UNIVERSITAT POLITÈCNICA DE VALÈNCIA

Gibberellins and ovule number: a molecular mechanism

PhD Thesis

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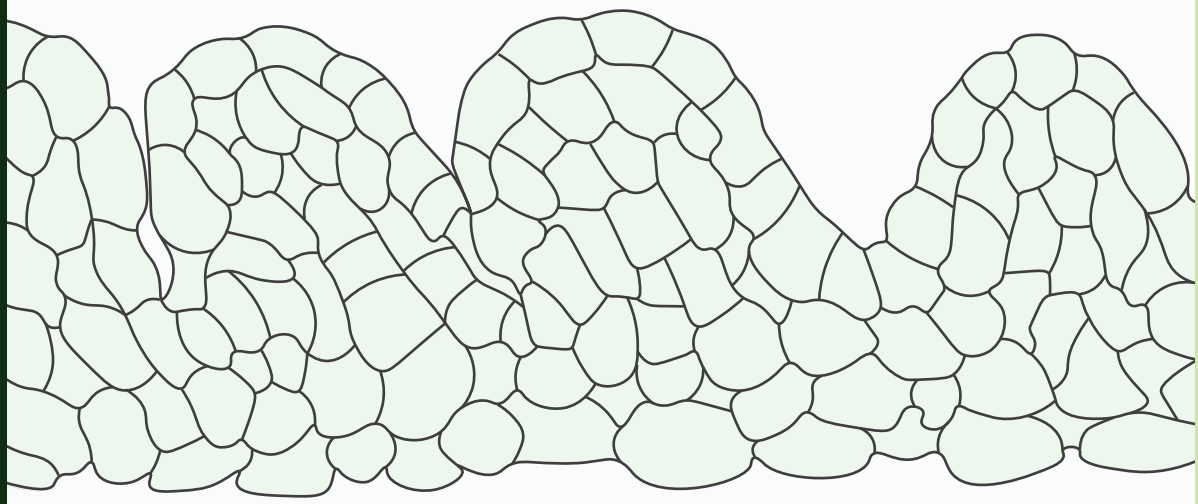

Advisors: Dr. Miguel Á. Pérez Amador
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SUMMARY

As precursors of seeds, ovules represent a fundamental organ during the plant life cycle. Due to their importance, ovule development has been studied for decades from a morphological and molecular point of view, allowing the elucidation of a complex and intricate gene regulatory network governing it. Specifically, ovule initiation is controlled by the plant hormones auxins, cytokinins and brassinosteroids (BRs), all of them being positive regulators of ovule number. Recently, we demonstrated that gibberellins (GAs) negatively module ovule number by the destabilization of DELLA proteins. However, how GAs and DELLA proteins fit in the regulatory model for ovule initiation still needs to be clarified. The work presented in this PhD thesis aims to clarify the molecular mechanism by which GAs act in ovule initiation. After a comprehensive introduction, we show in Chapter 1 that both GAs and BRs regulate ovule number in Arabidopsis regardless of the activity levels of the other hormone, suggesting that GAs and BRs act independently to control ovule initiation. In Chapter 2 we provide genetic and molecular evidence pointing to DELLA proteins participating in ovule initiation by the interaction with the CUC2 transcription factor in placental cells. Collectively, the findings presented here allowed us to integrate GAs and DELLA proteins in the gene regulatory network guiding ovule primordia initiation. A final discussion highlights open questions that still need to be addressed to fully understand the hormonal control of ovule initiation in plants.

RESUMEN

Como precursores de las semillas, los óvulos representan un órgano fundamental durante el ciclo de vida de las plantas. Debido a su importancia, el desarrollo del óvulo ha sido estudiado durante décadas desde un punto de vista morfológico y molecular, lo que ha permitido dilucidar la compleja e intrincada red de regulación genética que lo rige. En concreto, la iniciación del óvulo está controlada por las hormonas vegetales auxinas, citoquininas y brasinoesteroides (BRs), siendo todas ellas reguladoras positivas del número de óvulos. Recientemente demostramos que las giberelinas (GAs) modulan negativamente el número de óvulos mediante la desestabilización de las proteínas DELLA. Sin embargo, aún debe aclararse cómo encajan las GAs y las proteínas DELLA en el modelo regulador de la iniciación de los óvulos. El trabajo presentado en esta tesis doctoral tiene como objetivo aclarar el mecanismo molecular por el cual las GAs actúan en la iniciación del óvulo. Después de una introducción general, en el Capítulo 1 mostramos que tanto las GAs como los BRs regulan el número de óvulos en Arabidopsis independientemente de los niveles de actividad de la otra hormona, lo que sugiere que las GAs y los BRs actúan de forma independiente para controlar la iniciación del óvulo. En el Capítulo 2 proporcionamos evidencias genéticas y moleculares que apuntan a que las proteínas DELLA participan en la iniciación de los óvulos mediante su interacción con el factor de transcripción CUC2 en las células placentarias. En conjunto, los hallazgos presentados aquí nos han permitido integrar a las GAs y proteínas DELLA en la red genética que guía el inicio de los primordios de óvulos. Una discusión final destaca las preguntas abiertas que aún deben abordarse para comprender completamente el control hormonal de la iniciación de los óvulos en las plantas.

RESUM

Com a precursors de les llavors, els òvuls representen un òrgan fonamental durant el cicle de vida de les plantes. A causa de la seva importància, el desenvolupament de l'òvul ha estat estudiat durant dècades des d'un punt de vista morfològic i molecular, el que ha permès dilucidar la complexa i intricada xarxa de regulació genètica que el regeix. En concret, la iniciació del òvul està controlada per les hormones vegetals auxines, citoquinines i brasinoesteroides (BRs), sent totes elles reguladores positives del nombre d'òvuls. Recentment demostrem que les gibberel · lines (GAs) modulen negativament el nombre d'òvuls mitjançant la desestabilització de les proteïnes DELLA. No obstant, encara s'ha d'aclarir com encaixen les GAs i les proteïnes DELLA al model regulador de la iniciació dels òvuls. El treball presentat en aquesta tesi doctoral té com a objectiu aclarir el mecanisme molecular pel qual les GAs actuen a la iniciació de l'òvul. Després d'una introducció general, al Capítol 1 mostrem que tant les GAs com els BRs regulen el nombre d'òvuls a Arabidopsis independentment dels nivells d'activitat de l'altra hormona, cosa que suggereix que les GAs i els BRs actuen de forma independent per controlar la iniciació de l'òvul. Al Capítol 2 proporcionem evidències genètiques i moleculars que apunten que les proteïnes DELLA participen en la iniciació dels òvuls mitjançant la seva interacció amb el factor de transcripció CUC2 a les cèl · lules placentàries. En conjunt, els descobriments presentats ací ens han permès integrar les GAs i proteïnes DELLA a la xarxa genètica que guia l'inici dels primordis d'òvuls. Una discussió final destaca les preguntes obertes que encara cal abordar per comprendre completament el control hormonal de la iniciació dels òvuls a les plantes.

Para mis padres

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TABLE OF CONTENTS

LIST OF FIGURES

Introduction

Chapter 1

Chapter 2

General discussion

LIST OF TABLES

Chapter 1

Chapter 2

ABBREVIATIONS

ABA Abscisic Acid
ABI ABA INSENSITIVE
ANT AINTEGUMENTA
AP APETALA
ARF AUXIN RESPONSE FACTOR
ARR ARABIDOPSIS RESPONSE REGULATOR
ATAF ARABIDOPSIS TRANSCRIPTION ACTIVATOR FACTOR
ATS ABERRANT TESTA SHAPE
BAK BRI1-ASSOCIATED RECEPTOR KINASE
BES BRI1-EMS-SUPPRESSOR
bHLH basic Helix-Loop-Helix
BIN BRASSINOSTEROID INSENSITIVE
BR(s) Brassinosteroid(s)
BRI BRASSINOSTEROID INSENSITIVE
BRZ Brassinazole
BSU BRI1 SUPPRESSOR
bZIP basic leucine Zipper
BZR BRASSINAZOLE-RESISTANT
ChIP Chromatin Immunoprecipitation
CK(s) Cytokinin(s)
CKX CYTOKININ OXIDASE
CMM Carpel Medial Meristem
Co-IP Co-Immunoprecipitation
CRF CYTOKININ RESPONSE FACTOR
CUC CUP-SHAPED COTYLEDON
DET DE-ETIOLATED
EBR 24-Epibrassinolide
GA(s) Gibberellin(s)
GA20ox GIBBERELLIN 20-OXIDASE
GA2ox GIBBERELLIN 2-OXIDASE
GA3ox GIBBERELLIN 3-OXIDASE
GAI GIBBERELLIC ACID INSENSITIVE
GFP Green Fluorescent Protein
GID GIBBERELLIN INSENSITIVE DWARF
GRAS GAI/RGA/SCARECORW
GRF GROWTH-REGULATING FACTOR
HLL HUELLENLOS
MP MONOPTEROS
mRFP monomeric Red Fluorescent Protein
MS Murashige and Skoog
NAC NAM/ATAF/CUC
NAM NO APICAL MERISTEM
PBZ Paclobutrazole
PIF PHYTOCHROME INTERACTING FACTOR
PIN PIN-FORMED
REM REPRODUCTIVE MERISTEM
RGA REPRESSOR OF *ga1-3*
RGL RGA-LIKE
SAM SHOOT APICAL MERISTEM
SCL SCARECROW LIKE
SEP SEPALLATA
SLY SLEEPY
SPT SPATULA
SPY SPINDLY
STK SEEDSTICK
STM SHOOT MERISTEMLESS
TCP TEOSINTE BRANCHED1 CYCLOIDEA PCF1
TES Transcription End Site
TF(s) Transciption Factor(s)
TR(s) Transcriptional Regulator(s)
TSS Transcriptional Start Site
UBQ UBIQUITIN
UGT UDP-glucosyl transferase
UTR Untranslated region
VENUS Variant of YFP
Y2H Yeast Two-Hybrid
YFP Yellow Fluorescent Protein
YPet Variant of YFP

GLOSSARY OF MUTANTS

The mutants and lines used in this work were:

35S:ANT overexpression line (Barro-Trastoy et al. 2020b).

4xdella (*rgaT2 gaiT6 rgl1-1 rgl2-1*), quadruple loss-of-function *della* mutant (Achard et al. 2006)

ant-4 (*aintegumenta-4*), loss-of-function mutant. Derived from a ethyl-nitrosourea mutagenesis screening (Baker et al. 1997).

bzr1-1D (*brassinazole-resistant 1-1D*), gain-of-function mutant with increased BR response. It presents a single base pair change that results a proline-to-leucine substitution. This substitution leads to the accumulation of unphosphorylated BZR1 (Wang et al. 2002).

cuc1-1 (*cup-shaped cotyledon1-1*), loss-of-function mutant. It presents a single base pair change that results in a lysine-to-threonine substitution (Takada et al. 2001).

cuc2-1 (*cup-shaped cotyledon2-1*), loss-of-function mutant. It carries a Tag1 transposon insertion in the first exon (Aida et al. 1997).

cuc2-3 (*cup-shaped cotyledon2-3*), loss-of-function mutant. It carries a T-DNA insertion upstream of the ATG (Hibara et al. 2006).

det2-1 (*de-etiolated 2-1*), loss-of-function mutant, defective in early stage BR biosynthesis (Chory et al. 1991).

gai-1 (*gibberellic acid insensitive-1*), gain-of-function mutant with constitutive blockage of GA response (insensitive to GAs). It presents a deletion of 17 amino acids within the N-terminal region, which contain the DELLA motif (Peng and Harberd 1993; Peng et al. 1997).

gaiT6 (*gibberellic acid insensitive-T6*), loss-of-function mutant. It carries a Ds transposon insertion (Peng et al. 1997).

global (*rgaT2 gaiT6 rgl1-1 rgl2-1 rgl3-1*), pentuple loss-of-function *della* mutant (Feng et al. 2008).

pCUC1:CUC1m-GFP *miR164*-resistant version of *CUC1*. *CUC1* coding region presents eight silent mutations (Baker et al. 2005).

pCUC2:CUC2m-GFP *miR164*-resistant version of *CUC2*. *CUC2* coding region presents eight silent mutations (Sieber et al. 2007).

rgaT2 (*repressor of ga-T2*), loss-of-function mutant. It carries a Ds transposon insertion (Lee et al. 2002).

rgl1-1 (*rga-like 1-1*), loss-of-function mutant. It carries a Ds transposon insertion (Lee et al. 2002).

rgl2-1 (*rga-like 2-1*), loss-of-function mutant. It carries a Ds transposon insertion (Lee et al. 2002).

rgl3-1 (*rga-like 3-1*), loss-of-function mutant. It carries a T-DNA insertion (Feng et al. 2008).

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Takada et al. (2001). *Development*, (128), 1127–1135. doi: 10.1242/dev.128.7.1127

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LIST OF GENES

The locus IDs of the genes mentioned in this document are:

ABI3 *At3g24650*
ABI5 *At2g36270*
ANT *At4g37750*
AP2 *At4g36920*
ARF6 *At1g30330*
ARR1 *At3g16857*
ATAF1 *At1g01720*
ATAF2 *At5g08790*
ATS *At5g42630*
BAK1 *At4g33430*
BES1 *At1g19350*
BIN2 *At4g18710*
BRI1 *At4g39400*
BSU1 *At1g03445*
BZR1 *At1g75080*
CKX3 *AT5G56970*
CKX5 *At1g75450*
CRF2 *At4g23750*
CRF3 *At5g53290*
CRF6 *At3g61630*
CUC1 *At3g15170*
CUC2 *At5g53950*
CUC3 *At1g76420*
DET2 *At2g38050*
GA20ox1 *At4g25420*
GA20ox2 *At5g51810*
GA20ox3 *At5g07200*
GA20ox4 *At1g60980*
GA20ox5 *At1g44090*
GA2ox1 *At1g78440*
GA2ox2 *At1g30040*
GA2ox3 *At2g34555*
GA2ox4 *At1g47990*
GA2ox5 *At3g17203*
GA2ox6 *At1g02400*
GA2ox7 *At1g50960*
GA2ox8 *At4g21200*
GA3ox1 *At1g15550*
GA3ox2 *At1g80340*
GA3ox3 *At4g21690*
GA3ox4 *At1g80330*
GAI *At1g14920*
GID1A *At3g05120*
GID1B *At3g63010*
GID1C *At5g27320*
GRF1 *At2g22840*
HLL *At1g17560*
MIR164A *At2g47585*
MIR164B *At5g01747*
MIR164C *At5g27807*
MP *At1g19850*
NAM *At1g52880*
PIF4 *At2g43010*
PIN1 *At1g73590*
PIN3 *At1g70940*
REM23 *At2g35310*
RGA *At2g01570*
RGL1 *At1g66350*
RGL2 *At3g03450*
RGL3 *At5g17490*
SCL3 *At1g50420*
SEP2 *At2g21970*
SLY1 *At4g24210*
SPY *At3g11540*
STK *At4g09960*
STM *At1g62360*
UBQ10 *At4g05320*
UGT73C1 *At2g36750*
UGT85A3 *At1g22380*

Introduction

INTRODUCTION

In angiosperms, ovules are formed inside the gynoecium, the female reproductive part of flowers. Specifically in *Arabidopsis thaliana* (Arabidopsis thereafter), the gynoecium is composed of a single pistil formed by two congenitally fused carpels that emerge from the fourth whorl at the center of the flower. During pistil development, a group of meristematic cells located at the lateral margins of the carpels (named carpel margin meristem, CMM) expands toward the center and give rise to the septum and placentas. Ovules develop later from the placental tissues (Simonini and Østergaard 2019; Herrera-Ubaldo and de Folter 2022).

Mature ovules are composed of three different morphological structures. These structures are, from terminal to basal, the nucellus, the chalaza and the funiculus (Schneitz et al. 1995). The nucellus is where megasporogenesis and megagametogenesis occur to finally form the embryo sac. From chalaza, two integuments grow and encase the nucellus leaving an opening at the apex (the micropyle) through which the pollen tube can access the embryo sac and fertilize the egg cell and the two polar nuclei. The funiculus is a stalk-like and vascularized structure that connects the ovule to the placenta (Schneitz et al. 1995).

Ovule development has been extensively studied at the morphological level, specially in Arabidopsis (**Fig. I.1**). Nowadays, several qualitative descriptions (Robinson-Beers et al. 1992; Schneitz et al. 1995; Christensen et al. 1997) and some quantitative cellular characterizations have been published (Lora et al. 2017; Hernandez-Lagana et al. 2021; Vijayan et al. 2021, 2022). First, a mass of cells in the placenta divide and protrude to define and form ovule primordia (process named ovule initiation). Then, the incipient primordia grow and the protruded mass of cells is organized to define the nucellus, chalaza and funiculus (patterning). At later stages, outer and inner integuments differentiate and grow from the chalaza, and megasporogenesis and megagametogenesis take place (morphogenesis).

Once fertilized, mature ovules become into seeds. The zygote and trinuclear central cell become to the embryo and endosperm, respectively, and the integuments differentiate into a seed coat (Phillips and Evans 2020). Thus, seed development is intimately linked to proper ovule development prior to fertiliza-

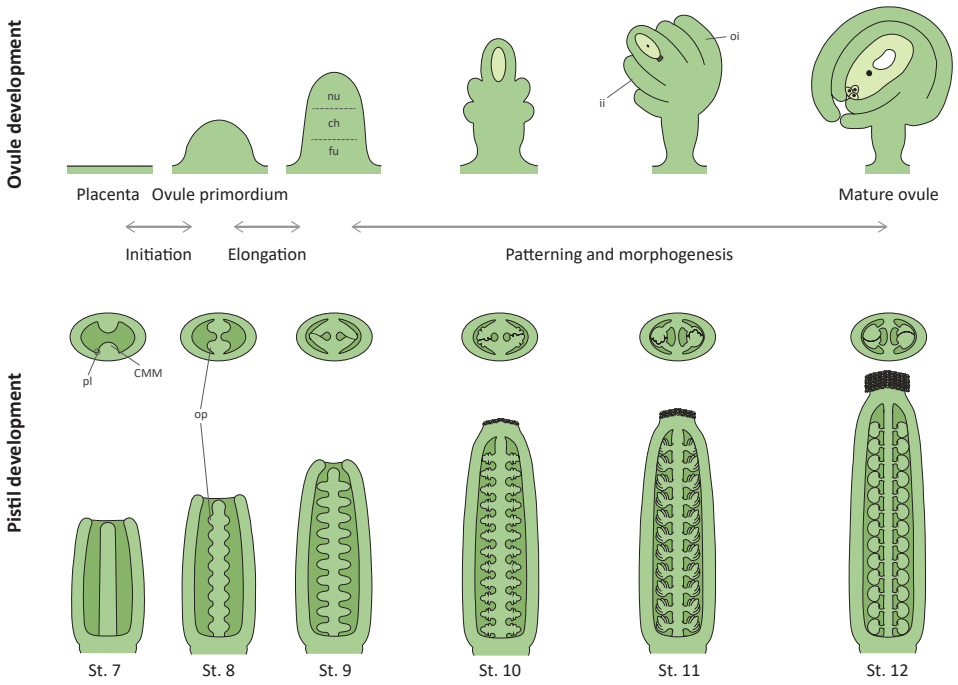

Fig. I.1: Schematic illustrations of ovule and pistil development in Arabidopsis. The illustrations represent ovule development stages (top), transversal sections (middle), and longitudinal sections (bottom) of stages 7–12 of pistil development. ch: chalaza; CMM: carpel margin meristem; fu: funiculus; ii: inner integument; nu: nucellus; op: ovule primordium; oi: outer integument; pl: placenta; St.: stage. Adapted from Barro-Trastoy et al. (2020a).

tion. For its part, seeds play a central biological role in the plant life cycle as they ensure plant reproduction, but also form the basis of agriculture. This highlight the importance of understanding the molecular mechanisms that govern seed and ovule development: seed number per fruit and seed quality will directly depend on proper ovule initiation and morphogenesis, and fine-tuning this processes could be an strategy for improving crop yields. In this thesis we are going to focus on the molecular mechanism that control ovule initiation and, consequently, ovule number, paying special attention at the role of plant hormones in this key developmental process.

OVULE INITIATION

Ovule primordia arise from the placenta as a consequence of the division of subepidermal placental cells. This process occur at stage 8-9 of flower development (Smyth et al. 1990; Schneitz et al. 1995) and is guided by a gene regulatory network that coordinates the action of several transcription factors (TFs) and hormonal signaling pathways (**Fig. I.2**) (Cucinotta et al. 2020; Herrera-Ubaldo and de Folter 2022; Barro-Trastoy et al. 2020a).

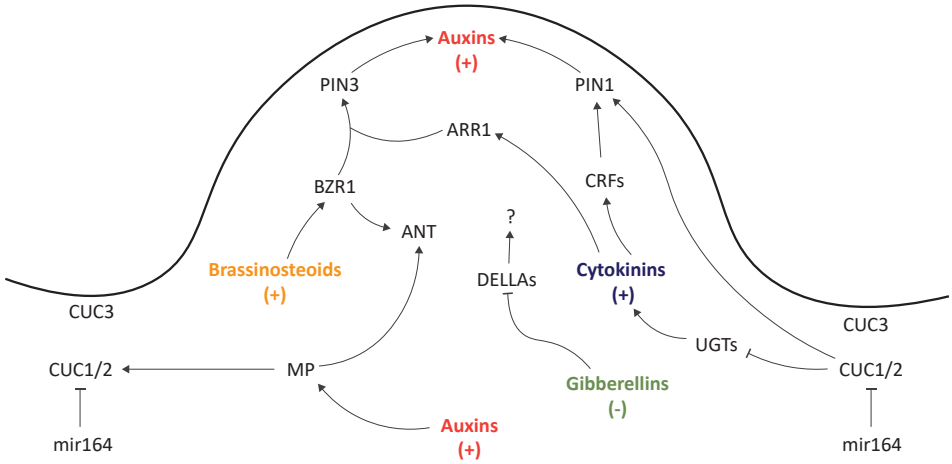

Fig. I.2: Simplified model for the regulation of ovule initiation in Arabidopsis. Different colors represent different plant hormones.

A key step of ovule initiation is the establishment of primordium boundaries and the definition of the zone of primordium outgrowth. Some TFs were involved in these processes. AINTEGUMENTA (ANT, an AP2 TF) is a key positive regulator of ovule primordia growth (Elliott et al. 1996; Klucher et al. 1996; Baker et al. 1997). *ANT* expression is found in the placenta and the ovule primordia, and *ant* mutations lead to a reduction of more than half of ovules in comparison to wild type, without a concomitant reduction in pistil length (Klucher et al. 1996; Liu et al. 2000). CUP-SHAPED COTYLEDON1 (CUC1), CUC2 and CUC3 (NAC TFs) have a role in ovule primordia boundaries definition (Ishida et al. 2000; Gonçalves et al. 2015). *CUC* genes are expressed in placenta and/or ovule primordia boundaries, and lack of both CUC1 and CUC2 show reductions in ovule

number as well as aberrant spacing between ovule primordia (Ishida et al. 2000; Galbiati et al. 2013; Gonçalves et al. 2015). Both ANT and CUC TFs are interconnected with hormone homeostasis and signaling pathways, mainly with auxins, cytokinins (CKs) and brassinosteroids (BRs).

As in other plant developmental processes, auxin accumulation in the ovule initiation site is essential to promote ovule primordia formation (Benková et al. 2003; Ceccato et al. 2013). The generation of this auxin maxima is led by polar auxin transport (Benková et al. 2003; Ceccato et al. 2013; Larsson et al. 2014; Hu et al. 2022) and probably by local auxin biosynthesis (Nole-Wilson et al. 2010a). PIN-FORMED1 (PIN1) is an auxin efflux carrier well known to direct auxin accumulation to the tip of ovule primordia (Benková et al. 2003; Ceccato et al. 2013; Yu et al. 2020). The weak *pin1-5* mutant produces pistils with a dramatic reduction in ovule density, suggesting that in fact PIN1 is required for ovule initiation (Bencivenga et al. 2012). It was shown that CUC1 and CUC2 are redundantly promoting *PIN1* expression and correct PIN1 membrane localization in ovule primordia (Galbiati et al. 2013). Additionally, both *CUC1* and *CUC2* are directly and positively regulated by MONOPOTEROS/AUXIN RESPONSE FACTOR 5 (MP/ARF5) (Galbiati et al. 2013), which also directly induces *ANT* expression (Galbiati et al. 2013; Yamaguchi et al. 2013). For its part, it was proposed that ANT could be regulating expression levels of several auxin biosynthesis genes, suggesting a role for ANT in auxin homeostasis at least in young pistils (Krizek et al. 2020; Nole-Wilson et al. 2010a).

CKs are also essential for ovule initiation. Several high order mutants with reduced CK signaling present severe reductions on ovule number (Bencivenga et al. 2012; Cucinotta et al. 2016; Zu et al. 2021). On the contrary, high CK levels induces both pistil growth and increased ovule density (Bartrina et al. 2011; Cucinotta et al. 2016). It has been shown that CKs directly regulate *PIN1* expression in ovule primordia through several CYTOKININ RESPONSE FACTORS (CRF), CRF2, 3 and 6 (Cucinotta et al. 2016). This supports an auxin-CK crosstalk guiding ovule initiation. What is more, CUC1 and CUC2 influence CK homeostasis as they repress the expression of *UGT73C1* and *UGT85A3*, genes that encode UDP-glucosyl transfesares that catalyse the reversible inactivation of CKs (Cucinotta et al. 2018). Indeed, pistils from plants lacking both *CUC1* and *CUC2* in

placenta have lower active CK levels (Cucinotta et al. 2018), and their lower ovule number is restored upon CK treatments (Galbiati et al. 2013). In summary, CKs are positive factors of ovule number determination through *PIN1*, whose levels are controlled by CUCs, and which in turn also are, as mentioned before, regulated by auxins.

BRs have been described as positive regulators of ovule initiation (Huang et al. 2013a). Ovule number is decreased in reduced BR-signaling mutants and plants with reduced BR levels, whereas it is increased in enhanced BR-signaling mutants (Huang et al. 2013a; Jia et al. 2020; Zu et al. 2021; Nole-Wilson et al. 2010b). It was proposed that BRs increase ovule initiation by inducing *ANT* expression (Huang et al. 2013a). New studies suggest that, indeed, CKs interact with BRs to coordinately regulate downstream genes expression, being *ANT* among them (Zu et al. 2021).

Recently, an asynchronous initiation of ovule primordia has been elucidated (Yu et al. 2020; Hu et al. 2022). During flower development, there are a group of ovule primordia that arise firstly from placenta (early initiation). Then, placenta elongates as pistil development go on, the boundaries between ovules enlarge, and a second group of primordia protrude in the space between ovule primordia already initiated (late initiation) (Yu et al. 2020). It was proposed that this asynchrony depends on proper polar auxin transport mediated by PIN3 (Hu et al. 2022). PIN3 is detected in some placental cell prior to ovule initiation (Larsson et al. 2014; Hu et al. 2022) and, as PIN1, in epidermal cells of ovule primordia with its polarity pointing toward the tips (Ceccato et al. 2013; Yu et al. 2020). However, unlike PIN1, lack of PIN3 lead to a slight reduction of ovule density. This reductions seems to be due to a lack of secondary initiated ovules, suggesting that PIN3 is needed for late ovule initiation (Hu et al. 2022). Furthermore, *PIN3* expression (but not PIN3 localization) is positively regulated by BRs at ovule primordia, auxin response at primordium tips increase upon BR treatments, and ovule number of *pin3* with enhanced BR-signaling is equal to that of single *pin3* mutant. These observations point that PIN3 is needed for BR-mediated ovule initiation, and highlight an auxin-BRs crosstalk occurring on this developmental process (Hu et al. 2022).

GAs, in addition to auxins, BRs and CKs, also play a key role in ovule ini-

tiation, as our laboratory has demonstrated (Gomez et al. 2018). In this thesis we study the molecular mechanism of gibberellins in determining ovule number in Arabidopsis.

