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EVOLUTION OF DELLA PROTEINS AS TRANSCRIPTIONAL HUBS IN PLANTS

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El **Dr. Miguel Ángel Blázquez Rodríguez**, Profesor de Investigación del CSIC, perteneciente al Instituto de Biología Molecular y Celular de Plantas (IBMCP, UPV-CSIC) de Valencia, CERTIFICA que **Asier Briones Moreno** ha realizado bajo su dirección en el Instituto de Biología Molecular y Celular de Plantas, el trabajo titulado «**Evolution of DELLA proteins as transcriptional hubs in plants**», y que autoriza su presentación para optar al grado de Doctor en Biotecnología de la Universitat Politècnica de València.

Y para que así conste, firma el presente certificado en Valencia a 27 de octubre de 2020.

Dr. Miguel Ángel Blázquez Rodríguez

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A principios de 2015, a mitad de un máster y sin saber muy bien qué pasaría después, decidí solicitar una beca FPU con un grupo que no conocía, pero del cual me habían hablado muy bien y había visto muchas publicaciones. Probé suerte, y como me pasa a menudo, la suerte me sonrió. Si no fuera por esa decisión, en parte precipitada por los plazos del Ministerio, hoy no estaría aquí. Y me alegro mucho de que haya sido así. Han sido unos años muy buenos, trabajando en un proyecto muy bonito, con un grupo genial, en el seno de un instituto y una universidad a los que tengo mucho cariño. No cambiaría nada de este tiempo, y se lo agradezco a muchas personas.

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Summary

DELLA proteins are central elements of the gibberellin (GA) signaling pathway, where they act as repressors of GA responses. In angiosperms, DELLAs have been shown to interact with hundreds of transcription factors and other transcriptional regulators, thereby modulating gene expression. Hence, the widespread involvement of GAs along the plant life cycle is a direct consequence of the promiscuity of DELLA proteins and their role as key transcriptional regulators. Although DELLAs can be found in all land plants, they are only regulated by GAs in tracheophytes, where most of the previous studies have been focused. The work presented here aims to decipher at which point in evolution did DELLAs acquired the molecular features that render them as 'hubs', and what biological advantages could be related with DELLA evolution. In the first chapter, we describe comparative analyses of DELLA-associated gene co-expression networks in vascular and non-vascular species and propose that DELLAs have a critical role in the conformation of transcriptional landscapes. Upon their emergence in the ancestor of land plants, they connected multiple transcriptional programs that would be independent without them, improved the efficiency of information transmission and increased the level of complexity in transcriptional regulation. We also observed that this effect was enhanced after their integration in GA signaling. In the second chapter, we provide stronger experimental evidence that extends this conclusion. Using a combination of targeted yeast two-hybrid screenings with DELLAs from different positions in the plant lineage, and heterologous complementation in Arabidopsis and Marchantia plants, we show that promiscuity is a conserved feature in all the examined DELLA proteins, which suggests that this property might have been encoded in the ancestral DELLA, and then maintained along evolution, with episodes of co-evolution between DELLAs and their partners. Finally, comparison of DELLA transcriptional targets in different species shows a striking conservation of a small set of functions regulated by DELLAs in vascular and non-vascular

plants –including the response to stress factors–, while comparative promoter analysis indicates that species-specific DELLA targets emerge through at least two mechanisms: establishment of novel DELLA interactions, and the access by conserved partners to new target promoters.

In summary, we propose that DELLAs are intrinsically promiscuous proteins, with hub properties in virtually all land plants, and the conservation of their transcriptional targets largely depends on the evolution of their interactors. The conservation of the hub properties of DELLA proteins makes them ideal biotechnological targets, as most of the knowledge generated in one species could be readily adapted to other relatively distant species.

Resumen

Las proteínas DELLA son elementos centrales de la ruta de señalización por giberelinas (GAs), donde actúan como represores de las respuestas a GAs. En angiospermas, se ha observado que las DELLAs interaccionan con cientos de factores de transcripción y otros reguladores transcripcionales, modulando de este modo la expresión génica. Por lo tanto, la participación generalizada de las GAs a lo largo del ciclo vital de las plantas es una consecuencia directa de la promiscuidad de las proteínas DELLA y de su rol como reguladores transcripcionales clave. Aunque las DELLAs se encuentran en todas las plantas terrestres, solo son reguladas por GAs en traqueofitas, en las cuales se han centrado la mayoría de los estudios previos. El trabajo aquí presentado pretende descifrar en qué punto de la evolución las DELLAs adquirieron las características moleculares que las convierten en “hubs”, y qué ventajas biológicas podrían estar relacionadas con la evolución de las DELLAs. En el primer capítulo, describimos análisis comparativos de redes de co-expresión génicas asociadas a DELLA en especies vasculares y no vasculares, y proponemos que las DELLAs tienen un papel crítico en la conformación de panoramas transcripcionales. Desde su aparición en el ancestro de las plantas terrestres, conectaron múltiples programas transcripcionales que serían independientes sin ellas, mejoraron la eficiencia de la transmisión de información y aumentaron el nivel de complejidad en la regulación transcripcional. También observamos que este efecto se incrementó tras su integración en la señalización por GAs. En el segundo capítulo, proporcionamos pruebas experimentales más sólidas que extienden esta conclusión. Usando una combinación de rastreos de doble híbrido en levadura dirigidos, con DELLAs de diferentes posiciones en el linaje vegetal, y complementación heteróloga en plantas de Arabidopsis y Marchantia, mostramos que la promiscuidad es una característica conservada en todas las proteínas DELLA examinadas; lo cual sugiere que esta propiedad puede haber estado codificada en la

DELLA ancestral, y después se mantuvo a lo largo de la evolución, con episodios de co-evolución entre las DELLAs y sus interactores. Finalmente, la comparación de dianas transcripcionales de las DELLAs en diferentes especies muestra la llamativa conservación de un pequeño conjunto de funciones reguladas por DELLAs en plantas vasculares y no vasculares -incluyendo la respuesta a factores de estrés-, mientras que análisis comparativos de promotores indican que las dianas específicas de cada especie aparecen mediante al menos dos mecanismos: el establecimiento de nuevas interacciones de la DELLA, y el acceso a nuevos promotores diana a través de interactores conservados.

En resumen, proponemos que las DELLAs son proteínas intrínsecamente promiscuas, con propiedades de “hub” en virtualmente todas las plantas, y la conservación de sus dianas transcripcionales depende en gran medida de la evolución de sus interactores. La conservación de las propiedades de “hub” de las proteínas DELLA las convierte en dianas biotecnológicas ideales, ya que la mayoría del conocimiento generado en una especie podría ser fácilmente adaptado a otras especies relativamente lejanas.

Resum

Les proteïnes DELLA són elements centrals de la ruta de senyalització per gibberel·lines (GAs), on actuen com a repressors de les respostes a GAs. En angiospermes, s'ha observat que les DELLAs interaccionen amb centenars de factors de transcripció i altres reguladors transcripcionals, modulant d'aquesta manera l'expressió gènica. Per tant, la participació generalitzada de les GAs al llarg del cicle vital de les plantes és una conseqüència directa de la promiscuïtat de les proteïnes DELLA i del seu rol com a reguladors transcripcionals clau. Tot i que les DELLAs es troben en totes les plantes terrestres, només són regulades per GAs en traqueofites, en les quals s'han centrat la majoria dels estudis anteriors. El treball ací presentat pretén desxifrar en quin punt de l'evolució les DELLAs van adquirir les característiques moleculars que les converteixen en "hubs", i quins avantatges biològics podrien estar relacionats amb l'evolució de les DELLAs. En el primer capítol, descrivim anàlisis comparatius de xarxes de co-expressió gèniques associades a DELLA en espècies vasculars i no vasculars, i proposem que les DELLAs tenen un paper crític en la conformació de panorames transcripcionals. Des de la seua aparició en l'ancestre de les plantes terrestres, van connectar múltiples programes transcripcionals que serien independents sense elles, van millorar l'eficiència de la transmissió d'informació i augmentar el nivell de complexitat en la regulació transcripcional. També observem que aquest efecte es va incrementar després de la seua integració en la senyalització per GAs. En el segon capítol, proporcionem proves experimentals més sòlides que estenen aquesta conclusió. Usant una combinació de rastrejos de doble híbrid en rent dirigits, amb DELLAs de diferents posicions en el llinatge vegetal, i complementació heteròloga en plantes d'Arabidopsis i Marchantia, vam mostrar que la promiscuïtat és una característica conservada en totes les proteïnes DELLA examinades; la qual cosa suggereix que aquesta propietat pot haver estat codificada en la DELLA

ancestral, i després es va mantenir al llarg de l'evolució, amb episodis de co-evolució entre les DELLAs i els seus interactors. Finalment, la comparació de dianes transcripcionals de les DELLAs en diferents espècies mostra la cridanera conservació d'un petit conjunt de funcions regulades per DELLAs en plantes vasculars i no vasculars -incloent la resposta a factors de estrès-, mentre que anàlisis comparatius de promotors indiquen que les dianes específiques de cada espècie apareixen mitjançant al menys dos mecanismes: l'establiment de noves interaccions de la DELLA, i l'accés a nous promotors diana a través d'interactors conservats.

En resum, proposem que les DELLAs són proteïnes intrínsecament promíscues, amb propietats de "hub" en virtualment totes les plantes, i la conservació de les seues dianes transcripcionals depèn en gran mesura de l'evolució dels seus interactors. La conservació de les propietats de "hub" de les proteïnes DELLA les converteix en dianes biotecnològiques ideals, ja que la majoria del coneixement generat en una espècie podria ser fàcilment adaptat a altres espècies relativament llunyanes.

Contents

Introduction 1
1. Introduction to the phytohormones gibberellins 3
2. Gibberellin signaling: GA-dependent degradation of DELLA proteins 4
3. Molecular mechanisms of DELLA-mediated transcriptional regulation 7
4. DELLAs as transcriptional hubs 9
5. Evolution of GA signaling: GA perception and DELLA degradation across land plants 12
6. Evolution of DELLA proteins 15
7. References 17
Objectives 31
Chapter 1 35
0. Abstract 37
1. Introduction 38
2. Results and discussion 41
Construction of networks and subnetworks 41
DELLA-associated subnetworks reflect increased relevance of DELLAs after being recruited by GA signaling 44
Efficiency of transcriptional regulation is a DELLA-associated parameter 46
The regulation of the stress response: a likely role of ancestral DELLA proteins 48
3. Conclusion 50
4. Materials and methods 51
Gene co-expression network inference 51
Data compilation and processing 52
Network analysis and visualization 53
Acknowledgments 53
5. Supplementary material 53
6. References 54

Chapter 2 61
0. Abstract 63
1. Introduction 63
2. Results and discussion 64
DELLA promiscuity is a conserved trait 64
DELLA promiscuity is modulated in a taxon-specific manner 67
DELLA function evolution reflects the life history traits of the interacting partners 72
3. Conclusion 76
4. Materials and Methods 76
DELLA interactome studies 76
Determination of 3D structure and conservation of GRAS domains 78
Heterologous complementation tests in *A. thaliana* 79
Heterologous complementation tests in *M. polymorpha* 83
Comparison of DELLA-regulated genes in different species 84
5. Supplementary material 87
6. References 99
General Discussion 105
1. DELLA promiscuity is a conserved ancestral property 107
2. Functional innovation in DELLAs is linked to the evolution of TFs and their targets 108
3. Conservation of core functions highlights a role for DELLA in stress responses 110
4. Biotechnological implications of DELLA evolutionary studies 112
5. References 113
Conclusions 115

Introduction

1. Introduction to the phytohormones gibberellins

As sessile organisms, it is especially difficult for plants to survive and prosper under adverse conditions. They need to respond to a constantly changing environment that poses challenges like predators, diseases and the scarcity of water and nutrients, without the possibility of moving to a different location. Despite that, they can successfully adapt thanks to a high level of plasticity, conferred to a large extent by complex signaling networks that they have acquired along evolution (Casal, 2004). A central element of these networks are phytohormones, biochemical signals involved in the regulation of plant growth, development, and response to stress. One of the most studied and long-known hormone types are gibberellins (GAs), a group of diterpenoid compounds found in vascular plants, as well as in fungi and bacteria (Salazar-Cerezo et al., 2018). GAs are especially relevant for adaptation, as their synthesis is tightly related to external stimuli, and they affect multiple processes throughout the whole plant life (Hedden and Thomas, 2012). In tracheophytes, there are several bioactive GAs, derived from *ent*-kaurene and produced in several cellular compartments, mostly by sequential oxidations (reviewed by Hedden, 2016). Multiple functions have been described for them since they were first isolated in the 1950s (Takahashi et al., 1955; West and Phinney, 1959); most of them in *Arabidopsis thaliana* and rice (*Oryza sativa*), but also in several other species. Even though they are mainly known as promoters of plant growth, because they stimulate the cell expansion and proliferation that supports root, hypocotyl and stem elongation (Ubeda-Tomás et al., 2008; Achard et al., 2009), GAs modulate multiple other developmental processes, from seed germination (Ogawa et al., 2003; Tyler et al., 2004) to flowering (Porri et al., 2012) or fruit set (Hu et al., 2008; Fuentes et al., 2012). They participate in all developmental stages and the transitions between them, as well as in the response mechanisms to various external stimuli like temperature (Yamauchi et al., 2004; Camut et al., 2019), light (de Lucas et

al., 2008; Feng et al., 2008), or even physical contact (Lange and Lange, 2015). Furthermore, GAs have an important role in the defense against biotic (Navarro et al., 2008; Wild et al., 2012) and abiotic stress (Achard, 2006; Achard et al., 2008a, 2008b). Many of these GA functions have been observed in some gymnosperms (Ross et al., 1984; Li et al., 2020b), and in numerous angiosperms –both dicots like tomato and poplar (Rappaport, 1957; Groot and Karssen, 1987; Zawaski et al., 2011), and monocots like rice and wheat (Suge and Yamada, 1965; Ayano et al., 2014)–, while some others are specific to certain plant clades. For example, GAs seem to control sex determination in ferns (Tanaka et al., 2014), nodule establishment in legumes (Fonouni-Farde et al., 2016) or fleshy fruit formation (Serrani et al., 2007; Mesejo et al., 2016). All the vast evidence collected for decades has proven the role of GA signaling as a central regulatory element in vascular plants.

2. Gibberellin signaling: GA-dependent degradation of DELLA proteins

GA signaling pathway depends mostly on the degradation or accumulation of DELLA proteins, growth repressors that antagonize GA responses. When levels of bioactive GAs are high, they promote DELLA degradation, releasing GA responses. Conversely, when bioactive GA levels are low, DELLAs accumulate and restrain growth (Fu et al., 2002; Sun, 2010). The mechanism for DELLA degradation (reviewed in Sun, 2011; Davière and Achard, 2013) has been elucidated mainly in *Arabidopsis* and rice, and the core elements of the general model are the GA receptor GIBERELLIN INSENSITIVE DWARF1 (GID1), the DELLA proteins, and the F-box protein SLEEPY1 (SLY1)/GA INSENSITIVE DWARF2 (GID2). GID1 is a soluble receptor present in the cytoplasm and the nucleus, composed of a N-terminal extension with a flexible structure, and a C-terminal domain with a GA-binding pocket. When bioactive GAs bind the receptor, an allosteric

change is induced, and the N-terminal domain folds like a lid over the binding pocket. This conformational change allows the interaction between the N-terminal extension of GID1 and the N-terminal domain of DELLA proteins (Figure 1A). DELLAs are soluble nuclear proteins that belong to the GRAS family and, like the other members, they possess a C-terminal GRAS domain that confers them the ability to interact with other proteins and regulate gene expression. However, unlike other GRAS proteins, their N-terminal domain contains the conserved motifs DELLA (for the amino acid sequence Asp-Glu-Leu-Leu-Ala), LEQLE and TVHYNP (Figure 1C), all of which directly interact with GID1. This physical interaction between GA-GID1 and DELLA promotes the recognition of the DELLA C-terminal by a F-box protein (SLY1 and SNE in *Arabidopsis*, and GID2 in rice) of the SCF ubiquitin E3 ligase complex. Once the whole $SCF^{SLY1/GID2}$ complex is recruited, it catalyzes the polyubiquitination of DELLAs, thus marked for degradation in the 26S proteasome (Figure 1B).

Despite being the main determinant, variation in GA levels is not the only element affecting DELLA stability. Like other hormonal pathways, GA signaling needs to be precisely modulated in order to trigger an adequate response; so other mechanisms participate, such as the transcriptional regulation of *GID1*. There is a direct relation between the circadian clock and GA signaling, as *GID1* genes are expressed at higher levels during the night, and repressed during the day; thus creating daily changes in GA sensitivity that depend on light (Arana et al., 2011). Additionally, DELLAs can be stabilized by physical interaction with GIGANTEA (GI), a regulator of the plant circadian clock that reduces DELLA degradation during the day by blocking their interaction with GID1 (Nohales and Kay, 2019).

Apart from GA concentrations and *GID1* expression levels, DELLA accumulation can also affect its own stability, as it has been shown that high levels of DELLAs lead to enhanced expression of genes involved in GA biosynthesis (Zentella et al., 2007; Fukazawa et al., 2017). Thereupon,

DELLAs are part of a feedback regulatory system, in which they promote their own degradation through an increase in GA levels.

DELLA stability seems to be regulated as well by certain post-translational modifications (PTMs) (reviewed by Blanco-Touriñán et al., 2020b). Several studies indicate that DELLA phosphorylation makes them resistant to proteolysis, while dephosphorylation allows their degradation (Wang et al., 2009; Dai and Xue, 2010; Qin et al., 2014). At the same time, SUMOylation of DELLAs seems to increase their stability through a GA-independent interaction between SUMO and GID1 (Conti et al., 2014). This interaction cannot completely prevent DELLA-degradation, but it entails a reduction of free GID1 molecules through a slight sequestration, and thus reduces the degradation of non-SUMOylated DELLAs. Other PTMs affect DELLA activity instead of its accumulation, like their O-GlcNAcylation by SECRET AGENT (SEC) and their O-fucosylation by SPINDLY (SPY), which respectively inhibit and enhance the ability of DELLAs to interact with certain TFs like PIFs and BZR1 (Zentella et al., 2016, 2017).

More recently, it has been shown that DELLAs can be degraded independently of GAs through physical interaction with the E3 ubiquitin ligase CONSTITUTIVELY PHOTOMORPHOGENIC1 (COP1) in *Arabidopsis*. Under shade or warm temperature conditions, COP1 directly interacts with DELLAs and catalyzes their polyubiquitination, thus marking them for degradation (Blanco-Touriñán et al., 2020a). Similarly, the F-box protein FLAVIN-BINDING KELCH REPEAT F-BOX 1 (FKF1) interacts with DELLA proteins and promotes their ubiquitination and degradation to promote flowering under long day conditions (Yan et al., 2020).

These discoveries broaden the idea of DELLA regulation beyond the only canonical mechanism accepted for years, and prove that DELLAs can integrate additional environmental information apart from the one provided by GAs.

