrojar más luz sobre las redes de regulación génica

(GRNs) que dirigen procesos de desarrollo clave. Sin embargo, la mayor parte del conocimiento sobre las bases moleculares de la morfogénesis del carpelo y del fruto proviene de estudios genéticos en *A. thaliana*. Si bien el conocimiento acumulativo derivado de estos trabajos está en constante crecimiento, todavía nos encontramos lejos de comprender completamente cómo se integran todos los componentes de las GRNs que dirigen el desarrollo del gineceo, y cómo variaciones en las funciones e interacciones entre estos componentes producen diferentes alteraciones en el desarrollo. Por estas razones, sería necesario complementar estos estudios previos realizando una investigación adicional en especies de diferentes categorías taxonómicas, los cuales todavía son escasos. De ese modo, podemos centrar nuestro interés principal en aquellos elementos que dirigen programas genéticos esenciales, como los procesos morfogenéticos que determinan la estructura del carpelo y el fruto, apertura de la vaina o la formación de estilo y estigma, ya que ambos tejidos solo se encuentran en las angiospermas y están estrechamente relacionados con el origen evolutivo del gineceo.

En esta tesis, nuestro propósito es contribuir a este objetivo general mediante el uso de enfoques alternativos y potencialmente complementarios a los análisis genéticos habitualmente más utilizados. En este contexto, hemos propuesto tres objetivos específicos:

Inicialmente, utilizamos la lógica booleana para modelar la GRN que dirige la diferenciación de la zona de dehiscencia en el fruto de Arabidopsis.

Los frutos se pueden dividir en dos grandes categorías: secos y carnosos. Mientras que los últimos han evolucionado para ser atractivos para los animales que los comen y, por lo tanto, actúan como vectores para la propagación de las semillas, los frutos secos generalmente dependen del viento u otras fuerzas mecánicas para dispersar las semillas. Muchos frutos secos se abren una vez maduros para liberar las semillas directamente al entorno. Para ello, deben someterse al llamado proceso de dehiscencia o rotura de la vaina, que generalmente implica el desarrollo de tejidos especializados, los cuales en última instancia, permiten la apertura controlada del fruto en la etapa óptima de maduración de la semilla. El modo de apertura del fruto es una propiedad ecológica y agronómica de interés para la mejora de los cultivos, por lo que, durante las últimas dos décadas, muchos esfuerzos de investigación se han centrado en comprender en mayor detalle las bases moleculares de este proceso, principalmente en la planta modelo *Arabidopsis thaliana*. En realidad, los componentes maestros de la GRN que dirigen la morfogénesis de la DZ en el fruto de Arabidopsis son bien conocidos desde hace bastante tiempo. Sin embargo, a pesar de la gran cantidad de datos experimentales generados hasta el momento, todavía no entendemos al completo la compleja dinámica que subyace a esta red transversal. Por ejemplo, no solo debemos aclarar las interacciones moleculares a través de las cuales factores como SHATTERPROOF (SHP) o INDEHISCENT (IND) determinan la diferenciación de las capas celulares adyacentes de lignificación y separación, sino también cómo se excluye la expresión de ALCATRAZ (ALC) de la capa de lignificación, limitándose solo a la capa de separación y considerando que no se ha identificado represor alguno hasta la fecha.

Por estas razones entre otras, en este estudio proponemos por primera vez, un modelo mínimo para identificar la GRN compuesta por el conjunto de componentes necesarios y suficientes que nos permitan simular computacionalmente y de manera satisfactoria la diferenciación de los diferentes tejidos que conforman el eje medio-lateral de *A. thaliana*. De este modo, consideramos las redes dinámicas booleanas sincrónicas como una eficiente aproximación mecanicista para proporcionar un marco de trabajo formal y sistémico, mediante la implementación de una herramienta estratégica para la inferencia de redes, la cual se ha utilizado con éxito en diferentes organismos y procesos biológicos. No obstante, a pesar de integrar todos los datos robustamente contrastados y publicados previamente en relación con la formación de la DZ a través de un modelo booleano discreto, estos datos conocidos hasta el momento no fueron suficientes para explicar la aparición de los patrones de expresión que conforman los cuatro destinos celulares esperados. Por lo tanto, estos resultados insatisfactorios nos hicieron plantearnos la necesidad de proponer nuevas interacciones y/o componentes hipotéticos adicionales, así como revisar la literatura reciente para incorporar elementos adicionales a la red. De hecho, cuando incorporamos nuevas interacciones y sometimos nuestra nueva red propuesta a exhaustivas pruebas de validación (líneas de simulación de mutantes de pérdida y ganancia de función, perturbaciones en las funciones booleanas y conversión a un modelo de aproximación continua), fuimos capaces de recuperar en gran medida el comportamiento dinámico esperado de los participantes de la DZ. Este nuevo modelo integrador nos sirvió por tanto para evaluar la robustez de los modelos genéticos actuales, inferidos a partir de información fragmentada, así como identificar posibles inconsistencias o ausencia de elementos necesarios no considerados hasta el momento. Esta estrategia nos permitió proponer un conjunto de reglas hipotéticas junto con la inclusión de un componente adicional, el factor de transcripción NO TRANSMITTING TRACT (NTT), como elemento crucial de esta GRN. Con esta nueva configuración, el modelo propuesto recupera los patrones de desarrollo observados experimentalmente y proporciona nuevas hipótesis a validar en futuros trabajos, lo que en última instancia puede facilitar la manipulación biotecnológica de caracteres en frutos de especies de cultivo de interés comercial.

En segundo lugar, utilizamos una aproximación transcriptómica para identificar elementos regulados por HECATE3 (HEC3), un factor de transcripción clave para el desarrollo del estigma y el tracto de transmisión, que, curiosamente, está altamente relacionado con INDEHISCENT (IND), un gen esencial para la diferenciación de la zona de dehiscencia.

De acuerdo con este enfoque, tres factores de transcripción bHLH estrechamente relacionados, HECATE1 (HEC1), HEC2 y HEC3, con funciones parcialmente redundantes, actúan como centro de integración para controlar diversos procesos del desarrollo a lo largo del ciclo de vida de *A. thaliana* y podrían conservar funciones biológicas similares en otras especies no pertenecientes a la familia de las brasicáceas. Por ejemplo, estos genes HEC desempeñan un papel esencial desde etapas tempranas del desarrollo, actuando como reguladores positivos de la fotomorfogénesis, hasta fases tardías del desarrollo del carpelo y el fruto, lo que propició el ya comentado éxito reproductivo de estas plantas. Así, tanto en el

contexto del SAM como en etapas posteriores del desarrollo del gineceo, HEC1 heterodimeriza con SPATULA (SPT) y ambos actúan como moduladores transcripcionales al regular las actividades antagónicas entre las fitohormonas auxinas y citoquinas. De esta forma, los factores HEC coordinan el ritmo entre la proliferación y diferenciación de las células madre, promoviendo las respuestas a citoquinas en la zona central del SAM y restringiendo el sistema de retroalimentación de auxinas en los flancos. En etapas posteriores del desarrollo, los triples mutantes de pérdida de función, hec1 hec2 hec3, muestran una acusada disminución de la fertilidad como consecuencia de graves defectos en el tracto de transmisión, septum y desarrollo del estigma, semejantes a los descritos en los mutantes spt. Por el contrario, la sobreexpresión de los genes HEC induce la aparición de fenotipos con formación de tejido estigmático ectópico, relacionados con la regulación directa de los transportadores de auxinas PIN-FORMED1 (PIN1) y PIN3, la cual es imprescindible para establecer la polaridad apical-basal y garantizar un correcto cierre apical tanto del estilo como del estigma. HEC y SPT no son los únicos reguladores maestros que especifican la identidad de estilo y estigma, también los factores de transcripción STYLISH (STY) y NGATHA (NGA) promueven la expresión de genes de biosíntesis de auxinas, como YUCCA4, con la consiguiente acumulación de estas en el dominio apical del pistilo. De este modo, los cuádruples mutantes nga de pérdida de función, así como diferentes combinaciones de mutantes de la familia SHORT INTERNODES (SHI)/STYLISH (STY)/SHI RELATED SEQUENCE (SRS), no forman tejidos apicales y muestran esterilidad femenina. En concordancia con estos resultados, los factores NGA y SHI/STY/SRS presentan patrones de expresión comparables y dianas comunes, de modo que solo la sobreexpresión simultánea de NGA3 y STY1 es suficiente para dirigir la formación de tejido estilar ectópico en toda la superficie del ovario. Además, una vez que el fruto está maduro, resulta imperativo el establecimiento de un mínimo local de auxinas para la especificación de la capa de separación del margen de valva a lo largo de la DZ de la silicua. Dentro de la familia de las brasicáceas, los genes similares a HEC son los homólogos más cercanos al factor de transcripción IND, cuya función parece estar conservada en esta familia y es indispensable en la formación de la DZ del fruto. Sin embargo, de acuerdo con los estudios filogenéticos que demuestran que los ortólogos de IND están confinados a la familia de las brasicáceas, cualquier esfuerzo por asignar locus de carácter cuantitativo con genes de tipo HEC, relacionados con el proceso de dehiscencia y fuera de esta familia, ha sido infructuoso. También resulta especialmente llamativo el papel de los genes HEC en Arabidopsis, participando en la deposición de lignina, dehiscencia de la antera o, más concretamente, la función de HEC3 en la abscisión de las semillas. Asimismo, los factores HEC e IND interactúan físicamente con SPT y comparten dianas comunes involucradas en procesos de separación celular como las poligalacturonasas ADPG1 y ADPG2. Por lo tanto, considerando las numerosas evidencias experimentales en las que estas GRN comparten componentes similares a la DZ, parece plausible la hipótesis de la neofuncionalización de IND a partir de ancestros similares a HEC para dirigir la especificación de la DZ, un papel que en otras especies puede depender de genes diferentes a los factores de tipo HEC.

Para comprender mejor el papel de HEC3 en el desarrollo del pistilo, decidimos hacer uso de la secuenciación masiva paralela de ARN (RNA-Seq), una potente tecnología basada en secuenciación masiva de nueva generación (NGS), la cual se

ha implementado con éxito en diversas especies de plantas para la obtención de perfiles transcripcionales. De esta manera, nuestros datos experimentales nos permitieron identificar una subfamilia B-6 de factores de respuesta al etileno (ERF), el clado SHINE (SHN) de factores de transcripción, incluidos dentro de la familia ERF/APETALA2 (AP2), como efectores regulados transcripcionalmente por HEC3 de manera positiva. De esta forma, revelamos una función hasta el momento desconocida de los genes SHN en el desarrollo del tracto de transmisión y ampliamos la información disponible para elucidar la función de HEC en las GRN implicadas en el desarrollo del gineceo.

Finalmente, en el tercer y último capítulo de esta tesis, llevamos a cabo una caracterización del proceso de morfogénesis del gineceo y el fruto, así como un ensamblaje *de novo* del transcriptoma de *Lepidium dydimum*, una especie filogenéticamente cercana a Arabidopsis, pero sin embargo, con una morfología de fruto muy diferente. De hecho, la arquitectura floral de las brasicáceas está mayormente conservada, aunque existe una gran diversidad en la forma de sus frutos, los cuales presentan diseños estructurales relativamente sencillos, con formas cilíndricas, discoidales o esféricas, hasta estructuras más complejas, por ejemplo, con forma de corazón. Las diferencias entre especies cercanas pueden surgir de la fijación de mutaciones de un número relativamente reducido de importantes genes morfogenéticos y las similitudes en la morfología del fruto no siempre están necesariamente vinculadas a la proximidad filogenética, encontrando especies estrechamente relacionadas con frutos muy dispares y viceversa. Esto sugiere que los procesos morfogenéticos que determinan la estructura del carpelo y el fruto exhiben una alta plasticidad y, hasta cierto punto, todavía son vagamente comprendidos, a pesar haberse identificado numerosos genes que dirigen el patrón estructural del fruto en la planta modelo *Arabidopsis thaliana*. Es más, los frutos de Arabidopsis son comparativamente simples en su estructura y, por tanto, un análisis complementario del desarrollo del fruto en parientes cercanos con diferentes morfologías frutales, podría proporcionar un marco de trabajo para profundizar en el estudio de los procesos de determinación de la morfología.

En el género Lepidium, uno de los géneros más numeroso de las brasicáceas con aproximadamente 250 especies, la reducción de órganos es un rasgo distintivo de más de la mitad de todas sus especies, a través de tres mecanismos diferentes, en los cuales la reducción localizada en la función B de los genes de identidad de órganos florales, junto con el aumento de la función C, podrían estar directa o indirectamente involucrados. En estas especies, los pétalos están ausentes y el número de estambres se reduce de seis a dos por flor. Además de su gran variación en la morfología del fruto, las estrategias de dispersión de semillas evolucionaron independientemente de frutos dehiscentes a indehiscentes varias veces dentro del género, en comparación con el típico fruto dehiscente de las brasicáceas, lo que lo convierte en un modelo altamente adecuado para el estudio del mecanismo de dehiscencia. Los análisis funcionales demostraron un alto grado de conservación en las rutas genéticas que dirigen el proceso de dehiscencia del fruto entre *Lepidium campestre* y *Arabidopsis thaliana*, junto con estudios previos que incluyeron especies de brasicáceas distintas de *A. thaliana*.

Una de las especies representantes con dos estambres en este género es *L. didymum* L. (sinónimo: *Coronopus didymus* (L.) Smith), una mala hierba autóctona de América del Sur y ampliamente distribuida en la mayoría de los países lecheros del mundo, que ha causado importantes pérdidas económicas para la industria durante muchos años. Las vacas que ingieren esta maleza producen leche contaminada con propiedades organolépticas desagradables para el consumidor, las cuales no se logran reducir sino que se intensifican mediante las técnicas convencionales de pasteurización al vacío. Además, esta herbácea dispersa miles de valvas de frutos por planta y forma abundantes y persistentes bancos de semillas no latentes en el suelo cultivable y en los pastizales. El conjunto de factores de transcripción, generalmente denominados genes de identidad del margen de valva, son bien conocidos por su función en la correcta especificación de la DZ del fruto. Sin embargo, la transferencia del conocimiento adquirido en los sistemas genéticos de desarrollo en Arabidopsis a otras especies de la familia Brassicaceae, se ve obstaculizada por la falta de recursos genómicos o transcriptómicos disponibles.

En este trabajo, emprendimos pues un doble enfoque para generar nuevas herramientas para el estudio de la diversidad morfológica y la evolución dentro de las brasicáceas, eligiendo a *L. didymum* como una especie modelo novedosa para estudios comparativos de evolución y desarrollo (evo-devo), considerando las notables diferencias con respecto a *A. thaliana*, a pesar de ser especies estrechamente emparentadas. Por un lado, realizamos una caracterización morfológica precisa de la ontogenia floral y el desarrollo del pistilo y el fruto, para revelar las similitudes y diferencias con especies bien estudiadas en la familia, como *A. thaliana*, *Cardamine hirsuta* u otras especies de Lepidium. Nuestra descripción detallada de los eventos de desarrollo que acontecen en los diferentes órganos, así como la disponibilidad de un primer transcriptoma ensamblado, proporcionará el conjunto de herramientas necesarias para emprender análisis genéticos moleculares en esta especie y, por extensión, ayudará a ampliar y profundizar en el conocimiento de los procesos evolutivos que han llevado a la amplia diversidad existente en la morfología del fruto en las brasicáceas. Por otro lado, optamos de nuevo por el uso del RNA-Seq como herramienta ampliamente contrastada, al haberse aplicado con éxito para la secuenciación y ensamblaje de transcriptomas completos, tanto en plantas modelo como en otras especies no consideradas modelo. No obstante, hasta la realización de este estudio, no se había publicado ningún transcriptoma completo de Lepidium. Para el ensamblaje del transcriptoma, aprovechamos la disponibilidad de un genoma secuenciado de una especie estrechamente relacionada, *L. meyenii*, que utilizamos como referencia para ensamblar los transcritos de hojas e inflorescencias de *L. didymum*. A su vez, nuestro ensamblaje podría ayudar a estructurar el genoma, corregir la anotación o refinar los modelos genéticos propuestos para el genoma de *L. meyenii*. Además, recientemente, se logró implementar con éxito una estrategia para estudiar las bases moleculares de la variación morfológica de la hoja entre *Cardamine hirsuta* y *A. thaliana*. Siguiendo un enfoque similar al de estos autores, nuestro ensamblaje del transcriptoma de *L. didymum*, combinado con los abundantes recursos genéticos disponibles para Arabidopsis, permitirá la identificación de los genes homólogos entre ambas especies con un papel importante como reguladores del desarrollo en base a la información ya conocida en *A. thaliana*. Este transcriptoma

debe ser un recurso útil para caracterizar un sistema modelo alternativo destinado a estudiar las bases moleculares subyacentes a los cambios en la morfología y la dehiscencia del fruto, junto con la aportación de los resultados anatómicos obtenidos.

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ABBREVIATIONS

AG	AGAMOUS
AHP6	ARABIDOPSIS HISTIDINE PHOSPHOTRANSFERASE6
ALC	ALCATRAZ
ANT	AINTEGUMENTA
AP2	APETALA2
ARF6	AUXIN RESPONSE FACTOR6
AS	ASYMETRIC LEAVES
BP	BREVIPEDICELLUS
ChIP-Seq	Chromatin Immunoprecipitation Sequencing
CHX	Cycloheximide
CK	Cytokinin
CRC	CRABS CLAW
DEG	Differential Expressed Gene
DET2	DE-ETIOLATED2
DEX	Dexamethasone
DVL	DEVIL
DWF1	DWARF1
DZ	Dehiscence Zone
ECM	Extracellular Matrix
ERF	ETILENE RESPONSE FACTOR
ETT	ETTIN
evo-devo	Evolution and Development
FAE	Formaldehide, Acetic Acid, Ethanol
FDR	False Discovery Rate
FIL	FILAMENTOUS FLOWER
FUL	FRUITFULL
GA	Giberellin
GAG	Glycosaminoglycan
GA3ox1	GIBERELLIN BIOSYNTHETIC ENZYME
GO	Gene Ontology
GOF	Gain-of-function
GR	Glucocorticoid Receptor
GRN	Gene Regulatory Network
HEC	HECATE
HMG	High Mobility Group
IND	INDEHISCENT
JAG	JAGGED
LD	Long day
LL	Lignified Layer
LOF	Loss-of-function
MP	MONOPTEROS
NASC	European Arabidopsis Stock Centre
NGA	NGATHA
NGS	Next Generation Sequencing
NPO	Natural Pericarp Opening
NTT	NO TRANSMITTING TRACT
ov	ovule

PCR	Polymerase Chain Reaction
PFL2	POINTED FIRST LEAF2
PIF	PHYTOCHROME INTERACTING FACTOR
PIN	PIN-FORMED
RNA-Seq	RNA Massive Parallel Sequencing
R	Replum
ROT	ROTUNDIFOLIA
RPL	REPLUMLESS
SAM	Shoot Apical Meristem
SEA	Singular Enrichment Analysis
SEM	Scanning electron Microscopy
SHN	SHINE
SHP	SHATTERPROOF
SHY	SHORT INTERNODES
SL	Separation Layer
sp	septum
SPT	SPATULA
SRS	SHI RELATED SEQUENCE
STY	STYLISH
SWP	STRUWWELPETER
TCP15	TEOSINTE BRANCHED-CYCLOIDEA-PCF15
TF	Transcription Factor
tt	transmitting tract
V	Valve
YAB	YABBY

INTRODUCTION

1. EVOLUTIVE IMPORTANCE OF ANGIOSPERMS GYNOECIUM.

Angiosperms are the largest and most diverse group of terrestrial plants, and conversely to gymnosperms, the oldest group of plants with seeds, their ovules are encased and protected during their development within the gynoecium instead of exhibiting usually naked structures (Ferrandiz et al. 2010). This ovule protection throughout this specialized floral organ is most probably responsible for the evolutionary success of plants with flowers (Scutt et al. 2006). In the flower, we can find gynoecia formed by a single carpel, by several unfused carpels or by a syncarpous structure of multiple fused carpels. Despite the morphological diversity of gynoecia along angiosperms, they all share a basic structural plan, where *Arabidopsis thaliana*, a little dicotyledonous angiosperm, has become the widely chosen organism model for basic and applied plant biology research (Somerville and Koornneef 2002).

Numerous studies during the last decades have focus the interest of their work on the study of both the origin an functional conservation of gynoecium identity modules (J. L. Bowman, Smyth, and Meyerowitz 1989; Bradley et al. 1993; Davies et al. 1999; Dreni et al. 2011; Fourquin and Ferrandiz 2012; Pan et al. 2010; Yellina et al. 2010). Fortunately, taking advantage of the increasing availability and development of new Next Generation Sequences (NGS) technologies (Mortazavi et al. 2008; Zhong Wang, Gerstein, and Snyder 2009; Nagalakshmi et al. 2008; Parchman et al. 2010; Zan Wang et al. 2014) in conjunction with pioneer systems biology approaches (Azpeitia et al. 2011; Wang, Saadatpour, and Albert 2012; Ortiz-Gutiérrez et al. 2015; García-Gómez, Azpeitia, and Álvarez-Buylla 2017), allowed us to reconstruct robust phylogenies including higher taxonomic sampling, aiming to propose novel evolutionary hypotheses (Pabon-Mora, Wong, and Ambrose 2014; Pfannebecker et al. 2017a, 2017b), as well as shed further light on Gene Regulatory Networks (GRNs) directing key development processes, such as pistil morphogenesis, including model and non-model species. However, most of the current knowledge is mainly based on genetic and molecular studies carried out in *Arabidopsis thaliana,* and we are still far away from complete an integrative and comprehensive overall network (Ferrandiz et al. 2010; Reyes-Olalde et al. 2013; Chávez Montes et al. 2015; Schaller, Bishopp, and Kieber 2015; Ballester and Ferrandiz 2017; Marsch-Martínez and de Folter 2016; Weijers and Wagner 2016).

For these reasons, it would be necessary to complement these previous studies by performing additional research on species of different taxonomic categories, which are still scarce, and emphasizing in those elements that direct essential genetic programs, such as morphogenetic processes determining carpel and fruit structure (Gu et al. 1998; Ferrandiz, Pelaz, and Yanofsky 1999; Roeder, Ferrandiz, and Yanofsky 2003; Liljegren et al. 2004; Dinneny, Weigel, and Yanofsky 2005; Balanzá et al. 2006; Alonso-Cantabrana et al. 2007; Trigueros et al. 2009; Seymour et al. 2013; Langowski, Stacey, and Ostergaard 2016), pod shattering (Ballester and Ferrandiz 2017 and references therein) or the formation of style and stigma, since

both tissues are only found in angiosperms and are closely related to the evolutionary origin of the gynoecium (Gomariz-Fernández et al. 2017).

1. 1. THE *ARABIDOPSIS* GYNOECIUM.

A bicarpelar pistil forms the gynoecium of *Arabidopsis thaliana*. In anthesis, the different functional modules are clearly differentiated. In the apical-basal axis, there is stigma, a short style, a bilocular ovary and in the basal position there is a short gynophore (Fig. I1).

The **stigma** is a unicellular layer of specialized elongated epidermal cells that form the stigmatic papillae; during fertilization, the stigma is involved in the adhesion and germination of pollen. The stigma also represents the beginning of the transmission tract (tt), a tissue that is organized in elongated cells connected by plasmodesmata, which secretes an extracellular matrix rich in polysaccharides (ECM) specialized in guiding the downward growth of the pollen tubes through the style and the ovary in direction to the ovules.

The **style** is a short and compact cylinder whose epidermal cells are arranged in short rows and exhibit deposits of wax; the central part consists of axially elongated cells of transmission tract tissue, along which the pollen tubes grow, and between this core and the epidermal cells there is a ring of vascular tissue and three rows of chlorophyll parenchyma cells. The origins of apical tissues (style and stigma) are medial, although they only occupy apical positions and show radial symmetry.

The **ovary** occupies most of the length of the gynoecium. Externally, we observe two valves that end in 2 or 3 rows of smaller cells that form a longitudinal groove called the **valve margin**, and the **replum**. In a cross section of the ovary we can see the distribution of the tissues along the medio-lateral axis: the valves, in a lateral position, correspond to the two carpels walls where several layers of cells are distinguished. The **septum (sp)** is found in a central or medial position, where the carpels merge, along the transmission tract runs through, and together with the placenta, both divides the ovary internally in two locules encasing the **ovules (ov)**. The ovules are complex structures comprissing a central nucleus that contains the embryonic sac; two teguments, which enclose the nucleus; and a stem or funiculus that connects them with the placenta tissue. All ovary tissues show adaxial-abaxial polarity. Thus, in the medial zone of the ovary, the replum is found in the abaxial position, forming a narrow layer between the valve margins, while the septum, tract, placenta and ovules are adaxial. The valves, in lateral positions, also show abaxial-adaxial polarity, which is reflected in the different cellular layers that constitute them (Balanzá et al. 2006).