GIBBERELLINS

Gibberellins (GAs) are broadly known as regulators of a wide range of plant developmental processes through the whole life cycle of plants (Davière and Achard 2013). They promote plant growth via cell proliferarion and elongation (Cowling and Harberd 1999; Achard et al. 2009), stimulate seed germination (Ogawa et al. 2003; Tyler et al. 2004), trigger floral transition (Blázquez et al. 1998), control male fertility (Plackett et al. 2011), participate in the control of ovule morphology (Gomez et al. 2016), and induce fruit set (Dorcey et al. 2009). Moreover, GAs are also involved in the response to enviromental stimuli (de Lucas et al. 2008; Stavang et al. 2009; Gallego-Bartolomé et al. 2011b) and the defense against abiotic (Achard et al. 2006) and biotic (Navarro et al. 2008) stresses.

Chemically, GAs are tetracyclic diterpenoid acids. In most of land plants, the first steps of GA biosynthesis take place mainly in plastids through the methylerythritol 4-phosphate pathway, which yields *ent*-kaurene (Kasahara et al. 2002; van Schie et al. 2007). Then, *ent*-kaurene is sequentially oxidized in plastids and endoplasmatic reticulum membranes to yield GA_{12}. Subsequent steps to form bioactive GAs in plants (GA_1 and GA_4) occur in the cytosol, and involve a multigenic family of 2-oxoglutarate-dependent dioxygenases (2-OGD) named GA20 oxidases (GA20ox) and GA3 oxidases (GA3ox) (Yamaguchi 2008). Bioactive GAs can be inactivated, among other processes, by another 2-OGDs, named GA2 oxidases (GA2ox) (Rieu et al. 2008). All these processes are tightly regulated by both environmental and endogenous signals to fine-tune GA levels in response to different stimuli (Yamaguchi 2008).

GA signaling pathway relies mostly on the degradation or accumulation of DELLA proteins, nuclear proteins that belong to the GRAS family of transcriptional regulators and antagonize GA responses (Silverstone et al. 2001). This mechanism begins with the binding of bioactive GAs to GIBERELLIN INSENSITIVE DWARF1 (GID1), a soluble receptor present in both the cytoplasm and the

nucleus (**Fig. I.3**) (Ueguchi-Tanaka et al. 2005; Murase et al. 2008). After this, GID1 suffers an allosteric change that blocks the GA molecule and induces the interaction between its N-terminal extension and the N-terminal domain of DELLA proteins, leading to a conformational change in DELLA structure (Willige et al. 2007; Shimada et al. 2008). The GA-GID1-DELLA complex can then promote the recruitment of a SCF E3 ubiquitin ligase complex through the F-box protein SLEEPY1 (SLY1)/GA INSENSITIVE DWARF2 (GID2), which causes the attachment of polyubiquitin chains to DELLA proteins for their subsequent degradation

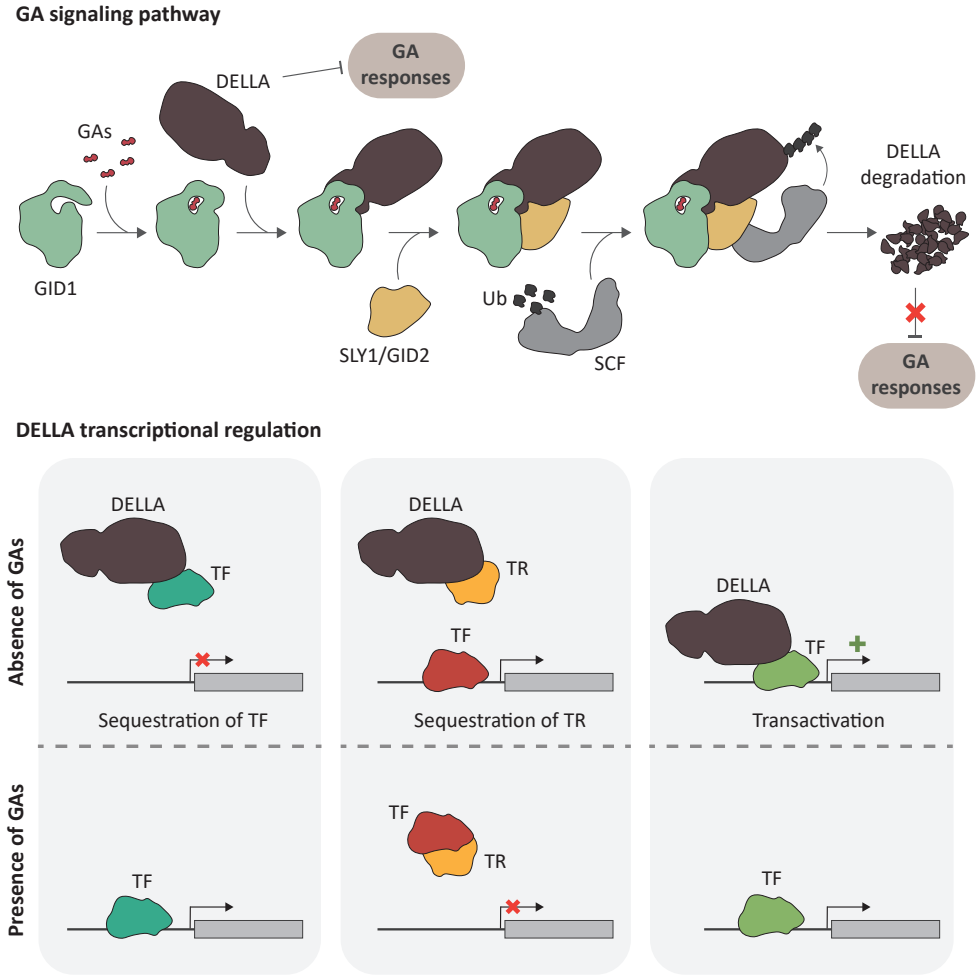

Fig. I.3: GA signaling and mechanisms of DELLA transcriptional regulation.

by the 26S proteasome (Ariizumi et al. 2011). As a result, when the bioactive GA levels are high, DELLA degradation is promoted and GA responses are released. On the contrary, when bioactive GA levels are low, DELLA proteins accumulate and restrain GA responses (**Fig. I.3**).

DELLA proteins present a conserved C-terminal GRAS domain involved in protein-protein interactions and a unique N-terminal regulatory domain that includes the DELLA (Asp-Glu-Leu-Leu-Ala) motif, which is crucial for the GA-induced GID1 binding (Vera-Sirera et al. 2016). Although DELLA proteins lack typical DNA-binding domains, they act as transcriptional regulators by interacting, through their GRAS domain, with a wide variety of TFs and transcriptional regulators (TRs) (Marín-de la Rosa et al. 2014; Hernández-García et al. 2020; Lantzouni et al. 2020). In some cases, DELLA interaction with TFs prevents their binding with the promoter of their target genes (sequestration of TFs, **Fig. I.3**). In other cases, DELLA interacts with TRs to inhibits their interaction with TFs, and thus allowing gene expression (sequestration of TRs, **Fig. I.3**). Also, DELLA proteins can associate with DNA through the binding with the TFs and act as transactivators (transactivation, **Fig. I.3**) (Davière and Achard 2016).

The ability of DELLA proteins to regulate gene expression through their interaction with hundreds of proteins allow them to coordinate the previously mentioned variety of regulatory processes, and connect GAs with other plant hormone signaling cascades and environmental clues (Davière and Achard 2016; Thomas et al. 2016). For instance, GA regulation of seed germination relies on the transactivation by DELLA proteins of ABA-insensitive3 (ABI3) and ABI5, TFs that are activated by abscisic acid (ABA) and inhibit germination (Lim et al. 2013). Root growth is controlled by DELLA proteins also by the transactivation of CK-regulated genes through type-B ARABIDOPSIS RESPONSE REGULATORs (ARRs) (Moubayidin et al. 2010; Marín-de la Rosa et al. 2014). Hypocotyl cell elongation, which is controlled by AUXIN RESPONSE FACTOR6 (ARF6), BRASSINAZOLE-RESISTANT1 (BZR1) and PHYTOCHROME-INTERACTING FACTOR4 (PIF4) (a TF regulated by auxins, a positive regulator of the BR-signaling and a TF regulated by light and temperature, respectively), is repressed by DELLA proteins as they sequestrate these factors (Oh et al. 2014).

The Arabidopsis genome encodes three GID1 receptors (GID1A, GID1B and

GID1C) (Nakajima et al. 2006) and five DELLA proteins (GIBBERELLIC ACID INSENSITIVE, GAI; REPRESSOR OF *ga1-3*, RGA; RGA-LIKE1, RGL1; RGL2 and RGL3) (Peng et al. 1997; Silverstone et al. 1998; Dill and Sun 2001; Wen and Chang 2002) with partially overlapping functions. Complete loss-of-function of DELLA activity causes a constitutive GA response, whereas mutant proteins lacking the N-terminal DELLA motif, such as in the *gai-1* allele (Peng and Harberd 1993) or the *pRGA:GFP-rgaΔ17* and *pRGL2:YPet-rgl2Δ17* lines (Dill et al. 2001; Gomez et al. 2019), that can not be binded by GID1 and consequently targeted for degradation, result in constitutive DELLA activity and blockage of the GA-mediated response.

Gibberellins and ovule development

GAs are involved in ovule development since the earliest stages. This is firstly evidenced by the presence of different components of the GA signaling pathway and GA metabolism in placenta and/or ovules. Specifically, *DELLA* genes *GAI*, *RGA*, *RGL1*, and *RGL2* expression is detected in placental tissues and outgrowing ovules (Gomez et al. 2018, 2019, 2020). Then, *RGA*, *RGL1* and *RGL2* are detected in mature ovules (Gomez et al. 2016, 2019, 2020). The GA receptors *GID1A* and *GID1B* and some GA metabolism genes (*GA20ox* and *GA3ox*) are also found in this tissues (Gallego-Giraldo et al. 2014; Ferreira et al. 2017; Gomez et al. 2018).

Genetic evidence point to GAs as negative regulators of ovule primordia initiation (**Fig. I.4**) (Gomez et al. 2018). The *global della* mutant, which lacks the activity of the five DELLA proteins of Arabidopsis (*rgaT2 gaiT6 rgl1-1 rgl2-1 rgl3-1*) produces fewer ovules compared to wild type. A similar reduction is observed in a quadruple *4xdella* (*rgaT2 gaiT6 rgl1-1 rgl2-1*) and triple *3xdella* (*rgaT2 gaiT6 rgl2-1*) mutants, which suggests that RGA, GAI, and RGL2 (but not RGL1 and RGL3) have a major role in ovule initiation. A reduction in ovule number is also observed in GA-treated plants, which phenocopy the null *della* mutants (Gomez et al. 2018). Oppositely, an increase in ovule number is found in the gain-of-function DELLA mutants *gai-1* (Gomez et al. 2018) and *pRGL2:YPet-rgl2Δ17* (Gomez et al. 2019) and in the double *gid1a gid1b* double mutant, which lacks GA perception in ovules (Gallego-Giraldo et al. 2014; Gomez et al. 2018). These results suggest that GAs are negative modulators of ovule number by promoting

the degradation of DELLA proteins, whose activity is necessary to regulate ovule primordia formation.

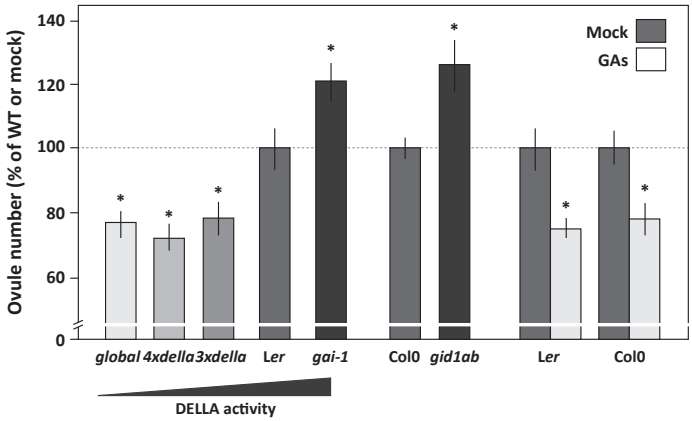

Fig. I.4: Ovule number in different *della* mutants and GA-treated plants. Data represent the mean $\pm$ SD. Significant differences (Student's *t*-test) with the corresponding wild type indicated by an asterisk ($P < 0.01$). Adapted from Gomez et al. (2018).

GAs were involved in ovule development also at later stages. Both *global* and *4xdella* (mentioned above) present two layer of cells in outer and inner integuments, while wild type ovules normally form three layers in the inner integument and two in the outer. As a result, *global* and *4xdella* plants form mature ovules with an irregular shape, suggesting a role of GAs in integument development (Gomez et al. 2016).

The GA-mediated molecular mechanism that guide ovule development is still not fully understood. It was proposed that DELLA proteins directly interact with ABERRANT TESTA SHAPE (ATS), a KANADI TF, to coordinate proper integument growth during late stages of ovule development (Gomez et al. 2016), but no mechanism was still clarified for GAs during ovule initiation. For this case, and taking into account what it is known about both the molecular mechanism that controls ovule initiation and the role of GAs during plant development, we can formulate several hypothesis. One plausible scenario is that GAs could crosstalk with other hormones, such as auxins, CKs or BRs, to properly regulate ovule primordia initiation. Another possibility (perhaps in addition to the first one) would be for DELLA proteins directly interacting with any of the key TFs involved in

ovule initiation, such as ANT, CUC1 and CUC2.

Regarding the first scenario, there are some evidence that point to GAs regulating ovule number independently of auxin distribution, as neither auxin response nor auxin transport is altered upon GA treatments or in the *gai-1* mutant (Gomez et al. 2018). In this thesis we are going to focus on the possible GA-BR interaction during ovule initiation. GAs and BRs share overlapping functions in growth, seed germination and flowering (Steber and McCourt 2001; Tanaka et al. 2003; Domagalska et al. 2010). Indeed, there is a complex molecular interaction between GAs and BRs that points to both the direct regulation of GA biosynthesis by BRs (Unterholzner et al. 2015) and the protein binding between DELLA proteins and BZR1, which results in the sequestration of BZR1 and, consequently, the inhibition of its transcriptional activity (Bai et al. 2012; Gallego-Bartolomé et al. 2012; Li et al. 2012). For the case of ovule initiation, it was reported that BR action relies on the regulation of *ANT*, but it is not known if GAs participate in this regulation through or together with BRs.

Regarding the second hypothesis, between ANT, CUC1 and CUC2, only CUC2 is found as a putative interactor of the DELLA protein GAI in yeast two-hybrid screenings (Marín-de la Rosa et al. 2014). Therefore, we studied if CUC proteins were a component of the DELLA-mediated ovule initiation mechanism.

BRASSINOSTEROIDS

BRs are a group of steroid plant hormones with roles in plant growth, plant development and stress (Nolan et al. 2019; Planas-Riverola et al. 2019).

BRs are perceived extracellularly by the membrane-localized receptor BRASSINOSTEROID INSENSITIVE 1 (BRI1). Upon BR binding, BRI1 forms an heterodimer with BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1) (Sun et al. 2013), which in turn activates an intracellular phosphorylation relay cascade that involves the phosphatase BRI1 SUPPRESSOR 1 (BSU1) and the kinase BRASSINOSTEROID INSENSITIVE 2 (BIN2) (Li and Nam 2002; Mora-García et al. 2004). This cascade eventually regulates the phosphorylation status of BRASSINAZOLE RESISTANT 1 (BZR1) and BRI1-EMS-SUPPRESSOR 1 (BES1), two TFs that can directely control the transcription of BR-responsive genes (Wang

et al. 2002; Yin et al. 2002). Thus, in absence of BRs, BIN2 phosphorylates and inactivates BZR1 and BES1, leading their binding to 14-3-3 proteins and, consequently, their cytoplasmatic retention and degradation by the 26S proteasome (Peng et al. 2008). When BR levels are high, BIN2 is inactivated by BSU1, dephophorilated BZR1 and BES1 translocate to the nucleus and BR responses are activated (Wang et al. 2002; Yin et al. 2002).

Brassinosteroids and ovule development

BRs are involved in ovule and seed development by regulating their number, size and shape (Huang et al. 2013a; Jiang et al. 2013; Jia et al. 2020). Regarding ovule number, it has been observed that loss-of-function mutants of BR receptors *bri1* and the gain-of-function mutant *bin2-1* have fewer ovules than wild type plants (Huang et al. 2013a; Jia et al. 2020). In addition, plants with low BR levels (as, for instance, the BR biosynthesis defective mutant *det2-1*, or plants treated with brassinazole, a BR biosynthesis inhibitor) present significant reductions in ovule number (Huang et al. 2013a; Nole-Wilson et al. 2010b). Oppositely, the BR-signal dominant mutant *bzr1-1D* has increased ovule number. This suggest that BRs positively regulate ovule number by activating BZR1.

BZR1 regulates expression levels of some ovule developmental genes (Huang et al. 2013a). It was found that BR treatments up-regulate *ANT* (**Fig. I.2**), *HUELLENLOS* (*HLL*) and *SEEDSTICK* (STK) and down-regulate *APETALA2* (*AP2*) in inflorescences (Huang et al. 2013a). The opposite behavior occurs in brassinazole-treated plants, being *ANT* and *AP2* BZR1-direct targets (Huang et al. 2013a). As mentioned before, ANT promote ovule primordia growth, but also integument development (Elliott et al. 1996; Klucher et al. 1996). HLL promote ovule growth redundantly with ANT (Schneitz et al. 1998). STK and AP2 affects ovule identity (Modrusan et al. 1994; Pinyopich et al. 2003). Therefore, BZR1, whose activity is induced by BRs, regulate some ovule development genes.

ANT, *HLL* and *STK* are also induced by CKs (Bartrina et al. 2011; Zu et al. 2021), indicating that BRs and CKs influence a common subset of ovule development genes. It has been found that CK response is higher in both placenta and ovule primordia of *bzr1-1D*, whereas it is lower in *bin2-1*. Some *ARRs* (*ARR5* and *7*) are up-regulated upon BR treatments (Zu et al. 2021). This indicates that

BRs enhance CK signaling. Oppositely, protein levels of nucleus-localized BZR1 is increased in ovules with high CK levels, which suggests that CKs, in turn, also regulate BR signaling (Zu et al. 2021). BZR1 can physically bind with ARR1 (Zu et al. 2021), pointing to a direct interaction of BR and CK signaling. Certainly, *arr1* loss-of-mutant have a slightly reduction in seed number that is not increased in a *arr1 bzr1-1D* double mutant, ARR1 induces both *STK* and *HLL* promoters activity, and this induction is strengthened by BZR1. However, high CK levels partially restores *bin2-1* seed number (Zu et al. 2021). Overall, it is concluded that BRs and CKs regulate ovule and seed number coordinately.

BRs also interact with auxins to regulate ovule initiation. Auxin response at the tip of ovule primordia is enhanced upon BR treatments and decreased in *bin2-1* (Hu et al. 2022). Similarly, *PIN3* expression is up-regulated in *bzr1-1D* and down-regulated in *bin2-1*. PIN3 protein levels are concordantly reduced in *bin2-1*, although its localitation is not altered (Hu et al. 2022). As *pin3* loss-of-function mutant presents reduced auxin response in ovule primordia (Hu et al. 2022), suggesting that BR-mediated auxin response in ovule initiation depends on PIN3. Ovule number in *pin3* mutant is reduced as a result of the failure of late ovule initiation, and this phenotype persists in the *bzr1-1D pin3* double mutant (Hu et al. 2022) confirming that PIN3 might function downstream of BRs in ovule primordia (**Fig. I.2**). Interestingly, *PIN3* is directly activated by ARR1, and CK treatments up-regulate *PIN3* in the medial domain of developing pistils (Reyes-Olalde et al. 2017), which aims the possibility that the BZR1-ARR1 complex could be also regulating PIN3 on ovule primordia (**Fig. I.2**).

CUP-SHAPED COTYLEDON PROTEINS

CUC proteins belong to the large plant-specific family of NAC TFs and play a boundary-defining role during plant development (Maugarny et al. 2016). As part of the NAC family (named after the identification of its first three members, the NO APICAL MERISTEM -NAM- of *Petunia hybrida* and the ARABIDOPSIS TRANSCRIPTION ACTIVATOR FACTOR1/2 -ATAF1/2- and CUC2 of *Arabidopsis thaliana*), the CUC proteins can be divided into two main functional domains: the amino-terminal NAC domain, which is subdivided into five highly conservated regions and is implicated in the DNA-binding, homo-/heterodimerization

and protein-protein interaction (Olsen et al. 2005), and the carboxyl-terminal domain, which is more variable and was shown to have transactivation activity (Ooka et al. 2003; Taoka et al. 2004). *CUC* genes are expressed in frontier regions in both the embryo and the mature plant, and its loss-of-function leads to fused organs and sometimes loss of organs (Maugarny et al. 2016). Additionally, *CUC* genes have partially redundant functions, so some of the strongest phenotypes are more noticeable in double *cuc* mutants and much less severe in single mutants. Some examples are described below.

The first described phenotype of the loss-of-function of *CUC* genes in Arabidopsis was the fusion of cotyledons accompanied by the lack of shoot apical meristem (SAM) in embryos and seedlings of the double *cuc1 cuc2* (currently redefined as *cuc1-1 cuc2-1*) (Aida et al. 1997). This phenotype is a result of the loss of growth inhibition ascribed to CUC1 and CUC2 activity in the boundary region between developing cotyledons (Aida et al. 1997) and the misexpression of *SHOOT MERISTEMLESS* (*STM*, a class I KNOX gene required for maintaining meristem activity) in the presumptive SAM cells (Aida et al. 1999). *CUC1* and *CUC2* are expressed in the presumptive SAM during early embryogenesis and becomes restricted to the boundaries between the cotyledons and the SAM in later stages, which goes in accordance with the observed phenotype and suggests that *CUC1* and *CUC2* have a role in separating cotyledons and specifying SAM initiation. Later, it was described that *CUC3*, which encodes a NAC TF highly similar to CUC1 and CUC2 (although it belongs to a different clade), also participates in SAM initiation and cotyledon boundary formation redundantly with *CUC1* and *CUC2* (Vroemen et al. 2003; Hibara et al. 2006).

CUC genes were also found to be expressed at the boundaries between the apical meristem and leaf primordia (Hibara et al. 2006; Raman et al. 2008), in the sinuses of rosette leaves (Nikovics et al. 2006; Maugarny-Calès et al. 2019), between the inflorescence and floral meristems (Ishida et al. 2000; Takada et al. 2001; Hibara et al. 2006; Peaucelle et al. 2007), in the axis of flower pedicels (Peaucelle et al. 2007; Burian et al. 2015), and between floral organs (Ishida et al. 2000; Takada et al. 2001; Hibara et al. 2006; Hasson et al. 2011; Galbiati et al. 2013; Gonçalves et al. 2015). In accordance, combinations of *cuc* mutants show organ fusion or reduced organ number in all this developmental contexts.

For instance, vegetative leaf fusions and cauline leaves or pedicels fused to the inflorescence stem were observed in *cuc2 cuc3* double mutants, resulting in altered phylotaxis (Hibara et al. 2006; Burian et al. 2015). The *cuc3-2* single mutant shows a strong reduction in the number of axillary buds originating from the axils of rosette leaves (Raman et al. 2008). Leaves of *cuc2* and *cuc3* mutants have smoother margins as they present reduced serrations (Nikovics et al. 2006; Bilsborough et al. 2011; Hasson et al. 2011). And regenerated shoots[1] of *cuc1-1 cuc2-1* double mutant show strong fusions between sepals and stamens, as well as reduced number of petals, stamens and ovules (Aida et al. 1997; Ishida et al. 2000; Hibara et al. 2006).

All these observations coupled with morphological analysis suggest that CUC proteins repress growth in frontier regions and, in consequence, allow organ separation. Indeed, it was reported that cells located at boundaries display reduced cell division (Breuil-Broyer et al. 2004) and there are some evidence pointing to a role of CUC1 (Sieber et al. 2007) and CUC2 (Larue et al. 2009) in cell division but not cell expansion regulation. Posterior works showed that some plant hormones involved in cell proliferation, such as BRs or auxins, repress *CUC* gene expression to control boundary formation. However, the underlying molecular mechanism by which CUC TFs control cell proliferation still need to be elucidated.

CUC1 and *CUC2* (but not *CUC3*) expression is post-transcriptionally regulated by *miR164*, which is encoded in Arabidopsis by three different genes (*MIR164A, B* and *C*) that are partially redundant in function. Plants expressing *miR164*-resistant versions of *CUC1* or *CUC2*, as well as *mir164* mutants (with increased *CUC1* and *CUC2* expression levels), show some phenotypes of extra organs or enlarged boundaries. For instance, *CUC1/2 miR164*-resistant alleles and *mir164c* mutant show extra petals, enlarged sepal boundaries and/or defects in carpel fusion. Both *mir164abc* triple mutant and *CUC1/2 miR164*-resistant variants induce the formation of accesory buds in leaf axils. And expression of *miR164*-resistant *CUC2* or loss of *MIR164A* lead to deeper serrations in leaves. Accordingly with these observations, *MIR164A, B* and/or *C* are expressed in the margins of the mentioned organs. Overall, it seems that *mir164* is needed to

[1] As *cuc1-1 cuc2-1* double mutations lack SAM, it is required to induced shoots from hypocotyls-derived calli to examine its effect on postembryonic development.

fine-tune *CUC1* and *CUC2* gene expression and allow proper organ separation.

CUC during gynoecium and ovule development

CUC1 and *CUC2* are expressed within the developing pistil firstly in the adaxial region of the medial wall, then in CMMs, and later in septum and placenta (Ishida et al. 2000; Takada et al. 2001; Nahar et al. 2012; Galbiati et al. 2013; Kamiuchi et al. 2014; Gonçalves et al. 2015). Upon initiation, *CUC2* and *CUC3* are expressed in the boundaries of ovule primordia (Ishida et al. 2000; Vroemen et al. 2003; Gonçalves et al. 2015). Interestingly, no *CUC* expression is detected in ovules themselves until integument primordia start to be differenciated, when *CUC1* and *CUC2* expression is again detected in the boundary between nucellus and chalaza (Ishida et al. 2000; Takada et al. 2001; Galbiati et al. 2013; Kamiuchi et al. 2014). *CUC2* and *CUC3* are also expressed in this region in mature ovules (Ishida et al. 2000; Vroemen et al. 2003). Concurrently with these expression patterns, gynoecia that lack or have reduced CUC1 and CUC2 activity present abnormal CMM and septum development, fewer ovules and some defects in integuments growth (Ishida et al. 2000; Galbiati et al. 2013; Kamiuchi et al. 2014; Gonçalves et al. 2015).

It was proposed that *CUC1* and *CUC2* promote the formation and positioning of CMMs as they activate and pattern *STM* expression domains along the carpel margins (Kamiuchi et al. 2014). Additionally, *CUC1* and *CUC2* are repressed by *SPATULA* (*SPT*) to ensure proper apical carpel fusion (Nahar et al. 2012). Then, during ovule initiation, *CUC1* and *CUC2* are linked with auxin signaling and transport (Galbiati et al. 2013) and CK inactivation (Galbiati et al. 2013; Cucinotta et al. 2018). Both *CUC1* and *CUC2* are directly transcriptionally activated by MP/ARF5, an auxin response factor (**Fig. I.2**) (Galbiati et al. 2013). *MP* shares expression pattern with *CUC1* and *CUC2* in the placenta before ovule primordia arise and with *CUC2* in ovule primordia boundaries, so probably MP is required for *CUC1* and *CUC2* expression during early stages of placenta development and ovule initiation (Galbiati et al. 2013). For its part, *CUC1* and *CUC2* promote *PIN1* expression and proper PIN1 membrane localization in ovule primordia (**Fig. I.2**) (Galbiati et al. 2013). Interestingly, it was reported that CK treatments also increase *PIN1* expression (Bencivenga et al. 2012), and *CUC1* and *CUC2* induce CK

responses by transcriptionally repressing *UGT73C1* and *UGT85A3*, which encode for two enzymes involved in CKs reversible inactivation (Cucinotta et al. 2018). These data suggest that CKs could act downstream from CUC1 and CUC2 to induce *PIN1* (**Fig. I.2**). It is worth mentioning that overexpression of *MIR164A* strongly reduces ovule number, indicating that *miR164* could be also regulating*CUC1* and *CUC2* during ovule initiation (**Fig. I.2**) (Gonçalves et al. 2015).

