DELLA proteins as hubs in signaling networks in plants

Nora Alicia Marín de la Rosa
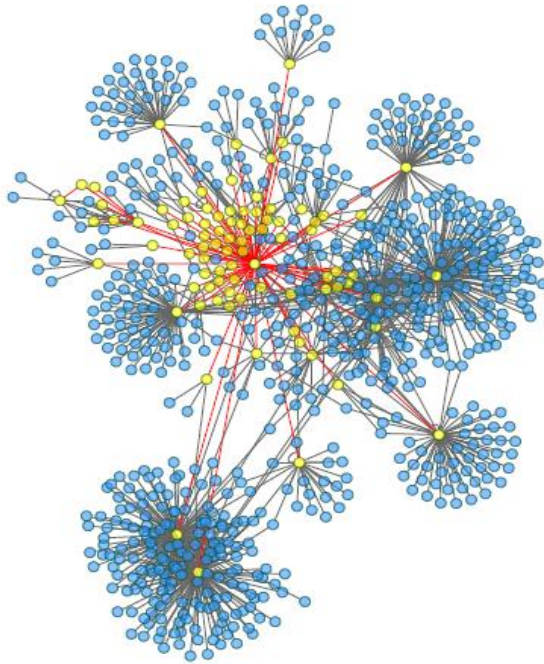
Advisors:
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El **Dr. Miguel Ángel Blázquez Rodríguez**, Investigador Científico del CSIC, y el **Dr. David Pablo Alabadí Diego**, Científico Titular del CSIC, ambos pertenecientes al Instituto de Biología Molecular y Celular de Plantas (IBMCP, UPV-CSIC) de Valencia,

CERTIFICAN que Nora Alicia Marín de la Rosa, ha realizado bajo su dirección en el Instituto de Biología Molecular y Celular de Plantas, el trabajo titulado ***"DELLA proteins as hubs in signaling networks in plants",*** y que autorizan su presentación para optar al grado de Doctor en Biotecnología.
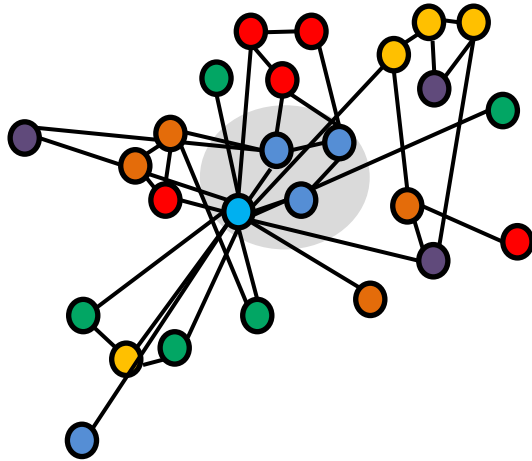
Y para que así conste, firman el presente certificado en Valencia a 10 de Marzo de 2014.

Dr. Miguel Ángel
Blázquez Rodríguez

Dr. David Pablo
Alabadí Diego

To my parents, my brother Jorge

and my entire social network…

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Summary

As sessile organism plants are extremely plastic. This feature is due their ability to integrate external and internal signals to modulate development. Therefore, understand the molecular mechanism underlying this feature is of great importance. Signals such as light, hormones and the circadian clock contribute to this plasticity. During this Thesis we use the plant model *Arabidopsis thaliana* to address how the circadian clock and the DELLA proteins, negative regulators of the gibberellin (GA) signaling integrate environmental signals and relay this information to transcriptional networks.

We have demonstrated that the circadian clock modulate transcriptional levels of the GAs receptors GIDs, which promote the degradation of DELLA proteins, this result in a daily oscillation of DELLA proteins, which reach a minimum at the end of the night. This oscillation is key to modulate hypocotyl rhythmic growth and to control transcription of many genes.

In this Thesis we present mechanisms of cross-talk among GAs and two plant hormones; ethylene and cytokinin. These mechanisms of cross-talk relies on the interaction between DELLA proteins and two transcriptional factors involved in signaling of these two hormones. The interaction with RAP2.3, prevents the transcription factor activity, this inactivation contributes to the regulation of apical hook opening by ethylene and GAs. On the contrary, the interaction with ARR1, a transcription factor that promotes cytokinin signaling, is positive for the activity of ARR1, thus contributing to the regulation of some developmental processes which are antagonistically regulated by GAs and cytokinin, for example root growth and photomorphogenesis. This interaction defines a new mechanism of DELLAs action. Additionally, by chromatin immunoprecipitation followed by massive sequencing, allowed us to show that DELLA proteins are near the promoter of many genes, this indicated that this mechanism is broad extensive.

Based in these results an in the identification of more than 50 transcription factors as interactors of the DELLA GAI, we propose that DELLAs act as "**hubs**" in

signaling networks. In particular we propose that this is the mechanism by which these proteins are key for the integration of internal signals and developmental processes.

Resumen

Como organismos sésiles, las plantas son extremadamente plásticas. Esta característica se debe a la habilidad de integrar las señales internas y externas para modular su desarrollo. Por tanto, entender el mecanismo de esta integración es de suma importancia. Señales como la luz, las hormonas y el reloj circadiano contribuyen a conferir esta plasticidad. En esta tesis hemos abordado, en la planta de referencia *Arabidopsis thaliana*, cómo el reloj circadiano y las proteínas DELLA, que son los reguladores negativos de la señalización por las hormonas giberelinas (GAs), integran señales del entorno para modular redes transcripcionales.

Hemos demostrado que el reloj circadiano regula los niveles de mensajero de los receptores de GAs GID1s, que promueven la degradación de las proteínas DELLA, lo que se traduce en una oscilación diaria en los niveles de las proteínas DELLA, que son mínimos al final de la noche. Esta oscilación es clave tanto para la regulación diurna del crecimiento rítmico del hipocótilo como para la regulación de muchos genes.

En esta Tesis también mostramos dos mecanismos por el cual la vía de las GAs interacciona con otras dos vías hormonales de la planta, la del etileno y la de las citoquininas. Estos mecanismos se basan en la interacción física de las proteínas DELLA con dos factores de transcripción que participan en esas dos vías. La interacción con RAP2.3 previene la unión del factor de trascripción a los promotores de sus genes diana, esta inactivación contribuye a la regulación de la apertura del gancho apical por etileno y GAs. Por el contrario, la interacción con ARR1, que promueve la señalización por citoquininas, es positiva para la actividad del factor de transcripción, contribuyendo de esta manera a la regulación de ciertos procesos fisiológicos controlados de manera antagónica por GAs y citoquininas, como la regulación del crecimiento de la raíz o de la escotomorfogénesis. Esta interacción define un mecanismo nuevo de acción de las proteínas DELLA. Además, un análisis de inmunoprecipitación de cromatina seguido de secuenciación masiva, nos ha

permitido mostrar que las proteínas DELLA se encuentran en los promotores de muchos genes, indicando que este mecanismo es extenso.

Basándose en estos resultados y en la identificación de más de 50 factores de transcripción como interactores de la proteína DELLA GAI, proponemos que estas proteínas actúan como "**hubs**" en redes de señalización. En particular, proponemos que éste es el mecanismo por el que estas proteínas son clave para la integración de señales externas y respuestas de desarrollo.

Resum

Com a organismes sedentaris, les plantes són extremadament plàstiques. Aquesta característica es deu a l'habilitat d'integrar els senyals endògens i externs per tal de modular el seu desenvolupament. Per tant, entendre el mecanisme d'aquesta integració és de màxima importància. Senyals com la llum, les hormones i el rellotge circadià contribueixen a conferir aquesta plasticitat. En aquesta tesi hem adreçat, a la planta de referència *Arabidopsis thaliana*, com el rellotge circadià i les proteïnes DELLA, que són els reguladors negatius de la senyalització per les hormones giberel•lines (GAs), integren senyals de l'entorn per a modular xarxes transcripcionals.

Hem demostrat que el rellotge circadià regula els nivells de missatger dels receptors de GAs GID1s, que promouen degradació de les proteïnes DELLA, el que es tradueix en una oscil•lació diària en els nivells d'aquestes, que són mínims al final de la nit. Aquesta oscil•lació és clau per a la regulació diürna tant de la velocitat de creixement del hipocòtil com de la regulació de molts gens.

En aquesta tesi també mostrem el mecanisme pel que la via de les GAs interacciona amb altres dos vies hormonals de la planta, la de l'etilé i la de les citoquinines, mitjançant la interacció física de les proteïnes DELLA amb dos factors de transcripció que participen en eixes vies. La interacció amb RAP2.3 inactiva al factor de transcripció evitant la unió als gens diana, el que és un cas més de inactivació per segrest, i contribueix a la regulació de l'obertura del ganxo apical per etilé i GAs. Pel contrari, la interacció amb ARR1, que promou senyalització per citoquinines, és positiva per a l'activitat del factor de transcripció i es produeix als promotors diana, contribuint d'aquesta manera a la regulació antagònica de certs processos fisiològics per GAs i citoquinines, com la regulació del creixement de l'arrel o l'escotomorfogènesi. Aquesta interacció defineix un mecanisme nou de regulació de factors de transcripció per proteïnes DELLA. A més, un anàlisi per immunoprecipitació de cromatina seguit de seqüenciació massiva, ens ha permés

mostrar que les proteïnes DELLA apareixen als promotors de molts gens, indicant que aquest mecanisme és extens.

Basant-se en aquests resultats i en la identificació de més de 50 factors de transcripció que hem identificat que interaccionen amb la proteïna DELLA GAI, proposem que aquestes proteïnes actuen com a "**hubs**" en xarxes de senyalització. En particular, proposem que aquest és el mecanisme pel que aquestes proteïnes són claus integrant senyals externs i respostes de desenvolupament.

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Introduction **1**

1.1 Plant hormones

Contrary to other organisms, plants cannot run away from the environmental conditions that compromise their survival. Therefore they have evolved an interlocked mechanism to adjust their growth and developmental processes accordingly to environment. This characteristic, also called "plasticity", provides plants with a big adaptability allowing them to survive. Among the factors that contribute to plasticity are hormones.

Plant hormones are a group of natural substances able to influence physiological processes, and only small concentrations are needed to produce significant effects. Contrary to animal hormones, plant hormones can be produced within each cell, and they either act locally or are transported where needed. For the moment, eight classes of plant hormones have been identified: auxin, gibberellins (GAs), cytokinins (CKs), ethylene, abscisic acid (ABA), brassinosteroids (BRs), jasmonates and, more recently, strigolactones.

Since their discovery, the role of each hormone has been extensively studied, especially to understand how their activity is integrated with environmental signals. Hormones participate in response to stimuli such as light and temperature. They also contribute during the response triggered by other types of external stimuli such as nutrient deficiency, salt stress and infection with pathogens (Achard et al.,2006; Bari and Jones, 2009; Jaillais and Chory, 2010). Due to this pervasive effect of plant hormones in plant development and physiology, big efforts have been made to elucidate their biosynthesis and signaling pathways. At present most components of signaling and biosynthesis are characterized, but the mechanism of action is not completely understood. Although each hormone has been attributed a different set of specific roles, it has also become clear that many of them share overlapping functions. So, one of the pressing questions that biologists are trying to answer is what is the molecular mechanism that underlies the interaction between the different hormones –or, more broadly, between the different environmental and endogenous signals.

1.2 An endogenous mechanism that regulates hormone signal transduction

Plants are able to compensate and anticipate daily changes. The "circadian clock" is the core mechanism that modulates plant responses in a timely manner, for instance adjusting plant growth to the most suitable time of the day (Dodd et al., 2005). This mechanism is widely conserved among organisms including algae, fungi, plants and animals. Its importance in plants can be perceived since one third of the *Arabidopsis thaliana* genes are regulated by the circadian clock (Pruneda-Paz and Kay, 2010), resulting in the control of many physiological processes such as cotyledon movement, hypocotyl elongation, stomata opening, photosynthesis, and seasonal processes such as flowering (Cumming and Wagner, 1968; McClung, 2006). Hormones modulate these developmental processes as well, and it is known that the clock has an influence in hormonal biosynthesis; for instance it controls the levels of ethylene, BRs, GAs, ABA and auxin related genes (Jouve et al., 1999; Blazquez et al., 2002; Thain et al., 2004; Bancos et al., 2006; Covington and Harmer, 2007; Legnaioli et al., 2009). In addition to biosynthesis, the circadian clock regulates hormone signal transduction; for example, control of auxin regulated genes by the circadian clock modifies the sensitivity of the plant to the hormone (Covington and Harmer, 2007). This indicates that hormone signaling can be regulated by the circadian clock -a question that is not completely understood.

The circadian clock can be entrained by external stimuli such as light and temperature, and one of the questions during this thesis was trying to integrate the circadian clock with GAs, since GAs play an essential role during temperature and light mediated growth (Alabadi and Blazquez, 2009; Stavang et al., 2009). During the next section we will introduce GAs.

1.3 Gibberellins

GAs were first discovered when the Japanese scientist Eiichi Kurisawa was studying the *bakanae* disease in rice. The pathogenic fungus *Gibberella fujikuroi* was

identified as the cause of the disease that produced big losses due to the exaggerated elongation and fall over of infected plants. Observations showed that a substance secreted by the fungus was responsible for this tallness (Yabuta and Sumiki, 1938), and soon thereafter this substance was identified as a form of GA. Since then, GAs have been widely identified in plants, and also in some fungi and bacteria (Phinney, 1983).

In plants, a large effort has been made to elucidate GA function, synthesis and signaling. The elucidation of the crystal structure of the fungus substance "*gibberellic acid*" help to classify GAs as a big family of diterpenoids carboxylic acids (Yabuta and Sumiki, 1938). Up to now, 130 GAs have been identified, however only a few of them are functional in plants (GA_1, GA_3, GA_4 and GA_7) (Hedden and Phillips, 2000). These functional GAs regulate several physiological processes such as germination, stem elongation, photomorphogenesis, pollen development, flowering, fruit induction, leaf expansion and root growth (Figure 1.1) (Wilson et al., 1992; Garcia-Martinez et al., 1997; Peng and Harberd, 1997; Blazquez et al., 1998; Lee et al., 2002; Ogawa et al., 2003; Alabadi et al., 2004; Yu et al., 2004; Achard et al., 2007; Ubeda-Tomas et al., 2008; Achard et al., 2009; Nelissen et al., 2012).
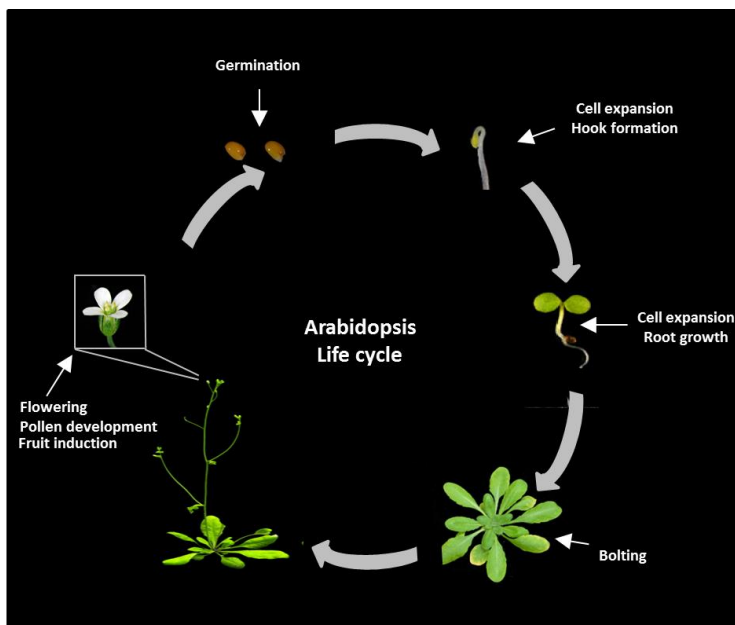
Figure 1.1 Gibberellin contribution during plant´s life cycle

The relevance of GA action for plant biology has been reflected in the numerous practical uses derived from GA application, the application of GA inhibitors, or the biotechnological manipulation of GA synthesis and signaling, like changing the stalk length of seedless grapes, or increasing sugar yield in sugarcane (Silverstone and Sun, 2000). Moreover the plants used during the so called "Green Revolution", which were rice and wheat varieties selected for increased yield that decreased the losses caused by lodging, turned out to be GA insensitive mutants.

1.4 Gibberellin metabolism

In higher plants GAs are usually produced through the methylerythritol phosphate (MEP) pathway. In this pathway, GAs are synthesized in three stages, each in a different cellular compartment (Figure 1.2): in the **plastid,** geranylgeranyl diphosphate (GGDP), a common precursor for diterpenes and tetraterpenes (carotenoids) (Lange, 1998; Hedden and Phillips, 2000), is converted into *ent*-kaurene in two steps catalyzed by two different enzymes: CPS (*ent*-copalyl diphosphate synthase) and KS (*ent*-kaurene synthase). *ent*-kaurene is then converted into GA_{12} or GA_{53} (inactive precursors) by hydroxylation carried out by two cytochrome P450 monooxygenases: KO (*ent*-kaurene oxidase) located in the membrane of the plastid, and KAO (*ent*-kaurenoic acid oxidase) located in the **endoplasmic reticulum.** GA_{12}, considered as the common precursor for all GAs in plants (Hedden and Phillips, 2000). In the **cytoplasm** active GAs are produced when GA_{12} is submitted to two subsequent oxidations catalyzed by GA 20-oxidase (GA20ox) and GA 3-oxidase (GA3ox) (Hedden and Phillips, 2000).

GAs can also be inactivated, and the best characterized mechanism is the *2β-*hydroxylation of GAs by GA 2-oxidases (GA2ox). Another inactivation mechanism is the formation of conjugates with glucose. The conjugating sugar is usually abundant in seeds, and the conjugates could be a storage form of GAs (Schneider and Schliemann, 1994), although this role has not been confirmed yet. Recently two additional mechanisms of inactivation have been reported. First, the cytochrome P450 mono-oxygenase, encoded by the *ELONGATED UPPERMOST INTERNODE* (*EUI*)

gene in rice, converts non hydroxylated GAs into 16α,17-epoxides, reducing the biological activity of GA_4 (Zhu et al., 2006). And second, the enzymes GAMT1 and GAMT2 catalyze the formation of GA methyl-esters. Their overexpression causes a GA deficient phenotype (Varbanova et al., 2007).

To respond effectively to environmental changes, plants need to be able to precisely regulate hormone homeostasis. GA levels can be regulated by several mechanisms. Most of the *GA20ox* and *GA3ox* genes are down-regulated through a GA-mediated feedback mechanism (Hedden and Phillips, 2000; Yamaguchi and Kamiya, 2000). In contrast, the genes encoding GA 2-oxidases are up-regulated by GA treatment (Thomas et al., 1999; Elliott et al., 2001). Apart from this mechanism of regulation, other hormones can alter GA levels. For instance, BRs promote the expression of the *GA20ox1* gene in *Arabidopsis* seedlings (Bouquin et al., 2001). Auxins have also been proposed to increase GA levels by transcriptional regulation of *GA20ox* and *GA3ox* genes in several plant species (Ross et al., 2000; Frigerio et al., 2006). In addition, the gaseous hormone ethylene promotes flowering through the regulation of GA metabolism (Achard et al., 2007). Moreover, there is also transcriptional regulation of GA metabolism genes triggered by environmental conditions. The seeds of some plant species require light to germinate, and this requirement can be overcome by external application of GAs, suggesting that light induces GA production during seed germination. In fact, it has been shown that light can induce transcript levels of *GA3ox* in seeds (Toyomasu et al., 1998). Interestingly however, the effect of light on seedlings stages is completely the opposite: it reduces the levels of *GA20ox* and *GA3ox* transcripts (O'Neill et al., 2000; Garcia-Martinez and Gil, 2001). Finally, temperature has been shown to have an impact: cold temperatures stimulate transcription of GA biosynthesis genes, whereas *GA2ox* genes are repressed, in species that require low temperatures for germination of dark imbibed seeds (Yamauchi et al., 2004). And again the effect of the temperature at seedling is the opposite (Stavang et al., 2009).
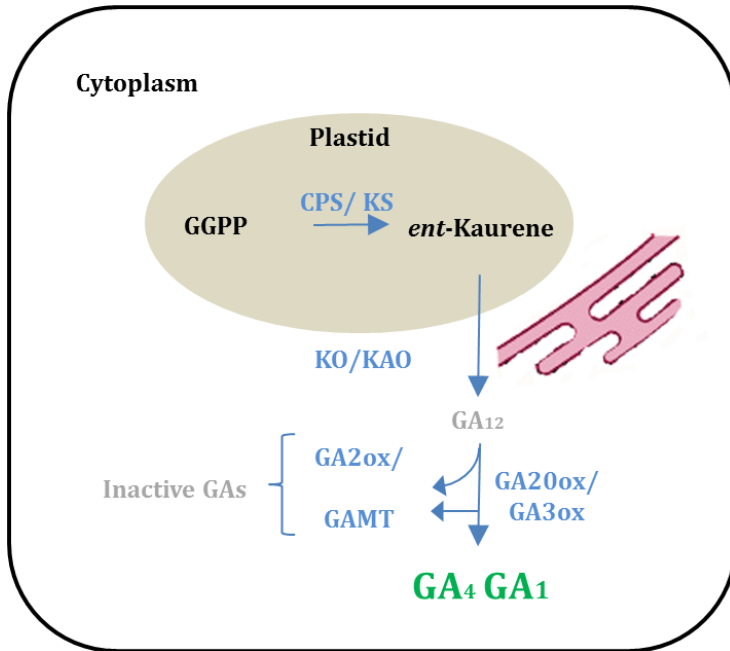
Figure 1.2 Gibberellin metabolism.

GGPP (geranylgeranyl diphosphate). Enzymes are represented in blue: **CPS** (*ent*-copalyl diphosphate synthase), **KS** (*ent*-kaurene synthase), **KO** (*ent*-kaurene oxidase), **KAO** (ent-kaurenoic acid oxidase), **GA20ox** (GA 20-oxidase), **GA3ox** (GA 3-oxidase), **GA2ox** (GA 2-oxidase), **GAMT** (gibberellin methyltransferase).

1.5 Gibberellin signaling

The mechanism of GA action is widely conserved among dicots and monocots. Current information of GA signaling has been mostly gathered from genetic screenings in *Arabidopsis* and rice. In summary, the GA signaling pathway consists of three elements; the **soluble receptors (GID1s)**, the **transcriptional regulators DELLA proteins (DELLAs)**, and **F-box proteins (GID2/SLY1)** that mediate GA-dependent degradation of the transcriptional regulators.

The GA-insensitive rice mutant *gibberellin insensitive dwarf1* (*gid1*), led to the discovery of GA receptors (Ueguchi-Tanaka et al., 2005). In *Arabidopsis* there are three ortholog genes coding for GA receptors: *GID1A, GID1B,* and *GID1C* (Griffiths et

8

al., 2006; Nakajima et al., 2006). Single *gid1* mutants develop normally, and only a triple knockout mutant displays the dwarf phenotype characteristic of GA signaling deficiency (Griffiths et al., 2006; Willige et al., 2007), it appears that the three receptors in *Arabidopsis* have overlapping biological functions. This is in concordance with the observation that the three paralogs are expressed in all tissues, but at different levels (Griffiths et al., 2006; Nakajima et al., 2006).

DELLAs are considered as "the master negative regulators" of GA signaling, and were discovered through the study of the *Arabidopsis gai-1* (*gibberellic acid insensitive-1*) mutant (Peng et al., 1997). GAI is one of the five DELLAs in *Arabidopsis*. Over the years all DELLAs in *Arabidopsis* have been identified, GAI, Repressor of *ga1-3* (RGA), RGA like1 (RGL1), RGL2, RGL3 (Silverstone et al., 1998; Lee et al., 2002). These proteins are able to downregulate all GA responses (Wilson and Somerville, 1995; Peng et al., 1999).

Basically all GA functions rely on promoting the degradation of these proteins; GA signaling starts when active GAs bind to the nuclear receptor GID1 (Ueguchi-Tanaka et al., 2005). Crystal structure revealed that GID1 contains a GA-binding pocket and a flexible N-terminal extension (Shimada et al., 2008). When the GA-GID1 complex is formed the flexible domain closes the pocket, and this conformational change allows GID1s to bind to the N-terminal part of DELLAs (Murase et al., 2008), this domain is necessary for interaction with GID1s (Willige et al., 2007). Once the GA-GID1-DELLA complex is formed, a SCF-dependent E3 ubiquitin ligase complex involving the F-box proteins GID2/SLY1 binds to DELLAs (Griffiths et al., 2006; Ariizumi et al., 2011), leading to their polyubiquitination and subsequent degradation by the 26S proteasome (Griffiths et al., 2006; Ueguchi-Tanaka et al., 2007; Willige et al., 2007; Schwechheimer, 2008) (Figure 1.3).
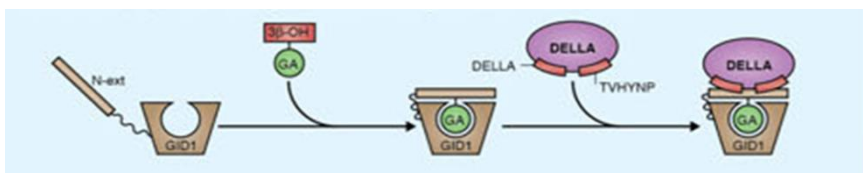
Figure 1.3 Gibberellin signaling (Daviere and Achard, 2013).

1.6 Structural features of DELLA proteins

DELLAs, the most extensively studied components of GAs signaling, are members of the GRAS protein family with nuclear localization (Bolle, 2004). Their ability to negatively regulate GA responses is based on the observation that *della* mutants in *Arabidopsis* and rice results in a constitutive GAs response (Dill and Sun, 2001; Ikeda et al., 2001; Lee et al., 2002; Cheng et al., 2004; Tyler et al., 2004), and on the contrary, gain of function mutants display the characteristic phenotype of gibberellin deficiency (Wilson and Somerville, 1995; Dill et al., 2001).

DELLAs are highly conserved among species (Peng et al., 1997; Peng et al., 1999; Ikeda et al., 2001; Chandler et al., 2002; Marti et al., 2007; Gallego-Bartolomé et al., 2010). Moreover, unlike *Arabidopsis* where there are five *DELLA* genes, in some species like rice or tomato there is a single *DELLA* gene (Peng et al., 1997; Peng et al., 1999; Ikeda et al., 2001; Chandler et al., 2002; Marti et al., 2007). Interestingly, the only DELLA is sufficient to regulate all GA responses in these species. In accordance with this, it has been proposed that functional diversification of *DELLA* genes, at least in Brassicaceae, is based on their differential expression pattern (Gallego-Bartolome et al., 2010). Therefore, the differential roles for the different DELLAs in *Arabidopsis* is caused by differential transcriptional regulation of the corresponding genes. In particular, RGA and GAI are the major GA repressors during vegetative growth and floral induction (Richards et al., 2001; Olszewski et al., 2002), RGA, RGL1 and RGL2 together modulate flower development (Lee et al., 2002; Cheng et al., 2004; Tyler et al., 2004), and RGL2 is the main DELLA regulating seed dormancy (Lee et al., 2002).

