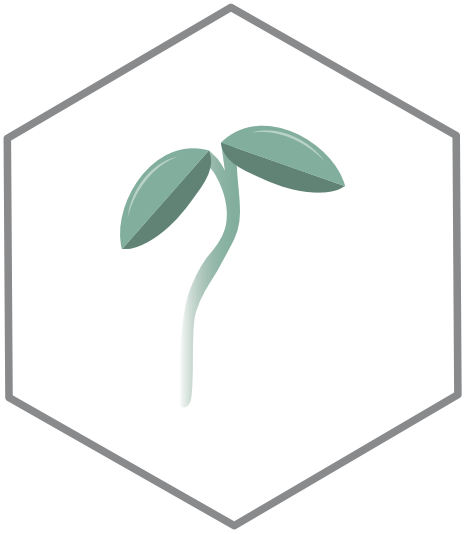
MODULATION OF PRIMARY MERISTEM ACTIVITY BY GIBBERELLINS THROUGH DELLA-TCP INTERACTION IN ARABIDOPSIS



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UNIVERSITAT POLITÈCNICA DE VALÈNCIA

MARCH 2017

UNIVERSITAT POLITÈCNICA DE VALÈNCIA

BIOTECHNOLOGY DEPARTMENT

PhD Thesis

Modulation of primary meristem activity by gibberellins through DELLA-TCP interaction in Arabidopsis

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March 2017

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CERTIFICAN que Amelia Felipo Benavent, ha realizado bajo su dirección en el Instituto de Biología Molecular y Celular de Plantas, el trabajo titulado ***"Modulation of primary meristem activity by gibberellins through DELLA-TCP interaction in* Arabidopsis",** y que autorizan su presentación para optar al grado de Doctor en Biotecnología.

Y para que así conste, firman el presente certificado en Valencia a 4 de febrero de 2017.

Dr. Miguel Ángel Blázquez Rodríguez

Dr. David Pablo Alabadí Diego

Dr. Ignacio Rubio Somoza

Para mis abuelos

Agradecimientos

Después de más de cinco años el momento agridulce ha llegado y toca dejar atrás esta etapa, que siempre recordaré con cariño. Ante todo, gracias a todos los que os habéis cruzado en mi camino este tiempo, porque de todos vosotros me llevo algo, una amistad, una enseñanza o unas risas.

En primer lugar, quiero dar las gracias a Miguel y David, por guiarme estos años con tanto humor y apoyarme cuando lo he necesitado. He tenido suerte con vosotros. También por confiar en mí y empujarme hacia retos que me han hecho crecer como profesional y como persona. Estoy muy contenta con la formación que me habéis dado y sobre todo por la pasión por el trabajo que trasmitís. Gracias por este aprendizaje, lo llevaré siempre conmigo.

A Nacho, por darme la oportunidad de trabajar con él y aprender tanto a su lado. Por estar siempre dispuesto a ayudarme en mi carrera científica, con sus sabios consejos y agudas observaciones, que me ayudan a ver otros puntos de vista y ser mejor profesional. También a nuestros colaboradores: Detlef Weigel en Alemania, Antonio y Robert Sablowski en Gran Bretaña y Simona Masiero en Italia, porque trabajar con ellos ha sido muy enriquecedor.

A Asier y Marta, no tengo palabras. O mejor dicho, tengo demasiadas, y si empiezo no paro. Soy muy afortunada de teneros. Gracias por ser mis remos.

A mis amigos y compañeros pasados y presentes del 2.07 y aledaños. A Juan Camilo y Moe, gracias por los cafés y la amistad. A Cris Úrbez, Francesca, Eugenio y David "nuevo", por cuidarme, animarme y siempre estar ahí cuando os necesitaba. A mis grandes colegas Jorge, Rodri, Noel y Marco, que me han hecho sentir como un tío más y ya puedo decir que estoy inmunizada para lo que venga después. Sobre todo a Noel, por su ayuda con los experimentos. Mención especial para Mª Ángeles y Juan, que son el alma del IBMCP, y me alegro de que retrasaran su jubilación y haber compartido con ellos estos años. A Nora y Paco, por ser unas grandes personas, siempre dispuestas a echar una mano. En definitiva, a todas las personas que a lo largo de estos años han compartido conmigo en mayor o menor grado momentos, charlas de pasillo, boleras, karaokes, horchatas, torrás, fiestas… Todo lo vivido con vosotros me ha llevado a ser la persona que hoy soy, disfrutando los buenos momentos y aprendiendo de los malos.

Quiero agradecer a mis viejos amigos, sobre todo a mis biotecnólogas, que hayan estado conmigo todo este tiempo. Han demostrado que no importa el tiempo que pase, la distancia o las responsabilidades laborales y familiares, siempre están ahí para lo que haga falta.

Por último, quiero dar las gracias a mi familia entera. Por muchísimas cosas, pero sobretodo, por su cariño y los valores que me han llevado hasta aquí y me permitirán seguir adelante pase lo que pase.

Ha sido una gran aventura,

¡Gracias!

Summary

Plant development is an iterative process of organ formation from the primary meristems of the plant. Meristem activity is driven by dynamic transcriptional programs that determine cell fate and identity as cells are displaced trough the meristematic tissue to initiate organ primordia. This regulatory network includes members of the TCP and KNOX family of transcription factors, and integrates external and intrinsic cues to efficiently adapt meristem activity to an ever-changing environment. However, how this integration occurs is not clear yet.

DELLA proteins have been proposed to modulate transcriptional circuits in plants in response to environmental signals. Although they do not show DNA binding capacity, DELLAs regulate transcription through physical interaction with a large number of DNA-binding transcription factors and other transcriptional regulators. Given the observed interaction between DELLAs and several members of the TCP family of transcription factors, we have explored the relevance of this interaction in the regulation of primary meristems. We have confirmed that DELLAs interact with members of both Class I and Class II TCPs, and prevent their ability to regulate downstream targets. In the embryonic roots, DELLAs maintain a dormant meristem by impairing TCP14/15-dependent activation of cell-cycle genes. On the other hand, DELLAs participate in the establishment of the shoot apical meristem domain that keeps an indeterminate fate, through the control of *KNAT1* gene expression by the TCP2/4-AS1 regulatory module. In summary, this Thesis provides a mechanistic framework to eventually explain environmental regulation of meristem activity.

Resumen

El desarrollo de las plantas es un proceso iterativo de formación de órganos a partir de los meristemos primarios de la planta. La actividad meristemática está dirigida por programas transcripcionales dinámicos que determinan el destino y la identidad celular conforme las células son desplazadas a través del tejido meristemático para iniciar el primordio del órgano. Esta red regulatoria incluye miembros de las familias de factores de transcripción TCP y KNOX, e integra señales externas e intrínsecas para adaptar eficientemente la actividad meristemática al medio ambiente, siempre cambiante. Sin embargo, la manera en que esta integración ocurre no se ha desvelado todavía.

Se ha propuesto que en plantas, las proteínas DELLA modulan los circuitos transcripcionales en respuesta a señales medioambientales. Aunque no muestran capacidad de unión al ADN, las DELLAs regulan la transcripción a través de su interacción física con un gran número de factores de transcripción capaces de unirse al ADN y otros reguladores transcripcionales. Dada la interacción observada entre las DELLA y varios miembros de la familia de factores de transcripción TCP, hemos explorado la relevancia de esta interacción en la regulación de los meristemos primarios. Hemos confirmado que las DELLA interaccionan con miembros de las dos clases de TCPs (Clase I y Clase II) e impiden su capacidad de regular dianas aguas abajo. En la raíz del embrión, las DELLAs mantienen el meristemo durmiente al impedir la activación de los genes de ciclo celular dependiente del módulo TCP14/15. Por otro lado, las DELLAs participan en el establecimiento del meristemo apical del tallo, que mantiene un estado indiferenciado, a través del control el módulo TCP2/4-AS1, el cual regula la expresión del gen *KNAT1*. En resumen, esta Tesis aporta un marco mecanístico para explicar, con el tiempo, la regulación medioambiental de la actividad meristemática.

Resum

El desenvolupament de les plantes consiteix en un procés iteratiu de formació d'órgans a partir dels meristems primaris. L'activitat meristemàtica està diridida per programes transcripcionals dinàmics que determinen el destí i la identitat cel.lular a mesura que les cèl.lules es van allunyant del meristem per formar els primordis d`órgans. Esta xarxa de regulació inclou membres de les famílies de factors de transcripció TCP i KNOX, i integra senyals externes i intrínseques per adaptar d'una manera eficient l'activitat del meristem als canvis del medi ambient. No obstant, no es coneix de quina manera la planta fa esta integració.

S'ha proposat que les proteïnes DELLA modulen estes xarxes transcripcionals en resposta a senyals del medi. Estes proteïnes no tenen capacitat d'unir-se a l'ADN, però regulen la transcripció mitjançant la interacció amb factors de transcripció i altres reguladors transcripcionals. Donada la interacció entre les proteïnes DELLA i alguns membres de la família de factors de transcripció TCP, hem explorat la rellevància d'esta interacció a la regulació dels meristems primaris. Hem confirmat que les DELLA interaccionen amb membres de les dos classes de TCPs (Classe I i Classe II) i els impedeixen regular les seues dianes. A l'arrel de l'embrió, les DELLA mantenen el meristem dorment al impedir l'activació de gens del cicle cel.lular depenent del mòdul TCP14/15. Per una altra banda, les DELLA participen a l'establiment del meristem apical de la tija, al que mantenen en un estat indiferenciat, mitjançant el control del mòdul TCP2/4-AS1, que regula l'expressió de *KNAT1*. En resum, esta Tesi aporta un marc mecanístic per poder explicar, més endavant, la regulació mediambiental de l'activitat meristemàtica.

Abbreviations

A. tumefaciens	*Agrobacterium tumefaciens*
ABA	ABscisic Acid
ACT8	*ACTINE8*
AD	Activation Domain
ALC	*ALCATRAZ*
Arabidopsis	*Arabidopsis thaliana*
ARR	*ARABIDOPSIS RESPONSE REGULATOR*
AS1	*ASYMMETRIC LEAVES1*
bHLH	*BASIC HELIX-LOOP-HELIX*
BiFC	Bimolecular Fluorescence Complementation
BME3	*BLUE MICROPYLAR END 3*
BP	*BREVIPEDICELLUS*
BR	Brassinosteroid
BZR1	*BRASSINAZOLE RESISTANT1*
CDKs	Cyclin-Dependent Kinases
cDNA	complementary DNA
ChIP	Chromatin ImmunoPrecipitation
CIN	*CINCINNATA*
CK	Cytokinin
Co-IP	Co-ImmunoPrecipitation
Col0	Columbia 0
CYC	*CYCLIN*
CZ	Central Zone
DBD	DNA binding domain
E. coli	*Escherichia coli*
EDZ	Elongation-Differentiation Zone
EMSA	Electrophoretic Mobility Shift Assay
ET	Ethylene
GA	Gibberellin
GA20OX	*GA20-OXIDASE*
GA2ox	*GA2-OXIDASE*
GA3OX	*GA3-OXIDASE*
GAI	*GIBBERELLIC ACID INSENSITIVE*
GFP	*GREEN FLOURESCENT PROTEIN*
GGDP	*GERANYLGERANYL DIPHOSPHATE*
GID	*GIBBERELLIN INSENSITIVE DWARF*

GO	Gene Ontology
GUS	*β-glucoronidase*
HDA	*HISTONE DEACETYLASE*
IAA	auxin/ Indol Acetic Acid
IDD	*INDETERMINATE DOMAIN*
IPT	*ISOPENTENYL TRANSFERASE*
JA	Jasmonic Acid
JAZ	*JASMONATE ZIM-DOMAIN*
KN1	*KNOTTED1*
KNAT	*KNOTTED1-LIKE HOMEOBOX IN ARABIDOSIS THALIANA*
KNOX	*KNOTTED1-LIKE HOMEOBOX*
KRP2	*KIP-RELATED PROTEIN 2*
LD	Long Day
Ler	*Landsberg erecta*
LHR	Leucine Heptad Repeats
LOX	*LIPOXYGENASE*
Luc	Luciferase
mRNA	messenger RNA
N. benthamiana	*Nicotiana benthamiana*
No-0	*Nossen-0*
OD	Optical Density
OSH1	*Oryza sativa homeobox1*
P_0	Plastochron 0
PAC	Paclobutrazol
PCNA	*PROLIFERATING CELL NUCLEAR ANTIGEN*
PCR	Polymerase Chain Reaction
PD	Physiological Dormancy
PIF	*PHYTOCHROME INTERACTING FACTOR*
PKL	*PICKLE*
PM	Proximal Meristem
PZ	Peripheral Zone
QC	Quiescent Center
qRT-PCR	quantitative-Real Time PCR
RAM	Root Apical Meristem
RBR1	*RETINOBLASTOMA-RELATED 1*
RGA	*RESPRESSOR OF ga1-3*
RGL	*RGA LIKE*

rTCP	dominant *miR319*-resistant gene version of CIN-TCP
RZ	Rib Zone
SA	Salicylic Acid
SAM	Shoot Apical Meristem
SCF	*SKP1, CULLIN, F-BOX*
SIM	*SIAMESE*
SL	Strigolactone
SLY1	*SLEEPY1*
SPL	*SQUAMOSA PROMOTER BINDING-LIKE*
STM	*SHOOT MERISTEMLESS*
TCA cycle	Tricarboxylic acid cycle
TCP	*TEOSINTE BRANCHED1, CYCLOIDEA, PROLIFERATING CELL FACTOR*
TF	Transcription Factor
TR	Transcriptional Regulator
TZ	Transition Zone
VAL	*VP1/ABI3-LIKE*
Y2H	Yeast Two-Hybrid
YFP	*YELLOW FLUORESCENT PROTEIN*

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9. Material and Methods

1. General Introduction

1. Plant hormones

Plants are sessile organisms that ensure their survival by adjusting their growth and developmental processes in accordance to environmental conditions. This trait is called “plasticity” and implies the perception and integration of external environmental conditions (such as changes in light quality and quantity, temperature, moisture, nutrient access, herbivorous feeding, etc.) with the intrinsic genetic programs of the plant (1). An important factor contributing to plasticity are hormones.

Phytohormones are small endogenous signaling molecules that can be produced in each cell and act locally or are transported to other cells or tissues. They play a dual role in the plant: as mediators that coordinate endogenous developmental processes and integrating environmental cues to conduct adaptive responses. Nine classes of phytohormones have been identified for the moment: the five classical hormones discovered in the mid 20th century [gibberellin (GA), auxin (IAA), cytokinin (CK), abscisic acid (ABA) and ethylene (ET)] and other hormones more recently recognized [jasmonic acid (JA), salicylic acid (SA), brassinosteroid (BR) and strigolactone (SL)] (2).

In the last decades, intense research in this field has unraveled the molecular mechanisms controlling the metabolism, perception and signal transduction of each hormone, although their mechanism of action is not completely understood. Specific roles for each hormone have been assigned. Nevertheless, increasing evidence shows that the interconnection between hormonal pathways to perform overlapping functions is common in plants (3, 4). These interconnections can lead to different outputs: additivity, synergism, antagonism or co-regulation (modulation of outcomes for a determined process mediated through independent pathways) (5).

In this context, current research is focused in finding out the molecular mechanisms by which hormones act, their underlying interactions and their relation with environmental and endogenous signals, such as the circadian clock.

2. Gibberellins

In this Thesis, we will focus on the role of GAs regulating *Arabidopsis thaliana* (*Arabidopsis*) primary meristems.

GAs were first discovered while studying the *bakanae* disease in rice caused by the fungus *Gibberella fujikuroi*, which produced big losses due to the elongation and fall over of infected plants. It was discovered that the fungus secretes a substance, they called gibberellin, responsible for this tallness (6). Since then, GAs have been broadly identified in plants, and also in some fungi and bacteria, as tetracyclic diterpenoids (6). Currently, there are 136 GAs identified, although only a few have biological activity in plants (GA_1, GA_3, GA_4 and GA_7) (7, 8).

In general, GAs are considered the "growth hormones", since they promote and are involved in the regulation of diverse key developmental processes during the life cycle of the plant (9-22), such as stem elongation or root growth (Figure 1.1). They also play an essential role integrating environmental cues and driving adaptive responses, for example temperature and light mediated growth (23, 24). Recent research has provided evidence of the individual function of GAs in these developmental processes, but also demonstrated that GA activities overlap with all other hormones at different levels (2). In the following sections of the Introduction, I will elaborate on key aspects of GA metabolism and the molecular events that occur from GA perception to the activation of the transcriptional networks for its signaling transduction, focusing on the pivotal signaling element, the DELLA proteins.

Remarkably, the study of GAs has been linked intimately with agriculture. For instance, several practical uses have derived from GA application, the application of GA inhibitors, or the biotechnological manipulation of GA synthesis and signaling, such as changing the stalk length of seedless grapes, or increasing sugar yield in sugarcane (25). Furthermore, during the "Green Revolution", certain varieties of rice and wheat were selected for increased yield, given that they decreased losses by lodging and were more resistant to pathogens. Later investigation demonstrated that these varieties were indeed GA insensitive mutants.

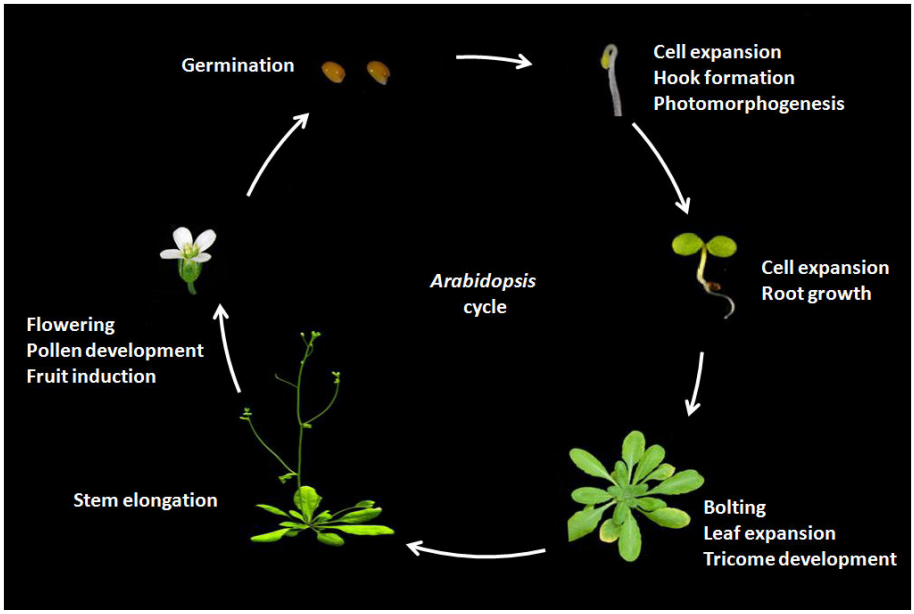

Figure 1.1. GAs regulate several developmental processes during the life cycle of the plant.

3. Gibberellin metabolism

As it has been above mentioned, GAs are diterpenoid acids that are synthesized by the terpenoid pathway in the plastid and then modified in the endoplasmic reticulum and cytosol until they reach their active form. In brief, GA synthesis occurs in three stages (each one in a different cellular compartment). We will focus on the last step of biosynthesis, which is the one that is regulated by a feedback mechanism in response to developmental and environmental cues.

In the plastid is where the first reactions take place, converting the geranylgeranyl diphosphate (GGDP) in *ent*-kaurene. This occurs in two steps catalyzed by two different terpene synthases (26, 27). Remarkably, the GA deficient *ga1* and *ga2* mutants of *Arabidopsis* are deficient in the first and the second step, respectively. In a second stage, that takes place in the plastid membrane and endoplasmic reticulum, the *ent*-kaurene is transformed to GA_{12} or GA_{53} by the action of two cytochrome P450 monooxygenases. GA_{12}, in turn can be converted to GA_{53} by further hydroxylation (Figure 1.2).

In the cytosol, the last stage in the production of biologically active GAs is catalyzed by two oxoglutarate–dependent dioxygenases, GA20-oxidase (GA20ox) and GA3-oxidase (GA3ox) (Figure 1.2). GA_{12} and GA_{53} are the inactive precursors of all biologically active GAs (27), and are converted in parallel pathways through three consecutive oxidations by GA20ox, leading to the production of GA_9 and GA_{20}, which are further oxidized by GA3ox to form the bioactive GAs GA_4 and GA_1 (27-29). This route is conserved in all plants, and in some of them there are alternative pathways to form the minority, biologically active compounds GA_3, GA_5, and GA_6; which can be derived from the precursor GA_{20} (Figure 1.2).

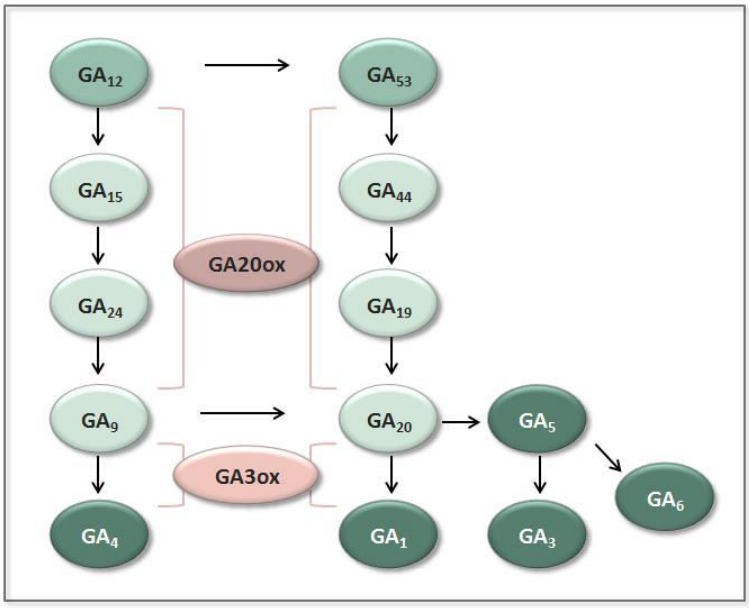

Figure 1.2. Reactions taking place in the last stage of GA biosynthesis in the cytosol. Two oxoglutarate–dependent dioxygenases, GA20ox and GA3ox (enzymes in mauve) catalyze the formation of different bioactive GAs (dark green) from the inactive precursors GA_{12} and GA_{53} (medium green). Inactive GAs are shown in light blue.

GA homeostasis does not only rely only on their synthesis but also on a delicate balance with their inactivation. Several mechanisms for GA inactivation have been reported. The best characterized is the addition of an hydroxyl group to GAs by the GA2-oxidase (GA2ox) (Figure 1.2) (27). This inactivation is irreversible, given that the resulting molecules are immediately degraded. This mechanism is biologically important because the expression of *GA2ox* genes is

transcriptionally regulated in response to environmental and developmental cues and it constitutes the main GA-deactivation pathway.

4. Regulation of gibberellin metabolism

To ensure their survival, plants need to respond effectively to environmental changes. That means being able to promptly and precisely regulate hormone homeostasis according to those changes. The *Arabidopsis* genome contains small gene families encoding the cytosolic enzymes GA20ox (five genes), GA3ox (four genes) and GA2ox (eight genes) (30). Remarkably, plants regulate transcriptionally the expression levels of members of these gene families by a feedback mechanism in order to regulate GA levels.

Then, when bioactive GA level is high, most of the biosynthetic *GA20ox* and *GA3ox* genes are down-regulated through this GA-mediated feedback mechanism (27, 28). In contrast, *GA2ox* genes are up-regulated by GA treatment (31). In a low GA level situation, the inverse regulation takes place. In some situations, however, the plant needs a higher level of GA, and this self-regulation does not occur. This is the case during germination, where the *GA3ox2* gene is uncoupled from the feedback and its continuous expression allows the constant production of bioactive GAs (32). Moreover, GA levels in the plant are also influenced by different elements: endogenous factors such as the circadian clock, GAs and other hormones such as BRs (33), IAA (34) or ET (19); and environmental conditions such as light (35-37), temperature (24, 38), pathogens, etc.

5. Gibberellin signaling

During the past twenty years, intense research has been made to unravel the molecular mechanisms of GA signaling, and most of its components have been identified by genetic screenings in *Arabidopsis* and rice and by the subsequent biochemical analysis of GA response mutants. It was also found that this signaling pathway is widely conserved among dicots and monocots (39).

In brief, GA signaling consists in three key elements: the soluble GA receptor *GIBBERELLIN INSENSITIVE DWARF1* (*GID1*), the transcriptional regulators DELLA proteins (DELLA) and the F-box proteins *SLEEPY1 (SLY1)* in *Arabidopsis* and

GIBBERELLIN INSENSITIVE DWARF2 (GID2) in rice (40) that mediate the GA-dependent degradation of DELLAs.

GA receptors were discovered while studying the GA-insensitive rice mutant *gibberellin insensitive dwarf1* (*gid1*) (41). In *Arabidopsis,* three orthologous genes have been found (*GID1A, GID1B* and *GID1C* (42, 43)) and they seem to have overlapping functions, since only the triple knockout mutant presents the dwarf phenotype characteristic of GA signaling deficiency (42, 44). Accordingly, the three genes are expressed in all tissues, but at different levels (42, 43), and the proteins localize to the nucleus and the cytoplasm (44).

DELLAs are the master negative regulators of the GA signaling pathway, since these proteins down-regulate all GA responses (45). They were discovered through the study of the *Arabidopsis gai-1* (*gibberellic acid insensitive-1*) mutant (13), a dominant version resistant to GA-dependent degradation. Over the years the five DELLAs in *Arabidopsis* have been identified: GAI, RGA (Repressor of *ga1-3*), RGL1 (RGA like1), RGL2 and RGL3 (15, 46). We will further describe key aspects of these proteins in section 6 of the Introduction, as they are the main protagonists of this Thesis.

The current model of GA signaling it is well established in the literature. Basically, DELLA proteins restraint all GA functions, whereas the GA signal overcomes this DELLA-mediated restriction by promoting DELLA degradation by the proteasome (40) (Figure 1.3).

The signaling cascade starts when a bioactive GA binds the nuclear receptor GID1 (41). Crystallization studies demonstrated that GID1 contains a GA-binding pocket and a flexible N-terminal extension (47). Upon formation of the GA-GID complex, the flexible domain closes the pocket, changing the three-dimensional conformation of the complex. This new conformation allows the N-terminal part of the DELLA proteins to bind the lid of the complex, generating a GA-GID1-DELLA complex (48). Remarkably, the N-terminal part of DELLAs, that contains the DELLA and TVHYNP motifs (see section 6.1 of the Introduction), is essential for the interaction with GID1 because their deletion results in an inability of DELLAs to interact, despite the presence of GA (44).

The resulting GA-GID1-DELLA complex induces conformational changes in the C-terminal half of the DELLA, called GRAS domain, that triggers its recognition by the F-box protein SLY1/GID2 (49). SLY is part of a SCF (SKP1, CULLIN1, F-BOX)-dependent E3 ubiquitin ligase complex that catalyzes the attachment of polyubiquitin chains to target proteins for their subsequent degradation by the *26S* proteasome (42, 50, 51). The union of both complexes lead to the polyubiquitination of DELLA and its subsequent degradation by the 26S proteasome, thereby relieving their GA function-restraining effects (42, 44, 52, 53) (Figure 1.3).

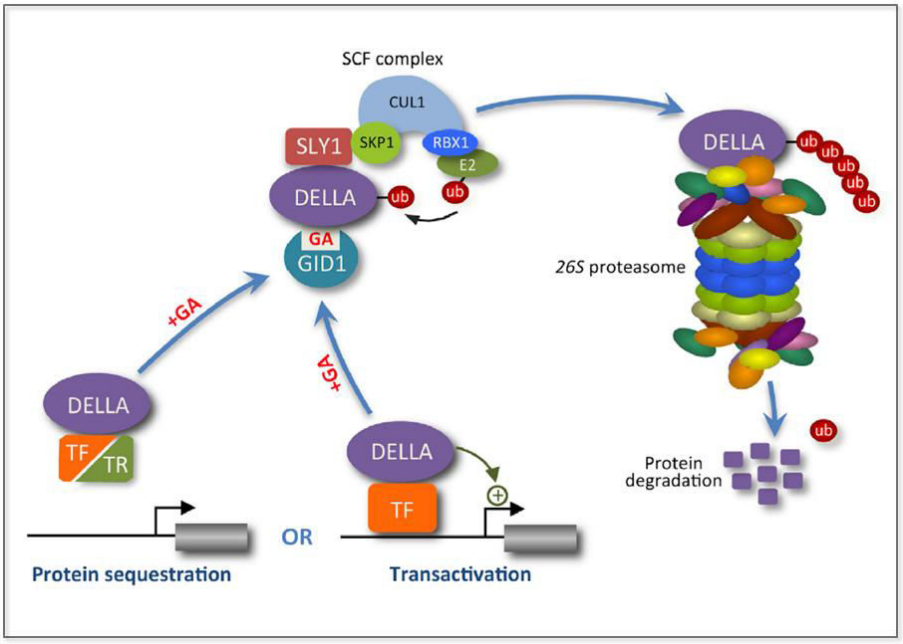

Figure 1.3. The GA signaling pathway. When GA level is low, DELLAs repress GA responses by two different mechanisms: (1) by protein sequestration, interacting with and inhibiting the activity of transcription factors (TF) or regulatory proteins (TR), or (2) by transactivation, activating the transcription of target genes associated with TF. On the other hand, when GA concentrations increase, GA binds to GID1 receptor, forming the GA-GID1-DELLA complex. This complex interacts with the SCF^{SLY1} complex through DELLA, which is consequently polyubiquitylated and degraded by the *26S* proteasome. This leads to the activation of GA responses. Extracted from Davière and Achard, 2015 (2).

Thus, GA promotes its responses, such as growth, by mediating the proteasome-dependent destabilization of DELLA proteins; on the contrary, in absence of GAs, DELLAs accumulate and repress GA responses (54). In the

following section, the mechanism by which DELLAs perform its repressing function is described.

6. DELLA proteins

Now, we will focus on the key signaling component of the pathway, the DELLA proteins, which are the most extensively studied elements of the GA signaling pathway. Several observations gathered during the last decades have led to the current model of GA and DELLA action (Figure 1.3). *della* mutants in *Arabidopsis* and rice results in a constitutive activated GA response (15, 55-58), with a slender or tall phenotype in these loss of function mutants. Inversely, DELLA gain of function mutants such as *gai-1* in *Arabidopsis*, present the phenotype of GA deficiency that includes dwarfism among other features (59, 60). Besides, exogenous GA treatments were associated with DELLA protein destabilization to rescue dwarfism of a GA-deficient mutant *ga1* (54).

DELLAs are highly conserved proteins among plant species, and several of them carry a single DELLA gene, such as tomato (61), grapevine (62), rice (56), barley (63) and wheat (45). Interestingly, that only DELLA is sufficient to regulate all GA responses in those species. On the other hand, the *Arabidopsis DELLA* gene has undergone amplification, and, as it has been previously mentioned, the *Arabidopsis* genome encodes five DELLAs: GAI, RGA, RGL1, RGL2 and RGL3 (39). Those DELLA genes have undergone sub-functionalization. Thus, distinct but overlapping functions have been reported for the five DELLAs, and it has been proposed that the functional diversification of *DELLA* genes is based on their differential expression patterns (64). Therefore, the differential transcriptional regulation of *DELLA* genes is the cause for the differential roles in *Arabidopsis*.

In particular, RGA and GAI are the major players during vegetative growth, since they control cell expansion and cell division in hypocotyls, shoot and root; and in induction of flowering (55, 65-70). RGA, RGL1 and RGL2 together modulate flower development (57, 58) and RGL2 is the main DELLA inhibiting seed germination (15, 58, 71). Finally, RGL3 contributes to plant fitness during environmental stress (72, 73).

6.1 Structural features of DELLA proteins.

It is well established in the literature that DELLAs are nuclear proteins and belong to the plant-specific GRAS family of transcription regulators (74). GRAS proteins are characterized by a conserved C-terminal GRAS domain involved in protein-protein interactions and transcriptional regulation of specific targets. Additionally, and in contrast with other GRAS proteins, DELLAs present a distinctive N-terminal domain that mediates interaction with the GA-loaded GID1 receptor, as mentioned earlier (Figure 1.4) (39).

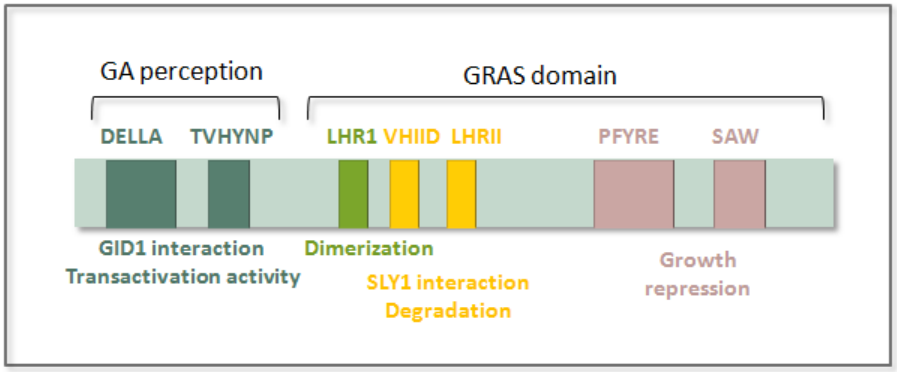

Figure 1.4. Structural features of the DELLA proteins. The important motifs of DELLA proteins and their associated functions are shown in the same color. Modified from Davière and Achard, 2015 (2).

The GRAS domain contains two leucine heptad repeats (LHRI and LHRII) and three conserved motifs, VHIID, PFYRE and SAW (Figure 1.4) (2). The VHIID and LHRs domains have been implicated in dimerization of the protein (75) and in their interaction with the F-box protein SLY1 in *Arabidopsis* and subsequent degradation (76). On the other hand, the function of PFYRE and SAW is still unknown, although it has been involved in growth repression (2).

DELLAs are distinguished from the rest of the GRAS family by its specific N-terminal sequence containing two conserved domains: the TVHYNP domain and the DELLA domain, which presents conserved amino acid sequences Asp-Glu-Leu-Leu-Ala (DELLA) that gives them their name. It has been demonstrated that mutations or deletions on DELLA or TVHYNP regions result in protein versions unable to interact with GID1. Thus, these versions are resistant to GA-promoted

degradation, the DELLA protein is stabilized and this leads to semi-dominant GA-insensitive dwarf phenotypes such as rga-Δ17 and gai-1 (41, 42, 44). Furthermore, recent research has demonstrated that the N-terminal DELLA/TVHYNP domain of the rice DELLA possesses transactivation activity, indicating additional functions for this domain that can be conserved in other species (Figure 1.4) (77).

6.2 Mechanisms of action of DELLA proteins

Since their discovery, DELLAs have been involved in the regulation of a great variety of developmental processes during the whole life of the plant (see section 2 of the Introduction). However, the mechanisms by which DELLAs repress GA responses were just recently unraveled, defining two different manners of regulation and mediated by protein-protein interactions in both cases.

It has been found that an important function of DELLAs relies on their ability to interact with key regulatory proteins such as transcription factors (TFs) or transcriptional regulators (TR) to modulate plant development. This first mechanism is called “protein sequestration”, since DELLAs impair the DNA binding ability of their target TFs upon interaction (2), as in the case of PHYTOCHROME INTERACTING FACTORS (PIFs) (68, 69) or BRASSINAZOLE-RESISTANT1 (BZR1) (78). DELLAs also interact with and inhibit the activity of TRs such as JASMONATE ZIM-DOMAIN (JAZ) proteins (79) (Figure 1.3). By this mechanism, DELLAs have been found to regulate a great variety of processes. They control hypocotyl elongation by interacting with PIFs (64, 68, 69) and BZR1 (78, 80); floral transition with SQUAMOSA PROMOTER BINDING-LIKE (SPL) (81); fruit patterning with ALCATRAZ (ALC) (82), plant defense with JAZ proteins (73, 79), etc. Remarkably, the list of DELLA interactors is still growing, highlighting the high promiscuity of these proteins. The great number and variety of DELLA interactors provide a molecular explanation for the fact that GAs regulate a wide variety of physiological processes (39). Therefore, DELLAs are central players and integrators of GA-dependent processes in a context-dependent manner.

On the other hand, DELLAs operate in the chromatin either as transactivation factors, as in the case of the interaction with ARABIDOPSIS RESPONSE REGULATOR 1 (ARR1) (83); or as co-repressors, as described for the interaction with SPL15 (84). Thus, DELLAs regulate the transcription of downstream genes to repress GA responses (Figure 1.3) (85). For instance, recent transcriptome analyses indicate

that the DELLA protein RGA is able to activate the transcription of target genes (83, 86-89). However, there is no evidence for direct binding of DELLAs to DNA, since DELLAs do not present a DNA binding domain, and chromatin precipitation assays show only a moderate enrichment of promoter targets. Thus, it has been proposed that the association of DELLAs with their target promoters might be rather indirect, including other elements (39).

Acccordingly, several studies strongly support this idea. Just in the last four years, DELLAs have been found to associate with the promoter of target genes trough the interaction with two families (up to now) of TFs, INDETERMINATE DOMAIN (IDD) and type-B ARR factors (83, 90). Therefore, these TFs are intermediate proteins between DELLA and DNA, allowing DELLAs to enhance the expression of target genes. In this sense, experiments in rice suggest that the transactivation activity may reside in the N-terminal DELLA/TVHYNP domain (Figure 1.4) (77). Besides, DELLAs have been found to interact with the core subunit of the chromatin remodeling factor SWI/SNF, resulting in an increased transcription of DELLA target genes in *Arabidopsis* (91).

7. The role of DELLAs in the primary meristems: interaction with TCPs

7.1 Looking for suitable DELLA interactors regulating meristem activity

According to literature, the levels of bioactive GAs are modulated in different tissues and developmental stages to regulate plant growth in an environmental-dependent manner. In particular, they seem to be decisive in two different biological contexts, as elaborated below.

The first developmental challenge plants have to overcome to ensure their survival is the germination process and GAs play a crucial regulatory role at this stage promoting embryo growth and reducing the physical restraint imposed by the endosperm and testa. Remarkably, activation of embryo growth, especially in the radicle allows radicle protrusion trough the covering layers, what constitutes the final step of the process (see introduction of Chapter II) (66). This implies the activation of the root apical meristem.