The **gynophore**, a short structure similar to a pedicel, joins the ovary to the base of the flower.

After fertilization of the ovules, the ovary cells divide and expand, the fruit lengthens and a series of different cell types are differentiated, allowing optimal

maturation and dispersion of the seeds (Ferrándiz 2002; Ferrándiz, Pelaz, and Yanofsky 1999; Robles and Pelaz 2005). The dried and dehiscent fruit of *Arabidopsis thaliana*, also called silique, is representative of more than three thousand species of the Brassicaceae family. This elongated and cylindrical silique, from one to two centimetres in length and one millimetre wide, harbours about fifty developed seeds (J. Bowman 1994).

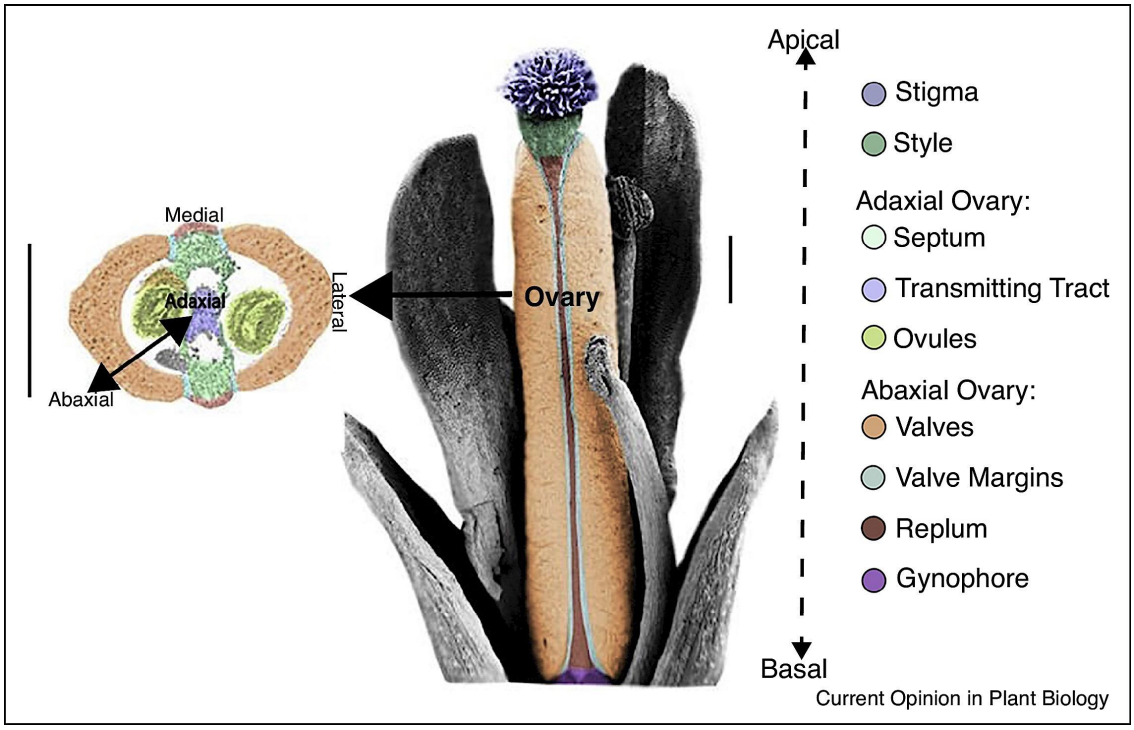


Figure I 1. *Arabidopsis thaliana* gynoecium. The different morphological axes of the gynoecium are shown. The left panel shows a gynoecium cross-section, while the right shows a scanning electron micrograph. Both are false-coloured to describe and distinguish the individual tissues (see colour code on the right). Scale bars, 100 µm. Image from Deb, Bland, and Østergaard (2018).

Both the proper development of the seeds inside the silique as well as the differentiation of the tissues required for fruit dehiscence are the main development processes that occurs after fructification (Robles and Pelaz 2005). This tissue differentiation involves mostly the valve margin and endocarp b, which is the inner cell layer of the valves. The valve margin is between the valves and the replum and once mature it becomes the dehiscence zone. This zone comprises a layer of small cells constituting the separation layer, and defines a longitudinal plane of rupture on both sides of the replum, together with an adjacent area of lignified cells extending towards the valve. Endocarp b is also lignified and these two regions of lignified cells provide, when the mature fruit dries, mechanical stresses that facilitate silique aperture (Ferrándiz 2002). Once the silique is opened, the mechanical force of the wind, rain or physical contact releases the seeds.

2. GRNs Driving Gynoecium Development

In recent years, the vast diversity and potential of the genetic tools available for *Arabidopsis* has made possible the identification of several genes involved in the correct differentiation of the tissues and structures shaping the gynoecium, which mostly encode transcription factors (TFs) (Balanzá et al. 2006; Ferrandiz et al. 2010). The work of several research groups, including ours, has made it possible to begin to unravel the GRNs that takes part in the differentiation and coordination of gynoecium development processes.

2.1 Key Genes Involved in Carpel Identity

The key gene that specifies the identity of the carpel is the MADS-box AGAMOUS (AG), whose role seems to be widely conserved in angiosperms. Once this identity is established, the different GRNs that control the gynoecium development and the differentiation of its functional modules are activated (Sundberg and Ostergaard 2009; Yanofsky et al. 1990). The MADS-box transcription factors SHATTERPROOF 1 (SHP1) and SHATTERPROOF 2 (SHP2) are involved in the specification of the margin identity and stigma (Favaro et al. 2003; Liljegren et al. 2000). SHP1/2 and AG belong to the same clade of MADS genes, being very similar at the sequence level. Other works, such as functional complementation studies, have shown that both SHP and AG proteins are basically redundant and can perform very similar functions (Fig. I2, A, B); however, the fact of their different roles during gynoecium development seems to rely on the differences in their expression patterning (Pinyopich et al. 2003).

Two other transcription factors, the YABBY (YAB) type CRABS CLAW (CRC) and the bHLH SPATULA (SPT), both are necessary for the development of the marginal tissues of the gynoecium (placentas, style, stigma and transmission tract), and seems to act downstream AG and SHP. CRC displays zinc-finger domain and a two alpha-helices homology domain to proteins of the HMG (High Mobility Group) type, which has a crucial function in style development and, to a lesser extent, in the stigma (Bowman et al. 1999). CRC restrains the radial growth of the development gynoecium but promotes its longitudinal growth, while the SPT function is mainly to ensure both the proper transmission tract and the stigma development (Alvarez and Smyth 1999). SPT is expressed in different cell types and organs throughout development and both in reproductive and vegetative structures (Heisler et al. 2001), while CRC is expressed specifically in nectaries and carpel (Bowman et al. 1999). The crc mutant (Fig. I2, C) exhibits absence of nectaries and a shorter and wider gynoecium as compared to the wild type, in which the apical zone is partially merged and underdeveloped (Bowman et al. 1999). In the double mutant crc spt (Fig. I2, D), the gynoecium is completely unmerged and shows a markedly reduction ovaries number and stigmatic and style tissues (Alvarez and Smyth 1999). These phenotypes strongly suggest that CRC and SPT are involved in carpel identity determination as well as AG and SHP (Alvarez and Smyth 1999).

Both SHP and AG appear to be acting at the beginning of the carpel identity pathway, and from this position could directly or indirectly activate SPT and CRC. In addition, CRC has been identified as a direct target of AG (Gomez-Mena et al. 2005). However, less is known about how SPT is regulated.

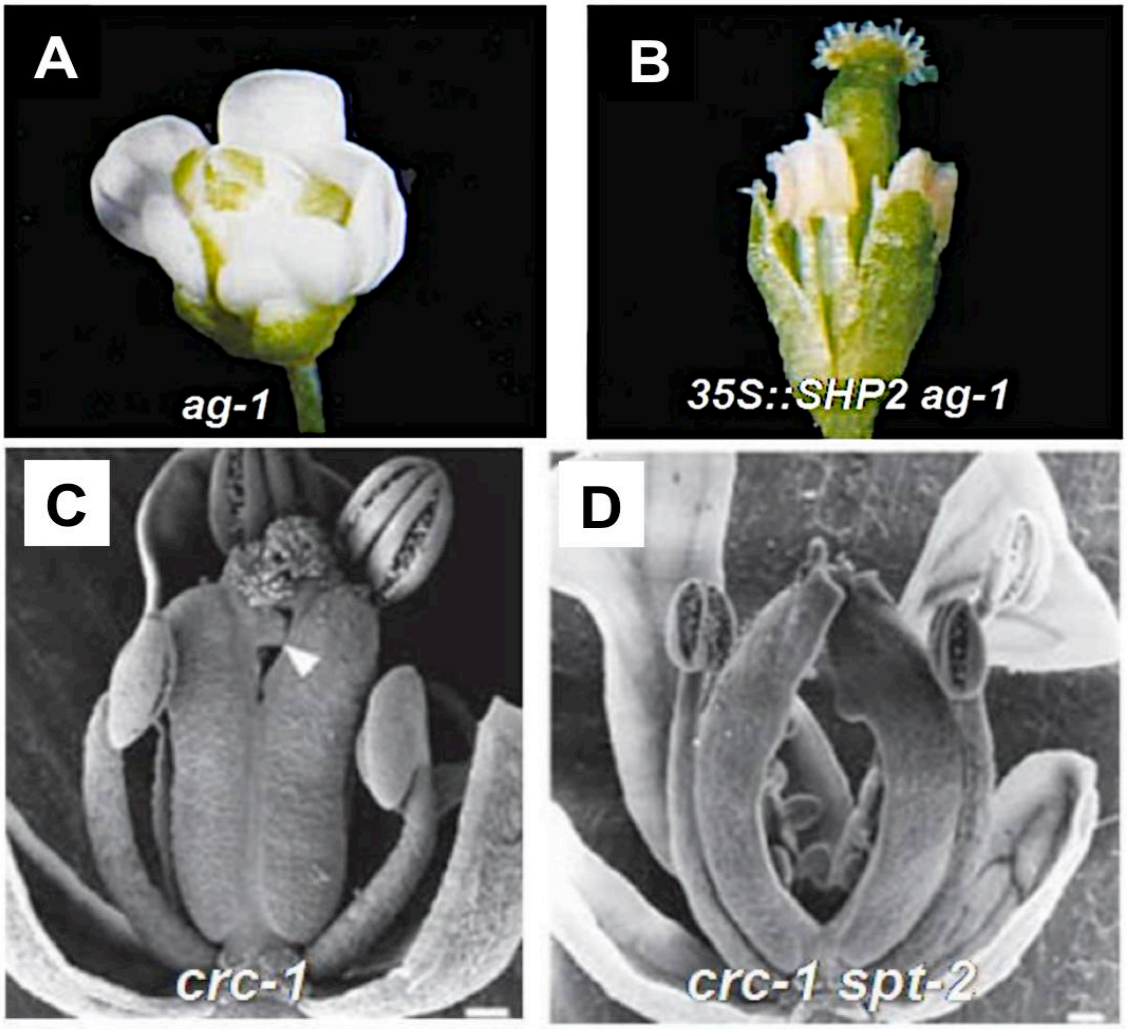


Figure I 2. Loss-of-function and gain-of-function mutant phenotypes of some genes involved in the gynoecium development. A) Low-magnification photograph of an ag-1 mutant flower (Pinyopich et al., 2003). **B)** Low-magnification picture of SHP2 overexpression in ag-1 mutant background (Pinyopich et al., 2003). **C-D)** Scanning Electron Microscope pictures (SEM), **C)** crc-1 mutant flower (E = 100µm) (Álvarez and Smyth, 1999), **D)** Double mutant crc-1 spt-2 flower. (E) = 100µm) (Álvarez and Smyth, 1999).

2.2 Genetic and Hormonal Interactions Establishing Apical-Basal Polarity

The specification of the apical-basal polarity seems to establish once medio-lateral region polarity has been acquired (Larsson et al. 2014; Zúñiga-Mayo et al. 2014). The guided auxin downward flux in the medial domain along the vasculature likely establishes the necessary concentration gradients for the onset of quasi-meristematic state of this territory (Girin, Sorefan, and Østergaard 2009). Moreover, auxin-mediated mechanisms might be involved in the specification of the medial-lateral polarity. The antagonistic action of the cytokinin signalling

repressor ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER-ASE6 (AHP6) in the lateral regions confines the cytokinin promoting action of the bHLH transcription factor, SPATULA (SPT) only to the medial domain (Reyes-Olalde et al. 2017)(Figure I3A).

In the framework of gynoecium development, auxin and cytokinin inputs act as positional information signalling for identity factors to establish accurate spatial expression patterns. Thus, along early gynoecium development, the tissue identity-factor genes such as INDEHISCENT (IND), SHATTERPROOF (SHP) and FRUITFULL (FUL) are expressed in a non-distinct overlapping manner in the gynoecium primordium (Flanagan, Hu, and Ma 1996; Gu et al. 1998; Girin et al. 2011). However, in later stages of gynoecium development, the emergence of precise hormonal distribution patterns allow a very precise expression regions of these genes and facilitate tissue differentiation in their specific domains (Figure I3B). The expression of SPT and HEC genes is confined to the medial domain of the gynoecium by ETT (Gremski, Ditta, and Yanofsky 2007; Heisler et al. 2001), and both SPT and HEC control PIN1 and PIN3 polarity to (Schuster, Gaillochet, and Lohmann 2015) ensure proper auxin-mediated positional information. Moreover, based on cytokinin signalling in the SAM, HEC and SPT may also regulate cytokinin levels to maintain medial boundaries (Figure I3). Moreover, the correct stylar domain expression of both IND and SPT relies on the establishment of positional information provided by an auxin maximum in this apical region (Moubayidin and Østergaard 2014; Girin et al. 2011). So, hormone boundary specification ensures the correct expression of identity determinants. Despite the current knowledge regarding the essential role of the cytokinin-signalling machinery during gynoecium development, most probably genetic redundancy makes difficult to identify specific factors. An example to illustrate these additional mechanisms is the cytokinin-mediated expression of TEOSINTE BRANCHED-CYCLOIDEA-PCF15 (TCP15) in the valves and replum which results in the repression of YUCCA auxin biosynthesis genes and auxin signalling (Figure I3), thus balancing both auxin and cytokinin levels in the medial and apical tissues (Lucero et al. 2015) and the consequent restriction of replum size and over-proliferation of medial tissues.

2.3 GRN along the medio-lateral axis

The master components of the gene regulatory network (GRN) driving the morphogenesis of the dehiscence zone (DZ) in the Arabidopsis fruit have been well known for quite some time. The core of this network can be majorly ascribed only to the concerted action of relatively few transcription factors. In summary, the expression of the functionally redundant MADS-box genes SHATTERPROOF 1 (SHP1) and SHP2, acting upstream and upregulating the basic helix-loop-helix (bHLH) INDEHISCENT (IND) and ALCATRAZ (ALC) factors, is indispensable for proper specification of the DZ in the valve margin territory. Thus, impaired function of SHP or IND results in entirely indehiscent mature fruits, with absence of both separation and lignification layers, whereas *alc* mutants are only deficient for the separation layer formation (Liljegren et al. 2000, 2004; Rajani and

Sundaresan 2001). In addition, two additional regulators act as repressors in the valves and replum respectively, FRUITFULL (FUL), another MADS-box gene, and the homeobox gene REPLUMLESS (RPL), restricting the expression of the SHP/IND/ALC module to the valve margin domain, and completing the basic GRN that substantially explains the emergence of the different cell types characterizing the DZ formation (Liljegren et al. 2004; Ferrandiz, Liljegren, and Yanofsky 2000; Roeder, Ferrandiz, and Yanofsky 2003).

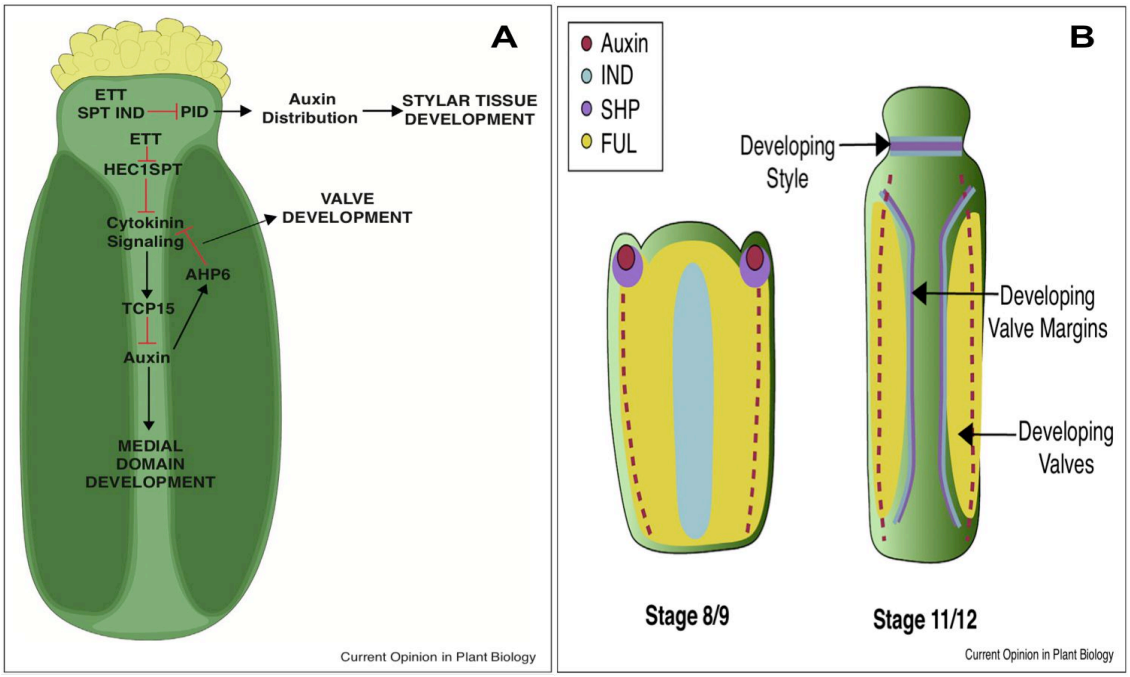


Figure I 3. A) Genetic and hormonal interactions during *Arabidopsis* gynoecium development. Inhibiting interactions are shown with red lines and positive interactions by black arrows. **B)** Overlapping versus distinct expression patterns of Arabidopsis fruit-tissue identity genes at early versus late stages of development. Expression domains for IND (light blue), SHP (purple) and FUL (yellow) are shown at gynoecium stages 8/9 and 11/12 with auxin maxima indicated in red. Modified image from Deb, Bland, and Østergaard (2018).

However, this simplified scenario becomes increasingly complex as we incorporate additional modulators identified in more recent works, which are not essential for DZ specification but seem to modify the extent and positioning of this domain in a partially redundant manner. Within these modulators, replum width is determined by meristem-related factors, acting at the medial domain of the gynoecium, as BREVIPEDICELLUS (BP) (Alonso-Cantabrana et al. 2007). The development of the two lateral pattern elements, valve and valve margin, is directed by the synergistic activity of the previously reported leaf-related genes JAGGED (JAG), FILAMENTOUS FLOWER (FIL) and YABBY3 (YAB3) (Dinneny, Weigel, and Yanofsky 2005). Accordingly, several authors have proposed reciprocal antagonistic activities among medial (BP/RPL) and lateral factors (JAG/FIL) in the gynoecium, mimicking the relationship between genes maintaining the undifferentated state of meristem and genes promoting the differentiation of leaves; in this same context, the ASYMMETRIC LEAVES 1 (AS1) and AS2 genes are expressed in lateral domains and when mutated, cause significant valve reductions and a concomitant replum expansion (Balanzá et al. 2006; González-Reig et al. 2012; Girin, Sorefan, and Østergaard 2009; Sundberg and Ferrándiz 2009).

Another of these recently uncovered newcomers is APETALA2 (AP2), better known as a perianth organ identity specification factor, which fine tune the expression of both DZ (SHP/IND) and replum (RPL/BP) factors to correctly delimit the expansion of these territories (Ripoll et al. 2011). To conclude this overview of experimentally well-supported participants building the elementary scaffold of this medio-lateral network, it is mandatory to consider posttranscriptional regulation as well as the intriguingly role of hormones. Thereby, the combined action of FRUITFULL (FUL) along with AUXIN RESPONSE FACTOR6 (ARF6) and ARF8 activates miR172, thus preventing ectopic AP2 activity in the valves, which results in reminiscent *ful* mutant fruit phenotypes (Ripoll et al. 2015). On the other hand, by directly regulating a discrete number of downstream targets, such as the gibberellin (GA) biosynthetic enzyme GA3ox1, IND promotes the establishment of opposite local hormone gradients, where minimum auxin and cytokinin levels versus a gibberellic acid maximum at the valve/replum boundary are cardinal for proper DZ development and pod shatter (Arnaud et al. 2010; Girin et al. 2011; Marsch-Martínez et al. 2012; Sorefan et al. 2009; Zúñiga-Mayo et al. 2014). In this manner, the separation layer differentiates as a consequence of this increment in GAs at the DZ domain, where IND becomes an indirect activator of ALC by degradation of DELLA repressor proteins, which in turns feedbacks negatively on IND expression levels to prevent consequent IND-promoted lignification (Arnaud et al. 2010; Lenser and Theissen 2013).

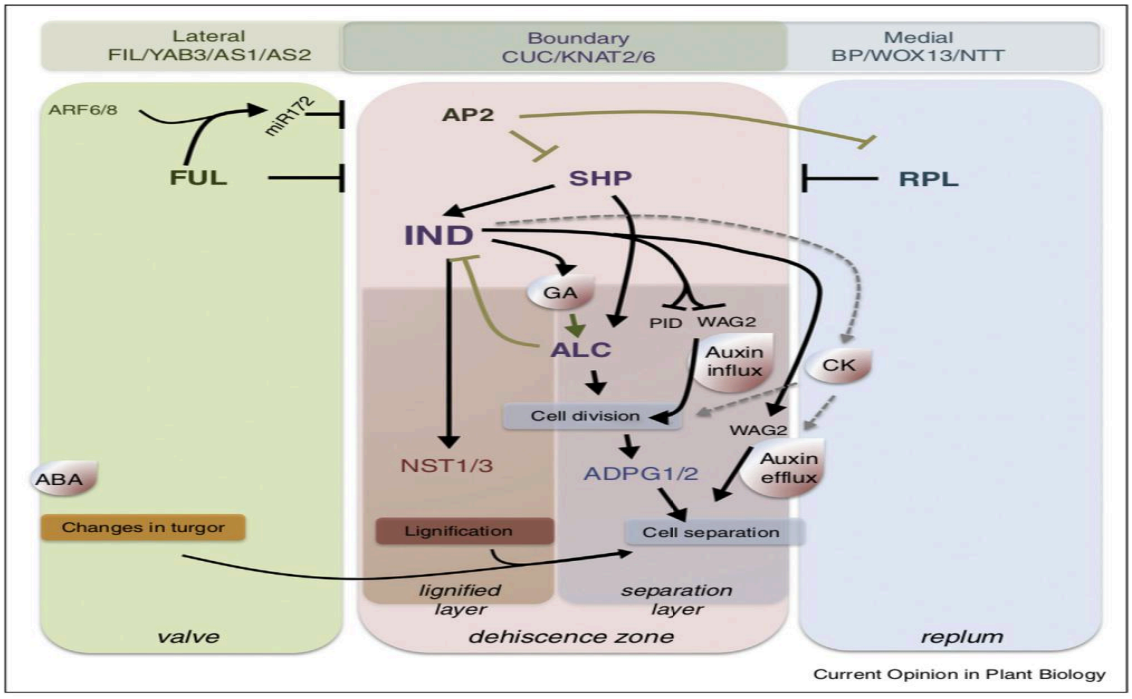


Figure I 4. Updated model of dehiscence zone development in Arabidopsis. The TFs and enzymatic functions with major roles in DZ formation are included, as well as their regulatory interactions. The role of hormones at different levels and how hormone signaling is regulated by the transcriptional network is described. Discontinuous grey arrows note hypothetical relationships not well supported by experimental data. Negative regulations represented by brownish lines indicate a modulating effect on expression levels, but not complete repression.