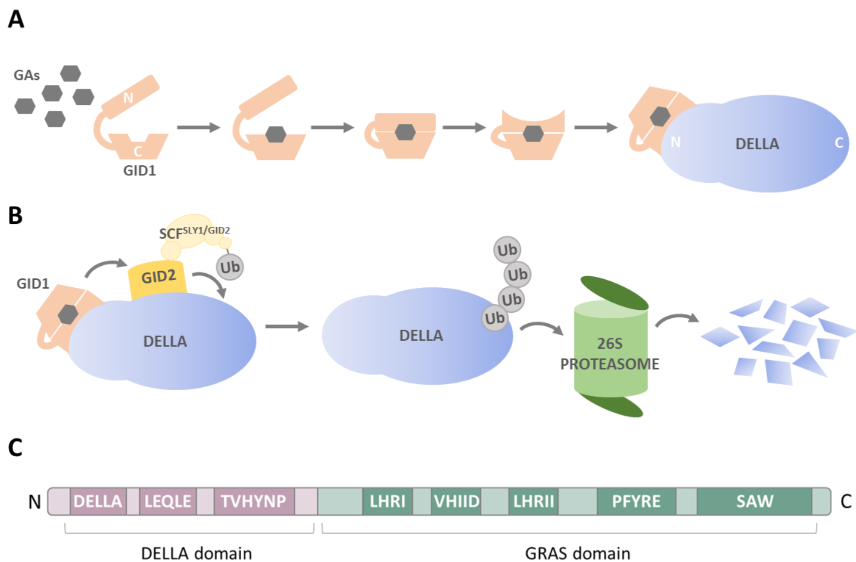

Figure 1. GA-dependent degradation of DELLA proteins. A, formation of the GA-GID1-DELLA complex. B, DELLA degradation via 26S proteasome, mediated by the F-BOX protein GID2 and the E3 ubiquitin ligase complex $SCF^{SLY1/GID2}$. C, domains and motifs present in DELLA proteins. The motifs in the N-terminal are responsible for the interaction with GID1. GID2 binds the VHIID and LHRII C-terminal motifs.

3. Molecular mechanisms of DELLA-mediated transcriptional regulation

GA-mediated DELLA proteolysis, as well as DELLA loss-of-function mutations, provoke dramatic changes in the plant transcriptome and severe phenotypical alterations, like an increased plant height, loss of seed dormancy, fruit parthenocarpy and susceptibility to stress (Ikeda et al., 2001; Cao et al., 2005; Achard, 2006; Livne et al., 2015). The known pleiotropic effects of GAs are due to the ability of DELLAs to modulate the expression of genes involved in multiple signaling pathways. However, DELLAs are unable to bind DNA, so they rely on the promiscuity of their GRAS C-

terminal domain to interact with a variety of transcription factors (TFs) and other transcriptional regulators (TRs). As a result of these interactions, DELLAs can both inhibit or promote gene expression. In some cases, the GRAS domain interacts with the DNA-binding motifs of a TF, thus preventing its association with its target gene promoters (Figure 2A). The best-known example of this mechanism is the interaction between DELLAs and the bHLH motif of Phytochrome Interacting Factors (PIFs), which results in the repression of cell expansion (de Lucas et al., 2008; Feng et al., 2008). In other cases, DELLAs sequester TRs like JASMONATE ZIM-domain (JAZ) proteins, interfering with their signaling cascade (Hou et al., 2013). In this case, DELLAs prevent the interaction between JAZs and MYC TFs, allowing MYCs to bind DNA and releasing the jasmonic acid (JA) responses (Figure 2B). Thereby, their interaction with a non-DNA-binding protein affects indirectly the TF's activity. In addition to sequestering TFs and TRs, DELLAs have also been shown to act as transactivators, associating with DNA through the interaction with TFs like *Arabidopsis thaliana* Response Regulators (ARRs) and INDETERMINATE DOMAINS (IDDs), to promote transcription (Yoshida et al., 2014b; Marín-de la Rosa et al., 2015). During these events, while the GRAS C-terminal domain of the DELLA interacts with the TF, the N-terminal region acts as a transcriptional activator (Figure 2C). Therefore, the functional versatility of DELLA proteins relies on a N-terminal domain, which aside from being essential for the recognition of the GA receptor GID1, confers them the ability to transactivate gene expression; and a C-terminal GRAS domain, capable of interacting with a variety of proteins, involved in many different processes.

Apart from their direct and indirect relation with multiple TFs to regulate transcription, DELLAs interact as well with proteins involved in chromatin-remodeling like PICKLE (PKL), needed in most GA-related developmental processes (Park et al., 2017); and SWI/SNF complex subunit SWI3C, which has an important role in GA biosynthesis and signaling (Sarnowska et al., 2013). Remarkably, they are also able to regulate cell elongation by

sequestering the chaperonin Prefoldin in the nucleus, thus hindering tubulin folding and affecting microtubule organization (Locascio et al., 2013).

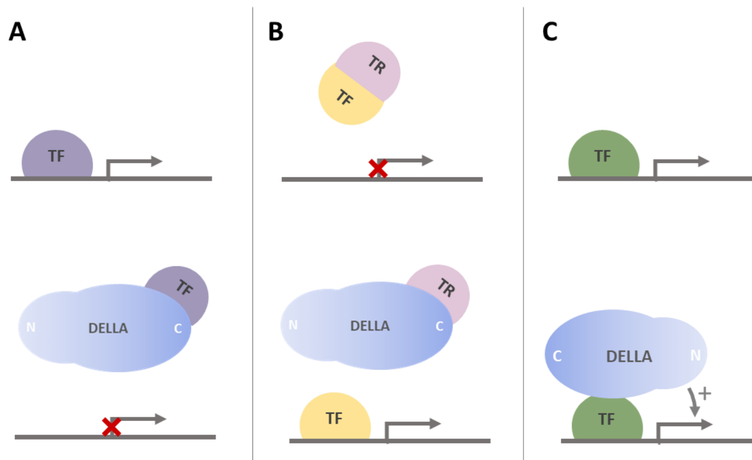

Figure 2. Main molecular mechanisms of DELLA-mediated transcriptional regulation. Case scenarios in the absence (above) and presence (below) of DELLAs. A, DELLA sequestration of a TF prevents it from binding the promoter of the target gene. B, DELLA sequestration of a TR inhibits its interaction with a TF, and thus permits the expression of the gene. C, DELLA indirectly binds DNA through the interaction between its C-terminal GRAS domain and a TF, while its N-terminal region promotes transcription.

4. DELLAs as transcriptional hubs

As mentioned above, DELLAs are widely known for repressing growth by sequestering PIFs and other TFs, but they can also inhibit seed germination by interacting with ABA-INSENSITIVE (ABIs) and Teosinte-branched1/Cycloidea/PFC (TCPs) (Lim et al., 2013; Davière et al., 2014; Resentini et al., 2015) and repress or delay flowering transition through interactions with FLOWERING LOCUS C (FLC), CONSTANS (CO) and SQUAMOSA promoter binding protein-like (SPLs) (Yu et al., 2012; Li et al.,

2016; Wang et al., 2016), among many other examples (reviewed by Van De Velde et al., 2017).

Ultimately, all functions attributed to GAs depend directly on the ability of DELLAs to interact with other proteins and regulate gene expression through them. Essentially, GA target genes are the genes controlled by DELLA interactors. Until now, hundreds of DELLA interactors from various protein families have been discovered, most of them through large-scale screenings (Marín-de la Rosa et al., 2014; Yoshida et al., 2014b; Lantzouni et al., 2020). Thanks to their promiscuity, DELLAs can participate in a variety of regulatory processes, and connect GAs with virtually all the other hormone signaling pathways (reviewed by Davière and Achard, 2016). Some phytohormones have overlapping or opposite roles in certain processes, and in the case of GAs most of them can be explained by interactions between DELLAs and other TRs or TFs. For example, the antagonism between ABA and GAs regarding seed germination relies mostly on the DELLA transactivation activity through ABI3 and ABI5, ABA-activated TFs that inhibit germination (Lim et al., 2013). DELLAs also transactivate gene expression of cytokinin-regulated genes through ARR TFs to control root growth (Marín-de la Rosa et al., 2015); interact with ETHYLENE INSENSITIVE 3 (EIN3) and other ethylene-related TFs like ETHYLENE RESPONSE FACTORS (ERFs) to control apical hook formation (Marín-de la Rosa et al., 2014); and sequester BRASSINAZOLE-RESISTANT 1 (BZR1) from the brassinosteroid pathway and the auxin-regulated TFs AUXIN RESPONSE FACTORS (ARFs) like ARF6 to repress cell expansion (Li et al., 2012; Gallego-Bartolomé et al., 2012; Oh et al., 2014). They can even modulate the balance between the signaling pathways of other two hormones, Salicylic Acid and Jasmonic Acid, thus altering the immune response of the plant against certain types of pathogens (Navarro et al., 2008). The relation between GA and JA signaling, mediated by interactions between DELLAs, JAZs and MYCs, seems to be key for maintaining the balance between growth and stress (Wild et al., 2012; Yang

et al., 2012). It has been proposed that DELLAs repress growth in response to stress, so that the plant resources are employed in the defense mechanisms (Claeys and Inzé, 2013; Claeys et al., 2014; Colebrook et al., 2014). That is a clear example of their behavior as hubs, central elements in the plant regulatory networks that integrate environmental information and transduce it into multiple transcriptional programs. In protein-protein interaction networks, hubs are proteins with a large number of interactions; and in regulatory networks, they are elements that regulate many targets (Zhang, 2013). Therefore, DELLAs can be considered hubs in both senses.

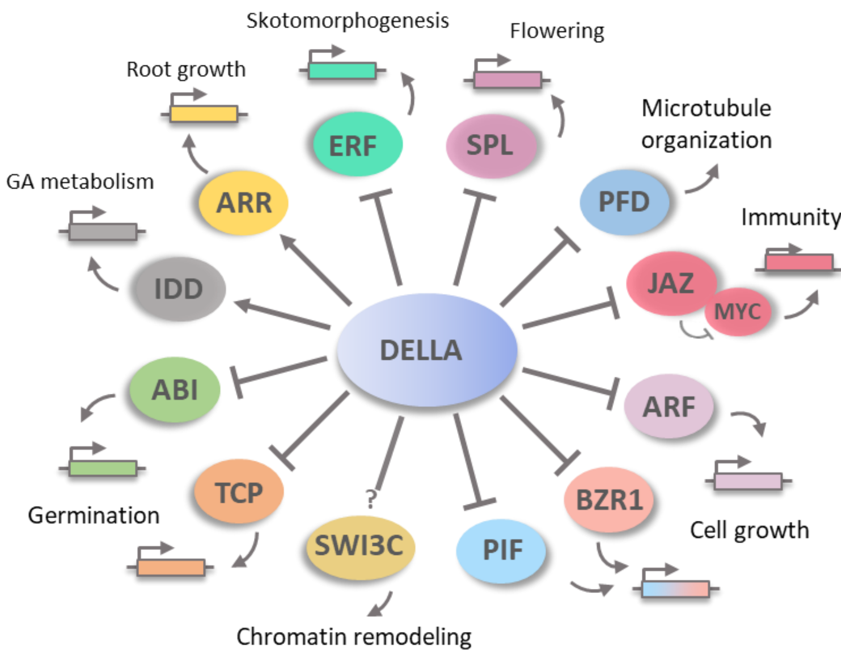

Figure 3. DELLA proteins act as regulatory hubs. Representative DELLA interactors and functions controlled through them are depicted. Enhancing or inhibitory effects of the interactions are represented with arrows and T-shaped lines, respectively. Adapted from Hernández-García et al., 2020.

5. Evolution of GA signaling: GA perception and DELLA degradation across land plants

The general model for GA signaling, discovered in angiosperms, includes bioactive GAs, their receptor GID1, DELLA proteins and the F-box SLY1/GID2. However, perception of GAs by GID1 seems to be functional in vascular plants exclusively. First of all, neither bioactive GAs nor genes encoding the necessary enzymes for their synthesis have been found in non-vascular species (Anterola and Shanle, 2008; Rensing et al., 2008; Bowman et al., 2017; Miyazaki et al., 2018), so the molecules that trigger the whole mechanism are absent in those clades. More importantly, only vascular plants present canonical GID1s, characterized by their ability to bind GAs in their C-terminal pocket, and recognize DELLA motifs when their N-terminal lid is closed (Figure 4). There are homologs of GID1 in non-vascular plants, but they lack the essential molecular features that allow the GA recognition or interaction with DELLAs (Hirano et al., 2007). It is still uncertain how GID1s became GA receptors and promoters of DELLA degradation, but studies in different species could provide some clues.

Despite their overall similarities, vascular GID1s have diverted into several clades, with different sensitivities towards GAs. For example, GID1-1 from the fern *Lygodium japonicum* shows a very high affinity, responding to very low concentrations of GA_4 (Tanaka et al., 2014); while GID1s from the lycophyte *Selaginella moellendorfii* display lower affinities than GID1s from spermatophytes, and are able to bind inactive GAs (Hirano et al., 2007). In dicots, two clades appeared following a duplication in a GID1 gene, GID1ac and GID1b, which show different expression patterns and sensitivities to GAs (Yoshida et al., 2018). Most of these GID1s can only interact with DELLAs after binding GAs, but some of them are able to interact in a GA-independent manner thanks to an intermediate disposition of their lid, that can be semi closed in the absence of GAs (Yamamoto et al., 2010). This could mean that GID1s were able to interact with DELLAs even before

acquiring the ability to bind GAs; and GAs were recruited later, making the regulation of this interaction more precise.

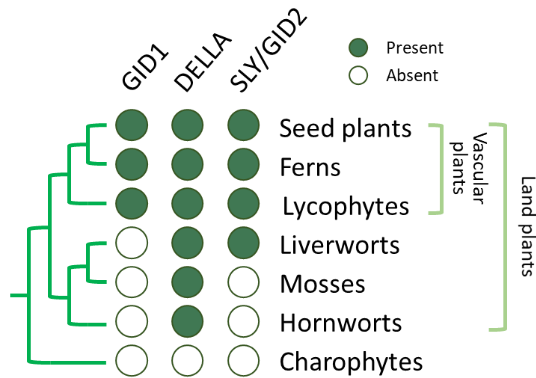

Figure 4. Evolution of GA-signaling genes. Presence or absence of genes encoding GA-signaling elements in different green plant lineages. Adapted from Hernández-García et al., 2020.

It is also uncertain at what point the GID1-DELLA interaction began to result in the degradation of DELLAs in the proteasome, promoted by SLY1/GID2. On one side, SLY1/GID2 genes are present in vascular plants but also in liverworts (Hernández-García et al., 2019)(Figure 4), so they could have regulated DELLA stability before bioactive GAs and canonical GID1s appeared. On the other side, GID1 can inhibit the DELLA transactivation activity in *sly1* and *gid2* mutants, so GID1 may have been able to inhibit DELLAs before SLY/GID2 was recruited (Ariizumi et al., 2008; Ueguchi-Tanaka et al., 2008).

Surprisingly, DELLAs seem to be present in all land plants, vascular or not (Figure 4). During the last years, *DELLA* genes have been found in several non-vascular species: one in the genome of the liverwort *Marchantia polymorpha* (Bowman et al., 2017), one in the hornwort *Anthoceros agrestis* (Li et al., 2020a), and two in the moss *Physcomitrella patens* (Yasumura et al., 2007), among others. In an extensive study using sequences from

numerous species, DELLAs were found in all analyzed land plants lineages, but not in algae (Hernández-García et al., 2019). Although other GRAS proteins were identified in some charophyte families, none of them belong to the DELLA subfamily. These results indicate that DELLAs originated in the ancestor of all land plants, and pose some immediate questions: How did DELLAs become part of the GA signaling pathway? How are DELLAs regulated in plants without GAs? What is the function of DELLAs in those species?

Interestingly, the N-terminal region of DELLA proteins, necessary for the interaction with GID1 that leads to their GA-dependent degradation, is highly conserved across land plants (Murase et al., 2008; Shimada et al., 2008). The preservation of this region in non-vascular plants, where there is no interaction with GID1s, is probably related to other functions residing in it. Apart from sequestering other proteins, DELLAs can act as transactivators of gene expression. Deletion assays have shown that this ability relies on their N-terminal domain, and it can be impaired by the interaction with GID1 in vascular plants (Hirano et al., 2012). However, transactivation activity has also been observed in DELLAs from non-vascular species, so it seems to be a conserved trait in all land plants (Hernández-García et al., 2019). It is possible that the interaction between GA-GID1 and DELLAs acted as a first mechanism of DELLA repression, blocking its transactivation effect. The later incorporation of SLY1/GID2 to the system, with the consequent ubiquitination and degradation of DELLAs, would have added an extra level of regulation, connecting GA levels with DELLA accumulation.

It is noteworthy that non-vascular plants not only lack GID1s able to bind DELLAs, but their DELLAs are also incapable of binding canonical GID1s. Even though a reconstructed ancestral DELLA protein and some DELLAs from bryophytes possess a conserved N-terminal domain, DELLAs from *M. polymorpha* and the moss *Takakia lepidozioides* are unable to interact with AtGID1s (Hernández-García et al., 2019). Furthermore, when expressed in

A. thaliana, the degradation of SkDELLA can be induced by GAs, while PpDELLAa seems to be resistant (Yasumura et al., 2007). In vascular plants, the specificity of GID1-DELLA interactions is variable, as the *S. kraussiana* DELLA can interact with AtGID1c in a GA-dependent manner, but SkGID1 is unable to bind AtRGA (Yasumura et al., 2007).

The main elements of the GA signaling pathway emerged in different events, until the mechanisms of GA perception and DELLA degradation where completely established in vascular plants, where they continued evolving. DELLAs have probably recruited the GA-GID1 module, and thus originated GA signaling.

6. Evolution of DELLA proteins

Aside from their main regulatory mechanism, studying the evolution of DELLA structure, characteristics and functions is crucial for understanding how they became a central element of the coordination of transcriptional responses.

Phylogenetically, DELLAs from early diverging land plants belong to an ancestral type (DELLA1/2/3) that duplicated in the ancestor of vascular plants (DELLA1/2 and DELLA3) and again in eudicots (DELLA1, DELLA2 and DELLA3) (Hernández-García et al., 2019). Therefore, all DELLAs in non-vascular plants belong to one clade, while two differentiated clades can be observed in vascular non-eudicots, and three in eudicots. It is also possible to associate the number of DELLA genes in each genome with the evolutionary history of each species. For instance, the loss of the DELLA domain in the DELLA3 clade left only one DELLA in *O. sativa* (Ikeda et al., 2001), *S. lycopersicum* has only one DELLA gene (Jasinski et al., 2008) because DELLA1 and DELLA3 clades were lost, and *A. thaliana* has five (Peng et al., 1997; Silverstone et al., 1998; Lee, 2002; Wen and Chang, 2002) as a result of duplications in DELLA1 and DELLA2, and the loss of

DELLA3. In species with several DELLAs, differentiated and overlapping functions can be attributed to each one (reviewed by Davière and Achard, 2013). That is the case of RGL2, the main DELLA controlling germination in *A. thaliana* (Lee, 2002). However, it has been shown that DELLAs from the same species share interactions with TFs, and their performances are similar when their promoters are exchanged (Gallego-Bartolome et al., 2010). Therefore, functional differences among DELLA paralogs seem to depend mostly on their expression patterns, and not on their intrinsic abilities.

Given that DELLA activity relies mainly on their interactions with other proteins, assessing the conservation of the DELLA interactome is essential for understanding how they got involved in multiple processes and connected them along evolution. Ideally, obtaining and comparing the whole set of DELLA interactions in different species through vast screenings would provide the required information, but TF collections are only available for *A. thaliana*. For that reason, extensive studies have only been performed in that species (Marín-de la Rosa et al., 2014; Yoshida et al., 2014a; Lantzouni et al., 2020); but many individual interactions have been discovered in others. In some of the particular cases studied until now, DELLA interactions and their effects are conserved. For example, the role of JAZ-DELLA association in the regulation of JA response is similar in *Arabidopsis* and rice (Yang et al., 2012; Um et al., 2018). In other cases, even though interactions are conserved, their outcomes are not. DELLA interaction with NF-Y proteins regulates nodulation in *Medicago truncatula* (Fonouni-Farde et al., 2016), while in *Arabidopsis* it is involved in the control of flowering time (Hou et al., 2014). This situation is probably due to changes in the interactors or their targets, independently of DELLAs.