After germination, during the vegetative growth, GAs are needed to promote stem elongation, leaf expansion and root growth, among other processes (92); and they are present mainly in rapidly developing tissues, such as the shoot tips, expanding leaves and petioles near elongating internodes, for example (93-95).

GA activity seems to be focused on regions where new cells and tissues are formed, where they can promote morphogenesis or cell growth. Importantly, this observation made us think that the GA signaling pathway might be involved in the regulation of the primary meristems of the plant: the root apical meristem (RAM) and the shoot apical meristems (SAM). As GAs perform their activity by promoting DELLA degradation, and DELLA proteins are the main regulatory elements of the pathway, we will focus on the role of DELLAs in the regulation of primary meristems of *Arabidopsis*. In particular, the aim of this Thesis is to unravel the regulatory role of DELLAs in the meristem in two different situations: in the SAM during vegetative growth (Chapter I), and in the RAM during germination (Chapter II).

Since DELLAs are known to repress GA responses trough the interaction with other proteins, mainly TFs, understanding the GA-mediated control of meristems would require the identification of its downstream transcriptional mediators. Therefore, the first step in our research was the identification of DELLA interactors capable to regulate jointly meristem activity. As a matter of fact, at the beginning of this Thesis, we took advantage of unpublished work in our laboratory, which unveiled a great amount of these interactors. In this work, that was published later on (96), the TF interactome of the *Arabidopsis* DELLA protein GAI was determined by yeast two-hybrid assays (Y2Hs).

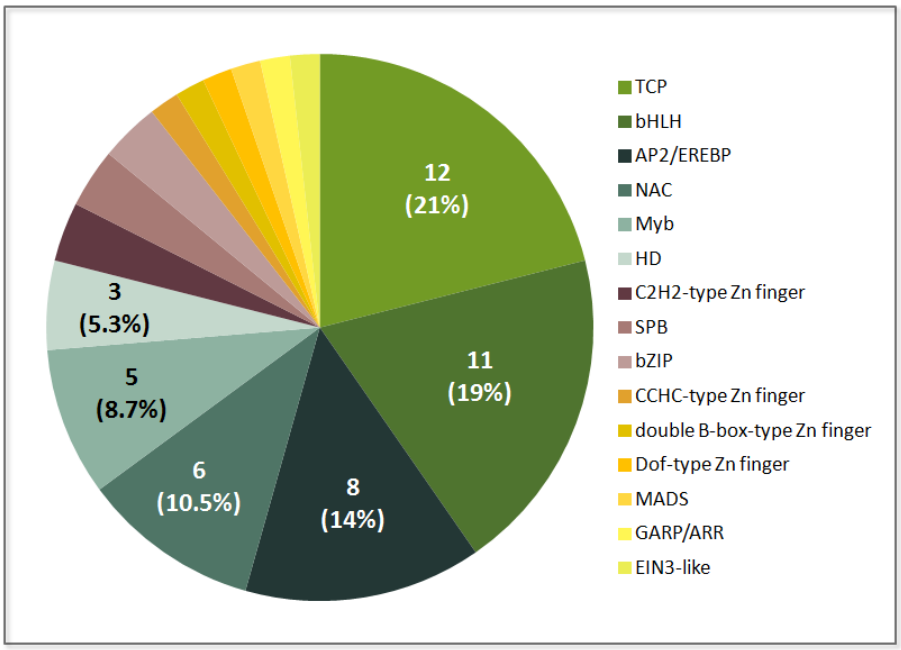

Figure 1.5. TF interactome of the DELLA protein GAI in *Arabidopsis*. The Y2H screening unraveled 57 interactors of GAI, that belonged to 15 different families of transcription factors (different sections in the circle). The number of interacting members and the percentaje of interactors that the family represents over the total 57 are shown. Mauve and yellow sections represent families with 2 and 1 interactors respectively. Data extracted from Marín-de la Rosa *et al.*, 2014 (96).

To do so, a library of approximately 1,200 TFs of *Arabidopsis* (97) was screened using the GRAS domain of GAI (M5GAI) as bait, and 57 novel interactors were found. Among the TFs found, there were two known interactors of GAI, PIF3 and PIF4 (68, 69), showing the reliability of the screening. The interactors found belonged to 15 out of 39 families of TFs represented in the library. This diversity is in accordance with the great variety of DELLA interactors reported in the literature (98) and demonstrates that there is not a strong bias to any particular family of TFs. Nevertheless, it is remarkable that approximately 21% of the interactors found belonged to the TEOSINTE BRANCHED1, CYCLOIDEA, PROLIFERATING CELL FACTOR (TCP) family of transcription factors (Figure 1.5); and 19% to their structurally close proteins basic helix-loop-helixes (bHLHs). Other categories were less represented (96).

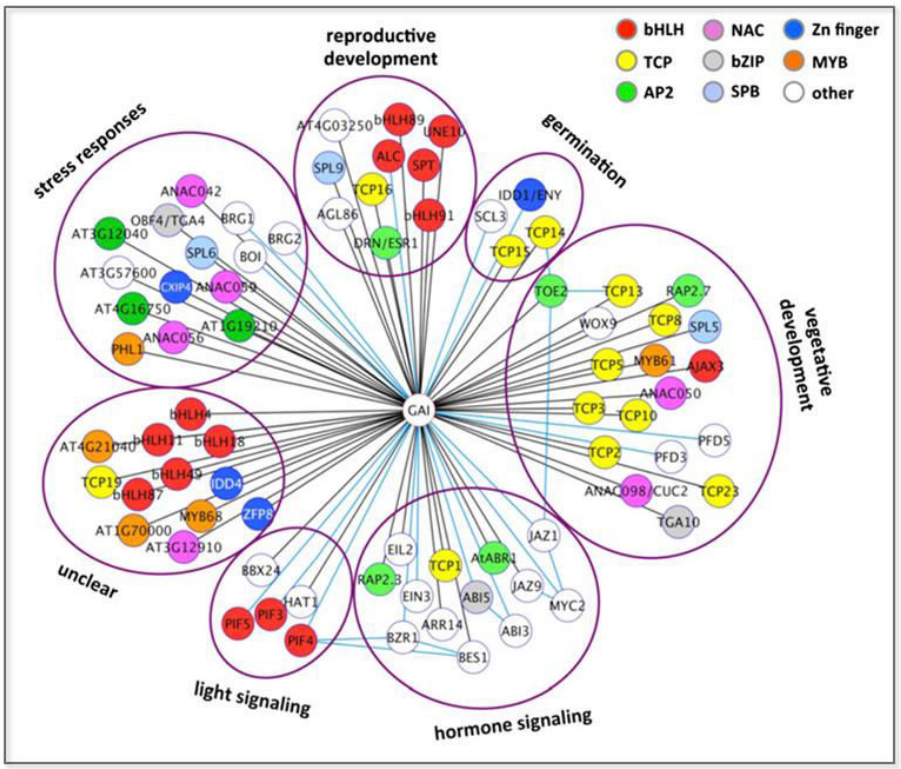

Figure 1.6. TF interactome of the DELLA protein GAI: biological processes. The graph shows all GAI partners found in the Y2H screening grouped by the biological processes in which they are involved. The most abundant TF families are represented in different colors (see legend in the figure). Notice the TCP (yellow) enrichment in the functional categories "germination" and "vegetative development". Extracted from Marín-de la Rosa *et al*., 2014 (96).

The GAI partners were also grouped based on the biological processes in which they participate (Figure 1.6) (96). Among the four partners of GAI associated with germination, two were TCPs (TCP14 and TCP15); and in the vegetative development category, seven out of eighteen (approximately 39%) belonged also to this family of TFs (TCP2, TCP3, TCP5, TCP8, TCP10, TCP13 and TCP23) (96). Besides the physical interaction, DELLAs and their TCP partners identified in the Y2H screening seemed to have a functional connection, as indicated by the co-expression analysis they performed (96).

Notably, out of the 23 members of the TCP family present in the library, 12 were identified as GAI partners. That constitutes a 52% of GAI interactors over the total TCP members present. However, due to the high-throughput approach followed in the screening, it is possible that not all the interactions were

identified, and that more members of the family constitute reliable GAI partners (see section 1 of Results and Discussion of Chapter I) (96).

Taking into account that TCP is the family of TFs for which more members have been found to interact with GAI by Y2H, and the enrichment in TCPs of the biological processes aim of our study (germination and vegetative development), we thought that the TCP family of TFs represent a promising target for additional research. Therefore, in this Thesis, we decided to focus in the study of **the relevance of DELLA-TCP interaction in the control of SAM and RAM activity during vegetative development and germination.**

7.2 The TCP family of transcription factors

The family was first discovered in 1999, and takes its name from its founding members: TEOSINTE BRANCHED1 (TB1), CYCLOIDEA (CYC) and PROLIFERATING CELL NUCLEAR ANTIGEN FACTOR1 (PCF1). TCP proteins are plant-specific TFs, closely linked historically to the control of cell proliferation and growth, therefore shaping plant morphology. They are known to have profound effects on the growth patterns of meristems and lateral organs, they regulate the transcription of cell cycle regulators such as cyclins and they are involved in the regulation of diverse growth-related processes, such as embryonic growth, branching, leaf development, floral and pollen development, germination, cell cycle regulation, etc (99, 100).

The family is characterized by a non-canonical basic helix-loop-helix (bHLH) motif, the TCP domain, which is responsible of DNA-binding and protein-protein interactions (dimerization). This domain is closely related to the canonical bHLH that can be found in bHLH proteins such as PIFs, but the basic region of the TCP is longer and contains helix-breaking amino acids (100).

In *Arabidopsis*, the TCP family is a group of 24 phylogenetically related members, and according to differential features within the TCP domain, can be distinguished in two types, class I and class II, whereas class II can be further subdivided in two groups: CYC/TB1 and CINCINNATA (CIN) (Figure 1.7) (101). In particular, sequence alignment of the conserved TCP domain of several TCP proteins reveals three differential features between the two classes: a 3-residue insertion in the basic region of the class II but not in the class I TCP domain (101),

the residue compositions in the loop and hydrophilic faces of helices I and II, and the length of helix II (99).

Besides, outside the TCP domain, a subset of five CIN-TCPs (indicated with an asterisk in Figure 1.7) possess a microRNA miR319 recognition sequence in their mRNA. In fact, the function of this class II TCP genes seems to be tightly controlled post-transcriptionally by this microRNA. This is not surprising given the strong effects on proliferation and growth of these CIN-TCPs, which at some developmental stages and tissues need to be finely tunned, for example in the SAM (100).

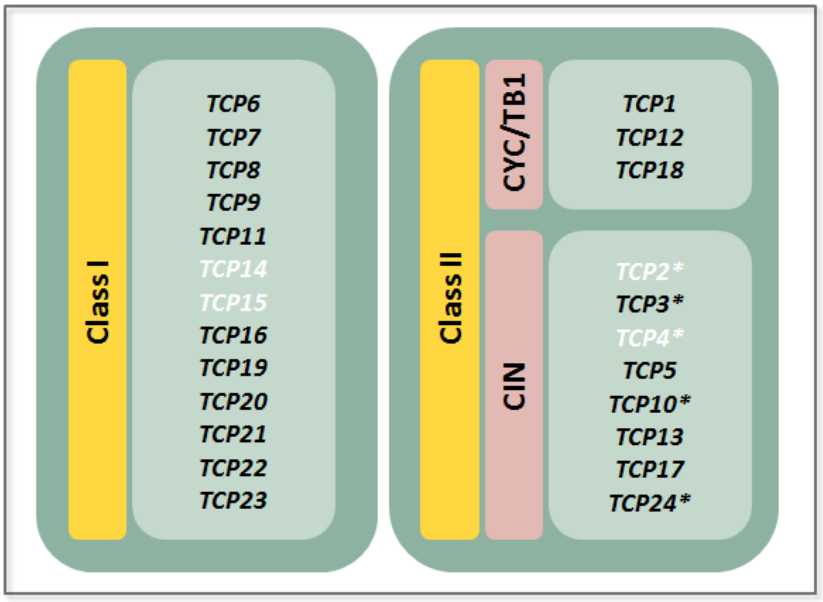

Figure 1.7. Classification of the *Arabidopsis* TCP family of transcription factors. The 24 members of the family in *Arabidopsis* are divided in two classes based on differences within their TCP domain. Class II TCPs can be further subdivided in two groups: CYC/TB1 and CIN. The five miR319-targeted CIN-TCPs are indicated with asterisks, and the TCPs target of our study in this Thesis are shown in white: the CIN-TCPs TCP2 and TCP4 in Chapter I and Class I TCPs TCP14 and TCP15 in Chapter II.

Research on TCPs has been always hindered by two characteristics: the considerable functional redundancy of its members and, in the case of CIN-TCP genes, their post-transcriptional down-regulation by miR319 (102).

Several studies reported that single, double and even triple *tcp* mutants and CIN-TCP over-expressor lines do not show strong phenotypes different from wild type plants, preventing functional gene analysis (excluding a delay in flowering in the *tcp4* mutant (103)). Nevertheless, two strategies have been used to overcome these difficulties. First, dominant-negative genetic approaches have been used, consisting in the fusion of EAR or SRDX repression domains with TCP coding sequences. This strategy has generated loss of function lines with strong mutant phenotypes (70, 104, 105). Second, some molecular strategies have been used to manipulate the regulatory effect of miR319 in CIN-TCPs. It was discovered that miR319 over-expressing lines and the *jaw-D* mutant (that is an activation tag dominant mutant of miR319) simultaneously down-regulated the five target CIN-TCPs (102-104, 106, 107). Inversely, the enhancement of CIN-TCPs expression has been possible, among other strategies, by inactivating miR319 (miR319 mimicry, MIM319) (108-110). Another successful way to obtain gain of function lines is by introducing synonym mutations within the microRNA target sequence in the TCP coding regions. Thus, the resulting TCPs escape miR319 regulation without altering their protein sequence. Those miR319-resistant gene versions of CIN-TCPs (rTCPs) were placed under the control of their natural promoters or ectopic promoters (102, 104, 108, 111). These versions lead to reductions in growth, fusion of cotyledons, lack of shoot apical meristem development and lethality, what remarks the importance of regulating these *TCP* genes for plant viability (102, 112).

In fact, the *tcp2 tcp3 tcp4 tcp10 tcp24* quintuple loss of function mutant, *jaw-D* plants, or plants expressing a fusion of any of the CIN TCPs to the dominant SRDX (EAR) repressor domain have similar phenotypes, with wavy petals and leaves due to excessive proliferation in the margin regions (113, 114). Although these approaches have been very useful to generate interesting, informative phenotypes, their results are usually interpreted with caution as they often lead to interference with several TCPs, especially when constitutive, ectopic or highly active promoters are used (115).

As for the DNA-binding properties of TCPs, intense research has been made, though a complete understanding of this feature is still lacking. Several DNA-binding site selection assays have unraveled the consensus-binding site of both

classes of TCPs: class I TCPs is “GTGGGCCCAC” (116) whereas class II is “GTGGTCCCA” (117).

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2. Objectives

2. Objectives

DELLAs have been involved in the regulation of a great variety of biological processes during the life cycle of the plant. However, little is known about their role modulating meristem activity. In this Thesis, we decided to focus in the regulation exerted by DELLAs on the primary meristems of *Arabidopsis thaliana* in two different processes: regulating the RAM during germination and the SAM during the vegetative phase. Thanks to the GAI TF interactome, TCP TFs were identified as suitable partners of DELLA in this regulation. Therefore, the two specific objectives pursued in this work are:

1. **Investigating the regulation of SAM by DELLAs in the vegetative phase through their interaction with CIN-TCPs, and more particularly, with TCP2 and TCP4 (Chapter I).** Previous evidence shows that CIN-TCPs and GAs promote cell differentiation in organ primordia. Accordingly, we hypothesized that DELLAs would promote the meristematic state of cells by inhibiting CIN-TCP activity through their physical interaction.

2. **Exploring the regulation of the RAM during the germination process by DELLA interaction with the class I TCPs TCP14 and TCP15 (Chapter II).** Cell division in the RAM has been found to be essential for completing germination, and TCP14 and TCP15 have been involved in promoting cell progression. Besides, TCP14 has a role promoting germination, whereas DELLAs are known to repress this process. With these data, we propose that DELLAs interact with these TCPs inhibiting their role favoring germination.

3. Introduction of Chapter I:

DELLAs' role on the vegetative shoot apical meristem

1. The vegetative shoot apical meristem

Plant development is an iterative process of organ initiation from meristems. The shoot apical meristem (SAM) is established during embryogenesis, and remains active during the whole life cycle of the plant, making it able to generate organs throughout every stage of its development. It is the structure from which all the aboveground organs originate, and generates simple leaves during the vegetative phase of *Arabidopsis*. It harbors a pool of self-renewing, un-differentiated pluripotent stem cells that are located in the central region of the meristem, a highly regulated microenvironment termed the stem cell niche (1); and their daughters, that fill the periphery and transition to differentiation and determinate growth to generate leaves at this stage (2). A great advantage of this continual organ initiation is that plant architecture is able to be flexible and respond to changing environmental conditions, contributing to their plasticity.

The SAM is a dome-shaped structure that is organized in distinct cell layers and subdivided into different zones or functional domains (Figure 3.1). In *Arabidopsis*, the SAM is defined by three cell layers (L1 to L3). An external L1 overlies the L2, both growing in a two-dimensional way by anticlinal cell division (originating cell walls are perpendicular to the apex surface) and constituting thr tunica layers (3, 4); and the most inner layer, L3, that constitutes the corpus and grows in a three-dimensional manner (with periclinal and anticlinal cell divisions) (Figure 3.1). Each layer generates different tissues. In general, epidermis is formed from the L1 layer while gametes from the L2 and internal tissue of organs from the L3 and further corpus cells.

More importantly for our study, there are three functional domains in the *Arabidopsis* SAM, characterized by different transcriptional and developmental programs directing each of them (5): the central zone (CZ), the peripheral zone (PZ) and the rib zone (RZ). The CZ includes L1, L2 and L3 cells, and is where the stem cells are located. The organizing center (OC) is formed by a small group of cells and sustains the stem cells above. It is known that stem cells from the CZ divide at a slow rate and the progeny produced from its division are used to replenish the stem cells themselves and are also displaced into the PZ, which is a ring of cells that surround the CZ. This PZ is characterized by high mitotic activity: cells start to divide more rapidly and it is where lateral organs are initiated. The

site of incipient organ formation is called the Plastochron 0 (P_0), used to index leaves at a point in development. Cells in the PZ undergo sequential differentiation as they distance from the CZ and give rise to organ primordia (Figure 3.1) (6, 7). On the other hand, daughter cells can also be displaced to the RZ that generates the inner tissues of the stem.

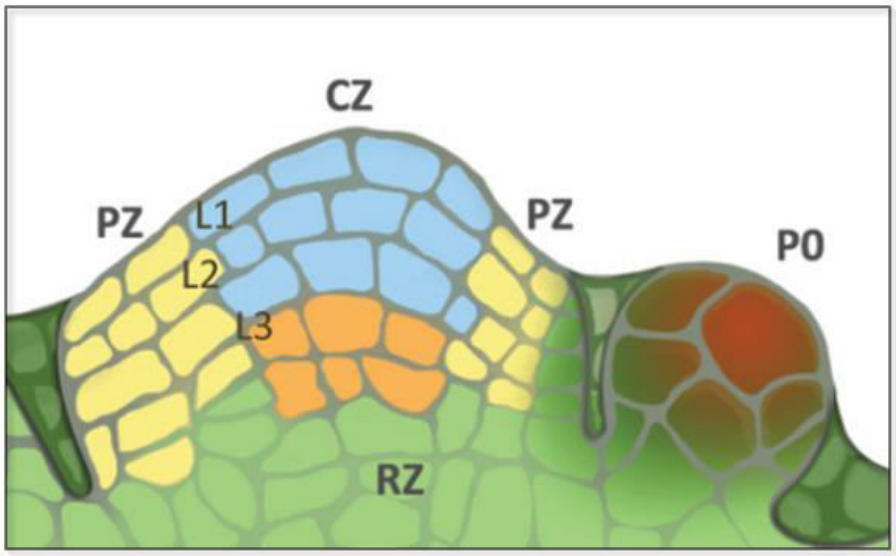

Figure 3.1. Layers and functional domains of the *Arabidopsis* shoot apical meristem. Cells from the SAM are disposed in three layers: L1, L2 and L3. Regarding functional domains, SAM architecture is organized in a central zone (CZ, blue), where the stem cell niche and the organizing center (orange) are localized; the peripheral zone (PZ, yellow) and the rib zone (RZ). The molecular events driving meristem maintenance are in tight coordination with organ differentiation processes. Leaf primordia in P0 developmental stage is indicated. Extracted from Bustamante *et al*., 2015 (1).

In this line, stem cell maintenance is accomplished by the balance between self-replacement and organ initiation. Therefore, unraveling the molecular mechanisms of meristem function, including meristem maintenance, is currently a major area of interest in plant development. As cells are passively displaced from the CZ to the periphery by cell division and growth, they need to integrate the information of its own position within the meristem and the transcriptional program directing its fate or identity and act accordingly. Thus, this changing nature of the SAM depends on two factors: intercellular communication by plasmodesmata to assess their position relative to the neighboring cells; and principally, dynamic transcriptional programs within all the area of the meristem

able to drive changes in cell fate and identity as cells are displaced through the meristematic tissue to initiate organ primordia and organogenesis (1, 8).

A key aspect for Chapter I of this Thesis is the transition of cells from the un-differentiated to differentiated state as they are displaced from the CZ to the periphery to form the organ primordia. This process is essential for plant development and implies a tight coordination of pro-meristematic and pro-differentiation regulators. In other words, there is a balance between the maintenance of the undifferentiated fate of cells in the shoot meristem and the promotion of cellular differentiation in organ primordia. The establishment of a boundary between both situations is essential for organogenesis and meristem maintenance. Unraveling the molecular mechanisms that establish this boundary is still a goal for current research, and we provide evidence in this Thesis that DELLAs might be involved in this point.

It is noteworthy that genes related to this regulation of the SAM and organ primordia are highly conserved among different plant species and their mutation result in severe developmental defects (9). This is the case for *Knotted1-like homeobox (KNOX)* genes, which are fundamental for meristem maintenance (10), and indirect targets of TCPs (11, 12). Given these data, we decided to focus our research on the role of DELLAs in this TCP/KNOX regulatory network. In the following section, an outline of this pathway is provided.

2. The TCP/KNAT1 module of regulation

KNOX genes constitute a small family of *TALE homeobox* genes found in all green plant lineages, and they are divided in two subclasses based on sequence similarity within the homeodomain, phylogenetic analysis and other aspects such as expression pattern (13, 14). Class I *KNOX* genes are the most widely studied and are expressed within the SAM (15), whereas Class II genes show diverse expression patterns and less known functions (16). As their function is involved in meristem maintenance and their expression primarily confined in the SAM, in this Thesis we focus on Class I *KNOX* genes.

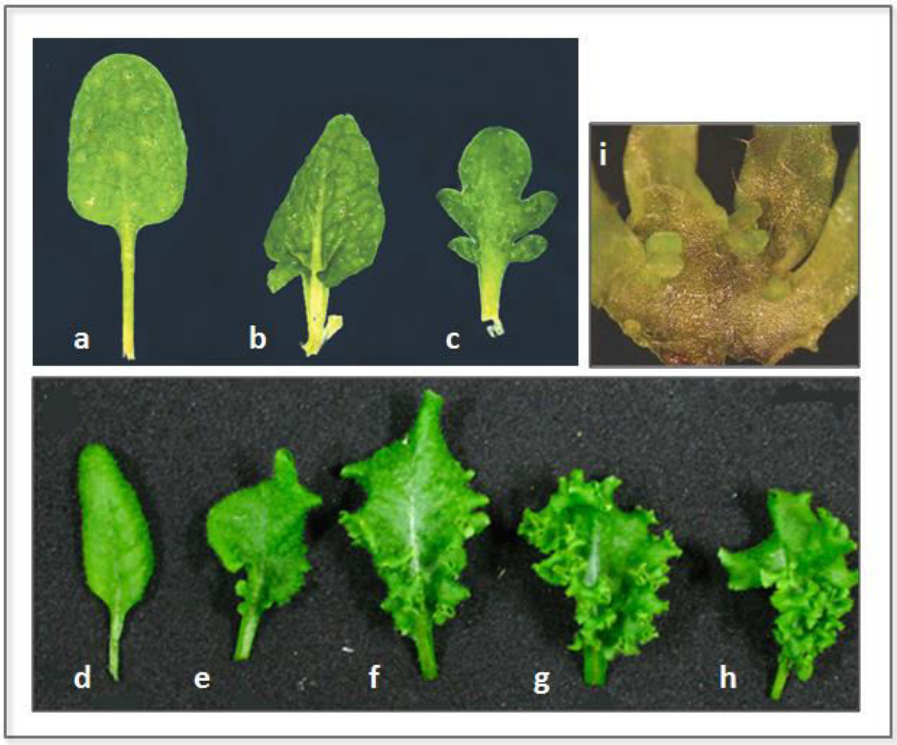

Figure 3.2. Leaf-phenotypes of *Arabidopsis thaliana* mutants defective in proper downregulation of class I KNOX gene expression in the leaf primordia. A representative leaf of each genotype is shown: WT (Ler)(a), *as1-1* (b), *35S::KNAT1* (c), WT (Col0) (d), *tcp3/4/10* (e), *tcp3/4/5/10* (f), *tcp3/4/5/10/13* (g), and *35S:miR319 tcp3/4/5/10/13* (h). Close-up of vegetative leaf of a severely lobed *35S::KNAT1* plant with ectopic shoot meristems in the sinus region (i). Extracted from Tsukaya, 2005 (a-c) (17), Koyama *et al*., 2010 (d-h) (11) and Chuck *et al*., 1996 (18).

The *Arabidopsis* genome encodes four class I *KNOX* (*KNOXI*) genes: *SHOOT MERISTEMLESS (STM), Kn1-like in Arabidopsis thaliana1 (KNAT1)* [also called *BREVIPEDICELLUS (BP]), KNAT2* and *KNAT6*. *STM* and *KNAT1* are the most extensively studied and more relevant for meristem function, since single mutations in *KNAT2* and *KNAT6* genes do not apparently affect shoot development (19, 20). In contrast, *stm-1* development is arrested at the seedling stage, showing fused cotyledons and consumed meristem (15). On the other hand, the architecture of the *KNAT1* mutant, named *bp-1*, is altered. They are plants with reduced height, irregularly shortened internodes, reduced apical dominance, and their pedicels are shorter and point downward. Cell wall defects are manifest in ectopic lignin accumulation in epidermis and cortex and in precocious lignifications in young plants, indicating a role of KNAT1 preventing

premature cell differentiation and promoting meristem maintenance (21) (Figure 3.2). It is important to remember that we will focus our attention on *KNAT1*, since it constitutes an indirect target of TCPs and can be relevant in our research (Figure 3.3).

In *Arabidopsis*, the expression of class-I *KNOX* genes is confined to meristem cells and excluded from the leaf founder cells in organ primordia (P_0); thus, they have been considered good markers for meristem versus lateral organ cell fate (22). The study of *KNOX* genes has allowed unraveling key aspects of the regulation of cell fate and tissue differentiation. These homeodomain transcription factors maintain the stem cells in the SAM in its un-differentiated state. Several reports support this affirmation. First, it has been demonstrated that KNOX activity is required to impede stem cells from adopting differentiated cell fates in maize and *Arabidopsis*, where mutants in *KNOX* genes *KNOTTED1 (KN1)* and *STM* fail to establish and maintain a SAM (23, 24). Second, in plants with simple leaves (*Arabidopsis*, maize and tobacco), *KNOX* genes are expressed exclusively in the meristem and their ectopic expression in differentiated tissues (such as leaves) alter drastically their development (25-27), producing differential growth at the leaf margin, creating new meristems in leaves and novel 'meristem-leaf' boundaries that lead to the formation of lobes (18, 25, 26) (Figure 3.2). Third, in plants with dissected leaves, such as tomato, *KNOX* are also expressed in leaf primordia, further supporting their role in the control of cell fate (differentiation or un-differentiation) on the leaf margins (15).

In this context, defining a boundary between *KNOX*-expressing and non expressing cells is crucial for a correct initiation of lateral organs and plant development (15). The establishment of this boundary requires the repression of *KNOX* genes outside the SAM and involves the coordination of the antagonistic actions of meristem determinants, such as *KNAT1*, and pro-differentiation determinants (*KNAT1* negative regulators) (Figure 3.3).

In this sense, several negative regulators of *KNOX* genes have been reported to confine KNOX activity in the SAM and to exclude it from organ primordia *(15)*. Among them, we will pay attention to *ASYMMETRIC LEAVES1 (AS1)*, whose expression is directly activated by TCPs, and therefore, interesting for our research.

AS1 is a MYB-type TF specifically expressed in the shoot lateral organs, that is, with a complementary expression pattern to that of the *KNOX* genes. Thus, AS1 promotes cell differentiation through the repression of *KNOX* expression (28). *AS1* mutation in *Arabidopsis* inhibits cell differentiation and induces ectopic expression of *KNAT1* in organ primordia, producing asymmetric leaves with lobed margins and occasional ectopic meristems (29-31), a phenotype that resembles the alterations in leaf patterning observed in transgenic plants over-expressing *KNAT1* (25). As for the mechanism of *KNAT1* repression, it has been reported that AS1 can bind the *KNAT1* promoter alone or interacting with the LOB domain protein AS2. Then, chromatin remodeling factors that make promoter regulatory sequences un-accessible are recruited: the histone deacetylase HDA6 (by AS1) and POLYCOMB-REPRESSIVE COMPLEX 2 (by AS1–AS2) (32, 33).

In this regulatory interplay between pro-meristematic (KNAT1) and pro-differentiation (AS1) factors in the SAM to establish the boundary of KNOX-expressing and non-expressing cells, CIN-TCPs play an important role:

Koyama *et al*., 2010 (11), demonstrated the mechanism by which CIN-TCPs promote cell differentiation in organ primordia in the SAM context: activating *AS1* expression, and therefore, indirectly repressing *KNOX* genes such *as KNAT1* (11) (Figure 3.3). A microarray experiment using a β-estradiol inducible TCP3SRDX line identified *AS1* as a putative direct target of CIN-TCPs. Further chromatin immuno-precipitation (ChIP) and transient expression assays demonstrated that CIN-TCPs are able to bind *AS1* promoter and activate its transcription (11). These results indicate that TCPs are located upstream of AS1 in the regulatory network controlling the boundary of *KNAT1*-expressing *vs* non-expressing cells; or, in other words the SAM *vs* the differentiating cells (Figure 3.3).

Several observations implicate CIN-TCPs in the regulation of the differentiation of cells in the SAM boundary and confirm its role promoting cellular differentiation. Plants with reduced expression of CIN-TCPs, such as *TCP* mutants (11), the *Arabidopsis jaw-d* mutant (which over-expresses miR319 that down-regulates five out of eight CIN-TCPs) and plants over-expressing *TCP-SRDX* in which all TCP target genes are repressed; exhibit a wavy-leaf phenotype, irregular vasculature, undifferentiated cells and ectopic formation of shoot

meristems in cotyledons; resembling the phenotype of *35S::KNAT1* (25, 34). And contrarily, over-expression of CIN-TCPs harboring mutations that prevent the binding of the miR319 (microRNA-resistant versions of TCPs) produces defects in the formation of a functional SAM and the fusion of cotyledons (34-36), also in tomato (37).

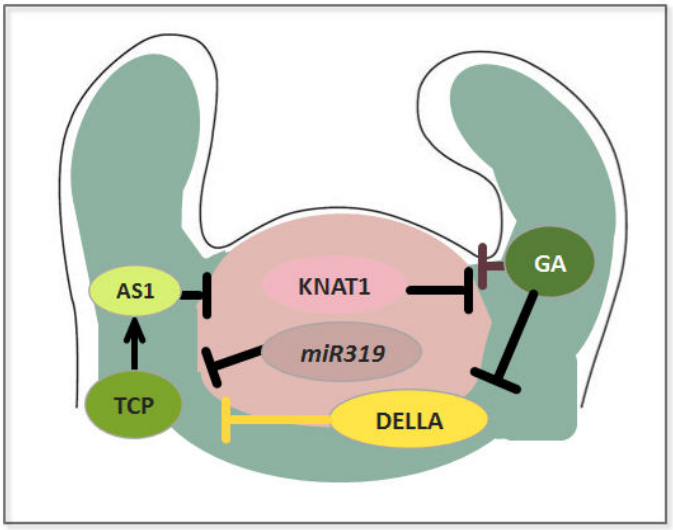

Figure 3.3. Regulatory network controlling the un-differentiate state of stem cells in the SAM and cell differentiation in organ primordia. The factors involved in the pathway are localized in their expression domains: pro-meristematic factors (in mauve, KNAT1 and miR319) in the SAM (mauve zone); and pro-differentiation factors (in green, CIN-TCPs, AS1 and GA) in organ primordia (blue zone). The working hypothesis is represented in yellow: DELLA-CIN-TCP physical interaction would inhibit TCP activity, therefore promoting *KNAT1* expression in the SAM.

It is important to take into account that the microRNA miR319 down-regulates five out of eight CIN-TCPs, as we have already mentioned. Interestingly, the precursor of this microRNA was more abundant in the SAM, and its expression decreased drastically in leaves (34). A complementary pattern of expression was found for the CIN-TCP *TCP4* by *in situ* hybridization assays. *TCP4* expression in the vegetative phase is restricted to leaf primordia, cotyledons and leaves with the highest levels near the tip, whereas TCP signal is excluded from the SAM. These domains of expression are consistent with the repressive effect of miR319 on *CIN-TCPs* in the SAM (34). This way, miR319 would impair the pro-differentiation effects of CIN-TCPs in the SAM, favoring meristem identity (Figure 3.3).

All in all, these reports show that there is an interplay between TCPs and AS1 promoting differentiation in organ primordia through *KNAT1* inhibition, and meristem maintenance by *KNAT1* and miR319. This regulatory network establishes the boundary between the *KNOX*-expressing and non-expressing cells (Figure 3.3). The implication of GAs on this pathway is presented in the following section.

3. The role of gibberelin in the vegetative shoot apical meristem

Several lines of evidence implicate plant hormones (including GAs) in the fine-tuning mechanism that determines cell fate and maintenance of stem cells. KNOX proteins favor meristem activity, in part by regulating the balance between both CK and GA activities. Specifically, low levels of GAs and high levels of the cell division promoter CKs seem to be necessary for meristem maintenance, while higher levels of GAs are associated to primordia outgrowth, mostly for cell expansion and differentiation (38-41). KNOX are known to guarantee this balance by repressing GA biosynthesis and inducing GA inactivation, while promoting CK synthesis in the SAM (42).

First, *KNOX* genes have been shown to repress the transcription of the GA biosynthetic genes *GA20ox* in several plant species (39-41, 43). Second, KNOX proteins activate the transcription of *GA2ox* genes, which encode GA catabolic enzymes, at the meristem-leaf primordia boundary (39, 44, 45). In this sense, it has been proposed that this localized *GA2ox* expression protects the stem cells in the SAM from the possible diffusion of GA from the adjacent organ primordia (40). Therefore, KNOX action is directed to exclude GAs from the SAM region (Figure 3.3), where the GA pro-differentiation effect could lead to detrimental impacts on SAM activity. In fact, genetic evidence supports this idea. The O-fucosyltransferase *SPINDLY* (*SPY*) is a negative regulator of GA responses, because mutation of this gene phenocopies plants treated with an overdose of bioactive GA and results in insensitivity to a GA inhibitor during seed germination (46, 47). Therefore, it is known that the *Arabidopsis* mutant *spindly* is characterized by constitutive activated GA signaling, and the combination of this mutant with weak *stm* alleles enhances its phenotypic defects (39).

And the third way by which KNOX regulates the CK/GA balance is by activating CK biosynthesis, as *KNOX*-overexpressing plants have elevated levels of CK (41) due to higher expression of CK biosynthetic gene *ISOPENTENYL TRANSFERASE7 (IPT7)* (40, 41). In this line, over-expression of CK biosynthetic IPT genes and exogenous application of CK can partially rescue the meristem defects of *stm* mutants (41), confirming that CK is required in the SAM for its correct activity (38).

This GA/CK balance reflects the complex way in which hormones are integrated in the regulatory network controlling the SAM that may serve to buffer the apex against hormone perturbations caused by the normal course of development or as a response to environmental changes (38). In fact, when this CK/GA balance is reversed, for instance combining *spindly* with an over-expressor of the CK catabolic enzyme CKX3, the SAM function is abolished (40).

To sum up, in the SAM, high CK/low GA balance promotes indeterminate growth, whereas in organ primordia (P0) high GA and low CK concentrations promote lateral organ initiation. As far as GAs are concerned, they are excluded from the SAM by KNOX and located in organ primordia (Figure 3.3) (in fact the biosynthetic gene *GA20ox1* is expressed there (39)), where they promote differentiation and cell elongation via reorienting microtubules (48). In fact, the lack of GAs in the SAM could be involved in maintaining the isodiametric shape that characterizes meristem cells (39).

4. Proposal of the hypothesis: DELLAs function in the SAM

The aim of this part of the Thesis (Chapter I) is to unravel the role of DELLAs regulating the vegetative SAM. As it has already been presented, in the meristem there is a balance between factors that promote a meristematic state in the central zone and factors that favor cell differentiation in external regions (primordia). Importantly, *KNAT1* expression is a distinctive trait in meristematic cells, and the establishment of the boundary between *KNAT1*-expressing and non-expressing cells defines cell fate and it is relevant for plant development. Currently, the mechanisms that determine the *KNAT1* expression domain remain to be understood.

Here, we challenge the hypothesis that DELLAs participate in the maintenance of the SAM by defining the *KNAT1* expression boundary at the transcriptional level, given that GAs do not accumulate in the SAM and that DELLAs interact with upstream regulators of *KNAT1* (the CIN-TCPs module).