OBJECTIVES

Most of the knowledge about the molecular basis of carpel and fruit morphogenesis comes from genetic studies in the model species *Arabidopsis thaliana*, a member of the Brassicaceae family. Complex gene regulatory networks (GRNs) have been proposed to explain the development of the different functional domains of carpels in Arabidopsis, which are also the base for comparative development studies within Brassicaceae or in distant clades that aim to explain the basis for fruit morphological and functional evolution. While the cumulative knowledge derived from these works is steadily growing, we still have to fully understand how all the components of the GRNs directing carpel development integrate and how variations in the functions and the interactions of these components result in different developmental outputs. In this thesis, we aim to contribute to this general purpose by using alternative approaches to the most widely used genetic analyses that could complement those. In this context, we have proposed **three specific objectives**:

First, we used Boolean logic to model the GRN directing the differentiation of the dehiscence zone in the Arabidopsis fruit. This model was used to assess the robustness of the current genetic models inferred from fragmented information and to identify potential inconsistencies or missing elements. Eventually, the model will be used to direct new research questions to be addressed in the future.

Second, we used a transcriptomics approach to identify downstream elements regulated by HECATE3 (HEC3), a key factor for the development of the stigma and the transmitting tract, that, interestingly, is highly related to INDEHISCENT, an essential gene for dehiscence zone differentiation.

Finally, we characterized gynoecium and fruit morphogenesis and generated molecular resources (i.e. a de novo assembly of a vegetative and reproductive tissues transcriptome) in *Lepidium dydymum*, a close relative of Arabidopsis with a highly divergent fruit shape and dehiscence. These tools will be the basis for future comparative developmental analyses.

MATERIALS AND METHODS

CHAPTER 1

Boolean Network Model

To study how the dehiscence zone of the *Arabidopsis* fruit is established, we construct a Boolean network from all experimental data available. The network consists of *n* number of genes $x_1, x_2, \ldots, x_n$ and regulatory interactions essential for the establishment of the dehiscence zone of the *Arabidopsis* fruit. Besides regulatory genes, nodes can be also non-coding RNA or proteins such as transcription factors. The edges that connect the nodes stand for the functional relation between two nodes. In Boolean networks, each node has one of two possible states, *'0'* or *OFF* when is been repressed or is inactive, or '*1'* or *ON* if the gene is been expressed or is active. The state of a node changes according to the state of all its regulators in the previous time step, which is generalized with the function $xi(t+1) = F(xi_1(t), xi_2(t) \ldots xi_n(t))$ that also has associated a Boolean function. The set of regulatory interactions included in the model were compiled in the Table 1, and the Boolean functions/logical rules are found in Table 1.

The set of nodes' states in a specific time step, defines the network configuration. All possible transition between network configurations are explored to identify when a configuration is transiting to itself, *i.e.* it is a steady state or *attractor*. When only one-network configurations is visited repeatedly, the attractor is of fixed-point, and when two or more network configurations are visited periodically, the attractor is cyclic. For this model, all nodes' states are updated simultaneously or synchronously.

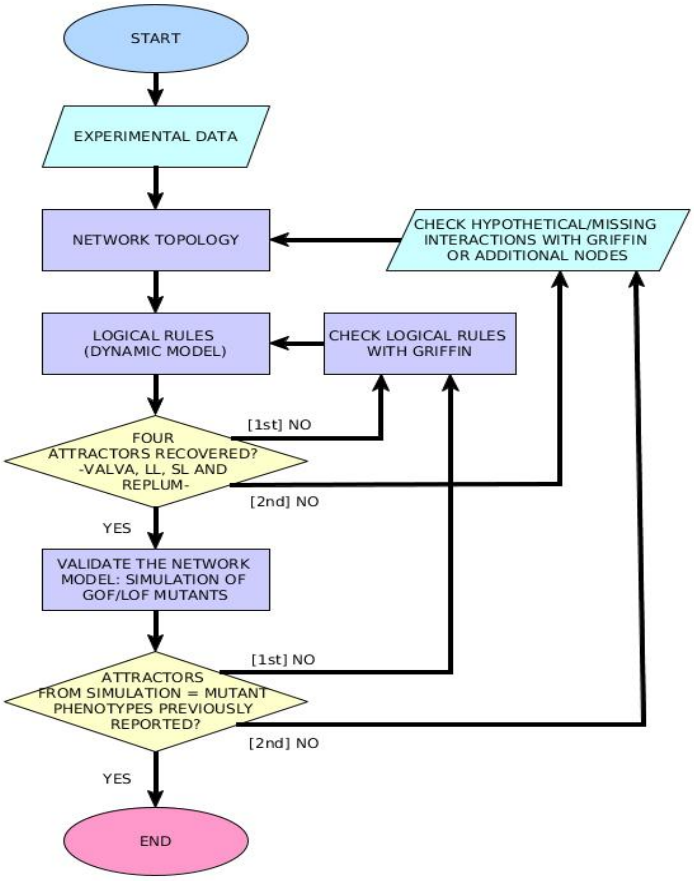


Figure 1. Diagram that summarizes the methodology.

Validation of the model

In order to evaluate the construction of the model, we simulate Loss and Gain of Function mutations (LOF and GOF, respectively) to compare them with the mutant phenotypes reported previously in other studies. The WT and mutant network dynamics were performed with functions of the *BoolNet* (Müssel et al. 2010) package for *R* programming language (R Core Team 2018). For the simulation of the LOF and GOF mutations, the Boolean function of the node is excluded and the node state is set to "*0*" or "*1*", respectively.

Queries for *Griffin*

In some cases, the known regulatory interactions or the set of regulatory genes characterized are not enough to retrieve all the attractors expected for the biological phenomenon under study. Also, it could be that the state of one or several nodes is not well characterized for all attractors. In order to solve these situations, we use *Griffin*, a computational tool that implements several algorithms to enhance the inference of the Boolean networks that satisfy a query (for further details see Muñoz et al. 2018). In particular, we used *Griffin*, first, for verify* if exist a Boolean network with the nodes and interactions known so far that can recover

the four fixed-point attractors of interest, and only these: no additional attractors are allowed (allow.additional.states = false; allow.additional.cycles = false). Second, we asked *Griffin* to find Boolean networks with known and hypothetical interactions that recover the four fixed-point attractors: the set of hypothetical interactions were hypothetical = {NTT->AP2, AP2-|ALC, ALC→NTT, AP2→NTT, IND-|NTT} (allow.additional.states = false; allow.additional.cycles = true; allow.hypotheses = true; topology.iterator.type = radial; topological.distance.radius = 1, 2, 3, 4, 5). The four attractors expected were set without ambiguity (i.e. all nodes' states were specified). It is important to note that *Griffin* only receives the interactions and their sign (-> for positive, -| for negative), while the space of potential Boolean functions is explored.

Continuous model

For the discrete nature of the Boolean networks, and the synchronous update mode used, a continuous version of the model is especially useful to discard artefactual cyclic attractors that can be obtained. However, straightforward methods have been developed to get the continuous approach of the Boolean model. In the continuous version, true* cyclic attractors are conserved while the artefactual ones have steady state dynamics. When we test hypothetical interactions with Griffin expecting exactly four fixed-point attractors, no Boolean networks were found, thus it was necessary to allow cyclic attractors to expand the search (parameter in Griffin allow.additional.cycles = true). In a further complementary analysis, all Boolean networks found with Griffin were filtered to select only those that reach the set of four fixed-point attractors and any number of cyclic attractors that eventually converges to a steady state equivalent to valve, LL, SL or replum attractors.

We attain the continuous version of the model with the methodology described in (Sánchez-Corrales et al. 2010, Di Cara et al. 2007) applied to the Boolean model. In the continuous version, the value of each node is its rate of change determined by the differential equation:

$$\frac{\mathbf{dx_i}}{\mathbf{dt}} = \frac{-\mathbf{e}^{\mathbf{0.5h_i}}+\mathbf{e}^{-\mathbf{h_i}*(\omega_\mathbf{i})}}{(\mathbf{1}-\mathbf{e}^{\mathbf{0.5h_i}})*(\mathbf{1}+\mathbf{e}^{-\mathbf{h_i}*(\omega_\mathbf{i}-\mathbf{0.5})})} - \gamma_\mathbf{i}\mathbf{x_i}$$

The term to the right is the production rate, which has a sigmoidal shape, and the term to the left represents the linear decay of node *xi at* γ_i *rate*. *h* determines the steepness of the production term, being similar to a straight line when h is close to and greater than 0, or resembles to a logistic curve when h is around 50, and it approaches to a step function for values of h close to and greater than 100. The initial conditions were set randomly from a network configuration of each cyclic attractor evaluated. 1 000 random combinations of γ_i's and h's values were tested

for each initial condition, between 1 and 100 for hi, and 1 and 2 for γ_i. ω_i stands for continuous form of the truth table set out the logical rule of node xi, and it is obtained with fuzzy logic as follows:

$x_j(t)$ AND $x_k(t) \rightarrow \min(x_j(t), x_i(t))$

$x_j(t)$ OR $X_k(t) \rightarrow \max(x_j(t), x_i(t))$

NOT $x_j(t) \rightarrow 1 - x_j(j)$

CHAPTER 2

Plant Material

Plants were stratified for 2 days at 4°C after sowing and grown at the greenhouse at 22°C under long-day conditions (LD). Col-0 seeds were obtained from the European Arabidopsis Stock Center (NASC ID N603775) and the *hec1/2/3* allele was previously described in Schuster et al. (2014). miR-SHN1/2/3 seeds were kindly provided by Asaph Aharoni (Shi et al. 2011) (Table M1).

Strain	Usage	Origin/Reference
Escherichia Coli Supercharge EZ10	Vector Cloning	Clontech
Agrobacterium tumefaciens C58 pMP90	Arabidopsis Transformation	(Koncz and Schell 1986)
Genotype	**NASC Code**	**Origin/Reference**
Col-0	N603775	(Redeí 1962)
Genotype	**Ecotype**	**Origin/Reference**
hec 1/2/3	Col-0	(Schuster et al. 2014)
miR-SHN1/2/3	Col-0	(Shi et al. 2011)
35S::HEC3–GR	Col-0	This work
Plasmid	**Features**	**Origin/Reference**
PCR8/GW/TOPO	$Spec^r$, binding sites for primers M13D, M13R, GW1 and GW2	Invitrogen
pMDC32	Kan^r (bacteria) and $Hygro^r$ (Plant)	Invitrogen
pGEM-T Easy	Amp^r, lacZ, binding sites for primers T7, SP6, M13D and M13R	Promega

Table M 1. Bacterial, Plant Material and Plasmids used in Chapter 2

Generation of Inducible Transgenic Lines

Transgenic lines carrying an inducible HEC3 construct were designed as a fusion protein consisting of the full-length HEC3 coding sequence fused to the rat glucocorticoid receptor (GR) domain and driven by the cauliflower mosaic virus 35S promoter (35Spro: HEC3–GR) (Kuusk et al. 2002). Coding sequences were cloned into PCR8/GW/TOPO (Invitrogen) and then transferred by Gateway reactions into the pMCD32 destination vector (Curtis and Grossniklaus 2003). Each vector was introduced into *Agrobacterium tumefaciens* strain PMP90 (Koncz and Schell 1986) for Arabidopsis transformation into the wild type Columbia background using standard floral dipping protocols (Clough and Bent 1998) (Table M2). The GR domain makes the protein cytoplasmic, but it is shuttled to the nucleus upon treatment with the synthetic ligand dexamethasone (DEX) (Lloyd et al. 1994; Schena, Lloyd, and Davis 1991). Transgenic plants were selected based on kanamycin selection. 10 days after bolting (DAB), inflorescences were manually sprayed and once treated with Dexamethasone (DEX) (10 μM) (Sigma), Mock (0.01% ethanol and 0.015% Silwet), a combined solution of DEX (10 μM) and CHX (10 μM) (Sigma), and only CHX, samples were collected three hours later.

Quantitative RT-PCR

Total RNA was extracted from 10 DAB inflorescences using the RNeasy plant mini kit (Qiagen). RNA concentrations were determined by spectrophotometer analysis using a NanoDrop 8000 (Thermo Scientific). 4 ug of total RNA from each sample were reverse transcribed to cDNA with oligo (dT) 20 primer using Superscript III reverse transcriptase (Invitrogen). RT-negative (no enzyme) controls were performed to monitor for contamination with genomic DNA .The cDNA was diluted to 2 ng/uL, and 5 uL of the diluted cDNA was used as template for amplification using SYBR Green PCR Master Mix (Applied Biosystems) on an ABI PRISM 7700 Sequence detection system (Applied Biosystems). Primers targeting *TIP41* were used to normalize the expression data for each gene. The efficiencies in the amplification of the *SHN* genes of interest and the corresponding reference gene were similar. At the end of the experiment, a dissociation kinetics analysis was performed to check the specificity of annealing. Five plants were pooled for each genetic line and three technical replicates were performed. *hec1 hec2 hec3* homozygous plants were selected based on phenotype analysis. See Table M2 for primer sequences used.

Aniline Blue Staining for Arabidopsis Pollen Tubes

Aniline Blue staining for pollen tubes was performed after emasculating flowers just prior to pollination (late stage 12) following the methodology described in Balanzà et al. (2014). We removed any siliques, open flowers, open buds (it is possible to see the stigma poking out through the top of the bud), meristem, and smaller buds from the inflorescence. Emasculated flowers were protected from undesired pollination by covering the pistil. The pollination was performed after 24 h to allow transmitting tract and ovule development to be completed, and then

hand-pollinating them maximally. After allowing 36 hours for pollen growth, they were fixed overnight in absolute ethanol:acetic acid (3:1). The fixing solution was replaced by a softening solution (8 M NaOH) and leave again overnight at room temperature. After softening solution was removed, the pistils were gently washed with distilled water. Finally, we replaced the water with Aniline Blue solution (0.1% w/v Aniline Blue in 0.1M K_2HPO_4-KOH, pH 11) (Jiang et al. 2005) and left the samples 2 h under dark conditions. The pollen tubes were examined under a Leica 5000 optical microscope with standard fluorescence microscope.

Alcian Blue Staining for Arabidopsis Transmitting Tract

Staining with Alcian Blue 8GX is one of the most widely cationic dyes for the demonstration of glycosaminoglycans (GAGs) and mucopolysacharides. We used it to visualize the transmitting tract since these cells secrete a complex extracellular matrix (ECM) very rich in acidic glycoproteins such as arabinogalactans. As a result, non-lignified cell walls are soft blue tonality and ECM cells are identified by an intense blue staining (Scott and Dorling 1965). Paraplast-embedded flowers and inflorescences were transversally sectioned at 8 um and fixed to slides. Slides were then de-waxed with Histoclear (National Diagnostics), rehydrated through a gradual ethanol series, rinsed, stained for 5 minutes with Alcian Blue pH 3.1, rinsed again, dried briefly at 37°C, then mounted directly in Permount (Fischer Scientific).

In situ hybridization.

RNA *in situ* hybridization with digoxigenin-labelled probes was performed as described in Ferrandiz et al. (2000). Tissue was fixed for 2 hours at room temperature in FAE solution (ethanol:acetic acid:formaldehyde:water, 50:5:3.5:41.5, v/v/v/v), dehydrated, embedded and sectioned to 8 µm. After dewaxing in histoclear and rehydration, sections were treated for 20 minutes in 0.2 M HCl, neutralized for 10 minutes in 2× SSC and incubated for 30 minutes with 1 µg/ml Proteinase K at 37°C. Proteinase action was blocked with 5 minutes incubation in 2 mg/ml Gly and 10 minutes postfixation in 4% formaldehyde. Tissue sections were washed in PBS, dehydrated through an ethanol series and dried under vacuum before applying the hybridization solution (100 µg/ml tRNA; 6× SSC; 3% SDS; 50% formamide, containing approx. 100 ng/µl of antisense DIG-labeled RNA probe). For *SHN1*, RNA antisense probes were generated using as substrate a 373-bp fragment of the *SHN1* cDNA (310–682 from ATG), amplified by PCR and cloned into the pGEM-T Easy vector (Promega). Sections were hybridized overnight at 52°C, washed twice for 90 minutes in 2× SSC; 50% formamide at 52°C and the antibody incubation and colour detection was performed according to the manufacturer instructions (Boehringer). Signal was detected as a purple precipitate when viewed under the light microscope. Sequence of *SHN1* probe can be found in Table M2.

Name	Sequence	Purpose	Target Sequence	Orientation
oPBF3	ATGAATAATTATAATATGAACCCAT	HEC3 CDS Amplification	HEC3	F
oJMC2	GATTTTTTTCTTTGTTTTTCGAGCTTCG	HEC3-GR CDS Amplification	HEC3-GR	R
GRfATG	GCCATGGAAGCTCGAAAAACAAAG	GR CDS Amplification	GR	F
oJMC3	TCATTTTTGATGAAACAGAAG	GR CDS Amplification	GR	R
SHN1f	GGGTCGCTGAGATTCGTCA	Real Time RT-PCR (Shi et al. 2011)	SHN1	F
SHN1r	TCGAACGTCCCTAGCCAAAT	Real Time RT-PCR (Shi et al. 2011)	SHN1	R
SHN2f	CCGCCAGCGACAATGG	Real Time RT-PCR (Shi et al. 2011)	SHN2	F
SHN2r	TCGAAAGTTCCAAGCCACACT	Real Time RT-PCR (Shi et al. 2011)	SHN2	R
SHN3f	TGTCCGCCAGCGTCAGT	Real Time RT-PCR (Shi et al. 2011)	SHN3	F
SHN3r	CCGCCGTGTCGAATGTTC	Real Time RT-PCR (Shi et al. 2011)	SHN3	R
qRT-TIP41 F	GTGAAAACTGTTGGAGAGAAGCAA	Real Time RT-PCR (housekeeping gene)	TIP41	F
qRT-TIP41 R	TCAACTGGATACCCTTTCGCA	Real Time RT-PCR (housekeeping gene)	TIP41	R
oSHN1_ISH_310_682_from_ATG	CAGCTTCGTCCACAATGTCATCCTCAACATCATCTTCATCGCTCTCTTCCATCCTCAGCGCCAAACTGAGGAAATGCTGCAAGTCTCCTTCCCCATCCCTCACCTGCCTCCGTCTTGACACAGCCAGCTCCCATATCGGCGTCTGGCAGAAACGGGCCGGTTCAAAGTCTGACTCCAGCTGGGTCATGACGGTGGAGCTAGGTCCCGCAAGCTCCTCCCAAGAGACTACTAGTAAAGCTTCACAAGACGCTATTCTTGCTCCGACCACTGAAGTTGAAATTGGTGGCAGCAGAGAAGAAGTATTGGATGAGGAAGAAAAGGTTGCTTTGCAAATGATAGAGGAGCTTCTCATAACAAACTAAATCTTATTTGC	In Situ Hybridization Probe	SHN1	AS

Table M 2. List of primers used in Chapter 2

Scanning Electron Microscopy

Samples were vacuum infiltrated with FAE (3.7% formaldehyde, 5% acetic acid, 50% ethanol [v/v]) for 10 min and fixed with fresh solution for 16 h at 4°C. Samples were dehydrated in an ethanol series and critical point dried in liquid CO2 (Polaron E300 apparatus). Dried samples were mounted on stubs and coated with gold palladium (4:1) in a Sputter Coater SCD005 (Baltec). Scanning electron microscopy was performed with a JEOL JSM-5410 microscope (10 kV).

Differential expression: RNA-Seq and bioinformatics analysis

2-4 ug of total RNA was purified from *Arabidopsis* inflorescences using the RNeasy Plant Mini Kit (Qiagen) with the RLC buffer following the manufacturer's instructions. Illumina Ribo-zero was used for rRNA removal and the libraries were constructed using the Epicentre ScriptSeq v2 RNA-Seq library preparation kit. Sequencing was carried out by BGI company (Denmark) on the HIseq 4000 100-bp pair-end reads. Sixteen different libraries were prepared and sequenced using a strand-specific protocol and Illumina's sequencing-by-synthesis technology. The sequence alignment and the quantification of gene expression levels were performed as previously described in Mandel et al. (2016) with some modifications. The reads were quality filtered and trimmed using Trimmomatic version 0.36 (Bolger, Lohse, and Usadel 2014) with the following options: -threads 4 -phred33 ILLUMINACLIP: TruSeq3-PE.fa:2:30:10 LEADING: 3 TRAILING: 3 SLIDINGWINDOW: 4:15 MINLEN:36. The resulting reads were then aligned to the TAIR10 version of the Arabidopsis thaliana genome sequence (https://www.arabidopsis.org/) using Hisat2 version 2.1.0 (Kim, Langmead, and Salzberg 2015) with the following options: -p 4 (number of threads) --phred33 --rna-strandness R --dta-cufflinks --no-discordant and default values for all other parameters. The resulting read alignments (in BAM format) were used for transcript quantification with cuffdiff program of the Cufflinks version 2.2.1 (Trapnell et al. 2013) package with the following options: -p 4 --library-type fr-firststrand, masking the rRNA, tRNA, snRNA and snoRNA genes for quantification purposes and default values for the rest of the parameters. Four biological replicates were used for each genotype. The resulting read alignments were visualized and explored using Tablet software (Trapnell et al. 2013) and CummeRbund R package version 2.23.0 (Goff, Trapnell, and Kelley 2012). Differentially expressed genes (DEGs) were subjected to Singular Enrichment Analysis (SEA) for the identification of overrepresented Gene Ontology (GO) terms using agriGO (Berardini et al. 2004) with the default options (statistical test: hypergeometric, multi-test adjustment method: Yekutieli, significance level: 0.01). Using the criteria of twofold up- or down regulation, DEGs were also mapped to metabolic pathways using the KEGG PATHWAY online tool (Aoki-Kinoshita and Kanehisa 2007).

CHAPTER 3

Scanning Electron Microscopy

Samples were collected in the wild from plants growing in the wild and vacuum infiltrated with FAE (3.7% formaldehyde, 5% acetic acid, 50% ethanol [v/v]) for 10 min and fixed with fresh solution for 16 h at 4°C. Samples were dehydrated in an ethanol series and critical point dried in liquid CO2 (Polaron E300 apparatus). Dried samples were mounted on stubs and coated with gold palladium (4:1) in a Sputter Coater SCD005 (Baltec). Scanning electron microscopy was performed with a JEOL JSM-5410 microscope (10 kV).

Lignin Staining

Fruits collected in the wild were fixed in formaldehyde-acetic acid-ethanol overnight and then embedded into paraffin. 12 micrometre sections were stained in a 0.2% (m/v) toluidine blue solution for 2 min and then washed in water. Alternatively, sections were stained in phloroglucinol 2.5% (m/v) for 30 min and then soaked 30 s in 50% HCl (v/v) before being photographed under a Leica 5000 optical microscope.

Histology

Samples for histological analyses, fixed in FAA (3.7% formaldehyde, 5% acetic acid, 50% ethanol) under vacuum and embedded into paraffin. Whole mount samples were stained in 0.1% toluidine blue solution, and observed under a Leica 5000 optical microscope. For the thin-resin sections, histological procedures were as described previously (Carbonell-Bejerano et al. 2010).

Vascular Clearing

Anthesis flowers were collected to compare vascular development in the gynoecium of *L. didymum* with *Arabidopsis* patterning. Samples were fixed (Absolute ethanol: acetic acid (6:1)) overnight at room temperature and then the fixing solution was replaced with absolute ethanol for tissue clearing for 30 min at room temperature. We repeated this step twice. Next, ethanol was replaced with clearing solution (Chloral hydrate: glycerol: H_2O (8 g: 1 mL: 2mL) and samples stayed for 48 hours at room temperature in darkness. Finally, we proceed to observation with a Leica 5000 optical microscope with dark-field illumination (Balanzà et al. (2014).

RNA sequencing and transcriptome assembly

2-4 ug of total RNA was purified from *Arabidopsis* leaves and infrutescences using the RNeasy Plant Mini Kit (Qiagen) with the RLC buffer following the manufacturer's instructions. Illumina Ribo-zero was used for rRNA removal and the libraries were constructed using the Epicentre ScriptSeq v2 RNA-Seq library preparation kit. Sequencing was carried out by BGI company (Denmark) on the HIseq 4000 100-bp pair-end reads. Six different libraries were prepared and sequenced using a strand-specific protocol and Illumina's sequencing-by-synthesis technology. The resulting paired-end reads were processed with several bioinformatic tools in order to improve the overall quality of the raw sequencing data: Rcorrector (Song and Florea 2015) was run with default in order to correct errors in the reads. Cutadapt (v. 1.18) and Trim Galore (v. 0.4.1; https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) were used for adapter trimming (with options --paired --retain_unpaired --phred33 --length 36 -q 5 --stringency 1 -e 0.1), and Trimmomatic (v. 0.36) (Bolger, Lohse, and Usadel 2014) was used for an additional step of quality trimming (with options PE -threads 32 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36). SortMeRNA (v. 2.1b; with options --paired_in -m 20000 -a 30) was used to filter out reads derived from ribosomal RNA molecules (Kopylova, Noe, and Touzet 2012). Reads from the chloroplast genome were filtered out using and Bowtie2 (v. 2.3.4) (Langmead and Salzberg 2012).