Although DELLAs have been mostly studied in angiosperms, some functional information has been obtained from other clades. In the lycophyte *S. moellendorfii*, growth is reduced upon treatment with the GA synthesis

inhibitor uniconazole, and the expression of its DELLA1 causes dwarfism in rice (Hirano et al., 2007). The same effect is observed when a *S. kraussiana* DELLA is expressed in *A. thaliana* (Yasumura et al., 2007), so growth restraint seems to be a conserved function in basal vascular plants. Regarding non-vascular species, expression of DELLAs from the bryophyte *P. patens* impairs growth in *A. thaliana* (Yasumura et al., 2007) but not in rice (Hirano et al., 2007). It seems that all DELLAs can restrain growth, but their performance depends on the regulation of each species. Moreover, the *Ppdella*AB double mutant shows an increased sensibility to salt stress (Yasumura et al., 2007), so stress response may be another conserved DELLA function in all land plants.

All these data are consistent with results from our group, obtained through the study of the only DELLA in the liverwort *M. polymorpha* (Hernández-García et al., unpublished). Although a knock-out mutation in MpDELLA seems to be lethal, lines overexpressing this protein show a remarkably reduced growth, increased resistance to salt stress and altered regulation of gemmae germination. Hence, there seems to be a certain functional conservation between distant species.

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Objectives

Contrary to what has been observed with other hormones that target different processes, the multiplicity of functions exerted by GAs does not seem to rely on a wide chemical diversity or the combinatorial action of multiple signaling elements (Blázquez et al., 2020)[1]. Rather, DELLA's promiscuity (i.e., the ability to interact with hundreds of TFs) seems to be the main cause of the wide range of aspects affected by GAs. However, the evolutionary origin of this promiscuity, the constraints and degrees of freedom that have shaped DELLAs evolution as hubs, and the influence of the emergence of GA perception on DELLA functions, are completely unknown.

Given the importance of these issues, not only from a basic perspective but also to assist in the design of future DELLA-based biotechnological strategies, we set out to answer the following questions: (i) when did the role of DELLA as coordinator of transcriptional programs emerge? (ii) was the ancestral DELLA a promiscuous protein? (iii) has DELLA promiscuity increased gradually during evolution, or through discrete changes? (iv) how does DELLA evolution explain the differences of GA functions in different plant species?

In particular, the specific objectives of this Thesis were:

1. **To analyze the role of DELLAs in the coordination of transcriptional programs along evolution.** We hypothesized that DELLAs have contributed substantially to the complexity of plant signaling networks. We tested it through the comparison of DELLA-associated gene co-expression networks from different species.

2. **To study the evolution of the DELLA interactome.** We hypothesized that the promiscuity of DELLAs, and hence their number of interactors, has increased gradually along evolution because it is a favorable trait. We tested it through the examination of the ability of DELLAs from multiple species to interact with TFs.
3. **To assess the functional conservation of DELLAs across species**. We hypothesized that DELLAs are key transcriptional regulators in all land plants with a core of common functions. We tested it by comparing transcriptional targets of DELLAs from different species, both in their own biological context and through heterologous complementation.

[1]Blázquez, M. A., Nelson, D. C., and Weijers, D. (2020). Evolution of Plant Hormone Response Pathways. Annu. Rev. Plant Biol. 71, 327–353. doi:10.1146/annurev-arplant-050718-100309.

Chapter 1

Evolutionary Analysis of DELLA-Associated Transcriptional Networks

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0. Abstract

DELLA proteins are transcriptional regulators present in all land plants which have been shown to modulate the activity of over 100 transcription factors in *Arabidopsis*, involved in multiple physiological and developmental processes. It has been proposed that DELLAs transduce environmental information to pre-wired transcriptional circuits because their stability is regulated by gibberellins (GAs), whose homeostasis largely depends on environmental signals. The ability of GAs to promote DELLA degradation coincides with the origin of vascular plants, but the presence of DELLAs in other land plants poses at least two questions: what regulatory properties have DELLAs provided to the behavior of transcriptional networks in land plants, and how has the recruitment of DELLAs by GA signaling affected this regulation. To address these issues, we have constructed gene co-expression networks of four different organisms within the green lineage with different properties regarding DELLAs: *Arabidopsis thaliana* and *Solanum lycopersicum* (both with GA-regulated DELLA proteins), *Physcomitrella patens* (with GA-independent DELLA proteins) and *Chlamydomonas reinhardtii* (a green alga without DELLA), and we have examined the relative evolution of the subnetworks containing the potential DELLA-dependent transcriptomes. Network analysis indicates a relative increase in parameters associated with the degree of interconnectivity in the DELLA-associated subnetworks of land plants, with a stronger effect in species with GA-regulated DELLA proteins. These results suggest that DELLAs may have played a role in the coordination of multiple transcriptional programs along evolution, and the function of DELLAs as regulatory 'hubs' became further consolidated after their recruitment by GA signaling in higher plants.

1. Introduction

Higher plants are characterized by a particularly flexible capacity to adapt to multiple environmental conditions. In other words, environmental signals are very efficient modulators of plant developmental decisions. This ability is generally assumed to be based on at least two mechanistic features: the presence of an extensive and sensitive repertoire of elements that perceive environmental signals (such as light photoreceptors covering a wide range of wavelengths), and the high degree of interconnectivity between the different signaling pathways to allow cellular integration of variable information (Casal et al., 2004).

Evidence has accumulated in recent years about the important role that plant hormones play in the translation of environmental signals into developmental decisions. On one hand, it has become evident that hormone pathways share common components with the pathways that transduce light and other environmental signals (Jaillais and Chory, 2010); and, on the other hand, hormones have been shown to participate in the regulation of developmental processes all throughout a plant's life cycle (Alabadi et al., 2009). In this context, gibberellins (GAs) and DELLA proteins are a paradigmatic example of the mechanisms that allow environmental signal integration. DELLA proteins constitute a small clade within the GRAS family of loosely defined plant specific nuclear proteins (Vera-Sirera et al., 2015). Their name was coined on the basis of a short stretch of amino acids (D-E-L-L-A) in their N-terminal region, which is tightly conserved among all higher plant species. They also present additional conserved motifs, such as the VHYNP domain, two leucine heptad repeats which may mediate protein–protein interactions, a putative nuclear localization signal, and a putative SH2 phosphotyrosine-binding domain, among others (Vera-Sirera et al., 2015). It has been shown in *Arabidopsis thaliana* and rice that recognition of GAs by their GID1 receptor allows physical interaction with DELLA proteins and promotes their degradation via the proteasome. In *A. thaliana*,

loss of DELLA function mimics the phenotype of plants treated with an excess of GAs, both anatomically and also at the transcriptional level (Schwechheimer, 2011; Locascio et al., 2013b). Work in the past few years has established that DELLAs regulate transcription through the interaction with more than 100 transcription factors (TFs) in *A. thaliana* (de Lucas et al., 2008; Feng et al., 2008; Crocco et al., 2010; Hou et al., 2010; Gallego-Bartolomé et al., 2012; Daviere et al., 2014; Marin-de la Rosa et al., 2014, 2015; Resentini et al., 2015). In some cases, interaction with the TF inhibits its ability to bind DNA, while in other cases DELLAs seem to act as co-activators (Locascio et al., 2013b; Daviere and Achard, 2016). For all the cases examined in detail, the DELLA region responsible for the interaction with the TFs is the C-terminal region of the protein, the GRAS domain. Given that GA levels are strongly regulated by environmental signals such as light, temperature and photoperiod (Hedden and Thomas, 2012; Colebrook et al., 2014), cellular DELLA levels seem to be a proxy for the environmental status faced by plants (Claeys et al., 2014). Changes in DELLA levels could in turn differentially modulate distinct sets of TFs and their target genes in various developmental contexts. The promiscuous interaction with TFs, and the observation that *A. thaliana dellaKO* mutants display constitutive growth even under stress, and suffer from increased sensitivity to several types of stress factors such as salinity, cold, or fungal attacks (Alabadí et al., 2004; Achard et al., 2006, 2007, 2008a,b; Cheminant et al., 2011) suggests that DELLAs are potentially important ‘hubs’ in the transcriptional network that regulates the balance between growth and stress tolerance in higher plants.

Previous interest in the evolution of DELLA proteins is restricted to the question on how they were recruited to mediate cellular signaling by GAs. Based on phylogenetic analyses and shallow molecular analysis with fern and moss orthologs, it seems that the GA/GID1/DELLA module originated with early diverging tracheophytes (Wang and Deng, 2014). For instance, the *Selaginella* genus possesses the ability to synthesize GAs, a GID1 GA receptor, and a DELLA protein (Wang and Deng, 2014), which is sensitive

to GA-induced degradation, even when introduced in an angiosperm, such as *A. thaliana* (Hirano et al., 2007; Yasumura et al., 2007). On the other hand, the DELLA proteins that existed in other land plants before the emergence of vascular plants were not involved in GA signaling. First, there are no bona-fide DELLA genes in algae and, second, the genomes of bryophytes like *Physcomitrella patens* encode DELLA proteins that lack the canonical 'DELLA motif' (Wang and Deng, 2014), and PpDELLAs are not sensitive to GAs when introduced in *A. thaliana* (Yasumura et al., 2007). However, the ability of DELLA proteins to modulate transcriptional programs relies on the GRAS domain through which interactions with TFs occur, and the evolution of this activity has not been addressed before.

In an attempt to identify the possible function of ancestral DELLAs and to delineate how evolution has shaped the functions of the GA/DELLA module in higher plants, we have addressed the analysis of the transcriptional networks potentially regulated by DELLAs in several species. For this reason, we have used gene co-expression networks, in which genes are represented as nodes, and if two genes exhibit a significant correlation value for co-expression, the corresponding nodes are joined by an edge. Importantly, if a node is a TF, first neighbors can be confidently taken as targets for that particular TF (Franco-Zorrilla et al., 2014). Therefore, the analysis of topological parameters of a gene co-expression network is an interesting tool that may reveal information about the function and evolutionary history of transcriptional programs (Aoki et al., 2007; Usadel et al., 2009).

Here we have investigated the properties of networks formed by DELLA-interacting TFs and their co-expressing genes in *A. thaliana*, and compared them with the orthologous networks in three other plant species: (i) *Solanum lycopersicum* (possessing a fully operative GA/DELLA module); (ii) *P. patens* (possessing GA-independent DELLA functions); and (iii) *Chlamydomonas reinhardtii* (without GA perception or DELLAs) (Figure 1A).

All the parameters examined suggest that the functions regulated by DELLA-interacting TFs (and thus DELLAs themselves) have increased their level of coordination along evolution.

2. Results and discussion

Construction of networks and subnetworks

Gene expression data from RNA sequencing (RNA-seq) experiments in *A. thaliana*, *S. lycopersicum*, *P. patens*, and *C. reinhardtii* were obtained from the Gene Expression Omnibus, and gene co-expression networks were inferred for each species from transcriptomic data as described in section "Materials and Methods." All four networks are scale-free networks (Supplementary Figure S1) (Romero-Campero et al., 2013, 2016) and have comparable sizes in terms of number of nodes, but there are remarkable differences in the way they are connected (Table 1). The *A. thaliana* network contains more than twice as many edges than the others, the average degree of its nodes (average number of connections) is one order of magnitude higher and its average shortest path length (average number of nodes between two random nodes) is lower. Even though the number of genes of each species represented in the networks is similar, in some species they are more connected, possibly due to differences in their endogenous regulation and the availability of experimental data. For that reason, we decided to do every comparative analysis between the different species in relative terms.

To be able to compare the co-expression networks of the different species, we first identified the orthologous nodes in each of them using the OrthoMCL method (Li et al., 2003). Up to 17,053 groups of genes were obtained. Genes in the same group were considered orthologs or paralogs if they belonged to different or the same species, respectively. The four species were represented unequally, as both *A. thaliana* and *S. lycopersicum* genes

were present in ca. 70% of the groups, while *P. patens* genes were found in little more than 50% of them, and only ca. 30% of the groups contained genes from *C. reinhardtii* (Figure 1B). This was already expected, given the evolutionary distance among these species and the genomic complexity of each one.

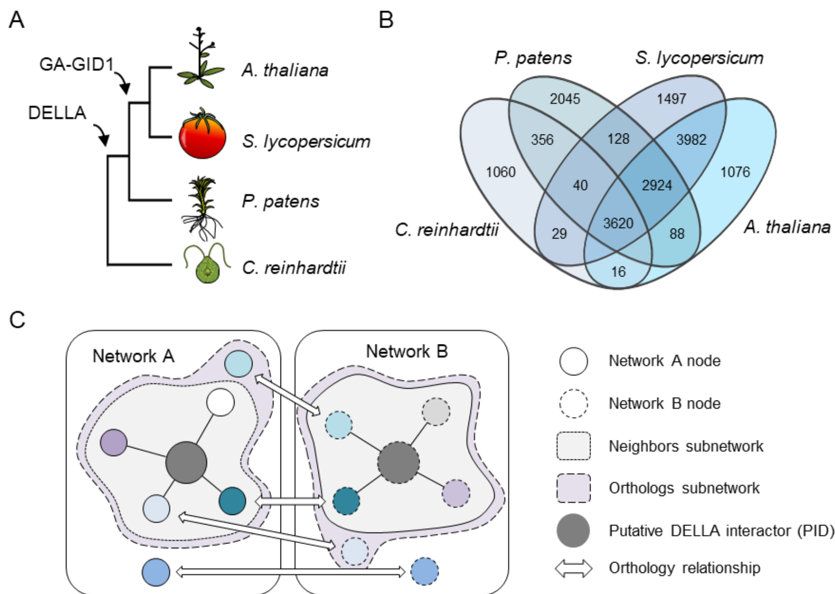

Figure 1. Phylogenetic relationships between the chosen species. **(A)** Representation of the species tree indicating the origin of key elements related to the gibberellin signaling pathway. **(B)** Venn's diagram showing the number of OrthoMCL groups in which genes of each species are present. **(C)** Schematic representation of the basis for subnetwork design.

To assess the possible contribution of DELLA proteins to co-expression networks architecture, we created subnetworks based on reported DELLA interactors known to act as transcriptional regulators. First, we compiled a list of all published DELLA interactors (Supplementary Table S1), obtained their orthologs for the four species, and localized them in their respective networks. Since most of the interactions have been described for *A. thaliana*, the corresponding orthologs in the other species are only "putative

interactors of the DELLA proteins" (PIDs), and the first neighbors of AtDELLA interactors and PIDs are their putative expression targets. Second, we built two different subnetworks using this information. The first one, called "Neighbors" subnetwork (abbreviated as AtNeigh, SlNeigh, PpNeigh, and CrNeigh), is composed of the DELLA interactors (or the corresponding PIDs) and their first neighbors (Figure 1C and Supplementary Table S2). The second one, called "Orthologs" subnetwork (abbreviated as AtOrtho, SlOrtho, PpOrtho, and CrOrtho), contains the orthologs of all the first neighbors of PIDs in all the species (Figure 1C and Supplementary Table S3). For a given species, the "Neighbors" subnetwork provides a good approximation to its actual DELLAdependent transcriptome, while the "Orthologs" subnetwork represents the full landscape of potential transcriptional targets for DELLAs, since it includes orthologs of genes that are DELLA transcriptional targets in other species (Figure 2).

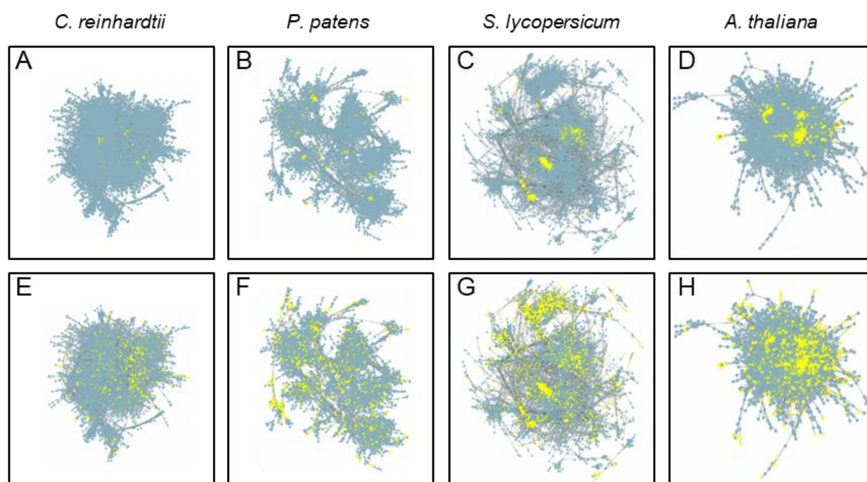

Figure 2. Gene co-expression networks. Full *C. reinhardtii* **(A, E)**, *P. patens* **(B, F)**, *S. lycopersicum* **(C, G)**, and *A. thaliana* **(D, H)** gene co-expression networks. Neighbors subnetworks are comprised of yellow-marked nodes in A-D. Orthologs subnetworks are comprised of yellow-marked nodes in E-H.

DELLA-associated subnetworks reflect increased relevance of DELLAs after being recruited by GA signaling

It is important to take into account a circumstance that affects the construction of subnetworks: OrthoMCL does not always retrieve orthologs for some of the genes, because either they do not exist in the other species, or the method does not provide high-confidence results. This results in a particular bias toward smaller subnetwork sizes with increasing phylogenetic distance (Table 1). However, the impact of this bias can be disregarded when analyzing relative parameters. Hence, regardless of the absolute sizes, we observed that the average degree in the Neighbor subnetworks increased dramatically in SlNeigh and AtNeigh with respect to their full networks (more than threefold and twofold, respectively), while this parameter did not change in PpNeigh, and it actually decreased in CrNeigh (Table 1). Similarly, the Orthologs subnetworks displayed an equivalent behavior as the Neighbors subnetworks: their diameter and average shortest path length decreased considerably more in SlOrtho and AtOrtho with respect to the full networks; and the same happened with the increase of the average degree. In summary, both subnetworks showed a higher compaction and interconnection of nodes in relative terms in the case of *S. lycopersicum* and *A. thaliana* compared with *P. patens* and *C. reinhardtii*, indicating that the putative interactors and targets of the DELLAs become more connected in those species presenting GA-regulated DELLAs.

A confirmation of the impact of GA regulation on the relevance of DELLA function is found in the analysis of neighborhood conservation. Figure 3A shows the percentage of genes with a significantly overlapping neighborhood in each comparison (see Materials and Methods). When comparing *P. patens* with the other species, there are no substantial differences between the full network and the Orthologs subnetwork. On the contrary, SlOrtho and AtOrtho contain a considerably higher proportion of genes with conserved neighborhood than their corresponding full networks

(15% vs. 10%). Between *S. lycopersicum* and *A. thaliana*, the regulation of the putative DELLA targets is more conserved than for the network in general, so this group of genes seems to have a cohesive element in the two species.