Finally, lack of *CUC3* does not affect ovule initiation and number, but it was described that, together with CUC2, CUC3 promotes proper ovule separation, suggesting that different CUC genes promote ovule initiation or ovule separation in a partially redundant fashion (Gonçalves et al. 2015).

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Objectives

OBJECTIVES

GAs negatively influence ovule initiation in Arabidopsis. In consequence, plants with low GA levels or enhanced DELLA activity have more ovules than wild type plants, with a slightly effect on pistil length, pointing to an specific role of DELLA proteins in the placenta to promote ovule initiation. However, how do GAs regulate this developmental process still needs to be elucidated. **Uncovering the interaction of GAs with other hormones or genetic factors in the placenta of developing pistils** would help to better understand ovule development, adding a new layer of complexity to the known gene regulatory network, and perhaps helping to develop biotechnological strategies aimed to fine-tune seed yield. To address this general objective, we propose two specific objectives:

- **To study the crosstalk between GAs and BRs in ovule initiation**. Previous studies demonstrated that GAs and BRs coordinately regulate a wide range of developmental processes, and we hypothesized that they could also co-regulate ovule initiation. We tested this genetically. This is the focus of Chapter 1.
- **To study the DELLA-CUC interaction and its role in ovule initiation**. Previous evidence suggest that CUC2 could be a putative interactor of GAI. We hypothesized that CUCs could be needed for the DELLA-mediated regulation of ovule initiation. We tested the DELLA-CUC interactions genetically and molecularly. This is the focus of Chapter 2.

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Regulation of ovule initiation by gibberellins and brassinosteroids in Arabidopsis

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CHAPTER 1

Regulation of ovule initiation by gibberellins and brassinosteroids in Arabidopsis

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Abstract

Ovule primordia formation is a complex developmental process with a strong impact on the production of seeds. In Arabidopsis this process is controlled by a gene network, including components of the signalling pathways of auxin, brassinosteroids (BRs) and cytokinins. Recently, we have shown that gibberellins (GAs) also play an important role in ovule primordia initiation, inhibiting ovule formation in both Arabidopsis and tomato. In contrast, BRs participate in the control of ovule initiation by promoting an increase on ovule primordia formation. Here we reveal that both GAs and BRs regulate ovule number independently of the activity levels of the other hormone in Arabidopsis. This mechanism is different from that described in tomato, where BRs also promote ovule formation but through the downregulation of GA biosynthesis. Taken together, our data strongly suggest that different molecular mechanisms could operate in different plant species to regulate identical developmental processes even, as for ovule primordia initiation, if the same set of hormones trigger similar responses.

Keywords: Arabidopsis thaliana, gibberellins, brassinosteroids, ovule, reproductive development, hormone interaction.

The data presented in this chapter are part of a published work about the role of GAs and BRs in both Arabidopsis and tomato (Barro-Trastoy et al. 2020b). DB-T, MDG, PT, JP-R and MAP-A performed experiments in Arabidopsis. EC, JB, OR-R and IL-D performed experiments in tomato. For this reason, the chapter was adapted to present only the results of the experiments performed in Arabidopsis. The results obtained from the experiments performed in tomato will be mentioned in the discussion.

INTRODUCTION

Seeds are extremely important in many ways. Besides their biological roles in the survival of the species, preserving embryonic life or being the vehicles for dispersal, seeds are the primary basis for human sustenance (Sabelli and Larkins 2009). Seeds are formed upon double fertilization of the ovule in the ovary, therefore seed number depends, among other things, on the number of viable ovules that are formed.

Ovule development in Arabidopsis has been extensively studied at the morphological, genetic and molecular levels (Schneitz et al. 1995; Cucinotta et al. 2014). Ovule primordia are formed in the carpel medial meristem (CMM) as lateral organs from the placenta and follow a well established developmental process (Schneitz et al. 1995). A key step in ovule development is determination of the position and number of ovule primordia in the CMM (Cucinotta et al. 2014). CMM and ovule primordia formation are both controlled by regulatory genes, as well as by several plant growth regulators, including auxin, cytokinin (CKs), and brassinosteroids (BRs) (Bartrina et al. 2011; Bencivenga et al. 2012; Galbiati et al. 2013; Huang et al. 2013a; Reyes-Olalde et al. 2013; Cucinotta et al. 2014, 2016; Müller et al. 2017). Recently, we have demonstrated that gibberellins (GAs) also play an important role in modulation of ovule number in Arabidopsis as well as in tomato and rapeseed (Gomez et al. 2018).

Key elements in GA signalling are DELLA proteins (coded by five genes in Arabidopsis), which repress GA responses (Sun 2010, 2011). In the presence of

GAs, DELLA proteins are degraded via the proteasome, releasing the repression of GA signalling. Degradation mainly depends upon the N-terminal domain of the DELLA protein (the so-called DELLA domain). Gain-of-function varieties of DELLA proteins, such as the *gai-1* allele of GIBBERELLIC ACID INSENSITIVE (GAI) in Arabidopsis (Peng et al. 1997), lack this domain and encode stable proteins that cannot be degraded by GAs, hence promoting a constitutive blockage of the GA response. Interestingly, DELLA proteins are transcriptional regulators that lack a canonical DNA-binding domain, and therefore exert their activity by binding to a wide variety of transcription factors (TFs) (Davière and Achard 2016; Vera-Sirera et al. 2016), such as ABERRANT TESTA SHAPE (ATS/KAN4) to regulate integument growth (Gomez et al. 2016). Regarding ovule initiation, constitutive repression of GA responses in the *gai-1* mutant produces more ovules than the wild type, whereas constitutive GA signalling in null della mutants or by GA treatment causes a strong decreased in ovule number in Arabidopsis (Gomez et al. 2018). A similar role for GAs in ovule initiation was confirmed in tomato and rapeseed. Therefore, DELLA protein activity is a positive factor in ovule formation, although the molecular mechanism of DELLA action in the CMM is still unknown.

Most of the BR signalling pathway relies on the BRASSINAZOLE-RESISTANT 1 (BZR1) and BRI1-EMS-SUPPRESSOR1 (BES1) TFs. In the presence of BR, the receptor complex BRI1/BAK1 mediates the dephosphorylation of BRZ1 and BES1 by a PP2A protein phosphatase that activates their transcriptional activity upon BR-regulated genes (Belkhadir and Jaillais 2015; Nolan et al. 2019). BR signalling positively regulates ovule and seed number in Arabidopsis (Huang et al. 2013a). The *bzr1-1D* mutant (a gain-of-function of BZR1) increases ovule and seed number, while the BR-deficient mutant *det2-1* (Fujioka et al. 1997) produces fewer ovules and seeds. It has been proposed that BRs control ovule number by the effects of BZR1 on expression of ovule development genes. Among these, expression levels of *HUELLENLOS* (*HLL*) and *AINTEGUMENTA* (*ANT*) are upregulated, whereas that of *APETALA2* (*AP2*) is downregulated by BRs and in *bzr1-1D* plants (Huang et al. 2013a). In addition, *ANT* and *AP2* are direct targets of BZR1 (Huang et al. 2013a).

GAs and BRs control similar developmental processes. Both hormones pro-

mote hypocotyl growth during skotomorphogenesis in Arabidopsis (Tanaka et al. 2003), or participate in the control of cell elongation to determine plant height in rice (De Vleesschauwer et al. 2012; Xiao et al. 2017; Tang et al. 2018). Moreover, mutants deficient in either GAs or BRs display dwarf plant architecture in Arabidopsis (Clouse 2011; Sun 2011) and tomato (Martí et al. 2006). BR mutants and GA-deficient plants show other phenotypes such as impaired germination (Unterholzner et al. 2015), reduced hypocotyl elongation, darker green leaves (Martí et al. 2006), late flowering, or reduced fertility (Clouse 2011). The complex interaction between GAs and BRs points to both the direct regulation of GAs biosynthesis by BRs in Arabidopsis (Unterholzner et al. 2015) and rice (Oryza sativa) (Tong et al. 2014) and the direct protein–protein interaction between DELLA proteins and BZR1 in Arabidopsis (Bai et al. 2012; Gallego-Bartolomé et al. 2012; Li et al. 2012). In the latter, DELLA proteins inhibit BZR1 transcriptional activity by protein–protein interaction, whereas GAs relieve the repression by promoting DELLA degradation via the ubiquitin proteasome mechanism, which allows BZR1 binding to target gene promoters.

For ovule primordia formation in Arabidopsis, GAs and BRs act antagonistically, BRs promoting and GAs reducing ovule number. Although it has been proposed that BR action rely on the transcriptional regulation of several TFs, including ANT, the mechanism by which GAs participate is unknown. In this study we focussed on the possible interaction of GAs with the BR signalling pathway in Arabidopsis. Our findings point GAs and BRs participating independently in ovule initiation in Arabidopsis. Moreover, ANT did not mediate an increment in ovule number by BZR1 or DELLAs.

RESULTS

GAs act independently of BRs to control ovule initiation

To determine whether an interaction between BRs and GAs during ovule primordia formation occurs in Arabidopsis, we studied the effect on ovule number of (1) GA application in BR signalling mutants, (2) BR application in GA signalling mutants, and (3) the *gai-1* and *bzr1-1D* gain-of-function mutation combinations. As expected, *bzr1-1D* and *det2-1* mutants produced higher and lower ovule number

respectively. Interestingly, GA treatment produced a similar reduction, approximately 20%, in ovule number in both *bzr1-1D* and *det2-1* mutants, and in the wild type plants (**Fig. 1.1**).

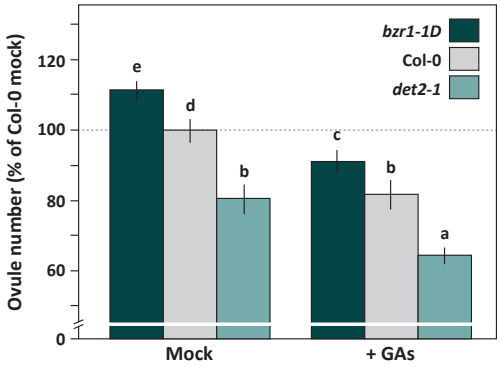

Fig. 1.1: Ovule number per pistil in non-treated (Mock) or GA-treated (+ GAs) plants of Col-0 and the BR mutants *bzr1-1D* and *det2-1*. Data represent the mean ± SD (n = 15). Letters indicate statistical significance as determined by ANOVA with a Bonferroni post hoc test for multiple comparisions. Data that are not significantly different are marked with the same letter.

Moreover, treatment of wild type plants with 2,4-epibrassinolide (EBR), an active form of BRs, or brassinazole (BRZ), a specific inhibitor of BR biosynthesis, produced an increase or decrease in ovule number in the wild type plant, respectively, which mimicked the changes observed in *bzr1-1D* and *det2-1* mutants. Importantly the effects of EBR and BRZ treatment on ovule number were similar in the GA mutant *gai-1* and in wild type plants (**Fig. 1.2**).

These results implied that BRs and GAs would act independently in the determination of ovule number in Arabidopsis. Additionally, the *gai-1* and *bzr1-1D* mutations had additive effects when combined. Whereas *gai-1* or *bzr1-1D* produced a significant increase in ovule number individually (15-25%), the combined *gai-1 bzr1-1D* mutant showed an additive effect, an increase in ovule number that was around 40%, very similar to the phenotype of the *gai-1* treated with EBR (**Fig. 1.3**).

These results reinforce the idea that BRs and GAs would act independently in the determination of ovule number in Arabidopsis. Taken together, our data strongly support a regulatory mechanism of ovule number by BRs that is indepen-

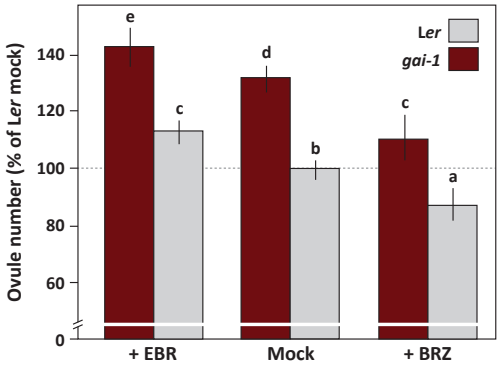

Fig. 1.2: Ovule number per pistil in non-treated (Mock), EBR- (+ EBR) or BRZ- (+ BRZ) treated plants of L*er* and the GA mutants *gai-1*. Data represent the mean ± SD (n = 15). Letters indicate statistical significance as determined by ANOVA with a Bonferroni post hoc test for multiple comparisions. Data that are not significantly different are marked with the same letter.

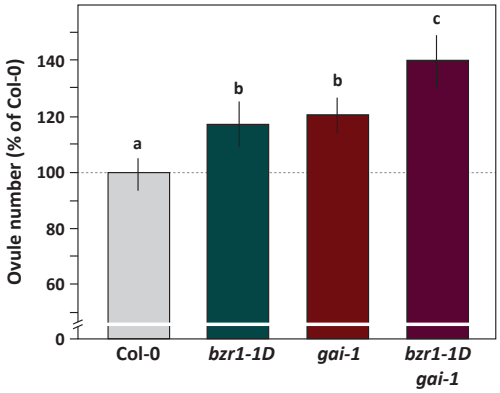

Fig. 1.3: Ovule number per pistil in *bzr1-1D*, *gai-1*, and double *gai-1 bzr1-1D* mutants. Data represent the mean ± SD (n = 15). Letters indicate statistical significance as determined by ANOVA with a Bonferroni post hoc test for multiple comparisions. Data that are not significantly different are marked with the same letter.

dent of GAs.

Increased ovule number per pistil could be due to increase in ovary length, which maintains ovule density in the placenta, rather than a specific increase in ovule density with no alteration in ovary length. In the pistil of *gai-1*, the signifi-

cant increase in ovule number is accompanied by a slight increase in ovary length, resulting in increased ovule density (Gomez et al. 2018). To test whether BRs could also control ovule number independently of ovary length, we determined ovule number, ovary length and the ratio of ovule number to ovary length from the single and double mutants of *gai-1* and *bzr1-1D* (**Sup. Fig. 1.1** and **Sup. Table 1.1**). While constitutive BR signalling in *bzr1-1D* produced a 15% increase in ovule number, it also had a slight increase (9%) in ovary length, resulting in a slight but significant (6%) increased ovule density (ratio of ovule number to ovary length). A similar effect was observed for *gai-1* on the Col-0 background. Moreover, the combined *gai-1 bzr1-1D* mutant showed a strong increase in ovule number of about 43% and 15% increase in ovary length, which resulted in a significant increase of 15% in ovule density. Therefore, the activities of both dominant versions of GAI and BZR1 had a similar effect in promoting an increase in ovule number, but they had a minor effect on ovary length. The data from this experiment also confirmed the additive effect of both mutations in ovule number, ovary length, and ratio (**Sup. Table 1.1**). Whether the effect of the DELLA and BZR1 activities on ovary length is a direct consequence of the increased ovule number or it occurs via a different molecular mechanism not linked to ovule number is unknown and requires further study.

Table 1.1: Levels of GAs in Col-0 and *bzr1-1D* inflorescences (ng · g^{-1} FW).

	Non-C-13 hydroxylation						
	GA_{12}	GA_{15}	GA_{24}	GA_{9}	GA_{51}	**GA_{4}**	GA_{34}
Col-0	4.23	0.60	3.42	5.78	1.03	**6.74**	3.31
	±0.29	±0.02	±0.34	±0.37	±0.16	±**0.07**	±0.22
bzr1-1D	3.00	0.68	3.06	5.05	0.71	**11.30**	4.46
	±0.12*	±0.05	±0.24	±0.38	±0.05	±**0.90***	±0.30*
	Early C-13 hydroxylation						
	GA_{53}	GA_{44}	GA_{19}	GA_{20}	GA_{29}	**GA_{1}**	GA_{8}
Col-0	0.34	0.23	1.51	0.05	nd	**0.37**	0.09
	±0.03	±0.00	±0.14	±0.00		±**0.04**	±0.02
bzr1-1D	0.28	0.26	3.02	0.04	nd	**0.36**	0.16
	±0.01	±0.03	±0.36*	±0.01		±**0.02**	±0.01*

In bold, the bioactive GAs (GA_4 and GA_1). nd, not detected. FW, fresh weight.

*Significant differences with Col-0 (Student's *t*-test, $P < 0.01$).

BRs promote an increase in GA levels in inflorescences

It has been proposed that BRs and GAs coordinate growth by the BR-mediated upregulation of GA biosynthesis genes to promote an increase in GA levels in seedlings (Tong et al. 2014; Unterholzner et al. 2015). We studied whether BRs also promote a change in GA levels in inflorescences. As shown in **Fig. 1.4** and **Table 1.1**, the levels of GA_4, the main bioactive GA in Arabidopsis, were significantly higher in *bzr1-1D* compared with the wild type, whereas GA_1 levels were not affected.

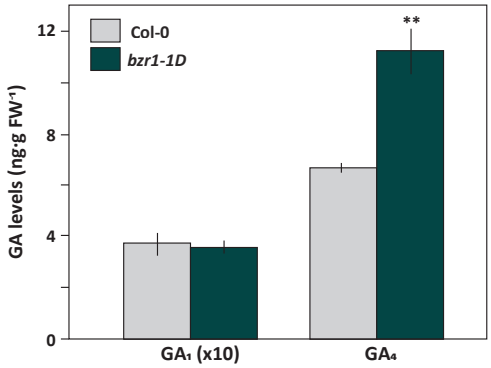

Fig. 1.4: Levels of the bioactive GAs (GA_1 and GA_4) in inflorescences of Col-0 and *bzr1-1D*. Significant differences (Student's *t*-test analysis) are indicated (**, $P < 0.001$). Data are the mean $\pm$ SD. For representation purposes, the levels of GA1 were multiplied 10-fold.

Increased GA levels in *bzr1-1D* would be the consequence of upregulation of expression of GA biosynthesis genes, especially *GA20ox3* and *GA3ox1* (**Fig. 1.5**). As a result of increased GA levels, DELLA protein levels were reduced, as observed for the GFP–RGA levels upon treatment with EBR (**Fig. 1.6**).

Interestingly, the increased GA level of *bzr1-1D* and the destabilized DELLA proteins upon EBR treatment should result in the reduction of ovule number, which was not the case. By contrast, *bzr1-1D* and EBR treatment produced an increase in ovule number. Based on these data, we consider that the mechanism by which BRs regulate ovule number is not mediated by the regulation of GA levels.

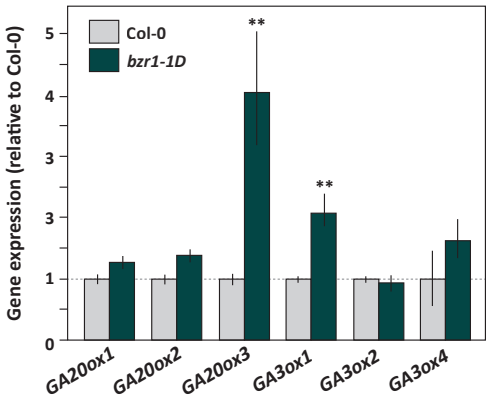

Fig. 1.5: Expression of GA biosynthesis genes in inflorescences of Col-0 and *bzr1-1D*. qPCR expression analysis was carried out for *GA20ox1* to *GA20ox5* and *GA3ox1* to *GA3ox4* in inflorescences. Expression of *GA20ox4*, *GA20ox5*, and *GA3ox3* was not detected. Expression was normalized to that of *UBQ10* (*At4g05320*) in Col-0. Data are the mean ± SD. Significant differences (Student's *t*-test analysis) are indicated (**, $P < 0.05$).

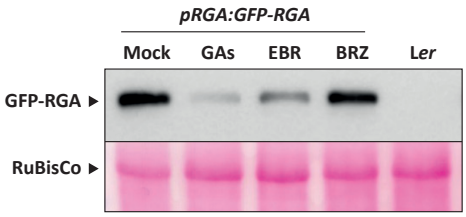

Fig. 1.6: Levels of GFP-RGA protein in GA-, EBR- or BZR-treated plants. Western blot analysis was carried out in inflorescences of the *pRGA:GFP–RGA* plants treated for 4 h with 20 µM GA_{4+7}, or 2 µM of EBR or BRZ. RuBisCo was used as a loading control using Ponceau staining.

GA promotion of ovule initiation does not rely on ANT

It has also been reported that the molecular mechanism of BRs signalling in ovule initiation is based on the activation of *ANT* expression in the placenta of developing pistils, as *ANT* a direct target of BZR1 (Huang et al. 2013a). To further test whether ANT also participates in the GA-dependent ovule formation, we studied the regulation of *ANT* expression by GAs and the genetic interaction between *gai-1* and the strong null *ant-4* mutant. Interestingly, qPCR analyses on inflorescences revealed a similar upregulation of ANT expression by both GAI and

BZR1 (**Fig. 1.7**).

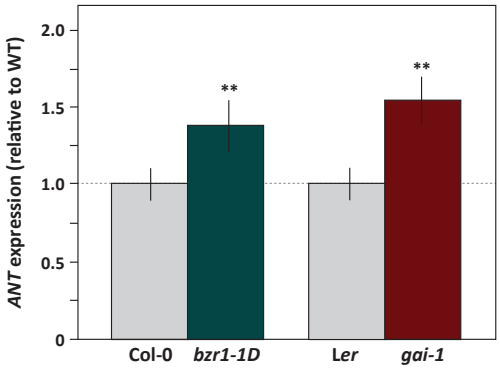

Fig. 1.7: Expression of *ANT* in inflorescences of Col-0, *bzr1-1D*, L*er*, and *gai-1*. Expression was normalized to that of *UBQ10* (*At4g05320*) in the corresponding Col-0/L*er* control. Data are the mean ± SD. Significant differences (Student's *t*-test analysis) are indicated (**, $P < 0.05$).

Therefore, a plausible scenario is that GAI could mediate ovule primordia initiation by promoting the expression of ANT, as was previously proposed for BRs (Huang et al. 2013a). To prove whether increased expression of *ANT* was sufficient to trigger an increase in ovule number, we generated an ANT overexpressing line, using the strong constitutive 35S promoter. As reported previously (Mizukami and Fischer 2000), overexpression of ANT caused an increase in the size of floral organs and ovules, but also impaired anther dehiscence and hence fertility (**Sup. Fig. 1.2**). Moreover, the number of ovules in the 35S:ANT line was not affected, compared

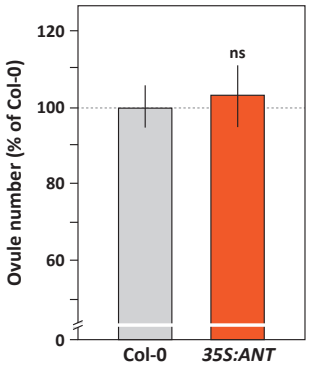

Fig. 1.8: Ovule number per pistil in *35S:ANT* plants. Data represent the mean ± SD (n = 15). ns: non-significant difference (Student's *t*-test).

with the wild type plant (**Fig. 1.8**), as occurred with other floral organs that were not altered in number by ANT ectopic expression (Mizukami and Fischer 2000). Therefore, the increased ANT expression by BRs and GAI does not explain the increase in ovule number observed in *gai-1* and *bzr1-1D*.

Next, by *in situ* mRNA hybridization, we analyzed the spatial expression pattern of ANT in two opposite GA mutants, *gai-1* and *global* (null mutant of the five Arabidopsis DELLA genes: *rgaT2 gaiT6 rgl1-1 rgl2-1 rgl3-1*) (**Fig. 1.9**), which produced high and low ovule number, respectively (Gomez et al. 2018). The spatial expression pattern of *ANT* was not altered in either *gai-1* or *global* mutants, as expression was localized in the placenta tissue, before the initiation of ovule primordia formation at stage 8 of pistil development (Elliott et al. 1996; Schneitz et al. 1998).

We also analyzed ANT protein levels using a translational fusion ANT-YPet driven by its own promoter in a *pANT:ANT-YPet* transgenic line. ANT-YPet

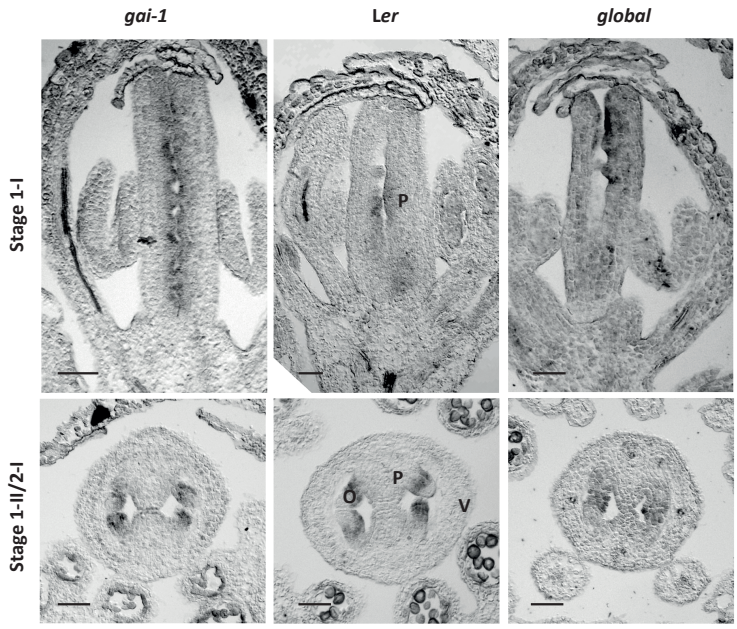

Fig. 1.9: *In situ* mRNA hybridization of *ANT* transcripts in *gai-1*, L*er*, and the *global* mutant at early pistil development before ovule primordia initiation and stage 1-I and ovule primordia at stage 1-II/2-I. Scale bars represent 20 µm in stage 1-I and 40 µm in stage 1-II/2-I. O, ovule primordium; P, placenta.

expression was able to complement the *ant-4* mutant allele. Inflorescences of *ant-4* plants showed defects in flower development similar to those reported in other *ant* mutants (Elliott et al. 1996; Klucher et al. 1996; Baker et al. 1997) and that included reduced number and width of the four whorls, as well as alteration in ovule morphology, causing complete sterility (Baker et al. 1997). Expression of ANT–YPet fully restored normal flower and ovule development, as well as fertility (**Sup. Fig. 1.3**). ANT-YPet was localized in the placental tissue at early developmental stages, before ovule initiation, or in ovule primordia (**Fig. 1.10**). Treatment of inflorescences with GAs or paclobutrazol (PBZ), an inhibitor of GA biosynthesis, did not alter ANT-YPet protein levels. In addition, levels and localization of ANT-YPet did not differ in the *gai-1* mutant and wild type plants (**Fig. 1.11**).

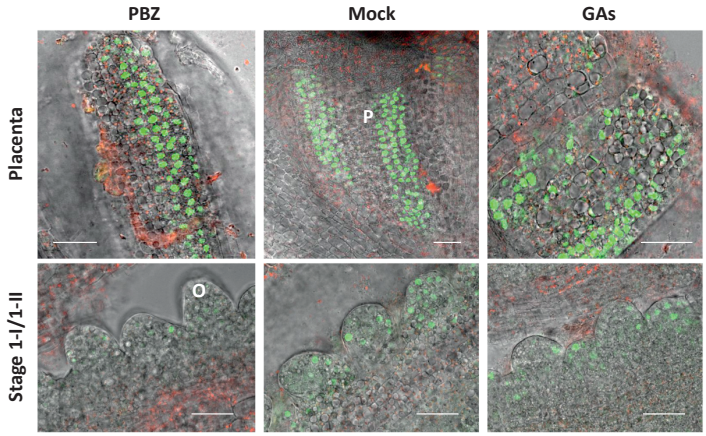

Fig. 1.10: Visualization of ANT–YPet protein in the placenta or ovule primordia upon PBZ, mock, or GA treatment. Scale bars represents 20 µm. O, ovule primordium.