High-quality paired-end reads passing all the above steps were aligned to the genome of L. meyenii (Zhang et al. 2016) using Hisat2 (v. 2.1.0) (D. Kim, Langmead, and Salzberg 2015) and then subjected to genome-guided transcriptome assembly using Trinity (v. 2.4.0) (Grabherr et al. 2011; Haas et al. 2013). The resulting transcript assemblies were subsequently processed the with CAP3 (X Huang and Madan 1999) and CD-HIT-EST (Li and Godzik 2006) to eliminate redundant transcripts.

RESULTS AND DISCUSSION

CHAPTER 1

PREFACE

The fruit is a key evolutionary innovation of flowering plants, responsible for protection and dispersal of the developing seeds. Fruits can be divided in two big categories: dry and fleshy. While the later have evolved to be attractive to animals that eat them and thus act as vectors for seed dissemination, dry fruits usually rely on wind or other mechanical forces to disperse the seeds. Many dry fruits open at maturity to release the seeds directly to the environment. For this, they have to undergo the so-called dehiscence or pod shatter process, which usually involves the development of specialized tissues that ultimately allow the controlled opening of the fruit at the optimal stage of seed maturation. The mode of fruit aperture is an important ecological and agronomic trait for crop improvement, so, over the last two decades, many research efforts have focused on understanding in greater depth the molecular basis of this process mainly in the model plant *Arabidopsis thaliana*. Actually, the master components of the gene regulatory network (GRN) driving the morphogenesis of the dehiscence zone (DZ) in the Arabidopsis fruit have been well known for quite some time. The core of this network can be majorly ascribed only to the concerted action of relatively few transcription factors. In summary, the expression of the functionally redundant MADS-box genes SHATTERPROOF 1 (SHP1) and SHP2, acting upstream and upregulating the basic helix-loop-helix (bHLH) INDEHISCENT (IND) and ALCATRAZ (ALC) factors, is indispensable for proper specification of the DZ in the valve margin territory. Thus, impaired function of SHP or IND results in entirely indehiscent mature fruits, with absence of both separation and lignification layers, whereas *alc* mutants are only deficient for the separation layer formation (Liljegren et al. 2000, 2004; Rajani and Sundaresan 2001). In addition, two additional regulators act as repressors in the valves and replum respectively, FRUITFULL (FUL), another MADS-box gene, and the homeobox gene REPLUMLESS (RPL), restricting the expression of the SHP/IND/ALC module to the valve margin domain, and completing the basic GRN that substantially explains the emergence of the different cell types characterizing the DZ formation (Liljegren et al. 2004; Ferrandiz, Liljegren, and Yanofsky 2000; Roeder, Ferrandiz, and Yanofsky 2003). However, this simplified scenario becomes increasingly complex as we incorporate additional modulators identified in more recent works, which are not essential for DZ specification but seem to modify the extent and positioning of this domain in a partially redundant manner. Within these modulators, replum width is determined by meristem-related factors, acting at the medial domain of the gynoecium, as BREVIPEDICELLUS (BP) (Alonso-Cantabrana et al. 2007). The development of the two lateral pattern elements, valve and valve margin, is directed by the synergistic activity of the previously reported leaf-related genes JAGGED (JAG), FILAMENTOUS FLOWER (FIL) and YABBY3 (YAB3) (Dinneny, Weigel, and Yanofsky 2005). Accordingly, several authors have proposed reciprocal antagonistic activities among medial (BP/RPL)

and lateral factors (JAG/FIL) in the gynoecium, mimicking the relationship between genes maintaining the undifferentated state of meristem and genes promoting the differentiation of leaves; in this same context, the ASYMMETRIC LEAVES 1 (AS1) and AS2 genes are expressed in lateral domains and when mutated, cause significant valve reductions and a concomitant replum expansion (Balanzá et al. 2006; González-Reig et al. 2012; Girin, Sorefan, and Østergaard 2009; Sundberg and Ferrándiz 2009). Another of these recently uncovered newcomers is APETALA2 (AP2), better known as a perianth organ identity specification factor, which fine tune the expression of both DZ (SHP/IND) and replum (RPL/BP) factors to correctly delimit the expansion of these territories (Ripoll et al. 2011). To conclude this overview of experimentally well-supported participants building the elementary scaffold of this medio-lateral network, it is mandatory to consider posttranscriptional regulation as well as the intriguingly role of hormones. Thereby, the combined action of FRUITFULL (FUL) along with AUXIN RESPONSE FACTOR6 (ARF6) and ARF8 activates miR172, thus preventing ectopic AP2 activity in the valves, which results in reminiscent *ful* mutant fruit phenotypes (Ripoll et al. 2015). On the other hand, by directly regulating a discrete number of downstream targets, such as the gibberellin (GA) biosynthetic enzyme GA3ox1, IND promotes the establishment of opposite local hormone gradients, where minimum auxin and cytokinin levels versus a gibberellic acid maximum at the valve/replum boundary are cardinal for proper DZ development and pod shatter (Arnaud et al. 2010; Girin et al. 2011; Marsch-Martínez et al. 2012; Sorefan et al. 2009; Zúñiga-Mayo et al. 2014). In this manner, the separation layer differentiates as a consequence of this increment in GAs at the DZ domain, where IND becomes an indirect activator of ALC by degradation of DELLA repressor proteins, which in turns feedbacks negatively on IND expression levels to prevent consequent IND-promoted lignification (Arnaud et al. 2010; Lenser and Theissen 2013). However, despite the exhaustive experimental data generated thus far, the complex dynamics underlying this transversal network is not fully understood. For instance, not only we need to clarify the molecular interactions through which SHP and IND drive the differentiation of lignified and separation layers in neighbouring cell stripes, but also how ALC expression is excluded from the lignification layer and confined only to the separation layer, considering that no repressors have been identified to date. For this reason, in this study we propose, for the first time, a minimal model to uncover the set of necessary and sufficient components and regulatory interactions in order to recover the expected attractors shaping the *A. thaliana* DZ. Hence, we considered synchronous Boolean dynamic networks as an efficient mechanistic approach to provide a systemic and formal working framework, by implementing a strategy tool for network-inference that have been successfully deployed in different organisms and biological processes (Azpeitia et al. 2011; Wang, Saadatpour, and Albert 2012). So, to this end, despite we integrated all the previously published meaningful data related to DZ formation into a discrete Boolean model, the regulatory interactions previously published were not sufficient to explain the emergence of the expression patterns which conform the four different resultant cell fates. Therefore, these unsatisfactory results suggested the need of proposing new additional hypothetical interactions

and/or components, as well as revisiting recent literature to incorporate additional elements to the network. Indeed, when we incorporated novel interactions, and subjected our proposed network to exhaustive validation tests (loss- and gain-of-function simulation lines, perturbations in the Boolean functions and conversion to a continuous approximation model), we largely recovered the expected dynamical behaviour of the DZ participants. Hereby we present the first integrative model to formally tackle the dynamic mechanism of the *A. thaliana* DZ specification and lead the way for future experimental and modelling studies with the aim of deepening the current understanding of the pod shatter process.

RESULTS AND DISCUSSION

Following recent publications that proposed models for GRN directing DZ formation in *Arabidopsis*, we aimed to build a minimal set of nodes corresponding to the genetic factors that were well characterized at the functional level and for which detailed experimental evidence describing expression patterns and molecular interactions was available in wildtype and mutant backgrounds. After extensive literature reviewing, the resulting set of nodes and interactions was compiled as it appears in Table 1 and graphically described in Figure 2. Following published patterns of expression or defined domains of activity, we also could generate a combination of expression profiles in the four functional territories at the medio lateral plane of the fruit: Valve (V), Lignified Layer (LL), Separation Layer (SL) and Replum (R) (Figure 3). This figure represents the observed configuration of the network, in which every of these four domains should correspond to an expected attractor of the model.

Regulator		Target	Description of the interaction	Refs.
JAG/FIL/YAB3	+	FUL	FIL and YAB3 promote *FUL* expression in the valves. In *fil yab3* mutants, FUL expression is absent from valves in both the apical and basal regions. In addition, *FUL* expression decreases in *jag* single mutants with reminiscent *ful* mutant phenotypes, which are accentuated to a greater extent in *jag fil* and *jag fil yab3*$^{+/-}$ fruits. These results suggest redundant JAG activity with FIL and YAB3 to promote *FUL* expression in the valves.	(Dinneny, Weigel and Yanofsky, 2005)
JAG/FIL/YAB3	+	SHP1;2	As it occurs with FUL, in *fil yab3* mutants, *SHP2* expression is lost during early development stages. In a redundant manner, JAG, together with FIL and YAB3, promote *SHP* expression, which is stronger reduced in *jag fil yab3* mutants when compared to *fil yab3* backgrounds.	(Dinneny, Weigel and Yanofsky, 2005)
JAG/FIL/YAB3	-	BP	Phenotypic likeness between loss of function *jag fil* mutants and 35S::BP fruits, as well as the partial suppression of *jag fil*	(González-Reig *et al.*, 2012)

			phenotype in a *bp*, background supports the negative regulation of *BP* by JAG/FIL/YAB3 lateral factors. Besides, an increased and expanded BP::GUS signal, along with higher expression levels of BP in pistils with compromised JAG/FIL activity confirmed these previous evidences.	
JAG/FIL/YAB3	-	RPL	RPL expression level is considerably enhanced in *fil yab3 bp* and *fil jag bp* pistils with respect to the wild type and *bp* genetic backgrounds, despite the low impact of defective BP activity on RPL function, thus revealing the repressive JAG/FILIYAB3 activity on this replum gene.	(González-Reig *et al.*, 2012)
BP	-	JAG/FIL/YAB3	Both decreased *JAG* and *FIL* expression is detected when BP is ectopically expressed. This negative regulation of BP on JAG/FIL activity was further confirmed by qRT-PCR mRNA quantification.	(González-Reig *et al.*, 2012)
AS	-	BP	Ectopic expression of BP is detected in lateral regions of *as1* carpels, together with fruit defects resembling 35S::BP plants. Furthermore, almost complete restoration of wild-type replum and valve size is evident in *as1 bp* fruits.	(Alonso-Cantabrana *et al.*, 2007)
RPL	-	JAG/FIL/YAB3	*rpl* mutation results in the expansion of FIL expression into the replum and its conversion into valve margin. Both impaired JAG or FIL activity in a *rpl* mutant background rescues replum development.	(Dinneny, Weigel and Yanofsky, 2005)
FUL	-	SHP1;2	*ful* mutants show ectopic *SHP1* and *SHP2* expression throughout the valves, contrary to the *SHP* down-regulation detected in 35S::FUL lines. ChIP-seq experiments demonstrate repression of SHP2 by direct FUL binding to CArG boxes located within 1000 bp at the start of the gene.	(Dinneny, Weigel and Yanofsky, 2005)(Ferrándiz, Liljegren and Yanofsky, 2000) (Bemer *et al.*, 2017)
FUL	-	IND	*IND* is ectopically expressed in *ful* mutant fruits and absent in 35S::FUL fruits	
FUL [+ARF6/8]	+	miR172	Physical interaction between FUL and ARF6/8 *in planta* promotes miR172C expression in fruit valves, most probably by directly binding to CArG and AuxREs motifs in the miR172C promoter. Decreased relative transcript levels of MIR172C as well as the dramatic reduction of miR172C::GUS expression in *ful* and *arf6/8* mutant combinations, confirmed the role of both FUL and ARF6/8 as positive regulators of the miR172C activity.	(José Ripoll *et al.*, 2015)
miR172	-	AP2	Proper valve growth depends on post-transcriptional limitation of AP2 activity by miR172 repression. Fruit phenotypes reminiscent of *ful* mutants were observed in transgenic plants expressing a miR172-	(José Ripoll *et al.*, 2015)

			resistant version of AP2 (FUL>>rAP2). Additionally, reduced activity of mature miR172 results in overall reduction of fruit size.	
AP2	-	RPL/BP	An enlarged replum comes up as a result of *ap2* mutation, in conjunction with both a prominent increase in the expression levels of RPL::GUS and BP::GUS reporters and the expansion of their expression domains. This direct or indirect repression is also supported by significantly higher level of RPL and BP transcripts in *ap2* carpels than in the wild-type background. The major role for AP2 as a suppressor of replum overgrowth is further confirmed as *rpl* and *bp* mutations mitigate *ap2* replum defects.	(Ripoll *et al.*, 2011)
AP2	-	SHP	Consistent with the increased size of the lignification layer in the valve margin of *ap2* mutants, the SHP2::GUS expression domain broadens and the higher expression levels in *ap2* mutants suggests that AP2 acts as a negative regulator of SHP activity.	(Ripoll *et al.*, 2011)
AP2	-	IND	The role of AP2 as an IND repressor is suggested by both increased levels and wider domain of *IND::GUS* expression. This repression is not mediated by SHP, since in a*p2 shp1 shp2* mutants IND expression is detected at the valve margin, as opposed to total absence in *shp1 shp2* mutants	(Ripoll *et al.*, 2011)
SHP	+	IND	IND expression is missing in *shp1 shp2* indehiscent fruits, which display remarkable phenotypic similarities to *ind* mutant alleles.	(Ferrándiz, Liljegren and Yanofsky, 2000)(Liljegren *et al.*, 2000) (Liljegren *et al.*, 2004)
SHP	+	ALC	ALC transcripts are not detected at the valve margin of *shp1 shp2* fruits.	(Liljegren *et al.*, 2004)(Rajani and Sundaresan, 2001)
IND	+	ALC	ALC transcripts are not detected at the valve margin of *ind* mutants.	(Liljegren *et al.*, 2000) (Lenser *et al.*, 2013)
ALC	-	IND	In *alc* mutants IND expression is elevated	(Lenser *et al.*, 2013)
NTT	-	FUL	Overexpression of NTT causes a strong reduction of FUL expression in valves	(Chung *et al.*, 2013) (Marsch-Martínez *et al.* 2012)
NTT	+	BP	Overexpression of NTT ectopically activates BP expression	

Table 1. Experimental Interactions and source references. NTT was not included in the first set of nodes used to generate the first version of the Boolean model, but the interactions shown in this table are well established and were used in subsequent steps.

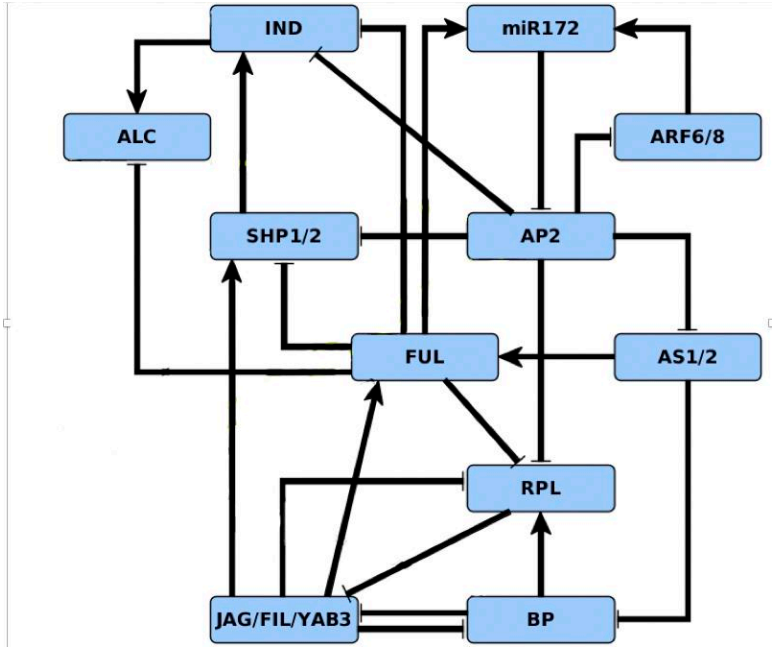
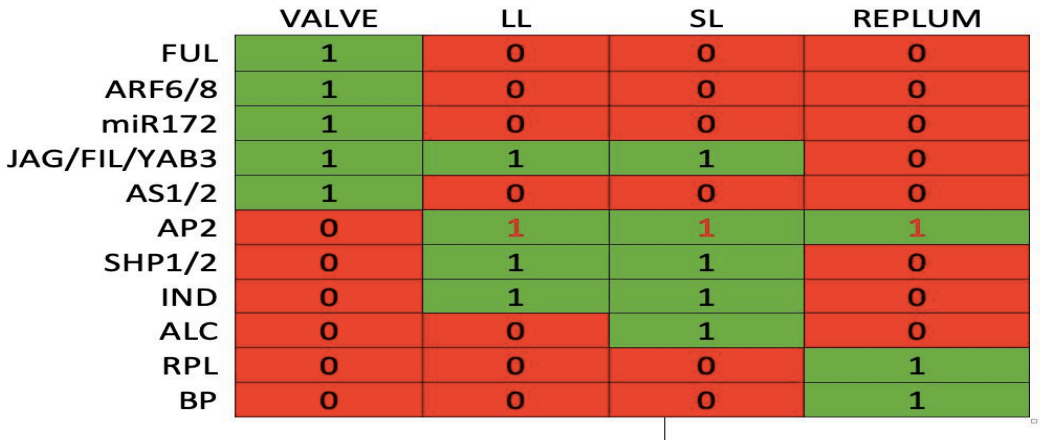


Figure 2. Gene regulatory network.

As presented in figure 2, our starting components are a set of 11 nodes and 23 experimentally validated interactions. If these nodes and interactions were sufficient to explain how the four functional domains required for DZ formation are specified, a boolean model generated with the logical rules derived from these nodes/interactions (Table 2) should recover the expected attractors represented in figure 3. However, after running the corresponding scripts in Boolnet, we were only able to obtain the configuration shown in figure 4. These results indicated that the set of experimental data that we were able to compile was insufficient to explain the genetic mechanisms driving the differentiation of the DZ. In addition, we tried the Griffin tool, which, as described in methods, is able to explore hypothetical interactions between the proposed nodes in an exhaustive way, in an attempt to identify potential missing interactions that could recover the expected attractors. However, Griffin output clearly showed that it was not possible to propose any hypothetical rule that reproduced the experimentally observed configuration (see *Queries for Griffin* in Methods).



	VALVE	LL	SL	REPLUM
FUL	1	0	0	0
ARF6/8	1	0	0	0
miR172	1	0	0	0
JAG/FIL/YAB3	1	1	1	0
AS1/2	1	0	0	0
AP2	0	1	1	1
SHP1/2	0	1	1	0
IND	0	1	1	0
ALC	0	0	1	0
RPL	0	0	0	1
BP	0	0	0	1

Figure 3. Expected attractors. The expression profiles experimentally observed for each component of the proposed network is noted as 1 (green) if present, or 0 (red) if absent. AP2 expression has not been unequivocally charcaterized but appears to be widely distributed. However, since miR172 is present in the valves, where it inhibits AP2 activity, we have considered AP2 inactive only in this domain.

TARGET,	**RULE**
FUL,	JAG/FIL/YAB3 & AS1/2
ARF6/8,	!AP2
miR172,	FUL & ARF6/8
JAG/FILYAB3,	!BP | !RPL
AS1/2,	!AP2
AP2,	!miR172
SHP1/2,	JAG/FIL/YAB3 & (!AP2 | !FUL)
IND,	(SHP1/2 | !AP2) & !FUL
ALC,	IND & !FUL
RPL,	!JAG/FIL/YAB3 & BP & (!FUL | !AP2)
BP,	!JAG/FIL/YAB3 & !AS1/2

Table 2. List of logical rules formulated from the experimental evidence compiled in Table 1. & stands for AND; ! for NOT; | for OR. () goup a condition that has to be fulfilled simultaneously.

Figure 4. Attractors obtained with the experimentally supported dynamical model.

At this point, we considered the possibility of including new interactions and even nodes that could be deduced from experimental data not extensively validated or even conflicting according to different publications, and/or proposed in base of indirect observations or interpretations of phenotypic effects of mutant combinations. In addition, we decided to include in the model the NO TRANSMITTING TRACT (NTT) factor. NTT function in fruit development has been described in different publications. Most interestingly, while *ntt* loss of function does not impact significantly the development of the DZ, replum or valves, NTT overexpression causes a major perturbation of the distribution and identity of these domains, with phenotypes resembling *ful* mutants, a concomitant reduction of FUL expression levels and ectopic expression of BP (See Table 1). However, NTT expression has not been well characterized and in different publications completely different expression patterns in the fruit are reported, making it difficult to adscribe its activity to specific domains in the expected set of attractors. We made use of a NTTpro:NTT:GFP line available in the lab to carefully inspect the localization of the NTT protein, detecting its presence mainly in the SL. Moreover, close inspection of the published characterization of the *ntt* mutant phenotypes in the fruit showed a slight shift of SL to LL in the *ntt* mutants, consistent with a role of NTT in correct SL specification, and thus we decided to include NTT as a functional node in the SL. Additional published studies also showed preferential expression of *NTT* in the replum and, since the positive regulation of BP (a replum factor) by NTT was well established, we also adscribed NTT as a functional node in the replum.

Interestingly, in previous experiments in the lab (Simon, TFM, unpublished) it had been explored a potential role of NTT as a modulator of FIL/YAB3 activity, as it had been observed that NTT and FIL proteins were able to physically interact. Since it has not been satisfactorily resolved how the positive regulatory effect of JAG/FIL/YAB3 on FUL and SHP results in opposite expression patterns of these factors in the valve and the DZ, we hypothesized that NTT could play a role in

establishing this differential output. The activity of FUL and SHP2 promoters were assayed in transient expression analyses in *N. bentamiana* leaves where the FUL or SHP2 reporters were coinfiltrated with different combinations of effectors. In these experiments, it was observed that FIL was able to induce the activity of both FUL and SHP2 promoters, but that when NTT was present, FUL promoter activity was repressed while the FIL+NTT combination resulted in a more efficient activation of the SHP2 promoter activity (Simón, TFM). If NTT is assumed to be excluded from the valves, these results could explain why FUL can be activated by FIL in this domain while repressed in the presence of NTT, that, in the other hand, does not prevent, but even enhances, SHP activation. In the context of our current work, these results also were used to propose more logical rules, namely that JAG/FIL/YAB3 are positive activators of FUL only if NTT is not present and that JAG/FIL/YAB3 are positive activators of SHP if NTT is present.

Finally, based on phenotypic observations, we proposed new hypothetical interactions as follows:

- NTT activates AP2 and/or AP2 activates NTT
- AP2 represses ALC
- ALC activates NTT
- IND represses NTT

We then redrew our starting set of nodes, interactions and expected attractors as shown in figure 5.

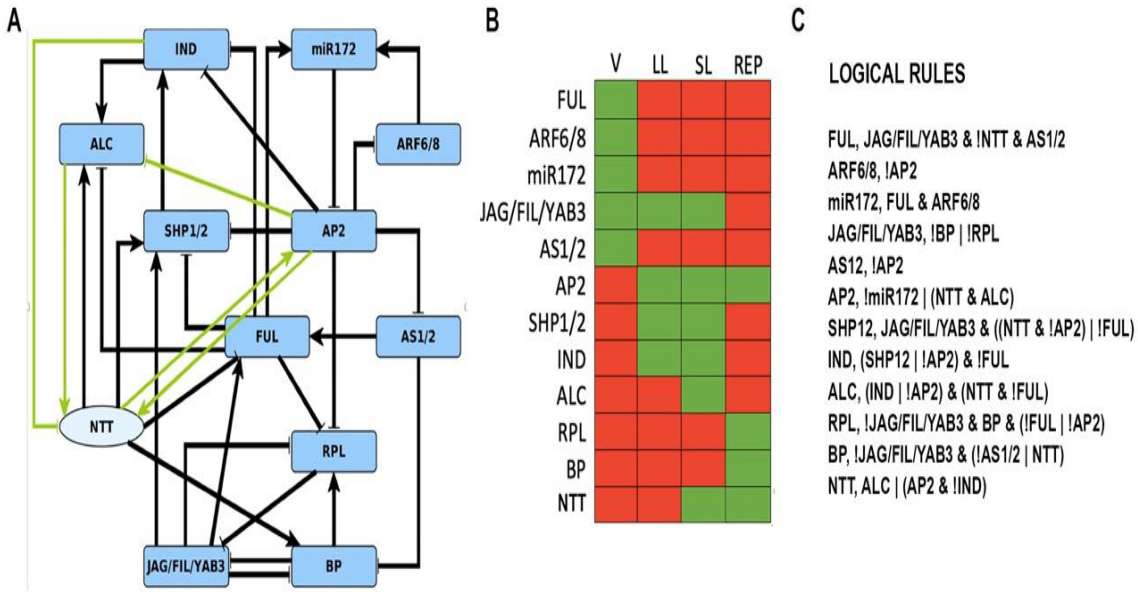


Figure 5. Redrawing of the starting set of nodes, interactions and expected attractors.