Besides, DELLAs present high sequence conservation, all DELLAs contain the intrinsically disordered N-terminal DELLA domain containing conserved amino acid sequences Asp-Glu-Leu-Leu-Ala (DELLA) and the TVHYNP and poly Ser/Thr domains (Figure 1.4). Deletions on DELLA or TVHYNP regions result in protein versions unable to interact with GID1 (Figure 1.3), and therefore are resistant to GA-promoted degradation, for example the *Arabidopsis* mutants *rga-Δ17* or *gai-1* (Willige et al., 2007).
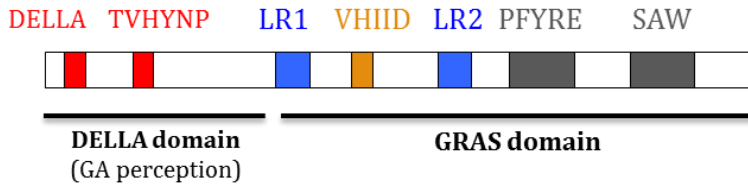
Figure 1.4. The domain structure of DELLA proteins.

On the other hand, the GRAS domain within the C-terminus contains two Leu heptads repeats (LR1 and LR2), a VHIID domain, and the subdomain SH2-like including PFYRE and SAW subunits (Hauvermale et al., 2012). The VHIID and LHRs domains have been implicated in dimerization of the protein (Itoh et al., 2002). While the function of PFYRE and SAW is still unknown. The C-terminal region interacts with the F-box protein SLY1 in *Arabidopsis* (and OsGID2 in rice) (Dill et al., 2004; Gomi et al., 2004).

Since their discovery, DELLAs have been implicated in the regulation of development during the whole plant life. For instance, they restrict vegetative growth and root elongation; they promote photomorphogenesis and seed dormancy; and they delay flowering, especially under non-inductive photoperiodic conditions (Blazquez et al., 1998; Lee et al., 2002; Alabadi et al., 2004; Penfield et al., 2006; Achard et al., 2007). They are also involved in the response to stress, increasing the resistance to necrotrophic fungi and promoting the tolerance to certain abiotic stress factors like drought and cold (Achard et al., 2006; Achard et al., 2008; Navarro et al., 2008).

It is well established that DELLAs act as transcriptional regulators to control all these responses (Ogawa et al., 2003; Cao et al., 2006; Zentella et al., 2007; Hou et al., 2008; Gallego-bartolome et al., 2011). However, DELLAs do not possess a DNA binding domain, and the mechanism by which they control transcription has been elusive until the recent discovery that RGA interacts with PIF4 (PHYTOCHROME INTERACTING FACTOR 4) and PIF3, DNA-binding transcription factors (TFs) of the bHLH family that promote the expression of genes involved in cell elongation (de Lucas et al., 2008; Feng et al., 2008). Through this interaction, DELLAs would block the DNA binding capacity of PIF4 and PIF3 to their target genes, resulting in the

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restriction of cell elongation (de Lucas et al., 2008). However since these interactions do not explain all processes regulated by DELLAs, a possibility is that the ability of DELLAs to interact with TFs could be extended to more than the two known at the beginning of my work. One of the goals of this thesis has been to explore this possibility.

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Objectives **2**

2. Objectives

As reviewed above, GAs contribute largely to the modulation of developmental processes through DELLA-mediated transcriptional regulation. All those processes are coincidentally regulated by additional signaling pathways (other hormones, light, the circadian clock, etc), and this Thesis addresses **the molecular mechanisms by which DELLAs integrate environmental signals and relay this information to transcriptional networks.**

The two specific objectives pursued in this work are:

1. **To investigate the connection between the circadian clock and DELLA abundance**. Previous evidence shows that DELLAs are essential regulators of cell expansion, while the circadian clock determines the timing for cell expansion during the day, so we hypothesized that the circadian clock could regulate GA signaling at one or several levels. This is the focus of Chapter 1.

2. **To find additional transcription factors through which DELLAs control gene expression.** Previous evidence indicates that DELLAs do not bind DNA directly, but modify the activity of other TFs with which they interact physically. The published interaction with PIF3 and PIF4 does not account for all the DELLA-dependent transcriptional changes, therefore we propose to screen for additional TFs and evaluate the biological significance of the partners found. This is the focus of Chapters 2, 3, and 4.

Chapter 1 | 3

Circadian oscillation of gibberellin signaling in Arabidopsis

María Verónica Arana, Nora A. Marín-de la Rosa, Julin N. Maloof, Miguel A. Blázquez, David Alabadí.

3.1 Abstract

Circadian clocks are endogenous time-keeping mechanisms that allow organisms to anticipate rhythmic, daily environmental changes. Temporal coordination of transcription results in a set of gene expression patterns with peak levels occurring at precise times of the day. An intriguing question is how a single clock can generate different oscillatory rhythms, and it has been proposed that hormone signaling might act in plants as a relay mechanism to modulate the amplitude and the phase of output rhythms. Here we show that the circadian clock gates GA signaling through transcriptional regulation of the GA receptors, resulting in higher stability of DELLAs during daytime and higher GA sensitivity at night. Oscillation of GA signaling appears to be particularly critical for rhythmic growth, given that constitutive expression of the GA receptor expands the daily growth period in seedlings, and complete loss of DELLA function causes continuous, arrhythmic hypocotyl growth. Moreover, transcriptomic analysis of a pentuple *della* KO mutant indicates that the GA pathway mediates the rhythmic expression of many clock-regulated genes related to biotic and abiotic stress responses and cell wall modification. Thus, gating of GA sensitivity by the circadian clock represents an additional layer of regulation that might provide extra robustness to the diurnal growth rhythm and constitute a regulatory module that coordinates the circadian clock with additional endogenous and environmental signals.

3.2 Introduction

The pervasive role of the circadian clock driving plant physiology is reflected by the extensive regulation it exerts on gene expression, since more than one-third of *Arabidopsis* genes are under circadian control (Hasty et al., 2001). Remarkably, the expression of almost every single gene of *Arabidopsis* cycles when plants are grown under more realistic situations, for example combinations of thermo- and photocycles (Michael et al., 2008b). This suggests that the entrainment of the circadian clock by light and temperature signals might allow plants to adapt to the daily changes in the environment by timing every physiological pathway to the specific time of day when it is more advantageous. For instance, the concerted action of the circadian clock and phyB-mediated light signaling allows the expression of a set of hormone-related genes towards dawn (Michael et al., 2008a) which may provide robustness to the rhythmic patterns of growth of the seedling under diurnal conditions (Nozue et al., 2007). Accordingly, a correlation exists between the oscillation of auxin-related genes and changes in the hypocotyl growth-rate of seedlings grown under free-running conditions (Nozue et al., 2007). Accordingly, a correlation exists between the oscillation of auxin-related genes and changes in the hypocotyl growth rate of seedlings grown under free-running conditions (Dowson-Day and Millar, 1999; Covington and Harmer, 2007) although the physiological significance of this correlation remains to be explored. In addition to light-mediated growth, the circadian clock controls the time of day that other environmental response pathways can be activated, often by triggering the oscillation of key signaling genes involved in these pathways (Harmer et al., 2000). This type of regulation is known as gating because the clock can be thought of as opening or closing a gate to control the flow of information through a signaling pathway. Through such gating the circadian clock regulates many physiological responses including the photoperiodic induction of flowering and stress responses (de Montaigu et al., 2010).

GA has a prominent role in the regulation of several developmental programs also affected by light and the circadian clock, including the establishment of

photomorphogenesis (Alabadí et al., 2004; Achard et al., 2007; Alabadí et al., 2008) and cell expansion (Cowling and Harberd, 1999), and the question arises of whether GA activity might mediate circadian regulation of clock targets. Given that such a regulatory mechanism would impact the robustness and flexibility of circadian regulation of development, we decided to explore this possibility and its physiological relevance

3.3 Results and discussion

3.3.1 Expression of GA receptors is controlled by the circadian clock

To investigate whether the circadian clock regulates GA signaling in *Arabidopsis*, we examined the daily expression pattern of all known GA signaling elements in the DIURNAL database (http://diurnal.cgrb.oregonstate.edu/) (Mockler et al., 2007; Michael et al., 2008b). Although a weak oscillation could be detected in some cases, mostly linked to temperature rhythms, only the *GID1* receptor genes displayed robust cycling under short days (Figure. S3.1A). The cycling of *GID1a* and *GID1b* was validated by real-time RT-qPCR in independent time-course experiments, whereas we were not able detect oscillation for *GID1c* transcript (Figure. 3.1A). The anticipation of changes in transcript levels to the light-to-dark and dark-to-light transitions and the oscillation under continuous light in entrained seedlings (Figure. S3.1B) suggested circadian rather than diurnal regulation. This was confirmed by analyzing mRNA levels in mutants defective for clock function, *toc1-1* (Boonsirichai et al., 2002) and *lhy* (Schaffer et al., 1998). The waveform of the oscillation in *toc1-1* seedlings was different from the wild-type, the peak was narrower and it was phased earlier The waveform of the oscillation in *toc1-1* seedlings was different from the wild-type, the peak was narrower and it was phased earlier (Figure. 3.1F). The phase advance is typical of *toc1-1* mutants, due to the deviation between their endogenous period (21 h) and the length of the day (24 h) (Strayer et al., 2000). Transcript levels of both genes were altered also in the arrhythmic mutant *lhy* (Figure. 3.1K).

The expression of GA receptor genes is known to respond to endogenous GA levels through a DELLA-mediated feedback mechanism, i.e. their expression increases

when GA levels are low and decreases when hormone levels are high (Griffiths et al., 2006). Hence, oscillation of *GID1* transcript levels might be a direct consequence of the circadian clock activity or, alternatively, it might respond to a putative oscillation of GA levels. However, *GID1a* expression was not altered in seedlings of the quadruple *della* mutant (*rga-t2 gai-t6 rgl1-1 rgl2-*1) (Cheng et al., 2004; Achard et al., 2006) (Figure. S3.2), indicating that the circadian clock controls the expression of *GID1* genes independently of the status of the GA pathway.
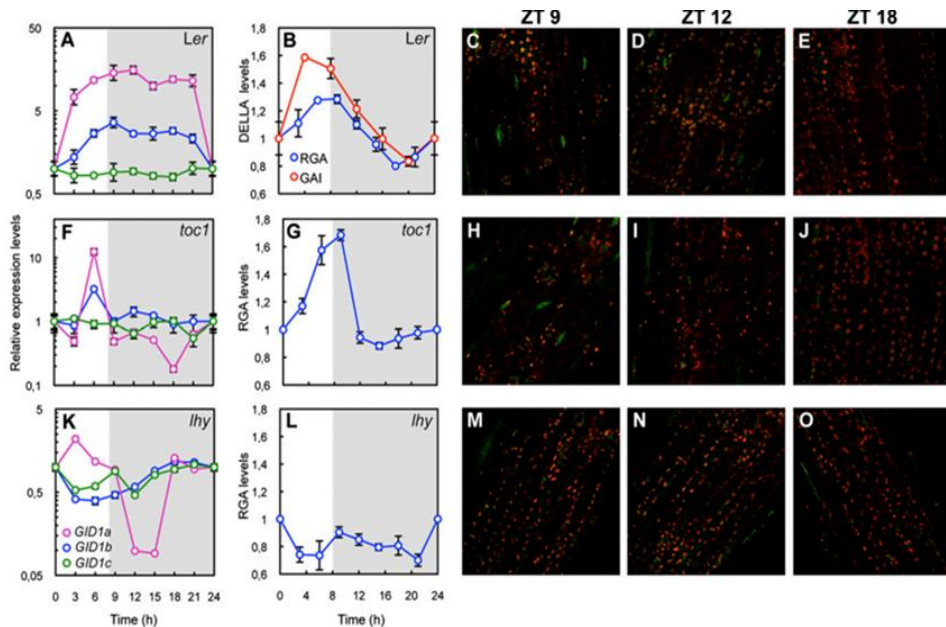
Figure. 3.1. The circadian clock controls the diurnal oscillation of DELLA proteins in the cell expansion zone of hypocotyls. (A,F,K) Expression of *GID1a*, *GID1b*, and *GID1c* in 5-day-old L*er* wild type seedlings (A), in *toc1-1* (F) and in *lhy* (K) mutants grown under short-day photocycles (8-h light/16-h dark). In (B-E, G-J, L-O), seedlings carrying the *35S::TAP-GAI* and *RGA::GFP-RGA* constructs were grown for 5 days under short-day photocycles (8-h light/16-h dark). DELLA protein levels in the L*er* WT (B) and in the *toc1-1* (G) and the *lhy* (J) mutants were determined by western-blot analysis. TAP-GAI and GFP-RGA proteins were detected with commercial antibodies against the *myc* tag and GFP, respectively. DELLA levels were normalized against levels of DET3, which was used as loading control. Data are average of three independent experiments and plotted as mean ± s.e.m. Protein level at ZT0 was set to one and used as reference for all other time points. White and grey areas represent day and night, respectively. Fluorescence of GFP-RGA oscillates in the upper part of hypocotyls of L*er* WT (C-E) and *toc1-1* mutant seedlings (H-J), but not in the *lhy* mutant (M-O). Fluorescence was detected by confocal microscopy. Images are representative of three independent biological repeats including 12-15 seedlings per time point and per genotype.

3.3.2 Levels of DELLA proteins oscillate with a daily rhythm

GID1 receptors are known to interact with DELLAs in a GA-dependent way, and promote their degradation (Griffiths et al., 2006). Thus, if the oscillation of *GID1* expression is physiologically relevant, it should cause coherent changes in DELLA accumulation with a daily rhythm. We focused our attention in two DELLAs, GA INSENSITIVE (GAI) and REPRESSOR OF *ga1-3* (RGA), which are the most abundant DELLAs in young seedlings and shoots (Tyler et al., 2004). To monitor the level of these proteins we used lines that express either the *RGA::GFP-RGA* (Silverstone et al., 2001) or *35S::TAP-GAI* (Feng et al., 2008) transgenes. Consistent with the clock regulation of *GID1* genes, both GFP-RGA and TAP-GAI protein levels oscillated in a diurnal manner, showing peak levels at the end of the light period (Figure. 3.1 B). Strikingly, GFP-RGA oscillation was detected in the growing region of the hypocotyls (Figure. 3.1 C-E and H-J). Fluorescence from the fusion protein accumulated in nuclei of the uppermost part of hypocotyls at ZT9, while it was below the detection limit late in the night, at ZT18 (Figure. 3.1 C-E), coinciding with a period of minimum and maximum growth rates, respectively (Nozue et al., 2007). The periodicity of RGA accumulation must largely be caused by the activity of GID1 receptors, as the RGA transcript did not show significant oscillation (Figure. S3.1C). Thus, given (i) the major role of RGA and GAI in controlling growth (Dill and Sun, 2001; King et al., 2001), and (ii) that seedling growth under diurnal conditions is gated by the circadian clock (Nozue et al., 2007), this result suggests that DELLAs are regulatory components for the control of the clock output, such as daily growth rhythm in young seedlings (Nozue et al., 2007).

The oscillation of GFP-RGA levels was affected also in clock mutants. The waveform of GFP-RGA oscillation in *toc1-1* seedlings was slightly different from the WT, the amplitude was higher and the peak narrower because of an advance in the phase of the trough (Figure. 3.1 G-J), according to the phase advance observed in the expression of GA receptor genes (Figure. 3.1F). On the contrary, GFP-RGA protein levels were constant and low in the *lhy* mutant (Fig. 3.1 L-O), which correlates with the long hypocotyl phenotype observed in this mutant when grown in short days (Michael et al., 2008a).

3.3.3 The circadian clock gates GA signaling activity

The observation that lower DELLAs levels coincide with higher growth rates at the end of the night suggests that they participate in the core mechanism that controls rhythmic growth of hypocotyls. To test this hypothesis, we examined the impact upon growth of an alteration of the normal rhythm of GA signaling with two complementary approaches. First, we used a transgenic line that expresses a dominant version of GAI under the control of a heat-shock inducible promoter, *HS::gai-1D* (Alabadí et al., 2008). This line allowed us to block GA signaling by applying a 10-min heat shock at 33ºC at two different times of the day: ZT5, when the growth rate is low and DELLA levels high, and ZT17, which coincides with the beginning of the growing phase and with the trough of DELLA levels (Figure. 3.2A). The effectiveness of the treatments was confirmed by expression analysis of *gai-1* and one of its known direct targets, *AtGA20ox2* (Figure. 3.2B). Interestingly, blocking GA activity at ZT5 during four consecutive days did not have any effect upon hypocotyl growth (Figure. 3.2C). On the contrary, the heat treatment had a strong inhibitory effect on the hypocotyl growth of *HS::gai-1D* seedlings when applied at ZT17 (Figure. 3.2C).

Figure. 3.2 Blocking GA signaling at night affects hypocotyl growth. Seedlings of the *HS::gai-1D* line were grown under short-day photocycles (8-h light/16-h dark) and received heat treatments of 33ºC for 10 min at either ZT5 or ZT17 as explained in Materials and Methods. In (A), shaded areas mark the period of the day during which *gai-1D* accumulates. (B) Expression of *gai-1D* (bars) and its target gene GA20ox2 (circles, scale on the right) after heat treatments at ZT5 (white symbols) and ZT17 (dark symbols). (C) Hypocotyl length of Col-0 WT and *HS::gai-1D* seedlings that did not receive heat treatments (grey bars) or that received treatments at ZT5 (white bars) or ZT17 (black bars). The experiment was repeated three times with similar results. Data represent the mean ± sd (n ≥ 15 seedlings), and asterisks indicate p<0.0001.
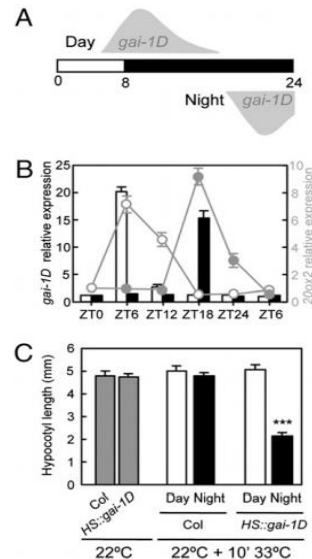
Second, we examined how transient application of GA$_4$ at two different times of the day would rescue the dwarfism caused by continuous incubation with paclobutrazol (PAC), a compound that causes accumulation of DELLAs (Figure. 3.3A and B). GA-application provoked rapid degradation of DELLAs that lasted for the next 10 hours: between ZT1 and ZT10 when the GA treatment was applied at dawn, and between ZT13 and ZT22 when applied at ZT12 (Figure. 3.3B). Importantly, GA treatment applied at ZT12 during two consecutive days (fourth and fifth) significantly alleviated the growth-repressing effects of PAC, whereas it had no effect when applied at dawn (Figure. 3.3C). In summary, these results confirm that there is a DELLA-sensitive period that overlaps the growing phase of the night, and that under short-day conditions the circadian clock might allow growth by preventing accumulation of DELLAs during that particular period.

Figure. 3.3 GA application at night releases the growth restrain imposed by DELLAs. *RGA::GFP-RGA* seedlings grown under short-day photocycles (8-h light/16-h dark) in the presence of 0.2 μM PAC were treated with 1 μM GA4 at either ZT0 or ZT12, or untreated, as explained in Materials and Methods. (A) Scheme of DELLA accumulation after GA$_4$-treatments, deduced from the GFP-RGA fluorescence of seedlings grown under the same conditions (B). Confocal images taken at the time of GA$_4$-treatment (ZT0 and ZT12), 1 h (ZT1 and ZT13), and 10 h later (ZT10 and ZT22), show that the maximum period with low DELLA levels spans less than 10 h. Images are representative of three independent biological repeats including 8-10 seedlings per time point. (C) Hypocotyl length of wild-type (L*er*) seedlings grown in the presence of PAC that did not receive any additional treatment (mock) or that were treated with GA4 at ZT0 (day) or ZT12 (night). The wild-type seedlings contain the RGA::GFP-RGA transgene. Data represent mean ± sd (n ≥ 15 seedlings). Asterisks indicate p<0.001
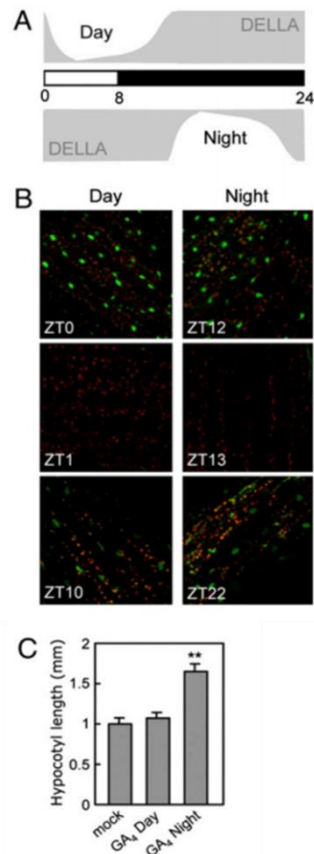
3.3.4 Oscillation of GA signaling refines rhythmic growth

If the oscillation of GA signaling constitutes part of the mechanism that ensures rhythmic growth, a prediction of this model is that GA signaling mutants should display not only a defect in the final size of the hypocotyl (Cowling and Harberd, 1999), but also an altered rhythmic growth pattern. In agreement with this, transgenic plants that expressed *GID1a* under the control of the *35S* promoter showed an expanded growth phase that started at the same time as in the wild type and extended well into daytime, almost spanning the whole light period (Figure. 3.4A). In agreement with this, plants overexpressing *GID1a, GID1b*, or *GID1c* displayed longer hypocotyls compared to the wild type ($P < 0.05$, Student t test; Figure. S3.3A), and seedlings of *gid1a-1, gid1b-1*, and *gid1c-1* loss-of-function mutants, and of the different double mutant combinations (Griffiths et al., 2006) had shorter hypocotyls than the WT ($P < 0.05$, Student t test; Figure. S3.3B). Taken together, these results indicate that *GID1* expression is limiting for promotion of hypocotyl elongation under diurnal conditions and that oscillation of *GID1* is necessary for the establishment of proper patterns of rhythmic growth.
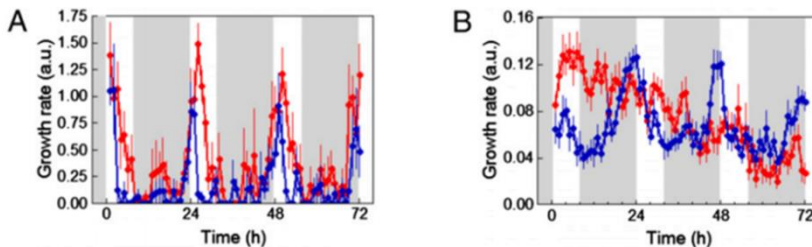
Figure. 3.4 GA activity regulates diurnal rhythms of hypocotyl elongation. Col-0 and *35S::GID1a* seedlings (A), and L*er* and quintuple *della* mutant seedlings (B) were grown under short-day photocycles (8-h light/16-h dark) for three days before they were imaged under the same conditions for three additional days. Blue and red symbols and lines denote the WT and mutant/transgenic seedlings, respectively. Seedlings growth-rate was measured as described in Materials and Methods. White and grey areas represent day and night, respectively. Data represent the mean ± sd (n ≥ 10 seedlings).

Nonetheless, seedlings overexpressing *GID1a* still exhibited robust rhythmic growth. Given that this behavior cannot be attributed to circadian regulation of GID1

protein stability (Figure. S3.4), it could be taken as an indication that GA levels oscillate in a diurnal or in a clock-controlled manner. In fact, expression of several genes encoding GA metabolic enzymes oscillate diurnally in *Arabidopsis* (Hisamatsu et al., 2005; Zhao et al., 2007), in a manner that would likely result in lower GA levels at dusk; and diurnal changes in GA levels has been described in sorghum (Lee et al., 1998).

More clear evidence for an integral role of GA signaling in the establishment of rhythmic growth was provided by the quintuple *della* KO mutant (Feng et al., 2008). Growth of this mutant was completely arrhythmic under short days, showing a relatively high growth rate during the first day examined, that progressively decreased over the next few days (Figure. 3.4B). This arrhythmic phenotype is not likely due to a dysfunction of the core clock mechanism, since the expression of the clock genes *TOC1* and *CCA1* was not significantly affected in the mutant (Figure. S3.5). DELLAs have been proposed to regulate cell expansion through the inhibition of PIF's activity (de Lucas et al., 2008; Feng et al., 2008). The observation that *pif4 pif5* double mutants are impaired in rhythmic growth (Nozue et al., 2007) suggests that the DELLA-PIF interaction might thus constitute the main output pathway that controls rhythmic growth. However, given that DELLAs exert part of their action also through the HY5 transcription factor (Alabadí et al., 2008) and *hy5* mutants display arrhythmic growth (Nozue et al., 2007) it is also possible that other components different from PIF might also be part of this regulatory module.

3.3.5 DELLA proteins mediate daily rhythms of gene expression

GA signaling participates in the regulation of multiple developmental and physiological processes other than cell expansion (Sun, 2010). It is therefore possible that the role of DELLAs as regulatory components of the output of the circadian clock extends beyond the control of rhythmic growth and affects other processes. To test this hypothesis, we performed a genome-wide search for genes that would oscillate in a circadian manner in WT seedlings, and whose oscillation would be affected in a *della* KO. For this purpose, we examined global gene expression in short-day-grown WT and *della* mutant seedlings at ZT9 (high DELLA levels) and ZT21 (low DELLA

levels). In this search, we found 5,087 genes whose expression varied between both time points in the WT. We found that this list was significantly enriched (66%; $P < 2.2$ x 10^{-16} by Fisher exact test) for genes differential expressed across time points in a previously published full short-day data set (Mockler et al., 2007; Michael et al., 2008b), indicating that our approximation to identify genes that oscillated with a specific diurnal phase was acceptable. To ask what role DELLA genes play in diurnal regulation of gene expression, we looked for genes that were differentially expressed when *della* mutants were compared to WT. We found 58 genes differentially expressed between *della* and WT; 37 of these were only found at ZT9 and four were found at ZT21, consistent with DELLAs being most important at ZT9 (Figure. 3.5A). Furthermore, the 37 genes affected by the *della* KO at ZT9 were significantly enriched for diurnally regulated genes (70%; $P = 0.001$ by Fisher exact test), whereas the remaining differentially genes were not enriched.