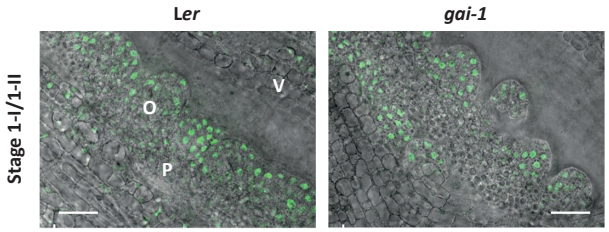

Fig. 1.11: Visualization of ANT–YPet protein in ovule primordia in L*er* or *gai-1*. Scale bars represents 20 µm. O, ovule primordium; P, placenta; V, valve.

Overall, our data indicated that neither GAs nor DELLA proteins significantly regulated levels or pattern of ANT expression at the mRNA or protein level, despite the small increase in the qPCR result observed in *gai-1*.

Finally, we also evaluated whether the *gai-1* mutation required ANT function to promote the increase in ovule number. *gai-1* was not able to significantly alleviate the developmental defects caused by *ant-4* mutation in the inflorescences, pistils or ovules (**Fig. 1.12**) (Elliott et al. 1996; Klucher et al. 1996; Baker et al. 1997).

More importantly, *gai-1* was not able to increase ovule number in *ant-4* (**Fig. 1.13**). The strong allele *ant-4* caused a 60% reduction in ovule number,

Fig. 1.12: Images of inflorescences, flowers, pistils at anthesis and mature ovules of L*er*, *ant-4*, *gai-1*, and the double *ant-4 gai-1*. Scale bars represent 2 mm in inflorescences and flowers, 1 mm in pistils, and 50 µm in ovules.

and similar reduction was also observed in the *ant-4 gai-1* double mutant.

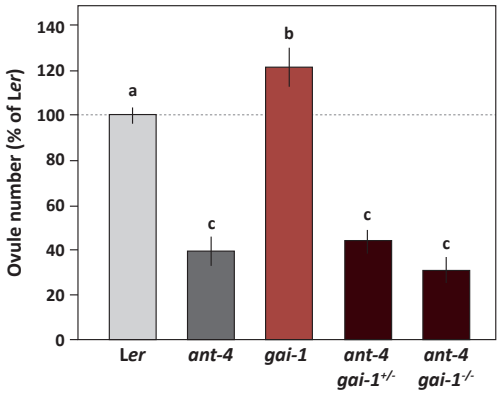

Fig. 1.13: Ovule number per pistils in L*er*, *ant-4*, *gai-1*, *ant-4 gai-1*$^{+/-}$, and *ant-4 gai-1*$^{-/-}$. Data represent the mean $\pm$ SD (n = 15). Letters indicate statistical significance as determined by ANOVA with a Bonferroni post hoc test for multiple comparisions. Data that are not significantly different are marked with the same letter.

In summary, our findings strongly imply that promotion of ovule primordia emergence by DELLA proteins in Arabidopsis is independent of BRs, and does not rely on changes in *ANT* expression. Despite this, ANT activity is required for GA effects in ovule initiation.

DISCUSSION

GAs and BRs regulate many aspects of plant growth and development. In most cases, both hormones act cooperatively. For example, in the photomorphogenesis-related hypocotyl elongation in Arabidopsis, BRs upregulate the expression of *GA20ox1* and *GA3ox1* involved in GA biosynthesis, and results in increased GA levels (Unterholzner et al. 2015). Moreover, DELLA proteins bind directly to BZR1, preventing its binding to target promoters, and thus blocking BZR1-mediated transcriptional activity (Bai et al. 2012; Gallego-Bartolomé et al. 2012; Li et al. 2012). Both mechanisms are not mutually exclusive as they act simultaneously in a strong feed-forward loop mode, at least in Arabidopsis. GAs and BRs also cooperate in the promotion of shoot elongation in other species, such as tomato (Martí et al. 2006), where the loss-of-function *procera* mutation (a null allele of

PROCERA, the only DELLA gene in tomato) enhanced growth in plants with low and high BRs content (Carrera et al. 2012).

Antagonistic functions of GAs and BRs, similar to those involved in the control of ovule initiation, have also been reported. In rice roots, BRs favoured fungal infection, whereas GA treatment enhanced resistance in a concentration-dependent manner (De Vleesschauwer et al. 2012). In this case, BRs promote a reduction of GA levels. More recently, Xiao et al. (2017) stated that BR-mediated GA repression and growth inhibition in rice is due to the activity of OFP1, which inhibits the expression of the GA biosynthesis genes. In addition to rice, downregulation of GA levels by BRs have also been reported in tomato, pea and sunflower (Jager et al. 2005; Kurepin et al. 2012; Li et al. 2016).

Here we have shown that in Arabidopsis there is no interaction between these hormones during ovule initiation as GAs reduced the ovule number regardless of BR content, or BRs can promote ovule number increase in plants with constitutive or impaired GA signalling (upon GA treatment or in the *gai-1* mutant, respectively). Interestingly, *bzr1-1D* promotes an increase in GA biosynthesis and, therefore, the destabilization of DELLA proteins in the inflorescences. If BRZ1 would regulate ovule number by promoting GA biosynthesis, a decrease in ovule number in *bzr1-1D* would be observed, which is not the case. Therefore, either the increase in GA levels is not localized in the placenta at ovule primordia initiation, or the additional effects of BRs are stronger and can overcome the effect of GAs. In the latter case, a synergistic effect in the *gai-1 bzr1-1D* would be observed. As the number of ovules in *gai-1 bzr1-1D* is not synergistic but additive, it is presumed that the increase of GAs observed in the *bzr1-1D* does not localize in the tissue where the ovules are formed, and that there is an independent mechanism of ovule initiation by BRs and GAs in Arabidopsis.

It is worth mentioning that in Micro-Tom tomato ovaries, BRs and GAs also positively and negatively regulate ovule number, respectively (Gomez et al. 2018; Barro-Trastoy et al. 2020b). However, in this case this process relies on a BR control of GA biosynthesis (Barro-Trastoy et al. 2020b). BRs can only modulate ovule number in tomato plants with normal GA signalling, but not in plants treated with GAs or in the *procera* mutant that have an activated GA response. In tomato unpollinated ovaries, the levels of the bioactive GAs are higher in Micro-

Tom (which harbours a *dwarf* mutation in the *DWARF4* gene, involved in BR biosynthesis) than those from Micro-Tom-D (a Micro-Tom isogenic line that carries the wild type *DWARF4* gene), suggesting that BRs have a negative role in GA biosynthesis. This would rely on the downregulation of *SlGA20ox1* expression at early phases of ovary development, when *DWARF4* is highly expressed (Montoya et al. 2005). The repression of GA biosynthesis by BRs may not be directly controlled by BRZ1, as the canonical Brassinosteroid Binding Responsive Elements of Arabidopsis (He et al. 2005) are not present in the promoter of the tomato *SlGA20ox1*. Nonetheless, it is also possible that a tomato-specific cis-element, different from the motif described in Arabidopsis, could be responsible for binding of BRZ1 to the *SlGA20ox1* promoter. Taken together, data suggest that, in tomato, BRs control ovule number by reducing the GA levels in the placenta, through the repression of *SlGA20ox1* expression, thus stabilizing PROCERA that would finally promote ovule primordia emergence.

Molecular mechanism to control ovule primordia formation by GAs and BRs

Based on the data presented here, we propose a working model involving GAs and BRs in the determination of ovule number (**Fig. 1.14**). In Arabidopsis, BRs and GAs act independently and antagonistically in ovule initiation, being the activity of both BZR1 and GAI positive factors. GAI, and probably other DELLA proteins, would bind to unknown TFs to modulate expression of genes that mediate the GA-dependent ovule initiation pathway. Moreover, BRs activate BZR1 activity

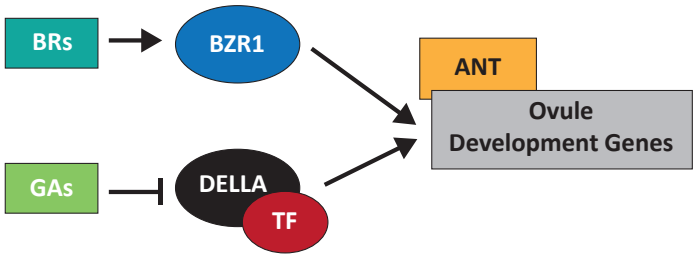

Fig. 1.14: Working model for the interaction between GAs and BRs in the regulation of ovule number in Arabidopsis.

to promote the formation of the ovule primordia. Although both *gai-1* and *bzr1-1D* showed increase in *ANT* expression in inflorescences, ovule number was not altered in *35S:ANT* plants (discussed below).

ANT would not be related to BR or GA pathways in ovule initiation

ANT is a key factor of the genetic control of carpel margin meristem formation, and a master regulator of ovule primordia initiation (Klucher et al. 1996; Galbiati et al. 2013; Cucinotta et al. 2014). In addition to defects in ovule development, *ant* mutants show a strong reduction in the number of ovules per carpel (Elliott et al. 1996; Liu et al. 2000; Azhakanandam et al. 2008; Galbiati et al. 2013). It has been proposed that BRs influence ovule development by regulating the transcription of genes such as *HLL*, *AP2*, and *ANT* (Huang et al. 2013a), with *HLL* and *ANT* being induced and *AP2* being repressed by BRs. Also, *AP2* and *ANT* would be direct targets of BRZ1, whereas *HLL* is regulated indirectly. Our data, however, clearly indicated that *ANT* is not directly related to the increase in ovule number, neither in *bzr1-1D* nor in *gai-1* mutant backgrounds. Although both mutants showed a slight but significant increase in *ANT* expression in inflorescences by qPCR analysis, GAs did not promote changes in *ANT* expression or ANT–YPet protein levels or distribution in the pistil during ovule initiation. In addition, the constitutive expression of *ANT* in the *35S:ANT* lines did not alter ovule number. This finding implies that the increase in ANT activity is not sufficient to promote an increase in ovule number, therefore it could not be the cause of the increased ovule number in *bzr1-1D*.

Nonetheless, ANT activity seems to be necessary in promoting ovule primordia formation. The *gai-1* mutant cannot mitigate the ovule phenotype of *ant-4* as ovule number and ovule development arrest are identical in *ant-4* and in the double mutant *gai-1 ant-4*. Interestingly, it has been proposed that ANT activity, per se, is not absolutely required for ovule initiation, as ovule primordia are initiated and continue to develop until the time of integument initiation in ant mutants (Azhakanandam et al. 2008). Most probably, ANT is required for proper placenta development, being a major regulatory player superimposed to other factors, such the GAs and BRs in ovule primordia initiation.

In summary, our results have provided a detailed analysis of the molecular

mechanism of BR and GA interactions in ovule initiation. In addition, ANT is probably not related mechanistically to the BR or GA pathways in this process. This experimental evidence adds one more layer of complexity to the working model of the gene network that governs the determination of ovule number and ovule primordia emergence.

EXPERIMENTAL PROCEDURES

Plant material assays

Arabidopsis thaliana plants used were on the L*er* or Col-0 backgrounds as indicated. Seeds were sterilized in ethanol and germinated in Murashige and Skoog (MS) medium plates (Murashige and Skoog 1962) (Duchefa Biochemie, Haarlem, The Netherlands) for 4 days at 4°C in the dark, followed by 7–8 days at 22°C under a long day photoperiod (16 h/8 h). Seedlings were then transferred to soil and grown in a chamber at 22°C under long day photoperiod (16 h/8 h). All chemicals and oligos were purchased from Sigma-Aldrich (Madrid, Spain) or Integrated DNA Technologies (IDT; Coralville, Iowa, USA), respectively, unless otherwise is stated.

Arabidopsis DELLA mutants have been previously described (Gomez et al. 2016). *ant-4* (Baker et al. 1997), *bzr1-1D* (Wang et al. 2002) and *det2-1* (Chory et al. 1991) were obtained from the European Arabidopsis Stock Centre (NASC). pRGA:GFP-RGA (Silverstone et al. 2001) was obtained from Tai-ping Sun (Duke University, Durham, NC, USA). The *gai-1* mutant allele, originally in L*er*, was transferred to a Col-0 background by means of three consecutive backcrosses. The combined *gai-1 bzr1-1D* mutant on the Col-0 background was obtained by genetic crossing between introgressed *gai-1* in Col-0 to *bzr1-1D*.

Hormonal treatments and ovule number determination

Ovule number in Arabidopsis was determined as previously described (Gomez et al. 2018). GA treatment was applied by watering every other day with 20 μM of GA_4 + GA_7 (Duchefa Biochemie) and ovules were counted after 2 weeks of treatment. EBR and BRZ treatments were carried out by spraying for 5–8 consecutive days

with either 2 µM of EBR (Apollo Scientific) or 2 µM of BRZ (TCI Chemicals), all in 0.01% (v/v) Tween-20 as the wetting agent. Mock solutions consisted of an equivalent dilution of methanol and Tween-20, as both chemicals were dissolved as 1 mM in methanol. Ovules were determined 7–10 days after the first treatment. Ovary size was determined in the same pistils used for ovule number determination, from images taken under a stereomicroscope.

Treatments of pANT:ANT-YPet plants were carried out using the floral-dip method, immersing the primary inflorescence for 5-10 sec in either 20 µM GA_4 + GA_7 or 1 µM PBZ (Duchefa Biochemie), all in 0.01% (v/v) Tween-20. Mock solutions consisted of an equivalent dilution of ethanol for GAs or acetone for PBZ, both supplemented with Tween-20. After 3 h, the inflorescences were harvested and hand dissected under a Zeiss LSM 780 confocal microscope to visualize the placenta of developing pistils.

Construction of pANT:ANT-YPet *and* 35S:ANT *and plant transformation*

pANT:ANT–YPet translational fusion was generated by bacterial homologous recombination system (recombineering) using a modified variety of the pBALU6 plasmid (Tursun et al. 2009). Recombineering-based DNA modification was done basically as described in Brumos et al. (2020), using universal adaptors at the 5' and 3' ends of the recombineering cassette. Oligonucleotides used are described in **Sup. Table 1.2**. A large genomic fragment containing *ANT* locus (*At4g37750*) in the JAtY57K20 TAC clone was used to introduce a variety of the YPet fluorescent protein at the C_t of *ANT* coding sequence. A modified JAtY57K20 clone with the YPet tag was trimmed at both ends to reduce clone length to stabilize the binary clone and facilitate transformation (Brumos et al. 2020).

For generation of *35S:ANT* lines, the cDNA of the *ANT* gene in the pDONR201 vector was obtained from the REGIA collection (Paz-Ares and REGIA Consortium 2002). The cDNA product was transferred to the pMDC32 binary vector using a LR Gateway reaction, and the construct was confirmed by sequencing.

Finally, *pANT:ANT–YPet* and *35S:ANT* were introduced in planta by

Agrobacterium-mediated floral-dip transformation (Clough and Bent, 1998). For *pANT:ANT–YPet*, the selected transgenic line was crossed to *ant-4* to confirm that it complemented the *ant-4* phenotypes. For *35S:ANT*, the phenotype produced by overexpression of *ANT* was similar to that described previously by Mizukami and Fischer (2000).

qPCR analysis of gene expression

Gene expression analysis was carried out by qPCR in Arabidopsis inflorescences as described in Dorcey et al. (2009). Total RNA was extracted using the NucleoSpin™ RNA Plant kit (Macherey-Nagel™). cDNA was synthesized using PrimerScript™ 1st strand cDNA Synthesis kit (TaKaRa). qPCR was performed using the TB Green Premix Ex Taq II kit (Tli RNase H Plus) (TaKaRa) on the 7500 Fast Real-Time PCR System (Thermo Fisher). The oligonucleotides used (**Sup. Table 1.3**) were designed with Primer Express v2.0 software (Applied Biosystems, Thermo Fisher) and were tested for efficiency. Expression levels were calculated according to the expression of the constitutive gene *UBQ10* (*At4g05320*). The data were normalized using the $\Delta\Delta C_T$ method.

In situ *RNA hybridization*

A 516-bp cDNA fragment of *ANT* that excluded the AP2 domain was amplified from inflorescences, using oligos described in **Sup. Table 1.2**, and cloned into the pGEM-T Easy vector (Promega). Sense and antisense DIG-labelled RNA transcripts were synthesized using the corresponding SP6 and T7 RNA polymerases in the vector. Inflorescences were embedded, sectioned and hybridized, as described by Weigel and Glazebrook (2002). No significant signal was detected using the sense probe. Images were obtained using a Nikon Eclipse E600 microscope and a Nikon Digital-Sight (DS-Ri) camera.

Quantification of GAs

Inflorescences of Arabidopsis of Col-0 and *bzr1-1D* plants were collected, once flowers at anthesis and two or three younger floral buds were removed. Three biological replicates were harvested and analyzed. Plant material were ground in liquid nitrogen in a mortar; 50 mg of frozen material were extracted with 80%

(v/v) methanol and 1% (v/v) acetic acid, including 17-$^{2}H_{2}$-labelled GA internal standards (Olchemim), and mixed by shaking for 1 h at 4°C . GA levels were quantified as described in Seo et al. (2011) and Gomez et al. (2018).

Western blot analysis of GFP-RGA

Levels of the GFP–RGA protein were determined from inflorescences of the transgenic *pRGA:GFP–RGA* line (Silverstone et al. 2001). Upon bolting, adult plants were treated with either mock, GAs (20 µM), EBR (2 µM) or BRZ (2 µM) by spray. Inflorescences were collected 4 h later, once flowers at anthesis as well as two or three floral buds were removed. Total protein from 50 mg frozen material was extracted in 1 volume of 2x Laemmli buffer (0.25 M Tris–HCl, pH 6.8, 10% [w/v] SDS, 25% [v/v] glycerol, 0.75% [w/v] bromophenol blue). Protein levels were determined using the DC™ Protein Assay method. Total protein (20 µg) was loaded onto SDS-PAGE 12.5% (w/v) acrylamide gels and run for 2–3 h at 120 V. Proteins in the gel were then transferred by blotting onto a membrane (Amersham Hybond 0.2 µm polyvinylidene fluoride, PVDF). Anti-GFP mouse Living Colors® A.v. Monoclonal Antibody (JL8) (TaKaRa) and anti-mouse Amersham ECL Mouse IgG (GE Healthcare) antibodies were used to detect GFP–RGA. Signals were detected using SuperSignal West FemtoMaximun Sensitivity Substrate and images were recorded on a Fujifilm LAS3000 Imager.

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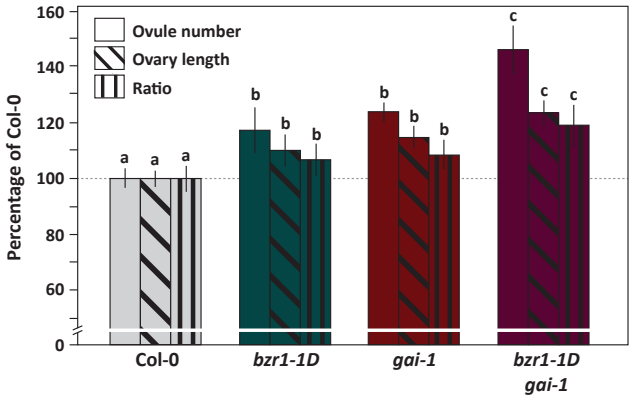
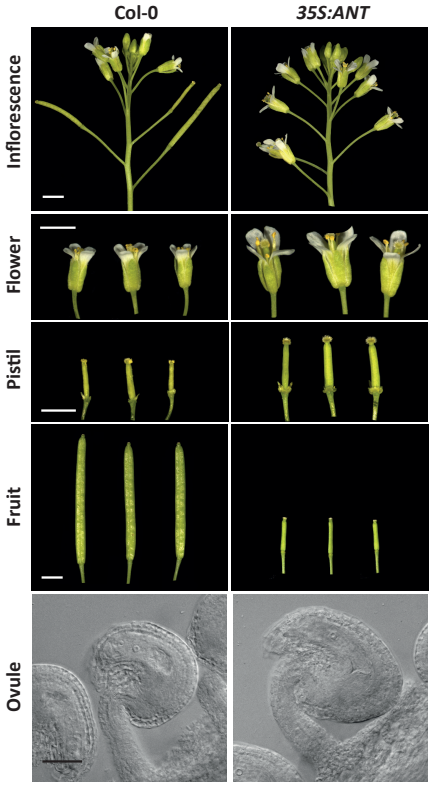
SUPPLEMENTARY INFORMATION

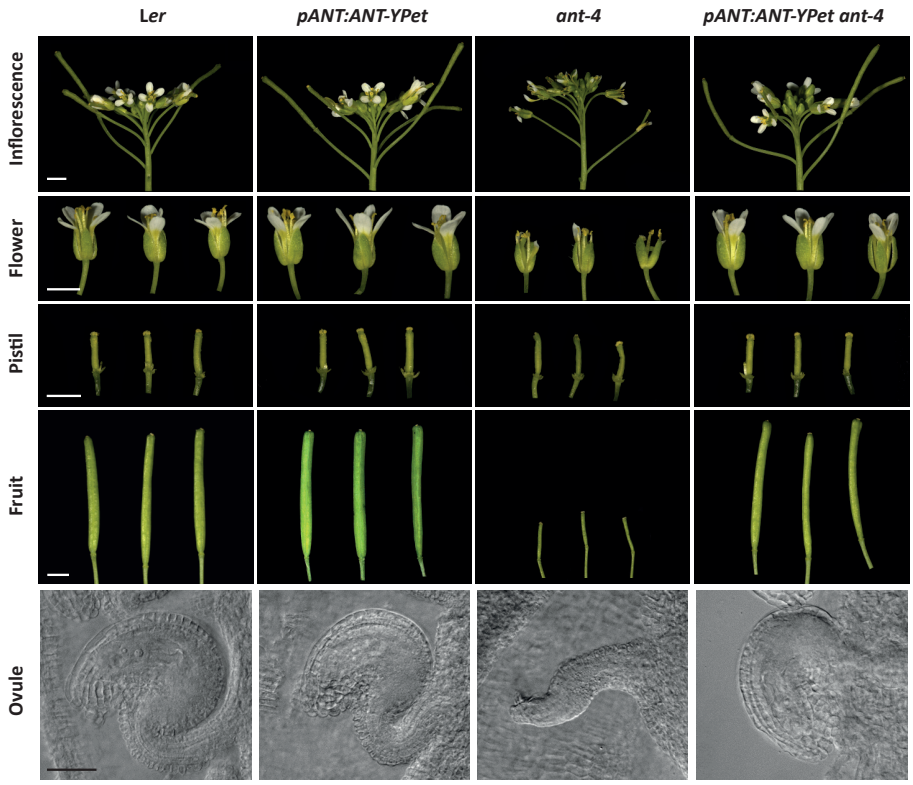
Supplementary figures



Sup. Fig. 1.1: Ovule number, ovary length and ratio of ovules per mm in *bzr1-1D*, *gai-1*, and double *gai-1 bzr1-1D* mutants. Data represent the mean $\pm$ SD (n $=$ 15). Letters indicate statistical significance as determined by ANOVA with a Bonferroni post hoc test for multiple comparisions. Data that are not significantly different are marked with the same letter.



Sup. Fig. 1.2: Effect of overexpression of *ANT* in floral organ size in Arabidopsis. Scale bars represent 2 mm in inflorescences and flowers, 1 mm in pistils and fruits, and 50 µm in ovules.



Sup. Fig. 1.3: Complementation of developmental defects of *ant-4* by *pANT:ANT-YPet* in Arabidopsis. Scale bars represent 2 mm in inflorescences and flowers, 1 mm in pistils and fruits, and 50 µm in ovules.

Supplementary tables

Sup. Table 1.1: Ovule number, ovary length and ratio of ovules per mm in *bzr1-1D*, *gai-1*, and double *gai-1 bzr1-1D* mutants.

	Ovule number	**p-value**	**Ovary length**	**p-value**	**Ratio**	**p-value**
Col-0	100.0 ± 3.7[a]		100.0 ± 3.2[a]		100.0 ± 4.7[a]	
gai-1	124.4 ± 3.6[b]	<0.001	115.1 ± 4.5[b]	<0.001	108.1 ± 5.5[b]	<0.001
bzr1-1D	116.8 ± 2.2[b]	<0.001	110.0 ± 5.9[b]	<0.001	106.4 ± 6.4[b]	<0.01
gai-1 bzr1-1D	146.1 ± 9.7[c]	<0.001	123.2 ± 5.0[c]	<0.001	118.6 ± 7.9[c]	<0.001

Data referenced to Col-0.

Values for Col-0: ovule number 61.2 ovules; ovary length 2.2 mm; ratio 27.5 ovules · mm^{-1}.

The letters for each genotype indicate statistical significance as determined by an ANOVA with a Bonferroni post hoc test for multiple comparisons.

Sup. Table 1.2: Oligonucleotides used as primers for constructs and probes generation.

Generation of *pANT:ANT-Ypet* by recombineering*	
ANTRec-Fw*	ACTTTGCCTCAGATGCCGGTTTTCGCTGCTTGGGCTGATTCTGGAGG TGGAGGTGGAGCT
ANTRec-Rv*	CTAAACCAAGAAAACCAGAGTTAGTCATTGAAGTTGATCAGGCCCCAG CGGCCGCAGCAGCACC
ANTTest-Fw	GACCAAACCGCTCTAAATGC
ANTTest-Rv	TCGAAAATACGTAACGAAGAGAGA
ANTDelLeft	TAAAATATTCGGATATTTTAAAGTCTGCATCCCTTTCCCATTACCAAT GCTTAATCAGTG
ANTDelRight	ATAAATGATATGAAAACAATTGAAAGGGATCAAAAAAATGTAGGAAC TTCCCCCTCTTGG
ANTTestDelL	CTGTTACTGGTTATCACACAAATCA
ANTTestDelR	CAGCAAAGCAAAAATCTTAATGG
Generation of the *ANT* molecular probe for *in situ* RNA hybridization	
ANT-Ins-Fw	GGTGGAGGATTTCTTTGGGACCC
ANT-Ins-Rv	AACGCCTCGGTATTGAGAAGTTCG

*, universal adaptor sequence is underlined.

Sup. Table 1.3: Oligonucleotides used for qPCR analysis.

GA biosynthesis genes	
GA20ox1 (*At4g25420*)	CTTCCATCAACGTTCTCGAGC
	CTTCCATCAACGTTCTCGAGC
GA20ox2 (*At5g51810*)	CGAGCAGTTTGGGAAGGTGTATC
	CCTAAACTTAAGCCCAGAAGCTCC
GA20ox3 (*At5g07200*)	TTCGTGGACAACAAATGGCA
	CCATTCGTTAGAGCCATGAAGG
GA20ox4 (*At1g60980*)	GGCGACACTTTAATGGCTCTAACG
	GTGTCTTCCTTGTCGTCTCGCC
GA20ox5 (*At1g44090*)	CCTGGTGCTCTTGTCGTCAAC
	CCATTTGACAATGCCATGAAGG
GA3ox1 (*At1g15550*)	GATCTCCTCTTCTCCGCTGCT
	GAGGGATGTTTTCACCGGTG
GA3ox2 (*At1g80340*)	GCCACCACCTCAAATACTGTGAA
	GGCTGCCAACTTTTGCATATGT
GA3ox3 (*At4g21690*)	CATGCCGAGTTCTGCAATGTGATGG
	GTGTTAGCCCTAACGAGCCCATC
GA3ox4 (*At1g80330*)	CACACCAAGTACTGCGGTATAATCC
	GCCCATTCAATGTCTTCCACGG
***ANT* gene**	
ANT (*At4g37750*)	GCGTTACAAGACATAGATGGA
	TGCAACATATTCTTGTCTAGTC
Housekeeping gene	
UBQ10 (*At4g05320*)	GGCCTTGTATAATCCCTGATGAATAAG
	AAAGAGATAACAGGAACGGAAACATAGT

2

Gibberellins regulate ovule number through a DELLA–CUC2 complex in Arabidopsis

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CHAPTER 2

Gibberellins regulate ovule number through a DELLA–CUC2 complex in Arabidopsis

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Abstract

Ovule development is a key process for plant reproduction, helping to ensure correct seed production. Several molecular factors and plant hormones such as gibberellins are involved in ovule initiation and development. Gibberellins control ovule development by the destabilization of DELLA proteins, whereas DELLA activity has been shown to act as a positive factor for ovule primordia emergence. But the molecular mechanism by which DELLA acts in ovule primordia initiation remained unknown. In this study we report that DELLA proteins participate in ovule initiation by the formation of a protein complex with the CUC2 transcription factor. The DELLA protein GAI requires CUC2 to promote ovule primordia formation, through the direct GAI–CUC2 interaction in placental cells that would determine the boundary regions between ovules during pistil development. Analysis of GAI–CUC2 interaction and co-localization in the placenta supports this hypothesis. Moreover, molecular analysis identified a subset of the loci for which the GAI protein may act as a transcriptional co-regulator in a CUC2-dependent manner. The DELLA–CUC2 complex is a component of the gene regulatory network controlling ovule primordia initiation in Arabidopsis.