	C. reinhardtii			*P. patens*			*S. lycopersicum*			*A. thaliana*		
	Full	**Neigh**	**Ortho**	**Full**	**Neigh**	**Ortho**	**Full**	**Neigh**	**Ortho**	**Full**	**Neigh**	**Ortho**
Nodes	8652	48	658	8564	448	1503	7851	1314	2885	5663	2070	2949
Edges	145903	78	1173	295317	15078	19828	287409	153396	169171	593730	460951	512042
Average degree	33.73	3.25	3.57	68.97	67.31	26.38	73.22	233.48	117.28	209.69	445.36	347.26
Average SPL	7.37	1.91	8.71	13.11	1.39	12.01	13.78	1.67	5.63	4.28	2.15	3.09
Diameter	23	4	24	46	4	41	44	6	25	20	9	12

Table 1. General parameters in co-expression networks. Parameters of networks and subnetworks used in this study. Full, full gene co-expression network; Neigh, first neighbors subnetwork; Ortho, orthologs subnetwork; *C. reinhardtii*, *Chlamydomonas reinhardtii*; *P. patens*, *Physcomitrella patens*; *S. lycopersicum*, *Solanum lycopersicum*; *A. thaliana*, *Arabidopsis thaliana*. Average SPL stands for Average Shortest Path Length.

Furthermore, we examined gene–gene co-expression values, as a measure of the conservation of individual edges. For every pair of linked genes in one species, if the corresponding orthologs are also linked in a second species, it is considered that gene–gene co-expression is conserved. Therefore, the calculation of conserved links between two subnetworks is a measure of functional conservation of a regulatory module. Interestingly, we observed that gene links between PpOrtho and SlOrtho were less conserved than in the full networks, and almost unaltered between PpOrtho and AtOrtho (Figure 3B). However, the gene–gene co-expression was three times more conserved between SlOrtho and AtOrtho than between their full networks (11% vs. 3.5%). In other words, these data are compatible with the proposition that the presence of GA-regulated DELLAs (in *S. lycopersicum*

and *A. thaliana*) provides stronger links between transcriptional programs, not detected in an organism with GA-independent DELLAs (*P. patens*).

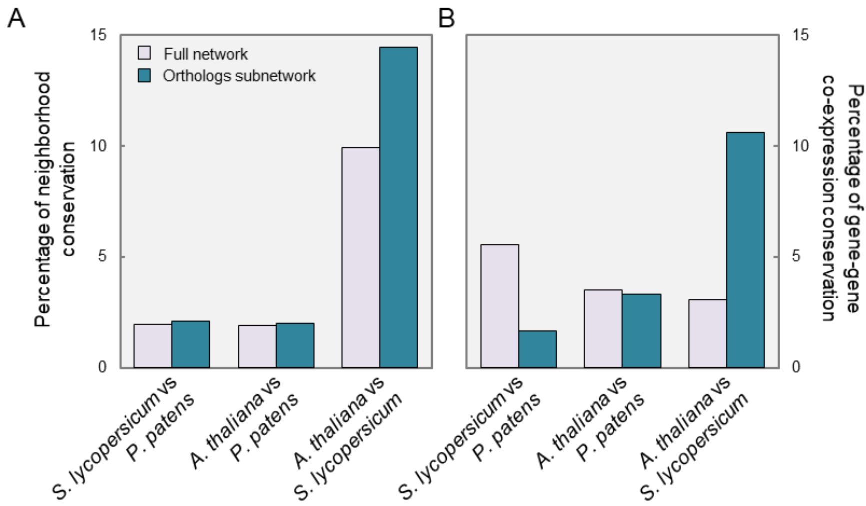

Figure 3. Gene connections are more conserved in species with GA-regulated DELLAs. Pairwise comparisons of *P. patens*, *S. lycopersicum* and *A. thaliana* Full networks and Ortho subnetworks regarding: **(A)** Percentage of genes with significantly overlapping neighborhoods; **(B)** Percentage of conserved gene-gene links.

Efficiency of transcriptional regulation is a DELLA-associated parameter

The efficiency of a transcriptional regulatory mechanism can be evaluated through two additional parameters in gene coexpression networks: shortest path length distribution and motif frequency. In network theory, average shortest-path length is defined as the average number of steps along the shortest paths for all possible pairs of network nodes. It is a measure of the efficiency of information propagation on a network, with a shorter average path length being more efficient (Vragovic et al., 2005). When we compared the distribution of shortest path lengths in full and Orthologs subnetworks,

we observed a clear tendency toward shorter path lengths in the Orthologs subnetworks of organisms possessing DELLAs (*S. lycopersicum*, *A. thaliana*, and *P. patens*) compared with the situation in an organism without DELLAs (*C. reinhardtii*) (Figure 4).

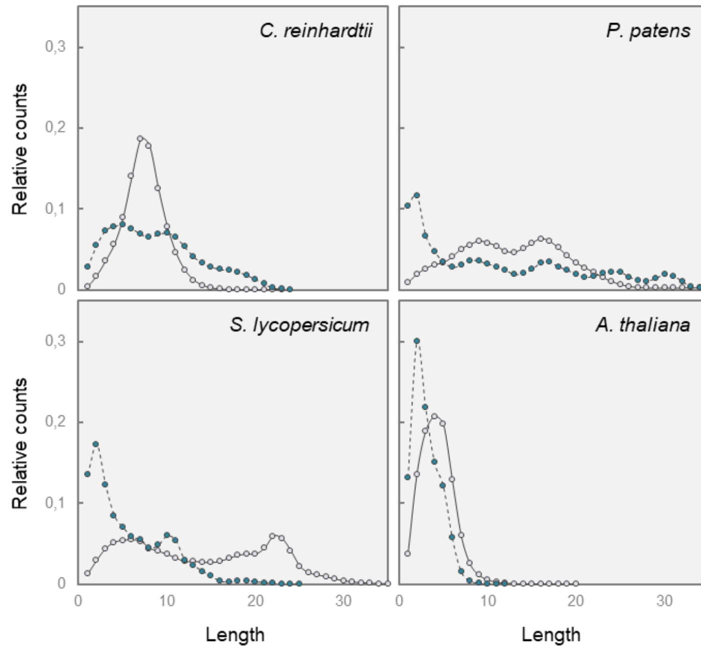

Figure 4. Paths are shorter in DELLA-associated subnetworks. Shortest path length distribution in Full networks and Orthologs subnetworks from the four species.

Network motifs are small recurring patterns involving a few nodes that appear more frequently in biological networks than in randomized ones. They consist of a certain level of regulation which connects small sets of nodes with a particular topology. Motifs characterize a network, as some of them are useful for the regulation of determined functions, and thus conserved along evolution (Kashtan and Alon, 2005). After measuring the frequency of the eight common motifs composed of three and four nodes in the full networks, we found that there was no relative enrichment of any

particular motif between species when comparing the full networks or the Orthologs subnetworks (Figure 5A). However, the AtOrtho, SlOrtho, and PpOrtho subnetworks displayed a clear enrichment in virtually every motif, compared with their respective full networks (Figure 5B). Given that the function of this sort of motifs is to allow coordinated expression of a group of genes with shared function (Alon, 2007), the increase in the proportion of small regulatory patterns among all the putative DELLA targets in species that do contain DELLAs indicates an increase in the complexity of gene regulation, in which DELLAs might mediate the coordination of transcriptional programs.

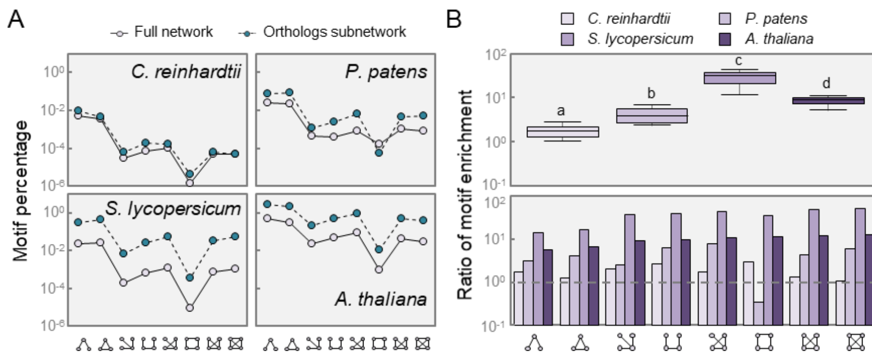

Figure 5. Network motifs are enriched in DELLA related networks. **(A)** Percentage of motifs found in each network compared to possible combinations of three and four nodes. **(B)** Ratio of motif enrichment comparing Orthologs subnetworks to Full networks per species (upper panel), and per motif (lower panel). Dashed lines in (B) mark a ratio of 1. Motifs are as depicted in X-axis. Letters indicate significant differences between groups, $p < 0.01$ (One way ANOVA, Tukey HSD Post Hoc test). Box-plot whiskers are Tukey-defined (extended 1.5 times the IQR from the box edges).

The regulation of the stress response: a likely role of ancestral DELLA proteins

The results shown above suggest that the origin of DELLAs in land plants would be associated to an increase in the co-expression between genes that

are putative targets of DELLA-interacting TFs, both in terms of size of the gene set and degree of the co-expression value. Therefore, DELLAs would have helped in the coordination of certain transcriptional circuits, and their recruitment to mediate GA signaling later in development would have further expanded their coordination capacity. To reveal the most likely functions ultimately regulated by DELLAs in the common ancestor of land plants, we carried out Gene Ontology (GO) analyses on each of the Neighbor subnetworks, with the idea that the terms shared by those in *S. lycopersicum*, *A. thaliana*, and *P. patens* could represent likely functions regulated by the ancestral DELLA proteins.

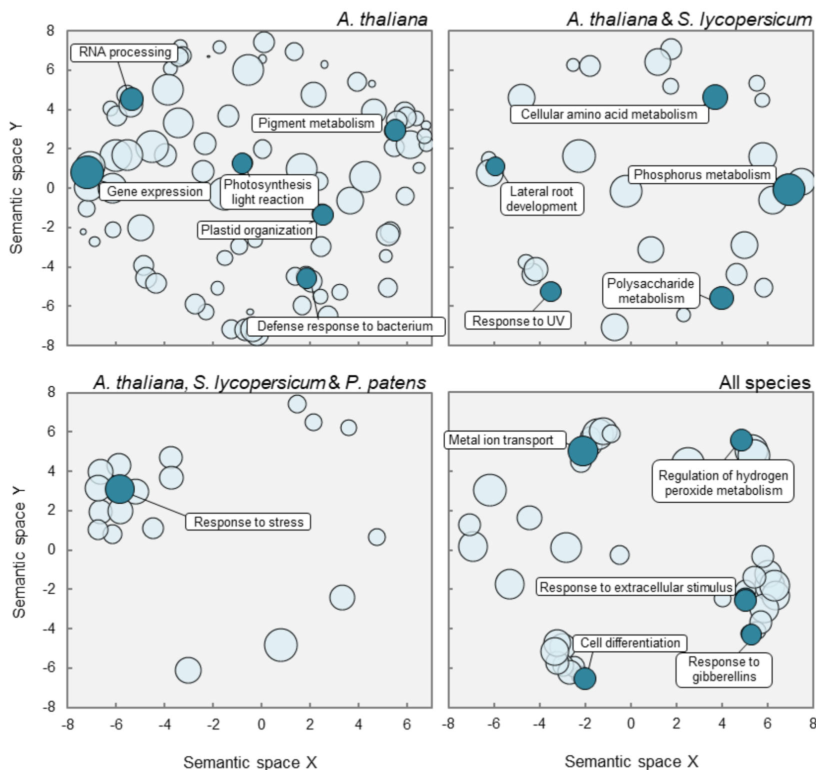

Figure 6. Gene Ontology terms enriched in Neighbors subnetworks. Scatterplots show cluster representatives after redundancy reduction in a two dimensional space derived by applying multidimensional scaling to a matrix of the GO categories semantic similarities. Bubble size is proportional to p-value significance of GO enrichment.

Not surprisingly, given the larger size of AtNeigh (Table 1), GO analysis rendered a much larger number of terms significantly enriched in this subnetwork, compared to those from the other three organisms (Supplementary Table S4). Terms referring to chloroplast function, such as plastid organization, photosynthesis, or pigment biosynthesis (including chlorophyll) were specifically enriched among the putative DELLA targets in *A. thaliana* only (Figure 6). This result might reflect functions whose regulation by DELLA has been acquired more recently, or it could simply be a bias of the analysis, caused by the big difference in size of the analyzed sets in the different species. On the contrary, the finding that terms comprised under general 'response to stress' were significantly over-represented in the subnetworks of the three land plants, but not *C. reinhardtii*, suggests that this function might have been the primary target of the regulation by ancestral DELLAs through their interaction with specific TFs.

3. Conclusion

Our analysis suggests that DELLAs may have contributed to the acquisition of an increasing degree of coordination between transcriptional programs during plant evolution. Although these results are consistent with the current view of DELLAs as 'hubs' in transcriptional programs in higher plants, and provide a plausible evolutionary scenario, it is important to remark that further experimental work is required to validate most of the conclusions from *in silico* network analysis. In fact, several reasonable assumptions have been made that would be relatively easy to confirm. For instance, actual transcriptomic data of *dellaKO* mutants in the different species, coupled to comparative analysis would help establish the role of ancestral DELLAs. Moreover, our current analysis would be strengthened by the experimentally obtained information of which PIDs are in fact bona-fide DELLA interactors in the different species. Finally, the conclusion that DELLAs have probably contributed to the establishment of new co-regulatory circuits during land-

plant evolution does not explain the molecular mechanism that supports this progressive acquisition, and it can be generated by changes in DELLA proteins, in their interactors, or in both.

4. Materials and methods

Gene co-expression network inference

The *C. reinhardtii* and *A. thaliana* networks were downloaded from the web resources of previous work (Romero-Campero et al., 2013, 2016). For the new networks, RNA-seq data were selected from equivalent experiments involving comparable tissues and environmental situations (Supplementary Table S5). The *P. patens* gene co-expression network was inferred from the RNA-seq data freely available from the Gene Expression Omnibus identified with accession numbers GSE19824, GSE33279, GSE36274, and GSE25237. The *S. lycopersicum* network was constructed based on the RNA-seq data identified with the accession numbers GSE45774, GSE64665, GSE64981, GSE68018, and GSE77340 in the Gene Expression Omnibus. In both cases, RNA-seq data was processed using the Tuxedo protocol (Trapnell et al., 2012) to obtain gene expression levels measured as FPKM. Briefly, short reads were mapped to the corresponding reference genome using Tophat, transcripts were assembled using Cufflinks and expression levels were computed using Cuffdiff. The Bioconductor R package cummerbund (Goff et al., 2013) was used for subsequent analysis of the results generated by the Tuxedo protocol. In order to reduce noise in our analysis only genes that were detected as differentially expressed in at least one of the studies integrated in this work were considered. Differentially expressed genes were determined comparing each condition with the corresponding control within each study using a fold-change threshold of two. For each species, a matrix containing the expression levels of the selected genes was extracted. The Pearson correlation coefficient between every pair of gene expression profiles was computed using the *cor*

function from the stats R package to generate a correlation matrix. Two genes were assumed to be co-expressed when the Pearson correlation coefficient between their expression profiles over the analyzed conditions was greater than 0.95. Following this criterion, the corresponding adjacency matrix was generated from the correlation matrix. Using the R package igraph1 (Csardi and Nepusz, 2006), each network was constructed from its adjacency matrix and exported in gml formal for subsequent analysis.

Data compilation and processing

The reference proteomes from *A. thaliana* TAIR10, *S. lycopersicum* iTAGv2.3, *C. reinhardtii* v5.5, and *P. patens* v3.3 were downloaded from Phytozome (Goodstein et al., 2012). From all the possible proteins from each locus tag only the longest protein was kept and assigned to its locus tag. These files were used to identify the orthologs among the four species with OrthoMCL (Li et al., 2003).

The networks were converted to SIF format and processed using the package igraph1 (Csardi and Nepusz, 2006) made with R2 (R Core Team, 2016). Only the edges between two non-identical nodes were conserved. If a given node was not identified in the proteome files, it was removed from the network. Afterward, components with fewer than seven elements were removed from the network to generate the complete network for each species. The orthologs for the set of manually curated DELLA interactors from *A. thaliana* were identified, and these nodes were selected from the complete networks. The first neighbors for all the selected nodes were identified and used to build a subnetwork. Finally, the orthologs on each species for all the genes in the previous subnetworks were identified and used to generate a new subnetwork for each species.

Network analysis and visualization

All networks were imported into the software package Cytoscape (Smoot et al., 2011) for their visualization using the Prefuse Force Directed layout.
The measures of network topology were calculated using both predefined and custom-made functions. The gene-gene co-expression and neighborhood conservation were determined following the approach described by Netotea et al. (2014), using Fisher exact tests to check for statistical significance.
Gene Ontology analysis on Neigh subnetworks was made with AgriGO (Du et al., 2010), and represented with ReviGO (Supek et al., 2011).

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5. Supplementary material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.00626/full#supplementary-material

TABLE S1 | Compilation of DELLA interactors used in this study.

TABLE S2 | Genes included in the ‘Neighbors’ subnetworks.

TABLE S3 | Genes included in the ‘Orthologs’ subnetworks.

TABLE S4 | Gene Ontology categories enriched in the 'Neighbors' subnetworks.

TABLE S5 | RNA-seq datasets used for the construction of the new gene co-expression networks in *Physcomitrella patens* and *Solanum lycopersicum*.

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Chapter 2

Evolution of DELLA Proteins as Transcriptional Hubs

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0. Abstract

DELLA proteins are land-plant specific transcriptional regulators that transduce environmental information to multiple processes all along a plant's life (Davière and Achard, 2013; Claeys et al., 2014; Vera-Sirera et al., 2015). The molecular basis for this essential function in angiosperms has been linked to their capacity to interact with hundreds of transcription factors (TFs) (Marín-de La Rosa et al., 2014; Lantzouni et al., 2020). However, it is not clear whether this promiscuity is an ancestral property of DELLA proteins or has been gradually acquired during plant evolution (Blázquez et al., 2020; Hernández-García et al., 2020). Here we show that representative DELLAs from the main plant lineages display a conserved ability to interact with multiple TFs, and we define a minimal set of common core functions controlled by DELLAs in all the species tested. We propose that promiscuity was encoded in the ancestral DELLA protein, and that this property has been maintained partly through TF coevolution, while the increase in complexity of the DELLA-dependent transcriptional network simply reflects the functional evolution of their interacting partners.

1. Introduction

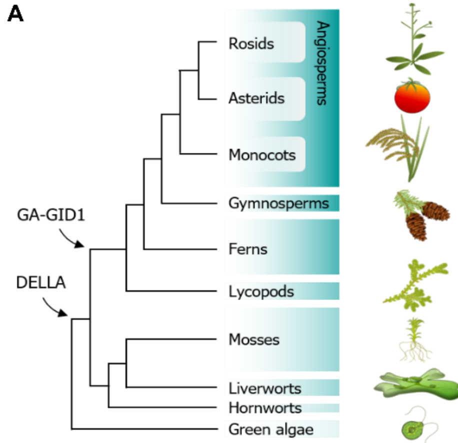
DELLA proteins were first identified in vascular plants as key elements in the signaling pathway triggered by the plant hormones gibberellins (GAs) (Sun and Gubler, 2004; Davière and Achard, 2013). The perception of GAs by GID1 promotes the ubiquitination and degradation of DELLAs, which regulate the activity of a large number of TFs and other transcriptional regulators through direct physical interaction (de Lucas et al., 2008; Marín-de La Rosa et al., 2014; Lantzouni et al., 2020). GA levels are a proxy for environmental information, so it has been proposed that DELLAs coordinate transcriptional programs in different tissues and organs in response to environmental fluctuations (Locascio et al., 2013; Claeys et al., 2014). Unlike other hormones whose action is based on the combinatorial activity

of a small set of paralogous TFs and their regulators, GA functional diversity largely relies on the capacity of DELLAs to interact with a wide variety of TFs from different families and expressed in a tissue- or organ-specific manner (Blázquez et al., 2020). Given that GA perception is restricted to vascular plants but DELLAs are also present in the genomes of non-vascular land plants (Hernández-García et al., 2020), it is unclear if this key capacity of DELLA proteins is an ancestral property, or was acquired during evolution in conjunction with the emergence of GA signaling. Therefore, we have undertaken a comparative molecular genetic and genomic study with representative species of the plant lineage (Figure 1A), with the goal of understanding the evolutionary circumstances that drive the evolution of a transcriptional hub.