Importantly, Gene Ontology analysis showed that, among the genes whose oscillation was altered in the *della* mutant, there was an enrichment in functional categories related to the response to stress and environmental signals, as well as in genes encoding proteins located in the cell wall and the endoplasmic reticulum (Figure. 3.5B). Although part of the genes represented in the "cell wall" category could include those with a function in growth and cell expansion, the enrichment of other growth-unrelated categories suggests that DELLAs mediate the regulation of a larger array of circadian-clock controlled processes. These results attribute a more general role to DELLAs in the modulation of the output of the clock. Moreover, meta-analysis of the DELLA targets at ZT9 (Figure. 3.5C) indicated that only 43% of these genes were either direct targets for HY5 (Lee et al., 2007) or genes also regulated by the PIF transcription factors (Leivar et al., 2009; Shin et al., 2009). This implies that DELLAs control gene expression through the interaction with additional transcription factors, which is in agreement with the observation that DELLAs can interact with several members of the bHLH family of transcription factors other than PIFs (Arnaud et al., 2010; Gallego-Bartolome et al., 2010).
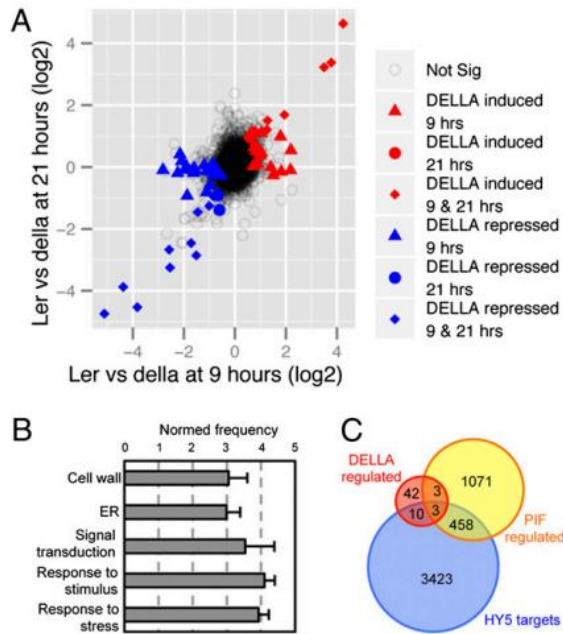
Figure. 3.5 DELLAs mediate circadian regulation of transcription. (A) Scatter plot of genes differentially regulated at ZT21 vs ZT9 in wild-type L*er* and *della* mutants. Genes showing statistically significant (FDR<0.1) differential expression between L*er* and *della* are displayed for each time point in blue and red. (B) Enrichment of Gene Ontology categories among genes regulated by DELLAs at ZT9 (p<0.0001 in all cases). (C) Venn diagram showing the overlap between genes regulated by DELLAs at ZT9; genes directly bound by HY5, as detected by ChIP-chip experiments in light-grown seedlings (Lee et al., 2007); and genes regulated by PIF transcription factors, as genes differentially expressed in the quadruple *pif1 pif3 pif4 pif5* mutant in darkness and in the light, with respect to the wild type (Leivar et al., 2009).

3.4 Concluding remarks

The enormous plasticity of plant growth and development is based on a web of interacting signaling pathways, which provides the plant with multiple entry points to adjust their physiology in response to frequent, unpredicted environmental changes (Casal et al., 2004). The circadian system, on the other hand, provides the plant with the ability to anticipate predictable, daily and seasonal environmental changes (Kirschner and Mitchison, 1986) and buffers plant responses against casual environmental variability (Troein et al., 2009). The circadian clock, therefore, provides stability to plant's life. Then, can responses regulated by the circadian clock

be plastic? We suggest that the regulation of GA activity by the circadian clock might provide such ability, acting as a link between two properties critical for plant growth and development, robustness and plasticity (Alabadí and Blázquez, 2009). Thus, the concurrency of clock and GA regulation of certain processes guarantees a precise and robust response to unpredicted, transitory, and above-noise changes in the environment that have an impact on the GA pathway, such as nutrient availability (Stelling et al., 2004), salt stress (Achard et al., 2006), ambient temperature (Stavang et al., 2009), or flooding (Benitez et al., 2008). The observation that the functioning of the circadian clock does not seem to be affected significantly by GA (Hou et al., 2008 and the present study), supports the suggested role of GA as a regulatory output module that fine-tunes clock-regulated gene expression in response to environmental signals.

3.5 Materials and methods

Plant material. *Arabidopsis thaliana* accessions Col-0 and L*er* were used as wild-types. Seeds of *gid1a-1, gid1b-1, gid1c-1, gid1a-1 gid1b-1, gid1b-1 gid1c-1, gid1a-1 gid1c-1, toc1-1, lhy, rga-t2 gai-t6 rgl1-1 rgl2-1, rga-t2 gai-t6 rgl1-1 rgl2-1 rgl3-1, RGA::GFP-RGA, 35S::TAP-GAI*, and *HS::gai-1D* have been previously described (Millar et al., 1995; Schaffer et al., 1998; Silverstone et al., 2001; Achard et al., 2006; Griffiths et al., 2006; Alabadí et al., 2008; Feng et al., 2008). *RGA::GFP-RGA*, *toc1-1* and *RGA::GFP-RGA, lhy* lines were obtained by genetic crosses and isolated from a F3 population.

Plasmid constructs and transgenic plants. The preparation of transgenic lines expressing either *35S::GID1a-YFP-HA*, *35S::GID1b-YFP-HA*, or 3*5S::GID1c-YFP-HA* was as follows. Coding sequences of GID1a-1c, excluding the stop codon, were PCR-amplified with Pwo polymerase (Roche) from cDNA obtained from 7-day-old, light-grown wild-type Col-0 seedlings. Oligonucleotides used as primers for PCR (Supporting Table I) included the attB sites needed for Gateway®-mediated cloning and were designed to allow expression of a C-terminal fusion. PCR products were first cloned into vector *pDONR-221* (Invitrogen) by BP recombination, and then

transferred to the binary vector *pEarleyGate-101* (Pierik et al., 2009) by LR recombination, to create a C-terminal fusion with YFP and HA-tag. The final constructs were transferred to wild-type Col-0 plants by Agrobacterium-mediated transformation. Primary transformants were selected in MS plates containing 50 mM glufosinate ammonium (Fluka). Transgenic lines with a 3:1 (resistant:sensitive) segregation ratio were selected and several homozygous lines were identified in the T3 generation for each construction.

Seedling growth assays. All seeds were surface sterilized with 70% (v/v) ethanol and 0.01% (v/v) Triton X-100 for 5 min, followed by 96% (v/v) ethanol for 5 min. Seeds were sown on plates of ½ MS medium (Duchefa), 0.8% (w/v) agar without sucrose, and stratified at 4°C in darkness for 5 d. Germination was induced by placing the plates under white fluorescent light (190–200 µmol m-2 s-1) at 22°C for 8 h. Seedlings were grown at 22°C under short-day photocycles, 8 h light (70–200 µmol m-2s-1, depending on the experiment)/16 h dark in a Percival E-30B growth cabinet (Percival).

For heat shock experiments, wild-type Col-0 and *HS::gai-1D* (Alabadí et al., 2008) seedlings grown in the same plate under short days (70 µmol m-2 s-1) received a heat treatment (10 min at 33°C in darkness) at either ZT5 or ZT17. Control seedlings of both genotypes were kept at 22°C. Heat treatments were applied at days 3, 4, 5, and 6. Hypocotyl length was measured on day 7.

For GA sensitivity assays, RGA::GFP-RGA seeds were sown on sterile filter papers placed on ½ MS, 0.8% (w/v) agar plates without sucrose, stratified, and induced to germinate as above. After induction of germination, filter papers harboring seeds were transferred to treatment plates containing 0.2 µM PAC (Duchefa) and grown under short days (190–200 µmol m-2 s-1) at 22°C for 3 d. Filter papers containing 4-day-old seedlings were transferred at either ZT0 or ZT12 for 1h to Petri dishes with 5 ml of ½ MS liquid media containing 0.1 µM GA4 (Sigma) plus 0.2 µM PAC, or just 0.2 µM PAC. Filter papers containing seedlings were then rinsed three times for 20 min in Petri dishes containing ½ MS with PAC 0.2 µM. After washes, seedlings were transferred into a new sterile filter paper, placed on fresh 0.2 µM PAC treatment-plate, and returned to short-day conditions. GA treatments were

given during two consecutive days (4th and 5th) and hypocotyl length was measured on day 6. Handling of seedlings during the dark period was performed under a safe green light.

To measure hypocotyl length, seedlings were placed on an acetate sheet, scanned at a resolution of 600 dots per inch, and the length was measured with the ImageJ software (http://rsb.info.nih.gov/ij/).

The time-lapse photography and image analysis to determine the hypocotyls growth-rate was performed as previously described (Nozue et al., 2007), except that the growth medium containted ½ MS and 1% sucrose.

RNA extraction and gene expression analysis by RT-qPCR. Total RNA was isolated from whole seedlings grown as described above (190–200 µmol m-2 s-1) by using the E.Z.N.A. Plant RNA Mini Kit (Omega Bio-tek) according to the manufacturer´s instructions. cDNA synthesis and quantitative PCR conditions were as described (Alabadí et al., 2008). Primers used are listed in Supporting Table II.

Microarray analysis. Wild-type L*er* and *rga-t2 gai-t6 rgl1-1 rgl2-1 rgl3-1* pentuple *della* mutant seedlings were grown under short days (190–200 µmolm-2sec-1) at 22°C, and seedlings were sampled at ZT9 and ZT12 of day 5. Total RNA from whole seedlings was extracted with RNeasy Plant Mini kit (Qiagen). RNA labeling and hybridization to Affymetrix ATH1 arrays were performed by the Nottingham Arabidopsis Stock Centre (NASC). Analysis was performed in R (Duek et al., 2004) and Bioconductor (Reimers and Carey 2006). Microarrays were normalized with the RMA procedure as implemented in the affy package (Gautier et al., 2004), and differential expression was determined using limma (Gentleman et al., 2005) with a FDR < 0.05). To determine genes whose expression varies in diurnal short day conditions, the previously published data set from (Mockler et al., 2007) was downloaded from array express (http://www.ebi.ac.uk/arrayexpress/; accession E-MEXP-1304), RMA normalized, and analyzed in limma using a one-way ANOVA model with time as the grouping variable. Samples from each of the two days of collection were used as replicates (so in total there were 2 replicates for each of 6 time points).

Gene annotations were based on the TAIR9 version of the Arabidopsis website (http://arabidopsis.org).

Protein extraction and western blots analysis. Protein extraction and western blot analysis from whole 5 d old seedlings grown under short days (190–200 µmol m-2 s-1) were performed as described (Stavang et al., 2009). The GFP, TAP, and HA fusion proteins were detected using anti-GFP (JL8, Clontech), anti-c-*myc* (9E10, Roche), and anti-HA (3F10, Roche) antibodies, respectively. Antibodies against DET3 were used to check protein loading (Duek et al., 2004). Signal from bound antibodies was revealed using ECL Advance Western Blotting detection Kit (GE Healthcare) and visualized and quantified using the Luminiscence Image Analyzer LAS-3000 (Fujifilm) and Image Gauge v4.0 (Fujifilm), respectively.

Confocal imaging. Fluorescence from the GFP-RGA fusion protein was detected using a Leica TCS SL confocal microscope (Leica Microsystems) as previously described (Stavang et al., 2009).

3.6 Supporting figures

Figure. S3.1 The circadian clock controls diurnal oscillation of *GID1* genes in *Arabidopsis*. (A) Transcript levels of *GID1* genes in short-day photocycles (8 h light/16 h dark). (B) Transcript levels of GID1 genes in continuous light after entrainment in short-day photocycles. (C) Expression of RGA in WT and *lhy* mutant seedlings in short-day photocycles. Data are taken from DIURNAL (http://diurnal.cgrb.oregonstate.edu/) and are normalized to the average value to facilitate comparison.
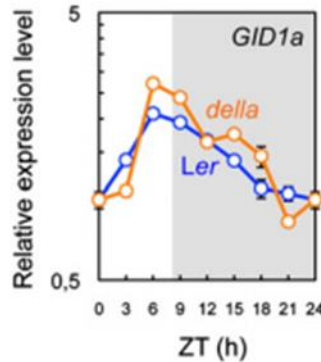
Figure. S3.2 Expression of *GID1*a in 5-d-old L*er* WT and in quadruple *della* mutant seedlings grown under short-day photocycles (8 h light/16 h dark). Values are expressed relative to *PP2a* expression. Data represent mean±SD of three technical replicates. Experiments were repeated twice with similar results. White and gray areas represent day and night, respectively.
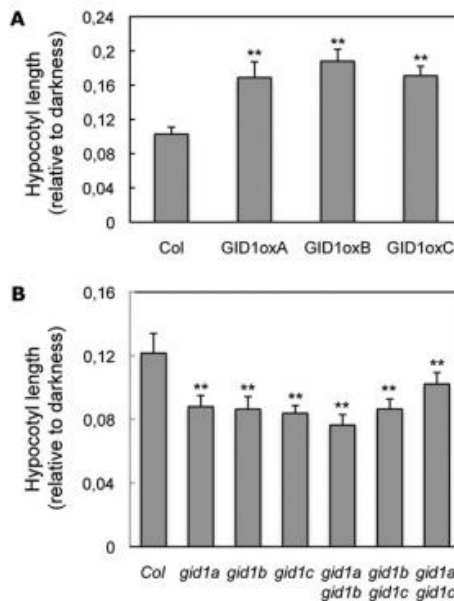
Figure. S3.3 GID1 activity controls hypocotyl elongation under short-day conditions. (A) Normalized hypocotyl length of Col-0 WT and *35S::GID1a*, *35S::GID1b* and *35S::GID1c* seedlings grown for 7 d in constant darkness and in short-day photocycles (8 h light/16 h dark). (B) Normalized hypocotyl length of Col-0 WT and *gid1* mutant seedlings grown for 7 d in constant darkness and in short-day photocycles (8 h light/16 h dark). Data are mean±SD (n≥15 seedlings, **P<0.001). Hypocotyl length under diurnal conditions was normalized to that in etiolated seedlings. Experiments were repeated twice with similar results; results from one representative experiment are shown.
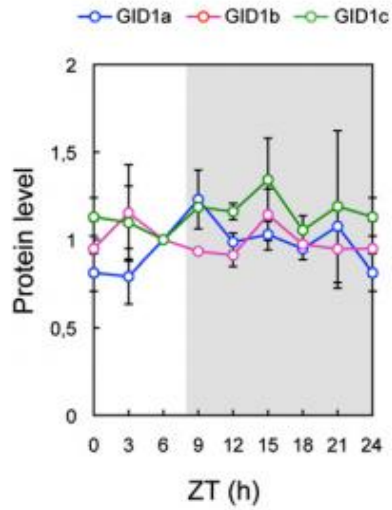
Figure. S3.4 GID1s accumulate constitutively in the overexpressing lines *35S::GID1a, 35S::GID1b, and 35S::GID1c*. Seedlings were grown for 5 d in short-dayphotocycles (8h light/16 h datk). GID1-GFP protein levels were determined by Western blot with commercial antibodies against GFP. GID1 levels were normalized against levels of DET3, which was used as loading control. Data are the mean of two independent experiments. White and gray areas represent day and night, respectively.
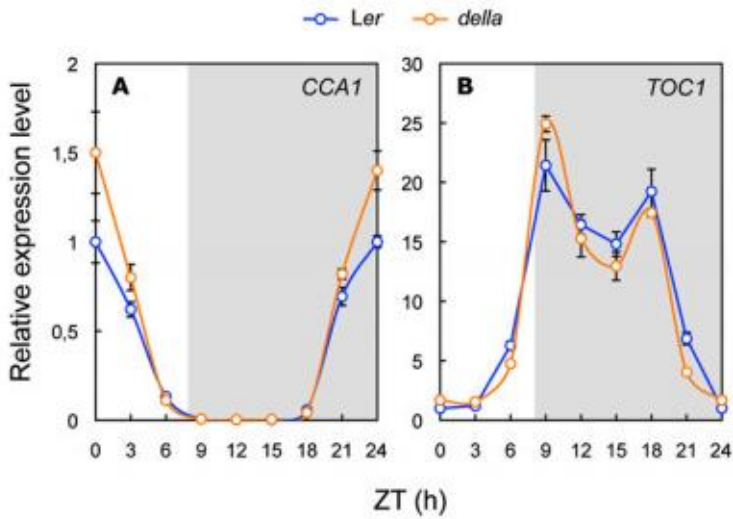
Figure. S3.5 The core clock mechanism is not affected in the pentuple della mutant.
WT L*er* and pentuple *della* seedlings were grown under short-day photocycles (8 h light/16 h dark) for 5 d. Expression of *CCA1* (A) and *TOC1* (B) clock genes is shown. Values are expressed relative to *PP2a*. Data represent mean and SD of three technical replicates. Experiments were repeated twice with similar results; results from one representative experiment are shown. White and gray areas represent day and night, respectively.

3.7 Supporting Tables

3.7.1 Table I Primers for RT-qPCR

Gene		
AtGID1a	Forward (5´to 3´)	GTGACGGTTAGAGACCGCGA
	Reverse (5´to 3´)	TCCCTCGGGTAAAAACGCTT
AtGID1b	Fw (5´to 3´)	TCGCCCTGACGGTTCTTTC
	Reverse (5´to 3´)	TTACGGTCAAGGAACTCGGC
AtGID1c	Fw (5´to 3´)	GAAAGCGGGTCAAGAGGTGA
	Reverse (5´to 3´)	CCAATAGTGGCTTGCTCCAAG
TOC1	Fw (5´to 3´)	TCTTCGCAGAATCCCTGTGAT
	Reverse (5´to 3´)	GCTGCACCTAGCTTCAAGCA
CCA1	Fw (5´to 3´)	CAGCCTTCTGCAACACCTGA
	Reverse (5´to 3´)	TCCTGCTCCATCTGAACCCTT

3.8.2 Table II Primers for GATEWAY cloning

Gene		
AtGID1a	Forward (5´to 3´)	**GGGGACAAGTTTGTACAAAAAAGCAGGCTA**TGGCTGCGAGCGATGAAGT
	Reverse (5´to 3´)	**GGGGACCACTTTGTACAAGAAAGCTGGGTT**ACATTCCG
AtGID1b	Fw (5´to 3´)	**GGGGACAAGTTTGTACAAAAAAGCAGGCTA**TGGCTGGTGGTAACGAAGT
	Reverse (5´to 3´)	**GGGGACCACTTTGTACAAGAAAGCTGGGTT**AGGAGTAA
AtGID1c	Fw (5´to 3´)	**GGGGACAAGTTTGTACAAAAAAGCAGGCTA**TGGCTGGAAGTGAAGAAGT
	Reverse (5´to 3´)	**GGGGACCACTTTGTACAAGAAAGCTGGGTT**TTGGCATT

Sequence in bold corresponds to attB recombination sites

3.8 References

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Chapter 2 | 4

DELLA proteins modulate ethylene signaling through the interaction with RAP2.3

In collaboration with Luis Oñate, Pilar Carbonero, Rishikesh Bhalerao and Michael Holdsworth

4.1 Introduction

The extraordinary plasticity that characterizes plant development is thought to rely in a complex network of interacting signaling pathways (Casal et al., 2004). Hormones play important roles in this network, acting in many instances as second messengers that connect environmental signals that represent cues to modify development or growth rate with the actual developmental and growth pathways (Lau and Deng, 2010; Rymen and Sugimoto, 2012). In addition, extensive cross-regulation between hormone pathways usually adds an extra layer of regulation to this network (Depuydt and Hardtke, 2011).

The GA pathway is very responsive to changes in the environmental conditions, both biotic and abiotic (Alabadí et al., 2004; Achard et al., 2006; Achard et al., 2007; Navarro et al., 2008; Stavang et al., 2009). Moreover, it is also modulated by endogenous factors such as the circadian clock (Arana et al., 2011), or other hormones like auxins, ethylene, or cytokinins (Jasinski et al., 2005; Frigerio et al., 2006; Achard et al., 2007). This places GAs, and thus DELLAs, as central players in integrating environmental cues with growth and development. Our current view of DELLA action indicates that these proteins exert their pervasive control on plant's life through regulating the activity of diverse TFs by physical interaction (Daviere and Achard, 2013). The identification of protein-protein interactions is key to understand from a mechanistic point of view the network of signaling cascades that governs plant's life (Arabidopsis, 2011). The determination of the topology of the interactome network in *Arabidopsis* and other model organisms has allowed to identify proteins with many more interacting partners than the average; thus, the particular position of these proteins within the network suggests that they act as hubs, performing important roles in signaling or other cellular processes (Dietz et al., 2010). Here, we have determined the TF-interactome of the *Arabidopsis* DELLA protein GAI by yeast two-hybrid assays (Y2H). Our results show that DELLAs interact with many TFs suggesting that they act as central signaling hubs in the plant connecting different signaling cascades. As follow up of this approach, we have further investigated the interaction with a particular TF that has provided new insights about the mechanism

of cross-regulation between the GA and ethylene pathways in controlling apical hook development.

4.2 Results and discussion

4.2.1 The DELLA protein GAI interacts with multiple transcription factors

In order to determine the TF-interactome of the DELLA protein GAI, we identified additional protein partners by screening by Y2H a normalized library containing 1,172 TFs from *Arabidopsis* (Castrillo et al., 2011), using the GRAS domain of GAI as bait (M5-GAI). After one round of testing all pairwise interactions between GAI and the TFs in the library, we identified 66 interactions that corresponded to 58 unique TFs (Supporting Table I), including 2 known interactors, PIF3 and PIF4 (de Lucas et al., 2008; Feng et al., 2008). Figure 4.1 shows the GAI interactome visualized with Cystoscape. The interacting TFs belong to 15 out of the 39 families represented in the library (Supporting Table II). The overall diversity of interactors found in this study is in line with the variety reported in the literature (Supporting Table III), and suggests that there is not a clear, strong bias for any particular type. Nonetheless, 20% of them belong to the TCP family (12 interactors out of 23 present in the library). TCPs share certain structural similarity with bHLHs that resides in their DNA binding domain (Aggarwal et al., 2010), and at least in the case of the bHLH PIF4, a region including this domain acts as interacting surface with DELLA proteins (de Lucas et al., 2008), suggesting that this structure might perform the same role also for the TCPs.

Currently, GAI can be classified as a major hub (between 50-100 interacting partners) (Geisler-Lee et al., 2007). Nonetheless, the number of interacting partners identified in this work is an underestimation given that (i) the library we screened contains 1,172 TFs out of the more than 1,500 encoded in the Arabidopsis genome (Riechmann et al., 2000), and (ii) DELLA proteins perform cellular roles by interacting with proteins that are not TFs, such as PFD3 and PFD5 (Locascio et al., 2013). Therefore, it is very likely that DELLA proteins are super hubs (more than 101 interacting partners) within the network, according to the classification by Geisler-

Lee et al. (2007). Indeed, only 11 out of 3,617 proteins were identified as super hubs in the predicted interactome for *Arabidopsis*, based in evolutionarily conserved interactions (Geisler-Lee et al., 2007). Interestingly, the GAI interactome network mostly held together when GAI was removed. This is similar to what happens when both major and super hubs are removed from the predicted *Arabidopsis* interactome network (Geisler-Lee et al., 2007) and indicates that it is sustained mainly by minor hubs that form a stratus structure, as coined for the yeast interactome (Batada et al., 2006), indicating that other proteins in the network also form part of multiple complexes being shared by several signaling modules (see Figure 7.1 in the "General Discussion" section).

The structural variety of the GAI-interacting TFs (Supporting Tables II and III) joins to the diversity of processes in which they are involved (Figure 4.1 and Supporting Table I) in supporting the notion that DELLA proteins act as signaling hubs, widely controlling plant's life (Claeys et al., 2013). Remarkably, the fact the GA metabolism, and hence DELLA levels, is very sensitive to changes in the environmental conditions (Sun, 2010), places DELLA proteins as potential signaling hubs connecting many aspects of plant's physiology with the ever changing environment. See the "General Discussion" section for a further discussion on the possible role of DELLA proteins as signaling hubs, including structural issues.

These results provide us with testable hypotheses about the molecular mechanisms by which GAs control these processes and interact with other signaling pathways. Our investigations about the physiological relevance for one of the novel interactions found in the screening, RELATED TO APETALA2.3 (RAP2.3), are presented in the next sections. We chose to pursue this interaction further for two reasons: (i) this TF belongs the group VII ERFs of the AP2/EREBP superfamily (Nakano et al., 2006), unrelated to any of the reported DELLA interactors (Supporting Table III) (Locascio et al., 2013); and (ii) it participates in the ethylene signaling cascade (Buttner and Singh, 1997), so it might represent an additional cross-regulatory point between both hormone pathways (An et al., 2012).
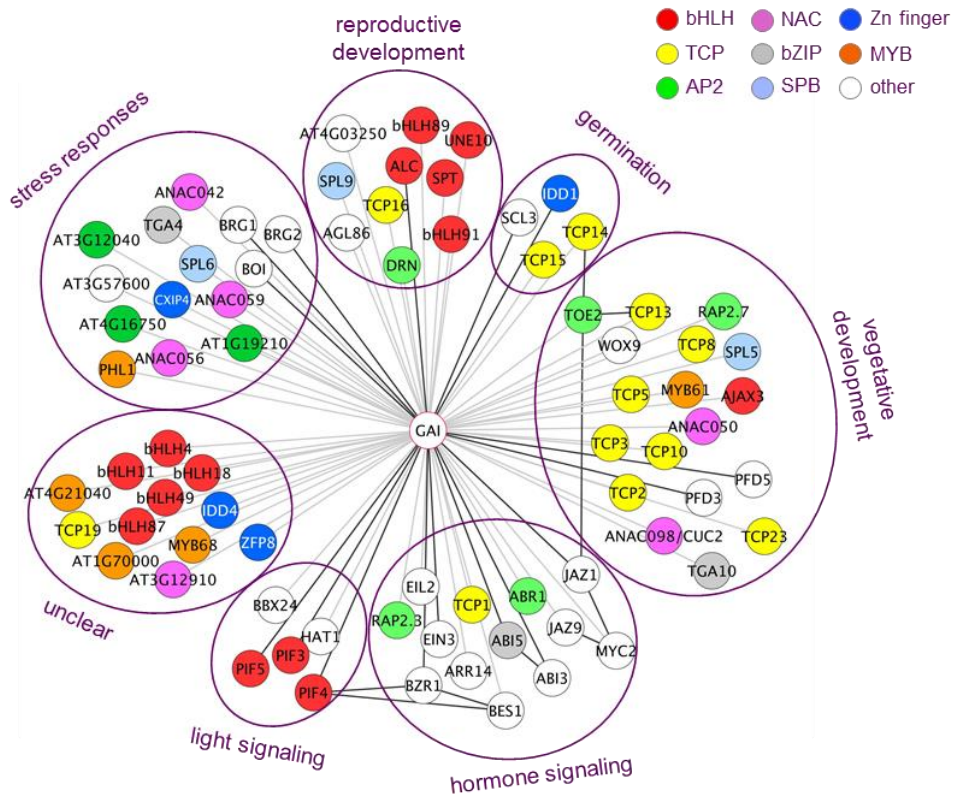
Figure 4.1 DELLA protein interactors. Cytoscape representation of protein-protien interaction among M5-GAI and all interactors found. Nodes were classified by transcription factors families (represented by different colors) and by biological function. Black edges represent reported interactions by other authors, while grey edges represent interactions found in our screening

4.2.2 GAI interacts with RAP2.3

First, we confirmed the interaction between GAI and RAP2.3 by Y2H (Figure 4.2A). Assaying deleted versions of the TF may be informative about the possible effects on the TF's activity that the interaction with the DELLA protein may cause. For instance, RGA interacts with PIF4 through a region encompassing its DNA binding domain, and accordingly the PIF4's ability to bind DNA is impaired upon interaction (de Lucas et al., 2008). We prepared four deleted versions of RAP2.3 (see Figure 4.2A) and tested their ability to interact with the GRAS domain of GAI (M5-GAI). Strikingly, only del1 was able to interact with M5-GAI. These results suggest that both the N-

terminal part and the AP2 domain are needed to support the interaction with M5-GAI. Other TFs also interact with DELLA through specific parts of the protein, for instance BZR1 (Gallego-Bartolomé et al., 2012), PIF4 (de Lucas et al., 2008), MYC2 (Hong et al., 2012), or ARR1 (see Chapter 4 of this Thesis). It is tempting to speculate that the ability of the RAP2.3 to bind DNA might be affected upon interaction with GAI, since del1 includes the AP2 domain, responsible of DNA binding. Interestingly, M5-GAI was also able to interact with a RAP2.12 (Figure 4.2A), a close relative of RAP2.3 (Nakano et al., 2006), suggesting that this ability might extend to all other members of the group VII ERFs.