Keywords: Arabidopsis, gibberellins, ovule, DELLA proteins, GAI, CUC2.

The data presented in this chapter were published (Barro-Trastoy et al. 2022). DB-T performed most experiments. MDG analyzed GAI spatial localization by confocal microscopy. DB-T, MDG, NB-T, PT, and MAP-A wrote and commented on the manuscript. MDG, PT, and MAP-A designed the study.

INTRODUCTION

Ovules are essential for reproductive development as they provide the foundation for seed development that will secure the perpetuation of the plant species. In the last few years, the molecular mechanisms that control ovule formation have attracted interest, and several key pieces of experimental evidence have allowed the construction of models of the molecular mechanism controlling ovule primordia initiation and development (recently reviewed in Erbasol-Serbes et al. (2019), Lora et al. (2019), Pinto et al. (2019), Cucinotta et al. (2020), and Barro-Trastoy et al. (2020a)). In fact, studying ovule development provides an interesting model for understanding organ formation in general during plant development. In addition, proper ovule initiation and development directly impact the production of seeds and grains, a key component of human and animal diets (Shirley et al. 2019; Cucinotta et al. 2020); hence, a full understanding of the players involved in ovule and seed development will contribute to designing additional approaches to increase crop yield.

In Arabidopsis, ovule primordia arise from the placenta, a meristematic tissue located in the medial domain of the pistil, at stage 8 of floral development (Cucinotta et al. 2014, 2020; Yu et al. 2020; Vijayan et al. 2021; Barro-Trastoy et al. 2020a). This is the first key step of ovule development and depends on the correct determination of ovule primordia position along the placenta. Regulatory genes found to have a role during ovule initiation include *AINTEGUMENTA* (*ANT*), which regulates cell proliferation and promotes ovule primordia growth, and *CUP-SHAPED COTYLEDON1* (*CUC1*) and *CUC2*, which establish the boundary regions between ovule primordia.

Additionally, several plant hormones are also involved, some of them being highly interconnected with *ANT*, *CUC1*, and *CUC2*. PIN-FORMED1 (PIN1) auxin efflux causes auxin accumulation, which is essential to promote ovule initiation (Benková et al. 2003; Ceccato et al. 2013; Galbiati et al. 2013). Cytokinins (CKs), whose levels are partially controlled by *CUC1* and *CUC2* (Cucinotta et al. 2018), promote *PIN1* expression to allow correct auxin efflux (Bencivenga et al. 2012; Galbiati et al. 2013). Finally, brassinosteroids (BRs) positively influence ovule number, probably through the regulation of *ANT* expression (Huang et al. 2013a).

Recently, we have demonstrated that gibberellins (GAs) negatively modulate ovule number in Arabidopsis, rapeseed (*Brassica napus*), and tomato (*Solanum lycopersicum*) through DELLA protein activity (Gomez et al. 2018, 2019; Barro-Trastoy et al. 2020b). GAs are a group of diterpenoid compounds involved in many developmental processes such as seed germination, stem and root elongation, flowering, and ovule and fruit development (Sun 2011; Gupta and Chakrabarty 2013; Gomez et al. 2016). GA signaling relies on the degradation of DELLA proteins, nuclear proteins that belong to the GRAS family of transcriptional regulators and act as GA signaling repressors.

GA-dependent DELLA degradation is mediated by the N-terminal domain of DELLA (Sun 2011). Deletion of this 17-aa domain is sufficient to prevent GA-mediated protein degradation, like in the *gai-1* mutant (Peng et al. 1997) allele, which encodes dominant DELLA proteins that constitutively block GA signaling. Both high GA levels and loss of-function of DELLA proteins release GA responses, while low GA levels or GA insensitive DELLA mutants restrain GA responses (Sun 2011; Davière and Achard 2016; Vera-Sirera et al. 2016; Hernández-García et al. 2020).

During pistil development, constitutive GA signaling in the null mutant *4xdella*, lacking four of the five DELLA proteins encoded by the Arabidopsis genome (i.e., lacking GA INSENSITIVE [GAI], REPRESSOR OF GA1-3 [RGA], RGA-LIKE1 [RGL1], and RGL2), produces a decrease in ovule number, with GAI, RGA, and RGL2 being those that have a major role (Gomez et al., 2018). In fact, these three DELLA proteins are expressed in ovule primordia at early developmental stages (Gomez et al. 2018). A similar ovule number phenotype

was also seen in GA-treated plants. On the contrary, constitutive blockage of GA responses in the *gai-1* mutant produces more ovules than in wild type plants (Gomez et al. 2018). Similarly, RGL2 is also involved in ovule initiation, as *pRGL2:rgl2Δ17-YPet* plants, which produce an RGL2 protein version that is not degraded by GAs, also show increased ovule number (Gomez et al. 2019). These pieces of evidence clearly indicate that DELLA proteins are positive factors in ovule formation.

However, the molecular mechanism underlying the regulation of ovule initiation by DELLA proteins remains unclear. Previous analyses suggested that DELLA-mediated ovule initiation does not seem to be related to either auxin or BR pathways in Arabidopsis. On the one hand, neither auxin transport nor the auxin response is altered in the placenta of GA mutants or upon GA treatment (Gomez et al. 2018). On the other hand, genetic analysis revealed that GAs and BRs act independently during ovule initiation (Barro-Trastoy et al. 2020b). Despite these analyses, there is no indication of how DELLA proteins promote ovule primordia formation.

At the molecular level, DELLA proteins are transcriptional regulators that lack a canonical DNA-binding domain, exerting their function by binding to a wide variety of transcription factors (TFs) (Davière and Achard 2016; Vera-Sirera et al. 2016; Hernández-García et al. 2020). Based on this, a plausible hypothesis is that DELLA proteins physically interact with any of the key TFs involved in ovule initiation, such as ANT, CUC1, and CUC2. In fact, CUC2 was found as a putative interactor with the DELLA protein GAI in a yeast two-hybrid (Y2H) screen (Marín-de la Rosa et al. 2014), suggesting CUC2 is a component of the DELLA-mediated ovule initiation mechanism.

CUC1, *CUC2*, and *CUC3* genes encode NAC-domain family TFs with a central and redundant role in organ boundary formation, e.g., in primary and axillary shoots, leaf serration, and floral organs including the gynoecium and ovules (reviewed in Maugarny et al. (2016)). In the placenta, *CUC1* and *CUC2* are known to positively regulate ovule initiation, whereas *CUC3* is involved, along with *CUC2* in a redundant manner, in ovule separation (Ishida et al. 2000; Galbiati et al. 2013; Gonçalves et al. 2015).

This work aims to uncover the molecular mechanism by which DELLA proteins

regulate ovule development. Our data point to CUC2 as the key component of the DELLA-mediated ovule initiation pathway. CUC2 is required for the DELLA protein GAI to promote ovule primordia formation. GAI participates in ovule initiation by its direct protein–protein interaction with CUC2 in cells of the boundary regions between ovules during pistil elongation. Direct analysis of GAI–CUC2 interaction and colocalization in the placenta supports this hypothesis. Furthermore, molecular analysis of the loci at which GAI may act as transcriptional co-regulator in a CUC2-dependent manner identified a subset of genes potentially regulated by the GAI–CUC2 complex and contributing to regulating ovule primordia emergence. Altogether, this analysis allows us to integrate GAs and DELLA proteins in the gene network that governs ovule primordia initiation.

RESULTS

GAI requires CUC2 to mediate ovule number

Previously, a Y2H screen of 1200 TFs from Arabidopsis identified CUC2 as a putative interactor of GAI (Marín-de la Rosa et al. 2014). To address whether CUC2 may mediate DELLA function in ovule initiation, we carried out a com-

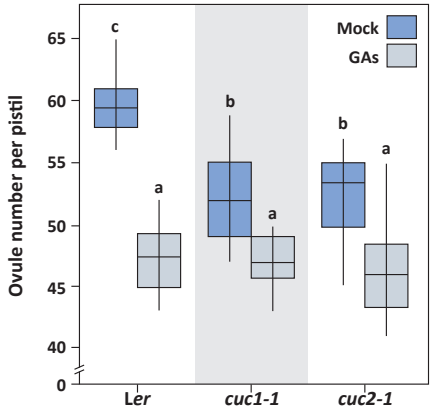

Fig. 2.1: Ovule number per pistil in L*er*, *cuc1-1*, and *cuc2-1* upon mock or GA treatment. Data are presented as boxplots (n = 12). Letters indicate statistical significance as determined by ANOVA and a Bonferroni post hoc test for multiple comparisions ($P < 0.01$). Data that are not significantly different are marked with the same letter.

prehensive analysis of ovule number phenotype in *cuc* mutants under the effect of treatments with GA and paclobutrazol (PBZ), an inhibitor of GA biosynthesis that promotes an increase in the levels of DELLA proteins. We also performed analyses of development under the dominant *gai-1* or null *4xdella* backgrounds.

In accord with previous results (Ishida et al. 2000; Galbiati et al. 2013), both *cuc1-1* and *cuc2-1* single null mutants showed a 10% reduction in ovule number (**Fig. 2.1**), which is consistent with CUC1 and CUC2 being positive regulators of ovule initiation. GA treatment, which reduces levels of DELLA proteins, caused a 20% decrease in ovule number in wild type plants (Gomez et al. 2018) and a 10% decrease in *cuc1-1* and *cuc2-1* mutants. Therefore, ovule number was similar in wild type and *cuc* single mutants upon GA treatment. Similarly, *cuc2-1* has no effect on ovule number in the *4xdella* background (**Fig. 2.2**).

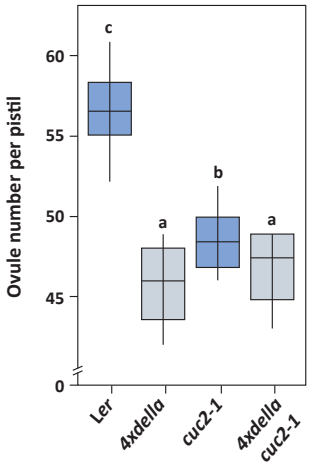

Fig. 2.2: Ovule number per pistil in L*er*, *4xdella*, *cuc2-1*, and *4xdella cuc2-1*. Data are presented as boxplots (n = 12). Letters indicate statistical significance as determined by ANOVA and a Bonferroni post hoc test for multiple comparisions ($P < 0.01$). Data that are not significantly different are marked with the same letter.

Next, we treated wild type, *cuc1-1*, and *cuc2-1* plants with PBZ. PBZ treatment slightly increased ovule number in wild type plants and the *cuc1-1* mutant (**Fig. 2.3**). Surprisingly, PBZ did not have any effect in *cuc2-1*, indicating that CUC2 but not CUC1 is required by DELLA activity to promote ovule primordia formation. This was confirmed when *cuc1-1* and *cuc2-1* were crossed to *gai-1*;

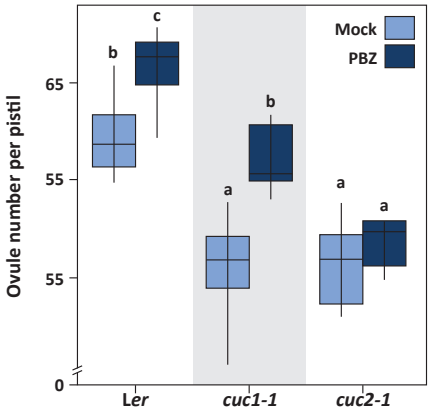

Fig. 2.3: Ovule number per pistil in L*er*, *cuc1-1*, and *cuc2-1* upon mock or PBZ treatment. Data are presented as boxplots (n = 12). Letters indicate statistical significance as determined by ANOVA and a Bonferroni post hoc test for multiple comparisions ($P < 0.01$). Data that are not significantly different are marked with the same letter.

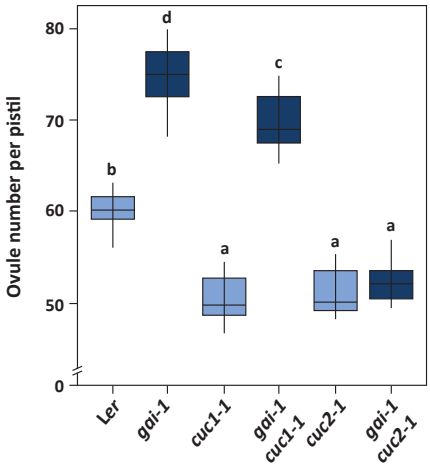

Fig. 2.4: Ovule number per pistil in L*er*, *gai-1*, *cuc1-1*, *gai-1 cuc1-1*, *cuc2-1*, and *gai-1 cuc2-1*. Data are presented as boxplots (n = 12). Letters indicate statistical significance as determined by ANOVA and a Bonferroni post hoc test for multiple comparisions ($P <0.01$). Data that are not significantly different are marked with the same letter.

whereas the *gai-1* mutant increases ovule number by around 20% in both wild type and *cuc1-1* backgrounds, no significant differences in ovule number were observed in the *gai-1 cuc2-1* double mutant compared to *cuc2-1* (**Fig. 2.4**).

These results clearly indicate that GAI activity requires the presence of CUC2 activity to promote the formation of ovule primordia, since *gai-1* was not able to increase the number of ovules in a *cuc2-1* background. It is worth mentioning that whereas *cuc2-1* suppressed the *gai- 1* ovule number phenotype, it did not alleviate the overall *gai-1* plant shape phenotype (**Sup. Fig. 2.1**), which is characterized by smaller and darker plants (Peng and Harberd 1993; Peng et al. 1997).

Next, we sought to find out whether the observed differences in *cuc* mutants in the presence of *gai-1* were a direct consequence of changes in ovule number rather than an indirect effect of the alteration of ovary length. Interestingly,

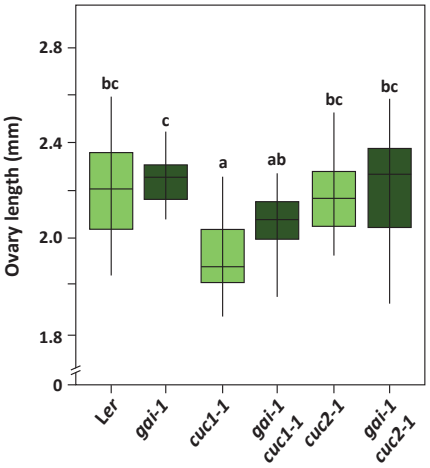

Fig. 2.5: Ovary length in L*er*, *gai-1*, *cuc1-1*, *gai-1 cuc1-1*, *cuc2-1*, and *gai-1 cuc2-1*. Data are presented as boxplots (n = 12). Letters indicate statistical significance as determined by ANOVA and a Bonferroni post hoc test for multiple comparisions ($P < 0.01$). Data that are not significantly different are marked with the same letter.

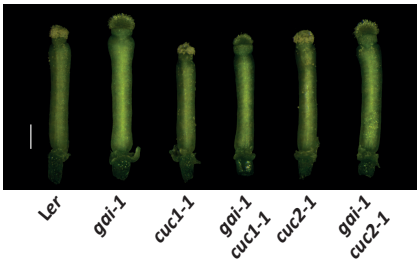

Fig. 2.6: Mature pistils of L*er*, *gai-1*, *cuc1-1*, *gai-1 cuc1-1*, *cuc2-1*, and *gai-1 cuc2-1*. Scale bar represents 500 µm.

the *cuc1-1* mutant developed slightly shorter ovaries (**Fig. 2.5** and **Fig. 2.6**). As a consequence, the ratio of ovule number to ovary length in *cuc1-1* was not significantly different compared to the wild type, as the ovule number was reduced to a similar extent (**Fig. 2.7** and **Fig. 2.8**). On the contrary, the length of *cuc2-1* ovaries was similar to those of the wild type, which, along with a reduction in ovule number, resulted in a decrease in the ratio of ovule number to ovary length. In other words, ovule density in *cuc2-1*, but not *cuc1-1*, was reduced. Finally, ovaries of the *gai-1* single mutant as well as the *gai-1* cuc double mutant were similar to those of their corresponding control plants (L*er* for *gai-1*, *cuc1-1* for *gai-1 cuc1-1*, and *cuc2-1* for *gai-1 cuc2-1*). Therefore, the ratio was clearly higher in *gai-1* and *gai-1 cuc1-1* but not in *gai-1 cuc2-1*. The parallel analysis of ovule number and ovary length allows us to conclude that both CUC1 and CUC2 participate in

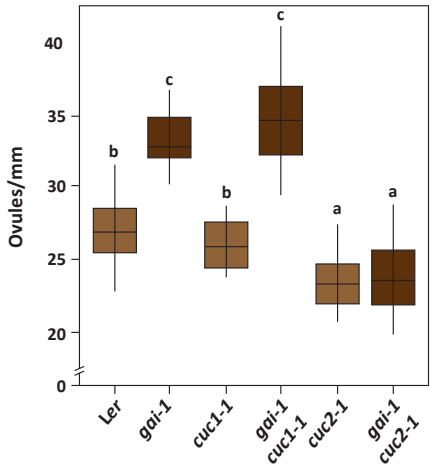

Fig. 2.7: Ratio of ovule number to ovary length in L*er*, *gai-1*, *cuc1-1*, *gai-1 cuc1-1*, *cuc2-1*, and *gai-1 cuc2-1*. Data are presented as boxplots (n = 12). Letters indicate statistical significance as determined by ANOVA and a Bonferroni post hoc test for multiple comparisions ($P < 0.01$). Data that are not significantly different are marked with the same letter.

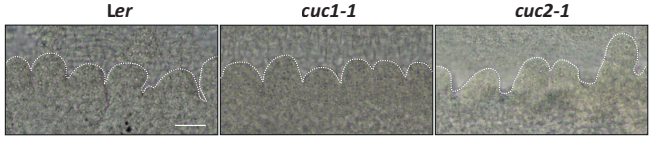

Fig. 2.8: Images of ovule primordia at stage 1-I from L*er*, *cuc1-1*, and *cuc2-1*. Scale bar represents 20 µm.

ovule number in different ways: *cuc1-1* has an indirect effect on ovule number by reducing ovary length, whereas *cuc2-1* has a direct effect on ovule number. These phenotypes were even clearer in the *gai-1 cuc2-1* mutant, where neither ovule number nor the ratio was altered compared to *cuc2-1*.

To further study the roles of CUC2 and CUC1 in ovule number determination, we counted ovules and scored ovary length in gain-of-function *pCUC1:CUC1m-GFP* and *pCUC2:CUC2m-GFP* lines. CUC genes are downregulated by miR164, and these constructs express miR164 cleavage-resistant versions of *CUC1* and *CUC2*, respectively (Baker et al. 2005; Sieber et al. 2007). Both lines developed more ovules per pistil, which is consistent with the positive role of CUCs in ovule initiation (**Sup. Fig. 2.2**). Moreover, in accordance with data from the knockout mutants, *pCUC1:CUC1m-GFP* generated longer ovaries, resulting in the same density of ovules as the wild type, whereas *pCUC2:CUC2m-GFP* did not alter ovary length, resulting in an increased density of ovules in the placenta.

Finally, ovule and ovary phenotypes were also tested in higher-order *cuc* mutants in combination with *gai-1*. The *cuc1-1 cuc2-1* double mutant completely lacks a shoot apical meristem and does not develop shoots and, consequently, inflorescences (Aida et al. 1997; Hibara et al. 2006). As an alternative, we used *cuc1-1 cuc2-1/+* and *cuc1-1/+ cuc2-1* sesquimutants. In *cuc1-1 cuc2-1/+*, a null *CUC1* with only one copy of *CUC2*, we observed a 20% reduction in the number of ovules (**Sup. Fig. 2.3**) and a 10% reduction in ovary length, which resulted in a decrease of ovule density. The difference in phenotype observed between *cuc1-1 cuc2-1/+* and the single mutant *cuc1-1* could be due to an additive effect of loss-of-function of *CUC1* regulating ovary length and the loss of one copy of *CUC2*, which further reduced the number of ovules that initiate from the placenta. The *cuc2-1* mutant has been described as potentially semi-dominant (Aida et al. 1997), and, therefore, the loss of one copy of *CUC2* in *cuc2-1/+* may contribute significantly to ovule number reduction. Interestingly, just one copy of wild type *CUC2* was sufficient to allow *gai-1* to increase the ovule number in the *cuc1-1 cuc2-1/+* background. On the other hand, the loss of one copy of *CUC1* in the *cuc1-1/+ cuc2-1* sesquimutant reduced the ovule number by around 25% but did not affect ovary length compared to *cuc2-1* (**Sup. Fig. 2.3**). In the *cuc1-1/+ cuc2-1* background, *gai-1* was not able to increase the ovule number,

due to the loss of both wild type copies of *CUC2*, unlike the case with the single mutant *cuc2-1*, which further confirms that CUC2 is an absolute requirement for GAI-dependent ovule primordia formation.

Taken together, these genetic analyses suggest that GAI requires CUC2 activity to directly regulate ovule number, whereas CUC1 indirectly contributes to ovule number determination by regulating ovary elongation, independent of GAs.

GAI interacts with CUC1 and CUC2

Interaction of CUC2 with GAI and with the other four DELLA proteins of Arabidopsis was confirmed by Y2H assays (**Fig. 2.9**). For this, the M5 truncated versions of DELLA, lacking the DELLA N-terminal domain that confer auto-activation in yeast when fused to the Gal4 DNA-binding domain (de Lucas et al. 2008), were used as baits.

To better characterize the GAI–CUC2 interaction, protein domains necessary for protein–protein interactions were mapped (**Sup. Fig. 2.4**). Several deletions of

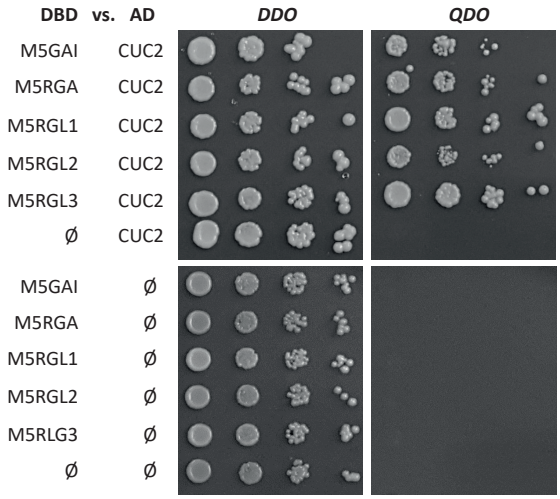

Fig. 2.9: Y2H assay of protein interaction between CUC2 and the five DELLA proteins in Arabidopsis. DBD, DNA-binding domain; AD, activation domain, Ø, empty vector. DDO, double dropout (SD/-Leu/-Trp); QDO, quadruple dropout (SD/-Ade/-His/-Leu/-Trp). Ten-fold serial dilutions were spotted onto SD media to test the interactions. M5 truncated versions of DELLA proteins (de Lucas et al. 2008) were used as baits.

GAI (previously described in Gallego-Bartolomé et al. (2012)) were tested against full-length CUC2. First, the DELLA regulatory domain was not involved in the interaction with CUC2, as its removal in the M5GAI version did not affect the interactions in yeast. Second, deletion of the first leucine heptad repeat (LHR1) in GAI (Hauvermale et al. 2012; Vera-Sirera et al. 2016) prevented GAI–CUC2 interaction. However, small fragments of GAI containing the LHR1 did not interact with CUC2, suggesting that the LHR1 is necessary but not sufficient for the interaction. On the other hand, serial deletions of CUC2 were tested against the complete GAI protein (**Sup. Fig. 2.5**). CUC2 is characterized by a NAC N-terminal domain divided into five sub-domains that mediate protein interactions and DNA binding (Ooka et al. 2003; Ernst et al. 2004). Serial deletions of these domains showed that elimination of the first NAC sub-domain prevented interaction with GAI, suggesting that this region is important for GAI–CUC2 interaction. Finally, the N-terminus of CUC2 containing the five subdomains is not sufficient to confer binding to GAI. Overall, deletion analysis indicated that full-length CUC2 and at least the GRAS functional domain of GAI are necessary for the formation of the GAI–CUC2 protein complex.

We tested whether CUC1 and GAI interact in a designed Y2H assay. CUC1 showed very little binding to M5GAI (compared to the M5GAI control, which showed residual auto-activation), whereas CUC2 showed strong interaction in the same assay (**Sup. Fig. 2.6**). CUC1–CUC2 and CUC2–CUC2 (forming homo- and hetero-dimers) were used as positive controls (Rubio-Somoza et al. 2014; Gonçalves et al. 2015).

In planta interaction of both CUC2 and CUC1 with M5GAI was confirmed by protein co-immunoprecipitation (Co-IP) assays in *Nicotiana benthamiana* leaves (**Fig. 2.10** and **Sup. Fig. 2.7**). In the assay, CUC2-HA or myc-CUC1 was transiently expressed together with YFP-M5GAI. Both CUC2-HA and myc-CUC1 were co-immunoprecipitated when YFP-M5GAI was pulled down in leaf extracts from plants that were co-infiltrated.

Finally, we also wanted to check if these proteins were co-localized in vivo in the placenta of Arabidopsis pistils, which would be key for determining whether the GAI–CUC interaction is a fundamental mechanism for GAI mediation in ovule formation. To test this, we created a *pGAI:mRFP-gaiΔ17* reporter line, which ex-

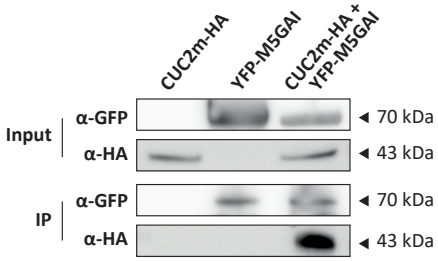

Fig. 2.10: Co-IP assay of YFP-M5GAI with CUC2m-HA in *N. benthamiana*. Western blot was performed with anti-GFP and anti-HA antibodies to detect YFP-M5GAI and CUC2m-HA, respectively, in both input and IP protein samples. A miR164-resistant version of CUC2 (CUC2m) was used.

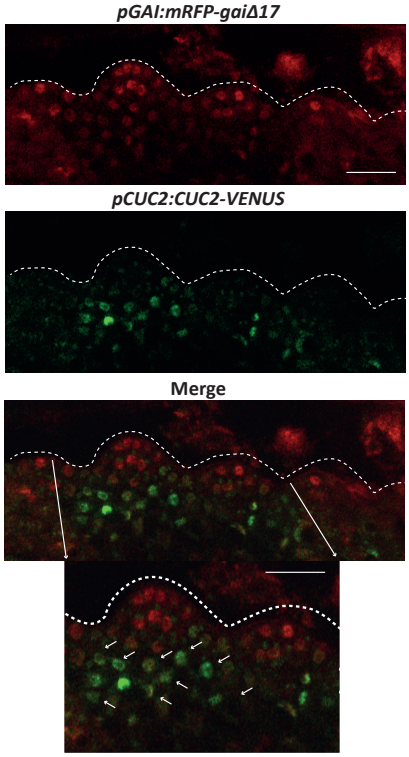

Fig. 2.11: Co-localization assay in placental tissue of GAI (*pGAI:mRFP-gaiΔ17*) with CUC2 (*pCUC2:CUC2-VENUS*) at stage 1-I of ovule development. White arrowheads point to nuclei where both mRFP-gaiΔ17 and CUC2-VENUS were co-localized. Scale bars represent 20 µm.

presses an mRFP fluorescence peptide fused to a stable version of GAI, identical to that in the *gai-1* mutant (Peng et al. 1997). GAI protein expression, monitored with the *pGAI:mRFP-gaiΔ17* line, was localized in the placental cells and primordia at stage 1-I of ovule development (**Fig. 2.11** and **Sup. Fig. 2.9**), with a similar gene expression pattern to that of the transcriptional line *pGAI:GUS* (Gomez et al. 2016). This line was crossed with *pCUC2:CUC2-VENUS* and *pCUC1:CUC1-GFP* transgenic lines (Gonçalves et al. 2015). Both mRFP-gaiΔ17 and CUC2–VENUS were observed in some nuclei in the placenta and ovule primordia boundaries. Similarly, mRFP-gaiΔ17 and CUC1-GFP also co-localized in some cells of the placenta. In summary, protein–protein interaction and co-localization studies of GAI, CUC1, and CUC2 strongly suggest that these proteins can form a complex in nuclei of placental cells during pistil development.