When we run Boolnet including NTT and the new list of hypothetical interactions, we were able to obtain the expected set of attractors, indicating that this set of rules is able to explain the emergence of the correct profiles of expression/activity in the four functional domains (Figure 6).

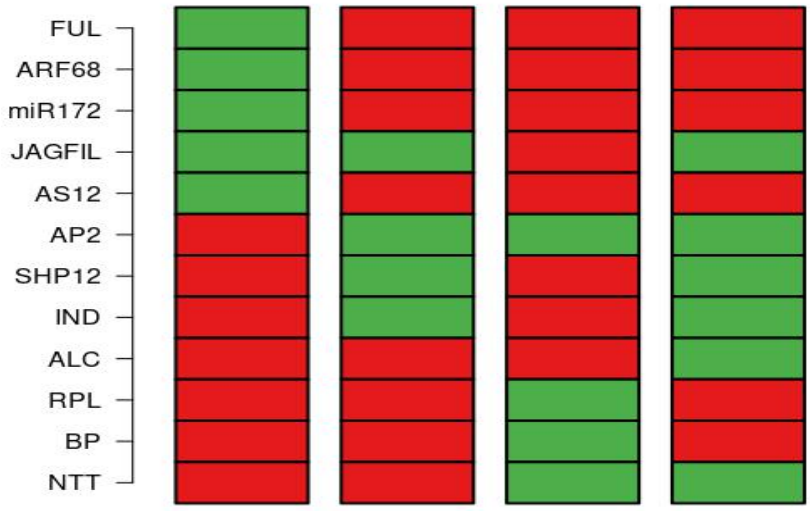


Figure 6. Attractors obtained with the dynamical model complemented with hypothetical interactions.

However, in addition to these four fixed-point attractors, we also obtained a cyclic attractor that does not correspond to any experimentally observed state (Figure 7a). The intrinsic characteristics of the Boolean models is responsible for occasional artifactual outputs, when transitions between the different configurations of the network are less robust and cause that one configuration could revert to a previous one. To distinguish this artifactual behaviour of the model and a real cyclic attractor, it is possible to use a continuous model approach (see methods) to evaluate the dynamic behaviour of the network using as initial condition one of the configurations of the putative cyclic attractor. In figure 7b, it is shown how all nodes in the network reach a stationary state from the 6th time step, approximately, eliminating the cyclic attractor, which corresponds instead to a stationary state equivalent to that of the SL.

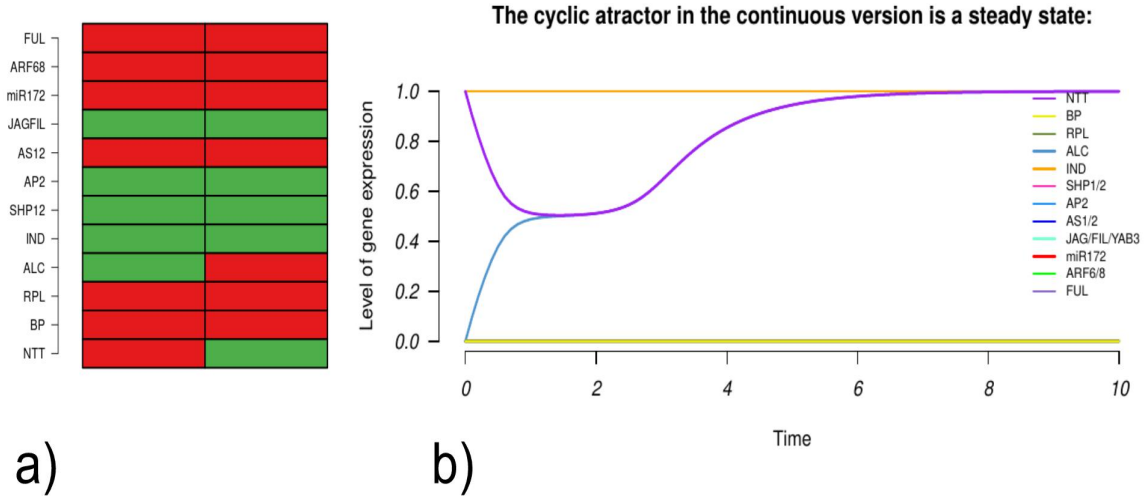


Figure 7. The artefactual cyclic attractor and its equivalence in the continuous version.

It is important to note here that, even though we proposed a minimal number of hypothetical interactions in addition to those experimentally validated, we introduced a highly arbitrary set of logical operators (AND/OR/ONLY/NOT) in the logical rules describing the relationship among the nodes to be able to recover the expected configuration. While they have limited significance at this point, they represent a valuable tool to test new hypothesis and to understand the complexity of the dynamic relationships of the network. Moreover, once the model is generated, it is possible to test in silico these and new hypothesis to simplify the experimental work required to confirm our predictions and to uncover new key elements or interactions required for the correct development of the DZ.

In summary, we have shown that the available contrasted experimental data that has been used to propose a genetic model for the differentiation of the DZ in Arabidopsis is not complete and lacks essential components. In this work, we have been able to propose a set of hypothetical rules and the incorporation of NTT as a crucial component of the GRN directing the differentiation of the DZ in Arabidopsis that is able to recover the observed developmental outputs. The model that we propose here still requires extensive experimental validation that will have to be undertaken in the future and that could lead to confirm or to reformulate our proposal. In any case, it provides a new framework to feed further work in the field and to identify new avenues for biotechnological manipulation of fruit characters in crop species.

CHAPTER 2

PREFACE

The origin of carpels, probably the most important evolutionary innovation of angiosperms, likely responsible for its adaptive success, is linked to the emergence of the defining structure of all their species, the flower. Flower development has been exhaustively studied in *Arabidopsis*. Floral meristems arise at the periphery of the shoot apical meristem (SAM), and subsequently, floral organ primordia start to differentiate arranged in whorls. At the centre of the Arabidopsis floral meristem, the congenital fusion of two carpels eventually originate the pistil or gynoecium, the female reproductive organ. Once mature, the pistil is apically crowned by specialized epidermal cells forming the stigma, whose function is the capture, discrimination and assistance for pollen grain germination. The ovary is the basal chamber, divided longitudinally by a septum, that accommodates the ovules and, after pollination, becomes the fruit and ensures seed maturation and, eventually, seed dispersal (Edlund, Swanson, and Preuss 2004; Ferrandiz, Pelaz, and Yanofsky 1999; Østergaard 2009; Smyth, Bowman, and Meyerowitz 1990; Ferrandiz et al. 2010). The stigma-ovary connection is mediated by the style, an intermediate short tubular structure, harbouring transmitting tract tissues to allow pollen tube downward growth toward the ovules. Thus, this specialized tissue not only requires the secretion by the transmitting tract cells of a complex acidic polysaccharide extracellular matrix (ECM), but also a program of developmentally controlled cell death (Lennon et al. 1998; H. Wang, Wu, and Cheung 1996; Crawford, Ditta, and Yanofsky 2007) and the involvement of numerous chemical gradients and/or signal molecules (Johnson and Preuss 2002; Palanivelu et al. 2003; Palanivelu and Preuss 2006); when transmitting tract development is impaired, pollen tube growth is limited and fertility is reduced (Crawford, Ditta, and Yanofsky 2007).

In recent years, mainly based on genetic and molecular studies carried out in *Arabidopsis thaliana*, complex gene regulatory networks (GRNs) have been proposed to direct the spatial-temporal pistil morphogenesis, and despite the identification of some of the master patterning orchestrators, specially phytohormones and transcription factors, additional next generation sequencing (NGS) data and system biology approaches are useful tools so as to complete an integrative and comprehensive overall network (Ferrandiz et al. 2010; Reyes-Olalde et al. 2013; Chávez Montes et al. 2015; Schaller, Bishopp, and Kieber 2015; Ballester and Ferrandiz 2017; Marsch-Martínez and de Folter 2016; Weijers and Wagner 2016).

According to this approach, the closely related bHLH transcription factors HECATE1 (HEC1), HEC2 and HEC3, with partially overlapping functions, act as an integration hub to control diverse developmental processes throughout the life cycle of *A. thaliana* and might conserve similar biological roles in other non-Brassicaceae species (Gremski, Ditta, and Yanofsky 2007; Schuster, Gaillochet, and

Lohmann 2015; Zhu et al. 2016; Ballester and Ferrandiz 2017; Gaillochet et al. 2017). Thus, these *HEC* genes play an essential role from early development stages, as positive photomorphogenesis regulators by interacting with the PHYTOCHROME INTERACTING FACTORS PIF1 and PIF3 (Zhu et al. 2016), to late roles in carpel and fruit development, consequently impacting reproductive success of the plant. As above-mentioned, phytohormones, such as auxin, play a determinant role in flower morphogenesis (Weijers, Nemhauser, and Yang 2018 and references therein). In the context of the shoot apical meristem (SAM), HEC factors coordinate the timing between stem cell proliferation and differentiation, while antagonising niche cell activity through physical interaction with SPATULA (SPT), thus promoting cytokinin responses at the central zone of the SAM and restraining the auxin feedback system at the flanks by transcriptional regulation and physical interaction with AUXIN RESPONSE FACTOR 5 (ARF5)/ MONOPTEROS (MP) (Schuster et al. 2014; Schuster, Gaillochet, and Lohmann 2015; Gaillochet et al. 2017).

In later development stages, loss-of-function in *hec1 hec2 hec3* triple mutants exhibit decreased fertility as a consequence of dramatic defects in the transmitting tract, septum and stigma development (Gremski, Ditta, and Yanofsky 2007), resembling those of the *spt* mutants (Heisler et al. 2001). On the contrary, overexpression of *HEC* genes induces the emergence of pin-like phenotypes with ectopic stigmatic tissue formation (Gremski, Ditta, and Yanofsky 2007), related to the HEC1 direct regulation of auxin efflux carriers PIN-FORMED 1 (PIN1) and PIN3, which is imperative to establish apical-basal polarity and ensure a correct style and stigma apical closure (Sundberg and Ostergaard 2009; Larsson, Franks, and Sundberg 2013; Larsson et al. 2014; Moubayidin and Østergaard 2014; Schuster, Gaillochet, and Lohmann 2015). In fact, as it occurs in the SAM, HEC1 heterodimerize with SPT, and both transcriptional modulators buffer the antagonistic activities between auxin and cytokinin phytohormones during gynoecium development, as specifically shown by *hec1 hec2 hec3* hypersensitivity to cytokinin during gynoecium development (Schuster, Gaillochet, and Lohmann 2015). HEC and SPT are not the only master regulators specifying style and stigma identity, also the STYLISH (STY) and NGATHA (NGA) transcription factors promote the expression of auxin biosynthesis genes, such as YUCCA4, with the resultant accumulation of auxin in the apical domain of the pistil (Eklund et al. 2010; Martinez-Fernandez et al. 2014). Thereby, the *nga* quadruple mutants, four redundant members of the RAV clade of the B3-domain transcription factor family, as well as different mutant combinations in the SHORT INTERNODES (SHI)/ STYLISH(STY)/ SHI RELATED SEQUENCE (SRS) family of zinc-finger transcription factors, share almost identical phenotypes, failing to form apical tissues and showing impaired female sterility (J. P. Alvarez et al. 2009; Trigueros et al. 2009; Kuusk et al. 2002, 2006). Concordantly, NGA and SHI/STY/SRS factors display comparable expression patterns and coincident targets, so that just simultaneous overexpression of *NGA3* and *STY1* is sufficient to direct ectopic style tissue formation on the entire surface of the ovary (Kuusk et al. 2002, 2006; Sohlberg et al. 2006; Trigueros et al. 2009; Ståldal et al. 2012; Martinez-Fernandez et al. 2014).

Once the fruit is mature, a local auxin minimum is essential for the specification of the valve margin separation layer along fruit dehiscence takes place (Sorefan et al. 2009). Within Brassicaceae, HEC-like genes are the closest homologs to the bHLH transcription factor INDEHISCENT (IND), which indispensable role in dehiscence zone (DZ) formation appears to be conserved in this family (Lenser and Theissen 2013; Girin et al. 2010; Kay et al. 2013). However, in agreement with phylogenetic studies proving IND orthologs confinement to the Brassicaceae, any effort to assign dehiscence-related quantitative trait loci with HEC-like genes outside this family has been fruitless (Pabon-Mora, Wong, and Ambrose 2014; Dong et al. 2014; Gioia et al. 2013). Strikingly, in *Arabidopsis*, *HEC* genes participate in lignin deposition or anther dehiscence (Kay et al. 2013; Balanza et al. 2016), and more specifically, HEC3 is required for seed abscission (Gremski, Ditta, and Yanofsky 2007; Ogawa et al. 2009; Balanza et al. 2016) and both HEC and IND factors interact physically with SPT (Gremski, Ditta, and Yanofsky 2007; Girin et al. 2011; Groszmann et al. 2011), and share common downstream targets involved in cell separation processes, such as the polygalacturonases ADPG1 and ADPG2 (Ogawa et al. 2009). So, considering multiple experimental evidences where these GRNs share similar participants to the DZ, it seems plausible the hypothesis of IND neofunctionalization from similar HEC ancestors to drive DZ specification, a role that in other species may rely on different genes than HEC-like factors.

To better understand the role of HEC3 in pistil development, in this work we propose a transcriptomics approach using RNA-Seq, a powerful NGS-based technology for transcription profiling which has been successfully implemented in both model and non-model plants (Mortazavi et al. 2008; Zhong Wang, Gerstein, and Snyder 2009; Nagalakshmi et al. 2008; Parchman et al. 2010; Zan Wang et al. 2014). Our experimental data allowed us to identify the SHINE (SHN) clade of AP2 domain transcription factors (Aharoni et al. 2004; Shi et al. 2011) as downstream effectors positively regulated by HEC3. We uncovered a novel role of SHN genes as crucial components in the development of the transmitting tract and provide deeper insights to decode the polyvalent HEC function in the GRNs of gynoecium development.

RESULTS

35Spro: HEC3–GR Inducible Lines display resembling phenotypes to those of 35Spro: HEC3

Transcriptomic analyses were performed on 35Spro::HEC3-GR lines once we validated the quality of these lines by assessing the phenotypic effecs of inducing HEC3-GR overexpression. For this validations, inflorescences of several lines harbouring a single insertion of the 35Spro::HEC3-GR transgene in homozygosis were manually sprayed every three days for 5 times, from approximately one week after bolting, with Dexamethasone or Mock solution for control groups and fruits were subsequently observed at the dissecting scope one week after the last treatment.

The resulting phenotypes in response to DEX induction were very similar to those reported for constitutively overexpression of HEC3 (Gremski, Ditta, and Yanofsky 2007), showing growth defects and ectopic production of stigmatic tissue among other morphological defects (Figure 8), while plants sprayed with mock solution were indistinguishable from wild type. Thereby, we confirmed that the transgene was functional and our induction experiments worked successfully.

Figure 8. Phenotype comparison between inducible 35S:HEC3-GR and constitutively overexpressing fruits of 35S:HEC3, both compared with mock control treatment. The resulting phenotypes in response to DEX induction are very similar to those reported for constitutively overexpression of HEC3, showing growth defects and ectopic stigma tissue formation among other morphological alterations.

Differential Gene Expression (DEG) of *Arabidopsis thaliana* in Response to HEC3 overexpression

To reveal overall transcriptomics changes and to identify genes with altered transcriptional activity in response to HEC3 overexpression, we sequenced RNA samples from inflorescences of our *Arabidopsis* inducible lines (35Spro: HEC3–GR) treated a single time with DEX, a combined solution of DEX and Cycloheximide (CHX) and only mock or CHX solutions for control groups respectively, collecting the samples three hours after treatment. Four biological replicates were processed for each of the four treatments. We decided to include an induction experiment in the presence of CHX to obtain an approximate idea about the putative direct targets out of the total DEGs, although we were aware of the likely presence of artifactual results. Upon DEX treatment in 35Spro::HEC3 lines, we found 3615 DEGs at a false discovery rate (FDR) threshold of 5%. Of these, 1874 were up-

regulated and 1741 down-regulated (Figure 9A). On the other hand, twice the number of DEGS were identified in response to the combined action of DEX together with CHX treatment (7385 genes). When we used different stricter criteria of twofold up- or down-regulation [Log_2FC (fold change) 1 or −1] in absence of CHX (Mock vs DEX), these numbers decreased up to 101 DEGs up-regulated and 52 down-regulated. The number of so-called high confidence genes, those that overlap when comparing DEGS in response to both treatments (presence or absence of CHX) amounts to 1894 genes (20.8%)(Fig 9B).

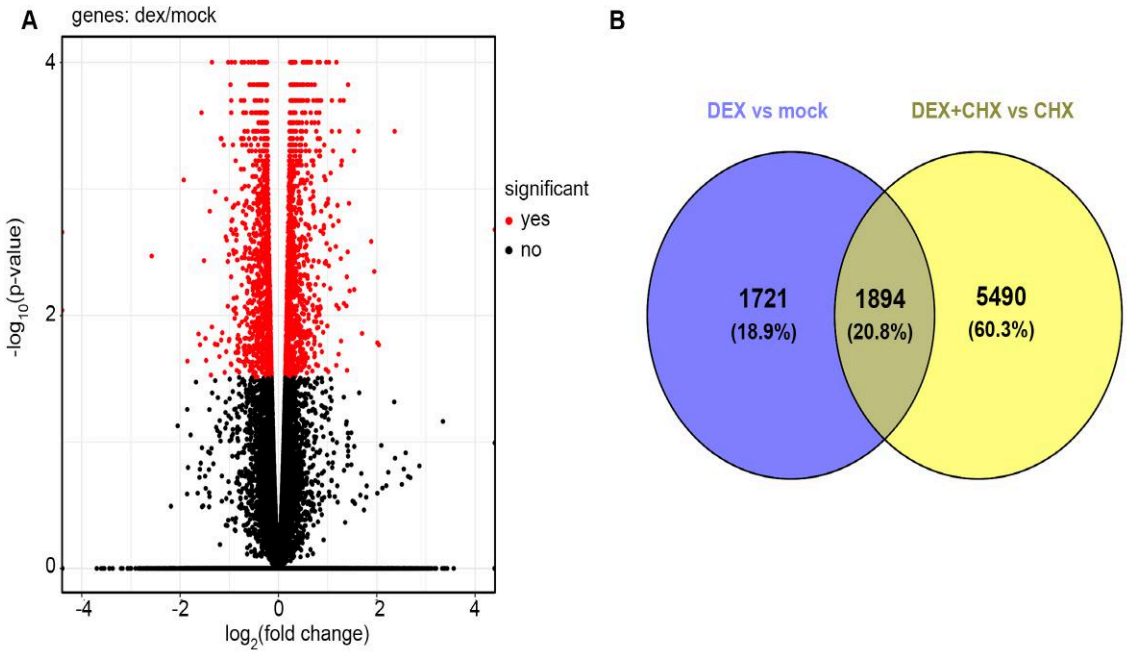


Figure 9. A) Volcano plot of the differentially expressed genes (DEGs) between the control (MOCK) and dexamethasone (DEX) treatment. B) High-confidence HEC3 early response genes as shown by overlap of 1894 DEGs between 35S:HEC3-GR RNA-seq experiments 3 h after dexamethasone DEX treatment in the presence or absence of the protein synthesis inhibitor Cycloheximide (CHX).

Gene Ontology (GO) Analysis of the Functional Annotation and Classification of the DEGs

DEGs from our DEX treatment were used as objects to perform singular enrichment analysis (SEA) for the GO terms assigned to the complete set of GO significantly regulated genes to identify the HEC3 response related functional processes in *A. thaliana* inflorescences.

31 GO terms in the 'cellular component' subontology were significantly enriched. The most significantly enriched term was 'cell periphery' (GO:0071944), as 22.6% out of the 811 genes containing this term in the background set (3586 reference

TAIR genome locus, TAIR10 2017) were differentially expressed. This term was followed by other highly significantly enriched terms related to cell matrix, such as 'extracellular region' (GO:005576), 'plasma membrane' (GO:005886), 'external encapsulating structure' (GO:0030312), 'cell wall' (GO:005618) and 'plant-type cell wall' (GO:0009505) (Fig 10A).

184 GO terms in the 'biological process' subontology were significantly enriched. The most significantly enriched term was 'metabolic process' (GO: 0008152) as 48.5 % out of the 1738 genes containing this term in the TAIR background set. Some of the highest representative metabolic processes were 'carbohydrate metabolic process' (GO:0005975), 'lipid metabolic process' (GO:0006629) and 'organic substance metabolic process' (GO:0017704). In line with the most significantly enriched terms in the 'cellular component' subontology, the prominent second most enriched term was 'single-organism process' as 44.5% out of the 1597 genes were also found within our reference set. Within this term the most enriched terms were 'single-organism cellular process' (GO:0044763), 'external encapsulating structure organization' (GO:0045229) and 'cell wall organization' (GO:0071555). Moreover, as expected for exogenous chemical treatments, we also identified other markedly enriched terms such as 'response to chemical' (GO:0042221), 'response to abiotic stimulus' (GO:0009628) or 'response to stress' (GO:0006950) among others.

To conclude our GO analysis, 52 terms were significantly enriched in the 'molecular function' subontology. This analysis provided us with more specific information about HEC3 transcription factor activity. In agreement with the results from 'biological process' and 'cellular component' subontology, as well as already known roles of this bHLH transcription factor, the most significantly enriched term was 'catalytic activity' (GO: 0003824), with 40.1% gene representation, including terms as 'oxidoreductase activity' (GO: 0016491), 'hydrolase activity' (GO: 0016787) or 'transferase activity' (GO: 0016740). And finally, and not surprisingly, the more enriched second and third most enriched terms were 'protein binding' (GO: 0005515) and 'nucleic acid binding transcription factor activity' (GO: 0001071) with enrichments of 16% and 8.5% assignment.

KEGG Pathway Analysis of the HEC3 Overexpression Responsive Genes

To further determine the specific metabolic pathways affected in HEC3 overexpression plants, we mapped our RNA-Seq DEGs against the KEGG pathway online tool database (http://www.genome.jp/kegg/pathway.html). The top 10 significantly enriched pathways are 'Metabolic pathways', 'Biosynthesis of secondary metabolites', 'Plant hormone signal transduction' (including PIF3, PIF4 and many auxin-related genes), 'Ribosome', 'Phenylpropanoid biosynthesis', 'Biosynthesis of amino acids', 'Starch and sucrose metabolism', 'Carbon metabolism', 'Amino sugar and nucleotide sugar metabolism' and 'Purine metabolism' (Figure 3B). Most DEGs (328 genes) were included in the The 'Metabolic pathways' cathegory, encoding many genes involved, for example, in the Glycosaminoglycan (GAG) metabolism, principal component secreted by the

transmitting tract to the complex extracellular matrix (ECM). We found also many genes involved in 'Energy metabolism', 'Carbohydrate and lipid metabolism', 'Nucleotide and amino acid metabolism' and 'Secondary metabolism'.