Next, we investigated if the interaction also occurs in plant cells. For that purpose, we performed co-immunoprecipitation assays (co-IP) in *Arabidopsis* protoplasts co-transfected with *myc*-GAI and HA-RAP2.3. As shown in Figure 4.2B, HA-RAP2.3 was efficiently pulled down from extracts by anti-*myc* antibodies only in the presence of *myc*-GAI, indicating that both proteins are able to interact in plant cells as well.
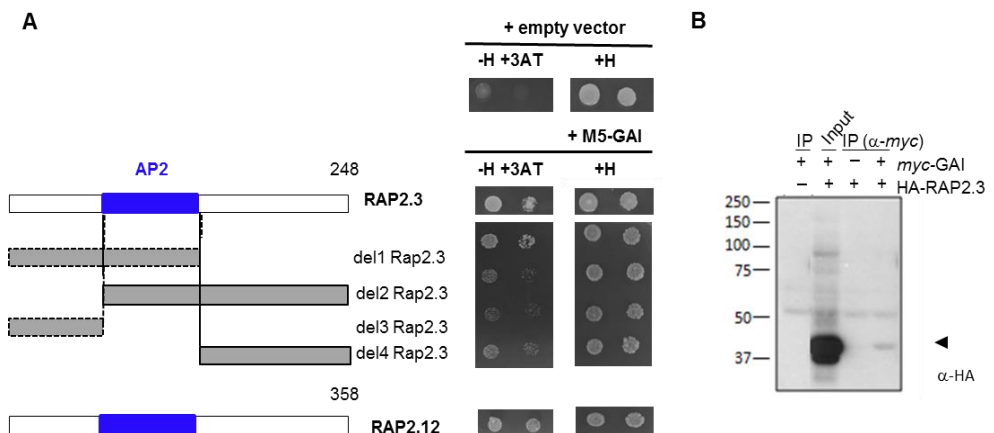
Figure 4.2 DELLA interact with RAP2.3. (A) Y2H assays analyzing the interaction between M5-GAI and full length of RAP2.12, RAP2.3, and truncated versions of RAP2.3 containing of not the DNA binding domain AP2 (H, Histidine; 3-AT, 5 mM 3-aminotriazol). (B) co-IP in *Arabidopsis* protoplasts expressing full length of GAI fusion with *myc*- tag and RAP2.3 fusion with HA-tag.

4.2.3 GAI inactivates RAP2.3 upon interaction

As mentioned above, the ability of several TFs to bind their DNA targets is inhibited upon interaction with DELLA proteins. For instance, this is the case of several PIFs (de Lucas et al., 2008; Feng et al., 2008; Cheminant et al., 2011; Gallego-Bartolomé et al., 2011) and BZR1 (Bai et al., 2012; Gallego-Bartolomé et al., 2012; Li et al., 2012). In other cases, the interaction with the TF occurs at the vicinity of the promoters of certain genes to grant their expression (Lim et al., 2013; Park et al., 2013). Nonetheless, the contribution of DELLA proteins to the activity of the latter group of TFs is unknown. The RAP2.3 binds in vitro and in vivo to the GCC-box (Buttner and Singh, 1997; Yang et al., 2009; Gibbs et al., 2014) and it is able to activate transcription when transiently overexpressed in *Arabidopsis* leaves (Yang et al., 2009). In order to test the effect that the interaction with GAI may cause on the transcriptional activation ability of RAP2.3, we performed transactivation assays in leaves of *Nicotiana benthamiana*. As reporter, we placed the LUCIFERASE gene under the control of a synthetic promoter containing 5 copies of a 29-nucleotide fragment from the promoter of the ethylene-induced gene *HOOKLESS1* (*HLS1*) that contains one GCC-box. A similar reporter construct has been used to demonstrate the ability of several RAP2.3-related TFs to activate or repress transcription (Fujimoto et al., 2000; Song et al., 2005). As shown in Figure 4.3, the luciferase activity strongly increased when HA-RAP2.3 was expressed together with the reporter construct, which is in agreement with previous results (Yang et al., 2009). Importantly, when YFP-GAI was co-expressed together with HA-RAP2.3 in the same leaves, the luciferase activity was significantly lower despite the TF accumulated to a higher level. Expression of YFP-GAI alone caused a slight increase in the activity of the reporter. These results suggest that GAI prevents either the DNA binding ability of RAP2.3 or its capacity to activate transcription.
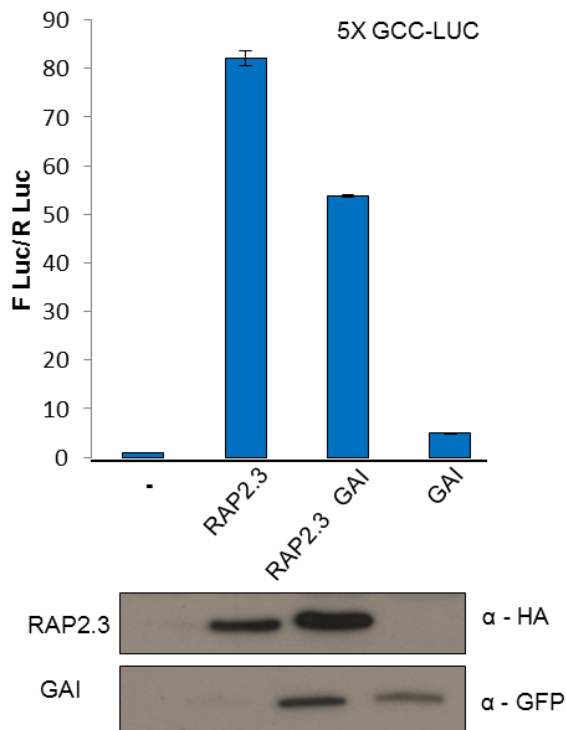
Figure 4.3 DELLA interaction inactivates RAP2.3. Transient expression assays in leaves of *N. Benthamiana* by agroinfiltration. Results represent the ratio between the activity of three technical replicates *firefly Luciferase* that carry out the synthetic promoter containing 5 copies of the GCC box. *Renilla* LUC under 35S promoter and in the same construct was used as control. Western blots were performed to ensure that samples contain the same amount of each protein.

4.2.4 DELLAs prevent the binding of RAP2.3 to the promoter of its target genes in vivo

Next, we sought to investigate the functional relationship between DELLA proteins and RAP2.3 in *Arabidopsis*. To identify genes potentially co-regulated by this

interaction, we first performed a meta-analysis between sets of genes regulated by the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) (Goda et al., 2008) and those misregulated in the *dellaKO* mutant (Arana et al., 2011), and selected four of them that were up-regulated both by ACC and in the *dellaKO* background: At5g44120, At4g31940, At2g41260, and At4g19690. The effect of a conditional over-accumulation of RAP2.3 on the expression of these genes was further assayed in dark-grown seedlings that accumulated high or low DELLA levels (see Figure 4.4A for the experimental design). To induce *RAP2.3* we used a transgenic line from the TRANSPLANTA collection expressing its ORF under the control of a β-estradiol-inducible promoter (Coego et al., 2014). The expression of the four target genes analyzed was higher when RAP2.3 accumulated in a context deprived of DELLA proteins, suggesting that DELLAs have a negative effect on the RAP2.3's activity (Figure 4.4B). These results are in line with those obtained with the transactivation assays in N. *benthamiana* (Figure 4.3).

To determine whether GAI affects RAP2.3's ability to bind target promoters or to activate their transcription, we studied the binding of the TF to promoters in vivo by chromatin IP (ChIP). We used a transgenic line overexpressing an HA-tagged, mutant version of the RAP2.3 (MA-RAP2.3-HA) that is resistant to the nitric oxide (NO) and oxygen-induced degradation through the N-end rule pathway (Gibbs et al., 2011; Gibbs et al., 2014). The four target genes used for the expression analysis contain at least one canonical GCC-box within the 1,500 nucleotides immediately upstream of their ATG (Figure 4.4C). We were not able to detect in vivo binding of the RAP2.3 to the region containing the GCC-box in the promoter of At2g41260 and At4g19690 in etiolated seedlings (data not shown). However, the two regions containing GCC-boxes in the promoter of At4g31940 and the region containing a cluster of four of them in the promoter of At5g44120 were efficiently co-immunoprecipitated with MA-RAP2.3-HA (Figure 4.4D). Importantly, the enrichment of these regions in the immunoprecipitated was significantly higher when seedlings were grown in conditions that favor DELLA degradation (PAC+GA) than in seedlings that accumulate DELLAs (PAC). Thus, all these results together suggest that DELLA proteins inactivate the RAP2.3 upon physical interaction by preventing its DNA binding activity, in what seems a common mode of regulating the activity of TFs by

DELLA proteins (de Lucas et al., 2008; Feng et al., 2008; Bai et al., 2012; Gallego-Bartolomé et al., 2012).
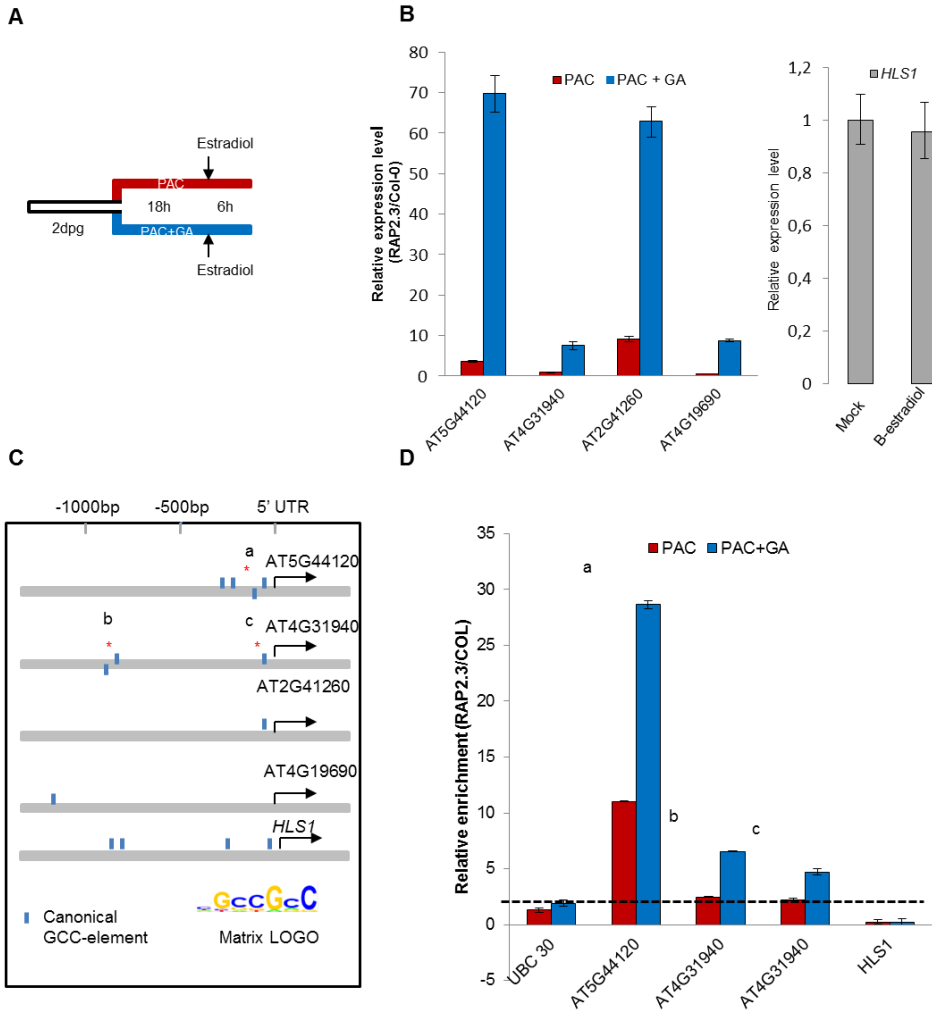
Figure 4.4 DELLAs prevent the binding of RAP2.3 in vivo. (A) Graphical representation of the procedures performed to obtain the results observed in Figure 4.4B, seedlings were grown in darkness for 2 days, and then were transferred for the times indicated in liquid MS containing 10µMPAC + 1µM GA4 or 10µMPAC. Then β-estradiol was added to a final concentration of 5 µM before harvest. (B) Expression levels of some RAP2.3 targets in the presence or absence of DELLAs. (C) Graphical representation of GCC-elements on the promoter of RAP2.3 targets from figure B. (D) Chromatin immunoprecipitation of *35S::MARAP2.3*-HA followed by qRT PCR of the promoter of selected genes, UBQ30 represent a control region not bound by RAP2.3.

4.2.5 The DELLA-RAP2.3 interaction mediates apical hook opening

Besides the newly identified role of RAP2.3 as part of the NO and oxygen sensor mechanism (Gibbs et al., 2011; Licausi et al., 2011; Gibbs et al., 2014), it is known for several years to be an ethylene-induced gene (Buttner and Singh, 1997). The development of the apical hook typical of etiolated seedlings is regulated by GAs and ethylene (Abbas et al., 2013). In particular, both pathways jointly prevent premature apical hook opening in darkness (Gallego-Bartolomé et al., 2011). Recently, it has been demonstrated that DELLA proteins counteract the effect of ethylene during apical hook development by inhibiting EIN3 activity through physical interaction, providing therefore a mechanism for the co-regulation of this process by GAs and ethylene (An et al., 2012). Interestingly, EIN3 binds in vivo to the *RAP2.3* promoter (Chang et al., 2013) and activates its expression in etiolated seedlings (Figure 4.5A), suggesting that RAP2.3 could also participate in the mechanism regulating apical hook development by GAs and ethylene. To unambiguously demonstrate the involvement of RAP2.3 in this process, we investigated the hook phenotype in the loss-of-function, T-DNA insertional mutant *rap2.3-1* (WiscDsLox247E11). A combined treatment with GAs and ACC provokes the formation of hooks with an exaggerated curvature (Figures 4.5B and 4.5C) (Gallego-Bartolomé et al., 2011; An et al., 2012). Remarkably, *rap2.3-1* mutant seedlings were partially resistant to treatment with ACC + GAs, and the hook angle was significantly smaller in the mutant than in the wild type (Figures 4.5B and 4.5C). Hooks of mutant and wild type seedlings were equally responsive to mock, ACC, and GA treatments, suggesting that under these conditions RAP2.3 activity is not limiting, most likely because of the genetic redundancy with other members of the group VII ERFs is more remarkable (Nakano et al., 2006). In agreement, a β-estradiol treatment that enhanced *RAP2.3* expression (Figure 4.5D) partially prevented PAC-induced hook opening (Figure 4.5E). All these results suggest that DELLA proteins might also regulate the apical hook development by inactivating the RAP2.3, in addition to EIN3.
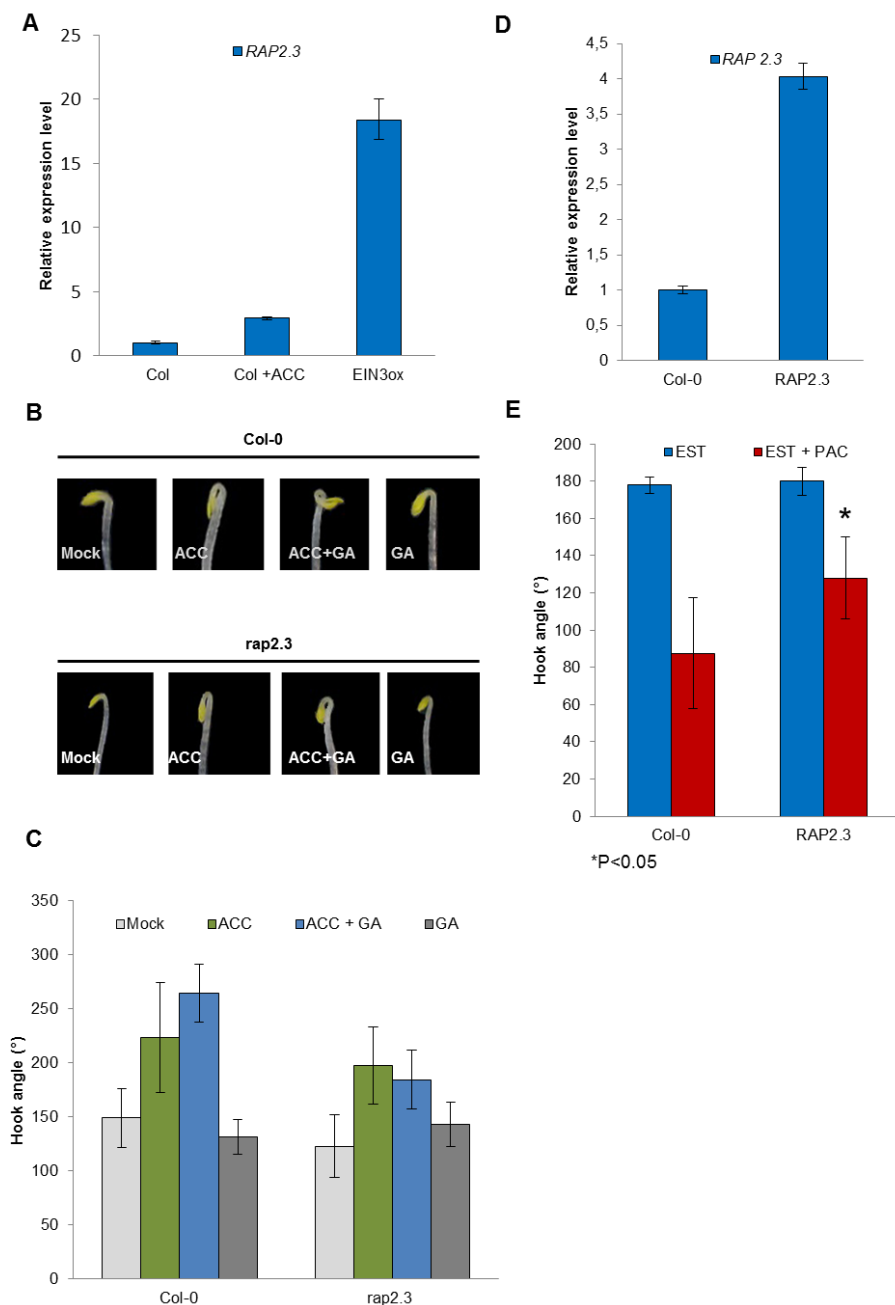
Figure 4.5 Physiological relevance of RAP2.3- DELLA interaction. (A) Expression of *RAP2.3* in 4days old dark grown seedlings of Col-0 in mock or with 12.5 μM ACC and EIN3ox. (B-C) Hook angle of Col-0 and *rap2.3-1* insertional mutant in the presence of ACC, ACC+GA₄, and GA₄. (D) *RAP2.3* expression in *RAP2.3* estradiol inducible line. (E) Hook angle of 2 day old seedlings of Col-0 and *RAP2.3* estradiol inducible line growing in MS medium containing 5μM of βestradiol with or without 0.2μM PAC. For C and E n=16; data are mean ± SD.

4.3 Concluding remarks

The identification of TFs that interact with DELLA proteins provides molecular mechanisms for the interaction between the pathways in which the TFs participate and the GAs, and at the same time mechanisms to explain the regulation of physiological processes by GAs. Our results add a novel piece to the mechanism that controls apical hook development by GAs and ethylene (Figure 4.6). RAP2.3 represents an EIN3-dependent branch of ethylene signaling, as formerly exemplified by ERF1 (Solano et al., 1998), participating in the transcriptional cascade triggered by the hormone in the control of this developmental process. RAP2.3 might regulate sets of genes that may not be directly regulated by EIN3, as is the case of the four genes sown in Figure 4.4 (Chang et al., 2013). Interestingly, *HLS1* that is a major target of EIN3 regulating hook development is not a target of RAP2.3 (Figures 4.4B-D) despite it binds to and regulates transcription from a fragment of its promoter (Figure 4.3).

The interaction of DELLAs with EIN3 and RAP2.3 indicates that GAs impinge at various levels on the ethylene-triggered transcriptional cascade and that it could be relevant at several stages of hook development (Figure 4.6). For instance, low levels of DELLAs during skotomorphogenesis will ensure the proper activity of both EIN3 and RAP2.3 to keep the hook closed. An additional scenario where the negative regulation of both TFs by DELLAs could be relevant is to promote hook opening during de-etiolation. DELLAs and EIN3 levels increase and decrease, respectively, during this transition (Achard et al., 2007; Zhong et al., 2009). Nonetheless, the kinetics of these changes is quite different, being the accumulation of DELLAs faster. Therefore, inactivating both TFs by interaction would be an efficient and rapid way to counteract the ethylene-dependent mechanism that maintains the hook closed.

The role of RAP2.3 as a NO and oxygen sensor (Gibbs et al., 2011; Licausi et al., 2011; Gibbs et al., 2014) opens up more physiological contexts in which the interaction with DELLAs might be relevant. RAP2.3 along with the other members of the group VII ERFs are degraded through the N-end rule pathway in response to the simultaneous presence of NO and oxygen (Gibbs et al., 2011; Licausi et al., 2011; Gibbs et al., 2014). Interestingly, DELLAs accumulation during de-etiolation is

dependent upon NO (Lozano-Juste and Leon, 2010), suggesting that under certain physiological conditions the plant ensures the proper inactivation and degradation of the RAP2.3. Thus, other aspects of skoto- and photomorphogenesis besides hook development might be also regulated by the DELLA and RAP2.3 interaction. Nonetheless, the functional relationship between DELLAs and RAP2.3 seems to be different under other physiological scenarios. For instance, both RAP2.3 and the DELLA protein RGL2 favor the ABA signaling in the endosperm that maintains the seed dormant (Lee et al., 2010; Gibbs et al., 2014).
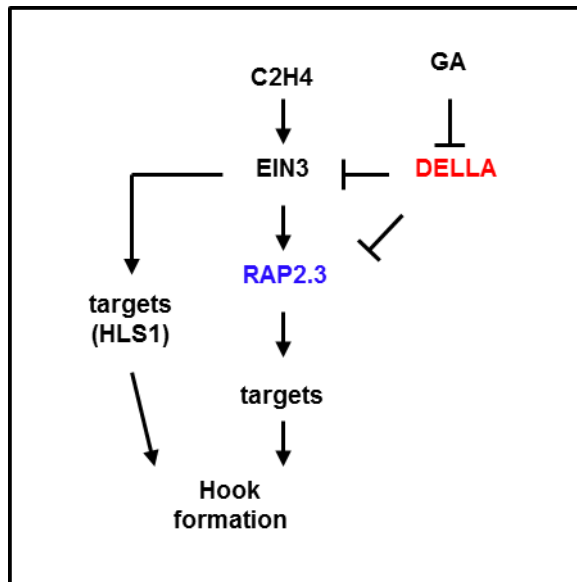
Figure 4.6 Model proposed for the molecular mechanism of action of DELLAs to mediate hook formation.

4.4 Material and methods

Plant Material. *MARAP2.3-HA* lines were gently provided by Michael Holdsworth. Heterozygous F2 T-DNA insertional mutant *rap2.3-1* lines (WiscDsLox247E11) were genotyping using the primers 5´-CCATCCCACCAACCAAGTTAACGTGA-3´ and 5´-GCAGATCTGGGAAGTTGAGCTTGGC-3´.

Experimental Conditions. Seedlings were grown in MS at 22°C in darkness. For hook angle experiments 2days old seedlings of WT and RAP2.3 estradiol inducible were grown in MS agar containing 5μM of β-estradiol with or without 0.2μM PAC. For the experiment that show the partially resistance of GAs during hook formation. the insertional mutant *rap2.3-1* Hook angle of Col-0 and *rap2.3-1* insertional mutant were germinated in MS agar and the transfer to medium containing 12.5 μM of ACC, 12.5μM ACC + 1 μM GA_4 or 1 μM GA_4.

Y2H screening. The M5-GAI (Gallego-Bartolome et al., 2012) in pDEST32 (Invitrogen) was used and to transform the haploid yeast strain YM4271. Then collection of TFs is in the haploid yeast strain PJ694α. The screening was performed by mating in liquid SD medium. Then positive clones were selected in medium without leucine, tryptophan and histidine, and with 1 μM of 3-Amino-1,2,4 triazole 3-AT (Sigma-Aldrich).

Y2H assays Full length and deletions of RAP2.3 were obtained by PCR amplification using the primers listed below to create *pENTR* the fragments were cloned into *pCR8/GW/TOPO* (Invitrogen), then transferred into *pDEST22* (Invitrogen) to create Gal4DNA binding domain fusion. GAI deletions have been described before (Gallego-Bartolome et al., 2012). Subsequent cotransformation of the yeast strain AH109 (Clontech) were performed. To select the interaction, clones were grown in SD plates without Leu, Trp and His, and with 5 μM 3-AT (Sigma-Aldrich). Primers used for amplified RAP2.3 deletions were:

	Forward	Reverse
Rap2.3	5'-ATGTGTGGCGGTGCTATTATTTC- 3'	5'-CTCATACGACGCAATGACATCAT-3'
del1Rap2.3	5'-ATGTGTGGCGGTGCTATTATTTCC-3'	5'-TTATGGGAAGTTGAGCTTGGCTTTATC-3'
del2Rap2.3	5'-ATGAAATGGGCGGCTGAGA-3'	5'-TTACTCATACGACGCAATGACATCAT-3'
del3Rap2.3	5'-ATGGATCTGCACCATCCTCCTCCTCC-3'	5'-TTACTCATACGACGCAATGACATCAT-3'
del4Rap2.3	5'-ATGTGTGGCGGTGCTATTATTTCC-3'	5'-TTATCCCCATGGACGCTTACGTA-3'

Nicothiana benthamiana *pENTR* of RAP2.3 and GAI (Gallego-Bartolome *et al.*, 2012) were transfer into *pEarleyGate-201*, *pEarleyGate-104* respectively (Earley et al., 2006), then *Nicotiana benthamian*a leaves were infiltrated as explain before. Protein extraction was performed as described (Gallego-Bartolome *et al.*, 2012).

Reporter Construct and Transcriptional Assays. Five copies of the GCC-box containing a 29 nucleotide flanking fragment of *HOOKLESS1* (*HLS*1) (AGCCGCCATTTATGAGTTAACGCAGACAT) upstream of the minimal 35S promoter and the Ω translational enhancer containing the restriction sites *PstI* and *NcoI* respectively, were synthetized by GenScript. Then the fragment was cloned into *pGreenII 0800-LUC* vector (Hellens *et al.*, 2005). **For Transcriptional Assays.** Leaves of 4 weeks-old *Nicotiana benthamina* were infiltrated with *Agrobacterium tumefaciens C58* cells carrying the constructs, the ratio of Agrobacterium-carrying reporter and effector constructs was 1:4. Firefly and the control Renilla LUC activities were assayed from leaf extracts with the Dual-Glo Luciferase Assay System (Promega) and quantified with a GloMax 96 Microplate Luminometer (Promega). To verify that protein amounts were equal, Western Blot analysis were performed with proteins extracted from the same experiment, the HA-RAP2.3, YPF-GAI fusions were detected with anti-HA (3F10; Roche), anti-GFP (ab290; Abcam), antibodies respectively.