5. References

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4. Results and Discussion of Chapter I: DELLAs regulate the shoot apical meristem through the activity of CIN-TCPs and KNAT1

1. GAI and RGA interact with CIN-TCP family members

Previous work using a yeast two hybrid (Y2H) screen with the GRAS domain of GAI as bait (M5GAI) against an arrayed library of approximately 1,200 TFs from *Arabidopsis* had identified 12 members of the TCP family as interactors (1). As stated above, 6 of these members are included in the class I TCPs (for example TCP14 and 15, Chapter II) and other 6 in the class II. Among them, 5 belong to the CIN subclass (TCP2, TCP3, TCP5, TCP10 and TCP13).

Given that out of the five *DELLA* genes in *Arabidopsis* (2), *GAI* and *RGA* are the two most strongly expressed in the shoot apex (3), our first aim was to further characterize the interaction of CIN-TCPs with these two DELLA proteins in particular. We carried out Y2H assays with the GRAS domain from GAI and we found that this DELLA protein was able to interact with all the CIN-TCP family members tested, as expected (1) (Figure 4.1A). Taking TCP2 and TCP4 as a model, we found that they are able to interact with the GRAS domain of RGA (called RGA52) as well (Figure 4.1B). According to these results it seems reasonable to think that RGA is also able to interact with the other members of the CIN-TCP subclass.

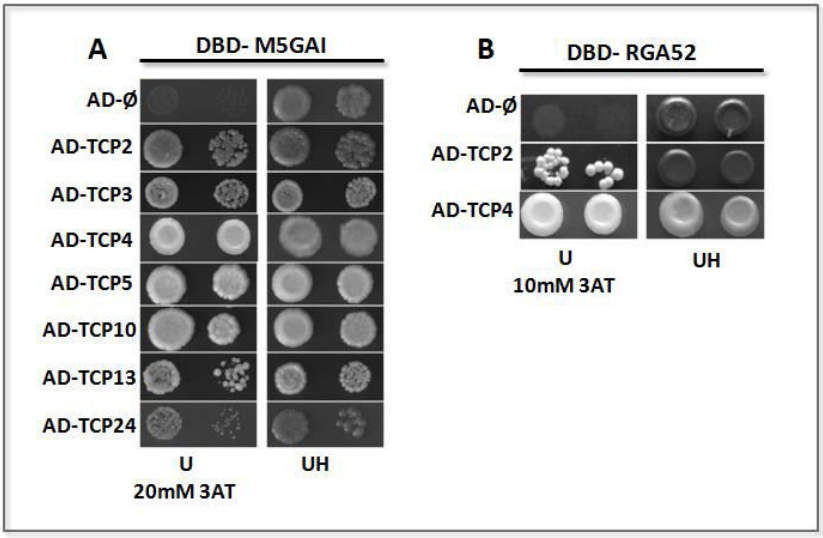

Figure 4.1. DELLA proteins interact with CIN-TCP family members. Y2H assay of the interaction between **(A)** a truncated version of GAI (M5GAI) and seven members of the CIN-TCP family; and **(B)** a truncated version of RGA (RGA52) and TCP2 and TCP4. (DBD, DNA binding domain; AD, activation domain; AD-ϕ, activation domain empty vector; U, uracil; H, histidine; 3-AT, 3-aminotriazol).

The identification of protein regions involved in a particular interaction might provide useful information regarding its effects on the activity of the TF. For instance, this sort of analysis showed that the interacting surface between PIFs and DELLAs includes the bHLH domain, providing this way the first hints pointing that the interaction would have a negative effect on the activity of the TF (4, 5). To determine the domains involved in the interaction between DELLAs and CIN-TCPs, we generated truncated versions of both TCP2 and GAI. We split TCP2 in roughly two halves, with the N-terminal one including the TCP domain (Figure 4.2A). This domain is a 59 amino acid non-canonical basic helix-loop–helix (bHLH) motif responsible of DNA binding and protein–protein interactions (6). In fact, this motif is very similar to the bHLH of PIF3 and PIF4 (4, 5). However, none of the deleted versions were able to interact with M5GAI. These results indicate either that both parts of the protein are needed to support the interaction in the native TCP protein, or that none of the truncated versions are expressed or folded correctly.

On the other hand, we tested the ability of TCP2 to interact with different regions of M5GAI, and remarkably all of them were able to interact, although the interaction seems weaker than with M5GAI (Figure 4.2.B). Similar results were obtained when we assayed TCP4 with the same deletions of M5GAI (Figure 4.2C). The interaction involves different regions of the GRAS domain, and none of them seems to be essential. This behaviour is not typical among the DELLA interactors, for which certain specificities in the domains involved in the interaction have been described. For instance, the LHR1 motif is essential for the interaction of GAI with BZR1, PIF4, and JAZ1 (4, 5, 7-11). However, ARR1 interacts with GAI even when the LHR1 motif is not present, but further deletion of the VHIID motif abolishes the union (12).

Next, we confirmed that the interaction also occurs *in planta*. For that purpose we decided to use two complementary techniques: Bimolecular Fluorescence Complementation (BiFC) and co-immunoprecipitation (co-IP). Both assays were performed in *Nicotiana benthamiana* leaves. As shown in the BiFC assay in Figure 4.3A, the fluorescence of the reconstituted YFP can be seen in the nuclei of epidermal cells co-expressing YFC-GAI and YFN-TCP4 and YFN-TCP2, while in the control it remains undetected. We also demonstrated the DELLA-CIN-TCP interaction by co-IP (Figure 4.3B). The c-myc-M5GAI protein was effectively

pulled down by anti-HA antibody coated beads only from extracts of leaves co-expressing HA-TCP2, demonstrating the interaction between both proteins.

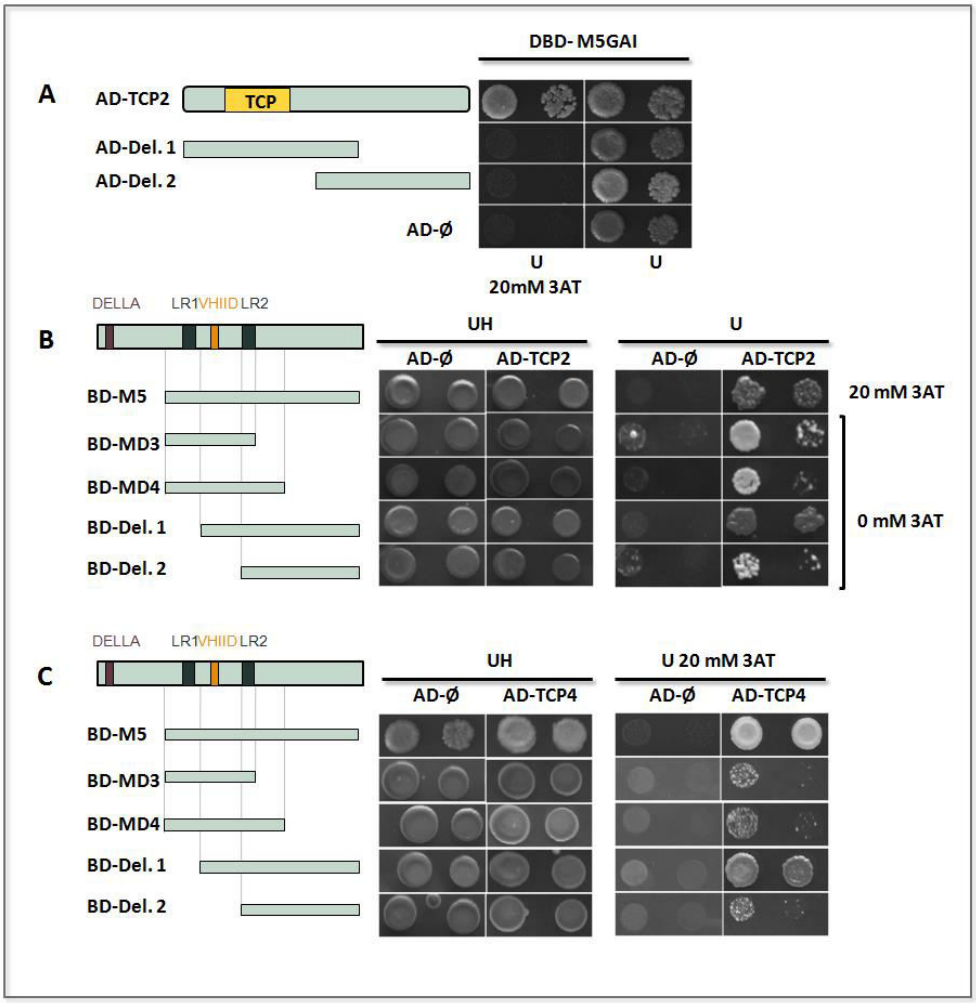

Figure 4.2. Domains involved in CIN-TCP-DELLA interaction. Y2H assay of the interaction between **(A)** M5GAI and truncated versions of TCP2, **(B)** TCP2 and truncated versions of GAI and **(C)** TCP4 and truncated versions of GAI. The different domains are indicated in colors. (DBD, DNA binding domain; AD, activation domain; AD-ϕ, activation domain empty vector; U, uracil; H, histidine; 3-AT, 3-aminotriazol).

All in all, those findings indicate that the DELLA-CIN-TCP interaction takes place *in planta* and that DELLAs can potentially regulate the activity of these TFs post-translationally. Moreover, the fact that there are 8 members of the CIN-TCP family and 5 different DELLAs suggests the possibility of an extensive and

combinatorial module of regulation involving different pairs of proteins that could interact depending on their expression patterns, the physiological contexts and/or environmental conditions, for instance.

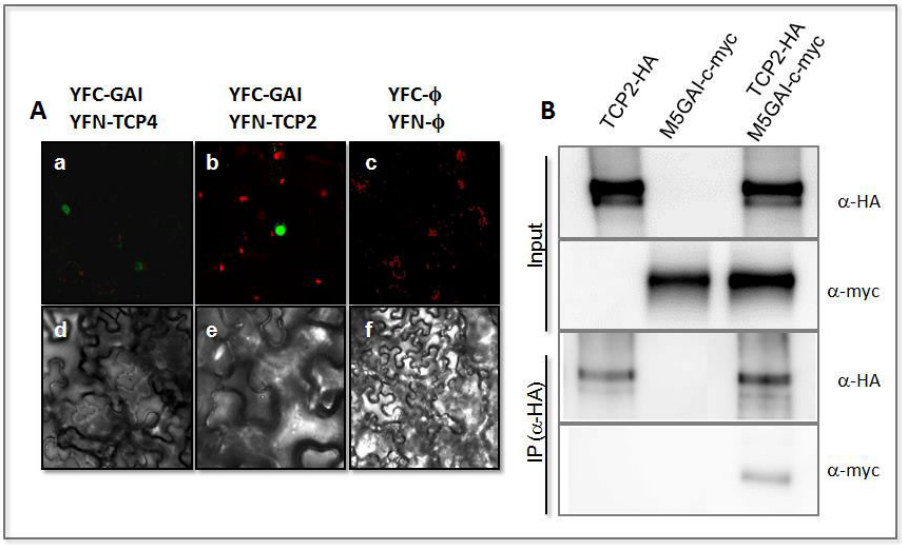

Figure 4.3. CIN-TCPs and DELLA proteins interact *in planta*. (A) Bimolecular fluorescence complementation analysis showing the interaction of TCP4 (a,d) and TCP2 (b,e) proteins with GAI in *nuclei* of *N. benthamiana* leaf cells. Empty vectors of each construction were used as a negative control (c, f). The lower panel shows the bright field of each picture. **(B)** Co-IP of M5GAI-myc and TCP2-HA expressed in *N. benthamiana leaves*. TCP2 was immunoprecipitated using anti-HA (α-HA) conjugated paramagnetic beads, and TCP2 and M5GAI were detected in immunoblots using anti-HA and anti-myc, respectively. Note that M5GAI was co-immunoprecipitated with TCP2. The sizes of the bands correspond to the expected sizes of the fusion proteins.

2. DELLAs do not regulate CIN-TCPs transcription

Besides the role of DELLAs regulating CIN-TCPs by physical protein-protein interaction, we wanted to check whether they are also modulating CIN-TCPs at the transcriptional level. To this end, we tried two different approaches, one chemical and one genetic.

2.1 Chemical approach

Treatments with GAs and with their biosynthesis inhibitor paclobutrazol (PAC) are widely used to decrease and increase, respectively, the level of DELLA

proteins in plants (see General Introduction). For this reason, we first treated wild type plants with PAC for 18 and 24 hours, and analyzed *TCP2* expression. As it can be seen in Figure 4.4, *TCP2* mRNA level does not significantly change in plants treated with PAC respect to the control.

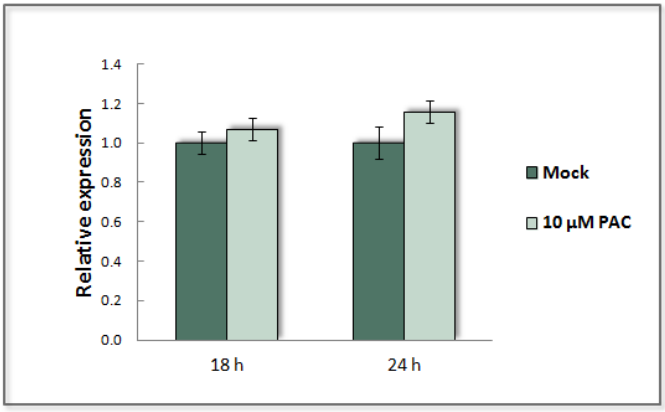

Figure 4.4. Chemical treatments with PAC do not affect *TCP2* mRNA level in wild type plants. RT-qPCR experiment indicating *TCP2* mRNA level in seven-day-old wild-type (*Ler*) seedlings treated with 10 µM PAC for 18 and 24 hours (light green bars) or mock-treated (dark green bars).

We further corroborated those findings in two types of genetic resources that we prepared as part of this work. On the one side, we used the transgenic lines *pTCP2::TCP2-GFP* and *pTCP4::TCP4-GFP* that express a translational fusion of the TCP protein with the GFP under the control of the their endogenous promoters (see Material and Methods). On the other side, we used miR319-resistant versions of the TCPs (rTCP) uncoupling then their expression from the down regulation imposed by the microRNA, *pTCP2::rTCP2-GFP* and *pTCP4::rTCP4-GFP* (see Material and Methods). Therefore, we evaluated *TCP2* (Figure 4.5B) and *TCP4* (Figure 4.5C) expression in these transgenic lines in response to GA and PAC treatments. We treated 7 day old seedlings of each genotype with 10 µM PAC for 18 hours, and then half of them were treated with 100 µM GA_3 for 5 hours whereas the other half remained in PAC for 5 hours (Figure 4.5A). None of the transgenic lines presented significant changes in one condition respect to the other, suggesting that, as in the wild type (Figure 4.4), DELLAs do not regulate *TCP2* and *TCP4* transcriptionally.

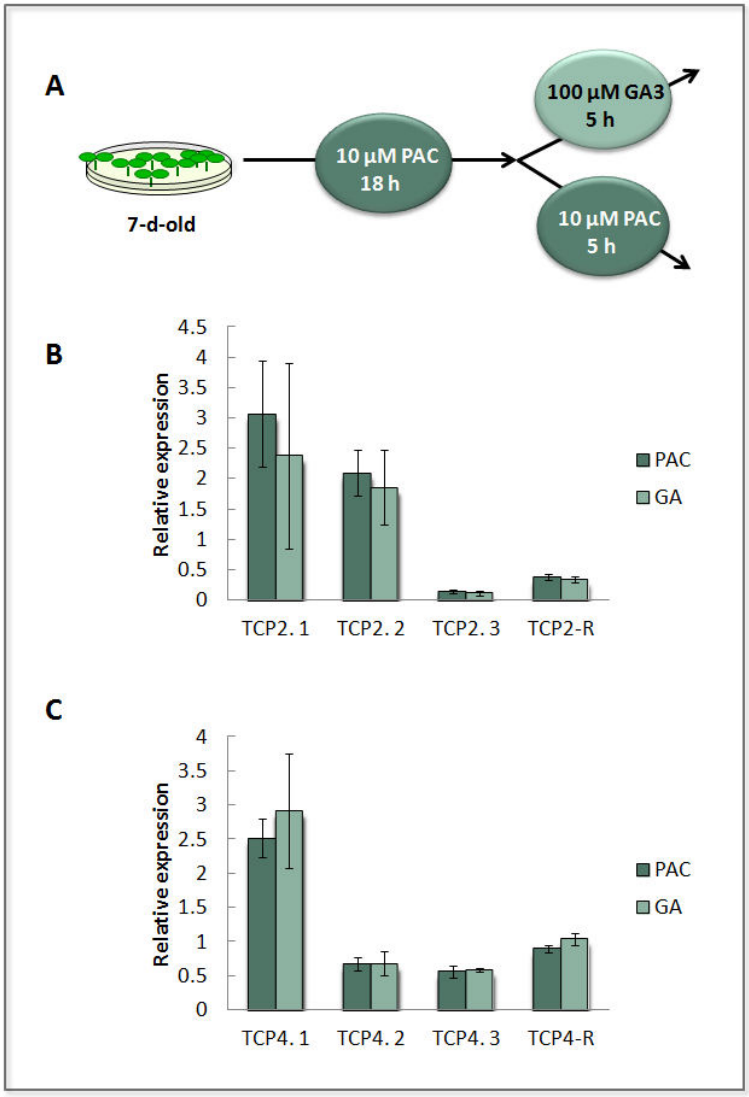

Figure 4.5. Chemical treatments with PAC and GA do not affect CIN-TCP mRNA level in transgenic lines. **(A)** Scheme of the experimental setting. Seven-day-old seedlings of each genotype are treated with 10 µM PAC for 18 hours, then half of them are treated with 100 µM GA_3 for 5 hours whereas the other half remain in PAC. Samples are collected and RT-qPCR analysis is performed. A transgenic line carrying the empty vector was used as a control of the experiment. **(B)** mRNA level of *TCP2* in three homozygous lines of the *pTCP2::TCP2-GFP* (TCP2.1, TCP2.2 and TCP2.3) and the microRNA-resistant *pTCP2::rTCP2-GFP* (*TCP2-R*) after PAC (dark green bars) or GA (light green bars) treatment. **(C)** *TCP4* expression level in three different *pTCP4::TCP4-GFP* (TCP4.1, TCP4.2 and TCP4.3) and *pTCP4::rTCP4-GFP* (TCP4-R) lines following the same treatments in (B).

2.2 Genetic approach

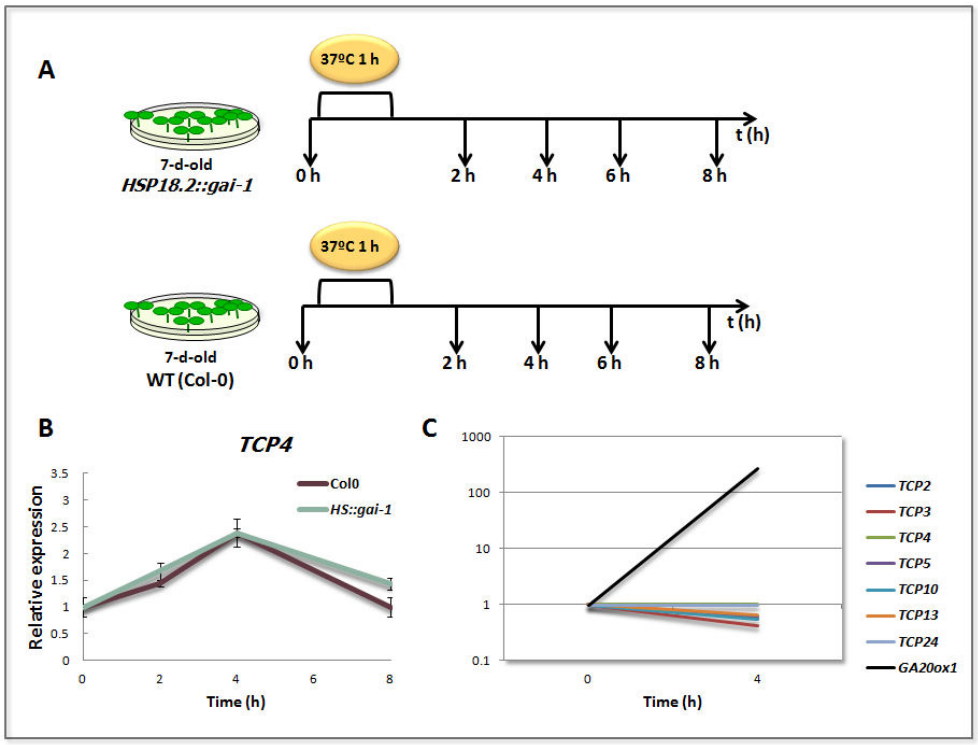

Figure 4.6. DELLA induction does not affect *CIN-TCPs* mRNA level. (A) Diagram of the experimental setting. In the transgenic line (*HSP18.2::gai-1*), the dominant version of the DELLA protein GAI (*gai-1*) is induced by a heat shock (1 hour at 37ºC). Samples were collected at several time points before and after the heat shock and RT-qPCR analysis was performed. Wild-type (Col-0) plants followed the same procedure as a control. Seven-day-old seedlings of each genotype were used. **(B)** mRNA level of TCP4 in the control (purple) and the transgenic line (*HS::gai-1*, green) within all the time-course. **(C)** Fold change of the mRNA level of several CIN-TCPs 4 hours after *gai-1* induction. Data was normalized first to time cero and then to the control line for each time point. *GA20ox1* gene is used as a positive control.

To further confirm our previous results, we used a genetic approach. To this end, we used the *HS::gai-1* transgenic line that expresses the dominant gain-of-function version of GAI (*gai-1*) under the control of a temperature inducible promoter (13). We subjected 7-day-old seedlings of control and transgenic lines to 37ºC for one hour to induce *gai-1* expression. This line has been demonstrated to accumulate *gai-1* quickly after the treatment (14), so we decided to test the expression of *TCP4* before the heat shock and shortly after it (Figure 4.6A and B). We did not observe differences between the transgenic line and the control

(Figure 4.6B). Next, we evaluated the expression of several CIN-TCPs at the 4 h time point, using the expression of the *GA20ox1*, a *bona fide* GAI early target (see General Introduction), as a control (Figure 4.6C). As it can be seen in the graph, the expression of none of the CIN-TCPs was severely altered upon gai-1 induction, whereas expression of *GA20ox1* clearly increased validating the approach.

All in all, these experiments demonstrate that CIN-TCP expression is not altered in response to changes of DELLA levels, indicating that DELLAs regulate CIN-TCP activity post-translationally through the physical interaction of the proteins. These results are in line with a whole body of investigation starting last decade, showing that DELLAs regulate TFs and other transcriptional regulators by interacting with them (15).

3. DELLAs inactivate CIN-TCPs upon interaction

DELLAs regulate its TF targets away of the chromatin by a sequestration mechanism or in the context of the target genes (15). Upon interaction, DELLAs can interfere with the DNA-binding capacity of their targets, and so, inhibit their activity by a sequestration mechanism. This is the case for example of the bHLH transcription factors PIFs (4, 5). On the other hand, DELLAs can operate in the chromatin as transactivation factors, which is the case of the interaction with ARR1 (12), or can be co-repressors, as described for the interaction with SPL15 (16).To test the effect of DELLA on TCP-mediated regulation of gene expression, we performed transactivation assays in *Nicotiana benthamiana* leaves.

For this purpose, we designed a synthetic promoter with 6 repeated copies of the Class II TCP consensus binding site (GTGGTCCCA) (17) with a 6-nucleotide spacer (AAAAAA). We cloned the synthetic promoter in a vector under the control of a minimal *Cauliflower mosaic 35S* promoter and the translational enhancer omega to control the expression of the *LUCIFERASE* (*LUC*) reporter gene (Figure 4.7A). Then, we co-expressed the synthetic promoter with a translational fusion of TCP4 with the transcriptional activator VP16, taking the basal expression level of the promoter as a control (Figure 4.7B.1). As it can be seen in the graph, VP16-TCP4 transcriptional activity caused an increase in the LUC activity respect to the control. Notably, this activation of LUC was partially reversed when GAI was co-expressed, whereas GAI alone did not alter the activity of the reporter. To test whether these results can be extended to other CIN-TCPs, we performed the

same experiment with VP16-TCP2, with similar results (Figure 4.7C.1). As TCP4 and TCP2 proteins were tagged with HA and the GAI with myc, we could perform a western blot of the same samples. The protein levels of TCPs and GAI were similar in all conditions, ruling out the possibility that changes in LUC activity were due to differences in the expression of the effectors rather than in their activity (Figure 4.7B.2, 4.7C.2).

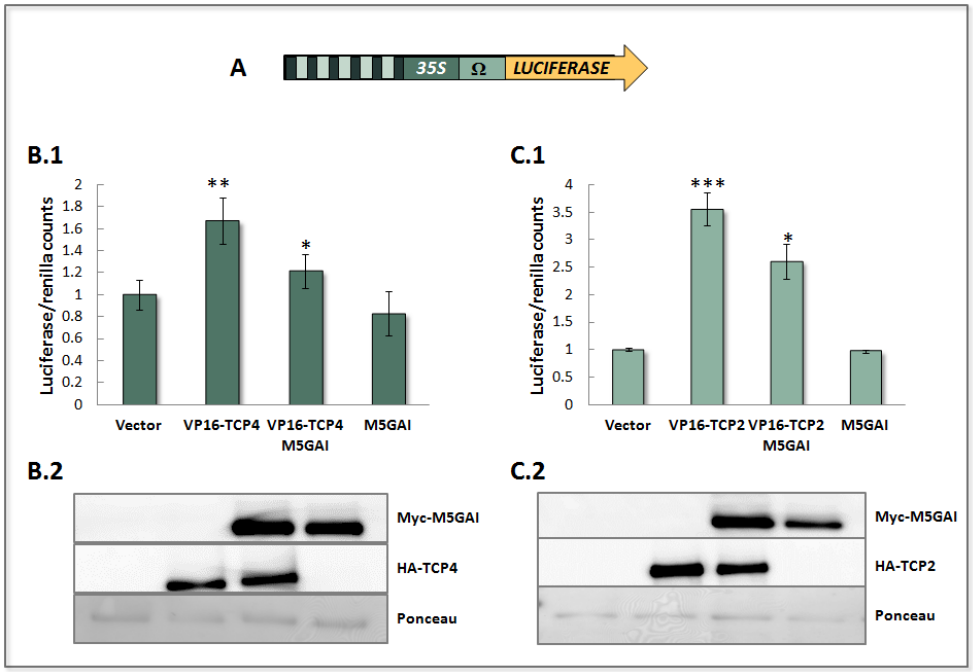

Figure 4.7. GAI inactivates CIN-TCPs upon interaction. (A) Design of the synthetic promoter used for the transient expression assays in *N. benthamiana* leaves. It consist of six repeated copies of the CIN-TCP consensus binding site (dark green boxes) separated by a 6-nucleotide spacer (AAAAAA, light green boxes) placed it upstream of a minimal 35S promoter (dark green box) and the translational enhancer *omega* (medium green box) to control the expression of the *Luciferase* reporter gene (orange arrow). *Renilla Luciferase* under 35S promoter in the same construct was used as control. **(B.1, C.1)** Transient expression assays for TCP4 (B.1) and TCP2 (C.1). (Vector, infiltration with the synthetic promoter alone used as a negative control; VP16, transcriptional activator). **(B.2, C.2)** Western blots corresponding to the transient expression assays for TCP4 (B.2) and TCP2 (C.2). TCPs and M5GAI were detected in immunoblots using anti-HA and anti-myc, respectively. Ponceau was used as a load control. Statistic differences are shown (P-value<0.05).

Altogether, these results indicate that DELLAs inhibit CIN-TCP transcriptional activity upon interaction, probably by preventing the binding to their target

promoters, in a similar way that they do with PIFs and other TFs (4, 5, 15). Furthermore, these observations are in line with the studies made with Class I TCPs that are described in Chapter II. What is more, recent work in the laboratory of Patrick Achard demonstrated that DELLAs impair TCPs capacity to bind their target promoters and so, their transcriptional activity (3).

4. Effect of the interaction in a known target of TCPs in the shoot apical meristem context

Once we had demonstrated the negative effect of DELLA-CIN-TCP interaction on CIN-TCP transcriptional activity, we investigated whether this regulation occurs in the SAM context. CIN-TCPs are expressed in the primordia (18), where they bind to *AS1* promoter and activate its transcription (19). AS1, in turn, represses *KNAT1* expression (20-22). In fact, CIN-TCP level in the meristem remain low by the action of miR319 that down-regulates 5 out of 8 CIN-TCPs (18). This way, we have TCPs and AS1 promoting differentiation in organ primordia through *KNAT1* inhibition and meristem maintenance by KNAT1 and miR319 (Figure 4.8).

In this context, we wanted to evaluate the effect of the DELLA-CIN-TCP interaction in a known target of TCPs in the SAM context, *AS1*, i.e. whether DELLA-imposed CIN-TCP inhibition reduces *AS1* expression. It is important to take into account that induction of *gai-1* expression in etiolated *HS::gai-1* seedlings caused a decrease in *AS1* expression (14), which is in line with our hypothesis. Then, we evaluated whether *AS1* expression was also altered upon *gai-1* accumulation in our experimental conditions. To do so, we took advantage of our previous experiment with the *HS::gai-1* line (section 2.2 of the Results of this Chapter). As it can be observed in Figure 4.9A, *AS1* mRNA level continuously decreased in the transgenic line after the heat treatment.

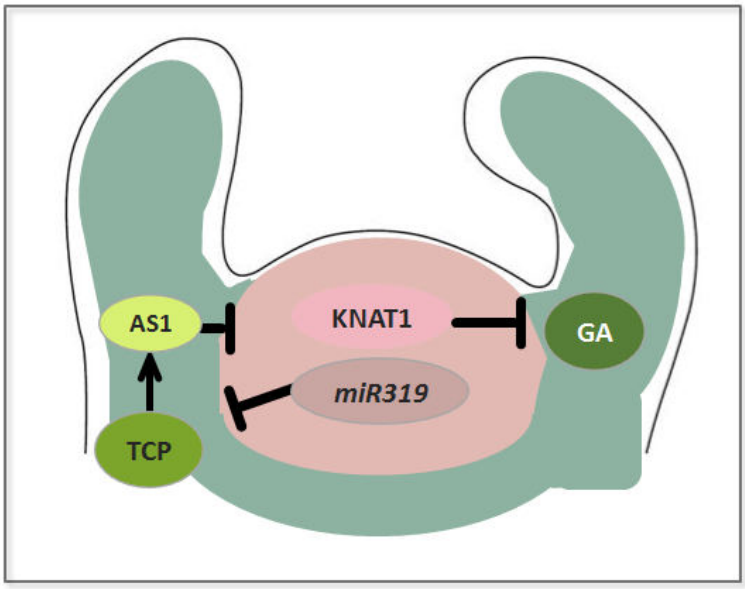

Figure 4.8. Interplay of pro-meristematic (mauve) **and pro-differentiation** (green) **regulators in the transition from the in-determinate SAM to differentiated lateral organs.**

To additionally confirm that the effect of DELLA-CIN-TCP interaction on *AS1* expression is indeed taking place within the SAM context, we used another strategy to test our hypothesis, we evaluated *AS1* expression pattern using the *pAS1::GUS* transgenic line (23). First, we treated 8 day old seedlings of the *pAS1::GUS* line with 10 µM PAC for 18 hours and then half of the samples followed a 100 µM GA_3 treatment whereas the other half remained in PAC for 5 hours. Seedlings subjected to mock treatment were used as a control (Figure 4.9B, a, d and g). A significant decrease in *AS1* expression can be observed after the PAC treatment, and this effect is reversed when plants were treated with GA. In fact, the expression domain of *AS1* after the GA treatment seems to be expanded compared to the mock.

Next, we tested our hypothesis genetically. We analyzed *AS1* expression in *pAS1::GUS p35S::GAI* and in *pAS1::GUS pTCP2::rTCP2-GFP* transgenic lines in response to the same treatments. In seedlings of the *pAS1::GUS p35S::GAI* transgenic line (Figure 4.9B, b, e and h), a decrease in the staining could be noticed even in the mock condition and hyper sensibility to the PAC treatment was observed. This effect was reverted by the GA treatment, although to a lesser

extent than in the wild type background line. In contrast, *AS1* expression in mock condition was increased in *pAS1::GUS pTCP2::rTCP2*-GFP, a transgenic line expressing a microRNA-resistant version of TCP2. Remarkably, this line was more resistant to the PAC treatment and GAs further expanded the *AS1* expression domain (Figure 4.9B, c, f and i).

Although it is possible that other mechanisms can be involved, these results are in agreement with our hypothesis, which is that DELLAs regulate *AS1* expression through the inhibition of CIN-TCPs.

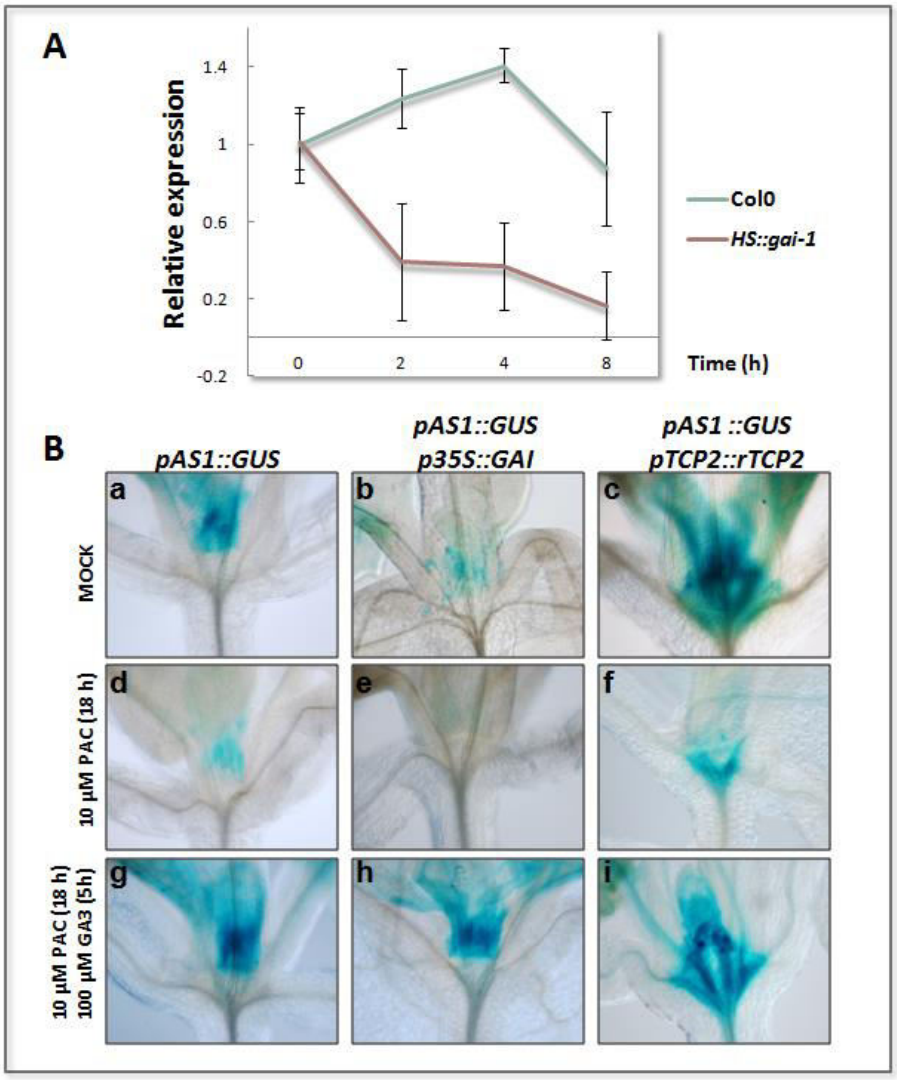

Figure 4.9. DELLAs regulate *AS1* expression through the inhibition of CIN-TCPs. (A) *AS1* mRNA level continuously decreases in the transgenic line (*HS::gai-1*, purple line) when the dominant version of the DELLA protein GAI (*gai-1*) is induced by a heat shock (for a diagram of the experimental setting see Figure 4.6A). Wild-type (Col-0) plants followed the same procedure as a control (light green line). Seven-day-old seedlings of each genotype were used. **(B)** The expression pattern of *AS1* depends on the activity of DELLA proteins. The transgenic lines *pAS1::GUS* (a,d,g); *pAS1::GUS p35S::GAI* (b, e, h) and *pAS1::GUS pTCP2::rTCP2* (c, f, i) are used to carry out GUS staining assays. *AS1* expression pattern is evaluated in three different conditions: mock (a, b, c); plants treated with PAC for 18 h (d, e, f) and plants in PAC for 18 h followed by a GA treatment for 5 h (g, h, i). Seven to ten-day-old seedlings of each genotype were used in both experiments. Images are representative of four independent biological repeats including 20 seedlings per genotype.

5. Effect of the interaction in a downstream target: a KNOX gene

According to previous results, the inhibition that DELLAs exert on CIN-TCP activity upon interaction seems to have a regulatory role in the SAM given the known role that CIN-TCPs and AS1 exert in this tissue. The fact that DELLAs impair the function of these pro-differentiation factors suggests that DELLAs would act as pro-meristematic factors in this scenario. This idea is line with the current knowledge that GAs promote cell differentiation in organ primordia and that they are excluded from the SAM (see Introduction of Chapter I). Therefore, we investigated whether the DELLA-CIN-TCP module could be affecting the activity of the pro-meristematic *KNOX* genes. Since *KNAT1* is a known target of AS1, we evaluated whether *AS1* regulation by DELLAs has consequences in *KNAT1* expression. We used the *pKNAT1::GUS* transgenic line (24) to check whether *KNAT1* expression depends on the level of DELLA proteins. To do so, two different approaches were taken. On the one hand, we tested our hypothesis genetically. The *pKNAT1::GUS p35S::GAI* transgenic line was generated, and GUS staining assays in these seedlings as well as in the *pKNAT1::GUS* line were carried out (Figure 4.10, a-f). On the other hand, *pKNAT1::GUS* seedlings were subjected to chemical treatments: plants were treated with PAC to induce DELLA accumulation and with a PAC plus GAs to reduce DELLA levels. Seedlings in mock were used as a control of the normal *KNAT1* expression domain (Figure 4.10, d-l). Interestingly, *KNAT1* was over-expressed and its expression domain seemed to be expanded in response to high DELLA level (*pKNAT1::GUS p35S::GAI* and plants treated with PAC), whereas *KNAT1* expression decreased when DELLA levels were low (GA treatment), which is in agreement with our hypothesis.