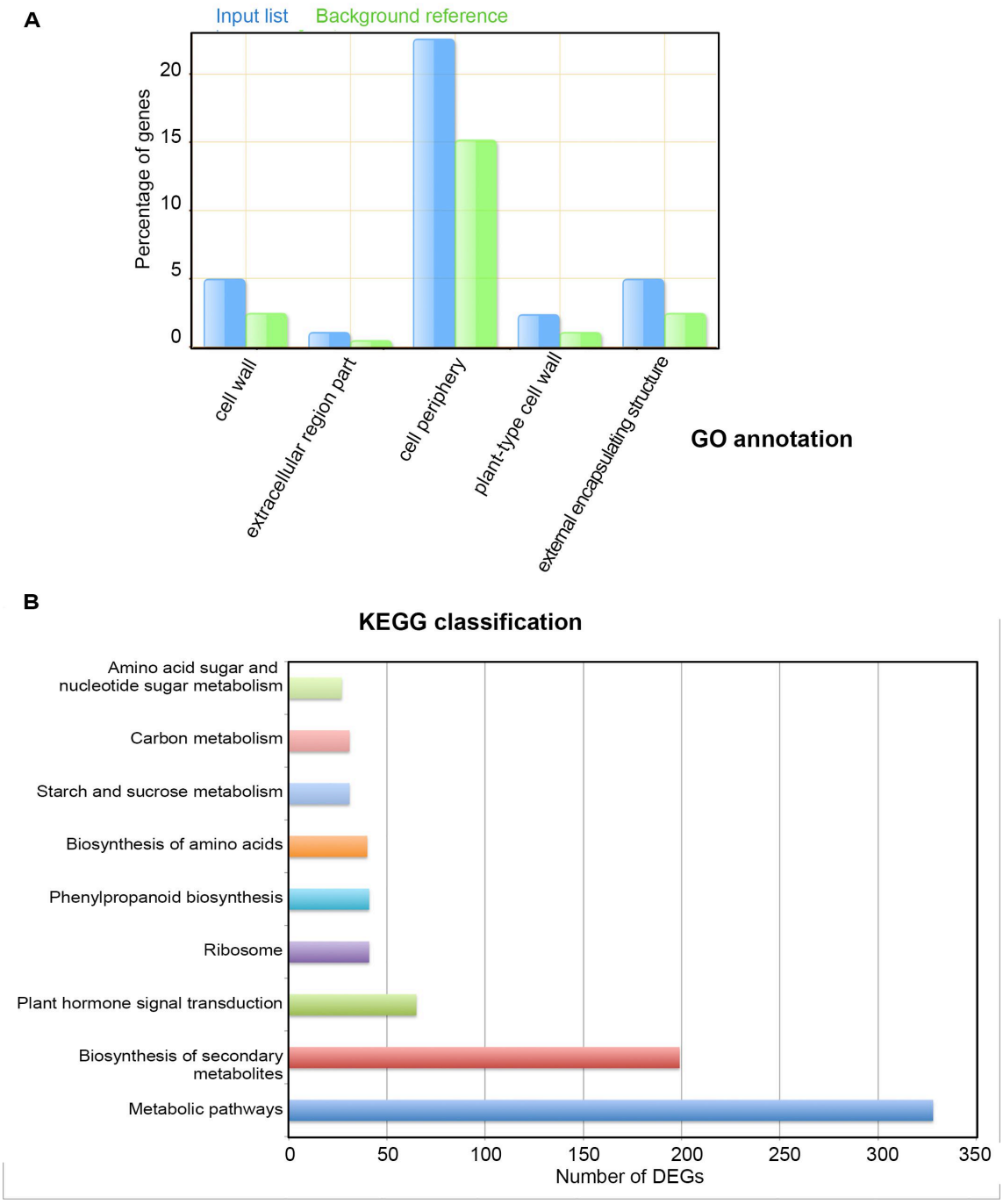


Figure 10. A) Significantly enriched GO terms in the 'cellular component' subontology in the reference TAIR10 (2017). **B)** Number of DEGs mapped in the top 10 metabolic pathways against the KEGG pathway database.

HEC3 and SHN Transcription Factors Share Common Downstream Effectors

As we analysed our list of DEGs in response to HEC3 overexpression, we found many common downstream targets with the SHN clade of AP2 domain transcription factors (Aharoni et al. 2004; Shi et al. 2011), including the *Arabidopsis* SHINE1/WAX INDUCER1 (SHN1/WIN1) (Aharoni et al. 2004; Broun et al. 2004; Kannangara et al. 2007) in response to our DEX treatment, but also SHN2, SHN3 and SHN1 itself in response to DEX together with CHX. Specifically, the 58% of reported genes that display up- or down-regulation expression in flower buds of a line where the three genes were downregulated by the overexpression of an synthetic microRNA targeting these clade (35S:amiR-SHN1/2/3) (Shi et al. 2011), were found to be within the 35Spro: HEC3–GR DEGs in response to DEX treatment (Table 3) (see Material and Methods for more detail). In this manner, our data suggested that SHN transcription factors could be HEC3 downstream targets.

35S:miR-SHN1/2/3 - HEC3 COMMON DOWNSTREAM TARGETS	Gene	Anotation	Functional Category
AT2G05540	GRP	Glycine-rich protein	Cell wall structure
AT1G70720	PMEI	Pectin methylesterase inhibitor	Cell wall structure
AT1G58430	RXF26	GDSL-motif lipase/hydrolase	Lipid metabolism
AT1G63710	CYP86A7	Cytochrome P450	Lipid metabolism
AT5G22500	FAR1	Fatty acid reductase	Lipid metabolism
AT4G14365		C3HC4-type RING finger	Transcription factor
AT1G15360	SHN1/WIN1	ERF/AP2 transcription factor	Transcription factor
AT4G15210	BAM5/RAM1	Beta-amylase	Starch metabolism
AT3G62950	GRXC11/ROXY4	Glutaredoxin-C	Redox regulation
AT5G60090		Protein kinase	Signaling
AT2G43620		Chitinase	Stress response
AT1G27940	PGP13/MDR15	Multidrug resistance P-glycoprotein	Transport
AT2G16760		Expressed protein	Unknown
AT1G58270		ZW9 mRNA	Unknown

Table 3. List of Common Downstream Effectors between HEC3 and SHN Transciption Factors

HEC3 Promotes the Expression of SHN Transcription Factors

HEC genes are expressed in the developing septum, transmitting tract and stigma (Gremski, Ditta, and Yanofsky 2007). On the other hand, the promoters of SHN1 and SHN3 have been shown to be active in the developing gynoecium, petals and the apical domain of the silique, while SHN2 promoter activity is confined only to the dehiscence zone in anthers and siliques (Aharoni et al. 2004). Since SHN1 expression appears to overlap more extensively with that of HEC genes, we analyzed the expression of SHN1 in wild type and loss-of-function *hec1 hec2 hec3* plants by RNA *in situ* hybridization. Our results showed a clear expression in petals and the upper domain of the wildtype style, from which stigma and transmitting tract develops, validating the reported activity of the published SHN1pro::GUS

lines (Aharoni et al. 2004). In contrast, no traces of expression were detected in the triple *hec* mutant, neither in the gynoecium nor in the epidermal cells of the petals (Fig 11A-B). Additionally, we carried out quantitative RT-PCR of *Arabidopsis* inflorescences to further confirm our results and also to include the rest of the SHN genes. Interestingly, we found a significant decrease not only for relative expression of SHN1 but also for SHN2 and SHN3 (Fig 11C). Our results strongly suggest that HEC3 (and likely other HEC factors) act as direct or indirect transcriptional activators of *SHN* genes.

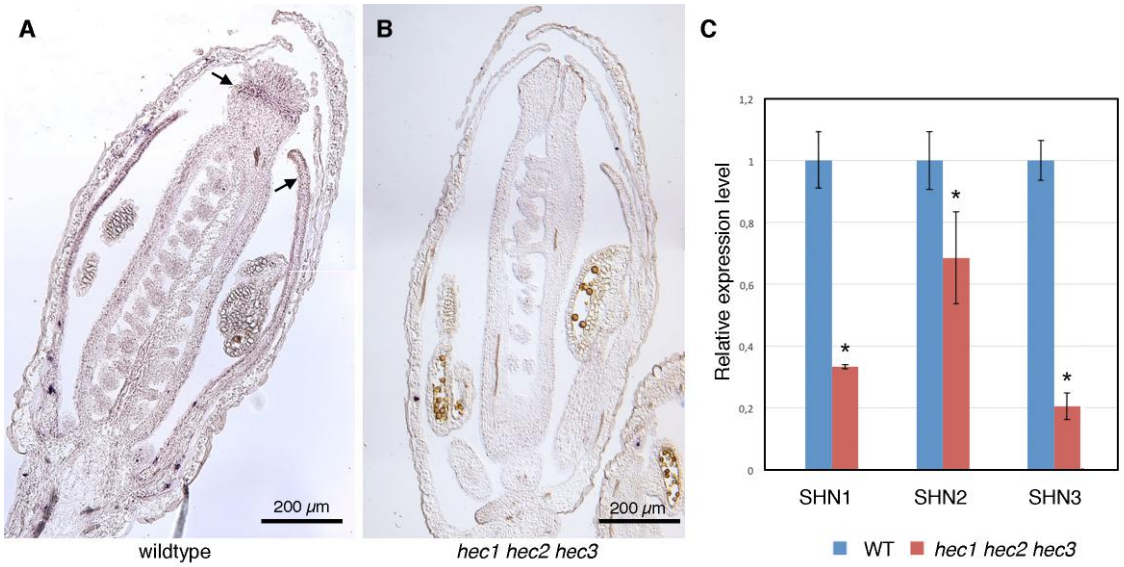


Figure 11. A-B) RNA in situ analysis of SHN1 expression at pre-anthesis stage in Arabidopsis flower buds. C) Quantitative Real Time-PCR for SHN transcripts in wild-type Arabidopsis genetic background. A) Longitudinal section of a wild-type flower bud showing expression signal at petals and upper domain of style (arrow). **B)** Longitudinal section of a *hec1 hec hec3* loss-of-function flower bud exhibiting no evident *SHN1* expression. **C)** *SHN1*, *SHN2* and *SHN3* relative expression levels significantly decreased *in hec1 hec2 hec3* loss-of-function *Arabidopsis* plants when compared with wild-type background.

Decreased Expression of SHN Genes Reduces Fertility

hec3 mutant plants have smaller fruits and a reduction in fertility (59% wild- type seed set) compared with wild type (Gremski, Ditta, and Yanofsky 2007). Considering HEC3 as a positive regulator of *SHN* genes, it would be possible that part of the HEC3 function was mediated by SHN function, and therefore, amiR-SHN1/2/3 and *hec* mutant plants should exhibit similar phenotypes to a certain extent. We then characterized the phenotypic alterations caused by down-regulation of the *SHN* genes in the pistils and fruits of the amiR-SHN1/2/3 lines kindly provided by Prof. Asaph Aharoni (Shi et al. 2011). We first compared fruits of wild type and amiR-SHN lines with back illumination, to reveal developing seeds in the mature silique. amiR-SHN fruits showed a dramatic fertility reduction, ranging from that comparable to *hec1 hec3* double mutants (17% wild-type seed set) (Gremski, Ditta, and Yanofsky 2007) to completely empty fruits, as in the case

of *HEC2-RNAi hec1 hec3,* which are totally sterile (Gremski, Ditta, and Yanofsky 2007) (Figure 12).

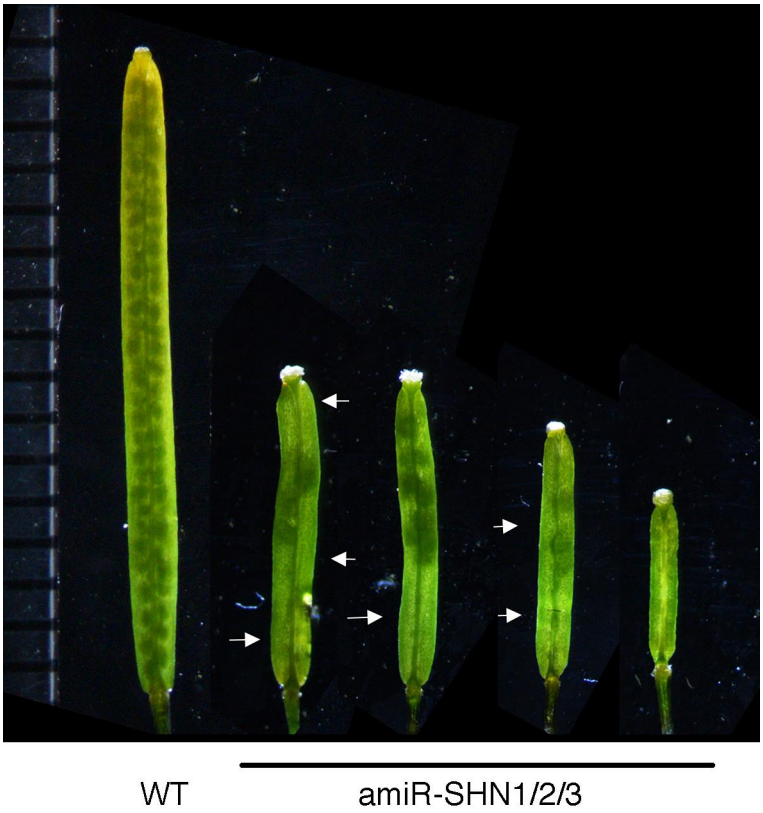


Figure 12. Decreased expression of SHN genes reduces fertility. miRSHN 1/2/3 show shorter fruits. Arrowheads point to significant empty spaces that should be occupied by the shadow produced by the developing seeds.

Defects in Pollen Tube Growth in the amiR-SHN1/2/3 Mutants

It has been shown that the loss of fertility in *Arabidopsis hec* mutants is caused by reduced pollen tube growth, since *HEC* genes are required for correct development of the stigma and transmitting tract, essential tissues to promote pollen germination and pollen tube elongation (Gremski, Ditta, and Yanofsky 2007). To test whether *SHN* down-regulation also affected these tissues or their performance, we used aniline blue staining to visualize pollen tube growth within the ovary (Balanzà et al. 2014) 36 hours after hand-pollinating previously emasculated carpels (see Methods).

We observed that wild-type gynoecia displayed an abundance of pollen tubes throughout the length of the transmitting tract and the entire ovary (Figure 13C). On the contrary, amiR-SHN1/2/3 carpels exhibited a severe reduction in the

density of pollen tubes growing downward throughout the style and significantly fewer pollination events, even more dramatically than in the case of *hec1 hec3* double mutants (Gremski, Ditta, and Yanofsky 2007)(Figure 13D). Thereby, conclude that SHN function is required for a proper pollen tube growth within the pistil.

amiR-SHN1/2/3 Mutants Display Increased Length Papillae and are essential for transmitting tract development

As the pistil matures, the stigma papillae lengthen to form cells receptive for pollination. Mutations that alter carpel structure also disrupt stigma structure (John Alvarez and Smyth 1998; J Alvarez and Smyth 1999). *hec1 hec3* double mutant itself shows smaller stigmatic papillae compared with *Arabidopsis* wild type and a slight tendency for the style to be somewhat longer (Gremski, Ditta, and Yanofsky 2007). Surprisingly, scanning electron microscopy (SEM) pictures revealed that amiR-SHN1/2/3 papillae were conspicuously longer than wild type (Figure 13A-B). The style, and by extension the entire carpel, was also visibly wider both as shown in our SEM and histology sections (Figure 13E-F).

Based on our previous results regarding decreased fertility in *SHN* mutants, we used Alcian blue staining, which detects acidic polysaccharides characteristic of the transmitting tract extracellular matrix (ECM), to examine staining intensity and cytology (Figure 13E-F). As previously described by Gremski et al. (2007), wild-type style sections showed a cell compact, intensely staining transmitting tract (Figure 13E). Conversely, the amiR-SHN1/2/3 pistils had very different transmitting tract morphology. The Alcian Blue staining was very reduced and the high cell density and intense staining above-mentioned in wild type had been replaced by few thin-wall bigger cells (Figure 13F). Thus, amiR-SHN carpels showed aberrant transmitting tract cells with a severely decreased production of ECM, which is also severely affected in *hec1 hec3* double mutants (Gremski, Ditta, and Yanofsky 2007).

Altogether, the phenotype of miR-SHN lines are consistent with SHN factors acting downstream of HEC genes to mediate part of their function.

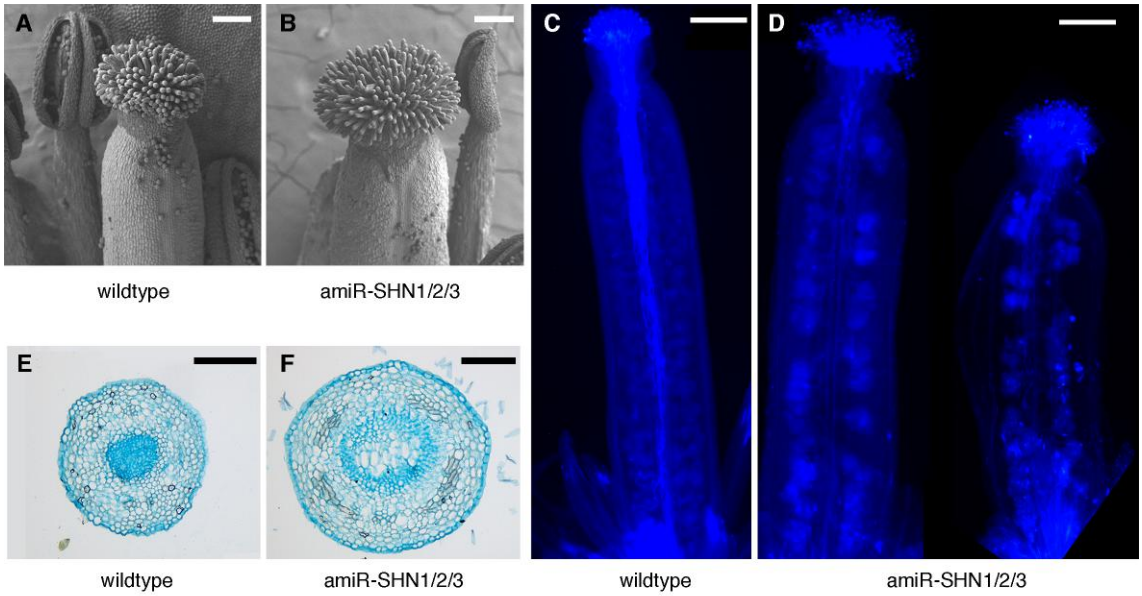


Figure 13. Phenotypic difference in amiR-SHN1/2/3 papillae and deficient transmitting tract development and pollination. A-B) Stigma of amiR-SHN123 plants show longer papillae than wild type fruits. **C-D**) amiR-SHN1/2/3 ovaries display a severe reduction in the density of pollen tubes growing downward throughout the style and significantly fewer pollination events compared to wild type carpels. **E-F)** In SHN down-regulated lines, transmitting tracts have almost no presence of Alcian Blue staining in contrast to wild type. Note that mutant style is visibly wider than wild type. Bars: 100μm in all panels.

DISCUSSION

During about the last two decades, the increasing access to NGS techniques, such as ChIP-Seq and RNA-Seq among others, has facilitated the study of the complex and partially overlapping roles of HEC factors as master regulators of diverse developmental processes during the *Arabidopsis* life cycle (Gremski, Ditta, and Yanofsky 2007; Schuster, Gaillochet, and Lohmann 2015; Zhu et al. 2016; Ballester and Ferrandiz 2017; Gaillochet et al. 2017; Gaillochet et al. 2018). In this line, our work provides the first transcriptomics profiling of HEC3 and further experimental assays to validate the identification of a novel clade of downstream targets with a crucial role in the development of the *Arabidopsis* transmitting tract.

Thereby, the Gene Ontology (GO) Analysis of the Functional Annotation and Classification of our DEGs is consistent with the key function of HEC factors in the proper specification of the transmitting tract (Gremski, Ditta, and Yanofsky 2007). This tissue secretes to the apoplast a complex acidic polysaccharide matrix (ECM) and we found that the most significantly enriched term in the 'cellular component' subontology in response to HEC3 overexpression was 'cell periphery', together with 'extracellular region', 'plasma membrane', 'external encapsulating structure', 'cell wall' and 'plant-type cell wall'. All the subsequent subontology analysis point out in the same direction: HEC3 appears to be a master regulator of cell wall properties, likely responsible not only of initial steps of transmitting tract specification, which can be deduced from the dramatic *hec1 hec2 hec3* triple mutant, with no transmitting tract development, but also of subsequent transmitting tract differentiation.

Analysing in detail our KEGG Pathway results, and within the 'Biological Process' subontology, we want to highlight the numerous genes included in the most enriched term, 'Metabolic Process', which encode for GAG metabolism (Figure 3). Glycosyltransferases are the enzymes that polymerize multiple sugars into chains (starch, cellulose and chitin are some examples of polysaccharides) or add single sugar molecules onto existing molecules, including carbohydrates, proteins, and lipids (Stavolone and Lionetti 2017). So, other biosynthetic processes included in this term as 'carbohydrate process', 'lipid processes' or 'transferase activity' in 'Molecular Function' subontology, suggest that HEC3 is a key participant in the proper formation of the ECM essential components, the GAGs, and most probably its loss-of-function is partially responsible of transmitting tract specification failure in a redundant manner with HEC1/2, as shown by Gremski et al. (2007).

So, in the framework of the importance of cell wall properties and the main role of GAGs metabolism for a proper ECM development, how do we integrate and relate the previously published studies about SHN transcription factors with their uncovered role as downstream HEC3 effectors? Now, with the data generated in this work maybe the question could be not so complicated to answer. We already knew that SHN factors control cuticle lipids metabolism and modify the epidermal cell walls by altering pectin metabolism and structural proteins. Moreover, in the published list of 35S:amiR-SHN1/2/3 downstream targets (Shi et al. 2011), we found two HEC3 common downstream effectors, CYP86A7 and a Chitinase. The first one is a Cytochrome P450 involved in lipid metabolism (Bak et al. 2011) and Chitinases display several functions during the plant life cycle, taking part in developmental processes such as pollination and embryo development (Passarinho and de Vries 2002). Besides, considering their demonstrated importance for fertilization success, as well as the phenotype convergence in transmitting tract differentiation with HEC factors, it suggests that HEC role in transmitting tract differentiation could be mediated by these SHN factors. However, since some SHN mutant phenotypes in the pistil are different from those of *hec* mutants (longer papillae, wider style), it is likely that part of SHN role in gynoecium development is independent of HEC. More extensive genetic analyses will be then required to test this hypothesis and to elucidate the precise interaction of HEC and SHN factors, for example, introducing the 35S::amiR-SHN1/2/3 construct in HEC overexpression genetic backgrounds, or conversely, expressing SHN in gynoecia of *hec* mutants to see whether some phenotypes are recovered. Moreover, it still remains to be addressed whether SHN only depends on HEC, if the regulation is direct (check for G-boxes in SHN promoters, ChIP), etc.

In addition to the presence of terms regarding its role as a cell wall orchestrator and bHLH transcription factor, within the HEC3 top five metabolic pathways we also find 'Phenylpropanoid biosynthesis' and 'Plant hormone signal transduction'. The phenylpropanoid pathway is responsible for the synthesis of numerous compounds important for plant growth and responses to the environment, also involved in cytokinin (CK) signalling and auxin-regulated plant growth and sensitivity (Kurepa et al. 2018).

In this context, we expected to find clusters of our DEGs that could reflect HEC3 specific functions as well as common regulatory modules with HEC1/2. Taking advantage of robust systems-level transcriptomics and ChIP-seq data already available for HEC1 (Gaillochet et al. 2018 and references therein), we compared our overlapping DEGs against five main HEC1-dependent regulatory modules: Light signalling, Flowering transition, Gynoecium, Auxin and HEC-TCP interaction. Our HEC3 results suggest a complete functional redundancy with the 'Light signalling modules', since all the HEC1 regulated genes (PIF3, PIF5, GAI, RGA, PAR1, PHYA, PHOT1, PORA, PORB and STH2) were differentially expressed in our experiment with the only exception of GAI. We also found many common genes, about 57% and 50% gene match respectively, in 'Auxin' (PIN3, PIN4 PIN7 and ARF11), and 'Gynoecium' modules (SPT, GAI, PIF5, PIN3, NGA1 and CRC). On the other hand, for the related TCP-participant regulatory modules 'Flower Transition' and 'HEC-TCP' we found and scarce gene concurrence, suggesting that this interactions might be mainly circumscribed to HEC1/2 factors.

Despite the close relation of these bHLH transcription factors, we previously knew about their partially overlapping function (Gremski, Ditta, and Yanofsky 2007; Schuster, Gaillochet, and Lohmann 2015; Zhu et al. 2016; Ballester and Ferrandiz 2017; Gaillochet et al. 2017), and with the generation of this novel transcriptomics resource, we found additional HEC3 specific roles not previously reported, as far as we now, in HEC1/2. Our data reveal a putative repression of the CK repressor CKX3, thus promoting CK expression, and a positive regulation of NTT, a gynoecium-related transcription factor involved, in the same way as HEC3, in control enzyme and transporter-encoding genes involved in cell wall polysaccharide and lipid distribution in gynoecial medial domain cells. The NTT protein interacts with several gynoecium-related transcription factors and loss-of-function mutant NTT phenotype also exhibits severe defects in the *Arabidopsis* transmitting development (Crawford, Ditta, and Yanofsky 2007).