DELLA and CUC2 are not reciprocally regulated

At this point, we could formulate a plausible working model in which GA-mediated ovule initiation may rely on the interaction of the DELLA protein GAI with the TF CUC2. To deepen this model, we checked whether DELLA proteins could also regulate CUC2 expression and *vice versa*. Quantitative PCR (qPCR) analysis indicated that *CUC2* mRNA levels did not significantly change in *4xdella* or *gai-1* inflorescences (**Fig. 2.12**). In addition, reanalysis of a transcriptomic assay of pistils of *global della* (lacking all five *DELLA* genes) and *gai-1* mutants at stage 8 of

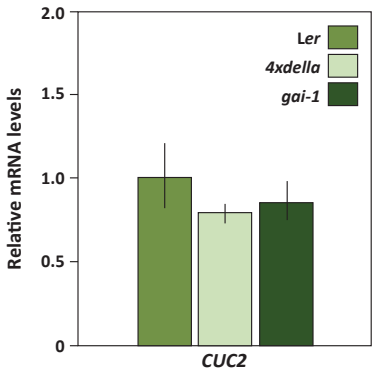

Fig. 2.12: Relative mRNA levels of *CUC2* in inflorescences of L*er*, *4xdella*, and *gai-1*. Data were normalized to *UBQ10* in L*er*. Data represent the mean ± SD. No significant differences (Student's *t*-test, $P < 0.01$) were detected.

floral development (Gomez et al. 2018) revealed that *CUC2* was not differentially expressed. Finally, CUC2 protein levels were not altered in *pCUC2:CUC2-VENUS* reporter lines when crossed with the *gai-1* mutant (**Fig. 2.13**).

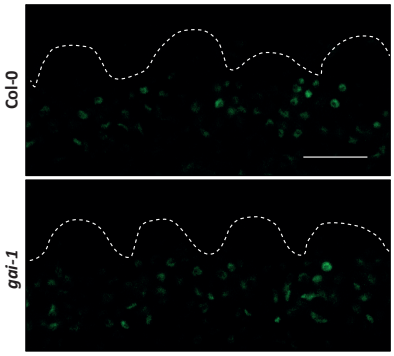

Fig. 2.13: Confocal microscopy images of ovule primordia at stage 1-I in plants *pCUC2:CUC2-VENUS* in Col-0 or *gai-1*. Scale bar in represents 20 µm.

With a similar rationale, we also checked whether CUC2 could regulate the expression of *DELLA* genes. First, mRNA levels of the four main DELLA genes involved in ovule development, *GAI*, *RGA*, *RGL1*, and *RGL2* (Gomez et al. 2018, 2019, 2020), did not show significant changes in *cuc2-1* mutant inflorescences (**Fig. 2.14**). Second, reanalysis of data reported by Cucinotta et al. (2018) of a *cuc2-1 pSTK:CUC1-RNAi* versus wild type transcriptomic assay revealed that DELLA genes were not differentially expressed. Taken together, the data indicate

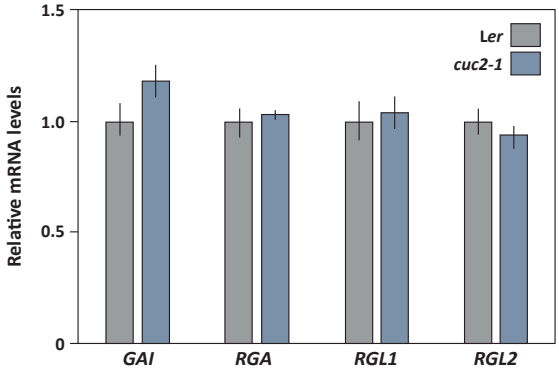

Fig. 2.14: Relative mRNA levels of *GAI*, *RGA*, *RGL1* and *RGL2* in inflorescences of L*er* and *cuc2-1*. Data represent the mean $\pm$ SD. No significant differences (Student's *t*-test, $P < 0.01$) were detected.

that GAI does not regulate CUC2 expression, and CUC2 regulates GAI at neither the mRNA nor the protein level in the placenta during ovule initiation.

CUC2 does not participate in BR-mediated ovule initiation

It has been shown that *CUC* genes are repressed by BRs (in a BZR1-dependent manner) during organ boundary formation in the shoot apical meristem (Gendron et al. 2012); therefore, *CUC* expression and boundary formation require reduced *BZR1* expression. In contrast, in the placenta both BZR1 and CUC are positive factors for ovule initiation. We asked whether BZR1 may require CUC2 to promote ovule primordia development, as in GAI-dependent ovule primordia formation. To do so, the ovule number was determined in the *bzr1-1D cuc2-3* double mutant in comparison with *bzr1-1D*, *cuc2-3*, and Col-0 wild type plants (**Fig. 2.15**). First, *cuc2-3* (Col-0 background) reduced the number of ovules by around 10%, like the *cuc2-1* mutant (L*er* background). Second, *bzr1-1D* pistils presented an increase in ovule number of around 20%, however, as previously described (Huang et al. 2013a; Barro-Trastoy et al. 2020b). More importantly, *bzr1-1D* can also increase

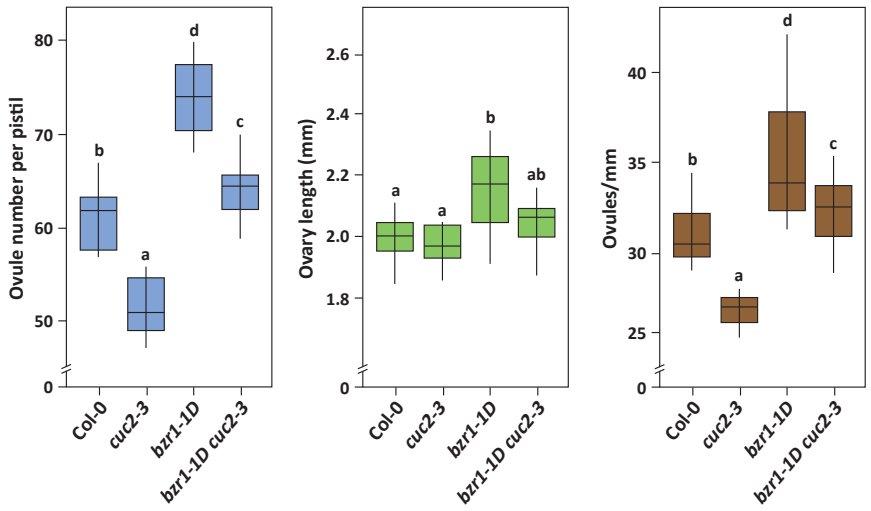

Fig. 2.15: Ovule number (left), ovary length (middle) and ratio of ovule number to ovary length (rigth) in Col-0, *cuc2-3*, *bzr1-1D*, and *bzr1-1D cuc2-3*. Data are presented as boxplots (n = 12). Letters indicate statistical significance as determined by ANOVA and a Bonferroni post hoc test for multiple comparisions (P <0.01). Data that are not significantly different are marked with the same letter.

the ovule number in the *cuc2-3* mutant similar to the situation in wild type plants. These results clearly suggest that BZR1, and probably BRs, act independently of CUC2 in ovule initiation.

Identification of GAI–CUC2 gene targets in ovule primordia initiation

If DELLA and CUC2 form a protein complex in placental cells, CUC2 would determine the specificity of target genes by its DNA-binding domain, while the DELLA protein (GAI) would transcriptionally co-activate the complex. But what are the direct target genes of the GAI–CUC2 complex? To identify these genes, a ChIP–sequencing (ChIP-Seq) assay of GAI using the *pGAI:gaiΔ17-3xYPet* reporter line in both wild type (L*er*) and *cuc2-1* genetic backgrounds was performed. The *pGAI:gaiΔ17-3xYPet* plants showed resemblance to the original *gai-1* mutant, indicating that the DELLA deletion in gaiΔ17 performed as expected. Moreover, gaiΔ17-3xYPet was located in the placenta and ovule primordia cells during early pistil development (**Sup. Fig. 2.9**), as in the *pGAI:mRFP-gaiΔ17* line (**Fig. 2.11**). The *pGAI:-gaiΔ17-3xYPet* line was crossed with *cuc2-1* to allow the identification of GAI targets dependent on CUC2, that is, true GAI–CUC2 complex targets.

In a wild type L*er* background, ChIP-Seq analyses identified 2194 loci that could be putative indirect GAI binding sites, located 1 kb upstream of the transcription start sites (TSSs) of 2087 genes (**Sup. Fig. 2.10**) (**link** to table). Cis-element enrichment analysis among these loci highlighted several potential binding motifs, most being canonical DELLA-interacting TF binding sites, e.g., the bHLH, TCP, bZIP, and MYB families (Marín-de la Rosa et al. 2014; Hernández-García et al. 2020). Among them, the CUC binding site CG/TTG/ANNNNNNAA/CGNNA, described by the Plant Cistrome project (O'Malley et al. 2016), was also enriched. A subset of 558 GAI-associated genes that had a sequence similar to the CUC binding site was selected. Of these, 381 genes were expressed in ovule primordia, according to Matias-Hernandez et al. (2010). Several Gene Ontology (GO) terms were enriched in this group of genes, including developmental processes (stamen development and regulation of meristem growth), response to stimulus (cytokinin, gibberellic acid, abscisic acid, and jasmonic acid-mediated signaling pathway), regulation of transcription (DNA-dependent), and metabolic processes (protein amino acid autophosphorylation and jasmonic acid metabolic process).

When ChIP-Seq was performed in the *cuc2-1* background, 1894 genes were identified (**link** to table), with 1886 being common in both wild type and *cuc2-1* and seven present only in the *cuc2-1* mutant (**Sup. Fig. 2.11**). More importantly, 201 out of the 2087 genes identified in the wild type were not present in *cuc2-1*, suggesting that these were CUC2-dependent GAI targets. Of these, 68 genes had a sequence similar to the CUC binding site and 46 were, in addition, expressed in ovule primordia (**Table 2.1**). Enriched GO terms in these 46 genes were related to meristem and root development, negative regulation of cellular processes, and regulation of transcription. Among this group of genes, putative candidates were those described as participating in ovule development, especially *SEPALLATA2* (*SEP2*), *CYTOKININ OXIDASE 5* (*CKX5*), and *CYTOKININ RESPONSE FACTOR 2* (*CRF2*) (Favaro et al. 2003; Brambilla et al. 2007; Bartrina et al. 2011; Cucinotta et al. 2016).

Table 2.1: Genes identified as GAI binding in a CUC2-dependent manner

AGI code	Name	D.TSS	Fold change		-Log_{10} *q*-value	
			Rep-1	Rep-2	Rep-1	Rep-2
At1g01110	*IQD18*	-242	3.52	2.69	19.40	21.32
At1g16720	*HCF173*	-208	2.50	1.79	8.24	7.54
At1g19310		-56	2.67	2.11	9.03	12.45
At1g21590		-445	2.31	3.13	5.93	29.96
At1g33420		-62	2.79	2.09	11.56	9.81
At1g62870		-243	5.15	4.41	39.58	69.41
At1g63010	*VPT1*	-790	2.25	2.90	5.32	21.74
At1g64620	*DOF1.8*	-463	3.10	2.37	12.64	11.87
At1g69260	*AFP1*	-349	2.28	2.23	5.26	10.67
At1g69600	*ZFHD1*	-427	3.68	3.39	19.16	33.04
At1g74430	*MYB95*	-802	2.63	2.55	8.47	14.64
At1g75450	*CKX5*	-749	2.91	2.12	10.68	8.30
At1g79420	*BDR2*	-646	3.02	2.34	11.92	12.46
At1g80360	*VAS1*	-944	1.85	2.43	3.31	15.17
At2g22840	*GRF1*	-269	2.28	2.97	5.16	22.21

D.TSS, Distance to TSS (bp).

Rep-1, biological replicate # 1; Rep-2, biological replicate # 2.

Gene annotations in **Sup. Table 2.1**

Continued on next page

AGI code	Name	D.TSS	Fold change		-Log$_{10}$ *q*-value	
			Rep-1	Rep-2	Rep-1	Rep-2
At2g24360	*STYK*	-124	3.45	3.02	18.91	30.66
At2g26520		-61	1.66	2.92	2.09	25.49
At2g28085	*SAUR42*	-899	3.20	3.22	13.67	27.17
At2g35310	*REM23*	-123	3.00	2.32	11.82	11.11
At2g41460	*MYB12*	-953	2.71	3.49	8.84	36.66
At3g02010		-632	3.29	2.90	14.69	22.75
At3g02310	*SEP2*	-728	2.19	2.22	6.41	13.15
At3g03450	*RGL2*	-103	2.49	2.40	7.10	15.12
At3g07760	*WEEP*	-2	2.86	2.32	12.09	16.96
At3g12440		-842	2.41	2.34	7.10	14.66
At3g19930	*STP4*	-768	2.57	2.17	7.68	9.44
At3g22750		-571	3.39	4.62	15.79	60.50
At3g24050	*GATA1*	-255	2.69	2.58	8.67	18.04
At3g53540	*TRM19*	-101	3.03	2.83	12.01	21.54
At3g56370	*IRK*	-156	2.34	3.07	6.32	28.62
At3g61260	*REM1.2*	-374	2.64	1.97	9.29	8.45
At3g62000		-971	3.18	2.50	13.93	15.37
At4g00150	*HAM3*	-842	2.62	2.17	7.96	8.98
At4g13160		-43	2.81	2.72	9.75	17.62
At4g14310		-256	2.34	1.82	6.58	10.19
At4g15620	*CASPL1E2*	-119	2.26	2.57	5.28	17.49
At4g18890	*BEH3*	-561	2.43	2.62	6.73	19.74
At4g23750	*CRF2*	-965	2.05	2.71	4.48	22.13
At4g27830	*BGLU10*	-102	2.00	2.47	5.06	19.64
At4g29040	*RPT2a*	-321	2.41	2.17	6.76	9.65
At4g34640	*SQS1*	-37	3.07	2.57	14.19	20.15
At5g11970		-912	2.29	2.25	5.57	13.36
At5g13240	*MAF1*	-873	2.60	2.27	8.75	10.39
At5g49230	*HRB1*	-97	2.56	2.31	8.32	15.70
At5g53130	*CNGC1*	-25	2.71	2.10	8.84	7.97

D.TSS, Distance to TSS (bp).

Rep-1, biological replicate # 1; Rep-2, biological replicate # 2.

Gene annotations in **Sup. Table 2.1**

To confirm that selected genes were indeed direct targets of GAI in a CUC2-

dependent manner, we performed ChIP-qPCR analysis to amplify chromatin derived from immunoprecipitation of L*er* and *cuc2-1*. *SCARECROW LIKE 3* (*SCL3*), a well-described direct DELLA target (Yoshida et al. 2014), was used as a control. As shown in **Fig. 2.16**, *CKX5*, *CRF2*, and *SEP2* displayed lower amplification in *cuc2-1* than in the L*er* background, strongly suggesting that *CKX5*, *CRF2*, and *SEP2* are truly CUC2-dependent GAI targets. On the contrary, *SCL3* displayed high enrichment in both *cuc2-1* and L*er* backgrounds. Taken together, these results support the hypothesis that the DELLA-dependent mechanism regulating ovule primordia formation may reside in the direct DELLA–CUC2 complex, regulating specific target genes, *CKX5*, *CRF2*, and *SEP2* being possible *bona fide* candidates.

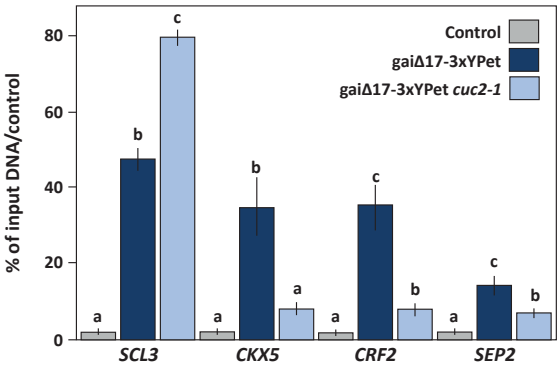

Fig. 2.16: ChIP-qPCR analysis of genes that are potentially indirectly bound by GAI in a CUC2-dependent manner. qPCR amplification of promoter regions of *SCL3*, *CKX5*, *CRF2*, and *SEP2* in immunoprecipitated chromatin using anti-GFP from inflorescences of control L*er*, *pGAI:gaiΔ17-3xYPet* and *pGAI:gaiΔ17-3xYPet cuc2-1* plants. Data were normalized using the Percent Input Method. Letters indicate statistical significance as determined by ANOVA and a Bonferroni post hoc test for multiple comparisions ($P < 0.01$). Data that are not significantly different are marked with the same letter.

DISCUSSION

CUC1 and CUC2 interactions with GAI have different biological significances

The Y2H and Co-IP data in *N. benthamiana* and the co-localization of expression in placental tissue strongly suggest that both CUC1 and CUC2 could interact *in vivo* with GAI in placental tissue. In contrast, genetic analysis of *cuc* mutants reveals that these GAI–CUC interactions may have very different biological significances. On the one hand, CUC2 but not CUC1 would require GAI to promote the formation of ovule primordia; elimination of CUC2 in *cuc2-1* prevents the increase in ovule number by GAI or upon PBZ treatment. On the other hand, CUC1 seems to be involved in pistil elongation, but in this case the effects of CUC1 would not be mediated by GAI. Therefore, most probably the GAI–CUC1 interaction in the placenta is not related to the role of CUC1 in ovary growth. For example, in the *cuc1-1* mutant, ovary length is reduced irrespective of the circumstance tested, such as the presence of *gai-1* or treatment with PBZ and GA. We conclude that the GAI–CUC1 interaction is unlikely to be significant in the control of ovule initiation in a *cuc2-1* background. More experiments should be designed to elucidate the biological significance of such interaction in planta.

CUC and GAs/BRs in ovule initiation

BRs are positive regulators of ovule number; *bzr1-1D* (a dominant mutant of the positive BR signaling regulator BZR1) and *det2-1* (mutant deficient in BR biosynthesis) produce more and fewer ovules, respectively (Huang et al. 2013a). In addition, the role of BRs in ovule number determination is independent of GAs (Barro-Trastoy et al. 2020b). GAs and BRs can negatively and positively modify ovule number in BR and GA signaling mutants, respectively. Moreover, the *gai-1* mutation had an additive effect on ovule number when combined with bzr1-1D (Barro-Trastoy et al. 2020b). The genetic evidence provided here clearly indicates that GAI but not BZR requires CUC2 to promote ovule primordia initiation, which is concordant with both hormones acting independently in Arabidopsis pistils.

Interestingly, BRs are also known to play a role in organ boundary formation in

the shoot apical meristem by repressing organ boundary genes like *CUC1*, *CUC2*, *CUC3*, and *LATERAL ORGAN FUSION 1* (*LOF1*) in a BZR1-dependent manner (Gendron et al. 2012). Moreover, reduced *BZR1* expression in the boundary cells is required for normal *CUC* gene expression and boundary formation. In fact, *bzr1-1D* exhibited similar fusion organ phenotypes as *cuc2-3* and *cuc2-3 cuc3-105* mutants, like fusion of cotyledons, fusion of the cauline leaves to the main stem, and fusion of stamens (Gendron et al. 2012). In this case, the action of BRs (by BZR1 activity) is to counteract CUC activity in the boundary regions. However, it seems that both *CUC* genes and BRs act as positive regulators of ovule number. Whereas *bzr1-1D* or dominant *pCUC2:CUC2m-GFP* produce more ovules (Huang et al. 2013a; Barro-Trastoy et al. 2020b), the loss of *CUC2* or *DET2* (of BR biosynthesis) results in the production of fewer ovules (Ishida et al. 2000; Galbiati et al. 2013; Huang et al. 2013a; Barro-Trastoy et al. 2022, 2020b). This discrepancy in the regulation by BRs and *CUC* genes points to the same molecular components acting through differential molecular mechanisms in these two developmental processes.

Working model of DELLA regulation of ovule primordia initiation

GAs are negative regulators in the formation of ovules, probably through the GA-mediated degradation of DELLA proteins (Gomez et al. 2018). Therefore, DELLA activity is a positive factor for ovule primordia emergence. In addition, in Arabidopsis DELLA activity does not alter auxin or BR pathways of ovule initiation, and DELLA seems to act independently of ANT in the placenta (Gomez et al. 2018; Barro-Trastoy et al. 2020b). On the other hand, DELLA proteins are transcriptional regulators that bind to different TFs, which provide the specificity for DELLA actions in plant growth and development (Vera-Sirera et al. 2016; Hernández-García et al. 2020). Based on the data presented here, as well as our previous data, a working model that includes DELLA proteins in the gene network that participates in ovule primordia determination can be proposed (**Fig. 2.17**). The data conclusively point to CUC2 as the DELLA protein interactor that mediates the role of DELLA in ovule primordia determination. Genetic and molecular analyses indicated that CUC2 is required for GAI and probably other DELLA proteins to promote the formation of more ovules. In this case, DELLA acts as a co-activator of CUC2, which would provide the specificity to regulate a subset

of genes that are responsible for ovule initiation. Therefore, genes regulated by the DELLA–CUC2 complex should contain the canonical cis-regulatory element of CUC in their promoter regions (O'Malley et al. 2016).

Among specific genes identified by genomic analyses, those differentially regulated by CUC2 provide good candidates to finally mediate ovule initiation. Future analysis of such genes with a specific role in ovule initiation may provide biotech tools to modify ovule and seed production by avoiding the detrimental effects on plant development that occur when altering higher-hierarchy regulatory genes, such as those from the *DELLA* or *CUC* families. *CUC2* is a gene described as a determinant of the boundary regions; therefore, a DELLA–CUC2 complex should

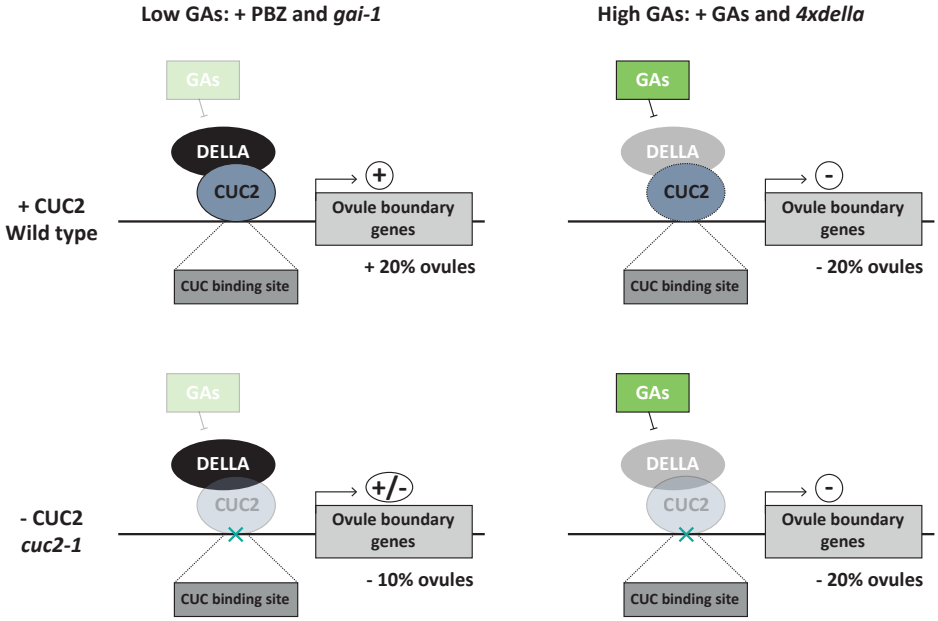

Fig. 2.17: Proposed working model of how GAI–CUC2 mediates ovule number in Arabidopsis. When GA levels are low, in either PBZ-treated or *gai-1* mutant plants, DELLA protein levels are high, which favors the formation of the DELLA–CUC2 complex, which regulates the expression of target genes involved in ovule initiation, promoting an increase in ovule number by 20%. When GA levels are high, upon GA treatment or in the *4xdella* mutant, DELLA protein levels are low and the DELLA–CUC2 complex is not formed, resulting in a 20% reduction of ovules. In contrast, in the *cuc2-1* mutant background, the DELLA–CUC2 complex is not formed either in the presence of high or low DELLA protein levels and, consequently, ovule number is reduced.

determine, through the transcriptional regulation of target genes, the boundary domain between ovule primordia to limit cell proliferation therein. The higher DELLA–CUC2 activity in *gai-1*, in *pCUC2:CUC2m-GFP*, or upon PBZ treatment should promote the formation of more boundaries, which ultimately results in more ovule primordia. On the contrary, null *DELLA* or *CUC* mutants (*4xdella* or *cuc2-1*, respectively, and other mutant combinations) and GA treatment result in absence of the complex activity and hence reduced ovule number.

GAI–CUC2 targets ovule primordia determination genes

At the molecular level, GAI protein is recruited by CUC2 to the promoters of target genes, where it acts as a transcriptional coactivator. ChIP-Seq analysis points to several candidate target genes regulated by GAI in a CUC2-dependent manner. Some of these have been described to have a role in ovule initiation, such as *CKX5*, *CRF2*, and *SEP2*, providing a clue about the downstream molecular events controlled by the DELLA–CUC2 complex. *CKX5* encodes an enzyme that catalyzes irreversible degradation of CKs. It was noted that the double loss of *CKX5* and *CKX3* induces a drastic increase in ovule number (Bartrina et al. 2011). For its part, the *crf2 crf3 crf6* triple mutant, which is less sensitive to CKs, presented a reduced ovule number (Cucinotta et al. 2016). The DELLA–CUC2 complex may transcriptionally regulate these two CK-related genes in a different manner to finally promote ovule initiation.

Another candidate gene is *SEP2*, belonging to the SEP MADS-box protein family (including SEP1 to 4) that are needed to properly establish ovule identity (Favaro et al. 2003; Brambilla et al. 2007). *SEP2*, together with another MADS-box gene, *AGAMOUS* (*AG*), defines carpel identity in the fourth whorl of the floral meristem (Thomson and Wellmer 2019). Lack of both activities in an *ag ap2* double mutant produced aberrant flowers with ectopic carpelloid structures instead of sepals that could also develop ectopic ovules; some of them converted into carpelloid structures themselves (Pinyopich et al. 2003).

RGL2 was also identified as a putative CUC2-dependent GAI target by ChIP-Seq analysis. It has been reported that DELLA proteins can regulate the expression of other DELLA genes in Arabidopsis (Gallego-Bartolomé et al. 2011a). In addition, RGL2 has a role in the control of ovule number (Gomez et al. 2019).

Therefore, it could be plausible to foresee a mechanism of GAI regulating *RGL2* during ovule initiation. Experimental analysis of this hypothesis, as well as the possible role of other DELLA proteins, will provide further details on the complex mechanism governing ovule initiation. Furthermore, the three DELLA proteins GAI, RGA, and RGL2 play a significant role in ovule number and show expression in ovule primordia at early stages of development (Gomez et al. 2018). Elucidation whether RGA and RGL2 also participate in ovule number determination by their interaction with CUC2 requires further analysis.

Other genes that have been described to play a role in ovule development are *REPRODUCTIVE MERISTEM 23* (*REM23*) (Mantegazza et al. 2014), *GROWTH-REGULATING FACTOR 1* (*GRF1*), and other TF-encoding genes in the major families like *MYB12*, *MYB95*, *ZFD1*, *ZF family*, *GATA*, *TF1*, and *Dof*. Interestingly, *GRF1* has been recently found to be involved in the DELLA pathway of the cold stress response (Lantzouni et al. 2020). The nine-membered GRF TF family, including GRF1, are DELLA interactors and also targets of DELLA-modulated transcription after exposure to cold stress. In this case, this DELLA–GRF regulatory module participates in the cold stress response by regulating a subset of target genes.