2. Results and discussion

DELLA promiscuity is a conserved trait

To gain insight into the conservation of the DELLA interactome in plants, we selected a core set of 42 proteins (covering all major families of TFs and transcriptional regulators) known to be DELLA partners in *A. thaliana*, and examined the ability of DELLAs from another angiosperm (*S. lycopersicum,* SlPRO), a lycophyte (*S. moellendorfii,* SmDELLA1), and a liverwort (*M. polymorpha*, MpDELLA) to interact in a yeast two-hybrid assay with the corresponding orthologs of these AtDELLA partners in each species. Given that all the interactions occur through the C-terminal GRAS domain, a truncated version of each DELLA without the N-terminal domain was used. As expected, all of them interacted with AtRGA, and the interactions were also conserved at a very high level in the case of the other three species: 74% for SlPRO, 71% SmDELLA1, and 85% for MpDELLA (Figure 1B). Moreover, 98% of the interactions were detected in at least two species, suggesting that the ability of DELLAs to interact with a large number of TFs has been extensively conserved during land plant evolution.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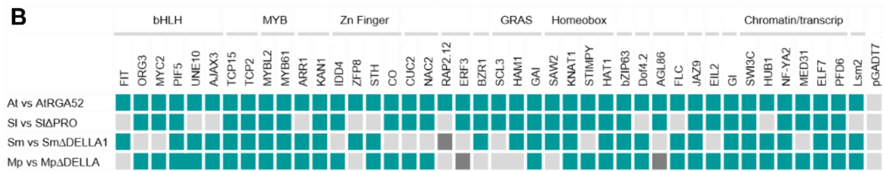
DELLA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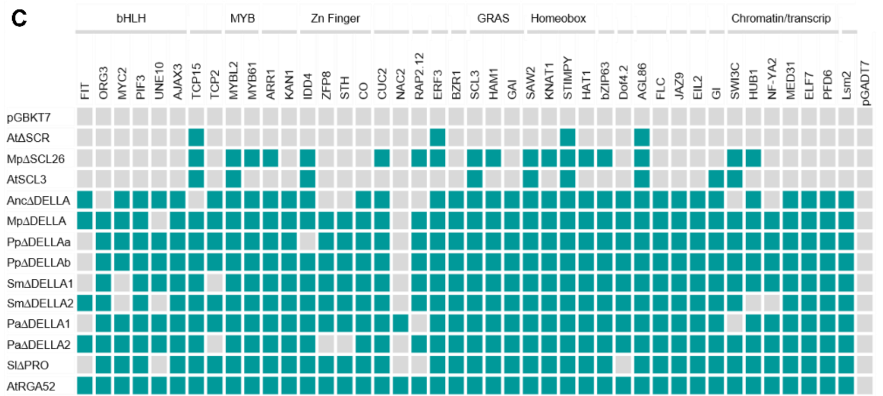
Sm vs SmΔDELLA1
Mp vs MpΔDELLA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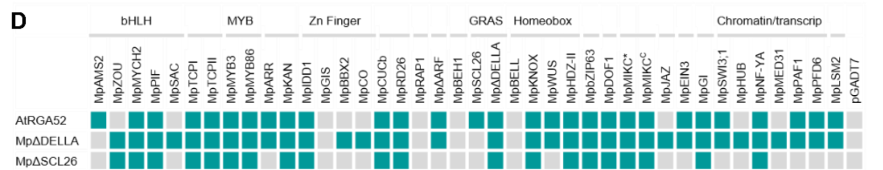
SlΔPRO
AtRGA52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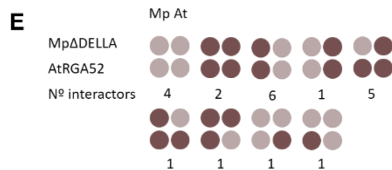
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Figure 1. Yeast two-hybrid screenings for DELLA protein-protein interactions. A, phylogenetic tree of the green lineage with depictions of the species used in this study; **B**, homologous interactions between DELLAs from four species and putative DELLA interactors in each species; **C**, heterologous interactions between DELLAs or other GRAS proteins from different species, and known DELLA interactors from *A. thaliana*; **D**, interactions between different DELLAs or other GRAS proteins, and putative DELLA interactors from *M. polymorpha*; **E**, strength of the interactions between DELLAs from *A. thaliana* and *M. polymorpha*, and DELLA interactors in both species. All interactions were determined by yeast two-hybrid. Rows in B-E correspond to DELLAs and other GRAS, and columns correspond to interactors. In B, C and D, colored squares indicate detected interaction, while light grey squares indicate no detected interaction, and dark grey squares imply lack of a clear ortholog in the genome of a certain species. In E, dark and light circles imply strong and weak interactions respectively; and numbers show the amount of interactors presenting each combination.

To investigate to which point the conservation of these protein-protein interactions depends on the conservation of the DELLA protein itself, or is the result of DELLA-TF coevolution, we tested the capacity of DELLAs from several lineages to establish heterologous interactions with the set of *A. thaliana* TFs (AtTFs). All the DELLA proteins analyzed in the previous experiment, as well as the two moss DELLAs (from *P. patens*), the two gymnosperm DELLAs (from *Picea abies*) and a second SmDELLA2 were able to interact with at least 86% of the AtTFs (Figure 1C). This result suggests that promiscuity is a property encoded in the ancestral GRAS domain of DELLA proteins, an idea further supported by two observations: (i) a resurrected GRAS domain (see Methods section for details) displayed the same ratio of interactions with this set of AtTFs (Fig. 1C); and (ii) reciprocal heterologous interactions were also conserved, as the AtRGA protein interacted with 73% of the MpTF set (Figure 1D). Importantly, although other non-DELLA GRAS proteins also showed a significant capacity of interaction with TFs, the highest ratio observed was only 20% for the closest GRAS paralogs of AtDELLA proteins, SCARECROW-LIKE3 (SCL3) vs the AtTF set (Figure 1C), and only 50% for MpSCL26 vs the MpTF

set (Figure 1D). Considering the significantly higher conservation level of the GRAS domain within the DELLA clade, compared with eight other clades in the GRAS family (Supplementary Figure 1), we propose that DELLAs' promiscuity is an advantageous property actively maintained during evolution, rather than a characteristic achieved by convergent evolution in different lineages.

Despite the conservation of a high interactive capacity in DELLA proteins during plant evolution deduced from the qualitative assays shown above (Figure 1B-D), there are indications that DELLA-TF coevolution has contributed to the specificity of the interactions in different lineages. By applying 3-aminotriazol (3-AT) to titrate homologous and heterologous interactions between DELLAs and TFs from *A. thaliana* and *M. polymorpha* (Figure 1E), we found that in 6/22 cases the strength of the interaction was equivalent for homologous and heterologous interactions; in 7/22, the strength was determined by the TF species; and in 9/22 cases the strength was determined by the combination of DELLA and TF, suggesting a relatively high level of fine-tuning of the DELLA-TF affinity in a species-dependent manner.

DELLA promiscuity is modulated in a taxon-specific manner

Angiosperm DELLA proteins have been shown to undergo different post-translational modifications in various environmental contexts which modulate their activity (Blanco-Touriñán et al., 2020). Thus, a reasonable scenario emerges in which species-specific regulatory mechanisms and species-specific differences in DELLA-TF relative affinities would have contributed to the optimization of DELLA function during evolution. To obtain a faithful picture of the relevance of such mechanisms *in vivo*, we decided to examine the capacity of distant DELLAs to complement the At*dellaKO* mutant. We introduced five DELLA proteins, each one from a different

species (*A. thaliana,* AtRGA; *S. lycopersicum*, SlPRO; *P. abies*, PaDELLA2; *S. moellendorfii*, SmDELLA1; and *M. polymorpha*, MpDELLA) fused to GFP, under the control of the At*RGA* promoter (Gallego-Bartolomé et al., 2010) to obtain native expression patterns. Two sets of lines were selected containing one independent line per species, in which all DELLAs accumulated at a similar level in the nuclei (Supplementary Figures 2 and 3). To focus on DELLA activity and avoid the interference of possible species-specific differences in the sensitivity towards GAs, all the experiments were performed in the presence of paclobutrazol (PAC), a GA synthesis inhibitor. Among the processes affected by DELLA proteins in *A. thaliana*, we evaluated the degree of heterologous complementation of At*dellaKO* in the control of plant size (Dill and Sun, 2001; King et al., 2001; Achard et al., 2009), seed germination (Cao et al., 2005), skototomorphogenic development (Alabadí et al., 2004), and salt stress resistance (Achard et al., 2008). While all DELLAs displayed certain degree of complementation that correlated with their expression levels, there was a marked decrease in the capacity to substitute endogenous DELLAs that was directly dependent on the evolutionary distance between *A. thaliana* and the corresponding species. In other words, the complementation achieved by AtRGA was almost matched by the angiosperm SlPRO and gymnosperm PaDELLA2, but the lycophyte SmDELLA1 and the liverwort MpDELLA were less efficient in the correction of the defects caused by DELLA loss of function with respect to hypocotyl elongation (Figure 2A), seedling fresh weight (Figure 2B and Supplementary Figure 4A), adult plant height (Figure 2C and Supplementary Figure 4B), apical hook opening (Figure 2D), seed germination (Figure 2E), and sensitivity to high salt concentration (Figure 2F).

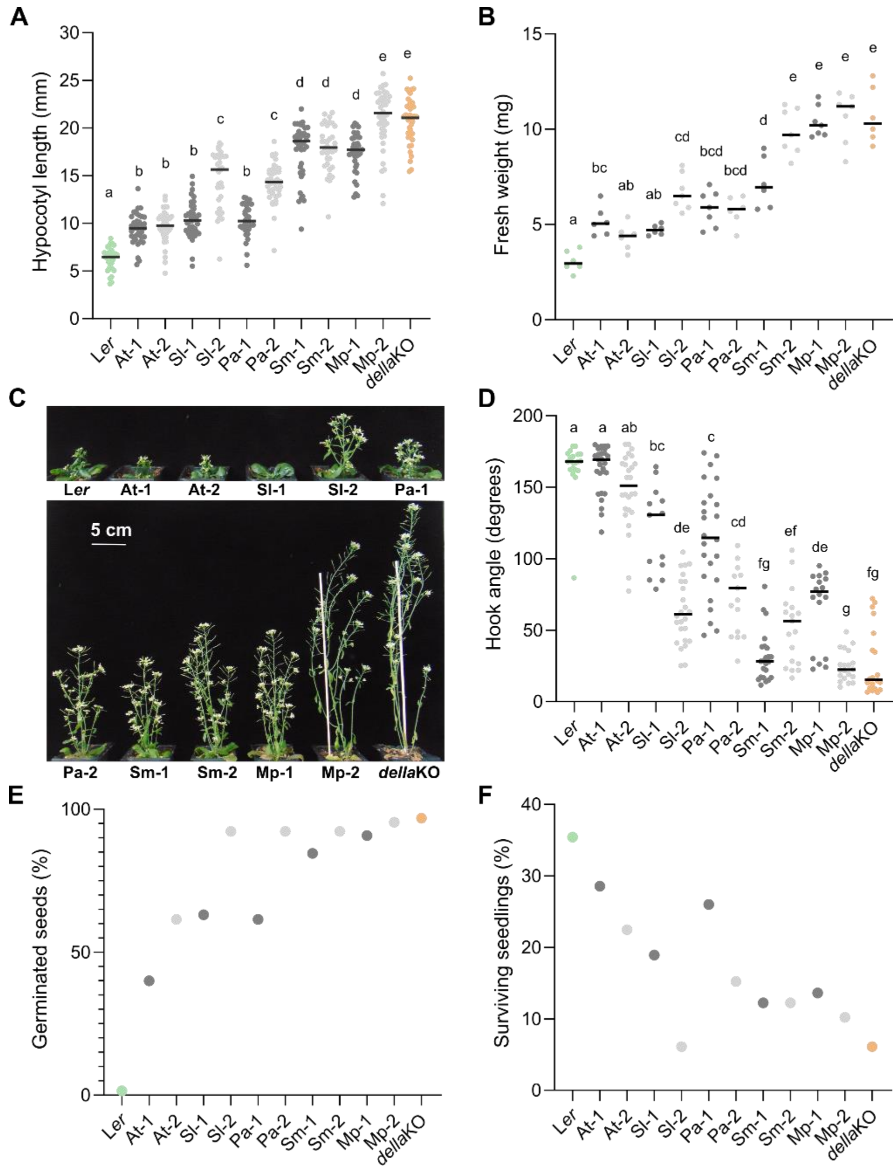


Figure 2. Phenotypical complementation of an *A. thaliana della*KO mutant with DELLAs from five species. A, hypocotyl length of 3-day-old etiolated plants; **B**, fresh weight of 7-day-old seedlings in sets of four; **C**, 30-day-old plants, one representative individual per line; **D**, apical hook angle of 3-day-old etiolated plants; **E**, percentage of germinated seeds after 24h in darkness, in the presence of 1µM PAC; **F**, percentage of surviving

seedlings after 6 days exposed to 250mM NaCl. In A, B and D, horizontal lines represent medians, and letters indicate statistically differentiated groups.

Given that DELLA function is mostly exerted through transcriptional regulation via the interaction with TFs, we investigated the complementation capacity in terms of transcriptomic changes, to obtain an extensive description of the processes and TFs differentially affected by distant DELLAs operating in the cellular context of *A. thaliana*. We therefore performed an RNA-seq analysis of 7-day-old seedlings grown in 0.5 μM PAC including the uncomplemented At*dellaKO* mutant, and one of each of the complemented lines expressing AtRGA, SlPRO, PaDELLA2, SmDELLA1 and MpDELLA (Supplementary Figure 5). All DELLAs were associated with a substantial number of differentially expressed genes (DEGs), with SlPRO being able to alter the expression of an even larger number of genes than AtRGA (Figure 3A). This result might reflect the fact that RGA is only one of five partially redundant DELLAs in *A. thaliana*, while PRO is the only DELLA in *S. lycopersicum*. On the other hand, differences in biological function of DELLA paralogs in *A. thaliana* has been attributed to differences in their expression patterns rather than in the ability to interact with partners (Ikeda et al., 2001a; Feng et al., 2008; Gallego-Bartolomé et al., 2010; Shinozaki et al., 2018; Lantzouni et al., 2020). Given that 50% of the SlDELLA-dependent DEGs are exclusive (Figure 3A), it is likely that this DELLA is able to interact with a larger set of TFs. More importantly, 86% of the AtRGA-dependent DEGs were also under the regulation of the DELLA of at least one other species, and the direction of the change was the same (Figure 3A and 3B). The highest overlap was detected with the evolutionarily closer species *S. lycopersicum* and *P. abies* and, consistently, a similar degree of overlap was found among the biological functions of the DEGs regulated by each DELLA (Figure 3C).

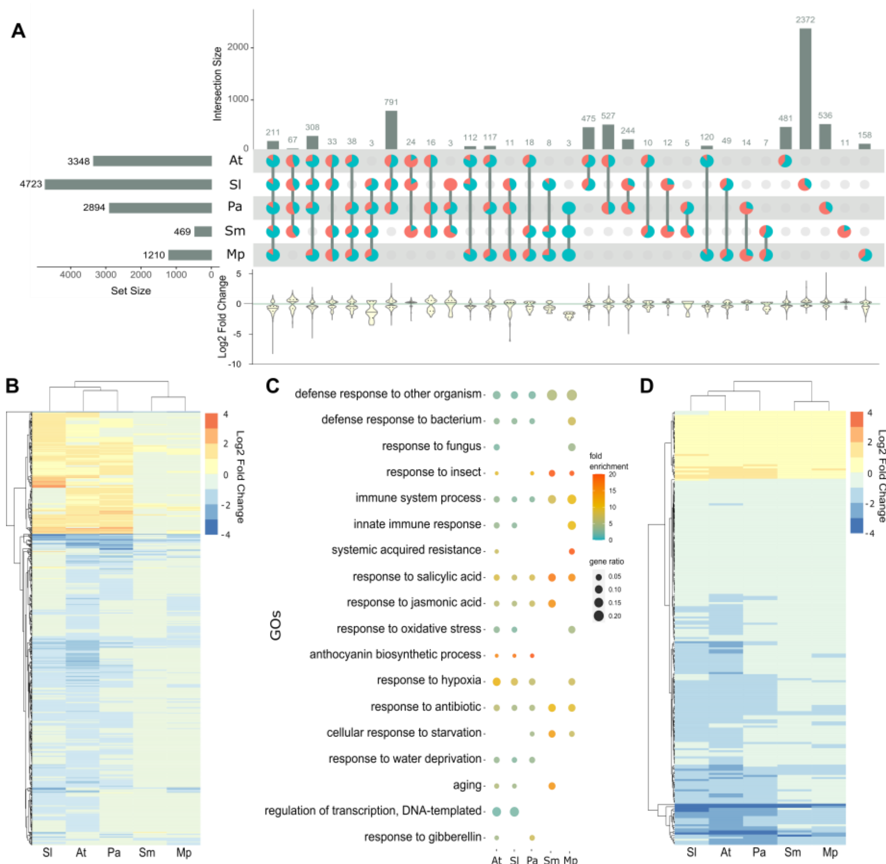


Figure 3. Analysis of DEGs in transgenic lines expressing DELLAs from different species. A, Overlapping DEGs between lines. Set Size indicates the total number of DEGs in each line and Intersection Size indicates the number DEGs overlapping between the lines included in each possible intersection. Pie charts show, for each species at each intersection, the percentage of up and down regulated genes, in red and blue respectively. Violin plots illustrate the Log2 Fold Change of all the genes contained in each intersection. **B**, heatmap with clustering of DEGs based on their Log2 Fold Change values. Rows correspond to genes differentially expressed in more than one of the lines, with a Log2 Fold Change value higher than 1 or lower than -1 in at least one of them. **C**, enriched GO categories among DEGs of the different lines. Categories with fold enrichment higher than 20 are shown in red. **D**, heatmap with clustering of DEGs based on their Log2 Fold Change values. Rows correspond to genes differentially expressed in all five lines.

Functions related to the response to pathogen infections are among the ones regulated by all DELLAs in *A. thaliana*, while other functions, like the response to water deprivation, are regulated only by DELLAs from Spermatophyta (Figure 3C and Supplementary Table 1). Particularly interesting is the overlap between the five species in the regulation of a set of 211 DEGs (Figure 3D) which define a set of functions that had not been previously attributed to DELLA regulation, like the response to hypoxia, heat, antibiotics, or to unfolded proteins in the endoplasmic reticulum (Supplementary Table 2). In summary, the partial complementation at the transcriptomic level of the At*dellaKO* mutant by DELLAs from other species suggests that, despite the general conservation of promiscuity among DELLAs, additional factors modulate DELLA-TF interactions in a species-specific manner. This conclusion is further supported by the observation that heterologous overexpression of At*RGA* in *M. polymorpha* provoked much weaker effects than the homologous Mp*DELLA* gene in terms of growth repression (Supplementary Figure 6).