According to these results, it is reasonable to think that DELLAs might regulate *KNAT1* expression through the inhibition of CIN-TCPs (and consequently *AS1*) within the SAM context. Therefore, DELLAs would act favoring meristem maintenance through the promotion of *KNAT1* expression.

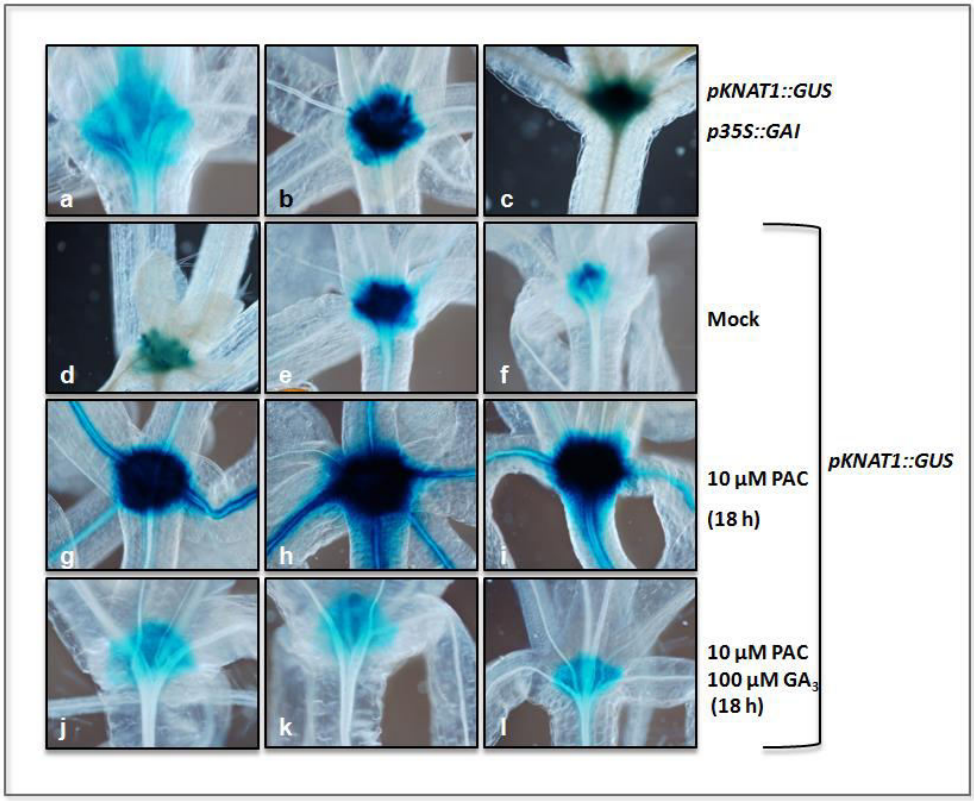

Figure 4.10. DELLAs regulate *KNAT1* expression. The expression pattern of *KNAT1* depends on the activity of DELLA proteins. The transgenic lines *pKNAT1::GUS* (d-l) and *pKNAT1::GUS p35S::GAI* (a-c) are used to carry out GUS staining assays in seven to ten-day-old seedlings. *KNAT1* expression pattern is evaluated in mock (a-f), 10 µM PAC (g-i) and 10 µM PAC plus 100 µM GA_3 for 18 hours. Three seedlings per genotype or treatment are shown. Images are representative of three independent biological repeats including 15 seedlings per genotype.

6. DELLAs *and* KNAT1 *expression patterns*

Our results indicate that DELLAs promote *KNAT1* expression, in agreement with the inhibition by DELLAs of negative regulators of *KNAT1*. However, for this effect to be biologically relevant, it needs to be placed in the context of the spatial accumulation of the corresponding proteins. For that reason, our next aim was to unravel the specific location of these transcripts and proteins in the vegetative SAM.

Our first step was to assess *GAI*, *RGA* and *KNAT1* expression patterns. To do so, we used GUS reporter lines lines *pKNAT1::GUS* (24), *pGAI::GUS* and *pRGA::GUS*

(25); we carried out GUS staining assays in 7 to 10 day old seedlings of the three genotypes (Figure 4.11). As it can be seen in the figure, *GAI* and *RGA* are expressed in the meristematic zone, cotyledons and primary leaves, and also in the root vasculature and especially near the root tip. However, *KNAT1* expression pattern seem to be restricted to the shoot apex meristematic zone. Focusing on this zone (Figure 4.11B), it can be seen that the overlapping zone is located in the vegetative meristem. Besides, the observed expression pattern of *KNAT1* is in agreement with the literature, that establishes that in species with simple leaves, such as *Arabidopsis*, Class I *KNOX* gene expression, such as that of *KNAT1*, is only found in the shoot meristem and subtending stem and is not found during any stage of leaf development. *KNAT1* expression disappears in the meristem cells that are destined to be the next leaf (26).

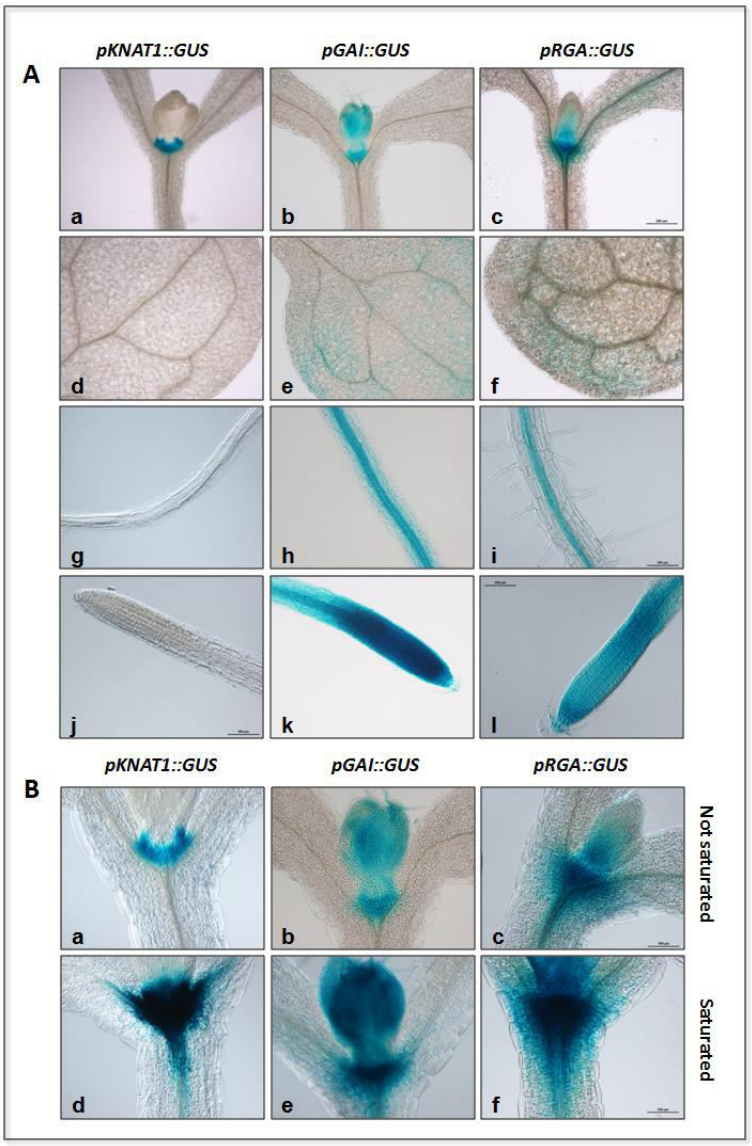

Figure 4.11. *DELLAs* and *KNAT1* expression patterns overlap. The expression domains of *KNAT1* (A. a, d, g, j; B. a, d), *GAI* (A. b, e, h, k; B. b, e) and *RGA* (A. c, f, i, l; B. c, f) were evaluated by GUS staining assays in seven to ten-day-old seedlings. **(A)** Meristem zone (a, b, c); cotyledon (d, e, f); middle part of the root (g, h, i) and root tip (j, k, l) are shown. **(B)** Zoom on the vegetative meristem in not saturated (a, b, c) and saturated (d, e, f) conditions. Images are representative of three independent biological repeats including 15 seedlings per genotype.

7. Localization of KNAT1 and RGA proteins in the vegetative shoot apical meristem

As described previously, there is a complex regulation between the pro-meristematic and pro-differentiation factors in the apical meristem (see Introduction of Chapter I). Stem cells in the central zone of the SAM undergo cell division and their daughter cells are displaced towards the periphery where they can start the differentiation process. In this situation, the boundaries between the cells expressing or not expressing *KNOX* genes become very important for plant development. Once we have established that *DELLAs* and *KNAT1* expression patterns overlap in the meristematic region, our next goal was to precisely localize the proteins in the SAM. Localizing DELLA and KNAT1 proteins in this region would provide us with important information about their possible function in this regulatory network.

To this end, we generated *pRGA::RGA-GFP pKNAT1::KNAT1-CFP* transgenic lines and analyzed the localization of both proteins in the vegetative SAM. For confocal microscopy observations, we established collaboration with Dr. Antonio Serrano-Mislata and Prof. Robert Sablowski in the John Innes Centre in Norwich, UK. As it can be seen in Figure 4.12, RGA (green) and KNAT1 (blue) indeed co-localized in the SAM but not uniformly. Thus, *pRGA::RGA-GFP* signal was clearly weaker in the central region, becoming stronger towards the periphery, while *pKNAT1::KNAT1-CFP* fluorescence accumulated preferably in the central zone of the shoot apical meristem.

These results point out the idea that DELLA proteins would primarily perform a role in the boundary regions of the SAM, where the DELLA-CIN-TCP interaction could take place. For instance, *in situ* hybridization experiments demonstrated that *TCP4* is mainly expressed in leaf primordia of *Arabidopsis* seedlings (18), suggesting that this boundary region could be where *DELLA* and *CIN-TCP* expression patterns mostly overlap. The activity of KNAT1 preventing GA accumulation in the boundary region will facilitate the presence of DELLAs. Then, in that frontier, DELLAs would inhibit the CIN-TCP/AS1 module, therefore promoting *KNAT1* expression in a region where its negative regulators start to be present and probably establishing the boundary zone from which *KNAT1* expression gets progressively stronger towards the center of the meristem. DELLAs, therefore, may help establishing the boundary between the meristematic

and the differentiation zone in the SAM guaranteeing the proper spatial *KNAT1* expression pattern.

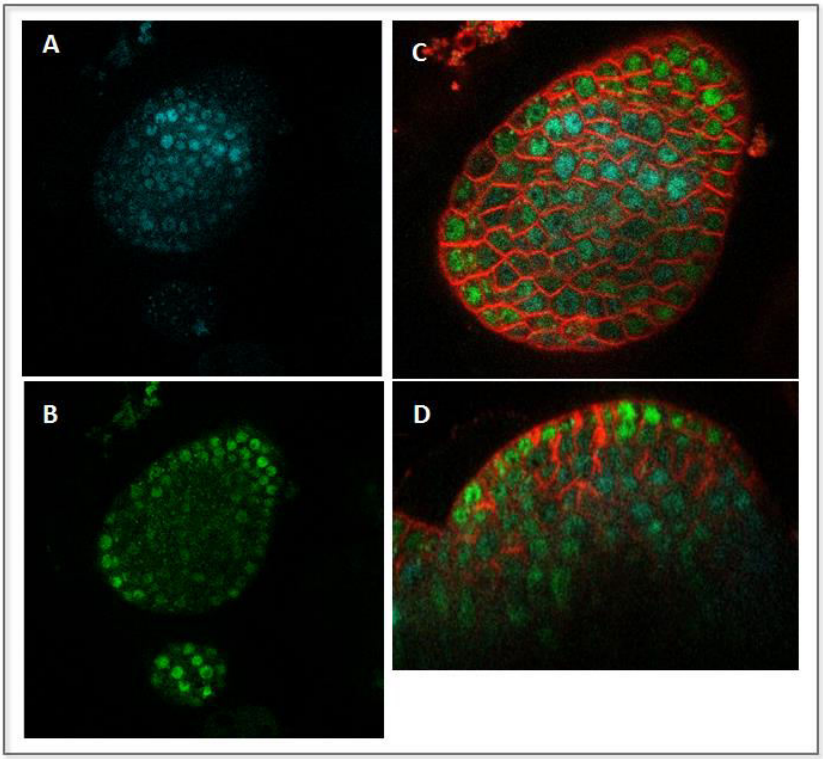

Figure 4.12. Localization of RGA-GFP and KNAT1-CFP proteins in the SAM. Fluorescence of *pRGA::RGA-GFP pKNAT1::KNAT1-CFP* lines, green and blue, respectively, was detected by confocal microscopy of shoot apices stained with FM4-64 (red). Meristems were from 9 day-old F1 seedlings grown at 22ºC and continuous light. **(A,B)** Splitted channels of the same stack for detection of KNAT1-CFP (a) and RGA-GFP (b). **(C)** Merge of both signals. **(D)** Orthogonal view, merged channels. Images are representative of three independent biological repeats.

Thus, our results suggest that there is a mutual negative regulation between KNAT1 and GAs to maintain meristem homeostasis, which is represented in Figure 4.13. The localization of the RGA and KNAT1 in the SAM implies a complex regulation between pro-meristematic and pro-differentiation factors in this context. It is also highlighted the possible role of DELLAs in the boundary of the meristem, where they would ensure *KNAT1* expression by inhibiting its negative regulators (CIN-TCPs and AS1).

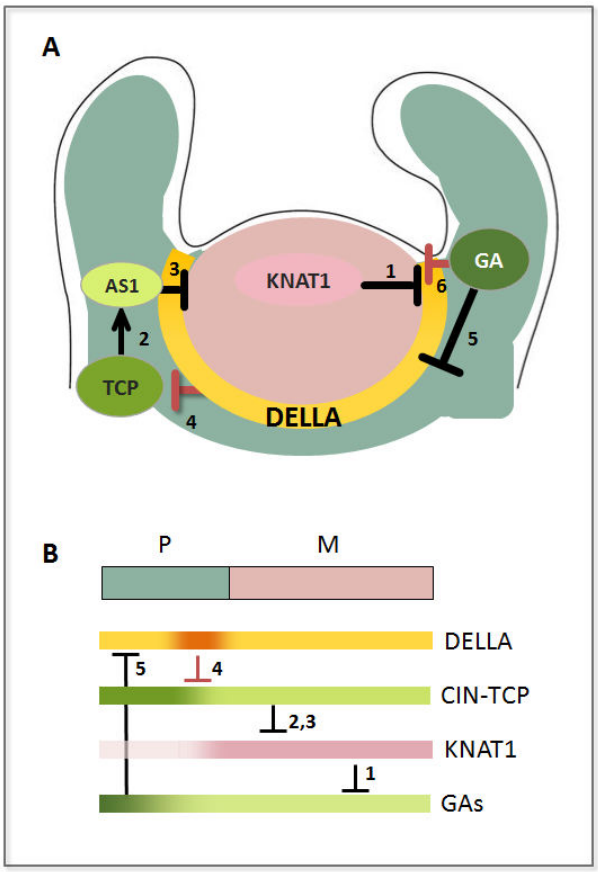

Figure 4.13. Summary of the molecular and genetic interactions observed at the SAM. Graphical **(A)** and schematic **(B)** representation of the GA/KNAT1 regulatory module. The pro-meristematic factor KNAT1 (in mauve) is found in the un-differentiated zone (mauve zone (M)), where it represses GA biosynthesis and promotes its deactivation (1). On the other hand, pro-differentiation factors GAs, CIN-TCPs and AS1 (in green) are located in the primordia (blue zone (P)), where they promote cell specification by repressing *KNAT1* expression. CIN-TCPs are known to activate *AS1* expression (2), which is a known repressor of *KNAT1* (3). Results in this Thesis indicate that DELLAs (in yellow) interact with CIN-TCPs, probably in the meristematic-differentiation frontier, inhibiting CIN-TCP transcriptional activity, and therefore, they could guarantee *KNAT1* expression in that boundary, where its negative regulators star to be present (4). As higher level of GAs (that lead to DELLA degradation (5)) is found in the primordia, DELLA function could be specially relevant controlling the extent of *KNAT1* expression in the boundary of the un-differentiated zone. This way, there is a mutual negative regulation between KNAT1 and GA (6) that could help to modulate the interplay between meristematic and differentiation factors to maintain meristem homeostasis. In **(B)**, intensity of the color indicates accumulation of the protein or molecule. Lines and arrows in black indicate literature data; in red, results of this Thesis.

On the other hand, it is remarkable that, as depicted in Figure 4.12, RGA protein accumulation decreased towards the center of the meristem. This suggests that there should be other mechanisms that operate in the center of the meristem taking over the role of DELLAs. For instance, the presence of miR319 causes a down-regulation of *CIN-TCPs* (18). Other interesting question derived from this observation was how RGA levels decreased in the center of the SAM. Since *KNAT1* guarantees a GA-free environment in the meristem, transcriptional but not post-transcriptional regulation of DELLAs should be important in this zone. Therefore, there must be factors in the meristematic zone that could inhibit *DELLA* expression.

8. The GA/KNAT1 balance regulates cell expansion in the shoot apical meristem

Once we have established that there seem to be a mutual negative interaction between KNAT1 and GAs in the meristem context to balance meristem homeostasis, our next aim was to unravel the SAM characteristics regulated by this GA/KNAT1 balance.

It has been described that GAs play a relevant role in the transition from vegetative to reproductive meristem, which is characterized by an increase in the size of the meristem (27). In this line, although a lot of recent work has been focused on unraveling the molecular pathways involving *KNOX* genes such as *KNAT1* (28), no attention has been paid to its possible role in the regulation of meristem size. Taking into account these observations and our previous results indicating the regulation that DELLAs exert on *KNAT1*, we wondered whether the GA/KNAT1 module could regulate meristem size.

To this purpose, we measured the cell number, and cell and meristem volume of 9 day old wild type and *KNAT1* mutant seedlings (called *bp-1* (29)) treated or not with GAs. Remarkably, thanks to our collaboration, we used a novel technique implemented in the laboratory of Prof. Sablowski (30). To track cell number and volume, we used confocal imaging of vegetative apices and then a package of Python scripts and Fiji macros were applied to the images to landmark, segment, locate and measure cells in 3D, covering all the volume of the meristem (Figure 4.14A). Another step of manual treatment of the data was applied to delete cells that were incorrectly segmented.

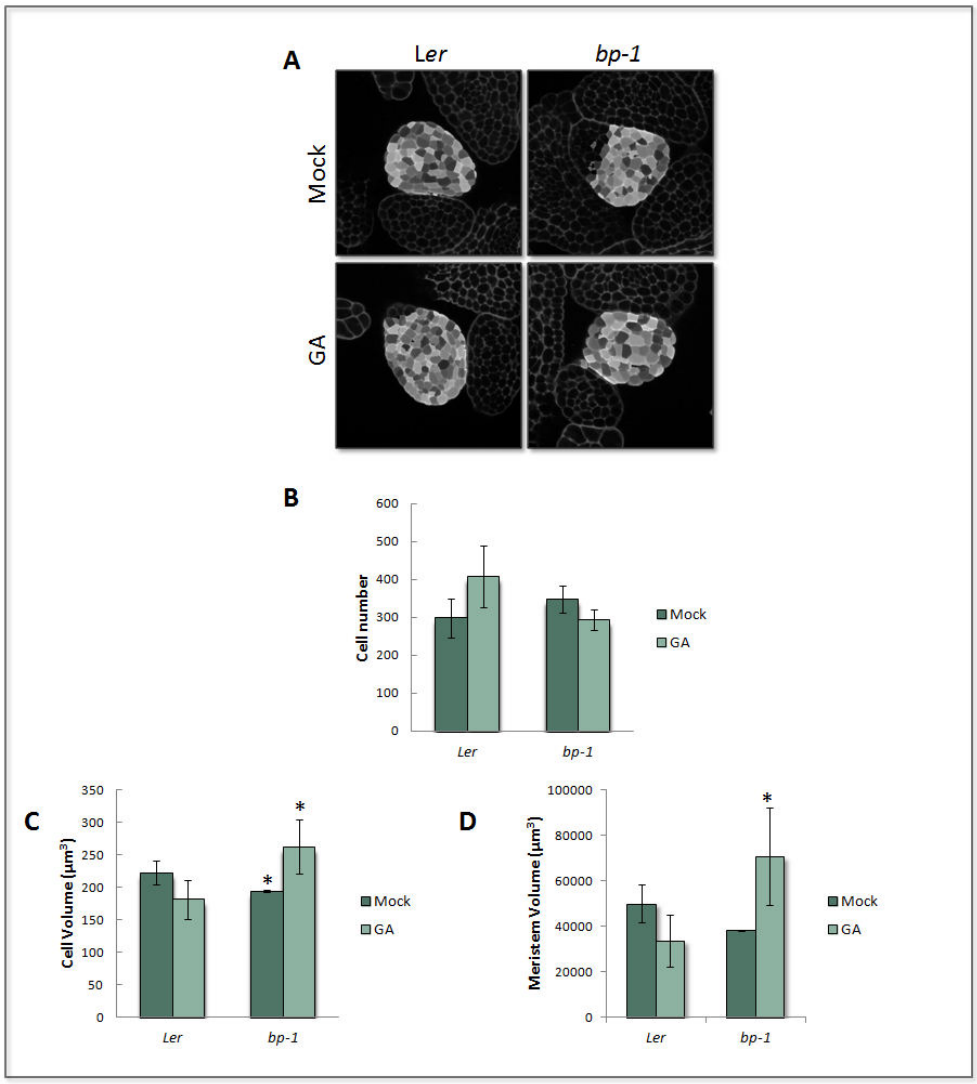

Figure 4.14. The GA/KNAT1 module regulate SAM size. Confocal microscopy images of vegetative meristems of 9 day old seedlings grown in continuous light of the depicted genotypes were *in silico* analyzed. GA-treated plants were maintained in 50 µM GA_3 since sowing. **(A)** Stack of each condition at the same point of the Z-axis (stack 30). Confocal microscopy image and *in silico* segmentation image overlap is shown. **(B)** Cell number, **(C)** cell volume and **(D)** meristem volume of the indicated genotypes and conditions are depicted. Three to five replicates per apex were analyzed. Statistic differences between mock conditions in both genotypes and between the GA and the mock condition in each genotype are shown (P-value<0.05).

As it can be seen in Figure 4.14B, neither KNAT1 nor GA treatment affected cell number in the meristem, suggesting that none of these factors regulates cell division in the context of SAM development. However, cell and meristem volume seem to be modulated by both factors. Meristem cells in the *KNAT1* mutant (*bp-1*) were slightly smaller than in the wild type (Figure 4.14C). Furthermore, GA treatment does not affect cell volume in the wild type, whereas it has an impact on cells of the *bp-1* mutant, as their cell volume is increased. This higher cell volume is reflected in an increased meristem volume in *bp-1* (Figure 4.14D). The fact that the *bp-1* mutant presents a slightly smaller meristem than the wild type indicates that KNAT1 could contribute to cell volume. This result is in line with the observation that *KNAT1* expression increases during the transition from vegetative to reproductive meristem (eFP Browser (31)), in parallel with a significant increase in meristem size (27).

On the other hand, KNAT1 renders the meristem insensitive to GAs, since only the *bp-1* mutant (deficient in KNAT1 activity) responded to applied GAs (Figure 4.14C and D). In this sense, it is important to remember that *KNAT1* represses GA biosynthesis and promotes GA deactivation in the SAM (see Introduction of this Chapter), excluding GAs from that zone. This way *KNAT1* would protect the meristem from the effect of GAs, maintaining an adequate meristem volume. On the other hand, results in this Thesis indicate that GAs have a negative effect on *KNAT1* expression, probably due to the fact that the DELLA-CIN-TCP interaction inhibits the transcriptional activity of TCPs and therefore, promotes *KNAT1* transcription. Hence, in the SAM, there is a balance between GA and KNAT1 to maintain meristem homeostasis in relevant aspects, such as meristem size (Figure 4.15).

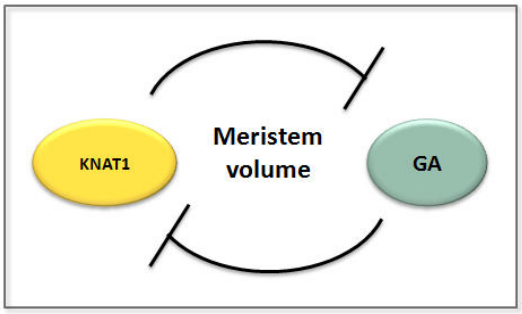

Figure 4.15. The GA/KNAT1 regulatory module ensures meristem homeostasis in relevant aspects such as meristem volume.

Interestingly, *KNAT1* seems to have a dual role in the regulation of cell expansion, and consequently, meristem size. On the one hand, it could promote cell expansion, as meristem volume of the mutant is significantly smaller than the wild type (Figure 4.14.1). On the other hand, it renders the meristem insensitive to GAs, which could be important to prevent excessive growth under conditions when GA flow towards the SAM increases, such as during the transition to flowering (27). All in all, these data strongly supports the idea that DELLAs and KNAT1 constitute an important regulatory network in the SAM context destined to maintain a correct meristem size by controlling cell expansion by a feedback mechanism (Figure 4.15).

Additionally, we evaluated whether DELLAs are found in inflorescence meristems, and therefore, the KNAT1/DELLA module of regulation could be taking place also in this stage. To do so, we performed *in situ* assays in inflorescences of three to four week old plants to evaluate the endogenous expression of the DELLA *GAI* (Figure 4.16). According to our results, *GAI* expression can be also found in the inflorescence meristem, suggesting that the mechanism described can be also functional at that stage and organ after the induction of flowering. In this line, *KNAT1* expression in inflorescence meristems has also been described (26, 31).

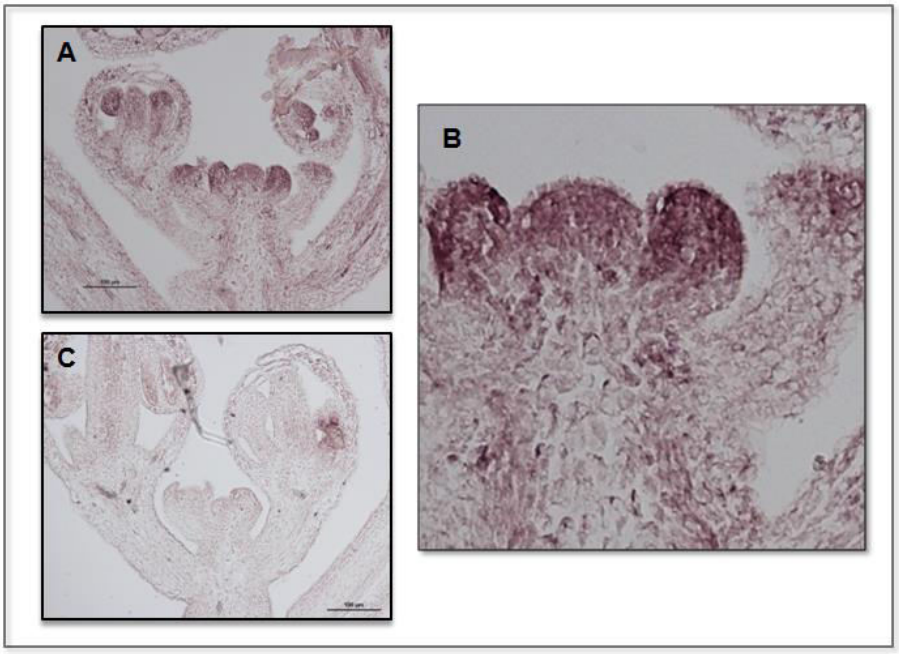

Figure 4.16. DELLA *GAI* is expressed in inflorescence meristems. *In situ* hybridization of *GAI* in longitudinal slices of inflorescences of 3 to 4 weeks Col-0 plants. Antisense **(a, b)** and sense **(c)** probes are shown. A zoom of the shoot apical meristem can be observed in **(b)**.

9. Unraveling KNAT1 function at the genomic level

Previous results point out the importance of *KNAT1* modulating meristem size. Although the implications of this gene in plant development have been extensively studied, information about its specific downstream targets or the molecular processes regulated by *KNAT1* is still lacking (28). To further unveil possible *KNAT1* biological functions, a RNA-sequencing (RNA-seq) experiment was done. Additionally, to test a possible implication of DELLAs in the *KNAT1*-driven regulation, the experiment was done in response to different DELLA levels.

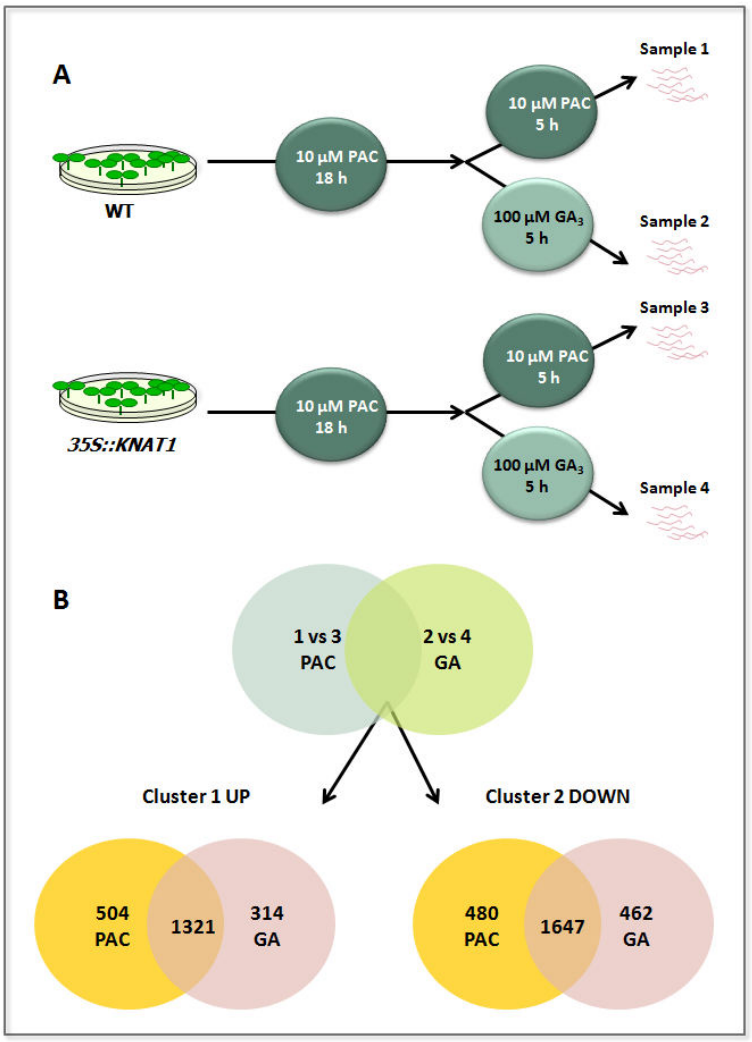

Figure 4.17. RNA-seq experiment. (A) Diagram of the experimental setting. Seven day old seedlings of wild type (*No-0*) and *35S::KNAT1* genotypes were treated with 10 µM of PAC for 18 hours. Then, half of them followed a 100 µM GA_3 treatment whereas the other half remained in PAC for 5 hours. Samples (named as sample 1 to 4) were collected and RNA-seq data was obtained. Three biological replicates were used for the analysis. **(B)** Scheme of the *in silico* analysis, which compared samples in the same chemical treatment (sample 1 *versus* sample 3 and sample 2 *versus* sample 4) to highlight *KNAT1* targets. This initial cluster is subdivided in two: targets up-regulated by the presence of *KNAT1* over-expressor were organized in Cluster 1 and the *KNAT1* down-regulated targets in Cluster 2. Each cluster renders three different datasets of *KNAT1* targets: DELLA-presence-dependent targets (504 UP and 480 down), DELLA-absence-dependent targets (314 UP and 462 DOWN) and *KNAT1* targets that are independent of DELLA presence or absence, that is the overlapping zone of both previous datasets (1321 UP and 1647 DOWN).

In particular, we treated seedlings of the *KNAT1* over-expressor transgenic line *35S::KNAT1* (32) and its wild-type control *No-0* with PAC for 18 hours to ensure the accumulation of DELLAs. At that point, half of them were subjected to GA treatment whereas the other half remained in PAC. This way it is possible to compare between two different situations, one with a high level of DELLA (PAC) and other where the degradation of DELLA is promoted (GA). As a result, 4 different samples were obtained and processed to obtain sequencing data for each one of them (see Material and Methods) (Figure 4.17.A). Regarding the *in silico* analysis, samples in the same chemical treatment (sample 1 *versus* 3 and sample 2 *versus* 4) were compared to detect *KNAT1* targets in each condition. Thus, the differential expression of these targets is due to the presence of the *KNAT1* over-expressor, not to the chemical compound. To simplify the analysis, this initial clustering is further subdivided in two attending to the up-regulation (Custer 1) or down-regulation (Cluster 2) of the targets in response to *KNAT1* over-expression (Figure 4.17.B). Thus, for each cluster, this analysis renders three different sets of *KNAT1* targets. First, targets altered only when DELLAs are present (PAC, DELLA-presence-dependent targets) that include 504 up- and 480 down-regulated genes. Second, a set of differentially expressed targets only when DELLAs are absent (GA, DELLA-absence-dependent targets), that renders 314 up- and 462 down-regulated targets. Finally, a third set of genes that are *KNAT1* targets independently of the presence or absence of DELLAs, that is the overlapping zone of both previous datasets (*KNAT1* DELLA-independent targets), with 1321 up- and 1647 down-regulated genes (Figure 4.17.B). The complete datasets can be found in the Supplemental Folders “Cluster 1” and “Cluster 2”.

Once the sequencing data was obtained and organized, we pursued three different goals in the following analysis, which we elaborate in the next sections. First, we compare our results with literature data, and then we analyze the DELLA-independent and DELLA- dependent KNAT1 targets found in our RNA-seq experiment.

9.1 Comparison with literature data

KNOX genes have been studied for decades and, despite the deep appreciation for the importance of these genes in plant development, we know very little about the genes under *KNOX* regulation. To gain further understanding of *KNOX* gene function and possible *KNAT1* biological roles, we decided to carry

out a bibliographical research on genome-wide studies in *KNOX* genes and compare this data with our results. Remarkably, two recent articles from the group of Dr. Hake have investigated on this topic (33, 34). In both articles, RNA-seq and ChIP-sequencing technologies are combined to identify genes differentially expressed in response to the *KNOX* genes *KNOTTED1* (*KN1*) in maize (34) and *Oryza sativa homeobox1* (*OSH1*) in rice (33). As a result, we could obtain three datasets: one from the maize ChIP-seq experiment, one from the ChIP-seq in rice and finally, one from the RNA-seq in rice.

As the genomic data was found in different species from *Arabidopsis*, before the comparison with our data we carried out a plant comparative genomic study with the tool PLAZA (35). This bioinformatic platform integrates numerous types of information (e.g. gene families, phylogenetic trees and genomic homology) along with structural and functional annotation, and, with this information, it provides the most probable ortholog among two species. Using this tool, we searched for the orthologs in *Arabidopsis* of the targets of *KNOX* genes in maize and rice. As the methodology holds some limitations, not all the targets were assigned, and sometimes the same *Arabidopsis* gene was assigned to various original targets (duplications). Nevertheless, a considerable amount of orthologs were found (Table 4.1). The complete datasets can be found in the Supplemental File "PLAZA orthologs".

Table 4.1. Comparative genomic study with PLAZA

		Original targets found	Assigned orthologs	Duplications	Unique targets in *Arabidopsis*
Maize	ChIP-seq	277	277	62	215
Rice	RNA-seq	1473	1192	282	910
	ChIP-seq	4662	4324	3715	609

Once the different data sets of orthologs are available, our aim in this first analysis was to evaluate which functions of *KNAT1* could be shared with other *KNOX* genes in other species. To do so, we searched for common targets among the orthologs of these published studies and our candidates in the RNA-seq. In particular, we included all the *KNAT1* targets found in our analyses, independently of the DELLA level. That is 504+1321+314 in Cluster 1 and 480+1647+462 in Cluster 2, meaning a total of 4728 genes. To find the common targets among all 4 datasets (ours and the three published ones) we used the bioinformatic tool Venny (36), that provides a graphical Venn diagram and the different lists of common and unique targets (Figure 4.18.A). Remarkably, among all the targets

found in our RNA-seq experiment, 125 (2,6% of our total dataset), 163 (3,4%) and 31 (0,65%) were shared with the ChIP-seq and RNA-seq in rice and the ChIP-seq in maize respectively. In other words, we found 319 common targets of *KNAT1* with *KN1* and *OSH1*. Next, we performed a Gene Ontology (GO) enrichment analysis of the shared targets with agriGO (37) and visualized it with REVIGO (38), which forms clusters of related terms and facilitates the identification of enriched categories (Figure 4.18.B). These datasets and the REVIGO table can be found in the Supplemental File 1.

As it can be seen in Figure 4.18.B, among the enriched GO functional categories, a great variety of biological processes can be found. These include metabolic and catabolic processes such as the metabolism of hormones, lipids, nitrogen compounds, glycosides and carbohydrates. Also, response to stimulus categories such as defense response, immune system processes, response to light, stress and hormones (regulation of hormone levels). Other processes highlighted are growth, cell wall modification, xylem and phloem formation, cell death, photosynthesis, lipid localization and reproduction. These results suggest that these biological processes can be commonly regulated by *KNOX* genes in different species, constituting key targets of KNOX function.

To further investigate this issue, we repeated the analysis taking the up- and down-regulated targets found in our RNA-seq separately and comparing each group with the same three published datasets (Table 4.2). For example, among the 125 shared targets of our dataset with the ChIP-seq in rice, 47 were up-regulated in our experiment and 78 (more than 60% of the total shared targets) were down-regulated. See Supplemental File 2. Similar proportions were obtained in the three comparisons, indicating that the targets more conserved among species are mainly down-regulated by *KNOX*.