Gene expression. For gene expression analysis, total RNA was extracted with E.Z.N.A. Plant RNA Mini Kit (Omega Bio-tek) according to the manufacturer´s instructions. cDNA synthesis was performed with SuperScript II First-Strand Synthesis System (Invitrogen). qRT-PCR was performed as describe before, and the

EF1-α gene was used for normalized (Frigerio *et al.,* 2006). Primers used to amplified transcripts are listed below:

	Forward	Reverse
AT3G16770	5'-CGATTATGCCCCTCTCGTCA-3'	5'-CCAGAGTTCCTCAGCCGTGA-3'
AT5G44120	5'-CACAACACCCGGTGTAGCAC-3'	5'-AGTGGTTCCTGTCCGTCGTT-3'
AT4G31940	5'-TTTTCGGGTTTGCCCCTTAT-3'	5'-CGATTTTACGCATCTCACGC-3'
AT2G41260	5'-TTTCTCCGTCGCTGTTTTCG-3'	5'-TCATCGTGTGTGGCATCGTT-3'
AT4G19690	5'-ATGTTCGAAGGCATGGGTCT-3'	5'-CGATCCCTAACGCTATTCCG-3'

Chromatin immunoprecipitation. 4 days *MARAP2.3-HA* seedlings were grown at 22°C in with MS containing PAC 0.5µM with and without 1µMGA4, a Col-0 in the same conditions was used as control. ChIP was performed as described previously (Saleh et al 2008), using Dynabeads Protein A (Invitrogen) and an anti-HA (hemagglutinin) polyclonal antibody (ab9110; Abcam). Relative enrichment was calculated by normalizing the amount of target DNA, first to the internal control gene *HSF* (At4g17740) and then to the corresponding amount in the input. Data are mean and SD of two technical replicates from a representative experiment from two biological replicates. Primers used to amplified the GCC-elements of the selected targets were:

	Forward	Reverse
AT5G44120	5'-GCCTATCTCAAAAGCTGATGTGT-3'	5'-CATTTCAATTTGCATGGCTTA-3'
AT4G31940-a	5'-CTCTTTGTGGGCTTTTTGGA-3'	5'-TTTTATTCATGGAAGCCCATT-3'
AT4G31940-b	5'-CTGCTCTTTTTGTTCATTGTCA-3'	5'-CCCCAATTTTAATTTTTATTAATGC-3'
UBQ30	5'-CAAATCCAAAACCCTAGAAACCGAA-3'	5'-AACGACGAAGATCAAGAACTGGGAA-3'
HSF	5´-GCTATCCACAGGTTAGATAAAGGA-3´	5´-GAGAAAGATTGTGTGAGAATGAAA-3´

4.5 Supporting tables

4.5.1 Supporting Table I List of DELLA interactors divided by biological function.

Shoot development

Gene	Name	Biological process
At5g60120	TOE2 (AP2-like)	flowering
At4g18390	TCP2	leaf differentiation
At1g53230	TCP3	leaf differentiation
At5g60970	TCP5	leaf differentiation
At2g31070	TCP10	leaf differentiation
At1g35560	TCP23	leaf differentiation
At5g09460	AJAX3	vascular development
At1g69690	TCP15	leaf differentiation
At3g10480	ANAC050	shoot architecture
At3g15270	SPL5	juvenile to adult shoot transition
At2g33880	STIMPY/WOX9	meristem growth
At1g58100	TCP8	leaf differentiation
At5g06839	bZIP65/TGA10	floral development
At3g02150	TCP13	leaf differentiation
At5g53950	CUC2/ANAC098	meristem formation/leaf serration
At2g28550	RAP2.7 (TOE1)	
At1g09540	MYB61	stomata closure
At2g42280	Flowering bHLH4	flowerig

Hormonal cross-regulation

At2g41940	ZFP8	Trichome initiation
At1g67260	TCP1	BR biosynthesis
At2g01760	ARR14	CK signaling
At3g16770	RAP2.3	ethylene responses
At5g21120	EIL2	ethylene signaling
At5g64750	AtABR1	repression of ABA responses

Embryo, flower, pollen development

At1g12980	DRN/ESR1	embryo development
At3g45150	TCP16	pollen development
At5g06839	bZIP65/TGA10	floral development
At1g31630	AGL86	female gametophyte
At1g06170	bHLH89	anther development
At2g31210	bHLH91	anther development
At4g03250	HDZIP	
At4g00050	UNE10	

Light signaling

At1g09530	PIF3	skotomorphogenesis
At2g31380	STH	photomorphogenesis
At4g17460	HAT1	shade avoidance
At2g43010	PIF4	skotomorphogenesis

Biotic and abiotic stress

At3g57600	DREB	cold acclimatation
AT3G15510	AtNAC2/ANAC056	salt-induced senescence
At5g10030	OBF4/TGA4	defence and flowering
At5g29000	PHR1-like1 (PHL1)	response to phosphate starvation
At3g13040	myb-like HTH	similar to PHR1, PHL1
At1g19210	DREB	
At1g69170	SPL6	response to pathogens
At4g16750	DREB	

Other processes or unknown

At3g47620	TCP14	germination
At3g29035	ANAC059	regulation of sensecence
At2g43000	ANAC042/JUNGBRUNNEN1	regulation of senesence
At2g28910	CXIP4 (CCHC-type Zn finger)	Ca2+ signaling

At2g22750	bHLH18	ER body formation
At5g65790	MYB68	lignification in root pericycle
At5g51910	TCP19	
At2g02080	ATIDD4 (C2H2-type Zn finger)	
At4g36060	bHLH11	
At3g21330	bHLH87	
At3g12910	NAC/NAM	
At4g21040	DOF	
At1g70000	myb-type HTH	
At1g68920	bHLH49	

4.5.2 Supporting Table II Transcription factors families representation of DELLA interactors.

TF family	
C2H2-type Zn finger	2
double B-box-type Zn finger	1
CCHC-type Zn finger	1
Dof-type Zn finger	1
TCP	12
bHLH	11
MADS	1
NAC	6
HD	4
Myb	5
AP2/EREBP	8
SPB	2
bZIP	3
GARP/ARR	2
EIN3-like	1

4.5.3 Supporting Table III Transcription factors that have been reported as DELLA interactors.

TF	Family	Physiological context	Reference
PIFs	bHLH	Hypocotyl elongation, apical hook development, Chl synthesis	De Lucas *et al.* 2008; Feng *et al.* 2008; Gallego-Bartolomé *et al.* (2010-2011); Cheminant *et al.* (2011)
SPT	bHLH	Seed germination	Gallego-Bartolomé *et al.* (2010); Josse *et al.* (2011)
ALC	bHLH	Fruit development	Amaud *et al.* (2010)
MYC2	bHLH	Gibberellin-jasmonic acid cross-talk	Hong *et al.* (2012)
EIN3	EIL	Gibberellin-ethylene cross-talk, apical hook development	An *et al.* (2012)
SPLs	SBP-box	Floral induction	Yu *et al.* (2012)
BES1, BZR1	BES1/BZR1	Gibberellin-brassinosteroid cross-talk	Bai *et al.* (2012); Gallego Bartolomé *et al.* (2012); Li *et al.* (2012)
SCL3	GRAS	Seed germination, hypocotyl elongation, root growth	Zhang *et al.* (2011)
IDD1/ENY	C2H2 Zinc finger	Seed germination	Feurtado *et al.* (2011)
JAZs	JAZ	Gibberellin-jasmonic acid cross-talk, biotic stress, root growth	Hou *et al* (2010); Wild *et al.* (2012); Yang *et al.* (2012)
BOIs	RING finger	Seed germination, floral induction	Park *et al.* (2012)
ABI3/ABI5	bZIP	Seed germination	Lim *et al.*,2013

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Chapter 3 **5**

DELLAs associate with DNA in vivo

In collaboration with Anne Pfeiffer and Jan U. Lohmann

5.1 Introduction

Plant hormones regulate developmental and stress-related responses mostly through the modulation of pre-wired gene regulatory networks. This type of regulation has been proposed as particularly important in short-term adaptation to changing environmental conditions (Casal et al., 2004). In the case of gibberellins (GAs), transcriptional regulation is based on the degradation of the nuclear-localized DELLA proteins, which act as negative elements in the GA signaling pathway. The role of DELLAs as transcriptional regulators is supported by the large changes in expression patterns observed in *dellaKO* mutants (Cao et al., 2006; Achard et al., 2008; Arana et al., 2011; Cheminant et al., 2011) and after the conditional induction of stable DELLA alleles (Willige et al., 2007; Zentella et al., 2007; Hou et al., 2008; Gallego-Bartolome et al., 2011). Although it has been reported that the rose DELLA protein RhGAI1 can bind DNA in vitro (Luo et al., 2013), there is no evidence regarding the physiological relevance of this characteristic. On the contrary, other mechanisms have been proved relevant, all of which involve physical interaction between DELLAs and other transcriptional regulators. These mechanisms can be summarized in three different non-exclusive models: (i) DELLAs interact with DNA-binding TFs and prevent them from binding their target promoters. This is the case of PIFs (de Lucas et al., 2008), EIN3 (An et al., 2012), BZR1 (Bai et al., 2012; Gallego-Bartolome et al., 2012; Li et al., 2012), (ii) DELLAs modulate the activity of DNA-binding TFs through the interaction of other transcriptional regulators, such as the JAZ proteins that mediate jasmonic acid (JA) signaling through MYCs (Hou et al., 2010; Fernandez-Calvo et al.,2011). And (iii) DELLAs affect the activity of DNA-binding TFs without impairing their target recognition, as shown for ABI3 and ABI5 (Lim et al., 2013).

This last mechanism of action does not explain the association of DELLAs with the promoter of some genes reported before such as *SCARECROW-LIKE* (*SCL3*), a gene that promotes GAs signaling, or *XERICO* that promotes accumulation of the hormone abscisic acid (ABA) (Zentella et al., 2007; Zhang et al., 2011; Lim et al., 2013; Park et al., 2013). Therefore it would be important to identify other TFs that mediate

the presence of DELLAs at the target promoters and find out how extensive this mechanism may be. To approach these questions, we decided to investigate the genome wide occupancy of DELLAs in *Arabidopsis*.

5.2 Results and discussion

5.2.1 Genome-wide regions bound by DELLAs

To determine the *in vivo* binding sites of DELLAs, we performed chromatin immunoprecipitation (ChIP) followed by massive sequencing using the *RGA::GFP-RGA* line (Silverstone et al., 2001). Seedlings were grown in continuous light (~50 μmol m^{-2} s^{-1}) for 10 days, and before cross-linking, with formaldehyde an 18-h treatment with 10μM PAC was performed to promote accumulation of GFP-RGA (See Materials and Methods). Sequences obtained from three biological replicates where mapped to the *Arabidopsis* genome (TAIR 10) using Bowtie 2 (Langmead and Salzberg, 2012) and the binding sites were determined by Model-based Analysis for ChIP-Seq (MACS) software (Zhang et al., 2008). A total of 842 reproducible binding sites were found, from which a surprisingly high proportion was found within a gene or in transposons (Figure 5.1A). All these binding sites were discarded for further analysis, which left us with 311 binding sites, a 36% of the total, within the proximal 2.5 kb of a transcriptional start site (TSS) (Figure 5.1A). Given the proximity between genes, it was not always possible to assign each peak to a single gene, so the number of genes with enrichment in "DELLA binding" in the final set was 421. This number of genes may seem low compared to other TFs (Winter et al., 2011), which could be caused by the circumstance that DELLAs do not bind DNA directly and all peaks must be due physical interaction with TFs.

As observed in Figure 5.1B, binding sites were regularly distributed along the five chromosomes, indicating no preference for specific genomic regions. To investigate if DELLAs would preferentially bind the promoters of genes involved in a particular process, we performed a Gene Ontology (GO) enrichment analysis with agriGO (Du et al., 2010) and visualized it with REVIGO (Supek et al., 2011), which forms clusters of related terms and facilitates the identification of enriched

categories. As shown in Figure 5.1C, there was a very significant enrichment among DELLA-bound genes of those involved in the response to stimuli, including light and abiotic stress. This result fits the observed implication of DELLAs in all these processes (Alabadi et al., 2008; Hou et al., 2010). Responses to hormones like GAs and JA are also represented in the clusters.
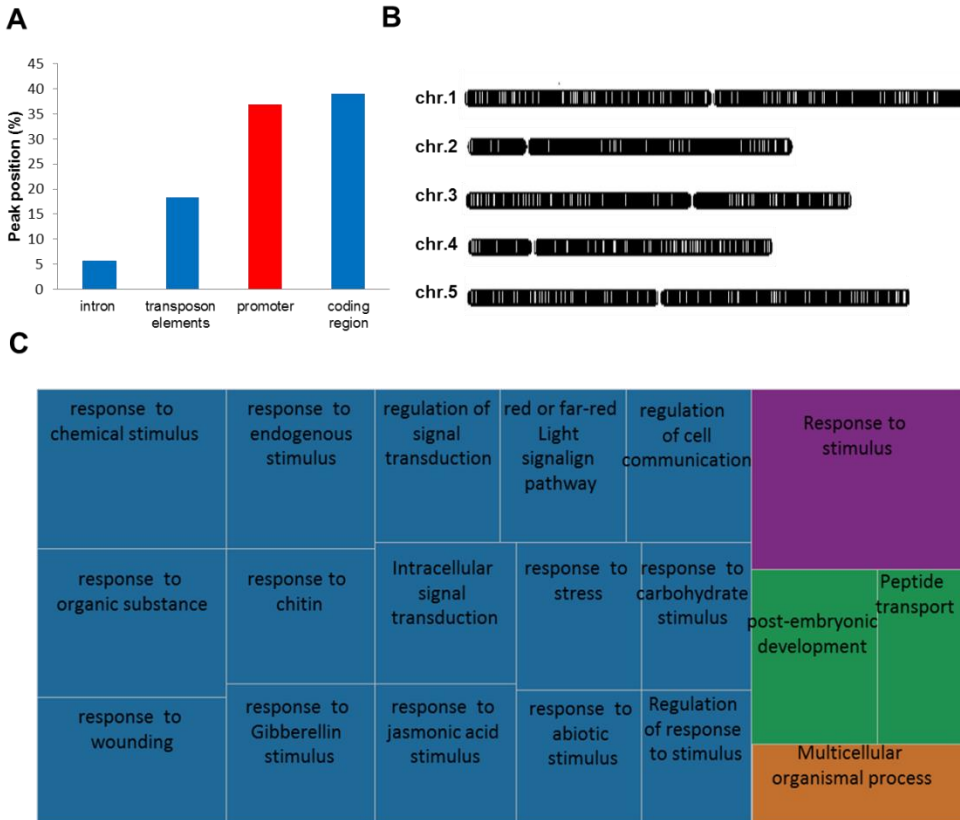
Figure 5.1 Genome wide occupancy of DELLAs. (A) Graphical representation of the location of the 842 peaks obtained, the peaks used for the analysis are represented in red. (B) Distribution of the 311 peaks within a promoter region. (C) GO for DELLA bound genes cluster by biological process. Each rectangle is a single cluster representative. Clusters were joined into "superclusters" of related terms, (visualized with different colors). Sizes of rectangles were adjusted to reflect the *p*-value.

5.2.2 *cis*-element enrichment of DELLAs bound genes

To search for TFs by which DELLAs are bound to each target sequence, we decided to find over-represented *cis*-elements within the peak region. Since most representative *cis*-elements are in a window of 200 bp next to the peak (Kaufmann et al., 2010; Winter et al., 2011), we extracted the sequence corresponding to 200 bp around each peak (100 bp on either side). Then we collected all the TF binding site matrices from the open-access libraries JASPAR and TRANSFAC (Matys et al., 2006; Portales-Casamar et al., 2010), as well the matrix available through the Spanish TRANSPLANTA consortium (Franco-Zorrilla et al., 2014). This provided us with access to more than 70 binding sites corresponding to 25 protein families, which we used to analyze the DELLA-bound regions with MotifLab (Klepper and Drablos, 2013). Interestingly, our results indicated that there is an over-representation of only 7 consensus binding sites, corresponding to 7 different TF families (ARR1, MYB46, DOF5, RAV1a, MYC4, SPL3 and KANADI4) (Figure 5.2A-B). It is important to remark that despite the *cis* elements were obtained for particular members of the TF families, proteins from the same family usually show similar DNA-recognition patterns (Franco-Zorrilla et al., 2014), then for now on we will refer each TF binding site by family.

Remarkably, we found at least one positive interactor in our Y2H screening from each of the families that could bind these sequences (Supporting Table I, Chapter 2); so it is reasonable to think that these motifs were found because of the interaction of DELLA with these proteins. An intriguing result is that in many peak regions there is more than one over-represented binding site. It may therefore be possible that DELLAs regulate transcription of some genes by interacting with two proteins, either forming a complex with two TFs of by independent interaction with each of them. It has been recently reported that PIF4 and BZR1 (both of them DELLA interactors) heterodimerize to control a set of genes (Oh et al., 2012). Nevertheless more work need to be done to completely support this hypothesis.
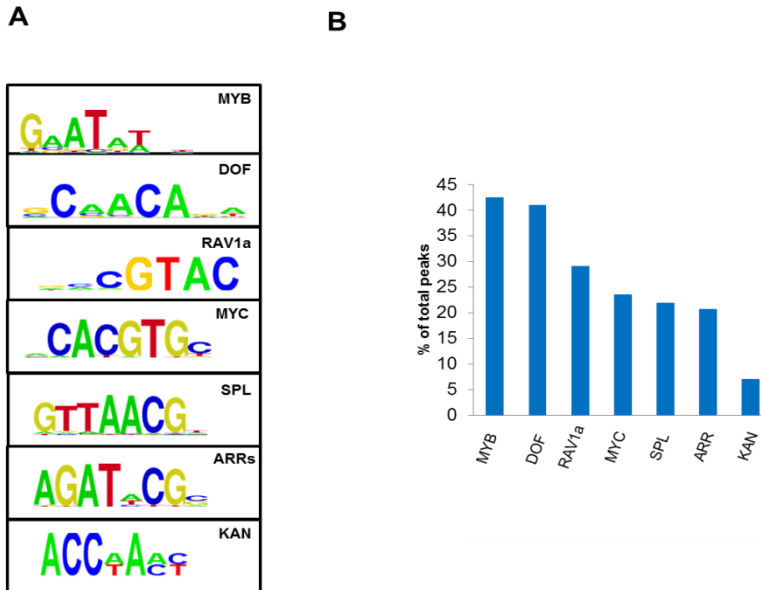
Figure 5.2. ***cis*-element enrichment.** (A)Position weight matrix (PWM) of each enriched *cis*-element (B) Representation of the of each *cis*-element found in the total of peak region.

5.2.3 Transcriptional regulation of genes bound by DELLAs

To investigate the extent to which DELLAs affect the expression of their bound genes, we decided to compile the data of all the publicly available transcriptome data (Supporting Table III), that encompass conditions with either low or high levels of DELLAs (Locascio et al., 2013). Data also represent different developmental stages, seedlings grown in dark and light, embryos and endosperms, root tips and inflorescences. We searched for differential expression of bound genes, and among the 421 genes, 135 showed changes during at least one condition. Then we sorted each gene depending on the presence of the different TFs binding sites. Our results show that the transcriptional behavior of each set of genes is basically the same, indicating that there is no preference of DELLAs for any particular type of TF to up- or down-regulate their target genes (Figure 5.3A). Interestingly a big proportion of genes present in the data set do not change in any condition (Figure 5.3B), this is

maybe due the diversity of samples, so probably with tissue specific experiments a differential expression pattern could be observe.
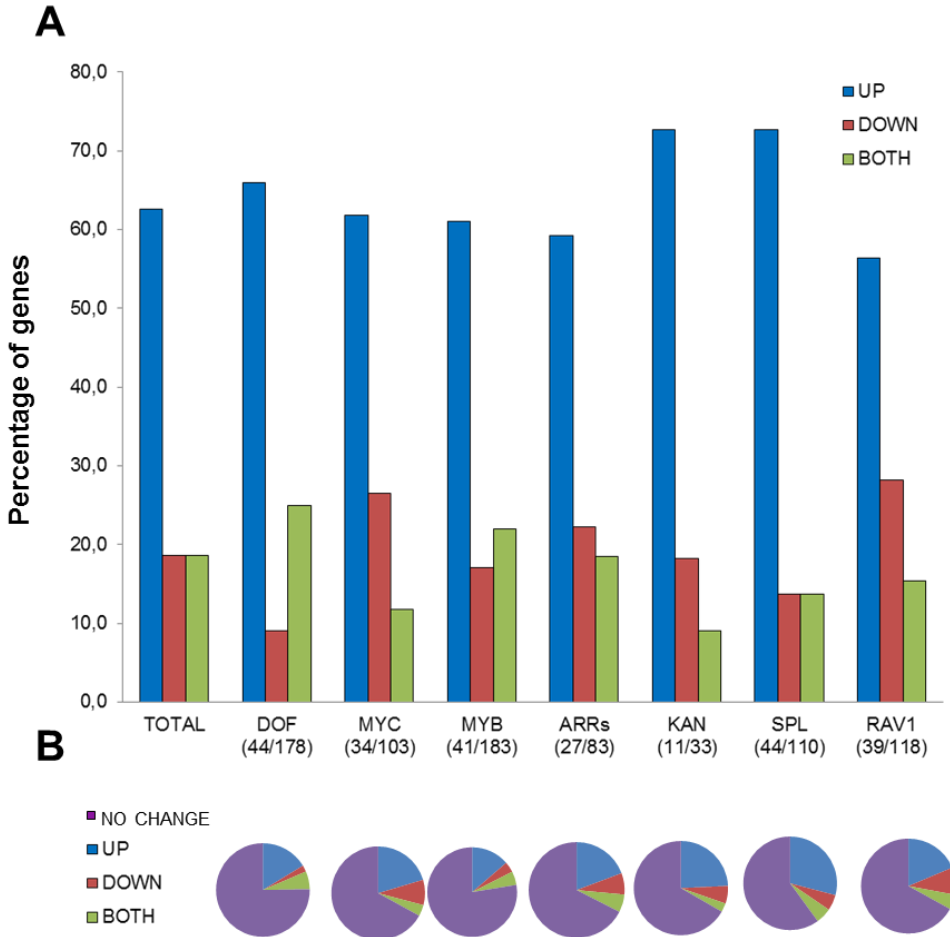
Figure 5.3 Representation of bound genes by DELLA. (A) Representation of all genes found to be up- regulated, down-regulated or both in dataset. Genes were divided by presence of TFs binding sites. Some genes are present in more than one group. Numbers represent total of genes that show a change from the total (B) Percentage of the total genes that change or not corresponding to each TF binding site.

5.3 Concluding remarks

During this section we have identified several DELLAs bound genes. Importantly, we have detected an enrichment of a low number of *cis*-element corresponding to 7 different TF families, providing a starting point to further investigate the association of DELLAs with DNA by one or more of TFs belonging to the families found. We also found that one third of the bounded genes are transcriptional regulated by DELLAs, however we could not attribute these changes to any particular TF.

Remarkably as observe in Figure 5.1C there is a particular enrichment if functional categories involved in response to stimuli, and this is not surprising if we observe the identity of bound genes (Supporting Table I). Interestingly as represented in Figure 5.4 some of the genes could provide a mechanism of cross-talk between light and the circadian clock, and hormone signaling pathways, for instance SHY2, an AUX/IAA which is a repressor of auxin signaling activated by ARR1, a Type-B response regulator that acts in cytokinin signaling pathway (Sakai et al., 2001;Dello Ioio et al.,2008). In Chapter 2 we show that DELLAs interact with ARR14, a Type-B response regulator, so it is reasonable to imagine that DELLAs interact with ARR1. Additionaly reports show that some developmental processes are controlled in common by GAs and CK (Chory et al., 1994; Alabadi et al., 2004; Jasinski et al., 2005; Dello Ioio et al., 2008; Ubeda-Tomas et al., 2008; Achard et al., 2009).
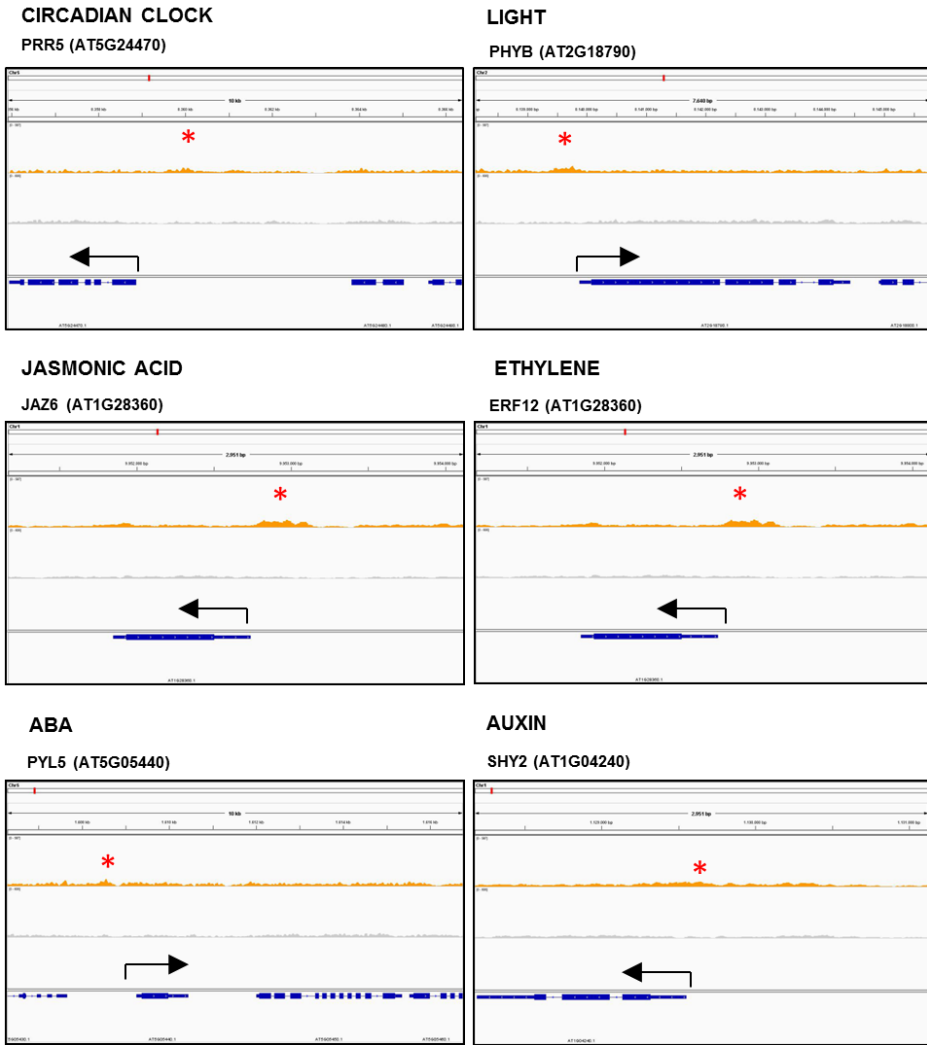
Figure 5.4 Examples of DELLA bound genes, visualized with IGV Browser (Thorvaldsdottir et al., 2012). The results of enrichment in the GFP -RGA line are shown in orange, while the results for Col-0 are in grey. The asterisks indicate the peaks corresponding to the next TSS (represented with arrows).

5.4 Material and methods

Plant material and growth conditions. Seedlings of wild type (L*er*) and *RGA::GFP-RGA* (Silverstone et al., 2001) were grown in solid MS for 10 days under continuous light (~50 µmol m^{-2} s^{-1}); then seedlings were submerged in liquid MS PAC to a final concentration of 10µM for 18 hours.