Evolution of DELLA function reflects the life history traits of the interacting partners

Although all previous results point to an intrinsic capacity of the ancestral DELLA protein to act as a transcriptional hub, they do not demonstrate that this function is indeed conserved across land plant evolution. To investigate the capacity of DELLAs to act as hubs in species other than *A. thaliana*, we decided to compare the DELLA-dependent transcriptomes in the dicots *A. thaliana* and *S. lycopersicum*, the monocot *O. sativa*, the lycophyte *S. kraussiana*, and the liverwort *M. polymorpha* –spanning an evolutionary distance of around 400 M years. For the first three species, DELLA loss-of-function mutants are available (Ikeda et al., 2001a; Feng et al., 2008; Livne et al., 2015), so the comparison between At*dellaKO*, Os*slr1* and Sl*pro* and their respective wild types would define the transcriptome mobilized by DELLAs in each species. Despite the lack of *della* mutants in *S. kraussiana*,

a GA treatment is an efficient way to remove DELLAs in *Selaginella* spp (Hirano et al., 2007; Yasumura et al., 2007); and the DELLA-dependent transcriptome in *M. polymorpha* is available through the comparison between wild-type and MpDELLA overexpressing lines (Jorge Hernández-García, unpublished results).

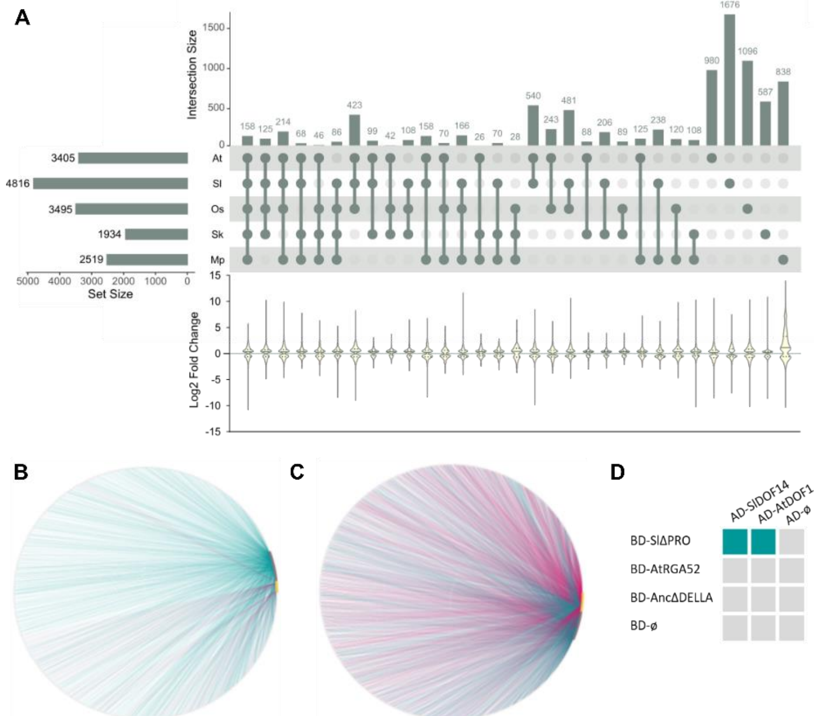


Figure 4. Analysis of DELLA-regulated genes in different species. A, shared DELLA-regulated orthogroups between species. Set size indicates the total number of orthogroups with at least one DEG in each species. Intersection size indicates the number of orthogroups with at least one DEG overlapping between the species included in each possible intersection. Violin plots illustrate the Log2 Fold Change values of all the genes contained in each intersection. **B**, TFs which possess significantly over-represented targets among the genes regulated by DELLAs only in *A. thaliana*, and their corresponding targets. Nodes in the border represent targets in white, *A. thaliana* enriched TFs in black and *S. lycopersicum* enriched TFs in yellow. Edges link each TF with its targets, in blue for *A. thaliana* and in red for *S.*

lycopersicum. **C**, same type of representation as B, but in this case for TFs with significantly over-represented targets among the genes regulated by DELLAs only in *S. lycopersicum*. **D**, protein-protein interactions between two DOF TFs from *A. thaliana* and *S. lycopersicum* with the same predicted targets, and DELLAs from the two species. Colored and light gray squares imply detected and non-detected interactions, respectively.

RNAseq analyses were performed (see Methods for details) and DEGs were determined between the conditions with high and low DELLA levels for each species. For inter-species comparison of the transcriptomes, orthogroups (OGs) were first defined between the five species. Similar numbers of genes were mobilized by DELLAs in each species, ranging from 1934 OGs in *S. kraussiana* to 4816 in *S. lycopersicum* (Figure 4A). Interestingly, only around 30% of the differentially expressed OGs in each species were unique to it, while a large set of the OGs were common to at least three species (42% in *A. thaliana*, 35% in *S. lycopersicum*, 42% in *O. sativa*, 44% in *S. kraussiana*, and 43% in *M. polymorpha*) (Figure 4A). These results are a strong indication of the extensive conservation in molecular targets for DELLAs across evolution, possibly caused by the largely conserved interactome. However, the identification of genes regulated in a species-specific manner denotes the existence of alternative mechanisms that have operated during evolution to optimize DELLA functions in extant plants. Species-specific DELLA transcriptional targets may have emerged from the loss or gain of particular DELLA-TF interactions, but also from the loss or gain of an interacting TF's capacity to regulate downstream targets. To explore these two possibilities, we searched for enriched regulatory elements in the promoters of exclusive *A. thaliana* DELLA targets and their corresponding *S. lycopersicum* orthologs (Jin et al., 2017; Tian et al., 2020). We found that only 25% of the genes shared the same set of putative TF regulators in both species (Figure 4B), suggesting that these genes are not DELLA targets in *S. lycopersicum* because they are regulated by different sets of TFs in each species. On the other hand, when we did the same analysis with the promoters of exclusive *S. lycopersicum* DELLA targets and their orthologs

in *A. thaliana*, we found that 80% of these genes were regulated by the same set of putative TF regulators in both species (Figure 4C), with DOF TFs explaining most of the coincidental regulation. This result illustrates the alternative mechanism by which a large number of targets would be unique for DELLA in *S. lycopersicum* because they are regulated by a particular DELLA-TF interaction happening only in this species. In agreement with this idea, we found that SlPRO was able to interact with SlDOF14 in a yeast two-hybrid assay, while AtRGA did not interact with its ortholog AtDOF1 (Figure 4D and Supplementary Figure 7).

In summary, genomic analysis of DELLA targets in several vascular and non-vascular species demonstrates that DELLAs have conserved their role as transcriptional hubs, and that the gain and loss of particular sets of transcriptional targets has been probably due not only to the establishment and loss of specific DELLA-TF interactions in certain clades, but also to evolutionary changes attributable to the TFs themselves. Given the scarcity of functional information derived from direct experimental evidence for most of the species used in this study, it is difficult to establish if the molecular conservation results in the conservation of biological processes regulated by DELLAs in the different species. Nevertheless, we focused our gene ontology enrichment analysis on the OGs that were DELLA-dependent in the four vascular plants with an active GA signaling pathway, and the OGs common to all five species. We found that the DEGs common to vascular plants were enriched in functions related to the response to biotic and abiotic stress, hormone signaling, cell growth and cell wall modification (Supplementary Table 3), while the genes mobilized by DELLAs in all the species tested represented more basic cellular processes, such as glycolysis, amino acid synthesis, and transcriptional regulation, as well as cell growth and the response to abiotic stress (Supplementary Table 4). Thus, the conservation of molecular targets during evolution seems to be paralleled by certain degree of conservation of basic DELLA-dependent

functions in extant plants. Interestingly, this experimental evidence supports previous observations using *in silico* network analyses that pointed to stress responses as a likely ancestral function of DELLAs (Briones-Moreno et al., 2017).

3. Conclusion

Our study reveals an evolutionary model in which the ancestral DELLA soon acquired an extensive capacity to interact with multiple TFs, which is now maintained in extant plants of vascular and non-vascular clades, irrespective of the presence of a GA perception module. Such a model – contrary to the gradual development of the high degree of connectivity– has multiple implications, both from basic and applied perspectives. For instance, it becomes evident that the diversity of functions regulated by GAs in vascular plants (many of which have profound impact in cultivated species) is a direct consequence of DELLA's conserved promiscuity. The fact that this property has been conserved for over 400 M years is a faithful measure of its physiological relevance and highlights the constraints under which this type of 'hub' proteins evolve.

4. Materials and Methods

DELLA interactome studies

To assess the conservation of the DELLA interactome through yeast two-hybrid screenings, collections of DELLA putative interactors expressed in yeast were created for four different species. To select the members of the collections, an exhaustive literature search was conducted on DELLA reported interactions; this information was compiled, and a few representative members of each protein family were chosen. Gateway entry clones were obtained for these DELLA known interactors in *Arabidopsis*, by resorting to existing transcription factor collections and manual cloning when

needed. The unavailable genes were amplified from *A. thaliana* (ecotype Landsberg erecta, L*er*) cDNA using *att*B-PCR primers and introduced in entry vectors through BP recombination reaction. Expression clones were created by transferring these genes to the destination vector pGADT7 through LR recombination reaction. This process results in the fusion of the CDS with the Gal-4 activation domain contained in the pGADT7 vector. A truncated version of the *Arabidopsis* DELLA RGA (RGA52) including only the GRAS C-terminal domain, responsible for most DELLA protein-protein interactions, was introduced in the destination vector pGBKT7 (Gal-4 binding domain) using the same procedures. DELLA interactors in pGADT7 and pGBKT7-RGA52 were transformed in the yeast haploid strains Y187 and Y2H-Gold (Clontech) respectively, by subjecting yeast cells to a 42ºC heat shock in the presence of polyethylene glycol and Lithium acetate. Transformants were grown in SD selective medium without leucine or tryptophan depending on the transformed vector (-L for pGADT7 and -W for pGBKT7). Diploid yeast containing both types of plasmids were obtained by yeast mating, induced by co-culture of both strains in liquid YPD medium. After selecting diploids in SD -L/W, they were grown in liquid until saturation and dropped in SD plates (-L/W as a growth control and -L/W/H). Interactions were considered positive when the respective drop grew visibly in SD -L/W/H after 4 days. Only the proteins which showed a clear interaction with RGA52 in this system were eligible for the collection. With this information, a definitive list of interactors was established for subsequent experiments. For the collections of DELLA putative interactors in *S. lycopersicum*, *S. moellendorfii* and *M. polymorpha*; a search for the most reliable orthologs was conducted using PLAZA Integrative Orthology Viewer (Proost et al., 2009), BAR expressolog identification (Patel et al., 2012), Phytozome (Goodstein et al., 2012), MarpolBase (http://marchantia.info), OrthoMCL-DB (Chen, 2006) and OneKP (Carpenter et al., 2019; Leebens-Mack et al., 2019). The retrieved gene sequences were synthesized and introduced in pGADT7 for direct transformation in

yeast. *DELLA* genes from *S. moellendorfii* (Sm*DELLA1* and Sm*DELLA2*) and *P. abies* (Pa*DELLA1* and Pa*DELLA2*) were also synthesized, while those from *M. polymorpha* (Mp*DELLA*), *P. patens* (Pp*DELLAa* and Pp*DELLAb*) and *S. lycopersicum* (*PRO*) were amplified from cDNA. The sequence of the ancestral *DELLA* gene was obtained as described in Hernández-García et al. (2019) and synthesized. Other three non-DELLA GRAS genes were also amplified from cDNA: SCARECROW (At*SCR*) and SCARECROW-LIKE 3 (At*SCL3*) from *A. thaliana*, and SCARECROW-LIKE 26 (Mp*SCL26*) from *M. polymorpha*. They were all introduced in Entry Vectors, and truncated versions were obtained by amplification of their C-terminal. Sequences of all truncated versions can be found in Supplementary Table 5. All Y2H screenings were performed in the same conditions, and the strength of the interactions was assessed by using SD -L/W/H plates supplemented with 2,5µM 3-Amino-1,2,4-triazole (3-AT); a competitive inhibitor of HIS3 that reduces histidine production by the yeast. Interactions were considered strong when diploids grew in the presence of 3-AT, and weak when they did not.

Determination of 3D structure and conservation of GRAS domains

The model for the 3D structure of the GRAS domain of AtGAI was obtained through the Protein Homology/analogy Recognition Engine (Phyre2) with a 99.9% confidence value (Kelley et al., 2015). Conservation indices for each residue were calculated after multiple sequence alignment of the GRAS domains of DELLA proteins and other GRAS proteins separately, using ProtSkin software (Ritter et al., 2004), which also generated the color-coded file for subsequent mapping on the GRAS domain PDB structure in PyMol (DeLano, 2020).

Heterologous complementation tests in *A. thaliana*

DELLAs from five different species were expressed in an *A. thaliana della*KO mutant under the promoter of an endogenous DELLA, to assess their ability to recover DELLA-associated phenotypes and gene expression profiles.

Plasmid constructions

All plasmids employed in the creation of transgenic lines for the complementation assays were obtained using a combination of GoldenBraid (GB) and Gateway systems. Each plasmid contains two transcriptional units (TUs); one of them to recreate native patterns of DELLA expression in *Arabidopsis*, and the other for the selection of transformed seeds. For the first TU, genomic context of the *Arabidopsis* DELLA *RGA* was amplified from L*er* genomic DNA, up and downstream of the gene (3.7 Kb of 5’ UTR and 2.8 Kb of 3’ UTR). Additionally, a Gateway recombination cassette containing the *ccd*B selection gene was amplified from a Gateway Destination Vector. This cassette would assume the position of the main CDS, so that *DELLA* genes from different species could be introduced in the final construct through a LR recombination reaction; thereby avoiding the time-consuming GB domestication (elimination of certain restriction sites). In all cases, primers were designed using GB online tools (Sarrion-Perdigones et al., 2013) to establish the order of each element inside the TU, and every amplicon was introduced in a pUPD vector. Finally, a pUPD vector containing a gene coding a Yellow Fluorescent Protein (designed for its use as a reporter gene in C-terminal fusions) was retrieved from the GB collection; and the four pieces were inserted together in a pEGB3-α2r vector. The second TU consists of a gene encoding the fluorescent protein DsRED under the regulation of the seed-specific At2S3 promoter and the 35S terminator, in a pEGB3-α1 vector (Aliaga-Franco et al., 2019). Both TUs were introduced in a pEGB3-Ω1 vector, placing the DELLA expression TU (pRGA::GW:YFP:tRGA) at the Right Border, and the seed selection TU

(pAt2S3::DsRED:t35S) at the Left Border. Once the final construct was obtained, full-length versions of *DELLA* genes from *A. thaliana* (*RGA*), *S. lycopersicum* (*PRO*), *P. abies* (*PaDELLA2*), *S. moellendorfii* (*SmDELLA1*) and *M. polymorpha* (*MpDELLA*) were introduced in the Gateway cassette by LR recombination.

Plant transformation and selection

Omega level plasmids containing each *DELLA* gene were introduced by electroporation in *Agrobacterium tumefaciens* C58 strain electrocompetent cells. Positive *Agrobacterium* transformants were verified by colony PCR and grown in liquid LB medium with antibiotics for their use in *Arabidopsis* transformation by the floral dip method. Pentuple *della* knock-out mutant (*dellaKO*) plants in L*er* ecotype (NASC ID: N16298) were used as background for the complementation assay. Once the mutations in the five *Arabidopsis DELLA* genes were checked by PCR (primers in Supplementary Table 6), *dellaKO* plants were grown in soil for two weeks under long-day conditions (16 hours of light and 8 hours of darkness) before transformation. Transgenic seeds were selected using DsRED fluorescence. 20 seeds with intense fluorescent signal were collected from among the offspring of each set of transformed plants (five sets, each one expressing a different DELLA). Adult plants grown from those seeds were genotyped to confirm the correct insertion of each *DELLA* gene, by amplifying the whole CDS from the end of the RGA promoter to the beginning of the YFP and Sanger sequencing of the PCR product (primers in Supplementary Table 6). Two more plant generations were obtained by permitting self-pollination. The proportion of fluorescent seeds was used as a measure of segregation, allowing the selection of transgenic lines with single-copy insertions in the first generation and homozygous for the transgene in the second. Among those, two independent lines were chosen from each set, considering a sufficient DELLA accumulation and localization in the nuclei as the main criteria. For this selection, seeds from each line

were sown in half strength MS medium supplemented with 0.5 µM paclobutrazol (PAC) to minimize DELLA degradation, stratified for 3 days at 4ºC, and grown in a phytotron for 10 days under long-day conditions. Some of the seedlings were used in confocal microscopy to detect YFP fluorescence in nuclei of the root tip. The rest of them were frozen in liquid Nitrogen, ground with mortar and pestle, and subjected to native protein extraction and protein quantification by Bradford assay; for subsequent protein electrophoresis in 12% polyacrylamide gels and Western Blot. DELLA-YFP and the control protein DET3 were detected using JL-8 and Anti-DET3 antibodies (Clontech), respectively.

Phenotypical analyses

To evaluate the performance of the 5 chosen *DELLA* genes when expressed in the *A. thaliana della*KO mutant, a series of DELLA-associated phenotypic traits were assessed in 12 plant genotypes: 2 independent transgenic lines for the expression of each different DELLA (all of them with a single-copy insertion of the transgene in homozygosis and sufficient DELLA accumulation in the nuclei), L*er* wild type as a positive control for DELLA activity, and *della*KO as a negative control. Considering the different susceptibility to GA-mediated degradation of the employed DELLA proteins, all tests were performed in the presence of PAC to maximize DELLA accumulation in every case. For all *in vitro* assays, seeds were sown in Petri dishes containing half strength MS medium supplemented with PAC (1 µM for the germination tests and 0.5 µM for the rest) and stratified for 3 days at 4ºC. Some of the plates were placed in a phytotron at 22ºC in light for 8h, and then covered in 3 layers of aluminum foil. After 3 days, etiolated seedlings were transferred to transparent plastic sheets and scanned. Hypocotyl length and apical hook angle were measured for 20-40 seedlings of each line using ImageJ (Abràmoff et al., 2004). Other plates were placed in light (long-day) for 7 days, then 28-32 seedlings of each line were weighed in a precision scale in sets of 4 to determine their fresh weight. Germination

rates were established by visual inspection of 75 seeds from each line after 24h at 22ºC in darkness. Seeds were considered germinated if emerging radicles were detected under binocular loupe. Tolerance to salt stress was assessed by transferring 7-day-old seedlings (50 seedlings per line) grown in light (long-day), to plates supplemented with 250 mM NaCl, and counting the number of surviving seedlings after 6 days. Seedlings were considered alive when green areas were observed. For size measurement in adult plants, seeds were sown in individual pots containing soaked soil mix (2:1:1 peat, vermiculite and perlite), stratified for 3 days at 4ºC and then grown in a growth chamber at 22ºC under long-day conditions. After 7 days, plants were watered once a week with 10 µM PAC dissolved in water. The length of the main stem, from the rosette to the tip, was measured in 30-day-old plants (18 plants per line). One-way ANOVA with post-hoc Tukey HSD test was employed to find statistically significant differences between phenotypes of different complemented lines.