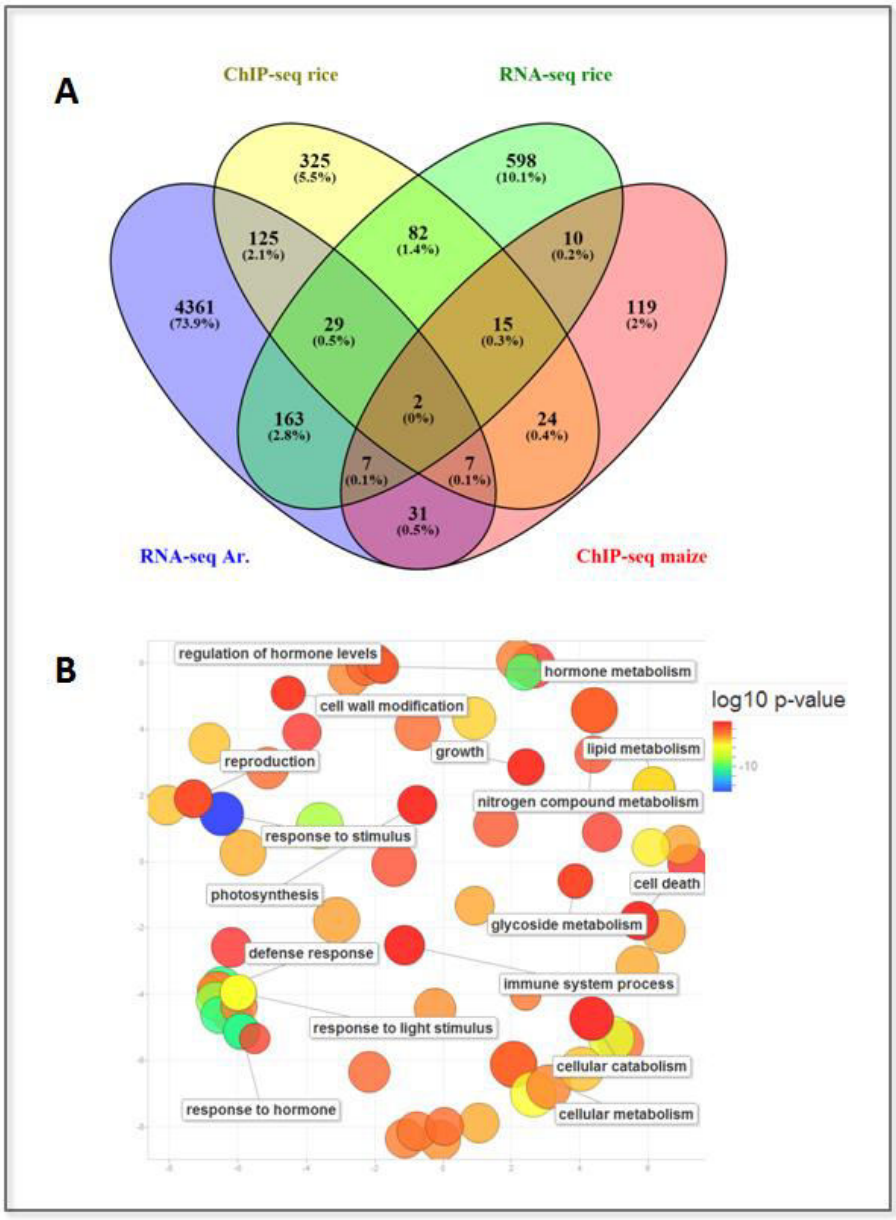

Figure 4.18. *KN1*, *OSH1* and *KNAT1* common targets. (A) Venn diagram showing the overlapping or unique targets among all 4 datasets. Numbers in the different sections indicate the number of targets and the percentage respect to the total amount of data. **(B)** REVIGO visualization of the GO analysis for the common targets clustered by biological process. Each circle is a category representative and its size reflects the level in the hierarchy (the smaller, the more specific). Different colors reflect the logarithmic *p*-value (legend).

Table 4.2. *KNOX* common targets				
		Total shared targets	UP shared targets	DOWN shared targets
Maize	ChIP-seq	31	7 (22,5%)	24 (77,5%)
Rice	RNA-seq	163	64 (39%)	99 (61%)
	ChIP-seq	125	47 (37,6%)	78 (62,4%)

Furthermore, taking into account that ChIP-seq analysis in rice and maize were available, we decided to look for likely direct targets of *KNAT1* among the targets found in our RNA-seq experiment. To do so, we searched for the overlapping targets among our dataset and both published ChIP-seq experiments and performed a GO enrichment analysis (Figure 4.19, Supplemental File 3). The 154 and 38 targets shared with rice and maize respectively are genes altered in our analysis by *KNAT1* over-expression and they could be direct targets of the *KNOX* gene *KNAT1*, as they are *KNOX* direct targets in other species. As for the enriched GO categories found, although most of them were also obtained in the previous analysis, new biological processes can be observed. Importantly, processes related to development and morphogenesis are highlighted, such as tissue and shoot system development, shoot system morphogenesis, regulation of anatomical structure size and regulation of cellular component size. These enriched GO categories are in line with observations in the literature of the developmental role of *KNAT1*, and remarkably, with our findings that *KNAT1* plays a relevant role in the modulation of meristem size. Further evaluation of these specific targets might be needed to unravel the underlying molecular mechanism in this regulation.

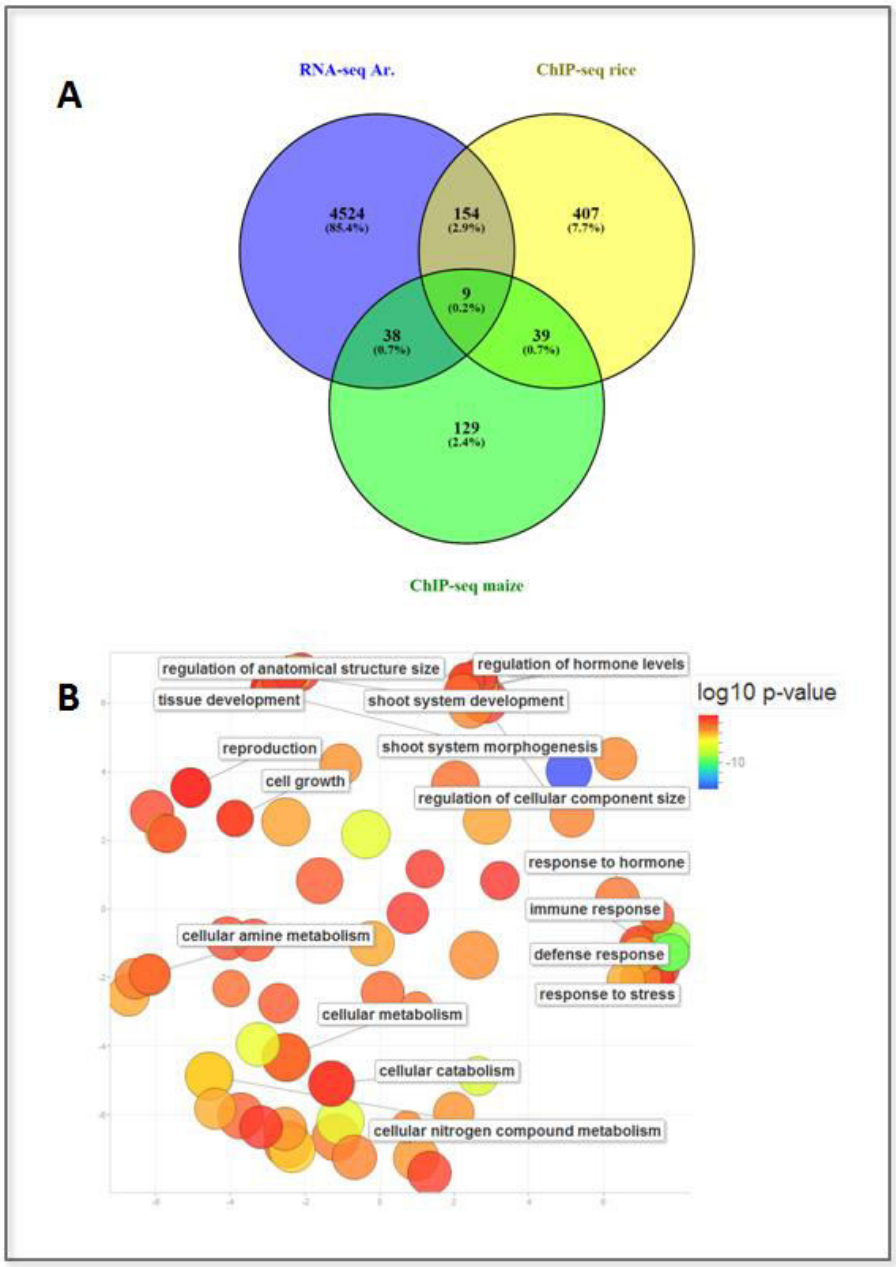

Figure 4.19. Processes regulated by likely direct targets of *KNAT1*. (A) Venn diagram showing the overlapping or unique targets among 3 datasets. Numbers in the different sections indicate the number of targets and the percentage respect to the total amount of data. **(B)** REVIGO visualization of the GO analysis for the common targets clustered by biological process. Each circle is a category representative and its size reflects the level in the hierarchy. Different colors reflect the logarithmic *p*-value (legend).

Notably, only two articles in the literature have investigated on the specific targets regulated by *KNAT1* in *Arabidopsis*. Both of them performed microarray analysis of gene expression profiling comparing wild type plants with the *KNAT1* mutants *bp-101* (39) and *bp-9* (40), obtaining 56 and 64 differentially expressed genes respectively. With the aim to unravel *KNAT1* specific functions in *Arabidopsis*, we looked for common targets among the three datasets and carried out a GO enrichment analysis of them (Figure 4.20, Supplemental File 4).

Despite the microarray datasets being extremely small, we found 9 and 24 shared targets among our data and the microarrays for *bp-9* and *bp-101* respectively; and outstandingly, the agriGO and REVIGO analysis rendered significant highlighted GO categories. Among them, metabolism processes (glucosinolate, sulfur compound and carbohydrate), development and response to stimulus such as stress, chemical compounds or abiotic stimulus. These results suggest that *KNAT1* specific targets must be related with these processes, being these the processes more directly regulated by *KNAT1* in *Arabidopsis*. A close view of these targets is required to further unravel the molecular mechanisms controlled by this gene. In this line, 6 genes of the lignin pathway have been found to be direct targets of *KNAT1* in *Arabidopsis* by electrophoretic mobility shift assays (EMSA) (40). However, only one of them, *AT4G35160* (*COMT2*), that encodes a cytosolic N-acetylserotonin O-methyltransferase involved in melatonin synthesis, was found in our RNA-seq data.

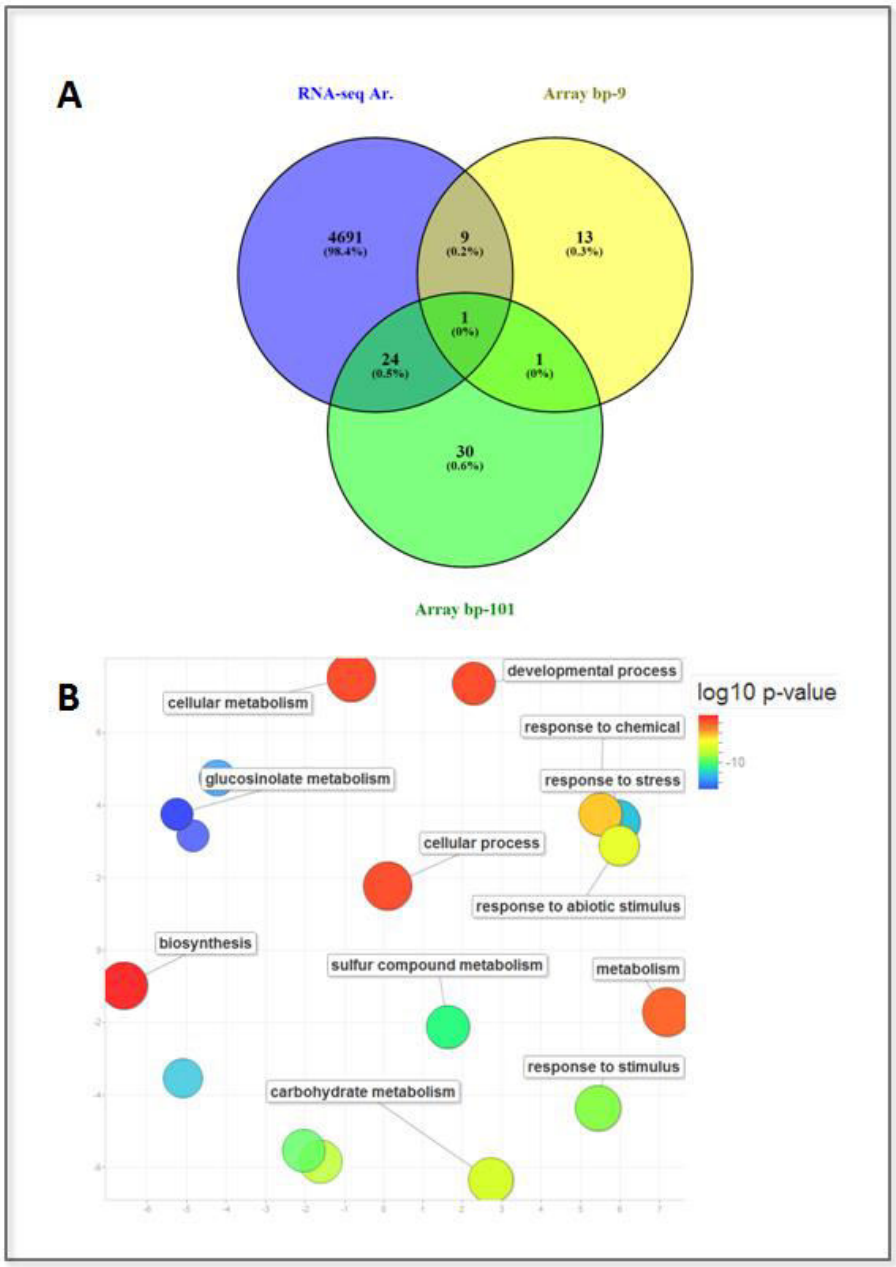

Figure 4.20. Specific *KNAT1* targets in *Arabidopsis*. (A) Venn diagram showing the overlapping or unique targets among 3 datasets. Numbers in the different sections indicate the number of targets and the percentage respect to the total amount of data. **(B)** REVIGO visualization of the GO analysis for the common targets clustered by biological process. Each circle is a category representative and its size reflects the level in the hierarchy. Different colors reflect the logarithmic *p*-value (legend).

9.2 Analysis of the KNAT1 targets found in the RNA-sequencing experiment

Focusing now on our RNA-seq experiment, we decided to analyze first the targets which regulation by *KNAT1* is independent of the presence or absence of DELLAs. This dataset consists in 1321 up-regulated and 1647 down-regulated genes in response to *KNAT1* over-expression, that is, 2968 total genes (Figure 4.17). First, we performed a GO enrichment analysis of the total dataset (2968 genes), that brought out several interesting GO categories. Besides, we carried out the same analysis with the up-regulated and down-regulated genes separately. Comparing the results obtained in these three tests, a general overview of the biological processes presumably promoted and inhibited by *KNAT1* can be observed (Table 4.3, Supplemental File 5).

In the table, the GO categories highlighted in the three analysis performed ("All", "UP"- and "DOWN"-regulated targets of *KNAT1*) can be observed, and they are indicated with a green, mauve or blue mark, respectively. Outstandingly, several processes seem to be either specifically promoted by *KNAT1* or specifically inhibited by this gene.

Among the processes favored by *KNAT1* over-expression, we can find some important metabolic processes and, interestingly, the seed germination category. Besides, some of these enriched categories seem to be functionally linked, as they are related to response to biotic stress. In this line, not only apoptosis is induced by *KNAT1*, but also immune system and some specific defense responses, such as callose deposition in the cell wall and cell wall thickening. These cell-wall-related processes are also enriched independently of the defense response, suggesting a developmental role of *KNAT1* controlling cell wall function. Interestingly, *KNAT1* has been found to regulate lignin biosynthesis (28) and to bind the promoters of some lignin-pathway-related genes (40). As for the biological processes down-regulated by *KNAT1*, light-related processes are emphasized. Photosynthesis, chlorophyll biosynthesis and electron transport chain are accentuated, and also response to light categories, such as response to light intensity, blue, red and far red response categories. These observations suggest a role of *KNAT1* integrating external light sensing with endogen molecular cues that regulate photosynthesis-related processes.

Table 4.3 GO terms highlighted in *KNAT1* DELLA-independent target dataset

Biological process		All	UP	DOWN
Metabolism	Nitrogen compound			
	Glucosinolate			
	Carbohydrate			
	Sulfur			
	Lipid transport/localization			
	Toxin catabolism			
	Starch			
	Aminoacid			
	Pigment			
	Lipid			
	DNA			
	Alcohol			
	Carbon fixation			
	Ketone			
Photosyntesis	Photosyntesis			
	Light reaction			
	Chlorophyll biosynthesis			
	Electron transport chain			
Death	Programmed cell death			
	Apoptosis			
Response to estimulus	Chemical			
	Stress			
	Biotic			
	Abiotic			
	Radiation			
	Light			
	Light intensity			
	Blue light			
	Red light			
	Far red light			
	Defense response			
	Defense response by callose deposition			
	Defense response by cell wall thickening			
	Immune system			
Wall processes	Cell wall modification			
	Cell wall thikening			
	Callose deposition in cell wall			
Other	Seed germination			

Notably, our RNA-seq results show that there are some targets of *KNAT1* that are exclusively modulated by this gene when DELLAs are present (Figure 4.17). In particular, this dataset consists in 504 up-regulated and 480 down-regulated genes in response to *KNAT1* over-expression in the presence of DELLA. Given this

observation, our next aim was to evaluate those biological processes regulated by *KNAT1* in a DELLA-dependent way. To do so, we performed a GO enrichment analysis of all the dataset and the up- and down-regulated subsets separately (Supplemental File 6). We found the most represented categories were related to metabolism, photosynthesis, response to stimulus, hormone regulation, and, interestingly, categories related to structural organization and development, and more specifically meristem organization (Table 4.4). Strikingly, almost all the categories highlighted are enriched in the subset of down-regulated genes in response to *KNAT1* over-expression in the presence of DELLAs, indicating that *KNAT1* could be a repressor TF.

Comparing these results with the ones obtained for *KNAT1* over-expression in a DELLA-independent manner (Table 4.3), it can be seen that the study renders some common processes, although DELLA presence seem to influence *KNAT1* in the control of some specific biological processes not present in the previous DELLA-independent study (Table 4.3). Among these processes targeted by *KNAT1* exclusively in the presence of DELLA, a new class of categories is emphasized, related to structural and developmental features. In this line we can find categories such as morphogenesis, shoot, leaf and tissue development and meristem organization and development (Table 4.4). Other specific categories are related to hormones, such as regulation of hormone levels, response to hormones and hormone transport. Remarkably, although these hormone categories are highlighted also when comparing our data to the published studies (section 9.1), this group is not highlighted in our dataset of DELLA-independent *KNAT1* targets; indicating that the regulation of these processes could be dependent of DELLA presence.

The fact that DELLA presence influences KNAT1 regulatory activity despite being expressed from a constitutive promoter opens up the suggestive possibility that DELLAs could regulate KNAT1 through a post-transcriptional mechanism. As it has been previously introduced, DELLAs perform their regulatory activity by protein-protein interaction (15). For this reason, we hypothesize that DELLAs could be modulating KNAT1 activity by physical binding.

Table 4.4 GO terms highlighted in *KNAT1* DELLA-dependent target dataset

Biological process		All	UP	DOWN
Metabolism	Nitrogen compound			
	Lignin			
	Glucosinolate			
	Glicoside			
	Carbohydrate			
	Sulfur			
	Amine			
	Aromatic compound			
	Nucleotide			
	Oxidation/reduction			
	Ribonucleoprotein complex biogenesis			
	Ribisome biognesis			
Photosyntesis	Photosyntesis			
	Light reaction			
	Cellular respiration			
	Electron transport chain			
	Precursor metabolites and energy			
Structure	Developmental growth involved in morphogenesis			
	Anatomical structure arrangement			
	Anatomical structure morphogenesis			
	Shoot system morphology			
	Shoot development			
	Leaf development			
	Tissue development			
	Meristem structural development			
	Meristem structural organization			
	System development			
Response to	Chemical			
	Abiotic			
	Radiation			
	Light stimulus			
	Hormone			
	Gravity			
Hormone	Regulation of hormone levels			
	Hormone transport			
Other	Cell wall modification			
	Chromatin assembly and disassembly			

9.3 Supplemental material

Next, an enumeration of the supplemental files derived of the RNA-seq experiment is listed. The documents (in PDF and Excel versions) are available on the following website http://www.ibmcp.upv.es/BlazquezAlabadiLab/, in the Download section.

Supplemental files derived of the RNA-seq experiment	
Folder	**Document**
Folder "Cluster 1"	Cluster 1 314 genes Cluster 1 505 genes Cluster 1 1321 genes
Folder "Cluster 2"	Cluster 2 462 genes Cluster 2 480 genes Cluster 2 1647 genes
PLAZA	PLAZA orthologs
Supplemental Files	Supplemental File 1 Supplemental File 2 Supplemental File 3 Supplemental File 4 Supplemental File 5 Supplemental File 6

10. GAI and RGA interact with KNAT1

We tested our hypothesis first by performing Y2H assays and results indicate that KNAT1 is able to interact with the GRAS domains of GAI and RGA (Figure 4.21.A-B). To determine the protein regions involved in the interaction, we used deleted versions of the GAI protein (separating the different regulatory domains of the protein) and tested their ability to interact with KNAT1 (Figure 4.21.C). As it can be seen in the figure, only M5GAI and deletion 1 are able to interact, suggesting that complete removal of the LHR1 motif of GAI does not impair interaction with KNAT1, whereas it is prevented by further deletion of the VHIID motif (del2). These results are similar to the interaction with ARR1 (12) and contrast with the requirement of the LHR1 motif to sustain interaction of GAI or RGA with BZR1, PIF4, and JAZ1 (4, 5, 7, 8). Besides, it seems to be a requirement for the region close to the C-terminus to support DELLA interactions. Indeed, it has been seen that point mutations in DELLA genes that create a premature stop codon close to the very end of the coding sequence, therefore producing truncated proteins, represent loss-of-function alleles most likely because of their incapacity to interact with downstream partners (41).

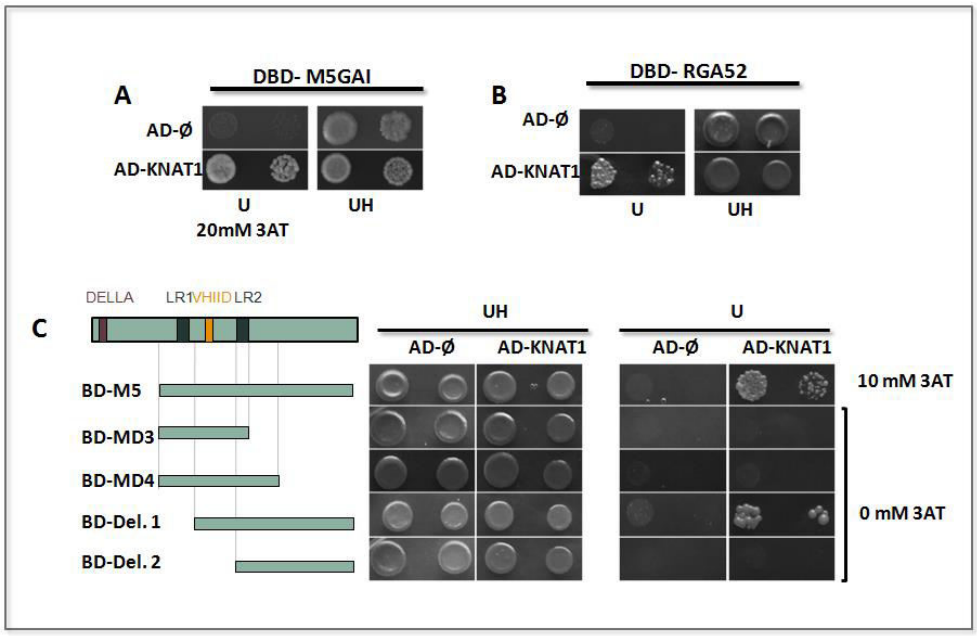

Figure 4.21. DELLA proteins interact with KNAT1. Y2H assay of the interaction between **(A)** a truncated version of GAI (M5GAI) and KNAT1 and **(B)** a truncated version of RGA (RGA52) and KNAT1. **(C)** Domains of GAI involved in the KNAT1-DELLA interaction. Y2H assay of the interaction between KNAT1 and truncated versions of GAI. The different domains are indicated in colored boxes and their names specified. (DBD, DNA binding domain; AD, activation domain; AD-ϕ, activation domain empty vector; U, uracil; H, histidine; 3-AT, 3-aminotriazol).

To confirm that the interaction between GAI and KNAT1 also occurs *in planta*, we performed a co-IP assay in *N. benthamiana* leaves transiently expressing HA-KNAT1 and c-myc-M5GAI (Figure 4.22). As it can be seen in the figure, the HA-tagged version of KNAT1 was co-immunoprecipitated with c-myc-M5GAI, using an anti-c-myc antibody, therefore verifying that DELLA-KNAT1 interaction takes place *in planta*.

These results provide a possible alternative mechanism by which DELLAs modulate KNAT1 activity, which will need to be further investigated. Indeed, by electrophoretic mobility shift assay (EMSA) technique, KNAT1 has been found to bind the promoter of some components of the lignin pathway (40). Using these promoters in transient luciferase assays would clarify the effect of this DELLA-KNAT1 interaction on *KNAT1* transcriptional activity. Besides, this molecular mechanism would explain our results of the RNA-seq assay, showing that there are some genes that depend on DELLA presence for their regulation by KNAT1.

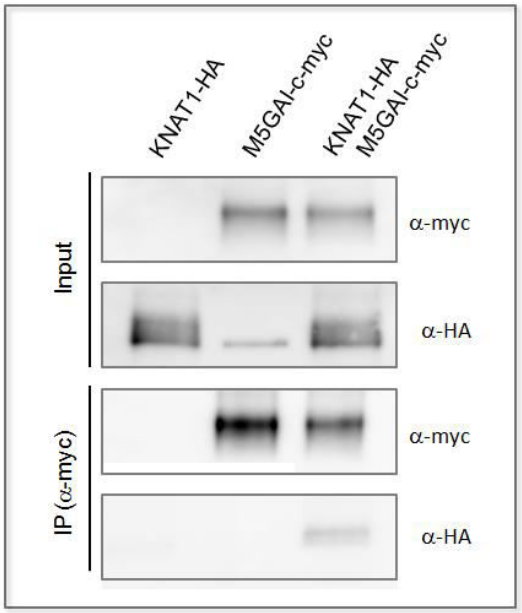

Figure 4.22. KNAT1 and DELLA proteins interact *in planta*. Co-immunoprecipitation of M5GAI-myc and KNAT1-HA expressed in *N. benthamiana leaves*. M5GAI was immunoprecipitated using anti-myc (α-myc) conjugated paramagnetic beads, and M5GAI and KNAT1 were detected in immunoblots using anti-myc and anti-HA, respectively. Note that KNAT1 was co-immunoprecipitated with M5GAI. The sizes of the bands correspond to the expected sizes of the fusion proteins.

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5. Introduction of Chapter II:

DELLAs' role on the germination process

1. The germination process

Seed germination is a major developmental transition in a plant's life that mostly consists on resuming embryo growth after a long quiescence imposed during seed maturation. In this section, a brief view of the key aspects of this process is depicted.

1.1 Seed structure and germination

In angiosperms such as *Arabidopsis*, the diploid embryo is surrounded by two different, protective layers. The inner is the triploid endosperm, formed by a single cell layer of nutritive tissue and mostly living cells; and in the outer layer, the diploid testa (also named seed coat) that is formed by maternal tissue and mostly dead cells. In *Arabidopsis* seeds most nutrients are translocated to and stored in the embryonic leaves, the cotyledons. The micropylar endosperm is the endosperm zone covering the radical tip (Figure 5.1) (1).

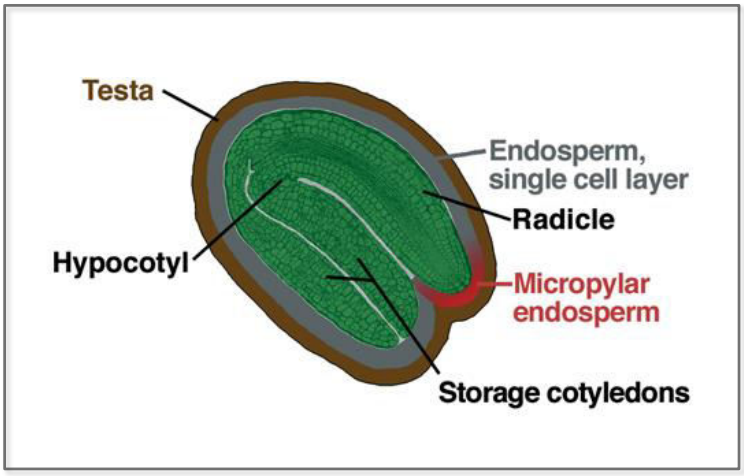

Figure 5.1. Mature seed of *Arabidopsis thaliana*. Different parts of the embryo and its two covering layers are depicted. The micropylar endosperm is shown in red. Extracted from Finch-Savage and Leubner-Metzger, 2006 (1).

The germination process involves the concerted action of several genetic and physiological pathways, where the environmental conditions play a determinant role (2). These internal and external cues determine the dormancy status

(incapacity of a viable seed to germinate under favourable conditions) and the potential for germination (percentage of emergence of the radicle through surrounding structures) (2).

Seed germination is sensitive to environmental conditions such as water, oxygen, temperature, light and nitrate. Only when these conditions are favorable, the germination commences with the uptake of water by imbibition by the dry seed, followed by embryo expansion. As a result, the embryo axis elongates and breaks through the covering layers to germinate (1). In particular, *Arabidopsis thaliana* seed germination is developed in two steps: testa rupture followed by endosperm rupture (see following section, Figure 5.1) (2). Cell elongation and division is also necessary for the completion of radicle protrusion (visible germination) (3).

1.2 Dormancy and dormancy releasing mechanisms

There are two different mechanisms imposing dormancy to the seed: embryo dormancy and coat dormancy, and their sum and interaction determine the degree of physiological dormancy (PD) (4, 5).

Embryo dormancy is characterized by a block that inhibits the inherent growth of the embryo (cell elongation and cell division). This dormancy is independent from the coat-induced one, and thus, embryos excised from their protective layers are not able to germinate. Importantly, in the case of *Arabidopsis*, its PD is classified as nondeep (1). That means that embryos excised from these seeds produce normal seedlings. In other words, their embryos are not dormant and the PD is imposed by the coat dormancy only. Remarkably, GA treatment, scarification and cold stratification can break this dormancy.

On the other hand, coat dormancy is conferred by the covering layers, and is the result of two forms of mechanical constraints to the protrusion of the radicle: the dormancy imposed by the testa and the endosperm. Germination requires that the embryo growth potential increases to overcome the mechanical resistance imposed by both layers. Besides, this resistance is reduced during germination by different releasing mechanisms (1). Two mechanisms release the dormancy in the testa: predetermined breaking points in the testa that facilitate

tissue rips and the hydrolytic enzymes released by the endosperm and/or the radicle, which promote cell-wall hydrolysis (6-8). The endosperm dormancy is characterized by other releasing mechanism called endosperm weakening, which is promoted by GA and inhibited by ABA. It consists in the decline of the mechanical resistance of the micropylar endosperm (the endosperm zone covering the radicle tip, Figure 5.1) and is required for radicle protrusion during seed germination (5).

2. GA involvement in germination

A handful of evidences demonstrate the implication of GA signaling in the germination process. First, the GA deficient mutant *ga1-3* is unable to germinate, but GA treatment rescues this defect (9). In this line, seeds lacking GA receptors also fail to germinate (10, 11). Second, the biosynthetic enzymes *GA3ox1* and *GA3ox2* are essential for GA-regulated germination, as mutations in both genes reduce germination (12, 13). In addition, both genes are expressed in the axis of the embryo during initial imbibition before germination. As for DELLA proteins, several reports show that RGL2 have a major role in regulating germination potential, although other DELLAs also influence (14). *rgl2* mutant seeds are known to be non-dormant (15), and insensitive to paclobutrazol (PAC, an inhibitor of GA synthesis (16)) repression of germination (17). Besides, the lack of RGL2 function in a *ga1* mutant restores germination, showing that RGL2 represses germination in the absence of GA biosynthesis (17, 18). Interestingly, DELLAs are thought to integrate environmental signals in germination, as seeds lacking four DELLAs show light-independent and cold-independent germination (14).

In the following sections, an overview of the involvement of GAs in the germination process is depicted. In this Thesis, we focus on the influence of GAs on cell cycle and cell division in the RAM of germinating embryos (section 3 of this Introduction), as it is the aspect that DELLAs and TCP proteins might co-regulate.

2.1 The ABA:GA balance

Among the internal cues required for seed germination, plant hormones play a central role in dormancy induction and maintenance and the promotion of germination. A major conclusion from recent studies is that the abscisic acid (ABA)

and GAs are the main contributors to the process. ABA is a positive regulator of seed maturation and dormancy, while GAs counteract the effects of ABA by releasing dormancy and promoting germination (2). Importantly, a dynamic balance of the ABA:GA ratio is established in the seed to direct signalling pathways that drive the replacement of maturation genetic programs (high ABA) with the ones promoting germination (high GA). In fact, in the seed, there is an intimate interplay between ABA and GA biosynthesis (*NCED* and *GA3ox1* gene expression) and catabolism (*GA2ox2* and *CYP707A2* expression) to determine active hormone levels and sensitivity to these hormones that is influenced by the environmental conditions. For example, sensitivity of the seeds to light and GA increases as PD is progressively released. When these requirements overlap with favorable environmental conditions, germination will take place (1).

In this context, taking in to account the influence of these hormones in dormancy, we can re-interpret the dormancy as follows:
A dormant embryo is characterized by a high ABA:GA ratio, with high ABA sensitivity and low GA sensitivity. As embryo dormancy is released, there is a remodeling of hormone biosynthesis and degradation towards a low ABA:GA ratio, decreasing and increasing ABA and GA sensitivity, respectively. A non-dormant embryo has increased growth potential, increased capacity for cell extension growth and increased cell division, as well as its ability to induce the release of coat dormancy. As for coat dormancy, it has been demonstrated that GAs can release it by increasing the embryo growth potential and/or by reducing the mechanical constraint of the covering layers, and ABA again plays the opposite role. Besides, as the endosperm is living tissue, it actively participates in influencing both the ABA:GA ratio and sensitivity to these hormones (1). A more detailed view of germination and GAs involvement in this process in exposed in the following section.

2.2 GA implication in the molecular networks regulating germination following seed imbibition

The transition between dormancy and germination is a critical stage in the life cycle of plants. Since the formation of the seed, different developmental phases occur: seed maturation, after-ripening and imbibition, when germination starts. After seed imbibition in suitable environmental conditions, the germination

is induced, and this is the stage when GAs are more important (2). Next, we will briefly present several aspects of the germination process in which GAs are known to play a determinant role. Finally, in the next section, we present the influence of GAs on cell cycle and cell division in the RAM of germinating embryos, which is a key aspect in this Thesis.

Endosperm weakening

Endosperm weakening is the decline of the physical resistance of the mycropilar endosperm by the action of hydrolytic enzymes released by the own endosperm and/or the embryo, and is required for radicle protrusion during seed germination (5). Endosperm weakening has been demonstrated in *Arabidopsis* and other *Brassicaceae* seeds (19). Remarkably, GAs induce this weakening, and this process is regulated by the GA:ABA ratio (see part 2.1 of this section) (19). In the embryo, several GA-related factors induce endosperm weakening. It has been reported that a signal originating from the embryo promotes endosperm weakening, and this signal can be replaced by exogenous GA (19). Other work demonstrates that GA is synthesized *de novo* in the embryo, as the biosynthetic genes *GA3ox1* and *GA3ox2* are strongly expressed in the axis of the imbibed embryo (13). Besides, the TF *Blue Micropylar End 3 (BME3)* is known to be a positive regulator of germination, and is expressed in the embryo at the radicle tip. It is required to facilitate endosperm rupture, as seeds mutant in *BME3* lacked endosperm rupture, although it could be restored by exogenous GA treatment (20, 21). On the other hand, GAs have been found to induce the expression of cell wall remodeling enzymes in the micropylar zone of the endosperm, which weakens the strength of the micropylar endosperm cell walls, thus facilitating endosperm rupture by the radicle (22, 23).

Repression of embryonic traits

During and after germination, it has been reported that genes that promote embryonic identity are repressed by the chromatin remodelling factor *PICKLE (PKL)* and the TFs *VP1/ABI3-LIKE (VAL)* in a GA-dependent manner (24-27).

Interaction with the environment

It has been described that low temperatures and exposure to light are the major environmental factors that release seed dormancy and allow the completion of germination. Cold temperatures (4ºC) are known to induce the biosynthetic *GA3ox1* gene in dark imbibed seeds (12) and repress the catabolic *GA2ox2*. Thus, GA level increases in these conditions. Also red light and GA deficiency increase the amount of *GA3ox1* transcript, suggesting a complex regulation integrating multiple signals. It has also been reported that the synergistic interaction of light and stratification regulates some bHLH TFs which, in turn, control *GA3ox1*, *GA3ox2*, *GAI* and *RGA* expression (2, 28).

Transcriptomic changes

Several publications indicate that there are very large changes in genome expression associated with the transition from dormancy to germination. Germination completion is associated with higher expression of *GA3ox1* in the presence of light. It is also known that the transcriptome of GA-treated *ga1-3* mutant seeds identified 230 genes up-regulated in response to this hormone (9). Interestingly, many of these genes are also induced in the after-ripening stage, indicating that GAs regulate the expression of these genes also at that stage. Remarkably, DELLAs have been involved in these transcriptomic changes as well, as half of the GA-regulated genes were regulated in a DELLA-dependent manner (29). This report suggests that there are GA-regulated genes in a DELLA-independent or DELLA-partially dependent manner.