Chromatin Immunoprecipitation and sequencing Immunoprecipitation with anti-GFP (ab290;AbCam) was performed as described (Saleh et al., 2008). Before construction of the ChIP DNA library, quality of the precipitated DNA was checked by PCR amplification of *SCL3*, a reported DELLA bound gene (Zentella et al., 2007). Then libraries were generated following Illumina ChIP-Seq manufacturer's instructions and subjected to ultra-high-through-put Solexa (Illumina) sequencing. All results represent three biological replicates. For sequence alignment bowtie2 (Langmead and Salzberg, 2012) was used, and enrichment were analyzed by Model-based Analysis (MACS) (Zhang et al., 2008).To visualize the sequence reads obtained among the chromosomes we use Integrative Genomics Viewer (IGV) (Thorvaldsdottir et al., 2012).

GO analysis For Gene ontology analysis only 421 genes corresponding to 311 peaks within 2.5 kb of gene promoter were used, the web tool agriGO (Du et al., 2010) was used to obtain biological function categories. Then to summarize, the GO terms and their *p*-values were extracted and analyzed with REVIGO (Supek et al., 2011), for visualization the "TreeMap" option was selected.

Gene expression analysis. For gene expression analysis raw data of microarrays listed in supporting table III (Chapter2) were collected. Statistical *Z*-score was calculated for each dataset (Cheadle et al., 2003).

Motif analysis. 200bp surrounding each peak was used to perform motif analysis. Marices from JASPAR, TRANSFAC (Matys et al., 2006; Portales-Casamar et al., 2010), and from Franco-Zorrilla et al., 2014 were imported into MotifLab (Klepper and Drablos, 2013), then motif scanning was performed using the MotifScanner method. For background noise a background model of order 2 was used.

5.5 Supporting Table

5.5.1 Table I Corresponding genes to the peaks within 2 kb of promoter region

AT1G01060	LATE ELONGATED HYPOCOTYL (LHY)
AT1G01490	Heavy metal transport/detoxification superfamily protein
AT1G01500	Erythronate-4-phosphate dehydrogenase family protein
AT1G03030	P-loop containing nucleoside triphosphate hydrolases superfamily protein
AT1G03800	ERF domain protein 10 (ERF10)
AT1G03810	Nucleic acid-binding, OB-fold-like protein
AT1G04240	SHORT HYPOCOTYL 2 (SHY2)
AT1G04990	Zinc finger C-x8-C-x5-C-x3-H type family protein
AT1G07000	exocyst subunit exo70 family protein B2 (EXO70B2)
AT1G07010	Calcineurin-like metallo-phosphoesterase superfamily protein
AT1G07630	pol-like 5 (PLL5)
AT1G07885	unknown protein
AT1G07890	ascorbate peroxidase 1 (APX1)
AT1G08100	nitrate transporter 2.2 (NRT2.2)
AT1G09130	ATP-dependent caseinolytic (Clp) protease/crotonase family protein
AT1G09140	SERINE-ARGININE PROTEIN 30 (ATSRP30)
AT1G09380	nodulin MtN21 /EamA-like transporter family protein
AT1G09390	GDSL-like Lipase/Acylhydrolase superfamily protein
AT1G09520	LOCATED IN: chloroplast
AT1G09570	phytochrome A (PHYA)
AT1G10360	glutathione S-transferase TAU 18 (GSTU18)
AT1G11180	Secretory carrier membrane protein (SCAMP) family protein
AT1G12330	unknown protein

AT1G13260   related to ABI3/VP1 1 (RAV1)

AT1G17420   lipoxygenase 3 (LOX3)

AT1G17495   max) (SIRE1) (Ty1_Copia-family)

AT1G18740   FUNCTIONS IN: molecular_function unknown

AT1G19180   jasmonate-zim-domain protein 1 (JAZ1)

AT1G19460   Galactose oxidase/kelch repeat superfamily protein

AT1G19660   Wound-responsive family protein

AT1G19870   IQ-domain 32 (iqd32)

AT1G20890   unknown protein

AT1G21080   DNAJ heat shock N-terminal domain-containing protein

AT1G21100   O-methyltransferase family protein

AT1G21410   SKP2A

AT1G21975   unknown protein

AT1G22275   ZYP1b

AT1G22280   phytochrome-associated protein phosphatase type 2C

AT1G22310   methyl-CPG-binding domain 8 (MBD8)

AT1G22320   tRNA-Met (anticodon: CAT)

AT1G22400   UGT85A1

AT1G24265   Protein of unknown function (DUF1664)

AT1G25560   TEMPRANILLO 1 (TEM1)

AT1G27540   Protein of unknown function (DUF295)

AT1G27540   Protein of unknown function (DUF295)

AT1G28360   ERF domain protein 12 (ERF12)

AT1G30110   nudix hydrolase homolog 25 (NUDX25)

AT1G31358   attenuating translation or by directing mRNA cleavage. Mature sequence:ATTAACGCTGGCGGTTGCGGCAGC

AT1G31360   RECQ helicase L2 (RECQL2)

AT1G31910   GHMP kinase family protein

AT1G32920   unknown protein

AT1G33250   Protein of unknown function (DUF604)

AT1G34315   unknown protein

AT1G34320   Protein of unknown function (DUF668)

AT1G36030   F-box family protein

AT1G36185   copia-like retrotransposon family

AT1G36622   unknown protein

AT1G39350   transposable element gene;

AT1G39430   similar to unknown protein [*Arabidopsis thaliana*] (TAIR:AT3G43100.1)

AT1G40097   Mutator-like transposase family

AT1G40113   gypsy-like retrotransposon family

AT1G41755   CACTA-like transposase family (Tnp2/En/Spm)

AT1G43145   unknown protein

AT1G43150   non-LTR retrotransposon family

AT1G43820   tRNA-Asp (anticodon: GTC)

AT1G49160   WNK7

AT1G49170   Protein of unknown function (DUF167)

AT1G49200   RING/U-box superfamily protein

AT1G49500   unknown protein

AT1G50420   scarecrow-like 3 (SCL3)

AT1G51172   unknown protein

AT1G51510   Y14

AT1G51520   RNA-binding (RRM/RBD/RNP motifs) family protein

AT1G52710   Rubredoxin-like superfamily protein

AT1G52720   unknown protein

AT1G52905   unknown protein

AT1G52960   similar to unknown protein [*Arabidopsis thaliana*] (TAIR:AT3G13250.1)

AT1G53560   Ribosomal protein L18ae family

AT1G53570   mitogen-activated protein kinase kinase kinase 3 (MAP3KA)

AT1G53830   pectin methylesterase 2 (PME2)

AT1G53840   pectin methylesterase 1 (PME1)

AT1G56010   NAC domain containing protein 1 (NAC1)

AT1G56140   Leucine-rich repeat transmembrane protein kinase

AT1G56145   Leucine-rich repeat transmembrane protein kinase

AT1G56630   alpha/beta-Hydrolases superfamily protein

AT1G58440   XF1

AT1G58450   Tetratricopeptide repeat (TPR)-like superfamily protein

AT1G58602   LRR and NB-ARC domains-containing disease resistance protein

AT1G59171   Inositol-pentakisphosphate 2-kinase family protein

AT1G61190   LRR and NB-ARC domains-containing disease resistance protein

AT1G63390   FAD/NAD(P)-binding oxidoreductase family protein

AT1G63400   Pentatricopeptide repeat (PPR) superfamily protein

AT1G67340   HCP-like superfamily protein with MYND-type zinc finger

AT1G67960   CONTAINS InterPro DOMAIN/s: Membrane protein,Tapt1/CMV receptor (InterPro:IPR008010)

AT1G69490   NAC-like, activated by AP3/PI (NAP)

AT1G69500   cytochrome P450, family 704, subfamily B, polypeptide 1 (CYP704B1)

AT1G70080   Terpenoid cyclases/Protein prenyltransferases superfamily protein

AT1G70090 glucosyl transferase family 8 (LGT8)

AT1G70520 cysteine-rich RLK (RECEPTOR-like protein kinase) 2 (CRK2)

AT1G71030 MYB-like 2 (MYBL2)

AT1G71080 RNA polymerase II transcription elongation factor

AT1G71090 Auxin efflux carrier family protein

AT1G71500 Rieske (2Fe-2S) domain-containing protein

AT1G71520 Integrase-type DNA-binding superfamily protein

AT1G72450 jasmonate-zim-domain protein 6 (JAZ6)

AT1G72460 Leucine-rich repeat protein kinase family protein

AT1G72920 Toll-Interleukin-Resistance (TIR) domain family protein

AT1G74780 Nodulin-like / Major Facilitator Superfamily protein

AT1G74790 catalytics

AT1G75820 CLAVATA 1 (CLV1)

AT1G76080 chloroplastic drought-induced stress protein of 32 kD (CDSP32)

AT1G76430 phosphate transporter 1

AT2G01160 tRNA-Asn (anticodon: GTT)

AT2G01180 phosphatidic acid phosphatase 1 (PAP1)

AT2G01180 phosphatidic acid phosphatase 1 (PAP1)

AT2G01670 nudix hydrolase homolog 17 (NUDT17)

AT2G04170 TRAF-like family protein

AT2G04180 , member 30 (LINE-element) (Mus musculus)

AT2G05210 Protection of Telomeres 1a (AtPOT1a)

AT2G05210 Protection of Telomeres 1a (AtPOT1a)

AT2G09830 copia-like retrotransposon family

AT2G12320 similar to unknown protein [*Arabidopsis thaliana*] (TAIR:AT5G32169.1)

AT2G18780 F-box and associated interaction domains-containing protein

AT2G18790 phytochrome B (PHYB)

AT2G19930 RNA-dependent RNA polymerase family protein
AT2G19940 oxidoreductases, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor

AT2G20570 GBF's pro-rich region-interacting factor 1 (GPRI1)

AT2G20580 26S proteasome regulatory subunit S2 1A (RPN1A)

AT2G20875 EPIDERMAL PATTERNING FACTOR 1 (EPF1)

AT2G20880 Integrase-type DNA-binding superfamily protein

AT2G21655 Protein of unknown function (DUF784)

AT2G25482 LOCATED IN: endomembrane system

AT2G25490 EIN3-binding F box protein 1 (EBF1)

AT2G28610 PRESSED FLOWER (PRS)

AT2G29080   FTSH protease 3 (ftsh3)

AT2G30032   unknown protein

AT2G30600   BTB/POZ domain-containing protein

AT2G31560   Protein of unknown function (DUF1685)

AT2G31570   glutathione peroxidase 2 (GPX2)

AT2G39710   Eukaryotic aspartyl protease family protein

AT2G41010   calmodulin (CAM)-binding protein of 25 kDa (CAMBP25)

AT2G41640   Glycosyltransferase family 61 protein

AT2G42420   tRNA-Gly (anticodon: GCC)

AT2G42955   unknown protein

AT2G42960   Protein kinase superfamily protein

AT2G43240   Nucleotide-sugar transporter family protein

AT2G43250   unknown protein

AT2G44120   Ribosomal protein L30/L7 family protein

AT2G45170   AUTOPHAGY 8E (ATATG8E)

AT2G45180   Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein

AT2G45310   UDP-D-glucuronate 4-epimerase 4 (GAE4)

AT2G46800   zinc transporter of *Arabidopsis thaliana* (ZAT)

AT2G46970   phytochrome interacting factor 3-like 1 (PIL1)

AT2G46980   unknown protein

AT3G02140   TWO OR MORE ABRES-CONTAINING GENE 2 (TMAC2)

AT3G02460   Ypt/Rab-GAP domain of gyp1p superfamily protein

AT3G02470   S-adenosylmethionine decarboxylase

AT3G02550   LOB domain-containing protein 41 (LBD41)

AT3G03150   unknown protein

AT3G03160   FUNCTIONS IN: molecular_function unknown

AT3G03370   BEST *Arabidopsis thaliana* protein match is: DegP protease 7 (TAIR:AT3G03380.1)

AT3G03380   DegP protease 7 (DegP7)

AT3G03870   unknown protein

AT3G04721   unknown protein

AT3G05110   CONTAINS InterPro DOMAIN/s: Molecular chaperone, heat shock protein, Hsp40, DnaJ (InterPro:IPR015609)

AT3G05120   GA INSENSITIVE DWARF1A (GID1A)

AT3G05810   FUNCTIONS IN: molecular_function unknown

AT3G05820   invertase H (INVH)

AT3G05830   FUNCTIONS IN: molecular_function unknown

AT3G05936   unknown protein

AT3G05937   unknown protein

AT3G07350   Protein of unknown function (DUF506)

AT3G09590   CAP (Cysteine-rich secretory proteins, Antigen 5, and Pathogenesis-related 1 protein) superfamily protein

AT3G10415   tRNA-Tyr (anticodon: GTA)

AT3G10420   P-loop containing nucleoside triphosphate hydrolases superfamily protein

AT3G11280   Duplicated homeodomain-like superfamily protein

AT3G11285   tRNA-Arg (anticodon: CCT)

AT3G11700   FASCICLIN-like arabinogalactan protein 18 precursor (FLA18)

AT3G12100   Cation efflux family protein

AT3G12110   actin-11 (ACT11)

AT3G12920   SBP (S-ribonuclease binding protein) family protein

AT3G13450   DARK INDUCIBLE 4 (DIN4)

AT3G13460   evolutionarily conserved C-terminal region 2 (ECT2)

AT3G13790   ATBFRUCT1

AT3G13800   Metallo-hydrolase/oxidoreductase superfamily protein

AT3G15320   similar to unknown protein [*Arabidopsis thaliana*] (TAIR:AT5G32620.1)

AT3G15450   Aluminium induced protein with YGL and LRDR motifs

AT3G15460   Ribosomal RNA processing Brix domain protein

AT3G15518   unknown protein

AT3G17390   METHIONINE OVER-ACCUMULATOR 3 (MTO3)

AT3G17400   F-box family protein

AT3G18524   MUTS homolog 2 (MSH2)

AT3G18530   ARM repeat superfamily protein

AT3G18535   tubulin-tyrosine ligases

AT3G18815   tRNA-Thr (anticodon: AGT)

AT3G18820   RAB GTPase homolog G3F (RAB7B)

AT3G19690   CAP (Cysteine-rich secretory proteins, Antigen 5, and Pathogenesis-related 1 protein) superfamily protein

AT3G19980   flower-specific, phytochrome-associated protein phosphatase 3 (FYPP3)

AT3G20030   F-box and associated interaction domains-containing protein

AT3G20820   Leucine-rich repeat (LRR) family protein

AT3G21420   2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein

AT3G23250   myb domain protein 15 (MYB15)

AT3G26520   tonoplast intrinsic protein 2 (TIP2)

AT3G29595   Mutator-like transposase family

AT3G29784   CACTA-like transposase family (En/Spm

AT3G29787   CACTA-like transposase family (Ptta/En/Spm)

AT3G30816   similar to unknown protein [*Arabidopsis thaliana*] (TAIR:AT5G28120.1)

AT3G44400   Disease resistance protein (TIR-NBS-LRR class) family

AT3G44630	Disease resistance protein (TIR-NBS-LRR class) family
AT3G44950	glycine-rich protein
AT3G44950	glycine-rich protein
AT3G45260	C2H2-like zinc finger protein
AT3G45640	mitogen-activated protein kinase 3 (MPK3)
AT3G45650	nitrate excretion transporter1 (NAXT1)
AT3G45775	copia-like retrotransposon family
AT3G45780	phototropin 1 (PHOT1)
AT3G46630	Protein of unknown function (DUF3223)
AT3G46640	PHYTOCLOCK 1 (PCL1)
AT3G47240	similar to unknown protein [*Arabidopsis thaliana*] (TAIR:AT1G54926.1)
AT3G47430	peroxin 11B (PEX11B)
AT3G49790	Carbohydrate-binding protein
AT3G50337	unknown protein
AT3G50340	unknown protein
AT3G50660	DWARF 4 (DWF4)
AT3G50800	unknown protein
AT3G52110	unknown protein
AT3G52115	gamma response gene 1 (GR1)
AT3G54440	glycoside hydrolase family 2 protein
AT3G54450	Major facilitator superfamily protein
AT3G56275	expressed protein
AT3G58750	citrate synthase 2 (CSY2)
AT3G58760	Integrin-linked protein kinase family
AT3G59765	other RNA;Unknown gene
AT3G59940	Galactose oxidase/kelch repeat superfamily protein
AT3G60130	beta glucosidase 16 (BGLU16)
AT3G60190	DYNAMIN-like 1E (DL1E)
AT3G60200	unknown protein
AT3G61150	homeodomain GLABROUS 1 (HDG1)
AT3G61470	photosystem I light harvesting complex gene 2 (LHCA2)
AT3G62090	phytochrome interacting factor 3-like 2 (PIL2)
AT3G63006	tRNA-Ala (anticodon: TGC)
AT3G63006	tRNA-Ala (anticodon: TGC)
AT3G63010	GA INSENSITIVE DWARF1B (GID1B)
AT3G63020	Protein of unknown function (DUF3049)
AT4G00720	shaggy-like protein kinase 32 (SK32)

AT4G00893   unknown protein

AT4G00895   ATPase, F1 complex, OSCP/delta subunit protein

AT4G01250   WRKY22

AT4G01720   WRKY47

AT4G03840   gypsy-like retrotransposon family

AT4G05616   similar to unknown protein [*Arabidopsis thaliana*] (TAIR:AT4G04155.1)

AT4G06742   gypsy-like retrotransposon family (Athila

AT4G07332   pseudogene, similar to putative AP endonuclease/reverse transcriptase

AT4G08400   Proline-rich extensin-like family protein

AT4G09466   Carbohydrate-binding X8 domain superfamily protein

AT4G09620   Mitochondrial transcription termination factor family protein

AT4G12420   SKU5

AT4G13395   ROTUNDIFOLIA like 12 (RTFL12)

AT4G13400   2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein

AT4G14310   Transducin/WD40 repeat-like superfamily protein

AT4G14315   unknown protein

AT4G14342   Splicing factor 3B subunit 5/RDS3 complex subunit 10

AT4G14345   tRNA-His (anticodon: GTG)

AT4G15545   unknown protein

AT4G15550   indole-3-acetate beta-D-glucosyltransferase (IAGLU)

AT4G15590   non-LTR retrotransposon family (LINE

AT4G15610   Uncharacterised protein family (UPF0497)

AT4G15755   Calcium-dependent lipid-binding (CaLB domain) family protein

AT4G15760   monooxygenase 1 (MO1)

AT4G17070   peptidyl-prolyl cis-trans isomerases

AT4G17230   SCARECROW-like 13 (SCL13)

AT4G19710   aspartate kinase-homoserine dehydrogenase ii (AK-HSDH II)

AT4G20520   RNA binding

AT4G20725   non-LTR retrotransposon family (LINE)

AT4G22285   Ubiquitin C-terminal hydrolases superfamily protein

AT4G23030   MATE efflux family protein

AT4G23630   VIRB2-interacting protein 1 (BTI1)

AT4G24110   unknown protein

AT4G24204   RING/U-box superfamily protein

AT4G24210   SLEEPY1 (SLY1)

AT4G24570   dicarboxylate carrier 2 (DIC2)

AT4G25490   C-repeat/DRE binding factor 1 (CBF1)

AT4G25500	arginine/serine-rich splicing factor 35 (RSP35)
AT4G25640	detoxifying efflux carrier 35 (DTX35)
AT4G26090	RESISTANT TO P. SYRINGAE 2 (RPS2)
AT4G26240	unknown protein
AT4G26250	galactinol synthase 6 (GolS6)
AT4G26940	Galactosyltransferase family protein
AT4G27300	S-locus lectin protein kinase family protein
AT4G27300	S-locus lectin protein kinase family protein
AT4G27730	oligopeptide transporter 1 (OPT6)
AT4G27740	Yippee family putative zinc-binding protein
AT4G27930	unknown protein
AT4G27940	manganese tracking factor for mitochondrial SOD2 (MTM1)
AT4G27970	SLAC1 homologue 2 (SLAH2)
AT4G27980	CONTAINS InterPro DOMAIN/s: Molecular chaperone, heat shock protein, Hsp40, DnaJ (InterPro:IPR015609)
AT4G28180	unknown protein
AT4G28181	unknown protein
AT4G28230	unknown protein
AT4G28240	Wound-responsive family protein
AT4G28540	casein kinase I-like 6 (CKL6)
AT4G29310	Protein of unknown function (DUF1005)
AT4G30270	xyloglucan endotransglucosylase/hydrolase 24 (XTH24)
AT4G31695	tRNA-Met (anticodon: CAT)
AT4G31700	ribosomal protein S6 (RPS6)
AT4G31800	WRKY DNA-binding protein 18 (WRKY18)
AT4G32208	heat shock protein 70 (Hsp 70) family protein
AT4G33050	embryo sac development arrest 39 (EDA39)
AT4G33060	Cyclophilin-like peptidyl-prolyl cis-trans isomerase family protein
AT4G33990	embryo defective 2758 (EMB2758)
AT4G34000	abscisic acid responsive elements-binding factor 3 (ABF3)
AT4G35110	*Arabidopsis* phospholipase-like protein (PEARLI 4) family
AT4G35760	NAD(P)H dehydrogenase (quinone)s
AT4G35770	SENESCENCE 1 (SEN1)
AT4G36010	Pathogenesis-related thaumatin superfamily protein
AT4G36970	Remorin family protein
AT4G38600	KAKTUS (KAK)
AT4G39070	B-box zinc finger family protein
AT4G39080	vacuolar proton ATPase A3 (VHA-A3)

AT4G39080   vacuolar proton ATPase A3 (VHA-A3)

AT4G39090   RESPONSIVE TO DEHYDRATION 19 (RD19)

AT4G39140   RING/U-box superfamily protein

AT4G39150   DNAJ heat shock N-terminal domain-containing protein

AT4G39390   nucleotide sugar transporter-KT 1 (NST-K1)

AT5G01849   unknown protein

AT5G01850   Protein kinase superfamily protein

AT5G02615   tRNA-Arg (anticodon: TCG)

AT5G02620   ankyrin-like1 (ANK1)

AT5G05440   PYRABACTIN RESISTANCE 1-LIKE 5 (PYL5)

AT5G06390   FASCICLIN-like arabinogalactan protein 17 precursor (FLA17)

AT5G06970   CONTAINS InterPro DOMAIN/s: Munc13 homology 1 (InterPro:IPR014770)

AT5G06980   unknown protein

AT5G08790   ATAF2

AT5G09810   actin 7 (ACT7)

AT5G11970   Protein of unknown function (DUF3511)

AT5G12990   CLAVATA3/ESR-RELATED 40 (CLE40)

AT5G13000   glucan synthase-like 12 (GSL12)

AT5G13180   NAC domain containing protein 83 (NAC083)

AT5G13700   polyamine oxidase 1 (PAO1)

AT5G13710   sterol methyltransferase 1 (SMT1)

AT5G13730   sigma factor 4 (SIG4)

AT5G13740   zinc induced facilitator 1 (ZIF1)

AT5G14110   Protein of unknown function (DUF 3339)

AT5G14120   Major facilitator superfamily protein

AT5G14800   pyrroline-5- carboxylate (P5C) reductase (P5CR)

AT5G14810   retrotransposon family

AT5G15260   Ribosomal protein L34e superfamily protein

AT5G15265   unknown protein

AT5G16560   KANADI (KAN)

AT5G17340   Putative membrane lipoprotein

AT5G18080   SAUR-like auxin-responsive protein family

AT5G18085   tRNA-Trp (anticodon: CCA)

AT5G18910   Protein kinase superfamily protein

AT5G18920   Cox19-like CHCH family protein

AT5G19110   Eukaryotic aspartyl protease family protein

AT5G19120   Eukaryotic aspartyl protease family protein

AT5G19130	GPI transamidase component family protein / Gaa1-like family protein
AT5G21940	unknown protein
AT5G21940	unknown protein
AT5G24470	pseudo-response regulator 5 (PRR5)
AT5G24870	RING/U-box superfamily protein
AT5G25290	CONTAINS InterPro DOMAIN/s: F-box domain, cyclin-like (InterPro:IPR001810)
AT5G27505	Mutator-like transposase family
AT5G28460	Pentatricopeptide repeat (PPR) superfamily protein
AT5G28463	unknown protein
AT5G28928	pseudogene, hypothetical protein
AT5G30545	CACTA-like transposase family (Ptta/En/Spm)
AT5G31807	Mutator-like transposase family, has a 1.4e-05 P-value blast match to GB:AAA21566 mudrA of transposon="MuDR" (MuDr-element) (Zea mays)
AT5G32520	pseudogene, expressed protein, predicted proteins, *Arabidopsis thaliana* and others
AT5G32525	gypsy-like retrotransposon family
AT5G34560	pseudogene, uridine kinase/uracil phosphoribosyl transferas -related, similar to uridine kinase/uracil phosphoribosyl transferas, putative
AT5G35065	copia-like retrotransposon family
AT5G35880	similar to unknown protein [*Arabidopsis thaliana*] (TAIR:AT1G32680.1)
AT5G37250	RING/U-box superfamily protein
AT5G39640	Putative endonuclease or glycosyl hydrolase
AT5G39645	Defensin-like (DEFL) family protein
AT5G40260	Nodulin MtN3 family protein
AT5G41060	DHHC-type zinc finger family protein
AT5G41220	glutathione S-transferase THETA 3 (GSTT3)
AT5G43755	BEST *Arabidopsis thaliana* protein match is: Polynucleotidyl transferase
AT5G43760	3-ketoacyl-CoA synthase 20 (KCS20)
AT5G45576	Mutator-like transposase family
AT5G45920	SGNH hydrolase-type esterase superfamily protein
AT5G46050	peptide transporter 3 (PTR3)
AT5G46060	Protein of unknown function, DUF599
AT5G46325	tRNA-Leu (anticodon: TAG)
AT5G46330	FLAGELLIN-SENSITIVE 2 (FLS2)
AT5G46770	unknown protein
AT5G46780	VQ motif-containing protein
AT5G47610	RING/U-box superfamily protein
AT5G47620	RNA-binding (RRM/RBD/RNP motifs) family protein
AT5G50380	exocyst subunit exo70 family protein F1 (EXO70F1)
AT5G51060	ROOT HAIR DEFECTIVE 2 (RHD2)

AT5G51070   EARLY RESPONSIVE TO DEHYDRATION 1 (ERD1)

AT5G53550   YELLOW STRIPE like 3 (YSL3)

AT5G55920   OLIGOCELLULA 2 (OLI2)

AT5G57550   xyloglucan endotransglucosylase/hydrolase 25 (XTH25)

AT5G58090   O-Glycosyl hydrolases family 17 protein

AT5G58100   unknown protein

AT5G58570   unknown protein

AT5G58575   CONTAINS InterPro DOMAIN/s: Sgf11, transcriptional regulation (InterPro:IPR013246)

AT5G59230   transcription factor-related

AT5G59730   exocyst subunit exo70 family protein H7 (EXO70H7)

AT5G60260   unknown protein

AT5G60270   Concanavalin A-like lectin protein kinase family protein

AT5G61590   Integrase-type DNA-binding superfamily protein

AT5G62090   SEUSS-like 2 (SLK2)

AT5G62090   SEUSS-like 2 (SLK2)

AT5G62100   BCL-2-associated athanogene 2 (BAG2)

AT5G62100   BCL-2-associated athanogene 2 (BAG2)

AT5G63870   serine/threonine phosphatase 7 (PP7)

AT5G63880   VPS20.1

AT5G65165   succinate dehydrogenase 2-3 (SDH2-3)

AT5G65305   tRNA-Met (anticodon: CAT)

AT5G65445   tRNA-Ser (anticodon: GCT)

AT5G65450   ubiquitin-specific protease 17 (UBP17)

AT5G66310   ATP binding microtubule motor family protein

AT5G66380   folate transporter 1 (FOLT1)

AT5G66631   Tetratricopeptide repeat (TPR)-like superfamily protein

AT5G66640   DA1-related protein 3 (DAR3)

AT5G67300   myb domain protein r1 (MYBR1)

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Chapter 4 6

Transcriptional co-activation by DELLA proteins during cytokinin signaling in Arabidopsis

In collaboration with Kristine Hill, Antonella Locascio, Rishikesh Bhalerao, Stephen G. Thomas, Malcolm J. Bennett and Jan U. Lohmann

6.1 Introduction

A unique feature of plant development is the highly plastic behaviour that it displays with respect to a changing environment. Depending on the environmental conditions, plants are able to trigger specific differentiation programs, promote growth over differentiation, or favour defense strategies over growth. Although the molecular mechanism by which plants integrate environmental and endogenous signals is not completely established, the high degree of connectivity between plant signaling pathways is undoubtedly a very useful feature, with certain unique signaling elements playing decisive roles in the coordination between multiple signaling pathways (Casal et al., 2004). One of such elements are DELLA proteins. These are nuclear-localized transcriptional regulators whose accumulation depends on the cellular levels of GAs, in such a way that higher GA levels promote the GID1 receptor-mediated polyubiquitination of DELLAs and their subsequent degradation by the proteasome (Sun, 2011).