Finally, comparison of ChIP-Seq data from gaiΔ17-3xYPet and those from RGA-GFP by Serrano-Mislata et al. (2017), both performed in inflorescences, revealed a high degree of overlap. As many as 35% of genes identified with GAI were also a target of RGA, including several CUC2-dependent genes, like *RGL2*, *CRF2*, and *SEP2*. Further characterization of these and other candidates will be needed to identify those genes that are regulated by the DELLA–CUC2 complex, acting downstream of GAs in the control of ovule primordia formation.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

Arabidopsis plants had L*er* or Col-0 backgrounds as indicated. Seeds were surface sterilized in ethanol and incubated in 1/2 Murashige and Skoog (MS) medium plates (Murashige and Skoog 1962) for 4 days at 4°C in the dark followed by

7 days at 22°C under a long-day photoperiod (16/8 h). Seedlings were then transferred into soil (a 2:1:1 mix of peat moss, vermiculite, and perlite) and grown in a chamber at 22°C under a long-day photoperiod (16/8 h).

Dominant mutants *gai-1* (Peng et al. 1997) and *bzr1-1D* (Wang et al. 2002), loss-of-function mutants *4xdella* (*rga-t2 gai-t6 rgl2-1 rgl1-1*) (Achard et al. 2006), *cuc1-1*, *cuc2-1* (Aida et al. 1997), and *cuc2-3* (Hibara et al. 2006), and lines *pCUC1:CUC1-GFP* (Gonçalves et al. 2015), *pCUC2:CUC2-VENUS* (Heisler et al. 2005), *pCUC1:CUC1m-GFP*, and *pCUC2:CUC2m-GFP* (Sieber et al. 2007) were previously described. All mutant combinations were generated by genetic crosses. F3 homozygous plants were selected by PCR genotyping of genomic DNA and/or antibiotic or herbicide resistance. All used primers are listed in **Sup. Table 2.2**.

Generation of constructs and transgenic plants

Both *pGAI:gaiΔ17-3xYPet* and *pGAI:mRFP-gaiΔ17* lines were generated by recombineering (Brumos et al. 2020) using the JAtY clone JAtY51J10 from the JIC (JAtY library, https://abrc.osu.edu/stocks/number/CD4-96) in the pYLTAC17 vector, which contains the *GAI* locus (*At1g14920*). All procedures for removal of the DELLA domain in GAI and tagging with 3xYPet or mRFP were basically as described in Gomez et al. (2019) and Gomez et al. (2020). All primers used are listed in **Sup. Table 2.3**. Final constructs were introduced into *Agrobacterium tumefaciens* strain GV3101 to transform L*er* Arabidopsis plants by floral dipping (Clough and Bent 1998). Transgenic plants were selected in ammonium glufosinate and T3 homozygous lines segregating as a single locus were selected.

GA and PBZ treatments and determination of ovule number and ovary length

GA and PBZ treatments were applied by watering the plants every other day with 20 µM of $GA_4 + GA_7$ (Duchefa Biochemie, Haarlem, Netherlands) or 1 µM PBZ (Duchefa Biochemie), respectively, starting at bolting. Stock solutions of GA and PBZ at 10 mM were prepared in absolute ethanol or acetone, respectively. Primers and chemical products were purchased from Sigma unless otherwise stated.

Ovule number was determined in pistils of flowers at stage 12 between posi-

tions 10 and 20 of the main inflorescence by hand dissection of each pistil under an SMZ-1270 Nikon stereomicroscope (Gomez et al. 2018). Ovary length was determined in each pistil from images taken with a digital camera attached to the stereomicroscope before ovule number determination using ImageJ software (Schindelin et al. 2012). At least 12 pistils were used for each genotype and treatment per assay. Pistil images were captured with a Leica MZ16 F stereomicroscope.

Histological procedures and confocal laser scanning microscopy

Ovule primordia morphology was studied using chloral hydrate clearing and differential interference contrast light microscopy according to Weigel and Glazebrook (2002). Confocal laser scanning microscopy was used to detect and image the distribution of the proteins translated from the *pCUC1:CUC1-GFP*, *pCUC2:CUC2-VENUS*, *pGAI:gaiΔ17-3xYPet*, and *pGAI:mRFP-gaiΔ17* plasmids in stably transformed lines in dissected stage 8 flowers (floral and ovule stages as described by Smyth et al. (1990) and Schneitz et al. (1995)). A Zeiss LSM 780 confocal microscope was used with excitation at 488 nm and detection at 510–530 nm for proteins fused to GFP, YPet, or VENUS and excitation at 561 nm and detection at 598–640 nm for mRFP.

Yeast two-hybrid assays

A synthetic DNA fragment corresponding to the full-length *CUC2* coding sequence (CDS) was ordered from IDT (Integrated DNA Technologies, Coralville, IA, USA) and cloned into the pDONR^{TM}221 entry vector (ThermoFisher Scientific; New York, NY, USA). For DELLA–CUC2 Y2H analysis, *CUC2* CDS was then transferred into the pGADT7 prey vector (Clontech, Takara Bio Europe, Saint-Germain-en-Laye, France) by Gateway and introduced into the yeast strain Y187 (Clontech). The M5-truncated versions of DELLA protein cloned in the pGBKT7 bait vector (Clontech) in the yeast strain Y2HGold (Clontech) (de Lucas et al. 2008) were used.

For CUC2–CUC1, CUC2–CUC2, and GAI–CUC1 interactions, synthetic full-length miR164-resistant versions of *CUC2* (*CUC2m*) and *CUC1* (*CUC1m*) CDSs, identical to those in Baker et al. (2005) and Sieber et al. (2007), were cloned into

pDONR^{TM}221 and moved into pDEST22 and/or pDEST32 vectors (ThermoFisher Scientific). Deleted versions of CUC2 were amplified by PCR from the synthetic *CUC2m* CDS, cloned into the pDONR^{TM}221 entry vector, transferred into the pDEST32 prey vector (ThermoFisher Scientific), and introduced into the yeast strain Y187. Primers used for deletion and cloning are listed in **Sup. Table 2.4**. The complete *GAI* cDNA cloned into the pDEST22 bait vector and introduced into the yeast strain Y2HGold and the GAI deletions cloned into the pDEST32 prey vector (Gallego-Bartolomé et al. 2012) in the yeast strain Y187 were obtained from Dr. Alabadí (IBMCP, Spain).

Y2H screening was performed via mating. Both strains containing the corresponding plasmids were mated overnight at 28°C , and diploids were selected on synthetic dextrose (SD)/-Leu/-Trp. Yeasts were then plated onto SD/-Leu/-Trp/-Ade/-His (Clontech protocol) or SD/-Leu/-Trp/-Ura/-His (ThermoFisher Scientific protocol) in 10-fold serial dilutions to test interactions. Empty vectors were used as negative controls.

qPCR analyses

For qPCR assays, dissected main inflorescences were collected when the plant developed 5–10 flowers and flash-frozen in liquid nitrogen. RNA was extracted with the NucleoSpin^{TM} RNA Plant kit (Macherey-Nagel^{TM}, Dueren, Germany); cDNA was synthesized from 1 µg of total RNA with the PrimerScript^{TM} 1st strand cDNA Synthesis kit (TaKaRa Bio Inc., Saint-Germain-en-Laye, France); and qPCR was performed as described in Dorcey et al. (2009) in a 7500 Fast Real-Time PCR System (Applied Biosystems, Thermo Fisher Sci., Waltham, MA, USA) with SYBR premix ExTaq (Tli RNaseH Plus) Rox Plus (Takara Bio Inc.). Primers for qPCR amplification are listed in **Sup. Table 2.5**. Expression levels were normalized to *UBQ10* (*At1g05320*) (Czechowski et al. 2005) and analyzed by the comparative $\Delta\Delta C_T$ method (Schmittgen and Livak 2008) to the values in the wild type.

Co-IP assays

Construct *35S:myc-CUC1m* was prepared by transferring the *CUC1m* CDS from pDONR^{TM}221 to the pEarleyGate203 vector (Earley et al. 2006) following the

Gateway method. For 3*5S:CUC2m-HA* preparation, a synthetic full-length *CUC2* CDS fused to an HA-tag sequence at the 3' end was cloned into the pDONR^{TM}221 entry vector and transferred to the pEarleyGate100 vector. Both constructs were introduced in *A. tumefaciens* strain C58 and tested against *35S:YFP-M5GAI* (Blanco-Touriñán et al. 2020a). The different combinations of *A. tumefaciens* C58 cells carrying vectors and the p19 silencing suppressor were infiltrated with a solution of 10 mM MES, 10 mM $MgCl_2$, and 1 mM acetosyringone in 4-week-old *N. benthamiana* leaves for transient coexpression. After 3 days, infiltrated leaf sections were frozen in liquid nitrogen and homogenized in extraction buffer containing 25 mM Tris-HCl (pH 7.5), 10% glycerol, 1 mM EDTA (pH 8), 150 mM NaCl, and 19 cOmplete EDTA-free protease inhibitor cocktail (Roche, Darmstadt, Germany).

Total proteins were quantified by Bradford assay (Bradford 1976). An aliquot of 160 µg of total proteins was denatured in Laemmli buffer (125 mM Tris-HCl [pH 6.8], 5% SDS, 12.5% glycerol, and 0.375% bromophenol blue) and set aside to be used as input controls. An aliquot of total proteins (800 µg) was incubated with anti-GFP-coated paramagnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany) for 2 h at 4°C and loaded into µColumns (Miltenyi) for immunoprecipitation. Immunoprecipitated proteins and inputs were separated by 12% SDS-PAGE, transferred to a PVDF membrane (GE Healthcare, Chicago, IL, USA), and immunodetected with anti-HA-HRP (3F10, 1:5000; Roche), anti-c-myc (9E10, 1:1000; Roche), anti-GFP (JL8, 1:5000; Clontech), and anti-mouse IgG HRP (NXA931, 1:10000; GE, Healthcare). Chemiluminescence signals were detected with SuperSignalTM West Femto (Thermo- Fisher Scientific) and imaged with a LAS-3000 imager (Fujifilm, Tokyo, Japan).

ChIP, ChIP-Seq, and ChIP-qPCR analyses

For the ChIP assay, 1.8 g of Arabidopsis inflorescences were collected from the main stem when it developed 5–10 flowers. Open/anthesis flowers, as well as two to three older floral buds, were removed before flash-freezing in liquid nitrogen. Two independent biological replicates for each genotype were recollected from *pGAI:gaiΔ17-3xYPet*, *pGAI:gaiΔ17-3xYPet cuc2-1*, and nontransgenic L*er* plants. The chromatin extraction and *in vitro* double crosslinking were performed

as previously described (Milhinhos et al. 2019). The chromatin was then sonicated using a Bioruptor (Diagenode, Seraing, Belgium) for nine cycles (30 sec on/30 sec off each). A 100 µl aliquot of each sample was kept as input. For immunoprecipitation, samples were incubated with anti-GFP (ab290; Abcam, Cambridge, UK) and magnetic Dynabeads^{TM} Protein A (ThermoFisher Scientific). Finally, chromatin was eluted, proteins were digested with Proteinase K, and DNA was purified with phenol:chloroform:isoamyl alcohol and precipitated with ethanol. Illumina Nextera library preparation and sequencing were carried out by the CRG Genomics Core Facility (Barcelona, Spain).

For ChIP-Seq analysis, reads from all different libraries were cleaned with CutAdapt and mapped to the TAIR10 Arabidopsis genome with Bowtie2 (Lagmead and Salzberg 2012). Data were sorted and indexed with SAMtools (Li et al. 2009). MACS2 (Zhang et al. 2008) was used for peak calling and to calculate fold enrichments and q-values using inputs as a control to detect enriched peaks in the IPs. Peaks from two biological replicates with a false discovery rate of <0.01 were intersected using BEDTools (Quinlan and Hall 2010), and only peaks that exhibited intersection beyond the two replicates were selected, obtaining 6554 peaks (**link** to table). Peaks were then attributed to gene models within 1 kb upstream of the TSS using PAVIS2 (Huang et al. 2013b). ChIP-Seq data were visualized using the IGV Browser (Robinson et al. 2011). GO enrichment of the genes obtained was performed using AgriGo (Tian et al. 2017). To detect enriched sequence motifs and for de novo motif discovery, MEME-ChIP (http://memesuite.org/tools/meme-chip) (Machanick and Bailey 2011) was used in discriminative mode.

ChIP-qPCR was performed in the same manner as the qPCR analyses, using the primers listed in **Sup. Table 2.4**. Enrichments were determined using the Percent Input Method (ThemoFisher Scientific).

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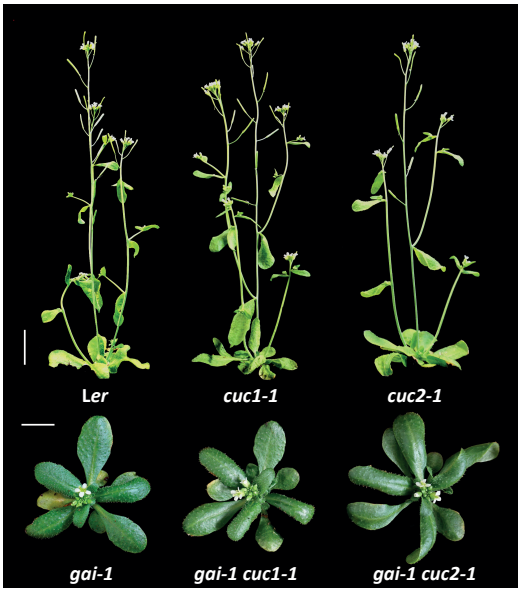
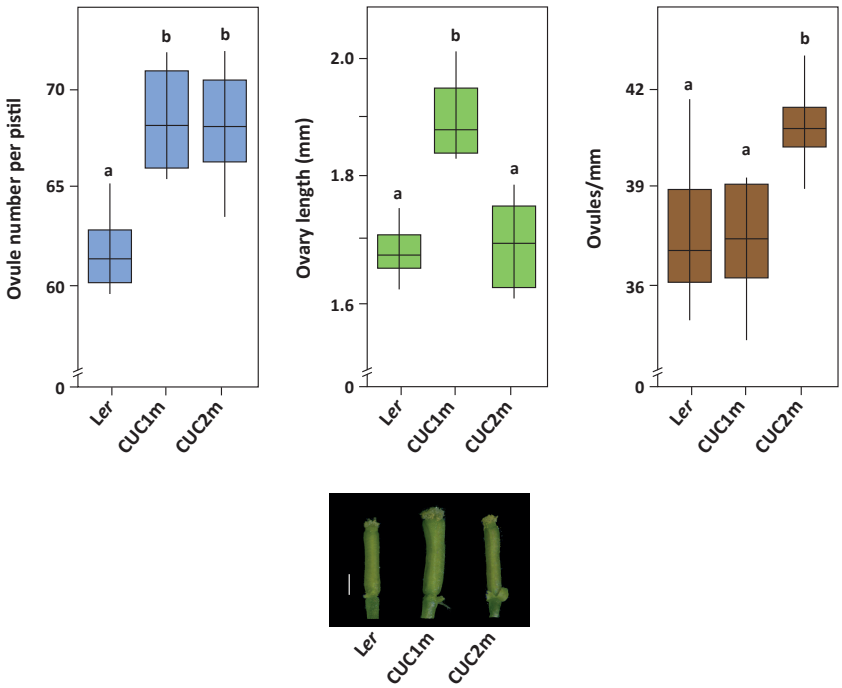
SUPPLEMENTARY INFORMATION

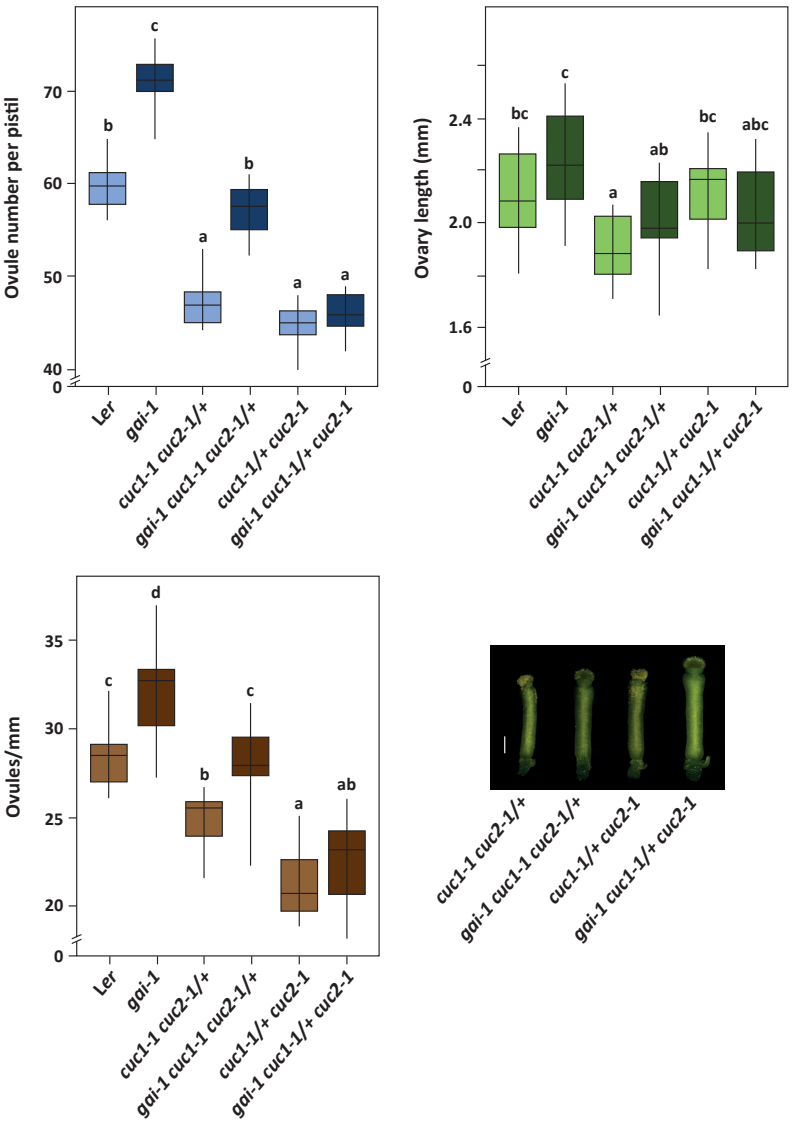
Supplementary figures



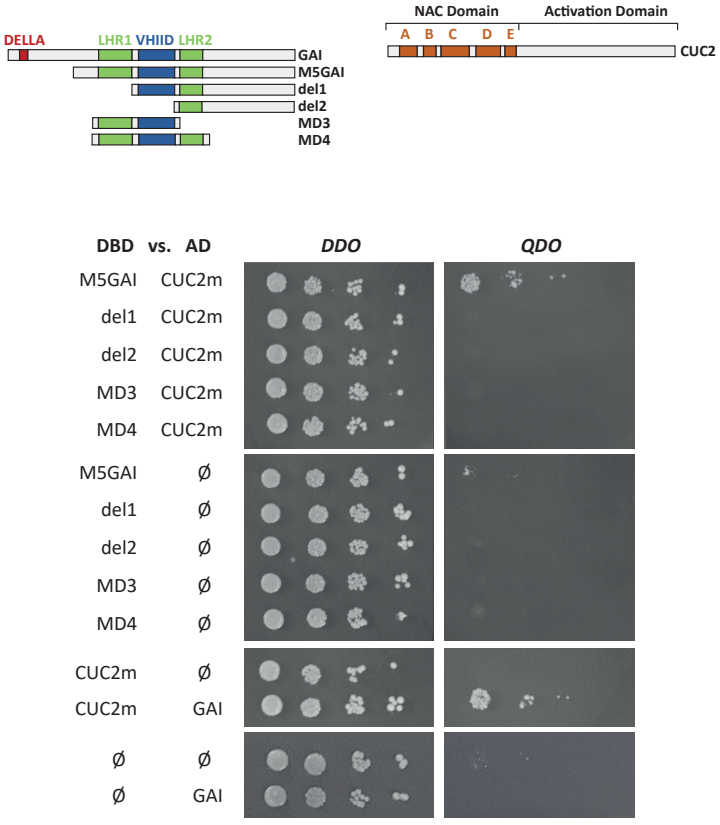
Sup. Fig. 2.1: Images of mature plants of L*er*, *cuc1-1*, *cuc2-1* (upper, lateral view) and *gai-1*, *gai-1 cuc1-1*, and *gai-1 cuc2-1* (lower, zenital view). All images are from 5-week old plants grown in parallel. Scale bars represent 4 cm (upper) and 1 cm (lower).



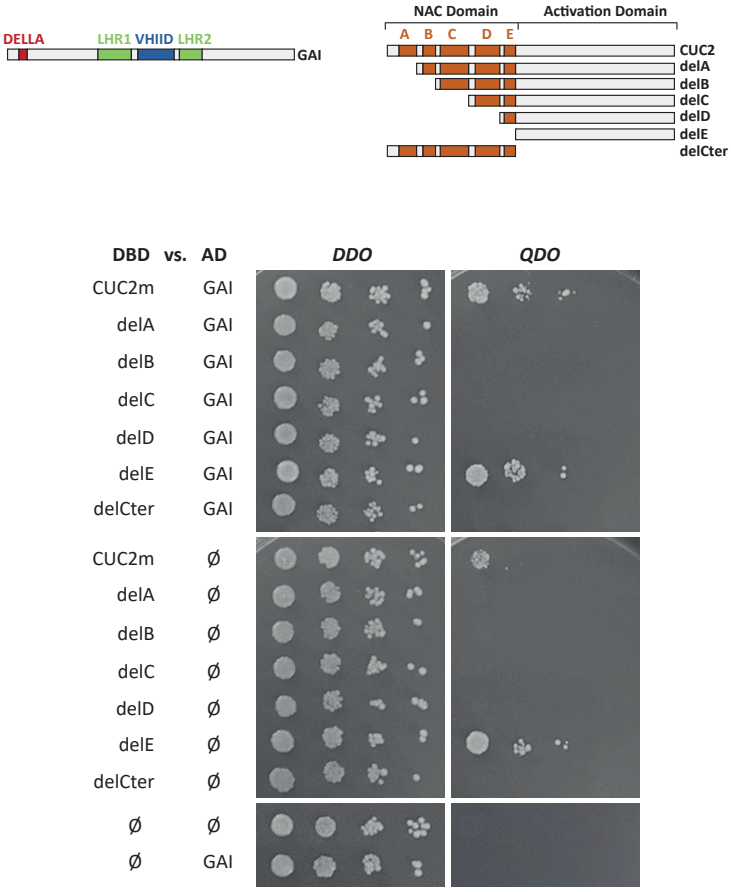
Sup. Fig. 2.2: Ovule number, ovary length and ratio ovule number to ovary length of L*er*, *pCUC1:CUC1m-GFP* (CUC1m), and *pCUC2:CUC2m-GFP* (CUC2m) lines expressing miR164-resistant versions of *CUC1* and *CUC2*, respectively. Data are presented as boxplots (n = 12). Letters indicate statistical significance as determined by ANOVA and a Bonferroni post hoc test for multiple comparisions ($P < 0.01$). Data that are not significantly different are marked with the same letter. Scale bar in images of mature pistils represents 500 µm.



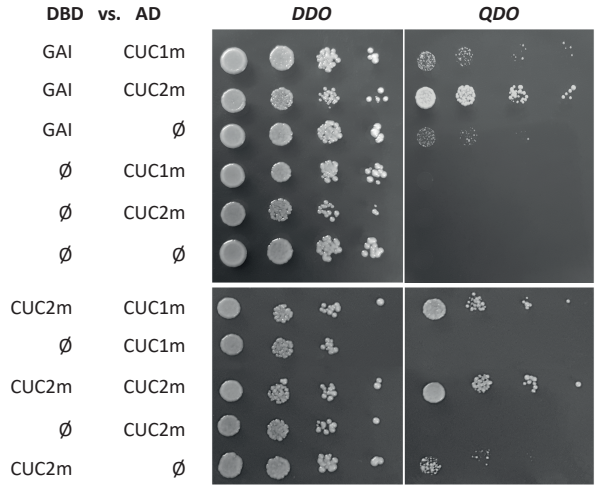
Sup. Fig. 2.3: Ovule number, ovary length and ratio ovule number to ovary length of L*er*, *gai-1*, *cuc1-1 cuc2-1/+*, *gai-1 cuc1-1 cuc2-1/+*, *cuc1-1/+ cuc2-1*, and *gai-1 cuc1-1/+ cuc2-1*. Data are presented as boxplots (n = 12). Letters indicate statistical significance as determined by ANOVA and a Bonferroni post hoc test for multiple comparisions (P <0.01). Data that are not significantly different are marked with the same letter. Scale bar in images of mature pistils represents 500 µm.



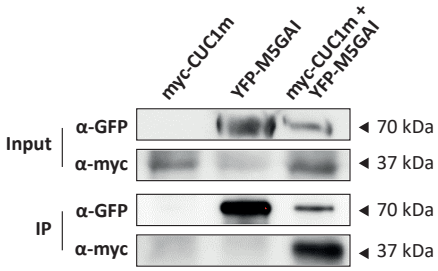
Sup. Fig. 2.4: Serial deletions of GAI in Y2H binding assays. Above, schematic representation of protein domains in GAI and CUC2 and deleted versions of GAI to map the domains required for binding. Below, Y2H assay of GAI and its deleted versions against CUC2. DBD, DNA-binding domain; AD, activation domain, Ø, empty vector. DDO, double dropout (SD/-Leu/-Trp); QDO, quadruple dropout (SD/-Ura/-His/-Leu/-Trp). M5GAI version at DBD and miR164-resistant version of CUC2 (CUC2m) were used. Ten-fold serial dilutions were spotted onto SD media to test the interactions.



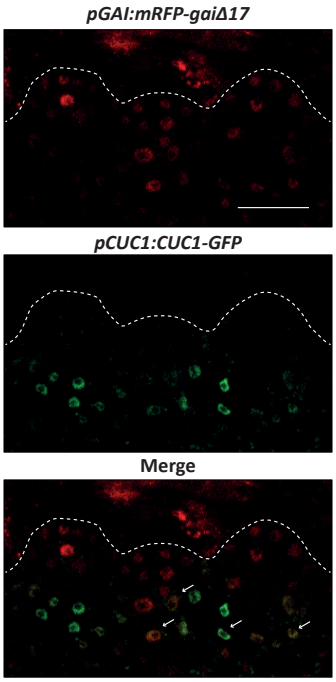
Sup. Fig. 2.5: Serial deletions of CUC2 in Y2H binding assays. Above, schematic representation of protein domains in GAI and CUC2 and deleted versions of CUC2 to map the domains required for binding. Below, Y2H assay of CUC2 and its deleted versions against GAI. DBD, DNA-binding domain; AD, activation domain, Ø, empty vector. DDO, double dropout (SD/-Leu/-Trp); QDO, quadruple dropout (SD/-Ura/-His/-Leu/-Trp). miR164-resistant version of CUC2 (CUC2m) were used. Ten-fold serial dilutions were spotted onto SD media to test the interactions.



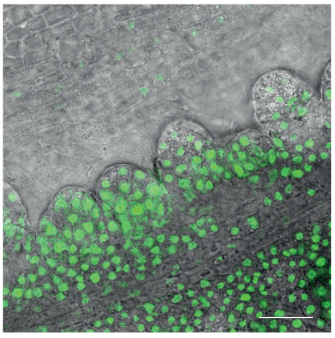
Sup. Fig. 2.6: Y2H assays of protein interaction between GAI-CUC2, GAI-CUC1, CUC2-CUC1 and CUC2-CUC2. DBD, DNA-binding domain; AD, activation domain, Ø, empty vector. DDO, double dropout (SD/-Leu/-Trp); QDO, quadruple dropout (SD/-Ura/-His/-Leu/-Trp). M5GAI version at DBD and miR164-resistant version of CUC1 (CUC1m) and CUC2 (CUC2m) were used. Ten-fold serial dilutions were spotted onto SD media to test the interactions.