To conclude, we can go back to one of the starting points of this work, the plausible hypothesis of IND neofunctionalization from similar HEC ancestors to drive DZ specification. So far, only a small number of IND direct targets have been described, and we interestingly found two genes in our HEC3 DEGs list, *NST1* and *ADPG2*, previously identified as direct or indirect IND targets (Ogawa et al. 2009; Mitsuda and Ohme-Takagi 2008). This suggests that HEC3 and IND share common targets, thus supporting the idea of ancestral common functions. We can also relate these results with the still-pending question on how IND is able to spacially specify the adjacent lignification and separation layers in the dehiscence zone, which are characterized, respectively by the up-regulation of the lignification and cell wall remodelling pathways. If IND regulates both NST1/ADPG1-2, its activity has to be modulated by other factors to obtain different spatial outputs. In this context, the data that we have obtained on *NTT* and *SHN* genes as HEC3 targets, point to a maybe hidden role of HEC3 in DZ formation, considering that NTT expression pattern is specifically located in the DZ domain (data obtained by our group but not published) in the same way as SHN2 (Shi et al. 2011). A further exhaustive analysis of HEC3 and IND genetic and molecular interactions will be needed to

explore the idea of HEC3 as a modulator of IND function and to better understand the GRN driving DZ formation that is not fully solved yet.

CHAPTER 3

PREFACE

The floral architecture in the Brassicaceae family is predominantly conserved (Endress 1992), although vast diversity exists in their fruit shape which increases its complexity ranging from cylindrical, disc-formed or spherical basic forms, to more complicated heart-shaped structures (Langowski, Stacey, and Ostergaard 2016). Differences between related species may arise from the fixation of mutations of relatively few major morphogenetic genes (Theissen et al. 2000; R. M. Bateman and DiMichele 2003) and similarities in fruit morphology are not always necessarily linked to phylogenetic proximity, finding closely related species with severely different fruits and vice versa (M. Koch, Al-Shehbaz, and Mummenhoff 2003; K Mummenhoff et al. 2005; Bailey et al. 2006; I. A. Al-Shehbaz, Beilstein, and Kellogg 2006). This suggests that morphogenetic processes determining carpel and fruit structure are highly plastic and to some extent vaguely understood, despite numerous genes driving fruit patterning in the model plant *Arabidopsis thaliana* have been identified (Gu et al. 1998; Ferrandiz, Pelaz, and Yanofsky 1999; Roeder, Ferrandiz, and Yanofsky 2003; Liljegren et al. 2004; José R Dinneny, Weigel, and Yanofsky 2005; Balanzá et al. 2006; Alonso-Cantabrana et al. 2007; Trigueros et al. 2009; Seymour et al. 2013). Furthermore, *Arabidopsis* fruits are comparatively simple in their structure and therefore complementary analysis of fruit development in close relatives with differently shaped fruits might provide a framework for depicting processes of morphology determination.

In the genus *Lepidium* (pepper cresses), one of the main genera of the Brassicaceae with about 250 species (I. A. Al-Shehbaz and Mummenhoff 2011), organ reduction is a distinct trait of more than half of all species (Ihsan A. Al-Shehbaz 1986; Hewson 1981) through three different mechanisms (John L Bowman and Smyth 1998), and localized reduction in B function floral organ identity genes, together with increased C function, might be directly or indirectly involved (J L Bowman, Smyth, and Meyerowitz 1991; Liu and Meyerowitz 1995; Jack, Sieburth, and Meyerowitz 1997). In these species, petals are absent and stamens are reduced from six to two per flower (Ihsan A. Al-Shehbaz 1986). In addition to their large variation in fruit morphology, seed dispersal strategies evolved independently from dehiscent to indehiscent fruits several times within the genus (Klaus Mummenhoff et al. 2009; Muhlhausen et al. 2013; A. Al-Shehbaz, Mummenhoff, and Appel 2002), versus the typical dehiscent Brassicaceae fruit type (Hall, Sytsma, and Iltis 2002), thus representing a highly suited model for the study of the dehiscence mechanism. Functional analyses revealed a high degree of conservation of fruit dehiscence pathways between *Lepidium campestre* and *Arabidopsis thaliana* (Lenser and Theissen 2013), concurring with previous studies involving Brassicaceae species other than *A. thaliana* (Petersen et al. 1996; Chauvaux et al. 1997; Ostergaard et al. 2006; Ogawa et al. 2009; Sorefan et al. 2009; Thomas et al. 2010; Avino et al. 2012; Muhlhausen et al. 2013).

One of the two-stamen representative species in the genus is *Lepidium didymum* L. (syn: *Coronopus didymus* (L.) Smith), a cruciferous weed indigenous to South America and extensively distributed in most dairying countries of the world, which has caused significant economic losses to the industry for many years (Klaus Mummenhoff et al. 2009; A. Al-Shehbaz, Mummenhoff, and Appel 2002). Cows which have ingested this weed produce tainted milk with burnt and displeasing flavour, which is not reduced but intensified by conventional vacuum pasteurization techniques (Walker and Gray 1970). Moreover, this weed scatters thousands of fruit valves per plant and forms abundant and persistent non-dormant seed banks in arable soil and grassland (Popay et al. 2006; Roberts 1986; H. and Bastow 2003; Thompson, Green, and Jewels 1994; Rahman, James Trevor, and Grbavac 2006; Kiffe 1990). A set of transcription factors, usually referred to as valve margin identity genes, is well known for their function in proper establishment of the dehiscence zone (Liljegren et al. 2000; Ferrandiz, Liljegren, and Yanofsky 2000; Rajani and Sundaresan 2001; Ferrándiz 2002; Liljegren et al. 2004). However, transferring the knowledge acquired from developmental genetic systems in *Arabidopsis* to other species of the Brassicaceae family is hindered due to the lack of sufficient genomic or transcriptomic resources.

On the other hand, the massively parallel sequencing of RNA (RNA-Seq or transcriptome profiling) (Wang, Gerstein, and Snyder 2009) is a powerful, cost-efficient tool that has been successfully applied for sequencing the full transcriptomes of both model and non-model plants (Parchman et al. 2010; Ashrafi et al. 2012; E. et al. 2013; Cardoso-Silva et al. 2014; Xiu Huang et al. 2016; Pan et al. 2016). Nonetheless, on the basis of data provided by literature, no *Lepidium* full transcriptomes have been published so far. In this study, we have generated a genome-guided transcriptome based on the sequencing of cDNA samples from *L. didymum* leaf and inflorescence tissues. Our main goal was to characterize an alternative model system aimed at studying the underlying molecular basis of changes in fruit morphology and dehiscence, building on anatomical and transcriptomic results.

RESULTS

Flower morphogenesis in *Lepidium didymium*

Flower morphogenesis and development has been studied and staged in detail in *A. thaliana* (Smyth, Bowman, and Meyerowitz 1990) and also in several other Brassicaceae species, such as *Brassica napus* (Polowick and Sawhney 1986) or other *Lepidium* species (John L Bowman and Smyth 1998). These studies have shown that, in general, it does not differ broadly within the family, particularly in early developmental stages. In this work, and for comparative purposes, we have used scanning electron microscopy (SEM), histological sections and optical microscopy of cleared material to describe in detail flower and fruit development of *L. didymum,* in the framework of the developmental landmarks defined for Arabidopsis.

Early development (stages 1-7)

Flower development was documented from the emergence of the floral meristem, which comes up as a subtle bump on the periphery of the apical meristem at stage 1 (Figure 14A). This primordium broadens and becomes bisected from the central apex by a narrow groove, thus marking the onset of stage 2 (Figure 14A). Stage 3 initiates with sepal primordia appearance, which forms at adaxial, abaxial and lateral positions of the floral meristem, which then adopts a rhomboid shape. Sepal primordia become distinct from the central dome of the floral meristem, at the beginning of stage 4 (Figure 14A). From this point on, the stage 5 distinctive landmark is the synchronously emergence of stamen and petal primordia (Figure 14A-B). Similarly to what is reported in *Arabidopsis* and other *Lepidium* species, four petal primordia differentiate at alternate positions to the sepals (Figure 14A-B) (Smyth, Bowman, and Meyerowitz 1990; John L Bowman and Smyth 1998). Despite petals are missing in several *Lepidium* species, including *L. didymum*, this absence appears to be due to the extreme reduction of petal growth, which may differ within a species and occasionally within a flower, but not to the failure to initiate the petal primordia (Ihsan A. Al-Shehbaz 1986; Hewson 1981; John L Bowman and Smyth 1998). In contrast, only two stamen primordia initiate from the floral meristem in medial positions (Fig 14B, D, E), while lateral stamen primordia are absent, similarly to what has been described in other two-stamen *Lepidium* species. Thus, each of the two individual stamens in *L. didymum* emerge from a wide primordium, located in the same region where two medial stamens develop in other six- or four-stamen species (John L Bowman and Smyth 1998).

By the time stage 6 initiates, medial and lateral sepals entirely overlie the bud (Figure 14C), while the gynoecium is first visible as a mound at the center of the bud (Figure 14C). Later on, the gynoecium primordium appears as a flattened mound surrounding a medial notch (Figure 14D-E). This hollow barrel-like primordium then undergoes an initial phase of anisotropic growth rate, lengthening preferably along the longitudinal axis during stage 7 (Figure 14F-G), as has previously been described both in *Capsella* and *Arabidopsis* fruits (Eldridge et al. 2016). Later in stage 7, anther locules are already visible, whereas petal primordia remain as small undifferentiated bumps at both sides of stamen primordia (Figure 14F-G).

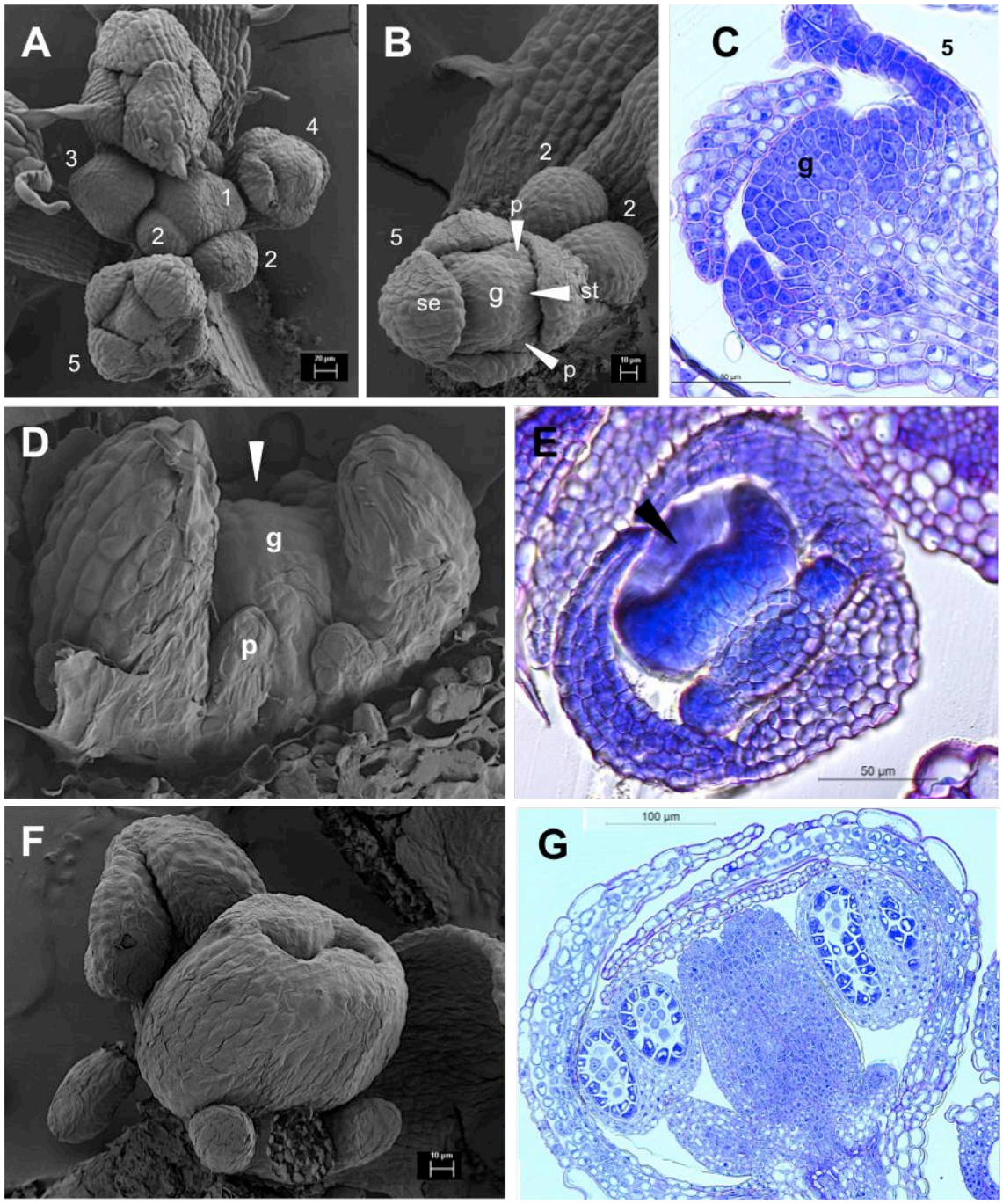


Figure 14. Early development (stages 1-7) series of SEM pictures and histology sections of Coronopus didymus species showing four petals of reduced development with two medial stamens. A) Flower development from the emergence of the floral meristem until the synchronously emergence of stamen and petal primordia (stages 1-5). **B)** Four petal primordia differentiate at alternate positions to the sepals and only two stamen primordia initiate from the floral meristem in medial positions (stage 5). **C)** Medial and lateral sepals entirely overlie the bud while the gynoecium is first visible as a mound at the centre of the bud (stage 6). **D-E**) The gynoecium primordium appears as a flattened mound surrounding a medial notch and is clearly visualized in sections with blue toluidine staining (right)(late stage 6). **F-G)** Hollow barrel-like primordium (late stage 7).

Late Development (stages 8-12)

At stage 8, *L. didymum* gynoecium proportions visibly deviate from the characteristic cylindrical shape of *Arabidopsis* pistils. This rapid growth in length appreciably slows down in favour of growth in width, resulting in an oblate spheroid pistil morphology, wider at the base and narrower at the distal portion (Figure 15A). As the valves enlarge throughout stages 9-10, the apex becomes progressively tapered and the style starts to be morphologically discernible from the ovary, along with the first emergence of rounded stigmatic papillar cells (Figure 15B). In consequence, the above-mentioned oblate spheroid turns into an inverted heart-shaped morphology, flattened in the medio-lateral plane (Figure 15B). The petal primordia elongate adopting a finger-like morphology, and, at the medial side of the petals base, small bump of cells appear, that later will develop as nectaries positioned between the stamens and the reduced petals. (Figure 15B).

At stage 11, the enlargement of the distal domain of both valves modifies again the overall pistil form, which results in a disc-shaped gynoecium, topped by a short style (Figure 15C). By the end of this stage, the style is totally crowned by incipient stigmatic papillae, and two lateral creases mark the style/valve different domains (Figure 15C). Already from stage 12 onward, the different tissues patterning the mature *L. didymum* pistil become clearly identifiable (Figure 15D-E). A wide replum is first apparent along the middle longitudinal axis and morphologically distinct from both adjacent mature valves (Figure 15D). In addition, the nectaries and the filamentous petals are clearly visible and elongated (Figure 15D). As the gynoecium approaches anthesis, the apical style and the stigmatic papillae complete their development, as well as the rest of inner structures such as both ovules and integuments, thus preparing the flower for self-pollination at the onset of stage 13 (Figure 15D-E). This pre-anthesis period also sets the final phase of petal and nectary development. The mature petals are relatively filamentous and tapered, resembling those described for *L. aschersonii* (John L Bowman and Smyth 1998)(Figure 15D). Nevertheless, the ultimate petal expansion may vary widely from above half the length of the ovary to other cases of dramatic early limited growth, as it is reported for *L. hyssopifolium* and *L. pseudo-hyssopifolium* (John L Bowman and Smyth 1998)(Figure 15D, F). The spatial patterning of nectaries is directly related to stamen number, consistent with previous reports for other two-stamen species (John L Bowman and Smyth 1998) (Figure 15D, F). Stage 13 is eventually the moment in which the anthers dehisce. At this point, the valve epidermal cells exhibit a miscellany of shapes and sizes, mostly isodiametric, with many interspersed clustered stomata (Figure 15F), where the medial replum starts to be buried by the rapid valve expansion also in the medial plane (Figure 15F). Post-anthesis stages are characterized by radial growth of the valves, mostly driven by cell expansion, and are concomitant with seed development at the two ovary locules (Figure 15F-I). Additionally around stage 17, second order vascular strands have completed their development connecting lateral and medial vascular bundles and covering the mature valves in a reticulate pattern reminiscent of that reported for *A. thaliana* (Roeder and Yanofsky 2006)(Figure 15I).

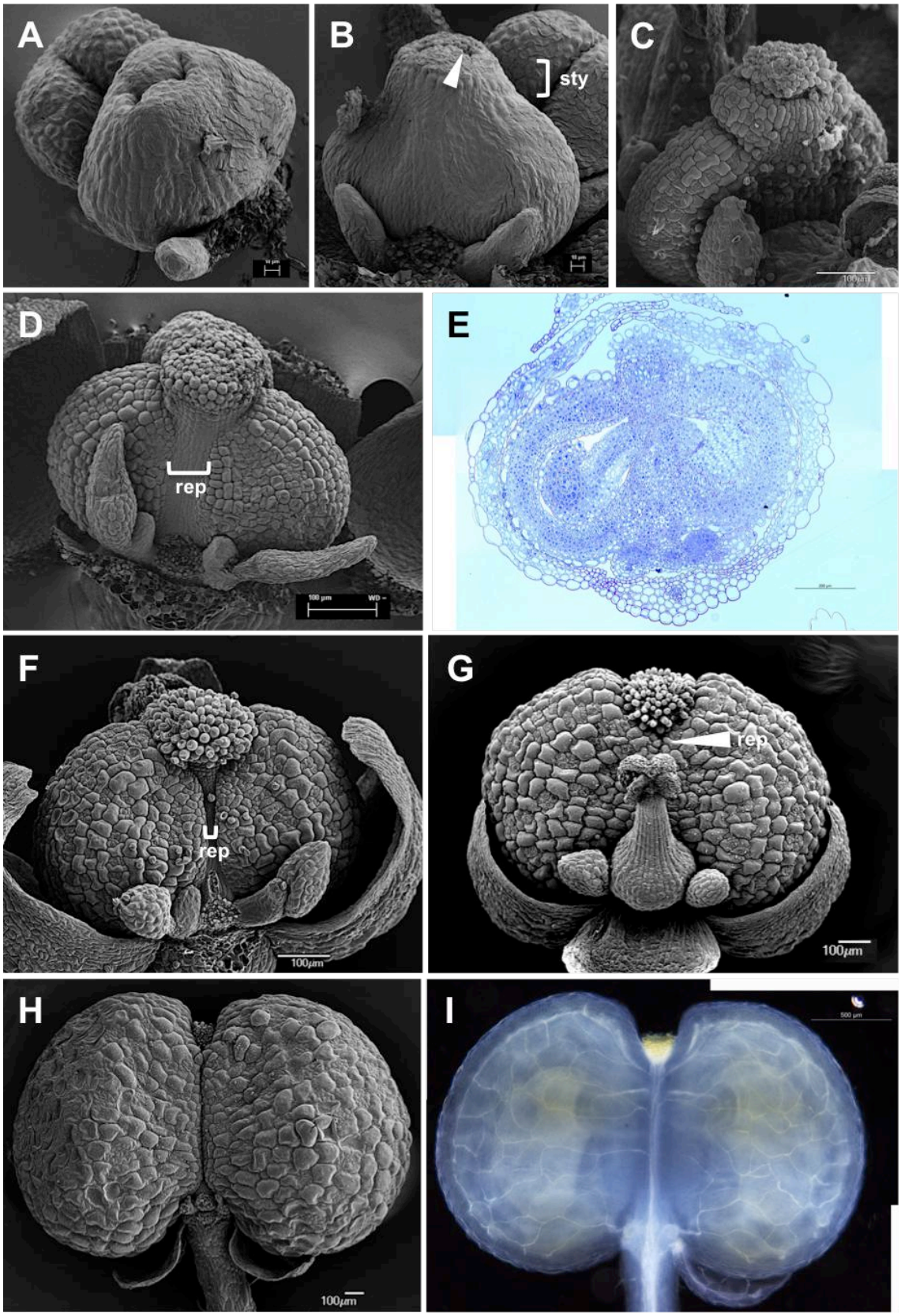


Figure 15. Late development (8-12) series of SEM, histology section and vascular clearing of Coronopus didymus fruit morphology A) Oblate spheroid pistil morphology (stage 8) **B**) Tapered morphology and the style starts to be morphologically discernible from the ovary. Arrowhead points out the first emergence of stigmatic papillar cells (stage 9-10) **C)** disc-shaped gynoecium topped by a short style (stage 11). D) Wide replum is first apparent between both adjacent mature valves **D-E**) The different tissues patterning the mature pistil become identifiable and the flower is prepared for self-pollination (stages 12-13) **F)** Stage 13 is

eventually the moment in which the anthers dehisce. The valve epidermal cells exhibit a miscellany of shapes and sizes with many interspersed clustered stomata. **F-I)** Post-anthesis stages (14-17). Radial growth of the valves and seed development at the two ovary locules. At stage 17 second order vascular strands have completed their development connecting lateral and medial vascular bundles and covering the mature valves.

Dehiscence zone morphology

So as to describe dehiscence zone morphology in *L. didymum*, transversal histological sections of fruits from early and late developmental stages were stained to reveal cell morphologies and lignification patterns. The number of cell layers in the valves is similar to those in Arabidopsis siliques for the outer layers: one cell-layered exocarp with big cells and 4-5 layers of mesocarp cells, numbers that do not increase with development (Figure 16A, B) (Roeder and Yanofsky, 2006). In contrast, the endocarp differs from that of Arabidopsis. In this latter species, the endocarp is composed of two single-celled layers: the inner endocarp*a,* with large cells facing the locule, and the outer endocarp*b*, with small cells that lignify at late stages of development (from mid stage 17), concomitantly with endocarp*a* break down (Roeder and Yanofsky, 2006). In *L. didymum*, several layers of very small endocarp cells are visible already in young post-anthesis fruits, being the number of cell layers at the valve margins highly increased. Moreover, no signs of the typical big cells of the endocarp*a* are observed at these early fruit developmental stages (Figure 16A), while in pistil sections at pre-anthesis stages, a distinct inner endocarp cell layer of big cells is present (Figure 16E), indicating that endocarp*a* degeneration may take place precociously. In addition, at early post-anthesis stages in *L. didymum*, it is already possible to identify large pre-lignified domains of small cells with thickened cell walls (Figure 16A). At later stages, lignification is extensive at the endocarp and especially at the valve margins (Figure 16B). This suggests a relative earliness in the commencement of the lignification events in comparison with *Arabidopsis* (Figure 16H, 16B).

At the valve-replum junction, two clearly visible dehiscence zones are observed already in young post-anthesis *L. didymum* fruits. The replum, formed by 7-8 files of small cells, is flanked by a separation layer of even smaller cells that leave a clear plane of separation. Towards the valve, the outer portion of the separation layer is in contact with the mesocarp, while the inner portion is connected to the pre-lignified endocarp. Two vascular bundles are visible in the replum already showing lignin deposition at the inner domain (Figure 16A). At late fruit development, extensive lignification of the endocarp is apparent, together with an increased lignin deposition at the replum that reaches the outer epidermal layers, and signals of separation layer degradation are observed, progressing from the outer cell layers to the inside. The lignification spatial-temporal pattern of *L. didymum* is very similar to other dehiscent *Lepidium* representatives, such as *L. campestre,* in terms of lignification of the vascular area and formation of a well-defined dehiscence zone along the valve margin (Rajani and Sundaresan 2001; Muhlhausen et al. 2013; Klaus Mummenhoff et al. 2009)(Figure 16B). In these closely related dehiscent species, the tension triggered by the presence of a stripe

of lignified cells (lignification layer) adjacent to a region of thin-walled cells (separation layer), whose middle lamellae degenerates as a result of hydrolytic enzyme secretion, is sufficient to detach the valves and release the encased seeds. However, the round shape of *L. didymum* fruits results in a different dehiscence strategy. The thickened lignified endocarp leaves a little hole or "Natural Pericarp Opening" (NPO) facing the replum and smaller than the non-dormant seed, as it has been already described for other didymous-type fruits (Sperber et al. 2017)(Figure 16B). In this manner, the dispersal units are the whole valves and this NPO permits water uptake and later opening by distal pericarp rupture (A. Al-Shehbaz, Mummenhoff, and Appel 2002; Sperber et al. 2017).