RNA-seq assay

The degree of transcriptional complementation conferred by different DELLAs was determined through RNA-sequencing of seven genotypes: one *A. thaliana* transgenic line for each expressed DELLA (RGA, PRO, PaDELLA2, SmDELLA1 and MpDELLA, in all cases line number 1), L*er* wild type and the *della*KO mutant. 30 seeds of each line were sown in triplicate in Petri dishes containing half strength MS medium supplemented with 0.5 µM PAC, stratified for 3 days at 4ºC and transferred to growth chambers at 22ºC under long-day conditions for 7 days. Three samples consisting of pools of whole 7-day-old seedlings were taken from each genotype, frozen in liquid nitrogen and ground with mortar and pestle. Total RNA was isolated using NucleoSpin™ RNA Plant Kit from Macherey-Nagel and sent to BGI Europe. Quality control, mRNA enrichment, cDNA library construction and sequencing on DNBseq platform were performed by BGI. Thus, 100bp paired-end reads were retrieved, and their quality was analyzed with

FastQC (v0.11.5 with default parameters). Then they were trimmed with Cutadapt (v1.18 with parameters '--minimum-length=20 --max-n=0.1 --quality-cutoff=30,30') (Martin, 2011) and mapped with HISAT2 (v2.1.0 with default parameters) (Kim et al., 2015) to the *A. thaliana* genome (TAIR10). The read count was carried out with htseq-count (v0.11.2 with parameters '--format=bam --order=name --stranded=no') (Anders et al., 2015), and RPKMs were calculated for their use as an indicator of gene expression. Genes with at least 1 RPKM in all three replicates of a sample were considered expressed and included in the analysis of differential expression with DESeq2 v1.24.0 (Love et al., 2014), where *della*KO was taken as the reference for the other six genotypes. Only differentially expressed genes (DEGs) whose adjusted P-values (padjust) were lower than 0.05 were used in all successive analyses. All DEGs for the six comparisons can be found in Supplementary Table 7.

Enriched functions among genes regulated by each DELLA were found using the GO Enrichment Analysis tool available at geneontology.org, connected to the analysis tool from the PANTHER Classification System (Thomas et al., 2003), with default settings. To highlight the most significant functions, only DEGs with a Log2 Fold Change value higher than 1 or lower than -1 were used in the analysis. Enriched GO terms with False Discovery Rate values lower than 0.05 were considered exclusively.

Heterologous complementation tests in *M. polymorpha*

M. polymorpha male accession Takaragaike-1 (Tak-1, Ishizaki et al., 2008), was cultured aseptically on half-strength Gamborg's B5 medium (Gamborg et al., 1968) containing 1% agar under long day conditions at 22ºC, and maintained asexually. To obtain lines overexpressing *RGA* and *MpDELLA*, both genes were transferred from the previously available full-length Gateway entry vectors, into the Gateway binary vector pMpGWB106 (Ishizaki et al., 2015) through LR reaction. The resulting vectors were

introduced into regenerating thalli of Tak-1 by *Agrobacterium tumefaciens* (C58) -mediated transformation as previously described (Kubota et al., 2013). Transformants were selected with 10 µg/ml hygromycin B and 100 µg/ml cefotaxime.

Whole thallus growth was quantified by measuring the projected area of 2-weeks old gemmallings using ImageJ (Abràmoff et al., 2004). Four independent lines for each DELLA overexpression and the Tak-1 wild type were analyzed, and One-way ANOVA with post-hoc Tukey HSD test was employed to find statistically significant differences between them.

Comparison of DELLA-regulated genes in different species

Plant Materials and Growth Conditions

In order to determine which genes and functions are regulated by DELLAs from different species in their own biological environment, we conducted RNA-sequencing assays where transcriptomic data was compared between samples with higher and lower accumulation of DELLA proteins in 5 plant species (*A. thaliana*, *S. lycopersicum*, *O. sativa, S. kraussiana* and *M. polymorpha*). For three of them, *della*KO mutants were available, so the comparisons were made between each mutant and their respective wild type. In *A. thaliana*, the employed genotypes were pentuple *della*KO mutant (NASC ID: N16298) and L*er* wild type; in *S. lycopersicum*, the complete loss-of-function mutant *pro*$^{\Delta GRAS}$ (Livne et al., 2015) and wild type cultivar M82; and in *O. sativa*, the *slr1-1* mutant (Ikeda et al., 2001b) and the wild type subsp. Japonica cv. Nipponbare. Both tomato and rice *della* mutants are sterile, so the seeds used in the assays were one fourth of the offspring of heterozygous individuals. Phenotypical differences were clear (Supplementary Figure 8), but their genotype was confirmed by PCR amplification and Sanger sequencing of the regions containing the mutations (primers in Supplementary Table 6). Seedlings of the three

species were grown *in vitro* in transparent plastic pots containing half strength MS medium supplemented with PAC, to increase DELLA accumulation in the wild types and consequently maximize their differences with *della*KO mutants. The concentration of PAC to be used in each species was established through previous tests, where seedlings were cultivated in the presence of different concentrations of PAC, and the length of their hypocotyls (*A. thaliana* and *S. lycopersicum*) or coleoptiles (*O. sativa*) was measured as an indirect indicator of DELLA accumulation. The lowest concentration to cause a maximum growth restraint was chosen for each one (0.5μM for *A. thaliana*, and 5μM for *O. sativa* and *S. lycopersicum*) (Supplementary Figure 9). Plant material from *S. kraussiana* was obtained from the botanical collection of the University of Valencia, grown in soil, and propagated by cutting. In this species, the accumulation and degradation of DELLA proteins was induced by treating young plants with PAC and GAs respectively, as described in Hirano et al. (2007). Samples for RNA-seq consisted of three replicates of 7-day-old whole seedlings from *A. thaliana*, *S. lycopersicum* and *O. sativa*; and young treated *S. kraussiana* plants, including stems, leaves and roots. In the case of *M. polymorpha*, generating a *della* knock-out mutant through CRISPR/Cas9 technology was not possible, as a loss-of-function mutation in *MpDELLA* seems to be lethal. As an alternative, transgenic lines overexpressing *MpDELLA* under the constitutive promoter *35S* were obtained as described previously. RNA was extracted from 1-month old plants with excised apical notch regions, from both the Tak-1 wild type and a *MpDELLA* overexpressing line. All species were cultivated in growth chambers at 22°C under long-day conditions. RNA isolation and sequencing were performed exactly like in the transcriptional complementation assay.

RNA-seq assay

For *A. thaliana, S. lycopersicum, O. sativa and S. kraussiana*, 100 bp paired-end reads were obtained from the 3 replicates of each genotype or

treatment. In the case of *M. polymorpha*, the available data were 75 bp single-end from 2 replicates of each genotype. The read qualities were explored using FastQC version 0.11.9. The adaptors were removed from the reads processing the paired-end files together using bbduk version 38.42 with the default adapters file and the following parameters: “ktrim=r k=23 mink=11 hdist=1”. Next, the reads were quality filtered using Trimmomatic (Bolger et al., 2014) version 0.39 with the following parameters: “-phred33 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:35” and the quality of the filtered files was assessed with FastQC.

For the *de novo* assembly of the full *S. kraussiana* transcriptome, all the available filtered reads were included, and Trinity version 2.9.1 (Grabherr et al., 2013) was used with default parameters.

For the differential expression analysis, the full genome and transcriptome were downloaded from NCBI for *A. thaliana, O. sativa, P. patens, S. lycopersicum* and *M. polymorpha*. A decoys file was created for each species using the genome and next the index was created using the index command from *Salmon* version 1.1.0 (Patro et al., 2017). The full transcriptome of *S. kraussiana* was indexed without a decoys file given the lack of a genome assembly. The number of reads per transcript was determined with *salmon quant* using the –validateMappings parameter and the filtered reads file. Both paired-end files while processing each replicate when available. Using the accessory scripts abundance_estimates_to_matrix.pl from Trinity a matrix with counts per transcript in all the replicates was obtained. Finally, the differential expression analysis using DESeq2 was performed using the run_DE_analysis.pl accessory script from Trinity. All the detected DEGs for the five species can be found in Supplementary Tables 8 to 12.

For the definition of orthologous genes, the full proteome was downloaded for the aforementioned species in NCBI and for *S. kraussiana*, the proteome was obtained from the TransDecoder (Haas and Papanicolaou, 2017)

version 5.5.0 output of the full transcriptome assembly. Next, the longest isoform was selected for each gene and the proteins were written on a single file per species in the same folder. Finally, OrthoFinder version 2.3.11 (Emms and Kelly, 2015) was run on the folder containing the proteomes with default parameters. The obtained orthogroups are listed in Supplementary Table 13.

GO enrichment analyses were executed as described before; and TF enrichment analysis was performed using the TF enrichment tool available at plantregmap.gao-lab.org (Tian et al., 2020), selecting the corresponding species for each dataset and "all" in the Method options. A TF is considered enriched if the number of possible targets for it on the input list of genes is higher than expected; and a gene is considered a target if there is experimental evidence or it has cis regulatory elements or binding motifs for the TF. The obtained data were represented using Cytoscape, and they can be found in Supplementary Tables 14 and 15.

5. Supplementary material

Supplementary Tables are available at

http://plasticity.ibmcp.csic.es/downloads.html

Supplementary Table 1. Enriched GO terms among DEGs of each complemented line. GO terms with the highest Log2 Fold Enrichment values were selected. The 'Species' column indicates DEGs were obtained by the expression of AtRGA (At), SlPRO (Sl), PaDELLA2 (Pa), SmDELLA1 (Sm) or MpDELLA (Mp).

Supplementary Table 2. Enriched GO terms among the 211 DEGs common to all complemented lines. GO terms with the highest Log2 Fold Enrichment values were selected.

Supplementary Table 3. Enriched GO terms among DEG-containing OGs in common to the four vascular species. GO terms with the highest Log2 Fold Enrichment values were selected.

Supplementary Table 4. Enriched GO terms among DEG-containing OGs in common to the five species. GO terms with the highest Log2 Fold Enrichment values were selected.

Supplementary Table 5. Sequences of the truncated versions of DELLAs and other GRAS proteins used in the yeast two-hybrid screenings, containing only the C-terminal GRAS domain.

Supplementary Table 6. Oligonucleotides used for genotyping and cloning.

Supplementary Table 7. DELLA-regulated genes in *A. thaliana* complemented lines. Differentially expressed genes (DEGs) obtained by comparing transcriptomic profiles from an *A. thaliana della*KO mutant and transgenic lines expressing DELLAs from different species, in the same background.

Supplementary Table 8. DELLA-regulated genes in *A. thaliana*. Differentially expressed genes obtained by comparing transcriptomic profiles from an *A. thaliana della*KO mutant and its corresponding wild type.

Supplementary Table 9. DELLA-regulated genes in *S. lycopersicum*. Differentially expressed genes obtained by comparing transcriptomic profiles from an *S. lycopersicum della*KO mutant and its corresponding wild type.

Supplementary Table 10. DELLA-regulated genes in *O. sativa*. Differentially expressed genes obtained by comparing transcriptomic profiles from an *O. sativa della*KO mutant and its corresponding wild type.

Supplementary Table 11. DELLA-regulated genes in *S. kraussiana*. Differentially expressed genes obtained by comparing transcriptomic profiles from *S. kraussiana* plants treated with GAs and PAC.

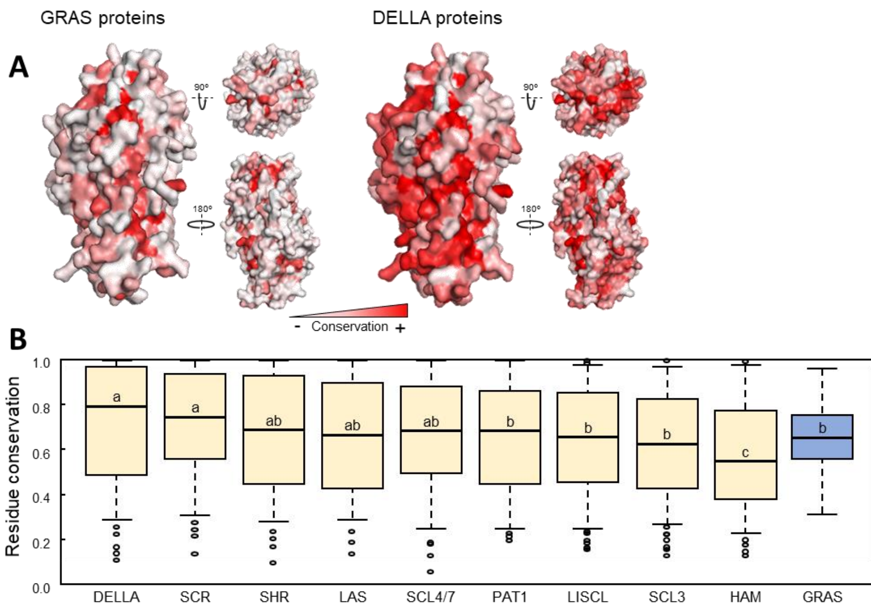
Supplementary Table 12. DELLA-regulated genes in *M. polymorpha*. Differentially expressed genes obtained by comparing transcriptomic

profiles from a *M. polymorpha* transgenic line overexpressing MpDELLA and its corresponding wild type.

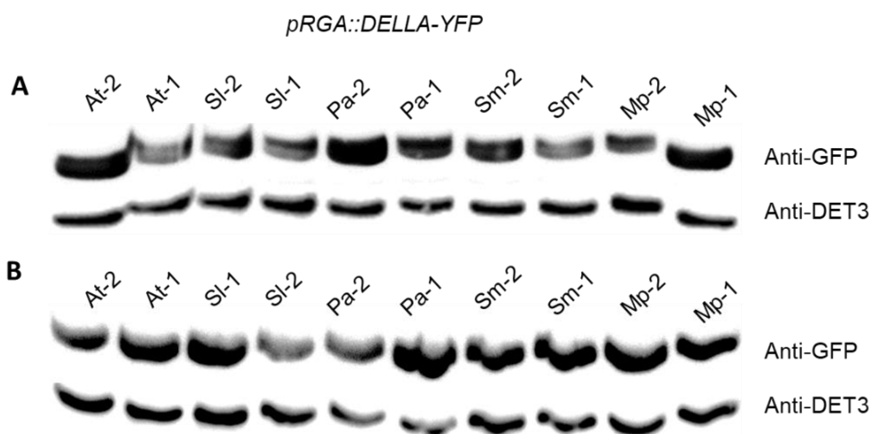
Supplementary Table 13. Orthogroups obtained for *A. thaliana*, *S. lycopersicum*, *O. sativa*, *S. kraussiana* and *M. polymorpha* using OrthoFinder.

Supplementary Table 14. Results of the TF enrichment analysis performed on genes regulated by DELLA only in *A. thaliana*.

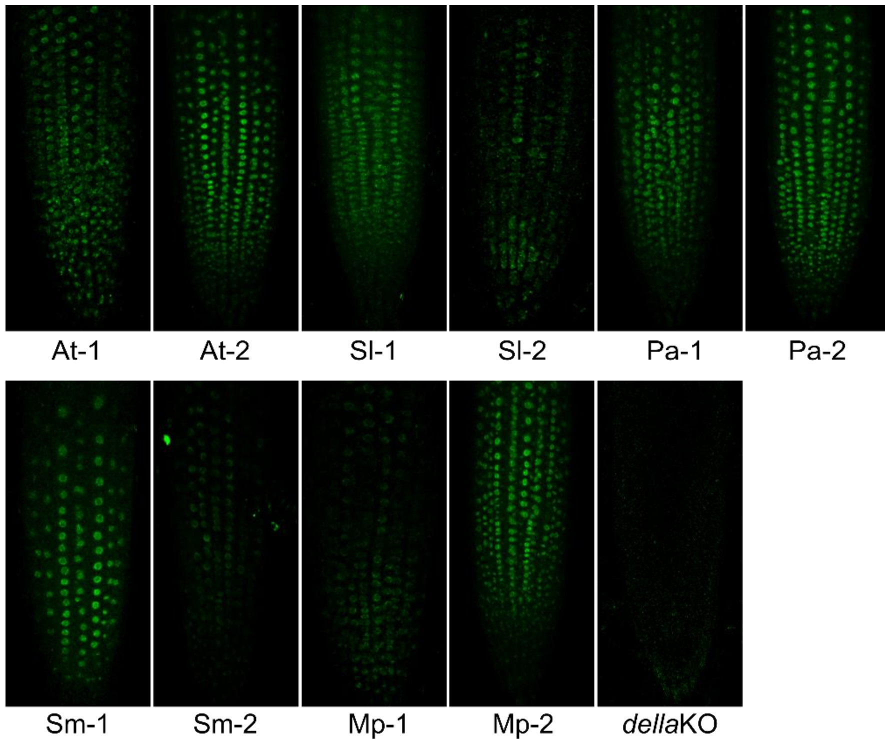
Supplementary Table 15. Results of the TF enrichment analysis performed on genes regulated by DELLA only in *S. lycopersicum*.



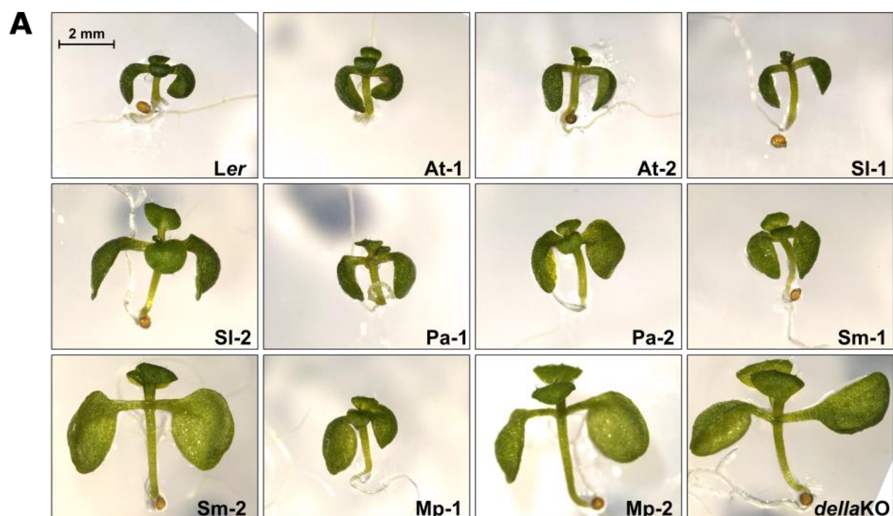
Supplementary Figure 1. 3D structure and conservation of the GRAS domain. A, predicted 3D structure of the GRAS domain of the *A. thaliana* DELLA protein GAI, and residue conservation across the whole GRAS family and across the DELLA subfamily. **B**, residue conservation in the GRAS domains of different GRAS subfamilies (in yellow) and the whole GRAS family (in blue).

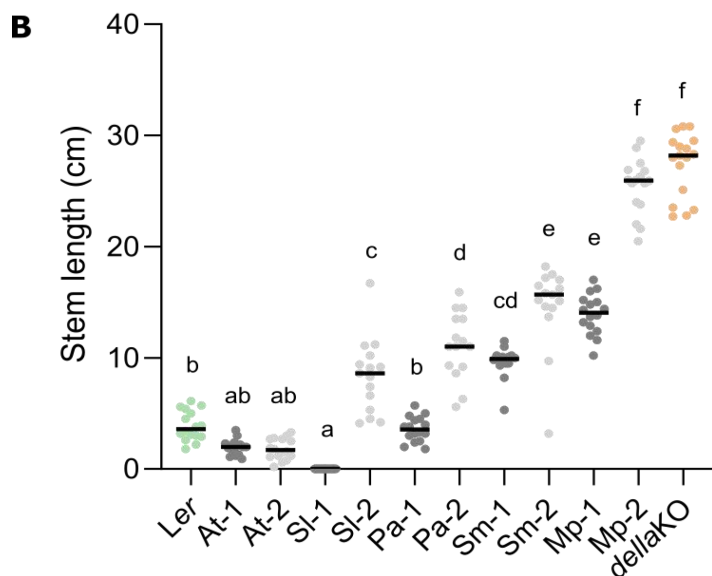


Supplementary Figure 2. DELLA accumulation in complemented lines (*pRGA::DELLA-YFP*). Western Blot assay of whole 7-day-old seedlings (**A**) and 30-day-old adult leaves (**B**). DELLA proteins fused to YFP were detected with the Anti-GFP antibody JL-8. DET3 protein is used as an internal control. Seedlings grown in half strength MS medium with 0.5μM PAC; adult plants watered with 10μM PAC once a week.