3. GAs role in cell division in the RAM during germination

Among all the aspects of germination in which GAs are involved, we decided to focus on the regulation of cell division in the RAM of germinating embryos. This is due to the fact that class I TCPs have been found to regulate cell proliferation (30), so it is likely that GAs and TCPs regulate jointly this aspect of germination (see part 5 of this section).

It has been described in the literature that the activation of cell division in the embryo is an integral part of germination that precedes the protrusion of the root through the seed coat. In *Arabidopsis*, this activation has been linked to

significant early changes in the expression of cell-cycle elements (3). In the dry *Arabidopsis* seed, embryonic cells are arrested in the G1 phase of the cell cycle. After imbibition, germination is initiated by water uptake and metabolic activity re-starts. This is followed by directional cell expansion, leading to radicle elongation and protrusion (3). In some species, cell division activation has been observed before root protrusion (31, 32). In fact, in *Arabidopsis* it has been demonstrated that cells transit from G1 into S phase and cell division is activated in a subset of cells in the root meristem just before radicle protrusion. Masubele *et al*., 2005 (3) demonstrated using a *pCYCB1;1::GUS* transgenic line (*CYCB1;1* is a mitotic marker that marks G2/M phase of the cell cycle (33)) that cell division initiates in the RAM, where its cells undergo mitotic cycles before root protrusion. This activation of division is then extended to the cotyledons, SAM, and secondary meristems after the embryo have been released from the coat. Besides, they describe that a high increment in the expression of cyclins (CYC) precede root emergence. Seeds with loss-of-function in these cyclins showed reduced division and delayed root emergence, whereas higher expression of CYCs increases cell cycle activation in the RAM and promotes embryonic radicle protrusion. Thus, CYCs regulate cell cycle reentry during RAM activation to promote cell division, radicle growth and germination (3).

Outstandingly, several publications demonstrate that in the RAM, GAs regulate cell division (34, 35). Before describing this role of GAs in detail, an overview of root structure and growth is presented.

Root length is determined by the number of dividing cells originated by the RAM and their final cell size (35). Root cell division is first originated within a population of stem cells surrounding the quiescent center (QC) at the root apex (36); being the QC a small group of organizing cells defining together with the stem cells the stem cell niche (Figure 5.2). Daughter cells that remain in contact with the QC still maintain stem cell identity (37). However, those daughter cells that lose contact with the QC undergo repeated rounds of mitotic divisions in the proximal meristem as they are displaced and reach the elongation zone, where they experience rapid cell expansion to definitely differentiate and acquire a determinate cell identity (35).

The *Arabidopsis* RAM structure can be appreciated in figure 5.2. It is composed of lateral root cap, epidermis, cortex, endodermis, and stele tissues

(35). Meristem size is considered to be the length of the meristematic zone and the number of cortex cells in a file between the QC and the first elongated cell exhibiting vacuolization (38) after the transition zone (TZ) (34). Importantly, root growth is maintained and regulated by the activity of the RAM, where a balance between cell production (cell division) and differentiation needs to be coordinated to allow the formation of a meristem of stable size (34, 38).

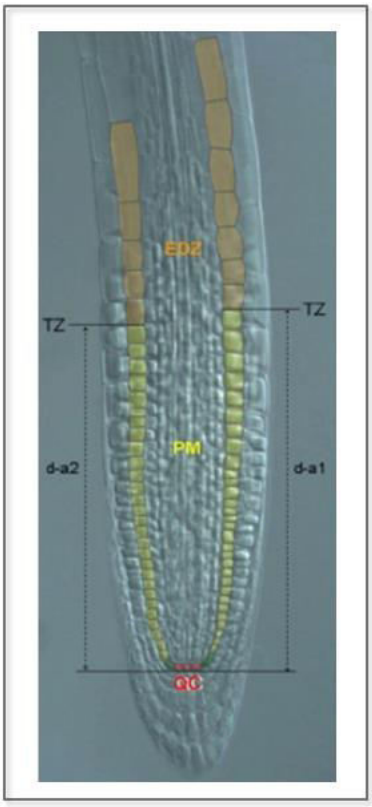

Figure 5.2. Structure of *Arabidopsis* root meristem. Bright-field microscopy image depicting the different zones of the *Arabidopsis* RAM: proximal meristem (PM) (with cortical cell files in yellow); elongation-differentiation zone (EDZ) (with cortical cell files in orange); transition zone (TZ); and quiescent center (QC) (cells in red). The distance (d-a1 and d-a2) between the TZ and the QC is the meristem size (µm). Extracted from Ubeda-Tomás *et al.,* 2009 (35).

In this context, GAs have been reported to regulate cell division in the RAM (34, 35). It has been established that root growth rate is proportional to the rate of root cell production (cell division of the RAM), RAM size and the number of

meristematic cells (35). Several evidences implicate the GA signaling pathway in the regulation of these processes.

First, plants treated with PAC or GA biosynthetic mutants show reduced cell production and root growth rate and a smaller meristem size compared to wild-type. Interestingly, GA treatment was able to rescue RAM size (35). Furthermore, using transgenic lines with the mitotic marker cyclin *CYCB1;1* fused to the GUS reporter, it has been demonstrated that PAC treatment significantly decreases mitotic cell number, and this phenotype is rescued by GA addition. These results indicate that GAs regulate RAM size by promoting mitotic activity. Thus, they are required to promote and maintain meristem size and therefore, root growth (35)

Second, it has been demonstrated that GAs promote root cell division in a DELLA-dependent manner, as the reduced cell division of the GA-deficient mutant *ga1-3* could be restored by mutating the DELLA proteins RGA and GAI (35). Redundantly, Achard *et al*., 2009 (34) showed that GAs modulate cell cycle activity in the RAM through DELLAs by visualizing the cell cycle marker *CYCB1;1::GUS* in GA-signaling mutants. In accordance, targeting the expression of the dominant *gai-1* in the RAM disrupts cell proliferation, because it reduced drastically the number of dividing cells and root growth (34).

Third, one of the molecular mechanisms DELLAs use to restrain cell production has been unraveled, as it has been reported that DELLAs enhance the levels of the cell cycle inhibitors *Kip-related protein 2 (KRP2)* and *SIAMESE (SIM)* (34). Nevertheless, GAs repress the expression of *KRP2* and members of the *SIM* family by overcoming DELLA-mediated promotion.

Thus, it seems clear that GA signaling controls cell division and meristem size in the RAM, at least in part by modulating the expression levels of cell cycle inhibitors, and therefore, root growth. Specifically, DELLAs restrain root growth by enhancing the accumulation of cell cycle inhibitors and so, cell proliferation. Besides, these results point out the importance of GAs during root development to maintain a correct root meristem size, by regulating cell proliferation through DELLA protein degradation. In the next section, we address the involvement of TCPs in germination, and specifically in cell proliferation.

4. Class I TCPs involvement in germination

As it is explained in section 7.1 of the General Introduction, one of the objectives of this Thesis is to unravel the role of DELLAs in germination trough their interaction with class I TCPs (*TCP14* and *TCP15*). In this sense, the involvement of *TCP14* in germination has been investigated by Tatematsu *et al*., 2008 (39). By transcriptomic analysis, they found that the transcript levels of 12 *TCP* genes are up-regulated during seed germination, suggesting a redundant function of TCPs in this process. Interestingly, they found out that among all the up-regulated genes prior to seed germination, the site II motif, a known *cis* element target of TCP TFs is over-represented. This indicates that induction of TCPs after imbibition could implicate the activation of their downstream pathways (39). Among the up-regulated *TCP* genes, *TCP14* transcript levels greatly increased after imbibition and it was the most abundantly expressed family member in 24 h-imbibed seed, so it was chosen for further studies. Indeed, germination of freshly harvested *tcp14* mutant seeds was significantly delayed (39), indicating that *TCP14* is involved in the regulation of seed germination.

Expression analysis of *TCP14* in dissected embryos and its surrounding tissues (endosperm and testa) and *in situ* and reporter gene analysis at 24 h of imbibition revealed that the transcript and protein was mostly present in embryos, specifically in the vascular tissues of hypocotyls. This indicates that *TCP14* acts primarily in the embryo rather than the endosperm. Remarkably, in the same article, it is reported the induction of site II motif-containing genes (putative targets of TCPs) in the root tip during germination. This could indicate that these genes in the root tip might be activated by other TCPs, or that *TCP14* regulates radicle growth in a non-cell-autonomous manner.

In this sense, they examined whether loss-of-function of *TCP14* affects gene expression in embryo radicles, and notably, they analyzed the expression pattern of a *CYCB1;1::GUS* reporter gene (direct target of TCP14 (40)) in the *tcp14* mutant after imbibition. As it has already mentioned, this CYC is widely used as a mitotic marker, since it marks G2/M phase of the cell cycle. Thus, the experiment also tests the involvement of *TCP14* in cell division in the root tip. They found GUS staining in the radicle of wild-type seedlings, whereas very weak signal was detectable in the *tcp14* mutant (39), indicating that *TCP14* regulates embryonic

growth potential, possibly by positively regulating cell division in the radicle even though it does not seem to be expressed in this region.

As a matter of fact, the role of class I TCPs, and specifically *TCP14* and *TCP15,* in the regulation of cell proliferation has been further addressed in the literature (30, 40).

Interestingly, disruption of *TCP14* and *TCP15* affects plant stature by reducing stem internode elongation as well. *tcp14*, *tcp15* and double *tcp14 tcp15* mutants display a significant reduction in inflorescence height and shorter internodes (30). This defect is associated with a reduction of cell proliferation, as *TCP14* and *TCP15* are expressed in the internodes, where they are found to repress the expression of five effectors of cell division. Specifically, the expression of the mitosis markers *CYCB1;1*, *CYCB1;2*, *CYCA1;1*, *CDC20* and *CDKB2;1* was reduced in double mutant apices relative to the wild-type (30). These results show that TCP14 and TCP15 act redundantly to promote cell proliferation in young stem internodes, and together modulate plant stature.

During the course of this Thesis, an article depicting class I TCPs regulation of cell proliferation in shoot apices was published (40). In this article, Davière *et al*., 2014, showed that DELLAs modulate cell division by directly binding and regulating the activity of class I TCPs (TCP14 and TCP15) in *Arabidopsis* inflorescence shoot apices. As the results of this article overlap the results of this Thesis, we will discuss it in the General Discussion.

Therefore, it seems clear that class I TCPs, and especially *TCP14* and *TCP15* promote cell cycle progression in different contexts on the plant, including root growth (30, 39, 40). On the other hand, cell division in the embryo RAM before radicle protrusion has been identified as one of the processes required for germination (39). In Chapter II of this Thesis we address the possibility that TCP14 and TCP15 regulate cell division in the RAM before germination and whether this aspect is modulated by DELLAs, given the physical interaction of both factors.

5. Proposal of the hypothesis: DELLAs function in the RAM

As previously presented, GAs have a crucial role promoting germination. In this process, the activation of cell division in the RAM of the embryonic radicle before germination is an integral part of germination, as it promotes embryo growth and radicle protrusion through the seed coat. In *Arabidopsis*, this activation implies the activation of cell-cycle elements (3). Although GAs have been found to regulate cell division in the RAM (3, 34, 35), the underlying molecular mechanism is still unknown. In this line, the Class I TCPs *TCP14* and *TCP15* have been involved in the control cell proliferation in root growth and other contexts of the plant (30, 39, 40). Moreover, the activity of *TCP14* is necessary to undergo seed germination (39). The finding that DELLA proteins physically interact with TCP14 and TCP15 suggests that DELLAs could restrain germination by inhibiting the activity of these TCPs activating cell cycle genes. This way, GAs could promote germination by releasing this inhibitory mechanism.

Therefore, we hypothesize that *TCP14* and *TCP15* mediate the GA-dependent activation of the cell cycle during germination by their physical interaction with DELLA proteins.

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6. Results and Discussion of Chapter II: Regulation of the root apical meristem by DELLAs trough Class I-TCPs

Seed germination involves the coordination of several genetic and physiological pathways (1) and hormones play important roles in the switch from dormant to germinating seeds. During seed maturation, a quiescent state is imposed on the embryo. One of the mechanisms that repress germination at this stage is the one controlled by abscisic acid (ABA). This way, the seed is able to resist adverse environmental conditions. Only when these conditions are favorable, the mechanisms that promote germination (such as GA biosynthesis and a decrease in ABA level) start working to resume embryo growth. This process implies the activation of cell division in the embryo and early changes in the expression of cell-cycle elements (2), that eventually leads to the protrusion of the root through the seed coat. GAs play an essential role in this process, given that they regulate cell division in the RAM (3, 4) and had been found to be required for the rupture of the testa and the endosperm (5).

Interestingly, the Class I TCP transcription factors TCP14 and TCP15 have been proposed to regulate cell proliferation (6) and TCP14 activity has been highly linked with the germination process (7).

Taking all these observations into account, we hypothesized that TCP14 and TCP15 could have a role mediating GA-dependent activation of the cell cycle during germination.

1. TCP14 and TCP15 are required for the promotion of the germination by gibberellin

TCP14 activity had been previously demonstrated to be needed for germination. It was shown that freshly harvested *tcp14* mutant seeds present a delay in germination. Furthermore, TCP14 is involved in the hormonal regulation of seed germination; given that its expression level is reduced in imbibed seeds of the *ga1-3* mutant and *tcp14* mutants are hypersensitive to the negative effects on germination of paclobutrazol (7). These observations suggest a functional relationship between this TF and GAs in this process.

In this sense, it has been proposed in the literature that TCP14 and TCP15 are closely related phylogenetically and can operate together in some processes (8),

such as internode length and leaf development (6). For this reason, we wanted to evaluate if TCP15 activity is also necessary for seed germination.

First, we performed a germination assay with freshly harvested seeds of wild type, two different loss-of-function mutants in *TCP14* and *TCP15*, and the *tcp14 tcp15* double mutants. As shown in Figure 6.1, the percentage of germination of both *tcp15-2* and *tcp15-3* was greatly reduced (52% and 41.5% respectively) 4 days after sowing, even more than the single *tcp14* mutants. Remarkably, the germination capacity in the double mutants is even smaller. These results suggest that both TCPs may act redundantly promoting germination.

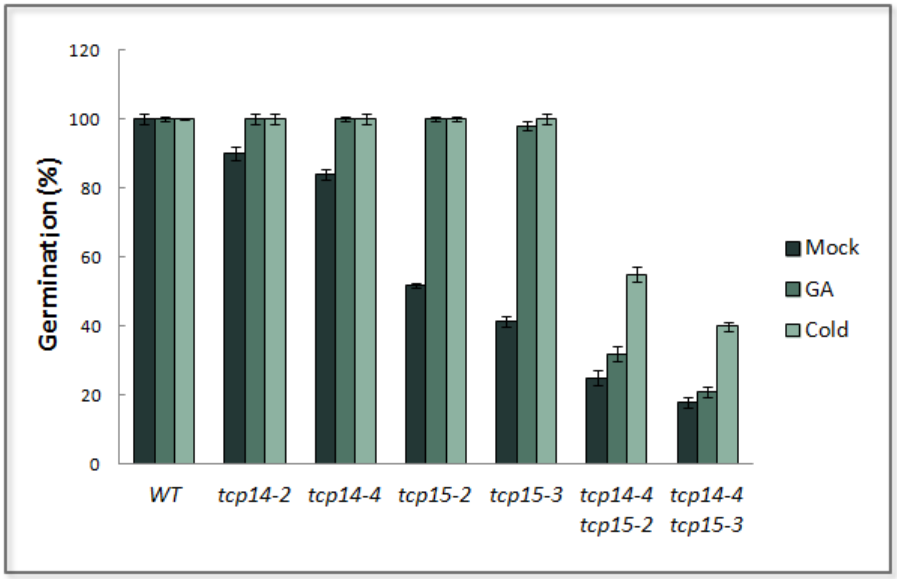

Figure 6.1. TCP14 and TCP15 are needed for *Arabidopsis* germination. Percentage of germination of wild type, simple and double mutants of *TCP14* and *TCP15* in three different conditions: mock (black bars), 1 mM GA_3 treatment (dark green bars) and stratification treatment, 5 days in dark at 4ºC (light green bars). Freshly harvested seeds were used in these assays.

To assess whether these TCPs and GAs are promoting seed germination in the same molecular pathway, we carried out germination assays of the same genotypes exposed to different treatments. On the one hand, we added 1 mM GA_3 to the medium, and on the other hand we maintained seeds in dark at 4ºC for 5 days before the exposure to light, since it had been previously demonstrated that cold triggers the accumulation of transcripts encoding GA biosynthetic enzymes (9) and enhances GA accumulation (10). Results in Figure 6.1 show that

neither GA application nor exposure to cold were able to efficiently overcome this defect in the double mutants while a full recovery was obtained in the case of single mutants. These results are compatible with the idea that GAs promote germination through the redundant activity of TCP14 and TCP15.

2. TCP15 is expressed in developing embryos

It had been previously demonstrated that TCP14 is expressed in developing embryos and in seeds (7). In particular, TCP14 is the most abundantly expressed TCP family member in 24 hour-imbibed seeds and is expressed preferentially in embryos rather than in the endosperm or testa (7). *In situ* hybridization and GUS staining assays in 24 h-imbibed seeds show that the transcript is mainly detected in the vascular tissues of hypocotyls and, in a lesser extent, in the vascular tissues of cotyledons and roots (7).

Given the relevance of TCP15 in germination, where it would act redundantly with TCP14, we decided to compare the expression patterns of TCP14 and TCP15 in *Arabidopsis* embryos.

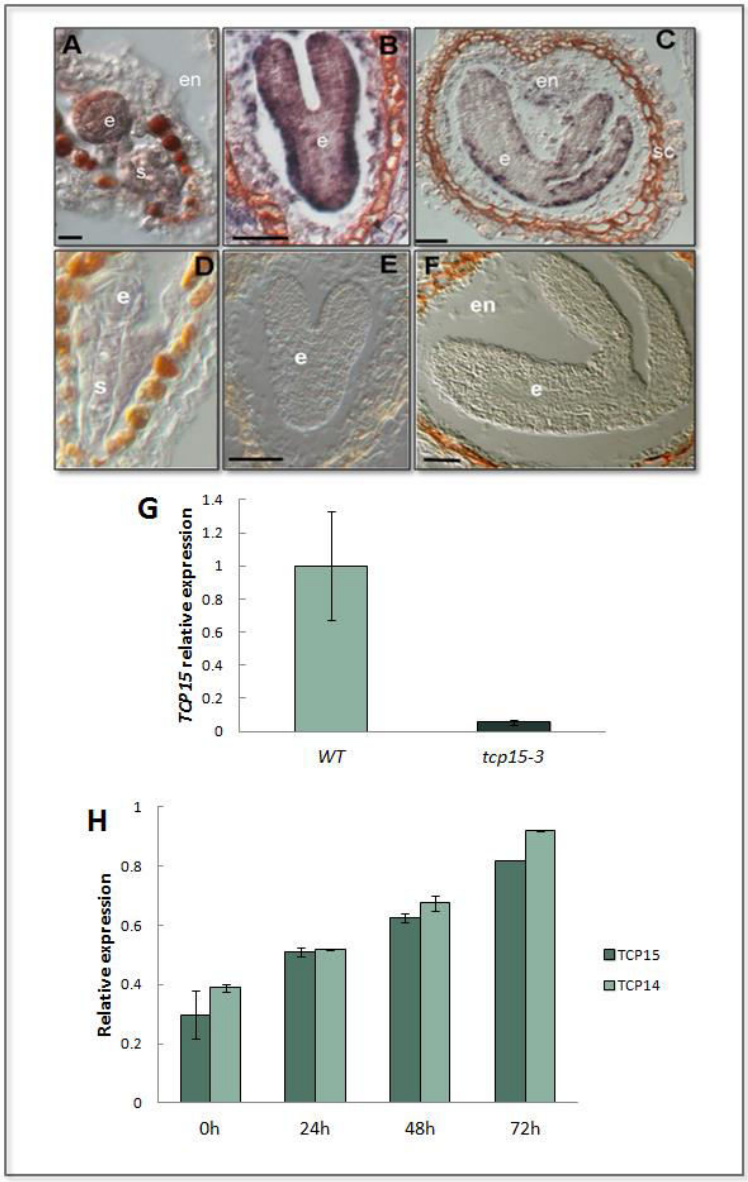

Figure 6.2. TCP14 and TCP15 are expressed in developing embryos. (A-F) *In situ* hybridizations of *TCP15* in *Arabidopsis* wild type (A-C) and *tcp15-3* seeds (D-F). Antisense probe was used in transversal sections of embryos in different developmental stages: globular (A,D), late heart/torpedo (B, E) and cotiledonary or mature (C, F). Scale bars: 20 µM (A,D); 50 µM (B,C,E,F). Letters: e, embryo; en, endosperm; s, suspensor; sc, seed coat. **(G)** Quantitative RT-PCR showing *TCP15* expression level in wild type and *tcp15-3* seeds imbibed for 24 hours **(H)** RT-qPCR experiment indicating TCP14 and TCP15 mRNA level at several time points during the germination of imbibed wild type seeds. Actin 2-8 is used as control gene.

We carried out *in situ* hybridization assays of *TCP15* in wild type and *tcp15-3* embryos at different developmental stages (Figure 6.2A-F). In wild type, *TCP15* mRNA was detected in developing embryos since globular stage, although its expression decreases in mature embryos. It is also expressed in the endosperm from the torpedo stage on. On the other hand, *TCP15* antisense probe was not detected in *tcp15* mutant embryos, indicating that the probe is specific. The very low *TCP15* expression level in the mutant was confirmed by RT-qPCR in *tcp15-3* seeds (Figure 6.2G).

The role of TCP14 and TCP15 in the germinating embryo was also supported by the observation that the level of expression of both genes increased progressively during at least the first three days after seed imbibition (Figure 6.2H).

3. DELLAs do not regulate TCP14 and TCP15 transcriptionally

Given that GAs seem to promote germination via TCP14 and TCP15 (Figure 6.1), we decided to investigate the mechanism by which these hormones would regulate TCP activity.

To test if *TCP* gene expression was under the control of GA signaling, we analyzed mRNA levels of both TFs in response to an increasing level of the DELLA protein GAI. To this end, we chose a genetic approach by using the *HS::gai-1* transgenic line (11) that expresses the dominant version of the DELLA protein GAI (*gai-1*) under the control of a temperature-inducible promoter. In this line a heat-shock treatment leads to *gai-1* accumulation.

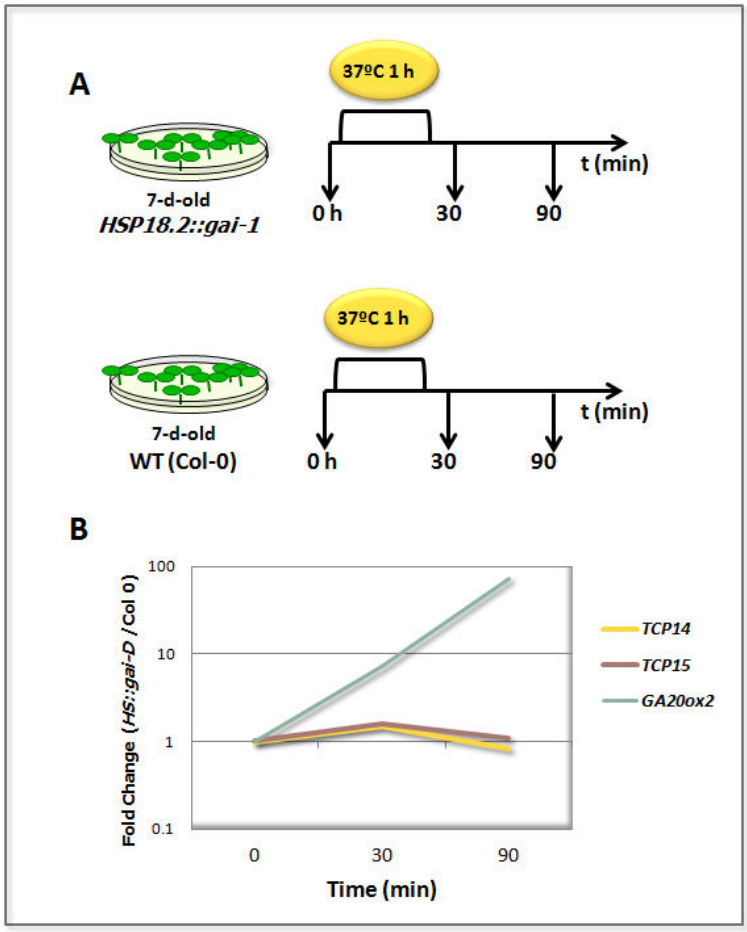

Figure 6.3. DELLA induction does not affect *TCP14* and *TCP15* mRNA levels in roots. **(A)** Diagram of the experimental setting. In the transgenic line (*HSP18.2::gai-1*), the dominant version of the DELLA protein GAI (*gai-1*) is induced by a heat shock (1 hour at 37ºC). Seven day-old seedlings of each genotype were used for the experiment. Root samples were collected at several time points before and after de heat shock and RT-qPCR analysis was performed. Wild-type (Col-0) plants followed the same procedure as a control. **(B)** RT-qPCR experiment showing the fold change of the mRNA level of *TCP14* (yellow) and *TCP15* (mauve) within all the time-course. *GA20ox2* (green) gene was used as a positive control.

As shown in Figure 6.3, the mRNA levels of *TCP14* and *TCP15* was not altered after the induction of *gai-1*, whereas the control gene *GA20ox1* was clearly induced even 30 minutes after the treatment. These results suggest that if DELLAs regulate TCP14 and TCP15 activity, it should be at the post-transcriptional level.

4. DELLAs interact with TCP14 and TCP15

It is widely acknowledged that DELLAs modulate multiple signaling pathways through protein-protein interaction with many TFs (reviewed for instance in Locascio et al., 2013 (12)). Therefore, the possibility that GAs promote germination by affecting DELLA-TCP14 and DELLA-TCP15 interactions was a very likely possibility.

Although the functions of the five DELLAs of *Arabidopsis* have been usually described as redundant, two DELLAs have been demonstrated to have a more relevant role in the regulation of germination: GAI and RGL2 (5). Consequently, we carried out yeast two hybrid assays to test the ability of these two DELLA proteins to interact with TCP14 and TCP15 (Figure 6.4). We found that both GAI and RGL2 are able to bind physically TCP14 and TCP15, indicating that a post-transcriptional regulation of these TF by DELLAs is plausible.

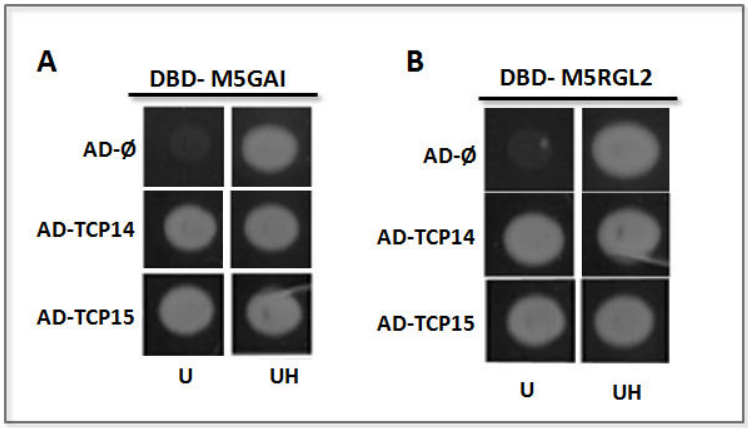

Figure 6.4. DELLA proteins interact with TCP14 and TCP15. Yeast two-hybrid assay of the interaction between truncated versions of GAI (M5GAI) **(A)** and RGL2 (M5RGL2) **(B)** with TCP14 and TCP15. (DBD, DNA binding domain; AD, activation domain; AD-ϕ, activation domain empty vector; U, uracil; H, histidine).

To test if the interactions would also take place *in planta*, we first performed Bimolecular Fluorescence Complementation assays (BiFC) in *Nicotiana benthamiana* leaves. This analysis showed that the fluorescence from the reconstituted YFP appeared in the nuclei of epidermal cells of leaves co-infiltrated with YFN-TCP14 or YFN-TCP15, and YFC-GAI or YFC-RGL2, whereas fluorescence in the control leaves was below the threshold level (Figure 6.5A). Similarly, myc-GAI

was efficiently pulled down with anti-HA antibodies in *Nicotiana benthamiana* leaves that co-expressed HA-TCP14, confirming the interaction between the TCPs and GAI and RGL2 (Figure 6.5B).

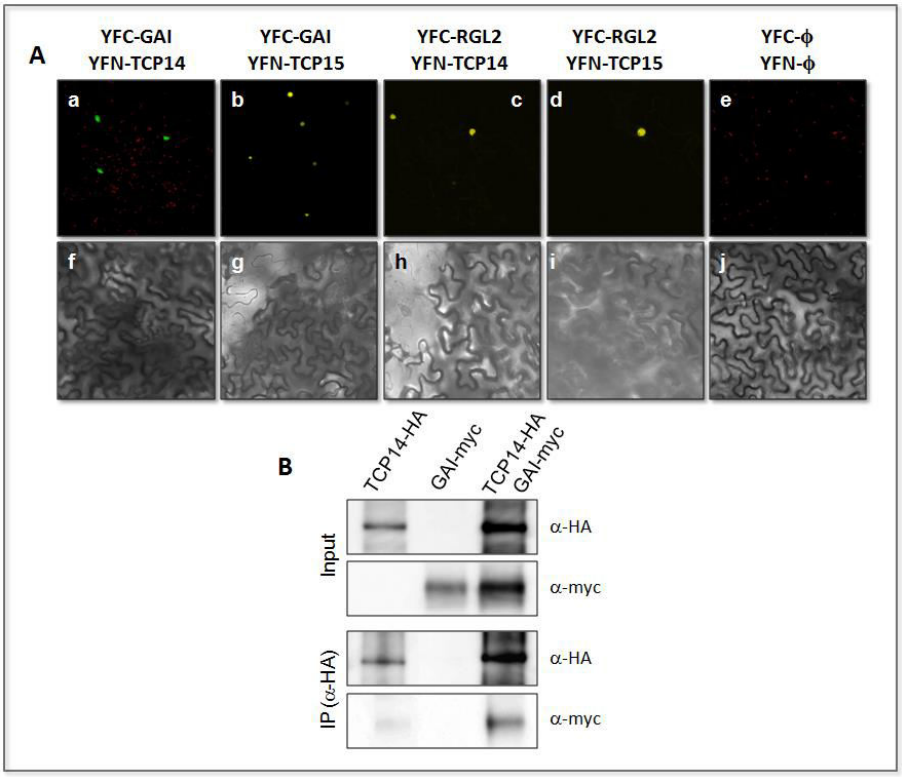

Figure 6.5. TCP14 and TCP15 interact with DELLA proteins *in planta*. (A) Bimolecular fluorescence complementation analysis showing the interaction of TCP14 (a,c) and TCP15 (b,d) proteins with GAI (a,b) and RGL2 (c,d) in *nuclei* of *N. benthamiana* epidermal leaf cells. The empty vectors of each construction were used as a negative control (e). The lower panel shows the visible chanel of each picture. **(B)** Co-immunoprecipitation of myc-GAI and HA-TCP14 expressed in *N. benthamiana leaves*. TCP14 was immunoprecipitated using anti-HA (α-HA) conjugated paramagnetic beads, and TCP14 and GAI were detected in immunoblots using anti-HA and anti-myc, respectively. Note that GAI was co-immunoprecipitated with TCP14. The sizes of the bands correspond to the expected molecular weight of the fusion proteins.

As a matter of fact, in the course of this Thesis work, it was reported that TCP14 is able to interact with the five DELLAs from *Arabidopsis*, and TCP15 was proved to interact with at least RGA (13). Further co-immunoprecipitation analysis demonstrated that TCP14 and RGA also interact *in planta*. Besides, a recent Y2H screen using the GRAS domain of GAI as bait against an arrayed library containing

approximately 1,200 TFs from *Arabidopsis* showed that DELLAs can interact with 12 members of the TCP family (14). Among them, TCP14, TCP15 and four other members of the Class I TCPs (TCP8, TCP16, TCP19 and TCP23).

5. GAI inactivates TCP14 upon interaction

DELLAs have been reported to operate by at least two different mechanisms. They can either repress their targets by binding to TFs and impairing their capacity to activate transcription (sequestration mechanism) or they can function in the chromatin either as transactivation factors (15) or as co-repressors (16).

As it is indicated in the introduction, TCP transcription factors have a bHLH-like domain that is very similar to the one of PIFs and other bHLH proteins (17) and it is well established in the literature that DELLAs are able to interact with and sequestrate this family of transcription factors (18, 19). Therefore, our hypothesis would be that DELLAs bind physically TCPs and inhibit their transcriptional activity, rather than acting as transactivation factors.

In order to test this hypothesis, we performed transactivation assays in *N. benthamiana* leaves. To this end, we prepared a synthetic promoter consisting of six repeated copies of the Class I TCP consensus binding site (GTGGGCCCAC) (20) separated by a 6-nucleotide spacer (AAAAAA), and placed it upstream of a minimal 35S promoter and the translational enhancer omega to control the expression of the *Luciferase* reporter gene (Figure 6.6A).

Next, we expressed this synthetic promoter alone and in the presence of a construct carrying a translational fusion of TCP14 with the transcriptional activator VP16, which caused an increase in the luciferase activity with respect to the control (Figure 6.6B). Remarkably, this activation was partially reversed when GAI was co-expressed, whereas GAI alone did not affect the activity of the reporter. In addition, we checked by western blot that the protein levels of TCP14 and GAI in all the conditions are comparable (Figure 6.6C).

In this context, our results are in line with the exhaustive studies made in the laboratory of Patrick Achard (13), where they performed electrophoretic mobility shift assay (EMSA), chromatin immunoprecipitation (ChIP) and transient expression assays and they demonstrated that DELLA-Class I TCP interaction inhibit TCP DNA-binding activity and therefore, its transcriptional function.

Moreover, they also give a mechanistic explanation for these results. It is well known that the TCP domain is responsible of the DNA-binding capacity of TCPs. They show that the DELLAs prevent TCP DNA-binding activity by interacting with their TCP domain, in a similar manner that they act with bHLH proteins such as PIF3 or PIF4 (18, 19).

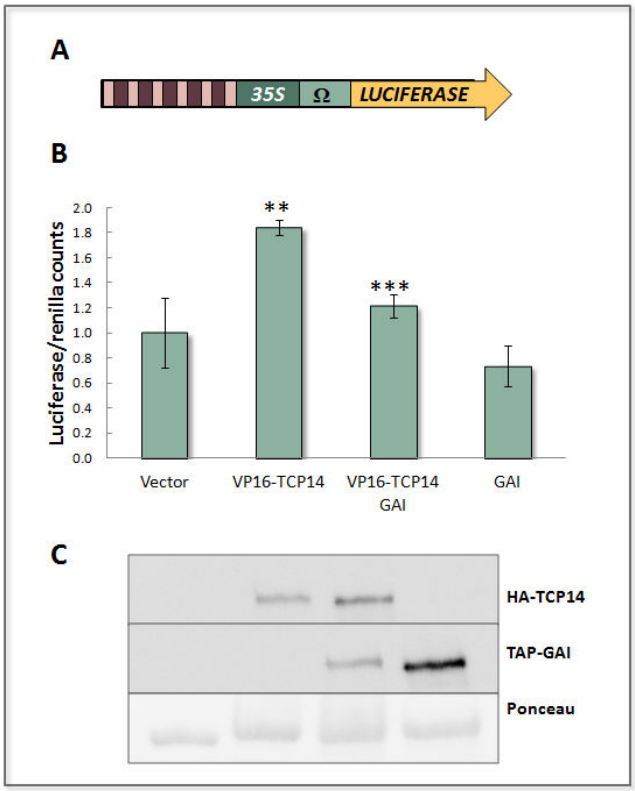

Figure 6.6. GAI inactivates TCP14 upon interaction. (A) Design of the synthetic promoter used for the transient expression assays in *N. Benthamiana* leaves. It consist of six repeated copies of the Class I-TCP consensus binding site (pink boxes) separated by a 6-nucleotide spacer (AAAAAA, red boxes) placed upstream of a minimal 35S promoter (dark green box) and the translational enhancer *omega* (light green box) to control the expression of the *Luciferase* reporter gene (orange arrow). *Renilla Luciferase* under 35S promoter in the same construct was used as control. **(B)** Transient expression assays for TCP14 (Vector, infiltration with the synthetic promoter alone used as a negative control; VP16, transcriptional activator). **(C)** Western blot corresponding to the transient expression assay for TCP14. TCP14 and GAI were detected in immunoblots using anti-HA and anti-myc, respectively. Ponceau was used as a load control. Statistic differences are shown (P-value<0.05).

6. Role of TCP14 and TCP15 regulating cell division in the root apical meristem

The physiological observation that TCP14 and TCP15 act downstream of GAs in the promotion of germination provides a possible biological framework to test the relevance of the molecular interaction between DELLAs and TCPs. But which aspect of germination would be directly regulated by the GA-TCP module?

It is well established that Class I TCPs stimulate cell proliferation by activating the transcription of several cell-cycle genes. This was first indicated by Kosugi *et al*. in 1997 (21), showing that two TCP proteins of rice, PCF1 and PCF2, bind a conserved region (GGNCCCAC) in the promoter of the *PROLIFERATING CELL NUCLEAR ANTIGEN (PCNA)* gene (21). Further research demonstrate that these TFs interact with promoters of several cell-cycle genes such as the cyclins CYCA2;3 and CYCB1;1, PCNA2, and RETINOBLASTOMA-RELATED 1 (RBR1) (22-25). In fact, TCP14 and TC15 have been found to jointly regulate plant stature by promoting cell proliferation in young internodes (6) and in the inflorescence shoot apex (13). What is more, TCP14 have been demonstrated to positively regulate *CYC1* in the radicle of 48 hours-imbibed seedlings (7).

Remarkably, the germination process requires active cell division at embryonic root tips to cause root protrusion (2), followed by a sequential activation of cell division in cotyledons, SAM and secondary meristems. In the dry seed, cells of the embryo are arrested in the G1 phase of the cell cycle, and activation of cell division is essential for the success of the germination. Moreover, transcriptional studies demonstrate that several cyclins (CYCs) are greatly induced in the RAM before root emergence and these early activated CYCs play key roles in regulating the extent of cell cycle activation and root emergence. In other words, CYCs regulate cell cycle reentry during RAM activation to promote radicle growth, root protrusion and a successful germination.