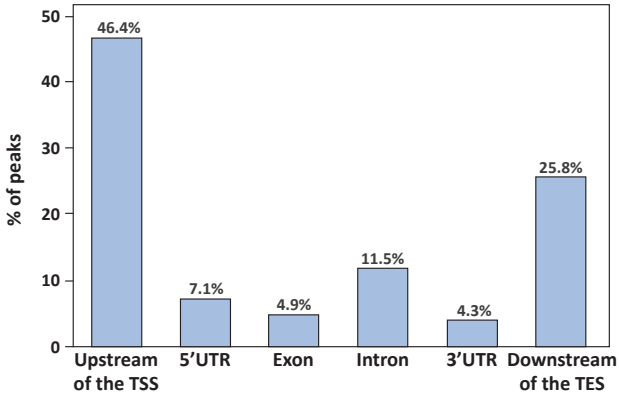
Sup. Fig. 2.7: Co-IP assay of YFP-M5GAI with myc-CUC1m in *N. benthamiana*. Western blot was performed with anti-GFP and anti-c-myc antibodies to detect YFP-M5GAI and myc-CUC1m, respectively, in both input and IP protein samples. A miR164-resistant version of CUC1 (CUC1m) was used.



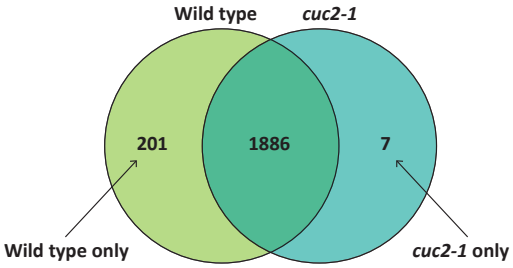
Sup. Fig. 2.8: Co-localization assay in placental tissue of GAI (*pGAI:mRFP-gaiΔ17*) with CUC1 (*pCUC1:CUC1-GFP*) at stage 1-I of ovule development. White arrowheads point to nuclei where both mRFP-gaiΔ17 and CUC1-GFP were co-localized. Scale bars represent 20 µm.



Sup. Fig. 2.9: Confocal microscopy image of ovule primordia at stage 1-II of *pGAI:gaiΔ17-3xYPet* plants used for ChIP-Seq analysis. Scale bars represent 20 µm.



Sup. Fig. 2.10: Percentage of peaks that are mapped upstream of the TSS, in 5'UTR, in exon, in intron, in 3'UTR or downstream of TES. TSS, transcription start site; TES, transcription end site; UTR, untranslated region.



Sup. Fig. 2.11: ChIP-seq Venn diagram showing the number of genes annotated that were identified in wild type but not in *cuc2-1*, in *cuc2-1* but not in wild type and common in wild type and *cuc2-1* backgrounds.

Supplementary tables

Sup. Table 2.1: Annotations of genes identified as GAI binding in a CUC2-dependent manner

AGI code	Name	Annotation
At1g01110	*IQD18*	IQ-DOMAIN 18
At1g16720	*HCF173*	HIGH CHLOROPHYLL FLUORESCENCE PHENOTYPE 173
At1g19310		RING/U-box superfamily protein
At1g21590		Kinase protein
At1g33420		RING/FYVE/PHD zinc finger superfamily protein
At1g62870		Hypothetical protein
At1g63010	*VPT1*	VACUOLAR PHOSPHATE TRANSPORTER 1
At1g64620	*DOF1.8*	Plant-specific Dof transcription factor
At1g69260	*AFP1*	ABI FIVE BINDING PROTEIN 1
At1g69600	*ZFHD1*	ZINC FINGER HOMEODOMAIN 1
At1g74430	*MYB95*	MYB DOMAIN PROTEIN 95
At1g75450	*CKX5*	CYTOKININ OXIDASE 5
At1g79420	*BDR2*	BOUNDARY OF ROP DOMAIN2
At1g80360	*VAS1*	REVERSAL OF SAV3 PHENOTYPE 1
At2g22840	*GRF1*	GROWTH-REGULATING FACTOR 1
At2g24360	*STYK*	SERINE/THREONINE/TYROSINE PROTEIN KINASE
At2g26520		Transmembrane protein
At2g28085	*SAUR42*	SMALL AUXIN UPREGULATED RNA 42
At2g35310	*REM23*	REPRODUCTIVE MERISTEM 23
At2g41550		Rho termination factor
At2g47460	*MYB12*	MYB DOMAIN PROTEIN 12
At3g02010		PPR superfamily protein
At3g02310	*SEP2*	SEPALLATA 2
At3g03450	*RGL2*	RGA-LIKE 2
At3g07760	*WEEP*	WEEP
At3g12440		Polynucleotidyl transferase, ribonuclease H-like superfamily
At3g19930	*STP4*	SUGAR TRANSPORTER 4
At3g22750		Protein kinase superfamily protein
At3g24050	*GATA1*	GATA TRANSCRIPTION FACTOR 1
At3g53540	*TRM19*	TON1 RECRUITING MOTIF 19
At3g56370	*IRK*	INFLORESCENCE AND ROOT APICES RECEPTOR KINASE
At3g61260	*REM1.2*	REMORIN 1.2

Continued on next page

AGI code	Name	Annotation
At3g62000		SAM-dependent methyltransferases superfamily protein
At4g00150	*HAM3*	HAIRY MERISTEM 3
At4g13160		zein-binding protein
At4g14310		Transducin/WD40 repeat-like superfamily protein
At4g15620	*CASPL1E2*	CASP-LIKE PROTEIN 1E2
At4g18890	*BEH3*	BES1/BZR1 HOMOLOG 3
At4g23750	*CRF2*	CYTOKININ RESPONSE FACTOR 2
At4g27830	*BGLU10*	BETA GLUCOSIDASE 10
At4g29040	*RPT2a*	REGULATORY PARTICLE AAA-ATPASE 2A
At4g34640	*SQS1*	SQUALENE SYNTHASE 1
At5g11970		ABC family ABC transporter
At5g13240	*MAF1*	Global repressor of RNA polymerase III
At5g49230	*HRB1*	HYPERSENSITIVE TO RED AND BLUE
At5g53130	*CNGC1*	CYCLIC NUCLEOTIDE GATED CHANNEL 1

Sup. Table 2.2: Oligonucleotides used for genotyping.

Genotyping of *cuc* mutants	
cuc1-1-wt-Fw	GTTACTGGAAAGCCACTGGTAAAGACAGA
cuc1-1-wt-Rv	GCTCGTCCTTTGTAAAAGACAAGAGCTT
cuc1-1-mut-Fw	AGACAAAATCACTTCTCGGGATGAAAAC
cuc1-1-mut-Rv	CTTAGCGGAGGAGGAAATGTAATGGTAA
cuc2-1-wt/mut-Fw	CGGAGGCTAAAGAAGTACCA
cuc2-1-wt-Rv	ATCCACATTATTACCACGCCC
cuc2-1-mut-Rv	CTCGAGAGATTGAGTCGCCGTTTG
cuc2-3-wt/mut-Fw	GGTCACGGAGGCTAAAGAAGTACCA
cuc2-3-wt-Rv	AGCCCATTCCTCGTTTCTTT
cuc2-3-mut-Rv (LB3)	ATCTGAATTTCATAACCAATCTCGATACAC
Genotyping of *della* mutants	
gai-1-Fw	GATCCGACATTGAAGGAAAAACC
gai-1-Rv	TTGTAGTATACGTATCTCCTCCG
gaiT6-wt/mut-Fw	CTAGATCCGACATTGAAGGA
gaiT6-wt-Rv	AGCATCAAGATCAGCTAAAG
gaiT6-mut-Rv	TCGGTACGGGATTTTCGCAT
rgaT2-wt/mut-Fw	GCCGGAGCTATGAGAAAAGTGG
rgaT2-wt-Rv	AAGAATTTTAAACAAGTCAACG
rgaT2-mut-Rv	CCGGTATATCCCGTTTTGG
rgl1-1-wt/mut-Fw	AAGCTAGCTCGAAACCCAAAT
rgl1-1-wt-Rv	CCACAGAGCGCGTAGAGGATAAC
rgl1-1-mut-Rv	CATGGGCTGGGCCTCAGTG
rgl2-1-wt/mut-Fw	GCTGGTGAAACGCGTGGGAACA
rgl2-1-wt-Rv	ACGCCGAGGTTGTGATGAGTG
rgl2-1-mut-Rv	CCGGTATATCCCGTTTTGG
Genotyping of *bzr1-1D* mutant[1]	
bzr1-1D-Fw	CCCTTTCCTCAGAAATGGTGGC
bzr1-1D-Rv	GTATCCTCTCTCCTTCCCAGGG

[1], the amplified fragment is then digested with HpaII, which produces 3 fragments for the WT allele and 2 for the *bzr1-1D* allele.

Sup. Table 2.3: Oligonucleotides used for recombineering.

Construction of *pGAI:mRFP-gaiΔ17* and *pGAI:gaiΔ17-3xYPet*	
Deletion of the DELLA domain[1]	
GAI-delTest-Fw	CTAAGCAGTCCTAACCGATCCCC
GAI-delTest-Rv	CGCCGTTTGAGCATTTCAACCGC
GAI-delFirst-Fw	ACTATGATGATGAATGAAGAAGACGACGGTAACGGCATGG ATGGAGGTGGAGGTGGAGCT
GAI-delFirst-Rv	AGACATCATAACTTCAAGCTGCTCGAGTTTCTGAGCAACA TCGGCCCCAGCGGCCGCAGC
GAI-delSecond-Fw[2]	TGATGATGAATGAAGAAGACGACGGTAACGGCA**TG**GAT GATGTTGCTCAGAAACTCGAGC
GAI-delSecond-Rv	CTGATTGAGAATCGCGTCACCGGG
Tagging 3xYPet at Ct of gaiΔ17[1]	
GAI-CTest-Fw	GTGATGGACCTGACCGAGTT
GAI-CTest-Rv	GCCTATCCAATTTACCCTCCA
GAI-CRec-Fw	CGACCGCTCATAGCCACCTCGGCTTGGAAACTCTCCACCA ATGGAGGTGGAGGTGGAGCT
GAI-CRec-Rv	ATAACCGGTTCAACAGATCAATTCATTGAGCCACCATCTAGG CCCCAGCGGCCGCAGC
Tagging mRFP at Nt of gaiΔ17[1]	
GAI-NTest-Fw	CTAAGCAGTCCTAACCGATCCCC
GAI-NTest-Rv	CTGATTGAGAATCGCGTCACCGGG
GAI-NRec-Fw	GAAAAACCTTTTAGATCCATCTCTGAAAAAAAACCAACC ATGGAGGTGGAGGTGGAGCT
GAI-NRec-Rv	AGTCTTCTTATCTTGATGATGATGATGATGATGATCTCTC TTGGCCCCAGCGGCCGCAGC
Trimming of genomic clones with *pGAI:mRFP-gaiΔ17* and *pGAI:gaiΔ17-3xYPet* constructs[1]	
GAI-TrimLeft	TATGAGAATAATGAGAAAACCACTTTCCCAAATTGCTTT TTTACCAATGCTTAATCAGTG
GAI-TrimRigth	TTACTCTGATTCTAACAACAAAAATCCCAAACCAAACA TATAGGAACTTCCCCCTCTTGG
GAI-TrimLeft-Test	TTCCCATATGTCCACGTCAG
GAI-TrimRigth-Test	TCTTTCTGAACGACCGGTTT

[1], upper case, sequence of *GAI* gene or genomic region close to the GAI locus; underlined, sequence of universal adaptors.

[2], bold and underlined are the nucleotides flanking the DELLA domain of *GAI* after deletion of the DELLA domain.

Sup. Table 2.4: Oligonucleotides used for Y2H constructs.

Deletion of *CUC2* for Y2H[1]	
delA-Fw	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCTCAAGCCGTG CCATCGC
delB-Fw	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGCTAAGATGGG AGAGAAA
delC-Fw	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCAAGACTTGTGCA CTTGTTG
delD-Fw	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCTCTTACCATTT CATCTCA
delE-Fw	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCACTTTAGCC AGCACCG
delA/B/C/D/E-Rv	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAGTAGTTC CAAATAC
delNter-Fw	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGACATTCC GTATTAC
delNter-Rv	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCATTTCTGG AAAACCC

[1], underlined are the nucleotides of the attB1 (for Fw sequences) and attB2 (for Rv sequences).

Sup. Table 2.5: Oligonucleotides used for qPCR assays.

qPCR analysis of the expression of constitutive genes	
UBQ10-qPCR-F1	GGCCTTGTATAATCCCTGATGAATAAG
UBQ10-qPCR-R1	AAAGAGATAACAGGAACGGAAACATAGT
qPCR analysis of the expression of endogenous *DELLA* genes	
GAI-qPCR-F1	CCTCCGTCGTCTAACGCC
GAI-qPCR-R1	GTTGACTCAGCCGTCGCTGTAG
RGA-qPCR-F1	AGAAGCAATCCAGCAGA
RGA-qPCR-F1	GTGTACTCTCTTCTTACCTTC
RGL1-qPCR-F1	GAGTTCATCAATGACGACGGT
RGL1-qPCR-F1	GTACTCTGAGTCAGGCTT
RGL2-qPCR-F1	CACCAAAACCACTACCAGC
RGL2-qPCR-F1	CTATCCACACAACTTCGGG
qPCR analysis of the expression of *CUC2*	
CUC2-qPCR-F5	TCCTGTTTCTCCACTGTCCCTAC
CUC2-qPCR-R5	CAAAGTCAAACCCTAGCGGCGG
ChIP-qPCR	
SCL3-ChIP-Fw	TGTAGAATCTCTCTGGTCAATGG
SCL3-ChIP-Rv	GTCTTCTTCTCTTCTTTCTCTCTC
CKX5-ChIP-Fw	AGAACGCCACGTGGATATTC
CKX5-ChIP-Rv	AGAGAAAAGGGCCCACTATG
CRF2-ChIP-Fw	ACGTCCTAGATTGTTGTCTGCC
CRF2-ChIP-Rv	AAGAGACCGTTACGGCTCGTAG
SEP2-ChIP-Fw	TTTTGCATCTTGGAGTGGTTAG
SEP2-ChIP-Rv	CTATCTACCACCACTTGGACAA

2

General discussion

GENERAL DISCUSSION

During the last years, some studies have highlighted the importance of developing new strategies focused on the improvement of plant yield. The demand for crop production, either for sustaining rising human population and livestock nutrition or for producing biofuel, is increasing. It is predicted that current crop yield will not meet the projected food demands in the relatively next few decades (Ray et al. 2013). In this context, the detailed study of the regulatory networks governing plant reproductive organ development holds considerable promise, as it could provide novel targets for genetic improvements focused on enhancements in seed (or grain) size, quality and/or number. Some of the crop-related traits that could help to develop these improvement strategies are those related to ovules, the precursors of seeds, as well as pistils, the precursors of fruits (Shirley et al. 2019; Cucinotta et al. 2020). The work presented in this thesis adds new elements to the known gene and hormone regulatory network controlling ovule development, specifically ovule initiation.

Updated model for the control of ovule primordia initiation: the role of gibberellins

We previously reported that GAs negatively module ovule number in plants (Gomez et al. 2018). In that work, we also described that GAs probably participate in this process independently of auxins, as auxin response at the tip of ovule primordia, and PIN1 levels at the membranes of primordia epidermal cells, are not altered regardless of GA or DELLA protein levels (Gomez et al. 2018).

In this thesis, we demonstrate that GAs also act independently of BRs (Barro-Trastoy et al. 2020b, Chapter 1). We found that GAs reduce ovule number regardless of BR levels. Moreover, BRs increase ovule number regardless of GA levels or DELLA proteins activity. Our data indicate that BRs promote an increase in GA synthesis and, consequently, the destabilization of DELLA proteins in inflorescences. However, if BRs would regulate ovule number by increasing GA levels, a decrease in ovule number should be observed in BR mutants such as *bzr1-1D*, which is not the case (Huang et al. 2013a). Taking into account that the *gai-1* and *bzr1-1D* mutants have additive effects in ovule number when combined, we

propose that the regulation of GA levels by BRs probably would not take place in the placenta. In addition, recent studies show that BRs enhance auxin response at the tips of ovule primordia, probably through the regulation of *PIN3* (Hu et al. 2022). As mentioned before, GAs do not alter auxin response at primordia tips, so this new observation goes in accordance with GAs and BRs acting independently during ovule initiation.

Likewise, the GA-mediated regulation of ovule number does not seem to rely on *ANT* (Barro-Trastoy et al. 2020b, Chapter 1). Firstly, qPCR analysis revealed that *ANT* is slightly up-regulated by DELLA proteins in inflorescences, in a similar extend that it is up-regulated by BZR1, as described by Huang et al. (2013a) (who proposed that the effect of BRs on ovule number should rely on the regulation of *ANT*, among other genes). However, *in situ* mRNA hybridizations and confocal analyses of ANT-YPet showed that neither *ANT* transcript nor ANT protein levels are altered by GAs or DELLA proteins in the placenta or ovule primordia. More important, overexpression of *ANT* (as in the *35S:ANT* line) did not affect ovule number, demonstrating that both GAs or BRs effects in ovule number would not rely on the regulation of *ANT* expression. *ANT* overexpression increased floral organs size, including mature ovules. Ovule size and shape is also altered by GAs. The *global* mutant present ovules with shorter integuments and reduced cell layers (Gomez et al. 2016), whereas the *gai-1* ovules are bigger than those of the wild type (unpublished data). We currently know that *ANT* is indeed up-regulated by GAI in later developing ovules, when integuments primordia are incipient (unpublished data). Therefore, we can hypothesize that the role of ANT during ovule development does not rely on the regulation of ovule number, but in the regulation of ovule size, and perhaps involving GAs and BRs. Nonetheless, ANT seems to be a superimposed requirement for proper placenta development, which could explain why a reduction in ovule number is observed in the *ant-4* loss-of-function mutant, and why the *gai-1* mutant can not alleviate its phenotype.

Genetic and molecular analyses indicate that GAI needs CUC2 to promote the formation of more ovules (Barro-Trastoy et al. 2022, Chapter 2). This is evidenced by the Y2H, Co-IP and co-localization assays, which demonstrate that GAI and CUC2 proteins can physically interact and that this interaction could take place in placental cells. Moreover, genetic analyses reveal that GAI activity requires CUC2

to promote an increase in ovule number, as the *gai-1* mutant did not increase ovule number in a *cuc2-1* background. Moreover, genetic analysis also suggest that BZR1 does not require CUC2 to promote ovule initiation, pointing to BRs acting independently of CUC2 (and independently of GAs) in this developmental process. Altogether, we propose that GAs modulate ovule number in Arabidopsis through a DELLA-CUC2 complex that should be regulating the expression of a set of genes related to ovule development. Molecular analysis allowed us to identify some loci that presumably are CUC2-dependent GAI targets, such as *CKX5*, *CRF2* or *SEP2*. However, among the identified loci, those confirmed to be differentially regulated by GAI and CUC2 would be good gene candidates to finally mediate ovule number determination. Future analysis of such genes and their role on ovule initiation should be performed to deepen on the knowledge of ovule development.

In order to reflect this new information, we propose an updated model for the control of ovule initiation detailing the possible molecular mechanism of GAs (**Fig. D.1**). The plausible hypotheses to conduct the following investigations are discussed down below.

Plausible hypotheses

From DELLA and CUC to CKs

CKX5 and *CRF2* are two genes related to CKs expressed in placenta and with a well known role in ovule initiation (Bartrina et al. 2011; Cucinotta et al. 2016). CKX5 is an oxidase/dehydrogenase enzymes that catalyse irreversible CKs degradation. It was described that CKX5, together with CKX3, could be regulating the activity of meristematic cells in the placenta, and thus modulating ovule number, through the control of CK levels (Bartrina et al. 2011). The *ckx3-1 ckx5-1* double mutant, with enhanced CK levels, present a strong increase in ovule number (it was described that it contains twice as many ovules as wild type) and significantly bigger pistils. The *ckx3-1* and *ckx5-1* single mutants also presents, although to a lesser extent, an increase in ovule number with a minor effect on pistil length, specially in *ckx5-1* (Bartrina et al. 2011). For its part, CRF2 is a CK response factor that mediate CK transcriptional responses functionally overlapping with type-B ARRs. In this case, it was reported that a *crf2-2 crf3-1 crf6-S2* triple

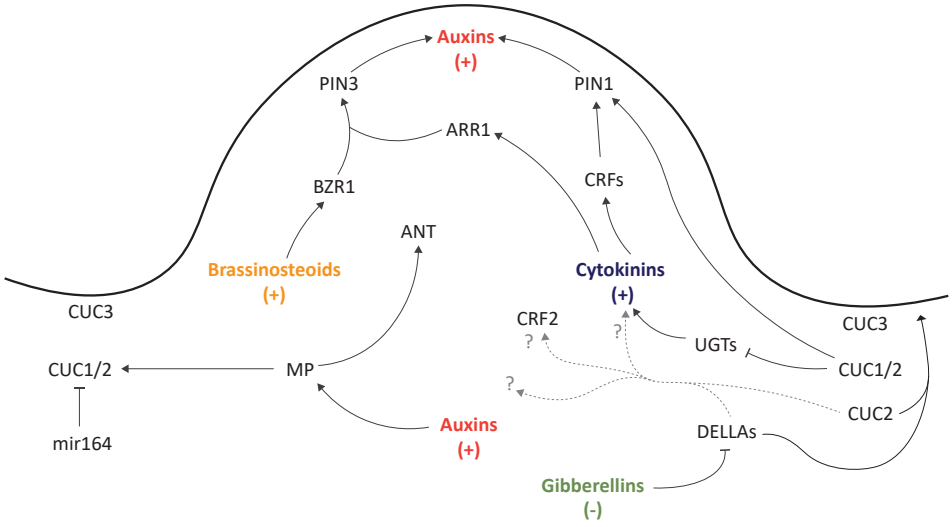


Fig. D.1: Updated model for the regulation of ovule initiation in Arabidopsis. Dashed light grey lines represent several possibilities for the DELLA-CUC2 downstream mechanisms. Different colors represent different plant hormones.

mutant have significantly fewer ovules than wild type and shorter pistils. This phenotype is probably partially due to a reduced CK response, as CK treatments on this mutant does not increase ovule number in a same extend as in wild type (Cucinotta et al. 2016). Interestingly, single *crf3-1* and *crf6-S2* single mutants do not present any significant difference in ovule number compared to wild type, but single *crf2-2* and double *crf2-2 crf3-1* present a similar slight but significant decrease in the number of ovules. Additionally, it was reported that CK levels are regulated by CUC1 and CUC2 to determine ovule number (Cucinotta et al. 2018). Indeed, *cuc2-1 pSTK:RNAi-CUC1* plants, which lack CUC2 activity and has silenced *CUC1* specifically in the placenta, have reduced levels of total active CKs (Cucinotta et al. 2018), and CK treatments rescue their reduced ovule number phenotype (Galbiati et al. 2013). All these considered, we may wonder whether GAs, through the DELLA-CUC2 complex, could be regulating CK levels or CK responses to modulate ovule number (**Fig. D.1**).

There are some observations to be considered in this scenario. CKs regulate

PIN1 expression through the binding of CRF2, CRF3 and CRF6 to its promoter (Cucinotta et al. 2016). *PIN1* expression is also regulated by CUC1 and CUC2 (Galbiati et al. 2013), but not by DELLA proteins (Gomez et al. 2018). In addition, recent studies propose that CKs and BRs coordinately regulate ovule development genes, such as *ANT*, and we have observed that GAs act independently of BRs and ANT. Altogether, we can figure that if GAs influence CKs during ovule initiation, their together downstream responses should be different than those observed for CKs (for instance, not altering *PIN1*). Therefore, we may conclude that, although CUC TFs regulate CK levels and hence ovule number, this may be a DELLA-independent CUC function. For its part, the DELLA-CUC2 complex could be controlling other molecular processes that are not related to CKs. In any case, further work must be done to finally confirm this hypothesis.

Fucosylation of DELLA proteins

It is known that boundaries act as frontiers between different cell populations, thus properly separating developing organs, but also participate in meristematic activity maintenance, thus regulating organ initiation (Aida and Tasaka 2006). For instance, at the developing embryo, *CUC1* and *CUC2* promote cotyledon separation, probably by repressing cell division in a cell-autonomous manner, but also induces *STM* expression in a non-cell-autonomous manner to promote SAM formation (Aida et al. 1999). In leaf margins, CUC2 restricts sinuses growth, but also promotes tooth outgrowth by modifying auxin levels in neighbouring cells (Bilsborough et al. 2011). For the case of ovule initiation, we have repeatedly mentioned along the introduction and this discussion that *CUC* genes modify CK levels and *PIN1* expression to induce primordia outgrowth, in a similar way to they do in leaf margins.

Recently, it was described that SPINDLY (SPY), an O-fucosyltransferase, is required to inhibit cell growth in leaf sinuses, and that CUC2 partially needs *SPY* activity to develop leaf serrations (Bouré et al. 2022). Indeed, SPY and CUC2 repress a common set of genes encoding for enzymes with functions on cell wall loosening, consequently increasing cell wall stiffness of boundary cells, and inhibiting their expansion (Bouré et al. 2022). It is known that DELLA activity is regulated by different post-translational modifications (Blanco-Touriñán et al.

2020b). One of these modifications is O-fucosylation, mediated precisely by SPY. O-fucosylation of DELLA proteins induce them to adopt an open conformation that is more active and enhance DELLA interaction with TFs (Zentella et al. 2017). Thereby, it is feasible to think that in a *spy* loss-of-function mutant, the DELLA-CUC2 complex would be more difficult to form. In addition, DELLA proteins are known to inhibit both cell proliferation and expansion (Achard et al. 2009). Altogether, we can consider that the DELLA-CUC2 could be specifically regulating ovule primordia boundary formation in a cell-autonomous manner by the repression of cell proliferation (**Fig. D.1**). Interestingly, it was described that SPY also promotes CK signaling, highlighting GA-CK crosstalk throughout plant development (Greenboim-Wainberg et al. 2005). Knowing this, we may consider a more complex scenario where a fine-tuned balance of GAs and CKs would be regulating growth dinamics during ovule initiation.

Downstream gene targets of DELLA in the placenta

Finally, we still have a wide range of possibilities that should also be considered. *SEP2*, *GRF1*, *REM23*, *RGL2*, and other genes (**Table 2.1**) were also identified as putative CUC2-dependent GAI targets (Barro-Trastoy et al. 2022, Chapter 2). SEP2 is a MADS-box protein involved in ovule identity definition (Favaro et al. 2003). GRF1 is a DELLA interactor that also regulated cell proliferation and expansion rates (Lantzouni et al. 2020). *REM23* belong to a extensive gene family that is preferentially expressed during ovule and seed development (Mantegazza et al. 2014). RGL2 is a DELLA protein with a known role in the control of ovule number (Gomez et al. 2019). Curiously, *RLG2* seems to be also a target of RGA (Serrano-Mislata et al. 2017). It was also reported that DELLA proteins can regulate the expression of other DELLA genes (Gallego-Bartolomé et al. 2011a), so probably DELLA proteins could be involved in a more complex mechanism governing ovule initiation than the described here. If other DELLA proteins, as RGA and RGL2, also participate in ovule number determination with CUC2, or if the mentioned genes act downstream of GAs in this developmental process, still need to clarified. In deep characterization of these downstream genes may provide new biotechnological tools to modify the production of ovule and seeds in crops and fullfill the requirements of increased population in an scenario of global warming.

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D

Conclusions

CONCLUSIONS

The work described in this thesis have allowed us to decipher a molecular mechanism by which GAs regulate ovule initiation. **The main conclusion is that the regulation of ovule initiation mediated by GAs acts independently of BRs, and resides in the formation of a DELLA-CUC2 complex**. In detail:

- In Arabidopsis, GAs and BRs act independently and antagonistically in ovule initiation.
- ANT would not be mediating the BR or GA pathways in ovule number determination.
- GAI requires CUC2 to regulate ovule number.
- GAI and CUC2 could interact physically in placental cells.
- *DELLA* and *CUC2* are not reciprocally regulated.
- CUC2 would not participate in BR-mediated ovule number determination.

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