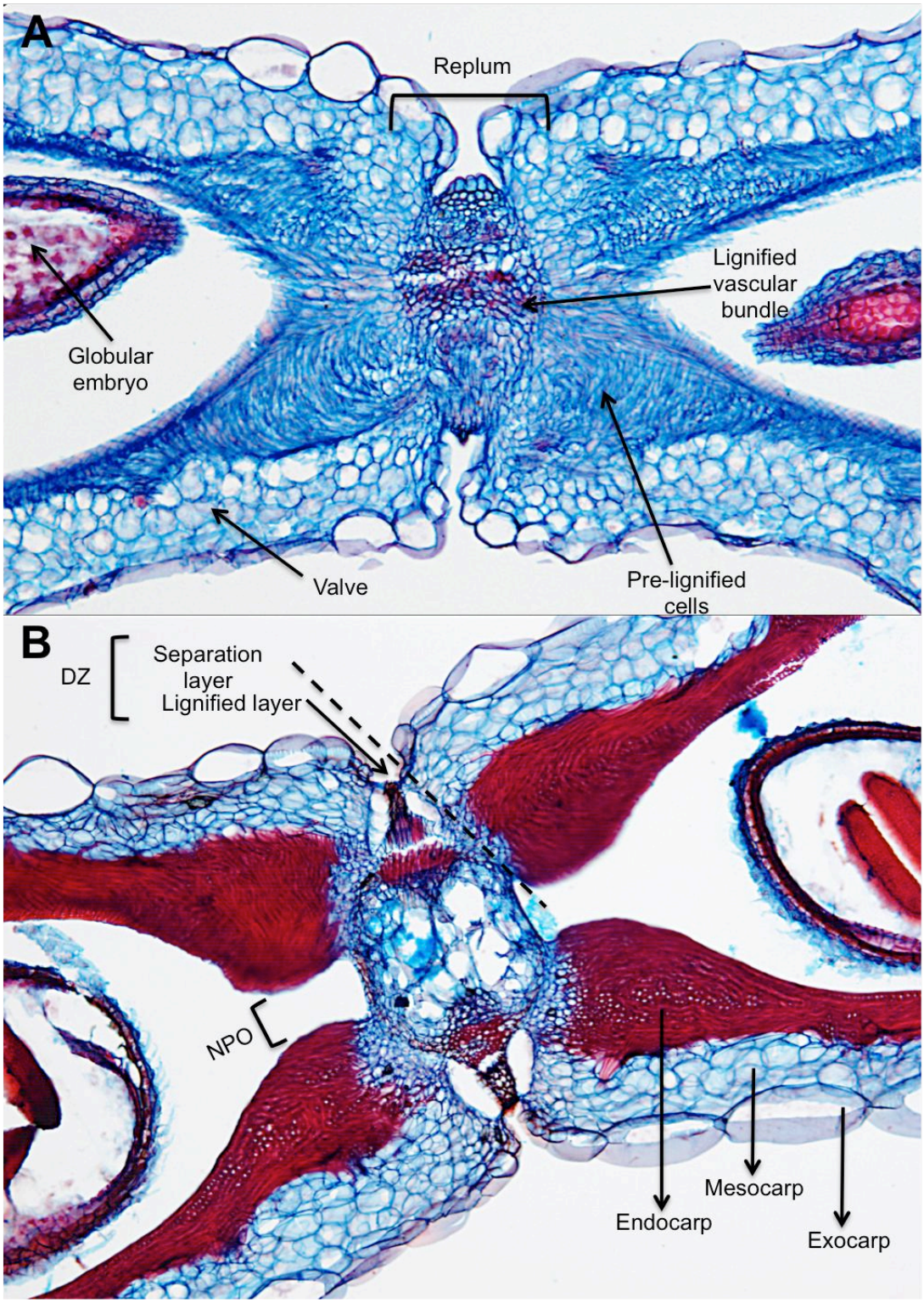


Figure 16. Stained transversal histological sections of fruits from A: early and B: late developmental stages to reveal cell morphologies and lignification patterns.

Genome-guided assembly of the *L. didymum* leaf and inflorescence transcriptomes

In order to develop *L. didymum* as a new model for molecular studies of leaf and fruit evolution and development, we obtained 612×10^6 paired-end strand-specific reads using an Illumina sequencer. Based on their highest quality, we selected 15.5% of the reads (i.e. 47,515,513 read pairs) for the subsequent assembly after applying consecutive filtering steps for removal of plastid-derived reads, correction of sequencing errors, adapter removal, quality trimming, and depletion of rRNA reads (see Methods and Table 4). The selected reads were pooled and used by Trinity (Haas et al. 2013) in a strand-specific genome-guided assembly. The initial assembly comprised 92,313 genes, collectively represented by 113,474 transcript isoforms. Because transcriptomes assembled by Trinity usually include a large number of redundant transcripts, we used CAP3 (X Huang and Madan 1999) and CD-HIT-EST (Li and Godzik 2006) to produce a low-redundancy reference transcriptome comprising 51,591 distinct nucleotide sequences. We performed local BLASTX searches using these sequences as queries against a local database containing the well-annotated transcriptomes of other Brassicaceae (e.g. *Camelina sativa*). The results of these searches show that ~15% of the sequences include a full-length coding sequence (i.e. 7431 assembled sequences contain an ORF matching at least 90% of the length of a known protein). Among these sequences, we found those of numerous genes encoding homologs of well-known developmental regulators, such as APETALA2 (Fig 17.), which provide a framework for future evolutionary and comparative functional studies. To assess the completeness of the assembly, we mapped back the set of filtered paired-end reads to our reference transcriptome using the Bowtie2 read mapper. The overall alignment rate was 72.29%, with over 60% of the reads aligning concordantly (as properly oriented pairs) to the reference sequence. Of these, less than 6% of the read pairs aligned concordantly to more than one transcript, reflecting the low levels of sequence redundancy in our assembly.

		Number of read pairs			
	Sample	Unprocessed	After removing plastid-derived reads	After removing adapters and quality trimming	After removing rRNA reads
Inflorescence	1	43,188,082	40,543,705	35,877,443	5,094,737
	2	29,951,606	27,764,538	24,214,238	4,740,103
	3	20,851,599	19,011,050	16,626,520	8,563,018
	11	37,930,992	35,727,117	31,977,022	3,996,173

	new	20,958,370	20,120,496	7,153,681	3,048,523
Leaf	2	41,071,132	27,217,257	22,911,917	4,051,259
	3	37,132,280	27,472,439	23,635,853	3,975,579
	4	30,859,409	22,709,760	19,599,820	10,917,187
	21	19,711,926	12,999,474	10,638,232	1,378,924
	31	24,441,702	18,458,820	15,197,399	1,750,010
	Total	306,097,098	252,024,656	207,832,125	47,515,513
	%	100	82.3	67.9	15.5

Table 4. Number of read pairs per sample

```
>12362
Length=1479

 Score = 625 bits (1612),  Expect = 0.0, Method: Compositional matrix adjust.
 Identities = 385/440 (88%), Positives = 395/440 (90%), Gaps = 23/440 (5%)
 Frame = +1

Query  1     MWDLNDAPHQTQREEESEEFCYSSPskrvgsfsnssssavvIEDGSDDDELNRVRPNNPL  60
             MWDLND+     REEE+EEF YSSPSK VGSFSNSSSSAVVIEDGSDDDE NRVRPNNPL
Sbjct  160   MWDLNDSV----REEETEEFSYSSPSKWVGSFSNSSSSAVVIEDGSDDDEPNRVRPNNPL  327

Query  61    VTHQFFPEMDSNGGGVASG-------FPRAHWFGVKFCQSDLATGSSAGKATNVAAAVVE  113
             +THQFFPEMDS+GG    G       FPRAHWFGVKFCQSDLATGS  GKATN+A  VVE
Sbjct  328   ITHQFFPEMDSSGGDGGGGSGDVGSGFPRAHWFGVKFCQSDLATGS-GGKATNLATTVVE  504

Query  114   PAQPLKKsrrgprsrssQYRGVTFYRRTGRWESHIWDCGKQVYLGGFDTahaaaraydra  173
             PAQPLKKSRRGPRSRSSQYRGVTFYRRTGRWESHIWDCGKQVYLGGFDTAHAAARAYDRA
Sbjct  505   PAQPLKKSRRGPRSRSSQYRGVTFYRRTGRWESHIWDCGKQVYLGGFDTAHAAARAYDRA  684

Query  174   aIKFRGVEAdinfniddydddLKQMTNLTKEEFVHVLRRQSTGFPRGSSKYRGVTLHKCG  233
             AIKFRGVEADINFNI+DYDDDLKQMTNLTKEEFVHVLRRQSTGFPRGSSKYRGVTLHKCG
Sbjct  685   AIKFRGVEADINFNIEDYDDDLKQMTNLTKEEFVHVLRRQSTGFPRGSSKYRGVTLHKCG  864

Query  234   RWEARMGQFLGKKYVYLGLFDTEVEAARAYDKAAIKCNGKDAVTNFDPSIYDEELNAESS  293
             RWEARMGQFLGKKYVYLGLFDTEVEAARAYDKAAIKCNGKDAVTNFDPSIYD+ELNAESS
Sbjct  865   RWEARMGQFLGKKYVYLGLFDTEVEAARAYDKAAIKCNGKDAVTNFDPSIYDDELNAESS  1044

Query  294   GNPTTPQDHNLDLSLGNSANSKHKSQDMRLRMNQQQQDSLHSNEVLGLGQTGMLNHTPNS  353
             GNP TPQDHNLDLSLGNSANSK KSQDMRLRMNQQQ      NE+LGLGQTGMLNH
Sbjct  1045  GNP-TPQDHNLDLSLGNSANSKQKSQDMRLRMNQQQ-----DNEILGLGQTGMLNH----  1194

Query  354   NHQFPgssnigsgggf-sLFPAAENHRFDGRASTNQVLTNAAASSGFSPHHHNQIFNSTS  412
             NHQFPGSSNIG GGG  SLFP AEN R+DGR +TNQVL NAAASSGFSPHHHNQIFNSTS
Sbjct  1195  NHQFPGSSNIGGGGGGFSLFPVAENQRYDGRTTTNQVLANAAASSGFSPHHHNQIFNSTS  1374

Query  413   TPHQNWLQTNGFQPPLMRPS  432
             T HQNWLQ NGFQ PLMRPS
Sbjct  1375  TSHQNWLQANGFQNPLMRPS  1434
```

Figure 17. AP2 coding sequence alignment between *A. thaliana* and *L. didymum*

DISCUSSION

Within the already inherent diversity characterizing the Brassicaceae genera, in terms of leaf and fruit shape among others, *Lepidium* is well-recognizable for its infrageneric systematic complexity, which makes it necessary to extend the available genetic resources to deepening the molecular bases of the morphological variances over evolutionary time (J L Bowman 2006). What is more, these fruit characters have proven to be highly homoplasious (M. A. Koch and Mummenhoff 2006), and severe phenotypic differences can be explained by mutations of master morphogenetic genes, not always mandatorily linked to corresponding changes in the molecular marker system (Kadereit 1994; Theissen et al. 2000; R. Bateman and Dimichele 2002), conversely for example to the case of dehiscence fruit capability, which was considered a key trait in the systematics of Brassicaceae for years, until phylogeny reconstructions revealed the recurrent emergence of indehiscent fruits (M. Koch, Al-Shehbaz, and Mummenhoff 2003; K Mummenhoff et al. 2005; I. A. Al-Shehbaz, Beilstein, and Kellogg 2006).

In this work we have undertaken a two-fold approach to generate new tools for the study of morphological diversity and evolution within Brassicaceae, choosing *Lepidium didymum* as our working model species. First, we have performed an accurate morphological characterization of floral ontogeny and pistil and fruit development, to reveal the similarities and differences with well-studied species in the family such as *Arabidopsis thaliana* (J L Bowman, Smyth, and Meyerowitz 1989; Alvarez and Smyth 2002; Roeder and Yanofsky 2006), *Cardamine hirsuta* (Hofhuis et al. 2016) or other *Lepidium species* (Muhlhausen et al. 2013; Bowman et al. 1999). Our results allow to pinpoint several major characteristic features of *Lepidium didymum* floral development that could be useful for comparative development studies and to propose molecular mechanisms of morphological variation. Second, we have generated a genome-guided assembly of *Lepidium didymum* transcriptome. This transcriptome should be a useful resource to undertake future comparative studies at the molecular level and fulfils a gap in the genus, for which no transcriptome has been published till date.

Ontogenetical characteristics of *Lepidium didymum* in the context of the genus and the Brassicaceae family

First, we have observed that the patterns of petal and stamen initiation and development are similar to those described for other *Lepidium* species (John L Bowman and Smyth 1998), including the Brassicaceae-typical complement of four petals being initiated, but generally failing to grow, and the initiation of only two stamen primordia instead of the four or six primordia that emerge in most Brassicaceae species. These observations reinforce the idea of petal and stamen reduction as a distinctive feature of the *Lepidium* genus, and, moreover, suggest an independent mechanism for reduction of both types of floral organs: reduced growth of correctly initiated petals vs. reduction in stamen primordia specification, but unaffected stamen growth or development. Different hypothesis can be

proposed based on our knowledge of the genetic of flower development in *Arabidopsis*.

Indeed, a relevant role of well-known *Arabidopsis* homologues, also in the rest of the Brassicaceae, has been specifically proposed to support petal reduction (or even suppression in some species of *Lepidium*) as a result of specific disruptions in the function on petal-specific genes (John L Bowman and Smyth 1998). An example is the *Lepidium* reminiscent filamentous petals in *Arabidopsis aintegumenta short integument1* double mutants (Lee et al. 1997), or the *unusual floral organs* (*ufo*) mutants, which shows reduced growth of floral organs, specially petals and stamens, as a consequence of the misregulation of B-class gene function (Lee et al. 1997), although, so far, none of them have been thoroughly tested. Interestingly, recent work in *Cardamine hirsuta*, another Brassicaceae species with variable number of petals, have revealed that, in this species, variation in petal number is controlled genetically by several QTLs (Monniaux, Pieper, and Hay 2016), and that the differential regulation of the A-class gene APETALA1, likely by genetic functions associated with these QTLs, is responsible of the stochastic nature of the petal initiation in *Cardamine* flowers (Monniaux et al. 2018) However, it is interesting to note that, in *Cardamine*, the variation in petal number is linked to the failure to initiate the corresponding primordia and not to defective petal growth (Monniaux, Pieper, and Hay 2016); therefore, it is likely that the molecular basis for petal reduction in *Lepidium didymum* are completely different. Still, it will be interesting to make comparative analyses on the regulation and sequence variation of all the putative genes potentially involved in the different developmental outputs leading to petal formation and growth.

The differential pattern of stamen primordia initiation appears to underlie the reduction in stamen number in *Lepidium didymum,* as it has been also noted for other two-stamen *Lepidium* species (John L Bowman and Smyth 1998). Again, several hypothesis have been or can be proposed to explain this, such as alterations in auxin distribution, or a collateral effect of A class organ identity gene mutations, causing an anomalous increased expression of the C-class gene AGAMOUS (AG), which expands its repressing function to the third whorl (stamen) positions, thus reducing the number of primordia that arise (Weigel and Meyerowitz 1994; J L Bowman, Smyth, and Meyerowitz 1991; Liu and Meyerowitz 1995). Taking into account that lateral stamens are absent in transgenic plants with ectopic AG expression, it has been suggested that incremented C activity might be directly related to this phenotype (Jack, Sieburth, and Meyerowitz 1997). Other plausible scenario would be alterations in the function of the cadastral gene SUPERMAN, which control primordia initiation and identity at the frontier of the third and fourth whorls of the Arabidopsis flower (Schultz, Pickett, and Haughn 1991; J L Bowman et al. 1992; Sakai, Medrano, and Meyerowitz 1995). Again, to test these hypotheses, it would be necessary to address comparative studies of expression and sequence of the relevant genes.

On the other hand, we also move our focus of interest towards candidate genes influencing pistil and fruit shape, although these are still mostly unknown and the

sparse data comes from studies in *Arabidopsis*. *Lepidium didymum* pistils are formed, as in Arabidopsis and other Brassicaceae, by two congenitally fused carpels that, at first stages of development, grow as an elongated hollow cylinder similar to what is observed in *Arabidopsis*. From stage 8 and subsequently, the shape of the growing pistil is markedly different from that of similarly staged Arabidopsis pistil primordia, resulting in a mature disc-shaped gynoecium, where the length and width of the ovary are comparable, as opposed to the elongated shape of the Arabidopsis mature pistil, where length is several times higher than width. According to our observations, this is likely caused by restricted cell division and elongation in the apical-basal axis of the ovary, so it would be interesting to trace in more detail the patterns and orientation of cell division events to propose potential molecular mechanisms that explain these variations. Other striking difference with the development of the Arabidopsis pistil is the number of ovules produced (2 in *L. didymum* vs around 60 in Arabidopsis), which suggest that the medial meristem that produces the placentae may be functionally very different form that of *Arabidopsis* (Reyes-Olalde et al. 2013). Apart from these marked differences in proportions or ovule number, the *L. didymum* gynoecium comprises all types of functional domains and tissues observed in Arabidopsis, namely valves, valve margin, replum, gynophore, style and stigma, suggesting that the genetic networks directing the formation of these tissues should be equivalent to those described in *Arabidopsis* (Balanzá et al. 2006; Sundberg and Ferrándiz 2009; Ballester and Ferrandiz 2017), although differences in levels of expression or expression domains could still explain the different proportions of these tissues (take, for example, the higher levels of lignification observed in *L. didymum* when compared to Arabidopsis). In contrast, the markedly different fruit shape could be related to genetic functions that have been proposed to control these aspects, based on mutant analyses mostly in Arabidopsis, but also in other species. For example, it has become obvious that alterations in FUL activity, the principal regulator of valve development (Gu et al. 1998), may be involved in overall shape. It is suggestive that Brassicaceae species, as *Capsella rubella* and *Lepidium campestre* with essentially different wild-type fruit morphologies, exhibit a resembling phenotype when compared with loss-of-function ful alleles in Arabidopsis (Langowski, Stacey, and Ostergaard 2016); in addition, when Arabidopsis FUL is converted into a constitutive transcriptional activator by the translational fusion of a VP16 domain, the fruits are heart-shaped and the length/width ration is significantly reduced (Balanza et al. 2018). This potential role of FUL shaping the fruit is further reinforced by its down-regulation by DEVIL1 (DVL1), a member of a family of 21 small lateral organ development peptides, whose overexpression mediated by DVL5 leads to a miscellaneous of silique morphologies encompassing wider, diamond, arrowhead, or horned fruits (Wen, Lease, and Walker 2004). Additionally, Cytochrome P450s enzymes, in particular CYP78 (Nelson 1999; Bak et al. 2011; Mizutani and Ohta 2010; Schuler et al. 2006; Werck-Reichhart, Bak, and Paquette 2002) can play a relevant role in fruit shape formation. Thus, overexpression of CYP78A9 in *Arabidopsis* displays fruits with a phenotype halfway between round Lepidium and heart-shaped Capsella (Sotelo-Silveira et al. 2013).

As it is generally assumed the evolutionary origin of fruits from modified leaves, is expected that both organs share common developmental mechanisms (Balanzá et al. 2006; Reyes-Olalde et al. 2013; Vialette-Guiraud and Vandenbussche 2012), also as well between leaves and petals (Coen and Meyerowitz 1991; Goto, Kyozuka, and Bowman 2001). For example, the antagonistic activities reported in the distal growth of petals, among the endoreduplication inhibitor FRILL1 and the zinc finger transcription factor JAG (Y Hase et al. 2000; Yoshihiro Hase et al. 2005; Jose R Dinneny et al. 2004; Sauret-Gueto et al. 2013), postulate these transcription factors as presumptive participants in distal growth and expansion of fruit valves in heart-shaped fruits such as *Capsella* or *Coronopus*.

Moreover, many polar cell expansion and proliferation leaf development regulators have been previously identified in *Arabidopsis*, such as aintegumenta (ant) (Autran et al. 2002), struwwelpeter (swp) (Ito, Kim, and Shinozaki 2000); pointed first leaf2 (pfl2) (Mizukami and Fischer 2000) or brassinosteroid-related de-etiolated2 (det2), dwarf1 (dwf1) (Nakaya et al. 2002) rotundifolia3 (rot3) and rotundifolia4 (rot4) (Narita et al. 2004). Nevertheless, their more tan likely role driving fruit size and shape, as in the case of the subtle phenotype showed by functionally defective ANGUSTIFOLIA *Arabidopsis* mutants (G.-T. Kim et al. 2002), has not been firmly contemplated, mainly due the disparate and relatively too simple silique structure of the *Arabidopsis* wild-type fruit (Y. Bai et al. 2010; B. Bai et al. 2013), and hence it may be more informative to study the eventual morphology alterations in heart-shaped fruit forms.

A new tool for molecular comparative studies in the Brassicaceae

In this work, we propose a novel model system for comparative evo-devo studies, which, despite being closely related to Arabidopsis thaliana, exhibit, marked morphological and developmental differences. Our detailed description of the developmental events occurring in several organs, as well as the availability of a newly assembled transcriptome, will provide the necessary toolkit to initiate molecular genetic analyses in this species and will help to further our understanding of the evolutionary processes that have led to the extant rich variation in fruit morphology in the Brassicaceae. For the transcriptome assembly, we took advantage of the availability of a sequenced genome of a closely related species, *L. meyenii* (Zhang et al. 2016), which we used as a reference to assemble the leaf and inflorescence transcriptomes of *L. didymum.* In turn, our assembly could help to scaffold the genome, correct the annotation or refine the proposed gene models in the *L. meyenii* genome. Previous authors successfully implemented a strategy to study the molecular bases of leaf morphological variation between *Cardamine hirsuta* and *A. thaliana* (Hay et al. 2014). Following a similar approach, our assembly of the *L. didymum* transcriptome, combined with the abundant genetic resources that are available for *Arabidopsis*, will enable the identification of species-specific factors involved in the morphological changes observed among closely related species (Vlad et al. 2014).

Despite exhibiting distinct fruit morphologies, previous phylogenetic analyses made with nuclear (ITS) and cpDNA markers have shown that all *Lepidium*-related genera are well nested within *Lepidium s. str*. (Klaus Mummenhoff et al. 2009), pointing to limited value of fruit characters in traditional systematic studies (I. A. Al-Shehbaz 1986; I. A. Al-Shehbaz, Beilstein, and Kellogg 2006). The sequence data generated in this work will enable functional studies of the *Coronopus* homologs of important developmental regulators, which were first identified using *Arabidopsis* as a model organism. The anatomical and transcriptomic data reported in this work will fuel future evo-devo studies at investigating the evolutionary trends and molecular bases of the extant variation in developmental processes and organ morphology occurring in nature.

CONCLUSIONS

CHAPTER 1

1. The available experimental data till date are not sufficient to explain the emergence of the four different tissues shaping the DZ in the Arabidopsis fruit.

2. The proposal of novel hypothetical interactions and the incorporation of NTT as a crucial component of the GRN directing the mediolateral axis, allowed us to largely recover the expected dynamical behaviour of the DZ components.

3. We present the first integrative model to formally tackle the dynamic mechanism of the *A. thaliana* DZ specification, thus feeding future experimental work and modelling studies, which the aim of deepening the current understanding of the pod shatter process and ultimately facilitate the biotechnological manipulation of fruit characters in crop species.

CHAPTER 2

1. Using a transcriptomics approach, we have identified the SHN clade of transcription factors as downstream effectors positively regulated by HEC3.

2. We uncovered a novel role of SHN genes in the development of the Arabidopsis transmitting tract.

3. This work provides the first transcriptomics profiling of HEC3 and sheds further light to decode HEC function in the GRNs directing gynoecium development.

4. HEC3 appears to be a master regulator of cell wall properties, likely responsible not only of initial steps of transmitting tract specification, but also of subsequent differentiation.

5. Our data reveal a novel role of HEC3 as a putative repressor of the CK inhibitor CKX3 and a positive regulator of the transcription factor NTT, whose protein interacts with several gynoecium-related transcription factors and loss-of-function NTT mutant phenotype displays severe defects in the Arabidopsis transmitting tract.

6. HEC3 could act as an IND modulator and might be involved in the DZ formation considering our obtained data on NTT and SHN genes as HEC3 targets.

CHAPTER 3

1. We propose *L. didymum* as an alternative model system to study the underlying molecular basis of changes in fruit morphology and dehiscence, building on anatomical and transcriptomic results.

2. Our accurate morphological characterization of floral ontogeny and pistil and fruit development revealed the similarities and differences with well-studied species in the Brassicaceae family, such as *A. thaliana*, *Cardamine hirsute* or other Lepidium species. These L. didymum characteristic features on floral development will be the basis for future comparative developmental analyses.

3. We obtained the first Lepidium transcriptome assembly and we found numerous genes encoding homologs of well-known developmental regulators, which provide a framework for future evolutionary and comparative functional studies.

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