Taking all these data into account, we hypothesized that TCP14 and TCP15 could mediate the activation of cell-cycle genes in the RAM early in the germination process and this would lead to root emergence. Therefore, we investigated whether these two TCPs are able to regulate cell division at the root apex of germinating embryos.

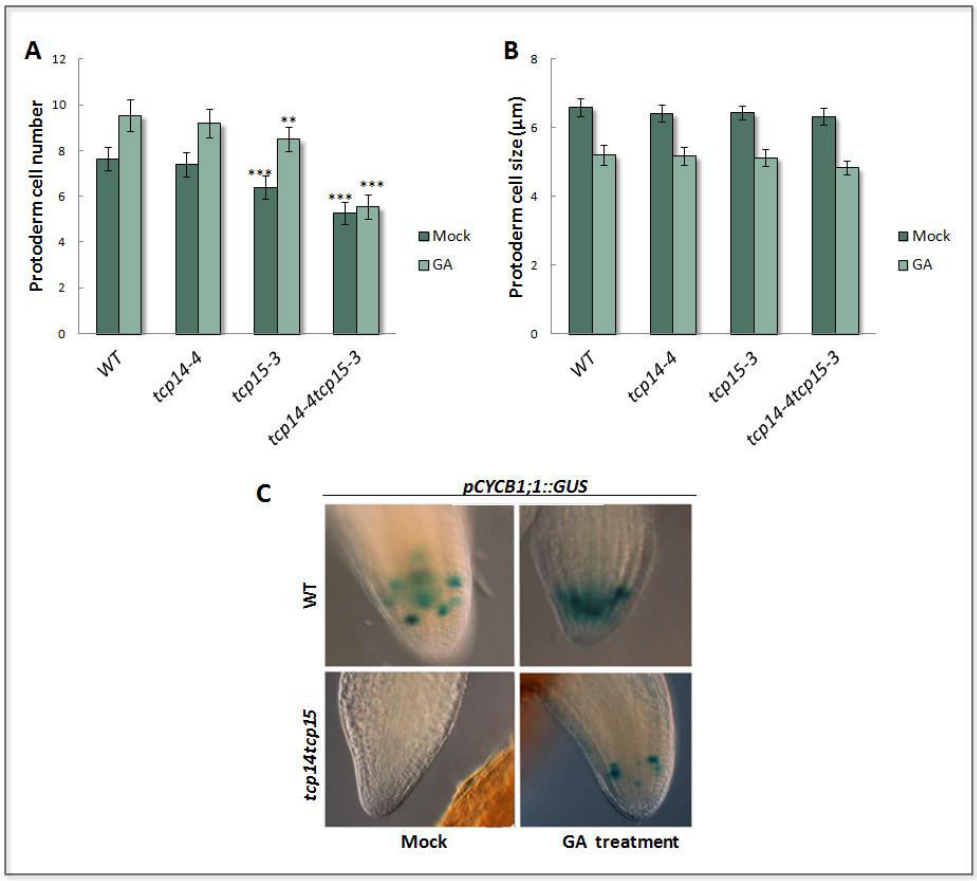

Figure 6.7. TCP14 and TCP15 regulate cell division but not cell expansion in the RAM. Graphs showing cell number **(A)** and cell size **(B)** of protoderm cells of the RAM at mature embryos in wild type, single and double mutants in two different conditions: mock (dark green bars) and 1 µM GA_3 treatment (light green bars). Statistic differences according with t test analysis are shown (***, P<0.0001; **, P=0.0023). **(C)** The transgenic line *pCYCB1;1::GUS* in wild type and *tcp14-4 tcp15-3* double mutant genetic backgrounds is used to carry out GUS staining assays in embryos. The expression pattern is evaluated in two different conditions: mock and 1 µM GA_3 treatment, as indicated.

We measured the number and size of protoderm cells in the RAM (Figure 6.7 A and B, dark green bars) of wild type, single and double mutants of TCP14 and TCP15 mature embryos. Whereas cell size was similar in all genotypes, cell number was greatly and significantly decreased in the double mutant *tcp14-4 tcp15-3* and, to a lesser extent, in the *tcp15-3* mutant. These results indicate that TCP15 and also TCP14 (its important role is depicted by the great reduction of cell number in the double mutant) are relevant factors promoting cell division and

proliferation in the RAM during germination and they act jointly constituting a specific module of regulation in the germination process. Remarkably, the module DELLAs/TCPs seems to exert its control specifically over cell proliferation but not over cell size.

To further evaluate the involvement of GA signaling in this module of regulation, we repeated the measurements after GA application (Figure 6.7 A and B, light green bars). Interestingly, GA application greatly increased the cell number in the *tcp15-3* mutant RAM, although it was not sufficient to restore it to control level. Moreover, GA treatment had no effect in the double mutant and does not affect cell size in the RAM.

Interestingly, transgenic lines such as *pCYCB1;1::GUS* have been extensively used as cell division reporters (26). In particular, this line maks cells at the G2/M phase of the cell cycle. Therefore, to further support the role of TCP14 and TCP15 controlling cell division and proliferation at the embryonic RAM, we assessed the activity of *CYCB1;1::GUS* in different genetic backgrounds and conditions (Figure 6.7C). Results show that GUS staining is completely abolished in the double mutant background in normal conditions, in accordance with our previous observations. When plants are exposed to a GA treatment, it is clearly seen an increase in cell division in the wild type background, and the ability of GAs to promote this increase in CYCB1;1 activity was mostly suppressed in double mutant embryos.

These results are in agreement with the independent observation by the Achard group that TCP14 binds TCP binding motives in the promoter of CYCA2;3, CYCB1;1, PCNA2, and RBR1 (13). What is more, in the same study, an *in silico* analysis defined the most probable Class I TCP consensus motif (KHGGGVC) and it was found that 39% of the core cell-cycle genes (29 of 74 genes) have at least one TCP consensus motif within their 1 kb promoter sequences.

In other words, TCP14 and TCP15 constitute an essential module of regulation of the germination process by promoting cell division and proliferation in the RAM, and DELLAs inhibit this activity most likely by a sequestration mechanism, given their physical interaction. This model is in agreement with the observation that GA application did not restore cell number in the double mutant.

Nevertheless, this DELLA-TCP module seems to exert its control specifically over cell proliferation, and not cell size. The fact that a little number of cells is stained in the double mutant in response to GA might be due to other GA-dependent mechanisms involved in the promotion of cell cycle progression. Therefore, TCP14 and TCP15 seem to promote cell division in the RAM of germinating embryos as opposed to the situation in the quiescent state of the seed previous to germination, where DELLAs impair TCP function by their physical interaction.

Regarding to this subject, we have to take into account again recent studies made in *Arabidopsis* inflorescences by the group of Dr. Achard (13), which are closely related to our subject. They showed that TCP14 capacity to bind the promoter of several cell-cycle genes (*CYCA2;3, CYCB1;1, PCNA2*, and *RBR1*) decreased in inflorescences accumulating DELLAs (after PAC treatment) in comparison with GA-treated inflorescences. Furthermore, they performed transient expression assays in *Nicotiana benthamiana* leaves using a *pCYCB1;1::GUS* construct as a reporter for TCP14 transcriptional activity. They showed an increase in the reporter activity by TCP14 expression, whereas this induction is inhibited by RGA co-expression. In addition, they measured the expression levels of *CYCA2;3*, *CYCB1;1, PCNA2*, and *RBR1* in dissected inflorescence shoot apices treated and not treated with PAC of wild type, *tcp14-4 tcp15-3* double mutant and a transgenic line mutant in *TCP14* and *TCP15* and with reduced expression of *TCP8* and *TCP22*. Upon PAC treatment, they found an expression decrease in all cell-cycle genes in wild type and the double mutant, whereas the transgenic line lost a great deal of sensibility to the treatment. These results further confirm our hypothesis that DELLAs repress cell-cycle genes by sequestrating Class I TCPs. Also, this mechanism seems to be universal, given that it is taking place in developmental moments so different such as roots of germinating seeds and in inflorescences.

7. Role of TCP14 and TCP15 regulating root growth in later stages of development

To investigate whether the regulation of cell division at the RAM by the GA-TCP module would also extend beyond germination, we also measured the lengths of roots of young seedlings of wild type, *tcp14* and *tcp15* single mutants and the double mutant, since this trait might be affected as a consequence of a defect in the cell division at the RAM (Figure 6.8 A and B, dark green bars).

Both the single and the double mutants presented a shorter root than that of the wild type in early stages of the development (four days after germination, Figure 6.8A). However, in the case of the single mutants, this difference in root length decreased with the age of the plant, becoming non significant in the case of *tcp14* (Figure 6.8B). Interestingly, the roots of the double mutant were still significantly shorter than wild-type roots even seven days after germination.

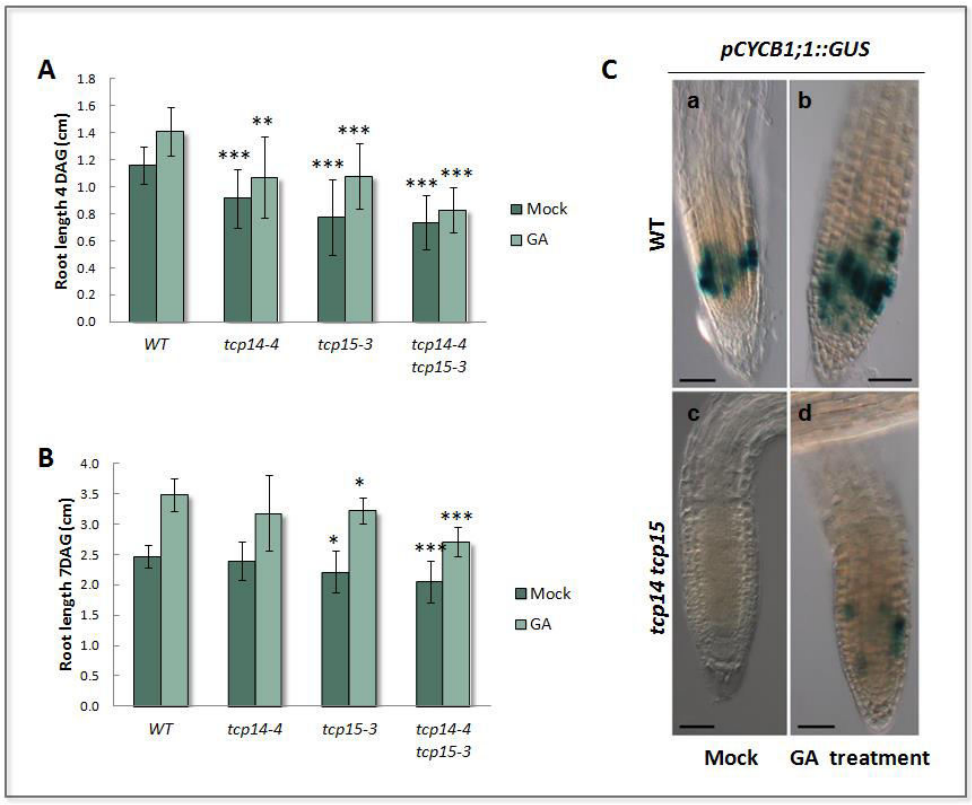

Figure 6.8. TCP14 and TCP15 regulate root growth. (A, B) Graphs showing root length of wild type, single and double mutant seedlings at 4 **(A)** and 7 **(B)** days after germination (DAG) in two different conditions: mock (dark green bars) and 1 µM GA_3 treatment (light green bars). Asterisks indicate significant statistical differences respect to the WT control according to the t test (P-value<0.05) **(C)** *pCYCB1;1::GUS* expression in 7 day-old seedling roots in wild type (a,b) and *tcp14-4 tcp15-3* double mutant (c,d) backgrounds. Staining of the mock (a, c) and 1 µM GA_3 treatment (b, d) are shown (Scale =100 µM).

Next, we evaluated the role of GA in this process, so we treated the plants with GA and performed the same experiment (Figure 6.8, light green bars). As it can be seen, GAs promoted root elongation in all the cases, especially in the single

mutants, and its effect was more notorious in later stages of root growth (seven days after germination), when GA application completely restored the phenotype in *tcp14* and the difference between *tcp15* and the control was reduced. It is important to remark that the double mutant was less sensitive to the treatment and its final root length was significantly shorter and seriously compromised even seven days after germination. The fact that there was a slight increase in root length in the double mutant after GA application could be due to additional mechanisms by which GAs facilitate this trait.

In agreement with the effect of GAs on root length, the expression of the *pCYCB1;1::GUS* marker was induced after GA application in the RAM of wild-type seedlings, but not in the *tcp14 tcp15* double mutant, also indicating that final root size is strongly influenced by cell divisions at the RAM (Figure 6.8C).

All in all, these results indicate that the TCP14/TCP15 module supports cell division in the *Arabidopsis* RAM by activating cell-cycle genes and is required for GAs to promote root growth post-embrionically, as well as embrionically. In recent years several works have provided genetic support for the involvement of the interactors of DELLAs in the control of seed germination (27, 28). Moreover, control of DELLAs over cell-cycle progression at the RAM has also been reported (3, 4). Nonetheless, a molecular mechanism explaining the action of DELLAs on seed germination has not been proposed. Work in this Thesis provide evidence for such a mechanism, showing that the transcriptional regulators DELLAs maintain the embryo in a quiescent state by restricting cell-cycle progression in the embryonic RAM through the inhibition of TCP14 and TCP15 activities upon interaction. Given the strong influence of environmental conditions on DELLA levels (29), the DELLA-TCP module acts as a relay for environmental information into the cell cycle at the RAM to coordinate root emergence with other events during seed germination.

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7. General Discussion

Plant development is a repetitive process of organ initiation from primary meristems (SAM and RAM). As plants are sessile organisms, they need to adjust their growth and developmental processes according to environmental conditions to guarantee their survival. To achieve this plasticity, plants perceive and integrate environmental conditions, and translate these signals to the intrinsic genetic programs of the plant (1). A paradigmatic example of the mechanisms that support this interplay between the plant and the environment is the regulation of DELLA stability by environmentally modulated GA metabolism, such that cellular DELLA levels turn out to be directly dependent on light, temperature and other external cues. Through physical interaction with a wide variety of TFs, DELLAs can modulate for instance the balance between growth and defense when plants are subjected to adverse conditions, such as cold, salt and osmotic stress (2). This is a critical issue for plants, since the ability to adaptively restrain or resume growth ensures plant survival. In this context, **the results shown in this Thesis provide an initial molecular framework to study how the activity of primary meristems is regulated by the environment**:

(i) GAs influence the size of the SAM by interfering with the mutual regulation between DELLAs and KNOX factors.

(ii) GAs trigger cell divisions in the RAM that permit root protrusion during germination, by relieving DELLA-dependent repression of Class I TCPs in the embryos.

1. DELLA-TCP interaction, an example of tissue specific signaling

The discovery the DELLAs can interact with multiple TFs in higher plants represented a molecular mechanism by which a single hormone could exert different effects in different spatial and temporal contexts. In other words, DELLAs would have access to different partner TFs in different tissues or developmental stages, and physical interaction with them would consequently affect very different transcriptomes. Here we have provided new evidence for differential effects of DELLAs in shoot and root tips, depending on the type of TCP transcription factors expressed in each region of the plant. Importantly, the molecular mechanism by which DELLAs act in both cases is the same: the inhibition of TCPs' ability to regulate their downstream targets. However, the

consequence of this regulation in the shoot apex is the control of meristematic cell size and the contribution to the establishment of boundaries between differentiated and undifferentiated regions of the meristem, while in the root apex the consequence is the regulation of cell divisions (3, 4).

With respect to the molecular mechanism by which DELLAs regulate TCP activity, we favour the model in which the interaction prevents TCP binding to target promoters for several reasons. Curiously, modelling studies of the three-dimensional structure of the DNA binding domain of TCPs conclude that it resembles the structure of bHLH domains with high confidence (5). This suggests that similar motifs recognized by DELLAs are present in the DNA binding domains of both TF families. Moreover, in support of our transactivation assays in *N. benthamiana*, the group of Dr. Patrick Achard found during the course of this Thesis using electrophoretic mobility shift assays (EMSA) and chromatin immunoprecipitation (ChIP) that the DELLA-Class I TCP interaction inhibits TCP DNA-binding activity (6).

2. DELLA function in meristems: a possible link with the environment

It has been widely documented how plant developmental decisions and growth rate are tightly influenced by the environment. Here we propose that DELLAs would transduce environmental information to the activity of at least the primary meristems. Indications that plant meristems change their activity depending on environmental cues are very clear. For instance, cell division at the RAM is triggered by exposure of seeds to germination-promoting signals such as the presence of water and particular light wavelengths (7). And cell division rate at the SAM and the size of the apical dome increase during the transition from the vegetative to the reproductive phase of growth, which is triggered by the inductive photoperiodic signals (8). Moreover, stress signals also modify the homeostasis of the primary meristems, to allow better adaptation to the different circumstances encountered by the plants. This is for example the case of high temperatures, which arrest the activity of the SAM while the plant undergoes physiological reprogramming to cope with these factors (9). Similarly, it has been shown that the RAM transiently decreases its activity upon exposure to high salt concentration, but later resumes growth even if the stress factor is still present (10). And phosphate availability critically alters the activity of the dormant RAMs

of secondary roots, thereby promoting changes in the root architecture that favour phosphate detection and assimilation (11, 12).

Does the interaction between DELLAs and TCPs mediate environmental signaling in the primary meristems, as we propose? In this Thesis, we have not explored experimentally the direct implication of DELLAs in the transduction of environmental signals specifically to meristematic cells. However, at least three lines of evidence support this hypothesis, which should be tested in the future.

First, *dellaKO* mutants have been shown to elude the growth arrest imposed by different types of stress, such as exposure to salt (13). In this case, most of the growth of the plant is supported by cell divisions at the meristems, so it is quite reasonable that DELLAs participate in this particular process depending on the environmental situation. Although other unidentified interactors of DELLAs might be the target for stress-imposed growth arrest, the observation that TCPs participate in the regulation of key cell-cycle genes seems to indicate that this particular interaction is probably critical for the control of meristematic activity under stress.

Second, our transcriptomic analysis of DELLA-dependent KNAT1 targets shows that this category of genes largely includes those involved in environmental regulation, or in processes directly regulated by the environment (such as response to light and abiotic stresses in Table 4.4), suggesting that the DELLA-TCP-KNOX module probably mediates adaptation of SAM activity to environmental conditions.

Third, the observation that GA levels increase dramatically (over 100-fold) at the *Arabidopsis* shoot apex during the transition to flowering (8) suggests that a mechanism to maintain SAM homeostasis must be in place, involving DELLAs. Remarkably, transcriptional analysis in the shoot apex demonstrated that the transcription of GA-negative feedback-regulated *GA20OX* and *GA3OX* genes were unchanged when the GA_4 levels started to increase, and expression of positively regulated *GA2OX* genes was increased (8). Importantly, the increase in size of the meristem during flowering is accompanied not only by an increase in GA input (8), but also by an increase in *KNOX* genes (*KNAT1* and *STM*) expression (eFP Browser (14)), that could explain the increase in *GA2OX* transcription. The observation that GA and KNAT1 levels increase in parallel during this stage supports that our model of KNAT1/GA balance might be also important during the transition from

vegetative to reproductive SAMs to guarantee a controlled growth at this temporal window and even later in the reproductive phase.

Finally, it will be interesting to test if the DELLA-TCP-KNOX module is also operating at the cambium during secondary growth. Unpublished observations in our lab have shown that DELLA genes are expressed in cambial cells, and *bp* mutants have been shown to display defects in vascular development (15). Considering that secondary growth (both in woody species and in herbaceous plants like Arabidopsis) is highly influenced by the environment, it is quite likely that at least DELLAs will participate in this regulation. If eventually proven, DELLAs would confirm and expand their role as regulatory players for environmental control of development.

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8. Conclusions

Results in this Thesis provide new evidence of the role of GAs controlling two different aspects of *Arabidopsis* primary meristems, cell expansion and cell division. In particular, GAs exert their action by releasing the inhibitory effect of DELLAs over two sub-classes of TCP TFs:

1. DELLAs inhibit CIN-TCP activity in the boundaries of the shoot apical meristem, guaranteeing *KNAT1* expression in that zone. KNAT1 and GAs promote cell expansion and, therefore, the balance between both activities is relevant to control meristem size.

2. The promotion of cell division by TCP14 and TCP15 in the root apical meristem before radicle protrusion is essential for germination, and, in later stages of development, for root growth. DELLAs impair cell division in the root meristem by inhibiting the activity of these TFs.

9. Materials and Methods

1. Plant material and growth conditions

Plant material

Arabidopsis thaliana accessions Col-0, L*er* and *No-0* were used as wild-types as indicated. A list of the previously described lines used in this work can be found in Tables I and II.

Table I: Previously generated lines used in Chapter I

Genotype	Background	Vector/Mutation	Reference
HS::gai-1 /HSP18.2::gai-1	Col-0	pTT101	Alabadí *et al*, The Plant Journal (2008)
pAS1::GUS	Col-0	pGreen	Iwakawa *et al* , The Plant Journal (2007)
pKNAT1::GUS	Col-0	Not described	Ori *et al*, Development (2000)
pGAI::GUS	Col-0	pBI101.1	Gallego-Giraldo *et al*, The Plant Journal (2014)
pRGA::GUS	Col-0	pOCA28	Gallego-Giraldo *et al*, The Plant Journal (2014)
pRGA::GFP-RGA	Col-0	pRG51	Provided by Dr. Salomé Prat
pKNAT1::KNAT1-CFP	Col-0	pMLBART	Rast-Somssich *et al*, Genes and Dev (2015)
bp-1	*Ler*	hyl methanesulfona	Venglat *et al*, PNAS (2001)
p35S::KNAT1	*No-0*	pBIN19	Lincoln *et al*, The Plant Cell (1994)

Table II: Previously generated lines used in Chapter II

Genotype	Background	Vector/Mutation	Reference
HS::gai-1 /HSP18.2::gai-1	Col-0	pTT101	Alabadí *et al*, The Plant Journal (2008)
tcp14-2	Col-0	T-DNA	Provided by Dr. Brendan Davies
tcp14-4	Col-0	T-DNA	Krysan *et al*, The Plant Cell (1999)
tcp15-2	Col-0	T-DNA	Provided by Dr. Brendan Davies
tcp15-3	Col-0	T-DNA	Alonso and Stepanova, Plant Functional Genomics (2003)
tcp14-4 tcp15-3	Col-0	T-DNA	Provided by Dr. Brendan Davies
tcp14-4 tcp15-2	Col-0	T-DNA	Provided by Dr. Brendan Davies
pCYCB1;1::GUS	Col-0	pCDG	Colon-Carmona *et al*, The Plant Journal (1999)

Seedling growth conditions

Seeds were surface sterilized with 70% (v/v) ethanol and 0.01% (v/v) Triton X-100 for 10 min, followed by 96% (v/v) ethanol for another 10 min. Then, seeds were sown on plates of growing medium (½ MS medium (Duchefa) 2.2 g/L, agar 0.8% (w/v), sucrose 20 g/L, at pH 5,7), and stratified at 4°C in darkness for 3-5 days. Plates were then moved to a Percival E-30B growth cabinet (Percival) under white fluorescent light (55–200 µmol m^{-2} s^{-1}) at 22°C for 8 hours. Seedlings were grown at 22°C under continuous light (55–65 µmol m^{-2} s^{-1}) the indicated time in most cases.

Plant growth conditions

Arabidopsis thaliana and *Nicotiana Benthamiana* plants were grown in the greenhouse, in 12 cm pots or in polystyrene growing pots. The soil was a mixture of peat moss, perlite and vermiculite in a 1:1:1 proportion. Plants were watered twice a week with the nutritive solution described in Table III. The photoperiod was long day (LD) with 16 hours light (22ºC, 130 µmol m-2s-1) and 8 hours dark (19ºC). Seeds were harvested when

plants had ceased flowering and siliques start to dehisce and stored in the dark at 4°C and 30% relative humidity.

Table III. Composition of the nutritive solution.

Compound	Dose (g/L)
H_3PO_4	0.15
$Ca(NO_3)_2$	0.82
KNO_3	0.2
K_2SO_4	0.35
$MgSO_4$	0.24
BMX (Micronutrients)	0.1

Hormonal treatments

Chemical treatments with GA_3 (Sigma) and the inhibitor of GA biosynthesis Paclobutrazol (PAC, Duchefa) were performed as indicated in each case (Results and Discussion). For long treatments, the compound was added to the solid growing medium in the moment of plate preparation, before it solidifies; whereas for short time treatments the chemical is added in the liquid growing medium, where plants were incubated.

2. Genetic engineering

During the course of this Thesis, several DNA constructs have been generated with different purposes. For this reason, diverse DNA manipulation and cloning techniques have been carried out.

2.1 Bacteria manipulation

The bacterial strains used in this Thesis are depicted in Table IV. They were grown at the indicated temperature in LB (Luria-Bertani) medium (1% tryptone, 0.5% yeast extract and 1% NaCl at pH 7). For solid medium, 1.5% Bacteriological Agar (Pronadisa) was added.

Table IV: Bacterial strains used

Microorganism	Strain	Transformation method	Growth temperature
Escherichia coli	TOP10	*Termo-competent*	37ºC
	DH5α	*Electro-competent*	
	DB3		
Agrobacterium tumefaciens	C58		28ºC

For bacteria transformation, approximately 200 ng of DNA were added to a frozen aliquot of competent cells (40 µL). Then, transformation of the microorganisms was

carried out. For termo-competent cells, the protocol of the manufacturer (Invitrogen®) was followed. In the case of the electro-competent strains, cells were transferred to an electroporation bucket (BioRad®) placed in ice and an electric pulse was applied (200 Ω, 25µF and 1.5 kV for *E. coli* and 440 Ω, 25µF and 1.44 kV for *A. tumefaciens*). Cells were recovered with 700 µL of liquid LB medium without antibiotics and placed at the growth temperature in continuous agitation for 1 hour, or in the case of *A. tumefaciens*, 4 hours. After incubation, cells (50-200 µL) were plated in solid LB medium supplemented with the selective combination of antibiotics to be later on incubated at the corresponding growth temperature. One day later (or three in the case of *A. tumefaciens*), resistant colonies carrying the desired plasmid were selected and inoculated in a 3 mL culture of liquid LB with the selection antibiotic. After 1-2 days, the culture was used for DNA extraction, or plant infiltration, in the case of *A. tumefaciens*.

2.2 Plasmid DNA extraction

For plasmid extraction, 3 mL of liquid cultures of the transformed bacteria grown in LB supplemented with the selective antibiotic were centrifuged 1 minute at 13.000 revolutions per minute (r.p.m.) and then the protocol of the E.Z.N.A plasmid miniprep Kit OMEGA BIO-tek was followed. DNA concentration, $A_{260/280}$ and $A_{260/230}$ was evaluated with a NanoDrop® ND-1000 spectrophotometer.

2.3 Plant genomic DNA extraction

For genomic extraction, 300 mg of fresh tissue were grinded and incubated in 500 µL of Extraction Buffer (0.2 M Tris-HCl pH 9.0, 0.4 M LiCl, 25 mM EDTA and 1% v/v SDS) for 5 minutes. After a 5 minutes centrifugation at 13.000 r.p.m., 350 µL of the supernatant were transferred to a new tube containing 350 µL of isopropanol. After a short shaking, the mixture was centrifuged for 10 minutes and the supernatant was discarded. The pellet was washed with ethanol 70% (v/v) twice and let dry for 20 minutes. The dried pellet was resuspended in 200 µL of distillated de-ionizated water (ddH_2O) and the quantification of genomic DNA was made using a NanoDrop® ND-1000 spectrophotometer.

2.4 DNA engineering

Different techniques for DNA manipulation were employed. Enzymes used to this purpose are depicted in Table V.

Table V. Enzymes used for DNA engineering

Application	Enzyme	Brand	Proof-reading	Ends generated
PCR-Cloning	Pfu DNA Polymerase	Biotools	Yes	blunt-ended products
	Ex Taq	Takara	Yes	3'-terminal adenosine
	Phusion® High-Fidelity DNA Polymerase	NEB	Yes	blunt-ended products
PCR-Genotyping	Taq DNA Polymerase	Quiagen	No	3'-terminal adenosine
	MyTaq™ DNA Polymerase	Bioline	No	Not specified
Digestion	Digestion enzymes	ThermoFisher		
Ligation	T4 DNA Ligase	Promega		
De-Phosphorylation	Shrimp Alkaline Phosphatase (SAP)	Takara		
Gateway Technology	pCR8/GW/TOPO TA	Invitrogen		
	LR Clonase	Invitrogen		

2.4.1 DNA amplification

Polymerase Chain Reaction (PCR) was used to amplify DNA fragments. Different commercial DNA polymerases (Table V) were used to amplify 50 ng of DNA according to the protocol of the manufacturer and depending on the application. Proof-reading polymerases were used to amplify fragments destined to be cloned in different constructs, whereas enzymes lacking this activity were used for genotyping or colony-PCR applications, for example. Reactions were carried out in thermocyclers following, in general, these conditions: initial denaturation step at 94ºC for 2 min, 30-35 cycles of amplification and final extension at the extension temperature of the given polymerase during 10 minutes.

2.4.2 DNA digestion, ligation and phosphorylation

For regular DNA restriction pattern by enzymatic digestion, 5-10 U of the restriction enzyme per µg of DNA were used (ThermoFisher, Table V). For normal cloning applications, the number of enzyme units was calculated with Formula 1. The protocol specified for the manufacturer was followed for each enzyme.

$$Units\ for\ 1h\ digestion = \left(\frac{48.5}{kb\ of\ target\ DNA}\right) * \left(\frac{cuts\ in\ target\ DNA}{cuts\ in\ reference\ DNA}\right) * \mu g\ of\ target\ DNA$$

Formula 1. Units of restriction enzyme needed for one hour of digestion reaction.

For DNA ligation and de-phosphorylation of the ends of the DNA molecule, the commercial T4 Ligase and SAP phosphatase (Table V) were used respectively, and the manufacturer protocol was followed in each case. For the ligation reaction, Promega recommends using a 1:1, 1:3 or 3:1 molar ratio of insert: vector DNA when cloning a fragment into a plasmid vector. The ng needed of insert can be obtained by using Formula 2. Usually, ligation is performed with 100 ng of destination vector. Ligation varied from 3 hours at room temperature to over-night at 4ºC. SAP phosphatase catalyzes the release of

5'- and 3'-phosphate groups from DNA. It dephosphorylated all types of DNA ends in 10 minutes at 37°C. The enzyme was inactivated in 5 minutes at 75°C.

$$Insert\ (ng) = \left(\frac{Vector\ (ng) * Insert\ (kb)}{Vector\ (kb)}\right) * Molar\ Ratio\ of\ \ Insert/Vector$$

Formula 2. Nanograms of insert needed for the ligation reaction.

2.4.3 DNA-end modification

For some applications, modification of DNA ends becomes indispensable. For example, Gateway technology requires the presence of a 3'-terminal adenosine (A) in the insert; or in some ligation reactions there is a requirement for the insert and vector to have blunt ends.

For the addition of adenosines in the 3'-terminal, 1 µl of a 1 mM dNTPs solution, 0.5 U of DNA polymerase and the required quantity of its buffer were added to the product of the PCR and incubated in a thermocycler for 10-30 min at the extension temperature of the DNA polymerase.

To transform DNA overhangs in blunt ends, we used the protocol for the Klenow enzyme. First, 0.2-5 µg of DNA were digested with the appropriate restriction enzyme in a final volume of 20 µl, then 1 µl of a 1 mM dNTPs solution and 1U of Klenow enzyme per µg of DNA were added and the mixture is incubated for 1 hour at room temperature. Finally, both the Klenow and the restriction enzyme were inactivated for 1 hour at 65ºC.

2.5 Electrophoresis and fragment purification

DNA fragments were separated by agarose gel electrophoresis 0.8-2% (w/v) in TBE 1X Buffer (Tris 0.89 M, boric acid 0.89 M, EDTA 2 mM (pH= 8)), used as electrophoresis buffer as well. Samples were mixed with Loading Buffer 6X (bromophenol blue 0.25% (w/v), xylencyanol blue 0.25% (w/v), glycerol 30% (v/v)) to a 1X final concentration. Electrophoresis run at constant voltage. Gels contained 0.5 µg/ml of ethidium bromide, so DNA could be observed by exposing it to UV light in a transilluminator.

To DNA fragment purification, the selected DNA bands observed under UV light were excised from the agarose gel and the QIAquick Gel Extraction Kit® system was used following the manufacturer protocol.

2.6 Gateway Technology

This technology is based on the site-specific recombination properties of the *lambda* phage, which includes well characterized recombination sites such as *att* (site-specific

ATTachment). First, the selected insert is cloned in an entry vector, the pCR8/GW/TOPO TA® (Invitrogen™), which contains the recombination sites attL1 and attL2 flanking the fragment that will be replaced by the insert. After transformation, selection of clones and digestion to verify the orientation of the insert, the selected construct was sequenced. Once selected the correct entry plasmid, a second reaction (LR reaction) catalyzed by the LR Clonase (Invitrogen™) was made to transfer the fragment that includes the insert to a destination vector containing two recombination sites, attR1 and attR2.

2.7 Sequencing

Sequencing of the fragments was carried out in the sequencing service of the *Instituto de Biología Molecular y Celular de Plantas (Universidad Politécnica de Valencia-CESIC)*, where a capilar sequenciator ABI 3100 (Applied Biosystems) was used.

2.8 Constructs and oligos used in the Thesis

In this work, several genetic constructs were used for different purposes. The ones used for Yeast two Hybrid are depicted in Table VI, whereas the ones for *in planta* expression are showed in Table VII. A list of the primers used according to their application can be found in Table VIII.

Table VI: Plasmid constructs for Y2H

Constructs generated in this Thesis

Chapter	Construct	Vector	Origin	Technology
Chapter I	*pADH1::GAL4* ***AD-TCP2***	pDEST22	*pENTR223.1-Sfi-Orf-TCP2*	Gateway (LR reaction)
	pADH1::GAL4 ***AD-TCP2 Del1***		*pENTR223.1-Sfi-Orf-TCP2*	
	pADH1::GAL4 ***AD-TCP2 Del2***		*pENTR223.1-Sfi-Orf-TCP2*	
	pADH1::GAL4 ***AD-TCP3***		*pENTR223.1-Sfi-Orf-TCP3*	
	pADH1::GAL4 ***AD-TCP4***		*pENTR223.1-Sfi-Orf-TCP4*	
	pADH1::GAL4 ***AD-TCP5***		*pENTR223.1-Sfi-Orf-TCP5*	
	pADH1::GAL4 ***AD-TCP10***		*pENTR223.1-Sfi-Orf-TCP10*	
	pADH1::GAL4 ***AD-TCP13***		*pENTR223.1-Sfi-Orf-TCP13*	
	pADH1::GAL4 ***AD-TCP24***		*Per8 GW TCP24*	PCR amplification, then Gateway
	pADH1::GAL4 ***AD-KNAT1***		*pENTR223.1-Sfi-Orf-KNAT1*	Gateway (LR reaction)
Chapter II	*pADH1::GAL4* ***AD-TCP14***	pGADT7	*pDONR207 TCP14*	
	pADH1::GAL4 ***AD-TCP15***		*pDONR207 TCP15*	
	pADH1::GAL4 ***DBD-M5GAI***	pGBKT7	*pDONR207 M5GAI*	
	pADH1::GAL4 ***DBD-M5RGL2***		*pDONR207 M5RGL2*	

Constructs previously described

Chapter	Construct	Vector	Reference
Chapter I and II	*pADH1::GAL4* ***DBD-M5GAI***	pDEST32	Gallego-Bartolomé *et al*., 2012
	pADH1::GAL4 ***DBD-RGA52***		
	pADH1::GAL4 ***DBD-MD3 GAI***		
	pADH1::GAL4 ***DBD-MD4 GAI***		
	pADH1::GAL4 ***DBD-Del1 GAI***		
	pADH1::GAL4 ***DBD-Del2 GAI***		

Table VII: Plasmid constructs for expression in plant				
Constructs generated in this Thesis				
Application	**Construct**	**Vector**	**Origin**	**Technology**
BiFC	*p35S::* ***YFN-TCP2***	pMDC43-YFN	*pENTR223.1-Sfi-Orf-TCP2*	Gateway (LR reaction)
	p35S:: ***YFN-TCP4***		*pENTR223.1-Sfi-Orf-TCP4*	
	p35S:: ***YFN-TCP14***		*pDONR207 TCP14*	
	p35S:: ***YFN-TCP15***		*pDONR207 TCP15*	
	p35S:: ***YFC-RGL2***	pMDC43-YFC	*pDONR207 RGL2*	
CoIP	*p35S::* ***HA-TCP2***	pEarleyGate201	*pENTR223.1-Sfi-Orf-TCP2*	
	p35S:: ***HA-KNAT1***		*pENTR223.1-Sfi-Orf-KNAT1*	
	p35S:: ***HA-TCP14***		*pDONR207 TCP14*	
Luciferase	*p35Sm-* ***Syn. Class II TCPbs::LUC***	pGreenII 0800-LUC	*pUC57 GeneScript*	Traditional cloning
	p35Sm- ***Syn. Class I TCPbs::LUC***			
	p35S::HA- ***VP16-TCP2***	pAlligator	*pENTR223.1-Sfi-Orf-TCP2*	Gateway (LR reaction)
	p35S::HA- ***VP16-TCP4***		*pENTR223.1-Sfi-Orf-TCP4*	
	p35S::HA- ***VP16-TCP14***		*pDONR207 TCP14*	
Transgenic plants	*p35S::* ***HA-GAI***	pEarleyGate201	*pDONR201 GAI*	
Constructs previously described				
Application	**Construct**	**Vector**	**Reference**	
BiFC	***p35S::YFC-GAI***	pMDC43-YFC	Provided by Antonella Locascio	
	p35S::YFC		Provided by Dr Alejandro Ferrando-IBMCP	
	p35S::YFN	pMDC43-YFN		
CoIP/ Luciferase	***p35S::myc-M5GAI***	pEarleyGate203	Provided by Antonella Locascio	
	p35S::GAI-TAP	pC- TAPa	Provided by David Alabadí	
Transgenic plants	***pTCP2::TCP2-GFP***	CHF3	Provided by Ignacio Rubio Somoza	
	pTCP4::TCP4-GFP			
	pTCP2::rTCP2-GFP	pXW74	Provided by Javier Palatnik	
	pTCP4::rTCP4-GFP			

Table VIII. Oligos used in this Thesis.			
Experiment	Gene	Forward	Reverse
RT-PCR	*TCP2*	GCATCACAATTCTTCCCGGAT	TTACCACCGGAAGGTCGTG
	TCP3	TTCTCCGGCCAGAGCAATAA	GCTTGCCGTCGATGAGTCA
	TCP4	CACTCACAAGCCGTCCTCTG	TTCGATTGTCAATGGCGAGA
	TCP5	GGTCCTACTCCTCCGGCAAT	AAACGAAGGGTATGTCGGGAA
	TCP10	GACGCAAAGACCGTCATAGCA	GGTCACGTGGACCCTTTGAT
	TCP13	CTTCGTGGATGAAATCCAAGG	AGGCGCGTGAAACCCTAAC
	TCP24	TCATGGCTACACCTCCACCTC	AGTCTCCGGAAGCTGGTGGT
	TCP14	CCAACATGATCAAATCGGAGG	CGCCACCAGTACCAATAGAA
	TCP15	CTCTCCAGCTTCTTGATTC	CGAAGAATGTGACTCGTTC
	AS1	GTTAGACAGTTCGGTCCGAGAGA	TGATCCCTGGCTTAAGATAATTCTTC
	GA20ox1	GATCCATCCTCCACTTTAGA	GTGTATTCATGAGCGTCTGA
	Actine8	AGTGGTCGTACAACCGGTATT	GAGGATAGCATGTGGAAGTGA
Deletions for Y2H	*TCP2 Del1*	ATGATTGGAGATCTAATGAAG	TCACGACCCGAGAATTAAACCCG
	TCP2 Del2	ATGACAACAGTTGCTGCTAATTC	TCAGTTCTTGCCTTTACCCTTA
Genotyping	*GUS*	CAACGTCTGGTATCAGCGCGAAGT	TATCCGGTTCGTTGGCAATACTCC
	GFP	ATGAGTAAAGGAGAAGAACTTTTC	GTATAGTTCATCCATGCCATGTG
	tcp14-4	GTTGTTGATGATGATGATGTCTCTGTG	GACGACAACCATCAACAACAACCTTC
	tcp15-3	CTCTCCAGCTTCTTGATTC	CGAAGAATGTGACTCGTTC
	SALK T-DNA	CGATTTCGGAACCACCATCAAACAGGA	
Sequencing	*M13*	GTAAAACGACGGCCAGT	CAGGAAACAGCTATGAC
	YFN	TCATGGCCGGTGGAGGTGGATCTGG	
	pGreen	CTAAAATCCAGATCGATAACATTAACG	GAACCAGGGCGTATCTCTTCATAGC
	Alligator	CGATCTGGACATGTTGGGGGA	GTAACATAGATGACACCGCGCGCG

3. Genetic transformation

During this Thesis, stable and transitory genetic transformation of *Arabidopsis thaliana* and *Nicotiana benthamiana* was carried out, respectively. In both cases, a liquid culture in selective medium (Gentamicin, Rifampicine and the selection antibiotic) of the *Agrobacterium tumefaciens* C58 strain carrying the selected construct in each case was used (see Section 2.1 of Materials and Methods).