Over the past 15 years, evidence has indeed accumulated on the multiple roles of DELLAs all along development and in the response to biotic and abiotic stress. For instance, genetic and genomic studies in *Arabidopsis* show that DELLAs: (1) promote the maintenance of seed dormancy (Penfield et al., 2006; Piskurewicz et al., 2008); (2) restrict cell elongation and division in almost all plant tissues and organs (Dill and Sun, 2001; King et al., 2001); (3) promote the gravitropic response in shoots and roots (Gallego-Bartolome et al., 2011; Lofke et al., 2013); (6) enhance the resistance to cold temperatures (Achard et al., 2008); (7) set up the program to prevent photo-oxidative damage (Achard et al., 2008); and (8) activate the defense against necrotrophic fungi (Navarro et al., 2008). These observations reinforce the idea that DELLAs are key elements that impinge on –and modulate– multiple cellular pathways (Claeys et al., 2013).

A likely explanation for the multiplicity of DELLAs' roles is their promiscuous ability to interact with many of TFs (Schwechheimer, 2011; Daviere and Achard, 2013; Locascio et al., 2013). In *Arabidopsis*, the DELLA proteins GAI and RGA were

first found to interact physically with PIF3 and PIF4, two bHLH TFs of the PIF family (de Lucas et al., 2008; Feng et al., 2008), and since then several additional TFs have been found as partners of DELLA proteins (Hou et al., 2010; Gallego-Bartolome et al., 2011; Josse et al., 2011; Zhang et al., 2011; An et al., 2012; Bai et al., 2012; Gallego-Bartolome et al., 2012; Li et al., 2012; Yu et al., 2012). Interestingly, this molecular mechanism can simultaneously explain two important features: the regulation of gene expression by DELLAs, and the long-standing observation of physiological crosstalk between GAs and other signaling pathways. However, in all these cases, physical interaction with DELLAs impairs the ability of the corresponding TF to bind the target genes, a mechanism that does not explain the observed enrichment of DELLA proteins at some loci (Zentella et al., 2007; Park et al., 2013).

Cytokinins and GAs are known to exert antagonistic regulation of multiple developmental processes (Weiss and Ori, 2007). For instance, shoot apical meristem activity (Jasinski et al., 2005), hypocotyl elongation in etiolated seedlings (Chory et al., 1994; Alabadi et al., 2004; Argyros et al., 2008) and root growth (Argyros et al., 2008; Achard et al., 2009; Ubeda-Tomas et al., 2009) are promoted by GAs and repressed by cytokinins. At least two mechanisms have been proposed to account for this antagonistic action: a marginal repression by GAs of the expression of type-B ARRs (the cytokinin-responsive TFs that mediate cytokinin signaling) (Moubayidin et al., 2010); and independent transcriptional regulation of common targets (Gan et al., 2007). However, the validity of these mechanisms to explain the antagonistic modulation of gene expression by GAs and cytokinins all along development has not been demonstrated. Here we identify a novel regulatory module involving physical interaction between DELLAs and ARR1 in which ARR1 mediates the presence of DELLAs in transcriptionally active target loci, and DELLAs act as transcriptional coactivators.

6.2 Results and discussion

6.2.1 DELLA proteins GAI and RGA interact with type-B *Arabidopsis* response regulators

In order to identify additional DELLA partners responsible for the crosstalk with other hormone signaling pathways, we screened several *Arabidopsis* cDNA libraries by the yeast two-hybrid (Y2H) approach, using the GRAS domain of both GAI and RGA as bait (see Experimental Procedures for details). Several cDNA clones encoding type-B ARABIDOPSIS RESPONSE REGULATORS (ARR) ARR1 (Figures 6.1A) and ARR2 were recovered (data not show). Type-B ARRs are TFs of the GARP family that mediate changes in gene expression in response to cytokinins (CKs) (Mason et al., 2005; Sakai et al., 2001). Further analysis by Y2H showed that complete removal of the LHR1 motif of GAI (del1) does not impair interaction with ARR1, whereas it was prevented by further deletion of the VHIID motif (del2; Figure 6.1A). These results contrast with the requirement of the LHR1 to sustain interaction of GAI or RGA with BZR1, PIF4, and JAZ1 (de Lucas et al., 2008; Hou et al., 2010; Bai et al., 2012), indicating that DELLAs show certain degree of differential specificity for each partner. On the other hand, this part of the protein was not sufficient to sustain the interaction (Figure 6.1A), as occurs with BZR1 (Gallego-Bartolome et al., 2012), in agreement with the requirement for the region close to the C-terminus to support DELLA interactions (Hong et al., 2012). Indeed, point mutations in DELLA genes that create a premature stop codon close to the very end of the coding sequence and that produce truncated proteins represent loss-of-function alleles (Silverstone et al., 1998; Ikeda et al., 2001; Chandler et al., 2002; Gubler et al., 2002; Dill et al., 2004), most likely because of their incapacity to interact with downstream partners.

Contrary to what has been observed for other DELLA interactors, the DNA binding (M) domain of ARR1 was not involved in the interaction, while the glutamine-rich region responsible for the transactivation activity of ARR1 (Sakai et al., 2000) was necessary and sufficient to sustain the interaction with GAI (Figure 6.1B). The modular nature of ARR1, demonstrated by the ability of the isolated M domain to bind DNA (Sakai et al., 2000) and by the hyperactivity of the DDK-deleted version of ARR1, would be compatible with the idea that the interaction with GAI does not interfere with the regulation of ARR1 by CKs through the DDK domain or with the binding of ARR1 to the promoters.
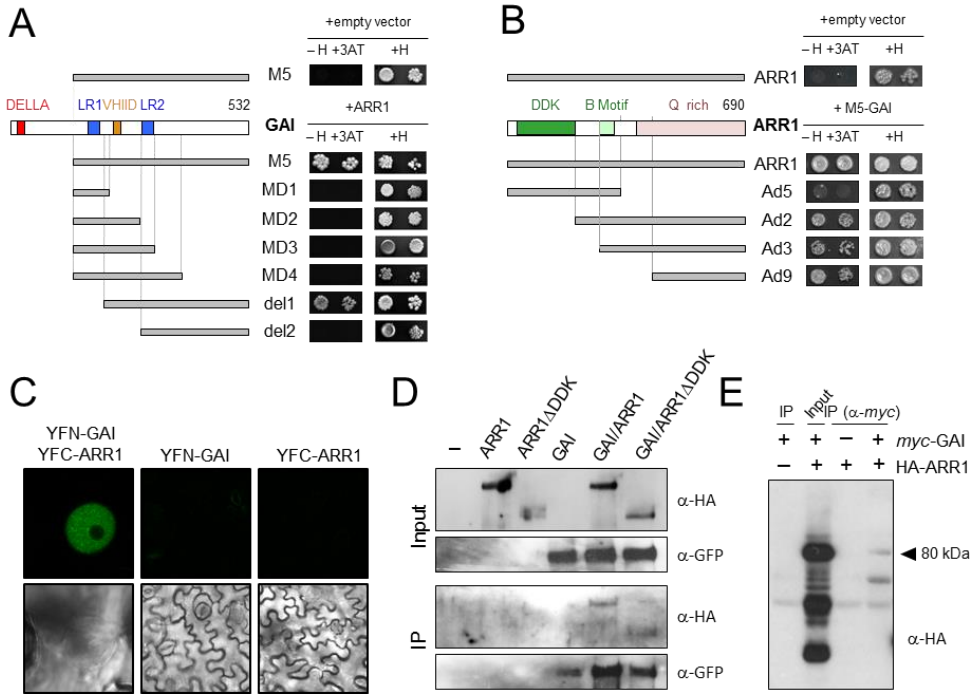
Figure 6.1. ARR1 and GAI interact physically in plants. (A) Yeast 2-hybrid assay of the interaction between ARR1 and truncated versions of GAI. (H, Histidine; 3-AT, 5mM 3-aminotriazol). (B) Yeast 2-hybrid assay of the interaction between M5-GAI and truncated versions of ARR1. (H, Histidine; 3-AT, 5 mM 3-aminotriazol). (C) Bimolecular Fluorescence Complementation assay of the interaction between GAI and ARR1 in agroinfiltrated *N. benthamiana* leaves. (D) Analysis of the interaction between HA-ARR1 or HA-ARR1ΔDDK with GFP:GAI by co-immunoprecipitation (co-IP) with anti-GFP in agroinfiltrated leaves of *N. benthamiana*. (E) Co-IP assay of the interaction between *myc*-GAI and HA-ARR1 in *Arabidopsis* protoplasts.

To confirm that the interaction between GAI and ARR1 also occurred in plant cells, we performed both Bimolecular Fluorescence Complementation (BiFC) and co-immunoprecipitation (co-IP) assays. The BiFC analysis showed that the fluorescence from the reconstituted YFP decorated nuclei of epidermal cells of leaves of *Nicotiana benthamiana* co-infiltrated with YFN-GAI and YFC-ARR1, whereas fluorescence in the control leaves was below the threshold level (Figures 6.1C). It is interesting to note that the fluorescence from the YFP presented a pattern of punctate speckles over a uniform and weak background. This particular pattern might be a consequence of the

interaction, given that either type-B ARRs or DELLAs show a nuclear, uniform distribution (Silverstone et al., 1998; Sakai et al., 2000; Hwang and Sheen, 2001; Lohrmann et al., 2001; Mason et al., 2004).

The co-IP experiments performed in leaves of *N. benthamiana* showed that HA-tagged versions of both full-length ARR1 and a deleted version lacking the DDK domain (ARR1ΔDDK) were pulled down by an anti-GFP antibody when YFP-GAI was co-expressed in the same leaves (Figure 6.1D). Similarly, HA-ARR1 was also pulled down by an anti-*myc* antibody in *Arabidopsis* protoplasts co-transfected with *myc*-GAI (Figure 6.1D). These results demonstrate that the interaction between GAI and ARR1 occurs in plant cells, and that the DDK domain of ARR1 is dispensable for the interaction, as shown by the Y2H assays (Figure 6.1B).

6.2.2 GAI enhances the transactivation ability of ARR1

Given that GAs are responsible for DELLA degradation and they antagonize the effect of CKs, a reasonable hypothesis is that the interaction with DELLAs would promote the activity of the CK-activated type-B ARRs. This idea is supported by the observation that the expression of a reporter construct in *Arabidopsis* roots harbouring GFP under the control of the type-B ARR responsive TCS synthetic promoter (Muller and Sheen, 2008; Chickarmane et al., 2012) was enhanced by an 18-h treatment with 0.5 μM of the CK *trans*-zeatin, but not when the plants had been pretreated with 1 μM GA_4 (Figures 6.2A). Therefore, to test if DELLAs act as transcriptional co-activators of ARR1, we prepared a reporter construct containing the firefly *LUCIFERASE* (*LUC*) gene under the control of the TCS synthetic promoter and assayed its activity by transient expression in leaves of *N. benthamiana*. As previously reported (Sakai et al., 2000; Muller and Sheen, 2008), HA-ARR1 increased the expression of the wild-type, but not a mutated version of the *TCS::LUC* reporter (Figure 6.2B). Remarkably, the expression of *TCS::LUC* resulted significantly higher when YFP-GAI was co-expressed with HA-ARR1 in the same leaves, but not when YFP-GAI was expressed by itself (Figure 6.2B). This result suggests that the transactivation ability of ARR1 is enhanced upon interaction with the DELLA protein.
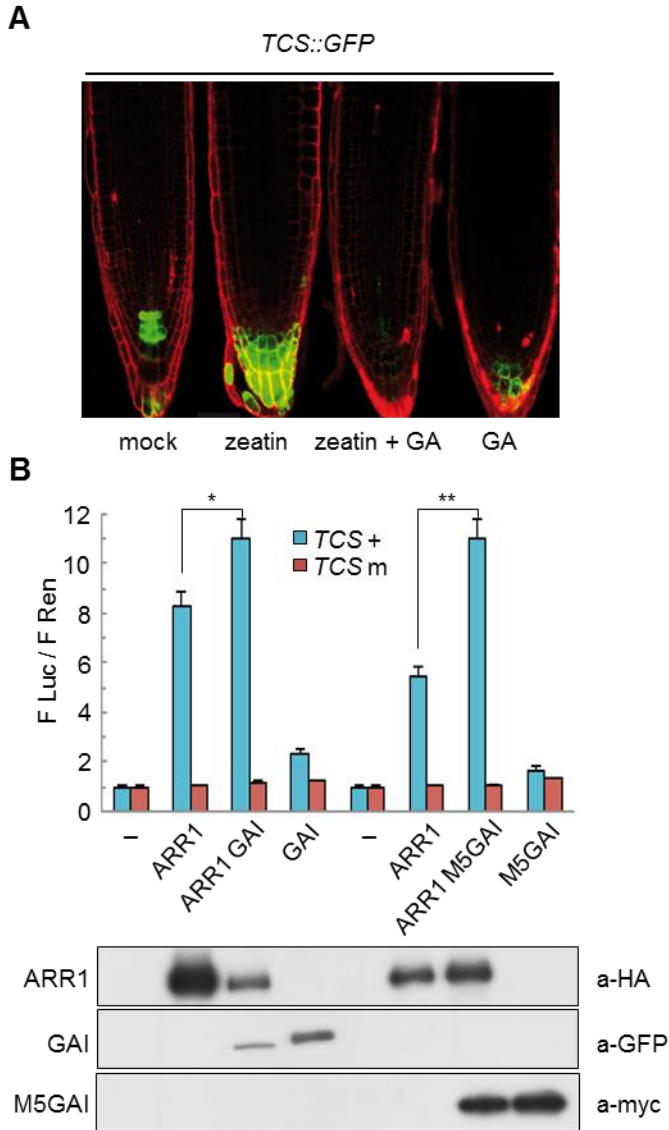
Figure 6.2. DELLAs promote ARR1 activity. (A) Expression in *Arabidopsis* roots of GFP under the control of the CK- and ARR1-responsive TCS element, after treatments with 0.5 µM zeatin and 1 µM GA$_4$. (B) Luciferase assays in *N. benthamiana* leaves agroinfiltrated with HA-ARR1, GFP:GAI, and *myc*-M5-GAI, using the *Luc* gene under the control of the wild-type and mutant versions of the TCS element. The lower panel contains the western-blot analysis of the samples used for the Luciferase assay.

Interestingly, DELLA proteins are endowed with transactivation ability that resides in the N-terminal part of the protein, including the DELLA and TVHYNP motifs

(Ogawa et al., 2000; Hirano et al., 2012). To test whether the enhanced activity of the *TCS:LUC* reporter when YFP-GAI and HA-ARR1 are co-expressed was due to the intrinsic transactivation of the DELLA protein, we decided to co-express a truncated version of GAI, M5-GAI, that lacks its N-terminal part but that still interacts with ARR1 (Figures 6.1A and 6.1B). As seen in Figure 6.2B, the activity of the reporter was enhanced when HA-ARR1 was co-expressed with *myc*-M5-GAI, indicating that the enhanced transactivation is not due to the N-terminal part of the DELLA protein. These results also suggest that either the DELLA protein recruits additional transcriptional co-activators to the complex or other regions of the DELLA acquire transactivation ability upon interaction with ARR1.

### 6.2.3 ARR1 mediates the presence of GAI at target promoters

CKs have been shown to regulate the expression of hundreds of different genes depending on the tissue and the developmental stage (Brenner and Schmulling, 2012). To identify putative targets for coregulation by ARR1 and DELLAs, we chose to perform a microarray analysis on seedlings that conditionally expressed the ARR1ΔDDK:GR allele under the 35S promoter (Sakai et al., 2001) in the presence and in the absence of PAC. In these seedlings, a treatment with dexamethasone (DEX) causes translocation of ARR1ΔDDK to the nucleus, where it regulates the transcription of its target genes even in the absence of CKs. Therefore, we searched for genes displaying differential expression after a 3 hour treatment with 5μM DEX depending on the presence of 10μM PAC (see experimental Procedures for details). In parallel, we also examined transcriptomic changes induced by 5μM benzyladenine (BA) both in the presence and in the absence of PAC, to identify those targets in which regulation by CKs would be primarily dependent on ARR1.

Statistical analysis of the transcriptomic data by *Z*-score (Cheadle et al., 2003) revealed a total of 638 and 1070 genes up- and downregulated by CKs irrespective of DELLA levels. From those, only 99 were upregulated both in high and in low DELLA levels, and included well-known targets of CK signaling, like ARR genes, CK Response Factors, or SHY2/IAA19 (Supporting Table I). More importantly, 140 genes were identified whose expression was induced by ARR1ΔDDK only when DELLA levels

were high, while 99 genes were repressed in those conditions (Figure 6.3A). Gene Ontology analysis of the genes induced by ARR1 in the presence of DELLAs indicates an statistically significant enrichment of several categories, with a preference for ribosome biogenesis, translation, and protein metabolism (Figure 6.3B). Given that ARR1 has been shown to act as a transcriptional activator, we selected six of the induced genes to further test the functional and molecular relationship between ARR1 and DELLAs. First we examined the consequence of short-term activation of ARR1ΔDDK:GR in seedlings that had high or low levels of DELLA proteins. Four of the six genes showed a much stronger induction by ARR1ΔDDK in seedlings with high DELLA levels (Figure 6.3C), in tune with the previous transcriptomic analyses in similar conditions. Then we did the reciprocal test in which we examined the influence of an activated CK pathway on the ability of *gai-1* to induce gene expression using *HS::gai-1* seedlings (Alabadí et al., 2008). In this case, five of the six genes displayed a stronger induction by *gai-1* in seedlings that had been pretreated with 5 μM BA, than in the untreated plants (Figure 6.3D), which supports the idea that type-B ARRs and DELLAs jointly promote transcription of the target genes.

If the coregulation of the target genes by DELLAs and ARR1 is mediated by physical interaction between those two proteins, DELLAs should be present at the target promoters. To test this prediction, we performed Chromatin Immunoprecipitation (ChIP) of the DELLA protein RGA in *RGA:GFP-RGA* seedlings. In fact, the presence of RGA was significantly enriched in the promoters of the six genes tested (Figure 6.3E) and, what is more important, the presence of GFP-RGA at the promoters of three of the six genes tested was much higher in seedlings when ARR1ΔDDK:GR is forced to accumulate in the nucleus with a DEX treatment (Figure 6.3F). The requirement for ARR1 in the binding of GFP-RGA was further supported by the loss of enrichment in the *arr1 arr12* double mutant (data not shown). Altogether, all the results shown here strongly suggest that ARR1 mediates the binding of DELLAs to the target promoters, and together they regulate their expression.
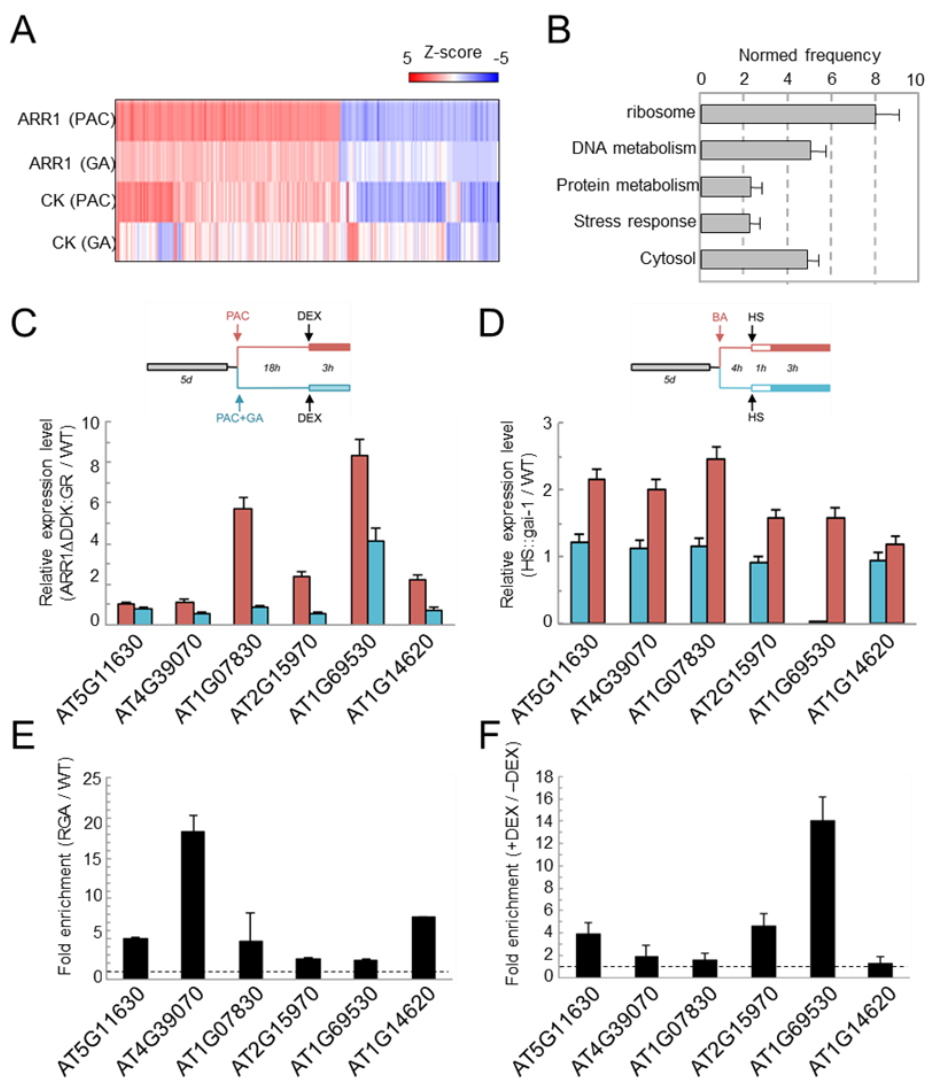
Figure 6.3. ARR1 and DELLA act as transcriptional co-regulators in *Arabidopsis.* (A) Heat map representation of the *Arabidopsis* gene set that is regulated by ARR1ΔDDK:GR in the presence, but not in the absence of DELLA proteins. The colour scale represents Z-scores. (B) Enrichment of Gene Ontology categories of direct ARR1 target genes in the presence of DELLAs(C) Gene expression analysis by RT-qPCR in response to short-term ARR1ΔDDK:GR induction with or without PAC. (D) Gene expression analysis by RT-qPCR in response to short-term induction of HS::gai-1 with or without benzyladenine. (E) ChIP analysis of RGA::GFP-RGA at the promoters of six representative common targets for ARR1 and DELLAs. (F) Fold enrichment of GFP-RGA at selected target promoters in the presence (+DEX) vs the absence (-DEX) of ARR1ΔDDK:GR, in F1 seedlings of a cross between RGA::GFP-RGA and 35S::ARR1ΔDDK:GR. For (C-F), data correspond to single biological samples analyzed in triplicates. A second biological sample showed very similar results.

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6.2.4 DELLA-ARR1 interaction is necessary for proper root meristem maintenance and skotomorphogenesis

Physical interaction between ARR1 and DELLAs provides a likely mechanism for the antagonistic effect of CKs and GAs in the regulation of gene expression, but to test the physiological relevance of this particular mechanism in the control of plant development we decided to test the impact of altering this interaction on two processes known to be regulated both by CKs and GAs. DELLA accumulation has been shown to reduce cell division at the root meristem (Achard et al., 2009) resembling the arrest caused by ARR1 overproduction (Dello Ioio et al., 2007; Dello Ioio et al., 2008). Indeed, it has been shown that ARR1 mediates the reduction of cell division by DELLAs, and the proposed mechanism involves the promotion of *ARR1* gene expression by DELLAs (Moubayidin et al., 2010). Therefore, to test the relevance of the interaction between the ARR1 and DELLA proteins in this context and separate the possible effect on ARR1 expression, we examined the ability of a constitutively expressed version of ARR1 (35S::ARR1ΔDDK:GR) to block root meristem growth depending on the presence of DELLAs. In agreement with previous reports, induction of ARR1ΔDDK:GR translocation into the nucleus after DEX treatment caused a reduction in meristem size (Figure 6.4A). And, more importantly, this effect was completely reverted by a GA treatment that depletes DELLAs from roots (Figure 6.4A), indicating that DELLAs are required for full ARR1 function, rather than for *ARR1* expression.