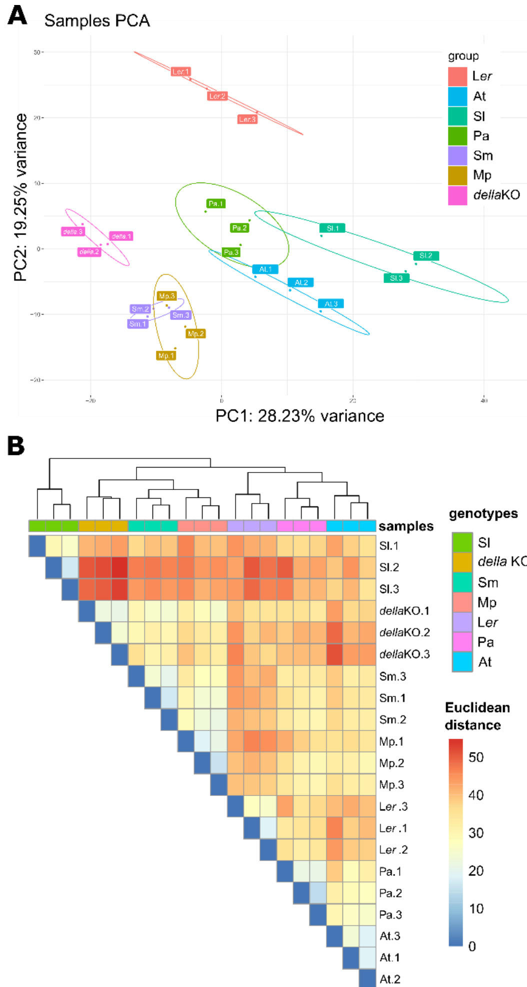


Supplementary figure 3. DELLA expression in the nuclei of complemented lines (*pRGA::DELLA-YFP*). Confocal microscopy images of 7-day-old seedling root tips. YFP fluorescent signal in green. Seedlings grown in half strength MS medium with 0.5μM PAC.

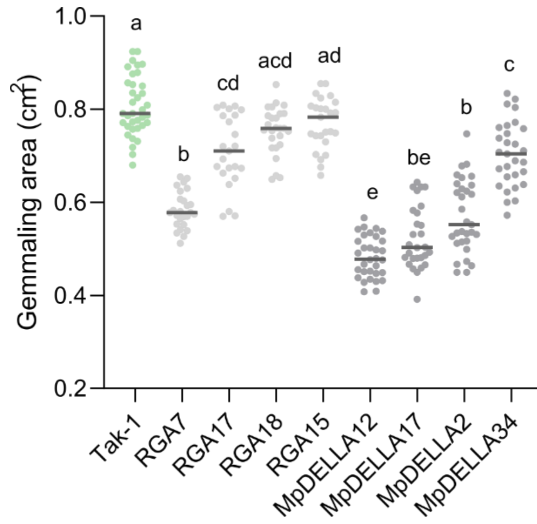




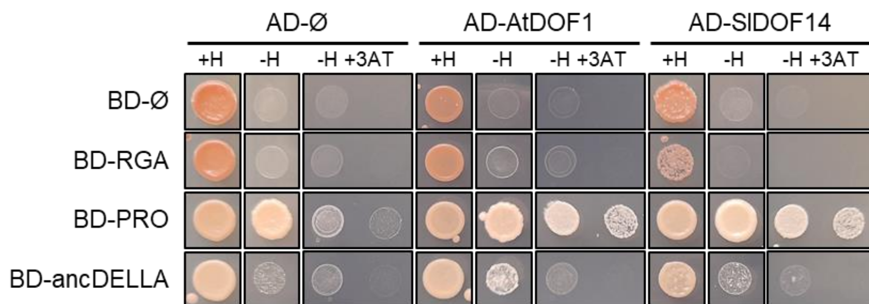
Supplementary Figure 4. Plant growth in complemented lines (*pRGA::DELLA-YFP*). A, representative photographs of 7-day-old seedlings grown in half strength MS medium with 0.5µM PAC under long day conditions. **B**, stem length of 30-day-old plants grown under long day photoperiod and watered with 10µM PAC once a week.



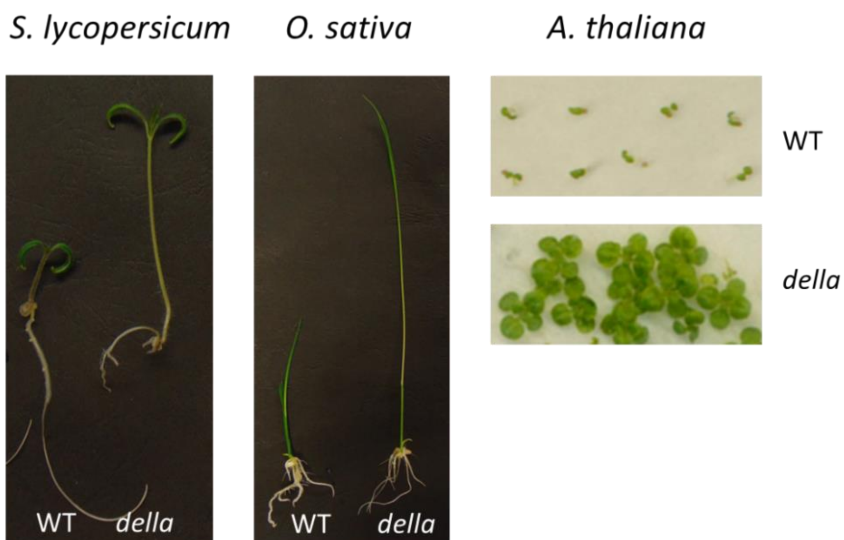
Supplementary Figure 5. Analysis of read counts from RNA-sequencing of complemented lines (*pRGA::DELLA-YFP*). A, Principal Component Analysis, where PC1 explains nearly 30% of the variation and seems to be linked to DELLA activity; while PC2 explains nearly 20% of the variation and separates the wild type L*er* from all the other lines, which have a *della*KO background. **B**, heatmap with clustering of the samples, based on Euclidean distance. Lines expressing DELLAs from basal species are clustered with the *della*KO mutant, while the ones expressing DELLAs from higher species are clustered with the wild type; except for Sl, which appears as an outlier due to its larger number of DEGs. For both analyses, data were normalized by variance-stabilizing transformation and all genes were considered. In all cases the three replicates of each sample group together.



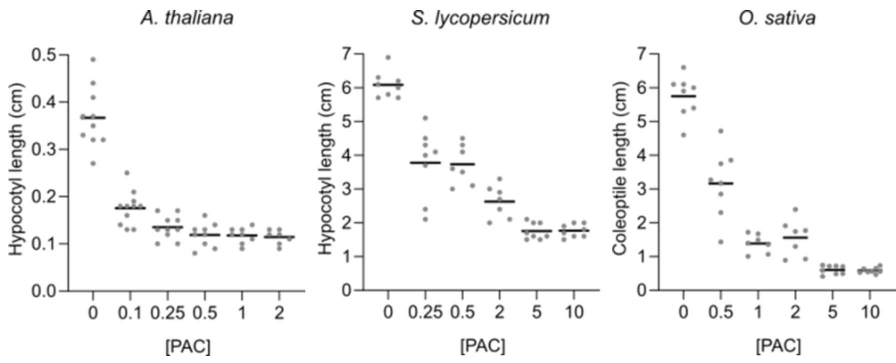
Supplementary Figure 6. Growth reduction in *M. polymorpha* transgenic lines overexpressing AtRGA and MpDELLA. Whole thallus area of 2-weeks-old gemmalings from independent transgenic lines.



Supplementary Figure 7. Interactions between two DOF TFs from *A. thaliana* and *S. lycopersicum* with the same predicted targets, and DELLAs from the two species. Results from yeast two-hybrid assay. PRO shows autoactivation, so interactions are only considered positive when yeast growth is observed in the presence of 3-AT.



Supplementary Figure 8. Phenotypical differences between the wild types and *della*KO mutants of *S. lycopersicum*, *O. sativa* and *A. thaliana* employed in our experiments. 7-day-old seedlings grown in the presence of PAC (5µM for *S. lycopersicum* and *O. sativa*, and 0.5µM for *A. thaliana*) under long-day conditions.



Supplementary Figure 9. Response curves to different concentrations of paclobutrazol in wild type seedlings of *A. thaliana, S. lycopersicum* and *O. sativa*.

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General Discussion

DELLA proteins have taken center stage in the past two decades, as ubiquitous coordinators of processes through the regulation of TF activity. Importantly, this coordination seems to be executed in response to environmental changes, thus optimizing plant adaptation. The high impact of DELLAs on these processes was our main motivation for the detailed analysis of DELLA functions and mechanisms from an evolutionary perspective, presented in this Thesis. As stated in the Introduction, we were particularly intrigued by questions such as the origin of the role of DELLAs as coordinators, the origin and evolution of their remarkable interacting capacity, and the way they have evolved functional novelties in different plant species. As a one-sentence summary of our findings, it is reasonable to say that **DELLAs have always been promiscuous since their emergence in the ancestor of land plants, and that their functional evolution is the evolution of their interactors**.

1. DELLA promiscuity is a conserved ancestral property

One of the central hypotheses of this Thesis was that the large capacity of DELLAs to interact with TFs was acquired gradually during evolution of land plants. However, our results are compatible with a different model, in which this capacity was rapidly encoded in DELLA after its inception from a GRAS protein ancestor, and all land plant lineages have inherited and maintained promiscuous DELLAs. The observation that GRAS proteins display certain intrinsic degree of interactivity with other TFs could suggest that the ancestral DELLA benefited from this tendency and quickly expanded the range and type of interacting TFs.

More important is the observation that this rapidly acquired capacity has been conserved in all land-plant DELLAs examined. A general trend in the evolution of enzymes and receptors is that an initial flexibility in substrate or ligand recognition, evolves into a narrower specificity (Khersonsky and

Tawfik, 2010; Siddiq et al., 2017). An immediate example is the evolution of the GID1 GA receptor, whose ortholog in early-diverging vascular plants is capable of recognizing a number of GA molecules including GA precursors –all with low affinity–, while the GID1 receptors of angiosperms have increased both the affinity and specificity for GA_4 and GA_1, to the detriment of GA precursor molecules (Yoshida et al., 2018). Why DELLA evolution has followed an opposite path might be related to its position as a 'hub' in transcriptional regulation. The evolution of hubs is logically constrained by their acquired roles because most changes would cause deleterious consequences in the activity of the interacting partners.

2. Functional innovation in DELLAs is linked to the evolution of TFs and their targets

Work with Arabidopsis had already pointed to a model by which GAs or DELLA proteins do not have what could be considered specific targets. All DELLA targets are the targets of their numerous interacting TFs (Gallego-Bartolomé et al., 2011; Marín-de la Rosa et al., 2015). How can we explain that DELLAs in different species have acquired or lost certain sets of target genes? Our comparative transcriptomic analyses, coupled with the interactomic studies, have shown that at least two general mechanisms have participated in these species- (or lineage-)specific innovations: (1) the incorporation of new TFs to the set of DELLA-interacting partners; (2) the loss or acquisition of *cis* regulatory elements for DELLA-interacting TFs in certain target genes (Figure 1). Again, this conclusion is compatible with the model that DELLA evolution is intimately linked to, and explained by, the evolution of its interacting partners.

Our work has not delved into particular functions and the molecular explanation for their origin in a given lineage. Nonetheless, it is reasonable to hypothesize that novel biological functions of DELLA proteins in a given lineage arise when new transcriptional targets are established by one of the

aforementioned mechanisms. An example would be the interaction between DELLA proteins and CYCLOPS in angiosperms to regulate nodulation and arbuscular mycorrhiza formation described in legumes and rice (Fonouni-Farde et al., 2016; Jin et al., 2016; Pimprikar et al., 2016). Orthologs of CYCLOPS are present in the whole green lineage, but new CYCLOPS target genes in certain angiosperms would explain the regulation of root symbiosis development by DELLAs.

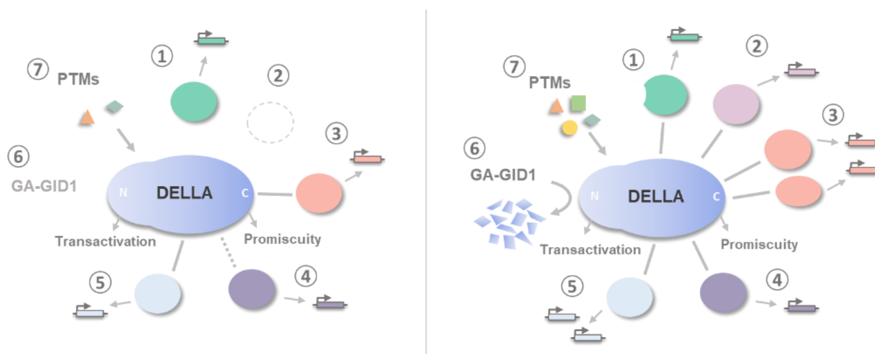

Figure 1. Factors involved in the evolution of DELLA-mediated signaling. On the left, DELLA regulation in a hypothetical non-vascular basal plant species; on the right, in a hypothetical vascular higher species. Although the transactivation activity of the N-terminal domain and the promiscuity of the C-terminal GRAS domain are conserved in DELLA proteins, other factors can affect their ability to modulate gene expression along evolution: 1, structural changes in TFs/TRs allow them to interact with DELLAs; 2, emergence of new TFs/TRs able to bind DELLAs; 3, expansion of an existing family of DELLA interactors; 4, changes in the affinity between DELLA and its partner modify the strength of the interaction; 5, acquisition or loss of transcriptional targets by the TF/TR; 6, establishment of the GA-GID1 module and the GA-dependent degradation of DELLAs in vascular plants; 7, variations in post-translational modifications of DELLA proteins, that affect their stability and their capacity to bind certain proteins.

Although species-specific changes in DELLA-TF affinity could explain our observation that the At*dellaKO* mutant was not fully complemented by distant DELLA orthologs, an additional source of functional differentiation

may rely on specific post-translational modifications (PTMs) happening in certain lineages. Sumoylation has been found to affect the ability of the target proteins to interact with other partners in plants (Conti et al., 2014). The same is true for glycosylation, whose effect has even been proven for AtDELLAs (Zentella et al., 2016, 2017). However, the SUMO sites in OsSLR1 and AtRGA are conserved in angiosperms and gymnosperms, but not in early divergent land plants (Blanco-Touriñán et al., 2020b), and the potentially glycosylated residues are only unevenly conserved. These differences support the idea that PTMs might be involved in the generation of functional diversification in DELLAs.

On the other hand, the fact that some species have more than one DELLA paralog does not seem to be a prime mechanism for multiplicity of functions in those species. Studies with Arabidopsis have shown that RGA and RGL2 can perform equivalent functions as long as they are expressed in the equivalent locations (Gallego-Bartolome et al., 2010), which is in agreement with the different Arabidopsis DELLAs sharing over 90% of their interacting partners (Lantzouni et al., 2020).

3. Conservation of core functions highlights a role for DELLA in stress responses

The *in silico* analysis of DELLA-associated targets (Chapter 1) and the transcriptomic analysis of plants with altered DELLA levels in several land plant species (Chapter 2) coincide in one important point: both studies underscore the relevance of the response to stress as one of the major and more ancient roles of DELLAs. Studies in Arabidopsis had already established the involvement of DELLAs in the protection against oxidative damage (Achard et al., 2008), and the conservation of this role seems to be extended to all the examined land plants, through the transcriptional regulation of genes responsible for antioxidants production, even in species lacking the GA perception module. Interestingly, the other function that is

characteristic of DELLA activity –the control of cell division and cell expansion, *i.e.*, growth– did not appear as a major function conserved at the transcriptional level. This is in contrast with the growth arrest caused by Mp*DELLA* overexpression in *M. polymorpha* reported in Chapter 2, but is in agreement with the lack of clear growth defects observed in the Pp*dellaKO* mutant of *P. patens* (Yasumura et al., 2007). Perhaps the regulation of growth in different species occurs through different mechanisms, or the scarce annotation of certain land plant genomes prevents this function from emerging in GO enrichment analyses.

Another important question directly related to the conservation of DELLA functions is whether their role in establishing the essential balance between optimal growth/development on one side, and the defense program on the other side, was already encoded in the ancestral DELLA. More experimental evidence needs to be gathered, for instance through molecular genetic analysis of DELLAs in non-vascular land plants or early diverging vascular lineages. Nonetheless, our *in silico* network analysis supports an increase in the coordination between transcriptional circuits in plants with DELLAs (Arabidopsis, tomato, *P. patens*) *vs* plants without DELLAs (*C. reinhardtii*), which further increased in the vascular (Arabidopsis, tomato) vs non-vascular plants (*P. patens*), correlating with the incorporation of DELLAs to GA regulation. An increase in coordination seems to be, intuitively, a beneficial trait for plant adaptation in changing environments, although this is only a theoretical consideration at this time. But if this were the case, it would be important to investigate how DELLA levels would vary in bryophytes exposed to different environmental cues, given that they lack the regulatory system for DELLA stability based on environmental control of GA metabolism. At least two possibilities can be discussed: that the levels of DELLAs in those plants are regulated at the transcriptional (and not the post-translational level), or that DELLA protein levels are regulated by a GA-independent mechanism in these species. AtDELLA gene expression is only mildly regulated by external cues (Gallego-Bartolome et al., 2010), but the

recent reports of the polyubiquitination of AtDELLAs by the COP1/SPA1 E3-Ub ligase complex (Blanco-Touriñán et al., 2020a) seem to leave that possibility open. Interestingly, MpCOP1 and MpSPA1 have been found to interact physically with MpDELLA (Blázquez-Alabadí Lab, unpublished results).

4. Biotechnological implications of DELLA evolutionary studies

The first straightforward conclusion of our work, in this respect, is that DELLAs are a major biotechnological target for the manipulation of GA-related traits in any vascular species, and that the knowledge generated at the molecular level in Arabidopsis can be confidently transferred to other plants because the basic function as a hub is conserved.

More importantly, our work also indicates that it is possible to specifically manipulate certain processes regulated by DELLAs without affecting others, as shown by the partial complementation of At*dellaKO* by the DELLAs of different origins. In a way, distant DELLAs acted in this heterologous complementation as edgetic alleles that have specifically lost efficient interaction with some TFs but not with others. This is an interesting application, given that the indiscriminate application of GAs or GA inhibitors in the field causes secondary unwanted effects in all kinds of cultivated plants. For instance, it has been described that GA application causes a reduction in chlorophyll (Williams and Arnold, 1964), a decrease in the biomass of aerial tissues (Blacklow and McGuire, 1971), or a tendency to flower feminization in male corn flowers (Nickerson, 1960). Similarly, application of GA inhibitors causes severe alterations in the flowering time of certain species, difficult to reconcile with the desired compactness of ornamental plants (Rademacher, 1995). Thus, there is a need to generate plant varieties that specifically improve certain agronomical aspects without

the secondary deleterious effects. It is reasonable to think that work like the one presented here can help set the foundations for this line of research.

5. References

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Conclusions

This study has revealed key aspects about the origin and evolution of the role of DELLA proteins as regulatory hubs:

1. DELLAs have notably contributed to the connection and coordination of transcriptional programs since their emergence, and their performance has improved after their integration in GA signaling.
2. DELLA promiscuity is a conserved trait, probably originated in the ancestor of all land plants, and maintained along evolution.
3. The functional conservation of DELLA proteins is partial, and it depends largely on the evolution of their interactors.
4. A likely ancestral function of DELLA proteins is the response to stress.