Transgenic Arabidopsis plants

For generation of *Arabidopsis* transgenic plants, the protocol of floral dip described by Clough and Bent (1998) (1) was used, with some modifications (2). First, 20 seeds of the selected genotype were sown in 12 cm of diameter pots (6 seeds per pot) grown in the greenhouse at long day (LD) conditions. After 4 weeks, when the first inflorescence appears, is the moment of the transformation.

Three days before the transformation, a pre-culture in selective medium of 5 ml of *A. tumefaciens* carrying the desired construct was prepared and incubated over-night at

28ºC. The following day, the pre-culture was inoculated in a flask containing 300 ml of selective medium and is incubated at 28ºC for 16 hours in agitation. In the moment of plant transformation, that culture must reach an OD_{600} of 0.6, corresponding to an inoculate concentration of 10^{-2}-10^{-3} CFU/ml. When the culture reached this OD_{600} value, it was centrifuged at 6000 r.p.m. for 20 min and the pellet was resuspended in 300 ml of infiltration medium (sucrose 5% (w/v), MES 0.5 g/L, Silwet L-77 0.03%, at pH 5,7). Plants were submerged upside-down in the culture for 15-60 s, and then the pots were deposited horizontally in trays with absorbent tissue. These trays were covered with plastic to preserve high humidity conditions favorable for infection and they were placed in the dark for a day. After a day, the plastic was removed and the pots were deposited vertically in the trays. Plants were grown in the greenhouse under LD conditions until seed harvesting. The collected seeds were dried at 37ºC for 2 days (except heat-shock lines) and saved.

For selection of the transformants, the collected seeds were surface-sterilized and sown in plates containing growing medium supplemented with the selection antibiotic of the vector at 100 mg/L (see Section 1 of Materials and Methods) and subjected to 3-7 days of stratification. Then the plates were moved to growing chambers in LD conditions. After 7-10 days, the antibiotic-resistant seedlings were transferred to pots containing soil and they were grown in the greenhouse until seed collection. Each one of these antibiotic-resistant plants was considered an independent transgenic line (T1).

In order to select transgenic lines carrying a unique insertion of T-DNA, 100 seeds of each T1 plant were sterilized and sown in selection medium. After 10 days, a ratio of Resistant: Sensitive seedlings was calculated for each T1 offspring and lines presenting a 3:1 Resistant: Sensitive segregation were selected. Approximately 12 resistant seedlings for each line (T2) were transferred to the greenhouse to obtain their seeds.

A third round of selection was performed to choose homozygous transgenic lines. 100 seeds of each T2 were sown in selective medium and followed the same protocol than in the previous step. In this case, only lines with 100% of resistant seedlings were selected (T3). In Table IX, a list of the transgenic lines generated in this Thesis can be found. In the case of the TCP transgenic lines, the constructs were provided by Dr. Javier Palatnik.

Table IX: Transgenic lines generated in this Thesis

Genotype	Background	Vector
pTCP2::TCP2-GFP	Col-0	CHF3
pTCP4::TCP4-GFP	Col-0	
pTCP2::rTCP2-GFP	*Ler*	pXW74
pTCP4::rTCP4-GFP	*Ler*	
pAS1::GUS;; p35S::HA-GAI	Col-0, *pAS1::GUS*	pEarleyGate201
pKNAT1::GUS;; p35S::HA-GAI	Col-0, *pKNAT1::GUS*	

Transitory expression in N. benthamiana

Transitory expression in 3 week old *N. benthamiana* leaves was used in this Thesis for different purposes such as Bimolecular Fluorescence Complementation (BiFC), co-immunoprecipitation or transient expression assays with the *Luciferase* reporter gene.

A day before the infiltration, we refreshed a pre-culture in 5 ml of selective medium of *A. tumefaciens* carrying the desired construct and we incubated it over-night at 28ºC. A pre-culture per each construct is needed. The following day, cultures were centrifuged at 3000 r.p.m. for 20 min and the pellet was resuspended in 15 ml of infiltration buffer (1mM MES pH 5.7, 0.1 mM $MgCl_2$, 200 µM acetosyringone). Then, cultures were incubated in dark and agitation at 28ºC for 2 hours. After this time, the OD_{600} was measured for each culture, and calculations are made to reach the desired concentration for each construct. Once the mixture of constructs was made, 3 leaves of *N. benthamiana* were agroinfiltrated per plant and combination of constructs, in the presence of the P19 protein (except BiFC assays) to suppress gene silencing. The third day after infiltration, samples can be obtained for the different applications previously mentioned.

4. Genetic crosses

Several genetic crosses were made to obtain different genotypes. In the case of the *pAS1::GUS pTCP2::rTCP2-GFP* genotype, the constructs to be crossed carry different selection genes, so it facilitates the posterior genotyping. In the transgenic line *pAS1::GUS* (3), the pGreen vector carries the *bar* gene, that confers resistance to the glufosinate herbicide. Pollen of *pAS1::GUS* (masculine parental) was used to fertilize flowers of the *pTCP2::rTCP2-GFP* line generated by us. Then, the F1 seeds were sown in growth medium supplemented with glufosinate (100 mg/L) to discard self-pollinated *pTCP2::rTCP2-GFP* seeds. Posterior selection combined the resistance to glufosinate and genotyping by PCR of the GFP gene (see Table VIII) to obtain plants homozygous for both transgenes in the F3 generation. For generating the *pRGA::RGA-GFP pKNAT1::KNAT1-CFP* transgenic line, crosses using pollen of the *pRGA::RGA-GFP* line (Col-0 background, provided by Dr. Salomé Prat, CNB, Madrid) to fertilize *pKNAT1::KNAT1-CFP* flowers (provided by Dr. Miltos Tsiantis (4)) and viceversa were made and F1 seeds were used for microscopy observations. Finally, to obtain the *tcp14-4 tcp15-3 pCYCB1;1::GUS* genotype genetic crosses of the double mutant and the transgenic line were performed and the homozygous line was selected by genotyping *GUS* and both mutant alleles with primers depicted in Table XII.

5. Germination assays

Wild type and mutant seeds were collected simultaneously from plants grown under the same conditions. Storage conditions were 22°C and 30% relative humidity in the dark for 1 week for freshly harvested seeds. For each genotype, approximately 100 seeds were placed onto filter papers (Whatman N°3), moistened with 2 ml of sterile water or aqueous solution of GA_3 1 µM (Duchefa) in 6 cm diameter Petri dishes and then transferred in a climate room (25°C, 16 h light/8 h dark). The percentage of germination was counted after 4 days (96 hours). Assays were carried out in triplicate with at least two independent seed batches.

6. Yeast two hybrid assays

For the yeast two hybrid assays (Y2H), the genetic constructs depicted in Table VI were used. Deleted versions of GAI (M5GAI, MD3, MD4, Del1 and Del2), RGA (RGA52) and RGL2 (M5RGL2) fused to the DNA binding domain of the GAL4 (DBD) were used to avoid the self-activation effect that confers de N-terminal region of the protein. On the other hand, the Class I TCPs TCP14 and TCP15, CIN-TCPs and KNAT1 were fused to the activation domain of GAL4 (AD). Constructs were co-transformed by pairs (one DBD and one AD) in the yeast strain AH109 according to the protocol of the manufacturer (Clontech). The *HIS3* reporter gene is stably integrated at the yeast genome of the AH109 strain, which is characterized by the presence of GAL4 binding sites in its promoter region.

The basis of the assay is the fact that if the tested proteins physically interact, the DBD and AD of GAL4 are in enough proximity and there is an induction of the *HIS3* reporter gene by two-hybrid-dependent transcriptional activation, which allows AH109 cells (auxotroph for histidine) growth on plates lacking histidine. The analysis of the interaction was made in an SD medium and positive colonies were selected in the absence of tryptophan, leucine and histidine. In some cases, the medium was supplemented with 3-amino-1, 2, 4 triazole (3AT, Sigma-Aldrich), an inhibitor of the reporter gene *HIS3* to test the strength of the interaction. As a control, each bait (the protein fused to the DBD of GAL4) was also co-transformed into the yeast strain with the empty AD vector.

7. Bimolecular Fluorescence Complementation assay

To perform the bimolecular fluorescence complementation assay (BiFC), the DELLAs *RGL2* and *GAI* were cloned in the pMDC43-YFC vector, whereas *TCP2, TCP4, TCP14* and *TCP15* were introduced in the pMDC43-YFN (Table VII). These vectors, pMDC43-YFN and pMDC43-YFC, constitutively express the selected proteins fused to the N-terminal and C-terminal of the yellow fluorescent protein (YFP), respectively. This way, when the tested

proteins are co-expressed, only if they physically interact both parts of the YFP proteins are close enough to reconstitute its fluorescence that can be observed by confocal miscroscopy.

Each construct was introduced in the *A. tumefaciens* C58 strain (section 2.1 of Material and Methods) and *N. benthamiana* leaves were agroinflitrated (section 3.2 of Material and Methods). Co-infiltration of the empty vectors was used as a negative control. Three days after infiltration, disks of leaves were taken and fluorescence was analyzed in the confocal microscope (section 12 of Material and Methods). Complementation was confirmed in two independent assays.

8. Co-immunoprecipitation assays

Preparation of the samples

To evaluate the physical interaction between two target proteins *in planta*, we performed co-immunoprecipitation (Co-IP) assays. First, we cloned the selected genes in specific vectors for expression in plants with different tags, the *pEarlyGate* (Table VII, (5)). Second, the constructs are introduced in the C58 *A. tumefaciens* strain and leaves of *N. benthamiana* were agroinfiltrated. Three conditions were tested, two with each one of the proteins to be tested alone (used as controls) and one co-expressing both constructs. Bacteria concentration was adjusted to guarantee that the expression of both proteins was similar. Three days after infiltration, leaves were collected and frozen in liquid nitrogen (N_2 (l)).

Protein extraction and immunoprecipation

The frozen tissue was grinded in a cold mortar to obtain a thin powder of each sample. Then 1 ml of the powder was mixed with the same volume of an extraction buffer that maintains the proteins in native conditions [Tris-HCl (pH 7.5) 50 mM, glycerol 10%, Nonidet P-40 0.1%] supplemented with Complete Protease Inhibitors 1X (Roche). After incubation in ice for 15-120 minutes, samples were centrifuged at 4ºC and 13000 g for 15 minutes twice and supernatant was transferred to a new tube. 250 µl of this supernatant were saved in a different tube, to serve as input. Then, the denaturing protein extraction buffer Laemli (1x) [Tris-HCl (pH 7.5) 50 mM, NaCl 150 mM, EDTA 1 mM, DTT 1 mM, glycerol 10%] was added, heated to 100ºC for 5 min and stored at -80ºC. The rest of the extract was incubated between 30 minutes and overnight in slow agitation at 4ºC with paramagnetic beads (Miltenyi Biotec) with antibodies that recognize the corresponding epitope tag. This step allows the beads to bind specifically to the epitope of the target protein (one of the both tested). After incubation, samples were loaded at room temperature in µ columns (Miltenyi Biotec) previously washed with extraction buffer. The

magnetically-labeled proteins are retained on the μ column placed in the magnetic field of a μMACS Separator. Then, two washing steps were made, one with 800 μl of extraction buffer without protease inhibitors and other with 100 μl of the washing buffer 2 provided by the manufacturer. Immunoprecipitated proteins in each one of the samples were eluted in 50 μl of pre-heated denaturing elution buffer (Miltenyi Biotec).

Western blot

The six samples (three inputs and three IPs) were heated at 95ºC for 5 minutes, centrifuged at 13000 g for another 5 minutes and loaded in separation SDS-PAGE 8.5% gels (Pierce). After protein separation, proteins were transferred to a PVDF membrane (Bio-Rad), stained with Ponceau Red to check that protein level in the different input samples were comparable and de-stained with water. Then, membranes were saturated with blocking buffer [TBS 0.1%, Tween-20 (0.1%), Blocking Reagent 20% (Roche®)] one hour at room temperature and incubated with the selected primary antibody diluted in blocking reagent overnight at 4ºC. The anti-*myc* (clone 9E10; Roche®) and anti-*HA-HRP* (clone 3F10; Roche®) antibodies were used. The following day, three washing steps of 15, 10 and 5 minutes with washing buffer (TBS 0.1%, Tween-20 (0.1%)) were performed. Incubation for 1 hour at room temperature with a secondary antibody followed by another three washing steps was needed in the case of non-conjugated antibodies. The SuperSignal West Femto Chemiluminiscent Substrate (ThermoFisher) was used and the signal was detected with a LAS 3000 imaging system.

9. Real-time quantitative RT-PCR

To analyze the expression level of different genes, real-time quantitative RT-PCR was performed. Preparation of the samples is specified in each case in Results and Discussion and at least two biological replicates were used. Once samples were collected in N_2 (l) and grinded in a mortar, total RNA was extracted following the protocol of the Plant RNA Mini Kit (Omega Biotek). These RNA samples were subjected to a treatment with the DNase Kit (Ambion) to further eliminate genomic DNA. Then, RNA concentration in the samples was measured with a NanoDrop® ND-1000 spectrophotometer and cDNA was synthesized from 2 μg of total RNA using the PimeScript™II Reverse Transcriptase kit (Takara).

Quantitative PCR reactions were prepared in MicroAmp® Fast Optical 96-Well Reaction plates (Applied Biosystems) using SYBR Green PCR Master Mix (Applied Biosystems). Three technical replicates were performed for each condition and the primers employed are depicted in Table VIII. Reactions were carried out in the 7500 Fast-Real Time (Applied Biosystems) quantitative PCR thermocycler. Quantitative PCR program was as follows: initial incubation for 2 minutes at 50ºC, initial denaturation step at 95ºC for 10 minutes and 40 amplification cycles of 15 seconds at 95ºC and 1 minute at 60ºC.

Data analysis was performed with the 7500 Software v2.0.6 (Applied Biosystems) using the CT comparative method (ΔΔCT). The expression levels obtained for the different genes were normalized respect to the expression levels obtained for the control gene, *ACTINE8* (ACT8).

10. Transactivation assays

For the transactivation assays in *N. benthamiana* leaves, we decided to use specific synthetic promoters for each class of TCPs studied: class I TCPs and Class II TCPs. To this purpose, we designed a synthetic promoter consisting of six copies of either the Class I TCP (GTGGGCCCAC) (6) or Class II TCP (GTGGTCCCA) (7) consensus binding site separated by a 6-nucleotide spacer (AAAAAA) upstream of a minimal *35S* promoter and the translational enhancer omega (Ω). We ordered the synthesis of these constructs to Genescript who provided each one of them in a pUC57 vector, with our synthetic promoters flanked by *PstI* and *NcoI* restriction sites. Therefore, we digested the pUC57 vectors and cloned the fragment of interest in a *pGreenII 0800-LUC* vector (8) by traditional cloning techniques (Table VII). This way, the synthetic promoter controls the expression of the *Luciferase* reporter gene.

The next step was to generate the constructs for expression *in planta* of the proteins to be tested. In particular, *TCP2*, *TCP4* and *TCP14* were cloned in the Alligator vector, to obtain a translational fusion of the TCP with the transcriptional activator VP16 and the 3xHA molecular tag. On the other hand, we used the previously generated constructs *p35S::myc-M5GAI* and *p35S::GAI-TAP* to express this DELLA protein *in planta* (Table VII).

The C58 *A. tumefaciens* strain was transformed to incorporate all constructs and 3 leaves of each *N. benthamiana* plant were infiltrated for each combination of constructs, as described in sections 2.1 and 3.2 of Material and Methods. Firefly and the control Renilla LUC activities were assayed from leaf extracts (made with the Lysis Buffer of Promega) with the Dual-Glo Luciferase Assay System (Promega) following the protocol of the manufacturer and quantified with a GloMax 96 Microplate Luminometer (Promega). To verify that protein levels were similar in all conditions, Western Blot analysis were performed with proteins extracted from the same experiment using the specific antibody required for each molecular tag (Section 8 of Material and Methods).

11. Analysis of the β-glucoronidase activity

β-glucoronidase (GUS) staining assays were carried out as established in Zadnikova et al, 2010 (9). Samples were collected and immediately fixed in cold, 90% acetone for 10-20 minutes at 4ºC [in the case of developing seeds (Chapter II of Results and Discussion), they were cleared before fixation according to Yadegari et al., 1994 (10)]. Then, they were

subjected to a brief vacuum pulse of 1-3 minutes and a washing step with the staining solution (Table X) without the substrate (X-gluc) for 20 minutes. After this time, the substrate was added to the solution to allow the staining reaction to start. Substrate concentration, temperature and time of incubation in each case are depicted in Table XI. After incubation, samples were de-stained with serial ethanol washing steps (15, 30, 50 and 70%). At this point, samples can follow a final incubation in chlorhydrate (Sigma) for 2 days and be directly observed in the microscope.

Table X. Composition of the staining solution.

Compound	Concentration	
$NaPO_4$ pH 7	50 mM	
Titron X-100 (Sigma)	0.1 % v/v	
Potassium Ferrocyanide	0.5 mM	K_3K_4 $Fe(CN)_6$ 1 mM
Potassium Ferricyanide	0.5 mM	
EDTA	10 Mm	
X-Gluc	Table XI	

Table XI. GUS staining conditions.

Experiment	Transgenic line	[X-Gluc] (mM)	Incubation time	Incubation temperature
Chapter I Section 4	***pAS1::GUS*** ***pAS1::GUS;;*** ***p35S::HA-GAI*** ***pAS1::GUS;;*** ***pTCP2::rTCP2-GFP***	0.5	30 min	Room T./37ºC
Chapter I Section 5	***pKNAT1::GUS*** ***pKNAT1::GUS;;*** ***p35S::HA-GAI***	2	Overnight	Room T.
Chapter I Section 7	***pKNAT1::GUS*** ***pGAI::GUS*** ***pRGA::GUS***		1-3 hours non saturated, o/n saturated	37ºC
Chapter II Section 6	***pCYCB1;1::GUS***	2	Overnight	37ºC
Chapter II Section 7	***pCYCB1;1::GUS***	2	Overnight	37ºC

12. Histological cuts and microscopy

Histological cuts

Once samples are in 70% ethanol, they were introduced in the tissue processor (Leica TP1020) that was programmed following the instructions of the manufacturer. This processor performed two different functions: first, samples were subjected to several de-hydration steps and then they were included in paraffin. For sample de-hydration, samples were incubated with sequential 70%, 95% and 100% ethanol (in the second of these incubations, they also were stained with Eosina-Y 0.2%) and different concentrations of a Histo-Clear® (National Diagnostics): ethanol solution [25:75, 50:50, 75:25 and 100:0 (%v/v)]. Finally, samples were included in 100% paraffin Paraplast® Plus (McCormick Scientific) at 58ºC in vacuum. After the inclusion, samples were placed in aluminium moulds with liquid paraffin, covered with plastic pieces and let solidify at 4ºC.

Longitudinal cuts of the samples (10 µm thickness) were made with the Microm HM330 microtom, placing them in polilysine coated slides. Next, paraffin was removed with histoclear. In the case of samples used for in situ hybridization assays, the slices were then re-hydrated with decreasing quantities of ethanol (100%, 75%, 50% and 25%) and water. This last step is important if samples are destined to be hybridized or stained.

Optical microscopy

Stained fresh tissue was observed with the Nikon Eclipse E600 optic microscope. Bright field illumination and Normaski technique (D.I.C., differential interface contrast) were used. Images were captured with a Nikon Digital-Sight (DS-Fi1) camera connected to the microscope and processed with the NIS-Elements F3.0 image analysis software.

In the case of the *CYCB1;1::GUS* transgenic line and *TCP15 in situ* hybridization assay in Chapter II of Results and Discussion, the experiments were performed in collaboration with Drs. Masiero and Resentini. Therefore, the samples were observed using a Zeiss Axiophot D1 microscope (Zeiss) equipped with DIC optics. Images were recorded with an Axiocam MRc5 camera (Zeiss) using the Axiovision program (4.1).

Confocal microscopy

For confocal microscopy observations, the Leica TCS SL (Leica) was used. This microscope consists in an inverted DMIR2 microscope and a confocal spectral unit SL with two simultaneous detectors of fluorescence and four laser lines (458 nm, 488 nm, 514 nm and 534 nm). To excite the YFP or GFP protein a laser of 488 nm was used and the emission was absorbed between 500 nm and 520 nm (maximum of emission: 509 nm). Chlorophyll was excited with the same laser and its emission was absorbed between 660

nm and 690 nm. Signal identity was confirmed in all cases by a scan of wavelengths (λ-scan), to visualize the intensity of the emission within all the range of wavelengths.

In the case of the experiments made in collaboration with Drs. Serrano-Mislata and Sablowski in the John Innes Centre, in UK, samples were treated differently. For co-localization of *pRGA::RGA-GFP* and *pKNAT1::KNAT1-CFP*, vegetative meristems of 9 day old seedlings were dissected and stained with FM4-64 (30 ug/ml) for 20 minutes. Next, the images were obtained with a Zeiss LSM780 confocal microscope, where the localization of *pRGA::RGA-GFP* and *pKNAT1::KNAT1-CFP* was sequentially obtained with a 488 nm laser followed by a 405 nm laser. For cell measurements, apices were dissected and left to recover in GM medium (0.1% glucose; 0.44% MS medium including vitamins, 0.9% agar [pH 5.7]) for 24 hours at 16ºC, continuous light. Then, they were stained with FM4-64 30 mg/ml (Invitrogen) for 10 minutes and imaged with the same Zeiss LSM780 confocal microscope with excitation at 488 nm, emission filter set to 572–625 nm for FM4-64.

13. Cell measurements in the shoot apical meristem

The experiment to measure cell number, cell volume and meristem volume was made in collaboration with the laboratory of Dr. Sablowski, and we used a novel technique implemented in his laboratory (11). It combines confocal microscopy with a package of Python scripts and Fiji macros to process the images to landmark, segment, locate and measure cells in 3D, covering all the volume of the meristem. This was followed by another step of manual treatment of the data to delete cells that were incorrectly segmented.

First, the preparation of the samples and imaging was made as described in section 12 of this Material and Methods. Second, the image analysis was performed. For 3D segmentation and cell measurements, confocal image stacks were processed using scripts written in Python 2.7.3 and Fiji macros were used to visualize images, select landmark points, and select cells to be removed from the analysis during manual quality control. Next, statistics of the measurements were calculated. For each treatment, measurements from three to five replicates per apex were pooled after filtering by cell layer and region of interest (meristem). The raw data were processed in a Python shell using the functions defined in Serrano-Mislata, 2015 (11) and the obtained measures were then analyzed statistically with a Student test.

14. Root length and cell measurements in the root apical meristem

To measure the root length, seedlings were grown for one week in continuous light at 22ºC in a vertical orientation on plates containing half strength MS medium (Duchefa) with 0.8% (w/v) phytoagar and without sucrose, and supplemented with either mock solution or 1µM GA3 (Duchefa). Root length was measured using Image J software.

To measure the size of protoderm cells in the RAM, the modified pseudo-Schiff propidium iodide staining technique (mPS-PI) was performed as described in Truernit *et al*., 2008 (12). In brief, the basis of this technique is the covalent labeling of fluorophores (in our case propidium iodide) to the cell wall, followed by clearing of the tissue with chloral hydrate. This results in samples with highly fluorescent cell walls for the visualization of internal tissue structures (such as the vasculature) that can be easily observed by confocal microscopy and render high resolution images. After capturing the images in the confocal microscope with excitation at 488 nm and emission collected at 520 to 720 nm (section 12 of Material and Methods), cell number and length in the protoderm was measured with the Image J software.

15. RNA-sequencing

Preparation of the samples

Three biological replicates for each condition were taken. Extraction and purification of the RNA samples was performed according to section 9 of this materials and methods. Samples were collected in N_2 (l) and grinded, and total RNA was extracted with the Plant RNA Mini Kit (Omega Biotek). These RNA samples were then treated with the DNase Kit (Ambion) to further eliminate genomic DNA. Following isolation, RNA concentration, quality and integrity was checked using a NanoDrop® ND-1000 spectrophotometer and an Agilent Technologies 2100 Bioanalyzer. All samples had an RNA Integrity Number (RIN) value bigger than 8. Additionally, a 1% agarose gel was carried out and RNA integrity was corroborated, as the 28S and 18S ribosomal RNA bands can be seen (Figure 9.1). Concentrations of all samples were equalized and adjusted to 4 ng.

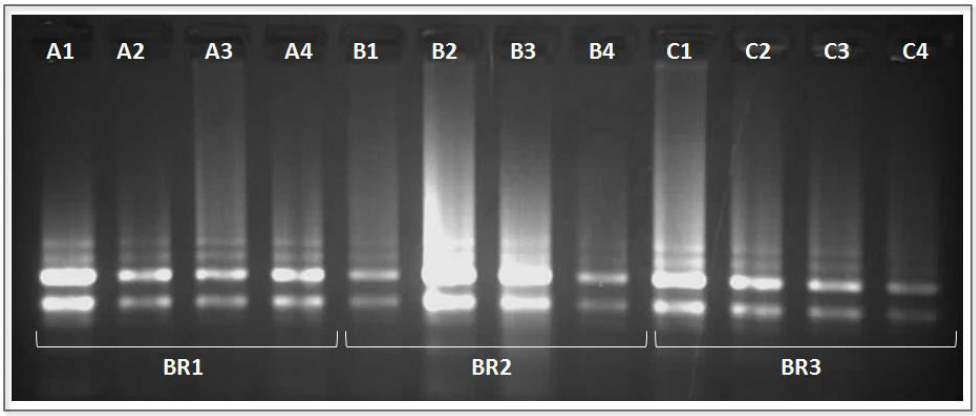

Figure 9.1. Agarose gel showing the 28S and 18S ribosomal RNA bands of the 12 samples analyzed. Samples are named from 1 to 4 (section 9 of Chapter I). Three biological replicates were used for the analysis: biological replicate 1 (BR1, A), biological replicate 2 (BR2, B) and biological replicate 3 (BR3, C).

cDNA library preparation for RNA-Sequencing

RNA-sequencing (RNA-seq) library preparation was done in the Genomic Service of the University of Valencia following the TruSeq Stranded mRNA Sample Preparation Low Sample (LS) Protocol from Illumina. Next, an overview of the process is depicted.

The first step involves the purification of the poly-A containing mRNA molecules using poly-T oligo attached magnetic beads. After purification, the mRNA was fragmented using divalent cations at high temperature. The cleaved RNA fragments were retrotranscribed into first strand cDNA using reverse transcriptase and random primers, and the original RNA template was removed. The second cDNA strand was then synthesized, in a process that incorporates dUTP instead of dTTP to quench the second strand during amplification, because the polymerase used does not incorporate past this nucleotide. At the end of this process, blunt-ended double stranded cDNAs were generated. After, a single adenine nucleotide was added to the 3' ends of the blunt fragments to prevent concatenation during the adapter ligation reaction. Then, multiple indexing adapters were ligated to the ends of the ds cDNA, purified and enriched with PCR to create the final cDNA library.

Sequencing and in silico analysis

At the Genomic Service of the University of Valencia, libraries were sequenced on a NextSeq 500 sequencer from Illumina and the reads of 10M deep and 75 nucleotides in length (paired-end) were pre-processed (edition and elimination of adapters) using the package Trim.fastq. All processed reads were mapped to the *Arabidopsis thaliana* genome

using the TopHat.v2.1.0 program, retrieving the results depicted in Table XII. Statistical analysis was also performed. Then, data was subjected to a quality control with QC program and the differential expression analysis was performed using DESeq2, SeqMonk 0.32.1 and EdgeR packages.

Table XII. Mapping analysis results.

Sample	Total reads	Mapped reads R1	% Mapped reads R1	Mapped reads R2	% Mapped reads R2	Mapped reads two pairs	% Concordant mapped reads
A1	14036014	13208686	94,1	13045202	92,9	12626913	87,5
A2	16204580	15240033	94	15025542	92,7	14534846	88,2
A3	13062704	12206959	93,4	12073960	92,4	11644403	88,3
A4	17624615	16683423	94,7	16495648	93,6	16021713	89,8
B1	12555265	11846667	94,4	11729055	93,4	11361129	89,5
B2	12029438	11256524	93,6	11086725	92,2	10711860	88,4
B3	13583652	12699855	93,5	12375077	91,1	11954168	86,9
B4	12178959	11399949	93,6	11253578	92,4	10879175	88,2
C1	12982278	12218083	94,1	12080876	93,1	11692410	88,9
C2	13366125	125875445	94,2	12418245	92,9	12022743	88,9
C3	10726383	10013715	93,4	9834489	91,7	9484603	87,5
C4	12804526	11987861	93,6	11795398	92,1	11392827	87,6

16. In situ hybridization

Generation of the digoxigenin (DIG) labeled RNA probe

Genomic DNA of Col-0 *Arabidopsis* plants was extracted and amplified with the *GAI* and *TCP15* primers specified in Table VIII to generate a 782 and 469 bp fragment, respectively, belonging to specific zones in the gene that constitute the mould for the RNA probe. These fragments were cloned in the *pGEM®-T* vector (Promega) that contains T7 and SP6 RNA polymerase transcription initiation sites flanking the insert. On both sides of the T7 and SP6 initiation sites, the vector contains specific primer sites M13 forward and M13 reverse, allowing the amplification of the fragment of interest by PCR (Table VIII). After the PCR, *in vitro* transcription with T7 provided the antisense probe, whereas with SP6 the sense probe was generated.

For the *in vitro* transcription reaction, a 20 µl mix was prepared with 200 ng of mould DNA, 2 µl of DIG RNA Labeling Mix (10 mM ATP, 10 mM CTP, 10 mM GTP, 6.5 mM UTP, 3.5 mM DIG-11-UTP. Roche®), 2 µl of Protector RNase Inhibitor (Roche®), 2 µl of T7 RNA polymerase (or SP6 polymerase) and 2 µl of 10X Buffer (Roche®). Reaction was incubated at 37ºC for 2 hours and then it was treated with 1 µl RNase-free DNase I (Roche®) at 37ºC for 15 minutes. To stop the reaction, 2 µl of 0.2M EDTA were added and 1 µl of yeast tRNA (10 µg/ µl, Roche®). Precipitation was made in 10 µl of 1M LiCl and 75 µl 100% ethanol and it was deposited in a freezer at -20ºC over night. The following day, probes were centrifuged at 13000 r.p.m. for 15 minutes, washed in 80% ethanol and let them dry around one hour. Probes were resuspended in 10 µl of RNase-free ddH_2O and 1 µl was

used for quantification. The remaining 9 µl were resuspended in 91 µl of hybridization buffer and saved at -20ºC.

Probe quantification

The quantification of the probe was made with the nucleic acid hybridization technique or dot blot. Taking 1 µl of the synthesized probe, different dilutions were made (1/25, 1/100 and 1/250) and 1 µl of each dilution was deposited in a nylon membrane (Hybond N+), dried and crosslinked with UV light. The same procedure was performed for the control of the quantification, a DIG labeled RNA of known concentration (Roche®). The membrane was incubated in TBS 1X (TBS 10X: Tris-HCl 1M, NaCl 4M at pH 7.5) for 2 minutes, blocked for 10 minutes in Blocking Reagent 0.5% (Roche®) diluted in TBS 1X , and washed in BSA solution (BSA 1%, Triton X-100 0.3%; in TBS 1X) for 5 minutes. Then, the membrane was incubated for 20 minutes with a 1:3000 dilution of the anti-Digoxigenin-AP, Fab fragments antibody (Roche®) diluted in BSA solution. The trace of antibody was washed twice for 4 minutes with BSA solution and detection buffer was added (Tris 100 mM, NaCl 100 mM, $MgCl_2$ 50M, at pH 9.5). The membrane was revealed by incubation in dark for 12 minutes in 2.5 ml of detection buffer 1X with substrate [3.75 µl of NBT (100mg/ ml) and 3.75 µl of BCIP (50 mg/ ml), Roche®). Then the membrane was washed with ddH_2O and let dry.

Deparaffinization, antigen retrieval and hybridization

Samples were included in paraffin and longitudinally cut as described in section 12 of Material and Methods. The slides were de-paraffinated with histoclear, re-hydrated with decreasing quantities of ethanol (100%, 75%, 50% and 25%) and water (2 minutes of incubation in each solution), and hydrolyzed in HCl 0.2M for 20 minutes. Then, samples were subjected to 4 washing steps of 5 minutes each: ultrapure water, twice with SSC 2X (SSC 20X: sodium citrate 0.3M, NaCl 3M, at pH 7) and again ultrapure water. After that, they were incubated with Proteinase K (1 µg/ml in buffer: Tris 100mM, EDTA 50 mM, at pH 8) at 37ºC for 18 minutes. After incubation, the slides were washed with PBS 1X (PBS 20X: NaCl 2.75M, KCl 50mM, Na_2HPO_4 200mM, KH_2PO_4, at pH 7.4) for 2 minutes and incubation in glycine 0.2% (2 mg/ml in PBS 1X) for 2 minutes followed by 2 washes of 2 minutes each in PBS 1X was used to block the Proteinase K. For the re-fixation of the tissue, slides were incubated for 10 minutes in a dilution of 4% formaldehyde in PBS 1X. After incubation, slides were washed with PBS and de-hydrated in 2 minutes incubation with ultrapure water, 30%, 50%, 70%, 95% and 100% ethanol and let them dried.

For the hybridization, probes were diluted in hybridization buffer (HB: SSC 6X, SDS 1.5%, formamide 50%, yeast tRNA 100µg/ml) in the following relation: 4 µl of the probe in 50 µl de HB. Probes were de-naturalized at 80ºC for 2 minutes, added in each slide

(50µl/slide) and covered with slide covers. Hybridization was carried out over night in a wet chamber at 50ºC.

Washing and colorimetric immunodetection

The excess of probe was removed with 3 washing steps in SSC 2X with formamide 50% (v/v) at hybridization temperature (50ºC): the first one for 30 minutes and the other two one hour and a half each. After washing, the slides were incubated in TBS 1X for 5 minutes and blocked in Blocking Reagent (Roche®) 0.5% in TBS 1X for 1 hour. Then, the slides were washed in BSA solution for 30 minutes and incubated with anti-Digoxigenin-AP, Fab fragments (Roche®) diluted in BSA solution (1:3000). After that, slides were washed with BSA solution three times (20 minutes each). Next, they were submerged for 5 minutes in detection buffer without the substrate (Tris 100mM, NaCl 100mM, $MgCl_2$ 50M, at pH 9.5) and then incubated in 50 ml of detection buffer supplemented with the substrate [75 µl of NBT (100mg/ ml) and 75 µl of BCIP (50 mg/ ml), Roche®] until the signal was observed, in our case, more than 18 hours. Finally, the reaction was stopped by placing the slides in water and let them dry. For the visualization of the signal, slides were prepared with Merckglass (Merck) and observed with the optical microscope (section 12 of Material and Methods).

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Esta tesis se llevó a cabo gracias a una beca de Formación de Personal Investigador (FPI) del Ministerio de Economía, Industria y Competitividad, Gobierno de España.