At a different developmental stage, CKs have been found to promote de-etiolation (Chory et al., 1994), while GAs repress photomorphogenesis in darkness (Alabadi et al., 2004; Achard et al., 2007; Alabadí et al., 2008). Accordingly, nuclear accumulation of ARR1ΔDDK:GR in dark-grown seedlings resulted in cotyledon opening and expansion, a well known photomorphogenic trait, but this effect was milder in GA-treated seedlings (Figure 6.4B), indicating that DELLAs enhance the photomorphogenic activity of ARR1. Conversely, DELLA-induced cotyledon opening in darkness was completely suppressed in the *arr1 arr12* double mutant (Figure

6.4C), supporting the idea that ARRs and DELLAs jointly regulate various physiologically relevant developmental processes.
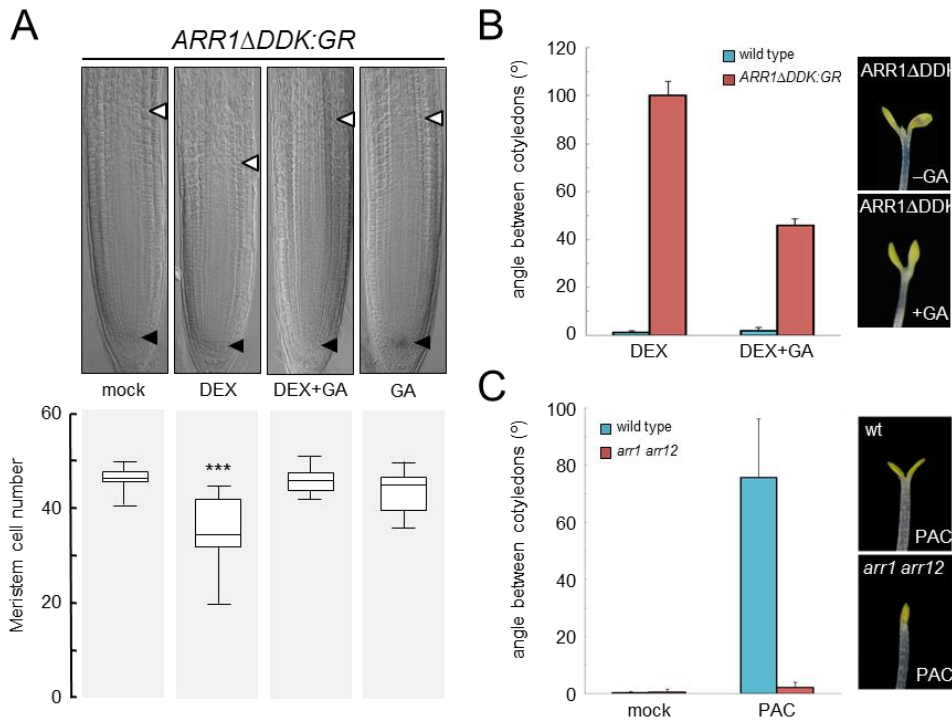
Figure 6.4. **Physical interaction between ARR1 and DELLAs regulates division at the root meristem and photomorphogenesis.** (A) Root meristem size of *35S::ARR1ΔDDK:GR* seedlings grown for 4 days with and without GA. (n=20; data are mean ± SD; ***p<0.001)**. (B) Angle between cotyledons of 35S::ARR1ΔDDK:GR seedlings grown for 4 days with and without GA in darkness. (n=18; data are mean ± SD). (C) Angle between cotyledons of wild-type and *arr1 arr12* seedlings grown for 4 days with and without PAC in darkness. (n=18; data are mean ± SD)

Taken together, our results expand the mechanism by which DELLA proteins regulate transcriptional programs in plants. The observation that DELLAs modulate not only the binding, but also the activity of TFs at target loci, together with the indications that they may also regulate chromatin remodelling through their interaction with SWI/SNF complexes (Sarnowska et al., 2013) delineates a landscape in which DELLA proteins act as molecular hubs in signaling networks, with a very

broad effect on development. Equivalent central roles have been found in other systems only for Mitogen-activated Protein kinases (MAPKs). For instance, mammalian p38 kinases and their yeast ortholog Hog1 modulate gene expression in a very wide sense by regulating the activity of DNA-binding TFs, transcriptional elongation, chromatin remodelling, and mRNA stability in response to environmental stress (de Nadal et al., 2011). However, the activity of DELLA proteins seems to rely on their intrinsic ability to interact with elements of the transcriptional regulation machinery.

Under this perspective, at least two relevant issues would need to be solved: the molecular features of DELLA proteins that allow them to display such a promiscuous set of interactors and activities; and the spatial requirements that may constrain the different DELLA interactions to specific cell-types.

6.3 Material and methods

Plant Material. *Arabidopsis thaliana* accessions Col-0 and L*er* were used as wild type. The transgenic lines *35S::ARR1ΔDDK-GR*, *TCS::GFP, RGA::RGA-GFP*, *HS::gai-1* and the mutant *arr1-3;arr12-1* have been described previously (Sakai et al., 2001; Silverstone et al., 2001; Alabadi et al., 2008; Argyros et al., 2008; Muller and Sheen, 2008).

Experimental Conditions. Seedlings were grown on MS at 22°C in continuous light ($\sim$50 μmol m^{-2} s^{-1}) unless otherwise indicated. For *TCS::GFP* activity 6 days old seedlings growing in MS were transfer to liquid MS containing the chemicals for the indicated times, and at the next concentrations, PAC 1μM, GA$_4$ 1μM and *trans*-zeatin 0.5 μM. For RAM growth 5day old seedlings were treated in liquid MS for 16 hours with the chemicals at the specified concentrations, dexamethasone 30 μM and GA$_4$ 1 μM and then transfer to MS agar for 2 days. For cotyledon opening assays, seedlings were germinated for 8 hours in MS agar and then transfer to darkness in MS with 0.1 μM dexamethasone with or without 1 μM GA$_4$ for 7 days.

Y2H Assays. A GAL4AD fusion library prepared from cDNA f 3-days old de-etiolated seedlings (CD4-22, ABRC) was used to screen for GAI interactors using a GAL4BD-M5-GAI fusion (Gallego-Bartolome et al., 2012), clones were selected with 5mM 3 AT.

Full length and deletions of ARR1 were obtained by PCR amplification using the primers listed in bellow were used to create *pENTR* the fragments were cloned into *pCR8/GW/TOPO* (Invitrogen), then transferred into *pDEST22* (Invitrogen) to create GAL4DNA binding domain fusion. GAI deletions have been described before (Gallego Bartolome *et al.,* 2012). Subsequent cotransformation of the yeast strain AH109 (Clontech) were performed. To select the interaction, clones were grown in SD plates without Leu, Trp and His, and with different concentrations of 3-aminotriazol (3-AT) (Sigma).

	Forward	**Reverse**
ARR1	5´-ATGATGAATCCGAGTCACGGAAGAG-3´	5´-AACCGGAATGTTATCGATGGAGTATG-3´
Ad2	5´-ATGGTTAGGAAGAGGAGAAGTG-3´	5´-AACCGGAATGTTATCGATGGAGTATG-3´
Ad3	5´-ATGAAACCGCGTGTCGTCTGGTC-3´	5´-AACCGGAATGTTATCGATGGAGTATG-3´
Ad5	5´-ATGATGAATCCGAGTCACGGAAGAG-3´	5´-CTGCGATACCCCTCCAAGCC-3´
Ad9	5´-ATGAGAAGTGGTTTCTCTGGAAGG-3´	5´-AACCGGAATGTTATCGATGGAGTATG-3´

Bimolecular Fluorescence Complementation. *pENTR* containing the full length of ARR1 and GAI were transferred into *pMDC43-YFC* and *pMDC43-YFN* vectors (provided by Dr Alejandro Ferrando-IBMCP) respectively, then introduced into *Agrobacterium tumefaciens C58.* For BiFC experiments, 4-week-old *Nicotiana benthamiana* leaves were coinfiltrated with *Agrobacterium tumefaciens C58* as describe before (Sparkes et al., 2006), then after 2 days confocal imaging was performed.

Co-Immunoprecipitation in *Nicothiana benthamiana* *pENTR* of ARR1, M5-GAI and GAI (Gallego-Bartolome et al., 2012) were transfer into *pEarleyGate-201*,

pEarleyGate-203 pEarleyGate-104 respectively (Earley et al., 2006), then *Nicotiana benthamiana* leaves were infiltrated as explain before. Protein extraction and IP was performed as described (Gallego-Bartolome et al., 2012).

Reporter Construct and Transcriptional Assays. The Type-B binding site (TCS) (AAAATCTACAAAATCTTTTTGGATTTTGTGGATTTTCTAGC) upstream of the minimal 35S promoter and the Ω translational enhancer were amplified from the construct in pUC18 vector using the primers 5´-AACTGCAGGTAAAACGACGGCCAGT-3´ and 5´-GGCCATGGTGTAATTGTAAATAGTAATTG-3´containing the restriction sites *PstI* and *NcoI* respectively, this was cloned into *pGreenII 0800-LUC* vector (Hellens et al., 2005). To obtain the mutated version of the reported (TCS*) (AA<u>AAT</u>G<u>T</u>ACAA<u>AAT</u>G<u>TTTTT</u>G<u>C</u>ATT<u>TTGT</u>G<u>C</u>ATT<u>T</u>TCTAGC) and overlapping PCR were performed to fusion the Ω translational enhancer and the TCS* with the minimal 35S promoter. The Ω translational enhancer was amplified by PCR from TCSpUC18 (provided by Bruno Müller) using the primers 5´-ATTTCATTTGGAGAGGTATTTTTACAAC-3´ and 5´-GGCCATGGTGTAATTGTAAATAGTAATTG-3´, meanwhile the TCS*m35S was amplified with the primers 5´-AACTGCAGGTAAAACGACGGCCAGT-3´ and 5´-CCTCTCCAAATGAAATGAACTTCCTTAT-3´ the fusion was cloned into *pCR8/GW/TOPO* (Invitrogen) and then into *pGreenII 0800-LUC* vector. **For Transcriptional Assays.** Leaves of 4 weeks-old *Nicotiana benthamina* were infiltrated with *Agrobacterium tumefaciens C58* cells carrying the constructs, the ratio of Agrobacterium-carrying reporter and effector constructs was 1:4. Firefly and the control Renilla LUC activities were assayed from leaf extracts with the Dual-Glo Luciferase Assay System (Promega) and quantified with a GloMax 96 Microplate Luminometer (Promega).To verify that protein amounts were equal, Western Blot analysis were performed with proteins extracted from the same experiment, the ARR1, GAI and M5-GAI fusions were detected with anti-HA (3F10; Roche), anti-GFP (ab290; Abcam), and anti-c-*myc* (9E10; Roche) antibodies respectively.

Microarray Analysis. Seedlings were grown under continues light (50 µmolm^{-2}S^{-1}) for 5 days before treatments. RNA was extracted with RNeasy Plant Mini

kit (Qiagen). Two biological replicates were labeling and hybridization to Affymetrix ATH1 arrays. Analysis was performed in R (Duek et al., 2004) and Bioconductor (Reimers and Carey 2006). Microarrays were normalized with the RMA procedure as implemented in the affy package (Gautier et al., 2004), and differential expression was determined using limma (Gentleman et al.,2005) with a Z score (Cheadle et el.,2003).

Gene expression. For gene expression analysis, total RNA was extracted with E.Z.N.A. Plant RNA Mini Kit (Omega Bio-tek) according to the manufacturer´s instructions. cDNA synthesis was performed with SuperScript II First-Strand Synthesis System (Invitrogen).And RT-PCR was performed as describe before, the *EF1-α* gene was used for normalized (Frigerio et al., 2006). Primers used to amplify transcripts were:

	Forward	Reverse
AT5G11630	5´-TCCCAAATCGGCTTCATCAT-3´	5´-GAAAATCCAGGCGAAGGAGG-3´
AT4G39070	5´-TTCCTCCTTACCGGCGTTAA-3´	5´-TCTTGGGTAGGCTGACGGG-3´
AT1G07830	5´-CTTCAGAGCTGCGTCTCAAGTC-3´	5´-CGTACCACAGCTTCTGAAGATCAT-3´
AT2G15970	5´-CTTCACACTCACTGGTTTAGGCTTT-3´	5´-GCAACCCATTCGAGGACAGA-3´
AT1G69530	5´-AAGCGATGGCCAAACCATT-3´	5´-GACCAGCCTGCGTTAGCAAC-3´
AT1G14620	5´-ATGCCGAGATCATCTTTGCG-3´	5´-CCTGCTCTCCAAGAGAGGCTT-3´

Chromatin immunoprecipitation 10 days seedling of L*er* and *RGA::RGA-GFP* growing at 22°C in continues light ($\sim$50 µmol m^{-2} s^{-1}) were treated with PAC 10µM for 18 hours after this, benzyladenine was added to a final concentration of 5 µM for 6 hours, a mock treatment was used as control. ChIP was performed as described previously (Saleh et al 2008), for immunoprecipitation Dynabeads Protein A (Invitrogen) and an anti-GFP (hemagglutinin) polyclonal antibody (ab290; Abcam) was used. Relative enrichment was calculated by normalizing the amount of target DNA, first to the internal control gene *HSF* (At4g17740) and then to the corresponding amount in the input. The same was done with 35S::ARR1ΔDDK:GR-

RGA::RGA-GFP F1 crosses. Data are mean and SD of three technical replicates from a representative experiment from two biological replicates.

	Forward	Reverse
AT5G11630	5´-AGACGTCGTGTGGTTTTTGG-3´	5´-AACGACGACGTTATCAAACAAA-3´
AT4G39070	5´-CCCGGTATGCTTTTGTTTAAG-3´	5´-CCCCCAAATTGACTTGTTTT-3´
AT1G07830	5´-TGCGTTGATCCGTGTGATTT-3´	5´-CATTGGATCAGTAATCAACGGTTG-3´
AT1G69530	5´-ACAACAGATTCTCATAATCATCTC-3´	5´-AGATCACATTTTGTGAAGCTAAA-3´
AT1G14620	5´-GAATGCATAGCAAACCGGAT-3´	5´-ATTGGTTTACATAACCAGAATCCG-3´
HSF	5´-GCTATCCACAGGTTAGATAAAGGA-3´	5´-GAGAAAGATTGTGTGAGAATGAAA-3´
UBC30	5´-CAAATCCAAAACCCTAGAAACCGAA-3´	5´-AACGACGAAGATCAAGAACTGGGAA-3´

6.4 Supporting Table I

https://www.dropbox.com/s/5rtcdnmbwxft111/GO%20DELLA%2BARR1.xlsx

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General Discussion **7**

7. General discussion

As outlined in the Introduction of this Thesis, a characteristic feature of GA action is the participation of this hormone in the regulation of a surprisingly large array of processes all along plant development, triggering different responses in an organ- and tissue-specific manner. Therefore, to understand the role of GAs in a plant's life, at least three outstanding questions need to be solved at the molecular level:

- How can a single hormone exert so many different actions in the same and in different cells? Or, expressed in molecular terms, how can a single molecule activate different transcriptional programs in different situations?

- How do GAs interact with other hormones to regulate gene expression? Through which molecular mechanism?

- How is GA activity regulated by environmental and other endogenous signals that modulate plant development?

The work presented in this Thesis, together with the important contributions made by other laboratories during the past few years, defines a molecular mechanism in which DELLA proteins act as **hubs** of a signaling network that modulates plant development in response to the environment. And, more importantly, this molecular mechanism provides a likely answer to the three questions stated above.

One of the key findings that explain the molecular activity of DELLA proteins is the observation that they are able to interact with an unexpectedly high number of DNA-binding TFs. Although most of the interactions found in this work have not been confirmed with techniques different to the yeast-two hybrid approach, the fact that many of them have been reported as relevant in different contexts in vivo (Supporting Table III and Figure 7.1) suggests that the final number of bona fide interactors will be high in any case.

The simplicity of a molecular mechanism based on the interaction between DELLAs and multiple TFs should not distract from the strong regulatory potential of such a mechanism. In fact, it may explain how DELLAs regulate different sets of genes in different contexts: by interacting with a different TF in each case. And it also

explains at the molecular level several physiological observations that connected GAs with many other hormones. For instance, we have shown here that DELLA-ARR1 interaction would underlie the antagonistic relationship between GAs and CKs, while DELLA-RAP2.3 interaction would be partly responsible for the crosstalk between GAs and ethylene, which also occurs through the interaction between DELLAs and EIN3 (An et al.,2012).
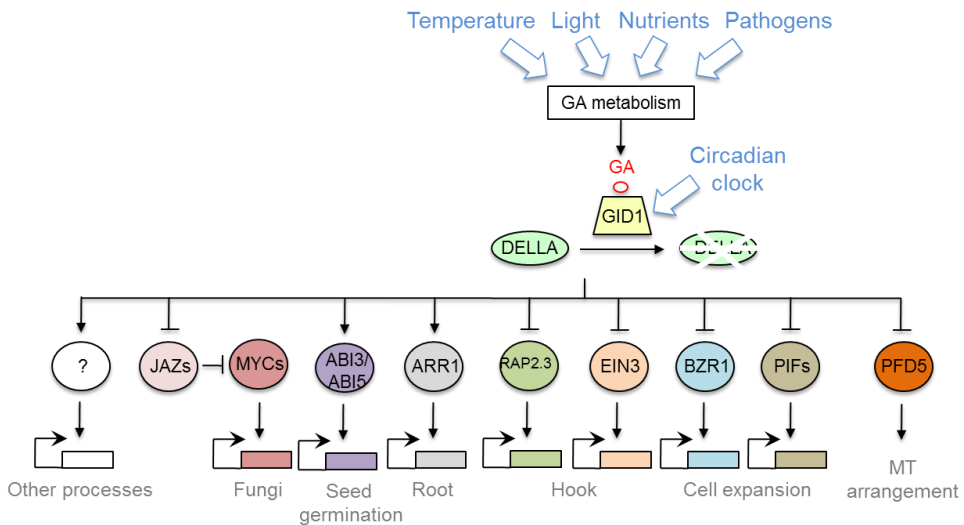
Figure 7.1 Mechanism of DELLA action to control development. DELLAs interact with diverse transcription factors to regulate transcription. However DELLAs also interact with no transcriptional means. For instance control microtubules disposition by the interaction with the chaperone Prefoldin 5 (PFD5) (Locascio et al., 2013).

The extraordinary capacity of DELLAs to interact with other proteins is an unusual feature for the average protein, and thus they can be considered as "hubs" in a transcriptional network. Interestingly, the GAI-interactome network is mostly maintained even in the absence of GAI (Figure 7.2), which is a behavior shared with the network of the predicted *Arabidopsis* interactome when major and super hubs are removed (Geisler-Lee et al., 2007), and that indicates that interactions between minor hubs also maintain the network together. This means that several proteins in the GAI interactome participate in different complexes that are likely shared by different signaling cascades, as described for the yeast interactome (Batada et al., 2006).
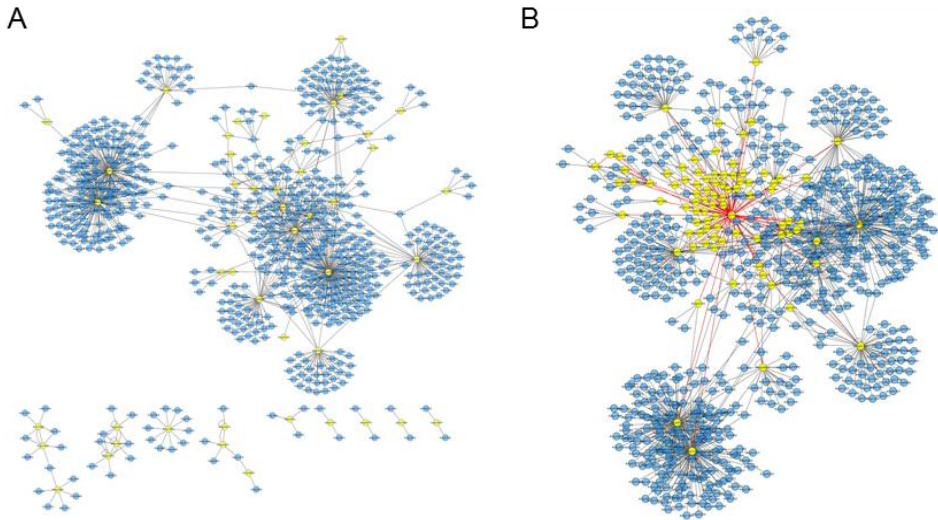
Figure 7.2 (A) Cytoscape representation of network formed by DELLA interactors (yellow), without DELLAs. (B) Network formed by DELLA interactors (yellow), including DELLAs (red edges). Blue nodes represent reported proteins that interact with each transcription factor.

Thanks to the study of signaling networks in different organisms, functional hubs have been attributed a series of properties (Gunasekaran et al., 2003), some of which can indeed be found in DELLAs. For instance, yeast hub proteins show a high importance in the control of regulatory networks. Accordingly, deletion of a hub protein is lethal in some cases, or at least causes an extremely pleiotropic phenotype (Fraser et al., 2002). The elimination of DELLA genes in different species has not been found to be lethal in any case, but the knockout mutants are strongly incapable of producing a correct response under stress. Plants have developed a defense mechanism that coordinates a general growth arrest when subjected to life threats like higher salinity or cold temperatures, while the appropriate defense response is triggered; however the *della*KO mutants continue growing under stress conditions and reduce their chance to survive (Achard et al.,2006).

A second feature of hub proteins is that they usually display structural characteristics that allow the physical recognition of many partners which do not share common structural domains between them. It has been proposed that some hub proteins lack a fixed tertiary structure and contain extensive intrinsically disordered regions (IDR), which serve as the basis for promiscuity and provide them with extraordinary flexibility. Bioinformatic analyses indicate that approximately 23% of *Arabidopsis* proteins are mostly disordered (Oldfield et al., 2005). In fact, the N-terminal part of DELLAs is intrinsically disordered, and its structure undergoes a conformational change upon binding to the GA- receptor GID1 (Sun et al., 2010; Sun et al., 2011). However, the region in DELLAs that interacts with their partners is the central and C-terminal domains, in which no IDR index is sufficiently high (Figure 7.3). How can DELLAs then recognize so many structurally different partners? At least two complementary possibilities can be taken into consideration. The first one is that the small "linkers" that connect the structured domains (indicated as * in Figure 7.3A) could probably favor globular domains to twist freely and recruit binding partners. In agreement with this, these regions are less structured (Figure 7.3A). In some cases these linkers could be as short as a few amino acids (Reddy Chichili et al., 2012).

The second possibility is that DELLAs act as structured hubs, as exemplified by 14-3-3 hub proteins, in which case the partners are the ones that contain IDRs (Oldfield et al., 2008). Taking into account the study presented by Liu et al., 2002 transcription factors usually contain extended disorder regions, we are able to observe a high propensity to contain IDRs (Figure 7.3C). However not all DELLA partners contain IDRs (Figure 7.3B). Thus, experimental structural analysis for several DELLAs-partner complexes will be necessary to answer solve this issue.
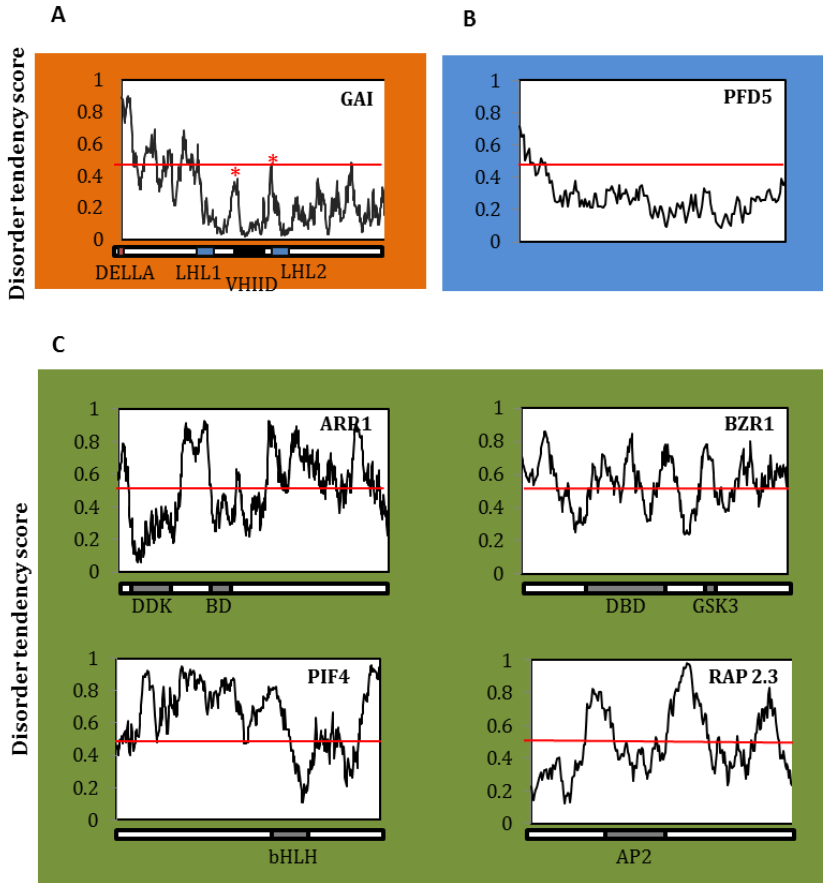
Figure 7.3 IDRs of GAI and some DELLAs partners. We use IUPRED to predict IDRs, scores above 0.5 indicates disorder. Protein domains are indicated above each plot.

A question that still remains to be solved is the biological advantage that can be conferred by DELLAs as hubs. One possibility is that when the interaction with several partners is possible within a single cell, DELLAs will establish differential interactions depending on the respective affinities. In this model, any alteration of DELLA levels will be not be translated equally into different transcriptional programs,

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providing an extra level of plasticity to transcriptonal programs governed by DELLAs. Today, techniques like FLIM (Fluorescence-Lifetime Imaging Microscopy) could help us to better understand the dynamics of DELLA proteins interactions within living cells (Truong and Ikura, 2001).

In any case, it is already clear that the activity of DELLAs is dependent on a large array of signals and temporal and spatial constrains. The major input pathway of environmental information has been shown to eventually modify GA levels (Yamaguchi et al., 1998; Oh et al., 2006; Seo et al., 2006; Yamauchi et al., 2007), which in turn will determine DELLA levels. In a different layer of regulation, the expression patterns of *GID1* and *DELLA* genes (Griffiths et al., 2006; Gallego-Bartolome et al., 2010) also determine the extent to which each tissue will respond to the environment, and GA transport over the phloem can also establish big differences in DELLA accumulation in different organs. Finally, the activity of the circadian clock adds a temporal control to GA sensitivity, altering DELLA levels in a rhythmic fashion (Chapter 1). This is why DELLAs can be defined as hubs not only from a topological point of view within an interactome network, but also as a functional hub that relays environmental signals to already pre-established transcriptional networks (Figure 7.1).

The view that emerges with the results presented here makes it more difficult to consider GA signaling as a single classical pathway with GA-specific transcriptional programs. On the other hand, it provides a very clear strategy for the biotechnological manipulation of agronomically important traits, without the side effects characteristic of GA or paclobutrazol applications. Such a strategy consists in the selection of edgetic DELLA alleles that disrupt one or only a few of the interactions, without affecting the rest of them (Figure 7.4).

Selection of such edgetic alleles using molecular approaches (Dreze et al., 2009), or screening for compounds that preferentially affect one of the interactions may represent a powerful agronomical tool in the next future.
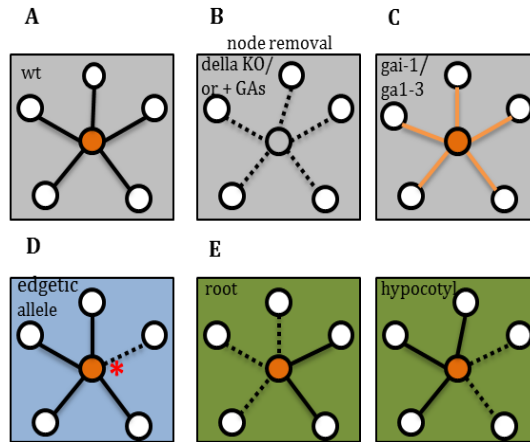
Figure 7.4 DELLAs network model. (A) Schematic representation on the network on a wild type. (B) Node removal on a *quintuple della* or by the degradation of DELLAs by GAs treatment. (C) Increase activity of DELLAs. (D) Representation of edgetic alleles and (E) contribution in different tissues.

7.2. References

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Conclusions **8**

8. Conclusions

The main conclusion of this Thesis is that **DELLA proteins act as genuine hubs that integrate environmental information and control transcription through the interaction with a large array of transcription factors**. In more detail:

1. The level of DELLA proteins is temporally controlled by the circadian clock through the circadian oscillation of the expression of the genes encoding the GA receptors. This mechanism helps restrict the growth period to the end of the night.

2. DELLA proteins interact with DNA-binding transcription factors of very different structural families. In some cases, such as RAP2.3 and other partners reported in the literature, interaction with DELLAs prevents the binding of the transcription factor to the target promoters. A novelty in this work is that in several other cases DELLAs are recruited to the target loci. In the case of ARR1, for instance, DELLAs act as transcriptional co-activators.